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Role of the highly structured 5'-end region of *MDR1* mRNA on P-glycoprotein expression

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Abbreviations: EBV, Epstein Barr virus; eIF, eukaryotic initiation factor; MAPK, mitogen-activated protein kinase; Mnk, MAP kinase-interacting kinase; mTOR, mammalian target of rapamycin kinase; PI3-K, phosphatidylinositol 3-kinase; RT-Q-PCR, reverse transcription followed by quantitative PCR; siRNA, small interfering RNA; TPA, phorbol ester; uORF, upstream open reading frame; UTR, untranslated region; 4E-BP, eIF4E-binding protein.

ABSTRACT

Overexpression of P-glycoprotein, encoded by the *MDR1* gene, is often responsible for multidrug resistance in acute myeloid leukemia. We have shown previously that *MDR1* (P-glycoprotein) mRNA levels in K562 leukemic cells exposed to cytotoxic drugs are up-regulated but P-glycoprotein expression is translationally blocked. Here we show that cytotoxic drugs down-regulate the Akt signaling pathway leading to hypophosphorylation of the translational repressor 4E-BP and decreased eIF4E availability. The 5'-end of *MDR1* mRNA adopts a highly-structured fold. Fusion of this structured 5'-region upstream of a reporter gene impeded its efficient translation, specifically under cytotoxic stress, by reducing its competitive ability for the translational machinery. The effect of cytotoxic stress could be mimicked *in vivo* blocking the phosphorylation of 4E-BP by mTOR using rapamycin or eIF4E siRNA, and relieved by over-expression of either eIF4E or constitutively-active Akt. Upon drug exposure *MDR1* mRNA was up-regulated, apparently stochastically, in a small proportion of cells. Only in these cells could *MDR1* mRNA compete successfully for the reduced amounts of eIF4E and translate P-glycoprotein. Consequent drug efflux and restoration of eIF4E availability results in a feed-forward relief from stress-induced translational repression and to the acquisition of drug resistance.

INTRODUCTION

Multidrug resistance, the phenomenon by which cancer cells treated with a single chemotherapeutic agent become cross-resistant to multiple functionally and structurally unrelated compounds, is most frequently associated with up-regulation of P-glycoprotein. This membrane protein, coded by the *MDR1* (*ABCB1*) gene, is an ATP-dependent efflux pump that maintains intracellular drug concentrations below cytotoxic levels [1]

We have used a K562 myelogenous leukaemia cell model to determine the mechanisms by which *MDR1*/P-glycoprotein-mediated multidrug resistance is acquired [2], with the aim of developing therapeutic strategies that will target up-regulation of P-glycoprotein expression. The K562 cell line is a useful model system as drug-resistant sub-lines can readily be derived by selection with clinically-relevant doses of chemotherapeutic agents [2]. Short-term (1-3 days) exposure of naïve K562 cells to a range of cytotoxic agents results in up-regulation of *MDR1* mRNA steady-state levels by stabilization of the otherwise short-lived *MDR1* mRNA. However, this stabilized mRNA is not associated with translating polyribosomes and P-glycoprotein is not synthesised. Selection for drug resistance, by long-term exposure to drug, leads to resistant cells in which this translational block is overcome. Translational repression of *MDR1* mRNA has also been described in EBV-transformed human B lymphocytes and in leukaemic CEM cells [2, 3], and the well-documented lack of correlation between *MDR1* mRNA and P-glycoprotein levels in leukaemia [4] implies that translational regulation represents a general regulatory step in P-glycoprotein expression.

The rate-limiting step in translation is initiation and is the most common target for translational control. For the majority of eukaryotic mRNAs, initiation of protein synthesis occurs via a cap-dependent mechanism [5]. The initial binding of the translation apparatus at the 5'-end of an mRNA begins with the formation of a ternary complex (eIF2, GTP and a tRNA initiator) which is recruited by the small ribosomal subunit (40S) together with its associated initiation factors to form the 43S pre-initiation complex which recognizes the 5'-cap of an mRNA. This requires the eIF4F protein complex which comprises eIF4E (which recognises and binds to the 5'-cap structure), eIF4A, and eIF4G. Since the levels of eIF4E are normally low, assembly of the eIF4F complex and its interaction with the 5'-mRNA cap provides the rate-limiting step in translation initiation. Once bound to the mRNA cap, the 43S

pre-initiation complex migrates along the 5'-untranslated region (UTR) in an ATP-dependent process known as scanning, until it reaches the translation initiation codon (AUG) [5,6]. For a proportion of cellular mRNAs, cap-independent translation involves internal ribosomal entry sites [7].

Several features of an mRNA molecule can influence its translation [5]. Loop structures involving the 5'-UTR of mRNAs with free energy values of less than between -30 to -50 Kcal/mol, generally inhibit translation [8]. *In vitro* RNase mapping has shown that the 5'-terminal region of *MDR1* mRNA (nucleotides 1-289) is highly structured [9]. This 289-nucleotide region includes the 140-nucleotide 5'-UTR which folds with the following 149 nucleotides of the *MDR1* coding sequence to form a stable structure with the initiation codon located in a hexanucleotide loop at its centre (Figure 1A). Here we show that the highly structured region at the 5'-end of *MDR1* mRNA affects translation of reporter transcripts and that this down-regulation is more pronounced under cytotoxic stress, when the Akt pathway is down-regulated and available levels of eIF4E low. Drug resistance is acquired by up-regulation, by an apparently stochastic mechanism, of *MDR1* mRNA in a small subpopulation of cells. This allows effective competition for eIF4E, translation of P-glycoprotein, and consequent removal of cytotoxic drugs from the cell restoring eIF4E availability. This positive feed-forward cycle leads to the development and maintenance of multidrug resistance.

EXPERIMENTAL

Cells

Human myelogenous leukaemic K562 cells and its colchicine-resistant derivative KC40, have been described before [2]. KC40 cells were routinely maintained in the presence of 20 ng/ml colchicine. Cells were transfected using an Amaxa Nucleofector, following the manufacturers' recommendations. For transient transfections, 4.5 µg of the test plasmid were cotransfected with 0.5 µg pHRTK (expressing *Renilla* luciferase to normalize for transfection efficiency) (Promega). For stable transfections, plasmids (2 µg) were linearised with *PvuI*, cotransfected as above with 0.1 µg pGK-puro, and transfectants selected with 2 µg/ml puromycin.

Generation of drug resistant K562 cells

Cells were seeded at 5×10^5 cells/ml in 50 ml culture medium containing 20 ng/ml colchicine. At days 3, 10 and 14, 50 ml of fresh culture medium without drug were added. At day 18 cells were spun down and resuspended in 160 ml culture medium with 20 ng/ml colchicine. From day 25-40 the colchicine-containing medium was changed routinely twice per week.

Plasmids

The constitutively-active Akt expression plasmid (pCAAKT) was from Upstate. The constitutively-active Mnk-1 expression plasmid (pCAMNK) [10], and the *MDR1* downstream promoter-luciferase reporter plasmid pMDR1(-1202) [11] have been described. The 289-nucleotide *MDR1* 5'- structure (consisting of the 140-nucleotide 5'-UTR and 149 nucleotides of proximal coding region) was amplified from reverse-transcribed KC40 mRNA using primers OLEY24bis and OLEY26 (all oligonucleotide sequences are listed in Supplementary Table 1). The 140-nucleotide 5'-UTR was amplified with primers OLEY24bis and OLEY25. PCR products were inserted into pGL3Control (Promega) upstream of the luciferase gene, using the *NcoI* and *EcoRI* sites, generating pGL-Loop and pGL-UTR, respectively. The luciferase translation initiation codon within pGL-UTR was then mutated to that found in the native *MDR1* using primers OLEY176 and OLEY177, and the Quikchange Site Directed mutagenesis kit (Stratagene), according to manufacturer's instructions. The translation start codon and two other out of frame-AUGs in the *MDR1* coding region,

but upstream from the luciferase gene in pGL-Loop, were sequentially mutated by site-directed mutagenesis as above with oligonucleotides OLEY27 and OLEY28, OLEY41 and OLEY42, and OLEY43 and OLEY44, respectively, to generate pGL-LoopM. Thus, pGL-UTR, pGL-Loop and pGL-LoopM had the same 5'-UTR length and sequence surrounding the translation initiation codon. T7 promoter sequences, produced by annealing the complementary oligonucleotides OLEY203 and OLEY204, were inserted into *EcoRI* –digested pGL-UTR and pGL-Loop to generate T7pGL-UTR and T7pGL-Loop, respectively. eIF4E cDNA was obtained from reverse transcribed K562 mRNA by PCR using high fidelity Expand (Roche Diagnostics) and primers OLEY184 and OLEY185. The resulting 1391-base pairs DNA fragment was inserted into the pEF6/V5-His-TOPO plasmid vector (Invitrogen) to generate peIF4E.

Genomic DNA and Southern blotting

Genomic DNA was digested with *MfeI* (for luciferase) or *EcoRI* (for β -globin), blotted and hybridized to radioactive probes, derived from pGL3control (luciferase coding sequence) or the β -globin locus as described [2].

Luciferase expression

A Dual-Luciferase Reporter Assay was used to measure both firefly and *Renilla* luciferase in lysates of transiently transfected cells 16 h after transfection (Promega). Protein content was measured using a *DC* protein assay (Bio-Rad).

***De novo* protein synthesis**

Incorporation of [35 S]Met into nascent proteins was determined by trichloroacetic acid precipitable counts as previously described [2].

RNA analysis

Luciferase mRNA analysis was performed by semi quantitative RT-PCR. Briefly, after reverse transcription [2], cDNA was amplified by PCR with OLEY218 and OLEY219 for luciferase, and OLEY8 and OLEY70 for *GAPDH* normalization [12] for 20 to 29 cycles. Detection of amplified cDNA was by Southern blotting and hybridization to radioactively end-labelled internal oligonucleotides, essentially as

described [2]. Quantification was with a PhosphorImager (Molecular Dynamics). *MDR1* mRNA analysis was by reverse transcription and real-time quantitative PCR (RT-Q-PCR), as described [13]. mRNA half-life was determined as described [2] except that *Id2* mRNA turnover was determined by RT-Q-PCR with primers Id2F and Id2R (PrimerBank ID: 31982933a1) [14] and normalized to 18S ribosomal RNA (18Sf and 18Sr primers).

m⁷GTP pull-down

eIF4E-containing protein complexes were isolated by binding to m⁷GTP Sepharose as described [15].

***In vitro* transcription, *in vitro* translation and RNA competition**

T7pGL-UTR and T7pGL-Loop plasmids were linearized downstream from the luciferase stop codon and *in vitro* transcribed using a mMessage mMachine T7 Ultra kit (Ambion). A cap analogue and a poly(A) tail were added to the 5'- and 3'-ends, respectively, following the manufacturer's recommendations. *In vitro* translation was carried out with 0.4 µg of *in vitro*-transcribed mRNA and 0-20 µg competitor RNA (isolated either from K562 cells or from yeast) in a rabbit reticulocyte cell-free system (Promega), and luciferase activity measured after 90 min at 30°C following manufacturer's recommendations. Where indicated, *in vitro* transcription and translation was also carried out using a TNT Quick Coupled Transcription and Translation Rabbit Reticulocyte System (Promega). All experiments were performed in triplicate on at least three independent occasions.

RNA interference

siRNA (2.7 µg) targeted to eIF4E (Cell Signalling Technology), or non-specific AllStars negative control (Qiagen), were transfected into cells using Amaxa Nucleofector Kit V. Decrease in cellular eIF4E protein levels were assayed by western blotting 24 h after transfection.

Antibodies and flow cytometry

All antibodies against translation factors were from Cell Signalling. Detection of plasma membrane P-glycoprotein with antibody UIC2, BODIPY-taxol efflux assays,

and western blotting were performed as previously described [2, 16]. Cell viability was determined by TOTO-3 exclusion.

RESULTS

The 5'-MDR1 mRNA structure down-regulates translation of a reporter gene

The structure of the 5'-end of the *MDR1* mRNA, nucleotides 1-289, [9] (Fig 1A), has a calculated free energy value of less than -100 Kcal/mol [17]. We hypothesized that if formed *in vivo*, such a structure would down-regulate translation from a linked reporter. The 289-base pairs region and the 140-base pairs region corresponding to the 5'-UTR were cloned upstream from the luciferase reporter gene in pGL3control, generating pGL-Loop and pGL-UTR, respectively (Fig 1B). The luciferase activity of pGL-UTR was approximately 2.5 fold above pGL3control in transiently transfected K562 cells (Figure 1C). This may be due to the longer 5'-UTR sequence in pGL-UTR transcripts (138 nucleotides compared to 35 nucleotides in pGL3control mRNAs), resulting in a greater translation efficiency of the luciferase mRNA [18]. The presence of the 289-bp structured region (pGL-Loop) resulted in a significant reduction in luciferase activity (5- to 7-fold) relative to pGL-UTR (Figure 1C) indicating that this region of *MDR1* mRNA is inhibitory to reporter gene expression. Changes in luciferase activity were due to changes at the translational level and not at the levels of transcription or mRNA stability since the steady state levels of luciferase mRNA, as detected by semi-quantitative RT-PCR, were comparable (Figure 1D). Equally, the small fusion protein formed between the first amino acids of P-glycoprotein and firefly luciferase in pGL-Loop did not affect luciferase enzymatic activity (data not shown). Following site-directed mutagenesis to eliminate AUGs in the loop structure (pGL-LoopM), the predicted fold and free energy was unchanged (data not shown). Therefore, we reasoned that if the loop at the 5'-end of *MDR1* mRNA were formed *in vivo*, luciferase translation from pGL-LoopM, when compared to pGL-Loop, would be greatly reduced. Transfection of pGL-LoopM into K562 cells showed that luciferase activity was completely absent in pGL-LoopM-expressing cells (Figure 1C). This suggests that the 43S pre-initiation complex cannot scan or shunt through the loop region present in pGL-LoopM, preventing translation of the downstream luciferase reporter. In contrast, in pGL-Loop-expressing cells, the initiation codon in the hexanucleotide loop at the centre of the structure improves the translatability of these transcripts, presumably because the 43S pre-initiation complex has to scan or shunt through a shorter region before reaching the starting AUG. Thus, the 5'-end of *MDR1* mRNA is highly structured *in vivo* and down-regulates translation of a downstream reporter gene.

The loop at the 5'-end of MDR1 mRNA specifically down-regulates translation of a luciferase reporter gene upon treatment with cytotoxic drugs

To test the effects of cytotoxic stress on the translation of transcripts with the 5'-*MDR1* structure, we generated stable K562 cell lines with similar numbers of copies of pGL3control, pGL-UTR or pGL-Loop, named K-pGL3, K-pGL-UTR and K-pGL-Loop cells, respectively (Figure 2A). Treatment of each of these cell lines with 1 μ M colcemid for 3 days resulted in a similar decrease in the incorporation of 35 S[Met] into nascent proteins (Figure 2B). Luciferase activity of K-pGL3 and K-pGL-UTR cells decreased by 25%. However, in K-pGL-Loop cells, which include the entire 5'-*MDR1* structure upstream of the luciferase reporter, luciferase activity decreased by 75% (Figure 2B). This effect was due to a decrease in translation, and not transcription of the transgenes, since the steady-state levels of the corresponding mRNAs did not change significantly upon drug treatment (Figure 2C). Cytotoxic stress results in a global reduction in translation [19]. While the observed decrease in luciferase activity in K-pGL3 and K-pGL-UTR cells following colcemid treatment probably reflects this generalized effect, the greater decrease observed in K-pGL-Loop cells indicates a specific effect of the *MDR1* 5'-mRNA structure on translation of the luciferase reporter under cytotoxic stress.

Down-regulation of signaling pathways due to cytotoxic stress

Since control of protein synthesis is generally achieved by changes in the phosphorylation state of translation initiation factors, or the regulators that interact with them, we examined several signaling pathways known to affect translation following cytotoxic stress. A number of kinases, activated by various types of stresses, can phosphorylate eIF2 α at Ser51 and down-regulate global translation [20]. Following exposure of K562 cells to 1 μ M colcemid for 3 days, an increase in the phosphorylation of eIF2 α was observed (Figure 3A left panel), which is probably responsible for the down-regulation of 35 S[Met] incorporation into nascent proteins reported by us previously [2] and shown in Figure 2B.

Extracellular stimuli also modulate the activities of both ERK1 and p38 MAP kinase (MAPK), which activate MAP kinase-interacting kinase 1 (Mnk-1) which phosphorylates eIF-4E at Ser209 [21, 22]. Colcemid treatment of K562 cells resulted

in down-regulation of phosphorylated Mnk and p38 MAPK, but not of Erk, leading to hypophosphorylation of eIF4E (Figure 3A middle panel).

Activation of the phosphatidylinositol 3-kinase (PI3-K)-Akt signalling pathway and phosphorylation by mTOR (mammalian target of rapamycin kinase) reduces the affinity of eIF4E-binding proteins (4E-BPs, a family of translational repressors that bind and sequester eIF4E) for eIF4E, allowing binding to eIF4G and translation initiation. Many cellular stresses also inhibit 4E-BP phosphorylation leading to increased binding of eIF4E, reduced availability of eIF4E and ultimately a decrease in the levels of translation [21, 23, 24]. Exposure of K562 cells to colcemid led to down-regulation of the PI3-K / Akt / mTOR signalling pathway and hypophosphorylation of 4E-BP (Figure 3A right panel). The effect on the phosphorylation status of eIF4E and 4E-BP was independent of the drug used, since doxorubicin and cytarabine produced similar down-regulation in phosphorylation (Figure 3B).

To confirm that the drug-induced hypophosphorylation of 4E-BP affected the formation of translation initiation complexes we pulled-down eIF4E and associated proteins from colcemid-treated K562 cell extracts using a 5'-end mRNA cap analogue (m⁷GTP-Sepharose). Bound proteins were analyzed by western blotting. Treatment with colcemid resulted in a time-dependent increase in the binding of 4E-BP to eIF4E, and a reduction in the binding of eIF4G (Figure 3C). Thus, cytotoxic stress in K562 cells leads to decrease in the ability of eIF4E to associate with eIF4G resulting in down-regulation of translation.

Down-regulation of available eIF4E following cytotoxic stress decreases the translation efficiency of transcripts with the *MDR1* mRNA 5'-structure

To ask how the changes in 4E-BP phosphorylation and eIF4E availability affect the translation of luciferase reporter genes in K-pGL-UTR and K-pGL-Loop cells following cytotoxic drug exposure, we transfected these lines transiently with plasmids expressing constitutively-active forms of Mnk1 (pCAMNK) and Akt (pCAAKT), singly or together, and measured luciferase activity following treatment of the cells with 1 μ M colcemid for 3 days. Expression of constitutively-active Mnk1 increased the phosphorylation of eIF4E after drug exposure in both cell lines, as expected (Figure 4A). Expression of constitutively-active Akt also increased 4E-BP

phosphorylation after drug exposure, but to a lesser extent. This is probably because, unlike Mnk1 which phosphorylates eIF4E directly, Akt phosphorylates and activates mTOR which then in turn phosphorylates 4E-BP (Figure 4B) [22]. There was no significant difference in the steady state-levels of the reporter mRNAs in each transfected cell line upon drug exposure (data not shown). Since expression of constitutively-active Mnk1 did not increase expression of luciferase from pGL-Loop under cytotoxic stress (Figure 4A, C, D) we can infer that, at least under these conditions, the phosphorylation status of eIF4E does not contribute to the down-regulation of translation of pGL-Loop transcripts. In contrast, translation of pGL-Loop transcripts following cytotoxic stress was enhanced significantly in cells expressing constitutively-active Akt (Figure 4D).

We confirmed the importance of Akt by down-regulating mTOR with rapamycin in K-pGL-UTR and K-pGL-Loop cells. Concentrations of rapamycin used in these assays were not toxic to K562 cells, as determined by proliferation analyses (data not shown). Western blot analysis demonstrated that treatment with rapamycin decreased the level of phosphorylated 4E-BP in cells, as expected (Figure 4E). Similarly, steady-state levels of luciferase reporter mRNAs (data not shown) and the translation of pGL-UTR transcripts (Figure 4F) did not change significantly. Nonetheless, rapamycin strongly repressed translation of pGL-Loop transcripts (Figure 4F). Thus, translational repression of transcripts containing the 5'- structure of *MDR1* mRNA is very likely due to a decrease in Akt activity and 4E-BP phosphorylation. In conclusion, inhibition of the PI3K/Akt/mTOR/4E-BP phosphorylation cascade, and subsequent decrease in the availability of eIF4E in drug exposed cells, specifically reduces the translation of transcripts including the structured 5'-end of *MDR1* mRNA.

The 5'-end MDR1 mRNA loop renders chimaeric luciferase reporter mRNAs poorly competitive for eIF4E

The limiting factor for translation initiation is eIF4E, and transcripts with long and structured 5'-UTRs are generally considered to be poorly competitive [8, 25]. We tested whether the 5'- *MDR1* mRNA structure affected mRNA competitiveness *in vitro*. Luciferase transcripts were synthesized *in vitro* with either the *MDR1* 5'-UTR or the intact 5'-*MDR1* loop structure from T7pGL-UTR and T7pGL-Loop, respectively. Translation of these transcripts in the presence of increasing amounts of

total K562 RNA was performed in a rabbit reticulocyte cell-free system. Luciferase mRNA with the 5'-UTR of *MDR1* mRNA (T7pGL-UTR) competed efficiently with increasing amounts of RNA up to 7 µg. Conversely, just a small increase (0.1 µg) in the amount of competitor RNA negatively affected translation from luciferase mRNA with the 5'-end *MDR1* mRNA structure (T7pGL-Loop). A progressive increase in the amount of competitor RNA, decreased luciferase translation from T7pGL-Loop (Figure 5A). Similar results were obtained when *in vitro* transcription and translation was performed in a single reaction using a TnT system (Promega) (data not shown). Equally, yeast RNA could be substituted for K562 RNA as competitor, thus ruling out the effect of any putative miRNA from K562 cells (data not shown).

We hypothesized that over-expression of eIF4E would compensate for loss of available endogenous eIF4E through repression by 4EBP, and so would increase the competitive ability of pGL-Loop transcripts under cytotoxic stress. We therefore transfected K-pGL-UTR and K-pGL-Loop cells with an eIF4E expression plasmid (Figure 5B) and treated them with 1 µM colcemid for 3 days. m⁷GTP pull-down of translation initiation complexes confirmed that, upon cytotoxic treatment, more eIF4G was associated with eIF4E in cells transfected with the eIF4E expression plasmid (Figure 5C). As before, luciferase activity in transfected K-pGL-UTR and K-pGL-Loop cells was measured to estimate the relative translation efficiencies of pGL-UTR and pGL-Loop transcripts. Although translation from pGL-UTR was not affected by over-expression of eIF4E, translation from pGL-Loop was increased by approximately 40%. There was no significant difference in the steady-state levels of luciferase mRNA in each transfected cell line upon drug exposure (data not shown).

Further confirmation of the poor competitiveness of pGL-Loop transcripts was obtained by RNA interference. Transfection of siRNA targeting eIF4E in K-pGL-UTR and K-pGL-Loop cells decreased eIF4E levels equally in both cells (Figure 5E). Although the eIF4E decrease in unstressed K562 cells did not affect global protein synthesis or translation from pGL-UTR transcripts, it decreased specifically the translation from pGL-Loop transcripts (by approximately 25%) (Figure 5F).

Thus, reporter transcripts with the 5'-structure from *MDR1* mRNA compete inefficiently for the limiting amounts of eIF4E in K562 cells such that, under cytotoxic stress which decreases the availability of eIF4E in the cells, translation from *MDR1* transcripts is diminished.

***MDR1* mRNA is translated when global translation is not down-regulated**

How do drug resistant K562 cells arise and overcome the translational block in *MDR1* mRNA to translate P-glycoprotein in conditions of cytotoxic stress when eIF4E is downregulated? A mechanism that would allow efficient translation of *MDR1* mRNA in drug resistant K562 cells would be by increasing the levels of phosphorylated 4E-BP, with the consequent increased availability of eIF4E and enhanced translation initiation. However, western blot analysis showed that the levels of phosphorylated 4E-BP are similar in both naïve and drug resistant K562 cells (data not shown). Therefore, to translate *MDR1* mRNA drug-resistant K562 cells must increase the steady-state levels of this transcript, enabling it to compete effectively for the translation machinery.

If the above competition model is correct, *MDR1* mRNA should be translated into P-glycoprotein in cells in which eIF4E availability has not been down-regulated (i.e. in cells not exposed to cytotoxic stress). Phorbol ester (TPA) treatment of K562 cells up-regulates *MDR1* mRNA steady-state levels to those found in P-glycoprotein-expressing, drug-resistant KC40 cells (Supplementary Figure 1A). This up-regulation is due both to an increase in transcription from the *MDR1* promoter (Supplementary Figure 2A) and to specific stabilization of *MDR1* mRNA, increasing its half-life to 20 h (Supplementary Figure 2B). TPA treatment did not affect the turnover of other short-lived mRNA like *Id2* [2] (Supplementary Figure 2C) or alter the *MDR1* transcriptional start point (data not shown), ensuring the same structured mRNA is formed as from P-glycoprotein-expressing drug-resistant K562 cells. Treatment with TPA did not increase the phosphorylation status of eIF2 α or alter the phosphorylation status of eIF4E and 4E-BP1, which remained similar to that of drug naïve cells, as opposed to drug-induced K562 cells (Supplementary Figure 1B).

After 16 h treatment with 32 nM TPA, approximately 30% K562 cells expressed P-glycoprotein on the plasma membrane, at 20% of the levels of KC40 cells (Supplementary Figure 1C). TPA-treated K562 cells were sorted by flow cytometry into P-glycoprotein positive and negative populations. These populations showed differential up-regulation of *MDR1* mRNA. Steady-state *MDR1* mRNA levels were up-regulated approximately 20-fold in P-glycoprotein positive cells when compared with P-glycoprotein negative cells (Supplementary Figure 1D). In addition,

although *MDR1* mRNA levels in P-glycoprotein negative TPA-treated K562 cells were 10-fold greater than in naïve cells, this degree of up-regulation was insufficient to increase the competitive ability of *MDR1* transcripts (Supplementary Figure 1D). Thus, only in the subpopulation of TPA-treated K562 cells that up-regulate levels of *MDR1* mRNA sufficiently to increase its competitive ability for eIF4E, is P-glycoprotein translated.

Drug resistant K562 cells originate from a small subpopulation which differentially up-regulates *MDR1* mRNA

We next asked whether differential up-regulation of *MDR1* mRNA in a subpopulation of drug-treated K562 cells might occur during selection for drug resistance. We followed cultures of cells for 37 days after treatment with colchicine, in the presence and absence of 10 μ M verapamil (a competitive inhibitor of P-glycoprotein). Four parameters were monitored: changes in *MDR1* mRNA levels were detected by RT-Q-PCR; the percentage of viable cells (TOTO-3 negative), P-glycoprotein expression (antibody binding) and P-glycoprotein activity (BODIPY-taxol efflux) were detected by flow cytometry. Treatment with 20 ng/ml colchicine resulted in progressive cell death (Figure 6A), reaching a maximum at 26 days when only approximately 5% of the cells were alive. After longer incubation times, colchicine-resistant clones began to emerge, and after an additional 10 days most of the culture was composed of viable, colchicine-resistant cells. The first clones of colchicine-resistant cells were discernible at day 12, consisting of at least 8 cells. Assuming that the growth rate of these cells was equivalent to that of KC40 (approx 2 days doubling time), we can estimate that the first colchicine-resistant cells appear as early as day 6, equivalent to approximately 5 cells per million in the original population. While addition of verapamil alone (10-25 μ M) had no effect on the growth of K562 cells, its inclusion during the selection process abolished the appearance of colchicine resistant cells, implying that P-glycoprotein function is essential for the development of drug resistance in these cells (Figure 6 and data not shown).

MDR1 mRNA levels increased slightly during the first 20 days of culture (from 0.1% to 3% of the level found in KC40), and dramatically thereafter reaching similar levels to those of KC40 cells at day 37 (Figure 6B). Similarly, the percentage of cells expressing surface P-glycoprotein (UIC2 positive), and the percentage of cells

showing P-glycoprotein-mediated drug efflux (BODIPY-taxol), remained at basal levels for the first 14 days and then increased progressively until day 37 where a population phenotypically similar to KC40 was obtained (Figure 6C, D). Verapamil abolished the appearance of P-glycoprotein and BODIPY-taxol efflux positive cells.

We sorted by flow cytometry the small percentage of UIC2 positive cells (3-4%) after 7 days of treatment with 20 ng/ml colchicine and determined their *MDR1* mRNA levels by RT-Q-PCR. As observed for TPA-treated K562 cells (Supplementary Figure 1D), there was a differential up-regulation of *MDR1* mRNA (approximately 5-fold) between P-glycoprotein positive and negative cells. Levels of *MDR1* mRNA in P-glycoprotein negative and positive cells were approximately 25- and 125- fold those of naïve K562 cells, respectively (Figure 6E).

We next asked whether the naïve K562 cell line was a heterogeneous cell population (i.e. not clonal) and the cells up-regulating *MDR1* mRNA were predetermined. For that we isolated, by serial dilution, 20 clones each derived from a single cell, exposed them to 1 μ M colcemid for 3 days and determined the percentage of P-glycoprotein-positive cells as above. The K562 cell line and its 20 single cell derived clones all expressed equivalent percentages of P-glycoprotein-positive cells following drug exposure (data not shown). This indicates that the small percentage of cells which differentially up-regulate *MDR1* mRNA and synthesize very small amounts of P-glycoprotein is not predetermined and is a stochastic process within a clonal population. Thus, differential up-regulation of *MDR1* mRNA occurs stochastically in a small subpopulation of K562 cells upon exposure to cytotoxic drugs.

DISCUSSION

Acquisition of the multidrug resistance phenotype appears to be a multistep process involving many different regulatory steps and may differ between cell types [26-28]. We have previously shown that, upon low level drug selection, multidrug-resistant K562 leukemic cells arise due to overexpression of P-glycoprotein. However, this only occurs in a small proportion of cells within the drug-exposed population which can overcome a translational block of P-glycoprotein synthesis. This block prevents *MDR1* mRNA associating with polysomes [2]. Here we determine how this translational block is overcome and define the mechanism, at least for this cell type, by which drug resistance develops.

Several features of an mRNA can influence its translation. mRNA transcribed from the *MDR1* downstream promoter, which is the only promoter active in K562 cells [12], does not have uORFs in the 5'-UTR, and other features influencing translation such as the poly(A) tail length and polyadenylation site are unchanged irrespective of the translational status of *MDR1* mRNA (data not shown). Secondary structures in the 5'-UTR, such as hairpins, are known to block translation [5]. Although the short 5'-UTR of *MDR1* mRNA appears to have no negative effects on translation, it has previously been shown, by RNase mapping, that the 5'UTR can fold with the first 149 nucleotides of coding region into three highly structured hairpins with several internal loops [9]. We have shown that, *in vivo*, when this entire 5'-*MDR1* structure is cloned upstream of a reporter gene it reduces translation, specifically under conditions of cytotoxic stress.

Following cytotoxic stress, we show that eIF2 α is phosphorylated and that there is a down-regulation in global cellular translation. In addition, the Akt signalling pathway is down-regulated, leading to hypophosphorylation of 4E-BP. Hypophosphorylation of 4E-BP increases its affinity for, and sequestration of, the cap binding protein eIF4E [5]. eIF4E is generally considered rate-limiting for translation. Crucially, not all mRNAs are affected equally by down-regulation in available eIF4E levels. It has been proposed that transcripts with long and structured 5'-UTRs are poor competitors for limiting eIF4E [23, 29], and it has long been known that mRNAs containing extensive secondary structure in their 5'-UTR translate efficiently when eIF4E is over-expressed [30]. We demonstrate that, under cytotoxic stress, there is a down-regulation of the available eIF4E levels, and that the highly structured 5'-end

MDR1 mRNA competes poorly for eIF4E and its translation is repressed, specifically. This poor competitiveness explains the translational block of P-glycoprotein synthesis under conditions of cytotoxic stress. This suggests that compounds that mimic the 5'-end cap of mRNA, such as ribavirin, or which inhibit mTOR or Akt, could decrease the competitive ability of *MDR1* mRNA, preventing translation of P-glycoprotein and thereby avoiding of drug resistance.

Activation of Akt had a greater effect on the translation of *MDR1* 5'-end reporter mRNAs than did over-expression of eIF4E. In the same way, inhibition of mTOR activity by rapamycin had a greater effect in the translation of the same transcripts than specifically targeting eIF4E with siRNA. This suggests that other targets of mTOR (such as eIF4G and p70 S6 kinase), whose role in the activation of translational initiation is well established [31,32], may also play a role in *MDR1* mRNA translational control.

The PI3K-Akt signalling pathway is over-activated in a wide range of tumour types promoting a variety of responses from cell growth and proliferation to survival and motility, leading to tumour progression [33]. Akt activation has also been associated with tumour metastasis and resistance to chemotherapy [34]. It has been suggested that activation of the PI3K –Akt pathway in tumours leads to an increase in the availability of eIF4E and this in turns activates the translation of poorly competitive mRNAs, many of which are important for cell growth [25, 29, 35]. Our data suggests a similar phenomenon for *MDR1* mRNA, implying a mechanistic link between uncontrolled growth and drug resistance.

The poor competitiveness of *MDR1* mRNA implies that, in order to develop drug resistance in the presence of drugs which reduce the availability of eIF4E, *MDR1* mRNA must be up-regulated above a certain threshold in order to compete effectively for eIF4E and translate P-glycoprotein. An alternative hypothesis would be that an inhibitory factor, such as a miRNA, is out-competed when the transcripts reach a critical level. We have found different putative miRNAs targeting *MDR1* 3'-UTR when using the miRBase (<http://microrna.sanger.ac.uk>) or TargetScanS (<http://genes.mit.edu/tscan/>) databases, but none of them are shared between the five vertebrate target sequences available (data not shown). Thus, although we cannot rule out the possibility that some as yet uncharacterized miRNA negatively regulates *MDR1* mRNA translation in K562 cells, the *in vitro* competition data using rabbit

reticulocyte lysates (Figure 5A, and data not shown) rule out that the effect of the 5'-end *MDR1* loop involves a miRNA.

Under conditions in which the levels of eIF4E are not affected, such as following TPA treatment, we have shown that for P-glycoprotein to be translated, *MDR1* mRNA levels must be increased at least 10-fold above those in naïve K562 cells. The threshold for *MDR1* mRNA translation would be expected to be even higher under conditions, such as cytotoxic drug exposure, in which eIF4E is sequestered. On a population basis, the increase in *MDR1* mRNA levels, due to increased stability, is 60 to 70-fold. Importantly, we show here that the up-regulation of *MDR1* mRNA in response to cytotoxic stress is not homogeneous but that, in a small percentage of cells (less than 5%) there is a large increase in mRNA levels of up to 125-fold. This is stochastic, in that fluctuation tests showed that the small subpopulation of cells in which *MDR1* mRNA is stabilised following drug treatment is not predetermined. The mechanism of this stochastic stabilisation is unknown. However, the consequence is that this subpopulation of cells produces sufficient *MDR1* mRNA to effectively compete for the reduced eIF4E, enabling a small amount of P-glycoprotein to be synthesised. The active P-glycoprotein reduces intracellular concentrations of the cytotoxic drug, leading to increased availability of eIF4E. This, in turn, increases the ability of *MDR1* mRNA to compete and translate more P-glycoprotein. This positive feed-back loop, which is abolished by inhibiting P-glycoprotein function, leads to the appearance and maintenance of drug resistance.

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FIGURE LEGENDS

Figure 1 The 5'-end of *MDR1* mRNA is highly structured

(A) Two-dimensional fold of the 5'-end of *MDR1* mRNA, based on *in vitro* RNA mapping data as described [9]. The four lines joining the two first loops represent a pseudo-knot. Nucleotide numbering is in italics with the translation initiation codon at the centre of the structure, indicated as +1 Met. AUG codons are shown in black. The location of the loop within *MDR1* mRNA is indicated in the diagram: white regions, UTRs; black region: *MDR1* coding region forming the loop with the 5'-UTR; grey region: remaining of the *MDR1* coding region. (B) The principal plasmids used in this study. In all cases, the position of the translation start codon is indicated (ATG). pGL-UTR has only the 5'-UTR of *MDR1* mRNA (140 nucleotides) in front of the luciferase gene, whereas in pGL-Loop the entire 5' - *MDR1* mRNA structure (comprising the 140-nucleotide 5'-UTR and the contiguous 149 nucleotides of coding sequence) is cloned in-frame with the luciferase gene. In pGL-LoopM, the ATGs in the loop structure have been mutated (marked as X) so that translation starts at the luciferase gene ATG. (C) Luciferase expression of K562 cells after transient transfection and phRGTK (expressing *Renilla* luciferase to normalize for transfection efficiency). Luciferase activity is expressed relative to that obtained with pGL3control. Data are the mean of three experiments; error bars represent the standard deviation. (D) RT-PCR analysis of luciferase transcripts from cells transfected with the luciferase reporter plasmids. Three different cycle numbers were used. As a control, *GAPDH* transcripts were also amplified by RT-PCR.

Figure 2 The structure at the 5'-end of *MDR1* mRNA down-regulates translation specifically upon treatment with cytotoxic drugs

Stably transfected K562 cell lines were generated using pGL3, pGL-UTR, and pGL-Loop plasmids to generate K-pGL3, K-pGL-UTR, and K-pGL-Loop cells, respectively. (A) Southern blot analysis of the luciferase and β -globin loci in the three stable cell lines. All lines had similar copy number of integrated luciferase plasmid (Luciferase: β -globin ratio). (B and C) K-pGL3, K-pGL-UTR, and K-pGL-Loop cells were treated with 1 μ M colcemid for 3 days. (B) Luciferase activity (white boxes) was used as a surrogate for the translation efficiency of the luciferase reporters whereas [35 S]Met incorporation (grey boxes) was used to determine global translation efficiency. Both are expressed as the percentage of those found in untreated cells. (C) Steady-state luciferase mRNA levels relative to those of untreated cells. The data are the mean of three experiments. Error bars represent the standard deviation. * indicates $p < 0.05$.

Figure 3 Down-regulation of signaling pathways controlling the phosphorylation of eIF4E and 4E-BP

(A) K562 cells were treated with 1 μ M colcemid for 3 days and the status of several pathways controlling the phosphorylation of the indicated translation factors analyzed by western blotting. (B) Western blot showing the phosphorylation status of eIF4E and 4E-BP after treatment of K562 cells with 0.35 μ M doxorubicin and 100 μ M cytarabine for 3 days. (C) m^7 GTP-sepharose pull-down and western blot analysis of initiation complexes from K562 cells treated with 1 μ M colcemid for the indicated time.

Figure 4 The phosphorylation status of 4E-BP controls the translation efficiency of transcripts with the structured *MDR1* mRNA 5'-end

K-pGL-UTR and K-pGL-Loop cells were transiently transfected with vector only or plasmids expressing constitutively-active forms of Mnk (pCAMNK), Akt (pCAAKT), or both, and treated with 1 μ M colcemid for 3 days. Western blot analyses (**A-C**) were used to verify the effect of the constitutive kinase activity on the phosphorylation status of eIF4E (Mnk) and 4E-BP (Akt). Luciferase activity (**D**) was used as a surrogate for translation efficiency and is expressed as the percentage of that found in colcemid-treated untransfected cells. (**E, F**) The mTOR inhibitor rapamycin down-regulates translation from pGL-Loop transcripts. K-pGL-UTR and K-pGL-Loop cells were treated with rapamycin for 8 hours. (**E**) Western blot analysis indicating the inhibition of 4E-BP phosphorylation by mTOR in both cell lines. (**F**) Relative luciferase activity (expressed as the percentage of that found in cells not treated with rapamycin) as a surrogate of pGL-UTR and pGL-Loop translation. All numerical data are the mean of three experiments; error bars represent the standard deviation. * indicates $p < 0.01$.

Figure 5 The 5'-MDR1 mRNA structure makes a reporter mRNA poorly competitive for eIF4E

(A) Translation of *in vitro* synthesized pGL-UTR (white circles) and pGL-Loop (black circles) mRNAs in a rabbit reticulocyte system in the presence of increasing amounts of competitor K562 total RNA. Luciferase activity is expressed as the percentage of that found without competitor RNA. (B-D) Over-expression of eIF4E increases the translation efficiency of pGL-Loop transcripts under conditions of cytotoxic stress. K-pGL-UTR and K-pGL-Loop cells were transfected with an eIF4E expression plasmid (peIF4E) and treated with 1 μ M colcemid for 3 days. Western blot analysis (B), m⁷GTP-sepharose pull down and western blot analysis of initiation complexes (C), and luciferase activity as a surrogate for translation efficiency (D), are shown. Luciferase activity is expressed as the percentage of that found in colcemid-treated untransfected cells. (E-F) Down-regulation of eIF4E reduces translation efficiency specifically from pGL-Loop transcripts. K-pGL-UTR and K-pGL-Loop cells were transfected with either siRNA specific for eIF4E or an irrelevant (control) siRNA, and eIF4E (western blot analysis, E) and translation efficiency (F) measured after 24 hours. Luciferase activity is used as a surrogate for the translation efficiency of the luciferase reporters and [³⁵S]Met incorporation is used to determine global protein synthesis. Both values are expressed as the percentage of those found in cells transfected with the control siRNA. All numerical data are the mean of three experiments; error bars represent the standard deviation. * indicates $p < 0.05$.

Figure 6 Drug resistant K562 cells arise from a small subpopulation of cells which differentially up-regulate *MDR1* mRNA upon drug treatment

K562 cells were treated with colchicine (20 ng/ml) according to the protocol described in Materials and Methods. Where indicated 10 μ M verapamil was included to inhibit P-glycoprotein activity. **(A)** Percentage of live cells (TOTO-3 negative), determined by flow cytometry. **(B)** Relative *MDR1* mRNA levels determined by RT-Q-PCR, expressed as percentage of the amount present in colchicine-resistant KC40 cells. **(C)** Relative P-glycoprotein expression determined by flow cytometry after binding with the phycoerythrin-conjugated antibody UIC2. Two parameters were used, number of UIC2-positive cells, and median fluorescence of the UIC2-positive cell population expressed as the percentage of those found in colchicine resistant KC40 cells. **(D)** P-glycoprotein activity, determined by BODIPY-taxol (BT) efflux, expressed as percentage of the value for colchicine-resistant KC40 cells. Two parameters were used, number of cells with a cyclosporin-inhibitable BT efflux, and efflux activity (measured as decrease in the median fluorescence from the BT-positive population of cells). **(E)** UIC2-labelled K562 cells were sorted by flow cytometry after 7 days in the presence of 20 ng/ml colchicine (relative P-glycoprotein values shown in C) and the *MDR1* mRNA levels determined by RT-Q-PCR in each of the two subpopulations, P-glycoprotein positive and negative. The level of *MDR1* mRNA in K562 cells was set to 1. Data are the mean of two sorting experiments; error bars represent the standard deviation.

Figure 1

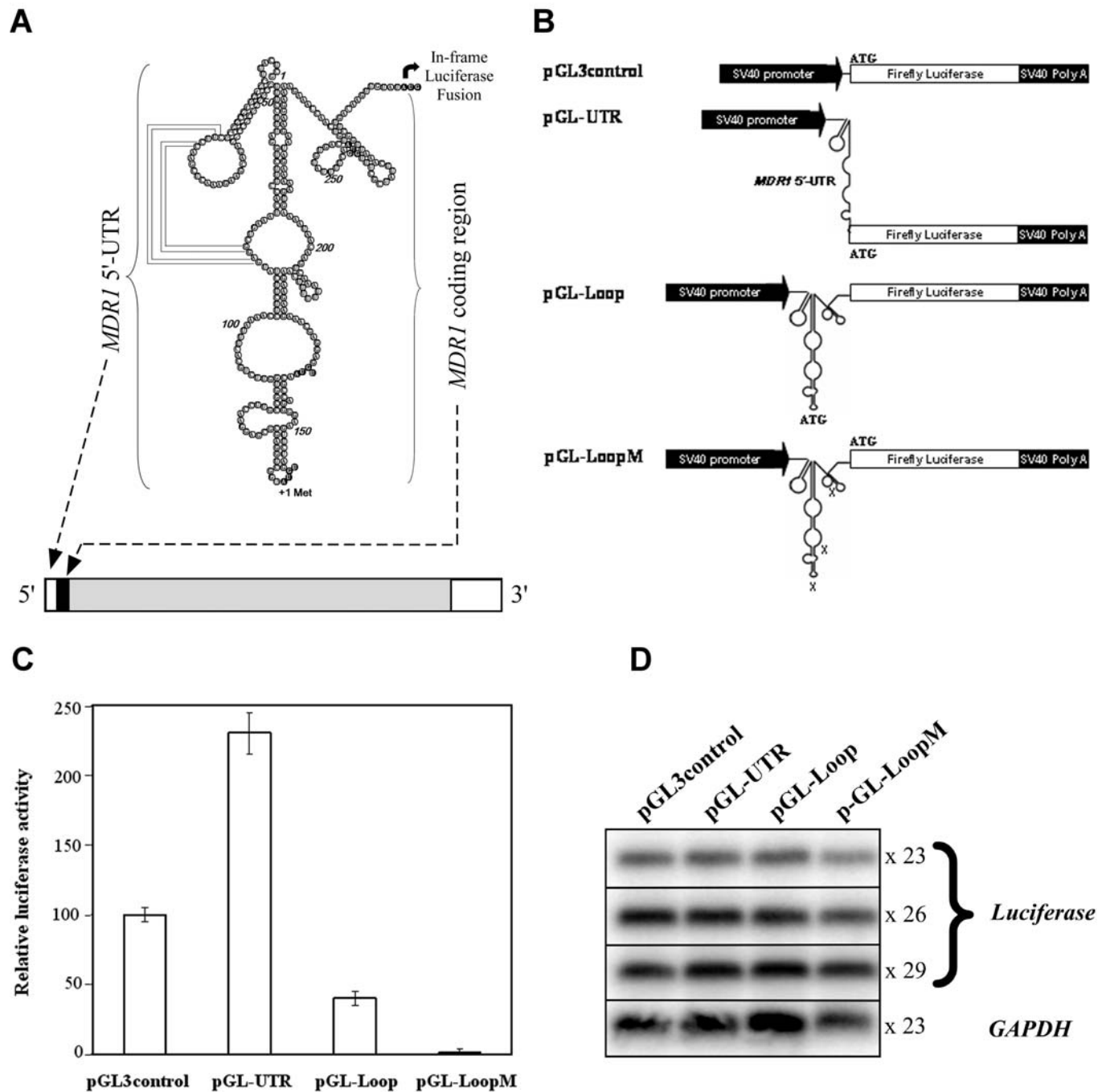
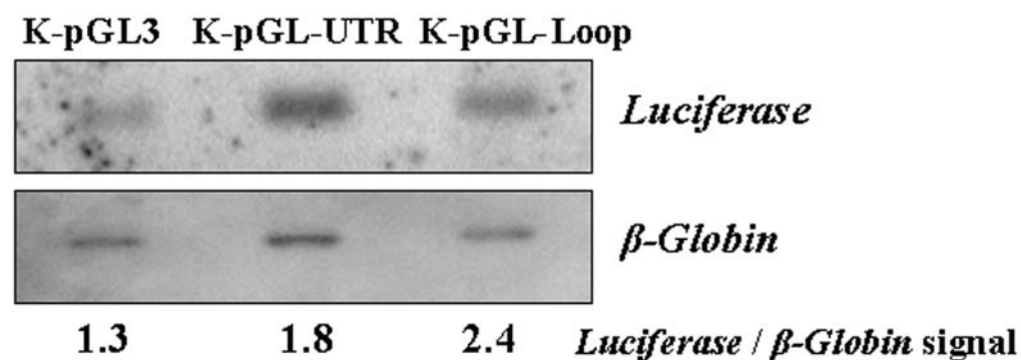
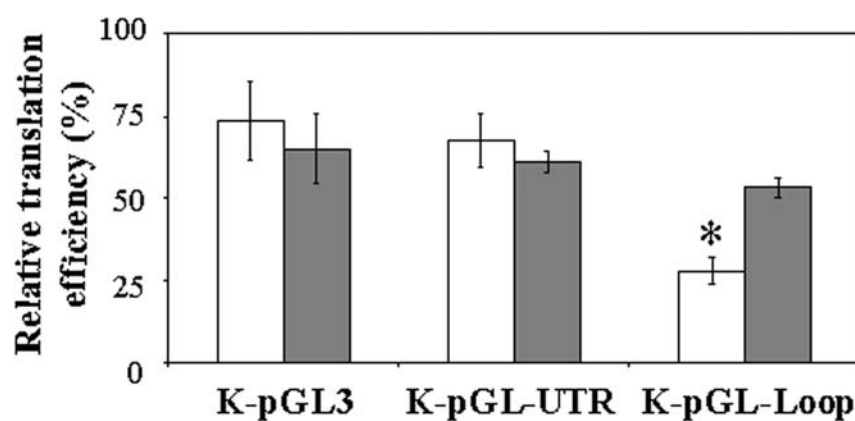


Figure 2

A



B



C

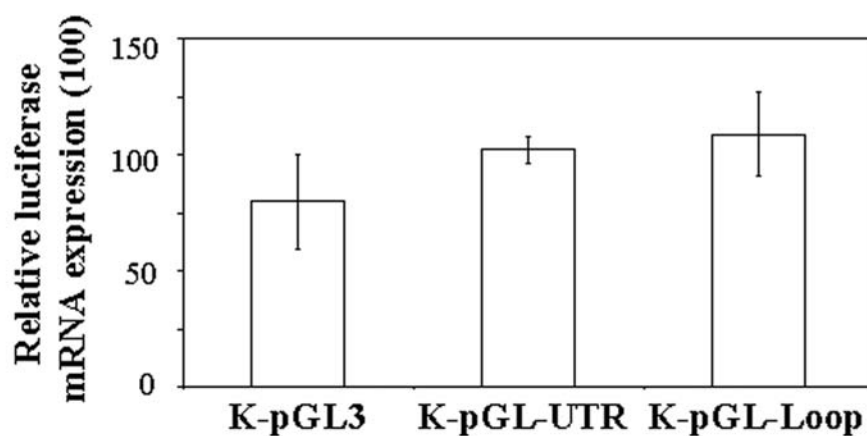


Figure 3

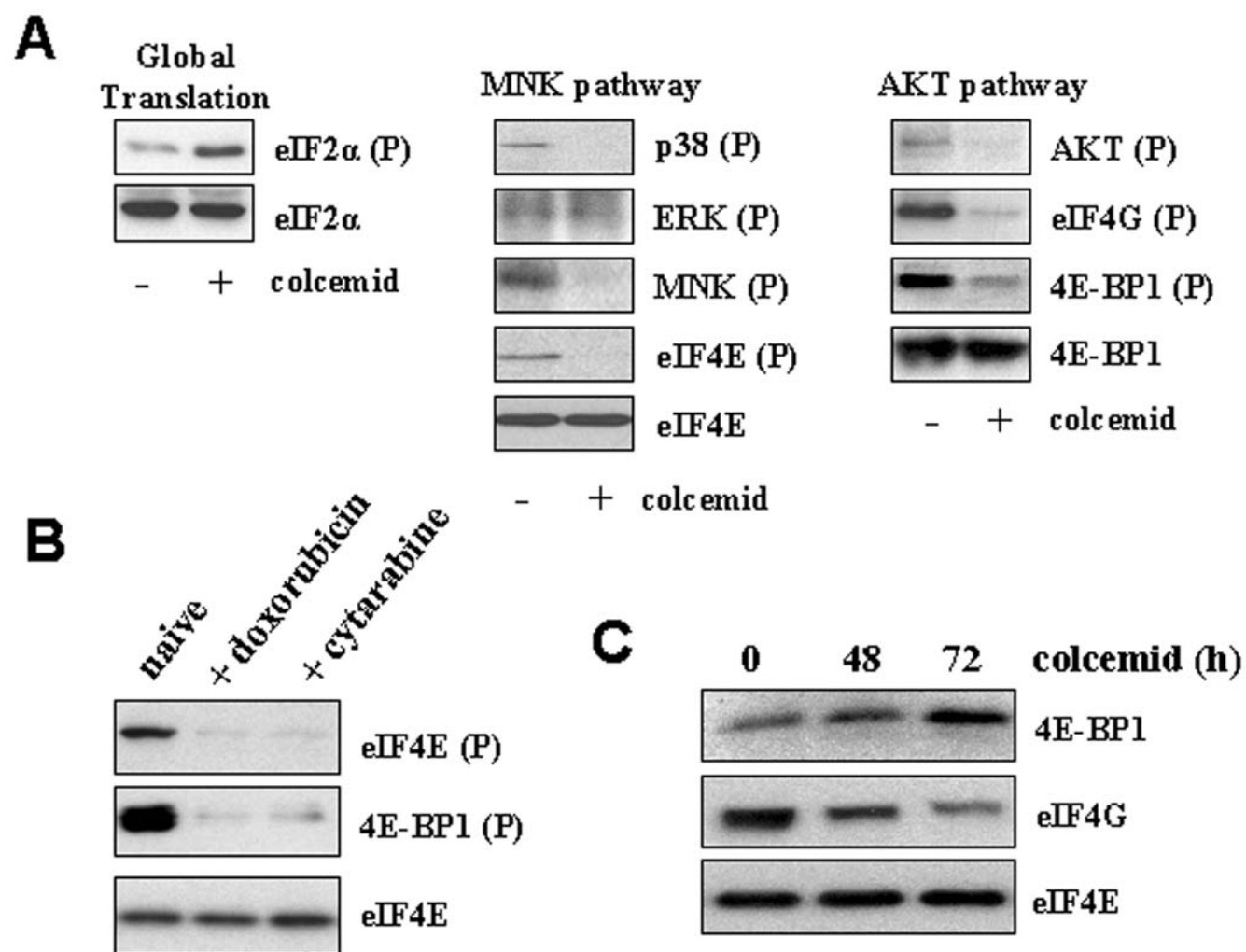


Figure 4

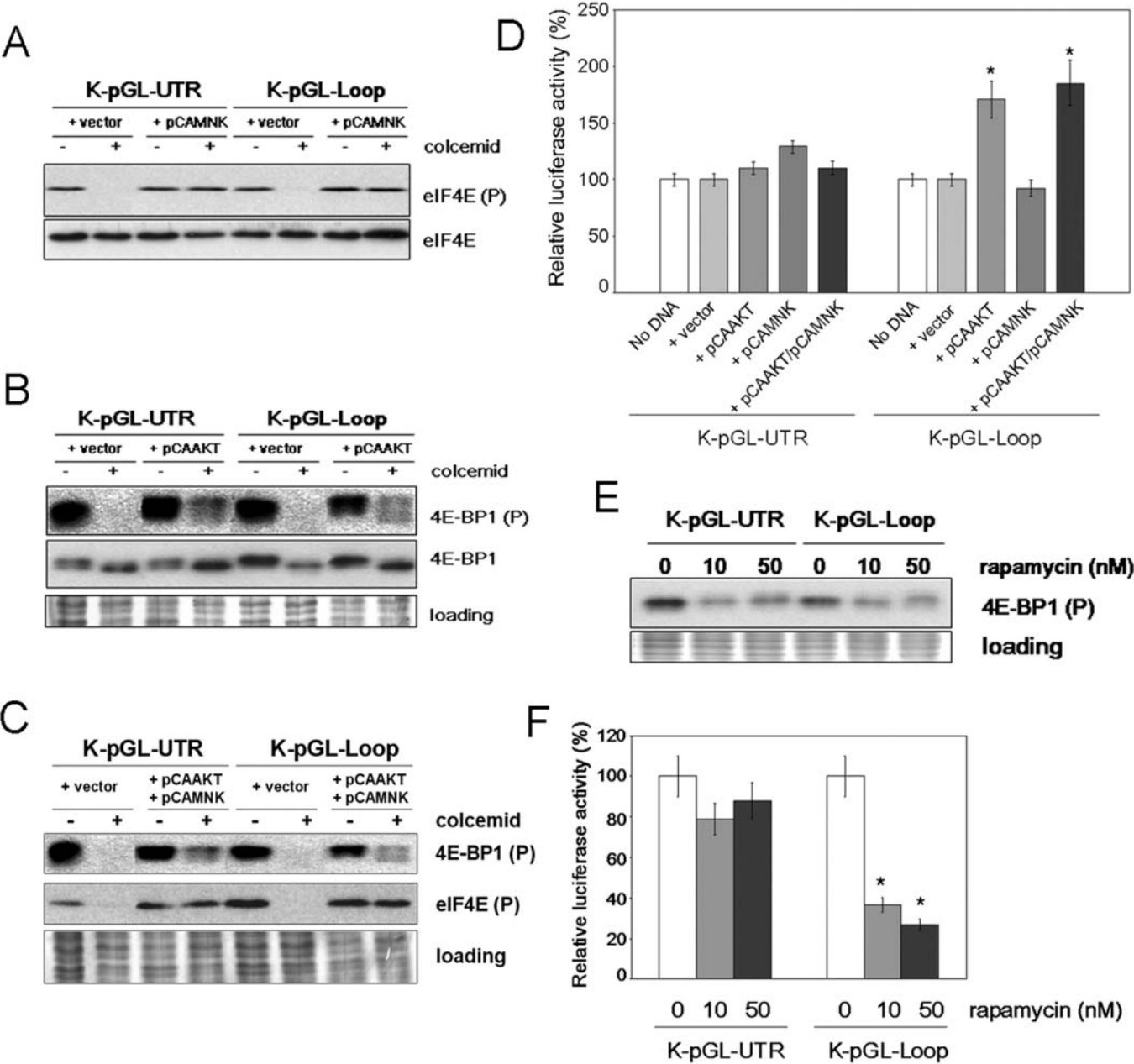


Figure 5

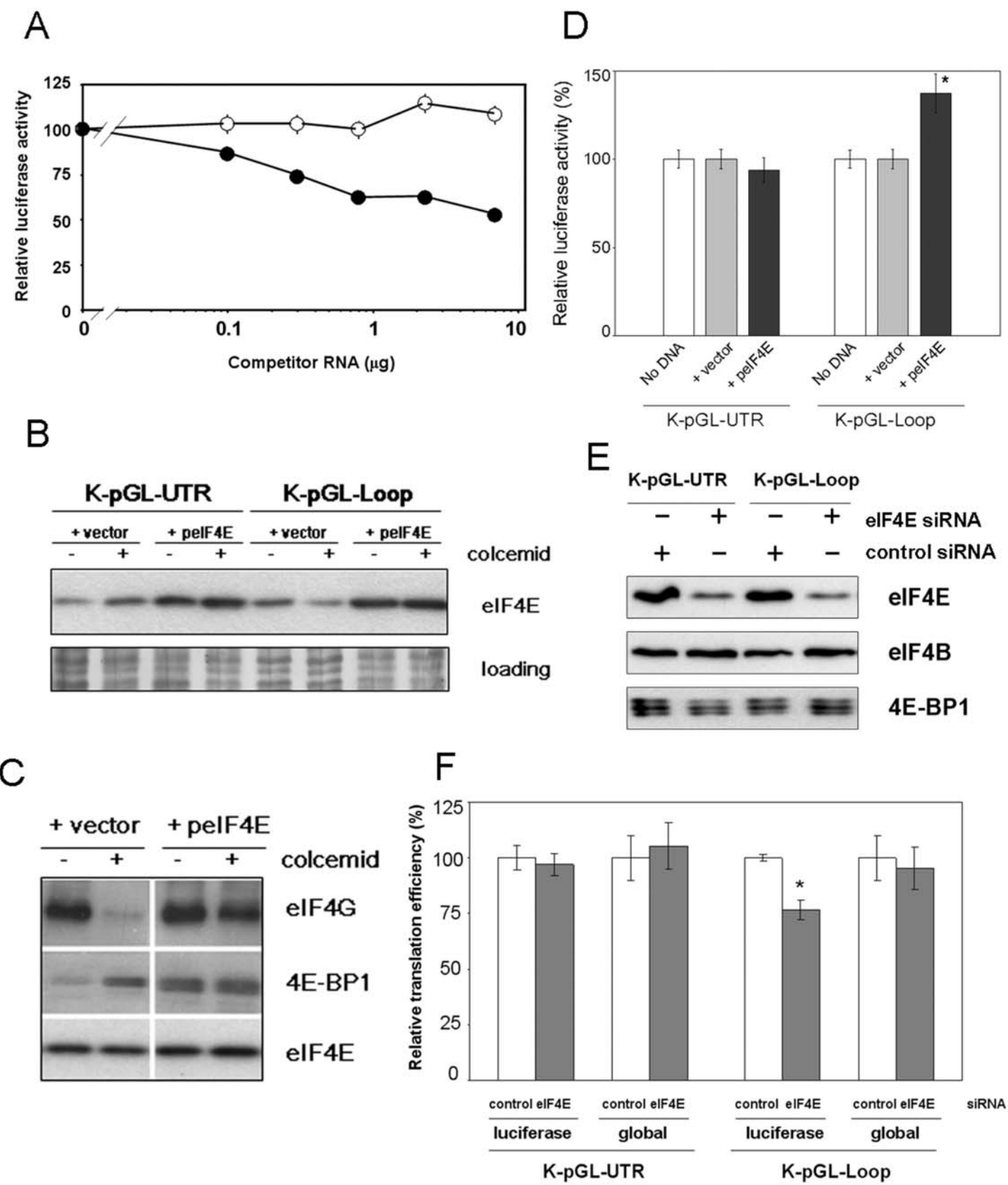


Figure 6

