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ABC50 modulates sensitivity of HL60 leukemic cells to endoplasmic reticulum (ER) stress-induced cell death

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ABSTRACT

ABC50 (aka ABCF1) is a member of the ATP Binding Cassette protein family. ABC50 stimulates complex formation between eIF2, GTP and Met-tRNA implicating it in translation initiation. Econazole (Ec) is an imidazole anti-fungal that induces endoplasmic reticulum (ER) stress in mammalian cells by promoting ER Ca^{2+} depletion and sustained protein synthesis inhibition. HL60 cells selected for Ec resistance were found to exhibit a multi-drug resistance phenotype associated specifically with ER stress. Differential Display was used to identify ABC50 as an overexpressed gene in resistant cells. ABC50 knockdown (KD) in Ec-resistant HL60 cells partially restored Ec sensitivity. In parental HL60 cells, ABC50 KD increased sensitivity to Ec, thapsigargin and tunicamycin but not to serum withdrawal or etoposide. ABC50 overexpression (OE) partially and specifically decreased sensitivity to ER stress agents. ABC50 KD or OE had no effect on ROS generation by Ec, ER Ca^{2+} stores or thapsigargin-stimulated influx. Increased eIF2α phosphorylation in response to ER stress was observed in the KD cells while decreased phosphorylation was observed in the OE cells. Ribosomal content was reduced in ABC50 KD cells and increased in OE cells. Knockdown suppressed protein synthesis while OE increased it. Protein synthesis was sustained in ABC50 OE cells exposed to Ec. ABC50 OE promoted ER stress resistance and increased antibody production in the hybridoma GK1.5 suggesting it may be useful for the overproduction of specific proteins. Taken together, these results indicate that ABC50 modulates sensitivity to Ec and other ER stress agents primarily through its effects on protein synthesis.

1. Introduction

ABC50 (aka ABCF1) is a member of the ATP Binding Cassette (ABC) family of proteins. ABC50 was first identified as a Tumor Necrosis Factor α-inducible gene in synoviocytes [1], and then re-discovered as a protein that purifies with the translation initiation factor eIF2 [2]. Biochemically, ABC50 binds to ribosomes and stimulates formation of complexes between eIF2, GTP and Met-tRNA, implicating it in translation initiation and control for both Cap-dependent and -independent translation [3]. ABC50 is a unique member of the ABC family in that it lacks transmembrane domains. Recently Paytubi et al. showed that the N-terminal region was responsible for eIF2 binding [4]. Binding was found to be regulated by Casein Kinase 2 phosphorylation in this domain.

Econazole (Ec) is an imidazole antifungal that also induces endoplasmic reticulum (ER) stress in mammalian cells by promoting ER Ca^{2+} depletion and sustained inhibition of protein synthesis. Ec's mechanism of action involves both Ca^{2+} influx blockade and stimulation of ER Ca^{2+} release [5]. The latter effect is mediated by reactive oxygen species (ROS) generation at the mitochondria [6]. Some cancer cells are extraordinarily sensitive to Ec [7,8]. Recently, we showed that transformation by the c-myc oncogene sensitizes cells to Ec by enhancing ROS generation at the mitochondria [9] providing at least one mechanism by which cancer cells exhibit sensitivity to Ec.

Previously, we generated and characterized variants of HL60 cells that were resistant to Ec [10]. Although selected for resistance to Ec, the cells also displayed resistance to other ER stress agents including thapsigargin, tunicamycin, DTT and cycloheximide, thus defining a novel phenotype of multi-drug resistance associated with ER stress. Resistance was found to be associated with increased store-operated Ca^{2+} influx capability and sustained

Abbreviations: ABC50, ATP Binding Cassette family protein 50; Ec, econazole; eIF, Eukaryotic Initiation Factor; ER, endoplasmic reticulum; Eto, etoposide; ROS, reactive oxygen species; shRNA, short hairpin RNA; Tg, thapsigargin; Tu, tunicamycin.

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protein synthesis after exposure to Ec. Microarray analysis of a resistant clone revealed increased expression of ribosomal protein genes. Biochemical analysis showed that this increased gene expression was associated with increased ribosomal content. Ribosome inactivating toxins partially reversed resistance to ER stress suggesting that the increased ribosomal content and function contributed to resistance.

To further identify genes associated with resistance and sensitivity to Ec, we performed Differential Display analysis [11] comparing the Ec-resistant cell line E2R2 with its parental HL60 cells. This analysis identified ABC50 as a gene overexpressed in Ec-resistant cells. Here we show that ABC50 modulates sensitivity to ER stress.

2. Experimental procedures

2.1. Cells and cell culture

Human HL60 promyelocytic leukemia cells, their E2R2 derivative and GK1.5 hybridoma cells [12] were cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

2.2. Growth curves

Cells were grown in duplicate cultures at the initial concentration of 0.4×10^6 in RPMI containing 10% FCS. Cells were collected and counted at the 24, 48 and 72 h time intervals.

2.3. Apoptosis and limiting dilution

To measure apoptosis induced by Econazole (Ec; Sigma-Aldrich, St. Louis, MO), cells were treated with Ec in RPMI containing 1% FBS for 2 h at 37 °C then further incubated overnight in RPMI containing 10% FBS. Apoptosis induced by thapsigargin (Tg; Sigma-Aldrich, St. Louis, MO), Tunicamycin (Tu; Sigma-Aldrich, St. Louis, MO) or etoposide (Eto; Sigma-Aldrich, St. Louis, MO) was determined after overnight incubation in RPMI containing 1% FBS. The cells were washed with PBS and stained with Annexin V-cy5 Apoptosis Detection kit (Biovision. Inc., Mountain View, CA)/PI and analysed by flow cytometry. Limiting dilution analysis was performed as described previously [7]. Cells were treated as above with Ec, Tg, Tu or Eto, resuspended in regular growth medium and then plated in 96 well plates with a calculated initial cell number of 1×10^5. Subsequent wells were seeded in a series with a serial dilution factor of 5. Wells were scored for growth 10 days later and initial viabilities were determined using the Spearman's estimator as described by Johnson and Brown [13].

2.4. Differential Display

Differential Display [11] comparing mRNA from HL60 vs. Ec-resistant E2R2 cells was performed using the Delta Differential Display Kit from Clontech (Mountain View, CA). All procedures were performed according to the manufacturer's instructions and involved using pairwise combinations of 10 Arbitrary primers with 9 Oligo dT primers. Differentially expressed bands were excised from the gel, re-amplified, TA-cloned and sequenced.

2.5. Reverse Northern analysis

3 μg of plasmid DNA from each sample was boiled, rapidly placed on ice, then dotted through a dot blot manifold onto duplicate pre-soaked nylon membranes. The membranes were U.V. cross-linked, incubated in pre-hybridization solution (5 × SSC, 5 × Denhardt's solution, 50 mM PBS (pH 7.0), 0.2%SDS, 500 μg/ml salmon sperm DNA, 50% formamide). The membranes were hybridized in hybridization solution (5 × SSC, 5 × Denhardt's solution, 50% formamide) containing 6.5×10^7 cpm of ^{32}PdCTP-labeled reverse-transcribed cDNA probe from either HL60 or E2R2 total RNA. The blots were hybridized overnight, washed in 2 × SSC and then 2 × SSC, 0.1% SDS until background radiation was reduced. The blots were exposed to X-ray film for visualization.

2.6. Construction of lentivirus vectors

The empty lentivirus vector pLEN (H1GFP), in which the H1 promoter drives expression of shRNA sequences was a gift from Dr. John Dick (University Health Network, Toronto, Canada). The sequences of the oligos used to knock down ABC50 expression were: 5′-TAAGCTGTCATCTGGCTTAATAAGGATCCTTATTAAGCCAGATGACAGCTTTTT-3′ and 5′-CTAGAAAAAGCTGTCATCTGGCTTAATAAGGATCCTTATTAAGCCAGATGACAGCTTAAT-3′. Each pair of oligos were mixed and annealed by incubating at 95 °C for 5 min and cooling slowly. The annealed mixture was ligated into pLEN vector that had been digested with PacI and XbaI.

2.7. Construction of lentivirus over-expressing ABC50

The ABC50 clone 7 (obtained from Dr. A. Beaulieu, University of Laval, Quebec, Canada, missing 4 nt from the 5′ end) (GenBank Accession number: AF027302; gi: 2522533) was used as the template for cloning the ABC structural gene by PCR amplification. To add the 4 nt at the 5′, two primers were used: Forward: 5′-AT CCCGGG ATGC CGA AGG CGC CCA AGC AGC AGC-3′ (contains XmaI site); Reverse: 5′-AT CTCGAG TCAC TCT CGG GGC CGG CTG ACC-3′ (contains XhoI site). The amplified ABC50 structural gene was first cloned into pCR4Blunt-TOPO vector (Invitrogen, Burlington, ON) then subcloned into the pCE lentivirus expression vector (Dr. John Dick, UHN, Toronto) that has been digested with XmaI and XhoI. The whole ABC50 gene was sequenced to confirm the lack of mutations.

2.8. Generation of the infective lentivirus

Lentivirus vectors harboring human ABC50 shRNA or the ABC50 structural gene were produced by transient transfection into 293T cells as previously described [14]. Briefly, the backbone plasmid vector construct (10 μg) was mixed with the accessory plasmids VSVG (3.5 μg), pRRE (6.5 μg) and pREV (2.5 μg) and transfected into 293T cells with the Calphos Mammalian Transfection Kit (Clontech, Mountain View, CA). The cell supernatant was replaced with 4 ml fresh Iscove MEM (10% FCS) at 24 h and virus was harvested at 48 h after the plasmid transfection.

2.9. Lentiviral infection

A total of 0.1×10^6 HL60 cells were infected with 2 ml lentivirus culture supernatant ($\sim 2 \times 10^6$ virus particles) in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) for 4 days. Up to 94% of cells were positive for GFP expression. GFP positive cells were sorted by fluorescence activated cell sorting and grown in RPMI (10% FBS) for further analysis.

2.10. Western blot

Cells were washed with PBS and lysed with Triple lysis buffer (50 mM Tris pH 7.0, 150 mM NaCl, 0.1% SDS, 1% NP-40 and 0.5% DOC). Proteinase inhibitor (Boehringer Ingelheim, Burlington, ON) was added to 10 ml lysis buffer before use. Protein concentration was determined with the Pierce BCA kit. 20 μg of total protein was loaded onto 10% SDS-PAGE, transferred onto filters and blotted with rabbit anti-human ABC50 polyclonal serum (kind gift from

Dr. C. Proud, Vancouver, Canada). eIF2α and its phosphorylated form (Ser51) were detected with rabbit polyclonal antibodies from Cell Signalling (Danvers, MA). Mouse anti-BiP/GRP78 antibodies were obtained from BD Biosciences (San Jose, CA) Anti-actin (pan Ab-5, Clone ACTN05) (Labvision/Neomarker, Fremont, CA) was used as a loading control.

2.11. Mitochondrial ROS generation

To determine the generation of mitochondrial ROS in response to Ec, the indicated cells were incubated in Tyrode's buffer containing 5 μM MitoSox Red fluorescent dye (Invitrogen, Burlington, ON). MitoSox accumulates in the mitochondria, is oxidized by superoxide and emits at 580 nm. We showed previously that this dye is relatively specific for mitochondrially generated ROS [6]. Accumulation of ROS was measured by flow cytometry. Ec (15 μM) was added and emission was followed over time.

2.12. Ca^{2+} measurement

$[Ca^{2+}]c$ measurements were performed by flow cytometry. Cells (5×105 cells/ml) were serum-deprived for ~2 h in Tyrode's buffer [HEPES (10 mM), NaCl (100 mM), KCl (5 mM), $CaCl_2$ (1.4 mM), $MgCl_2$ (1 mM), glucose (5.6 mM), BSA (0.05%)]. Cells were then incubated in Indo-1 loading buffer (30 min, 37 °C; 5 μM Indo-1AM, 0.03% pluronic F-127 in Tyrode's buffer), washed (2 times) and incubated at room temperature (greater than 15 min) to allow for the complete removal and/or conversion of Indo-1AM to Ca^{2+}-sensitive Indo-1. Measurements were performed using a laser tuned to 338 nm while monitoring emissions at 405 nm and 450 nm. The concentration of intracellular free Ca^{2+} was calculated according to the following formula [15]:

$$[Ca^{2+}]_i = K_d \times \left(\frac{F_{min}}{F_{max}}\right) \times \left(\frac{R - R_{min}}{R_{max} - R}\right),$$

where R is the ratio of the fluorescence intensities measured at 405 nm and 450 nm during the experiments and F is the fluorescence intensity measured at 450 nm. R_{min}, R_{max}, F_{min} and F_{max} were determined from *in situ* calibration of unlysed cells using 4 μM ionomycin in the absence (R_{min} and F_{min}; 10 mM EGTA) and presence of (R_{max} and F_{max}) of Ca^{2+}. Kd (250 nM) is the dissociation constant for Indo-1 at 37 °C. R_{min}, R_{max}, F_{min} and F_{max} varied depending upon settings and were determined at the beginning of each experimental procedure.

2.13. Protein synthesis

Cells (2×10^5/sample) were collected, washed with PBS and then re-suspended in RPMI supplemented with fatty acid-free bovine serum albumin (BSA; 0.05%; Sigma, St. Louis, MO). Cells were treated with Ec (0, 15 μM) for 15 min. After centrifugation (2500 rpm; 5 min), cells were pulse-labeled with [^{3}H]-leucine (50 μCi/ml) for 10 min (37 °C; 5% CO_2) in leucine-free RPMI. After two washes in RPMI, pellets were lysed with Triton X-100 (0.5% in PBS) followed by trichloroacetic acid (TCA, 10%, w/v; 4 °C). Samples were washed in TCA (5%, w/v), and the protein pellets were re-suspended in microscintillant (Packard, CT, USA) and measured using a microplate scintillation counter (Packard, CT, USA).

2.14. Ribosomal purification

5×10^7 HL-60 cells growing in log phase were collected, washed with cold PBS, and fractionated according to the method described by Greco and Madjar [16]. The ribosomal fraction was isolated through centrifugation of post-mitochondrial supernatants on top of a 1 M sucrose cushion at 245,000 × *g* to pellet the ribosomes. The ribosome pellets were resuspended in 300 μl of RIPA buffer and disrupted by incubation in 60 mM EDTA on ice for 30 min. The concentration of the total ribosomal protein was calculated based on the absorbance of the samples (A280). Ribosomal RNAs were extracted with TRIzol and the concentration was measured by a spectrophotometer at A260.

2.15. IgG measurements

IgG levels produced by the rat hybridoma GK1.5 (ATCC no. TIB-207) were measured by Western blotting and ELISA. For Western blotting, cells were plated at a concentration of 1×10^6 cells/ml in growth medium for 24 and 48 h. The cells were then collected, counted, pelleted and cell lysates were prepared in RIPA buffer with protease inhibitors (Sigma, St. Louis, MO). Lysates and cell supernatants were resolved on 10% SDS-PAGE and transferred to PVDF membranes. Antibody heavy and light chains were detected with HRP-conjugated rabbit anti-rat IgG (H + L) (Zymed; San Franciso, CA). For ELISA measurements, Goat anti-rat IgG or normal control IgG from Goat serum (Sigma) were diluted to 5 μg/ml in coating buffer (50 mM Tris, 150 mM NaCl, pH 9.5), placed into a 96 well ELISA plate in 50 μl volume and incubated for 40 min at room temperature. The plate was washed for 8 times with distilled water and incubated with 50 μl of PBS containing 3% FBS for additional 40 min at room temperature. Empty vector and ABC50 over-expressed lentivirus transfected GK1.5 hybridoma cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS. Cell culture supernatant was collected and diluted in same media and 50 μl diluted samples were added into the 96-well plate. Normal rat IgG from rat serum (Sigma, St. Louis, MO) was used for determining the standard curve. After incubating for 2 h at room temperature, the wells were washed 8 times with distilled water. HRP conjugated Goat anti-rat IgG (Sigma, St. Louis, MO) was diluted 1:2000 in IMDM and 50 μl reagent was added, incubated for another 40 min and washed as described above. 100 μl of substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, St. Louis, MO) was added and the reaction was stopped with 0.5 M H_2SO_4 when a yellow color developed (5–10 min). The plate was read at 450 nM with an ELISA reader.

2.16. Statistical analysis

Where indicated, statistical significance was determined using the unpaired Student's *t*-test. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were as indicated.

3. Results

3.1. Differential Display of Ec-resistant vs. sensitive HL60 cells

In order to identify additional genes associated with Ec resistance, we performed Differential Display analysis [11] comparing Ec-resistant E2R2 cells with parental HL60 cells. This analysis identified approximately 200 gene fragments that appeared to be overexpressed in E2R2 cells compared to Wild Type. We cloned these gene fragments and employed Reverse Northern analysis to confirm differential expression. 50 of the 200 genes had expression levels above the detection limit of the Reverse Northern. Of the 50, 15 genes were confirmed to be differentially expressed. Sequence analysis identified these genes as follows: Two of the 15 encoded ribosomal protein genes, three encoded Alu-containing sequences, two were mitochondrial genes and one gene encoded the integrin CD11a. Two genes were identified that are classified as TNFα inducible. These were HLA gene (Bw-62), and ABC50 (NM_001090; aka ABCF1), a member of

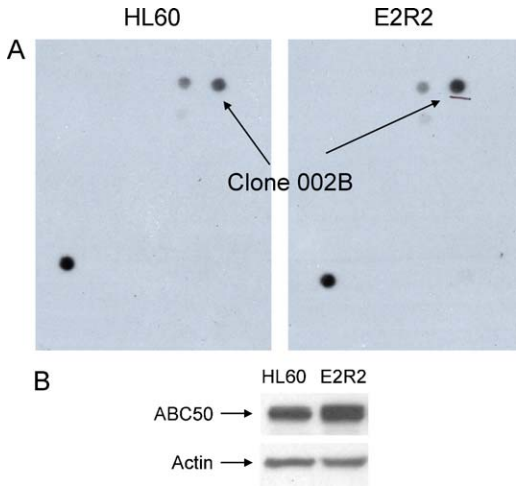

Fig. 1. Enhanced expression of ABC50 in Ec-resistant E2R2 cells. (A) Reverse Northern analysis of genes identified by Differential Display was performed as described in Experimental Procedures. Clone 002B, identified in this analysis as having increased expression was sequenced and found to be the ABC50 gene. (B) Western blot of ABC50 in HL60 vs. E2R2 cells. Actin expression was also evaluated to allow normalization between the two samples.

the ATP binding cassette family (Fig. 1A). Two additional genes of unknown function with no known homology or similarity to any other gene (AC114546, AC012358) were identified. One codes for hypothetical protein FLJ12363 (XP_043979) with no known function. The final gene identified in this screen was polyubiquitin C (AB009010).

Given our previous identification of differences in ribosome content and protein synthesis between HL60 and Ec-resistant variants [10], and the known role for ABC50 in translation initiation, we further investigated this gene product in Ec sensitivity.

3.2. ABC50 protein levels in E2R2 cells

We first confirmed that ABC50 was overexpressed in E2R2 cells. As shown in Fig. 1B, increased levels of ABC50 protein were detected in E2R2 cells compared to HL60 cells. Densitometric analysis of Western blots indicated an 80% increased expression (relative to actin) of ABC50 in E2R2 compared to HL60 cells.

3.3. ABC50 knockdown in E2R2 cells

The observed increase in ABC50 levels in Ec-resistant cells, coupled with its role in protein synthesis suggested that the protein might play a role in sensitivity to the drug. We investigated the association of ABC50 with the Ec-resistance phenotype of E2R2 cells by knocking down its expression in these cells. The cells were infected with a lentiviral vector expressing shRNA specific for ABC50 and sorted based on GFP expression. As shown in Fig. 2A, ABC50 knockdown was successful in these cells (decreased to 36% relative to vector control). Furthermore, as shown in Fig. 2B, ABC50 knockdown in E2R2 cells partially reversed their resistance to Ec (21.4% combined early and late apoptosis compared to 7.6% combined early and late apoptosis in the control cells), consistent with a role for ABC50 in the Ec resistance phenotype.

3.4. ABC50 knockdown in HL60 cells increases sensitivity to ER stress agents

To investigate further the consequences of manipulating ABC50 levels in cells, we infected parental HL60 cells with the lentiviral vector expressing shRNA specific for ABC50 and sorted infected cells based on GFP expression. As shown in Fig. 3A, the shRNA

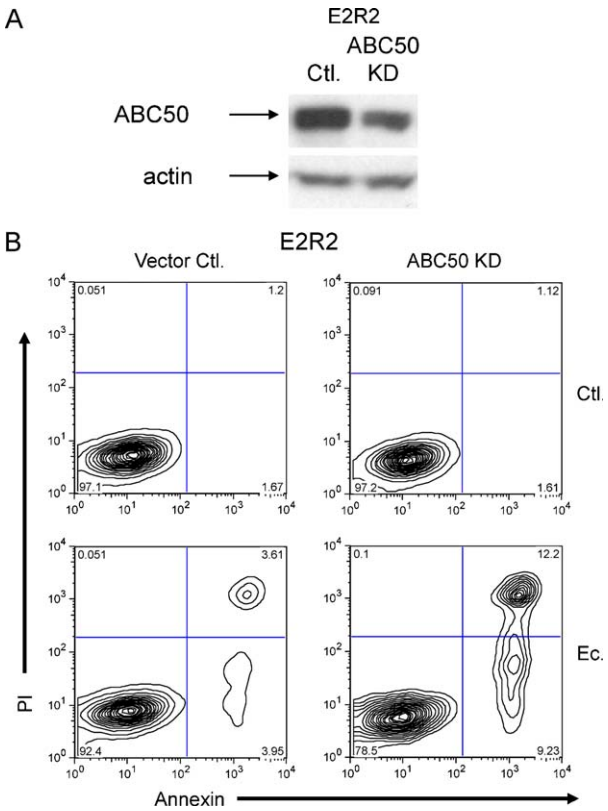

Fig. 2. ABC50 knockdown partially reverses resistance to Ec in E2R2 cells. (A) Western blot of ABC50 expression in E2R2 cells infected with vector control or ABC50 shRNA. (B) Apoptosis induction by Ec in E2R2 vector control and ABC50 knockdown cells. Cells were exposed to 15 μM Ec for 2 h followed by overnight recovery as described in Section 2. The following day, cells were stained with PI and AnnexinV and analysed by flow cytometry. AnnexinV positive, PI negative cells represent early apoptotic cells, AnnexinV positive, PI positive cells represent late apoptotic or necrotic cells.

knocked down expression of ABC50 by 89% compared to vector control. Light microscopic observation revealed that the cells had no obvious morphological differences. We also found that the knocked-down cells grew at a rate that was not significantly different from the control cells (Fig. 3B).

We next measured the effect of ABC50 knockdown on sensitivity to Ec and other apoptosis-inducing agents. Tg is a classic inducer of ER stress and HL60 cells selected for resistance to Ec were also found to be resistant to Tg. We also tested sensitivity to Tunicamycin (Tu), an inhibitor of protein glycosylation and another classic inducer of ER stress. As shown in Fig. 3C, ABC50 knockdown significantly increased the sensitivity of HL60 cells to Ec, Tg and Tu as measured by apoptosis induction. In contrast, ABC50 KD did not affect sensitivity to serum withdrawal or the topoisomerase inhibitor etoposide. As an independent measure of sensitivity, we also examined the effect of the drugs on long term viability as measured by the ability of cells to grow for an extended period of time. Cell growth was quantitated by limiting dilution analysis. As shown in Fig. 3D, increased sensitivity in ABC50 KD cells was also observed in this assay. These observations suggest that ABC50 knockdown specifically increases sensitivity to ER stress-inducing agents.

3.5. ABC50 overexpression in HL60 cells decreases sensitivity to ER stress agents

The observation of increased ABC50 expression in the Ec-resistant E2R2 cells suggested that overexpression of the gene might promote resistance. To investigate this possibility, we infected HL60

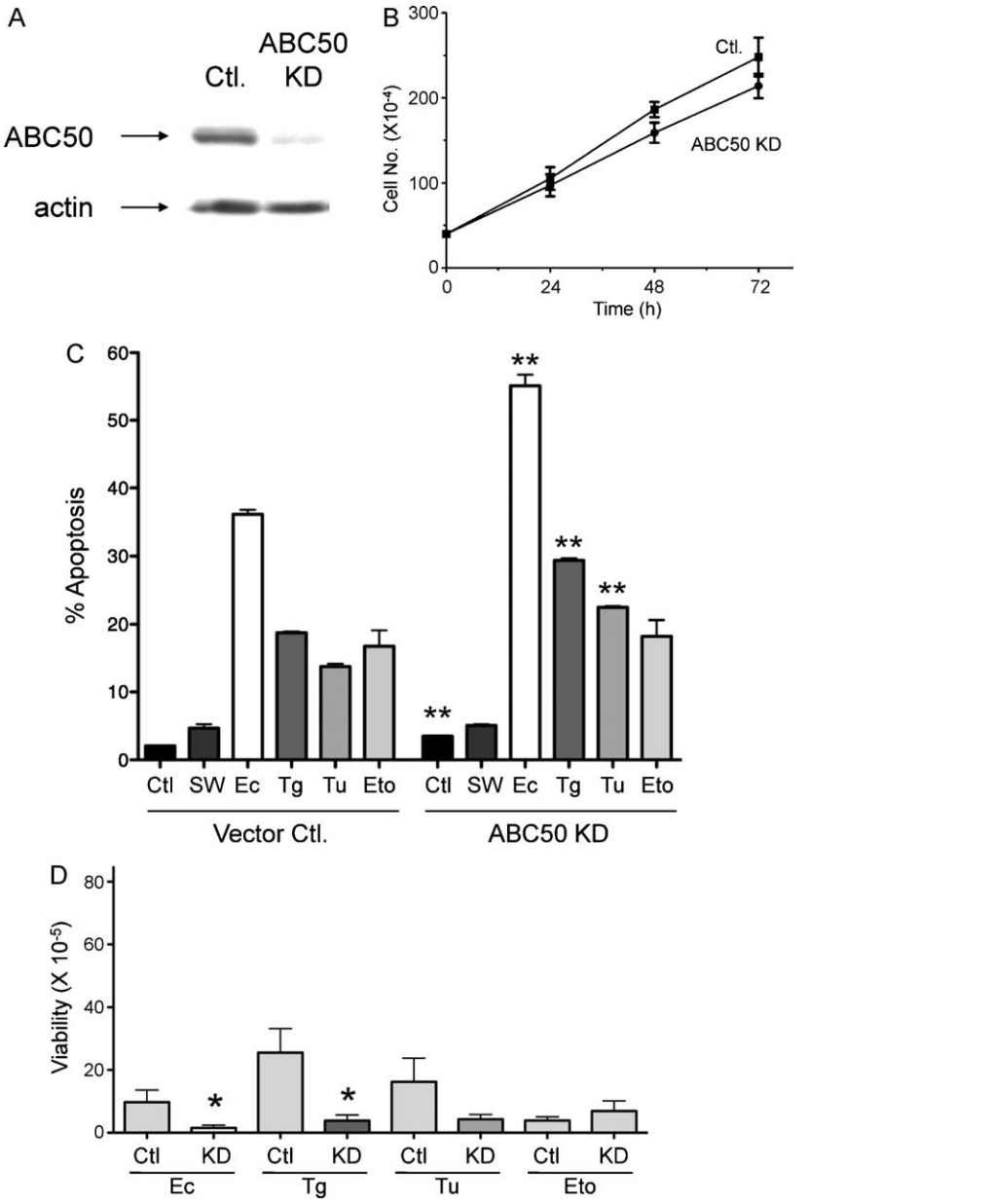

Fig. 3. ABC50 knockdown alters growth rate and sensitivity to Ec in HL60 cells. (A) Western blot of ABC50 expression in HL60 cells infected with vector control or ABC50 shRNA. (B) Cell growth kinetics of control and ABC50 knocked-down cells. Values are means and standard errors determined from triplicate cultures and are representative measurement from a series of three independent experiments. ***Indicates $p < 0.001$ at 48 h for ABC50 KD cells vs. control. (C) Apoptosis induction by serum withdrawal (SW), Ec, Tg, Tu and etoposide (Eto) in HL60 vector control and ABC50 knockdown cells. Cells were exposed to 15 μM Ec for 2 h followed by overnight recovery as described in Section 2. Cells were incubated overnight in the absence of serum, 200 nM Tg, 1 μM Tu or 5 μM etoposide. The following day, cells were stained with PI and AnnexinV and analysed by flow cytometry. AnnexinV positive, PI negative cells represent early apoptotic cells, AnnexinV positive, PI positive cells represent late apoptotic or necrotic cells. Plotted is early and late apoptotic cells combined. D: Long term viability. Cells were treated as in C then plated under limiting dilution conditions as described in Section 2. Viability was normalized to the untreated cells to facilitate direct comparison. A representative experiment is shown. This experiment was performed twice with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$ comparing knockdown cells to their vector control.

cells with a lentiviral vector expressing the full ABC50 gene, sorted infected cells as above using the GFP marker, and analysed the cell phenotype. As shown in Fig. 4A, infection with the ABC50 lentiviral vector significantly increased expression of the protein (68% relative increase compared to vector control). We measured cell growth properties and found that the ABC50 overexpressing cells had no significant differences in growth kinetics compared to control HL60 cells infected with vector alone (Fig. 4B). However as shown in Fig. 4C and D, ABC50 overexpressing cells displayed decreased sensitivity to the ER stress agents Ec, Tg and Tu whereas their sensitivity to serum withdrawal or etoposide was unchanged compared to control cells. Taken together, we conclude that ABC50 expression levels specifically affect sensitivity to ER stress.

3.6. Exposure to ER stress does not directly alter ABC50 protein levels

Ec-resistant E2R2 cells were originally selected by exposing HL60 cells to multiple rounds of increasing drug concentrations followed by a recovery phase. In considering the mechanism of how resistance develops, it is possible that Ec itself or other ER stress agents may induce expression of the protein, leading to increased levels and thus resistance. To examine this possibility, we exposed HL60 cells to Ec, Tg or Tu and measured ABC50 protein levels. As shown in Fig. 5, exposure to these agents had no direct effect on ABC50 protein levels. This observation therefore indicates that the original protocol most likely selected for rare variants in the population of cells that

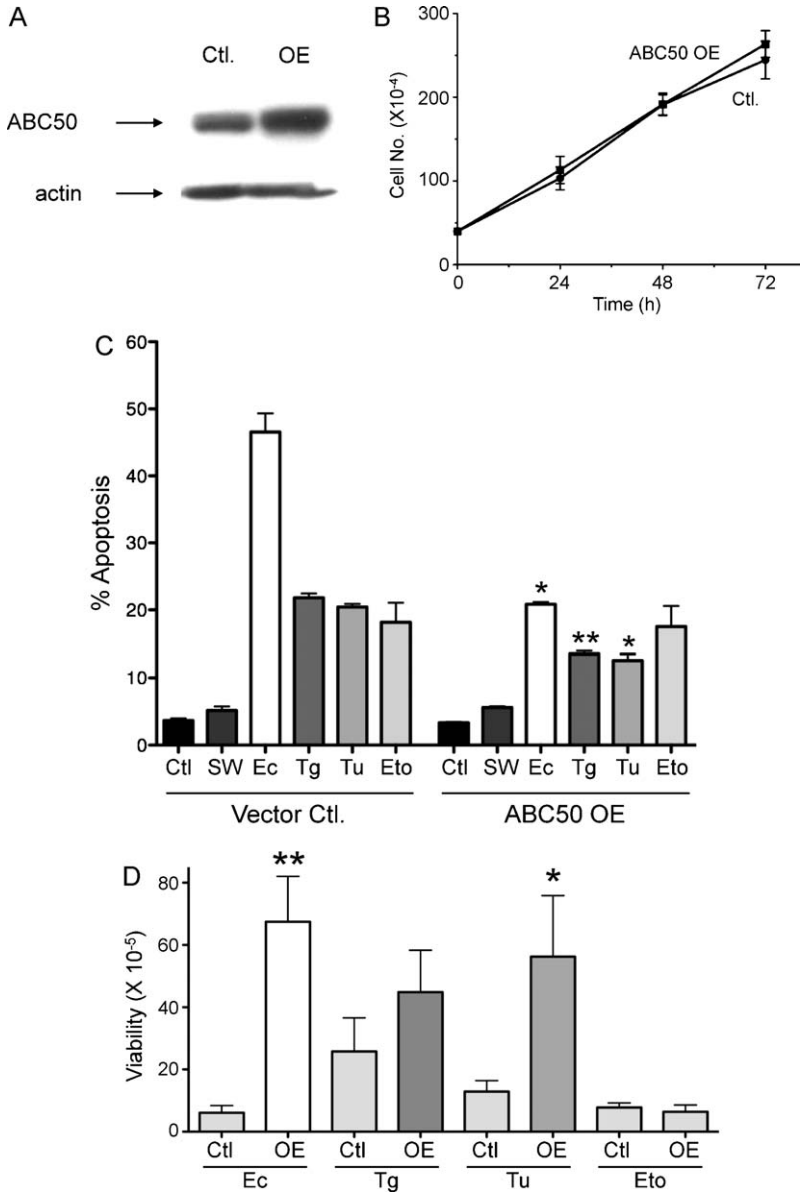

Fig. 4. Effect of ABC50 overexpression on growth rate and sensitivity to ER stress agents in HL60 cells. (A) Western blot of ABC50 expression in HL60 cells infected with vector control or ABC50 OE vector. (B) Cell growth kinetics of control and ABC50 overexpressing cells. Values are means and standard errors determined from triplicate cultures and are representative measurement from a series of three independent experiments. (C) Apoptosis induction by serum withdrawal (SW), Ec, Tg, Tu and etoposide (Eto) in HL60 vector control and ABC50 overexpressing cells. Cells were exposed to 15 μM Ec for 2 h followed by overnight recovery as described in Section 2. Cells were incubated overnight in the absence of serum, 200 nM Tg, 1 μM Tu or 5 μM etoposide. The following day, cells were stained with PI and AnnexinV and analysed by flow cytometry. AnnexinV positive, PI negative cells represent early apoptotic cells, AnnexinV positive, PI positive cells represent late apoptotic or necrotic cells. Plotted is early and late apoptotic cells combined. (D) Long term viability. Cells were treated as in C then plated under limiting dilution conditions as described in Section 2. Viability was normalized to the untreated cells to facilitate direct comparison. A representative experiment is shown. This experiment was performed twice with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$ comparing overexpressing cells to their vector control.

overexpressed ABC50 rather than inducing expression through drug exposure.

3.7. ROS generation in ABC50 knockdown and overexpressing cells

We have previously shown that Ec stimulates ER Ca^{2+} depletion through the joint activities of Ca^{2+} influx blockade and ROS generation at the mitochondria. ROS stimulates ER Ca^{2+} emptying while influx blockade prevents refilling. Since both events are required for full lethality, we first determined if altered ABC50 expression had any effect on ROS generation. As shown in Fig. 6, we observed that ABC50 knockdown or overexpression had no effect

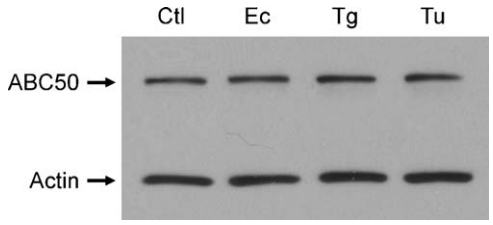

Fig. 5. ER stress agents have no direct effect on ABC50 protein levels. HL60 cells were incubated with Ec (15 μM), Tg (200 nM) or Tu (200 ng/ml) for 2 h (Ec) or 4 h (Tg and Tu). Cells were collected, lysed in RIPA buffer, resolved by SDS-PAGE and probed for ABC50 or actin expression.

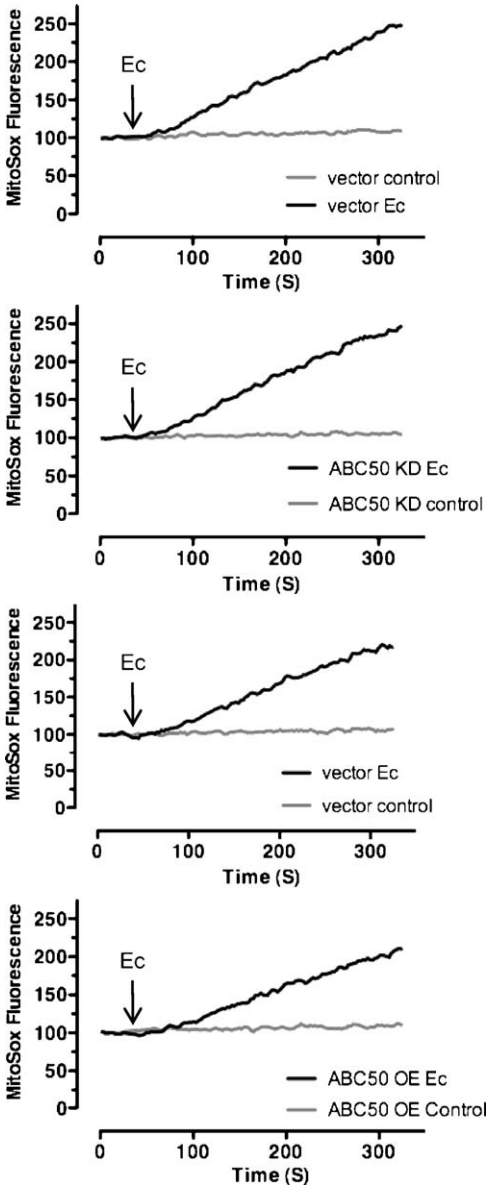

Fig. 6. Effect of ABC50 knockdown or overexpression on ROS generation by Ec. The indicated cells were incubated with MitoSox Red and Fluorescence was followed over time following addition of Ec (15 μM). Ctl traces are for cells not treated with Ec.

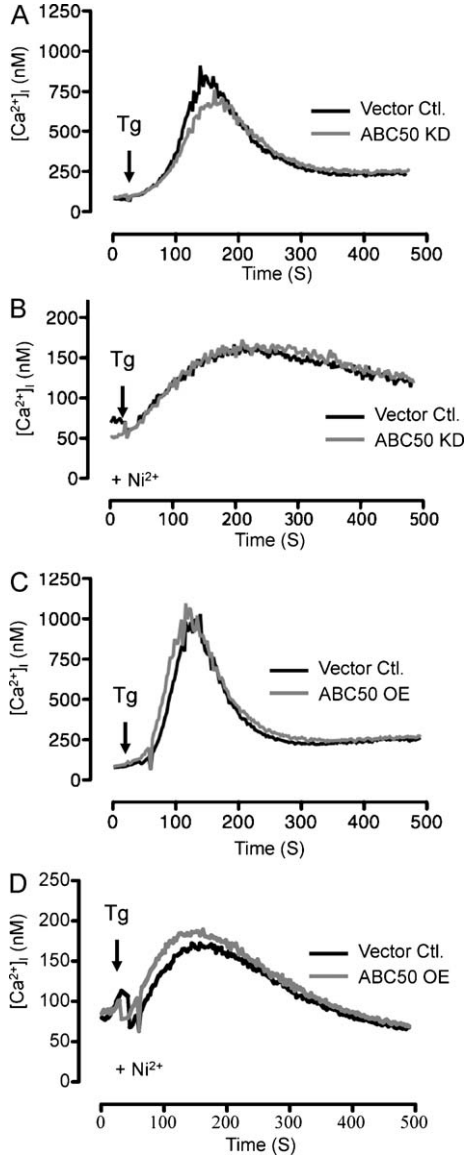

Fig. 7. Effect of ABC50 knockdown or overexpression on ER Ca^{2+} stores and influx in HL60 cells. HL60 cells were loaded with the Ca^{2+}-sensitive dye Indo-1 as described in Section 2. Cells were incubated (or not) in 5 mM Ni^{2+} to non-specifically block all Ca^{2+} influx and then exposed to thapsigargin to release ER Ca^{2+} and stimulate Ca^{2+} influx. Cytoplasmic Ca^{2+} levels were followed over time. Tg releases ER Ca^{2+} in all cases but subsequent store-operated Ca^{2+} influx is blocked in cells pre-incubated with Ni^{2+}. (A) HL60 cells infected with vector control or ABC50 shRNA treated with Tg. (B) HL60 cells infected with vector control or ABC50 shRNA pre-incubated in Ni^{2+} to block influx, and then treated with Tg. (C) HL60 cells infected with vector control or ABC50 overexpressing virus treated with Tg. D: HL60 cells infected with vector control or ABC50 overexpressing virus pre-incubated in Ni^{2+} to block influx, and then treated with Tg.

on the ability of Ec to generate mitochondrial ROS. This observation suggests that ABC50 does not modulate sensitivity through any effect on ROS generation.

3.8. ER Ca^{2+} content and influx in ABC50 knockdown and overexpressing cells

We previously demonstrated that the Ec resistance phenotype of E2R2 cells was associated with altered Ca^{2+} physiology. Specifically, E2R2 cells displayed unchanged ER Ca^{2+} store content, but increased Ca^{2+} influx in response to ER Ca^{2+} store depletion by the ATPase ER Ca^{2+} pump inhibitor thapsigargin [10]. To investigate the effect of altered ABC50 expression on Ca^{2+} physiology, we measured ER Ca^{2+} content and influx in ABC50 knockdown and overexpressing cells. As shown in Fig. 7, no differences in either ER Ca^{2+} content (Fig. 7B,and D) or Tg-stimulated Ca^{2+} influx (Fig. 7A and C) were observed in ABC50 KD or overexpressing cells. These observations indicate that ABC50 does not directly affect Ca^{2+} physiology in HL60 cells.

3.9. ER stress response in ABC50 knockdown or overexpressing cells

Ec, Tg and Tu are all potent inducers of ER stress. To compare the ER stress response of cells with altered ABC50 expression, cells were treated for 60 min with the ER stress agents and levels of phosphorylated eIF2α and the chaperone BiP, two classic indicators of ER stress, were determined by Western blot. As shown in Fig. 8A, increased levels of phosphorylated eIF2α were observed in treated ABC50 knockdown cells compared to vector control. Ec and Tg were particularly effective at inducing increased levels of eIF2α. Induction of BiP expression by ER stress agents was not affected by ABC50 knockdown (Fig. 8B) although basal levels were slightly increased compared to control. In contrast, ABC50

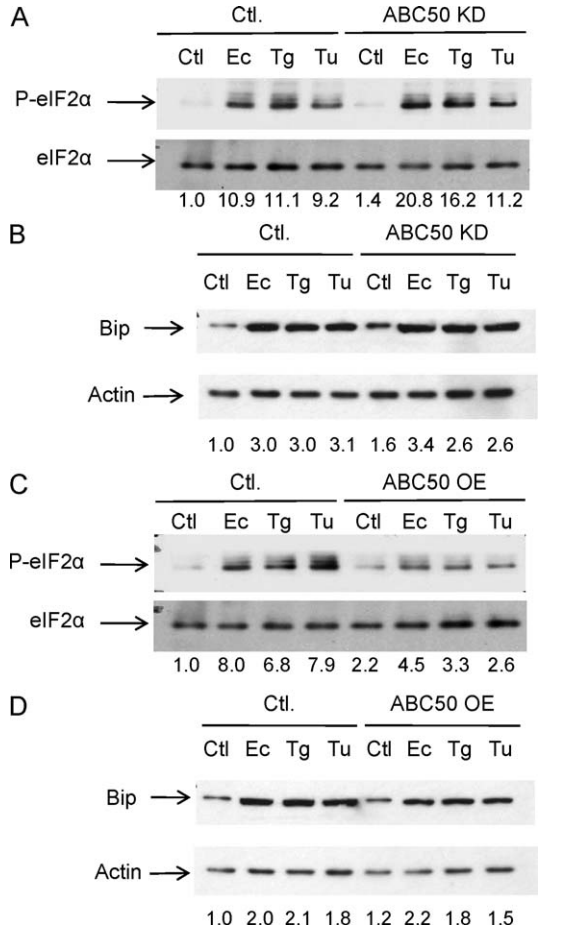

Fig. 8. ABC50 knockdown or overexpression alters the ER stress response. Vector controls, knockdown (A and B) or overexpressing cells (C and D) were exposed to Ec (15 μM), Tg (200 nM) or Tu (200 ng/ml) for 60 min. The cells were collected, lysed in RIPA buffer, resolved by SDS-PAGE and analysed with anti-sera specific for (A and C) ser51-phosphorylated eIF2α or total eIF2α and (B and D) BiP or actin. Numbers represent relative expression levels compared to control normalized to either total eIF2α or actin. The blots are representative of two independent experiments.

overexpressing cells displayed reduced levels of phosphorylated eIF2α when exposed to Ec, Tg and Tu (Fig. 8C). BiP expression was little changed in response to the ER stress agents compared to control (Fig. 8D) with no observed difference in background expression. Taken together, the divergence of response between eIF2α phosphorylation and BiP induction suggests that the effect of ABC50 is specific for the eIF2α response.

3.10. Ribosomal content and Protein synthesis in ABC50 knockdown or overexpressing cells

Two major biochemical differences observed previously in Ec-resistant cells were increased ribosomal content and sustained protein synthesis in response to Ec [10]. As shown in Fig. 9A and B, we observed a trend toward decreased ribosomal RNA and Protein in ABC50 knockdown cells and increased levels in ABC50 over-expressing cells. To test the effect of altered ABC50 expression on protein synthesis, we exposed ABC50 knocked-down or over-expressing cells to Ec and measured global protein synthesis rates. As shown in Fig. 10A, exposure of control cells to Ec resulted in a significant decrease in protein synthesis levels. Interestingly, ABC50 knocked down cells displayed a lower base rate of protein synthesis compared to control. Addition of Ec reduced protein synthesis rates even further. In contrast, ABC50 overexpressing cells displayed a slightly higher level of protein synthesis

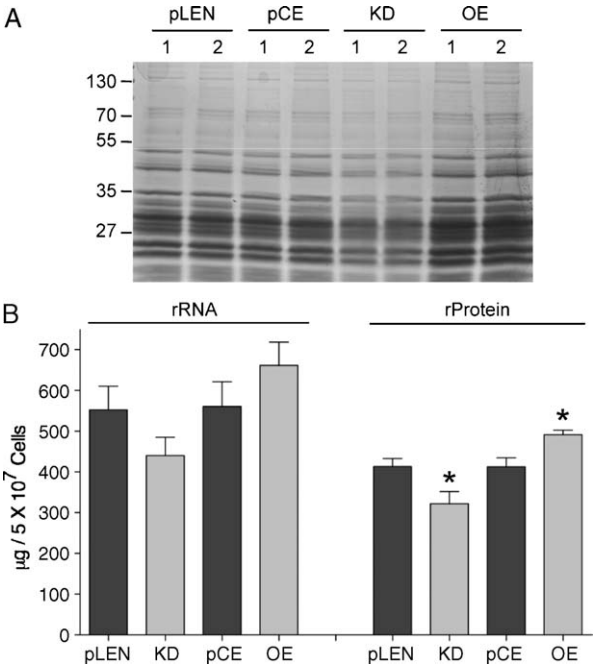

Fig. 9. Effect of ABC50 knockdown or overexpression on ribosomal RNA and Protein content. Ribosomes were purified as described in Section 2. (A) Total ribosomal proteins obtained from 2 independent cultures and extractions were analysed by electrophoresis on a 12% SDS-PAGE gel. The gel was then stained with Coomassie Brilliant Blue to visualize the protein bands. Molecular weight marker migration is indicated on the left. (B) rRNA and rProtein content as measured by absorbance.

compared to control cells and this level was significantly less reduced after exposure to Ec (Fig. 10B). Taken together, these observations indicate that altered ABC50 expression affects ribosomal content, basal protein synthesis and modifies the cellular response to Ec on protein synthesis.

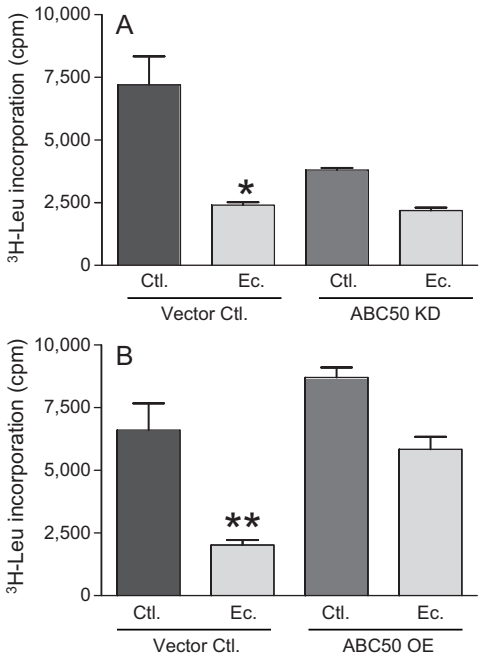

Fig. 10. Effect of ABC50 knockdown or overexpression on global protein synthesis. Cells were incubated with 15 μM Ec for 15 min, pulse-labeled with ^{3}H-leucine and incorporation was measured as described in Section 2. (A) Vector control vs. ABC50 knock-down. (B) Vector control vs. ABC50 overexpression. The values are averages and standard errors from 4 replicates. This experiment was repeated six times. $^{*}p < 0.05$, $^{**}p < 0.01$ comparing knockdown or overexpressing cells to their vector control.

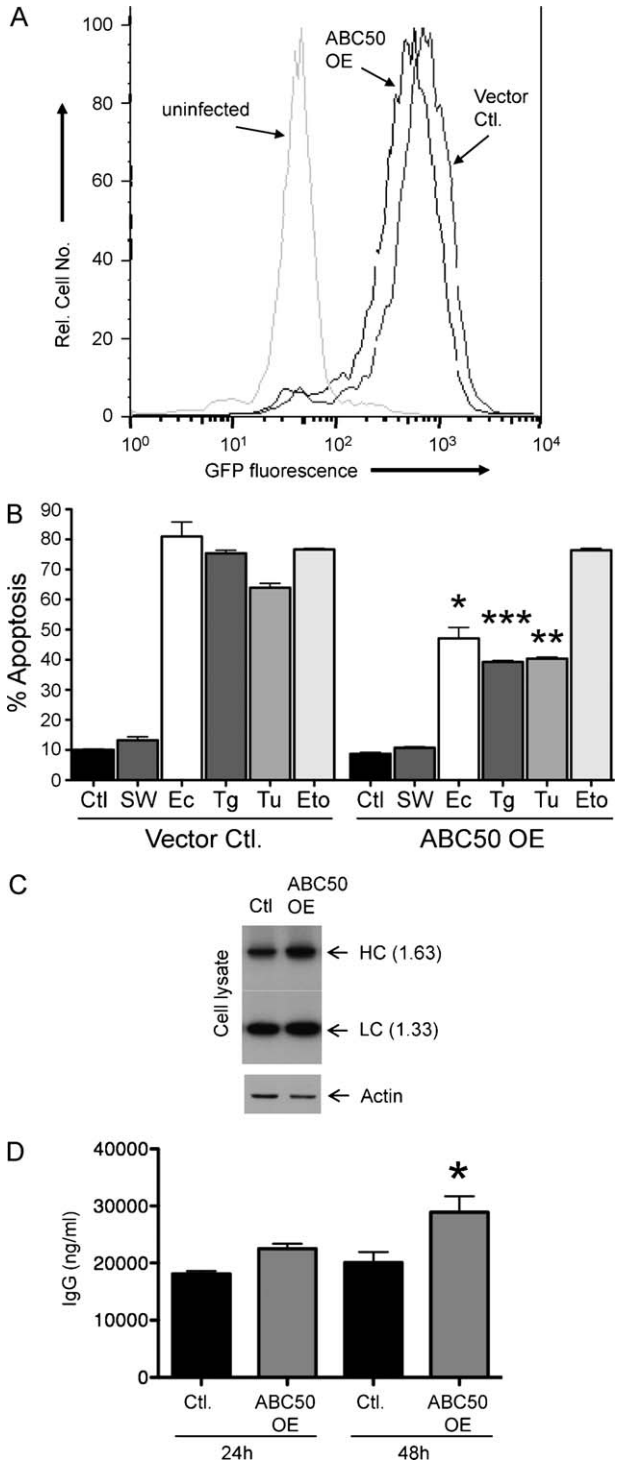

Fig. 11. ABC50 overexpression increases IgG production in hybridoma GK1.5. (A) GK1.5 cells were infected with empty vector control or ABC50 overexpressing virus, then sorted for GFP expression. GFP expression levels were measured by flow cytometry. (B) Apoptosis induction by serum withdrawal (SW), Ec, Tg, Tu and etoposide (Eto) in GK1.5 vector control and ABC50 overexpressing cells. Cells were exposed to 15 μM Ec for 2 h followed by overnight recovery as described in Section 2. Cells were incubated overnight in the absence of serum, 200 nM Tg, 1 μM Tu or 5 μM etoposide. The following day, cells were stained with PI and AnnexinV and analysed by flow cytometry. AnnexinV positive, PI negative cells represent early apoptotic cells, AnnexinV positive, PI positive cells represent late apoptotic or necrotic cells. Plotted is early and late apoptotic cells combined. (C) Control or ABC50 overexpressing cells were seeded at 1×10^6 cells/ml, cultured for 24 h, the cells were pelleted, lysed in RIPA buffer with protease inhibitors and cell lysates were resolved by SDS-PAGE and blotted with rabbit anti-sera specific for heavy and light chains. H: antibody heavy chain, L: antibody light chain. The numbers in brackets represent the ratio of band intensities (as determined by densitometry) for ABC50 overexpressing vs. control. The ratio is the average of three independent measurements. (C) Cell supernatants were collected at 24 and 48 h and IgG levels were measured by ELISA. The values are averages of two determinations. This experiment was repeated three times. $^*p < 0.05$ comparing overexpressing cells to their vector control.

3.11. Enhanced IgG production in ABC50 overexpressing hybridoma cells

The observation that ABC50 expression influenced global protein synthesis levels suggested that it might also affect expression of individual proteins. This property might be of utility in enhancing production of useful proteins, particularly in cells expressing high amounts of specific proteins such as hybridomas. To test this possibility, we infected the hybridoma cell line GK1.5 with the ABC50-expressing lentivirus, sorted infected cells using GFP expression as a marker of infection (Fig. 11A), verified that ABC50 OE decreased sensitivity to ER stress agents (Fig. 11B) and measured antibody production by Western blotting and ELISA. As shown in Fig. 11C, GK1.5 cells infected with the ABC50 expressing virus produced significantly more antibody heavy and light chains compared to vector control. ELISA analysis of antibody concentrations secreted into the supernatant indicated that antibody production was 44% higher at 48 h in ABC50 overexpressing cells compared to control cultures (Fig. 11D). This result suggests that ABC50 overexpression might be useful in boosting protein expression of specific gene products.

4. Discussion

Ec induces ER stress and cell death through the sustained depletion of ER Ca^{2+} stores. This is caused by blocking Ca^{2+} influx at the plasma membrane and stimulating ER Ca^{2+} release through ROS generation at the mitochondria. One consequence of this Ca^{2+} depletion effect is profound and sustained inhibition of protein synthesis [5]. The generation and characterization of Ec-resistant mutants further supported the importance of Ca^{2+} depletion and protein synthesis inhibition by demonstrating increased influx and increased ribosomal content and function in resistant cells. Here we have investigated a role for the protein ABC50 in Ec resistance. ABC50 was identified as an overexpressed gene in Ec-resistant E2R2 cells. Western blot analysis demonstrated that protein levels were increased by 80% compared to WT cells. Knockdown of ABC50 in both HL60 and E2R2 cells increased sensitivity to Ec indicating that ABC50 contributes to resistance. ABC50 was also found to modulate sensitivity to Tg and Tu, other ER stress agents, but not serum withdrawal or etoposide. ABC50 knockdown had no effect on ROS generation, ER Ca^{2+} content or influx, but reduced ribosomal content and protein synthesis in knocked-down cells and increased ribosomal content and protein synthesis in HL60 cells overexpressing the protein. Taken together, these results indicate that ABC50 affects sensitivity to Ec and other ER stress agents, likely through its effects on protein synthesis.

It is of interest to contrast the effect of ABC50 knock-down with ABC50 overexpression. While the knock-down significantly increased the ER stress indicator phospho-eIF2α, decreased protein synthesis and increased sensitivity to Ec, overexpression relieved ER stress induction of phospho-eIF2α and protein synthesis, and had a modest but significant effect on ER stress sensitivity. The observation of decreased protein synthesis after knockdown, and increased protein synthesis through ABC50 overexpression differs somewhat from the recent work of Paytubi et al. who observed a lack of effect on protein synthesis after overexpression in HEK293 cells [4]. These observations indicate that a reduction in its protein level may make ABC50 rate-limiting for protein synthesis while the variable effect from overexpression

indicates that ABC50 may not be rate-limiting in all cells. As well, while ABC50 overexpression did partially prevent full inhibition of protein synthesis by Ec, this effect was insufficient to provide full protection from Ec-induced apoptosis. This observation may indicate that full resistance to Ec requires both altered Ca^{2+} influx as well as increased protein synthesis.

We showed that manipulating ABC50 expression levels also altered sensitivity to the classic ER stress inducers Tg and Tu. Tg, like Ec, depletes the ER of Ca^{2+}. However unlike Ec which blocks Ca^{2+} influx, ER depletion by Tg overstimulates influx resulting in very high cytoplasmic Ca^{2+} levels (Fig. 7A and C). This Ca^{2+} overload response likely contributes significantly to Tg-induced apoptosis, as we documented previously in mast cells [5]. Therefore the partial effect of ABC50 knockdown on Tg sensitivity may reflect the relative importance of Ca^{2+} overload compared to ER stress in Tg toxicity. Tu is a glycosylation inhibitor and induces ER stress through the Unfolded Protein Stress Response [17,18]. Since one consequence of ER stress induction is suppression of protein synthesis, it is possible that ABC50 knockdown promotes Tu toxicity through a combined effect on protein synthesis. Nevertheless, the fact that ABC50 overexpression partially protects cells from Ec, Tg and Tu indicates that its overexpression contributed to the multi-drug resistance phenotype of E2R2 cells.

As shown in Fig. 8, we observed increased phosphorylation of eIF2α in response to ER stress when ABC50 was knocked down, and decreased levels when ABC50 was overexpressed. This effect may be associated with the known binding of ABC50 with eIF2α. Tyzack et al. [2] previously commented that they did not observe any effect of ABC50 on eIF2α phosphorylation by RNA PK *in vitro*. Therefore, our observation may reflect the unique environment of ER stressed cells. Alternatively, the effects of ABC50 on eIF2α phosphorylation may be an indirect effect associated with altered cellular stress due to insufficient (or excess) ABC50. The fact that BiP induction is little changed when ABC50 expression is altered argues against a general effect on ER stress. Additional studies will be required in order to resolve this issue.

Ribosomal biogenesis is tightly regulated during growth through the mTOR pathway [19,20]. Cellular stress can also influence ribosome biogenesis through both mTOR and JNK-mediated phosphorylation of the TIF-IA transcription factor [21], resulting in inhibition of rDNA transcription. Our observation of reduced and increased ribosomal content in ABC50 KD or overexpressing cells respectively is unlikely to reflect growth conditions, since growth rate in both cases appeared to be similar to WT. It is possible that altered ribosomal content reflects differences in basal stress levels, as indicated by increased Bip and phospho-eIF2α levels in ABC50KD cells. Future work should address this interesting possibility.

Although modest, the increased protein synthesis due to ABC50 overexpression translated into a significant increase in antibody production by the hybridoma GK1.5. Interestingly, the observed increase of 44% compares favourably with increases in antibody production observed in CHO cells when the ER stress transcription factor XBP1 is overexpressed [22]. Therefore, increasing ABC50 expression as well as other elements associated with protein translation may be useful for boosting expression of specific proteins of interest. Ota et al. [23] previously identified a genetic linkage between the ABC50 gene locus and increased susceptibility to autoimmune pancreatitis. Since the phenotype of these patients includes increased serum titers of IgG_4, it is possible that ABC50 polymorphisms may contribute to this disease by enhancing antibody production.

In conclusion, ABC50 contributes significantly to Ec resistance. Its mechanism of action appears to be primarily through its modulation of protein synthesis. Future work will address the role of other translation initiation components in mediating Ec resistance or sensitivity.

Acknowledgements

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