

1 **Eukaryotic initiation factor 5B (eIF5B) regulates temozolomide-mediated apoptosis in brain**
2 **tumor stem cells (BTSCs).**

3

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23

24 **Abstract**

25 Glioblastoma multiforme (GBM) is among the deadliest cancers, owing in part to complex inter-
26 and intra-tumor heterogeneity and the presence of a population of stem-like cells called brain tumor
27 stem cells (BTSCs/BTICs). These cancer stem cells survive treatment and confer resistance to the
28 current therapies—namely, radiation and the chemotherapeutic, temozolomide (TMZ). TMZ
29 induces cell death by alkylating DNA, and BTSCs resist this mechanism via a robust DNA damage
30 response. Hence, recent studies aimed to sensitize BTSCs to TMZ using combination therapy, such
31 as inhibition of DNA repair machinery. We have previously demonstrated in established GBM
32 cell lines that eukaryotic initiation factor 5B (eIF5B) promotes the translation of pro-survival and
33 anti-apoptotic proteins. Consequently, silencing eIF5B sensitizes these cells to TRAIL-induced
34 apoptosis. However, established cell lines do not always recapitulate the features of human glioma.
35 Therefore, we investigated this mechanism in patient-derived BTSCs. We show that silencing
36 eIF5B leads to increased TMZ sensitivity in two BTSC lines, BT25 and BT48. Depletion of eIF5B
37 decreases levels of anti-apoptotic proteins in BT48 and sensitizes these cells to TMZ-induced
38 activation of caspase-3, cleavage of PARP, and apoptosis. We suggest that eIF5B represents a
39 rational target to sensitize GBM tumors to the current standard-of-care.

40

41 **Keywords:** Eukaryotic initiation factor 5B (eIF5B), Brain tumor stem cells (BTSCs), Apoptosis,
42 Temozolomide (TMZ), Glioblastoma multiforme (GBM)

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47 **Introduction**

48 Glioblastoma multiforme (GBM) is the most lethal form of brain tumour in adults, with a
49 median survival of just 15 months (Stupp et al. 2005). Several factors—including complex tumour
50 heterogeneity and a sub-population of stem-like glioma cells, termed brain tumour stem cells
51 (BTSCs) (Cusulin et al. 2015; Kelly et al. 2009)—promote resistance to conventional therapies,
52 including radiotherapy and the standard-of-care chemotherapeutic agent temozolomide (TMZ)
53 (Bao et al. 2006; Salmaggi et al. 2006). TMZ is an alkylating agent that produces toxic methyl
54 adducts at O6-guanine, leading to activation of the mismatch repair pathway and ultimately, lethal
55 DNA breaks (Mojaś et al. 2007). Intrinsic TMZ resistance is primarily mediated by O6-
56 methylguanine-DNA methyltransferase (MGMT), which removes the methyl adducts from the
57 DNA (Kitange et al. 2009). Other mechanisms for resistance to alkylating agents include base-
58 excision repair and homologous recombination (Gil Del Alcazar et al. 2016; Trivedi et al. 2005).
59 In preclinical studies, combination therapy has been shown to improve the efficacy of TMZ by
60 overcoming these resistance mechanisms (Lun et al. 2016; Yuan et al. 2018).

61 We have previously shown that in established GBM cell lines, eIF5B is required for
62 optimal translation of anti-apoptotic and pro-survival proteins from internal ribosome entry site
63 (IRES)-encoding mRNAs, including X-linked inhibitor of apoptosis (XIAP), B-cell lymphoma
64 extra-large (Bcl-xL), cellular inhibitor of apoptosis protein 1 (cIAP1), and the short isoform of
65 cellular FLICE-like inhibitory protein (c-FLIPs) (Ross et al. 2019). Consequently, depletion of
66 eIF5B by RNAi sensitizes these cells to TNF-related apoptosis-inducing ligand (TRAIL),
67 enhancing apoptosis by a caspase-8/9-dependent pathway (Ross et al. 2019). As both extrinsic (i.e.
68 receptor mediated) and intrinsic (i.e. DNA damage-mediated) apoptotic pathways converge on
69 caspases-9, we reasoned that eIF5B depletion would also sensitize GBM cells to TMZ. As

70 established cell lines do not always recapitulate the features of human glioma, we investigated this
71 mechanism in patient-derived BTSCs. We show here that siRNA-mediated depletion of eIF5B
72 sensitizes two BTSC lines, BT25 and BT48, to TMZ. We show in BT48 cells that silencing eIF5B
73 leads to enhanced TMZ-mediated activation of caspase-3, cleavage of PARP, and apoptosis.
74 Moreover, eIF5B depletion in these cells leads to modestly decreased levels of XIAP and cIAP1,
75 and substantially decreased levels of Bcl-xL and c-FLIPs proteins. We suggest that eIF5B
76 represents a novel target to sensitize BTSCs to temozolomide.

77

78 **Materials and Methods**

79

80 **Cell culture, transfection, and reagents**

81 BT48 (previously called 48EF) and BT25 (previously called 25M) have been previously described
82 (Sarkar et al. 2014). Cells were cultured in serum-free media supplemented with epidermal growth
83 factor, fibroblast growth factor, and heparan sulphate in a humidified incubator (37°C, 5% CO₂)
84 as described elsewhere (Kelly et al. 2009). To propagate these cells in monolayer, cells were
85 dissociated with Accutase (Innovative Cell Technologies, San Diego, USA) and seeded into plates
86 coated with laminin (Millipore Sigma, Toronto, Canada) at 1 µg/cm². U343 cells were propagated
87 in Dulbecco's high modified Eagle's medium (DMEM, HyClone, Logan, USA) with 4 mM L-
88 glutamine, 4500 mg/L glucose, and 1 mM sodium pyruvate supplemented with 10% fetal bovine
89 serum (Gibco, Waltham, USA) and 1% penicillin-streptomycin (Gibco). Transfections were
90 carried out using Opti-MEM (Gibco) and Lipofectamine RNAiMAX (Invitrogen, Madison, USA)
91 according to manufacturer's instructions. Non-specific control siRNA and siRNAs targeting

92 eIF5B (HSS114469/70/71) were obtained from Qiagen (Hilden, Germany) and Invitrogen,
93 respectively. TMZ was obtained from Active Biochem Ltd (Hong Kong, China).

94

95 **Immunoblotting**

96 Cells were seeded at 400,000 cells/well in 6-well plates, incubated 24 hours, and forward
97 transfected. After a further 24 hours, TMZ or DMSO (vehicle control) were added where indicated.

98 After a final 72 hours, cells were harvested, and immunoblotting was performed as previously
99 described (Ross et al. 2019).

100

101 **In vitro viability assay**

102 Cells were seeded at 13,000 cells/well in 96-well plates, incubated 24 hours, and forward
103 transfected. After a further 24 hours, cells were treated with a vehicle control (DMSO) or TMZ.

104 Where indicated, cells were pre-treated for 2 hours with z-VAD-fmk (R & D Systems,

105 Minneapolis, USA) or Necrostatin-1 (Millipore-Sigma) before adding TMZ. After a further 96

106 hours, cell viability was determined by alamarBlue assay (resazurin sodium salt; Sigma-Aldrich,

107 Oakville, Canada) as previously described (Ross et al. 2019).

108

109 **Microscopy**

110 Cells were grown and treated the same as immunoblotting, except that instead of harvesting, a

111 general DNA stain (1 µg/mL Hoechst 33342; Thermo Scientific, Waltham, USA) was added to

112 the cells. After a 30-minute incubation, the cells were imaged in a Cytation 5 plate imager (BioTek,

113 Winooski, USA). For fluorescence microscopy, cells were imaged using a DAPI filter to analyze

114 Hoechst-stained nuclear DNA. The percent of Hoechst-stained nuclei per field-of-view
115 demonstrating fragmentation were quantified using the onboard Cytation 5 analysis software.

116

117 **Statistical analyses**

118 All quantitative data represent the mean \pm standard error on the mean (SEM) for at least 3
119 independent biological replicates. Statistical significance was determined by an unpaired, two-
120 tailed t-test. The significance level was set at a p-value of 0.05. Data were analyzed using
121 GraphPad Prism, version 8.

122

123 **Bioinformatics analyses**

124 Kaplan-Meier survival curves were plotted using PROGgeneV2-Prognostic database
125 (<http://watson.compbio.iupui.edu/chirayu/proggene/database/?url=proggene>) (Goswami and
126 Nakshatri 2014). *EIF5B* mRNA levels across 20 cancer types were examined in The Cancer
127 Genome Atlas (TCGA) cBioportal (<http://www.cbioportal.org/>) (Cerami et al. 2012).

128

129

130 **Results**

131

132 **Depletion of eIF5B enhances TMZ-induced apoptosis in BTSCs.**

133 We have previously demonstrated that depletion of eIF5B leads to increased apoptosis in GBM
134 cell lines (Ross et al. 2019). However, established cell lines do not always recapitulate the features
135 of human glioma. We therefore tested whether eIF5B depletion has a similar effect in BTSC cell
136 lines (BT48 and BT25), which normally grow as neurospheres in liquid suspension (Figure 1A).

137 In order to deplete eIF5B by RNAi, we grew BT48 or BT25 cells in a monolayer on laminin-
138 coated plates as previously described (Pollard et al. 2009), before transfecting with a non-specific
139 control siRNA or a pool of eIF5B-specific siRNAs. We successfully depleted levels of the eIF5B
140 protein by ~90% in both cell lines, leading to no significant effect on alamarBlue activity (Figure
141 1B). We next tested whether eIF5B depletion increases the sensitivity of BTSCs to a frontline
142 therapeutic agent, TMZ. We measured the effects of various concentrations of TMZ on control or
143 eIF5B-depleted BT25 or BT48 cells by alamarBlue assay. Indeed, silencing eIF5B decreased the
144 IC₅₀ of TMZ approximately 3- and 2-fold in BT25 and BT48, respectively (Figure 1C, D).
145 Moreover, pre-treatment of the cells with a pan-caspase inhibitor (z-VAD-fmk) prevented the
146 enhancement of TMZ sensitivity by eIF5B depletion, consistent with apoptotic cell death;
147 conversely, the TMZ sensitivity phenotype was not reversed by a RIP1-kinase inhibitor
148 (Necrostatin-1), indicating that eIF5B does not affect the ability of TMZ to induce necroptosis
149 (Figure 1E).

150 To confirm that decreased alamarBlue activity is due to increased apoptosis, we performed
151 a series of microscopy experiments in BT48. First, brightfield microscopy confirmed that TMZ-
152 treated, eIF5B-silenced BT48 cells grew to a lower density compared to untreated control cells
153 (Figure 2A, left panels). Moreover, the combination of eIF5B depletion and TMZ treatment led to
154 an altered cell morphology (Figure 2A, left panels). Specifically, a large proportion of the cells
155 shrank and became rounded, as opposed to the elongated adherent cells observed for untreated
156 control cells. Consistent with apoptotic cell death, Hoechst live-cell nuclear staining revealed
157 increased nuclear fragmentation and condensation in TMZ-treated, eIF5B-depleted cells (Figure
158 2A, middle and right panels, and Figure 2B). Finally, we observed a significant increase in cleaved
159 caspase-3 and cleaved PARP in eIF5B-depleted, TMZ-treated cells (Figure 2C-F). Moreover,

160 depletion of eIF5B did not lead to decreased levels of the stemness marker, SRY-box 2 (SOX2),
161 indicating that stemness was maintained in BT48 upon silencing eIF5B (Figure 2C). Taken
162 together, the data indicate that depletion of eIF5B enhances sensitivity of BT48 cells to TMZ-
163 induced apoptosis.

164

165 **Depletion of eIF5B leads to reduced levels of anti-apoptotic proteins in BTSCs.**

166 We have previously demonstrated that depletion of eIF5B leads to decreased translation of anti-
167 apoptotic proteins in GBM cell lines (Ross et al. 2019). We therefore tested whether silencing
168 eIF5B decreases the expression of such proteins in BT48 cells. Indeed, depletion of eIF5B lead to
169 a modest but significant reduction in the levels of XIAP, cIAP1, and c-FLIP_L, and a more robust
170 reduction (~2-fold) in Bcl-xL and c-FLIPs levels (Figure 3). This indicates that, consistent with
171 our previous findings, eIF5B is important for the optimal expression of anti-apoptotic proteins.

172

173 **Discussion**

174

175 We show in this work that eIF5B depletion leads to decreased anti-apoptotic protein
176 expression and increased TMZ sensitivity in BT48 BTSCs. Our findings are consistent with a
177 recent body of work suggesting that eIF5B is involved in regulating pro-growth pathways (Jiang
178 et al. 2016), central carbon metabolism and hypoxia adaptation of glioblastoma (Ho et al. 2018),
179 and non-canonical translation by IRES- and uORF-mediated mechanisms (Joseph A. Ross 2018;
180 Ross et al. 2019; Thakor and Holcik 2012).

181 Notably, the effects of eIF5B depletion on Bcl-xL and c-FLIPs levels were larger in
182 magnitude than those observed for XIAP, cIAP1 and c-FLIP_L (Figure 3). It is not surprising that

183 c-FLIP_L levels decreased modestly upon eIF5B depletion, as we previously observed a similar
184 phenotype in an established GBM cell line (U343) which we attributed to the lack of an IRES
185 element in c-FLIP_L (Ross et al. 2019). XIAP is translated from two alternative transcripts, only
186 one of which encodes an IRES; as we have previously demonstrated that eIF5B is important for
187 IRES-dependent translation of XIAP (Thakor and Holcik 2012), this could explain the relatively
188 modest drop in XIAP levels reported here. Members of the inhibitor of apoptosis protein (IAP)
189 family (e.g. XIAP, cIAP1) function as competitive inhibitors of caspases (Roy et al. 1997). XIAP,
190 the most potent of the IAPs, plays a key role in suppressing the activity of caspase-9 and the
191 executioner caspases (Silke and Meier 2013). Bcl-xL inhibits pore formation by Bim, Bid, Bax,
192 and Bak, which would otherwise cause mitochondrial outer membrane permeabilization (MOMP)
193 (Lomonosova and Chinnadurai 2008). c-FLIP is expressed in humans as a short isomer (c-FLIP_S)
194 and a long isomer (c-FLIP_L), both of which can inhibit caspase-8 activation by death receptors
195 (Safa 2013).

196 Depletion of eIF5B did not lead to decreased levels of SOX2, a transcription factor
197 essential for self-renewal or pluripotency (Figure 2C). The effect of eIF5B depletion on TMZ
198 sensitivity in BTSCs is therefore unlikely due to any loss of stemness. The robust levels of SOX2
199 detected here are in line with Pollard et al. (2009), who demonstrated that glioma stem cells
200 expanded in adherent culture on laminin-coated plates recapitulated the features of neural stem
201 cells—including the expression of stemness markers like SOX2 (Pollard et al. 2009).

202 Importantly, the increase in TMZ sensitivity upon eIF5B depletion was prevented by z-
203 VAD-fmk but not by Necrostatin-1, suggesting that eIF5B promotes resistance to caspase
204 activation (Figure 1E). This notion was supported by microscopy, which indicated increased
205 nuclear condensation and fragmentation—indicative of apoptotic cell death (Tsujiimoto 2012)—in

206 TMZ-treated, eIF5B-depleted BT48 cells (Figure 2A). We also observed significantly increased
207 caspase-3 activation and PARP cleavage upon eIF5B depletion (Figure 2C). Notably, we used an
208 antibody specific for the 89 kDa C-terminal fragment of PARP classically generated by caspase-3
209 cleavage (Chaitanya et al. 2010). Cleavage of PARP by caspases separates its catalytic domain
210 from its DNA-binding domain, attenuating DNA repair (Chaitanya et al. 2010). Interestingly, the
211 PARP cleavage observed here did not depend entirely upon TMZ treatment, as eIF5B depletion
212 also enhanced PARP cleavage in the absence of TMZ (Figure 2C). This suggests that eIF5B
213 protects PARP from cleavage resulting from a pro-apoptotic stimulus besides TMZ. We previously
214 demonstrated that eIF5B depletion leads to decreased levels of the master regulator of oxidative
215 stress response, NRF2, leading to increased ROS accumulation in an established GBM cell line
216 (Ross et al. 2019). It is possible that eIF5B depletion has a similar effect in BT48. Increased
217 oxidative stress would cause increased MOMP and, hence, increased caspase activation (Sinha et
218 al. 2013), which would be further enhanced by decreased levels of Bcl-xL and other anti-apoptotic
219 proteins. Thus, we propose that eIF5B depletion sensitizes BT48 cells to intrinsic apoptosis
220 stimulated by TMZ-induced DNA damage due to decreased levels of anti-apoptotic proteins.
221 Moreover, the increased cleavage of PARP would lead to a less robust repair response to the DNA
222 damage caused by TMZ.

223 Transcript levels are routinely measured from tumors and are correlated with patient
224 survival. In line with this notion, using The Cancer Genome Atlas (TCGA) data, Yi et al. (2018)
225 correlated decreased levels of *EIF5B* mRNA with worse outcome for patients with glioblastoma
226 (Yi et al. 2018). eIF5B at the protein level does not work alone, and its dynamic interactions with
227 eIF1A, eIF2A, and eIF5 play a critical role in regulating non-canonical translation (Kim et al.
228 2018; Lin et al. 2018; Nag et al. 2016). For example, eIF5B interacts with eIF1A to stabilize

229 initiator tRNA into the ribosome (Lin et al. 2018; Nag et al. 2016). Its interaction with eIF5
230 regulates the displacement of initiator tRNA from ternary complex (eIF2-GTP-Met-tRNA_i).
231 However, eIF1A and eIF5 have conserved eIF5B-binding sequences and they compete to bind to
232 eIF5B (Lin et al. 2018). Moreover, eIF2A (not to confuse with eIF2 α) binds and cooperates with
233 eIF5B to deliver initiator tRNA to the ribosome, specifically during IRES-mediated translation
234 initiation (Kim et al. 2018). We have demonstrated that the stoichiometry between eIF5B and its
235 interacting partner(s) is critical for uORF-mediated non-canonical translation initiation (Joseph A.
236 Ross 2018). These findings suggest that eIF5B is a critical scaffolding protein for regulating non-
237 canonical translation initiation via its dynamic interactions with eIF1A, eIF2A, and eIF5.
238 Therefore, *EIF5B* mRNA levels alone should not be used as a prognostic marker for GBM patient
239 survival. To this end, we have looked at the association between ratios of *EIF5B/EIF2A*,
240 *EIF5B/EIF1AX*, and *EIF5B/EIF5* mRNAs and overall survival of GBM patients using various
241 patient cohorts (Supplemental Figure S1). These data suggest that higher levels of *EIF5B* mRNA
242 over *EIF1AX*, *EIF2A*, and *EIF5* was correlated with worse patient outcomes. Further, Yi et al.
243 (2018) also showed that eIF5B levels decrease in TMZ-treated U87 cells. Moreover, our data
244 suggest that *EIF5B* is not lost in GBM (Supplemental Figure S2), in fact, eIF5B protein levels are
245 modestly enhanced in TMZ-treated U343 cells and brain tumor initiating cells (BTICs)
246 (Supplemental Figure S3 and Figure 2D, respectively). Additionally, the ratios of eIF5B/eIF1A,
247 eIF5B/eIF5, and eIF5B/eIF2A proteins are also modestly enhanced (Supplemental Figure S3)
248 under TMZ treatment in U343 cells. It is known that TMZ-mediated genotoxic stress induces the
249 production of reactive oxygen species (ROS) (Jiang et al. 2012; Lee et al. 2014) that leads to eIF2 α
250 phosphorylation (Supplemental Figure S3). TMZ enhances the unfolded protein response (UPR)
251 in GBM cells (Jiang et al. 2012; Lee et al. 2014; Yuan et al. 2012; Zeeshan et al. 2016). Under this

252 condition canonical translation would be attenuated due to eIF2 α phosphorylation. However, the
253 enhanced levels of eIF5B would execute the switch from canonical to non-canonical translation
254 and would likely support the cell survival by facilitating non-canonical translation XIAP, Bcl-xL,
255 cIAP1, and cFLIPs. Interestingly, eIF5B depletion leads to downregulation of these anti-apoptotic
256 proteins and sensitizes GBM cells to pro-apoptotic agents. Therefore, in contrast to Yi. G. Z., et
257 al. (2018), our data warrants a study on validating eIF5B as a therapeutic target for GBM tumors.

258
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263
264 **Conflict of Interest**

265 The authors declare no competing interests.

266
267 **References**

268
269 Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., et al. 2006. Glioma stem cells
270 promote radioresistance by preferential activation of the DNA damage response. *Nature*
271 **444**(7120): 756-760. doi:10.1038/nature05236.
272
273 Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., et al. 2012. The cBio cancer
274 genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer*
275 *Discov* **2**(5): 401-404. doi:10.1158/2159-8290.CD-12-0095.
276
277 Chaitanya, G.V., Steven, A.J., and Babu, P.P. 2010. PARP-1 cleavage fragments: signatures of cell-death
278 proteases in neurodegeneration. *Cell communication and signaling* : **CCS** **8**: 31.
279 doi:10.1186/1478-811x-8-31.

281 Cusulin, C., Chesnelong, C., Bose, P., Bilenky, M., Kopciuk, K., Chan, J.A., et al. 2015. Precursor States
282 of Brain Tumor Initiating Cell Lines Are Predictive of Survival in Xenografts and Associated
283 with Glioblastoma Subtypes. *Stem cell reports* **5**(1): 1-9. doi:10.1016/j.stemcr.2015.05.010.

284
285 Gil Del Alcazar, C.R., Todorova, P.K., Habib, A.A., Mukherjee, B., and Burma, S. 2016. Augmented HR
286 Repair Mediates Acquired Temozolomide Resistance in Glioblastoma. *Molecular cancer research*
287 : **MCR 14**(10): 928-940. doi:10.1158/1541-7786.mcr-16-0125.

288
289 Goswami, C.P., and Nakshatri, H. 2014. PROGgeneV2: enhancements on the existing database. *BMC*
290 *Cancer* **14**: 970. doi:10.1186/1471-2407-14-970.

291
292 Ho, J.J.D., Balukoff, N.C., Cervantes, G., Malcolm, P.D., Krieger, J.R., and Lee, S. 2018. Oxygen-
293 Sensitive Remodeling of Central Carbon Metabolism by Archaic eIF5B. *Cell reports* **22**(1): 17-
294 26. doi:10.1016/j.celrep.2017.12.031.

295
296 Jiang, X., Jiang, X., Feng, Y., Xu, R., Wang, Q., and Deng, H. 2016. Proteomic Analysis of eIF5B
297 Silencing-Modulated Proteostasis. *PLoS One* **11**(12): e0168387.
298 doi:10.1371/journal.pone.0168387.

299
300 Jiang, Y., Sun, Y., and Yuan, Y. 2012. [Mechanism of temozolomide-induced anti-tumor effects on
301 glioblastoma cells in vitro is via ROS-dependent SIRT1 signaling pathway]. *Zhonghua Zhong*
302 *Liu Za Zhi* **34**(10): 734-738. doi:10.3760/cma.j.issn.0253-3766.2012.10.004.

303
304 Joseph A. Ross, K.R.B., Nehal Thakor. 2018. Eukaryotic Initiation Factor 5B (eIF5B) Cooperates with
305 eIF1A and eIF5 to Facilitate uORF2-Mediated Repression of ATF4 Translation. *International*
306 *Journal of Molecular Sciences* **19**(12): 4032.

307
308 Kelly, J.J., Stechishin, O., Chojnacki, A., Lun, X., Sun, B., Senger, D.L., et al. 2009. Proliferation of
309 human glioblastoma stem cells occurs independently of exogenous mitogens. *Stem Cells* **27**(8):
310 1722-1733. doi:10.1002/stem.98.

311
312 Kim, E., Kim, J.H., Seo, K., Hong, K.Y., An, S.W.A., Kwon, J., et al. 2018. eIF2A, an initiator tRNA
313 carrier refractory to eIF2alpha kinases, functions synergistically with eIF5B. *Cell Mol Life Sci*
314 **75**(23): 4287-4300. doi:10.1007/s00018-018-2870-4.

315
316 Kitange, G.J., Carlson, B.L., Schroeder, M.A., Grogan, P.T., Lamont, J.D., Decker, P.A., et al. 2009.
317 Induction of MGMT expression is associated with temozolomide resistance in glioblastoma
318 xenografts. *Neuro-oncology* **11**(3): 281-291. doi:10.1215/15228517-2008-090.

319
320 Lee, S., Truesdell, S.S., Bukhari, S.I., Lee, J.H., LeTonqueze, O., and Vasudevan, S. 2014. Upregulation
321 of eIF5B controls cell-cycle arrest and specific developmental stages. *Proc Natl Acad Sci U S A*
322 **111**(41): E4315-4322. doi:10.1073/pnas.1320477111.

323

324 Lin, K.Y., Nag, N., Pestova, T.V., and Marintchev, A. 2018. Human eIF5 and eIF1A Compete for
325 Binding to eIF5B. *Biochemistry* **57**(40): 5910-5920. doi:10.1021/acs.biochem.8b00839.

326

327 Lomonosova, E., and Chinnadurai, G. 2008. BH3-only proteins in apoptosis and beyond: an overview.
328 *Oncogene* **27 Suppl 1**: S2-19. doi:10.1038/onc.2009.39.

329

330 Lun, X., Wells, J.C., Grinshtein, N., King, J.C., Hao, X., Dang, N.H., et al. 2016. Disulfiram when
331 Combined with Copper Enhances the Therapeutic Effects of Temozolomide for the Treatment of
332 Glioblastoma. *Clinical cancer research : an official journal of the American Association for
333 Cancer Research* **22**(15): 3860-3875. doi:10.1158/1078-0432.ccr-15-1798.

334

335 Mojás, N., Lopes, M., and Jiricny, J. 2007. Mismatch repair-dependent processing of methylation damage
336 gives rise to persistent single-stranded gaps in newly replicated DNA. *Genes & development*
337 **21**(24): 3342-3355. doi:10.1101/gad.455407.

338

339 Nag, N., Lin, K.Y., Edmonds, K.A., Yu, J., Nadkarni, D., Marintcheva, B., et al. 2016. eIF1A/eIF5B
340 interaction network and its functions in translation initiation complex assembly and remodeling.
341 *Nucleic acids research* **44**(15): 7441-7456. doi:10.1093/nar/gkw552.

342

343 Pollard, S.M., Yoshikawa, K., Clarke, I.D., Danovi, D., Stricker, S., Russell, R., et al. 2009. Glioma stem
344 cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for
345 chemical and genetic screens. *Cell stem cell* **4**(6): 568-580. doi:10.1016/j.stem.2009.03.014.

346

347 Ross, J.A., Dungen, K.V., Bressler, K.R., Fredriksen, M., Khandige Sharma, D., Balasingam, N., et al.
348 2019. Eukaryotic initiation factor 5B (eIF5B) provides a critical cell survival switch to
349 glioblastoma cells via regulation of apoptosis. *Cell Death Dis* **10**(2): 57. doi:10.1038/s41419-018-
350 1283-5.

351

352 Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. 1997. The c-IAP-1 and c-IAP-2
353 proteins are direct inhibitors of specific caspases. *The EMBO journal* **16**(23): 6914-6925.
354 doi:10.1093/emboj/16.23.6914.

355

356 Safa, A.R. 2013. Roles of c-FLIP in Apoptosis, Necroptosis, and Autophagy. *J Carcinog Mutagen Suppl*
357 **6**. doi:10.4172/2157-2518.S6-003.

358

359 Salmaggi, A., Boiardi, A., Gelati, M., Russo, A., Calatozzolo, C., Ciusani, E., et al. 2006. Glioblastoma-
360 derived tumorspheres identify a population of tumor stem-like cells with angiogenic potential
361 and enhanced multidrug resistance phenotype. *Glia* **54**(8): 850-860. doi:10.1002/glia.20414.

362

363 Sarkar, S., Doring, A., Zemp, F.J., Silva, C., Lun, X., Wang, X., et al. 2014. Therapeutic activation of
364 macrophages and microglia to suppress brain tumor-initiating cells. *Nat Neurosci* **17**(1): 46-55.
365 doi:10.1038/nn.3597.

366

367 Silke, J., and Meier, P. 2013. Inhibitor of apoptosis (IAP) proteins-modulators of cell death and
368 inflammation. *Cold Spring Harb Perspect Biol* **5**(2). doi:10.1101/cshperspect.a008730.

369
370 Sinha, K., Das, J., Pal, P.B., and Sil, P.C. 2013. Oxidative stress: the mitochondria-dependent and
371 mitochondria-independent pathways of apoptosis. *Archives of toxicology* **87**(7): 1157-1180.
372 doi:10.1007/s00204-013-1034-4.

373
374 Stupp, R., Mason, W.P., van den Bent, M.J., Weller, M., Fisher, B., Taphoorn, M.J., et al. 2005.
375 Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England*
376 *journal of medicine* **352**(10): 987-996. doi:10.1056/NEJMoa043330.

377
378 Thakor, N., and Holcik, M. 2012. IRES-mediated translation of cellular messenger RNA operates in
379 eIF2alpha- independent manner during stress. *Nucleic acids research* **40**(2): 541-552.
380 doi:10.1093/nar/gkr701.

381
382 Trivedi, R.N., Almeida, K.H., Fornisaglio, J.L., Schamus, S., and Sobol, R.W. 2005. The role of base
383 excision repair in the sensitivity and resistance to temozolomide-mediated cell death. *Cancer*
384 *research* **65**(14): 6394-6400. doi:10.1158/0008-5472.can-05-0715.

385
386 Tsujimoto, Y. 2012. Multiple ways to die: non-apoptotic forms of cell death. *Acta oncologica*
387 (Stockholm, Sweden) **51**(3): 293-300. doi:10.3109/0284186x.2011.648340.

388
389 Yi, G.Z., Xiang, W., Feng, W.Y., Chen, Z.Y., Li, Y.M., Deng, S.Z., et al. 2018. Identification of Key
390 Candidate Proteins and Pathways Associated with Temozolomide Resistance in Glioblastoma
391 Based on Subcellular Proteomics and Bioinformatical Analysis. *Biomed Res Int* **2018**: 5238760.
392 doi:10.1155/2018/5238760.

393
394 Yuan, A.L., Ricks, C.B., Bohm, A.K., Lun, X., Maxwell, L., Safdar, S., et al. 2018. ABT-888 restores
395 sensitivity in temozolomide resistant glioma cells and xenografts. *PloS one* **13**(8): e0202860.
396 doi:10.1371/journal.pone.0202860.

397
398 Yuan, Y., Xue, X., Guo, R.B., Sun, X.L., and Hu, G. 2012. Resveratrol enhances the antitumor effects of
399 temozolomide in glioblastoma via ROS-dependent AMPK-TSC-mTOR signaling pathway. *CNS*
400 *Neurosci Ther* **18**(7): 536-546. doi:10.1111/j.1755-5949.2012.00319.x.

401
402 Zeeshan, H.M., Lee, G.H., Kim, H.R., and Chae, H.J. 2016. Endoplasmic Reticulum Stress and
403 Associated ROS. *Int J Mol Sci* **17**(3): 327. doi:10.3390/ijms17030327.

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410 **Figure Captions**

411

412 **Figure 1. Depletion of eIF5B sensitizes BTSCs to TMZ.** (A) BT25 and BT48 neurospheres were
413 grown for 1 week in liquid suspension and imaged at 10x magnification. (B-D) BT25 or BT48
414 cells were transfected with a non-specific control siRNA (siC) or an eIF5B-specific siRNA pool
415 (si5B) and treated with a vehicle control (0.5% DMSO) or various concentrations of TMZ.
416 AlamarBlue activity was measured after a further 96 hours. In (C) and (D), the resulting
417 fluorescence readings are expressed as percent alamarBlue activity, where the readings for control
418 or eIF5B-depleted cells were each normalized to the vehicle treatment. (E) Control or eIF5B-
419 depleted BT48 cells were pre-treated with a vehicle control (0.5% DMSO), z-VAD-fmk (20 μ M),
420 or Necrostatin-1 (100 μ M) for 2 hours before adding 62.5 μ M TMZ or a further 0.5% DMSO
421 (vehicle control). Data are expressed as mean \pm SEM for three independent biological replicates.
422 *, $p < 0.05$; **, $p < 0.01$.

423

424

425 **Figure 2. Depletion of eIF5B enhances TMZ-induced apoptosis.** (A) Control or eIF5B-depleted
426 BT48 cells were treated with 62.5 μ M TMZ or a vehicle control (0.5% DMSO) for 24 hours and
427 stained with Hoechst 33342 for 30 minutes. The cells were then imaged at 20x magnification (left)
428 by brightfield microscopy, or at 20x magnification (middle) and 40x magnification (right) by
429 fluorescence microscopy to image DNA (blue). Images from a representative experiment are
430 shown in the left and middle panels; images from a separate experiment are shown in the right

431 panels. **(B)** The percent of Hoechst-stained nuclei per field-of-view demonstrating fragmentation
432 were quantified using the onboard Cytation 5 analysis software. The percent of Hoechst-stained
433 nuclei from (A) displaying fragmentation (white arrows) were quantified. **(C-F)** Control or eIF5B-
434 depleted BT48 cells were treated with 62.5 μ M TMZ or a vehicle control (0.5% DMSO) for 72
435 hours, harvested in RIPA lysis buffer, and 30 μ g of total protein resolved by SDS-PAGE before
436 performing immunoblotting. **(C)** Representative immunoblots probing for eIF5B, caspase-3 (Pro-
437 Cas-3), cleaved caspase-3 (Cas-3), cleaved PARP (Clv PARP), SOX2 (n=1), and β -actin (internal
438 control). **(D-F)** Quantitation of eIF5B **(D)**, cleaved- versus pro-caspase-3 **(E)**, or cleaved PARP
439 **(F)**, all normalized to β -actin. Data are expressed as mean \pm SEM for three independent biological
440 replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

441

442

443 **Figure 3. Depletion of eIF5B leads to decreased levels of anti-apoptotic proteins.** Control or
444 eIF5B-depleted cells were harvested in RIPA lysis buffer and 25 μ g of total protein resolved by
445 SDS-PAGE before performing immunoblotting. **(A)** Representative images of immunoblots
446 probing for eIF5B, XIAP, cIAP1, Bcl-xL, c-FLIP_L, c-FLIP_S, or β -actin (internal control). **(B)**
447 Quantitation of eIF5B, XIAP, cIAP1, Bcl-xL, c-FLIP_L, or c-FLIP_S, all normalized to β -actin. Data
448 are expressed as mean \pm SEM for three (cIAP1, c-FLIP_S, c-FLIP_L) and four (eIF5B, XIAP, Bcl-
449 xL) independent biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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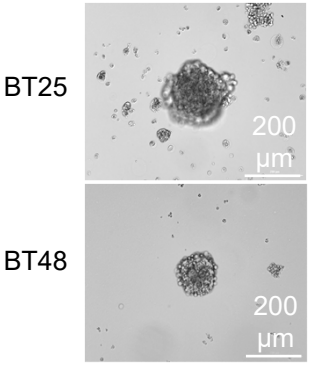
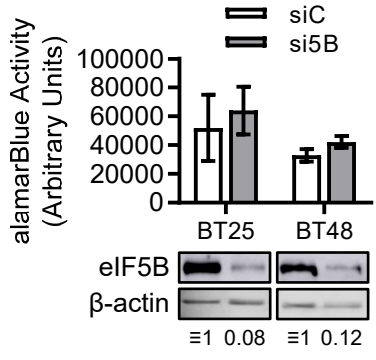
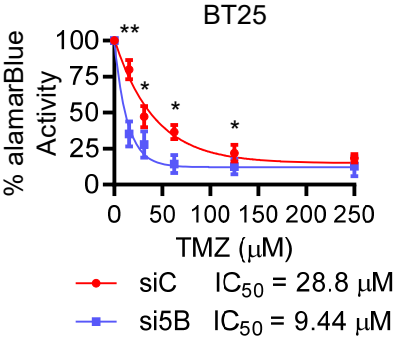
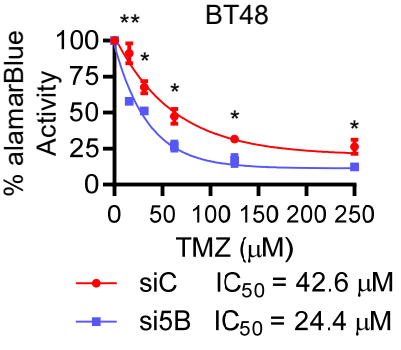
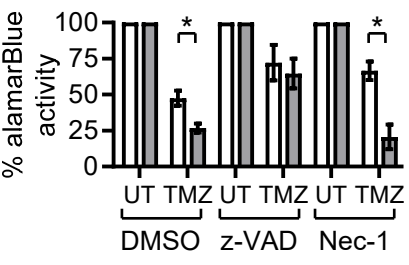
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Figure 1

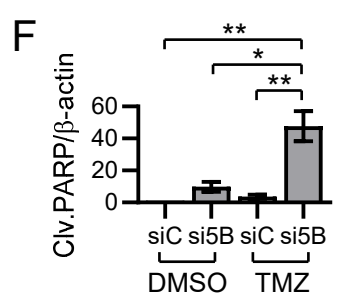
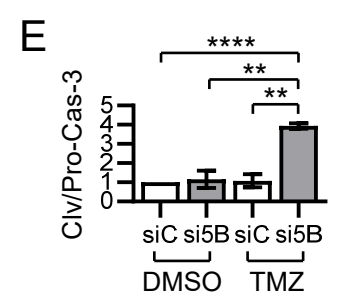
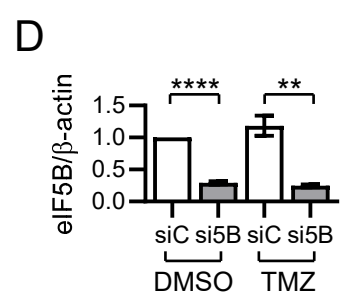
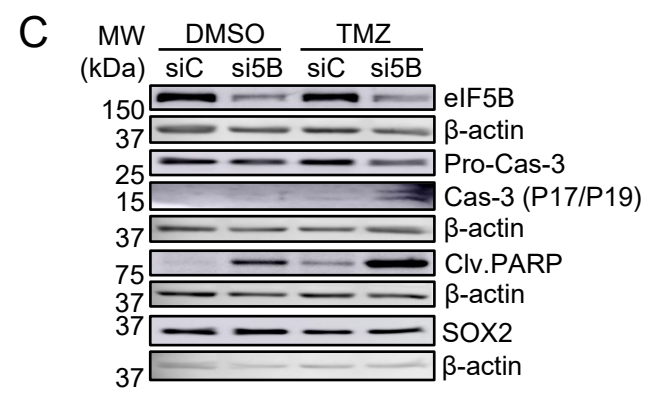
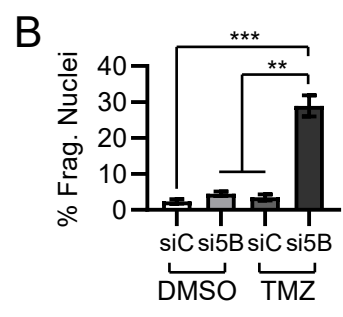
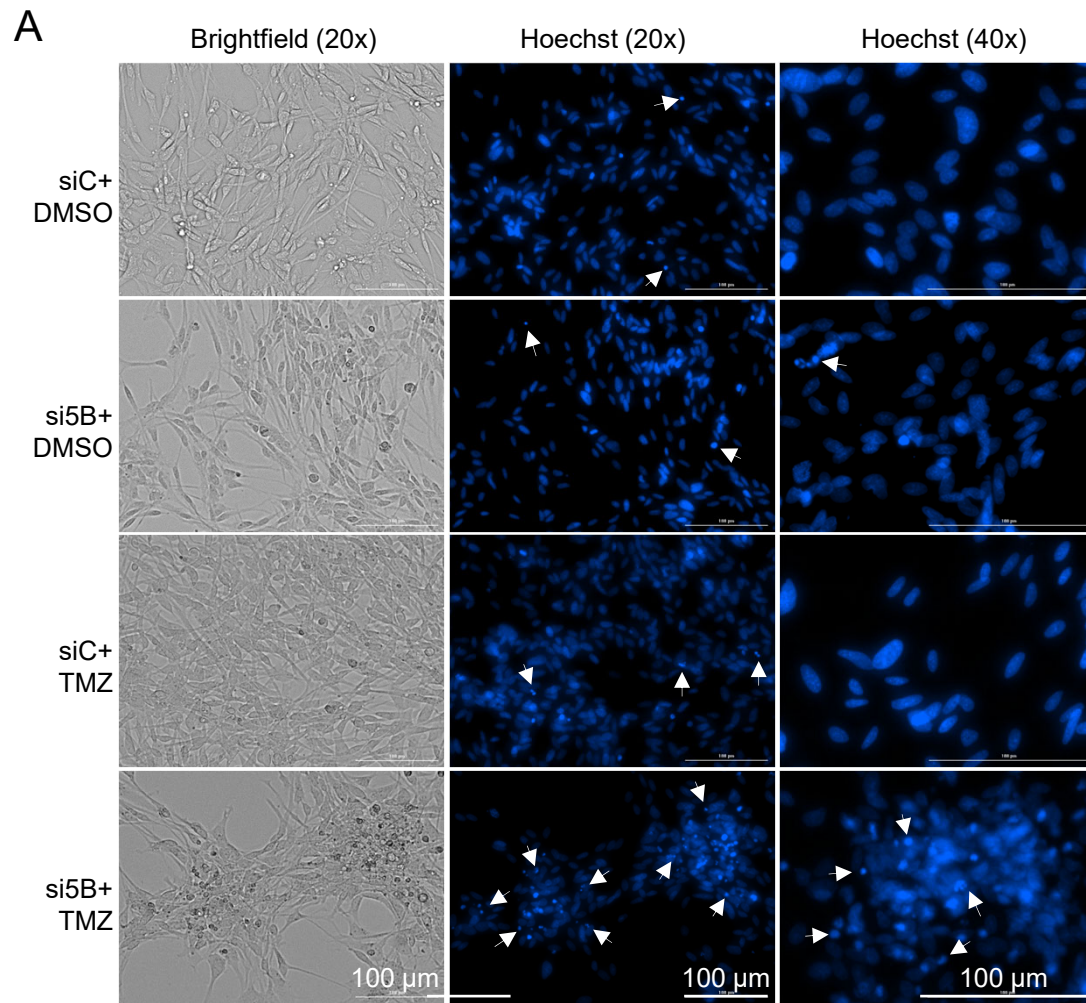


Figure 2

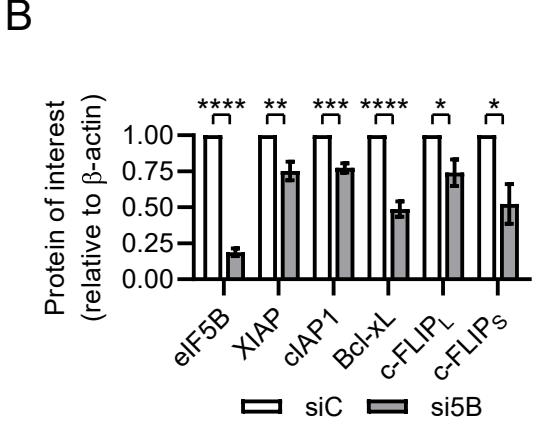
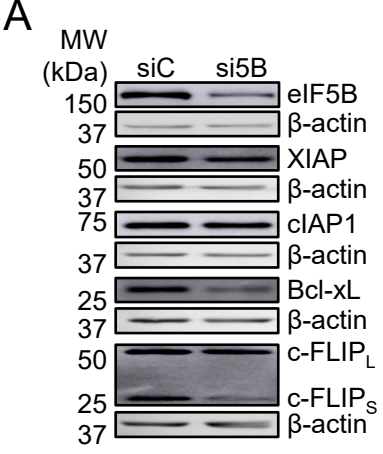
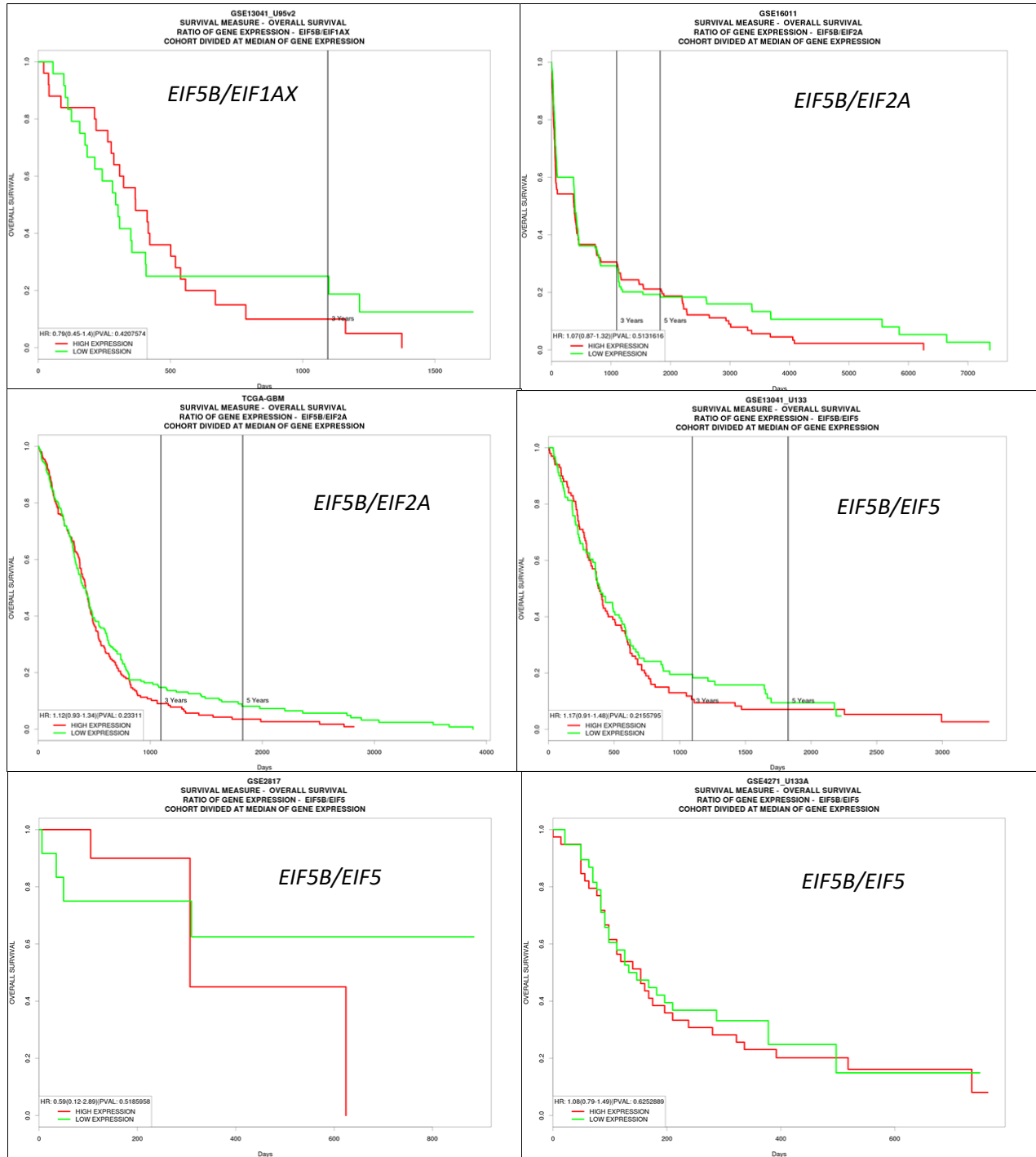
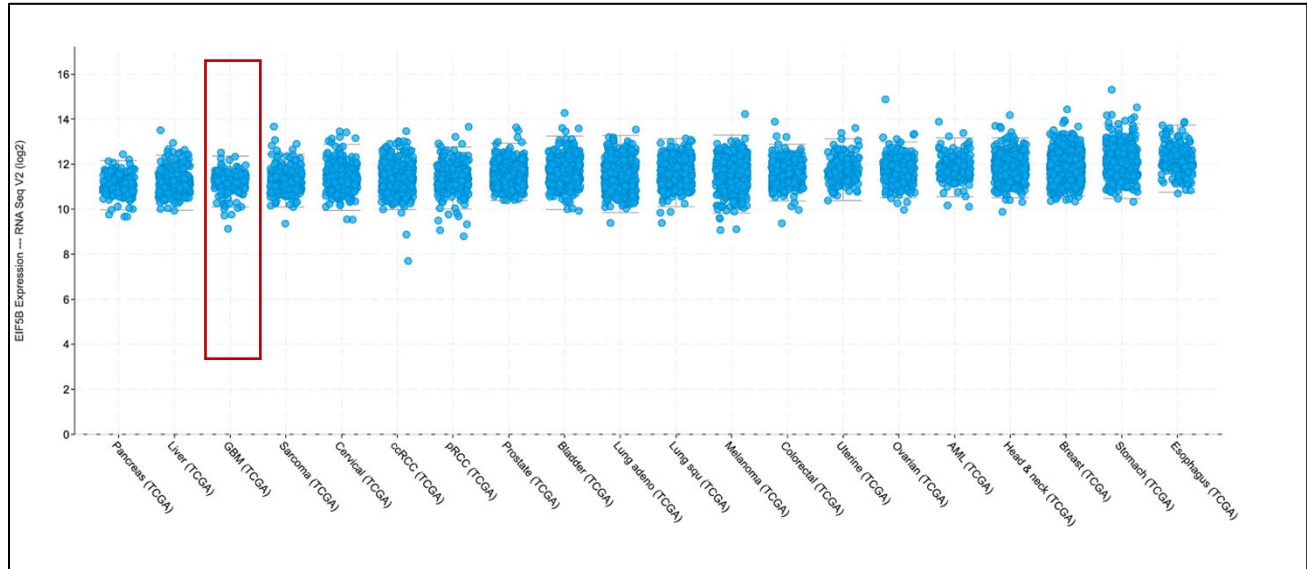


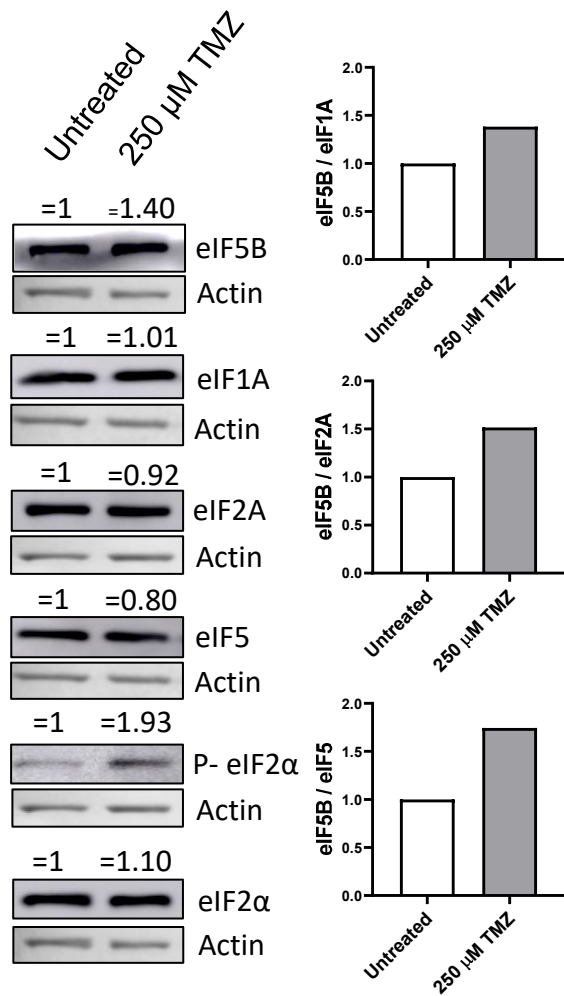
Figure 3



Supplemental Figure S1: We have looked at the association between ratios of *EIF5B/EIF2A*, *EIF5B/EIF1AX*, and *EIF5B/EIF5* mRNAs and overall survival of GBM patients using various patient cohorts. These data suggest that higher levels of *EIF5B* mRNA over *EIF1AX*, *EIF2A*, and *EIF5* were correlated with worse patient outcomes.



Supplemental Figure S2: Increasing median expression of EIF5B mRNA in various cancer types, suggesting that EIF5B is not lost in GBM.



Supplemental Figure S3: The protein level of eIF5B modestly increases in TMZ-treated U343 cells. The ratios of eIF5B/eIF1A, eIF5B/eIF2A, and eIF5B/eIF5 are modestly enhanced upon TMZ treatment. U343 cells were treated with 250 μM TMZ for 24 hours, harvested in RIPA lysis buffer and 25 μg of total protein was resolved by SDS-PAGE before performing immunoblotting.