Eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) expression in glioblastoma is driven by ETS1- and MYBL2-dependent transcriptional activation

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INTRODUCTION

Eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) encodes 4EBP1, a negative regulator of mRNA translation and a substrate of the mechanistic target of rapamycin (mTOR), whose function and relevance in cancer is still under debate. Here, we analyzed EIF4EBP1 expression in different glioma patient cohorts and investigated its mode of transcriptional regulation in glioblastoma cells. We verified that EIF4EBP1 mRNA is overexpressed in malignant gliomas, including isocitrate dehydrogenase (IDH)-wildtype glioblastomas, relative to non-neoplastic brain tissue in multiple publicly available datasets. Our analyses revealed that EIF4EBP1 overexpression in malignant gliomas is neither due to gene amplification nor to altered DNA methylation, but rather results from aberrant transcriptional activation by distinct transcription factors. We found seven transcription factor candidates co-expressed with EIF4EBP1 in gliomas and bound to the EIF4EBP1 promoter, as revealed by chromatin immunoprecipitation (ChIP)-sequencing data. We investigated the ability of these candidates to activate the EIF4EBP1 promoter using luciferase reporter assays, which supported four transcription factors as candidate EIF4EBP1 regulators, namely MYBL2, ETS1, HIF-1A, and E2F6. Finally, by employing transient knock-down experiments to repress either of these transcription factors, we identified MYBL2 and ETS1 as the relevant transcriptional drivers of enhanced EIF4EBP1 expression in malignant glioma cells. Taken together, our findings confirm enhanced expression of EIF4EBP1 in malignant gliomas relative to non-neoplastic brain tissue and characterize the underlying molecular pathomechanisms.

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Glioblastoma is the most common and most malignant primary glial tumor type of the central nervous system (CNS) that according to the World Health Organization (WHO) classification of CNS tumors corresponds to CNS WHO grade 4 [19, 20]. This tumor entity nowadays comprises only isocitrate dehydrogenase (IDH)-wildtype tumors [20], as opposed to the previous WHO tumor entity which also included IDH-mutant tumors [19]. IDH-wildtype glioblastomas are diffuse astrocytic gliomas that grow invasively in the brain parenchyma, are highly proliferative and angiogenic, and are characterized by the presence of hypoxic and necrotic regions [21]. Median survival time is around 15 months after diagnosis [19], despite standard of care treatment [22, 23]. The initiation and progression of IDH-wildtype glioblastomas are driven by genetic alterations found in malignant gliomas. Specifically, we analyzed EIF4EBP1 mRNA expression levels in EGFR-amplified and EGFR-non-amplified as well as in O6-methylguanine DNA methyltransferase (MGMT) promoter-methylated and promoter-unmethylated IDH-wildtype glioblastoma patient samples using publically available datasets [32]. We found that EIF4EBP1 mRNA level is not impacted by either of these alterations (Fig. S1C, D). We also investigated the potential association of EIF4EBP1 expression with the IDH mutation status in primary glioma samples and found that EIF4EBP1 mRNA expression is not dependent on the IDH mutation status in three independent datasets (Fig. S1E–G). Among IDH-mutant gliomas, there was no difference in EIF4EBP1 expression levels in 1p/19q-codeleted oligodendrogliomas versus 1p/19q-intact astrocytomas included in the FRENCH cohort dataset [28] (Fig. S1H) or TCGA dataset [32] (Fig. S1I).

Next, we asked whether EIF4EBP1 overexpression in malignant gliomas might be caused by EIF4EBP1 gene amplification. Analyzing the copy number status of EIF4EBP1 in 507 malignant glioma samples did not reveal any amplification of EIF4EBP1 (Fig. 1B). This observation stands in contrast to a previous report stating that EIF4EBP1 is amplified in approximately 13% of breast cancers [11]. While approximately 8.5% of TCGA malignant glioma cases analyzed here exhibited a low-level gain of EIF4EBP1 [33, 34], there was no association with higher EIF4EBP1 mRNA expression as compared to tumors without EIF4EBP1 copy number gain (Fig. 1B and Table S1). We then assessed whether EIF4EBP1 mRNA overexpression is due to differential promoter methylation in non-neoplastic brain versus malignant glioma tissues. We analyzed the
DNA methylation level of 12 CpG sites within the EIF4EBP1 promoter region (hg19; Chr8: 37,886,520–37,889,020), which showed that non-neoplastic brain tissues and malignant glioma tissues exhibited a very similar methylation profile (Fig 1C). This goes along with a previous study reporting no difference of EIF4EBP1 promoter methylation in glioma compared to control samples [35]. Based on these analyses, we can exclude EIF4EBP1 gene amplification or altered EIF4EBP1 promoter methylation as possible mechanisms driving EIF4EBP1 overexpression in malignant gliomas.

**Identification of potential transcription factors driving enhanced transcription of EIF4EBP1 in malignant gliomas**

We next reasoned that the increased EIF4EBP1 mRNA expression in malignant gliomas might be driven by specific transcription factors. To identify potential transcription factor candidates, we searched for transcription factors that are positively co-expressed with EIF4EBP1 in malignant gliomas, overexpressed in these tumors as compared to non-neoplastic brain tissues, and known to bind the endogenous EIF4EBP1 promoter by ChIP. This allowed us to uncover seven transcription factors that fulfilled these criteria. We searched for transcription factors that are positively co-expressed with EIF4EBP1 in gliomas and found EIF4EBP1 mRNA expression to be significantly and positively associated with the mRNA expression levels of MYBL2, FOXM1, ETS1, HIF-1A, JUN, E2F1, and E2F6 in the REMBRANDT dataset [26] (Fig 2A–G). These associations were validated for each of these transcription factors, excluding E2F1, in at least three additional glioma cohorts, including the SUN [27] (Fig 2A–G), KAWAGUCHI [36], FRENCH [28], or FREUJE [37] datasets (Table S2). In support of the co-expression data, we analyzed the expression of these transcription factors in malignant glioma tissues using TCGA datasets [32, 38] and the REMBRANDT dataset [26] datasets, as well as non-neoplastic brain tissues [39].

This demonstrated a significant overexpression of MYBL2, FOXM1, ETS1, HIF-1A, and JUN in both glioma cohorts compared to non-neoplastic brain tissues (Fig S3A, B). Expression of E2F1 and E2F6 was previously reported to be higher in glioblastomas (using TCGA dataset) compared to non-neoplastic brain tissues [40], which we validated in the REMBRANDT dataset [26] (Fig S3B). Of note, the expression of these transcription factors was independent of the IDH mutation status in malignant gliomas, except for ETS1 (Fig S3C). Finally, we analyzed existing ChIP-seq data [41, 42] in glioblastoma cell lines. In accordance, ChIP-seq data from the Encode consortium [41, 42], which demonstrated distinct H3K4 trimethylation signals (Fig.2H). In contrast, transient knock-down of either ETS1 or MYBL2 resulted in a significant decrease of 4EBP1 mRNA and protein levels in both glioblastoma cell lines. In contrast, transient knock-down of either ETS1 or MYBL2 resulted in a significant decrease of 4EBP1 mRNA and protein levels in both glioblastoma cell lines. In contrast, transient knock-down of either ETS1 or MYBL2 resulted in a significant decrease of 4EBP1 mRNA and protein levels in both glioblastoma cell lines (Fig.4C–F). Based on these results, we identified two transcription factors, ETS1 and MYBL2, that regulate EIF4EBP1 expression in glioblastoma cells.

**EIF4EBP1 is co-expressed with MYBL2, but not with ETS1, in other non-CNS cancer types**

We further analyzed the potential co-expression of EIF4EBP1 and either ETS1 or MYBL2 at the mRNA level in multiple different cancer types using datasets available in R. For each analyzed cancer type, we observed a decrease of ETS1 and MYBL2 in U-118 MG and U-87 MG, and of MYBL2 in U-118 MG upon knock-down. However, while the knock-down of MYBL2 in U-87 MG was strong at the mRNA level, we could not detect it at the protein level due to low endogenous MYBL2 levels in this cell line. These analyses proved that ETS1 and MYBL2, but not with ETS1, regulate EIF4EBP1 expression in glioblastoma cells.
represent a more general regulatory mechanism driving EIF4EBP1 expression in different cancer entities.

DISCUSSION

EIF4EBP1 gene expression and its clinical relevance in cancer are highly tumor-type specific [47]. We found that EIF4EBP1 is overexpressed in glioblastoma tissue samples in different patient cohorts as compared to non-neoplastic brain tissues, thus extending previous observations made in the TCGA cohort [12]. Elevated mRNA expression may lead to increased active 4EBP1 protein levels in glioblastoma, as it was reported that mTOR activity is reduced regionally in this tumor entity, thus leading to 4EBP1 activation in poorly vascularized areas [48]. We searched for the underlying causes of increased EIF4EBP1 mRNA expression in malignant gliomas and observed that the EIF4EBP1 gene is not amplified in glioblastomas although amplification of 8p11.23, which encompasses EIF4EBP1, has been reported in other cancer entities, such as lung squamous cell carcinoma, bladder cancer, and breast cancer, and correlated with higher EIF4EBP1 expression [49]. By bioinformatic analysis, we identified seven transcription factors that may potentially drive overexpression of EIF4EBP1 in gliomas. Each of these transcription factors harbors oncogenic or tumor-promoting functions and some of them were reported to be overexpressed in cancer, including overexpression of E2F1, E2F6 [40], FOXM1, and MYBL2 [50] in glioblastomas. Among the
Fig. 3  Induction of EIF4EBP1 promoter activity by E2F6, ETS1, HIF-1A, and MYBL2. A Scheme of the luciferase reporter construct containing the EIF4EBP1 promoter, exon 1, and part of intron 1 (−661; +705), coupled to Firefly luciferase, with the indicated binding sites of transcription factor candidates. B–H HEK293-T cells were transfected with the −661; +705 EIF4EBP1 promoter reporter construct, together with increasing amounts of plasmids expressing either one of the indicated transcription factors and a vector expressing Renilla luciferase. Luciferase activities were detected using the Dual-Luciferase Reporter Assay. Firefly luciferase activity was normalized to Renilla luciferase activity and the ratio was normalized to the corresponding 0 ng condition. Data represent the mean of three independent replicates ± standard deviation (SD). Significance was calculated using an unpaired and one-tailed parametric t-test (*p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001). Below each diagram, a representative immunoblot analyzing overexpression of each of the indicated transcription factors is presented.
Fig. 4 Regulation of EIF4EBP1 mRNA and protein expression by MYBL2 and ETS1 in glioblastoma cells. A–F U-118 MG and U-87 MG glioblastoma cells were transiently transfected with negative control siRNAs (NC), and an siRNA pool targeting (A, B) E2F6 (siE2F6) or two different siRNAs each targeting either (C, D) ETS1 (si 2 and si 3) or (E, F) MYBL2 (si 4 and si 5). Cells were re-transfected after 96 h with their corresponding siRNA and incubated for a total of 192 h. mRNA and protein were harvested to determine the expression levels of EIF4EBP1/4EBP1 and (A, B) E2F6, (C, D) ETS1 or (E, F) MYBL2 by qRT-PCR and immunoblot. Data obtained by qRT-PCR represent the mean of three independent replicates ±SD and the fold change in expression was normalized to the negative control. Results of representative immunoblot are depicted on the right-hand side of the diagrams representing the qRT-PCR results. Significance was calculated using an unpaired and one-tailed parametric t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).
Fig. 5  Co-expression of EIF4EBP1 and MYBL2 or ETS1 in different cancer entities. A Correlation between the mRNA expression levels of EIF4EBP1 and ETS1 (light blue dots) or MYBL2 (yellow dots) in the indicated human cancer types (Table S3). Co-expression levels were quantified by calculating the Pearson correlation coefficient. Each dot represents the $R$-value for one cohort. The dotted line corresponds to an $R$-value of 0.3, chosen as the cut-off for positive correlation. B–H Representative co-expression analysis between EIF4EBP1 mRNA and B, D, F ETS1 (light blue dots) or C, E, G MYBL2 (yellow dots) mRNA levels in the indicated tumor type and cohort. The represented cohorts are (B, C) glioma (KAWAGUCHI cohort; $n = 50$) [36], D, E breast cancer (BLACK cohort; $n = 107$) [45], and F, G lung cancer (CHUANG cohort; $n = 60$) cohort [46]. Co-expression levels were quantified by calculating the Pearson correlation coefficient. GIT, gastrointestinal tract.
seven transcription factor candidates, we found that HIF-1A, E2F6, ETS1, and MYBL2 activated the EIF4EBP1 promoter in vitro while E2F1, JUN, and FOXM1 did not. Surprisingly, E2F1 a transcriptional activator repressed EIF4EBP1 promoter activity while E2F6, which is a transcriptional repressor, induced EIF4EBP1 promoter activity. Of note, E2F1 has been shown to repress transcription of YAP1 by binding to the transcription factor TEAD [51], so we cannot exclude that E2F1 may repress the endogenous EIF4EBP1 promoter. While JUN was not validated as a transcriptional regulator of EIF4EBP1 promoter with our assays, this may be explained by the absence of a consensus binding motif (5′-TGAC/GTCA-3′) [52] within the −661;+705 EIF4EBP1 promoter construct we used. Of note, the endogenous EIF4EBP1 promoter contains two JUN consensus binding motifs, which are located further upstream and downstream of the −661;+705 promoter region, suggesting that JUN is still a possible candidate that might regulate the EIF4EBP1 promoter.

By functional knockdown experiments, we uncovered that ETS1 and MYBL2 regulate the transcription of endogenous EIF4EBP1 in glioblastoma cells, highlighting novel regulators of EIF4EBP1 transcription that complement the transcription factors previously reported, including MYC [15], the androgen receptor [16], ATF4 [15], ATF5 [17], and HIF-1A [18]. Since ETS1 and MYBL2 as well as EIF4EBP1 are overexpressed in other cancer entities, for instance in colorectal cancer [12, 13, 53, 54] or breast cancer [12, 53, 55], these transcription factors might also regulate EIF4EBP1 expression in cancers outside the CNS. In support of this assumption, we found that MYBL2, but not ETS1, is co-expressed with EIF4EBP1 at the mRNA level in a variety of non-CNS cancer entities, suggesting that MYBL2 might represent a general transcriptional driver of EIF4EBP1 overexpression in human cancers while ETS1-dependent regulation of EIF4EBP1 may be more restricted to CNS tumors. The molecular mechanisms underlying MYBL2 and ETS1 overexpression in malignant gliomas are to date unknown. In the case of MYBL2, this may be due to EGFR signaling, which is frequently amplified and overexpressed in IDH-wildtype glioblastomas [56] and was reported to activate the MYBL2 promoter in association with E2F1 [57]. ETS1 activity is directly induced by the RAS/RAF/MEK/ERK pathway [53], which is overactive in a large number of IDH-wildtype glioblastomas [58] and leads to ETS1 promoter activation [53].

Given that we found EIF4EBP1 to be a target gene of the ETS1 and MYBL2 oncoproteins in malignant gliomas, 4EBP1 may possibly contribute to ETS1 and MYBL2 tumorigenic functions in these tumors. Functions of both transcription factors as well as 4EBP1 have been linked to support angiogenesis. Indeed, ETS1 is known to regulate the VEGF promoter and its transcription [59], and ETS1 is associated with a higher density of microvessels in tumors [60]. MYBL2 expression was reported to be induced under ischemic conditions in rat brains [61], stabilized by HIF-2α [62], and to protect cells toward hypoxia-induced apoptosis [63]. Additionally, 4EBP1 has been shown to promote the selective translation of VEGF or HIF-1A mRNAs in response to hypoxia [7]. Taken together, this raises the possibility that the induction of EIF4EBP1 expression by ETS1 and MYBL2 in glioblastoma cells may be a previously unrecognized mechanism mediating angiogenesis in this tumor type. Independently of ETS1 or MYBL2, 4EBP1 may exhibit other functions in glioblastomas. It has been reported that 4EBP1 is required for oncogenic Ras transformation of mouse embryonic fibroblasts in vitro and in vivo [64], pointing to a tumor-supporting role of 4EBP1. Thus, it is possible that 4EBP1 may also contribute to glioma tumorigenesis by supporting oncogenicity.

In summary, we elucidated molecular mechanisms of enhanced EIF4EBP1 levels in glioblastoma cells, revealing the oncogenic transcription factors ETS1 and MYBL2 as responsible transcriptional regulators.

**MATERIALS AND METHODS**

**Data availability and bioinformatics analysis**

We used publically available cancer datasets (Table S3) as well as glioma and non-neoplastic brain tissue datasets derived from various cohorts for correlative analyses of RNA expression data. Table S4 provides an overview of the glioma datasets that were used including accession numbers, patient numbers, original diagnoses, and information on IDH mutation status, if available. As these datasets were generated before the current WHO classification, the provided diagnoses are mostly based on histological classification only. RNA expression data were analyzed with the Gepia website [38] using the publicly available GTEX non-neoplastic brain tissue and TCGA [32] (tumor tissues) datasets or obtained from the R\(^2\) Genomic Analysis Visualization Platform (R\(^2\) AMC; http://r2.amc.nl) using the REMBRANDT [26] datasets to analyze the expression levels of EIF4EBP1, MYBL2, FOXM1, ETS1, HIF-1A, JUN, E2F1, and E2F6 in non-neoplastic brain tissue versus malignant glioma patient samples. Additionally, the expression levels of EIF4EBP1 were analyzed with R\(^2\) AMC using the SUN dataset [27], FRENCH [28], HEGI [29], DONSON [31] (microarray platforms u133p2 and TUYUSUZ [30] (microarray platform huGene21st) datasets. For co-expression analyses, the above-mentioned cohorts as well as the KAWAGUCHI [36], FREIEJ [37], and PAUGH [65] cohorts were used. Expression data of IDH-wildtype glioblastoma patient samples according to the MGMT promoter methylation status were retrieved from cbioportal [33, 34] (TCGA [32]) and data related to the EGFR amplification status in IDH-wildtype glioblastomas were retrieved with R\(^2\) AMC using the FRENCH [28] cohort. Expression data according to 1p/19q co-deletion were obtained for IDH-mutant CNS WHO grade 2, 3, and 4 gliomas from R\(^2\) AMC using the FRENCH [28] cohort or from https://portal.gdc.cancer.gov using TCGA datasets for lower-grade glioma and glioblastoma [32]. MRNA expression data according to IDH mutation status were analyzed using the CGGA [66], FRENCH [28], and TCGA [32] datasets for EIF4EBP1 expression and TCGA dataset [32] for the expression of the transcription factors. TCGA data were accessed using cbioportal [33, 34]. Copy number variations for EIF4EBP1 and corresponding EIF4EBP1 expression in glioma patient samples were acquired from cbioportal and R\(^2\) AMC, respectively [33, 34] (TCGA [32]) and compared to expression data of EIF4EBP1 in non-neoplastic brain tissue [67] from R\(^2\) AMC. DNA methylation data were downloaded from R\(^2\) AMC (GSE12179 [68] and GSE156374 [69] for non-neoplastic brain tissue and GSE19774 [70] for tumor tissues). CpG sites included within the −1500 to +1000 region of EIF4EBP1 (human genome GRCh 38/hg38; Chr8:38,029,034−38,031,534) were selected for analysis and the mean was determined for each group and CpG site. ChiP-seq data for H3K27ac (UCSC; Accession: wgEncodeEH000030, wgEncodeEH000097, wgEncodeEH001111, wgEncodeEH000055, wgEncodeEH000043, wgEncodeEH000064, wgEncodeEH000097, H3K4me3 (wgEncodeEH000013, wgEncodeEH000099, wgEncodeEH002876, wgEncodeEH001182), ETS1 (wgEncodeEH0002290; wgEncodeEH0011580), FOXM1 (wgEncodeEH0002529), JUN (wgEncodeEH000746, wgEncodeEH000719, wgEncodeEH002805, wgEncodeEH000620), E2F1 (wgEncodeEH000699, wgEncodeEH000688, wgEncodeEH000693) and E2F6 (wgEncodeEH000692 wgEncodeEH0000676, wgEncodeEH000055, ETS1 and ETS1; [57]) were retrieved from the UCSC Genome Browser (hg19 [66]). Promoter methylation data were retrieved from cBioportal [33, 34] and compared to expression data of ETS1 in non-neoplastic brain tissue and glioblastoma [32]. A bootstrapping approach was applied to estimate significance levels for the overrepresented CpG sites.

**Statistical analyses**

Unpaired t-tests were performed when comparing gene expression in gliomas versus non-neoplastic brain tissues samples, as well as between IDH-mutant glioma and control samples. For the R\(^2\) AMC dataset, we used the Wilcoxon rank-sum test (Mann-Whitney U test). Multiple testing corrections were performed using the Hochberg method (all values) and the Benjamini-Hochberg method (all values) was applied. To test for correlation of expression levels of transcription factors, Spearman’s correlation coefficient was used with p-values corrected by the Benjamini-Hochberg method (all values). The co-expression data were visualized using the R package ‘s依旧[41]’ and the ‘ggplot2’ package. The Wilcoxon rank-sum test (Mann-Whitney U test) was performed to compare gene expression in glioma and non-neoplastic brain tissues samples, as well as between IDH-mutant glioma and control samples. The significance level was set to p<0.05. For all statistical analyses, the R software (version 3.5.0) was used.

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Cell culture
HEK 293-T embryonic kidney cells as well as the human glioblastoma cell lines U-118 MG and U-87 MG were originally obtained from American Type Culture Collections (ATCC). Cells were maintained in Dulbecco’s modified Eagle Medium (10569010, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (10270-106, Thermo Fisher Scientific) and 1% penicillin/streptomycin (10790-013, Thermo Fisher Scientific) and cultured in a humidified incubator at 37 °C with 5% CO2. The cell lines were confirmed to be mycoplasma-free by Venor GeM Classic (11-1050, Minerva Biolabs, Berlin, Germany) kit and validated by STR-profiling (Genomics & Transcriptomics Labor (GTL), Heinrich Heine University, Düsseldorf, Germany).

siRNA transfection
Cells were transfected in 6-well plates at 70% confluence with 25 nM control siRNA (D-001206-14-50, Dharmacon, Cambridge, UK) or negative control siPool (siTools Biotech, Planegg, Germany) or siRNAs targeting ETS1 (D-003887-02-0010 & D-003887-03-0010, Dharmacon), MYBL2 (D-01444-04-0005 and D-01444-05-0005, Dharmacon) or E2F6 (siTools Biotech) using siLentFect transfection reagent (1703362, Biolud, Hercules, CA, USA) (see Table S5 for siRNA sequences). Briefly, a master mix containing 125 µl Opti-MEM (31985-070, Thermo Fisher Scientific) and 3 µl siLentFect was prepared and incubated for 5 min at room temperature (RT). Meanwhile, 125 µl Opti-MEM were mixed with 25 nM of siRNA for each well. The siRNA mix was mixed 1:1 with the master mix, incubated for 20 min at RT, and added dropwise onto the cells. The medium was changed the day after transfection. Cells were re-transfected after 96 h. At 192 h following the first transfection, RNA and protein were harvested for further analysis.

Plasmid construction
The promoter region of the human EIF4EBP1 gene, spanning from nucleotide –661 to +705 (human genome GRCH 38/hg38; Chr8: 38,029,873–38,031,239), was inserted into the SacI and BglII restriction sites of the Firefly Luciferase expressing pGL4.22 plasmid (E6771, Promega, Madison, WI, USA). Cloning was performed by GENEWIZ Germany GmbH (Leipzig, Germany).

Luciferase reporter assays
HEK 293-T cells were seeded in 12-well plates to reach 50% confluence on the day of transfection. Cells were transfected with 125 ng of the Eif4ebp1 promoter Firefly luciferase plasmid, 2 ng of Renilla luciferase-expressing pRL SV40 plasmid (E2231, Promega), as internal control, and 3–373 ng of either of the transcription factor expressing plasmids, completed to 500 ng total DNA with pCMV-Neo-Bam (16440, Addgene) or pcDNA3.1 (V79020, Thermo Fisher Scientific) plasmids using CalFectTM Cell Transfection Reagent (SL100478, SigmaGen Laboratories; Frederick, MD, USA) according to the manufacturer’s guidelines. The used transcription factor expressing plasmids were pcDNA3 E2F1 (kind gift from Dr. Tony Kouzarides, University of Cambridge, UK), pSG3.1 ETS1 (kindly provided by Dr. Lawrence Michtosh, University of British Columbia, Vancouver, Canada), pcDNA3 FoxM1 (kindly provided by Dr. Pradipt Raychaudhuri, University of Illinois Cancer Center, Chicago, IL, USA), pcDNA3 HA-HIF-1A (gift from Dr. William Kaelin [Addgene plasmid # 18949; http://n2t.net/addgene:18949; RRID: Addgene_18949; [72]]), pcDNA3 MYBL2 (gift from Dr. Rob Lewis [Addgene plasmid # 25965; http://n2t.net/addgene:25965; RRID:Addgene_25965; [73]]), pCMV6 JUN (kind gift of Dr. Marguerite Buzza, University of Maryland, College Park, MD, USA). Cells were harvested 48 h post-transfection and the activity of Firefly and Renilla luciferases were sequentially determined using the Dual-Luciferase Reporter Assay System (E1980, Promega) and analyzed with Beckman Coulter microtiter plate reader (Beckman Coulter, Krefeld, Germany). All samples were performed in triplicate and the final luciferase quantification was formulated as the ratio of Firefly luciferase to Renilla luciferase luminescence.

RNA extraction, cDNA synthesis, and qRT-PCR
RNA was extracted using the RNeasy Plus Mini Kit (74136, Qiagen, Hilden, Germany). The extraction was performed according to the protocol provided by the manufacturer. Isolated RNA was retro-transcribed to cDNA using 1 µg of RNA per reaction with either of the QuantiTect Reverse Transcription Kit (205311, Qiagen) or the High-Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems, Waltham, MA, USA) according to the manufacturer’s protocol. Real-time PCR was performed in triplicates using 1 µl cDNA and 9 µl master mix consisting of 5 µl SYBR Green PCR Mix (430915S, Applied Biosystems), 3 µl H2O, and 1 µl of forward and reverse primers (0.5 µM final concentration). PPIA, GusB, and β-actin were used as housekeepers. For primer sequences, see Table S6.

Protein extraction and immunoblot analysis
Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 1% Triton X100, 0.5% Sodium deoxycholate, and 0.1% SDS) supplemented with protein inhibitor cocktail (1187358001, Roche, Basel, Switzerland) and phosphatase inhibitor (0490637001, Roche). Cell lysates were centrifuged at 14,000 × g for 15 min at 4 °C and supernatants were collected. Protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific) according to the manufacturer’s protocol. Twenty micrograms of total protein were loaded either on a 12% polyacrylamide-SDS gel or on a NativePAGE™ 4–12%, Bis-Tris Gels (NP0336 BOX, Thermo Fisher Scientific) and transferred to a 0.2 µm nitrocellulose membrane (Novo10600001, GE Healthcare; Chicago, IL, USA). Membranes were blocked with 5% bovine serum albumin (BSA) (8076.3, Carl Roth, Karlsruhe, Germany) TBS-Tween (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and probed with primary antibodies (as detailed in table S7) diluted 1:1000 in 5% BSA TBS overnight at 4 °C if not stated otherwise. Membranes were then incubated with a corresponding anti-mouse (926-32210, Li-Cor, Bad Homburg, Germany) or anti-rabbit (926-32211, Li-Cor) fluorescent secondary antibody diluted 1:10000. The fluorescent signal was visualized with the Li-COR Odyssey® CLx system (Li-Cor).

Statistical analysis of experimental data
All experiments were carried out in three biological replicates. Data are represented as mean ±/− standard deviation (SD). A one- or two-sided Student’s t-test was used to compare differences between control and experimental groups. Results were considered as being statistically significant at p<0.05. Statistical tests were calculated with GraphPad Prism version 7.04.

DATA AVAILABILITY
The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES
1. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. Cell. 2017;168:960–76.
2. Hoffarth A, Maden S, Pause A, Sonenberg N. Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J. 1995;14:701–9.
3. Wang Z, Feng X, Molinolo AA, Martin D, Vitale-Cross L, Nohata N, et al. 4E-BP1 is a tumor suppressor protein reactivated by mTOR inhibition in head and neck cancer. Cancer Res. 2019;79:1438–50.
4. Ding M, Van der Kwast TH, Yellanki RN, Foltz WD,McKee TD, Sonenberg N, et al. The mTORC1 targets ETS1 to maintain tumor growth and promote hypoxia tolerance in PTEN-driven prostate cancer. Mol Cancer Res. 2018;16:682–95.
5. Dowling RJ, Topisirovic I, Alain T, Bidinosti M, Fonseca BD, Petroulakis E, et al. mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. Science. 2010;328:1172–6.
6. Monia M, Gravel SP, Chenard V, Sikstrom K, Zheng L, Alain T, et al. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent transcriptional regulation. Cell Metab. 2013;18:698–711.
7. Braunstein S, Karpshева K, Pola C, Goldberg J, Hochman T, Yee H, et al. A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer. Mol Cell. 2007;28:501–12.
8. Dubois L, Magaquin MG, Cleven AM, Wepppler SA, Grenacher B, Landuyt W, et al. Inhibition of 4E-BP1 sensitizes U87 glioblastoma xenograft tumors to irradiation by decreasing hypoxia tolerance. Int J Radiat Oncol Biol Phys. 2009;73:1219–27.
9. Graff JR, Koniecck BW, Lynch RL, Dumstorf CA, Dowless MS, McNulty AM, et al. elf4E activation is commonly elevated in advanced human prostate cancers and significantly related to reduced patient survival. Cancer Res. 2009;69:1866–73.
10. Karlsson E, Waltersson MA, Bostner J, Perez-Tenorio G, Olsson B, Hallbeck AL, et al. High-Resolution genomic analysis of the 11q13 amplicon in breast cancers identifies synergy with 8p12 amplification, involving the mTOR targets SK62 and 4EBP1. Genes Chromosomes Cancer. 2011;50:775–87.
11. Rutkovsky AC, Yeh ES, Guest ST, Findlay VJ, Muise-Helmericks RC, Armeson K, et al. Eukaryotic initiation factor 4E-binding protein as an oncogene in breast cancer. BMC Cancer. 2019;19:491.
37. Freije WA, Castro-Vargas FE, Fang Z, Horvath S, Cloughesy T, Liu LM, et al. Gene expression profiling of gliomas strongly predicts survival. Cancer Res. 2004;64:6503–10.

38. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res. 2017;45:e998–W102.

39. GT Consortium. The Genotype-Tissue Expression (GTEx) project. Nat Genet. 2013;45:580–5.

40. Liao P, Han S, Qu H. Expression, prognosis, and immune infiltrates analyses of EZFs in human brain and CNS cancer. Biomed Res Int. 2020;2020:6281635.

41. Davis CA, Hitz BC, Sloan CA, Chan ET, Davidson JM, Gabdank I, et al. The Encyclopedia of DNA elements (ENCODE): data portal update. Nucleic Acids Res. 2018;46:D794–801.

42. Consortium EP. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;489:57–74.

43. Mimura I, Nangaku M, Kanki Y, Tsutsumi S, Inoue T, Koho T, et al. Dynamic change of chromatin conformations in response to hypoxia enhances the expression of GLUT1 (SLC2A3) by cooperative interaction of hypoxia-inducible factor 1 and KDM3A. Mol Cell Biol. 2012;32:2018–32.

44. Musa J, Cidre-Aranaz F, Aynaud MM, Orth MF, Knott MML, Mirabeau O, et al. Cooperation of cancer drivers with regulatory germine variants shapes clinical outcomes. Nat Commun. 2019;10:4128.

45. Caldwell CE, Sergio CM, Kang J, Muthukaruppa A, Boersma MN, Stone A, et al. Cyclin EZ expression is associated with endocrine resistance but not insensitivity to CDK2 inhibition in human breast cancer cells. Mol Cancer Ther. 2012;11:1488-99.

46. Lu TP, Tsai MI, Lee JM, Hsu CP, Chen PC, Lin CW, et al. Identification of a novel biomarker, SEMA5A, for non-small cell lung carcinoma in nonsmoking women. Cancer Epidemiol Biomark Prev. 2010;19:2590–7.

47. Musa J, Orth MF, Dallmayer M, Baldauf M, Pardo C, Rotblat B, et al. Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) gene expression: a master regulator of mRNA translation involved in tumorigenesis. Oncogene. 2016;35:4675–88.

48. Kumar S, Sharife H, Kreisel T, Mogilevsky M, Bar-Lev L, Grunewald M, et al. Intratumoral metatational zonation and resultant phenotypic diversification are dictated by blood vessel proximity. Cell Metab. 2019;30:201.e1–11.e6.

49. Voutsadakis IA. 8p11.23 Amplification in breast cancer: molecular characteristics, prognosis and targeted therapy. J Clin Med. 2020;9:3079.

50. Zhang X, Lv QL, Huang YT, Zhang LH, Zhou HH. Akt/FoxM1 signaling pathway-mediated upregulation of MYC regulates closure of the M phase by promoting progression of human glioma. J Exp Clin Cancer Res. 2017;36:105.

51. Zhang P, Pei C, Wang X, Xiang J, Sun BF, Cheng Y, et al. A balance of Yki/5d activator and E2F1/5d repressor complexes controls cell survival and affects organ size. Dev Cell. 2017;43:630–17. e5.

52. Li M, Ge Q, Wang W, Wang J, Lu Z. J-c-Jun binding site identification in K562 cells. J Genet Genomics. 2013;40:235–42.

53. Dittmer J. The role of the transcription factor Ets1 in glioma. Semin Cancer Biol. 2015;35:20–38.

54. Ren F, Wang L, Shen X, Xiao X, Liu Z, Wei P, et al. MYBL2 is an independent prognostic marker that has tumor-promoting functions in colorectal cancer. Am J Cancer Res. 2015;5:1542–52.

55. Thorner AR, Hoadley KA, Parker JS, Winkel S, Millikan RC, Perou CM. In vitro and in vivo analysis of the role of B-Myc in basal-like breast cancer. Oncogene. 2006;25:742–51.

56. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. Cell. 2013;155:462–77.

57. Hanada N, Lo HW, Day CP, Pan Y, Nakajima Y, Hung MC. Co-expression of GLUT3 (SLC2A3) by cooperative interaction of hypoxia-inducible factor 1 and KDM3A. Mol Cell Biol. 2009;29:201.e1–11.e6.

58. Voutsadakis IA. 8p11.23 Amplification in breast cancer: molecular characteristics, prognosis and targeted therapy. J Clin Med. 2020;9:3079.

59. Zhang X, Lv QL, Huang YT, Zhang LH, Zhou HH. Akt/FoxM1 signaling pathway-mediated upregulation of MYC regulates closure of the M phase by promoting progression of human glioma. J Exp Clin Cancer Res. 2017;36:105.

60. Zhang P, Pei C, Wang X, Xiang J, Sun BF, Cheng Y, et al. A balance of Yki/5d activator and E2F1/5d repressor complexes controls cell survival and affects organ size. Dev Cell. 2017;43:630–17. e5.

61. Li M, Ge Q, Wang W, Wang J, Lu Z. J-c-Jun binding site identification in K562 cells. J Genet Genomics. 2013;40:235–42.

62. Dittmer J. The role of the transcription factor Ets1 in glioma. Semin Cancer Biol. 2015;35:20–38.

63. Ren F, Wang L, Shen X, Xiao X, Liu Z, Wei P, et al. MYBL2 is an independent prognostic marker that has tumor-promoting functions in colorectal cancer. Am J Cancer Res. 2015;5:1542–52.

64. Thorner AR, Hoadley KA, Parker JS, Winkel S, Millikan RC, Perou CM. In vitro and in vivo analysis of the role of B-Myc in basal-like breast cancer. Oncogene. 2006;25:742–51.

65. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. Cell. 2013;155:462–77.

66. Hanada N, Lo HW, Day CP, Pan Y, Nakajima Y, Hung MC. Co-expression of GLUT3 (SLC2A3) by cooperative interaction of hypoxia-inducible factor 1 and KDM3A. Mol Cell Biol. 2009;29:201.e1–11.e6.

67. Voutsadakis IA. 8p11.23 Amplification in breast cancer: molecular characteristics, prognosis and targeted therapy. J Clin Med. 2020;9:3079.

68. Zhang X, Lv QL, Huang YT, Zhang LH, Zhou HH. Akt/FoxM1 signaling pathway-mediated upregulation of MYC regulates closure of the M phase by promoting progression of human glioma. J Exp Clin Cancer Res. 2017;36:105.

69. Zhang P, Pei C, Wang X, Xiang J, Sun BF, Cheng Y, et al. A balance of Yki/5d activator and E2F1/5d repressor complexes controls cell survival and affects organ size. Dev Cell. 2017;43:630–17. e5.

70. Li M, Ge Q, Wang W, Wang J, Lu Z. J-c-Jun binding site identification in K562 cells. J Genet Genomics. 2013;40:235–42.

71. Dittmer J. The role of the transcription factor Ets1 in glioma. Semin Cancer Biol. 2015;35:20–38.
65. Paugh BS, Broniscer A, Qu C, Miller CP, Zhang J, Tatevossian RG, et al. Genome-wide analyses identify recurrent amplifications of receptor tyrosine kinases and cell-cycle regulatory genes in diffuse intrinsic pontine glioma. J Clin Oncol. 2011;29:3999–4006.
66. Zhao Z, Zhang KN, Wang Q, Li G, Zeng F, Zhang Y, et al. Chinese Glioma Genome Atlas (CGGA): a comprehensive resource with functional genomic data from Chinese glioma patients. Genomics Proteomics Bioinformatics. 2021;19:1–12.
67. Berchtold NC, Cribbs DH, Coleman PD, Rogers J, Head E, Kim R, et al. Gene expression changes in the course of normal brain aging are sexually dimorphic. Proc Natl Acad Sci USA. 2008;105:15605–10.
68. Pai S, Li P, Killinger B, Marshall L, Jia P, Liao J, et al. Differential methylation of enhancer at IGF2 is associated with abnormal dopamine synthesis in major psychosis. Nat Commun. 2019;10:2046.
69. Kobov K, Jabari S, Pieper T, Kudernatsch M, Polster T, Woermann FG, et al. Mosaic trisomy of chromosome 1q in human brain tissue associates with unilateral polymicrogyria, very early-onset focal epilepsy, and severe developmental delay. Acta Neuropathol. 2020;140:881–91.
70. Mack SC, Singh I, Wang X, Hirsch R, Wu Q, Villagomez R, et al. Chromatin landscapes reveal developmentally encoded transcriptional states that define human glioblastoma. J Exp Med. 2019;216:1071–90.
71. Robinson JT, Thorvaldsdottir H, Winckler W, Gutman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol. 2011;29:24–6.
72. Kondo K, Klco J, Nakamura E, Lechpammer M, Kaelin WG Jr. Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell. 2002;1:237–46.
73. Johnson TK, Schweppre RE, Septer J, Lewis RE. Phosphorylation of B-Myb regulates its transactivation potential and DNA binding. J Biol Chem. 1999;274:36741–9.

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