P53 independent activation of the *hdm2*-P2 promoter through multiple transcription factor response elements results in elevated hdm2 expression in estrogen receptor-α positive breast cancer cells

Monika Phelps, Matthew Darley, John N. Primrose and Jeremy P. Blaydes[2]

Cancer Sciences Division
School of Medicine, University of Southampton
MP 824, Southampton General Hospital,
Southampton SO16 6YD, UK

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[2]To whom requests for reprints should be addressed at Cancer Sciences Division, School of Medicine, University of Southampton, MP 824, Southampton General Hospital, Southampton SO16 6YD, U.K. Phone: +44 (0)23 8079 4582, Fax: +44 (0)23 8079 5152, email: j.p.blaydes@soton.ac.uk

[3]The abbreviations used are: ERα, estrogen receptor-α; p53-RE, p53 response element; DMSO, dimethyl sulphoxide; RPA, ribonuclease protection assay; mAb, monoclonal antibody; RLU, relative luciferase units.

ABSTRACT

The negative-regulatory feedback-loop between p53 and hdm2 forms part of a finely-balanced regulatory-network of proteins that controls cell-cycle progression and commitment to apoptosis. Expression of hdm2, and its mouse orthologue mdm2, is known to be induced by p53, but recent evidence has demonstrated mdm2 expression can also be regulated via p53 independent pathways. However the p53 independent mechanisms that control transcription of the human *hdm2* gene have not been studied. Differential levels of *hdm2* mRNA and protein expression have been reported in several types of human malignancy, including breast cancers, in which hdm2 expression correlates with positive estrogen-receptor-α (ERα) status. Experimental models have demonstrated that hdm2 over-expression can promote breast cancer development. Here we show that the elevated level of hdm2 protein in $ER\alpha^{+ve}$ breast cancer cell lines such as MCF-7 and T47D is due to transcription from the p53 inducible P2 promoter of *hdm2*. The P2 promoter is inactive in $ER\alpha^{-ve}$ cell lines such as SKBr3. *Hdm2*-P2 promoter activity in T47D cells is independent of p53, as well as of known regulators of the mouse *mdm2*-P2 promoter, including ERα and ras-raf-MEK-MAPK signalling. We show that *hdm2*-P2 activity in T47D cells is dependent on the integrity of both an evolutionarily conserved AP1-ETS element and a non-conserved upstream $(nnGGGGC)_5$ repeat sequence. Lack of *hdm2*-P2 activity in $ER\alpha^{-ve}$ cells is shown to be a consequence of reduced transcriptional activation through the AP1-ETS element. Over-expression of ETS2 in SKBr3 cells reconstitutes AP1-ETS element-dependent *hdm2*-P2 promoter activity, resulting in increased levels of hdm2 protein in the cells. Our findings support the hypothesis that the elevated levels of hdm2 expression reported in cancers such as $ER\alpha^{+ve}$ breast tumors play an important role in the development of these tumors.

INTRODUCTION

Sporadic human breast tumors show a high degree of genotypic and phenotypic diversity (1, 2). Despite this diversity, the sub-classification of tumors on the basis of their expression of the steroid hormone receptor, estrogen receptor-α (ERα)[3], and its transcriptional target, progesterone receptor, has proven to be a useful predictor of prognosis and therapeutic response (3). ERα is detectable in approximately two-thirds of breast cancers, and its expression correlates with a well-differentiated phenotype (4) and a dependence on the mitogenic action of estrogen for tumor growth (2). Recent gene expression profiling studies have clearly demonstrated that ERα status is associated with a distinct pattern of transcription of several hundred genes (5, 6).

In common with several other human tumor types (7), resistance to chemotherapy in breast cancer has been correlated with the presence of inactivating mutations in the *p53* tumor suppressor gene (8, 9). This is consistent with the central role played by p53 in the stress-induced up-regulation of transcription of genes that induce cell cycle arrest and apoptosis. (10). *P53* gene mutation occurs in approximately 18 % of breast tumors according to a meta-analysis of published reports (11), and shows a strong correlation with ERα^{-ve} tumor status (9, 12). The frequency of *p53* mutation in tumors expressing both ERα and progesterone receptor has been shown to be as low as 9% (12), which compares to an overall rate of 50-55% in all human cancers (13), indicating that the selection pressure for the acquisition of *p53* mutation is relatively low in these breast tumors (14). Several lines of evidence now indicate that this reduced selection pressure is a consequence of multiple genotypic and phenotypic characteristics of these tumor cells. Firstly, it has been demonstrated that in a proportion of these cancers, the p53 protein is sequestered in the cytoplasm of the cell where it is unable to function as a transcription factor (15). Secondly, levels of transcription of *p53* mRNA are low in some breast cancers due to reduced HOXA5 transcription factor activity (16). Furthermore,

the p53 dependent apoptotic response is reduced in some breast tumors due to reduced expression of ASPP proteins (17). Finally, several reports have also demonstrated that the expression of hdm2, which is the major negative-regulator of p53 protein levels and activity in the cell (18-21), is up-regulated in ERα^{+ve} breast cancers at the protein and mRNA level (22-27). Mdm2 promotes breast cancer formation in murine models (28), and over-expression of hdm2 in human tumors such as sarcomas, in which the *hdm2* gene is amplified (29), results in a reduced rate of p53 mutation in these cancers. The mechanism of increased hdm2 expression in ERα^{+ve} breast tumors is currently unknown and, because transcription of *hdm2* is itself up-regulated by p53 (30, 31), it is not known whether this increased expression is merely a consequence, rather than a cause, of the retention of wild-type p53 in these tumors.

Hdm2 expression is regulated by transcription from two distinct promoters, P1 and P2 (30). Transcription from P1 occurs at low levels in most cells, whereas P2 is highly induced by p53 due to the presence of evolutionarily conserved p53 response elements (p53-RE) in both the murine *mdm2*-P2 (32, 33) and human *hdm2*-P2 (30) promoters. Studies dissecting the mechanisms that control transcription of *mdm2* and *hdm2* have focussed on the P2 promoter, primarily because the human P1 promoter dependent transcript is poorly translated (34). With the exception of the study by Zauberman *et al* (30), which identified the p53-responsive elements in the human *hdm2*-P2 promoter, mechanistic studies have to date been limited to the analysis of the murine P2 promoter. A number of functional, p53 independent response elements have been identified in this promoter, notably a thyroid hormone response element which is active in pituitary cells (35), and a combination of a 5' ETS binding site and composite AP1-ETS site that is required for activation of the promoter by growth factor dependent ras-raf-MEK-MAPK signalling pathways (36, 37). The mechanism whereby mdm2 expression can be regulated by other factors, most notably ERα in ras-transformed murine fibroblasts (38) has yet to be defined. It is important to note, however, that levels of

murine P2 transcript in non-stressed adult tissues has been demonstrated to be essentially p53 independent (39).

In this paper we have investigated the mechanism underlying the increased levels of hdm2 expression in ERα^{+ve} breast tumor cells. We have demonstrated that both P1 and P2 promoter derived mRNA transcripts are differentially expressed in a panel of breast cancer cell lines, and that the P2 promoter is activated by a p53 independent pathway in ERα^{+ve} cell lines such as MCF-7 and T47D. T47D cells provide a useful experimental system to study the role of estrogens on the proteins involved in p53-dependent cell cycle control (40). We have therefore used these cells to dissect the transcription factor response elements in the human *hdm2*-P2 promoter which drive hdm2 expression in ERα^{+ve} cells, and have subsequently identified a transcription factor which is able to restore *hdm2*-P2 promoter activity in ERα^{-ve} cells.

MATERIALS AND METHODS

Culture of human breast cancer cell lines. Breast cell lines were selected for this study based on a previous report (23). All lines were cultured in Dulbecco's modified Eagles medium (Invitrogen) supplemented with 10% fetal calf serum (Autogen Bioclear). The following reagents, dissolved in dimethyl sulphoxide (DMSO), were added to the medium where indicated: ICI 182780 (Tocris), U0126 (Promega), and PD98059 (Promega). Cells were exposed to ionising radiation using a modified 225 kV X-ray unit (Gulmay Medical, UK) at a dose rate of 1.15 Gy/min.

RNA analysis. Total RNA extraction was performed using RNAzol B (Biogenesis Inc.). For RT-PCR analysis of *hdm2* transcripts 1 μg of RNA was reverse-transcribed in a 40 μl volume using MMLV-reverse transcriptase (Promega) and O3 primer 5'-

CTGCCTCGAGTCTCTTGTTCCGAAGCTGG-3'. 2 µl cDNA product was used as target in 50 µl PCR reactions containing O1b/O3 or O2/O3 primer pairs for the amplification of *hdm2*-P1 and -P2 promoter derived transcripts respectively (O1b 5'-CTGGGGAGTCTTGAGGGACC-3', O2 5'-CCTGTGTGTCGGAAAGATGG-3'). PCR conditions were the same for both products (95°C 30 s, 58°C 30 s, 72 °C 1 min), except that cycle number was optimised to ensure that amplification was terminated in the exponential phase: P1 transcript, 30 cycles; P2 transcript, 25 cycles. Products were of the predicted molecular size, and were verified by sub-cloning into pGEMTeasy (Promega) and sequencing. RT-PCRs using oligo dT and *ß actin* primers were used to control for levels of input mRNA, and were also terminated in the exponential phase of PCR (22 cycles). Ribonuclease protection assay (RPA) was performed according to the manufacturers instructions (Ambion), using cloned O2/O3 PCR product as a probe. PCR and RPA results were quantified using a Kodak KDS1D imaging system.

Protein analysis. Cells were washed with phosphate buffered saline, pelleted by centrifugation at 1000 g, snap frozen and stored at -80°C. For immunoblotting, pellets were lysed for 15 min at 4°C in denaturing urea buffer (7 M urea, 0.1 M dithiothreitol, 0.05% Triton X-100, 25 mM NaCl, 20 mM HEPES pH7.6) then clarified by centrifugation at 13000 g for 10 min. Protein concentration was determined by the method of Bradford (Biorad). Immunoblotting was performed by standard procedures, as described previously (41) and membranes were probed for hdm2 using monoclonal antibody (mAb) 2A9 or 2A10 (42), p53 (mAb DO-1 or DO-12, Serotec) or ETS2 (Rabbit polyclonal C-20, Santa Cruz Biotech). Equal protein loading was confirmed on all immunoblots using rabbit anti-actin antibody (Sigma). Bands were visualised by chemiluminescence (Supersignal, Pierce) and quantified using a Fluor-S MAX system (Biorad) .

Plasmids, transfections and reporter gene assays. Genomic *hdm2* sequence was amplified from normal human liver DNA and ligated into pGL3-Basic using the *Mlu*I/*Xho*I sites (Promega) to generate reporter construct hdm2luc01. The sequence of the inserted 895 b.p. region ((-602) to (+293) relative to the start of exon 2) was identical to that generated by the human genome project (AC026121.10) with the exception of two single base pair differences (C-470T, which is a documented polymorphism, and G-133A). Further constructs containing deletions of the *hdm2* promoter (luc23, luc06, luc02 and luc03) (Fig. 4A) were generated by proof-reading PCR of hdm2luc01 using primers containing *Mlu1* and *Xho1* sites, followed by ligation into pGL3-Basic. Analysis of potential transcription factor binding sites in the *hdm2*-P2 promoter was performed using MatInspector (http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl). Mutations and deletions were introduced into hdm2luc01 using the QuickChange mutagenesis kit (Stratagene) and all constructs were verified by sequencing (MWG Biotech). Forward site-directed mutagenesis primers used are as follows (complementary reverse primers not shown):

ΔEBOX 5'-GGGGCATGGGGCAGGCTTTGCGGAGG-3' (3 b.p. deletion),

ΔETSb/c 5'-GCTTCGGCGCGGTGATCGCAGGTGCC-3' (10 b.p. deletion),

ΔAP1 5'-GTGGGCAGGTACACTCAGCTTTTC-3' (2 b.p. substitution),

ΔETSa 5'-CTCAGCTTTAGCTCTTGAGCTGGTC-3' (2 b.p. substitution).

Other vectors used in this study have been described previously; pCDNA3.1mychislacZ for β-galactosidase (Invitrogen), pC53SN3 for wild-type p53 (43), pRKETS2 (in which ETS2 is expressed from a CMV promoter, and which was provided by Eiji Hara, Paterson Institute), $pSG_5ER\alpha HEGO$ (44), from which we generated the dominant negative ERα mutant S554fs by site directed mutagenesis, pERE-Tkluc contains 3 copies of

the estrogen receptor binding element 5' to a minimal promoter, and the mouse *mdm2*-P2 promoter reporter vector, mdm2luc (30). md2.9CAT0 (45) was used to generate murine *mdm2*-P2 promoter sequence. For reporter assays, cells in 60 mm dishes were transfected using Transfast reagent (Promega) with 2 μg reporter plasmid, 0.25 μg pCDNA3.1mychislacZ and 0.05-1 μg of other expression vectors where indicated. Cells were assayed 44 h later using Luclite reagent and a Topcount plate reader (Packard Bioscience) for luciferase reporter gene activity, and a colorimetric ß-galactosidase assay to control for transfection efficiency. Luciferase activity was normalised to ß-galactosidase and results are expressed as mean relative luciferase units (RLU). Each transfection was performed in duplicate and results are presented either as mean RLU $\pm$ SD (n=2) for a representative experiment or, where indicated, results were calculated as a percentage of the activity of the full length hdm2luc01 reporter vector in the cell line, and data from multiple experiments were pooled and expressed as mean $\pm$ SEM. Statistical analysis was performed using a paired two-tailed t-test.

RESULTS

Expression of the *hdm2*-P2 promoter dependent mRNA is elevated in ERα^{+ve} breast cancer cell lines. The breast cancer cell lines for this study were selected from a panel described in a previous report (23). MCF-7, ZR75.1, T47D and BT474 are ERα^{+ve} cell lines, whereas SKBr3 and MDAMB-231 are ERα^{-ve}. MCF-7 and ZR75.1 express wild-type p53 protein whereas the other four lines express p53 protein containing inactivating point mutations at codons 194, 285, 175 and 280 in T47D, BT474, SKBr3 and MDAMB-231 respectively. The study by Gudas *et al* (23) demonstrated that hdm2 protein levels, detected by western blotting with mAb IF2, were consistently higher in the ERα^{+ve} than ERα^{-ve} cancer cell lines. Hdm2 protein is known to be highly phosphorylated at a number of sites that can

affect its immunoreactivity with several antibodies (46), and therefore we first sought to confirm hdm2 expression levels in the breast cancer cell lines using a second mAb, 2A9 (42), the epitope for which (amino acids 155-222) is not known to be sensitive to post-translational modification. As shown in Fig. 1A (top panel), levels of the p90 form of hdm2 were highest in the 4 $ER\alpha^{+ve}$ cell lines, with MCF-7 cells having the lowest levels of this group, and ZR75.1 the highest. Hdm2 protein levels in both of the $ER\alpha^{-ve}$ cell lines (SKBr3 and MDAMB-231) were determined, in two independent experiments, to be <10% of levels in $ER\alpha^{+ve}$ T47D cells, thus confirming the previous report (23). As shown in Fig. 1A, centre panel, in this panel of cell lines elevated levels of p53 protein correlate with the presence of an inactivating mutation in the *p53* gene.

To examine *hdm2* mRNA expression levels in the breast cancer cell lines we used a RPA using an exon 2-exon 3 fragment of *hdm2* cDNA as a probe (Fig. 1B). This probe is complementary to the 5' region of the mRNA transcribed from the *hdm2*-P2 promoter (Fig. 1B) and we were therefore able to quantitatively determine the levels of this transcript. The *hdm2*-P2 transcript was readily detectable in all of the $ER\alpha^{+ve}$ cell lines, whereas in the $ER\alpha^{-ve}$ cell lines it was absent (MDAMB-231) or expressed at very low levels (levels in SKBr3 are <5% of those in MCF-7). The transcript from the *hdm2*-P1 promoter only partially protects the probe and therefore gives rise to a smaller fragment corresponding to part of exon 3 (upper panel). This fragment was detected in all of the breast cancer cell lines at higher levels than the P2 transcript. *Hdm2*-P1 transcript expression was independent of ERα status, though P1 levels were 3-4 fold higher in T47D and BT474 cells than any of the other lines. It is theoretically possible that the exon 3 fragment is derived from mRNA other than the expected *hdm2*-P1 transcript, so semi-quantitative exon-specific RT-PCR was used to confirm *hdm2* transcript levels in the cell lines (Fig. 1B). The RT-PCR data for both the *hdm2*-P1 and -P2 transcripts were comparable to that obtained with the RPA, with small differences in the

relative levels of the P1 transcript being due to the semi-quantitative nature of the RT-PCR assay.

These data demonstrate that *hdm2* mRNA expression in breast cancer cells can be regulated by differential expression from both the P1 and P2 promoters. It is known, however, that whilst the P1 mRNA transcript is expressed at higher levels than the P2 transcript in most cell types, it is translated approximately 8 fold less efficiently (34), and therefore contributes less to hdm2 protein expression levels. Furthermore we found that expression of the P2 transcript in the ERα^{+ve} breast cancer cells correlated with the elevated hdm2 protein levels in these cells, whereas the 3-4 fold increased levels of the P1 transcript in T47D and BT474 compared to MCF-7 and ZR75.1 were not associated with increased hdm2 protein expression. We therefore proceeded to study in detail the mechanisms that regulate *hdm2*-P2 promoter activity in the breast cancer cell lines.

Of the panel of cell lines we have used, MCF-7 and ZR75.1 are representative of the most common class of breast cancers, which both express ERα and retain wild-type p53. However it is not possible to study p53 independent *hdm2*-P2 promoter activity in these cells by conventional reporter assays as the transfection procedure induces a DNA damage response, and consequent activation of p53-responsive promoters such as *hdm2*-P2 (47). T47D cells, however, express inactive mutant p53 protein, and therefore provide a good model to study p53 independent regulation of the *hdm2*-P2 promoter. To confirm that the mutant p53 in these cells cannot be activated by DNA damage, cells were exposed to 5 Gy γ-irradiation and levels of *hdm2*-P2 transcript determined 3 h later. Whilst a strong p53 dependent induction of *hdm2*-P2 transcripts occurred in MCF-7 cells, levels in T47D cells were unaffected by the radiation (data not shown).

***Hdm2*-P2 promoter activity is confined to ERα^{+ve} cell lines, but is independent of ERα function.** Our first aim was to establish whether *hdm2*-P2 transcript levels correlate with ERα expression because the P2 promoter is dependent on ERα activity, or whether it may belong to the class of genes that are estrogen independent, but co-expressed with ERα (5). We therefore generated a luciferase reporter construct (hdm2luc01) containing 895 b.p. of DNA sequence flanking the *hdm2*-P2 promoter, including part of exon 1 through to sequence 3' to the start ATG in exon 3 (Fig. 4A). As shown in Fig. 2Ai, the activity of this promoter mirrored the cell-type specific expression of the P2 derived mRNA transcript, the reporter gene being efficiently expressed in ERα^{+ve} T47D cells, but not in the ERα^{-ve} SKBr3 line. To confirm that the reporter vector was able to function in SKBr3 cells if relevant activating transcription factors were present, we performed co-transfections with wild-type p53, which binds the tandem p53 response elements in the promoter, and demonstrated that the promoter was active in SKBr3 cells when wild-type p53 was expressed (Fig. 2Aii).

This reporter vector was then used to determine the role of estrogen receptor function in driving *hdm2*-P2 promoter activity in T47D cells. The pure ERα antagonist ICI 182780 is able to effectively inhibit transcription from a *bona fidae* estrogen responsive promoter in T47D cells (Fig. 2B, pERE-Tkluc), whereas we demonstrate that it has no effect on expression from the hdm2luc01 vector (Fig. 2B). It is known, however, that in addition to its effect on promoters containing estrogen response elements, ERα is also able to enhance transcription through its ability to interact with, and increase the activity of, AP1 transcription factors, and this activity is not repressed by ERα antagonists (48). Indeed this function of ERα has previously been implicated in up-regulating mdm2 expression in transformed mouse fibroblasts (38). We therefore generated a dominant negative mutant of human ERα (ERα-S554fs), which inhibits this activity of ERα and also down-regulates mdm2 expression in murine fibroblasts (38). ERα-S554fs had no effect on the activity of hdm2luc01 in T47D

cells, despite blocking expression from the known ERα-responsive reporter in the same cells (Fig. 2C). Finally we demonstrate that forced over-expression of ERα in the ERα^{-ve} SKBr3 cell line has no significant effect (P>0.05) on *hdm2*-P2 promoter activity, despite it being functionally active in the cells, as demonstrated by its ability to activate transcription from the *bona fidae* estrogen responsive reporter vector (Fig. 2D).

Role of ras-raf-MEK-MAPK signalling in regulating *hdm2*-P2 promoter activity in T47D cells. Previous studies have identified a role for growth factor signalling through the ras-raf-MEK-MAPK cascade in the p53-independent regulation of mdm2/hdm2 expression (36, 37). The mechanism of raf-induced mdm2 expression has been dissected in the context of the murine *mdm2* promoter, and is dependent on the integrity of both a 5' ETS site (ETSA), and a composite ras-response element (AP1-ETSB) (36). An alignment analysis of the *hdm2* and *mdm2* promoter regions (Fig. 3) showed that, whilst the AP1-ETSB element is conserved between species (labelled as AP1-ETSa in the human promoter), the ETSA site, which was demonstrated by Ries *et* al (36), to be necessary for raf-responsiveness, is not conserved. Consistent with this lack of conservation, we found (data not shown) that treatment of T47D cells with inhibitors of MEK (U0126 and PD98059) inhibited neither the expression of the endogenous *hdm2*-P2 transcript, nor transcription from the hdm2luc01 reporter vector. Expression from a murine *mdm2* promoter vector was inhibited by U0126 in T47D cells. Consistent with the findings of Ries *et al*, however, we did find that U0126 reduced levels of expression of hdm2 protein in human cancer cells, and we are currently examining the mechanism whereby this occurs.

Dissection of transcription factor response elements responsible for *hdm2*-P2 promoter activity. We next proceeded to identify response elements within the hdm2luc01 reporter that

are required for its activity in T47D cells. Two approaches were undertaken: 1) deletion mapping and 2) inactivation of candidate transcription factor binding sites. The reporter vectors used in these studies are summarised in Fig. 4A.

Deletion mapping of the hdm2luc01 vector (Fig. 4B) determined that 55% of the total activity was lost when the 5' region (-602 to –376) was deleted (compare hdm2luc01 with hdm2luc06, P=0.006). Deletion of the region -375 to -133 resulted in a smaller, but significant reduction in activity (compare hdm2luc02 with hdm2luc03, P=0.001), whereas the reduction in activity observed by the deletion of sequences 3' of +33 did not reach the level of statistical significance (compare hdm2luc06 with hdm2luc02, P=0.063). Therefore, whilst it is clear that multiple sequence elements are required for the full activity of the *hdm2*-P2 promoter, at least one positive acting element is present between –602 and -376. A further deletion mutant (hdm2luc23) located this element to the sequence between –418 and –376 (Fig. 4C, compare hdm2luc23 with hdm2luc06, P=0.006)). This 42 b.p. sequence consists almost entirely of a $(nnGGGGC)_5$ repeat sequence (-416 to -381). A potential EBOX (CACGTG) is also present (-381 to -376), however destruction of this element by an internal 3 b.p. deletion had no effect on promoter activity in either the hdm2luc01 (Fig. 4C, hdm2luc01ΔEBOX) or hdm2luc23 (not shown) backgrounds, and therefore the positive-acting element is contained within the $(nnGGGGC)_5$ repeat.

The –375 to +293 region of the promoter (hdm2luc06 vector) retains 35-55% activity, and therefore we employed a candidate site approach to identify the positive acting elements in this region. Based on studies of the murine P2 promoter (36), three separate mutations were made in the hdm2luc01 vector, either singly or in combination, to inactivate potential transcription factor binding sites (Fig. 4A). The mutated sites were: 1) the conserved AP1 site (2 b.p. substitution), 2) the adjacent ETS response element (ETSB in mouse, ETSa in human, Fig. 3) (2 b.p. substitution), which together form an AP1-ETS ras response element in the

murine promoter, and 3) the non-conserved (ETSb/c) sequence containing two potential ETS binding sites (10 b.p. deletion). As shown in Fig. 4D, deletion of the ETSb/c element had no effect on promoter activity in T47D cells, whereas mutation of the AP1 site resulted in a 52.5% loss of promoter activity (hdm2luc01 vs hdm2luc01ΔAP1, $P<0.001$), and the ETSa site deletion resulted in a loss of 29.6% of activity (hdm2luc01 vs hdm2luc01ΔETSa, $P=0.003$). A vector containing mutations in both AP1 and ETSa elements had the same activity as the AP1 site mutant (46.6% vs 47.5%).

Having determined that the AP1-ETSa site in the *hdm2*-P2 promoter confers approximately half of its p53-independent promoter activity in T47D cells, we were then able to determine to what extent transcriptional activation through this element contributes to the level of *hdm2*-P2 promoter activity in the MCF-7 cell line (Fig. 4D). Whilst overall hdm2luc01 activity was approximately 40 fold higher in MCF-7 cells than T47D (in Fig. 4D, promoter activity is presented as a percentage of hdm2luc01 activity in each cell line), due in part to the activation of p53 by the transfection process (data not shown), inactivation of the AP1-ETSa element also reduced promoter activity to approximately 50% in MCF-7 cells.

ETS2 over-expression reconstitutes *hdm2*-P2 promoter activity in SKBr3 cells through the same elements that drive constitutive *hdm2*-P2 expression in T47D cells. Having identified the *cis*-acting elements necessary for *hdm2*-P2 promoter activity in ERα^{+ve} T47D and MCF-7 cells, we then wished to determine why the promoter is inactive in the ERα^{-ve} cell lines. Expression from the murine P2 promoter is known to be dependent on the levels of transcriptional activation through the AP1-ETS element and its activity can be induced by the transient over-expression of AP1 or ETS transcription factors (36). We therefore transfected SKBr3 cells with an expression vector encoding ETS2 in order to determine whether the lack of *hdm2*-P2 transcription in these cells is due to limiting activity of the AP1-ETS binding

transcription factors. ETS2 is known to be able to activate transcription of the murine *mdm2*-P2 promoter when over-expressed in fibroblasts (36) and is normally expressed in all of the breast cancer cell lines used in this study at similar levels (Fig. 5A) (49). Transfection of SKBr3 cells with the ETS2 expression vector resulted in an elevation in ETS2 protein expression levels (Fig. 5A), and a significant (P=0.007) 5-fold activation of the hdm2luc01 reporter vector (Fig. 5B). ETS2 also activated the hdm2luc01 reporter vector in the other ERα^{-ve} cell line, MDAMB-231 (not shown). *Hdm2*-P2 activation by ETS2 in SKBr3 cells was dependent of the integrity of the AP1-ETS element in the promoter as there was no significant activation of hdm2luc01ΔAP1ETSa by ETS2 (P=0.073) (Fig. 5B). Consistent with the requirements for basal *hdm2*-P2 promoter activity in T47D cells, effective ETS2 activation of the promoter in SKBr3 cells was also dependent on the GC-rich repeat sequences present in hdm2luc01, and -23, but not -06 reporter vectors (Fig. 5B).

Finally we wished to confirm that ETS2 over-expression in ERα^{-ve} cells was capable of inducing the expression of functional hdm2 protein. SKBr3 cells were therefore transfected with increasing amounts (0-1 μg) of pRKETS2 plasmid, and levels of endogenous hdm2 and p53 proteins determined by western blotting (Fig. 5C). ETS2 transfection resulted in a clear increase in hdm2 protein levels, and a concomitant decrease in the levels of mutant p53 protein, consistent with increased rates of hdm2 dependent degradation of p53 by the proteosome.

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DISCUSSION

In this study we have confirmed previous findings (23) that elevated levels of hdm2 protein expression correlate with ERα^{+ve} status in a panel of 6 breast cancer cell lines. We have therefore used these cell lines to investigate the mechanisms whereby hdm2 expression is regulated in breast cancer cells. We firstly determined that high hdm2 protein expression correlated with increased levels of transcription from the *hdm2*-P2 promoter, and identified T47D as a cell line in which the p53 inducible *hdm2*-P2 promoter is constitutively active, despite p53 being expressed in a functionally compromised, mutant form in the cells. We show that this activity is dependent on at least three different predicted transcription factor binding sites: an AP1 site, an ETS site, which together form a bi-partite AP1-ETS element at -120 to -99, and a series of 5 consecutive nnGGGGC repeats at –415 to –381. We also demonstrate that transcriptional activation by the AP1-ETS element drives expression of the *hdm2*-P2 promoter in the ERα^{+ve} cell line, MCF-7. These cells express wild-type, functional p53 protein and are widely used as a model system that is representative of the majority of human breast cancers, which are both ERα^{+ve} and p53 wild-type.

In contrast, however, the *hdm2*-P2 transcript was not efficiently expressed in the ERα^{-ve} cell lines SKBr3 or MDAMB-231. Several lines of evidence demonstrate that this lack of expression is due to a loss of transcriptional activation through the AP1-ETS element in these cell lines: 1) in contrast to results from T47D cells, mutation of the AP1-ETS element has no effect on the low basal activity of the *hdm2*-P2 promoter in SKBr3 cells, demonstrating that there is no activation of transcription through this element in SKBr3 2) removal of the nnGGGGC repeats does result in a loss of the residual promoter activity in SKBr3 cells (Fig. 5B, hdm2luc23 vs hdm2luc06), demonstrating that the mechanism whereby promoter activity is stimulated through these repeats is functional in SKBr3, and 3) the over-expression of an ETS factor, ETS2, is able to reconstitute *hdm2*-P2 promoter activity in SKBr3 cells in an

AP1-ETS element dependent manner. As was found to be the case for the constitutive *hdm2*-P2 promoter activity in T47D cells, maximal ETS2 induced P2 activity in SKBr3 cells was dependent on the integrity of both the AP1-ETS and nnGGGGC repeat sequence. The precise mechanism whereby this nnGGGGC element contributes to transcription remains to be elucidated. Whilst there is no clearly identifiable transcription factor-binding element in the sequence, it bears similarity to the consensus for factors such as SP1, which binds a direct repeat of GGGGC without a spacer (50). Both SV40 LT and PyLT viral proteins direct transcription through GGGGC direct repeats similar to the one in the *hdm2*-P2 promoter, and can inhibit the expression of cellular genes by displacing factors, such as the recently identified Rnf6, from such elements (51).

Whilst over-expression of ETS2 is able to reconstitute *hdm2*-P2 promoter activity in SKBr3 cells, we show that the lack of P2 activity in these cells is unlikely to be simply as consequence of lack of ETS2 protein expression, as ETS2 is expressed at similar levels in all the breast cancer cell lines examined. Activation of transcription through composite AP1-ETS elements normally involves the co-operative activation by both AP1 and ETS family members. Not only are these families comprised of multiple members with differing activities (over 20 in the case of ETS) (52), many of which can form hetero-dimers with other family members, but both protein families are subject to control by post-translational modification, and the interaction with both positive and negative regulatory factors (53-55) . We have considered pathways which have been previously shown to regulate *mdm2*-P2 promoter activity to determine whether they may account for the activity of the AP1-ETS element in the *hdm2*-P2 promoter in ERα^{+ve}, but not ERα^{-ve} cells. However, the activity in ERα^{+ve} cells is neither due to the stimulation of AP1 factors by interaction with ERα (38), or the activation of AP1 or ETS factors by ras-raf-MEK-signalling (36). Additionally, using electrophoretic mobility shift analysis with the AP1-ETS sequence as a probe, we detect specific binding

complexes of similar mobilities in the nuclear lysates of both T47D and SKBr3 cells (data not shown). The expression of a large number of genes are known to be differentially regulated between $ER\alpha^{+ve}$ and $ER\alpha^{-ve}$ breast tumors (6), and both AP1 (data not shown) (56) and ETS factor (57) expression shows considerable variation between different breast cancer cells. Based on our cell line study and a recent analysis of breast tumor samples (27), the *hdm2*-P2 promoter may be included in the group of genes whose expression is co-ordinately regulated with ERα in breast cancers.

During the course of our investigations, we have also identified striking differences between human and murine hdm2/mdm2 in the ability of the ras-raf-MEK-MAPK signalling cascade to regulate P2 promoter activity. This finding is critical to our understanding of how hdm2 expression is regulated in a wide range of normal and malignant human cells. Specifically, whilst *mdm2* mRNA and protein expression can be induced by this signalling pathway in murine fibroblasts (36), and in human cancer cells both hdm2 protein expression and transcription from the murine *mdm2*-P2 promoter reporter vector, are sensitive to inhibition of MEK activity, transcription from the human *hdm2*-P2 promoter is not MEK dependent in the human cancer cell lines. This species-specific difference can be explained by a lack of conservation of the transcription factor binding site in the two promoters, as a single ETS factor binding site (ETSA) that is required for ras-raf- MEK-MAPK induction of the murine promoter (36) is not present in the human, and instead constitutive activity of the human promoter in T47D cells is dependent on the nnGGGGCC repeat element. Ras-raf-MEK-MAPK signalling must therefore utilise an *hdm2*-P2 promoter independent mechanism to regulate hdm2 protein levels in human cells, as has recently been described for the inhibition of hdm2 expression by hypoxia (58).

Our study has a number of important implications regarding how levels of p53 activity may be regulated during the development of breast, and most probably other,

malignancies. Firstly, inactivation of p53 by its exclusion from the nucleus, which occurs in approximately 30% of breast cancers and also in normal breast tissue (15), is known to be dependent on the expression of hdm2 (59) and it is therefore probable that the p53-independent transcription of *hdm2* we have described will promote the nuclear exclusion of wild-type p53 in ERα^{+ve} breast cancer cells. Secondly, the widely reported correlation between the presence of inactivating mutations of *p53*, and elevated p53 protein levels, is a consequence of the inactive p53 mutant protein no longer driving the expression of the hdm2 protein required to target their own degradation (60, 61). The loss of this correlation which is observed in significant numbers of breast cancers (8, 9) is likely to be due in part to p53-independent expression of hdm2 in a proportion of these tumors.

Finally, our demonstration that the elevated levels of hdm2 expression in ERα^{+ve} breast cancer cells are not merely a consequence of transcriptional activation by wild-type p53, confirms the presence of a p53-independent mechanism whereby the p53-hdm2 negative-regulatory feedback loop is modified in these cells to reduce levels of cellular p53 activity. These data clearly strengthen the previously un-investigated hypothesis (14) that differences between cancer cells, such as cell-lineage- or differentiation- dependent activity of transcription factors like AP1 or ETS, may determine the frequency at which p53-mutations are observed in individual tumor types, through the regulation of hdm2 expression levels.

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FIGURE LEGENDS

Fig. 1. **Hdm2 protein and mRNA transcript levels in a panel of breast cancer cell lines**. (A) Whole cell protein lysates were analysed by western blotting and probed with antibodies mAb 2A9 for hdm2 and DO-1 for p53. MCF-7, ZR75.1, T47D and BT474 are $ER\alpha^{+ve}$ cell lines, SKBr3 and MDAMB-231 are $ER\alpha^{-ve}$. (B) Levels of *hdm2*-P1 and -P2 mRNA transcripts were determined by quantitative RPA (upper panels) using an *hdm2* exon2-exon3 probe (see diagram). Protected fragment sizes are 128 b.p. for the P2 transcript (containing exons 2 and 3), and 81 b.p. for the P1 transcript, which only protects the exon 3 portion of the probe. The ladders below the main bands are due to 'breathing' of the ends of the probes. A GAPDH probe was used to control for input mRNA levels. Results from the RPA were confirmed using exon-specific RT-PCR (lower panels) using O1b/O3 and O2/O3 primer pairs for the P1 and P2 promoter transcript respectively (see diagram). β actin primers were used to control for input mRNA levels. All PCR reactions were initially optimised to ensure that they were stopped in the exponential phase (not shown).

Fig. 2. **Role of ERα in regulating *hdm2*-P2 promoter activity.** (A) $ER\alpha^{+ve}$ T47D (solid bars) and $ER\alpha^{-ve}$ SKBr3 (open bars) cells were transfected with 2 µg of either pGL3-basic or the *hdm2*-P2 reporter vector hdm2luc01. (Ai) Reporter vector only; (Aii) reporter vector plus 50 ng wild-type p53 expression vector pC53SN3. (B) T47D cells were transfected with either hdm2luc01 or the pERE-Tkluc reporter plasmid. Cells were then cultured for 40 h before assay in the presence of either DMSO control (open bars) or 20 nM of the ERα antagonist ICI 182780 (solid bars). (C) T47D cells were transfected with either hdm2luc01 or pERE-Tkluc in the presence of 1 µg of either pcDNA3.1 control vector (open bars) or dominant negative ERα (ERα-S554fs) expression vector (solid bars). (D) SKBr3 cells were transfected with either hdm2luc01 or pERE-Tkluc in the presence (solid bars) or absence (open bars) of 0.2 µg

ERα expression vector, $pSG_5ER\alpha HEGO$. (A-D) Each experiment was repeated at least twice, and data from a representative experiment is shown. Data are mean $\pm$ SD for duplicate assays.

Fig. 3. **Alignment of human and mouse *hdm2/mdm2*-P2 promoter region.** Human intron 1 sequence was obtained from the human genome database (AC026121.10). Mouse P2 promoter sequence was compiled from published promoter sequence (45) as well as sequencing of the plasmid md2.9CAT0 for the region 5' to the start of the mdm2luc plasmid. Alignment was performed using MacVector and homologous regions are shaded. The upper row is the mouse sequence. The 5' ends of the hdm2luc reporter vectors described in Fig. 4A are indicated, with the exception of hdm2luc01 which also encompasses part of exon 1, and luc02, which starts at the same position as luc06. Potential transcription-factor binding sites indicated were identified using MatInspector, using a cut-off of 0.95/0.95 for core and matrix fits. Additional sites shown are the p53 response elements (P53 RE) 1 and 2, mouse ETSB (36)and the homologous human ETSa site (both with a matrix fit of <0.9), and the potential human ETSb/c sites (matrix fit 0.92).

Fig. 4. **Dissection of *hdm2*-P2 promoter response elements**.

(A) *Hdm2*-P2 promoter map and reporter vectors. Boxes I-III represent *hdm2* exons 1-3. Numbering is relative to the start of exon 2. Solid boxes indicate the p53 response elements. The deletion series of *hdm2* reporter vectors is shown relative to the full construct hdm2luc01. Potential transcription factor response elements where mutations were introduced are indicated. The sequence of the 42 b.p. region at the 5' end of luc23 (difference between luc06 and luc23) contains 5 GC-rich repeats adjacent to an EBOX site (underlined) as

follows: GC(TGGGGGC)(TCGGGGC)(GCGGGGC)(GCGGGGC)(ATGGGGC)ACGTG. The $(nnGGGGC)_5$ repeats are represented by a solid triangle on the diagram. (B) T47D cells were transfected with hdm2luc01 reporter construct and the hdm2luc06, 02 and 03 deletion constructs as indicated. Results are expressed as a percentage of hdm2luc01 activity (mean ± SEM), with data pooled from 5 independent experiments. (C) T47D cells were transfected with the reporter constructs indicated. (D) T47D (solid bars) cells were transfected with the constructs indicated. Open bars represent the results from a separate experiment in which MCF-7 cells were transfected with hdm2luc01 or hdm2luc01ΔAP1-ETSa reporter vectors. Note that the activity of hdm2luc01 in MCF-7 cells was approximately 40 fold higher than in T47D due to the activity of wild-type p53. Data in (C) and (D) are pooled from two or more independent experiments (C, n=4; D, n=6) and expressed as in (B).

Fig. 5. **Reconstitution of *hdm2*-P2 promoter activity in SKBr3 cells.** (A) Left panel; levels of ETS2 protein in the six breast cancer cell lines were determined by western blotting. Right panel; SKBr3 cells were transfected with 2 μg carrier DNA (hdm2luc01 plasmid) plus either 0 or 100 ng of pRKETS2 expression vector as indicated. 48 h after transfection cells were lysed and analysed for ETS2 protein expression by western blotting. (B) SKBr3 cells were transfected with the reporter constructs indicated, either with (solid bars) or without (open bars) 100 ng pRKETS2 over-expression vector. Data are derived from two independent experiments, (n=4). (C) SKBr3 cells were transfected with 0–1 μg pRKETS2 as indicated. Total plasmid transfected was made up to 1 μg with pcDNA3.1. Whole cell lysates were prepared after 36 h. Western blots were probed with antibodies mAb 2A10 for hdm2 and DO-12 for p53.

Fig. 1

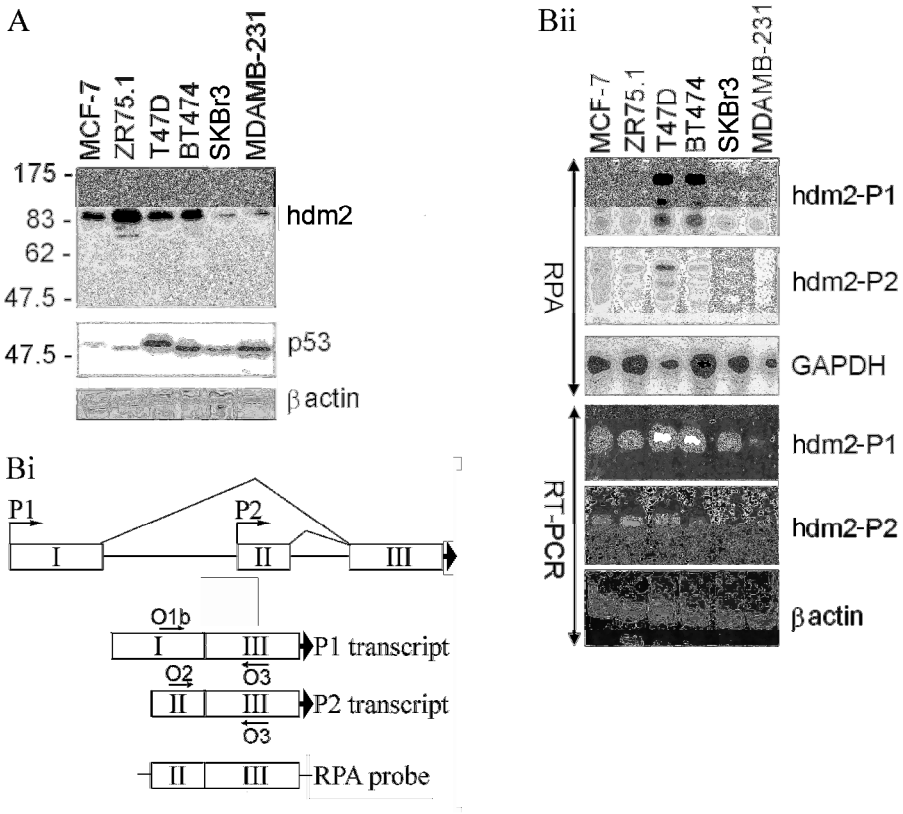
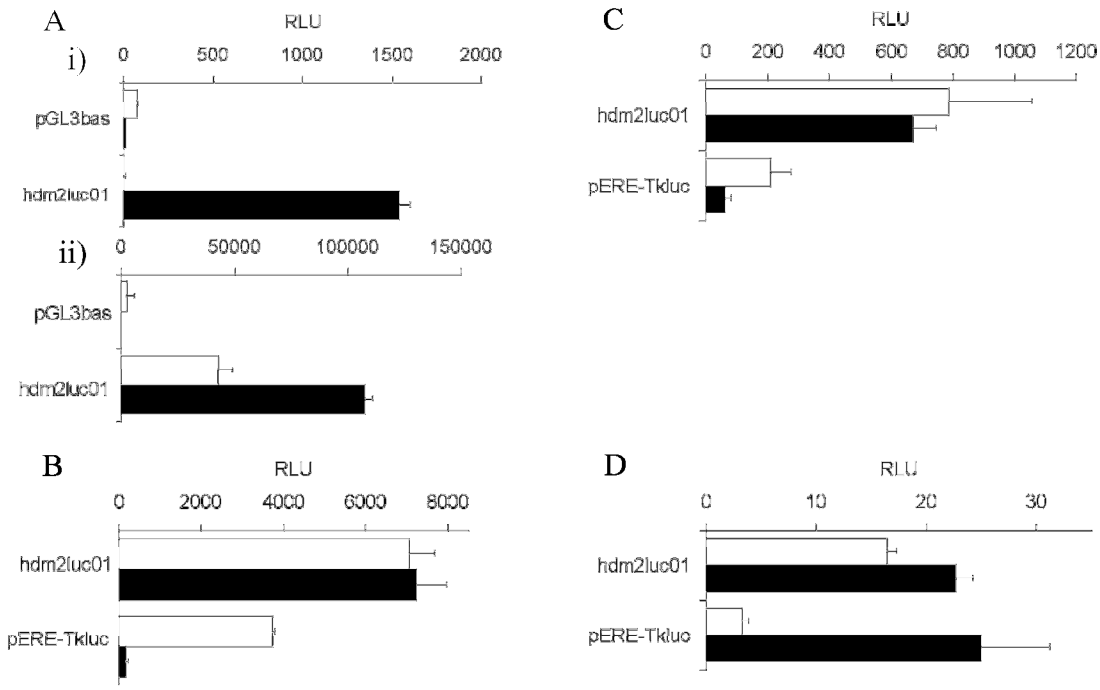

A
MCF-7
ZR75.1
T47D
BT474
SKBr3
MDAMB-231
175 -
83 -
62 -
47.5 -
hdm2
47.5 -
p53
β actin
Bi
P1
P2
I
II
III
O1b
P1 transcript
O2
O3
P2 transcript
RPA probe
Bii
RPA
RT-PCR
hdm2-P1
hdm2-P2
GAPDH
hdm2-P1
hdm2-P2
β actin

Fig. 2


A
i)
RLU
0
500
1000
1500
2000
pGL3bas
hdm2luc01
ii)
0
50000
100000
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pGL3bas
hdm2luc01
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200
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RLU
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20
30
hdm2luc01
pERE-Tkluc

Fig. 3

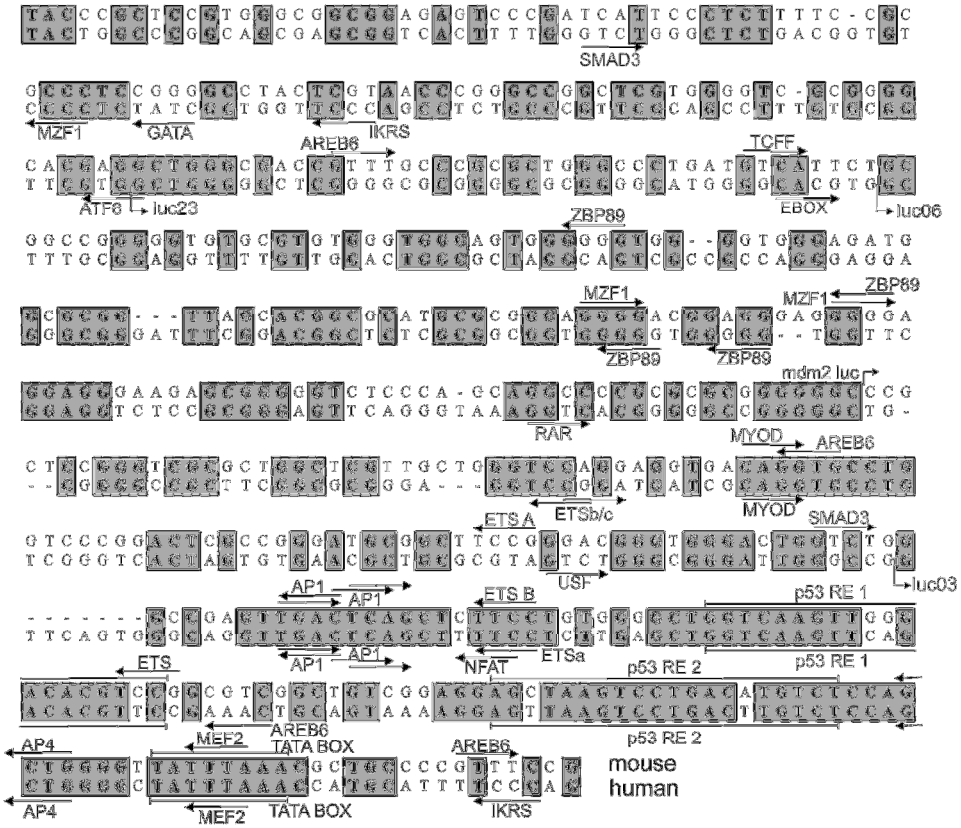

SMAD3
MZF1
GATA
IKRS
AREB6
TCFF
ATF6
luc23
EBOX
luc06
ZBP89
MZF1
ZBP89
ZBP89
ZBP89
mdm2 luc
RAR
MYOD
AREB6
ETS A
ETSb/c
MYOD
SMAD3
USF
luc03
AP1
AP1
ETS B
p53 RE 1
AP1
AP1
NFAT
ETSa
ETS
p53 RE 2
p53 RE 1
AREB6
TATA BOX
MEF2
p53 RE 2
AP4
AREB6
mouse
human
AP4
MEF2
TATA BOX
IKRS

Fig. 4

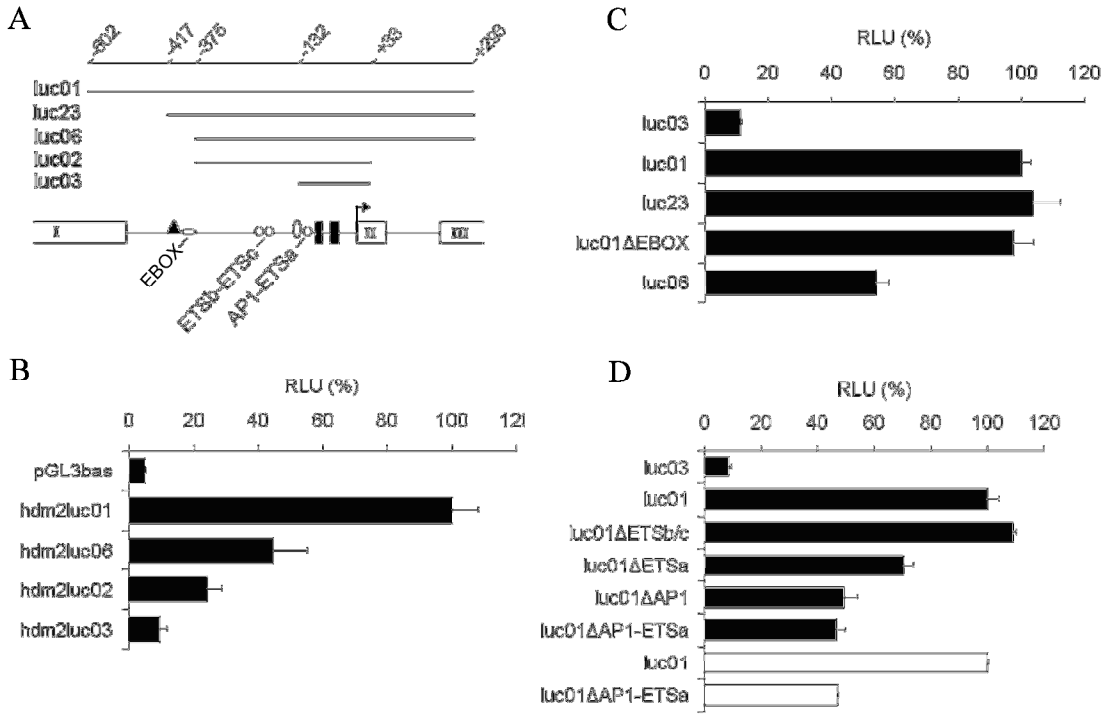
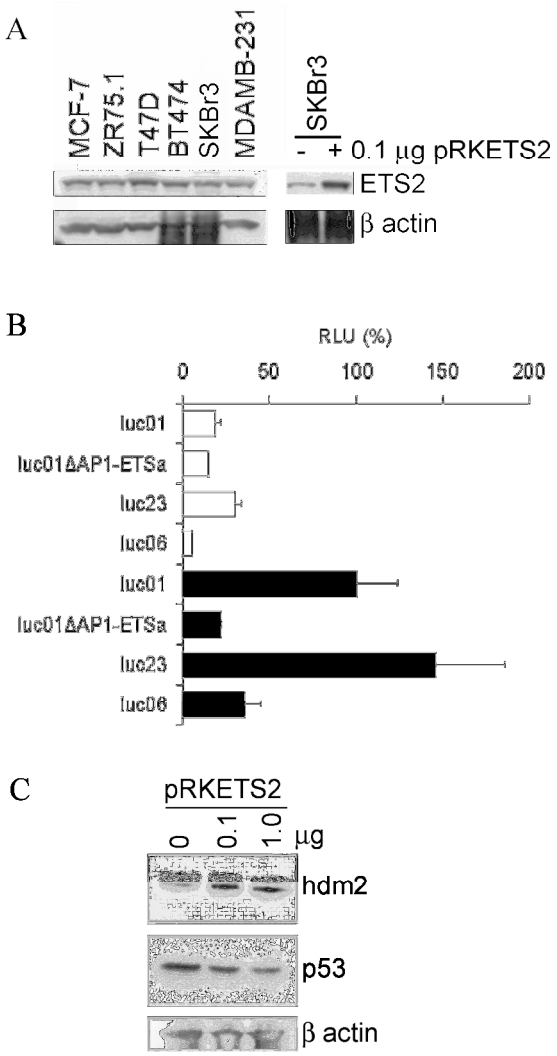

A
-802
-417
-376
-132
+55
+269
luc01
luc23
luc06
luc02
luc03
I
II
III
EBOX
ETSb-ETSc
AP1-ETSa
B
RLU (%)
0
20
40
60
80
100
120
pGL3bas
hdm2luc01
hdm2luc06
hdm2luc02
hdm2luc03
C
RLU (%)
0
20
40
60
80
100
120
luc03
luc01
luc23
luc01ΔEBOX
luc06
D
RLU (%)
0
20
40
60
80
100
120
luc03
luc01
luc01ΔETSb/c
luc01ΔETSa
luc01ΔAP1
luc01ΔAP1-ETSa
luc01
luc01ΔAP1-ETSa

Fig. 5


A
MCF-7
ZR75.1
T47D
BT474
SKBr3
MDAMB-231
SKBr3
- + 0.1 μg pRKETS2
ETS2
β actin
B
RLU (%)
0
50
100
150
200
luc01
luc01ΔAP1-ETSa
luc23
luc06
luc01
luc01ΔAP1-ETSa
luc23
luc06
C
pRKETS2
0
0.1
1.0
μg
hdm2
p53
β actin