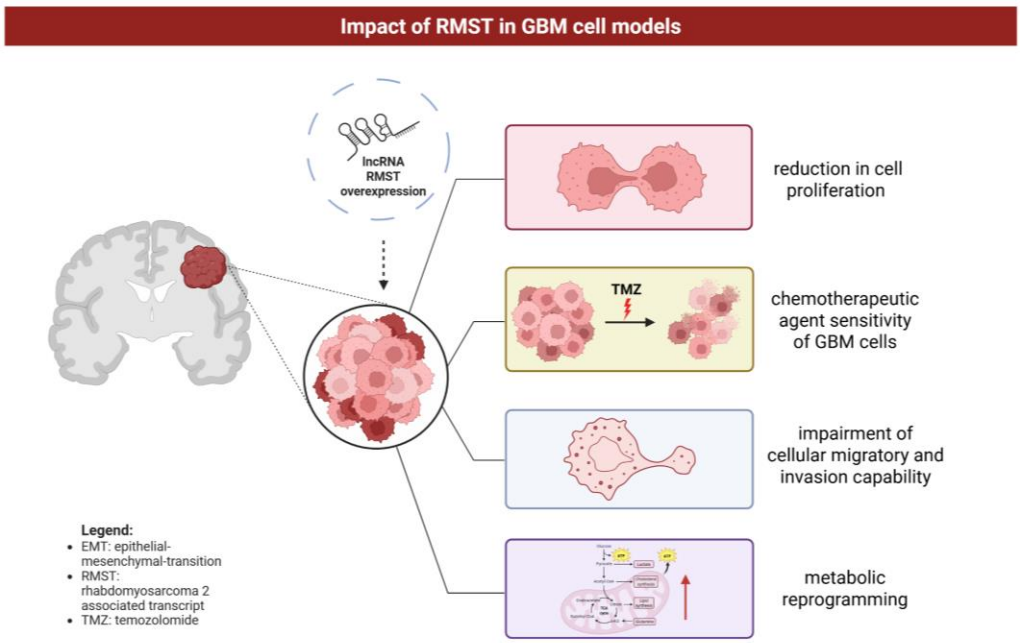
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXVIII CYCLE

Antonella Napolitano

Understanding the role of the long non-coding RNA Rhabdomyosarcoma 2-Associated Transcript in Glioblastoma



2022-2025

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Understanding the role of the long non-coding RNA Rhabdomyosarcoma 2-Associated Transcript in Glioblastoma

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2022-2025

STATEMENT

I hereby declare that this Thesis is the product of my own work, and, to the best of my knowledge, it contains no material previously published or written by another person, except where due acknowledgement is provided. I also declare that this Thesis has been successfully screened through the software Turnitin.

Any contribution made by others is explicitly acknowledged.

INDEX

ABBREVIATIONS

Ac – Acetylation

ALT – Alternative Lengthening of Telomeres

BBB – Blood-Brain Barrier

BSA – Bovine Serum Albumin

ceRNA – Competing Endogenous RNA

CNS – Central Nervous System

Ct – Cycle threshold (PCR)

cDNA – Complementary DNA

DEGs – Differentially Expressed Genes

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic Acid

DNMT – DNA Methyltransferase

ECM – Extracellular Matrix

EDTA – Ethylenediaminetetraacetic Acid

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

EMEM – Eagle's Minimum Essential Medium

EMT – Epithelial-to-Mesenchymal Transition

ENCODE – Encyclopedia of DNA Elements

FGF / FGFR – Fibroblast Growth Factor / Fibroblast Growth Factor Receptor

FBS – Fetal Bovine Serum

GBM – Glioblastoma Multiforme

GSCs – Glioma Stem Cells

GO – Gene Ontology

HGF – Hepatocyte Growth Factor

hnRNPK – Heterogeneous Nuclear Ribonucleoprotein K

IDH / IDH1/2 – Isocitrate Dehydrogenase 1/2

IGF2 – Insulin-like Growth Factor 2

IL-10 – Interleukin-10

lncRNA – Long Non-Coding RNA

LOH – Loss of Heterozygosity

MAPK – Mitogen-Activated Protein Kinase

Me3 – Trimethylazione

MGMT – O6-Methylguanine-DNA Methyltransferase

miRNA – microRNA

MMR – Mismatch Repair

mRNA – Messenger RNA

mTOR – Mechanistic Target of Rapamycin

NF-κB – Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NHEJ – Non-Homologous End Joining

NTC – No Template Control

OS – Overall Survival

PBS – Phosphate Buffered Saline

PCR / qPCR / RT-PCR – Polymerase Chain Reaction / Quantitative PCR / Reverse Transcription PCR

PDGF / PDGFR – Platelet-Derived Growth Factor / Receptor

PI – Propidium Iodide

PI3K – Phosphoinositide 3-Kinase

PTEN – Phosphatase and Tensin Homolog

REST / NRSF – RE1-Silencing Transcription Factor / Neuron-Restrictive Silencer Factor

RNP – Ribonucleoprotein

RMST – Rhabdomyosarcoma 2-Associated Transcript

RT – Reverse Transcription

SD – Standard Deviation

SF – Scatter Factor

SDS-PAGE – Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SOX2 – SRY-Box Transcription Factor 2

STAT3 – Signal Transducer and Activator of Transcription 3

SYBR – SYBR Green fluorescent dye

TAK1 – Transforming Growth Factor-β-Activated Kinase 1

TCGA – The Cancer Genome Atlas

TGF-β – Transforming Growth Factor Beta

TERT / TERTp – Telomerase Reverse Transcriptase / TERT promoter

TME – Tumor Microenvironment

TMZ – Temozolomide

TNBC – Triple-Negative Breast Cancer

VEGF / VEGFR – Vascular Endothelial Growth Factor / Receptor

WHO – World Health Organization

ABSTRACT

Glioblastoma (GBM) is the most aggressive primary brain tumor in adults, characterized by rapid proliferation, extensive invasion, and resistance to standard therapies. Long non-coding RNAs (lncRNAs) have emerged as critical regulators of cancer progression, yet the functions of many remain poorly understood. This study investigates the role of the brain-enriched lncRNA Rhabdomyosarcoma 2-Associated Transcript (RMST) in GBM pathogenesis. Transcriptomic analyses and qPCR validation revealed a marked downregulation of RMST across various histological types of brain tumours, from oligodendroglioma to GBM, parallel to increasing tumour grade. RMST overexpression correlated positively with patient overall survival, supporting its potential as a prognostic biomarker. Functional assays demonstrated that RMST overexpression significantly inhibited GBM cell proliferation, clonogenicity, migration, and invasion, while enhancing sensitivity to the chemotherapeutic agent temozolomide (TMZ). RMST overexpression also suppressed the expression of key Extra Cellular Matrix (ECM) and epithelial-to-mesenchymal transition (EMT)-associated genes, including SNAIL, SLUG and N-cadherin, while upregulating E-cadherin, suggesting a reversal of the mesenchymal phenotype. Interestingly, RNA-seq analysis revealed that RMST overexpression is also associated with alterations in metabolic gene expression, indicating that this lncRNA may contribute to metabolic reprogramming in GBM cells. These findings support a model whereby RMST functions as a tumor suppressor lncRNA in GBM by attenuating malignant phenotype and enhancing therapy responsiveness. Although further mechanistic and in vivo studies are needed, these data indicate that RMST represents a promising candidate for biomarker development and therapeutic targeting in high-grade gliomas.

1 BACKGROUND

1.1 Glioblastoma Multiforme: A molecular and clinical overview

Glioblastoma multiforme (GBM) is one of the most malignant types of central nervous system (CNS) tumors. The aggressive nature of this brain cancer, characterized by rapid growth and invasiveness, represents a main challenge for patient survival and quality of life (Fig. 1) [Darlix 2017, Tykocki and Eltayeb 2018]. Despite multimodal approaches combining surgery, radiation, and chemotherapy, the prognosis remains extremely poor, with high recurrence rates shortly after treatment [Tamimi and Juweid 2017].

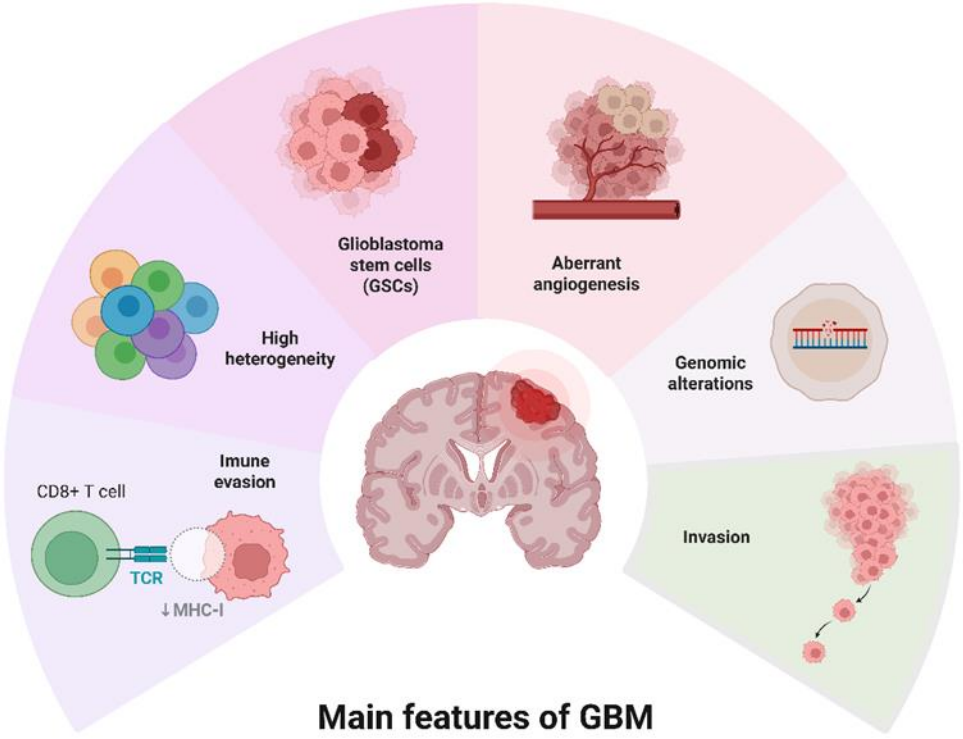


Fig.1. Characteristics feature of glioblastoma multiforme (GBM). This cartoon illustrates the key pathological and clinical characteristics of GBM, highlighting its highly aggressive nature and resistance to therapy.

Gliomas represent the most common tumors of the CNS, comprising approximately 80% of all malignant primary brain tumors [Agnihotri

2013]. They are categorized according to cellular origin, encompassing astrocytic tumors – including astrocytoma, anaplastic astrocytoma, and glioblastoma- as well as oligodendrogliomas, ependymomas, and mixed gliomas. These tumor types constitute the predominant varieties of primary CNS neoplasms, each exhibiting unique histological features and clinical behaviors [Maher 2001, Schwartzbaum 2006].

GBM is a diffuse glioma, posing considerable treatment obstacles due to its highly infiltrative properties. In contrast to circumscribed gliomas, which are characterized by well-defined borders and more favourable prognosis, diffuse gliomas feature infiltration into adjacent healthy brain tissue. This characteristic considerably restricts the effectiveness of surgical excision [Louis 2016]

GBM, the most aggressive type of diffuse glioma, constitutes about 50% of all new brain tumors and is recognized as the most lethal glioma [Louis 2021]. The prevailing international standard for the nomenclature and diagnosis of gliomas is the WHO (World Health Organization) classification. This classification system organizes gliomas into grades I to IV according to their malignancy, as established by precise histological criteria. Grade I gliomas exhibit minimal proliferative capacity and are frequently amenable to complete surgical resection. Conversely, grade II to IV gliomas exhibit increased malignancy and invasiveness. GBM, designated as Grade IV is recognized as the most aggressive and undifferentiated form of gliomas, distinguished by its rapid proliferation and ability to infiltrate adjacent brain tissue [Jovcevska 2013, Louis 2007].

GBM can be categorized into primary and secondary types, with around 90% of instances being primary, generally arising *de novo* in older individuals. Secondary glioblastomas originate from lower grade astrocytomas and are more common in younger patients. A prevalent genetic modification in primary and secondary GBMs is the loss of heterozygosity (LOH) on the 10q chromosome arm, detected in 60–90% of cases [von Deimling 1992, Waugh 2016]. Furthermore, roughly 85.3–87% of GBMs display alterations to the p53 gene, a crucial tumor suppressor that governs cell cycle and apoptosis in reaction to DNA damage. This enables malignancies to circumvent standard cell cycle checkpoints, resulting in unregulated proliferation [Furnari 2007, Wang 2015].

The genetic characteristics of primary and secondary GBMs exhibit significant differences, as mutations in the isocitrate dehydrogenase (IDH1/2) gene are commonly found in secondary GBMs, whereas they are absent in primary tumors (Fig. 2) [Ohgaki and Kleihues 2013]. Mutations in the IDH enzymes play a crucial role in glioma classification and have a substantial impact on tumor progression. IDH catalyses the oxidative decarboxylation of isocitrate and is an essential enzyme that participates in metabolic processes affecting cellular metabolism, cancer biology, and oncogenesis (Fig. 3).

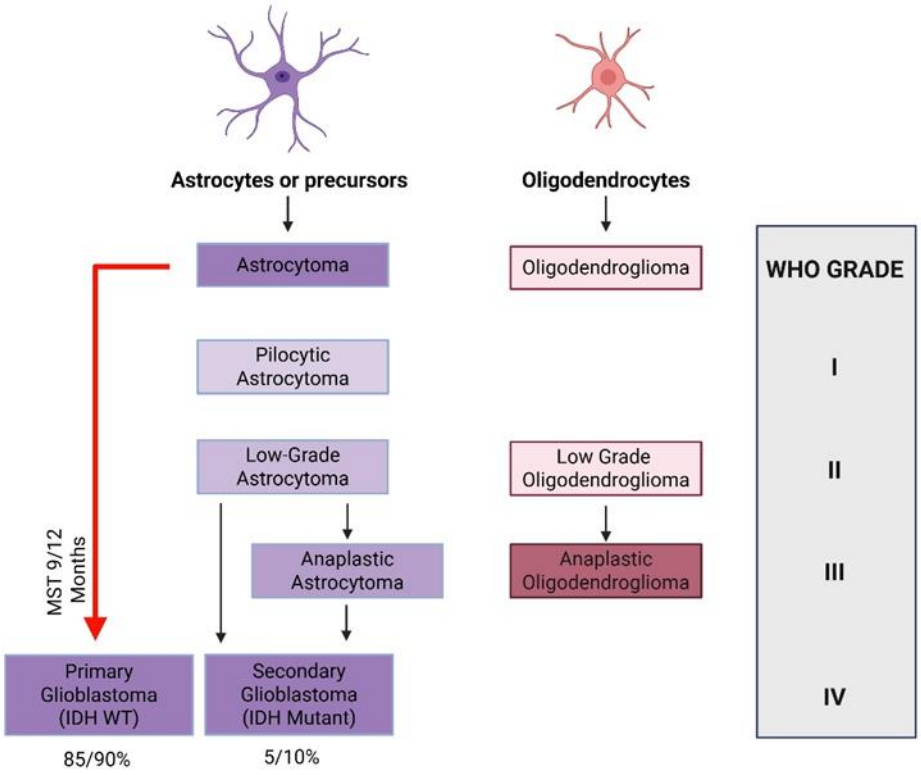


Fig. 2. Glioma classification. This diagram summarizes glioma classification and progression based on their cellular origin—astrocytic or oligodendroglial—and corresponding WHO grades (I–IV). Astrocytomas may evolve from low-grade lesions to anaplastic astrocytoma (Grade III) and secondary glioblastoma (IDH-mutant, Grade IV), or present de novo as primary glioblastoma (IDH wild-type, Grade IV), which accounts for 85–90% of glioblastomas. Oligodendrogliomas progress from low-grade (Grade II) to anaplastic forms (Grade III).

Primary GBMs often exhibit abnormalities such as EGFR amplification or PTEN loss rather than TP53 mutations [Ohgaki and Kleihues 2013]. Mutations in EGFR and PDGFR are crucial for the development of GBM.

EGFR, a receptor tyrosine kinase that regulates cell proliferation, differentiation, and survival, is mutated in 40–60% of GBM of cases. These mutations frequently cause persistent receptor activation, resulting in amplified signaling that fosters tumor proliferation and resistance to apoptosis.

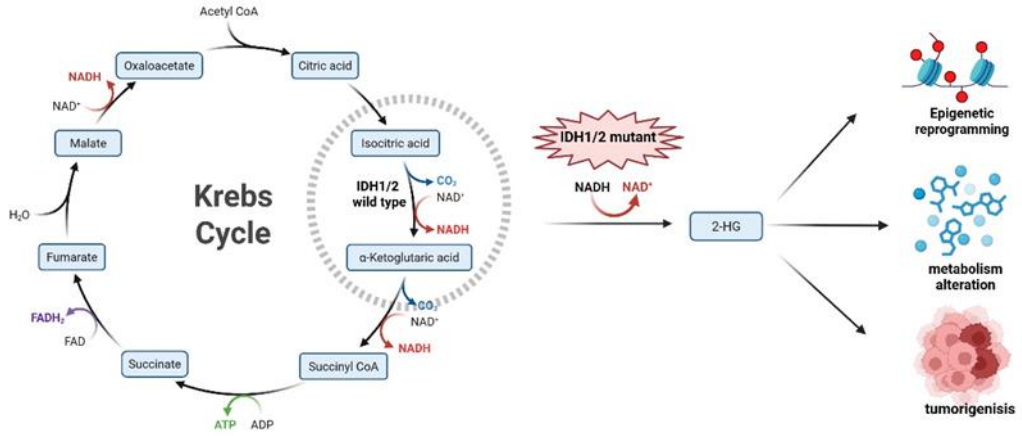


Fig. 3. Isocitrate dehydrogenase mutation in gliomas. Isocitrate dehydrogenase (IDH) enzymes catalyse the oxidative decarboxylation of isocitrate, thereby playing key roles in the Krebs cycle and cellular homeostasis. Mutation of IDH in glioma alters normal enzymatic activity, disrupts metabolic pathways, drives epigenetic modifications, and contributes to tumorigenesis.

In addition, PDGFRA amplification, a receptor implicated in cellular proliferation, motility, and angiogenesis, accounts for approximately 25–30% of cases, facilitating aberrant signaling pathways that promote tumor progression. Therapies aimed at inhibiting aberrant signaling in GBM concentrate on targeting mutations in EGFR and PDGFR. Nonetheless, resistance to these targeted medicines is a substantial obstacle owing to the tumors' intrinsic complexity and heterogeneity [Brennan 2013, Desai 2016, Ozawa 2010]. Further mutations are observed in the MDM2 gene (10–15% of cases) and the PTEN gene (20–34% of cases) [Duerr 1998, Smith 2001].

The advancement of molecular classification has enhanced GBM subtyping, beyond histological grading, to offer a more profound comprehension of its genetic and epigenetic framework (Table 1).

Verhaak's classification method (2010) categorizes GBM into four subtypes: proneural, neural, classical, and mesenchymal. Proneural GBM exhibits resistance to conventional treatment, even though it is

characterized by a high frequency of IDH1 mutations and elevated expression of platelet-derived growth factor receptor alpha (PDGFR-α), potentially conferring a survival benefit. Neural subtypes that have gene expression similarities with normal neurons (e.g., SYT1, GABRA1, and NEFL) exhibit increased vulnerability to radiation and chemotherapy [Verhaak 2010].

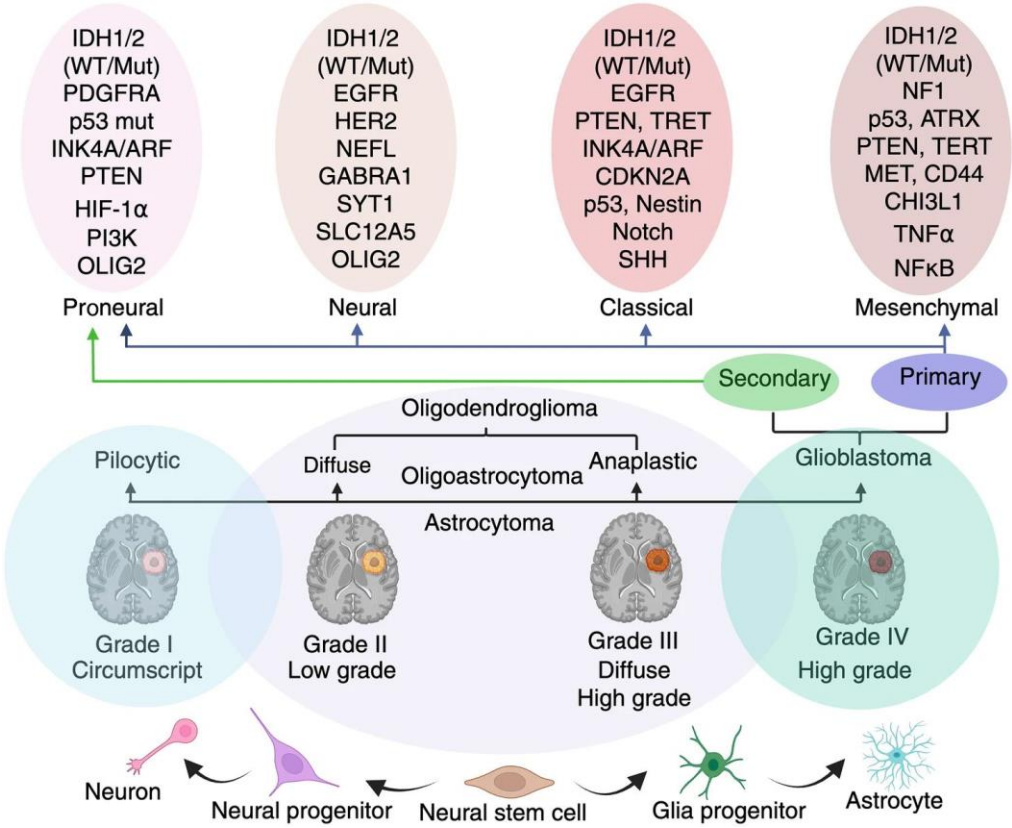


Fig. 4. Histopathological and molecular classification of gliomas. This scheme integrates the histopathological grading and molecular classification of gliomas with their cellular origins and progression. The lower panel depicts glioma development along neural lineages, from neural stem cells to differentiated neurons and glial cells. Grade I pilocytic astrocytomas are typically circumscribed and low-grade, while Grades II–IV gliomas (diffuse, anaplastic, and glioblastoma) show increasing malignancy and invasiveness. The upper panel outlines the four molecular subtypes of glioblastoma—proneural, neural, classical, and mesenchymal—each defined by distinct genetic alterations (e.g., *IDH1/2*, *EGFR*, *TP53*, *NF1*). These subtypes correlate with either primary or secondary glioblastoma origins and emphasize the clinical importance of integrating molecular markers for accurate diagnosis, prognosis, and treatment strategies [Singh 2025].

Mesenchymal GBM, the most aggressive subtype with unfavorable treatment outcome, is characterized by extensive necrosis, inflammatory markers, upregulation of interstitial and angiogenesis-related genes, frequent deletions of tumor suppressor genes such as TP53, PTEN, and NF1, as well as elevated expression of genes including VEGF-A, VEGF-B, ANG1, and ANG2. In contrast, the classical subtype exhibits greater vulnerability to aggressive treatment and it is associated to the activation of Notch (NOTCH3, JAG1, LFNG) and Sonic Hedgehog (SMO, GAS1, GLI2) signaling pathways, alongside EGFR amplification, alterations in the RB pathway, amplification of chromosome 7, and deletion of chromosome 10 [Sharma 2017].
These molecular subgroups underscore the heterogeneity of GBM and indicate potential targets for precision medicine (Fig. 4).
Genomic investigations, including those conducted by the Cancer Genome Atlas Research Network, have uncovered further modifications in critical signaling networks linked to the initiation and progression of GBM [Pearson and Regad 2017]. These findings are essential for understanding the molecular mechanisms behind GBM and uncovering possible therapeutic targets.

Table 1. Clinical grading and molecular subtypes of GBM

Grading	Circumscript		Diffuse	
	WHO grade-I (G1)	WHO grade-II (G2)	WHO grade-III (G3)	WHO grade-IV (G4)
	Low grade		High grade	
Characteristics	Benign, slow growing tumor associated with long-term survival and amenable to surgical resection	Increased hypercellularity, no vascular proliferation, no mitosis and no necrosis	High rate of hypercellularity, high rate of mitosis, high rate of recurrence, no necrosis and no vascular proliferation	Very high rate of hypercellularity, presence of vascular proliferation, presence of necrosis and nuclear atypia
Types	Policystic and subependymal astrocytomas	Astrocytoma (IDH-mutant) and oligodendroglioma (IDH- mutant and 1p/19q-codeleted)	Anaplastic astrocytoma (IDH-mutant) and anaplastic oligodendroglioma (IDH-mutant and 1p/19q-codeleted)	GBM (IDH-wild-type), GBM (IDH-mutant) and diffuse midline glioma (H3K2M mutant)
Molecular subtypes of GBM				
GBM subtypes	Proneural	Mesenchymal	Classic	Neural
Markers	SOX2, DCX, DLL3, ASCL1, TCF4, PDGFRA, NKK2-2, OLIG2, PDGFRA	CD44, MERTK	NES, NOTCH3, JAG1, LFNG, SMO, GAS1, GLI2	NEFL, GABRA1, SYT1 and SLC12A5
Alterations/ mutations	Alteration and IDH1 point mutation	Lower NF1 expression, NF1 PTEN co-mutation and high expression of TRADD, RELS, TNFRSF1A	Chromosome 7 amplification paired with chromosome 10 loss, high EGFR amplification, EGFR mutation and lack of p53 mutation	Neuron projection, axon and synaptic transmission

The table summarizes the WHO classification of gliomas by histological Grade (I–IV) and growth pattern (circumscribed vs. diffuse), highlighting key characteristics, tumor types, and associated molecular features. Low-grade gliomas (Grades I–II) are typically slow-growing and surgically resectable, while high-grade gliomas (Grades III–IV) show increased cellularity, mitotic activity, vascular proliferation, and necrosis. The table also outlines the four molecular subtypes of glioblastoma (Proneural, Mesenchymal, Classical, Neural), including their expression markers and key genetic alterations.

1.2 Regulatory mechanisms in glioblastoma and their role in pathogenesis

GBM is driven by a complex interplay of genetic and epigenetic alterations. These factors regulate tumor initiation, progression, and therapeutic resistance. Among them, epigenetic mechanisms—including histone modifications, DNA methylation, chromatin remodeling, and mutations in regulatory networks—play a central role. They modulate gene expression, maintain genomic stability, and influence tumor behaviour (Fig. 5).

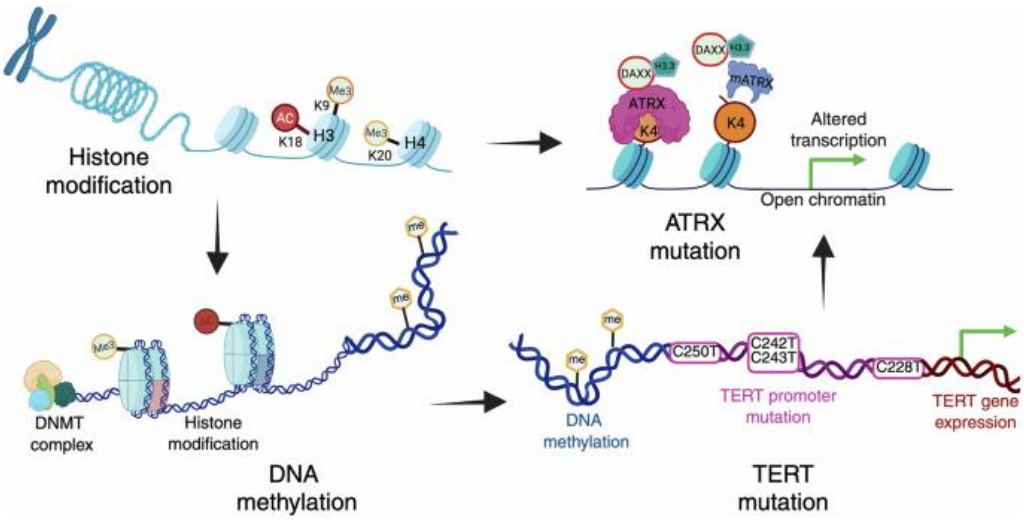


Fig. 5. The role of epigenetic mechanisms in glioblastoma pathogenesis. The figure describes the key epigenetic mechanisms implicated in glioblastoma (GBM) include histone modifications, DNA methylation, and mutations affecting chromatin remodeling and telomerase regulation. Histone methylation (Me3) and acetylation (Ac) regulate chromatin accessibility and gene expression. DNA methylation by DNA methyltransferases (DNMTs) can silence or activate genes, with aberrant patterns linked to tumor progression. ATRX mutations disrupt the ATRX-DAXX complex, impairing H3.3 histone deposition and altering chromatin structure. TERT promoter mutations lead to abnormal telomerase activation, supporting cellular immortality [adapted from Singh 2025].

Histone modifications significantly affect chromatin architecture and transcriptional activity. Acetylation of histones usually promotes gene activation by relaxing chromatin structure. In contrast, methylation can

activate or repress transcription depending on the residue and methylation state [Kim 2014].

Dysregulation of these modifications contributes to tumor aggressiveness and therapy resistance [Kreth 2014]. For example, reduced acetylation of histone H3 at lysine 18 (H3K18Ac) has been associated with improved survival in primary GBM. Increased trimethylation of histone H4 at lysine 20 (H4K20me3) is linked to better prognosis in secondary GBM. In addition, H3K9 trimethylation (H3K9me3), a repressive mark, is enriched in IDH-mutant gliomas. It serves as a distinguishing feature from IDH wild-type GBM [Venneti 2013].

DNA methylation is another key epigenetic mechanism in GBM pathogenesis. Promoter hypermethylation of tumor suppressor genes, especially the O^6-methylguanine-DNA methyltransferase (MGMT) gene, leads to gene silencing and sensitivity to alkylating chemotherapy like temozolomide (Fig. 6). MGMT promoter methylation has become a key predictive and prognostic biomarker in clinical practice, influencing therapeutic decision-making and patient outcome [Butler 2020].

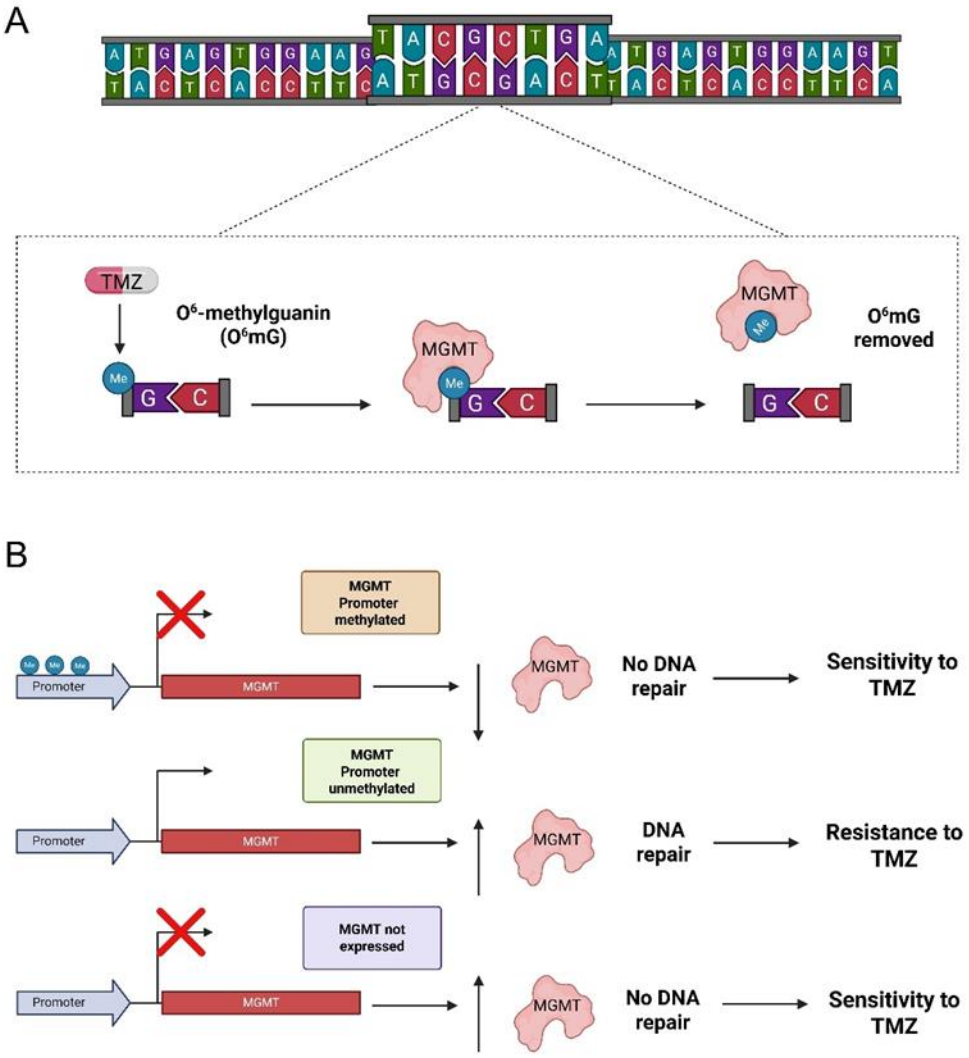


Fig. 6. Role of MGMT promoter methylation in temozolomide response in glioblastoma. This figure illustrates the molecular mechanism underlying the role of O^6-methylguanine-DNA methyltransferase (MGMT) in glioblastoma response to temozolomide (TMZ) therapy. Panel A: TMZ induces methylation at the O^6 position of guanine (O^6-MeG), which leads to G:C mismatches during DNA replication. MGMT can repair this lesion by removing the methyl group, reversing the damage. If MGMT is inactive or absent, the lesion persists, leading to cytotoxicity. Panel B: Shows three scenarios based on MGMT promoter methylation and mismatch repair (MMR) status: MGMT promoter methylation silences MGMT expression. Without MGMT, O^6-MeG persists, leading to DNA damage that cannot be repaired, especially in MMR-proficient cells, resulting in TMZ sensitivity (Top). Unmethylated MGMT promoter allows MGMT expression and DNA repair, leading to TMZ resistance (Middle). In some glioblastomas cases (bottom), MGMT is not expressed despite the absence of promoter methylation, leading to increased sensitivity to TMZ.

Chromatin remodeling alterations further contribute to the epigenetic complexity of GBM, particularly through mutations in the ATRX gene. ATRX is critical for telomere maintenance, DNA repair, and genomic integrity. Loss-of-function mutations in ATRX are closely associated with the alternative lengthening of telomeres (ALT), a telomerase-independent maintenance mechanism that increases genomic instability.

Although ATRX mutations are rare in primary adult GBM, they are more frequently observed in secondary GBM and lower-grade gliomas, particularly in the context of IDH mutations and absence of 1p/19q codeletion [Haase 2018]. ATRX loss has been associated with improved prognosis in these subtypes, but also with impaired DNA repair capacity, particularly in the non-homologous end-joining (NHEJ) pathway, which may contribute to enhanced tumor progression and therapeutic resistance [Nandakumar 2017].

Mutations in the promoter region of the telomerase reverse transcriptase (TERT) gene represent another important epigenetic regulatory event in GBM (Fig. 7). These mutations, typically occurring at positions C228T and C250T, create *de novo* binding sites for Ets/TCF transcription factors, resulting in aberrant TERT expression and constitutive telomerase activation [Spiegl-Kreinecker 2015]. Reactivation of telomerase allows cell immortalization and resistance to apoptosis.

TERT promoter (TERTp) mutations are highly prevalent in GBM and other diffuse gliomas, emphasizing their role in tumor maintenance and progression. Clinically, TERTp mutations are associated with reduced overall survival. However, their prognostic impact varies depending on tumor grade and the presence of other molecular alterations. For example, in lower-grade gliomas (WHO Grade II and III), the impact of TERTp mutations varies with co-occurring IDH mutations, MGMT methylation, and 1p/19q codeletion.

This highlights the complexity of molecular interactions that shape clinical outcomes [Kim 2018].

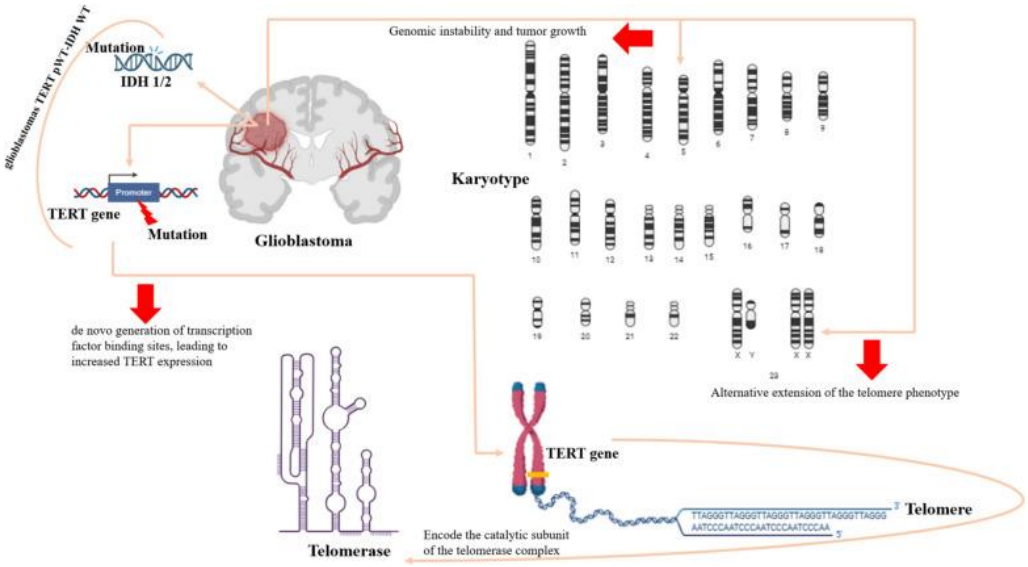


Fig. 7. The Role of TERT and telomere maintenance in glioblastoma progression. Mutations in the *IDH1/2* and *TERT* genes play a significant role in the development of *TERT* promoter wild-type/*IDH* wild-type glioblastoma (GBM), primarily by maintaining telomere length during DNA replication, an essential process for sustained tumor growth. *TERT*, located on chromosome 5, encodes the catalytic subunit of the telomerase enzyme complex, which is crucial for telomere elongation and contributes to genomic instability and tumor progression. TERT promoter (TERTp) mutations are among the most frequent genetic alterations in GBM. These mutations generate novel binding sites for transcription factors, resulting in aberrantly increased *TERT* expression and enhanced telomerase activity, thereby promoting cellular immortality and gliomagenesis. Although *TERTp* mutations are well established in GBM biology, their prognostic significance remains controversial [adapted from Pouyan 2025].

A comprehensive understanding of the regulatory mechanisms underlying epigenetic alterations is essential for addressing the intrinsic therapeutic resistance of GBM and improving clinical outcomes. Mutations in GBM frequently affect multiple molecular pathways simultaneously, underscoring the complexity of this malignancy.

1.3 Tumor microenvironment

The GBM microenvironment is highly complex and dynamic. It is characterised by various interacting components that significantly contribute to tumour formation and invasion. This intricate network includes cancer cells, glioma stem cells (GSCs), immune cells, endothelial cells, and extracellular matrix components, which interact

in ways that promote tumour growth and aggressiveness (Fig. 8) [Prasetyanti and Medema 2017].

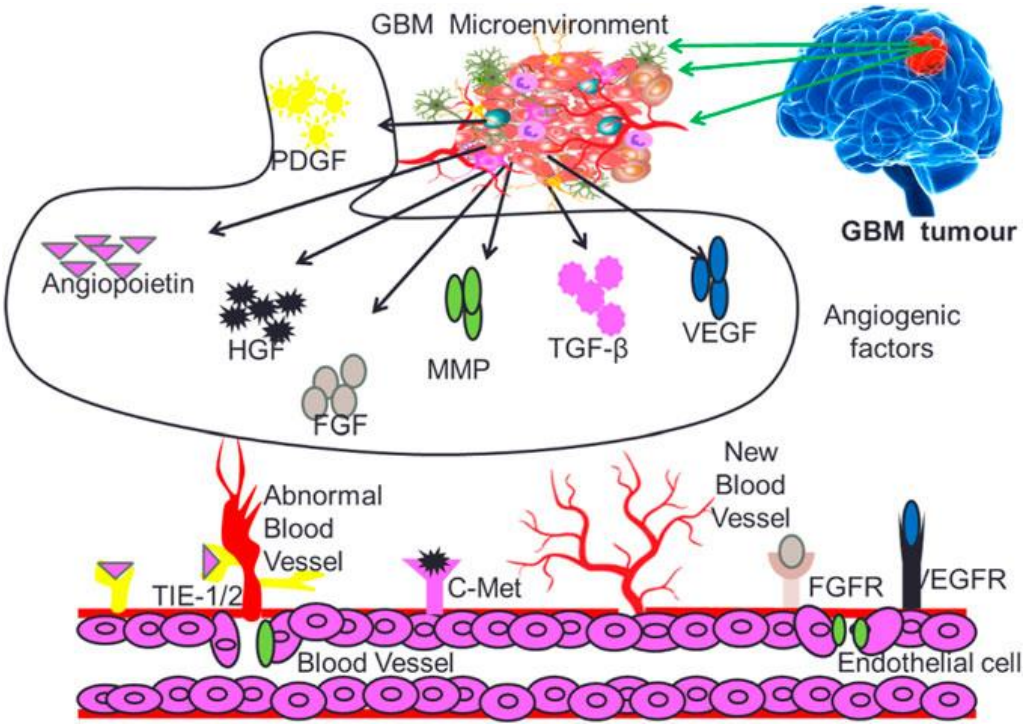


Fig. 8. Key growth factors that support GB tumor proliferation and growth [adapted from Tripathy 2024].

Tumor heterogeneity, a hallmark of GBM, significantly impacts gene expression, modulation of metabolic pathways, and evasion of the immune system, complicating treatment strategies and limiting the effectiveness of standard therapeutic approaches. The treatments fail mainly due to the unique molecular characteristics of GBM (Fig. 9). The presence of a population of stem-like cells, termed glioma stem cells (GSCs), with the ability to self-renew and exhibit tumorigenicity, makes them resistant to chemotherapy and radiotherapy [Bao 2006, Liu 2006]. A core set of neurodevelopmental transcription factors, including POU3F2, SOX2, SALL2, and OLIG2, fully regulates the tumor-propagating properties of these GSCs [Suva 2014].
The invasive potential of GSCs is driven by the upregulation of different signaling pathways, including L1CAM, ephrin-B2, and epithelial–mesenchymal transition (EMT)-associated factors, such as twist-related

protein 1 (TWIST1), SOX2, and signal transducer and activator of transcription 3 (STAT3) [Carro 2010, Krusche 2016]. GSCs influence the tumor microenvironment (TME) by promoting immunosuppressive mechanisms. They facilitate the M2 differentiation of glioma-associated macrophages (GAMs) through the secretion of periostin and the activation of interleukin-10 (IL-10) signaling, thereby contributing to immune evasion [Lin 2023]. Furthermore, pericytes derived from GSCs enhance angiogenesis, which can lead to vascular abnormalities and disruption of the blood-brain barrier (BBB) [Cheng 2013].

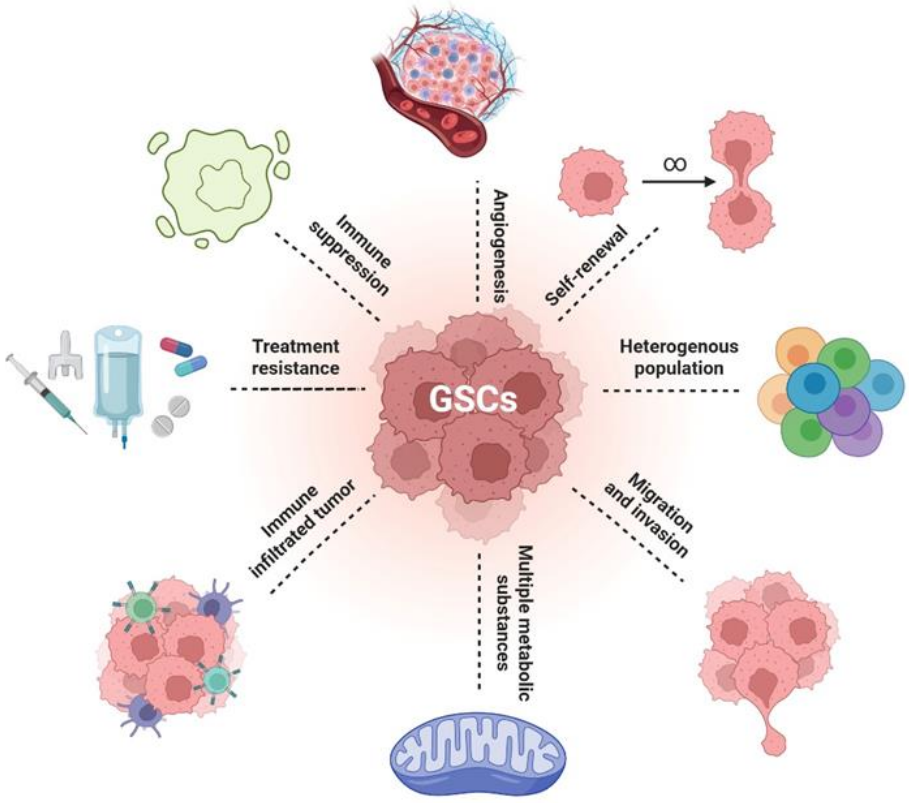


Fig. 9. Key features of glioma stem cells. Glioma stem cells (GSCs) exhibit unique characteristics that distinguish them from non-stem tumor cells. These features play a pivotal role in glioma initiation, progression, and treatment resistance. These hallmarks are critical for their ability to sustain tumor growth and adapt to hostile microenvironments.

The Wnt and Sonic Hedgehog signaling pathways are critical for maintaining GSC self-renewal and resistance to therapy. Aberrant activation of the Wnt pathway, which is influenced by mutations in FAT

atypical cadherin 1 (FAT1), accelerates tumor progression. Conversely, Sonic Hedgehog signaling promotes the expression of Nanog and enhances the activity of drug efflux transporters, leading to increased chemoresistance [Biserova 2021].

Angiogenesis is a critical process in the progression of GBM and is regulated by different growth factors and signaling pathways. Vascular endothelial growth factor (VEGF) is a primary regulator of this process, with its expression increasing in tandem with tumor grade, thereby promoting vascular proliferation and tumor progression [Hatva 1995]. The activation of VEGFR-1 and VEGFR-2 has distinct roles in the initiation and malignancy of GBM [Plate and Risau 1995]. Notably, the overexpression of VEGF and VEGFR-1 in low-grade astrocytomas is associated with a poor prognosis, suggesting their potential as prognostic biomarkers [Yao 2001].

The fibroblast growth factor receptor (FGFR) signaling, mediated by FGF ligands, supports endothelial cell migration, proliferation, and angiogenesis via the PI3K/AKT/mTOR pathway [Batchelor 2014]. FGF-2 plays a significant role in extracellular matrix (ECM) remodeling and works synergistically with VEGF and platelet-derived growth factor (PDGF) to enhance neovascularization, underlining the collaborative role of these factors in tumor vascularization [Kanda 1996].

Angiopoietins such as Ang-1, Ang-2, and Ang-4 further contribute to GBM vascularization. Ang-2, in particular, destabilizes blood vessels and stimulates neovascularization, while Ang-4 enhances tumor angiogenesis [Brunckhorst 2010]. Ang-1 plays a crucial role in promoting endothelial cell survival, proliferation, migration, and tube formation by activating the Tie2 receptor tyrosine kinase, which stabilizes existing blood vessels [Scholz 2016].

Transforming growth factor-beta (TGF-β) exerts context-dependent effects on angiogenesis, promoting the expression of VEGF, FGF, and PDGF, while also inducing EMT in GBM-derived endothelial cells [Bruna 2007]. Matrix metalloproteinases (MMPs), particularly MMP-9, play a role in degrading the endothelial basement membrane, promoting angiogenic switching, and contributing to tumor vascularization through the release of VEGF (Fig. 10) [Bergers 2000].

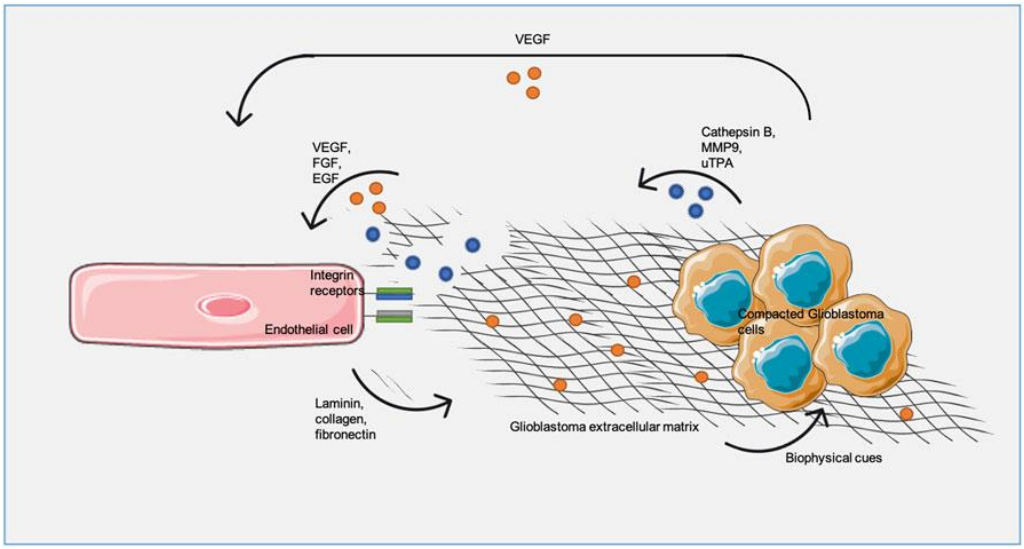


Fig. 10. Communication between glioblastoma extracellular matrix (ECM) and endothelial cells (ECs) drives matrix remodeling and angiogenesis. ECs produce laminin, collagen, and fibronectin, which increase ECM density and stimulate tumor cells to produce and release pro-angiogenic factors like vascular endothelial growth factor (VEGF). Additionally, tumor-derived ECM is degraded by enzymes such as cathepsin B, metalloproteinase 9 (MMP9), and urokinase-type plasminogen activator (uPA), releasing anchored pro-angiogenic factors—including VEGF, fibroblast growth factor (FGF), and epidermal growth factor (EGF)—that promote vascular proliferation (adapted from Pacheco et al., 2022).

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a heparin-binding cytokine derived from mesenchymal tissues, which is associated with the GBM angiogenic processes [Abounader and Laterra 2005]. Recent studies have established the significant role of HGF/SF in promoting tumor angiogenesis by enhancing endothelial cell proliferation, migration, and survival [Abounader and Laterra 2005].

1.4 Long non-coding RNAs

The ENCODE (Encyclopedia of DNA Elements) project has profoundly reshaped our understanding of the human genome, particularly concerning what was once referred to as "junk DNA." Traditionally, non-coding regions of the DNA were considered non-functional, leading to the dismissal of their significance. However, these non-coding regions, which constitute a significant portion of the genome, play critical roles in regulating gene expression and maintaining

cellular function. Multiple functional elements have been identified within these non-coding regions, including enhancers, promoters, and regulatory RNAs, highlighting their importance in gene regulation and the overall complexity of the genome [Pennisi 2012].
Long non-coding RNAs (lncRNAs) are increasingly recognized as critical regulators of various aspects of cell differentiation and development, particularly in the brain [Mercer 2008, Mercer 2010]. Defined as RNA molecules greater than 200 nucleotides in length, lncRNAs are involved in diverse biological networks [Mattick 2023, Mercer 2009, Quinn and Chang 2016, Schmitt and Chang 2016, Zhang 2017]. They play essential roles in regulating chromosomal architecture and dynamics, transcription processes, post-transcriptional modifications, RNA editing, as well as cell growth, differentiation, death, motility, and invasion. Interestingly, a large portion of tissue-specific lncRNAs is expressed in the CNS, with precisely regulated temporal and spatial expression patterns [He 2019, Slack and Chinnaiyan 2019, Zhang and Guo 2019].
The various functions of lncRNAs underscore the complexity of gene regulation and their potential implications for understanding health and disease, particularly in the context of brain function and neurodevelopment.

1.5 Function of lncRNAs

LncRNAs can be classified into distinct groups according to their structure, location, and function.

Guides. LncRNAs can function as molecular guides by directing ribonucleoprotein (RNP) complexes to specific genomic loci. This targeting occurs through the direct interaction between lncRNAs and protein components of the RNPs, allowing the precise localization of these complexes to particular regions of the genome. By guiding RNPs to these sites, lncRNAs promote the modulation of gene expression, either by activating or repressing transcription depending on the nature of the recruited regulatory complexes (Fig.11 A). These complexes encompass a diverse array of factors, including histone modifiers, chromatin remodelers, and transcription factors, which collectively influence chromatin state and transcriptional output [Bonasio 2010, Hung and Chang 2010, Mercer and Mattick 2013].

Scaffold. In addition to their guiding role, lncRNAs can act as scaffolds, serving as organizational hubs that promote the interaction of multiple protein effectors to form functional regulatory assemblies. This scaffolding function relies on the presence of specific structural domains within the lncRNA molecule which allow for the simultaneous binding of various proteins, including both transcriptional activators and repressors. Through these interactions, lncRNAs coordinate the assembly of multiprotein complexes in a highly regulated temporal and spatial manner, which is critical for regulating gene expression programs (Fig.11 A). By facilitating the formation of such complexes, lncRNAs contribute to diverse biological processes, including chromatin remodeling, transcription initiation, and elongation, as well as post-transcriptional regulation [Mercer and Mattick 2013, Spitale 2011].

Signaling. LncRNAs function as dynamic molecular signals that play a crucial role in the initiation and modulation of transcriptional programs. Their expression is both spatially and temporally regulated, exhibiting distinct patterns across different tissues, developmental stages, and physiological conditions. This specificity allows lncRNAs to act as finely tuned regulators of gene expression in response to diverse cellular signals. Upon exposure to various stimuli—such as stress, differentiation cues, or signaling molecules—lncRNAs can be rapidly transcribed and localized to specific subcellular compartments, including the nucleus, cytoplasm, or chromatin-associated regions (Fig.11 B). The tightly regulated transcriptional timing and subcellular distribution enable them to interface with key molecular pathways, including those involving chromatin modifiers, transcription factors, and RNA-binding proteins. Therefore, lncRNAs operate not only as downstream effectors of signaling cascades but also as integrators that coordinate complex transcriptional responses, ensuring that gene expression is contextually appropriate and precisely controlled [Guttman 2009].

Decoys. LncRNAs can regulate gene expression by acting as molecular decoys or "sponges" that bind and sequester RNA-binding proteins, including transcription factors and chromatin-associated regulators (Fig.11 B). Guenther demonstrated that lncRNAs associate with components of the transcriptional machinery, effectively competing with DNA or other RNA targets for binding. As a result, lncRNAs modulate the availability of these regulatory proteins, thereby positively or negatively influencing transcriptional activity. This decoy

mechanism enables lncRNAs to fine-tune gene expression programs by regulating the access of key factors to their target sites on chromatin, thereby introducing additional levels of regulation beyond direct DNA binding or protein modification [Guenther 2007].

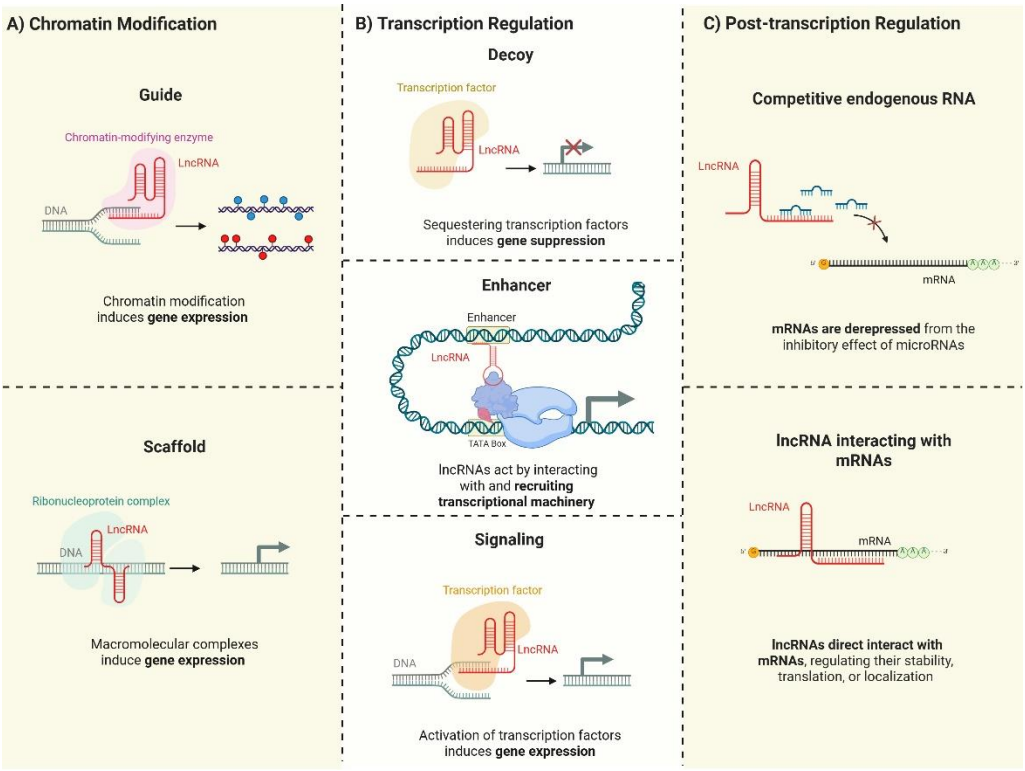


Fig. 11. Epigenetic and transcriptional functions of lncRNAs. (A) Long non-coding RNAs (lncRNAs) can regulate gene expression by modifying chromatin structure. *Guide lncRNAs* interact with chromatin-modifying enzymes and direct them to specific genomic loci, altering local chromatin states to either activate or repress transcription. *Scaffold lncRNAs* serve as platforms that facilitate the assembly of ribonucleoprotein (RNP) complexes through their interactions with multiple proteins. (B) LncRNAs also modulate gene expression at the transcriptional level. *Decoy lncRNAs* inhibit transcription by sequestering transcription factors (TFs), preventing them from binding to their DNA targets. In contrast, *enhancer lncRNAs* and *signal lncRNAs* can promote transcription. Enhancer lncRNAs act by interacting with and recruiting transcriptional machinery, while signal lncRNAs serve as indicators of specific signaling events and can act as transcription factor co-activators. (C) Post-transcriptionally, lncRNAs can influence gene expression through various mechanisms. One key role is as *competing endogenous RNAs (ceRNAs)*—they act as molecular sponges for microRNAs (miRNAs), binding them and preventing their interaction with target mRNAs, thereby relieving repression. Additionally, some

lncRNAs can directly bind to mRNAs to modulate their stability, translation, or localization.

Enhancer. Enhancers are critical regulatory DNA elements that define cellular identity by controlling the precise spatiotemporal patterns of gene expression during development and differentiation. The human genome harbors up to 400,000 enhancers, many of which cluster into regions known as "super-enhancers" (Fig.11 B). These super-enhancers are particularly important in driving high levels of expression of genes that govern cell identity and function [Arnold 2020].

A distinctive feature of active enhancers is their ability to transcribe non-coding RNAs (ncRNAs), often referred to as enhancer RNAs (eRNAs). These enhancer-derived ncRNAs are typically produced exclusively in cells where the enhancer is active, indicating a tightly regulated expression pattern. The ncRNAs transcribed from enhancers are believed to play pivotal roles in the function of these enhancers. They promote chromatin looping, a process that brings enhancers into close spatial proximity with their target gene promoters, thereby enhancing transcriptional activation. Furthermore, these ncRNAs contribute to the establishment and maintenance of topologically associating domains (TADs), which are 3D chromatin structures that ensure proper gene regulation [Arnold 2019, Ntini and Marsico 2019].

Competitive endogenous RNA. LncRNAs also function as competitive endogenous RNAs (ceRNAs), acting as natural sponges for microRNAs (miRNAs). Through complementary base pairing, lncRNAs bind to specific miRNAs, effectively sequestering them and preventing their interaction with mRNA targets (Fig.11 C). The sequestration reduces miRNA-mediated repression, leading to the de-repression or increased expression of the miRNA target genes. This ceRNA activity enables lncRNAs to regulate gene expression post-transcriptionally, playing significant roles in various biological processes and diseases [Tay 2014]. Together, these functions highlight the multifaceted roles of lncRNAs and enhancer-derived ncRNAs in fine-tuning gene expression through both transcriptional and post-transcriptional mechanisms, thereby influencing cellular identity, development, and disease.

1.6 Brain tumors and lncRNA

As discussed above, brain tumors represent one of the most aggressive and challenging forms of cancer, frequently evading standard diagnostic and therapeutic approaches due to the complex and poorly characterized nature of neural tissues. This clinical and biological heterogeneity underscores the urgent need for novel molecular biomarkers to improve early detection, prognostic accuracy, and treatment efficacy. In this context, non-coding RNAs (ncRNAs), and, in particular, lncRNAs, have obtained considerable attention for their regulatory roles in tumorigenesis. Emerging evidence highlights the involvement of lncRNAs in key oncogenic processes, including cell proliferation, invasion, and metastasis, positioning them as potential molecular targets and diagnostic indicators in brain tumor management [Latowska 2020].

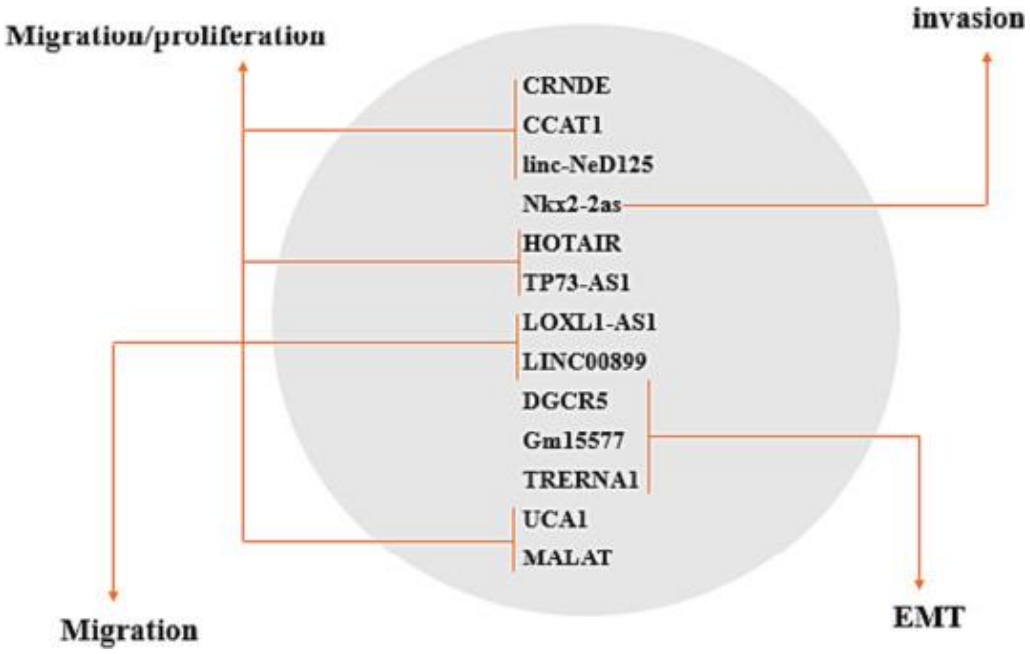


Fig. 12. LncRNAs correlate with brain tumors. (adapted from Pouyan A et al., 2024)

LncRNAs have emerged as important prognostic biomarkers in GBM, providing insights into tumor progression, survival prediction, and resistance to therapy. As outlined in Table 2, several lncRNAs have been identified as biomarkers associated with GBM [Amer 2022, Ho

2022, Shen 2018, Sun 2021, Tan 2018, Wang 2022, Xie 2019]. Numerous studies have demonstrated strong correlations between specific lncRNAs and clinical parameters, including tumor grade, patient survival, and treatment response, thereby underscoring their clinical relevance [Mei 2020, Yang 2023].

Table 2. Long non-coding RNAs as prognostic biomarkers in GBM

Long non-coding RNA	Source	Status	Expression	References
ANRIL	Serum	Upregulated	Tumor grade and prognosis	Sun *et al.*, 2021
GAS5	Serum	Upregulated	Prognosis	Shen *et al.*, 2018
HOTAIR	Serum	Upregulated	Prognosis	Tan *et al.*, 2018; Wang *et al.*, 2022
linC00565	Serum	Upregulated	Prognosis	Amer *et al.*, 2022
linC00641	Serum	Upregulated	Prognosis	Amer et al., 2022
MIR210HG	Serum	Upregulated	Diagnosis	Ho *et al.*, 2022
SAMMSON	Plasma	Upregulated	Diagnosis	Xie *et al.*, 2019

The table presents a summary of several long non-coding RNAs (lncRNAs) identified as upregulated in serum or plasma samples and associated with cancer prognosis or diagnosis. These lncRNAs are implicated in several clinical outcomes, including tumor grade, prognosis, and diagnosis, highlighting their potential utility as biomarkers [modified from Singh 2025].

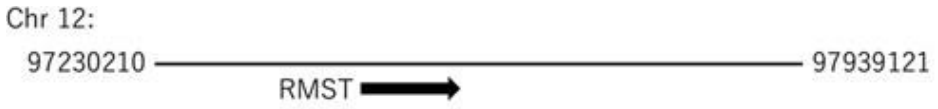
1.7 Long non-coding RNA rhabdomyosarcoma 2-Associated Transcript

LncRNAs have emerged as critical regulatory molecules involved in multiple biological networks, including development, differentiation, and disease. Among these, the Rhabdomyosarcoma 2-Associated Transcript (RMST) is a crucial regulator in various biological processes, particularly in neurogenesis and cancer biology [Cai 2024, Chen 2023, Ng 2012].

RMST was originally identified in the context of alveolar rhabdomyosarcoma due to its genomic proximity to chromosomal rearrangements associated with this malignancy [Chan 2002]. However, subsequent studies have demonstrated that RMST is a brain-specific lncRNA, significantly upregulated during neuronal differentiation of human embryonic stem cells (hESCs). Functional analyses demonstrated that depletion of RMST significantly impairs neuronal lineage commitment, resulting in a shift in cell differentiation toward a glial fate. These findings were among the first to propose a direct regulatory role for RMST in neural development, establishing it as a pivotal factor in determining cell fate specification within the CNS [Ng 2012].

RMST is a multi-exon transcript that gives rise to different alternatively spliced isoforms in human cells [Ng 2012].

In human cells, over ten RMST isoforms have been identified, with transcript lengths ranging from the relatively short RMST-207 (60 nucleotides) to the considerably longer RMST-209 (3,269 nucleotides) located on chromosome 12 (Fig. 13) [Alaqeeli 2021]. RMST exhibits dual intracellular localization, being present in both the nucleus and cytoplasm [Alaqeeli 2021, Chen 2023, Zhang 2022], a characteristic that may contribute to its functional heterogeneity.



Name	Type	Length (nt)	Exon No.	Expression Tissues
RMST-201	lncRNA	2,598	4	brain
RMST-202	lncRNA	2,099	4	brain, thyroid
RMST-203	retained intron	687	10	–
RMST-204	retained intron	755	11	brain
RMST-205	lncRNA	1,047	12	brain, thyroid
RMST-206	retained intron	656	2	muscle
RMST-207	miscellaneous RNA	60	–	–
RMST-208	lncRNA	2,099	–	heart, muscle
RMST-209	lncRNA	3,269	4	brain
RMST-210	lncRNA	2,844	–	–

Fig. 13. Characterization of RMST isoforms and tissues expression. The figure illustrates the genomic location and transcript variants of the RMST (Rhabdomyosarcoma 2 Associated Transcript) gene located on chromosome 12. The arrow indicates the transcriptional direction of the RMST gene.

The table provides detailed information about different RMST transcript isoforms, including their names, RNA types, lengths in nucleotides (nt), exon counts, and tissue expression patterns [Adapted from Tani 2025].

Notably, in triple-negative breast cancer cells, RMST is predominantly cytoplasmic [Alaqeeli 2021, Yakushina 2021], whereas in vascular endothelial cells, all detectable isoforms are exclusively nuclear [Arakawa 2023]. Importantly, the RMST orthologs are highly conserved across vertebrate species, including frogs, particularly at promoter regions, first exons, and canonical splice sites [Chodroff 2010].
This level of conservation is uncommon among lncRNAs, suggesting that RMST serves some evolutionarily preserved function in vertebrate neurodevelopment.
The transcriptional regulation of RMST further underscores its distinct role in the CNS. This lncRNA is positively regulated by PAX2, a transcription factor required for the formation of the midbrain–hindbrain boundary during early neural patterning [Bouchard 2005]. Additionally, binding motifs for REST (RE1-silencing transcription factor) have been identified upstream of the RMST locus based on ENCODE data, suggesting a role for REST in repressing RMST expression outside of neural contexts. REST, also known as neuro-activating repressor silencing factor (NRSF), functions as a master repressor of neuronal gene expression in non-neuronal tissues and is known to dissociate from RE1 sites to facilitate neuronal gene activation during differentiation [Abrajano 2009, Ballas 2005, Gao 2011].
Mechanistically, it has been reported that RMST could operate through direct interaction with the transcription factor SOX2, a core regulator of neural stem cell identity and fate determination (Fig.14). This interaction promotes the recruitment of SOX2 to target promoters of neurogenic genes, thereby enhancing their transcription and promoting the commitment of progenitor cells to the neuronal lineage [Ng 2012]. Studies have further substantiated RMST's role, demonstrating that its overexpression increases the expression of neuronal markers such as TUJ1 and the proportion of differentiated neurons *in vitro* [Ng 2013].

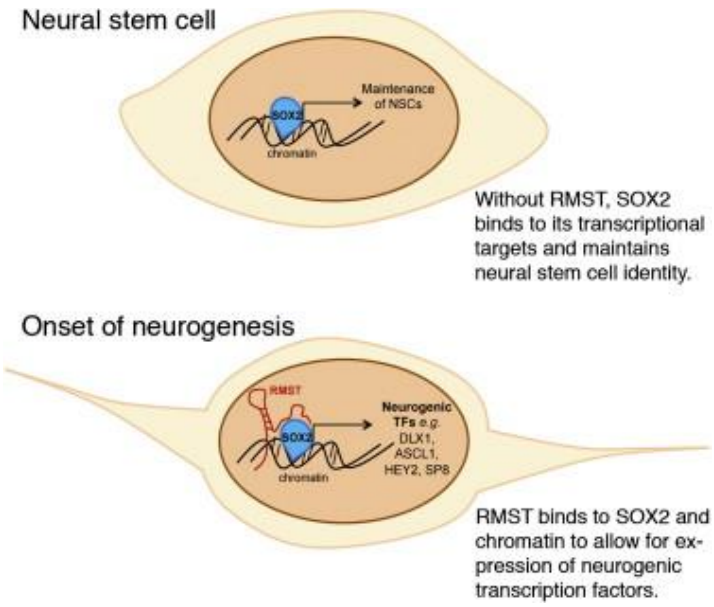


Fig. 14. RMST modulates neurogenesis through its direct interaction with the transcription factor SOX2. [from Ng 2013]

RMST has also been demonstrated to cooperate with SOX2 in the regulation of miR-1251 expression, thereby modulating the progression of Hirschsprung's disease [Zhou 2022]. Moreover, RMST acts as a positive regulator of DNA methyltransferase 3 (DNMT3) in cancer by enhancing the stability of DNMT3 mRNA [Guo 2022]. RMST further participates in the regulation of cellular processes through interactions with diverse proteins and RNA molecules. For example, it promotes microglial cell activation by promoting transforming growth factor-beta-activated kinase 1 (TAK1)-mediated nuclear factor kappa B subunit 1 (NF-κB) signaling via competitive, binding to heterogeneous nuclear ribonucleoprotein K (hnRNPK) [Alaqeeli 2021, Ma 2023].

1.8 RMST regulatory role as a competing endogenous RNA

RMST functions as a competing endogenous RNA (ceRNA) for multiple microRNAs (miRNAs), playing a pivotal role in the regulation of gene expression [Izuogu 2018, Kaur 2018, Sun 2019]. This regulatory mechanism involves RMST binding to and sequestering specific miRNAs, thereby inhibiting their interaction with target mRNAs (Fig.

15)[Ma 2023]. A significant example is the interaction between RMST and miR-24-3p, which is implicated in the regulation of cardiac fibrosis following myocardial infarction [Ma 2023]. Additionally, RMST indirectly regulates the expression of vascular cell adhesion molecule 1 in brain vascular endothelial cells and semaphorin 3A in neurons by sponging miR-204-5p and miR-377, respectively [Arakawa 2023, Cai 2024]. In the context of Hirschsprung's disease, RMST acts as a transcriptional co-regulator with SOX2 to modulate miR-1251 expression [Zhou 2022]. RMST has also been reported to regulate miR-5692 in myocardial infarction [Zhang 2021] and to be associated with tumor progression and prognosis in gastric cancer through targeting miR-204-5p [Cai 2024]. Moreover, RMST contains miRNA recognition elements for several other miRNAs, including miR-27a, miR-27b, miR-33a, miR-132-3p, miR-139-5p, and miR-212-3p [Alaqeeli 2021].

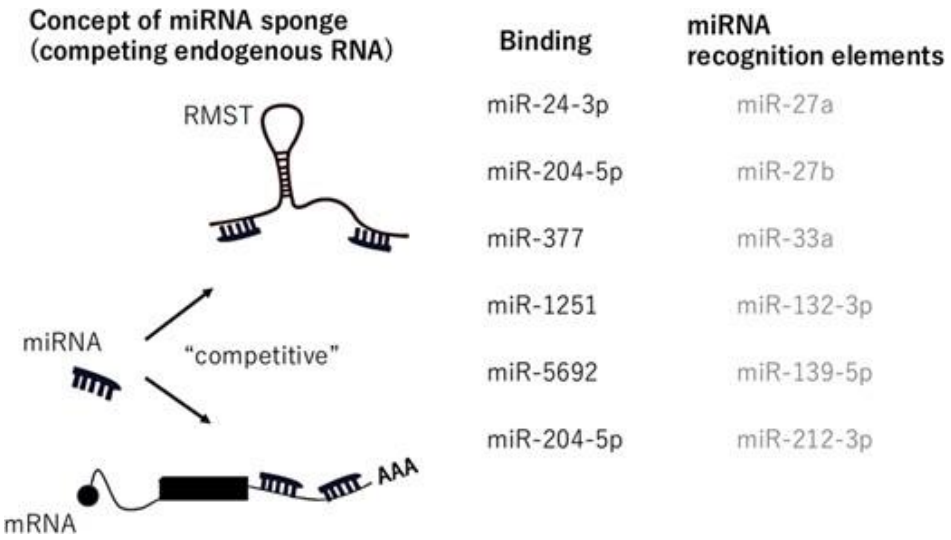


Fig. 15. RMST interactions with microRNAs as competing endogenous RNAs. [Adapted from Tani 2025]

Given that these miRNAs negatively regulate cellular processes such as survival, proliferation, and migration, RMST may promote endothelial cell viability, proliferation, and migration by sponging these miRNAs. Furthermore, RMST has been implicated in antagonizing the Wnt signaling pathway during colorectal cancer progression by regulating the miR-27a-3p/retinoid X receptor alpha axis [Chen 2023].

Collectively, these diverse interactions underscore the critical role of RMST as a ceRNA, influencing multiple biological processes and pathological conditions through miRNA-mediated gene regulation.

1.9 The role of RMST in disease progression

RMST is implicated in the development and progression of neurological disorders, cardiovascular diseases, and Hirschsprung's disease.

Neurological disorders. RMST plays a critical role in neurogenesis, particularly the deficiency of this lncRNA in neural stem cells results in their differentiation into glial cells, highlighting its essential function in proper brain development and neuronal lineage specification [Ng 2013]. Elevated RMST expression has been reported in the central nervous system in neurological disorders such as stroke and Parkinson's disease [Ma 2021, Xu and Zhang 2022]. Notably, suppression of RMST expression has been proposed to inhibit neuronal apoptosis and enhance neurological outcomes. Furthermore, RMST is implicated in neuropathic pain, where its upregulation contributes to pain modulation by stabilizing DNA methyltransferase 3α (DNMT3α) mRNA [Guo 2022].

Cardiovascular diseases. RMST plays a significant role in the development of cardiac fibrosis following myocardial infarction [Ma 2023]. Cardiac fibrosis, characterized by the activation of cardiac fibroblasts and excessive extracellular matrix deposition, is a common pathological consequence of myocardial infarction. Studies based on mouse and pig models of myocardial infarction have demonstrated an upregulation of RMST expression correlated with the progression of cardiac fibrosis. Notably, both *in vitro* and *in vivo* knockdown of RMST has been shown to attenuate cardiac fibrosis and improve cardiac function [Ma 2023].

Hirschsprung's disease. RMST is critically involved in the pathogenesis of Hirschsprung's disease [Zhou 2022], a congenital disorder marked by impaired migration and proliferation of enteric neural crest cells. In this context, downregulation of RMST disrupts its cooperative interaction with SOX2 in the regulation of miR-1251. Reduced RMST expression diminishes SOX2-mediated repression of miR-1251, resulting in elevated levels of this miRNA. Consequently,

increased miR-1251 suppresses AHNAK expression, thereby inhibiting the development and migration of enteric ganglion cells. This molecular pathway involving RMST, SOX2, miR-1251, and AHNAK supports the pathogenesis of Hirschsprung's disease, underscoring the essential role of RMST in maintaining normal enteric nervous system development [Zhou 2022].

1.10 RMST and cancer

RMST acts as a cancer-related lncRNA, exhibiting context-dependent functions across distinct cancer types (Fig. 16).

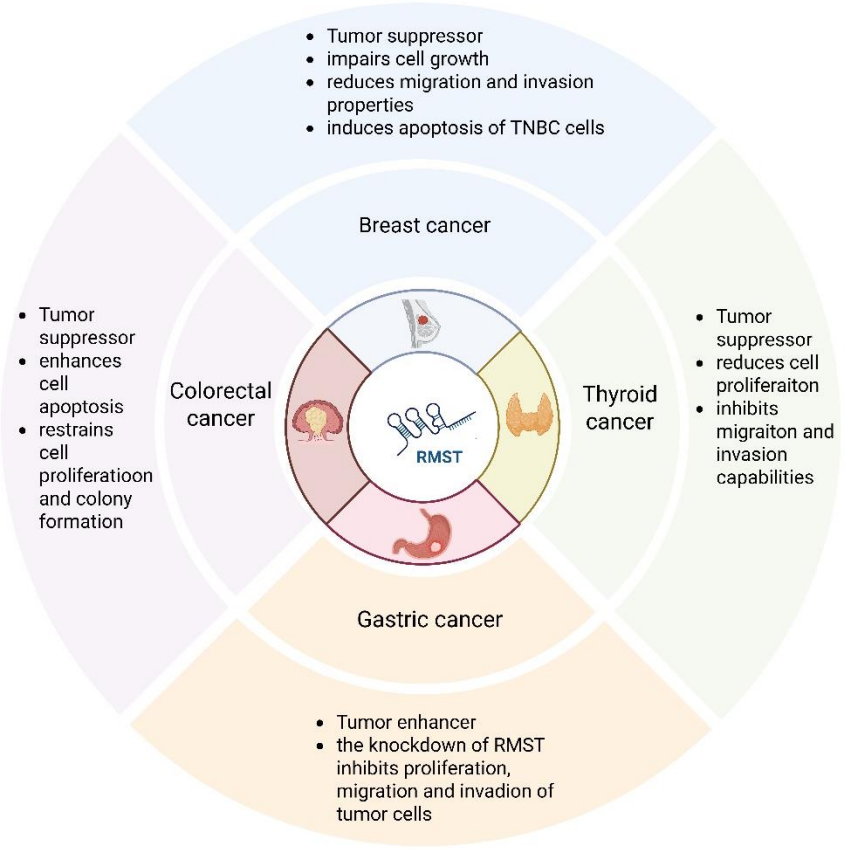


Fig. 16 The role of RMST in human cancer. In oncological contexts, RMST primarily acts as a tumour suppressor. However, its function varies depending on the specific cancer type.

Its expression is frequently reduced in several tumors, including triple-negative breast cancer (TNBC) and anaplastic thyroid carcinoma (ATC). In ATC, a highly aggressive and poorly differentiated thyroid malignancy, RMST re-expression significantly inhibits tumorigenic properties. It has been demonstrated that RMST suppresses cell proliferation, migration, invasion, and epithelial–mesenchymal transition (EMT), and reduces cancer cell stemness—each of which contributes to disease progression and therapeutic resistance [De Martino 2023].

In triple-negative breast cancer (TNBC), RMST expression is significantly downregulated, suggesting a tumor suppressive role [Alaqeeli 2021, Zhang 2025]. RMST is proposed to exert anti-tumor effects by inhibiting the proliferation, migration, and invasion of TNBC cells. Experimental overexpression of RMST has been demonstrated to suppress malignant phenotype while promoting apoptosis. Predominantly localized in the cytoplasm, RMST is believed to modulate cellular behavior through the regulation of mRNA and protein expression. Collectively, these findings indicate that RMST serves as a critical tumor suppressor in TNBC, with its downregulation potentially contributing to disease progression.

These anti-tumor effects are likely mediated through RMST's regulatory influence on transcriptional and post-transcriptional pathways, although the precise molecular mechanisms remain to be fully elucidated. Given its selective expression and functional specificity, RMST presents as a potential biomarker and therapeutic target in both developmental disorders and malignancies characterized by impaired differentiation and uncontrolled proliferation.

Circular form (circRMST) of RMST is highly expressed and evolutionarily conserved and plays a pivotal role in enabling adenocarcinomas (prostate and lung) to transdifferentiate into aggressive neuroendocrine tumors (NEPC and SCLC). It exerts this effect by physically binding to and stabilizing transcription factors NKX2-1 and SOX2—thus sustaining ASCL1 expression. Loss of circRMST disrupts this regulatory network, causing NKX2-1 degradation, altering SOX2 binding patterns, reducing ASCL1 levels, and ultimately impeding neuroendocrine transdifferentiation [Teng 2025].

2 AIM

Glioblastoma (GBM) is an aggressive and treatment-resistant primary brain tumor characterized by high heterogeneity, invasive potential, and poor prognosis. Emerging evidence highlights the involvement of long non-coding RNAs (lncRNAs) in tumor progression, therapeutic resistance, and the maintenance of stem-like features in GBM. Among these, the lncRNA Rhabdomyosarcoma 2-Associated Transcript (RMST) has been previously implicated in neural development and transcriptional regulation through its interaction with key factors such as SOX2. However, the functional role and clinical significance of RMST in glioblastoma remain largely unexplored. To this purpose, I first examined whether RMST expression was altered in distinct glioma subtypes of different histological grades and assessed its prognostic value by correlating expression levels with patient survival. I combined *in silico* analyses of publicly available datasets with experimental validation in GBM cell lines to determine whether RMST downregulation was a consistent feature of high-grade gliomas. Subsequently, I investigated the functional impact of RMST overexpression in GBM cells characterized by low endogenous RMST levels (LN18 and LN229). These experiments aimed to evaluate how RMST influenced key cancer-related phenotypes, including cell proliferation, clonogenic potential, apoptosis, and sensitivity to temozolomide (TMZ), the standard chemotherapeutic agent used in GBM treatment. Given the importance of cellular plasticity and EMT in GBM invasiveness and recurrence, I examined the effects of RMST on migration, invasion, and EMT-related transcription factors. Although the underlying mechanisms remain unclear, RMST overexpression consistently reduced several ECM- and EMT-associated markers—including collagens, SNAIL, SLUG, and N-cadherin—while increasing epithelial markers, indicating its involvement in EMT regulation. Interestingly, RNA-seq analysis further revealed broad RMST-dependent changes in metabolic gene networks, providing insight into RMST's contribution to metabolic reprogramming in GBM cells. Through these investigations, the study aimed to define the tumor-suppressive potential of RMST in GBM, as well as its relevance as a prognostic indicator and modulator of tumor cell behavior. This work provides a framework for future research aimed at elucidating the molecular mechanisms of RMST's action.

3 MATERIALS AND METHODS

3.1 Cell culture and Transfection

The human glioblastoma cell lines A172, U251, T98G, U87, DBRTG, LN229 and LN18 (ATCC, LGC Standards, UK) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, USA), 2 mM glutamine (Gibco, USA) and 25 µg of streptomycin/ml (Gibco, USA). Human immortalized glial cell line SVGp12 were obtained from ATCC and cultured in EMEM (Eagle's Minimum Essential Medium) (Gibco, USA) with the addition of 10% FBS (Gibco, USA), 2 mM glutamine (Gibco, USA) and 25 µg of streptomycin/ml (Gibco, USA). All cell lines were maintained at 37 °C in a humidified atmosphere with 95% air and 5% CO2.

LN229 and LN18 cells were stably transfected with pLenti-GIII-CMV-RMST (encoding RMST cDNA) or an empty vector control using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Post-transfection, cells were selected with puromycin at 0.75 µg/mL for LN18 and 1 µg/mL for LN229. RMST expression was verified by RT-PCR.

3.2 Growth curve assay

For each experimental point 3×104 cells were plated in a 60 mm plate. Cells were cultured under standard conditions, and at the indicated time points, cells were harvested and counted to assess proliferation. Cell counts were performed in triplicate using a Bürker hemocytometer chamber under a light microscope. Viable cells were distinguished by trypan blue exclusion and only viable cells were included in the analysis.

3.3 Migration assay

Migration assay was performed in Transwell chambers with 8-µm pores (Corning, USA). Cells were seeded in 100 µl serum-free medium (50× 10^4 for LN229, 100 × 10^4 for LN18) on the upper insert. The lower

chamber contained 600 µl complete medium as a chemoattractant. After incubation (48 hours for LN18, 72 hours for LN229), migrated cells on the lower membrane surface were stained with 0.05% crystal violet in 20% methanol. Images were captured at 20× magnification.

3.4 Invasion assay

Cell invasion was assessed using 24-well Transwell chambers with Matrigel-coated membranes. Matrigel was diluted (1:2 for LN18, 1:4 for LN229), applied at 100 µl per filter, and polymerized at 37°C for 60 minutes. Cells were resuspended in serum-free medium (50×10^4/ml for LN18, 100×10^4/ml for LN229) and 100 µl was added to each upper chamber. After 72 hours at 37°C and 5% CO2, filters were fixed and stained. Non-migrated cells were removed with cotton swabs. Experiments were performed in triplicate. Migrated cells were imaged at 20× magnification in five random fields per filter.

3.5 Wound healing assay

Glioblastoma cells were seeded in 100-mm plates and cultured to full confluence. Linear wounds were generated in the monolayer with a 200 µL pipette tip. After wounding, cells were washed to remove debris and incubated in serum-free DMEM. Migration was monitored for up to 96 hours at 37 °C. Phase-contrast microscopy captured images at defined time points. Wound closure was analyzed using Wound Healing Size Tool, an ImageJ/Fiji® plugin (NIH, USA) that allows the quantification of wound area in images obtained from a wound-healing assay.

3.6 RNA extraction and real-time PCR

Total RNA was extracted with Trizol reagent (Life Technologies, USA). RNA concentration was determined using a Nanodrop spectrophotometer. cDNA was synthesized from 1 µg total RNA using the M-MLV Reverse Transcriptase (Invitrogen, USA). Real-time PCR was performed with Sybr Green (Bio-Rad, USA), 200 nM primers, and 20 ng cDNA on a CFX96 thermocycler (Bio-Rad, USA). The following primers have been used:

hRMST-Fw 5′-GCCCAGCCCATTTCATTCAC-3′

hRMST-Rv 5′-GCTCTGTCGTTCCACCTTGA-3′

hCOL1A2-Fw 5′-AGAGTGGAGCAGTGGTTACTA -3′

hCOL1A2-Rv 5′- GATACAGGTTTCGCCAGTAGAG-3′

hCOL3A1-Fw 5′-GCTCTGCTTCATCCCACTATTA-3′

hCOL3A1-Rv 5′-CTGGCTTCCAGACATCTCTATC-3′

hCOL4A2-Fw 5′- CTGGTCGACCACCAT-3′

hCOL4A2-Rv 5′-CATGCACACCTGGCA -3′

hGAPDH-Fw 5′-AGCCACATCGCTCAGACAC-3′

hGAPDH-Rv 5′-GCCCAATACGACCAAATCC-3′

GAPDH was used as the normalization control. Relative quantification used cDNA standard curves, and results were expressed as fold change ($2^{-\Delta\Delta Ct}$) [Livak and Schmittgen 2001] and analyzed with Bio-Rad CFX Manager.

3.7 Western Blot

Cells were lysed in lysis buffer containing 1% NP40, 1 mM EDTA, 50 mMTris-HCl (pH 7.5) and 150 mM NaCl, supplemented with complete protease inhibitors mixture (Roche Branford, CT, USA). Total proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham, Rainham, UK) by elettroblotting. Membranes were blocked with 5% non-fat dry milk and incubated with primary antibodies. The primary antibodies used were anti-N-Cadherin (D4R1H) #13116; anti-β-Catenin (D10A8) #8480; anti-Snail (C15D3) #3879; E-Cadherin (24E10) #3195; anti-Slug (C19G7) #9585. All the antibodies were purchased from Cell Signaling (MA, USA).

3.8 PI/Annexin V FITC assay

Apoptosis of LN18 and LN229 cells was assessed using propidium iodide staining (PI)/Annexin V-FITC kit (BD Biosciences). Cells grown in 60-mm dishes were treated with 500 μM temozolomide for 72 hours

(LN229) or 120 hours (LN18). After treatment, cells were harvested, washed with phosphate-buffered saline (PBS) and stained with PI and Annexin V-FITC for 15 minutes in the dark. The apoptosis rate was analyzed by flow cytometry. All experiments were performed in triplicate.

3.9 Colony formation assay

Colony formation assays were performed in 60 mm plates. Cells were trypsinized to obtain a single-cell suspension and seeded at low density. After incubation for 2–3 weeks, resulting colonies were fixed and stained with crystal violet for 30 minutes. Colonies were counted, and plates were photographed.

3.10 RNA-Seq analyses

Genomix4life S.R.L. (Baronissi, Salerno, Italy) performed the next generation sequencing analysis, including samples quality control and Bioinformatics analysis. Following the producer's guidelines, indexed libraries were obtained from 500 ng/ea RNA through TruSeq Stranded total RNA Sample Prep Kit (Illumina, San Diego, CA, USA). The libraries quantification was performed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Qubit fluorometer (Thermofisher, Waltham, MA, USA), then combined in order that every index-tagged sample was in equimolar amounts, with 2 nM pooled samples final concentration. Illumina HiSeq. 2500 System (Illumina, San Diego, CA, USA) sequenced the pooled samples with a format of 2 × 100 paired-end at 8 pmol final concentration. FastQC tool was utilized for the quality control analysis of the generated raw sequence files (.fastq files). Cutadapt was used to eliminate the adapter sequences. Paired-end reads were mapped using STAR [Dobin 2013] on reference genome assembly mm10 acquired from Ensembl [Hubbard 2002]. The quantification of transcripts expressed for each replicate of the sequenced samples was performed using HTSeq-Count algorithm [Anders 2015]. Differential expression analysis was conducted in R using the DESeq2 package [Love 2014]. Genes with an adjusted p-value < 0.05 (Benjamini–Hochberg correction) and an absolute $\log_2$ fold change ≥ 1 were considered significantly regulated. Biological significance of DEGs was explored by GO term enrichment analysis

including biological process (BP), based on Bioconductor packages enrichR (https://cran.r-project.org/package=enrichR), using R version 4.3.1. Heatmaps were generated by GraphPad Prism, volcano plots were generated using ggplot2 in R. Statistical procedures followed standard methods implemented within each bioinformatics package.

3.11 Statistical Analysis

Data were analysed using a two-sided unpaired t test (GraphPad Prism, GraphPad Software, Inc.). Values of $P < 0.05$ were considered statistically significant. Regression analysis, correlation coefficients and statistical analysis were generated using GraphPad Prism, GraphPad Software, Inc.

4 RESULTS

4.1 RMST downregulation predicts better prognosis in glioma

In order to investigate the expression of RMST and its clinical relevance in glioma, a comprehensive analysis was conducted using Rembrandt dataset through GlioVis (https://gliovis.bioinfo.cnio.es/) a web-based tool designed for brain tumor research. Bioinformatic analysis displayed that RMST expression consistently decreases across various histological types of brain tumors, from oligodendroglioma to glioblastoma (GBM), parallel to increasing tumor grade (Fig. 17A and B). These findings indicate an inverse correlation between RMST expression and tumor grade. Accordingly, Kaplan–Meier survival analysis revealed that patients with high RMST expression levels have significantly longer overall survival (OS) than those with low expression in both glioma cohorts (Fig. 17C, p = 0.0122) and in GBM patients (Fig. 17D, p = 0.0722). These data collectively suggest that RMST may serve as a favourable prognostic biomarker in glioma.

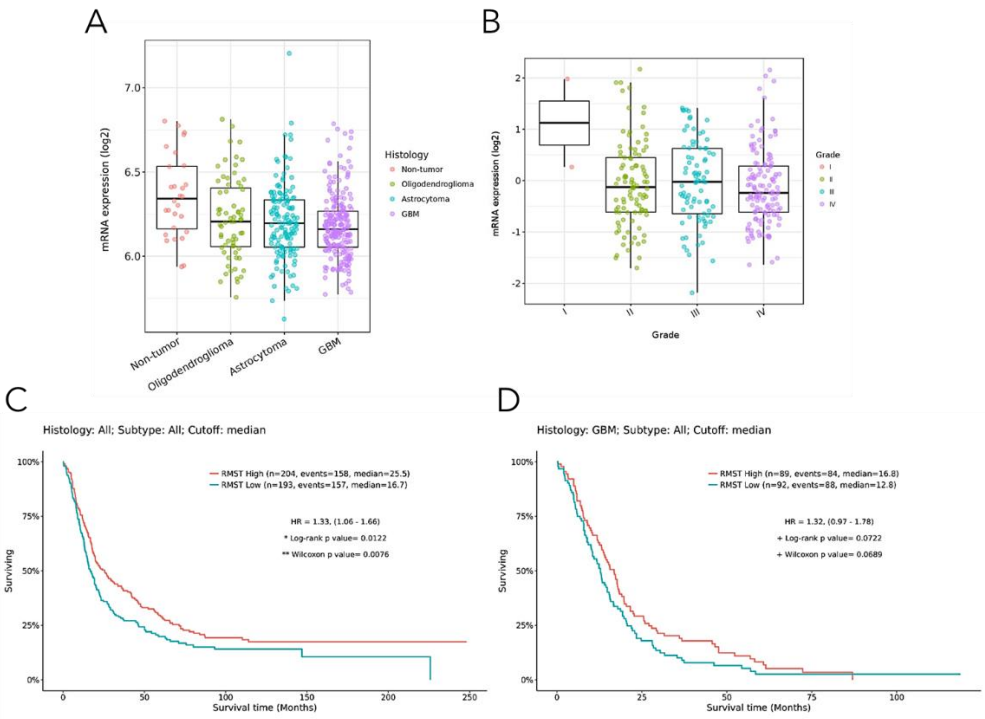


Fig.17. RMST expression is associated with glioma grade and predicts a poor prognosis for patients. (A) RMST mRNA expression (log2 scale) across different histological types of brain tissues, including oligodendroglioma (N=67), astrocytoma (N=147), and glioblastoma (N=219) compared with 28 non-tumoral samples. (B) RMST expression stratified by tumor grade (Grade I (N=2), Grade II (N=98), Grade III (N=85), Grade IV (N=130), showed a progressive decrease with increasing malignancy. The lowest expression was observed in grade IV tumours (GBM). (C) Kaplan-Meier survival curve analysis of 397 samples of brain tumors and 181 GBMs. (D) Stratified for the expression of high (red, median OS = 16.8 months) and low (green median OS = 12.8 months) levels of RMST.

To proceed further, the differentially expressed genes (DEGs) associated with RMST were analyzed using bioinformatic methods. A hierarchical clustering heatmap of genes co-expressed with RMST (Fig. 18A) displays distinct expression patterns between high- and low-RMST groups, highlighting genes potentially related to RMST expression.

The Gene Ontology (GO) analysis of glioma samples in the Rembrandt database indicated that biological processes and molecular functions associated to RMST expression are related to tumor progression, invasion, metastasis and epithelial-mesenchymal transition (EMT), processes that are relevant for tumor aggressiveness and therapeutic response (Fig. 18B and C).

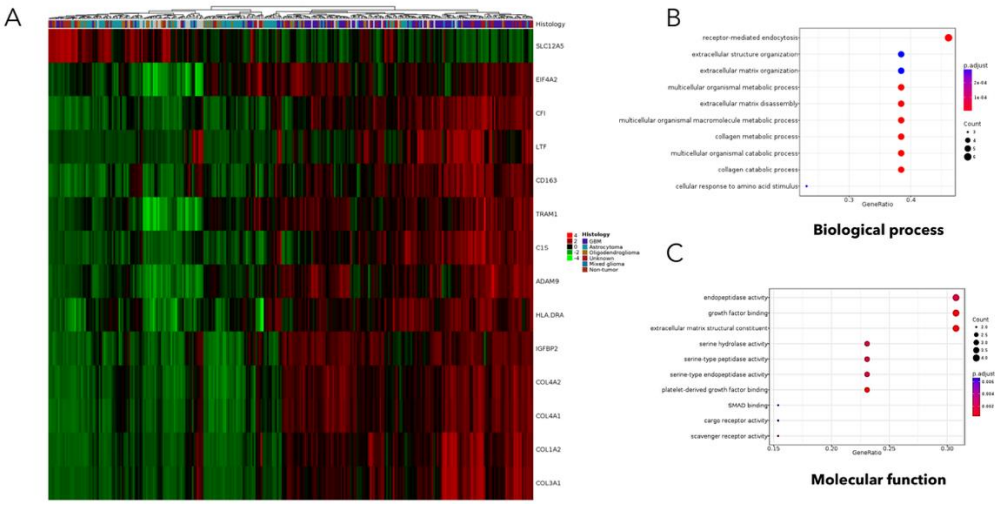


Fig. 18. Gene expression profiling of RMST-associated key differentially expressed genes and enriched biological functions and processes. (A) Cluster analysis showing association between expression of RMST-correlating genes and tumoral histology (GBM, Astrocytoma, Oligodendroglioma, Unknown, Mixed Glioma, Non-tumor) in brain tumoral samples from Rembrandt dataset. Red denotes high gene expression and green, low gene expression. (B) Bubble plot for Gene Ontology (GO) function enrichment analysis for biological processes and (C) molecular function. The y-axis shows pathway terms, whereas the x-axis denotes gene ratio. The dot size represents the number of genes, and the color scale indicates adjusted p-values (p.adjust). Data analyzed in panel A-C are from Rembrandt Dataset.

4.2 RMST overexpression inhibits GBM cell growth

To further explore the role of RMST in GBM, I analyzed its expression in a panel of human GBM cell lines (A172, U251, T98G, LN229, LN18, U87 and DBRTG) alongside human immortalized glial cell line (SVGp12) using qRT-PCR analysis. Notably, as shown in Figure 19A, RMST levels are considerably reduced in GBM cells compared to the SVGp12. This suggests that the downregulation of RMST may be a critical player in GBM pathogenesis. In order to evaluate the involvement of RMST in GBM proliferation, two GBM cell lines, LN18 and LN229, featuring CSC-like characteristicsY, with a strong downregulation of RMST levels compared to the control SVGp12, were selected for the analysis. These two GBM-derived cellular models were transfected with empty vector (pLenti) and pLenti-RMST one. After puromycin selection, the resulting cell populations displayed markedly higher RMST expression levels compared with both their respective parental GBM cell lines (Fig 19B) and the non-tumorigenic control cell line SVGp12. LN18 and LN229 cells overexpressing RMST, in parallel with the appropriate controls, were characterized in relation to their growth rate. As shown in Fig. 19C, GBM cells transfected with pLenti-RMST exhibited a substantial decrease in growth rate when compared with those carrying the corresponding empty vector (pLenti). Accordingly, RMST overexpression significantly also restrained colony formation in both GBM cell lines (Fig. 19D).

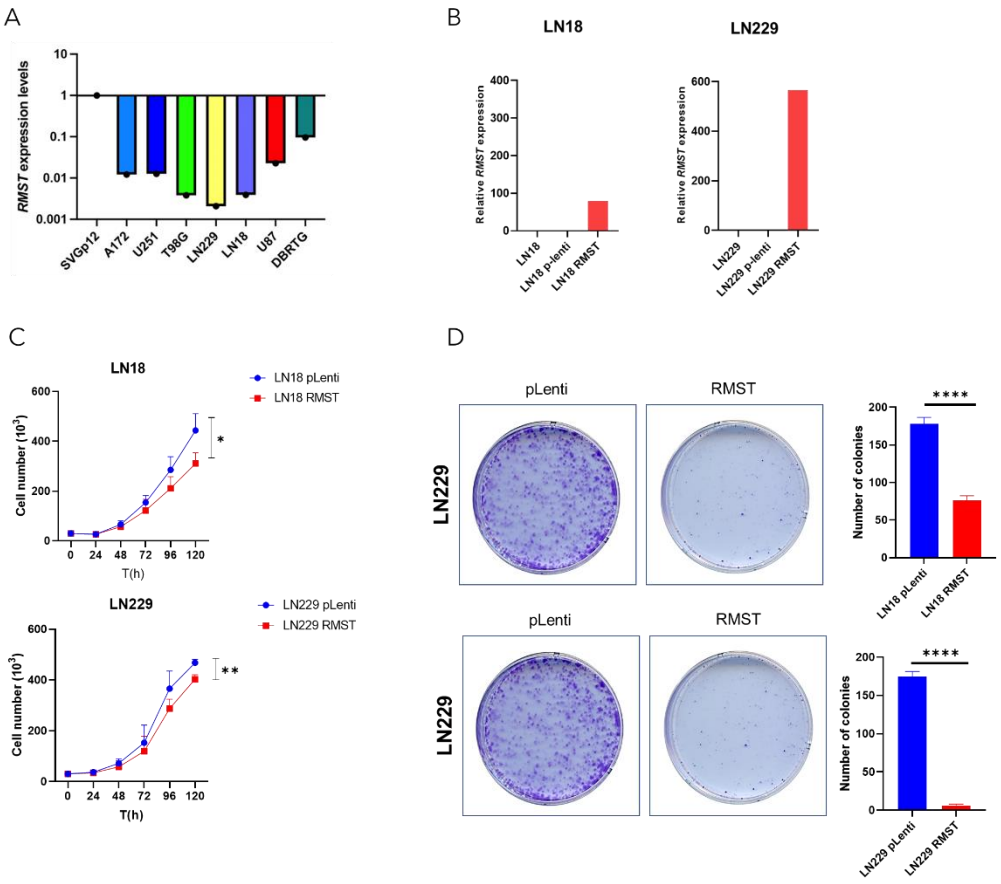


Fig. 19. *RMST* overexpression inhibits cell proliferation in GBM cell lines. (A) qRT-PCR analysis of RMST levels across a panel of glioma cell lines (A172, U251, T98G, LN229, LN18, U87, DBTRG) and normal glial cells (SVGp12). (B) qRT-PCR analysis of LN18 and LN229 GBM cell lines stably expressing RMST in comparison to cells transfected with the empty vector (pLenti). Data were compared to pLenti, normalized to 1, and expressed as $2^{(-\Delta\Delta Ct)}$ values ± SD. (C) Analysis of cell proliferation in LN18 and LN229 cells stably expressing RMST or transfected with the corresponding control vector (pLenti). Cells were counted at 24, 48, 72, 96, and 120 hours post-seeding. Values were derived from three independent experiments. Data are presented as mean ± SD. (D) Representative colony formation assays and quantification showed an impairment in colony-forming ability in LN18 and LN229 cells following RMST overexpression. The Student's t-test has been applied* $p < 0.05$; *** $p < 0.001$.

4.3 RMST promotes temozolomide sensitivity in GBM cells

To address whether RMST exerts its effects exclusively on tumor cell proliferation or also cell survival, I subsequently evaluated the impact of RMST on apoptosis. To this aim, Annexin V/PI staining was performed on RMST- and pLenti-transfected GBM cells. RMST overexpression alone induced only a modest increase in apoptosis (Fig 20A), indicating a positive trend without statistical significance; however, its pro-apototic effect was markedly enhanced following temozolomide (TMZ) treatment.

TMZ is an alkylating agent and the standard chemotherapeutic for glioblastoma, acting by methylation of the DNA at the O^6 position of guanine, which induces DNA damage and apoptosis. However, resistance to TMZ remains a major clinical barrier, limiting its long-term efficacy. In this context, RMST transfection significantly elevated the apoptotic rate of GBM cells treated with 500 μM of TMZ, compared to controls, as reflected by the increased percentage of Annexin V^+/PI^- (early apoptotic) cells (Fig. 20B). These findings suggest that RMST suppresses cell growth and enhances GBM cell sensitivity to chemotherapy-induced apoptosis.

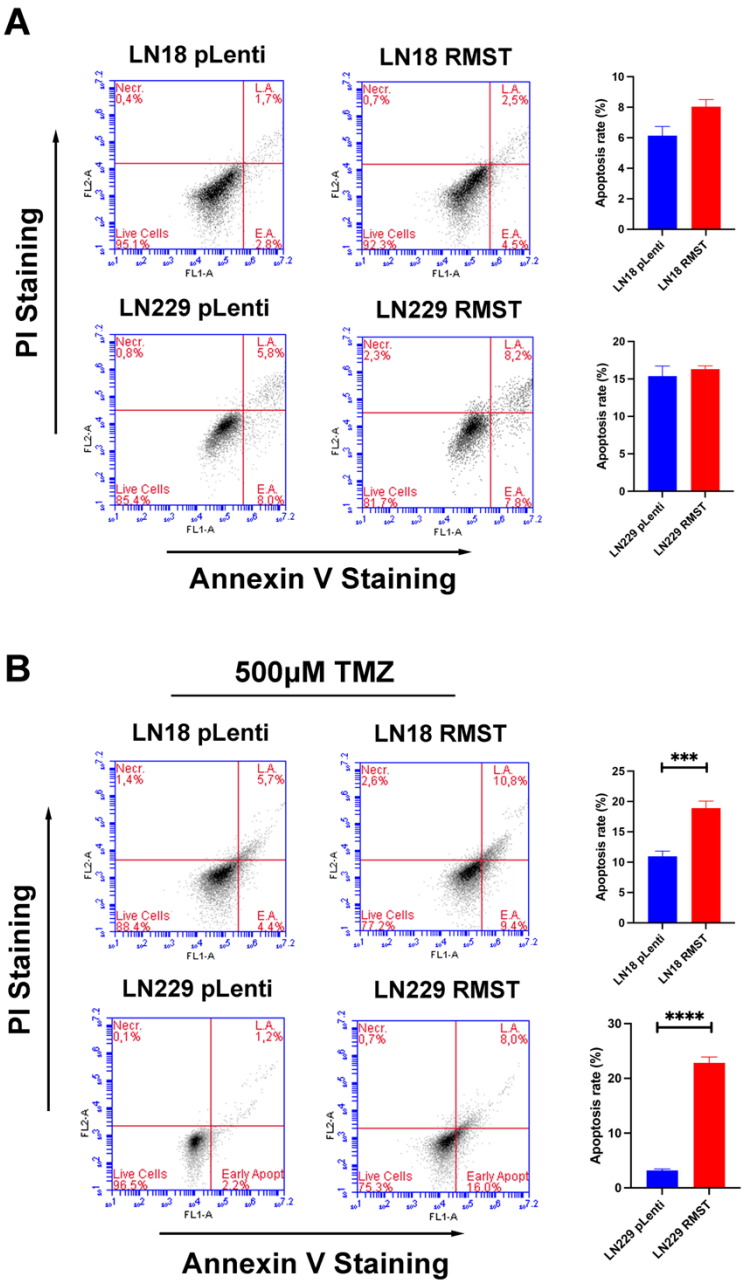


Fig. 20. RMST enhances apoptosis rate in GBM cells treated with temozolomide. (A) Annexin V-FITC/PI staining of LN18 cells (48h post-transfection) and LN229 cells (72h post-transfection), overexpression of RMST resulted in a moderate increase in early and late apoptotic populations compared to control cells (pLenti). (B) The GBM cells treated with TMZ (500 µM) were stained by Annexin V-PI and analyzed using flow cytometry. TMZ treatment significantly increased the rate of apoptotic cells at 96h (LN18) and 72h (LN229) compared to control cells (pLenti). Data are represented as mean ± SD of three independent determination, $*p < 0.05$ compared with the control cells (pLenti) by Student's *t*-test.

4.4 RMST overexpression reduces GBM cell migration and invasion in vitro

Glioblastoma (GBM) is characterized by its highly invasive nature, which enables tumor cells to diffusely infiltrate surrounding brain parenchyma. This infiltrative growth pattern significantly compromises the efficacy of conventional therapeutic approaches, particularly surgical resection and localized radiotherapy, as residual invasive cells often lead to tumour recurrence. Unlike many other malignancies, GBM rarely metastasizes beyond the central nervous system; thus, its lethality is predominantly attributed to its aggressive local invasion. Given that invasion constitutes an early and critical step in glioma progression, a more profound understanding of this process may lead to innovative strategies to reduce tumor spread and improve clinical outcomes in patients with GBM.

Thus, I evaluated RMST role in the motile phenotype of GBM cells. I performed a wound-healing assay to determine if GBM cells transfected with RMST exhibited reduced migration capability (Fig. 21). Compared to pLenti-GBM cells, RMST cells were less effective at triggering wound closure in culture medium supplemented with 0.5% fetal bovine serum. To confirm these data, I also assessed migration ability using the Transwell assay (uncoated filters) on RMST-overexpressing LN18 and LN229 cells. The results indicated that the RMST overexpression significantly inhibited the migration properties of LN18 and LN229 cells compared to the control. More importantly, RMST also suppressed the invasive phenotype of GBM cells. In an invasion assay using Transwell chambers coated with Matrigel, a reconstituted basement membrane, both RMST-overexpressing LN18 and LN229 cells featured a strong reduction of their invasion properties compared to the control cells.

These findings suggest that RMST may play a crucial role in regulating the migratory and invasive capabilities of GBM cells. Further elucidation of the mechanisms by which RMST exerts its effects may uncover novel regulatory pathways and identify potential targets for therapeutic intervention.

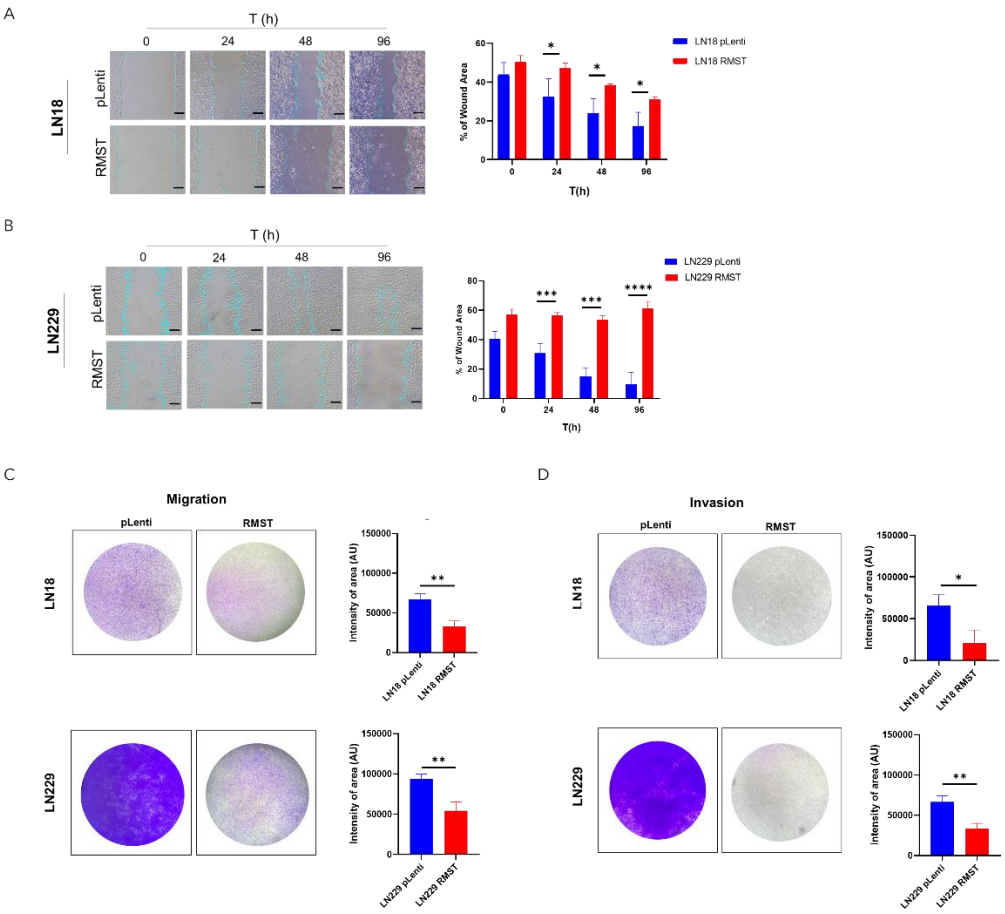


Fig. 21. RMST overexpression impairs the migration and invasion capability in LN18 and LN229 cells. Wound-healing assays of LN229-RMST (A) and LN18-RMST (B) cells, compared to the corresponding LN229-pLenti and LN18-pLenti control cells, in 0.5% of fetal bovine serum. Quantifications of wound closure (right panel) are shown together with photograms of migration (left panel; scale bar 50 mm). Values represent the average of triplicate experiments ± SD, *p < 0.05, compared to the control cells (pLenti) by Mann-Whitney t-test. (C) Representative photographs of transwell migration assay (uncoated wells) and the number of transmembrane GMB cells panel (magnification 20×). (D) Invasion assay (Matrigel coasted wells) performed in LN18 and LN229 cells stably expressing *RMST* or carrying the corresponding pLenti vector are showed in the representative photographs (magnification 20 ×). Migrating and invading GBM cells were fixed and stained with 0.05% of crystal violet solution. Statistical analysis of triplicate experiments was calculated by Student's t-test with * p 0.05 (versus control cells).

4.5 RMST overexpression strongly reduces the expression of EMT-associated genes in GBM cell lines

GBM exhibits an EMT (epithelial-mesenchymal transition)-like phenotype, characterized by the loss of cell adhesion and the gain of migratory and invasive properties. The EMT transition is a key driver of GBM aggressiveness, enhancing tumor invasiveness, therapy resistance, and acquisition of stem-like features. During EMT, the remodeling of the ECM (extracellular matrix) is crucial for enabling tumor cell migration and invasion; in particular, collagen genes, which encode structural components of the ECM, play a significant role in regulating EMT [Fujisaki and Futaki 2022, Tan 2022].
Previous transcriptomic analysis revealed a clear EMT-related signature in GBM, marked by the upregulation of several ECM-related genes, including COL1A2, COL4A2 and IGFBP2 [Szabo 2023, Wang 2023, Wu 2025, Zheng 2024] (Fig. 18A).
Therefore, to investigate the functional role of RMST in EMT process, I performed a quantitative PCR (q-PCR) analysis of EMT-related gene expression in GBM cells following RMST overexpression. The analysis revealed a consistent downregulation of multiple EMT markers in response to RMST overexpression compared to control cells (p-Lenti) (Fig. 22A-B). This reduction in gene expression suggests a potential role for RMST as a negative regulator of the EMT process in GBM cells, highlighting its possible involvement in the modulation of tumor cell plasticity and invasiveness.

Table 3. ECM-related genes from Rembrandt dataset analysis

Gene Name	EMT involvement	References
COL1A2	EMT promoter	Wang *et al.*, 2022
COL3A1	EMT promoter	Szabo *et al.*, 2023
COL4A2	EMT promoter	Wu *et al.*, 2025
IGFBP2	EMT promoter	Zheng *et al.*, 2024

Several transcription factors and signaling pathways are critically involved in regulating EMT in GBM. Among these, SNAIL (SNAI1) and SLUG [Medici 2008] play central role by repressing epithelial markers such as E-cadherin and promoting mesenchymal gene expression. In addition, EMT is also associated with upregulation of mesenchymal markers such N-cadherin, which facilitate cytoskeletal reorganization and cell motility [Loh 2019]. Another important factor, β-Catenin [Xue 2024] is a transcriptional regulator frequently overexpressed during EMT and are associated with increased invasiveness, therapeutic resistance, and poor prognosis in GBM.

Table 4. Main EMT-markers deregulated by RMST overexpression

EMT-related genes	Function	Description	References
SNAI1/Snail	Transcription Factor	Core EMT transcription factor	Medici *et al.*, 2008
SNAI2/Slug	Transcription Factor	Core EMT transcription factor	Medici *et al.*, 2008
CDH1/E-Cadherin	Adhesion Molecule	Often downregulated in EMT	Loh *et al.*, 2019
CDH2/N-Cadherin	Adhesion Molecule	Often upregulated in EMT	Loh *et al.*, 2019
β-Catenin	Adhesion Molecule and Transcription Factor	Often downregulated in EMT	Xue *et al.*, 2024

Thus, to examine more in depth the role of RMST in EMT and related biological processes, I conducted a Western Blot analysis on a set of genes linked to EMT and related processes in LN18 and LN229 GBM cells after RMST upregulation. The results revealed a global downregulation of key EMT regulators, including SNAIL, SLUG, β-catenin, and N-cadherin (Fig. 22C), and the upregulation of E-cadherin in both LN18- and LN229-RMST overexpressing cells, supporting a shift towards an epithelial-like phenotype.

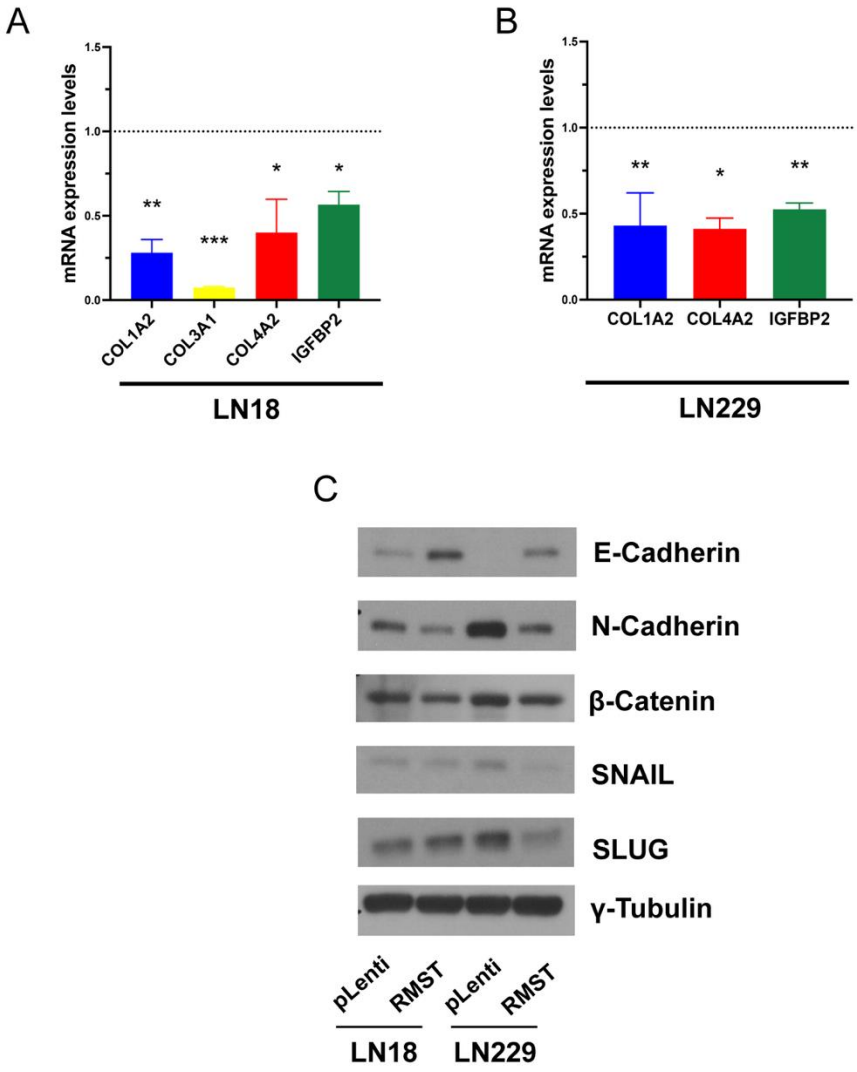


Fig. 22. RMST overexpression suppresses EMT-associated gene expression in GBM cells. (A) The figure presents a panel of qPCR (quantitative PCR) bar graphs showing relative ECM-related gene expression levels in LN18-RMST compared to LN18-pLenti. Data were compared to control cells (pLenti), set equal to 1, and reported as $28^{(-\Delta\Delta Ct)}$ values ± SD. (B) Panel of qPCR (quantitative PCR) bar graphs showing relative ECM-related gene expression levels in LN229-RMST compared to LN229-pLenti. Data were compared to control cells (pLenti), set equal to 1, and reported as $2^{(-\Delta\Delta Ct)}$ values ± SD. (C) Western blot analysis of EMT-related genes in RMST-overexpressing LN18 and LN229 cells compared to the control (p-Lenti).

These findings indicate that RMST may act as a negative regulator of the EMT program. Thus, its downregulation in GBM cell may potentially contribute to their invasive and stem-like properties. This highlights a novel role for RMST in the molecular network controlling glioblastoma progression and provides a rationale for further functional studies.

4.6 RNA-seq analysis identifies RMST-induced metabolic reprogramming in Glioblastoma by enhancing mitochondrial oxidative phosphorylation

In order to investigate the transcriptomic changes induced by RMST overexpression and to elucidate its potential regulatory functions in GBM cell biology, RNA-seq analysis of LN18 GBM cells following RMST overexpression revealed extensive transcriptional reprogramming across multiple functional domains (Fig. 23). Consistently with our previous results, RNA-seq revealed marked downregulation of genes involved in cytoskeletal organization, extracellular matrix (ECM) composition, and cell adhesion, including several collagen isoforms (COL1A2, COL3A1, COL4A2) and other ECM-related regulators. GO analyses linked these changes to processes governing cytoskeletal remodeling, focal adhesion, and ECM–receptor interactions, suggesting that RMST overexpression disrupts structural and migratory pathways that support GBM invasiveness.
Conversely, among the most prominent changes was the upregulation of genes associated with mitochondrial activity and metabolic processes, including key components of the oxidative phosphorylation machinery such as MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ATP6, MT-ATP8, MT-ATP6P1, and MT-TV. Consistent with previous evidence demonstrating profound metabolic plasticity in GBM and its reliance on altered mitochondrial function and enhanced glycolysis, gene ontology enrichment in our dataset highlighted significant activation of pathways related to cellular energy metabolism, mitochondrial respiratory chain function, and reactive oxygen species homeostasis. These results collectively indicate that RMST may drive a transcriptional shift toward enhanced mitochondrial oxidative metabolism, counteracting the glycolytic bias characteristic of the Warburg effect, in which GBM cells preferentially utilize aerobic glycolysis even under normoxic conditions.

Overall, these RNA-seq findings indicate that RMST orchestrates a coordinated transcriptional program that enhances mitochondrial oxidative metabolism while suppressing ECM-associated and cytoskeletal pathways involved in tumor invasion. By promoting oxidative phosphorylation and potentially mitigating the Warburg effect, RMST may exert tumor-suppressive effects through combined regulation of metabolic plasticity and invasive behavior [Agnihotri and Zadeh 2016, Cortes Ballen 2024]. GBM exhibits significant metabolic plasticity, enabling tumor cells to sustain rapid proliferation, adapt to hypoxic microenvironments, and resist therapeutic interventions. In contrast to normal glial cells, GBM cells frequently display increased glycolysis, altered mitochondrial function and oxidative phosphorylation, resulting in a metabolic state that efficiently supports tumor growth and biosynthetic demands. This metabolic reprogramming is closely associated with tumor aggressiveness, stemness, and invasion [Agnihotri and Zadeh 2016, Cortes Ballen 2024].

Transcriptomic profiling of the LN18 GBM cell line following RMST overexpression demonstrated extensive reprogramming of gene expression across multiple functional domains. Notably, a subset of upregulated genes was linked to mitochondrial function and metabolic pathways, including components of the oxidative phosphorylation machinery such as MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ATP6, MT-ATP8, MT-ATP6P1, and MT-TV (Fig. 23A and C). Gene ontology pathway analyses revealed a significant enrichment of processes related to cellular energy metabolism, mitochondrial respiratory chain activity, and reactive oxygen species homeostasis (Fig. 23C). These findings indicate that RMST overexpression promotes a shift toward enhanced mitochondrial oxidative metabolism, counteracting the glycolytic bias characteristic of the Warburg effect, in which cancer cells preferentially utilize aerobic glycolysis even under normoxic conditions. This transcriptional profile underscores the potential of RMST to modulate metabolic plasticity, fostering a more oxidative and energetically efficient phenotype that may affect cell survival and response to chemotherapeutic stress. Although other experiments are needed, these results suggest that RMST may orchestrate a coordinated transcriptional program in GBM cells, enhancing mitochondrial metabolism while suppressing structural and ECM-associated pathways. By promoting oxidative phosphorylation and potentially reducing the Warburg effect, RMST may exert tumor-

suppressive effects by simultaneously modulating metabolic and invasive properties.

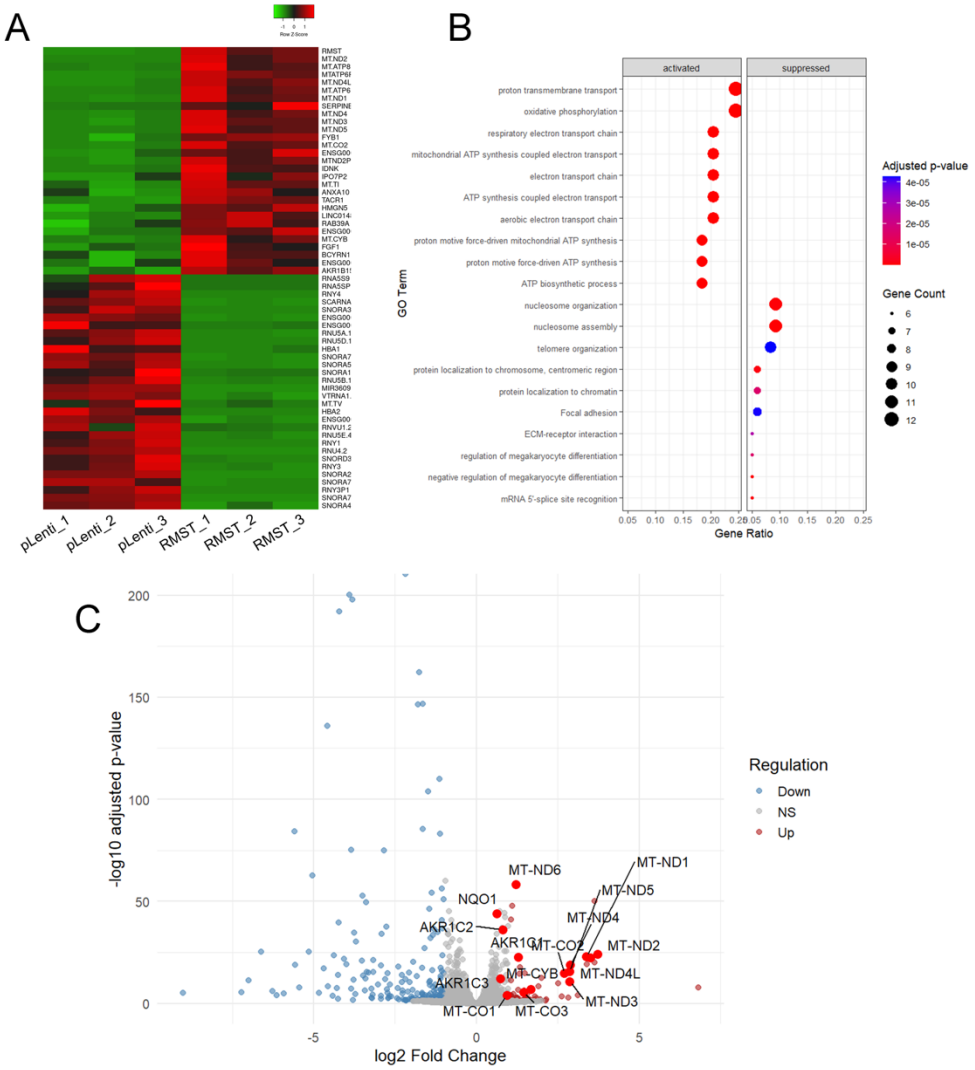


Fig. 23. Differential Gene Expression and Pathway Enrichment in RMST-Overexpressing GBM cells. (A) Heatmap of differentially expressed genes comparing LN18 glioblastoma cells overexpressing RMST with the corresponding LN18 cell line carrying the empty vector (EV). RNA-seq data were normalized and scaled across rows to highlight relative expression differences for each gene (green = decreased expression; red = increased expression). Hierarchical clustering of both genes and samples reveals a clear segregation of the RMST-overexpressing cells from EV controls, indicating that RMST induction drives a distinct transcriptional program. Among the most affected transcripts are multiple mitochondrial respiratory chain components and regulators of redox homeostasis. (B) Functional enrichment analysis of Gene Ontology (GO) terms associated with significantly upregulated ("activated") and downregulated ("suppressed") genes in RMST-overexpressing LN18 cells (cutoff: $|\log_2$ fold change$| > 1$, adjusted p-value < 0.05). Dot size reflects

the number of differentially expressed genes annotated to each GO term, whereas dot color denotes the significance of enrichment after multiple-testing correction. Activated pathways were strongly enriched for mitochondrial processes, including ATP synthesis coupled to electron transport, mitochondrial protein import, inner membrane organization, and NADH dehydrogenase (complex I) activity. Conversely, suppressed pathways involved also extracellular matrix organization, cytoskeletal rearrangement, and cell–substrate adhesion, suggesting that RMST modulates both metabolic and structural programs. (C) Volcano plot displaying $\log_2$ fold changes versus $-\log_{10}$ adjusted p-values for all transcripts detected in RNA-seq analysis. Upregulated genes are shown in red, downregulated genes in blue, and non-significant genes in grey. Notably, RMST overexpression leads to a coordinated induction of mitochondrial respiratory chain genes, including multiple subunits of NADH dehydrogenase (e.g., MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ND6) and cytochrome c oxidase (e.g., MT-CO1, MT-CO3). These expression patterns align with the enriched GO terms and suggest that RMST enhances mitochondrial electron transport chain activity at the transcriptional level.

5. DISCUSSION

The conventional view of the transcriptomic landscape, once predominantly focused on protein-coding mRNAs, has been redefined with the advent of high-throughput genome and transcriptome sequencing technologies [Carninci 2005, Mattick and Makunin 2006, Pennisi 2012]. These advances have revealed that the human genome encodes a wide array of non-coding RNAs (ncRNAs), including long non-coding RNAs (lncRNAs), which exert regulatory functions in several cellular processes such as proliferation, differentiation, apoptosis, migration, invasion, and epigenetic regulation.

Aberrant expression of lncRNAs has been implicated in various pathological conditions, particularly cancer. In multiple tumor types, dysregulation of lncRNA expression disrupts essential cellular functions and drives carcinogenesis [Gutschner and Diederichs 2012, Huarte 2015, Liu 2018]. LncRNAs display cell-type-specific expression patterns and are highly enriched in the brain, especially during neurodevelopment, underlining their potential role in CNS function and pathology.

In particular, lncRNAs have emerged as important prognostic biomarkers in glioblastoma (GBM), providing insights into tumor progression, patient survival, and therapeutic resistance. Accordingly, numerous studies have demonstrated that specific lncRNAs are significantly associated with tumor grade, overall survival, and treatment response [Mei 2020, Yang 2023].

Among lncRNAs, *Rhabdomyosarcoma 2-Associated Transcript* (RMST) has emerged as a key player in neural development and transcriptional regulation, particularly through its interaction with SOX2, a transcription factor essential for stem cell maintenance. In this framework, the current study aimed exploring the role of RMST in GBM, a highly aggressive brain tumor characterized by poor prognosis, high recurrence, and resistance to conventional therapies.

Preliminarly, bioinformatic analyses of publicly available datasets, corroborated by qPCR validation in a panel of GBM cell lines, revealed a consistent downregulation of RMST across various histological types of brain tumors, with the most pronounced decrease observed in glioblastoma cell models such as LN18 and LN229 (Fig. 17A and B, 19A). RMST expression levels were inversely correlated with glioma grade, thereby decreasing progressively from oligodendroglioma to

GBM. The decrease in RMST expression, parallel to increasing tumor grade, is consistent with the hypothesis that RMST may act as a favourable prognostic biomarker. Importantly, high RMST expression levels were associated with improved overall survival, thus reinforcing the prognostic value of RMST expression. However, it is important to note that these correlations were derived only from *in silico* analyses, and further validation using patient-derived samples matched to clinical outcomes will be essential to firmly establish RMST's prognostic and predictive value.

To assess the functional impact of RMST on GBM, two GMB cell lines, LN18 and LN229, both featuring a strong downregulation of RMST, were used for functional studies. Overexpression of RMST by using a pLenti-*RMST* vector, resulted in significant reductions in cell proliferation and clonogenic capacity (Fig. 19A-D). In addition, while RMST alone induced only a modest increase in apoptosis, its overexpression significantly sensitized GBM cells to temozolomide (TMZ), the standard chemotherapeutic agent for GBM (Fig. 20).

Given the critical role of cell migration and invasion in GBM progression and recurrence, the effects of RMST on these malignant hallmarks were investigated. Wound healing and Transwell migration assays revealed that RMST-overexpressing GBM cells exhibited significantly decreased migratory capacity compared to controls (Fig. 21A). Furthermore, invasion assays employing Matrigel-coated Transwell chambers revealed a marked inhibition of invasive behavior in RMST-overexpressing cells (Fig. 21C and D). EMT has been recognized as a key mechanism underlying GBM aggressiveness. By involving loss of cell adhesion and the acquisition of enhanced migratory, invasive, and stem-like properties, EMT may drive tumor progression and therapeutic resistance [Iser 2017, Tian 2025]. In particular, extracellular matrix (ECM) remodeling plays a pivotal role in facilitating EMT and tumor cell dissemination. Collagen genes, including COL1A2, COL4A2 and other ECM-related components such as IGFBP2, have been identified as critical mediators of this process [Szabo 2023, Wang 2023, Wu 2025, Zheng 2024]. Transcriptomic analyses have confirmed an EMT-related signature in GBM, marked by the upregulation of these ECM genes, underscoring their contribution to the invasive phenotype (Fig. 18A). Notably, quantitative PCR analysis revealed that RMST overexpression leads to a consistent downregulation of such key ECM-associated genes (Fig. 22A-B). Multiple transcription factors and signaling pathways drive the EMT

program in GBM. SNAIL (SNAI1) is a key inducer, as it suppresses epithelial markers like E-cadherin (CDH1) and activates mesenchymal gene expression [Batlle 2000]. The expression of mesenchymal markers, such as N-cadherin (CDH2) and β-catenin, further supports cytoskeletal changes and cell motility [Noh 2017]. In particular, β-catenin is a key mediator of EMT, promoting mesenchymal gene expression and enhancing invasive behavior in human cancer [Xue 2024]. Interestingly, western blot analysis of LN18 and LN229 GBM cell lines following RMST overexpression showed a significant decrease in the protein levels of mesenchymal markers, including SNAIL, SLUG, N-cadherin, and β-catenin, as well as the upregulation of the epithelial marker E-cadherin. These results indicate a shift toward a more epithelial-like state upon RMST expression, supporting its role in suppressing the mesenchymal program in GBM cells (Fig. 22C).

Transcriptomic analyses in the LN18 GBM cell line revealed a multifaceted role for the lncRNA RMST, encompassing regulation of extracellular matrix components, signaling pathways, and potentially metabolic reprogramming. Consistent with prior reports, RMST overexpression led to downregulation of collagen genes (COL1A2, COL3A1, COL4A2) and ECM-associated factors, including IGFBP2, which are critical mediators of tumor invasiveness and mesenchymal hallmarks. These findings suggest that RMST contributes to a less invasive transcriptional profile, potentially counteracting EMT-like processes that facilitate GBM progression. In addition to modulating ECM remodeling and signaling, RMST appears to have a significant impact on GBM metabolism. Transcriptomic analysis revealed that upregulated genes are predominantly associated with metabolic functions and pathways, including multiple components of the mitochondrial oxidative phosphorylation machinery.

Together with suppression of cell proliferation and increase in pro-apoptotic response, the reversal of EMT-related genes with RMST overexpression further suggests that RMST could act as a tumor suppressor in GBM. Consistently, RMST has been found to exhibit tumor-suppressive function across distinct cancer types. In triple-negative breast cancer (TNBC), RMST overexpression significantly inhibited cell proliferation and migration, promoted apoptosis, and enhanced autophagy [Zhang 2025]. Similarly, in colorectal cancer (CRC), RMST was shown to be downregulated in tumor tissues, and its ectopic expression reduced proliferation and promoted apoptosis by sponging miR-27a-3p and modulating the RXRα/Wnt signaling axis

[Chen 2023]. Consistently, De Martino et al. (2023) reported that RMST is drastically downregulated in anaplastic thyroid carcinomas, where it exerts tumor-suppressive functions by impairing epithelial–mesenchymal transition (EMT) and reducing cancer stemness. These findings further support the notion that RMST acts as a negative regulator of tumor aggressiveness across distinct cancer types [De Martino 2023]. However, recent studies show that lncRNAs can function as either oncogenes or tumor suppressors, depending on the context [Huang 2021, Zhang 2013]. At this stage, only some speculations can be done in terms of molecular mechanisms of RMST tumor - suppressive effects in GBM. The enhanced apoptotic response observed upon RMST overexpression in combination with TMZ suggests a potential interaction, likely mediated through converging effects on cell cycle progression and DNA damage response pathways. TMZ is an alkylating agent that induces cytotoxicity primarily through methylation at the O^6 position of guanine. The oxidized guanine mispairs with thymine during replication, leading to repeated cycles of mismatch repair (MMR) and ultimately to double-strand breaks and apoptosis [Hegi 2005, Stupp 2005]. Resistance to TMZ is frequently mediated by elevated expression (through reduced promoter methylation) of the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT). MGMT directly reverses O^6-methylguanine adducts, thereby counteracting the therapeutic efficacy of TMZ [Esteller 2000].

How, RMST may exerts these effects is still unknown. Accumulating evidence in other cancers suggests that RMST may function as a competitive endogenous RNA (ceRNA) able to modulate apoptotic and DNA repair pathways [Lou 2021]. In colorectal cancer, RMST was shown to sponge miR-27a-3p, thereby regulating the *RXRα/Wnt* signaling axis and promoting apoptosis [Chen 2023]. Similarly, in TNBC RMST binds to miR-4295 to regulate *ITPR1*, a key modulator of autophagy and apoptosis [Zhang 2025]. These findings suggest that RMST may influence the expression of genes involved in the cellular response to DNA damage, either by miRNA sequestration or by modulating the activity of transcriptional complexes. It is possible that, in GBM, RMST may downregulate MGMT expression or interfere with MMR or DNA damage response (DDR) pathways such as ATM/ATR, p53, or CHK1/2, thereby sensitizing cells to TMZ-induced cytotoxicity [Zou 2023].

Regardless the specific mechanism, our data indicate that the combination of RMST overexpression and TMZ exposure exceeds the cellular capacity for repair and survival. These findings position RMST as a potential therapeutic sensitizer that may enhance the efficacy of standard chemotherapy.

Our data show that the effects on cell motility/migration is probably mediated by the ability of RMST to downregulate N-cadherin, SNAIL and SLUG, along with upregulation of E-cadherin as observed at the protein level. This raises further questions about the molecular circuitry in which RMST is involved and how its loss contributes to the reprogramming of GBM into a more invasive state. It is unclear whether RMST acts through a direct interaction with EMT transcription factors, or indirectly through chromatin remodeling, epigenetic changes, or miRNA-related mechanisms. Alternatively, RMST may function indirectly *via* epigenetic regulation or miRNA sponging, mechanisms that have been extensively reported for other lncRNAs. For example, RMST might act as a competing endogenous RNA (ceRNA), sequestering EMT-promoting microRNAs such as miR-21 or miR-155, both of which are implicated in glioma progression and EMT regulation [Estridge 1989, Zhu 2008]. Furthermore, given the critical involvement of TGF-β, Wnt/β-catenin, in promoting EMT in GBM [Fan 2023, Hu 2018, Nie 2025, Pouyan 2025], it is plausible that RMST intersects with one or more of these pathways to exert its suppressive effects.

Metabolic reprogramming is a hallmark of glioblastoma (GBM), enabling tumor cells to sustain rapid proliferation, survive under hypoxic conditions, and resist therapy [Agnihotri and Zadeh 2016]. GBM cells exhibit profound metabolic plasticity, including a reliance on glycolysis even in the presence of oxygen (Warburg effect) [DeBerardinis 2008, Vander Heiden 2009], coupled with mitochondrial dysfunction and altered tricarboxylic acid cycle activity. Such adaptations support tumor growth, survival under stress, and therapeutic resistance [Faubert 2020]. While multiple mechanisms contribute to this plasticity, long non-coding RNAs (lncRNAs) have emerged as central regulators of metabolic programs in cancer, influencing glycolysis, mitochondrial function, and lipid metabolism via interactions with transcription factors, chromatin modifiers, or microRNAs [Liu 2019, Tan 2021]. In this context, RMST, a lncRNA previously implicated in neural differentiation and tumor biology, may similarly coordinate metabolic pathways in GBM, potentially modulating both mitochondrial activity and glycolytic flux to influence

tumor bioenergetics and growth [Zheng 2015]. RNA-seq analysis enables the systematic identification of genes and pathways altered by RMST overexpression, providing a transcriptomic framework to infer its potential effects on mitochondrial function, oxidative phosphorylation, and glycolytic activity [De Paepe 2018]. Based on these paradigms, we propose that RMST may similarly modulate metabolic reprogramming in GBM. RMST could interact with transcriptional regulators or chromatin remodeling complexes to enhance the expression of mitochondrial genes, such as components of the electron transport chain and TCA cycle, while concomitantly repressing glycolysis-promoting genes [Fang 2024]. This action would potentially counteract the Warburg effect, promoting oxidative phosphorylation and increasing mitochondrial ATP production. Alternatively, RMST could act as a competitive endogenous RNA (ceRNA), sequestering microRNAs that normally inhibit mitochondrial biogenesis and metabolic enzymes. By relieving repression of factors such as PGC-1a, NRF1, or TFAM, RMST might restore mitochondrial function and bioenergetic balance, reducing GBM dependence on aerobic glycolysis [Long 2016].

Taken together, these transcriptomic and mechanistic insights suggest a model in which RMST coordinates multiple aspects of GBM biology. By simultaneously downregulating ECM remodeling genes, modulating stress- and proliferation-related signaling, and potentially enhancing mitochondrial function, RMST may act as a tumor suppressor that limits invasion, proliferation, and metabolic adaptation.

While the data presented herein strongly support a tumor-suppressive role for RMST in GBM, this study has several limitations. The role of RMST was primarily evaluated through *in vitro* experiments using the LN18 and LN229 cell lines. This model system does not reflect the full biological complexity of tumours *in vivo*. Ongoing *in vivo* experiments are currently being conducted to further validate the functional impact of RMST on GBM growth and progression. Future research should employ *in vivo* models, such as patient-derived xenografts to validate RMST's impact on tumour invasiveness, growth, and therapeutic response. The precise molecular mechanisms through which RMST regulates EMT, apoptosis, and proliferation remain unclear. It is unknown whether RMST exerts its functions by directly interacting with transcription factors, modulating chromatin structure, or functioning through microRNAs or other epigenetic regulators or a combination

thereof. Similarly, it remains to be determined whether RMST acts by sponging specific miRNAs or through interactions with regulatory proteins. To elucidate these mechanisms, future studies should identify RMST's molecular partners using techniques such as RNA immunoprecipitation (RIP), RNA pull-down assays, and advanced OMICS approaches to characterize the RMST interactome and the corresponding signaling. In parallel, RNA sequencing analyses are in progress to comprehensively identify downstream transcriptional targets and pathways modulated by RMST in GBM cells. Another limitation concerns the restricted set of candidate EMT-related genes that have been analyzed and the inter-patient variability in RMST expression observed across GBM samples. Broader transcriptome-wide analyses could uncover additional RMST-regulated genes and clarify its role within wider regulatory networks. Finally, integrating RMST into multi-biomarker panels alongside other known lncRNAs and mRNAs may improve diagnostic and prognostic precision, facilitating the development of more effective, personalized treatment strategies for GBM.

6. CONCLUSIONS

This study explored the functional role and prognostic potential of the long non-coding RNA RMST in glioblastoma (GBM), the most aggressive and lethal primary brain tumor. RMST emerged as a putative tumor suppressor with crucial functions in GBM progression. Its expression was consistently downregulated in GBM samples and cell lines. RMST up-regulation exerted an inhibitory effect on cell growth and sensitized GBM cells to temozolomide (TMZ)-induced apoptosis. RMST overexpression also suppressed cell motility and invasion, likely through interference with epithelial-to-mesenchymal transition (EMT)-like programs.

In conclusion, these findings strongly support the hypothesis that RMST acts as a tumour suppressor in GBM. RMST represses key EMT-associated transcription factors and, as suggested by RNA-seq analyses, may also contribute to metabolic reprogramming within GBM cells. Through these combined effects, RMST reduces cellular invasiveness and enhances sensitivity to therapeutic interventions. Its ability to suppress migration and invasion further points to a potential role in limiting early steps of tumour dissemination and recurrence. Future studies will be essential to delineate the molecular interactions and signalling pathways through which RMST mediates its tumour-suppressive and therapy-enhancing functions.

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