Original Article

Downregulation of p68 RNA Helicase (DDX5) Activates a Survival Pathway Involving mTOR and MDM2 Signals

(p68 RNA helicase / mTOR / MDM2 / IRES / c-MYC)

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Abstract. The DEAD box p68 RNA helicase (DDX5) is required to manipulate RNA structures implicated in mRNA/rRNA processing and transcript export, and acts as a co-activator for a range of transcription factors. Previous research has indicated that p68 RNA helicase may also be important in tumour development. Wild-type HeLa and stable HeLa (clone 13) cell cultures containing RNAi-mediated depletion of p68 RNA helicase induced by doxycycline (DOX) were used to study how the p68 RNA helicase affects the mTOR cell signalling pathway. Relevant results were repeated using transient transfection with pSuper/pSuper-p68 RNA helicase, containing RNAi-mediated depletion of p68 RNA helicase, to avoid DOX interference. Here we provide strong evidence for the participation of p68 RNA helicase in mTOR regulation. In detail, depletion of this helicase decreases cell growth and activates the mTOR/MDM2 cell survival mechanism, which ultimately leads to inhibition of the pro-apoptotic activity. p68 RNA helicase downregulation strongly stimulates 4E-BP1 phosphorylation, thereby provoking activation of cap-dependent translation. In contrast, the IRES-dependent translation of c-myc is reduced when p68 RNA helicase is depleted, thus indicating that at least this specific translation requires p68 RNA helicase activity to manipulate the complex 5' end of this mRNA. Interestingly, p68 RNA helicase depletion decreases cell growth while activating the mTOR/MDM2 cell survival mechanism. As MDM2 is a known negative regulator of p53, we infer that the activation of the cell survival mechanism may result in inhibition of the pro-apoptotic factor p53. Finally, p68 RNA helicase depletion activates cap-dependent translation and inhibits c-MYC IRES-mediated translation.

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Abbreviations: AML – acute myeloid leukaemia, ANOVA – analysis of variance, DOX – doxycycline, FSC – forward scatter, SSC – side scatter.

Introduction

The DExD/H box RNA helicase family consists of a group of proteins that play important roles in many cellular processes and whose participation is clearly linked to the modulation of secondary/tertiary RNA structures during RNA processing, RNA export, as well as ribosome assembly and translation. It has also been postulated that these helicases play an important role in transcription regulation (Nicol and Fuller-Pace, 2010; Fuller-Pace, 2013; Nicol et al., 2013; Zonta et al., 2013).

Numerous studies have implicated p68 RNA helicase to be involved in oncogenic processes, see review by Dai et al. (2014). Specifically, p68 RNA helicase has been shown to be overexpressed in colorectal tumours and post-translational changes or modification of p68 expression may play a role in tumour development (Causevic et al., 2001). In addition, tyrosine phosphorylation on Tyr593 of the p68 RNA helicase has been shown to be associated with cellular transformation and epithelial-mesenchymal transition in colon cancer (Yang et al., 2005, 2006, 2007). p68 RNA helicase has also been found to be overexpressed in prostate cancer (Clark et al., 2008) and multiple myeloma (Felix et al., 2009). Moreover, acute myeloid leukaemia (AML) was reported to be dependent on p68 RNA helicase, and inhibition of DDX5 expression slows AML proliferation (Mazurek et al., 2014). Additionally, one study proposed a model in which the p68 RNA helicase and p53 interplay regulates PLK1 expression in human breast cancer (Iyer et al., 2014). Finally, in HCT116 cells and human diploid fibroblasts, p53 interacts with the Drosha processing complex through association with the p68 RNA helicase (Suzuki et al., 2009).

Our previous results have shown that p68 RNA helicase is an important factor for alternative splicing of proto-oncogene *H-ras* (Guil et al., 2003a; Camats et al., 2008, 2009). This oncogene regulates two proteins, namely p21 and p19 H-Ras. The former induces growth, whereas the latter causes a delay at the G1/S stage, thus

maintaining cells in a reversible quiescence state (Guil et al., 2003b; Camats et al., 2009). Collectively, these findings and those detailed above provide compelling evidence that p68 RNA helicase may play an important role in tumour development.

Cell survival and death decisions are normally controlled by antagonistic signalling pathways, which results in a balance between pro- and anti-apoptotic signals. One good example of this are the antagonistic properties of the p53 and PI3K pathways (Vivanco and Sawyers, 2002; Trotman and Pandolfi, 2003), where a balance between p53 and PI3K activation is vital for the control of cell survival during development and differentiation, as well as in various pathologies (Vivanco and Sawyers, 2002; Trotman and Pandolfi, 2003; Sarkar et al., 2015).

Activation of the PI3K/Akt/mTOR signalling pathway results in a major intracellular crosstalk that affects both growth and survival. Phosphatase PTEN, which can act as both a pro- and anti-apoptotic factor, is one example of a crosstalk factor between PI3K/Akt/mTOR signalling and pro-apoptotic factor p53; the p53 protein activates transcription of *PTEN*, which in turn antagonizes PI3K activation and decreases Akt phosphorylation and activity, and can even modulate p53 activity (Stambolic et al., 2001; Vivanco and Sawyers, 2002; Freeman et al., 2003). Moumen et al. (2007) studied how PI3K/Akt/mTOR signalling prevents p53-promoted cell death and described a novel pathway involving mTOR and MDM2 signals that promote cell survival. Thus, after Hgf stimulation, Met activates the nuclear translocation of MDM2 by stimulating Akt-mTOR via PI3K. Finally, MDM2 represses p53, thereby altering the cell survival/death balance in embryonic cells (Moumen et al., 2007).

The regulatory circuits that control the two different targets of rapamycin complexes, namely TORC1 and TORC2, are some of the most important pathways involved in the regulation of metabolism as well as cell growth, survival, proliferation and differentiation (Bhaskar and Hay, 2007). These circuits have also been implicated in the biogenesis of several diseases, including cancer, diabetes, ageing and degenerative disorders (Bhaskar and Hay, 2007). In brief, activated growth factor receptors themselves activate PI3K, which modulates downstream Akt activation. Activated Akt then phosphorylates TSC2, thus inhibiting the TSC1/2 GAP activity towards small GTPase Rheb, which is required to activate mTORC1. Growth factors can also inhibit TSC1/2 via ERK and RSK (Bhaskar and Hay, 2007). Induction of mRNA translation by mTORC1 is mediated by activation of p70S6K and the eukaryotic translation factor 4E (eIF4E). mTOR regulates translation mainly by phosphorylation/inactivation of mRNA translation repressors 4E-BPs. 4E-BP1 inactivates eIF4E by binding directly to it when hypophosphorylated (Bhaskar and Hay, 2007).

Our previous studies have clearly demonstrated that downregulation of the p68 RNA helicase does not provoke apoptosis in human cells (Camats et al., 2008). To complement these previous studies, we now show that p68 RNA helicase downregulation induces less cell growth and activates a survival mechanism involving mTOR/MDM2 activation, which finally represses the p53 pro-apoptotic factor. Specific inhibition of this survival mechanism increases apoptosis in cells in which p68 RNA helicase activity is minimized.

Material and Methods

Cell culture and transfections

The HeLa cell cultures used herein were described previously (Guil et al., 2003a; Camats et al., 2009). Stable HeLa cells containing p68 RNA helicase RNAi induced by doxycycline (DOX), named clone 13: these stable cells were obtained by co-transfecting pTER-p68 RNA helicase RNAi/pCDNA$_6$/TR. Initial selection was performed with zeocin/blasticidin (200/105 μg/ml) and secondary selection by indirect immunofluorescence detection with anti-p68 RNA helicase antibody in cells induced with 2 μg/ml DOX. Clone 13 was maintained at a final zeocin/blasticidin concentration of 100/5 μg/ml, respectively. Since DOX interfered in some experiments, we used a DOX concentration range of 0.2–2 μg/ml to ensure the quality and reliability of the results. To exclude the possibility of DOX interference, relevant results were repeated with transient transfection with pSuper/pSuper-p68 RNA helicase containing RNAi-mediated depletion of p68 RNA helicase (not induced by DOX). Transient transfection with pSuper and pSuper-p68 RNA helicase has been described previously (Guil et al., 2003a).

Antibodies

Sources of antibodies: anti-GAPDH (Abcam, Bristol, UK); anti-p68 RNA helicase and clone PAb204 (Upstate, Merck Millipore, Darmstadt, Germany); anti-MDM2 and anti-DDX3 (AnaSpec, Fremont, CA); anti c-Raf-1, pTYr^{340} pTYr ^{341}, (Enzo Life Sciences, Farmimgdale, NY); anti-Raf-1(C-12) and sc-133-G (Santa Cruz Biotechnology Inc, Dallas, TX); anti-p-ERK1/2 T202/Y204 and anti-Akt, pan,11E7 (Cell Signalling, Danvers, MA); anti-p-Akt1/2/3 (Thr308)-R and anti-p-Akt1/2/3 (Ser473)-R (Santa Cruz Biotechnology Inc.); anti-phospho-p70S6 kinase (Thr389) and anti-4E-BP proteins #9955 (Cell Signalling, Danvers, MA); anti-c-MYC (Sigma-Aldrich Química SL, Madrid, Spain); MDM2 antagonist and Nutlin-3 (Racemic, # 444143) were obtained from Calbiochem® (EMD Millipore).

Cell proliferation assays

HeLa cells were transiently transfected with pSuper or pSuper-p68 RNA helicase containing the RNAi-mediated depletion of p68 RNA helicase with a small interfering RNA (siRNA) duplex (see Guil et al., 2003a). After three days, cells were collected and plated in 96 wells (500 cells/well) on six microplates in sextuplicate

and incubated at 37 °C, with 5% CO_2 and DMEM/10% FBS. After the appropriate time, the cell culture medium was discarded and the microplates were frozen at –80 °C. Cells were quantified using the green fluorescent dye CyQuant (Invitrogen, in Thermo Fisher Scientific Inc. Waltham, MA) according to the manufacturer's instructions. Fluorescence measurements were performed using a microplate reader with excitation at 485 nm and detection at 530 nm in the Turner BioSystems TD-700 Laboratory Fluorometer (Turner Designs, San Jose, CA). The siRNA duplex sequences were obtained from the following sources: siRNA B negative control siRNA duplex, sc-44230 (Santa Cruz Biotechnology Inc.) and siRNA duplex to H, sc-35579 (Santa Cruz Biotechnology Inc.).

Real-time PCR

MDM2 POWER SYBR-green real-time PCR was performed under standard conditions, as described above, but with oligonucleotides CAG TGA ATC TAC AGG GAC GCC and CTT ACA CCA GCA TCA AGA TCC G.

Apoptosis assays

Clone 13 was treated under the different conditions described in the legend to Fig. 4. The half maximal inhibitory concentration (IC50) of Nutlin-3 in cell culture is 8 µl/ml. Therefore, a stock solution of Nutlin-3 was prepared in DMSO solvent (1 mg/40 µl). Apoptotic cells were detected by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich Química SL) according to the manufacturer's instructions. Flow cytometric experiments were performed using an Epics XL flow cytometer (Coulter Corporation, Miami, FL). Samples were excited using an air-cooled argon-ion laser set at 488 nm and 15 mW power with other parameters as for the standard configuration. Forward scatter (FSC), side scatter (SSC), as well as green fluorescence (525 nm) for annexin-FITC-conjugated antibody and red fluorescence (675 nm) for PI were determined. Green fluorescence was collected with a 550 nm dichroic long-pass filter and 525 nm band-pass filter, whereas red fluorescence was collected with a 645 nm dichroic long-pass filter and a 675 nm band-pass filter. Optical alignment was based on an optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division, Coulter Corp. Miami, FL). Apoptotic (FITC), dead-cell (IP-labelled) and non-altered (i.e., annexin and IP) populations were quantified simultaneously in FITC/IP dot plots.

Rluc/Fluc assay

The Dmp68-containing clone was a generous gift from Dr. Akira Ishizuka (University of Molecular Biology, Tokyo, Japan), whereas pRK5-Dmp68 and pRK5-Dmp68T were obtained by amplifying the sequences containing specific deoxyoligonucleotides and subsequent cloning in pRK5 vector. Dmp68T contains a deletion of the last 32 amino acids and has been shown to be more active in some heterologous HeLa cell complementation assays. PRF-cMYC-IRES was a generous gift from Dr. Debbie (University of Nottingham, UK). This assay was performed using a Dual-Glo™ Luciferase Assay System (Promega Corporation, Madison, WI), according to the manufacturer's instructions. Samples were incubated for 10 min at 22 °C, then measured in a The Turner Designs 20/20 Luminometer (Turner Designs, San Jose, CA).

Statistical analysis

The assays were analysed by Student *t*-test and statistical significance differences calculated by one-way analysis of variance (Anova).

Other methods

Protein extraction for SDS gels and Western blots was performed as described previously (Guil et al., 2003b; Camats et al., 2009). Chemiluminescence reaction was detected for 5 min in LAS-4000 Image analyser (GE Healthcare Life Sciences, Pittsburgh, PA) and quantified using Fujifilm Multi Gauge V3.0 imaging software (GE Healthcare Life Sciences).

Results

Downregulation of p68 RNA helicase induces less cell growth

Previously, we have demonstrated that p68 RNA helicase regulates pre-mRNA H-Ras alternative splicing (Guil et al., 2003a). Indeed, p68 RNA helicase is a negative effector of p19 H-Ras protein expression (Guil et al., 2003a; Camats et al., 2008). Consequently, RNAi-mediated depletion of p68 RNA helicase upregulates endogenous p19 H-Ras protein (Guil et al., 2003b). Previous *in vivo* studies have also shown that p19 H-Ras expression induces cell quiescence (Camats et al., 2009) and that p68 RNA silencing does not induce apoptosis (Camats et al., 2008). In the present study, we made use of these previous findings and we now show that RNAi-mediated depletion of p68 RNA helicase (Fig. 1A) also results in a clear decrease in HeLa cell growth, thus indicating that this helicase is necessary for cell growth.

Our previous studies concluded that hnRNP H is a positive effector for p19 H-Ras expression (Camats et al., 2008). Moreover, cooperation between p68 RNA helicase and hnRNP H was established in epithelial- and myoblast-specific splicing programmes (Dardenne et al., 2014). Therefore, we performed assays containing RNAi-mediated depletion of hnRNP H guided by specific siRNA duplex in HeLa cells, to determine how the downregulation of hnRNP H influences cell growth. Our results showed that the RNAi-mediated depletion of hnRNP H increases cell growth (Fig. 1B, labelled as siRNA) and that hnRNP H downregulation reverses the RNAi-mediated depletion of p68 RNA helicase guided by a specific siRNA duplex: compare Fig. 1A (with 4.6 times cell growth decrease with p68 downregulation by

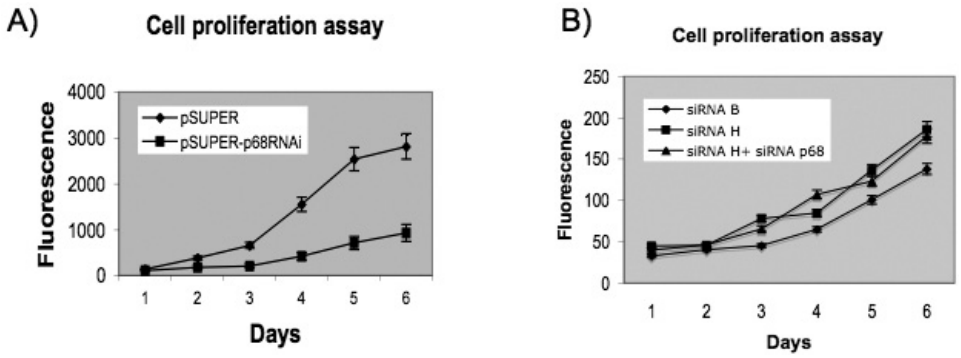


Fig. 1. RNAi-mediated depletion of p68 RNA helicase prevents cell proliferation. **A**) RNAi-mediated depletion of p68 RNA helicase was performed with pSuper vectors as detailed in the Material and Methods section. Empty pSuper vector was used as negative control. **B**) RNAi-mediated depletion of p68 RNA helicase or hnRNP H was performed with specific siRNA duplexes (to hnRNP H and p68 RNA helicase) in a similar manner to panel "A". The negative control, siRNA B duplex from Santa Cruz Biotechnology, was also assayed in parallel on the same day using a microplate as negative control. Three independent experiments were done, each of them per triplicate (N = 9; statistical significance difference calculated by Anova, $P > 0.05$, P value = 0.042).

RNAi-mediated depletion) with Fig. 1B (hnRNP H RNAi-mediated depletion with siRNA duplex + p68 RNAi-mediated depletion with siRNA duplex), thus maintaining cell growth. Taken together, these findings allow us to suggest that the splicing factors p68 RNA helicase and hnRNP H have opposite effects on the cell growth. Indeed, hnRNP H RNAi-mediated depletion reverses the cell growth downregulation induced by RNAi-mediated depletion of p68 RNA helicase.

mTOR signalling is activated after RNAi-mediated depletion of p68 RNA helicase

We observed that p68 RNA helicase downregulation decreases cell growth, but the cells do not proceed to apoptosis/necrosis (Camats et al., 2008). Our previous findings therefore allowed us to postulate that a survival mechanism may have been activated to maintain the cells alive. Moumen et al. (2007) reported a novel pathway involving mTOR and MDM2 signals that regulates the cell survival/death balance. This mechanism involves several mTOR effectors, which finally lead to inhibition of the p53 pro-apoptotic factor (Moumen et al., 2007). In light of this proposed mechanism, we developed new experiments to establish whether this pathway is implicated in the observed cell survival after RNAi-mediated depletion of p68 RNA helicase. Thus, we began by analysing phosphorylation of the 4E-BP1 mTOR effector after RNAi-mediated depletion of p68 RNA helicase. Figure 2 shows that 4E-BP1 is inactivated by phosphorylation (see lanes "+") in cells containing RNAi-mediated depletion of p68 RNA helicase.

This observation indicates that cap-dependent translation is activated after RNAi-mediated depletion of p68 RNA helicase. We subsequently checked other phosphorylation regulations that could activate mTOR. Figures 3A and 3B show that Akt and ERK were not activated after RNAi-mediated depletion of p68 RNA helicase. However, we found that p70S6K was activated

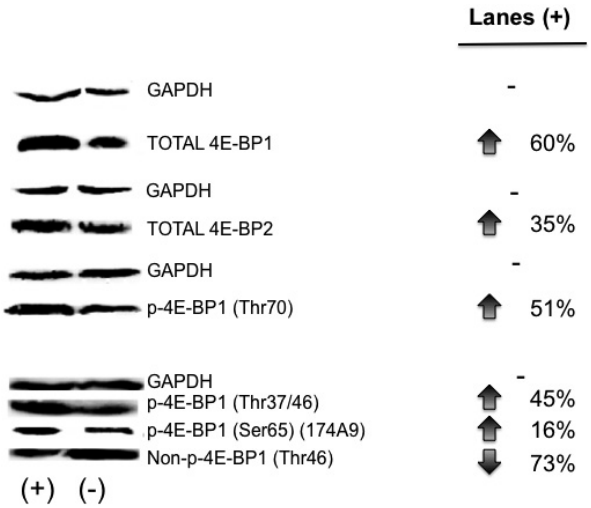


Fig. 2. 4E-BP1 action is inhibited after RNAi-mediated depletion of p68 RNA helicase. HeLa cells were transiently transfected with pSuper empty vector (–) or pSuper-p68 RNA helicase containing the RNA sequences that RNAi-mediated depletion of p68 RNA helicase (+). After three days, the protein extracts obtained from these cells were analysed by Western blot with different anti-4E-BP antibodies. GAPDH antibody was used as internal control for further quantifications. Quantification was performed as detailed in the Material and Methods section. The following proteins were found to be upregulated when p68 RNA helicase was downregulated (on the right): 4E-BP1, 60 %; 4E-BP2, 35 %; p-4E-BP1 (Thr70), 51 %; p-4E-BP1 (Thr37/46), 45 %; and p-4E-BP1 (Ser65), 16 %. Non-phosphorylated 4E-BP1 (Thr46) was downregulated by 73 % (also on the right). Chemiluminescence reaction was detected for 5 min and quantified.

(Fig. 3C, lanes 1 and 2), probably due to the activation of other signalling signals independent of Akt and ERK (see Discussion below). To further determine whether the proposed mTOR survival pathway is implicated in

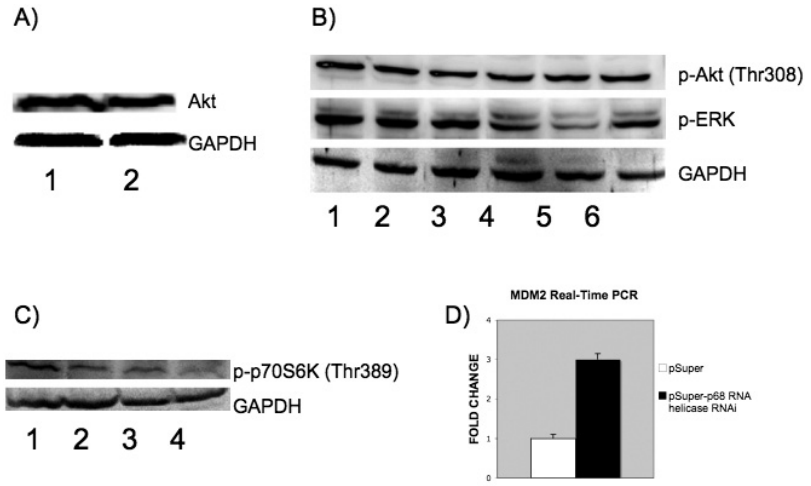


Fig. 3. p68 RNA helicase regulates a cell survival pathway involving mTOR and MDM2 signals. **A–C**) Western blots of cell extracts incubated with the different antibodies listed on the right of the panel. Two cell extracts were used in these experiments, HeLa cells and stable clone 13 containing the RNAi-mediated depletion of p68 RNA helicase activated by DOX. Anti-GAPDH was used as internal control for further quantifications. **A**) HeLa cells transiently transfected with (lane 1) pSuper-p68 RNA helicase that contains the RNA sequences that RNAi-mediated depletion of p68 RNA helicase and (lane 2) pSuper empty vector. **B**) Lanes 1–3: HeLa cells (control cells): lane 1, without DOX; lane 2, with 2 μg/ml DOX and lane 3, with 0.2 μg/ml DOX. Lanes 4–6: stable clone 13 containing the RNAi-mediated depletion of p68 RNA helicase activated by DOX: lane 4, without DOX; lane 5, with 2 μg/ml DOX and lane 6, with 0.2 μg/ml DOX. The downregulation of p-ERK in Panel B lane 5 was quantified as 50 % lower. **C**) HeLa cells were transiently transfected with (lane 1) and (lane 3) pSuper-p68 RNA helicase that contains the RNA sequences that RNAi-mediated depletion of p68 RNA helicase (lane 2) and (lane 4) pSuper empty vector. The upregulation of p-p70S6K in Panel C lanes 1 and 3 was quantified as a twofold change. **D**) Real-time PCR of MDM2 mRNA in HeLa cells transiently transfected with pSuper-p68 RNA helicase containing the RNA sequences that RNAi-mediated depletion of p68 RNA helicase and pSuper empty vector (Student's *t*-test, P = 0.0341). Chemiluminescence reaction was detected for 5 min and quantified.

the observed cell survival/death balance, we studied the MDM2 mRNA levels. As shown in Fig. 3D, MDM2 is upregulated (threefold) when p68 RNA helicase is downregulated. This finding, in combination with the activation of cap-dependent translation, indicates that p53 is inhibited by these higher levels of MDM2 (Moumen et al., 2007). In summary, p68 RNA helicase downregulation activates the mTOR and MDM2 cell survival signals.

MDM2 inhibitor Nutlin-3 increases the amount of apoptotic/necrotic cells

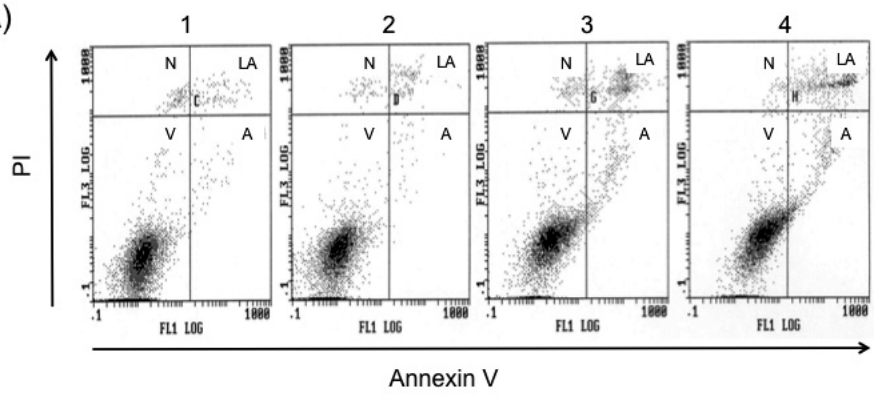
To further assess the mTOR cell survival mechanism when p68 RNA helicase is downregulated, we additionally treated cells containing the RNAi-mediated depletion of p68 RNA helicase with MDM2 inhibitor Nutlin-3. As shown in Fig. 4 A and B, Nutlin-3 clearly increased the amount of apoptotic/necrotic cells present when p68 RNA helicase was downregulated (treatment 4 in panels A and B) compared to control (treatment 3, no DOX plus Nutlin-3). These observations suggest that the mTOR and MDM2 signalling pathway (that is also activated when p68 RNA helicase is downregulated) is essential to prevent the cells to enter in primary/secondary apoptosis when RNAi-mediated depletion of p68 RNA helicase is activated. Then, in this experiment when MDM2 was inhibited, a higher amount of cells progressed into apoptosis (Fig. 4 A and B treatment 4). This observation clearly agrees with the result in Fig. 4 A and B (treatment 2), which shows that when RNAi-mediated depletion of p68 RNA helicase is activated (without Nutlin-3), no apoptosis is induced.

These observations allow us also to conclude that MDM2 upregulation observed in Fig. 3D, when p68 RNA helicase was downregulated, was reverted by the presence of Nutlin-3.

C-Myc is downregulated when p68 RNA helicase is silenced

As cap-dependent translation is activated when p68 RNA helicase is silenced, we next investigated whether the downregulation of p68 RNA helicase also has an effect on IRES-dependent translation. Thus, following similar experiments involving RNAi-mediated depletion of p68 RNA helicase in HeLa cells, we analysed the level of IRES-dependent protein c-Myc. Figure 5 shows that the c-Myc protein is strongly downregulated when p68 RNA helicase is silenced, thus indicating that this helicase might also be necessary for the translation machinery to access the complex secondary/tertiary IRES structure. In order to further support this hypothesis, we performed an *in vivo* assay, with and without DOX, with a reporter Rluc/Fluc plasmid containing the c-Myc IRES sequence with clone 13. As shown in Fig. 5B (col-

A)



B)

		V (%)	A (%)	LA (%)	N (%)
Negative Control (-)DOX (+)DMSO	1	93,2	1,4	2,6	2,6
Negative Control (+)DOX (+)DMSO	2	93,2	1,26	3,51	2,07
(-)DOX and (+)Nutlin-3 in DMSO	3	77,6	7,06	12	3,32
(+)DOX and (+)Nutlin-3 in DMSO	4	67,6	13	18	1,45

Fig. 4. The MDM2 inhibitor Nutlin-3 induces higher apoptosis in cells with downregulated p68 RNA helicase. Nutlin-3 was added to the cell culture as a DMSO solution. Clone 13 treated with (1) no DOX and plus DMSO alone, (2) plus both DOX and DMSO, (3) no DOX plus Nutlin-3 (in DMSO) and (4) plus both DOX and Nutlin-3 (in DMSO). **A**) Flow cytometry experiments (in triplicate) estimating apoptosis levels in clone 13 with the above-described treatments and staining with annexin V-FITC (Y axis) and propidium iodide (X axis). A representative experiment is shown in the figure. Live cells (**V**) are both annexin V and PI negative. At early stage of apoptosis (**A**), the cells bind annexin V while still excluding PI. At late stage of apoptosis (**LA**), they bind annexin V-FITC and stain brightly with PI. Primary necrotic and some very late apoptotic cells (**N**) stain with PI only. **B**) Table representation of the data shown in A), where the percentage of **V, A**, **LA** and **N** cells is detailed.

umn 4; clone 13 with DOX), c-Myc IRES has a lower translation activity when human p68 RNA helicase activity is depleted. We therefore complemented the depleted human 68 RNA helicase activity with Drosophila p68 RNA helicase (Dmp68 and Dmp68T).

Initially we selected p68 RNA helicase from *Drosophila melanogaster* (Dmp68) to complement clone 13 plus DOX, since we observed that the human RNA target for RNAi-mediated depletion of the p68 RNA helicase sequence in clone 13 is quite different from that for Dmp68. Subsequently we obtained the pRK5-Dmp68 human expression vector, along with a second pRK5-Dmp68T vector containing a C-terminal truncated form of Dmp68 (deletion of the last 32 amino acids) and found that the Dmp68T form was more active in some HeLa cell complementation assays. We therefore used both Dmp68 and Dmp68T in specific experiments. Figure 5B shows that both Dmp68T and Dmp68 increased the c-myc IRES-dependent translation (columns 5 and 6, respectively) in clone 13 with DOX, thus indicating that helicase activity is necessary for this specific translation, probably to unwind specific structures of the complex 5' end of the c-MYC mRNA.

Discussion

PI3K/AKT/mTOR is a cell signalling pathway that plays a major role in the regulation of cell growth, the cell cycle and apoptosis (Bhaskar and Hay, 2007). This pathway has been found to be deregulated in human cancers and has been extensively studied as a target for anti-tumour therapy. We have previously shown that p68 RNA helicase inhibits the alternative splicing of H-Ras to render p19 H-Ras and that p19 H-Ras plays quite a different role to p21 H-Ras (Guil et al., 2003b; Camats et al., 2009). Thus, overexpression of p19 induces a delay at the G1/S stage and maintains cells in a reversible quiescence state. We have also shown that p19 H-Ras regulates the mTOR pathway (Camats et al., 2009). The results reported here complement these previous observations by directly assessing the effect of p68 RNA helicase depletion on the mTOR mechanism.

Indeed, we have now demonstrated that RNAi-mediated depletion of p68 RNA helicase induces activation of cap-dependent translation by 4E-BP1 phosphorylation (Fig. 2), thus indicating that p68 RNA helicase downregulation activates the mTOR pathway. We have also successfully demonstrated that RNAi-mediated depletion of p68 RNA helicase does not activate Akt and ERK. However, it does specifically induce phosphorylation of p70S6K, and therefore may activate the mTOR pathway downstream of Akt. For example, other authors showed that amino acids are known to activate mTORC1 independently of TSC1/2 via the class III PI3K, hVps34, and that hypoxia induces expression of REDD1 and REDD2 to activate TSC2 and inhibit mTORC1 (Bhaskar

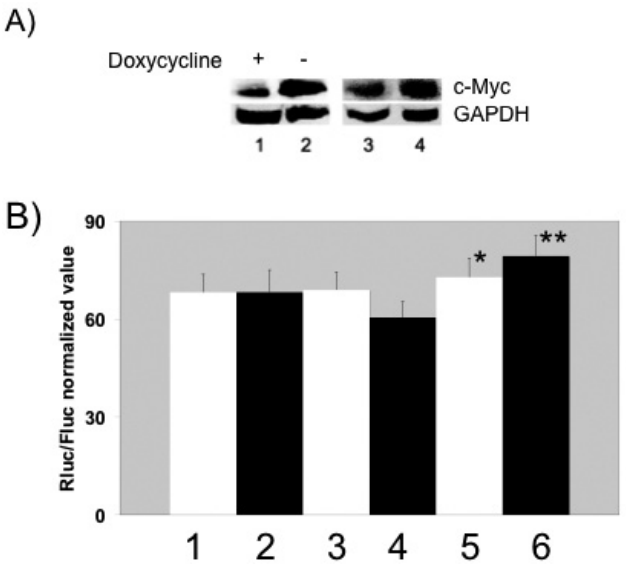


Fig. 5. RNAi-mediated depletion of p68 RNA helicase inhibits IRES-dependent translation. Western blots of protein extracts obtained from different cells and incubated with anti-c-MYC or anti-GAPDH. **A**) lanes 1 and 2, clone 13 treated or not with DOX; lanes 3 and 4, HeLa cells transiently transfected with pSuper-p68 RNA helicase that contains the RNA sequences that RNAi-mediated depletion of p68 RNA helicase or pSuper empty vector, respectively. Chemiluminescence reaction was detected for 5 min and quantified. Quantification of the protein bands indicates that lanes 1 and 3 contained 68-74 % less c-Myc. **B**) pRK5-Dmp68 or pRK5-Dm68T (truncated terminal form, see Material and Methods) and PRF-cMYC-IRES Rluc/Fluc vectors were co-transfected in clone 13 in the presence or absence of DOX. Column 1: PRF empty vector without DOX; column 2: PRF empty vector with DOX; column 3: PRF-cMYC-IRES without DOX; column 4: PRF-cMYC-IRES with DOX; column 5: PRF-CMYC-IRES+ pRK5-Dmp68T with DOX; column 6: PRF-cMYC-IRES+ pRK5-Dmp68 with DOX. Samples were incubated for 10 min at 22 ºC, then measured and quantified. *$P < 0.05$ and **$P < 0.01$, with respect to column 4 (Student's *t*-test).

and Hay, 2007). The ERK hypophosphorylation shown in Fig. 3B may indicate that MAPK signalling is also inhibited when p68 RNA helicase is downregulated, and this question should be addressed in the future.

Our previous results suggested that p68 RNA helicase downregulation does not induce apoptosis (Camats et al., 2008). To complement this previous work, we have shown here that RNAi-mediated depletion of p68 RNA helicase clearly results in less cell growth (Fig. 1). Both these observations prompted us to postulate that a cell survival mechanism might therefore be activated when p68 RNA helicase is depleted. Cell survival is controlled by communication between pro- and anti-apoptotic signals. As we had demonstrated previously that p68 RNA helicase regulates the mTOR pathway, we decided to search for an mTOR-dependent cell survival mechanism. Moumen et al. (2007) described a novel pathway implicating mTOR and MDM2 signals in cell survival. In the present study, we now provide strong evidence for activation of this mTOR-dependent cell survival mechanism when p68 RNA helicase is depleted: MDM2 is expressed at a higher level (Fig. 3), mTOR is activated (Fig. 3) and Nutlin-3 treatment (an MDM2 inhibitor) induces higher apoptosis when p68 RNA is depleted (Fig. 4). This cell survival mechanism, which results in inhibition of the p53 apoptotic factor (Moumen et al., 2007), therefore appears to be activated when p68 RNA helicase is downregulated.

Cap-dependent translation is activated when p68 RNA helicase is depleted (Fig. 2). Interestingly, however, IRES-dependent translation is inhibited when p68 RNA helicase is reduced (Fig. 5) – at least in the case of c-MYC mRNA. Of note is that the helicase activity has been postulated to be necessary for the translation of mRNAs with complex RNA secondary structures at the 5' end (Linder, 2003). One of the helicases implicated in this IRES-dependent translation is DDX3, which enhances hepatitis C virus IRES-mediated translation (Schroder, 2010). We therefore verified whether the DDX3 level is affected by RNAi-mediated depletion of p68 RNA helicase. Our preliminary data indicated that the DDX3 level is unaffected when p68 RNA helicase is downregulated, thereby providing strong evidence for the participation of p68 RNA helicase in IRES-mediated translation. For example, the fact that Dmp68 complements the missing human activity in the HeLa clone 13 (Fig. 5 B) indicates that p68 RNA helicase is necessary, at least for c-MYC IRES-mediated translation, and that this activity is conserved from Drosophila to humans.

Our results provide strong evidence for the participation of the p68 RNA helicase in the regulation of the mTOR pathway. For example, p68 RNA helicase depletion decreases cell growth, but activates the mTOR/MDM2 cell survival mechanism, which ultimately results in inhibition of pro-apoptotic factor p53. Finally, p68 RNA helicase depletion activates cap-dependent translation and inhibits IRES-mediated translation of c-MYC.

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Ethical guidelines statement

This study does not require ethical guidelines statement.

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