**Introduction**

Diffuse gliomas are the most common primary brain tumors in adults, with an incidence of 5.95 per 100,000 in the United States[1](https://www.zotero.org/google-docs/?bih04f). The classification of these tumors is increasingly guided by molecular diagnostics, as highlighted in the WHO 2021 classification of central nervous system (CNS) tumors (REF). The primary treatment approach for most gliomas is surgical resection to the greatest extent that is safely possible, and early resection is now also recommended for patients with radiological signs of histologically low-grade tumors. In adult-type diffuse gliomas, the standard treatment consists of a combination of radiotherapy and chemotherapy. While durable responses are rarely achievable, multimodal treatment has led to long-term survival (over 10–20 years) in some patients with isocitrate dehydrogenase (IDH) mutant tumors[2](https://www.zotero.org/google-docs/?3Xiiuj). In August 2024, the FDA approved the mutant IDH inhibitor vorasidenib as the first targeted therapy for IDH-mutant diffuse gliomas, specifically for patients with Grade 2 astrocytoma or oligodendroglioma with IDH1 or IDH2 mutations, following surgery. This approval was based on findings from the phase 3 INDIGO trial (REF). To date, targeted therapies based on molecular alterations have played a limited role in the management of diffuse gliomas. In March 2023, the FDA approved the combination of dabrafenib and trametinib for patients with low-grade glioma (LGG) carrying a BRAFV600E alteration. This is the first FDA-approved systemic therapy for the initial treatment of LGG with this specific mutation but is not yet approved for high-grade gliomas (HGG) harboring BRAFV600E.

Despite these advancements in molecularly targeted therapies, many diffuse gliomas remain without effective precision treatments. One such subset includes sporadic gliomas driven by the loss of *Neurofibromin 1* (*NF1*), a tumor suppressor gene altered in ~22% of adult-type diffuse gliomas[7](https://www.zotero.org/google-docs/?mMpWzx). While germline *NF1* mutations underlie neurofibromatosis type 1 (NF1)-associated gliomas, sporadic cases with somatic *NF1* loss occur independently of this syndrome and contribute to a distinct subset of gliomas. Loss of NF1 leads to hyperactive RAS-MEK-ERK and PI3K-AKT signaling, promoting proliferation and survival. Loss of tumor suppressors including PTEN, TP53, CDKN2A, and ATRX, can modulate tumor progression. These tumors often exhibit aggressive behavior and are enriched in mesenchymal-like transcriptional programs, which are associated with treatment resistance and poor outcomes.

ATRX is a member of the SWI/SNF family of chromatin remodeling proteins[12](https://www.zotero.org/google-docs/?GgesJT). The loss of ATRX in glioma aids in the development of malignant tumors by stabilizing the genome through telomere extension during cancer development[13,14](https://www.zotero.org/google-docs/?4n9jUu). This occurs in a mutually-exclusive manner with TERT promoter mutation in glioma, which also contributes to immortalization in cancer tumorigenesis[15](https://www.zotero.org/google-docs/?ELTIZe). ATRX mutations frequently co-occur with loss of TP53 in astrocytoma and can co-occur with CDKN2A and PTEN alterations in glioblastoma[7](https://www.zotero.org/google-docs/?fapuiZ). Furthermore, genetic profiling suggests that NF1 loss may occur more prevalently in gliomas lacking ATRX than in those with wildtype ATRX[15](https://www.zotero.org/google-docs/?5qg3I1). Germline heterozygous loss of *Atrx* in combination with *Nf1* and *p53* loss in zebrafish has been shown to induce different tumors with a more diverse histology compared to *Nf1* and *Pten* loss alone[16](https://www.zotero.org/google-docs/?Vg6Cre).

Genetically Engineered Mouse Models (GEMMs) have been developed to study the role of Nf1 loss in sporadic gliomagenesis (reviewed in REF). The *Nf1flox/flox*;GFAP-Cre model leads to biallelic *Nf1* loss in astrocytes or neural progenitors, recapitulating gliomas of varying grades depending on additional mutations including TP53. The *Nf1flox/flox*;hGFAP-CreERT2 model introduces a tamoxifen-inducible system, allowing temporal control of *Nf1* loss, making it valuable for investigating tumor initiation and progression. The *Nf1flox/flox;Ptenflox/flox*;GFAP-Cre model combines *Nf1* and *Pten* loss, producing aggressive high-grade gliomas characterized by enhanced PI3K-AKT signaling. Although loss of *Nf1,* *Cdkn2a, Pten,* and *Atrx* have all been linked to glioma formation and progression, the cooperativity of these alterations to induce gliomagenesis has not been extensively explored. Furthermore, there is limited data regarding gliomas harboring *Atrx* loss of function mutations due to their rarity, resulting in a need for clinically relevant models that can characterize these tumors[7](https://www.zotero.org/google-docs/?t48g1h).

In this study, we utilized an *in vivo* CRISPR/Cas9 approach in the RCAS/TVA glioma mouse model to investigate *Nf1* loss in cooperation with *Cdkn2a*, *Pten*, and/or *Atrx* loss. [Describe the results of the tumor incidence figure here]. While Atrx WT and KO [resulted in tumorigenesis at similar rates?]

**Results:**

F1

Loss of Nf1, Atrx, Pten, and Cdkn2a resulted in equal proportions of nerve sheath tumors, spindle/epithelioid tumors, and gliomas.

—Loss of Nf1, Pten, and Cdkn2a with WT Atrx had the highest proportion of the otherwise rare glioneuronal tumor type. Notably, Nf1, Pten, and Cdkn2a loss with or without Atrx loss resulted in similar proportions of tumor types.

Nf1 loss by itself was sufficient to lead to tumor development in ~20% of mice, while the majority of the cohort exhibited pretumorigenic signs of disease.

Knockout of Pten, Cdkn2a, and Atrx resulted in some tumor development, but the majority of animals were disease free at 150 days. Histologically, the majority of these tumors were gliomas, with minor inclusions of nerve sheath and spindle/epithelioid tumor types.

–While knockout of Pten and Cdkn2a in combination produced few tumors with most mice histologically presenting pretumorigenic lesions(?) or without evidence of disease.

Notably, knockout of ATRX

F2

F3

F4

**Discussion:**

Hariharan and colleagues also observed an increase in survival in Atrx KO mice due to enhanced T-cell infiltration and innate immune signaling [12](https://www.zotero.org/google-docs/?QTvaz1) .

We did not assess whether TERT promoter mutations as an alternative to ATRX knockout result in a similar phenotype.

**Methods:**

**Mice and Genotyping**

All mice were maintained on a mixed C57Bl/6 and FVB/N background by random interbreeding. DNA from tail biopsies was used to genotype for the TVA transgene, Cdkn2aflox/flox, H11LSL-CAS9, Ptenflox/flox, Atrxflox/flox, and wild-type alleles as described previously[17,18](https://www.zotero.org/google-docs/?0m1BNQ). Both male and female newborn through adult mice were used in this study. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah before experimentation. H11LSL-CAS9 mice were obtained from Jackson Laboratories and bred with N::TVA, N-TVA::Cdkn2aflox/flox, N-TVA::Ptenflox/flox;Cdkn2aflox/flox and N-TVA::Ptenflox/flox;Cdkn2aflox/flox;Atrxflox/flox mice. Mice harboring the H11LSL-CAS9 gene express Cas9 in the presence of Cre recombinase. Nf1 knockout was accomplished by delivery of sgRNA targeting exon 2 of the Nf1 gene.

**Viral Constructs and propagation**

Mouse tumorigenesis was induced using the RCAS-TVA system to deliver Cre, with or without an sgRNA targeting exon 2 of the Nf1 gene, to neural stem and progenitor cells. This RCAS-Cre system has been described in depth previously [19](https://www.zotero.org/google-docs/?i8hA0Y). To generate a construct simultaneously encoding Cre and Nf1 sgRNA, the pX330 plasmid (Addgene, Watertown, MA) encoding the Nf1 exon 2 guide was digested with Bbs I (New England Biolabs, Ipswich, MA). Subsequently, annealed/phosphorylated 38 nucleotide oligos possessing 5’ and 3’ Sap I restriction sequences were ligated into the vector to generate pX330-SapI (5’ – CACCGGAAGAGCTTAATTAGATCTTATAAGCTCTTCT; 3’ – AAACAGAAGAGCTTATAAGATCTAATTAAGCTCTTCC). Subsequent PCR amplification of the U6 promoter and TRACR elements (440 bp) possessing the Sap I insert were cloned into Eco RI and Not I digested pENTR-2B (Invitrogen, Waltham, MA) via standard Gibson cloning (NEB) (5’ – CCAATTCAGTCGACTGGATCCGGTACCGAATTCTTTTGCTCACATGTGAGGG; 3’ – GGGTCTAGATATCTCGAGTGCGGCCGCAAAACAAAAAAGCACCGACTC) to generate pENTR-2B U6 Sap I. Cre recombinase (1075 bp) was PCR amplified and cloned upstream of the U6 promoter in digested pENTR-2B U6 Sap I by Gibson cloning (5’ – GCAGGCTGGCGCCGGAACCAATTCAGTCGACATGTCCAATTTACTGACCG; 3’ – CCCTCACATGTGAGCAAAAGAATTCCTAATCGCCATCTTCCAGC) to generate the modular pENTR-2B Cre U6 Sap I plasmid. Annealed/phosphorylated Nf1 exon 2 directed guide oligos were ligated into the Sap I digested pENTR-2B Cre U6 Sap I vector to generate pENTR-2B Cre Nf1 exon 2 (5’ – ACCGTTGTGCTCGGTGCTGACTT; 3- AACAAGTCAGCACCGAGCACAAC), which was recombined via LR Clonase II (Invitrogen) with the RCAS-Y DV-A destination vector to generate RCAS Cre Nf1 exon 2. Viral infection was initiated by calcium phosphate transfection of proviral DNA into DF-1 avian fibroblasts. Viral spread was monitored by expression of the p27 viral capsid protein as determined by western blot [35].

**Cell Culture**

DF-1 cells were grown in DMEM-high glucose media (Thermo Fisher, Waltham, MA) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 0.5 µg/mL Gentamicin (Thermo Fisher), and maintained at 39°C. Viral infection was initiated by calcium phosphate transfection of proviral DNA into DF-1 avian fibroblasts. Viral spread was monitored by expression of the p27 viral capsid protein as determined by western blot [35]. Cells from a confluent 10cm dish were resuspended in 100ul of Hank’s Balanced Salt Solution (Life Technologies, Waltham, MA) and newborn mice were injected intracranially 2 mm ventral of Bregma with 5 µl of cell suspension using a gas-tight Hamilton syringe as described previously[20](https://www.zotero.org/google-docs/?Rqbu27).

**Histological analysis**

Brain tissue from injected mice was isolated, fixed in neutral buffered formalin (VWR, Radnor, PA), and paraffin embedded. 5 µm sections from tissue blocks were adhered to glass slides and stained by H&E or left unstained for further analysis. Slides were scored for tumor type by <<name of the pathologist>> using the <<pathology standard that he would have used to determine this>>.

**Immunohistochemistry**

Tissue sections from formalin-fixed paraffin embedded blocks were deparaffinized at 65˚C for 10 minutes, incubated in xylene, and rehydrated using decreasing concentrations of ethanol. Antigen retrieval was performed in a decloaking chamber at 120˚C for 20 minutes using citrate buffer (pH 6.0) Peroxidase activity was quenched in 3% H2O2 for 10 minutes and slides were incubated in 5% normal goat serum in TBS-T (0.05% Tween-20) for 1 hour inside a humidity chamber. Anti-rabbit primary antibodies (P-ERK, PTEN, Ki67) were diluted in SignalStain Antibody Diluent (Cell Signaling Technology, Danvers, MA), added to slides, and incubated overnight at 4˚C. Slides were incubated in SignalStain Boost Detection Reagent (Cell Signaling Technology) for 30 minutes in a humidity chamber and the SignalStain DAB Substrate Kit (Cell Signaling Technology) was used to detect the presence of each epitope. Slides were counterstained with hematoxylin and dehydrated with increasing concentrations of alcohol and xylene prior to the addition of coverslips. Antibodies used: Ki67 (1:300; UM800033 Origene, Rockville, MD), Phospho-ERK1/2 (T202/Y204) (1:400; 4370 Cell Signaling Technology), PTEN (1:125; 9188 Cell Signaling Technology). Slide scanning and cell quantification was completed through CaseViewer software using a nucleic counter program.

**RNA sequencing**

FFPE slides were prepared and stained with H&E to visualize tumor area. Circled tumor areas on stained slides were matched to freshly cut, unstained FFPE slides sections and microdissected. RNA was isolated from microdissected FFPE sections using miRNeasy Kit (Qiagen, Germantown, MD) and treated with DNase. RNA libraries were prepared from total RNA samples (5-100 ng) using reagents from the Illumina Stranded mRNA Prep (cat# 20020189) and TruSeq RNA UD Indexes (20040534) for reverse transcription, adapter ligation and pcr amplification. Amplified libraries were hybridized to biotin-labeled probes from the Illumina Exome Panel (cat# 20020183, San Diego, CA) using the Illumina RNA Fast Hyb Enrichment kit (20040540) to generate strand specific libraries enriched for coding regions of the transcriptome. Exon-enriched libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay (cat# 5067-5582 and 5067-5583, Santa Clara, CA). The molarity of adapter-modified molecules was defined by quantitative PCR using the Kapa Biosystems Kapa Library Quant Kit (cat#KK4824, Roche, Rotkreuz, Switzerland). Individual libraries were normalized and pooled in preparation for Illumina sequence analysis. Sequencing libraries were chemically denatured in preparation for sequencing. Following transfer of the denatured samples to an Illumina NovaSeq X instrument, a 151 x 151 cycle paired end sequence run was performed using a NovaSeq X Series 10B Reagent Kit (20085594).

**Sequencing Analysis**

FASTQ reads were assessed for quality using FastQC.[21](https://www.zotero.org/google-docs/?SE3b6t) Reads were aligned to the GRCm39 genome using STAR[22](https://www.zotero.org/google-docs/?j9G4Y6). Mapped reads were assigned using featureCounts[23](https://www.zotero.org/google-docs/?WsriHe). Genes were filtered based on at least one sample with an RPKM of ≥ 1. Differential expression analysis was performed using edgeR[24](https://www.zotero.org/google-docs/?gbzLom) with significant genes meeting a <<GO threshold info>> cutoff.

**Pathway analysis (GO)**

Need a list of all of the tools used

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