NOVEL RELATIONSHIPS OF SPLICING FACTORS AND REGULATION IN TMZ-RESISTANT GLIOBLASTOMA

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ABSTRACT
Glioblastoma (GBM) is a devastating cancer, due to both our narrow understanding of its molecular drivers and limited therapeutic strategies. The blood-brain barrier (BBB) limits the chemotherapeutic options for the treatment of GBM, but the DNA-damaging agent temozolomide (TMZ) can penetrate the BBB and is the first-line standard of care treatment together with surgery and radiation for this tumor. However, most patients’ tumors rapidly become resistant to TMZ, and TMZ-resistant GBM is uniformly fatal. The overall premise of my thesis research was to identify strategies for the second-line treatment of GBM through the study of models and mechanisms of TMZ resistance. To this end, I established two TMZ-resistant GBM cell line pairs. Molecular and phenotypic characterization these GBM TMZ-resistant cell lines indicated that one, 42MGBA-TMZ, captured the “grow” of the “go or grow” model for GBM-resistance. By contrast, the second, 8MBGA-TMZ, exhibited increased in migration, encompassing the “go” aspect of TMZ resistance. One potential mechanistic driver of TMZ resistance is alternative splicing, as the brain contains the most alternatively spliced transcripts of any organ, and the GBM tissue exhibits the deregulated expression of some splicing factors compared with healthy brain tissue. The orphan nuclear receptor - estrogen-related receptor beta (ERRβ) expresses multiple isoforms, and using the 42MGBA-TMZ and 8MBGA-TMZ, I assessed the expression and function of two ERRβ isoforms - ERRβ short form (ERRβ-sf) and ERRβ-2 in GBM. Although ERRβ is an orphan nuclear receptor with no known endogenous ligand, the synthetic agonist DY131 can potently activate the
receptor. Previous studies have shown ERRβ-2 to be pro-apoptotic when activated by DY131. I therefore used in silico and in vitro methods to determine proteins required for the expression of the pro-apoptotic ERRβ-2 isoform. I found that the serine/arginine (SR) rich splicing factor SRSF6 may function in the generation of the ERRβ-2 isoform, and that inhibition of cdc2-like kinases (CLKs, which phosphorylate SR proteins) with TG-003 in combination with the ERRβ synthetic agonist DY-131 potently inhibits GBM cell migration and growth in vitro. Overall, these studies add two new cell lines to the limited repertoire of TMZ-resistant GBM cell culture models and begin to define the importance of studying ERRβ isoform function and switching as a potential strategy to target TMZ-resistant GBM cells.
DEDICATION

I would like to dedicate this work to my family. My family has been my rock and cornerstone in all my academic endeavors, and they support me constantly and completely. Extending these thanks to all my family members and friends who have called with encouraging words or sent letters, this has truly changed my overall experience, and I am truly grateful to you for all the encouragement. I love and cherish you all.

Next, I would like to dedicate this work to all of those who have succumbed to both GBM and other cancers. To all my grandparents who have died of liver, lung, breast, and pancreatic cancers. Your dedication to raising my wonderful supportive family will never be forgotten. I would especially like to dedicate this to the family of Mr. Langendorf, and my dear friend Megan. I’m sorry I was unable to help you. I hope this work may be another chip away in assisting patients to a better tomorrow. Also, to my cousin Gracie, may you continue to prevail in your fight against brain cancer. I hope you know how much we are all fighting for and with you.
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1.1 Glioblastoma Treatment Options and Outcomes

Glioblastoma (GBM) is a fatal brain cancer that accounts for about 55% of all diagnosed gliomas¹. The cell type of origin of GBM is still a debated topic, but researchers hypothesize that discovering this will allow us to target tumor cells more effectively. Currently, the three cell types that are candidates due to their receptor markers, gene profiles, and in vivo genetic modeling that resemble GBM are neural stem cells (NSCs), NSC-derived astrocytes, and oligodendrocyte precursors. As these cells differ significantly in their functions within the CNS, attempting to determine the cell type of origin has not brought us closer to better treatments for GBM². Furthermore, our lack of understanding of both the biological basis and targetable dependent signaling pathways in GBM is apparent, as it was one of only four cancers that saw an increase in the estimated mortality rates in 2019³. The current median overall survival for patients newly diagnosed with GBM is about 15 months, with less than 5% surviving five years¹.

Since the 1960s, four FDA-approved first-line therapies have been approved for GBM that have increased median overall survival by about three months⁴. First was DNA-damaging radiation, then DNA-damaging lomustine⁵, followed by two different formulations of DNA-damaging carmustine (both intravenous) and wafers implanted into the affected brain tissue)⁶, and finally the current standard of care, the DNA-damaging temozolomide (TMZ)⁷. The treatment of many tumor-types uses radiation that breaks the DNA backbone phosphodiester bond, creating single and double-stranded DNA breaks⁸. Both lomustine and carmustine, nitrosourea
chemotherapeutics also damage DNA by acting as alkylating agents that target guanine deoxyribonucleotides.\(^9\)

TMZ is a prodrug that is activated in a more alkaline environment, which the brain provides. The prodrug spontaneously breaks down into highly reactive methyldiazonium cations that preferentially methylate DNA bases at the N7-guanine, N3-adenine, and O6-guanine positions. The most cytotoxic adduct is the latter, which can be reversed by the O6-methylguanine methyltransferase (MGMT). MGMT is a suicide DNA repair protein that can remove the O6-guanine adduct, allowing for proper DNA repair.\(^10\) Cytosine-phosphate-guanine (CpG) islands in the promoter of MGMT are heavily methylated in healthy brain tissue, repressing its expression.\(^11\) However, after TMZ treatment, tumor cells show loss of methylation at the MGMT promoter.\(^12\) The resulting expression of MGMT blocks the DNA damaging effects of O6-guanine adducts, and patients stop responding to TMZ treatment.\(^12\) Accordingly, a logical second-line treatment would be to target and inhibit MGMT to allow for an extended response to TMZ. Despite considerable efforts, the results of studies of MGMT inhibition have proven underwhelming.\(^13\) Other available “targeted” options for the treatment of GBM are the monoclonal antibody against VEGF, Avastin, and a noninvasive extracorporeal tumor treating wave treatment that disrupts microtubules within the brain.\(^14\) However, neither of these show a significantly better overall survival than TMZ and cost substantially more.\(^14\) Therefore, the current standard of care includes surgery, radiation, and TMZ until failure. TMZ failure happens quickly, and with no useful second-line therapeutic option, resistance to TMZ is uniformly fatal.
The lack of targeted therapies for GBM to complement or replace broad DNA-damaging agents like TMZ is not for lack of trying. Thousands of patients have succumbed to the disease while testing a multitude of targeted agents in over 400 clinical trials\textsuperscript{15}. So, what makes this cancer so resistant to successful intervention? GBM differs from most cancers as it disseminates individual cells and extends long finger-like projections within the brain, making complete resection nearly impossible. Efficiency of removing bulk tumor area does correlate with longer overall survival, but even when most of the tumor is removed, disseminated cells throughout the brain are left behind, and the outcome is tumor recurrence\textsuperscript{4}. The second impediment unique to GBM treatment is a physical barrier to treatment: the blood-brain barrier (BBB). The BBB is a necessary barrier to protect the brain from everyday insults and is covered in transporters that pump out toxins, which include most chemotherapeutic agents. Countless studies have shown the strength of the BBB in preventing drugs from passing through this rigorous first-line defense\textsuperscript{16}. Therefore, a successful first or second-line therapy must be able to traverse the BBB and not destroy it in the process\textsuperscript{17}.

1.2 Splicing Regulation

The discovery of the human genome changed our perception of cell biology. It was clear that a code was orchestrating all the moving parts within a cell. From this genome code came the central dogma of biology: DNA $\rightarrow$ RNA $\rightarrow$ Protein\textsuperscript{18}. However, this incorrectly suggests the stoichiometry is 1:1:1. In 1977, Phil Sharp and Robert Roberts discovered “split genes”, or genes that contained more DNA sequence than was present in their coded RNA sequence\textsuperscript{19,20}. They had discovered splicing. Splicing is the process of multiple ribonucleoproteins coming together to form the spliceosome (Figure 1-1). This large machine of RNA and protein work with other splicing factors
to dictate the final mRNA transcript. There are three necessary sequences in an intron to alert the spliceosome for its removal, the 5’ GU, branch point A, and 3’ AG. The U1 splicing small nuclear ribonucleoproteins (snRNP) scans the intron to find the 5’ GU where the G is cleaved by transesterification and attaches to the branch point A, forming an intron lariat. Next the U2 and U4/6 snRNPs come in to define the 3’ AG and assist in the transesterification of the 3’ end with the U5 snRNP. The intron is then released with U2 U5/6 snRNPs still attached\textsuperscript{21}. While this intron lariat used to be thought of as “junk DNA” this theory has recently been disproven with stable introns being polyadenylated and necessary for yeast growth regulation in saturated conditions\textsuperscript{22}. In this way, one multi-exon gene can become multiple RNA products.

However, genes can also be alternatively spliced, which allows for multiple proteins to be produced from a single gene. \textit{Cis-} sequences can act as enhancers or silencers for nearby exon inclusion or exclusion\textsuperscript{23}. These sequences can be defined through bioinformatic motif recognition, where both exonic and intronic splicing \textit{cis}-sites have been defined for the serine/arginine rich (SR) protein family\textsuperscript{24}. These enhances can also be antagonized by \textit{cis}-repressor sequences of which the heterogenous nuclear ribonucleoprotein particles (hnRNPs) are a major family that bind these elements\textsuperscript{25}. While the SR and hnRNP families can regulate isoform fate across many diverse tissues, tissue-specific RNA binding proteins also play an important role in determining the final protein product. Accessory non-spliceosomal RNA binding proteins, like NOVA or Rbfox3 in the brain, can act as either activators or suppressors of alternative splicing depending on both the location of the binding site, and their overall abundance compared to other RNA binding proteins\textsuperscript{26}. The regulation of alternative splicing is incredibly complex, and further study will continue to advance our understanding of how it contributes to both normal biology and the disease state.
Figure 1-1. Splicing schematic. Top of the figure shows exons as boxes and a line depicting an intron which is excised over the course of the splicing reactions below. The splicing small nuclear ribonucleoproteins (snRNPs) associate with the pre-mRNA in a step-wise sequence to form the spliceosome. Two transesterification reactions happen to excise out the intron and join the exons together. Adapted from 27
1.3 Role of the Estrogen-related Receptor Beta in Glioblastoma

One understudied protein that may function in GBM biology is the orphan nuclear receptor estrogen-related receptor beta (ERRβ). A previous study showed an association of shorter overall survival in GBM patients with deletion of the region of chromosome 14 (14q24.3) that includes the ESRRB locus (Figure 1-2A). There are three known isoforms of the ERRβ protein; ERRβ-sf, ERRβ-2, and ERRβ-Δ10 encoded by three transcript variants (Figure 1-2B). The ERRβ-sf transcript variant encompasses ESRRB exons 3-9 and part of intron 9. The ERRβ-2 variant consists of ESRRB exons 1-11. The ERRβ-Δ10 variant includes ESRRB exons 3-9, skips exon 10, and then has a frameshift from the β2 variant to include distinct ESRRB exons 11 and 12. Interestingly, while ERR-βsf is evolutionarily well-conserved, only primates express the ERRβ-2 and ERRβ-Δ10 transcript variants and isoforms.
Figure 1-2. ERRβ transcript variants and isoforms. Adapted from 28A. The ESRRB gene chromosome location is 14q24.3 with the known splicing diagram shown. B. Splicing diagrams of each known ERRβ mRNA with UCSC Genome Browser identifiers to the left of each transcript. C. Amino acid (aa) differences between the three known isoforms with the Online Mendelian Inheritance in Man (OMIM) identifiers to the left. D. Functional domains of each isoform where amino acids 1-432 encode well-established DNA binding domain (DBD) and ligand-binding domains (LBD). The amino acid and structural predictions of the divergent F domains in ERRβ-2 and ERRβ-Δ10 are predicted by Phyre30.
Two of the ERRβ protein isoforms – ERRβ-sf and ERRβ-Δ10 – localize in the nucleus and bind DNA to activate transcription of downstream target genes. In contrast, ERRβ-2 resides in both the nucleus and cytoplasm, and antagonizes the transcription factor activity of ERRβ-sf. ERRβ is an orphan nuclear receptor, meaning its endogenous ligand is unknown. DY131 (DY) is a small molecule that can bind and activate ERRβ. Treatment with DY in a ERRβ-sf-dependent system elicits a G1 arrest and senescence, whereas in a ERRβ-2-dependent system it elicits a G2/M arrest and apoptosis. Creation of these ERRβ-sf- and ERRβ-2-dependent systems required small hairpin RNA (shRNA) to deplete each isoform, an approach that has proven difficult to translate to the clinical setting. An alternative approach, currently under investigation, is the use of splicing modulatory drugs to alter the processing of the pre-mRNA and favor the expression of a specific transcript variant and thus a particular isoform.

1.4 Targeting Alternative Splicing through cdc2-like Kinase Inhibition

The processing of nascent pre-mRNAs is a tightly regulated process that requires cooperation between the core splicing factors and accessory proteins required for alternative splicing. The serine/arginine-rich (SR) proteins contain a protein domain rich in arginine and serine dipeptides (RS), as well as at least one RNA recognition motif (RRM). The role of SR proteins in alternative splicing is complex and position-dependent. SR proteins can bind enhancer splicing elements within exons to facilitate exon inclusion, or they may bind introns and act as a suppressor. SR proteins undergo many changes in serine phosphorylation that determine sub cellular localization. SR phosphorylation also mediates SR protein interaction with the mRNA transport machinery, and affects downstream splicing regulation. One group of kinases that facilitate nuclear hyperphosphorylation of SRs is the cdc2-like kinase (CLK) family. This
hyperphosphorylation releases SR proteins from nuclear speckles/cajal bodies, allowing them to bind pre-mRNAs and dictate transcript fate\textsuperscript{41}. The CLK family is composed of four members (CLK1-4) known as dual-specificity kinases as they are able to phosphorylate serine, tyrosine, and threonine residues on substrate proteins\textsuperscript{42}. The founding member, CLK1, was originally discovered through its ability to autophosphorylate itself on tyrosine residues\textsuperscript{43}. As kinases are a viable therapeutic target in several cancers, multiple groups have discovered or designed CLK family inhibitors for potential cancer therapeutics\textsuperscript{44-46}. These tool compounds have facilitated greater insight into the role of CLKs in alternative splicing. Both CLK inhibitors and RNAi-mediated depletion of CLKs modify alternative splicing, specifically an increase in skipped exons\textsuperscript{47,48}. Genetic analyses of cancer have identified increased alternative splicing in many contexts, and therefore targeting the CLK family may provide therapeutic benefits in the future\textsuperscript{49}.

1.5 Research Goals

The goal of this research was to examine essential and targetable pathways that arise after TMZ treatment and resistance has occurred in GBM. GBM has a low survival rate and is a universal death sentence with few therapeutic options. We still do not understand what causes GBMs to form, early detection is rare, and resistance to first-line therapy is rapid. Therefore, I chose to focus on identifying pathways that can be targeted, like alternative splicing control, for a potential therapeutic benefit in the future. I first established two novel, isogenic models of TMZ-resistant GBM, characterizing the changes in chromosome number, cell growth, and motility in the resistant vs. sensitive parental cells (Chapter 2). I then used these and a pre-existing cell model with \textit{de novo} resistance to TMZ to better define the role of the pro-apoptotic ERRβ-2 isoform in GBM (Chapter 3)
CHAPTER 2
ALTERATIONS IN CHROMOSOME NUMBER, CELL MOTILITY, AND PROLIFERATION IN NOVEL MODELS OF ACQUIRED TEMOZOLOMIDE RESISTANT GLIOBLASTOMA

2.1 Rationale

Glioblastoma (GBM) is the most common glioma among adults and confers an abysmally low overall survival with only 5% of patients surviving at the 5-year mark\(^1\). One reason may be that resistance to the standard of care (Temozolomide, TMZ) is rapid, and there is no effective second line of treatment option\(^5\). Cell line models have been invaluable in elucidating the molecular mechanisms behind the uncontrolled growth of cancer cells. As resistance to TMZ is rapid in clinical models, cell lines were created to observe phenotypic changes and attempt to better understand the mechanism behind the initial efficacy of TMZ sensitivity and changes that occur with acquired TMZ-resistance. In this study, we present two unique TMZ-resistant GBM cell lines derived from the 42MGBA and 8MGBA parental lines. First characterized in 1997, the 42MGBA line was derived from a temporal lobe tumor resected from a 63-year old male, and the 8MGBA line was derived from a frontal lobe tumor resected from a 54-year old female\(^5\). After continual exposure to TMZ in culture, both cell lines no longer undergo TMZ-mediated G2/M arrest. As the cell lines acquired resistance to TMZ, both cell lines gained expression of MGMT, showed an increase in nuclear size, chromosome number, and early endosomes. However, as they differed in their growth and migratory capacity, these cell lines offered insights into the “go or grow” model for GBM recurrence\(^5\). This “go or grow” model has been suggested as the driver of two distinct outcomes of GBM resistance, where cells that no longer respond to TMZ either have increased migratory capacity (“go”) or increased proliferation (“grow”) \(^5\).

**Citation:** Tiek, DM *et al.* Alterations in cell motility, proliferation, and metabolism in novel models of acquired temozolomide resistant glioblastoma. *Scientific Reports.* 8, 7222 (2018).
The 42MGBA-TMZ resistant cell line had an increase in growth capturing the “grow” of the “go or grow” model for resistance. By contrast, the 8MBGA-TMZ resistant cell line had an increase in migration, encompassing the “go” aspect of resistance. These TMZ-resistant models from both a male and female patient have evolved distinct resistant phenotypes and therefore serve as two new resources to investigate the molecular mechanisms of acquired TMZ-resistance.

2.2 Results

2.2.1 Acquired temozolomide resistance decreases sensitivity to BCNU

To recapitulate acute temozolomide (TMZ) resistance, we generated cell lines that were resistant to 200 µM TMZ and were challenged in 100 µM TMZ for all subsequent experiments. The overall time to acquired resistance varied from approximately two months for the 8MBGA cell line to about three months for the 42MBGA cell line. Cells were defined as resistant when each cell line no longer showed a G2/M arrest or cell death, shown here as sub-G1 fragmented DNA content, in response to TMZ treatment (Figure 2-1a-d). While both parental cell lines (WT) exhibited a significant increase in the sub-G1 fraction following TMZ treatment, the TMZ-res variants lost this sensitivity to drug (Figure 2-1e). However, the shape of the sub-G1 curves differed between the two cell lines – 8MGBA-WT cells treated with TMZ showed a defined sub-G1 peak, classically associated with caspase-mediated apoptosis, while 42MGBA-WT cells did not (Sup Figure 2-1a, b). This was corroborated by immunoblotting for cleaved poly (ADP-ribose) polymerase (PARP), a second indicator of caspase-mediated apoptosis, which was observed in 8MGBA-WT but not 42MGBA-WT cells (Figure 2-1g, h). We detected expression of the O6-methylguanine methyltransferase (MGMT) in both resistant cell lines, but not in the parental lines (Figure 2-1f).

Acquired resistance to the previous standard of care for GBM, bis-chloroethylnitrosourea
(BCNU), leads to increased sensitivity to TMZ\textsuperscript{54}. Because BCNU can still be a component of second-line treatment for GBM\textsuperscript{55-58}, we tested whether our TMZ-res cells exhibited altered responsiveness to BCNU. The TMZ-res cells still showed a statistically significant G2/M arrest upon BCNU treatment. However, the biological effect is dramatically reduced, with a 74% increase in G2/M versus an 8% increase in G2/M for the 42MBGA-WT versus 42MBGA-TMZ-res cells compared to controls, respectively (Figure 2-1a, b). We observed a similar response in the 8MGBA-WT and 8MGBA-TMZ-res cells, where the increase in G2/M fraction was 86% versus 7% compared to controls, respectively (Figure 2-1c, d). Overall we show that our two new acquired TMZ-resistant models no longer respond to TMZ treatment and now express MGMT, the current clinical marker of resistance.

\subsection*{2.2.2 Increase in nuclear size and chromosome number with TMZ resistance}

The mechanism of action for TMZ is to induce DNA damage and give rise to cell cycle arrest in the G2/M phase\textsuperscript{50}. As cells have already copied their DNA at this point in the cell cycle, if a cell is to survive TMZ-treatment they may also be retaining the extra copies of chromosomes that have already been duplicated. The 42MGBA-WT cell line has a hyper-tetraploid karyotype (88-95 chromosomes) with 8% polyploidy, while the 8MGBA-WT cell line has a hyper-diploid karyotype with 15% polyploidy and contains 47-52 chromosomes (DSMZ, https://www.dsmz.de/). 42MGBA-TMZ-res cells showed a significant increase in total nuclear area (Figure 2-2a), and their nuclear morphology became multi-lobed (Figure 2-2b). Total nuclear area was also significantly increased in 8MGBA-TMZ-res cells, though their nuclei retained an oblong or circular morphology (Figure 2-2a, b).

To determine whether the increase in the total nuclear area in TMZ resistant cells was
associated with chromosomal gain, we assessed the overall chromosomal number in metaphase spreads from the TMZ-res cell lines compared to their respective parental lines (WT). We observed an increase in overall chromosome number for most of the 42MBGA-TMZ-res cell population, in contrast to the 8MBGA-TMZ-res cells where only a small subpopulation of cells had an increase in overall chromosome number (Figure 2-3b). Since the distribution of chromosome number we observe in the TMZ-res cells is not completely encompassed within their parental counterparts, I hypothesize that there are now distinct TMZ-res populations that have emerged as resistance evolved. However, it is worth noting that where overlap is present, there may have been enrichment of a pre-existing TMZ-resistant population. We also assessed the change in copy number of a representative chromosome in each cell line pair (chromosome 17 in the 8MGBA pair and chromosome X in the 42MGBA pair), in interphase nuclei and metaphase spreads (200/cell line) using fluorescent in situ hybridization (FISH) (Figure 2-3a). The choice of the two representative chromosomes was made based on reported karyotype analysis of the two parental cell lines, which show a mostly diploid count for chromosome 17 in the 8MGBA line and the X chromosome in 42MGBA (DSMZ, https://www.dsmz.de/). We observed that 96% of the 42MBGA-TMZ-res cells had three or more copies of the X chromosome compared to only 7% of the 42MBGA-WT cells (93% of those cells had two copies). In contrast, this dramatic shift was not observed in 8MBGA-TMZ-res cells, where only a small subpopulation of cells showed an increase in the number of chromosomes 17 (18% had 3 or more copies) compared to the parental cells (6% had 3 or more copies).
2.2.3 Changes in proliferation, migration, and actin cytoskeleton

We then determined how TMZ-resistance affected cell size and proliferative versus migratory phenotypes. 42MBGA-TMZ-res cell size was not changed vs 42MGBA-WT, though their basal growth rate was dramatically increased (Figure 2-4c; Sup Figure 2-3a, b). They also showed a modest numeric but nonsignificant reduction in cell migration (Figure 2-4a, images in Sup Figure 2-4). In contrast, 8MBGA-TMZ-res cell size was significantly increased when compared to its parental cell line, while the basal growth rate was unchanged (Figure 2-4d; Sup Figure 2-3a, b). 8MGBA-TMZ-res cells were significantly more migratory than 8MGBA-WT cells (Figure 2-4b). Enhanced cell migration correlated with increased F-actin stress fiber thickness in both TMZ-res models. There was no significant change in F-actin thickness in the 42MBGA-TMZ-res compared to 42MGBA-WT cells, while it was significantly increased in the more migratory 8MGBA-TMZ-res when compared to 8MBGA-WT cells (Figure 2-4e, f).

2.2.4 TMZ resistance leads to an increase in intracellular vesicles

An advantage of selecting these resistant models was the ability to observe changes that occurred during and continued to persist after resistance was acquired. One observation was the number of intracellular puncta and extracellular vesicles that were expelled into the media as the cells became resistant to TMZ (Figure 2-5c). As there was no longer a dramatic increase in the sub-G1 fraction (Figure 2-1e, Sup Figure 2-1), or cleaved PARP (Figure 2-1h) upon resistance, we concluded that these were not apoptotic bodies. We therefore wanted to test if there was an increase in overall vesicle production in the TMZ-res vs WT cell lines. To accomplish this, we stained for the endosomal marker early endosome antigen 1 (EEA1). We observed a significant increase in the total number of endosomes in both TMZ-res cell lines compared to their parental lines (Figure 2-
Interestingly in two independent datasets – the Chinese Cancer Genomics Consortium (CCGC) and the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) – *EEA1* expression is increased in GBM versus healthy brain tissue, and survival is significantly enhanced for patients with tumors that express lower levels of *EEA1* (Figure 2-5d-f).
2.3 Figures

**Figure 2-1. Acquired TMZ resistance.** a and c) Cell cycle analysis of the parental 42MGBA (a) and 8MGBA cell lines (c) with 100 μM TMZ and 50 μM BCNU treatment for 72 hr. One-way ANOVA p = 0.0003; p = <0.0001. b and d) Cell cycle analysis of 42MGBA- (b) and 8MBGA-TMZ-res cell lines (d) with 100 μM TMZ and 50 μM BCNU treatment for 72 hours. One-way ANOVA p = <0.0001; p = 0.0010. e) Cell cycle analysis of sub-G1 fraction for parental and resistant cell lines. t-test of treatment versus DMSO 42MGBA-WT p = 0.0046, 42MBGA-TMZ-res p = 0.0065, 8MBGA p = 0.0011. f) TMZ-res cells express MGMT. g and h) TMZ and BCNU induce PARP cleavage in 8MGBA-WT cells, but not 8MGBA-TMZ-res, 42MGBA-WT, or 42MGBA-TMZ-res cells. Cells were treated for 72 hours prior to lysis and Western blot analysis. All experiments are representative of three biological replicates.
Supplementary Figure 2 - 1. Gating and sub-G1 peak for cell cycle analysis for a) 42MBGA-WT and –TMZ-res; b) 8MBGA-WT and –TMZ-res. M1 is the cut-off for sub-G1 fragmented DNA; red box. M2 shows gating of live cells, with the first peak being the G1 fraction; blue box. The second peak represents the G2 fraction; black box, with the space in the middle showing S phase content; green box. All experiments are representative of three biological replicates.
Figure 2-2. Changes in nuclear size and shape upon acquired TMZ resistance. a) Quantification of nuclear area by pixel size. AU = arbitrary units. Two-tailed t-test 42MBGA p = 0.0035; 8MBGA p = 0.0034. b) Representative image of nuclear size, quantified in a. Change in nuclear structure in 42MGBA-TMZ-res in comparison to 42MBGA-WT. All experiments are representative of three biological replicates.
Acquired TMZ resistance is associated with chromosomal copy number increase. a) Top four panels: interphase nuclei from TMZ-res cells showing multiple copies of chromosomes 17 (8MGBA-TMZ-res, red signal) and X (42MGBA-TMZ-res cells, green signal) and two copies in the respective parental cells. Bottom four panels: metaphase spreads from TMZ-res cells showing overall chromosomal copy number gain compared to parental cells, and multiple copies of chromosomes 17 (8MGBA-TMZ-res, red signal, arrows) and X (42MGBA-TMZ-res, green signal, arrows). Metaphase spreads from the parental cells show 2 copies of the respective chromosomes. b) Quantification of chromosomes from a, bottom 4 panels 42MGBA-WT vs –TMZ-res p = <0.0001. c) Quantification of probe signal from a, top 4 panels. Chi-squared test 8MGBA p = 0.03; 42MGBA p = <0.0001.
Supplementary Figure 2-3. Cell size and proliferation rates. a) Cell size in microns measured by the Countess 2. t-test 8MBGA p = 0.0121 b) Crystal Violet assay of basal proliferation. Area under the curve 42MBGA-TMZ-res p = <0.0001. All experiments are representative of three biological replicates.
Figure 2-4. Changes in cell growth, migration, and the actin cytoskeleton. a and b) Scratch-wound analysis for 2D migration over 48 hours. t-test at 48 hours 8MBGA p = 0.04. c and d) Trypan blue dye exclusion assay to measure cell growth over 72 hours; 42MBGA p = 0.0066. e) Mean thickness of F-actin filaments assessed by FIJI plug-ins as denoted in Methods section. Mann-Whitney U test 8MBGA p = 0.001. f) Representative image of quantification in e. All experiments are representative of three biological replicates.
Supplementary Figure 2- 4. Representative images from scratch wound assays. Cell line depicted on the left with time after scratch on the top. Yellow lines denote the edges of open area. All experiments are representative of three biological replicates.
Figure 2-5. Increase of intracellular vesicles with TMZ resistance. a and b) Number of intracellular vesicles stained positive for the early endosomal marker EEA1 quantified between sensitive and resistant cell lines by immunofluorescence. t-test a) 42MGBA p = <0.0001; b) 8MBGA p = <0.0001. c) Representative image of EEA1 immunofluorescence. d) REMBRANDT dataset Kaplan-Meier overall survival plot with median expression of EEA1 in all brain cancer patients. e) EEA1 expression in the Chinese Cancer Genome Consortium (CCGC) dataset by brain cancer type. A = astrocytoma; AA = anaplastic astrocytoma; AO = anaplastic oligodendroglioma; AOA = anaplastic oligoastrocytoma; O = oligodendroglioma; OA = oligoastrocytoma; GBM = glioblastoma. r(cancer type) = recurrent, s(cancer type) = secondary. f) EEA1 expression in the REMBRANDT dataset by cancer type. One-way ANOVA p = <0.0001. All experiments are representative of three biological replicates.
Figure 2-6. Summary figure of changes with TMZ-resistance.
2.3 Materials and Methods

2.3.1 Cell Lines and Culturing Conditions

42MBGA and 8MGBA cell lines were provided by Dr. Jeffrey Toretsky (Lombardi Comprehensive Cancer Center (LCCC), Georgetown University, Washington DC). 42MBGA-TMZ-res and 8MGBA-TMZ-res were developed by our lab with constant exposure of the parental cell lines to increasing concentrations of TMZ, from 12.5, 25, 50, 100, 200 µM, over the course of two to three months. Cells were maintained in a humidified incubator with 95% air: 5% carbon dioxide. All cells tested negative for *Mycoplasma* and cell lines were fingerprinted by the LCCC Tissue Culture Shared Resource to verify their authenticity using the standard 9 STR loci and Y-specific amelogenin. Both the 42MBGA-TMZ-res and 8MGBA-TMZ-res fingerprinted the same as their parental cell line. 42MGBA and 8MGBA cells were grown in DMEM with 10% FBS. 42MBGA-TMZ-res and 8MBGA-TMZ-res cells were grown in DMEM with 10% FBS and 100 µM TMZ. TMZ (Selleckchem, Catalog No. S1237) was dissolved in DMSO to 130 mM and used at concentrations indicated. Bis-chloroethylnitrosourea (BCNU), a kind gift from Dr. Esther Chang, was dissolved in ethanol to 100 mM before use at concentrations indicated.

2.3.2 Cell Cycle Analysis

On day 0, cells were seeded at 100,000 – 150,000 cells per well in 6-well plastic tissue culture dishes one day prior to treatment with the indicated concentrations of drug. For experiments with TMZ or BCNU cells were treated for 72 hours. After 72 hours, cells were collected, ethanol-fixed, stained with propidium iodide, and analyzed for cell sub-G1 (fragmented) DNA content and cell cycle profile by fluorescence activated cell sorting.
2.3.3 Western Blotting

Cells were lysed in RIPA buffer for protein extractions and separated by a 4-12% gradient gel (Novex by Life Tech, NP0321BOX). Lysates were transferred onto Nitrocellulose membranes (Invitrogen, IB23001) using the iBlot 2 system and probed with antibodies against MGMT (1:1000, Cell Signaling, 2739S), β-tubulin (1:10,000, Sigma Aldrich, T7816), PARP (1:1000, Cell Signaling, 9542L), β-actin (1:5000, Sigma Aldrich, A5316), and Electron Transport Chain antibody cocktail (1:500, Abcam, ab110413). Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences) and enhanced chemiluminescent detection HyGLO Quick Spray Chemiluminescent (Denville) for dark room development.

2.3.4 Evaluation of chromosomal copy number changes induced by TMZ treatment

Metaphase and interphase slide preparations were performed using a standard protocol59. For metaphase chromosomal copy number determination, chromosomes were stained with 4’,6-diamidino-2-phenylindole (DAPI). FISH analysis was performed using a standard protocol60-62. A 17q21.1-21.2 locus-specific FISH probe (Stat5A/B locus) was used to evaluate chromosome 17 copy number change in the 8MGBA-TMZ-res and WT cell lines, as described earlier60. For the X chromosome enumeration in the 42MGBA-TMZ-res and WT cell lines, an X chromosome centromeric probe obtained from Empire Genomics (Buffalo, NY) was used according a manufacturer’s protocol. Scoring of cells and digital image acquisition were performed using a Leica DMRBE microscope (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with the appropriate optical filters and a CCD camera59.
2.3.5 Scratch Wound Assays

Cells were plated at 150,000-200,000 cells/well and grown for 48 hours to create a monolayer. After monolayer formation, a P200 tip was used to make the scratch. Images were taken at 0 hr, 24 hr, and 48 hr time points. Analysis was done in ImageJ\textsuperscript{63} to determine percent closed with 0% being at 0 hr.

2.3.6 F-actin measurements

FIJI (ImageJ \textsuperscript{63}) was used to measure the thickness of F-actin fibers. Grayscale images of phalloidin-stained cells were converted to binary using the Threshold tool (black/white). The BoneJ plugin \textsuperscript{64} was then used to measure fiber thickness in pixels. The mean thickness per cell is reported for 28-29 individual cells per cell line.

2.3.7 Immunofluorescent Staining

8MGBA-WT and 8MGBA-TMZ-res cells were seeded at a density of 50,000 cells onto 18mm coverslips in 12-well dishes. On the following day, the media was removed and cells were fixed and permeabilized in 3.2\% paraformaldehyde (PFA) with 0.2\% Triton X-100 in PBS for 5 minutes at room temperature. Three washes were performed with PBS in the 12-well plate, then coverslips were inverted onto 100 \( \mu l \) of primary antibody in the antibody block (0.1\% gelatin with 10\% normal donkey serum in PBS) on strips of parafilm and incubated for one hour. Coverslips were first stained with EEA1 (Cell Signaling, 3288), an early endosomal marker (1:100 dilution, 1 hr). Phalloidin (polymerized F-actin, Invitrogen, A12379, 1:200 dilution), and DAPI (DNA, 1:500 dilution) stains were added to the secondary antibody mixtures. Each coverslip was washed three times with PBS after primary incubation and then were inverted onto 100 \( \mu l \) of the appropriate
primary antibody, DAPI dihydrochloride, and (where appropriate) ActiStain-488-phalloidin (Cytoskeleton, Denver, CO) in antibody block in the dark for 20 minutes. Coverslips were again washed 3x with PBS, then gently dipped three times into molecular biology-grade water before inversion onto one drop of FLUOROGEL (Electron Microscopy Sciences, Hatfield, PA) then allowed to air-dry in the dark for at least 10 minutes. Slides were stored at 4°C until image collection on the LCCC Microscopy & Imaging Shared Resource’s Leica SP8 microscope with the 63X oil objective.

2.3.9 Crystal violet assay

Cells were seeded at a density of 1,000 cells per well in three, 96-well plastic tissue culture dishes per cell line on day 0. On day 1, one plate was stained with crystal violet (Sigma, C0775). For staining, plates were rinsed 1 time with 1X PBS to remove excess cellular debris. After, 100 µl of 3.2% PFA was added to each well and incubated at room temperature for 5 minutes followed by three washes in 1X PBS. Then, 200 µl of 0.5% crystal violet in 25% methanol was added to each well and incubated at 4C for 10 min. The stain was then removed and the plate was rinsed 4–6X with diH2O to remove excess stain. The plates were left to air-dry overnight. On day 8 and 10, all plates were rehydrated with 100% methanol and read at an absorbance of 550 nm.

2.3.10 Cell size quantification and growth

Cell size was determined by the Countess 2 (Invitrogen) using trypan blue dye exclusion of dead cells. The Countess 2 was also used to determine number of cells for 72-hour growth assays. 100,000 cells were seeded per 6-well on day 0. 24, 48, and 72 hours post-seeding, cells were collected and total cell number was determined by trypan blue dye exclusion.
2.3.11 Statistical Analysis

Results are represented as mean values ± standard error (SD) and considered statistically significant where p-value <0.05. Three independent biological replicates were done for each experiment. Statistical significance was calculated using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla California USA (www.graphpad.com) for the Student’s t-test, Mann Whitney U test, one-way ANOVA with Dunnett’s correction, or χ2 analysis of 2x3 contingency table. The RStudio packages survival, GGally, ggplot2, readr, and magrittr were used for the generation of violin plots and the analysis of large data sets (CCGA, REMBRANDT)65.
2.4 Discussion and Limitations

In the present study, we generated two GBM cell lines that are resistant to the standard of care drug temozolomide (TMZ). True to the heterogeneous nature of clinical GBM, the phenotypes displayed by these resistant cells were similar, but not identical. TMZ treatment no longer induced G2/M arrest in either of these cell lines, and both were markedly less responsive to BCNU, the previous standard of care for GBM. Both resistant cell lines also gained expression of the MGMT protein, which is responsible for removing one of the DNA adducts caused by TMZ treatment50.

An important finding of the present study is that both TMZ-res models have severely impaired response to BCNU. For decades BCNU was the standard of care for GBM, and it is still a component of some second-line regimens. Findings from clinical studies examining the efficacy of BCNU in recurrent GBM post-TMZ treatment are inconsistent, with some suggesting BCNU remains active while others demonstrate no benefit56-58. These TMZ-res cell line pairs may therefore also prove to be valuable resources for defining contexts in which BCNU is or is not appropriate second-line treatment, and for identifying other, more appropriate therapies for TMZ-resistant GBM.

There was an increase in nuclear size in both resistant cell lines when compared with their parental cell lines. In the 42MGBA-TMZ-res cell line there was also a change in nuclear morphology which has been described in other GBM cell lines that have acquired resistance to TMZ66,67. This may be associated with stability of the resistant phenotype, as we observed that the 42MBGA-TMZ-res cell line resistant phenotype can be maintained without continuous TMZ treatment. However, the 8MGBA-TMZ-res cell line, which has not undergone nuclear structure reorganization, and showed two distinct populations with respect to metaphase spreads, regained partial sensitivity when cultured in the absence of TMZ.
Increases in nuclear size can be driven by increased chromosome numbers, impaired transport of mRNA or proteins out of the nucleus\textsuperscript{68} or altered cytokinesis, defective mitosis and many other processes. In these TMZ resistant models, we hypothesized that the increase in nuclear size was a function of retained chromosomes. This is consistent with the mechanism of action of TMZ, which induces a G2/M arrest in cells. Cells which escape TMZ-mediated cell cycle arrest and death have already replicated their DNA, and may therefore retain sister chromatids that were not able to separate during anaphase. Because both parental GBM cell lines were initially polyploid with distinct karyotypes, we assessed two different chromosomes that were diploid in the parental cells – 17 for the 8MBGA-WT and –TMZ-res cell lines, and X for the 42MBGA-WT and –TMZ-res cell lines – as an indicator of chromosomal copy number alterations. Both TMZ resistant variants showed significant copy number gains. Of note, our chromosome 17 locus specific probe targets the \textit{STAT5} gene, which has already been implicated as a pro-tumorigenic factor in GBM\textsuperscript{69}. The increased copy number of the \textit{STAT5} locus in the 8MBGA-TMZ-res cell line is consistent with previous work and suggests that the observed increase in nuclear size is at least partially attributable to an increase in chromosome number. Because chromosome 17 existed in multiple copies in the 42MBGA-WT cell line, we chose an X-centromeric probe to test chromosomal copy number changes in the 42MBGA-TMZ-res line. Again, we saw an increase in X-chromosome copy number in the 42MBGA-TMZ-res line, and an overall increase in the total number of chromosomes. While it can be energetically disadvantageous to increase ploidy, we hypothesize that, as for \textit{STAT5}, there may be X chromosome genes that when duplicated give a proliferative advantage to these cells.

Two major challenges in the clinical management of GBM, particularly after TMZ resistance has developed, are rapid tumor cell proliferation and widespread local migration or
invasion. However, these phenotypes are rarely displayed by the same cells, giving rise to the “go or grow” hypothesis to explain aggressive behavior and drug resistance. This is not to say heterogeneity is lost within a fast-growing tumor or a more migratory tumor, but that broadly in previous glioma studies these are the two main phenotypes noted post resistance. For this reason, our two cell line models of TMZ-resistant GBM, which provide examples of both phenotypes, are unique. More research is still necessary, especially on patient samples, to truly understand the mechanism behind these differing resistant phenotypes, but the creation of these two cell line models is an important start. The 42MBGA-TMZ-res cell line did not exhibit any significant changes in migration, cytoskeletal structure, or cell size when compared to its parental cell line, but it did proliferate significantly faster. In contrast, the 8MBGA-TMZ-res cell line had a significantly increased migratory capacity, F-actin thickness, and overall cell size relative to 8MGBA-WT cells, but no accompanying increase in proliferation. These divergent alterations in basal growth rate and migratory capacity provide two unique models – one adapting the “go” (8MGBA-TMZ-res) and the other the “grow” (42MBGA-TMZ-res) strategy of TMZ resistance. There have been many hypotheses as to what determines the switch between “go or grow”, including mutational status, hypoxia, and influences of the surrounding cell secretome. Activation of the FAK and MAPK/ERK pathways have been shown to be differentially regulated between a “go” and “grow” phenotype, respectively. Others have also hypothesized that remaining cells from the “go” population lead to recurrence of GBM. Therefore, more insight needs to be gained into potential common pathways between the “go or grow” phenotypes that could be used as therapeutic targets for both cell populations. Candidate strategies to revert “go” phenotypes could include inhibition of the integrin signaling axis, either through blockade of integrin engagement or inhibition of FAK, while MEK inhibition could suppress the “grow”
phenotype. Further studies employing the wild-type and resistant pairs of cell lines generated in this study have the potential to provide important insight into the differential mechanisms of TMZ resistance.

Acquisition of TMZ resistance was associated with a marked increase in the number of intracellular and extracellular vesicles in our models. While there are always potential limitations in an in vitro setting, Oliveira et al showed that the in vitro secretome can affect the “go or grow” phenotype of murine GBM cells. Therefore, it is important to identify potential in vitro mechanisms that can be further extrapolated to in vivo models in the future. The increase in early endosome marker EEA1 expression is interesting, as others have shown EEA1 to interact directly with RRAD, STAT3, and EGFR to regulate EGFR’s subcellular localization and resistance to TMZ. Clinically, higher EEA1 mRNA expression is associated with poor survival. EGFR and its structural variants have been targeted extensively in GBM, but with limited success. We suggest further study of the mechanisms by which EEA1 contributes to the TMZ resistant phenotype could provide a new way to target EGFR by modifying its subcellular localization rather than activation.

In conclusion, we present two TMZ-resistant GBM cell lines, one originally generated from a male and one from a female patient that have distinct proliferative and migratory capacities while sharing certain GBM resistance characteristics such as increased chromosome number, nuclear size, and vesicle formation (Figure 7). These models are a potential resource for the field to better define the essential drug resistance mechanisms and therapies in TMZ-resistant GBM.

Limitations

Cell line models are widely used to mechanistically test molecular phenotypes of cancer, as well
as to validate *in silico* and test tube experimental findings. They have also played an important role in being the biological workhorse for basic science research for many decades. This being said, they have obvious limitations when comparing to clinical samples and patient tumors, most notably two-dimensional growth on plastic and the absence of the tumor microenvironment. The pitfalls of these limitations are most clearly illustrated, in my opinion, by the multiple failed clinical trials where potential novel therapies are effective in cell culture systems but fail when put to the test in a real tumor environment. The goal of this project was to create two independent TMZ-resistant cell lines in which to perform future experiments designed to test potential molecular mechanisms behind TMZ-resistance. It is well understood that this is simply a start in understanding the immensely complex disease of glioblastoma and the surrounding brain tumor microenvironment.

The characteristics of resistant cells found in this project, while focused on TMZ-resistance in GBM as that is the area of focus for my thesis, may not be specific to only TMZ-resistant GBM. Changes in chromosome number, cell growth, and cell motility are also hallmarks of other drug-resistant malignancies. The goal of this project was not to identify new features that were GBM-specific. Rather, the goal was to corroborate what others have found in TMZ-resistant models previously created or TMZ-resistant patient samples in my novel cell lines, and create a strong foundation from which to ultimately move into more clinically relevant *in vivo* studies. The use of these two models does not recapitulate a true tumor setting, but they do share key characteristics of resistance that have been established by other TMZ-resistant models.
CHAPTER 3

ESTROGEN-RELATED RECEPTOR BETA ACTIVATION AND isoFORM SHIFTING BY CDC2-LIKE KINASE INHIBITION RESTRICTS MIGRATION AND INTRACRANIAL TUMOR GROWTH IN GLIOBLASTOMA

3.1 Rationale

Dysregulation of alternative mRNA splicing is one potential mechanistic driver of GBM\textsuperscript{81}, given that the brain contains the most alternatively spliced transcripts of any organ and the expression of proteins that function in the processing of RNA are upregulated in GBM versus healthy brain\textsuperscript{82,83}. Serine/arginine rich (SR) proteins are a prominent group of splicing regulatory factors that are phosphorylated and thereby regulated by the \textit{cdc}2-like kinases (CLKs)\textsuperscript{84}, some of which have been mechanistically implicated in GBM\textsuperscript{45}. One inherent challenge to developing effective therapeutic strategies for GBM is the blood brain barrier (BBB), which occludes many established and experimental therapeutic agents. While CLK inhibitors have not yet entered clinical trials, preclinical studies of TG-003 (a pan-CLK inhibitor) show that this agent can cross the BBB in mouse models of autism\textsuperscript{45}.

Given that improved therapeutic options are an urgent clinical need for GBM, the nuclear receptor superfamily - members of which are highly successful targets in breast and prostate cancers - provide a possible alternative strategy. Estrogen-related receptor beta (ERR\textbeta, - also referred to in the literature as ESRRB, NR3B2) is the founding orphan member of the nuclear receptor superfamily\textsuperscript{72,73}. By definition, orphan nuclear receptors lack known endogenous ligands, though their function can be modified by coregulatory proteins or synthetic ligands that increase or decrease their transcription factor activity\textsuperscript{52}. DY131 is one synthetic agonist that has been shown to enhance ERR\textbeta transcription factor activity in the murine arcuate nucleus\textsuperscript{74,75}, which is strongly suggestive of BBB penetrance. The pre-mRNA expressed from the \textit{ESRRB} gene is alternatively
spliced at the 3’ end, leading to the production of three transcript variants and resulting protein isoforms: ERRβ short form (ERRβ-sf), ERRβ-2, and ERRβ exon 10-deleted (ERRβ-Δ10) (Figure 3-1A, 76). The 3’ splicing events that generate these three transcript variants are unique to primates, with all lower vertebrate organisms expressing only the ERRβ-sf isoform. The inclusion of additional sequences in 3’ of the ERRβ-2 and ERRβ-Δ10 transcripts results in the expression of protein isoforms that include 67 and 75 amino acids that the carboxyl terminal when compared to the ERRβ-sf isoform. The additional amino acids affect the nuclear receptor F domain that can modify transcription factor function and recruit distinct coregulatory proteins77,78.

We previously identified the ERRβ-2 isoform as a cytoplasmic and centrosome-adjacent protein72, and showed that activation of this isoform can delay mitosis and partially repress the transcription factor activity of the ERRβ-sf isoform79. The goal of this study was to better define the function and interacting partners of the pro-apoptotic ERRβ-2 isoform in GBM and test the ability of inhibitors of kinase regulators of splicing factors to shift isoform balance towards the ERRβ-2 isoform. We found that the cytoplasmic ERRβ-2 isoform suppresses GBM cell migration and interacts with the actin nucleation-promoting factor cortactin, and that an ERRβ agonist remodels the actin cytoskeleton, and suppresses cell migration. CLK inhibition with TG-003 in combination with the ERRβ agonist DY131 shifts isoform expression in favor of ERRβ-2 and leads to suppression of growth and migration in TMZ-resistant GBM cells. Finally, we use a zebrafish model to show that the combination of TG-003 and DY131 has anti-tumor activity in vivo in a setting where the BBB is intact.
3.2 Results

3.2.1 ERRβ isoforms are expressed in GBM

Nuclear receptors are attractive drug targets, and successful inhibitors to nuclear receptors like estrogen receptor alpha (ERα) have fundamentally changed cancer treatment outcomes for breast cancer patients. However, targeting ERα with tamoxifen in GBM has been unsuccessful\(^8\), perhaps because its expression in the brain is quite low relative to other nuclear receptors, including the orphan nuclear receptor ERRβ (Supplementary Figure 3-1A). Two ERRβ isoforms – ERRβ-2 and ERRβ-sf - are expressed across multiple TMZ sensitive and resistant GBM cell lines\(^8\), as well as primary normal human astrocytes (NHAs, Figure 3-1B) and immortalized human oligodendrocytes (MO3.13, Supplementary Figure 3-1B). DY131 is a small molecule agonist of ERRβ that has anti-proliferative activity in several preclinical cancer models\(^8\). DY131 is growth inhibitory in GBM cell lines, but not NHAs or immortalized oligodendrocytes where it elicits a slight increase in growth (Figure 3-1C).

A limitation of conventional GBM cell lines is that they do not always recapitulate essential GBM molecular features or pathobiology\(^8\). Patient-derived xenografts (PDXs) from primary tumors have been established to address this limitation, and we show that ERRβ-2 and ERRβ-sf protein are broadly expressed across PDX models that are representative of GBM molecular subtypes and clinical/pathological features. EGFR wild type and vIII-mutant amplified tumors are more frequently categorized in the Classical molecular subtype\(^8\), while PARP1 expression is enriched in Classical and Proneural subtypes\(^8,9\), and CDKN1A/p21 expression is indicative of primary vs. secondary GBM\(^9,10\) (Figure 3-1D).

Studies in COS-1 cells transfected with transcript variant specific ERRβ cDNAs show that ERRβ-sf localizes to the nucleus, while ERRβ-2 localizes to the cytosol and nucleus, suggesting
differential functions. We also observed this pattern of subcellular localization of endogenous ERRβ-sf (nucleus) and ERRβ-2 (cytosol and nucleus) in NHAs, oligodendrocytes, and GBM cells (Figure 3-2A, Supplementary Figure 3-1C), and in primary tumor specimens (Figure 3-2B). Collectively, these data demonstrate expression of ERRβ isoforms in TMZ sensitive and resistant GBM models and growth inhibition of these models systems when exposed to a ERRβ agonist.

3.2.2 The long ERRβ2 isoform suppresses GBM cell migration and interacts with the actin nucleation-promoting factor cortactin

Prior studies implicate ERRβ-sf as a transcription factor with activity at multiple DNA response elements, but suggest that ERRβ-2 has little or no transcription factor activity and may partially repress ERRβ-sf-mediated gene transcription. Based on these observations, coupled with the localization of ERRβ-2 expression adjacent to centrosomes and diffusely throughout the cytoplasm (Figure 2A) we next tested whether ERRβ-2 might have additional functions in GBM cell migration. Using shRNAs that selectively target the ERRβ-2, but not the ERRβ-sf transcript variant, and vice versa, we observed significantly enhanced T98G cell migration in wound healing (Figure 3-3A and B) and transwell migration assays (Supplementary Figure 3-2).

The F-domains of nuclear receptor proteins are carboxyl terminal extensions of the ligand binding domain that can modify transcription factor function and recruit distinct coregulatory proteins. Inspection of the 67 amino acid extended carboxyl-terminal F domain unique to ERRβ-2 shows a number of putative protein-protein interaction motifs and post-translational modification sites. Of these, a proline-rich region consisting of amino acids 467-472 (PLPPPP) forms the core of the consensus binding motif for the Src homology 3 (SH3) domain of the cytoskeletal protein and actin nucleation-promoting factor cortactin. Cortactin (CTTN) mRNA
expression is increased with increasing severity of gliomas, including GBM (Supplementary Figure 3-3A), and is significantly enriched at the leading edge of tumors (Supplementary Figure 3-3B, 45). Cortactin has previously been implicated in GBM cell migration34, and is expressed across all GBM cell lines (Figure 3-1B). Transfection of GBM cells with transcript variant specific ERRβ cDNAs showed the ERRβ-2 isoform, but not the ERRβ-sf isoform, interacts with endogenous cortactin (Figure 3-3C). Together, these data suggest that the ERRβ-2 isoform plays a role in cell migration and is uniquely capable of binding cortactin.

3.2.3 ERRβ activation by DY131 suppresses GBM cell migration and remolds the actin cytoskeleton

We next examined the impact of activating endogenous ERRβ on actin cytoskeletal remodeling and cell migration in GBM cells. Treatment with the ERRβ agonist DY131 had no effect on ERRβ-2, ERRβ-sf, cortactin, or MGMT protein expression, but modestly increased the cleavage of PARP in 42MBGA-TMZ-res and T98G cell lines, which can be indicative of caspase-mediated apoptosis (Figure 3-4A). However, DY131 induced a marked redistribution of cortactin away from the cell membrane and towards the center of the cell body in multiple GBM cell lines (Figure 3-4B, white arrowheads versus asterisks). In two of three TMZ resistant GBM cell lines (42MGBA-TMZ-res and T98G), DY131 also significantly increased actin polymerization, as measured by fluorescence-activated cell sorting for F-actin (Figure 3-4C). Loss of cortactin membrane localization coupled with increased actin polymerization can be indicative of impaired cell movement94, and consistent with this we show that DY131 significantly inhibits the migration of multiple GBM cell lines (Figure 3-4D).
3.2.4 Inhibition of Cdc2-like kinases (CLKs) shifts ERRβ isoform expression and potentiates DY131-mediated inhibition of growth and migration in GBM cells

ERRβ-2 and ERRβ-sf have distinct roles in transcription factor activity and differentially suppress tumor cell growth and motility, but how splicing of the ESRRB pre-mRNA is regulated to produce these isoforms is unknown. The spliceosome is the primary driver of differential splicing, but heterogeneous nuclear ribonucleoprotein (hnRNP) and serine/arginine rich splicing factor (SRSF, SR protein) families cooperate with the spliceosome to enhance the exclusion versus inclusion (respectively) of specific exons (Figure 3-5A). SR proteins broadly promote exon inclusion by binding cis exon splicing enhancers (ESEs), while hnRNPs are able to antagonize SR proteins, which promotes exon skipping (e.g. 95,96). The ERRβ-2 transcript variant is defined by inclusion of ESRRB exon 10). Computational analysis of ESRRB exon 10 predicts multiple high-confidence consensus ESEs sites for the SR protein SRSF6 (Figure 3-5B) 97,98.

Serine phosphorylation (p) of SR proteins modifies their ability to promote exon inclusion. Hyper-phosphorylated SR proteins are recruited to sites of active transcription and pre-mRNA processing, where they can bind ESEs and strengthen splicing recognition sites29. After exon-exon joining in the now-mature transcript, SR proteins become hypo-phosphorylated and are exported from the nucleus99. Using a pan-pSR antibody, we show there is a significant positive correlation between the presence of pSRSF6 and the expression of the ERRβ-2 isoform in GBM PDX models (Figure 3-5C), but no correlation between pSRSF6 and ERRβ-sf or the expression of the ERRβ-2 isoforms the phosphorylation of other SR proteins.

One of the kinase families responsible for catalyzing nuclear phosphorylation of SR proteins is the cdc2-like kinase (CLK) family31. TG-003 is a pan-CLK-1, -2, and -4 inhibitor that suppresses the phosphorylation of multiple SR proteins, leading to differential splicing of a broad
range of responsive mRNAs\textsuperscript{32}. We therefore tested TG-003 in two TMZ-resistant GBM models as a strategy to shift the relative expression of the ERRβ-2 and ERRβ-sf isoforms (Figure 3-5D, E). In 42MGBA-TMZ-res cells, the combination of TG-003 and DY131 markedly upregulates ERRβ-2 expression (Figure 3-5D, asterisk and bars), thereby increasing the ERRβ-2:ERRβ-sf ratio. In T98G cells, the combination of TG-003 and DY131 also increases the ratio of ERRβ-2:ERRβ-sf, although here this is achieved through decreased expression of the ERRβ-sf isoform (Figure 3-5E, asterisk and bars). Differential patterns of SR protein expression and phosphorylation are observed in these cell models (Supplementary Figure 3-4). In both cell lines, the combination of TG-003 and DY131 enhances PARP cleavage and Serine 10 phosphorylation of histone H3, which are suggestive of increased apoptosis and of mitotic arrest, respectively (Figure 3-5D, E).

As pan-CLK inhibition shifts isoform expression in favor of ERRβ-2, and ERRβ-2 has a more pronounced pro-apoptotic, anti-mitotic, and anti-migratory activity (\textsuperscript{32}, and Figures 3-3B, 3-5D, 3-5E, and Supplementary Figure 3-2), we tested whether combination treatment with TG-003 and DY131 would more robustly induce G2/M arrest, apoptosis, and inhibit cell migration across all of our GBM models. Multiple TMZ sensitive and resistant GBM cell lines exhibit significantly enhanced cell death and mitotic arrest in response to combination treatment with TG-003 and DY131 (Figure 3-6A, Supplementary Figure 3-5), with 42MGBA-TMZ-res and T98G cells being the most sensitive. Importantly, T98G cells treated with the combination of TG-003 and DY131 undergo equivalent G2/M arrest to T98G cells in which we depleted the expression of ERRβ-sf isoform selectively (Supplementary Figure 3-5), further suggesting that this phenotype is driven predominantly by ERRβ-2. By contrast, MO3.13 oligodendrocytes showed no significant induction of G2/M arrest or cell death by either drug alone or the combination. In scratch-wound assays using reduced concentrations of TG-003 and DY131 to isolate specific effects on migration
versus general cell viability, the combination treatment significantly inhibited cell migration in multiple TMZ sensitive and resistant GBM cell lines (Figure 3-6B).

3.2.5 Combination treatment with TG-003 and DY131 inhibits the growth of GBM intracranial xenografts

A key limitation of novel treatment strategies for GBM is the failure of therapeutic agents to penetrate the blood-brain barrier (BBB) effectively. The BBB protects the central nervous system from a range of endogenous and exogenous insults, and while this can be locally compromised by the primary tumor, disseminated GBM cells remaining after surgical resection are largely shielded from systemic therapies. DY131 and TG-003 are both reported to be brain penetrant in mice\(^{100}\), so we tested these drugs individually and combined using a zebrafish intracranial xenograft model (Figure 3-7A). The zebrafish BBB forms at about three days post-fertilization (dpf) and is functionally similar to that of higher organisms\(^{101}\). We modified published intracranial xenograft procedures (Figure 3-7B, \(^{31,102}\), injecting labeled 42MGBA-TMZ-res cells into 1.5 dpf zebrafish embryos. Two to three days after the BBB forms (by 6 dpf), tumors were imaged, fish were treated daily by addition of test compounds to the fish water, and tumors were imaged again at 10 dpf. A blinded comparison of the area of pre- and post-treatment confocal tumor images shows that 54% of tumors in control-treated fish increased in size, but five days of treatment with the dose-reduced combination of TG-003 (15 μM) and DY131 (1.5 μM) reduced this to 32% (Figure 3-7C, Cochran-Armitage p=0.09). While this is not a significantly significant result, it shows a trend for the combination of DY131 and TG-003 to affect tumor growth in an in vivo intracranial model. Cascade Blue-conjugated dextran imaging at 10 dpf was used to assess the integrity of the BBB.
In both DMSO control- and combination-treated fish, signal is occluded from the brain (Figure 3-7D).

The zebrafish intracranial model was chosen over subcutaneous flank injection in a mouse model (a more typical initial in vivo model), because this preserves the main barrier to treatment: the blood brain barrier. Many studies have shown therapy-induced tumor shrinkage in treated flank tumors, but these same drugs fail to shrink tumors that were implanted into the brain, behind the BBB. Our data provide a potential proof of principle that the combination treatment of an ERRβ agonist and splicing modulator may effective for GBM treatment in the future, though it is important to note that these two tool compounds have poor bioavailability. Second-generation compounds are being made for TG-003 and (in our lab) for ERRβ. Therefore, the goal of this initial study is to provide a rationale for using the combination of the second-generation compound in in vivo studies in the future.
Figure 3-1. **ERRβ isoforms are expressed in GBM** (A) Differential splicing at the 3’ end of the ESRRB pre-mRNA leads to the production of three known ERRβ transcripts and protein products—the short form (ERRβ-sf), and two longer forms called beta2 (ERRβ-2), and exon 10-deleted (Δ10). The ERRβsf isoform is conserved in zebrafish and mice, with percent identity for each ortholog compared to the human sequence. AF-1 = activation function-1, aa = amino acid. (B) ERRβ-2 and ERRβ-sf protein isoforms are expressed in primary normal human astrocytes (NHAs) as well as in multiple TMZ sensitive (8MGBA, 42MGBA) and resistant (8MGBA-TMZ-res, 42MGBA-TM-Zres, T98G) GBM cell lines. The expression of the O6-methylguanine methyltransferase (MGMT), a known marker of therapy resistance in GBM, is upregulated in the TMZ resistant cell lines. (C) DY131, a small molecule agonist of ERRβ, inhibits cellular proliferation
in TMZ sensitive and resistant cell lines but not in NHAs or normal human oligodendrocytes. Data in the growth curves are presented as the mean (point) ± standard deviation (SD, error bars), and data in the inset are presented as the mean (line) ± minimum/maximum values (box) for 3 (NHA), 6 (oligodendrocytes, T98G), or 8 (all other cell lines) technical replicates, which were analyzed by two-way analysis of variance (ANOVA) with post hoc Dunnett’s multiple comparisons test. Data are representative of at least two independent biological replicates. **, *** denote p<0.01 and p<0.0001, respectively, versus DMSO control. (D) Expression of ERRβ-2 and ERRβ-sf across a panel of patient-derived xenografts (PDXs) of GBM. Also shown are the expression of wild type (wt) and vIII mutant EGFR, the DNA damage marker PARP, and the cyclin-dependent kinase inhibitor, p21. Lanes labeled β2 and βsf denote lysate from T98G cells transfected with the indicated cDNA construct.
Supplementary Figure 3-1. ERRβ expression in healthy brain. (A) ESRRB mRNA expression in human brain as compared to estrogen receptor α (ERα, gene = ESR1) and OLIG1, a key regulator of neural progenitor cells. (B) ERRβ-2 and ERRβ-sf protein isoforms are expressed in immortalized human MO3.13 oligodendrocyte cells. (C) Immunofluorescent staining of ERRβ-2 and ERRβ-sf depicts nuclear localization of ERRβ-sf, and nuclear and cytoplasmic localization of ERRβ-2, in MO3.13 oligodendrocyte cells.
Figure 3-2. **ERRβ expression across cell lines and patient samples** (A) Immunofluorescent staining of ERRβ-2 and ERRβ-sf demonstrates the nuclear localization of ERRβ-sf, and nuclear and cytoplasmic localization of ERRβ-2 in NHAs and TMZ sensitive and resistant GBM cell lines. (B) Immunohistochemistry of ERRβ-2 and ERRβ-sf protein isofrom expression demonstrates both are expressed in primary GBM tumor samples.
Supplementary Figure 3-2. Effect of ERRβ-2 on migration. The selective silencing of the ERRβ-2 transcript variant significantly enhances T98G cell migration, as measured by transwell migration assay. Data are presented as the median (line) ± minimum/maximum for 3-5 fields of view per transwell filter, in each of at least two independent biological replicates. Data were analyzed by Mann-Whitney test at each time point, where **** denotes p<0.0001.
Figure 3-3. (A, B) The long ERRβ2 isoform suppresses GBM cell migration. The silencing of ERRβ-2, but not ERRβ-sf, significantly enhances T98G cell migration, as measured by scratch wound assay. In A, cells stably transduced with the indicated shRNA are compared to parental cells, or those stably transduced with a scrambled shRNA control. Lanes labeled Δ10, β2, and βsf denote lysate from cells transfected with the indicated cDNA construct. In B, data are presented as the median (line) ± minimum/maximum for 3-8 fields of view, in each of at least two independent biological replicates. Data were analyzed by one-way ANOVA with post hoc Tukey’s multiple comparisons test. ***, **** denote p<0.001 and p<0.0001, respectively. n.s. denotes not significant. (C) ERRβ-2, but not ERRβ-sf or ERRβ-Δ10, interacts with endogenous cortactin as analyzed by immunoprecipitation. - denotes control immunoprecipitation of ERRβ2-transfected cell lysates.
Supplementary Figure 3-3. Cortactin mRNA expression. (A) Cortactin mRNA expression (gene = CTTN) increases with increasing severity of glioma. RNAseq data from the Chinese Glioma Genome Atlas, obtained through the International Cancer Genome Consortium, were plotted in R. A = astrocytoma, AA = anaplastic astrocytoma, AO = anaplastic oligodendrogliaoma, AOA = anaplastic oligoastrocytoma, O = oligodendrogliaoma, OA = oligoastrocytoma, GBM = glioblastoma, r = recurrent, s = secondary. (B) Cortactin mRNA expression is significantly increased at the invasive front of GBM. RNAseq expression Z scores from micro dissected tumors obtained from the Ivy Glioblastoma Atlas Project through the Allen Brain Institute were analyzed by Mann-Whitney test comparing the cellular tumor to the leading edge. * denotes p<0.05.
**Figure 3-4. ERRβ activation by DY131 suppresses GBM cell migration.** (A). Western blot analysis of PARP cleavage, cortactin, ERRβ-2, ERRβ-sf, and MGMT expression in the indicated cell lines treated with DY131 or DMSO control (denoted as -) for 24 hours. (B). Immunofluorescent staining of cortactin in the indicated cell lines treated with 5 μM (42MBGA and 42MBGA-TMZ-res) or 2.5 μM DY131 (T98G) or DMSO control for 24 hours prior to fixation, staining, and imaging. DY131 treatment causes a redistribution of cortactin away from the membrane (DMSO, white arrowheads vs. DY131, white asterisks). (C). Changes in F-actin polymerization were measured by flow cytometry analysis of cells stained with Acti-Stain 488 phalloidin treated with 5 μM DY131 or DMSO control for 24 hours. Fold increase in F-actin polymerization in each DY131-treated cell line relative to its own DMSO control are presented as mean ± SD for 3-4 independent biological replicates. Data were analyzed by Mann-Whitney test, where * denotes p<0.05. (D). Scratch-wound analysis of two-dimensional migration over 72 hours of treatment with the indicated concentration of DY131 or DMSO control. Data are presented as mean ± SD for 3 independent
biological replicates. Data were analyzed by Mann-Whitney test at each time point, where *,**,***,**** denote p<0.05, p<0.01, p<0.001, and p<0.0001, respectively.
Supplementary Figure 3-4. Expression and phosphorylation of SR proteins. (A, B) in 42MGBA-TMZres (A) and T98G cells (B) treated with 50 μM TG-003, the combination of 50 μM TG-003 and 5 μM DY131, or DMSO for 24 hours.
Figure 3-5. Inhibition of Cdc2-like kinases (CLKs) shifts ERRβ (A) Simplified schematic showing interplay between SR proteins and hnRNPs, which broadly promote exon inclusion or exclusion, respectively. CLK = cdc2-like kinase, star = phosphorylation. U1,2 = spliceosomal components. ESE = exon splicing enhancer, ESS = exon splicing suppressor. (B) RBPmap prediction of high-confidence SRSF6 binding sites in ERRβ-2-specific exon 10. (C) Representative Western blot of pSRs (1H4), ERRβ-2 and ERRβ-sf expression, and Spearman rank-order correlation for pSRSF6 with ERRβ-2, but not ERRβ-sf, in GBM PDXs. Arrows indicate ERRβ-2 and pSRSF6. (D, E). Changes in ERRβ-2 and ERRβ-sf expression, PARP cleavage, and Serine 10 phosphorylation of histone H3 were measured in 42MGBA-TMZ-res (D) and T98G (E) cells treated with 50 μM TG-003, the combination of 50 μM TG-003 and 5 μM DY131, or DMSO for 24 hours. Asterisks indicate increased ERRβ-2 (D) or decreased ERRβ-sf expression (E), both of which serve to increase the relative abundance of ERRβ-2. Bar charts depict the normalized ratio of ERRβ-2 to ERRβ-sf protein expression by densitometry.
Supplementary Figure 3-5. Full cell cycle profile. Immortalized oligodendrocyte MO3.13 and GBM cell lines treated with 5 μM DY131, 50 μM TG-003, the combination of 5 μM DY131 + 50 μM TG-003 (TGDY), or DMSO control for 24 hours. For each panel, data are presented as the mean ± SD for 3 independent biological replicates. For T98G cells, data show an identical magnitude of G2/M arrest caused by the combination of 10 μM DY131 + silencing of ERRβ-sf (adapted from ref. 105) as that seen in response to the combination of 5 μM DY131 + 50 μM TG-003 (TG/DY).
**Figure 3-6.** (A). Inhibition of Cdc2-like kinases (CLKs) potentiates DY131-mediated inhibition of growth and migration in GBM cells

Flow cytometric cell cycle analysis of sub-G1 (fragmented DNA) and G2/M fractions of immortalized oligodendrocyte MO3.13 and GBM cell lines treated with 5 μM DY131, 50 μM TG-003, the combination of 5 μM DY131 + 50 μM TG-003 (TGDY), or DMSO control for 24 hours. For each panel, data are presented as the mean ± SD for 3 independent biological replicates. Data were analyzed by one-way ANOVA with post hoc Tukey’s multiple comparisons test. **,** ***,** **** denote p<0.05, p<0.01, p<0.001, and p<0.0001, respectively. (B). Scratch-wound analysis of two-dimensional migration over 72 hours of treatment with dose-reduced concentrations of DY131 and TG-003, as indicated in the legend. For each panel, data are presented as the mean ± SD for 3 independent biological replicates. Data were analyzed by one-way ANOVA at each time point with post hoc Tukey’s multiple comparisons test. **,** ***,** **** denote p<0.05, p<0.01, p<0.001, and p<0.0001, respectively.
Figure 3-7. Treatment with TG+DY stunts intracranial xenograft growth. (A). Brightfield (left) and fluorescent (right) images of 5 day post-fertilization (dpf) zebrafish bearing xenografts of 42MGBA-TMZ-res cells. Dil-labeled tumor cells are red and the vasculature is green. (B). Experimental schematic for in vivo zebrafish model with representative images of Dil-labeled 42MBGA-TMZ-res cells pre- and post-treatment. (C). Growth or shrinkage of 42MGBA-TMZ-res xenografts following 5 days of treatment with 1.5 μM DY131, 15 μM TG-003, the combination of 1.5 μM DY131 + 15 μM TG-003, or DMSO shows a trend towards tumor shrinkage in the combined treatment group. Data are presented as fraction of fish showing tumor growth vs. tumor shrinkage for 19-26 fish per group. Data were analyzed by χ² test for trend.
(Cochran-Armitage), \( p = 0.09 \). Scoring was performed by investigators blinded to treatment group/status. (D). Fluorescent images of 10 day post-fertilization (dpf) zebrafish bearing xenografts of 42MGBA-TMZ-res cells following 5 days of treatment with DMSO control (left) or the combination of 1.5 \( \mu \text{M} \) DY131 + 15 \( \mu \text{M} \) TG-003 (right). Dil-labeled tumor cells are red, the vasculature is green, and Cascade Blue-conjugated dextran shows integrity of the blood-brain barrier (BBB). (E). Summary schematic of key findings. CLK inhibition shifts isoform expression in favor of ERRβ-2, and potentiates ERRβ agonist-mediated inhibition of growth and migration in GBM.
### Supplementary Table 1- ERRβ F Domain Motif Analysis

#### ERRβ2

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3.3 Materials and Methods

3.3.1 Cell Lines and Culturing Conditions

Primary normal human astrocytes (NHAs) were purchased from Lonza (#CC-2565). Immortalized human oligodendrocyte MO3.13 cells were a kind gift from Dr. Alexandra Taraboletti (Lombardi Comprehensive Cancer Center, LCCC). Temozolomide (TMZ) sensitive 42MGBA and 8MGBA cell lines were provided by Dr. Jeffrey Toretsky (LCCC), and the de novo TMZ resistant T98G cell line was provided by Dr. Todd Waldman (LCCC). Acquired TMZ resistant 42MGBA-TMZ-res and 8MGBA-TMZ-res cell line variants were developed by our laboratory and described previously (20). Cells were maintained in a humidified incubator with 95% air: 5% carbon dioxide. All cell lines tested negative for Mycoplasma and were fingerprinted by the LCCC Tissue Culture Shared Resource to verify their authenticity using the standard 9 STR loci and Y-specific amelogenin. Both the 42MGBA-TMZ-res and 8MGBA-TMZ-res are documented to be of the same origin as their respective parental cell lines. NHAs were used within one passage and maintained in astrocyte growth medium (AGM, Lonza #CC-3187) supplemented with L-glutamine, gentamicin sulfate, ascorbic acid, human epidermal growth factor (HEGF), insulin and 3% fetal bovine serum (FBS) (Lonza, #CC-4123). MO3.13, 42MGBA, 8MGBA, 42MGBA-TMZ-res, and T98G cells were grown in Dulbecco's Modified Eagle Medium (DMEM, high glucose, ThermoFisher, #11965092) with 10% FBS. 8MGBA-TMZ-res cells were grown in DMEM with 10% FBS and 100 µM TMZ. TMZ (Selleckchem, #S1237) was dissolved in dimethyl sulfide (DMSO, Sigma, #D8418) to 130 mM and used at concentrations indicated. DY131 (Tocris, #2266) was dissolved in DMSO to 10 mM and used at the concentrations indicated.
3.3.2 Western blotting

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, #4906837001) for protein extractions and separated by polyacrylamide gel electrophoresis using 4-12% gradient gels (Novex by Life Tech, #NP0321BOX) as described previously (19). To detect ERRβ2, 55-65 µg of protein was loaded. Lysates were then transferred onto nitrocellulose membranes (Invitrogen, #IB23001) with the iBlot2 (Invitrogen, #IB21001) and probed with the following antibodies: ERRβ-2 (1:500, RNDSystems, #PP-H6707-00), ERRβ-sf (1:1000, RNDSystems, #PP-H6705-00), PARP (1:1000, Cell Signaling, #9542L), CDKN1A/p21 (1:300, Santa Cruz Biotechnology, #sc-756), EGFR (1:1000, Cell Signaling, #2232S), MGMT (1:1000, Cell Signaling, #2739S), Cortactin (1:1000, Upstate, #05-180), phosphorylated SR proteins (clone 1H4, 1:500, Millipore, #MABE50), total SR proteins (1:1000, Sigma-Aldrich, #MABE126), phosphorylated histone H3 Serine 10 (1:1000, Cell Signaling, #3377S), and total histone H3 (1:1000, Cell Signaling, #9715S). Beta-Tubulin (1:5000, Sigma Aldrich, #T7816) and Beta-Actin (1:5000, Sigma-Aldrich, #A5316) were used as a loading controls. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, #NA931-1ML (Mouse) or #NA934-1ML (Rabbit)) and enhanced chemiluminescent detection HyGLO Quick Spray Chemiluminescent (Denville Scientific, #E2400) using film (Denville Scientific, #E3212).

3.3.3 Immunofluorescence

Cells were seeded at a density of 40,000-50,000 cells onto 18 mm diameter #1.5 round coverslips (VWR, #101413-518) in 12-well dishes. On the following day, the media was removed and cells were fixed and permeabilized in 3.2% paraformaldehyde (PFA) with 0.2% Triton X-100 in PBS
for 5 minutes at room temperature. Three washes were performed with PBS in the 12-well plate, then coverslips were inverted onto 120 µl of primary antibody in the antibody block (0.1% gelatin with 10% normal donkey serum in PBS) on strips of parafilm and incubated for one hour. Coverslips were first incubated with either ERRβ-2 (1:150) or ERRβ-sf (1:200) for 1 hour. After incubation with primary antibodies, coverslips were washed three times with PBS. Then coverslips were inverted onto 100 µl of antibody block with secondary antibodies (Alexa Fluor 488 anti-mouse - 1:200, Life Technologies #A11029) and DAPI (DNA, 1:500 dilution) for 20 minutes in the dark. Coverslips were again washed 3x with PBS, then gently dipped four times into molecular biology-grade water before inversion onto one drop of Fluoro-Gel (with TES Buffer, Electron Microscopy Sciences, #17985-30) then allowed to air-dry in the dark for at least 10 minutes. Slides were stored at 4°C until image collection on the LCCC Microscopy & Imaging Shared Resource’s Leica SP8 microscope with the 63X oil objective.

3.3.4 Cell Growth Assays

Cells were seeded in 96-well plastic tissue culture plates at 1000 cells/well one day prior to treatment with the indicated concentrations of DY131. Cells were treated for a total of 8-10 days, with media changed and drug replenished on Days 4 or 5. Staining with crystal violet, resolubilization, and analysis of staining intensity as a proxy for cell number was performed as described previously106.
3.3.5 Immunohistochemistry on Human GBM Tumor Samples

Immunohistochemical staining of GBM human tumor samples was performed for ERRβ-2 or ERRβ-sf. Five-micron sections from formalin-fixed paraffin-embedded tissues were de-paraffinized with xylenes and rehydrated through a graded alcohol series.

For ERRβ-2, heat induced epitope retrieval (HIER) was performed by immersing the tissue sections at 98°C for 20 minutes in 10 mM citrate buffer (pH 6.0) with 0.05% Tween. Immunohistochemical staining was performed using a horseradish peroxidase labeled polymer from Agilent (#K4001, #K4003) according to manufacturer’s instructions. Briefly, slides were treated with 3% hydrogen peroxide and 10% normal goat serum for 10 minutes each, and exposed to primary antibody at a 1:125 dilution overnight at 4°C. Slides were exposed to the appropriate HRP labeled polymer for 30min and DAB chromagen (Dako) for 5 minutes.

For ERRβ-sf, HIER was performed by immersing the tissue sections at 98°C for 20 minutes in 110 mM Tris, 1m M EDTA pH 9.0 buffer (Genemed). Immunohistochemical staining was performed using the VectaStain Kit from Vector Labs according to manufacturer’s instructions. Briefly, slides were treated with 3% hydrogen peroxide, avidin/ biotin blocking, and 10% normal goat serum and exposed to primary antibody at a 1:200 dilution overnight at 4°C. Slides were exposed to appropriate biotin-conjugated secondary antibodies (Vector Labs), Vectastain ABC reagent and DAB chromagen (Dako).

For both primary antibodies, slides were counterstained with Hematoxylin (Fisher, Harris Modified Hematoxylin), blued in 1% ammonium hydroxide, dehydrated, and mounted with Acrymount. Consecutive sections with the primary antibody omitted were used as negative controls.
3.3.6 ERRβ Silencing

Short hairpin RNA (shRNA)-mediated silencing of ERRβ-2 and ERRβ-sf in T98G GBM cells was reported previously\textsuperscript{107}. shRNA sequences targeting *ESRRB* are as follows: shESRRB-1: TGAGGACTACATCATGGAT shESRRB-2: TGCAGCACTTCTATAGGT.

3.3.7 Migration Assays

For scratch wound assays, cells were plated at 150,000-200,000 cells/well depending on the cell line and allowed 48 hours to create a monolayer. After monolayer formation, a P200 tip was used to make a scratch and images were taken at 0 hr, 24 hr, 48 hr, and 72 hr time points. Analysis was done in ImageJ (21) to determine percent closed with 0% being at 0 hr. For transwell migration assays, cells were cultured in low serum conditions (0.5% FBS) overnight. The following day, 100,000 cells in 200 μl of 0.5% DMEM were loaded into the top of a modified Boyden chamber (24-well, 8 μm cell culture insert, Becton Dickinson #353097), which was then placed in a 24-well plate containing 500 μl of DMEM supplemented with full serum (10% FBS). Cells were incubated for 4 and 24 hours at 37°C, at which time the nonmigratory cells were removed from the top of the membrane with cotton swabs and the bottom of the membrane was stained with crystal violet as described for cell growth assays. After staining the membranes were excised from the insert, mounted on glass slides using Fluoro-Gel, and allowed to air dry. Cells per field at 20X magnification were counted by light microscopy.

3.3.8 Immunoprecipitation

On day 0, cells were seeded at 200,000/well in 6-well dishes. On day 1, 2.5 μg plasmid DNA (psg5, ERRβ-2, ERRβ-sf, or ERRβ-Δ10), 2.5 μl PLUS reagent (from Lipofectamine LTX, Life
Technologies, #15338100), and Opti-MEM (Life Technologies, #31985070) were used for make a transfection mixture. The transfection reagent was added drop-wise to the cells. 4-6 hours later, medium was changed back to 10% DMEM overnight. On day 2, medium was changed again with fresh 10% DMEM. In the afternoon of day 2, cells were lysed in RIPA for protein extraction. Protein was quantified, and amounts were normalized between all samples to be used in the immunoprecipitation (IP). Total volume for each IP was brought up to 500 µl of RIPA with inhibitors. An aliquot was also set aside for input for each sample. In the evening of day 2, 1 µl of the cortactin antibody (Abcam, #ab81208 - 1:500 dilution) was added to each sample and allowed to rotate overnight at 4°C. The next morning, Protein A/G beads (Pierce, #20421) were vortexed, and 30 µl of beads were added to an eppendorf tube per sample. Beads were then washed in cold RIPA buffer with inhibitors and centrifuged at 4°C for 5 minutes. Supernatant was aspirated, and cold RIPA buffer with inhibitors was added to bring up the volume. Then 30 µl of washed beads were added to each IP tube and allowed to rotate again at 4°C for 1 hour. Tubes were then centrifuged for 5 minutes at 10,000 rpm at 4°C. Supernatant was aspirated, and 500 µl of cold RIPA with inhibitors was used to wash IPs. Tubes were then centrifuged again for 5 minutes at 10,000 rpm at 4°C. IPs were washed 2x more in tris-saline buffer (50 mM tris base pH 7.5, 150 mM NaCl in diH₂O). After the last wash, aspirate supernatant and add 30 µl of 2X loading buffer. Samples were then vortexed and boiled for 9 minutes. Samples were then spun down in a centrifuge at room temperature for 1-2 minutes. Finally, both IP and input samples were loaded onto a 4-12% gradient gel and followed the Western blotting protocol from above.
3.3.9 F-actin Flow Cytometry

Cells were seeded at 100,000 - 200,000 cells/well in 6-well plastic tissue culture dishes then treated with 5 µM DY131 the next day. After 24 hours, cells were collected by trypsinization, combined with nonadherent cells in the culture media, then washed with PBS prior to fixation in 4% PFA for 5 minutes. Fixed cells were permeabilized using 0.2% Triton X-100 in PBS for 5 minutes, then blocked in 0.1% gelatin with 10% normal donkey serum in PBS for 30 minutes at room temperature. Cells were stained in suspension with a 1:300 dilution of Anti-Stain 488 Fluorescent Phalloidin (Cytoskeleton, #PHDG1), gently mixed, and incubated in the dark at room temperature for 30 minutes. Stained cells were washed twice with PBS, then 30,000 cells were acquired by flow cytometry on a BectonDickinson Fortessa. Data was analyzed using FCSExpress 6 (DeNovo Software, Glendale, CA).

3.3.10 Cell Cycle Analysis

On day 0, cells were seeded at 200,000 cells per well in 6-well plastic tissue culture dishes one day prior to treatment with the indicated concentrations of drug. For experiments with TG-003 +/- DY-131, treatment time was 24 hours. After 24 hours, cells were collected, washed with PBS, ethanol-fixed, stained with propidium iodide, and analyzed for cell sub-G1 (fragmented/apoptotic) DNA content and cell cycle profile. 30,000 cells were acquired by flow cytometry on a BectonDickinson Fortessa. Files were modeled using ModFit software (Verity Software, Topsham, ME) to determine sub-G1, G1, S, and G2/M cell cycle stage.
3.3.11 Zebrafish Intracranial Xenografts

The zebrafish model used is a transgenic line with green blood vessels in a double mutant, transparent, background with the genotype Tg(kdrl:GRCFP)zn1; mitfab692/ b692 ; ednrb1b140/ b140. This line is propagated in house by raising equal numbers of fry from five independent group matings, each consisting of three females and three males, in order to maintain background genetic diversity. Correct genotype is determined by visual inspection; the fish lack all pigment except for the eyes and the blood vessels fluoresce bright green. A new generation is raised biennially and the oldest generation is discontinued when they reach two years of age. All procedures were performed in accordance with NIH guidelines on the care and use of animals and were approved by the Georgetown University Institutional Animal Care and Use Committee, Protocol #2017-0078.

Tumor cells were labeled in suspension with a 1:100 dilution of Vibrant CM-DiI cell-labeling solution (ThermoFisher, #V22885) at a density of 5 x 10⁶ cells/ml for 20 min at 37°C. The labeled cells were washed 5 times in PBS, suspended in PBS at 5 x 10⁷ cells/ml, and then back-loaded into a pulled borosilicate microinjection needle (David Kolf Instruments). The needles were placed in a vertical position with the point facing down for 10 min to allow the cells to settle toward the tip. Thirty-six (36) hours post fertilization (hpf) embryos were anesthetized in 160 μg/ml buffered tricaine solution (Sigma-Aldrich). The embryos were loaded onto an injection plate at 37°C and covered with 1.5% low melting point agarose (Fisher Scientific) where they were positioned for injection. The plate was cooled to room temperature and then was covered in fish water (0.3 g/L sea salt) containing 160 mg/ml tricaine. The embryos were injected with 1-2 nl of cell suspension (50-100 cells) into the area of the midbrain-hindbrain border. Following injection, the embryos
were manually freed from the agarose and allowed to recover at 28°C for one hour, followed by incubation at 33°C. At 5 days post fertilization (dpf), tricaine-anesthetized embryos were mounted in 3% methyl cellulose and imaged on an Olympus XI-71 Inverted epifluorescence microscope. Following imaging, the fry were transferred to 24-well plates in 1ml fish water per well. At 10 dpf, fry were anesthetized with 112 μg/ml tricaine, mounted in 3% methyl cellulose, and imaged on an Olympus XI-71 Inverted epifluorescence microscope, or, mounted in 1.5% low melting point agarose for imaging on a Leica SP8 confocal microscope.

Treatment groups were assigned to achieve similar numbers of larvae containing similar distributions of tumor sizes. The larvae were treated starting at 6 dpf. Final drug concentrations were made using 1:1000 dilution of stock in DMSO. Control larvae were treated with 0.1% DMSO. The drugs were refreshed daily by removing most of the solution, leaving ~100 μl to keep the fry wet, followed by the addition of 1 ml of fresh drug solution. For blood-brain barrier (BBB) imaging experiments, Cascade Blue dextran, 10,000 MW (ThermoFisher, #D1976) at 25 mg/ml was back-loaded into a pulled borosilicate microinjection needle. 10 dpf larvae were anesthetized in 112 μg/ml buffered tricaine solution and loaded onto an injection plate at 37°C and covered with 1.5% low melting point agarose, where they were positioned for injection. Approximately 2 nl of Cascade Blue dextran solution was injected into the common cardinal vein. The injected larvae were then mounted in 1.5% low melting point agarose and imaged on a Leica SP8 confocal microscope at 1 hour post injection.

### 3.3.12 Computational, Image, and Statistical Analysis

Images and figures were compiled using either Adobe Photoshop or Illustrator. Densitometry of the ERRβ2:ERRβsf ratio was calculated using ImageJ. Calculation of zebrafish intracranial xenograft tumor size was carried out in Illustrator as follows, by investigators blinded to treatment group: Zebrafish images
were imported into Illustrator. The red channel was selected, which highlighted the GBM cells as DiI fluoresces red. The “magic wand” tool was chosen to select the red fluorescent tumor, where after selection area was calculated by Illustrator’s “measure area” function. These measurements were exported and pre- and post-treatment tumor areas were compared. Motif analysis of ERRβ2 F domain sequences was performed using ScanSite3.0. Statistical analysis and graphing were performed using GraphPad Prism 8.0, with the following exceptions. Supplementary Figure 1A was generated using RNAseq data from EMBL-EBI (dataset E-MTAB-4840). Supplementary Figure 3A was generated using RNAseq data from the Chinese Glioma Genome Atlas through the International Cancer Genome Consortium (https://icgc.org) analyzed in R using GGally and ggplot2 packages. Supplementary Figure 3B was generated and analyzed in GraphPad Prism 8.0 from RNAseq data obtained through the Ivy Glioblastoma Atlas Project. SRSF6 consensus binding sites in ESRRB exon 10 were predicted using RBPmap. Data are presented as the mean ± standard deviation (SD) unless otherwise indicated. The number of biological, technical replicates, and specific statistical tests performed are reported in the figure legend for each figure. Statistical significance is defined as α ≤ 0.05.
3.4 Discussion and Limitations

Here, we show that the ERRβ agonist, DY131, is growth inhibitory in multiple GBM cell lines. The cytoplasmic and nuclear ERRβ-2 isoform is expressed in multiple GBM cell lines, PDX models, and human tumors, where it suppresses GBM cell migration, and interacts with the actin nucleation-promoting factor cortactin. Treatment with the ERRβ agonist DY131 remolds the actin cytoskeleton and suppresses migration. Furthermore, we show that broad CLK inhibition shifts ERRβ isoform expression in favor of ERRβ-2 and shows a non-significant trend towards enhancement of ERRβ agonist-mediated inhibition of growth and migration in GBM cells and intracranial tumors (Figure 7E).

ERRβ-2, unlike ERRβ-sf, is specifically expressed in primates and localizes to both the cytoplasm and the nucleus. Altered subcellular localization of splice variants is a common phenotype, especially in brain cancer and brain development (e.g. 112), and suggests a specialized function of the cytoplasmic primate-specific ERRβ-2 nuclear receptor isoform. Inclusion of a unique F domain may allow ERRβ-2 to bind cortactin, an essential regulator of the actin cytoskeleton and cell motility in GBM, gives further insight into its specific function. GBM is a highly migratory tumor in which cortactin plays a critical role113,114. We show that the ERRβ agonist DY131 decreases migration, while shRNA-mediated silencing of ERRβ-2, but not ERRβ-sf, increases the migration of GBM cells in the absence of this agonist.

In the absence of established nucleotide-based strategies, we looked to splicing modulatory drugs, specifically splicing kinase inhibitors, to increase the ERRβ-2:ERRβ-sf ratio. We show that the pan-CLK inhibitor TG-003 in combination with DY131 increases the relative expression of the ERRβ-2 isoform in two TMZ-resistant GBM cells and causes a more robust inhibition of G2/M arrest and migration which have previously been shown to be ERRβ2-dependent. Coupled with
the knowledge that differential splicing is a critical driver of phenotypic diversity and plasticity in the malignant brain\textsuperscript{115}, our results suggest a new paradigm in which splicing modulatory drugs could be an effective approach to inhibit GBM migration and invasion by changing the balance of pro- versus anti-migratory isoforms of ERRβ and other proteins. TG-003 is a broad spectrum CLK inhibitor, and we do not yet know which of the CLKs are most important for shifting isoform expression in favor of ERRβ-2. Sakuma \textit{et al.} showed that the strength of uracil-rich polypyrjdine tracts, exon length, and abundance of splicing factor binding sites characterize TG-003-responsive exons in human and mouse skeletal muscle cells\textsuperscript{116,117}. Additional studies are necessary to determine how \textit{ESRRB} sequence features and the repertoire of RNA binding factors beyond SR proteins dictate context-dependent pre-mRNA processing and the expression of distinct ERRβ isoforms. Nevertheless, we provide evidence that ERRβ, specifically the ERRβ-2 isoform, has pro-apoptotic and anti-migratory functions in GBM, and that splicing modulatory drugs such as CLK inhibitors are a novel strategy for shifting the balance of ERRβ isoforms to potentiate ERRβ agonist-mediated inhibition of growth and migration in GBM cells and intracranial tumors.

Poor brain penetrance is also a common cause of failure for therapeutic agents in GBM clinical studies despite apparently robust anti-tumor activity \textit{in vitro}\textsuperscript{35}, which is why intracranial GBM models are an essential preclinical test of candidate small molecules and treatment strategies. We show that the combination of DY131 and TG-003 restricts the growth of TMZ-resistant intracranial GBM xenografts in a context in which the BBB remains intact. Zebrafish are an ideal model organism for early-phase screening of GBM intracranial xenografts, due to their evolutionarily conserved BBB structure, capacity for intrinsic neovascularization of implanted tumors, and in pigmentation-deficient transgenic animals, tumor development and response to treatment can be tracked in real time in live animals\textsuperscript{41}. While others\textsuperscript{41} show that DY131 and TG-
003 are brain penetrant and our data demonstrate that these small molecules have anti-tumor activity in combination, further optimization and drug development efforts will be necessary to improve the efficacy of these and other splicing modulatory drugs and nuclear receptor ligands.

A potential limitation of our study is that, aside from co-immunoprecipitation studies in Figure 3-3C, it does not address the contribution of ERRβ-Δ10. The field currently lacks reagents that are selective enough to study the endogenous function of the ERRβ-Δ10 isoform. The extended carboxyl-terminal F domain of the exon 10-deleted (ERRβ-Δ10) isoform has a proline-rich sequence that conforms more closely to the consensus binding motif for the SH3 domain-containing protein amphiphysin (Supplementary Table 1), yet we show that exogenously expressed ERRβ-Δ10 is unable to interact with endogenous cortactin (Figure 3-3C). Previously published studies in cells transfected with cDNA for ERRβ-Δ10 show that this isoform, like ERRβ-sf, localizes to the nucleus (and not the cytoplasm), and has transcription factor activity. Another limitation is that ERRβ agonist ligands can have activity against other members of the estrogen-related receptor family, or off-target effects. DY131 is also an agonist for the closely related ERRγ, and a prior report implicates this compound as an antagonist of Hedgehog signaling. However, we have shown previously that shRNA-mediated silencing of ERRγ does not abrogate DY131-mediated phenotypes, and the Hedgehog pathway inhibitors cyclopamine and vismodegib do not phenocopy DY131 in GBM cells.

Overall our current work builds upon prior studies that first established the more robust anti-tumor effects of the ERRβ-2 isoform. This suggests that changing the balance between ERRβ-2 and ERRβ-sf could successfully limit GBM migration and local invasion. However, si/shRNA-dependent strategies have limitations and few clinical successes. Splice-switching antisense oligonucleotides (AONs) designed to modify isoform expression are emerging as useful
approaches in musculoskeletal disorders\textsuperscript{32}, but to our knowledge they have not yet been tested in GBM. Therefore, we looked to change the isoform ratio by using small molecule inhibitors, and then target ERR\(\beta\) with its known agonist DY131. Future studies will need to be completed to determine more specific splicing modulating drugs, but this study provides a potential framework to use small molecule splicing modulators in place of si/shRNA-dependent strategies for isoform shifting.

**Limitations**

One of the key limitations to this study is the lack of data demonstrating a direct interaction of pSRSF6 with the pre-mRNA of ERR\(\beta\)-2. I attempted several *in silico* or bioinformatic studies correlating the expression of both ERR\(\beta\) transcript variants with overall survival, but it quickly became apparent that these two transcripts are not abundantly expressed in cell lines or patient samples. Using qRT-PCR methods, both transcripts are detected between cycle 37-39, so I would not be confident in drawing conclusions from these results compared to background noise. In the mouse, which only expresses ERR\(\beta\)-sf, transcript half-life is less than 3 hours\textsuperscript{123}. Therefore, working with the RNA was largely dropped due to technical difficulties, and my focus was shifted towards changing the protein ratios towards the more pro-apoptotic ERR\(\beta\)-2 isoform. ERR\(\beta\)-2 protein can be reproducibly detected across our cell models, though it is necessary to load 55-65 \(\mu\)g of whole cell lysate. Identifying the cause(s) of discrepancy between ERR\(\beta\) RNA levels and protein production are a future goal of our laboratory.

Another limitation of this study is that we also only show overexpression data for the interaction of ERR\(\beta\)-2 and cortactin. Overexpression of ERR\(\beta\)-2 is used as a negative control to determine the “stickiness” of the protein to the agarose beads, but more rigorous studies using site-
directed mutagenesis will be needed to determine the actual interacting domain(s) of ERRβ-2 with cortactin, as will studies that test the interaction of endogenous ERRβ-2 and cortactin.

A third limitation of the study was the lack of statistically significant effect of the DY131 + TG-003 combination in the intracranial in vivo model. Even though zebrafish models for intracranial tumor engraftment have been used previously for GBM, the timeframe in which we wanted to inject cells – pre BBB formation – was not well tolerated by the fish. It is worth noting that because zebrafish viability is optimal at 28°C and our cell lines are adapted to grow at 37°C, a typical compromise employed by zebrafish xenograft studies (including ours) is to maintain the fish at 33°C. This may explain both the poor tolerance of the embryos to intracranial injection and the shrinkage shown in the DMSO treated cells. Orthotopic injections in immunocompromised mouse models are an alternate approach and future strategy that will be considered to circumvent this limitation.
CHAPTER 4
CONCLUSIONS

As approximately 98% of current drugs on the market are unable to cross the BBB\textsuperscript{124}, continued dependence on TMZ is the reality for brain cancers where TMZ resistance is a major clinical problem. While TMZ with surgery and radiation is the standard of care for GBM, it is also used in other brain cancers like astrocytomas and oligodendrogliomas\textsuperscript{125}. Newly diagnosed anaplastic astrocytoma, and recurrent anaplastic astrocytoma are treated with both radiation and concurrent adjuvant TMZ, like GBM patients\textsuperscript{126}. TMZ treatment has also shown clinical benefit for both newly diagnosed and recurrent oligodendrogliomas with the addition of radiation\textsuperscript{127}. Also like GBM, cytosine methylation of CpG islands in the MGMT promoter has been proposed as a prognostic marker in other central nervous system cancers\textsuperscript{128}. Contrary to these findings in brain cancers, MGMT promoter methylation does not seem to have the same prognostic value in colorectal cancer\textsuperscript{129} and non-small-cell lung\textsuperscript{130} cancers in terms of their response to TMZ\textsuperscript{131}. As TMZ is broadly used in many central nervous system cancers, and drug resistance is a major clinical problem, further studying molecular mechanisms of TMZ-resistance may be broadly beneficial for multiple brain cancers.

Genetically diverse TMZ-resistant models are lacking in the scientific literature. Therefore, I wanted to create new cell culture model systems of acquired TMZ-resistance to both observe the phenotypes associated with the acquisition of resistance and use these models in future studies. Using both \textit{in vitro} and \textit{in vivo} markers of resistance that have been previously described\textsuperscript{50}, I observed and characterized robust changes in nuclear morphology, proliferation, and cell motility. While the use of cell lines will not directly translate to the clinic, adding more model systems to the already defined cell line, patient-derived xenograft, and mouse models of GBM may aid future
studies to determine the dependent pathways of TMZ-resistance and how this may be targeted in the future. One emerging model system is primary, patient-derived glioblastoma stem-like cells (GSCs). Recent work has implicated GSCs as having a high baseline rate of DNA damage response and repair, which is thought to drive resistance to radiotherapy and potentially TMZ.

An area of research that has shown increasing importance in both cancer and brain development is splicing. The brain has the most diverse transcriptome of any organ, but the functional consequences of this diversity are poorly studied in both normal brain and cancer. Our lab has already characterized two isoforms of the ERRβ protein that have opposite effects on cell cycle regulation and transcription factor activity. As my goal was to provide potential avenues for second-line treatment of TMZ-resistant GBM, I began to determine the splicing factors that may dictate ERRβ isoform fate. Choosing to focus on the pro-apoptotic ERRβ-2 isoform, I found SRSF6 binding sites in the ERRβ-2-specific exon 10 and pSRSF6 abundance correlated with ERRβ-2 expression in patient samples. Using multiple GBM cell line models, patient samples, and an in vivo zebrafish model, I showed that the combination treatment of an ERRβ agonist and splicing modulator decreases tumor cell migration, increases G2/M cell cycle arrest and apoptosis, and trends toward shrinking tumor size in vivo. The use of these two drugs mimics the effect of genetic manipulation with shRNAs that increases expression of the pro-apoptotic ERRβ-2 isoform. Alternatively-spliced isoforms commonly elicit different functions or have different cellular localizations. One well-known alternatively spliced gene with functional relevance in cancer is the Bcl-X gene where the short isoform, Bcl-xS, is pro-apoptotic and the long isoform, Bcl-xL is anti-apoptotic. Shifting the isoform ratio towards the short pro-apoptotic isoform by many different methods, including splice-switching oligonucleotides and engineered splicing factors, is an active area of research for treating cancers and other pathologies. Not only can
isoforms have differential roles in cell death, but also differential localization within the cell. One well-studied protein in brain development and autism is Rbfox1. Rbfox1 is known to be alternatively spliced where the exclusion of exon19 gives rise to a nuclear-localized protein and inclusion of exon 19 translates to a cytoplasmic-located protein. These different cellular localizations dictate function, whereby the nuclear Rbfox1 plays a role in splicing regulation, but the cytoplasmic Rbfox1 predominately affects mRNA stability and translation.

There are many different avenues for targeting alternative splicing in cancer. In GBM specifically there was a recent report showing a dependency on the arginine methyltransferase PRMT5 in a murine model of glioma (GL261 cells). PRMT5 has been suggested to regulate splicing upstream as part of the methylosome, which modifies small nuclear ribonucleoprotein (snRNP) assembly. Using both immune competent and immunodeficient mouse models with mouse and human glioma cells, respectively, Braun et al. showed a significant benefit in overall survival in flank tumors treated with the PRMT5 inhibitor, EPZ015666 (EPZ). When tumors were implanted intracranially, EPZ no longer showed therapeutic efficacy, again showing the impact the BBB has on GBM treatment failure. However, when PRMT5 was genetically silenced in GBM cells that were implanted intracranially, these mice saw significant increases in overall survival and even some “long-term survivors”. This study suggests that targeting the regulation of alternative splicing in GBM may produce significant therapeutic effects, if therapeutic strategies are able to cross the BBB.

In conclusion, the first goal of this body of work was to add two unique TMZ-resistant cell culture models to aid in determining molecular mechanisms of TMZ-resistance in GBM. As many brain cancers are treated with TMZ, understanding drug resistance may have broad effects for glioma patients in the future. Secondly, increasing our knowledge of the functional role of
antagonistic isoforms, like ERRβ, in GBM will help with our understanding of the biological roles differential isoform functions and localizations play as well as untapped treatment potential in the future.
APPENDIX A

TARGETING DESTABILIZED DNA G-QUADRUPLEXES AND ABERRANT SPlicing IN AMYLOID-LIKE AGGREGATE POSITIVE DRUG-RESISTANT GLIOBLASTOMA

A.1 Rationale

TMZ causes DNA damage by adding mutagenic adducts to DNA, favoring O6-methyl guanine. Methyl guanine methyl transferase (MGMT) is a suicide DNA repair protein that removes the O6methyl adduct from guanine. The overall survival benefit provided by TMZ is approximately four months and this rapid development of TMZ resistance occurs at least in part through increased expression of MGMT\(^{143}\). However, many attempts to inhibit the activity of MGMT to re-sensitize tumors to TMZ have been unsuccessful\(^{144}\). While this seems like a sound path for second-line treatment, TMZ preferentially targets guanines, like most chemotherapeutic options\(^{81}\), which are critical nucleotides in many DNA and RNA secondary structures\(^{145}\). Mechanistic studies in other central nervous system disorders and neurodegenerative diseases have shown that disruption of DNA and RNA secondary structures can cause nucleolar stress, change alternative splicing decisions, and alter RNA binding protein localization\(^{146}\). Nucleolar stress, which is determined by changes in nucleolar size and circularity, correlate with cancer outcomes\(^{147}\). Alternative splicing changes are widespread in GBM, with Braun et al suggesting that GBM tumors are “addicted to splicing”\(^{148}\). Many RNA binding proteins have also been shown to be upregulated during cancerous transformation where higher expression is linked to worse overall survival\(^{149}\). These splicing decisions are made in part by proper regulation of RNA binding proteins. One family with essential functions in cancer and central nervous system disorders is the FET family of proteins that consists of FUS, EWSR1, and TAF15\(^{150}\). The aggregation-prone properties of this family have led to FUS being used as a model to study protein aggregation, and a biomarker for ALS and FTLD\(^{151}\). EWSR1 has also been shown to have the same aggregation-
prone properties as its family members, but is commonly studied as the translocation partner of FLI1 in the EWS-FLI1 fusion in Ewing’s sarcoma. FET family members have also been shown to be involved in DNA repair, able to bind the G-quadruplex secondary structure of DNA and RNA, and play a role in isoform fate.

Here, we exploit the known mechanism of action of TMZ, a mutagenic O6-methyl guanine adduct, to study the role of guanine mutations in changing DNA and RNA secondary structures in TMZ-resistant GBM. We further propose two potential second-line therapeutic strategies for TMZ-resistant GBM. We found a differential staining pattern of G-quadruplexes (G4s) and mutations at splice sites that can be specifically targeted in TMZ-resistant cell lines with a G4-stabilizing drug (TMPyP4) and a novel splicing modulator (CLK2i), respectively. We also found mislocalization of the G4-binding and RNA-binding protein EWSR1, which forms amyloid-like cytoplasmic aggregates in the TMZ-resistant cells specifically.

A.2 Results
A.2.1 TMZ-induced guanine mutations
Temozolomide (TMZ; Temodar) is the FDA-approved standard of care first line therapy for glioblastoma (GBM) and is known to add mutagenic adducts to guanine - the most prevalent being O6-methyl guanine. To address the role of guanine (G) mutations in TMZ-resistance we performed whole genome sequencing (WGS) on TMZ-sensitive (42MBGA), acquired TMZ-resistant (42MBGA-TMZ-res), and intrinsically TMZ-resistant (T98G) cell lines. Both acquired and intrinsic TMZ-resistant lines had increased overall mutations as compared to the TMZ-sensitive line (Figure A-1a). Further analysis showed that the two mutations that had the largest increase between TMZ-sensitive and -resistant lines were G>A, and C>T (Figure A-1b). These mutations
corroborate what others have defined as mutational signature 11, or increased C>T mutations, that are enriched in tumors treated with alkylating agents. Two G-rich regions that interested us were G-quadruplex forming regions and splice sites, as their presence and functional impact can be validated by immunofluorescence (IF) staining with a monoclonal antibody and RNA sequencing, respectively. We first focused on G-quadruplexes (G4s) as they can be easily detected by IF, and we hypothesized that the G adducts from TMZ may disrupt G4-structure (Figure A-1c). Confirming what others have previously published, the monoclonal anti-G4 antibody BG4 recognizes DNA-based G4s in vivo, since treatment of 42MBGA cells with DNAseI abrogated specific nuclear BG4 staining (Figure A-1d; Sup fig A-1a). In the TMZ-sensitive line, obvious puncta can be seen by IF with the G4-specific antibody (BG4) as well as discrete nucleolar staining (Figure A-1e; white arrows). However, in the acute TMZ-treated and both TMZ-resistant cell lines, there is no longer a punctate G4 nuclear staining pattern (Figure A-1f-h). This stain only tells us that the BG4 antibody can no longer react with its epitope in the TMZ-resistant cells. While we could do BG4 ChIP-seq, which has been previously published, this would only tell us what sites the antibody is bound to in the TMZ-sensitive cells, as there is reduced specific reactivity in the TMZ-resistant cells. We also know from our WGS data that matching regions between the cell lines, if we were to map the BG4 ChIP-seq reads, is bioinformatically difficult because the resistant cells have acquired many mutations, insertions and deletions. Therefore, we decided to treat with a G4-targeting drug to show a potential difference in the functional dependence on G4s between the cell lines. We hypothesized that if disruption of G4 sequences played a role in TMZ-resistance, a G4-stabilizing drug may be more effective in TMZ-resistant cells (Figure A-1c). TMPyP4, a G4 stabilizing drug (Sup Figure A-1b,c.), treatment showed a significant G2/M arrest, decrease in cell proliferation, and increase in sub-G1 fraction in both the acquired and intrinsic TMZ resistant
cell lines, but had minimal effect on growth or cell cycle profile in the TMZ sensitive cell line (Figure A-1 i-k). This supported our hypothesis of G mutations being a potential target in TMZ-resistant GBM. G4s are abundant in nucleoli which function as major stress organelles in cells undergoing DNA damage and can be used as a predictive marker for cancer outcomes. Therefore, we next probed nucleolar changes induced by TMZ treatment and resistance.

A.2.2 Acquired nucleolar changes with TMZ-induced mutations in g-quadruplexes
Nucleoli are major stress organelles formed in part by regions of 5 chromosomes: 13, 14, 15, 21, and 22. The iNO score is a method of quantifying nucleolar stress that takes into account both size and roundness of nucleoli. Using IF detection of the nucleolar binding protein nucleolin (NCL) to determine nucleolar size and circularity changes (Figure A-2a), we see significant changes to nucleolar size in relation to TMZ treatment and intrinsic resistance (Figure A-2b), as well as significant changes in circularity, or the nucleolar roundness, of TMZ treated and both acquired and intrinsic drug resistant nucleoli (Figure A-2c). Within the nucleoli, DNA polymerase I transcribes ribosomal RNAs (rRNA), which when in a GC-rich region are able to hybridize back to their antisense ribosomal DNA (rDNA) to form an rRNA:rDNA r-loop, often opposite of a G-quadruplex (Figure A-2d). Continuing to focus on nucleic acid structure changes, we used the r-loop specific antibody S9.6 to stain cells for nucleolar r-loop changes when treated with TMZ (Figure A-2f), compared to the nucleolar staining in 42MBGA-WT (Figure A-2e). However, TMZ-resistant cell lines showed a robust increase in nucleolar r-loop accumulation, rather than nucleoplasmic puncta (Figure A-2g-i). A potential explanation of increased nucleolar r-loops is an increase in the rDNA and rRNA. To test rRNA changes, we used the Click-IT reaction.
between a Uracil-azide and fluorescent alkyne to label nascent RNA and visualize by IF (Sup Figure A-2a). While we do see an increase in nascent nucleolar rRNA in the TMZ-resistant cell line (Sup Figure. A-2b), this only holds true for the small ribosomal subunit at the protein level (Sup Figure A-2c), and no broad changes in translation (Sup Figure A-2d,e). These data suggest more of a DNA and RNA-mediated role for the rRNA/r-loop changes vs. downstream ribosomal protein translation effects, though this would require further study to confirm.

A.2.3 CLK2 inhibition specifically targets TMZ-resistant cells

5’ to 3’ splice sites (GU- [branch point A] - AG) consist of 50% G’s. TMZ-induced G mutations led to increased splice site mutations (Figure A-3a). Splicing mutations have previously been discovered in many cancers, but targeting splicing broadly has proven to be difficult\textsuperscript{161}. For this reason, we looked at splicing factor families that may be targetable and important in TMZ resistance. Serine/arginine rich proteins (SRs) are a major splicing factor family whose activity is dependent on their phosphorylation status and have been shown to change localization after UV-induced DNA damage\textsuperscript{145}. Looking at changes in phosphorylated SRs (pSRs) pre- and post-TMZ treatment, we observed a link between splicing and TMZ-induced DNA damage with a decrease in the phosphorylation of pSR proteins only in the TMZ-sensitive cells (Figure A-3b). pSR proteins are one family of splicing regulatory proteins which can bind to both introns and exons to determine transcript variant fate through exon inclusion/exclusion status. SR proteins that are not hyperphosphorylated may not be able to modify transcript variant abundance\textsuperscript{7}. Therefore, we sought to recapitulate the decrease of pSRs we observed in the TMZ-treated sensitive cells in the resistant cells by targeting the cdc2-like kinases (CLKs). CLKs catalyze the hyperphosphorylation of multiple SR proteins\textsuperscript{162}, with clinical data suggesting a role specifically for CLK2 in brain...
cancers (Figure A-3c,d). Our cell line models also show an increase in CLK2 expression with both acquired and intrinsic resistance (Figure A-3e). Treatment with a novel and selective CLK2 inhibitor (CLK2i) shows a decrease in SR phosphorylation at 30 minutes (Sup Figure A-3a-c), and specifically causes a significant G2/M arrest, apoptosis, and decrease in cell growth in the TMZ-resistant cells (Figure A-3f-h). In an immortalized oligodendrocyte cell line, MO3.13, CLK2i treatment caused no significant changes to growth rates or cell cycle, with a modest increase in the apoptotic fraction (Figure A-3f-h).

### A.2.4 EWSR1 mislocalization and amyloid-like aggregation in TMZ-resistant cells

Critically evaluating the changes in G4s, nucleoli, and splicing we sought to find a potential biomarker to predict the efficacy of our novel CLK2i in TMZ-resistant cells. EWSR1 has been shown to bind G4 structures\(^\text{15}\), relocate to the nucleolus under UV-induced stress by DNA damage\(^\text{163}\), and plays a role in splicing. EWSR1 is member of the FET family of proteins, which also includes FUS and TAF15, where FUS is an established biomarker in many neurodegenerative diseases\(^\text{164}\). Therefore, we determined EWSR1 localization between sensitive and resistant cell lines. A basal stain of EWSR1 showed a dramatic difference between nuclear staining in TMZ-sensitive cells and cytoplasmic amyloid-like aggregates in TMZ-treated and resistant cells (Figure A-4a), while overall expression of EWSR1 did not increase with TMZ resistance (Figure A-4d). High resolution stimulated emission depletion microscopy (STED) showed discreet proteins in the cytoplasmic aggregates found in both resistant cell lines (Figure A-4b,c). We also noted that the amyloid-like aggregates were specific to the cytoplasm, but not present in the nucleus. This suggested a potential role for RNA buffering, as has been previously shown\(^\text{165}\). Using Actinomycin D (ActD) to inhibit RNA synthesis, we see induction of flower-like droplets of EWSR1 that
accumulated around nucleoli (Figure A-4e). Further investigation via STED imaging shows distinct EWSR1 nucleolar structures. The 42MBGA cell line horseshoe shaped staining patterns do not link to each other (Figure A-4f), whereas in both acquired and intrinsic resistant cells EWSR1 forms linked structures (Figure A-4g,h). This may suggest a role for RNA buffering that should be studied further in the future. A co-stain of EWSR1 and the DNA G4 antibody showed colocalization of EWSR1 and G4 in the TMZ-sensitive cell line which was gradually lost with TMZ treatment and resistance suggesting DNA secondary structure changes to perturb G4-binding proteins (Sup Figure A-4a-c). However, the EWSR1 amyloid-like aggregates do not seem to be necessary for TMZ-resistance, as silencing of EWSR1 has minimal effect on cell cycle profile or cell death in the TMZ-resistant cell line (Sup Figure A-5a-d). EWSR1 can also be trafficked to and from the cytoplasm efficiently in both TMZ-sensitive and -resistant lines as shown with cytoplasmic:nuclear fractionation or treatment with Leptomycin B (LMB), an inhibitor of the nuclear export protein CRM1 (Sup Figure A-3f-i).

### A.2.5 EWSR1 amyloid-like aggregates in patient samples

A major limitation of the study thus far is the dependence on created or acquired cell lines which have been in culture for decades. Therefore, we screened a cohort of 21 brain cancer patient samples from Lombardi Cancer Center. After selecting for GBM patient samples, we found 9 of the 15 samples to have EWSR1 cytoplasmic amyloid-like aggregates in tumors that are MGMT positive (Figure A-5). Future studies are needed to determine the role of EWSR1 amyloid-like aggregation in TMZ-resistance.
Figure A-1. Effect of guanine mutations on sensitivity to g-quadruplex drugs. A. Mutation changes by type by whole genome sequencing. B. Mutation burden of G>A and C>T transition mutations. C. Schematic of my hypothesis targeting g-quadruplexes (G4s). D. Schematic of the BG4 antibody binding epitope. E-H Immunofluorescent staining of the g-quadruplex antibody BG4 in E.TMZ-sensitive 42MBGA, F. 72hr-treated 42MBGA, and TMZ-resistant G. 42MBGA-TMZ-res, and H. T98G with surface plots of the yellow nuclear outline of the BG4 staining below. I. Cell cycle analysis of 50 µM TMPyP4 or DMSO in 42MBGA, 42MBGA-TMZ-res, and T98G cells. J. Same conditions as I, growth rates using FACS. K. Same conditions as I,J sub-G1 fraction from cell cycle analysis.
Supplementary Figure A-1. BG4 and TMPyP4 controls. a. DNase 1 treatment of 42MGBA cells ameliorates the epitope of BG4. b. Treatment with 50 µM TMPyP4 for 24 hours in 42MBGA-TMZ-res increases the discreet puncta of BG4 staining. Surface plot depicted on the right.
Figure A-2. Nucleolar changes upon TMZ treatment and resistance. a. Representative images of endogenous nucleolin (NCL) IF. b. Quantification of the nucleolar area from images in a. c. Quantification of nucleolar circularity from images in a. d. Graphic depicting potential g-quadruplex and r-loop structures and their corresponding antibodies. IF of the r-loop binding s9.6 in e. 42MBGA, f. 72hr TMZ treatment in 42MGBA, g. 42MBGA-TMZ-res, h. T98G. I Quantification of r-loop intensity in e, g and h.
Figure A-3. Splice-site mutations and CLK2i sensitivity in TMZ-resistant cells. a. Quantified splice-site mutations between TMZ-sensitive and -resistant cells. b. Quantified IF of pSR (1H4) intensity in cells with DNA damage (γH2AX+) or without (γH2AX-). c. Overall survival for REMBRANDT brain cancers. d. CLK2 expression in the same REMBRANDT cohort. e. Western blot of CLK2 expression. f. Cell cycle analysis of 5 µM CLK2i for 24 hours. g. Apoptotic fraction from f. h. Relative cell numbers with 24 hr treatment of 5 µM CLK2i.
Supplementary Figure A-3. Specificity of CLK2i. a. NanoBRET assay of CAF-022 (CLK2i) with other CLK family members in vivo. b. Western blot of pSR changes with a timecourse of CLK2i treatment. c. Quantification of pSRSF5 in b to determine the lowest phosphorylation status and optimal effect of CLK2i on pSR abundance.
Figure A-4. EWSR1 aggregation in TMZ-resistant GBM. a. Basal IF of EWSR1 in depicted cell lines, with the nucleus outlined in yellow. Stimulated emission depletion microscopy (STED) imaging of EWSR1 cytoplasmic aggregates in b. 42MBGA-TMZ-res and c. T98G cells. d. Western blot of EWSR1 basal expression between cell lines. e. Movement of EWSR1 to the nucleoli after 3 hr 10nM Actinomycin D treatment. STED imaging of EWSR1 nucleolar movement post 3 hr ActD treatment in f. 42MBGA, g. 42MBGA-TMZ-res, h. T98G cells.
Supplementary Figure A-4. EWSR1 movement from G4s. Co-stain of the G4 antibody BG4 with EWSR1 in a. 42MBGA, b. 42MBGA-TMZ-res, c. T98G cells. Merge has an inset of BG4 and EWSR1.
Supplementary Figure A-5. Characterization of EWSR1. a. Western blot of EWSR1 with four different siRNAs. Cell cycle analysis with 72 hours of EWSR1 depletion in b. 42MBGA, c. 42MBGA-TMZ-res, d. T98G cells. e. Sub-G1 from b-d. Fractionation of f. TMZ-sensitive cell line and g. 42MBGA-TMZ-res showing EWSR1 in both cytoplasm and nucleus. 24 hour treatment of 10 nM of the nuclear import/export inhibitor Leptomycin B (LMB) in h. 42MBGA, and i. 42MBGA-TMZ-res cells.
Figure A-5. EWSR1 aggregates in patient samples. Representative IHC of EWSR1 in GBM patient samples with EWSR1 cytoplasmic aggregates. Inset shows zoomed version of EWSR1 aggregate + cells.
A.3 Materials and Methods

A.3.1 Cell Lines and Culturing Conditions

Immortalized human oligodendrocyte MO3.13 cells were a kind gift from Dr. Alexandra Taraboletti (Lombardi Comprehensive Cancer Center, LCCC). Temozolomide (TMZ) sensitive 42MGBA and 8MGBA cell lines were provided by Dr. Jeffrey Toretsky (LCCC), and the de novo TMZ resistant T98G cell line was provided by Dr. Todd Waldman (LCCC). Acquired TMZ resistant 42MGBA-TMZ-res and 8MGBA-TMZ-res cell line variants were developed by our lab and previously described (20). All cells tested negative for Mycoplasma contamination and were maintained in a humidified incubator with 95% air: 5% carbon dioxide. All cell lines were fingerprinted by the LCCC Tissue Culture Shared Resource to verify their authenticity using the standard 9 STR loci and Y-specific amelogenin. Both the 42MGBA-TMZ-res and 8MGBA-TMZ-res are documented to be of the same origin as their respective parental cell lines. MO3.13, 42MGBA, 8MGBA, 42MGBA-TMZ-res, and T98G cells were grown in Dulbecco's Modified Eagle Medium (DMEM, high glucose, ThermoFisher, #11965092) with 10% FBS. 8MGBA-TMZ-res cells were grown in DMEM with 10% FBS and 100 µM TMZ. TMZ (Selleckchem, #S1237) was dissolved in dimethyl sulfide (DMSO, Sigma, #D8418) to 130 mM and used at concentrations indicated.

A.3.2 Immunofluorescence

Cells were seeded at a density of 25,000-30,000 cells onto 18mm diameter #1.5 round coverslips (VWR, #101413-518) in 12-well dishes (day1). They were allowed to attach to the coverslips for a full day (day2). On the following day (day3), the media was removed, cells were washed 3x with PBS, and then fixed and permeabilized in 3.2% paraformaldehyde (PFA) with 0.2% Triton X-100
in PBS for 5 minutes at room temperature. Three washes were performed with PBS in the 12-well plate, then coverslips were inverted onto 120 μL of primary antibody in the antibody block (0.1% gelatin with 10% normal donkey serum in dH2O) on strips of parafilm and incubated for two hours. Coverslips were first incubated with either BG4 (1:150), EWSR1 (1:600), s9.6 (1:150), or NCL (1:500) for 2 hours. The donkey serum, non-confluent cells, and day 3 staining is vital for visualizing consistent EWSR1 cytoplasmic staining. After incubation with primary antibodies, coverslips were washed three times with PBS. Then coverslips were inverted onto 100 μl of antibody block with secondary antibodies (Alexa Fluor 488 anti-mouse - 1:200, Life Technologies #A11029; Alexa Fluor 594 anti-rabbit – 1:200, Life Technologies A11037) and DAPI (DNA, 1:500 dilution) for 20 minutes in the dark. Coverslips were again washed 3x with PBS, then gently dipped four times into molecular biology-grade water before inversion onto one drop of Fluoro-Gel (with TES Buffer, Electron Microscopy Sciences, #17985-30) then allowed to air-dry in the dark for at least 10 minutes. Slides were stored at 4°C until image collection on the LCCC Microscopy & Imaging Shared Resource’s Leica SP8 microscope with the 63X oil objective at 1.52 magnification.

A.3.3 DNAseI treatment

Cells were seeded at a density of 25,000-30,000 cells onto 18mm diameter #1.5 round coverslips (VWR, #101413-518) in 12-well dishes (day1). They were allowed to attach to the coverslips for a full day (day2). On the following day (day3), the media was removed, cells were washed 3x with PBS, and then fixed and permeabilized in 3.2% paraformaldehyde (PFA) with 0.2% Triton X-100 in PBS for 3 minutes at room temperature. 10 μl of a 1000 U of DNAseI stock was added to 500 μl of DNAseI buffer on cells for 10 minutes. Cells were washed 3x with PBS and 3.2% PFA with
0.2% Triton X-100 was added again for 5 minutes post-DNAsel treatment. Immunofluorescent staining was completed as stated above.

A.3.4 Cell cycle analysis
For TMPyP4 treatment, on day 0 cells were seeded at 100,000 cells per well in 6-well plastic tissue culture dishes one day prior to treatment with the indicated concentrations of drug. 50 µM TMPyP4 was added on day 1, treatment time was 48 hours. After 48 hours, cells were collected, washed with PBS, ethanol-fixed, stained with propidium iodide, and analyzed for cell sub-G1 (fragmented/apoptotic) DNA content and cell cycle profile. 20,000 cells were acquired by flow cytometry on a BectonDickinson Fortessa. Files were modeled using ModFit software (Verity Software, Topsham, ME) to determine sub-G1, G1, S, and G2/M cell cycle stage. For CLK2i treatment, on day 0 cells were seeded at 200,000 cells per well in 6-well plastic tissue culture dishes one day prior to treatment with the indicated concentrations of drug. 5 µM CLK2i was added on day 1, treatment time was 24 hours. After 24 hours, cells were collected, washed with PBS, ethanol-fixed, stained with propidium iodide, and analyzed for cell sub-G1 (fragmented/apoptotic) DNA content and cell cycle profile. 20,000 cells were acquired by flow cytometry on a BectonDickinson Fortessa. Files were modeled using ModFit software (Verity Software, Topsham, ME) to determine sub-G1, G1, S, and G2/M cell cycle stage.

A.3.5 Nucleolar size and circularity detection and graphing
Images taken from the Leica SP8 microscope were opened in FIJI, split into the channel to be analyzed, and converted from RGB to 8-bit images for analysis. In FIJI, the intensity threshold was set via Image -> Adjust -> Threshold (over/under 95). Next, the image was made into a binary
form (Process -> Binary -> Make binary). Finally, particles that met the threshold were quantified for area and circularity via Analyze -> Analyze particles. These readouts were then imported into R and graphed/analyzed using the R markdown file “Raincloud_Area” in the supplemental files.

A.3.6 Nascent transcription assay

On day 0, 35,000 cells were seeded on coverslips in a 12-well dish. The following day cells were cultured with 1 mM EU for 30 minutes and followed the manufactures protocol for immunofluorescent staining of the Click-IT RNA 488 kit. Post-488 labeling, cells were washed and stained with DAPI for 20 minutes in antibody block (0.1% gelatin with 10% normal donkey serum in dH2O). Coverslips were again washed 3x with PBS, then gently dipped four times into molecular biology-grade water before inversion onto one drop of Fluoro-Gel (with TES Buffer, Electron Microscopy Sciences, #17985-30) then allowed to air-dry in the dark for at least 10 minutes. Slides were stored at 4°C until image collection on the LCCC Microscopy & Imaging Shared Resource’s Leica SP8 microscope with the 63X oil objective at 1.52 magnification.

A.3.7 Nascent translation assay

Cells were seeded at 200,000 cells per well in a 6-well dish one day prior to treatment. The following day, cells were treated with 10 µM Puromycin for 10 minutes, media was removed, and cells were washed 3x with cold PBS. Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, #4906837001) for protein extractions, sonicated, and separated by polyacrylamide gel electrophoresis using 4-12% gradient gels (Novex by Life Tech, #NP0321BOX) as described previously (19). They were then transferred onto Nitrocellulose membranes (Invitrogen, #IB23001) with the iBlot2 (Invitrogen, #IB21001) and probed with an
anti-puro antibody (Sigma, MABE343). Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, #NA931-1ML (Mouse) or #NA934-1ML (Rabbit)) and enhanced chemiluminescent detection HyGLO Quick Spray Chemiluminescent (Denville Scientific, #E2400) using film (Denville Scientific, #E3212).

**A.3.8 Western blot analysis**

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, #4906837001) for protein extractions and separated by polyacrylamide gel electrophoresis using 4-12% gradient gels (Novex by Life Tech, #NP0321BOX) as described previously (19). They were then transferred onto Nitrocellulose membranes (Invitrogen, #IB23001) with the iBlot2 (Invitrogen, #IB21001) and probed with the following antibodies: RPS3 (1:100, SCBT, sc-376098), RPS7 (1:100, SCBT, sc100834), RPSL13a (1:100, SCBT, sc-390131), RPLP0 (sc-293260), EWSR1 (1:1000, abcam, ab133288), CLK2 (1:1000, sigma, HPA055366-100UL), PARP (1:1000, Cell Signaling, #9542L), MGMT (1:1000, Cell Signaling, #2739S), phosphorylated SR proteins (clone 1H4, 1:500, Millipore, #MABE50), total SR proteins (1:1000, Sigma-Aldrich, #MABE126), phosphorylated histone H3 Serine 10 (1:1000, Cell Signaling, #3377S), and total histone H3 (1:1000, Cell Signaling, #9715S). Beta-Tubulin (1:5000, Sigma Aldrich, #T7816) and Beta-Actin (1:5000, Sigma-Aldrich, #A5316) were used as a loading controls. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, #NA931-1ML (Mouse) or #NA934-1ML (Rabbit)) and enhanced chemiluminescent detection HyGLO Quick Spray Chemiluminescent (Denville Scientific, #E2400) using film (Denville Scientific, #E3212).
A.3.9 High resolution stimulated emission depletion microscopy (STED)

STED images were acquired using a Leica SP8 3X STED microscope, a white-light laser for fluorescence excitation (470-670nm), time-gated hybrid-PMTs, and a Leica 100x (1.4 N.A.) STED White objective (Leica Microsystems, Inc.). [Dye 1] was excited with XXXnm excitation, depleted with XXXnm, and the fluorescence emission was collected over a bandpass of XXX-XXXnm. [Dye 2] was excited with XXXnm excitation, depleted with XXXnm, and the fluorescence emission was collected over a bandpass of XXX-XXXnm. Time-gating of the emission signal from the PMT was set to a range of 0.7-6.5ns for experiments involving the 775nm depletion laser. Z-stacks were taken with an interslice distance of XX microns and pixel sizes were XXnm. The pinhole was set to a value of 0.7 airy units for all images. Image deconvolution was performed using Hyugens software (Scientific Volume Imaging B.V., Netherlands) assuming an idealized STED point spread function.

A.3.10 Silencing of EWSR1

Four EWSR1 siRNAs (2 nmol each) were purchased (LQ-005119-02-0002, Dharmacon) and resuspended in RNA reconstitution buffer provided. On day 0, 250 µl of Opti-MEM was warmed and added to 5 µl of siRNA (stock = 20 µM) and 7.5 µl of Trans-IT X2. The mixture was left to form micelles for 25 minutes. This was then added dropwise to 6-wells with 1% FBS media and 100,000 freshly plated cells. The cells then adhered overnight and media was changed the following morning. Cells were collected 48 hours later for either cell cycle analysis or Western blot, which allowed for 72 hours of transfection total.
A.3.11 Nuclear and cytoplasmic fractionation

On day 0, cells were seeded at 750,000 in a 10 cm dish. The following day, the media was aspirated and the plate was washed with cold PBS 3x. 500 µl of Buffer A (250 mM sucrose, 20 mM HEPES, 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2 with 10 µl each of inhibitor: 1 M NaF, 200 mM NaOrthovanadate, 0.1 M PMSF, 0.1 M DTT per 1mL of Buffer A made), was added to each dish and the cells were scraped into a labeled Eppendorf tube. Lysate was then passed through a 26 5/8 gauge needle 10x and left on ice for 20 minutes. An aliquot was then taken out for whole cell lysate, the rest was centrifuged at 720 xg for 5 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 720 x g for 3 minutes at 4°C to clean up remaining nuclei. While the supernatant is spinning, the nuclear pellet was washed with 500 µl of Buffer A plus inhibitors and pass through a new 26 5/8 gauge needle 10 x more. Centrifuge the nuclear fraction at 3000 rpm for 10 minutes. As the nuclear fraction is spinning, the supernatant was collected from the cytoplasmic fraction and moved to a final tube. After the centrifugation of the nuclear fraction was finished, it was resuspended in 500 µl of 0.1% SDS in TBS and sonicated. Western blots were then run on the whole cell lysate, nuclear and cytoplasmic fractions.

A.3.12 Immunohistochemistry on human GBM patient samples

Immunohistochemical staining of GBM was performed for EWSR1. Five-micron sections from formalin fixed paraffin embedded tissues were de-paraffinized with xylenes and rehydrated through a graded alcohol series. Heat induced epitope retrieval (HIER) was performed by immersing the tissue sections at 98°C for 20 minutes in 10 mM citrate buffer (pH 6.0) with 0.05% Tween. Immunohistochemical staining was performed using a horseradish peroxidase labeled
polymer from Agilent (K4003) according to manufacturer’s instructions. Briefly, slides were treated with 3% hydrogen peroxide and 10% normal goat serum for 10 minutes each and exposed to primary antibodies for EWSR1 (1/400, Abcam, ab133288) overnight at 4°C. Slides were exposed to the appropriate HRP labeled polymer for 30min and DAB chromagen (Dako) for 5 minutes. Slides were counterstained with Hematoxylin (Fisher, Harris Modified Hematoxylin), blued in 1% ammonium hydroxide, dehydrated, and mounted with Acrymount. Consecutive sections with the primary antibody omitted were used as negative controls.

A.3.13 RNA isolation for full-length ONT sequencing

750,000 cells were seeded on day 0. The following day cells were washed 3x with PBS, trypsinized, and pelleted into an Eppendorf tube. Then, 4 mL of TRIzol was added to each tube and incubated at room temperature for 5 minutes. 1 mL of each sample was aliquoted into 4 separate tubes. 200 µl of choloform was added to each tube and vortexed briefly to mix. Samples incubated at room temperature for 5 minutes, were briefly vortexed, and then centrifuged at 12,000g for 10 min at 4°C. 500 µl of the clear upper layer was collected from each tube and pooled into two 1 mL Eppendorf tubes where 500 µl of isopropanol was added and mixed thoroughly by multiple inversions. Samples were incubated for 15 minutes at room temperature, and then centrifuged for 15 minutes at 12,000 g at 4°C. Supernatent was then discarded, and pellet was washed with 750 µl 80% ethanol by multiple inversions. Sample was centrifuged for 5 minutes at 4 °C at 12,000 g. Pellet was again washed with 80% ethanol by multiple inversions and centrifuged the same way as before. The supernatant was removed and discarded, and a second quick spin was done to remove any residual contaminating liquid. The RNA pellet was then air dried for 10
minutes before being resuspended in 100 µl of nuclease-free water. Total RNA was quantified using a NanoDrop 2000.

A.3.14 DNA isolation for Whole genome sequencing

750,000 cells were collected for each cell line, where DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen; Cat. No. 69504). Briefly, cells were centrifuged and resuspended with proteinase K and buffer AL for 10 min at 56°C. 200 µl of ethanol was then added and mixed thoroughly by vortexing. DNA was added to the DNeasy Mini spin column, centrifuged, and flow-through discarded. DNA was washed in the DNeasy column with buffer AW1 once, and AW2 once as well. Membrane was then dried by centrifugation with all flow-through being discarded. DNA was eluted using 200 µl buffer AE after 1 min incubation at room temperature.

A.3.15 WGS using Illumina TruSeq DNA PCR-Free Library prep Kit

Illumina TruSeq DNA PCR-Free Library prep Kit was used per manufactures instructions. Paired-end, indexed libraries for human whole genome sequencing were constructed from 1.0 µg gDNA using the TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego) according to manufacturer’s instructions. Briefly, DNA was fragmented using a Covaris M220 focused ultrasonicator (Covaris, Woburn, MA) using settings for a 350bp insert size. Library quality was assessed with a BioAnalyzer 2100 using the High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA). The libraries were quantified using the Kapa Library Quantification Kit Illumina Platforms (Kapa Biosystems, Boston, MA). The denatured and diluted libraries were sequenced on a NextSeq 550 System (Illumina) using v2.5 High Output 300 cycle kit with 1% PhiX to an average sequencing depth of 50X coverage.
**A.4 Discussion and Limitations**

Temozolomide (TMZ) is the current standard of care first line treatment for glioblastoma (GBM) along with surgery and radiation. TMZ has been almost exclusively used in brain cancers since the 2005 pivotal clinical trial which changed GBM clinical care to the Stupp regimen, albeit only adding a few months to median overall survival\(^{166}\). As there are currently no successful FDA-approved second-line interventions, I wished to explore potential dependent pathways after TMZ-resistance had occurred to determine avenues forward for successful second-line options\(^{167}\). However, as TMZ is rarely used in other cancers, and the 400+ clinical trials that have been performed to repurpose other chemotherapeutic or targeted agents from other cancers have failed\(^{168}\), I looked to other central nervous system (CNS) disorders for insight. As research efforts have currently expanded the focus of neurodegenerative diseases, multiple studies have shown the dependence of these diseases on DNA and RNA secondary structures\(^{169}\), splicing\(^{152}\), and localization of RNA binding proteins\(^{170}\). We therefore reframed our strategy for tackling TMZ-resistance to test the effect of TMZ-induced mutations on disrupting DNA and RNA secondary structures, as well as RNA processing and RNA binding proteins for potential second-line therapeutic treatments.

We started by exploring the known mechanism of action of TMZ to add mutagenic O6 methyl adducts to guanines\(^{144}\). Guanines are necessary nucleotides in many DNA and RNA secondary structures adding stability\(^{156}\) and are often the target of chemotherapeutic agents\(^{154}\). It has also been previously shown that TMZ-treated GBM has a characteristic mutational signature of C>T\(^{34}\). We also observed this C>T mutation rate, but were more interested in the G>A, which were just as prevalent, and could be hypothesized to be the cause or effect of a C>T mutation. By widely and broadly characterizing guanine changes induced by TMZ treatment, we came to focus
on the G-rich regions of G-quadruplexes (G4s) and splice sites for further functional roles in TMZ-resistance. Our data show changes in G4s through immunofluorescence between TMZ-sensitive and TMZ acute treatment, acquired, and intrinsic resistance, as well as the G4 stabilizing ligand being more effective in TMZ-resistant lines. Others have also tested the efficacy of different G4 ligands in multiple cancer cell lines with those deriving from the CNS being the most sensitive. However, I would be surprised to only have G4 secondary structure changes with TMZ treatment. Therefore, this avenue of targeting nucleotide secondary structure changes post-chemotherapy should be investigated further in the future, requiring more insight and drugs that are able to cross the BBB.

G4s were not the only secondary structure changes in TMZ-resistant cells. I also observed changes to nucleolar r-loops. Nucleoli are major stress organelles whose size and circularity have been correlated to cancer grade. This increase in r-loops is interesting, as a normal DNA-damage response would have caused punctated r-loops throughout the nucleus, like we see in the TMZ-sensitive cell line 42MBGA. This suggests that there may be a differential DNA damage response between TMZ-sensitive and -resistant cells. Nucleolar r-loop changes have also been correlated with rDNA mutations. As rDNA is G-rich, this also fits along with our hypothesis of guanine mutations affecting multiple processes within the cell. If the mutant rDNA is transcribed to unfit rRNA, this may give insight to the discrepancy between the increase in global rRNAs but not global ribosomal proteins.

As splice sites are G-rich, I then focused on the splicing mutations I found to be enriched in the TMZ-resistant cell lines. Taking a more global look at alternative splicing regulation, I focused on the CLK family as they regulate SR proteins which I found to be differentially phosphorylated after TMZ treatment. Targeting splicing broadly is an active area of research with
clinical trials underway in SF3B1 mutant cancers. It is imperative to understand the model system in which to test a splicing inhibitor as this can have grotesque side effects if not in the proper patient population. In this way, I propose looking at CLK2 status in TMZ-resistant patients to determine the therapeutic window for CLK2i.

However, I do not find EWSR1 aggregates to be the cause of resistance as knockdown of EWSR1 is not more lethal in TMZ-resistant cells where the aggregates are observed. I also see proper trafficking of EWSR1 between the cytoplasm and nucleus in all cell lines, suggesting that it is not a transport defect. However, I do observe a role for DNA damage as acute TMZ treatment starts inducing EWSR1 aggregation that remains post-resistance. RNA buffering may explain part of the aggregation phenotype, since the concentration of RNA is higher in the nucleus compared to the cytoplasm. This argument is strengthened by acute ActD treatment inducing ribbon-like strands of EWSR1 around the nucleolus, though the local concentration of EWSR1 would presumably be increasing as well. The last factor I explore is the ability of DNA secondary structures to alter protein localization and therefore function. The inability of EWSR1 to bind G4s in the mutated TMZ-resistant lines may affect the function and proper regulation of EWSR1. Overall, my data suggests some overlapping functions of the cytoplasmic aggregates of EWSR1 with other neurodegenerative diseases, and further studies may provide insight on different pathways to non-malignant protein aggregation.

Lastly, gleaning knowledge from the relationship between DNA damage and RNA binding proteins, I discovered cytoplasmic amyloid-like aggregates of EWSR1 in TMZ-resistant cells and patient samples. My observations hint that CNS cancers may be more related to other CNS diseases than cancers and that significantly more research need to be conducted to determine what part
DNA and RNA secondary structures and amyloid-like aggregates play in GBM maintenance and TMZ-resistance.

**Limitations**

As there are many limitations with any study, one of the main limitations here is the lack of an *in vivo* model. It is well understood that the BBB is the major cause of treatment failure for GBM patients. Future studies will be necessary to test the efficacy of the novel CLK2i we developed for this project. While it is exciting to have developed a novel compound to test in TMZ-resistant cell lines based on previous studies suggesting alternative splicing to be a dependency for GBM, our novel CLK2i still in development and thus not yet mass produced or commercially available, making it unrealistic to test in a mouse model at this time.

There is also the issue of patient sample numbers. Though the first report of mutant FUS cytoplasmic aggregation only used 20 patient samples, these were enriched for ALS type 6 from a cohort of 197 cases\(^{174}\). As there are limited resources for studying GBM, especially TMZ-resistant GBM, future studies will need to be done on a larger cohort and preferably matched patient samples of pre- and post-TMZ treatment/resistance. There is also the issue of no clinical data for the patient samples that we have presented in this work. New cohorts with proper annotation, like treatment and overall survival, will really assist in interpreting the role of EWSR1 cytoplasmic aggregates in GBM.
REFERENCES


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