MNK1 AND EIF4E ARE DOWNSTREAM EFFECTORS OF MEKs IN THE REGULATION OF THE NUCLEAR EXPORT OF *HDM2* mRNA

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Key words: p53, MDM2, mRNA export, breast cancer, eIF4E

Running title: Regulation of HDM2 by eIF4E

ABSTRACT

Regulation of the synthesis, function and degradation of HDM2 (Mdm2 in mouse) plays a key role in controlling the abundance and activity of the transcription factor p53, with consequent implications for the proliferation and survival of normal and cancer cells. We have previously identified the regulation of export of *HDM2* mRNA from the nucleus as a novel point of control of HDM2 synthesis. This process is dependent on activity of the growth-factor regulated MEK kinases. Here we provide evidence that the eIF4E kinase, MNK1 is a key downstream effector of MEKs in this regulatory pathway. We show that *HDM2* mRNA export in breast cancer cells is promoted by over-expressed eIF4E in a MEK- and MNK1- dependent manner, and inhibition of MNK1 suppresses endogenous *HDM2* mRNA export pathways. This MNK1- and eIF4E- dependent *HDM2* regulation occurs through sequences in the 3'UTR of *HDM2* mRNA, and consequently *HDM2* mRNA transcripts from both the constitutive P1 and inducible P2 promoters are regulated by this pathway. eIF4E is a known oncogene that is over-expressed in human tumours, including the majority of breast cancers. This pathway, therefore, may play an important role in the dysregulation of HDM2 oncoprotein expression that occurs in many human tumours.

HDM2 is a key regulator of p53 function in the cell (Brooks and Gu, 2006; Vogelstein *et al.*, 2000). Domains within the N-terminus and central acidic region of HDM2 interact directly with p53 to inhibit the interaction of p53 with transcriptional co-activators, and target it for ubiquitination, export from the nucleus, and degradation by the proteasome (Brooks and Gu, 2006; Wallace *et al.*, 2006). Experimental disruption of the p53-HDM2 interaction can be sufficient to induce the activation of p53-dependent cell cycle arrest and apoptotic pathways (Blaydes et al., 1997; Vassilev et al., 2004). Multiple stress-response pathways induce p53 activity in cells by regulating HDM2 and the HDM2-p53 interaction (Vogelstein *et al.*, 2000; Wahl and Carr, 2001). The rate of HDM2 protein synthesis is critical in determining p53 function in both normal stress responses and during tumorigenesis; increased HDM2 expression due to *HDM2* gene amplification is associated with some cancers such as osteosarcoma (Oliner et al., 1992), and a single nucleotide polymorphism in the *HDM2* promoter can result in increased HDM2 protein levels and increased susceptibility to tumorigenesis (Bond and Levine, 2007). Whilst *HDM2* gene amplification is uncommon in most cancers, HDM2 protein levels are frequently increased in many common malignancies including breast, prostate, lung, colon and melanoma (Onel and Cordon-Cardo, 2004). We have therefore studied how the expression of the human *HDM2* gene is regulated at the transcriptional and post-transcriptional level in normal and cancer cells (Phelps et al., 2003; Phelps et al., 2005; Phillips et al., 2006a; Phillips et al., 2006b).

HDM2 has two promoters, which generate transcripts with alternate 5'UTRs (exons 1 or 2), but a common coding region and 3'UTR (exons 3-12). The P1 promoter is constitutively expressed (Phillips et al., 2006a), whereas the P2-promoter is highly

inducible by p53 (Zauberman et al., 1995). Growth factor signalling through RAS-RAF-MEK-ERK can also regulate HDM2 protein synthesis, with readily identifiable consequences for cancer cell survival (Ries et al., 2000; Shaulian et al., 1997). This regulation occurs both through transcriptional regulation at the P2-promoter (Phelps et al., 2005; Ries et al., 2000) and, as we have discovered recently, control of the export of *HDM2* mRNA from the nucleus to its sites of translation in the cytoplasm (Phelps et al., 2005). Bulk export of polyadenylated mRNAs through nuclear pores occurs in metazoan cells through an evolutionarily pathway involving the TAP/NXF1/Mex67p and NXT1/Mtr2p transporter complex (Cullen, 2003; Erkmann and Kutay, 2004). However recent studies have demonstrated that certain mRNAs can be organised and exported from the nucleus as functionally related groups, or regulons, by RNA binding proteins (Culjkovic et al., 2007; Keene, 2003). Examples of such RNA-binding proteins in mammalian cells are HuR (Gallouzi and Steitz, 2001) and eIF4E (Culjkovic et al., 2006; von der Haar et al., 2004). This provides the potential for gene-selective control of mRNA export, through at present little is known regarding how such pathways may be regulated in response to extra-cellular signals. In order to better understand the mechanisms of MEK-dependent *HDM2* mRNA nuclear export, we have now delineated some of the molecular pathways downstream of MEK which are involved in this process.

A recent report identified *HDM2* as one of a number of genes that are regulated at the level of mRNA export by eIF4E (Culjkovic et al., 2006). As the ability of eIF4E to regulate mRNA export is known to be regulated by its state of phosphorylation (Topisirovic et al., 2004), we set out to investigate whether it might be involved in the MEK-dependent *HDM2* mRNA export we had previously identified in breast cancer

cells (Phelps et al., 2005). Initial characterisation of a panel of breast cancer cell lines (data not shown) determined that the T47D line had an approximately 2 fold greater ratio of cytoplasmic to total *HDM2* mRNA than most cell lines, including MCF-7, and therefore we chose to use MCF-7 for experiments aimed at promoting eIF4E-dependent mRNA export, and T47D for studies of the inhibition of endogenous *HDM2* mRNA export pathways. We first established that transient over-expression of eIF4E in MCF-7 resulted in an increase in Cyclin D1 (Figure 1a), a protein whose mRNA is well-characterised as a target for eIF4E-dependent nuclear export (Culjkovic et al., 2005; Rousseau et al., 1996)). The abundance of HDM2 protein also clearly increased with increasing eIF4E. We then performed an analysis of the subcellular distribution of mRNAs in control- and eIF4E- over-expressing MCF-7 cells to establish whether eIF4E can promote the nuclear export of *HDM2* mRNA in these cells (Figure 1b). When eIF4E was over-expressed there were no significant changes in the overall abundance of *CYCLIN D1* or *HDM2* transcripts in total cell extracts, however eIF4E caused increases in the cytoplasmic pools of these mRNAs; for *HDM2*-P1 and –P2 transcripts these increases were to 174.4 ± 22.3% and 180.8 ± 3.6% of controls respectively. A reduction in the nuclear fraction was also observed, though this was rather more modest. The difference in the relative changes in transcript levels between nuclear and cytoplasmic fractions is most likely because a high proportion of the *HDM2* transcripts are present in the nucleus; in control cells the relative abundance of *HDM2-P1* and *-P2* transcripts in the cytoplasm compared to the nucleus was only 15.6 ± 5.4% and 7.4 ± 0.4% respectively.

We then examined the role of kinases involved in the phosphorylation of eIF4E in the regulation of nuclear export of *HDM2* mRNA. MEK1 and MEK2 kinases

phosphorylate and activate ERK1 and ERK2, which in turn phosphorylate MNK1, an eIF4E serine 209 kinase (Mamane et al., 2004; Waskiewicz et al., 1997). This is a key post-translational modification of eIF4E that can enhance its ability to promote mRNA transport (Topisirovic et al., 2004). T47D cells, as well as having a higher cytoplasmic to total *HDM2* mRNA ratio than MCF-7, also exhibited a higher level (169.0 ± 22.6 %, n=4) of constitutive phosphorylation of eIF4E on serine 209 (Figure 2a), and were therefore used to examine the effect of pharmacological inhibition of kinase activity on eIF4E phosphorylation and the subcellular localisation of *HDM2* mRNA. Inhibition of MEKs with U0126 resulted in decreased eIF4E phosphorylation and a reduction in the abundance of HDM2 and Cyclin D1 proteins (Figure 2b). We have already demonstrated that this reduction in HDM2 involves both an inhibition of *HDM2*-P2 promoter activity, and reduced nuclear export of both *HDM2* mRNA transcripts (Phelps et al., 2005). ERK1 and ERK2, key enzymes immediately downstream of MEKs in the signalling cascade, phosphorylate multiple substrates including MNK1. To examine whether MNK1 activity is required for optimal HDM2 synthesis, we treated cells with the MNK inhibitor 4-amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidine (CGP57380) (Knauf et al., 2001; Topisirovic et al., 2004) (Figure 2b). Results were similar to those obtained following inhibition of MEKs, the inhibitor causing reduced eIF4E phosphorylation at serine 209, and a decrease in HDM2 and Cyclin D1 protein abundance. CGP57380 has recently been shown to inhibit MNK2 as well as MNK1, however MNK2 activity is MEK-independent and, as U0126 was nearly as effective as CGP57380 at inhibiting eIF4E phosphorylation, it can be concluded that the effects of CGP57380 that we observe are likely to be primarily due to its activity towards MNK1 (Buxade et al., 2005). Figure 2c shows that inhibition of MNK1 also has similar effects on *HDM2* mRNA to those we have

previously reported for U1026 (Phelps et al., 2005). There was an overall reduction in expression of *HDM2*-P1 and –P2 transcripts in total cellular RNA (decrease to 53.3 ± 5.5% and 33.1 ± 4.5% of controls respectively), presumably due to inhibition of transcription through an as-yet undefined pathway, and a greater decrease when cytoplasmic mRNA was analysed (to 27.6 ± 3.1% and 15.5 ± 1.4% of controls respectively) indicating *HDM2* mRNA export was also inhibited. Nuclear *HDM2*-P1 and –P2 transcripts were reduced to a lesser extent than in the whole cell fraction (to 73.7 ± 3.4% and 54.3 ± 4.4% of controls respectively), indicating retention of the synthesised *HDM2* transcripts in the nucleus.

eIF4E-dependent mRNA export has been shown to be mediated by a structurally conserved 4E-sensitivity element present in the 3'UTR of transcripts (Culjkovic et al., 2005; Culjkovic et al., 2006). As such an element can be identified in the structure of the 3'UTR of *HDM2* (Culjkovic et al., 2006), we cloned the *HDM2* 3'UTR (-15 to +2763 relative to the end of the *HDM2* coding sequence) 3' of the luciferase gene in the *HDM2*-P2 promoter reporter construct hdm2luc01 and examined its effects on luciferase expression in MCF-7 cells. We first assayed expression from hdm2luc01 or hdm2luc01-3'UTR, in the absence or presence of an eIF4E expression vector (Figure 3a). There was a clear increase (to 171.6 ± 15.3 % of control) in relative luciferase activity when eIF4E was over-expressed, only when the reporter vector contained the *HDM2* 3'UTR. This is strong evidence that the *HDM2*-3'UTR does contain a functional 4E response element which, in this assay, directs the eIF4E-dependent nuclear export of the chimeric mRNA, and consequently enhanced luciferase expression when active eIF4E is present. Note that, as well as promoting mRNA export, eIF4E also enhances the translation of a specific subset of mRNAs,

however the RNA elements involved in eIF4E-regulated initiation of translation are present in the 5'UTR (Mamane et al., 2007; von der Haar et al., 2004) and, combined with our previous finding that inhibition of MEKs has no transcript-selective effect on the association of *HDM2* mRNA with polyribosomes, it is unlikely that the effects of the 3'UTR are through control of translation.

We then examined the effect of inhibitors of eIF4E phosphorylation on the activity of the reporter gene constructs. As shown in Figure 3bi, whilst U0126 inhibited *HDM2*-P2 promoter activity, as we have shown previously (Phelps et al., 2005), in the absence of the *HDM2* 3'UTR eIF4E over-expression did not confer any additional sensitivity to inhibition of MEKs or MNK1. In contrast, the eIF4E-induced increase in activity of the hdm2luc01 3'UTR vector was ablated by both kinase inhibitors (Figure 3bii). Therefore eIF4E-dependent export of *HDM2* mRNA to the cytoplasm is dependent upon the activity of both MEK and MNK1 kinases, consistent with the known role of phosphorylation of eIF4E in its regulation of mRNA export (Topisirovic et al., 2004).

Activation of growth factor-dependent signalling cascades results in the up-regulation of p53-activating signalling pathways (Harvey et al., 1993). In the absence of sufficient activity of its negative-regulatory partner, HDM2, growth factor-stimulated cells will therefore cease proliferating due to the activation of p53-dependent cell cycle arrest or pro-apoptotic pathways (Blaydes and Wynford-Thomas, 1998). The co-ordinated increase in expression of HDM2 in response to the same stimuli is therefore critical in maintaining the dynamic equilibrium of the p53-HDM2 auto-regulatory feedback loop. We have previously identified a MEK-dependent mRNA

export pathway as an important component of this regulation in cancer cell lines (Phelps et al., 2005), and now identify MNK1 and eIF4E as downstream effectors of MEKs that promote *HDM2* mRNA export. Our finding that the 3'UTR of *HDM2* is sufficient to render it sensitive to this export pathway is consistent with the prior identification of eIF4E sensitivity elements that interact with eIF4E in the 3'UTRs of other mRNAs, notably *CYCLIN D1* (Culjkovic et al., 2005; Culjkovic et al., 2006), and also our finding that both *HDM2*-P1 and P2 mRNA transcripts, which differ in their 5'UTRs, but contain a common coding sequence and 3'UTR, can both be exported through a MEK-dependent pathway. Relatively minor perturbations in the normal regulation of HDM2 protein synthesis is sufficient to promote tumour development (Bond et al., 2004). eIF4E is over-expressed in tumours including a majority of breast carcinomas, and is a known oncogene (Mamane et al., 2004). Its oncogenic properties are believed to be attributable, at least in part, to its role in mRNA-selective nuclear export pathways (Topisirovic et al., 2003; Topisirovic et al., 2004). It seems highly probable that the ability of eIF4E to promote the production of the p53 antagonist, HDM2, will be an important contributor to the abnormal proliferation of these tumour cells.

ACKNOWLEDGEMENTS

Supported by a grant (#04-422) from the Association for International Cancer Research. We are grateful to Prof. Katherine Borden for the sharing of data prior to its publication, N. Sonenberg for making available the eIF4E expression vector, and Dr. Monika Phelps for cloning of the *HDM2* 3'UTR.

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LEGENDS TO FIGURES

Figure 1. Over-expression of eIF4E increases nuclear export of HDM2 transcripts. **(a)** MCF-7 cells in 6-well plates were transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) with 0 to 2 μg of eIF4E expression vector pMV7-4E. DNA in all transfections was kept constant using empty vector pMV7. 48 h post-transfection, western blotting was performed using the following antibodies; HDM2 (monoclonal antibody 2A9 (Chen et al., 1993)), Cyclin D1 (Calbiochem, La Jolla, CA, USA), eIF4E (BD Biosciences, Oxford, UK), rabbit anti β–actin antibody (Sigma Aldrich Co., Poole, UK). **(b)** MCF-7 cells in 100 mm plates were transfected with 18 μg pMV7 or pMV7-4E 48 h before cellular fractionation by hypotonic lysis (Phillips et al., 2006a). Following RNA extraction using RNABee (Biogenesis Inc.), cDNA was synthesised using Superscript II reverse transcriptase (Invitrogen) and Taqman quantitative PCR analysis was performed using Taqman Universal PCR Mastermix (Applied Biosystems). *CYCLIN D1* transcripts were detected using a pre-designed

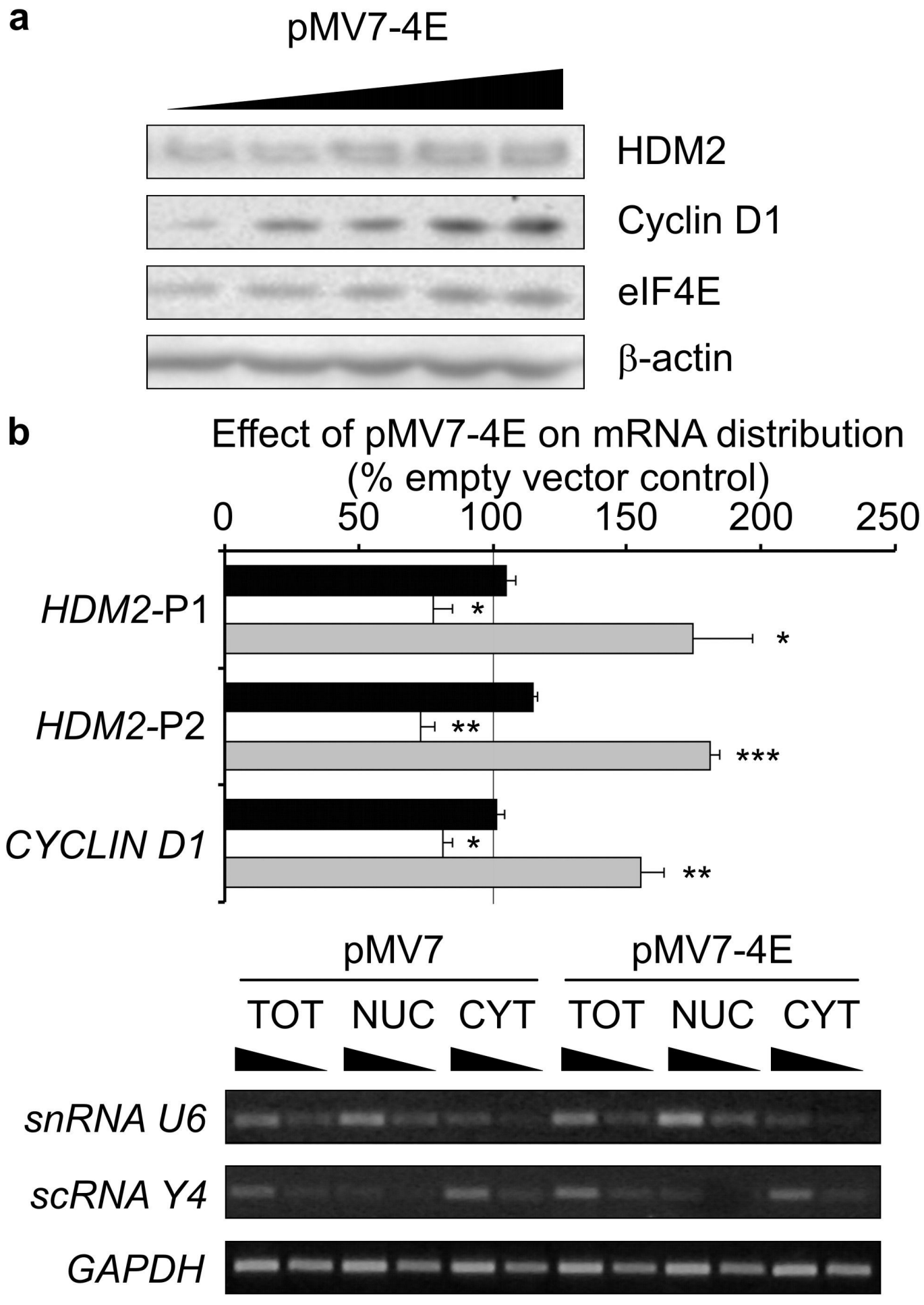
Taqman gene expression assay (Applied Biosystems) and *HDM2* transcripts were determined as described previously (Phelps et al., 2005). Data are normalised to *GAPDH*. Results are expressed as percentage of transcripts in pMV7-transfected fraction. Black bars, total cell; white bars, nuclear fraction; grey bars, cytoplasmic fraction. Data is representative of three independent experiments. Error bars are S.E.M. of triplicate PCR assays. Asterisks indicate the significance of the change in transcript abundance in the subcellular fraction, compared to the total cell fraction (*$p<0.05$, **$p<0.01$, ***$p<0.001$. Unpaired, two-tailed Students t test). Compared to pMV7-transfected controls, *GAPDH* levels in pMV7-4E transfected cells were 118.8 ± 6.57, 80.8 ± 6.88 and 76.0 ± 7.28% in total, nuclear and cytoplasmic fractions respectively. Cellular fractionation was validated using semi-quantitative RT-PCR for *snRNA U6*, *scRNA Y4* and *GAPDH* as described previously (Phelps et al., 2005; Phillips et al., 2006a). Two PCRs for each fraction are shown, with a threefold difference in the amount of input cDNA to confirm PCRs have not plateaued.

Figure 2. Inhibitors of eIF4E phosphorylation reduce HDM2 protein expression and inhibit nuclear export of *HDM2* transcripts. **(a)** MCF-7 and T47D cell lysates were analysed by Western blotting. Phospho-Ser-209 eIF4E antibody was purchased from Cell Signaling Technology, Beverly, MA, USA. Data is representative of four independent experiments. **(b)** T47D cells were cultured for 24 h in the presence of 0.1% DMSO vehicle control, 25 μM U0126 (Promega) or 20 μM MNK inhibitor (Calbiochem) before being analysed for expression of the indicated proteins. Quantification of blots from four independent experiments by densitometry demonstrates that, relative to controls, U0126 and the MNK inhibitor reduce phosphorylated eIF4E by 70.6 ± 2.6% and 92.8 ± 4.2% respectively, and HDM2 by

69.6 ± 4.0% and 66.6 ± 7.9%. **(c)** T47D cells were cultured for 24 h in the presence of 0.1% DMSO or 20 μM MNK1 inhibitor before cellular fractionation by hypotonic lysis. Transcript levels were determined by qPCR and presented as in Figure 1b. Results are expressed as percentage of transcripts in DMSO-treated fraction. Black bars, total cell; white bars, nuclear fraction; grey bars, cytoplasmic fraction. Data is representative of three independent experiments. Error bars are S.E.M. of triplicate qPCR assays. Compared to DMSO-treated controls *GAPDH* levels in MNK inhibitor-treated cells were 68.9 ± 8.09, 82.4 ± 4.60 and 80.0 ± 4.10% in total, nuclear and cytoplasmic fractions respectively. Cellular fractionation was validated by PCR for *snRNA U6*, *scRNA Y4* as in Figure 1b.

Figure 3. eIF4E-dependent *HDM2* transport requires the *HDM2* 3'-UTR. **(a)** MCF-7 cells in 96-well plates were transfected with 120 ng hdm2luc01 (Phelps et al., 2003) or hdm2luc01-3'UTR (*HDM2* 3'UTR cloned by 3'RACE and ligated into *Xba1* site 3' of the luciferase gene in hdm2luc01) and 80 ng pMV7 (solid bars) or pMV7-4E (open bars) 48 h before reporter gene activity was assayed and normalised to expression from a co-transfection *Renilla* luciferase expression plasmid. Results are expressed as a percentage of activity in hdm2luc01- and pMV7-transfected cells (mean ± S.E.M.). Data is pooled from three independent experiments containing duplicate transfections each (n=6). **(b)** MCF-7 cells in 96-well plates were transfected with 120 ng hdm2luc01 **(i)** or hdm2luc01-3'UTR **(ii)** and 80 ng pMV7 (solid bars) or pMV7-4E (open bars), and 24 h later exposed to 0.1 % DMSO, 25 μM U0126 or 20 μM MNK1 inhibitor for a further 24 h before reporter gene activity was assayed. Results are expressed as percentage of activity in DMSO-treated pMV7-transfected

cells (mean ± S.E.M.). Data is pooled from two independent experiments containing duplicate transfections each (n=4).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Effect of pMV7-4E on mRNA distribution
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HDM2-P1
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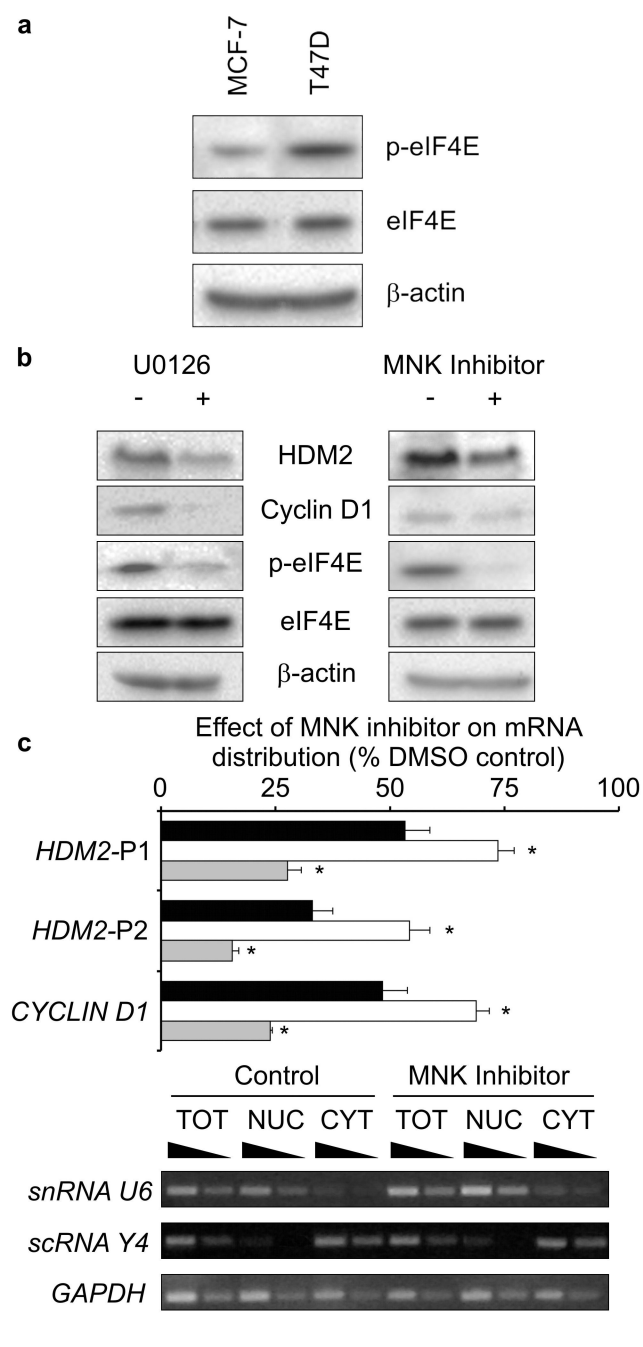
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Effect of MNK inhibitor on mRNA distribution (% DMSO control)
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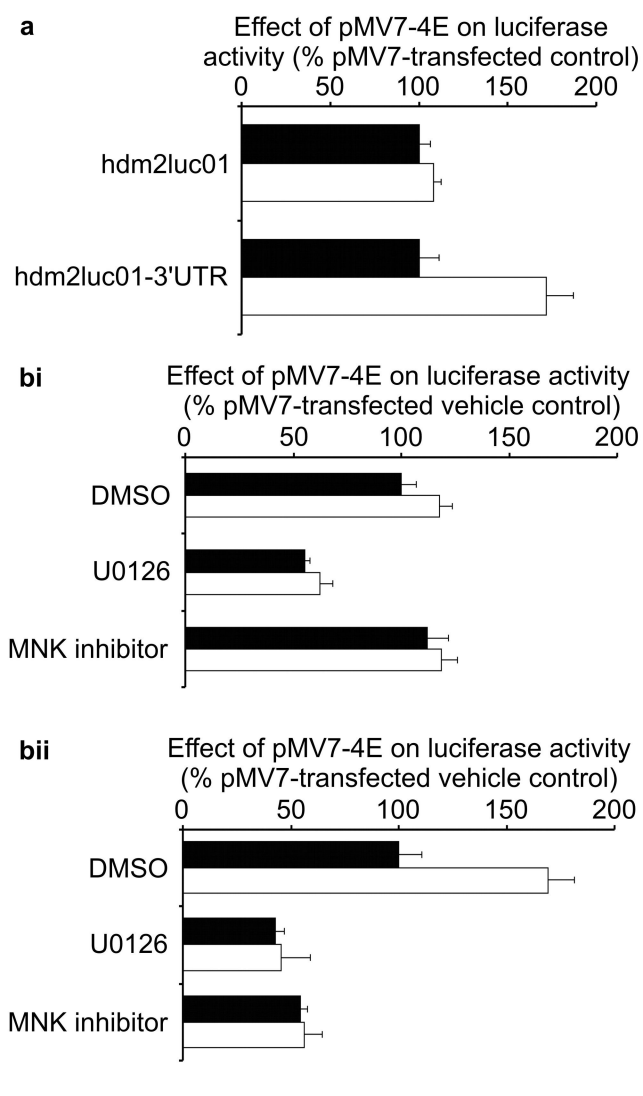
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Effect of pMV7-4E on luciferase activity (% pMV7-transfected vehicle control)
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MNK inhibitor
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Effect of pMV7-4E on luciferase activity (% pMV7-transfected vehicle control)
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