nature cell biology

Chromatin signatures of pluripotent cell lines

Véronique Azuara[1,2,7], Pascale Perry[1], Stephan Sauer[1], Mikhail Spivakov[1], Helle F. Jørgensen[1], Rosalind M. John[1,3], Mina Gouti[1,4], Miguel Casanova[5], Gary Warnes[6], Matthias Merkenschlager[1] and Amanda G. Fisher[1,7]

Epigenetic genome modifications are thought to be important for specifying the lineage and developmental stage of cells within a multicellular organism. Here, we show that the epigenetic profile of pluripotent embryonic stem cells (ES) is distinct from that of embryonic carcinoma cells, haematopoietic stem cells (HSC) and their differentiated progeny. Silent, lineage-specific genes replicated earlier in pluripotent cells than in tissue-specific stem cells or differentiated cells and had unexpectedly high levels of acetylated H3K9 and methylated H3K4. Unusually, in ES cells these markers of open chromatin were also combined with H3K27 trimethylation at some non-expressed genes. Thus, pluripotency of ES cells is characterized by a specific epigenetic profile where lineage-specific genes may be accessible but, if so, carry repressive H3K27 trimethylation modifications. H3K27 methylation is functionally important for preventing expression of these genes in ES cells as premature expression occurs in *embryonic ectoderm development* (*Eed*)-deficient ES cells. Our data suggest that lineage-specific genes are primed for expression in ES cells but are held in check by opposing chromatin modifications.

Transcriptional profiling of stem cells has allowed the identification of genes that are important for stem-cell biology[1–3], but a lack of consensus between data sets has called into question whether a common molecular identity or 'signature' can be accurately defined[4–6]. Parallel studies using epigenetic markers have suggested that epigenetic profiles may be valuable indicators of cell identity[7–13]. ES cells, unlike tissue-specific stem-cell populations, have a broad range of lineage options, consistent with their origin from pluripotential cells within the inner cell mass of the developing embryo[14]. Here, we examine whether ES cells have epigenetic features that distinguish them from stem cells with more restricted developmental potential, such as haematopoietic stem cells (HSCs). Early replication during S phase is widely considered a feature of gene-rich, transcriptionally active regions of the genome and correlates with accessible chromatin carrying acetylated histones, whereas late replication is characteristic of constitutive heterochromatin and some facultative heterochromatin[15–17]. The timing of DNA replication of a panel of transcriptional regulatory genes (which are important for early development or for the specification of neural, haematopoietic or muscle lineages) was compared in pluripotent ES cells, multipotent haematopoietic cell lines and unipotent T lymphocytes (Fig. 1 and see Supplementary Information, Fig. S1). Replication timing was assessed using a previously described method[16] in which cell populations are pulse-labelled with 5-bromo-2′-deoxyuridine (BrdU), fractionated according to cell-cycle stage and the abundance of candidate sequences within newly synthesized DNA determined using semi-quantitative PCR or real-time quantitative (q) PCR. This allows locus replication to be compared in different cell types without the need to synchronise cells. Representative cell-cycle profiles of ES, HSC and T cells with appropriate early (*α-globin*) and late (*Amylase 2–1* and *X141*) replicating controls[16] are shown in the Supplementary Information, Fig. S1a. Replication timing is shown as a gradation of colour from green (earliest) to red (latest; Fig. 1a, b). The replication profiles for each of the three independently derived ES cell lines CCE, OSG and OS25, were remarkably similar. Most genes replicated within the first quarter (65%) or first half (90%) of S phase, including *Oct4*, *Rex1* and *Nanog*[10,11] and genes that are expressed in blood or neural lineages (such as *Ikaros* and *PU.1*, *Sox1* and *neurogenin,* respectively) but not in ES cells. Some genes (*Myf5*, *Neurod* and *Mash1*) and constitutive heterochromatin (*K-satellite* and *X141*) consistently replicated late in ES cells, confirming that the analysis was not inadvertently skewed towards early replication.

The replication profiles of two different sources of haematopoietic stem cells (the IL-3 dependent FDCP (factor-dependent cell-paterson)-mix A4 clone[18] and *Pax5*-deficient progenitors[19]) and of T lymphocytes were distinct from ES cells. Although a substantial proportion

[1]Lymphocyte Development Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK. [2]Current address: Stem Cell Initiative, Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK. [3]Current addres: Cardiff School of Biosciences, Cardiff CF10 3US, UK. [4]Current address: Developmental Biology Laboratory, Foundation for Biomedical Research of the Academy of Athens, Soranou Ephessiou 4, Athens 11527, Greece. [5]Developmental Epigenetics Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK. [6]FACS Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.
[7]Correspondence should be addressed to V.A. or A.G.F. (e-mail: v.azuara@imperial.ac.uk; amanda.fisher@csc.mrc.ac.uk)

Received 22 November 2005; accepted 16 March 2006; published online 29 March 2006; DOI: 10/1038 ncb1403

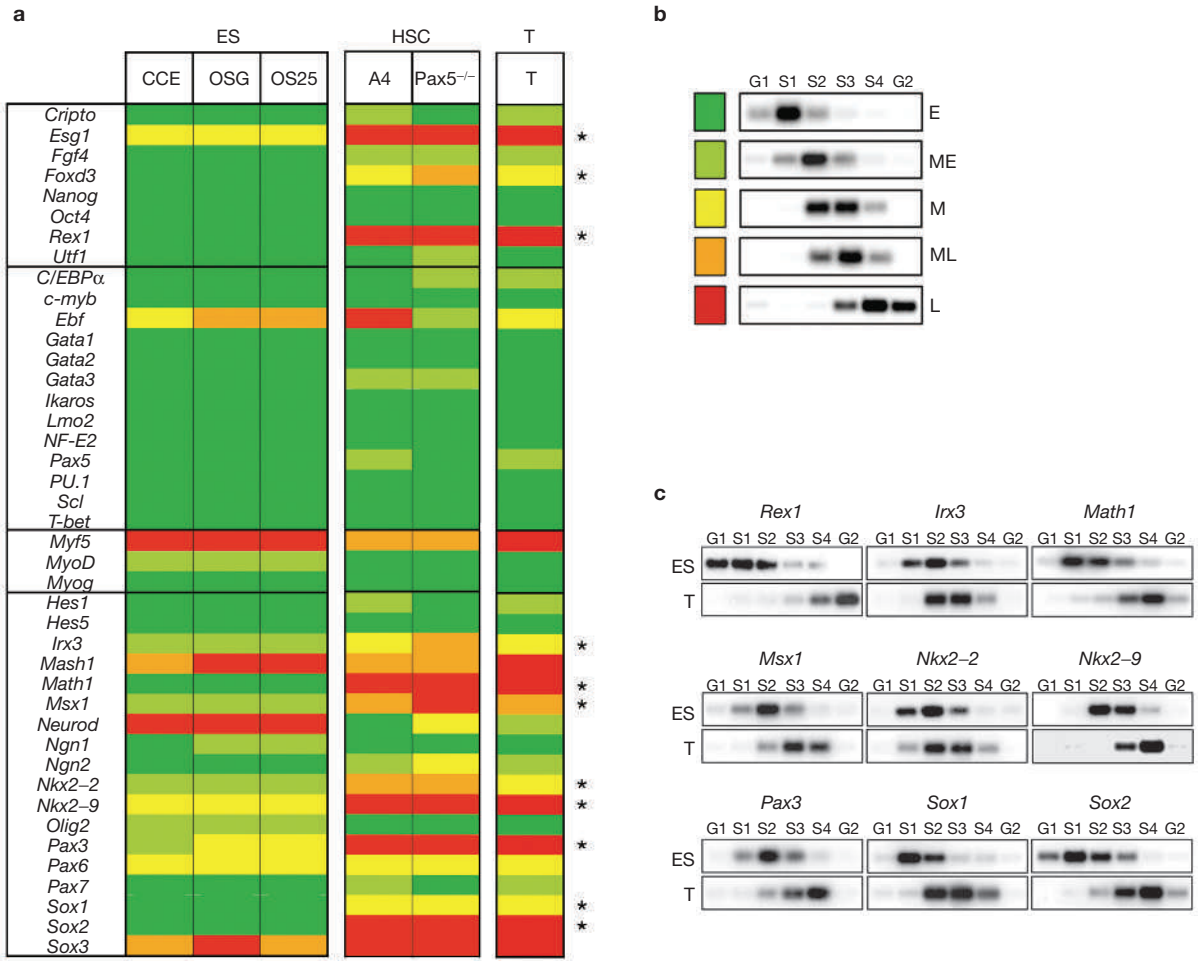


Figure 1 Pluripotent ES cells, multipotent HSC and unipotent T lymphocytes have distinct replication timing profiles. (**a**, **b**) The replication timing comparisons of candidate genes between three embryonic stem cell lines (CCE, OSG, and OS25), the FDCP-mix A4 clone and *Pax5*-deficient pro-B cells representing two populations of haematopoietic progenitors (HSC), and primary T cells are shown (**a**). The replication timing of each gene was defined according to its peak abundance in G1–S1 phase (early, E), S2 phase (middle–early, ME), S2 and S3 phase (middle, M), S3 phase (middle–late, ML) or S4–G2 (late, L), determined in at least two independent experiments. To facilitate a comparison between different cell types, replication timing assignments are represented in a gradation of colours from dark green (E), to light green (ME), to yellow (M), to orange (ML) to red (L) as shown in **b**. Genes marked by asterisks consistently replicate later in HSC and T cells than in ES cells. (**c**) Southern blot comparisons of the abundance of PCR product for *Rex1*, *Irx3, Math1*, *Msx1*, *Nkx2-2*, *Nkx2-9*, *Pax3*, *Sox1* and *Sox2* loci in ES and T cell fractions.

of genes replicated within the first quarter (42–47%) or half of S phase (69–76%), the proportion of late-replicating genes increased to approximately 20% in these haematopoietic cells (Fig. 1a and see Supplementary Information, Fig. S2). Loci that replicated later in HSC and T cells than in ES cells (asterisked in Fig. 1a) could be divided into two groups. The first group, as exemplified by *Rex1,* is expressed early in embryonic development and was previously shown to shift to later replication during ES cell differentiation and transcriptional shut down[10,11]. The second group is comprised of a subset of neural-specific genes that are not thought to be expressed by ES cells, HSC or lymphocytes (*Irx3*, *Math1*, *Msx1*, *Nkx2–2*, *Nkx2–9*, *Pax3* and *Sox1*), but are selectively replicated earlier in ES cells (which retain neural potential) than in haematopoietic cells (which have lost neural potential; Fig. 1c). This observation implies that these epigenetic changes may reflect differences in transcriptional 'competence' and lineage affiliation, rather than overt changes in gene transcription.

To investigate whether replication-timing profiles distinguish different stem cells, a subset of genes in two embryonic carcinoma cell lines, F9 and P19, which share the expression of numerous markers with ES cells but have a much narrower range of lineage potential, was examined[20]. Replication timing was different between the two embryonic carcinoma cell lines and distinct from that of ES cells (Fig. 2). Several neural genes replicated slightly later in F9 cells than in ES cells. F9 cells have a propensity to differentiate towards endoderm lineages[21]. In contrast, a shift to earlier replication of several neural genes was seen in P19 cells, resulting in a profile that was more similar to ES-derived neural precursors[10]. P19 cells preferentially differentiate towards neuronal lineages[22]. Therefore, the replication profiles observed reflect the different potentials of these embryonic carcinoma cell lines.

The later replication of several neural-specific genes in T cells than in ES cells (Fig. 1) may indicate underlying differences in the chromatin structure. To investigate this possibility, chromatin immunoprecipitation analysis was performed using a panel of antibodies that recognise the histone modifications that are characteristic of accessible or repressed chromatin. For this analysis, genes that are selectively expressed in ES

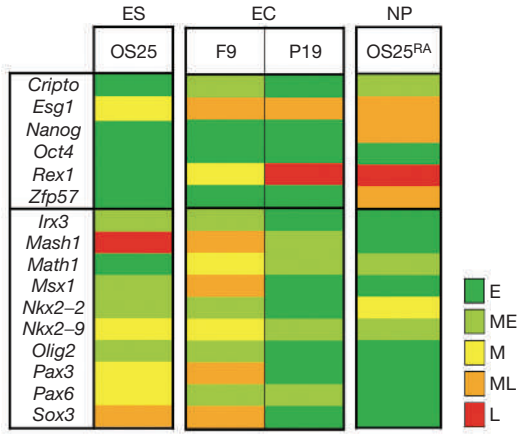


Figure 2 Replication timing profiles of ES and embryonic carcinoma (EC) cells reflect their distinct lineage potential. Replication timing profiles of genes in ES cells (OS25), EC cells (F9 and P19) and in ES-derived ($OS25^{RA}$) neural progenitors (NP) are shown. Neural differentiation was induced as previously described[10]. Colours indicate replication timing as in Fig. 1.

cells (*Oct4* and *Sox2*) or T cells (*Ikaros*) were used as indicators of active chromatin. Genes that are not expressed by ES or T cells, but replicated early (*Hoxa1*) or late (*Myf5*) in both cell types, provided additional controls. The abundance of modified histones at the promoter region (or, where the promoter was not known, 200–400 base pairs (bp) upstream of the transcriptional start site) of each gene was assessed and expressed as a ratio of total H3 detected (relative abundance). As illustrated in Fig. 3, *Math1*, *Sox1*, *Nkx2-2*, *Msx1*, *Irx3*, *Nkx2-9*, *Pax3* and *Sox3* were enriched for acetylated H3K9 in ES cells (coloured bars, top graph), as compared to T cells (open bars, top graph). Other genes that are inactive and early replicating (green) in ES cells such as *Ikaros* and *Hoxa1* also displayed high levels of acetylated H3K9, whereas *Myf5*, a gene that replicated late (red) in both ES and T cells, was not appreciably acetylated. Other markers of accessible chromatin including abundant H3K4 dimethylation (Fig. 3) and trimethylation (see Supplementary Information, Fig. S3) were also detected at several inactive early-replicating genes in ES cells. Levels of acetylated H3K9 and methylated H3K4 detected at these promoters in ES cells were comparable, or only slightly lower, than those seen at actively transcribed genes (such as *Sox2* and *Oct4*). In contrast, significant levels of trimethylated H3K9 were not detected. Unexpectedly, ES cells had high levels of trimethylated H3K27 at the *Math1*, *Sox1*, *Nkx2-2*, *Msx1*, *Irx3*, *Nkx2-9*, *Pax3*, *Sox3* and *Ikaros* promoters (Fig. 3, bottom graph), whereas lower levels were detected at *Myf5* and *Hoxa1*. Trimethylated H3K27 was absent from the promoters of expressed *Oct4* and *Sox2* genes, suggesting that this modification was selectively marking non-expressed genes. Evidently, some loci that are silent in ES cells, but potentially expressed in their differentiated progeny, were simultaneously marked by acetylated H3K9, di- and trimethylated H3K4 and trimethylated H3K27 — an unusual combination of apparently conflicting chromatin modifications. This configuration was not seen at all inactive loci in ES cells as *Myf5* (Fig. 3), *Mash1* (ref. 23), *Pl1* and *Tpbpa* (J. Santos and V. A., unpublished observations) were hypoacetylated and displayed lower levels of H3K27 methylation.

The functional significance of this chromatin profile was examined using ES cells that were genetically deficient in *Eed*. Eed associates with and is required for Ezh2 histone methyltransferase (HMTase) function and H3K27 methylation[24,25] (see Supplementary Information, Fig. S4). ES cells lacking Eed can differentiate, to some extent, both *in vitro* (as embryoid bodies) and *in vivo* (within chimeric embryos)[26] but development is compromised as *Eed*-deficient embryos die shortly after gastrulation[27]. Two independent *Eed* null ($Eed^{-/-}$) ES cell lines (G8.1 and B1.3) were generated and RT–PCR was used to compare the expression of embryonic genes and neural-associated genes in these cells. Both lines expressed *Oct4* and *Rex1* transcripts at comparable levels to wild-type ES cells, whereas *Sox2* expression was slightly reduced (Fig. 4a). In contrast, the neuronal genes *Msx1* and *Pax3* were upregulated 3–10-fold in *Eed*-deficient ES cells, and *Math1* and *Nkx2–2* were increased more than 15-fold (Fig. 4a). To verify that this increased expression is the consequence of *Eed* loss rather than a reflection of the different genetic backgrounds of these cell lines, *Eed* heterozygous ES cells were also derived from genetically matched blastocysts. As shown in Fig. 4b, *Oct4*, *Rex1, Sox1, Sox2* and *Sox3* genes were expressed at similar levels in *Eed*-deficient (G8.1, B1.3) and *Eed* heterozygous ES cells (2.2). However, expression levels of *Msx1*, *Pax3*, *Math1* and *Nkx2.2* were consistently lower in *Eed* heterozygous than *Eed*-deficient ES cell lines (Fig. 4b), confirming that loss of *Eed* (and H3K27 methylation) results in the upregulation of several neural genes in ES cells.

To assess whether loss of methylated H3K27 in *Eed*-deficient ES cells results in global changes in chromatin accessibility, the replication profiles of $Eed^{-/-}$ ES cells was examined. *Rex1*, a locus that switches from early to late replication when ES cells commit to neural progenitor fate[10], and six neighbouring loci that span a further 4.4 Mb, replicated at comparable times in *Eed*-deficient and wild-type ES cells (Fig. 5a). Appropriate early replication of *Oct4,* and late replication of *Mash1* and constitutive heterochromatin (*γ-satellite* and *X141*), was also preserved in *Eed*-deficient cells (Fig. 5b). The replication of neural-specific genes (Fig. 5c), including those genes that we had previously shown to be upregulated in mutant ES cells, was slightly retarded in *Eed*-deficient (G8.1, B1.3) cells when compared with wild-type ES cells, but was clearly different from ES cell-derived neuronal progenitors (Fig. 2 and ref. 10). Thus, loss of H3K27 methylation resulted in the premature expression of several neural-specific genes in ES cells, but had modest effects on the timing of locus replication.

In this study we show that several inactive, neural-specific genes replicate earlier in ES cells than in HSC and lymphocytes and are also enriched for acetylated H3K9 and methylated H3K4. Unexpectedly, these genes also have trimethylated H3K27 — a potential mediator of Polycomb group-dependent gene repression and frequent correlate of silent chromatin[25]. Although the Polycomb repressor complex (PRC) 2 is known to be abundant in ES cells, and is implicated in the silencing of the inactive X chromosome in differentiating ES cells[28,29], its function in undifferentiated ES cells is unclear. Here, we show that in the absence of *Eed* and H3K27 methylation, several neural-specific genes are expressed, implicating PRC2 in preventing the inappropriate expression of some neural-specific genes in ES cells. Several genes that are required for the specification of alternative lineages — including mesoderm (*Flk-1)*, endoderm (*Gata4* and *HNF4*) and extraembryonic tissue (*Gata6* and *Cdx2*) — are also upregulated in *Eed*-deficient ES cell lines (S. Giadrossi and H. J., unpublished observations). Although insufficient for global predictions of the role of PRC2, the finding that markers of active (acetylated H3K9 and dimethylated H3K4) and inactive chromatin (trimethylated H3K27) coexist at the promoters of lineage-specific genes in ES cells is important as it suggests that lineage induction may

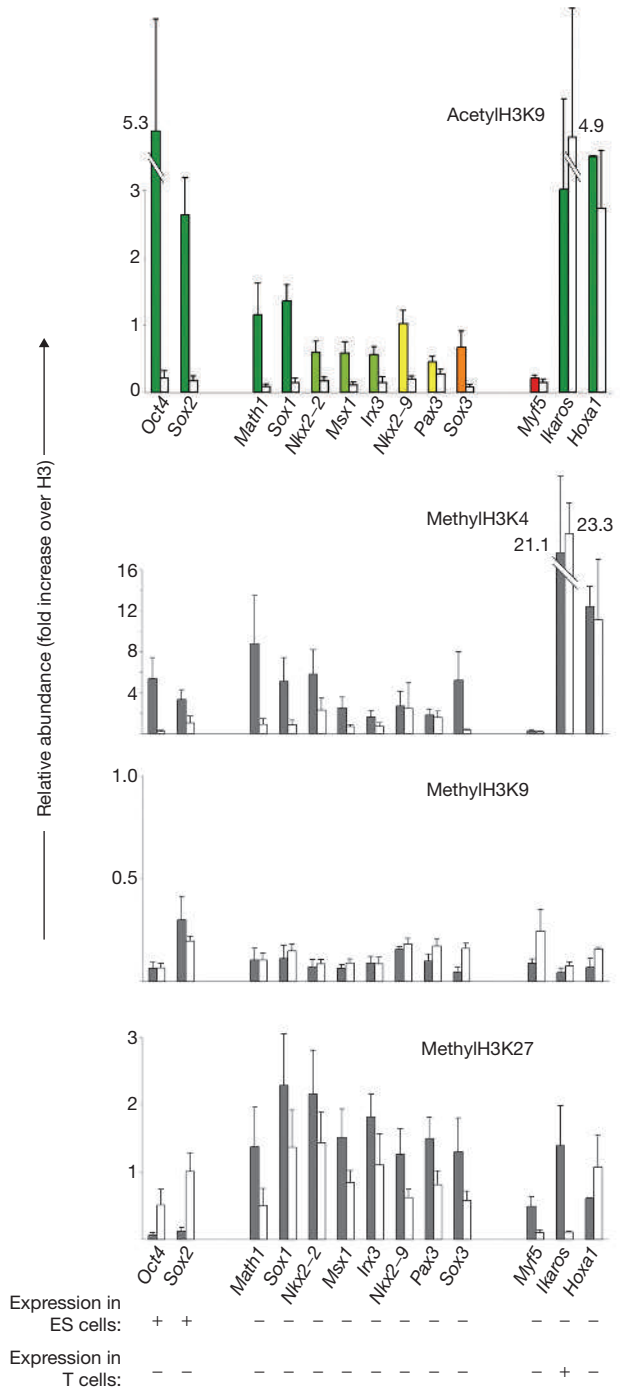


Figure 3 Markers of active and repressed chromatin are simultaneously present at silent tissue-specific genes in undifferentiated ES cells. The abundance of acetylH3K9, methylH3K4, methylH3K9 and methylH3K27 was assessed in undifferentiated ES cells (coloured or grey bars) or in T cells (open bars) using chromatin immunoprecipitation and qPCR. Values shown are the mean of four independent chromatin immunoprecipitation experiments. Relative abundance indicates the ratio of modified H3 (acetylated or methylated) to unmodified H3 detected at each promoter region, and allows the relative levels of modified histones to be compared in different cell types. Genes are ordered according to their replication timing in ES cells (left to right) and colour-coded as in Fig. 1. Chromatin immunoprecipitation for trimethylated H3K9 was validated using primers for K-satellite repeats (data not shown). Error bars indicate the s.d. of 3–4 individual experiments.

a

	WT ES		*Eed*$^{-/-}$ ES	
	OS25	OSG	G8.1	B1.3
Oct4	100	96	96	127
Rex1	100	152	86	120
Sox2	100	115	46	66

	Embryo head (E15)	WT ES		*Eed*$^{-/-}$ ES	
		OS25	OSG	G8.1	B1.3
Msx1	100	<1	<1	7	3
Pax3	100	<1	<1	10	3
Sox1	100	7	5	7	5
Sox3	100	13	3	9	7
Math1	100	2	2	32	33
Nkx2–2	100	2	1	33	55

b

	Eed$^{-/-}$ ES		*Eed*$^{+/-}$ ES
	G8.1	B1.3	2.2
Oct4	100	180	130
Rex1	100	170	90
Sox2	100	160	120
Msx1	100	80	5
Pax3	100	90	1
Sox1	100	60	110
Sox3	100	120	160
Math1	100	100	10
Nkx2–2	100	120	20

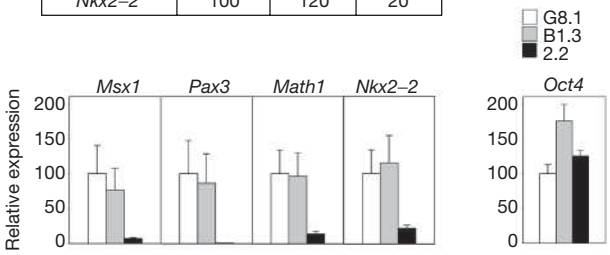


Figure 4 *Eed* is required for repressing neural-specific gene expression in undifferentiated ES cells. The expression of early embryonic genes and neural-specific genes in *Eed*-deficient and wild-type (WT) ES cell lines was assessed by RT–PCR. **(a)** *Oct4, Rex1* and *Sox2* expression is shown in *Eed*$^{-/-}$ ES cells (G8.1, B1.3) relative to OS25-ES (100%, top) and *Msx1, Pax3, Sox1, Sox3, Math1* and *Nkx2.2* expression is shown relative to the signal obtained with total RNA extracted from the head of E15 embryos (100%, bottom). **(b)** Expression of *Oct4, Rex1, Sox2, Msx1, Pax3, Sox1, Sox3, Math1* and *Nkx2.2* in *Eed*$^{-/-}$ and *Eed*$^{+/-}$ ES cells was compared using RT–PCR. Values shown are relative to G8.1 (*Eed*$^{-/-}$; 100%, top). The histograms show that expression of *Msx1, Pax3, Math1* and *Nkx2.2* genes is consistently higher in *Eed*$^{-/-}$ ES cells (G8.1, B1.3) than in *Eed*$^{+/-}$ ES cells (2.2), whereas *Oct4* expression is comparable in all ES cells. Error bars indicate the s.d. of 3–5 independent experiments.

be accomplished, in part, by a mechanism of selective gene derepression. Further work is required to resolve whether active and repressive histone modifications coexist within the same nucleosome, or whether they occupy alternate nucleosomes, and to understand how this gene derepression may function. The observation that replication of some neural-specific genes is delayed in cells that no longer have the capacity for neural differentiation (such as HSC and T cells), compared with cells where neural potential is retained (ES cells and P19), also suggests a role

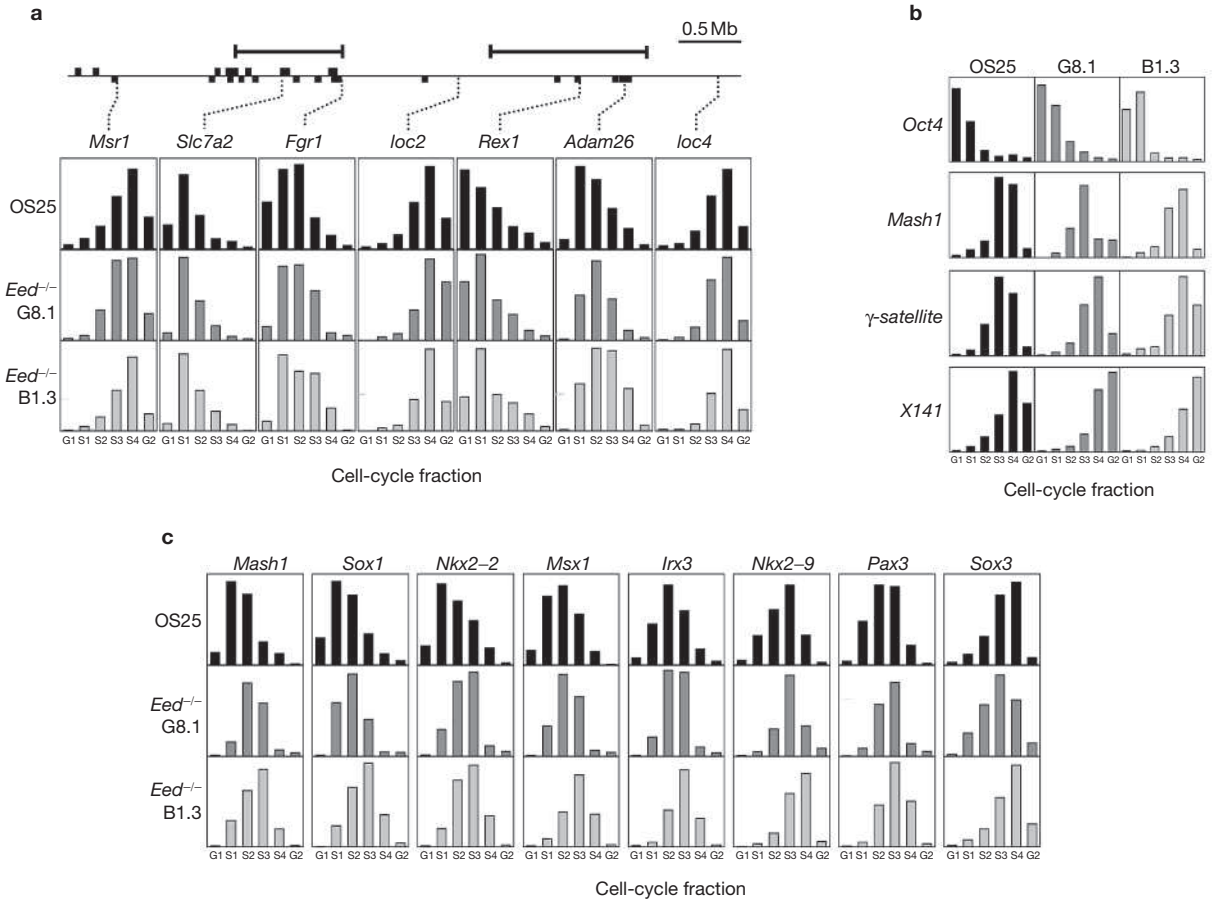


Figure 5 Replication timing is not significantly altered in *Eed*-deficient ES cells despite gene derepression. (**a**) The profile of replication timing across a large genomic region spanning the *Rex1* locus was compared between the wild-type OS25 ES cell line (WT, black), G8.1 (*Eed*$^{-/-}$, dark grey) and B1.3 (*Eed*$^{-/-}$, pale grey). Two subregions that change their replication timing on ES cell differentiation (ref. 10 and P. P. and V. A., unpublished observations) are indicated by horizontal bars and the position of annotated genes by are indicated by boxes on the schematic representation of the locus. (**b**) Early (*Oct4*) and late (*Mash1*, *X141* and K-*satellite*) replication of control loci in wild-type (OS25) and *Eed*-deficient ES cell lines (G8.1 and B1.3). (**c**) The replication timing of neural-specific genes (*Math1*, *Sox1*, *Nkx2-2*, *Msx1*, *Irx3*, *Nkx2-9*, *Pax3*, *Sox3*) in OS25 (WT, black), G8.1 (*Eed*$^{-/-}$, dark grey) and B1.3 (*Eed*$^{-/-}$, pale grey) ES cell lines. Histograms show the relative abundance of locus-specific DNA within each cell cycle fraction (G1, S1–S4 and G2–M phases) as assessed by qPCR.

for epigenetic mechanisms in progressively restricting lineage potential during normal development. Additional loci that replicate later in haematopoietic-restricted cells than ES cells have been identified by microarray analyses (A. Allen and M. Harrison, Oxford Gene Technology, Oxford, UK; personal communication). These include genes that are selectively expressed in the early embryo (*Pramel6*, *Pramel7* and *Dppa4*), or selectively in neural cells (*Otx2*, *Gbx2*, *En1* and *olfactory receptor 128–136*). Taken together, these data suggest that some lineage-specific genes that are accessible in ES cells become late replicating (and less accessible) in committed cells where alternative differentiation pathways are no longer an option.

The precise relationship between DNA replication, chromatin structure and transcription is complex and remains largely unresolved. Our results indicate that early replication correlates with histone acetylation, consistent with results from other studies[10,30–32]. In particular, genes showing high levels of acetylated H3K9 replicated early in S phase regardless of whether they were expressed or not, whereas low levels of acetylated H3K9 were associated with late replication. Previously, we have shown that *Mash1*, a gene that similar to *Myf5* replicates late in undifferentiated ES cells, is also H3K9 hypoacetylated[23]. However, in this case, high levels of di- and trimethylated H3K4 were detected at the *Mash1* promoter in ES cells (reviewed in ref. 33 and S. S., unpublished observations), implying that although H3K4 methylation is a frequent correlate of accessible chromatin, it may be insufficient to predict or determine the early replication of a locus. Similarly, no obvious relationship between several other conventional markers of repressed chromatin (such as H3K9 or H3K27 trimethylation) was evident in our analysis. However, given the small number of loci tested, this does not exclude a function for H3K27 methylation in replication timing, but argues that it may be rare.

Despite loss of H3K27 methylation and transcriptional derepression of several neural specific genes in *Eed*-deficient ES, we show that the replication timing of many genes is not significantly disturbed in mutant ES cells. *Eed*-deficient embryos are compromised in their ability to successfully develop and to execute gastrulation[26,27], even though *Eed*-deficient ES cells contribute to most tissues in chimeric animals[26], indicating that they retain considerable lineage potential. Our study shows that DNA replication profiles may be useful as indicators of cellular differentiation stage and intrinsic lineage potential, are capable of discriminating ES

cells from embryonic carcinoma cells and accurately reflect the lineage preference of individual embryonic carcinoma lines. Replication profiles determined by independent laboratories have also been very consistent[10,11,34,35], suggesting that this robust assay may offer some additional advantages over conventional transcription-based profiling. As replication profiling distinguishes mouse ES cells from progenitors with a more restricted lineage potential (embryonic carcinoma, HSC, neural precursors[10,11] and lymphocytes), generating a reliable epigenetic signature for ES cells seems to be a realistic goal. In this context, the analysis of other sources of pluripotent cells (including cells of the pre- and post-implantation epiblast and embryonic germ cells) could provide important comparisons against which the value of replication and epigenetic profiling in predicting cellular potential can be further interrogated. This will become increasingly important for human ES cells, where the functional verification of embryonic potential *in vivo* is not possible. □

METHODS

Cell culture. ES cells were grown and maintained in an undifferentiated state in GMEM–BHK21 medium plus 10% fetal calf serum (FCS), nonessential amino acids, sodium pyruvate, sodium bicarbonate, L-glutamine, 2-mercaptoethanol, antibiotics and 1000 U ml^{-1} of leukaemic inhibitory factor (ESGRO–LIF), either on irradiated SNL cells as feeder layers (CCE-ES cell line) or directly on gelatin-coated surfaces (CGR8-derived OSG and E14Tg2a-derived OS25 ES cell lines). F9 and P19 embryonic carcinoma cells were grown in ES cell culture conditions. G8.1, B1.3 (*Eed*-deficient) and 2.2 ($Eed^{+/-}$) ES cell lines of normal karyotype were derived using standard methods. The culture medium consisted of DMEM-F12 medium plus 10% FCS, nonessential amino acids, 2-mercaptoethanol, antibiotics and 2000 U ml^{-1} of ESGRO–LIF. Established ES cell lines were genotyped by loss of an *Alu*I restriction site within the mutation[36]. FDCP-mix A4 cells were maintained in IMDM medium plus 10% FCS, antibiotics and 5% (v/v) conditioned medium from the IL-3-expressing myeloma WEHI-3B cell line. Pro-B cell clones derived from *Pax5*-deficient mice were cultured in IMDM medium plus 2% FCS, 2-mercaptoethanol, L-glutamine, antibiotics and 0.03% (w/v) primatone in the presence of IL-7. T lymphocytes isolated from lymph nodes of 6–8 week old C57BL/6 mice were prepared and cultured as previously described[16].

Replication timing analysis. BrdU labelling, fixation in 70% cold ethanol, cell cycle fractionation by flow cytometry and isolation of BrdU-labelled DNA by immunoprecipitation were carried out as previously described[16], with the following modifications to BrdU-pulse labelling times: 30 min (ES and embryonic carcinoma cells), 45 min (T cells) and 60 min (FDCP-mix A4 cells and *Pax5*-deficient pro-B cells). The abundance of newly replicated DNA was determined using 500–1000 cell equivalents of BrdU-labelled DNA by semi-quantitative PCR amplification and Southern blot hybridization as previously described[16] or by real-time qPCR amplification. Primer sequences used for this analysis are available on request.

To confirm uniform recovery of BrdU labelled DNA, fractions were 'spiked' with *Drosophila melanogaster* BrdU-labelled DNA before immunoprecipitation. Using primers for the *D. melanogaster Gbe* locus and PCR conditions within the dynamic range, *Gbe* DNA was detected in each of the cell-cycle fractions at similar levels.

Chromatin immunoprecipitation analysis. Approximately 1×10^8 OS25-ES cells and T cells were harvested and crosslinked with 1% paraformaldehyde for 10 min at room temperature. After quenching of paraformaldehyde with 125 mM glycine, whole-cell extracts were prepared for use in chromatin immunoprecipitation assays. The size of sonicated chromatin was around 300–1000 bp as analysed on agarose gels. Fragmented chromatin (140 µg) was subjected to immunoprecipitation with 3 µl anti-acetyl-H3K9 antibody (07–352; Upstate Biotechnology, Lake Placid, NY), 2.5 µl anti-dimethyl-H3K4 (07–030; Upstate), 2.5 µl anti-trimethyl-H3K4 (ab8580; Abcam Ltd., Cambridge, UK), 5 µl anti-trimethyl-H3K9 (07–442; Upstate), 5 µl anti-trimethyl-H3K27 (07–449; Upstate), 2 µl of a rabbit anti-mouse-IgG antiserum (negative-control; Dako Inc., Carpinten, CA) and 2 µl of anti-H3-carboxy terminal antibody (1791; Abcam). After elution of immune complexes, DNA was resuspended in 80 µl TE (10 mM Tris, 1 mM EDTA at pH 8.0) solution. After elution of immune complexes, DNA was resuspended in 80 µl TE solution. Quantification of precipitated DNA was performed using real-time qPCR amplification. Acetyl-H3K9, methyl-H3K4, methyl-lH3K9 or methyl-H3K27 levels were normalised against total H3 detected, and the ratio of modified-H3 to H3 was denoted as 'relative abundance'. Primer sequences used in this analysis are available on request.

RT–PCR analysis. RNA extraction from G8.1, B1.3 ($Eed^{-/-}$), 2.2 ($Eed^{+/-}$) ES cells, OS25 and OSG wild-type ES cells was performed using RNeasy protect mini kit (Qiagen, Venlo, The Netherlands) and RNase-free DNase set (Qiagen) for digestion of residual DNA. Total RNA (1.5 µg) was then reverse transcribed using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) and cDNA of interest quantified using real-time qPCR amplification. The data were normalized to at least two housekeeping genes including *Hmbs*, *GAPD*, *Ubc* and *Ywhaz* as previously described[37]. Primer sequences used for this analysis are available on request.

Real–time qPCR analysis. Real-time PCR analysis was carried out on a Chromo4 DNA engine (Biorad, Hercules, CA), running the following program: 94 °C for 15 min, then 40 cycles of 94 °C for 30 s, 60 °C (or 55 °C) for 30 s, 72 °C for 1 min, followed by plate read. PCR reactions included 1× SYBR green PCR master mix (Qiagen), 300 nM primers and 2 µl of template in a 50 µl reaction volume.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGMENTS

We thank A. Smith for ES cell lines, M. Dexter for the FDCP-mix A4 clone, M. Busslinger for *Pax5*-deficient pro-B cells, R. Lovell-Badge for the F9-EC line and V. Episkopou for the embryonic carcinoma cell lines F9 and P19. E. O'Connor, R. Brough, G. Reed and R. Newton are thanked for help and advice and A. Allen, M. Harrison, T. Reed and Z. Szarka (at Oxford Gene Technology, Oxford, UK). J. Santos and S. Giadrossi for communicating unpublished information. S. Giadrossi, N. Brockdorff and M. Raff are acknowledged for advice and critical reading of the manuscript. This work was supported by the Medical Research Council (MRC) and by a MRC Collaborative Career Development Fellowship in Stem Cell Research funded by the Parkinson's disease Society (V.A.).

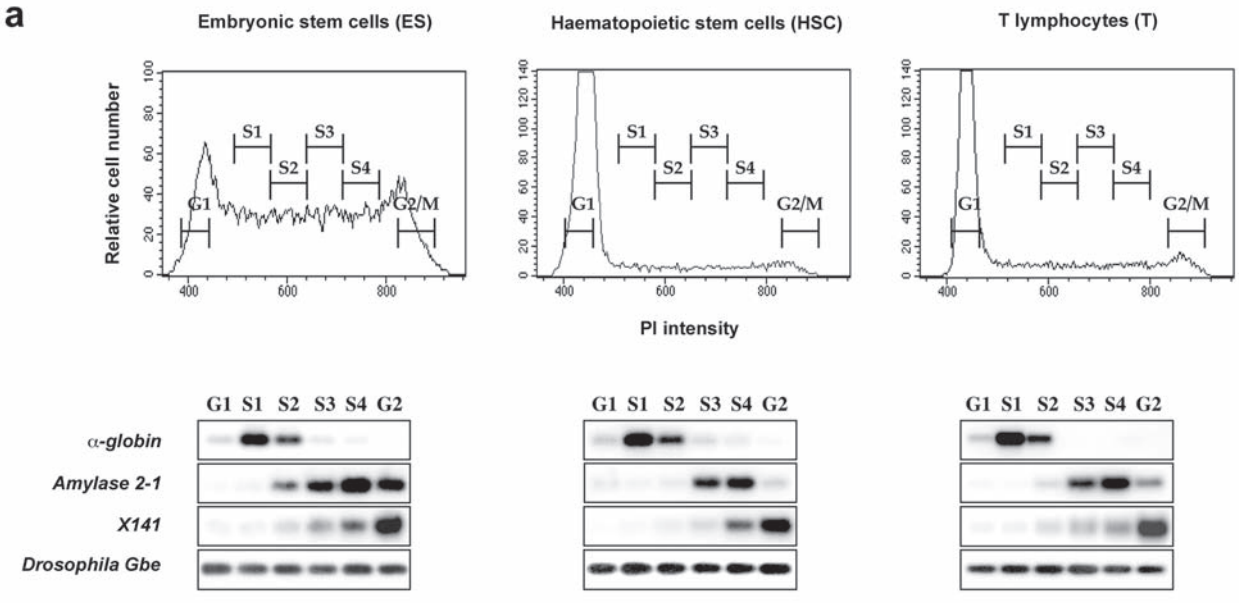
COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturecellbiology
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Terskikh, A.V. *et al.* From hematopoiesis to neuropoiesis: evidence of overlapping genetic programs. *Proc. Natl Acad. Sci. USA* **98,** 7934–7939 (2001).
2. Ivanova, N.B. *et al.* A stem cell molecular signature. *Science* **298,** 601–604 (2002).
3. Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C. & Melton, D.A. "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* **298,** 597–600 (2002).
4. Fortunel, N.O. *et al.* Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". *Science* **302,** 393 (2003).
5. Evsikov, A.V. & Solter, D. Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". *Science* **302,** 393 (2003).
6. Vogel, G. Stem cells. 'Stemness' genes still elusive. *Science* **302,** 371 (2003).
7. Bernstein, B.E. *et al.* Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell* **120,** 169–181 (2005).
8. Schubeler, D. *et al.* The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes Dev.* **18,** 1263–1271 (2004).
9. Woodfine, K. *et al.* Replication timing of the human genome. *Hum. Mol. Genet.* **13,** 191–202 (2004).
10. Perry, P. *et al.* A dynamic switch in the replication timing of key regulator genes in embryonic stem cells upon neural induction. *Cell Cycle* **3,** 1645–1650 (2004).
11. Hiratani, I., Leskovar, A. & Gilbert, D.M. Differentiation-induced replication-timing changes are restricted to AT-rich/long interspersed nuclear element (LINE)-rich isochores. *Proc. Natl Acad. Sci. USA* **101,** 16861–16866 (2004).
12. Gilbert, N. *et al.* Chromatin architecture of the human genome: gene-rich domains are enriched in open chromatin fibers. *Cell* **118,** 555–566 (2004).
13. Rugg-Gunn, P.J., Ferguson-Smith, A.C. & Pedersen, R.A. Epigenetic status of human embryonic stem cells. *Nature Genet.* **37,** 585–587 (2005).
14. Evans, M. & Hunter, S. Source and nature of embryonic stem cells. *C. R. Biol.* **325,** 1003–1007 (2002).
15. Schubeler, D. *et al.* Genome-wide DNA replication profile for *Drosophila melanogaster*: a link between transcription and replication timing. *Nature Genet.* **32,** 438–442 (2002).

16. Azuara, V. *et al*. Heritable gene silencing in lymphocytes delays chromatid resolution without affecting the timing of DNA replication. *Nature Cell Biol.* **5,** 668–674 (2003).
17. Gilbert, D. M. In search of the holy replicator. *Nature Rev. Mol. Cell Biol.* **5,** 848–855 (2004).
18. Spooncer, E., Heyworth, C. M., Dunn, A. & Dexter, T. M. Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors. *Differentiation* **31,** 111–118 (1986).
19. Nutt, S. L., Heavey, B., Rolink, A. G. & Busslinger, M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* **401,** 556–562 (1999).
20. Chambers, I. & Smith, A. Self-renewal of teratocarcinoma and embryonic stem cells. *Oncogene* **23,** 7150–7160 (2004).
21. Hogan, B. L., Taylor, A. & Adamson, E. Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* **291,** 235–237 (1981).
22. Jones-Villeneuve, E. M., Rudnicki, M. A., Harris, J. F. & McBurney, M. W. Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Mol. Cell Biol.* **3,** 2271–2279 (1983).
23. Williams, R. R. *et al*. Neural induction promotes large-scale chromatin reorganisation of the *Mash1* locus. *J. Cell Sci.* **119,** 132–140 (2006).
24. Montgomery, N. D. *et al*. The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. *Curr. Biol.* **15,** 942–947 (2005).
25. Peters, A. H. & Schubeler, D. Methylation of histones: playing memory with DNA. *Curr. Opin. Cell Biol.* **17,** 230–238 (2005).
26. Morin-Kensicki, E. M., Faust, C., LaMantia, C. & Magnuson, T. Cell and tissue requirements for the gene *eed* during mouse gastrulation and organogenesis. *Genesis* **31,** 142–146 (2001).
27. Faust, C., Lawson, K. A., Schork, N. J., Thiel, B. & Magnuson, T. The Polycomb-group gene *eed* is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development* **125,** 4495–4506 (1998).
28. Silva, J. *et al*. Establishment of histone H3 methylation on the inactive X chromosome requires transient recruitment of Eed—Enx1 polycomb group complexes. *Dev. Cell* **4,** 481–495 (2003).
29. Plath, K. *et al*. Role of histone H3 lysine 27 methylation in X inactivation. *Science* **300,** 131–135 (2003).
30. Vogelauer, M., Rubbi, L., Lucas, I., Brewer, B. J. & Grunstein, M. Histone acetylation regulates the time of replication origin firing. *Mol. Cell* **10,** 1223–1233 (2002).
31. Lin, C. M., Fu, H., Martinovsky, M., Bouhassira, E. & Aladjem, M. I. Dynamic alterations of replication timing in mammalian cells. *Curr. Biol.* **13,** 1019–1028 (2003).
32. Zhang, J., Xu, F., Hashimshony, T., Keshet, I. & Cedar, H. Establishment of transcriptional competence in early and late S phase. *Nature* **420,** 198–202 (2002).
33. Ballas, N. & Mandel, G. The many faces of REST oversee epigenetic programming of neuronal genes. *Curr. Opin. Neurobiol.* **15,** 500–506 (2005).
34. Hansen, R. S., Canfield, T. K., Lamb, M. M., Gartler, S. M. & Laird, C. D. Association of fragile X syndrome with delayed replication of the *FMR1* gene. *Cell* **73,** 1403–1409 (1993).
35. Gomez, M. & Brockdorff, N. Heterochromatin on the inactive X chromosome delays replication timing without affecting origin usage. *Proc. Natl Acad. Sci. USA* **101,** 6923–6928 (2004).
36. Wang, J. *et al*. Imprinted X inactivation maintained by a mouse Polycomb group gene. *Nature Genet.* **28,** 371–375 (2001).
37. Vandesompele, J. *et al*. Accurate normalization of real-time quantitative RT—PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, DOI: 10.1186/gb-2002-3-7-research0034 (2002).



b

Embryonic	
Cripto	(9, F2)
Esg1	(X, A1.2)
Fgf4	(7, F5)
Foxd3	(4, C6)
Nanog	(6, F2)
Oct4	(17, B2)
Rex1	(8, B1.3)
Utf1	(7, F5)
Zfp57	(17, B3)

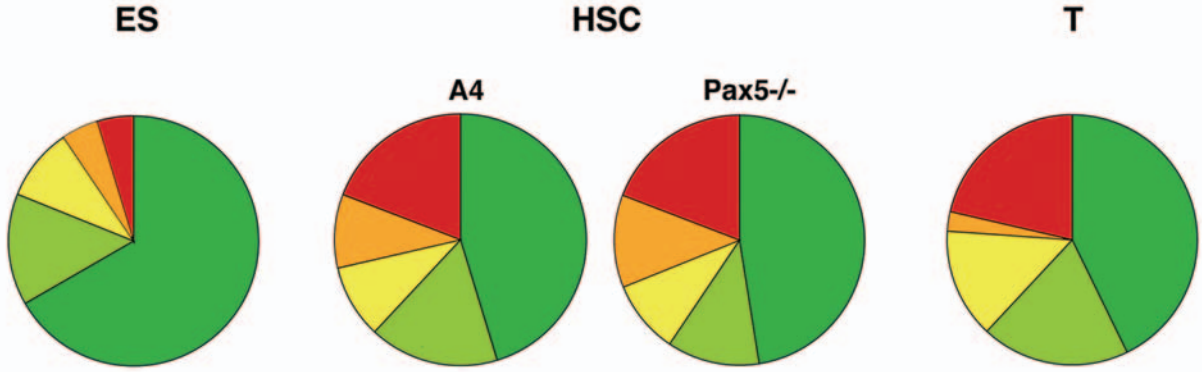
Myogenic	
Myf5	(10, D1)
MyoD	(7, B2)
Myog	(1, E4)

Haematopoietic	
C/EBP α	(7, B1)
c-Myb	(10, A3)
Ebf	(11, B1.1)
Gata1	(X, A1.1)
Gata2	(6, D2)
Gata3	(2, A1)
Ikaros	(11, A2)
Lmo2	(2, E2)
NF-E2	(15, F3)
Pax5	(4, B2)
PU.1	(2, E1)
Scl	(4, C7)
T-bet	(11, D)

Neuronal	
Hes1	(16, B2)
Hes5	(4, E2)
Irx3	(8, C5)
Mash1	(10, C2)
Math1	(6, C1)
Msx1	(5, B2)
Neurod	(2, D)
Ngn1	(13, B2)
Ngn2	(3, H1)
Nkx2-2	(2, H1)
Nkx2-9	(12, C2)
Olig2	(16, C4)
Pax3	(1, C4)
Pax6	(2, E3)
Pax7	(4, D3)
Sox1	(8, A2)
Sox2	(3, B)
Sox3	(X, A5)

Figure S1 Replication timing analysis of genes using flow cytometry and BrdU-labelling. ES (CCE-ES), haematopoietic progenitors (HSC, FDCP-Mix A4 clone) and activated T cells were pulse-labelled with BrdU, stained with propidium iodide (PI) and separated by cell sorting into six fractions (G1, S1, S2, S3, S4 and G2/M) according to DNA content. (a) Typical profiles of PI intensity for each cell population (top). The timing of locus replication was determined by analysing the abundance of locus-specific DNA within newly synthesised (BrdU-containing) strands isolated from each cell fraction following anti-BrdU immunoprecipitation. Southern blots of PCR products from each cell population (bottom). Early-replicating (*α-globin*) and late-replicating loci (*Amylase 2-1* and X141) are indicated. The *D. melanogaster Gbe* gene provides a control to ensure uniform recovery of BrdU-labelled DNA. (b) 43 candidate genes selected for this analysis are listed. These genes mostly encode transcription factors, which are either exclusively expressed in ES cells or important for different cell fate specifications. The chromosomal and band assignments for each locus are indicated in brackets according to ENSEMBLv20 (www.ensembl.org).

a



b

	CCE	OSG	OS25	A4	Pax5-/-	T
	66.7%	64.3%	64.3%	45.2%	47.6%	42.8%
	14.3%	14.3%	14.3%	16.7%	11.9%	19.1%
	9.5%	9.5%	9.5%	9.5%	9.5%	14.3%
	4.75%	2.4%	4.75%	9.5%	11.9%	2.4%
	4.75%	9.5%	7.15%	19.1%	19.1%	21.4%

Figure S2 Comparison of replication timing profiles between cells at different stages of development. Shown in (a) are the proportion of genes that replicate during early (E), middle/early (ME), middle (M), middle/late (ML) or late (L) S-phases in ES (CCE-ES), in haemopoietic progenitors FDCP-Mix A4 (A4) and *Pax5-/-* pro-B (Pax5-/-) and activated T cells, represented as a pie-chart. The proportion of candidate loci that replicate in each S-phase fraction, expressed as a percentage of the total, for ES cell lines (CCE, OSG and OS25), haematopoietic progenitors (A4 and Pax5-/-) and T lymphocytes are summarised in (b).

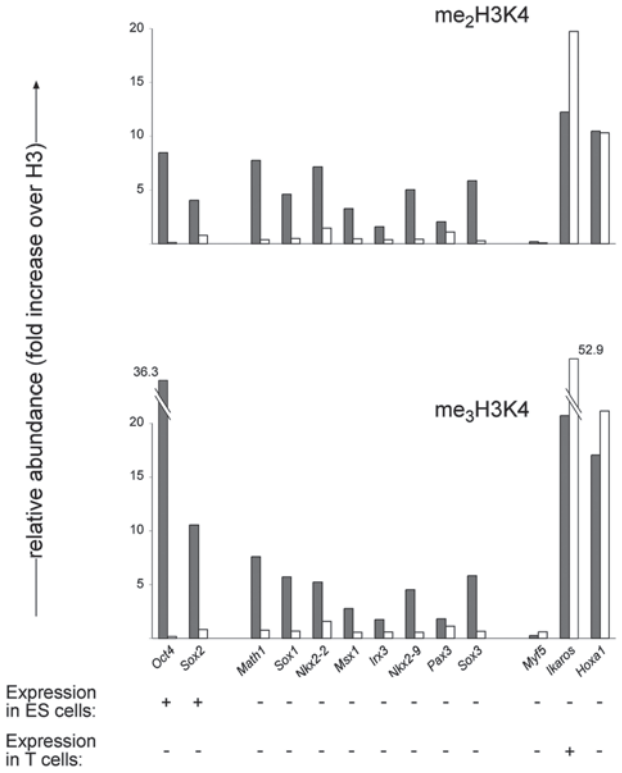


Figure S3 Comparison of me_2H3K4 and me_3H3K4 levels at the promoter regions of silent tissue-specific genes in ES and T cells. Levels of me_2H3K4 and me_3H3K4 were assessed using ChIP and qPCR. Results show the abundance of di- and tri-methylated H3K4 (expressed relative to unmodified histone H3) detected at each locus, where values for undifferentiated ES cells (grey bars) and for T cells (open bars) are shown. Primers were designed to recognise the promoters (or where not known, 200-400bp upstream of the transcriptional start site) of *Oct4*, *Sox2*, *Math1*, *Sox1*, *Nkx2-2*, *Msx1*, *Irx3*, *Nkx2-9*, *Pax3*, *Sox3*, *Myf5, Ikaros* and *Hoxa1* genes. A close correlation between me_2H3K4 and me_3H3K4 levels at active (*Oct4* and *Sox2*) and inactive genes in ES cells (*Math1*, *Sox1*, *Nkx2-2*, *Msx1*, *Irx3*, *Nkx2-9*, *Pax3*, *Sox3*, *Myf5, Ikaros* and *Hoxa1*) indicates that H3K4 trimethylation does not selectively mark actively expressed genes.

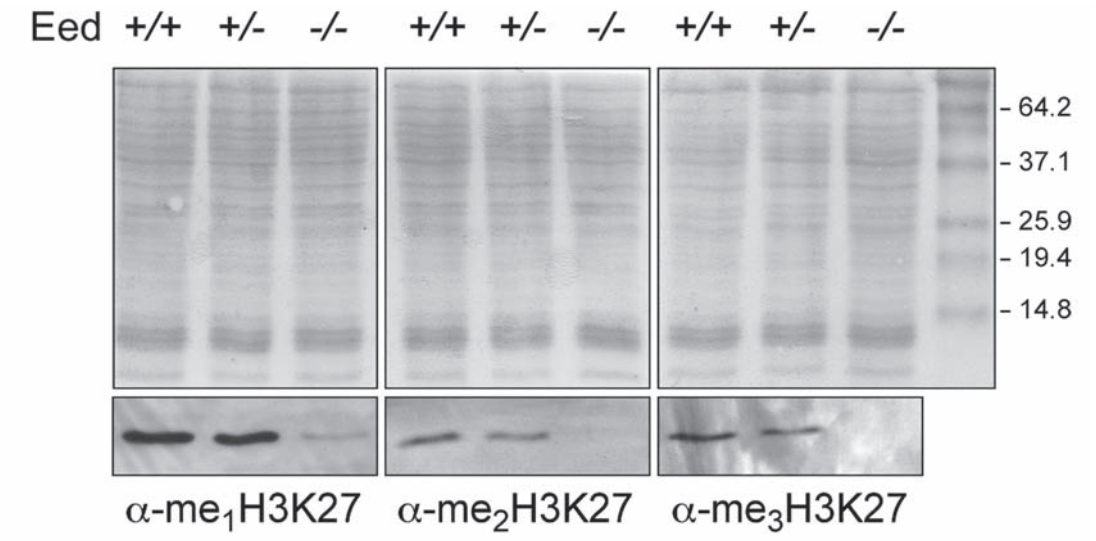


Figure S4 Global reduction of H3K27 methylation levels in Eed deficient ES cells. Whole cell lysates from wild type, heterozygote and Eed-deficient ES cells (Eed+/- line 2.2, Eed -/- line G8.1) were analysed by Western blotting using antibodies to mono-, di, or trimethylated H3K27. Equivalent protein loading is shown by staining with ponceau red (top panel), and markers indicate approximate molecular size (in kD). Trimethylated and dimethylated H3K27 was not detected in Eed null ES cells, and levels of monomethylated H3K27 were markedly reduced.

SUPPLEMENTARY INFORMATION METHODS

Western Blot analysis. Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Bio-Rad minigel system, with 12% Polyacrylamide (PAA) separating gels and 5% PAA stacking gels. Proteins were blotted onto nitrocellulose membranes and incubated for 30 min with blocking buffer (3% (w/v) fat free milk powder in 220mM NaCl, 10mM Tris base, pH 7.4). Primary antibodies (anti-monomethyl-Histone H3K27 [Upstate, 07-448], anti-dimethyl-Histone H3K27 [Upstate, 07-452] and anti-trimethyl-Histone H3K27 [Upstate, 07-449]) were applied to the blot at a concentration of 1:500 for 2 hours at room temperature, blots were washed 3 times for 5 min with blocking buffer, followed by incubation with a secondary anti rabbit peroxidase-coupled antibody (according to manufacturers instructions) for 30 min at room temperature and then ECL detection was used to visualize antibody binding.