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Mutations in a translation initiation factor identify the target of a memory-enhancing compound

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The integrated stress response (ISR) modulates mRNA translation to regulate the mammalian unfolded protein response (UPR), immunity and memory formation. A chemical ISR inhibitor, ISRIB, enhances cognitive function and modulates the UPR in vivo. To explore mechanisms involved in ISRIB action we screened cultured mammalian cells for somatic mutations that reversed its effect on the ISR. Clustered missense mutations were found at the N-terminal portion of the delta subunit of guanine nucleotide exchange factor (GEF) eIF2B. When reintroduced by CRISPR-Cas9 gene editing of wildtype cells, these mutations reversed both ISRIB-mediated inhibition of the ISR and its stimulatory effect on eIF2B GEF activity toward its substrate, the translation initiation factor eIF2, in vitro. Thus ISRIB targets an interaction between eIF2 and eIF2B that lies at the core of the ISR.

The integrated stress response (ISR) is a widely conserved mechanism for coupling diverse upstream stresses to the phosphorylation of serine 51 in the α subunit of eukaryotic translation initiation factor 2 (eIF2α) (*1*) (*2*). Underlying the eIF2α phosphorylation-dependent ISR is a potent attenuation in translation of most mRNAs and selective upregulation of translation of a few special mRNAs that encode transcriptional regulators. The ISR thus activates a broad translational and transcriptional program involved in resistance to unfolded protein stress in the endoplasmic reticulum (ER stress) (*3*), intermediary metabolism (*2*), memory (*4*) and immunity (*5*).

A small molecule ISR inhibitor (ISRIB) exerts potent effects on the outcome of stress and on memory (*6*, *7*). As expected, ISRIB interfered with the ISR without blocking phosphorylation of eIF2α (Fig. 1A), suggesting that its molecular target(s) lie between eIF2(αP) and its effects on the translational machinery.

We tested ISRIB's effects on mRNA translation in an in vitro assay - cell-free translation in reticulocyte lysates. Both ISRIB and the eIF2α kinase inhibitor GSK2606414 (*8*) increased the luminescent signal of reticulocyte lysates programmed with luciferase-encoding mRNA (fig. S1A). The effect of ISRIB was enhanced further through eIF2α phosphorylation, which was promoted by pre-incubating the lysate at 30°C before adding the luciferase mRNA (fig. S1B-S1C). ISRIB's EC_{50} for stimulating translation, 35 nM, was similar to that in vivo (*6*) and was restricted to the *trans* geometric isomer (Fig. 1B). Thus, the ISR imparted by resident eIF2α kinase(s) in the reticulocyte lysate could be reversed by ISRIB.

Phosphorylation of eIF2α inhibits protein synthesis by inhibiting eIF2B, a guanine nucleotide exchange factor (GEF), which accelerates the exchange of GDP for GTP in the eIF2 complex (*9*, *10*). To measure the effects of ISRIB on eIF2B GEF activity, we established an assay in which the GEF activity in cell lysates (*11*) promoted the release of BODIPY-FL conjugated GDP (hereafter [b]GDP) from purified eIF2, with an attendant decrease in fluorescent intensity. The eIF2 substrate was purified from Chinese Hamster Ovary (CHO) cells that also expressed a conditionally active eIF2α kinase [Fv2E-PERK (*12*)] and eIF2 with low or high levels of phosphorylation was generated by treating the cells briefly with the Fv2E-PERK activator, AP20187 (fig. S2A-S2C). A lysate protein concentration- and time-dependent decrease in fluorescence intensity of eIF2-[b]GDP was observed (Fig. 1C), consistent with lysate-induced release of the bound nucleotide. The fluorescent signal declined more slowly in eIF2-[b]GDP with higher levels of phosphorylated eIF2α (Fig. 1C and fig. S2D), consistent with the inhibitory effect of eIF2(αP) on eIF2B GEF activity (*13*). ISRIB compensated for the inhibitory effect of eIF2(αP) on the GEF activity in cell lysate, with an EC_{50} of 27 nM; similar to ISRIB's action in intact cells (Fig. 1D).

ISRIB-mediated acceleration of GEF activity was maintained using an eIF2(α^{S51A})-[b]GDP substrate that could not be phosphorylated (Fig. 1E, samples 1-4) and was observed in lysates from both wildtype (eIF2$\alpha^{+/+}$) and mutant (eIF2$\alpha^{S51A/S51A}$) mouse embryonic fibroblasts (*14*) (Fig. 1E, samples 5-8). Furthermore, ISRIB stimulated the GEF activity of purified eIF2B on both phosphorylated and non-phosphorylated eIF2 (Fig. 1F-1G), suggesting that the molecular target of ISRIB is present in the pure complex and functions independently of eIF2 phosphorylation.

To isolate ISRIB-resistant cells (ISRIB^r) we used a CHO-K1 based cell line (CHO-C30) with the ISR-activated promoter of the mouse *Ddit3/CHOP* gene fused to green fluo-

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rescent protein (*CHOP::GFP*) (*15*). Activation of *CHOP::GFP* by unfolded protein stress in the endoplasmic reticulum was only partially inhibited by ISRIB, while activation by histidinol, a competitive inhibitor of histidine tRNA synthetase, [that activates the eIF2α kinase GCN2 (*16*)] was strongly inhibited (fig. S3). Chemically-induced mutations that reversed the ISRIB-mediated suppression of the histidinol-induced ISR, generated ISRIB^r CHO-C30 cells (fig. S4A-S4B).

We isolated numerous clones with strong or weak ISRIB^r phenotypes (Fig. 2A and fig. S4C-S4E). The ISRIB^r mutation(s) reversed both the sensitivity of the ISR reporter gene to ISRIB and the ability of ISRIB to promote protein synthesis in stressed cells (Fig. 2B-2C and fig. S5). Furthermore, the eIF2-directed GEF activity in lysates from the mutant clones was not stimulated by ISRIB in vitro (Fig. 2D).

ISRIB targets the interaction of eIF2B with eIF2. Therefore we examined the coding region of the genes encoding the subunits of eIF2B and eIF2. But for one exception, the coding regions of eIF2B subunits α, β, γ & ε and eIF2α had no mutations (table S1). This bland mutational landscape contrasted dramatically with that of *Eif2b4*, encoding eIF2Bδ. The majority of ISRIB^r clones isolated had one or more non-synonymous mutations affecting three closely-spaced codons, R171, V178 and L180 (table S1 and fig. S6). These mutations cluster in a unique N-terminal region of eIF2Bδ that is not conserved in the other two regulatory subunits of the GEF, but is well conserved among vertebrate eIF2Bδ (Fig. 3A and fig. S7).

To determine if the mutations in these clustered residues of eIF2Bδ were sufficient to impart an ISRIB^r phenotype, we promoted homologous recombination at the *Eif2b4* locus of parental CHO-C30 cells by CRISPR Cas9-directed editing, offering a homologous directed repair template with either the eIF2Bδ^{R171Q} or eIF2Bδ^{L180F} mutation (fig. S8A). With either repair template, a population of ISRIB^r cells emerged after co-transfection of the CRISPR guide and Cas9 nuclease. A single round of enrichment by sorting, delivered clones with weak and strong ISRIB^r phenotypes (fig. S8B-S8D and table S2). Clones with the weak ISRIB^r phenotype retained a wildtype copy of the gene encoding eIF2Bδ, whereas clones with the strong ISRIB^r phenotype had gained the mutation and lost both wildtype alleles (Fig. 3B-3E).

In vitro, the baseline GEF activity in lysates from eIF2Bδ^{R171Q} mutant cells was two-fold lower, while that of the eIF2Bδ^{L180F} was indistinguishable from wildtype (Fig. 3F). Yet both mutations similarly attenuated the effect of ISRIB on lysate GEF activity (Fig. 3G and fig. S9). Thus, mutations in eIF2Bδ can selectively compromise ISRIB action without affecting other aspects of eIF2B function.

Here we used a chemical genetic approach to identify proteins implicated in ISRIB action. We found that a small segment of eIF2Bδ is involved in the response to ISRIB providing a molecular clue to the how ISRIB might work. Though it is not clear if ISRIB binds eIF2B directly, ISRIB's ability to promote GEF activity in vitro together with the identification of a clustered set of mutations in the δ subunit that selectively eliminate this response (imparting an ISRIB^r phenotype on cells), suggest that direct modulation of the GEF lies at the heart of ISRIB-mediated reversal of the ISR. The active form of eIF2B is a dimer of pentamers (*17–19*), whereas the active, *trans*-isomer of ISRIB has perfect two-fold symmetry. Perhaps stabilization of the eIF2B decamer by binding of a symmetric molecule across the interface of its constituent pentamers is important for ISRIB's action and the ISRIB^r mutations, identified here, interfere with this process.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S9
Tables S1 to S3
References (*20*–*29*)

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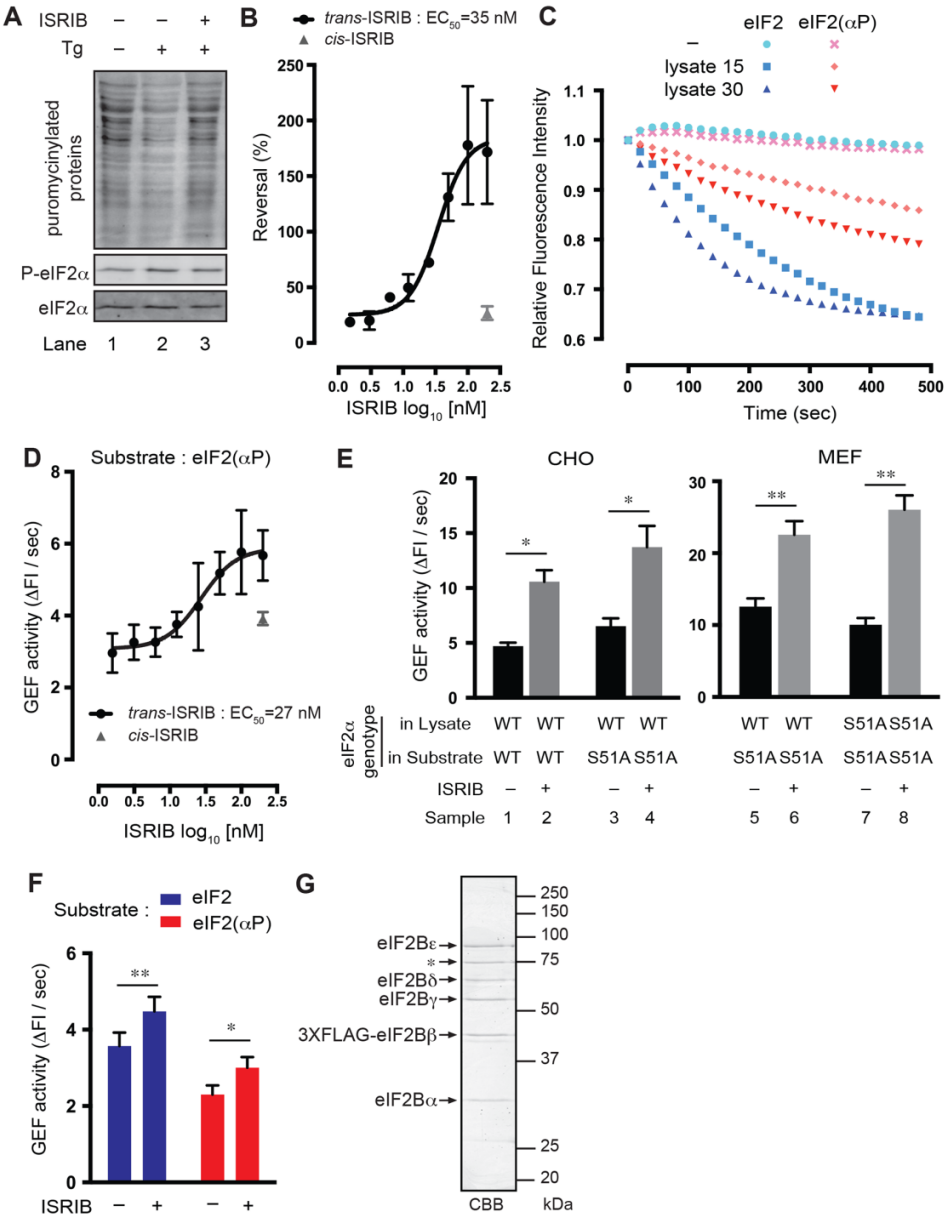


Fig. 1. ISRIB reverses attenuated translation and accelerates eIF2B GEF activity toward eIF2(αP) in vitro. (**A**) Immunoblot of newly-synthesized puromycinylated proteins in extracts of untreated CHO cells, cells were exposed to the ISR-inducing agent thapsigargin (Tg 300 nM, 30 min) in the presence or absence of *trans* ISRIB (100 nM). Phosphorylated (P-eIF2α) and total eIF2α were detected in the immunoblots below. Quantified signal intensities are shown in fig. S5. (**B**) Dose-response of ISRIB-stimulation of translation in reticulocyte lysate fitted to a non-linear trace. Mean ± SEM (n = 3) and EC_{50} (for active *trans*-ISRIB). Note the inactivity of *cis*-ISRIB. (**C**) GEF activity as reflected in time dependent decrease in fluorescence of weakly and heavily phosphorylated eIF2 loaded with Bodipy-FL-GDP and incubated with unlabeled GDP in the presence or absence of cell lysate (μg). Mean of three independent measurements. (**D**) Relation between the initial velocities of the release of Bodipy-FL-GDP from heavily phosphorylated eIF2 and ISRIB concentration, fitted to a non-linear trace. Mean ± SEM (n = 3) and EC_{50} for *trans*-ISRIB. (**E**) GEF activity reflected in the initial velocities of GDP release reactions with CHO cell lysate (samples 1-4), wildtype or mutant eIF2$\alpha^{S51A/S51A}$ mouse embryonic fibroblast lysate (MEFs, samples 5-8) and Bodipy-FL-GDP loaded eIF2 of the indicated eIF2α genotype. Mean ± SEM (n = 3 for samples 1-4 and n = 6 for samples 5-8). *P < 0.05, **P < 0.01 (Student's *t* test). (**F**) As in "E", but with purified eIF2B and Bodipy-FL-GDP loaded non-phosphorylated and phosphorylated eIF2. Mean ± SEM (n = 8). *P = 0.012, **P = 0.0054 (Student's *t* test). (**G**) Coomassie-stained SDS-PAGE of the purified eIF2B used in "F". The five subunits of eIF2B and PRMT5 (*, a non-specific contaminant) are noted.

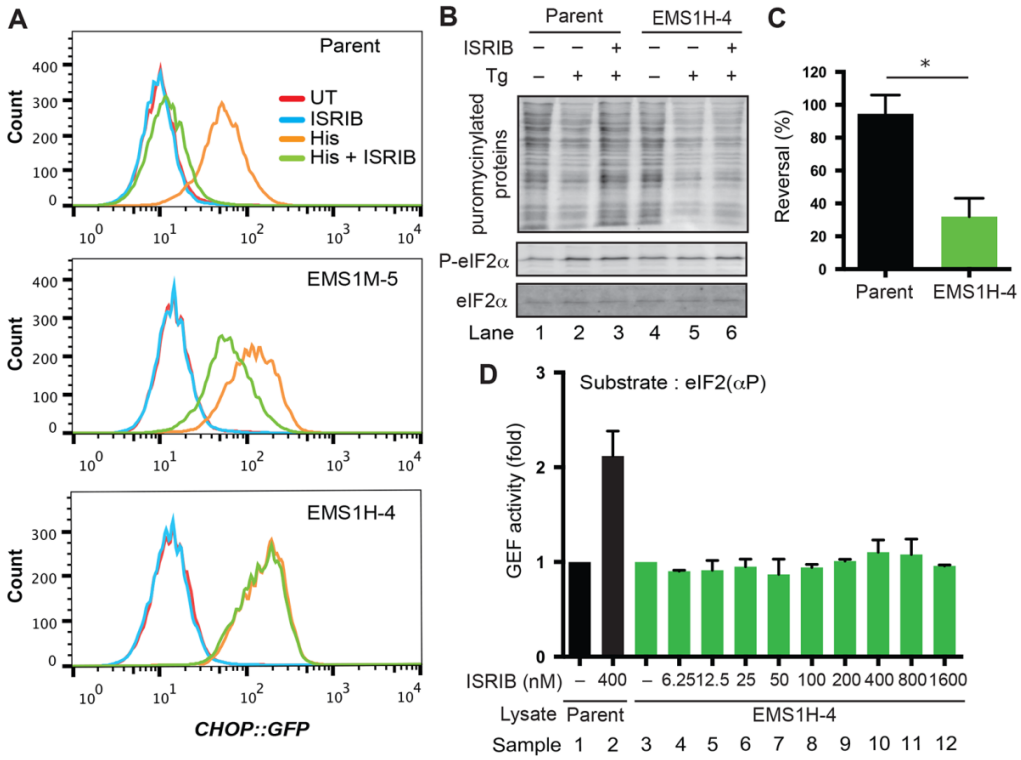


Fig. 2. Selection of ISRIB resistant (ISRIB^r) mutations. (**A**) Histograms of the distribution of GFP fluorescence arising from an ISR-inducible *CHOP::GFP* reporter gene in parental CHO-C30 cells and clones bearing the indicated mutations. The cells were left untreated or treated with histidinol (His; 0.5 mM), ISRIB (100 nM) or both. EMS1M-5 exemplifies a class of clones with a weak and EMS1H-4 a class with a strong ISRIB^r phenotype. (**B**) Immunoblot of puromycinylated proteins in extracts of parental CHO-C30 cells or a representative strong ISRIB^r clone (EMS1H-4) following exposure to thapsigargin (Tg) in the presence or absence of ISRIB (as in Fig. 1A). The images are representative of all three independent experiments that yielded similar results. Quantified signal intensities are shown in (fig. S5). (**C**) Bar diagram, displaying the reversal of translation attenuation by ISRIB in "C" above: Reversal = $[(P^{Tg+ISRIB}-P^{Tg}) \div (P^{UT}-P^{Tg})]$ X 100, ($P^{Tg+ISRIB}$, P^{Tg} and P^{UT} are the puro signal from the sample treated with Tg and ISRIB (Lanes 3 or 6), Tg alone (Lanes 2 or 5) and the untreated sample (Lanes 1 or 3), respectively. Mean ± SEM (n = 3). * $P < 0.05$ (Student's *t* test). (**D**) Bar diagram of the GEF activity of lysates from parental and strong ISRIB^r mutant cells with Bodipy-FL-GDP-loaded eIF2(αP) as a substrate in the absence or presence of ISRIB, as indicated. Mean ± SEM of the initial velocity of the decline in Bodipy-FL-GDP fluorescence upon adding lysate, normalized to the rate in the untreated sample (n = 4).

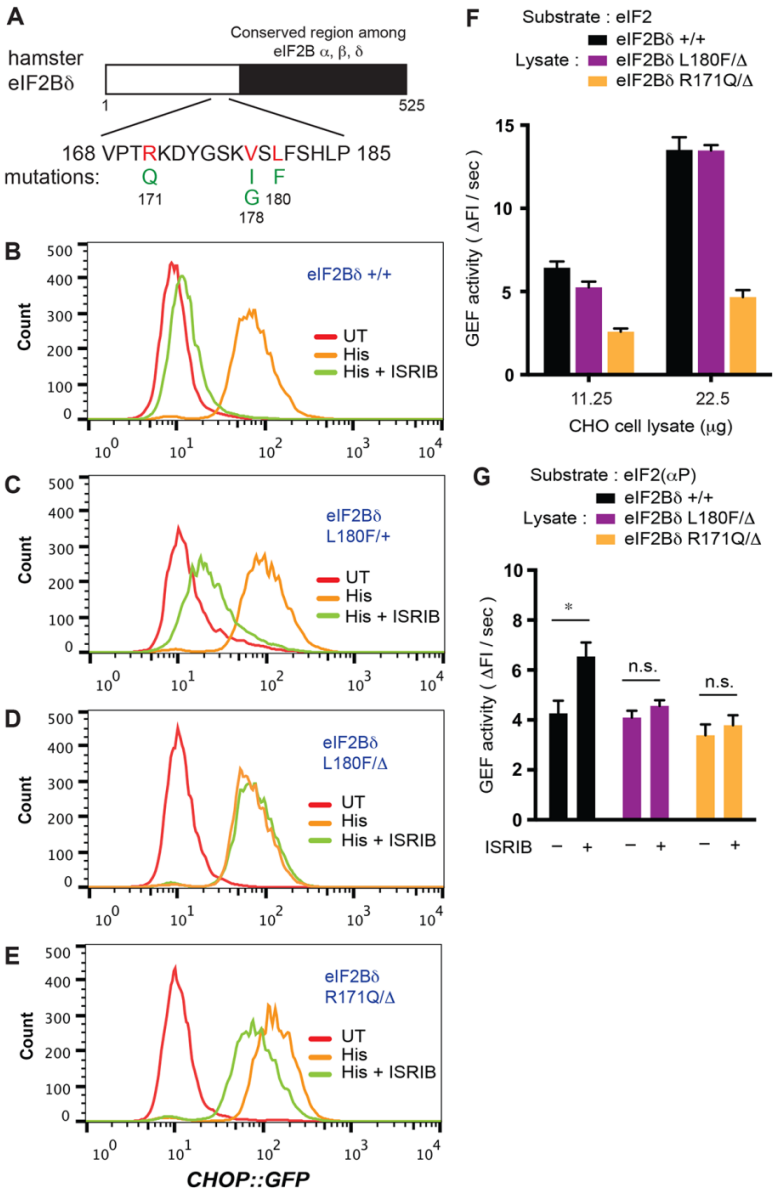


Fig. 3. Clustered mutations in *Eif2b4* impart ISRIB resistance. (**A**) Schema of eIF2Bδ with the position of the mutations associated with an ISRIB^r phenotype showing. These are clustered at the unique N-terminal region that is not conserved in the other regulatory subunits (α, β) of eIF2B. (**B-E**) Distribution of *CHOP::GFP* reporter gene activity in parental CHO-C30 cells and derivative sub-clones bearing the indicated mutations (induced by CRISPR-Cas9 targeted homologous recombination at the *Eif2b4* locus). The cells were left untreated or treated for 24 hours with histidinol (His; 0.5 mM) alone or with ISRIB (100 nM). (**F-G**) Bar diagram of the GEF activity of lysates from parental or CRISPR-Cas9-induced ISRIB^r mutant cells with Bodipy-FL-GDP-loaded eIF2 or eIF2(αP) as substrates in the absence or presence of ISRIB, as indicated. Mean ± SEM (n = 6, for "F"; n=5 for "G"). * $P < 0.05$, n.s.; not significant (Student's *t* test).