eIF2B localisation and its regulation during the integrated stress response is
cell type specific

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Abstract:

Eukaryotic initiation factor 2B (eIF2B) is a master regulator of translation control. eIF2B
recycles inactive eIF2-GDP to active eIF2-GTP. Under transient/acute cellular stress,
a family of kinases phosphorylate the alpha subunit of eIF2 (eIF2α-P[S51]) activating
the integrated stress response (ISR). This response pathway inhibits eIF2B activity
resulting in overall translation attenuation and reprogramming of gene expression to
overcome the stress. The duration of an ISR programme can dictate cell fate wherein
chronic activation has pathological outcomes. Vanishing white matter disease
(VWMD) is a chronic ISR-related disorder linked to mutations in eIF2B. eIF2B is vital
to all cell types, yet VWMD eIF2B mutations primarily affect astrocytes and
oligodendrocytes suggesting cell type-specific functions of eIF2B. Regulation of the
cytoplasmic localisation of eIF2B (eIF2B bodies) has been implicated in the ISR. Here,
we highlight the cell type specific localisation of eIF2B within neuronal and glial cell
types. Our analyses revealed that each cell type possesses its own steady-state
repertoire of eIF2B bodies with varied subunit composition and activity. We also
demonstrate that neural and glial cell types respond similarly to acute induction of the
ISR whilst a chronic ISR programme exerts cell type-specific differences. Regulatory
composition of eIF2B bodies is suggested to be differentially modulated in a manner
that correlates to the action of acute and chronic ISR. We also highlight a cell type
specific response of the ISR inhibitor ISRIB on eIF2B localisation and activity.

Introduction

All biological processes are intrinsically dependent upon the highly conserved and hierarchical process of mRNA translation. A key protein complex involved in ensuring that efficient translation initiation takes place is the eukaryotic initiation factor 2, eIF2. eIF2 is a heterotrimeric G-protein made up of the subunits α, β, and γ (Naveau et al., 2013; Schmitt et al., 2012). In its active GTP-bound state, eIF2 is complexed with initiator methionyl transfer RNA (eIF2-GTP-Met-tRNAi) and forms a ternary complex (TC) whose key role is to locate the first start codon to the ribosome (Hinnebusch & Lorsch, 2012). Following codon recognition, eIF2-GTP is hydrolysed to eIF2-GDP through the action of the canonical GTPase-activating protein eIF5 (Paulin et al., 2001). Crucial for successive rounds of translation is the regeneration of GTP-bound eIF2 which is catalysed by the guanine nucleotide exchange factor (GEF) eIF2B. Once released from the scanning ribosome, eIF5 stays associated with eIF2-GDP and hinders any spontaneous GDP release (GDP dissociation inhibitor, GDI) from eIF2. In addition to its GEF function, eIF2B acts as a GDI displacement factor (Jennings et al., 2013), removing eIF5, followed by GDP release from eIF2 (Williams et al., 2001). These functions highlight eIF2B as a powerful control checkpoint for the availability of TCs.

In its native form, eIF2B is a heterodecameric complex composed of two copies of 5 non-identical subunits (termed eIF2Bα-ε). The γ and ε subunits catalyse the GEF activity, whereas the α, β and δ subunits regulate this activity in response to different cellular stress insults (Bogorad et al., 2014; Kimball et al., 1998; Pavitt et al., 1997; Pavitt et al., 1998). Structurally, eIF2B decameric conformation is comprised of an eIF2B(αβδ)$_2$ hexameric regulatory core laid between two eIF2B(γε) catalytic heterodimers (Tsai et al., 2018; Zyryanova et al., 2018). In mammalian cells, eIF2B has been reported to exist in different sub-complexes arrangements with varying subunit composition (Liu, et al., 2011; Wortham et al., 2014).

At the hub of translational control is the regulation of eIF2B activity by the integrated stress response (ISR) (Pakos-Zebrucka et al., 2016; Hanson et al., 2022). During acute or transient stress, the ISR activates stress-sensing kinases (PERK, PKR, GCN2, HRI) which phosphorylate the α subunit of eIF2 at serine 51 (eIF2α-P[S51]). Phosphorylated eIF2α acts as a competitive substrate to its unphosphorylated

cognate, blocking GEF activity of decameric eIF2B by inhibiting the interaction of eIF2γ

with the eIF2Bε subunit (Schoof et al., 2021; Zyryanova et al., 2021; Adomavicius et

al., 2019; Kashiwagi et al., 2017; Kashiwagi et al., 2019; Kashiwagi et al., 2016).

Attenuated eIF2B activity limits TC levels and reduces global protein synthesis.

Concomitantly, a specific subset of mRNAs harbouring upstream ORFs bypass this

translation attenuation. These include activating transcription factor 4, ATF4, and

C/EBP homologous protein, CHOP (Harding et al., 2000). In contrast, transition to a

chronically activated ISR is widely reported as adaptive to prolonged stress, ultimately

pro-apoptotic when cells are unable to overcome sustained stress with pathological

consequences (Bond et al., 2020).

In yeast cells, eIF2B localises to stable cytoplasmic foci termed 'eIF2B bodies' where

GEF activity takes place and are targeted for regulation (Campbell et al., 2005;

Campbell & Ashe, 2006; Egbe et al., 2015; Moon & Parker, 2018; Norris et al., 2021;

Nüske et al., 2020; Taylor et al., 2010). These studies were further extended in

mammalian cells where heterogeneous populations of different-sized bodies

correlating to their eIF2B subunit makeup were observed (Hodgson et al., 2019).

Larger bodies contained all eIF2B subunits, whilst small bodies predominantly

consisted of the γ and ε catalytic subunits. Upon acute endoplasmic reticulum (ER)

stress, it was demonstrated that the ISR differentially modulates these eIF2B body

subpopulations, decreasing the GEF activity of larger bodies and inversely increasing

GEF activity within small bodies. This increase in GEF activity was concomitant with

a redistribution of eIF2Bδ to small bodies, suggesting the existence of a previously

unidentified eIF2Bγδε heterotrimeric sub-complex. ISR-targeting drugs (*e.g.* ISRIB)

which boost translation recapitulated this eIF2Bδ redistribution to small bodies in

unstressed cells (Hodgson et al., 2019), thus implying that this action might be an

innate response to the ISR to allow low baseline levels of translation. Nonetheless, the

functional relevance of eIF2Bδ redistribution is still unknown.

Despite eIF2B's ubiquitous role in the ISR across all cell types (Pakos-Zebrucka et

al., 2016), mutations in any of the five subunits of eIF2B result in the neurological

disorder leukodystrophy with vanishing white matter disease (VWMD) (van der Knaap

et al., 2006). VWMD mutations are selectively detrimental to astrocytes, cause

defective maturation and mitochondrial dysfunction in oligodendrocytes and,

ultimately, lead to neuronal death due to axonal de-myelination (Bugiani et al., 2011;

Dooves et al., 2016; Dooves et al., 2018; Herrero et al., 2019; Klok et al., 2018;
Leferink et al., 2018). Surprisingly, studies have shown that cultured neurons are
unaffected by eIF2B VWMD mutations, implying that cell type-specific features of
eIF2B function and regulation may exist at least in brain cell types, which remains to
be understood. We previously showed that eIF2B bodies are sites of eIF2B GEF
activity as eIF2 can shuttle into these bodies in a manner that correlates with ISR
activation   (Hodgson et al., 2019). Here, we investigated steady-state eIF2B
localisation dynamics and subsequent changes upon cellular stress and classical ISR-
targeting drugs in neuronal and glial cell lines. We report that eIF2B localisation to
eIF2B bodies is tailored in a cell type-specific manner. We also demonstrate that the
regulatory composition of eIF2B bodies is tightly modulated by cellular stress in a cell
type-manner. We further showcase a novel cell type-sensitivity feature of ISRIB in the
regulation of eIF2B body composition and eIF2 shuttling.

<u>Results</u>

eIF2B localises to eIF2B bodies in a cell type dependent manner

eIF2B localisation has been reported in yeast (Campbell et al., 2005; Moon & Parker,

2018; Taylor et al., 2010) and, more recently, in mammalian cells (Hodgson et al.,

2019), however the latter shows a higher degree of complexity. To further our

knowledge of cellular eIF2B localisation, we transiently transfected the catalytic ε

subunit (eIF2Bε) tagged with a monomeric green fluorescent protein (mGFP) into

neuroblastoma (SH-SY5Y), astrocytoma (U373) and hybrid primary oligodendrocytes

(MO3.13) cell lines and observed different patterns of eIF2B localisation in all 3 cells

lines (**Figure 1A and Figure S1A**). Cells expressing eIF2Bε-mGFP exhibited either

eIF2B bodies or the localisation was fully dispersed throughout the cytoplasm (**Figure**

1Bi). We observed that the percentage (%) cells localising eIF2B significantly differs

across cell types (**Figure 1Bii**). U373 cells showed the highest percentage of cells

containing eIF2B bodies (53.50%) followed by MO3.13 (33.25%) and SH-SY5Y

exhibiting the lowest percentage (19.25%). Because eIF2B overexpression could

potentially impact the observed localisation pattern across cell types, we examined

endogenous eIF2Bε and observed a similar trend (**Figure S1B**). Next, given the

heterogenous populations of different sized eIF2B bodies, we subcategorised them

into small eIF2B bodies ($<1\mu^2$) or large eIF2B bodies ($\geq1\mu^2$) (**Figure 1Ci**). Small

eIF2Bε-mGFP bodies were the predominant subpopulation across all cell types. U373

and MO3.13 cells exhibited a similar percentage per cell (88.19% and 89.34%,

respectively), and both were slightly higher in comparison to SH-SY5Y cells (71.46%).

In contrast, SH-SY5Y cells displayed an increased average percentage of large

eIF2Bε-mGFP bodies per cell (30.54%) in comparison to U373 and MO3.13 cells

(13.81% and 12.66%, respectively). Here, we show that eIF2B localisation is

fundamentally cell type specific: each brain cell type harbours its own prevalence of

eIF2B bodies although abundance of each body size group is suggested to be similar

amongst glial cell types.

Subunit composition of eIF2B bodies is cell type-specific

eIF2B exists as a decameric complex. eIF2Bε alone can carry out GEF activity,
however the rate of this exchange is enhanced upon joining of other eIF2B subunits
(Gomez & Pavitt, 2000). Regulatory subunits increase GEF activity, modulate levels
of eIF2B activity upon cellular stress and, more recently, colocalise to eIF2B bodies in
a size-dependent manner (Liu et al., 2011; Hodgson et al., 2019). Having shown eIF2B
localisation is different between cell types (**Figure 1B and Figure 1C**), we next
investigated whether subunit co-localisation to eIF2Bε-mGFP bodies also exhibits cell
type specific features. We performed immunocytochemistry on the regulatory (eIF2Bα,
eIF2Bβ, eIF2Bδ) and catalytic (eIF2Bγ) subunits of eIF2B in SH-SY5Y, U373 and
MO3.13 cells (**Figure 1Di**). Previous data using U373 cells revealed that small eIF2B
bodies predominantly contain catalytic subunits, whilst larger eIF2B bodies
additionally contain a mixture of regulatory subunits (Hodgson et al., 2019). We
confirmed that this trend is observed across all cell types by measuring the percentage
(%) of small and large eIF2Bε-mGFP bodies that co-localise with the remaining
subunits (eIF2Bα-γ). eIF2Bγ co-localisation with eIF2Bε-mGFP showed the highest
mean percentage in small eIF2B bodies, although slightly increased in neuronal cells
when compared to glial cells (SH-SY5Y: 51.99%; U373: 31.86%; MO3.13: 31.63%)
(**Figure 1Dii**). Moreover, neuronal cells also displayed a significantly higher
percentage of small bodies containing regulatory subunits eIF2Bα (SH-SY5Y: 27.58%;
U373: 7.72%; MO3.13: 8.13%), eIF2Bβ (SH-SY5Y: 17.33%; U373: 5.94%; MO3.13:
0.68%) and eIF2Bδ (SH-SY5Y: 20.83%; U373: 10.63%; MO3.13: 9.03%). Large eIF2B
bodies showed similar catalytic eIF2Bγ co-localisation across all cell types (SH-SY5Y:
91.23%; U373: 93.22%; MO3.13: 77.02%) with drastic cell-type disparities on
regulatory subunit make-up (**Figure 1Dii**). Oligodendrocytic cells displayed slightly
lower eIF2Bα co-localisation albeit no statistically significant difference compared to
the other cell types (SH-SY5Y: 60.26%; U373: 59.02%; MO3.13: 38.25%) and near
absence of eIF2Bβ co-localisation (SH-SY5Y: 38.38%; U373: 41.13%; MO3.13:
0.62%) even though endogenous eIF2Bβ localises to cytoplasmic foci (**Figure 1Dii).**
eIF2Bδ co-localisation to large eIF2B bodies was overall similar across cell types (SH-
SY5Y: 62.39%; U373: 67.48%; MO3.13: 65.00%). These results demonstrate that our
previous findings correlating eIF2B body size to subunit composition (Hodgson et al.,
2019) is somewhat exerted on a cell type basis: astrocytic and neuronal cells follow

this size:subunit pattern whilst eIF2B bodies of oligodendrocytes are largely depleted

of a regulatory eIF2B subunit.

The rate of eIF2 shuttling within eIF2B bodies is cell type specific

eIF2B controls the levels of ternary complexes by regulating the available pool of GTP-

bound eIF2. Previous studies have shown that the shuttling rate of eIF2 through eIF2B

bodies can infer eIF2B GEF activity (Campbell et al., 2005; Campbell & Ashe, 2006;

Hodgson et al., 2019; Norris et al., 2021). We co-transfected eIF2α-tGFP iand eIF2Bε-

mRFP in SH-SY5Y, U373 and MO3.13 cells and performed fluorescence recovery

after photobleaching (FRAP) on small and large eIF2B bodies. We first confirmed that

all sized eIF2Bε-RFP bodies co-localised with eIF2α-tGFP (**Figure 2A**). Next, FRAP

analysis showed that eIF2α-tGFP recovery of small eIF2B bodies was relatively similar

across cell types, although slightly higher for U373 cells despite not being statistically

significant (SH-SY5Y, 34.21%; U373, 42.32%; MO3.13, 34.16%) (**Figure 2Bi and ii**).

Surprisingly, large eIF2B bodies showed drastic discrepancies. SH-SY5Y and U373

cells exhibited similar eIF2α-tGFP recovery (SH-SY5Y: 36.13%; U373: 37.08%) whilst

MO3.13 cells have significantly lower recovery (22.51%) (**Figure 2Bii**). Hence. these

data demonstrate that small eIF2B bodies displaying similar % recoveries are

functionally similar across all cell types whilst large eIF2B bodies display cell type

specific differences.

The acute ISR is similar across cell types while chronic ISR displays cell type

specific features

eIF2B localisation is modulated upon induction of an acute ISR in astrocytes

(Hodgson et al., 2019). Here we further characterised eIF2B localisation during the

transition to a chronic ISR by firstly characterising the acute *vs* chronic ISR activation

in neuronal and glial cell types. To test induction of the acute ISR we used thapsigargin

(Tg) and sodium arsenite (SA) to trigger ER stress and oxidative stress, respectively

(**Figure 3A**). We performed western blot analysis using canonical ISR markers

(PERK-P, eIF2α-P, CHOP and GADD34) (**Figure 3Bi**). As expected, short-term

treatment with either Tg (1µM 1h) or SA (125µM 0.5h) led to increased eIF2α

phosphorylation (eIF2α-P) and eIF2α-P-dependent protein synthesis shutdown across

all cell types (**Figure 3Bii**). Next, cells were exposed to Tg at a lower concentration

(300 nM) for 24h to monitor ER stress during the chronic ISR adaptation phase (Smith

et al., 2020) (**Figure 3A**). As expected, PERK remained partially phosphorylated

(shifted PERK band) and ISR markers (CHOP, GADD34) were expressed (**Figure**

3Bi). ATF4 expression was no longer detected at the 24h time point, however temporal

monitoring during this 24h period showed that it peaked 4-8h post-Tg treatment across

all cell types (**Figure S2**). A Tg treatment for 24h showed partial translation recovery

in comparison to 1h Tg treatment, confirming the transition to a chronic ISR program

(Guan et al., 2017).

VWMD is predominantly characterised by an abnormal chronic-like ISR which

selectively targets glial function exhibited by progressive white matter deterioration

upon acute stress episodes *(e.g.* head traumas and infections). However, this glial

vulnerability remains poorly understood. To provide insight into this cell type

specificity, we devised a VWMD-mimicking environment in SH-SY5Y, U373 and

MO3.13 cells whereby cells exposed to a chronic ISR are subsequently exposed to an

acute insult. Cells were treated with 300nM Tg for 24h and then 1 µM Tg or 125 µM

SA in the final 1h or 30 minutes, respectively. Interestingly, the additional Tg treatment

did not affect ISR markers nor translation levels, suggesting that an on-going chronic

ER stress is protective against a new ER stress insult (**Figure 3Bi and ii**). To confirm

that this observed unresponsiveness was not due to Tg saturation or ISR-independent

cellular effects of Tg, we treated cells with tunicamycin (Tm; which inhibits *N*-linked

glycosylation of ER proteins and leads to an ER stress activated ISR like Tg) in the

last 2h of a 24h treatment with 300 nM Tg. Tm treatment alone induced eIF2α

phosphorylation and suppressed protein synthesis, while the additional Tm treatment

to Tg preconditioned cells did not further impact protein synthesis when compared to

Tg 24h alone (**Figure S3**). However, when the cells were subsequently treated with

an acute oxidative stress (SA: 125 µM 0.5h), a decrease in *de novo* protein synthesis

akin to SA-only levels was observed (**Figure 3Bii**), suggesting that cells reset the

acute ISR program following chronic ER stress when exposed to different stressors.

This decrease in protein synthesis was linked to a significant increase in eIF2α

phosphorylation in U373 and MO3.13 cells. Unexpectedly, this eIF2α phosphorylation

increase was not as dramatic in SH-SY5Y cells; suggesting that the suppression of

protein synthesis observed here may be less dependent on eIF2α phosphorylation

(**Figure 3Bii**). To test whether this was the case, we employed the same chronic stress
conditions (Tg 24h, Tg 24h + Tg last 1h; Tg 24h + SA last 0.5h) in the presence or
absence of the ISR inhibitor ISRIB and performed puromycin incorporation assay
(**Figure 3C**). ISRIB which reverses inhibitory effects of eIF2α phosphorylation
(Sidrauski et al., 2013) was unable to fully restore protein synthesis in SH-SY5Y cells
compared to the glial cell types (**Figure 3C**). Taken together, these results suggest
that subsequent oxidative stress in chronically ER-stressed neuronal cells is partially
uncoupled from eIF2α-mediated translational control while glial cells trigger a
sequential acute ISR program.

Regulatory remodelling of small eIF2B bodies is specific to the acute phase of
the ISR and partially modulated by eIF2α phosphorylation

To investigate the impact of cellular stress in eIF2B localisation, we transiently
transfected SH-SY5Y, U373 and MO3.13 cells with eIF2Bε-mGFP and treated with
the previously described acute (Tg 1h, SA 0.5h) and chronic (Tg 24h, Tg 24h + Tg last
1h, Tg 24h + SA last 0.5h) treatments. We observed an overall increase of eIF2B
localisation in all cell types although astrocytic cells displayed a higher degree of
stimulation (**Figure S4**). Furthermore, SH-SY5Y and MO3.13 cells showed
significantly increased cells harbouring localised eIF2B when treated with a VWMD-
devised condition (Tg 24h + SA last 0.5h) (**Figure S4**).

We previously reported increased eIF2Bδ localisation to small eIF2B bodies (mainly
composed of catalytic γ and ε subunits) upon acute ISR in astrocytes, suggesting the
presence of novel eIF2Bγδε subcomplexes (Hodgson et al., 2019). This implies that
eIF2Bδ redistribution may play a functional role during cellular ISR, however the
specific role is unknown. Given the similarities in the response to acute ISR observed
in neuronal and glial cell lines (**Figure 3B**), we wanted to investigate whether the
redistribution of eIF2Bδ was also similarly regulated. We performed
immunofluorescence analysis using an eIF2Bδ antibody in SH-SY5Y, U373 and
MO3.13 cells expressing eIF2Bε-mGFP (**Figure 4Ai**). As expected, short-term Tg and
SA treatment increased eIF2Bδ localisation to small eIF2B bodies in all cell types
whilst large eIF2B bodies remained predominantly unchanged (**Figure 4Aii**). During a
chronic ISR treatment (Tg 24h), eIF2Bδ composition of small bodies return to control

levels (**Figure 4Aii**) suggesting that this stress-induced eIF2Bδ movement is specific
to an acute ISR. Surprisingly, the additional SA treatment after chronic ER stress
exposure mirrored the cell type pattern observed for eIF2α phosphorylation (**Figure**
3Bii). U373 and MO3.13 cells which showed an induction of the acute ISR, also
displayed a redistribution of eIF2Bδ to small eIF2B bodies resembling their respective
SA-only treatment (**Figure 4Aii**). In neuronal cells, this acute SA insult after chronic
ER stress, which did not induce high levels of eIF2α phosphorylation, failed to
significantly enhance eIF2Bδ localisation to small eIF2B bodies when compared to
levels treated with SA only (**Figure 4Aii**).

eIF2Bδ redistribution has been previously hypothesized to be modulated by levels of
eIF2α-P (Hodgson et al., 2019), and here we further strengthened this hypothesis by
observing a mirrored pattern of increased eIF2α-P levels and increased eIF2Bδ to
small bodies (**Figure 3B and Figure 4A**). To investigate whether levels of eIF2α-P
influence eIF2Bδ redistribution, we subjected cells to acute Tg treatment in the
presence or absence of GSK2606414, a highly selective inhibitor of PERK (PERKi)
(Halliday et al., 2017). In line with this, PERKi completely blocked eIF2α
phosphorylation and inhibited translation suppression when co-treated with Tg across
all cell types (**Figure 4Bi**). We again performed an immunofluorescence analysis using
eIF2Bδ antibody in SH-SY5Y, U373 and MO3.13 cells expressing localised eIF2Bε-
mGFP under the previously described Tg and PERKi conditions (**Figure 4Bii**).
Unexpectedly, while we observed a slight increase of eIF2Bδ localisation to small
bodies in SH-SY5Y and U373 cells when co-treated with Tg and PERKi (thus in the
absence of eIF2α phosphorylation), it was significantly lower than when compared to
Tg alone treated cells (**Figure 4Biii**). Moreover, co-treatment of PERKi and Tg
treatment exhibited similar levels of eIF2Bδ in small bodies of MO3.13 cells when
compared to Tg alone (**Figure 4Biii**). These data indicate that eIF2Bδ localisation to
small eIF2B bodies is partially dictated by eIF2α phosphorylation in a cell type-specific
manner.

ISRIB's mode of action on eIF2B localisation is cell type specific

ISRIB is an eIF2B activator that attenuates eIF2α-P-dependent translation
suppression by promoting decamer formation and enhancing eIF2B GEF activity

(Schoof et al., 2021; Zyryanova et al., 2021; Sidrauski et al., 2013). ISRIB does not
impact levels of eIF2α phosphorylation *per se* but rather rescues its downstream
inhibitory effect on protein synthesis. Previously, we have shown that eIF2Bδ
localisation to small eIF2B bodies increased as a direct effect of ISRIB's binding to
eIF2Bδ (Hodgson et al., 2019). To test whether this is a general cellular feature, we
treated SH-SY5Y, U373 and MO3.13 cells expressing eIF2Bε-mGFP with ISRIB (200
nM) for 1h and performed an immunofluorescence analysis using eIF2Bδ antibody
(**Figure 5Ai**).   As before, large eIF2B bodies showed no changes in eIF2Bδ
composition when exposed to ISRIB alone or in combination with preconditioned Tg
treatment for 24h (**Figure 5Aii**).   In contrast, ISRIB treatment showed increased
eIF2Bδ localisation in small bodies of U373 and MO3.13 cells while a complete
absence of eIF2Bδ redistribution was observed in SH-SY5Y cells (**Figure 5Aii**).
Moreover, preconditioning cells to chronic ER stress abrogated eIF2Bδ movement in
MO3.13 cells upon ISRIB treatment, whereas it had no impact on U373 cells which
showed eIF2Bδ redistribution in all ISRIB conditions (**Figure 5Aii**). These data provide
evidence that ISRIB's mechanism of action may involve cell type specific regulation of
eIF2B localisation. Given this cell type specific impact of ISRIB in the eIF2B
composition of small bodies, we next aimed to investigate if this mirrored a cell type-
specific rescue of protein synthesis. Puromycin incorporation assay revealed that
adding ISRIB restored protein synthesis in all cell types pre-treated with Tg for 23h
(**Figure 5Bi and ii**). Taken together, ISRIB's mode of action is suggested to not be
linked to eIF2Bδ remodelling of small eIF2B bodies in neuronal and oligodendrocytic
cells but may be involved in astrocytic cells.

ISRIB and cellular stress selectively modulates activity of eIF2B bodies in a cell
type-manner

In addition to the remodelling of eIF2Bδ composition in small eIF2B bodies, we have
previously described that both acute stress and ISRIB result in increased shuttling of
eIF2 in astrocytic cells (Hodgson et al., 2019). Therefore, we next turned to assess
whether there was any cell-specific regulation of eIF2 shuttling in the different cell
types upon acute and chronic cellular stress and in the presence or absence of ISRIB
treatment. We treated SH-SY5Y, U373 and MO3.13 cells with ISRIB alone or with an

acute Tg stress (1h) in the presence or absence of ISRIB and performed FRAP
analysis on small and large eIF2B bodies. Intriguingly, cell type disparities were
observed in the % recovery of eIF2 in both small and large bodies. For small bodies
treated with ISRIB, a significant increase in the % recovery of eIF2 was observed for
U373 cells (in line with previously published data; Hodgson et al., 2019) but not for the
SH-SY5Y or MO3.13 cells (**Figure 6Ai**). Upon acute Tg stress, U373 cells displayed
an increase in the % recovery of eIF2 into small bodies and this increase was
sustained but not increased upon co-treatment with ISRIB (**Figure 6Ai**). Again, this
increase is similar to our previous observations (Hodgson et al 2019). This increase
in recovery of eIF2 in small bodies was unique to U373 cells and was not observed for
either the SH-SY5Y or MO3.13 cells (**Figure 6Ai**). For large eIF2B bodies, ISRIB
treatment alone did not impact on eIF2 recovery of any cell lines (**Figure 6Aii**).
However, when cells were treated with an acute Tg stress (1h), a decrease in the %
recovery of eIF2 was observed for both U373 and SH-SY5Y cells but not for the
MO3.13 cells (**Figure 6Aii**). Furthermore, co-treatment of ISRIB and acute Tg
reversed the Tg-induced inhibitory effects on eIF2 shuttling of large eIF2B bodies in
U373 cells, while showing no effect on eIF2B bodies of SH-SY5Y and MO3.13 cells
(**Figure 6Aii**). These data show that acute cellular stress and ISRIB predominantly
regulate small and large eIF2B bodies of U373 cells amongst the cell lines used in this
study.

We next sought to observe cells with chronic ISR treatment in the presence and
absence of ISRIB. In line with the recovery of protein synthesis post-chronic stress,
eIF2 recovery was similar to the vehicle control cells post-24h treatment (**Figure 6Bi**
and ii). Moreover, ISRIB treatment in the last hour of the 24h exposure to Tg
significantly increased eIF2 recovery of small eIF2B bodies in U373 cells but not in
SH-SY5Y or MO3.13 cells (**Figure 6Bi**), while altogether having no enhancing impact
of the activity of large eIF2B bodies (**Figure 6Bii**). These data suggest that the activity
of eIF2B bodies is transiently regulated upon cellular stress and ISRIB modulates
small eIF2B bodies during chronic ISR in a cell type manner.

<u>Discussion</u>

We have previously reported that eIF2B bodies represent steady-state autonomous
clusters of GEF active eIF2B complexes (Campbell et al., 2005; Hodgson et al., 2019;
Norris et al., 2021; Taylor et al., 2010). Here we show that the prevalence of eIF2B
bodies is cell type specific in unstressed conditions (**Figure 1A-C**). Amongst the cell
types used in this study, astrocytic cells showed increased number of cells displaying
eIF2B bodies (~54%) in comparison to oligodendrocytic (~33%) and neuronal (~19%)
cells. Because localised eIF2B accounts for only a certain portion of total eIF2B, with
the remaining GEF exchange occurring elsewhere in the cytoplasm, we hypothesize
that the degree of eIF2B localisation differs depending on the cellular requirement for
regulated GEF activity, both for steady state purposes and ability to respond to stress.

A correlation between eIF2B body size and subunit composition was previously
reported (Hodgson et al., 2019) and now we extend these studies by demonstrating
that this correlation is cell type specific (**Figure 1D**). Firstly, for the small eIF2B bodies,
neuronal cells harboured increased levels of regulatory subunits (eIF2Bα,β,δ) in
comparison to both types of glial cells. These data indicate that small eIF2B bodies
within astrocytic and oligodendrocytic cells mainly contain eIF2Bγε heterodimers,
while in neuronal cells these small bodies contain eIF2B tetrameric and decameric
complexes. Secondly, neuronal and astrocytic cells followed the size:subunit
relationship as all four subunits (eIF2Bα-γ) showed a higher degree of co-localisation
to large bodies compared to the small bodies; while oligodendrocytes exhibited the
surprising absence of eIF2Bβ and a slightly decreased % of eIF2Bα. These results
suggest the full eIF2B decameric complex reside in large eIF2B bodies of neuronal
and astrocytic cells but may not in oligodendrocytic cells. Given that increased GEF
activity of eIF2B correlates with the presence of regulatory eIF2B subunits (Dev et al.,
2010; Fabian et al., 1997; Liu et al., 2011; Williams et al., 2001), this decreased
association of eIF2Bβ with large eIF2B bodies may account for the decreased basal
% eIF2 recovery observed for oligodendrocytes when compared to other cell types
(**Figure 2B**).

eIF2B bodies are targeted during the acute ISR (Hodgson et al., 2019) however it
remains unknown what their significance is upon transition to a chronic ISR. We first
aimed to characterise the acute and chronic ISR of neuronal and glial cell lines used

in this study. We report that the acute ISR is a general cellular feature as assessed by

the canonical eIF2α-dependent pathway of translation shutdown in agreement to a

plethora of other cell types (Guan et al., 2014; Spaan et al., 2019; Teske et al., 2011).

Interestingly, we observed a cell type specific ability to reset an acute-like ISR

depending on whether faced with repeated stresses or treated with a different stressor

(**Figure 3B**). In all cells, an initial chronic ER stress was protective towards a second

ER stress treatment. This has been shown by others where preconditioning cells to

mild eIF2α phosphorylation, either through inhibition of PP1c or stress-inducing agents

(Lu et al., 2004), has been shown to be cytoprotective. Strikingly, replacing the second

stress with an oxidative stress elevated eIF2α phosphorylation in glial cells but not in

neuronal cells, however protein synthesis was still targeted in neuronal cells

suggesting that this second stress may be regulated via an eIF2α-independent

mechanism. Our observations in neuronal cells were strengthened by the fact that

ISRIB (which reverses inhibitory effects of eIF2α phosphorylation) was unable to

restore translation under these stress conditions (chronic Tg + acute SA) (**Figure 3C**),

but not when treated with Tg alone for 24h in neuronal cells (**Figure 5B**). Therefore,

chronically ER stressed neurons redirect towards an eIF2α-independent mechanism

when exposed to oxidative stress. These results are quite unexpected given that

GADD34 expression levels are still elevated in these cells (**Figure 3B**), as GADD34

mRNA levels are known to serve as a molecular memory damper to subsequent

stresses (Batjargal et al., 2022; Klein et al., 2022; Shelkovnikova et al., 2017). This

apparent ability of (at least) glial cells to reset the ISR in the presence of GADD34

while neuronal cells seem to "forget" how to respond brings an important question:

was it even meant to be remembered? Given this lack of activation of a subsequent

ISR in neuronal cells we consider three possible reasons, but not mutually exclusive,

by order of likelihood. (1) The transition to a chronic ISR highlights the inability of

neuronal cells to re-shape cell adaptation solely through the ISR, hence shifting

towards alternative and/or parallel signalling pathways (*e.g.,* mTOR (Guan et al.,

2014; Terenzio et al., 2018), eIF2A (Kim et al., 2011), eIF3d (Guan et al., 2017),

eEF1A2 (Mendoza et al., 2021). Recent evidence supports that translation repression

is maintained in PERK-deficient neurons by complementary eIF2α-independent

mechanisms (tRNA-cleaving RNase) (Wolzak et al., 2022). (2) Secondly, a cell non-

autonomous trigger of the acute ISR in neuronal cells undergoing chronic stress thus

signalled by eIF2-dependent glial cells, supported by recent work were targeting PERK

the eIF2α axis of PERK in astrocytes rescues prion-causing neuronal dysfunction
(Smith et al., 2020). (3) Thirdly, multiple eIF2α kinases might be activated in neuronal
cells (Alvarez-Castelao et al., 2020; Wolzak et al., 2022) during chronic ER stress,
thus less susceptible to re-start an acute ISR when subsequently challenged with a
different stressor, whereas glial activation of eIF2α kinases may be stimuli-specific.
ISR 'exhaustion' has also been recently appreciated where translational-demanding
cell types (in this study, pancreatic β cells) are susceptible to ATF4-mediated
transcriptome decay when faced with frequent ER stress insults (Chen et al., 2022).

Activation of the acute ISR increases eIF2Bδ localisation to small eIF2B bodies
(containing γ and ε) with increased eIF2 shuttling in astrocytic cells, suggesting the
stress-induced formation and clustering of eIF2Bγδε subcomplexes with increased
GEF activity (Hodgson et al., 2019). Here we observed that while eIF2Bδ redistributes
to small bodies during acute Tg (1h) and SA (0.5h) treatments it returns to basal levels
upon chronic Tg treatment (24h) (**Figure 4A**); thus, suggesting subunit remodelling of
small eIF2B bodies is a transient event and specific to the acute ISR independent of
cell types, and reversed during the chronic ISR. Interestingly, a subsequent SA
treatment to Tg pre-treated cells, which selectively re-elevates eIF2α phosphorylation
in U373 and MO3.13 cells (**Figure 3B**), is also accompanied by increased eIF2Bδ
localisation of small bodies in these cells but not in SH-SY5Y cells (**Figure 4A**).
Surprisingly, we show that eIF2Bδ localisation to small eIF2B bodies in the presence
of acute ER stress is only partially dictated by eIF2α phosphorylation (**Figure 4B**),
suggesting that non-ISR mechanisms are at play in eIF2B body remodelling. We do
not discard the possibility that chemical stressors such as Tg may trigger multiple
pathways (Li & Hu, 2015; Wink et al., 2017) that could influence our observations.
Given that ER stress-induced eIF2Bδ remodelling exists upon PERK inhibition (yet at
a lower level), we speculate that these other pathways could serve as an activator of
eIF2B body remodelling, further enhanced and/or maintained by eIF2α
phosphorylation.

As shown previously, ISRIB mimicked the acute ISR program response in astrocytic
cells (Hodgson et al., 2019) and oligodendrocytic cells, however chronic ER stress
hindered the action of ISRIB for the latter (**Figure 5A**). However, this "stress-
mimicking" feature of ISRIB was not recapitulated in neuronal cells. ISRIB alone or in
combination with chronic ER stress did not increase eIF2Bδ localisation in neuronal

small eIF2B bodies (**Figure 5A**). Unexpectedly, despite the differences in eIF2Bδ

movement, ISRIB treatment of chronic ER-stressed cells had a unanimous restorative

effect on translation across all cell types (**Figure 5B**), thus suggesting that ISRIB-

mediated translational rescue does not require increased eIF2Bδ-containing small

bodies as a general feature. However, our data suggest that eIF2Bδ regulatory

remodelling may be functionally relevant to astrocytic cells.

eIF2B regulatory subunits control eIF2B activity upon stress (Krishnamoorthy et al.,

2001; Pavitt et al., 1997) whilst catalytic subunits remain desensitized to stress when

uncoupled from regulatory subunits (Liu et al., 2011; Wortham et al., 2014). Indeed,

FRAP analysis identified a cell type specific correlation between eIF2B subunit

composition and eIF2 recovery upon acute ISR and ISRIB treatment. (1) In neuronal

cells, acute ER stress had a predominantly inhibitory effect on the eIF2 recovery of

small and large bodies (**Figure 6A**). This may be a result of a more homogenous

composition of both small and large bodies hence functionally similar. This agrees with

our previous observations that neuronal small bodies have increased regulatory

subunit composition (**Figure 1D**). (2) In contrast, astrocytic cells displayed distinctive

functional responses for small and large eIF2B bodies as previously reported

(Hodgson et al., 2019): small bodies exhibited enhanced eIF2 recovery upon acute

ER stress and ISRIB treatment, while acute ER stress repressed the eIF2 recovery of

large bodies which is rescued by the addition of ISRIB (**Figure 6A**). (3) Finally, acute

ER stress and ISRIB had no impact on small and large eIF2B bodies of

oligodendrocytic cells. This unresponsiveness is likely related to the lack of eIF2Bβ

from eIF2B bodies (**Figure 1D**), hence supporting that non-decameric eIF2B

subcomplexes may predominantly localise to eIF2B bodies in oligodendrocytic cells.

While acute ER stress shows cell type-dependent discrepancies in the % recovery of

eIF2 of eIF2B bodies, these changes are unanimously reversed upon sustained Tg

treatment (**Figure 6B**), further suggesting that eIF2B localisation is normalised during

chronic ISR. Guan *et al.* recently provided evidence that recovery of eIF2B activity

may not be required upon transition to chronic stress and may be alternatively

mediated via eIF3 (Guan et al., 2017).

A cell-specific relationship between eIF2Bδ redistribution and eIF2 recovery was

observed in astrocytic cells as illustrated in Figure 7. Indeed, ISRIB treatment has a

dominant effect of increasing eIF2δ composition of small bodies, either alone or in
combination with chronic ER stress (**Figure 5A**), accompanied by enhanced %
recovery of eIF2 (**Figure 6B**). This relationship is not recapitulated in the other cell
types used in this study, which requires further *in vitro* studies to investigate the cell
specific GEF activity of eIF2Bγδε subcomplexes.

Collectively, our results demonstrate that cells display cell type specific localisation
and regulation of eIF2B bodies. The existence of different eIF2B subcomplexes of
eIF2B bodies may allow unique rates of ternary complexes levels and adaptability to
stress which overall might make translation more efficient and/or more easily
regulated. More importantly, we provide evidence for cell type-specific fine-tuning of
eIF2B function and regulation, the core event of the ISR; further emphasizing the need
to tailor therapeutic interventions in a cell type-manner.

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Material and Methods

Cell culture

Human U373 astrocytoma cells were cultured in Minimum Essential Medium (MEM),
supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 1 % (w/v)
Non-essential amino acids, 1 % (w/v) sodium pyruvate, 2 mM L-glutamine and 1 %
(w/v) penicillin/streptomycin. Human SH-SY5Y dopaminergic neuroblastoma cells
were cultured in Dulbecco's modified Eagle's medium:F-12 (DMEM:F-12; 1:1)
containing high glucose (3.151 g/L) (Lonza), supplemented with 10% (v/v) heat-
inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% (w/v)
penicillin/streptomycin. Human MO3.13 hybrid primary oligodendrocytes were
cultured in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented
with 10 % (v/v) heat-inactivated foetal bovine serum (FBS) and 2 mM L-glutamine. All
experiments were done with passage number no higher than 25. All media and
supplements were purchased from Life Technologies Co. (New York, USA), except
when indicated otherwise. All cell lines were maintained at 37°C under 5% CO_2 and
were routinely tested for contamination with MycoAlert™ Mycoplasma Detection Kit
(Lonza).

Cell treatments

For acute/transient induction of the ISR, cells were treated with 1 µM thapsigargin (Tg)
(Sigma-Aldrich) for 60 minutes; 3 µg/mL tunicamycin (Tm) (Cayman Chemical) for 2h;
and 125 µM sodium arsenite (SA) (Sigma-Aldrich) for 30 minutes. For chronic
induction of the ISR, cells were treated with 300 nM Tg for 24h. For acute/transient
cellular stress previously challenged with a chronic induction of the ISR, cells were
treated with 300 nM Tg for 24h where 1 µM Tg, 3 µg/mL Tm or 125 µM SA were added
in the last 1h, 2h and 30 minutes, respectively. For ISRIB treatment, cells were added
with 200 nM ISRIB (Sigma, Dorset, UK) for 1h. For PERK inhibition treatment, cells
were treated with 500 nM GSK2606414 (#5107, Tocris) for 1h. As control, cells were
treated with vehicle solution (DMSO) with the highest volume and treatment duration
depending on its respective experimental setup.

Plasmids

pCMV6-AC-tGFP plasmid vector encoding EIF2B5 (#RG202322) and EIF2S1 (#RG200368) was purchased from OriGene (Rockville, Maryland, USA). The coding open reading frame of EIF2B5 from the pCMV6-AC-tGFP vector was cloned into an empty pCMV6-AC-mGFP (#PS100040, OriGene) and empty pCMV6-AC-mRFP (#PS100034, OriGene) vectors. The constructs were verified by sequencing.

Transient transfection procedures

U373, SH-SY5Y and MO3.13 cells were seeded at a density of $3x10^5$, $5x10^5$ and $2.5x10^5$ cells, respectively, in a 6-well plate for at least 24 hours before transfection. For U373 cells, transient transfection was performed with transfection reagent 25-kDa polyethylenimine, branched (PEI) (1 mg/mL) (Sigma-Aldrich). 1 μg of plasmid DNA was diluted in 100 μL of serum- and antibiotic-free medium and incubated with 4 μg PEI for 10 minutes. 600 μL of antibiotic-free media was added to the transfection mixture, added to cells, and incubated for 4 hours at 37°C. Media was removed and replaced with complete media and incubated for 24-48h at 37°C prior to confocal imaging. SH-SY5Y and MO3.13 cells were transfected with Lipofectamine 3000 according to manufactures instructions.

Immunoblotting

$5x10^5$ cells were cultured on 6-well plates. Whole-cell protein lysates were prepared in CelLytic M lysis buffer (Sigma) with 1% protease/phosphatase inhibitors (Sigma) and other supplements (17.5 mM β-glycerophosphate, 1 mM PMSF, 10 mM NaF). Lysates were incubated on ice for 10 minutes and centrifuged (13,000 rpm, 10 min, 4°C) to remove cellular debris. Protein concentrations were determined with Qubit™ Protein Assay Kit (Thermo-Fisher) and subjected to SDS-PAGE electrophoresis. For western blots, samples were run on 7.5 or 10 % polyacrylamide gel and transferred using Trans-Blot Turbo Mini-nitrocellulose Transfer packs (Bio-Rad) on a Trans-Blot Turbo apparatus. When necessary, membranes were subjected to Revert™ Total Protein Stain (LiCor) following manufacturer's instructions. Membranes were blocked in 5 % milk or 5 % BSA and probed with primary antibodies diluted in 5 % milk or 5 % BSA, overnight at 4°C. The following antibodies were used: eIF2α (1:500, ab5369, Abcam), phosho-eIF2α[ser51] (1:500, ab32157, Abcam), PERK (1:1000, 20582-1-AP, Proteintech), GADD34 (1:500, 10449-1-AP, Proteintech), CHOP (1:1000, 15204-1-

AP, Proteintech), ATF4 (1:750, ab184909, Abcam), GAPDH (1:5,000, #2118, Cell

Signalling). Membranes were then washed 3 times for 5 minutes/each in TBST,

followed by probing with secondary antibodies diluted in 5 % milk or 5 % BSA in TBST

for 1h at RT: goat-anti-rabbit IRDye 680RD (1:10,000, 925-68071, LiCor) and goat-

anti-mouse IRDye 800CW (1:10,000, 925-32210, LiCor) and washed 3 times for 5

minutes/each in TBST. Membranes were visualised and quantified on a LiCor

Odyssey Scanner with Image Studio Lite software.

Puromycin incorporation assay

For puromycin (PMY) integration, 91 µM puromycin dihydrochloride (Thermo Fisher

Scientific) was added to media 5 minutes prior to harvesting and incubated at 37°C.

Cells were washed twice with ice-cold PBS supplemented with 355µM cycloheximide

(Calbiochem), lysed and immunoblotted as described previously. Primary PMY-

specific antibody (1:500, clone 12D10, MABE343, Sigma-Aldrich) was used to detect

puromycinylated proteins. GAPDH was used as loading control.

Immunocytochemistry

22x22 mm squared glass coverslips (Sigma-Aldrich) were rinsed with 100% IMS

(Fisher Scientific), added to 6-well plates, and left until IMS fully evaporated. Cells

were seeded and transfected as described previously. U373 and SH-SY5Y cells were

fixed in ice-cold methanol (Fisher Scientific) at -20 °C for 15 min, and MO3.13 cells in

4 % (w/v) paraformaldehyde (Alfa Aesar) at RT for 20 minutes. For methanol fixation,

cell membranes were washed with PBS supplemented with 0.5 % (v/v) Tween 20

(PBST), 3 times for 3 minutes and then blocked in PBS supplemented with 1% (w/v)

bovine serum albumin (BSA) for 60 minutes at RT, or overnight at 4°C, under gentle

shaker. For paraformaldehyde fixation, cells were washed 3 times with PBST for 3

minutes, permeabilized with 0.1 % (v/v) X-Triton for 5 minutes at RT and blocked in 1

% (w/v) BSA in PBST for 60 minutes at RT or overnight at 4°C, under gentle shaker.

Cell membranes were probed with primary antibodies diluted in 1 % (w/v) BSA in PBS,

overnight at 4°C under gentle shaker, as following: eIF2Bα (1:25, 18010-1-688 AP,

Proteintech), eIF2Bβ (1:25, 11034-1-AP, Proteintech), eIF2Bγ (1:50, sc-137248,

Santa Cruz), eIF2Bδ (1:50, sc-271332, Santa Cruz), eIF2Bε (1:25, HPA069303,

Sigma-Aldrich). Cells were then washed 3 times with PBST for 5 minutes, followed by

probing with the appropriate host species Alexa Fluor conjugated secondary antibody

(Thermo Fisher Scientific), diluted in 1 % (w/v) BSA in PBS, for 60 minutes at RT.
Following secondary antibody incubation, cells were washed with PBST, 3 times for 5
minutes, and mounted with ProLong™ Gold Antifade Mountant with DAPI (Invitrogen,
Thermo Fisher Scientific). Cells were visualised on a Zeiss LSM 800 confocal
microscope.

Confocal imaging and fluorescence recovery after photobleaching (FRAP)

Imaging was performed using a Zeiss LSM 800 confocal microscope combined with
Zeiss ZEN 2.3 (blue edition) software for data processing and analysis. Both 40x and
63x plan-apochromat oil objectives were used and a laser with maximum output of
10mW at 0.2 % (488 nm) and 5.0 % (561 nm) laser transmissions. Fluorescence
crosstalk was minimal and bleed-through was not observed. Image acquisition was
performed by orthogonal projection of a Z-stack of automatically calculated increments
for complete single cell imaging. FRAP analysis was performed to quantify the
shuttling rate of eIF2 through localised eIF2B as described in the methodology by
(Hodgson et al., 2019). FRAP experiments were carried out by live cell imaging in an
incubation chamber with appropriate temperature and CO_2 levels. Specific areas
containing an entire cytoplasmic eIF2α-tGFP foci were manually marked for bleaching
using 23 iterations at 100 % laser transmission (488 nm argon laser). Pre-bleaching
image and intensity of targeted foci (ROI – region of interest) was captured followed
by 44 images captured every 151 ms for a total of 7.088 s. In-cell fluorescence intensity
was captured to normalise against ROI. Out-of-cell fluorescence, or background
intensity (B), was measured and subtracted from ROI and T values to provide
corrected measurements. Normalised data was fitted to a one-phase association
curve using GraphPad Prism to quantify rate of recovery. The relative percentage of
eIF2 recovery was determined as the plateau of the normalised FRAP curve.

Statistical analysis

All statistical assessments were made in GraphPad Prism 7 software, with a
significance at $p < 0.05$. All data is presented as means ± standard errors of the mean
(s.e.m.). Data was subjected to Shapiro-Wilk normality test. If parametric, data was
analysed by one-way ANOVA test for comparison of three of more groups followed by
Tukey's correction post-hoc test. If non-parametric, data was analysed by Kruskal-

Wallis test for comparison of three of more groups followed by Dunn's correction post-

hoc test.

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Figure 1. eIF2B localisation is cell type-specific. (A) SH-SY5Y, U373 and MO3.13
cells subjected to transient transfection and expressing eIF2Bε-mGFP. Scale bar: 10
µm. **(B) (i)** Cells express dispersed eIF2B or localised eIF2B (eIF2B bodies). **(ii)** Mean
percentage of cells displaying dispersed eIF2B or localised eIF2B in a population of
100 transfected cells (mean±SEM; *N*=4; ****$p≤0.001$, ***$p≤0.001$ according to two-way
ANOVA). **(iii)** eIF2B bodies were categorised as small bodies ($<1µ^2$) and large bodies
($≥1µ^2$). **(ii)** Within the transfected cells exhibiting localised eIF2B, the mean percentage
of small and large eIF2B bodies in a population of 50 cells (mean±SEM; *N*=3;
****$p≤0.001$, ***$p≤0.001$, *ns*: non-significant according to two-way ANOVA). **(D) (i)**
Confocal images of SH-SY5Y, U373 and MO3.13 expressing eIF2Bε-mGFP and
immunolabelled with primary anti-eIF2Bα, anti-eIF2Bβ, anti-eIF2Bδ and anti-eIF2Bγ.
Scale bar: 10 µm. **(ii)** Mean percentage of transfected cells displaying co-localisation
of eIF2B(α-γ) subunits to small (*top panel*) and large (*bottom panel*) eIF2Bε-mGFP
bodies of at least 30 cells per repeat (mean±SEM; *N*=3; **$p≤0.01$, *$p<0.05$ according
to one-way ANOVA).

Figure 2. eIF2 shuttling of eIF2B bodies is cell type-specific. Cells were co-
transfected with eIF2α-tGFP to carry out fluorescence recovery after photobleaching
(FRAP) analysis, and eIF2Bε-mRFP to locate the eIF2B body. **(A)** Representative live
cell imaging of a cell co-expressing eIF2α-tGFP and eIF2Bε-RFP. Scale bar: 10 µm.
(B) (i) Quantification of normalised FRAP curves for eIF2α-tGFP of at least 10 bodies
of each size category of SH-SY5Y, U373 and MO3.13 cells. The data were graphed
and shown as the mean and SEM bands (*N=3*). **(ii)** Mean percentage of eIF2α-tGFP
recovery determined from normalised FRAP curves (mean±SEM; *N*=3; *$p<0.05$
according to one-way ANOVA).

Figure 3. ER stress-preconditioned cells do not respond to additional acute ER
stress treatment but do respond to acute oxidative stress in a cell type-manner.
(A) Schematic diagram of stress treatments. **(B) (i)** Representative western blot of the
ISR expression profile (PERK-P, PERK, eIF2α-P[S51], pan-eIF2α, CHOP and
GADD34) and global newly synthesized proteins (puromycin incorporation assay) in
SH-SY5Y, U373 and MO3.13 cells treated with vehicle (DMSO), acute stress inducers
(Tg 1µM for 1h and SA 125µM for 30min) or chronic ER stress (Tg 300nM for 24h)
subsequently challenged with previously described acute stress treatments. **(ii)** Mean
expression levels of eIF2α-P[S51] normalised to total eIF2α levels (*top panel*) and

puromycin-labelled nascent proteins normalised to housekeeping GAPDH levels (*bottom panel*) upon the previously described stress conditions. Fold-change relative to vehicle-treated cells was calculated and analysed using one-way ANOVA (mean±SEM; *N*=3-9; *$p<0.05$, *ns*: non-significant). Chronic ER stress conditions are highlighted in green. **(C) (i)** Representative western blot of eIF2α-P[S51], pan-eIF2α and global newly synthesized proteins (puromycin incorporation assay) in SH-SY5Y, U373 and MO3.13 cells treated with ISRIB (200nM) for 1h alone, Tg 300nM for 24h added with SA 125μM in the last 30min, or combination of both. DMSO for 24h was used as vehicle. **(ii)** Mean expression levels of puromycin-labelled nascent proteins normalised to housekeeping GAPDH levels. Fold-change relative to vehicle-treated cells was calculated and analysed using one-way ANOVA (mean±SEM; *N*=3-4; ****$p≤0.001$, ***$p≤0.001$, *$p<0.05$, *ns*: non-significant).

Figure 4. eIF2Bδ remodelling of small eIF2B bodies is transient during cellular stress and partially dictated by eIF2α-P[S51] in a cell type-dependent manner. **(A) (i)** Confocal images of SH-SY5Y, U373 and MO3.13 expressing eIF2Bε-mGFP and immunolabelled with primary anti-eIF2Bδ subjected with acute stress inducers (Tg 1μM for 1h and SA 125μM for 30min) or chronic ER stress (Tg 300nM for 24h) subsequently challenged with previously described acute stress treatments. Scale bar: 10 μm. **(ii)** Mean percentage of transfected cells displaying co-localisation of anti-eIF2Bδ to small (*top panel*) and large (*bottom panel*) eIF2Bε-mGFP bodies of a population of 30 cells per biological repeat. Fold-change relative to vehicle-treated cells was calculated and analysed using one-way ANOVA (mean±SEM; *$p<0.05$; *ns*, non-significant). **(B) (i)** Representative western blot of the ISR expression profile (PERK-P, PERK, eIF2α-P[S51], pan-eIF2α, CHOP and GADD34), global newly synthesized proteins (puromycin incorporation assay) and loading control GAPDH in SH-SY5Y, U373 and MO3.13 cells treated with vehicle (DMSO), GSK2606414/PERKi (500 nM), Tg (1μM) or co-treated with PERKi and Tg (PERKi + Tg) for 1h. **(ii)** Confocal images of SH-SY5Y, U373 and MO3.13 cells expressing eIF2Bε-mGFP and immunolabelled with primary anti-eIF2Bδ subjected to previous treatments. Scale bar: 10 μm. **(iii)** Mean percentage of transfected cells displaying co-localisation of anti-eIF2Bδ to small (*left panel*) and large (*right panel*) eIF2Bε-mGFP bodies of a population of 30 cells per biological repeat. Fold-change relative to vehicle-treated

cells was calculated and analysed using one-way ANOVA (mean±SEM; N=3;
*p<0.05).

Figure 5. ISRIB restores translation during chronic ER stress while increasing
eIF2Bδ composition of small eIF2B bodies predominantly in astrocytic cells. (A)
(i) Confocal images of SH-SY5Y, U373 and MO3.13 expressing eIF2Bε-mGFP and
immunolabelled with primary anti-eIF2Bδ subjected to ISRIB (200nM) alone 1h or in
combination with preconditioned chronic ER stress treatment (Tg 300nM 24h + ISRIB
last 1h). Scale bar: 10 μm. **(ii)** Mean percentage of transfected cells displaying co-
localisation of anti-eIF2Bδ to small (*top panel*) and large (*bottom panel*) eIF2Bε-mGFP
bodies. Fold-change relative to vehicle-treated cells was calculated and analysed
using one-way ANOVA (mean±SEM; N=3; **p≤ 0.01, *p<0.05). **(C) (i)** Western blotting
of global newly synthesized proteins (puromycin incorporation assay) and loading
control GAPDH in SH-SY5Y, U373 and MO3.13 cells treated with same conditions as
described previously (*left panel*). **(ii)** Mean expression levels of puromycin-labelled
nascent proteins normalised to housekeeping GAPDH levels. Fold-change relative to
vehicle-treated cells was calculated and analysed using one-way ANOVA
(mean±SEM; N=5-9; ****p≤0.001, ***p≤0.001, *p<0.05).

Figure 6. ISRIB modulates the eIF2 shuttling of eIF2B bodies in astrocytic cells.
Cells were co-transfected with eIF2α-tGFP to carry out fluorescence recovery after
photobleaching (FRAP) analysis, and eIF2Bε-mRFP to locate the eIF2B body. **(A)**
Cells were then treated with vehicle (DMSO), ISRIB (200 nM) alone for 1h, Tg (1μM)
alone for 1h or both treatments in combination (Tg + ISRIB) for 1h. Quantification of
normalised FRAP curves for eIF2α-tGFP of at least 10 bodies of small (*right panel*)
and large (*left panel*) eIF2Bε-mRFP bodies of SH-SY5Y, U373 and MO3.13 cells. The
data were graphed and shown as the mean and S.E.M. bands (N=3). The mean
percentage of eIF2α-tGFP recovery was determined from normalised FRAP curves
(mean±SEM*; N*=3; ***p≤ 0.001, *p<0.05 according to one-way ANOVA). **(B)** Cells
were then treated with vehicle (DMSO), Tg (300nM) alone for 24h or both treatments
in combination where ISRIB was added on the last hour of the 24h period exposure to
Tg. Quantification of normalised FRAP curves for eIF2α-tGFP of at least 10 bodies of
small (*right panel*) and large (*left panel*) eIF2Bε-mRFP bodies of SH-SY5Y, U373 and
MO3.13 cells. The data were graphed and shown as the mean and S.E.M. bands

(*N=3*). Mean percentage of eIF2α-tGFP recovery was determined from normalised

FRAP curves (mean±SEM*; N*=3; ***$p \leq$ 0.001, *$p < 0.05$ according to one-way ANOVA).

Figure 7. Working model for the impact of cellular stress and ISRIB in eIF2B
979

bodies of astrocytes. (A) eIF2B localises to small eIF2B bodies containing catalytic

subcomplexes and larger eIF2B bodies containing a variety of regulatory

subcomplexes (including decameric eIF2B). **(B)** Upon activation of the acute ISR

program, eIF2Bγδε subcomplexes are formed and localised to small eIF2B bodies

which we hypothesize to have a regulatory role in eIF2B GEF activity; whilst large

eIF2B bodies are negatively impacted. **(C)** During transition to a chronic ISR, eIF2Bδ

distribution in small bodies is reversed and GEF activity is restored to basal rates,

whereas ISRIB treatment bypasses transient eIF2Bδ distribution by prompting

extended eIF2Bγδε formation by direct interaction with eIF2Bδ.

Figure S1. Endogenous eIF2B bodies are expressed in SH-SY5Y, U373 and

MO3.13 cells. (A) Confocal images of SH-SY5Y, U373 and MO3.13 cells

immunostained for neural marker *NeuN*, astrocytic marker *GFAP* and oligodendrocytic

marker *MBP*, respectively. **(B)** Confocal images of SH-SY5Y, U373 and MO3.13 cells

immunostained with α-eIF2Bε (*left panel*). Mean percentage of cells displaying at least

one eIF2Bε-containing body in a population of 100 cells (mean±SEM*; N*=3;

****$p \leq 0.0001$, ***$p \leq 0.001$ according to one-way ANOVA) (*right panel*). Scale bar: 10

μm.

Figure S2. ATF4 expression peaks before 24h time point of Tg exposure.

Representative western blot of the ISR expression profile (eIF2α-P[S51], pan-eIF2α,

ATF4, CHOP and GADD34) and total protein staining (loading control) in SH-SY5Y,

U373 and MO3.13 cells treated with vehicle (DMSO) for 24h or thapsigargin for 1, 4,

8 and 24 hours.

Figure S3. Tunicamycin treatment prior Tg preconditioning for 24h does not

further impact on eIF2α-P[S51] levels and protein synthesis. Western blot of the

ISR expression profile (eIF2α-P[S51], pan-eIF2α) and global newly synthesized

proteins (puromycin incorporation assay) in SH-SY5Y, U373 and MO3.13 cells treated

with vehicle (DMSO), acute ER stress inducer (Tm 3 μg/mL for 2h), chronic ER stress

(Tg 300nM for 24h) and chronic ER stress further challenged with acute ER stress

inducer (Tg 300nM for 24h + Tm 3 μg/mL on the last 2h).

Figure S4. eIF2B localisation increases with cellular stress. SH-SY5Y, U373 and
MO3.13 cells were subjected to acute stress inducers (Tg 1µM for 1h and SA 125µM
for 30min); chronic ER stress (Tg 300nM for 24h) or subsequently challenged with
previously described acute stress treatments (Tg 24h + Tg last 1h; Tg 24h + SA last
0.5h). Mean percentage of cells displaying localised eIF2B (at least one eIF2B body
present) in a population of 100 transfected cells (mean±SEM*; N*=3).

eIF2B localisation and its regulation during the integrated stress response is cell type-specific

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Running title: eIF2B localisation is cell type-specific.

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Figure 1

A

B (i)

Small bodies

Large bodies

— SH-SY5Y
— U373
— MO3.13

(ii)

Small bodies

Large bodies

Figure 2

Figure 3

Figure 4

A (i)

(ii)

B (i)

(ii)

Figure 5

Figure 6

Figure 7

A

Scale bar: 20μm

Figure S1

	SH-SY5Y	U373	MO3.13	
eIF2α-P[S51]:				37
Fold-change:	1.00 4.41 2.02 2.11 2.03	1.00 2.64 2.68 4.01 2.88	1.00 2.74 1.96 1.31 1.49	
eIF2α:				37
ATF4:				50
CHOP:				30
GADD34:				100
Fold-change:	1.00 1.21 7.56 14.44 8.28	1.00 0.74 1.72 4.74 7.10	1.00 1.21 4.07 20.14 24.54	
Total protein stain				
Tg:	- 1h 4h 8h 24h	- 1h 4h 8h 24h	- 1h 4h 8h 24h	

Figure S2

Figure S3

Figure S4