

BAPTA-AM significantly reduced PKC activity following PI3K inhibition. These data highlight the power of phosphoproteomic profiling for the rational design of drug combination strategies, which need to be tested *in vivo* prior to clinical trials for DIPG.

#### DIPG-31. MOLECULAR MECHANISMS AND FUNCTIONAL IMPACT OF ABERRANT SPLICING IN DIFFUSE MIDLINE GLIOMAS

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Fewer than 1% of children diagnosed with diffuse-midline glioma (DMG) survive for more than 5 years, because no effective therapies exist for these patients. Here, we sought to identify and characterize mechanisms of aberrant splicing (AS) in primary DMG tumors. We observed transcriptome-wide AS (9,805 differential splicing variations in 4,734 genes), and identified a DMG-specific splicing signature, that included known cancer genes. We hypothesize that AS of cancer genes play a role in DMG tumor formation. Assessing whether splicing factor dysregulation impacted known cancer transcripts, we discovered several splicing factors, including *SRRM4*, *SRRM3* and *RBFOX3* to be down-regulated in DMG. Additionally, we found an enrichment of binding motifs for these proteins within flanking regions of these mis-spliced exons. We also observed recurrent significant exon inclusion in tumor suppressor *SMARCA4*, an integral member of the SWI/SNF family of proteins involved in chromatin remodeling. Further, we identified AS in 16 of the 27 members of the SWI/SNF complex, including increased skipping of exon 7 in *DPF2*, representing a complete mRNA transcript switch in DMG. Since *SRRM4*, *SRRM3* and *RBFOX3* are known regulators for neural-specific microexons, we focused on microexon splicing changes, hypothesizing that these regulators may be driving microexon mis-splicing in these tumors. We identified 245 known microexons lost or gained in DMG. Moreover, a quarter of which were observed in known cancer genes, with the most frequent splice event causing gain of a clathrin-binding site in the tumor suppressor *BIN1* with a concurrent loss of an out-of-frame microexon in the oncogene *BAK1*, presumably activating it. Altogether, our results suggest that aberrant splicing may be an alternative mechanism driving DMG tumorigenesis and we are currently molecularly validating a subset of these events with the overall goal of identifying novel therapeutic targets for DMG tumors.

#### DIPG-32. AKT SIGNALING DRIVES RESISTANCE TO ONC201 IN DIFFUSE INTRINSIC PONTINE GLIOMA (DIPG)

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Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive, childhood brainstem cancer with a median overall survival of 10 months post diagnosis. Remarkably, 80–90% of patients harbor recurring point mutation in histone H3, which induces a lysine for methionine substitution at amino acid 27 (H3K27M) in either H3.1 (*HIST1H3B* ~25%) or H3.3 (*H3F3A* ~65%) variants. Using the blood-brain barrier (BBB) permeable DRD2 antagonist, ONC201 (in clinical trials for DIPG and H3K27M-mutant gliomas – NCT03416530), we hypothesized that DRD2 antagonism would induce TRAIL expression via indirect inhibition of AKT and ERK signaling, to drive apoptosis in both H3.1K27M and H3.3K27M patient-derived DIPG cell lines alike. For the first time, we reveal that ONC201 shows efficacy in 100% of WT-H3 and H3.1K27M mutant DIPG cell lines (n=5), compared to 50% of H3.3K27M mutant DIPGs (n=6). Investigations to identify the mechanisms of resistance to ONC201, revealed that cell lines with decreased sensitivity upregulated the PI3K/AKT/MTOR signaling axis to drive phosphorylation of AKT and increase metabolic activity. Combined administration of ONC201 and the BBB-permeable PI3K/AKT inhibitor, paxalisib (previously GDC-0084, in clinical trials for newly diagnosed DIPG – NCT03696355), showed synergistic cytotoxicity, reduced PI3K/AKT signaling and metabolic reprogramming to drive apoptosis in all DIPG cell lines tested. This combination was used to treat a 3-year-old DIPG patient, commencing 14 weeks post disease progression, completing 40 weeks of

therapy prior to her passing, December 2019. These studies highlight the potential of combined administration of two safe, BBB penetrant, oral targeted therapies and supports testing under clinical trial conditions.

#### DIPG-33. CHARACTERIZING THE NEURO-VASCULAR UNIT IN DIFFUSE INTRINSIC PONTINE GLIOMA

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Diffuse intrinsic pontine glioma (DIPG) is a childhood brainstem tumor with a median overall survival of eleven months. Lack of chemotherapy efficacy may be related to an intact blood-brain-barrier (BBB). In this study we aim to compare the neuro-vascular unit (NVU) of DIPG to healthy pons tissue. End-stage DIPG autopsy samples (n=5) and age-matched healthy pons samples (n=22), obtained from the NIH NeuroBioBank, were immunohistochemically stained for tight-junction proteins claudin-5 and zonula occludens-1 (ZO-1), basement membrane component laminin, and pericyte marker PDGFR $\beta$ . Claudin-5 stains were also used to determine vascular density and diameters. In DIPG, expression of claudin-5 and ZO-1 was reduced, and claudin-5 was dislocated to the abluminal side of endothelial cells. Laminin expression at the glia limitans was reduced in both pre-existent vessels and neovascular proliferation. In contrast to healthy pons, no PDGFR $\beta$  expression was detected. The number of blood vessels in DIPG was significantly reduced compared to healthy pons, 13.9 $\pm$ 11.8/mm<sup>2</sup> versus 26.3 $\pm$ 14.2/mm<sup>2</sup>, respectively ( $P<0.01$ ). Especially the number of smaller blood vessels (<10 $\mu$ m) was significantly lower ( $P<0.01$ ). Distribution of larger blood vessels ( $\geq 10\mu$ m) did not differ between groups ( $P=0.223$ ). Mean vascular diameter was 9.3 $\pm$ 9.9 $\mu$ m for DIPG versus 7.7 $\pm$ 9.0 $\mu$ m in healthy pons ( $P=0.016$ ). Our study demonstrates evidence of structural changes in the NVU in end-stage DIPG. Chemotherapeutic inefficacy could be the result of reduced vascular density. However, further research is needed to determine meaning and extent of these changes and to determine whether these observations are caused by the tumor or the result of treatment.

#### DIPG-34. SUPER ELONGATION COMPLEX AS A TARGETABLE DEPENDENCY IN H3K27M+ DIFFUSE MIDLINE GLIOMA

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Mutations in the histone 3 gene (H3K27M) are the eponymous driver in diffuse intrinsic pontine gliomas (DIPGs) and other diffuse midline gliomas (DMGs), aggressive pediatric brain tumors for which no curative therapy currently exists. To identify specific epigenetic dependencies within the context of the H3K27M mutation, we performed an shRNA screen targeting 408 genes classified as epigenetic/chromatin-associated molecules in patient-derived DMG cultures. This identified AFF4, a component of the super elongation complex (SEC), as necessary for DMG cells to maintain growth and self-renewal. We hypothesized that aberrant SEC expression occurs as a consequence of the H3K27M mutation and that this dysregulated SEC signaling overcomes repressive transcriptional regulation in order to suppress differentiation and promote self-renewal of DMG tumor stem cells. We interrogated the role of AFF4 in DMG using an shRNA lentiviral approach. We demonstrate a significant decrease in *in vitro* clonogenicity and stem cell maintenance following AFF4 depletion. We employed RNA-seq-based gene set enrichment analysis to delineate differentiation programs under SEC regulatory control. Finally, we sought to determine whether CDK9, the catalytic subunit of the SEC, represents a therapeutic vulnerability in DMG. Using RNA polymerase II ChIP-seq, we demonstrate that CDK9 pharmacologic inhibition restores regulatory Pol II pausing, promotes cellular differentiation, and leads to potent anti-tumor effect both *in vitro* and in patient-derived xenograft models. These studies present a biologic rationale for the translational exploration of CDK9 inhibition as a promising therapeutic approach.

#### DIPG-35. BIOLOGICAL MEDICINE FOR DIFFUSE INTRINSIC PONTINE GLIOMA (DIPG) ERADICATION: RESULTS OF THE THREE ARM BIOMARKER-DRIVEN RANDOMIZED BIOMEDE 1.0 TRIAL

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