The translational response of the human mdm2 gene in HEK293T cells exposed to rapamycin: a role for the 5'-UTRs

Genolet, Raphaël; Rahim, Gwendoline; Jaquier-Gubler, Pascale; Curran, Joseph

How to cite

GENOLET, Raphaël et al. The translational response of the human mdm2 gene in HEK293T cells exposed to rapamycin: a role for the 5′-UTRs. In: Nucleic acids research, 2011, vol. 39, n° 3, p. 989–1003. doi: 10.1093/nar/gkq805

This publication URL: https://archive-ouverte.unige.ch/unige:24260

Publication DOI: 10.1093/nar/gkq805

Published online 28 September 2010

Nucleic Acids Research, 2011, Vol. 39, No. 3 ***989–1003***
doi:10.1093/nar/gkq805

The translational response of the human *mdm2* gene in HEK293T cells exposed to rapamycin: a role for the 5′-UTRs

Raphael Genolet, Gwendoline Rahim, Pascale Gubler-Jaquier and Joseph Curran*

Department of Microbiology and Molecular Medicine, University of Geneva Medical School (CMU) 1, rue Michel Servet, CH-1205 Geneva, Switzerland

Received May 19, 2010; Revised July 26, 2010; Accepted August 28, 2010

ABSTRACT

Polysomal messenger RNA (mRNA) populations change rapidly in response to alterations in the physiological status of the cell. For this reason, translational regulation, mediated principally at the level of initiation, plays a key role in the maintenance of cellular homeostasis. In an earlier translational profiling study, we followed the impact of rapamycin on polysome re-seeding. Despite the overall negative effect on transcript recruitment, we nonetheless observed that some mRNAs were significantly less affected. Consequently, their relative polysomal occupancy increased in the rapamycin-treated cells. The behaviour of one of these genes, *mdm2*, has been further analysed. Despite the absence of internal ribosome entry site activity we demonstrate, using a dual reporter assay, that both the reported mdm2 5′-UTRs confer resistance to rapamycin relative to the 5′-UTR of β-actin. This relative resistance is responsive to the downstream targets mTORC1 but did not respond to changes in the La protein, a reported factor acting positively on MDM2 translational expression. Furthermore, extended exposure to rapamycin in the presence of serum increased the steady-state level of the endogenous MDM2 protein. However, this response was effectively reversed when serum levels were reduced. Taken globally, these studies suggest that experimental conditions can dramatically modulate the expressional output during rapamycin exposure.

INTRODUCTION

Studies suggest that the differential recruitment onto polysomes of messenger RNA (mRNA) populations may be sufficient to initiate and maintain tumour formation (1). Nonetheless, the rules that govern these events remain largely unresolved. Translation is regulated principally at the step of initiation (i.e. mRNA recruitment onto polysomes). One of the key targets for this regulation is the cap binding protein eukaryotic initiation factor 4E (eIF4E). It is frequently rate limiting (2), and can also be sequestered into an inactive complex by a family of 4E-binding proteins (4EBP1/2/3). The affinity of these proteins for eIF4E is modulated by phosphorylation in response to cell growth signals. This releases eIF4E increasing its availability for the formation of the active cap binding complex, eIF4F (3). The phosphorylation event is mediated by the PI3K/AKT pathway that impacts on translation initiation via mammalian target of rapamycin (mTOR) (4–6). This kinase exists within two distinct complexes referred to as complex 1 (mTORC1) and complex 2 (mTORC2). The best-characterized downstream effectors of the mTORC1, namely, the 70-kDa ribosomal protein S6 kinase 1 (S6K1) and the 4EBP1 pathways, act in parallel to regulate mRNA translation (7–9).

Because of its cardinal role in cell growth regulation, a great deal of research has been directed towards the development of mTOR inhibitors for cancer therapy (10,11). One of these, rapamycin, exerts its action by binding and inactivating the mTORC1 via its RAPTOR subunit (11–13) which, in turn, leads to a global down-regulation of protein synthesis. Recently, we reported on the impact of rapamycin on the re-seeding of the polysome populations in the non-tumoural cell line MRC-5. We identified transcripts that exhibited

*To whom correspondence should be addressed. Tel: +41 22 379 5799; Fax: +41 22 379 5702; Email: joseph.curran@unige.ch
Present address:
Raphael Genolet, LICR, Chemin des Boveresses 155, Case postale 34, 1066 Epalinges, Switzerland.

altered sensitivities to the drug, thus creating a molecular signature within this cellular context and growth conditions (14). One of the genes whose relative polysomal occupancy increased was *mdm2*. Its most-studied function is the regulation of the tumour suppressor protein P53. The *mdm2* gene is transcribed from two promoters: the upstream promoter (P1) ensures constitutive expression and a second downstream promoter (P2) is activated by P53 (15). This serves as an auto-regulatory feedback loop in which P53 modulates the expression of a protein that inhibits its own function. The two *mdm2* gene promoters generate mRNA transcripts with different 5′-UTRs. The longer, P1-derived 5′-UTR (L-mdm2), possesses two upstream open reading frames (ORFs) which repress translational initiation from the MDM2 start codon. The shorter P2-derived 5′-UTR (S-mdm2) carries no such elements and is generally translated more efficiently. The probe sets used in our original microarray were 3′ on the mRNA and so were unable to distinguish between the two 5′-UTRs. An additional problem associated with studying *mdm2* gene expression arises due to the complexity of the alternatively spliced transcripts. At least 40 such variants have been reported. The p90 full-length protein is frequently detected with other isoforms (generally p85, p75, p57 and p46), which retain part(s) of the P53 binding site and nuclear import/export signals. However, one feature shared by all spliced forms is the presence of either the L-mdm2 or S-mdm2 5′-UTR.

In this work, we have examined the role of the two 5′-UTRs in the rapamycin resistance associated with the polysomal recruitment of the mdm2 transcript. We have been unable to detect discernable internal ribosome entry site (IRES) activity in either of the alternative 5′-UTRs. Nonetheless, both regions confer relative drug resistance in a dual reporter assay performed in HEK293T cells. Results indicate that this is associated with alterations in the downstream mTORC1 targets. Furthermore, extended rapamycin exposure under conditions of high serum (conditions under which our earlier studies were performed) results in an increase in the steady-state levels of the endogenous MDM2 protein in both HEK293T and MRC-5 cell lines. However, this response is effectively reversed when serum levels are reduced. Taken globally, these studies suggest that the cellular context and experimental conditions can dramatically modulate the 5′-UTR-mediated expressional output during rapamycin exposure.

MATERIALS AND METHODS

Cell culture

HEK293T cells (ATTC, CRL-11268) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Brunschwig), 1% penicillin/streptomycin (Gibco), in a humidified atmosphere containing 5% CO_2. MRC-5 cells (Coriell Cell Repository) were cultured in Minimal Essential Medium (Gibco) supplemented with 1 mM sodium pyruvate (Sigma), 0.1 mM non-essential amino acids, 10% fetal calf serum, 1% penicillin/streptomycin, in a humidified atmosphere containing 5% CO_2. HCT116 cells were obtained from Dr André Fedier (University Hospital Zurich, Department of Gynecology) and were cultured in McCoy's 5A medium (Sigma) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin. Rapamycin was purchased from LC laboratories and stored as a 50-mM stock in dimethyl sulphoxide (DMSO) at −20°C.

DNA constructions

The bicistronic clones were generated in a pcDNA3 backbone. The 5′-UTRs were inserted into the intercistronic region as XhoI/NcoI fragments produced by reverse transcription-polymerase chain reaction (RT–PCR) from total cell RNA as outlined in (14). The encephalomyocarditis virus (EMCV) IRES construct has already been described (16). The primer sets used were as follows:

QKI: GTCGAGCTCCATGGATTCCAGGCTCCGCA GCTCACT, CCGCTCGAGTGAACTGAGGCGAA CTCCGCC
THAP2: TCGAGCTCCATGGCCCTTTCCCACTCAT TCAGCGCCC, CCGCTCGAGACTTCCTGCGACA CGCAGGAAACA
FAS: CGGGATCCATGGGGTTGTTGAGCAATCCT CCGAAGT, CCGCTCGAGTCTGTGAGCCTCTCA TGTTGCA
FASL: GTCGAGCTCCATGGGGCAGCTGGTGAGT CAGGCCAGCC, CCGCTCGAGAGAATCAGAGA GAGAGAGATAG
FAF1: GTCGAGCTCCATGGGGGCGGCCGCCGAG TTCCGCGGCT, CCGCTCGAGCAGGGGCGGAG GCGGCGGCCA
L-MDM2: GTCGAGCTCCATGGTTGCCTGCTCCTC ACCATCCGGGGT, CCGCTCGAGCCGAGGCAC CGCGGCGAGCTT
S-MDM2: Was generated as two overlapping primers carrying XhoI and NcoI extremities.
VCME: CATGCCATGGCGAATTCCCCCTCTCC, CC GCTCGAGCGCGTGTTTTTCAAAGGAAAACC

The pGL3-Enhancer monocistronic vector carrying the human β-actin 5′-UTR and the RLuc reporter was generated by RT–PCR cloning the former from HEK293T total cell RNA, and amplifying the latter from the bicistronic plasmid clone. The primers used are listed below. The fragments were subsequently ligated and then used to replace the original FLuc reporter in the plasmid.

β-Actin: CCGAAGCTTCGCGTCCGCCCCGCGAGCA CAGA, CACCATGGTGGCTAGCGGTGAGCTGG CGGCGGGTG,
RLuc: GCCACCATGGCTTCGAAAGTTTTTG, GCC TCGAGTTATTGTTCATTTTTGTG

The plasmid expressing the siRNA against RLuc (pBS/U6-RLi) and the empty vector control (pBS/U6ApaI) were supplied by Dr Richard Lloyd (Houston, USA). The Flag-La, GSTLa226-348 and eIF4E plasmids were

provided by Dr. Nahum Sonenberg (Montreal, Canada). The 4EBP1 expressing plasmid was provided by Dr. Chris Proud (Southampton, England). The His/myc tagged S6K1 constructs were provided by Dr. George Thomas (Cincinnati, USA).

Firefly reporter assays

Calcium phosphate-mediated DNA transfection was performed as described in ref. 17. Transfections were performed in triplicate. Rapamycin was applied as indicated in the figure legends. Extracts were prepared in passive lysis buffer according to the supplier (Promega). The activity of firefly (FLuc) and renilla (RLuc) luciferases were measured using the dual-luciferase reporter assay system (Promega) on a Gloma 20/20 luminometer (Promega).

Western blot

Cytoplasmic extracts were prepared by solubilizing the monolayer in 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 10 mM EDTA, 0.6% NP-40, and the complete mini protease inhibitor cocktail (Roche). Nuclei were removed by pelleting at 20 000*g* for 5 min. Thirty micrograms of protein was resolved on a polyacrylamide-sodium dodecyl sulphate (SDS) gel and electro-transferred to polyvinylidene fluoride (PVDF). Antibodies used in this study were anti-MDM2 (2A10, Calbiochem), anti-actin (Chemicon), anti-4EBP1 (Cell Signalling), anti-eIF2α (Cell Signalling), anti-phospho-eIF2α (Invitrogen), anti-eIF4E (Cell Signalling), anti-P53 (PAb240, Calbiochem), anti-phospho S6K1 (Cell Signalling), anti-Flag (polyclonal, Sigma-Aldrich), anti-Myc (9E10, Covance) and anti-ELK1 (Santa Cruz). A polyclonal rabbit anti-GST antibody was kindly provided by Dr. Dominique Soldati-Favre (Geneva, Switzerland). Blots were developed using the Super Signal Substrate (Thermo Scientific) and quantitated using the Quantity One software package (Bio-Rad).

Pulse-chase experiments

For protein labelling, cells were starved for 30 min in DMEM minus methionine and cysteine (Gibco). This was then replaced with the same medium containing 100 μCi/ml ^{35}S-translabel for 1 h (Amersham). For the chase period, the labelling medium was removed and replaced with normal growth medium containing 10% serum (with either DMSO or rapamycin) for the times indicated. Cytoplasmic extracts were prepared by solubilizing the monolayer in 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 10 mM EDTA, 0.6% NP-40, 1 mM AEBSF (Boehringer) and 1% aprotinin (Sigma). Nuclei were removed by pelleting at 20 000*g* for 5 min. Extracts were immunoprecipitated overnight at 4°C with 2.5 vol. of RIPA buffer (150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris pH 7.8) containing the MDM2 antibody 2A10. The immune complexes were recovered on protein-A Sepharose (Pharmacia) for 1 h at room temperature (RT), washed three times with RIPA buffer. Samples were boiled with sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.5% SDS, 60 mM Tris–HCl pH 6.8, bromophenol blue) and proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE).

RT–PCR and real-time PCR

The RT–PCR was performed using the One Step RT–PCR kit (Qiagen) according to the manufacturer's instructions. The number of amplifications cycles was first determined for each set of primer, and corresponded to the exponential phase of the different products (16). For the semi-quantitative RT–PCR on the reporter transcripts, the flowing primer sets were used:

L-mdm2 FLuc: CCGAGGCACCGCGGCGAGCTT, GC TCTCCAGCGGTTCCATCTTCCAG
S-mdm2 FLuc: GGGCACGGACGCACGCCACTTT, G CTCTCCAGCGGTTCCATCTTCCAG
β-Actin RLuc: CTGACGGCCAGGTCATCACCATTG, GAGGCCATGATAATGTTGGACGACG

For the real-time PCR, 1 μg of total RNA was reverse transcribed using random hexamers (Gibco). A 1/10 dilution of the cDNA was used to perform the PCR with the SYBR Green Reagent (Roche). The primers used were:

L-mdm2: CCCGACTCCAAGCGCGA, GGTGACACC TGTTCTCACTCACAGA
S-mdm2: GGGCACGGACGCACGCCACTTT, GGTG ACACCTGTTCTCACTCACAGA
β-Actin: CTGACGGCCAGGTCATCACCATTG, GCC GGACTCGTCATACTCCTGCTTG

Polysome analysis

Cells in the growth phase were hypertonically shocked by shifting to medium containing 300 mM NaCl for 50 min. The cells were then placed in normal isotonic medium for 30 min. When rapamycin was used, 100 nM drug or 0.01% DMSO (the negative control) was added during the hypertonic shock, 20 min before the transfer back to isotonic conditions. Rapamycin and DMSO were kept on the cells throughout the recovery period. After treatment, cells were scraped into the culture medium and pelleted for 4 min at 800 r.p.m. The pellets were lysed for 15 min on ice in 100 mM KCl, 50 mM Tris–Cl pH 7.4, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mg/ml heparin, 1.5% NP-40, 100 μM cycloheximide, 1% aprotinin, 1 mM AEBSF and 100 U/ml of RNasin. Nuclei were pelleted by centrifugation, 10 min at 12 000 r.p.m. The supernatant was loaded on a 20–60% sucrose gradient (in 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES pH 7.4 and 2 mM DTT). Extracts were fractionated for 3 h 30 min at 35 000 r.p.m. at 4°C in a Beckman SW41 rotor. The gradient was analysed and the fractions collected with the ISCO UA-6.

RNA was isolated by adding the same volume of TriZol (Invitrogen) to the fractions. Samples were mixed and incubated for 15 min on ice, and 0.3 vol of chloroform was added. After centrifugation, the upper phase was collected and the RNA precipitated with 0.7 vol of isopropanol. The pellet of RNA was re-suspended in water.

RESULTS

Translational properties of the *mdm2* 5′-UTR

The starting point for the current study was a high-throughput translational profiling screen that followed ribosome-transcript re-association in the presence and absence of rapamycin (14). Data mining revealed that the relative polysomal occupancy of 3.6% of the transcripts was affected by the drug. One feature that could impact on the sensitivity to rapamycin is the presence of an IRES within the 5′-UTR. We therefore tested for the presence of such an element in six different genes, three that were up-regulated (qki, mdm2 and thap2) and three that were down-regulated (fas, fasl and faf1). Surprisingly, the results indicated that 5′-UTRs both from the up and down-regulated sets, had normalized second cistron activities significantly higher than the VCME negative control (Figure S1A). However, the bicistronic assay is not sufficient to unambiguously define an IRES (18–22), and further controls indicated that all constructs, with the exception of mdm2, presented significant cryptic promoter activity (Figure S1B).

Despite the low activity of the mdm2 5′-UTR in the bicistronic assay, we considered that the result warranted further investigation. However, the expression of the *mdm2* gene is complex. There are at least 40 alternatively spliced transcripts and two 5′-UTRs. The form we tested above was the constitutively expressed long form (L-mdm2) driven from the P1 promoter (Figure 1A). A second shorter 5′-UTR, the S-mdm2, arises from a second p53 responsive P2 promoter (Figure 1A). Semi-quantitative RT–PCR analysis confirmed that both 5′-UTRs were present in HEK293T (Figure 1B) and MRC-5 (Figure S2B) cell lines, and there relevant abundance was essentially unaltered following rapamycin exposure. When tested in the bicistronic assay, the S-mdm2 gave second cistron activities higher than the L-mdm2; however, it also had significant cryptic promoter activity (Figure 1C and D). Furthermore, promoter activity within both the 5′-UTRs of S-mdm2 and qki was confirmed by co-transfection of a siRNA directed against the RLuc first cistron (Figure 1E) (18). Therefore, in a final attempt to assign IRES activity to the L-mdm2, we tested the bicistronic construct in the presence of rapamycin or 4EBP1 (His/myc tagged). The over-expression of 4EBP1 has been demonstrated to down-regulate cap-dependent, but not IRES-driven, translation. (23). As IRES controls, we also tested the viral IRES EMCV and a reported cellular IRES Dap5 (24). Consistent with its inhibition of the mTORC1 kinase, rapamycin treatment increased the levels of endogenous hypophosphorylated 4EBP1 (Figure 1G) and stimulated relative second cistron activity in the EMCV construct (Figure 1F). However, the effect on both the L-mdm2 and Dap5 constructs was negative (Figure 1). Furthermore, whereas 4EBP1 expression had a marked positive effect on the viral IRES, its effect was once again negative with both cellular 5′-UTRs (Figure 1F and H). One curious observation in the 4EBP1 transfected cells was a marked shift to the hyperphosphorylated forms of the endogenous 4EBP1 (Figure 1H; as if the cell is desperately trying to detour the 4EBP1 block by activating mTOR).

The mdm2 5′-UTRs and rapamycin

As none of the results unambiguously suggested IRES activity within the mdm2 5′-UTRs, we asked if the 5′-UTRs alone conferred relative rapamycin resistance to a monocistronic reporter. For this, we generated FLuc constructs carrying either the L-mdm2 or S-mdm2 5′-UTRs. As a control, we fused the 5′-UTR of the β-actin housekeeping gene to RLuc because the relative polysomal occupancy of this mRNA was not affected by rapamycin in the microarray screen (14) and its expression is considered a paradigm of 5′- cap-dependent translation (25). The RLuc/FLuc constructs were co-transfected into cells in the presence or absence of the drug. Examination of reporter activity 24 h post-transfection revealed that both the mdm2 5′-UTRs conferred resistance relative to β-actin (Figure 2A). Semi-quantitative RT–PCR performed on RNA isolated from these cells demonstrated that rapamycin treatment reduced transcript levels from all reporter plasmids equally, indicating that the observed changes in the normalized values reflects a translational response (Figure 5C; values obtained in 10% serum). Dosing the concentration of rapamycin from 100 to 0.1 nM demonstrated that this response correlated with the activity of mTORC1 as monitored by the phosphorylation status of 4EBP1 (Figure 2B). This confirms the microarray results, despite the fact that in the earlier experiments we followed mRNA-ribosome re-association following short drug exposure (50 min). However, we could not mimic the effect of the drug by simply over-expressing tagged versions of 4EBP1 (Figure 2A), despite the fact that this tends to repress cap-dependent initiation (23). This suggests that the effect cannot simply be explained by competitive advantage under conditions of limiting eIF4E availability. It has been reported that the rapamycin effect of 4EBP1 activity is temporally regulated implicating a second cellular kinase (26). However, the drug-induced enhancement from the mdm2 constructs remained essentially unchanged between 4 and 48 h post-transfection (Supplementary Figure S3), an observation that once again suggests that the effect is not principally via 4EBP1 and hence eIF4E availability.

As rapamycin is thought to act mainly via the inactivation of mTORC1 kinase activity, we asked if we could reverse the rapamycin effect by co-expressing active versions of its downstream targets. For this we selected eIF4E, S6K1 T389E and the S6K1 T389A control. The T389E mutation mimics mTORC1-mediated phosphorylation rendering the S6K1 more active under basal conditions, and partially rapamycin resistant. The T389A mutant has essentially the opposite effect (27,28). With S-mdm2, we again observed the positive effect of rapamycin on the normalized reporter value (Figure 3A). This was reversed upon over-expression of T389E but less so by eIF4E. Co-expression of both reduced levels to below the rapamycin minus control. In addition, the presence of the S6K1 T389A accentuated

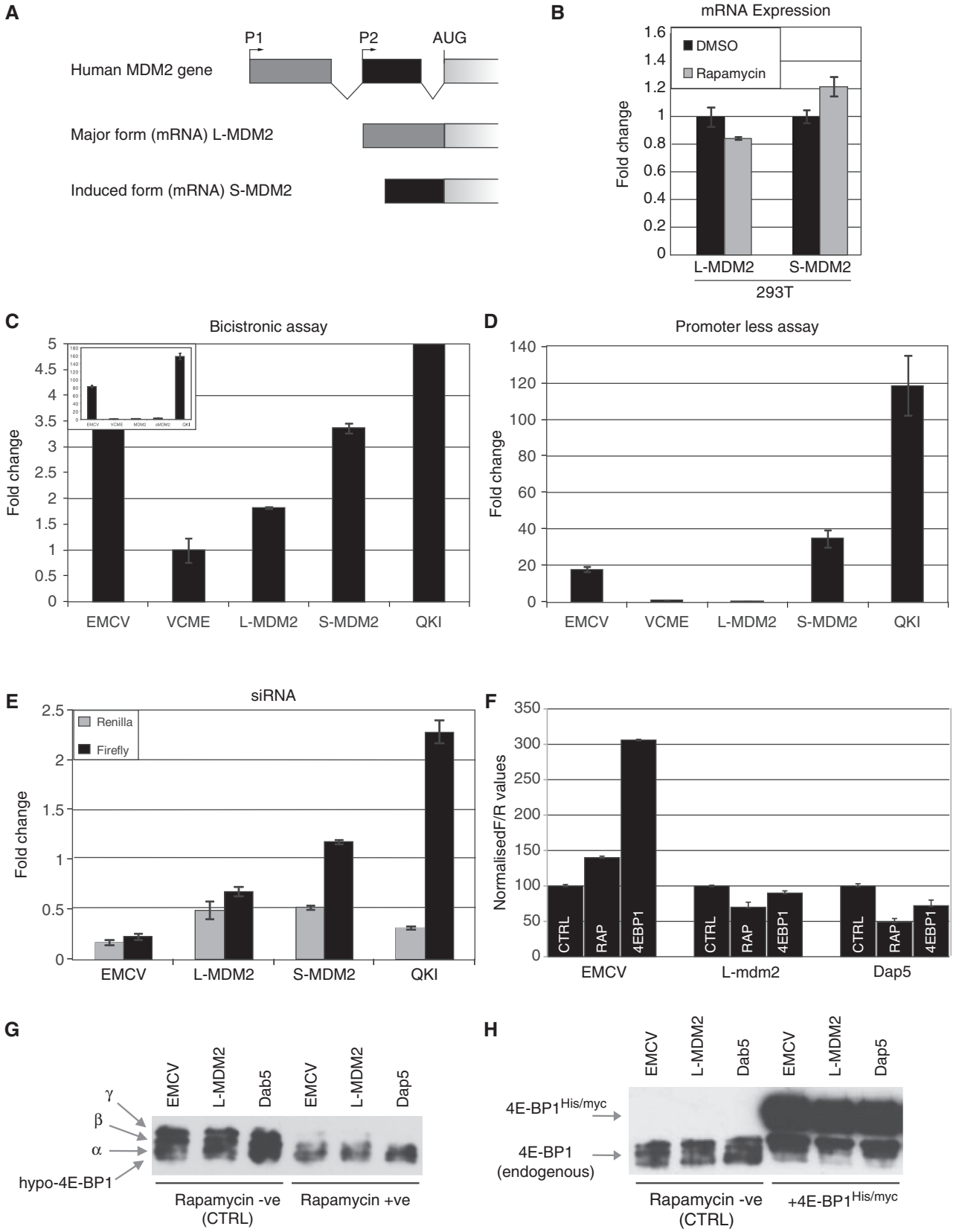


Figure 1. Controls for IRES activity within the mdm2 5′-UTRs. (**A**) A schematic representation of the organization of the human L-mdm2 and S-mdm2 5′-UTRs. The rectangles refer to exons, P1/P2 are the alternative promoters and AUG indicates the MDM2 start codon. (**B**) Semi-quantitative RT–PCR that follows L-mdm2 and S-mdm2 levels in HEK293T cells in the absence (DMSO, 24 h) or the presence of rapamycin (100 nM, 24 h). In each case the value of the DMSO control was set as 1. (**C**) Bicistronic assays were performed using the two alternative mdm2 5′-UTRs. (**D**) Cryptic promoter activity was assayed in the promoter-less pGL3-Basic monocistronic background. Values for the VCME control were

(continued)

the effect of the drug on the normalized reporter activities. With the L-mdm2 construct, the results were very similar (Figure 3B). One noticeable exception was that the S6K1 T389A construct had only a marginal effect on the readout hinting at possible differences in the regulation of the two 5′-UTRs. Nonetheless, these results appear to confirm that the relative drug resistance associated with the mdm2 5′-UTRs is coupled to mTORC1 signalling.

The MDM2 protein in HEK293T cells

It has been reported that rapamycin treatment can impact on the intracellular levels of MDM2. Indeed, in our HEK293T cells, we observed that protein levels increased after rapamycin exposure (Figure 4A). This increase could not be attributed to changes in mRNA levels (Figure 4B), suggesting that it was translational. That the drug had impacted on mTORC1 signalling was confirmed by analysing the phosphorylation status of S6K1 (Figure 4A). To further characterize the specificity of the rapamycin response, we analysed the expression of other regulatory proteins in these extracts; namely, ELK-1, eIF4E and p53 (Figure 4A). Whereas ELK-1 and eIF4E levels were reduced by the drug (the effect being more pronounced with the former), P53 was not perturbed. Changes in the steady-state MDM2 protein without a concomitant impact on P53 levels have been reported by others (29).

We also examined if rapamycin altered protein stability. MDM2 is generally considered to be an unstable protein at least in part due to its self-polyubiquitinylation (30). Furthermore, its half-life in certain cell lines has been reported to be reduced in the presence of rapamycin (31). However, pulse-chase experiments in HEK293T cells demonstrated no change in protein turnover as a consequence of drug exposure (Figure 4C). The measured $t_{1/2}$ (~2.5 h) was very similar to that reported recently by others in the same cellular background (32).

Initiation from both murine 5′-UTRs has been reported to be up-regulated by the La auto-antigen (33). An La binding site was mapped upstream of the murine MDM2 AUG codon within the exon II conserved in both long and short forms. Despite the fact that the human exon II is removed in the L-mdm2 isoform (Figure 1A), alignment suggested a similar sequence was partially conserved in both human 5′-UTRs. (33). Furthermore, a recent microarray study suggested that La-mediated recruitment of mRNA populations onto polysomes was coupled to its phosphorylation mediated via AKT (34). As the PI3K/AKT pathway is tightly coupled to mTOR, we decided to examine if La expression could provide insights into the behaviour of the human 5′-UTRs in the presence of rapamycin. We obtained an N-terminally FLAG tagged human La clone, and a GST tagged deletion mutant (GST-La226-348) which retains the La oligomerization domain and is dominant negative for the La-mediated stimulation from the murine mdm2 (35,36). These were transferred to the mammalian expression vector pEBS-PL, which expresses EBNA1 and carries an EBV oriP (37). This ensures that the plasmid is retained in dividing cells. Forty eight hours after transfecting the La constructs, the cells were re-transfected with the actin/mdm2 dual reporters and rapamycin was applied. Extracts were analysed 24 h later. This approach ensured high levels of La expression at the moment of reporter gene transfection and drug treatment. This was confirmed by immunoblotting (Supplementary Figure S4A). However, we observed no effect on our dual reporter assay in the presence of rapamycin (Supplementary Figure S4B; results obtained with the L-mdm2 were identical). Furthermore, the observed increase in cellular MDM2 protein levels after rapamycin exposure was not perturbed by the over-expression of the dominant negative GST-La226-348 (Figure 4A).

What modulates the mdm2 response?

Although the increased levels of endogenous MDM2 protein observed after extended exposure to rapamycin would not be inconsistent with our reporter assays, it does fly in the face of other work. Using a cancer model system (the HCT116 cell line), it was reported that rapamycin decreased MDM2 levels by translational inhibition (38). However, this was also at conflict with other results that reported a down-regulation of MDM2 levels in an OVCAR-3 cell line exclusively by changes in protein half-life (31). Upon analysis of the results reported in the HCT116 cell line, we noted that the conditions employed were somewhat different. Whereas during rapamycin exposure we maintained serum concentrations at 10%, the HCT116 work reduced the serum to 2%. They also employed a rapamycin concentration 10-fold higher (1 μM). In HEK293T cells, concentrations as low as 1 nM were sufficient to inhibit downstream signalling from mTORC1 as monitored by 4EBP1 phosphorylation (Figure 2B, lower panel). In a similar vein, a rapamycin dose experiment using the mdm2/β-actin reporter assay demonstrated that the mdm2 5′-UTRs conferred relative resistance at drug concentrations as low as 0.1 nM (Figure 2B, upper panel). Therefore, we repeated our HEK293T experiment at two serum concentrations. Analysis of MDM2 levels 24 h post-drug exposure demonstrated a striking difference in the response

Figure 1. Continued
set at 1. (**E**) As a further control the bicistronic constructs were co-transfected with a plasmid expressing either the siRNA against RLuc (pBS/U6-RLi) or the empty vector control (pBS/U6ApaI). Reporter activity in the cell extracts was measured 24 h post-transfection. The graph plots reporter activities in the presence of the siRNA RLuc relative to the empty vector control. A value of 1 indicates no change. (**F–H**) Bicistronic clones carrying the EMCV, L-mdm2 or Dap5 intercistronic regions were expressed in HEK293T cells in the presence of either DMSO (CTRL), rapamycin (100 nM added to the growth medium 30 min before transfection), or a second vector expressing a His/myc tagged 4EBP1 (co-transfected with the bicistronic construct). Cell extracts were prepared 24 h post-transfection. Normalized reporter activities were plotted (F) with the control (bicistronic alone) for each construct being set at 100. Rapamycin activity was confirmed by immunoblotting with an anti-4EBP1 antibody (G), as was expression of the tagged 4EBP1 transgene (H). Bars indicate the SEM from triplicate transfections.

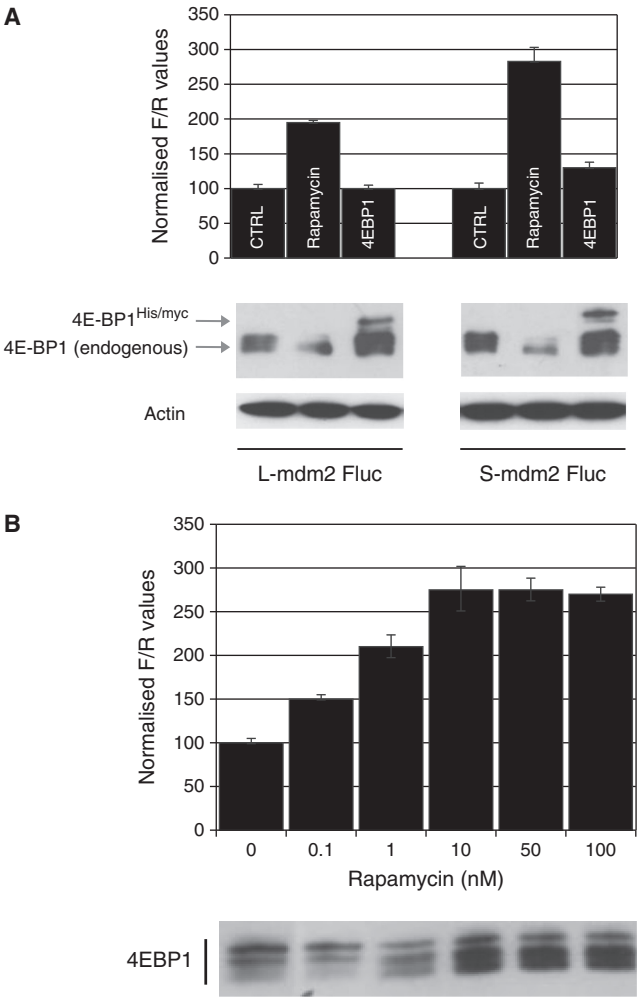


Figure 2. Both mdm2 5′-UTRs confer relative rapamycin resistance to a monocistronic construct. (**A**) pGL3-Enhancer constructs carrying either the L-mdm2 or S-mdm2 5′-UTRs fused to FLuc were expressed in HEK293T cells in the presence of rapamycin (100 nM added to the growth medium 30 min before transfection) or a second vector expressing $^{His/myc}$4EBP1. As an internal control, all cells were co-transfected with a pGL3-Enhancer vector that carried the 5′-UTR of β-actin fused to an RLuc reporter. Cells were harvested 24 h post-transfection. Normalized reporter activities were plotted with the CTRL (the two reporter constructs alone) set at 100 (upper panels). The effect of rapamycin and the expression of the 4EBP1 transgene were confirmed by immunoblotting with an anti-4EBP1 antibody. An immunoblot for actin provided a loading control (lower panels). (**B**) HEK293T cells were co-transfected with the S-mdm2 FLuc and β-actin RLuc reporter plasmids 30 min after addition of rapamycin at the concentrations indicated (0 indicates a DMSO control). Cells were harvested 24 h post-transfection and the normalized reporter activities plotted. The value for the rapamycin minus control was set at 100 (upper panel). The rapamycin response was confirmed by immunoblotting using an anti-4EBP1 antibody (lower panel). Bars indicate the SEM from triplicate transfections.

(Figure 5A). In the presence of 2% serum, rapamycin reduced MDM2 levels. In addition, reducing serum levels in the absence of the drug tended to mimic the rapamycin effect observed under high serum concentrations (compare lanes 1/3 with lanes 1/2). We asked if serum concentrations impacted on the readout from the dual reporter assay. Only a very marginal change was observed when the serum was reduced to 2% (compare Figure 5B with Figure 2A); however, the effect was significantly attenuated when the assay was performed under serum-free conditions (Figure 5B). Once again, RT–PCR analysis demonstrated that although rapamycin impacted on transcription at all serum concentrations tested, it was equally repressive for the entire reporter construct set (Figure 5C), indicating that the observed changes in the readout reflected alterations at the translational level. The absence of a marked effect on the reporter readout at 2% serum, despite a clear impact on intracellular MDM2 protein levels in the same cells, suggests that other elements in the mature mdm2 mRNA may also, in conjunction with the 5′-UTRs, fine-tune the serum response. Reports on both the human and murine genes indicate that the 3′-UTRs do not affect translational efficiency (39,40), suggesting that elements within the ORF itself may modulate the response. Regulatory elements within the ORF of an mRNA that respond to growth and stress signalling pathways have already been reported (41,42).

The serum effect observed with the S-mdm2 5′-UTR was more marked than that observed with the L-mdm2. An explanation for this becomes apparent if one examines how the normalized reporter activities responded to changes in serum concentrations alone (i.e. in the absence of the rapamycin: Figure 6A). The increase in the normalized reporter readout observed with the L-mdm2 in the absence of serum probably reflects changes in eIF2α activity that arise during these stress conditions (43), coupled to the presence of uORFs uniquely in the L-mdm2 5′-UTR (but neither in S-mdm2 or β-actin) (44). This interpretation is supported by the observation that mutation of the two uAUGs to GCG codons ablated the serum response (Figure 6A). It should also be noted that these mutations did not alter the relative rapamycin resistance observed in the dual reporter assay (Figure 6B), indicating that this phenomena is not coupled to translational re-initiation.

Despite the fact that one can extract useful information using values normalized to an internal control, the results can nonetheless be misleading. This is highlighted in Figure 5D, in which the rapamycin/serum response of the S-mdm2 and β-actin reporters (the normalized values depicted in the right-hand panel of Figure 5B) have been analysed independently. One observes that expression from the S-mdm2 reporter is clearly less sensitive to the drug in the presence of serum (the 2.5-fold difference corresponds to that observed with the normalized values) but becomes as sensitive as the β-actin reporter when serum is removed.

A re-recruitment polysomal assay in HEK293T cells confirms relative drug insensitivity

The starting point for this work was a high-throughput analysis of the impact of rapamycin on polysomal seeding in a human primary cell line MRC-5 (14). Studies focusing on the role of the 5′-UTRs were performed in HEK293T cells for purely technical reasons (they transfect well); nonetheless, these studies led to the demonstration of enhanced MDM2 protein levels after extended exposure.

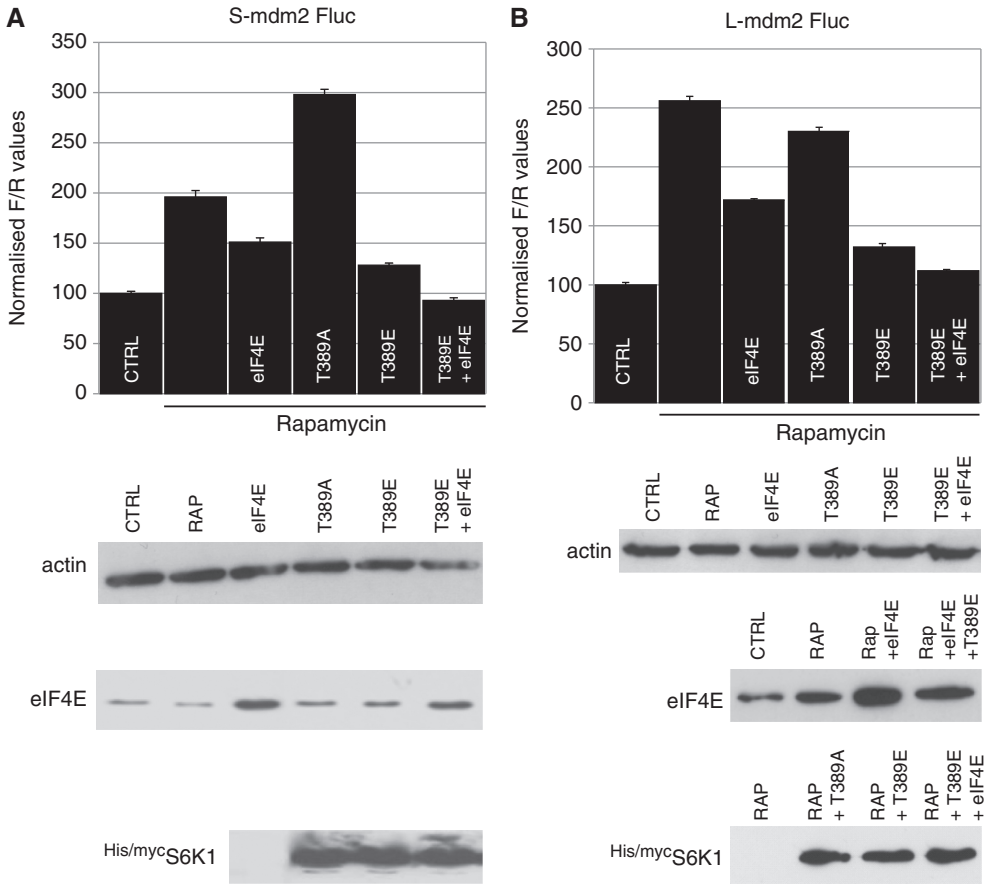


Figure 3. The relative rapamycin resistance conferred by the mdm2 5′-UTRs responds to the over-expression of the downstream mTORC1 effectors. (**A**) The S-mdm2 FLuc and β-actin RLuc reporter plasmids were transfected into HEK293T cells with combinations of eIF4E, S6K1T389E and S6K1T389A as indicated in the figure. Rapamycin was applied to the cells 24 h post-transfection. A series of non-treated cells provided the control (RAP-ve). Cell extracts were prepared 24 h after drug treatment and reporter ratios were normalized to the control which was fixed at 100 (upper panel). S6K1 and eIF4E expression were confirmed by immunoblotting and an anti-actin blot provided a loading control (lower panels). (**B**) The assays outlined above were repeated with the L-mdm2 reporter and the constructs as indicated. The bars in both graphs indicate the SEM from triplicate transfections.

Immunoblots indicated that expression from the *mdm2* gene was also increased in MRC-5 cells following rapamycin exposure. This cell line produced both the p90 and p46 MDM2 proteins (with traces of p75) in almost equal amounts and the expression of both was marginally increased after 24 h of rapamycin treatment without any marked changes in the S-mdm2 and L-mdm2 transcript levels (Supplementary Figure S2). As a final control, it seemed warranted to examine what happens in HEK293T cells during a reseeding of the polysomal transcript populations in the presence and absence of the drug (with 10% serum as performed in the MRC-5 cell line). We therefore repeated the hypertonic shock re-recruitment assay exactly as outlined previously (14). Sucrose gradient profiles demonstrated a clearly discernable reduction in the polysomal fraction in the presence of the drug (Figure 7A). Real-time RT–PCR quantification was performed across the gradient using primer sets specific for L-mdm2, S-mdm2 and β-actin. Evaluation of the percentage of each transcript in the heavy polysomal fraction (≥5 ribosomes; this is considered an indicator of efficient translational expression) demonstrated that both mdm2 transcripts increased in the presence of rapamycin whilst actin was slightly reduced (Figure 7B). These results essentially mirror those observed in the MRC-5 cell line.

DISCUSSION

The oncoprotein MDM2 acts as an ubiquitin ligase whose main, although not exclusive, function is to target P53 for degradation. Upon activation, P53 up-regulates the transcription of MDM2 from the P2 promoter (45), establishing a negative feedback loop that will limit the growth suppressive activities of P53. A further level of complexity arises because MDM2, via its RING domain, also binds to the p53 mRNA. These both impair E3 ligase activity whilst promoting p53 mRNA translation (42). In some tumours, the over-expression of MDM2 was not associated with an increase of RNA expression or protein stability, suggesting that regulation was at the level of translation. However, these studies are frequently frustrated by the plethora of transcript isoforms. For example, increased expression from the P2 promoter is

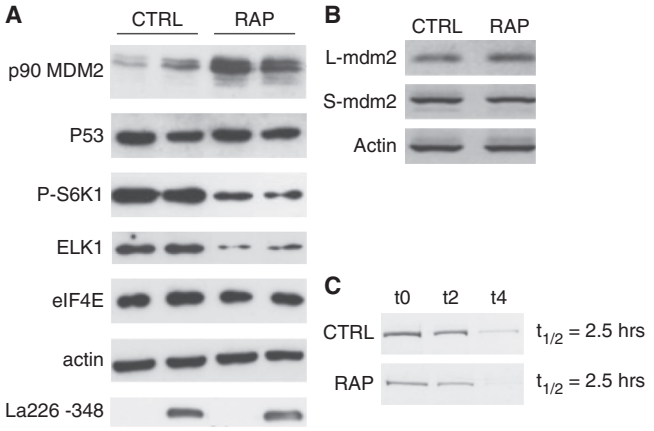


Figure 4. The endogenous MDM2 protein levels. (**A**) HEK293T cells were transfected with a pEBS-PL vector expressing a GST-tagged La226-348 (a dominant negative deletion mutant) or an empty plasmid vector. At 48 h post-transfection cells were either treated with DMSO (CTRL) or rapamycin (100 nM). Extracts were prepared 24 h later and analysed by immunoblotting using the antibodies indicated. (**B**) Semi-quantitative RT–PCR was performed on cells that had been treated or non-treated with rapamycin (lanes 1 and 3 in A) to monitor changes in the levels of the two mdm2 5′-UTRs. Actin provided the internal control. (**C**) Pulse-chase experiments were performed in HEK293T cells to monitor MDM2 stability after 24 h exposure to rapamycin. DMSO provided the negative control. Radiolabelled cell extracts were immunoprecipitated with an anti-MDM2 antibody and immune complexes were recovered on protein-A sepharose beads. Proteins were resolved by SDS–PAGE. The times of the chase and the estimated protein half-life are indicated.

frequently observed generating transcripts carrying the S-mdm2 5′-UTR. This element tends to produce more MDM2 products than the L-mdm2 5′-UTR (46,47). In addition, studies using polysomal gradient analysis reported that the S-mdm2 was more efficiently translated in tumoural compared to non-tumoural cells (48). Therefore, a switch in promoter usage without a marked change in overall transcript levels can have a profound effect on the translational readout, an effect that may be further fine-tuned by the cellular context.

Clearly, *mdm2* gene expression is tightly controlled at all levels. An additional layer of complexity arises when one examines this control in the presence of drugs that specifically target cellular physiology. With regard to our own work, the *mdm2* gene was selected from a translational profiling analysis performed with rapamycin. This demonstrated that the drug reduced the overall polysomal fraction in the cell (consistent with its global negative effect on initiation) and this uniformly affected the majority (~96%) of cellular transcripts. However, some mRNA populations did change (some exhibited a hyper-sensitivity and others a hypo-sensitivity to the drug). The *mdm2* gene formed part of the latter group. Drug-induced changes in the polysomal mRNA populations will ultimately impact on the proteomic composition of the cell (e.g. during extended clinical therapy). Although one of rapamycin's principal effects is to reduce eIF4E availability, our experiments appear to exclude the presence of an IRES-like element in either the S-mdm2 or L-mdm2 5′-UTRs. Indeed, IRESes are frequently cited as elements that assure continued translational expression under stress conditions that modulate the activity and/or availability of the cap binding complex. They are generally associated with genes which play key roles in cell growth control (49). For example, the p53 5′-UTR has also been reported to carry IRES activity and its translation is auto-repressed by the binding of the P53 protein. Upon DNA damage P53 migrates from the cytoplasm to the nucleus, thereby de-repressing the translation of its own mRNA (50–52). However, it is increasingly evident that other cap-dependent mechanisms can enter into play under conditions of limiting eIF4E, mechanisms that may well explain the relative rapamycin resistance observed with the mdm2 5′-UTRs. These may involve the recruitment of *trans*-acting cellular factors to the 5′-UTRs that then serve to facilitate pre-initiation complex (PIC) recruitment via the 5′-cap. These cap dependent *trans*-acting factors (CTAFs by analogy to the ITAFs that regulate IRES activity) (53) may interact with other initiation factors (eIF4G, eIF3, etc.) or even the 40S ribosomal subunit, thereby effectively increasing the local concentration of these components close to the mRNA 5′-end (25). MDM2 may itself function as a CTAF as its binding to the 5′- end of the p53 mRNA promotes translation (42). With regard to the mdm2 mRNA, one attractive CTAF candidate was the La protein which had been reported to modulate translational expression from the murine mRNA (33) and has also been described to function as an ITAF on both cellular and viral IRESes (36,54). However, we observed no effects of over-expressed La or the dominant negative La226-348 either in our dual reporter assay or on endogenous MDM2 expression levels in HEK293T cells. Other groups have also reported differences in the translational response of the murine and human mdm2 5′-UTRs. For example, unlike murine transcripts, the P53-activated P2 promoter in humans generates preferentially the p90 protein product (55). As the protein isoforms are functionally distinct this hints at important differences in MDM2 function in these different species.

Many aspects of our results are clearly at conflict with published data. Using a cancer model system (HCT116 cell line), it was reported that rapamycin decreased MDM2 levels by translational inhibition (29). This group reported that the drug also induced a P53-mediated apoptotic program. However, this is also at conflict with other results that reported a down-regulation of MDM2 levels exclusively by changes in protein half-life (31) (in a OVCAR-3 cell line), and studies showing the anti-apoptotic properties of rapamycin (56,57). Our current study suggests that some of this variation may reflect the conditions under which the assays were performed. However, results in the lab indicate that the situation may be even more complexed. We very recently examined the rapamycin response of our dual reporter assay in the HCT116 cellular background. No relative resistance was observed under any of the serum conditions tested and this correlated with little or no change in the intracellular MDM2 levels (Supplementary Figure S5). The reason for this discrepancy with previously published data is unclear. However,

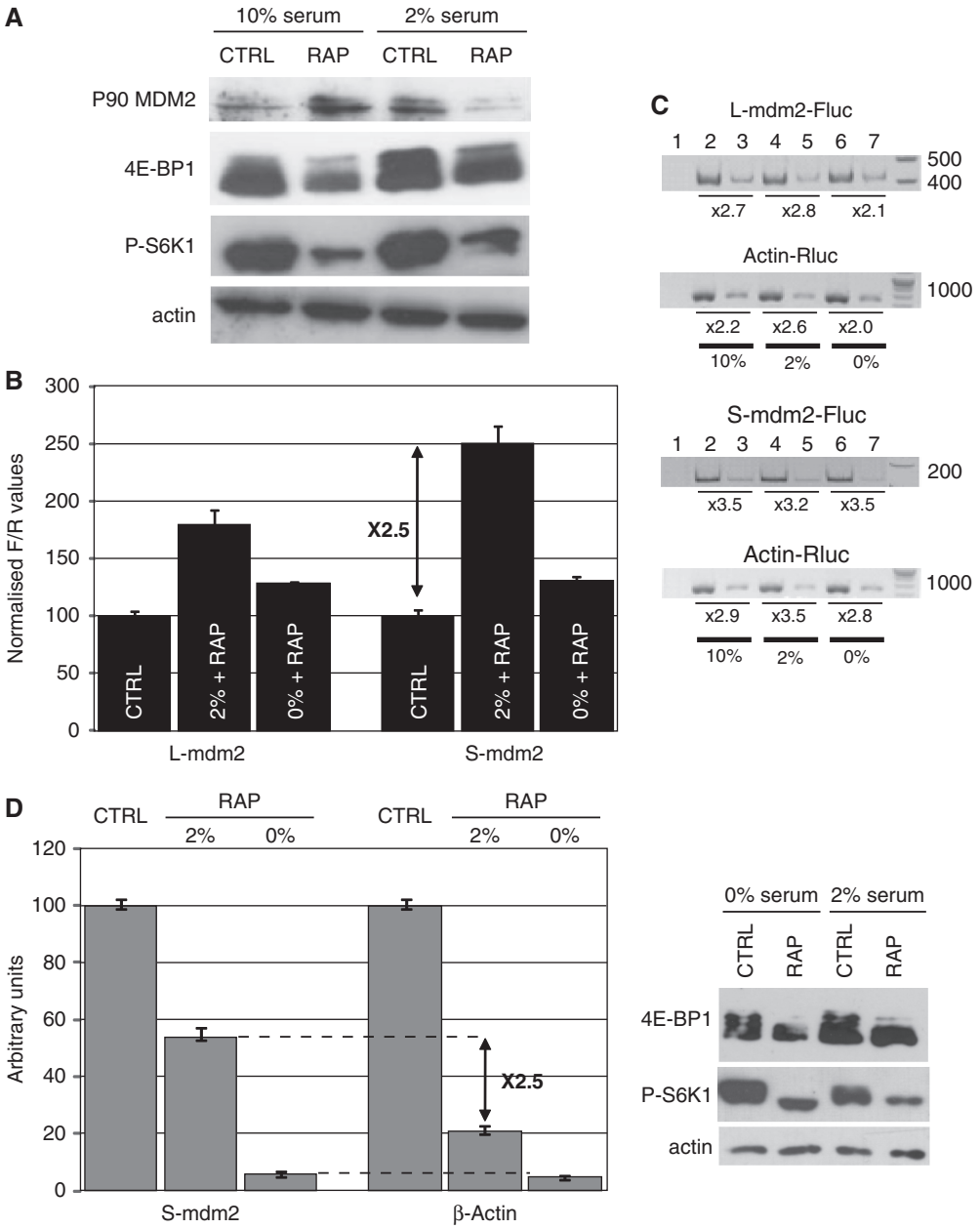


Figure 5. What modulates the mdm2 response? (**A**) HEK293T cells were treated with either DMSO or rapamycin (100 nM) for 24 h in the presence of either 2% or 10% fetal calf serum. Changes in the intracellular levels of the indicated proteins were determined by immunoblotting. (**B**) The FLuc/RLuc dual reporter assay was performed as outlined in Figure 3. In experiments performed in 2% serum, cells were grown at this serum concentration overnight prior to addition of DMSO/rapamycin (100 nM) and transfection. In the serum-free experiment, cells were grown overnight in 10% serum before starving in serum-free medium for 8 h before DMSO/rapamycin addition and transfection. In both cases the cells were harvested 24 h post-rapamycin addition. Reporter activities were normalized with the DMSO control in each experiment being set at 100. The vertical arrow in the S-mdm2 panel indicates the normalized 2.5-fold increase observed in the presence of 2% serum and rapamycin. (**C**) Semi-quantitative RT–PCR was performed on total RNA (500 ng) isolated from cells transfected with the reporter plasmid sets in the presence and absence of rapamycin (indicated as + and –) under the serum concentrations indicated at the bottom of each panel. Products were resolved on a 6% polyacrylamide gel (for the L-mdm2 FLuc transcript), an 8% polyacrylamide gel (for the S-mdm2 FLuc transcript) and a 1% agarose gel (for the β-actin RLuc transcript). The values indicated in brackets represent the change in transcript levels observed as a consequence of rapamycin exposure. In each gel, the lane 1 is the RT minus control. (**D**) The non-normalized values for the S-mdm2 Fluc and β-actin Rluc reporters [right-hand side of (B)] are depicted graphically with the 2% serum, rapamycin negative value being set at 100. The X2.5-fold value reflects the difference in the normalized reporter values observed in the right-hand side of (B). This difference effectively vanishes when the drug is applied in the absence of serum (indicated as the lower hatched line). Note that both rapamycin and the removal of serum are negative for expression of both reporters. The right-hand panel is an immunoblot using the antibodies indicated. The bars in all graphs indicate the SEM from triplicate transfections.

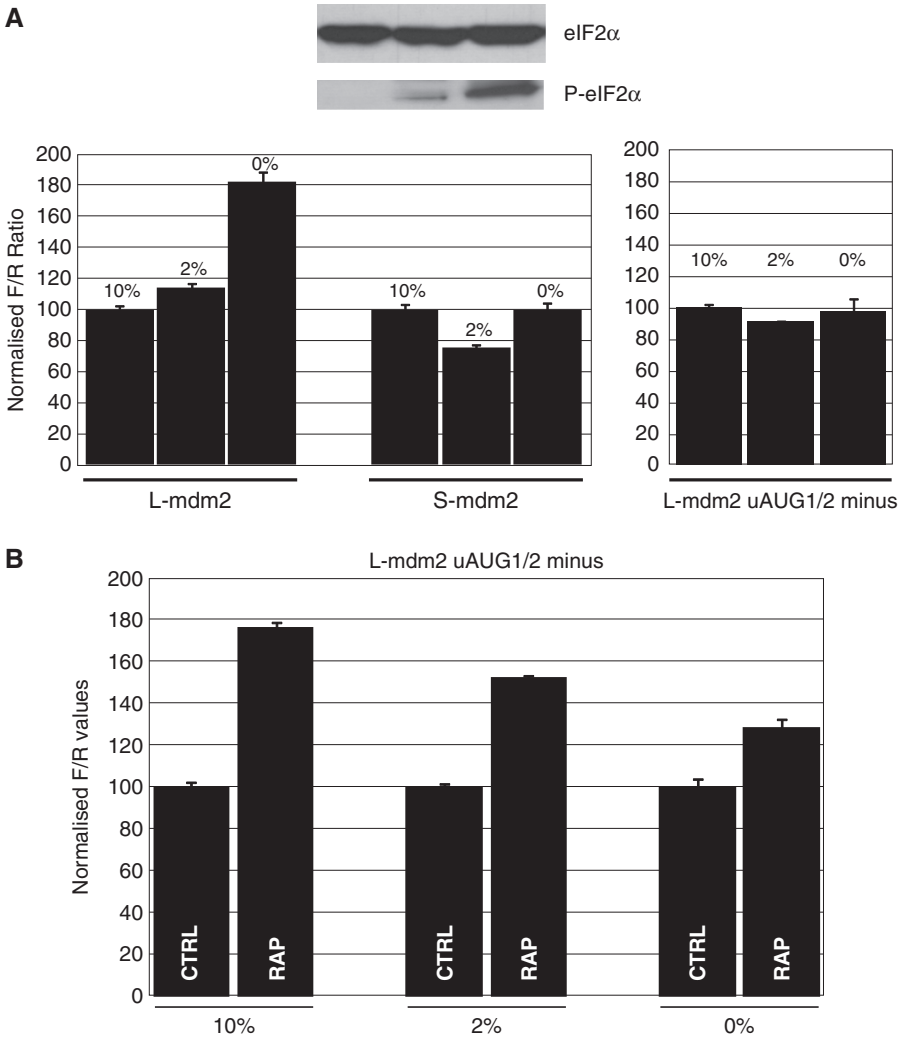
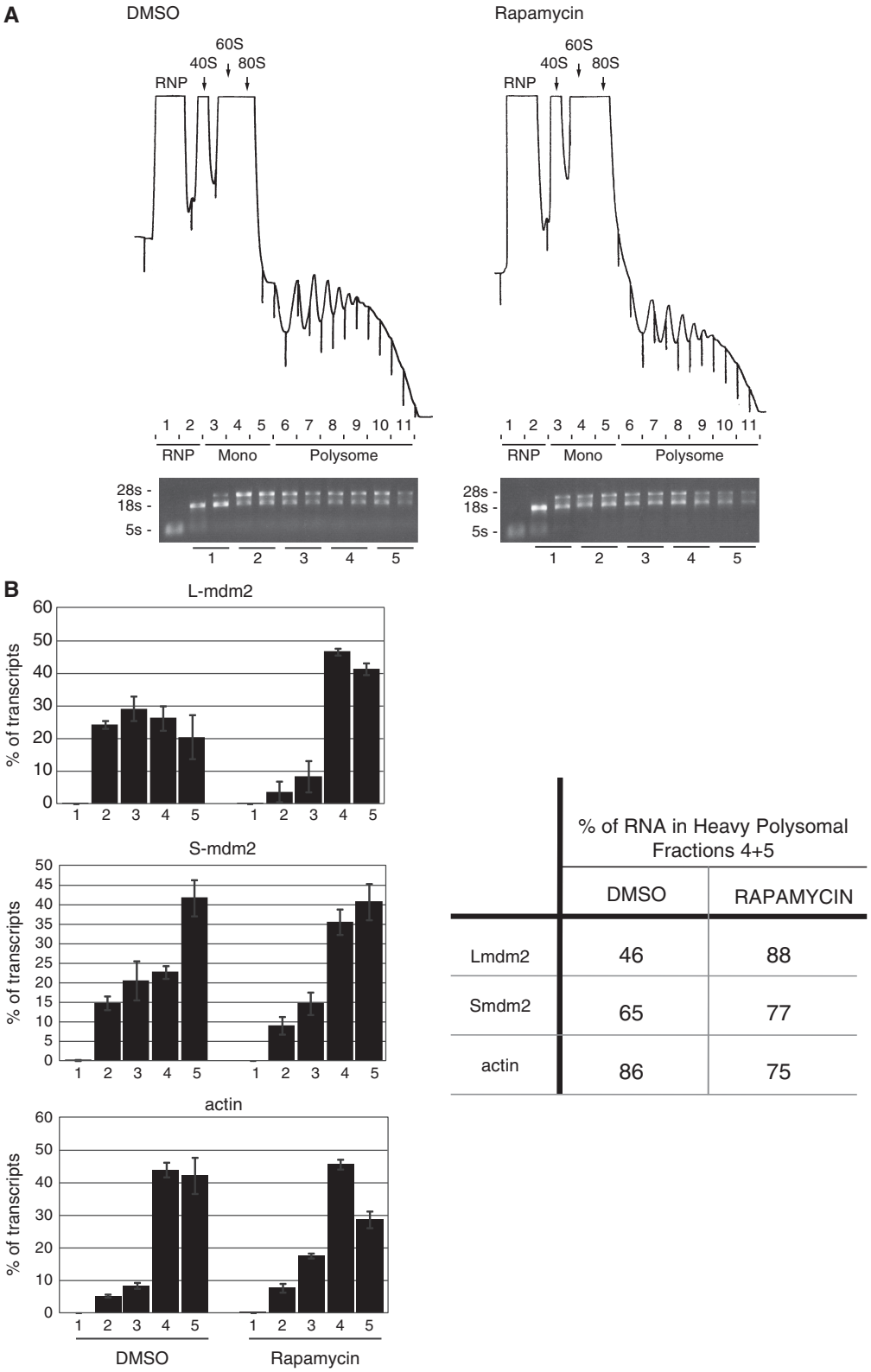


Figure 6. (**A**) HEK293T cells were transferred to DMEM containing the serum concentrations indicated at the top of each column 30 min prior to transfecting with vectors expressing either L-mdm2/Fluc, S-mdm2 Fluc or an L-mdm2/Fluc construct in which the two uAUGs were changed to GCG codons. As an internal control cells were co-transfected with the second Rluc reporter carrying the 5′-UTR of β-actin. Extracts were prepared 24 h later and the dual reporter activities measured. The FLuc/RLuc ratio obtained in the presence of 10% serum was then set at 100 and the values obtained at 2 and 0% serum were normalized relative to this. The upper panel is a series of immunoblots that follow eIF2α and phospho-eIF2α levels under the different serum concentrations employed in the assay. (**B**) The normalized reporter ratios were plotted for cells co-transfected with the β-actin RLuc and L-mdm2 uAUG1/2-ve FLuc reporter constructs in the absence (CTRL) and presence of rapamycin (100 nM) at the serum concentrations indicated at the bottom of each column set. Bars indicate the SEM from triplicate transfections.

despite rapamycin concentrations of 1 μM and in the total absence of serum we observed no effect on the downstream targets of mTORC1 (namely 4EBP1 and P-S6K1: Supplementary Figure S5C) or changes in the readout from the β-actin RLuc internal control (Supplementary Figure S5B), neither did we observe any signs of a stress response as evidenced by changes in the phosphorylation status of eIF2α; (Supplementary Figure S5C). This cell line is highly rapamycin resistant with an IC_{50}>12 μM (58), and it has been reported that drug treatment did not alter the phosphorylation status of the rpS6 protein (59), suggesting a relative resistance in the mTORC1. Furthermore, these cells express active mutant forms of K-RAS and PI3K (60), which may in part offer an explanation for the absence of a serum effect. The cell specificity of the rapamycin translational response is also apparent if one analyses the various translational profiling studies (performed in different cellular backgrounds) that have been reported (14,61,61–64). Despite overlaps in the various outputs, in particular the marked down-regulation of the highly rapamycin-sensitive TOP mRNAs (65), there was nonetheless considerable variation with some genes being up-regulated in one background and down-regulated in another (14).

Why would one observe an increase in MDM2 in response to rapamycin in certain cellular contexts? It has been reported that the MDM2 protein facilitates G1 to S-phase transition by activation of E2F-1 (66), a response that could serve to counteract the cell cycle block induced by rapamycin treatment. Likewise, evidence has accumulated linking P53 to the regulation of mTOR activity (67). In particular, it has been



	% of RNA in Heavy Polysomal Fractions 4+5	
	DMSO	RAPAMYCIN
Lmdm2	46	88
Smdm2	65	77
actin	86	75

Figure 7. Polysomal re-recruitment assays in the presence of rapamycin. **(A)** HEK293T cells were hypertonically shocked to induce polysomal disaggregation. Polysomes were then allowed to reform under normal growth conditions in the absence (DMSO) or the presence of rapamycin (100 nM). The gradients were fractionated and RNA isolated from each fraction. The positions of the RNP, monosomal and polysomal regions of each gradient are indicated. RNA from each fraction was analysed on a denaturing agarose gel (lower panels). To have sufficient material for the subsequent RT–PCR analysis gradient fractions containing rRNA (this excluded the RNP fractions) were pooled to generate five aliquots (indicated as 1 to 5 below the agarose gel). **(B)** Real time RT–PCR was performed starting with 1 μg of total RNA from each aliquot with primer sets specific for L-mdm2, S-mdm2 and β-actin. The results were then plotted as a percentage of the total in each of the five aliquots. The right-hand panel is a table that indicates the percentage of each transcript found in heavy polysomes (≥5 ribosomes). The bars in the graphs indicate the SEM from triplicate assays.

demonstrated that inactivation of P53 leads to mTOR activation via TSC1–TSC2 inhibition (68). Over-expression of MDM2 may therefore represent a specific cell type drug-induced response whose aim is to boost the mTOR pathway. The only caveat in this final scenario is our failure to observe clear changes in P53 levels during rapamycin treatment, despite increases in MDM2. However, a similar phenomenon has been noted by others (29).

Our results indicate that the relative rapamycin resistance associated with the mdm2 5′-UTRs is linked to the downstream targets of mTORC1, namely S6K1 and eIF4E, with the effect being more tightly coupled to the activity of the former at least with respect to the S-mdm2 5′-UTR. The effect of S6K1 on mRNA translation is largely mediated via its downstream effectors. One of these, eIF4B, plays an important role in the efficient recruitment of ribosomes to the mRNA (69). It assists eIF4A-mediated unwinding of RNA secondary structure and it has been suggested that its activity is increased by phosphorylation (27,70). Recently, footprinting assays have demonstrated that eIF4B is required for ribosome binding on mRNAs containing secondary structure (71), indicating that changes in eIF4B activity will alter the polysomal transcript populations. However, the length and %GC content of the β-actin and S-mdm2 5′-UTRs are similar, suggesting that the rapamycin response may not reside simply in changes in eIF4B activity. It may be that the S-mdm2 5′-UTR is less dependent on a downstream S6K1 effector(s) because it can recruit other *trans*-acting factors that substitute in PIC complex recruitment. If such a CTAF or cap-dependent translational enhancer (72) was to show cell specific expression patterns, it would also provide insights into the cell-specific responses that we observed with our dual reporter system. Likewise, the additive effect of the S6K1 T389A construct (Figure 3A) may just reflect an enhanced negative effect on global translation that serves to increase the pool of active PIC complexes that the CTAF-carrying S-mdm2 5′-UTR can recruit.

Our current work confirms and extends the earlier translational profiling studies with regard to the *mdm2* gene. Despite the fact that each of the alternative mdm2 5′-UTRs differ considerably in terms of primary sequence, they each confer a rapamycin resistance phenotype relative to the equivalent region in β-globin. One consequence of this is a change in the relative polysomal abundance of the mdm2 transcripts which foreshadows a change in the relative abundance of the protein in the cell. At least within the HEK293T cell background this response can be modulated by growth conditions, namely the serum concentrations employed during drug exposure. Clearly, a more detailed molecular dissection of the interplay between the serum signalling pathways, mTOR and mdm2 translation is now called for. With regard to the former, one interesting target is RSK1 as its activity is modulated positively by serum and it shares a common target with the S6K1, namely rpS6 (73). Furthermore, interplay between the RAS/ERK (serum) and PI3K/mTOR pathways have already been implicated in the selective recruitment of mRNAs to ribosomes (1). Intriguingly, these tended to code for proteins playing key roles in growth control and apoptosis, features that can be assigned to MDM2.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We would like to thank Dr Jean-Dominique Vassalli and Dr Beatrice Conne for use of the gradient apparatus and the input of Dr Tanguy Araud and Dr Bernadino Conrad.

FUNDING

The Ligue Genevoise Contre le Cancer; and the Swiss National Science Foundation (No. 310000-116170). Funding for open access charge: Swiss National Science Foundation and the Ligue Genevoise Contre le Cancer.

Conflict of interest statement. None declared.

REFERENCES

1. Rajasekhar,V.K., Viale,A., Socci,N.D., Wiedmann,M., Hu,X. and Holland,E.C. (2003) Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. *Mol. Cell*, **12**, 889–901.
2. Duncan,R., Milburn,S.C. and Hershey,J.W. (1987) Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. *J. Biol. Chem.*, **262**, 380–388.
3. Haghighat,A., Mader,S., Pause,A. and Sonenberg,N. (1995) Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.*, **14**, 5701–5709.
4. Lawrence,J.C. Jr and Abraham,R.T. (1997) PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem. Sci.*, **22**, 345–349.
5. Gingras,A.C., Raught,B. and Sonenberg,N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.*, **68**, 913–963.
6. Gingras,A.C., Gygi,S.P., Raught,B., Polakiewicz,R.D., Abraham,R.T., Hoekstra,M.F., Aebersold,R. and Sonenberg,N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.*, **13**, 1422–1437.
7. Gingras,A.C., Raught,B., Gygi,S.P., Niedzwiecka,A., Miron,M., Burley,S.K., Polakiewicz,R.D., Wyslouch-Cieszynska,A., Aebersold,R. and Sonenberg,N. (2001) Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.*, **15**, 2852–2864.
8. Gingras,A.C., Raught,B. and Sonenberg,N. (2001) Regulation of translation initiation by FRAP/mTOR. *Genes Dev.*, **15**, 807–826.
9. Fingar,D.C., Richardson,C.J., Tee,A.R., Cheatham,L., Tsou,C. and Blenis,J. (2004) mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. *Mol. Cell. Biol.*, **24**, 200–216.
10. Faivre,S., Kroemer,G. and Raymond,E. (2006) Current development of mTOR inhibitors as anticancer agents. *Nat. Rev. Drug Discov.*, **5**, 671–688.
11. Schmelzle,T. and Hall,M.N. (2000) TOR, a central controller of cell growth. *Cell*, **103**, 253–262.
12. Abraham,R.T. (2002) Identification of TOR signaling complexes: more TORC for the cell growth engine. *Cell*, **111**, 9–12.
13. Shamji,A.F., Nghiem,P. and Schreiber,S.L. (2003) Integration of growth factor and nutrient signaling: implications for cancer biology. *Mol. Cell*, **12**, 271–280.

14. Genolet,R., Araud,T., Maillard,L., Jaquier-Gubler,P. and Curran,J. (2008) An approach to analyse the specific impact of rapamycin on mRNA-ribosome association. *BMC. Med. Genomics*, **1**, 33.
15. Barak,Y., Gottlieb,E., Juven-Gershon,T. and Oren,M. (1994) Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.*, **8**, 1739–1749.
16. Araud,T., Genolet,R., Jaquier-Gubler,P. and Curran,J. (2007) Alternatively spliced isoforms of the human elk-1 mRNA within the 5′ UTR: implications for ELK-1 expression. *Nucleic Acids Res.*, **35**, 4649–4663.
17. Jordan,M., Schallhorn,A. and Wurm,F.M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.*, **24**, 596–601.
18. Van Eden,M.E., Byrd,M.P., Sherrill,K.W. and Lloyd,R.E. (2004) Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedures. *RNA*, **10**, 720–730.
19. Byrd,M.P., Zamora,M. and Lloyd,R.E. (2005) Translation of eukaryotic translation initiation factor 4GI (eIF4GI) proceeds from multiple mRNAs containing a novel cap-dependent internal ribosome entry site (IRES) that is active during poliovirus infection. *J. Biol. Chem.*, **280**, 18610–18622.
20. Sherrill,K.W., Byrd,M.P., Van Eden,M.E. and Lloyd,R.E. (2004) BCL-2 translation is mediated via internal ribosome entry during cell stress. *J. Biol. Chem.*, **279**, 29066–29074.
21. Bert,A.G., Grepin,R., Vadas,M.A. and Goodall,G.J. (2006) Assessing IRES activity in the HIF-1alpha and other cellular 5′ UTRs. *RNA*, **12**, 1074–1083.
22. Kozak,M. (2005) A second look at cellular mRNA sequences said to function as internal ribosome entry sites. *Nucleic Acids Res.*, **33**, 6593–6602.
23. Tee,A.R. and Proud,C.G. (2002) Caspase cleavage of initiation factor 4E-binding protein 1 yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. *Mol. Cell. Biol.*, **22**, 1674–1683.
24. Henis-Korenblit,S., Strumpf,N.L., Goldstaub,D. and Kimchi,A. (2000) A novel form of DAP5 protein accumulates in apoptotic cells as a result of caspase cleavage and internal ribosome entry site-mediated translation. *Mol. Cell. Biol.*, **20**, 496–506.
25. Andreev,D.E., Dmitriev,S.E., Terenin,I.M., Prassolov,V.S., Merrick,W.C. and Shatsky,I.N. (2009) Differential contribution of the m7G-cap to the 5′ end-dependent translation initiation of mammalian mRNAs. *Nucleic Acids Res.*, **37**, 6135–6147.
26. Choo,A.Y., Yoon,S.O., Kim,S.G., Roux,P.P. and Blenis,J. (2008) Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc. Natl Acad. Sci. USA*, **105**, 17414–17419.
27. Holz,M.K., Ballif,B.A., Gygi,S.P. and Blenis,J. (2005) mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell*, **123**, 569–580.
28. Schalm,S.S., Fingar,D.C., Sabatini,D.M. and Blenis,J. (2003) TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr. Biol.*, **13**, 797–806.
29. Kao,C.L., Hsu,H.S., Chen,H.W. and Cheng,T.H. (2009) Rapamycin increases the p53/MDM2 protein ratio and p53-dependent apoptosis by translational inhibition of mdm2 in cancer cells. *Cancer Lett.*, **286**, 250–259.
30. Stommel,J.M. and Wahl,G.M. (2004) Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation. *EMBO J.*, **23**, 1547–1556.
31. Fang,J., Meng,Q., Vogt,P.K., Zhang,R. and Jiang,B.H. (2006) A downstream kinase of the mammalian target of rapamycin, p70S6K1, regulates human double minute 2 protein phosphorylation and stability. *J. Cell. Physiol.*, **209**, 261–265.
32. Saha,A., Murakami,M., Kumar,P., Bajaj,B., Sims,K. and Robertson,E.S. (2009) Epstein–Barr virus nuclear antigen 3C augments mdm2-mediated p53 ubiquitination and degradation by deubiquitinating mdm2. *J. Virol.*, **83**, 4652–4669.
33. Trotta,R., Vignudelli,T., Candini,O., Intine,R.V., Pecorari,L., Guerzoni,C., Santilli,G., Byrom,M.W., Goldoni,S., Ford,L.P. *et al.* (2003) BCR/ABL activates mdm2 mRNA translation via the La antigen. *Cancer Cell*, **3**, 145–160.
34. Brenet,F., Socci,N.D., Sonenberg,N. and Holland,E.C. (2008) Akt phosphorylation of La regulates specific mRNA translation in glial progenitors. *Oncogene*, **28**, 128–139.
35. Craig,A.W., Svitkin,Y.V., Lee,H.S., Belsham,G.J. and Sonenberg,N. (1997) The La autoantigen contains a dimerization domain that is essential for enhancing translation. *Mol. Cell. Biol.*, **17**, 163–169.
36. Holcik,M. and Korneluk,R.G. (2000) Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry site element: role of La autoantigen in XIAP translation. *Mol. Cell. Biol.*, **20**, 4648–4657.
37. Takebe,Y., Seiki,M., Fujisawa,J., Hoy,P., Yokota,K., Arai,K., Yoshida,M. and Arai,N. (1988) SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.*, **8**, 466–472.
38. Kao,C.L., Hsu,H.S., Chen,H.W. and Cheng,T.H. (2010) Rapamycin increases the p53/MDM2 protein ratio and p53-dependent apoptosis by translational inhibition of mdm2 in cancer cells. *Cancer Lett.*, **286**, 250–259.
39. Mendrysa,S.M., McElwee,M.K. and Perry,M.E. (2001) Characterization of the 5′ and 3′ untranslated regions in murine mdm2 mRNAs. *Gene*, **264**, 139–146.
40. Phillips,A. and Blaydes,J.P. (2007) MNK1 and EIF4E are downstream effectors of MEKs in the regulation of the nuclear export of HDM2 mRNA. *Oncogene*, **27**, 1645–1649.
41. Figueroa,C. and Vojtek,A.B. (2003) Akt negatively regulates translation of the ternary complex factor Elk-1. *Oncogene*, **22**, 5554–5561.
42. Candeias,M.M., Malbert-Colas,L., Powell,D.J., Daskalogianni,C., Maslon,M.M., Naski,N., Bourougaa,K., Calvo,F. and Fahraeus,R. (2008) p53 mRNA controls p53 activity by managing Mdm2 functions. *Nat. Cell. Biol.*, **10**, 1098–1105.
43. Scheuner,D., Patel,R., Wang,F., Lee,K., Kumar,K., Wu,J., Nilsson,A., Karin,M. and Kaufman,R.J. (2006) Double-stranded RNA-dependent protein kinase phosphorylation of the {alpha}-subunit of eukaryotic translation initiation factor 2 mediates apoptosis. *J. Biol. Chem.*, **281**, 21458–21468.
44. Harding,H.P., Novoa,I., Zhang,Y., Zeng,H., Wek,R., Schapira,M. and Ron,D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell*, **6**, 1099–1108.
45. Zauberman,A., Flusberg,D., Haupt,Y., Barak,Y. and Oren,M. (1995) A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res.*, **23**, 2584–2592.
46. Landers,J.E., Cassel,S.L. and George,D.L. (1997) Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. *Cancer Res.*, **57**, 3562–3568.
47. Capoulade,C., Bressac-de,P.B., Lefrere,I., Ronsin,M., Feunteun,J., Tursz,T. and Wiels,J. (1998) Overexpression of MDM2, due to enhanced translation, results in inactivation of wild-type p53 in Burkitt's lymphoma cells. *Oncogene*, **16**, 1603–1610.
48. Brown,C.Y., Mize,G.J., Pineda,M., George,D.L. and Morris,D.R. (1999) Role of two upstream open reading frames in the translational control of oncogene mdm2. *Oncogene*, **18**, 5631–5637.
49. Stoneley,M. and Willis,A.E. (2004) Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene*, **23**, 3200–3207.
50. Yang,D.Q., Halaby,M.J. and Zhang,Y. (2006) The identification of an internal ribosomal entry site in the 5′-untranslated region of p53 mRNA provides a novel mechanism for the regulation of its translation following DNA damage. *Oncogene*, **25**, 4613–4619.
51. Ray,P.S., Grover,R. and Das,S. (2006) Two internal ribosome entry sites mediate the translation of p53 isoforms. *EMBO Rep.*, **7**, 404–410.
52. Halaby,M.J. and Yang,D.Q. (2007) p53 translational control: a new facet of p53 regulation and its implication for tumorigenesis and cancer therapeutics. *Gene*, **395**, 1–7.

53. Pilipenko,E.V., Pestova,T.V., Kolupaeva,V.G., Khitrina,E.V., Poperechnaya,A.N., Agol,V.I. and Hellen,C.U. (2000) A cell cycle-dependent protein serves as a template-specific translation initiation factor. *Genes Dev.*, **14**, 2028–2045.
54. Domitrovich,A.M., Diebel,K.W., Ali,N., Sarker,S. and Siddiqui,A. (2005) Role of La autoantigen and polypyrimidine tract-binding protein in HCV replication. *Virology*, **335**, 72–86.
55. Cheng,T.H. and Cohen,S.N. (2007) Human MDM2 isoforms translated differentially on constitutive versus p53-regulated transcripts have distinct functions in the p53/MDM2 and TSG101/MDM2 feedback control loops. *Mol. Cell. Biol.*, **27**, 111–119.
56. Ravikumar,B., Berger,Z., Vacher,C., O'Kane,C.J. and Rubinsztein,D.C. (2006) Rapamycin pre-treatment protects against apoptosis. *Hum. Mol. Genet.*, **15**, 1209–1216.
57. Noh,W.C., Mondesire,W.H., Peng,J., Jian,W., Zhang,H., Dong,J., Mills,G.B., Hung,M.C. and Meric-Bernstam,F. (2004) Determinants of rapamycin sensitivity in breast cancer cells. *Clin. Cancer Res.*, **10**, 1013–1023.
58. Huang,S., Bjornsti,M.A. and Houghton,P.J. (2003) Rapamycins: mechanism of action and cellular resistance. *Cancer Biol. Ther.*, **2**, 222–232.
59. Pencreach,E., Guérin,E., Nicolet,C., Lelong-Rebel,I., Voegeli,A.C., Oudet,P., Larsen,A.K., Gaub,M.P. and Guenot,D. (2009) Marked activity of irinotecan and rapamycin combination toward colon cancer cells in vivo and in vitro is mediated through cooperative modulation of the mammalian target of rapamycin/ hypoxia-inducible factor-1+¦ axis. *Clin. Cancer Res.*, **15**, 1297–1307.
60. Buck,E., Eyzaguirre,A., Brown,E., Petti,F., McCormack,S., Haley,J.D., Iwata,K.K., Gibson,N.W. and Griffin,G. (2006) Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in nonGÇôsmall-cell lung, pancreatic, colon, and breast tumors. *Mol. Cancer Therap.*, **5**, 2676–2684.
61. Gera,J.F., Mellinghoff,I.K., Shi,Y., Rettig,M.B., Tran,C., Hsu,J.h., Sawyers,C.L. and Lichtenstein,A.K. (2004) AKT Activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression. *J. Biol. Chem.*, **279**, 2737–2746.
62. Grolleau,A., Bowman,J., Pradet-Balade,B., Puravs,E., Hanash,S., Garcia-Sanz,J.A. and Beretta,L. (2002) Global and specific translational control by tapamycin in T cells uncovered by microarrays and proteomics. *J. Biol. Chem.*, **277**, 22175–22184.
63. Mamane,Y., Petroulakis,E., Martineau,Y., Sato,T.A., Larsson,O., Rajasekhar,V.K. and Sonenberg,N. (2007) Epigenetic activation of a subset of mRNAs by eIF4E explains its effects on cell proliferation. *PLoS ONE.*, **2**, e242.
64. Bilanges,B., rgonza-Barrett,R., Kolesnichenko,M., Skinner,C., Nair,M., Chen,M. and Stokoe,D. (2007) Tuberous sclerosis complex proteins 1 and 2 control serum-dependent translation in a TOP-dependent and -independent manner. *Mol. Cell. Biol.*, **27**, 57465764.
65. Meyuhas,O. and Hornstein,E. (2000) Translational control of TOP mRNAs. In Mathews,M.B. (ed.), *Translational Control of Gene Expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 671–694.
66. Teoh,G., Urashima,M., Ogata,A., Chauhan,D., DeCaprio,J.A., Treon,S.P., Schlossman,R.L. and Anderson,K.C. (1997) MDM2 protein overexpression promotes proliferation and survival of multiple myeloma cells. *Blood*, **90**, 1982–1992.
67. Jiang,B.H. and Liu,L.Z. (2008) Role of mTOR in anticancer drug resistance: perspectives for improved drug treatment. *Drug Resist. Updat.*, **11**, 63–76.
68. Feng,Z., Zhang,H., Levine,A.J. and Jin,S. (2005) The coordinate regulation of the p53 and mTOR pathways in cells. *Proc. Natl Acad. Sci. USA*, **102**, 8204–8209.
69. Rogers,G.W. Jr, Richter,N.J., Lima,W.F. and Merrick,W.C. (2001) Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. *J. Biol. Chem.*, **276**, 30914–30922.
70. Manzella,J.M., Rychlik,W., Rhoads,R.E., Hershey,J.W. and Blackshear,P.J. (1991) Insulin induction of ornithine decarboxylase. Importance of mRNA secondary structure and phosphorylation of eucaryotic initiation factors eIF-4B and eIF-4E. *J. Biol. Chem.*, **266**, 2383–2389.
71. Dmitriev,S.E., Terenin,I.M., Dunaevsky,Y.E., Merrick,W.C. and Shatsky,I.N. (2003) Assembly of 48S translation initiation complexes from purified components with mRNAs that have some base pairing within their 5′ untranslated regions. *Mol. Cell. Biol.*, **23**, 8925–8933.
72. Gilbert,W.V. (2010) Alternative ways to think about cellular internal ribosome entry. *J. Biol. Chem.*, **285**, 29033–29038.
73. Roux,P.P., Shahbazian,D., Vu,H., Holz,M.K., Cohen,M.S., Taunton,J., Sonenberg,N. and Blenis,J. (2007) RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J. Biol. Chem.*, **282**, 14056–14064.