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Translational control of the activation of transcription factor NF-κB and production of type I interferon by phosphorylation of the translation factor eIF4E

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Abstract

Type I interferon is an integral component of the antiviral response, and its production is tightly controlled at the levels of transcription and translation. The translation-initiation factor eIF4E is a rate-limiting factor whose activity is regulated by phosphorylation of Ser209. Here we found that mice and fibroblasts in which eIF4E cannot be phosphorylated were less susceptible to virus infection. More production of type I interferon, resulting from less translation of ***Nfkbia*** mRNA (which encodes the inhibitor IκBα), largely explained this phenotype. The lower abundance of IκBα resulted in enhanced activity of the transcription factor NF-κB, which promoted the

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AUTHOR CONTRIBUTIONS

B.H., M.J. and D.W. designed, did and analyzed experiments and wrote the manuscript; Y.V.S. and A.B.R. provided intellectual input and wrote the manuscript; M.K., D.W., T.A., N.R., I.T. and L.F. designed and did experiments; P.S., R.J.O.D., A.S., L.R. and J.H.F. contributed to experiments; R.C. critically reviewed the manuscript; M.C-M. designed experiments and critically reviewed the manuscript; M.O. and E.B. provided reagents; and I.M. and N.S. designed experiments and wrote the manuscript.

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production of IFN-β. Thus, phosphorylation of eIF4E has a key role in antiviral host defense by selectively stimulating the translation of mRNA that encodes a critical suppressor of the innate antiviral response.

The host innate immune system is the first line of defense against invading pathogens, which encompasses viruses[1]. Type I interferon, which includes interferon-α (IFN-α) and IFN-β, is a pivotal component of this system. Rapid synthesis and secretion of these cytokines is crucial for a potent antiviral and immunomodulatory response. The initial induction of type I interferon is dependent on pathogen recognition by pattern-recognition receptors, which survey the extracellular and intracellular milieu. DNA and RNA viruses are recognized by pattern-recognition receptors, including Toll-like receptors, which are present on the cell surface and in endosomes, and several cytoplasmic receptors[2]. The presence of a virus triggers a cascade of events that ultimately results in the activation of several transcription factors, including IRF3, IRF7, ATF2–c-Jun and NF-κB. Those factors, together with the transcription factor IRF1, the transcriptional coactivators CBP and p300 and the architectural protein HMGI(Y), form the IFN-β enhanceosome, which activates transcription of the gene encoding IFN-β (*Ifnb1*)[3]. The NF-κB pathway is particularly crucial in the control of various arms of the innate immune response and is phylogenetically conserved from insects to vertebrates[4]. Under basal conditions, the short-lived inhibitor IκBα binds to NF-κB in the cytoplasm, which prevents translocation of NF-κB to the nucleus and subsequent activation of transcription[5]. After infection with virus or stimulation with tumor necrosis factor, type I interferon or the synthetic RNA duplex poly(I:C), IκBα is phosphorylated by IκB kinases, which results in ubiquitination of IκBα and its degradation by the proteasome[6]. NF-κB then translocates to the nucleus and facilitates transcription of NF-κB-regulated genes, including *Ifnb1* (ref. 7). The synthesis of most components the type I interferon pathway, including regulators and interferon itself, requires stringent control, which is accomplished at transcriptional and translational levels[8,9].

Translational control enables the cell to instantly adjust to its environment by regulating the translation rate of selected mRNAs. It is thus ideally suited for the rapid responses required for host defense against viruses, which must utilize the cellular translation machinery to produce viral proteins. Under most circumstances, translational control is exerted at the initiation step, at which the ribosome is recruited to the 5′ end of an mRNA bearing the cap structure m^7GpppN (where 'm^7' indicates and 'N' is any nucleotide). The interaction between the ribosome and the mRNA is facilitated by the heterotrimeric eIF4F complex that consists of eIF4E, which directly binds the mRNA 5′-cap structure; eIF4G, a scaffolding protein; and eIF4A, a DEAD-box RNA helicase[10]. The subunit eIF4G interacts with eIF3, which is bound to the small ribosomal subunit, thereby establishing the critical link between the mRNA and the ribosome.

Among translation-initiation factors, eIF4E is the least abundant, and it is thought to be limiting for translation[11]. Thus, regulating eIF4E activity is critical for cellular function. The mitogen-activated protein kinase–interacting kinases Mnk1 and Mnk1 phosphorylate Ser209 of eIF4E[12]. Although the function of eIF4E phosphorylation in various biological contexts remains unclear, it has been shown to control the translation of certain mRNAs that encode

proteins associated with inflammation and cancer[13]. Mnk1 and Mnk1 are the sole kinases known to phosphorylate eIF4E in mice[14]. Although Mnk2 is constitutively active, Mnk1 is regulated by signaling cascades of the mitogen-activated protein kinases p38 and Erk in response to mitogens, growth factors and hormones[15,16].

Phosphorylation of eIF4E is altered during viral infection. Dephosphorylation of eIF4E occurs during infection with influenza virus, adenovirus, encephalomyocarditis virus (EMCV), poliovirus or vesicular stomatitis virus (VSV)[17–20]. In contrast, infection with herpesvirus or poxvirus stimulates Mnk1-dependent phosphorylation of eIF4E[21–24]. Although inhibition of Mnk1 suppresses the replication of herpesvirus and poxvirus[21–24], direct involvement of eIF4E phosphorylation in infection by DNA viruses has not been established. Furthermore, it is unclear how dephosphorylation of eIF4E affects the replication of RNA viruses. To address those issues, we studied mouse embryonic fibroblasts (MEFs) derived from mice in which the serine at position 209 of eIF4E was replaced with alanine (eIF4E(S209A) mice), which prevented phosphorylation of eIF4E at this critical regulatory site. We found that loss of eIF4E phosphorylation in eIF4E(S209A) mice and cells resulted in an enhanced type I interferon immune response that protected against viral infection. We also found that the phosphorylation status of eIF4E controlled IFN-β production by regulating translation of *Nfkbia* mRNA, which encodes IκBα, the repressor of NF-κB. Impaired infection of eIF4E(S209A) cells with RNA or DNA viruses demonstrated direct involvement of eIF4E phosphorylation in viral pathogenesis and host defense.

RESULTS

Impaired VSV replication in cells lacking eIF4E phosphorylation

To understand how eIF4E phosphorylation affects the replication of VSV, a negative-stranded RNA virus, we used spontaneously immortalized MEFs derived from wild-type or eIF4E(S209A) mice[13]. VSV-encoded mRNAs are translated via a cap-dependent mechanism[25]. By immunoblot analysis, we detected strong signals for most viral proteins in wild-type MEFs at 10 h after infection but detected only small amounts of VSV-specific nucleocapsid protein and phosphoprotein in eIF4E(S209A) MEFs (**Fig. 1a**). To exclude the possibility that the lower abundance of viral proteins in infected eIF4E(S209A) MEFs was a consequence of impaired global protein synthesis, we determined that new protein synthesis in uninfected eIF4E(S209A) MEFs was similar to that in wild-type MEFs (**Supplementary Fig. 1a**). In contrast to infected wild-type MEFs, eIF4E(S209A) cells did not show a detectable cytopathic effect (**Fig. 1b**). Plaque assays showed a ~1,000-fold lower viral yield in eIF4E(S209A) MEFs than in wild-type MEFs (**Fig. 1c**). The delayed VSV replication in eIF4E(S209A) MEFs was independent of the genetic background of the host, as we observed less was observed of viral proteins in eIF4E(S209A) MEFs from either C57BL/6 mice (**Supplementary Fig. 1b**) or BALB/c mice (**Supplementary Fig. 1c**). To confirm that the lower susceptibility of eIF4E(S209A) MEFs to VSV infection was a consequence of the S209A substitution rather than an unintended consequence of the gene-targeting manipulation, we reintroduced wild-type eIF4E into eIF4E(S209A) MEFs. Expression of wild-type eIF4E together with eIF4E(S209A) enhanced VSV replication, as shown by more

accumulation of viral protein (**Fig. 1d**) and more production of infectious virus (**Fig. 1e**). These data demonstrated that the impaired replication of VSV in eIF4E(S209A) MEFs was directly associated with the mutated *Eif4e* allele encoding the S209A substitution that precludes eIF4E phosphorylation.

Substitution of the serine at position 209 with phosphomimetic glutamic or aspartic acid residues does not recapitulate the activity of wild-type eIF4E. Phosphomimetic mutants of IF4E have less cap-binding ability[26], less ability to transport mRNA and substantially attenuated ability to transform cells[27,28]. Thus, these mutants do not mimic phosphorylated serine and therefore could not be used to show that the restricted replication of VSV in eIF4E(S209A) MEFs resulted from the inability of eIF4E to be phosphorylated rather than from nonspecific effects of the S209A substitution. Thus, we used MEFs deficient in the eIF4E kinases Mnk1 and Mnk2. Phosphorylation of eIF4E is not observed in cells that lack both kinases[14]. We obtained spontaneously immortalized MEFs derived from wild-type mice and mice doubly deficient in both Mnk1 and Mnk2 (Mnk1-Mnk2-deficient) and infected these MEFs with VSV. As noted for eIF4E(S209A) cells, accumulation of VSV protein was considerably impaired in infected Mnk1-Mnk2-deficient MEFs (**Supplementary Fig. 2a**). Although we detected a pronounced cytopathic effect in infected wild-type cells, Mnk1-Mnk2-deficient MEFs remained unaffected (**Supplementary Fig. 2b**). In sum, these results suggested that precluding phosphorylation of eIF4E via the S209A substitution or by deletion of eIF4E kinases impeded the replication of VSV.

Impaired replication of RNA viruses in eIF4E(S209A) cells

To investigate whether replication of other RNA viruses was similarly impaired in eIF4E(S209A) MEFs or if the replication defect was specific to VSV, we infected wild-type and eIF4E(S209A) MEFs with the alphavirus Sindbis virus or with the picornavirus EMCV. Sindbis virus–encoded mRNA has a 5′-cap structure and is translated in an eIF4E-dependent manner[29], similar to that of VSV[25]. EMCV mRNA lacks a 5′-cap structure and is translated in an eIF4E-independent manner via an internal ribosome entry site[30]. Infection of eIF4E(S209A) MEFs with Sindbis virus or EMCV resulted in less production of viral protein than that did infection of wild-type MEFs, as determined by metabolic [^{35}S]methionine labeling (**Fig. 2a,b**). Because translation of EMCV proteins is cap independent, these results suggested that the lower replication of EMCV in eIF4E(S209A) MEFs was probably a consequence of translational changes in host mRNA(s) whose protein product(s) is (are) essential for the regulation of virus infection.

More production of type I interferon in cells lacking eIF4E phosphorylation

To determine if the lower virus replication in eIF4E(S209A) cells could have been due to enhanced production of type I interferon, we treated wild-type and eIF4E(S209A) MEFs with poly(I:C), a potent inducer of type I interferon (**Supplementary Fig. 3a**). New grew naive wild-type MEFs in the presence of conditioned medium from poly(I:C)-treated wild-type or eIF4E(S209A) cells and then infected the MEFs with VSV. Conditioned medium from poly(I:C)-treated eIF4E(S209A) cells resulted in a VSV yield ~1,300-fold lower than that of control medium from mock-treated cells (**Fig. 3a**), whereas conditioned medium from poly(I:C)-treated wild-type cells resulted in a VSV yield only 50% as much as that of

control medium (**Fig. 3a**). Thus, conditioned medium from poly(I:C)-treated eIF4E(S209A) cells had potent antiviral activity.

To investigate whether IFN-β production was responsible for the antiviral activity detected, we used semiquantitative RT-PCR to measure *Ifnb1* mRNA. Stimulation of eIF4E(S209A) MEFs for 6 h with poly(I:C) produced approximately 12-fold more *Ifnb1* mRNA than did stimulation of wild-type cells the same way (**Fig. 3b**). We also detected much more *Ifih1* mRNA and *Irf7* mRNA, whose transcription is induced by type I interferon and whose protein products are required for poly(I:C)-dependent induction of IFN-β[31,32], after treatment of eIF4E(S209A) MEFs with poly(I:C) (~16-fold for *Irf7* mRNA and ~4-fold for *Ifih1*; **Fig. 3b**), whereas secretion of IFN-β was sixfold greater from eIF4E(S209A) MEFs than from wild-type cells (**Fig. 3c**). These data demonstrated that eIF4E(S209A) MEFs produced more IFN-β.

To show that the greater abundance of IFN-β in the medium of infected eIF4E(S209A) MEFs antagonized viral replication, we cultured cells in the presence of neutralizing antibodies to type I interferon before and during VSV infection. The addition of antibody to IFN-β (anti-IFN-β) alone or in combination with anti-IFN-α enhanced the accumulation of VSV protein (**Fig. 4a**) and viral titers (**Fig. 4b**) in eIF4E(S209A) MEFs, albeit not to the same degree observed in wild-type MEFs (21% of wild-type). The amount of anti-IFN-β used (25 μg/ml) was sufficient to neutralize the secreted IFN-β, as we found that a concentration of this antibody as low as 0.25 μg/ml was saturating for the enhancement of VSV replication (**Supplementary Fig. 3b**). In addition, in EMCV-infected wild-type and eIF4E(S209A) MEFs, control rat immunoglobulin G or isotype-matched monoclonal antibody to IFN-γ failed to cause more replication of EMCV, whereas the addition of an anti-IFN-β enhanced EMCV viral titers by ~100-fold (**Fig. 4c**). Thus, eIF4E(S209A) MEFs produced and secreted more IFN-β than did wild-type MEFs and this greater production of type I interferon in eIF4E(S209A) cells antagonized the replication of two different RNA viruses.

Lower susceptibility of eIF4E(S209A) mice to VSV infection

To assess the susceptibility of eIF4E(S209A) mice to infection with VSV, we infected female wild-type and eIF4E(S209A) mice intranasally with VSV and monitored the mice for 12 d. In this model, VSV is neurovirulent and results in fatal encephalitis, which is preceded by hindlimb paralysis. Although only ~50% of the wild-type mice survived this infection, 87% of the eIF4E(S209A) mice survived this infection ($P = 0.0243$; **Fig. 5a**). Wild-type mice secreted less type I interferon into the serum than did eIF4E(S209A) mice at 48 h after infection ($P = 0.0507$; **Fig. 5b**). These data established that eIF4E(S209A) mice produced more type I interferon in response to viral infection than did wild-type mice and were more resistant to the neurovirulent effects of infection with VSV.

Less translation of *Nfkbia* mRNA due to the eIF4E(S209A) substitution

To investigate the molecular mechanism by which the production of type I interferon was enhanced in eIF4E(S209A) MEFs, we used genome-wide polysome analysis to compare wild-type and eIF4E(S209A) MEFs[13]. Notably, *Nfkbia* mRNA was one of the mRNAs

whose translation was lower in eIF4E(S209A) MEFs than in wild-type cells and was the only mRNA that encodes a protein conspicuously involved in signaling pathways that regulate the expression of type I interferon. IκBα inhibits NF-κB, which is an essential nuclear factor in the activation of transcription[3]. Less IκBβ in eIF4E(S209S) cells would thus be expected to result in more translocation of NF-κB to the nucleus. The amount of IκBα in eIF4E(S209A) MEFs was approximately 50% of that in wild-type cells, as determined by immunoblot analysis (**Fig. 6a**). Ribosome loading of *Nfkbia* mRNA was much lower in eIF4E(S209A) MEFs, as indicated by the prominent shift of *Nfkbia* mRNA toward lighter polysomal fractions (fractions 3–5 in eIF4E(S209A) MEFs, compared with fractions 6–9 in wild-type MEFs; **Fig. 6b**). This indicated that initiation of *Nfkbia* mRNA translation was lower in eIF4E(S209A) MEFs than in wild-type MEFs. In contrast, we detected no apparent change in the polysome distribution of 'eIF4E-insensitive' *Actb* mRNA (which encodes β-actin; **Fig. 6b**). Cap-dependent translation of the VSV mRNA, which encodes the virus matrix protein, and translation of luciferase reporter mRNA driven by an EMCV internal ribosome entry site were not diminished by the eIF4E(S209A) substitution, as determined by polysome profiling (**Supplementary Fig. 4a–c**) and luciferase assay (**Supplementary Fig. 4d**). In addition, depletion of IκBα through the use of short hairpin RNA (shRNA) in wild-type MEFs diminished VSV replication (**Fig. 6c,d**), which provided evidence that diminishing IκBα was sufficient to diminish the production of VSV protein and VSV replication. Together these data demonstrated that translation of *Nfkbia* mRNA was selectively impaired as a consequence of dephosphorylation of eIF4E, which resulted in less IκBα protein.

More NF-κB activity in eIF4E(S209A) cells

Less IκBα in eIF4E(S209A) MEFs would be expected to result in enhanced NF-κB activity[6,7]. We used electrophoretic-mobility shift assay (EMSA) to monitor the translocation of NF-κB nucleus and its DNA-binding activity in nuclear extracts of wild-type and eIF4E(S209A) MEFs and found about threefold more NF-κB [Author: Which figure has this result (threefold)? Note 6e is a blot with no numerical data.] in nuclear extracts of eIF4E(S209A) MEFs than in those of wild-type MEFs under basal conditions (**Fig. 6e**). We detected an increase in the binding of NF-κB to DNA in nuclear protein extracts of wild-type and eIF4E(S209A) cells at 15 and 30 min after incubation with tumor necrosis factor, a potent inducer of NF-κB. However, the increase was threefold greater in extracts of eIF4E(S209A) MEFs than in those of wild-type MEFs. As a controlwe determined that binding of the transcription factors SP1 and AP-1 to DNA was similar in extracts of wild-type and eIF4E(S209A) cells. The difference between wild-type and eIF4E(S209A) MEFs in formation of the NF-κB complex was due to lack of eIF4E phosphorylation in eIF4E(S209A) cells, as the expression of wild-type eIF4E in these cells resulted in 44% less DNA-binding activity of NF-κB (**Supplementary Fig. 5a**). In addition, through the use of an NF-κB-specific reporter plasmid, we confirmed that eIF4E(S209A) MEFs had more NF-κB transcriptional activity under basal conditions (**Supplementary Fig. 5b**). Consistent with those results, expression of the NF-κB target genes *Ccl11*[33] and *Il1b*[34] was enhanced 4-fold and 139-fold, respectively, in untreated eIF4E(S209A) MEFs relative to their expression in wild-type cells (**Supplementary Fig. 5c**). However, the greater abundance of IL-1β did not contribute to the antiviral effect detected in eIF4E(S209A)

MEFs (**Supplementary Fig. 6a,b**). Thus, eIF4E(S209A) MEFs had greater NF-κB activity than did wild-type MEFs, which resulted in enhanced induction of NF-κB target genes.

To show that the greater abundance of IFN-β was due to enhanced transcription via NF-κB, we transfected luciferase-reporter plasmids containing the complete mouse *Ifnb1* promoter or the positive regulatory domain II (PRDII), which is a distinct part of the *Ifnb1* promoter that shares 80% nucleotide homology with the binding site for NF-κB, into eIF4E(S209A) and wild-type MEFs. We observed more luciferase expression for each promoter in eIF4E(S209A) MEFs than in wild-type MEFs (**Supplementary Fig. 7a,b**), which suggested that the greater production of IFN-β in eIF4E(S209A) MEFs was caused by transcriptional activation of *Ifnb1*. We detected no difference between wild-type and eIF4E(S209A) MEFs in the translation of *Ifnb1* mRNA (**Supplementary Fig. 4c**). Together these data indicated that eIF4E phosphorylation modulated the type I interferon response by controlling translation of *Nfkbia* mRNA and, subsequently, NF-κB activation.

Virus-induced eIF4E dephosphorylation and NF-κB activation

To demonstrate the physiological importance of eIF4E dephosphorylation, we assessed the translation of *Nfkbia* mRNA in wild-type MEFs infected with viruses (VSV or EMCV) known to dephosphorylate eIF4E[19,20]. Ribosomal loading of *Nfkbia* mRNA was much lower in VSV-infected wild-type MEFs than in uninfected wild-type MEFs, as indicated by the shift of *Nfkbia* mRNA toward lighter polysomal fractions (**Fig. 7a**, fractions 11–12). We obtained similar results with wild-type cells infected with EMCV (**Fig. 7b**, fractions 8–10). In addition, VSV infection caused NF-κB activation in wild-type cells (**Fig. 7c**) and wild-type mice (**Fig. 7d**). These results suggested that the cellular antiviral response to RNA viruses that dephosphorylate eIF4E involved less translation of *Nfkbia* mRNA and activation of NF-κB.

We next sought to determine whether infection with DNA viruses would result in a similar phenotype. We infected wild-type and eIF4E(S209A) MEFs with the following two DNA viruses known to phosphorylate eIF4E: vaccinia virus (a poxvirus) and herpes simplex virus 1 (HSV-1; a member of the herpesviridae)[21,22]. The replication of vaccinia virus (**Fig. 8a–c**) and HSV-1 (**Fig. 8d**) was impaired in eIF4E(S209A) MEFs relative to their replication in wild-type cells. Hence, absence of the eIF4E-phosphorylation site Ser209 resulted in less replication of DNA and RNA viruses in MEFs, which demonstrated that this post-translational modification of eIF4E acted as a determinant of host susceptibility to a broad spectrum of viruses.

DISCUSSION

The production of type I interferon is critical in the innate host response to limit viral replication. A key cellular component that regulates the production of type I interferon is the transcriptional activator NF-κB[3,35]. Here we have established that activation of NF-κB was regulated by a mechanism that involved phosphorylation of the cap-binding protein eIF4E. Translation of the mRNA encoding IκBα, the short-lived inhibitor of NF-κB, was selectively lower in MEFs from mice with a S209A substitution of eIF4E that prevents eIF4E phosphorylation. Less IκBα resulted in more basal activated NF-κB in

eIF4E(S209A) cells, which in turn enhanced the production of type I interferon. Not only was the replication of several RNA viruses impaired in eIF4E(S209A) MEFs but also eIF4E(S209A) mice were more resistant to the neurological damage caused by VSV infection. Together our results have demonstrated a key role for the phosphorylation of eIF4E in viral pathogenesis and host defense against VSV through its effect on the translation of mRNA that encodes a critical suppressor of the innate antiviral response.

The production of IFN-β is dependent on several transcription factors, including NF-κB, that assemble on the DNA at a region immediately upstream of the *Ifnb1* promoter to form the IFN-β enhanceosome[3,7,36]. As the enhanceosome components exist in suboptimal concentrations in a cell, many cells do not produce IFN-β. Only 20% of an infected cell population reportedly expresses *Ifnb1* mRNA[37,38]. Diminishing the abundance of phosphorylated eIF4E provides a means to rapidly increase NF-κB activity and thereby promote assembly of the IFN-β enhanceosome, especially in cells that would otherwise fail to activate *Ifnb1* transcription. The existence of virus-encoded functions, including the poliovirus protein 3C, which cleaves the RelA subunit of NF-κB[39] and the influenza virus protein NS1, which prevents the activation of NF-κB mediated by double-stranded RNA[40], further illustrates the importance of neutralizing NF-κB-mediated production of type I interferon for efficient propagation of virus.

Diminishing the abundance of phosphorylated eIF4E promoted the activation of NF-κB and production of type I interferon by inhibiting the translation of *Nfkbia* mRNA. Notably, eIF4E dephosphorylation occurs late in infection with several viruses[19,20]. Our data have suggested that dephosphorylation of eIF4E enabled cells to rapidly produce type I interferon via a translational-control mechanism that selectively diminished IκBα and stimulated activation of NF-κB. Although this would be of limited benefit to the cell during the first cycle of viral replication, it could effectively establish an antiviral state in the surrounding cells. Thus, diminishing eIF4E phosphorylation might be a cellular response aimed at limiting the spread of viral infection and protecting the host.

Although enhanced production of type I interferon was the main mechanism that rendered eIF4E(S209A) MEFs more resistant to infection with RNA viruses, the production and secretion of other antiviral cytokinesmight also be involved, as the addition of neutralizing anti-IFN-β to infected eIF4E(S209A) MEFs failed to fully restore VSV replication. Even unstimulated eIF4E(S209A) MEFs secreted more of the NF-κB-dependent cytokines CCL11 (eotaxin) and IL-1β than wild-type MEFs. CCL11 (eotaxin) is a chemokine known to attract eosinophils, and IL-1β is a proinflammatory cytokine that mediates a wide range of immune and inflammatory responses. Despite the enhanced secretion of IL-1β from eIF4E(S209A) cells, neutralization of IL-1β in combination with neutralization of IFN-β did not render eIF4E(S209A) MEFs more susceptible to VSV infection. This observation further demonstrated that IFN-β was the main cytokine that limited viral replication in eIF4E(S209A) MEFs. Systemically, however, secretion of these cytokines could further augment the antiviral phenotype observed in eIF4E(S209A) mice, as recruitment of several cells of the immune response to the site of infection could clear the virus more effectively.

Viruses use various strategies to attenuate the type I interferon response. Indeed, both herpesvirus and poxvirus produce proteins that stimulate the assembly of eIF4F and promote the phosphorylation of eIF4E, which successfully maintains a high concentration of phosphorylated eIF4E[21–23]. Accordingly, eIF4E(S209A) MEFs were more refractory to infection with vaccinia virus or HSV-1 than were wild-type MEFs. These viruses also use many strategies to prevent phosphorylation of eIF2α and are more resistant to type I interferon than are RNA viruses[41,42]. Thus, phosphorylation of eIF4E represents another example of viral strategies to control the type I interferon response by post-translational modification of critical host translation-initiation factors.

Our data have suggested that dephosphorylation of eIF4E acts as an arm of the host response to VSV and EMCV infection. In nature, the interaction between host defense mechanisms and virus countermeasures is balanced and results in limited acute viral replication and persistence in immunocompetent hosts. The S209A substitution of eIF4E distorted this balance by diminishing the synthesis of IκBβ, which resulted in more production of type I interferon. One possibility is that an antiviral mechanism does exist that diminishes the activity of eIF4E. Notably, natural mutations in the gene encoding eIF4E in some plants confer resistance to viral infection[43,44]. Together our findings establish a previously unknown translation-control mechanism involved in the production of type I interferon, a fundamental aspect of innate host defense. Furthermore, they raise the possibility that interfering with the phosphorylation of eIF4E in virus-infected cells could be an additional means of augmenting the cellular innate immune response to viral infection.

ONLINE METHODS

Chemicals and antibodies

Chemicals, poly(I:C) and poly(dI:dC) were from Sigma. FuGENE 6 and RNaseIN were from Roche. Tumor necrosis factor was a gift from M. Olivier (McGill University). [^{35}S]methionine-[^{35}S]cysteine and [γ-^{32}P]dATP were from PerkinElmer. Antibodies were as follows: anti-eIF4E (; BD Biosciences); anti-β-actin (; Sigma); anti-IκBα (; Cell Signaling); antibody to phosphorylated eIF4E (; Novus Biologicals), control rat antibody immunoglobulin G () and neutralizing antibody to mouse IFN-α () or IFN-β (; all from PBL Biomedical Laboratories); goat immunoglobulin G antibody to mouse () and neutralizing anti-IL-1β (; both from R&D Systems); antibody to vaccinia virus (; Virostat); and antibody to VSV proteins (a gift from J. Bell). Superscript III reverse transcriptase was from Invitrogen. The retroviral vectors MSCV-GFP (empty vector control) and MSCV-IF4E[27] were provided by J. Pelletier. The shRNA lentiviral plasmids were from Sigma (pLKO.1-puro control shRNA and IκBα shRNA clone 939 (5′-CCGGGAGTCAGAATTCACAGAGGATCTCGAGATCCTCTGTGAATTCTGACTCTTTTTG-3′). The PRDII luciferase reporter *Ifnb1*PRDII-Luc was provided by M. Gale. The *Ifnb1*-pGL3 and *Nfkb1*-luciferase reporters were provided by D.A. Muruve.

Cells, mice and viruses

Mice were kept in pathogen-free housing. Research involving animals was done according to the regulations of the Canadian Council of Animal Care and with the approval of the

McGill University Animal Care Committee. Wild-type and eIF4E(S209A) mice and MEFs have been described[13]. Mice were backcrossed seven times onto a pure BALB/c background. Mice were born at normal Mendalian ratios and developed normally, without an obvious phenotype. Wild-type and Mnk1-Mnk2-deficient mice were from R. Fukunaga[14]. BHK21, Vero cells and BSC40 cells were from the American Type Culture Collection. The Indiana serotypes of VSV and EMCV have been described[45,46]. Sindbis virus was a gift from J. Berlanga. VSV-GFP was from J. Bell. Vaccinia virus (Western Reserve strain)[22] and HSV-1 Us11-GFP have been described[47].

Viral infection and metabolic radiolabeling

Viral infection and metabolic radiolabeling of cells have been described[46]. For *in vivo* experiments, female BALB/c mice were anesthetized with 2 μl rodent 'cocktail' (5 ml ketamine, 2.5 ml xylazine, 1 ml acepromazine and 1.5 ml sterile saline) per 1 g body weight and were infected intranasally with VSV at a dose of 1×10^4 PFU per mouse. Mice were monitored for 12 d after infection and were killed when hindlimb paralysis developed. Blood was collected 48 h after infection by cardiac puncture and the serum concentration of IFN-β was determined by B16-Blue assay (InvivoGen).

Rescue experiments

The retroviral vector MSCV-IRES-GFP (empty vector control) or MSCV-eIF4E-IRES-GFP[27] was transfected into phoenix-293-T packaging cells. Virus-containing medium was used for infection of eIF4E(S209A) MEFs. Cells were sorted by flow cytometry for expression of green fluorescent protein after 48 h.

Isoelectric focusing

Vertical-slab isoelectric focusing was done as described[21].

Plaque assays

Plaque assays were done as described with confluent monolayers of BHK-21 or Vero cells[46]. Titers of vaccinia virus were determined as described[22].

Poly(I:C) treatment, virus-infection-protection assay and enzyme-linked immunosorbent assay

Wild-type and eIF4E(S209A) MEFs were seeded at a density of 0.7×10^5 cells per well. Cells were treated for 6 h with the appropriate concentration of poly(I:C) through the use of the FuGENE 6 transfection reagent according to the manufacturer's protocol (Roche). Cells were lysed and total RNA was isolated, followed by reverse transcription and semiquantitative PCR. The intensity of bands was quantified with Image J software. Conditioned supernatants were collected and the production of mouse IFN-β was detected by enzyme-linked immunosorbent assay according to the manufacturer's protocol (PBL Biomedical Laboratories). For measurement of antiviral activity, wild-type cells were overlaid for 24 h with conditioned cell medium and were infected for 17–24 h with VSV at an MOI of 0.1 PFU/cell.

Polysome profiles

Cells from five plates 15 cm in diameter at ~80% confluence were used for the preparation of polysomal fractions as described[9]. RNA was isolated from half of each fraction with TRIzol reagent (Invitrogen).

RNA extraction and semiquantitative RT-PCR

RNA was extracted from 0.7×10^5 cells with an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). Superscript III reverse transcriptase, oligo(dT) and 1 μg of total RNA or RNA of polysomal fractions was used according to the manufacturer's protocol for reverse transcription. Each cDNA template (1 μl) was analyzed by semiquantitative PCR with specific primers and Taq DNA polymerase according to the supplier's protocol. The reaction products were in quantifiable range, as determined by serial dilution of mRNA isolated from the cytoplasmic fraction of wild-type MEFs. Primers for *Ifnb*, *Irf7* or *Actb* have been described[9]. Primers for *Ifih1*, *Nfkbia* and VSV-specific M protein were as follows: *Ifih1* sense (TGTCTGTTCTGCAGAGGACAGCTT) and antisense (ACAGAAAGATGAGGTGGTCCAGCA); *Nfkbia* sense (GACGCAGACCTGCACACCCC) and antisense (TGGAGGGCTGTCCGGCCATT); and M protein sense (GTGGCAGCCGCTGTATCCCATT) and anti-sense (CGCAGTGAGCGTGATACTCGGG).

Preparation of nuclear extracts and EMSA

Lungs of mice infected with VSV were isolated and nuclear proteins were extracted as described[48]. Cells grown to a confluence of ~70% in a dish 10 cm in diameter were treated for the appropriate time with tumor necrosis factor (1 ng/ml). Nuclear extracts were prepared and EMSA was done as described[49]. Nuclear extracts were incubated with [γ-^{32}P]dATP-radiolabeled oligonucleotides containing a consensus binding site for NF-κB–c-Rel homodimeric and heterodimeric complexes (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Santa Cruz Biotechnology) or containing a consensus binding site for AP-1–c-Jun homodimer and Jun-Fos heterodimeric complexes (5′-CGCTTGATGACTCAGCCGGAA-3′; Santa Cruz) or a consensus binding site for SP1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′). DNA-protein complexes were resolved from free-labeled DNA by electrophoresis through native 4% (wt/vol) polyacrylamide gels and the dried gels were analyzed by autoradiography.

Cytokine assay

Supernatants of wild-type or eIF4E(S209A) MEFs (1×10^5 cells) were analyzed by the MILLIPLEX MAP Mouse Cytokine/Chemokine according to the manufacturer's protocol (Millipore) 8 h after being seeded.

Luciferase reporter assays

Plasmid DNA (1 μg) and the renilla luciferase plasmid pRL-TK (0.01 μg) were transfected into 3×10^5 cells in a six-well plate through the use of Lipofectamine 2000 (Invitrogen). *In vitro*–transcribed EMCV–IRES–firefly luciferase[46] mRNA (0.5 μg) was transfected together with capped-renilla luciferase mRNA (0.05 μg) into 3×10^5 cells. Luciferase activity was

measured 48 h after transfection of plasmids or 3 h after transfection of mRNA as described[9].

Metabolic labeling and TCA precipitation

Wild-type and eIF4E(S209A) MEFs were grown for 2 h in methionine- and cysteine-free medium supplemented with 10% dialyzed serum. Medium was replaced with methionine-free DMEM containing ^{35}S protein-labeling mix (10 μCi/ml) and 1% FBS. After 30 min, cell monolayers were lysed and the radioactivity incorporated into – material insoluble in 5% trichloroacetic acid was measured. Total protein content was determined with a Bio-Rad Protein Assay. Incorporation was normalized to protein content and is presented as counts per minute.

Nitric oxide production

B10R bone marrow–derived macrophages (3×10^4 cells per well in 96-well plates) were stimulated for 24 h with the appropriate treatment. The production of nitric oxide was assessed by measurement of the accumulation of nitrites in the cell culture medium with the colorimetric Griess Reagent System according to the manufacturer's protocol (Promega).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Olivier (McGill University) for tumor necrosis factor; J. Bell (University of Ottawa) for antibody to VSV proteins and VSV-GFP; J. Pelletier (McGill University) for the retroviral vectors MSCV-GFP and MSCV-IF4E; M. Gale (University of Washington) for the PRDII luciferase reporter *Ifnb1*PRDII-Luc; D.A. Muruve (University of Calgary) for the *Ifnb1*-pGL3 and *Nfkb1* luciferase reporters; R. Fukunaga (Kyoto University) for wild-type and Mnk1-Mnk2-deficient mice; J. Berlanga (Universidad Autónoma de Madrid) for Sindbis virus; and C. Lister for assistance. Supported by the Canadian Institutes of Health Research (MOP-7214 to N. S.), the US National Institutes of Health (I.M., and T32 AI007647 to M.K.) and the Irma Hirschl Trust (I.M.).

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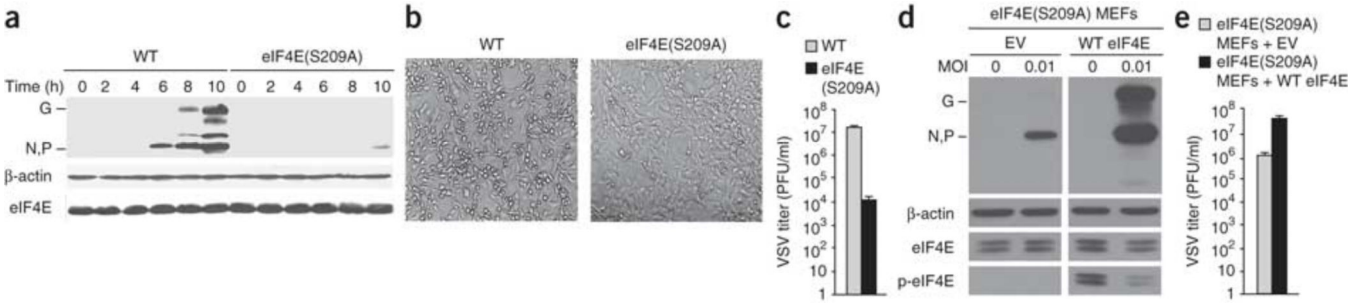

Figure 1.

Replacement of the serine at the eIF4E-phosphorylation site at position 209 with alanine impairs VSV replication. (**a**) Immunoblot analysis of VSV glycoprotein (G nucleocapsid protein (N) and phosphoprotein (P), as well as β-actin (loading control throughout) and eIF4E, in lysates of wild-type and eIF4E(S209A) MEFs mock infected (0) or infected for 2–10 h (above lanes) with VSV at a multiplicity of infection of 1 plaque-forming unit (PFU) per cell. (**b**) Cytopathic effect of VSV in wild-type and eIF4E(S209A) MEFs at 10 h after infection **[**as in **a**. Original magnification, ×00. (**c**) Plaque assay of VSV titers 10 h after infection as in **a**. (**d**) Immunoblot analysis of VSV proteins (as in **a**), β-actin, total eIF4E and phosphorylated (p-) eIF4E in lysates of eIF4E(S209A) MEFs transfected with empty vector (EV) or vector encoding wild-type eIF4E, mock infected (0) or infected for 20 h with VSV at a multiplicity of infection (MOI) of 0.01 PFU/cell. (**e**) Plaque assay of VSV titers in eIF4E(S209A) MEFs transfected and infected as in **d**. Data are representative of three independent experiments (error bars, s.e.m.).

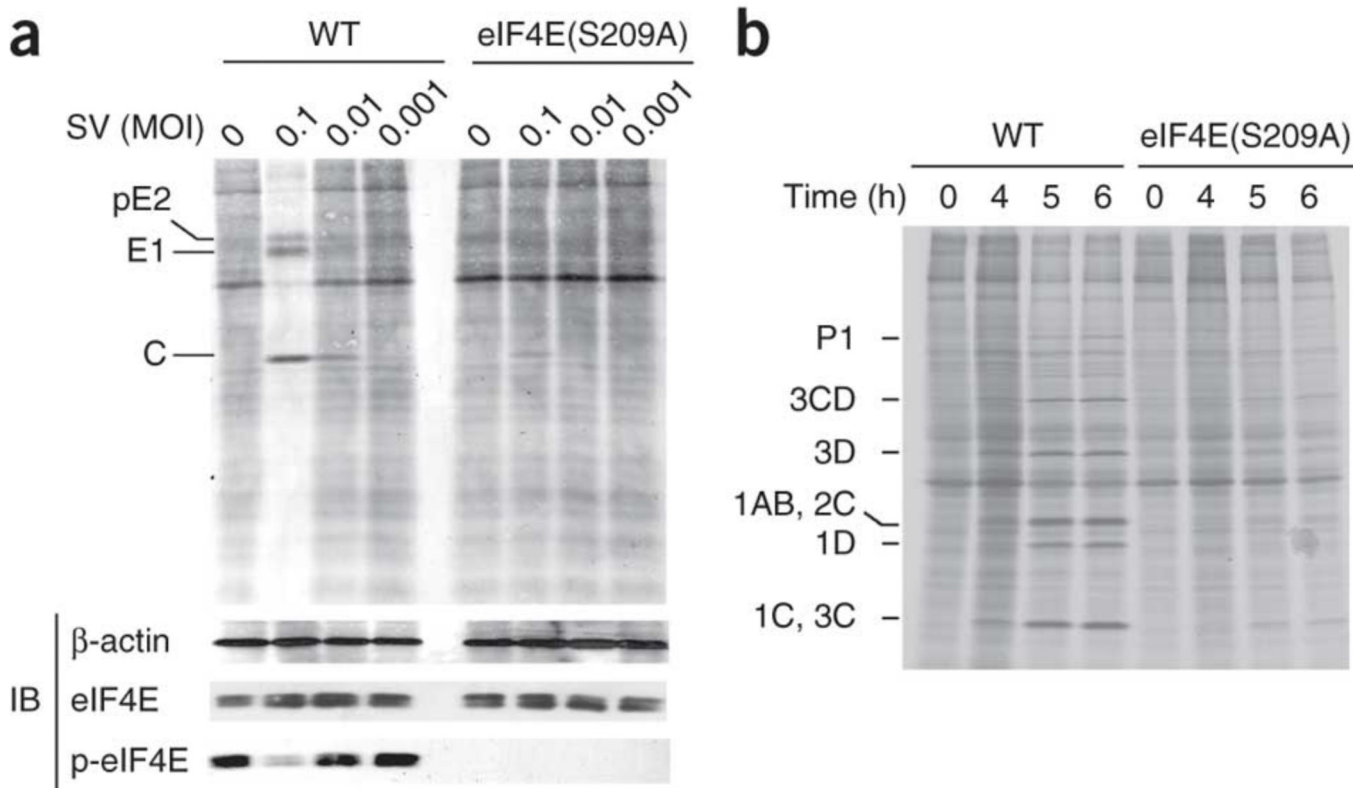

Figure 2.

Restricted viral protein synthesis in eIF4E(S209A) MEFs infected with Sindbis virus or EMCV. (**a**) Autoradiogram (top) of new protein synthesis in wild-type and eIF4E(S209A) MEFs pulse-labeled with [^{35}S]methionine and mock infected (0) or infected for 17 h with Sindbis virus (SV; MOI above lanes); left margin, viral proteins. Below, immunoblot analysis of total and phosphorylated eIF4E and β-actin. (**b**) Autoradiogram of wild-type and eIF4E(S209A) MEFs pulse-labeled with [^{35}S]methionine, deprived of serum for 72 h and mock-infected or infected for 4, 5 or 6 h (above lanes) with EMCV at an MOI of 10 PFU/cell; left margin, viral proteins. Data are representative of three independent experiments.

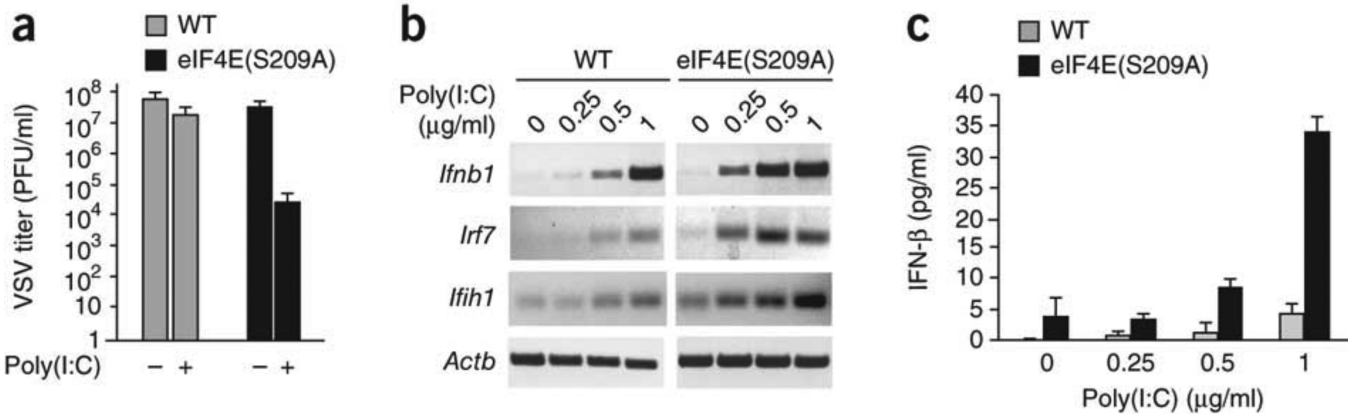

Figure 3.

More production of type I interferon in eIF4E(S209A) MEFs after poly(I:C) stimulation. (**a**) VSV titers in naive wild-type MEFs grown overnight in the presence of conditioned medium from wild-type or eIF4E(S209A) cells (key) left untreated (–) or treated for 6 h (+) with poly(I:C) (0.5 μg/ml); after that overnight incubation, MEFs were infected for 12 h with VSV at an MOI of 0.1 PFU/cell. (**b**) Semiquantitative RT-PCR of *Ifnb1*, *Ifih1* and *Irf7* mRNA among total RNA isolated from wild-type and eIF4E(S209A) MEFs treated for 6 h with various concentrations (above lanes) of poly(I:C). *Actb* encodes β-actin (loading control). (**c**) Enzyme-linked immunosorbent assay of IFN-β in conditioned medium of wild-type and eIF4E(S209A) MEFs treated for 6 h with various concentrations (horizontal axis) of poly(I:C). Data are representative of two independent experiments (error bars, s.e.m.).

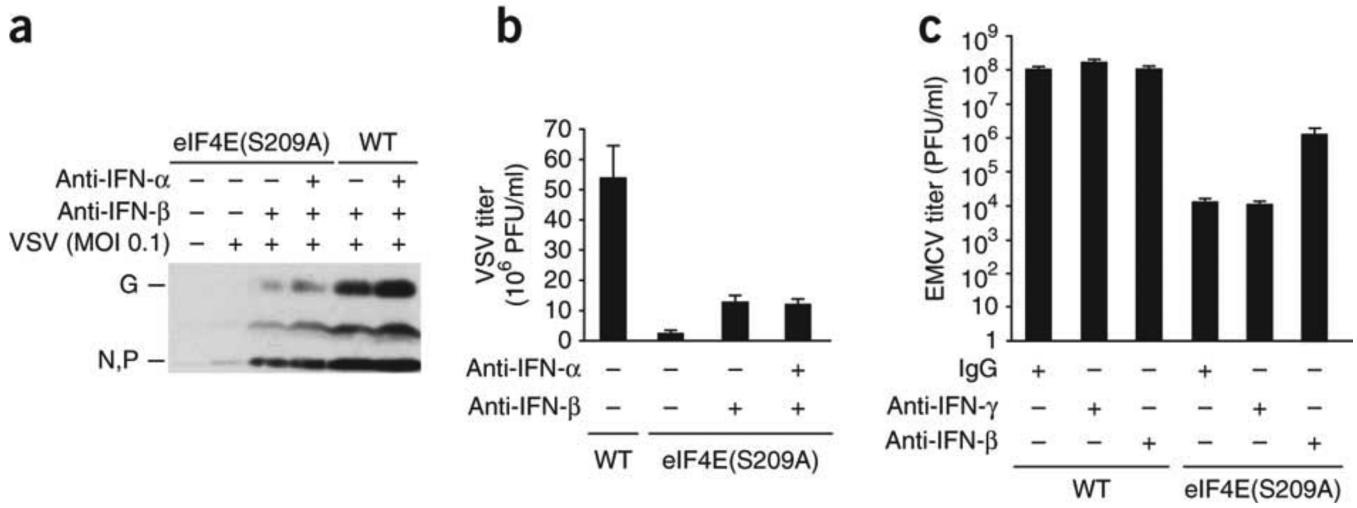

Figure 4.

The addition of neutralizing antibody to IFN-β enhances viral replication in eIF4E(S209A) MEFs. (**a**) Immunoblot analysis of VSV proteins in lysates of wild-type or eIF4E(S209A) MEFs mock treated (–) or treated with anti-IFN-β (25 μg/ml) alone or in combination anti-IFN-α (20 μg/ml), followed by infection for 12 h with VSV at an MOI of 0.1 PFU/cell. (**b**) VSV titers in wild-type or eIF4E(S209A) MEFs infected for 12 h with VSV at an MOI of 0.1 PFU/cell in the presence or absence of neutralizing anti-IFN-β and anti-IFN-α. (**c**) EMCV titers in wild-type or eIF4E(S209A) MEFs deprived of serum (0.5% FBS in DMEM) for 72 h, then treated for 24 h with control antibody (rat immunoglobulin G (IgG; 2.5 ug/ml)) or neutralizing anti-IFN-β and anti-IFN-α and then infected for 48 h with EMCV at an MOI of 0.0005 PFU/cell. Data are representative of two (**a**) or three (**b**,**c**) independent experiments (error bars, s.e.m.).

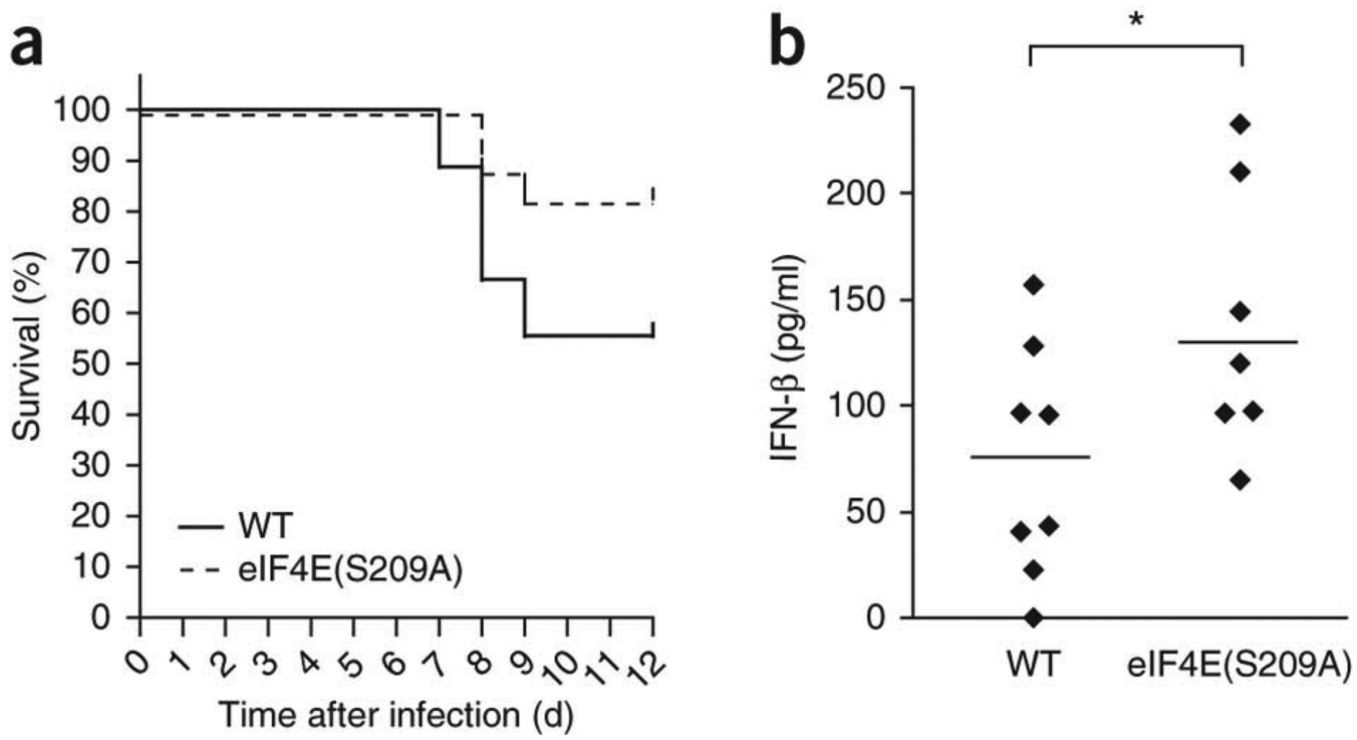

Figure 5.

Enhanced survival of eIF4E(S209A) mice after intranasal infection with VSV. (**a**) Survival of female wild-type mice (*n* = 14) and eIF4E(S209A) mice (*n* = 17) infected intranasally with VSV at a dose of 1×10^4 PFU per mouse and monitored for 12 d (mice were killed when they developed hindlimp paralysis); results are presented as a Kaplan-Meier plot. *P* = 0.0243 **[**IFN-β in blood from wild-type mice (*n* = 8) and eIF4E(S209A) mice (*n* = 7) infected for 48 h with VSV at a dose of 1×10^4 PFU per mouse. Each symbol represents an individual mouse; small horizontal lines indicate the mean. *P* = 0.0507 (). Data are representative of two independent experiments.

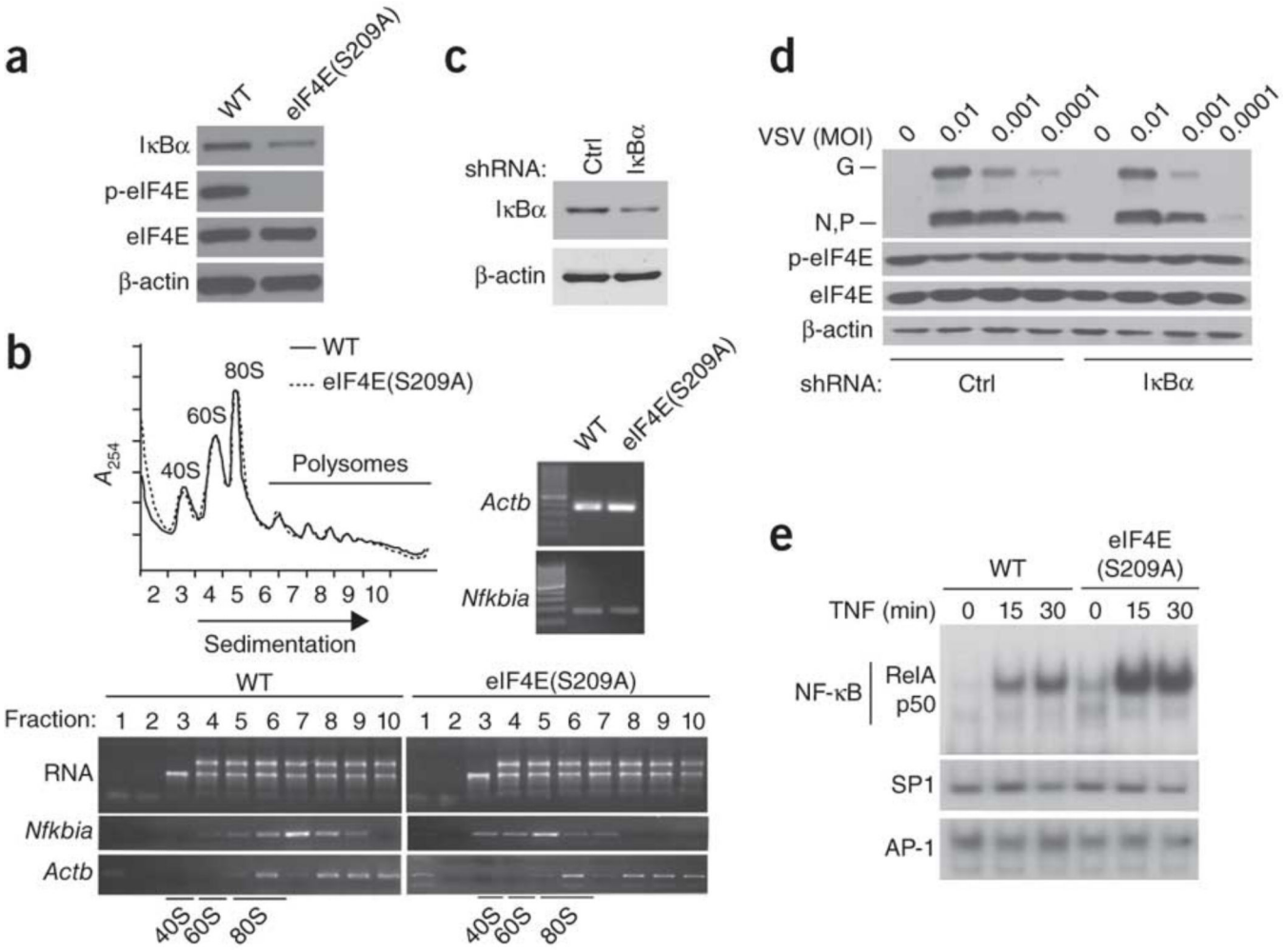

Figure 6.

Less of translation *Nfkbia* mRNA in eIF4E(S209A) MEFs leads to more activation of NF-κB. (**a**) Immunoblot analysis of IκBα, total and phosphorylated eIF4E, and β-actin in wild-type and eIF4E(S209A) MEFs deprived of serum overnight and then stimulated with serum as described[13]; results are presented as absorbance at 254 nm (A_{254}). (**b**) Polysome profiles of wild-type and eIF4E(S209A) MEFs (top left); semiquantitative RT-PCR analysis of cytoplasmic *Actb* and *Nfkbia* mRNA among total RNA isolated from wild-type and eIF4E(S209A) cells (top right); and distribution of ribosomal RNA in individual fractions collected after sucrose-gradient (10– 50%) centrifugation of cytoplasmic extracts of wild-type and eIF4E(S209A) MEFs, with semiquantitative RT-PCR analysis of the distribution of *Nfkbia* and *Actb* mRNA (bottom). (**c**) Immunoblot analysis of IκBα and β-actin in extracts of wild-type MEFs expressing control (Ctrl) shRNA or shRNA targeting IκBα. (**d**) Immunoblot analysis of VSV protein, total and phosphorylated eIF4E, and β-actin in lysates of wild-type MEFs expressing control or IκBα-specific shRNA, mock infected (0) or infected for 17 h with VSV (MOI, above lanes). (**e**) EMSA of NF-κB subunits RelA and p50, as well as of SP1 and AP-1 (controls), in nuclear extracts of wild-type and

eIF4E(S209A) MEFs treated for 0, 15 or 30 min (above lane) with tumor necrosis factor (TNF; 1 ng/ml). Data are representative of two independent experiments.

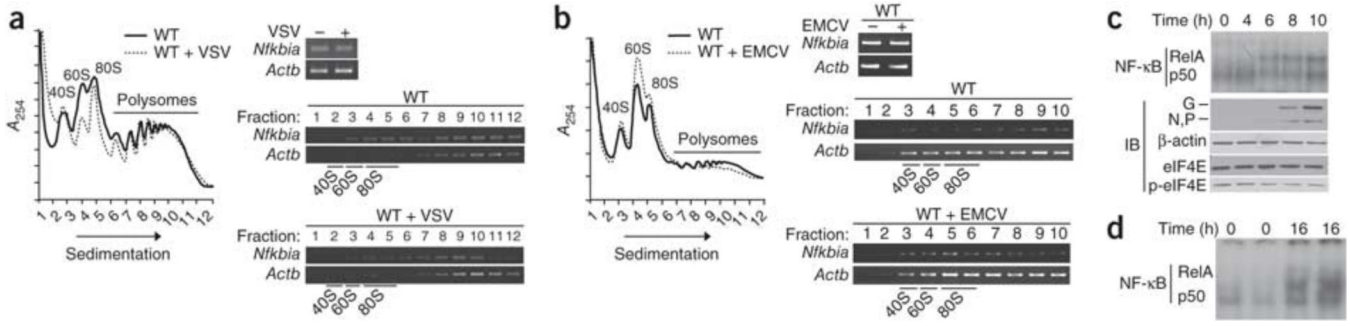

Figure 7.

Less translation of *Nfkbia* transcripts in wild-type MEFs infected with VSV or EMCV. (**a**) Polysome profiles of wild-type MEFs mock infected or infected for 8 h with VSV at an MOI of 1 PFU/cell (left), and semiquantitative RT-PCR analysis of cytoplasmic *Actb* and *Nfkbia* mRNA among total RNA isolated from mock-infected (–) or VSV-infected (+) wild-type MEFs (right, top) and of the distribution of *Nfkbia* and *Actb* mRNA in fractions (collected as in **Fig. 6b**) of cytoplasmic extracts of mock-infected or VSV-infected (+ VSV) wild-type MEFs (right, bottom). (**b**) Polysome profiles of wild-type MEFs mock infected or infected for 6 h with EMCV at an MOI of 0.1 PFU/cell in medium supplemented with 2% serum (left), and semiquantitative RT-PCR analysis of cytoplasmic *Actb* and *Nfkbia* mRNA among total RNA from mock- or EMCV-infected wild-type MEFs (right, top) and of the distribution of *Nfkbia* and *Actb* mRNA in fractions (collected as in **Fig. 6b**) of cytoplasmic extracts of mock- or EMCV-infected wild-type MEFs (right, bottom). (**c**) EMSA of NF-κB in nuclear extracts of wild-type MEFs infected for 0–10 h (above lanes) with VSV at an MOI of 1 PFU/cell (top), and immunoblot analysis (IB) of VSV proteins, β-actin and total and phosphorylated eIF4E in cytoplasmic extracts of those cells (below). (**d**) EMSA of NF-κB in nuclear extracts of lungs from wild-type mice mock infected (0) or infected for 16 h with VSV at a dose of 2×10^6 PFU per mouse; each lane is from the lung of one mouse. Data are representative of two independent experiments.

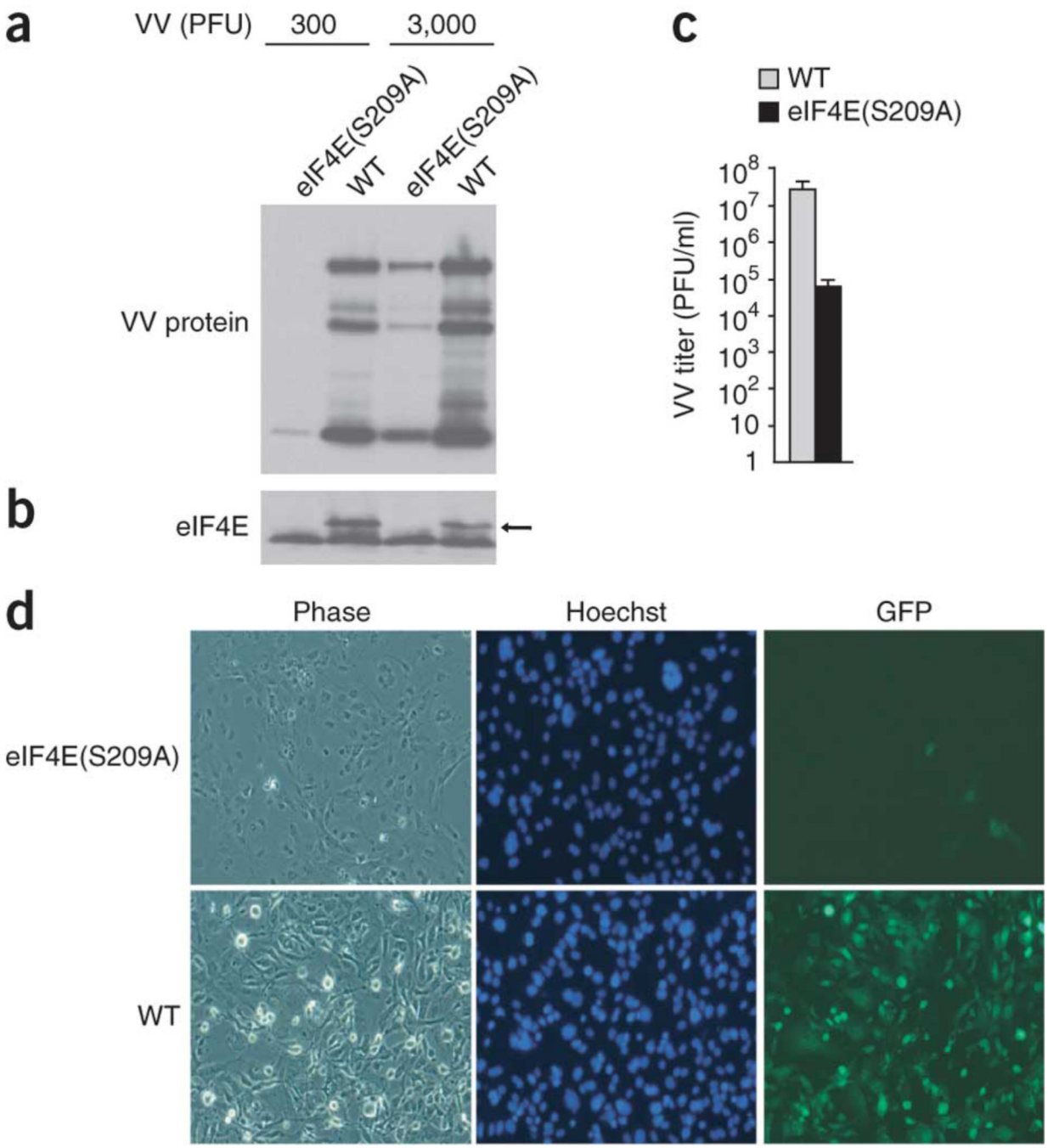

Figure 8.

Less replication of DNA viruses in eIF4E(S209A) MEFs. (**a**) Immunoblot analysis of vaccinia virus proteins (VV) in lysates of wild-type and eIF4E(S209A) MEFs (4×10^5) infected for 65 h with VV (300 or 3,000 PFU). (**b**) Isoelectric focusing of total proteins from **a** and immunoblot analysis of eIF4E; arrow indicates phosphorylated eIF4E. (**c**) Vaccinia virus titers in wild-type and eIF4E(S209A) MEFs (4×10^5) infected for 65 h with vaccinia virus (300 PFU). (**d**) Phase-contrast images (left) and fluorescence images of green fluorescent protein (GFP) and Hoechst staining (middle and right) of wild-type and

eIF4E(S209A) MEFs infected with an HSV-1 strain expressing green fluorescent protein fused to Us11. Original magnification, ×00. Data are representative of two independent experiments (error bars, s.e.m.).