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Integrative cross-platform analyses identify enhanced heterotrophy as a metabolic hallmark in glioblastoma

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Abstract

Background. Although considerable progress has been made in understanding molecular alterations driving gliomagenesis, the diverse metabolic programs contributing to the aggressive phenotype of glioblastoma remain unclear. The aim of this study was to define and provide molecular context to metabolic reprogramming driving gliomagenesis.

Methods. Integrative cross-platform analyses coupling global metabolomic profiling with genomics in patient-derived glioma (low-grade astrocytoma [LGA; $n = 28$] and glioblastoma [$n = 80$]) were performed. Identified programs were then metabolomically, genomically, and functionally evaluated in preclinical models.

Results. Clear metabolic programs were identified differentiating LGA from glioblastoma, with aberrant lipid, peptide, and amino acid metabolism representing dominant metabolic nodes associated with malignant transformation. Although the metabolomic profiles of glioblastoma and LGA appeared mutually exclusive, considerable metabolic heterogeneity was observed in glioblastoma. Surprisingly, integrative analyses demonstrated that O^6-methylguanine-DNA methyltransferase methylation and isocitrate dehydrogenase mutation status were equally distributed among glioblastoma metabolic profiles. Transcriptional subtypes, on the other hand, tightly clustered by their metabolomic signature, with proneural and mesenchymal tumor profiles being mutually exclusive. Integrating these metabolic phenotypes with gene expression analyses uncovered tightly orchestrated and highly redundant transcriptional programs designed to support the observed metabolic programs by actively importing these biochemical substrates from the microenvironment, contributing to a state of enhanced metabolic heterotrophy. These findings were metabolomically, genomically, and functionally recapitulated in preclinical models.

Conclusion. Despite disparate molecular pathways driving the progression of glioblastoma, metabolic programs designed to maintain its aggressive phenotype remain conserved. This contributes to a state of enhanced metabolic heterotrophy supporting survival in diverse microenvironments implicit in this malignancy.

Key Points

1. Integrative analyses define and provide molecular context to metabolic reprogramming driving gliomagenesis.
2. A metabolic adaption of enhanced heterotrophy supports survival in the diverse ecology implicit in this malignancy.

Importance of the Study

Glioblastoma is an aggressive brain tumor with limited treatment options. Although progress has been made in understanding molecular alterations in these tumors, this level of knowledge has not translated to clinical improvements. Metabolic reprogramming represents a hallmark of cancer and may serve as a therapeutic target. We performed integrative cross-platform analyses coupling global metabolomic profiling with genomics in patient-derived tumors to both identify metabolic nodes specific to glioblastoma and understand their molecular context. In addition to delineating metabolic reprogramming contributing to the aggressive phenotype of this malignancy, defining its transcriptional framework, and recapitulating these findings in preclinical models, this work may serve as a powerful resource for investigators to explore novel metabolic vulnerabilities and therapeutic targets in glioblastoma.

Glioblastoma is an aggressive brain tumor with limited treatment options. Considerable progress has been made in understanding molecular alterations unique to these tumors in an effort to improve therapeutic gains. For example, glioblastoma is one of the first cancer types systematically studied at the genomic and transcriptomic level revealing a landscape of intertumoral heterogeneity and distinct molecular subtypes that offer the promise of tumor-specific treatment strategies.[1–3] These include the proneural and mesenchymal molecular subtypes, which have been identified most consistently in glioblastoma. Their transcriptional profiles are mutually exclusive and can be applied to approximately one half of tumors; other subtypes include classical and neural, which are characterized by epidermal growth factor receptor and the expression of neuronal markers, respectively.[1,4] Promoter methylation of O^6-methylguanine–DNA methyltransferase (MGMT) has been established as a key prognostic factor in glioblastoma, allowing for stratification in ongoing clinical trials. More importantly, MGMT status is now recognized as a predictive factor to the otherwise standard chemotherapeutic agent temozolomide, providing the framework for the development of alternate, personalized treatment strategies in these particularly aggressive tumors.[5,6] In addition, the discovery of mutations of the metabolic enzyme isocitrate dehydrogenase 1 (IDH1) and its biologic consequence has been one of the most notable findings in the field. IDH mutation, which occurs in approximately 20% of these tumors and was previously referred to as secondary glioblastoma, represents an early event in gliomagenesis. Mutation results in the formation of a neomorphic enzyme with the capacity of generating the oncometabolite 2-hydroxyglutarate (2-HG), which subsequently drives global epigenetic programs in this malignancy.[7–9] Unfortunately, despite these steadfast gains in the understanding of molecular pathways associated with gliomagenesis, clinical advancements have remained limited.

Metabolic reprogramming represents a hallmark of cancer and results as a direct and/or indirect consequence to oncogenic signaling, allowing cells to produce sufficient energy and biosynthetic building blocks to promote malignant cellular proliferation, thereby offering the potential to serve as a therapeutic target.[10] However, the diverse metabolic programs driving the aggressive phenotype of glioblastoma are only beginning to be recognized. As glioblastoma represents an archetypal example of a heterogeneous malignancy, harboring regions of invasion, necrosis, and vascularization,[11] focused investigations on metabolic reprogramming may be particularly relevant, as dynamic metabolic remodeling is likely required to allow for continued growth in these unique microenvironments. To begin to understand the diverse metabolic consequences of this complex tumor ecology, we performed integrated cross-platform analyses coupling metabolomic profiling with genomics to both identify metabolic nodes specific to glioblastoma and understand their molecular context.

Materials and Methods

Tumor Samples

This study was institutional review board approved. Low-grade astrocytoma (LGA; $n = 28$) and glioblastoma ($n = 80$) tumors were obtained from the Moffitt Cancer Center. Written informed consent was obtained from patients and histology confirmed by a pathologist. Tumors were flash-frozen and a schema summarizing the performed analyses is provided in Supplementary Fig. 1.

Metabolomic Profiling

Global metabolomic profiling of tumors and cell lines was performed by Metabolon as previously described[12] and additional details are provided in Supplementary Methods.

Gene Expression Analysis

Five hundred nanograms of total RNA was used for analysis on an Affymetrix Clariom-D Human expression array (Life Technologies). Manufacturer's protocol was followed. Detailed protocol, data processing, and subtyping are provided in Supplementary Methods. Raw data are available in ArrayExpress (accession number: E-MTAB-7116).

MGMT Methylation and IDH1 Mutation Status

MGMT promoter methylation was determined by methylation-specific PCR. IDH mutation status was determined using allele-specific PCR. Additional details are provided in Supplementary Methods.

Glioblastoma Tumor-Initiating Cells

Mesenchymal (MES83, MES326) and proneural (PN19, PN84) glioblastoma tumor-initiating cells were cultured as previously described.[13] Evaluation of fatty acid uptake was performed using fatty acid–bovine serum albumin (BSA) boron-dipyrromethene (BODIPY). Peptide uptake was evaluated using fluorescein isothiocyanate (FITC)–conjugated BSA with/without inhibition by tetramethylrhodamine (TMR)-dextran. The detailed protocol is provided in Supplementary Methods.

Data Analysis

To compensate for mass spectrometer thresholding of data, missing values were imputed with values based on the average of values across all samples. Statistical analysis and hierarchical clustering was performed using Metaboanalyst 3.0. Additional details of data analysis are provided in Supplementary Methods.

Results

Metabolic Reprogramming in Gliomagenesis

To provide insight into metabolic programs associated with gliomagenesis, global metabolomic profiling was performed on patient-derived, newly diagnosed glioma, specifically evaluating for differences between LGA ($n = 28$) and glioblastoma ($n = 80$). From a biochemical library consisting of over 3000 standards, a total of 757 compounds were detected. Of these, over 60% demonstrated differential accumulation between LGA and glioblastoma, suggesting clear metabolic changes associated with malignant transformation (Supplementary Fig. 2A). Hierarchical clustering of this panel of metabolites resulted in distinct signatures distinguishing the metabolic profile of glioblastoma from LGA (Fig. 1A) with a predictive accuracy of 92% on Random Forest classification (Supplementary Fig. 2B). This was further corroborated by principal component analysis (PCA), which, in addition to distinct signatures, demonstrated considerable metabolic heterogeneity in glioblastoma compared with LGA (Supplementary Fig. 2C).

A panel of biochemicals representing diverse metabolic pathways differentiated glioblastoma from LGA (Fig. 1B, Supplementary Table 1). Although an overwhelming majority of pathways appeared activated in glioblastoma, metabolites involved in the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, histidine metabolism, and phospholipid synthesis were enriched in LGA. Considerable metabolic reprogramming in amino acid and lipid metabolism was observed in glioblastoma, along with an accumulation of dipeptides, which represented the most dominant class of metabolites identified. For example, of the >30 dipeptides identified, a differential abundance score of almost 1 indicates that nearly all of them demonstrated aberrant accumulation in glioblastoma compared with LGA. Interestingly, an overwhelming majority of the dipeptides included an essential amino acid, and 2/3 included branched chain amino acids, which represented a metabolic pathway activated in glioblastoma (Supplementary Fig. 3A/B). A biochemical

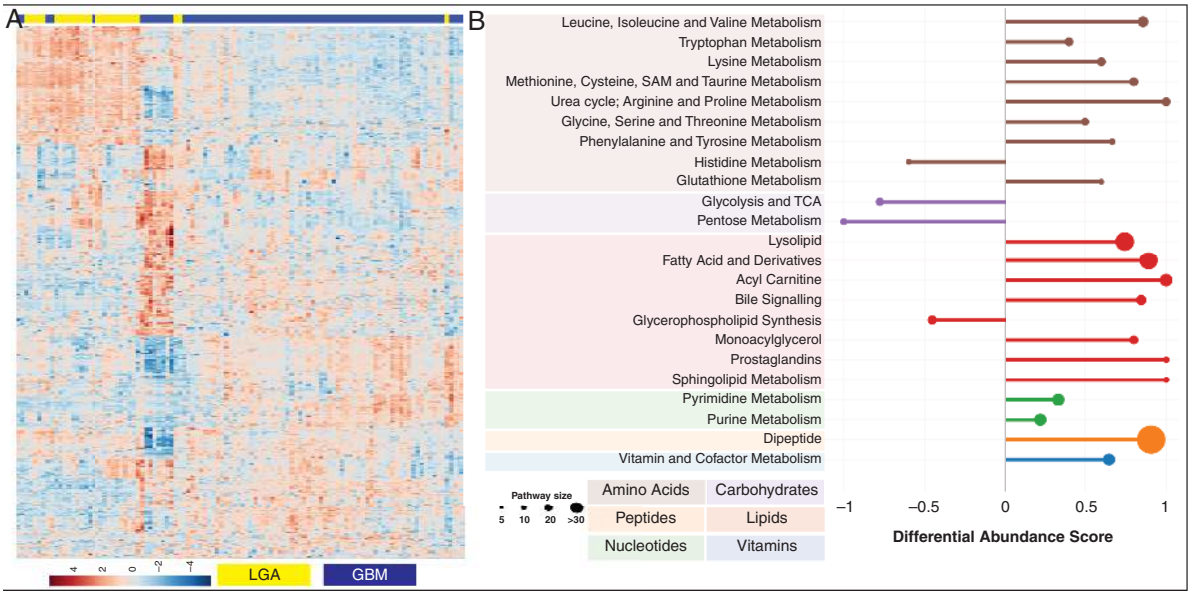

Fig. 1 Metabolic reprogramming driving gliomagenesis involves aberrant amino acid, lipid, and peptide metabolism. (A) Hierarchical clustering of biochemicals identified by global metabolomic profiling of LGA ($n = 28$) and GBM ($n = 80$). Tumors and metabolites are represented in columns and rows, respectively. (B) Significantly altered metabolites in GBM compared with LGA were classified into major metabolic pathways and the differential abundance scores of each pathway were plotted.

importance plot (BIP) designed to rank individual metabolites based on their capacity to discriminate between LGA and glioblastoma represented the metabolic pathways described above, and the previously established markers of LGA—myo-inositol and 2-HG—emerged as top-ranking metabolites (Supplementary Fig. 4A). In addition, these investigations uncovered novel metabolites associated with gliomagenesis. This included an accumulation of the advanced glycation end product N(6)-carboxymethyllysine in LGA, a potential marker for oxidative stress,[14] which emerged as the top ranking metabolite on the BIP, followed by the carnitine precursor deoxycarnitine in glioblastoma. Although ranking higher on BIP than 2-HG, their relative magnitude was lower, reiterating the clear importance of this oncometabolite in gliomagenesis (Supplementary Fig. 4B).

As aberrant fatty acid metabolism, amino acid metabolism, and accumulation of dipeptides emerged as dominant metabolic pathways differentiating LGA from glioblastoma, we studied these pathways in further detail. To determine molecular pathways that may be driving these programs, a panel of genes defined as having specific roles in these metabolic pathways in UniProt was assembled and their expression analyzed using The Cancer Genome Atlas (TCGA). Consistent with our metabolomic findings, clear separation between LGA and glioblastoma was observed when evaluating the expression of genes involved in these metabolic pathways (Fig. 2A–C). Specifically, from the panel of 590 genes involved in lipid metabolism, glioblastoma demonstrated an expression profile supporting a phenotype of lipid utilization, consisting of increased expression of genes associated with lipid and phospholipid uptake and catabolism coupled with a decrease in enzymes associated with lipid and glycerophospholipid synthesis (Fig. 2A). Next, we performed pathway level, cross-platform analyses integrating these genomic findings with our metabolomic profiles, uncovering a tightly orchestrated and highly redundant transcriptional program designed to drive metabolism and accumulation of free fatty acids that was concordant with metabolomic findings (Fig. 3A). This included increased free fatty acid stores through both fatty acid uptake and an accumulation of its metabolic precursors in glioblastoma, including acyl glycerols and phospholipids, along with subsequent lipid catabolism along fatty acid oxidation and arachidonic acid metabolism. This appeared to be coordinated with a decrease in de novo fatty acid synthesis, reflected by decreases in malonyl CoA members and metabolites involved in phospholipid synthesis. Consistent with an accumulation of dipeptides in glioblastoma, increased expression of numerous genes associated with lysosomal protein endocytosis and degradation was observed compared with LGA. Interestingly, genes associated with metallopeptidases and ubiquitin mediated protein lysis appeared lower in glioblastoma

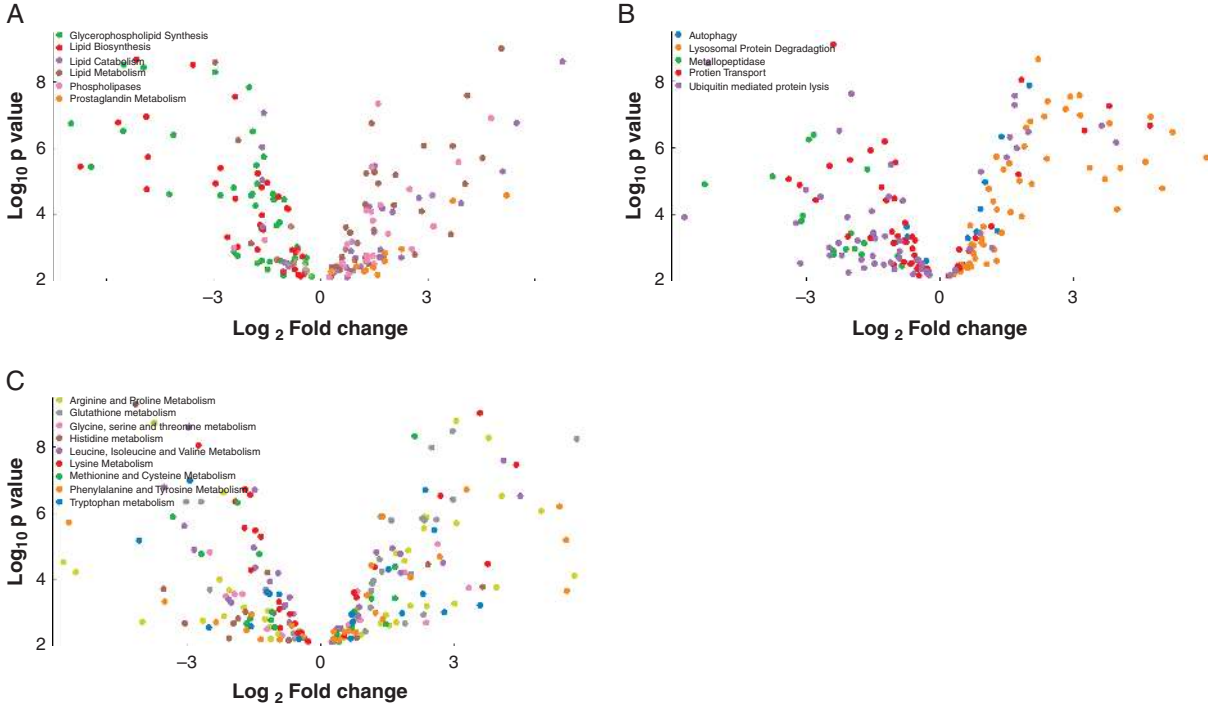

Fig. 2 Gene expression profiles of LGA and glioblastoma reflect metabolic reprogramming involved in gliomagenesis. Transcriptional signatures of genes (curated from UniProt) associated with (A) lipid, (B) peptide, and (C) amino acid metabolism from TCGA dataset: TCGA Lower Grade Glioma and Glioblastoma (GBMLGG) was compiled. Expression values for these genes were compared between LGA and glioblastoma (Benjamini–Hochberg corrected *P*-value <0.05) and log2 fold-change (LGA as baseline) was used to generate volcano plots.

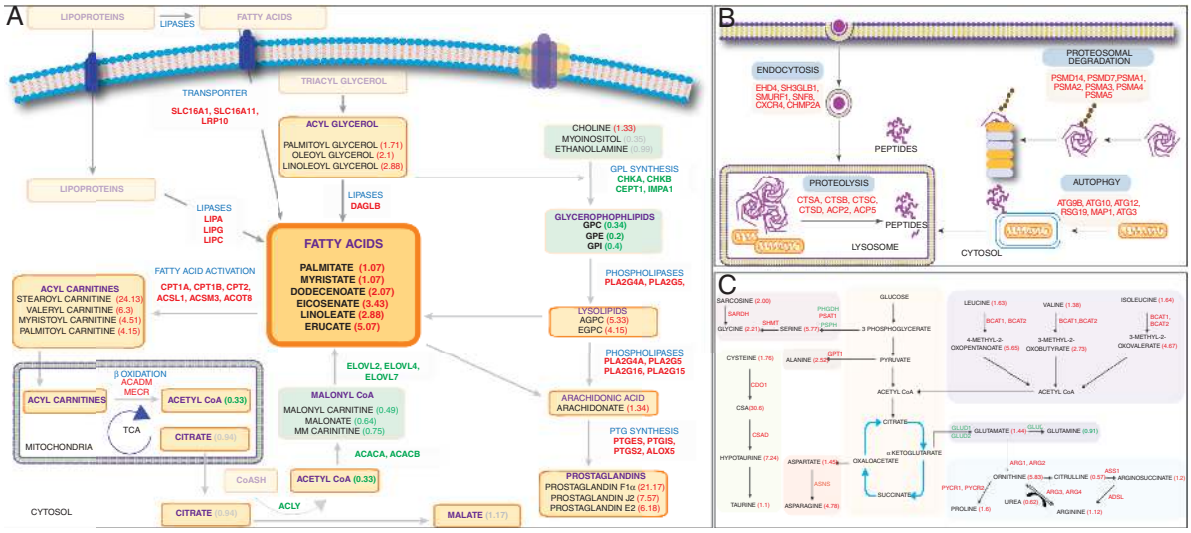

Fig. 3 Integrative analyses combining metabolomic and transcriptional profiles of LGA and glioblastoma. Schematic representation of (A) lipid, (B) peptide, and (C) amino acid metabolism in glioblastoma, integrating differential accumulation of metabolites identified in Fig. 1 with the expression of enzymes involved in their intermediary metabolism identified in Fig. 2. Values following metabolites in red and green indicate fold change increases and decreases, respectively, in glioblastoma compared with LGA. Values in gray were not statistically significant (Benjamini–Hochberg corrected *P*-value <0.05). Genes represented in red and green indicate higher and lower expression, respectively, in glioblastoma compared with LGA. GPC = glycerophosphocholine, GPE = glycerophosphoethanolamine, GPI = glycerophosphoinositol, AGPC = arachidoylglycerophosphocholine, EGPC = eicosatrienoylglycerophosphocholine.

(Figs. 2B, 3B). Similar findings were observed when evaluating aberrant amino acid metabolism in glioblastoma, which involved divergent pathways of glycolysis and the TCA cycle, including serine and ornithine metabolism, respectively, cysteine, and the metabolism of the branched chain amino acids (Figs. 2C, 3C).

Metabolic Heterogeneity in Glioblastoma

As our initial analyses suggested considerable metabolic heterogeneity in glioblastoma, we extended investigations to begin to understand these metabolic phenotypes in further detail. Hierarchical cluster analysis confirmed clear metabolic heterogeneity in these tumors (Fig. 4A). We next sought to determine if the observed metabolic heterogeneity in glioblastoma could be a direct consequence of established molecular subtypes. To accomplish this, we performed cross-platform analyses using RNA and DNA isolated from 56 samples where a matched aliquot of tumor tissue was available. MGMT promoter methylation status and IDH1 mutation represent two of the strongest prognostic factors in glioblastoma.[6–9] We therefore went on to determine if these molecular subtypes were driven by specific metabolic programs. Interestingly, both MGMT methylated and IDH1 mutated tumors appeared to be evenly distributed within the metabolic subtypes. As mutations in IDH1 represent a gain of function, forming a neomorphic enzyme that generates the oncometabolite 2-HG,[7] we extended investigations by defining the remaining tumors where matched tissue was not available as "high" or "low" 2-HG (representing IDH mutant and wild-type tumors, respectively) using thresholds defined by known samples (Supplementary Fig. 5). These tumors (n = 80) were again evenly distributed between metabolic subtypes (Fig. 4A). Similar findings were observed when evaluating IDH status of glioblastoma tumors that clustered with LGA in Fig. 1 (Supplementary Fig. 6). We next hypothesized that although IDH1 mutation may not lead to global changes in metabolism, this mutation may result in specific metabolic programs unique to these favorable tumors. Interestingly, following evaluation of the over 300 identified biochemicals, no consistent metabolic pathways and only a handful of individual metabolites were statistically significant between IDH1 mutant and wild-type tumors (Supplementary Table 2). Collectively, these findings suggest that despite clearly different molecular pathways driving gliomagenesis, metabolic reprogramming required to promote growth in this unique microenvironment remains highly conserved.

As transcriptional profiling has identified distinct molecular tumor subtypes in glioblastoma, we extended studies by performing gene expression profiling on matched tissue to define tumors as proneural, neural, classical, or mesenchymal.[4] Interestingly, although IDH1 mutation and MGMT methylation status were distributed evenly, transcriptional subtypes tightly clustered by their metabolomic profile (Fig. 4A). PCA analysis further demonstrated the mutual exclusivity of the proneural (n = 12) and mesenchymal (n = 21) metabolic profiles, which is consistent with their molecular signatures (Supplementary Fig. 7A). Similar to above-described metabolic reprogramming contributing to malignant transformation, aberrant lipid

Fig. 4 Metabolic heterogeneity in glioblastoma. (A) Hierarchical clustering of biochemicals identified by global metabolomic profiling of GBM (n = 80). Tumors and metabolites are represented in columns and rows, respectively. Genomics was performed on matched tissue to define molecular subtypes (PN = proneural, N = neural, CL = classical, M = mesenchymal), MGMT status (MET = MGMT promoter methylated, UNM = unmethylated), and IDH1 status (WT = wild type, MT = mutant). NA in all classifications indicates matched tissue was not available. 2-Hydrogyglutarate (2-HG) levels of samples are represented as brown (high) and gray (low) bars, reflective of IDH mutated and WT tumors based on thresholds determined using known samples, as described in Supplementary Fig. 5. (B) Metabolites from GBMs classified as PN and MES were further analyzed and those with a Benjamini–Hochberg corrected P-value <0.05 were classified based on metabolic pathways. Data are depicted as scatter plots generated using Z scores calculated with PN serving as baseline.

and amino acid metabolism and an accumulation of dipeptides differentiated proneural and mesenchymal subtypes (Fig. 4B, Supplementary Fig. 7B). Integrating the transcriptional signatures of these tumors with their metabolomic profiles again supported a molecular program driving these observed phenotypes. Although not as striking, a shift from lipid synthesis to catabolism was again observed in mesenchymal tumors (Supplementary Fig. 8A), with a clear emphasis on ubiquitin mediated proteolysis, protein transport (Supplementary Fig. 8B), and amino acid metabolism, with molecular machinery driving arginine, proline, tryptophan, and tyrosine metabolism differentially expressed in these tumors (Supplementary Fig. 8C).

We have recently shown that rather than *inter*tumoral heterogeneity, molecular subtypes in glioblastoma were reflective of *intra*tumoral heterogeneity, with infiltrating cells harboring a proneural signature while the mesenchymal subtype was enriched in perinecrotic regions.[11] We therefore extended our investigations to the Ivy Glioblastoma Atlas Project database, which contains transcriptional signatures from geographically distinct regions within an individual tumor that were isolated and enriched using laser-capture microdissection, to determine if the observed metabolic heterogeneity in glioblastoma could be a direct consequence of its diverse tumor microenvironment.[11,15] We therefore went on to apply the comprehensive gene panels assembled to evaluate lipid, amino acid, and peptide metabolism described above (Fig. 2, Supplementary Fig. 8A–C), to determine if genomic signatures of the observed metabolic phenotypes were recapitulated in these distinct regions. Although different transcriptional platforms were utilized in these studies, common themes continued to emerge, including a shift from lipid synthesis to catabolism, an emphasis on protein transport and ubiquitin-mediated protein transport, and amino acid metabolism in the perinecrotic region. In addition, autophagy signaling appeared specifically enriched in this region compared with infiltrative cells (Supplementary Fig. 8D–F). Collectively, these findings suggest considerable intratumoral metabolic heterogeneity in glioblastoma driven by transcriptional programs supporting diverse reprogramming of lipid, protein, and amino acid metabolism.

Enhanced Heterotrophy Represents a Metabolic Hallmark of Mesenchymal Glioblastoma

Aberrant fatty acid and amino acid/peptide metabolisms were identified as dominant metabolic nodes in the mesenchymal subtype of glioblastoma, and integrative genomics suggested that molecular machinery designed to promote uptake of these metabolites might contribute to driving this phenotype. We therefore extended investigations to novel, subtype-specific glioblastoma preclinical models to determine if this phenotype was functionally recapitulated, contributing to a state of enhanced metabolic heterotrophy

in glioblastoma. Integrating global metabolomic and transcriptional profiles of mesenchymal and proneural tumor-initiating cell lines closely mirrored results obtained from clinical specimens. This included an accumulation of dipeptides, acyl carnitines, and branched chain amino acids in mesenchymal glioblastoma cells that was molecularly driven by complementary transcriptional programs (Fig. 5A). For example, the branched chain amino acids leucine, isoleucine, and valine demonstrated a differential abundance (DA) score of nearly 1 in both metabolomic studies (*x*-axis) and gene expression profiling (*y*-axis), indicating that nearly all genes and metabolites associated with branch chain amino acid metabolism are aberrantly active or expressed in mesenchymal compared with proneural cells. Cysteine metabolism, on the other hand, had a DA score of 1 for metabolites and 0 for gene expression, indicating that the aberrant accumulation of these metabolites in glioblastoma did not appear to correspond with enzymes driving their metabolism/accumulation. As these cell lines are grown in identical media, these findings further support the potential for mesenchymal glioblastoma cells to co-opt machinery to promote import of these biochemicals from the microenvironment. As an initial investigation, we focused on fatty acid metabolism. Using BODIPY labeling to evaluate fatty acid uptake, the unique ability of mesenchymal glioblastoma cells to import fatty acids from the media was validated (Fig. 5B, C), which was inhibited by the fatty acid transport inhibitor cannabinoid receptor type 2 (CB2) (Fig. 5D). Mitigating fatty acid uptake in mesenchymal cells inhibited proliferation (Fig. 5E, Supplementary Fig. 9), supporting biologic consequence to this metabolic phenotype.

Next, we extended studies to investigate the novel observation of dipeptide accumulation in glioblastoma. As the identified dipeptides appeared diverse and relatively nonspecific, we tested the hypothesis that mesenchymal cells co-opted machinery to allow import of protein products from the environment to serve as a source of requisite amino acids in nutrient-deprived conditions. As an initial investigation, we evaluated whether macropinocytosis could serve as one potential mechanism to drive this phenotype.[16] Using FITC conjugated albumin, we again demonstrated the unique capacity of mesenchymal glioblastoma tumor-initiating cells to import exogenous albumin from media, which colocalized with the macropinocytosis marker TMR-dextran[16] and was inhibited by the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA)[16] (Fig. 5F, G). We went on to demonstrate that exogenous albumin rescued mesenchymal cells from the anti-proliferative effects of amino acid deprivation, which was mitigated by EIPA (Fig. 5H), validating the survival advantage offered by this metabolic adaptation.

Discussion

Integrative cross-platform analyses coupling global metabolomic profiling with genomics were performed to both define and provide molecular context to metabolic reprogramming driving gliomagenesis. We identified distinct metabolic signatures when comparing glioblastoma with LGA and uncovered a tightly orchestrated and highly redundant transcriptional program designed to support the observed metabolic phenotype. Aberrant lipid and amino acid metabolism emerged as dominant metabolic nodes in glioblastoma, and these tumors appear to have co-opted a variety of mechanisms to actively import these biochemical substrates from microenvironment, contributing to a metabolic state of enhanced heterotrophy that supports survival in the diverse tumor ecology of this malignancy. It is important to note that aerobic glycolysis and glutamine metabolism, which undoubtedly play key roles in gliomagenesis, were not captured in the static picture of metabolism offered by metabolomics. This is likely based on the rate of metabolism and the subsequent catabolism of their metabolic intermediaries. Therefore, the unique window into metabolic reprogramming in cancer offered by metabolomics is complementary to more targeted, flux-based approaches, which together can collectively provide a comprehensive insight into metabolic reprogramming in cancer.

One particularly striking finding these integrative analyses offered was that despite clearly different molecular pathways contributing to gliomagenesis and clinical outcome, MGMT methylated and IDH mutated glioblastoma appeared to be evenly distributed within the metabolic subtypes. This was particularly unexpected in IDH mutated tumors, as this represents an alteration in a metabolic enzyme resulting in the formation of an oncometabolite,[17] reinforcing that the pathogenesis in this unique molecular subtype is likely governed by epigenetic, rather than metabolic, processes.[18,19] Although IDH mutation and MGMT methylation status did not appear to influence metabolic heterogeneity in glioblastoma, clear clustering was observed based on transcriptionally defined molecular subtypes. Specifically, the metabolic profiles of mesenchymal and proneural subtypes were mutually exclusive, which is consistent with their transcriptional signatures. However, rather than *inter*tumoral heterogeneity, we have recently demonstrated that these molecular subtypes reflect *intra*tumoral heterogeneity in glioblastoma, with mesenchymal and proneural subtypes enriched in perinecrotic and infiltrative regions, respectively, within an individual tumor.[11] We therefore hypothesized that the observed metabolic subtypes in glioblastoma were a direct consequence of the diverse tumor microenvironment in this archetypal example of a heterogeneous malignancy. This was supported by extending identified genomic signatures of metabolic subtypes to a unique genomic dataset generated from commonly identified intratumoral regions in glioblastoma. Collectively, these findings support the concept that despite disparate molecular pathways that may be driving gliomagenesis, metabolic programs designed to maintain the aggressive phenotype of this malignancy appear conserved within its complex tumor ecology. Investigations designed to define metabolic profiles between specific regions within an individual tumor to definitively validate these findings and a more focused evaluation of different metabolic subtypes observed in glioblastoma coupled with their global gene expression profiles represent ongoing investigations in our laboratory.

A metabolic switch from de novo fatty acid synthesis to catabolism was observed in glioblastoma that was

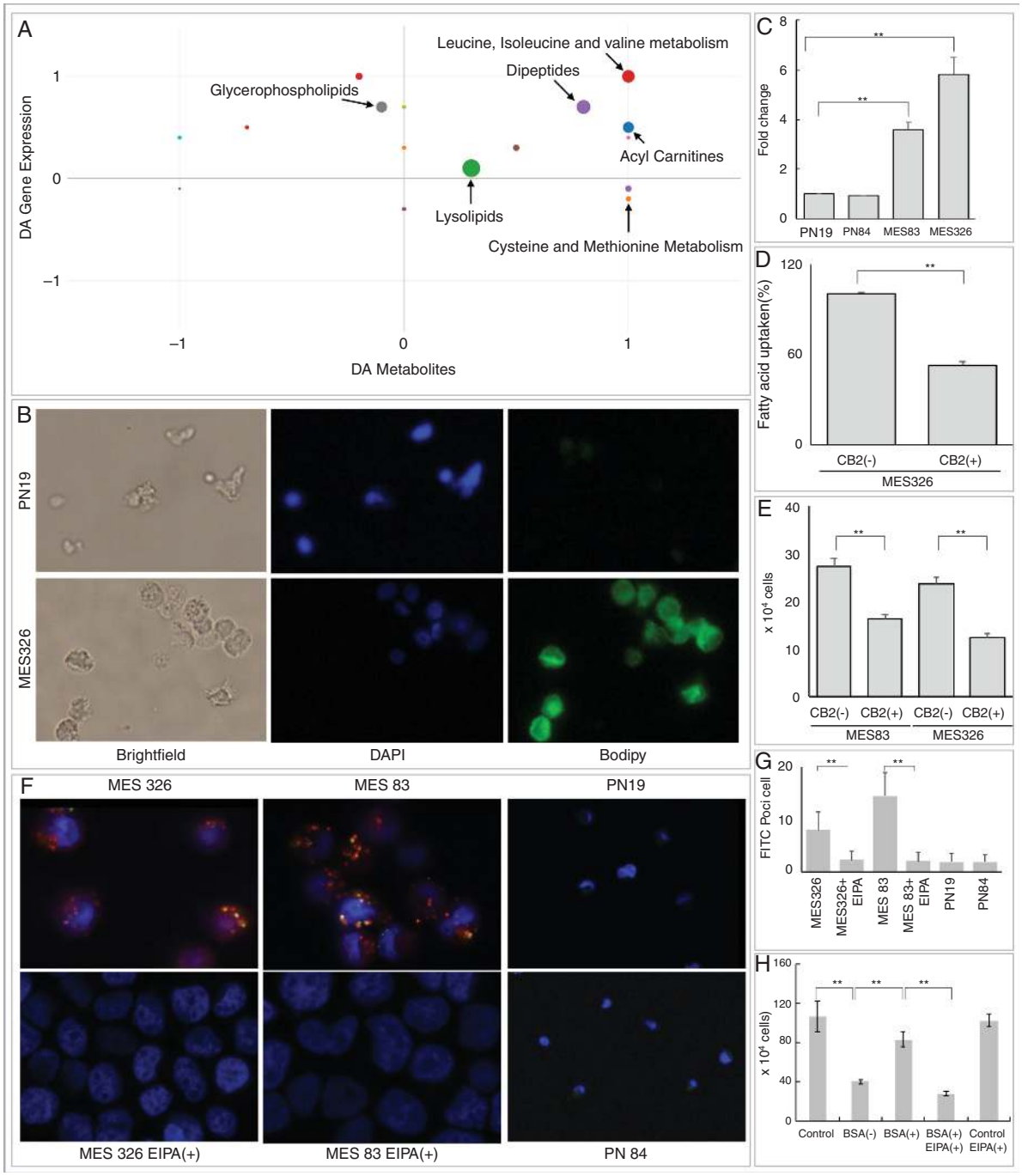

Fig. 5 Mesenchymal tumor-initiating cells recapitulate metabolic reprogramming in glioblastoma and demonstrate enhanced metabolic heterotrophy. (A) Global metabolomic profiling was performed on mesenchymal (MES326) and proneural (PN19, PN84) glioblastoma tumor-initiating cells and their associated gene expression profiles were obtained from Mao et al.[13] Significantly altered metabolites and genes between MES and PN were classified into major metabolic pathways and differential abundance (DA) scores were calculated. DA scores of metabolites versus gene expression were plotted for pathways having more than 5 metabolites represented. (B–D) fatty acid (FA) uptake in the described cell lines was (B) visualized with fluorescent microcopy using labeled BODIPY (green), (C) quantified at baseline using fluorescence spectroscopy (using PN19 as baseline) and (D) with or without the FA uptake inhibitor CB2. (E) The FA uptake inhibitor CB2 (5 μM, 48 h) inhibits proliferation of MES cells. (F) Peptide uptake by MES and PN cells was determined using FITC conjugated BSA (green) and the macropinocytosis marker TMR-dextran (red) and visualized by fluorescence microscopy. Colocalization of these 2 markers is represented as yellow. (G) Quantification of albumin uptake by MES cells with or without the macropinocytosis inhibitor EIPA. (H) Exogenous BSA rescues MES83 cells from the anti-proliferative effects of amino acid deprivation, which is reverted by EIPA (75 μM, 48 h). $^{**}P < 0.05$.

genomically supported and metabolomically captured as an accumulation of fatty acids, acyl carnitines, and lysolipids in concert with decreased malonyl CoA and glycerophospholipid metabolism. Although increased fatty acid synthesis has long been recognized for playing an active role in tumorigenesis and actively investigated as a therapeutic target,[20,21] our findings are consistent with more contemporary studies highlighting the complex interplay between fatty acid synthesis and catabolism in cancer and the multiple roles their intermediaries may play in tumorigenesis beyond lipid membrane synthesis, including their potential to drive oncogenic signaling and/or serve as alternate substrates for energy production.[22–24] Therefore, these findings provide a framework to further examine the interface between these divergent aspects of lipid metabolism, including studies designed to determine the source of these intermediaries, mechanisms driving this phenotype, its biologic consequence in different metabolic states, and potential to serve as a therapeutic target. Interestingly, an accumulation of choline-derived lysolipids coupled with decreased glycerophospholipid synthesis appeared to contribute to metabolic heterogeneity in glioblastoma. Recent work has identified a wide variety of roles this specific class of lipids may play beyond structural support, including its capacity to serve as a mediator of cell signaling, marker of demyelination, and activator of endothelial cells.[25,26] Therefore, further work designed to understand how these lysolipids contribute to the complex lipid dynamics uncovered in glioblastoma is warranted.

In addition to energetic and lipid requirements, amino acids play important roles in maintaining cellular function and continued proliferation in different microenvironments and have been a common metabolic alteration observed in cancer.[27–29] For example, glutamine metabolism has been deemed a metabolic hallmark in cancer,[30] contributing to diverse roles in tumorigenesis and continued proliferation, including serving as a substrate of carbon and nitrogen for macromolecular biosynthesis, a regulator of bioenergetics, modulator of redox stress, and gene expression. Accordingly, tumor cells have co-opted several mechanisms to provide requisite levels of this multi-faceted amino acid in nutrient-deprived microenvironments, including de novo biosynthesis and proteolytic scavenging. The amino acid serine plays a similar role, supporting several metabolic processes crucial for the growth and survival of proliferating cells, including protein, amino acid, and glutathione synthesis.[31] Further, as an important one-carbon donor to the folate cycle, serine contributes to nucleotide synthesis, methylation reactions, and the generation of NADPH for antioxidant defense. Cysteine and tryptophan metabolism also have established roles in a variety of malignancies, including glioblastoma.[32,33] Our studies reinforce the clear importance that aberrant amino acid metabolism plays in gliomagenesis, with numerous amino acids contributing to diverse metabolic functions enriched in glioblastoma. This included intermediates of serine, glutathione, cysteine, tryptophan, and urea metabolism that have been implicated in many oncogenic roles, including energy production, maintenance of redox balance, protein and nucleotide synthesis, anaplerosis, and immune modulation. In addition, perhaps some of the most consistent metabolomic and genomic findings included an emphasis on enhanced metabolism of the branched chain essential amino acids leucine, isoleucine, and valine in glioblastoma.[27] Recent work has demonstrated the potential for branched chain amino acids to serve as a nitrogen substrate in non–small cell lung cancer[34] and even suggests that cells may rely more heavily on these amino acids for de novo amino acid and nucleotide synthesis than glutamine when studied in vivo.[35–37] Therefore, further work is warranted to delineate the role this family of amino acids may play in gliomagenesis.

We propose that enhanced heterotrophy, a term used to describe a cell's capacity for co-opting mechanisms to actively import diverse macromolecules from the microenvironment, supports survival in the unique ecology of this tumor and therefore represents a metabolic hallmark of glioblastoma. This phenotype consisted of an accumulation of diverse intermediates involved in fatty acid catabolism and a seemingly nonspecific amino acid pool that was defined by an accumulation of a wide array of dipeptides. Although increased expression of such transporters has been previously described in glioblastoma,[29,38,39] including increased expression of the xCT transporter[40,41] involved in providing intermediaries for cysteine and glutathione metabolism and the L-type amino acid transporter 1 involved in the import of large neutral amino acids, including tryptophan and branched chain amino acids,[38,39,42] we propose that this is a reflection of a larger metabolic phenotype. As this metabolic reprogramming appeared particularly relevant in the mesenchymal subtype, which corresponds to the perinecrotic region in an individual tumor, we postulated that enhanced heterotrophy represents an essential metabolic adaptation that provides requisite biochemical intermediates to maintain growth and survival in these nutrient-depleted regions. These findings were recapitulated in preclinical models metabolomically, transcriptionally, and functionally, with mesenchymal tumor-initiating cells demonstrating the unique capacity of importing both fatty acids and albumin from the media. Importantly, inhibiting these processes had biologic consequence, including anti-proliferative effects following inhibition of fatty acid uptake and the import of BSA allowing mesenchymal glioblastoma cells to survive and adapt to amino acid–deprived conditions. Although experiments designed to determine the source of these intermediates were not performed, we speculate they represent recycled cellular components derived from dying tumor and/or normal cells unable to survive this microenvironment. Unfortunately, these metabolic adaptations reinforce the clear challenges implicit in developing effective treatment strategies for these aggressive tumors. For example, tumor cells that have co-opted modes of survival in these nutrient-depleted regions would likely be sheltered from systemic therapies and, even if biologically relevant concentrations of an agent were achieved in these regions, the highly redundant transcriptional machinery driving this phenotype would be a formidable opponent to a molecularly targeted agent.

In summary, these integrated metabolomic and genomic studies provide a window into the complex metabolic

programs contributing to gliomagenesis. In addition to identifying metabolic hallmarks of glioblastoma, this work serves as a powerful resource to identify novel metabolic vulnerabilities and therapeutic targets in this invariably fatal malignancy.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

genomics | glioblastoma | integrative analyses | intratumoral heterogeneity | metabolomics

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Development of Methodology—A. Prabhu, P. Chinnaiyan
Analysis and interpretation of data—A. Prabhu, S. Kant, P. Chinnaiyan

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