Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein

Judith Roth^{1,2}, Matthias Döbelstein^{3,4}, Deborah A. Freedman¹, Thomas Shenk³ and Arnold J. Levine^{1,5}

¹Department of Molecular Biology and ²Howard Hughes Medical Institute, Princeton University, Lewis Thomas Laboratory, Princeton NJ 08544-1014, USA

³Present address: Klinikum der Universität Marburg, ZIM, Gastroenterologie, Baldinger Str. 35033 Marburg, Germany

⁴Present address: Institut für Virologie, Universität Marburg, Robert-Koch-Str. 17, 35037 Marburg, Germany

⁵Corresponding author

e-mail: alevine@molbio.princeton.edu

J. Roth and M. Döbelstein contributed equally to this work

The *hdm2* gene is overexpressed in a variety of human tumors. Its gene product localizes predominantly to the nucleus, where it acts as an inhibitor of the p53 tumor suppressor gene product. It is shown here that the *hdm2* oncoprotein constantly shuttles between the nucleus and the cytoplasm. Shuttling of *hdm2* does not depend on its interaction with p53. Nuclear export of *hdm2* is mediated by a signal sequence similar to the nuclear export signal of the rev protein from human immunodeficiency virus and other lentiviruses. Mutation of this signal sequence abolishes detectable nucleo-cytoplasmic shuttling. When fused to a carrier protein, the *hdm2* signal sequence can mediate nuclear export after intranuclear microinjection into HeLa cells. The export of *hdm2* can be blocked by a competitive inhibitor of rev export, arguing that the export pathways for *hdm2* and rev are either overlapping or identical. Inhibition of its export modifies the ability of *hdm2* to block p53-mediated transcriptional activation, and *hdm2*'s export function is required to accelerate the degradation of p53. Thus the rev nuclear export pathway may be used to regulate an oncogene product's activity and modulate cellular growth.

Keywords: *hdm2*/HIV/nucleo-cytoplasmic transport/oncogene/p53

Introduction

The *hdm2* oncoprotein is a cellular antagonist of the p53 tumor suppressor gene product (Momand *et al.*, 1992). It antagonizes transcriptional activation by p53 (Momand *et al.*, 1992; Chen *et al.*, 1995) as well as p53-induced growth inhibition (Finlay, 1993), G₁ arrest and apoptosis (Chen *et al.*, 1996). This is accomplished by binding of *hdm2*'s N-terminal region to the N-terminal transactivation domain of p53 (Chen *et al.*, 1993; Oliner *et al.*, 1993; Haines *et al.*, 1994; Lin *et al.*, 1994; Kussie *et al.*, 1996). On the other hand, p53 activates transcription of *hdm2*,

creating an autoregulatory feedback loop (Wu *et al.*, 1993). *hdm2* is overexpressed in a variety of human tumors (Oliner *et al.*, 1992; Bueso-Ramos *et al.*, 1993; Ladanyi *et al.*, 1993; Leach *et al.*, 1993; Reifemberger *et al.*, 1993; Cordon-Cardo *et al.*, 1994), due to gene amplification, enhanced transcription and/or augmented translational efficiency (Barak *et al.*, 1994; Berberich and Cole, 1994; Landers *et al.*, 1994; Zauberman *et al.*, 1995). *hdm2* not only inactivates p53 but also accelerates its intracellular degradation, and overexpression of *hdm2* reduces the amount of intracellular p53; this degradation is apparently mediated, at least in part, by the ubiquitin-proteasome pathway (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). In addition to p53, other cellular proteins have been identified that physically interact with *hdm2*, including the L5 ribosomal protein (Marechal *et al.*, 1994), the Rb tumor suppressor gene product (Xiao *et al.*, 1995) and the E2F-1 growth-regulating transcription factor (Martin *et al.*, 1995). *hdm2* has also been found to interact with RNA through its C-terminal RING finger region (Elenbaas *et al.*, 1996). These multiple interactions suggest that *hdm2* could play a role in growth regulation and oncogenicity using pathways other than its inactivation of p53 transcriptional activity. However, mice deficient for *mdm2*, the murine homolog of *hdm2*, are not viable unless they lack the p53 gene as well (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995), arguing that the most crucial function of *mdm2* and *hdm2* during early development is the regulation of p53.

The adenoviral E1B-55 kDa protein interacts with the same region on p53 as *hdm2* (Lin *et al.*, 1994) but, during adenovirus infection, it has additional functions. Together with the adenoviral E4-34 kDa protein, it forms a complex (Sarnow *et al.*, 1984) and modulates the transport of mRNA, up-regulating the movement of viral mRNA from the nucleus to the cytoplasm, but simultaneously halting the export of most cellular mRNA species into the cytoplasm (Babiss *et al.*, 1985; Halbert *et al.*, 1985; Pilder *et al.*, 1986; Shenk, 1996). It has been shown recently that the E4-34 kDa protein mediates the nucleo-cytoplasmic export of a complex that consists of E1B-55 kDa, E4-34 kDa and possibly cellular proteins and RNA molecules. This nucleo-cytoplasmic export is mediated by a nuclear export signal (NES) on a pathway that overlaps with the export mechanism for the rev proteins of the human immunodeficiency virus (HIV) and other lentiviruses (Döbelstein *et al.*, 1997). These findings lead to the speculation that the analogy between *hdm2* and E1B-55 kDa might go beyond their association with the p53 protein and include the possibility that *hdm2* undergoes a nuclear export-import cycle.

The nuclear export of the lentiviral proteins rev and rex is mediated by discrete signal sequences (Fischer *et al.*, 1995; Palmeri and Malim, 1996), analogous to previously

identified nuclear import signals (Boulikas, 1993). Such NESs are used by a number of viral and cellular proteins (Gerace, 1995; Izaurrealde and Mattaj, 1995; Simos and Hurt, 1995; Gorlich and Mattaj, 1996; Moore, 1996). Their mechanism of action is currently under intense investigation, and a number of candidates for mediators of nuclear export have been identified (Bogerd *et al.*, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995; Bevec *et al.*, 1996). The nuclear export mediated by a rev-like NES occurs on a pathway that is used to export 5S rRNA but not mRNA or tRNA (Guddat *et al.*, 1990; Jarmolowski *et al.*, 1994; Fischer *et al.*, 1995). An export signal with apparently different structure and functional activities was identified within the mRNA-associated hnRNP A1 protein (Michael *et al.*, 1995), and presumably this contributes to the export of mRNA. Two vertebrate cellular proteins have been reported to be a substrate of the rev pathway nuclear export: the TFIIIA protein which contains an NES (Fridell *et al.*, 1996) and can mediate the export of 5S rRNA (Guddat *et al.*, 1990) and the protein kinase inhibitor PKI (Wen *et al.*, 1995) which regulates cAMP-dependent protein kinase activity. Thus the use of NESs and the rev-related export pathway has so far been implicated in RNA export and kinase regulation, but it is unclear if other regulatory functions (e.g. growth regulation) are linked to rev-like nuclear export.

This report demonstrates that the hdm2 oncoprotein undergoes nucleo-cytoplasmic export and continuously shuttles between the nucleus and the cytoplasm. An NES sequence confers on hdm2 the ability to shuttle, and this export signal functions autonomously when placed on a carrier protein. The export of hdm2 can be blocked with a competitive inhibitor of the rev export pathway, suggesting that hdm2 exits the nucleus on a pathway similar or identical to that of the HIV and human T cell lymphotropic virus (HTLV) RNA export mechanism. Inhibition of hdm2's export strongly reduces its ability to destabilize p53 and modulates its inhibitory effect on p53-mediated transactivation, arguing that nuclear export might serve to regulate hdm2's oncogenic potential.

Results

hdm2 shuttles between the nucleus and cytoplasm

When hdm2 is transiently expressed in mammalian cells, it localizes predominantly to the nucleus (Chen *et al.*, 1995). To determine whether hdm2 remains stably in the nucleus or shuttles between the nucleus and cytoplasm, a heterokaryon assay was performed (Pinol-Roma and Dreyfuss, 1992). HeLa cells were transfected with an hdm2 expression plasmid (Oliner *et al.*, 1993) using electroporation and were allowed to synthesize hdm2 protein. These cells were subsequently fused to murine cells (Balb/c 3T3) and incubated at 37°C while blocking *de novo* protein synthesis with cycloheximide 15 min prior to the fusion and throughout the experiment. Forty five minutes after fusion, the cells were fixed and stained for hdm2 (Figure 1A, a). To distinguish between human and murine nuclei, the cells were stained simultaneously with an antibody that recognizes human but not murine Ku antigen (Figure 1A, b), a nuclear autoantigen that is part of the DNA-dependent protein kinase enzyme

Fig. 1. hdm2 shuttles between the nucleus and cytoplasm. (A) Nucleo-cytoplasmic shuttling of hdm2. HeLa cells were transiently transfected with an expression plasmid for hdm2 (Oliner *et al.*, 1993). Eighteen hours after transfection, the cells were fused to murine Balb/c 3T3 cells, while *de novo* protein synthesis was blocked by cycloheximide. Forty five minutes after the fusion, the cells were fixed and stained with antibodies against hdm2 (a) and human Ku antigen (b). The heterokaryon cells were photographed using immunofluorescence (a and b) and phase contrast (c) optics. (B) Effect on shuttling of mutations in hdm2 that abolish p53 binding. Expression constructs encoding wild-type (a–c) or mutant hdm2 with the amino acid changes G58A (d–f), D68A (g–i) and V75A (j–l) were assayed for shuttling as described above.

(Gottlieb and Jackson, 1993). hdm2 could be found in those murine nuclei that were part of a heterokaryon containing a transfected human nucleus, as judged by phase contrast microscopy (Figure 1A, c). This indicates that hdm2 had moved from the HeLa nuclei to the cytoplasm and subsequently entered the murine nuclei. Thus, hdm2 shuttles across the nuclear membrane in both directions.

Next, a set of hdm2 mutants was assayed for nucleo-cytoplasmic shuttling. The point mutations G58A, D68A and V75A in the hdm2 primary structure each abolish the interaction of hdm2 with p53 (Freedman *et al.*, 1997). Nonetheless, all three mutants shuttled from the human to the murine nuclei in heterokaryon assays, with no observable difference to wild-type hdm2 (Figure 1B).

Fig. 2. Nuclear export of hdm2 is mediated by an export signal. (A) A putative nuclear export signal (NES) in hdm2. A part of the hdm2 primary structure is shown that resembles various known NESs. This sequence element is aligned with known NESs. The hydrophobic residues critical for NES function are shown in bold. Dots are inserted to equalize the pairing between conserved hydrophobic residues in the different motifs. Numbers indicate the position of the amino acids within the proteins. The corresponding sequence elements of the dm proteins from mouse and hamster are shown. A mutant hdm2 was created with the indicated amino acid changes (L205A, I208A). The references for the NES elements are: cAMP-dependent protein kinase inhibitor (PKI) (Wen *et al.*, 1995); HIV-1 rev (Fischer *et al.*, 1995); HTLV-1 rex (Palmeri and Malim, 1996); transcription factor TFIIIA (Fridell *et al.*, 1996); yeast mRNA transport protein Gle1 (Murphy and Went, 1996). (B) Influence of a mutation in the putative NES on hdm2 shuttling. The NES mutant hdm2 was transiently expressed in HeLa cells and assayed for nucleo-cytoplasmic shuttling as described in the legend to Figure 1.

Therefore, hdm2's nuclear export is independent of its ability to bind p53.

hdm2 contains a nuclear export signal that is necessary for shuttling

The hdm2 primary structure contains a sequence element that resembles some of the previously characterized NESs (Gorlich and Mattaj, 1996; Murphy and Went, 1996) and is in close proximity to hdm2's putative nuclear localization signal (NLS) (Chen *et al.*, 1995), as shown in Figure 2A. Hydrophobic residues can be found at identical positions in the mouse and hamster hdm2 homologs. To test whether this sequence element plays a role in hdm2 export, point mutations were introduced into the corresponding codons to change two of the hydrophobic residues within it (Leu205 and Ile208) to alanines (Figure 2A, last line). Such hydrophobic residues have been shown to be crucial for NES function in HIV rev (Fischer *et al.*, 1995), HTLV rex (Bogerd *et al.*, 1996), the human protein PKI (Wen *et al.*, 1995) and yeast Gle1 (Murphy and

Fig. 3. Autonomous function of the hdm2 signal sequence. GST was purified from *E.coli* either unmodified (a and b), fused to the NES from HIV rev (c and d) or fused to the putative export signal sequence from hdm2 (e and f). The proteins were microinjected into the nuclei of HeLa cells along with FITC-coupled dextran 150 (as a marker of the injection site). The injected GST fusion proteins were visualized by immunostaining and a Texas Red-coupled secondary antibody (a, c and e). The co-injected dextran was visualized by the FITC coupled to it (b, d and f).

Wente, 1996). When tested in a heterokaryon assay as described above, this mutant hdm2 was greatly impaired in its ability to shuttle from a human nucleus to the cytoplasm and into a murine nucleus (Figure 2B, a–c). This shows that the sequence outlined in Figure 2A is necessary for, or at least largely contributes to, hdm2's ability to undergo nuclear export.

The hdm2 signal sequence can mediate nuclear export of a carrier protein

To test whether the signal sequence from hdm2 outlined in Figure 2A is not only necessary, but also sufficient to allow nuclear export, we fused this sequence to the carboxy-terminus of a carrier protein, glutathione *S*-transferase (GST). The fusion protein was purified from *Escherichia coli* and microinjected into the nuclei of HeLa cells along with a marker for the injection site, fluorescein isothiocyanate (FITC)-dextran 150. Eight hours after injection, the cells were fixed and stained with an antibody against GST, followed by a Texas Red-coupled secondary antibody. Native GST largely remained in the nucleus, and only a small proportion of the injected protein was found in the cytoplasm (Figure 3a and b), presumably due to slow passive diffusion (Gorlich and Mattaj, 1996). In contrast, GST fused to the NES from rev had undergone virtually complete relocation from the nuclear injection site to the cytoplasm (Figure 3c and d), as shown previously (Meyer *et al.*, 1996). When the hdm2 signal sequence was fused to GST, the resulting fusion protein was exported

from the nucleus to an extent resembling GST fused to the rev NES (Figure 3e and f), indicating that the hdm2 signal sequence can function as an autonomous NES. There were, however, differences in the kinetics of this export; the NES from rev could mediate nearly complete export in less than 1 h, whereas fusion constructs containing the hdm2 NES were completely exported only 5–6 h after injection (not shown). Despite the slower kinetics, the export driven by the hdm2 NES eventually reached completion, with little or no fusion protein left in the injected nuclei (Figure 3e).

The nuclear export of hdm2 occurs on a rev-like pathway

Given the similarities between the NESs of rev and hdm2 (cf. Figure 2A), experiments were designed to find out whether the export of hdm2 and rev occurs on overlapping or even identical pathways. To test this, a known competitive inhibitor of rev export was employed (Katahira *et al.*, 1995), in which the RNA-binding domain of the HTLV1 rex protein is replaced by an NLS from SV40 T antigen (Figure 4A). This construct expresses a fusion protein, NLS–rex, that contains strong signals for nuclear import as well as export. The NLS–rex fusion protein was shown to inhibit rev-mediated export (Katahira *et al.*, 1995), presumably by shuttling rapidly between the nucleus and the cytoplasm and competing for one or several of the components constituting the nuclear export machinery. When co-transfected with a rev expression vector and a rev-dependent reporter construct, NLS–rex markedly inhibited rev-mediated export, as determined by reporter expression (not shown). The inhibitory activity of this fusion construct can be greatly reduced by introducing a point mutation (M90) into the endogenous rex NES sequence (Katahira *et al.*, 1995). NLS–rex was expressed together with an hdm2 expression construct, followed by a heterokaryon assay (Figure 4B). When co-expressed with NLS–rex, the shuttling of hdm2 was inhibited (Figure 4B, d–f). After evaluation of a large number of heterokaryons by an individual who had no knowledge of the identity of the transfected plasmids, the inhibition of hdm2 shuttling by NLS–rex was judged to be even more pronounced than the inhibition by the NES mutation within

hdm2 (not shown). In contrast, the ‘empty’ expression vector for NLS–rex (pCFN) did not detectably inhibit hdm2 shuttling (Figure 4B, a–c), and the mutated inhibitor NLS–rexM90 had only a slight suppressing effect upon hdm2 shuttling (Figure 4B, g–i). The same expression plasmids were also co-injected into HeLa cell nuclei together with the GST fusion protein carrying the hdm2 NES (Figure 4C). The cells were then assayed for nuclear export as described above. The NLS–rex expression construct markedly inhibited nuclear export of GST fused to hdm2 NES (Figure 4C, c and d), whereas the vector alone (Figure 4C, a and b) showed little, if any, inhibition of export. Co-injection of the same protein with a plasmid encoding the mutated inhibitor NLS–rexM90 resulted in

Fig. 4. hdm2 and HIV rev are exported from the nucleus on overlapping pathways. (A) Schematic depiction of fusion proteins that inhibit the rev export pathway. The RNA-binding domain of HTLV-1 rex was replaced by the nuclear localization signal from SV40 T antigen. This fusion protein still retains the rex NES and competitively inhibits nuclear export on the rev pathway. A point mutation within the NES, M90, renders this fusion protein inactive for export and competition, thus serving as a negative control. (B) Effect of an inhibitor of rev-mediated export on hdm2 shuttling. hdm2 was expressed in HeLa cells and assayed for nucleo-cytoplasmic shuttling as described in the legend to Figure 1. Together with the hdm2 expression plasmid (8 µg), the following expression plasmids were co-transfected (2 µg each): pCFN (a–c), an ‘empty’ expression vector (Zhu *et al.*, 1995); the expression construct for the rev inhibitor NLS–rex (d–f); the expression construct for the inactive inhibitor NLS–rexM90 (g–i). (C) Effect of a rev inhibitor on export driven by the hdm2 NES. The fusion protein of GST and the hdm2 NES sequence was microinjected into the nuclei of HeLa cells and assayed for export as described in the legend to Figure 3. Together with the fusion protein and FITC–dextran, the following plasmids were co-injected: pCFN (a and b); the NLS–rex expression plasmid (c and d); the expression plasmid for the inactive inhibitor NLS–rexM90 (e and f).

only a slightly decreased efficiency of export (Figure 4C, e and f). Taken together, these data suggest that hdm2 is exported from the nucleus with slower kinetics than, but on a similar or identical pathway to, the retroviral rev and rex proteins.

Inhibition of the rev export pathway modulates hdm2's ability to negatively regulate transcriptional activation by p53

Because hdm2 has the ability to inhibit the transcriptional activity of p53, the question of whether its rev-like nuclear export function might influence its ability to inhibit p53 was explored. The transcriptional activity of p53 was assayed in Saos-2, a p53-deficient osteosarcoma cell line, using the reporter construct pCOSX1-CAT (Wu *et al.*, 1993), in which the CAT reporter is driven by a synthetic, p53-dependent promoter derived from the mdm2 promoter enhancer element. As a p53 transcriptional activator, a mutant form of p53 lacking the PXXP motifs near the amino-terminus of p53 (Walker and Levine, 1996) was employed. This mutant, p53 Δ pro, can fully activate transcription but is greatly reduced in its ability to suppress cell growth and possibly apoptosis, thus avoiding indirect effects caused by growth inhibition and cell death in this reporter assay. Mapping the interaction domains between p53 and hdm2 (Lin *et al.*, 1994) as well as the crystal structure of these interaction domains (Kussie *et al.*, 1996) strongly suggest that the Δ pro mutation of p53 does not affect hdm2 binding. The co-transfection of a p53 Δ pro expression construct strongly increased reporter expression (Figure 5A, compare lanes 1 and 2). The addition of an hdm2 expression construct decreased p53-dependent transcription (lane 3). Next, the nuclear export pathway used by rev and hdm2 was blocked through the expression of the competitive inhibitor NLS–rex. When NLS–rex was co-expressed with these proteins, the inhibition of p53-mediated transcription was markedly enhanced (lane 4). This was not observed when an inactive export inhibitor, NLS–rexM90, was co-transfected (lane 5).

The effects of a mutation in the NES of hdm2 were assayed in 174-1 3T3 cells (McMasters *et al.*, 1996) derived from knock-out mice lacking p53 as well as mdm2 genes. Thus, the induction of endogenous mdm2 by transiently expressed p53 was avoided, which may otherwise have led to the presence of both wild-type and mutant hdm2 proteins in the transfected cells. When the NES mutant of hdm2 was used instead of wild-type hdm2, the inhibition of p53 by hdm2 was enhanced (Figure 5B, compare lanes 3 and 4). There was no detectable difference in expression and stability of wild-type and NES mutant hdm2, as judged by immunoprecipitation from transiently transfected and radiolabeled cells (not shown). Taken together, these data suggest that inhibition of hdm2 export can enhance hdm2's ability to inhibit p53-mediated transcription.

The effect of hdm2 on p53-mediated transactivation was also followed using wild-type p53 instead of p53 Δ pro. Here, too, the inhibition of hdm2 export by NLS–rex led to a more pronounced inhibition of wild-type p53 by hdm2 (Figure 5C). Similarly, the NES mutant of hdm2 showed a stronger inhibition of p53 transactivation compared with the wild-type hdm2 (Figure 5D).

The ability of both wild-type and Δ pro mutant p53 to

Fig. 5. Export blockage enhances hdm2's ability to inhibit p53 transcriptional activity. **(A)** Competitive down-regulation of rev-related export and its effect on hdm2-mediated inhibition of p53 Δ pro. One μ g of a p53-responsive reporter construct, pCOSX1-CAT (Wu *et al.*, 1993), was co-transfected into Saos-2 cells alone (lane 1) or along with 100 ng of pRcp53 Δ pro (lane 2). The latter plasmid expresses p53 Δ pro, a mutant form of p53 lacking the amino-terminal PXXP motif that is strongly impaired for growth suppression but fully capable of transcriptional activation (Walker and Levine, 1996). In addition, the cells were co-transfected with the hdm2 expression plasmid (0.5 μ g, lanes 3–5) and the following plasmids (1 μ g each): the 'empty' expression vector pCFN (lane 3), the expression plasmid for NLS–rex (lane 4) or the expression plasmid for the inactive rex inhibitor NLS–rexM90 (lane 5). CAT activity was determined 18 h after electroporation of the cells. The results of at least three independent experiments are shown as percentages with the standard errors (bars), as compared with reporter expression in the presence of p53 but absence of hdm2. **(B)** Effect of wild-type and NES mutant hdm2 on transcriptional activation by p53 Δ pro, as assayed in 174-1 3T3 cells. One μ g of pCOSX1-CAT was co-transfected alone (lane 1) or along with 100 ng of pRcp53 Δ pro (lane 2). In addition, the cells were co-transfected with an expression plasmid for hdm2 (0.5 μ g, lane 3) or the same amount of an otherwise identical plasmid expressing hdm2 with an NES mutation (lane 4). In the transfections without an hdm2 expression plasmid, the 'empty' vector pCMVneoBam was co-transfected instead. CAT activity was determined as above. **(C)** Competitive down-regulation of rev-related export and its effect on hdm2-mediated inhibition of wild-type p53. One μ g of pCOSX1-CAT was transfected into Saos-2 cells alone (lane 1) or along with 30 ng of pRcp53 (lanes 2–5), an expression plasmid for wild-type p53 (Lin *et al.*, 1994). In addition, the cells were co-transfected with the hdm2 expression plasmid (2.5 μ g, lanes 3–5) and the following plasmids (1 μ g each): the 'empty' expression vector pCFN (lane 3), the expression plasmid for NLS–rex (lane 4) or the expression plasmid for the inactive rex inhibitor NLS–rexM90 (lane 5). **(D)** Effect of wild-type and NES mutant hdm2 on transcriptional activation by wild-type p53. One μ g of pCOSX1-CAT was transfected into 174-1 3T3 cells alone (lane 1) or along with 30 ng of pRcp53 (lanes 2–4). In addition, the cells were co-transfected with the hdm2 expression plasmid (2.5 μ g, lane 3) or the same amount of the plasmid expressing NES-mutated hdm2 (lane 4).

activate transcription was inhibited by hdm2, and this effect is enhanced when nuclear export of hdm2 is blocked by a point mutation or by a competitive inhibitor. Similar

results were obtained using different p53-responsive reporter constructs (not shown), arguing that the observed results are not restricted to the particular promoter sequence used here.

The export function of hdm2 is required to accelerate p53 degradation

Given the recent findings on p53 destabilization by hdm2 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997), we asked whether nuclear export might influence hdm2's ability to trigger the degradation of p53. Saos-2 cells were transfected to express p53 and hdm2 (either in wild-type or NES mutant form) simultaneously. Subsequently, p53 and hdm2 were detected by immunoblotting. As shown in Figure 6A (compare lanes 2 and 3), hdm2 drastically reduced the level of p53 expression, confirming previously reported results. However, hdm2 with a mutant NES loses most if not all of its ability to affect the detectable amount of p53 (lane 4). Immunodetection of hdm2 showed that similar amounts of hdm2 (either wild-type or mutant) were expressed (Figure 6B, compare lanes 3 and 4). Also, the immunostainings (Figure 1) and cell fractionations (not shown) suggest that the steady-state distribution of hdm2 between the nucleus and cytoplasm is not affected detectably by the NES mutation. In both cases, the vast majority of the protein is found in the nucleus. When cells were transfected with the same plasmids but were treated with the proteasome inhibitor MG132 (*N*-carbobenzoxyleucyl-leucyl-leucinal) for 2 h prior to harvesting, neither wild-type nor mutant hdm2 reduced the amount of detected p53 (Figure 6C, compare lanes 3 and 4 with lane 2). This observation suggests that the reduction in the p53-derived signal (observed in the absence of MG132) is due to the accelerated degradation of p53. Taken together, these data suggest that nuclear export of hdm2 is necessary for triggering the efficient degradation of p53.

Next we co-transfected the export inhibitor NLS–rex

along with hdm2 and p53. As shown in Figure 6D, NLS–rex, but not the empty expression vector pCFN nor the inactive export inhibitor NLS–rexM90, reversed the ability of wild-type hdm2 to decrease p53 levels in these cells. In contrast, neither NLS–rex nor NLS–rexM90 affected the amount of p53 in the absence of transfected hdm2 (compare lanes 2 and 3 with lane 1). These data support the hypothesis that it is specifically the export function of hdm2 that is required for p53 destabilization.

In an effort to see if p53 destabilization is accompanied

Fig. 6. Effect of hdm2 export on p53 stability. (A) Immunodetection of p53 in transfected cells. A p53 expression plasmid (0.3 µg pRcp53, lanes 2–4) or a control plasmid (0.3 µg pCMVEGFP, lane 1) were co-transfected into Saos-2 cells along with a control plasmid (2.7 µg pCMVEGFP, lanes 1 and 2) or expression plasmids for wild-type hdm2 (2.7 µg pCMVhdm2, lane 3) or hdm2 containing the NES mutation (2.7 µg pCMVhdm2mtNES, lane 4). At 24 h after transfection, total cell protein was separated by SDS–PAGE and electroblotted, followed by immunodetection with an antibody against p53. (B) Expression of hdm2 in wild-type and mutant forms. Cells were transfected and blots were prepared as described in (A), followed by immunodetection with an antibody against hdm2. (C) Effect of proteasome inhibition on p53 levels. Cells were processed as described in (A), but treated with the proteasome inhibitor MG132 (100 µM) 2 h prior to harvest. p53 was detected as in (A). (D) Effect of export inhibition on p53 levels. A p53 expression plasmid (0.2 µg pREp53, lanes 1–6) or a control plasmid (0.2 µg pCMVEGFP, lane 7) were co-transfected into Saos-2 cells along with a control plasmid (1.8 µg pCMVEGFP, lanes 1, 2, 3 and 7) or an expression plasmid for wild-type hdm2 (1.8 µg pCMVhdm2, lanes 4 and 6). In addition, 1 µg of the plasmids pCFN (lanes 1, 4 and 7), pCFNrex expressing NLS–rex (lanes 2 and 5) or pCFNrexM90 expressing NLS–rexM90 (lanes 3 and 6) were transfected. Subsequently, p53 was detected as described in (A). (E) Transcriptional activity of p53. Cells were transfected as described in (A), but in each case a reporter plasmid with a p53-responsive promoter regulating luciferase expression (pBP100-GL2) (Freedman *et al.*, 1997), 0.5 µg, was added to the transfection. After 24 h, luciferase expression was determined. The results are shown as percentages of the value obtained when expressing p53 in the absence of hdm2.

by reduced transcriptional activity, we transfected Saos-2 cells using the same conditions and plasmid amounts as in the immunoblot experiments but co-transfected with a p53-responsive luciferase reporter plasmid. It should be noted that p53 was present in far greater amounts than during the reporter assays described above (cf. Figure 5). In the previous experiment, the p53 expression plasmid represented only 0.4% of transfected plasmid DNA. In contrast, during the experiment described here, the same plasmid was used at a proportion of 10%, i.e. at a 25-fold higher relative amount. As before, p53 was observed to activate luciferase expression strongly (Figure 6E, compare lanes 1 and 2), and hdm2 reduced the transcriptional activity of p53 (lane 3). Surprisingly, the NES mutation almost completely abolished hdm2's effect on p53-mediated transcription (lane 4). This observation suggests that under conditions of high-level p53 expression, the destabilization by hdm2 is needed to reduce p53's transcriptional activity, and that the export function of hdm2 is then largely contributing to its ability to block p53-mediated transcriptional activation.

Discussion

The experiments presented here demonstrate that the hdm2 oncoprotein is subject to nuclear export and shuttles between the nucleus and the cytoplasm (Figure 1A). Nuclear export of hdm2 is independent of its ability to bind p53 (Figure 1B). This export is driven, at least in part, by the presence of an NES that is similar to other export signals described previously (Figure 2). It can be inhibited specifically by a competitor that also interferes with the nuclear export of the rev protein from HIV (Figure 4). When p53 is transfected in low abundance, inhibition of the rev export pathway by a co-transfected competitor increases hdm2's inhibitory effect on p53-mediated transcriptional activation, and a similar effect is achieved when the NES in hdm2 is mutated (Figure 5). Thus, preventing hdm2's nuclear export enhances its inhibition of p53 under these circumstances. On the other hand, larger amounts of p53 can be destabilized and inactivated only in the presence of an intact nuclear export function. Apparently, nuclear export is part of the mechanism that allows hdm2 to accelerate p53 degradation.

The hdm2-derived NES sequence can direct the complete nuclear export of a carrier protein (Figure 3), but this is accomplished more slowly than with the rev NES. While it is not clear what slows down the export driven by the hdm2-derived NES, it is conceivable that a range of strong to weak NESs exist. These NESs of variable strength may thus fit the previously defined consensus sequences (Hope *et al.*, 1991; Bogerd *et al.*, 1996) to variable degrees. It seems reasonable that a viral-encoded export signal might evolve to be stronger than a cellular homolog to provide the virus with an advantage as it competes with cellular systems. The use of NESs could be more widespread among cellular proteins than previously anticipated.

hdm2 associates with the L5 ribosomal protein (Marechal *et al.*, 1994), and L5 has been implicated in the nuclear export of 5S rRNA (Guddat *et al.*, 1990; Michael and Dreyfuss, 1996; Rudt and Pieler, 1996). One

could therefore speculate that L5 might mediate the export of hdm2. After transient expression of wild-type hdm2 or its NES mutant (L205A/I208A), both of these hdm2 proteins bound equal amounts of L5 protein as shown by co-immunoprecipitation (results not shown). This indicates that a mutation in the NES region does not influence the degree of L5 binding. In addition, the region on hdm2 interacting with L5 has been mapped to sequences located further toward the carboxy-terminus of hdm2 than the NES (Elenbaas *et al.*, 1996). Thus the hdm2 NES does not appear to facilitate an L5-hdm2 interaction, and it probably plays a direct role in hdm2 export from the nucleus. However, it remains possible that L5 binding can facilitate NES-mediated export of hdm2.

Nuclear export of an oncogene product raises the possibility that growth regulation could be mediated by the rev-related export machinery. A detailed study of this export pathway may therefore lead not only to a deeper understanding of retroviral RNA export but might also contribute to the concept of cellular growth regulation. There are previous examples of growth regulatory proteins that might be influenced in their activity by subcellular distribution, even though nuclear export is not known to influence their localization; for example, the Rb tumor suppressor protein accumulates in two different biochemically defined nuclear compartments, depending on its phosphorylation (Mittnacht and Weinberg, 1991). Another example is the p53 tumor suppressor gene which can only carry out its transcriptional function when in the nucleus (Gannon and Lane, 1991; Shaulsky *et al.*, 1991; Knippschild *et al.*, 1996). Some cells show a differential distribution of p53 between the nucleus and the cytoplasm, in some cases depending on the stage in the cell cycle or even the tumorigenic state of the cells (Shaulsky *et al.*, 1990; Moll *et al.*, 1992, 1995).

What are the mechanisms that link the nuclear export function of hdm2 with its influence on p53's transcriptional activity? The destabilization of p53 after binding hdm2 seems to occur mainly through the ubiquitin-proteasome pathway, as suggested by our (Figure 6C) and previous (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997) results. In human fibroblasts, proteasome inhibition leads to p53 accumulation and apoptosis (Dietrich *et al.*, 1996), suggesting that this pathway is generally responsible for rapid turnover of p53. When degraded through this pathway, proteins are first tagged for degradation by being covalently linked to ubiquitin peptides, and they subsequently undergo proteolysis catalyzed by the proteasome (Jentsch and Schlenker, 1995). Proteins from intracellular compartments lacking proteasomes, like the endoplasmic reticulum, can be transported to the proteasome through specialized mechanisms (Plempner *et al.*, 1997). Even though proteasomes can be found in both the nucleus and the cytoplasm of most cells, the intracellular distribution of the proteasome is cell cycle dependent (Wojcik *et al.*, 1995), and proteasomes accumulate in cytoplasmic aggregates after pharmacological inhibition (Wojcik *et al.*, 1996). Different components of the proteasome predominate in the nucleus and the cytoplasm (Wojcik *et al.*, 1995; Palmer *et al.*, 1996), suggesting functional differences between the nuclear and cytoplasmic proteasomes. Taken together, these studies raise the possibility that certain nuclear proteins might be degraded preferentially through the

Table I. Parallels between hdm2 and the adenoviral E1B–E4 complex

Activity	hdm2	E1B–E4
p53 binding	Binds amino-terminal portion of p53 (Momand <i>et al.</i> , 1992; Lin <i>et al.</i> , 1994) Inhibits transactivation (Momand <i>et al.</i> , 1992)	E1B binds the amino-terminal portion of p53 (Sarnow <i>et al.</i> , 1982; Kao <i>et al.</i> , 1990; Lin <i>et al.</i> , 1994) E4 binds the carboxy-terminal portion of p53 (Dobner <i>et al.</i> , 1996) E1B and E4 inhibit transactivation (Yew and Berk, 1992; Dobner <i>et al.</i> , 1996)
Oncogenicity	Mediates cell transformation (Finlay, 1993) Enhanced expression in human tumors (cf. Introduction)	Both E1B and E4 mediate cell transformation together with adenovirus-E1A (Moore <i>et al.</i> , 1996) Both found in adenovirus-induced rodent tumors (E1B more frequently than E4) (Moore <i>et al.</i> , 1996)
Nuclear export	Mainly localized in the nucleus (Olson <i>et al.</i> , 1993) Nucleo-cytoplasmic shuttling (this report) Nuclear export on a rev-like pathway Nuclear export signal	E4 relocates E1B from the cytoplasm to the nucleus (Goodrum <i>et al.</i> , 1996) The complex of E1B and E4 undergoes nucleo-cytoplasmic shuttling (Dobbelstein <i>et al.</i> , 1997) Nuclear export on a rev-like pathway (ibid) E4 contains a functional nuclear export signal (ibid)
Interaction with RNA	Binds RNA <i>in vitro</i> through its carboxy-terminal RING finger domain (Elenbaas <i>et al.</i> , 1996) Associates with the RNA-binding ribosomal protein L5 (Marechal <i>et al.</i> , 1994)	Both proteins together enhance the export of viral mRNA and suppress the export of cellular mRNA during adenovirus infection (Babiss <i>et al.</i> , 1985; Halbert <i>et al.</i> , 1985; Pilder <i>et al.</i> , 1986) E1B suppresses mRNA export in yeast when directed to the nucleus (Liang <i>et al.</i> , 1995)

cytoplasmic proteasome after nucleo-cytoplasmic export. Therefore, it is tempting to speculate that hdm2 can carry p53 from the nucleus to the cytoplasm, thus facilitating p53's degradation. This transport would depend on a functional NES and the rev export pathway, as suggested by our results (Figure 6).

On the other hand, failure to export hdm2 seems to have the opposite effect on p53-mediated transcription when p53 is present in low amounts. hdm2 binds to the same amino acids in p53 that are used for transcriptional activation (Lin *et al.*, 1994). Thus excess hdm2 should block p53-mediated transcription even without nuclear export and p53 degradation. In some cells, free p53 and hdm2 molecules are present as well as p53–hdm2 complexes (Momand and Zambetti, 1996). This could arise from a distinct nuclear sublocalization for p53 (transcriptionally active sites) and hdm2 (with the nuclear export machinery) as suggested by the data in this report.

It remains an open question whether the nuclear export of hdm2 regulates biological functions other than the inhibition of p53. Nuclear export may also influence hdm2's effect on Rb (Xiao *et al.*, 1995), E2F (Martin *et al.*, 1995; Xiao *et al.*, 1995) and MyoD (Fiddler *et al.*, 1996) activity. Both the retinoblastoma protein pRb and p53 have been reported to interact physically with hdm2 (Momand *et al.*, 1992; Oliner *et al.*, 1993; Xiao *et al.*, 1995). One could therefore speculate that hdm2 might carry one or both of these proteins to the cytoplasm after binding to them in the nucleus. It is also certainly possible that hdm2, like rev or rex, might modulate the export of certain cellular RNA molecules. The RING finger region of hdm2 was shown recently to be capable of interacting with RNAs of a specific sequence or conformation *in vitro* (Elenbaas *et al.*, 1996). After forming a complex with hdm2, particular species of cellular RNA might be trans-

ported more efficiently from the nucleus to the cytoplasm when hdm2 is highly expressed, resulting in a novel post-transcriptional mechanism by which an oncogene can influence gene expression. hdm2 is an oncogene, but it also belongs to the group of genes inducible by the tumor suppressor p53. Thus, if hdm2 mediates export of certain mRNA species, the expression of these mRNAs might promote cellular growth; alternatively, the hdm2-mediated export of particular mRNA molecules after p53-mediated hdm2 induction might be part of p53's growth suppressing activity and halt progression through the cell cycle.

There are remarkable similarities between the hdm2 and adenovirus E1B-55 kDa proteins (Table I); both hdm2 and E1B-55 kDa proteins bind to p53 (Sarnow *et al.*, 1982; Momand *et al.*, 1992). In fact, the very same amino acid residues in p53 (Leu22 and Trp23) are involved in binding to hdm2 and the E1B-55 kDa protein, with the same result: the inhibition of p53 transcriptional activity. It has now become clear that the adenovirus E1B-55 kDa–E4–34 kDa complex (Sarnow *et al.*, 1984), like hdm2, shuttles between the nucleus and the cytoplasm and uses the HIV rev pathway to exit the nucleus (Dobbelstein *et al.*, 1997). This complex apparently regulates which mRNA can leave the nucleus in the late phase of adenoviral infection. Viral mRNA is exported efficiently whereas host mRNA largely remains nuclear (Halbert *et al.*, 1985; Pilder *et al.*, 1986). Because hdm2 has the ability to bind specifically to RNAs, it remains possible that hdm2 mediates some of its growth regulatory effects via nuclear export of certain RNA species.

To identify cellular RNA molecules that can interact with hdm2, a cDNA-based *in vitro* selection procedure, cDNA-SELEX (Dobbelstein and Shenk, 1995), was employed. By this method, a portion of human 28S rRNA (nucleotides 328–406) was found to be part of the majority

of the selected RNA ligands of hdm2, even when the initial pool of RNA used for the selection experiment was highly enriched for polyadenylated RNA (data not shown). Like previously identified synthetic RNA ligands for hdm2 (Elenbaas *et al.*, 1996), the 28S rRNA fragment was found to interact specifically with the RING finger domain of hdm2 (unpublished observations). Intriguingly, p53 has been reported to bind p53-encoding mRNA and to regulate the translation of certain mRNAs (Mosner *et al.*, 1995). The hdm2 protein might contact the large ribosomal subunit after leaving the nucleus by interacting with 28S rRNA through its RING finger domain and with the L5 protein through its central portion (Marechal *et al.*, 1994; Elenbaas *et al.*, 1996). Simultaneously, hdm2 could bind p53 through its amino-terminal portion (Chen *et al.*, 1993), thus forming a bridge between the ribosome and p53 and enhancing p53's putative ability to regulate translation.

Based upon the data presented here, nuclear export of hdm2 serves to (i) modulate the hdm2-mediated inhibition of p53 transcriptional activity and accelerate the degradation of p53. Furthermore, hdm2 might (ii) transport RNAs or proteins to the cytoplasm that regulate cellular growth or (iii) act at the ribosome to complex p53 and regulate translation of mRNA. Nuclear export by the rev pathway might thus contribute to the control of cellular proliferation and tumor development.

Materials and methods

Cells and antibodies

All cell lines were from ATCC and propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Monoclonal antibodies were obtained from hybridoma cultures and used as crude supernatants, unless stated otherwise.

Plasmids

pCMVhdm2 (Oliner *et al.*, 1993) was mutated within the putative NES coding region according to the instructions of the QuikChange kit (Stratagene) for site-directed mutagenesis, using the complementary oligonucleotides GAAAGCCTGGCTGCATGCGTAGCAAGGGAG-ATATG and CATATCTCCCTTGCTACGCATGCAGCCAGGCTTTC. This resulted in a mutation of the amino acids L90 and I92 to alanine residues and the introduction of a novel *SphI* site into the hdm2 cDNA.

The plasmids pCFNrex and pCFNrexM90 have been described (Dobbelstein *et al.*, 1997). The plasmid pGexNESrev was constructed by hybridizing the oligonucleotides CGCGATCCCTGCAGCTGCC-GCCGCTGGAACGTCTGA and GGATTCGTCGACGGGCCCTCAC-AGAGTACAGCGTTCCAG to each other, performing a fill-in reaction with Klenow DNA polymerase fragment, and treatment with the restriction enzymes *Bam*HI and *Eco*RI. The vector pGex4T-1 (Pharmacia) was treated with the same enzymes, followed by ligation to the fragments. pGexNEShdm2 was generated by PCR-amplifying the putative NES region of hdm2 using the primers CGGGATCCTCTAGATCTGATG-TATTTCCCTTTC and GGGGTACCGCGGCCGCTCACAACATAT-CTCCCTTATTACAC. The PCR product and the vector pGex4T-1 were treated with *Bam*HI and *Not*I, followed by ligation. These plasmids were used to express NES sequences fused to the carboxy-terminus of GST, namely LQLPLRLTL for pGexNESrev and SISLSFDESALCVIRE-ICCE for pGexNEShdm2.

Heterokaryon assays

Nucleo-cytoplasmic shuttling was detected by using a heterokaryon assay (Pinol-Roma and Dreyfuss, 1992). HeLa cells were transfected with the indicated expression plasmids by electroporation as described (Dobbelstein and Shenk, 1996). Briefly, 10 µg of the expression plasmid was mixed with 400 µl of a HeLa cell suspension (3×10^6 cells/ml) in DMEM/10% FBS and subjected to a voltage pulse in a cuvette of 0.4 cm gap width using a Bio-Rad Gene Pulser with capacitance extender at 230 V and 950 µF. Immediately after transfection, the cells from one cuvette were seeded on a 3.5 cm tissue culture dish containing a

2.2×2.2 cm glass coverslip together with 1×10^6 (murine) Balb-c 3T3 cells. After 18 h, the cells were treated with DMEM/10% FBS containing 50 µg/ml cycloheximide (Sigma) for 15 min. The cells on the coverslip were then covered with a solution of 50% (w/v) polyethylene glycol 8000 (Sigma) in DMEM for 2 min at 37°C to induce cell fusion. Subsequently, the coverslips were transferred back to DMEM/10% FBS containing cycloheximide (50 mg/l). Forty five minutes later, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, followed by permeabilization with 0.2% Triton X-100 in PBS (20 min). During and after fixation, all incubations were done at room temperature. The cells were incubated with a blocking solution (10% FBS in PBS with 0.01% w/v sodium azide) for 30 min, followed by the primary antibody 2A9 (Chen *et al.*, 1993) (IgG2a hybridoma supernatant 1:10 in blocking solution containing 1:40 000 diluted anti Ku-86 kDa mouse monoclonal IgG1, Santa Cruz J15) for 1 h, three washes for 5 min in PBS, secondary antibodies [FITC-conjugated goat anti mouse IgG2a and Texas Red-conjugated goat anti-mouse IgG1 (Caltag), both diluted 1:500 in blocking solution] for 1 h, three washes as above and mounting in Aquamount solution (Lerner). The cells were examined by fluorescence microscopy using a confocal microscope in a blinded fashion.

Microinjection

GST fusion proteins were prepared from *E.coli* strain DH10B containing the appropriate pGex-derived construct as described previously (Dobbelstein and Shenk, 1995). The proteins were adjusted to a concentration of 3 mg/ml in PBS containing FITC-conjugated dextran 150 000 (Sigma FD 150) at 3 mg/ml. When indicated, expression plasmids were added to a final concentration of 200 µg/ml. This solution was microinjected as described (Dobbelstein *et al.*, 1992) into the nuclei of HeLa cells that were seeded in a semiconfluent monolayer onto glass coverslips coated with polylysine (Becton Dickinson) using an automated injection system (AIS, Zeiss/Eppendorf) and an Axiovert 35M microscope (Zeiss). Eight hours after injection, the cells were fixed and stained with a monoclonal mouse antibody against GST (diluted 1:200, Santa Cruz) and a Texas Red-conjugated antibody against murine IgG (1:500, Jackson) as described above, and evaluated by confocal microscopy in a blinded fashion.

Reporter assays

Saos-2 cells were transfected with the indicated plasmids by electroporation as described above. In all experiments, 5 µg of the plasmid pGem7Zf(+) (Promega) was added as a carrier. At 18 h after transfection, the cells were harvested in reporter buffer (Promega); CAT assays were performed using the appropriate reagents (Promega) according to the manufacturer's instructions and quantified after thin-layer chromatography using a PhosphorImager (Molecular Dynamics).

Immunodetection of p53 and hdm2

Saos-2 cells were seeded on 3.5 cm dishes and transfected using Superfect reagent (Qiagen) according to the manufacturer's instructions. At 24 h after transfection, the cells were harvested and total protein was separated on an SDS-polyacrylamide gel. After transfer to nitrocellulose, p53 was detected by the antibody Pab 1801 (Ab-2, Calbiochem) and hdm2 by the antibody IF2 (Ab-1, Calbiochem). The proteins were visualized using a peroxidase-coupled secondary antibody (Jackson) and a chemiluminescent substrate (Pierce).

Luciferase assays

The cells were transfected as described above and processed for luciferase quantitation using an established protocol (Promega). Light emission was detected using an automated luminometer (Lumistar).

Acknowledgements

This paper is dedicated to Wolfgang Roth, who gave continuous support to J.R. and M.D. as a father, a scientist and a friend. We are grateful to Joe Goodhouse for expert assistance with confocal microscopy. We thank Hisatoshi Shida for plasmids and Randy Talbott and Nicolai Kley for generous permission to use their microinjection device. J.R. received a postdoctoral fellowship from the German Research Foundation, and M.D. was supported by the Stipendium für Infektionsbiologie of the German Cancer Research Center during the course of this work. T.S. is an American Cancer Society Professor and an Investigator of the Howard Hughes Medical Institute.

References

- Babiss, L.E., Ginsberg, H.S. and Darnell, J.E., Jr (1985) Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Mol. Cell. Biol.*, **5**, 2552–2558.
- Barak, Y., Gottlieb, E., Juven-Gershon, T. and Oren, M. (1994) Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.*, **8**, 1739–1749.
- Berberich, S. and Cole, M. (1994) The mdm-2 oncogene is translocated and overexpressed in a murine plasmacytoma cell line expressing wild-type p53. *Oncogene*, **9**, 1469–1472.
- Bevec, D. et al. (1996) Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A. *Science*, **271**, 1858–1860.
- Bogerd, H.P., Fridell, R.A., Madore, S. and Cullen, B.R. (1995) Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell*, **82**, 485–494.
- Bogerd, H.P., Fridell, R.A., Benson, R.E., Hua, J. and Cullen, B.R. (1996) Protein sequence requirements for function of the human T-cell leukemia virus type 1 rex nuclear export signal delineated by a novel *in vivo* randomization-selection assay. *Mol. Cell. Biol.*, **16**, 4207–4214.
- Boulikas, T. (1993) Nuclear localization signals (NLS). *Crit. Rev. Eukaryot. Gene Expr.*, **3**, 193–227.
- Bueso-Ramos, C.E., Yang, Y., deLeon, E., McCown, P., Stass, S.A. and Albitar, M. (1993) The human MDM-2 oncogene is overexpressed in leukemias. *Blood*, **82**, 2617–2623.
- Chen, J., Lin, J. and Levine, A.J. (1995) Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. *Mol. Med.*, **1**, 142–152.
- Chen, J., Wu, X., Lin, J. and Levine, A.J. (1996) mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. *Mol. Cell. Biol.*, **16**, 2445–2452.
- Chen, J., Marechal, V. and Levine, A.J. (1993) Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.*, **13**, 4107–4114.
- Cordon-Cardo, C. et al. (1994) Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res.*, **54**, 794–799.
- Dietrich, C., Bartsch, T., Schanz, F., Oesch, F. and Wieser, R.J. (1996) p53-dependent cell cycle arrest induced by N-acetyl-L-leucyl-L-leucyl-L-norleucinal in platelet-derived growth factor-stimulated human fibroblasts. *Proc. Natl Acad. Sci. USA*, **93**, 10815–10819.
- Dobbelstein, M. and Shenk, T. (1995) *In vitro* selection of RNA ligands for the ribosomal L22 protein associated with Epstein-Barr virus-expressed RNA by using randomized and cDNA-derived RNA libraries. *J. Virol.*, **69**, 8027–8034.
- Dobbelstein, M. and Shenk, T. (1996) Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. *J. Virol.*, **70**, 6479–6485.
- Dobbelstein, M., Arthur, A.K., Dehde, S., van Zee, K., Dickmanns, A. and Fanning, E. (1992) Intracistronic complementation reveals a new function of SV40 T antigen that co-operates with Rb and p53 binding to stimulate DNA synthesis in quiescent cells. *Oncogene*, **7**, 837–847.
- Dobbelstein, M., Roth, J., Kimberly, W.T., Levine, A.J. and Shenk, T. (1997) Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. *EMBO J.*, **16**, 4276–4284.
- Dobner, T., Horikoshi, N., Rubenwolf, S. and Shenk, T. (1996) Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. *Science*, **272**, 1470–1473.
- Elenbaas, B., Dobbelstein, M., Roth, J., Shenk, T. and Levine, A.J. (1996) The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol. Med.*, **2**, 439–451.
- Fiddler, T.A., Smith, L., Tapscott, S.J. and Thayer, M.J. (1996) Amplification of MDM2 inhibits MyoD-mediated myogenesis. *Mol. Cell. Biol.*, **16**, 5048–5057.
- Finlay, C.A. (1993) The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol. Cell. Biol.*, **13**, 301–306.
- Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W. and Luhrmann, R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, **82**, 475–483.
- Freedman, D.A., Epstein, C.B., Roth, J.C. and Levine, A.J. (1997) A genetic approach to mapping the p53 binding site in the MDM2 protein. *Mol. Med.*, **3**, 248–259.
- Fridell, R.A., Fischer, U., Luhrmann, R., Meyer, B.E., Meinkoth, J.L., Malim, M.H. and Cullen, B.R. (1996) Amphibian transcription factor IIIA proteins contain a sequence element functionally equivalent to the nuclear export signal of human immunodeficiency virus type 1 Rev. *Proc. Natl Acad. Sci. USA*, **93**, 2936–2940.
- Fritz, C.C., Zapp, M.L. and Green, M.R. (1995) A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature*, **376**, 530–533.
- Gannon, J.V. and Lane, D.P. (1991) Protein synthesis required to anchor a mutant p53 protein which is temperature-sensitive for nuclear transport. *Nature*, **349**, 802–806.
- Gerace, L. (1995) Nuclear export signals and the fast track to the cytoplasm. *Cell*, **82**, 341–344.
- Goodrum, F.D., Shenk, T. and Ornelles, D.A. (1996) Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. *J. Virol.*, **70**, 6323–6335.
- Gorlich, D. and Mattaj, I.W. (1996) Nucleocytoplasmic transport. *Science*, **271**, 1513–1518.
- Gottlieb, T.M. and Jackson, S.P. (1993) The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell*, **72**, 131–142.
- Guddat, U., Bakken, A.H. and Pieler, T. (1990) Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell*, **60**, 619–628.
- Haines, D.S., Landers, J.E., Engle, L.J. and George, D.L. (1994) Physical and functional interaction between wild-type p53 and mdm2 proteins. *Mol. Cell. Biol.*, **14**, 1171–1178.
- Halbert, D.N., Cutt, J.R. and Shenk, T. (1985) Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J. Virol.*, **56**, 250–257.
- Haupt, Y., Maya, R., Kazanietz, A. and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature*, **387**, 296–299.
- Hope, T.J., Bond, B.L., McDonald, D., Klein, N.P. and Parslow, T.G. (1991) Effector domains of human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex are functionally interchangeable and share an essential peptide motif. *J. Virol.*, **65**, 6001–6007.
- Izaurrealde, E. and Mattaj, I.W. (1995) RNA export. *Cell*, **81**, 153–159.
- Jarmolowski, A., Boelens, W.C., Izaurrealde, E. and Mattaj, I.W. (1994) Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell. Biol.*, **124**, 627–635.
- Jentsch, S. and Schlenker, S. (1995) Selective protein degradation: a journey's end within the proteasome. *Cell*, **82**, 881–884.
- Jones, S.N., Roe, A.E., Donehower, L.A. and Bradley, A. (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature*, **378**, 206–208.
- Kao, C.C., Yew, P.R. and Berk, A.J. (1990) Domains required for *in vitro* association between the cellular p53 and the adenovirus 2 E1B 55K proteins. *Virology*, **179**, 806–814.
- Katahira, J., Ishizaki, T., Sakai, H., Adachi, A., Yamamoto, K. and Shida, H. (1995) Effects of translation initiation factor eIF-5A on the functioning of human T-cell leukemia virus type I Rex and human immunodeficiency virus Rev inhibited trans dominantly by a Rex mutant deficient in RNA binding. *J. Virol.*, **69**, 3125–3133.
- Knippschild, U., Oren, M. and Deppert, W. (1996) Abrogation of wild-type p53 mediated growth-inhibition by nuclear exclusion. *Oncogene*, **12**, 1755–1765.
- Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) Regulation of p53 stability by Mdm2. *Nature*, **387**, 299–303.
- Kussie, P.H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A.J. and Pavletich, N.P. (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*, **274**, 948–953.
- Ladanyi, M., Cha, C., Lewis, R., Jhanwar, S.C., Huvos, A.G. and Healey, J.H. (1993) MDM2 gene amplification in metastatic osteosarcoma. *Cancer Res.*, **53**, 16–18.
- Landers, J.E., Haines, D.S., Strauss, J.F.R. and George, D.L. (1994) Enhanced translation: a novel mechanism of mdm2 oncogene overexpression identified in human tumor cells. *Oncogene*, **9**, 2745–2750.
- Leach, F.S. et al. (1993) p53 Mutation and MDM2 amplification in human soft tissue sarcomas. *Cancer Res.*, **53**, 2231–2234.
- Liang, S., Hitomi, M. and Tartakoff, A.M. (1995) Adenoviral E1B-55kDa protein inhibits yeast mRNA export and perturbs nuclear structure. *Proc. Natl Acad. Sci. USA*, **92**, 7372–7375.
- Lin, J., Chen, J., Elenbaas, B. and Levine, A.J. (1994) Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev.*, **8**, 1235–1246.
- Marechal, V., Elenbaas, B., Piette, J., Nicolas, J.C. and Levine, A.J. (1994)

- The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. *Mol. Cell. Biol.*, **14**, 7414–7420.
- Martin, K., Trouche, D., Hagemeier, C., Sorensen, T.S., La Thangue, N.B. and Kouzarides, T. (1995) Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature*, **375**, 691–694.
- McMasters, K.M., Montes de Oca Luna, R.M., Pena, J.R. and Lozano, G. (1996) mdm2 deletion does not alter growth characteristics of p53-deficient embryo fibroblasts. *Oncogene*, **13**, 1731–1736.
- Meyer, B.E., Meinkoth, J.L. and Malim, M.H. (1996) Nuclear transport of human immunodeficiency virus type 1, visna virus, and equine infectious anemia virus Rev proteins: identification of a family of transferable nuclear export signals. *J. Virol.*, **70**, 2350–2359.
- Michael, W.M. and Dreyfuss, G. (1996) Distinct domains in ribosomal protein L5 mediate 5S rRNA binding and nucleolar localization. *J. Biol. Chem.*, **271**, 11571–11574.
- Michael, W.M., Choi, M. and Dreyfuss, G. (1995) A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell*, **83**, 415–422.
- Mittnacht, S. and Weinberg, R.A. (1991) G1/S phosphorylation of the retinoblastoma protein is associated with an altered affinity for the nuclear compartment. *Cell*, **65**, 381–393.
- Moll, U.M., Riou, G. and Levine, A.J. (1992) Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proc. Natl Acad. Sci. USA*, **89**, 7262–7266.
- Moll, U.M., LaQuaglia, M., Benard, J. and Riou, G. (1995) Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc. Natl Acad. Sci. USA*, **92**, 4407–4411.
- Momand, J. and Zambetti, G.P. (1996) Analysis of the proportion of p53 bound to mdm-2 in cells with defined growth characteristics. *Oncogene*, **12**, 2279–2289.
- Momand, J., Zambetti, G.P., Olson, D.C., George, D. and Levine, A.J. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, **69**, 1237–1245.
- Montes de Oca Luna, R., Wagner, D.S. and Lozano, G. (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature*, **378**, 203–206.
- Moore, M.S. (1996) Protein translocation: nuclear export—out of the dark. *Curr. Biol.*, **6**, 137–140.
- Moore, M., Horikoshi, N. and Shenk, T. (1996) Oncogenic potential of the adenovirus E4orf6 protein. *Proc. Natl Acad. Sci. USA*, **93**, 11295–11301.
- Mosner, J., Mummenerbrauer, T., Bauer, C., Sczakiel, G., Grosse, F. and Deppert, W. (1995) Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J.*, **14**, 4442–4449.
- Murphy, R. and Wenthe, S.R. (1996) An RNA-export mediator with an essential nuclear export signal. *Nature*, **383**, 357–360.
- Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L. and Vogelstein, B. (1992) Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*, **358**, 80–3.
- Oliner, J.D., Pietenpol, J.A., Thiagalingam, S., Gyuris, J., Kinzler, K.W. and Vogelstein, B. (1993) Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature*, **362**, 857–860.
- Olson, D.C., Marechal, V., Momand, J., Chen, J., Romocki, C. and Levine, A.J. (1993) Identification and characterization of multiple mdm-2 proteins and mdm-2-p53 protein complexes. *Oncogene*, **8**, 2353–2360.
- Palmer, A., Rivett, A.J., Thomson, S., Hendil, K.B., Butcher, G.W., Fuentes, G. and Knecht, E. (1996) Subpopulations of proteasomes in rat liver nuclei, microsomes and cytosol. *Biochem. J.*, **316**, 401–407.
- Palmer, D. and Malim, M.H. (1996) The human T-cell leukemia virus type 1 posttranscriptional trans-activator Rex contains a nuclear export signal. *J. Virol.*, **70**, 6442–6445.
- Pilder, S., Moore, M., Logan, J. and Shenk, T. (1986) The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.*, **6**, 470–476.
- Pinol-Roma, S. and Dreyfuss, G. (1992) Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature*, **355**, 730–732.
- Plempner, R.K., Bohmler, S., Bordallo, J., Sommer, T. and Wolf, D.H. (1997) Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature*, **388**, 891–893.
- Reifenberger, G., Liu, L., Ichimura, K., Schmidt, E.E. and Collins, V.P. (1993) Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. *Cancer Res.*, **53**, 2736–2739.
- Rudt, F. and Pieler, T. (1996) Cytoplasmic retention and nuclear import of 5S ribosomal RNA containing RNPs. *EMBO J.*, **15**, 1383–1391.
- Sarnow, P., Ho, Y.S., Williams, J. and Levine, A.J. (1982) Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kDa cellular protein in transformed cells. *Cell*, **28**, 387–394.
- Sarnow, P., Hearing, P., Anderson, C.W., Halbert, D.N., Shenk, T. and Levine, A.J. (1984) Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J. Virol.*, **49**, 692–700.
- Shaulsky, G., Ben-Ze'ev, A. and Rotter, V. (1990) Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. *Oncogene*, **5**, 1707–1711.
- Shaulsky, G., Goldfinger, N., Tosky, M.S., Levine, A.J. and Rotter, V. (1991) Nuclear localization is essential for the activity of p53 protein. *Oncogene*, **6**, 2055–2065.
- Shenk, T. (1996) In Fields, B.N., Knipe, D.M. and Howley, P.M. (eds), *Adenoviridae*. The Viruses and Their Replication. Lippincott-Raven, Philadelphia, PA, pp. 979–1016.
- Simos, G. and Hurt, E.C. (1995) Nucleocytoplasmic transport: factors and mechanisms. *FEBS Lett.*, **369**, 107–112.
- Stutz, F., Neville, M. and Rosbash, M. (1995) Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell*, **82**, 495–506.
- Walker, K.K. and Levine, A.J. (1996) Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc. Natl Acad. Sci. USA*, **93**, 15335–15340.
- Wen, W., Meinkoth, J.L., Tsien, R.Y. and Taylor, S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.
- Wojcik, C., Paweletz, N. and Schroeter, D. (1995) Localization of proteasomal antigens during different phases of the cell cycle in HeLa cells. *Eur. J. Cell. Biol.*, **68**, 191–198.
- Wojcik, C., Schroeter, D., Wilk, S., Lamprecht, J. and Paweletz, N. (1996) Ubiquitin-mediated proteolysis centers in HeLa cells: indication from studies of an inhibitor of the chymotrypsin-like activity of the proteasome. *Eur. J. Cell. Biol.*, **71**, 311–318.
- Wu, X., Bayle, J.H., Olson, D. and Levine, A.J. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.*, **7**, 1126–1132.
- Xiao, Z.X., Chen, J., Levine, A.J., Modjtahedi, N., Xing, J., Sellers, W.R. and Livingston, D.M. (1995) Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature*, **375**, 694–698.
- Yew, P.R. and Berk, A.J. (1992) Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature*, **357**, 82–85.
- Zauberman, A., Flusberg, D., Haupt, Y., Barak, Y. and Oren, M. (1995) A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res.*, **23**, 2584–2592.
- Zhu, H., Shen, Y. and Shenk, T. (1995) Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J. Virol.*, **69**, 7960–7970.

Received March 25, 1997; revised October 9, 1997;
accepted October 29, 1997