A novel role for IGF-1R in p53-mediated apoptosis through translational modulation of the p53-Mdm2 feedback loop

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Insulin-like growth factor 1 receptor (IGF-1R) is important in cancer cell growth and survival and has been implicated in cancer pathophysiology and treatment. Here we report a novel function for IGF-1R in p53-dependent apoptotic response. We show that inhibition or loss of IGF-1R activity reduces translational synthesis of p53 and Mdm2 protein. Notably, IGF-1R inhibition increases p53 protein stability by reducing p53 ubiquitination and maintains p53 at low levels by decreasing p53 synthesis, thus rendering p53 insensitive to stabilization after DNA damage. The accumulation and apoptosis of DNA-damage– induced p53 is therefore reduced in *Igf-1r*$^{-/-}$ mouse embryonic fibroblasts or tumor cells treated with the IGF-1R inhibitor. Furthermore, we find that inhibition of IGF-1R reduces p53 and Mdm2 translation through a gene-specific mechanism mediated by the respective 5′ untranslated region of p53 and mdm2 messenger RNA. The eukaryotic translation initiation factor 4F complex is also involved in this translational inhibition. These results demonstrate an unexpected role for translational control by IGF-1R in p53-mediated apoptosis.

Introduction

A hallmark of cancer is evasion of apoptosis (Hanahan and Weinberg, 2000), which links cancer genetics and cytotoxic chemotherapies inextricably together (Johnstone et al., 2002). Apoptosis induced by chemotherapeutic agents has been attributed to the induction of DNA damage. One of the key molecules involved in response to DNA damage is the tumor suppressor protein p53 (Lakin and Jackson, 1999; Vousden and Lu, 2002). The loss of p53 response is thought to promote genomic instability (Yin et al., 1992) that can lead to increased resistance to chemotherapeutic agents. In normal unstressed cells, the p53 protein is present at very low levels because of continuous degradation mediated by Mdm2, a protein that is also transcriptionally activated by p53 (Wu et al., 1993). Thus, p53 and Mdm2 are linked to each other through an autoregulatory negative feedback loop (Prives, 1998). Disruption of the p53–Mdm2 complex is the pivotal event in p53 activation after DNA damage (Prives, 1998; Lakin and Jackson, 1999; Vousden and Lu, 2002). In addition, recent papers have suggested that enhanced translation of p53 mRNA is also an important step in the induction of p53 in stressed cells (Giaccia and Kastan, 1998; Mazan-Mamczarz et al., 2003; Takagi et al., 2005), although the mechanisms remain largely unknown.

Translation of eukaryotic mRNAs is predominantly regulated at the level of initiation (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002), when the ribosome is recruited to the mRNA. The eukaryotic translation initiation factor (eIF) complex eIF4F is required for this multistep process and is composed of the cap-binding protein eIF4E; the RNA helicase eIF4A; and the scaffold protein eIF4G, which provides binding sites for eIF4E, eIF4A, and the poly(A)-binding protein (PABP; Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002). eIF4A is required to unwind the second structure in the 5′ untranslated region (UTR). The helicase activity of eIF4F should be proportional to the amount of the secondary structure in the 5′ UTR, which would otherwise affect translational efficiency

(Gray and Wickens, 1998; Raught et al., 2000). The efficiency of translation initiation is tightly coupled with cell cycle progression and cell growth, with translational induction occurring in response to mitogenic stimulation (Raught et al., 2000; Dever, 2002). Such changes in translation are normally mediated by alterations in the expression or phosphorylation status of the various translation initiation factors involved (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002). Hypophosphorylated eIF4E–binding protein 1 (BP1) competes with eIF4G for binding to eIF4E and prevents formation of the eIF4F complex (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002). In addition, the interaction of eIF4E with its partners can be regulated by the availability of free eIF4G, which may be regulated at the levels of synthesis and turnover (Morley et al., 1997). Despite suggestions that the control of translation may be regulated by growth-factor signaling (Dever, 2002; Rajasekhar et al., 2003), the relative contribution of translational effects of these signaling pathways in their corresponding cellular activities and the mechanisms involved have remained unclear.

Insulin-like growth factor 1 receptor (IGF-1R) is a membrane-associated tyrosine kinase receptor that plays an important role in cell growth, transformation, and protection of cells from a variety of apoptotic stimuli (LeRoith and Roberts, 2003; Pollak et al., 2004; Samani et al., 2007). IGF-1R signaling protects cells from apoptosis mainly through the phosphoinositide-3 kinase (PI-3K)–Akt and Ras–Raf–MAPK pathways (Párrizas et al., 1997; Gooch et al., 1999; Peruzzi et al., 1999). Inhibition of IGF-1R has been shown to block tumor growth and sensitize cells to antitumor treatments (Samani et al., 2007), indicating that IGF-1R is a promising target for cancer therapeutics (De Meyts and Whittaker, 2002). In other situations, however, IGF-1R signaling contributes to cell death (Kooijman, 2006). Overexpression of the C terminus of the IGF-1R β subunit induced apoptosis in culture cells (Hongo et al., 1998; Liu et al., 1998), suggesting that IGF-1R has intrinsic proapoptotic features. Remarkably, a potential role of IGF-1R in mediating cell death in vivo was suggested by the findings that $Igf\text{-}1r^{+/-}$ mice exhibited enhanced resistance to oxidative damage compared with wild-type mice (Holzenberger et al., 2003). Furthermore, it has been reported that IGF-1R signaling may be able to potentiate p53 induction (Wang et al., 1998; Macaulay et al., 2001), which can induce apoptosis. Although the antiapoptotic functions of IGF-1R have been well established (Pollak et al., 2004; Samani et al., 2007), the mechanism by which IGF-1R sends a proapoptotic signal is not well known. A better understanding of the proapoptotic function of IGF-1R may reveal more rational approaches for cancer therapies targeting IGF-1R signaling.

In this paper, we have used $Igf\text{-}1r^{-/-}$ mouse embryonic fibroblasts (MEFs) and a specific IGF-1R inhibitor, AG1024, to decipher the role of IGF-1R in regulating cellular apoptosis induced by the chemotherapeutic agent etoposide and the mechanisms involved. We found that inhibition of IGF-1R reduces DNA-damage–induced apoptosis through translational inhibition of p53 and Mdm2 expression. Our results not only provide insights into the role for IGF-1R in the p53-induced apoptotic response but also reveal a critical role for translational regulation of the p53–Mdm2 feedback loop by IGF-1R signaling.

Results

Attenuation of etoposide-induced apoptosis and p53 induction in MEFs lacking *Igf-1r*

We observed that R⁻ MEFs, in which the *Igf-1r* gene has been knocked out (Miura et al., 1995), were insensitive to apoptosis induced by the DNA-damage agent etoposide compared with R⁺ MEFs (Fig. 1 A; see Materials and methods). Detection of the cleavage of apoptotic markers caspase-3 and poly (ADP-ribose) polymerase (PARP) supported this observation (Fig. 1 B). Additionally, treatment with the IGF-1R kinase inhibitor AG1024, which suppressed both the autophosphorylation activity of IGF-1R and its downstream signaling (Fig. 1 D), reduced apoptosis in response to etoposide in R⁺ MEFs (Fig. 1 C). Furthermore, transient expression of plasmids encoding the wild-type IGF-1R (IGF-1R-WT) but not the kinase-inactive IGF-1R (IGF-1R-YF) in R⁻ MEFs resulted in an increased apoptotic response to etoposide (Fig. 1 E). Collectively, these results suggest that functional IGF-1R renders MEFs more susceptible to etoposide-induced apoptosis.

Because p53 is a key mediator of apoptosis induced by DNA damage (Vousden and Lu, 2002), we examined whether apoptosis of MEFs induced by etoposide depended on functional p53. Both R⁺ and R⁻ MEFs transfected with dominant-negative p53 (GFP-p53DD) exhibited a reduced apoptotic response to etoposide (Fig. 2 A), indicating that p53 is required for the apoptotic response of MEFs to etoposide. Given that p53 transcriptional activity is required for p53-dependent apoptosis after DNA damage (Chao et al., 2000), we next investigated whether IGF-1R inhibition could impair p53 activation. To this end, we performed luciferase assays using p53-reponsive elements (p53bs-luc) and unstimulated elements (p53ms-luc). The p53bs-luc reporter had higher relative luciferase activity in R⁺ than in R⁻ MEFs after DNA damage (Fig. 2 B), implying that DNA-damage–induced p53 activation is impaired in R⁻ MEFs.

Because p53 activation after DNA damage is associated at least in part with p53 accumulation (Lakin and Jackson, 1999), we next analyzed the induction of p53 protein levels in R⁺ and R⁻ MEFs. Titration experiments revealed a substantial increase in the amount of p53 protein as well as its downstream targets p21 and Mdm2 in response to etoposide in R⁺ compared with R⁻ MEFs (Fig. 2 C). Furthermore, AG1024 attenuated p53 induction followed by etoposide treatment in R⁺ but not in R⁻ MEFs (Fig. 2 D), suggesting that IGF-1R–mediated sensitization of MEFs to p53 accumulation was dependent on IGF-1R kinase activity. In agreement with p53 expression, p21 and Mdm2 induction in response to etoposide treatment was also impaired in R⁺ but not in R⁻ MEFs after AG1024 treatment (Fig. 2 D). To test the generality of our observations, we next examined whether the lack of IGF-1R could reduce p53 induction in response to other anticancer agents, such as doxorubicin and Taxol. We found that in R⁻ MEFs, the induction of p53 and p21 in response to doxorubicin or Taxol was impaired (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200703044/DC1). However, despite impaired p53 induction, R⁻ MEFs exhibited enhanced apoptotic responses to doxorubicin and Taxol (Fig. S1 B), suggesting that impaired p53 induction in R⁻ MEFs may
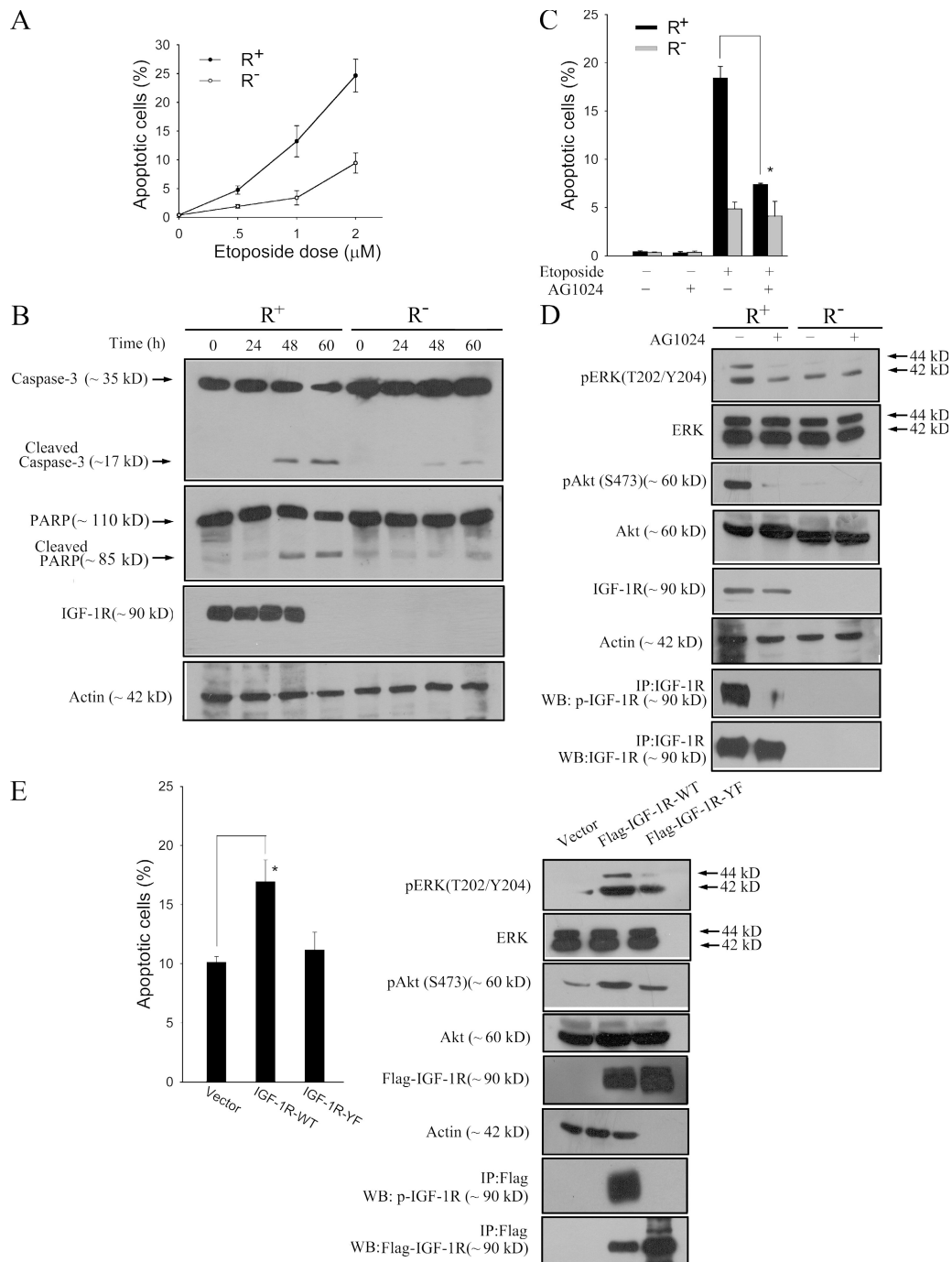
Figure 1. **Requirement of IGF-1R for etoposide-induced apoptosis in MEFs.** (A) Dose-dependent etoposide-induced apoptosis in MEFs. R$^+$ and R$^-$ MEFs were subjected to different doses of etoposide for 60 h, and apoptosis was analyzed by flow cytometry. Values are mean ± SD from three independent experiments. (B) Etoposide-induced cleavage of caspase-3 and PARP in MEFs. Cells were treated with etoposide at 2 μM for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis with the indicated antibodies. Actin was used as a loading control. (C) Reduction of etoposide-induced apoptosis in R$^+$ MEFs by AG1024 treatment. R$^+$ and R$^-$ MEFs were pretreated with or without 20 μM AG1024 for 18 h and then subjected to etoposide for 48 h. Three independent experiments were performed (mean ± SD; *, P < 0.01). (D) Inhibitor specificity. R$^+$ and R$^-$ MEFs were treated with AG1024 as indicated for 18 h. Cell lysates were probed with the indicated antibodies for phosphorylation of endogeneous proteins. (E) Reconstitution with functional IGF-1R sensitizes R$^-$ MEFs to apoptosis. R$^-$ MEFs were transiently cotransfected with either an empty vector, IGF-1R-WT, or IGF-1R-YF and a plasmid encoding EGFP, and then treated with etoposide for 48 h. The percentage of apoptotic cells was determined by flow cytometry as the fraction of GFP-positive cells with sub-G1 DNA content (left; mean ± SD; *, P < 0.05). The expression and activity of IGF-1R-WT and IGF-1RYF were confirmed using the indicated antibodies (right).

not always translate into reduced apoptosis. Because p53 induction may also result in G1 cell cycle arrest in response to DNA damage (Lukas et al., 2004), we next examined the cell cycle

profiles of R$^+$ and R$^-$ MEFs after DNA damage. Treatment with etoposide induced cell cycle arrest at the G1/S and G2/M checkpoints in R$^+$ MEFs, whereas R$^-$ MEFs exhibited a reduced G1
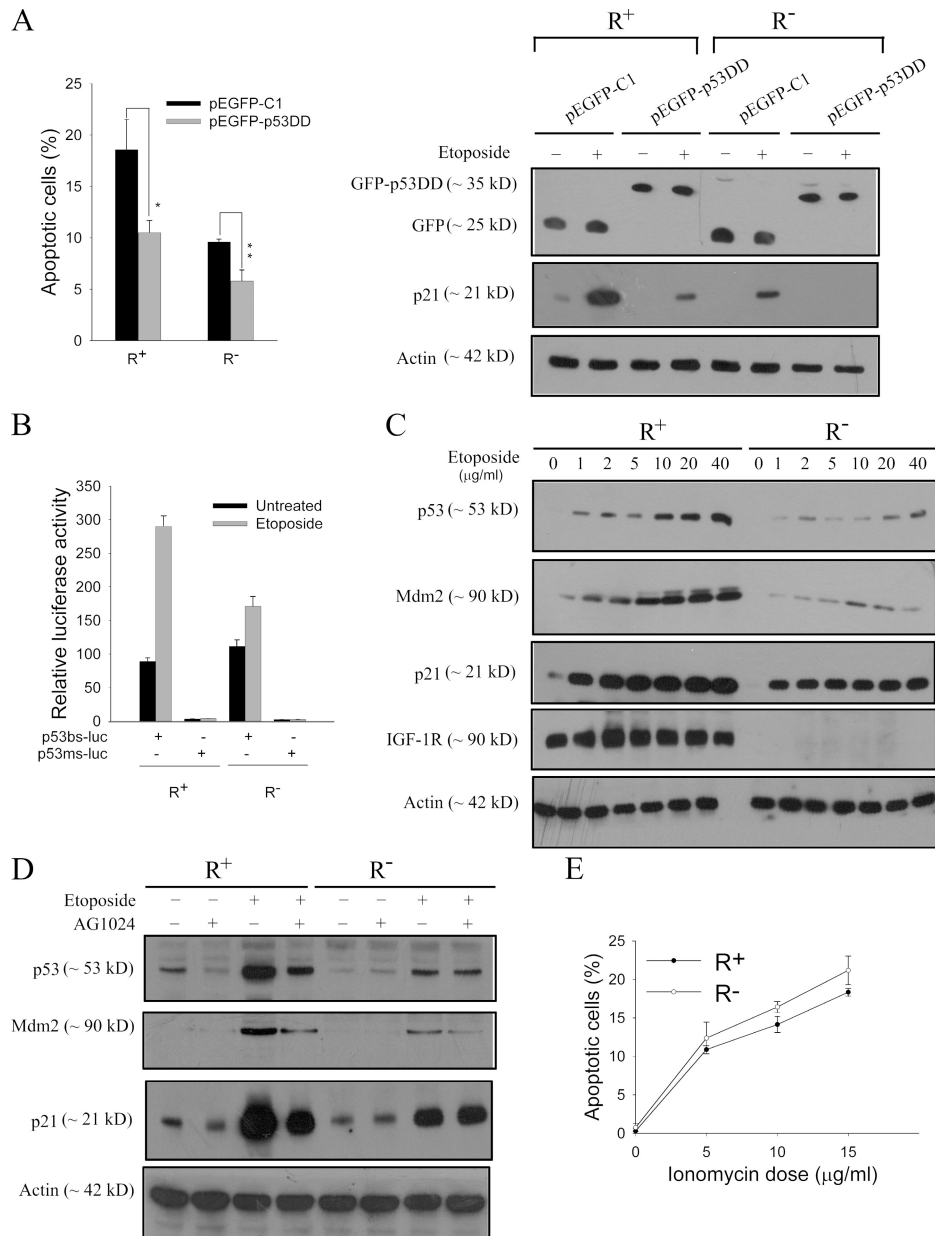
Figure 2. **Requirement of IGF-1R for p53 accumulation and activation in response to etoposide in MEFs.** (A) Etoposide-mediated apoptosis is reduced in MEFs upon transfection with GFP-p53DD. R$^+$ and R$^-$ MEFs were transfected with either an empty vector or GFP-p53DD and treated with etoposide for 48 h. Apoptotic cell death was measured as described in Fig. 1 E. Values are mean ± SD from three independent experiments. *, P < 0.05; **, P < 0.01. The expression and activity of GFP-p53DD were determined using antibodies against GFP and p21. (B) Detection of p53 activation in MEFs. R$^+$ and R$^-$ MEFs were cotransfected with p53bs-luc (wild-type responsive elements) or p53ms-luc (mutant responsive elements) and a plasmid expressing renilla that served as an internal control. After etoposide treatments, luciferase activity was measured. Relative luciferase activity is expressed as light units normalized for renilla luciferase activity. Data shown are from one out of three independent experiments with comparable results (mean ± SD). (C) Induction of p53 and its downstream targets Mdm2 and p21 after etoposide treatment in R$^+$ and R$^-$ MEFs. R$^+$ and R$^-$ MEFs were treated with the indicated doses of etoposide for 12 h, and whole cell lysates were analyzed by Western blot analysis with the indicated antibodies. (D) AG1024 treatment impairs etoposide-induced p53 induction and activation in R$^+$ but not R$^-$ MEFs. R$^+$ and R$^-$ MEFs were pretreated with AG1024 before etoposide treatment. Whole cell lysates were harvested and subjected to Western blot analysis with the indicated antibodies. (E) Dose course of apoptosis induced by ionomycin in R$^+$ and R$^-$ MEFs. MEFs were subjected to different doses of ionomycin and collected after 36 h, and apoptosis was analyzed by flow cytometry. Values are mean ± SD from three independent experiments.

arrest (Fig. S1 C), which is consistent with the impaired p53 induction observed in R$^-$ MEFs.

To determine whether IGF-1R inhibition could impair p53 accumulation and apoptosis in human tumor cells, we treated human hepatocellular carcinoma SK-hep1 and human colon cancer HCT116 cells with AG1024. Treatment of these cells

with AG1024 impaired p53 accumulation as well as apoptosis in response to etoposide (Fig. S2, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200703044/DC1). Notably, AG1024-treated and -untreated *p53$^{-/-}$* cells showed a similar level of apoptosis in response to etoposide (Fig. S2 B), indicating that IGF-1R inactivation cannot protect these cells against
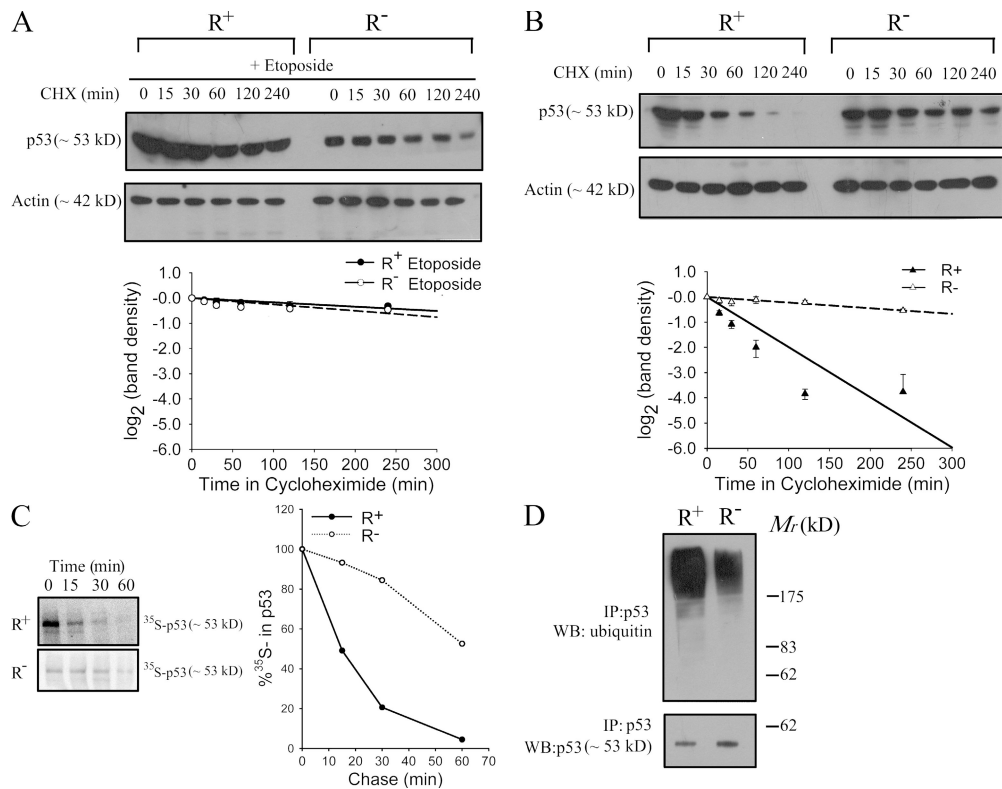
Figure 3. **IGF-1R inhibition increases p53 protein stability.** (A) Measurement of p53 protein stability in etoposide-treated MEFs. R^+ and R^- MEFs were treated with etoposide for 24 h before exposure to CHX. Extracts prepared at the indicated times after the addition of CHX were analyzed by Western blot analysis (top). The stability of p53 protein was quantified by ImageQuant software (bottom). p53 band density was normalized to actin density, and then expressed relative to the $t = 0$ controls and plotted on a semilogarithmic scale by a linear regression program against the times of CHX treatments. Each decreased unit of $\log_2$ (band density) is equivalent to one half life. (B) Measurement of the p53 protein stability in unstressed MEFs. R^+ and R^- MEFs were treated with CHX for the indicated times. Quantitation of the stability of p53 protein was performed as described in A. Values are mean ± SD from three independent experiments. (C) Lack of IGF-1R leads to enhanced p53 protein stability. MEFs were pulse labeled with [^{35}S]methionine/cysteine and chased as described in Materials and methods. p53 protein was immunoprecipitated and resolved by SDS-PAGE (left) and the amount of ^{35}S was quantified by PhosphorImaging (right). (D) Conjugation of ubiquitin to p53 protein is reduced upon IGF-1R loss. R^+ and R^- MEFs were harvested for immunoprecipitation. Equal amounts of immunoprecipitated p53 proteins were subjected to Western blot analysis with antibodies against ubiquitin.

DNA-damage–induced apoptosis in the absence of p53. In addition, we tested whether IGF-1R inhibition could protect cells from p53-independent apoptotic stimuli such as ionomycin, which causes calcium flux. Fig. 2 E demonstrates that the ability of ionomycin to induce apoptosis was unaffected in R^- MEFs. Similarly, ionomycin induced comparable levels of p53-independent cell death in both untreated and AG1024-treated HCT116 cells (Fig. S2 C). Thus inactivation of IGF-1R antagonizes the ability of etoposide to increase p53 abundance and activity and thereby impairs p53-dependent functions including apoptosis and cell cycle arrest.

Enhancement of p53 protein stability in *Igf-1r*$^{-/-}$ MEFs

To define the mechanisms that underlie attenuated p53 response to etoposide in R^- MEFs, we next investigated the integrity of DNA-damage checkpoint pathways in R^- MEFs. Phosphorylation of p53 on ser18 (corresponding to serine 15 in human p53) contributes to p53 activation after DNA damage through increased binding to the p300 coactivator protein (Dumaz and Meek, 1999). We found that etoposide treatment induced similar levels of ser18 phosphorylation of p53 in both R^+ and R^-

MEFs (unpublished data). In addition, the experiment to detect p53 localization revealed that the etoposide-induced p53 protein in both R^+ and R^- MEFs was localized in the nuclei (unpublished data), again indicating that inactivation of IGF-1R impairs p53 induction without affecting the DNA-damage signaling to p53.

Because DNA damage increases p53 protein levels mainly by up-regulating p53 protein stability (Prives, 1998; Lakin and Jackson, 1999), we reasoned that IGF-1R inhibition might regulate p53 accumulation in response to DNA damage by influencing p53 protein stability. Indeed, treatment of R^+ MEFs with etoposide increased p53 stability (Fig. 3, A and B). Importantly, there was no measurable difference in p53 stability in etoposide-treated R^+ and R^- MEFs (Fig. 3 A). Likewise, p53 protein was stable in untreated and AG1024-treated SK-hep1 cells after etoposide treatment (Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200703044/DC1). Surprisingly, we detected a higher stability of p53 protein in untreated R^- MEFs than in untreated R^+ MEFs (Fig. 3 B). Similarly, the IGF-1R inhibitor also stabilized p53 protein in SK-hep1 cells (Fig. S3 B). To confirm that a lack of IGF-1R activity can stabilize p53 protein, R^+ and R^- MEFs were pulse labeled with [^{35}S]methionine/

Figure 4. IGF-1R inhibition down-regulates Mdm2 expression at the translational level. (A) Measurement of Mdm2 expression in MEFs. Cell lysates were harvested and subjected to Western blot analysis with the indicated antibodies. (B) Down-regulation of Mdm2 by IGF-1R inhibition is independent of p53. $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells were incubated with or without AG1024. Expression of Mdm2 protein was determined by immunoblotting with the indicated antibodies. (C) Measurement of mdm2 mRNA levels upon AG1024 treatment. After AG1024 treatment, total cellular RNA was prepared and levels of mdm2 transcripts were revealed by semiquantitative RT-PCR. (D) IGF-1R inhibition reduces translation of mdm2 mRNA in a p53-independent manner. After AG1024 treatments, HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were pulse labeled and newly synthesized. Mdm2 was immunoprecipitated from cells and analyzed by PhosphorImaging (top). Analysis of total cellular proteins by SDS-PAGE showed equal amounts of loading (bottom). (E) AG1024 treatment does not affect Mdm2 protein stability. HCT116 $p53^{+/+}$ cells were treated as described in C, and then exposed to CHX for the indicated times. Quantitative analysis of Mdm2 stability was performed as described in Fig. 3 A. Values are mean ± SD from three independent experiments. (F) HCT116 $p53^{+/+}$ cells were pulse labeled and chased for the indicated periods of time, followed by immunoprecipitation. The amount of ^{35}S was quantified by PhosphorImaging.
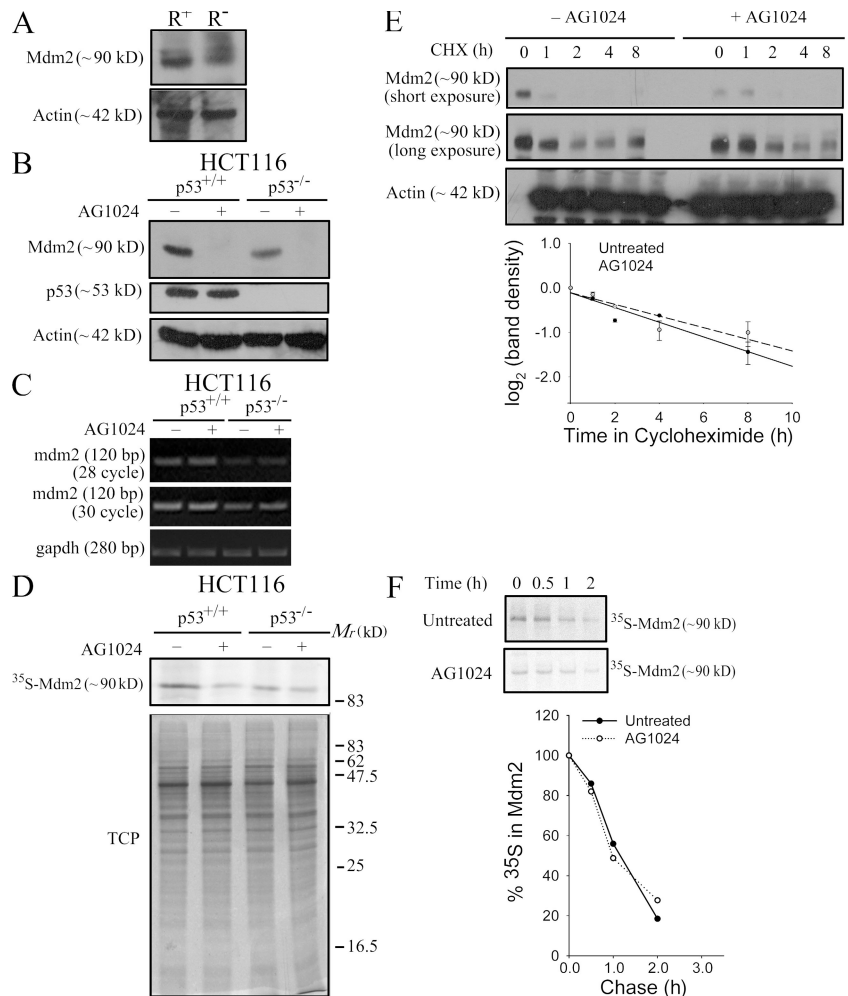
cysteine followed by a 4-h chase. The results showed that the half-life of p53 protein was ~15 and 60 min in R⁺ and R⁻ MEFs, respectively (Fig. 3 C), again demonstrating an increased half-life of p53 protein upon IGF-1R inhibition.

Because the degradation of p53 is mediated by the ubiquitin–proteasome pathway, we next examined the amount of ubiquitin that is conjugated to p53 for degradation. The results showed a remarkable decrease in p53–ubiquitin complexes in R⁻ MEFs and AG1024-treated SK-hep1 cells (Fig. 3 D and Fig. S3 C), implying that IGF-1R inhibition may increase p53 stability by reducing p53 ubiquitination.

Reduction of mdm2 and p53 mRNA translation by IGF-1R inhibition

Because the ubiquitin ligase Mdm2 is a key regulator of p53 protein turnover (Prives, 1998), we tested whether Mdm2 was involved in the regulation of p53 stability by IGF-1R inhibition. R⁻ MEFs as well as AG1024-treated Sk-hep1 cells expressed lower levels of Mdm2 protein compared with R⁺ MEFs and untreated Sk-hep1 cells, respectively (Fig. 4 A and Fig. S3 D). Furthermore, AG1024 treatment led to the down-regulation of Mdm2 protein in wild-type HCT116 cells and HCT116 $p53^{-/-}$ cells (Fig. 4 B), implying that Mdm2 expression is down-regulated in a p53-independent manner in response to IGF-1R inhibition.

RT-PCR analysis revealed no detectable difference in mdm2 mRNA levels in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells upon IGF-1R inhibition (Fig. 4 C), suggesting a translational or posttranslational role of IGF-1R signaling in regulating Mdm2 expression. We therefore examined Mdm2 protein synthesis by metabolic labeling assay. The ^{35}S-labeling experiments revealed a reduced synthesis of ^{35}S-labeled Mdm2 in either $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells upon AG1024 treatment (Fig. 4 D). The reduction in ^{35}S incorporation was not caused by the reduced stabilization of Mdm2 because treatment of HCT116 $p53^{+/+}$ cells with AG1024 did not alter the half-life of Mdm2 protein (Fig. 4, E and F). In fact, using a ^{35}S-pulse label analysis, we demonstrated that the half-life of Mdm2 protein in untreated and AG1024-treated HCT116 $p53^{+/+}$ cells was ~55 and 60 min, respectively (Fig. 4 F). Thus, these results suggest that inhibition of IGF-1R activity decreases the translational rate of mdm2 transcripts and consequently the expression levels of Mdm2 protein, therefore increasing p53 protein stability.

It should be noted that IGF-1R inhibition did not up-regulate the steady-state levels of p53 protein in either of the examined MEFs or tumor cells (Figs. 2 D and 4 B, and see Fig. 7 A), although degradation of p53 protein had been severely attenuated. It is therefore conceivable that, despite decreased p53 turnover, IGF-1R inhibition might maintain low levels of p53
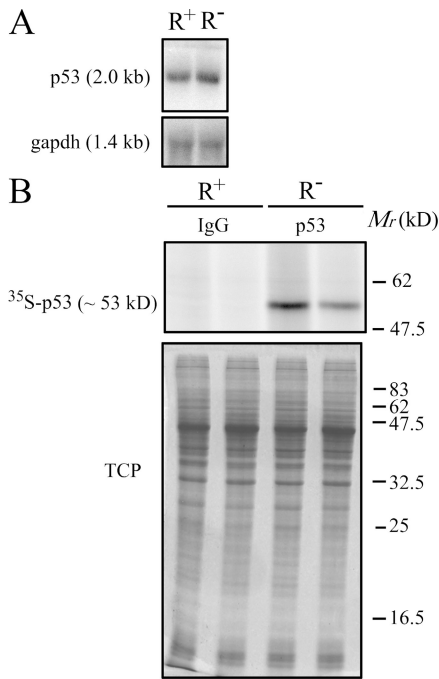
Figure 5. **Reduced translational synthesis of p53 in R⁻ MEFs.** (A) Measurement of p53 mRNA levels in MEFs by Northern blot analysis. p53 mRNA levels were detected by Northern blot analysis in R⁺ and R⁻ MEFs. gapdh levels were shown as loading controls. (B) Reduced translation of p53 mRNA in R⁻ MEFs. p53 protein was immunoprecipitated from R⁺ and R⁻ MEFs labeled with [³⁵S]methionine/cysteine and analyzed as described in Fig. 4 D (top, lanes 3 and 4). An SDS-PAGE gel confirmed equal loading of total cellular proteins.

Figure 6. **IGF-1R inhibition disrupts translation initiation complex formation.** (A) Total protein synthesis rate is reduced in R⁻ MEFs. Pulse-labeled R⁺ and R⁻ MEFs were lysed and analyzed by 20% trichloroacetic acid precipitation as described in Materials and methods. The incorporation of [³⁵S]methionine/cysteine was measured by using a liquid scintillation counter. ³⁵S incorporation for R⁺ MEFs was set to 100% in the set of comparisons. Values are mean ± SD from three experiments. *, $P < 0.05$. (B) Impaired assembly of the eIF4F complex in R⁻ MEFs. Cell extracts from R⁺ and R⁻ MEFs were prepared in NLB buffer (see Materials and methods), followed by incubation with m⁷GTP Sepharose 4B. Proteins eluted from Sepharose 4B were analyzed by Western blot analysis (top). Whole cell lysates were also subjected to Western blot analysis to determine the overall levels of proteins (bottom).

protein by reducing p53 synthesis. Northern blot analysis revealed similar levels of p53 mRNA in R⁺ and R⁻ MEFs (Fig. 5 A); therefore, we reasoned that IGF-1R inhibition might counterbalance the effects of the enhancement of p53 protein stability by reducing p53 synthesis at the translational level. We did observe a reduction in [³⁵S]methionine/cysteine–labeled p53 in R⁻ MEFs (Fig. 5 B). Similarly, treatment of SK-hep1 cells with IGF-1R inhibitor also decreased synthesis of ³⁵S-labeled p53 (Fig. S4 B, available at http://www.jcb.org/cgi/content/full/jcb .200703044/DC1), whereas p53 mRNA levels remained constant (Fig. S4 A). Collectively, these results suggest that decreased p53 mRNA translation may neutralize reduced p53 degradation in response to IGF-1R inhibition. Thus, our analyses indicate that R⁻ MEFs and AG1024-treated cells are refractory to p53 induction after DNA damage because of the prolonged half-life of p53 and reduced p53 synthesis.

Disruption of translation initiation complex and gene-selective impairment in translation efficiency by IGF-1R inhibition

The observation that protein synthesis of Mdm2 and p53 proteins is reduced after IGF-1R inhibition suggests a possible role for IGF-1R in translational regulation of gene expression. It has been reported that growth-factor signaling could regulate mRNA translation by modulating the general translation initiation factors (Rajasekhar et al., 2003; Kelleher et al., 2004). We therefore tested whether the lack of IGF-1R activity altered overall protein synthesis and activity of the eIF4F complex.
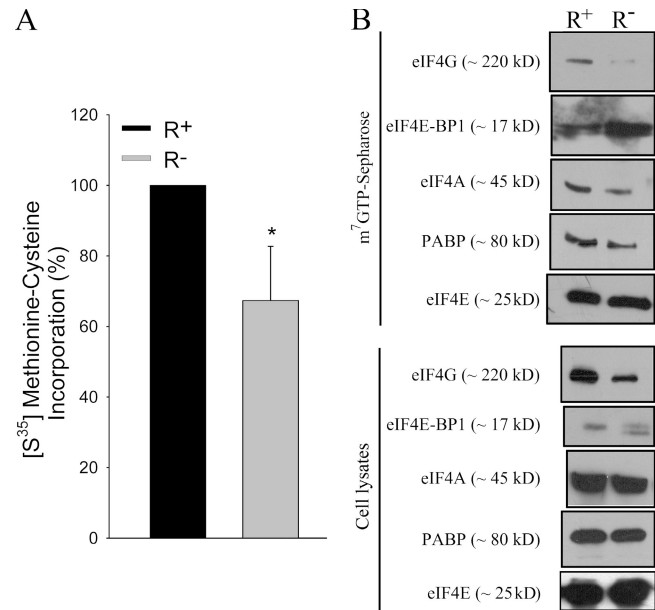
Compared with R⁺ MEFs, R⁻ MEFs had a slower rate of incorporating amino acids into protein (Fig. 6 A). Similar levels of inhibition were obtained in SK-hep1 cells with the administration of AG1024 (Fig. S4 C). Furthermore, IGF-1R inhibition had no measurable effect on the levels of eIF4A, PABP, and eIF4E proteins, but resulted in a reduction in eIF4G abundance (Fig. 6 B, bottom). In addition, the hyperphosphorylated form of eIF4E–BP1 was also reduced upon IGF-1R loss (Fig. 6 B, bottom). To determine whether these modulations could disrupt the eIF4F complex, we next examined the association of eIF4E with other translation initiation factors by pull-down on m⁷GDP–sepharose resin. The precipitation assay showed reduced association of eIF4G, eIF4A, and PABP with eIF4E, whereas the amount of eIF4E–BP1 in the precipitate was increased in R⁻ MEFs (Fig. 6 B, top). The IGF-1R inhibitor induced similar alterations of translation initiation factors and impaired the formation of the translation initiation complex in SK-hep1 cells (Fig. S4 D). Together, these results suggest an important role for IGF-1R signaling in the regulation of translation initiation processes.

Cellular mRNAs differ hugely in the amount of eIF4F required for efficient translation (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002). Alterations of the general translational apparatus may preferentially affect the translation of weak mRNAs with extensive secondary structure in their 5′ UTR (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002; Rajasekhar et al., 2003; Moerke et al., 2007). We next investigated whether

the modulations of the basal translational machinery by IGF-1R inhibition could evoke a selective translational effect. To this end, we examined the translation levels of several proteins with short half-lives upon IGF-1R inhibition because the levels of short-lived proteins are believed to be more sensitive to translational inhibition (Beuvink et al., 2005). We observed no change in the translation levels of short-lived proteins after IGF-1R inhibition, including p27 and c-fos (Fig. 7 A, bottom panels). These results indicate that the translational depression in response to IGF-1R inhibition might be caused by an mRNA-specific mechanism.

Although it is likely that the attenuated translation initiation induced by the impaired eIF4F system contributes to decreased p53 and mdm2 mRNA translation in response to IGF-1R inhibition, there might be additional mechanisms, including the regulation of translation elongation or termination on mRNA, for the observed effects of IGF-1R inhibition on p53 and mdm2 mRNA translation. We examined the impact of eIF4F complex disruption on translation using a dicistronic mRNA construct that contains the FLAG-tagged p53, Mdm2, or c-fos coding region flanked by the corresponding 5′ and 3′ UTRs and a GFP coding sequence (Fig. 7 C, left). The respective coding region was translated in a cap-dependent manner, whereas the translation of the gfp sequence is driven by the cricket paralysis virus (CrPV) internal ribosome entry site (IRES), which is independent of translation initiation factors (Pestova and Hellen, 2003). We found that the expression of p53 and Mdm2 was down-regulated by AG1024 treatment, whereas the levels of c-fos were unaltered (Fig. 7 C, right). The cytomegalovirus (CMV) promoter in the constructs drove similar levels of gfp mRNA expression under all conditions (Fig. 7 C, right), thus excluding the possibility that there are differences in the promoter activity or transfection efficiency in AG1024-treated and -untreated cells. Importantly, GFP protein levels were unaltered after IGF-1R inhibition (Fig. 7 C, right), indicating that the initiation factor–independent translation is not inhibited. Interestingly GFP expression driven by the control vector (pIRES-GFP) was higher than that driven by other constructs, presumably because of the interference of the insert sequence (Fig. 7 C, right). Together, these findings suggest that translational control of p53 and Mdm2 expression by IGF-1R signaling is regulated at the level of initiation.

Modulation of p53 and mdm2 mRNA translation through 5′ UTRs

Weak mRNAs are subjected to gene-specific regulation under conditions that reduce the efficiency of translation initiation owing to the presence of long, highly structured 5′ UTRs (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002). We therefore predicted the secondary structures of the 5′ UTRs of p53, mdm2, and c-fos mRNA using the program MFOLD (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi; Zuker, 2003). Consistent with the idea that the weak mRNA has a highly structured 5′ UTR, the sequences of the p53 and mdm2 5′ UTR but not the c-fos 5′ UTR were predicted to form several highly structured stem loops (unpublished data).

To determine whether the UTRs of p53 or mdm2 mRNA are sufficient on their own to mediate IGF-1R signaling–

dependent translational regulation, we generated a series of constructs that contain a reporter sequence encoding firefly luciferase flanked by the UTRs of p53, mdm2, or c-fos mRNA (Fig. 7 B) and then transfected the constructs into SK-hep1 cells. We found that in the absence of the flanking UTRs or the presence of c-fos UTRs, AG1024 does not inhibit the translation of the reporter mRNA (Fig. 7 D). In contrast, the translatability of reporter mRNA containing p53 or mdm2 UTRs was decreased by AG1024 treatment (Fig. 7 D).

Because the mechanisms by which the 5′ and 3′ UTRs confer translational control of specific mRNAs may be different (Wilkie et al., 2003), we examined the impact of the 5′ and 3′ UTRs of p53 and mdm2 mRNA on translational efficiency using chimeric luciferase reporter constructs (Fig. 7 B). We found that the three reporter mRNAs (p53–CUTR–luc, Mdm2–CUTR–luc, and c-fos–CUTR–luc) lacking their respective 5′ UTRs were less translated (Fig. 7 E). Nonetheless, AG1024 inhibited the reporter mRNA translation in the presence of p53 or mdm2 5′ UTR but not in the presence of their respective 3′ UTRs (Fig. 7 E). In contrast, IGF-1R inhibition did not influence the luciferase activity of the reporter construct c-fos–NUTR–luc and c-fos–CUTR–luc (Fig. 7 E). Moreover, the IGF-1R inhibitor attenuated the translatability of hybrid reporter mRNA containing p53 or mdm2 5′ UTR and c-fos 3′ UTR (Fig. 7 E), further demonstrating that the translational control of p53 and mdm2 by IGF-1R inhibition is mediated by the respective 5′ UTR. Collectively, these data indicate that the 5′ UTR of p53 or mdm2 mRNA is sufficient to enable the IGF-1R signaling-dependent control of protein translation.

The PI-3K–Akt–mTOR (molecular target of rapamycin) pathway has been demonstrated to regulate general protein synthesis and translation of selected mRNAs (Gingras et al., 2001; Levine et al., 2006). We found that inhibition of PI-3K by LY294002, or mTOR by rapamycin, had no effect on p53 and Mdm2 expression (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200703044/DC1), which suggests an mTOR-independent mechanism for IGF-1R–mediated mRNA- specific translational regulation. Extracellular signal-regulated kinase (ERK) signaling has also been shown to promote translation by facilitating assembly of the translation initiation complex (Kelleher et al., 2004). PD98059, a specific inhibitor of MAPK and ERK kinase, did not alter the amount of p53 and Mdm2 (Fig. S5 A). Furthermore, treatment of cells with LY294002 (rapamycin) or PD98059 did not affect luciferase activity driven by p53–UTR–luc or Mdm2–UTR–luc (Fig. S5 C). It therefore appeared that the PI-3K–Akt–mTOR and ERK pathway, although inactivated after IGF-1R inhibition, may not be involved in reducing p53 and mdm2 translation. It has been suggested that active glycogen synthase kinase (GSK)-3β phosphorylates and inhibits the translation initiation factor eIF2B (Welsh et al., 1998). Because IGF-1 signaling inactivates GSK-3β and promotes protein synthesis (Quevedo et al., 2000), we examined whether inhibition of IGF-1R activity could reduce p53 and mdm2 translation through activation of GSK-3β. The reduction of Mdm2 levels in AG1024-treated SK-hep1 cells was not inhibited by GSK-3β inhibitors SB216763 or SB415286, which blocked β-catenin degradation (Fig. S5 B). Likewise, GSK-3β
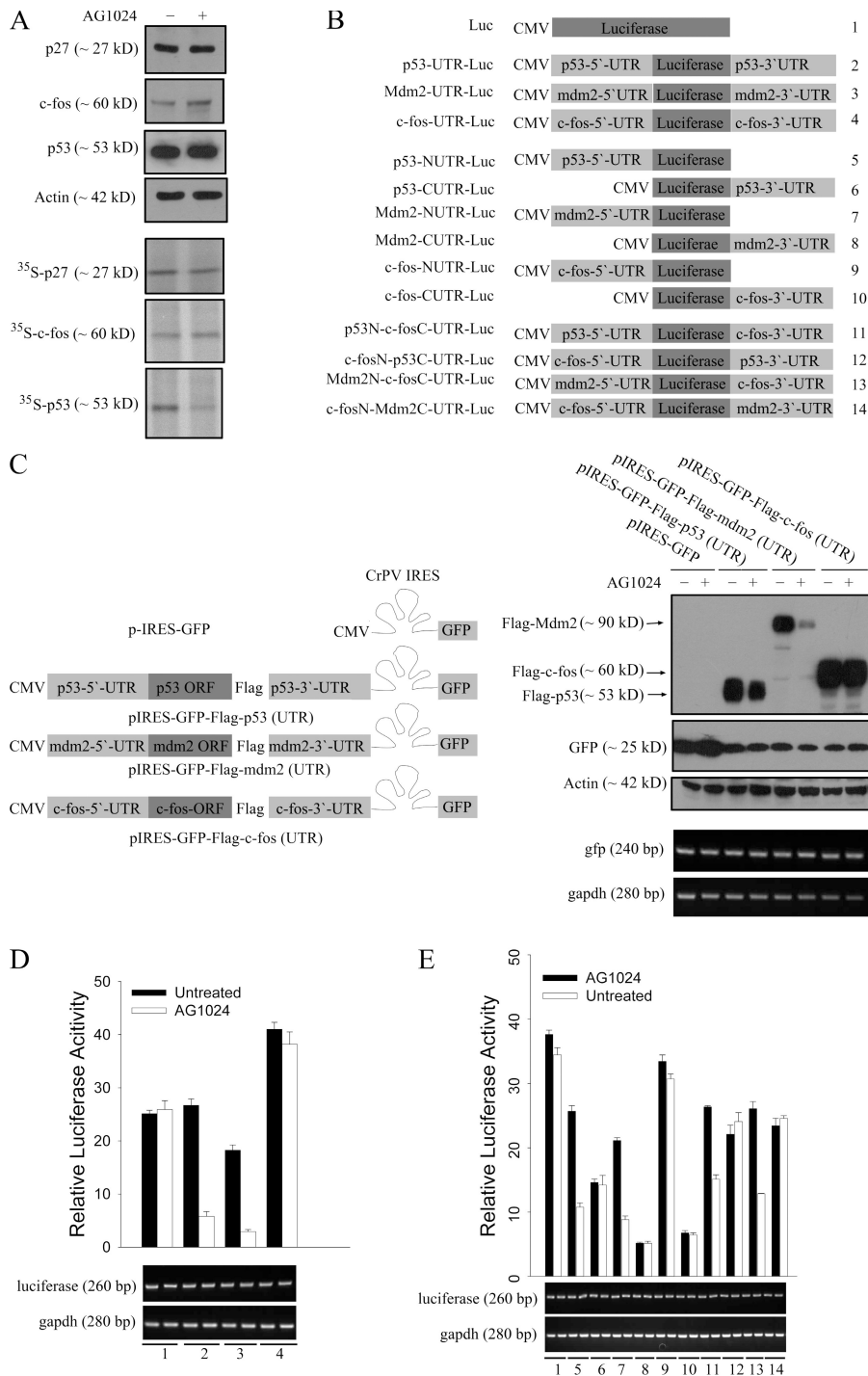
Figure 7. **The 5′ UTR of p53 and mdm2 transcript mediates IGF-1R–dependent regulation of p53 and Mdm2 translation.** (A) AG1024 specifically reduces translational levels of p53. [^{35}S]methionine/cysteine pulse-labeled p27, c-fos, and p53 were immunoprecipitated from SK-hep1 cells treated with or without AG1024 and analyzed by PhosphorImaging (bottom). Expression levels of p27, c-fos, and p53 proteins were monitored by immunoblotting (top). Adjustment of brightness and contrast (^{35}S-p27 panel) was performed with Photoshop 8.0 software. (B) Chimeric UTR-luc constructs carrying the 5′ and/or 3′ UTR of p53, mdm2, and c-fos mRNA. Each construct is labeled with the corresponding number at the right side. (C) AG1024 inhibits p53 and Mdm2 translation at the level of translation initiation; a schematic presentation of the FLAG-p53, FLAG-Mdm2, and FLAG–c-fos constructs containing the corresponding UTRs, CrPV IRES, and EGFP (left). SK-hep1 cells were transfected with the indicated expression vectors. Cells were treated with or without AG1024 and analyzed for protein expression by immunoblotting with antibodies against FLAG and GFP. gfp mRNA levels were determined by semiquantitative RT-PCR. (D and E) 5′ UTRs of the p53 and mdm2 transcript impose IGF-1R signaling–mediated translational regulation. SK-hep1 cells were transiently cotransfected with the chimeric UTR-luc constructs and pRL–SV40–renilla as described in Material and methods. After AG1024 treatment, luciferase activity was measured. Relative luciferase activity is expressed as light units normalized for renilla luciferase activity. Data are presented as mean ± SD of three independent experiments performed in quadruplicate. Levels of luciferase transcripts were revealed by semiquantitative RT-PCR. The number at the bottom of the panel is representative of the corresponding chimeric construct shown in B.

inhibitors had no effect on the luciferase activity of the chimeric reporter constructs (Fig. S5 D), further indicating that GSK-3β plays no part in the translational inhibition of p53 and Mdm2 by IGF-1R inactivation.

Discussion

Opposing effects of IGF-1R signaling on p53

Although p53 is frequently mutated in >50% of human cancers (Hainaut et al., 1997), a large fraction of cancers express wild-type p53, which may be regulated by other mechanisms such as amplification of Mdm2 (Oliner et al., 1992) or deregulation of growth-factor signaling (Brown and Benchimol, 2005; Levine et al., 2006). In this study, we demonstrate that inactivation of IGF-1R signaling impairs p53 accumulation after DNA damage through translational modulation of the p53–Mdm2 feedback loop. On the one hand, the translation of both p53 and mdm2 mRNA is attenuated upon IGF-1R inhibition. On the other hand, p53 protein becomes stabilized in response to IGF-1R inhibition because of reduced Mdm2 protein levels and is thus

insensitive to further up-regulation of protein stability. IGF-1R inhibition therefore acts on p53 through two competing pathways (decreasing p53 protein synthesis and increasing p53 protein stability).

It is conceivable that p53 protein levels are determined by a balance between the opposing effects of IGF-1R signaling. In different cell types, the balance of the two competing pathways is likely to be different. Consistent with this idea, a lack of IGF-1R activity led to reduced p53 protein levels in MEFs (Fig. 2 D), whereas in HCT116 and SK-hep1 cells there was no detectable difference in p53 expression levels upon IGF-1R inhibition (Figs. 4 B and 7 A). Moreover, in MCF-7 cells the IGF-1R inhibitor up-regulated p53 protein levels with reduced p53 and mdm2 mRNA translation (unpublished data), further supporting the notion that the opposing effects of IGF-1R signaling on p53 are dependent on cell type.

Previous papers showing that activation of IGF-1R signaling decreases p53 expression in many systems are not contradictory to our findings of translational regulation of p53 by IGF-1R signaling, as these papers do not reveal whether IGF-1R signaling could regulate p53 mRNA translation (Leri et al., 1999; Héron-Milhavet and LeRoith, 2002; Jackson et al., 2006). In fact, our results indicate that a reduction in p53 mRNA translation by itself induced by IGF-1R inhibition may not always reflect and/or translate into a decline in p53 expression. Furthermore, IGF-1 signaling has been reported to be able to up-regulate p53 expression (Wang et al., 1998). Thus, it is possible that the down-regulation of p53 expression upon IGF-1R activation that was observed in previous studies is cell-context dependent and additionally might be associated with an increase in p53 translation. Our results also provide a possible explanation for previous observations that Mdm2 expression is up-regulated by IGF-1 signaling (Leri et al., 1999; Héron-Milhavet and LeRoith, 2002).

Mechanisms of translational regulation of p53 and Mdm2 by IGF-1R

There are two general forms of translational control: mRNA-specific regulation and global control of protein synthesis (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002). Importantly, these two forms of regulation are not mutually exclusive (Gray and Wickens, 1998). We found that despite a reduction in global translation, the effect of IGF-1R inhibition on p53 and mdm2 mRNA translation is mRNA specific because the 5′ UTR of p53 and mdm2 mRNA rather than the 5′ UTR of c-fos mRNA imposed the translational regulation by IGF-1R signaling (Fig. 7, D and E), nor did we observe a change in c-fos and p27 mRNA translation after IGF-1R inhibition (Fig. 7 A). mRNA-specific regulation is either acquired by alterations of the general translational machinery or conferred by specialized mRNA binding factors (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002). Previous papers have documented a translational regulation of p53 and Mdm2 expression through the interactions of mRNA binding factors with the corresponding mRNAs (Mazan-Mamczarz et al., 2003; Trotta et al., 2003; Takagi et al., 2005). Our findings from this study suggest a different mechanism by which IGF-1R signaling regulates p53 and mdm2 mRNA translation. We showed that IGF-1R inhibition led to reduced eIF4G

expression and decreased eIF4E–BP1 phosphorylation (Figs. 6 B and S4 D), both of which in turn attenuated the formation of the eIF4F complex and may impair cap-dependent translation initiation. Consistently, repression of p53 and mdm2 mRNA translation by IGF-1R inhibition was at the level of initiation, not elongation or termination, because there was no decrease in CrPV IRES–driven EGFP translation (Fig. 7 B). However, although it is likely that these observed inhibition effects are at least in part mediated by impairing the activity of the eIF4F complex, there could be additional mechanisms for the attenuated translation of p53 and mdm2 mRNA upon IGF-1R inhibition.

Many growth regulators are encoded by weak mRNAs, translation of which is highly eIF4F dependent and more sensitive to small perturbations in eIF4F complex formation (Gray and Wickens, 1998; Raught et al., 2000; Dever, 2002; Rajasekhar et al., 2003; Moerke et al., 2007). The mechanisms of gene-specific translational regulation by IGF-1R signaling presented in this paper may therefore not be limited to regulation of p53 and Mdm2 but may rather be of general significance in translational regulation of gene expression. It will be interesting to determine how many genes could be regulated at the translational level by IGF-1R signaling and how many physiological effects of IGF-1R signaling could occur through translational effects. Although in our studies we show that IGF-1R signaling regulates p53 and mdm2 translation independent of Ras and the PI-3K–Akt–mTOR pathway, we cannot exclude the possibility that these pathways may be involved in IGF-1R–dependent translational regulation of other weak mRNAs.

The role of IGF-1R in cell survival and cell death

Two well-documented hallmarks of cancer are deregulation of cell proliferation and evasion of apoptosis (Hanahan and Weinberg, 2000). IGF-1R not only transmits mitogenic growth signals but also governs survival pathways, both of which are conducive to increased tumor growth (Pollak et al., 2004; Samani et al., 2007). However, IGF-1R signaling has also been proposed to be involved in inducing contradictory signals, including proapoptotic signaling (Kooijman, 2006), on malignancy in different environments (Baserga, 2000; Samani et al., 2007), though how IGF-1R functions as a proapoptotic factor is unclear. The findings presented in this paper implicate IGF-1R as a proapoptotic factor by modulating the response of p53 to DNA damage.

Because p53 is involved in cellular responses to oxidative damage (Finkel and Holbrook, 2000), our findings provide an explanation for the increased resistance observed in $Igf-1r^{+/-}$ mice when challenged with oxidants (Holzenberger et al., 2003). Our data is also consistent with the notion that growth signals have the potential to sensitize cells to apoptosis (Evan and Littlewood, 1998). IGF-1R has been shown to be involved in TNF-α–induced apoptosis (Niesler et al., 2000) and in a non-apoptotic form of cell death (Sperandio et al., 2000), both of which seem not to depend on p53 function. Thus IGF-1R signaling can participate in both p53-dependent and -independent cell death. Together, these results provide an interesting contrast to other papers that showed that inactivation of IGF-1R sensitizes

cells to apoptosis induced by chemotherapeutic drugs (Samani et al., 2007). Yet as shown in our studies, IGF-1R inhibition not only impairs p53-dependent apoptosis but also inactivates the PI-3K–Akt and ERK pathways, which have been shown to be important for the antiapoptotic activity of IGF-1R signaling (Párrizas et al., 1997; Gooch et al., 1999; Peruzzi et al., 1999). Therefore, upon IGF-1R inhibition, it is the balance between attenuated p53-dependent apoptosis and inactivated survival pathways that determines whether a cell survives or dies in response to stress. One might expect that the inclination of the balance would be dependent on cell type and the nature of apoptotic stimuli. Consistent with this idea, the loss of IGF-1R sensitized cells to doxorubicin- and Taxol-induced apoptosis (Fig. S2 B), although p53 induction was attenuated (Fig. S2 A).

Our findings may have important implications for the design of therapeutic protocols that involve the targeting of IGF-1R signaling. In tumors with functional p53, where p53 is critical for chemotherapeutic response (Johnstone et al., 2002), small molecular therapy targeting IGF-1R, when used together with chemotherapy, may lead to the attenuation of cytotoxicity of chemotherapeutic drugs. However, because IGF-1R is important for cancer cell growth and survival, such therapy between courses of chemotherapy may well be useful (LeRoith and Helman, 2004).

In summary, we have shown that inactivation of IGF-1R leads to a specific inhibition of p53 and mdm2 mRNA translation. Studies of the expression of chimeric constructs demonstrate an essential role of the 5' UTR of p53 and mdm2 mRNA in the translational regulation by IGF-1R inhibition. IGF-1R signaling therefore regulates p53 through competing pathways that involve decreasing p53 translation and enhancing p53 protein stability, which lead to impaired p53 induction in response to DNA damage. This may have important implications in cancer therapy.

Materials and methods

Cell culture and chemicals
R⁻ MEFs lacking *Igf-1r* have been described previously (Miura et al., 1995). R⁺ MEFs were obtained from R⁻ MEFs stably transfected with a plasmid containing human IGF-1R cDNA. Both cell lines were provided by R. Baserga (Thomas Jefferson University, Philadelphia, PA) and cultured in DME medium supplemented with 10% FBS (Invitrogen). SK-hep1, HCT116 *p53*⁺ᐟ⁺, and *p53*⁻ᐟ⁻ cells (provided by B. Vogelstein, Johns Hopkins University, Baltimore, MD) were maintained in standard medium.

AG1024, LY294002, PD98059, rapamycin, etoposide, doxorubicin, Taxol, and cycloheximide (CHX) were obtained from Calbiochem. SB216761, SB415286, and ionomycin were obtained from Sigma-Aldrich. 7-methyl GTP (m⁷GTP) and m⁷GTP-Sepharose 4B were obtained from GE Healthcare.

Constructs and transfections
Full lengths of IGF-1R-WT and IGF-1R-YF were isolated from pBPV–IGF-1R-WT and pBPV–IGF-1R-YF (provided by R. Baserga) and subcloned into pCMV-Tag2B vector (Stratagene). p53DD lacking the transactivation and DNA-binding domain (amino acids 15–301) was derived from mouse cDNA by PCR and inserted downstream of GFP in a pEGFP-C1 vector (CLONTECH Laboratories, Inc.). p53bs-luc and p53ms-luc were provided by S.E. Kern (Johns Hopkins University). A 194-unit long oligonucleotide corresponding to the sequence of CrPV IRES was synthesized chemically and inserted upstream of GFP in the pEGFP-N3 vector (CLONTECH Laboratories, Inc.) and named pIRES-GFP. The full length of p53, mdm2, or c-fos cDNA that contains 2,629, 2,357, and 2,084 nucleotides, respectively,

was amplified from human cDNA by PCR and cloned into the pIRES-GFP vector with a FLAG tag attached to the C terminus of the corresponding coding region under the CMV promoter. The UTRs of p53, mdm2, or c-fos were subcloned from the corresponding cDNA into the pCMV-luc vector containing a CMV-driven luciferase sequence.

For determination of the sub-G1 population, 10⁶ MEFs were transfected with 8 µg of the indicated plasmids and combined with or without 1 µg cDNA coding for GFP. Transfections were performed using a transfection system (Nucleofector; Amaxa) according to the manufacturer's instructions. 70% transfection efficiency of cells was obtained using solutions and programs recommended by the manufacturer. For reporter assay, cells were transfected with the indicated reporter plasmids by jetPEI transfection reagent (Polyplus). The empty pCMV-Tag2B vector was added to adjust total DNA amount to 1 µg per well.

Western blot and immunoprecipitation
After electrophoresis and transfer of samples onto Immobilon membrane (Millipore), the blots were probed with the following antibodies: anti-Mdm2 (SMP14; Santa Cruz Biotechnology, Inc.; 2A10; Oncogene Research Products); anti–caspase-3, anti-PARP, anti–α-catenin, and anti-eIF4E (BD Biosciences); anti–eIF4E–BP1, anti-PABP, anti-pERK (T202/Y204), anti–p-p70 S6k (T389), anti-p70 S6k, anti-akt, anti–p–IGF-1R (Y1131), and anti-p27 (Cell Signaling Technology); anti-p53 (FL-393), anti–c-fos (H-125), anti–IGF-1R (C-20), anti–p-akt (Ser473), anti-ubiquitin (FL-76), anti-GFP (FL), and anti-actin (I-19; Santa Cruz Biotechnology, Inc.); anti-FLAG (Sigma-Aldrich); anti-eIF4A (provided by H. Trachsel, University of Bern, Bern, Switzerland); anti-eIF4G (provided by S. Morley, University of Sussex, Brighton, UK). The membranes were exposed to x-ray film (Kodak), which was scanned (Scanjet 3570c; Hewlett-Packard) using software (Photo and Imaging 2.0; Hewlitt-Packard). The analysis of the images was performed with imaging software (Photoshop 8.0; Adobe).

Cell-death assays
Cell death was determined according to the percentage of sub-G1 DNA content by flow cytometry. For untransfected cells, cells were collected and fixed with 70% cold ethanol overnight at −20°C. In transfected cells, after drug treatments for the indicated times, transfectants were collected and resuspended in 1% paraformaldehyde at room temperature for 10 min, centrifuged, and fixed in 70% cold ethanol at –20°C overnight. Fixed cells were then incubated in PBS containing 50 µg ml⁻¹ RNase A (Sigma-Aldrich) for 1 h at 37°C, followed by 30 µM propidium iodide (Sigma-Aldrich) staining. In each assay, either 10,000 (untransfected) or 50,000 (transfected) cells were collected by FACScan (BD Biosciences) and analyzed with software (WinMDI version 2.8; provided by J. Trotter, Scripps Research Institute, La Jolla, CA).

Reporter assays
Cells cotransfected with the indicated constructs and the PRL-SV40 vectors were harvested in lysis buffer and analyzed using a luciferase assay reagent according to the manufacturer's instructions (Dual-Luciferase reporter assay system; Promega). The reporter activity was expressed as arbitrary luciferase units (firefly/renilla).

Northern blot analysis and semiquantitative RT-PCR
RNA was isolated using TRIzol (Invitrogen) and subjected to Northern blot analysis with indicated probes. Primers used were as follows: human p53 (5'-TCCTACACCGGCGGCCCCTGCAC-3' and 5'-GCGGAGATTCTCTTC-CTCTGTG-3') and mouse p53 (5'-TGCCCCAGGATGTTGAGGAGTTTT-3' and 5'-CCCCCC ATGCAGGAGCTATTACACA-3'). The Northern blot hybridization bands were scanned into the computer using a scanner (PhosphorImager SI; Molecular Dynamics) and analyzed using software (ImageQuant; GE Healthcare).

cDNA was synthesized from total RNAs with the SuperScript preamplification system (Invitrogen). Primers used for semiquantitative RT-PCR analysis were as follows: for mdm2 mRNA, a 5' (5'-CGCCCCGTGAAG-GAAAC-3') and 3' primer (5'-CAGCACCATCAGTAGGTACAGACAT-3'); for gfp mRNA, a 5' (5'-CCCGCGCCGAGGTGAAGT-3') and 3' primer (5'-CGTCGCCGATG GGGGTGTT-3'); for luciferase mRNA, a 5' (5'-GGC-GCGGTCGGTAAA GTTGTT-3') and 3' primer (5'-AGCGGGAGCCACCT-GATAGC-3'). The images of agarose gels were captured using the G:Box system (Syngene) and analyzed using software (Genesnap; Syngene).

Metabolic labeling and quantitative analysis of protein synthesis
For [³⁵S]methionine/cysteine label analysis, cells were incubated in methionine/cysteine–free DME (Invitrogen) and supplemented with 10% dialyzed

FBS (Invitrogen) for 1 h. 0.3 mCi [^{35}S]methionine/cysteine (GE Healthcare) was next added in 0.5 ml of free medium for 30 min. Lysates were prepared for the immunoprecipitation assay. Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by PhosphorImaging. For quantitative analysis of total protein synthesis, lysates were prepared by standard protocols and the incorporation of ^{35}S for cells was measured by using a liquid scintillation counter (LS 6500; Beckman Coulter).

For [^{35}S]methionine/cysteine pulse-label analysis cells were starved for methionine/cysteine for 1 h, and then pulse labeled with 0.75 mCi [^{35}S]methionine/cysteine for 1 h and chased for the indicated times with unlabeled methionine/cysteine (1 mg ml^{-1}) added. The ^{35}S-labeled p53 or Mdm2 in the immunoprecipitates from each time point was resolved and quantified by PhosphorImaging and normalized to that of the zero time point.

Analysis of eIF4E-binding proteins

The m^{7}GTP pull-down assay was performed as described previously (Walsh and Mohr, 2004). In brief, after washing with cold PBS, 2×10^6 cells were lysed in 1 ml NLB (50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM Na$_3$VO$_4$, protease inhibitor cocktail, and 0.5% NP-40) and then extracts were clarified by centrifugation at 10,000 g (4°C for 10 min). Supernatants were then incubated with 1 ml NLB including m^{7}GTP-Sepharose 4B (60 μl of 50/50 slurry) at 4°C for 1 h. The beads were centrifuged at 2500 g, and then washed with NLB. The m^{7}GTP-agarose was resuspended in 100 μl NLB containing 100 μM m^{7}GTP at 4°C for 30 min. The elute was collected and diluted with an equal volume of 2× SDS sample buffer and boiled. The eIF4E-bound proteins were analyzed by SDS-PAGE and immunoblotting.

Online supplemental material

Fig. S1 presents the expression analysis of p53 and p21 and the apoptosis analysis of R$^+$ and R$^-$ MEFs upon doxorubicin or Taxol treatment and the cell cycle distribution of R$^+$ and R$^-$ MEFs after etoposide treatment. Fig. S2 shows that inhibition of IGF-1R attenuates etoposide-induced p53 accumulation and apoptosis in tumor cells and has no effect on ionomycin-induced p53-independent apoptosis. Fig. S3 shows that inactivation of IGF-1R leads to enhanced p53 protein stability in tumor cells. Fig. S4 shows that inhibition of IGF-1R activity results in a reduced p53 translation. Fig. S5 shows that IGF-1R inhibition impairs p53 and Mdm2 translation through an ERK- and GSK-3β–independent and probably PI-3K–Akt–mTOR–independent mechanism. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200703044/DC1.

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