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Delineating the glioblastoma stemness by genes involved in cytoskeletal rearrangements and metabolic alterations

Kałuźńska-Kołat Z *et al.* Cytoskeleton and metabolism in glioblastoma stemness

Abstract

Literature data on glioblastoma ongoingly underline the link between metabolism and cancer stemness, the latter one responsible for potentiating the resistance to treatment, *inter alia* due to increased invasiveness. In recent years of glioblastoma stemness research, a key aspect of cytoskeletal rearrangements has been bashfully introduced, whereas the impact of the cytoskeleton on invasiveness is well-known. Although non-stem glioblastoma cells are less invasive than glioblastoma stem cells (GSCs), these cells also acquire stemness with greater ease if characterized as invasive cells and not tumor core cells. This suggests that glioblastoma stemness should be further investigated for any phenomena related to the cytoskeleton and metabolism, as they may provide new invasion-related insights. Previously, we proved that interplay between metabolism and cytoskeleton exists in glioblastoma. Despite searching for cytoskeleton-related processes in which the investigated genes might have been involved, not only did we stumble across the relation to metabolism but also reported genes that were found to be implicated in stemness. Thus, dedicated research on these genes in the subject of GSCs seems justifiable and might reveal novel directions and/or biomarkers that could be utilized in the future. Herein, we review the previously identified cytoskeleton/metabolism-related genes through the prism of glioblastoma stemness.

Key Words: Glioblastoma; Stemness; Cytoskeleton; Metabolism; Biomarkers; Therapy

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Core Tip: Glioblastoma stemness intensifies the resistance to treatment *via* increased invasiveness. Among the processes crucial for glioblastoma stem cells, metabolism is known to influence invasion. However, the cytoskeleton is currently negligent in glioblastoma stemness research, while it also regulates invasion. Herein, we review the link between stemness and cytoskeleton/metabolism-related genes that we previously identified in glioblastoma. These genes influence stemness *via* numerous biological processes; for some genes, clinical trials are currently ongoing. Others were connected to glioblastoma stemness for the first time. Future glioblastoma-related research should delve into the cytoskeleton since the concept is already encouraging.

INTRODUCTION

Despite decades, glioblastoma (GBM) remains an incurable condition with increasing incidence in many countries^[1,2]. Although GBM is less prevalent than, *e.g.*, breast, colon, or lung cancer, it outperforms other tumors by affecting patients in the prime of their lives and causing them to lose many years of life^[3]. The initial intervention in newly diagnosed GBM includes a surgical approach, with post-surgery temozolomide (TMZ) and radiation therapy^[4]. Adding tumor-treating electric fields (TTFields) to maintenance TMZ chemotherapy was found to prolong progression-free and overall survival but is currently limited due to the lack of a method to predict or quantify the efficacy of TTFields (the imaging features associated with treatment response are unclear and there are no predictive neuroimaging markers). Moreover, the treatment device is required to be worn for a predetermined period (typically approximately 75% of the time) or until there is a clinical progression of the disease, which introduces a delay in getting used to the device and makes patients anxious with regard to the intended therapy effect^[5]. Strong motivation to predict TTField efficacy in a patient-specific manner was provided^[6]. Nevertheless, glioblastoma recurrence is practically inevitable which, combined with a grim prognosis and ineffective treatment, underlines the importance of further research into this one of the deadliest tumors^[3,7].

One of the GBM traits that implicate the lack of effective treatment is the heterogeneity that can be explained by both clonal evolution and the presence of stem cells^[8]. Stemness refers to the molecular events that underlie the essential characteristics of self-renewal and differentiation into daughter cells^[9]. On the cellular level, some processes were indicated as crucial for GBM stemness, namely epigenomic regulation, posttranscriptional regulation, and metabolism^[10]. In recent years of glioblastoma stemness research, a key aspect of cytoskeletal rearrangements has also been bashfully introduced^[11,12] while it is long time since this machinery is well-known for controlling two processes that influence cancer malignant behavior, *i.e.*, cellular division and invasion^[13]. The stemness itself is also responsible for potentiating the resistance to treatment^[14,15], *inter alia* due to increased invasiveness^[16]. In addition, more recent studies have identified the role of metabolism in GBM invasion^[17]. Although non-stem glioblastoma cells are less invasive than GBM stem cells (confirmed by, *e.g.*, sevenfold reduced cell migration through the Matrigel, or 3.8-times and 6.8-times lower expression of matrix metalloproteinase-14 and -16)^[18], the same cells also acquire stemness with greater ease if they are characterized as invasive cells and not tumor core cells^[19,20].

The above-mentioned data implies that GBM stemness should be further explored for any phenomena related to the cytoskeleton and metabolism, as they may provide the missing puzzle from the point-of-view of invasion. Moreover, the cytoskeleton and metabolism are related; for instance, the cytoskeleton is involved in carbohydrate metabolism^[21] and at the same time the actin and tubulin require energy from nucleotide hydrolysis to maintain structural dynamics^[22]. Cytoskeletal rearrangements and metabolic alterations are important not only for GBM cells but also for neuronal and glial progenitors. For example, cytoskeleton dynamics underlie the cellular asymmetry while metabolic reprogramming ensures a transition in energy production from glycolytic to oxidative^[23,24]. Nevertheless, it is possible to discriminate normal glial cells from glioblastoma; the cancerous cells present decreased cortical but increased intracellular stiffness, as well as preferentially metabolize glucose into lactate despite

the abundance of oxygen^[17,25]. Stiffness and metabolic adaptations can also influence stem cell differentiation^[26,27]. Moreover, the cellular cross-talk that utilizes cytoskeleton or metabolites affects physical dynamics and signaling of various cell types including astrocytes, neurons, and oligodendrocytes^[28,29]. In cancer, such cross-talk renders abnormal protrusions or extensions termed as tumor microtubes that contribute to, *e.g.*, glioma resistance^[30]. These structures are rich in cytoskeletal proteins like actin and tubulin, as well as are known to modify energetic metabolism of the receiving cells *via* transport of mitochondria^[31].

Our previous research has proved that interplay between metabolic alterations and cytoskeletal rearrangements exists in GBM^[32]. Of genes described below in the present review (some previously identified genes were not included if their implication in stemness was not found in the literature; Supplementary Table 1^[33-37]), the example of a relationship between metabolism and cytoskeleton can be visualized (Figure 1) using literature on methylenetetrahydrofolate dehydrogenase 2 (MTHFD2)^[38-41] and ribonucleotide reductase subunit M2 (RRM2)^[42-45]. In our previous research, despite searching for cytoskeleton-related processes in which the investigated genes might have been involved, not only did we stumble across the relation to metabolism, but we also reported some genes which were found to be implicated in glioblastoma stemness. Thus, the dedicated work on these genes in the subject of GBM stem cells (GSCs) seems justifiable and might reveal novel therapeutic directions and/or biomarkers that could be utilized in the future. Herein, we review the previously identified cytoskeleton/metabolism-related genes through the prism of GBM stemness. Literature screening allowed the decision to split these genes based on whether their role in stemness is known from GBM or another tumor, the latter suggesting an urgent need to experimentally verify the observations in the glioblastoma context.

GENES WITH CONFIRMED ROLE IN GLIOBLASTOMA STEMNESS

Bone morphogenetic protein 4

Based on the literature abundance, the best-known from its implication in glioblastoma stemness is *bone morphogenetic protein 4* (BMP4). The bone morphogenetic proteins are growth factors from the TGF- β superfamily that undergo expression during embryogenesis and control development. Initially denoted as crucial for osteogenesis, they are now described as regulators of gastrulation, neurulation, mesoderm patterning, proliferation, and differentiation in many tissues^[46]. About 15 years ago, it was found that the signaling *via* BMPs and their cognate receptors (BMPRs) influences the activity of normal brain stem cells but can also inhibit the cancer-initiating GBM stem-like cells^[47]. Later the same year, these authors confirmed that *in vivo* delivery of BMP4 blocks the tumor growth and associated mortality, which occur in all mice following intracerebral grafting of human glioblastoma^[48]. This protein was suggested as a non-cytotoxic therapeutic agent that can be utilized in combination with stem cell-based therapy^[49]; this complements its usage as an agent used to differentiate GSCs into normal glial cells^[50]. BMP4 has been found promising to the extent that it entailed the development of novel therapies. For example, one that utilizes the oncolytic vaccinia virus was developed to alleviate glioblastoma and prevent its recurrence^[51]. Later on, the cell-based treatment option of BMP4-secreting human adipose-derived mesenchymal stem cells was found to reduce proliferation and migration both *in vitro* and *in vivo*, as well as prolonged survival in a murine model^[52]. Still, Videla Richardson *et al*^[53] admitted that little is known about this morphogen regarding triggered cellular events, which prompted the authors to establish several GSC-enriched cell lines growing as adherent monolayers and not floating neurospheres. Distinct lineage preferences were noticed depending on the expression pattern of BMP signaling-astrocyte fate or neuronal commitment was noticed and, under certain conditions, even a smooth muscle-like phenotype^[53]. Providing new findings to the available data, BMP4-overexpressing neural stem cells were found to promote, *e.g.*, GSCs apoptosis *via* Smad1/5/8 signaling^[54]. Moreover, recent studies indicate a formerly underestimated link between BMP4 and metabolism or mechanotransduction which affects oxygen consumption or matrix stiffness^[55]. The latter is known to be associated with

cytoskeletal remodeling^[56,57]. With regard to the cytoskeleton, *BMP4* was found to reorganize actin dynamics *via* activation of Rac1, Rho, and Cdc42^[58]. The impact of *BMP4* in inducing asymmetric cell division was also noted, limiting the GSCs expansion^[59]. The newest literature data on *BMP4* consider it on a broader scale, either evaluating other GBM aspects and referring to *BMP4*, or investigating upstream/downstream molecules. Ciechomska *et al*^[60] explored *EGFR* alterations in glioblastoma since GSCs with various *EGFR* levels respond differently to therapy; the authors found that *EGFR*/FOXO3a/BIM signaling pathway determines chemosensitivity of *BMP4*-differentiated GSCs to TMZ. On the other hand, Wu *et al*^[61] identified BIRC3 as an inducer of glioblastoma stemness *via* downstream *BMP4* inactivation. At last, the most recent paper by Verploegh *et al*^[62] summarized the cellular viability variance in response to *BMP4* and proposed early-response markers for sensitivity to *BMP4*. Three cultures with the highest sensitivity for *BMP4* revealed a new cell subpopulation that presented a reduced cell proliferation but an elevation of apoptosis. These changes in composition correlated with treatment efficacy; the latter was found to be predicted using *OLIG1/2* expression. Furthermore, upregulated *RPL27A* and *RPS27* were considered early-response markers. Interestingly, *RPS27* is one of the genes identified in our previous study that prompted us to investigate the aspects issued in this review. This gene will be described below in a separate subsection.

20 **Glutamate ionotropic receptor NMDA type subunit 2B**

Glutamate ionotropic receptor NMDA type subunit 2B (GRIN2B) encodes one subtype of glutamate-binding GluN2 subunit, which is a part of the N-methyl-D-aspartate receptor (NMDAR). Ionotropic glutamate receptors from this family mediate Ca²⁺, *i.e.*, the permeable component of excitatory synaptic transmission in the central nervous system (CNS)^[63]. NMDARs assemble from four subunits: two GluN1 and two GluN2. The former subunits are widely expressed in the nervous system, while four subtypes of GluN2 subunits (from “A” to “D”) are characterized by various expression patterns^[64]. *GRIN2B* encodes the GluN2B subunit, which is abundantly expressed in the prenatal

period, then declines in most brain parts^[65]. The presence of GluN2B in such an early stage implies that it contributes to brain development, circuit formation, synaptic plasticity, as well as migration, and differentiation^[66]. Glutamate-dependent synaptic transmission is frequently dysfunctional in gliomas^[67], and regarding this specific subunit, an enrichment of expression was noticed in GSCs^[68]. In our previous research, with the use of literature data, we related this gene with the cytoskeleton since GluN2B interacts with cytoskeletal protein α -actinin-2 *via* the carboxyl-terminal domain^[63]. It might be of importance as α -actinin-2 is closely associated with multimerins which are possible markers and therapeutic targets in low-grade glioma^[69]. Moreover, one of the multimerins encoded by the *MMRN1* gene was found to be correlated to stemness and chemoresistance, although these observations were based on the leukemia model^[70]. Nevertheless, *GRIN2B* is confirmed to influence stemness not only in glioblastoma but also in lung cancer-She *et al*^[71] identified *GRIN2B* expression to be higher in primary tumors than in normal tissues, and at the same time elevated in metastatic lesions than in primary tumors which contributed to poorer prognosis. Moreover, the same authors observed inhibition of tumorsphere formation during *GRIN2B* silencing.

Homeobox protein A10 and A1

The homeotic genes, in vertebrates denoted as homeobox, are highly conserved and regulate the proper development of various body segments during ontogeny^[72]. *Homeobox protein A10* (*HOXA10*) is implicated in the embryogenesis of the uterine epithelium, stroma, and muscle^[73]. In response to hormones, it undergoes periodical expression in the mature endometrium, controlling receptivity during the implantation window^[74]. Concerning GBM stemness, the functionality of *HOXA10* was presented as a direct result of the activation of protein from the *Trithorax* family, which serves as a histone methyltransferase, *i.e.*, MLL. Afterward, *HOXA10* activated other *HOXA* genes, *e.g.*, *HOXA7* and *HOXC10*^[75]. In another study, *HOXA10* was marked as one of the strongest candidates (alongside the *HOX -A9*, *-C4*, and *-D9* genes), having value as a therapeutic target and biomarker for both GBM and GSCs^[76]. Our previous research

echoed the data that *HOXA10* facilitates cytoskeleton remodeling (*via* *CK15*)^[77], promotes tumorigenesis in glioma^[78], and regulates homologous recombinant DNA repair and subsequently TMZ resistance in GBM^[79]. Since stemness also contributes to treatment resistance^[14], the last two events complement each other mutually. Another homeotic gene that we identified in our previous study was *HOXA1*, a homeobox that is abundantly expressed in the mesoderm and neuroectoderm at the level of the brainstem precursor^[80]. Upregulation of *HOXA1* was noted in GBM, which inversely correlated with the survival of patients^[81]. This homeotic member was also implicated in regulating the cytoskeleton *via* E-cadherin. Namely, *CDH1*-dependent signaling was found to increase *HOXA1* expression through Rac1, *i.e.*, the same pathway that regulates actin cytoskeleton at cadherin adhesive contacts^[79]. With regard to GBM stemness, Schmid *et al*^[82] observed upregulated HoxA locus (encompassing, *e.g.*, *HOXA1*) after they dedifferentiated murine astrocytes into GSCs *via* Rb knockout, Kras activation, and Pten deletion. These cells were sufficient to form GBMs in their transplant mouse model. Although the insights did not provide further mechanistic details, the regulation loop of *HOXA1* and *HOXA* transcript antisense RNA (*HOTAIRM1*) was found to be involved in stemness maintenance^[81,83]. This was presented in colorectal carcinoma and uveal melanoma. Still, taking into account the Schmid *et al*^[82] study, the profound investigation of *HOXA1* in GSCs in this aspect should be considered.

Matrix metalloproteinase 13

Matrix metalloproteinases are constituents of extracellular matrix (ECM) belonging to the zinc-containing endopeptidases family that encompasses 23 members^[84]. Functionally, these calcium-dependent molecules are responsible for the degradation and remodeling of other proteins that constitute ECM. Moreover, their role in various biological and physiological processes dependent on hormones, growth factors, and cytokines were described^[85]. It is known that different ECM components modulate cancer stem cells' properties; regarding glioblastoma, the confirmed ones were type I

collagen, laminin $\alpha 2$, fibronectin, periostin, decorin, and lumican^[86]. *Matrix metalloproteinase 13 (MMP13)* is a collagenase almost universally upregulated in the pancreatic view^[87]; in GBM, its overexpression increases migration and invasion^[88], as well as confers poor prognosis^[89]. The relationships between *MMP13* and the cytoskeleton^[33] or metabolism^[90] are known. In terms of stemness, Inoue *et al*^[91] suggested that highly invasive potential GSCs depended on *MMP13* enzymatic activity; the authors also proposed *MMP13* as a potential therapeutic target.

MTHFD2

The folate cycle is responsible for appropriate cellular metabolism by regulating ATP production, methylation reactions for DNA/protein synthesis, or developing immunomodulatory molecules that orchestrate signaling and cytotoxicity^[92]. The differences between *MTHFD1* and *MTHFD2*, two enzymes implicated in the folate pathway, include the use of different co-enzyme (NADP *vs* NAD), functionality (*MTHFD1* has three distinct enzymatic activities while *MTHFD2* is bifunctional), and location (cytoplasm *vs* mitochondria). Compared to *MTHFD1*, which generates NADPH and formate for purine biosynthesis, *MTHFD2* is overexpressed in rapidly proliferating malignant tumors. It is considered the “main switch” that enables mitochondria to produce additional growth-facilitating one-carbon units and generates NADH necessary for protection from reactive oxygen species^[93]. *MTHFD2* is also an excellent example to present the link between metabolism and cytoskeleton; Lehtinen *et al*^[39] found that *MTHFD2* depletion leads to vimentin organization defects and identified this gene as a regulator of cell migration and invasion. Regarding glioma, *MTHFD2* was found to be associated with tumor grade and prognosis^[38]. Inhibition of this enzyme in GSCs induced apoptosis and affected not only central carbon metabolic pathways (*e.g.*, glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle) but also unfolded protein response, highlighting a novel connection between one-carbon metabolism and reaction to cellular stress^[94]. Nishimura *et al*^[95] suggested that the purine synthesis pathway, as well as folate-mediated one-carbon metabolism, seem to be crucial for the

5 maintenance of tumor-initiating cells. The same authors also concluded that EGF-5 induced expression of *MTHFD2* may be mediated by Myc, with the latter regulating the expression of metabolic enzymes 5 for the maintenance of brain tumor-initiating cells.

Plant homeodomain finger-like domain-containing protein 5A

Alternative splicing maintains post-transcriptional gene regulation, which enables a single gene to be transcribed into various RNAs, diversifying the proteome. Abnormal splicing function can lead to tumor-related processes, *e.g.*, proliferation, angiogenesis, and metastasis^[96]. Spliceosome, a dynamic machinery responsible for splicing, is made of small nuclear ribonucleoproteins (snRNPs; five molecules are known: U1, U2, U4, U5, 26 and U6) and numerous non-snRNP proteins^[97,98]. U2 snRNP comprises 9 U2 snRNA, SF3a complex, and SF3b complex, which are responsible for recognizing branchpoint sequences during initial spliceosome assembly stages^[99]. Splicing factors comprising the SF3b complex include, *e.g.*, 36 plant homeodomain (PHD) 9 finger-like domain-containing protein 5A (*PHF5A*), which facilitates interactions between the U2 snRNP and RNA helicases^[100] but can also bind chromatin via its PHD that is composed of a small zinc finger structural fold^[101,102]. The knockdown of *PHF5A* results in reduced GBM viability and cell cycle arrest^[103]. Trappe *et al*^[104] revealed that systematic deletion of its yeast homolog is lethal, showing that *PHF5A* is crucial for cell viability. The flagship paper on *PHF5A* in brain tumor^[105] indicates that the gene is required to expand GSCs and that in these tumor-initiating cells, but not untransformed neural stem cells, *PHF5A* contribute 21 to the identification of exons having unusual C-rich 3' splice sites in thousands of 18 essential genes. The same authors inhibited *PHF5A*, which reduced GSCs-driven tumor formation *in vivo* and inhibited the growth of established GBM patient-derived xenograft tumors.

Ribosomal protein S27

One of the most dynamic and largest molecular motors (driven by a complex thermal ratchet translocation mechanism) are ribosomes^[106]. Metallopanstimulin-1, also known

as ribosomal protein S27 (*RPS27*), is a constituent of the human 40S ribosome¹⁴ that is mainly found in the cytoplasm while it can also relocate to the nucleus^[107] or even extracellular space^[108]. Regarding the nuclear location, it is able to interact with DNA via its C4-type zinc finger^[109]. In glioblastoma, *RPS27* was found to be correlated with age in IDH-mutated glioma patients and with Ki67 in GBM patients. Interestingly, it is detected in astrocytic tumors but not in normal astrocytes unless the tissue was inflamed^[109]. This allowed the same authors to emphasize that in comparison to inflammatory tissue (in which only a small number of macrophages were positive for *RPS27*), almost all macrophages in tumor tissue were distinctly enriched in *RPS27* expression. As for GSCs, the ribosomes and related proteins were generally found to reprogram glioma cells to induce plasticity and stemness^[110]. Among these molecules, *RPS27* was considered oncogenic with higher expression at the GSC-dominant area^[111]. Inquisitive findings revealed that *RPS27* is also detected in the microvascular proliferation area and pseudopalisading cells around necrosis^[110]. It is worth underlining that aberrant vessels are crucial for the development of pseudopalisading necrotic regions that provide shelter for residing cancer stem cells from anti-tumor agents, which enable these cells to expand and promote proliferation and growth^[112]. As mentioned above, upregulated *RPL27A* and *RPS27* were considered to be early-response markers related to the presence of *BMP4*, suggesting a link that should be further investigated. This is especially since the signaling of ribosome translation was found to be overexpressed during the response to stress in glioblastoma^[62].

RRM2

A balanced supply of deoxyribonucleotide triphosphates (dNTPs) is a prerequisite of DNA synthesis. Still, *de novo* synthesis of dNTP is also possible via the reaction catalyzed by the ribonucleotide reductase (RR) that reduces the C2'-OH bond of the four ribonucleotides triphosphates to form corresponding dNTPs^[113]. ⁴²*RRM2* encodes the β subunit of RR; ¹each *RRM2* monomer contains the tyrosyl radical and non-heme iron^[114]. Since ¹a sufficient supply of dNTPs drives an uncontrolled DNA replication in

cancer^[115], it is not surprising that *RRM2* was frequently subjected to molecular therapy^[116,117]. Currently, several *RRM2* inhibitors have been developed, *e.g.*, radical scavengers, iron chelators, subunit polymerization inhibitors, or expression silencers^[118-120]; this is to inhibit proliferation, division, but also invasion^[32]. In glioblastoma, *RRM2* is responsible for the advancement of GBM tumorigenicity and protection from endogenous replication stress *via* the BRCA1-*RRM2* axis^[45]. For glioma in general, regulation of proliferation and migration *via* ERK1/2 and AKT signaling was noted^[44]. Available literature also links the *RRM2* to the cytoskeleton *via* hPLIC1; the latter decreases during *RRM2* downregulation, which entails actin cytoskeleton reorganization^[42]. Perrault *et al*^[121] suggested that *RRM2* can be a chemoresistance driver that dictates how GBM cells respond to TMZ. The same authors further verified that *RRM2*-overexpressing cells had enhanced DNA repair efficiency. Moreover, the use of a selective FDA-approved *RRM2* inhibitor, 3-AP Triapine, enabled Perrault *et al*^[121] to observe that in comparison to both TMZ and control, glioblastoma treated with the 3AP + TMZ formed fewer neurospheres that were also significantly smaller. Another group found that *RRM2* expression dramatically declined after 12 d of dasatinib treatment compared to naïve GSCs of the GSC8 cell line^[122].

Serum amyloid A protein 2

In order to re-establish homeostasis, both adaptable and primordial mechanisms exist; the latter comprises the acute-phase response (APR) that is a set of changes that occur after, *e.g.*, inflammation, infection, or trauma^[123]. During APR, the changes include the altered levels of serum proteins, with the most notable being C-reactive protein and serum amyloid A (SAA)^[124]. Being an apolipoprotein, SAA is related to plasma high-density lipoprotein and is implicated in the cholesterol transport to the liver for excretion as bile^[125]. Its other functions include regulation of amyloidogenesis, tumor pathogenesis, anti-bacterial events, and inflammatory response^[126]. The role of SAA in tumor progression was suggested owing to its cytokine-like properties that influence the course of inflammation^[127]. SAA2 is one of the paralogs of the family and was

investigated as a lung cancer biomarker a few years ago^[128]. The description of its role in glioblastoma is limited, yet it is already known that SAA2 increases GBM proliferation and invasion^[129]. Knebel *et al*^[130] confirmed that SAA production occurs not only in the liver but also in tumor cells; the authors emphasized that exploring the SAA influence on the cytoskeleton and invasiveness using more complex assays is needed. In terms of GBM stemness, Adamski *et al*^[131] recently compiled the literature data and stated that SAA2 is implicated in a drug-promoted cellular dormancy, with the latter being closely connected to stem cell characteristics. The group also indicated the ability of SAA2 to sustain inflammatory conditions in the brain, which consequently supports TMZ resistance and induces the expression of stemness markers in glioblastoma.

Wilms' tumor protein 1

The 5-methylcytosine (5mC) and its derivatives have altered patterns in a range of tumors. 5mC can be recognized and ²oxidized to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine by Ten-Eleven translocation (TET) ²²enzymes^[132,133]. One of the transcription factors that directly interacts with TET proteins is ²²Wilms' tumor protein 1 (WT1)-a master regulator ²²essential for urogenital, ²²epicardium, and kidney development that can act as a tumor suppressor or oncoprotein in multiple tumors^[134,135]. Initially cloned as a suppressor of Wilms' tumor, WT1 is now considered to be an oncoprotein in hematologic malignancies and a variety of solid tumors, as well as the ³⁰protein with the highest potential for cancer immunotherapy^[136-138]. According to the ³⁰phase I/II clinical trial, WT1 peptide-based vaccine among glioblastoma ³⁰patients was considered safe and induced cellular and humoral immune response^[139]. This is important due to the fact that WT1 is involved in GBM tumorigenicity *via* increasing proliferation and decreasing apoptosis^[140]. As for the impact on the cytoskeleton, this protein was found to interact with actin both in the cytoplasm and nucleus, as well as supposedly binds to RNA in a cytoskeleton-dependent regulation manner^[141]. Focusing on GBM stemness, Mao *et al*^[142] found WT1

to be expressed predominantly in mesenchymal GSCs which, compared to proneural stem cells subtype, are characterized by higher proliferation, greater radioresistance, and implication in worse patients' prognosis. Uribe *et al*^[143] reviewed that mesenchymal GSCs develop tumors having more blood vessels, hemorrhagic lesions, and necrotic areas; the expression pattern in these stem cells generally facilitates inflammation, angiogenesis, migration, invasion, and glycolysis-mediated metabolism. Undoubtedly, more insights are needed concerning GBM molecular pathways in which *WT1* is implicated.

GENES WITH STILL UNCONFIRMED ROLE IN GLIOBLASTOMA STEMNESS

Chemokine-like factor superfamily 6

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Cytokines are soluble proteins that are secreted by immune and non-immune cells in response to stimulants such as immunogens or mitogens; this allows them to maintain the immune response and homeostasis^[144]. Chemokines constitute a specific type of small (8-13 kDa) cytokines that promote the directed chemotaxis of nearby cells^[145]. Consisting of nine members, the chemokine-like factor superfamily (CMTM) is expressed throughout the human tissues and regulates immune, circulatory and muscular systems, as well as the hematopoiesis^[146-149]. The aberrant *CMTM* expression is implicated in various diseases, *e.g.*, rheumatoid arthritis, atopic dermatitis, focal cerebral ischemia, male infertility, as well as tumorigenesis and metastasis^[150-153]. The influence of *CMTM6* on glioblastoma is known, but the research in this entity seems to be in the initial state. Guan *et al*^[154] revealed that the highest *CMTM6* expression was noted in the glioblastoma (WHO grade IV) compared with WHO grade II and III gliomas. Enrichment was also observed in both microvascular proliferation and hyperplastic blood vessels, which are both essential for tumor progression. In GBM, *CMTM6* was also associated with one of the genes of immune checkpoints, *i.e.*, *TIM-3*. From a broader glioma scale, the same authors summarized it as a molecule diminishing T-lymphocyte-dependent anti-tumor immunity, reducing patient survival and indicating poor prognosis. However, it is still yet to be elucidated what role

CMTM6 may play in the GBM stemness. Currently, its contribution to such characteristics is confirmed on the basis of data from head-and-neck squamous cell carcinoma. Chen *et al*^[155] observed poorer patient prognosis during *CMTM6* overexpression that correlated with overactive Wnt/ β -catenin signaling, *i.e.*, the pathway crucial for tumorigenesis, epithelial-to-mesenchymal transition (EMT) and cancer stem cells maintenance. Silencing of *CMTM6* led to PD-L1 downregulation, decreased tumor growth, and increased CD8⁺ and CD4⁺ T-cell infiltration. Eventually, the authors not only suggested the therapeutic suitability of *CMTM6* but also concluded that this protein is implicated in EMT, stemness, and T-cell dysfunction. Similar research in the glioblastoma context is advisable, especially since *CMTM6* can stabilize PD-L1 protein to impair T-cell function^[156,157], as well as their combined expression had prognostic significance in pancreatic ductal adenocarcinoma and triple-negative breast cancer^[158]. Nowadays, the role of PD-L1 in cancer and immunotherapy is unquestionable^[159]; focusing on another protein related to this well-established molecule might bring novel strategies.

Dual specificity phosphatase 7 (DUSP7)

Signal transduction is based on phosphorylation and dephosphorylation events performed by kinases and phosphatases, leading to a cellular program relevant to the encountered stimulus^[160]. Dual specificity phosphatases are responsible for the dephosphorylation of threonine and tyrosine residues on mitogen-activated protein kinases, rendering them inactive^[161]. Even if *DUSP7* was only noted as downregulated in glioblastoma, whereas *DUSP1*, *DUSP5*, and *DUSP6* were induced within pseudopalisading and perinecrotic GBM regions^[162], the role of *DUSP7* in preserving the pluripotency of non-cancerous stem cells was certified in a murine model^[163]. However, its contribution could be distinct from *DUSP1*, *DUSP5*, and *DUSP6* but similar to *DUSP2*, *DUSP8*, and *DUSP9* which were clustered together with *DUSP7* in the study of Mills *et al*^[162]. At last, it is worth noting that *DUSP7* guides chromosome dynamics which is known for being regulated by cytoskeletal proteins^[164,165]. The study

linking this phosphatase to metabolism revealed that ²⁵ *DUSP7* knockout accelerates metabolic disorder and insulin resistance in mice with a high-fat diet^[166].

Kinesin family member 20A

Cytoskeletal elements that act as scaffolds for intracellular cargo transport are microtubules. Motor proteins known as kinesins and dyneins orchestrate microtubule-related transport that is essential for cell differentiation or survival^[167]. Kinesins constitute a large superfamily responsible for cargo trafficking, as well as controlling microtubule growth and stability^[168]. Increased expression of kinesin superfamily representatives KIF4A, -9, -18A, and -23 was associated with poor prognosis in low-grade glioma and glioblastoma^[169]. The pro-cancerous characteristics of *Kinesin family member 20A* (*KIF20A*) were noted more than 15 years ago in pancreatic cancer, which presented a reduction of proliferation once *KIF20A* was downregulated^[170]. Currently, accumulating evidence shows that this kinesin is overexpressed in multiple tumors^[171]. In glioblastoma, *KIF20A* downregulation ⁷ induces cell cycle arrest and apoptosis *via* suppressing PI3K/AKT pathway^[172]. Regarding cytoskeleton-related events, it is not only essential for cytokinesis but also interacts with Rab6 to regulate Golgi-related vesicle trafficking^[173]. Although the role of *KIF20A* in GBM stemness has not yet been confirmed, it was suggested outside of the glioblastoma context in a study by Qiu *et al*^[174]. The authors conceived the importance of *KIF20A* in controlling proliferation *vs* differentiation of tumor-initiating cells, based on both the fact that cancer stem cells share many mechanisms with neural progenitors, as well as their observations where *KIF20A* was implicated ¹⁷ in balancing symmetric and asymmetric divisions during cerebral cortical development^[175]. The *KIF20A* inactivation affected cortical neural progenitor cells that switched ¹⁷ from proliferative to differentiative mode. During divisions, daughter cell-fate specification was controlled by *KIF20A* in coordination with *RGS39* and *SEPT710*^[174,176].

Neurofibromatosis type 2 protein

Neurofibromatosis (type 1, type 2, schwannomatosis) are distinct, dominantly inherited disorders that have in common the occurrence of nerve sheath tumors^[177]. Type 1 neurofibromatosis presents with neurofibromas, cafe-au-lait spots/macules, freckling, and optic gliomas, whereas type 2 neurofibromatosis is characterized by bilateral vestibular schwannomas, ependymomas, and meningiomas^[178]. Each disease has a different underlying genetic alteration: Type 1 neurofibromatosis is related to the *neurofibromatosis type 1 protein* (*NF1*) gene, type 2 is linked to *NF2*, while schwannomatosis to integrase interactor 1 (*INI1*, also known as *SMARCB1*). The protein product of *NF2* has the same name as its gene but can also be referred to as Merlin. Although this tumor suppressor is not mutated in GBMs, it exhibited oncogenic properties in glioblastoma when phosphorylated at serine 518; this post-translational modification inactivates Merlin's anti-cancer capabilities, which affects the expression of EGFR or Notch1 and its downstream targets, *i.e.*, *HES1* or *CCND1*^[179]. Other authors demonstrated that upon *NF2* re-expression, a regulation of YAP, cIAP1/2, and the Hippo signaling pathway led to the inhibition of glioma growth and progression^[180]. Merlin is also known for regulating cell morphology or motility, and its loss renders dramatic changes in cellular adhesion and cytoskeleton organization^[181,182]. Specifically, this protein is closely related to ezrin, radixin, and moesin (collectively denoted as "ERM"), *i.e.*, critical proteins that enable the anchorage between membrane proteins and cortical cytoskeleton^[183]. Ultimately, the link between *NF2* and stemness might be related to CD44, the receptor of which cytoplasmic tail can interact with both Merlin and "ERM" proteins^[184,185]. Literature data states that *NF2* exhibits tumor suppressor function, *e.g.*, via negative regulation of CD44^[186], whereas this receptor has been repeatedly indicated as a marker of cancer stem cells in various tumors, *e.g.*, leukemia and carcinoma of breast, colon, ovarian, prostate, or pancreas^[187-191]. Knowing that CD44 is also an upstream regulator of the aforementioned Hippo signaling pathway^[192], of which components regulate the stem cell niche, self-renewal, maintenance, and differentiation^[193-196], one could investigate Merlin in the GBM stemness context taking into the account the *NF2*-ERM-CD44-Hippo regulation network.

Retinoid X receptor gamma

The signal transduction molecules being vitamin A derivatives are retinoids—they regulate cellular differentiation and proliferation *via* members of the nuclear receptors superfamily, *i.e.*, retinoic acid receptors (RARs) and retinoid X receptors (RXRs)^[197]. The RXR family members (RXRA, RXRB, and RXRG) form heterodimers within the superfamily, *e.g.*, with vitamin D, retinoic acid, or peroxisome proliferator-activated types of receptors^[198,199]. RXRs have tumor suppressor properties and, as partners of *RARA* and *RARB*, they are implicated in the anti-proliferative effects of retinoic acid^[197]. *RXRG* was found to modulate differentiation and apoptosis in various tumors, indicating its function in cancer pathogenesis^[200]. Glioblastoma-related research certifies the general view that *RXRG* contributes to anti-neoplastic effect *via* its ligands; in study by Papi *et al*^[201], the treatment of GBM with 6-OH-11-O-hydroxyfenantrene led to anti-proliferative and anti-invasive effects. However, the literature data on glioblastoma stemness seems to be focused on RARs rather than RXRs. Ying *et al*^[202] evaluated the cellular and molecular responses of GSCs to all-*trans* retinoic acid; this treatment changed cells morphology (*e.g.*, decreased neurosphere-forming capacity), caused growth arrest at G₁/G₀ to S transition, reduced cyclin D1 expression, and elevated p27 expression. Moreover, differentiation markers such as Tuj1 and GFAP were induced, while stem cell markers, *e.g.*, CD133, Msi-1, Nestin, and Sox-2, had decreased expression. Friedman *et al*^[203] provided similar observations with regard to Nestin level or neurosphere formation but also indicated that GBM differentiation induced by all-*trans* retinoic acid is executed *via* the ERK1/2 pathway. Evidently, retinoid-related research in the GBM context frequently focuses on all-*trans* retinoic acid while this isomer is bound only by RARs and not by both RARs and RXRs, as is the case with another retinoic lipid: 9-*cis*^[204]. Even if two of the best-known retinoid receptors (*RARA* and *RXRA*) are described in detail by Rodriguez *et al*^[205] in the GBM stemness context, the literature is still lacking data on *RXRG* and should begin with, *e.g.*, evaluation of

whether 9-*cis* retinoid acid is able to manifest the anti-glioblastoma effects *via* RXRG and subsequently ERK1/2 pathway.

SPARC/Osteonectin, CWCV, Kazal-like domains 1

ECM is a component containing elastin, collagen, laminins, glycoproteins, fibronectin, and proteoglycans. Together, these elements bind *via* cell adhesion receptors and form a complex macromolecular network^[206]. Matricellular proteins are made of matrix-binding proteins and cytokines that can be located within the cell or secreted outside^[207]. SPARC/Osteonectin, CWCV, Kazal-like domains 1 (SPOCK1), also referred to as testican-1, is an ECM proteoglycan from a matricellular family of proteins that regulate matrix remodeling and affects tumor progression^[208-210]. As the interplay between ECM and cytoskeleton is known^[211], it is not surprising that changes in *SPOCK1* lead to alterations in cytoskeletal components. For example, Schulz *et al*^[212] noticed that *SPOCK1* upregulation paralleled that of *EPB41L4B*, the latter being a cortical cytoskeleton protein underlying cellular membrane. With regard to brain tumors, testican-1 contributes to GBM metastasis and resistance to TMZ, as well as promotes glioma invasion, migration, and proliferation *via* Wnt/ β -catenin and PI3K/AKT pathways^[213,214]. Mediating TMZ chemoresistance *via* *SPOCK1* in GBM was independently confirmed by Sun *et al*^[215]. Although not yet directly concluded by any scientific group, it is conceivable that the impact of *SPOCK1* on TMZ resistance renders a similar GSCs-related effect as *SAA2* which was described in one of the previous sections.

Ubiquitin-like with PHD and ring finger domains 1 (UHRF1)

The proteins' turnover and degradation depend on ubiquitination that is orchestrated by the ubiquitin-proteasome system (UPS)^[216], of which alterations can lead to several tumor types^[217,218]. One of the ubiquitin-protein ligases responsible for the UPS specificity is ubiquitin-like with PHD and ring finger domains 1 (UHRF1)^[219], a molecule also interacting with DNA methyltransferase 1, which together constitute the main

regulatory axis of cellular senescence^[220]. *UHRF1* was already identified as a novel oncogene and/or druggable epigenetic target for various tumors^[221-223], and Jung *et al*^[220] suggested its role as a switch molecule between senescence and cancer. In GBM, *UHRF1* is overexpressed by upstream CD47 and regulates downstream silencing of tumor suppressor gene *p16^{INK4A}*, leading to increased proliferation^[224]. Regarding cytoskeleton, *UHRF1* contributes to microtubule organization through its downstream targets: *BRCA2*, *HOOK1*, *KIF11*, and *KIF18A*^[225]. The role of *UHRF1* in different types of stem cells is documented but overlooks GSCs. Namely, it was found to be required for the proliferative potential of basal stem cells in response to airway injury^[226], as well as regulated the transcriptional marks at bivalent domains in pluripotent stem cells^[227]. On the other hand, *UHRF1* decrease was found to be a major cause of DNA demethylation in embryonic stem cells^[228] and led to the activation of retroviral elements and delayed neurodegeneration^[229]. It is evident that research in the glioblastoma context should be pursued in the future, especially since some epigenetic features, next to transcriptional ones, are unique in GSCs compared to neural stem cells and may include druggable targets for new therapeutic approaches^[230].

DISCUSSION

Despite molecular advancements, there is still a considerable need for glioblastoma biomarkers^[231], especially since the relatively ineffective treatment leaves the patients with a very dismal chance of survival^[232]. One of the glioblastoma traits involved in the absence of effective treatment is tumor heterogeneity which can be explained by clonal evolution and the presence of stem cells^[8].

Literature data states that many independent studies on various tumor types have reported common genes as potential therapeutic or diagnostic biomarkers^[233]. Al-Fatlawi *et al*^[234] contemplated that biomarker signatures for different cancer types should be similar, owing to the fundamental mechanisms shared between tumors, *e.g.*, survival, tumor growth, or invasion. Thus, we presume that our description of stemness-related genes, especially those still unconfirmed in GBM, brings significant

value to the current knowledge and can enable novel therapeutic or diagnostic directions.

For clarity, a graphical presentation was prepared to emphasize the role of described genes specifically in stem cells, setting aside the rest of the information provided for each gene (Figure 2). At first glance, the most frequently regulated processes are proliferation and chemoresistance, followed by differentiation, tumor growth, invasion, and apoptosis. Except for *BMP4* (increase in asymmetric cell division and apoptosis), *NF2* (reduced self-renewal, tumor growth, stemness maintenance), *RXRG* (decrease in invasion and proliferation), and *DUSP7* (insufficient data for a definite conclusion), the remaining genes exhibit pro-cancerous properties. This corresponds to what was described in subsections, separately for each gene. Interestingly, two genes that promote invasiveness of stem cells (*SPOCK1*, *MMP13*) are known to affect the cytoskeleton^[33,212] and, in terms of *MMP13*, also the metabolism^[90]. Two genes that were also found to regulate both the cytoskeleton and metabolism were *MTHFD2* and *RRM2*. On the one hand, they control the organization of vimentin and actin; these proteins are known for influencing glioblastoma migratory potential^[235,236]. On the other hand, the contribution of *MTHFD2* and *RRM2* to metabolism is related to folate and glutathione cycles that are implicated in the resistance of GBM to therapy^[237,238].

In order to gravitate towards the link between metabolism, cytoskeleton, and GBM stemness, the appropriate representatives of each process (including the most frequently regulated processes that were mentioned above), were compiled into a cross-talk network. This allowed us to integrate the aim of our review with the main processes that are regulated by genes described in this work, additionally with the inclusion of GBM biomarkers (acquired from review by Sasmita *et al*^[231]). Prevalent interaction types include co-expression and physical interaction between these representatives, there is also a high interconnectivity of the entire network, confirming that these molecular events are related. The cross-talk is visualized in Supplementary Figure 1, whereas the datasets used in the workflow are summarized in Supplementary Table 2.

The narrative of this review was intended to elaborate on the background of the biological machinery in which each successive gene is involved, then proceed with details regarding the regulation of glioblastoma, cytoskeleton/metabolism, and stemness (GBM-related or, if not present in the literature, any available). It is worth emphasizing that the herein described genes constitute more than half of the “top genes” that we established in our previous *in silico* study *via* a multi-stage methodology that included, *e.g.*, enrichment analysis, machine learning algorithm, and differential expression analysis^[32]. The remainder was not presented due to a lack of stemness-related literature data (Supplementary Table 1). For the part available in this paper, the majority of genes (*BMP4*, *GRIN2B*, *HOXA10*, *HOXA1*, *MMP13*, *MTHFD2*, *PHF5A*, *RPS27*, *RRM2*, *SAA2*, *WT1*) were confirmed to influence GSCs. The attempt to associate *CMTM6*, *DUSP7*, *KIF20A*, *NF2*, *RXRG*, *SPOCK1*, and *UHRF1* with glioblastoma stemness revealed the promising implication in crucial biological processes that should be validated in future experiments. For *BMP4*, *WT1*, and *RXRG*, their contribution to novel therapeutic strategies was above-mentioned on the basis of literature data, prompting us to investigate whether any clinical trials utilize the products of described genes as drug components or targets. According to the ClinicalTrials website (<https://clinicaltrials.gov/>), cancer-related data can be found for six genes (Table 1); however, the seventh trial on *GRIN2B* was also included because it focused on brain research and highlights that selective *GRIN2B* antagonist is already developed. Moreover, the details on NF2-related intervention are not yet disclosed^[239]. Collectively, these studies are in the early phases, certifying that there is still a room for further research.

CONCLUSION

Taken together, a promising set of genes involved in cytoskeletal rearrangements and metabolic alterations were found to influence glioblastoma stemness *via* a plethora of biological processes. Most of the described genes exhibit pro-cancerous properties; among them, clinical trials on *GRIN2B*, *RRM2*, *WT1*, and *KIF20A* are ongoing and focus

on selective inhibitors or peptide-based vaccines. Concerning tumor suppressors, the anti-cancer effect can also be achieved *via* delivery of recombinant proteins (*BMP4*), ligands for tumor suppressors (*RXRG*), or counteracting the pathways that become hyperactive following an anti-oncogene loss (*NF2*). The cytoskeletal phenomena currently linked to the described genes require experimental verification of their contribution to GSCs expansion. Future GBM stemness-related research should generally delve into cytoskeleton and related molecular events, since the concept is already encouraging.

Figure 1 Example of the interplay between cytoskeleton and metabolism using the biological function of methylenetetrahydrofolate dehydrogenase 2 and ribonucleotide reductase subunit M2 enzymes. Typically, methylenetetrahydrofolate dehydrogenase 2 (*MTHFD2*) dehydrogenase is known for its activity in folate metabolism, whereas ribonucleotide reductase subunit M2 (*RRM2*) reductase is known for the conversion of ribonucleotide triphosphates to deoxyribonucleotide triphosphates which requires metabolic resources supplied by reduced glutathione. However, these two enzymes (encircled in red) are also involved in cytoskeletal rearrangements that are summarized on the right side of the figure. Literature data indicate that they also affect the same pathway (*i.e.*, ERK1/2 signaling) but render various outcomes. Moreover, their role in glioma has already been proposed (bottom-right panel). Figure created using Inkscape and GeneMania (*MTHFD2* and *RRM2* as query genes; five “resultant” genes included to highlight interconnectivity; exemplary metabolism-related processes included from the built-in functional analysis). NTP: Ribonucleotide triphosphates; dNTPs: Deoxyribonucleotide triphosphates; *MTHFD*: Methylenetetrahydrofolate dehydrogenase; *RRM2*: Reductase subunit M2.

Figure 2 Impact of described genes on biological processes related to stem cells. The “↑” or “↑↑” symbol indicates activation of the process while “↓” denotes inhibition. The impact of genes on processes (numbered from 1 to 19) is either directly confirmed (solid arrow next to the number) or recapitulated based on available data from various literature sources (dashed arrow next to the number). The “↓↓” symbol was not required as any gene inhibited the given process in an indirect manner. The white dashed line dividing the stem cell into two halves separates the genes with a confirmed role in glioblastoma stem cells (above the line) from those involved in cancer stemness outside the glioblastoma context (below the line). Figure created using Inkscape. NF2: Neurofibromatosis type 1 protein; BMP4: Bone morphogenetic protein 4; RXRG: Retinoid X receptor gamma; MMP13: Metalloproteinase 13; RRM2: Reductase subunit M2; SPOCK1: SPARC/Osteonectin, CWCV, Kazal-like domains 1; ECM: Extracellular matrix; CMTM: Chemokine-like factor superfamily.

Table 1 Clinical trials that utilize the products of described genes as drug components or targets

Gene	Compound	Condition	Trial number and phase	Intervention details
<i>BMP4</i>	hrBMP4	Glioblastoma	NCT02869243 (phase I)	Delivery of human recombinant BMP4
<i>GRIN2B</i>	EVT 101	Healthy volunteers (brain function assessment)	NCT00526968 (phase I)	Delivery of selective GRIN2B antagonist
<i>RRM2</i>	COH29	Solid tumors	NCT02112565 (phase I)	Delivery of ribonucleotide reductase inhibitor
<i>WT1</i>	DSP-7888	Gliomas (incl. GBM)	NCT02750891 (phase I/II)	Delivery of WT1 peptide-based cancer vaccine
<i>KIF20A</i>	KIF20A peptide	Small cell lung cancer	NCT01069653 (phase I)	Delivery of KIF20A peptide-based vaccination
<i>NF2</i>	IAG933	Solid tumors	NCT04857372 (phase I)	Not yet disclosed (the drug presumably counteracts the YAP/TAZ hyperactivity that occur following NF2 loss)
<i>RXRG</i>	9- <i>cis</i> retinoic acid	Breast cancer	NCT00001504 (phase I)	Delivery of RXRG ligand

NF2: Neurofibromatosis type 1 protein; BMP4: Bone morphogenetic protein 4; RXRG: Retinoid X receptor gamma; MMP13: Metalloproteinase 13; RRM2: Reductase subunit M2; SPOCK1: SPARC/Osteonectin, CWCV, Kazal-like domains 1; ECM: Extracellular matrix; WT1: Wilms' tumor protein 1; KIF20A: Kinesin family member 20A; GRIN2B: Glutamate ionotropic receptor NMDA type subunit 2B.

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