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The impact of the phosphomimetic eIF2αS/D on global translation, reinitiation and the integrated stress response is attenuated in N2a cells

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ABSTRACT

A plethora of stresses trigger a rapid downregulation of protein synthesis. However, a fraction of mRNAs continue to be recruited onto polysomes and their protein products play a key role in deciding cell fate. These transcripts are characterized by the presence of uORFs within their 5′ TL coupling protein expression to reinitiation. The translational brake arises due to the activation of a family of kinases targeting the α subunit of the trimolecular eIF2(αβγ) initiation factor. Phosphorylation of eIF2αSer51 inhibits ternary complex regeneration reducing the pool of 43S ribosomes. It is popular to mimic this event, and hence the integrated stress response (ISR), by the expression of the phosphomimetic eIF2αS51D. However, we report that whereas the ISR is reproduced by eIF2αS51D expression in human HEK293T cells this is not the case in N2a mouse neuroblastoma cells. With regards to translational downregulation, this arises due to the failure of the phosphomimetic protein to assemble an eIF2 complex with endogenous eIF2β/γ. This can be compensated for by the transient co-expression of all three subunits. Curiously, these conditions do not modulate reinitiation and consequently fail to trigger the ISR. This is the first demonstration that the inhibitory and reinitiation functions of eIF2αS/D can be separated.

INTRODUCTION

The cellular phenotype is in large part determined by protein composition, with the steady-state protein levels being the product of synthesis rate and turnover. Translation is the most energy dependent event in gene expression and is consequently under tight regulatory control (1). This occurs principally at the step of initiation, a process that involves the recruitment of the small 40S ribosomal subunit to the mRNA and the subsequent location of the initiation codon. Prior to loading, the free 40S must associate with a number of eukaryotic initiation factors (eIFs) including eIF3, eIF1A, eIF1, eIF5 and eIF2.GTP.tRNAi^{Met} to form the 43S pre-initiation complex (PIC). Association of the PIC with the mRNAs' 5′ is mediated by protein-protein interactions between eIF3 and eIF4G, the latter forming part of the trimolecular eIF4F cap binding complex. The specificity of eIF4F cap binding resides within its eIF4E subunit. After 43S loading, the PIC scans the mRNA 5′ transcript leader (TL). Codon-anticodon pairing within the P-site leads to activation of eIF5, a GTPase activating protein (GAP) and hydrolysis of the GTP within the eIF2.GTP.tRNAi^{Met} ternary complex (TC) to GDP and Pi. This triggers the release of the 40S-associated initiation factors, including eIF2.GDP, revealing sites on the small ribosomal subunit that permit 60S attachment. Hydrolysis of the eIF2 bound GTP and Pi release therefore marks the end of the initiation phase and the entry into elongation. For further rounds of initiation, eIF2.GDP must be recycled into its GTP form via the guanine nucleotide exchange factor (GEF), eIF2B (2–5).

Many intracellular signalling pathways modulate the translational readout of the cell and perturbations in this control are frequently associated with human pathologies, particularly cancer (6). Global translational regulation generally targets two key initiation factors, namely eIF4E and eIF2α. The eIF2α forms part of the trimolecular eIF2 (α/β/γ) within the TC. Cellular stresses, such as the accumulation of mis-folded proteins within the endoplasmic reticulum (the Unfolded Protein Response, UPR), viral infection or the accumulation of uncharged tRNAs, induce the activation of a number of cellular kinases that phosphorylate the Ser51 of eIF2α. In mammals there are four such kinases (PKR, PERK, GCN2 and HRI) (7). Since their activation leads to similar effects within the cell, they are collectively referred to as the integrated stress response (ISR). Phosphorylation of eIF2α leads to the accumulation of phospho-eIF2.GDP (eIF-2(P).GDP) as an end-product of initiation. This is a potent competitive inhibitor of the GEF

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because it has at least a 150-fold greater affinity for eIF-2(P).GDP than for eIF-2.GDP (8). Because most cell types have higher levels of eIF2 than those of eIF2B, even small changes in the phosphorylation status of eIF2α can impact significantly on global translation rates (9). The phosphorylation of eIF2α is a dynamic process that permits the cell to fine-tune its protein readout in response to changing environmental signals. The phospho-Ser51 can be dephosphorylated by the catalytic subunit of protein phosphatase I (PP1c). This effect is mediated via two eIF2α specific regulatory subunits, CReP and GADD34, which serve to target the phosphatase to its substrate (10). The *GADD34* gene is itself stress-induced as part of a feedback loop that ensures recovery of protein synthesis at the late phase of a stress response during which the eIF2α kinases have been activated (11,12).

Apart from quantitatively regulating protein expression within a cell, eIF2α phosphorylation can also qualitatively change the protein readout. This occurs via a mechanism referred to as delayed reinitiation (13). As many as 40% of mammalian genes harbour upstream open reading frames (uORFs) within their 5′ TLs and recent ribosomal profiling studies indicate that many of these are expressed (14,15). Because they trap the scanning PIC, uORFs are generally repressive for initiation events at the principle downstream AUG of the mRNA (the AUG^{GENE}). The magnitude of this effect is generally determined by the nature of the Kozak consensus sequence around the AUG of the uORF(16). However, during translation of small uORFs not all the initiation factors are released prior to termination. In particular, post-termination 40S subunits carrying eIF3 can remain associated with the mRNA and resume scanning (17–19). The subsequent site at which they reinitiate translation will be determined both by the intracellular levels of active TC, since they must re-recruit the eIF2.GTP.$tRNAi^{Met}$ from the free pool, and the distance in nucleotides that they scan (both parameters are actually two sides of the same coin since distance refers to the time available to recruit the TC) (20). As such, lowering TC levels will tend to displace the optimal reinitiation window towards the 3′. The quantitative and qualitative changes in the protein readout that can arise due to the presence of small uORFs can serve as a proliferation/differentiation switch that is coupled to changes in TC levels, as observed with the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) (21,22).

In cell culture model systems, it is relatively simple to induce the ISR. This can be achieved by chemical treatment (e.g. arsenite), drug treatment (e.g. thapsigargin), transfection of a dsRNA mimic (polydI:dC), thermal shock or serum deprivation. However, the effect of these stresses can often be pleomorphic. As an alternative, it has become popular to specifically target the TC by transfecting a cDNA that expresses the phosphomimetic eIF2αS51D (23,24). To mimic ISR this transgene product must associate with the endogenous eIF2β/γ subunits, assemble an active TC complex and complete one round of initiation to generate $eIF2^{S/D}$.GDP ($eIF2^{S/D}$ refers to the trimolecular eIF2 complex carrying the eIF2αS/D), the competitive inhibitor of the GEF. Failure at any of these steps would generate an attenuated or null phenotype. We have been using extensively this construct and noted that whereas it mimicked cellular stress in HEK293T cells (human embryonic kidney) its effect on both global translation and reinitiation was attenuated in N2a cells (mouse neuroblastoma) (20,25). In this article, we demonstrate that the attenuated phenotype observed in N2a cells with regards to global translation arises due to the failure of the transiently expressed phosphomimetic to form a significant amount of an eIF2 complex with the endogenous eIF2β/γ subunits. This can be compensated for by its co-expression with cDNA clones expressing eIF2β and eIF2γ. Curiously, eIF2αS/D alone or co-expressed with eIF2β/γ failed to modulate reinitiation in N2a cells and, as a consequence, the ISR. The implications of this are discussed.

MATERIELS AND METHODS

Cell culture and transfection

Experiments were performed in HEK293T cells, a human embryonic kidney cell line, cultured in Dulbecco's modified Eagle's medium (Sigma) and in N2a cells, a mouse neuroblastoma cell line, cultured in Dulbecco's modified Eagle's without pyruvate. Medium for both cell lines was supplemented with 10% fœtal bovine serum (Brunschwig) and 1% penicillin / streptomycin (Gibco). Cells were grown in a humidified atmosphere at 37°C with 5% CO_2. Transfections were performed using Lipofectamine 2000 (Life technologies) when the cells were 50–60% confluent. Eight hours later, the medium was replaced with fresh normal growth medium and lysates were prepared 24 h post-transfection.

DNA constructions

All clones were prepared in a pcDNA3 backbone. The human ^{HA}eIF2αS/D and ^{FLAG}eIF2αS/D have been previously described (36).

The human and mouse $eIF2\beta^{HA}$ and $eIF2\gamma^{FLAG}$ were cloned by RT-PCR starting from total cell RNA Trizol extracted from HEK293T and N2a cells. The primer sets employed were:

eIF2β forward: 5′-GATGGTACCATGTCTGGGGACG AGATGATT-3′
eIF2β reverse: 5′-AAAGCTAGCAGCTTTGGCACGG AGCTGTGC-3′
eIF2γ forward: 5′-GATGGTACCATGGCGGGCGGAG AA-3′
eIF2γ reverse: 5′-AAAGCTAGCATCATCTACTGTTGG CTTGAT-3′

These constructs were cloned between the KpnI/NheI in pcDNA3.

The primer sets used for the RT-PCR analysis of the ISR were as follows:

CHOP forward: 5′-CAGAACCAGCAGAGGTCACA-3′
CHOP reverse: 5′-AGCTGTGCCACTTTCCTTTC-3′
Actin forward: 5′-CTGACGGCCAGGTCATCACCAT TG-3′
Actin reverse: 5′- GCCGGACTCGTCATACTCCTGCTT G-3′

The β-actin renilla luciferase (RLuc) and ΔKpn 5′ TL bicistronic constructs have been previously described

(20,25,26). The γ34.5 construct was obtained from the lab of Dr David A. Leib (Geisel School of Medicine at Dartmouth, UK) and the mouse eIF2α clones from Dr R. Kaufman and Dr Janet Mitchell (HHMI, USA).

Luciferase reporter assay

Extracts were prepared in passive lysis buffer according to the instructions of the supplier (Promega). The activities of FLuc (firefly luciferase) and RLuc (renilla luciferase) were measured using a dual-luciferase reporter assay system (Promega) on a GloMax 20/20 luminometer (Promega). All transfections were performed in triplicate. Data are presented as the mean ± SEM. Statistical analysis was performed using the Student's t test. All experiments were repeated at least three times.

Immunoblotting

Protein extracts were prepared in a lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA and 0.5%(v/v) NP40. Protein concentrations were determined by Bradford (Cytoskeleton, USA). Twenty μg of protein was resolved on a polyacrylamide-SDS gel and electrotransferred to a PVDF membrane. Antibodies used in this study were anti-HA (clone 16B12, Covance), anti-FLAG (M2 antibody, Sigma), anti-phospho (Ser51) eIF2α (GenTex, #61039), anti-eIF2α (Invitrogen, #44728G), eIF2β (Santa Cruz, #9978), and goat anti-mouse or rabbit HRP secondary antibodies (Bio-Rad). Blots were developed using the WesternBright^{TM} Quantum (Advansta), and quantified using the Quantity One software package (Bio-Rad).

Glycerol gradient fractionation

After transfection with ^{HA}eIF2αS/D, HEK293T and N2a cells were lysed in a gradient fractionation buffer containing 100 mM KCl, 50 mM Tris-HCl pH 7.4 1.5 mM $MgCl_2$, 1 mM DTT, 1 mg/mL heparin, 1.5%(v/v) NP40, protease inhibitor (Roche) and phosphatase inhibitor (Roche). Lysates were incubated 30 min on ice followed by a centrifugation at 12000 xg at 4°C for 15 min. Supernatants were loaded onto a 5–20%(v/v) glycerol gradient in a buffer containing 0.2 M KCl, 10 mM $MgCl_2$, 40 mM Hepes pH7 and centrifuged at 40000 rpm at 4°C for 22 h in SW60 rotor (Beckman). 10 × 400 μl fractions were collected for each gradient. Gradient fractions were concentrated by methanol-chloroform precipitation and analysed by western blotting.

Co-immunoprecipitation

After transfection with pcDNA3, ^{HA}eIF2αWT, ^{HA}eIF2αS/D, HEK293T and N2a cells were harvested 48 h post-transfection in a co-IP buffer containing 100 mM KCl, 50 mM Tris pH 7.4, 1.5 mM $MgCl_2$, 0.5%(v/v) NP40, protease inhibitor (Roche) and phosphatase inhibitor (Roche). Cells were incubated 30 min on ice then centrifuged at 12000 xg for 15 min at 4°C. The supernatants were incubated overnight with 50 μl of pre-saturated and pre-washed anti-HA Affinity Matrix beads (Roche #11815016001). Three bead washes were performed in the co-IP buffer. Proteins were eluted in a buffer containing 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 5% SDS, 20% (v/v) glycerol, 200 mM DTT and 0.05% bromophenol blue. Fifteen μl of protein was loaded on a SDS-polyacrylamide gel and the immunoprecipitation was analysed by immunoblotting using the anti-HA and anti-eIF2β antibodies.

RESULTS

The impact of the phosphomimetic eIF2αS/D is attenuated in N2a cells

We monitor global cellular translation using a transiently transfected vector expressing an RLuc reporter carrying the 5′ TL of β-actin (20,26). This TL has little RNA structure and no uAUGs. In HEK293T cells, its co-transfection with a vector expressing a N-terminally HA-tagged version of human eIF2αS/D (^{HA}eIF2αS/D) resulted in a major inhibition of the reporter readout (Figure 1A/B). This effect was even more marked than that observed by treating the cells with the drug thapsigargin, a known ER stress agent that induces phosphorylation of the endogenous eIF2α (27). However, when these experiments were repeated in the N2a cell background, ^{HA}eIF2αS/D had only a marginal effect on the measured reporter readout despite robust expression of the phosphomimetic protein (Figure 1A/B). This was confirmed using a second reporter assay that followed protein expression by immunoblotting (Supplementary Figure S1). That N2a cells still responded to the cellular stress response was confirmed by thapsigargin treatment; in fact the reporter assays suggested that these cells were even more sensitive than HEK293T to this drug (Figure 1A), a result that may reflect the high endogenous levels of eIF2α phosphorylation that we observed in exponentially growing (sub-confluent) N2a cells (the conditions at the time of transfection), even in the absence of any stress (Figure 1C). This cell-specific difference in eIF2α phosphorylation levels was generally less marked at confluence (conditions at the time of cell lysis in our reporter assays). We next asked if the intrinsic phospho-eIF2α levels in the N2a cells could explain the attenuated S/D phenotype. We achieved this by co-transfecting a HA-tagged version of the Herpes Simplex virus (HSV) γ34.5 protein whose presence serves to de-phosphorylate eIF2α via the recruitment of the cellular protein phosphatase 1 (PP1) (28). However, despite a marked drop in the endogenous phospho-eIF2α levels in the presence of ^{HA}γ34.5, no significant change was observed in the eIF2αS/D phenotype (Note that in this experiment we employed the N-terminally FLAG-tagged version of eIF2αS/D) (Figure 1D/E).

Reinitiation

Phosphorylation of eIF2α effectively reduces TC levels in the cell due to a block in the recycling of eIF2.GDP. This not only reduces global translation but also delays reinitiation events mediated by uORFs within the mRNA 5′ TL. Since global translation in N2a cells was resistant to the effect of ^{HA}eIF2αS/D we asked if it impacted on reinitiation. For this we used two different reinitiation reporters developed in the lab. The first contains part of the 5′ TL from the human *ELK1* gene (referred to as ΔKpn) fused to the first cistron in

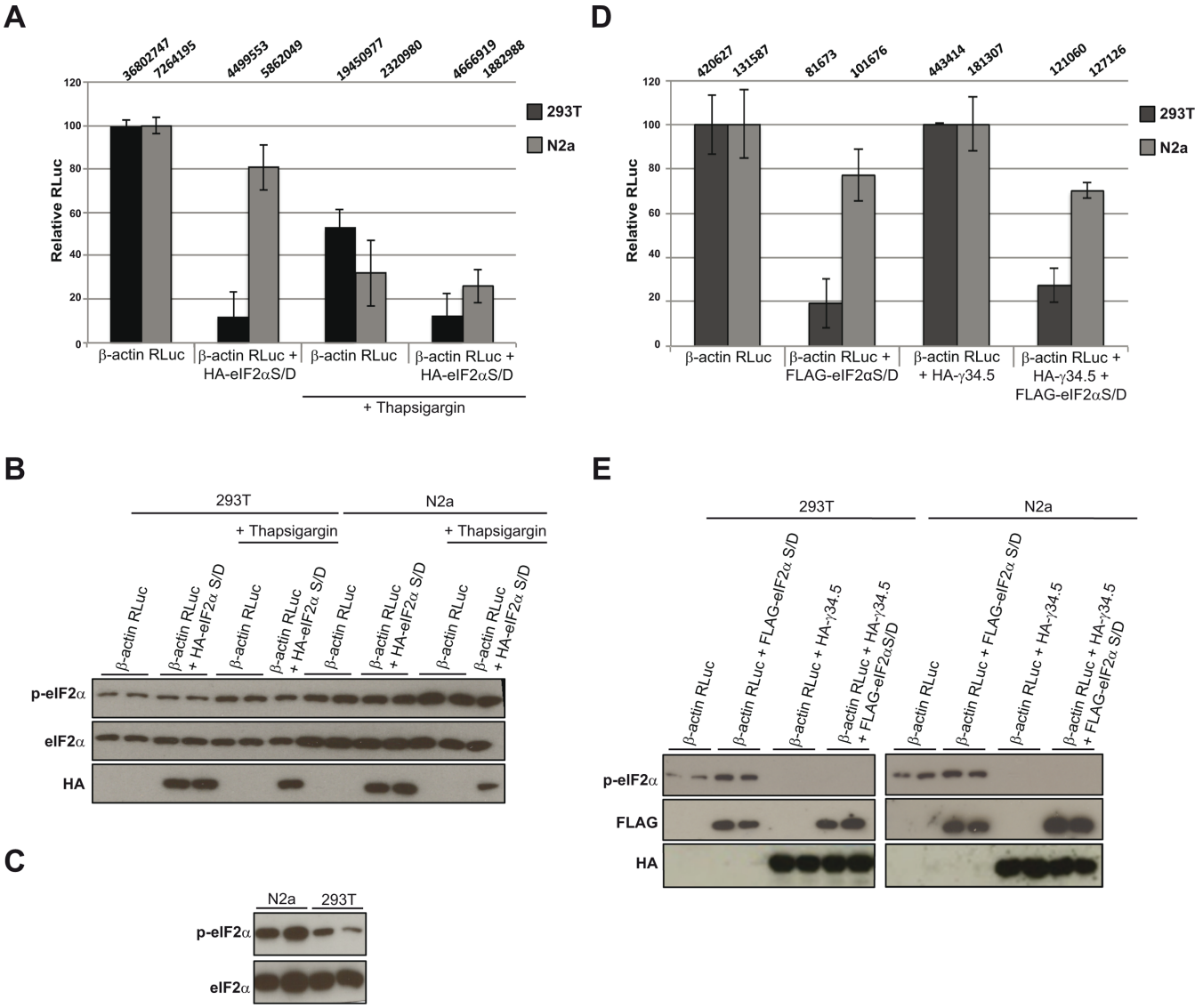

Figure 1. The negative effect of eIF2αS/D is attenuated in N2a cells. (**A**) Sub-confluent N2a and HEK293T cells were co-transfected with a βact-RLuc reporter vector and either an empty vector control or a clone expressing ^{HA}eIF2αS/D. These same transfections were also performed in the presence of the drug thapsigargin (300 nM) which was added 8 h after the transfection. Cells were harvested at 24 h post-transfection and RLuc activity was measured in 15 μg of the cell extract. Values have been normalized to the βact-RLuc control which has been given a value of 100. All transfections were performed in triplicate and the SEMs are indicated as bars. The numbers indicated above the bars are the average RLuc values obtained from each triplicate. (**B**) Immunoblots performed using two of the three transfected cell extracts. The Ab's employed (anti-phospho-eIF2α, anti-eIF2α and anti-HA) are indicated on the left. (**C**) Immunoblots performed on duplicate extracts prepared from sub-confluent N2a and HEK293T cells probed using the anti-phospho-eIF2α and anti- eIF2α Ab's. (**D**) The βact-RLuc reporter assay was repeated in the presence of vectors expressing ^{FLAG}eIF2αS/D, the herpes virus ^{HA}γ34.5 and both ^{FLAG}eIF2αS/D and ^{HA}γ34.5. The RLuc values recorded for the transfections expressing ^{FLAG}eIF2αS/D were normalized relative to the corresponding S/D minus control which was set as 100 (the βact-RLuc and βact-RLuc+γ34.5 columns). All transfections were performed in triplicate and the SEMs are indicated. The numbers indicated above the bars are the average RLuc values obtained from each triplicate. (**E**) Immunoblots performed using two of the three transfected cell extracts. The Ab's employed (anti-phospho-eIF2α, anti-FLAG and anti-HA) are indicated on the left.

a FLuc-EMCV-RLuc bicistronic reporter (Figure 2A, upper panel). The *ELK1* gene expresses a transcriptional activator whose translational expression is regulated by multiple elements within the 5′ TL (26,29). The ΔKpn 5′ TL contains one of these key elements, namely a short 2 codon out-of-frame uORF (referred to as uORF2) positioned only 14 nts upstream of the AUG^{ELK1} which has been fused to FLuc. We previously demonstrated in HEK293T that this uORF is permissive for reinitiation (20). In this dual reporter assay, the FLuc activity is the product of both leaky scanning and reinitiation whereas RLuc arises by IRES-mediated initiation. The EMCV IRES employed in the bicistronic does not require the eIF1, eIF1A and eIF4E initiation factors but does require eIF2 (30). In a second construct, the uORF2 UGA stop codon in the 5′ TL was changed to UGC (ΔKpn UGA 5′ TL). This mutation extends the uORF such that it overlaps with that of FLuc (Figure 2A, lower panel). In this context, FLuc activity is only the product of leaky scanning through the uAUG. Assuming that the UGA/UGC change does not modify the leaki-

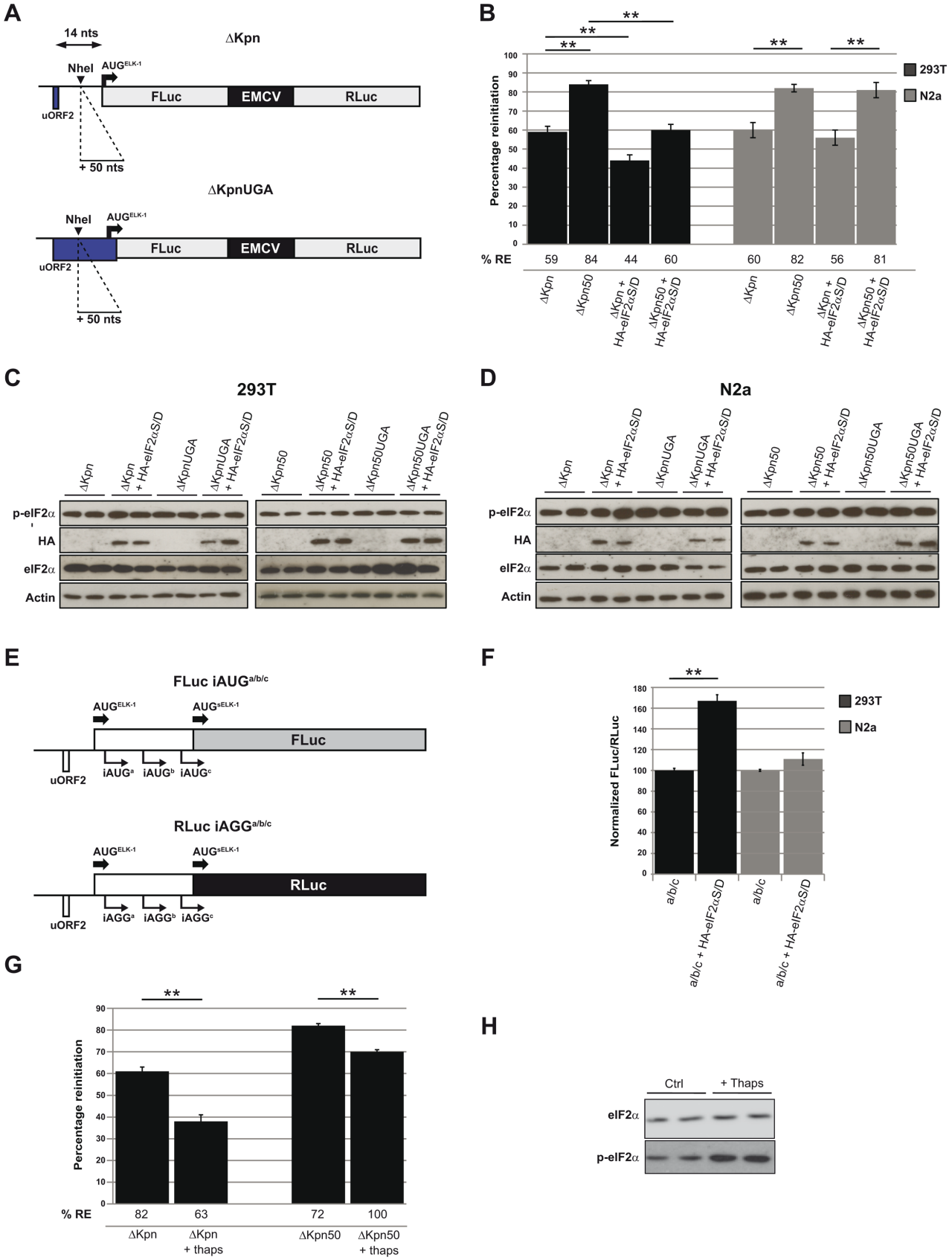

Figure 2. Expression of ^{HA}eIF2αS/D does not modulate reinitiation in N2a cells. (**A**) Schematic representation of the FLuc-EMCV-RLuc bicistronic reporter. Upper Panel: the ΔKpn 5′ TL has been fused to the first cistron. It carries a small uORF terminating 14 nts upstream of the AUG^{Elk1} which

ness of the uAUG (the Kozak context remains unchanged) or that leaky scanning itself is not responding to the transient expression of eIF2αS/D, comparison of the two normalized FLuc/RLuc data sets gives a measure of uORF-mediated reinitiation events at the AUG^{ELK1} (20). However, the very short distance (14 nts) between the uORF2 and the AUG^{ELK1} is not optimal for reinitiation. It can be significantly enhanced by the introduction of a 50 nts spacer just downstream of the uORF2 stop codon (ΔKpn50) (20). In the HEK293T background, the addition of the spacer increased reinitiation efficiency (RE) from ∼59% to ∼84, an observation in-line with our previously published data. The presence of ^{HA}eIF2αS/D reduced RE. Indeed, its presence in the ΔKpn50 5′ TL transfections caused the measured RE to return to the levels measured in the original ΔKpn 5′ TL (60%) (Figure 2B/C). In N2a cells, the spacer also significantly increased reinitiation frequency, with the results essentially mirroring those observed in HEK293T cells. However, these values remained largely unchanged when the phosphomimetic protein was co-expressed (Figure 2B/D).

The second reinitiation reporter is also derived from our previous work on the *ELK1* gene. The second AUG codon in the Elk1 ORF was proposed to generate an N-terminally truncated form of the protein referred to as sElk1. However, access to this start site by ribosomes in the delayed reinitiation mode that arise from the uORF2 is blocked by a series of three out-of-frame internal AUG codons ($iAUG^{a/b/c}$) positioned between the AUG^{ELK1} and AUG^{sELK1} (Figure 2E) (20,25). The scattering of these iAUGs through this region ensures tight repression of AUG^{sELK1} initiation across a range of TC levels. In this context, we fused the AUG^{sELK1} to FLuc (FLuc-$iAUG^{a/b/c}$ in Figure 2E). In a second construct carrying the RLuc reporter, the iAUGs were changed to iAGG thereby removing translational repression for initiation events at the AUG^{sELK1} (RLuc-$iAGG^{a/b/c}$ in Figure 2E). These monocistronic reporters were co-expressed in both cell backgrounds in the absence or presence of ^{HA}eIF2αS/D and normalized FLuc/RLuc reporter values recorded. In HEK293T cells, the presence of eIF2αS/D significantly increased the FLuc/RLuc ratio but this effect was absent in N2a cells (Figure 2F). Therefore, in HEK293T cells, the global negative effect of the eIF2αS/D phosphomimetic (which is cancelled out in the normalized reporter values) is compensated for in the FLuc-iAUG reporter by the displacement of delayed reinitiation events towards the 3′ AUG^{sELK1}. This serves to bypass the internal translational repressors. This effect is not observed in the RLuc background because the iAUG repressors had been removed.

Thus two independent assays indicate that despite the presence of apparently normal reinitiation in N2a cells (at least with regards to the ΔKpn50 spacer response: Figure 2B) the presence of ^{HA}eIF2αS/D is largely silent. However, to confirm that reinitiation in N2a was responsive to changes in endogenous TC levels we performed the ΔKpn 5′ TL and ΔKpn50 5′ TL bicistronic assays (as depicted in Figure 2A) in the absence or presence of thapsigargin (Figure 2G). In both reporters, the drug induced a marked increase in eIF2α phosphorylation (Figure 2H) and significantly reduced measured RE levels. As expected, the effect was more marked in the spacer-minus construct. We previously published similar observations in HEK293T cells (20).

Formation of an $eIF2^{S/D}$ trimolecular complex

Presumably, for a transiently expressed ^{HA}eIF2αS/D to effectively mimic phosphorylation of the endogenous protein it must first assemble the trimolecular eIF2 complex by association with the *de-novo* made endogenous eIF2β/γ subunits. This $eIF2^{S/D}$ complex must in-turn form an active TC, and complete one round of initiation to generate the $eIF2^{S/D}$-GDP that will sequester the exchange factor. The attenuated phenotype that we observed in N2a cells could arise due to a block at any of these steps. We therefore first examined if the transiently expressed ^{HA}eIF2αS/D formed an eIF2 complex. Transfected cell extracts were prepared in polysomal lysis buffer; conditions that we know maintain these complexes intact. Extracts were then divided in two, with one half being heated in the presence of SDS and DTT to induce complex dissociation. Both samples were subsequently fractionated on glycerol gradients. In HEK293T cells under native conditions, the ^{HA}eIF2αS/D sedimented into the lower end of the gradient, namely fractions 3 and 4. In these fractions we also observed the endogenous eIF2β protein. Upon denaturation both proteins moved up the gradient although the sedimentation profiles were slightly different (Figure 3A). Nonetheless, the results are consistent with the efficient assembly of an $eIF2^{S/D}$ complex in

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initiates FLuc expression. In a second construct, a 50 nts spacer sequence has been inserted at a NheI restriction site just downstream of the uORF2 (ΔKpn50). Lower Panel: the UGA termination codon of the uORF2 has been changed to UGC (ΔKpnUGA and ΔKpn50UGA). The extended reading frame now overlaps that of FLuc. The position of the small two codon uORF2, the insertion site for the +50nts spacer sequence and the UGA/UGC mutation that extends the uORF to overlap with that of FLuc are all indicated. (**B**). HEK293T and N2a cells were co-transfected with either the ΔKpn plus ΔKpn UGA or the ΔKpn50 plus ΔKpn50UGA bicistronic constructs in the presence or absence of ^{HA}eIF2αS/D. Cells were harvested after 24 h, and reporter activities measured. Reinitiation frequency (RE) at the AUG^{Elk1} codon was determined as indicated in the text. These values are plotted graphically. All transfection assays were performed in triplicate and the SEM is indicated. The arrows connect data points whose difference is statistically significant (**$P < 0.01$). The%RE is indicated below each column. (**C/D**) Immunoblots performed on two of the three cell extracts derived from the HEK293T and N2a experiments outlined above. The Ab's used (anti-phospho-eIF2α, anti-HA, anti-eIF2α and anti-actin) are indicated on the left. (**E**) Schematic representation of the monocistronic FLuc-$iAUG^{a/b/c}$ and Rluc-$iAGG^{a/b/c}$ dual reporters that monitor the ability of ribosomes to bypass the iAUG translational repressors for initiation events at the AUG^{sElk1}. Both reporters carry a 5′ TL with the small uORF2 derived from the *ELK1* gene. (**F**) The FLuc-$iAUG^{a/b/c}$ and Rluc-$iAGG^{a/b/c}$ reporters were co-transfected into HEK293T and N2a cells with either an empty vector or a vector expressing ^{HA}eIF2αS/D. FLuc/RLuc activities were recorded 24 h post-transfection and the FLuc/RLuc ratios plotted. For each cell line the empty vector control value was set as 100. Transfections were performed in triplicate and the SEM is indicated. Arrows point to data sets whose difference is considered statistically significant (**$P < 0.01$). (**G**) RE efficiencies in N2a cells were measured using the ΔKpn and ΔKpn+50 5′ TL bicistronic reporter assays in the absence or presence of thapsigargin. Arrows point to data sets whose difference is considered statistically significant (**$P < 0.01$). (H) An immunoblot performed on duplicate extracts using the anti-eIF2α and phospho-eIF2α Ab's.

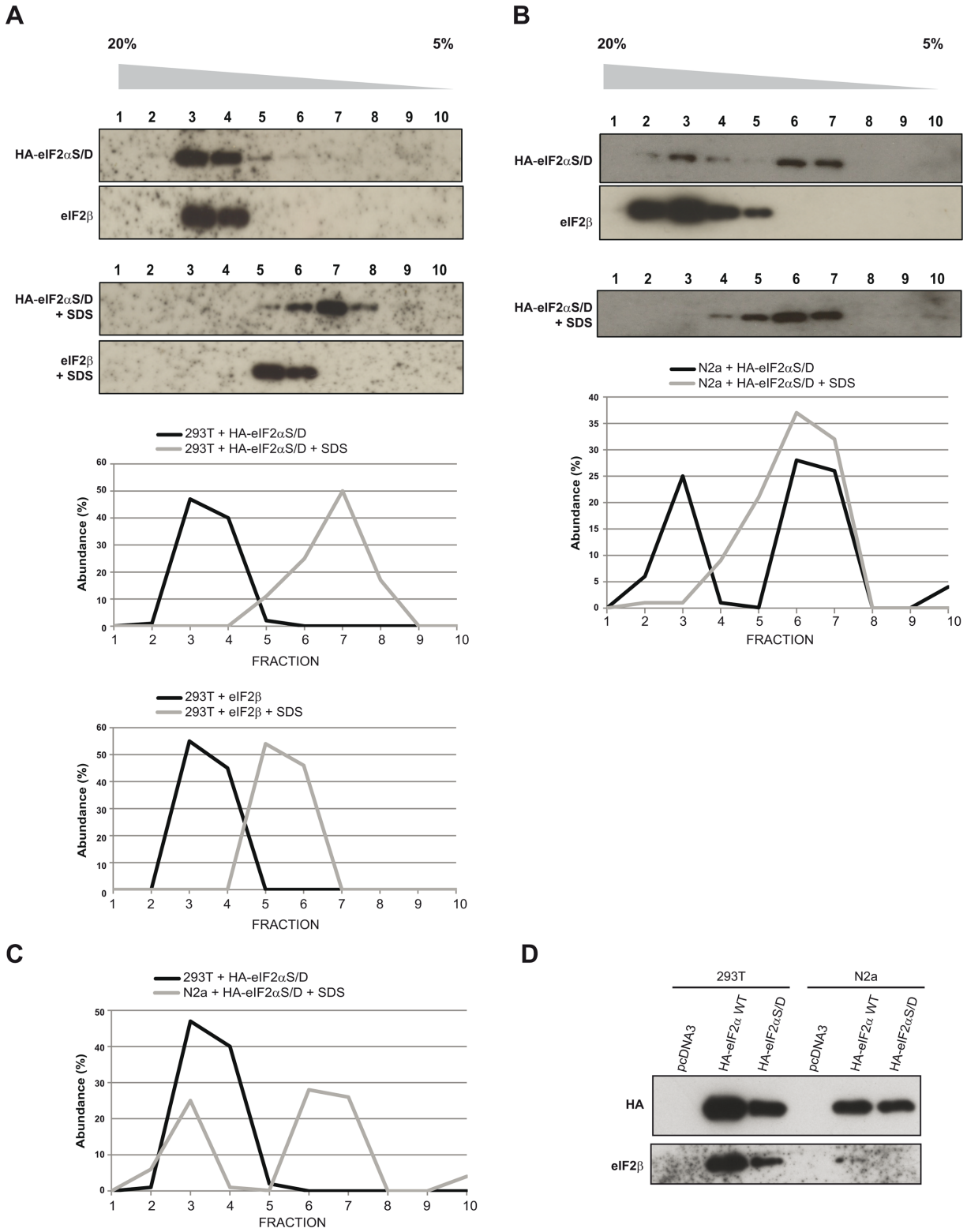

Figure 3. Sedimentation analysis of the eIF2 complex. (**A**) HEK293T cells were transfected with a vector expressing ^{HA}eIF2αS/D. Cytoplasmic extracts were prepared at 24 h post-transfection in polysome lysis buffer. Half of the extract was denatured by heating in SDS/DTT. Both native and denatured extracts were then fractionated on 5–20% glycerol gradients. The fractions from each gradient were analysed by immunoblotting using an anti-HA and an anti eIF2β antibody (upper panels). These blots were quantitated and plotted graphically as a percentage of the total protein expressed per fraction (lower panels). (**B**) Analysis of extracts prepared from transfected N2a cells. (**C**) Comparison of the sedimentation profiles for the transiently expressed ^{HA}eIF2αS/D under native conditions in HEK293T and N2a cells. (**D**) A co-IP experiment performed on extracts from either ^{HA}eIF2αWT or ^{HA}eIF2αS/D transfected HEK293T and N2a cells. Proteins were selected with the anti-HA Ab and analysed by immunoblotting with both anti-HA and anti-eIF2β. The negative control (−) was provided by mock-transfected cells.

HEK293T cells. When the analysis was repeated in N2a cells, only a small fraction of the transiently expressed ^{HA}eIF2αS/D was observed in the lower fractions suggesting only limited formation of an eIF2$^{S/D}$ complex (Figure 3B/C). This limited capacity to form the eIF2 complex with eIF2αS/D was confirmed by co-immunoprecipitation (Figure 3D).

Co-expression of eIF2β^{HA} and 2γ^{FLAG} enhances ^{HA}eIF2αS/D-mediated reporter inhibition in N2a cells

The studies above suggest that the failure of the phosphomimetic protein to impact on global translation rates and reinitiation in N2a cells correlated with its failure to form a significant amount of the eIF2$^{S/D}$ trimolecular complex. Assuming that the transiently expressed ^{HA}eIF2αS/D cannot exchange with the pre-existing α subunit within eIF2 but can only assemble into a trimer with nascent newly synthesized subunits, this could arise due to low level expression of the endogenous eIF2β and 2γ subunits in N2a cells during the period of the transient transfection. We attempted to measure endogenous eIF2β levels by metabolic labelling coupled to immunoprecipitation but without success (we were unable to identify specific bands on the gels) despite the fact that the steady-state levels of eIF2β were easily measured by immunoblotting with the same antibody (Supplementary Figure S2). As an alternative approach we asked if the ^{HA}eIF2αS/D negative phenotype could be enhanced by the co-transfection of cDNA clones expressing eIF2β and 2γ. C-terminal HA and FLAG tagged versions of both mouse and human eIF2β and 2γ were RT-PCR cloned from total N2a and HEK293T RNA and inserted into the pcDNA3 expression vector. Using the β-act-RLuc reporter assay in N2a cells, co-expressing ^{HA}eIF2αS/D and the murine eIF2β^{HA}/2γ^{FLAG} clones produced a significant drop in the luciferase signal with levels approaching those observed in HEK293T cells (Figure 4A/B). This was not attributable to overexpression of either eIF2β^{HA} or eIF2γ^{FLAG} alone, and was only observed when all three eIF2 subunits were co-transfected (Figure 4C/D). Transient expression of the human eIF2β/2γ clones in HEK293T cells was neutral with regards to the reporter read-out (Figure 4A/B). Therefore, the failure of ^{HA}eIF2αS/D to significantly inhibit global translation rates in N2a cells appears to reside in its inability to associate with the endogenous eIF2β/2γ subunits of the eIF2 complex.

Co-expression of eIF2β^{HA}, 2γ^{FLAG} and ^{HA}eIF2αS/D does not alter reinitiation in N2a cells

After translation of a uORF, those ribosomes that remain associated with the mRNA can continue to scan downstream. However, to subsequently reinitiate they must first recruit the TC (and possibly other initiation factors) from the free pool. The enhanced inhibition of global translation observed in N2a cells upon transient co-expression of the ^{HA}eIF2αS/D with eIF2β^{HA}/2γ^{FLAG}, suggested that under these conditions an eIF2$^{S/D}$.GTP.tRNA^{Met} TC was assembled, a single round of translation occurred and the released eIF2$^{S/D}$.GDP sequestered eIF2B. In this scenario, ^{HA}eIF2αS/D/2β^{HA}/2γ^{FLAG} co-expression should also modulate delayed reinitiation. We tested this using the two reporter assays depicted in Figure 2. Using the FLuc-iAUG/RLuc-iAGG dual monocistronic reporters we once again observed an increase in the normalized FLuc/RLuc ratios in HEK293T cells transiently expressing ^{HA}eIF2αS/D. This remained essentially unchanged upon ^{HA}eIF2αS/D and eIF2β^{HA}/2γ^{FLAG} co-expression (Figure 5A/B). However, in N2a cells, and under all the conditions tested, FLuc/RLuc ratios remained static suggesting no change in the behaviour of the reinitiating ribosomes (Figure 5A). This non-response in N2a cells was further examined using the ΔKpn50 FLuc reporter (Figure 5C/D). As reported earlier, in HEK293T cells ^{HA}eIF2αS/D expression significantly reduced the RE compared to the control (82% to 63%: Figure 5C). Curiously, ^{HA}eIF2αS/D and eIF2β^{HA}/2γ^{FLAG} co-expression produced an intermediate RE value (72%) in these cells. This was nonetheless statistically different relative to both the control and the ^{HA}eIF2αS/D. This may reflect the fact that in HEK293T cells we efficiently and continually form an eIF2$^{S/D}$.GTP.tRNA^{Met} TC from the three co-transfected expression vectors. This process is insensitive to the sequestration of eIF2B. However, once again in the N2a background RE values remained unchanged (Figure 5C). Therefore, despite the fact that in N2a cells one can restore the negative effect of ^{HA}eIF2αS/D on protein expression by the transient co-expression of the eIF2β^{HA}/2γ^{FLAG} subunits, this same approach fails to modulate the reinitiation response.

The expression of ^{HA}eIF2αS/D fails to induce a robust ISR in N2a cells

The ISR leads to the translational upregulation of ATF4, a response that is coupled to the presence of uORFs in its 5′ TL that promote downstream reinitiation events (13,31). Since our reporter assays in N2a cells indicated that ^{HA}eIF2αS/D expression did not modulate reinitiation we reasoned that it would also not alter ATF4 expression. ATF4 is a transcriptional activator of genes involved in metabolism, cellular redox status and regulation of apoptosis (32). One of these target genes is another transcription factor, the cEBP homologous protein (CHOP). CHOP expression plays a key role in determining subsequent cell fate (33,34). Initially, we confirmed that cellular stress, as induced by thapsigargin treatment, induced a strong ISR response in both HEK293T and N2a cells as determined by the transcriptional upregulation of CHOP mRNA expression (Figure 6). However, whereas ^{HA}eIF2αS/D ± eIF2β^{HA}/γ^{FLAG} upregulated CHOP expression in HEK293T cells all these conditions were silent in N2a cells (Figure 6).

DISCUSSION

Phosphorylation of eIF2α is the key event in the ISR. As such, it serves as a brake on *de-novo* protein expression giving the cell time to respond to the insult that generated the stress. However, stress also promotes the recruitment of specific mRNA sub-populations onto polysomes, an event that appears to correlate with the presence of a small uORF

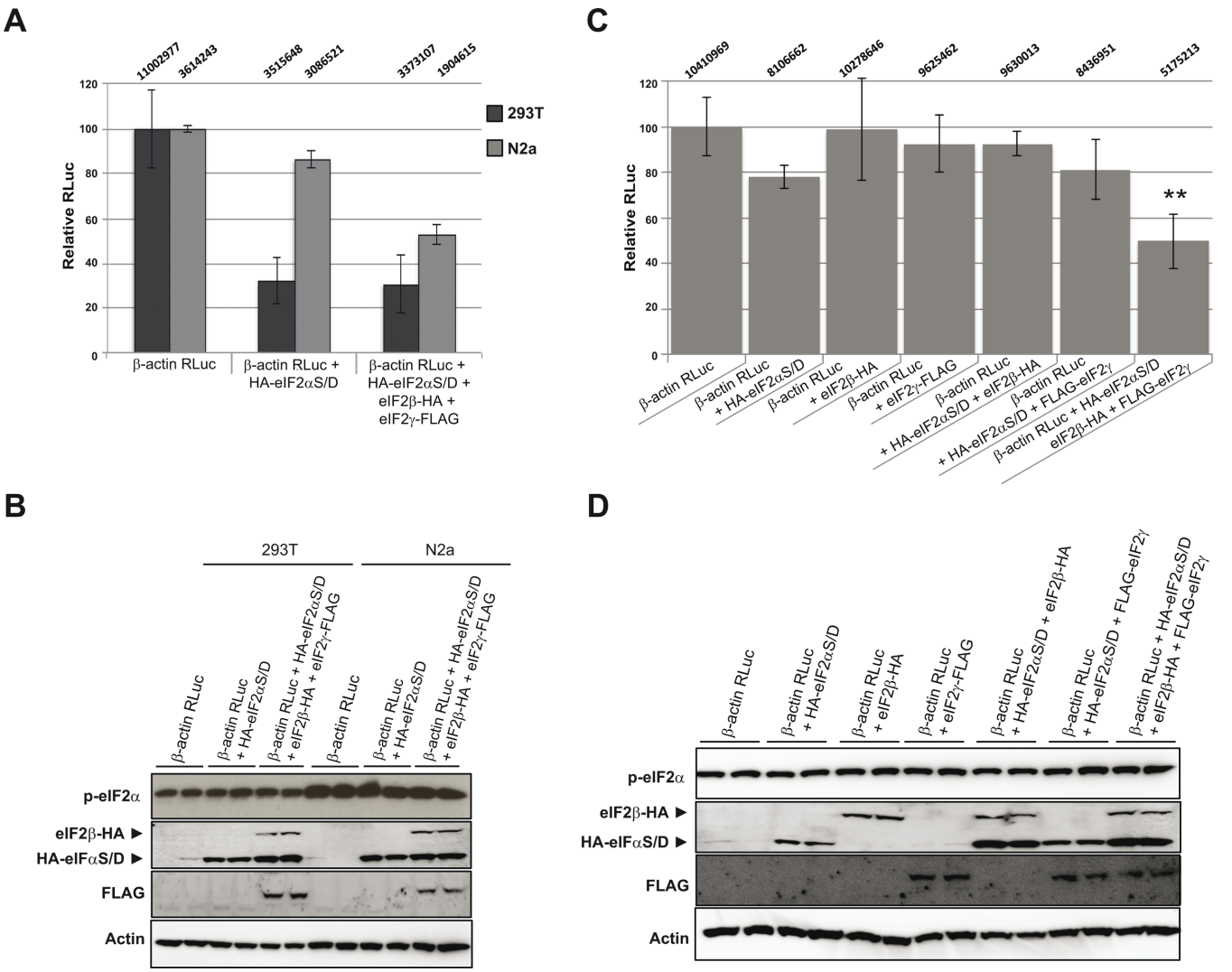

Figure 4. Co-expression of eIF2β/γ enhances the negative effect of eIF2αS/D in N2a cells. (**A**) HEK293T and N2a cells were transfected with the β-actin RLuc reporter in the presence of ^{HA}eIF2αS/D or ^{HA}eIF2αS/D plus eIF2β^{HA}/2γ^{FLAG}. RLuc activity was recorded 24 h post-transfection. Bars indicate the SEM determined from the transfection triplicates. The numbers indicated above the columns are the average RLuc values obtained from each triplicate. (**B**) Protein expression levels from two of the biological triplicates were monitored by immunoblotting using the Ab's indicated on the left (anti-phospho-eIF2α, anti-HA, anti-FLAG and anti-actin). (**C**) Different combinations of the eIF2 subunits were tested for their negative effect on β-actin RLuc reporter activities in transiently transfected N2a cells. Values have been normalized to the β-actin RLuc control which is set at 100. Bars indicate the SEM from triplicate assays. The ** indicates a statistically significant difference relative to the βactin RLuc control (**$P < 0.01$). The numbers indicated above the columns are the average RLuc values obtained from each triplicate. (**D**) Protein expression levels from two of the biological triplicates were monitored by immunoblotting using the anti-phospho-eIF2α, anti-HA, anti-FLAG and anti-actin Ab's (as indicated on the left).

within their 5′ TL (13,32,35,36). Sustained high levels of eIF2α phosphorylation induce cell cycle arrest via the inhibition of cyclin D1 translation (37,38), modulate gene expression by the translational upregulation of transcription factors such as ATF4 (39,40) and trigger apoptosis (7,41). Furthermore, the dynamics of eIF2α phosphorylation has been proposed to play a role in the inflammatory and immune response, long-lasting synaptic plasticity and long-term memory in the brain (42,43) and in pathologies such as cancer (6). How the phosphorylation status of eIF2α impacts on the tumoural phenotype remains unresolved. For example, studies in mice suggested that decreased phosho-eIF2α levels promote tumourigenesis (44,45), whereas reduced PKR levels, and hence reduced phosho-eIF2α levels, have been correlated with less aggressive human cancers (6).

With this spectrum of biological activity, the regulation of eIF2α phosphorylation has gained considerable interest. In cell culture model systems, it has been attractive to target the endogenous TC complex directly by expressing epitope tagged versions of the WT eIF2α, the phospho-deficient eIF2αS51A or the phosphomimetic eIF2αS51D (46). However, several groups have reported conflicting effects of these transgenes depending on the experimental system employed. For example, eIF2αS51A was alone able to transform NIH3T3 cells but not 3T3L1 (44,47). In a similar vein, eIF2αS51D expression induced apoptosis in NIH3T3 cells but not in 3T3L1 cells (23,47). This may reflect the na-

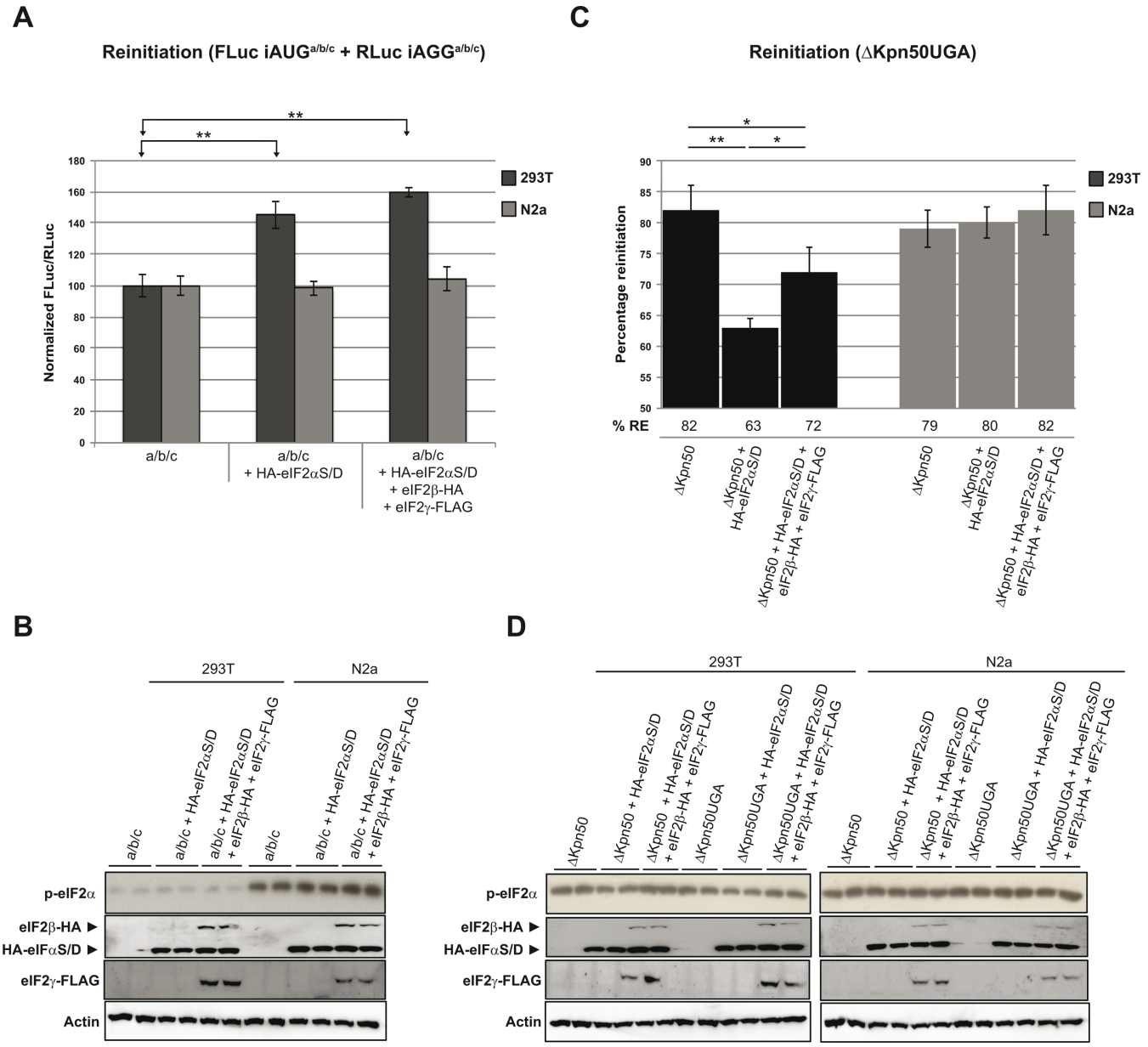

Figure 5. Co-expression of ^{HA}eIF2αS/D with eIF2β^{HA}/γ^{FLAG} does not alter reinitiation in N2a cells. (**A**) Reinitiation was monitored using the FLuc-iAUG$^{a/b/c}$/RLuc-iAGG$^{a/b/c}$ dual monocistronic reporter assay performed in the absence (columns abc) or presence of either a co-expressed ^{HA}eIF2αS/D or ^{HA}eIF2αS/D plus eIF2β^{HA}/2γ^{FLAG} (see Figure 2E). The normalized FLuc/RLuc ratios are depicted graphically, with the value for the abc control set at 100. Arrows point to data sets whose difference is considered statistically significant (**$P < 0.01$). (**B**) Protein expression levels from two of the biological triplicates were monitored by immunoblotting using the anti-phospho-eIF2α, anti-HA, anti-FLAG and anti-actin Ab's (as indicated on the left). (**C**) Reinitiation efficiency (RE) was measured using the ΔKpn50-FLuc-EMCV-RLuc and ΔKpn50UGA-FLuc-EMCV-RLuc bicistronic reporters (see Figure 2A). Plasmids were expressed in either HEK293T or N2a cells with ^{HA}eIF2αS/D or ^{HA}eIF2αS/D plus eIF2β^{HA}/2γ^{FLAG} and reinitiation frequency at the AUG^{Elk1} was determined as indicated in the text. Bars indicate the SEM from biological triplicates. Arrows point to data sets whose difference is considered statistically significant (**$P < 0.01$, *$P < 0.05$). (**D**) Protein expression levels from two of the biological triplicates were monitored by immunoblotting using the anti-phospho-eIF2α, anti-HA, anti-FLAG and anti-actin Ab's.

ture of the intricate signalling pathways operating in each cellular context. However, our current study points to another possible interpretation. The attenuated effect of the eIF2αS51D on reporter expression in N2a cells (in comparison with HEK293T cells), despite high expression levels, correlated with its inability to form a significant amount of the eIF2 complex (and hence TC) via association with the endogenous eIF2β/γ subunits. This interpretation was supported both by sedimentation studies, co-IP and by the observation that the negative effect could be enhanced (to levels similar to those observed in HEK293T cells) by the transient co-expression of eIF2β/γ. In contrast, sedimentation studies and co-IP indicate that in HEK293T cells a significant fraction of the transiently expressed eIF2αS51D formed a TC, an event that correlated with significant global downregulation.

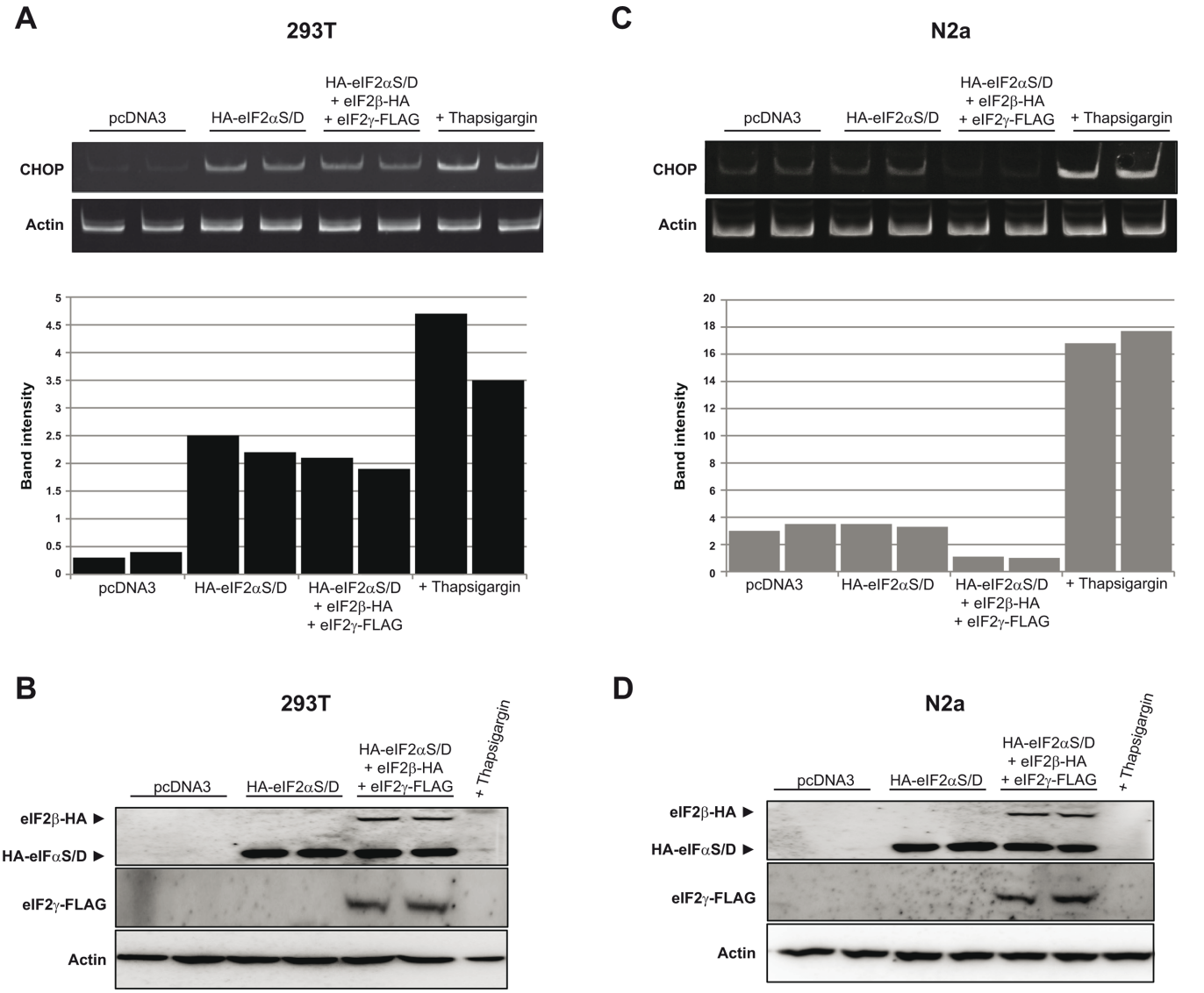

Figure 6. Induction of the ISR as monitored by CHOP mRNA expression. The ISR was monitored by measuring the transcriptional upregulation of the CHOP gene by RT-PCR. Both HEK293T (**A**) and N2a cells (**C**) were transfected with the plasmid constructs as indicated above the two panels or treated with thapsigargin (300 nM). All transfections were performed in duplicate. RT-PCR was performed on total cell RNA with primer sets specific for CHOP and actin (upper panel). The CHOP-specific bands were quantitated and this is plotted graphically below the gels (lower panel). Immunoblots were performed on the cell extracts from HEK293T cells (**B**) and N2a cells (**D**) using the anti-HA, anti-FLAG and anti-actin Ab's.

However, one curious observation was that reinitiation in N2a cells was not only insensitive to the presence of eIF2αS/D alone, but also in combination with transiently expressed eIF2β/γ, despite the fact that the latter condition impacted negatively on the global reporter readout. This was further confirmed by the absence of an ISR in N2a cells transiently expressing eIF2αS/D ± eIF2β/γ, conditions that produced a robust response in HEK293T cells (Figure 6). Assays performed in the absence of eIF2αS/D indicated no apparent differences in the behaviour of the reinitiating ribosome in both cell backgrounds. For example, using the ΔKpn-FLuc assay, RE frequency in N2a and HEK293T cells were essentially identical (60% versus 59%, respectively) and both increased after addition of the 50 nts spacer (82% versus 84%) (Figure 2). The response to increases in the endogenous phospho-eIF2α levels induced by thapsigargin treatment was also similar to that previously reported in HEK293T cells and the drug induced a strong ISR in both cell backgrounds (Figure 6) (20). So why this non-response to eIF2αS/D even in the presence of transiently co-expressed eIF2β/γ, conditions that clearly impact on the global readout, and what does this mean with regards to the utilisation of the phosphomimetic construct? With regards to the former, we can envisage at least two possible scenarios/models. In the first, the 40S ribosome paused after translation of an uORF in N2a cells is unable to recruit TC complexes carrying eIF2αS/D whereas this is not the case for free 40S subunits that have been released from the mRNA (hence the global downregulation upon co-expression of ^{HA}eIF2αS/D plus eIF2β^{HA}/γ^{FLAG}).

The difference between the two may reside with the continued presence of initiation factors on the RNA-associated 40S that is in the 'reinitiation mode'; factors that the free 40S subunit has lost and must recruit from the cytoplasmic pool. Indeed, work from several eukaryotic systems suggests that not all the initiation factors are immediately released at the entry into the elongation phase of translation. Rather, these factors, and in particular eIF1A and eIF3, are shed in a stochastic manner as the 80S subunit decodes the mRNA (48). Furthermore, the presence of eIF3 on ribosomes post-termination of a small uORF has been proposed to anchor the 40S to the mRNA and thereby promote reinitiation (17–19). Apart from the TC, it is unclear what other eIFs (and in what order) the reinitiating ribosome must recruit before it can scan downstream to the next start codon (49). With regards to the free 40S, it has been reported that eIF1/1A/3 are required for the formation of a stable PIC although it is unclear to what extent this can be extrapolated to the 40S paused after translation of a uORF (50). This is further complicated by the possible presence of the multi-factor complex (MFC), a pre-assembled complex composed of the TC with eIF3.eIF1.eIF1A.eIF5 (51). Could this be the major form in which eIF2αS/D assembles in N2a cells and can this form load onto reinitiating ribosomes that may already carry eIF3 and eIF1A? In the second model, the $eIF2^{S/D}$ complexed generated during transient co-expression of the $\alpha^{S/D}/\beta/\gamma$ subunits is defective at one step of the initiation process upstream of start site selection (i.e. prior to generation and release of the $eIF2^{S/D}$-GDP that will sequester eIF2B). This could explain the observed repression of global protein expression without an impact on reinitiation.

The failure to modify the reinitiation phenotype, even under conditions which induce a major inhibition in the global translational readout, is confirmed by the absence of an ISR response in N2a cells expressing $^{HA}eIF2\alpha S/D \pm eIF2\beta^{HA}/\gamma^{FLAG}$. Taken together our studies in the N2a cell background indicate that the phosphomimetic construct does not always faithfully mimic the response associated with eIF2α phosphorylation. Such a conclusion may explain some of the conflicting reports associated with the use of Ser51 mutants in different cellular backgrounds.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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