MEK-ERK Signaling Controls Hdm2 Oncoprotein Expression by Regulating hdm2 mRNA Export to the Cytoplasm*

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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 signaling pathways that regulates 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 signaling 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, p53-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 ~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.

The tumor suppressor p53 and oncoprotein Hdm2 function within an auto-regulatory feedback loop that is a point of convergence of signaling 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 translational machinery (12) and targeting it for ubiquitination, nuclear export, and proteasomal 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 signaling pathways can also impinge upon p53 through either the positive or negative regulation of Hdm2 function. v-akt murine thymoma viral oncogene homologue kinase, a key enzyme in pro-survival signaling pathways, phosphorylates Hdm2, which results in elevated Hdm2 levels in the nucleus (18–20). The growth factor-induced Ras-Raf-MEK1-ERK signaling pathway can have context dependent effects on proliferation and survival (21), and its regulation of the p53-Hdm2 axis appears to be similarly complex. Signaling via this cascade can induce the expression of p14ARF, an Hdm2 antagonist, resulting in p53 activation. This can be an important block to cancer progression, and p14ARF expression is lost in many tumors (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).

In murine breast cancer cells, MEK activity is necessary for the expression of hdm2-P1 transcript (26). MEK-ERK Signaling Controls Hdm2 Oncoprotein Expression by Regulating hdm2 mRNA Export to the Cytoplasm.

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 tumor types (28). In a proportion of cancers, this is a consequence of hdm2 gene amplification (29), although 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 signaling (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, it 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 signaling (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 signaling 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% fetal calf serum (Autogen Biolearc) as described previously (37). The following reagents were dissolved in dimethyl sulfoxide (MeSO) 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.

**Amine solutions.** Nutlin-3 (Alexis Biochemicals) was dissolved in eth-

**Cellular fractionation over a sucrose gradient, 1200 g/ml cycloheximide containing 700 g of protein was layered over a 3.6-mg sucrose gradient (15–55% in buffer B containing 150 g/ml cycloheximide). This was centrifuged at 130,000 × g for 2.5 h at 4 °C.**

**Analysis of polyribosome-associated mRNA (40), cycloheximide (10 µg/ml) was added to the serum-free medium used to wash the dishes. Cytoplasmic lysates were then layered over a cushion of 30% 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 was rinsed twice in buffer B before fractionation over a sucrose gradient, 1200 µl of cytoplasmic lysate containing 700 µg of protein was layered over a 3.6-mg 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 × 50-µl fractions were removed for analysis.

**For in situ hybridization, a 202-base pair region of the hdm2 coding sequence (within exons 7–9) was generated using GGTGGGAGTGATCCAGTCAAATTTAGCAGT and CCAGGCTTTCTACCATAGAAGAAAGAGA and cloned into pGEMTeasy (Promega). Sense and antisense digoxigenin-labeled RNA probes were generated using a digoxigenin RNA-labeling kit (Roche Diagnostics).** Cells growing on glass coverslips were fixed for 20 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline and dehydrated in ethanol before storage at −70 °C. After rehydration, in situ hybridization was performed essentially as described previously (41).

**Plasmids, Transfections, and Reporter Gene Assays—**The hdm2ac3 reporter vector (37) contains 165 bp 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 Ala-143 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 in 96-well plates, with normalization to pRLSV40 (Promega). The normalized data is presented as relative transcript levels using a standard curve. Semiquantitative PCR was performed as described previously (37); primers used were

**Regulation of Hdm2 Expression**

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 signaling 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.
MEK activity on the levels of Hdm2 protein in proliferating T47D cells. Consistent with studies in other cell types (24, 25), reduced Hdm2 levels by up to 95% (Fig. 1A). Loss of the phosphorylated form of ERK1 and ERK2 confirmed that MEK was effectively inhibited by 25 μM U0126 in these cells (Fig. 1B). Removal of U0126 and the addition of fresh medium led to the recovery of Hdm2 to greater than pretreatment levels within 2–3 h (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 after the inhibition of protein synthesis by cycloheximide (Fig. 2A). 12 h of exposure to 25 μM U0126 reduced Hdm2 protein levels by 70%; however, the half-life of Hdm2 was ~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 proteasome inhibitor MG132 (this method was used in preference to conventional metabolic labeling studies due to the extremely short half-life of Hdm2 and our concern that radio-labeling, 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 ~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 mmdm2 gene the activity of the P2-promoter is inducible by Ras-Raf-MEK-ERK signaling (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 toward a region within the hdm2 coding sequence. RPA did not detect any large changes in hdm2-P2 mRNA levels after U0126 treatment (Fig 3Ai). However, quantitative analysis of multiple independent experiments (Fig. 3Aii) identified a small, but significant difference in P2 transcript levels between Me2SO- and U0126-treated cells (100 ± 24.2% compared with 70.6 ± 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 ± 7.0% (n = 5) after U0126 treatment (Fig. 3B). We had previously been unable to detect this relatively small change using semiquantitative 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 with 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 Me2SO control and then subjected to a gentle hypotonic lysis to generate cytoplasmic extracts suitable for Western blotting.
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, after U0126 treatment, both hdm2 mRNA transcripts are significantly under-represented in these cytoplasmic extracts compared with their levels in total mRNA. Specifically, although total cellular levels of the hdm2 P1 transcript were unaffected by U0126, levels in the cytoplasmic fraction were decreased by 64.2%. Likewise, although U0126 decreased overall hdm2-P2 expression by ~40% as before, the levels of this transcript were further under-represented in the cytoplasmic lysates, being reduced by 71.3% compared with 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.

First, 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 with total cell lysates (T). A corresponding increase in hdm2 transcript levels was seen in the nuclear pellets (P) after U0126 exposure. Semiquantitative 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. After U0126 treatment, hdm2 mRNA clearly becomes associated with the insoluble pellet. The hypotonic lysis buffer was, therefore, modified to gain insight into the nature of this association. Solubilization 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, the 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 hybridization 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 antisense 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). After U0126 treatment, levels of \( hdm2 \)-P1 transcript in the polyribosome-associated fraction were reduced by 75.6%, and the \( hdm2 \)-P2 transcript by 83.7% compared with 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), although the effect on polyribosome association is slightly greater than that on subcellular localization, suggesting that a small degree of regulation at the level of translation might also occur.

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 after 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 localization of the message (Fig. 4A). MEK kinase signaling is known to regulate protein translation (46), possibly accounting for the apparent shift in the \( gapdh \) transcript on the profile after U0126 treatment.

**Fig. 5.** Effect of MEK inhibitors on the levels of polyribosome-associated \( hdm2 \) mRNA. T47D cells were incubated with either 25 \( \mu \)M U0126, or \( Me_{SO} \) (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 \( Me_{SO} \)-treated cells and are the mean \( \pm \) S.D. for two independent experiments. Hdm2 protein levels were analyzed in parallel and were reduced by 86% 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 synthesize cDNA, which was then analyzed 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. The y axis is the same for both \( Me_{SO} \)- 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 after incubation with 25 \( \mu \)M U0126 or \( Me_{SO} \) (DMSO) for the indicated times. B and C, 1.5 \( \times \) 10\(^5\) MCF-7 cells were plated per 90-mm dish and 48 h later were re-fed with medium containing 25 \( \mu \)M U0126 or \( Me_{SO} \). After a further 24 h 200 \( \mu \)M 5-FU was added where indicated, all dishes were incubated for a further 4 h before cell pellets were made for Western blotting analysis or 6 h before plates were trypsinized, and 2 \( \times \) 10\(^4\) cells were 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 (\( hdm2\text{Luc03} \)) and refed with medium containing 25 \( \mu \)M U0126 (solid bars) or \( Me_{SO} \) carrier (open bars) plus the indicated concentration of 5-FU. Reporter gene activity was assayed 40 h later. Results are the mean \( \pm \) S.D. of duplicate dishes and are representative of two independent experiments.
transfection cells were incubated for 48 h in the presence of 25 μM MutantTIP (M-TIP), or dominant negative (Ala-143) mutant p53. After 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 (Ala-143) mutant p53. After transfection cells were incubated for 48 h in the presence of 25 μM U0126 (solid bars) or Me2SO control (open bars) before reporter activity was assayed. Data are the mean ± S.D. for duplicate dishes. Results are representative of greater than three separate experiments. RLU, relative luciferase units.

**FIG. 8.** MEK signaling 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 as follows. 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 2) transcription and 3) nuclear export 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 signaling cascade. Note that this diagram does not include the MEK-dependent activation of the Hdm2 antagonist, p14ARF, which is not expressed in MCF-7 breast cancer cells.

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; first, transfection 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); second, the up-regulation of the p53-induced protein, p21WAF1, by a chemical inhibitor of the Hdm2-p53 interaction, Nutlin-3, was inhibited by U0126, the Nutlin-3-induced increase in Hdm2 protein levels also being partially attenuated. The stabilization of p53 protein by Nutlin-3 was not inhibited by U0126, suggesting that the effects of U0126 on p53 function occur at the post-translational level. Consequently, signaling through MEK kinase impinging 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 signaling pathways. Increased stability and nuclear localization of Hdm2 protein after its phosphorylation by v-akt murine thymoma viral oncogene homolog 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 and Deppert (51) 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 signaling 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 signaling (25). Therefore, the findings of Mosner and Deppert (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 signaling or MEK activity has also been shown to down-regulate Hdm2 protein expression in human cells (25, 47). However, although 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 partially 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% after 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 after 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 characterized (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 organized and exported from the nucleus as functionally related groups by RNA-binding proteins (57). Whether such pathways might be dependent on MEK signaling 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 signaling to modulate chemo- and radio-sensitivity in cancer cells. MEK-dependent expression of proteins such as Hdm2, Bel-X<sub>L</sub>, and inhibitors of apoptosis proteins 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<sup>ARF</sup> 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 that 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, because 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), although the inhibition of other post-translational modifications, or co-operating transcriptional activators, should also be considered. Although MEK inhibitors can also inhibit transcription of p53 mRNA (61), this is unlikely to be the mechanism of p53 inactivation in our experiments, because 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 signaling through the Ras-Ras-MEK-ERK signaling 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 G<sub>1</sub> arrest phase (data not shown); this is associated with a loss of p53 activity, possibly due to reduced serine 15 phosphorylation 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. After activation of Ras-Raf-MEK-ERK signaling 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 signaling 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 tumor 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<sup>α</sup>−<sup>α</sup>, compared with ER<sup>α</sup>−<sup>α</sup>, phenotypes, correlates with transcription from the P2 promoter of the hdm2 gene in the ER<sup>α</sup>−<sup>α</sup> cells (37). In breast tumor samples, Hdm2 protein is overexpressed compared with 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 tumor (34). Additionally, one study determined that, of the tumors in which hdm2 mRNA was up-regulated, only 69% showed Hdm2 protein overexpression (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 tumor environment, which affects signaling through the Ras-Raf-MEK-ERK cascade, superimposes upon the transcriptional phenotype by regulating Hdm2 protein expression at the level of nuclear export of its mRNA. Although our study has focused specifically on breast cancer cell lines, it is likely that this pattern of Hdm2 regulation will be prevalent among many cancer types in which Hdm2 protein is highly expressed.

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