The GC selective DNA-binding antibiotic, Mithramycin A, reveals multiple points of control in the regulation of Hdm2 protein synthesis

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ABSTRACT

The primary role of the Hdm2 / Mdm2 oncoprotein is to regulate the levels and activity of the transcription factor p53. Hdm2 synthesis is itself tightly controlled and, as demonstrated by a recently described SNP (SNP309) in the *hdm2*-P2 promoter, minor variations in Hdm2 expression have phenotypic consequences on radiation sensitivity and cancer predisposition. To further define mechanisms regulating Hdm2 expression, we have investigated the effects of the GC selective DNA binding drug, Mithramycin A (MA) on *hdm2* mRNA transcription, trafficking, and translation. Firstly we show that the constitutive *hdm2*-P1 promoter is inhibited by MA. We define, for the first time, the minimal sequence elements that are required for P1-promoter activity and identify those which confer MA sensitivity. Secondly, MA induces p53-dependent transcription from the *hdm2*-P2 promoter. Thirdly, and critically, MA also inhibits Hdm2 synthesis at the posttranscriptional level, with negative effects on *hdm2* mRNA nuclear export and translation. This study highlights the complex interplay between the pathways that regulate Hdm2 protein synthesis in cancer cells, and furthermore emphasises the export of *hdm2* mRNA from the nucleus to the cytoplasm as a key point of control in this process.

INTRODUCTION

The oncoprotein Hdm2 (Mdm2 in mouse) is the primary negative regulator of the activity of the transcription factor p53 in proliferating cells (Momand *et al*., 2000; Vogelstein *et al*., 2000). Hdm2 regulates p53 function through multiple mechanisms, including concealing its activation domain from the transcriptional machinery (Oliner *et al*., 1993), and targeting it for ubiquitination, nuclear export and proteosomal degradation (Michael & Oren, 2003). In the absence of functional Hdm2 / Mdm2, cells in the early embryo undergo spontaneous p53-dependent apoptosis (Jones *et al*., 1995; Montes de Oca Luna *et al*., 1995), normal human fibroblasts in culture undergo cell cycle arrest (Blaydes & Wynford-Thomas, 1998), and the *in vivo* proliferation of tumour cells which retain a wild-type p53 gene is inhibited (Vassilev *et al*., 2004). However, the sensitivity of the p53-Hdm2 regulatory module is such that even small changes in the levels of Hdm2 / Mdm2 can have profound effects on cellular biology. Most notably, the transgenic manipulation of mice to incorporate a hypomorphic allele of *mdm2* that results in as little as a 20% reduction in Mdm2 protein levels, renders the animals markedly more sensitive to ionising radiation (Mendrysa *et al*., 2003). In humans, a single nucleotide polymorphism (SNP309) in the *hdm2* gene which results in increased Hdm2 expression has been shown to correlate with accelerated rates of tumorigenesis in a subset of individuals (Bond *et al*., 2004; Bond *et al*., 2005).

Regulation of the levels and activity of Hdm2 therefore has the potential to play a major role in the control of proliferation and survival of both normal and cancer cells. It is already clear that regulation of Hdm2 activity at the post-translational level is

critically important for the control of its function, with Hdm2 phosphorylation and protein:protein interactions being a critical point of convergence for both stress- and survival-induced signalling pathways (Meek & Knippschild, 2003; Michael & Oren, 2003). Of particular note is the stress-induced increase in Hdm2 protein turnover that contributes to the activation of p53 following DNA-damage (Stommel & Wahl, 2004), the phosphorylation of Hdm2 by Akt kinase, which promotes the accumulation of Hdm2 in the nucleus and is an important component of the effects of pro-survival factors such as Insulin-like Growth Factor–1 (Ashcroft *et al*., 2002; Feng *et al*., 2004; Mayo & Donner, 2001; Shaulian *et al*., 1997), and the induction of the Hdm2 antagonist, $p14^{ARF}$ by oncogenic stress (Sherr & Weber, 2000; Stott *et al*., 1998).

Perhaps less thoroughly investigated are the mechanisms whereby the rates of Hdm2 protein synthesis are regulated. However it is clear, not least from the numerous studies in which elevated levels of *hdm2* mRNA and protein expression have been shown to occur in human tumours (Onel & Cordon-Cardo, 2004), that this is an equally important point of control. Transcription of *hdm2* mRNA encoding the full length, p53-binding form of Hdm2 (p90) occurs from two promoters, P1 and P2. The mRNA transcript generated from the P1-promoter is translated approximately 10 fold less efficiently than the P2 transcript, due to the presence of two upstream open reading frames (uORFs) in the first exon (Brown *et al*., 1999; Jin *et al*., 2003). The P1-promoter is thought to be responsible for the low level, basal expression of Hdm2 / Mdm2 in unstressed cells (Mendrysa *et al*., 2003), though its activity has recently been found to be regulatable in response to Akt kinase signalling in some cells (Chang *et al*., 2004). Transcription from the P2-promoter is highly induced by p53, due to the presence of two p53-binding sites <100 b.p. 5' to the transcriptional start site

(Zauberman *et al*., 1995). This response to p53 is key in regulating the duration of the p53-response to genotoxic stress (Lev Bar-Or *et al*., 2000). A number of other signalling pathways and transcription factors also impinge upon the activity of the p53-Hdm2 module via the regulation of the activity of the P2-promoter, including thyroid hormone receptors (Qi *et al*., 1999), HEY1 and HES1 transcriptional repressors (Huang *et al*., 2004), MYCN (Slack *et al*., 2005) and Ras-Raf-MEK-ERK signalling (Phelps *et al*., 2003; Phelps *et al*., 2005; Ries *et al*., 2000). Hdm2 protein synthesis is also subject to post-transcriptional control; the export of *hdm2* message from the nucleus to the cytoplasm is controlled by cellular MEK activity (Phelps *et al*., 2005), and *hdm2* mRNA translation rates are elevated in some cancer cells (Landers *et al*., 1997; Trotta *et al*., 2003).

There remain, however, many examples in the literature where distinct growth- or stress-induced signalling pathways have been shown to regulate the levels of expression of Hdm2, and as a consequence cellular p53 activity, through non-defined and potentially novel mechanisms. In the recent paper describing the SNP309 in the *hdm2*-P2 promoter (Bond *et al*., 2004), the authors made use of the GC selective DNA binding anti-tumour antibiotic, Mithramycin A (MA), to show increased activity of the P2-promoter in cells homozygous for the SNP309 (G/G) was dependent on Sp1 transcription factors that bind GC-rich sequences. Given that MA is a genotoxic compound that is able to strongly activate the DNA-binding activity of p53 (Koutsodontis & Kardassis, 2004), we were intrigued by the Bond *et al*'s data that MA did not noticeably induce p53-dependent Hdm2 expression in cell lines with an intact p53-responsive pathway, irrespective of their SNP309 status. In this manuscript we have further investigated the mechanistic basis for the effects of MA on Hdm2

synthesis. We demonstrate that MA does strongly induce transcription from the p53-dependent *hdm2*-P2 promoter, however it also inhibits transcription from the constitutive *hdm2*-P1 promoter, which we show is critically dependent on a GC rich element for its activity. MA also reduces the export of both *hdm2* mRNA transcripts from the nucleus to the cytoplasm, further inhibiting Hdm2 protein synthesis

RESULTS

Mithramycin A inhibits Hdm2 protein synthesis in T47D breast cancer cells

The study by Bond *et al*., 2004 demonstrated that MA decreased levels of SMP14 antibody-reactive Hdm2 protein in a number of cell lines which were G/G homozygous for SNP309. We therefore initially used one of these lines, breast cancer-derived T47D cells, to confirm the dose response (Fig. 1a) and time course (Fig. 1b) of the effects of MA on Hdm2 protein synthesis. For these studies we used antibody 2A9, which unlike several antibodies which recognise Hdm2 including SMP14 (Zhang & Prives, 2001), is not known to be affected by post-translational modification of Hdm2. Consistent with the previous study, MA at concentrations above 100 nM reduced Hdm2 levels after 24 h exposure, with the maximum effect being observed at 150-200 nM. Therefore all future experiments were carried out at 200 nM MA over 24 h.

Hdm2 is a highly labile protein, with a half-life of ~15 min in T47D cells (Phelps *et al*., 2005). The half life-of *mdm2* mRNA has also been reported to be relatively short in some cells (Hsing *et al*., 2000). This appeared to be incompatible with the proposed

mechanism of inhibition of Hdm2 synthesis by MA in these cells, i.e. direct binding of the *hdm2*-P2 promoter to inhibit transcription (Bond *et al*., 2004), as this would be expected to result in a rapid decrease in Hdm2 protein levels. We therefore examined the turnover of *hdm2* mRNA following blockade of mRNA synthesis by Actinomycin D (Fig. 1c). Compared with the labile *c-myc* mRNA, *hdm2* mRNA was relatively stable in T47D cells, not decreasing until 7-18 h exposure to Actinomycin D. This would, therefore, account for a delayed decrease in Hdm2 protein levels following the inhibition of *hdm2* mRNA synthesis by MA. Finally, we confirmed that MA does, in fact, inhibit Hdm2 protein synthesis, rather than promoting its proteosomal-mediated degradation, by demonstrating that treatment with the proteosome inhibitor, MG132, does not increase Hdm2 protein levels in T47D cell previously exposed to MA (Fig. 1d).

Mithramycin A down-regulates expression of mRNA from the 'constitutive' Hdm2-P1 promoter, but not the 'inducible' P2-promoter

Having confirmed that MA had the expected effects on Hdm2 protein synthesis in T47D cells, we then examined its effects in a cell line that retains a functional stress-induced, p53-dependent transcriptional response. MCF-7 breast cancer cells exposed to MA for 24 h showed a strong induction of p53 protein by MA (Fig. 2a). This was accompanied by a large increase in the levels of mRNA from the p53-inducible *hdm2*-P2 promoter (750 % ($P<0.05$), Fig. 2b). Despite this induction, Hdm2 protein levels were reduced in MA-treated MCF-7 cells (Fig. 2a), albeit not to as great an extent as in T47D cells.

Whilst it is the regulation of the P2-promoter that is normally responsible for the induction of Hdm2 expression in response to stress-induced p53 activation, the mRNA from the P1-transcript is actually more abundant than the P2 transcript in many cell types, including unstressed MCF-7 cells (Phelps *et al*., 2003). MA caused a reduction in *hdm2*-P1 transcript levels to approximately 35 % of control levels in MCF-7 cells ($P<0.05$, Fig. 2b), and hence the net effect of MA on levels of *hdm2* coding sequence containing transcripts was a decrease (Fig. 2b). As it is likely that this down-regulation of P1-transcript levels contributes to the overall effects of MA on Hdm2 protein levels, we next sought to establish the mechanistic basis underlying this observation.

Sequence elements required for hdm2-P1 promoter activity

We first confirmed that the effect of MA on *hdm2*-P1 transcript levels was seen in cell lines other than MCF-7 (Fig. 3a). MA reduced P1-transcript levels by 74.8 % ($P<0.05$) and 33.1 % ($P<0.05$) in T47D and MDA-MB231 breast cancer cell lines respectively. We also observed that, whilst both these cell lines express functionally inactive mutant p53 and hence MA did not cause the same striking increase in P2-transcripts as in MCF-7, the drug did still induce a modest increase in the P2-transcript levels in both cell lines (206 % ($P<0.05$) and 272 % ($P<0.05$) respectively).

The *hdm2*-P1 promoter has not been previously subjected to functional analysis, Therefore, to investigate the regulation of *hdm2*-P1 observed in all cell lines examined, we amplified a 1057 b.p. region of the *hdm2*-P1 promoter from normal human genomic DNA, and cloned it into the pGL3Basic luciferase reporter vector, to

produce the vector hdm2P1luc01. This region included 61 b.p. of sequence 3' to the expected transcriptional start site (Oliner *et al*., 1992) including an ATG from one of the two uORFs in exon 1 which inhibit translation of the *hdm2*-P1 message (Brown *et al*., 1999). In order to confirm that transcription was initiating, as would be expected, from 5' to this ATG, as well as to improve translation of the luciferase reporter gene, this ATG codon was mutated (ΔATG vectors). This resulted in an approximately 3-fold enhancement in reporter activity in all cell lines tested (data not shown), so all subsequent experiments were performed using reporter vectors with this mutation. We then made progressive 5' deletions of the inserted genomic sequence to identify the minimal region of the promoter that was required for constitutive activity (Fig. 3b). In MDA-MB231 cells, the plasmid hdm2P1luc05ΔATG, which contains only 221 b.p. of genomic DNA sequence, had comparable activity to the original 1057 b.p. containing vector. Deletion of a further 120 b.p. of 5' sequence (hdm2P1luc06ΔATG) resulted in loss of >90 % of the promoter activity.

To facilitate further dissection of this promoter region we performed an alignment analysis of this 221 b.p. region of the human P1-promoter with the comparable region of the murine promoter (Fig. 3c). It has previously been observed that the 5'UTR region encoded by exon 1 is relatively poorly conserved between species (Zauberman *et al*., 1995) and therefore we reasoned that any conserved elements between the species in the promoter regions may well be functionally important. The human sequence was also analysed for potential transcription factor binding sites. The first observation was that there is no TATA box element to provide a specific transcriptional start site, and BLAST analysis of both human and mouse EST databases identified sequences with different 5' ends in both species, which is

suggestive of multiple start sites. Based on this analysis, a further set of deletion mutants was then generated, and transfected into MDA-MB231 and MCF-7 cells (Fig. 3d). The activity of hdm2P1luc05ΔATG in each cell line was set as 100 %, and the other vectors compared to this. In MCF-7 cells, as in MDA-MB231 cells, hdm2P1luc01ΔATG and hdm2P1luc05ΔATG vectors have approximately equal activity, and therefore the minimal active region is similar in both cell lines. A deletion of 50 b.p. from the 5' end of hdm2P1luc05ΔATG to produce hdm2P1luc09ΔATG did not result in loss of activity in MDA-MB231 cells, and actually resulted in significantly ($P<0.01$) increased activity in MCF-7 cells. A further deletion of the 18 b.p. sequence between -110 and -92 resulted in a reduction to 21.4 % and 12.5 % of hdm2luc05ΔATG activity in MCF-7 and MDA-MB231 cells respectively. This region contains a CCAAT box consensus sequence. Deletion of 39 b.p. from the 3' end of hdm2P1luc05ΔATG resulted in a significant ($P<0.01$) reduction in activity to 45.3 % in MDA-MB231. In MCF-7 cells, whether or not this deletion had a significant effect was dependent on the absence or presence of the 50 b.p. between -160 and -110 at the 5' end of the inserted sequence.

Having established the minimal region required for P1-promoter activity, we then set out to establish the mechanism whereby MA inhibits expression of the P1-transcript. MA is thought to inhibit transcription by binding GC-rich regions in promoter, and preventing them from recruiting activating transcription factors, such as Sp1. We therefore made and analysed a series of hdm2P1luc09ΔATG-based vectors containing small mutations in GC rich motifs in the promoter. One of these mutations (GCII) resulted in a >85 % loss of promoter activity in both cell lines (Fig. 4a). Three other mutations (GCIII, IV, V) resulted in smaller, though significant ($P<0.05$), reductions

in activity in MCF-7 cells. In MDA-MB231 cells only one of these other mutants, GCIV, had a significant effect (P=0.001), though, interestingly the GCIV mutation resulted in a >2-fold increase in reporter activity in these cells, compared to a 42.8 % decrease in MCF-7.

The GCII region that is necessary, though not sufficient, for >85% of promoter activity in both cell lines is also well conserved between the human and mouse promoters. We therefore generated further mutants to characterise this region, and individually disrupted a potential ETS family binding site (defined here as site ETSb), and a consensus binding site for the ubiquitously expressed ZF5F factors, which overlaps with the 5' part of the GCII region. The effects of all these mutants of the GCII regions were examined in MDA-MB231, MCF-7 and T47D cells (Fig. 4b). A 2 b.p. substitution that destroyed the ETSb site resulted in significant (all $P<0.01$) 71.7 %, 83.0 % and 88.0 % reductions in promoter activity in MDA-MB231, MCF-7 and T47D cells respectively. The 6 b.p. substitution to destroy the ZF5F site also reduced promoter activity by 61.6 % ($P<0.001$) in MDA-MB231, 70.8 % ($P<0.001$) in MCF-7 and 65.5 % ($P<0.01$) in T47D cells. In all cell lines, the effect of the GCII mutation was slightly greater than either the ETSb or ZF5F mutations, suggesting there may be more than one functional transcription factor binding sites in this region, which the GCII mutant is more effective at inactivating. To investigate whether the GCII region could be the functionally relevant target for MA in the *hdm2*-P1 promoter, we exposed cells transfected with various reporter vectors to MA (Fig. 4c). MA significantly inhibits activity of hdm2P1luc09ΔATG to 19.8 % ($P<0.01$) of control levels; this inhibition is not seen with the GCII mutated vector. As MA is generally regarded as an inhibitor of Sp1 transcription factor activity, we attempted to

determine the role of Sp1 in transcription from the P1-promoter. However these experiments proved inconclusive as, whilst ~90 % knockdown of Sp1 protein was achieved, we did not observe any decrease in endogenous *hdm2*-P1 message levels, nor was a known Sp1-responsive reporter vector affected by the Sp1 siRNA. Potentially, other members of the Sp transcription factor family may be compensating for the loss of Sp1 in these experiments.

Finally, because the *hdm2*-P1 transcript is poorly translated, it is not clear to what extent it may contribute to the overall levels of Hdm2 protein in unstressed cells. MCF-7 cells were transfected with siRNAs targeting the unique 5'UTR in the *hdm2*-P1 transcript, and the effects compared to an siRNA targeting the Hdm2 coding sequence. (Fig. 4d). Compared to a control siRNA, Hdm2-CDS siRNA reduced Hdm2 protein expression by approximately 85 %, and *hdm2*-P1 siRNA caused a 30% reduction. This confirms that transcription from the P1-promoter does contribute significantly to the levels if Hdm2 protein in these cells, and therefore MA is likely to exert its effects on Hdm2 protein levels by, at least in part, inhibiting transcription from this promoter.

Regulation of the nuclear export of hdm2 mRNA by MA

The above results do not, however, satisfactorily explain why the large induction in *hdm2*-P2 transcripts in MCF-7 cells treated with MA does not result in elevated Hdm2 protein levels in these cells. Previous studies in our laboratory have indicated the importance of mRNA trafficking and translation as a regulatable point of control of Hdm2 protein synthesis (Phelps *et al*., 2005). We therefore set out to investigate

whether MA affects Hdm2 synthesis at the post-transcriptional level. Cells were incubated with MA or DMSO carrier control and then subjected to hypotonic lysis and homogenisation to obtain nuclear and cytoplasmic extracts. The fractionation was verified by comparison of semi-quantitative PCR detection of the small nuclear RNA *snRNA U6,* which is enriched in the nuclear compartment, compared to *gapdh* mRNA which is present in both nucleus and cytoplasm. Part of the cytoplasmic extract was then subjected to separation through 30 % sucrose buffer to isolate polyribosome-bound mRNA. In MCF-7 cells (Fig. 5a) MA again caused a large induction of P2-transcript when total cellular RNA was analysed (1530%). However when cytoplasmic mRNA was analysed, the *hdm2*-P2 transcript was under represented relative to *gapdh* mRNA, compared to the total RNA analysis. Following MA, levels of cytoplasmic P2-transcript did increase, but the relative increase was only 620%. In contrast, there was a 2560% increase in the levels of P2-transcript in the nucleus. This provides strong evidence that MA treatment leads to the preferential retention of *hdm2*-P2 transcripts in the nucleus. Furthermore, when the association of P2-transcripts with polyribosomes was analysed, the increase following MA treatment was only 213%. In support of these observations, MA caused a reduction in levels of total *hdm2*-P1 transcripts in the cells, as before (43%), whereas the reduction in cytoplasmic P1-mRNA was again greater (83%), indicating that the nuclear export of this transcript is similarly inhibited. The reduction in polyribosome-associated P1-transcript was nearly 90%. Overall, the combination of a large reduction in translated P1 message, with only a small increase in translated P2 message, could account for the modest decrease in Hdm2 protein levels following exposure of MCF-7 cells to MA.

Fig. 5b shows that this effect of MA is not restricted to MCF-7 cells, as in MA-treated T47D cells both P1- and P2- transcripts show an accumulation in the nuclear compartment, and an up to 50% under-representation in the cytoplasmic RNA, compared to RNA extracted from whole cells. In T47D cells, there was a reduction in both P1- and P2- transcripts in the polyribosome fraction following MA treatment, consistent with the greater effect of the drug on Hdm2 protein synthesis in these cells. Finally, the effects of MA on the nuclear export of *hdm2* mRNA in T47D cells are similar to the consequences of inhibiting MEK kinase activity that we reported previously (Phelps *et al*., 2005). We therefore examined whether inhibition of MEK kinase activity occurred following 24 h exposure of T47D cells to MA (Fig. 5c). MA did not affect phosphorylation of ERK1/2 by MEK, demonstrating that MA acts independently of MEK to inhibit *hdm2* mRNA nuclear export.

DISCUSSION

Both the basal and inducible levels of expression of Hdm2 protein are critical in determining the phenotypic response to cellular stress (Lev Bar-Or *et al*., 2000; Ma *et al*., 2005; Mendrysa *et al*., 2003; Vogelstein *et al*., 2000). An inherited polymorphism in *hdm2*, can result in variations in radiation responsiveness, and tumour susceptibility between individuals within a population (Bond *et al*., 2004; Harris *et al*., 2005), tissue-specific pathways which elevate Hdm2 expression may affect the selection pressure for the acquisition of p53 mutations in different types of cancer (Phelps *et al*., 2003; Wynford-Thomas & Blaydes, 1998), tumour associated genetic aberrations that increase Hdm2 protein levels can promote cancer progression (Momand *et al*., 1998; Oliner *et al*., 1992; Onel & Cordon-Cardo, 2004), and the differential effects of

genotoxic chemotherapeutics on the expression of Hdm2 may contribute to their ability to induce p53-dependent cell death pathways (Arriola *et al*., 1999; Inoue *et al*., 2001). Therefore it is critically important to understand the mechanistic basis for the regulation of Hdm2 expression. Mithramycin A is an anti-tumour antibiotic which inhibits transcription from promoters containing GC rich DNA sequences (Blume *et al*., 1991; Miller *et al*., 1987). MA is licensed for clinical use, and therefore the elucidation of its mechanisms of action is of interest in that respect (Ferrante *et al*., 2004; Remsing *et al*., 2003). In this report, however, we have focussed on its application as a useful tool to probe the mechanisms whereby the expression of Hdm2 is regulated in cancer cells.

MA is a potent inducer of sequence-specific DNA-binding competent p53 (Koutsodontis & Kardassis, 2004). The p53 target genes *WAF-1* and *PUMA* are not, however, induced by MA. This is because p53-dependent transcription of these genes requires co-operation between p53 and Sp1 transcription factors. MA, by interacting with GC rich DNA, prevents Sp1 from binding to the *WAF-1 and PUMA* promoters (Blume *et al*., 1991; Koutsodontis & Kardassis, 2004). The *hdm2*-P2 promoter contains two adjacent p53-responsive elements in close proximity to the transcription start site, and is highly sensitive to activation by p53 (Zauberman *et al*., 1995). Here we have shown that MA strongly induces expression of the *hdm2*-P2 transcript in wild-type p53 expressing cells, and therefore *hdm2*-P2 promoter activation by p53 is not Sp1 dependent. This differential dependence on Sp1 for p53-dependent transcription between *hdm2* and certain other p53-responsive genes may potentially play a role in defining the amplitude and longevity of p53 activation following distinct stimuli in different cell types.

The *hdm2*-P2 promoter is also regulated by factors other than p53, the extent of this p53-independent transcription again being an important regulator of cellular p53 activity (Ries *et al*., 2000). T47D breast cancer cells, despite expressing functionally inactive mutant p53, express similar levels of the *hdm2*-P2 transcript to wild-type p53 expressing MCF-7 cells (Phelps *et al*., 2003). We have previously shown that the activity of a *hdm2*-P2 promoter reporter construct in these cells is partially dependent on a highly GC rich repetitive sequence, which is a likely binding site for MA. Subsequently, Bond *et al*, 2004, showed that T47D cells were homozygous (G/G) for the SNP309 in the P2-promoter region. They provided compelling evidence that SNP309 acts to extend an Sp1 site, resulting in increased, Sp1-dependent, activation of this promoter. To support this interpretation, they showed that MA strongly down-regulates Hdm2 protein expression in T47D cells, and proposed that this was due to an inhibition of Sp1-dependent P2-promoter activity by MA in these cells. Here we show that MA actually causes a modest increase in the levels of P2 transcript in T47D cells. It is important to note, however, that this does not exclude a role for these GC rich sequences in the basal activity of the promoter in these cells, as MA may also prevent transcriptional repressors from binding to the promoter, potentially over-riding any effects on activation by Sp1, to result in a net up-regulation of activity. Clearly, however, the down-regulation of Hdm2 protein expression by MA in these T47D cells in not due to inhibition of transcription from the P2-promoter.

We did however, discover, at least two distinct mechanisms whereby MA does inhibit the synthesis of Hdm2 protein; inhibition of transcription from the constitutive P1-promoter, and inhibition of the nuclear export of both P1- and P2- *hdm2* mRNA

transcripts. Both of these effects occurred in MCF-7 and T47D cells lines, the less striking reduction in Hdm2 levels in MCF-7 being accounted for by the MA-induced up-regulation of p53-dependent P2-promoter activity in these cells. Mechanisms regulating transcription from the *hdm2*-P1 promoter have, to date received little attention. This is primarily because the promoter has not, until recently (Chang *et al*., 2004), been show to be regulatable, and also because the transcript is poorly translated (Brown *et al*., 1999; Jin *et al*., 2003). However, murine models have shown that Mdm2 protein synthesis is dependent almost exclusively on the P1-transcript in many unstressed normal tissues *in vivo* (Mendrysa *et al*., 2003), as well as in tumour cells in which the *mdm2* gene is amplified (Barak *et al*., 1994). Using siRNA directed towards the unique 5' UTR in the P1-transcript, we show here that that transcript does contribute significantly to the levels of Hdm2 protein in the MCF-7 breast cancer cell line, though we cannot define the extent of this contribution precisely due to differing efficacies between siRNAs.

We have defined a 171 b.p. minimal region of the P1-promoter that is required for its constitutive activity. Further deletions and mutations identified two regions, loss of either of which results in >80% reduction in promoter activity. These are the 18 b.p. sequence between -110 and -92, which contains an evolutionarily conserved CCAAT box, and a GC-rich region, which we have defined as GCII, which is also highly conserved between human and murine promoters. The residual activity of a reporter vector in which this GCII element is mutated is insensitive to MA, indicating that this is the key binding site for MA that is responsible for inhibition of P1-promoter activity by the drug. Further analysis of the promoter revealed that whilst the above two regions were essential for promoter activity in all three cells lines examined,

mutation of other sequences had differential effects between cell lines. These included the 50 b.p. region between -160 and -110, removal of which had no effect in MDA-MB231 cells, but approximately doubled activity in MCF-7 cells, and the highly conserved GCIV region immediately 5' to the transcriptional start sites, deletion of which either enhanced or reduced activity depending on the cell line examined. These results demonstrate that, whilst this promoter may be ubiquitously expressed, it is likely to be regulated by different sets of transcription factors depending on the cell type. Potentially this may account for our findings (data not shown) that, whilst P1 promoter activity in murine fibroblasts is dependent on an active Akt signalling pathway (Chang *et al*., 2004), the >90% reduction of Hdm2 protein levels that results from 48 h inhibition of Akt activity in MCF-7 cells is not associated with any decrease in *hdm2*-P1 transcripts. Further work will be required to determine the extent to which regulation of transcription from the P1 promoter contributes to the elevated levels of Hdm2 protein expression in human cancers (Onel & Cordon-Cardo, 2004).

We have demonstrated recently that the export of *hdm2* mRNA is a rate-limiting for the synthesis if Hdm2 protein, and dependent upon the activity of MEK-dependent signalling pathways (Phelps *et al*., 2005). We show now that MA treatment also reduces the cytoplasmic to nuclear ratio of *hdm2* mRNA and that this effect contributes to the reduced rates of Hdm2 protein synthesis in MA treated cells. Of particular note was our finding that, although MA-induced p53 activation and a strong up-regulation of *hdm2*-P2 transcript levels in MCF-7 cells, these transcripts accumulated to a greater extent in the nucleus, rather than being exported to the cytoplasm. As a consequence of this, and potentially additional effects of MA on

hdm2 mRNA translation, levels of *hdm2*-P2 transcripts being actively translated in polyribosome complexes increased only ~2 fold following MA treatment.

The majority of cellular mRNAs, in the form of ribonucleoprotein complexes, utilise common pathways for their export from the nucleus to cytoplasm (Erkmann & Kutay, 2004). More recently, however, it has become clear that a subset of cellular mRNAs utilise more selective processes for their export, through the interaction of RNA sequences, often in their 3'UTR, with RNA sequence- or structure-selective binding proteins that direct the export of these mRNAs (Keene, 2003). The translation initiation factor eIF4E for example has, in addition to its well described role in the cytoplasm, a nuclear role in the regulation of export of a subset of mRNAs including *cyclin D1* (Culjkovic *et al*., 2005). This function may be involved in the oncogenic function of eIF4E, and both this (Kentsis *et al*., 2004), and possibly other (O'Shea *et al*., 2004), selective mRNA export pathways are potential targets for cancer therapy. The proteins involved in the selective export of *hdm2* mRNA remain to be identified, however our finding that *hdm2* mRNA export is not only dependent upon MEK kinase activity, but is also sensitive to the anti-tumour antibiotic Mithramycin A in a MEK-independent manner, not only highlight the importance of regulated mRNA export in controlling Hdm2 synthesis, but should also provide useful in the elucidation of its mechanism.

MATERIALS AND METHODS

Cell Culture

T47D, MDA-MB231 and MCF-7 breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % fetal calf serum (Autogen Bioclear) as described previously (Phelps *et al*., 2003). The following reagents were dissolved in dimethyl sulphoxide (DMSO) at the indicated concentration before adding to the medium where stated; 200 μM Mithramycin A (Sigma), 10 mM MG132 (Sigma). Actinomycin D (Sigma) was dissolved in ethanol at a concentration of 5 mg/ml.

Protein Analysis

Cells were washed with phosphate-buffered saline, pelleted by centrifugation at 1000 X *g*, snap frozen and stored at -70 °C. Western blotting was performed as described previously (Phelps *et al*., 2005), and membranes were probed for Hdm2 (monoclonal antibody 2A9 (Chen *et al*., 1993)), p53 (DO-1, Serotec), phospho-Thr-202/Tyr-204 ERK 1 and ERK2 (E10), total ERK1 and ERK2 (both from Cell Signaling Technology). Equal protein loading was confirmed on all immunoblots using rabbit anti β-actin antibody (Sigma). Bands were visualised by chemiluminescence (Supersignal, Pierce) using a Fluor-S MAX system (Bio-Rad).

RNA analysis

RNA extraction from cell pellets was performed using either RNABee (Biogenesis Inc.) or RNeasy (Qiagen). Semi-quantitative RT-PCR analysis was performed as described previously (Phelps *et al*., 2003), using oligo dT (Promega) for the reverse transcriptase reaction. Primers for amplification of *c-myc* were c-mycU 5'-CCATCGATTTCTTCCTCATCTTC-3' and c-mycD 5'-TGAGGAGACACCGCCCAC-3'. Taqman quantitative polymerase chain reaction

(qPCR) analysis of *hdm2* transcripts was performed as described previously (Phelps *et al*., 2005).

Plasmids

A 1057 b.p. region of the *hdm2*-P1 promoter (-997 to +61 relative to the major transcriptional start site) was amplified from normal human genomic DNA and ligated into pGL3basic using the *MluI/XhoI* sites (Promega). Mutation of the ATG codon present in this sequence (+57 to +59) to TTG was introduced using a single base change in the reverse primer to generate hdm2P1luc01ΔATG. Additional constructs containing deletions of the *hdm2*-P1 promoter (luc03 – luc10) were generated by proof-reading PCR of hdm2P1luc01ΔATG using primers containing *MluI* and *XhoI* sites, followed by ligation into pGL3basic. Analysis of potential transcription factor binding sites was performed using MatInspector (www.genomatix.de/cgi-bin/matinspector/matinspector.pl). Further mutations were introduced using site-directed mutagenesis and all constructs were verified by sequencing (MWG Biotech). Forward site-directed mutagenesis primers used are as follows (complementary reverse primers not shown): ΔGCI 5'-TTGGCGGAAGCGTCTAGACGGTTGTGTGCG-3'; ΔGCII 5'-GCGCGCSCSSSTTCTAGAGATGCGCCGCGA-3'; ΔGCIII 5'-CGCGCTCCCCTCTTCTAGAGGTSGGGGGCGC-3'; ΔGCIV 5'-CTCGGGCGGTAGTCTAGAGCGCACCGAGGC-3'; ΔGCV 5'-TGGCTGCTTCTGTCTAGACAGAAGCAGCCA-3'; ΔETSb 5'-CACAAATGCCCGCTTGCGCCGCGACG-3'; ΔZF5F 5'-CCGGTTGTGTGCTCTAGAACAAATGCCCGG-3'.

Transfections

Cells were transfected with reporter vectors using Lipofectamine 2000 reagent (Invitrogen), and reporter assays were preformed using a Dual-Glo^{TM} luciferase assay (Promega) on cells transfected in 96-well plates, with normalisation to *Renilla* luciferase expressed from pRLSV40 (Promega). The normalised data is presented as relative luciferase units (RLU). For transfection of siRNA, cells in 6-well plates were transfected with 100 pmol of either control (non-silencing) siRNA or siRNA targeting different *hdm2* transcripts: the coding sequence of *hdm2* was targeted using validated siRNA from Qiagen (Hs_mdm2; will target both P1 and P2 transcripts); *hdm2*-P1 siRNA was targeted to the following sequence in exon 1, 5'-AAGATGGAGCAAGAAGCCGAG-3' (will target *hdm2*-P1 transcript only). Cells were harvested and analysed 72 h after transfection.

Cellular Fractionation

Cellular fractionation before RNA extraction or polyribosome analysis was performed using hypotonic lysis. Cells were washed twice with serum-free medium containing 10 µg/ml cycloheximide, then scraped and pelleted by centrifugation at 365 X *g*. Pellets were resuspended in hypotonic lysis buffer (10 mM Tris-HCl pH 7.6, 1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, 1 unit/µl RNAse inhibitor) and lysed on ice using a Dounce homogeniser. Lysates were centrifuged at 12 000 X *g* to separate the cytoplasmic extract from the nuclear pellet. For polyribosome analysis, aliquots of the cytoplasmic lysates were layered over a cushion of 30 % sucrose in hypotonic lysis buffer and centrifuged at 130 000 X *g* for 2.5 h at 4 °C. The supernatant was removed and the remaining polyribosome-bound RNA pellet was used for RNA extraction.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Mithramycin A blocks Hdm2 protein synthesis**.** (a) T47D cells were cultured in the presence of the indicated concentration of MA for 24 h before Hdm2 protein levels were determined by Western blotting using 2A9 antibody. As internal control, the same membrane was re-probed with an antibody to β-actin. (b) T47D cells were cultured in the presence of 200 nM MA for the indicated time before Hdm2 protein levels were determined by Western blotting using 2A9 antibody. A second Hdm2 antibody, 2A10, gave similar results (data not shown). (c) T47D cells were cultured in 5 μg/ml Actinomycin D for the indicated time before RNA was extracted and semi-quantitative PCR used to determine relative levels of *hdm2*-P1, *hdm2*-P2, *c-myc* and *β–actin* transcripts. (d) T47D cells were cultured with DMSO carrier or 200 nM MA for 24 h before 50 μM MG132 was added to the medium to block proteasome-mediated degradation of Hdm2. Cells were cultured for a further 0-60 min before Hdm2 protein levels were determined by Western blotting.

Figure 2 Mithramycin A inhibits Hdm2 expression in MCF-7 breast cancer cells**.** (a) MCF-7 cells were cultured in the presence of DMSO carrier or 200 nM MA for 24 h before Hdm2 protein levels were determined by Western blotting using 2A9 antibody. (b) MCF-7 cells were cultured in the presence of DMSO carrier or 200 nM MA for 24 h before total cellular RNA was extracted and analysed by qPCR. *hdm2* mRNA levels were normalised to *gapdh* housekeeping gene expression, and normalised *hdm2*

mRNA levels are expressed as percentage of levels in DMSO-treated cells. Open bars, DMSO-treated; Solid bars, MA-treated. Data are mean ± S.E.M (n=3).

Figure 3 Definition of minimum sequences required for hdm2-P1 promoter activity**.** (a) T47D and MDA-MB231 cells were treated and analysed as in Fig 2b. Open bars, DMSO-treated; Solid bars, MA-treated. Data are mean ± S.E.M (n≥3). (b) MDA-MB231 cells were transfected with reporter vectors that have progressive truncations from the 5' end of Hdm2P1luc01ΔATG, a vector that contains a 1057 b.p. region of the *hdm2*-P1 promoter. The 5' limit compared to the major transcriptional start site is 01 -997, 03 -510, 04 -330, 05 -160, 06 -30. The 3' end is at +61 in all vectors. Results are mean RLU ± S.E.M (n=4) expressed as a percentage of Hdm2P1luc01ΔATG. (c) The human genomic DNA sequence present in the minimally active vector, Hdm2luc05ΔATG, was aligned with the murine *mdm2* P1-promoter region, using ClustalW (Thompson *et al*., 1994). Highlighting of conserved regions was performed using BOXSHADE. The suspected major transcriptional start site in *hdm2* is shown with a black arrow, other potential start sites identified as ESTs are shown as grey arrows. uORF indicates the position of the ATG that we have mutated. The 5' end of the different reporter vectors used is indicated. Matinspector (http://www.genomatix.de/) was used to identify potential transcription factor binding sites (Core fit >0.95, Matrix fit >0.925) in the human sequence. Boxed annotations are positions of potential transcription factor binding sites, or GC rich regions I-V, that we have modified by site-directed mutagenesis in certain vectors. (d) MDA-MB231 (solid bars) and MCF-7 (open bars) cells were transfected with the indicated reporter vectors. Results are mean RLU ± S.E.M (n≥4) expressed as a percentage of

Hdm2P1luc05ΔATG. Annotations are: box – exon 1, triangle – CCAAT box, circles – GC rich regions I-V, X – mutated ATG.

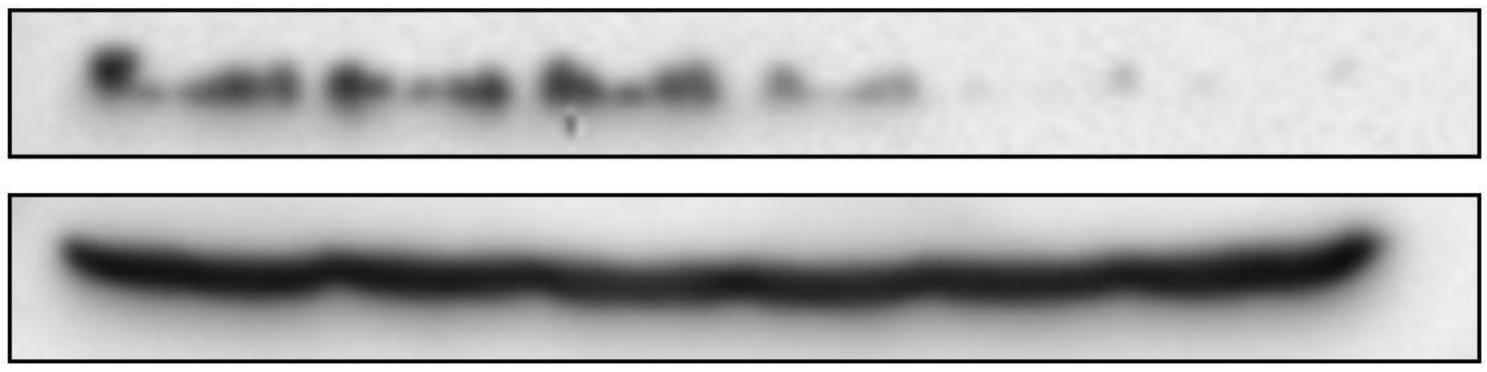
Figure 4 Identification of potential transcription factor binding sites required for P1 promoter activity**.** (a) MDA-MB231 (solid bars) and MCF-7 (open bars) were transfected with the reporter vectors shown. Results are mean RLU ± S.E.M (n=4) expressed as a percentage of P1luc09ΔATG. Annotations are as for Fig. 3d, except that an open symbol indicates that the site has been mutated. (b) Experiments were performed exactly as in Fig. 4a, except that T47D cells were also analysed (grey bars) and different reporter vectors were used. Additional symbols represent ETSa and ETSb (inverted triangles) and ZF5F (diamond). (c) T47D cells were transfected with the reporter vector indicated, then cultured for 24 h before assay in the presence of either DMSO carrier (solid bars) or 200 nM MA (open bars). Results are mean RLU ± S.E.M (n=2) expressed as a percentage of Hdm2P1luc09ΔATG. (d) MCF-7 cells were transfected with the indicated siRNA for 72 h before Hdm2 was determined by Western blotting.

Figure 5 Mithramycin A reduces the export of *hdm2* transcripts from the nucleus**.** (a) MCF-7 cells were cultured for 24 h in the presence of either DMSO carrier or 200 nM MA before RNA was extracted from whole cell pellets, nuclear, cytoplasmic or polyribosomal-associated fractions and levels of *hdm2* transcripts analysed by qPCR. Data are normalised to *gapdh*. Solid bars, DMSO-treated; Open bars, MA-treated. Error bars are S.D. of duplicate qPCR assays. Cellular fractionation was validated using semi-quantitative PCR for *snRNA U6* and *gapdh*. Two PCRs for each fraction are shown, with a 3-fold difference in the amount of input cDNA to confirm PCRs

have not plateaued. (b) T47D cells were treated and analysed as in Fig 5a. (c) T47D cells were cultured in the presence of either DMSO carrier or 200 nM MA for 24 h before the expression of the indicated proteins was determined. p =phosphorylated at Thr-202/Tyr-204.

a

0 25 50 100 150 200 nM MA

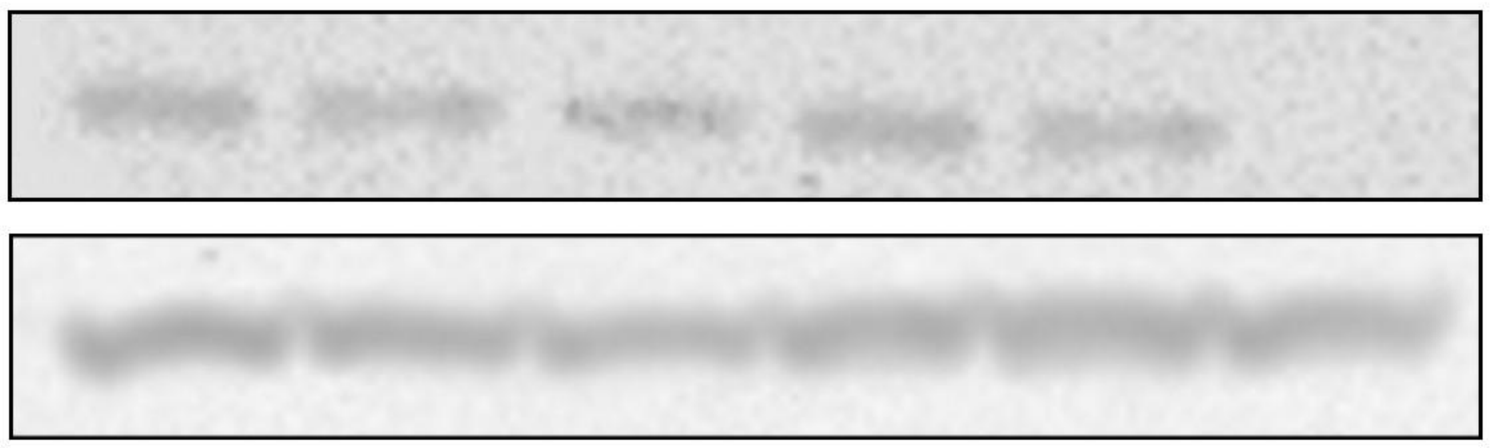


Hdm2

β-actin

b

0 1 2 4 8 24 h MA

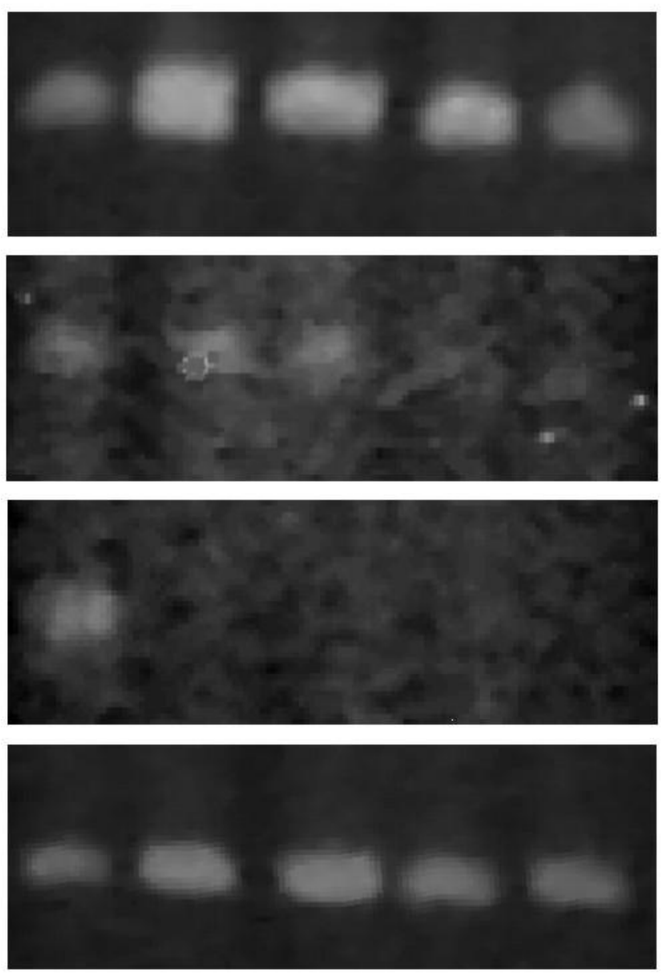


Hdm2

β-actin

c

0 3 7 18 24 h act. D



hdm2-P1

hdm2-P2

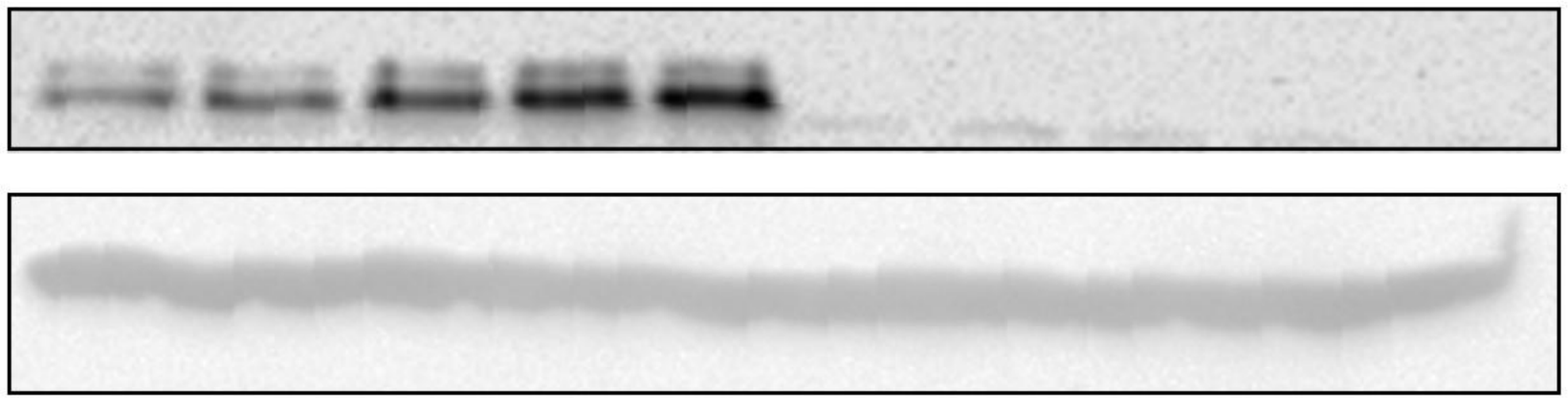
c-myc

β-*actin*

d

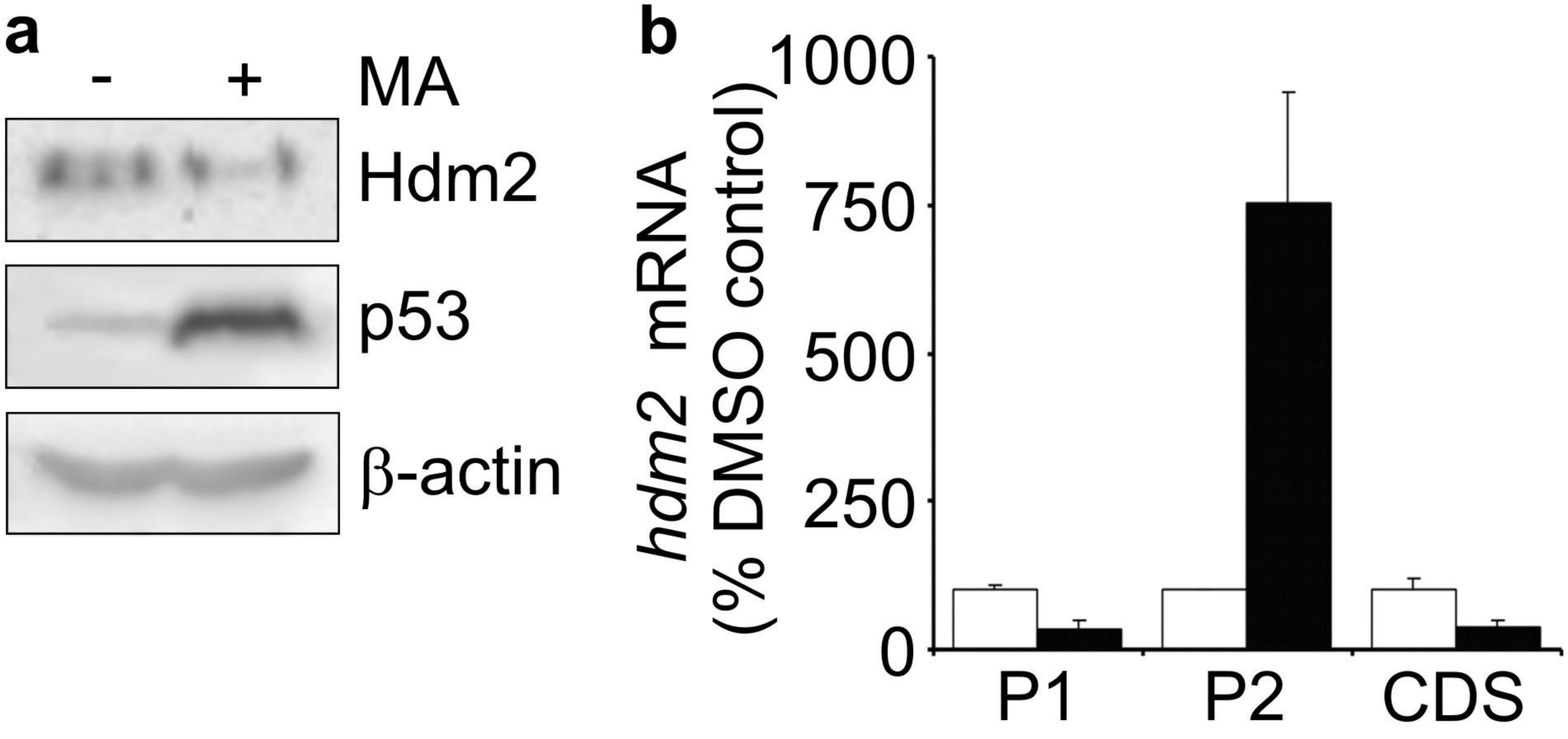
DMSO Mithramycin A

0 15 30 45 60 0 15 30 45 60 min MG132

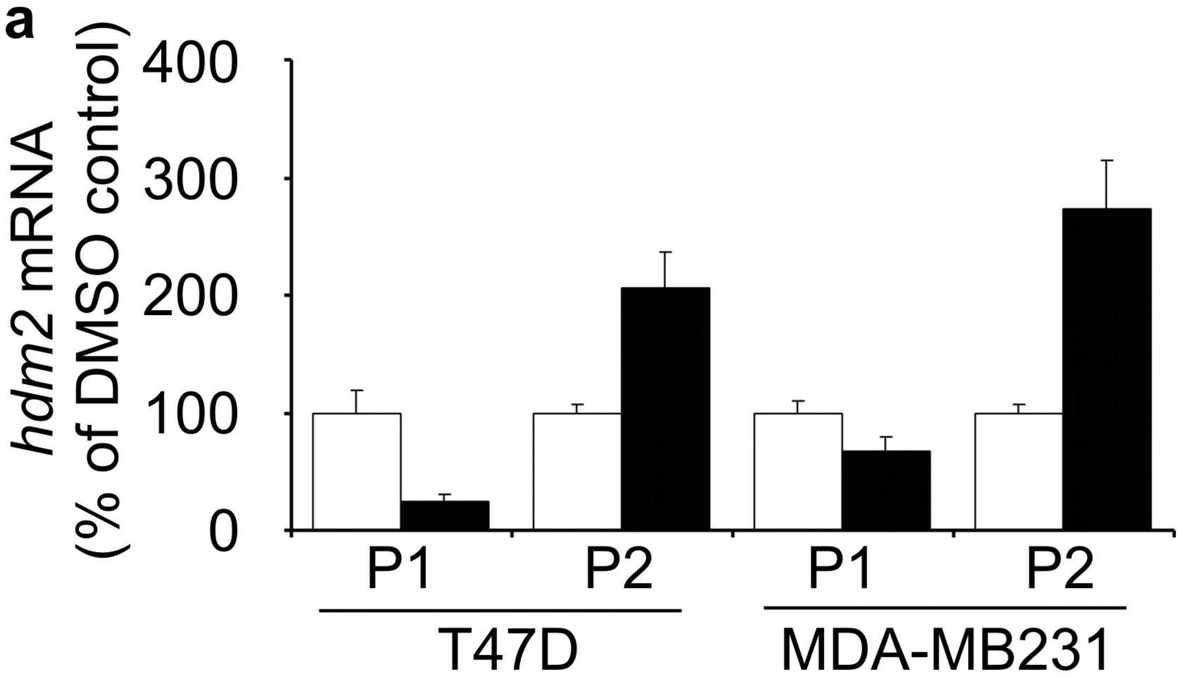


Hdm2

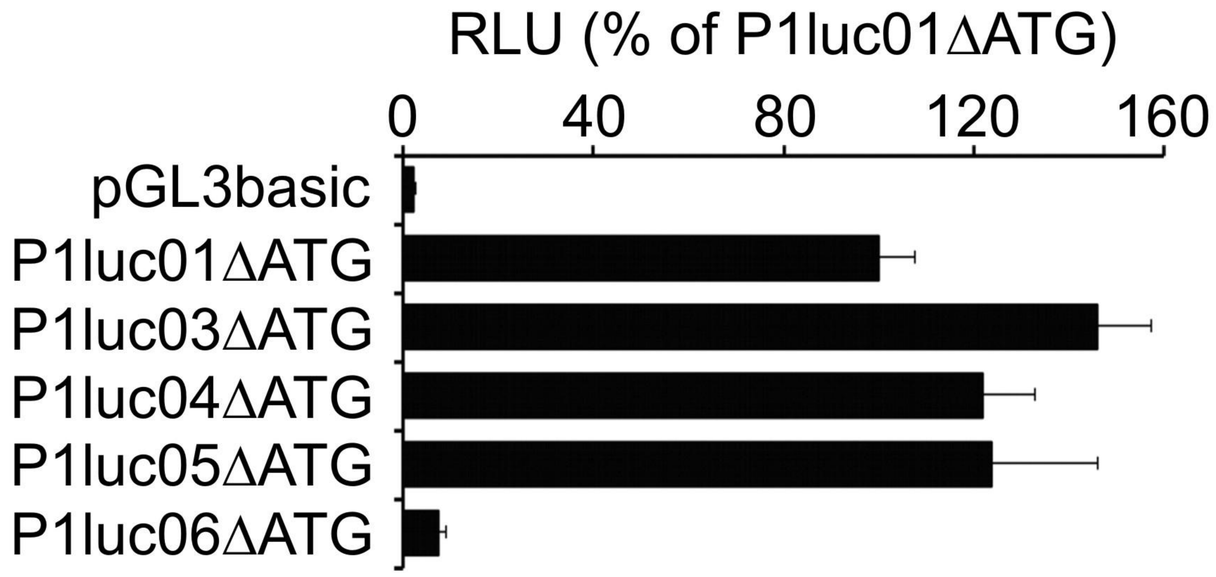
β-actin


a
-
+
MA
Hdm2
p53
β-actin
b
hdm2 mRNA
(% DMSO control)
1000
750
500
250
0
P1
P2
CDS

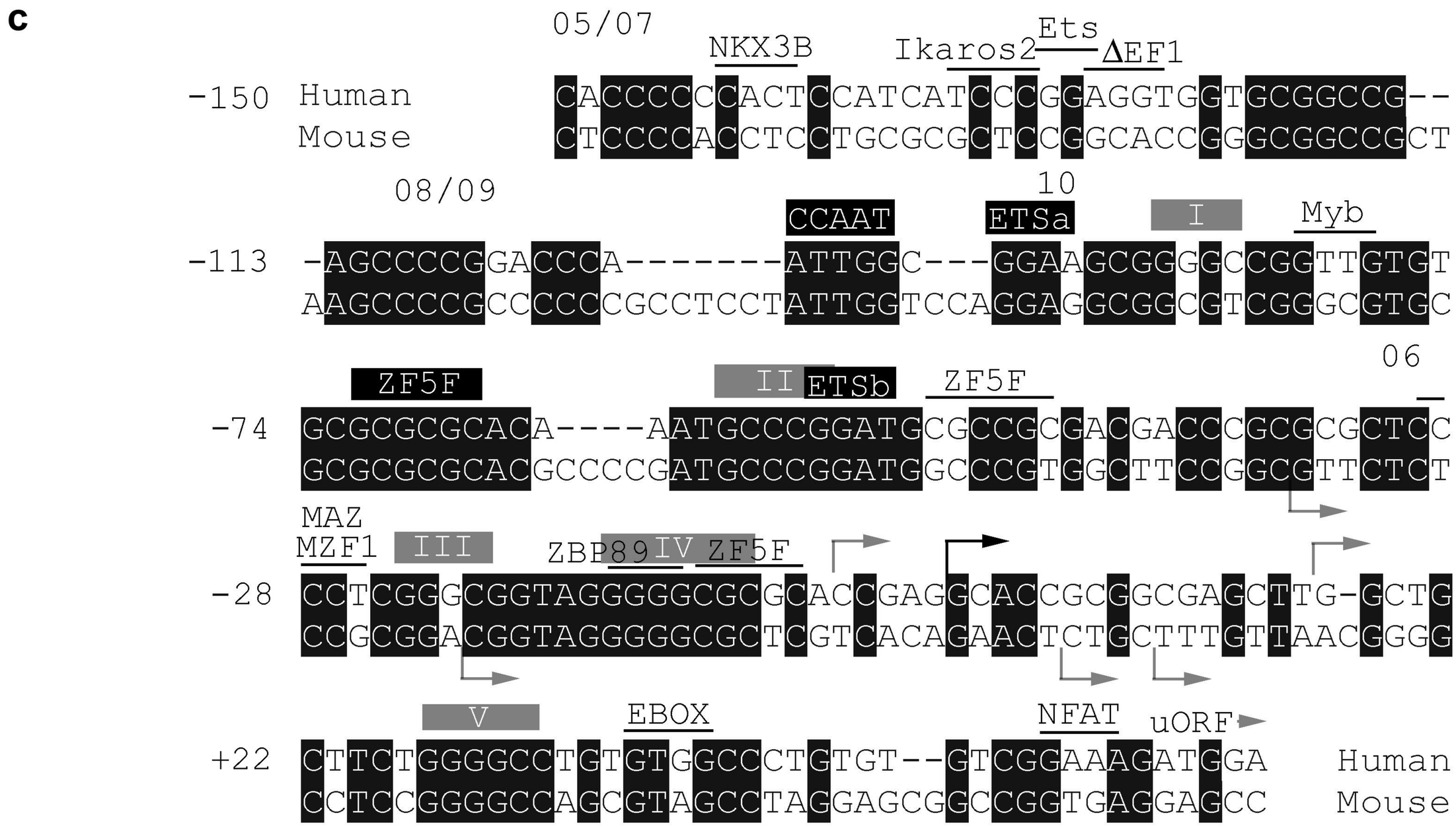
a


hdm2 mRNA
(% of DMSO control)
400
300
200
100
0
P1
P2
P1
P2
T47D
MDA-MB231

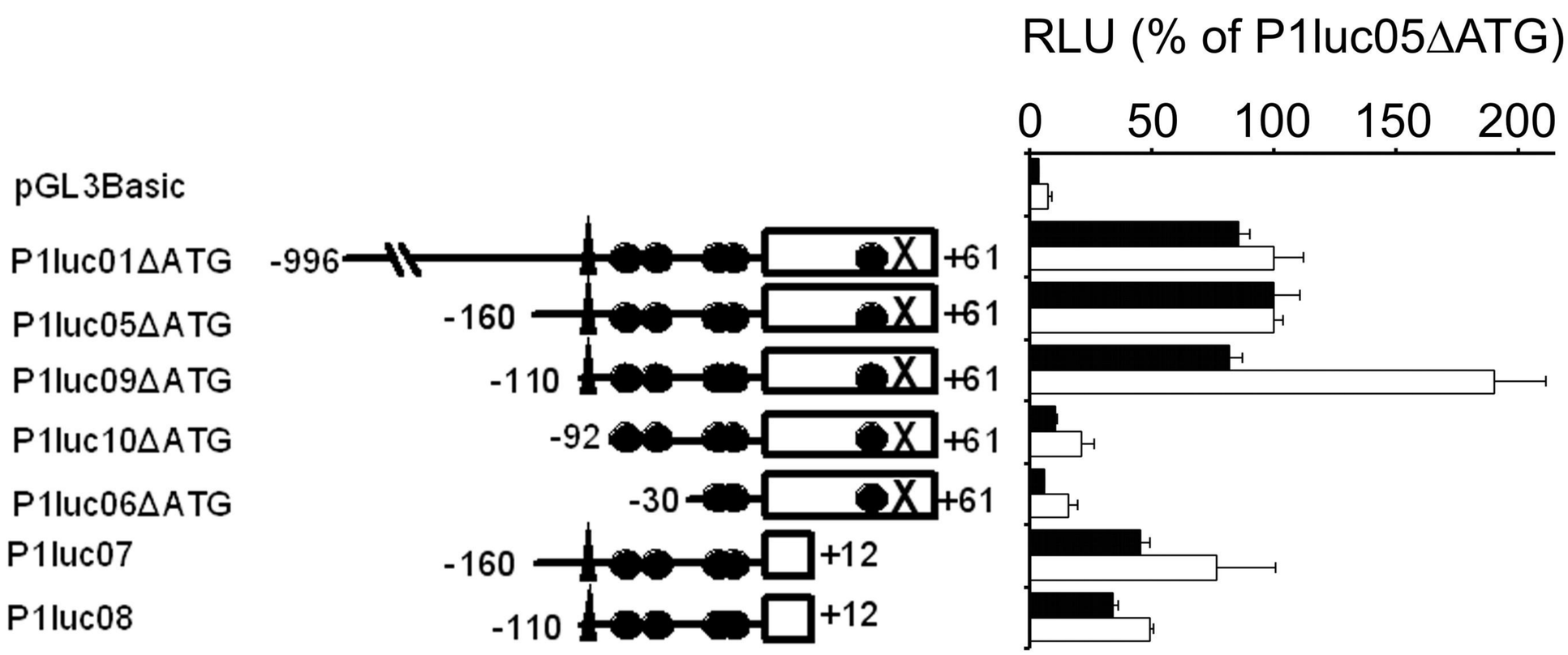
b


RLU (% of P1luc01ΔATG)
0
40
80
120
160
pGL3basic
P1luc01ΔATG
P1luc03ΔATG
P1luc04ΔATG
P1luc05ΔATG
P1luc06ΔATG

c


05/07
NKX3B
Ikaros2
Ets
ΔEF1
-150 Human CACCCCCCACTCCATCATCCCGGAGGTGGTGCGGCCG--
Mouse CTCCCCACCTCCTGCGCGCTCCGGCACCGGGCGGCCGCT
08/09
10
CCAAT
ETSa
I
Myb
-113 -AGCCCCGGACCCA-------ATTGGC---GGAAGCGGGGCCGGTTGTGT
AAGCCCCGCCCCCGCCTCCTATTGGTCCAGGAGGCGGCGTCGGGCGTGC
06
ZF5F
II
ETSb
ZF5F
-74 GCGCGCGCACA-----AATGCCCGGATGCGCCGCGACGACCCGCGCGCTCC
GCGCGCGCACGCCCCGATGCCCGGATGGCCCGTGGCTTCCGGCGTTCTCT
MAZ
MZF1
III
ZBP89
IV
ZF5F
-28 CCTCGGGCGGTAGGGGGCGCGCACCGAGGCACCGCGGCGAGCTTG-GCTG
CCGCGGACGGTAGGGGGCGCTCGTCACAGAACTCTGCTTTGTTAACGGGG
V
EBOX
NFAT
uORF
+22 CTTCTGGGGCCTGTGTGGCCCTGTGT--GTCGGAAAGATGGA Human
CCTCCGGGGCCAGCGTAGCCTAGGAGCGGCCGGTGAGGAGCC Mouse

d


RLU (% of P1luc05ΔATG)
0
50
100
150
200
pGL3Basic
P1luc01ΔATG
-996
+61
P1luc05ΔATG
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+61
P1luc09ΔATG
-110
+61
P1luc10ΔATG
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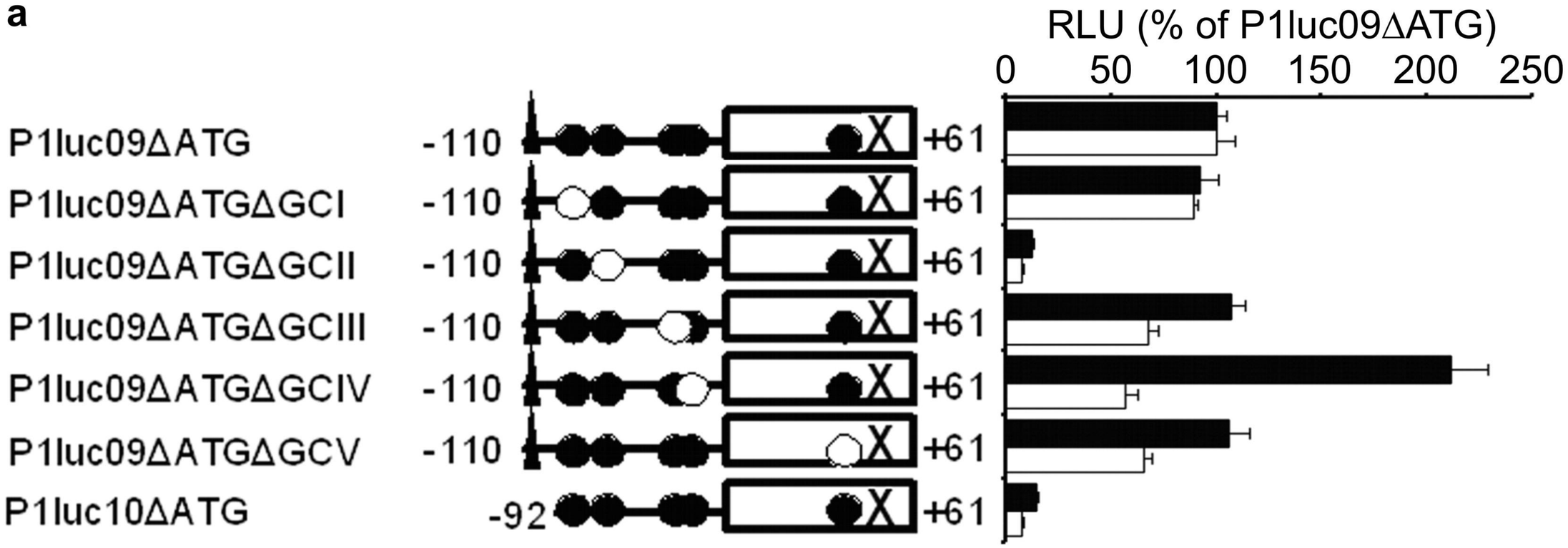
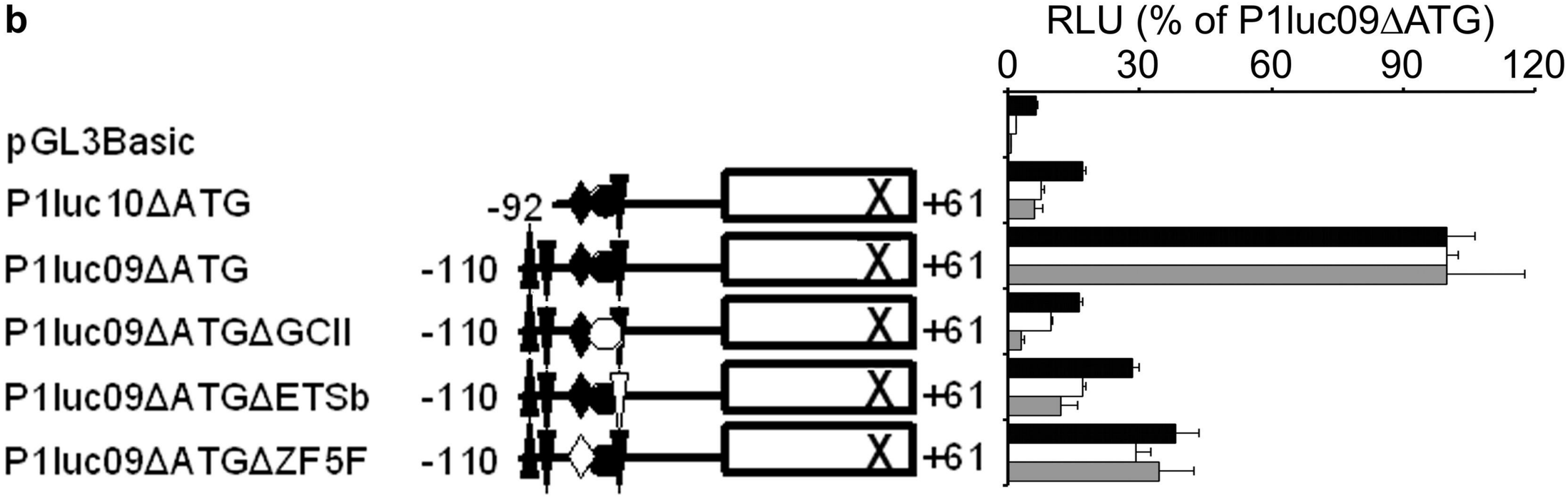
-160
+12
P1luc08
-110
+12

a


RLU (% of P1luc09ΔATG)
0
50
100
150
200
250
P1luc09ΔATG
P1luc09ΔATGΔGCI
P1luc09ΔATGΔGCII
P1luc09ΔATGΔGCIII
P1luc09ΔATGΔGCIV
P1luc09ΔATGΔGCV
P1luc10ΔATG
-110
-92
+61

b


RLU (% of P1luc09ΔATG)
0
30
60
90
120
pGL3Basic
P1luc10ΔATG
P1luc09ΔATG
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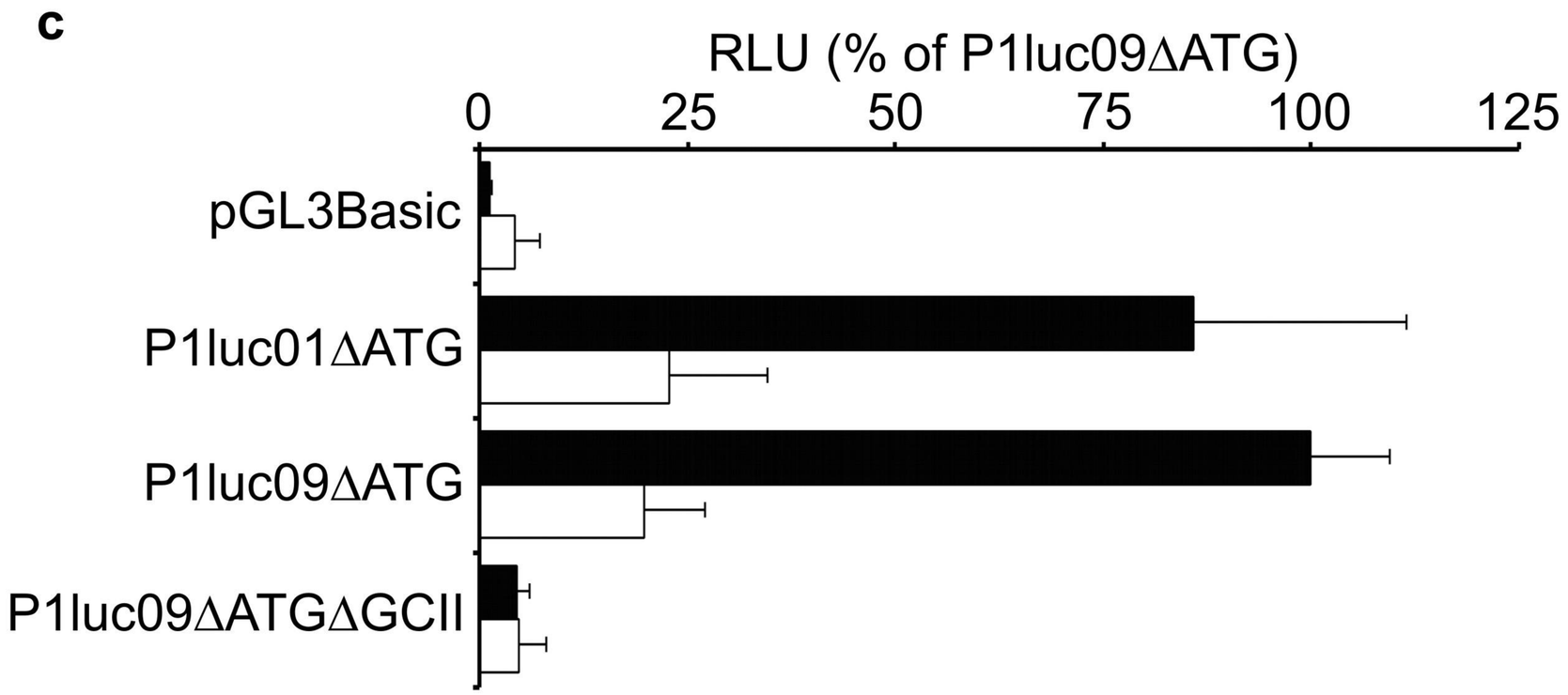
P1luc09ΔATGΔETSb
P1luc09ΔATGΔZF5F
-92
-110
+61

c


RLU (% of P1luc09ΔATG)
0
25
50
75
100
125
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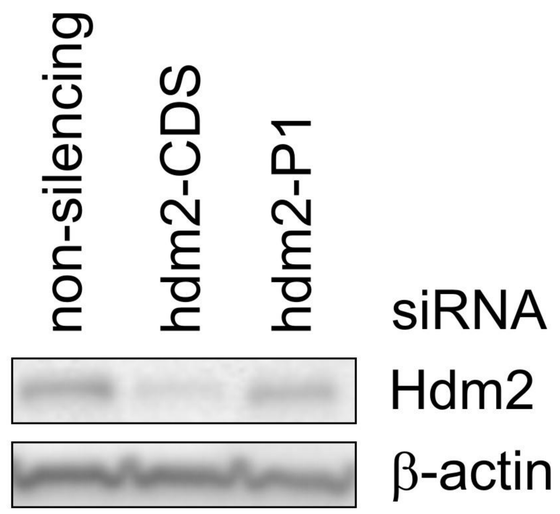
P1luc01ΔATG
P1luc09ΔATG
P1luc09ΔATGΔGCII

d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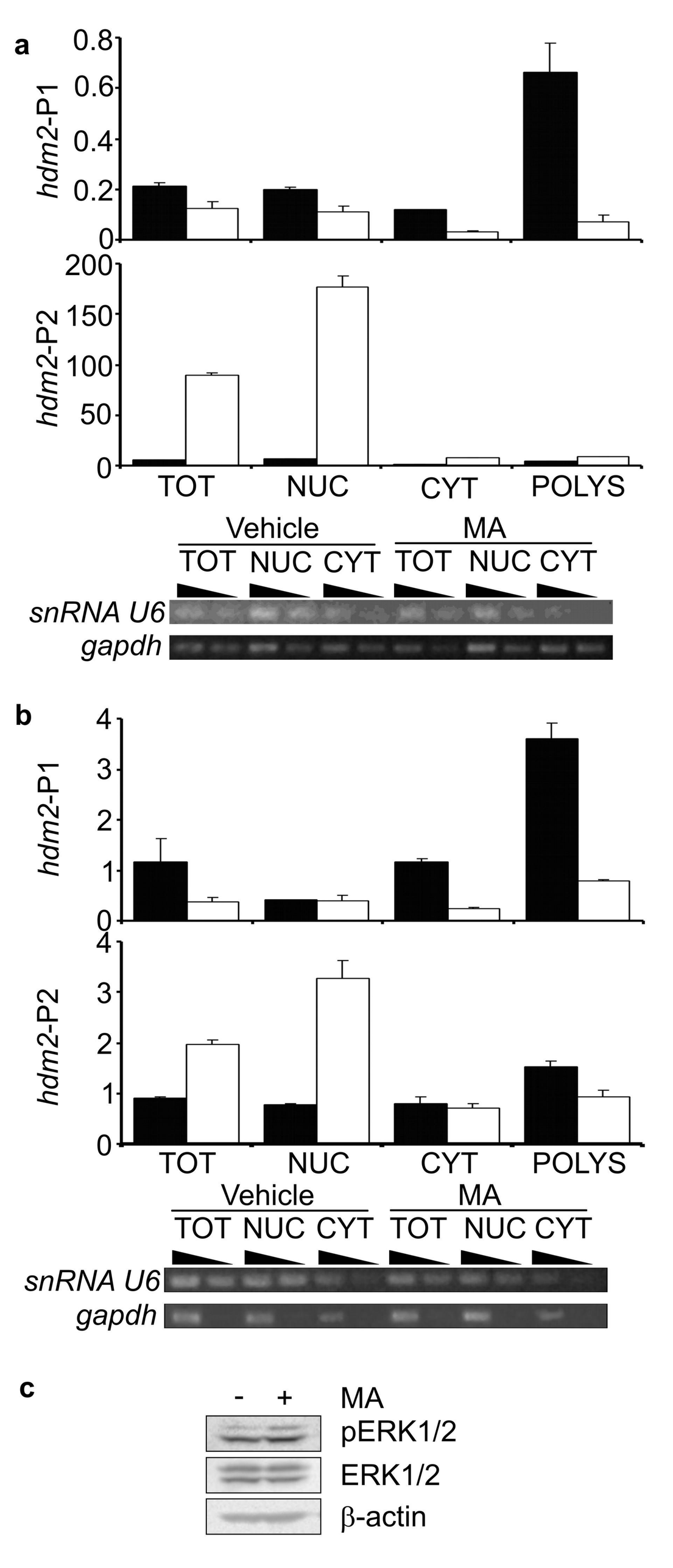
siRNA
Hdm2
β-actin


a
hdm2-P1
0.8
0.6
0.4
0.2
0
hdm2-P2
200
150
100
50
0
TOT
NUC
CYT
POLYS
Vehicle
MA
TOT NUC CYT TOT NUC CYT
snRNA U6
gapdh
b
hdm2-P1
4
3
2
1
0
hdm2-P2
4
3
2
1
0
TOT
NUC
CYT
POLYS
Vehicle
MA
TOT NUC CYT TOT NUC CYT
snRNA U6
gapdh
c
- +
MA
pERK1/2
ERK1/2
β-actin