



ESRRB IS A PIVOTAL TARGET OF THE GSK3/TCF3 AXIS REGULATING EMBRYONIC STEM CELL SELF-RENEWAL

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ESRRB はマウス ES 細胞の自己複製を制御する GSK3/TCF3 経路の
主要な標的因子である

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Key words:embryonic stem cell, pluripotency, self-renewal, nuclear receptor, Tcf

ESRRB IS A PIVOTAL TARGET OF THE GSK3/TCF3 AXIS REGULATING EMBRYONIC STEM CELL SELF-RENEWAL

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Running Title: Esrrb sustains pluripotency

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SUMMARY

Inhibition of glycogen synthase kinase-3 (Gsk3) supports mouse embryonic stem (ES) cells by modulating Tcf3, but the critical targets downstream of Tcf3 are unclear. We analysed the intersection between genome localisation and transcriptome datasets to identify genes repressed by Tcf3. Among these, manipulations of Esrrb gave distinctive phenotypes in functional assays. Knockdown and knockout eliminated response to Gsk3 inhibition, causing extinction of pluripotency markers and loss of colony forming capability. Conversely, forced expression phenocopied Gsk3 inhibition or Tcf3 deletion by suppressing differentiation and sustaining self-renewal. Thus the nuclear receptor Esrrb is necessary and sufficient to mediate self-renewal downstream of Gsk3 inhibition. Leukaemia inhibitory factor (LIF) regulates ES cells through Stat3, independently of Gsk3 inhibition. Consistent with parallel operation, ES cells in LIF accommodated Esrrb deletion and remained pluripotent. These findings highlight a key role for Esrrb in regulating the naïve pluripotent state and illustrate compensation among the core pluripotency factors.

Keywords: embryonic stem cell/pluripotency/self-renewal/nuclear receptor/Tcf

HIGHLIGHTS

- Esrrb is the principal target of Tcf3 repression in the pluripotency network
- Esrrb is essential for self-renewal downstream of Gsk3 inhibition
- Esrrb potently suppresses differentiation and sustains ES cell self-renewal
- Esrrb is a core pluripotency factor but can be compensated by LIF/Stat3

INTRODUCTION

Since the original derivation of mouse ES cells in 1981 (Evans and Kaufman, 1981; Martin, 1981), culture conditions for sustaining pluripotency *ex vivo* have been progressively refined. Following the demonstration that the cytokine leukaemia inhibitory factor (LIF) could replace feeder cells (Smith et al., 1988; Williams et al., 1988) and that ES cell differentiation is suppressed by inhibition of mitogen activated protein kinase (Erk) signalling (Burdon et al., 1999; Kunath et al., 2007), further addition of an inhibitor of glycogen synthase kinase-3 (Gsk3) has enabled robust ES cell propagation in well-defined conditions (Ying et al., 2008). When cultured using the two inhibitors (2i), ES cells display rather uniform marker expression (Wray et al., 2010) and exhibit distinctive gene expression and epigenetic features (Marks et al., 2012). A practical consequence is that it has become facile to establish ES cells from different strains of mice and also rats (Blair et al., 2011). It is noteworthy that, while the triple combination of 2i/LIF appears optimal, mouse ES cells can be propagated by providing any two of these three components (Wray et al., 2011; Wray et al., 2010), implying complementary inputs to a flexible gene regulatory circuit. However, understanding how intracellular signalling pathways engage with the core transcription factor circuitry to maintain or extinguish pluripotency remains fragmentary, (Chen et al., 2008; Jaenisch and Young, 2008; Nichols and Smith, 2012; Niwa et al., 2009).

Gsk3 is a negative regulator of many different proteins (Doble and Woodgett, 2003), including transcription factors such as cMyc (Singh and Dalton, 2009). Nonetheless, the effect of Gsk3 inhibition on ES cell self-renewal is mediated primarily via β -catenin since ES cells lacking β -catenin do not respond productively to Gsk3 inhibitors (Lyashenko et al., 2011; Wray et al., 2011). Consistent with action through intracellular β -catenin, mutation of *Apc* or expression of stabilised β -catenin variants can reduce ES cell differentiation (Kielman et al., 2002; Sato et al., 2004). Furthermore, Wnt3a can partially substitute for Gsk3 inhibition and support ES cell propagation in conjunction with LIF (Berge et al., 2011; Hao et al., 2006; Ogawa et al., 2006; Yi et al., 2011).

It has been suggested that β -catenin might interact directly with Oct4 to promote pluripotent gene expression (Kelly et al., 2011). On the other hand, genetic evidence is incontrovertible that a definitive β -catenin partner, Tcf3, is a major negative regulator of ES cell self-renewal (Guo et al., 2011; Pereira et al., 2006). Indeed, ablation of Tcf3 phenocopies deletion or inhibition of Gsk3 (Wray et al., 2011).

Genome location analyses indicate that Tcf3 binds in proximity to many core pluripotency genes (Cole et al., 2008; Marson et al., 2008; Tam et al., 2008). Although it has been proposed that β -catenin is recruited to stimulate transcription at these sites (Cole et al., 2008), this model seems inconsistent with the *Tcf3* loss of function phenotype. Furthermore, available evidence indicates that Tcf3 functions in a repressor complex (Pereira et al., 2006; Sokol, 2011; Yi et al., 2011), activity of which can be abrogated by β -catenin without requirement for its transactivation domain (Wray et al., 2011).

Tcf3 has previously been proposed to act via repression of *Nanog* (Pereira et al., 2006). However, *Nanog* null ES cells retain responsiveness to Gsk3 inhibition (Silva et al., 2009), and knockdown of *Nanog* in *Tcf3* null ES cells does not prevent up-regulated expression of other pluripotency genes (Yi et al., 2011). It is therefore unclear whether Tcf3 functions as a general repressor of pluripotency genes or acts via a selective target or subset of targets. To resolve this issue we integrated available high resolution genome location data with transcriptome profiles from Tcf3 perturbation studies. This enabled identification of a small number of transcription factor candidates that were subjected to loss and gain of function analyses.

RESULTS

Genome-scale identification of candidate genes directly regulated by Tcf3

Genome-wide mapping of transcription factor binding events is achievable by chromatin immunoprecipitation and deep sequencing (ChIP-seq). Integrated analysis of multiple transcription factor ChIP-seq studies (Hannah et al., 2011), has the potential to provide new mechanistic insights (Ouyang et al., 2009) (Wilson et al., 2010). Given the dominant role of transcriptional regulation in ES cell function (Jaenisch and Young, 2008; Nichols and Smith, 2012; Niwa, 2007; Smith, 2009; Young, 2011) we generated a compendium of transcription factor ChIP-seq analyses (see Figure S1A and B, Table S1 and http://bioinformatics.cscr.cam.ac.uk/ES_Cell_ChIP-seq_compendium.html). We used the ChIP-seq compendium to explore candidate target genes for the Tcf3 transcriptional repressor. We focussed on those Tcf3 ChIP-seq targets also bound by other pluripotency factors. As previously indicated from promoter-based ChIP-on-chip analysis (Cole et al., 2008), extensive co-localisation of Tcf3 with core

pluripotency transcription factors Oct4 and Nanog was evident in the genome-wide ChIP-seq datasets (Figure 1B).

We then analyzed published gene expression profiles for ES cells in which *Tcf3* has been inactivated, either by gene targeting (Yi et al., 2011) or by RNAi (Cole et al., 2008). *Tcf3* null and *Tcf3* knock-down ES cells show similarly enhanced self-renewal so we reasoned that genes differentially expressed in both studies are likely to underlie this phenotype. *Tcf3* appears to function in ES cells primarily, if not exclusively, as a transcriptional repressor (Pereira et al., 2006; Wray et al., 2011; Yi et al., 2011). We therefore focussed on genes with increased expression after *Tcf3* inactivation and found 379 genes up-regulated in *Tcf3* null cells and 1972 in *Tcf3* knock-down cells (Figure 1C). Of these, 120 genes were common to both groups and therefore were considered the most likely specific responders.

We intersected the list of *Tcf3* target genes from the ChIP-seq compendium with the 120 upregulated genes and found a statistically significant enrichment (P-value= 0.0034), with 50 genes identified as candidates for direct regulation, being both bound by *Tcf3* and de-repressed after *Tcf3* ablation. The same analysis for genes down-regulated following *Tcf3* inactivation did not show significant enrichment for *Tcf3* binding (P-value= 0.57, Figure S1C), indicating indirect regulation. To survey possible biological roles of *Tcf3* direct targets we performed Gene Ontology analysis and found an over-representation of developmental processes such as cell differentiation, anatomical structure development, cell morphogenesis and embryonic development (Figure S1D). This is in line with the role of *Tcf3* as a repressor of pluripotency (Wray et al., 2011) and mediator of axial patterning (Merrill et al., 2004).

For functional validation, we focussed on genes associated with cell differentiation and development and among them selected transcription factors. This gave a short-list of 9 genes (in bold in Figure S1E). We used gene expression analysis to test responsiveness to the Gsk3 inhibitor Chiron99021 (CH). Four genes showed very low expression in with or without CH (data not shown). The other 5 transcription factor genes exhibited significantly increased expression in response to CH (Figure 2D, orange bars). *Esrrb*, *Klf2*, *Nanog*, *Nr0b1* and *Tcfcp2l1* thus emerge as candidate mediators of Gsk3/*Tcf3*-mediated ES cell self-renewal. Figure 2E shows gene tracks of *Tcf3*, *Nanog* and Oct3/4 binding at these 5 gene loci.

We also analyzed *Tcf3* null cells and found an increased level of expression of the 5 genes, similar to that in wild-type cells treated with CH (Figure 2D, blue bars). Importantly, when *Tcf3* null cells were treated with CH we did not observe any further increase in expression (Figure S1F). These data indicate that CH promotes the transcription of *Esrrb*, *Klf2*, *Nanog*, *Nr0b1* and *Tcfcp2l1* through *Tcf3* inhibition without requirement for canonical activation through other *Tcf/Lef* family members (Yi et al., 2011).

***Esrrb* is critical for self-renewal downstream of the *Gsk3/Tcf3* axis.**

Gsk3 deletion or inhibition is sufficient to maintain ES cell self-renewal in bulk culture for several passages, albeit with ongoing differentiation (Doble et al., 2007; Wray et al., 2010; Ying et al., 2008). This condition provides a stringent assay for downstream gene requirement. To obtain a quantitative readout we used *Rex1*GFPd2 cells, in which a destabilized GFP protein is expressed from the *Rex1* (*Zfp42*) locus (Wray et al. 2011). *Rex1* is expressed specifically in ground state pluripotent cells and is down-regulated within 24 hours at the onset of differentiation (Toyooka et al., 2008; Wray et al., 2010). Consequently the fraction of undifferentiated ES cells in a population can be quantified by flow cytometric analysis for GFP (Wray et al., 2011). We knocked down the five transcription factor genes in *Rex1*GFPd2 cells by siRNA transfection (Figure 2A). Two different siRNAs were used for each gene (Figure S2A). Cells were maintained in the presence of CH and evaluated by flow cytometry (Figure 2B). ES cells treated with control siRNA are mostly *Rex1*GFP positive with a small shoulder of negative cells. This profile is unaltered by siRNA against *Nr0b1* while *Nanog* knockdown induces only a small increase in the negative fraction. The effect of *Klf2* and *Tcfcp2l1* siRNAs is rather more pronounced, but in both cases the majority of cells remain GFP positive. In contrast knock-down of *Esrrb* caused a dramatic shift from *Rex1*GFP positive to negative (Figure 2B and C). This is not a selective elimination effect because viable cell numbers determined by live/dead gating were similar in all conditions. To exclude non-specific effects of siRNAs on GFP expression we confirmed a reduction in *Rex1* transcript (Figure 2D). Further qPCR analyses showed a marked down-regulation in other pluripotency markers and a concomitant increase in the early differentiation marker *Fgf5* upon treatment with si*Esrrb*.

To address whether decreased expression of *Rex1* and pluripotency genes following *Esrrb* knock-down is associated with compromised self-renewal, we tested ability to form undifferentiated colonies 48 hours after siRNA transfection. Cells were plated at

single cell density in 2i+LIF and after 5 days stained for alkaline phosphatase (AP). AP activity is a classical marker of undifferentiated ES cells but does not reliably discriminate them from post-implantation epiblast stem cells (EpiSCs) (Bao et al., 2009). However, EpiSCs differentiate or die in 2i (Guo et al., 2009), therefore in this condition the marker is reliably indicative of ES cell status. Cells transfected with a control siRNA (siControl) or siRNA targeting *eGFP* (siGFP) gave rise to numerous AP-positive colonies, while knockdown of *Nr0b1*, *Nanog*, *Klf2*, or *Tcfcp2l1* caused up to two-fold reduction (Figure 2E). Knock-down of *Esrrb* had a much stronger effect, however, almost entirely eliminating AP-positive colonies (Figure 2E, green bars).

Previously we reported that CH promotes self-renewal by relieving Tcf3 repression (Wray et al. 2011). The expression of each of the 5 candidate genes is elevated in *Tcf3* null cells (Figure 1D). We therefore examined their importance for the ability of ES cells lacking Tcf3 to self-renew efficiently without requirement for CH. We knocked down the genes in *Tcf3* null cells and tested ability to form colonies in the presence of the Mek inhibitor PD0325901 (PD) alone. Null cells transfected with either siControl or siGFP readily generated AP-positive colonies (Figure 2F, blue bars). *Klf2* and *Tcfcp2l1* siRNA treatment resulted in slightly fewer colonies, while knockdown of *Esrrb* or *Nanog* almost eliminated colony formation (Figure 2F, green and purple bars). We also used titrated siRNA to partially reduce *Esrrb* expression to levels close to those in wild type ES cells (Figure S2B and S2C). This resulted in restoration of colony formation to similar numbers as for wild type ES cells (Figure S2D). Taken together, these results suggest that *Esrrb* is necessary to mediate self-renewal downstream of Gsk3 inhibition and Tcf3 derepression.

Esrrb expression reproduces the effect of Gsk3 inhibition on self-renewal

We then examined whether expression of *Esrrb* or the other factors may be sufficient to substitute for Gsk3 inhibition. Rex1GFPd2 cells maintained in 2i were transfected with Piggybac expression vectors (Guo et al., 2009). Following hygromycin selection for 2 passages in 2i, the response to inhibitor withdrawal was tested in colony forming assays and in bulk culture (Figure 3A and B). PB-vector and PB-*Tcfcp2l1* transfectants yielded negligible colonies when plated at clonal density in N2B27 alone. PB-*Nr0b1*, PB-*Nanog* and PB-*Klf2* produced some colonies, but much fewer than in the presence of CH. PB-*Esrrb*, however, generated undifferentiated colonies in comparable number and size to those obtained in the presence of CH. The absence of any significant additive effect when *Esrrb* is overexpressed in the

presence of CH (Figure 3A) suggests that *Esrrb* is capable of fully recapitulating the effect of Gsk3 inhibition on ES cell clonogenicity.

In bulk culture in the presence of PD alone, PB-vector and PB-Tcfcp2l1 cells collapsed within two passages. Other transfectants could be maintained longer but progressively lost Rex1GFP expression (Figure 3B) and completely differentiated or died by 5 passages. By contrast, PB-*Esrrb* cells could easily be expanded in PD for more than 12 passages. Their Rex1GFP profile showed only a small shoulder of GFP low cells and the cells retained undifferentiated morphology (Figure 3B and 3C). PB-*Esrrb* cells in these conditions expressed Oct4, Sox2 and Nanog at a level similar to control cells in 2i (Figure 3D, orange bars). They showed reduced Klf4 and Klf5, but increased Klf2.

We then tested whether expression of *Esrrb* could confer clonal self-renewal in the presence of serum, which is normally dependent on LIF. Colonies were scored as undifferentiated, mixed or differentiated according to the AP staining pattern because serum supports the survival and proliferation of differentiated cells. PB-vector cells formed undifferentiated colonies only in the presence of LIF, whereas PB-*Esrrb* cells were equally capable of generating AP-positive colonies without LIF (Figure 3E), similarly to PB-Nanog cells that serve as a positive control. Strikingly, the combination of *Esrrb* overexpression and LIF caused a further increase in the number of undifferentiated colonies, indicating that *Esrrb* is in a parallel pathway to LIF/Stat3. We also used this assay to examine whether *Esrrb* could rescue the differentiation phenotype induced by overexpression of Tcf3. Indeed the generation of undifferentiated colonies is diminished by transfection with PB-Tcf3 alone and regained by co-transfection with PB-*Esrrb* (Figure 3E). Consistent with the colony assay, Tcf3 transfectants showed reduced expression of Nanog that was restored by *Esrrb* (Figure S3A).

Esrrb expressing cells formed undifferentiated AP-positive colonies when LIF or Wnt signalling were blocked by a Jak inhibitor or DKK respectively (Figure S3B and S3C). Furthermore, neither the LIF target *Socs3* nor the Wnt target *Axin2* showed elevated expression (Figures S3D and S3E), confirming that *Esrrb* does not act by stimulating these pathways. We also co-cultured GFP labelled *Esrrb* transfectants with unlabelled wild type cells. After 3 passages in serum without LIF, the entire population was GFP positive (Figure S3F), demonstrating that *Esrrb* acts cell autonomously.

In unscreened PB-Esrrb cells the expression of Esrrb is typically up to 6-8 fold higher than endogenous levels. This creates the possibility of neomorphic effects. We therefore transfected Rex1GFPd2 ES cells with an alternative PB-Esrrb vector allowing capture of low expressors using G418 (Figure 3F). We screened for level of Esrrb expression and identified a pool in which Esrrb mRNA and protein are constitutively expressed at levels comparable to those found in control vector transfectants in 2i (Figure 3G and 3H). These PB-Esrrb-neo cells recapitulate the phenotype of Esrrb over-expressing cells: in the absence of CH, or LIF, they can be cultured for multiple passages; they express pluripotency markers (Figure 3G and 3I); and they self-renew at clonal density (Figure 3J). We conclude that constitutive expression of Esrrb at endogenous levels is sufficient to sustain ES cell self-renewal.

Since constitutive expression of Esrrb blocks differentiation, transgene excision (Chambers et al., 2003; Niwa et al., 2009) was necessary to determine whether ES cells maintained by Esrrb retain pluripotency. After transfection with an excisable vector cells were clonally selected and expanded in serum without LIF for 1 month. They were then transiently transfected with a Cre expression vector followed by sub-cloning of DsRed-positive cells in the presence of LIF (Figure 3K). These sub-clones no longer expressed the *Esrrb* transgene and re-acquired dependency on LIF (bottom panels of Figure 3L and Figure S3H). Reverted cells were injected into blastocysts and gave rise to chimaeric embryos in which DsRed-expressing cells contributed widely (Figure 3M).

Collectively these results indicate that constitutive expression of Esrrb can replace Gsk3 inhibition and furthermore maintain self-renewal and pluripotency independently of LIF/Stat3.

Esrrb is dispensable for self-renewal in the presence of LIF

Although Esrrb appears essential for ES cell propagation downstream of Gsk3 inhibition, the forced expression studies indicate that it does not lie downstream of LIF/Stat3. Therefore *Esrrb* might be dispensable in the presence of LIF, just as *Stat3* can be deleted when Gsk3 is inhibited (Ying et al., 2008). We tested this hypothesis by knocking down *Esrrb* in Rex1GFPd2 cells cultured in the presence of LIF and PD (Figure 4A). Under these conditions we found that *Esrrb* siRNA had a modest effect on the naïve pluripotency marker Rex1 (Figure 4B and 4C) and caused only a partial reduction in the ability to form undifferentiated colonies (Figure 4D). These findings

are in stark contrast with the near elimination of self-renewal by *Esrrb* siRNA in the absence of LIF (Figure 2).

To substantiate these findings we generated ES cells in which the *Esrrb* gene can be conditionally inactivated. Through serial gene targeting we inserted loxP sites to flank the second exon of both alleles of *Esrrb* (Figure S4A). The second exon encodes the start codon and part of the DNA-binding domain. *Esrrb*^{Δ/Δ} ES cells generated by Cre-mediated recombination are therefore expected to be functionally null. Tamoxifen-regulatable Cre was used to effect deletion. Homozygous deletion was confirmed in a clonally expanded population by immunostaining and qPCR (Figure 4E and 4F). These *Esrrb* null ES cells remained morphologically undifferentiated in serum-containing medium with LIF over multiple passages. Expression of some pluripotency-associated genes, *Klf4* and *Tbx3*, was reduced (Figure 4F) and the null cells exhibited a rather more flattened morphology (Figure 4H), but they showed similar proliferation to wildtype ES cells. Interestingly, *Nanog*^{-/-} ES cells also show reduced expression of *Klf4* (Figure 4G). From examination of the ChIP-seq compendium *Klf4* is a likely direct target of both *Esrrb* and *Nanog* which may explain its lower level in null cells.

When *Esrrb* null ES cells were cultured in serum-free conditions they expanded in 2i+LIF and LIF+PD. They were less compact than parental cells but remained undifferentiated (Figure 4H) with no overt compromise of proliferation or viability. However, on LIF withdrawal the null cells collapsed within one passage. Thus in the absence of *Esrrb* ES cells lose the ability to respond effectively to Gsk3 inhibition and their propagation appears strictly dependent on LIF. Clonal analysis of *Esrrb*^{Δ/Δ} cells confirmed these findings, showing a dramatic reduction in the number of AP positive colonies in the absence of LIF (Figure 4I, compare 2i+LIF to 2i and LIF+PD to CH).

We carried out gene expression analysis after 48h of culture either in LIF+PD or in CH (Figure 4K and L). In LIF+PD, *Esrrb*^{loxP/loxP} and *Esrrb*^{Δ/Δ} cells showed a comparable profile, apart from reduced *Klf4* and *Tbx3* in the null cells (Figure 4K). In contrast, after 48 hours in CH *Esrrb*^{Δ/Δ} cells showed lower expression of all pluripotency markers (Figure 4L). These deletion findings are fully consistent with results obtained after *Esrrb* knockdown (Figure 2 and 4A-D), confirming that *Esrrb* is essential for self-renewal downstream of CH, but can be compensated for by LIF stimulation. In contrast, *Nanog* null cells could be propagated under all conditions

described above, consistent with previous observations (Silva et al., 2009) that Nanog is not required for responsiveness to Gsk3 inhibition (Fig 4J).

To explore why loss of *Esrrb* has more severe consequences than deletion of Nanog, we utilised the ChIP-seq compendium (Figure S1) to compare profiles of Nanog, Oct4, Sox2, Tcf3 and *Esrrb*. As shown in Figure 4M using the *Tbx3* locus as an example, there are regions bound by Nanog, Oct4, Sox2, Tcf3 (O/S/T) together with *Esrrb* (see blue box), as well as regions bound only by *Esrrb* (see green box). Global analysis revealed that a majority of the genomic regions bound by Nanog are co-occupied by at least one of Oct4, Sox2, Tcf3 (O/S/T) as well as *Esrrb*. However, only a minority of *Esrrb*-bound regions are occupied by any of the other 4 pluripotency factors (Figure 4N and S4C). To investigate whether *Esrrb*-specific occupancy translates into *Esrrb*-specific candidate target genes, we mapped binding peaks to genes (see Table S1 and Methods section) and intersected the resulting gene lists. While over 90% (2921 out of 3230) of the predicted Nanog target genes were also targets of O/S/T and/or *Esrrb*, only ~55% (1992 out of 3647) of *Esrrb* candidate targets were shared with any of the other factors (Figure 4O and S4D). The extensive overlap of Nanog with Oct4, Sox2, Tcf3 and/or *Esrrb* candidate targets may explain why Nanog deletion can be tolerated in established ES cells. Conversely, the wider occupancy of *Esrrb* is consistent with the observation that *Esrrb* function is less readily dispensable.

We then examined whether ES cells lacking *Esrrb* and maintained using LIF are pluripotent. Cells grafted under the kidney capsule gave rise within 6 weeks to large multi-differentiated tumours that contained neural, mesodermal and endodermal tissues along with undifferentiated embryonal carcinoma (Figure 5A). The tumours showed no overt differences in size or differentiation from teratocarcinomas generated by *Esrrb*^{loxP/loxP} cells. Most significantly, the embryonic identity and developmental potential of *Esrrb* null cells was confirmed by integration into the inner cell mass after aggregation with morulae (Figures 5B and 5C) and widespread contribution to the mid-gestation embryo after blastocyst injection (Figure 5D and S5).

Direct regulation of *Esrrb* expression by the Gsk3/β-catenin/Tcf3 axis

In keeping with several other core pluripotency factors, *Esrrb* is expressed in a mosaic fashion in ES cells cultured in serum. When CH is added to the cultures, however, *Esrrb* immunostaining becomes more uniform and increased in intensity (Figure 6A, B). To confirm binding of Tcf3 to the *Esrrb* gene we carried out single

point ChIP assays at 5 regions detected in previous ChIP studies (Cole et al., 2008; Marson et al., 2008). Marked enrichment was observed in wildtype ES cells compared with *Tcf3* null cells (Figure 6C). As noted above, *Tcf3* null ES cells show elevated expression of *Esrrb* that is not further induced by CH alone. We repeated this analysis in the presence of LIF and PD and obtained similar results (Figure 6D). *β-catenin* null ES cells also fail to up-regulate *Esrrb* in response to CH, consistent with their inability to self-renew without LIF (Wray et al., 2011). These data confirm that both *Tcf3* and *β-catenin* are necessary for *Esrrb* induction by Gsk3 inhibition. We found that *Wnt3a* can induce *Esrrb*, although to lower levels than CH (Figure S6), likely reflecting the operation of negative feedback in the canonical Wnt pathway that can be short-circuited by Gsk3 inhibition. Finally we investigated the possibility that induction of *Esrrb* may involve *Nanog*. *Nanog* is modestly up-regulated by both CH and *Tcf3* ablation (Figure 1D) and it has been shown to bind to the *Esrrb* promoter region (Chen et al., 2008). We found that *Esrrb* is fully induced by CH in *Nanog* null ES cells (Figure 6E). Steady state expression is lower in the absence of *Nanog*, however (see also Figure 4G). We conclude that *Nanog* plays a role in maintenance of *Esrrb* expression level, but that induction by Gsk3 inhibition is mediated directly by abrogation of *Tcf3* repression (Figure 6F).

DISCUSSION

Several mechanisms have been proposed through which inhibition of Gsk3 may stabilise the naive state of mouse ES cells (Cole et al., 2008; Kelly et al., 2011; Sokol, 2011; Wray et al., 2011; Yi et al., 2011). The present analyses clarify this debate by identifying *Esrrb* as a direct functional target of *Tcf3* that is de-repressed downstream of Gsk3 inhibition. Perturbation studies demonstrate that *Esrrb* activity is both necessary and sufficient for the response to Gsk3 inhibition. Notably, *Esrrb* exhibits markedly more potent capacity than other *Tcf3* targets to suppress differentiation, sustain propagation and maintain key pluripotency genes. While recognising that *Tcf3* also regulates other key genes in the pluripotency circuitry, these findings pinpoint *Esrrb* as the main effector through which the Gsk3/*β-catenin*/*Tcf3* axis modulates ES cell self-renewal.

We generated a compendium of publicly available ChIP-seq data for 38 transcriptional regulators in mouse ES cells (Figure S1A and B). This integrated resource with a unified data structure enables streamlined cross-referencing of individual datasets from different laboratories. Distinct clusters of binding profiles can be discerned that appear to partition the ES-cell transcriptional programme into

several major sub-compartments. The compendium thus provides a powerful analytical tool with the potential to fuel new hypotheses on the transcriptional control of ES cell function. Tcf3 binds at a large number of sites, including many genes implicated in ES cell biology (Cole et al., 2008; Marson et al., 2008; Tam et al., 2008). We used the compendium resource to examine in greater detail genes bound by both Tcf3 and core pluripotency factors and generate a refined list of candidate targets, which included *Esrrb*.

Esrrb is an orphan nuclear receptor related to the oestrogen receptor (Luo et al., 1997). A potential role in ES cells was previously suggested from results of an RNAi screen by Ivanova and colleagues who found that knock down of *Esrrb* reduced ES cell self-renewal efficiency and promoted differentiation (Ivanova et al., 2006). Subsequently *Esrrb* was reported to replace Klf4 in fibroblast reprogramming, albeit with reduced efficiency (Feng et al., 2009). Through protein interaction studies *Esrrb* was found to bind to Oct4 and cooperate in transcriptional regulation of *Nanog* (van den Berg et al., 2008). *Esrrb* is also proposed to interact with *Nanog* and thereby play a reciprocal role in regulation of *Oct4* expression (Zhang et al., 2008). These authors also reported that overexpression of *Esrrb* can support formation of alkaline phosphatase colonies in serum in the absence of LIF (Zhang et al., 2008). However, the identity of the colonies was not characterised further and neither self-renewal nor retention of pluripotency were investigated. Nor is there any evidence that *Esrrb* is induced by LIF. In the embryo, deletion of *Esrrb* causes mid-gestation lethality due to placental defects (Luo et al., 1997), but mutant embryos develop through implantation and gastrulation. Furthermore, tetraploid complementation rescued embryo development at least to mid-gestation (Luo et al., 1997), confirming that there is no defect in the ICM or epiblast. Hence the functional significance of *Esrrb* in ES cells and its position in the regulatory hierarchy have been uncertain.

Here, through loss-of-function studies by siRNA knockdown and definitive gene deletion via homologous recombination, we established that *Esrrb* is specifically required for the self-renewal effect of Gsk3 inhibition. Furthermore, *Esrrb* gain-of-function can replace Gsk3 inhibition and support long-term ES cell propagation in the presence of Mek inhibition alone. Constitutive expression of *Esrrb* can also confer clonogenic LIF-independent self-renewal in serum. Importantly, ES cells propagated under the direction of *Esrrb* remain pluripotent and capable of colonising chimaeras when the transgene is removed.

Mouse ES cell self-renewal is highly responsive to LIF, acting through Jak kinase and the downstream transcription factor Stat3 (Matsuda et al., 1999; Niwa et al.,

1998). Neither LIF nor Gsk3 inhibition alone can fully suppress ES cell differentiation, but the combination of both together is very effective (Wray et al., 2010). Furthermore, *Stat3* null ES cells can be derived and maintained using 2i and are functionally non-responsive to LIF (Ying et al., 2008) while β -catenin null ES cells self-renew in the presence of LIF but do not respond to Gsk3 inhibition (Lyashenko et al., 2011; Wray et al., 2011). These observations indicate that Gsk3 inhibition/Tcf3 de-repression supports self-renewal in parallel with LIF/Stat3 and inputs independently into the core pluripotency network (Figure 6F). Indeed, although *Esrrb* expression can act dominantly to confer LIF independence, addition of LIF further augments clonogenic capacity. Consistent with parallel pathways, the requirement for *Esrrb* is not absolute. ES cells lacking *Esrrb* remain undifferentiated if cultured in the presence of LIF with either PD or serum. Thus the need for *Esrrb*, just as for Gsk3 inhibition or β -catenin (Lyashenko et al., 2011; Wray et al., 2011), is conditional on whether ES cells receive other pro- and anti-differentiation stimuli, in particular LIF. Parallel compensatory capacity could explain why the pluripotent lineage in the early embryo can develop without *Esrrb* (Luo et al., 1997).

When *Esrrb* knockdown or knockout ES cells are cultured without LIF, they down-regulate pluripotency genes and exit self-renewal. We therefore conclude that *Esrrb* is a component of the core transcription factor network that underpins pluripotency. *Esrrb* appears to be highly interconnected with other pluripotency factors by both protein interactions and transcriptional cross-regulation (van den Berg et al., 2008; van den Berg et al., 2010; Zhang et al., 2008). Interestingly, examination of the ChIP-seq compendium reveals that although *Esrrb* binds at many of the same genes as other core pluripotency factors (Nanog, Oct4, Sox2, Tcf3) it often occupies different sites. In addition, *Esrrb* is located at a large group of genes not bound by the other factors. These observations are consistent with the functional evidence that *Esrrb* makes a distinctive contribution to ES cell self-renewal. Indeed we find that *Esrrb* binding is detected at ~70% of genes (8149 out of 12051) that show significant expression by RNA-seq (>0.5 RPKM) in ground state ES cells (Marks et al., 2012). This suggests that it may play a generalised role in ES cell transcription, particularly when considered with evidence that *Esrrb* interacts physically with components of the basal transcriptional machinery (van den Berg et al., 2010).

In summary, these findings place *Esrrb* on a par with Nanog, Klf4, Klf2 and Tbx3 in the pantheon of intrinsic pluripotency factors that together with Oct4 and Sox2 establish and sustain naive ES cells (Figure 6F). Specifically, *Esrrb* is a direct target of Tcf3 repression and the principal factor mediating the self-renewal response to

Gsk3 inhibition and stabilization of β -catenin. Esrrb acts independently of LIF/Stat3, conferring flexibility and robustness to naïve ES cell self-renewal. Future work will explore the mutual compensation and plasticity in the pluripotency gene regulatory network that allows LIF stimulation to accommodate deletion of Esrrb. It will also be of interest to examine epiblast development in blastocysts doubly deficient for *Esrrb* and *Stat3*. Finally we note that Esrrb is not significantly expressed in mouse post-implantation epiblast stem cells (EpiSCs), nor in currently available human pluripotent stem cells, neither of which show a self-renewal response to Gsk3 inhibition.

Experimental procedures

Generation of ChIP-seq compendium

Processed data files (density maps, wig format; peak coordinates, bed format) were acquired from the NCBI Gene Expression Omnibus (GEO) and Short Sequence Read Archive (SRA). They were displayed in the UCSC Genome Browser allowing a visual inspection of the ChIP-seq data and peak calling quality. Where the published peak coordinates appeared to over- or under-predict, or were unavailable, creation of density maps and peak calling was performed de novo using the mapped/aligned reads as described (Hannah et al., 2011). The specific peak parameters used are indicated in Table S1.

A binary peak matrix was generated as described (Hannah et al., 2011), analysed by unsupervised hierarchical clustering using Pearson correlation coefficients, and displayed using the heatmap function in R (Figure S1A).

Lists of candidate target genes for all factors have been generated by peak-to-gene mapping using the same uniform parameters for all studies which may be different from the parameters used in the individual original studies. The requirements used are: if a TF peak is within 100bp of the TSS it is associated with that gene alone; all other peaks can be associated with up to two genes, by examining 50kb of flanking sequence either side of the peak.

The lists of candidate targets have been analyzed by unsupervised hierarchical clustering using Pearson correlation coefficients, and displayed using the heatmap function in R (Figure S1B).

All raw and processed datafiles used for this study are available for download from http://bioinformatics.cscr.cam.ac.uk/ES_Cell_ChIP-seq_compendium.html

The gene tracks have been generated by loading the density maps of the indicated factors into the UCSC genome browser as custom tracks.

The ChIP-seq data used for peaks intersection, target gene intersection and gene tracks (in Figures 1 and 4) are: Esrrb - GSE11431 (GSM288355), Nanog - GSE11724 (GSM307140 & GSM307141), Oct4 - GSE11724 (GSM307137), Sox2 - GSE11724 (GSM307138 & GSM307139), Tcf3 - GSE11724 (GSM307142 & GSM307143).

Embryonic stem cell culture

ES cells were cultured without feeders on plastic coated with 0.1% gelatine (Sigma, cat. G1890) and replated every three days at a split ratio of 1 in 10 following dissociation with Accutase (PAA, cat. L11-007). Cells were cultured either in the GMEM (Sigma, cat. G5154) supplemented with 10% FCS (Sigma, cat. F7524), 100 μ M 2-mercaptoethanol (Sigma, cat. M7522), 1x MEM non-essential amino acids (Invitrogen, cat. 1140-036), 2 mM L-glutamine, 1 mM sodium pyruvate (both from Invitrogen), and 100 units/ml LIF, or in the serum-free media N2B27 (NDiff N2B27 base medium, Stem Cell Sciences Ltd, cat. SCS-SF- NB-02) supplemented, as indicated, with small-molecule inhibitors PD (1 μ M, PD0325901) and CH (3 μ M, CHIR99021) and LIF prepared in-house. Colony forming assays were carried out by plating 600 ES cells per well on plates coated with laminin (Sigma, cat. L2020). Plates were fixed and stained for alkaline phosphatase (Sigma, cat. 86R-1KT) according to the manufacturer's protocol. Plates were scanned using a CellCelector (Aviso) and scored manually.

Gene expression analysis by quantitative PCR with reverse transcription

Total RNA was isolated using the RNeasy Kit (Qiagen) and cDNA was made from 500ng using SuperScriptIII (Invitrogen) and oligo-dT primers. For Real-time PCR we used TaqMan Fast Universal Master Mix and TaqMan probes (Applied Biosystems) or the Universal Probe Library (UPL, Roche) system. In Figures 4F,J and K we used SYBR green system. Primers and UPL probe numbers are detailed in Supplementary Table S2. Technical replicates were carried out for all quantitative PCR reactions. An endogenous control (Gapdh, glyceraldehyde-3-phosphate dehydrogenase, Applied Biosystems 4352339E) was used to normalize expression.

RNAi experiments

siRNAs were transfected at a final concentration of 40nM using Dharmafect 1 (Dharmacon, cat. T-2001-01), following the protocol for reverse transfection. For a

12 well plate (4cm²) we used 2 microliters of transfection reagent, 2 microliter of 20 microM siRNA solution and 30,000 ES cells in 1 ml of N2B27 medium. The medium was changed after overnight incubation; 48h after transfection the cells were analyzed as indicated. See Supplementary table S3 for sequences of the siRNAs used.

Flow cytometry

After treatment with Accutase, live ES cells were resuspended in PBS with 3% FCS and ToPro-3 (invitrogen) was added at a concentration of 0.05nM to detect dead cells. Flow cytometry analyses were performed using a Dako Cytomation CyAn ADP high-performance cytometer with Summit software.

Esrrb gene targeting

LoxP sites were inserted to flank exon2 of *Esrrb* using a promoter-trap vector containing an FRT flanked SA-IRES- β geo selection cassette (Figure S4). Subsequent transfection with the FLPe expression vector removed this cassette to generate *Esrrb*^{loxP:Δ/+} ES cells. Targeting was confirmed by genomic PCR. This strategy was repeated for the second allele to create homozygous *Esrrb*^{loxP:Δ/loxP:Δ} ES cells. To enable efficient conditional deletion, cells were stably transfected with an expression vector for the tamoxifen-inducible form of Cre (MerCreMer). Null cells were obtained by treatment with 200ng/ml of Tamoxifen for 3 days and expanded from single cells in medium containing serum and LIF.

Supplemental Information

Supplemental Information includes six figures, five tables and Supplemental Experimental Procedures.

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Author contributions: GM and TS performed and interpreted ES cell experiments; ET, AJ and RH generated the ChIP-seq compendium; SO produced chimaeras; BG, HN and AS supervised the study and wrote the paper with GM.

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FIGURE LEGENDS

Figure 1. Identification of Tcf3 direct targets in mouse ES cells

- A) Flow chart illustrating the approach used to identify candidate genes that mediate self-renewal downstream of Tcf3.
- B) Venn diagram showing overlap of Nanog, Oct3/4 and Tcf3 bound genes (see Methods section).
- C) Venn diagram showing overlap between genes up-regulated (>1.4 fold change) in Tcf3 null cells, genes up-regulated in Tcf3 knockdown, and genes bound by Tcf3, Oct3/4 and Nanog. 50 genes are identified as candidate Tcf3 direct targets.
- D) Gene expression analysis of wild type ES cells treated with the Gsk3 inhibitor (CH) for the indicated times (orange bars), and of *Tcf3* null ES cells (blue bars). All cultures were in LIF+serum. The fold change expression relative to wild type ES cells treated with vehicle (DMSO) is shown on a logarithmic scale. Mean and SD of 2 independent experiments is shown.
- E) Gene tracks represent binding of Nanog, Oct3/4 and Tcf3 at the indicated gene loci. The X axis represents the linear sequence of genomic DNA and the Y axis represents the total number of mapped reads (see Methods section).

Figure 2 - *Esrrb* is required to mediate self-renewal downstream of GSK3

- A) Experimental scheme for testing the functional requirement of the candidate genes identified in Figure 2. Rex1-GFPd2 cells were cultured in presence of the Gsk3 inhibitor (Chiron-N2B27) for 2 passages and transfected with 2 independent siRNAs for each candidate gene. Cells were harvested 48h after transfection and analyzed by flow cytometry (Rex1-GFP profile), quantitative PCR, and colony formation.
- B) Rex1-GFPd2 cells were transfected with the indicated siRNAs and analyzed after 48h by flow cytometry. One representative plot for knockdown of each gene is shown. The dashed line indicates the threshold used to separate GFP positive and GFP negative cells.
- C) Quantification of the flow cytometry data for Rex1-GFPd2 cells transfected with the indicated siRNAs. Bars show the number of GFP positive cells normalized to the negative control siRNA (siControl). Mean and SD of 6 independent experiments is shown.
- D) Gene expression analysis of Rex1-GFPd2 cells transfected with 2 independent siRNAs targeting *Esrrb* or 2 negative control siRNAs (siControl and siGFP). GAPDH was used as endogenous control and data are normalized to the siControl sample. Mean and SD of 4 independent experiments is shown.
- E) Clonogenicity assay on Rex1-GFPd2 cells transfected with the indicated siRNAs. 48h after transfection cells were replated at clonal density in 2i + LIF and stained for alkaline phosphatase (AP) after 5 days. Bars show the number of AP+ve colonies normalized to the siControl. Mean and SD of 4 independent experiments is shown.
- F) Clonogenicity assay of *Tcf3* null cells transfected with the indicated siRNAs. *Tcf3* null cells cultured in PD were transfected with the indicated siRNAs; after 48h they were replated at clonal density in PD, and stained for alkaline phosphatase after 5 days. Bars show the number of AP+ve colonies

normalized to the negative control siRNA (siControl). Mean and SD of 3 independent experiments is shown. See also Figure S2B, S2C and S2D.

Figure 3. Esrrb recapitulates the effect of GSK3 inhibition on ES cell self-renewal

- A) Rex1-GFPd2 cells were co-transfected with pBase helper plasmid and a piggyBac vector containing Esrrb, Klf2, Nanog, Nr0b1, Tcfcp2l1 or no cDNA (PB-vector); transfected cells were selected for 2 passages with Hygromycin in 2i. 600 cells were then plated at clonal density in the basal media N2B27 with (+) or without (-) Chiron and stained for alkaline phosphatase (AP) after 5 days. Bars show the number of AP+ve colonies. Mean and SD of 3 independent experiments is shown.
- B) Flow cytometry analysis of Rex1-GFPd2 transfectants cultured in the indicated conditions. PB-vector cells could not be maintained for more than 2 passages in presence of the Mek inhibitor PD without CH, while PB-Klf2, PB-Nanog and PB-Nr0b1 cells could not be maintained for more than 4 passages. Only PB-Esrrb cells showed robust self-renewal under these conditions, expanding continuously for more than 12 passages.
- C) Phase contrast pictures of PB-vector transfected cells in 2i media and PB-Esrrb cells in presence of the Mek inhibitor PD.
- D) Gene expression analysis of Rex1-GFPd2 cells transfected with either an empty vector or PB-Esrrb and cultured in the indicated conditions. Two independent PB-Esrrb transfections were carried out, generating two independent cell lines named "Rex1-GFPd2 PB-Esrrb A" and "Rex1GFPd2 PB-Esrrb B". GAPDH was used as endogenous control and data are normalized to PB-vector cells cultured in 2i media.
- E) Clonogenicity assay of Rex1-GFPd2 cells transfected with the indicated plasmids and selected for 2 passages. 600 cells were plated at clonal density in Serum media alone or with LIF and stained for alkaline phosphatase (AP) after 5 days. Bars show the number of wholly AP+ve, mixed or wholly differentiated (AP-ve) colonies. Mean and SD of 3 independent experiments is shown. See also Figure S3A.

- F) Rex1-GFPd2 cells in 2i were co-transfected with pBase helper plasmid and a piggyBac vector containing Esrrb-IRES-Neo (PB Esrrb IN); after 48h of culture in 2i, selection was applied and CH was withdrawn. Selection in low G418 (200 mg/ml) allowed isolation of transfectants expressing the Esrrb transgene at a level similar to endogenous expression in CH treated cells. Cells were cultured for >3 passages and analyzed as indicated.
- G) Gene expression analysis of Rex1GFP cells transfected with an empty vector (PB-vector) cultured in 2i, in PD for 2 passages or Rex1GFP Esrrb IN cells cultured in PD for 3 passages. Note that in Esrrb IN cells cultured in PD (orange bars) the expression of Esrrb is maintained at levels comparable to control cells cultured in 2i (blue bars). ActinB served as an internal control.
- H) Western blot of Rex1GFP cells transfected with an empty vector (PB-vector) cultured in 2i, and Rex1GFP Esrrb IN cells cultured in PD for 3 passages. For each sample 3 different amounts of total proteins (equivalent to 50,000 , 25,000 and 12,500 cells) were loaded. Note that Esrrb protein levels are comparable in the 2 samples. GAPDH served as a loading control.
- I) Rex1GFP cells transfected with an empty vector (PB-vector) cultured in 2i for 3 passages, and Rex1GFP Esrrb IN cells cultured in PD for 3 passages have been analyzed by flow cytometry. 79% of Esrrb IN cells were GFP positive.
- J) Rex1GFP cells transfected with an empty vector (PB-vector) cultured in 2i for 3 passages, and Rex1GFP Esrrb IN cells cultured in PD for 3 passages have been plated at clonal density in N2B27+PD and stained for alkaline phosphatase after 5 days. As expected, PB-vector cells could not form AP+ colonies (top panel), while Rex1GFP Esrrb IN cells did (bottom panel).
- K) Cre excisable construct used for Esrrb overexpression.
- L) Representative pictures of cells transfected with the Esrrb excisable construct before (Esrrb+) and after excision (Esrrb-). Note that only Esrrb+ cells can be

cultured in serum containing media without LIF. Successful excision was confirmed by PCR using transgene-specific primers (Figure S3H).

- M) *Esrrb* excised ES cells contribute to chimeric embryos. The DsRed signal is specifically detected in the injected embryos at mid-gestation and not in a sibling embryo with no chimeric contribution (control).

Figure 4. *Esrrb* is not required for self-renewal in the presence of LIF

- A) Rex1-GFPd2 cells were cultured in N2B27 plus LIF and PD03 for 2 passages and transfected with 2 independent siRNAs targeting *Esrrb*, or 2 negative control siRNAs (siControl and siGFP). Cells were harvested 48h after transfection and analyzed by flow cytometry (Rex1-GFP profile), quantitative PCR and clonal analysis.
- B) Rex1-GFPd2 cells were transfected with the indicated siRNAs and analyzed after 48h by flow cytometry. Bars show the number of GFP positive cells normalized to the negative control siRNA (siControl). Mean and SD of 3 independent experiments is shown.
- C) Gene expression analysis of Rex1-GFPd2 cells transfected with 2 independent siRNAs targeting *Esrrb* or 2 control siRNAs (siControl and siGFP). GAPDH was used as endogenous control and data are normalized to the control siRNA (siControl). Mean and SD of 3 independent experiments is shown.
- D) Quantification of clonogenicity assay of Rex1-GFPd2 cells transfected with the indicated siRNAs. 48h after transfection cells were replated at clonal density in 2i media and stained for alkaline phosphatase (AP) after 5 days. Bars show the number of AP+ve colonies normalized to the control siRNA (siControl). Mean and SD of 3 independent experiments is shown.
- E) Immunostaining of *Esrrb*^{-/-} and *Esrrb*^{fl/fl} cells confirming absence of *Esrrb* protein in the *Esrrb*^{-/-} cells.
- F) Gene expression analysis of *Esrrb*^{-/-} and *Esrrb*^{fl/fl} cells cultured in LIF+Serum. Note the absence of *Esrrb* transcript. GAPDH was used as endogenous control and data are normalized to *Esrrb*^{fl/fl} cells.
- G) Gene expression analysis of *Nanog*^{-/-} and *Nanog*^{+/-} cells cultured in LIF+Serum. Note the absence of *Nanog* transcript. GAPDH was used as endogenous control and data are normalized to *Nanog*^{+/-} cells.

- H) Phase contrast images of *Esrrb*^{fl/fl} and *Esrrb*^{-/-} cells under the indicated culture conditions.
- I) and J) Colony forming assay on *Esrrb*^{fl/fl} and *Esrrb*^{-/-} (I), and *Nanog*^{-/-} and *Nanog*^{+/-} (J) cells. Cells were cultured in 2i+LIF, plated at clonal density (300 cells per well) under the indicated conditions and stained for alkaline phosphatase after 5 days. Bars show the number of AP+ve colonies. Mean and SD of 3 independent experiments is shown.
- K) and L) *Esrrb*^{fl/fl} and *Esrrb*^{-/-} ES cells were cultured for 48h in LIF+PD (K) or CH (L) and analyzed by qPCR for the indicated pluripotency markers. GAPDH was used as endogenous control and data are normalized to *Esrrb*^{fl/fl}. Mean and SD of 2 independent experiments is shown.
- M) Gene tracks representing binding of Nanog, Oct3/4, Sox2, Tcf3 and *Esrrb* at the *Tbx3* gene locus. The X axis represents the linear sequence of genomic DNA and the Y axis represents the total number of mapped reads. A blue box highlights a region where the 5 factors co-localize while the green box highlights a region bound only by *Esrrb*.
- N) Venn diagram showing the intersection between the genomic regions bound by at least one factor among Oct3/4, Sox2 and Tcf3 (O/S/T in blue), by Nanog (in red) and by *Esrrb* (in green). For this diagram only the top 5000 ChIP-Seq peaks of each factor have been used, in order to account for differences in ChIP efficiency among different factors. See also Figure S4C.
- O) Venn diagram showing the intersection between the predicted target genes bound by at least one factor among Oct3/4, Sox2 and Tcf3 (O/S/T in blue), and the predicted target genes of Nanog (in red) or *Esrrb* (in green). Only the top 5000 peaks for each factor have been used to predict target genes in order to account for differences in ChIP efficiency. See also Figure S4D and Table S5.

Figure 5. *Esrrb* null cells are pluripotent

- A) *Esrrb*^{-/-} ES cells cultured in LIF+serum were injected into the kidney capsule and produced teratocarcinomas containing tissues representative of the three germ layers (epidermis for ectoderm, kidney and striated muscle for mesoderm and gut-like epithelia for endoderm) along with undifferentiated embryonal carcinoma (EC) cells.
- B) *Esrrb*^{-/-} ES cells contribute to chimeric embryos: summary of the experiments performed with GFP-labelled *Esrrb*^{-/-} ES cells. See also Figure S5A and B.
- C) GFP-labelled *Esrrb*^{-/-} ES cells cultured in either 2i+LIF or LIF/PD, were combined with 8-cell stage embryos, cultured in vitro for 48h, and scored for the presence of GFP-positive cells in the ICM. A representative image is shown.
- D) Blastocyst injection was performed followed by embryo transfer and embryos were scored at mid-gestation (E12.5) for the presence of GFP-positive cells. *Esrrb* fl/fl served as a positive control. See also Figure S5A and B.

Figure 6. Mechanism of *Esrrb* regulation by Tcf3

- A) Fluorescence micrographs showing immunostaining for *Esrrb* of wild-type ES cells cultured under the indicated conditions.
- B) Histogram showing the distribution of *Esrrb* immunostaining intensity under the indicated conditions. >3000 single ES cells for each condition were analyzed and divided into three categories based on staining intensity (see Methods section).
- C) Top: Gene tracks represent binding of Tcf3 at the *Esrrb* gene locus. The 5 red boxes indicate the regions analysed by ChIP for Tcf3. Bottom: ChIP for Tcf3 followed by qPCR for the indicated regions performed in either wild-type or Tcf3 null cells. Enrichment over a mock ChIP is shown. Mean and SD of 3 independent experiments is shown.
- D) *Esrrb* expression analysis of the indicated ES cell lines, cultured in LIF/PD and treated with the Gsk3 inhibitor (CH) for 24h (orange bars). The fold change expression relative to LIF/PD is shown; ActinB served as an internal control. Mean and SD of 2 independent experiments is shown.
- E) Gene expression analysis of Nanog +/- and -/- cells, cultured in LIF/PD and treated with the Gsk3 inhibitor (CH) for 8h or 24h (orange bars). GAPDH served as an internal control. Mean and SD of 2 independent experiments is shown.
- F) Schematic representation of core pluripotency transcription factor circuit with parallel input from LIF/Stat3 and GSK3 inhibition/Tcf3 de-repression.

Figure 1 - Identification of TCF3 direct targets in mouse ES cells

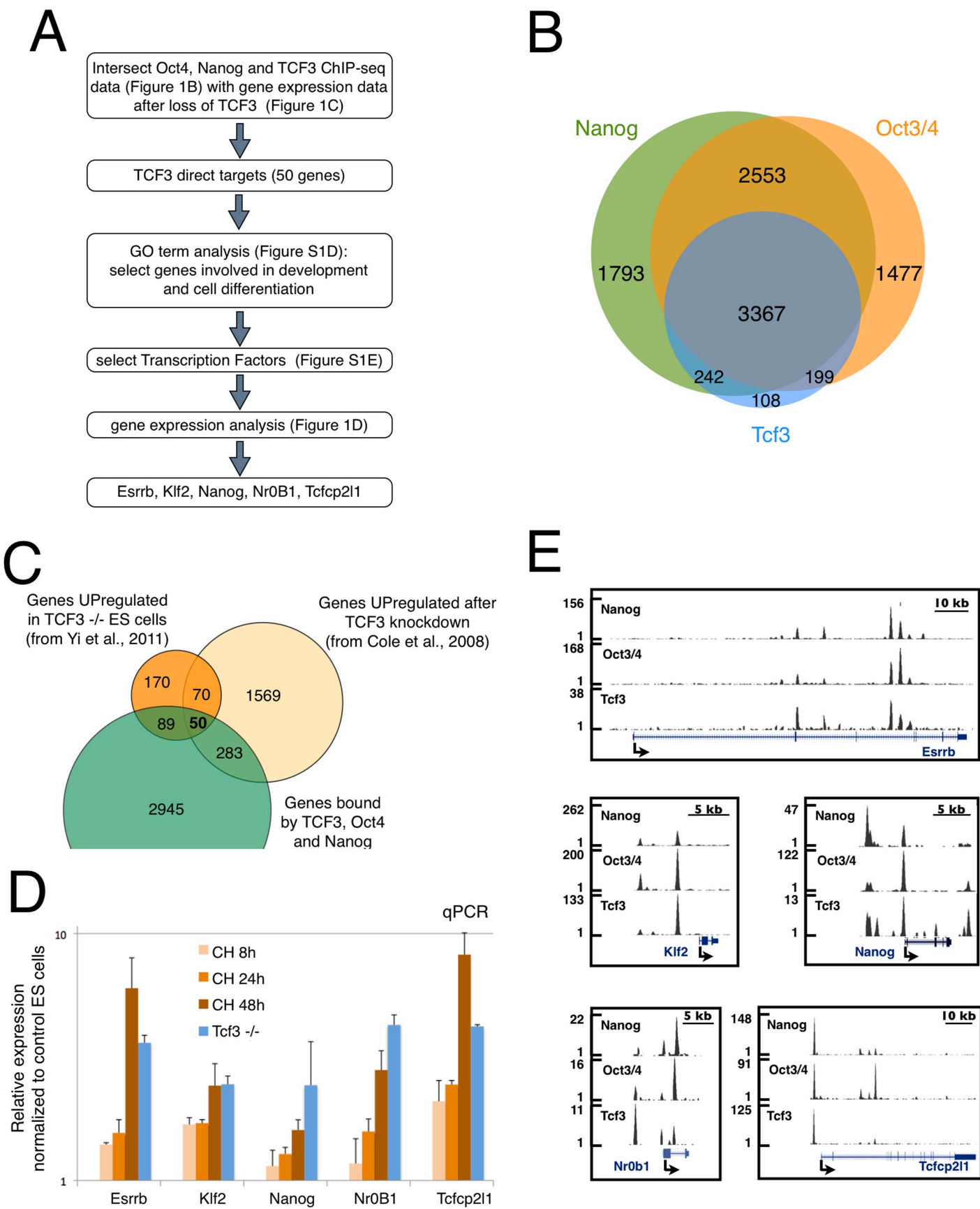


Figure 2 - Esrrb is required to mediate self-renewal downstream of the GSK3/Tcf3 axis

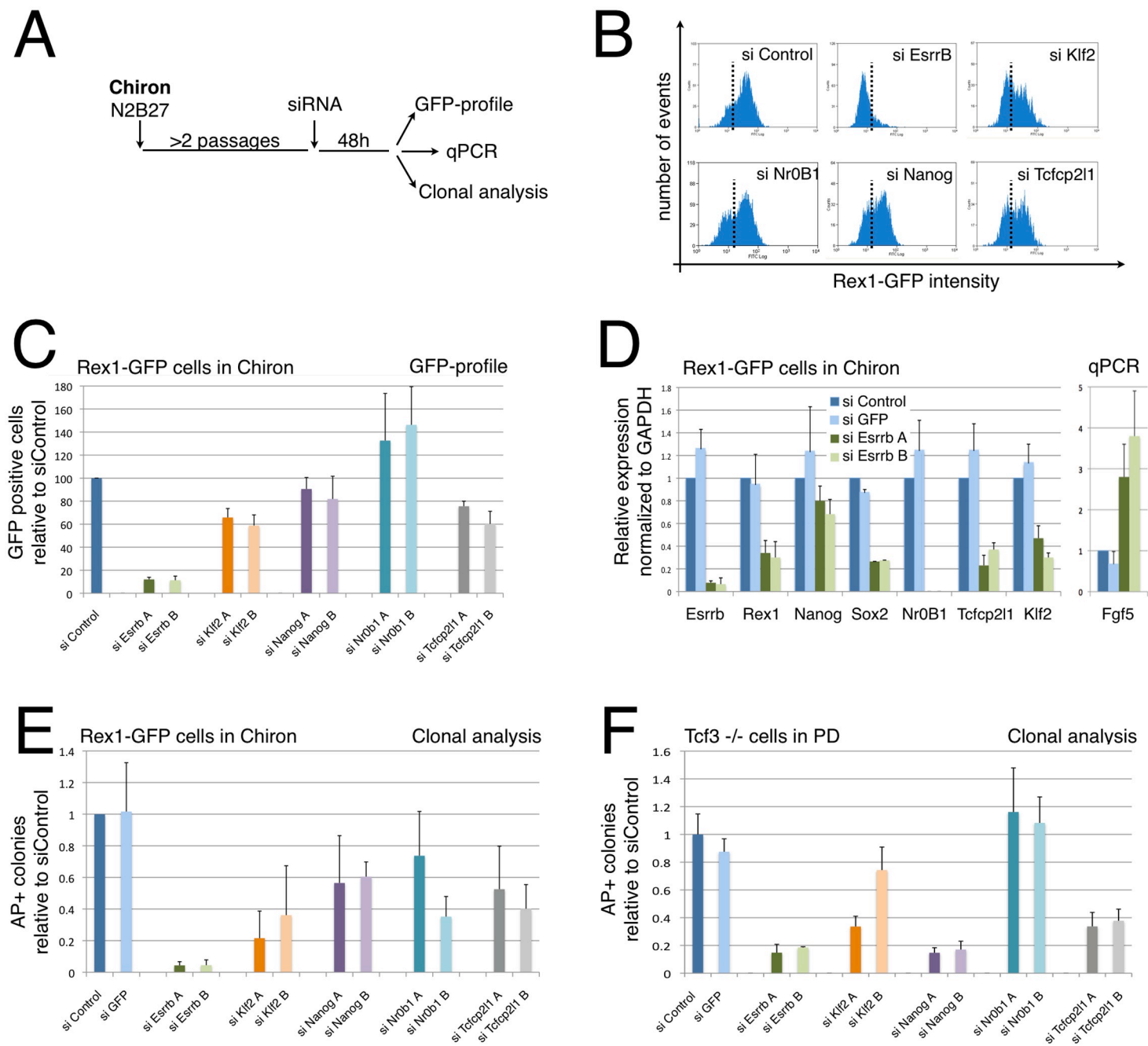


Figure 3 - Esrrb promotes self-renewal similarly to GSK3 inhibition

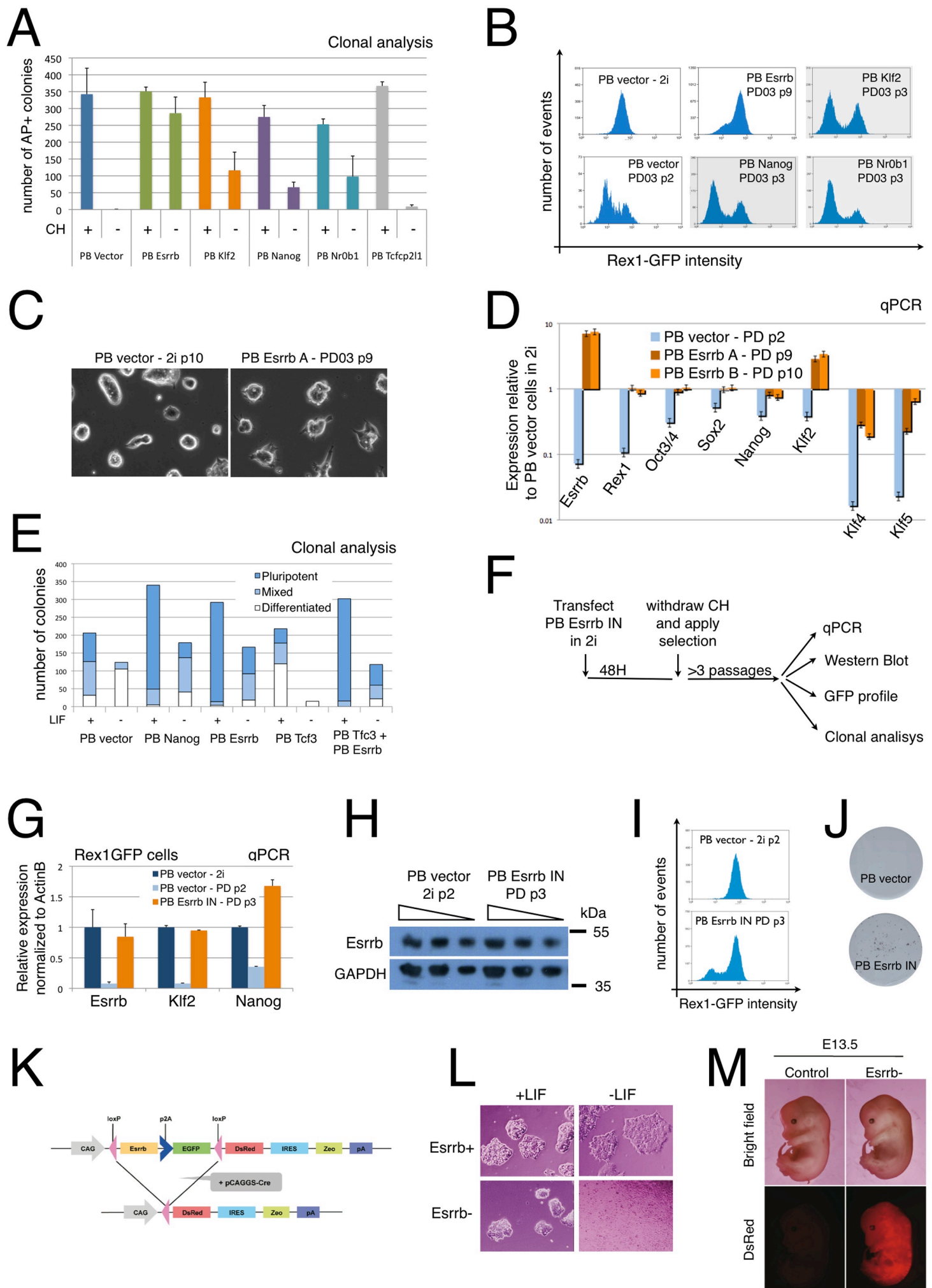


Figure 4 - *Esrrb* is not required for self-renewal in the presence of LIF

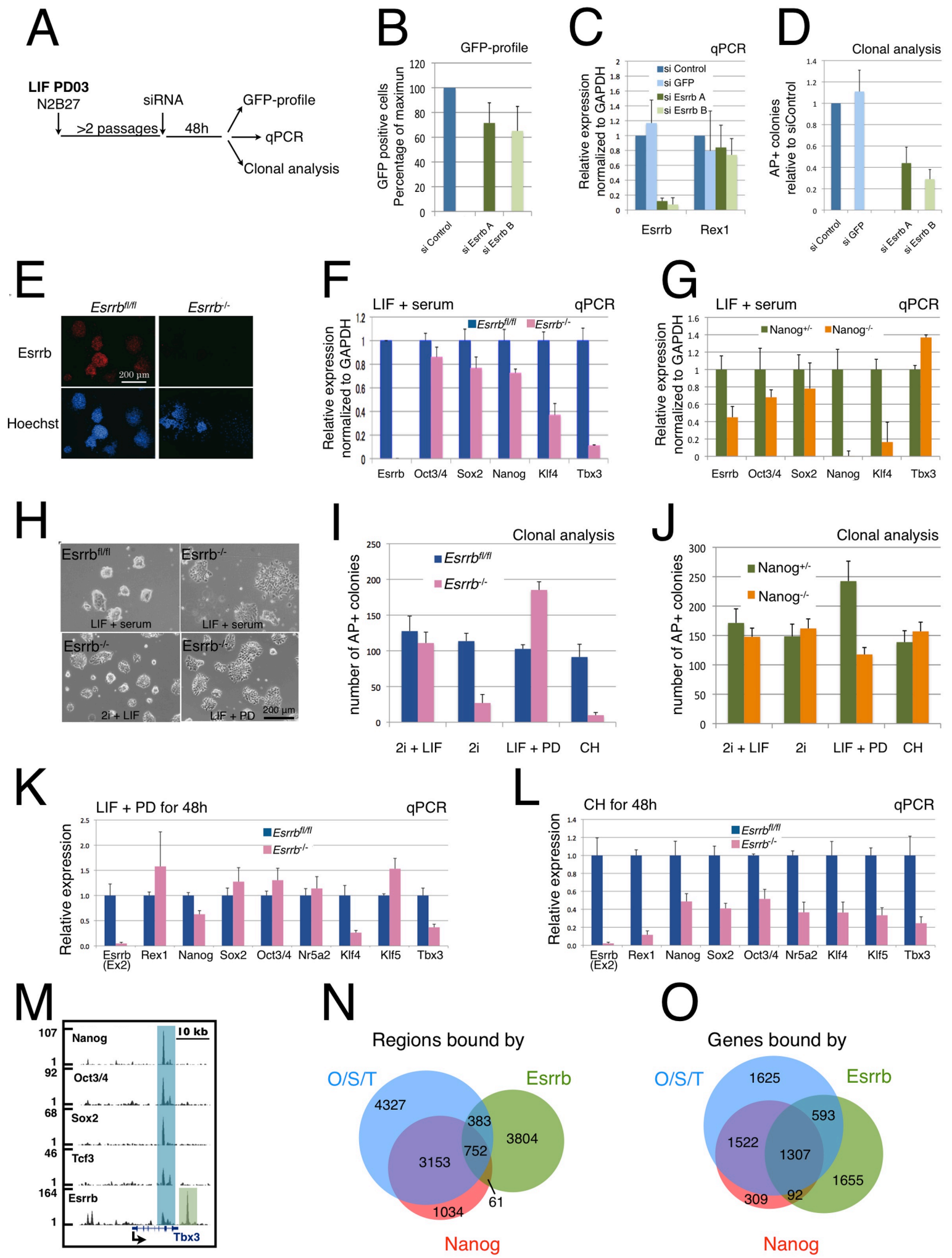
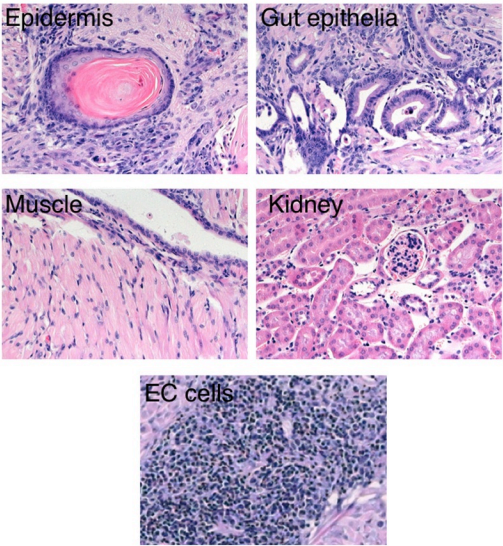


Figure 5 - Esrrb null cells are pluripotent

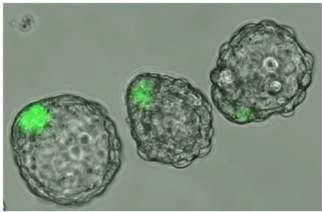
A



B

Experiment	Culture conditions	GFP+ embryos
Morula aggregation	2i + LIF	12/13
	LIF/PD	14/16
Blastocyst injection	2i + LIF	8/26
	LIF/PD	3/9

C



D

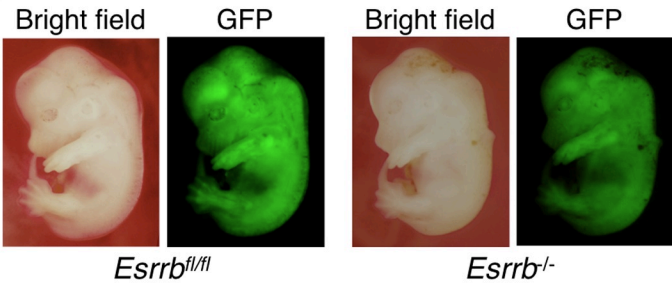
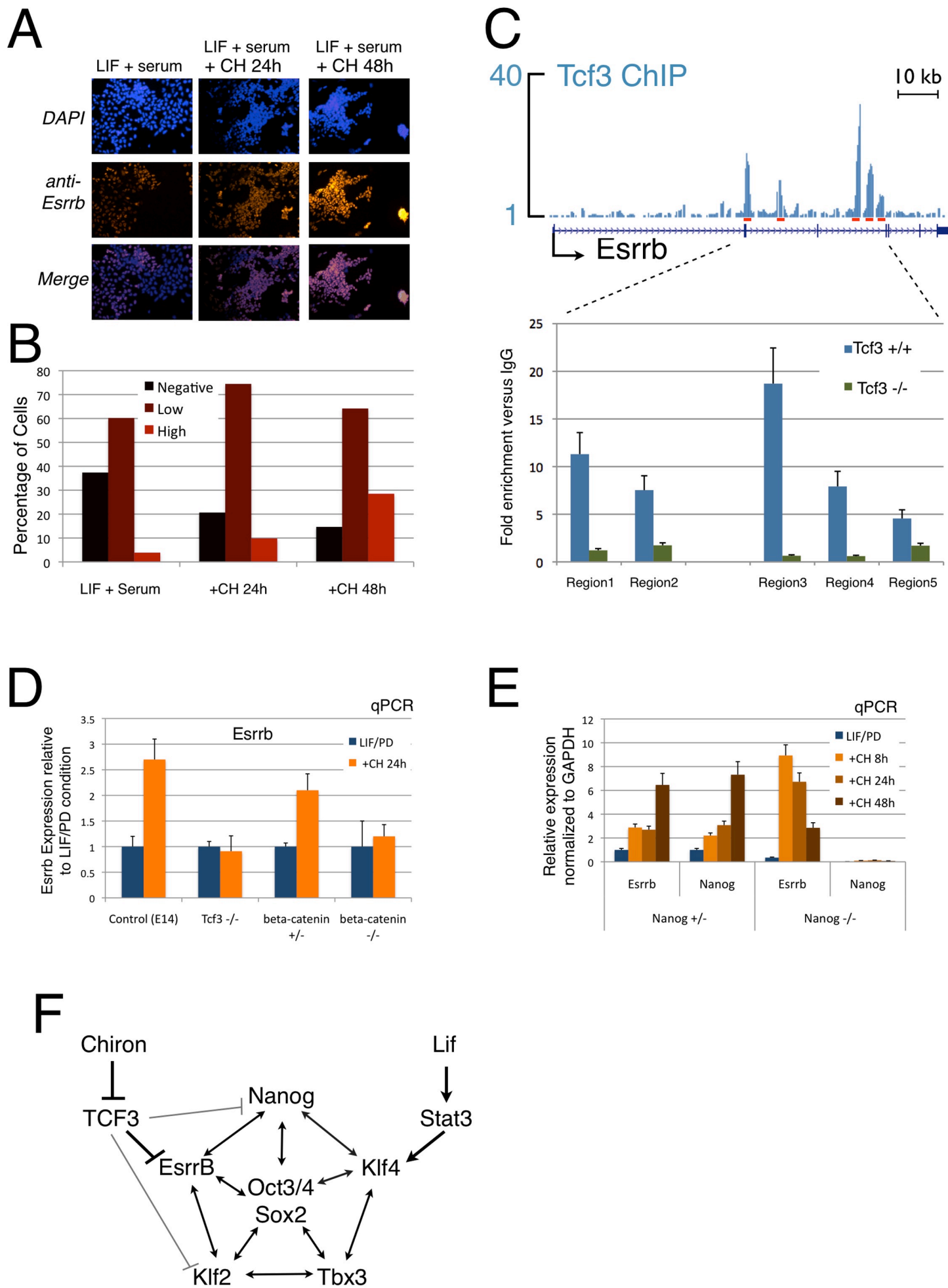


Figure 6 - Mechanism of Esrrb regulation by Tcf3



INVENTORY OF SUPPLEMENTAL INFORMATION

5 Supplemental Figures

Supplemental Figure 1, related to Figure 1 in paper

Supplemental Figure 2, related to Figure 2 in paper

Supplemental Figure 3, related to Figure 3 in paper

Supplemental Figure 4, related to Figure 4 in paper

Supplemental Figure 5, related to Figure 5 in paper

Supplemental Figure 6, related to Figure 6 in paper

4 Supplemental Tables

Supplemental Table 1, ChIP-seq studies included in the Compendium.

Supplemental Table 2, qPCR primers sequences used for qPCR

Supplemental Table 3, siRNAs used in this study

Supplemental Table 4, qPCR primers sequences used for Tcf3 ChIP.

Supplemental Table 5, list of genes analyzed in Figure 1, 4, S1 and S4

Supplemental Experimental Procedures

SUPPLEMENTAL INFORMATION

ESRRB IS THE PIVOTAL TARGET OF THE GSK3 /TCF3 AXIS REGULATING EMBRYONIC STEM CELL SELF-RENEWAL

Graziano Martello, Toshimi Sugimoto, Evangelia Diamanti, Anagha Joshi, Rebecca Hannah, Satoshi Ohtsuka, Berthold Gottgens, Hitoshi Niwa and Austin Smith

5 Supplemental Figures

Supplemental Figure 1, related to Figure 1 in paper

Supplemental Figure 2, related to Figure 2 in paper

Supplemental Figure 3, related to Figure 3 in paper

Supplemental Figure 4, related to Figure 4 in paper

Supplemental Figure 5, related to Figure 5 in paper

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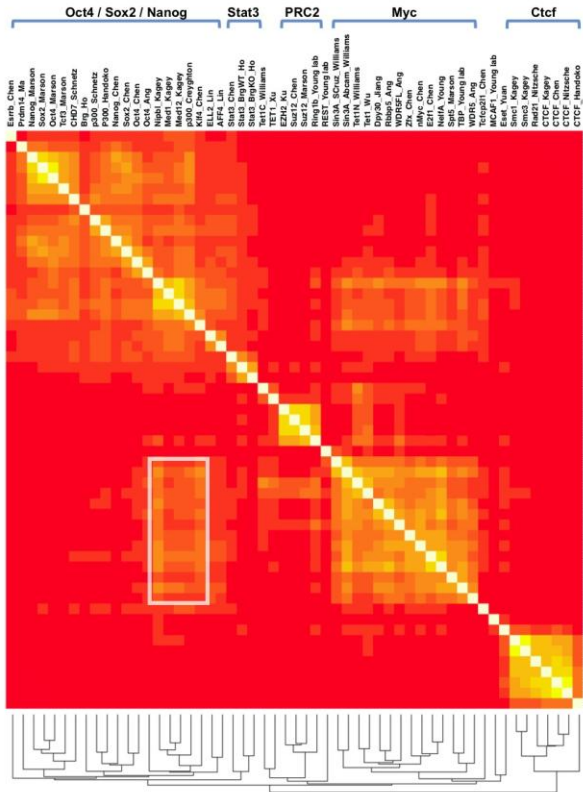
Supplemental Table 4, qPCR primers sequences used for Tcf3 ChIP.

Supplemental Table 5, list of genes analyzed in Figure 1, 4, S1 and S4

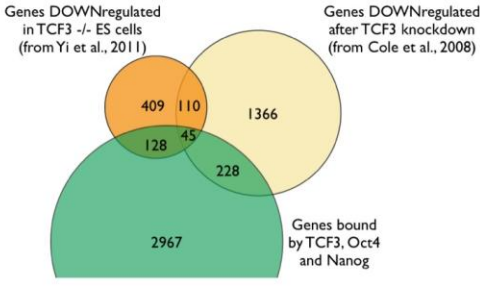
Supplemental Experimental Procedures

Figure S1 - Related to Figure 1

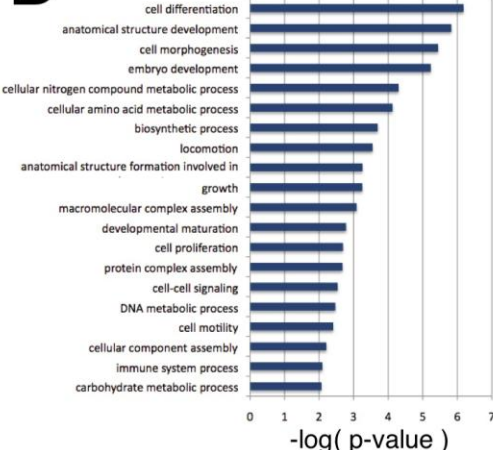
A



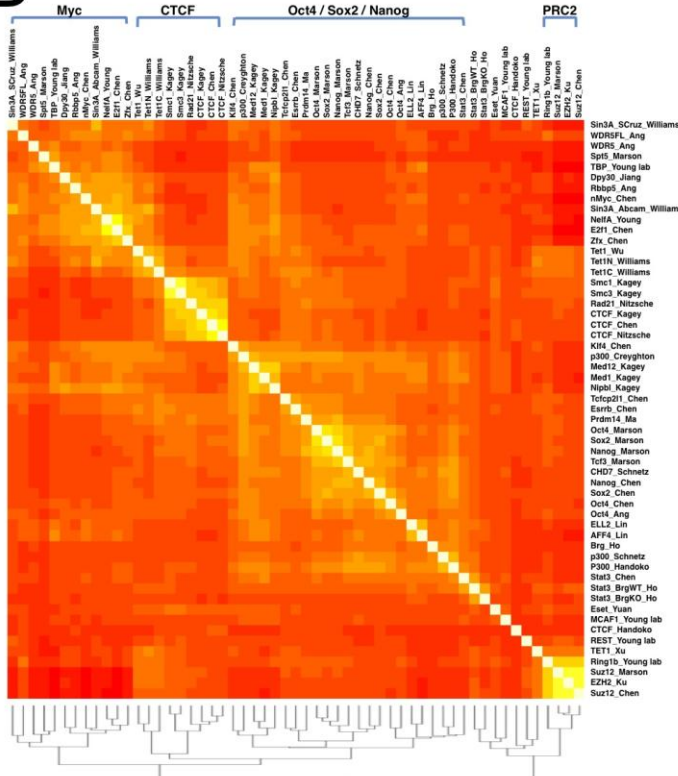
C



D



B



E

Gene (TF in bold)	cell differentiation	anat. struct. development	cell morphogenesis
COBL		X	
ESRRB	X	X	X
ETV4	X	X	X
FUT9		X	
GLI2	X	X	X
GRSF1		X	
KLF15	X	X	
KLF2	X	X	X
LIFR	X	X	X
MAPT	X	X	X
MCF2	X	X	
MORC1	X		
NANOG	X	X	
NR0B1	X	X	
TCF3	X	X	X
TUBB2B	X	X	
VEGFC	X	X	

F

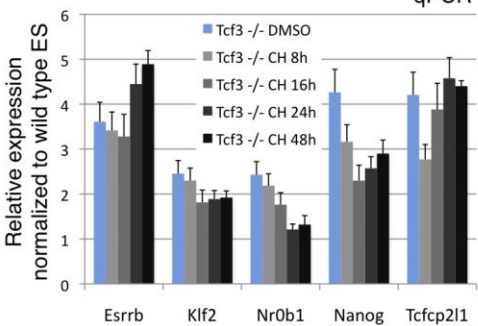


Figure S1 – related to Figure 1

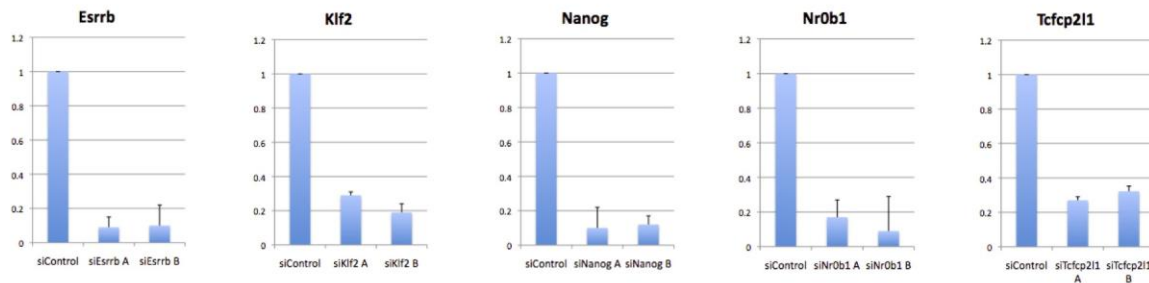
- A) Hierarchical clustering of 55 genome-wide binding maps. For analysis of combinatorial binding patterns, a unified data matrix was generated based on the 165,607 unique peak regions, indicating for each factor whether it was bound or not to each of the 165,607 unique regions. To investigate global relationships, hierarchical clustering and Pearson's correlation coefficients were used to display all pairwise comparisons in a clustered heatmap. Colours in the heatmap show the level of correlation for all pairwise comparisons (white is correlation=1; red is correlation=0). Factors have been clustered along both axes according to the level of correlation. The five major clusters identified are indicated at top of the figure by blue brackets. The first cluster contains “classical” pluripotency genes such as Oct4 and Nanog, together with more general transcriptional regulators such as p300. The second cluster comprises three STAT3 studies, while the third cluster contains polycomb repressor complex 2 (Prc2) components. The fourth is centred around N-Myc and contains NelfA and Spt5, which like Myc are involved in the control of transcriptional pause release (Rahl et al., 2010), plus a subcluster of Trithorax components (Dpy30, Rbpb5 and Wdr5). The final cluster contains CTCF and other factors thought to be involved in chromosome domain structure. Binding maps can show correlation with more than one major cluster, for example the Nipl/Med1/Med12/p300 cluster shows similarities to both the Oct4/Sox2/Nanog cluster and the Myc cluster (see white box).
- B) Hierarchical clustering of 55 factors based on predicted target genes. For each factor a list of candidate target genes has been compiled and used to calculate the correlation for all pairwise comparisons (see Methods section for details). Heatmap analysis generated the same 5 major clusters (compare Figures S1A and S1B). For most transcription factors therefore, the distinct clustering observed in Figure S1A is not attributable to binding different regions within the same gene loci, but instead reflects different gene targets. However, a small number of factors such as Esrrb that did not belong to any major cluster in figure 1A, were placed into the Oct4/Sox2/Nanog cluster in the target-gene based heatmap (Figure S1B). This suggests that such factors control target genes overlapping with the core pluripotency targets, but often do so through distinct binding sites. Data are represented as in Figure S1A.
- C) Venn diagram showing overlap between genes down-regulated in Tcf3 null cells, genes downregulated in Tcf3 knockdown and genes bound by Tcf3, Oct3/4 and Nanog.
- D) Gene ontology (GO) term analysis of up-regulated candidate Tcf3 direct targets revealed a statistically significant enrichment for genes involved in embryonic development and cell differentiation. GO categories are ranked according to the p-value. Similar results

were obtained using *the 3367 genes bound by Nanog, Oct4 and Tcf3 as the background, thereby explicitly asking whether any GO categories are specifically enriched in the 50 genes when considered as a subset of the 3367 genes (data not shown).*

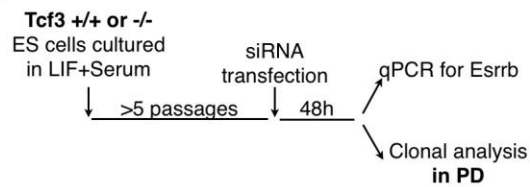
- E) Table containing the Tcf3 targets found in at least 1 of the 3 most represented GO categories. The genes in bold encode transcription factors.
- F) Gene expression analysis of *Tcf3* null ES cells, cultured in LIF+serum, treated either with the Gsk3 inhibitor (CH) for the indicated time (gray bars) or with DMSO (vehicle, blue bars). The fold change expression relative to wt ES cells treated with vehicle (DMSO) is shown. Mean and SD of 2 independent experiments is shown.

Figure S2 - related to Figure 2

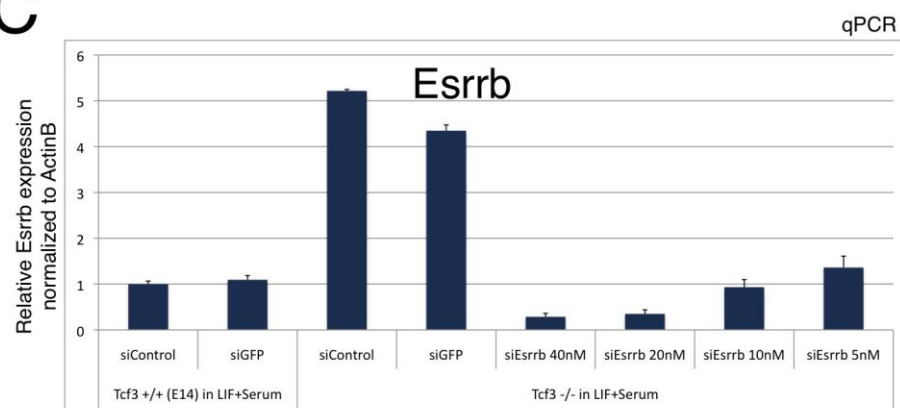
A



B



C



D

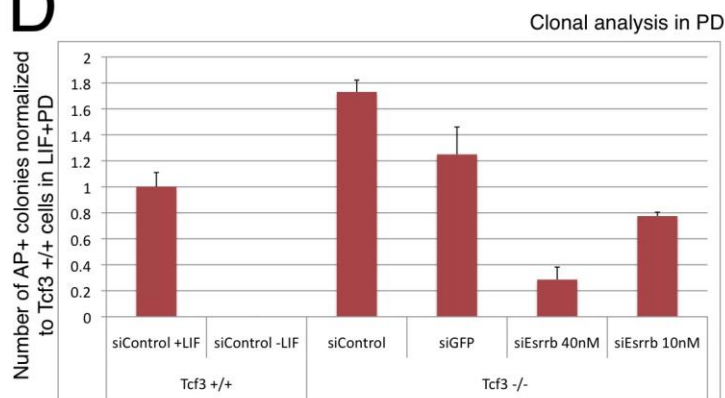


Figure S2 – related to Figure 2

- A) Validation of the siRNAs used in this study. ES cells were transfected with the indicated siRNAs and analyzed after 24h by qPCR for the 5 indicated genes. For each genes 4 independent siRNAs have been tested and the 2 showing the best knockdown efficiency have been used. Data in the figure are average and standard deviation of 2 independent experiments.
- B) *Tcf3* ^{-/-} cells, or *Tcf3* ^{+/+} (E14) , were cultured in LIF+serum media and transfected with the indicated siRNAs. After 48h cells were analyzed by qPCR and replated at clonal density in N2B27 media in presence of PD.
- C) Gene expression analysis of *Tcf3* ^{-/-} and ^{+/+} cells transfected with 2 control siRNAs (siControl and siGFP) or with different concentrations of siEsrrb. As expected, Esrrb expression is elevated in *Tcf3* ^{-/-} cells, compared to *Tcf3* ^{+/+}; trasfection of siEsrrb at 10 nM concentration reduced Esrrb espression to levels found in control cells (*Tcf3*^{+/+}) transfected with negative control siRNAs. ActinB was used as endogenous control and data are normalized to *Tcf3* ^{+/+} cells trasfected with the control siRNA (siControl). Mean and SD of 2 independent experiments is shown.
- D) Clonogenicity assay of *Tcf3* ^{-/-} and ^{+/+} cells transfected with the indicated siRNAs. Cells cultured in LIF+serum were transfected with the indicated siRNAs at the indicated concentration; after 48h they were replated at clonal density in PD, and stained for alkaline phosphatase after 5 days. Bars show the number of AP+ve colonies normalized to *Tcf3* ^{+/+} cells trasfected with the negative control siRNA (siControl) plated in LIF+PD, that also served as a positive control. Note that siEsrrb at 40nM concentration abolished colony formation (see also Figure 2F), while at the 10nM concentration colony formation was restored to levels comparable to *Tcf3*^{+/+} cells in LIF+PD. Mean and SD of 2 independent experiments is shown.

Figure S3 - related to Figure 3

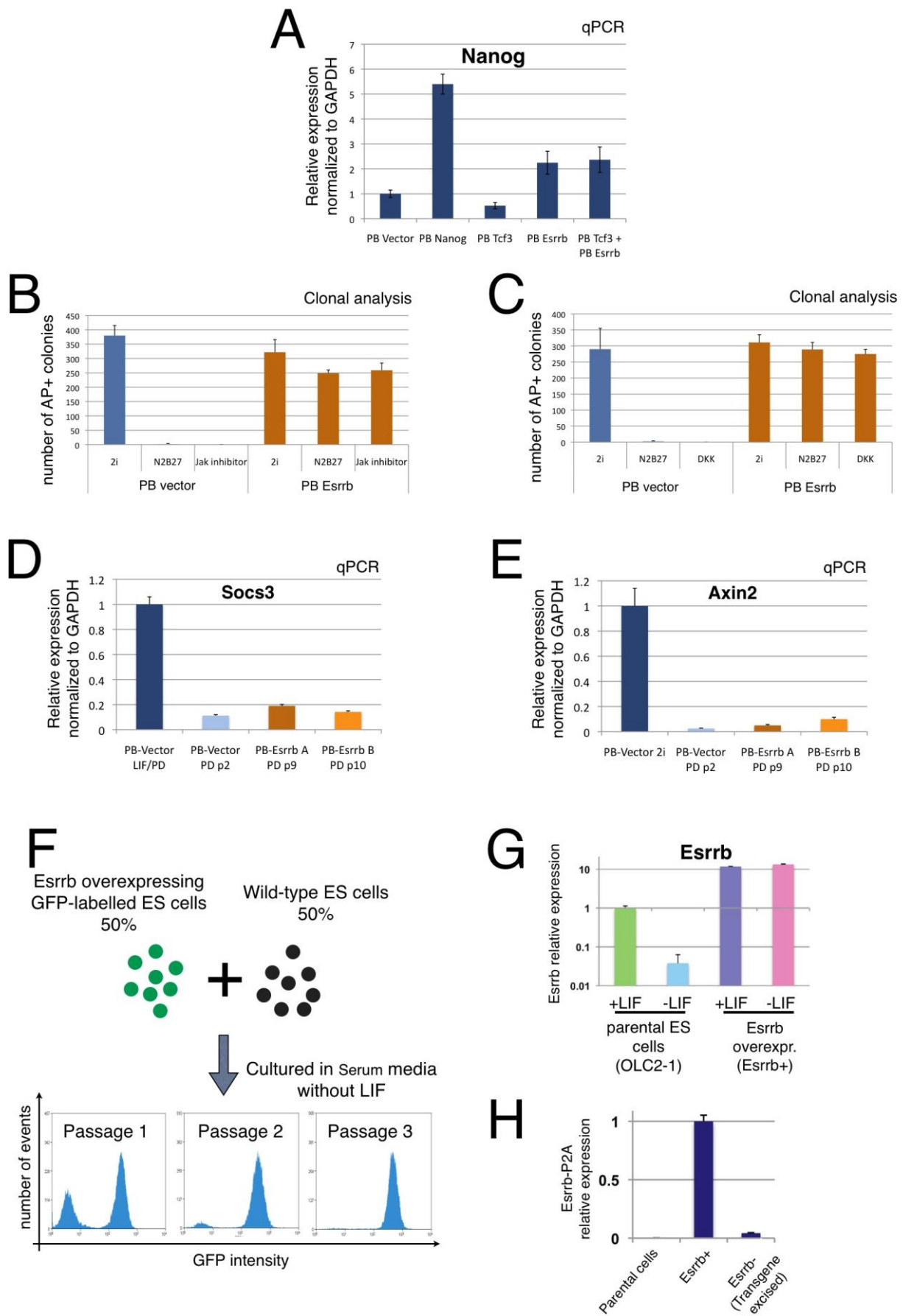
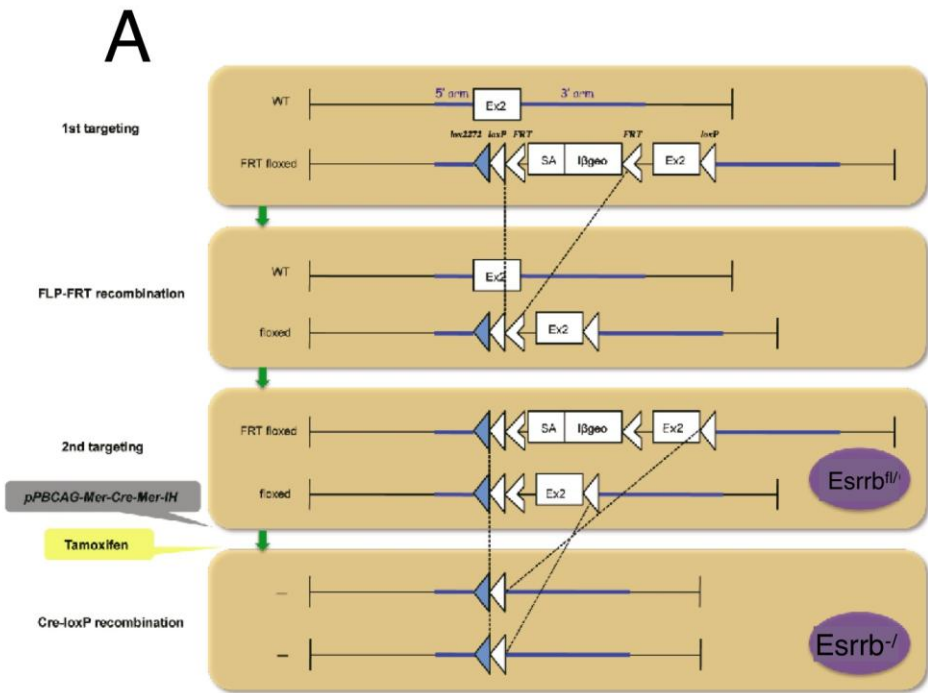


Figure S3 – related to Figure 3

- A) Expression levels of Nanog measured by qPCR in the indicated cell lines. Expression is normalized to empty vector transfected ES cells cultured in LIF and serum. Gapdh was used as internal control. Mean and SD of 2 biological replicates are shown.
- B) Representative pictures (bottom) and quantification (top) of clonogenicity assay on Rex1-GFPd2 cells transfected with either PB-vector or PB-Esrrb and cultured in the indicated conditions. 600 cells were plated at clonal density in 2i, in the basal media N2B27 or in N2B27 + Jak inhibitor (0.6µM), and stained for alkaline phosphatase (AP) after 5 days. Bars show the number of AP+ve colonies. Mean and standard deviation of 3 replicates of 2 independent experiments are shown.
- C) Representative pictures (bottom) and quantification (top) of clonogenicity assay on Rex1-GFPd2 cells transfected with either PB-vector or PB-Esrrb and cultured in the indicated conditions. 600 cells were plated at clonal density in 2i, in the basal media N2B27, or in N2B27 plus the Wnt inhibitor DKK (200ng/ml), and stained for alkaline phosphatase (AP) after 5 days. Bars show the number of AP+ve colonies. Mean and SD of 3 replicates from 2 independent experiments are shown.
- D) Expression levels of the LIF target Socs3. Socs3 levels were measured by qPCR and normalized to parental cells cultured in LIF/PD. Gapdh was used as internal control. Mean and SD of 2 experiments are shown.
- E) Expression levels of the Wnt target Axin2. Axin2 levels were measured by qPCR and normalized to parental cells cultured in 2i. Gapdh was used as internal control. Mean and SD of 2 experiments are shown.
- F) Esrrb sustains self-renewal in a cell autonomous manner. Wild-type ES cells were mixed with GFP-labelled Esrrb overexpressing cells in a 1:1 ratio. The mixed population of cells were cultured in the absence of LIF and the GFP intensity was measured by flow cytometry over 3 passages. Note that the wild-type, GFP negative, cells were rapidly lost indicative of differentiation, confirming that Esrrb overexpressing cells maintain self-renewal in a cell-autonomous manner. Similar results were obtained by culturing the mixed population in N2B27 media in presence of PD (data not shown).
- G) Gene expression analysis of parental and Esrrb overexpressing (Esrrb+) cells cultured in the indicated conditions. Total Esrrb expression levels were measured by qPCR and normalized to parental cells cultured in presence of LIF. Gapdh was used as internal control.
- H) Gene expression analysis of parental (OLC2.1), Esrrb overexpressing cells before (Esrrb+) and after (Esrrb-) excision of the transgene. The levels of exogenous Esrrb expression were measured using primers specifically recognising the Esrrb-P2A region

of the transgene. Levels are normalized to *Esrrb*⁺ cells and *Gapdh* was used as internal control.

Figure S4 - related to Figure 4



B

Esrrb cKO vector

Ex2	Left Primer (118846-)	ATctcgagagatctAATTCAAAGTTTCTAGAACTCTG	XhoI, BglII
	Right Primer (-119562)	ACgctagcGGAGACTTCTAGAGCACCTCTGCC	NheI
5' arm	Left Primer (116863-)	ATgcggccgcGGTGGTTAGACAAGGTAGCCATAC	NotI
	Right Primer (-118845)	CTaccgggTCTgtcgcacCGTAAACTTCAAATCAGAGACCCCT	AgeI, SalI
3' arm	Left Primer (119563-)	CTgtcgacccatggTGATCTGTCTCTGGGATCCTAG	SalI, NcoI
	Right Primer (-125809)	GTatcgatCTTGAAACACCTGAAGTTTCCTCT	ClaI

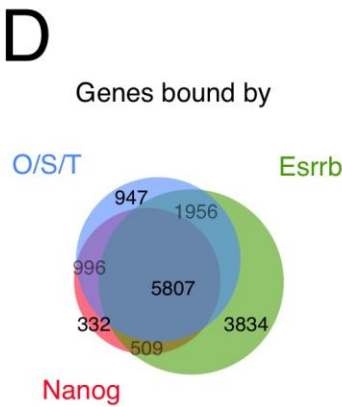
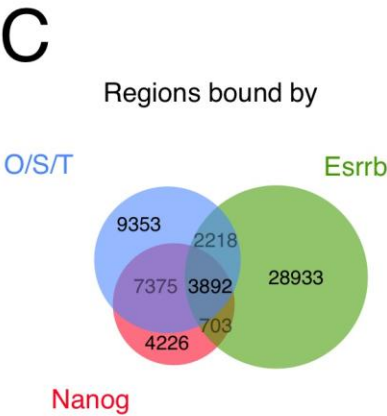


Figure S4 – related to Figure 4

- A) Schematic of strategy used to generate the *Esrrb*^{fl/fl} and *Esrrb*^{-/-} cells.
- B) Primers used for genotyping (see also Methods).
- C) Venn diagram showing the intersection between the genomic regions bound by at least one factor among Oct3/4, Sox2 and Tcf3 (O/S/T in blue), by Nanog (in red) and by Esrrb (in green). In contrast to Figure 4N, all the ChIP-seq peaks have been used for the analysis.
- D) Venn diagram showing the intersection between the predicted target genes bound by at least one factor among Oct3/4, Sox2 and Tcf3 (O/S/T in blue), and the predicted target genes of Nanog (in red) or Esrrb (in green). In contrast to Figure 4O, all the ChIP-seq peaks have been used for the analysis.

Figure S5 - related to Figure 5

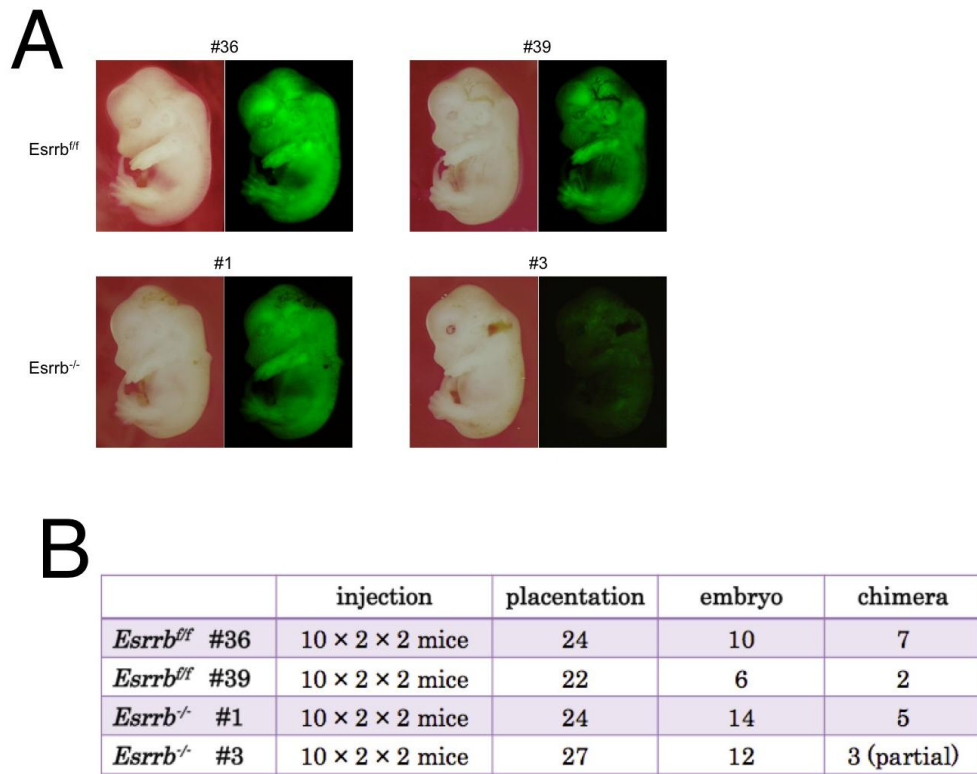


Figure S5 – related to Figure 5

- A) Representative pictures of chimeric embryos generated after blastocyst injection of 2 independent *Esrrb* ^{-/-} lines and 2 control fl/fl lines.
- B) Statistics of blastocyst injection experiments performed. All ES cells were cultured in 2i+LIF before blastocyst injection and embryos were analyzed at E12.5 .

Figure S6 - related to Figure 6

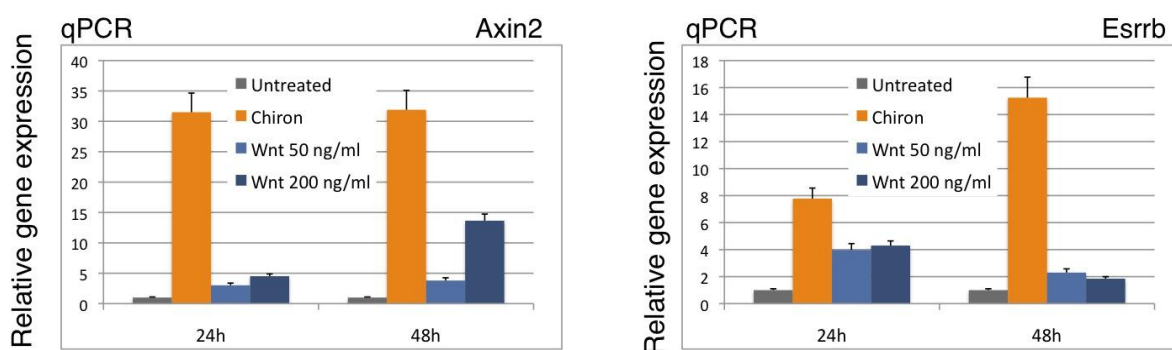


Figure S6 – related to Figure 6

Expression levels of the Wnt target *Axin2* (left) and *Esrrb* (right) after treatment with recombinant Wnt protein or the Gsk3 inhibitor CH for the indicated time. Gene expression levels were measured by qPCR and normalized to cells cultured in LIF/PD. *Gapdh* was used as internal control. Mean and SD of 2 experiments are shown.

SUPPLEMENTAL TABLE S1 – ChIP-seq studies included in the Compendium

Factor	Antibody	Peaks	Gene Expression Omnibus accession	Publication first author, year (PMID)	Peak source
Aff4	Custom made (see Lin et al. 2010, Mol. Cell)	2659	GSM749810	Lin, 2011 (21764852)	MACS (1e-9)
Brg	Custom made (Clone J1, Khavari et al., 1993)	642	GSM359413	Ho, 2009 (19279218)	MACS (1e-15)
CHD7	Abcam (AB31824)	3428	GSM558674	Schnetz, 2010 (20657823)	Consensus (1)
CTCF	Upstate (07-729)	27843	GSM288351	Chen, 2008 (18555785)	MACS (1e-9)
CTCF	Upstate (07-729)	25121	GSM699165	Handoko, 2011 (21685913)	Consensus (3)
CTCF	Upstate (07-729)	19784	GSM560352	Kagey, 2010 (20720539)	MACS (1e-9)
CTCF (GFP tag)	Custom made (anti-GFP)	33788	GSM634247	Nitzsche, 2011 (21589869)	Originals
Dpy-30	Bethyl Labs (unreleased)	6308	GSM651192	Jiang, 2011 (21335234)	MACS (1e-12)
E2f1	Upstate (05-379)	20557	GSM288349	Chen, 2008 (18555785)	MACS (1e-9)
Ell2	Custom made (see Lin et al. 2010, Mol. Cell)	1949	GSM749809	Lin, 2011 (21764852)	MACS (1e-9)
Eset	Custom made	2577	GSM440256	Yuan, 2009 (19884257)	MACS (1e-9)
Esrrb	Custom made	35808	GSM288355	Chen, 2008 (18555785)	MACS (1e-15)
Ezh2	Active Motif (39103)	4380	GSM327668	Ku, 2008 (18974828)	MACS (1e-9)
Klf4	Custom made	11150	GSM288354	Chen, 2008 (18555785)	MACS (1e-9)
MCAF1	Bethyl Labs (A300-169A)	974	GSM656526	Young lab, 2011 (unpublished)	MACS (1e-9)
Med1	Bethyl Labs (A300-793A)	5447	GSM560347 & GSM560348	Kagey, 2010 (20720539)	MACS (1e-9)*
Med12	Bethyl Labs (A300-774A)	8318	GSM560345	Kagey, 2010 (20720539)	MACS (1e-9)
n-Myc	Custom made	6759	GSM288357	Chen, 2008 (18555785)	MACS (1e-9)
Nanog	Cosmo Bio (REC- RCAB0002PF)	4957	GSM288345	Chen, 2008 (18555785)	MACS (1e-9)
Nanog	Bethyl Labs (A300-397A)	16198	GSM307140 & GSM307141	Marson, 2008 (18692474)	MACS (1e-9)

NelfA	Santa Cruz (sc-23599)	14595	GSM515664	Rahl, 2010 (20434984)	MACS (1e-9)
Nipbl	Bethyl Labs (A301-779A)	11070	GSM560350	Kagey, 2010 (20720539)	MACS (1e-9)
Oct4	Santa Cruz (sc-8628)	2330	GSM566277	Ang, 2011 (21477851)	MACS (1e-12)
Oct4	Santa Cruz (sc-8628)	2768	GSM288346	Chen, 2008 (18555785)	MACS (1e-9)
Oct4	Santa Cruz (sc-8628)	15701	GSM307137	Marson, 2008 (18692474)	MACS (1e-9)
p300	Santa Cruz (sc-585)	14355	GSM594600	Creyghton, 2010 (21106759)	MACS (1e-12)
p300	Santa Cruz (sc-585)	628	GSM558675	Schnetz, 2010 (20657823)	Consensus (1)
p300	Santa Cruz (sc-585)	2575	GSM699164	Handoko, 2011 (21685913)	Consensus (2)
Prdm14 (FLAG and HA tag)	Sigma (A2220 & A2095)	26559	GSM623989	Ma, 2011 (21183938)	MACS (1e-15)
Rad21 (GFP tag)	Custom made (anti-GFP)	15311	GSM591469	Nitzsche, 2011 (21589869)	Originals
Rbbp5	Custom made (Allis Lab)	5326	GSM566278	Ang, 2011 (21477851)	MACS (1e-12)
REST	Upstate (07-579)	1547	GSM656525	Young lab, 2011 (unpublished)	MACS (1e-9)
Ring1b	Custom made	5167	GSM656523	Young, 2011 (unpublished)	MACS (1e-9)
Sin3A	Abcam (AB3479)	8047	GSM611196	Williams, 2011 (21490601)	MACS (1e-15)
Sin3A	Santa Cruz (sc-994X)	3423	GSM611197	Williams, 2011 (21490601)	MACS (1e-15)
Smc1	Bethyl Labs (A300-055A)	9777	GSM560341 & GSM560342	Kagey, 2010 (20720539)	MACS (1e-9)*
Smc3	Abcam (AB9263)	9715	GSM560343 & GSM560344	Kagey, 2010 (20720539)	MACS (1e-9)*
Sox2	Santa Cruz (sc-17320)	3683	GSM288347	Chen, 2008 (18555785)	MACS (1e-9)
Sox2	R&D Systems (AF2018)	16721	GSM307138 & GSM307139	Marson, 2008 (18692474)	MACS (1e-9)
Spt5	Custom made (see Wada et al., 1998)	3232	GSM515665	Marson, 2010 (20434984)	MACS (1e-9)
Stat3	Upstate (sc-482)	1353	GSM288353	Chen, 2008 (18555785)	MACS (1e-12)
Stat3_BrgKO	Santa Cruz (sc-482 X)	185	GSM686674	Ho, 2011 (21785422)	Consensus (4)
Stat3_BrgWT	Santa Cruz (sc-482 X)	992	GSM686673	Ho, 2011 (21785422)	Consensus (4)
Suz12	Abcam (AB12073)	3900	GSM288360	Chen, 2008 (18555785)	MACS (1e-9)
Suz12	Abcam	4793	GSM307144 &	Marson, 2008	MACS (1e-9)

	(AB12073)		GSM307145	(18692474)	
TBP	Abcam (AB818)	3629	GSM555160 & GSM555162	Young lab, 2010 (unpublished)	MACS (1e-12)*
Tcf3	Santa Cruz (sc-8635)	5755	GSM307142 & GSM307143	Marson, 2008 (18692474)	MACS (1e-9)
Tcfcp2l1	Custom made	20666	GSM288350	Chen, 2008 (18555785)	MACS (1e-15)
Tet1	Custom made	2969	GSM706672	Xu, 2011 (21514197)	MACS (1e-12)
Tet1	Custom made	24033	GSM659799	Wu, 2011 (21451524)	MACS (1e-9)
Tet1-C	Custom made	26912	GSM611192	Williams, 2011 (21490601)	MACS (1e-15)
Tet1-N	Custom made	14644	GSM611194	Williams, 2011 (21490601)	MACS (1e-15)
Wdr5	Bethyl Labs (A302-429A)	1327	GSM566279	Ang, 2011 (21477851)	MACS (1e-12)
Wdr5 (FLAG tag)	Sigma (F3165)	4331	GSM566280	Ang, 2011 (21477851)	MACS (1e-12)
Zfx	Custom made	14187	GSM288352	Chen, 2008 (18555785)	MACS (1e-9)

Notes: The table summarizes the ChIP-Seq studies currently included which totals 55 independent experiments covering 38 different factors; read density profiles, peak files and lists of candidate target genes for all 55 factors are available for download at (http://bioinformatics.cscr.cam.ac.uk/ES_Cell_ChIP-seq_compendium.html) **(1)** Originals and MACS (1e-9); **(2)** MACS (1e-9) and PeakSeq (0.05); **(3)** MACS (1e-15) and PeakSeq (0.01); **(4)** MACS (1e-15) and PeakSeq (0.05); * Intersect of replicates.

SUPPLEMENTAL TABLE 2

UPL PRIMERS

Gene Name	Forward	Reverse	UPL probe
Fgf5	aaaacctggtgcaccctaga	catcacattcccgaattaagc	29
Klf2	ctaaaggcgcatctgcgta	tagtgcgggtaagctcgt	48
Klf4	cggaaggagagagacact	gagttctcagccaacg	62
Klf5	ccggagacgatctgaaacac	cagatacttctccattcacatcttg	17
Nanog	ttctgcttacaagggtctgc	agaggaaggcgaggaga	110
Nr0B1	cgfgctctttaaccagacc	ccggatgtgctcagtaagg	3
Pou5f1	gttgagaaagggaaccaa	ctcctctgcagggtcttc	95
Rex1	tcttctctcaatagatgagtggtgc	gcttcttctgtgtgcaggga	71
Sox2	tccaaaaactaatcacaacaatcg	gaagtgcattgggatgaaaa	63
Tcfcp2l1	gaccatttcaagcactgaccta	caaagcatggaagaacatgg	4

SYBR Green primers (used in figures 4F, K and L and S4)

Gene name	FORWARD	REVERSE
Esrrb-P2A	CCTGCTGAGGCAGACAGCCG	GGTTCTCCTCCACGTCGCCG
Esrrb (endogenous or Tg)	CTCGCCAACCTCAGATTCGAT	AGAAGTGTGTCACGGCTTGT
Esrrb (Ex2)	CATGGAGGACTCCGCCATC	CTTGAATGGTTCTCTTGAAG
Oct3/4	CACGAGTGGAAAGCAACTCA	AGATGGTGGTCTGGCTGAAC
Sox2	AAGGGTTCTTGCTGGGTTTT	AGACCACGAAAACGGTCTTG
Nanog	ACCTGAGCTATAAGCAGGTTAAGAC	GTGCTGAGCCCTTCTGAATCAGAC
Klf4	CCAGCAAGTCAGCTTGTGAA	GGGCATGTTCAAGTTGGATT
Klf5	GCCAGTTAATTCGCCAACTC	CCCGTATGAGTCCTCAGGTG
Tbx3	ATCTGCCAGTGCACCTTGTTAGATG	TGTTCTTCAGCCCCGACTTCCATAC
Nr5a2	ATGGGAAGGAAGGGACAATC	ATACAACTCCCGCTGATCG
Zfp42	GACAGAGGCACTGGGGATAC	CGTATGCAAAAAGTCCCCATC

SUPPLEMENTAL TABLE 3

Name	Cat. From Qiagen
siControl	1027280
si Esrrb A	SI02672110
si Esrrb B	SI02739569
si Klf2 A	SI01083523
si Klf2 B	SI01083544
si Nanog A	SI04460869
si Nanog B	SI01323357
si Nr0B1 A	SI02666125
si Nr0B1 B	SI02685809
si Tcfcp2l1 A	SI01444296
si Tcfcp2l1 B	SI04401558
siGFP	custom (target sequence GCAAGCTGACCTGAAGTTCA)

SUPPLEMENTAL TABLE 4

Region1 F	aaaggtaggcccgattcac
Region1 R	aaaatagcccagagcttcag
Region2 F	atcagttcaggacagttgagag
Region2 R	cctaaagctcagagcacatgg
Region3 F	ccaactggagtaaagatgtcca
Region3 R	cattcactgcaggcacctc
Region4 F	tcattggcaaaggagacaaa
Region4 R	cggatctgtggaattcgtg
Region5 F	gaagtgttcttgccggtta
Region5 R	gtaactaacatcctctccctgagc

Supplemental Experimental Procedures

Chromatin Immunoprecipitation

Embryonic stem cells (3×10^6 for each sample) were fixed for 10 min in 1% formaldehyde, collected in cold PBS and incubated for 20 min on ice in swelling buffer (5 mM HEPES at pH 8.25, 85 mM KCl and 0.5% NP-40). Nuclei were pelleted in a microfuge and resuspended in lysis buffer (10 mM Tris at pH 8.0, 1 mM EDTA, 0.5 mM EGTA and 1% SDS). Lysates were sonicated to obtain an average DNA fragment size of ~500 base pairs. Lysates were diluted 1:10 in chromatin immunoprecipitation dilution buffer (50 mM Tris-HCl at pH 8.0, 167 mM NaCl, 1.1% Triton X-100 and 0.11% Na deoxycholate), pre-cleared for 2 h at 4 ° C with protein-G Sepharose beads (Amersham) and incubated overnight at 4 ° C with 2 μ g Tcf3 antibody (Santa Cruz, sc8635) or an isotype IgG antibody as a control (Santa Cruz, sc2028). Lysates were then incubated for 30 min at 4 ° C with blocked protein-G Sepharose beads, beads were washed twice each in radioimmunoprecipitation assay buffer (RIPA; 100 mM Tris-HCl at pH 8.0, 300 mM NaCl, 2 mM EDTA at pH 8.0, 2% Triton X-100, 0.2% SDS and 0.2% Na deoxycholate), RIPA/150 mM NaCl and Tris-EDTA buffer. Chromatin was eluted for 30 min at room temperature in elution buffer (100 mM Tris-HCl at pH 8.0, 300 mM NaCl, 5 mM EDTA at pH 8.0 and 0.5% SDS), Samples were incubated overnight at 65°C to reverse the cross-linking and purified using QIAquick PCR Cleanup kit (Qiagen). Chromatin was analysed by SYBR green real-time PCR (see primers in Supplementary Table S4). Enrichment was calculated relative to the IgG ChIP.

Immunostaining

Cells were fixed for 10 minutes in 4% PFA at Room Temperature (RT), permeabilized for 5 minutes in PBS+0.2% TritonX at RT, and blocked for 30 minutes in PBS+3% donkey serum at RT. Cells were incubated overnight at 4° C with an anti-Esrrb monoclonal antibody (Perseus, pp-H6705-00, 1:500 in PBS). After washing in PBS the cells were incubated with a donkey anti-Mouse IgG Alexa Fluor-555-conjugated antibody (A31570 from Invitrogen, 1:500 in PBS), for 30 minutes at RT. After DAPI staining, more than 10 images for each sample (>3000 cells) were acquired using a Zeiss AxioObserver D1 microscope. DAPI labelled cells were identified using Velocity software (Perkin Elmer) and intensity of 455-labelled Esrrb measured. Intensity measurements were expressed as a frequency distribution using Excel (Microsoft) to categorize negative, intermediate and positive cells.

Immunoblotting

Immunoblotting was performed as previously described in Yang et al., 2010. Antibodies used: mouse monoclonal anti-Esrrb ((Perseus Proteomics, pp-H6705-00, 1:1000 dilution in 5% milk); mouse monoclonal anti-GAPDH (Sigma-Aldrich, G8795, 1:1000 in 5% milk).

