The proteasome activator complex, PA28αβ, regulates stemness in glioblastoma.

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The Proteasome and Glioblastoma

Glioblastoma (GBM) is the most common adult primary brain tumor plagued by inevitable recurrence and poor survival[1]. The proteasome is a multimeric protein complex that degrades cellular proteins contributing to homeostatic proteostasis, stress response, and antigen presentation[2,3]. Most proteasomal subunits are essential for GBM stem cell growth in vitro, however, they are also essential for non-malignant neural cells, suggesting that inhibition of those subunits may lead to toxicity[4]. Indeed, adverse neurological symptoms were prevalent in phase III clinical trials for the brain penetrant proteasome inhibitor, Marizomib[5], which may be linked to the vital role of proteasome subunits in non-malignant neural counterparts. Proteasome inhibitors target the catalytic subunits of the proteasome[2,3]; however, the role of individual non-canonical proteasome activators have not been fully elucidated in GBM. Here, we examined the functionality of one proteasome activator complex in GBM brain tumour stem cells (BTSCs) in vitro and in vivo. Surprisingly, despite lack of growth changes in vitro, we observed abrogated stem-cell self-renewal in vitro and improved survival in vivo in orthotopic xenograft models following targeting of specific activator subunits. Molecular profiling of targeted cells revealed an upregulation of interferon-γ signaling and upregulation of antigen presentation machinery. Thus, targeting specific activator subunits may inhibit malignant growth in vivo while sparing normal neural counterparts from proteotoxic stress. We are further investigating enhanced antigen presentation by targeting these proteasome activator subunits in syngeneic immunocompetent models of GBM and examining changes in the tumor microenvironment. We also aim to determine if this mechanism is conserved when targeting other non-canonical proteasome activator complexes in GBM. Further understanding of this mechanism may provide novel targets for GBM treatment or improve immunotherapies.

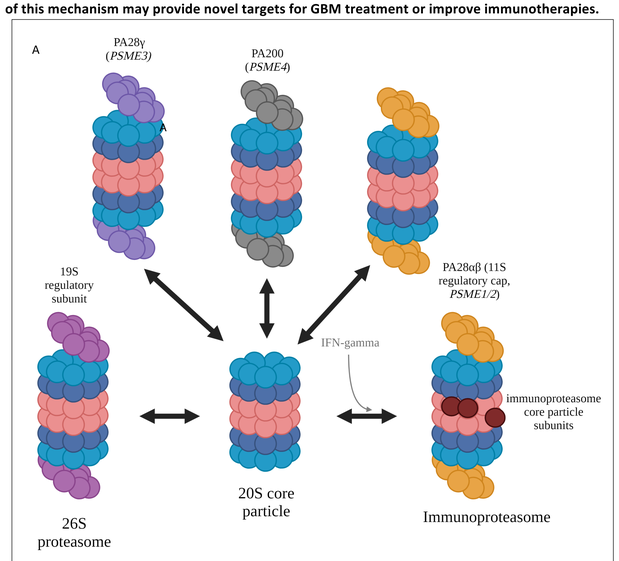


Figure 1. A) Model of different proteasome species that can be present within cells. The proteasome is a multi-subunit complex composed of a 20S core particle that can bind to various regulator caps. Canonically, in the presence of immune signaling, the immunoproteasome subunits and the PA28αβ regulatory cap are induced. B) Expression of the 4 non-canonical proteasome activators in GBM tissue compared to normal brain tissue from the TCGA. (Unpaired t-test, * p < 0.05, ** p < 0.01, **** p < 0.0001. C) Relative essentiality for the 4 non-canonical proteasome activators in BTSCs grown *in vitro* compared to human fetal derived neural stem cells[4].

References: 1. Stupp, R. et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N. Engl. J. Med. 352, 987–996 (2005). 2. Tanaka, K. The proteasome: overview of structure and functions. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **85**, 12–36 (2009). 3. Raule, M. *et al.* PA28αβ Reduces Size and Increases Hydrophilicity of 20S Immunoproteasome Peptide Products. *Chem. Biol.* **21**, 470–480 (2014). 4. Graham MacLeod, Danielle A. Bozek, *et al.* Genome-Wide CRISPR-Cas9 Screens Expose Genetic Vulnerabilities and Mechanisms of Temozolomide Sensitivity in Glioblastoma Stem Cells, Cell Reports, Volume 27, Issue 3, 2019. 5. Patrick Roth, Thierry Gorlia, Jaap C. Reijneveld, et al. EORTC 1709/CCTG CE.8: A phase III trial of marizomib in combination with temozolomide-based radiochemotherapy versus temozolomide-based radiochemotherapy alone in patients with newly diagnosed glioblastoma. Journal of Clinical Oncology 2021 39:15_suppl, 2004. 6. Shen, Y. et al. Comprehensive genomic profiling of glioblastoma tumors, BTICs, and xenografts reveals stability and adaptation to growth environments. Proc. Natl. Acad. Sci. U. S. A. 116, 19098–19108 (2019) 7. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A. 102, 15545–15550 (2005). 8. Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504 (2003). 9. Yang Liao, Gordon K Smyth, Wei Shi, The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads, Nucleic Acids Research, Volume 47, Issue 8, (2019). 10. Love, M.I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550 (2014). 11. Monitoring the Immunoproteasome in Live Cells Using an Activity-Based Peptide–Peptoid Hybrid Probe. Breanna L. Zerfas and Darci J. Trader. Journal of the American Chemical Society 2019 141 (13), 5252-5260; DOI: 10.1021/jacs.8b12873.

Results

PA28αβ is upregulated in recurrent BTSCs

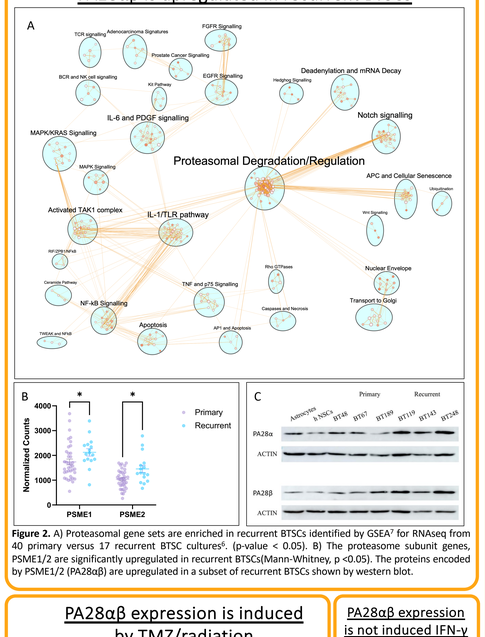


Figure 2. A) Proteasomal gene sets are enriched in recurrent BTSCs identified by GSEA[7] for RNAseq from 40 primary versus 17 recurrent BTSC cultures[6]. (p-value < 0.05). B) The proteasome subunit genes, PSME1/2 are significantly upregulated in recurrent BTSCs(Mann-Whitney, p <0.05). The proteins encoded by PSME1/2 (PA28αβ) are upregulated in a subset of recurrent BTSCs shown by western blot.

PA28αβ KO leads to down regulation of and interacts with NCAM1

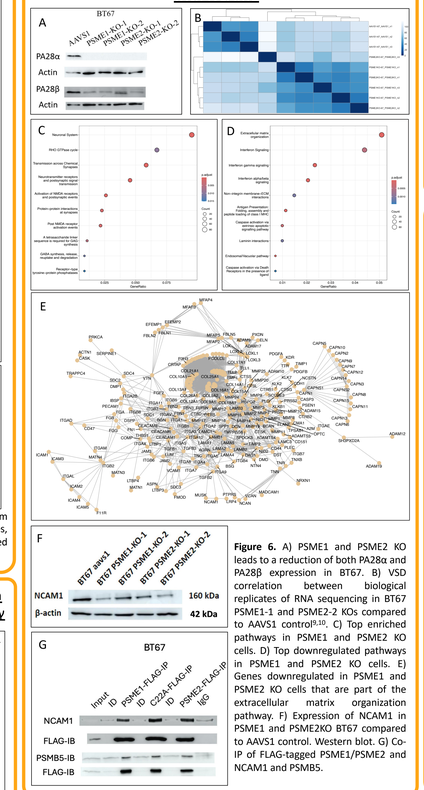


Figure 6. A) PSME1 and PSME2 KO leads to a reduction of both PA28α and PA28β expression in BT67. B) VSD correlation between biological replicates of RNA sequencing in BT67 PSME1-1 and PSME2-2 KOs compared to AAVS1 control[9,10]. C) Top enriched pathways in PSME1 and PSME2 KO cells. D) Top downregulated pathways in PSME1 and PSME2 KO cells. E) Genes downregulated in PSME1 and PSME2 KO cells that are part of the extracellular matrix organization pathway. F) Expression of NCAM1 in PSME1 and PSME2KO BT67 compared to AAVS1 control. Western blot. G) Co-IP of FLAG-tagged PSME1/PSME2 and NCAM1 and PSMB5.

PA28αβ KD/KO reduces sphere formation without altering growth *in vitro*

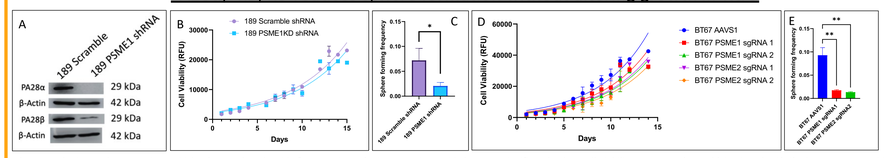


Figure 7. A) PSME1 shRNA KD in BT189 leads to reduced PA28αβ expression. B) PSME1 KD in BT189 does not significantly alter cell viability over time (growth) *in vitro*. (n=3) C) PSME1 KD in BT189 reduced sphere forming frequency (t-test p <0.05) (n=3). D) PSME1/2 KO in BT67 does not significantly alter cell viability over time (growth) *in vitro* (n=3). E) PSME1/2 KO in BT67 reduced sphere forming frequency (t-test p <0.01) (n=3).

PA28αβ KD/KO alters standard proteasome and immunoproteasome activity

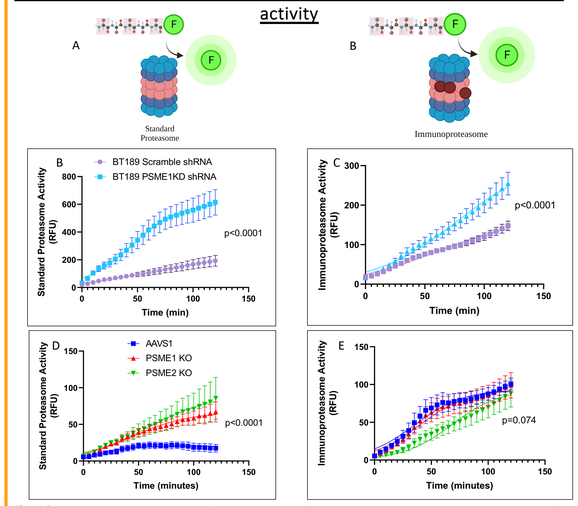


Figure 8. A) Model of the standard proteasome fluorescent probe assay[11]. B) Model of the immunoproteasome fluorescent probe assay[11]. C) PSME1 shRNA KD in BT189 increases the cleavage of the standard proteasome activity probe. (logistic growth model, f-test p<0.0001). D) PSME1 shRNA KD in BT189 increases the cleavage of the immunoproteasome activity probe. (logistic growth model, f-test p<0.0001). E) PSME1/2 KO in BT67 increases the cleavage of the standard proteasome activity probe. (logistic growth model, f-test p<0.0001). F) PSME1/2 KO in BT67 does not significantly alter the cleavage of the immunoproteasome activity probe.

PA28αβ KD/KO prolongs survival in xenograft models

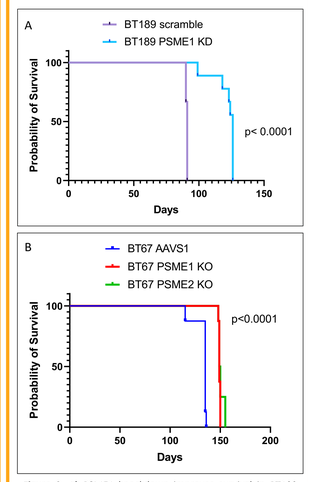


Figure 9. A) PSME1 knockdown improves survival in BT189 orthotopic xenograft model (log rank test p<0.0001, n=10). B) PSME1/2 KO improves survival in BT67 orthotopic xenograft model (log rank test p<0.0001, n=10).

PA28αβ expression is induced by TMZ/radiation

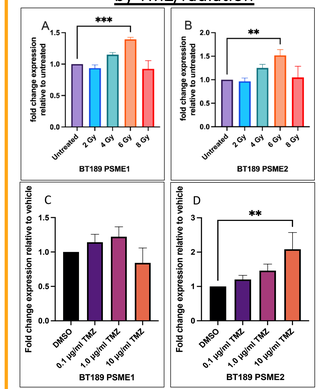


Figure 3. A) PSME1 RNA expression (RT-qPCR) in response to specified IR doses. (ANOVA p < 0.0005, n=3). B) PSME2 RNA expression (RT-qPCR) in response to specified IR doses. (ANOVA p < 0.005, n=3). C) PSME1 RNA expression (RT-qPCR) in response to specified TMZ doses. (n=3). D) PSME2 RNA expression (RT-qPCR) in response to specified TMZ doses. (ANOVA p < 0.005, n=3).

PA28αβ expression is not induced IFN-γ

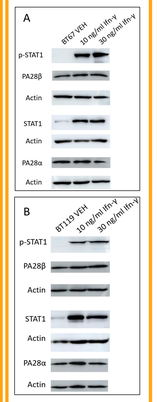


Figure 4. A) PA28αβ expression (Western) and p-STAT1 activation in response to Ifn-γ in BT67. B) PA28αβ expression (Western) and p-STAT1 activation in response to Ifn-γ in BT119.

PA28αβ knockdown (KD) or knockout (KO) increases susceptibility to ionizing radiation

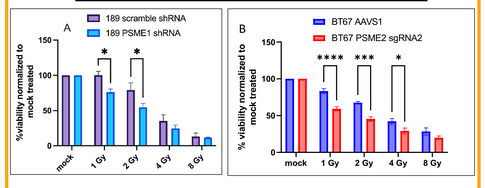
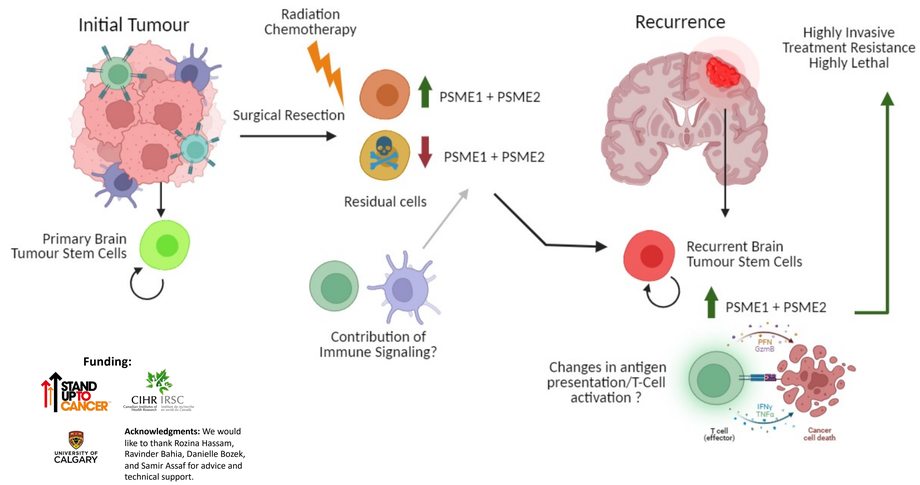


Figure 5. A) PSME1 KD in BT189 leads to increased susceptibility to ionizing radiation (IR) at specific doses. 7d. n=3. ANOVA p<0.05. B) PSME2 KO in BT67 increased susceptibility to IR at specific doses. 7d. n=3. ANOVA * p<0.05, *** p<0.001, **** p<0.0001.

Model and Summary



Summary

1. **The IFN-γ inducible proteasome activator complex, PA28αβ, appears to be upregulated in GBM and in BTSCs upon recurrence.**
2. **PA28αβ can be upregulated by TMZ and radiation in BTSCs, however BTSCs fail to upregulate PA28αβ in response to canonical Ifn-γ signaling, suggesting a mechanism of immune evasion.**
3. **PA28αβ KO leads to down regulation of extracellular matrix genes, specifically NCAM1.**
4. **PA28αβ interacts with NCAM1 and may contribute to its stability.**
5. **PA28αβ (*PSME1/2*) KD and KO leads to increased radiation susceptibility, reduced sphere formation, and altered proteasome activity.**
6. **PA28αβ KD/KO improved overall survival in orthotopic xenograft models**

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