Small molecule ISRIB suppresses the integrated stress response within a defined window of activation

Huib H. Rabouw\textsuperscript{a,1}, Martijn A. Langereis\textsuperscript{a,1}, Aditya A. Anand\textsuperscript{b,c}, Linda J. Visser\textsuperscript{a}, Raoul J. de Groot\textsuperscript{a}, Peter Walter\textsuperscript{b,c,2}, and Frank J. M. van Kuppeveld\textsuperscript{a,2}

\textsuperscript{a}Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, The Netherlands; \textsuperscript{b}Howard Hughes Medical Institute, University of California, San Francisco, CA 94143; and \textsuperscript{c}Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143

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Activation of the integrated stress response (ISR) by a variety of stresses triggers phosphorylation of the α-subunit of translation initiation factor eIF2. P-eIF2α inhibits eIF2B, the guanine nucleotide exchange factor that recycles inactive eIF2α•GDP to active eIF2α•GTP. eIF2 phosphorylation thereby represses translation. Persistent activation of the ISR has been linked to the development of several neurological disorders, and modulation of the ISR promises new therapeutic strategies. Recently, a small-molecule ISR inhibitor (ISRIB) was identified that rescues translation in the presence of P-eIF2α by facilitating the assembly of more active eIF2B. ISRIB enhances cognitive memory processes and has therapeutic effects in brain-injured mice without displaying overt side effects. While using ISRIB to investigate the ISR in picornavirus-infected cells, we observed that ISRIB rescued translation early in infection when P-eIF2α levels were low, but not late in infection when P-eIF2α levels were high. By treating cells with varying concentrations of poly(C) or arsenite to induce the ISR, we provide additional proof that ISRIB is unable to inhibit the ISR when intracellular P-eIF2α concentrations exceed a critical threshold level. Together, our data demonstrate that the effects of pharmacological activation of eIF2B are tuned by P-eIF2α concentration. Thus, ISRIB can mitigate undesirable outcomes of low-level ISR activation that may manifest neurological disease but leaves the cytoprotective effects of acute ISR activation intact. The insensitivity of cells to ISRIB during acute ISR may explain why ISRIB does not cause overt toxic side effects in vivo.

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1H.H.R. and M.A.L. contributed equally to this work.

2To whom correspondence may be addressed. Email: peter@walterlab.ucsf.edu or F.J.M.v.K. vanKuppeveld@uunl.nl.

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The integrated stress response (ISR) protects cells from a variety of harmful stressors by temporarily halting protein synthesis. However, chronic ISR activation has pathological consequences and is linked to several neurological disorders. Pharmacological inhibition of chronic ISR activity emerges as a powerful strategy to treat ISR-mediated neurodegeneration but is typically linked to adverse effects due to the ISR’s importance for normal cellular function. Paradoxically, the small-molecule ISR inhibitor ISRIB has promising therapeutic potential in vivo without overt side effects. We demonstrate here that ISRIB inhibits low-level ISR activity, but does not affect strong ISR signaling. We thereby provide a plausible mechanism of how ISRIB counters toxic chronic ISR activity, without disturbing the cytoprotective effects of a strong acute ISR.

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**Significance**

The integrated stress response (ISR) protects cells from a variety of harmful stressors by temporarily halting protein synthesis. However, chronic ISR activation has pathological consequences and is linked to several neurological disorders. Pharmacological inhibition of chronic ISR activity emerges as a powerful strategy to treat ISR-mediated neurodegeneration but is typically linked to adverse effects due to the ISR’s importance for normal cellular function. Paradoxically, the small-molecule ISR inhibitor ISRIB has promising therapeutic potential in vivo without overt side effects. We demonstrate here that ISRIB inhibits low-level ISR activity, but does not affect strong ISR signaling. We thereby provide a plausible mechanism of how ISRIB counters toxic chronic ISR activity, without disturbing the cytoprotective effects of a strong acute ISR.

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*U*ukaryotic cells respond to intrinsic stress [e.g., endoplasmic reticulum (ER) stress or oncogene activation] as well as extrinsic stress (e.g., glucose or amino acid deprivation, hypoxia, or virus infection) by activating the integrated stress response (ISR). The ISR comprises a complex, cytoprotective signaling pathway aimed at reducing global protein synthesis while allowing translation of a few select mRNAs to promote cell recovery and survival (1, 2).

A key factor in translation initiation is eIF2, a heterotrimeric complex composed of α, β, and γ-subunit. eIF2 γ binds GTP and initiator Met-tRNA{\text{Met\textsuperscript{tRNA}}\text{Met\textsuperscript{tRNA(i)}}\text{Met\textsuperscript{tRNA(i)}}} ternary complex (TC). The TC, together with other translation initiation factors and the 40S ribosomal subunit, scans the mRNA for AUG start codons. Upon base pairing of Met-tRNA\textsubscript{Met} to the start codon, eIF2-bound GTP is hydrolyzed and eIF2α•GDP is released from the translation complex. To reactivate eIF2, GDP is displaced by eIF2B, a guanine nucleotide exchange factor (GEF). eIF2B is a large decaemer composed of a homodimer of a heteropentamer protein complex (4). The interplay between eIF2 and eIF2B is targeted by the ISR to regulate translation efficiency. In response to stress, eIF2α kinases are activated and subsequently phosphorylate a single, conserved Ser51 residue in eIF2α. Four eIF2α kinases have been identified: protein kinase R (PKR), which is activated by recognition of “nonsense” (e.g., viral) RNA (5, 6); PKR-like endoplasmic reticulum kinase (PERK), which responds to an accumulation of misfolded proteins in the ER (7); general control...
the regulation of translation during mitosis and thereby in cytokinesis and cell proliferation (15–17).

Dysregulation of the ISR has been linked to cancer, diabetes, and inflammation (18–20). Moreover, there is growing evidence of persistent, smoldering ISR activation in neurodegenerative diseases and conditions exhibiting memory consolidation defects, such as traumatic brain injury (18–21). Pharmacological modulation of the ISR has been proposed as a promising therapeutic strategy to treat these neurological conditions that are characterized by chronic eIF2α phosphorylation. Recently, a small-molecule ISR inhibitor, ISRIB, was identified to rescue protein translation and prevent SG formation in the presence of P-eIF2α (19, 22). Structural, genetic and biochemical evidence revealed that ISRIB targets eIF2B (23–26). ISRIB enhances eIF2B’s GEF activity by promoting the assembly of the fully active heterodimeric eIF2B complex from smaller subcomplexes (24, 25). ISRIB’s ability to restore the cellular translational capacity upon ISR activation implicated it as a promising tool to modulate ISR-regulated neurological processes and diseases. Indeed, ISRIB enhances cognitive memory processes (19), has beneficial effects in prion-diseased mice (27), and remedies cognitive defects resulting from brain injuries (21). Remarkably, ISRIB does so without causing the side effects that were previously observed upon suppressing the ISR by approaches that directly targeted eIF2α kinases in vivo (27–29).

In this study, we initially set out to investigate the effect of ISRIB in cells infected with an ISR-inducing recombinant picornavirus lacking its PKR antagonist (30, 31). We observed that ISRIB suppressed the ISR early in infection, when the amount of viral dsRNA and the level of P-eIF2α were relatively low, but not late in infection, when dsRNA and P-eIF2α levels were relatively high. This prompted us to investigate more systematically ISRIB’s ability to rescue translation at varying levels of P-eIF2α. To this end, we treated cells with different concentrations of poly(I:C) or arsenite, which trigger the ISR by activating PKR or HRI, respectively. The results show that ISRIB inhibits the ISR when P-eIF2α levels are below a critical threshold (i.e., 45–70% of the maximum phosphorylation), but not when P-eIF2α levels exceed this threshold level. These findings are consistent with in vitro studies (24) and demonstrate that potentially negative effects of pharmacological eIF2B assembly may be sidestepped under conditions of enhanced phosphorylation of eIF2. The observation that ISRIB is only functional within a narrow range of P-eIF2α concentrations may explain the lack of toxic side effects that ISRIB displays in vivo.

**Results**

**ISRIB Inhibits the ISR Induced by a Recombinant Picornavirus only Early in Infection, When P-eIF2α Levels Are Relatively Low.** Picornaviruses, like other RNA viruses, synthesize dsRNA in an indispensable intermediate step of their replication process. These dsRNAs are detected by PKR to activate the ISR and limit the production of viral proteins. As a countermeasure, many viruses have evolved strategies to delay or suppress this antiviral response. We set out to test the ability of ISRIB to inhibit the ISR during infection with a recombinant encephalomyocarditis virus (EMCV) lacking its PKR antagonist (EMCV-L-Zn) (32, 33). To assess the effect of ISRIB on the ISR in virus-infected cells, we treated HeLa-R19 cells with ISRIB for 1 h from 5 h postinfection (p.i.) until 6 h p.i. and monitored active translation using a ribopuromycylation assay for 15 min at the 6-h time point p.i. (34). This time point is relatively late in the infection cycle, as a single round of replication of this virus takes only 6–8 h (Fig. L4). Viral dsRNA replication intermediates are readily detected at ~4 h p.i. and reach a maximum level at ~6 h p.i. (Fig. 1B). As a positive control, we treated cells with 50 μM sodium arsenite, a commonly used method to trigger the ISR via HRI activation (35, 36). In both arsenite-treated and virus-infected cells, we observed eIF2α phosphorylation and concomitant translational repression.

Remarkably, ISRIB treatment failed to restore translation efficiency in infected cells, while translation in arsenite-treated cells was largely rescued (Fig. 1C). Similar results were obtained in U2OS cells, suggesting that this effect was not cell-type specific (SI Appendix, Fig. S1). We noted that the level of eIF2α phosphorylation in virus-infected cells at 6 h p.i. was higher than in cells treated with 50 μM arsenite, suggesting a correlation between ISRIB’s ability to inhibit the ISR and the extent of eIF2α phosphorylation.

We hypothesized that ISRIB is only functional when P-eIF2α levels are relatively low. To test this notion, we monitored the effects of ISRIB on SG formation in EMCV-L-Zn-infected cells at earlier time points p.i., when smaller amounts of dsRNA were present. ISRIB suppressed SG formation at the earliest time point at which SGs were detected (4 h p.i.) but not later in infection (Fig. 24), suggesting that ISRIB’s ability to antagonize the ISR indeed depends on the concentration of the stress trigger. We next quantified intracellular P-eIF2α levels by flow cytometry. Indeed, the level of P-eIF2α in virus-infected cells increased gradually from 3 to 6 h p.i. (Fig. 2B), correlating with the timing of dsRNAs accumulation (Fig. 1B) and the appearance of SGs (Fig. 24). Furthermore, our data indicated that a plateau level of P-eIF2α was reached late in infection (5–6 h p.i.). To rule out that this observed maximum level of P-eIF2α was caused by a detection limit of our flow cytometry approach, we compared flow cytometry (SI Appendix, Fig. S2A) and Western blotting (SI Appendix, Fig. S2B) as readout methods for P-eIF2α levels in cells stressed with increasing arsenite concentrations. The arsenite concentration at which the plateau level of P-eIF2α level was reached (~250 μM) was similar between the two detection methods. By comparing Western blot band concentrations in EMCV-L-Zn–infected cells at various times p.i., we identified intracellular P-eIF2α levels.

**Fig. 1.** ISRIB does not inhibit virus-induced ISR activity late in infection. (A and B) HeLa-R19 cells were infected at MOI 20 with EMCV-L-Zn. (A) At the indicated time points, EMCV-L-Zn genome copies per cell were quantified by qPCR. A representative of two independent experiments is shown. Error bars indicate SEM of triplicate measurements. (B) At the same time points, dsRNA content in EMCV-L-Zn–infected cells was analyzed by flow cytometry (n = 3). (C) Cells were infected with EMCV-L-Zn for 6 h or treated with 50 μM arsenite for 1 h. One hour before harvesting, cells were treated with 200 nM ISRIB or left untreated. Fifteen minutes before harvesting, all samples were treated with 20 μg/mL puromycin. Arsenite and EMCV-L-Zn were kept the cells during these treatments. Subsequently, cells were harvested and analyzed by Western blot, using the indicated antibodies. A representative of two independent experiments is shown.
ISRIB inhibits only the virus-induced ISR early in infection, when P-eIF2α levels are relatively low. HeLa-R19 cells were infected at MOI 20 with EMCV-Lzn. (A) At the indicated time points, cells were fixed in PFA and analyzed by an immunofluorescence assay using antibodies specific to SG marker G3BP1. Percentages of SG positive cells were quantified from at least four images. Representative images are shown on the Left; quantification is shown on the Right. Error bars indicate SEM. Statistical significance was analyzed by a two-way ANOVA, with Bonferroni post hoc test (**P < 0.001). (B) At the indicated time points, cells were harvested and the level of P-eIF2α was analyzed by flow cytometry. Results are shown as histograms (Left) and the percentage increase in mean fluorescence intensity is shown on the Right. Mock-infected cells are set at 0% induction; maximum P-eIF2α level was set at 100% induction. Shown is a representative of two independent experiments.

ISRIB inhibits only the virus-induced ISR early in infection, when P-eIF2α levels are relatively low.

ISRIB also Fails to Antagonize High P-eIF2α Levels Induced by Arsenite Treatment. Thus far, we tested ISRIB in cells exposed to EMCV-Lzn infection or poly(I:C) transfection, both of which induce the ISR via activation of the dsRNA sensor PKR. Since ISRIB acts downstream of P-eIF2α, we expected similar results irrespective of which stress sensor was activated. To provide further support for this notion, we used multiple arsenite concentrations to induce HRI-mediated ISR activation. Again, we correlated the ability of ISRIB to counteract SG formation (Fig. 4A) to P-eIF2α levels (Fig. 4B). In the absence of ISRIB, SG formation was observed in cells treated with 50 μM arsenite or more. The presence of ISRIB increased the arsenite concentration required to induce SG formation to 200 μM or more (Fig. 4C). The highest arsenite concentration at which ISRIB blocked SG formation (100 μM) resulted in ~40% of the maximum P-eIF2α level. At 200 μM arsenite, which induced ~75% P-eIF2α, ISRIB showed no effect. Importantly, the P-eIF2α threshold level above which ISRIB no longer antagonized the ISR (between 40% and 70%) was similar to that observed in poly(I:C)-transfected cells (i.e., between 45% and 70%).

To directly determine ISRIB’s influence on translation rates, we performed [35S]methionine pulse labeling to monitor active translation in cells exposed to different arsenite concentrations in the presence or absence of ISRIB (Fig. 4C). In the absence of ISRIB, arsenic concentrations of 25 μM and higher were sufficient to inhibit translation. ISRIB largely rescued translation in cells treated with low arsenite concentrations (between 25 μM and 100 μM). However, upon treatment with higher arsenite concentrations (>250 μM), translation was severely impaired, even in the presence of ISRIB. These data are in line with our immunofluorescence data (Fig. 4C) showing the formation of P-eIF2α concentration and rendered ISRIB ineffective. Together, these data suggest that a threshold level of P-eIF2α exists in the range of 45–70% of the maximum, above which ISRIB can no longer antagonize the ISR.

ISRIB Fails to Antagonize the Effects of High P-eIF2α Levels Induced by Poly(I:C). Virus infections cause extensive changes in multiple processes in the host cell. Hence, we could not exclude that some virus-induced change(s) in one way or the other affected the ISRIB’s ability to suppress the ISR in infected cells. To provide more direct support for the link between the level of P-eIF2α and the ability of ISRIB to counteract the dsRNA-induced ISR, we next tested the efficacy of ISRIB in cells transfected with increasing concentrations of poly(I:C), a dsRNA mimic that—like EMCV-Lzn infection—triggers the PKR branch of the ISR (Fig. 3). In the absence of ISRIB, we observed SG formation in cells transfected with 0.25 ng of poly(I:C) or more. ISRIB prevented SG formation only in cells transfected with relatively low poly(I:C) concentrations (1 ng or less) but not when larger amounts of poly(I:C) were used (Fig. 3A). At the highest concentration of poly(I:C) at which ISRIB could counteract the ISR (i.e., 1 ng), the P-eIF2α level was ~45% of the maximum (Fig. 3B). Transfection of 2 ng of poly(I:C) induced >70% of the maximum P-eIF2α concentration and rendered ISRIB ineffective. Together, these data suggest that a threshold level of P-eIF2α exists in the range of 45–70% of the maximum, above which ISRIB can no longer antagonize the ISR.

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SGs in ISRIB-treated cells exposed to arsenite concentrations of 200 μM and higher. Besides the inhibitory effect on general translation, the ISR mediates enhanced translation of a subset of mRNAs that contain uORFs, such as ATF4 mRNA. To test the effects of ISRIB on the expression of these stress-induced proteins, we used a HEK293T reporter cell line that expresses firefly luciferase under the control of the ATF4 uORFs (19). To activate the ISR, we treated cells with the indicated arsenite concentrations for 2 h. In the absence of ISRIB, we observed increased ATF4 reporter expression upon treatment with arsenite concentrations of 32 nM and higher (Fig. S4). This is in line with a previous report which shows that less P-eIF2α is required for the induction of ATF4 than for translational inhibition (37). At low arsenite concentrations (32 nM–4 μM), ISRIB prevented the enhanced expression of the ATF4 reporter (Fig. S5B). In contrast, we found that upon treatment with higher concentrations of arsenite (≥20 μM), ATF4 reporter expression was induced even in the presence of ISRIB.

Discussion

In this study, we provide evidence that ISRIB antagonizes the ISR only when P-eIF2α levels are below a critical threshold. By analyzing translation efficiency and stress granule formation in HeLa or U2OS cells infected with a recombinant picornavirus lacking its PKR antagonist, we showed that ISRIB inhibited the ISR early in infection, when levels of viral dsRNA and P-eIF2α were relatively low, but not at later time points when levels of dsRNA and P-eIF2α were high. To extend this observation, we performed a detailed analysis of the P-eIF2α levels and the ability of ISRIB to inhibit the ISR upon treatment of HeLa cells with varying concentrations of poly(I:C) or arsenite. We found that the level of P-eIF2α correlated with the concentration of stress trigger used but reached a plateau under severe stress conditions. Thus, the extent of eIF2α phosphorylation is graduated, quantitatively reflecting the severity of the stress situation. Importantly, the P-eIF2α concentration continued to increase even beyond the concentration necessary to suppress protein synthesis. Irrespective of the stress inducer used, ISRIB antagonized the ISR only when P-eIF2α levels were below a critical threshold. This threshold was determined to be somewhere between 45% and 70% of the maximum P-eIF2α level that could be observed in HeLa cells and was similar in cells infected with virus, transfected with poly(I:C), or treated with arsenite. Using an ATF4 reporter cell line, we also showed that ISRIB failed to block the expression of stress-induced proteins in the presence of high intracellular P-eIF2α levels. Taken together, our data show that ISRIB is effective only under conditions of limited stress.

Early studies of translation repression by P-eIF2α have shown that partial eIF2α phosphorylation could efficiently block translation. In reticulocyte lysates, translation initiation was suppressed when the fraction of phosphorylated eIF2α was increased from ∼10% under basal conditions to 20–40% (38). In line with these data, our results show that only ∼20% of the maximum level of P-eIF2α is sufficient to block translation and induce the formation of SGs in living cells. In the presence of ISRIB, this threshold level of P-eIF2α was increased to 45–70% of the maximum. Importantly, the threshold level of P-eIF2α appeared independent of the stress trigger, and hence of the eIF2α kinase involved. These data are in line with published in vitro data showing that ISRIB increased the GEF activity of eIF2B in the presence of P-eIF2α, but failed to do so when the P-eIF2α:eIF2α ratio was increased further (24). Taken together, the data from these in vitro GEF assays and our data from assays in live cells suggest that ISRIB desensitizes cells to P-eIF2α, unless the P-eIF2α concentration exceeds a critical threshold level.

Formation of decameric eIF2B requires dimerization of eIF2B (β/γ/ε) subcomplexes. The resulting octamers contain an interface for association of an eIF2α dimer (25). Since eIF2α is essential for P-eIF2’s ability to inhibit eIF2B (39), P-eIF2 likely binds only the full eIF2B decamer, not its subcomplexes. Thereby, P-eIF2 likely promotes eIF2B decamer formation and mediates sequestration of eIF2B subcomplexes into inactive P-eIF2α:eIF2B complexes. Consequently, high P-eIF2α concentrations may deplete the
cytoplasmic pools of eIF2B building blocks. This provides a plausible explanation for ISRIB’s lack of effect in the presence of high P-eIF2 levels, since the absence of eIF2B subcomplexes prevents ISRIB from assembling active eIF2B decamers.

In most studies that investigate the ISR, eIF2α phosphorylation is induced by exposing cells to sodium arsenite, thapsigargin, tunicamycin, DTT, MG132, poly(LC), or heat shock. It is unclear to what extent these treatments reflect physiologically relevant stress situations. In this study, we used a virus lacking its PKR antagonist to assess the ability of ISRIB to antagonize the ISR induced by a viral stress trigger (i.e., viral dsRNA) with natural intracellular localization and in physiological quantities. We showed that ISRIB inhibits the ISR early in infection, when little viral dsRNA has been produced and P-eIF2α levels are relatively low, but not late in infection, when viral dsRNA and P-eIF2α levels are high. Thus, levels of P-eIF2α that can no longer be antagonized by ISRIB can be reached in living cells under natural conditions.

While the ISR protects cells from stressful situations, dysregulated ISR signaling may have pathological consequences in vivo, and may be involved in the presentation of cognitive defects in neurological disorders like Parkinson’s disease, Alzheimer’s disease, Huntington’s disease, amyotrophic lateral sclerosis (ALS), and prion diseases (40–44). ISRIB provided significant beneficial effects in a mouse model of prion disease, and it also reversed neurological damage caused by traumatic brain injury (21), all without exhibiting overt toxic side effects (27, 45). By contrast, pharmacological inhibition of PERK caused pancreatic toxicity in mice, and knockdown of PKR had an aberrant effect on cytokinesis (15, 28).

Paradoxically, ISRIB did not display such adverse side effects in vivo (27). Our observation that ISRIB is functional only within a narrow range of P-eIF2α levels resolves this paradox. According to this notion, ISRIB does not negatively affect high(e) P-eIF2α levels that may be relevant for certain stages during cell growth or proliferation. The fact that ISRIB has beneficial effects in vivo against several neurological disorders and other stress-induced pathologies suggests that P-eIF2α levels would be relatively modest under these conditions. These considerations stress the importance to obtain quantitative data on how much eIF2α phosphorylation occurs in neurological disorders. More insight into levels of eIF2B, eIF2, and P-eIF2α and the assembly state of these multiprotein complexes in different cell/tissue types exposed to different stress and disease conditions will be invaluable to predict and/or evaluate effects of ISRIB treatment.

Materials and Methods

Chemical Inhibitors and RNA Ligands. ISRIB (SML0843) and puromycin (P9620) were purchased at Sigma-Aldrich and used at 200 μM and 20 μg/mL, respectively, unless indicated otherwise. Poly(I:C) was purchased at GE Healthcare. Sodium arsenite was purchased at Riedel-de Haën.

Cells and Viruses. HEK293T, HeLa-R19, U2OS, and BHK-21 cells were maintained in DMEM (Lonza) supplemented with 10% FCS and penicillin-streptomycin (100 units/mL and 100 μg/mL). Recombinant EMCV with Zn-finger domain mutant pG-Tf2 (Takara Bio) and, for ex vivo exclusion chromatography. Details are included in SI Appendix.

Poly(I:C) Transfection. Semiconfluent monolayers of HeLa-R19 cells grown in 24-well clusters were transfected with poly(I:C) using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer’s instructions. For each transfection, the indicated amounts of poly(I:C) were combined with total cellular RNA from resting HeLa-R19 to a constant 100 ng per well.

ATF4 Reporter Assay. HEK293T cells carrying an ATF4 luciferase reporter (19) were plated on polylissine-coated 96-well plates (Greiner Bio-One) at 25,000 cells per well. Cells were simultaneously treated with or without 200 nM ISRIB, and with sodium arsenite at increasing concentrations. Luminescence was measured using One Glo (Promega) as specified by the manufacturer.

Ribopuromycilation Assay. Cells in 10-cm dishes were either mock treated, treated with 50 μM arsenite, or infected with EMCVΔL (multiplicity of infection (MOI) = 20) in the presence or absence of 200 nM ISRIB. After the indicated incubation time, puromycin (20 μg/mL) was added to the medium and incubated for another 15 min. Cells were collected and used for Western blot analysis.

The 35S Pulse Labeling of Active Translation. Semiconfluent cell monolayers were first starved in medium lacking methionine and cysteine for 30 min and then treated with the indicated arsenite concentrations for 30 min with or without ISRIB. Subsequently, newly synthesized proteins were labeled with 50 μCi/mL 35S Met/Cys (Perkin-Elmer) for another 90 min. Cells were then lysed, and proteins were separated using SDS/PAGE. Subsequently, gels were dried on Whatman paper and analyzed using a phosphor imager.

Immunofluorescence Assay. Immunofluorescence assays were performed as described previously (46), using primary antibodies rabbit-α-puromycin (MABE343, 1:1,000; Merck Millipore), rabbit-α-eIF2α (9722, 1:2,000; Cell Signaling), rabbit-α-eIF2α-P (ab32157, 1:1,000; Abcam), or mouse-α-tubulin (T9026, 1:5,000; Sigma-Aldrich), and secondary antibodies goat-α-mouse-IRDye680 (1:15,000; LI-COR) or goat-α-rabbit-IRDye800 (1:15,000; LI-COR).

Flow Cytometry Analysis of eIF2α Phosphorylation. Cells were released using trypsin and fixed with paraformaldehyde (2% in PBS) for 20 min. Cells were then washed once with FACS buffer (PBS + 1% BSA) and incubated in ice-cold methanol for 10 min. Cells were then washed once with FACS buffer and incubated for 45 min with primary rabbit-α-eIF2α-P (ab32157, 1:100; Abcam) and then for 45 min with donkey-α-rabbit-Alexa647 (A-31573, 1:200; Thermo Fisher Scientific) diluted in FACS buffer at room temperature. In between and after the incubations, the cells were washed, twice each time, with FACS buffer. Finally, the cells were suspended in PBS + 1% paraformaldehyde and analyzed on the FACSCanto II (BD Biosciences).

Purification of eIF2α and Phosphorylated eIF2α. Human eIF2α, codon optimized for Escherichia coli, was cloned into a PET28a expression vector. This plasmid was cotransformed with the chaperone plasmid pG-T2F (Takara Bio) and, for phosphorylated eIF2α, an additional plasmid expressing PERK kinase domain. eIF2α was purified by sequential nickel-affinity, cation-exchange, and size-exclusion chromatography. Details are included in SI Appendix.

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