Development 136, 715-721 (2009) doi:10.1242/dev.028548

REST selectively represses a subset of RE1-containing neuronal genes in mouse embryonic stem cells

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REST is a transcriptional repressor that targets a group of neuronal genes in non-neuronal cells. In embryonic stem (ES) cells, REST has been implicated in controlling the expression of transcription factor genes that are crucial for lineage determination and for maintaining ES cell potential. Here, we asked whether REST directly regulates neural-specifying genes in mouse ES cells using siRNA-mediated REST knockdown and ES cells that lack functional REST protein as a result of gene targeting. Loss of REST did not affect the expression of any of ten transcription factor genes known to promote neural commitment and did not affect the expression of several microRNAs, including *miR-21*, a putative REST target in ES cells. REST-deficient ES cells retained the ability to self-renew and to undergo appropriate differentiation towards mesoderm, endoderm and ectoderm lineages upon LIF withdrawal. Genome-wide expression profiling showed that genes that were deregulated in the absence of REST were preferentially expressed in the brain and highly enriched for the presence of canonical REST binding sites (RE1). Chromatin immunoprecipitation studies confirmed these genes as direct targets of REST in ES cells. Collectively, these data show that REST selectively silences a cohort of neuronal genes in ES cells.

KEY WORDS: REST (NRSF), Embryonic stem cells, Gene silencing, Neurogenesis

INTRODUCTION

Neural fate specification is controlled by the interplay of transcription factors and signalling networks that cooperate to establish a temporal and spatial identity of cells in the nervous system (Guillemot, 2007; Levine and Brivanlou, 2007). Many aspects of neurogenesis can be recapitulated in vitro using mouse or human embryonic stem (ES) cell differentiation systems (Eiraku et al., 2008; Giadrossi et al., 2007). This includes the correct induction of proneural bHLH transcription factors such as MASH1 (ASCL1 – Mouse Genome Informatics), NEUROG1/2 (NGN1/2) and MATH1 (ATOH1) and of other key transcription factors (e.g. PAX6, SOX1) that are crucial for neural patterning, commitment and differentiation (Bertrand et al., 2002). In undifferentiated ES cells, the genes encoding these factors appear to be functionally 'primed' (reviewed by Spivakov and Fisher, 2007) such that phosphorylated RNA polymerase II is bound throughout the promoter and coding regions but the genes are prevented from being productively expressed by the action of repressors, including those of the Polycomb group family (Guenther et al., 2007; Stock et al., 2007).

The neuronal repressor REST (RE1-silencing transcription factor, also known as NRSF) has been proposed to negatively regulate lineage-specific gene expression in undifferentiated ES cells (Ballas et al., 2005; Singh et al., 2008). REST is abundant in ES cells, where its expression is regulated by pluripotency factors such as OCT4 (POU5F1) and NANOG (Boyer et al., 2005; Kim et al., 2008; Loh et al., 2006). The REST protein binds in a sequence-specific manner to a 21-bp motif referred to as an RE1 element (Chong et al., 1995; Schoenherr and Anderson, 1995). Using the RE1 consensus sequence, REST binding sites have been predicted computationally (Bruce et al., 2004; Wu and Xie, 2006), and REST binding close to many genes that are expressed by mature neurons has been demonstrated by genome-wide chromatin immunoprecipitation analysis as well as by candidate studies in mouse and human cells (Johnson et al., 2007; Johnson et al., 2008; Otto et al., 2007; Sun et al., 2005). Despite this, the function of REST at individual sites in ES cells remains largely unresolved, as does the question of whether tissue-specific occupancy of RE1 sites accounts for a selective function for REST in different cell types (Johnson et al., 2008; Sun et al., 2005).

REST was initially described as a transcriptional repressor in non-neuronal tissues (Chong et al., 1995; Schoenherr and Anderson, 1995). Subsequent biochemical studies revealed that REST interacts with several different co-repressors, implying that REST might mediate transcriptional repression by a variety of distinct mechanisms (Ballas and Mandel, 2005). A biological function for REST during embryonic development has been implied from the analysis of *Rest*-null mice, in which development appears largely normal until embryonic day (E) 9, when forebrain malformation becomes evident and the embryos die (at E9.5-10.5) from unidentified causes (Chen et al., 1998). In ES cells, REST has been implicated in diverse functions, including the repression of lineage-specific genes [e.g. *Mash1*, *Ngn2*, brachyury (*Bry*, *T*), *Gata4*, *Sox18*, *Calb* (*Calb1*)], microRNA genes (Ballas et al., 2005; Singh et al., 2008), and in maintaining the expression of pluripotency genes in undifferentiated ES cells (Singh et al., 2008). To clarify the role of REST in ES cells we have used homozygously targeted *Rest* mutant ES cells and RNAi-mediated REST knockdown. We show that lowering REST levels in ES cells results in the derepression of a subset of neuronal genes that are highly enriched for the canonical RE1 elements and that directly bind REST protein in wild-type ES cells. By contrast, the expression of genes crucial for neural determination, or that regulate stem cell potential, was unaffected in REST-depleted ES cells.

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Accepted 5 January 2009

MATERIALS AND METHODS

Cells and antibodies

Wild-type, *Rest*$^{+/-}$ and *Rest*$^{-/-}$ ES cells (see Jorgensen et al., 2009) were cultured on a layer of mitotically inactivated embryonic fibroblasts in the presence of LIF (1000 U/ml). Karyotype analysis of wild-type and *Rest*$^{-/-}$ ES cells showed normal chromosome content (2*n*=40). For embryoid body differentiation, 7×10^6 cells were plated in non-adherent plates in ES cell medium without LIF, with or without retinoic acid (5 μM) from day 4. Wild-type ES cell lines used for RNAi experiments [46C (Ying et al., 2003) and OS25 (Billon et al., 2002)] were cultured on gelatinised plates without feeder cells as described (Jorgensen et al., 2007). For knockdown by siRNA or shRNA and transfection of Flag-REST, see Figs S1 and S5 in the supplementary material. For the heterokaryon reprogramming assays, human B lymphocytes were fused with mouse ES cells and analysed as described (Pereira et al., 2008).

Antibodies used were as follows. For ChIP: anti-IgG (DAKO, Z0259); anti-Histone H3 (Abcam, ab-1791-100); anti-H3K9ac (Upstate/Millipore, 07-352); anti-H3K4me2 (Upstate/Millipore, 07-030); anti-H3K4me3 (Abcam, ab-8580-50); anti-H3K27me3 (Upstate/Millipore, 07-449). For western blot: anti-lamin B (Santa Cruz, C-20 sc-6216); anti-goat-HRP (Santa Cruz, sc-2020); anti-rabbit-HRP (GE Healthcare, NA934V). For ChIP and western blot: anti-REST (Upstate/Millipore, 07-579). For FACS: anti-SSEA1-APC (R&D, FAB2155A); anti-B220-APC (BD Pharmingen, RA 3-6B2); anti-goat-Alexa568 (anti-Oct4 staining; Invitrogen/Molecular Probes, A11057). For western blot and FACS: anti-Oct4 (Santa Cruz, N-19 sc-8628).

Expression analysis

RNA was isolated using the RNeasy Kit (Qiagen, Crawley, West Sussex, UK) and either reverse transcribed [using SuperScriptIII as recommended by the manufacturer (Invitrogen)] and analysed by real-time PCR as described (Azuara et al., 2006; Jorgensen et al., 2007), or labelled (using 8 μg RNA with the One-Cycle cDNA Synthesis Kit and IVT Labelling Kit) and hybridised to Mouse 430 2.0 Arrays (all from Affymetrix). For analysis of microarray data, see Fig. S4 in the supplementary material; primer sequences for the RT-PCR analysis are available upon request. To analyse microRNA levels, RNA was extracted using the mir*VANA* Kit (Ambion, Warrington, UK), reverse transcribed and analysed using miRNA assays as described by the provider (Applied Biosystems, Foster City, CA, USA).

Epigenetic profiling and 3D FISH analysis

The replication timing analysis was carried out as described (Azuara, 2006). Three-dimensional (3D) FISH analysis was performed using a BAC probe spanning the *Mash1* locus [RP24-130P7, prepared and labelled as described (Williams et al., 2006)]. Cells were trypsinised, washed in PBS and left to attach onto poly-L-lysine-coated coverslips. Fixation, denaturation, hybridisation and washing were as described (Brown et al., 1997). After mounting, nuclei were viewed with a Leica TCS SP5 laser-scanning confocal microscope fitted with a 63× oil-immersion objective. Optical sections through the nuclei were captured with a LAS AF 6000 camera every 0.24 μm to create *z*-stacks for analysis. The position of *Mash1* loci relative to the nuclear periphery was determined on single focal plane sections using ImageJ. For each allele, the focal plane where the FISH signal was most intense was selected for measurements and the distance d=nuclear centre to FISH signal was divided by the distance r=nuclear centre to periphery; FISH signals with a d/r-ratio ⩾0.80 were considered peripheral (Kosak et al., 2002). Only nuclei containing two visible *Mash1* alleles were scored (36 cells for REST wild-type, 33 for *Rest*$^{-/-}$ ES cells, 29 for undifferentiated 46C ES cells, and 18 for 46C-derived neural stem cells).

Chromatin immunoprecipitation (ChIP) analyses were performed as described (Azuara et al., 2006) using 100 μg of chromatin per sample. Primer sequences are available upon request.

RESULTS AND DISCUSSION

ES cells lacking REST appropriately repress neural determinants

Loss of REST function in the mouse results in embryonic death around day 10 of gestation, but does not appear to affect early developmental processes such as gastrulation and body axis formation (Chen et al., 1998). To assess whether REST is required to repress the expression of neural-specifying genes in pluripotent ES cells, we analysed the mRNA levels of transcription factors known to promote neural commitment, in ES cells that lack REST. Fig. 1A shows a comparative analysis of *Sox1*, *Math1*, *Mash1*, *Ngn1*, *Ngn2*, *Pax3*, *Pax6*, *Pax7*, *Msx1* and *Nkx2-2* gene expression in mouse ES cells homozygous for a targeted REST allele (*Rest*$^{-/-}$) (upper panel) or in wild-type ES cells in which REST protein levels were substantially reduced by RNAi-mediated knockdown, relative to matched controls (lower panel and see Fig. S1A in the supplementary material). As anticipated, undifferentiated ES cells expressed very low levels of each of these genes as compared with control tissue (quantitative RT-PCR, see Table S1 in the supplementary material). In REST-deficient ES cells, expression of neural-specifying genes was either comparable to that in the wild type (9/10) or slightly reduced (*Ngn1*). Similarly, RNAi-mediated REST knockdown did not significantly enhance the expression of *Sox1*, *Mash1*, *Math1*, *Ngn1*, *Ngn2*, *Pax3*, *Pax6*, *Pax7*, *Msx1* or *Nkx2-2* in ES cells (Fig. 1A, lower panel). Two established REST target genes, *Syt4* and *Calb* (Ballas et al., 2005), were, by contrast, consistently upregulated both in *Rest*$^{-/-}$ ES cells and following RNAi-mediated REST knockdown (Fig. 1A), a result that is consistent with REST-mediated derepression. Collectively, these data suggest that in ES cells, REST is not required to silence crucial transcription factor genes known to promote neural commitment.

As REST was previously implicated in the silencing of *Mash1* in ES cells by binding to a putative RE1 element located 49 kb downstream of the transcription start site (Fig. 1B, top panel) (Ballas et al., 2005; Wu and Xie, 2006), we examined whether the epigenetic status of the *Mash1* locus was altered in REST-deficient ES cells. In earlier studies, we showed that the *Mash1* locus replicates late in S-phase in wild-type ES cells, preferentially localises to the nuclear periphery and is hypoacetylated at the promoter (features that are consistent with a repressed chromatin state), whereas the locus switches to earlier replication, becomes acetylated and relocates to the nuclear interior when the *Mash1* gene is productively transcribed upon neural induction (Williams et al., 2006). As shown in Fig. 1B, we found that *Mash1* alleles had a similar propensity to localise at the nuclear periphery in wild-type and REST-deficient ES cells (middle panel), and that REST-deficiency did not alter the timing of *Mash1* locus replication in ES cells (bottom panel and see Fig. S2 in the supplementary material). Likewise, we did not detect any differences in the levels of active or repressive histone modifications at the *Mash1* promoter between REST-deficient and wild-type ES cells (see Fig. S3 in the supplementary material). These data indicate that REST is required neither to silence nor to maintain the repressive epigenetic environment of the *Mash1* locus in undifferentiated ES cells.

As regulation of microRNAs has been proposed as an alternative mechanism underlying REST-mediated gene repression in ES cells (Singh et al., 2008), we asked whether the expression of a selected panel of microRNAs was significantly altered in *Rest*$^{-/-}$ and *Rest*$^{+/-}$ ES cells, as compared with wild-type cells. As shown in Table 1, expression of *miR-30* and *miR-16*, two ubiquitously expressed microRNA species (Landgraf et al., 2007), was similar in REST-

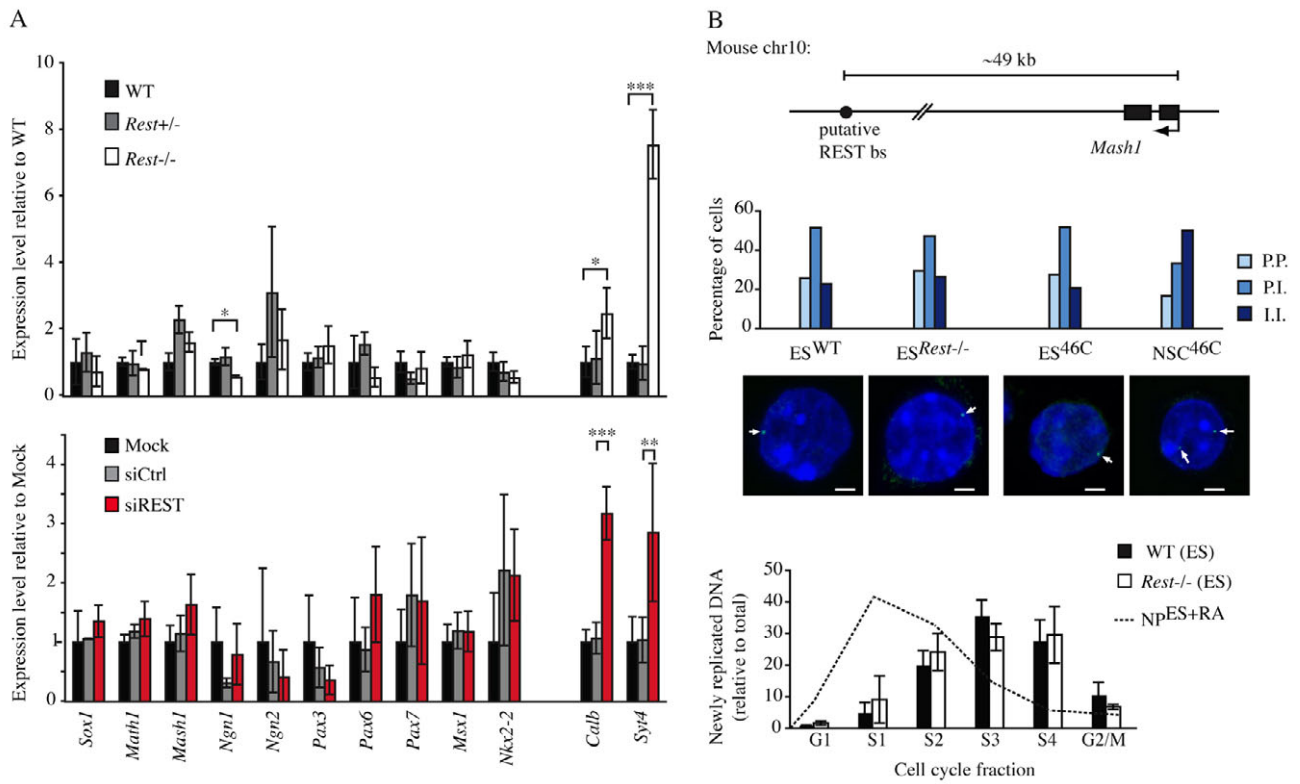


Fig. 1. Repression of neural determinants is not compromised in REST-deficient mouse ES cells. (**A**) The top panel shows transcript levels in $Rest^{+/-}$ and $Rest^{-/-}$ relative to wild-type (WT) ES cells, as assessed by real-time quantitative PCR. The lower panel compares gene expression in wild-type ES cells transfected with siRNA targeting either a control sequence (siCtrl) or *Rest* (siREST), relative to mock-transfected cells. Values were normalised to house keeping genes (*Ywhaz*, *Hmbs*, *Gapdh*). Error bars indicate the s.d. from three to six experiments. Significant differences (two-tailed Student's *t*-test) between wild-type and $Rest^{-/-}$ (top) or between siREST and siCtrl (bottom) samples are indicated: $*P<0.05$, $**P<0.005$, $***P<0.0005$. (**B**) Schematic representation of a 49 kb region flanking mouse *Mash1* (top panel). Arrow, transcription start site; black boxes, exons; the putative REST binding site (REST bs) is indicated. The subnuclear location of *Mash1* in wild-type ES cells (ES^{WT}), $Rest^{-/-}$ ES cells ($ES^{Rest-/-}$), undifferentiated wild-type 46C ES cells (ES^{46C}) and neural stem cells derived from 46C ES cells (NSC^{46C}) is shown in the middle panel. The bar chart shows the percentage of cells with two peripheral *Mash1* alleles (P.P.), one peripheral and one internal allele (P. I.) or two internal alleles (I.I.), as assessed in 3D FISH analysis. Representative confocal images of a single optical section are shown beneath for each cell type. Arrows mark *Mash1* FISH signals. Scale bars: 2 μm. The bottom panel shows a replication timing analysis of *Mash1* in wild-type and $Rest^{-/-}$ ES cells. The relative amount of newly synthesised (BrdU-labelled) locus-specific DNA in G1, four sequential S-phase fractions and G2–M is shown. Data for control genes are shown in Fig. S2 in the supplementary material. Error bars indicate s.d. from two experiments. For comparison, the replication profile of *Mash1* in neural progenitor cells (NP^{ES+RA}) is included (Williams et al., 2006).

deficient and wild-type ES cells. Likewise, the brain-specific microRNAs *miR-9*, *miR-124a* (Chen et al., 2007), *miR-152* [upregulated upon ES cell differentiation (Chen et al., 2007)] and *miR-21* [which has been suggested to be a target of REST in ES cells (Singh et al., 2008)] were detected at similar levels in REST-deficient and wild-type ES cells (Table 1). This analysis does not, therefore, provide any evidence of a generalised role for REST in regulating microRNA expression in ES cells.

REST selectively represses a subset of RE1-containing neuronal genes in ES cells

Based on our data indicating that REST is important for repressing *Syt4* and *Calb*, but not neural determinants, we used genome-wide expression profiling to identify other genes regulated by REST in ES cells. In order to avoid off-target effects that can complicate RNAi experiments and adaptive changes in gene expression that might occur in REST knockout cells, we focused our analysis on transcripts that were deregulated by both knockdown and knockout of REST (Fig. 2A). We found an over-representation of genes expressed in brain (P=0.02, Bonferroni corrected) and a highly significant enrichment ($P=2\times10^{-20}$, one-tailed Fisher's exact test) of canonical RE1 REST binding sites around the transcription start site (–1 kb to +5 kb) of genes that were deregulated by >1.4-fold in both data sets (Fig. 2B and see Fig. S4 in the supplementary material). ChIP experiments readily confirmed the presence of endogenous REST in wild-type ES cells at five out of five genes examined within this subset (Fig. 2C), and by comparison with a recent genome-wide study of REST binding in ES cells (Johnson et al., 2008), this was extended to include >60% of all upregulated genes. By contrast, no REST binding was detected at the negative control provided by the *Math1* promoter or at the putative REST binding site downstream of the *Mash1* gene (Fig. 2C). Most differentially expressed genes (84/87) and all RE1-containing genes (25/25) identified in our analysis were upregulated in the absence of REST. Real-time RT-PCR analysis confirmed the upregulation of the neuronal REST target genes *Scg3*, *Stmn3*, *Celsr3*, *Syp*, *Cplx1* and *Syt4*, and not of the proneural genes *Mash1* and *Math1*, in REST knockout (Fig. 2D) and REST knockdown cells (Fig. 2E). Transfection experiments in which we reconstituted REST knockout ES cells with Flag-tagged REST provided additional evidence that

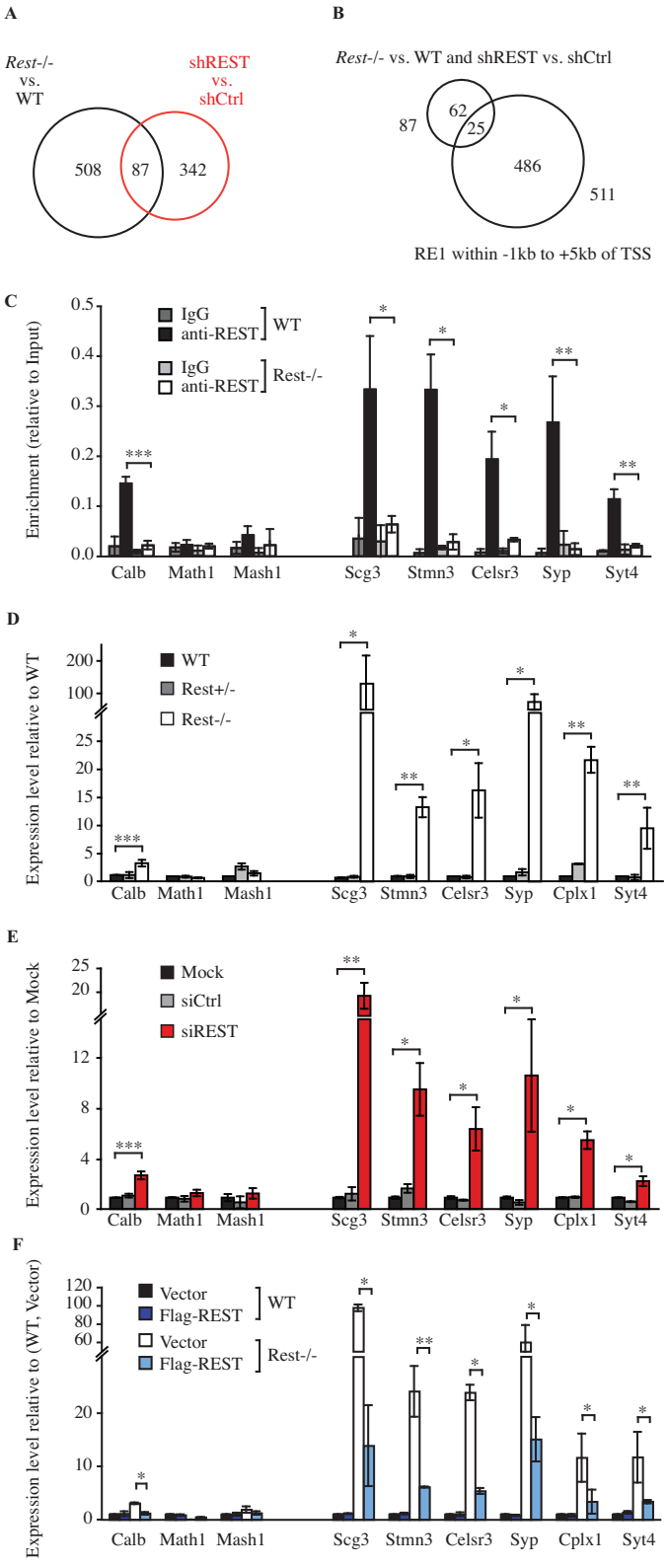


Fig. 2. Loss of REST in mouse ES cells causes upregulation of RE1-containing neuronal genes. (**A**) Venn diagram showing the number and intersection of genes that are at least 1.4-fold up- or downregulated between *Rest*$^{-/-}$ and wild-type (WT) ES cells or between shREST- and shCtrl-transfected ES cells ($P<0.05$). (**B**) Overlap between the 87 genes that are misregulated in both *Rest*$^{-/-}$ and shREST cells as defined in A and the 511 genes that contain RE1 sites within –1 kb to +5 kb relative to the transcription start site (TSS) (Otto et al., 2007). (**C**) ChIP analysis of REST binding in wild-type and REST-deficient ES cells. IgG, control. The average and s.d. of three to five experiments is shown. Significant REST-enrichment in wild-type relative to *Rest*$^{-/-}$ ES cells (one-tailed Student's *t*-test) is indicated: *$P<0.05$, **$P<0.005$, ***$P<0.0005$. (**D**,**E**) Confirmation that six candidate REST target genes are upregulated in the absence of REST. Gene expression detected by RT-PCR in *Rest*$^{+/-}$ and *Rest*$^{-/-}$ ES cells is shown relative to that in the wild type (D). Gene expression in siREST- and siCtrl-transfected ES cells is presented relative to that in mock-transfected controls (E). The expression levels were normalised to house keeping genes (*Ywhaz*, *Hmbs*). Bars show the average of three to six experiments and error bars indicate s.d. Significantly higher expression (one-tailed Student's *t*-test) in *Rest*$^{-/-}$ relative to wild type (D) and in siREST relative to siCtrl samples (E) is indicated: *$P<0.05$, **$P<0.005$, ***$P<0.0005$. (**F**) Reconstitution of *Rest*$^{-/-}$ ES cells with full-length Flag-tagged REST protein. Expression analysis of REST-dependent genes after ectopic expression of Flag-tagged full-length REST in wild-type or *Rest*$^{-/-}$ ES cells as compared with cells transfected with the control construct. The expression levels relative to 'WT, Vector' were normalised to house keeping controls. Bars show the average of two experiments and error bars indicate s.d. Significantly reduced expression (one-tailed Student's *t*-test) in *Rest*$^{-/-}$ ES cells transfected with Flag-REST relative to vector-transfected cells is indicated: *$P<0.05$, **$P<0.005$. A western blot verifying overexpression of REST is shown in Fig. S5 in the supplementary material.

REST directly represses *Scg3*, *Stmn3*, *Celsr3*, *Syp*, *Cplx1* and *Syt4* (Fig. 2F and see Fig. S5 in the supplementary material). The elevated expression of each of these candidate genes in the absence of REST (Fig. 2F white bars, *Rest*$^{-/-}$ Vector) was at least partially reduced by transfection of Flag-REST (blue bars), whereas *Mash1* and *Math1* expression remained unaffected.

Dominant reprogramming and multi-lineage potential of ES cells are retained in the absence of REST

REST expression in ES cells is regulated by pluripotency factors including OCT4 and NANOG (Boyer et al., 2005; Kim et al., 2008; Loh et al., 2006). Although it has been claimed that REST itself may regulate the expression of *Nanog*, *Oct4* and *Sox2* in ES cells (Singh et al., 2008), we have recently shown that normal levels of these transcripts are present in *Rest*$^{-/-}$ mutant ES cells and in siREST-transfected ES cell lines (Jorgensen et al., 2009). Consistently, REST-deficient ES cells expressed the stem cell markers OCT4 and SSEA1 (FUT4) (detected by FACS analysis, see Fig. S6 in the supplementary material) and had similar morphology, growth rate and colony-forming characteristics as their wild-type counterparts (data not shown) (Jorgensen et al., 2009). To investigate whether REST-deficient ES cells were capable of inducing mesoderm, endoderm and ectoderm lineage differentiation, we cultured *Rest*$^{-/-}$ ES cells under conditions that allow the formation of embryoid bodies. Upon LIF withdrawal, REST-deficient and wild-type control ES cells showed declining levels of *Oct4* (and of *Nanog*) transcripts, and a progressive increase in the expression of neural genes such as *Sox1* (and *Pax6*, *Ngn1*) that was enhanced by the addition of retinoic acid (Fig. 3A and see Fig. S7 in the supplementary material). Likewise, withdrawal of LIF resulted in an upregulation of *Bry* (and *Mixl1*) and *Gata4* (and *Sox17*) in both REST-deficient and wild-type samples (Fig. 3A and see Fig. S7 in the supplementary material), reflecting the induction of mesoderm and endoderm lineages, respectively. These results indicate that REST-deficient ES cells

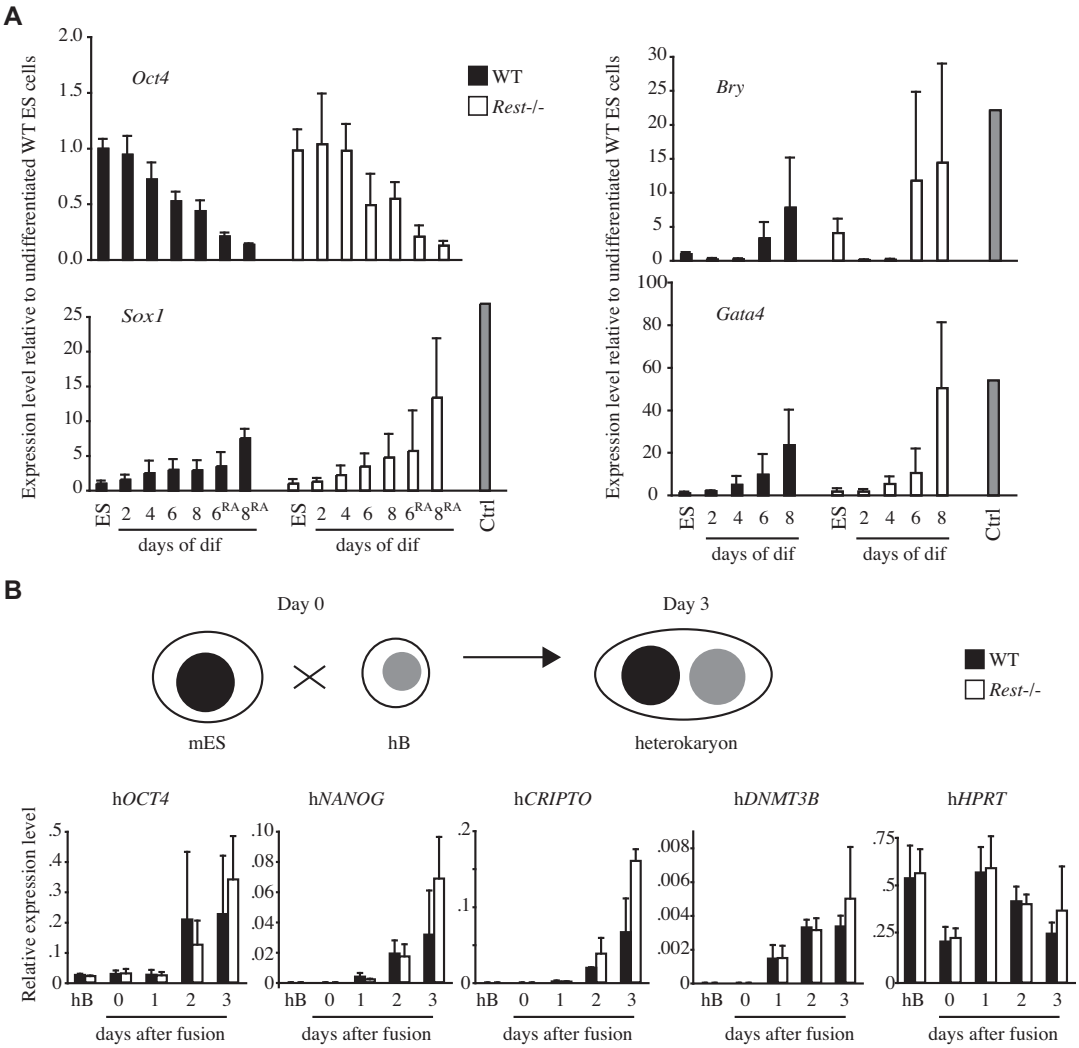


Fig. 3. REST-deficient ES cells retain multi-lineage potential and reprogramming ability. (**A**) The kinetics of induction of differentiation-associated transcripts (*Sox1*, *Bry*, *Gata4*) and loss of *Oct4* expression in wild-type or *Rest*$^{-/-}$ undifferentiated ES cells following LIF withdrawal and embryoid body formation. Retinoic acid was added at day 4 of differentiation where indicated (RA). For comparison, gene expression levels in control tissues (Ctrl, grey), E15 heads (*Sox1*), ES cell-derived mesoderm (*Bry*) and E15 liver (*Gata4*) are provided. Transcript levels were normalised to house keeping controls (*Hmbs*, *Ywhaz*, *Gapdh*). The average and s.d. from two to three experiments are shown. (**B**) Reprogramming activity of wild-type and *Rest*$^{-/-}$ ES cells assessed by heterokaryon formation after fusion of mouse ES cells with human B cells (hB). The ability of mouse ES cell lines to reprogram human B cells is indicated by the kinetics of induction of human (h) *OCT4*, *NANOG*, *CRIPTO* and *DNMT3B* transcripts detected 0, 1, 2 and 3 days after fusion. Bars show the average of two experiments and error bars indicate s.d.

can appropriately upregulate markers of each of the three germ layers and suggest that the multi-lineage potential of ES cells is not critically dependent on the REST repressor.

To investigate whether loss of REST impairs the ability of ES cells to dominantly reprogram somatic cells towards an induced pluripotent stem (IPS)-like state (Jaenisch and Young, 2008), we compared the reprogramming capacity of *Rest*$^{-/-}$ and wild-type ES cells when fused with human B cells in experimental heterokaryons (Fig. 3B). This assay tests the ability of stem cells to redirect the fate of differentiated cells (lymphocytes in this example), and successful reprogramming is indicated by the upregulation of the human genes *OCT4*, *NANOG*, *CRIPTO* (*TDGF1* – HUGO) and *DNMT3B* as

Table 1. Expression of microRNAs in wild-type and *Rest* mutant ES cells

	Expression level					
Genotype	*miR-9*	*miR-16*	*miR-21*	*miR-30b*	*miR-124a*	*miR-152*
Wild type	0.21±0.01	21.9±4.8	187±29	15.2±3.2	3.4±1.0	8.8±2.6
Rest$^{+/-}$	0.25±0.04	22.5±3.7	249±52	15.0±1.7	2.6±0.7	10.4±1.2
Rest$^{-/-}$	0.20±0.06	20.5±3.5	214±37	13.7±1.1	2.7±0.8	10.7±1.9

Expression of microRNAs is shown relative to control embryonic tissue (E15 head, expression level=100), using *miR-16* (a ubiquitously expressed microRNA) as standard. Values (±s.d.) shown are from three independent experiments.

described previously (Pereira et al., 2008). Dominant reprogramming of this nature is a feature of pluripotent stem cell lines and previous studies have shown that mouse ES cells lacking crucial pluripotency factors, such as OCT4, are unable to reprogram lymphocytes in heterokaryon assays (Maherali et al., 2007; Pereira et al., 2008). By comparing the kinetics of induction of human pluripotency-associated transcripts in heterokaryons formed between human B cells and either wild-type or REST-deficient ES cells (Fig. 3B), we found that REST-deficient ES cells were capable of dominantly reprogramming lymphocytes to a similar extent as their wild-type counterparts. These results show that ES cells lacking REST retain the capacity for multi-lineage differentiation and dominant reprogramming of somatic cells, two functional properties that are associated with pluripotency (Jaenisch and Young, 2008).

REST function in ES cells

Here we show that REST directly represses a subset of RE1-containing neuronal genes in ES cells: in the absence of REST, a cohort of genes important for the terminal differentiation and function of neuronal cells are inappropriately expressed. This derepression does not appear to be a consequence of unscheduled neural differentiation because we found no evidence that REST regulates the expression of genes encoding any of the transcription factors thought to be crucial for promoting neural commitment in ES cells. These include *Mash1*, a proneural factor that was previously thought to be a REST target in ES cells (Ballas et al., 2005), and *Ngn2*, also purported to be regulated by REST (Singh et al., 2008). Recent studies in which a dominant-negative form of REST was used to inhibit REST function support the idea that only a proportion of RE1-containing genes are in fact REST-dependent in ES cells (Johnson et al., 2008), and many of those identified overlap with target genes defined here. Importantly, our results show that misexpression of these brain-specific genes (including *Scg3*, *Cplx1* and *Stmn3*) by REST-deficient ES cells does not appear to abrogate stem cell function: REST-deficient ES cells express the same level of many pluripotency-associated genes [*Oct4*, *Nanog* and others (Jorgensen et al., 2009)] and display similar functional properties, including multipotency and reprogramming capacity, as their wild-type counterparts. Recently, REST ablation has been shown to compromise the generation of neurons from ES cells through dysregulation of laminin genes (Sun et al., 2008). Collectively, these studies argue that REST might be important for the correct execution of neuronal differentiation programmes, but is not required for neural commitment per se or for maintaining the multipotent status of ES cells.

We thank Natalie Ryan for excellent technical assistance; Eric O'Connor and Eugene Ng for FACS; Mikhail Spivakov for advice on statistics; Luca Mazzarella for providing reagents; and Maria Dvorkina for advice and suggestions on the manuscript. Hybridisation to Affymetrix arrays was performed by the Clinical Sciences Centre/Imperial College Microarray Centre. This work was funded by the MRC. Deposited in PMC for release after 6 months.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/5/715/DC1

References

Azuara, V. (2006). Profiling of DNA replication timing in unsynchronized cell populations. *Nat. Protoc.* **1**, 2171-2177.

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jorgensen, H. F., John, R. M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M. et al. (2006). Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* **8**, 532-538.

Ballas, N. and Mandel, G. (2005). The many faces of REST oversee epigenetic programming of neuronal genes. *Curr. Opin. Neurobiol.* **15**, 500-506.

Ballas, N., Grunseich, C., Lu, D. D., Speh, J. C. and Mandel, G. (2005). REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell* **121**, 645-657.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Billon, N., Jolicoeur, C., Ying, Q. L., Smith, A. and Raff, M. (2002). Normal timing of oligodendrocyte development from genetically engineered, lineage-selectable mouse ES cells. *J. Cell Sci.* **115**, 3657-3665.

Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G. et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122**, 947-956.

Brown, K. E., Guest, S. S., Smale, S. T., Hahm, K., Merkenschlager, M. and Fisher, A. G. (1997). Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* **91**, 845-854.

Bruce, A. W., Donaldson, I. J., Wood, I. C., Yerbury, S. A., Sadowski, M. I., Chapman, M., Gottgens, B. and Buckley, N. J. (2004). Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc. Natl. Acad. Sci. USA* **101**, 10458-10463.

Chen, C., Ridzon, D., Lee, C. T., Blake, J., Sun, Y. and Strauss, W. M. (2007). Defining embryonic stem cell identity using differentiation-related microRNAs and their potential targets. *Mamm. Genome* **18**, 316-327.

Chen, Z. F., Paquette, A. J. and Anderson, D. J. (1998). NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat. Genet.* **20**, 136-142.

Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., Altshuller, Y. M., Frohman, M. A., Kraner, S. D. and Mandel, G. (1995). REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell* **80**, 949-957.

Eiraku, M., Watanabe, K., Matsuo-Takasaki, M., Kawada, M., Yonemura, S., Matsumura, M., Wataya, T., Nishiyama, A., Muguruma, K. and Sasai, Y. (2008). Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* **3**, 519-532.

Giadrossi, S., Dvorkina, M. and Fisher, A. G. (2007). Chromatin organization and differentiation in embryonic stem cell models. *Curr. Opin. Genet. Dev.* **17**, 132-138.

Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R. and Young, R. A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* **130**, 77-88.

Guillemot, F. (2007). Cell fate specification in the mammalian telencephalon. *Prog. Neurobiol.* **83**, 37-52.

Jaenisch, R. and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* **132**, 567-582.

Johnson, D. S., Mortazavi, A., Myers, R. M. and Wold, B. (2007). Genome-wide mapping of in vivo protein-DNA interactions. *Science* **316**, 1497-1502.

Johnson, R., Teh, C. H., Kunarso, G., Wong, K. Y., Srinivasan, G., Cooper, M. L., Volta, M., Chan, S. S., Lipovich, L., Pollard, S. M. et al. (2008). REST regulates distinct transcriptional networks in embryonic and neural stem cells. *PLoS Biol.* **6**, e256.

Jorgensen, H. F., Azuara, V., Amoils, S., Spivakov, M., Terry, A., Nesterova, T., Cobb, B. S., Ramsahoye, B., Merkenschlager, M. and Fisher, A. G. (2007). The impact of chromatin modifiers on the timing of locus replication in mouse embryonic stem cells. *Genome Biol.* **8**, R169.

Jorgensen, H. F., Chen, Z.-F., Merkenschlager, M. and Fisher, A. G. (2009). Is REST required for ESC pluripotency? *Nature* doi:10.1038/nature07783.

Kim, J., Chu, J., Shen, X., Wang, J. and Orkin, S. H. (2008). An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* **132**, 1049-1061.

Kosak, S. T., Skok, J. A., Medina, K. L., Riblet, R., Le Beau, M. M., Fisher, A. G. and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science* **296**, 158-162.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A. O., Landthaler, M. et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**, 1401-1414.

Levine, A. J. and Brivanlou, A. H. (2007). Proposal of a model of mammalian neural induction. *Dev. Biol.* **308**, 247-256.

Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J. et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* **38**, 431-440.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R. et al. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* **1**, 55-70.

Otto, S. J., McCorkle, S. R., Hover, J., Conaco, C., Han, J. J., Impey, S., Yochum, G. S., Dunn, J. J., Goodman, R. H. and Mandel, G. (2007). A new binding motif for the transcriptional repressor REST uncovers large gene networks devoted to neuronal functions. *J. Neurosci.* **27**, 6729-6739.

Pereira, C. F., Terranova, R., Ryan, N. K., Santos, J., Morris, K. J., Cui, W., Merkenschlager, M. and Fisher, A. G. (2008). Heterokaryon-based reprogramming of human B lymphocytes for pluripotency requires Oct4 but not Sox2. *PLoS Genet.* **4**, e1000170.

Schoenherr, C. J. and Anderson, D. J. (1995). The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. *Science* **267**, 1360-1363.

Singh, S. K., Kagalwala, M. N., Parker-Thornburg, J., Adams, H. and Majumder, S. (2008). REST maintains self-renewal and pluripotency of embryonic stem cells. *Nature* **453**, 223-227.

Spivakov, M. and Fisher, A. G. (2007). Epigenetic signatures of stem-cell identity. *Nat. Rev. Genet.* **8**, 263-271.

Stock, J. K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A. G. and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat. Cell Biol.* **9**, 1428-1435.

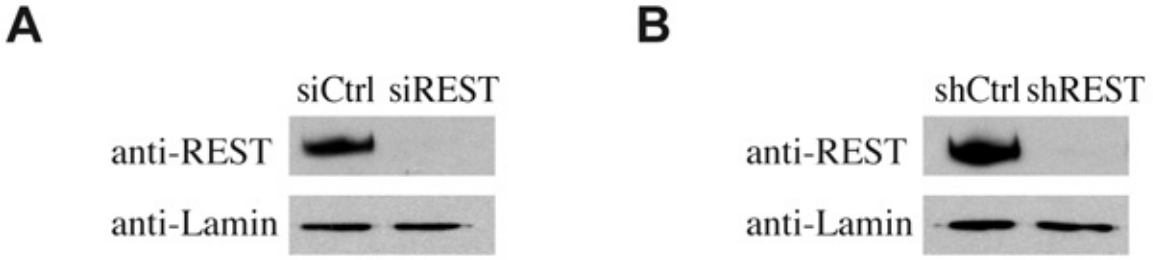
Sun, Y. M., Greenway, D. J., Johnson, R., Street, M., Belyaev, N. D., Deuchars, J., Bee, T., Wilde, S. and Buckley, N. J. (2005). Distinct profiles of REST interactions with its target genes at different stages of neuronal development. *Mol. Biol. Cell* **16**, 5630-5638.

Sun, Y. M., Cooper, M., Finch, S., Lin, H. H., Chen, Z. F., Williams, B. P. and Buckley, N. J. (2008). Rest-mediated regulation of extracellular matrix is crucial for neural development. *PLoS ONE* **3**, e3656.

Williams, R. R., Azuara, V., Perry, P., Sauer, S., Dvorkina, M., Jorgensen, H., Roix, J., McQueen, P., Misteli, T., Merkenschlager, M. et al. (2006). Neural induction promotes large-scale chromatin reorganisation of the Mash1 locus. *J. Cell Sci.* **119**, 132-140.

Wu, J. and Xie, X. (2006). Comparative sequence analysis reveals an intricate network among REST, CREB and miRNA in mediating neuronal gene expression. *Genome Biol.* **7**, R85.

Ying, Q. L., Stavridis, M., Griffiths, D., Li, M. and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat. Biotechnol.* **21**, 183-186.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anti-REST
anti-Lamin
B
shCtrl
shREST
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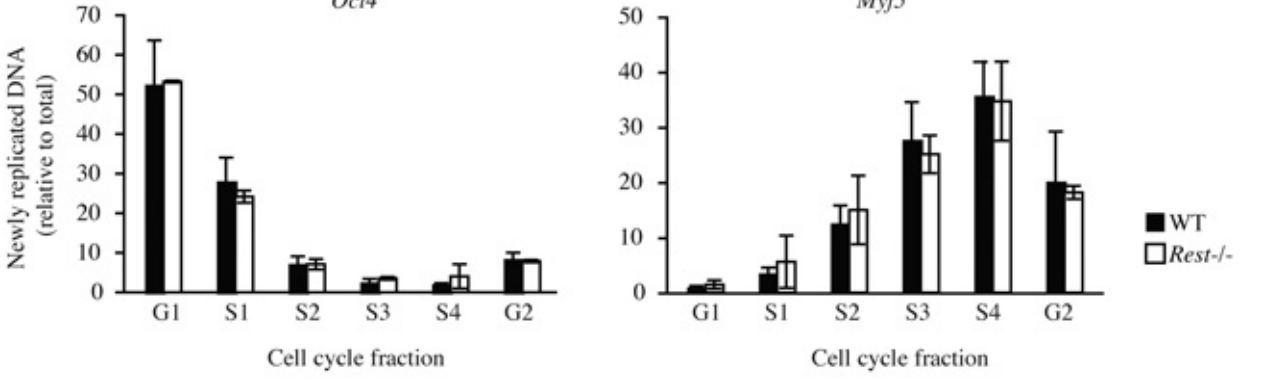
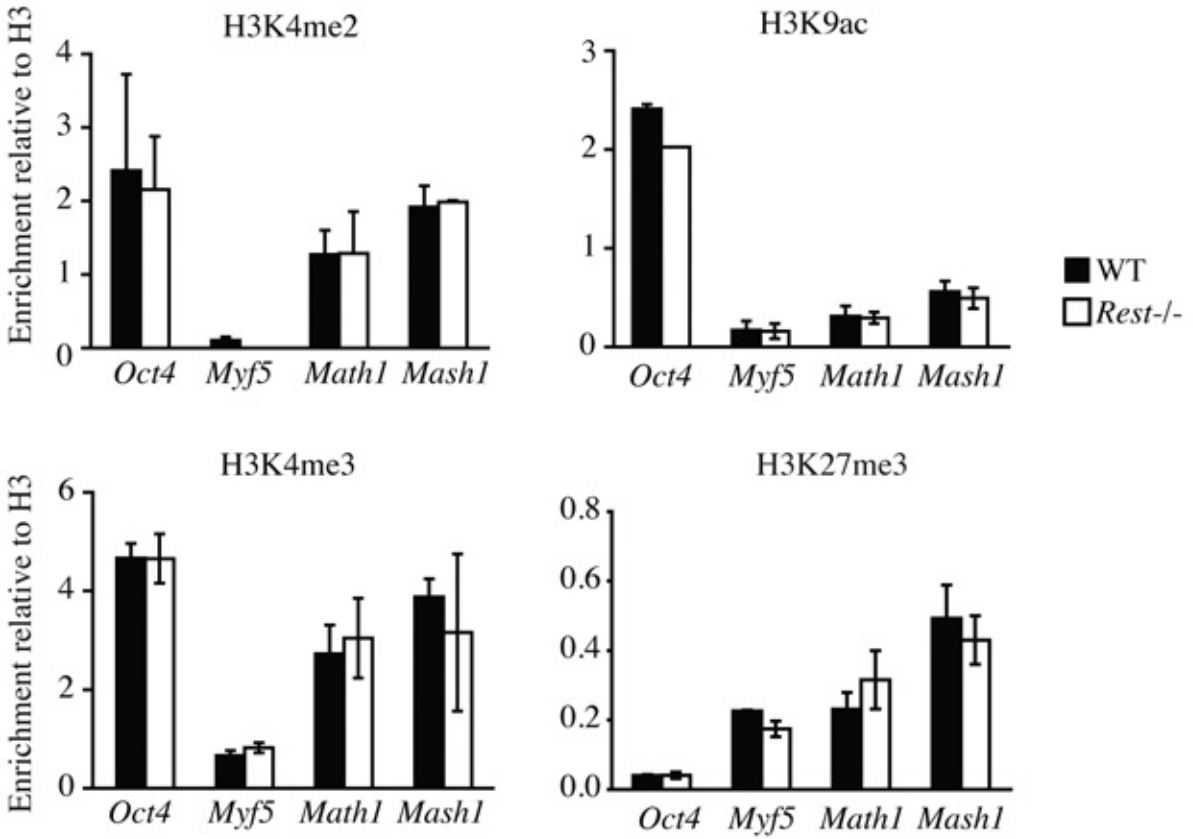
anti-Lamin


Oct4
Myf5
Newly replicated DNA (relative to total)
70
60
50
40
30
20
10
0
50
40
30
20
10
0
G1
S1
S2
S3
S4
G2
Cell cycle fraction
WT
Rest-/-


H3K4me2
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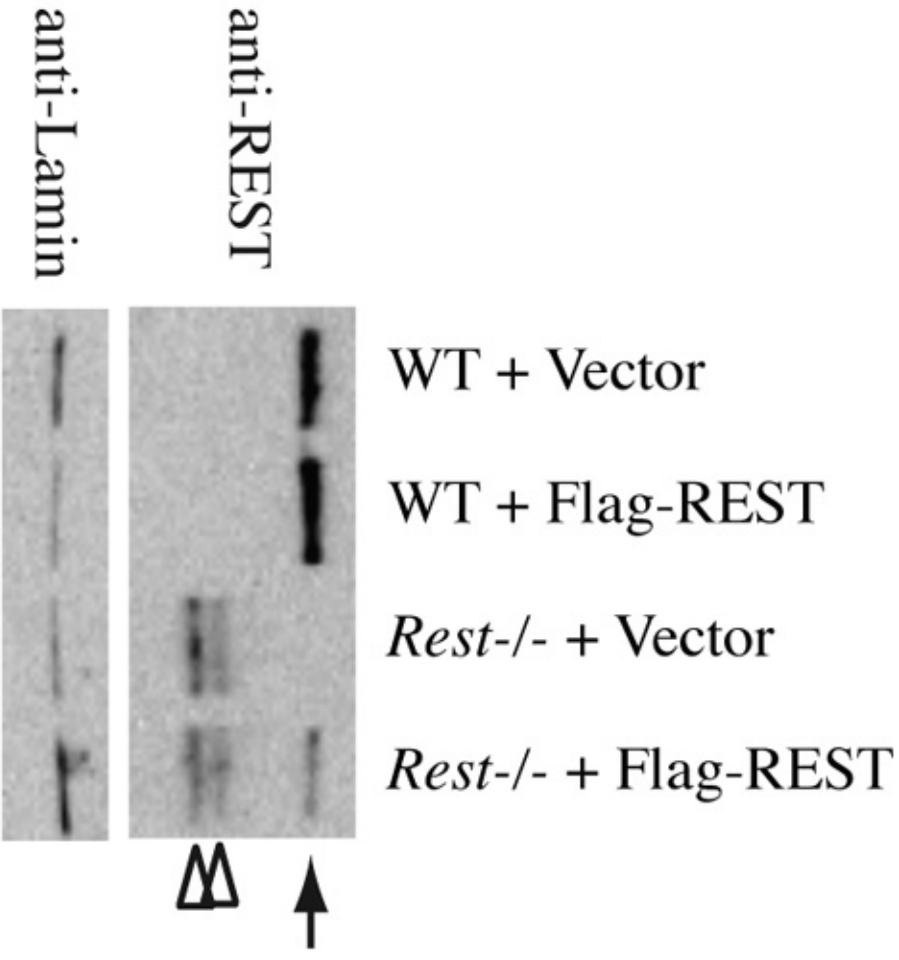
H3K4me3
H3K27me3
Enrichment relative to H3
Enrichment relative to H3
WT
Rest-/-
Oct4
Myf5
Math1
Mash1
0
1
2
3
4
0.0
0.2
0.4
0.6
0.8
6

A

Gene Symbol	RE1	Probe ID	*Rest-/-* vs. WT FC	*Rest-/-* vs. WT p-value	shREST vs. shCtrl FC	shREST vs. shCtrl p-value
1200015N20Rik		1437062_s_at	2.99	5E-05	3.11	4E-06
1500016O10Rik		1452807_s_at	1.80	4E-02	1.62	2E-03
2600009P04Rik		1435871_at	1.80	2E-04	2.16	2E-04
2610024G14Rik		1418222_at	2.32	6E-06	1.70	4E-04
3632451O06Rik		1450770_at	2.01	5E-04	1.57	1E-02
6330407J23Rik		1429628_at	2.79	2E-04	2.74	1E-04
Abcb6		1422524_at	2.34	1E-05	1.44	3E-03
Acsl6	X	1451257_at	2.46	7E-05	1.60	8E-03
AU040320		1452150_at	2.12	6E-05	1.58	9E-03
Bex2	X	1417388_at	2.07	7E-03	2.34	1E-05
Brunol4		1452240_at	1.61	3E-04	1.46	2E-02
Car3	X	1449434_at	2.87	3E-03	5.37	4E-05
Ccdc64		1426816_at	1.75	1E-04	1.89	1E-04
Cdk5r1		1433451_at	1.74	4E-04	1.50	6E-03
Celsr3	X	1425067_at	5.70	2E-07	4.29	8E-07
Chga		1418149_at	6.95	1E-08	2.75	2E-05
Chrnb2	X	1436428_at	2.79	6E-06	1.46	9E-03
Ckmt1		1417089_a_at	3.29	2E-05	2.27	7E-04
		1432418_a_at	2.84	7E-04	2.08	5E-03
Cplx1	X	1417746_at	3.52	3E-06	2.05	1E-04
Crip2	X	1417311_at	1.71	3E-04	1.72	2E-04
Crmp1		1448289_at	1.70	5E-02	2.46	4E-05
Cyr61		1457823_at	1.45	8E-03	1.46	5E-02
Dpp4		1416697_at	0.63	7E-03	0.52	8E-03
Dppa3		1424295_at	2.25	3E-03	1.90	2E-03
E130309D14Rik		1433673_at	2.28	1E-04	1.63	7E-03
Elavl3		1452524_a_at	1.42	3E-02	1.45	6E-03
Elovl2		1416444_at	3.86	7E-06	3.02	8E-06
Epas1		1435436_at	1.54	3E-03	1.47	2E-02
Epha4		1429021_at	0.19	1E-07	0.59	3E-03
Exoc3l		1436536_at	2.74	9E-06	1.65	3E-02
Galnt9		1434055_at	2.86	1E-06	2.40	8E-05
Gdap1	X	1423328_at	7.66	8E-07	3.67	3E-05
Gdap1l1	X	1424935_at	2.08	1E-03	1.51	4E-03
Gm50		1455202_at	1.45	2E-03	1.49	9E-03
Gnptg	X	1436526_at	4.04	8E-06	2.16	6E-05
Gps2		1417796_at	1.75	1E-03	1.66	8E-04
Hagh		1424171_a_at	1.43	3E-03	1.43	4E-03
Hcn3	X	1451023_at	1.96	2E-04	1.72	6E-04
Igfbp3		1423062_at	1.66	6E-03	1.63	4E-04
Ina		1448991_a_at	2.09	3E-03	2.37	8E-04
Kcnb1		1423179_at	1.46	5E-03	1.63	6E-04
Ldoc1		1435815_at	1.67	3E-02	1.43	7E-03
Letmd1		1418860_a_at	2.01	3E-04	1.89	1E-02
LOC100039489		1433507_a_at	1.56	5E-04	1.44	3E-03
Madd		1455502_at	1.45	8E-03	1.55	8E-04
Mapk11	X	1421926_at	1.56	3E-03	1.57	5E-03
Mapk8ip2	X	1449225_a_at	1.83	7E-03	1.96	8E-05
March11	X	1455947_at	1.86	4E-04	1.43	1E-02
Mterfd3		1455700_at	1.51	2E-03	1.49	3E-03
Nefh		1424847_at	1.58	2E-02	1.73	1E-02
Nefl		1426255_at	1.61	2E-02	2.02	3E-03
Neurod1	X	1426413_at	1.67	5E-02	1.60	2E-02
	X	1426412_at	1.51	2E-02	1.63	2E-02
Obfc2b		1451291_at	1.77	3E-04	1.60	8E-04
Pank1		1431028_a_at	0.63	5E-03	0.68	3E-03
Pclo		1419392_at	2.20	6E-03	1.60	3E-03
Phf21b		1454999_at	2.02	3E-05	1.64	6E-03
Phyhipl		1427023_at	2.29	2E-04	2.34	2E-05
Pias3		1451115_at	1.63	2E-04	1.42	9E-03
Rab28		1423990_at	1.62	2E-04	1.74	1E-04
Rab39		1437762_at	3.07	4E-06	2.80	3E-05
Rab3c		1432432_a_at	2.26	5E-04	1.87	2E-03
Rell2		1434147_at	1.79	2E-03	1.86	4E-03
Rgs17		1450693_at	1.58	2E-02	1.64	4E-04
Rhbdf1	X	1424138_at	1.61	2E-03	1.71	1E-03
Rims2		1450761_s_at	1.57	4E-04	1.56	3E-02
		1422809_at	1.55	2E-03	1.89	1E-02
Rtn2	X	1419056_at	1.78	3E-03	1.99	9E-04
Rundc3a	X	1449246_at	2.19	5E-06	1.52	8E-04
	X	1436674_at	1.50	3E-03	1.85	2E-04
Scap		1433520_at	1.50	1E-03	1.49	2E-03
Scg3	X	1448628_at	18.68	3E-08	4.57	4E-07
Scg5		1423150_at	6.28	4E-07	2.81	6E-06
Scn3b	X	1435767_at	2.26	3E-04	2.72	4E-05
Sgcb		1419668_at	3.08	8E-07	1.75	2E-03
Sirt7		1424238_at	1.61	1E-03	1.66	4E-04
Slc40a1		1417061_at	1.95	2E-03	2.04	1E-04
Smpd3		1438665_at	1.45	2E-02	1.71	3E-04
Snap25		1416828_at	9.54	3E-09	1.71	8E-04
Snx1		1416260_a_at	2.37	1E-05	1.98	2E-05
Stmn3	X	1460181_at	6.26	5E-07	4.65	3E-06
	X	1435113_x_at	6.26	2E-07	5.04	5E-07
Stx1a		1437390_x_at	1.61	8E-03	1.51	2E-02
Syp	X	1448280_at	4.23	1E-05	3.27	2E-05
Syt4	X	1415845_at	1.75	7E-05	1.46	2E-03
Tcerg1l		1431835_at	1.97	2E-03	1.56	2E-03
		1457133_at	1.75	2E-03	1.68	2E-04
Tmhs	X	1429266_at	2.79	5E-05	1.96	3E-04
Tob2		1448666_s_at	1.49	2E-03	2.01	5E-04
Ttc9b		1429416_at	1.80	7E-04	2.33	1E-05
Unc13a	X	1437472_at	4.02	8E-07	3.63	1E-05
Vgf		1436094_at	14.23	2E-06	3.52	1E-06

B

Category	Term	RT	Genes	Count	%	P-Value	Bonferroni
UP_TISSUE	Brain	RT		38	43.7	5.6E-5	2.4E-2
UP_TISSUE	Hippocampus	RT		12	13.8	1.2E-4	4.9E-2
UP_TISSUE	Cerebellum	RT		17	19.5	2.7E-4	1.1E-1
UP_TISSUE	Eye	RT		15	17.2	5.1E-4	1.9E-1
UP_TISSUE	Brain cortex	RT		9	10.3	9.5E-4	3.3E-1
UP_TISSUE	Retina	RT		9	10.3	2.6E-3	6.7E-1
UP_TISSUE	Olfactory bulb	RT		4	4.6	1.7E-2	1.0E0
UP_TISSUE	Spinal cord	RT		7	8.0	1.7E-2	1.0E0


anti-Lamin
anti-REST
WT + Vector
WT + Flag-REST
Rest-/- + Vector
Rest-/- + Flag-REST

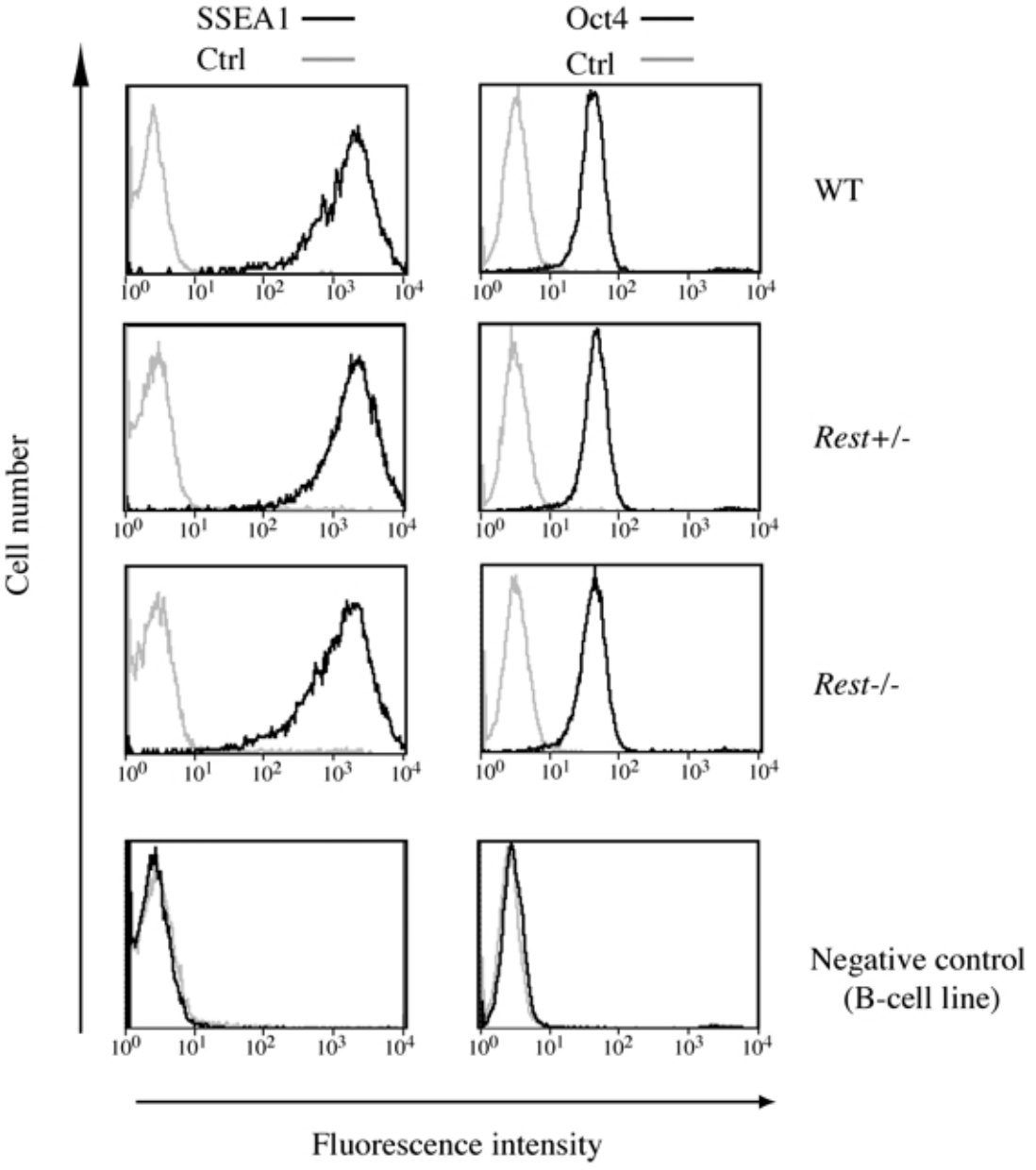
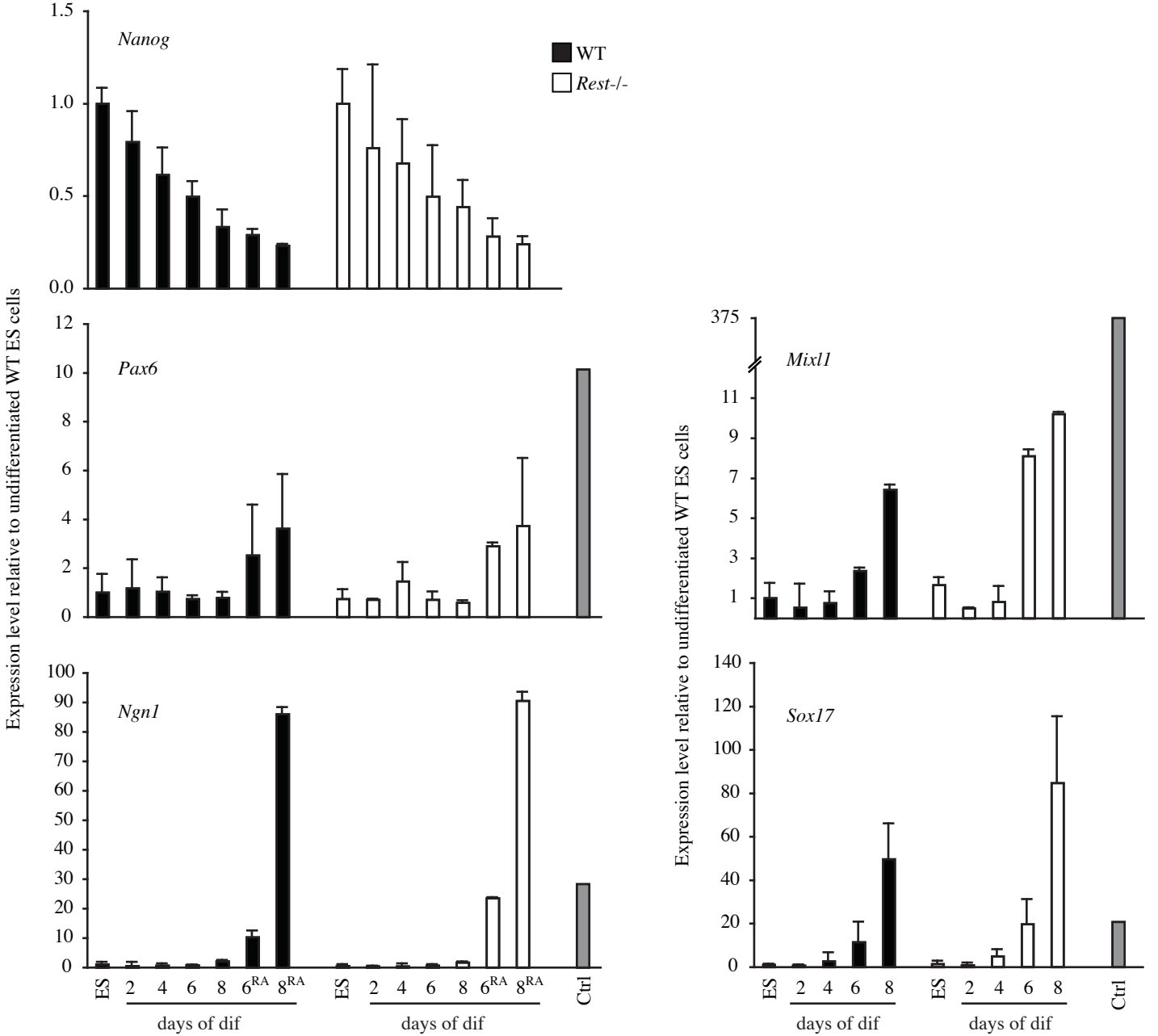

SSEA1
Ctrl
Oct4
Ctrl
WT
Rest+/-
Rest-/-
Negative control
(B-cell line)
Cell number
Fluorescence intensity
10^0
10^1
10^2
10^3
10^4

Table S1. Average Ct values for neural determinants

Gene	Ct in wild-type ES cells	Ct in E15 brain
Sox1	24	22
Math1	30	25
Mash1	29	21
Ngn1	26	23
Ngn2	33	22
Pax3	33	24
Pax6	27	23
Pax7	33	23
Msx1	32	25
Nkx2-2	29	25
Calb	30	23
Syt4	26	21
*Sox2**	18	21
*Gapdh**	13	14

Ct was determined by real-time PCR of cDNA using Qiagen SYBR Green Master Mix on a MJ Engine using Opticon4 software. Programme: 94°C for 15 minutes; 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds with a plate-read at the end of each cycle. The melting curve was recorded to ensure that a single product was formed. For each biological replicate, duplicate reactions were performed. All primer pairs were carefully selected for specificity, linearity and efficiency over a 64- to 125-fold range of template concentrations prior to the analysis of gene expression. All primer pairs that did not amplify a single band of the expected size were discarded.
*Expressed in ES cells.


Nanog
WT
Rest-/-
Pax6
Ngn1
Mixl1
Sox17
Expression level relative to undifferentiated WT ES cells
Expression level relative to undifferentiated WT ES cells
ES
2
4
6
8
6RA
8RA
Ctrl
days of dif
days of dif