T antigen transformation reveals Tp53/RB-dependent route to PLAC1 transcription activation in primary fibroblasts

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INTRODUCTION

PLAC1 (placenta-specific 1) gene expression is extraordinarily tissue specific. Among normal tissues, PLAC1 is uniquely expressed in the placenta, at a high level from 6.5 to 13.5 days post coitum in mouse and throughout pregnancy in humans, and is thus of interest to reproductive biologists. However, in spite of its selectivity in expression in the normal body, it is also expressed at high levels in a wide variety of cancers and, as a result, in a range of commonly used laboratory cell lines. Especially because inhibition of PLAC1 expression in cell lines derived from a breast cancer led to loss of invasiveness and growth, how the gene is derepressed in cancer is of particular interest; knowledge of this process might thereby suggest salutary ways to block PLAC1 expression in cancer cells. Consequently, based on its immunogenicity and oncogenic ranking, it is now on the National Cancer Institute list of ‘tumor antigens’ that could serve as candidate targets for development of vaccines against breast cancer.

How can PLAC1 achieve its intriguing selectivity of expression? Tissue-restricted expression is often attributable to the action of tissue-specific transcription factors such as hepatocyte nuclear factor 4 (HNF4) in liver, kidney and pancreas. Such factors explain the proximal regulation of the gene, but raise questions of ‘infinite regress’: that is, how are the tissue-specific transcription factors themselves regulated? And how are the initiating factors for those transcription factors regulated? Here we have tried to analyze possible activation and derepression pathways for the gene.

The gene structure is conserved between mouse and human, with two promoters, P1 and P2, separated by ~100 Kb but producing the same protein from a terminal exon. Defining the gene structure has facilitated the demonstration that cancer cell lines show a range of preferential usage of P1 and P2, and the activity of both promoters—both endogenously and from transfected reporter constructs—is stimulated by nuclear receptors retinoid X receptor-α (RXRα) and liver X receptor-α (LXRA) or LXRB. The receptors were shown to bind to cognate promoter binding sites and to lose their activity when the binding sites were mutated.

On the other hand, the question of how the gene is derepressed in cancer cells (and in placenta) was left open. To find a model to investigate the cancer-based gene activation, we turned to a classical approach, comparing primary cells with the same cells transformed by SV40. The SV40-transformed primary cell lines indeed showed activation of PLAC1, and further transfection studies with SV40 early region or large T (LT) antigen DNA constructs showed that cellular Tp53 and RB have critical—although opposing—effects in determining PLAC1 P1 promoter dynamics and transcription state.

RESULTS

LT activates the P1 promoter in primary cells

As expected from Northern and quantitative reverse transcription–PCR (qRT–PCR) analyses of normal tissues, WI38 or IMR-90 primary fibroblast cultures do not express PLAC1 (Figure 1a). However, established SV40-transformed cell lines of both cell types express PLAC1 (Figure 1a). The expression in established cell lines was not a secondary effect occurring during outgrowth, because transfection with SV40 early region or just the LT antigen encoded within the early region was sufficient to induce comparable levels of PLAC1 expression (Figure 1a).

Figure 1b confirms the expected expression of LT and small T (ST) in IMR-90 cells transfected with the early region (left panel), and the comparable amount of LT expressed in cells transfected with LT.

Figure 1c also shows the level of endogenous PLAC1 expression from both promoters in the choriocarcinoma-derived BeWo cell line, in MCF-7 cells and in placenta. In placenta, P2 is the preferred promoter, as it is in MCF7 cells; but induction at
P1 was preferential in BeWo and highly preferred in transformed WI38 and IMR-90.

Role of LT in derepression of P1 promoter
We used the previously described \( P1 \) promoter luciferase fusion construct P1-Luc (Figure 1c) for further characterization of LT action in \( PLAC1 \) induction. Studies were primarily carried out in BeWo cells, and critical differences were then confirmed in transfected primary IMR-90 cells.

When the P1-Luciferase plasmid (P1-Luc) was transfected into BeWo cells with and without SV40 constructs, the early region stimulated luciferase reporter activity 2500-fold over control, and LT stimulated the promoter activity up to 600-fold (Figure 1c). ST had very little effect, and co-transfection with both ST and LT together stimulated no more than did LT alone. Therefore, we focused further on the effects of LT.

LT is a multidomain protein that helps to subvert the normal cellular response to viral infection by preventing apoptosis, activating DNA replication and cell division and promoting viral replication. It functions via interactions with cellular tumor suppressors Tp53 and RB and a DNA J domain that interacts with HSPA8/HSC70. To further examine its role in stimulating P1 promoter activity, we co-transfected the P1-Luc construct into BeWo cells along with LT that carried mutations in Tp53-binding sites. Normally, Tp53 is at very low levels in cells and is kept in check by MDM2, an E3 ubiquitin ligase that targets Tp53 for proteasomal degradation. When the LT-Luciferase plasmid (LT-Luc) was transfected into BeWo cells, expressed (Figure 1e, lane ‘c’), and at a much higher level than LT-Wild type (lane ‘b’). Figure 1d shows that when LT, Tp53 forms a complex that simultaneously stabilizes it and elevates its cellular concentration; the complex formation likely helps to deplete it from promoter sites. Mutation in the LT-binding site for TP53 abrogates their interaction, permitting TP53 to bind unimpeded to TP53-responsive promoters and inhibit their transcription. Figure 1d shows that when LT lost its interaction with TP53 while maintaining intact pRB and DNA J domains, the LT promoter indeed was no longer stimulated in BeWo cells. We confirmed by western blot analysis that after transient transfection of BeWo cells, the early region induced the \( PLAC1 \) promoter 2500-fold; LT or LT together with ST stimulated luciferase levels up to 600-fold, and ST had only a small effect. P1-Luc construct features are diagrammed below. (d) Effect of mutations in LT on transcription from P1-Luc in BeWo cells. Expression constructs driven by a CAG promoter, carrying mutations in LT at either the Tp53-binding site (p53*), Retinoblastoma protein-binding site (Rb*) or DNA J interaction site (DNAJ*), were co-transfected with P1-Luc construct, and luciferase levels measured thereafter. For comparison, transfections with ER and wild-type LT are shown. Mutation in the Tp53-binding site of LT renders LT unable to induce transcription from P1, whereas mutations in the RB-binding site activate P1 expression to the level seen with wild-type LT. Western blots showing comparative expression of wild-type LT (lane ‘b’) and LT with mutant p53 binding site (lane ‘c’).
conditions, RB is expected to carry out its normal cellular functions, including the stimulation of transcription at many sites. P1 promoter activity was in fact activated up to levels seen by transfection with the entire early region (Figure 1c). The more robust upregulation by RB when Tp53 binding to LT is intact may also reflect the absence of additional modulating modifications on pRB, like phosphorylation, that occur when it is bound to LT,\textsuperscript{17,19} and this function could be influenced by additional factors such as the 17K protein that is also encoded by the early region.

Finally, mutations in the DNA J\textsuperscript{9,18} interaction site completely abolished activation of the PLAC1 promoter (Figure 1d). This is a surprising observation, because the J domain has been found to be dispensable for p53 binding.\textsuperscript{19} The finding raises the possibility that the J domain has a significant but uncharacterized function independent of RB binding.

Overall, these experiments indicated that LT exerts a net influence on P1 promoter through interactions with Tp53 and RB, and we thus assessed the direct effects of expression of Tp53 and RB on the promoter in BeWo cells. Figure 2a shows that luciferase levels from the P1-Luc construct transfected into BeWo cells were inhibited when P53 was expressed and were upregulated when RB was transfected. Tp53 and RB are thus confirmed to influence promoter activity independent of the presence of LT.

Notably, several cancer cell lines that express PLAC1 carry mutations in Tp53. When co-transfected with P1-Luc and compared with intact Tp53, Tp53 carrying any of the four frequently found mutations\textsuperscript{20} was unable to repress promoter expression (Figure 2b). These results imply that the Tp53 mutations most frequently found in cancer are likely sufficient to derepress the promoter (see Discussion).

As with Tp53, various RB mutations are found in cancer. Wild-type RB stimulated the P1 promoter 3.5 fold, whereas the more frequently found RB mutants, pRB;567L, pRB;661W, del22 and pRBΔex4,\textsuperscript{11} had far less effect, supporting a requirement for wild-type RB for maximum stimulation of the promoter (Figure 2c). Furthermore, because these mutants are specifically unable to bind to E2F,\textsuperscript{11} it suggests that the PLAC1 promoter is not responsive to E2F1 (see below), and RB would then necessarily affect the promoter through an alternate pathway.

Tp53 occupies a P1 promoter site in IMR-90 cells, whereas Rb shows no direct interaction

The P1 promoter contains a consensus binding site for Tp53 at -275 base pairs 5’ to the transcription start site, which was thus a candidate site to mediate interaction. To assess whether the site was truly occupied by Tp53, chromatin was immunoprecipitated with anti-Tp53 and anti-RB antibodies from IMR-90 cells and from IMR-90 cells transfected with the SV40 early region. In SV40-

transfected cells the promoter region showed enrichment with anti-Tp53 antibody but not anti-RB antibody, consistent with direct association of Tp53 but not RB (Figure 3a).

Because the level of promoter-bound Tp53 in untransfected IMR-90 cells is low (Figure 3a) we tested further whether Tp53 inhibition was nevertheless a major determinant of PLAC1 repression. If so, direct inhibition of Tp53 formation should relieve the repression. Transfection of short hairpin RNA (shRNA) against Tp53 in IMR-90 cells indeed significantly reduced Tp53 transcript and protein levels (Figures 3b and c). In addition, Tp53 inhibition by shRNA did result in derepression of the endogenous PLAC1 gene from the P1 promoter (Figure 3d).

RB acts in concert with nuclear receptor LXR and co-activator NCOA2

Unlike Tp53, inhibition of RB RNA and protein by shRNA (Figures 3b and c) resulted in no change in the levels of P1 PLAC1 activity (Figure 3d) in IMR-90 cells. Instead, RB could act indirectly in any of several established ways.

One route to RB involvement has been established from findings that the pRB family of proteins (pRB, p130, p107), through their shared A and B domains, recruit histone deacetylases and modify chromatin to bring about transcriptional repression.\textsuperscript{21} However, RB is apparently a positive activator of PLAC1, ruling out such a repressive action.

In an alternative route, RB is bound to E2F, rendering E2F-responsive promoters inactive. When RB dissociates from the RB/ E2F (1–4) complex following LT expression, E2F is free to activate E2F-responsive promoters.\textsuperscript{17,18} However, the PLAC1 promoter is apparently not E2F responsive (see above), because we see PLAC1 derepression only when LT interaction with RB is disrupted. This would leave a repressive RB/E2F (1–4) interaction intact—the opposite of what is observed.

A more likely route to positive action of RB is consistent with results when the effect of RB expression was tested in BeWo cells (Figure 4a). A direct test for transfected RB action in primary cells is not feasible, because expression of RB in primary cells inhibits growth: DNA replication ceases and cells become senescent.\textsuperscript{22} In BeWo cells, RB transfected by itself gave a fourfold stimulation of luciferase activity. When RXRα and LXRβ agonists were also added, a further 2.5-fold stimulation was seen. Given that either agonist alone gave some additional stimulation (less than twofold) compared with controls in the absence of RB, it remained possible that the nuclear receptor effect was independent of RB action, involving another pathway. However, additional experiments showed that RB and nuclear receptors act jointly, as follows.

**Figure 2.** Effects of expression of wild-type and mutant forms of Tp53 and RB on P1-Luc activity in BeWo cells. (a) BeWo cells were co-transfected with a P1-Luc construct and a vector carrying no insert or p53 or RB gene. p53 inhibited but RB stimulated the P1 promoter. (b) BeWo cells were co-transfected with P1-Luc and various mutant forms of Tp53 from cancer cell types. Compared with wild-type Tp53, mutated Tp53 failed to repress the promoter. (c) BeWo cells were co-transfected with P1-Luc and mutant forms of RB from cancer cells. Mutated RB failed to activate the promoter as much as wild-type RB.
From literature reports, joint action of RB with nuclear receptors would require co-activators. NCOA2 and other members of its co-activator class have histone acetyltransferase activity and recruit CAAAT binding protein/p300 (CBP/p300). We therefore tested the effect of expression of the P1-Luc construct in BeWo cells when nuclear co-receptor NCOA2 and CBP were expressed along
with RB. An increase in luciferase activity over RB transfection alone was seen, and increased further to over eightfold when RXRα and LXRβ agonists were also added. These results are consistent with stimulation of P1 promoter activity by RB in conjunction with RXRα and LXRβ and co-activator NCOA2 in the presence of CBP (Figure 4b).

Stimulation by RB is seen even for a shortened version of the minimal promoter, P173-Luc, which contains a single nuclear receptor-binding site (Figure 4c); again, expression was further enhanced twofold in the presence of RXRα and LXR agonists. Decisive linking of RB to nuclear receptors was seen when the promoter was mutated in the RXRα-binding site (Figure 4c, Mut2 construct), which prevents interaction with the nuclear receptors. The mutation sharply reduced stimulation by both RB and nuclear receptors, supporting the essentiality of the presence of nuclear receptors at the promoter for RB interaction.

Repressive histone methylation patterns are erased following transformation by SV40 early region

It seemed likely that histone methylation and/or acetylation at specific positions, resulting in heterochromatin formation and repression, could be seen at the endogenous P1 promoter in nonexpressing cells. We checked the chromatin status of the endogenous P1 promoter by immunoprecipitation with antibodies for multiple histone methylation and acetylation modifications in IMR-90, WI38, and BeWo cells. We found that the P1 PLAC1 promoter is enriched for H3K9 and H3K27 trimethyl modifications in IMR-90 and WI38 cells, consistent with repressed promoter status. Upon SV40 early region transfection of IMR-90 and WI38 cells, the promoter was depleted of these modifications (Figure 5a), as expected for transcriptional activation. Basal methylation levels between IMR-90 and WI38 cells differ by approximately threefold, whereas the methylation status in BeWo cells, which already express PLAC1, was similar to the hypomethylated levels in IMR-90 and WI38 cells following SV40 early region transfection; no significant changes in histone acetylation status were observed (results not shown). The changes in repressive methylation status are thus an index of SV40-induced promoter activation, possibly reflecting global LT effects across much of the genome.

To check whether Tp53 and RB had a direct role in bringing about the changes in histone methylation, we checked histone methylation status by inhibiting Tp53 or RB in IMR-90 cells. Results showed some (less than twofold) reduction in H3K9 and H3K27 trimethylation when either Tp53 or RB was inhibited (Figure 5b). However, in contrast, methylation was reduced nearly 70-fold in SV40 or LT transfected primary cells (Figure 5a). This suggests that LT transfection may affect histone demethylation by a process that requires additional proteins or is even independent of Tp53 and RB; alternatively, loss of a small number of critical modifications that are dependent on Tp53 and RB may be sufficient to provide a chromatin conformation adequate for gene expression.

DISCUSSION

PLAC1 knockout mice show substantial placental defects, with placental layers showing abnormal differentiation. Because the placenta has invasive properties, it has been suggested that cancer cells might hijack placental invasive mechanisms. But whatever the possible function of PLAC1 in cancer cells, activation of its promoter is an indispensable first step.

We find that PLAC1 expression follows a paradigm in which highly tissue-restricted transcription involves alleviation of any repression followed by positive amplification of transcription by auxiliary factors. We have shown that SV40 transformation of primary cells WI38 and IMR-90 or transfection with SV40 early region in fact derepresses transcription, and LT is sufficient. A schematic model (Figure 6) outlines a possible mechanism. Consistent with derepression, we see that repressive histone H3K9/K27 trimethylation declines as PLAC1 is activated. A major repressive transcription factor alleviated by SV40 action is Tp53, and we find that Tp53 indeed acts as a repressor of PLAC1 transcription in primary fibroblasts. Tp53 is normally expressed in the cells, and LT expression binds both Tp53 and RB. This could effectively reduce the local concentration at the P1 promoter or render it unable to act effectively as a repressor. The latter option is more likely, because even when transcription was derepressed chromatin immunoprecipitation (ChIP) assays still found Tp53 enriched at the promoter.

WT Tp53 apparently functions in normal fibroblasts independent of SV40 transformation, because PLAC1 expression increased by 25-fold when primary cells were transfected with shRNA against Tp53 (Figure 6). In addition, transfected WT Tp53 inhibited P1-Luc expression, whereas mutant Tp53 variants stimulated transcription (Figures 2a and b). Do all cancer cells with Tp53 mutations derepress PLAC1? In a relevant study, Silva et al. surveyed a number of cancer cell lines representing various cancers for expression of PLAC1, and ranked its expression level (see Table 2 in Silva et al.). We checked these cell lines in the Tp53 mutation database at http://p53.fr/ for the presence of Tp53 mutations, and found that a majority of the cancer cell lines that express high levels of PLAC1 indeed carry mutation in Tp53 (or, as in H929 cells, express high levels of MDM2, leading to loss of Tp53 expression). There are, however, also examples of cell lines that carry Tp53 mutation(s) but express very low levels of PLAC1 (for example, MOLT4 and U251). Possibly not all mutations in Tp53 lead to derepression of PLAC1 in cancer cells,

Figure 5. PLAC1 promoter enrichment following chromatin immunoprecipitation with antibodies against histone-modifying enzymes. Antibodies against histone 3 lysine 9 or lysine 27 trimethyl modifications were used to immunoprecipitate chromatin from (a) IMR-90 and WI38 cells with or without SV40 transformation compared with BeWo cells. (b) IMR-90 cells following transfection with shRNAs against Tp53 and RB. The immunoprecipitated chromatin fraction was tested for PLAC1 promoter enrichment by qPCR. Fold enrichment of the promoter was calculated as a ratio compared with control immunoprecipitations with IgG.

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or additional events are necessary for gene derepression. Nevertheless, activation of PLAC1 when Tp53 is specifically depleted with shRNA argues for direct involvement of Tp53 lesions in PLAC1 derepression. Inhibition of RB interaction with LT was necessary to bring about its full positive effect on PLAC1 transcription. pRB can act in association with several transcription factors to overcome repression by a negative regulator; for example, in the activation of MyoD during myogenesis and CBFA1/Runx2 during osteogenesis. In some instances, a positive promoter response is brought about indirectly by blocking the action of differentiation inhibitors such as E12-1, ID2 and RBP2.

In contrast to promoter derepression involving the disarming of Tp53, RB is rather involved in the second, activating phase of expression. Among various possible routes to positive RB action on PLAC1, a likely candidate is based on a co-stimulation model. pRB has been shown to potentiate transcription by interacting with nuclear receptors such as HNF4, SF-1 and ERα and ERβ in conjunction with NCOA2 as a cofactor. We previously found that nuclear receptor RXRα and LXRβ bind to cognate sites in the promoter to stimulate P1 and P2 promoters in BeWo cells; and we find here that RB, analogous to other instances, activates P1 and P2 promoters in the presence of NCOA2, leading to upregulation of basal transcription levels. pRB is free to interact with the nuclear receptors in the presence of NCOA2, leading to upregulation of basal transcription levels. pRB is free to interact with the nuclear receptors in the presence of NCOA2, leading to upregulation of basal transcription levels. (5) When shRNA against RB is expressed, Tp53 still occupies the promoter with less than a twofold change in histone trimethylations. PLAC1 remains repressed. (6) When shRNA against Tp53 is expressed, Tp53 occupancy at the promoter is lost, leading to promoter derepression, with assembly of a transcription initiator complex and binding of nuclear receptors. Once again, there is no drastic change in trimethylation status (reduced by less than twofold). These events suggest that complete erasure of trimethylation marks at the promoter is not strictly required, although that might further facilitate the opening of chromatin structure.

Figure 6. Model for events during PLAC1 derepression by SV40/LT. (1) The repressed status of P1 promoter chromatin in primary cells with histone 3 trimethylated at lysine 3 and lysine 9 position and Tp53 occupying its binding sites. At this stage, PLAC1 is not expressed, with no transcription initiator complex formed. RXRα and LXRβ may or may not occupy their binding sites. (2) Upon direct expression of LT, transfection of SV40-ER, or after SV40 transformation, histone trimethylations are erased, opening the chromatin. RB and Tp53 are bound to LT, resulting in increased stabilization of Tp53 and its cellular sequestration by LT, along with modification(s). This allows transcription machinery to assemble at the promoter; and in cells expressing nuclear receptors RXRα and LXRβ, they would bind to their cognate sites and further boost PLAC1 transcription. (3) When LT with a Tp53-binding site mutation is expressed, it can only interact with RB. Histone methylation patterns are modified, but transcription is still repressed, with Tp53 still bound at the promoter. (4) When LT with an RB-binding domain is expressed, it interacts with Tp53 and removes Tp53 from promoter site. RB is free to interact with the nuclear receptors in the presence of NCOA2, leading to upregulation of basal transcription levels. (5) When shRNA against RB is expressed, Tp53 still occupies the promoter with less than a twofold change in histone trimethylations. (6) When shRNA against Tp53 is expressed, Tp53 occupancy at the promoter is lost, leading to promoter derepression, with assembly of a transcription initiator complex and binding of nuclear receptors.

Because PLAC1 is immunogenic and its induction is associated with changes in histone methylation patterns, agents affecting methylation might upregulate its expression in cancers, thereby provoking an augmented immune response with therapeutic potential. However, direct experiments are required to determine whether any increase in PLAC1 levels might also increase tumor aggressiveness or have other negative side effects.
To summarize, a probable scenario for the overall regulation of the gene (Figure 6) now infers that the PLAC1 promoter region in somatic tissues is in a heterochromatin state. In various types of cancer, oncogetic transformation could have an effect like that of LT in fibroblasts, with RB and Tp53 affected in similar ways. For the transcription factor Cdx2, which triggers trophoblast lineage differentiation, 30 from which the placenta is derived, opens chromatin access at many loci; 31 again having an effect comparable to that of LT (though any involvement of Tp53 and RB has not been investigated in that case). In subsequent transcription initiation, expression would again be augmented in the placenta, as in fibroblasts and BeWo cells, by the positive interactions of co-activators with nuclear receptors, which are localized at the maternal/placental interface. 32

MATERIALS AND METHODS

Cell culture

The human placental-derived choriocarcinoma cell line BeWo (CCL-98), human lung epithelial cell line WI38 (CCL-75), SV40 transformed WI38 (VA-13, CCL-75.1) and human breast adenocarcinoma MCF-7 (HTB-22) cells were purchased from ATCC (Manassas, VA, USA). The human lung fibroblast cell line IMR-90 (I90) and SV40 transformed IMR-90 (AG02804) were obtained from Coriell Institute (Camden, NJ, USA). BeWo cells were cultured in F-12k medium with 10% fetal bovine serum, and the other human cell lines in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum and 0.01 mg/ml bovine insulin.

Luciferase assay

Luciferase assays were performed as previously described. 3 Briefly, BeWo cells in 24-well plastic dishes were transfected with a human PLAC1 P1 promoter construct (P1-Luc) along with indicated cDNA plasmids, and a Renilla Luciferase construct (10 ng/transfection) as an internal control of transfection efficiency. In other controls, vector without insert was co-transfected with the P1 construct. The relative luciferase activity was measured from cells 48 h after transfection, using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) on a Victor 1420 multi-label counter (Perkin Elmer, Waltham, MA, USA). To measure the effect of agonists, T091713 (100 nM) and/or 9-Cis-retinoic acid (1 M) (Sigma, St Louis, MO, USA), 1% for 10 min at room temperature. The crosslinking reaction was performed with formaldehyde at a final concentration of 10% for 15 min at 4 °C, in a Misonix Sonicator XL2020 (Misonix Incorporated, Farmingdale, NY, USA). The beads were washed five times with Lysis Buffer. Crosslinking was reversed by incubating the fraction overnight at 67 °C. The beads were removed by centrifugation. The supernatant was extracted with phenol/chloroform/isooamy alcohol (25:24:1), vortexed thoroughly, and the aqueous phase (DNA) precipitated with ethanol. The resulting DNA was measured in Real-time PCR reactions and quantitated using SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA). The sequence of the primers used to detect the promoter enrichment was as follows: forward: 5′-CAAGGTAACCCACCAGCTCTA-3′, and reverse: 5′-GTITTCTCGGGAATGCTGTG-3′. The primers amplify a 240-bp fragment between -163 and +76 bases with respect to the transcription start site; this region spans the RRxR-binding sites. 8

Quantitation of PLAC1, Tp53 and RB transcripts in human cancer cell lines by one step real-time RT-PCR

RNA was extracted from cells with TRI reagent (Sigma), quantified by measuring A260 absorbance and quality determined by measuring 260/280 absorbance ratios.

Real-time RT-PCR was performed with Taqman one step RT-PCR kits (Life Technologies, Grand Island, NY, USA) on an Applied Biosystems 7900. To distinguish differences in RNA expression levels from P1 and P2 promoters, the following set of primers were used, synthesized by Applied Biosystems: Ex1-realF: 5′-GAGGAGTCTGTCAGGAGTGC-3′, Ex4-realF: 5′-AAACGCCCACCCCTTCCTCAGT-3′, Ex6-realF: 5′-AGGCTGCTCAGGAATTCTCCT-3′, with the reporter oligonucleotide sequence 6Fam-5′-CTCCAGCTGCCAGAAG-3′-Tamra. TP53 and RB transcript levels were determined using TP53- or RB-specific oligonucleotides (Cat. no. HS.PT.51.22574907G and Cat. no. Rb: HS.PT.51.787090, respectively, from Integrated DNA Technology, Coralville, IA, USA). Standard 52 microglobulin primers were used as an endogenous control (Life Technologies, Cat. no. 4310886E) in all cases. The differences in expression levels were calculated by using the 2 -ΔΔCT method and normalized to actin levels.

List of plasmid constructs used in this study

All of the following plasmids were purchased from Addgene (Cambridge, MA, USA), and their descriptions are detailed in the corresponding references (Table 1).

| Addgene ID | Plasmids |
|------------|----------|
| 22883 | pRSVBeNeo early region SV40 9 |
| 9054 | pSGS Large T delta 434-444 10 |
| 9055 | pSGS Large T K12 11 |
| 16434 | pCMV-Neo-Bam p53 WT 10 |
| 16436 | pCMV-Neo-Bam p53 R175H 10 |
| 16438 | pCMV-Neo-Bam p53 R249W 10 |
| 16439 | pCMV-Neo-Bam p53 R249S 10 |
| 10720 | pGSGL HA RB 11 |
| 10725 | pGSGL HA RB 567L 11 |
| 10731 | pGSGL HA RB 661W 11 |
| 10730 | pGSGL HA RB del ex111 |
| 10721 | pGSGL HA RB del ex111 |
| 10730 | pGSGL HA RB del ex111 |
| 13970 | pBABE-puro SV40 LT 11 |
| 9048 | pBABE-neo large T H42Q 11 |
| 8583 | pBABE-zeo small T 13 |
| 16701 | pBABE HA 13 |

The left column gives the plasmid ID numbers in Addgene website and the column on the right gives a brief description of the vector and the gene content.

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Inserts in pBabe vector containing LT, LTH4Q2 or ST encoding DNA fragments were PCR amplified using, for LT, forward primer: 5'-ctctcgagcaccATGGATAAAGTTTTAAACAGAGAGG-3' and reverse: R 5'-gcggcgcgcgcgcgcgctTTAGTTAGCTTCCGAGGGG-3' and for ST, reverse primer ST_R 5'-gcggcgcgcccgcgcggcTCTAGAGCTTAAAATCTGTAGG-3' in combination with the forward primer for LT. The recovered products were gel-purified and cloned into the pCyCAG mammalian expression vector (http://www.cdb.riken.jp/pcs/protocol/vector/vector_top.html).

Table 2. List of antibodies used in this study, the type of assay in which they were used and their source

| Antibodies | Assay     | Company                                      |
|-----------|-----------|----------------------------------------------|
| s-SV40 T Ag N-terminus (Pab 108) | WB       | Santa Cruz Laboratories, Santa Cruz, CA, USA |
| s-SV40 T Ag C-terminus (Pab 101) | WB       | Santa Cruz Laboratories                      |
| s-p53 (FL-393) | WB/ChIP  | Santa Cruz Laboratories                      |
| s-RB (C-15) | ChIP     | Santa Cruz Laboratories                      |
| h-H3K9me3 (ab8898) | ChIP     | Abcam, Cambridge, MA, USA                   |
| h-H3K27me3 (07449) | ChIP     | Millipore, Billerica, MA, USA               |
| h-H3K9ac (ab10812) | ChIP     | Abcam                                        |
| h-H3K27ac (ab4729) | ChIP     | Abcam                                        |

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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