Fellowship Progress Report 2022-2023

Contents

1. Overview of timeline and progress

1.1 Timeline

This report summarizes the project objectives completed in the years 1-3 of the fellowship (**Table 1**):

Table 1. Project timeline

Year	0-1	1-2	2-3	3-4
Objective 1	- GBM scRNA-seq - human-mouse GBM comparisons			
Objective 2	- bioinformatic identification of GTFs - *in vitro* CTL-killing CRISPR screen			
		- *in vitro* validation of GTFs		
Objective 3		- generation of GTF-perturbed GBM cells	- *in vivo* experiments	
Publications				Manuscript prep.

1.2 Progress Update

Data collection for **objectives 1-3** is now complete, and I am focusing on bioinformatic analyses and manuscript preparation. In addition to the proposed in vitro CTL-killing CRISPR screens that were proposed in objective 2 to identify CTL immune evasion genes in glioma cells, I have now also evaluated immune evasion mechanism for microglia, macrophages and NK cells. Results from objectives 1-3 are currently being analyzed, and I anticipate that the resulting manuscripts will be drafted and ready for submission in early 2024.

2. Results

2.2 Objective 1 | Characterize murine GL261 and CT2A glioma models

Please refer to prior progress reports for more detailed results pertaining to objective 1. Interval progress in 2022-2023 involved finalization of the GL261/CT2A CRISPR Cas-9 screens. In brief, we

compared the genetic dependencies of murine GBM to human GBM. We performed pooled loss-of-function genetic screens in CT2A and GL261 cells and identified essential fitness genes using BAGEL (**Fig 1A-B,** BF > 5 threshold). 1392 genes were essential in both murine models, while 408 genes were GL261-specific and 250 were CT2A-specific (**Fig 1C**). Notably, among the GL261-specific hits, *Kras* and *Sox6* were top differential fitness genes, consistent with *Kras* being a known GL261 oncogene and *Sox6* being a transcriptional regulator of the OPC-like GBM phenotype (**Fig 1D**). Functional annotation of CT2A- and GL261-specific fitness genes further revealed that CT2A-specific fitness genes were enriched for processes involved in cell division and epigenetic and post-translational regulation of gene expression (e.g., RNA processing, spliceosome, cell division, histone modification) whereas GL261-specific genes were associated with metabolic processes (e.g., TCA cycle/ETC, nucleotide/flavin/cholesterol biosynthesis) (**Fig 1E**).

We next evaluated the fitness landscape in human GBM cells (data from *Project Score* database)[1,2]. Comparison of gene essentiality profiles from 41 human GBM cell-lines and 1031 human non-CNS cell lines identified 1625 common essential genes and 124 GBM-specific genes; notable GBM-specific human fitness genes included *JUN*, *FERTM2*, *FGFR1*, *WWTR1* and *ADAR* (**Fig 1F-G**). Of the 124 GBM-specific fitness genes identified in human cell lines, 44 (35%) and 54 (44%) genes were essential in CT2A and GL261 cells, respectively (**Fig 1H**). However, by comparison, 51/123 (41%) and 68/123 (55%) of non-CNS-specific fitness genes were also essential in CT2A and GL261 cells. This suggests that CT2A and GL261 have unique genetic dependency profiles that resemble GBM in some ways, but not others. These findings were consistent across different essentiality thresholds and supported by precision-recall analysis (**Fig 1I**, **Fig S1A**). Using the subset of GBM-specific fitness genes that were recovered by CT2A and GL261 cells (**Fig 1J**), we performed pathway enrichment to identify functionally-coherent clusters of genes (**Fig S1B**). Notably, we found that GL261 and human GBM cells, but not CT2A or non-CNS cell-lines, were dependent on the ufmylation pathway, including *Ufc1*, *Ube2g2* and *Ufl1*. Similarly, CT2A and human GBM cells shared dependencies related to epigenetic regulation (*Dnmt1*, *Ttf1*), DNA damage response (*Brat1*, *Rnf8*) and RNA modification (*Trmt6*, *Adat3*) that were otherwise absent in GL261 and non-CNS cell-lines. Together our analyses provide insight into the genetic fitness landscape in CT2A and GL261 glioma models, and highlight dependencies that are uniquely shared with human GBM

Results from this objective will be written up into a manuscript that provides a comprehensive characterization of GL261 and CT2A as translational murine models of GBM.

2.2 Objective 2 | Identifying immune-associated therapeutic vulnerabilities in murine gliomas

Please refer to prior progress reports for more detailed results pertaining to objective 2. Interval progress in 2022-2023 involved performing additional immune cell co-culture screens to identify mechanisms of immune evasion in glioma cells. Originally the plan was to perform this using cytotoxic T cells only, but given the success of those experiments, we expanded to include other immune cells.

To identify the genetic mechanisms regulating glioma-intrinsic immune evasion, we performed mTKO genome-scale pooled CRISPR screens. CRISPR-mutagenized CT2A cells were propagated in the present or absence of various immune cell lines (microglia; BV-2, non-phagocytic macrophages; Raw 264.7 and J774.1, phagocytic macrophages; J774.1 treated with anti-CD29 or anti-CD9, cytotoxic T-lymphocytes, or natural killer cells). Following a period of co-culture (i.e., selective pressure), CT2A cells were subjected to deep sequencing to identify gRNA that were enriched or depleted, i.e., genetic perturbations that conferred resistance or sensitivity to immune cell killing, respectively (**Fig 2A**).

In the upcoming year, we will analyze these results in depth, and describe the pathways and mechanisms involved in immune evasion of gliomas, with the aim of identifying therapeutic vulnerabilities that can be exploited in immunotherapy.

2.2 Objective 3 | Characterization of GTF-perturbed glioma cells

Objective 3 was to characterize GBM transcription factor (GTF)-perturbed glioma cell lines. I previously identified candidate GTFs bioinformatically and selected 4 for experimental validation: mesenchymal-regulators (*Wwtr1* and *Prrx1*), and developmental regulators (*Tcf4* and *Nfia*) (**Fig 3**). For each of the candidate GTFs, monoclonal GTF-perturbed CT2A lines were generated using CRISPR-Cas9 and engrafted into murine brains. At humane end point, mice were sacrificed and brain tissue was sampled and subject to scRNA-seq profiling to evaluate the effect of each GTF perturbation on glioma biology. As predicted bioinformatically, we found that perturbation of developmental *Nfia* and *Tcf4* promoted a mesenchymal phenotype in CT2A cells. Similarly, perturbation of mesenchymal *Wwtr1* promoted a developmental state and *Prrx1* perturbation reduced the degree of tumor inflammation and invasion.

Having confirmed that perturbation of GTFs can reprogram glioma cells to different canonical GBM phenotypes, we are now in the process of evaluating how these changes influence the surrounding microenvironment, specifically what implications it has on the infiltrating immune populations.

3. Relevant contributions

In addition to the funded project, my contributions to brain tumor research during my funding period have amounted to 9 peer-reviewed publications (2 first author, 7 co-author), and an additional 1 first author publication in review/revision. I've also presented findings from the funded project at AANS in April 2023.

Conferences

1. **Mikolajewicz N.**, Dasgupta K., Wei J., Savage N., Dimitrov V., Tatari N., Venugopal C., Brown K., Han H., Singh S., Moffat J. (2023) Evaluation of GL261 and CT2A murine gliomas as models of human glioblastoma. *Oral presentation at AANS 2023*.

Publications

1. **Mikolajewicz N.**, Yee PP., Bhanja D., Trifoi M., Miller AM., Metellus P., Bagley SJ., Balaj L., de Macedo Filho LJM., Zacharia BE., Aregawi D., Glantz M., Weller M., Ahluwalia MS., Kislinger T., Mansouri A. (2023) A systematic review of CSF biomarker discovery in neuro-oncology: a roadmap to standardization and clinical application. *In review* (at *Journal of Clinical Oncology*).
2. **Mikolajewicz N**., Khan S., Trifoi M., Skakdoub A., Ignatchenko V., Mansouri S., Zuccatto J., Zacharia BE., Glantz M., Zadeh G., Moffat J., Kislinger T., Mansouri A. (2022) Leveraging the CSF proteome toward minimally-invasive diagnostics and biological characterization of brain malignancies. *Neuro-Oncology Advances*.
3. Gwynne WD., Suk Y., Custers S., **Mikolajewicz N**., Chan JK., Zador Z., Zaslaver O., Bakhshinyan D., Chokshi C., Burns I., Chaudhry I., Nachmani O., Mero P., Brown K., Quaile AT., Venugopal C., Moffat J., Montenegro-Burke JR., Singh SK. (2022). Cancer-selective metabolic vulnerabilities in MYC-amplified medulloblastoma. *Cancer Cell*. 40(12):1488-1502.e7. doi: 10.1016/j.ccell.2022.10.009.
4. Zuccato JA., Patil V., Mansouri S., Voisin MR., Chakravarthy A., Shen SY., Nassiri F., **Mikolajewicz N**., Trifoi M., Skakdoub S., Zacharia BE., Glantz M., De Carvalho DD., Mansouri A., Zadeh G. (2022) Diagnostic utility of cerebrospinal fluid methylome-based liquid biopsies for malignant brain neoplasms. *Neuro Oncol*. 25(8):1452-1460. doi: 10.1093/neuonc/noac264.
5. **Mikolajewicz N**., Brown KR., Moffat J., Hong H. (2022). Multi-level cellular and functional annotation of single-cell transcriptomes. *Commun Biol*. 5(1):1142. doi: 10.1038/s42003-022-04093-2.
6. Tatari N., Khan S., Livingstone J., Mckenna D., Ignatchenko V., Chokshi C., Gwynne WD., Singh M., Revill S., **Mikolajewicz N**., Zhu C., Chan J., Hawkins C., Lu JQ., Provias JP., Ask K., Morrissy S., Brown S., Weiss T., Weller M., Greenspoon JN., Moffat J., Venugopal C., Boutros PC., Singh

S., Kislinger T. (2022) The proteomic landscape of glioblastoma recurrence reveals novel and targetable immunoregulatory drivers. *Acta Neuropathol.* 144(6):1127-1142. doi: 10.1007/s00401-022-02506-4.

7. Han H.*, Best AJ.*, Braunschweig U.*, **Mikolajewicz N.**, Li J., Roth., Chowdhury F., Mantica F., Nabeel-Shah S., Parada G., Brown K., O'Hanlon D., Wei J., Yao Y., Abou Zid A., Comsa E., Jen M., Wang J., Datti A., Gonatopoulos-Pournatzis T., Weatheritt RJ., Greenblatt JF., Wrana JL, Irimia M., Gingras A, Moffat J., Blencowe BJ. (2022). Systemic exploration of dynamic splicing networks reveals conserved multistage regulators of neurogenesis. *Mol Cell.* doi: 10.1016/j.molcel.2022.06.036. *contributed equally.
8. Seyfrid M., Maich WT., Shaikh VM., Tatari N., Upreti D., Piyasena D., Subapanditha M., Savage N., McKenna D., **Mikolajewicz N**., Han H., Chokshi C., Kuhlmann L., Khoo A., Salim SK., Archibong-Bassey B., Gwynne W., Brown KR., Murtaza N., Bakhshinyan D, Vora P., Venugopal C., Moffat., Kislinger T., Singh SK. (2021). CD70 as an actionable immunotherapeutic target in recurrent glioblastoma and its microenvironment. *J Immunother Cancer.* 10(1):e003289. doi: 10.1136/jitc-2021-003289.
9. Qazi M., Salim SK., Brown KR., **Mikolajewicz N**., Savage N., Hong H., Subapanditha MK., Bakhshinyan D., Nixon A., Vora P., Desmond K., Chokshi C., Singh M., Khoo A., Macklin A., Khan S., Tatari N., Winegarden N., Richards L., Pugh T., Bock N., Mansouri A., Venugopal C., Kislinger T., Goyal S., Moffat J., Singh SK. (2022). Characterization of the minimal residual disease state reveals distinct evolutionary trajectories of human glioblastoma. *Cell Reports.* doi: 10.1101/2022.01.28.478232
10. Tang Y., Qazi MA., Brown KR., **Mikolajewicz N**., Moffat J., Singh SK., McNicholas PD. (2021). Identification of five important genes to predict glioblastoma subtypes. *Neuroncol Adv.* 3(1):vdab144. doi: 10.1093/noajnl/vdab144

4. Financial Statement

Table 2. Financial Expense Report

Term	**Description**	**Amount ($)**
2020-2021	Salary support	20625.00
2020-2021	Conference Registration	25.85
2020-2021	Bioinformatics Computer	3460.81
2021-2022	Salary support	22500.00
2022-2023	AANS conference	2036.23
2022-2023	Salary support	20730.42
	Total	**69379.31**

5. Figures

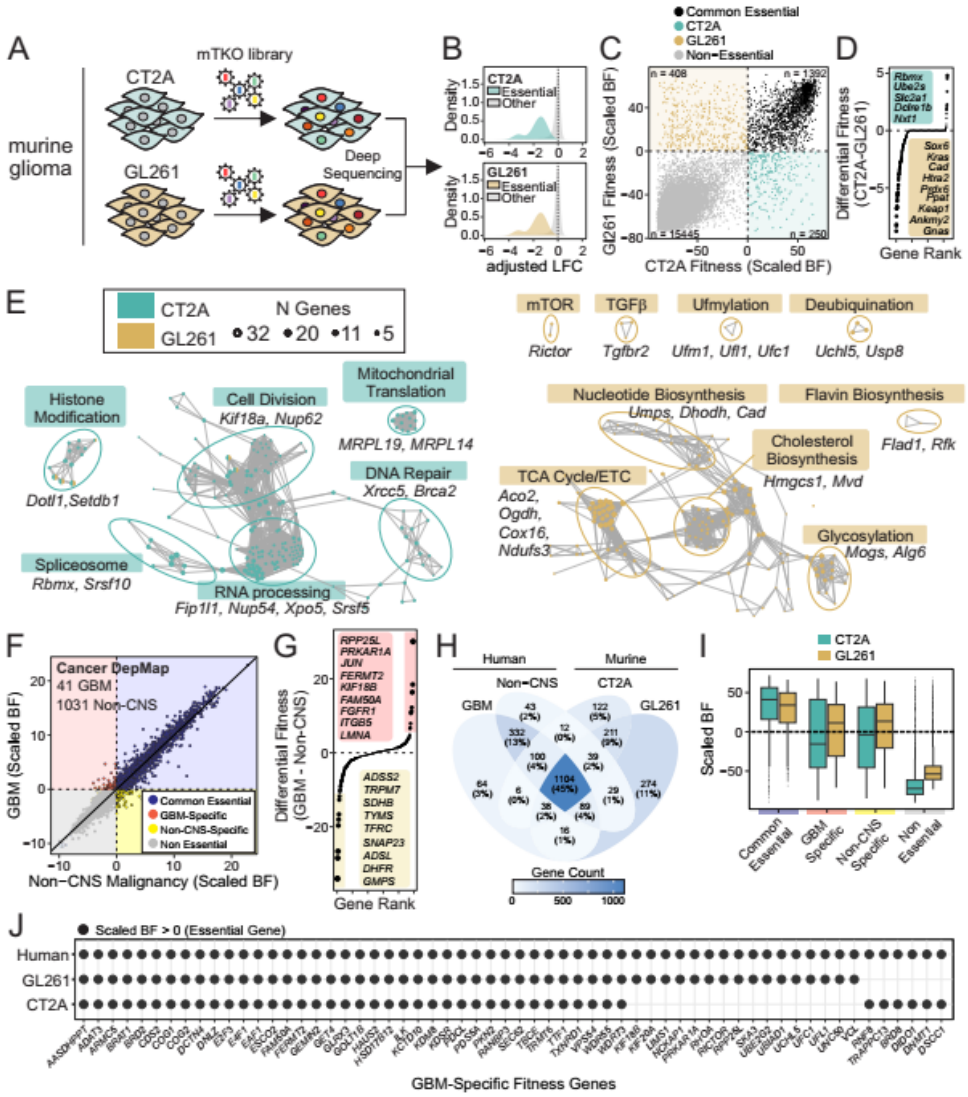


Figure 1. Genetic dependencies in murine and human glioblastoma. **(A)** Workflow for mTKO genome-scale pooled CRISPR screens to identify fitness genes in CT2A and GL261 cells. **(B)** Distribution of gene-level differential logFC of gRNAs in CT2A (*top*) and GL261 (*bottom*), stratified by essentiality. Genes fitness was scored using BAGEL and essential genes were classified using BF > 5 threshold. **(C)** Comparison of CT2A and GL261 gene-level fitness. Scatter plot shows CT2A and GL261 scaled BFs. Scaled BF was calculated as BF – 5 such that scaled BF > 0 represents essential genes. **(D)** Ranked differential fitness between GL261 and CT2A. Y-axis for differential fitness is signed log10(FDR) derived from difference between scaled BF scores. **(E)** Enrichment map illustrating CT2A and GL261-specific dependencies. *Nodes* represent gene sets, and *edges* represent Jaccard similarities. **(F)** Scatter plot of scaled BF scores for human GBM cells and non-CNS cells. Scores were retrieved from Project Score Database (*see methods*). **(G)** Ranked differential fitness between human GBM and non-CNS cell lines. Genes were ranked by signed log10(FDR) derived from difference between scaled BF scores. **(H)** Venn diagram of human (GBM and non-CNS) and murine (CT2A and GL261) essential genes (scaled BF > 0). **(I)** Boxplot of scaled BFs from CT2A and GL261 screens grouped by human essentiality gene sets (*as defined in panel **F***). **(J)** Dot plot of GBM-specific fitness genes that are common to human GBM and murine gliomas. BAGEL; Bayesian Analysis of Gene Essentiality, BF, Bayes factor; ETC, electron transport chain; logFC, log fold-change.

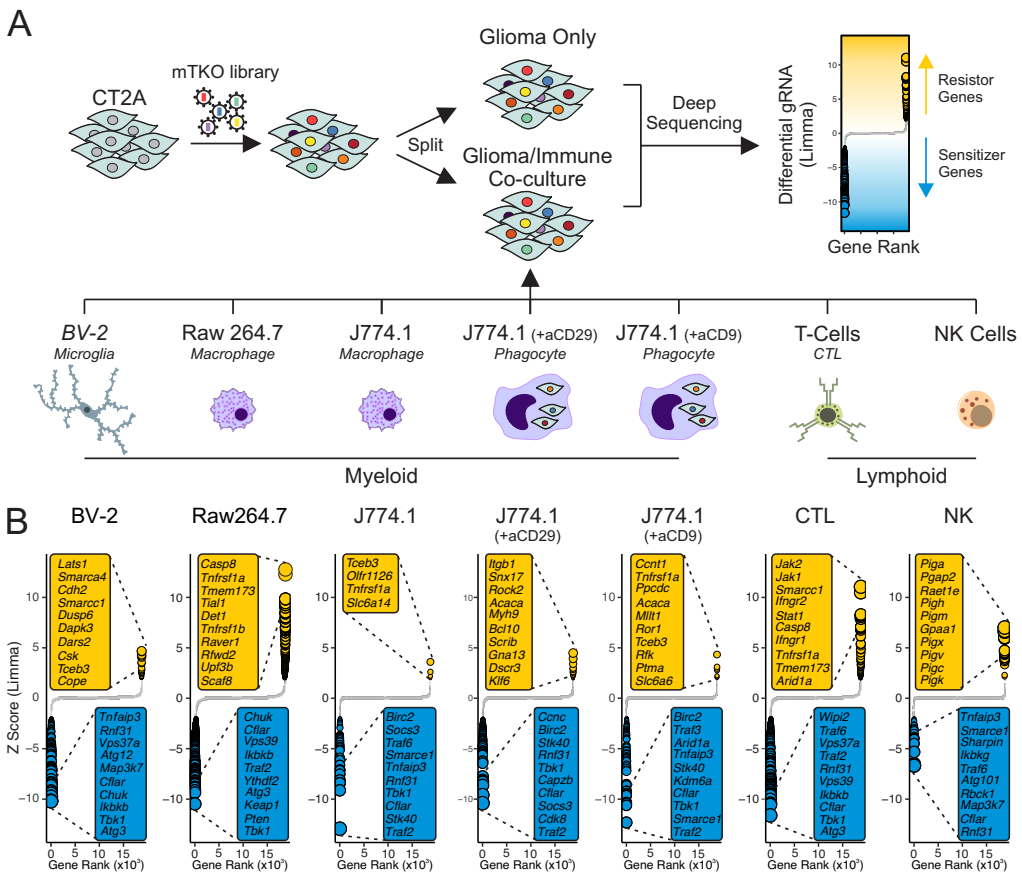


Figure 2. Mechanisms of CT2A-intrinsic immune evasions. (A) Workflow for mTKO genome-scale pooled CRISPR screens to identify immune-evasion genes. CRISPR-mutagenized CT2A cells were propagated in the present or absence of various immune cell lines (microglia; BV-2, macrophages; Raw 264.7 and J774.1, phagocytes; J774.1 treated with anti-CD29 or anti-CD9, cytotoxic T-lymphocytes, or natural killer cells) to apply selective pressure and CT2A cells were subjected to deep sequencing to identify gRNA that were enriched (i.e., resistor genes) or depleted (i.e., sensitizer genes) relative to untreated cells. **(B)** Rank-ordered z-score of gRNA enriched/depleted in mutagenized CT2A cells after exposure to immune cells. Hits at FDR <5% are highlighted in yellow (resistor genes) and blue (sensitizer genes). Point size is inversely scaled by FDR.

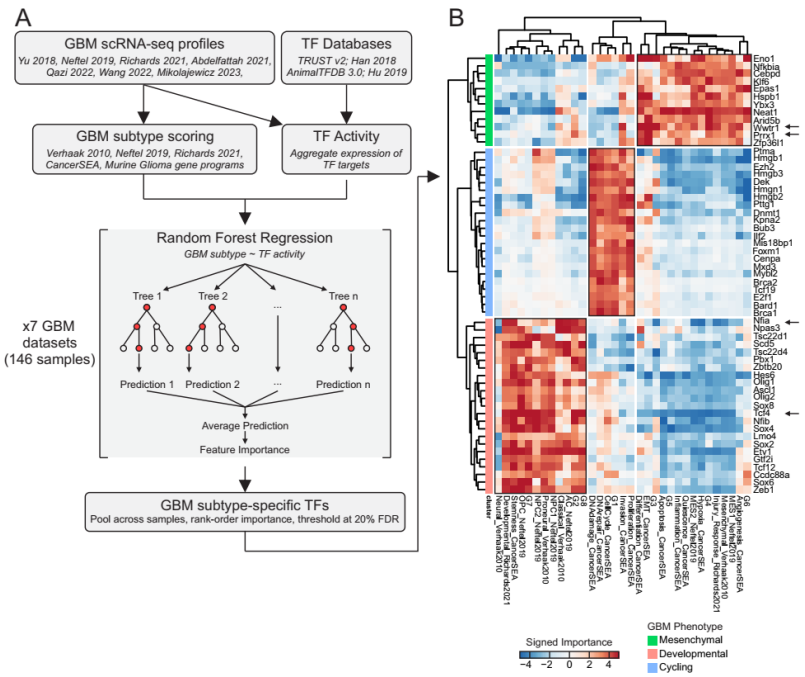
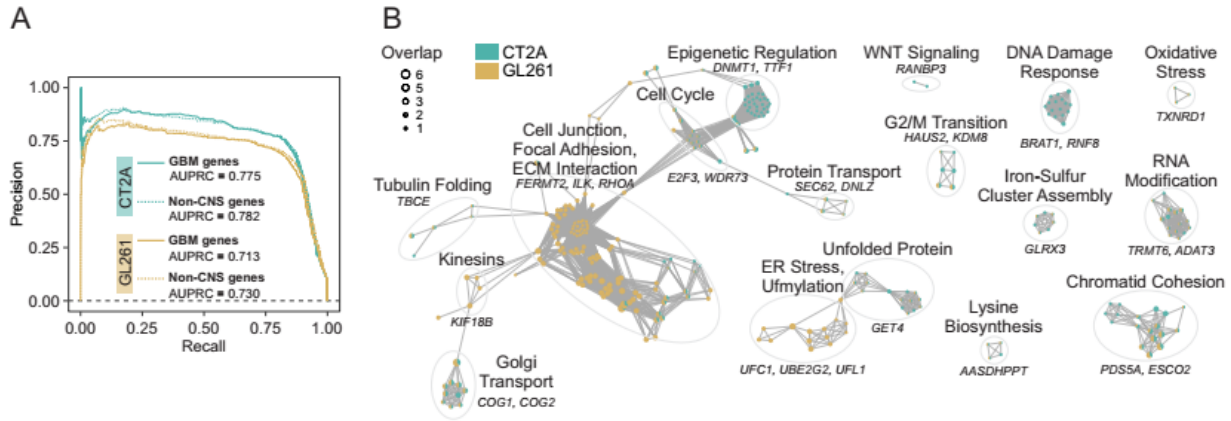


Figure 3. Evaluation of transcriptional regulators of human GBM in murine glioma cells. (A) Schematic illustrating approach to GBM subtype-specific transcription factor (TF) discovery. For each GBM scRNA-seq profile, *a priori* GBM signatures and TF targets were scored, and random forest regression was performed to identify TF activity patterns that are associated with each GBM phenotype, as quantified by feature importance (*see methods*). Feature importance scores were then pooled across datasets (GL261 and CT2A samples were omitted), and significant TFs were determined at 20% FDR. **(B)** Heatmap showing predicted relationships between TF activity (*rows*) and GBM signatures (*columns*). Heatmap values are signed feature importance scores. TFs were hierarchically clustered and GBM phenotypes (*green*; mesenchymal, *red*; developmental, *blue*; cycling) were inferred from signature consensus within each cluster.

6. Supplemental Figures



Supplemental Figure 1. Comparison of murine and human genetic dependencies. (A) Precision-Recall analysis of essential gene recovered using CT2A (*green*) and GL261 (*brown*) CRISPR screens. Human GBM and non-CNS essential genes were used as ground-truths. **(B)** Enrichment map illustrating shared human-murine genetic dependencies. *Nodes* are gene sets, *edges* are Jaccard similarities between gene sets. AUPRC; area under precision-recall curve.

7. References

1 Dwane, L. *et al.* Project Score database: a resource for investigating cancer cell dependencies and prioritizing therapeutic targets. *Nucleic Acids Research* **49**, D1365-D1372 (2021).

2 Pacini, C. *et al.* Integrated cross-study datasets of genetic dependencies in cancer. *Nature communications* **12**, 1661 (2021).