Combined expression of FOXO and Tribbles proteins predicts survival of glioma patients

Bruno F. Santos[1,2,3,4], Ana-Teresa Maia[5,6], Bibiana I. Ferreira[1,2,5]* and Wolfgang Link[7]*

[1] Algarve Biomedical Center Research Institute- ABC-RI University of Algarve, Campus of Gambelas, 8005-139 Faro, Portugal

[2] Algarve Biomedical Center (ABC), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[3] PhD Program in Biomedical Sciences, University of Algarve, University of Algarve, Faro, Portugal)

[4] Unidade Local de Saúde do Algarve (ULSAlg). Rua Leão Penedo, 8000-386 Faro, Portugal

[5] Faculty of Medicine and Biomedical Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[6] RISE-Health, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[7] Sols-Morreale Biomedical Research Institute (IIBM), Spanish National Research Council (CSIC), Universidad Autónoma de Madrid (UAM), Madrid, Spain

*Correspondence to: Bibiana I. Ferreira. E-mail: biferreira@ualg.pt, Wolfgang Link. E-mail: walink@iib.uam.es

1. Abstract

Glioma, a primary tumor of the central nervous system originating from glial cells, continues to pose significant therapeutic challenges, particularly in its high-grade variants. Genetic analyses have revealed that the majority of gliomas exhibit significant alterations in the RTK/RAS/PI3K/AKT signaling cascade. Forkhead box protein O (FOXO) transcription factors and their upstream regulator protein family Tribbles have been characterized as important components of the downstream effector network of this pathway. Here, we investigated the role of the FOXO and Tribbles family members mRNA levels in the clinical outcomes of patients with gliomas. We show that while high expression of FOXO1 and low expression of FOXO3 and FOXO4 is associated with worse survival of glioma patients, low expression of TRIB1, TRIB2, and TRIB3 improves prognosis. We also found that FOXO3 and FOXO4 levels are downregulated in patients with grade 4 gliomas, compared to those with lower tumor grades, while FOXO1 and all three Tribbles isoforms are overexpressed in the same patients. We also show that transcriptional profiles of FOXO and Tribbles can predict the clinical outcome of glioma patients better than MGMT status, one of the most important biomarkers currently used in glioblastoma

treatment. These findings highlight the potential of FOXO and Tribbles mRNA as predictive biomarkers and their proteins as therapeutic targets, paving the way for more personalized and effective glioma treatments.

2. Introduction

According to the latest data from the Global Cancer Observatory, cancer of the brain and central nervous system (CNS) is the 19th most common malignancy and the 11th most frequent cause of cancer death in both sexes worldwide (*Brain, Central Nervous System*, 2020). Glioblastoma (GB) is the most common and aggressive primary brain tumor in adults, accounting for almost half of the primary malignant brain tumors (Wild et al., 2020). GB belongs to a broader category called gliomas, which refers to tumors arising from glial cells, the supportive cells in the nervous system. In 2021, the World Health Organization (WHO) updated its classification of the central nervous system (CNS) tumors (WHO CNS 5th edition classification, WHO CNS5) to incorporate molecular data, simplifying the system for improved diagnostic accuracy and prognostic assessment (Brat et al., 2021). Diffuse gliomas are assessed for mutations on the Isocitrate dehydrogenase (IDH) gene and 1p/19q codeletion and are now classified into only three types: (i) astrocytoma, IDH-mutant; (ii) oligodendroglioma, IDH-mutant with 1p/19q-codeleted; and (iii) GB, IDH-wildtype. This classification system reflects key genetic differences that help guide diagnosis and treatment. Astrocytomas are now graded as grade 2, 3, or 4, oligodendrogliomas as grade 2 or 3, and this classification is no longer entirely histological. GBs are exclusively grade 4 tumors, and in addition to being IDH-wildtype, they must exhibit additional features such as microvascular proliferation, necrosis, TERT promoter mutation, EGFR gene amplification, or a + 7/-10 chromosome copy-number alteration (Brat et al., 2021). GB treatment includes surgical resection followed by radiation therapy and Temozolomide (TMZ) chemotherapy. Nonetheless, GB remains an incurable disease with a median survival of only 15 months due to therapy resistance and recurrence (Poon et al., 2020). Therefore, there is an urgent need for new treatments that can overcome drug resistance, along with predictive biomarkers to guide therapy.

Several studies revealed a prominent role of the PI3K/AKT signaling pathway in the formation and progression of gliomas, and it is possible to find changes in this pathway in approximately 90% of GB (Brat et al., 2021; Ohgaki & Kleihues, 2007). We and others have characterized FOXO transcription factors as major transcriptional effectors of the PI3K/AKT pathway (Brunet et al., 1999; Hill et al., 2014) and identified the Tribbles homologue 2 (TRIB2) as a member of the Tribbles family of pseudokinases as a potent repressor of FOXO proteins (Zanella et al., 2010). Here we explore the role of FOXO and Tribbles proteins in driving aggressiveness in gliomas. FOXO proteins, a family of transcription factors including FOXO1, FOXO3, FOXO4 and FOXO6, (Calnan & Brunet, 2008) play a key role in regulating the expression of genes involved in cell proliferation, metabolism, apoptosis, autophagy, and stress resistance (Link, 2019; Link & Fernandez-Marcos, 2017) and are often inactivated in human cancers (Dansen & Burgering, 2008). Additionally, variants of FOXO3 have been associated with extreme human

longevity (Martins et al., 2016). A breakthrough in the functional characterization of FOXO transcription factors was achieved by the generation of triple knockout mice lacking FOXO1, 3 and 4, that established FOXO as *bona fide* tumor suppressors (Paik et al., 2007). Conversely, an oncogenic role of FOXOs in tumor progression, metastasis, and therapy resistance has emerged (Hornsveld et al., 2018). These proteins are regulated by reversible post-translational modifications that convert external stimuli into specific transcriptional programs (Brown & Webb, 2018). Under stress conditions or in the absence of growth factors, FOXOs are active in the nucleus. Yet, in the presence of growth factors, or in cancer cells where the PI3K/AKT pathway is constitutively active, FOXOs are exported to the cytoplasm where they remain inactive (Eijkelenboom & Burgering, 2013; Link & Fernandez-Marcos, 2017). Thus, the pharmacological modulation of FOXO is considered an attractive therapeutic approach for treating cancer and age-related diseases (Orea-Soufi et al., 2022). The extensive diversity of roles of these proteins suggests that the benefit of FOXO activation depends on the isoform and cellular context. While the different FOXO isoforms bind to the same consensus DNA sites in target gene promoters, it is increasingly evident that they also exert non-redundant functions in specific tissues (Santos et al., 2023). The Tribbles protein family consists of three isoforms, TRIB1, TRIB2 and TRIB3. Instead of phosphorylating target proteins, the members of this family function as protein scaffolds that integrate and modulate signals of important signaling cascades involved in proliferation, survival, and differentiation (Eyers et al., 2017). Our group established TRIB2 as an oncogene in melanoma and found that high levels of this protein confer resistance to several drugs used for cancer treatment (Link, 2015; Machado et al., 2020; Zanella et al., 2010). While TRIB2 has been implicated in cancer progression and therapy resistance across numerous cancer types (Mayoral-Varo et al., 2021), and TRIB1 is also considered as an oncogene in various malignancies, the role of TRIB3 is more complex and context-dependent. Studies indicate that TRIB3's contribution to cancer progression varies, potentially acting as either a tumor suppressor or an oncogene depending on the specific cellular environment (Ferreira et al., 2021; Foulkes et al., 2018; Hong et al., 2019; Richmond & Keeshan, 2020; Salazar et al., 2009). However, the clinical relevance of these observations has yet to be fully established.

Here, we reveal a relationship between survival and mRNA levels of FOXO and Tribbles family members in patients with gliomas. We show that specific FOXO/Tribbles signatures are highly predictive for clinical outcome, with high FOXO1 and low FOXO3/4 plus high TRIB1/2/3 levels associated with a worse prognosis. Furthermore, this signature could be used to select patients with glioma who would benefit from new potential therapeutic targets.

3. Materials and Methods

3.1. Datasets

RNA-seq Datasets

RNA-seq data was obtained from two independent databases, The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) and The Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/). Two independent datasets (TCGA-GBM and TCGA-LGG), comprising a total of 705 samples, were directly downloaded from the TCGA database into RStudio (version 2023.3.0.386) in September 2024 using the TCGAbiolinks R package (Colaprico et al., 2016). TCGA data was reclassified according to the WHO CNS5 standards (Zakharova et al., 2023). Data from the CGGA database (1018 samples) was obtained from two independent datasets (mRNAseq_693 and mRNAseq_325), were downloaded from the CGGA portal in September 2024 and uploaded to RStudio. For both TCGA and CGGA datasets, read counts (STAR-counts) were converted to transcript per million (TPM) to reduce technical variability between datasets. Suppl. Table 1 provides an overview of the demographic characteristics and disease features for both RNA-seq datasets.

Expression levels of FOXOs and Tribbles genes were analyzed by plotting Log2 transformed TPM values using the ggplot2 R package (Hadley Wickham, 216 C.E.). Boxplots were compared using the ANOVA test.

Microarray datasets

REMBRANDT (GSE68848) and the Gravendeel (GSE16011) microarray cohorts (Affymetrix HG-U1333Plus2) were available from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). Both databases were downloaded in September 2024 and uploaded into RStudio using GEOquery (Sean & Meltzer, 2007) and Biobase (Falcon et al., 2006) R packages. In cases where multiple probes were used for one gene, their average expression levels were used. Matching clinical data for REMBRANDT and Gravendeel datasets was obtained through GEO or GlioVis (https://gliovis.bioinfo.cnio.es/), respectively. Suppl. Table 1 provides an overview of the demographic characteristics and disease features for both microarray datasets.

3.2. Survival Analysis

Survival analysis was performed with Kaplan-Meier, using the survival (Therneau, 2024) and survminer (Kassambara et al., 2024) R packages. The primary endpoint was death from any cause, with surviving subjects censored at the date of their last known contact. Kaplan–Meier survival curves were compared using the log-rank test. To quantify the correlation between risk predictions and event times, maximizing the ability to better discriminate patient survival, their Concordance Index was determined using the concordance function from survival R package (Therneau, 2024).

3.3. Code availability

The filtered data and code for the survival analysis of gliomas according to FOXOs and Tribbles expression levels can be publicly accessed at https://github.com/maialab/enduring.

4. Results

4.1. High FOXO1, low FOXO3/FOXO4, and elevated TRIB1/2/3 mRNA levels form a molecular signature predictive of clinical outcomes

To investigate if mRNA levels of FOXO family members were associated with the prognosis of glioma patients, we analyzed the mRNA levels of a total of 705 samples (534 Low-Grade Gliomas (LGG) and 171 GB) from the TCGA database, using RStudio. Quartiles were established as the cut-off to define low expression levels under the first quartile and high levels above the third quartile. Kaplan-Meier curves show that the patient survival rate is dependent on FOXO mRNA levels. High levels of FOXO1 ($p<0.0001$, Fig. 1A) and low levels of FOXO3 ($p<0.0001$, Fig. 1B), FOXO4 ($p<0.0001$, Fig. 1C) or FOXO6 ($p=0.027$, Fig. 1D) are significantly associated with a worse prognosis. Interestingly, FOXO6 mRNA levels have a considerably lower capacity to separate patients according to survival than the rest of FOXOs (Fig. 1D). Given that FOXO expression is tissue-specific, and their functions can overlap, with one isoform potentially compensating for another, we assessed whether the combined expression of various FOXO isoforms was associated with patient prognosis. We observed that patients with poorer survival rates share the characteristic of having high mRNA levels of FOXO1, along with low levels of FOXO3 and FOXO4, irrespective of their FOXO6 levels (Suppl. Fig. 1A). We validated our findings using an independent dataset of glioma RNA-seq data (CGGA), comprising 1018 samples, and found that high expression of FOXO1 ($p<0.0001$, Suppl. Fig. 1B) or low expression of FOXO4 ($p<0.0001$, Suppl. Fig. 1D) was significantly associated with poorer patient survival. In contrast, transcript levels of FOXO3 ($p=0.25$, Suppl. Fig. 1C) and FOXO6 ($p=0.45$, Suppl. Fig. 1E) did not show a significant correlation with patient survival.

Next, we sought to investigate if Tribbles transcript levels would also be associated with different survival rates. We found that high mRNA levels of TRIB1 ($p<0.0001$, Fig. 1E), TRIB2 ($p<0.0001$, Fig. 1F) or TRIB3 ($p<0.0001$, Fig. 1G) were significantly associated with worse patient prognosis. Moreover, patients who expressed combined high levels of all three Tribbles members were associated with the worst survival rates (Suppl. Fig. 1F). Importantly, elevated mRNA expression of TRIB1 ($p<0.0001$, Suppl. Fig. 1G), TRIB2 ($p<0.0001$, Suppl. Fig. 1H), or TRIB3 ($p<0.0001$, Suppl. Fig. 1I) in CGGA patients was associated with significantly worse survival, aligning with our results from the TCGA dataset.

FOXO transcription factors are downstream effectors of the PI3K/AKT pathway whose activity is modulated by TRIB2 and/or TRIB3 levels in many cancers (Eyers et al., 2017). Therefore, we sought to investigate if combined expression of these proteins would have a major impact on prognosis in gliomas. Our analysis revealed that patients who simultaneously expressed high levels of FOXO1 and low levels of FOXO3/4 plus high levels of TRIB1/2/3 represent the worst prognosis group of gliomas patients, for both TCGA ($p<0.0001$, Fig. 1H) and CGGA ($p<0.0001$, Suppl. Fig. 1J) datasets. These findings suggest a potential functional interplay between FOXO isoforms and Tribbles family proteins in promoting glioma progression and highlight this specific molecular signature as a powerful prognostic indicator for poor clinical outcomes in glioma patients.

4.2. Independent glioma datasets validate the mRNA signature and its robustness

To further validate our findings, we analyzed two independent microarray datasets: REMBRANDT (GSE68848) and Gravendeel (GSE16011). In the REMBRANDT dataset, which includes 552 glioma samples, the expression patterns of FOXO family members showed to be consistent with the predictive signature described above. Specifically, elevated FOXO1 expression levels (p=0.032, Fig. 2A) and reduced levels of FOXO3 (p<0.0001, Fig. 2B), FOXO4 (p=0.00065, Fig. 2C), and FOXO6 (p=0.00042, Fig. 2D) were significantly associated with poorer patient prognosis. Regarding the Tribbles family members, high expression levels of TRIB2 (p=0.0091, Fig. 2F) and TRIB3 (p<0.0001, Fig. 2G) correlated with worse survival rates. However, no significant association was found between TRIB1 transcript levels and patient survival (p=<0.25, Fig. 2E). The combined expression of these proteins with the worst impact on prognosis revealed to be in the patients who simultaneously expressed low levels of FOXO1/3/4/6 plus high levels of TRIB1/2/3 (↓FOXOs/↑TRIBs) (p<0.0001, Fig. 2H).

In the Gravendeel microarray dataset, which includes 268 glioma samples, the association between expression levels and survival was consistent with our findings from the TCGA dataset. Specifically, low levels of FOXO3 (p<0.0001, Fig. 2J) and FOXO4 (p<0.0001, Fig. 2K), along with high levels of FOXO1 (p<0.0001, Fig. 2I), TRIB1 (p<0.0001, Fig. 2L), TRIB2 (p=0.00035, Fig. 2M) and TRIB3 (p=0.0024, Fig. 2N) are significantly correlated with poorer prognosis. Notably, this dataset did not include expression levels for FOXO6. For these samples, high levels of FOXO1 and low levels of FOXO4 plus high levels of TRIB1/3 and low levels of TRIB2 (↑FOXO1/↓FOXO4/↑TRIB1/3/↓TRIB2) represent the worst prognosis group of glioma patients (p<0.0001, Fig. 2O).

Taken together, these findings show that, despite some variability across different populations, patients with low FOXO3, FOXO4 and FOXO6, and with high FOXO1, TRIB1, TRIB2 and TRIB3 mRNA levels have significantly worse survival rates. Discrepancies among FOXO3, FOXO6 and TRIB1 suggest that further studies are needed to fully establish their relationship with glioma patient survival. Importantly, the data suggests that combined transcriptional profiles of FOXO and Tribbles family members could be used to predict the clinical outcome of gliomas patients.

4.3. High grade tumors express ↑ FOXO1/↓FOXO3/4/↑TRIB1/2/3 mRNA levels

Next, we sought to determine whether certain glioma tumor types were overrepresented among those exhibiting a high FOXO1 and high Tribbles expression levels accompanied by low FOXO3/4 expression levels. We found that the ↑FOXO1/↓FOXO3/4/↑TRIBs expression pattern was exclusively observed in patients diagnosed with glioblastoma (GB), or grade 4 astrocytoma (Fig. 3A and 3B). In fact, the majority of GB cases exhibited either the ↑FOXO1/↓FOXO3/4 or ↑TRIBs profile, or both. This finding suggests that this particular mRNA expression signature is strongly associated with higher-grade gliomas known to be associated with poorer prognosis and

diminished overall survival (Suppl. Fig. 2A). Consistently, we hypothesized that the mRNA levels of FOXO1, FOXO3, FOXO4, TRIB1, TRIB2, and TRIB3 varied according to tumor grade. To address this question, we evaluated the mRNA expression levels, from the TCGA database, of each individual FOXO or Tribbles gene between GB and other gliomas. We found that FOXO1 ($p<2.2e-16$, Fig. 3C), TRIB1 ($p<2.2e-16$, Fig. 3F), TRIB2 ($p<2.2e-16$, Fig. 3G) and TRIB3 ($p<2.2e-16$, Fig. 3H) are overexpressed in GBs compared to patients with other type of gliomas, whilst FOXO3 ($p=1.5e-09$, Fig. 3D) and FOXO4 ($p<2.2e-16$, Fig. 3E) mRNA levels are underexpressed.

4.4. FOXOs and Tribbles expression enable enhanced stratification of grade 4 glioma patients

The presence of the IDH mutation in gliomas is a favorable prognostic marker ($p<0.0001$, Fig. 4A) and targeted therapeutics are currently tested in clinical trials to treat patients with mutated IDH (Pirozzi & Yan, 2021). However, patients without mutations in the IDH genes (i.e. patients with GB) are of greater concern as they exhibit worse survival rates and face a lack of viable treatment options. Therefore, it is paramount to find new biomarkers that further stratify these patients and ultimately develop new drugs capable of targeting specific drivers of these tumors. We hypothesized that the signature characterized by high FOXO1 and low FOXO3/4 combined with high TRIB1/2/3 could be used to select patients who might benefit from therapeutics against these two new potential targets. The majority of patients with high FOXO1 and low FOXO3/4 signature, elevated TRIB1/2/3 levels, or both signatures combined, are IDH wild-type (Fig. 4B). This is unsurprising since these signatures are enriched in GB (Fig. 3A). These findings suggest patients that lack viable treatment options may benefit from evaluating FOXO and Tribbles protein levels as potential therapeutic markers

Accordingly, we aimed to analyze the robustness of the FOXO/Tribbles signature in stratifying GB patients, as well as grade 4 Astrocytomas. Given the aggressive nature of these high-grade tumors both patient groups typically receive similar treatment regimens, making this comparison particularly relevant (Mohile et al., 2022). We first analyzed the distribution of TCGA patients with grade 4 tumors (astrocytomas or glioblastomas) across different combinations of FOXO and Tribbles signatures. We found that approximately 70% of grade 4 glioma patients express high levels of TRIB1/2/3 and/or decreased FOXO3/4 levels compared to 30% of patients who show no alteration on mRNA levels of both TRIBBLEs and FOXO (Fig. 4C). This means that the majority of grade 4 patients, approximately 55%, would potentially benefit from Tribbles inhibition while 32% could benefit from FOXO modulation. Of these patients eligible for target therapy, 25% would profit from both FOXO and Tribbles therapeutics.

We next compared the effectiveness of the FOXO/Tribbles signature to that of well-established glioma biomarkers. One of the most common predictive biomarkers determined in GB patients is the methylation status of the MGMT promoter as it is associated with improved response to TMZ (Stupp et al., 2009). MGMT is a DNA repair enzyme that removes alkyl groups from the O6 position of guanine and, as a result, can reduce the effects of alkylating agents like TMZ. If MGMT promoter is silenced through methylation, tumor cells are less capable of

repairing the DNA damage caused by TMZ and thus, better overall survival compared to those with an unmethylated promoter (Hegi et al., 2005). Likewise, in astrocytomas MGMT promoter is frequently methylated, although its clinical relevance has not yet been as well established as in GB (Brat et al., 2021). In order to compare the predictive power of the MGMT methylation status versus the FOXO/Tribbles signature, we performed Kaplan-Meier analysis on both and analyzed their Concordance Index. The Concordance Index is an indicator of discrimination between the two parameters, the higher the value, the better the discrimination. For the MGMT methylation status, TCGA patients with grade 4 were separated into methylated and unmethylated (p=0.018, Fig. 4D). For the FOXO/Tribbles signature, TCGA patients with grade 4 were separated into patients hypothetically eligible for FOXO and/or Tribbles therapeutics against else/else subgroup of patients (p=0.023, Fig. 4E).

This analysis revealed that the FOXO/Tribbles signatures offered significantly better discrimination of grade 4 patients in terms of survival compared to MGMT methylation status. High FOXO1 and low FOXO3/4 signature, high levels of TRIB1/2/3, or both signatures combined, collectively show a median survival difference of 4.5 months compared to other signature combinations, with a Concordance Index of 0.55. This contrasts with the 2.7-month difference observed for MGMT methylation status (methylated vs. unmethylated), with a Concordance Index of 0.46.

Taken together, these results underscore the clinical relevance of the FOXO/Tribbles signature in selecting patients for potential targeted therapies. Notably, 70% of grade 4 gliomas exhibit either a high FOXO1 and low FOXO3/4 signature, elevated TRIB1/2/3 levels, or a combination of both—signatures that have a greater impact on patient survival compared to one of the most commonly used predictive biomarkers, MGMT methylation status. This highlights the potential of FOXO/Tribbles signatures as a more effective tool for guiding treatment strategies.

5. Discussion

GB are the most lethal primary brain tumors and are associated with poor response to treatments (Poon et al., 2020). A better understanding of the mechanisms responsible for the progression of the disease revealing therapeutic targets and biomarkers capable of stratifying patients are of utmost importance to improve the clinical outcome. In this study, we established a signature of members of the FOXO and Tribbles protein family members capable of predicting the survival of glioma patients. We analyzed four independent datasets: two RNA-seq datasets, one from TCGA (Weinstein et al., 2013) containing 698 samples and the other from CGGA (Zhao et al., 2021) with 1018 samples of glioma tumors, and two microarray datasets, the REMBRANDT (Madhavan et al., 2009) with 580 samples and the Gravendeel (Gravendeel et al., 2009) with 284. We used the TCGA datasets to discover a transcriptional signature that links poor survival of glioma patients to high expression of the transcription factor FOXO1 alongside low expression of FOXO3 and FOXO4, combined with high expression of the pseudokinases TRIB1, TRIB2 and TRIB3. This data is in line with the characterization of FOXO proteins as context

dependent tumor suppressor proteins (Dansen & Burgering, 2008) and studies reporting Tribbles proteins as oncogenic proteins (Richmond & Keeshan, 2020). Validation efforts using CGGA, REMBRANDT and Gravendeel data revealed the compelling association of Tribbles expression with poor clinical outcome and a dramatic effect of FOXO1 and FOXO4 but did not replicate the effect of FOXO3, FOXO6 and TRIB1, in every dataset. Ethnic differences and genetic backgrounds within the datasets could account for the different findings. In addition, the difference in the methodologies being used for sample processing and analysis across the several studies might explain inconsistencies. It is important to note that the tumor suppressor function of the members of the FOXO family of proteins is context dependent with significant oncogenic potential under certain circumstances (Hornsveld et al., 2018). In particular, a study showed that FOXO1 inhibition in GB triggers cancer cells apoptosis, demonstrating its oncogenic role in these tumors (Flores et al., 2023). Our data also highlight the non-redundant functions of the different FOXO isoforms. The mechanisms underlying isoform-specific functions of FOXO proteins are poorly understood but might be due to different structural features of these proteins or their levels of expression in different tissues (Santos et al., 2023). The observation that there is no clear trend among the levels of FOXO3 and FOXO6 in different grades of gliomas may be due to their low range of expression in these tissues. Accordingly, the predominant tumor suppressor effect of FOXO4 on the survival of glioma patients may reflect the prevalent FOXO4 expression over the other three isoforms in the human brain supportive cells (Fig. 2B-C and supplementary Fig. 2A-B). Interestingly, a recent study reported a downregulation of FOXO4 expression in GB tissues and cell lines (Qi et al., 2020). These authors also showed that ectopic FOXO4 overexpression significantly inhibited GB cell proliferation, migration, and invasion. In agreement with this data, we found FOXO4 levels correlated inversely with tumor grade. Similarly, we show a correlation increasing FOXO1, TRIB1, TRIB2 and TRIB3 expression with tumor grade. We previously established a similar correlation between the expression of TRIB2 with disease stage and clinical prognosis in melanoma patients (Hill et al., 2015). Interestingly, a recent study showed that a combined elevation of TRIB2 and MAP3K1 expression indicates poor prognosis and chemoresistance to TMZ in GB (Wang et al., 2020). Similarly, TRIB3 was shown to facilitate GB progression by restraining autophagy (Tang et al., 2020). Taken together, these observations with our data suggest that FOXO1, FOXO4, TRIB2 and TRIB3 are drivers of glioma progression and therefore should be considered as potential therapeutic targets for this disease. Although non-liganded transcription factors such as FOXO proteins and the pseudokinases of the Tribbles family have been considered undruggable (Passirani et al., 2022) recent progress provides a variety of strategies to modulate the activity of these proteins (Calissi et al., 2021; Erazo et al., 2016; Foulkes et al., 2018).

In summary, we provide evidence for a potent tumor suppressor role of FOXO4 and a significant oncogenic action of FOXO1 and Tribbles protein family in gliomas. To a lesser degree, the tumor suppressor role of FOXO3 and FOXO6, and oncogenic of TRIB1 might be important for predicting gliomas survival. The FOXO/Tribbles signature can be helpful to stratify patients with glioma predicting the clinical outcomes. An

improvement of 4.5 months in medium survival not only is significant but can be meaningful for these patients. We propose to explore the therapeutic potential of FOXO1, FOXO4, TRIB2 and TRIB3 as drug targets to improve the treatment of patients with glioma. Drugs targeting these novel targets, combined with the FOXO/Tribbles signature as companion biomarkers, hold the potential to enhance GB treatment outcomes while sparing patients from ineffective therapies. TRIB2 has been shown to be druggable by approved kinase inhibitors and in the last years several companies have emerged to develop FOXOs therapeutics. This suggests that our results can have an immediate impact by establishing new potential therapeutic targets for several cancer types, in particular gliomas. However, the clinical relevance of these observations and the mechanistic of these proteins in gliomas has yet to be fully established.

Acknowledgments: This work was supported by a grant from Spanish Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación and the European Regional Development Fund (PID2022-136654OB-I00 financed by MCIN/AEI /10.13039/501100011033 / FEDER, UE), to W.L., by Fundação para a Ciência e Tecnologia - FCT through the grant PTDC/MED-ONC/4167/2020 and Algarve Biomedical Center (ABC) to B.I.F. and the R&D Units funding RISE - LA/P/0053/2020 and UIDB/4255/2020 - CINTESIS to A.T.M., Algarve Biomedical Center (ABC) to B.I.F and by STRATAGEM COST Action, CA17104.

Conflict of Interest

W.L. is cofounder of Refoxy Pharmaceuticals GmbH, Cologne, Germany and required by his institution to state so in his publications. Ana Teresa Maia, co-founder and CEO of expressTEC, Lda, declares no conflict of interest related to this study. The other authors declare no conflict of interest.

Fig. 1| High FOXO1, low FOXO3/FOXO4, and elevated TRIB1/2/3 mRNA levels form a molecular signature predictive of clinical outcomes. A-G: Kaplan-Meier plots of FOXO and Tribbles for TCGA glioma samples. The lower quartile, in blue, and the upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **A:** FOXO1; **B:** FOXO3; **C:** FOXO4; **D:** FOXO6; **E:** TRIB1; **F:** TRIB2; **G:** TRIB3. **H:** Kaplan-Meier plot of a combination of high levels of FOXO1 and low levels of FOXO3/4 plus high levels of Tribbles for TCGA glioma samples. The median value was set as the cut-off. "Else" means every other possible combination of expressions than the stated in comparison. High FOXO1 and low FOXO3/4 mRNA levels combined with high levels of TRIB1/2/3 (↑FOXO1/↓FOXO3/4/↑TRIBs) are represented in pink. All p-values refer to log-rank test.

Fig. 2| REMBRANDT and Gravendeel microarray datasets validate the mRNA signature and its robustness. A-G: Kaplan-Meier plots of FOXO and Tribbles for REMBRANDT glioma samples. The lower quartile, in blue, and the

upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **A:** FOXO1; **B:** FOXO3; **C:** FOXO4; **D:** FOXO6; **E:** TRIB1; **F:** TRIB2; **G:** TRIB3. All p-values refer to log-rank test. **H:** Kaplan-Meier plot of a combination of low levels of FOXOs plus high levels of Tribbles for REMBRANDT glioma samples. The median value was set as the cut-off. "Else" means every other possible combination of expressions than the stated in comparison. Low FOXO1/3/4/6 mRNA levels combined with high levels of TRIB1/2/3 (↓FOXOs/↑TRIBs) are represented in pink. All p-values refer to log-rank test. **I-N:** Kaplan-Meier plots of FOXO and Tribbles for Gravendeel glioma samples. The lower quartile, in blue, and the upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **I:** FOXO1; **J:** FOXO3; **K:** FOXO4; **L:** TRIB1; **M:** TRIB2; **N:** TRIB3. All p-values refer to log-rank test. **O:** Kaplan-Meier plot of a combination of high levels of FOXO1 and low levels of FOXO4 plus high levels of TRIB1/3 and low levels of TRIB2 for Gravendeel glioma samples. The median value was set as the cut-off. "Else" means every other possible combination of expressions than the stated in comparison High FOXO1 and low FOXO4 mRNA levels combined with high levels of TRIB1/3 and low of TRIB2 (↑FOXO1/↓FOXO4/↑TRIB1/3/↓TRIB2) are represented in pink. All p-values refer to log-rank test.

Fig. 3| High grade tumors express ↑ FOXO1/↓FOXO3/4/↑TRIB1/2/3 mRNA levels. A: Percentage of the different types of tumors for each combination of high levels of FOXO1 and low of FOXO3/4 (↑FOXO1/↓FOXO3/4) and high levels of Tribbles (↑TRIBs) for TCGA glioma samples. "Else" means every other possible combination of expressions than the stated in comparison. **B:** Number of cases of the different types of tumors for each combination represented in **A**. **C-H:** Boxplots of FOXO and Tribbles mRNA levels among glioblastoma and other glioma types of TCGA samples. N represents the number of samples in each group. **C:** FOXO1; **D:** FOXO3; **E:** FOXO4; **F:** TRIB1; **G:** TRIB2; **H**: TRIB3. All p-values refers to ANOVA test. **** $p<0.001$

Fig. 4 – FOXOs and Tribbles expression enable enhanced stratification of grade 4 glioma patients. A: Kaplan-Meier plot of IDH mutation status for the TCGA glioma samples. Mutated in pink and wild type (wt) in blue. **B:** Percentage of patients with different IDH mutation status for each combination of high levels of FOXO1 and low of FOXO3/4 (↑FOXO1/↓FOXO3/4) and high levels of Tribbles (↑TRIBs) for TCGA glioma samples. "Else" means every other possible combination of expressions than the stated in comparison. **C:** Number of patients for each combination of high FOXO1 and low FOXO3/4 levels plus high levels of Tribbles for TCGA grade 4 glioma samples. **D:** Kaplan-Meier plot of MGMT promoter methylation status for the TCGA grade 4 glioma samples. Methylated in pink and unmethylated in blue. **E:** Kaplan-Meier plot of a combination of FOXOs and Tribbles levels in TCGA grade 4 glioma samples. Subgroups with high FOXO1 and low FOXO3/4 mRNA levels (↑FOXO1/↓FOXO3/4/else), high levels of TRIB1/2/3 (else/↑TRIBs), or both (↑FOXO1/↓FOXO3/4/↑TRIBs) represented together in pink. "Else" means every other possible combination of expressions than the stated in comparison. All p-values refer to log-rank test.

References

Brain, central nervous system. (2020). https://gco.iarc.fr/today

Brat, D. J., Ellison, D. W., Figarella-Branger, D., Hawkins, C. E., Louis, D. N., Ng, H. K., Perry, A., Pfister, S. M., Reifenberger, G., Soffietti, R., von Deimling, A., & Wesseling, P. (2021). *Central Nervous System Tumours* (WHO Classification of Tumours Editorial Board, Ed.). International Agency for Research on Cancer.

Brown, A. K., & Webb, A. E. (2018). Regulation of FOXO Factors in Mammalian Cells. *Curr Top Dev Biol*, *127*, 165–192. https://doi.org/10.1016/bs.ctdb.2017.10.006.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., & Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, *96*(6), 857–868. https://doi.org/10.1016/S0092-8674(00)80595-4

Calissi, G., Lam, E. W. F., & Link, W. (2021). Therapeutic strategies targeting FOXO transcription factors. *Nature Reviews. Drug Discovery*, *20*(1), 21–38. https://doi.org/10.1038/S41573-020-0088-2

Calnan, D. R., & Brunet, A. (2008). The FoxO code. *Oncogene*, *27*, 2276–2288. https://doi.org/10.1038/onc.2008.21

Colaprico, A., Silva, T. C., Olsen, C., Garofano, L., Cava, C., Garolini, D., Sabedot, T. S., Malta, T. M., Pagnotta, S. M., Castiglioni, I., Ceccarelli, M., Bontempi, G., & Noushmehr, H. (2016). TCGAbiolinks: An R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Research*, *44*(8), e71. https://doi.org/10.1093/NAR/GKV1507

Dansen, T. B., & Burgering, B. M. T. (2008). Unravelling the tumor-suppressive functions of FOXO proteins. *Trends in Cell Biology*, *18*(9), 421–429. https://doi.org/10.1016/j.tcb.2008.07.004

Eijkelenboom, A., & Burgering, B. M. T. (2013). FOXOs: Signalling integrators for homeostasis maintenance. *Nature Reviews Molecular Cell Biology*, *14*(2), 83–97. https://doi.org/10.1038/nrm3507

Erazo, T., Lorente, M., López-Plana, A., Munoz-Guardiola, P., Ferńandez-Nogueira, P., García-Martínez, J. A., Bragado, P., Fuster, G., Salazar, M., Espadaler, J., Hernández-Losa, J., Bayascas, J. R., Cortal, M., Vidal, L., Gascón, P., Gómez-Ferreria, M., Alfón, J., Velasco, G., Doménech, C., & Lizcano, J. M. (2016). The New Antitumor Drug ABTL0812 Inhibits the Akt/mTORC1 Axis by Upregulating Tribbles-3 Pseudokinase. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *22*(10), 2508–2519. https://doi.org/10.1158/1078-0432.CCR-15-1808

Eyers, P. A., Keeshan, K., & Kannan, N. (2017). Tribbles in the 21st Century: The Evolving Roles of Tribbles Pseudokinases in Biology and Disease. *Trends in Cell Biology*, *27*(4), 284–298. https://doi.org/10.1016/J.TCB.2016.11.002

Falcon, S., Morgan, M., & Gentleman, R. (2006). *An Introduction to Bioconductor's ExpressionSet Class*. http://bioconductor.org

Ferreira, B. I., Santos, B., Link, W., & De Sousa-Coelho, A. L. (2021). Tribbles Pseudokinases in Colorectal Cancer. *Cancers*, *13*(11). https://doi.org/10.3390/CANCERS13112825

Flores, D., Lopez, A., Udawant, S., Gunn, B., & Keniry, M. (2023). The FOXO1 inhibitor AS1842856 triggers apoptosis in glioblastoma multiforme and basal-like breast cancer cells. *FEBS Open Bio*, *13*(2), 352–362. https://doi.org/10.1002/2211-5463.13547

Foulkes, D. M., Byrne, D. P., Yeung, W., Shrestha, S., Bailey, F. P., Ferries, S., Eyers, C. E., Keeshan, K., Wells, C., Drewry, D. H., Zuercher, W. J., Kannan, N., & Eyers, P. A. (2018). Covalent inhibitors of EGFR family protein

kinases induce degradation of human Tribbles 2 (TRIB2) pseudokinase in cancer cells. *Science Signaling*, *11*(549). https://doi.org/10.1126/SCISIGNAL.AAT7951/SUPPL_FILE/AAT7951_SM.PDF

Gravendeel, L. A. M., Kouwenhoven, M. C. M., Gevaert, O., De Rooi, J. J., Stubbs, A. P., Duijm, J. E., Daemen, A., Bleeker, F. E., Bralten, L. B. C., Kloosterhof, N. K., De Moor, B., Eilers, P. H. C., Van Der Spek, P. J., Kros, J. M., Sillevis Smitt, P. A. E., Van Den Bent, M. J., & French, P. J. (2009). Intrinsic gene expression profiles of gliomas are a better predictor of survival than histology. *Cancer Research*, *69*(23), 9065–9072. https://doi.org/10.1158/0008-5472.CAN-09-2307

Hadley Wickham. (216 C.E.). ggplot2: Elegant Graphics for Data Analysis. *Journal of the Royal Statistical Society Series A: Statistics in Society*, *174*(1), 245–246.

Hegi, M. E., Diserens, A.-C., Gorlia, T., Hamou, M.-F., de Tribolet, N., Weller, M., Kros, J. M., Hainfellner, J. A., Mason, W., Mariani, L., Bromberg, J. E. C., Hau, P., Mirimanoff, R. O., Cairncross, J. G., Janzer, R. C., & Stupp, R. (2005). *MGMT* Gene Silencing and Benefit from Temozolomide in Glioblastoma. *New England Journal of Medicine*, *352*(10), 997–1003. https://doi.org/10.1056/NEJMoa043331

Hill, R., Kalathur, R. K. R., Callejas, S., Colaço, L., Brandão, R., Serelde, B., Cebriá, A., Blanco-Aparicio, C., Pastor, J., Futschik, M., Dopazo, A., & Link, W. (2014). A novel phosphatidylinositol 3-kinase (PI3K) inhibitor directs a potent FOXO-dependent, p53-independent cell cycle arrest phenotype characterized by the differential induction of a subset of FOXO-regulated genes. *Breast Cancer Research: BCR*, *16*(6). https://doi.org/10.1186/S13058-014-0482-Y

Hill, R., Kalathur, R. K. R., Colaço, L., Brandão, R., Ugurel, S., Futschik, M., & Link, W. (2015). TRIB2 as a biomarker for diagnosis and progression of melanoma. *Carcinogenesis*, *36*(4), 469–477. https://doi.org/10.1093/CARCIN/BGV002

Hong, B., Zhou, J., Ma, K., Zhang, J., Xie, H., Zhang, K., Li, L., Cai, L., Zhang, N., Zhang, Z., & Gong, K. (2019). TRIB3 promotes the proliferation and invasion of renal cell carcinoma cells via activating MAPK signaling pathway. *International Journal of Biological Sciences*, *15*(3), 587–597. https://doi.org/10.7150/IJBS.29737

Hornsveld, M., Dansen, T. B., Derksen, P. W., & Burgering, B. M. T. (2018). Re-evaluating the role of FOXOs in cancer. *Seminars in Cancer Biology*, *50*, 90–100. https://doi.org/10.1016/J.SEMCANCER.2017.11.017

Kassambara, A., Kosinski, M., & Biecek, P. (2024). Drawing Survival Curves using "ggplot2" [R package survminer version 0.5.0]. *CRAN: Contributed Packages*. https://doi.org/10.32614/CRAN.PACKAGE.SURVMINER

Link, W. (2015). Tribbles breaking bad: TRIB2 suppresses FOXO and acts as an oncogenic protein in melanoma. *Biochemical Society Transactions*, *43*, 1085–1088. https://doi.org/10.1042/BST20150102

Link, W. (2019). Introduction to FOXO Biology. *Methods in Molecular Biology (Clifton, N.J.)*, *1890*, 1–9. https://doi.org/10.1007/978-1-4939-8900-3_1

Link, W., & Fernandez-Marcos, P. J. (2017). FOXO transcription factors at the interface of metabolism and cancer. *International Journal of Cancer*, *141*, 2379–2391. https://doi.org/10.1002/ijc.30840

Machado, S., Silva, A., Sousa-Coelho, A. L. De, Duarte, I., Grenho, I., Santos, B., Mayoral-Varo, V., Megias, D., Sánchez-Cabo, F., Dopazo, A., Ferreira, B. I., & Link, W. (2020). Harmine and Piperlongumine Revert TRIB2-Mediated Drug Resistance. *Cancers*, *12*(12), 1–20. https://doi.org/10.3390/CANCERS12123689

Madhavan, S., Zenklusen, J. C., Kotliarov, Y., Sahni, H., Fine, H. A., & Buetow, K. (2009). Rembrandt: helping personalized medicine become a reality through integrative translational research. *Molecular Cancer Research: MCR*, *7*(2), 157–167. https://doi.org/10.1158/1541-7786.MCR-08-0435

Martins, R., Lithgow, G. J., & Link, W. (2016). Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. *Aging Cell*, *15*, 196–207. https://doi.org/10.1111/acel.12427

Mayoral-Varo, V., Jiménez, L., & Link, W. (2021). The Critical Role of TRIB2 in Cancer and Therapy Resistance. *Cancers*, *13*(11). https://doi.org/10.3390/CANCERS13112701

Mohile, N. A., Messersmith, H., Gatson, N. T., Hottinger, A. F., Lassman, A., Morton, J., Ney, D., Nghiemphu, P. L., Olar, A., Olson, J., Perry, J., Portnow, J., Schiff, D., Shannon, A., Shih, H. A., Strowd, R., van den Bent, M., Ziu, M., & Blakeley, J. (2022). Therapy for Diffuse Astrocytic and Oligodendroglial Tumors in Adults: ASCO-SNO Guideline. *Journal of Clinical Oncology*, *40*(4), 403–426. https://doi.org/10.1200/JCO.21.02036

Ohgaki, H., & Kleihues, P. (2007). Genetic Pathways to Primary and Secondary Glioblastoma. *The American Journal of Pathology*, *170*(5), 1445–1453. https://doi.org/10.2353/AJPATH.2007.070011

Orea-Soufi, A., Paik, J., Bragança, J., Donlon, T. A., Willcox, B. J., & Link, W. (2022). FOXO transcription factors as therapeutic targets in human diseases. *Trends in Pharmacological Sciences*. https://doi.org/10.1016/J.TIPS.2022.09.010

Paik, J.-H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J. W., Carrasco, D. R., Jiang, S., Gilliland, D. G., Chin, L., Wong, W. H., Castrillon, D. H., & Depinho, R. A. (2007). FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. *Cell*, *128*, 309–323. https://doi.org/10.1016/j.cell.2006.12.029

Passirani, C., Vessières, A., La Regina, G., Link, W., & Silvestri, R. (2022). Modulating undruggable targets to overcome cancer therapy resistance. *Drug Resistance Updates*, *60*, 100788. https://doi.org/10.1016/J.DRUP.2021.100788

Pirozzi, C. J., & Yan, H. (2021). The implications of IDH mutations for cancer development and therapy. *Nature Reviews Clinical Oncology 2021 18:10*, *18*(10), 645–661. https://doi.org/10.1038/s41571-021-00521-0

Poon, M. T. C., Sudlow, C. L. M., Figueroa, J. D., & Brennan, P. M. (2020). Longer-term (≥ 2 years) survival in patients with glioblastoma in population-based studies pre- and post-2005: a systematic review and meta-analysis. *Scientific Reports*, *10*(1). https://doi.org/10.1038/S41598-020-68011-4

Qi, M., Sun, L. A., Jiang, X. C., Han, Y. L., Wang, L., Niu, W. H., Fei, M. X., Zhaba, W. dui, Zheng, L. R., & Zhou, M. L. (2020). FOXO4 expression associates with glioblastoma development and FOXO4 expression inhibits cell malignant phenotypes in vitro and in vivo. *Life Sciences*, *247*. https://doi.org/10.1016/J.LFS.2020.117436

Richmond, L., & Keeshan, K. (2020). Pseudokinases: a tribble-edged sword. *The FEBS Journal*, *287*(19), 4170–4182. https://doi.org/10.1111/FEBS.15096

Salazar, M., Carracedo, A., Salanueva, Í. J., Hernández-Tiedra, S., Lorente, M., Egia, A., Vázquez, P., Blázquez, C., Torres, S., García, S., Nowak, J., Fimia, G. M., Piacentini, M., Cecconi, F., Pandolfi, P. P., González-Feria, L., Iovanna, J. L., Guzmán, M., Boya, P., & Velasco, G. (2009). Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. *The Journal of Clinical Investigation*, *119*. https://doi.org/10.1172/JCI37948

Santos, B. F., Grenho, I., Martel, P. J., Ferreira, B. I., & Link, W. (2023). FOXO family isoforms. *Cell Death & Disease*, *14*(10), 702. https://doi.org/10.1038/s41419-023-06177-1

Sean, D., & Meltzer, P. S. (2007). GEOquery: A bridge between the Gene Expression Omnibus (GEO) and BioConductor. *Bioinformatics*, *23*(14), 1846–1847. https://doi.org/10.1093/BIOINFORMATICS/BTM254

Stupp, R., Hegi, M. E., Mason, W. P., van den Bent, M. J., Taphoorn, M. J., Janzer, R. C., Ludwin, S. K., Allgeier, A., Fisher, B., Belanger, K., Hau, P., Brandes, A. A., Gijtenbeek, J., Marosi, C., Vecht, C. J., Mokhtari, K., Wesseling, P., Villa, S., Eisenhauer, E., … Mirimanoff, R.-O. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *The Lancet Oncology*, *10*(5), 459–466. https://doi.org/10.1016/S1470-2045(09)70025-7

Tang, Z., Chen, H., Zhong, D., Wei, W., Liu, L., Duan, Q., Han, B., & Li, G. (2020). TRIB3 facilitates glioblastoma progression via restraining autophagy. *Aging*, *12*(24), 25020–25034. https://doi.org/10.18632/AGING.103969

Therneau, T. M. (2024). Survival Analysis [R package survival version 3.8-3]. *CRAN: Contributed Packages*. https://doi.org/10.32614/CRAN.PACKAGE.SURVIVAL

Wang, J., Zuo, J., Wahafu, A., Wang, M. de, Li, R. chun, & Xie, W. fu. (2020). Combined elevation of TRIB2 and MAP3K1 indicates poor prognosis and chemoresistance to temozolomide in glioblastoma. *CNS Neuroscience & Therapeutics*, *26*(3), 297–308. https://doi.org/10.1111/CNS.13197

Weinstein, J. N., Collisson, E. A., Mills, G. B., Mills Shaw, K. R., Ozenberger, B. A., Ellrott, K., Shmulevich, I., Sander, C., & Stuart, J. M. (2013). The Cancer Genome Atlas Pan-Cancer analysis project. *Nature Publishing Group*. https://doi.org/10.1038/ng.2764

Wild, C. P., Weiderpass, E., & Stewart, B. W. (2020). World Cancer Report: Cancer Research for Cancer Prevention. In *International Agency for Research on Cancer 2020*. https://linkinghub.elsevier.com/retrieve/pii/S004578251000068X

Zakharova, G., Efimov, V., Raevskiy, M., Rumiantsev, P., Gudkov, A., Belogurova-Ovchinnikova, O., Sorokin, M., & Buzdin, A. (2023). Reclassification of TCGA Diffuse Glioma Profiles Linked to Transcriptomic, Epigenetic, Genomic and Clinical Data, According to the 2021 WHO CNS Tumor Classification. *International Journal of Molecular Sciences*, *24*(1), 157. https://doi.org/10.3390/IJMS24010157/S1

Zanella, F., Renner, O., García, B., Callejas, S., Dopazo, A., Peregrina, S., Carnero, A., & Link, W. (2010). Human TRIB2 is a repressor of FOXO that contributes to the malignant phenotype of melanoma cells. *Oncogene 2010 29:20*, *29*(20), 2973–2982. https://doi.org/10.1038/onc.2010.58

Zhao, Z., Zhang, K. N., Wang, Q., Li, G., Zeng, F., Zhang, Y., Wu, F., Chai, R., Wang, Z., Zhang, C., Zhang, W., Bao, Z., & Jiang, T. (2021). Chinese Glioma Genome Atlas (CGGA): A Comprehensive Resource with Functional Genomic Data from Chinese Glioma Patients. *Genomics, Proteomics & Bioinformatics*, *19*(1), 1–12. https://doi.org/10.1016/J.GPB.2020.10.005

Fig. Supl. 1| High FOXO1, low FOXO3/FOXO4, and elevated TRIB1/2/3 mRNA levels form a molecular signature predictive of clinical outcomes. A: Kaplan-Meier plot of all possible combinations of low and high levels of FOXOs for TCGA glioma samples. Median value was set as the cut-off Profiles with worse survival are highlighted in red. **B-E:** Kaplan-Meier plots of FOXO for CGGA glioma samples. The lower quartile, in blue, and the upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **B:** FOXO1; **C:** FOXO3; **D:** FOXO4; **E:** FOXO6. **F:** Kaplan-Meier plot of all possible combinations of low and high levels of Tribbles levels for TCGA glioma samples. Median value was set as the cut-off. Profile with worse survival is highlighted in red. **G-I:** Kaplan-Meier plots of Tribbles for CGGA glioma samples. The lower quartile, in blue, and the upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **G:** TRIB1; **H:** TRIB2; **I:** TRIB3. **J:** Kaplan-Meier plot of a combination of high levels of FOXO1 and low levels of FOXO3/4 plus high levels of Tribbles for CGGA glioma samples. The median value was set as the cut-off. "Else" means every other possible combination of expressions than the stated in comparison. High FOXO1 and low FOXO3/4 mRNA levels combined with high levels of TRIB1/2/3 (↑FOXO1/↓FOXO3/4/↑TRIBs) are represented in pink. All p-values refer to log-rank test.

Fig. Supl. 2| Kaplan-Meier plot of the different types of tumors for TCGA glioma samples. p-value refers to log-rank test.

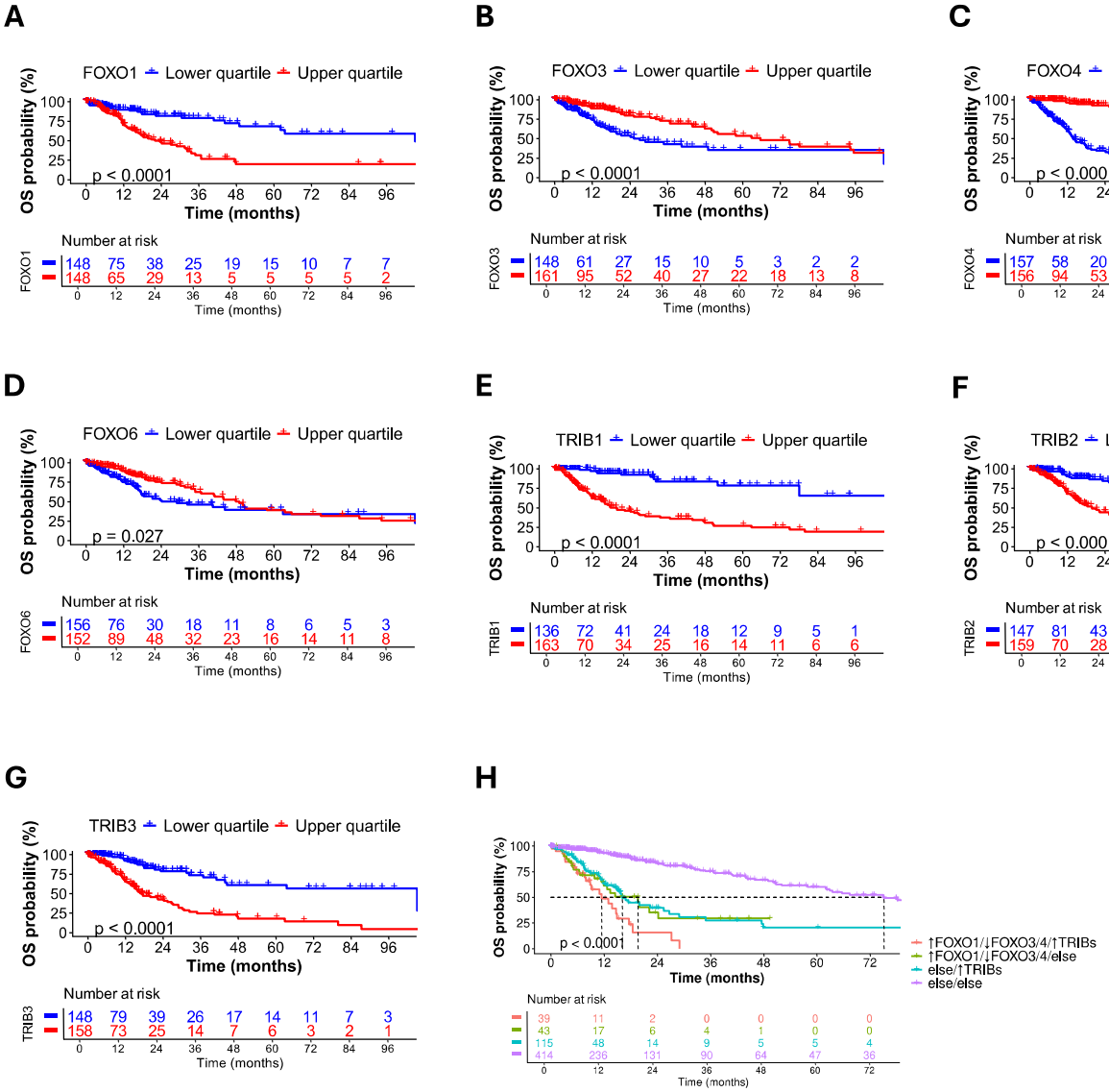


Fig. 1| High FOXO1, low FOXO3/FOXO4, and elevated TRIB1/2/3 mRNA levels form a molecular signature predictive of clinical outcomes. A-G: Kaplan-Meier plots of FOXO and Tribbles for TCGA glioma samples. The lower quartile, in blue, and the upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **A:** FOXO1; **B:** FOXO3; **C:** FOXO4; **D:** FOXO6; **E:** TRIB1; **F:** TRIB2; **G:** TRIB3. **H:** Kaplan-Meier plot of a combination of high levels of FOXO1 and low levels of FOXO3/4 plus high levels of Tribbles for TCGA glioma samples. The median value was set as the cut-off. "Else" means every other possible combination of expressions than the stated in comparison. High FOXO1 and low FOXO3/4 mRNA levels combined with high levels of TRIB1/2/3 (↑FOXO1/↓FOXO3/4/↑TRIBs) are represented in pink. All p-values refer to log-rank test.

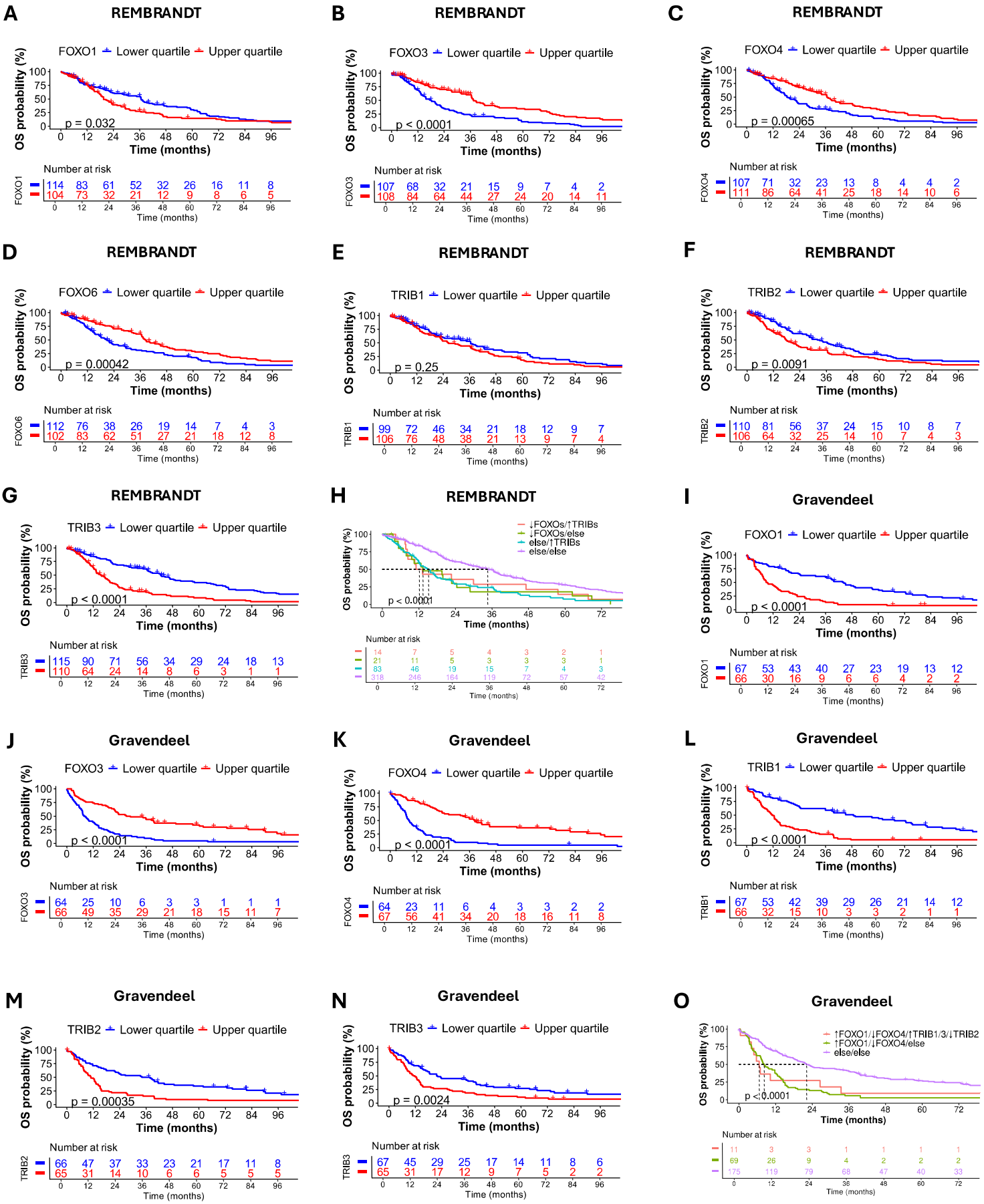
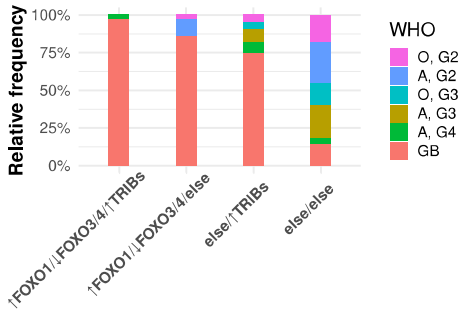


Fig. 2| REMBRANDT and Gravendeel microarray datasets validate the mRNA signature and its robustness. A-G: Kaplan-Meier plots of FOXO and Tribbles for REMBRANDT glioma samples. The lower quartile, in blue, and the upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **A:** FOXO1; **B:** FOXO3; **C:** FOXO4; **D:** FOXO6; **E:** TRIB1; **F:** TRIB2; **G:** TRIB3. All p-values refer to log-rank test. **H:** Kaplan-Meier plot of a combination of low levels of FOXOs plus high levels of Tribbles for REMBRANDT glioma samples. The median value was set as the cut-off. "Else" means every other possible combination of expressions than the stated in comparison. Low FOXO1/3/4/6 mRNA levels combined with high levels of TRIB1/2/3 (↓FOXOs/↑TRIBs) are represented in pink. All p-values refer to log-rank test. **I-N:** Kaplan-Meier plots of FOXO and Tribbles for Gravendeel glioma samples. The lower quartile, in blue, and the upper quartile, in red, were established as the cut-off for low and high expression levels, respectively. **I:** FOXO1; **J:** FOXO3; **K:** FOXO4; **L:** TRIB1; **M:** TRIB2; **N:** TRIB3. All p-values refer to log-rank test. **O:** Kaplan-Meier plot of a combination of high levels of FOXO1 and low levels of FOXO4 plus high levels of TRIB1/3 and low levels of TRIB2 for Gravendeel glioma samples. The median value was set as the cut-off. "Else" means every other possible combination of expressions than the stated in comparison High FOXO1 and low FOXO4 mRNA levels combined with high levels of TRIB1/3 and low of TRIB2 (↑FOXO1/↓FOXO4/↑TRIB1/3/↓TRIB2) are represented in pink. All p-values refer to log-rank test.

A



B

	O, G2	A, G2	O, G3	A, G3	A, G4	GB
↑FOXO1/↓FOXO3/4/↑TRIBs	0	0	0	0	1	41
↑FOXO1/↓FOXO3/4/else	1	5	0	0	0	37
else/↑TRIBs	5	1	5	8	8	84
else/else	75	107	65	90	15	59

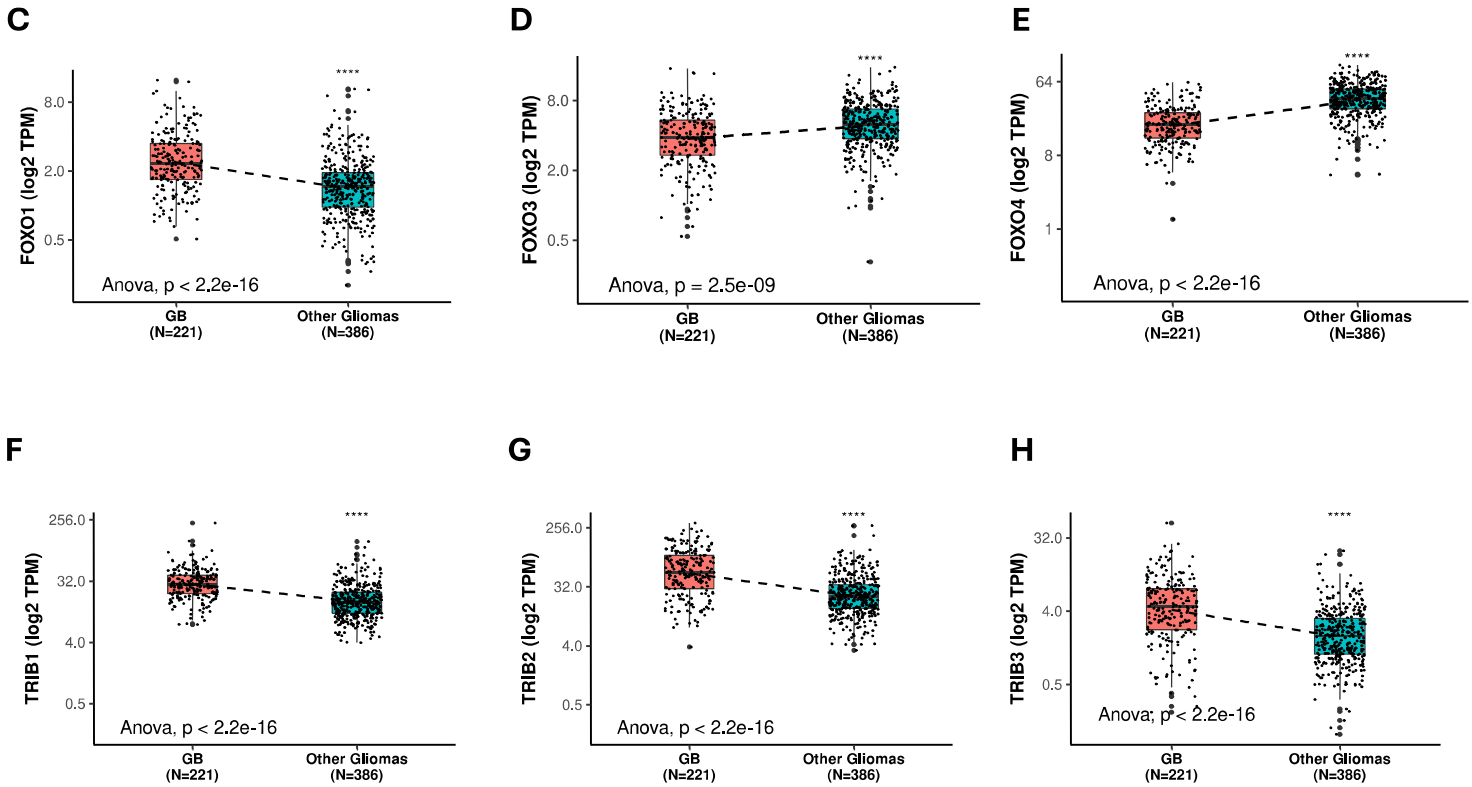


Fig. 3| High grade tumors express ↑ FOXO1/↓FOXO3/4/↑TRIB1/2/3 mRNA levels. A: Percentage of the different types of tumors for each combination of high levels of FOXO1 and low of FOXO3/4 (↑FOXO1/↓FOXO3/4) and high levels of Tribbles (↑TRIBs) for TCGA glioma samples. "Else" means every other possible combination of expressions than the stated in comparison. **B:** Number of cases of the different types of tumors for each combination represented in **A**. **C-H:** Boxplots of FOXO and Tribbles mRNA levels among glioblastoma and other glioma types of TCGA samples. N represents the number of samples in each group. **C:** FOXO1; **D:** FOXO3; **E:** FOXO4; **F:** TRIB1; **G:** TRIB2; **H**: TRIB3. All p-values refers to ANOVA test.

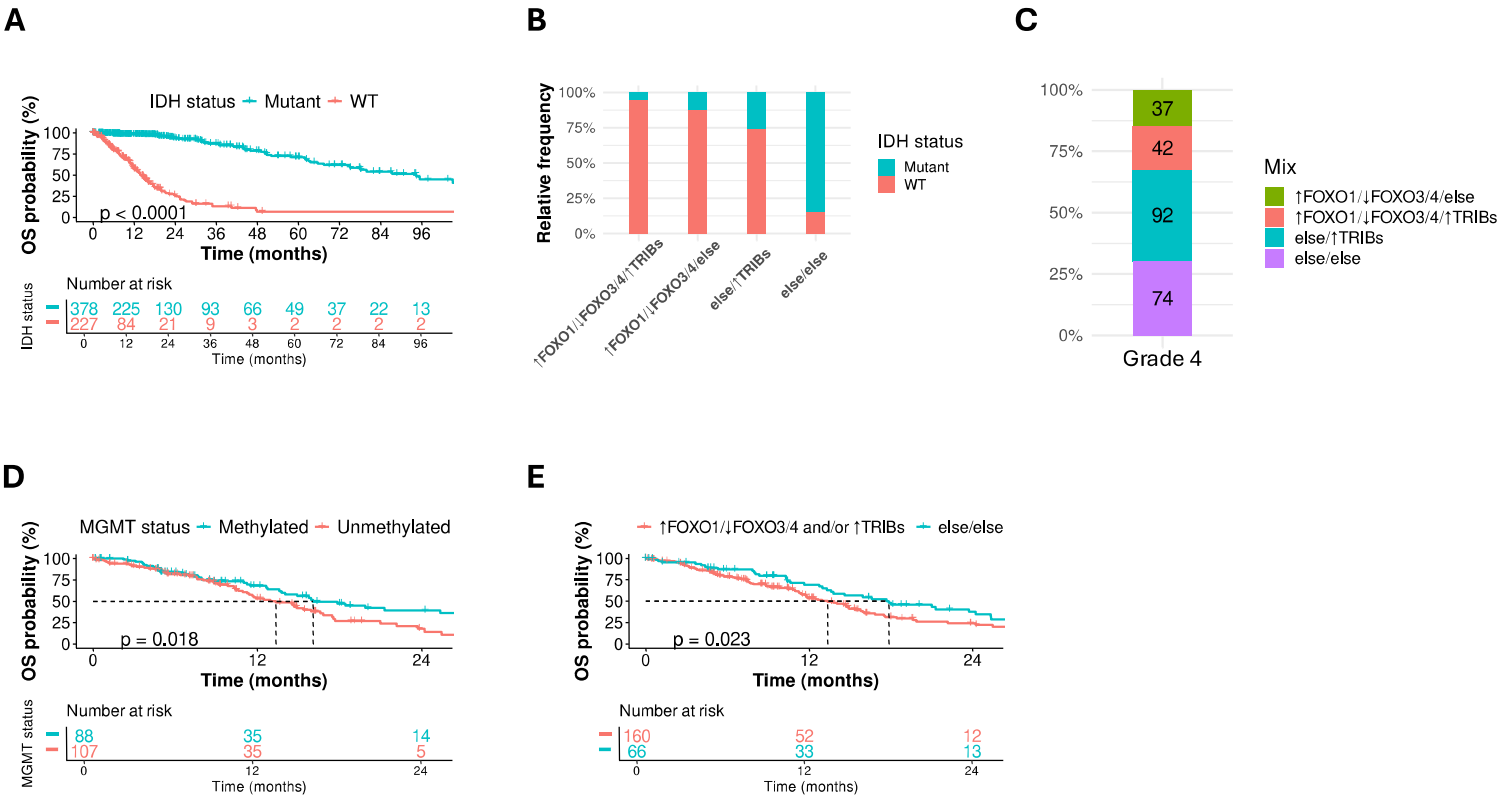


Fig. 4 – FOXOs and Tribbles expression enable enhanced stratification of grade 4 glioma patients. A: Kaplan-Meier plot of IDH mutation status for the TCGA glioma samples. Mutated in pink and wild type (wt) in blue. **B:** Percentage of patients with different IDH mutation status for each combination of high levels of FOXO1 and low of FOXO3/4 (↑FOXO1/↓FOXO3/4) and high levels of Tribbles (↑TRIBs) for TCGA glioma samples. "Else" means every other possible combination of expressions than the stated in comparison. **C:** Number of patients for each combination of high FOXO1 and low FOXO3/4 levels plus high levels of Tribbles for TCGA grade 4 glioma samples. **D:** Kaplan-Meier plot of MGMT promoter methylation status for the TCGA grade 4 glioma samples. Methylated in pink and unmethylated in blue. **E:** Kaplan-Meier plot of a combination of FOXOs and Tribbles levels in TCGA grade 4 glioma samples. Subgroups with high FOXO1 and low FOXO3/4 mRNA levels (↑FOXO1/↓FOXO3/4/else), high levels of TRIB1/2/3 (else/↑TRIBs), or both (↑FOXO1/↓FOXO3/4/↑TRIBs) represented together in pink. "Else" means every other possible combination of expressions than the stated in comparison. All p-values refer to log-rank test.