MEK-ERK signalling controls Hdm2 oncoprotein expression by regulating *hdm2* mRNA export to the cytoplasm*

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Running Title: *Regulation of Hdm2 expression*

*Supported by grants to JPB from the Association for International Cancer Research (#01-070) and (#04-422)

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[1]The abbreviations used are: DMSO, dimethyl sulphoxide; 5-FU, 5-fluorouracil; PBS, phosphate-buffered saline; DTT, dithiothreitol; RPA, ribonuclease protection assay; qPCR, Taqman quantitative polymerase chain reaction; CDS, coding sequence; SFM, serum free medium; RLU, relative luciferase units; ERα, oestrogen receptor-α.

SUMMARY

The physical and functional interaction between the transcription factor p53 and its negative regulatory partner protein Hdm2 (Mdm2 in mouse) is a key point of convergence of multiple signalling pathways that regulate cell proliferation and survival. *Hdm2* mRNA transcription is induced by p53, forming the basis of an auto-regulatory feedback loop. Growth and survival factor-activated Ras-Raf-MEK-ERK signalling can also regulate Hdm2 expression independently of p53, contributing to the pro-survival effect of these factors. In murine fibroblasts, this occurs through the regulation of *mdm2* mRNA transcription. Here we show that, in human breast cancer epithelial cells, MEK-dependent regulation of Hdm2 expression also occurs at a post-transcriptional level. Pharmacological blockade of MEK activity in T47D cells inhibits Hdm2 protein synthesis by 80-90%. This occurs in the absence of changes in the expression of the major *hdm2*-P1 mRNA transcript, and only an approximately 40% reduction in *hdm2*-P2 transcript levels. The amounts of both transcripts that are associated with polyribosomes, and are hence being actively translated, are reduced by >80% by the MEK inhibitor, U0126. We show here that this is due to the inhibition of *hdm2* mRNA export from the nucleus when MEK activity is inhibited. In MCF-7 breast cancer cells that express wild-type p53, Hdm2 is required to suppress p53-dependent transcription when MEK kinase is active. Regulation of the nuclear export of *hdm2* mRNA provides, therefore a mechanism whereby mitogen stimulated cells avoid p53-dependent cell cycle arrest or apoptosis by maintaining the dynamic equilibrium of the Hdm2-p53 feedback loop.

INTRODUCTION

The tumour-suppressor p53 and oncoprotein Hdm2 function within an auto-regulatory feedback loop that is a point of convergence of signalling pathways that regulate cellular proliferation and survival (1). p53 primarily functions as an activating transcription factor, and key p53 target genes include the cyclin-dependent kinase inhibitor *WAF1* (2), *PUMA*, which encodes a BH3-domain containing pro-apoptotic molecule (3,4), and *hdm2* (5). Cellular p53 activity is inhibited in proliferating cells and is activated under situations such as cellular stress, as part of a growth arrest or apoptosis response. Hdm2 functions principally as the primary negative regulator of p53 function (6) and its correct expression and function can be essential for the prevention of spontaneous p53-dependent apoptosis or cell cycle arrest (7-11). Mechanisms whereby Hdm2 down-regulates p53 function include concealing its activation domain from the transcriptional machinery (12), and targeting it for ubiquitination, nuclear export and proteosomal degradation (13).

Stress-induced activation of p53 almost invariably involves post-translational modifications to both p53 and Hdm2, which can inhibit Hdm2 function (14) or the Hdm2 - p53 interaction (15-17). Proliferative and pro-survival signalling pathways can also impinge upon p53 through either the positive or negative regulation of Hdm2 function. AKT kinase, a key enzyme in pro-survival signalling pathways, phosphorylates Hdm2, which results in elevated Hdm2 levels in the nucleus (18-20). The growth factor-induced Ras-Raf-MEK-ERK signalling pathway can have context dependent effects on proliferation and survival (21), and its regulation of the p53 – Hdm2 axis appears to be similarly complex. Signalling via this cascade can induce the expression of $p14^{ARF}$, an Hdm2 antagonist, resulting in p53 activation. This can be an important block to cancer progression, and $p14^{ARF}$ expression is lost in many tumours (22,23). In contrast, induction

of Hdm2 expression by the Ras-Raf-MEK-ERK can play an important role in the proliferative and pro-survival response to growth factors (24,25). Indeed, this increased expression of Hdm2 may dampen the p53 response to activating signals such as genotoxic cancer therapies, particularly in tumour cells in which $p14^{ARF}$ expression is lost (24,25). Conversely, transgenic mutations that result in as little as a 20% loss of Mdm2 expression in adult mice result in increased sensitivity to radiation (26).

Hdm2 is normally present at very low levels in cells, as the protein directs its own auto-ubiquitination and is rapidly degraded (27). Elevation of Hdm2 expression occurs at varying frequencies in diverse tumour types (28). In a proportion of cancers, this is a consequence of *hdm2* gene amplification (29), though in many cases, alternative mechanisms must underlie the increase (28). *Hdm2* expression is regulated by transcription from two promoters, P1 and P2 (5). Transcription from P1 is considered to be constitutive in most cells (30), whereas P2-promoter activity is highly induced by p53 (5). The murine *mdm2* P2-promoter is also induced by Ras-Raf-MEK-ERK signalling (25). Both *hdm2* transcripts include the full length coding sequence, but the P1 transcript contains a long 5' untranslated region with two upstream open reading frames, and is poorly translated (31). In addition to mRNA transcription, Hdm2 protein levels can also be controlled by mRNA translation (31,32) as well as protein turnover (33). Hdm2 expression is elevated in as many as 50% of breast carcinomas (34-36) and we have provided evidence for a role for p53-independent transcription from the P2-promoter in this increased expression (37). In this present study we have investigated the role of Ras-Raf-MEK-ERK signalling in controlling the levels of Hdm2 protein in breast cancer cells, and demonstrate a key role for this pathway in regulating the export of *hdm2* mRNA to the cytoplasm.

EXPERIMENTAL PROCEDURES

Culture of human breast cancer cell lines- MCF-7 and T47D breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% foetal calf serum (Autogen Bioclear) as described previously (37). The following reagents were dissolved in dimethyl sulphoxide (DMSO[1]) at the indicated concentrations before adding to the medium where stated; 10 mM U0126 (Promega), 20 mM PD98059 (Promega), 10 mM MG132 (Sigma). Cycloheximide (Sigma), and 5-fluorouracil (5-FU) (David Bull Laboratories) were in aqueous solutions. Nutlin-3 (Alexis Biochemicals) was dissolved in ethanol at 5 mM.

Protein analysis- cells were washed with phosphate-buffered saline (PBS), 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 (DTT), 0.05% Triton X-100, 25 mM NaCl, 20 mM HEPES pH 7.6) 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 (38), and membranes were probed for Hdm2 (monoclonal antibody 2A9 (39)), p53 (DO-1, Serotec), phospho Thr202/Tyr204 ERK 1 and ERK 2 (E10), total ERK 1 and ERK 2 (both from Cell Signalling Technology) or $p21^{WAF-1}$ (Clone SX118, Pharmingen). 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 (Biorad), and quantified using Quantity One software (Biorad).

For labelling of newly synthesised proteins, cells were washed twice with labelling medium (RPMI medium without methionine, cysteine or L-glutamine (Sigma), supplemented with dialysed and heat inactivated foetal calf serum), then incubated with labelling medium for 30 min before the addition of fresh labelling medium containing 0.25 mCi/ml TranS-label (Amersham Biosciences) and incubation at 37 °C for 90 min. Cells were then processed as for protein analysis. After the gel was stained using Coomassie blue, it was dried and exposed to a phosphor image plate for 5 h. Bands were visualised using a Personal Molecular Imager FX (Biorad).

RNA analysis- RNA extraction from cell pellets, and cell fractions, was performed using either RNABee (Biogenesis Inc.) or RNAeasy (Qiagen). Ribonuclease protection assay (RPA) for *hdm2* was performed as previously described (37). For Taqman quantitative polymerase chain reaction (qPCR) analysis of *hdm2* transcripts, 0.5 - 1 μg of RNA was used to generate cDNA, using Superscript II RNAse H^- reverse transcriptase (Invitrogen) and oligo dT primers, in a 20 μl volume. Oligonucleotide primer pairs and probes were designed using Primer Express, version 2.0 (Perkin Elmer Applied Biosystems), and were synthesised by MWG Biotech. Each primer sequence lies within a different exon, and the fluorogenic probe spans the junction between the two exons. *hdm2* coding sequence (CDS) primers amplify a region around the exon 9-10 boundary. Primers and probe for *gapdh*, which was used as an endogenous control gene, were purchased as a Pre-Developed TaqMan® Reagent Assay (Perkin Elmer Applied Biosystems). Analysis was performed using the ABI PRISM 7900HT Sequence Detection System instrument and software (Perkin Elmer Applied Biosystems). qPCR was performed in 20 μl reaction volumes containing 1X qPCR™ Mastermix (Eurogentec), cDNA (from 5 ng of RNA) and

one of the following sets of primers and probe: *hdm2*-CDS-F (TCTACAGGGACGCCATCGA) and *hdm2*-CDS-R (CTGATCCAACCAATCACCTGAA) (300 nM each) and *hdm2*-CDS probe (FAM-TTCACTTACACCAGCATCAAGATCCGGA–TAMRA) (100 nM); *hdm2*-P1-F (GACTCCAAGCGCGAAAACC) and *hdm2*-P1-R (CCATCAGTAGGTACAGACATGTTGGT) primers (900 nM each) and *hdm2*-P1 probe (FAM-TGCACATTTGCCTGCTCCTCACCAT-TAMRA) (200 nM); *hdm2*-P2-F (CGGACGCACGCCACTT) and *hdm2*-P2-R (CAGTAGGTACAGACATGTTGGTATTGC) primers (900 nM each) and *hdm2*-P2 probe (FAM-TTCTCTGCTGATCCAGGCAAATG-TAMRA) (200nM). Ct values were converted to relative transcript levels using a standard curve. Semi-quantitative RT-PCR was performed as described previously (37); primers used were: *gapdh*- RT oligo dT, forward GAAGGTGAAGGTCGGAGT, reverse GAAGATGGTGATGGGATTTC; *scRNA hY4*- RT AAAAAGCCAGTCAAATTTAGCA, forward GGTCCGATGGTAGTGGGTTA, reverse AAAGCCAGTCAAATTTAGCAGT.

Cellular fractionation prior to RNA extraction or polyribosome analysis was performed using hypotonic lysis (40). Cells were washed twice with serum free medium (SFM), then scraped in SFM and pelleted by centrifugation at 365 *g*. Pellets were resuspended in hypotonic lysis buffer B (10 mM Tris HCl pH 7.6, 1 mM CH_3COOK, 1.5 mM CH_3COOMg, 2 mM DTT, RNAse inhibitor 1 unit/µl) and cells were lysed on ice using a Dounce homogeniser. Lysates were centrifuged at 12,000 *g* to separate the cytoplasmic extract from the nuclear pellet. For some extractions, the lysis buffer was modified where indicated (B1, B2, B3).

For analysis of polyribosome associated mRNA (40), cycloheximide (10 μg/ml) was added to the SFM used to wash the dishes. Cytoplasmic lysates were then layered over a cushion of 30% (w/v) sucrose in buffer B and centrifuged at 130,000 *g* for 2.5 h at 4°C. After removing the supernatant, the remaining polyribosome-bound RNA pellet was rinsed twice in buffer B before RNA extraction. For fractionation over a sucrose gradient, 1200 μl cytoplasmic lysate containing 700 μg of protein was layered over a 3.6 ml sucrose gradient (15%-55% in buffer B containing 150 μg/ml cycloheximide). This was centrifuged at 130,000 *g* for 2.5 h at 4°C, and 12 x 400 μl fractions were removed for analysis.

For *in situ* hybridisation a 202 base pair region of the *hdm2* coding sequence (within exons 7-9) was generated using GGTGGGAGTGATCAAAAGGA and CCAGGCTTTCATCAAAGGAA primers, and cloned into pGEMTeasy plasmid (Promega). Sense and antisense digoxigenin-labelled RNA probes were generated using a digoxigenin RNA labelling kit (Roche Diagnostics Ltd.). Cells growing on glass coverslips were fixed for 20 min at room temperature with 4% paraformaldehyde in PBS, and dehydrated in ethanol before storage at -70 °C, desiccated. Following rehydration, *in situ* hybridisation was performed essentially as described previously (41). Bound probe was detected using anti-digoxigenin-rhodamine, Fab fragments (Roche Diagnostics Ltd.), and cell nuclei were stained with 4',6-Diamidine-2'phenylindole dihydrochloride (Roche Diagnostics Ltd.).

Plasmids, transfections and reporter gene assays- the hdm2luc03 reporter vector (37) contains 165 b.p. of the *hdm2*-P2 promoter region, including two p53-responsive elements, in pGL3Basic (Promega). SuperTIP, which encodes an inhibitor of the p53 –

Hdm2 interaction, and the inactive control MutantTIP, have been described previously (11). pC53SCX3, which encodes ala143 mutant p53, was a gift of Professor Bert Vogelstein. T47D and MCF-7 cells were transfected using Lipofectamine 2000 reagent (Invitrogen), and reporter assays were performed 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, reagents were obtained from Qiagen. Cells in 60 mm dishes were transfected with 5.5 μg of either control (non-silencing) siRNA, or validated siRNA for Hs-mdm2 (*hdm2).* Transfection was for 24 h, and the cells were lysed a further 24 h later.

RESULTS

Pharmacological inhibition of MEK activity suppresses Hdm2 protein synthesis in T47D breast cancer cells- compared to a panel of breast cancer cell lines, oestrogen receptor-α positive (ERα^{+ve}) lines such as T47D and MCF-7 express relatively high levels of Hdm2 protein (37). T47D cells express only a transcriptionally inactive mutant p53 protein, and therefore this cell line provides a useful model for the study of p53-independent mechanisms which regulate the expression of Hdm2. Findings made in this simple model can then be extrapolated into the more complex situation present in cell lines such as MCF-7, which are more representative of the majority of ERα^{+ve} breast cancers, in which changes in Hdm2 expression and p53 activity are dampened by a negative feedback loop (42). We determined the effect of pharmacological inhibition of MEK activity on the levels of Hdm2 protein in proliferating T47D cells. Consistent with studies in other cell types (24,25), 24 h exposure to 25 μM U0126 or 50 μM PD98059, two specific and structurally unrelated inhibitors of MEK activity (43), reduced Hdm2 levels by up to 95%

(Fig. 1A). Loss of the phosphorylated form of ERK 1 and ERK 2 confirmed that MEK was effectively inhibited by 25 μM U0126 in these cells (Fig. 1B). Removal of U0126, and the addition of fresh medium, lead to the recovery of Hdm2 to greater than pre-treatment levels within 2-3 hours (Fig. 1C). This recovery did not occur if U0126 was included in the medium (data not shown).

Hdm2 protein is rapidly turned over in most cells. The reduction in Hdm2 protein levels in U0126-treated T47D cells is not due to a further increase in the rate of Hdm2 protein turnover, as assayed by the rates at which Hdm2 protein levels decrease following the inhibition of protein synthesis by cycloheximide (Fig. 2A). 12 h exposure to 25 μM U0126 reduced Hdm2 protein levels by 70%, however the half-life of Hdm2 was approximately 15 min in both control- and U0126-treated cells. We then investigated whether U0126 affects Hdm2 protein synthesis by determining the rate at which Hdm2 protein accumulates when its primary degradation pathway is blocked by a proteosome inhibitor MG132 (this method was used in preference to conventional metabolic labelling studies due to the extremely short half life of Hdm2 and our concern that radio-labelling, like other inducers of DNA damage (44), would induce post-translational modifications of Hdm2 that could affect its activity and hence turnover). The results shown in Fig. 2B clearly demonstrate that the rate of Hdm2 synthesis is reduced by approximately 85% when MEK activity is inhibited by U0126. Global synthesis of new proteins was not inhibited by U0126 in these cells (Fig. 2C).

The effects of inhibiting MEK activity on hdm2 mRNA expression levels- in the murine *mdm2* gene, the activity of the P2-promoter is inducible by Ras-Raf-MEK-ERK signalling (25). We therefore determined the effects of U0126 on the levels of endogenous *hdm2* P1- and P2-transcript mRNA using two independent techniques; RPA

(Fig. 3A) and qPCR on cDNA generated from polyadenylated mRNA (Fig. 3B). Both RPA and qPCR clearly show that the levels of the predominant P1 transcript is not affected by U0126 treatment and, consistent with this, no U0126-induced changes were detected when qPCR analysis was directed towards a region within the *hdm2* coding sequence. RPA did not detect any large changes in *hdm2*-P2 mRNA levels following U0126 treatment (Fig 3Ai). However, quantitative analysis of multiple independent experiments (Fig. 3Aii) identified a small, but significant, difference in –P2 transcript levels between DMSO- and U0126 treated cells (100 $\pm$ 24.2% compared to 70.6 $\pm$ 15.8%, $P<0.05$). This effect could be more reliably detected by the more sensitive qPCR assay, which showed a decrease in the levels of the –P2 transcript by 41.1 $\pm$7.0% (n=5) following U0126 treatment (Fig. 3B). We had previously been unable to detect this relatively small change using semi-quantitative assays (37). The decrease is unlikely to be sufficient to account for the 95% reduction in Hdm2 protein levels that are observed in U0126 treated cells as siRNA-mediated knock-down of the *hdm2*-P2 transcript by approximately the same amount as occurs following U0126 treatment, results in only a 45% reduction in Hdm2 protein levels (Fig. 3C).

MEK inhibitors reduce Hdm2 levels by regulating the export of hdm2 mRNA to the cytoplasm- the data described above suggest that inhibition of MEK activity can affect Hdm2 protein synthesis at a post-transcriptional level. We therefore set out to establish whether this might occur at the level of mRNA trafficking or translation. T47D cells were incubated with either U0126, or DMSO control, and then subjected to a gentle hypotonic lysis in order to generate cytoplasmic extracts suitable for the subsequent analysis of polyribosomes. Initially, qPCR was performed on these extracts to determine levels of *hdm2* mRNA transcripts (Fig. 4A). This analysis showed that, following U0126

treatment, both *hdm2* mRNA transcripts are significantly under-represented in these cytoplasmic extracts, compared to their levels in total mRNA. Specifically, whilst total cellular levels of the *hdm2* P1 transcript were unaffected by U0126, levels in the cytoplasmic fraction were decreased by 64.2%. Likewise, whilst U0126 decreased overall *hdm2*-P2 expression by approximately 40% as before, the levels of this transcript were further under-represented in the cytoplasmic lysates, being reduced by 71.3% compared to mock-treated controls. These results suggest that U0126 treatment regulates the export of *hdm2* mRNA from the nucleus to the cytoplasm. We undertook two further experiments to substantiate these findings.

Firstly a more extensive fractionation analysis was performed (Fig 4B). Cell lysis was performed with the same hypotonic lysis buffer B as used in the experiment shown in Fig. 4A and U0126 again resulted in a marked under-representation of both *hdm2* transcripts in the soluble cytoplasmic extract (S) compared to total cell lysates (T). A corresponding increase in *hdm2* transcript levels was seen in the nuclear pellets (P) following U0126 exposure. Semi-quantitative PCR detection of the small cytoplasmic RNA *scRNA hY4* (45) confirmed that the cell lysis technique efficiently separated cytoplasmic RNA from the nuclear pellet, whereas *gapdh* mRNA was present in both fractions. Following U0126 treatment, *hdm2* mRNA clearly becomes associated with the insoluble pellet. The hypotonic lysis buffer was therefore modified in order to gain insight into the nature of this association. Solublisation of lipid membranes with 0.5 % IGEPAL CA-630 (B2) was unable to reverse the U0126-dependent association of *hdm2* mRNA with the pellet. However addition of 100 mM NaCl to the buffer (B1 and B3) was sufficient to release this *hdm2* from the nuclear pellet, this being most apparent in the presence of detergent (compare buffers B2 with B3).

In situ hybridisation experiments were then performed to detect *hdm2* mRNA (Fig. 4C). In mock-treated control cells, *hdm2* mRNA is detectable in the cytoplasm of the cell using anti-sense *hdm2* RNA probe, but not the control, sense probe. However, in U0126-treated cells, cytoplasmic *hdm2* message can no longer be detected. We were unable to detect nuclear *hdm2* mRNA in these assays, possibly due to poor access of the probe to the target message in the nuclei.

The effects of MEK inhibition on translation of hdm2 mRNA- we next wished to establish whether this reduction in the levels of cytoplasmic *hdm2* mRNA was reflected in an equivalent reduction in rates of *hdm2* translation, as assessed by the association of *hdm2* mRNA with high molecular weight polyribosome complexes. Cytoplasmic cell extracts were separated through 30% sucrose buffer to isolate polyribosome bound- from free cytoplasmic- mRNA (40), and mRNA from total cell extracts and the polyribosome-associated pellet was then assayed by qPCR (Fig. 5A). Following U0126 treatment, levels of *hdm2*-P1 transcript in the polyribosome-associated fraction was reduced by 75.6%, and the –P2 transcript by 83.7%, compared to mock-treated cells. This degree of reduction in actively translated *hdm2* mRNA transcripts is in good agreement with the 85% reduction in the rate of Hdm2 protein synthesis shown in Fig. 2B. The effect of U0126 on the amount of polyribosome-associated *hdm2* mRNA is largely accounted for by the decrease in levels of cytoplasmic message (Fig. 4A), though the effect on polyribosome association is slightly greater than that on subcellular localisation, suggesting that a small degree of regulation at the level of translation might also be occurring.

A more detailed analysis of polyribosome association was then performed using sucrose density gradient ultracentrifugation (Fig. 5B). This analysis did not detect any obvious shift in the *hdm2* message from high to low molecular weight fractions following U0126 exposure, indicating that U0126 does not inhibit the association of cytoplasmic *hdm2* mRNA with ribosomes in these cells. Rather, there is a reduced level of *hdm2* transcripts in all fractions in the gradient due primarily to the effect of U0126 on cytoplasmic localisation of the message (Fig. 4A). MEK kinase signalling is known to regulate protein translation (46), possibly accounting for the apparent shift in the *gapdh* transcript on the profile following U0126 treatment.

Regulation of the p53-Hdm2 auto-regulatory feedback loop by MEK signalling in wild-type p53-expressing MCF-7 breast cancer cells- inhibiting signalling through the Ras-Raf-MEK-ERK signalling cascade has previously been shown to have diverse effects in the p53 stress response pathway, (25,47-49) and we therefore sought to establish the role that control of Hdm2 levels by this pathway has in regulating the p53 response in breast cancer cell lines. Firstly, we determined that 24 h exposure to 25 μM U0126 also reduces Hdm2 protein levels by >75% in the wild-type p53-expressing cell line, MCF-7 (Fig. 6A). U0126 also prevented the induction of Hdm2 protein expression by the p53-activating chemotherapeutic agent, 5-FU (Fig. 6B). Interestingly, however, this inhibition did not result in increased levels of p53 protein (Fig. 6B), nor was there any clear synergistic effect on long term clonogenic survival of co-treatment with 5-FU and U0126 (Fig. 6C), that might have indicated that inhibition of Hdm2 expression by U0126 was leading to a hyper-activation of the p53 response to genotoxic stress. In fact, we found that U0126 actually attenuated the activation of p53-dependent transcription by 5-FU in MCF-7 cells (Fig. 6D). This inhibition of p53 activity was completely independent of functional Hdm2

protein, as demonstrated by two experiments: firstly transcription from a p53-dependent reporter vector (Fig. 7A) and an endogenous p53-induced transcript, *hdm2*-P2 mRNA (Fig. 7B) was inhibited by U0126 in cells in which p53 had been activated by a rationally designed inhibitor of the Hdm2-p53 interaction, SuperTIP (11); and secondly the upregulation of the p53-induced protein, $p21^{WAF-1}$, by a chemical inhibitor of the Hdm2-p53 interaction, Nutlin-3 (50) was inhibited by U0126, the Nutlin-3 induced increase in Hdm2 protein levels also being partially attenuated. The stabilisation of p53 protein by Nutlin-3 was not inhibited by U0126, suggesting that the effects of U1026 on p53 function occur at the post-translational level. Consequently, signalling through MEK kinase impinges on the p53-Hdm2 axis via at least three independent pathways in these cells (Fig. 8).

DISCUSSION

Regulation of Hdm2 expression and function is a key component of several growth factor activated pro-survival and proliferative signalling pathways. Increased stability and nuclear localisation of Hdm2 protein, following its phosphorylation by AKT kinase, is an important component of the anti-apoptotic response to survival factors (18-20). Growth factor stimulation also induces Hdm2 / Mdm2 expression (24,51) in a MEK kinase dependent manner (25,52). Mosner *et al* demonstrated that Mdm2 protein expression is induced in serum re-stimulated fibroblasts without a detectable increase in total *mdm2* mRNA by Northern blotting (51), suggesting that *mdm2* may be regulated at the post-transcriptional level under these circumstances. However, *mdm2* was identified as an early response gene induced by platelet-derived growth factor receptor-β signalling in murine fibroblasts (53), and AP1 and ETS factor binding sites were identified in the murine *mdm2* -P2 promoter that regulate its activity in response to Ras signalling (25).

Therefore, the findings of Mosner *et al* (51) could be explained by induction of the efficiently-translated *mdm2*-P2 transcript, which would have been masked on northern blots by the more highly expressed constitutive P1-derived message.

Inhibiting Ras signalling or MEK activity has also been shown to down-regulate Hdm2 protein expression in human cells (25,47). However, whilst the activity of murine *hdm2*-P2 reporter vector in human colon carcinoma cells is inhibited by MEK inhibitors, the effects of these interventions on the endogenous human *hdm2* transcripts, or the human P2-promoter, have not been demonstrated previously. In proliferating MCF-7 and T47D breast cancer cell lines, a conserved AP1-ETS element in the *hdm2*-P2 promoter is required for the p53-independent expression of the P2-transcript that occurs in these cells (37). As we show in this current paper, however, the activity of the *hdm2*-P2 promoter in T47D cells is only partialy dependent on MEK kinase activity. This is in marked contrast to the Hdm2 protein synthesis in these cells, which is reduced by up to 95% following exposure to MEK inhibitors. Further biochemical and histochemical analysis demonstrated that MEK inhibition results in greatly reduced export of both *hdm2* -P1 and -P2 transcripts from the nucleus to the cytoplasm.

Regulation of the nuclear export of mRNA is a key point of control for the expression of a number of cellular proteins, and is one of the most elaborate nuclear transport pathways (54). All nuclear mRNAs exist in relatively large complexes with proteins, and it is these complexes which interact with components of the nuclear export pathways that are responsible for transporting the mRNA from the site of transcription to the cytoplasm. Assembly of these mRNA-protein complexes begins during mRNA processing, and the complexes include cap binding proteins, splicing factors, and other proteins involved in

pre-mRNA processing. During mRNA processing, the mRNA-protein complexes remain tightly attached to the nuclear matrix, but at some point following processing, this interaction is weakened, and the mRNA can be released from the nucleus by salt extraction (55). As far as we are aware, the molecular basis of this observation has not been elucidated, and the question of how mRNAs are transported from the site of transcription to the nuclear pore complex remains only partly characterised (54). Inhibition of MEK activity results in the export of *hdm2* mRNA being blocked in this nuclear, salt extractable, compartment. At present we can only speculate that this represents the regulation, by MEK, of proteins involved in a nuclear export pathway that is required for the transport of a subset of mRNAs. Experimental manipulation of specific nuclear export processes can be shown to inhibit the expression of early response genes such as *c-fos* (56), and there is an increasing body of evidence suggesting that cellular mRNAs can be organised and exported from the nucleus as functionally related groups by RNA binding proteins (57). Whether such pathways might be dependent on MEK signalling has not been described. It will be of interest to determine what other mRNAs are similarly affected by MEK inhibitors, which are known to inhibit the synthesis of a number of key regulators of cell cycle progression and apoptosis at a post-transcriptional level (58,59).

Another issue raised by our findings is what the effect of this control mechanism might be on the ability of Ras-Raf-MEK-ERK signalling to modulate chemo- and radio-sensitivity in cancer cells. MEK-dependent expression of proteins such as Hdm2, Bcl-X_L, and IAPs, at the level of gene transcription and translation, is required for growth and survival factors, such as fibroblast growth factor-2, to protect cells from genotoxic agents (24,25,58,59). In the case of Hdm2, this is most likely to be through the suppression of

the p53 response, and, in cancer cells in which expression of the Hdm2 antagonist $p14^{ARF}$ is lost, MEK inhibitors can enhance the cytotoxicity obtained with γ-irradiation (25). We therefore examined whether, in the MCF-7 cell line model, there would be a synergistic effect between U0126 and the p53-activating anti-metabolite 5-FU, a chemotherapeutic agent which can be dependent on p53 activity to kill cancer cells (60), and also shows a strong induction of Hdm2 expression at cytotoxic doses. No synergism was observed in the colony-forming assays and, instead, we found that U0126 treatment resulted in inactivation of p53 function. This is consistent with other previous data showing that MEK activity can be required for both the expression of p53 at the transcriptional level (61) and the activation of p53 by genotoxic agents (48,49). We also showed that this inhibition of p53 activity did not require functional Hdm2 in the cell, as it occurred in the presence of the Hdm2 inhibitors SuperTIP and Nutlin-3. This is again consistent with the reports that MEK inhibitors inhibit stress-induced phosphorylation of p53 at serine 15, which is required for the interaction of p53 with its co-activator, p300 (62), though the inhibition of other post-translational modifications, or co-operating transcriptional activators, should also be considered. Whilst MEK inhibitors can also inhibit transcription of p53 mRNA (61), this is unlikely to be the mechanism of p53 inactivation in our experiments, as U0126 does not prevent the accumulation of p53 protein in response to Nutlin-3. From a broader perspective, inhibitors of the Ras-Raf-MEK-ERK pathway, and particularly MEK inhibitors, have good potential as anti-cancer agents (63). However, their ability to potentiate the effects of genotoxic chemotherapeutic agents appears to be highly dependent on the target cell type and agent being studied (64,65).

Together, our data suggest a model by which the p53-Hdm2 feedback loop is regulated in response to mitogenic or anti-apoptotic signalling through the Ras-Ras-MEK-ERK

signalling pathway, at least in the breast cancer cell lines we have studied here. In the absence of MEK activity, cells reduce their proliferation rate and enter a G1 arrest phase (data not shown); this is associated with a loss of p53 activity, possibly due to reduced serine 15 phoshorylation and p300 binding. Hdm2 expression is therefore not necessary to inhibit p53 function, and Hdm2 protein synthesis is decreased, due to both reduced transcription from the P2 promoter, and through reduced nuclear export of *hdm2* mRNA. Following activation of Ras-Raf-MEK-ERK signalling by growth factors, the block to *hdm2* nuclear export is released, allowing expression of the Hdm2 protein, which is now required as p53 activity increases. This helps establish a dynamic equilibrium between the p53 and Hdm2 proteins in proliferating cells that is exquisitely sensitive to regulation by other signalling pathways, such as those induced by cellular stress.

Hdm2 shows promise as a target for anti-cancer therapies (66), and small molecule inhibitors of the p53-Hdm2 interaction that have good efficacy against wild-type p53-expressing tumour cells in pre-clinical modes have recently been described (50). Presumably, such compounds will only be effective in cells in which the target molecule is expressed, and in which its activity is required for cellular survival or proliferation. In the specific case of breast cancer, which may well be a suitable target for such interventions, we have previously demonstrated that elevated levels of Hdm2 protein in proliferating cultures of breast cancer cell lines with $ER\alpha^{+ve}$, compared to $ER\alpha^{-ve}$, phenotypes, correlates with transcription from the P2-promoter of the *hdm2* gene in the $ER\alpha^{+ve}$ cells (37). In breast tumour samples, Hdm2 protein is over-expressed compared to normal cells in as many as 50% of cases (34-36), however in most cases in which Hdm2 expression is observed in breast cancer, it is limited to small patches of cells in the tumour (34). Additionally, one study determined that, of the tumours in which *hdm2*

mRNA was up-regulated, only 69% showed Hdm2 protein over-expression (35). Our data support a model in which differences in Hdm2 expression between cancers with different phenotypes and differentiation status is defined at the transcriptional level. However either specific mutations, or the local tumour environment, that affect signalling through the Ras-Raf-MEK-ERK cascade, superimpose upon the transcriptional phenotype by regulating Hdm2 protein expression at the level of nuclear export of its mRNA. Whilst our study has focussed specifically on breast cancer cell lines, it is likely that this pattern of Hdm2 regulation will be prevalent amongst many cancer types in which Hdm2 protein is highly expressed.

Acknowledgements- We are grateful to Professors A.J.Levine, D.P.Lane and B.Vogelstein for making available antibody and plasmid reagents. The manuscript was critically read by Dr. Sandra Campbell.

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FIGURE LEGENDS

FIG. 1. **MEK kinase activity is required for Hdm2 protein expression in T47D breast cancer cells.** (A) T47D cells were cultured in the presence of DMSO control (D, 7 h) or MEK inhibitors PD98059 (50 μM) or U0126 (25 μM) for the indicated time before Hdm2 proteins levels were determined by western blotting using 2A9 antibody (a second Hdm2 antibody, 2A10, gave similar results (data not shown)). For quantification, Hdm2 levels were normalised to the β actin loading control (open bars – DMSO, grey bars – PD98059, black bars – U0126). (B) T47D cells were incubated with DMSO or 25 μM U0126 for 18 h before expression of the indicated proteins was determined. (C) Cells were incubated as in Fig. 1B, then refed with drug-free medium and incubated for a further 0-5 h before lysis and western blotting analysis.

FIG. 2. **MEK inhibitors block Hdm2 protein synthesis.** (A) T47D cells were cultured with DMSO carrier or 25 μM U0126 for 12 h before 100 μg/ml cycloheximide (CHX) was added to the medium to block new protein synthesis. Normalised Hdm2 protein

levels are shown in the bar graph. The line graph shows the percentage of Hdm2 levels in either DMSO- or U0126-treated cells relative to levels prior to the addition of cycloheximide. Black graphics – DMSO treated, grey graphics – U0126 treated. Representative of three independent experiments. (B) T47D cells were cultured with DMSO or 25 μM U0126 for 18 h before 50 μM MG132 was added to the medium to block proteosome-mediated degradation of Hdm2. Cells were cultured for a further 0-90 min, then lysed and analysed by quantitative western blotting. Normalised Hdm2 protein levels are shown in the bar graph as in Fig. 2A. Lines show linear regression of these data over the first 60 min after MG132 addition. Rate of Hdm2 protein accumulation over this period in U0126-treated cells was 13.6% of controls. Representative of two independent experiments. (C) T47D cells were exposed to 25 μM U0126 (U) or DMSO (D) for 24 h before being labelled with ^{35}S-methionine/cysteine for 90 min in the presence of the same treatment. Equal protein amounts were run on SDS-PAGE gels that were then analysed by Coomassie blue staining for total protein and autoradiography for newly synthesised protein. Similar results were obtained with a 30 min labelling period (not shown). Cells treated identically in parallel were analysed by western blotting for Hdm2.

FIG. 3. **Effects of inhibiting MEK activity on *hdm2* mRNA levels.** (A) Total cellular RNA was analysed by RPA using an *hdm2* exon 2 – exon 3 probe, with a *gapdh* probe as a loading control. (Ai) shows a representative experiment. Lanes are: 1, non-digested probe; 2, digested probe; m, size markers; 5 and 6, mRNA from T47D cells which had been treated for 24 h with DMSO or 25 μM U0126 respectively. (Aii) Shows quantitation of RPA analysis of 4 independently treated sets of cells. Open bars – DMSO controls, black bars – U0126 treated. Data is normalised to *gapdh* mRNA levels, and is expressed as mean $\pm$ SEM (B) Total cellular RNA from T47D cells was analysed by qPCR. *hdm2*

mRNA levels were normalised to *gapdh* housekeeping gene expression, and normalised *hdm2* mRNA levels in U0126-treated (25 μM, 24 h) cells are expressed as a percentage of levels in DMSO-treated cells. Data are mean ± S.E.M. for five independently treated sets of samples. Western blots confirmed that Hdm2 protein levels decreased in cells treated in parallel to the experiments shown in Fig. 3A and B (not shown). (C) T47D cells were transfected with control, non-silencing siRNA (lanes- C), or *hdm2* specific siRNA (lanes –h). 48 h after transfection, cells were analysed for levels of Hdm2 protein (open bars, and western blot) and *hdm2* mRNA (solid bars). The *hdm*- P2 transcript levels are shown, however the *hdm2*-specific siRNA targets both -P1 and -P2 transcripts.

FIG. 4. **Inhibition of MEK reduces levels of *hdm2* mRNA transcripts in the cytoplasm.** T47D cells were cultured for 24 h with either DMSO carrier, or 25 μM U0126. (A) RNA was extracted from either whole cell pellets, or cytoplasmic lysates prepared using hypotonic buffer B, and levels of *hdm2*-P1 and –P2 transcripts determined by qPCR. Data is normalised to *gapdh*. Open bars- DMSO treated, solid bars- U0126 treated. Error bars are S.D. of duplicate qPCR assays. (B) Cells were extracted using either buffer B, or buffer B with the following modifications - B1 (B + 100 mM NaCl), B2 (B + 0.5% IGEPAL CA-630), B3 (B + 100 mM NaCl + 0.5% IGEPAL CA-630). *Hdm2* transcript levels in the soluble cytoplasmic extract (S), or the insoluble nuclear pellet (P), as well as in total cell extracts (T), were determined by qPCR. Data are presented as in Fig. 4A. Cellular fractionation was validated using semi-quantitative PCR for *scRNA hY4*, and *gapdh.* Two PCRs for each extraction method are shown, with a two-fold difference in the amount of input cDNA to confirm the PCRs have not plateaued. (C) *hdm2* mRNA was detected by *in situ* hybridisation using antisense *hdm2* probe. Control, sense, probe is also shown. Dotted white lines outline the position of the cell nuclei.

FIG. 5 **Effect of MEK inhibitors on the levels of polyribosome-associated *hdm2* mRNA.** T47D cells were incubated with either 25 μM U0126, or DMSO carrier for 24 h. (A) Polyribosome-associated RNA was then isolated by lysis in buffer B, followed by centrifugation of the cytoplasmic lysate through 30% sucrose buffer. qPCR analysis of *hdm2* transcript levels was performed on total-cell (solid bars) and polyribosome-associated (open bars) RNA. Data are presented as a percentage of levels in DMSO-treated cells, and are mean ± S.D. for two independent experiments. Hdm2 protein levels were analysed in parallel and were reduced by approximately 90% in U0126-treated cells (data not shown)**.** (B) Cytoplasmic extracts (from the experiment shown in Fig. 4A) containing equal amounts of protein were separated on a 15-55% sucrose gradient. 12 consecutive fractions were taken from the gradient. 12.5% of the RNA from each fraction was used to synthesise cDNA, which was then analysed by qPCR. Upper panel- relative protein concentration (Bradford assay); second panel- agarose gel electrophoresis to detect the major ribosomal RNAs; lower panels- levels of the indicated mRNA transcripts in each fraction. y axis is the same for both DMSO and U0126-treated cells.

FIG. 6. **Effect of inhibiting MEK activity on the p53-Hdm2 feedback loop in wild-type p53-expressing breast cancer cells.** (A) Hdm2 expression in MCF-7 cells following incubation with 25 μM U0126 or DMSO for the indicated times. (B and C) 1.5 x 10^6 MCF-7 cells were plated per 90 mm dish and 48 h later re-fed with medium containing 25 μM U0126, or DMSO. After a further 24 h, 200 μM 5-FU was added where indicated, and all dishes incubated for a further- (B) 4 h before cell pellets were made for western blotting analysis or- (C) 6 h before plates were trypsinised, 2 x 10^4 cells plated in a new dish, and cultured for a further 14 days before cells were fixed and stained with

Giemsa. (D) MCF-7 cells were transfected with a minimal p53-responsive reporter construct (hdm2luc03) and refed with medium containing 25 μM U0126 (solid bars) or DMSO carrier (open bars) plus the indicated concentration of 5-FU. Reporter gene activity was assayed 40 h later. Results are mean ± S.D. of duplicate dishes and are representative of two independent experiments.

FIG. 7. **Inhibition of MEK kinase in MCF-7 cells inhibits p53-dependent transcription independently of Hdm2 protein function.** (A) MCF-7 cells were transfected with hdm2luc03 or pGL3basic reporter plasmids plus plasmids encoding either SuperTIP (S-TIP, an inhibitor of the Hdm2:p53 interaction), the inactive control vector, MutantTIP (M-TIP), or dominant negative (ala143) mutant p53. Following transfection, cells were incubated for 48 h in the presence of 25 μM U0126 (solid bars) or DMSO control (open bars) before reporter activity was assayed. Data are mean ± S.D. for duplicate dishes. Representative of >3 separate experiments. (B) MCF-7 cells were transfected with either SuperTIP or MutantTIP plasmids and then cultured for 24 h in the presence of 25 μM U0126 (solid bars) or DMSO (open bars) for 24 h. qPCR was performed for the p53-responsive *hdm2*-P2 transcript, levels of which are normalised to *gapdh* control. Data are mean of duplicate assays. (C) MCF-7 cells were cultured in the presence of vehicle control (DMSO) or U0126 (25μM) for 24 hours, before Nutlin-3 (5μM) or vehicle control (ethanol) was added to the media and the cells harvested after the indicated time. Hdm2, p53 and p21 protein levels were determined by Western blotting.

FIG. 8 **MEK signalling maintains the p53-Hdm2 feedback loop in a state of dynamic equilibrium in cancer cells.** The three distinct points of regulation described in this

manuscript are: [1] MEK activity promotes the Hdm2-independent activation of p53 as a sequence-specific transcriptional activator of genes that promote cell cycle arrest and apoptosis. This activation is counteracted by a MEK-dependent increase in both the transcription [2] and nuclear export [3] of *hdm2* mRNA, which together prevent spontaneous p53-dependent growth arrest or apoptosis occurring in cancer cells following activation of the growth factor–Ras-Raf-MEK signalling cascade. Note this diagram does not include the MEK-dependent activation of the Hdm2 antagonist, $p14^{ARF}$, which is not expressed in MCF-7 breast cancer cells (67).

FIGURES

Fig. 1

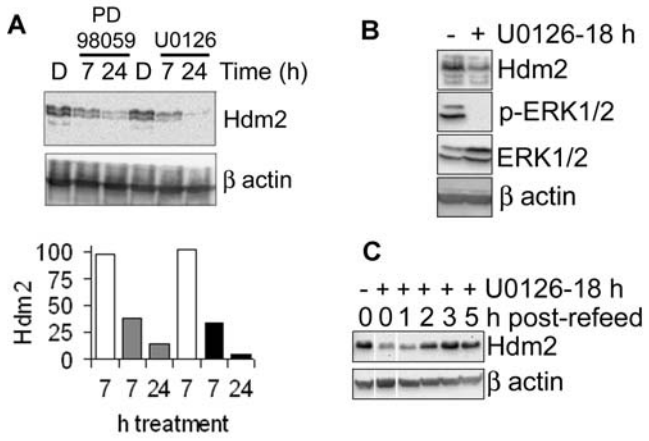


Fig. 2

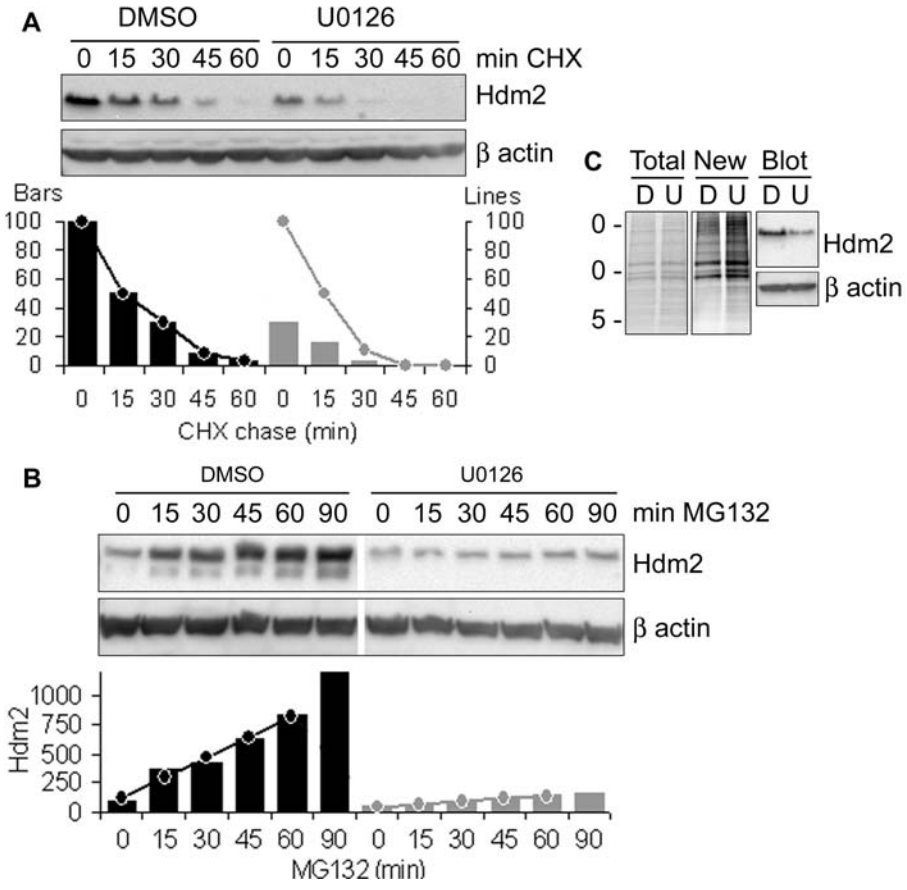


Fig. 3

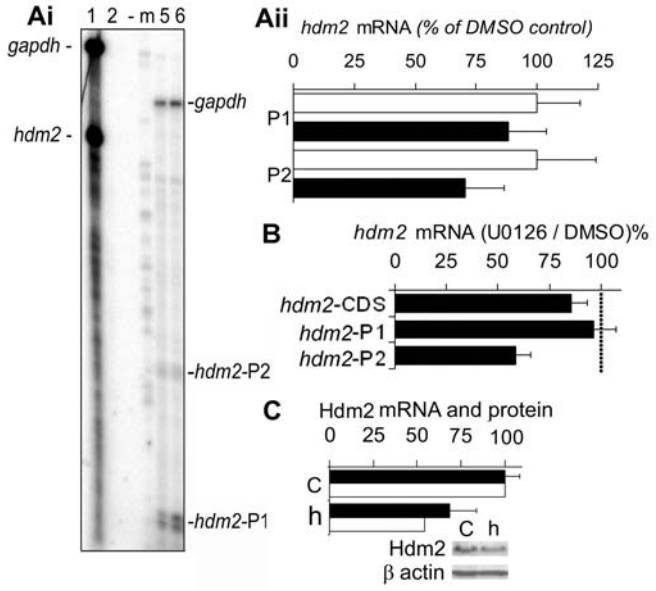

Ai
1 2 - m 5 6
gapdh -
hdm2 -
-gapdh
-hdm2-P2
-hdm2-P1
Aii
hdm2 mRNA (% of DMSO control)
0 25 50 75 100 125
P1
P2
B
hdm2 mRNA (U0126 / DMSO)%
0 25 50 75 100
hdm2-CDS
hdm2-P1
hdm2-P2
C
Hdm2 mRNA and protein
0 25 50 75 100
C
h
C h
Hdm2
β actin

Fig. 4

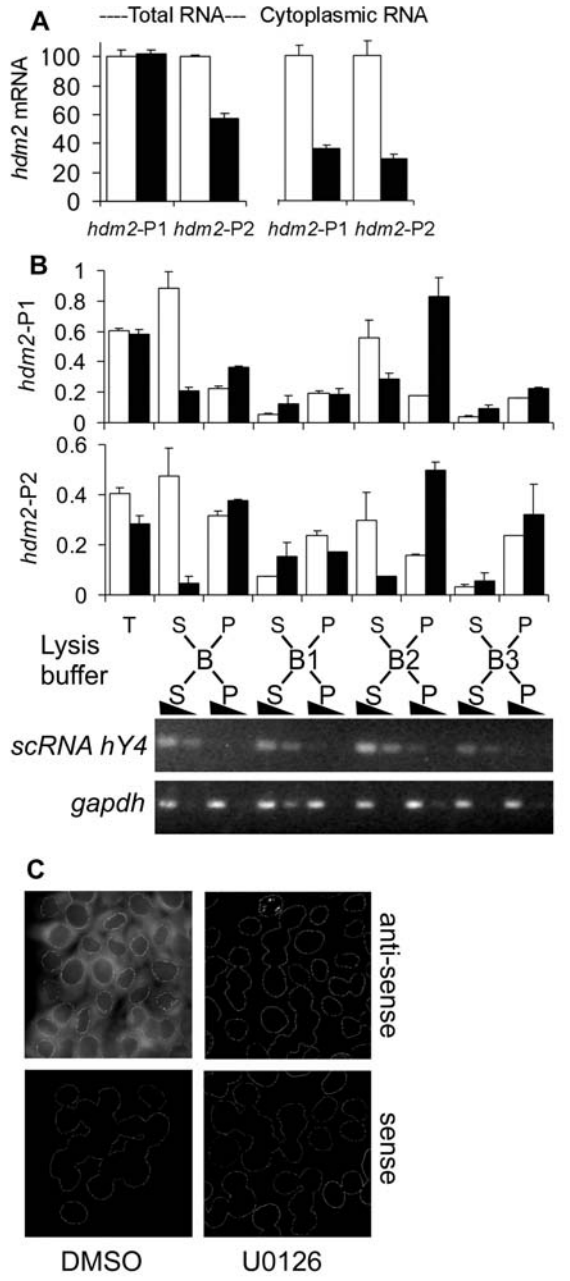

A
----Total RNA---
Cytoplasmic RNA
hdm2 mRNA
100
80
60
40
20
0
hdm2-P1
hdm2-P2
hdm2-P1
hdm2-P2
B
hdm2-P1
1
0.8
0.6
0.4
0.2
0
hdm2-P2
0.6
0.4
0.2
0
T
S
P
S
P
S
P
S
P
Lysis buffer
B
B1
B2
B3
S
P
S
P
S
P
S
P
scRNA hY4
gapdh
C
anti-sense
sense
DMSO
U0126

Fig. 5

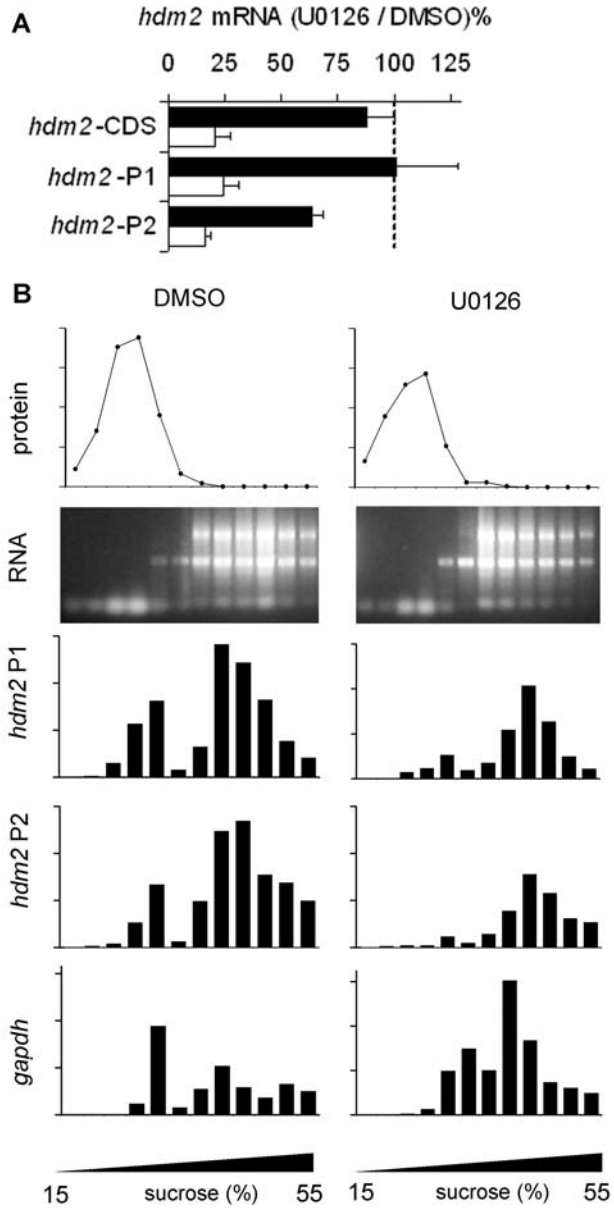

A
hdm2 mRNA (U0126 / DMSO)%
0 25 50 75 100 125
hdm2-CDS
hdm2-P1
hdm2-P2
B
DMSO
U0126
protein
RNA
hdm2 P1
hdm2 P2
gapdh
15
sucrose (%)
55
15
sucrose (%)
55

Fig. 6

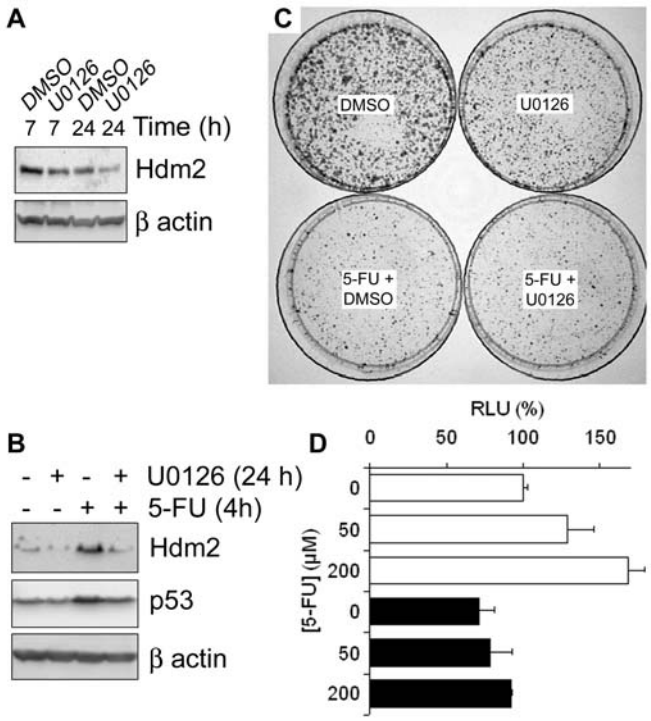

A
DMSO
U0126
DMSO
U0126
7 7 24 24 Time (h)
Hdm2
β actin
C
DMSO
U0126
5-FU + DMSO
5-FU + U0126
B
- + - + U0126 (24 h)
- - + + 5-FU (4h)
Hdm2
p53
β actin
D
RLU (%)
0 50 100 150
[5-FU] (μM)
0
50
200
0
50
200

Fig. 7

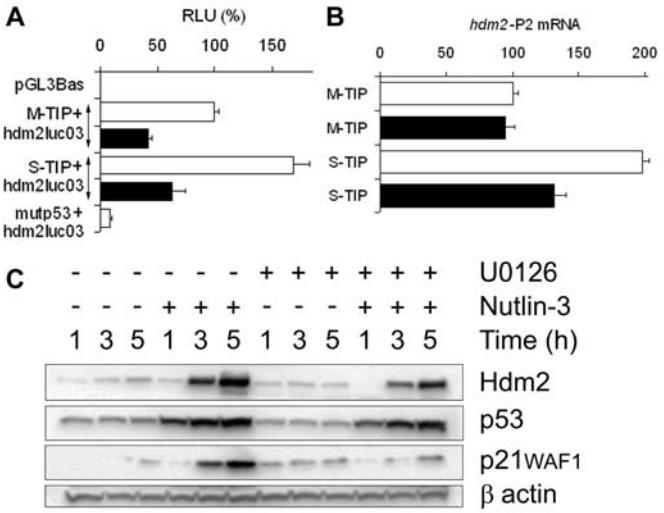
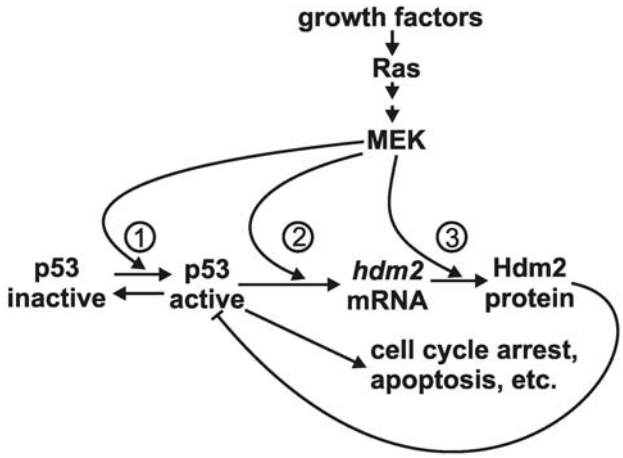

A
RLU (%)
0 50 100 150
pGL3Bas
M-TIP+ hdm2luc03
S-TIP+ hdm2luc03
mutp53+ hdm2luc03
B
hdm2-P2 mRNA
0 50 100 150 200
M-TIP
M-TIP
S-TIP
S-TIP
C
U0126
Nutlin-3
Time (h)
Hdm2
p53
p21WAF1
β actin

Fig. 8


growth factors
Ras
MEK
①
②
③
p53 inactive
p53 active
hdm2 mRNA
Hdm2 protein
cell cycle arrest, apoptosis, etc.