Suppression of human Mnk1 by small interfering RNA increases the eukaryotic initiation factor 4F activity in HEK293T cells☆

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Abstract Short-interfering RNAs (siRNAs) have proved to be a useful tool in studying gene function in plants, invertebrates and mammalian systems. Herein, we report the use of siRNAs for targeting the human MAP kinase-interacting kinase Mnk1 gene. This study demonstrates the efficacy of the designed siRNA in silencing Mnk1 in the human cell line HEK293T and shows that Mnk1 suppression decreases eukaryotic initiation factor 4E phosphorylation without causing any change in global protein synthesis rate and cell proliferation. Interestingly, suppression of Mnk1 results in a significant increase in eukaryotic initiation factor 4F complex formation after 72 h of transfection.

Keywords: Eukaryotic initiation factor 4E; Eukaryotic initiation factor 4F; MAP kinase-interacting kinase 1; Translation

1. Introduction

Translation is an important target for regulation of gene expression in response to a large array of extracellular stimuli playing a key role in controlling cell growth and proliferation. A predominant step in translational regulation is the recruitment of the 40S ribosomal subunit to the mRNA. This occurs through 5′ cap structure recognition (m^7GpppX, where "X" is any nucleotide) by the eukaryotic initiation factor 4F (eIF4F). In higher eukaryotes, eIF4F consists of three subunits: eIF4E, the cap-binding subunit, eIF4A, an ATP-dependent RNA helicase, and eIF4G, that serves as a scaffold protein for assembly of eIF4E and eIF4A into the eIF4F complex.

eIF4E is one of the main regulatory initiation factors because it is present in limiting molar amounts in the cell [1,2]. eIF4E has been reported to be phosphorylated on serine 209 following treatment of cells with growth factors, hormones and mitogens [3–5]. It has also been found that inhibition of extracellular signal-regulated kinase (ERK) or p38 mitogen-activated protein kinase (MAP kinase) pathways can inhibit the phosphorylation of eIF4E [6]. MAP kinase-interacting kinase 1, Mnk1, [7,8] is phosphorylated and activated by Erk1/2 and p38 MAP kinases both in vitro and in vivo [7,8] and phosphorylates eIF4E on Ser209 in vitro [8]. Studies conducted in vivo suggest that Mnk1 is the physiological kinase of eIF4E [6,9,10]. Nevertheless, there is conflicting evidence about the role of eIF4E phosphorylation in translational control. Under different situations, a correlation exits between increased phosphorylation and enhanced protein synthesis [11,12]. However, in others, eIF4E phosphorylation has no effect [13,14], or even induces a decrease in the rate of total protein synthesis [15].

The effect of eIF4E phosphorylation on eIF4F activity is also controversial; thus, phosphorylated eIF4E was reported to have higher binding affinity for the cap [16] and to form a more stable eIF4F complex [17]. However, another recently published paper reported that eIF4E phosphorylation markedly reduces its affinity for capped mRNA [18]. In addition, previous studies conducted to determine the role of human Mnk1 in the cells have used several approaches such as overexpression of Mnk1 and/or its constitutively active or inactive mutants and the recently found specific inhibitor of Mnk1, CGP57380 [15]. However, the use of either protein overexpression or pharmacological agents has the potential pitfall of non-physiological or non-specific drug actions, respectively. The double-stranded short-interfering RNAs (siRNA)-mediated RNA interference (RNAi) is emerging as a powerful tool to characterize gene function by genetic loss-of-function analysis. This novel post-transcriptional gene silencing mechanism was first demonstrated with great success in plants, *Caenorhabditis elegans*, *Drosophila* and more recently in cultured mammalian cells as well as in animal models [19–22]. Because of its high efficacy, selectivity and ease of application, we have investigated the functional importance of Mnk1 using this more specific RNAi strategy.

2. Materials and methods

2.1. T7 siRNA design and synthesis

Desalted DNA oligonucleotides were ordered from Sigma-Genosys Ltd. Following the procedure described by Elbashir et al. [20], we designed several double-stranded siRNAs to human Mnk1 with 3′

☆ When this paper was under revision, Ueda et al. [35] have published the generation of mice lacking Mnk1, Mnk2 or both. In this paper, the authors demonstrate that, even though eIF4E phosphorylation is not detected, protein synthesis rate and development are not affected. These findings strongly support our results.

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Abbreviations: 4E-BP, 4E-binding proteins; eIF, eukaryotic initiation factor; ERK, extracellular signal-regulated kinase; IEF, isoelectric focusing; MAP kinase, mitogen-activated protein kinase; Mnk, MAP kinase-interacting kinase; siRNA, short-interfering RNA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RNAi, RNA interference

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overhanging uridine dimers. Target sequences were aligned to the human genome database in a BLAST search to eliminate those with significant homology to other genes. The negative control for Mnk1 was designed scrambling the nucleotide sequence of the gene-specific siRNA and was analyzed in a BLAST search to make sure it lacks homology to any other gene. Four target sequences for Mnk1 gene were selected for testing (see Fig. 1A). For each gene, siRNAs corresponding to sequences located in the 5′, 3′, or central regions of each transcript were deliberately chosen to assess whether different regions are more or less susceptible to siRNA induced degradation. siRNAs were prepared by in vitro transcription using the Ambion Silencer™ siRNA Construction Kit and quantified by standard techniques.

2.2. Cell cultures and RNA interference transfection

Human embryonic kidney (HEK293T) cells were maintained in 75-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified 5% CO_2/95% air incubator at 37 °C. HEK293T cells were subcultured either in 24-well plates at a density of 10^4cells/well for cell viability and protein synthesis rate determination, or in 6-well plates (5×10^4 cells/well) for all other experiments. To suppress Mnk1 expression, cells were transfected 48 h after plating using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the supplier's instructions. Twenty or 100 pmol/well of siRNA in 24-well plates or 6-well plates, respectively, was used.

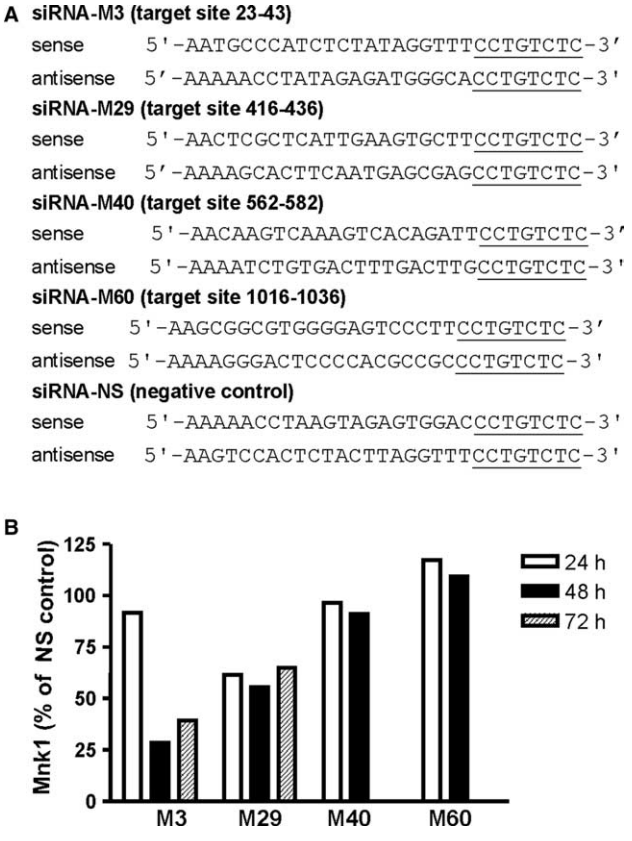

Fig. 1. Mnk1 suppression by siRNA. (A) Template sequences for siRNA duplexes formation. A pair of sense and antisense templates was designed to generate siRNA duplexes. The partial T7 promoter sequence (underlined) was also included in each template. In vitro transcription, annealing and purification of siRNA duplexes were performed using the protocol supplied with the silencer siRNA kit (Ambion). (B) HEK293T were transfected with siRNA as described in Section 2 and total lysates (60 μg) were resolved by 10% SDS–PAGE, transferred onto PVDF membranes and immunoblotted with anti-Mnk1(Ct) antibody. Bars represent the average of 1–3 different experiments. Data are expressed as the percentage of Mnk1 levels with respect to siRNA-NS transfected cells (NS control) (set at 100%).

2.3. Cell viability assay

We have measured cell viability by the methylthiazol tetrazolium MTT reduction assay, as described elsewhere [23]. Briefly, the mitochondrial function was determined by measuring the methylthiazol tetrazolium (MTT) reduction ability of HEK293T under the different experimental conditions, using a Cell Proliferation Kit (Boehringer–Mannheim [MTT]). MTT reduction was measured spectrophotometrically at 540 nm.

2.4. Measurement of protein synthesis rate

Protein synthesis rate was assayed in 24-mm diameter multi-well dishes with fresh medium containing 8 μCi/ml of [^{3}H]methionine for 30 min at 37 °C. Cells were harvested and the incorporation of methionine into protein was determined by trichloroacetic acid precipitation as described previously [23].

2.5. Cell lysates preparation

Cells cultured on 35-mm multi-well dishes were washed twice with ice-cold buffer A (20 mM Tris–HCl, pH 7.6, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 2 mM sodium molybdate, 2 mM sodium β-glycerophosphate, 0.2 mM sodium orthovanadate, 120 mM KCl, 1 μg/ml leupeptin and pepstatin A, and 10 μg/ml antipain) and lysed in the same buffer containing 0.5% NP-40 and 0.1% Triton X-100. Cell lysate was centrifuged at $12\,000 \times g$ for 10 min and the supernatants were kept at −80 °C until used. Protein determination was performed by the Bradford method [24].

2.6. SDS–PAGE, isoelectric focusing and immunoblotting

Cell lysates (60 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), at the conditions indicated in the legends of figures, and analyzed by immunoblotting as described previously [25]. Monoclonal GAPDH antibody was from Ambion and anti-Mnk1 antibodies (against amino and carboxy terminus) were obtained from Santa Cruz Biotechnology. To determine eIF4E phosphorylated levels, cell lysates (50 μg) were resolved by horizontal isoelectric focusing (IEF) slab gels and analyzed by immunoblotting as described [25]. Bands were scanned and quantified with an image analyzer equipped with Diversity One software (PDI).

2.7. RT-PCR expression analysis

Mnk1a and Mnk1b mRNAs expression pattern was assayed by reverse transcription-PCR using the Access RT-PCR System (Promega) as described elsewhere [26]. 10 and 50 ng of total RNA obtained from untransfected (UC) or siRNA-NS and siRNA-M3 transfected cells using TRIZOL® Reagent (Invitrogen) were used as template. The primers used in these reactions were 5MNK1b (5′-AAGGACTGGGCACAC-3′) and 3MNK1 (5′-GGCGAATTCTCA-GAGTGCTGTGGGCGG-3′). GAPDH mRNA and specific primers were used as a control. Bands were scanned and quantified as above.

2.8. eIF4F activity

eIF4F complex formation was measured as the amount of eIF4G recovered when eIF4E was purified using m^7GTP–Sepharose as described previously [25]. eIF4G and eIF4E levels in the complex were detected by immunoblot analysis using a polyclonal anti-eIF4G antibody generously provided by Dr. S.J. Morley and the monoclonal anti-eIF4E antibody (BD Biosciences) and quantified as above.

3. Results and discussion

To determine the functional role of Mnk1 in the cells, genetic loss-of-function analysis was performed using siRNA. Four siRNAs were designed by targeting different positions within the coding region of the kinase (Fig. 1A) according to the procedure described by Elbashir et al. [20]. As a negative control, a non-specific siRNA (NS) was also designed. To examine the ability of siRNAs to suppress endogenous Mnk1 expression, human embryonic kidney (HEK293T) cells were transfected with siRNAs. Cell lysates were harvested 24, 48 and 72 h posttransfection and the levels of Mnk1 protein

expression were determined by Western blotting (Fig. 1B). Mnk1 expression strongly decreased in cell transfected with siRNA-M3 and siRNA-M29 (72% and 45%, respectively) after 48 h of transfection, while Mnk1 expression was not suppressed in siRNA-M40 or siRNA-M60 transfected cells. No additional silencing effect was observed in siRNA-M3 and siRNA-M29 transfected cells after 72 h of transfection. Thus, siRNA-M3 was chosen for the study.

To test both the specificity and efficacy of siRNA-M3, we have used siRNA-NS and untransfected cells as controls. Mnk1 expression decreased significantly in siRNA-M3 transfected cells with respect to untransfected or siRNA-NS transfected cells after 48 and 72 h of transfection (Fig. 2A and B). We have recently identified a splice variant of human Mnk1 which has been named as Mnk1b. Human Mnk1b mRNA is homologous to human Mnk1 mRNA but lacks a region corresponding to exon 19, which causes a change in the reading frame generating a stop codon [26]. Because the resulting protein lacks the last 89 amino acids at the C-terminal region that are replaced by 12 amino acids with an entirely new sequence, this variant cannot be detected with the antibody against C-terminal region of human Mnk1 [26]. We have checked Mnk1b levels in siRNA-M3 and siRNA-M29 transfected cells with the antibody against the N-terminal end of Mnk1, which detects both isoforms a and b. As expected, a 30–40% reduction in Mnk1b levels was also observed with both siRNAs (Fig. 2C). RT-PCR analysis of Mnk1a and Mnk1b mRNAs also revealed a 40% reduction in Mnk1a/b mRNA levels with siRNA-M3 treatment as compared to both siRNA-NS treatment and untransfected cells (Fig. 2D), indicating that siRNA-M3 effectively degrades Mnk1 transcripts, which subsequently reduces Mnk1 expression. Altogether, the above results demonstrate that the siRNA designed is specific against human Mnk1.

Next, we have studied the effect of the reduction in the level of Mnk1 on eIF4E phosphorylation, protein synthesis rate, cell proliferation and eIF4F activity. Up to date, four Mnks human eIF4E kinases have been characterized, Mnk1a, Mnk1b, Mnk2a and Mnk2b [7,8,26,27]. Because Mnk2a and Mnk1b have very high cellular basal activities, they are likely to have important consequence for cellular levels of phosphorylated eIF4E and could mask our results [26,28]. However, the low phosphorylated eIF4E levels in HEK293T cells [26] suggest a low abundance of Mnk2a and Mnk1b and make this cell line a good candidate for our studies. Besides, as far as Mnk1b levels are concerned, we demonstrated that they significantly decreased in siRNA-M3 transfected cells in parallel with the decrease observed in Mnk1a levels (Fig. 2). Mnk1 downregu-

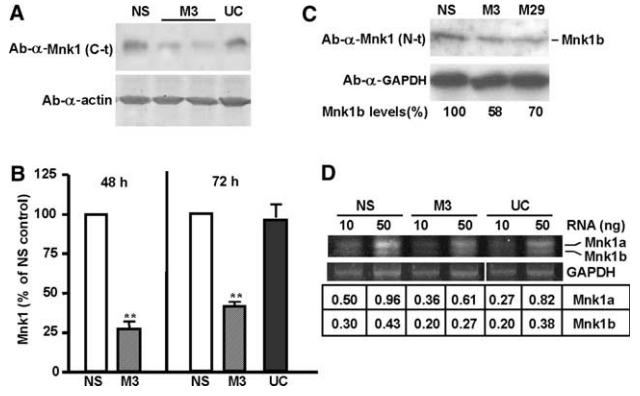

Fig. 2. Mnk1 suppression in HEK293T cells transfected with siRNA-M3. (A) Mnk1 expression in cells transfected with siRNA-M3 was determined as in Fig. 1B. siRNA-NS transfected and untransfected (UC) cells were used as controls. A representative blot from several experiments is shown. As a control of loading, the membranes were reprobed with β-actin antibody. (B) Quantification of the Mnk1 levels. Bars represent means ± S.E.M. of four different experiments. Data are expressed as the percentage of Mnk1 levels with respect to NS control (set at 100%). Statistical significance (calculated by Student's t test) compared to NS control, $^{**}P < 0.01$. (C) Mnk1b expression in cells transfected with siRNA-M3 and siRNA-M29 was determined by immunoblotting at 48 h posttransfection using anti-Mnk1 (Nt) antibody. Membranes were reprobed with anti-GAPDH for control of loading. Numbers at the bottom of the figure show the percentage of Mnk1b with respect to NS control (set at 100%). The figure shows a representative experiment. Similar results were obtained in three separate experiments. (D) RT-PCR analysis of Mnk1a and Mnk1b mRNAs expression was performed using total RNA (10 and 50 ng) from siRNA-NS and siRNA-M3 transfected cells and untransfected cells. GAPDH mRNA levels were used as control. Bands were quantified and the average values (OD × mm^2) from two separate experiments are shown at the bottom of the figure. Keys: Untransfected cells (UC); cells transfected with siRNA-NS (NS), siRNA-M3 (M3), siRNA-M29 (M29).

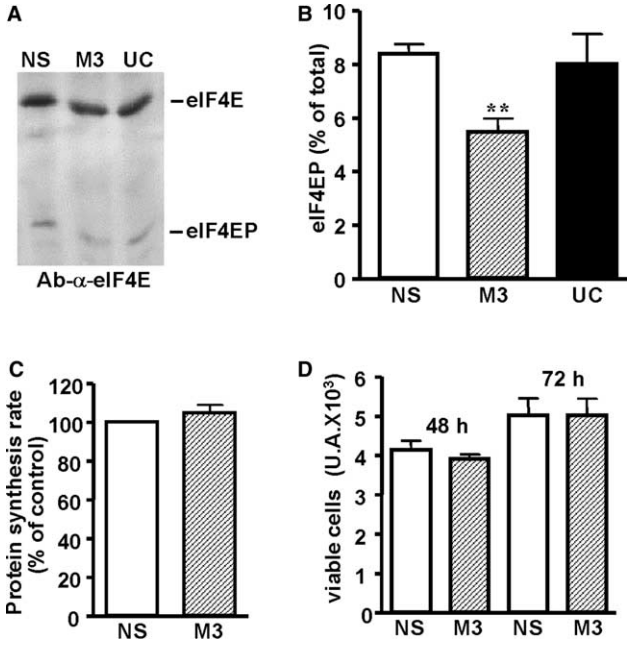

Fig. 3. Effect of Mnk1 suppression on eIF4E phosphorylation (A and B), protein synthesis rate (C) and cell proliferation (D). (A) Cell lysates (50 μg) of HEK293T cells previously transfected with siRNA-NS or siRNA-M3 or untransfected cells (UC) were obtained as described in Section 2 and subjected to IEF. Bands corresponding to unphosphorylated eIF4E (eIF4E) and phosphorylated eIF4E (eIF4EP) were analyzed by protein immunoblot as described in Section 2. Blot is representative of a typical experiment. (B) Results are expressed as percentage of eIF4E P over total eIF4E and represent means ± S.E.M. of 3–5 different experiments. Statistical significance compared to NS control, $^{**}P < 0.01$. (C) Protein synthesis rate was determined as described in Section 2. Results are expressed as the percentage of NS control values and represent means ± SEM of three different experiments. (D) Cell proliferation was determined at 48 and 72 h posttransfection as described in Section 2. Data are expressed in arbitrary units and represent the means ± S.E.M. of four different experiments. No differences were observed in protein synthesis rate (C) and cell proliferation (D) between untransfected cells and siRNA-NS or siRNA-M3 transfected cells (not shown).

lation by siRNA induced a significant reduction of basal eIF4E phosphorylation (Fig. 3A and B), but it did not have any effect on protein synthesis rate (Fig. 3C). This finding is in accordance with the notion that eIF4E phosphorylation does not seem to be essential for the activation of global translation [29]. Cell proliferation, as measured by MTT reduction, was not affected by Mnk1 suppression as well (Fig. 3D), consistent with the findings of Knauf et al. [15] that show no effect on cell proliferation when Mnk1 is inhibited by CGP57380.

The role of eIF4E phosphorylation on eIF4F activity remains to be elucidated. In HEK293 cells, the increased eIF4F activity induced by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate correlates with an increase in eIF4E phosphorylation, while insulin, which also enhances eIF4F formation, does not increase eIF4E phosphorylation [30]. Moreover, several studies have shown that increased eIF4E phosphorylation caused by overexpression of Mnk1 does not induce any change in eIF4E/eIF4G complex formation [15,30,31], but slightly decreases total protein synthesis rate [15,31]. Thus, it is hypothesized that the sustained increase in eIF4E phosphorylation may induce a decreased cap-binding affinity [18,32]. We have examined the effect of Mnk1 suppression on eIF4F activity by measuring the amount of eIF4G recovered when eIF4E was purified using m^7GTP–Sepharose. As shown in Fig. 4, eIF4G bound to eIF4E in cells transfected with siRNA-M3 increased with respect to that found in siRNA-NS, being significant after 72 h posttransfection. However, although total eIF4E and eIF4G levels in transfected cells slightly decreased with respect to untransfected cells (10% and 25%, respectively), no changes between siRNA-NS and siRNA-M3 transfected cells were observed (Fig. 4A). Our results support the hypothesis that the sustained dephosphorylation of eIF4E induced by Mnk1 suppression might generate a slower turnover of eIF4E on capped mRNA, resulting in an increased eIF4F complex formation. In fact, studies from our laboratory performed in primary neuronal culture show that IGF-1 activates eIF4F complex formation in parallel with a decrease in eIF4E phosphorylation levels [33]. eIF4E may not be the only Mnk1 substrate, in fact, eIF4G appears to be a substrate of Mnk1 [34]. Consequently, the dephosphorylation of other Mnk1 substrates possibly involved in eIF4F formation should not be discarded. Increased eIF4F activity could get the cells ready for changing the program of synthesized proteins in response to the different stimuli, i.e., by allowing the translation of specific transcripts with long and highly structured 5′ untranslated regions, which are extremely dependent on high eIF4F levels.

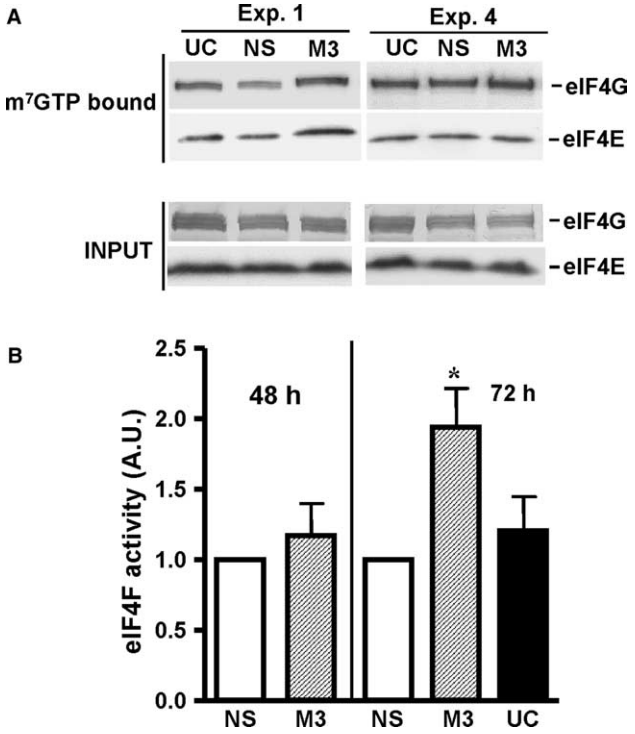

Fig. 4. Effect of Mnk1 suppression on eIF4F complex formation. (A) Cell lysates (100 μg) of HEK293T cells, previously transfected with siRNA-NS or siRNA-M3 during 48 or 72 h, and untransfected cells were subjected to m^7GTP–Sepharose chromatography following immunoblot using antibodies against eIF4E and eIF4G. Blots, corresponding to two different cultures, show eIF4G and eIF4E bound to m^7GTP–Sepharose (upper panel). No differences were found for eIF4E bound to resin (92.2 ± 6.2, 81.3 ± 14.8 and 84.5 ± 20.2 A.U., for untransfected cells (UC), siRNA-NS and siRNA-M3 transfected cells, respectively). Total levels of eIF4G and eIF4E corresponding to 50% input are shown (lower panel). (B) eIF4F complexes were measured as the ratio between eIF4G and eIF4E and were compared to the ratio in NS control cells, which was defined as 1. Bars represent the means ± S.E.M. of four different experiments. Abbreviations and statistical significance are as in Figs. 2 and 3, $^*P < 0.05$.

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References

[1] Duncan, R., Milburn, S.C. and Hershey, J.W. (1987) J. Biol. Chem. 262, 380–388.
[2] Hiremath, L.S., Webb, N.R. and Rhoads, R.E. (1985) J. Biol. Chem. 260, 7843–7849.
[3] Flynn, A. and Proud, C.G. (1995) J. Biol. Chem. 270, 21684–21688.
[4] Joshi, B., Cai, A.L., Keiper, B.D., Minich, W.B., Mendez, R., Beach, C.M., Stepinski, J., Stolarski, R., Darzynkiewicz, E. and Rhoads, R.E. (1995) J. Biol. Chem. 270, 14597–14603.
[5] Makkinje, A., Xiong, H., Li, M. and Damuni, Z. (1995) J. Biol. Chem. 270, 14824–14828.
[6] Wang, X., Flynn, A., Waskiewicz, A.J., Webb, B.L., Vries, R.G., Baines, I.A., Cooper, J.A. and Proud, C.G. (1998) J. Biol. Chem. 273, 9373–9377.
[7] Fukunaga, R. and Hunter, T. (1997) EMBO J. 16, 1921–1933.
[8] Waskiewicz, A.J., Flynn, A., Proud, C.G. and Cooper, J.A. (1997) EMBO J. 16, 1909–1920.
[9] Morley, S.J. and McKendrick, L. (1997) J. Biol. Chem. 272, 17887–17893.
[10] Waskiewicz, A.J., Johnson, J.C., Penn, B., Mahalingam, M., Kimball, S.R. and Cooper, J.A. (1999) Mol. Cell Biol. 19, 1871–1880.
[11] Rhoads, R.E. (1999) J. Biol. Chem. 274, 30337–30340.
[12] Morley, S.J. (2001) Prog. Mol. Subcell. Biol. 27, 1–37.
[13] McKendrick, L., Morley, S.J., Pain, V.M., Jagus, R. and Joshi, B. (2001) Eur. J. Biochem. 268, 5375–5385.
[14] Morley, S.J. and Naegele, S. (2002) J. Biol. Chem. 277, 32855–32859.
[15] Knauf, U., Tschopp, C. and Gram, H. (2001) Mol. Cell Biol. 21, 5500–5511.

[16] Bu, X., Haas, D.W. and Hagedorn, C.H. (1993) J. Biol. Chem. 268, 4975–4978.
[17] Minich, W.B., Balasta, M.L., Goss, D.J. and Rhoads, R.E. (1994) Proc. Natl. Acad. Sci. USA 91, 7668–7672.
[18] Scheper, G.C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D.J. and Proud, C.G. (2002) J. Biol. Chem. 277, 3303–3309.
[19] Crathew, R.W. (2001) Cell Opin. Cell Biol. 13, 244–248.
[20] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature 411, 494–498.
[21] Shi, Y. (2003) Trends Genet. 19, 9–12.
[22] Yang, Y., Kaul, S., Zhang, D., Anantharam, V. and Kanthasamy, A.G. (2004) Mol. Cell Neurosci. 25, 406–421.
[23] Muñoz, F., Martin, M.E., Manso-Tomico, J., Berlanga, J., Salinas, M. and Fando, J.L. (2000) J. Neurochem. 75, 2335–2345.
[24] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[25] Martin, M.E., Muñoz, F.M., Salinas, M. and Fando, J.L. (2000) Biochem. J. 351, 327–334.
[26] O'Loghlen, A., Gonzalez, V.M., Piñeiro, D., Perez-Morgado, M.I., Salinas, M. and Martin, M.E. (2004) Exp. Cell Res. 299, 344–355.
[27] Slentz-Kesler, K., Moore, J.T., Lombard, M., Zhang, J., Hollingsworth, R. and Weiner, M.P. (2000) Genomics 69, 63–71.
[28] Scheper, G.C., Morrice, N.A., Kleijn, M. and Proud, C.G. (2001) Mol. Cell Biol 21, 743–754.
[29] Scheper, G.C. and Proud, C.G. (2002) Eur. J. Biochem. 269, 5350–5359.
[30] Herbert, T.P., Kilhams, G.R., Batty, I.H. and Proud, C.G. (2000) J. Biol. Chem. 275, 11249–11256.
[31] Saghir, A.N., Tuxworth Jr., W.J., Hagedorn, C.H. and McDermott, P.J. (2001) Biochem. J. 356, 557–566.
[32] Zuberek, J., Wyslouch-Cieszynska, A., Niedzwiecka, A., Dadlez, M., Stepinski, J., Augustyniak, W., Gingras, A.C., Zhang, Z., Burley, S.K., Sonenberg, N., Stolarski, R. and Darzynkiewicz, E. (2003) RNA 9, 52–61.
[33] Quevedo, C., Salinas, M. and Alcázar, A. (2002) Biochem. Biophys. Res. Commun. 291, 560–566.
[34] Pyronnet, S., Imataka, H., Gingras, A.C., Fukunaga, R., Hunter, T. and Sonenberg, N. (1999) EMBO J. 18, 270–279.
[35] Ueda, T., Watanabe-Fukunaga, R., Fukuyama, H., Nagata, S. and Fukunaga, R. (2004) Mol. Cell Biol 24, 6539–6549.