

ORIGINAL ARTICLE

The nuclear receptor NR2E1/TLX controls senescence

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The nuclear receptor NR2E1 (also known as TLX or tailless) controls the self-renewal of neural stem cells (NSCs) and has been implied as an oncogene which initiates brain tumors including glioblastomas. Despite NR2E1 regulating targets like p21^{CIP1} or PTEN we still lack a full explanation for its role in NSC self-renewal and tumorigenesis. We know that polycomb repressive complexes also control stem cell self-renewal and tumorigenesis, but so far, no formal connection has been established between NR2E1 and PRCs. In a screen for transcription factors regulating the expression of the polycomb protein CBX7, we identified NR2E1 as one of its more prominent regulators. NR2E1 binds at the CBX7 promoter, inducing its expression. Notably CBX7 represses NR2E1 as part of a regulatory loop. Ectopic NR2E1 expression inhibits cellular senescence, extending cellular lifespan in fibroblasts via CBX7-mediated regulation of p16^{INK4a} and direct repression of p21^{CIP1}. In addition NR2E1 expression also counteracts oncogene-induced senescence. The importance of NR2E1 to restrain senescence is highlighted through the process of knocking down its expression, which causes premature senescence in human fibroblasts and epithelial cells. We also confirmed that NR2E1 regulates CBX7 and restrains senescence in NSCs. Finally, we observed that the expression of NR2E1 directly correlates with that of CBX7 in human glioblastoma multiforme. Overall we identified control of senescence and regulation of polycomb action as two possible mechanisms that can join those so far invoked to explain the role of NR2E1 in control of NSC self-renewal and cancer.

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INTRODUCTION

The orphan nuclear receptor NR2E1 (also known as tailless or TLX) is critical for maintaining the undifferentiated characteristics and proliferative potential of neural stem cells (NSCs).^{1–3} NR2E1 is expressed in the vertebrate forebrain⁴ and at elevated levels in a number of brain cancers, such as astrocytomas, ependymomas and gliomas, suggesting a role in tumorigenesis.^{5–11}

By analogy to tailless in *Drosophila*, NR2E1 has generally been viewed as a transcriptional repressor that is able to recruit corepressors such as histone deacetylase complexes¹² and the histone demethylase LSD1¹³ to consensus binding sites in target gene promoters. Prominent examples include the *Pten* tumor suppressor and *Cdkn1a*, which encodes the p21^{CIP1} cyclin-dependent kinase inhibitor.^{12,14} De-repression of *Cdkn1a* could in part explain the proliferative impairment of NSCs observed following conditional knockout of *Nr2e1/Tlx* in the mouse. However, crossing the *Nr2e1*^{−/−} mice into a *Cdkn1a*^{−/−} background does not fully rescue the phenotype.¹¹ NR2E1 also participates in a feedback loop with the brain-specific microRNA, miR-9.¹⁵ Thus, miR-9 downregulates NR2E1 via a target sequence in the 3'-untranslated region, whereas NR2E1 represses the expression of miR-9-1 by binding to consensus site(s) in the adjacent DNA.

A broader view of NR2E1 function has emerged from expression profiling of the *Nr2e1* knockout cells, which revealed equivalent numbers of up and downregulated genes.³ It was subsequently confirmed that NR2E1 activates the *Wnt7a* gene via two consensus binding sites in the promoter, and that Wnt/β-catenin signaling can partially rescue the defect in NSC proliferation

caused by NR2E1 knockdown.¹⁶ However, the possibility that NR2E1 regulates additional targets relevant to its function in NSC self-renewal and cancer clearly remains open.

The polycomb group (PcG) protein CBX7 is also implicated in the maintenance of stem cell characteristics and cancer. CBX7 is one of the five mammalian orthologues of *drosophila* Pc and participates in Pc repressive complex 1 (PRC1) along with members of the posterior sex combs, polyhomeotic and sex combs extra families.¹⁷ CBX7 is the predominant Pc orthologue in ES cells and upon differentiation its levels decline and are replaced by CBX4 and CBX8.^{18,19} This downregulation of CBX7 is in part orchestrated by micro-RNAs from the miR-125 and miR-181 families and by feedback loops with PRC complexes.^{18–20}

In contrast to ES cells, human diploid fibroblasts express multiple PRC1 components, including CBX4, CBX6, CBX7 and CBX8.²¹ CBX7 was first identified in a screen for the bypass of replicative senescence, the state of profound cell cycle arrest that occurs when cells reach replicative exhaustion or are exposed to stress caused by oncogene activation or DNA damaging agents.²² Although traditionally studied in cultured human fibroblasts (HFs), senescence is relevant in several physiological contexts including development, ageing and premalignant lesions *in vivo*.^{23–27} In particular, senescence limits tumor progression and escape from senescence is one of the hallmarks of cancer.²⁸ The role of CBX7 and PRC1 complexes in senescence is largely explained by repression of the *CDKN2A* tumor suppressor locus, and its primary product, the CDK inhibitor p16^{INK4a}.²⁹ Although this regulation contributes to explain, at least in part, the oncogenic properties of

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CBX7 in prostate cancer or follicular lymphomas,^{30,31} the role of CBX7 in cancer is context dependent as it can behave as a tumor suppressor in lung and pancreatic cancer.^{32,33}

Here, we identify NR2E1 in a screen for regulators of CBX7 expression and show that NR2E1 can downregulate p16^{INK4a} via effects on CBX7. As a consequence, NR2E1 expression inhibits senescence. In addition to maintain p16^{INK4a} repressed, NR2E1 also directly repressed p21^{CIP1} with the downregulation of both CDK inhibitors contributing to the ability of NR2E1 to control senescence. Besides identifying a novel pathway regulating CBX7 expression, our work suggests that modulation of polycomb function and control of senescence are additional mechanisms by which NR2E1 might regulate NSC self-renewal and cancer.

RESULTS

A reporter-based screen for regulators of CBX7 transcription

To identify novel factors controlling CBX7 expression, we screened a library of 704 complementary DNAs (cDNAs) encoding known

transcription regulators for their ability to regulate a reporter in which a region of the mouse *Cbx7* promoter was cloned upstream of the luciferase gene (Figures 1a and b). Among the top *Cbx7* activators we identified several members of the E2F family, the homeobox protein PITX2, and the nuclear receptor NR2E1. Re-testing of candidates with either the mouse *Cbx7* promoter (Figure 1c) or an equivalent reporter based on the human *CBX7* promoter (Supplementary Figure S1a) confirmed these observations.

As E2F family members are already known to regulate the expression of PcG genes, such as *EZH2*, *SUZ12* and *BMI1*,³⁴ we chose to focus on NR2E1, an orphan nuclear receptor without any previously known connections to PcG regulation.³⁵ In common with other nuclear receptors, NR2E1 has an amino-terminal DNA binding domain and a ligand-binding domain in the carboxy terminal region (Figure 1d). Whereas wild-type NR2E1 activates the CBX7 promoter, this effect is not seen with a mutated form of NR2E1 (NR2E1 Δ 40) that lacks 40 residues from the amino-terminal DNA binding domain (Supplementary Figures S1b and c). We

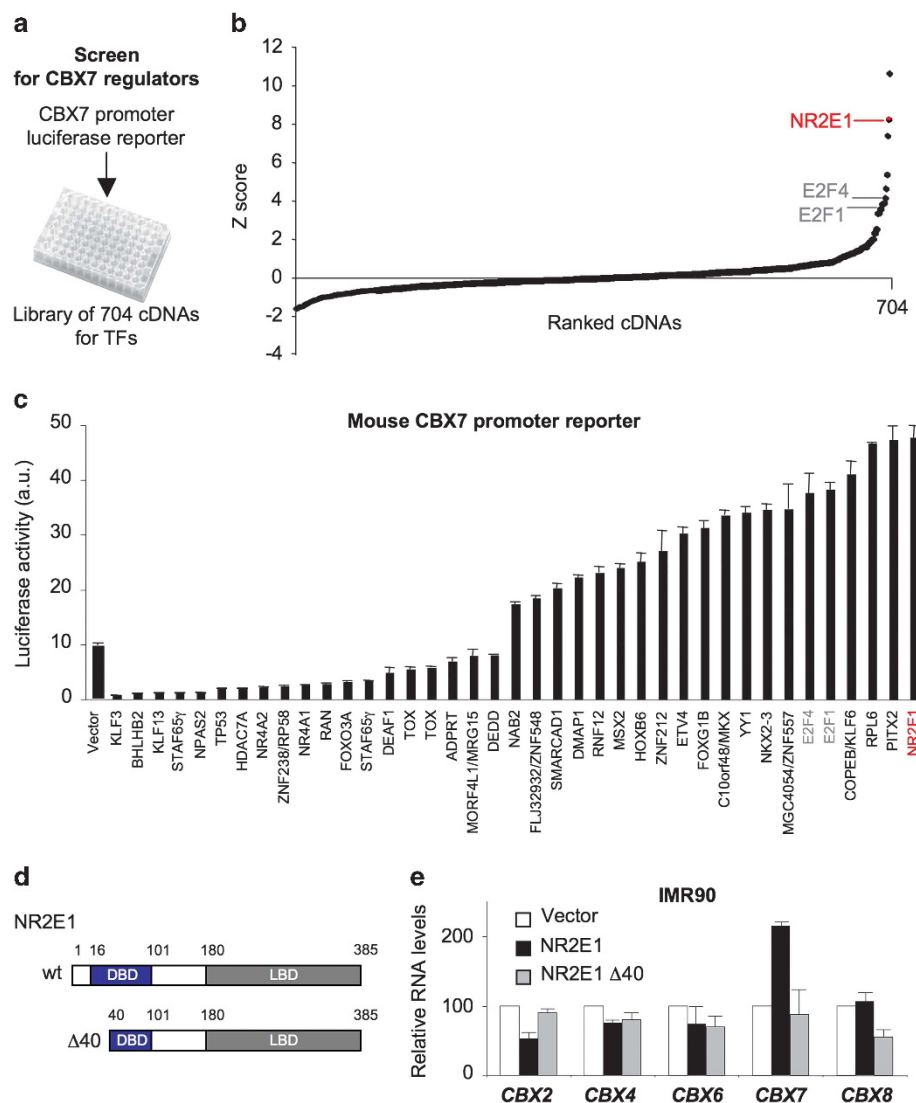


Figure 1. A screen for transcription factors regulating CBX7 expression identifies NR2E1/TLX. **(a)** Scheme depicting the screen. A mouse CBX7 promoter luciferase reporter was co-transfected in HEK293T cells with a library of cDNAs of transcription factors to screen for cDNAs regulating CBX7 transcription. **(b)** Results of the screen. **(c)** Re-testing of candidates for CBX7 regulation identifies E2F family proteins and NR2E1 as activators of CBX7 transcription. **(d)** Cartoon showing the structure of NR2E1 wt and a DNA binding domain mutant (NR2E1 Δ 40). DBD, DNA binding domain; LBD, ligand binding domain. **(e)** NR2E1 induces CBX7 but not other CBX family genes. qRT-PCR for CBX family members in IMR90 cells infected with expression vectors for NR2E1 wt or NR2E1 Δ 40 or with a control vector.

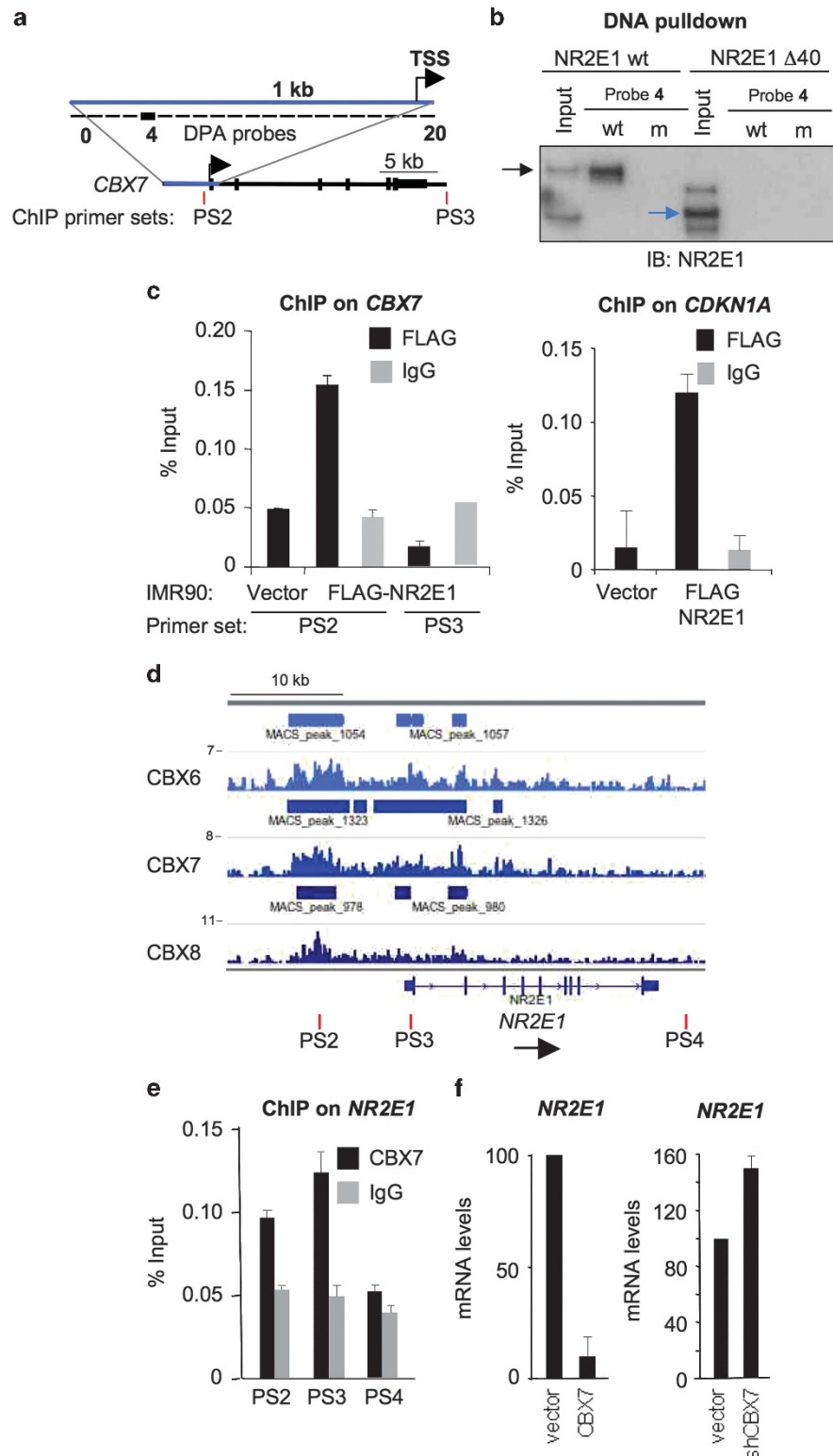


Figure 2. A regulatory feedback loop between NR2E1 and CBX7. **(a)** Scheme of the *CBX7* gene showing the location of primers sets used for ChIP and probes used for DNA pull-down assay. **(b)** DNA pull-down analysis. DNA pull-down assay confirmed NR2E1 binding to a DNA motif upstream of *CBX7* transcription start site. Lysates of HEK293T cells overexpressing NR2E1 wt or the DNA binding domain mutant NRE1 Δ 40 were incubated with probe 4, harboring a wt or mutated NR2E1 DNA binding motif. NR2E1 binding was assessed by immunoblot (IB). Black arrow, NR2E1 wt; blue arrow, NRE1 Δ 40. **(c)** NR2E1 is a direct transcriptional regulator of *CBX7*. ChIP with FLAG antibody or control immunoglobulin-G were performed on *CBX7* (left) or *CDKN1A* (right, as positive control) in IMR90 cells infected with FLAG-NR2E1 overexpressing vector or with an empty vector. **(d)** CBX6, CBX7 and CBX8 associate with NR2E1 promoter. ChIP-seq data in Hs68 HFs showing CBX6, CBX7 and CBX8 binding profiles on NR2E1. The location of primer sets used for ChIP on NR2E1 is also depicted. **(e)** ChIP in IMR90 cells confirming that CBX7 binds to NR2E1 promoter. **(f)** CBX7 regulates *NR2E1* expression, as shown by qRT-PCR upon CBX7 overexpression (left) or knockdown (right) in IMR90 cells.

recently showed that the expression of the five Pc orthologues in mammalian cells is differentially regulated.¹⁹ In agreement with these observations, NR2E1 wt, but not NR2E1 Δ 40, induced CBX7 in IMR90 primary HF cells without affecting the levels of other Pc orthologues (Figure 1e).

NR2E1 binds directly to the CBX7 promoter

A search for NR2E1 consensus binding sites in the human *CBX7* locus revealed multiple candidates, including a site in the promoter region used for the screening. To better define this, we performed a DNA pull-down assay using 21 probe sets spanning a region of 1 kb upstream of the *CBX7* TSS (Figure 2a). Probe 4 resulted in a specific pull down of NR2E1 (Supplementary Figure S2a). We observed that a consensus NR2E1 binding sequence was present in that probe, and its mutation resulted in abrogation of NR2E1 binding (Figure 2b). However, we did not observe a significant reduction in NR2E1 activation of a mouse *CBX7* reporter lacking the equivalent region (Supplementary Figure S2b). This suggests the presence of alternative NR2E1 binding sites or indirect mechanisms by which NR2E1 regulated *CBX7*. To confirm that NR2E1 was binding at the *CBX7* locus on native chromatin, we performed chromatin immunoprecipitation

(ChIP). Human fibroblasts were infected with a retrovirus encoding a FLAG-tagged version of NR2E1 and FLAG immunoprecipitates were interrogated using two PCR primer sets (PS) that sample the 5' and 3' ends of *CBX7* (Figure 2c). Clear enrichment, relative to the immunoglobulin-G control, was observed with the PS2 from the promoter region but not with the PS3, which served as a specificity control (Figure 2d). In addition, we also observed NR2E1 binding to the *CDKN1A* promoter (Figure 2d), consistent with previous reports showing direct repression of *CDKN1A* by NR2E1.¹²

Evidence for a feedback loop between CBX7 and NR2E1

We recently compared the genome-wide binding profiles of several CBX proteins in HF cells.²¹ Strikingly, we found that CBX6, CBX7 and CBX8 co-localize at multiple sites in the genome and, as others have reported, NR2E1 was among the potential PRC1 target genes^{18,20} (Figure 2d). In common with many other loci, the binding profile at NR2E1 has a complicated architecture, with several peaks located upstream, across and downstream of the predicted TSS. To confirm the ChIP-seq data, we prepared a series of PCR PS that can discriminate between regions of high and low/no binding in the NR2E1 gene and used these for conventional

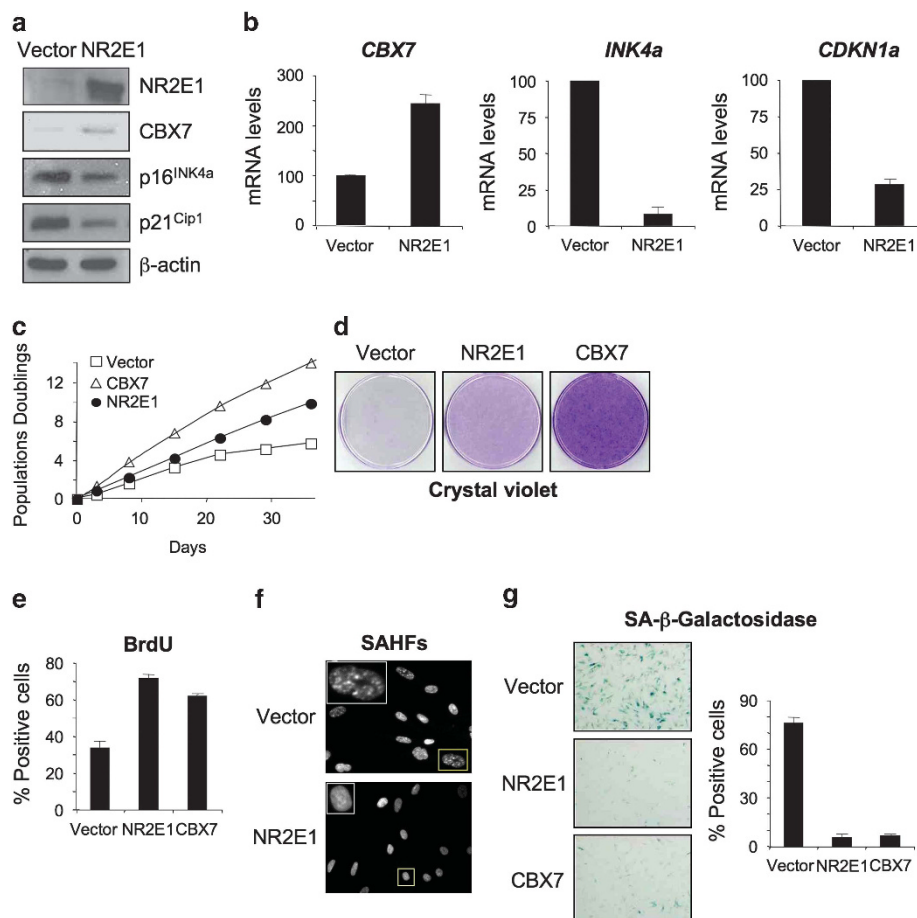


Figure 3. Expression of NR2E1 inhibits senescence. **(a, b)** NR2E1 overexpression in IMR90 results in an increase in CBX7 and decrease in p16^{INK4a} and p21^{CIP1} levels, as shown by immunoblot **(a)** and qRT-PCR **(b)**. **(c, d)** NR2E1 overexpression extends the lifespan of IMR90 cells. IMR90 cells were infected with vectors expressing CBX7 or NR2E1 or a control vector and growth curves were performed **(c)**. Crystal violet staining is also shown **(d)**. **(e)** BrdU incorporation is increased in NR2E1 overexpressing cells. BrdU incorporation was assessed at passage 24 in IMR90 cells infected with the indicated vectors. **(f)** NR2E1 overexpression results in prevention of senescence-associated heterochromatin foci (SAHF) formation. IMR90 cells infected with NR2E1 expression vector or control vector were fixed and stained with DAPI at passage 24. Representative images are shown. **(g)** SA-β-galactosidase activity is decreased in cells overexpressing NR2E1. IMR90 cells infected with the indicated vectors were stained for SA-β-galactosidase at passage 24. Representative pictures (left) and the percentage of SA-β-Gal-positive cells (right) are shown.

ChIP assays (Figure 2d). In line with our previous findings, we could show that CBX7 binds at the *NR2E1* locus (Figure 2e).

The presence of PRC1 proteins at the locus implies that NR2E1 expression could be regulated by CBX7. Consistent with this idea, overexpression of CBX7 resulted in downregulation of NR2E1 and conversely knockdown of CBX7 upregulated NR2E1 (Figure 2f). Taken together, the data imply that NR2E1 and CBX7 expression levels are intimately linked by regulatory feedback mechanisms.

NR2E1 expression inhibits senescence

To investigate whether NR2E1 has a role in controlling senescence, we first expressed NR2E1 in late passage IMR90 cells. Upon NR2E1 expression, we observed upregulation of CBX7 accompanied by downregulation of p16^{INK4a} (Figure 3a); a more pronounced effect was observed at the RNA level (Figure 3b). Importantly, the expression of NR2E1 also reduced the levels of p21^{CIP1} protein and RNA (Figures 3a and b) as previously reported that NR2E1 can do in other contexts,¹² and in agreement with our data showing binding of NR2E1 to the *CDKN1A* promoter (Figure 2b).

IMR90 cells were then passaged until the empty vector controls reached senescence. NR2E1 expression resulted in an appreciable extension of replicative lifespan, as judged by cumulative population doublings and colony formation assays (Figures 3c and d). Consistent with NR2E1 counteracting senescence, a higher percentage of the NR2E1-expressing cells incorporated BrdU (Figure 3e) and a smaller percentage stained positively for SA- β -Gal activity, or showed evidence of senescence-associated heterochromatin foci (Figures 3f and g). The effects of NR2E1 on senescence were dependent on DNA binding and were not observed with the NR2E1 Δ 40 mutant (Supplementary Figures S3a

and c). In addition NR2E1 expression also affected replicative senescence in a different strain of fibroblasts, WI-38 (Supplementary Figure S3d).

Next, we investigated a possible role of NR2E1 in oncogene-induced senescence (OIS). To this end, we overexpressed NR2E1 wt or a mutant version in IMR90 ER:RAS cells, a model of OIS. IMR90 ER:RAS express a chimeric protein that upon 4-hydroxy-tamoxifen (4OHT) addition becomes activated, triggering senescence.³⁶ The expression of NR2E1 partially prevented the growth arrest and senescence observed upon RAS induction (Figures 4a and b). Expression of NR2E1 also blunted the induction of p16^{INK4a} and p21^{CIP1} during OIS (Figure 4c). All together the above results suggest that one of NR2E1 functions is to suppress senescence.

Loss of NR2E1 expression induces premature senescence

To extend these observations, we investigated whether depletion of NR2E1 would be sufficient to induce premature senescence. To this end, we used recombinant retroviruses to deliver short hairpin RNAs (shRNAs) that target NR2E1 (Supplementary Figure S4a). IMR90 infection with shNR2E1.2, which was capable of reducing NR2E1 expression by over 90%, led to decreased cell proliferation and premature senescence (Figures 5a and b and Supplementary Figure S3b). These observations were confirmed using shNR2E1.3, another shRNA targeting NR2E1 (Supplementary Figure S4b). Similar results were obtained following transfection of IMR90 cells with an NR2E1-specific short interfering RNA (siRNA). Although the knockdown was less efficient, there was a significant decrease in the proportion of BrdU-positive cells (Figure 5c). As anticipated, NR2E1 knockdown resulted in downregulation of CBX7 levels and

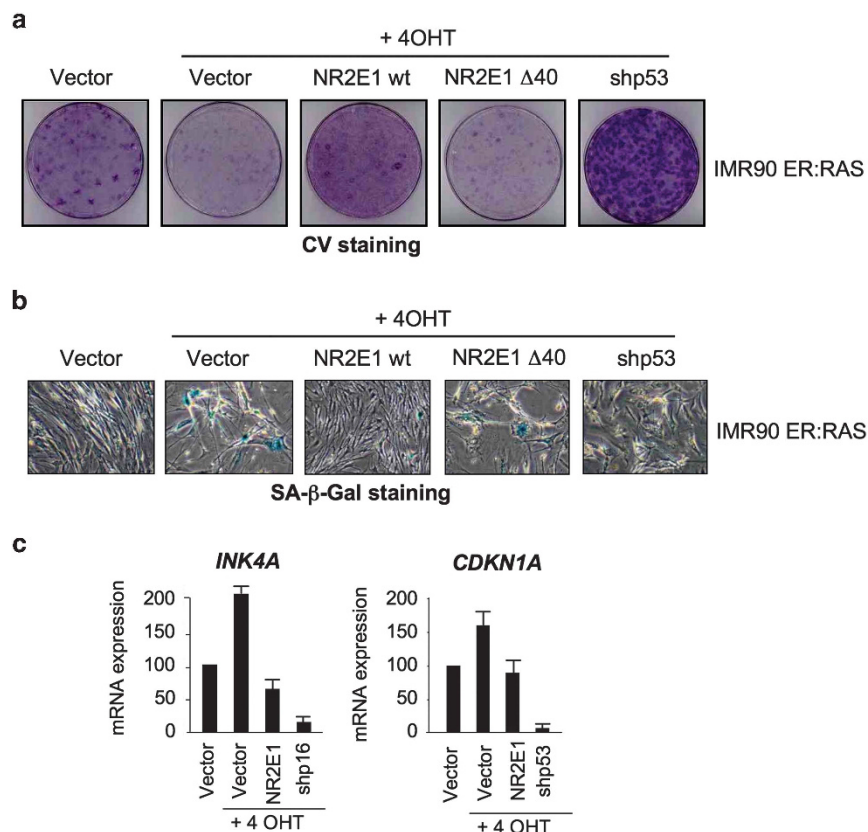


Figure 4. NR2E1 expression controls oncogene-induced senescence (OIS). (a, b) NR2E1 expression prevents OIS. (a) IMR90 ER:RAS cells were infected with the indicated vectors. ER:RAS was induced by 200 nM 4-hydroxy-tamoxifen (4OHT). Twelve days later, cell growth was evaluated by crystal violet staining (a) and senescence induction by SA- β -Gal staining (b). (c) NR2E1 expression prevents the induction of *INK4a* and *CDKN1a* during OIS. IMR90 ER:RAS cells were infected with the indicated vectors, the expression of *INK4a* and *CDKN1a* was monitored by qRT-PCR.

upregulation of p16^{INK4a} and p21^{CIP1} (Figure 5d). Further extending the generality and significance of our results, knockdown of NR2E1 in primary human prostate epithelial cells also causes a senescence-like response accompanied by upregulation of p16^{INK4a} and p21^{CIP1} (Supplementary Figures S4c and d).

To investigate the contributions of NR2E1 regulation of p16^{INK4a} and p21^{CIP1} to senescence, we used previously validated shRNAs³⁷ to knockdown the expression of these genes (Supplementary Figure S4e) and asked what effects they had on the premature senescence caused by depletion of NR2E1. In accordance with our previous data, the proliferation block was partially dependent on both p16^{INK4a} and p21^{CIP1} (Figure 5e).

NR2E1 controls CBX7 and prevents senescence in NSCs

NR2E1 has been primarily implicated in the regulation and function of NSC and initiation of brain tumors.⁵ We therefore sought to investigate whether the mechanisms that we have uncovered in primary fibroblasts also operates in neural cells. We used two different siRNAs to knock down Nr2e1 expression in post-natal mouse NSCs (Supplementary Figure S5a). Although the knockdown was partial, both siRNAs resulted in reduced expression of *Cbx7* and the upregulation of both *Ink4a* and *Cdkn1a* (Figure 6a). The cells also underwent a senescence-like arrest as judged by increased numbers of SA- β -Gal-positive cells (Figures 6b and c). Similar results were obtained using lentiviral vectors to express different shRNAs targeting mouse *Nr2e1*

(Supplementary Figures S5a and b), suggesting that NR2E1 can also regulate CBX7 and senescence in NSCs.

Finally, we took advantage of a collection of samples from glioblastoma multiforme (GBM) patients and asked whether there was a link between the expression levels of *NR2E1* and *CBX7*, as judged by quantitative reverse transcriptase PCR (qRT-PCR). Interestingly, we observed a direct correlation, suggesting that enhanced levels of *NR2E1* associated with glioblastoma multiforme result in a concomitant increase in *CBX7* expression (Figure 6d). The above results suggest that control of CBX7 and senescence could contribute to explain the role of NR2E1 in NSC self-renewal and its role in gliomagenesis.

DISCUSSION

There has been a great deal of interest in the mechanisms by which PRC complexes regulate gene expression and how they are recruited to target loci, but relatively little is known about the transcriptional regulation of the PcG genes themselves. In particular CBX7 is a PcG important to control senescence,²² stem cell self-renewal¹⁹ and cancer.^{30,31,38,39} Recently others and we described that CBX7 expression is subjected to repression by PRCs and is controlled by miR-125 and miR-181,^{18,19} but not much is known about its regulation. Here, we identify the orphan nuclear receptor NR2E1 as a novel regulator of CBX7 expression.

There are intriguing parallels between CBX7 and NR2E1 as both are implicated in the maintenance of stem cell characteristics and

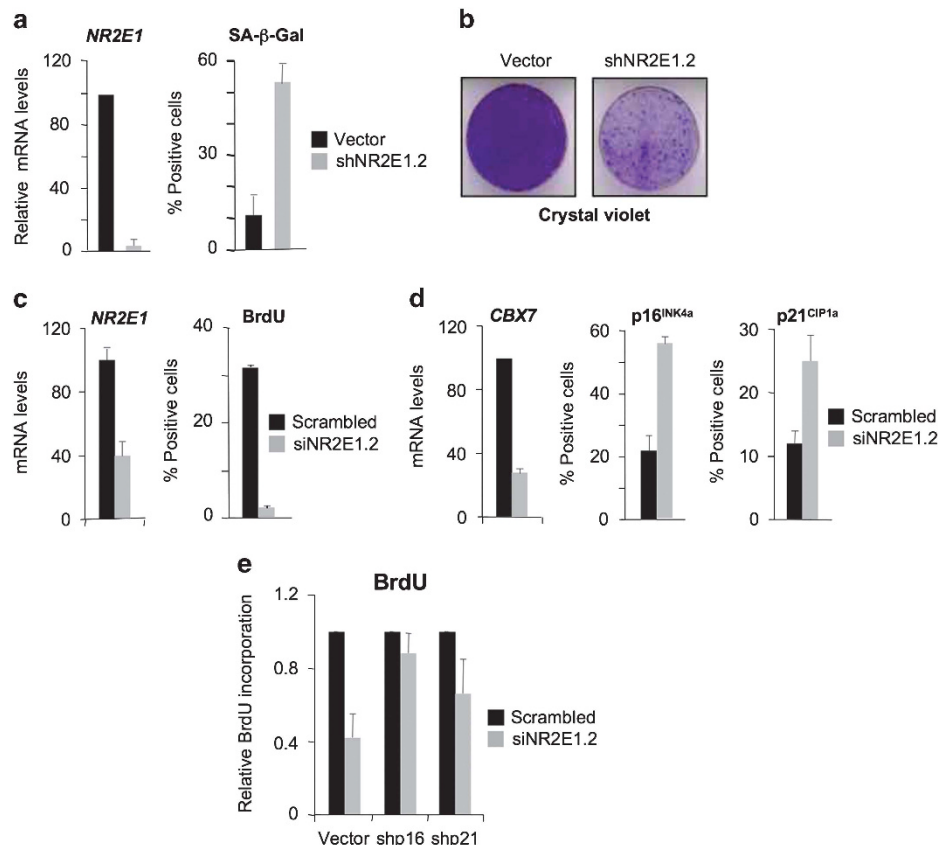


Figure 5. NR2E1 depletion causes premature senescence. **(a, b)** NR2E1 knockdown by shRNA induces cellular senescence in IMR90. Knockdown efficiency of NR2E1 shRNA **(a, left)** is shown. The effect of NR2E1 knockdown on the percentage of SA- β -galactosidase-positive cells **(a, right)** and cell growth **(b)** is shown. **(c)** NR2E1 knockdown by siRNA inhibits cell proliferation in IMR90, as shown by BrdU incorporation in IMR90 transfected with a scrambled siRNA or a siRNA targeting NR2E1 **(right)**. The efficiency of NR2E1 knockdown was assessed by qRT-PCR **(left)**. **(d)** NR2E1 knockdown results in a decrease in CBX7 level and an increase in p16^{INK4a} and p21^{CIP1} levels in IMR90, as shown by CBX7 qRT-PCR **(left)**, p16^{INK4a} immunofluorescence (IF; middle) and p21^{CIP1} IF **(right)**. **(e)** The effect of NR2E1 on senescence is dependent on p16^{INK4a} and p21^{CIP1}. BrdU incorporation was assessed in IMR90 cells infected with an empty vector (vector) or vectors knocking down p16^{INK4a} (shp16) or p21^{CIP1} (shp21) and then transfected with a scrambled siRNA or a siRNA targeting NR2E1.

both were historically viewed as transcriptional repressors. However, there is increasing evidence that they can have both positive and negative influences on gene expression, *albeit* in different ways. NR2E1 is a DNA binding transcription factor that recruits histone modifying enzymes to regulate transcription of target genes, whereas CBX7 operates within a multicomponent complex that associates with and modifies histone tails, rather than interacting directly with DNA. Whereas NR2E1 operates predominantly in neural lineages, CBX7 is more widely expressed. The role of CBX7 in the regulation of *INK4a* has been well-documented in human fibroblasts, the classical model for cellular senescence.²² Although the endogenous levels of NR2E1 in HF are low, ectopically expressed NR2E1 associates with the CBX7 locus and activates its expression. Conversely, CBX7 binds to the NR2E1 locus as a component of PRC1 complexes, and represses it as part of a regulatory feedback loop.

Our work shows how high levels of NR2E1 restrain senescence by keeping p16^{INK4a} and p21^{CIP1} expression downregulated. Therefore, besides regulating p16^{INK4a}, NR2E1 also impacts on p21/p53 as suggested from previous work showing that p21^{CIP1} is a direct NR2E1 target¹² and confirmed by us in the context of senescence. In addition, the engagement of p16^{INK4a} potentially explains why p21^{CIP1} knockout does not fully rescue the proliferation of *Nr2e1*^{-/-} cells.¹¹

As most of the previous literature on NR2E1 relates to its role in neural cells and brain-derived tumors,⁵ we investigated whether the regulation of CBX7 and senescence by NR2E1 that we have uncovered here also operates in these systems. Analysis of the gene expression data from human gliomas provided tentative evidence for a correlation between NR2E1 and CBX7. This is interesting, as a previous work has suggested that Cbx7 behaves as a tumor suppressor in gliomas.⁴⁰ However, in that study the effect of Cbx7 was analyzed on *Cdkn2a*^{-/-} mice, which don't express p16^{INK4a} or p19^{Arf}, key effectors of senescence. More importantly, knockdown of *Nr2e1* in NSCs recapitulated our observations in HF, suggesting that the ability of NR2E1 to prevent senescence is relevant for its role in self-renewal of NSCs as well as in cancer. Preliminary results with NSCs derived from *Cdkn2a*^{-/-} mice, suggest that similar to that observed in HF, the *Ink4a/Arf* locus contributes to mediate senescence caused by NR2E1 loss (data not shown).

In summary, we show that NR2E1 regulates CBX7 expression. As well as adding to our understanding of the transcriptional control of PcG genes, the data suggest that CBX7 can mediate some of the effects of NR2E1 in NSC and cancer. By controlling senescence via CBX7 dependent and independent mechanisms, NR2E1 adds additional functions to its already long repertoire that could explain its function in controlling NSC self-renewal and its role in gliomagenesis.

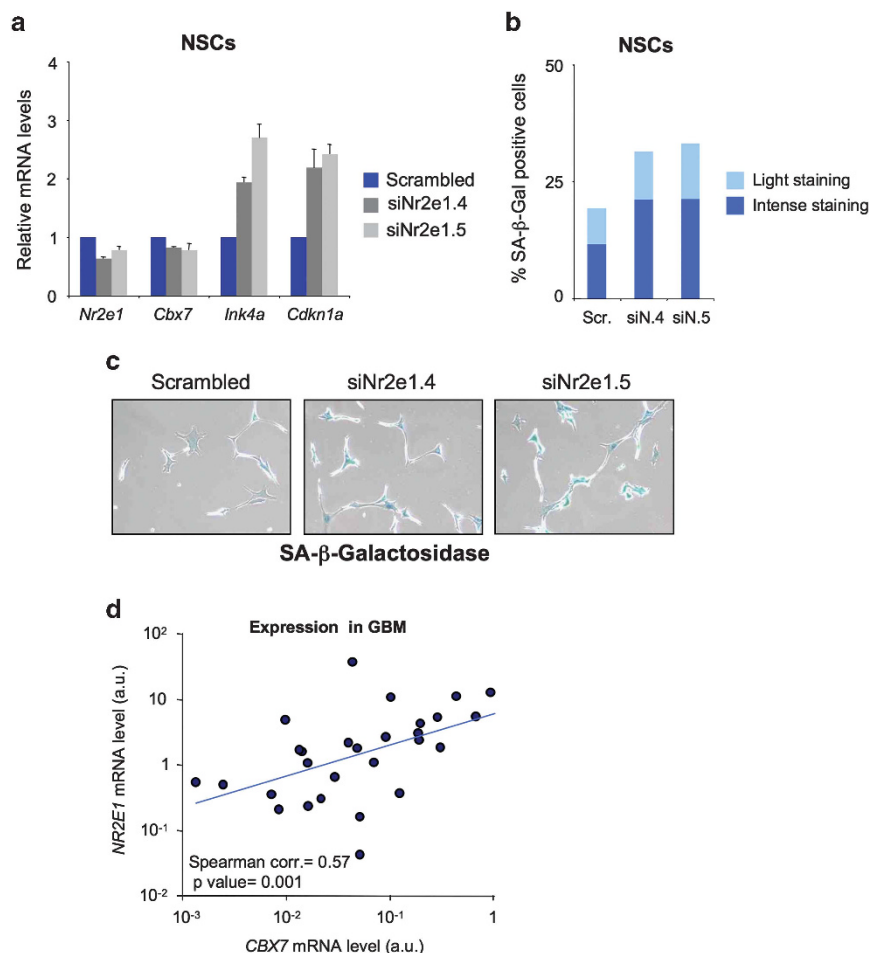


Figure 6. NR2E1 and CBX7 levels are linked in gliomas and neural stem cells. **(a)** *Nr2e1* knockdown by siRNA in mouse NSC downregulates *Cbx7* and upregulates *Ink4a* and *Cdkn1a*. NSC were transfected with two different siRNA targeting NR2E1 or a scrambled sequence and qRT-PCR were performed 2 days later. All differences in expression of *Nr2e1*, *Cbx7*, *Ink4a* and *Cdkn1a* between the scrambled and siNr2e1.4 and scrambled and siNr2e1.5 are $P < 0.05$ as calculated using paired *t*-test. **(b, c)** *Nr2e1* knockdown by siRNA in NSC causes senescence. NSC were subjected to SA-β-Gal staining. Quantification **(b)** and representative pictures **(c)** are shown. **(d)** The expression of CBX7 and NR2E1 correlate in glioblastoma multiforme (GBM), as shown by qRT-PCR in samples from GBM patients. a.u., arbitrary units.

MATERIALS AND METHODS

Cell culture and retroviral infection

HEK293T, IMR90 and WI-38 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Leiden cells were described previously.⁴¹ Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (PAA, Amersham, UK) and 1% antibiotic-antimycotic solution (Invitrogen). Primary human prostate epithelial cells were obtained from Lonza (Basel, Switzerland) and maintained in prostate epithelial cell growth medium (Lonza). Methods used for retrovirus production and infection have been previously described.⁴²

NSC isolation and culture of NSC

For isolation of NSCs, the SVZ was dissected from P6-P10 mouse brains as described previously.⁴³ Briefly, following isolation, the SVZ was dissociated in HBSS medium (Invitrogen) containing 0.05% trypsin (Invitrogen) and 60 U/ml Dnase I (Sigma, St Louis, MO, USA), washed and plated on poly-L-lysine-coated plates in SVZ explant medium (DMEM/F12 (Invitrogen), 3%FBS, 20 ng/ml EGF (Peprotech, Rocky Hill, NJ, USA)). The next day NSCs were purified by fractionation and the medium was replaced with SVZ culture medium (DMEM/F12 (Invitrogen), 0.25% FBS (Invitrogen), N2 (Invitrogen), 20 ng/ml EGF (Peprotech), 10 ng/ml bFGF (Peprotech) and 35 µg/ml bovine pituitary extract) modified from the study by Scheffler *et al*.⁴⁴ NSC were grown to confluency and subcultured in adhesion under the same conditions for up to 8–10 passages. All experiments were performed on freshly isolated NSCs.

Transcription factor library and plasmids

The Transcription Factor library was obtained from Origene (Rockville, MD, USA). The library contains 704 full-length human cDNAs cloned in the pCMV6-XL5 vector. NR2E1 was cloned into pMIV retroviral vector. NR2E1 Δ40 mutant and FLAG-NR2E1 were generated by PCR. Human and mouse CBX7 promoters were cloned into a pGL3-basic luciferase reporter vector by PCR using a BAC or FOSMID plasmid as a template. Plasmids expressing CBX7 have been described previously.¹⁹

Reverse transfection and luciferase assay

For the luciferase screen, reverse transfection using polyethylenimine (Polysciences, Warrington, PA, USA) was performed in HEK293T cells to individually transfect 704 clones from the transcription factor library in a 96-well plate format. A 20:1 ratio of promoter *Luciferase reporter* to *Renilla* construct was used. pGL3-basic was used as control vector. *Firefly* and *Renilla* luciferase activities were evaluated using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) 48 h after transfection. Each luciferase value was expressed as the number of median-adjusted s.d. (Z-score value) and a threshold was established to Z-score value ≤ 2.

BrdU assay and crystal violet staining

BrdU labeling and crystal violet staining were performed as previously described.⁴²

Senescence-associated β-Galactosidase staining

IMR90 fibroblasts or NSC were seeded in six-well plates. Two days later, the cells were fixed with 0.5% glutaraldehyde (w/v) for 15 min and then washed twice with 1 mM MgCl₂ in PBS (pH 6.0). X-Gal staining solution (1 mg/ml X-Gal, 5 mM K₃[Fe(CN)₆] and 5 mM K₄[Fe(CN)₆] in 1 mM MgCl₂/PBS (pH 6.0)) was added to the cells for 2–24 h, after which the cells were washed with water and stored at 4 °C, in the dark. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 30 min. Bright field and DAPI images were taken and the percentage of SA-β-Gal-positive cells was determined upon counting at least 100 cells per condition.

Antibodies, immunofluorescence and immunoblotting

Immunofluorescence was performed using an automated high throughput microscope (InCell Analyzer 2000, GE Healthcare, Amersham, UK). Image processing and quantification were performed using InCell Investigator software (GE). Immunoblot was performed following standard procedures. Primary antibodies used in this study are summarized in Supplementary Table 1.

qRT-PCR analysis

Total RNA was extracted using miRCURY RNA isolation kit (Exiqon, Vedbaek, Denmark). cDNAs were generated using SuperScript II reverse transcriptase (Invitrogen). PCR reactions were performed in a Real-Time PCR Detection System (BioRad, Hercules, CA, USA) using Power SYBR Green Master Mix or TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Expression was normalized to ribosomal protein S14 or TATA box binding protein expression. Primers and Taqman probes used are listed in Supplementary Table 2.

RNA interference

IMR90 cells, human prostate epithelial cell and NSC were transfected with 20–30 nM siRNA (Qiagen), miR or anti-miR (Ambion, Carlsbad, CA, USA) in 96-well or 6-well plates. A 3.5% solution of HiPerFect transfection reagent (Qiagen) was prepared in serum-free medium and then mixed with the siRNA. The mix was incubated for 30 min at room temperature and then added to the cells. The cell culture medium was replaced the following day and cells were either fixed for immunofluorescence or harvested for RNA extraction 48–120 h later. The Cy 3-labeled siGLO cyclophilin B siRNA (Dharmacon, Rockford, IL, USA) was used to monitor transfection efficiency. Sequences specifying shRNAs against human NR2E1 were cloned in pREtroSuper (NKL, Amsterdam, Netherlands) as previously described.²² Lentiviral pLKO-based shRNA targeting mouse NR2E1 (pLKO-shNR2E1.4 and pLKO-shNR2E1.4) were obtained from Sigma (TRCN0000026019 and TRCN0000026030). The sequences of siRNA and shRNAs used are listed in Supplementary Table 3. Knockdown of p16^{INK4a} and CBX7 was achieved using validated shRNA constructs.^{19,45}

Chromatin immunoprecipitation (ChIP)

ChIP experiment were performed as described previously.⁴⁶ Immunoprecipitation of cross-linked chromatin was conducted with antibodies listed above. After immunoprecipitation, DNA was extracted using the QIAquick PCR purification kit (Qiagen) and an aliquot amplified by real-time qPCR using primers described in Supplementary Table 4. To confirm target enrichment each PCR product was evaluated first by standard end point PCR.

ChIP-seq and bioinformatics analysis

Parallel ChIP were performed using ~5 µg of antibody with 500 µg chromatin. The recovered material was pooled and concentrated to a minimum of 0.2 µg/µl. Input DNA was used as control for the ChIP-seq analysis. Library preparation and Solexa genome-wide sequencing were performed as recommended by the manufacturer. The alignments were performed using novoalign (version 2.07.14; <http://novocraft.com>) allowing for a single mismatch per read. Duplicates were removed using the Picard MarkDuplicates program (picard-tools package version 1.48; <http://picard.sourceforge.net>) and peak calling was performed using MACS (version 1.4.0rc2;⁴⁷).

DNA pull-down assay

Lysates from HEK293T cells overexpressing wild-type or mutant NR2E1 were prepared in HKMG buffer (10 mM Hepes pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.5% NP40, 1 mM DTT and protease inhibitors (Complete EDTA-free, Roche, Basel, Switzerland) and pre-cleared with pre-equilibrated streptavidin-coupled Dynabeads (Invitrogen). Pairs of complementary oligonucleotides (44 bp-long, with the sense oligonucleotide biotinylated at the 5' end, Sigma, sequences described in Supplementary Table 5) were annealed and incubated overnight at 4 °C with the cell lysates. DNA-bound proteins were collected by incubation for 1 h at 4 °C with streptavidin-coupled Dynabeads (Invitrogen), washed four times in HKMG buffer and separated by SDS-PAGE. NR2E1 was detected by immunoblot.

Glioblastoma samples

Samples from 28 patients with newly diagnosed, untreated, histologically proven glioblastoma multiforme according to the World Health Organization (WHO) classification, were used. Samples were collected at the Clínica Universidad de Navarra (CUN; Pamplona, Spain). The study protocol was approved by the Institutional Review Board, and all the participants signed the informed consent form approved by the respective Institutional Review Boards or Ethical Committees. NR2E1 and CBX7 transcript levels were measured by RT-PCR using an ABI 7700 sequence detection system

(Applied Biosystems). The expression levels relative to GAPDH were calculated and normalized relative to expression in normal brain RNA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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