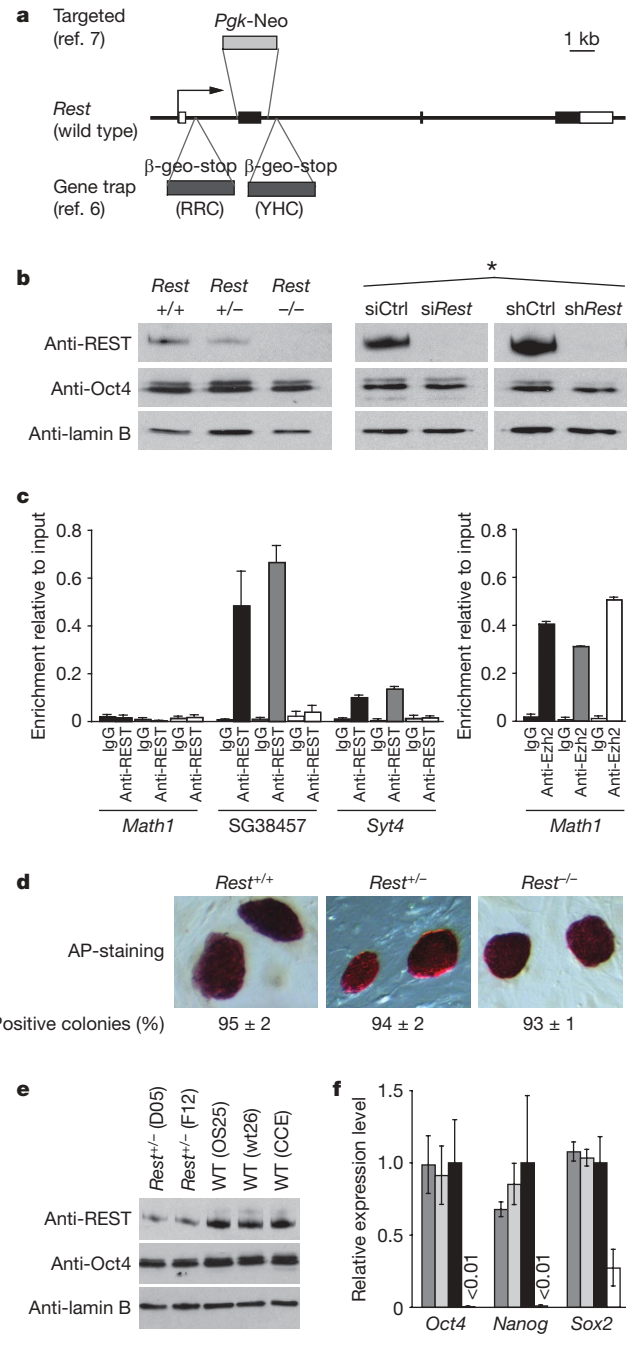
Is REST required for ESC pluripotency?

Arising from: S. K. Singh, M. N. Kagalwala, J. Parker-Thornburg, H. Adams & S. Majumder *Nature* **453**, 223–227 (2008)

The DNA-binding protein REST (also called NRSF) is a transcriptional repressor that targets many neuronal genes[1,2] and is abundant in human and mouse pluripotent embryonic stem cells (ESCs)[3–5]. In a recent Letter to *Nature*, Singh *et al.*[6] suggested that REST controls the self-renewal and pluripotency of ESCs, because they found that ESCs in which a single REST allele was disrupted (Fig. 1a, β-geo-stop insertion) had reduced alkaline phosphatase activity and expressed lower levels of several pluripotency-associated genes[6]. Here we show that partial or complete loss of functional REST protein does not abrogate ESC potential as reflected by marker gene expression. These data are consistent with earlier reports[7,8], and argue that REST is not required for maintaining ESC pluripotency.



Heterozygous *Rest*-targeted ESCs (Fig. 1a) that were isolated from a previously published mouse line[7] displayed a reduced level of full-length REST, and no full-length REST protein was observed in homozygous *Rest* mutant ESCs (Fig. 1b). The truncated REST peptide encoded by the mutated allele lacks DNA-binding capacity[7] and fails to bind REST target genes, as demonstrated by chromatin immunoprecipitation (Fig. 1c). We detected no substantial change in Oct4 (also called Pou5f1) protein levels or alkaline phosphatase activity in heterozygous (+/−) or homozygous (−/−) *Rest* ESCs relative to matched wild-type (+/+) controls (Fig. 1b, d). Analysis by quantitative polymerase chain reaction after reverse transcription (RT–PCR) demonstrated that the transcript levels of the pluripotency markers *Oct4*, *Nanog* and *Sox2* were similar in wild-type, $Rest^{+/-}$ and $Rest^{-/-}$ ESCs (Table 1). The REST target genes *Syp* and *Syt4* were de-repressed in the absence of REST. In contrast, genes that are induced on differentiation to endoderm (*Gata4*), mesoderm (*Bry*, also known as *T*) or ectoderm (*Sox1*) were expressed at low levels in both wild-type and *Rest* mutant ESCs compared to differentiated control tissues (Table 1). Hence, the expression of pluripotency markers and differentiation-associated genes was unaffected in REST-deficient ESCs. This is in contrast to the results reported by Singh *et al.*[6], but it is consistent with the phenotype of two independent, publicly available $Rest^{+/-}$ ESC lines (Fig. 1e, f). To address the possibility that the discrepancy between their results[6] and ours reflects either differences in the targeting approaches or selection for secondary events that compensate for REST deficiency, we performed REST knockdown experiments in feeder-independent wild-type ESCs using culture conditions and protocols similar to those described by Singh *et al.*[6]. The RNA interference analysis confirmed that markers of pluripotency and differentiation are not reproducibly deregulated in ESCs depleted of REST by either short hairpin RNA (shRNA) or short interfering RNA (siRNA) knockdown (Fig. 1b and Table 1, marked by asterisks).

Our results do not rule out that REST mutant ESCs may have a subtle phenotype; however, the conclusion by Singh *et al.*[6] that REST is required to maintain ESC self-renewal and pluripotency appears inconsistent with the results presented here.

Figure 1 | ESCs deficient in REST retain stem cell characteristics. a, Genetic alterations to the *Rest* locus analysed here (phosphoglycerate kinase 1-neomycin resistance gene (*Pgk*-Neo) insertion[7]) and by Singh *et al.*[6] (β-geo-stop insertions in the gene trap ESC lines RRC and YHC). Rectangles represent exons; coding regions are in black. **b**, REST, Oct4 and lamin B protein levels in wild-type (+/+), homozygous (−/−) and heterozygous (+/−) *Rest* ESC lysates, and (marked by an asterisk) in wild-type ESCs transfected with siRNA or shRNA constructs targeting *Rest* (si*Rest* and sh*Rest*) or a control sequence (siCtrl and shCtrl). **c**, Chromatin immunoprecipitation of $Rest^{+/+}$ (black), $Rest^{+/-}$ (grey) and $Rest^{-/-}$ (white) ESCs using anti-REST (left panel) and anti-Ezh2 (right panel) versus control antibody (immunoglobulin G, IgG). REST binds SG38457 (also known as *Fam70b*) and *Syt4* (which both contain a RE1 motif) but not *Math1* (also known as *Atoh1*; RE1-negative). No significant binding of REST was detected in $Rest^{-/-}$ ESCs. Ezh2-binding at *Math1* (ref. 10) confirmed that chromatin fragments were intact. Error bars represent the standard deviation of three experiments. **d**, Alkaline phosphatase (AP) activity of mutant ESCs (percentage AP-positive colonies ± standard deviation). **e**, REST, Oct4 and lamin B protein levels in two additional $Rest^{+/-}$ (D05 and F12) and three wild-type ESC lysates. **f**, Transcript levels in D05 (dark grey), F12 (light grey) and wild-type (black) ESCs are shown (and, for comparison, retinoic-acid-treated embryoid bodies (white)) relative to wild type. Values were normalized to housekeeping genes and error bars show standard deviation of 4–6 experiments.

Table 1 | Gene expression by REST-deficient ESCs

	Transcript levels†								
	Rest	*Oct4*	*Nanog*	*Sox2*	*Syp*	*Syt4*	*Gata4*	*Bry*	*Sox1*
Rest$^{+/+}$	100	100	100	100	100	100	100	100	100
Rest$^{+/-}$	62 ± 0.4	77 ± 30	48 ± 11	87 ± 13	208 ± 91	64 ± 52	76 ± 15	118 ± 47	157 ± 44
Rest$^{-/-}$	1 ± 0.5	96 ± 13	82 ± 18	95 ± 3	8,356 ± 2,678	1,291 ± 327	147 ± 19	337 ± 106	93 ± 13
Controls‡	9 ± 5	<1	<1	21 ± 10	10,000	2,700	1,500	2,300	2,000
siCtrl*	100	100	100	100	100	100	100	100	100
si*Rest**	15 ± 2	107 ± 5	143 ± 26	96 ± 6	1,840 ± 279	336 ± 58	96 ± 14	128 ± 28	114 ± 24
shCtrl*	100	100	100	N.A.	100	100	100	100	100
sh*Rest**	17 ± 7	85 ± 0.1	86 ± 6	N.A.	1,602 ± 39	352 ± 109	91 ± 10	77 ± 26	91 ± 17

* RNAi-mediated REST knockdown experiments.
† Transcipt levels were detected by real-time RT–PCR (primers available on request) and values were normalized to housekeeping genes (*Hmbs*, *Gapdh*, *Ubc*). Results are shown relative to wild-type ESCs (*Rest*$^{+/+}$) or control ESC samples (siCtrl, shCtrl). The average and standard deviation of 3–6 independent experiments are displayed.
‡ Differentiated ESCs (retinoic-acid-treated embryoid bodies) provide negative controls for *Rest*, *Oct4*, *Nanog* and *Sox2*, whereas detection of *Syp*, *Syt4*, *Sox1* (in E15 embryo head), *Gata4* (in E16 embryo liver) and *Bry* (in ES-cell-derived mesoderm) provide positive controls.

METHODS

Wild-type, *Rest*$^{+/-}$ and *Rest*$^{-/-}$ ESCs were derived from mutant *Rest* animals[7] by standard methods[9] and cultured on mitotically inactivated embryonic fibroblasts in the presence of leukaemia inhibitory factor (LIF, 1,000 U ml^{-1}). No difference in the phenotype of early passage (P5–P8) and late passage (>P30) cells was observed (data not shown). Two independent *Rest*$^{+/-}$ ESC lines (D034D05 (D05) and D034F12 (F12)) were obtained from the German Genetrap Consortium and cultured as described above. Culturing of wt26 and CCE wild-type ESC lines has been described[10,11]. E14Tg2A-derived 46C (ref. 12) and OS25 (ref. 13) ESC lines were cultured in the presence of LIF on gelatinized plates throughout. 46C ESCs were transfected with siRNA targeting REST or with negative control siRNA and analysed two days later. OS25 ESCs were transfected with shRNA constructs targeting REST or a control sequence and collected after three days as described previously[11]. Alkaline phosphatase staining, western blotting, chromatin immunoprecipitation and gene expression analyses were performed as described[10,11].

Helle F. Jørgensen[1], Zhou-Feng Chen[2], Matthias Merkenschlager[1] & Amanda G. Fisher[1]

[1]Lymphocyte Development Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN, UK.
e-mail: helle.jorgensen@imperial.ac.uk
e-mail: amanda.fisher@csc.mrc.ac.uk
[2]Departments of Anesthesiology, Psychiatry and Developmental Biology, Washington University School of Medicine Pain Center, Saint Louis, Missouri 63110, USA.

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Is REST a regulator of pluripotency?

Arising from: S. K. Singh, M. N. Kagalwala, J. Parker-Thornburg, H. Adams & S. Majumder *Nature* 453, 223–227 (2008)

Establishment and maintenance of the pluripotent state of ESCs is a key issue in stem cell biology and regenerative medicine, and consequently identification of transcription factors that regulate ESC pluripotency is an important goal. Singh *et al.*[1] claim that the transcriptional repressor REST is such a regulator and that a 50% reduction of REST in ESCs leads to activation of a specific microRNA, miR-21, and that this subsequently results in loss of pluripotency markers and a reciprocal gain in some lineage-specific differentiation markers. In contrast, we show that, in haplodeficient *Rest*$^{+/-}$ ESCs, we detected no change in pluripotency markers, no precocious expression of differentiated neuronal markers and no interaction of REST with miR-21. It is vital that identification of factors that regulate pluripotency is based on robust, consistent data, and the contrast in data reported here undermines the claim by Singh *et al.*[1] that REST is such a regulator.

Singh *et al.*[1] use mouse ESCs in which one allele of *Rest* was knocked out by insertion of gene traps (YHC334 and RRC160 cells) and by use of *Rest* siRNA. Both manipulations result in a 50% loss of REST protein. Using these two experimental systems, Singh *et al.*[1] then go on to characterize expression of pluripotent and lineage-specific markers in self-renewing E14 ESCs, and during their differentiation into embryoid bodies. They observe that decreased *Rest* levels lead to a loss of pluripotency markers such as Oct4 and Nanog, and an increase in expression of lineage-specific differentiation markers including induction of neuronal progenitor markers Ngn2 and Mash1. Most significantly, the authors conclude that these changes are a consequence of direct transcriptional repression of a microRNA, miR-21.

However, these conclusions are in marked contrast to previous results that have shown that RNA-interference-mediated reduction of *Rest* levels in ESCs does not induce loss of pluripotency markers[2].

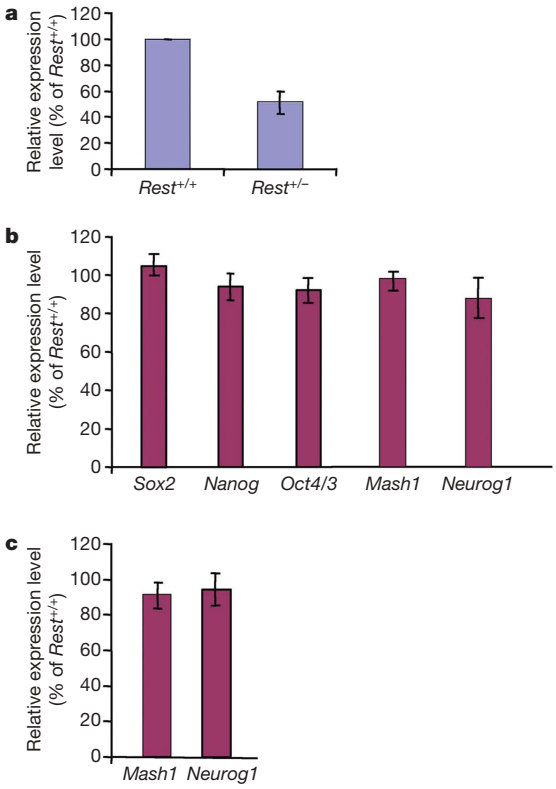

Figure 1 | *Rest*$^{+/-}$ ESCs retain pluripotent markers and show no precocious induction of neural marker genes. **a**, Relative expression levels of *Rest* in wild-type (*Rest*$^{+/+}$) and haplodeficient (*Rest*$^{+/-}$) mouse ESCs assessed using real-time PCR analysis. **b**, Relative levels of pluripotency genes *Sox2*, *Nanog* and *Oct4/3* and neuronal progenitor genes *Mash1* and *Neurog1* in undifferentiated *Rest*$^{+/-}$ compared to wild-type *Rest*$^{+/+}$ cells. No significant differences were detected using Student's two-tailed *t*-test at $P < 0.05$. **c**, Relative levels of neuronal progenitor genes *Mash1* and *Neurog1* in *Rest*$^{+/-}$ compared to wild-type *Rest*$^{+/+}$ cells after four days of monolayer differentiation. No significant differences were detected using Student's *t*-test at $P < 0.05$. Error bars indicate standard error of the mean of three independent experiments.

Furthermore, loss of either one or both *Rest* alleles does not lead to any discernible dysfunction in gastrulation in transgenic mice[3]. Here we present additional evidence, using ESCs with one allele of *Rest* deleted (*Rest*$^{+/-}$), that argues against a role of REST in regulating pluripotency genes. In our *Rest*$^{+/-}$ ESCs, despite a 50% reduction in *Rest* levels (Fig. 1a), we see no change in expression levels of *Sox2*, *Nanog* or *Oct4/3* between wild-type ESCs or in *Rest*$^{+/-}$ cells (Fig. 1b), nor do we observe precocious induction of neuronal progenitor markers *Mash1* and *Neurog1* (Fig. 1c). In support of these findings, DNA microarray analysis of ESCs treated with either a dominant-negative REST construct[4] or siRNA (data not shown) does not show downregulation of any pluripotency genes.

The second strand of data in Singh *et al.*[1] pertains to the repression of miR-21 by REST, and the suggestion that it mediates the pluripotency function of REST in ESCs through its repression of transcripts of *Nanog*, *Sox2*, *Tbx3* and *c-Myc*. Several facts argue against this interpretation. First, neither ChIP-PET (ChIP paired-end diTag) nor ChIP-PCR analysis of E14 mouse ESC chromatin provides any evidence for recruitment of REST at the miR-21 locus[4]. This absence of REST binding is also evident in other genome-wide ChIP analyses[5,6]. Second, another study showed that in human ESCs miR-21 levels actually drop during embryoid body differentiation[7] arguing against a role for miR-21 in the downregulation of pluripotency markers and contradicting the data of Singh *et al.*[1], who report an increase in miR-21 levels in embryoid bodies compared with ESCs. Third, REST does not regulate levels of miR-21 in a transformed embryonic striatal cell line[8] where both are robustly expressed[9]. Although, there may be some cell and/or context specificity to REST occupancy, most genome-wide ChIP studies show a remarkable overlap of target genes and, as yet, no such study has indicated miR-21 as a REST target gene. Further, we emphasize that, as with Singh *et al.*[1], all our data are derived using feeder-free conditions[4].

In light of these conflicting lines of diverse evidence from multiple laboratories, we feel that, at present, it is premature to conclude that REST "is a newly discovered element of the interconnected regulatory network that maintains the self-renewal and pluripotency of mouse ES cells"[1].

METHODS

A conditional knockout targeting vector was used to flox one of the *Rest* alleles in HM1 ESCs followed by transient expression of *Cre* recombinase to delete the floxed allele. ESCs were differentiated into neural progenitor cells and subsequently to mature neurons using a monolayer culture in N2B27 medium[10]. Gene expression analysis was carried out using real-time PCR and data analysed using Student's *t*-test and $P < 0.05$ and $P < 0.01$. ChIP-PET analysis[11] of chromatin was carried out as described using anti-REST antibody (Upstate 07-579). All ESCs were cultured feeder-free; full details can be found elsewhere[4].

Noel J. Buckley[1], Rory Johnson[2], Yuh-Man Sun[1] & Lawrence W. Stanton[2]
[1]King's College London, Institute of Psychiatry, Centre for the Cellular Basis of Behaviour, James Black Centre, 125 Coldharbour Lane, London SE5 9NU, UK.
e-mail: noel.buckley@iop.kcl.ac.uk
[2]Stem Cell and Developmental Biology, Genome Institute of Singapore, 60 Biopolis Street #02-01, Genome Building, Singapore 138672.

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Singh et al. *reply*

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In contrast to the comments made by Jørgensen *et al.*[1] and Buckley *et al.*[2], our experiments showed that REST maintains the self-renewal and pluripotency of mouse embryonic stem cells (mESCs)[3]. Two recent papers support our work: ref. 4 indicated that REST is indeed in the network that regulates ESC self-renewal and pluripotency and ref. 5 showed that mESCs with lower REST levels derived from a mouse model of Down's syndrome have decreased levels of self-renewal markers and a higher propensity towards differentiation, even when cultured in the presence of LIF. We note that Buckley and Stanton also recently concluded that REST is part of the Oct4–Sox2–Nanog regulatory network and has "a key role in the maintenance of the ESC phenotype"[6]. We proposed that REST represses a set of microRNAs that potentially target self-renewal genes. At least one of them, miR-21, represses self-renewal, probably by destabilizing the messenger RNAs of Sox2 and/or Nanog (not Tbx3 or c-Myc as suggested by Buckley *et al.*[2]). In this model, changes in the cellular environment that counter this function of REST or stimulate the mRNA levels of Sox2 or Nanog could minimize the effect of REST. For this reason, in our study we used mESCs with a low passage number, and we cultured them without feeder cells to avoid possible contributions of the feeder cells or an adaptive response to high passage.

One possible reason for the discrepancies between our study and the studies by Jørgensen *et al.*[1] is the use of feeder cells in most of their experiments (see Fig. 1d of Jørgensen *et al.*). Indeed, our additional experiments showed that culturing mESCs with feeder cells severely dampens the requirement of REST-mediated maintenance of mESC self renewal and pluripotency (S.K.S., manuscript in preparation). However, to rule out adaptive responses, Jørgensen *et al.*[1] also performed REST knockdown experiments in feeder-independent "wild-type" mESCs (46C and OS25) using either si*Rest* or sh*Rest* (Fig. 1e of Jørgensen *et al.*[1]). However, 46C and OS25 are not wild-type cells and were generated from the E14TG2a wild-type cells by genetic manipulation (46C has an insertion in *Sox1* gene and OS25 has an insertion in *Sox2* and *Oct4* genes)[7]. It is unclear how these manipulations affected REST function and the use of these cells and not the wild-type cells probably explains their contradictory results (we used wild-type E14TG2a mESCs). In some experiments, Jørgensen *et al.*[1] used retinoic acid (see Fig. 1e of Jørgensen *et al.*). Additionally, we did not use retinoic acid in our experiments. Buckley *et al.*[2] mentioned that their data (mostly relative RT–PCR) are derived using feeder-free conditions. One potential reason for the discrepancy between our data and theirs could be high cell passage number or different cell type, among other experimental variations. For instance, their earlier report[6] mentioned above, in which they did find a critical role of REST in mESC self-renewal, used E14 ESCs instead of the HM1 cells used here. The absence of relative difference in *Mash1* and *Neurog1* levels in wild-type versus $Rest^{+/-}$ mESCs is not surprising because neuronal differentiation will cause a notable reduction in REST protein levels[8,9] (and consequent high *Mash1* and *Neurog1* expression) in both wild-type and $Rest^{+/-}$ cells. Our REST-miR-21 ChIP and functional data are specific, reproducible and significant. Buckley *et al.*[2] cited their high-throughput ChIP-PET and ChIP-qPCR analysis using our published primers[3] to indicate that REST does not bind to the miR-21 gene chromatin in mESCs. We published multiple locus-specific primer sets only for conventional ChIP but not ChIP-qPCR analysis (although these were available on request). The primers used in our ChIP-qPCR studies would provide good controls for binding[3], because binding of REST is weaker on miR-21 than on miR-124 chromatin and requires higher substrate. REST-miR-21 functional assays will also help resolve the discrepancies.

Buckley *et al.*[2] cited reports that used cell types other than mESCs. Moreover, their cited references 8 and 9 measure neither REST protein levels nor the direct effect of REST on miR-21. In contrast to Buckley *et al.*'s cited ref. 7, we note that our ref. 10 found that miR-21 expression was higher in differentiated than in undifferentiated mESCs, supporting our conclusions. Both communications cited Loh *et al.*[11] to suggest that knockdown of *Rest* using si*Rest* did not reduce self-renewal and did not induce loss of self-renewal markers in mESCs. This paper neither measured percentage self-renewal nor the level of the self-renewal regulators after si*Rest* treatment and cannot be used to counter our conclusions. Both communications also cited Chen *et al.*[12] to indicate that $Rest^{+/-}$ or $Rest^{-/-}$ mutant mice show germline transmission/gastrulation. This situation is similar to many other self-renewal regulators, such as LIF, LIF-receptor β, gp130 (also known as Il6st), Stat3 and c-Myc[13] and could be relevant during diapause[14] (see Supplementary Information of our paper for details).

Sanjay K. Singh[1,5], Mohamedi N. Kagalwala[1,5]†, Jan Parker-Thornburg[2], Henry Adams[1] & Sadhan Majumder[1,3,4,5,6]

[1]Department of Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA.
e-mail: smajumder@mdanderson.org
[2]Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA.
[3]Department of Neuro-Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA.
[4]The Brain Tumor Center, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA.
[5]Center for Stem Cell and Developmental Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA.
[6]Program in Genes and Development, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas 77030, USA.
†Present address: Laboratory of Genetics, Salk Institute for Biological Studies, La Jolla, California 92037, USA.

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