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MDMX regulation of p53 response to ribosomal stress

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Ribosomal stress such as disruption of rRNA biogenesis activates p53 by release of ribosomal proteins from the nucleoli, which bind to MDM2 and inhibit p53 degradation. We found that p53 activation by ribosomal stress requires degradation of MDMX in an MDM2-dependent fashion. Tumor cells overexpressing MDMX are less sensitive to actinomycin D-induced growth arrest due to formation of inactive p53–MDMX complexes. Knockdown of MDMX increases sensitivity to actinomycin D, whereas MDMX overexpression abrogates p53 activation and prevents growth arrest. Furthermore, MDMX expression promotes resistance to the chemotherapeutic agent 5-fluorouracil (5-FU), which at low concentrations activates p53 by inducing ribosomal stress without significant DNA damage signaling. Knockdown of MDMX abrogates HCT116 tumor xenograft formation in nude mice. MDMX overexpression does not accelerate tumor growth but increases resistance to 5-FU treatment *in vivo*. Therefore, MDMX is an important regulator of p53 response to ribosomal stress and RNA-targeting chemotherapy agents.
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Introduction

The p53 tumor suppressor plays key roles in monitoring genomic stability and preventing malignant transformation. In unstressed cells, the MDM2 ubiquitin E3 ligase regulates p53 turnover by targeting it for rapid degradation. DNA damage and mitogenic stress use several well-characterized signaling mechanisms to induce p53 activation. DNA damage induces phosphorylation of p53 and MDM2 on multiple residues, which weaken p53–MDM2 binding, stimulates MDM2 degradation, and suppresses MDM2 E3 ligase function (Prives and Hall, 1999; Maya *et al*, 2001; Goldberg *et al*, 2002; Stommel and Wahl, 2004). Mitogenic stress induces expression of ARF, which can bind to MDM2 and prevent p53 degradation (Zhang and Xiong, 2001).

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MDMX is a p53-binding protein with sequence homology to MDM2 (Shvarts *et al*, 1996; Marine and Jochemsen, 2005). Unlike MDM2, MDMX does not have intrinsic E3 ligase activity and does not promote p53 degradation (Stad *et al*, 2001). However, MDMX forms a heterodimer with MDM2 through C-terminal RING domains (Sharp *et al*, 1999; Tanimura *et al*, 1999), and stimulates the ability of MDM2 to ubiquitinate and degrade p53 (Gu *et al*, 2002; Linares *et al*, 2003). Owing to self-ubiquitination, MDM2 has a short half-life; whereas MDMX is relatively stable in the absence of stress. MDMX can be ubiquitinated and degraded by MDM2 (de Graaf *et al*, 2003; Kawai *et al*, 2003; Pan and Chen, 2003), this may be an important mechanism for controlling MDMX level.

MDMX knock out mice die *in utero* despite having endogenous MDM2 (Parant *et al*, 2001; Finch *et al*, 2002; Migliorini *et al*, 2002). This suggests that MDMX has a unique role in regulating p53 during development. MDMX overexpression has been found in a number of tumors or tumor cell lines with wild-type p53 (Ramos *et al*, 2001; Danovi *et al*, 2004), suggesting that MDMX may contribute to p53 inactivation during tumorigenesis. MDM2 is regulated at the transcriptional level by p53, creating a negative feedback loop. Unlike MDM2, MDMX is not transcriptionally activated by p53 nor is it induced by DNA damage.

MDM2 and MDMX are both targeted by stress signaling pathways that activate p53. DNA damage induces phosphorylation of MDM2 by ATM and c-Abl kinases, and inhibits p53 degradation (Maya *et al*, 2001; Goldberg *et al*, 2002). DNA damage also induces MDMX phosphorylation at the C-terminal region by ATM (Pereg *et al*, 2005), Chk1 (Jin *et al*, 2006), and Chk2 (Chen *et al*, 2005a). MDMX phosphorylation stimulates 14-3-3 binding (Chen *et al*, 2005b; Okamoto *et al*, 2005; Jin *et al*, 2006), and promotes MDMX nuclear translocation and degradation by MDM2 (Li *et al*, 2002; Lebron *et al*, 2006). Mitogenic stress also induces MDMX degradation through induction of ARF expression. ARF binding to MDM2 selectively blocks p53 ubiquitination, but promotes ubiquitination of MDMX (Pan and Chen, 2003).

Recent studies revealed a connection between ribosomal stress and p53-dependent cell cycle arrest, suggesting that aberrant rRNA and ribosome biogenesis are sensed by p53 (Marechal *et al*, 1994; Pestov *et al*, 2001; Lohrum *et al*, 2003; Zhang *et al*, 2003). Ribosomal stress induced by actinomycin D, serum starvation, or contact inhibition cause p53 stabilization and activation (Bhat *et al*, 2004). These studies suggest a mechanism involving the translocation of ribosomal proteins, L5, L11, and L23 from the nucleolus to the nucleoplasm where they bind to MDM2 and prevent p53 degradation (Bhat *et al*, 2004; Dai *et al*, 2004; Jin *et al*, 2004). Each of these L proteins when overexpressed can inhibit MDM2 degradation of p53. The effect of ribosomal proteins on MDMX has not been determined.

P53 response to ribosomal stress may be an important tumor suppression mechanism. Inherited mutation in ribo-

somal protein S19 is associated with predisposition to cancer in humans (Draptchinskaia *et al*, 1999). A genetic screen for tumor suppressors in zebra fish has identified a large number of ribosomal protein genes, suggesting that they function as haploid-insufficient tumor suppressors (Amsterdam *et al*, 2004). Therefore, in addition to their roles in protein synthesis, ribosomal proteins may also be important signaling molecules in regulating cell proliferation and stress response. Their expression and signaling upon stress may be important for preventing malignant transformation.

Results described in this report suggest that activation of p53 by ribosomal stress requires downregulation of MDMX. This process can be blocked by MDMX overexpression. As a result, tumor cells expressing high-level endogenous MDMX have less-efficient p53 activation and growth arrest during ribosomal stress. Furthermore, we found that the widely used chemotherapy agent 5-fluorouracil (5-FU) activates p53 in part through inducing ribosomal stress. As such, MDMX overexpression can cause significant resistance to 5-FU in cell culture and tumor xenograft models. These observations suggest that MDMX plays a unique and important role in regulating p53 response to perturbations in ribosome biogenesis.

Results

Ribosomal proteins selectively bind MDM2 but not MDMX

In experiments aimed at identifying MDM2- and MDMX-binding proteins, we performed affinity purification of MDM2 and MDMX from stable or transiently transfected cells. As reported by others, MDM2 co-purified with several ribosomal proteins, the most prominent being L5, L11, and L23. This binding pattern was observed with transfected MDM2 (Figure 1A), or endogenous MDM2 from SJSA cells (not shown). In contrast, MDMX co-purified with casein kinase 1α and 14-3-3 under the same washing conditions (Figure 1B) (Chen *et al*, 2005b). Reproducibly absent from the MDMX IP was any co-precipitation of ribosomal proteins. These results indicated that ribosomal proteins directly target MDM2 but not MDMX.

To further confirm the results from Coomassie staining, U2OS stably expressing tetracycline-regulated MDMX and MDM2 were immunoprecipitated using MDMX and MDM2 antibodies, followed by Western blot for L11. MDMX and MDM2 expression were induced to ~10-fold above endogenous levels using tetracycline. Co-precipitation between MDM2 and L11 was detected when MDM2 was induced, whereas MDMX–L11 interaction was not detectable (Figure 1C). This result suggested that MDMX–L11 interaction was negligible even in overexpression conditions. The dramatic difference in ribosomal protein binding suggested that MDMX is regulated differently by ribosomal stress compared to MDM2.

Ribosomal stress induces MDMX degradation

To determine the effect of ribosomal stress on MDMX, we used actinomycin D (ActD) to inhibit ribosome biogenesis. ActD is a chemotherapeutic agent that can induce DNA damage and inhibit general transcription at high concentrations (>30 nM), but at low concentrations (5 nM) it selectively inhibits RNA polymerase I and induces ribosomal stress (Lohrum *et al*, 2003; Zhang *et al*, 2003).

When HCT116 and U2OS cells were treated with 5 nM ActD for 8–20 h, significant activation of p53 was observed, resulting in the induction of p21 and MDM2. In contrast, MDMX level decreased significantly after ActD treatment (Figure 2A). MDMX was also downregulated to the same degree in HCT116-p53−/− cells despite much weaker induction of MDM2 (Figure 2A), suggesting that additional mechanisms contributed to reduction in MDMX level. When HCT116-p53−/− cells were treated with ActD and MG132, MDMX downregulation was partially inhibited (Figure 2B). These results suggested that ActD promotes degradation of MDMX.

Recent studies showed that phosphorylation of MDMX C terminus by ATM and Chk2 promote MDMX degradation by MDM2 (Chen *et al*, 2005a; Pereg *et al*, 2005). We found that ActD (5 nM) and 5-FU (50 μM) did not induce significant phosphorylation of p53 S15 (Figure 6B) or phosphorylated histone gamma H2A.X (Supplementary Figure S5a). Phosphorylation-specific antibody against a Chk2 target site on MDMX (S367) did not reveal increased phosphorylation after ribosomal stress (Supplementary Figure S5b). Furthermore, Chk2 deficiency prevented MDMX degradation after irradiation, but had no effect on ActD and 5-FU (Supplementary

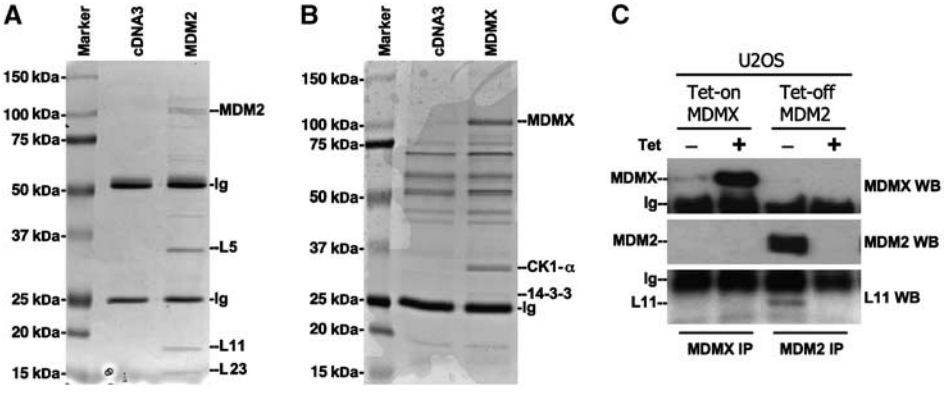

Figure 1 Differential binding of ribosomal proteins to MDM2 and MDMX. (**A**) MDM2 expression plasmid was transfected into 293 T cells for 2 days. MDM2 complex was immunoprecipitated using 2A9 antibody and stained with Coomassie blue. (**B**) FLAG-tagged MDMX stably expressed in Hela cells was purified using M2-agarose beads. MDMX complex was eluted with FLAG epitope peptide and stained with Coomassie blue. The MDM2 and MDMX-binding proteins were determined by mass spectrometry. (**C**) U2OS cell lines expressing Tet-on MDMX or Tet-off MDM2 were treated with tetracycline for 16 h to modulate expression levels, followed by MDMX or MDM2 IP and L11 Western blot.

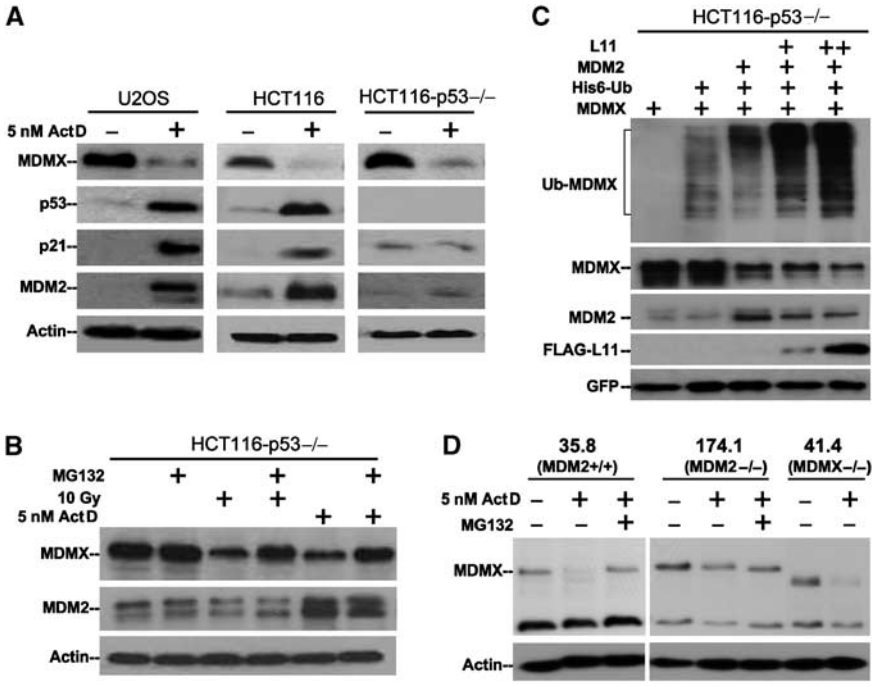

Figure 2 Downregulation of MDMX by ribosomal stress. (**A**) Cells were treated with 5 nM ActD for 16 h and analyzed by Western blot. (**B**) HCT116-p53−/− cells were treated with 5 nM ActD for 8 h, with or without 30 μM MG132 for the last 4 h and analyzed by Western blot. (**C**) HCT116-p53−/− cells were transiently transfected with His6-ubiquitin, MDMX, MDM2, and L11 plasmids. MDMX ubiquitination was detected by Ni-NTA purification followed by MDMX Western blot. (**D**) Mouse embryo fibroblasts with indicated genotypes were treated with ActD for 9 h with MG132 for the last 6 h and analyzed by Western blot with the 7A8 antibody.

Figure S5c). These results suggested that ribosomal stress induces MDMX degradation without causing DNA damage.

L11 promotes MDMX degradation by binding MDM2

Release of L11 from the nucleolus during ribosomal stress and binding to MDM2 was implicated in p53 activation. Therefore, we tested whether L11 stimulates MDMX ubiquitination by MDM2. The results showed that in HCT116-p53−/− cells, exogenous L11 stimulated MDMX polyubiquitination by MDM2 (Figure 2C). L11 expression did not increase MDM2 level, suggesting that the E3 ligase function of MDM2 was stimulated by L11. Although L5 and L23 also interact with MDM2, they did not stimulate MDMX ubiquitination (Figure 3E and data not shown). These results suggested that L11–MDM2 interaction during ribosomal stress promotes MDMX degradation.

Next, the role of MDM2 was tested using MDM2-null MEF (McMasters *et al*, 1996). ActD induced significant proteasome-dependent degradation of MDMX in MDM2+/+ MEFs compared to MDM2−/− control, suggesting that degradation of MDMX required MDM2 (Figure 2D). Knockdown of MDM2 in HCT116-p53−/− also blocked MDMX degradation after ActD and 5-FU treatment (Supplementary Figure S3c). In cotransfection assays, MDMX degradation was induced by L11 in MDM2+/+ cells (Figure 3B), but not in MDM2−/− cells unless MDM2 was restored by transfection (Figure 3A). Partial knockdown of L11 by siRNA also reduced the ability of ActD to downregulate MDMX (Figure 3C). Overall, these results showed that L11 and MDM2 are important for MDMX degradation after ribosomal stress.

To further test the specificity of L11 regulation of MDM2 and MDMX, we generated the MDM2-C305S mutant with a mutated zinc-finger in the L11-binding region. A similar mutation on MDMX (C306S) completely abrogated binding to casein kinase 1, revealing the structural importance of the zinc-finger (Chen *et al*, 2005b). As expected, MDM2-C305S did not bind L11, but retained binding to L5, L23, and ARF (Figure 3D and data not shown). The ability of MDM2-C305S to ubiquitinate and degrade MDMX was no longer stimulated by L11, but remained responsive to ARF as expected (Figure 3E). This result indicated that L11 stimulates MDMX degradation by binding to MDM2 and activating its ability to ubiquitinate MDMX.

Lack of MDM2 did not completely prevent MDMX downregulation by ActD (Figure 2D), suggesting additional mechanisms for MDMX regulation. Quantitative RT–PCR analysis showed that ActD also induced ~20% reduction in MDMX mRNA level (Supplementary Figure S1a). The activity of a 1 kb human MDMX promoter-luciferase construct was also inhibited ~30% by ribosomal stress, but not by DNA damage (Supplementary Figure S1b). Therefore, MDM2-mediated degradation played a major role in the rapid downregulation of MDMX by ribosomal stress, whereas transcriptional repression also contributed to the effect.

MDMX overexpression correlates with reduced p53 response to ribosomal stress

As MDM2 and MDMX showed different expression and binding to ribosomal proteins, they may have distinct effects on p53 response to ribosomal stress. To test this hypothesis, we compared tumor cell lines with different levels of MDMX and MDM2. In this panel, MDMX level can be ranked from highest to lowest in the order of JEG-3, MCF-7, U2OS, HCT116, A549, H1299 and SJSA. JEG-3 and SJSA have the

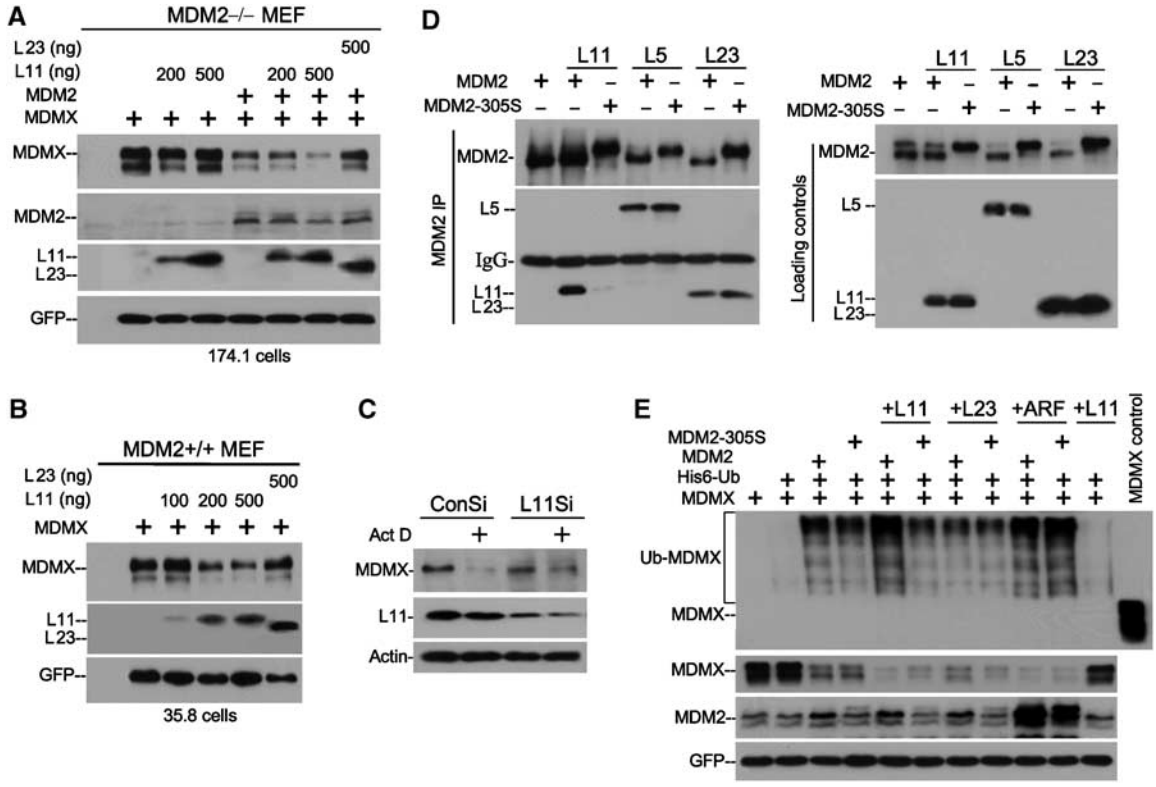

Figure 3 MDM2 and L11 mediate MDMX downregulation by ribosomal stress. (**A**, **B**) MEFs with and without MDM2 were transfected with 0.5 μg MDMX, 0.1 μg MDM2, and the indicated amounts of L11 plasmids and analyzed by Western blot. (**C**) HCT116 were transfected with 100 nM L11 siRNA for 48 h and treated with 5 nM ActD for 18 h, followed by Western blot analysis. (**D**) H1299 cells cotransfected with MDM2-C305S and FLAG-tagged L11, L5, and L23 were analyzed by MDM2 IP followed by FLAG Western blot for co-precipitation of L proteins. Expression was verified by MDM2 and FLAG Western blot of whole-cell extract. (**E**) HCT116-p53−/− cells transfected with the indicated plasmids were analyzed for MDMX ubiquitination, showing the loss of MDM2-C305S regulation by L11.

highest MDM2 levels due to gene amplification or increased translation (Leach *et al*, 1993; Landers *et al*, 1997). H1299 is p53 null and served as a control. After treatment with ActD, all cell lines showed p53 stabilization irrespective of MDM2 level. However, induction of p21 correlated inversely with the level of MDMX, but not MDM2 (Figure 4A), suggesting that high MDMX levels kept the stabilized p53 in an inactive state.

When the cells were analyzed for cell cycle arrest after ActD treatment, cell lines with low MDMX level (SJSA, A549, U2OS, HCT116) showed more significant reduction of S-phase than cells with high level MDMX (MCF-7, JEG-3) (Figure 4B, Supplementary Figure S2a). SJSA showed strong response to ActD despite expressing the highest level of MDM2. As expected, p53-null H1299 did not respond to ActD. Therefore, cell cycle sensitivity to ActD also correlated with MDMX level, but not MDM2 level. These results suggested that MDMX overexpression has significant impact on p53 activation by ribosomal stress.

Modulation of MDMX expression affects p53 activation by ribosomal stress

To further confirm that MDMX overexpression at a physiological level inhibits p53 activation and cell cycle arrest after ribosomal stress, HCT116 cells were infected with MDMX cDNA lentivirus and siRNA retrovirus. Polyclonal cell lines expressing MDMX, scrambled siRNA, or MDMX siRNA were analyzed. MDMX lentivirus provided ~5-fold increase of MDMX level in HCT116, below the levels of MDMX in MCF-7 (Supplementary Figure S3b). MDMX overexpression reduced the sensitivity, whereas MDMX knockdown sensitized cells to ActD induction of p21 (Figure 4C). Furthermore, ActD did not induce p21 in HCT116-p53−/− cells, and MDMX overexpression or knockdown had no effect on p21 expression (data not shown). Manipulation of MDMX level did not affect p53 stabilization by ActD. These results showed that MDMX overexpression blocked p53 activation, whereas MDMX knockdown increased sensitivity to ribosomal stress.

Next, the effect of MDMX on cell cycle arrest was analyzed. Treatment with 1–2 nM ActD for 18 h caused significant reduction of S-phase population in FACS analysis. HCT116 cells with MDMX overexpression were efficiently protected from cell cycle arrest by ActD, and knockdown of MDMX caused more efficient arrest (Figure 4D, Supplementary Figure S2b). Similar results were also obtained using U2OS with MDMX overexpression or knockdown (Figure 5A, B). These results demonstrated that MDMX expression level has significant impact on p53 response to ribosomal stress.

MDMX overexpression sustains cell proliferation after ribosomal stress

Cells contain a stockpile of ribosomes that can sustain normal protein synthesis for at least 24 h after inhibition of rRNA processing (Pestov *et al*, 2001). Therefore, overcoming p53-mediated arrest should permit cell proliferation until depletion of the ribosomes. To determine the maximum potential of MDMX in maintaining cell proliferation during ribosomal

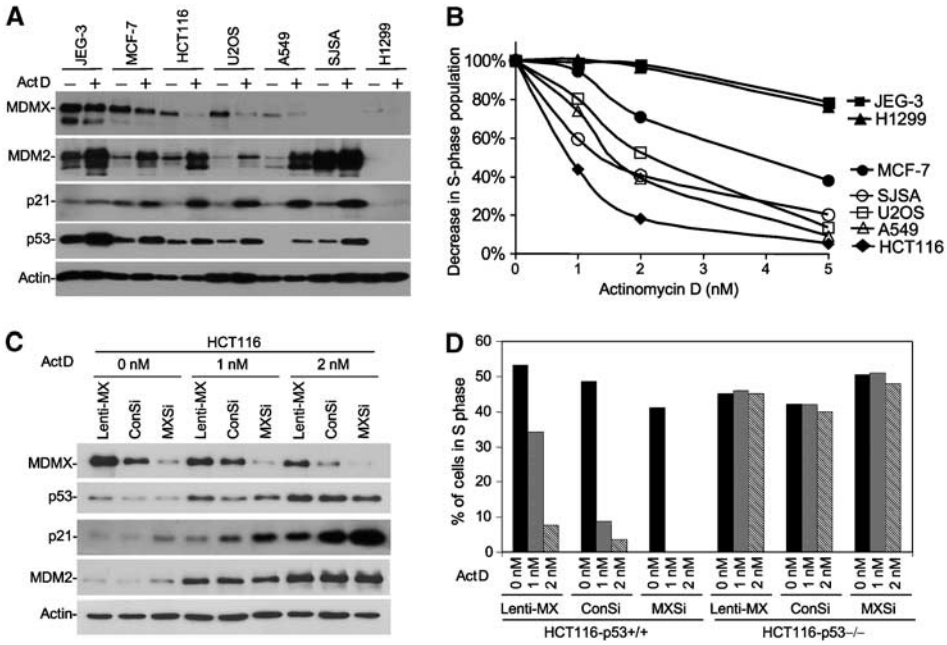

Figure 4 MDMX overexpression correlates with actinomycin D resistance. (**A**) Cell lines were treated with 5 nM ActD for 18 h and analyzed by Western blot. (**B**) Cells were treated with ActD for 18 h and analyzed for cell cycle distribution by FACS. The degree of growth arrest was shown as the decrease of S-phase population compared to untreated controls. (**C**) HCT116 cells were infected with MDMX lentivirus, scrambled siRNA, and MDMX siRNA retrovirus. Pooled colonies were treated with ActD for 18 h and analyzed by Western blot. (**D**) HCT116 cell lines expressing different levels of MDMX were treated with ActD for 18 h and analyzed for cell cycle distribution by FACS. The percent of cells in S-phase population are shown.

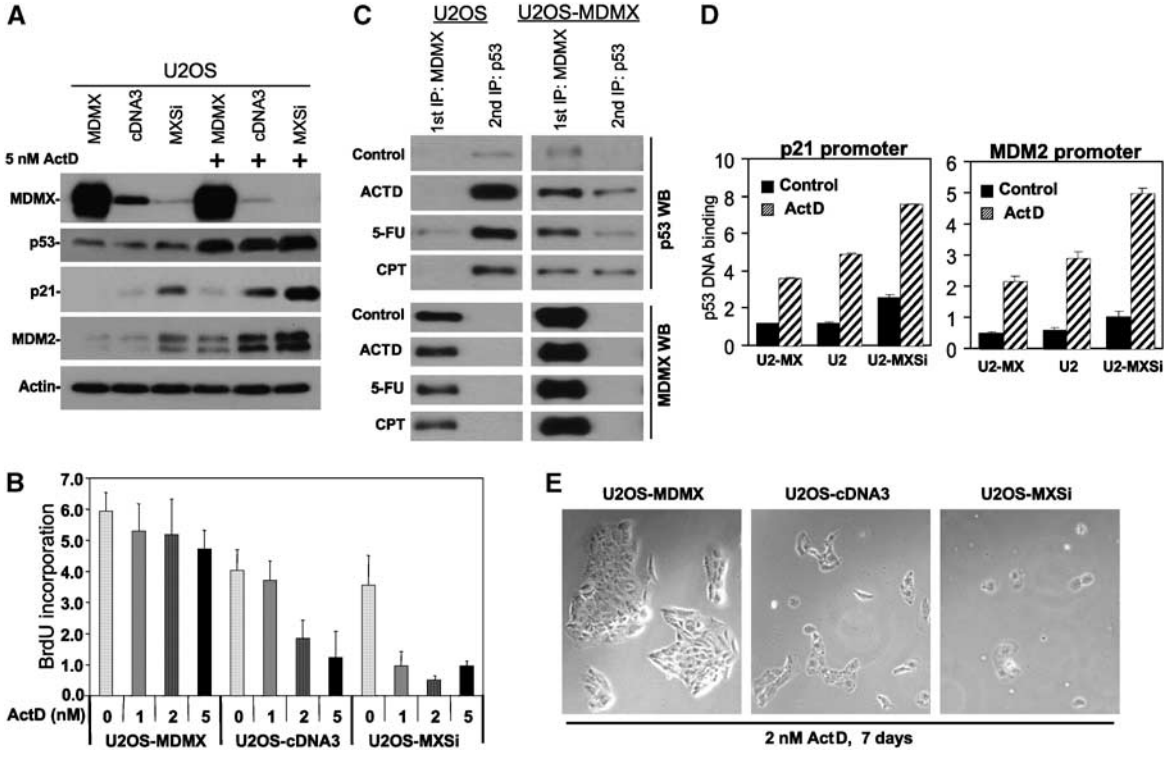

Figure 5 MDMX level affects growth arrest by actinomycin D. (**A**) U2OS cells stably transfected with MDMX cDNA or siRNA plasmids were treated with 5 nM ActD for 16 h and analyzed by Western blot. (**B**) U2OS expressing different levels of MDMX were treated with ActD for 18 h and analyzed for DNA synthesis by BrdU incorporation. (**C**) Lysate of U2OS-MDMX treated with drugs for 16 h was immunodepleted with MDMX antibody to detect MDMX–p53 complex, followed by IP with p53 antibody to detect free p53. The precipitates were analyzed by p53 Western blot. (**D**) U2OS expressing different levels of MDMX were treated with 5 nM ActD for 16 h and analyzed by ChIP to detect p53 binding to the MDM2 and p21 promoters. (**E**) Colony size of U2OS cell lines after continuous 2 nM actinomycin D treatment for 7 days.

stress, we generated a U2OS cell line expressing MDMX at ~30-fold above endogenous level (Figure 5A). BrdU labeling after 18 h of ActD treatment showed that cells overexpressing MDMX continued to synthesize DNA (Figure 5B). Conversely, MDMX knockdown caused more efficient shutdown of DNA synthesis even when treated with the lowest concentration (1 nM) of ActD.

Next, we tested the effect of MDMX overexpression on cell proliferation during ribosomal stress. Starting at ~10% confluency, cells cultured in the continuous presence of ActD were analyzed by MTT assay over 4 days. After 1 day of treatment with ActD, U2OS cells expressing MDMX siRNA stopped proliferating, indicating activation of cell cycle checkpoints. Conversely, U2OS cells overexpressing MDMX continued to proliferate at a significant rate, ultimately reaching confluency in the presence of ActD (Supplementary Figure S3a). As expected, cell proliferation sustained by MDMX overexpression would eventually reach a limit as ribosomes were depleted. When cells were given unlimited space to proliferate by plating at low density, MDMX-overexpressing cells were only able to give rise to micro-colonies before stopping completely in 2 nM ActD (Figure 5E). However, the arrest was reversible, as removal of ActD after 7 days of treatment allowed MDMX-overexpressing cells to form large colonies. MDMX siRNA significantly reduced long-term viability after ActD treatment (data not shown). These results suggested that MDMX overexpression abrogated p53-mediated growth arrest and allowed cells to proliferate through multiple cycles after inhibition of ribosome biogenesis.

To test the growth advantage from having moderate MDMX overexpression, HCT116 and HCT116-Lenti-MDMX cells were mixed at 20:1 ratio. Cells were treated with 3 nM ActD for 4 days followed by normal medium for 4 days. After the treatment cycle was repeated for a total of 30 days, the ActD-resistant colonies were pooled and MDMX expression was determined. The results showed that the survivors were predominantly HCT116-Lenti-MDMX cells (data not shown). This suggests that cells overexpressing MDMX have a clear survival advantage under conditions of ribosomal stress.

MDMX sequesters p53 into inactive complexes

As MDMX does not significantly affect p53 stability, we investigated the mechanism by which p53 is inactivated by MDMX overexpression. The fractions of free p53 and p53–MDMX complex were analyzed by MDMX immunodepletion followed by p53 IP. The results showed that overexpression of MDMX in U2OS sequestered the majority of p53 into MDMX–p53 complexes. After treatment with ActD or 5-FU, the majority of p53 remained bound to MDMX. In contrast, DNA damage by CPT released ~50% of p53 into a free form (Figure 5C). This assay also revealed that >50% of p53 in MCF-7 and JEG-3 cells can be co-precipitated with endogenous MDMX after ActD treatment (Supplementary Figure S5d, data not shown), confirming that physiological MDMX overexpression is sufficient to quantitatively sequester p53.

To further test whether MDMX interferes with p53 binding to DNA, U2OS expressing different levels of MDMX were analyzed by chromatin immunoprecipitation (ChIP) assay using p53 antibodies and PCR primers for MDM2 and p21 promoters. The results of p53 ChIP showed that MDMX overexpression reduced p53 DNA binding after ActD treatment, whereas MDMX knockdown increased p53 DNA binding in both untreated and ActD-treated cells (Figure 5D). These results suggested that MDMX inhibits the DNA-binding activity of p53. However, the difference in p53 binding to the p21 promoter appeared insufficient to account for the large difference in p21 expression level (Figure 5A). This suggests that MDMX may also function by blocking p53 interaction with basal transcription factors at the promoter. We currently cannot confirm or rule out the presence of MDMX–p53 complex on DNA because ChIP assay using MDMX antibodies was inconclusive.

MDMX prevents p53 activation by serum starvation and contact inhibition

To test the role of MDMX in p53 response to other types of ribosomal stress, we expressed MDMX in primary human foreskin fibroblasts (HFF) using lentivirus vector. Infection of HFF with MDMX lentivirus increased expression to a level similar to that of U2OS (data not shown). Therefore, this represents a physiologically achievable level of MDMX upregulation. Normal human fibroblasts undergo p53 activation and G1 arrest during serum starvation or contact inhibition. Recent study showed that inhibition of rRNA expression and release of L11 was responsible for p53 activation during serum starvation (Bhat *et al*, 2004). Other studies have shown that contact inhibition of normal fibroblasts causes a decrease in rRNA synthesis by inhibiting the recruitment of UBF to the rDNA promoter (Hannan *et al*, 2000a, b).

HFF and HFF-Lenti-MDMX cells were compared for p53 activation after culturing in 0.5% serum for 18 h (serum starvation), maintained at 100% density for 3 days (contact inhibition), or treated with 2 nM ActD for 18 h. Western blot showed that all three treatments resulted in increase of p53 and p21 levels in control HFF. However, p21 induction was significantly weaker in HFF-lenti-MDMX cells (Supplementary Figure S4a), indicating ineffective p53 activation. Furthermore, growth curve and FACS assays showed that HFF-Lenti-MDMX cells were desensitized to growth inhibitory conditions and did not undergo efficient cell cycle arrest (Supplementary Figure S4b, c). These results demonstrated that a tumor-equivalent level of MDMX overexpression in normal cells was sufficient to interfere with p53 response to abnormal ribosomal biogenesis.

MDMX overexpression confers resistance to 5-FU

To investigate the relevance of MDMX overexpression in cancer chemotherapy, we tested its effect on sensitivity to 5-FU. Inhibition of thymidylate synthase and DNA metabolism was thought to be responsible for the cytotoxicity of 5-FU (Parker and Cheng, 1990). However, recent studies suggested that inhibition of RNA metabolism is responsible for its proapoptotic activity (Ghoshal and Jacob, 1994; Longley *et al*, 2002). Cell death by 5-FU can be prevented by uridine but not thymidine (Pritchard *et al*, 1997). Numerous reports showed that 5-FU at 100–500 μM induce p53 phosphorylation at serine 15, possibly through DNA damage and ATM activation. However, it has also been suggested that lower concentrations of 5-FU (10–100 μM) activates p53 through mechanisms independent of DNA damage or ATM activation (Longley *et al*, 2002; Kurz and Lees-Miller, 2004). We hypothesized that 5-FU may activate p53 by inhibiting rRNA synthesis and inducing ribosomal stress.

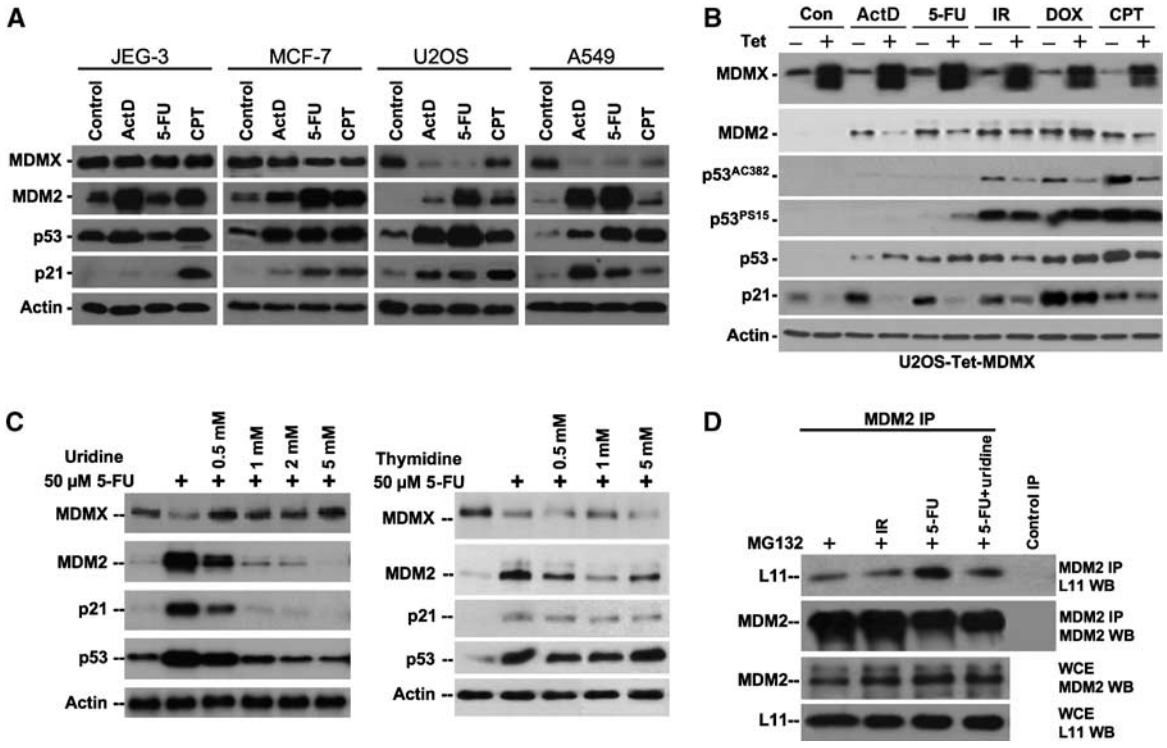

Figure 6 MDMX overexpression confers resistance to 5-fluorouracil. (**A**) Tumor cell lines were treated with 5 nM ActD, 50 μM 5-FU, and 0.5 μM CPT for 18 h and analyzed by Western blot. (**B**) U2OS cells expressing tetracycline-inducible MDMX were treated with 1.0 μg/ml tetracycline and 5 nM ActD, 50 μM 5-FU, 1 μM doxorubicin or 0.5 μM CPT for 18 h, or 10 Gy IR for 4 h and analyzed by Western blot. (**C**) U2OS cells were treated with 5-FU and uridine or thymidine for 8 h and analyzed for activation of p53. (**D**) U2OS cells were treated with 5-FU and uridine for 8 h or irradiated with 10 Gy for 4 h and analyzed for MDM2–L11 binding by MDM2 IP and L11 Western blot. MG132 was added for 4 h to obtain similar levels of MDM2.

Tests using unmodified tumor cell lines showed that high endogenous MDMX level was associated with reduced p21 induction after 5-FU treatment (Figure 6A). This pattern was similar to ActD, and different from the DNA-damaging drug camptothecin (Figure 6A). 5-FU also induced proteasome-dependent degradation of MDMX that can be partially rescued by MG132 (data not shown). Using a U2OS cell line expressing tetracycline-inducible Lenti-MDMX, we found that expression of MDMX five-fold above endogenous level resulted in significant inhibition of p21 induction by 5-FU and ActD, but had little effect on response to several DNA-damaging agents (Figure 6B). MDMX overexpression also sustained DNA replication in the presence of 5-FU, while MDMX knockdown increased sensitivity (data not shown). Compared to DNA-damaging agents, 50 μM 5-FU induced very little p53 serine 15 phosphorylation, gamma H2A.X phosphorylation, and MDMX S367 phosphorylation (Figure 6B, Supplementary Figure S5a, b), confirming the absence of significant DNA damage.

The effects of MDMX overexpression on 5-FU and ActD responses suggested that low concentrations of 5-FU mainly act by inducing ribosomal stress. To confirm that 5-FU activates p53 by inhibiting RNA metabolism, HCT116 cells were treated with 5-FU in the presence of uridine, which bypassed inhibition of uridine synthesis by 5-FU (Longley *et al*, 2003). Addition of uridine but not thymidine prevented p53 stabilization and p21 induction by 5-FU in a dose-dependent fashion (Figure 6C), suggesting that inhibition of RNA metabolism and ribosomal biogenesis was responsible for p53 activation. Treatment with 5-FU also increased the amount of endogenous binding between MDM2 and L11 (Figure 6D), and induced release of nucleolin from the nucleolus similar to ActD (Figure S6), consistent with nucleolar stress. These results suggested that low concentrations of 5-FU activate p53 by inducing ribosomal stress.

5-FU is a major chemotherapy agent for colorectal cancer. When HCT116 cells with overexpression and knockdown of MDMX were treated with 50 μM 5-FU, p21 expression was induced in a p53-dependent fashion. Similar to ActD response, MDMX expression level showed an inverse correlation with p21 induction (Figure 7A). HCT116 undergoes apoptosis after 5-FU treatment. Knockdown of MDMX resulted in enhanced cell death, whereas MDMX overexpression blocked apoptosis in the presence of 5-FU (Figure 7C). MDMX overexpression also increased resistance against the DNA-damaging drug doxorubicin in short-term MTT assay (Figure 7D). However, the impact of MDMX on 5-FU sensitivity was more significant, particularly at low drug concentrations (compare Figure 7C and D). In colony formation assays, MDMX overexpression improved long-term survival after treatment with 5-FU, but not doxorubicin (Figure 7B). These results suggested that MDMX is an important determinant of sensitivity to 5-FU.

MDMX regulates tumor formation and drug resistance in vivo

To test the role of MDMX in tumor formation *in vivo*, HCT116 cells expressing scrambled or MDMX siRNA (Figure 4C) were inoculated subcutaneously on the dorsal flanks of athymic nude mice. Each animal received both control and test cell

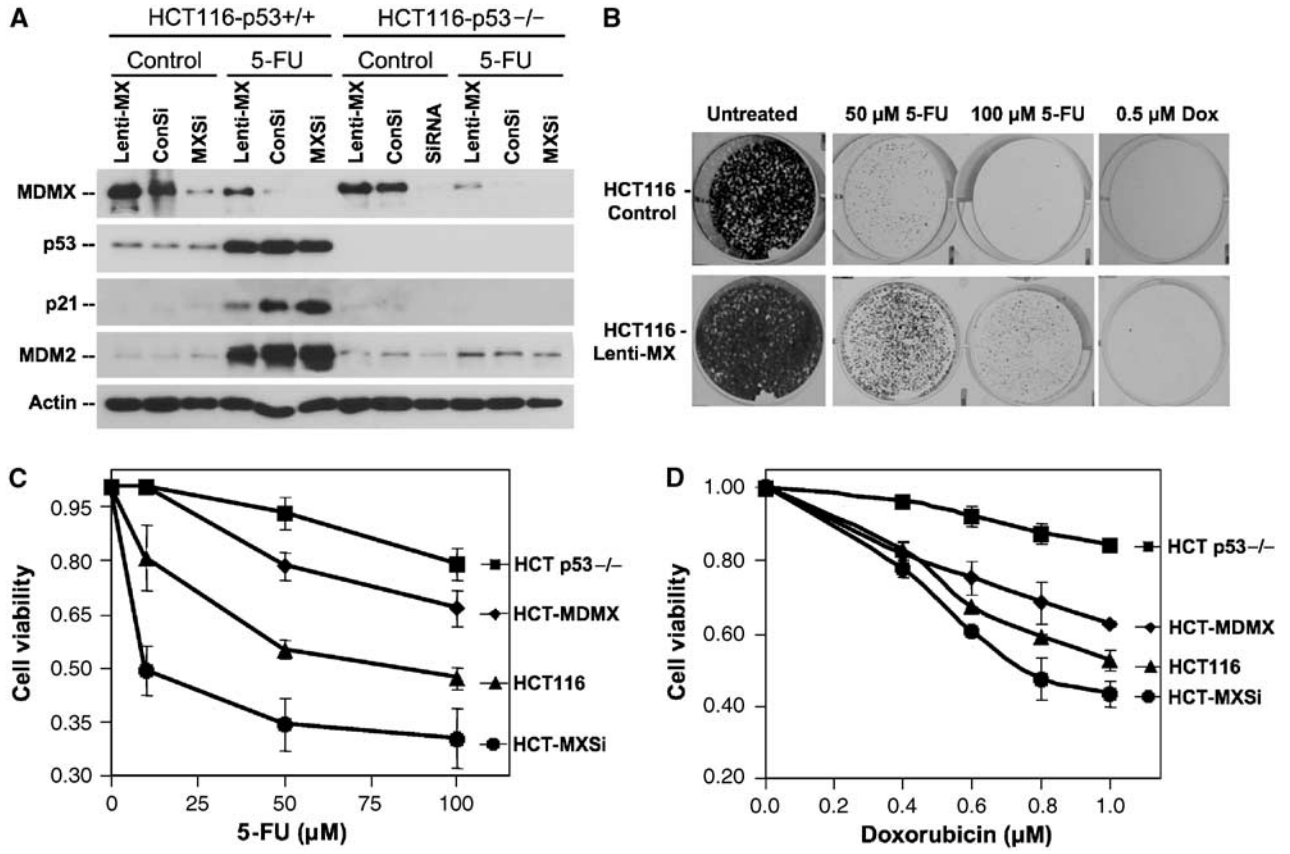

Figure 7 MDMX level determines p53-mediated cell death by 5-fluorouracil (5-FU). (**A**) HCT116 cell lines expressing different levels of MDMX were treated with 50 μM 5-FU for 18 h and analyzed by Western blot. (**B**) Control and MDMX-overexpressing HCT116 were plated at 5000/well for 24 h, treated with drugs for 24 h, incubated in drug-free medium for 7 days, and stained for colony formation efficiency. (**C**) HCT116 cell lines were treated with 5-FU for 48 h and analyzed for cell viability by MTT assay. (**D**) HCT116 cell lines were treated with doxorubicin for 48 h and analyzed for cell viability by MTT assay.

lines. The scrambled siRNA had no effect on tumor formation compared to the unmodified HCT116 cells (data not shown). In contrast, MDMX siRNA-expressing cells showed significantly reduced tumorigenic potential ($n=13$, $P=0.0005$, Figure 8A and B). A repeat of the experiment also generated similar results (not shown). When dissectable tumors formed by MDMX siRNA-expressing cells were analyzed, they showed MDMX expression level similar to control HCT116 tumors (Figure 8C). As the MDMX siRNA cell line was a polyclonal pool of retrovirus-infected colonies, it is likely that some of the cells regained normal MDMX expression and tumorigenic potential. These results demonstrated that partial knockdown of MDMX effectively blocked tumor formation *in vivo*. The results also suggested that the tumor environment caused unknown physiological stress that required suppression of p53 by MDMX.

To further test the effects of MDMX overexpression on tumor growth and treatment response *in vivo*, mice were inoculated with HCT116-vector and HCT116-Lenti-MDMX cells. The mice were treated with 5-FU when tumors developed to $\sim 0.1\,cm^3$ in size. In untreated animals, HCT116-Lenti-MDMX cells did not show increased tumor growth compared to HCT116-vector control (Figure 8D), suggesting that the level of endogenous MDMX was sufficient for growth *in vivo*. However, MDMX overexpression resulted in statistically significant tumor resistance (*P*-value 0.01) to 5-FU treatment (Figure 8D). These results further demonstrated that MDMX inhibits tumor response to RNA-targeting chemotherapy drugs *in vivo*.

Discussion

Results described above show that MDMX is an important regulator of p53 activation by ribosomal stress. MDMX overexpression at physiologically relevant levels significantly desensitizes cells to ribosomal stress-inducing agents. In contrast, physiological level of MDM2 overexpression (from gene amplification) does not confer resistance to ActD. Our results also demonstrated that endogenous MDMX expression in HCT116 cells is necessary for tumor formation, suggesting that MDMX is a useful drug target.

Differences in structure and function of MDM2 and MDMX may be responsible for their distinct effects on ribosomal stress response. MDM2 is an ubiquitin ligase that functions mainly by promoting p53 degradation. This mechanism may be highly sensitive to inhibition by ribosomal proteins. Therefore, physiological levels of MDM2 overexpression are effectively neutralized during ribosomal stress, resulting in p53 stabilization. In contrast, MDMX is a stable protein that regulates p53 mainly by sequestering p53 into complexes (Francoz *et al*, 2006; Toledo *et al*, 2006). Because ribosomal stress does not induce p53 phosphorylation or block p53–MDMX binding, MDMX overexpression will trap p53 in inactive complexes and prevent p21 induction, sustaining

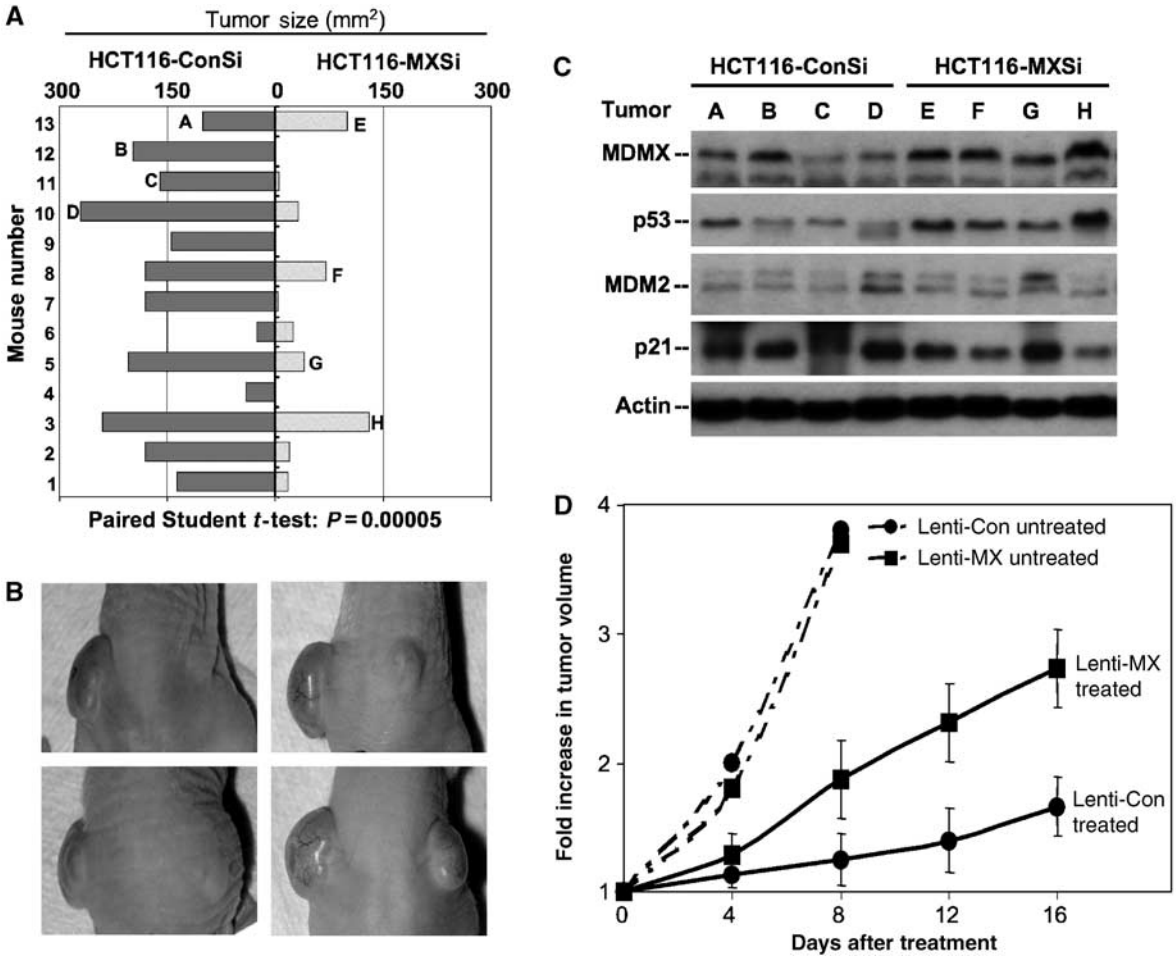

Figure 8 MDMX expression is required for tumor formation. (**A**) HCT116 cells expressing control and MDMX siRNA were inoculated into athymic nude mice (5×10^6/site). Tumor growth was measured after 14 days. Tumors marked with A–H were analyzed for MDMX expression. (**B**) Representative pictures of tumor-bearing animals. Left side: HCT116-control siRNA. Right side: HCT116-MDMX siRNA. (**C**) Tumor samples recovered 14 days after inoculation were analyzed by Western blot for MDMX and indicated markers. (**D**) HCT116 cells stably infected with lentivirus vector or lenti-MDMX were inoculated into nude mice. Mice with $\sim 0.1\ cm^3$ size tumors were treated with 5-FU at 50 mg/kg/day for 4 days and tumor growth were measured during the indicated time frame.

cell proliferation. We should note that our results do not rule out p53-independent effects of MDMX on p21 expression, such as by targeting it for degradation.

The biological significance of ribosomal stress in regulating cell proliferation *in vivo* is still not clearly defined. The ability of MDMX to attenuate p53 activation and cell cycle arrest during growth factor deprivation and other ribosomal stress conditions may provide an advantage in a tumor environment. It is possible that different regions of a tumor undergo cycles of proliferation, growth arrest, and cell death due to imbalance in the supply of growth factors and nutrients. MDMX overexpression would suppress p53 activation by ribosomal stress, allowing additional rounds of cell division. The cumulative effect of such limited growth would be significant after repeated cycles of stress selection, as suggested by our mixing experiment.

MDMX overexpression may also interfere with p53 activation by other growth regulators. It has been shown that the retinoblastoma protein pRb inhibits RNA polymerase I-mediated transcription by binding to the UBF factor, thus inhibiting rRNA expression (Voit *et al*, 1997). This function should lead to ribosomal stress and contribute to growth arrest by pRb during contact inhibition (Hannan *et al*, 2000b). In addition, p53 itself has been shown to inhibit rRNA transcription (Budde and Grummt, 1999), which would have a positive feedback effect through release of ribosomal proteins. Abnormal expression of MDMX may block p53 activation and weaken the effects of multiple tumor suppressor pathways.

The ability of MDMX to abrogate p53 activation by 5-FU may have significant clinical relevance. This drug is a mainstay compound in the chemotherapy of colon cancer. 5-FU cytotoxicity depends on conversion to 5-fluoro-UTP, 5-fluoro-dUMP, and 5-fluoro-dUTP. Binding of 5-fluoro-dUMP to the enzyme thymidylate synthase inhibits the synthesis of thymidine nucleotides, giving rise to DNA strand breaks (Parker and Cheng, 1990), this was believed to be the major mechanism of cytotoxicity. However, 5-FU also inhibits rRNA processing (Ghoshal and Jacob, 1994). *In vitro* studies have shown that 5-FU incorporation into RNA but not DNA was associated with cell death (Geoffroy *et al*, 1994). Incorporation into RNA is responsible for the gastrointestinal toxicity of 5-FU in mice (Houghton *et al*, 1979). A study using p53-null mice showed that intestinal epithelial apoptosis induced by 5-FU is p53-dependent, and involves interference of RNA metabolism (Pritchard *et al*, 1997). Experiments using

HCT116 cells also suggested a p53-dependent cytotoxicity of 5-FU through inhibition of RNA metabolism (Bunz *et al*, 1999). Here we show that 5-FU activation of p53 is abrogated by uridine but not thymidine, and is highly sensitive to MDMX overexpression. These results suggest that induction of ribosomal stress and p53 activation is an important mechanism of 5-FU cytotoxicity, although DNA damage may also be a contributing factor at high drug doses.

In light of the findings described above, it will be important to investigate whether there is a correlation between MDMX expression level and tumor response to 5-FU or other RNA-directed drugs in the clinic. MDMX overexpression has been observed in both tumor cell lines and primary tumor biopsies (Ramos *et al*, 2001; Danovi *et al*, 2004). MDMX gene amplification does not appear to be the major mechanism of overexpression (~5% in breast tumors) (Danovi *et al*, 2004). Analyses of MDMX promoter suggested that MDMX expression level in tumor cell lines correlates with promoter activity (unpublished observations). The drug sensitization and antitumor effects of MDMX siRNA suggest that targeting MDMX–p53 interaction with small molecules may have therapeutic value. To this end, it is noteworthy that the MDM2 inhibitor Nutlin 3 does not target MDMX–p53 binding (Vassilev, 2004; Patton *et al*, 2006), suggesting a need to develop novel MDMX inhibitors.

Materials and methods

Cell lines and plasmids

Tumor cell lines H1299 (lung, p53-null), A549 (lung), U2OS (bone), SJSA (bone, MDM2 amplification), MCF-7 (breast), JEG-3 (placenta, MDM2 overexpression) were maintained in DMEM medium with 10% fetal bovine serum. HCT116-p53−/− and HCT116-Chk2−/− cells were kindly provided by Dr Bert Vogelstein and maintained in McCoy 5A medium with 10% fetal bovine serum. Normal human skin fibroblast HFF was provided by Dr Jack Pledger. MDMX and MDM2-null MEFs were provided by Dr Gigi Lozano. To inhibit MDMX in human cell lines by RNAi, double-stranded oligonucleotide (5′GATCCCGTGATGATACCGATGTAGATTCAAGAGATCTACATCGGTATCATCACTTTTTTTGGAAA, MDMX sequence underlined) was cloned into the pSuperiorRetroPuro vector (OligoEngine). Cells were infected with the MDMX shRNA retrovirus and selected with 0.5–1 μg/ml puromycin. Drug-resistant colonies were pooled for analysis. A virus expressing a scrambled shRNA (5′GATCCCGCCGTCGTCGATAAGCAATATTTGATATCCGATATTGCTTATCGACGACGGCTTTTTTA) was used as control. L11 siRNA pool was purchased from Dharmacon.

Protein analysis

Western blot was performed as described previously (Chen *et al*, 2005a). The following monoclonal antibodies were used: 3G9 and 2A9 for MDM2 Western blot and IP (Chen *et al*, 1993); DO-1 (Pharmingen) p53 Western blot; 8C6 monoclonal or a rabbit polyclonal serum for MDMX Western blot and IP (Li *et al*, 2002). MDMX in mouse cell lines was detected using a new monoclonal antibody 7A8. L11 was detected using a rabbit polyclonal antibody provided by Dr Yanping Zhang. The filter was developed using ECL-plus reagent (Amersham).

Affinity purification of MDMX and MDM2

Purification of MDMX complex was performed as described before (Chen *et al*, 2005b). To purify MDM2 complexes, human MDM2 cDNA expression plasmid was transiently transfected into 293T cells. At 2 days after transfection, cells ($\sim 2 \times 10^8$) were treated with 30 μM MG132 for 4 h, lysed in a total of 10 ml lysis buffer and centrifuged for 5 min at 10 000 *g*. The lysate was precleared with protein A Sepharose beads for 30 min, and then incubated with 40 μl protein A Sepharose beads and 0.5 ml 2A9 hybridoma supernatant for 4 h at 4°C. The beads were washed with lysis buffer and boiled in SDS sample buffer. The eluted proteins were fractionated on SDS–PAGE and stained with Coomassie Blue. Proteins co-purified with MDMX and MDM2 were identified by mass spectrometry.

ChIP

ChIP assay was performed using standard procedure. P53 complexes were immunoprecipitated with a mixture of Pab1801 and DO-1 antibodies. Samples were subjected to SYBR Green real-time PCR analysis using forward and reverse primers for the p53-binding sites in the MDM2 promoter (5′-CGGGAGTTCAGGGTAAAGGT and 5′-CCTTTTACTGCAGTTTCG) and p21 promoter (5′-TGGCTCTGATTGGCTTTCTG and 5′-TCCAGAGTAACAGGCTAAGG).

In vivo ubiquitination assay

HCT116-p53−/− cells in 6 cm plates were transfected with combinations of 0.5 μg His6-ubiquitin expression plasmid, 1 μg MDMX, 0.2 μg MDM2, and 0.1–0.2 μg FLAG-L11 expression plasmids using Lipofectamine Plus reagents (Life Technologies). At 24 h after transfection, cells were collected and MDMX ubiquitination was detected as described previously (Chen *et al*, 2005a).

Expression of MDMX by lentiviral vector

Lentivirus vector expressing MDMX was generated using the ViraPower™ T-REx™ system following instructions from the manufacturer (Invitrogen). Overexpression of MDMX in primary HFF cells was achieved by infecting with the MDMX lentivirus and selection with Zeocin to obtain a pool of colonies. Tetracycline inducible expression of MDMX in U2OS was achieved by first infecting with the T-REX regulator lentivirus and selection with Blasticidin, followed by infection with the MDMX lentivirus and selection with Zeocin. MDMX expression was induced with 0.1–1 μg/ml tetracycline.

Tumor xenograft assay

Athymic-NCr-nu female mice between 7 and 8 weeks were inoculated s.c. on both flanks with 5×10^6 of HCT116 cells. Tumors were measured after 14 days with calipers. For 5-FU treatment response, control and Lenti-MDMX-expressing tumors were grown for 10 days to ~0.1 cm^3 on both flanks. Mice were treated with 5-FU at 50 mg/kg/day for 4 days by tail vain injection. Tumor growth was measured for 16 days after initiation of treatment.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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