Insulin-like growth factor (IGF)-I receptor activation leads to enhanced proliferation and cell survival via the MAP kinase and phosphatidylinositol 3-kinase-signaling pathways. Upon stimulation by IGF-I, the Hdm2 oncprotein is phosphorylated by AKT, leading to its rapid nuclear translocation and subsequent inhibition of p53. We now show that IGF-I stimulation regulates the nuclear export of Hdm2 and p53 via the MAP kinase pathway. Inhibition of p38 MAPK or MEK via pharmacological means or expression of dominant negative proteins inhibited the cytoplasmic accumulation of Hdm2 and increased Hdm2 and p53 protein levels, whereas constitutively active p90Rsk promoted the nuclear export of Hdm2.

Expression of constitutively active p90Rsk with E1A, oncogenic H-Ras, and hTERT resulted in the anchorage-independent growth of normal human fibroblasts. Our findings link p90Rsk-mediated modulation of Hdm2 nuclear to cytoplasmic shuttling with the diminished ability of p53 to regulate cell cycle checkpoints that ultimately leads to transformation.

It is well accepted that loss of the p53 signaling pathway, either by mutation or loss of upstream or downstream signaling components, occurs in the vast majority of human cancers. Loss of p53 eliminates a number of barriers that prevent transformation and tumor progression, including protective apoptotic or arrest signals (depending upon cell type) and repression of genes involved in angiogenesis and metastasis (1). The p53 protein is regulated in normal human cells by an autoregulatory feedback loop with Hdm2. The hdm2 gene is a transcriptional target of p53, and the subsequent p53-dependent increase in the levels of Hdm2 protein destabilizes p53 by conjugating ubiquitin to p53, leading to its proteosomal degradation (2).

An ever increasing number of signaling events are being implicated in regulating the p53-Hdm2 autoregulatory feedback loop. First, Hdm2 must undergo nuclear translocation, a process mediated by the PI3K/Akt-dependent phosphorylation of the nuclear localization sequence of Hdm2 (3–5). Once phosphorylated by AKT, the entrance of Hdm2 into the nucleus occurs rapidly. Second, Hdm2 must be able to exit the nucleus, a process mediated by CRM1 (6, 7). Although the activity of CRM1 is necessary for Hdm2 export, the events regulating CRM1-mediated Hdm2 export remain unclear. Finally, in response to DNA damage, both p53 and Hdm2 become phosphorylated at numerous residues, leading to the disruption of the feedback loop, stabilization of p53, and execution of p53-dependent responses (8). It is estimated that 50% of human malignancies have inactivating or gain-of-function mutations in the p53 gene itself, leaving 50% that maintain wild-type p53, but a functionally inactive signaling pathway. In many cases, the Hdm2 protein is overexpressed, leading to an imbalance in the autoregulatory loop, increased p53 degradation, and decreased tumor suppression (9). For this reason, Hdm2 remains an attractive target for therapeutic intervention in tumors harboring wild-type p53, as inhibition of Hdm2 would increase p53-dependent tumor suppression activities.

Similarly to p53, the aberrant activation of receptor-mediated signal transduction pathways also plays an integral role in the establishment and maintenance of many types of tumors. Hyperactive signaling is accomplished by the overexpression of growth factors or receptors that feed into the RAS pathway leading to hyperproliferation and a diminished ability to undergo apoptosis (10). Increasing attention is being given to insulin-like growth factor (IGF) signaling, as recent epidemiological studies indicate that increased circulating IGF-I levels put individuals at higher relative risk for developing numerous types of cancer including colon, prostate, breast, lung, and bladder cancer with enhanced growth and metastatic potential (11, 12).

Links between IGF-1 signaling and p53 continue to emerge. For example, IGF-I-induced cell division correlates with the phosphorylation and nuclear exclusion of p53 (13). Importantly, activation of signaling molecules downstream of the IGF-I receptor regulate various aspects of the p53-Hdm2 axis. As described above, PI3K-AKT activation regulates Hdm2 nuclear import (3–5). In addition, activation of p38 MAPK disrupts the interaction between Mdm2, the murine form of Hdm2, and p19ARF leading to enhanced p53 degradation in response to DNA damage signaling (14). Recently, MAPKAP kinase 2 (MK2) was also shown to contribute to the activation of Hdm2 (15). The phosphorylation of Hdm2 on serines 157 and 166 by MK2 increased the degradation of p53, making cell less susceptible to DNA damage-induced apoptosis. Ras-mediated signaling has also been reported to control the transcription of p53, Mdm2, and p14ARF (16–18). An imbalance in the opposing tumor suppression and oncogenic signaling cascades leads to...
incremental changes in the ability of a cell to respond to insults, leading to tumorigenesis when an oncogenic change is favored.

We show here that IGF-I stimulation leads to the accumulation of cytoplasmic Hdm2. The redistribution of Hdm2 and p53 to the cytoplasm from the nucleus promotes p53 degradation and is dependent upon the activation of MAP kinases in primary and cancer cell lines. We demonstrate that the signal through the MAP kinase pathway converges on p90Rsk, a downstream mediator of ERK signaling. The expression of a constitutively active p90Rsk alone is sufficient to promote the nuclear to cytoplasmic shuttling of both Hdm2 and p53. Furthermore, in conjunction with E1A, oncogenic H-Ras and hTERT, constitutively active p90Rsk, but not kinase dead p90Rsk, promotes the anchorage-independent growth of human fibroblasts. Thus, the IGF-I/MAPK/p90Rsk pathway, mediating Hdm2-p53 nuclear to cytoplasm shuttling, links p90Rsk activation with increased tumorigenic potential in cells with wild-type p53.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—U2OS and H1299 cells were grown in a humidified atmosphere containing 5% CO2 in Dulbecco’s minimal essential medium (Invitrogen), supplemented with antibiotics and 10% bovine growth serum (Hyclone, Logan UT). IMR90 fibroblasts were grown similarly with the exception that 10% fetal bovine serum and 10% bovine growth serum (Hyclone, Logan UT). IMR90 fibroblasts were grown in a humidified atmosphere containing 0.5% CO2 in MCDB 170 medium (HEPES based, US Biological, MA) supplemented with epidermal growth factor, hydrocortisone, insulin, bovine pituitary extract, transferrin, isoproterenol, ethanolamine, O-phosphoethanolamine, and antibiotics as described (Hammond et al. 25). Additional details of HME media components can be provided on request.3 PD98059 and SB 203580 were purchased from LC laboratories (Woburn, MA) and used at final concentrations of 25 and 20 μM, respectively.

**Confocal Microscopy**—MCF-7 Cells were fixed in 3% paraformaldehyde in PBS for 15 min, washed with PBS, and permeabilized in 1% Triton X-100 in PBS for 15 min. Slides were blocked with 5% fetal bovine serum in PBS-Tween and incubated with a polyclonal antibody to Hdm2 (H-221, Santa Cruz biotechnology, Santa Cruz, CA), followed by an anti-rabbit Cy5 secondary antibody (Jackson Labs). Slides were washed with PBS-T following primary antibody and secondary antibody, incubated with Syto 16 as described previously (4). For Fig. 4 confocal MCF7 cells were fixed as described above, and Hdm2 was detected by a mixture of monoclonal antibodies 2a10 and IF2 (oncogene sciences) and a secondary anti-mouse Alexa 488 (Molecular Probes). Nuclei were stained with Draq5 (Alexandria Platform), and slides were mounted with non-fading gel-mount prior to visualization on a Zeiss multiphoton microscope.

**Transfections and Western Blotting**—Whole cell extracts were prepared using lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (octylphenoxy)polyethoxyethanol (IGEPAL), 10 μl/ml protease inhibitor mixture, and 1 μM sodium metavanadate). Nuclear and cytoplasmic extracts were isolated by using Buffer A (50 mM Heps, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1% Nonidet P-40 lysis buffer 10 μl/ml protease inhibitor mixture, and 1 μM sodium metavanadate) to isolate the cytoplasmic fraction and Buffer C (50 mM Heps, pH 7.4, 10% glycerol, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 μl/ml protease inhibitor mixture, and 1 μM sodium metavanadate) to isolate the nuclear fraction.

To examine Hdm2-mediated p53 degradation, H1299 cells were transiently transfected using calcium phosphate precipitation of p53 (0.5 μg). Hdm2 (5 μg), kd-p90Rsk or empty vector (7 μg). Cells were harvested 24 h after transfection, and whole cell extracts were prepared as described above. To examine the effect of dn-Erk1, dn-p38, ca-p90Rsk, and kd-p90Rsk on endogenous Hdm2, H1299 cells were transiently transfected with 20 μg of each plasmid (empty vector used for control). Cells were harvested 24 h after transfection, and nuclear and cytoplasmic extracts were prepared as described above.

Extracts containing equal quantities of proteins, determined by the Bradford method, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8–12.5% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Antibodies to p53 (DO-1), tubulin (TU-02), PARP (F-2), and anti-hemagglutinin (F-7) were purchased from Santa Cruz (Santa Cruz, CA). Hdm2 antibodies 2A10, IF2, and 4B11 were from Oncogene Research Products (La Jolla, CA), and anti-FLAG M2 was purchased from Sigma. Primary antibodies were detected with goat-anti-mouse or goat-anti-rabbit antibody conjugated to horseradish peroxidase (Hoffman-La Roche), using enhanced chemiluminescence (PerkinElmer Life Sciences).

**Amphotropic Retroviral Production and Anchorage-independent Growth Assays**—To create stable lines of IMR90 fibroblasts expressing H-Ras-V12 (pBabe-Bleo), E1A (pLPCX), ca-p90Rsk, and/or kd-p90Rsk (cloned into pLNCX2), amphotropic retroviruses encoding the cDNA of interest were packaged in Phoenix-Ampho cells by Lipofectamine Plus-mediated transient transfection. Virus containing supernatants were collected at 36–48 h, supplemented with 4 μg/ml Polybrene, and filtered through a 0.22-μm filter. The filtered supernatants were added to cells overnight. Uninfected cells were removed by selection with G418 (400 μg/ml), puromycin (1 μg/ml), or zeocin (200 μg/ml). Selected populations of infected cells were resuspended in 0.6% Type VII-agarose (Sigma) and plated onto agar on a bottom layer of 1.0% agar. 1 × 10⁵ cells were used for each 60-mm dish.

**RESULTS**

**IGF-I Stimulation Promotes Hdm2-mediated p53 Export and Degradation, Dependent upon the MAP Kinase Pathway**—Although recent studies have demonstrated that PI3K-Akt signaling promotes the phosphorylation and movement of Hdm2 into the nucleus, leading to the down-regulation of p53, little is known about the mechanisms that may regulate the export of Hdm2. To more closely examine the kinetics of Hdm2 nuclear-cytoplasmic regulation we treated serum-starved MCF7 cells with IGF-I and monitored Hdm2 localization by confocal microscopy either 5 or 30 min after IGF-I addition. As previously described, treatment with IGF-I induced the rapid nuclear accumulation of Hdm2 (4). In contrast, when Hdm2 was examined 30 min after IGF-I stimulation, cytoplasmic accumulation was observed in ~20% of the cells (Fig. 1a). In agreement with the immunostaining data, IGF-I stimulation resulted in a time-dependent increase in cytoplasmic Hdm2 as measured by Western analysis of nuclear and cytoplasmic extracts isolated from normal human mammary epithelial cells immortalized by the hTERT subunit of telomerase (hTERT-HME1) and primary human foreskin fibroblasts (BJ; Fig. 1, b and c). Upon IGF-I stimulation, Hdm2 cytoplasmic localization increased within 30–60 min in both cell lines and began to decrease between 2 and 4 h (Fig. 1, b and c). Interestingly, the localization of HdmX, a protein with significant homology to Hdm2, remains mostly cytoplasmic and relatively stable throughout the time course of IGF-I stimulation of BJ fibroblasts indicating that HdmX is not involved in the translocation of Hdm2 in response to IGF-I (Fig. 1c).
IGF-I-mediated Hdm2 Nuclear Export

The increase in cytoplasmic Hdm2 could result from either an increase in Hdm2 nuclear export or a decrease in import; we examined whether leptomycin B, an inhibitor of CRM1-mediated nuclear export, could prevent the IGF-I-mediated cytoplasmic accumulation of Hdm2. We also assessed whether MAP kinase pathway inhibitors could prevent the cytoplasmic accumulation of Hdm2, because growth factor receptor activation leads to activation of Ras and its downstream effectors, including the MAPK pathway. Serum-starved U2OS cells were stimulated with IGF-1 alone or following pretreatment with SB 203580, a p38 MAPK inhibitor, or leptomycin B, which inhibits CRM1-mediated nuclear export. With both inhibitors, the localization of Hdm2 remained predominantly nuclear, indicating that Hdm2 nuclear export is dependent upon MAPK signaling and CRM1-dependent nuclear export (Fig. 2a). We confirmed this finding in hTERT-HME1 cells. Again, treatment of growth factor-depleted hTERT-HME1 cells with IGF-1 for 60 min increased the amount of cytoplasmic Hdm2 (Fig. 2b). In contrast, movement of Hdm2 into the cytoplasm was inhibited when cells were pretreated with SB 203580 or PD98059, a MEK inhibitor, for 60 min prior to IGF-1 stimulation. Importantly, Akt activation as measured by anti-phospho-473 remained robust in the cells treated with PD98059. The continued activation of Akt indicates that inhibitors of the MAP kinase pathway do not alter the signal known to induce Hdm2 nuclear import. In addition, treatment with a proteasome inhibitor further increased the fraction of cytoplasmic Hdm2 relative to IGF-1 alone indicating that Hdm2 degradation occurs in the cytoplasm following IGF-I stimulation (Fig. 2a).

Because PD98059 and SB 203580 could inhibit the cytoplasmic accumulation of Hdm2 following IGF-I stimulation, we examined whether dominant negative forms of the MAP kinase signaling proteins could affect Hdm2 localization and function. H1299 cells were transiently transfected with a dominant negative Erk1 (dn-Erk1) or dominant negative p38 (dn-p38), and nuclear and cytoplasmic extracts were prepared consistently observed that, after 4 h of IGF-1 stimulation, cytoplasmic Hdm2 decreased to levels seen under normal growth conditions.
for Western analysis to examine endogenous Hdm2 levels. Following transfection of either dn-Erk1 or dn-p38, Hdm2 became disproportionately localized within the nucleus, suggesting that Hdm2 export is inhibited by disruption of MAPK signaling (Fig. 2c).

Hdm2 Is Phosphorylated by MAP Kinases—One explanation for the ability of p38, Erk1, and Erk2 to alter Hdm2 nuclear export and inhibit p53 degradation is that a MAPK pathway-dependent phosphorylation event changes the conformation of Hdm2 and prevents efficient nuclear export. To test whether Hdm2 serves as a substrate for MAPKs, recombinant Hdm2 protein in which serines 166 and 186 adjacent to the nuclear localization signal were mutated to alanine (Hdm2-2xSA) was incubated with immunoprecipitated Erk1/2, p38, or MK2, together with p32ATP and physiologic buffer. Neither Erk1/2 nor p38 MAPK could directly phosphorylate Hdm2, only MK2 kinase, which is downstream of p38 and Erk1/2, phosphorylated Hdm2 in vitro (Fig. 2d). In agreement, MK2 was recently shown to phosphorylate Hdm2 at serines 157 and 166 in vitro (15). Additional studies examined Hdm2 phosphorylation by downstream effectors of Erk1/2 and p38 including p90Rsk, Msk, and MK3. Recombinant Abl was included as a positive control (19). Again, each of the precipitated kinases efficiently phosphorylated recombinant Hdm2 in vitro (Fig. 2d).

Having shown that Hdm2 was phosphorylated by members of the MAP kinase family, we set out to determine which MAPKAP kinase family member may be responsible for the nuclear-cytoplasmic shuttling of Hdm2 in cells. To determine whether p90Rsk, Msk, and MK2 were activated, we used phospho-antibodies (Ser-363 p90Rsk, Ser-376, Thr-581 Msk1, Thr-334 MK2) to monitor activation following stimulation of H1299 cells with combinations of plasmids encoding p53, Hdm2, and a series of dominant negative (dn) mutants involved in MAPK signaling. Expression of dn-p38, dn-Erk1, dn-Erk2, or kinase-dead p90Rsk all inhibited the Hdm2-mediated degradation of p53 (Fig. 5, a and b). In immortalized mouse embryo fibroblasts lacking both p53 and Mdm2, transient expression of p53 was not altered by the dominant negative proteins upon cotransfection, arguing that their effect requires Hdm2/Mdm2 (Fig. 5c).

To determine the consequence of inhibiting Hdm2 nuclear export on p53 levels, p53 degradation assays were performed by transient transfection of H1299 cells with combinations of plasmids encoding p53, Hdm2, and a series of dominant negative (dn) mutants involved in MAPK signaling. Expression of dn-p38, dn-Erk1, dn-Erk2, or kinase-dead p90Rsk all inhibited the Hdm2-mediated degradation of p53 (Fig. 5, a and b). In immortalized mouse embryo fibroblasts lacking both p53 and Mdm2, transient expression of p53 was not altered by the dominant negative proteins upon cotransfection, arguing that their effect requires Hdm2/Mdm2 (Fig. 5c).

To better understand the biological consequence of p90Rsk-signaling to Hdm2, we examined whether constitutively active p90Rsk could cooperate to transform normal human fibroblasts. It was recently shown that coexpression of E1A, oncogenic H-Ras (V12), and Mdm2 resulted in anchorage-independent growth and Mdm2-dependent tumor formation in nude mice (20). Additional studies from our laboratory indicate that the oncogenic activity of Mdm2 in this model system in specifically because of inhibition of p53-dependent apoptosis.
To examine whether hyperactive p90Rsk signaling can enhance the activity of endogenous Hdm2, making it more potent at inhibiting p53 and thus more tumorigenic, we coexpressed ca-p90Rsk or kd-p90Rsk in IMR90 fibroblasts expressing E1A, H-ras-V12, and hTERT. Selected populations of cells were plated into soft agar and grown for 2 weeks. Only the constitutively active form of p90Rsk cooperated to permit anchorage-independent growth in this system (Fig. 6). This observation indicates that the endogenous Hdm2 is sufficient to inhibit p53-dependent apoptosis and drive tumorigenesis when inappropriate signaling events are activated.

We have performed the same experiments using Mdm2 overexpression, finding that Mdm2 yields ~10-fold more colonies of about twice the size as those described here with ca-p90Rsk. Thus, the balance of nuclear and cytoplasmic Hdm2 may well decide the fate of a cell on the verge of transformation and will probably be decided by signaling through the MAPK pathway.

**DISCUSSION**

Tumorigenesis is a multistep process in which the checkpoints that regulate cellular division and survival become compromised or dismantled. The p53 pathway prevents the formation and proliferation of aberrant cells by inducing cell cycle arrest or apoptosis in response to various stress signals. Loss of p53 function can occur by mutation of the gene, inactivation of upstream activators, or overexpression or hyperactivation of negative regulators. In this study, we identified a novel function for the MAPK pathway, regulation of the nuclear export of Hdm2, and ultimately p53 inhibition. We showed that Hdm2 accumulates in the cytoplasm within 30 min of IGF-I stimulation and that pharmacological blockade or expression of certain dominant negative proteins prevents the accumulation of cytoplasmic Hdm2. Although it is possible that the regulation of Hdm2 could result from increased synthesis or decreased cytoplasmic degradation, the observations presented here using cells stably expressing Rsk as well
as cells transiently treated with IGF-1 (for 30 min), very different treatments, make this explanation improbable.

Our findings extend the observation that IGF-1 signaling induces Hdm2 function, arguing that PI3K-AKT signaling is not sufficient to activate Hdm2 and that MAPK signaling is an additional requirement for Hdm2-mediated p53 inactivation. Understanding the mechanisms that govern the down-regulation of p53 in response to growth factor signaling will help identify possible targets of therapy in cells harboring wild-type p53 or hyperactive Hdm2 activities because of constitutive receptor signaling.

We found that p38 and Erk1/2 were unable to phosphorylate Hdm2 directly, but that downstream kinases p90Rsk, Msx, and MK2 could. However, p90Rsk was the only kinase activated during the time course of IGF-1 stimulation used in our studies. Indeed, we found that Hdm2 interacts with p90Rsk and that overexpression of constitutively active p90Rsk leads to greater Hdm2 nuclear cytoplasmic shuttling, whereas kinase-dead p90Rsk maintains a greater portion of Hdm2 within the nucleus.

We extended the observation made using transient stimulation of serum-starved cells with purified growth factors to cells with stable expression of activated p90Rsk. Importantly, activated p90Rsk accentuates the functions of endogenous Hdm2, promoting the anchorage-independent growth of normal IMR90 fibroblasts expressing E1A, Ras, and hTERT, although less efficiently than Mdm2 overexpression itself. Our previously published results using this model system indicate that p53 is responsible for preventing anchorage-independent growth and that the transformation observed with Hdm2 can be inhibited upon treatment with Nutlin-3, an Hdm2 antagonist (21). Thus, we hypothesize that changes in the activities of receptor-mediated signaling networks, such as those activated by IGF-1, directly influence the tumor suppression networks involving p53 and perhaps others, such as p73, Rb/E2F, and HIF1a via modulation of Hdm2 activities.

It is important to note that our experiments uncovered three enzymes capable of phosphorylating Hdm2, including p90Rsk, Msx, and MK2. Although we have examined p90Rsk more closely because of its activation kinetics upon IGF-1 stimulation, one can imagine that different stimuli may lead to alternative levels of activation for each of these kinases, with the ultimate outcome being down-regulation of p53 and predisposition to carcinogenesis. Indeed, a recent paper demonstrates that phosphorylation of Hdm2 by MAPKAP kinase 2 enhances p53 degradation, although the authors noted only a slight decrease in Hdm2 serine 166 phosphorylation upon treatment with SB 203580 (15). Our data are consistent with this observation in that SB 203580 inhibits Hdm2 nuclear export rather than nuclear import. The diverse pathways that may modulate various molecules involved in MAP kinase signaling will undoubtedly have an effect on Hdm2 functions by regulating its subcellular localization.

Recent work has also shown that the MAPK pathway mediates hdm2 gene expression via AP1-Ets factors and regulates the localization of hdm2 mRNA (22, 23). This phenomenon requires a much longer time to develop and is not likely to impact the results presented here, which occur in a matter of hours. However, taken together these data illustrate the importance of MAPK signaling in regulating Hdm2 and p53 protein levels during normal cell cycle progression. Furthermore, Hdm2 has been shown to associate with components of the translational machinery and to bind to RNA. One can envision an integrated MAPK pathway in which cytoplasmic-to-nuclear shuttling of Hdm2 regulates the stability of p53, with an additional level of regulation relating to the translational machinery necessary to synthesize Hdm2 and other components. Further work will be needed to demonstrate such a mechanism.

Studies have linked high circulating levels of IGF-1 with an increased cancer incidence (11, 12), an observation likely because of the ability of IGF-1 receptor activation to transduce signals through both the MAP kinase and PI3K/Akt pathways. Previous studies from our laboratory and others have demonstrated that PI3K signaling to Akt promotes the nuclear import of Hdm2 by phosphorylation of serines 166 and 186 within the nuclear localization signal (3–5). The results presented here argue that activation of the MAPK pathway by IGF-1 plays an equally important role to promote Hdm2 nuclear export. We are currently working to decipher the sites affected by the downstream effectors of the MAPK pathway. Thus, the ultimate regulation of Hdm2 lies in the overall balance between PI3K/Akt activation and MAP kinase pathway activation.

Recent evidence that a SNP within the Hdm2 promoter results in a modest increase in Hdm2 protein that correlates with significant increase in cancer incidence underlies the point that a small shift in the import/export pathway in favor of Hdm2 export may have drastic consequences on the ability of p53 to eliminate aberrant cells (24). This point was clear when we examined the anchorage-independent growth of normal human fibroblasts expressing E1A, Ras, and hTERT. When combined with E1A and Ras, Mdm2 promotes the anchorage-independent growth of normal diploid fibroblasts (20). The contribution of Mdm2 to human transformation likely centers around its ability to inhibit p53 activity, although p53-independent functions for Mdm2 have been reported that likely also influence tumorigenesis. In our experiments, numerous small colonies were observed in cells expressing E1A, Ras, and hTERT only when active p90Rsk was present as well. Based on additional work with this transformation system, we know that anchorage-independent growth can also occur by specific inhibition of p53 by stable RNA interference, indicating that p53 alone is the final constraint preventing cells expressing E1A, Ras, and hTERT from growing in soft agar.

Unfortunately, most human cells will not tolerate knock-down or ablation of Hdm2 without also inhibiting p53. For the cells described in Fig. 6, our recently published findings show that treatment with an Hdm2 antagonist (Nutlin-3) results in p53-dependent apoptosis when grown on plastic or in soft agar (21). Although indirect, this provides evidence that Hdm2 is a major component preventing p53-dependent apoptosis and argues, collectively with the other data presented; that constitutively active Rsk augments Hdm2 activity to permit anchorage-independent growth. Thus, our work provides a mechanism whereby elevated MAPK signaling will dramatically affect the p53-Hdm2 axis in favor of attenuating p53 activity, which contributes to the progression of tumor formation and may provide a novel point for therapeutic intervention.

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**IGF-I-mediated Hdm2 Nuclear Export**

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