Regulation of HDM2 activity by the ribosomal protein L11

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Summary

The HDM2 protein plays an important role in regulating the stability and function of the p53 tumor suppressor protein. In this report, we show that the ribosomal protein L11 can interact with HDM2 and inhibit HDM2 function, thus leading to the stabilization and activation of p53. The inhibition of HDM2 activity by L11 shows some similarity to the previously described activity of ARF, and expression of either ARF or L11 can induce a p53 response. Enhancement of the interaction between endogenous L11 and HDM2 following treatment of cells with low levels of actinomycin-D suggests that the HDM2/L11 interaction represents a novel pathway for p53 stabilization in response to perturbations in ribosome biogenesis.

Introduction

The p53 tumor suppressor protein is a potent inhibitor of cell growth, inducing cell cycle arrest and apoptosis (reviewed in Vousden, 2000). Although p53 function is not absolutely required for normal growth and development, p53 plays an important role in preventing tumor development. p53 function, which is kept tightly regulated in normal cells, is rapidly induced in response to many kinds of stress signals (Ryan et al., 2001). This activation of p53 leads to inhibition of cell growth in cells undergoing oncogenic changes, and combined with a direct contribution of p53 in allowing repair of DNA damage, plays a critical role in preventing tumor development. The importance of p53 in tumor suppression is illustrated by the observation that around half of all human cancers have lost p53 activity through mutation within the *p53* gene, while many others show defects in the ability to activate or respond to p53. Taken together, it is apparent that most, if not all, cancers are defective in the normal p53 response.

The murine double minute 2 protein (Mdm2 or HDM2 in human) was originally described in transformed mouse cells and shown to have oncogenic activity (Fakharzadeh et al., 1991), and *HDM2* is found to be amplified in some human sarcomas (Oliner et al., 1992). The best understood function of HDM2 is its ability to negatively regulate p53, both by directly binding to p53 and through its activity as an E3 ligase that can target p53 for ubiquitination (Fang et al., 2000; Honda et al., 1997; Momand et al., 1992; Oliner et al., 1993; Wadgaonkar and Collins, 1999). The ubiquitination of p53 by HDM2 contributes to the proteasomal degradation of p53 and also to the export of p53 from the nucleus to the cytoplasm (Boyd et al., 2000; Geyer et al., 2000; Gu et al., 2001; Haupt et al., 1997; Kubbutat et al., 1997; Lohrum et al., 2001). p53 is a transcription factor that induces expression of many genes that mediate the apoptotic and cell cycle arrest response to p53 induction (Vousden and Lu, 2002). Importantly, p53 can also activate transcription of *HDM2*, establishing a regulatory loop in which p53 induces expression of its own negative regulator (Barak et al., 1993; Wu et al., 1993). The importance of the p53/HDM2 regulatory loop in allowing normal development has been demonstrated in mice, where loss of *Mdm2* results in early embryonic lethality that is the consequence of p53-mediated apoptosis (de Rozieres et al., 2000; Jones et al., 1995; Montes de Oca Luna et al., 1995). Deletion of *p53* with *Mdm2* rescues the lethal phenotype, allowing *p53/Mdm2* double null mice to develop essentially normally.

Stress-induced activation of p53 depends in part on the inactivation of HDM2 and the inhibition of HDM2-mediated ubiquitination of the p53 protein. The inhibition of HDM2 can be achieved through several mechanisms, with different stress signals utilizing different pathways to allow stabilization of p53 (Ashcroft et al., 2000). DNA damage-induced phosphorylation of p53 or HDM2, for example, has been shown to regulate the formation of the p53/HDM2 complex or the ability of HDM2 to target p53 for degradation (Blattner et al., 2002; Buschmann et

SIGNIFICANCE

The p53 tumor suppressor protein is activated by several cellular stress signals associated with tumor development. Activation of p53 is accompanied by stabilization of the p53 protein following inhibition of HDM2, the ubiquitin ligase for p53. We show here that the ribosomal protein L11 can interact with HDM2 and inhibit the degradation of p53, and provide evidence that the HDM2/L11 interaction is enhanced in response to perturbations in ribosome biogenesis. This process is sensitive to abnormalities in metabolism and growth - conditions that may exist during tumor progression. Our results therefore identify a novel pathway for p53 induction under these conditions and provide a target for the design of drugs to reactivate p53.

al., 2001; Chehab et al., 2000; Goldberg et al., 2002; Hirao et al., 2000; Maya et al., 2001; Shieh et al., 2000). HDM2 activity can also be regulated by interaction with other proteins. The small ARF protein binds to HDM2 and inhibits HDM2s ability to target p53 for degradation (Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). This function of ARF is important in activating a p53 response to abnormal proliferation driven by activation of oncogenes such as *Myc* and *Ras* (Sherr and Weber, 2000), although ARF does not seem to be required for the stabilization of p53 in response to other types of stress (Ashcroft et al., 2000; Khan et al., 2000).

Although the interaction of ARF with HDM2 can directly inhibit HDM2's E3 activity (Honda and Yasuda, 1999; Midgley et al., 2000), the interaction of HDM2 and ARF can also result in relocalization of HDM2 to the nucleolus (Lohrum et al., 2000; Rizos et al., 2000; Weber et al., 1999, 2000). This relocalization of HDM2 is not essential for the inhibition of HDM2 function by ARF in all cells (Korgaonkar et al., 2002; Llanos et al., 2001), but it seems likely that sequestration of HDM2 may contribute to the inactivation of HDM2 under some conditions. Nucleolar localization of HDM2 in response to ARF depends on a nucleolar localization signal within HDM2 itself (Lohrum et al., 2000; Weber et al., 2000), leading to the suggestion that other signals may also affect HDM2 function by driving nucleolar localization of HDM2. Interestingly, ARF-independent colocalization of HDM2 with nucleolar proteins has also been seen during the stabilization of p53 following treatment of cells with low levels of actinomycin-D or proteasome inhibitors (Ashcroft et al., 2000; Xirodimas et al., 2001). Previous studies have described an interaction of HDM2 with a ribosomal protein L5 and ribosomal RNA (Marechal et al., 1994), and it is possible that interactions with components of the ribosome may contribute to the localization and/or retention of HDM2 to the nucleolus.

In this study, we describe a novel interaction between HDM2 and the ribosomal L11 protein. Interaction with L11 can lead to nucleolar localization of HDM2, which is dependent on the NoLS in the C terminus of HDM2. Like ARF, L11 inhibits the ability of HDM2 to degrade p53 and expression of L11 induces a p53 response. The interaction between HDM2 and L11 is enhanced in response to treatment with low levels of actinomycin-D, which selectively inhibit the activity of RNA polymerase I. Our results therefore suggest that L11 may play a role in the inactivation of HDM2 and induction of the p53 in response to perturbations in ribosomal synthesis or assembly.

Results

Interaction of HDM2 with L11

Using a fragment of HDM2 encompassing amino acids 210–491 as bait, we carried out a yeast two-hybrid screen to identify HDM2-interacting proteins. One of the interacting clones identified in this way encoded the ribosomal L11 protein. The interaction between HDM2 and L11 was confirmed by coprecipitation of the two proteins following transient overexpression in U2OS cells (Figure 1) and in vitro translation (Figure 3). HDM2 has been shown to bind to p53 through an N-terminal domain, and we confirmed that p53 could form a complex with wild-type HDM2, an HDM2 protein carrying a point mutation in the C-terminal RING finger (HDM2Ala464) and an HDM2 protein deleted of the central region (HDM2Δ222–437) (Figure 1B) (Kubbutat et al., 1999). By contrast, although Flag-tagged L11 was

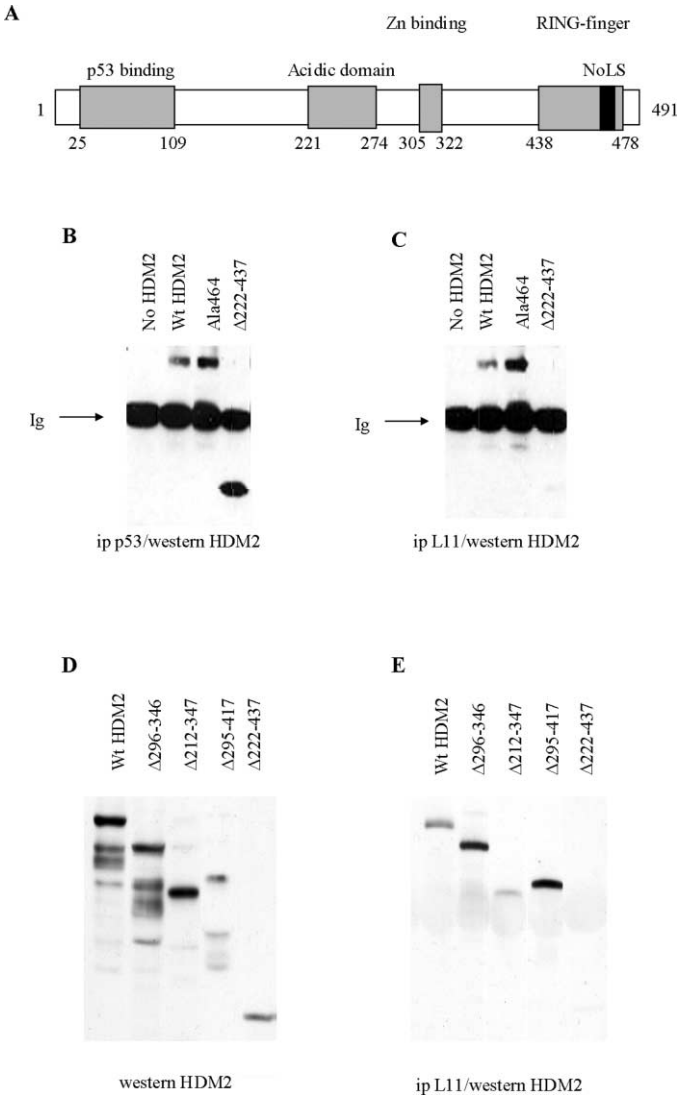

Figure 1. Association of L11 with HDM2

A: Cartoon of the HDM2 protein, indicating the position of principal domains.
B: Coprecipitation of the indicated HDM2 proteins with p53 following transfection into U2OS cells. Twenty-four hours posttransfection, the cells were lysed and p53 immunoprecipitated using the monoclonal antibody DO-1. Associated HDM2 protein was detected by Western blotting using AB1 and AB2.
C: Coprecipitation of the indicated HDM2 proteins with L11 following transfection into U2OS cells as described above. L11 protein was immunoprecipitated through the Flag-tag.
D: Expression of the indicated HDM2 proteins following transfection into U2OS cells.
E: Coprecipitation of the indicated HDM2 proteins with L11 following cotransfection into U2OS cells. L11 protein was immunoprecipitated through the Flag-tag.

able to coprecipitate wild-type HDM2 and HDMAla464, HMD2Δ222-437 was defective in the interaction with L11 (Figure 1C). These results demonstrated that p53 and L11 bound to different regions on HDM2. Further analysis of mutations within the central region of HDM2 showed that deletion of the region between amino acids 212–347 significantly reduced the binding between L11 and HDM2, although deletion of amino acids 295–

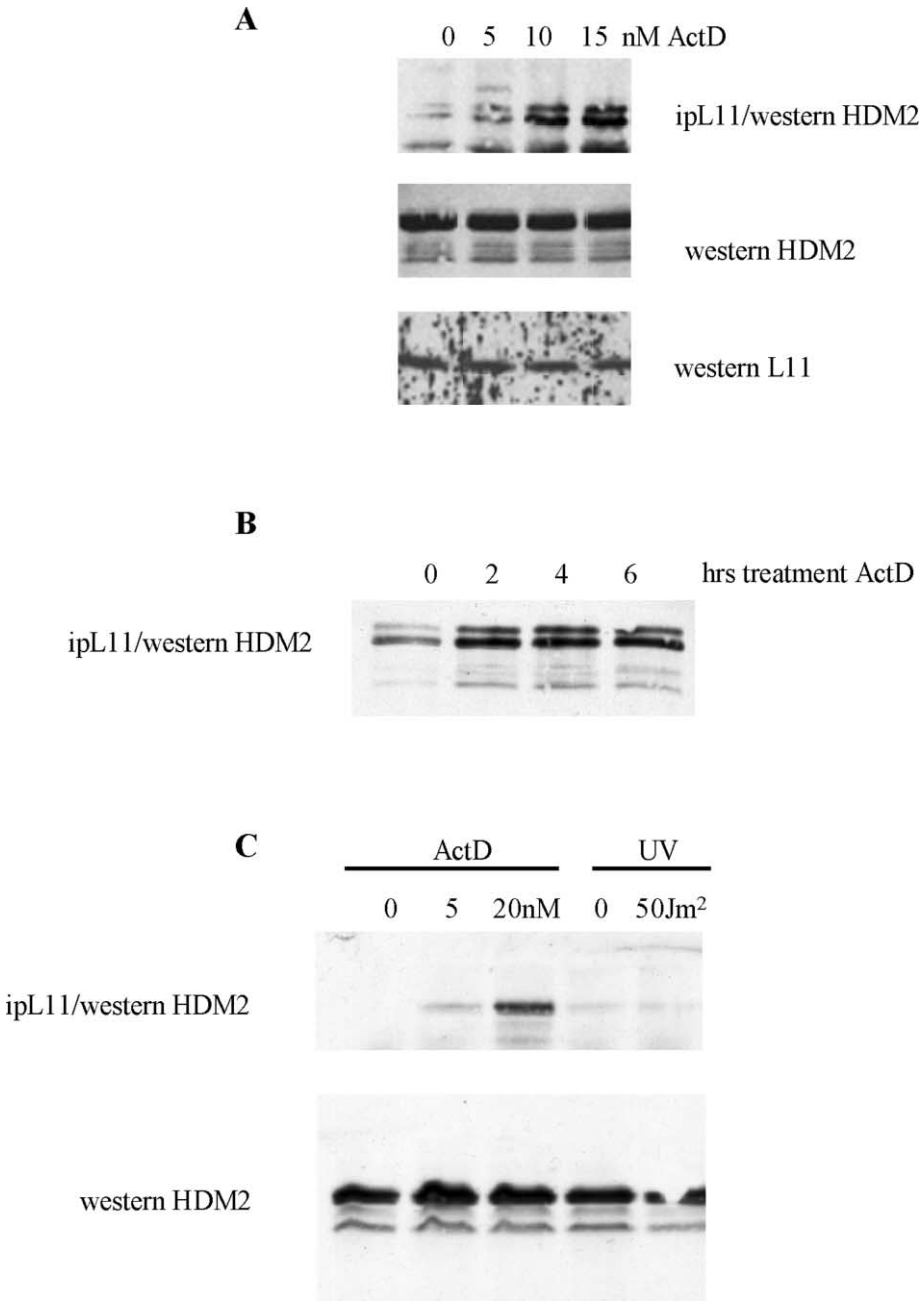

Figure 2. Interaction of L11 with HDM2 is enhanced in response to actinomycin-D

A: Coprecipitation of endogenous HDM2 with endogenous L11 from RKO cells. Cells were treated with 20 μM MG132 (Calbiochem) for 6 hr, with addition of the indicated concentrations of actinomycin-D for the final 2 hr. L11 was immunoprecipitated using polyclonal anti-L11 antiserum. Coprecipitated HDM2 proteins were detected by Western blotting using a mixture of AB1 and AB2. Western blots were also carried out to show total levels of HDM2 and L11 in each lysate.

B: Coprecipitation of endogenous HDM2 with endogenous L11 from untransformed human retinal pigment epithelial cells treated with MG132 for 6 hr, with the addition of 5 nM actinomycin-D for the indicated times. Immunoprecipitations were carried out as above.

C: Coprecipitation of endogenous HDM2 with endogenous L11 from RKO cells treated with MG132 for 6 hr, with the addition of actinomycin-D or exposure to UV after 4 hr. Immunoprecipitations and Western blotting were carried out as above.

417 or 296–346 did not prevent the interaction (Figures 1D and 1E). A similar region of HDM2 is required for ARF binding (Bothner et al., 2001).

To determine whether endogenous L11 and HDM2 could form an association, we raised a rabbit polyclonal antibody against a C-terminal L11 peptide and examined lysates from MCF-7 and RKO cells. Immunoprecipitation of L11 showed no clear evidence of association with HDM2, although this may reflect difficulties in detection since in unstressed cells HDM2 is a rapidly degraded protein and so is expressed at relatively low levels. Therefore, in order to increase the overall levels of HDM2, we treated the cells with the proteasome inhibitor MG132 (to stabilize the HDM2 protein). Although this treatment did not increase the levels of L11, HDM2 levels were enhanced and we were able to detect an interaction between these increased levels of endogenous HDM2 and L11 in RKO, RPE, U2OS, and MCF-7 cells (data not shown).

Our results indicated that although the interaction between endogenous HDM2 and L11 was not easily seen in unstressed cells, a detectable interaction could form when HDM2 levels were increased by proteasome inhibition—which has also been shown to allow nucleolar accumulation of HDM2 (Xirodimas et al., 2001). We therefore considered the possibility that the HDM2/L11 interaction might be enhanced in response to stress. Treatment of cells with low levels of actinomycin-D has previously been shown to stabilize p53 without phosphorylation of p53 or downregulation of HDM2 (Ashcroft et al., 2000), and we found that treatment of RKO cells with actinomycin-D led to an

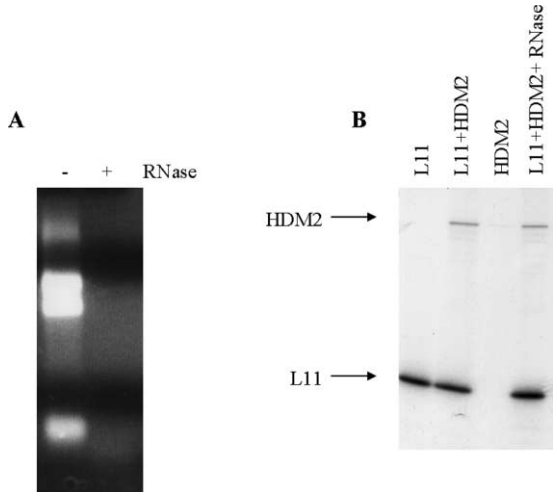

Figure 3. The HDM2/L11 interaction is not mediated by RNA

A: Ethidium bromide stained agarose gel showing the effect of RNase treatment of rabbit reticulocyte lysates containing in vitro translated HDM2 and L11.

B: Coprecipitation of HDM2 with L11 expressed in reticulocyte lystates with or without RNase treatment. The indicated ^{35}S-labeled proteins were allowed to associate, then L11 immunoprecipitated through the Flag-tag. L11 and associated HDM2 proteins were visualized by autoradiography.

increase in the amount of HDM2 coprecipitated with L11 (data not shown). However, although L11 levels were not affected by actinomycin-D treatment, HDM2 levels did increase (possibly in response to activation of p53), and so we sought to distinguish enhanced HDM2/L11 binding from an increase in the overall levels of available HDM2 by first stabilizing HDM2 and then exposing the cell to actinomycin-D. Using this approach, we found that the HDM2/L11 interaction seen following MG132 treatment was significantly enhanced following treatment of cells with low levels of actinomycin-D that would inhibit RNA polymerase I, but have less effect on the activity of RNA polymerase II or III (Perry and Kelley, 1970). The increase in HDM2/L11 interaction was seen within 2 hr in both the RKO tumor cell line (Figure 2A) and untransformed human epithelial cells (Figure 2B). To test whether the enhancement of the interaction between L11 and HDM2 was a general consequence of genotoxic stress, we compared the effect of actinomycin-D treatment with UV treatment of RKO cells (Figure 2C) and RPE cells (data not shown). In each case, only actinomycin-D treatment resulted in a significant increase in the HDM2/L11 interaction. Interestingly, UV has been shown to induce the ribotoxic stress response (Iordanov et al., 1998), which interferes specifically with translational elongation. Although it is possible that longer or more severe UV treatment would induce increased HDM2/L11 binding, our results suggest that the enhancement of the L11/MDM2 interaction shows some specificity to stress signals that can affect ribosome synthesis or assembly.

Previous studies have shown that HDM2 can interact directly with rRNA, and we therefore sought to determine whether the HDM2/L11 interaction is mediated by RNA. In order to address this point, we examined the interaction of in vitro translated L11 and HDM2 before or after treatment of the lysates with RNase (Figure 3). Significant amounts of RNA could be detected in the lysates by ethidium bromide staining and this was efficiently digested by treatment with RNase (Figure 3A). However, the RNase treatment had no effect on the coprecipitation of HDM2 by L11 (Figure 3B), suggesting that the HDM2/L11 interaction is not mediated by RNA.

L11 stabilizes both HDM2 and p53

Both L11 and ARF bind within the central region of HDM2 (Stott et al., 1998). Since ARF has been shown to inhibit HDM2 ubiquitin ligase function, preventing HDM2-mediated degradation of both itself and p53, we considered the possibility that L11 might have the same effect. Coexpression of L11 with HDM2 and p53 prevented degradation of p53 in a manner analogous to that seen following ARF expression (Figure 4A). Expression of L11 also resulted in stabilization of HDM2, once again showing similarity to the previously described effect of ARF (Figure 4B). Furthermore, in U2OS cells, which express wild-type p53 and HDM2, but no ARF, elevation of endogenous p53 and HDM2 levels was seen following L11 expression (Figure 4C). Taken together, these results suggest that the interaction of L11 with HDM2 inhibits the ability of HDM2 to target both p53 and itself for ubiquitination and degradation.

We next examined whether the overexpression of HDM2 could result in degradation of L11, like p53. Although ARF is not degraded by HDM2, the ARF protein does not contain lysine residues for ubiquitination. However, although L11 does contain lysines, under conditions where HDM2 clearly leads to the degradation of p53, no degradation of L11 was observed (Figure 4D).

Although HDM2 plays a major role in regulation of p53 turnover, other mechanisms that can contribute to the stability of p53 have also been described (reviewed in Woods and Vousden, 2001). While L11 could inhibit p53 degradation targeted by cotransfected HDM2, L11 expression had no effect on the stability of a p53 mutant that lacks the HDM2 binding site and is not degraded by HDM2 (data not shown), supporting a role for L11 in the abrogation of HDM2-mediated degradation of p53. We noted that expression of L11 could stabilize wild-type p53 in U2OS cells beyond levels seen in the absence of exogenous HDM2 (Figure 4A), possibly reflecting inhibition of the endogenous HDM2 in these cells. To clarify the role of HMD2 in mediating the effect of L11 on p53 levels, we examined the activity of L11 in mouse embryo fibroblasts lacking endogenous Mdm2 and p53 (Figure 4E). In these cells, L11 also inhibited the ability of exogenous HDM2 to degrade transfected p53. In the absence of HDM2, we reproducibly noted a very slight increase in p53 levels following expression of L11, raising the possibility that L11 may function to target an additional, unidentified regulator of p53 stability, such as the recently identified ubiquitin ligase Pirh2 (Leng et al., 2003).

Colocalization of HDM2 and L11

Both L11 and ARF are nucleolar proteins and under some conditions, expression of ARF has been shown to result in the relocalization of HDM2 to nucleoli (Weber et al., 1999). We therefore investigated the possibility that L11 can also relocalize HDM2. Initial experiments were carried out by cotransfecting L11 and HDM2 into U2OS cells. Expression of HDM2 alone in these cells resulted in localization of HDM2 in the nucleoplasm, with occasional cytoplasmic staining, while L11 expressed alone was found to be localized to the nucleolus, with overall staining

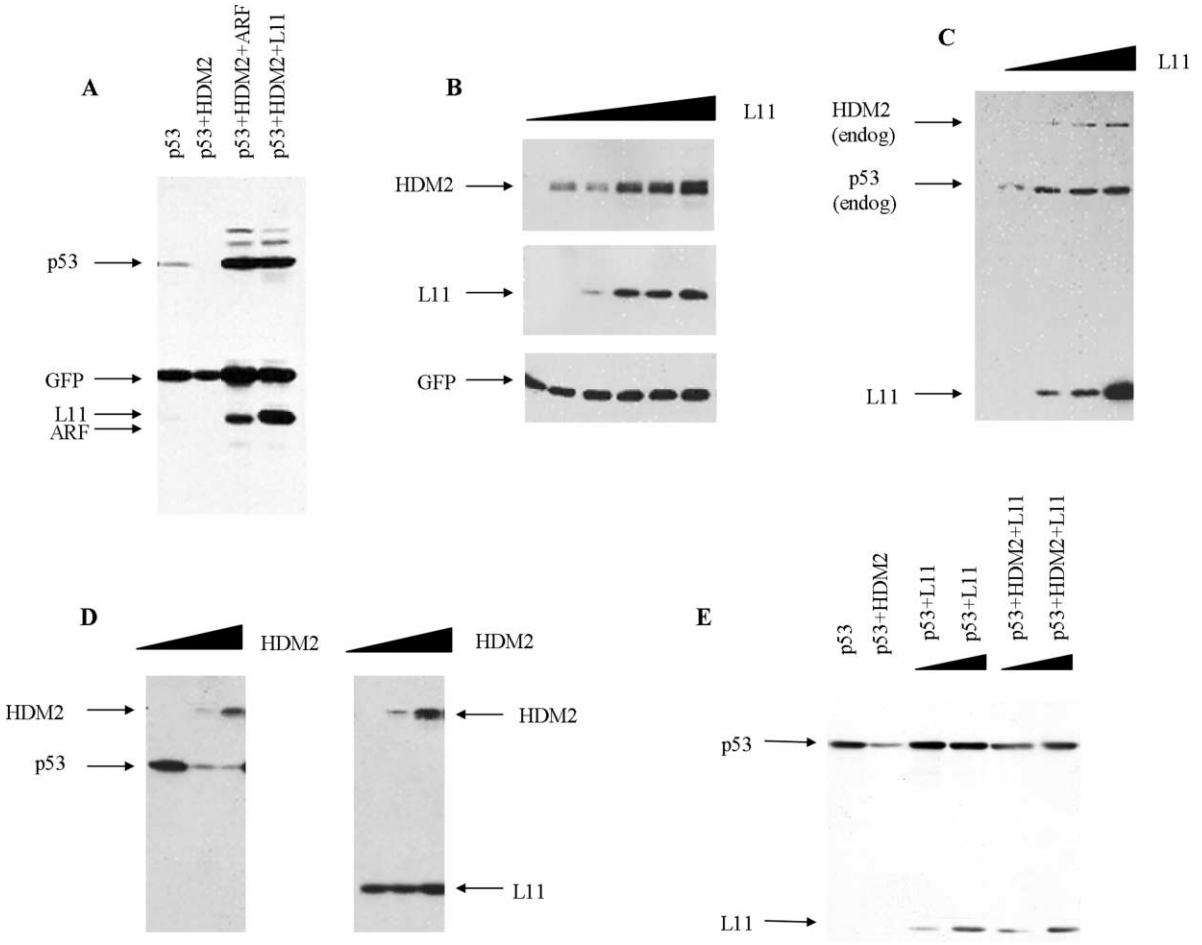

Figure 4. L11 inhibits HDM2-mediated degradation of p53

A: Stabilization of transfected p53. The Western blot shows expression of Flag-p53, HDM2, L11, and ARF following transient cotransfection of the indicated plasmids into U2OS cells. HDM2 expression leads to the degradation of p53, which is inhibited by either L11 or ARF expression. Equal amounts of GFP were added to each transfection to control for transfection efficiency and loading.

B: Stabilization of transfected HDM2. The Western blot shows expression of transfected HDM2 and L11 in transiently transfected U2OS cells. Equal amounts of HDM2 were included in each transfection with increasing amounts of L11. Equal amounts of GFP were added to each transfection to control for transfection efficiency and loading.

C: Stabilization of endogenous HDM2 and p53. The Western blot shows the effect of increasing amounts of transfected L11 on the levels of endogenous HDM2 and p53 in U2OS cells.

D: HDM2 expression leads to the degradation of p53, but not L11. Levels of HDM2, Flag-p53, and L11 protein were detected by Western blot following transient cotransfection of equal amounts of p53 or L11, with increasing amounts of HDM2.

E: HDM2 dependence of the stabilization of p53 by L11. The Western blot shows expression of p53 and L11 following transient cotransfection of p53, HDM2, and increasing levels of L11, as indicated, into *p53/Mdm2* double null mouse embryo fibroblasts.

throughout the cell in cases of high L11 expression levels (Figure 5A). When transfected alone, nucleolar HDM2 was detected in less than 1% of the cells. Following coexpression of L11 and HDM2, we were able to detect colocalization of the two proteins in the nucleolus in those cells showing nucleolar localization of L11. Titration experiments showed that this result was dependent on the relative levels of L11 and HDM2; at low L11 concentrations, both proteins remained in the nucleoplasm while at higher levels of L11 expression, both proteins relocalized to the nucleolus. Under these conditions, nucleolar HDM2 could be detected in up to 90% of cells cotransfected with L11 and HDM2. We also found that ectopic expression of L11 in U2OS cells can result in relocalization of endogenous HDM2 to the nucleolus (Figure 5B), although this was not seen in all cells in the population.

Previous studies examining relocalization of HDM2 to the nucleoli with ARF defined HMD2 mutants that have lost the HDM2 NoLS and fail to relocalize (Lohrum et al., 2000; Weber et al., 2000). Coexpression of HDM2 1-440, which has lost the NoLS but retains the ability to bind L11 (data not shown), did not relocalize to the nucleolus with L11, and L11 in this case is also nucleoplasmic rather than nucleolar (Figure 6A). This observation suggests that the NoLS in HDM2 contributes to the relocalization of the protein by L11, as it does to the relocalization of HDM2 by ARF (Lohrum et al., 2000). The HDM2Δ222-437 mutant, as previously described, is constitutively nucleolar, and under these conditions, L11 is also nucleolar. To test whether binding of L11 to HDM2 is required for the relocalization, we examined some of the smaller HDM2 deletion mutants shown in Figure 1D. Both HDM2Δ296-346 and HDM2Δ212-347

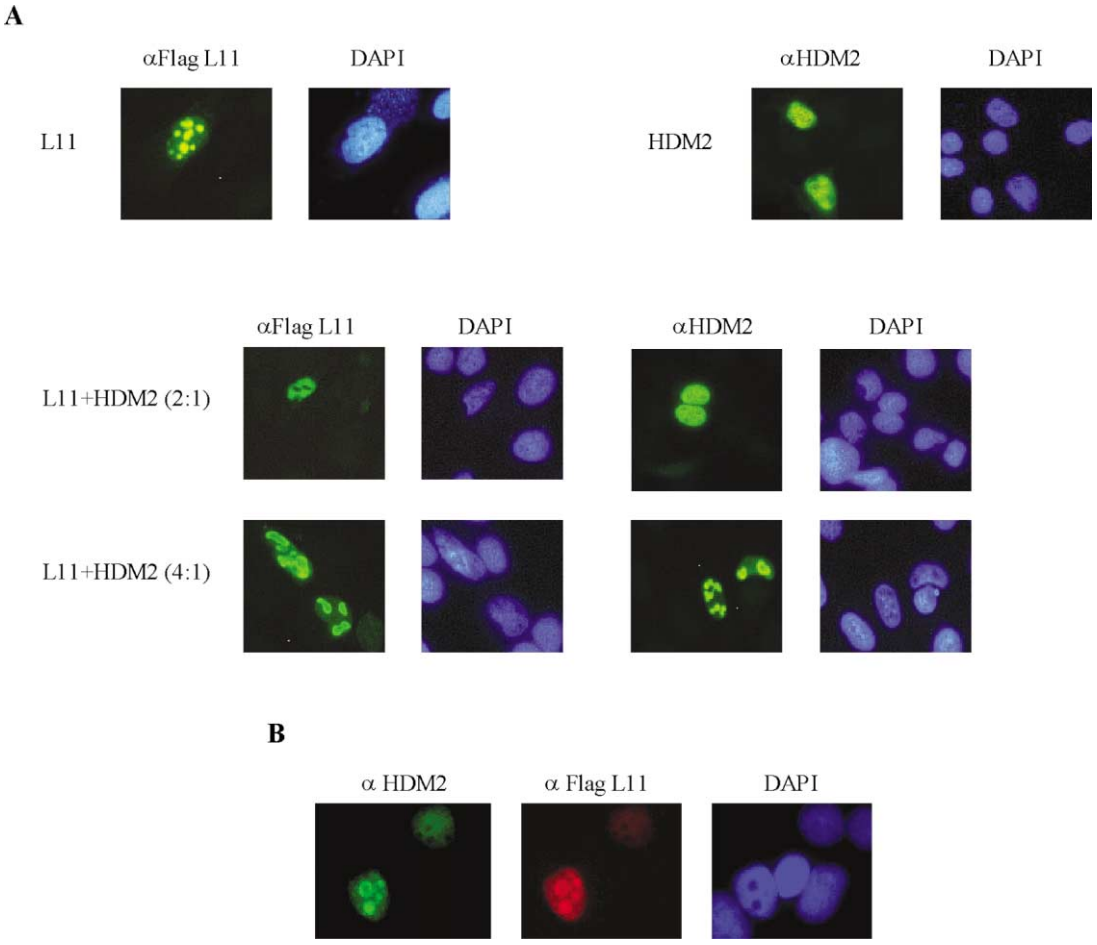

Figure 5. Subcellular localization of HDM2 and L11

A: Subcellular localization of transfected L11 and HDM2 in U2OS cells. When expressed alone, L11 is predominantly nucleolar and HDM2 confined to the nucleoplasm. Twenty-four hours after cotransfection, L11 and HDM2 are both localized to the nucleoplasm, when the L11:HDM2 ration is low (2:1), or both localized to the nucleolus when L11 levels are higher (4:1).

B: Relocalization of endogenous HDM2 to the nucleolus following transfection of L11 in U2OS cells. Twenty-four hours after transfection of L11 into U2OS cells, nucleolar localization of endogenous HDM2 is seen in around 40% of the L11-expressing cells.

localized to the nucleoplasm in the absence of cotransfected L11 (Figure 6B). Upon coexpression of L11, the HDM2Δ296-346 mutant, which retains binding to L11 (Figure 1E), was relocalized to the nucleolus (Figure 6B). However, HDM2Δ212-347, which is defective for L11 binding (Figure 1E), was significantly less efficiently relocalized to the nucleolus with L11 (Figure 6B), although a small proportion of the cotransfected cells still showed some evidence of nucleolar HDM2. These results support a role for the binding of HDM2 to L11 in allowing relocalization or retention of HDM2 in the nucleolus.

The ability of ectopic L11 to drive HDM2 to the nucleolus suggested that L11 may play a role in relocalizing HDM2 in the absence of ARF. We previously showed that treatment of cells with low concentrations of actinomycin-D could result in relocalization of HDM2 to distinct nuclear locations also occupied by the nucleolar protein B23, even in cells lacking ARF (Ashcroft et al., 2000). In light of the results presented here, it is possible that this relocalization reflects the activation of the HDM2/L11 interaction following actinomycin-D treatment (Figure 2). Although nucleoli appear to remain at least partially intact in the presence of very low levels of actinomycin-D, previous studies have shown that higher concentrations of actinomycin-D can result in the release of some components of the nucleolus into the nucleoplasm (Yokoyama et al., 1992). To determine whether L11 and HDM2 remain colocalized under such conditions, we examined the localization of both L11 and HDM2 in cells treated with concentrations of actinomycin-D sufficient to release the B23 proteins from the distinctive nucleolar location within 7 hr of treatment (Figure 7). Immediately after treatment with actinomycin-D, where nucleolar structure remains intact as measured by B23 localization, both L11 and HDM2 colocalized with B23 to discrete subnuclear bodies. However, after longer treatment (Figure 7), or at higher actinomycin-D concentrations (data not shown), both L11 and HDM2 were found to be colocalized with B23 in the nucleoplasm (Figure 7). Taken together, these results suggest that the HDM2/L11 complex could be formed in both the nucleolus and the nucleoplasm, like the ARF/HDM2 complex.

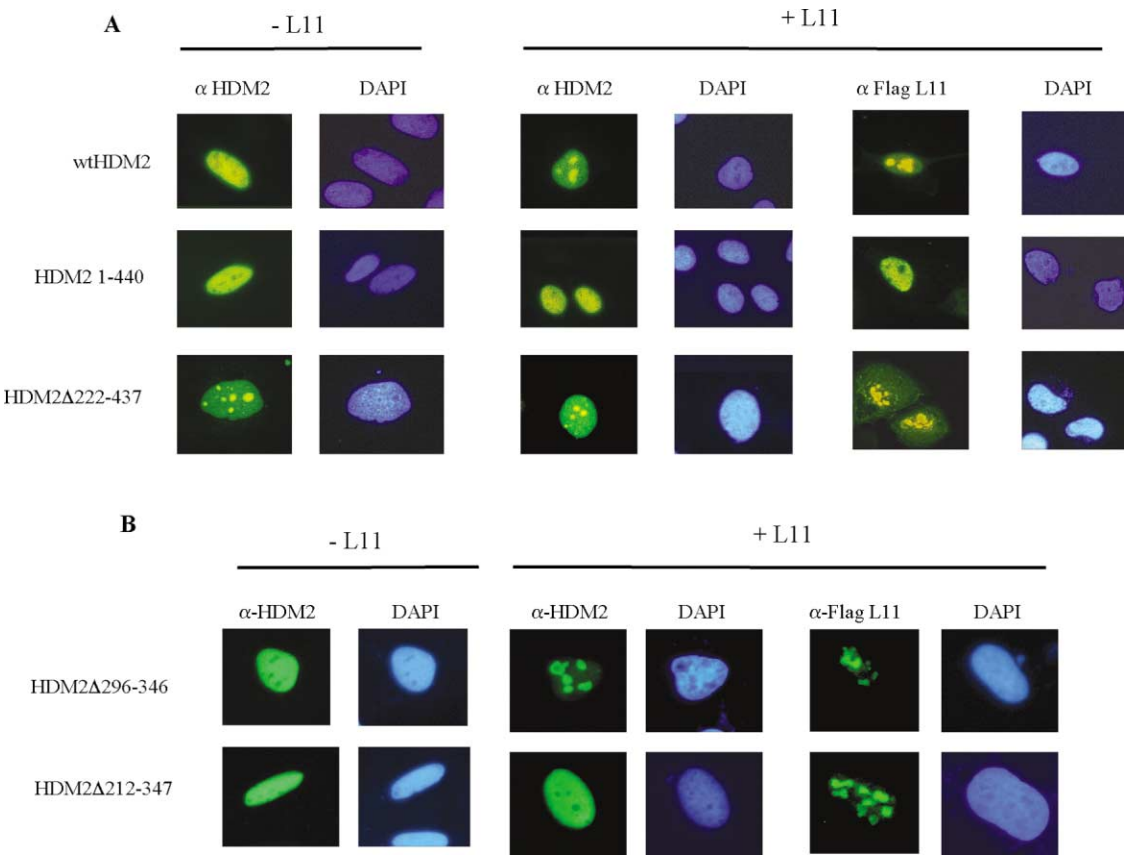

Figure 6. Relocalization of HDM2 to the nucleolus by L11 depends on the NoLS in HDM2 and binding of L11 to HDM2

A: Subcellular localization of wild-type HDM2, HDM2 1–440 (containing only amino acids 1–440 and therefore lacking the NoLS), and HDM2Δ222–437 (lacking amino acids 222–437). In the absence of cotransfected L11, wild-type HDM2 and HDM2 1–440 are localized to the nucleoplasm, while HDM2Δ222–437 is constitutively localized to the nucleoli, as previously described (Lohrum et al., 2000). Wild-type HDM2 relocalizes to the nucleolus following L11 expression, while HDM2 1–440 remains nucleoplasmic. HDM2 1–440 also causes relocalization of L11 to the nucleoplasm.

B: Subcellular localization HDM2Δ296–346 (that retains the ability to bind L11) and HDM2Δ212–347 (with reduced L11 binding activity) in the absence and presence of coexpressed L11.

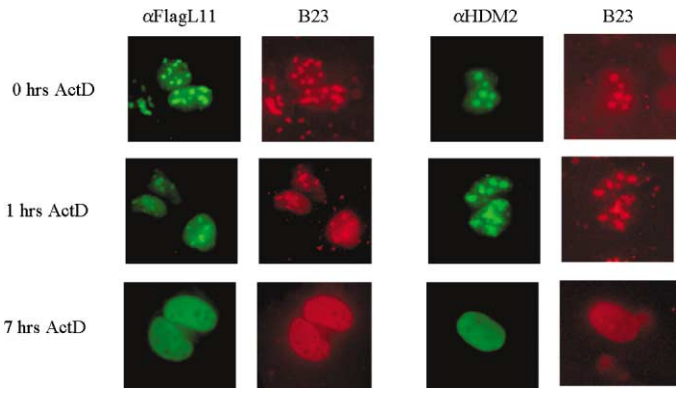

Figure 7. Subcellular localization of L11 and HDM2 in response to actinomycin-D

U2OS cells were transfected with L11 and HDM2 at a ratio of 4:1 and treated with 10 nM actinomycin-D for the indicated times. Localization of each transfected protein was then compared to the localization of endogenous B23, a nucleolar protein.

L11 activates p53-dependent cell cycle arrest

The ability of L11 to bind HDM2 and allow stabilization of p53 suggests that L11 may be able to participate in a signaling pathway that has been shown to induce a p53 response following nucleolar stress (Pestov et al., 2001). We therefore examined whether L11 could signal to activate p53-dependent cell cycle arrest. Transfection of L11 into ARF null U2OS cells led to stabilization of the endogenous p53 (Figure 4), and flow cytometric analysis of these transfected cells showed that expression of L11 and stabilization of p53 led to a G1 arrest similar to that seen following transfection of ARF (Figure 8A). To demonstrate that this arrest was the result of p53 activation, we examined the effect of coexpression of E6, a human papillomavirus protein that targets p53 for degradation and inhibits the p53 response (Vousden, 1995). As shown previously for ARF (Stott et al., 1998), E6-mediated inhibition of p53 markedly decreased the induction of the G1 arrest in response to L11 (Figure 8B), indicating that the stabilization of p53 by L11 correlates with an activation of p53-dependent cell cycle arrest. These results are consistent with the previously described ability of actinomycin-D to stabilize p53 and induce cell cycle arrest in wild-type p53-expressing cells (Nelson and Kastan, 1994). For example, treat-

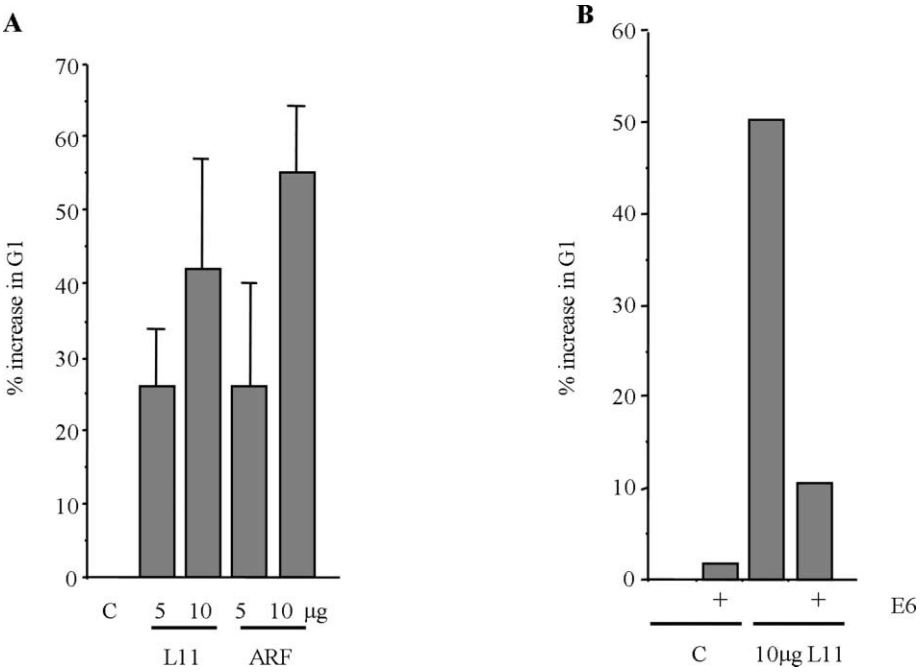

Figure 8. L11 activates p53 function

A: L11 and ARF activate endogenous p53 to induce cell cycle arrest. Expression of L11 increases endogenous p53 levels in U2OS cells (Figure 4), resulting in an elevation of the number of cells in the G1 phase of the cell cycle, as measured by flow cytometric analysis of transfected cells. The graph shows the percentage increase in G1 cells compared to vector only transfected cells as control (C) following expression of increasing amounts of L11 or ARF.

B: The G1 arrest induced by L11 is p53 dependent. U2OS cells were transfected with vector or L11 in the absence or presence of E6, which targets p53 for degradation and inhibits activation of a p53-dependent response. The graph shows the percent of the transfected cell population in G1, compared to vector only controls (C). Western blotting confirmed a reduction in p53 levels in the E6-expressing cells (data not shown).

ment of the U2OS cells used in Figure 8 with 5 nM actinomycin-D for 24 hr led to a 59% increase in the G1 population.

Discussion

We have identified an interaction between the ribosomal L11 protein and HDM2, with evidence that this interaction is enhanced in response to inhibition of RNA polymerase I and can lead to the stabilization and activation of p53. Overall, the effect of L11 on HDM2 function shows some similarities to the previously described activities of ARF, which also binds to the central region of HDM2, inactivating HDM2 function and inducing a p53 response. Our results suggest that there may be several proteins that bind to HDM2 to regulate HDM2 and p53 function in response to disparate stress signals.

In bacteria, L11 protein is an rRNA binding protein that has been shown to play an important role in the ribosome, being involved in several steps in protein synthesis. In both bacteria and plant cells, L11 has also been shown to participate in stress response pathways (Handa et al., 2001; Zhang et al., 2001), and other ribosomal proteins exhibit apoptotic activities in mammalian cells (Chen and Ioannou, 1999). Our results suggest that in mammalian cells, L11 may be part of a stress response pathway that can stabilize p53. Previous studies have shown that perturbations in ribosomal biogenesis can result in "nucleolar stress," leading to the activation of p53 (Pestov et al., 2001), and it is possible that L11 plays a role in mediating this response. Treatment of cells with low levels of actinomycin-D, which would selectively inhibit RNA polymerase I and so perturb ribosome biogenesis, can efficiently stabilize p53. We have now shown that these levels of actinomycin-D also enhance the interaction between endogenous HDM2 and L11 and result in colocalization of the HDM2 and L11 proteins in the cell. Stabilization of p53 in response to DNA damage or oncogene activation involves pathways that lead to p53 phosphoryation or ARF activation (Ryan et al., 2001). Interestingly, however, the ability of low levels of actinomycin-D to signal stabilization of p53 does not require ARF nor the efficient N-terminal phosphorylation of p53 (Ashcroft et al., 2000), although at increased actinomycin-D concentrations—sufficient to significantly inhibit RNA polymerase II—p53 phosphorylation can also be detected (Ljungman et al., 2001). These results indicate that nucleolar stress results in the stabilization of p53 through a novel pathway, potentially involving the activation of the HDM2/L11 interaction. Several stress signals, including lack of nutrients (Maden et al., 1969), reduced protein synthesis (Pederson and Kumar, 1971), drugs (Snyder et al., 1971), and induction of Arf (Sugimoto et al., 2003), can lead to changes in ribosomal biogenesis and result in the inhibition of proliferation. The importance of p53 in the response to these stress signals was recently highlighted by a study showing that this cell cycle arrest is not the direct result of ribosome depletion, but rather a response to the activation of p53 (Pestov et al., 2001). It is possible that perturbations in rRNA synthesis or ribosome assembly results in the release of an excess of free L11—which is normally a critical part of the 60S ribosomal subunit (Kressler et al., 1999)—to allow HDM2/L11 binding. Alternatively (or additionally), stress-induced posttranslational modifications of L11 or HDM2 may play a role in enhancing the interaction between the two proteins. Furthermore, other ribosomal components have been shown to bind HDM2 (Marechal et al., 1994), and these may also play a role in the stabilization of p53. Interestingly, p53 can also directly inhibit RNA polymerase I transcription (Zhai and Comai, 2000), suggesting that p53 may play a role in both induction and response to changes in ribosome biogenesis.

Examination of the subcellular localization of L11 and HDM2 suggests that L11 can, under some circumstances, direct the localization of HDM2 to the nucleoli. Under overexpression conditions, the localization of the two proteins clearly depends on the relative ratio of HDM2 to L11—with high L11 concentrations being required for nucleolar localization of both proteins. In unstressed cells, the endogenous proteins do not obviously

colocalize since L11 is primarily a nucleolar protein and HDM2 is localized to the nucleoplasm. Nevertheless, many nucleolar proteins have been shown to cycle rapidly between the nucleoplasm and nucleolus in unstressed cells (Chen and Huang, 2001), and this shuttling has been suggested to play a role in the regulation of the interaction between nucleolar proteins and their predominantly nucleoplasmic modifiers (Leary and Huang, 2001). Potentially, both L11 and HDM2 are cycling rapidly between nucleolus and nucleoplasm in unstressed cells, but since our experiments examine only single time points, they may not reveal a dynamic colocalization of a subfraction of L11 and HDM2, which could play a role in determining levels of p53 in response to fluctuations in growth conditions. However, in response to nucleolar stress induced by low levels of actinomycin-D, the activation HDM2/L11 binding is mirrored by the colocalization of the two proteins with B23, either in the nucleolus or in the nucleoplasm. These results are also concordant with our previous observations that low levels of actinomycin-D can drive relocalization of endogenous HDM2 to subnuclear structures containing B23 (Ashcroft et al., 2000). Interestingly, actinomycin-D-induced changes in the nucleolar-nucleoplasm exchange rate have been described for other nucleolar proteins (Chen and Huang, 2001), and it is possible that in addition to the release of L11 from ribosomal subunits as suggested above, stress-induced changes in the rate of shuttling of L11 or HDM2 could contribute to the enhanced interaction between the two proteins. Finally, it is possible that L11 also plays a role in stabilizing p53 in response to signals other than nucleolar stress. Although ARF has been shown to contribute to the stabilization of p53 in response to the activation of oncogenes such as *Myc* and *E2F1*, p53 stabilization can occur in response to these signals in the absence of ARF, and might reflect a contribution of L11. In the cell systems studied so far, we were unable to detect a reproducible increase in L11 expression or HDM2/L11 binding in response to either direct activation of E2F1 or deregulated proliferation induced by the adenoviral oncogene *E1A* (data not shown). However, studies showing that members of the Myc family can elevate expression of nucleolar proteins, including L11 (Boon et al., 2001) and Bop1 (Shiio et al., 2002), suggest that L11 may also play a role in mediating the induction of p53 in response to Myc.

Our results highlight the functional similarities between L11 and ARF in their interaction with HDM2 and their ability to induce the relocalization of HDM2 and activate a p53-dependent cell cycle arrest. These studies reveal a mechanism for the inhibition of HDM2 and activation of p53 through interaction with L11. This mechanism to stabilize p53 may play a role in the response to abnormalities in ribosomal biogenesis induced by abnormal metabolic conditions that could occur during tumor development. Mutations in HDM2 that perturb this response pathway by preventing the HDM2/L11 interaction may therefore contribute to cancer development by preventing the activation of p53 in response to these types of stress signals. A more complete understanding of how L11 is activated may reveal novel targets for the development of therapies aimed at reactivating p53 in some of those cancers that retain a wild-type *p53* gene.

Experimental procedures

Plasmids

A yeast two-hybrid screen (Fields and Song, 1989) was carried out to identify proteins that interact with *HDM2*. A cDNA fragment of *HDM2* encoding amino acids 210 to 491 was cloned in frame with the DBA binding domain of *Gal4* in pGBT9 (Clontech) and transformed into Y190 yeast (Clontech) with a human embryonal kidney cDNA library cloned into pGAD (Clontech). Yeast containing interacting proteins were selected on medium lacking leucine, tryptophan, and histidine, and selected colonies confirmed by assaying for β-galactosidase activity. Positive clones were regrown and DNA from single colonies used as a template for PCR amplification using pGAD424-specific primers (Clontech). The amplified fragments were then subcloned into the TA cloning vector pCR3.1 (Invitrogen) and sequenced using the T7 primer. Two inserts examined in this way encoded the human ribosomal L11 protein, one containing the full L11 coding sequence while the other encoded an L11 protein deleted of the first ten amino acids.

The insert encoding full-length *L11* was then subcloned with an N-terminal Flag-tag and Kozak box into pCDNA3.1 (Invitrogen) using the PCR strategy previously described (Bates et al., 1998). For this, the forward primer used was GATCAAGCTTGCACCATGGACTACAAGGACGACGATGACAAGGCGCAGGATCAAGGTGAA and the reverse primer GATCGAATTCTTATTTGCCAGGAAGGATGATCC.

Plasmids were verified by sequencing. Plasmids encoding human wild-type p53, Flag-p53, wild-type HDM2, HDM2 1-440, HDM2Δ222-437, HDM2 Ala 464, and p14ARF have been described previously (Chen et al., 1995; Marston et al., 1994; Stott et al., 1998). The HDM2 internal deletion mutants were generated using the ExSite PCR-based site-directed mutagenesis kit (Stratagene).

Cell culture

Wild-type p53 expressing human U2OS and RKO cells, and *p53/Mdm2* null mouse embryo fibroblasts, were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Untransformed human retinal pigment epithelial (RPE) cells were grown in DMEM:F12 medium supplemented with 10% fetal calf serum at 37°C, as described by Clontech. Cells were transfected using calcium phosphate coprecipitation and harvested for protein analysis 24 hr posttransfection. Where appropriate, 1 μg of pEGFP-N1 (Clontech) was included in each transfection to monitor for equal transfection efficiency and protein loading.

In vitro translation

In vitro translations of L11 and HDM2 were carried out in rabbit reticulocyte lysates using the TNT coupled reticulocyte lysates system (Promega). To test the role of RNA in the HDM2/L11 interaction, lysates were treated with 500 μg/ml RNase for 30 min prior to immunoprecipitation.

Protein analysis

Rabbit polyclonal antiserum was raised against a C-terminal peptide of human L11, corresponding to amino acids 156–170 (RISKEEAMRWFQQKY). The specificity of the antibody was tested by Western blot detection of overexpressed Flag-L11, and by detection of Flag-L11 proteins immunoprecipitated with the L11 antiserum and then Western blotted for Flag or L11.

To assess L11, HDM2, or p53 protein levels, proteins from whole-cell extracts were separated by SDS 12% polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-p53 DO-1 or 1801 (Pharmingen), anti-HDM2 AB1, AB2, and SMP14 (Oncogene Science), anti-Flag (Sigma), polyclonal anti-L11, and anti-GFP (Clontech) antibodies.

To assay for association between HDM2 and p53 or L11, cells were washed three times in ice-cold PBS and lysed in 500 μl (per 10 cm diameter dish) of NP40 lysis buffer (100 mM NaCl, 100 mM Tris [pH 8.0], 1% NP40) for 30 min at 4°C. p53 protein was immunoprecipitated overnight at 4°C by incubation with a mixture of anti-p53 protein A Sepharose beads equilibrated in NP40 lysis buffer, Flag-L11 by incubation with a mixture of anti-Flag protein A Sepharose beads equilibrated in NP40 lysis buffer, and endogenous L11 by incubation with a mixture of polyclonal anti-L11 protein A Sepharose beads equilibrated in NP40 lysis buffer. The immunoprecipitated protein was washed three times with NP40 buffer, and samples were resuspended in 50 μl of 2× SDS sample buffer and incubated at room temperature for 10 min. The samples were then separated by SDS 12% polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-Flag or anti-L11 and anti-HDM2 antibodies. Proteins expressed in vitro were allowed to associate on ice for 30 min before dilution in 500 μl NP40 buffer and immunoprecipitation as above. Following polyacrylamide gel electrophoresis, these ^{35}S-labeled proteins were detected by autoradiography.

Immunofluorescence

U2OS cells were plated on dishes containing 1 cm diameter glass coverslips and transfected as described. Twenty-four hours after transfection, cells on

the coverslips were washed three times with phosphate-buffered saline (PBS), and then fixed in 4% paraformaldehyde for 10 min at room temperature. After fixation, cells were washed in PBS three times and then permeabilized in ice-cold PBS containing 0.2% Triton X-100 for 5 min. After blocking in PBS containing 0.5% bovine serum albumin at room temperature for 30 min, the cells were incubated overnight at 4°C with anti-B23 (Santa Cruz) or anti-HDM2 AB1 (Oncogene Science) antibody, or for 2 hr at RT with anti-Flag (Sigma) antibody in blocking solution. Cells were washed three times with PBS and incubated for 1 hr at room temperature with fluorescein isothiocyanate (FITC-conjugated rabbit anti-mouse antibody [1:100; Sigma], FITC-conjugated donkey anti-rabbit antibody [1:100; Amersham], Cy3-conjugated donkey anti-rabbit antibody [1:500; Jackson ImmunoResearch], Cy3-conjugated donkey anti-mouse antibody [1:500; Jackson ImmunoResearch], or Cy-3 conjugated donkey anti-goat antibody [1:100; Sigma]) in blocking solution, containing 1 μg of DAPI (Sigma)/ml. Following three washes with PBS, the slides were mounted with PBS/glycerol mount.

Flow cytometry

U2OS cells were transfected using calcium phosphate coprecipitation and washed the following day. The cells were then harvested for FACS at the times indicated in the figure legends. Cells were stained and flow cytometric analysis (FACScalibur, Becton Dickinson) carried out as previously described (Rowan et al., 1996; Zhu et al., 1993).

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