Mitotic binding of Esrrb marks key regulatory regions of the pluripotency network

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Pluripotent mouse embryonic stem cells maintain their identity throughout virtually infinite cell divisions. This phenomenon, referred to as self-renewal, depends on a network of sequence-specific transcription factors (TFs) and requires daughter cells to accurately reproduce the gene expression pattern of the mother. However, dramatic chromosomal changes take place in mitosis, generally leading to the eviction of TFs from chromatin. Here, we report that Esrrb, a major pluripotency TF, remains bound to key regulatory regions during mitosis. We show that mitotic Esrrb binding is highly dynamic, driven by specific recognition of its DNA-binding motif and is associated with early transcriptional activation of target genes after completion of mitosis. These results indicate that Esrrb may act as a mitotic bookmarking factor, opening another perspective to molecularly understand the role of sequence-specific TFs in the epigenetic control of self-renewal, pluripotency and genome reprogramming.

Embryonic stem (ES) cell lines are derived from the pre-implantation blastocyst and can be maintained indefinitely in culture in an undifferentiated and pluripotent state¹. Despite undergoing virtually infinite divisions, ES cells do not lose their biological identity nor do they compromise their developmental potential. This phenomenon, known as self-renewal, is strictly dependent on a network of transcription factors (TFs) centred on Oct4, Sox2, Nanog and Esrrb²,³. Although these factors act collaboratively⁴, they also display unique functions. While Nanog⁻/⁻ cells exhibit low self-renewal efficiency⁵, Oct4⁻/⁻ or Sox2⁻/⁻ undifferentiated cells cannot be maintained⁶,⁷ and pure undifferentiated Esrrb⁻/⁻ cultures can be propagated only when differentiation is inhibited⁸,⁹. Moreover, whereas Oct4 and Sox2 upregulation triggers the loss of pluripotency⁵,¹⁰, that of Nanog and Esrrb abolishes the ability of ES cells to differentiate¹¹. Therefore, even though Oct4 is perhaps the only obligatory and general regulator of pluripotency, the function of Esrrb and Nanog is more specifically dedicated to the control of self-renewal.

ES cells are highly proliferative and present an atypical cell-cycle structure characterized by a short G1 phase lacking a G1/S checkpoint¹²–¹⁴. Therefore, ES cells effectively exist only in two continuously alternating states of DNA replication and cell division. Generally, mitosis is accompanied by the eviction of TFs and global transcriptional silencing¹⁶. Given that pluripotency TFs are permanently required to promote self-renewal and prevent differentiation, their activity must be promptly reinstated after each division. Moreover, the inactivation of major chromatin regulators associated with the epigenetic inheritance of gene expression programmes does not have severe detrimental effects on the propagation of the undifferentiated state¹⁷–²⁰. This large independence from classical epigenetic pathways has recently been recognized as one of the most intriguing hallmarks of ES cells²¹, raising the question of how ES cells maintain the TF-based regulatory continuity from mother to daughter cells and preserve their transcriptional status over indefinite periods of frequent and rapid divisions.

Increasing observations suggest that some transcriptional regulators can resist chromatin condensation and remain bound to their targets during mitosis. This process, known as mitotic bookmarking²², has been reported for general TFs²³, coactivators²⁴ and chromatin modifiers²⁵, but also for sequence-specific TFs such as Runx2 (ref. 26), Hnf1 (ref. 27), FoxA1 (ref. 28) and Gata1 (ref. 29). Therefore, mitotic bookmarking by one or more pluripotency TFs could propagate efficiently the regulatory information required to maintain the transcriptional identity of ES cells.

Given the intimate connection existing between their expression and the efficiency of self-renewal, we initially sought to determine whether Nanog or Esrrb could act as mitotic bookmarking factors.
Using fluorescence microscopy we demonstrate that Esrrb, but not Nanog, is retained on mitotic chromosomes, both in ES cells and during early embryogenesis. Furthermore, we report the compendium of genomic locations bound by Esrrb during mitosis, and demonstrate that mitotic Esrrb retention relies on dynamic interactions with its cognate DNA-binding motif. Finally, we identify the genes that are either rapidly activated or kept silent after mitosis in response to Esrrb activity. Altogether, we propose that Esrrb may act as a mitotic bookmarking factor in ES cells.

RESULTS
Esrrb decorates the mitotic chromosomes in ES cells and early embryos

To study whether Nanog and/or Esrrb are retained on mitotic chromosomes, we generated two cell lines expressing either Nanog-GFP or Esrrb-GFP fusion proteins. A Nanog-GFP expression vector was stably integrated into wild-type E14Tg2a ES cells; Esrrb-GFP was introduced in EKOiE ES cells, in which the two endogenous Esrrb alleles were previously deleted and Esrrb is expressed from a doxycycline (Dox)-inducible transgene (Methods and Supplementary Fig. 1a). After confirming that the transgenic GFP-fusions and Dox-inducible Esrrb proteins are functional (Supplementary Fig. 1b), we monitored their mitotic behaviour. While Esrrb-GFP was systematically localized to mitotic chromosomes, Nanog-GFP was excluded (Fig. 1a and Supplementary Fig. 2a,b). We then established an immunofluorescence protocol on metaphase spreads prepared from EKOiE cells cultured with Dox. We found that Esrrb was clearly associated with the metaphasic chromosomes (Fig. 1b). We did not observe any signal when Dox was withdrawn for 48 h, demonstrating the specificity of this assay (Supplementary Fig. 2d). Moreover, a similar coating of the chromosomes was observed in metaphase spreads prepared from EKOiE cells expressing an Esrrb-Snap-tagged variant, further supporting our observations (Supplementary Fig. 2e).

To exclude that the mitotic retention of Esrrb is due to the high levels of expression obtained either by Dox induction or by the CAG-driven Esrrb-GFP or Esrrb-Snap transgenes, we fused both endogenous Esrrb alleles to tdTomato (Methods). We found that Esrrb-tdTomato decorates the chromosomes throughout all phases of mitosis (Fig. 1c and Supplementary Fig. 2c), as also observed in time-lapse imaging of Esrrb-GFP (Supplementary Video). Moreover, when we performed immunofluorescence for Nanog and Esrrb using metaphase spreads prepared from E14Tg2a cells, we observed that Esrrb, but not Nanog, decorates the mitotic chromosomes in wild-type cells (Supplementary Fig. 2f). Overall, we have documented the chromosomal retention of Esrrb in all mitotic ES cells observed so far using diverse microscopy tools.

Next, since Esrrb messenger RNA is abundant in fertilized oocytes and is expressed during early embryogenesis, we asked whether the mitotic retention of Esrrb also occurs in vivo when pluripotency is established. To do this, in vitro transcribed Esrrb-tdTomato and GFP mRNAs were co-injected into single blastomeres of two-cell embryos. The embryos were allowed to develop in vitro for 48 h, reaching the compacted morula stage. We observed that Esrrb-tdTomato coats the mitotic chromosomes of GFP-positive cells (Fig. 1d). This indicates that the mitotic retention of Esrrb is operational during early embryogenesis.

Mitotic Esrrb is specifically enriched at individual genomic regions

To determine whether Esrrb is enriched at specific genomic regions during mitosis, we established a ChIP-Seq assay in interphasic and mitotic ES populations obtained by nocodazole treatment and mitotic shake-off (Methods). Binding profiles of Esrrb were produced in wild-type E14Tg2a and in transgenic EKOiE cells cultured with Dox. EKOiE cells depleted of Esrrb (−Dox for 48 h) were used as negative controls. We found that Esrrb remains bound at a subset of its interphase targets during mitosis, suggesting that it acts as a bookmarking factor (Fig. 2a).

Using statistical and reproducibility parameters (Methods and Supplementary Fig. 3), we identified nearly 14,000 Esrrb-binding regions in interphase and 1,980 in mitosis (Supplementary Table 1). Albeit at different levels (Supplementary Figs 3 and 4), mitotic binding regions were also bound by Esrrb in interphase (thereafter,
Figure 2 Esrrb binds a subset of its interphase targets in mitosis. (a) Illustrative binding profile of Esrrb across the Sox2 extended locus (controls are EKOiE cells cultured without Dox for 48 h to deplete Esrrb). (b) Histogram showing the distribution of Esrrb binding levels at all regions in interphase (Int., blue line) and mitosis (Mit., red line). The red filled area of the mitosis line corresponds to the bookmarked regions; the white area to the lost regions. RPKM, read counts per kilobase per million. (c) Average Esrrb binding profiles at bookmarked and lost regions. (d,e) Examples of regions (6.7 kb) retaining Esrrb in mitosis (d) or losing its binding (e). The closest gene to each peak is indicated. The y axes in panels a,c–e represent coverage in bases per kilobase per million.

bookmarked; Fig. 2b–d). Nine regions were used for validation by quantitative PCR and titration experiments, excluding that remnant interphase cells in our mitotic preparations (<5%; Methods) may affect the observed Esrrb binding profiles (Supplementary Fig. 4a–c).

We observed that Esrrb binding levels at its interphase targets followed a bi-modal distribution in mitosis (Fig. 2b), with ~6,000 regions showing very low levels. At these regions Esrrb binding was never detected in mitosis (Supplementary Fig. 3), indicating that Esrrb is efficiently evicted (thereafter, lost; Fig. 2e). The remaining ~8,000 regions include the 1,980 regions identified as bookmarked. Excluding those, the levels of mitotic Esrrb binding did not significantly differ from the negative controls and the detection of Esrrb failed in at least one of our four independent replicates (Supplementary Fig. 3). Even though a subset of these regions may be inefficiently bound in mitosis, we adopted a conservative approach and considered them as lost for subsequent analyses.

We conclude that Esrrb remains robustly bound to 10–15% of its interphase targets (bookmarked regions; Fig. 2c), and shows no or labile mitotic binding at other regions (lost regions; Fig. 2c). Of note, ~55% of all ES-specific super-enhancers31 such as those of key pluripotency factors (Sox2, Tbx3 and Klf4), together with other regulatory regions of important genes are mitotically bound by Esrrb (Supplementary Fig. 4d).

Relationships between mitotic Esrrb binding and chromatin status in interphase

In mitosis, Esrrb binding is generally reduced at bookmarked regions compared with interphase (Fig. 2b,c). Moreover, bookmarked regions show higher Esrrb binding in interphase than lost regions (Fig. 2c). Using published data sets3,32,33, both bookmarked –B– and lost –L– regions could be divided into two clusters (Fig. 3a) according to their chromatin status in interphase: either particularly enriched for binding of other TFs and active histone marks (B1 and L1) or not (B2 and L2; Supplementary Fig. 5a). However, direct quantitative comparisons showed that, whilst B2/L2 display homogeneously low levels (Supplementary Fig. 5a), B1 promoters and enhancers display higher enrichment for TFs, the transcriptional machinery and euchromatin-associated histone marks than L1 regions (box plots in Fig. 3a). For instance, both B1 promoters and enhancers display higher levels of Nanog, RNAPII and H3K27ac than their L1 counterparts losing Esrrb in

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Figure 3 Mitotic Esrrb binding at regions of high activity in interphase. (a) Hierarchical clustering of Esrrb-binding regions (numbers on the left) based on enrichment for the indicated marks or factors. The corresponding box plots (median, and 25th and 75th percentiles) and respective whiskers (median ± the interquartile range multiplied by 2) for B1 (red; n = 1,344) and L1 (grey; n = 9,680) regions are shown below the heatmaps. The P value of the difference between B1 and L1 for each factor is indicated (Kruskal–Wallis ANOVA followed by Dunn’s multiple comparison test). (b) Average binding profile of Nanog, Ser5-phosphorylated RNAPII (RNAPII-S5) and H3K27ac (H3K27ac) at B1 (red) versus L1 (black) promoters and enhancers. The y axis represents coverage in bases per kilobase per million. TSS, transcriptional start site.

Mitotic Esrrb binding is driven by specific DNA binding to its motif

Using de novo motif discovery algorithms we found that the canonical extended oestrogen receptor half-site characteristic of Esrrb was strongly enriched at bookmarked regions, with nearly 90% of these regions containing at least one motif (Fig. 5a). Conversely, only 32% of lost regions had Esrrb motifs, suggesting that specific DNA–protein interactions are required for mitotic binding of Esrrb. To test this, we mutated three residues of Esrrb that mediate the principal base-specific interactions with DNA (Methods). This binding-defective mutant (Supplementary Fig. 6a) did not decorate the mitotic chromosomes to the extent of wild-type Esrrb-GFP (Fig. 5b and Supplementary Fig. 6b).

An undescribed longer Esrrb motif extended by three bases (AGT) was prominent in bookmarked regions (63%). Only 14% of lost regions, however, displayed a long motif and of lower quality (Fig. 5a and Supplementary Fig. 6c). Hence, the long motif may play a specific role in Esrrb bookmarking. To test this, we used CRISPR to turn the single long Esrrb-binding motif present under the major Esrrb peak...
Mitotic Esrrb binding is dynamic

The retention of Esrrb on mitotic chromatin could result either from the permanence of Esrrb protein already loaded on chromatin at the end of G2, or from de novo and dynamic binding of Esrrb in mitosis. To discriminate between these two possibilities, we performed FRAP (fluorescence recovery after photobleaching) experiments (Fig. 6a), a strategy that has been widely used to infer TF binding dynamics.\textsuperscript{15–17}; whilst unbound TFs as well as those engaged in rapid ON/OFF binding transitions lead to a fast fluorescent recovery, a slow recovery results from a decreased turnover of photobleached and fluorescent molecules, indicating more stable interactions. In interphase, Esrrb-GFP signal promptly recovered after bleaching (80% recovery in 5s, Fig. 6a,b), indicating that Esrrb binding is dynamic. Strikingly, the specific spatial localization of the GFP signal resulting from the decoration of mitotic chromosomes by Esrrb-GFP was also recovered after bleaching, and with faster dynamics than in interphase (80% in 2s, Fig. 6a,b). As a control, DNA-binding Esrrb mutant showed even faster GFP recovery rates (Fig. 6b), confirming that the delay in recovery observed for wild-type Esrrb results from its DNA-binding ability both in interphase and mitosis.

These results indicate that Esrrb is able to bind de novo to mitotic chromatin and that the observed retention probably results from continuous cycles of transient and fast interactions of Esrrb with DNA. Together with the previous analysis of Esrrb-binding sites, this suggests that Esrrb behaves as a canonical TF in mitosis in terms of its binding to DNA.

Mitotic Esrrb binding correlates with transcription activation in early G1

To explore the functional consequences of mitotic Esrrb binding on gene expression we introduced a cell-cycle reporter into Dox-inducible EKOiE cells (Methods). This reporter system is based on the fusion of residues 1–156 of murine cyclin A2 (Ccna2) to GFP, driving its mitotic degradation starting from prometaphase\textsuperscript{38,39}, compensating viability (Supplementary Fig. 7a). Ccna2-GFP cells were grown in the presence or absence of Dox (24 h withdrawal), allowing sorting of cells as they proceed through G1, S and G2, without compromising viability (Supplementary Fig. 7a). Ccna2-GFP cells were grown in the presence or absence of Dox (24 h withdrawal), isolated by fluorescence-activated cell sorting (FACS) in early-G1, late-G1 and G2 fractions (Fig. 7a), and analysed by RNA-Seq using a pipeline enabling quantification of both mRNA and pre-mRNA levels (Methods and Supplementary Table 2).
### Figure 5

The Esrrb DNA-binding motif drives mitotic binding. (a) The Esrrb consensus is shown together with the main motifs discovered in lost and bookmarked regions. The histogram shows the fraction of bookmarked and lost regions containing long (red) or short (blue) Esrrb motifs. (b) Representative images of mutant (mut) Esrrb-GFP fusions in live cells cultured with Hoechst, imaged in parallel to that shown in Fig. 1a. Scale bar, 10 μm. Images are representative of multiple fields acquired in four independent repeat experiments. (c) Average Esrrb ChIP results shown as percentage of immunoprecipitation (%IP; n=4 independent chromatin preparations; error bars represent s.d.) at a bookmarked (Pdgfa) and a lost (Twistnb) region (see Fig. 2d,e), and at synthetic binding sites constituted of four tandem repetitions of short (upper panel) or long (lower panel) motifs. ‘Non-binding’ represents the average of two genomic positions not bound by Esrrb (Actin promoter and a region 3’ to the Esrrb gene). In each cell line (clone no. 1 and no. 2), the short and long synthetic binding sites were introduced at identical genomic positions. (d) Percentage of Esrrb mitotic retention calculated from c for the long and short synthetic binding sites in two independent clones (n=4). Error bars represent s.d.

Overall, around 500 genes were up/downregulated by Esrrb at both mRNA and pre-mRNA levels (Fig. 7b and Supplementary Table 3). Of these, 168 and 206 genes showed prompt positive or negative response already in early G1 and were coherently affected by the loss of Esrrb during all cell-cycle phases (Supplementary Fig. 7b). The number of Esrrb-responsive genes increased during cell-cycle progression (Fig. 7b), with around half showing changes only in G2. Early-G1 responsive genes associated with a bookmarked region include pluripotency TFs (Tfcp2l1, Tbx3 and Klf4) and regulators of differentiation (Id2-3), chromatin (Dnmt3L) and embryonic cell–cell interactions (Jam2; Fig. 6c). Notably, Esrrb-up/downregulated genes in early G1 are also up/downregulated in ground-state conditions of systematic self-renewal80 (Supplementary Fig. 7c).

Almost 75% of genes activated by Esrrb in early G1 were located within topological associated domains41 (TADs) containing at least one Esrrb bookmarked region (Fig. 7d). In contrast, only 55% of the genes regulated by Esrrb exclusively in G2 were located within bookmarked TADs, as observed for an equivalent set of random genes. Similarly, nearly 25% of the genes upregulated by Esrrb in early G1 were within 25 kilobases (kb) of a bookmarked region (10% for random genes). In contrast, genes downregulated by Esrrb tend to be further away from bookmarked regions (Fig. 7d).

Overall, the integration of our ChIP-Seq and RNA-Seq results indicates that mitotic Esrrb binding transcriptionally activates a subset of key genes in early G1, including pluripotency regulators that in turn may control additional genes during the cell cycle. This strongly suggests that Esrrb behaves as a canonical bookmarking factor.

### DISCUSSION

We report here that Esrrb, a major pluripotency regulator8,9,42, decorates the mitotic chromosomes of ES cells, an ability not shared by Nanog. We further demonstrate that Esrrb is specifically enriched at a subset of its interphase targets. Recently, other sequence-specific TFs have been shown to bind mitotic chromatin, suggesting that the mitotic retention of TFs may have been generally overlooked. Several common observations can be identified among recent studies27–29,43.

First of all, only a subset of TF binding regions are effectively bound in mitosis, albeit at lower levels than in interphase. Whether this is due to technical reasons or reflects a particular behaviour of mitotic binding is unclear, but our FRAP analyses suggest that mitotic Esrrb binding is hyper-dynamic and probably less amenable to chemical crosslinking and robust detection by ChIP-Seq. Secondly, regions bound in mitosis tend to exhibit particularly robust binding in interphase and contain canonical DNA-binding sites (Fig. 7e). Similarly to Gata1, FoxA1 and Hnf1, Esrrb requires its DNA-binding domain to interact with mitotic chromatin. Moreover, we demonstrate that the experimental introduction of ectopic recognition motifs is sufficient to drive Esrrb binding in interphase and in mitosis. Additional mechanisms of mitotic retention may however exist, as we identified around 10% of mitotically bound regions lacking clear motifs. These regions exhibit high enrichment for other pluripotency TFs, suggesting that additional regulators may bind mitotic chromatin in ES cells, a possibility that needs to be comprehensively assessed (Fig. 7e). Thirdly, the mitotic retention of TFs is associated with widespread coating of the chromosomes, which is hardly explained by binding at a subset of enhancers and promoters. The mutation of FoxA1 DNA-binding

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**Figure 5** The Esrrb DNA-binding motif drives mitotic binding. (a) The Esrrb consensus is shown together with the main motifs discovered in lost and bookmarked regions. The histogram shows the fraction of bookmarked and lost regions containing long (red) or short (blue) Esrrb motifs. (b) Representative images of mutant (mut) Esrrb-GFP fusions in live cells cultured with Hoechst, imaged in parallel to that shown in Fig. 1a. Scale bar, 10 μm. Images are representative of multiple fields acquired in four independent repeat experiments. (c) Average Esrrb ChIP results shown as percentage of immunoprecipitation (%IP; n=4 independent chromatin preparations; error bars represent s.d.) at a bookmarked (Pdgfa) and a lost (Twistnb) region (see Fig. 2d,e), and at synthetic binding sites constituted of four tandem repetitions of short (upper panel) or long (lower panel) motifs. ‘Non-binding’ represents the average of two genomic positions not bound by Esrrb (Actin promoter and a region 3’ to the Esrrb gene). In each cell line (clone no. 1 and no. 2), the short and long synthetic binding sites were introduced at identical genomic positions. (d) Percentage of Esrrb mitotic retention calculated from c for the long and short synthetic binding sites in two independent clones (n=4). Error bars represent s.d.
domain leads to the loss of binding at regions containing binding sites, but not to the loss of the chromosome-wide coating. Conversely, mutations in the Hnf1 DNA-binding domain abolish chromatid coating but not site-specific DNA binding in interphase. In the case of Esrrb, we show that the mutation of its DNA-binding domain abolishes both binding at specific loci in interphase and the general coating of mitotic chromosomes. Hence, the decoration of mitotic chromosomes is mediated by different types of molecular interactions depending on the TF under study. This differential behaviour of TFs needs to be directly investigated, as it calls into question the idea that mitotic chromosomes are intrinsically refractory to scanning by TFs. A better understanding of the nature of mitotic chromatin needs to be attained should we want to uncover the exact nature and relevance of mitotic bookmarking.

We have observed that, in interphase, regions bound by Esrrb tend to be either highly enriched for signatures of active chromatin (B1/L1 regions) or not marked by any specific feature (B2/L2), a chromatin state reminiscent of naive chromatin. However, direct comparison of B1 and L1 regions clearly shows that mitotic Esrrb binding is associated with regions of particularly high activity in interphase. Most notably, H3K27ac and RNAPII enrichment show the highest fold difference between bookmarked and lost regions. That Esrrb interacts with several members of the basal transcription complex, coactivators and histone acetyl-transferases may provide a molecular understanding of the consequences of mitotic binding. Together with previous reports, our findings indicate that mitotic binding of Esrrb and of other factors enables the maintenance of active chromatin properties at key regulatory regions that, in turn,
activate important genes during interphase. Esrrb binding in mitosis seems to result in the rapid upregulation of important pro-self-renewal genes (Klf4, Tcf21 and Tbx3) as ES cells re-enter into interphase (Fig. 7e). The fact that alterations in both transcriptional output and mature mRNA accumulation are evident already in early G1 argues in favour of an almost immediate resumption of transcription. Therefore, Esrrb should be considered as a mitotic bookmarking factor contributing to the long-term maintenance of pluripotency by jumpstarting the post-mitotic resuscitation of the pluripotency network and counterbalancing the inherent propensity of ES cells to undergo differentiation from G1 (refs 15,50,51).

As shown for Gata1<sup>29</sup>, taking into consideration only mitotic binding allows a contraction of the gene ontology associations of Esrrb-binding regions to particularly relevant genes, those highly expressed during pre-implantation development when pluripotency is established. Hence, the observation that in early embryos mitotic Esrrb binding is operational opens the possibility of a direct involvement of this process when Esrrb expression becomes restricted to the...
pluripotent inner cell mass. Similarly, the generation of induced pluripotent stem cell has been shown to transit from a stochastic to a more deterministic process coinciding with the reactivation of endogenous Esrb expression. Thus, naive pluripotency may be attained only when the regulatory network becomes mitotically self-sustainable through Esrb binding.

Whilst ES cells self-renew indefinitely, in the embryo pluripotency is a transient state that is rapidly extinguished following differentiation. We speculate that Esrb bookmarking might be a key phenomenon enabling the transient epigenetic maintenance of pluripotency in the early embryo without the need to establish mitotically heritable chromatin marks, which, although reversible, impose further molecular constraints on the timely extinction of pluripotency. More generally, we believe that mitotic bookmarking by TFs has enormous advantages over other systems of mitotic inheritance in transiently expanding cell types such as activated stem cells and progenitors.

### METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

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

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**AUTHOR CONTRIBUTIONS**

This study was conceived by N.F. and P.N. All experiments were carried out by N.F. with the exception of embryonic work performed by S.V.-P., S.B. and M.C.-T. In addition, A.D., E.G.T. and A.M. provided technical help and F.M. contributed with the exception of embryo work performed by S.V.-P., S.B. and M.C.-T.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Cell lines. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. All of the cell lines generated for this study were derived from E14Tg2a, tested for mycoplasma and their identity authenticated by several means including resistance to drug selection, PCR and sequencing of specific genomic regions.

EKOEp and EKOEp Mut. The first exon of both Esrrb alleles of E14Tg2a cells was flanked by Loxp sites as described previously38 and transfected with a CAG-RTA-IRES-1G4A-HPRT1- 在 a doxycycline (Dox)- inducible tdTomato transgene flanked by heterotypic FRT sites. Flp recombinase-mediated cassette exchange (RMCE) was then used to exchange tdTomato with an Esrrb-IRES-Purmycin cassette (wild-type or mutant Esrrb, see below). Both Esrrb alleles were then deleted by Cre recombinase.

EKOEp derivatives. EKOEp cells were stably transfected with a CAG-promoter-driven wild-type Esrrb-5xGly-GFP, mutant Esrrb-5xGly-GFP, SNAP-Esrrb or Ccnara-5xGly-GFP-IRES-purmycin transgenes and homogeneously expressing clones were selected. In mutant Esrrb, Glu121, Lys124 and Lys128 are mutated to glycine. Cnara-GFP includes only the mitotic degnor of murine cyclin A2 (residues 1-156).39,40

NanoGFP. E14Tg2a cells were stably transfected with a CAG-NanoGFP-IRES-purmycin transgene.

EtdT. tdTomato-IRES-Blasticidin and tdTomato-IRES-Hygromycin were knocked-in downstream of the Esrrb ORF at each allele of TNG cells.

ES cells carrying ectopic Esrrb-binding sites. Two E14Tg2a clones bearing an FRT-flanked Dox-inducible tdTomato transgene and showing high and homogenous expression were selected. tdTomato was exchanged for a 4× binding site-IRES-purmycin cassette by RMCE (long or short motifs). The 4× binding sites, derived from the Esrrb enhancer (3′-AGAAAGAGCACAAGTCGAGGTCGTTAGCTGCTGCTTCTCTA-TGAGTGAAGTTGTTGGCAGTATA-3′, AGT was mutated to CCC to generate a short motif), follow one another in the same orientation and spaced apart 30 bp. Multi-copy integration of the binding site arrays was excluded by genomic DNA qPCR. Clones were cultured in Dox to avoid transgene heterochromatization.

ES cells edited at the Esrrb enhancer. E14Tg2a cells carrying a Ccnara-GFP transgene were lipofected with a ssDNA oligonucleotide corresponding to mmnchr12:87846218-87847236 with three bases of the Esrrb motif mutated (3′-TGACCTTGAGGG-5′), pG3L-U6-spRNA-PGK-Purmycin vector (Addgene no. 51133) driving expression of the gRNA 3′-ACAGAGGGAGCACAAGGCTTCA-5′ (NGG) and the CAS9/GFP expression vector (Addgene no. 44719). Twenty-four hours later GFP+ cells were sorted (FacsARIA III; Becton-Dickinson) and single clones were screened by genomic DNA qPCR using primers specific for the WT or mutated allele. Four hundred base pairs centered on the motif were sequenced and three clones carrying the desired substitution at both alleles were selected. We deleted deletion of the genomic region extending beyond the PCR-screened region by plating 600 cells per well of a 6-well plate and after 6 days an Alkaline Phosphatase kit (Sigma, 86R1KT) was used to identify undifferentiated colonies using a stereomicroscope (Nikon-SMZ1500).

Microinjection. All experiments were conducted according to the French and European regulations (EC Directive 86/609, French Law 2001-486) and were approved by the Institut Pasteur ethics committee (no. 2012-0011). (C57BL/6xSJL) F1 female mice were purchased from Janvier and co-injected with CDI-IGS male mice purchased from Charles River Laboratories. Fertilized eggs were obtained from 6 superovulated 3-week-old females submitted to intraperitoneal injection of Chorionest PMSG (2.5 units per mouse, Centravet) followed by Chorulon (5 units per mouse, Intervet) 42-48 h later and then mated overnight with 6 males. Single blastomere microinjection was performed in 2-cell stage embryos in M2 medium using an IMT-2 inverso inverted microscope (Olympus), micromanipulators (Leica Microsystems) and a manual micromanipulation system. Embryos were co-injected with mRNAs for Esrrb-tdTomato (275 or 550 ng/µl) and mGFP (350 ng/µl) and cultured in KSO4+AA+ D-glucose (EmbyroMax, Millipore) for 48 h. No statistical method was used to predetermine sample size; the experiments were not randomized; the investigators were not blinded to allocation during experiments and outcome assessment.

Imaging. Culture conditions. Unless otherwise specified, cells were plated on IBIDI dishes and cultured overnight in DMEM non-essential amino acids (Gibco, 15212-012), trypsinized and resuspended in 10 ml of hypotonic solution (NaCtrate 0.017 M, KCl 0.03 M) for 10 min at room temperature. The cells were resuspended in 2.5 ml of ice-cold hypotonic solution. Five milliliters of cold fixative acetic acid–methanol 1:3 solution were added dropwise at 4 °C. Cells were collected and resuspended in 2.5 ml of the supernatant and the above procedure was repeated three times. Fixed cells were spread by dropping on pre-heated glass slides. Slides were incubated in PBST (PBS 0.1% Triton X-100; 10 min), blocked in PBST/5% donkey serum (30 min) and incubated (2 h, room temperature) with primary antibodies (1:500 Esrrb from Perseus Proteomics H6-705-00 and/or 1:200 Nanog from Cosmobio RCA0001P). After three washes with PBST, samples were incubated with goat anti-mouse or anti-rabbit secondary antibody (1:500; Alexa Fluor 488 or 594; Jackson ImmunoResearch, 715-545-151; 715-545-152) overnight at 4 °C and washed, mounted (Vectashield; VectorLab, H1200) and imaged as wide-field fluorescence.

Preparation of mitotic populations. E14tg2a or EKOEp ES cells were grown to 70-80% confluency in 4 to 6 T150 flasks per preparation. Gigotic shake-off was performed to detach debris, dying cells or poorly attached cells colonies, medium

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discarded and flasks washed twice in PBS. After 4 h in medium containing 50 ng/ml nocodazole (Sigma, M1404), flasks were gently washed with PBS. Gentle shake-off was performed in 10 ml of medium, monitoring the process under a microscope to avoid detaching clumps of cells in interphase. The cell suspension was filtered through a double layer of 20 μm filters (Millipore, NY2004700) and spun down. Typically, 10^5 cells were obtained from each preparation. Purity of mitotic preparations was systematically checked under a fluorescence microscope after fixation, cytopsinning and DAPI staining. Preparations with more than 5% contamination from interphase were discarded.

Chromatin preparation for Esrrb-binding analysis. In previous reports describing Esrrb ChIP analysis a dual crosslinking strategy with DSG and FA was used. Therefore, we initially applied similar conditions. However, to prove that Esrrb binding is not necessarily indirect, as it may be the case when DSG is required, we established an alternative protocol using only FA and digested chromatin with MNase rather than by sonication. Similar ChiP-Seq profiles were observed (Bioinformatic analyses section).

Chromatin preparation (DSG + FA/sonication). ES cells (10^5) were crosslinked in 2 ml of freshly prepared PBS-DSG 2 mM at pH 7.0 (Sigma, 80424-5 mg) for 50 min at room temperature with occasional shaking. After pelleting and washing in PBS, cells were incubated for 10 min in 2 ml PBS 1% formaldehyde (Thermo, 28908). Crosslinking was stopped with 0.125 mM glycine for 5 min at room temperature. Cells were pelleted, washed with ice-cold PBS and resuspended in 2 ml of swelling buffer (25 mM Hapes pH 7.95, 10 mM KCl, 10 mM EDTA) freshly supplemented with 1× protease inhibitor cocktail (PIC-Roche, 04 693 116 001) and 0.5% NP-40. After 30 min on ice, the suspension was passed 5 times in a bouncer (only for asynchronous populations), centrifuged and resuspended in 300 μl of TSE150 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl) buffer, freshly supplemented with 1× PIC. Samples were sonicated in 1.5 ml tubes (Diagenode) for 7–9 cycles divided into 30 s ON-30 s OFF sub-cycles at maximum power, in circulating ice-cold water. After centrifugation (30 min, full speed, 4°C), the supernatant was stored at −80°C. Five microlitres was used to quantify the chromatin concentration and check DNA size (typically 200–500 bp).

Chromatin preparation (FA/Mnase). ES cells (10^5) were resuspended in 3 ml of prewarmed DMEM/FCS/LIF and crosslinked for 10 min at room temperature adding 1% formaldehyde (Thermo, 28908). Cells were processed as above until nuclei were centrifuged, resuspended in 500 μl of KN buffer (20 mM Tris-HCl pH 7.5, 15 mM KCl, 60 mM NaCl, 0.34 M sucrose, 1 mM CaCl2 and 60 U MNase (Thermo, EN0181). After 7 min at 37°C, the reaction was stopped on ice with 4 μl of 500 mM EDTA. Samples were sonicated in ice-cold water (Bioruptor, Diagenode; 4 cycles of 20 s ON–40 s OFF, maximum power). The chromatin was centrifuged (30 min, full speed, 4°C), the supernatant was stored at −80°C until use. Five microlitres was used to quantify the chromatin and check DNA size (typically a highly prevalent mono-nucleosome band).

Chromatin immunoprecipitation (ChiP). For ChiP-qPCR and ChiP-Seq experiments, chromatin from 2.10^6 and 10^5 cells was used respectively. Chromatin was pre-cleared for 90 min rotating on-wheel at 4°C in 300 μl of TSE150 containing 50 μl of pG Sepharose beads (Sigma, P3296-5 ML) 50% slurry, previously blocked with 1% BSA (500 μg/ml–1; Roche, 9391665103) and yeast tRNA (1 μg/ml–1; Invitrogen, AM7119). Immunoprecipitations with anti-Esrrb mouse monoclonal (Perseus Proteomics, H6-705-00) (1 μg per 2.10^6 cells) were performed overnight rotating on-wheel at 4°C in 500 μl of TSE150. Twenty microlitres was set aside for input DNA extraction and preparation. Twenty-five microlitres of blocked pG beads 50% slurry was added for 4 h rotating on-wheel at 4°C. Beads were pelleted and washed for 5 min rotating on-wheel at room temperature with 1 ml of buffer in the following order: 3× TSE150, 1× TSE500 (as TSE150 but 500 mM NaCl), 1× washing buffer (10 mM Tris-HCl pH 8, 0.25M LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA), and 2×TE (10 mM Tris-HCl pH 8, 1 mM EDTA). Elution was performed in 100 μl of elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8) for 15 min at 65°C after vigorous vortexing. Eluates were collected after centrifugation and beads rinsed in 150 μl of TE-SDS1%. After centrifugation, the supernatant was pooled with the corresponding first elute. For both immunoprecipitated and input chromatin, the crosslinking was reversed overnight at 65°C, followed by proteinase K treatment, phenol/chloroform extraction and ethanol precipitation.

RNA isolation and reverse transcription. Total RNA was isolated in Triozol (Invitrogen, 15596026) and digested with DNase I (Qiang, 79254). Reverse transcription reactions were performed with random hexamers on 1 μg of total RNA (First Strand CDNA kit, Roche, 04379012001). Quantitative real-time PCR. Real-time PCR reactions were performed in quadruplicate for pre-mRNA expression analysis and duplicate for ChIP analysis in 384-well plates with a 480 LightCycler (Roche) using LightCycler 480 SYBR Green I Master (Roche, 40707516001). Standard and melting curves were generated to verify the amplification efficiency (>85%) and the production of single DNA species. PCR primer sets are listed in Supplementary Table 4. Values for gene expression were normalized to the levels of Tbp mRNA.

FACS. Ccna-GFP EKOiE ES cells were incubated with 20 μM Hoechst-33342 for 45 min, filtered through a 40 μm cell strainer and kept on ice. ES cells were sorted on the basis of Hoechst and GFP levels in three populations (early G1, late G1 and G2) using a FacsARIA III (Becton-Dickinson), keeping samples and collection tubes at 4°C. Data were analysed using the FlowJo software suite (Tree Star). Among 10^5 cells were sorted per population before RNA extraction. For replicating experiments, sorted cells were cultured for 7 h, incubated for 45 min with Hoechst, trypsinized and analysed with a LSR II Fortessa (Becton-Dickinson).

Bioinformatic analyses. ChiP-Seq sequencing and alignment. ChiP-Seq libraries (Supplementary Table 5) were generated using a MicroPlex kit (Diagenode) and sequenced (50 bp, HiSeq 2500 instrument, Illumina). Reads were mapped to the mm9 genome assembly using Bowtie and allowing three mismatches using GeneProF. Aligned reads corresponding to interphase, mitosis and respective controls were separately pooled. Unless otherwise specified all analyses were performed with SeqMonk (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk). We generated 500 bp-long windows sliding every 250 bp and quantitatively read counts per kilobase per million (RPKM) across the genome after excluding the Y chromosome (lost in EKOiE). Interphase_rep4 was a clear outlier in terms of fold-covering and global correlation to other interphase replicates (Supplementary Table 5).

Identification of candidate peaks and statistical analysis. Peaks were called with MACS as implemented in SeqMonk using EKOiE – Dox controls as background, a 10^-3–value threshold, and fragment sizes of 200, 350 and 300 for MNase, sonicated and pooled data sets respectively. Candidate peaks from pooled data sets (excluding Y chromosome) were combined and subject to a DESEQ2 statistical filter using raw read counts of each individual replicate (excluding interphase_rep4). Multiple testing correction and independent filtering were enabled leading to 20.151 peaks (FDR < 0.2 in interphase or mitosis versus the −Dox controls). Peaks were simplified such that no two peaks overlap by more than 1%, giving 14,559 binding regions.

Categorization of DESEQ2 filtered peaks as bookmarked or lost regions. A total of 14,559 regions passing the DESEQ2 filter were divided into 5 classes (Supplementary Fig. 3) according to the statistical significance of their enrichment levels (versus −Dox) and the reproducibility of their identification in individual replicates:

Class_1 (450 peaks). Passed DESEQ only in mitosis. However, most (437) were identified as peaks in at least two interphase replicates and all but one were identified in at least one of our interphase replicates or in an independent study. Therefore, these peaks cannot be rigorously qualified as mitotic-specific. ChiP-qPCR on 3 Class_1 peaks, including the single peak that was not detected in any interphase replicate (Supplementary Fig. 4), confirmed this interpretation.

Class_2 (1216). Passed the DESEQ filter both in mitosis and in interphase. They represent regions bound by Esrrb in interphase and in mitosis.

Class_3 (314). Passed DESEQ only in interphase but were identified as peaks in all 4 mitosis replicates. These regions are characterized by robust Esrrb binding in interphase and low but reproducible binding in mitosis.

Class_4 (6621). Passed DESEQ only in interphase and were identified as peaks in the pooled mitosis data set but not in all mitosis replicates.

Class_5 (5958). Passed DESEQ only in interphase and were never detected in any of the mitosis replicates. They represent clear interphase-specific Esrrb-binding regions.

Although Class_4 regions may represent weak binding in mitosis, we followed a conservative approach and combined them with Class_5 regions into the list of lost regions. In contrast, Classes_1,2,3 represent regions that bind Esrrb in mitosis and interphase (although at different degrees, see Supplementary Fig. 3 and 4); they were combined into the list of bookmarked regions. Coordinates and class of each individual peak, together with the quantities in all data sets, are available in Supplementary Table 1.
ChIP-Seq binding profiles. The ChIP-Seq profiles shown in Fig. 2 and Supplementary Fig. 4 were obtained in SeqMonk designing 100 bp windows every 10 bp and quantitating the base coverage normalized to the total number of aligned reads of the pooled data sets. A smoothing window of 25 was then implemented. The profiles shown in Supplementary Fig. 5 were obtained similarly but using 100 bp windows sliding every 50 bp.

Average profiles. All metaplots were calculated in SeqMonk using base coverage per million reads every 100 bp. All Esrrb peaks overlapping with a ±500 bp region flanking mRNA 5′-ends were considered promoters, leaving the rest as enhancers.

Hierarchical clustering and heatmaps. The heatmaps shown in Fig. 3 and Supplementary Fig. 3 were obtained in SeqMonk (RPKM quantification) using either 5-kb-long regions centred on the middle of each peak or the exact peak coordinates, respectively. To generate B1, B2, L1 and L2 groups a Euclidean clustering based on the smallest absolute difference between region quantification values was used.

Expression of genes associated with Esrrb-binding sites. All four clusters of binding regions identified by hierarchical clustering (Fig. 3) were used as inputs for GREAT using standard ‘basal plus extension’ parameters. The first five tissues showing expression of the selected genes according to the Mouse Gene Informatics database were used in Fig. 3c. In Fig. 3d all genes located within 25 kb of each Esrrb-binding region were selected. If a gene was near two or more peaks belonging to distinct clusters, it was associated with one only in a prioritized manner (B1 > B2 > L1 > L2). Next, RPKM values were calculated in several data sets corresponding to nuclear RNA-Seq, Ser2-phosphorylated RNAPII and H3K36me3.

De novo motif discovery. The RSAT peak-motif tool was used on a virtual machine (rsat-vm-2015-10) to identify motifs enriched at bookmarked or lost regions using 6, 7 or 8-bp-long seeds on both strands. The significance of the motifs was established using a background model of Markov order adapted to the length of the sequences. The corresponding TFs were identified on cisBP and JASPAR databases as implemented in RSAT. To search for putative Esrrb-binding sites in all of the regions a background model was used with a Markov order of 1.

RNA-Seq sequencing and alignments. Libraries for RNA-Seq (Supplementary Table 5) were prepared using the TrueSeq Stranded miRNA Sample preparation kit (Illumina) with rRNA-depleted samples (Ribozero Gold kit—Illumina). Strand-specific sequencing (50 bp) was performed on a HiSeq 2500 instrument (Illumina). Reads were aligned to the mm9 genome using Bowtie (allowing 50 hits per read; Supplementary Table 5).

RNA-Seq quantification and identification of differentially expressed genes. Read counts were quantified as RPKM in SeqMonk. High Pearson correlations were observed between all data sets (Supplementary Table 5). BAM/SAM files were generated for each aligned library and used as inputs for the iRNA-Seq pipeline. Raw read counts of exons (mRNA) and introns (pre-mRNA) were normalized and compared using edgeR as implemented in iRNA-Seq. Fold changes and associated FDR derived from multiple testing were generated for each annotated mRNA and pre-mRNA. We considered genes as upregulated in a given cell-cycle phase if displaying an exonic FDR < 0.1, an exonic mRNA FC > 0.5 and an intronic pre-mRNA FC > 0.3; for downregulated genes exonic FC < 0.5, intronic FC < −0.3 and exonic FDR < 0.1. From genes up/downregulated in G2 we subsampled those displaying a −0.6 < FC < 0.6 and FDR > 0.1 in both early and late G1 to identify genes responding to Esrrb levels only in G2.

Correlation with Esrrb-binding regions. A TAD (http://chromosome.sdsc.edu/mouse/hc-rc/download.html) was considered bookmarked when it included at least one bookmarked peak. The fraction of Esrrb-responsive genes at each cell-cycle phase located in a bookmarked TAD or located within 25 kb of a bookmarked peak was scored and compared with randomly selected genes (20 lists of 200 genes).

Statistics and reproducibility. The retention of Esrrb on mitotic chromosomes has been systematically observed by independent researchers (N.F., E.G.T., A.D. and A.M.). Statistical analyses of genome-wide studies were conducted with DESeq, EdgeR and Prism 5.00. Normality was assessed with D´Agostino and Pearson’s test followed by one-way ANOVA using Kruskal-Wallis and Dunn’s multiple comparison tests or Student t-tests. Contingency tables were analysed with Fisher’s exact test.

Data availability. Primary NGS data sets have been deposited at the Gene Expression Omnibus (GEO) under accession number GSE75966. Previously published data sets are available under accession numbers GSE11431, GSE29218, GSE34518, GSE23943 and GSE57092. ChIP-Seq and RNA-Seq results as well as all primer sequences used in this study have been provided in Supplementary Tables 1–5. All other data supporting the findings of this study are available from the corresponding author on request.

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Supplementary Figure 1 Cell lines used in this study. (a) Esrrb immunofluorescence on EKOiE cells cultured in the presence or absence of Dox (24h withdrawal). Scale bars represent 30µm. Images are representative of multiple field acquired in parallel. (b) The self-renewal efficiency of wild-type E14Tg2a, TNG, EKOiE and the respective derivative cell lines was assessed by Alkaline Phosphatase staining of cell colonies obtained 6 days after clonal plating in the indicated conditions. The graph shows the average and s.d of two independent experiments, each performed in duplicate wells (n=4). E14Tg2a cells self-renew exclusively in the presence of LIF. Upon introduction of a Nanog-GFP transgene, self-renewal efficiency is unaffected. EKOiE cells cultured without Dox show extremely severe defects in self-renewal efficiency as compared to Dox-treated cells, demonstrating the importance of Esrrb. In the presence of Dox, EKOiE cells also exhibit LIF-independent self-renewal. Upon the introduction of a constitutive Esrrb-GFP transgene, EKOiE cells are able to self-renew both in presence or absence of Dox; they also exhibit LIF-independent self-renewal. We conclude from this analysis that all the GFP and TdT fusion proteins (Nanog and Esrrb) are functional.
Supplementary Figure 2  Validation of Esrrb retention on mitotic chromosomes. (a) Representative live-imaging of nocodazole-treated EKOiE cells carrying an Esrrb-GFP transgene and cultured in the absence of Dox. DNA was counterstained with Hoechst. Note that all mitotic cells display chromosomal retention of Esrrb. (b) Representative live-imaging of E14Tg2a cells expressing a Nanog-GFP transgene. DNA was counterstained with Hoechst. All mitotic cells display exclusion of Nanog from the chromosomes. (c) Representative live-imaging of nocodazole-treated Esrrb-TdTomato cells. DNA was counterstained with Hoechst. All mitotic cells show enrichment of Esrrb on the chromosomes. (d) Representative Esrrb immuno-staining on a full karyotype from EKOiE cells cultured in the presence (top) or absence (bottom) of Dox. DNA was counterstained with DAPI. (e) SNAP-Cell TMR-Star staining on a karyotype from EKOiE cells constitutively expressing Snap-tagged Esrrb and cultured without Dox. (f) Simultaneous immuno-staining for Nanog and Esrrb on a karyotype obtained from wild-type E14Tg2a cells. Scale bars represent 10µm. Images are representative of multiple fields acquired in 1 (a), 3 (b), 2 (c), 6 (d), 2 (e) and 3 (f) independent repeat experiments.
Supplementary Figure 3 Additional information on Esrrb binding regions.

(a) The table highlights the criteria used to categorise peaks as bookmarked or lost. Classes_1, 2, 3 were considered as bookmarked (see Methods for further details). * Note that in class_1 all peaks (with one exception) were detected in at least one interphase dataset, they cannot be considered as mitotic-specific. (b) Esrrb average binding profile at the 5 classes of regions shown in (a) in both Interphase (Int.) and Mitosis (Mit.). All 5 classes show clear enrichment in interphase. Note that in mitosis Class_5 exhibits a complete depletion of Esrrb, whilst Class_4 shows low levels of Esrrb binding. The Y-axis represents alignment coverage expressed in bases per kb per million. (c) Aligned heat map of Esrrb density across the 5 classes of Esrrb binding regions. Note the focal accumulation of Esrrb at the centre of regions from all 5 classes in interphase (blue). In contrast, only Classes_1, 2, 3 show clear Esrrb binding in mitosis. (d) Heat-map of Esrrb enrichment levels (Log2_RPKM) at all binding regions in interphase (I) and mitosis (M) in all our datasets (E: Esrrb ChIP-Seq; C: Control obtained from Esrrb ChIP-Seq in EKOiE cultured without Dox; Mean represents the average of all shown replicates; Pooled represents enrichment levels after pooling together reads derived from all the replicates; Chen shows the enrichment levels in an independent ChIP-Seq for Esrrb in interphase (E: Esrrb ChIP-Seq; C: negative ChIP control against GFP). Note that across all regions the levels of Esrrb enrichment are significantly higher in interphase than in mitosis and all the controls. Also, note that in mitosis only Classes_1, 2, 3 show consistently high levels of enrichment. (e) Scatter plot representing enrichment levels in interphase (X-axis) versus mitosis (Y-axis) for the 5 classes of Esrrb binding regions. Note that Classes_1, 2 and 3 cluster together whilst Class_4 localises between Classes_1, 2 and 3 and Class_5. These analyses strongly support grouping together Classes_1, 2, 3 as Bookmarked and Classes_4, 5 as Lost, even though a subset of Class_4 regions may display low levels of mitotic binding.
Supplementary Figure 4 ChIP-Seq profiles and validation by qPCR. (a) Six Bookmarked positions selected for spanning different levels of mitotic binding were selected along with five Lost positions and analysed by ChIP-qPCR for Esrrb binding (n=3; error bars represent s.d) in interphase (blue) and mitosis (red) in EKOiE cells cultured with Dox. Interphasic EKOiE cells cultured in the absence of Dox were used to control for the specificity of our assay (dark grey). Moreover, 5 and 10% of chromatin prepared from Dox-treated EKOiE cells was spiked into -Dox chromatin to test whether the contaminant interphasic cells (<5%) in our mitotic preparations (see Methods online) are sufficient to produce a level of enrichment similar to that observed in mitosis. At bookmarked positions we observe mitotic enrichment levels (percentage IP, Y-axis) significantly higher than at Lost positions. Moreover, even after introducing 10% of contaminant chromatin to EKOiE -Dox controls, the observed %IP remains much lower than in mitotic cells for all Bookmarked positions tested. (b) Binding profile at three loci belonging to Class_1 (putative mitotic-specific peaks, see Supplementary Figure 3) illustrating that even though the peak detected in interphase is not statistically significant, it is of similar magnitude to that observed in mitotic cells. (c) ChIP-qPCR validation of interphasic and mitotic binding at the 3 loci shown in (b), presented as in (a). (d) Bookmarked regions include ~55% of ES-specific super-enhancers (e.g. Tbx3 and Klf4 or Sox2 in Figure 2a). Other noticeable regions bookmarked by Esrrb correspond to developmental genes (Otx2), chromatin remodelers (Ino80), Polycomb Group proteins (Jarid2), regulators of DNA methylation (Tet1), signalling mediators (Lif receptor) and metabolic genes (Eno1). Mitosis and interphase are shown at the same scale.
Supplementary Figure 5 B1 regions are more active than L1 regions. (a) Enrichment levels of the indicated factors and chromatin modifications at the 4 clusters identified in Figure 3a. The upper and lower extremities of each shaded area are the 25th and 75th percentile of each dataset across the 4 clusters. (b) Average profiles of the indicated factors and marks across promoters and enhancers belonging to B1 and L1 clusters as shown in Fig. 3.
Supplementary Figure 6 Additional information on the relationships between Esrrb bookmarking and the presence of Esrrb binding motifs. (a) ChIP-qPCR of wild-type (WT) and Mutant (Mut) Esrrb at several genomic positions (the graph shows the average of 2 independent experiments). The two variants are expressed in EKOiE or EKOiE Mut cells under the control of Dox (see methods; cells growing without Dox and not expressing Esrrb are used as a negative control – Ctl). (b) Representative live-imaging of mutant Esrrb-GFP carrying three mutations in residues known to establish specific contacts with the bases of the motif. DNA is counterstained with Hoechst. Note that no mitotic cell displays focal accumulation on the chromosomes. Scale bars represent 10µm. Images are representative of multiple fields acquired in 4 independent repeat experiments. (c) The best motifs discovered are shown together with the percentage of regions containing at least one. Note that the regions containing a long motif may also contain a short motif. (d) ChIP-Seq profile of Esrrb in interphase and mitosis across the Esrrb locus (coverage represents bases per kb per million). The arrow and dashed box indicate the position of the major enhancer of Esrrb shown at higher resolution on the right. The sequence of the long motif identified at this peak is shown; the 3 bases mutated to CCC are underlined. (e) Esrrb ChIP-qPCR at the Esrrb enhancer expressed as percentage of immunoprecipitation (for E14Tg2a n=7 for interphase and n=3 for mitosis; for EEt –edited to short– n=17 for interphase and n=8 for mitosis; error bars represent s.d.) in E14Tg2a and in cells where the long motif was edited into a short motif (AGTTCAGGTCA -> CTGTCAGGTCA). No alteration of Esrrb binding in interphase or mitosis is observed (unpaired t-test). (f) Global quantification (RPKM) of Esrrb enrichment at Bookmarked regions depending on whether they contain a long motif (red; n=1126), only a short motif (blue; n=537), or no Esrrb motif at all (grey; n=197). Note that regions without short or long motifs display significantly higher enrichment in interphase and lower in mitosis. (g) Calculation of the percentage of mitotic retention at the three groups of Esrrb binding regions shown in (f). The presence of a long or a short binding site is associated with increased bookmarking efficiency. (h) Quantification of enrichment levels (RPKM) of several pluripotency TFs in the regions described in (f). All factors are statistically over-enriched at Esrrb binding regions lacking Esrrb motifs. The p-value of the difference between regions with long, only short or no motifs is indicated (Kruskal-Wallis ANOVA followed by Dunn’s multiple comparison test).
**Supplementary Figure 7** Additional information of the Ccna-GFP reporter system and Esrrb-responsive genes. (a) A transgene expressing GFP fused to the domain of Cyclin A2 (Ccna) responsible for its mitotic degradation\(^{41,42}\) was introduced in EKOiE cells that were stained with Hoechst to quantify DNA content. Ccna-GFP shows low fluorescence in M and early G1 and accumulates gradually before the cells enter into S phase. Asynchronous cells (before sorting) are presented along with sorted Early-G1, Late-G1 and G2 cells replated and cultured for additional 8 hours. Approximate sorting gates are shown along with the percentages of cells falling in each gate to illustrate how EKOiE Ccna-GFP cells are able to resume cell cycle progression after sorting; the majority of cells sorted in early-G1 progressed to the beginning of the S phase; cells sorted in late-G1 largely entered S and many reached G2; cells in G2 were delayed but could enter mitosis. (b) Relative expression (Log2) of early-G1 responsive genes (Early-G1 cultured without Dox were set to 0) along the cell cycle. (c) Boxplot showing the log2 fold change of Esrrb-responsive early-G1 genes (n=168 for upregulated genes; n=206 for downregulated genes) in cells cultured in ground state conditions (2i) versus serum-containing medium\(^{43}\). The distribution of the fold changes followed a normal distribution (D’Agostino & Pearson normality test); therefore the p-values were calculated with a one-way Student test against a theoretical fold change of 0.

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\(^{42}\) Gradinaru, C. et al. Nat. Methods 6, 429–430 (2009).

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Supplementary Video Legend

Supplementary Video 1 Time-lapse of Esrrb-GFP cells. The movie shows Esrrb-GFP cells and illustrates the continuous association of Esrrb with the mitotic chromosomes as cells enter, progress and exit mitosis.

Supplementary Table Legends

Supplementary Table 1 Esrrb enrichment at its binding regions. In this table the coordinates of all annotated peaks can be found along with the RPKM values calculated in each independent replicate.

Supplementary Table 2 Genome-wide expression analysis of EKOiE cells in early G1, late G1 and G2. In this file the results of the RNA-Seq conducted for this study are presented in 3 tables (exonic counts, intronic counts, gene expression changes as established using edgeR within the iRNA-seq pipeline).

Supplementary Table 3 Esrrb-dependent genes in early G1, late G1 and G2. In this file 6 tables describing the differentially expressed genes identified at each analysed cell-cycle phase are presented along with their associated FC and FDR.

Supplementary Table 4 Primer sequences. Sequences of all primer pairs used to validate the ChIP-Seq and RNA-Seq are provided.

Supplementary Table 5 Global statistics of NGS analyses. In this file we provide global statistic parameters of our ChIP-Seq (2 tables) and RNA-Seq results (2 tables).