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Study of the Role of NRF2 in the Progression and Metabolic Adaptation of Glioblastoma

Maya Moubarak

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Maya Moubarak. Study of the Role of NRF2 in the Progression and Metabolic Adaptation of Glioblastoma. Human health and pathology. Université de Bordeaux, 2024. English. NNT : 2024BORD0230 . tel-04788234

HAL Id: tel-04788234

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE

**DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE : Sciences de la Vie et de la Santé
SPÉCIALITÉ : Biologie du Cancer

Par **Maya MOUBARAK**

**Étude du rôle de NRF2 dans le développement et l'adaptation
métabolique du glioblastome**

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Soutenue le 22 Octobre 2024

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Abstracts in French and English

Étude du rôle de NRF2 dans le développement et l'adaptation métabolique du glioblastome

Le glioblastome (GB), la forme la plus agressive de tumeur cérébrale, présente d'importants défis thérapeutiques en raison de son comportement invasif, de sa résistance aux traitements et de son hétérogénéité génétique. Le stress oxydatif, régulé par le facteur nucléaire lié à l'érythroïde 2 (NRF2), joue un rôle crucial dans la progression du cancer. NRF2, un facteur de transcription central pour l'homéostasie redox, présente un rôle double dans le cancer, agissant à la fois comme suppresseur et promoteur de tumeur. Cette étude visait à clarifier le rôle de NRF2 dans le développement du GB, son adaptation métabolique et sa résistance aux thérapies en utilisant un modèle de GB dérivé de patients (P3), cliniquement pertinent. Nous avons utilisé la technologie CRISPR-Cas9 pour générer des lignées cellulaires déficientes en NRF2 et induit une surexpression de NRF2 pour évaluer son impact sur la biologie du GB.

Nos résultats montrent que l'inactivation de NRF2 (KO) n'a pas significativement affecté la croissance des sphères P3 *in vitro*, tandis que les expériences *in vivo* ont démontré des effets inverses sur la croissance tumorale entre les deux clones invalidés. La tumeur du clone C7 KO a montré une croissance tumorale accrue, suggérant un rôle anti-tumoral de NRF2, tandis que celle du clone C16 KO a présenté une croissance réduite, indiquant un effet pro-tumoral. De plus, l'inactivation de NRF2 a significativement diminué la capacité invasive des cellules de GB *in vitro*, mais cette réduction n'a pas été observée *in vivo*, ce qui implique que le microenvironnement tumoral pourrait moduler l'activité de NRF2. Cependant, la surexpression de NRF2 induite par le tert-butylhydroquinone (TBHQ) n'a eu aucun impact, ni sur la prolifération, ni sur l'invasion des sphères P3 *in vitro*.

Par ailleurs, bien que l'inactivation de NRF2 n'ait pas altéré le potentiel de membrane mitochondriale, la biogenèse ou la machinerie d'importation des protéines, elle a entraîné une reprogrammation métabolique significative. Un déplacement notable vers la glycolyse a été mis en évidence par l'augmentation des niveaux de lactate intracellulaire, la surexpression de LDHA et la sous-expression de LDHB, soutenant l'effet Warburg. De plus, l'inactivation de NRF2 a augmenté le stockage des lipides en favorisant la formation de gouttelettes lipidiques.

Bien que NRF2 soit connu pour conférer une résistance aux agents chimiothérapeutiques comme le témozolomide (TMZ), notre étude a révélé que l'inactivation de NRF2 n'a pas augmenté la sensibilité au TMZ dans notre modèle. De manière inattendue, la déficience en NRF2 a entraîné une surexpression des marqueurs SOX2 et OLIG2, indiquant une possible augmentation du caractère souche des cellules invalidées pour NRF2, bien que cela ne se soit pas traduit par une capacité accrue d'auto-renouvellement des sphères P3.

En conclusion, cette étude souligne le rôle multiple de NRF2 dans la progression, l'invasion et l'adaptation métabolique du GB. Cependant, des recherches futures devraient explorer les mécanismes sous-jacents par lesquels NRF2 influence les voies moléculaires et les réseaux de gènes liés à la progression et au métabolisme du GB.

Mots clés : Glioblastome, NRF2, Stress oxydatif, Invasion cellulaire, Reprogrammation métabolique, Caractère souche, Résistance à la thérapie.

Study of the Role of NRF2 in the Progression and Metabolic Adaptation of Glioblastoma

Glioblastoma (GB), the most aggressive form of brain tumor, poses substantial therapeutic challenges due to its invasive behavior, treatment resistance, and genetic heterogeneity. Oxidative stress, regulated by Nuclear Factor Erythroid 2-related Factor 2 (NRF2), plays a critical role in cancer progression. NRF2, a central transcription factor for redox homeostasis, exhibits a dual role in cancer, acting as both a tumor suppressor and promoter. This study aimed to clarify NRF2's role in GB development, metabolic adaptation, and therapy resistance using a clinically relevant patient-derived GB model (P3). We employed CRISPR-Cas9 technology to generate NRF2-deficient cell lines and induced NRF2 overexpression to assess its impact on GB biology.

Our findings reveal that NRF2 knockout (KO) did not significantly affect P3 sphere growth *in vitro*, while *in vivo* experiments demonstrated contrasting tumor growth patterns between the different knockout clones. The tumor of the C7 KO clone exhibited enhanced tumor growth, suggesting an anti-tumoral role for NRF2, while that of C16 KO showed reduced growth, indicating a pro-tumoral effect. Additionally, NRF2 knockout significantly decreased the invasive capacity of GB cells *in vitro*, but this reduction was not observed *in vivo*, implying that the tumor microenvironment may modulate NRF2's activity. However, tert-Butylhydroquinone (TBHQ)-induced NRF2 overexpression had no impact on the proliferation or invasiveness of P3 spheres *in vitro*.

Moreover, although NRF2 KO did not alter mitochondrial membrane potential, biogenesis, or protein import machinery, it caused significant metabolic reprogramming. A significant shift toward glycolysis was evidenced by the increased intracellular lactate levels, upregulation of LDHA, and downregulation of LDHB expression, supporting the Warburg effect. Moreover, knocking out NRF2 increased lipid storage by favoring lipid droplet formation.

Although NRF2 is known to confer resistance to chemotherapeutic agents like temozolomide (TMZ), our study found that NRF2 knockout did not increase TMZ sensitivity. Unexpectedly, NRF2 deficiency resulted in elevated expression of stemness markers, such as SOX2 and OLIG2, indicating a potential enhancement of stem-like traits, though this did not correlate with the P3 sphere's increased self-renewal capacity.

Overall, this study underscores the multifaceted role of NRF2 in GB progression, invasion, and metabolic adaptation. However, future research should explore the underlying mechanisms

through which NRF2 influences the molecular pathways and gene networks related to GB progression and metabolism.

Keywords: Glioblastoma, NRF2, Oxidative stress, Cell invasion, Metabolic reprogramming, Stemness, Therapy Resistance.

Acknowledgment

“We must find time to stop and thank the people who make a difference in our lives.” — John F. Kennedy.

Completing this PhD has been one of the most significant and transformative experiences of my life. This experience has been far more than just a scholarly achievement; it has been a profound journey of personal growth and self-discovery. I have come to realize that success is not merely about reaching a goal; it is about the persistence, adaptability, and resilience one demonstrates throughout the process. As I reflect on these past three years, I am filled with an overwhelming sense of accomplishment and deep gratitude.

First and foremost, I owe an immense debt of gratitude to my advisor, Dr. Océane Martin, for granting me the opportunity to undertake this PhD. Choosing to pursue this challenge in a foreign country was a pivotal moment in my life, and thanks to you, it is a decision I will forever cherish. Your warm welcome and personal support, particularly during the early days of adjustment to a new environment, were invaluable. The trust you placed in me, combined with your essential guidance, instilled a profound sense of responsibility and commitment to the pursuit of knowledge. Your mentorship has been the foundation of my progress; I am deeply grateful for your time, effort, and insightful feedback. This dissertation is a testament to our shared dedication, and I am profoundly appreciative of the transformative experience I have had under your guidance.

I extend my deepest gratitude to Dr. Thomas Daubon, our team leader. Your leadership, marked by empathy and clarity, created an environment in which I constantly felt supported and motivated to push beyond my perceived limits. Your genuine concern for the well-being of our team, and your thoughtful counsel ensured continuous progress, even during the most challenging times. I am truly grateful for your help and contributions, which have not only guided our research but also helped me grow into a more capable and resilient individual.

I am also sincerely thankful to the members of the jury who agreed to examine and evaluate my work. My heartfelt thanks go to Dr. Laurence Huc from the University of Rennes, Dr. Elias El Habr from the University of Sorbonne, and Dr. Valerie Coronas from the University of Poitiers for

serving as the reviewers of this dissertation. I am honored by your willingness to engage with my research and deeply appreciate the valuable feedback, critiques, and advice you will provide.

To the incredible GBmetabo team members, thank you for being such an integral part of my journey. I am grateful for the positive energy and team spirit you brought into every aspect of our daily work. The camaraderie, hard work, and countless good times we shared made this experience unforgettable. My special gratitude goes to Dr. Antonio Christian Pagano Zottola. You have undoubtedly been a guiding light throughout my PhD journey. I am deeply thankful for our shared moments, the scientific knowledge we exchanged, and your support. I will always cherish you as an exceptional friend. I do not doubt that you will inspire your future students and be a mentor they will look up to with admiration. I also extend a heartfelt thanks to Dr. Claire Larrieu, my lab and office roommate for the past three years. Your kindness, friendship, and the long, insightful discussions we had made our shared space not just a place for science, but a place of warmth and understanding. To Doriane and Manon, your willingness to help whenever needed made all the difference, and your friendship added warmth and positivity to this journey. For Joana (mustache), Sebastian, Sarah, Audrey, Mathis (RT-qPCR king), Mathieu, Johanna, and Gauthier, thank you for all your help, the events we enjoyed, and the good moments we shared. This PhD experience would not have been the same without each of you. Your support and the joyful times we had together added much-needed balance to the intensity of this journey. Lastly, thank you, Sylvain, you are deeply missed!

My PhD journey also led me to the B2 team at the Bordeaux Institute of Oncology BRIC-U1312, where I spent several months conducting experiments. I am deeply grateful for the welcoming environment and the support I received from everyone there. A special thank you to Dr. Arnaud Mourier and his entire team—Claudine, Manuel, Philippe, Orane, Guillaume, Rose, Chloé, and Camille. Your scientific and technical support, along with our coffee breaks, and discussions, were a source of comfort and motivation throughout my thesis.

I would also like to express my sincere thanks to the technical staff of IBGC and the platform managers who supported my research throughout this journey. My gratitude extends to the teams at CRISP'EDIT, Celloxia, Histopathology, and the Metabolic Analysis Service of Bordeaux at the TBM core facility of the University of Bordeaux. I am deeply appreciative of the assistance, expertise, and tools you provided. Additionally, I would like to acknowledge with deep appreciation the funding bodies including the Ministry of Higher Education and Research for their

financial support and necessary resources to complete this work. For that, I am profoundly grateful.

To my family and friends who have always believed in me and stood by me throughout this journey. Your unwavering love and encouragement have been the foundation upon which I have built my strength and a constant source of comfort and inspiration. To my family, this dissertation is my gift to you. You have taught me how to embark on and persevere through the 1,000-mile journey of life with courage, resilience, and hope. Your unconditional support has been my anchor, providing the stability and reassurance I needed to keep moving forward, even when the path seemed uncertain. Thank you for believing in me, for guiding me, and for showing me that every journey, no matter how long, begins with a single step. To my friends, your companionship, and the joy and laughter we shared kept me grounded and reminded me of the importance of balance in life. Your friendship has made this journey not only possible but truly meaningful.

Finally, to all who have been part of this journey, whether named here or not, my deepest thanks. Your support, in its many forms, has made this achievement possible.

" And that man shall have nothing but what he strives for (39). And that his striving shall soon be seen - (40). Then shall he be rewarded for it with the fullest reward-(41)."

— Quran Sura 53: AN-NAJM (THE STAR):39-41.

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List of Abbreviations

- **ABC:** ATP-binding cassette
- **ABCC1:** ATP-Binding Cassette Subfamily C Member 1
- **AD:** Alzheimer's Disease
- **AKRs:** Aldo-keto reductases
- **ALS:** Amyotrophic Lateral Sclerosis
- **AMPK:** AMP-Activated Protein Kinase
- **AMPK:** 5' AMP-activated protein kinase
- **ANLS:** Astrocyte-Neuron Lactate Shuttle
- **APE1:** Apurinic/Apyrimidinic Endonuclease 1
- **ARE:** Antioxidant response element
- **ATF1:** Activating transcription factor 1
- **ATF3:** Activating transcription factor 3
- **A β :** Beta-Amyloid
- **BACH1:** BTB and CNC homology 1 transcription factor
- **BBB:** Blood-Brain Barrier
- **BCRP:** Breast cancer resistance protein
- **Bcl-2:** B-Cell Lymphoma 2
- **BMI-1:** B-Cell Specific Moloney Murine Leukemia Virus Integration Site 1
- **BLVRA/B:** Biliverdin Reductase A/B
- **BRG1:** Brahma-related gene 1
- **BTB:** Blood-Tumor Barrier
- **C/EBP :** CCAAT/enhancer-binding protein
- **CAT :** Catalase
- **CBP :** CREB binding protein
- **CBRs:** Carbonyl reductases
- **CIDs:** Chronic inflammatory disorders
- **CK-2:** Casein kinase 2
- **CHD6:** Chromodomain helicase DNA-binding protein 6
- **CNS:** Central Nervous System
- **CRIF1:** Growth arrest and DNA damage-inducible proteins-interacting protein 1
- **CRLs:** Cullin-RING E3 ubiquitin ligase
- **CRISPR/Cas9:** Clustered Regularly Interspaced Short Palindromic Repeats/Crispr-associated protein 9
- **CTC:** Circulating Tumor Cell
- **CVDs:** Cardiovascular diseases
- **CXCL10:** C-X-C Motif Chemokine Ligand 10
- **CYPs:** Cytochrome P450s
- **Cyclin E :** Cyclin E
- **D2-HG :** D2-Hydroxyglutarate
- **DC:** Dendritic Cell
- **DGR:** Double glycine repeat
- **DLG :** DLG motif
- **EGFR:** Epidermal Growth Factor Receptor
- **ER:** Endoplasmic reticulum
- **ETGE:** ETGE motif
- **FAO:** Fatty Acid Oxidation
- **FPN1:** Ferroportin 1
- **FTH1:** Ferritin Heavy Chain 1
- **GABA:** Gamma-Aminobutyric Acid
- **G6PD:** Glucose-6-Phosphate Dehydrogenase
- **GCLM :** Glutamate-Cysteine Ligase Modifier Subunit
- **GB :** Glioblastoma
- **GSC :** Glioblastoma Stem Cell
- **GEPIA2:** Gene Expression Profiling Interactive Analysis 2
- **GPx :** Glutathione peroxidase
- **GSH :** Glutathione
- **GSS :** Glutathione synthetase
- **GSK3 :** Glycogen synthase kinase-3

- **H₂O₂** : Hydrogen peroxide
- **HIF-1 α** : Hypoxia-Inducible Factor 1 Alpha
- **HO-1** : Heme oxygenase-1
- **HRD1**: 3-Hydroxy-3-methylglutaryl reductase degradation
- **HTT**: Huntingtin
- **ICB** : Immune Checkpoint Blockade
- **IDH** : Isocitrate Dehydrogenase
- **IFN- γ** : Interferon Gamma
- **IVR**: Intervening region
- **JDP2**: Jun dimerization protein
- **KEAP1**: Kelch-like ECH-associated protein 1
- **Ki-67**: Antigen identified by the Ki-67 monoclonal antibody
- **Kras**: Kirsten Rat Sarcoma Viral Oncogene Homolog
- **MAPKs**: Mitogen-activated protein kinases
- **MB**: Medulloblastoma
- **MCT**: Monocarboxylate Transporter
- **MDR**: Multidrug resistance-associated proteins
- **MEL**: Melatonin
- **MGMT**: O⁶-Methylguanine-DNA Methyltransferase
- **miRNAs**: MicroRNAs
- **MSC**: Mesenchymal stem cells
- **mRNA**: Messenger RNA
- **mTOR**: Mammalian Target of Rapamycin
- **MVD** : Microvessel Density
- **NADPH**: Nicotinamide adenine dinucleotide phosphate
- **NF- κ B**: Nuclear Factor Kappa B
- **NFE2L2**: Nuclear Factor Erythroid 2
- **NQO1** : NAD(P)H Quinone Dehydrogenase 1
- **NRF2**: Nuclear Factor Erythroid 2-Related Factor 2
- **NSC** : Neural Stem Cell
- **O₂ \bullet^-** : Superoxide radical
- **OH \bullet** : Hydroxyl radical
- **OPC** : Oligodendrocyte Precursor Cell
- **PAHs**: Polycyclic aromatic hydrocarbons
- **PD**: Parkinson's Disease
- **PI3K/Akt**: Phosphoinositide 3-Kinase/Protein Kinase B
- **PKC**: Protein kinase C
- **PON2**: Paraoxonase 2
- **PPAR- α** : Peroxisome proliferator-activated receptor α
- **PPP**: Pentose Phosphate Pathway
- **PRX**: Peroxiredoxin
- **RAR**: Retinoic acid receptor
- **ROC**: Receiver Operating Characteristic
- **RLRs**: Cytosolic RIG-I-like receptors
- **RNS**: Reactive nitrogen species
- **ROS**: Reactive Oxygen Species
- **RT** : Radiotherapy

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I. Introduction

I. Introduction

A. NRF2 in Health and Disease

A.1 Cellular Oxidative Stress and Antioxidant Response

A.1.a.i Generation and Function of Reactive Oxygen Species

Organisms are continually exposed to exogenous and endogenous sources of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other oxidants that have both beneficial and deleterious effects on cells (1). ROS are oxidants that are primarily generated as byproducts of cellular metabolism and biochemical processes occurring within cells. Mitochondria is a major source of endogenous ROS, particularly through aerobic respiration, which uses oxygen oxidation to produce ATP. ROS include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\bullet}), and singlet oxygen (i.e., a molecule that removes electrons from other molecules) (1–3). Also, peroxisomes produce ROS from aerobic metabolism (4) as well as phagocytic neutrophils and macrophages produce ROS to eliminate invading pathogens (5). Evidence shows that various cellular enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases also significantly contribute to ROS production (6,7) (**Figure 1**). Moreover, exogenous or environmental sources may trigger ROS production, including air pollutants, tobacco smoke, ionizing and nonionizing radiations, foods, drugs, xenobiotics, heavy metals, such as lead, arsenic, mercury, chromium, and cadmium, organic solvents, and pesticides (8).

In terms of function, ROS act as a signaling intermediate required for a variety of physiological processes. For example, ROS regulate cell fate and differentiation for the human mesenchymal stem cells (MSC) (9) and human pluripotent stem cells (hPSCs) (10), induces autophagy (11), and aide in sensing oxygen levels via stabilizing the hypoxia-inducible factors (HIF) responsible for orchestrating the transcriptional response to hypoxia (12,13). Also, ROS act as signaling molecules in regulating skeletal muscle glucose uptake, modulating gene stability and transcription via affecting chromatin stability, in addition to regulating vascular diameter and signal transduction, as reviewed by Alfadda and colleagues (14). ROS are also needed for NLRP3 inflammasome activation (15,16), inflammatory cytokines signaling (17), and the activation and assembly of inflammasomes (18). Additionally, ROS regulate immunity as they are essential to multiple toll-like receptors (TLR)-initiated pathways, cell surface TLRs (19), the cytosolic RIG-I-like receptors (RLRs) signaling (20) in addition to adaptive immune cell functioning during early stages

of T cell activation (21). Therefore, ROS play essential roles in numerous physiological processes within cells, highlighting their importance in maintaining cellular function and homeostasis.

A.1.a.ii Impact of Oxidative Stress

Extensive or prolonged exposure to ROS result in oxidative stress, a state of an imbalance between the production of ROS and the ability to clear them by the cellular antioxidant defense mechanisms (22). Chronic exposure to ROS is detrimental to cells, with consequences varying depending on the specific context. Excessive ROS-mediated oxidative stress can lead to aberrant cell cycle reentry due to a defect in DNA damage detection and activation of the transcription factor and tumor suppressor protein p53 (23). Additionally, oxidative stress can cause protein damage, including a reduction in histone deacetylase activity and impairment of cellular proteins' enzymatic activity (24). Lipid peroxidation, resulting from oxidative stress, further damages cell membranes and lipoproteins (25), and impairment cellular proteins' enzymatic activity (26).

DNA is particularly susceptible to oxidative stress-induced lesions, such as the formation of 8-oxo-2'-deoxyguanosine (8-OHdG) (26), a recognized tissue biomarker of oxidative stress (27). This damage can lead to both mutagenesis (28) and the loss of epigenetic information by impairing CpG island methylation within gene promoters (29). Additionally, oxidative stress results in various DNA modifications, including oxidized bases, strand breaks (both single and double) (30), and hydrolyzed bases, all of which are significant events in carcinogenesis (28,31). This process can trigger the onset of cancer by causing mutations that activate oncogenes or inactivate tumor suppressor genes (32,33). Additionally, oxidative stress contributes to the development of other diseases, including cardiovascular diseases (CVDs) (34), neurological diseases (35), respiratory diseases (36), chronic inflammatory disorders (37), and kidney diseases (38).

A.1.a.iii Antioxidant Defense Mechanisms

Due to its reactive nature, ROS production and elimination must be strictly regulated by antioxidants to preserve redox homeostasis and the delicate balance between the beneficial and harmful effects of ROS (39). Cellular antioxidant defenses comprise antioxidants that directly eliminate ROS, such as glutathione (GSH), vitamin C, and vitamin E, and antioxidant systems like superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (1). For example, SOD, present in both the cytosol and mitochondria, breaks down superoxide radicals into molecular

oxygen and hydrogen peroxide (40). Catalase, primarily located within peroxisomes but also found in mitochondria and the nucleus, facilitates the decomposition of hydrogen peroxide into molecular oxygen and water (41). Paraonase 2 (PON2) prevents oxidation and alteration of low-density lipoproteins, countering lipid peroxidation in the plasma membrane (42,43). GSH, a tripeptide composed of cysteine, glutamic acid, and glycine, is the most abundant antioxidant protein. It shields cells from oxidative harm either through direct antioxidant action or by coupling with GPx enzymatic function (**Figure 1**). GPx degrades hydrogen peroxide by oxidizing a GSH cofactor (44). GPx1 is a widely expressed isoform found in both the cytosol and mitochondria, while GPx2 is an extracellular enzyme primarily found in the intestine, GPx3 is located extracellularly, and GPx4 exhibits a preference for lipid peroxides (45).

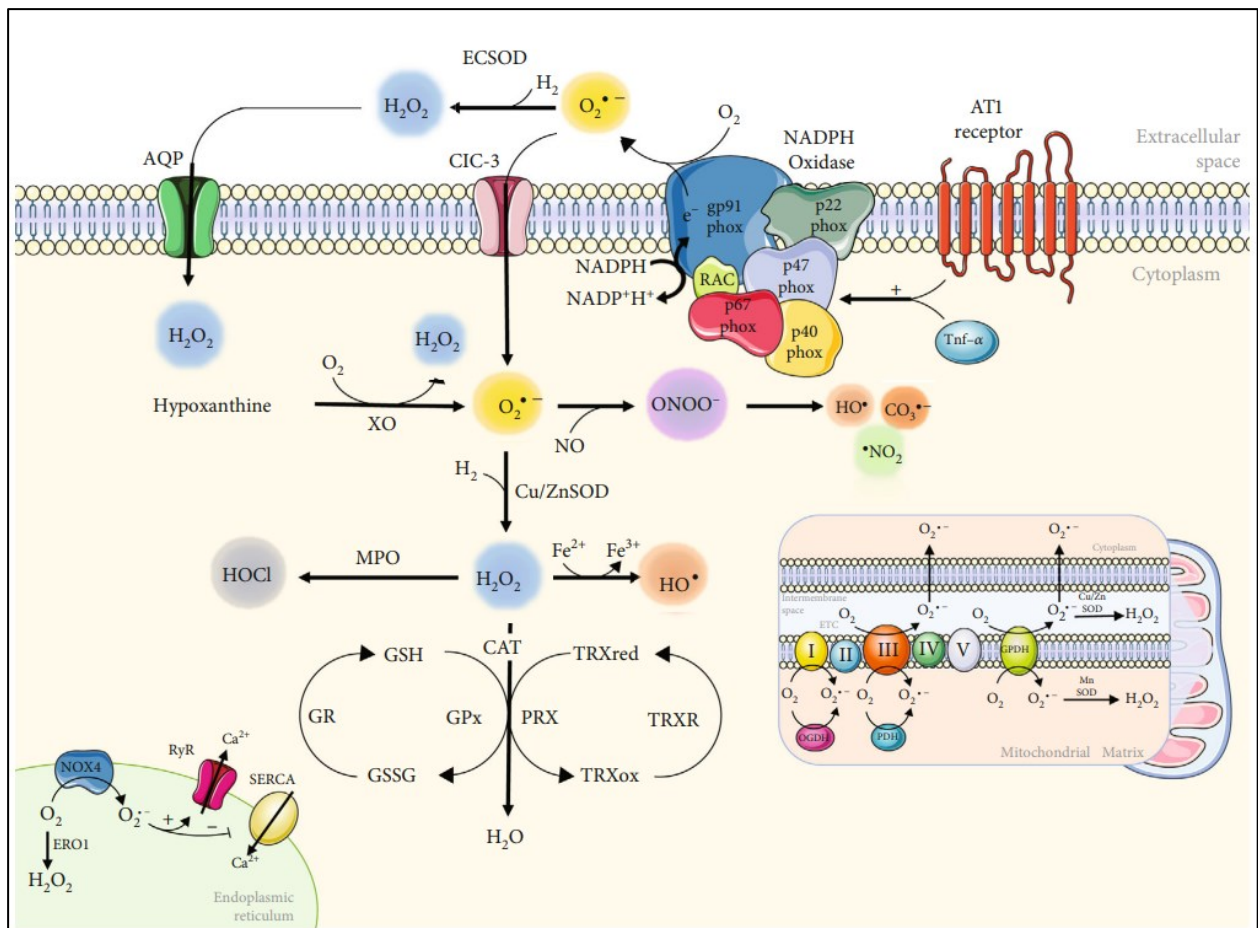


Figure 1. Schematic Overview of ROS Production and Elimination (46).

NOX, located in the plasma membrane, produces $O_2^{\bullet-}$ in the extracellular space by transferring an electron from cytoplasmic NADPH to O_2 . $O_2^{\bullet-}$ can be targeted by the ecSOD enzyme and converted into H_2O_2 , which can permeate the plasma membrane by aquaporins or be transported to the intracellular space by CIC-3. In the cytoplasm, $O_2^{\bullet-}$ can be produced by XO. In addition, $O_2^{\bullet-}$ reacts with NO to form $ONOO^-$, whose decomposition results in the formation of some very reactive species, such as OH^\bullet , $^{\bullet}NO_2$, and $CO_3^{\bullet-}$. However, the cytoplasmic isoform Cu/ZnSOD can act in $O_2^{\bullet-}$, producing H_2O_2 targeted by MPO, forming HOCl, or by CAT, GPx, and peroxiredoxins (PRX), forming H_2O . However, through the Fenton reaction, H_2O_2 is reduced to OH^\bullet , a highly toxic radical in the presence of iron. In the mitochondria electron transport chain (ETC) complexes I and III are the main sites of oxidant production, with $O_2^{\bullet-}$ production occurring both on the mitochondrial matrix side and in the intermembranous space of the mitochondria. Other important sources of ROS include the endoplasmic reticulum (ER), which impacts calcium signaling and proteostasis directly. Abbreviations: NADPH oxidase (NOX); extracellular superoxide dismutase (ECSOD); chloride channel-3 (CIC-3); xanthine oxidase (XO); myeloperoxidase (MPO); endoplasmic reticulum oxidoreductase 1 (ERO1); ryanodine receptors (RyRs); sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA); oxoglutarate dehydrogenase (OGDH); pyruvate dehydrogenase complex (PDH); tumor necrosis factor α (Tnf- α); reduced glutathione (GSH); oxidized glutathione (GSSG); glutathione reductase (GR); thioredoxin (TRX); and thioredoxin reductase (TRXR).

Key message: ROS are typical byproducts of cellular metabolism, playing a role as secondary messengers. Although excessive amount of reactive oxygen species (ROS) is associated with cellular damage through oxidative stress, they play a crucial role in regulating normal physiological processes. Both ROS and antioxidant systems, that counteract ROS effects, cooperate to maintain redox homeostasis and regulate the balance and consequences of redox status.

A.2 NRF2 Structure, Mechanisms of Regulation, and Downstream Targets

A.2.a Nuclear Factor Erythroid 2-Related Factor 2 (NRF2)

Maintaining homeostasis is vital for organismal health and survival, but cellular and environmental stress consistently challenges cell functions. NRF2 stands out as a master regulator, orchestrating cellular responses to oxidative stress and xenobiotics (46). Encoded by the nuclear factor (erythroid-derived 2)-like 2 genes (*NFE2L2*) located on chromosome 2q31.2 in humans (gene ID: 4780) (47), *NFE2L2* has a complex structure comprising five exons and four introns, resulting in eight transcript variants that encode six isoforms of NRF2 (48) (listed in **Table 1**). The NRF2 protein in humans is a 605-amino-acid protein (47), belonging to the cap 'n' collar (CNC) subfamily of basic leucine zipper (bZIP) transcription factors (49).

Table 1. List of NFE2L2 Transcript Variants and Encoded Protein Isoforms (47).

mRNA				Protein			
Transcript Variant	NCBI Reference Sequence	No of Base Pairs	Information	Isoform	NCBI Reference Sequence	No of Amino Acids	Information
1	NM_006164.5	2446 bp	The longest transcript variant encoding the longest isoform.	1	NP_006155.2	605 aa	The isoform has the canonical sequence.
2	NM_001145412.3	2988 bp	Uses an alternate promoter, 5' UTR and a downstream start codon vs. var. 1. It has a shorter N-terminus than isoform 1.	2	NP_001138884.1	589 aa	Protein lacks the Keap1 interaction domain, resulting in Nrf2 stabilization. Found in the lung and head-and-neck cancers.
3	NM_001145413.3	2967 bp	Uses an alternate promoter, 5' UTR, downstream start codon, and an alternate in-frame splice site in the 3' coding region vs. var. 1. It has a shorter N-terminus and is missing an internal segment than. isoform 1.	3	NP_001138885.1	582 aa	Protein lacks the Keap1 interaction domain, resulting in Nrf2 stabilization.
4	NM_001313900.1	2862 bp	Uses an alternate promoter, 5' UTR and a downstream start codon vs. var. 1. It has a shorter N-terminus than isoform 1.	2	NP_001138884.1	589 aa	Protein lacks the Keap1 interaction domain, resulting in Nrf2 stabilization. Found in the lung and head-and-neck cancers.
5	NM_001313901.1	2954 bp	Uses an alternate promoter, 5' UTR and a downstream start codon vs. var. 1. It has a shorter N-terminus than isoform 1.	2	NP_001138884.1	589 aa	Protein lacks the Keap1 interaction domain, resulting in Nrf2 stabilization. Found in the lung and head-and-neck cancers.
6	NM_001313902.1	2769 bp	Lacks an alternate in-frame exon in the 3' coding region vs. var. 1. It is shorter than isoform 1.	4	NP_001300831.1	575 aa	Computationally mapped isoform.
7	NM_001313903.1	2640 bp	Uses an alternate in-frame splice site in the 3' coding region vs. var. 1. It is shorter than isoform 1.	5	NP_001300832.1	532 aa	Computationally mapped isoform.
8	NM_001313904.1	2917 bp	Uses an alternate promoter, 5' UTR and an alternate in-frame splice site in the 3' coding region, vs. variant 1. It is shorter than isoform 1.	6	NP_001300833.1	505 aa	Computationally mapped isoform.

In terms of structure, NRF2 encompasses seven conserved domains known as NRF2-ECH homology (Neh) domains, labeled Neh1 through Neh7 (1) (**Figure 2**). These domains can be classified based on their functions: activation of NRF2 activity, NRF2 ubiquitination, and transactivation.

Activation of NRF2 Activity

- The Neh1 domain encompasses the DNA-binding region and is crucial for NRF2's interaction with small musculoaponeurotic fibrosarcoma proteins (sMAF) K, G, and F, as well as other bZip proteins. This interaction facilitates the recognition of antioxidant response elements (ARE) for gene transcription activation (50).

- The Neh2 domain acts as a redox-sensitive degron. It contains ETGE and DLG motifs that specifically interact with the Kelch domain of Kelch-like-ECH-associated protein 1 (KEAP1), negatively regulating NRF2 activity. It also includes seven lysine residues upstream of the ETGE motif, which are targets for ubiquitination by the Cul3 E3 ubiquitin ligase (51). This interaction mediates NRF2 ubiquitination and degradation.

NRF2 Ubiquitination

- The Neh6 domain acts as a redox-insensitive degron, providing Keap1-independent negative regulation of NRF2. Within Neh6, two conserved peptide motifs, DSGIS and DSAPGS, are recognized by the β -transducing repeat-containing protein (β -TrCP). The DSGIS motif contains a phosphorylation site for glycogen synthase kinase-3 (GSK3), which enhances β -TrCP activity upon GSK3-mediated phosphorylation of NRF2 (1). This phosphorylation facilitates the efficient binding of β -TrCP to Neh6 and promotes the recruitment of the Skp1-Cul1-F-box protein (SCF) ubiquitin ligase complex, leading to subsequent proteasomal degradation of NRF2 (52).

Transactivation Domains

- The Neh3 domain serves as a transactivation domain and interacts with chromodomain helicase DNA-binding protein 6 (CHD6), contributing to chromatin remodeling processes (53).
- The Neh4 and Neh5 domains also function as transactivation domains, working synergistically to achieve maximum activation of reporter gene expression (54).
- The Neh7 domain serves as the binding site for retinoid X receptor α (RXR α). Upon binding, RXR α disrupts the recruitment of cofactors to Neh4 and Neh5, essential for transactivation, thereby suppressing transcriptional activation of NRF2 (1).

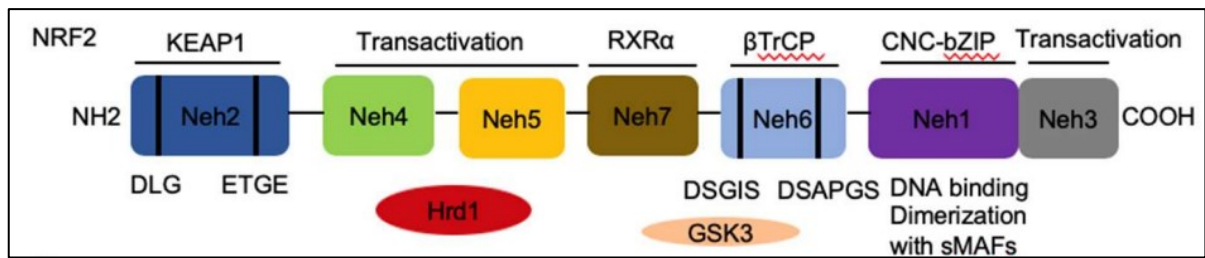


Figure 2. The Architecture of the Nuclear Factor Erythroid 2-Related Factor 2 (NRF2), adapted from (50).

NRF2 contains seven conserved NRF2-ECH homology (Neh) domains, Neh1-Neh7. Neh1 contains a basic leucine zipper (bZip) motif, where the basic region is responsible for DNA binding, and the Zip dimerizes with other binding partners such as sMAFs. Neh2 contains ETGE and DLG motifs, which are required for the interaction with KEAP1 and subsequent KEAP1-mediated proteasomal degradation. Neh3, 4, and 5 domains are transactivation domains of NRF2. Neh4 and 5 domains also interact with HRD1, which mediates NRF2 degradation. Neh6 contains two β TrCP degrons, DSGIS and DSAPGS, that are responsible for the β -TrCP mediated proteasomal degradation.

The cap 'n' collar (CNC) subfamily includes, alongside NRF2, other members such as NRF1, and NRF3 (55). The emergence of three closely related NRF family members in vertebrates resulted from genomic duplications, as NRF1, NRF2, and NRF3, are all located near related homeobox genes (i.e., HOXB, HOXD, HOXA, and HOXC, respectively) (56).

Unlike NRF2, the functional importance of other NRF transcription factors is less well understood. Recent studies have shown that NRF1 mediates proteasome homeostasis (57). In contrast to NRF2, which shuttles in the cytosol and the nucleus based on the oxidative stress status of the cell, NRF1 is primarily localized to the endoplasmic reticulum (ER). For NRF1 to become transcriptionally active and nuclear-localized, it must be actively transported across the ER membrane, deglycosylated, and proteolytically cleaved to release a transcriptionally active and nuclear-localized form of the transcription factor. This process allows NRF1 to regulate gene expression in the nucleus by binding to Antioxidant Response Elements (ARE) sequences. Despite the recognized importance of NRF1 in physiology, the complete range of genes under its transcriptional control has yet to be fully elucidated.

NRF3, the least explored member of the NRF transcription factor family, lacks obvious phenotypes in knockout animals and does not contribute significantly to the effects seen in animals lacking NRF2. However, recent reports have demonstrated NRF3's essentiality in cancer cells to maintain their basal proteasome activity and regulate the gene expression of the cell cycle regulator U2AF homology motif kinase 1 (UHMK1) for cell proliferation. Nevertheless, its role in normal physiology remains largely unknown (58,59).

A.2.b Mechanisms of NRF2 Regulation

A.2.b.i Regulation of NRF2 protein stability: KEAP1-dependent and independent mechanisms

(1) *KEAP1 dependent or canonical NRF2 activation*: Normally, cytoplasmic NRF2 has a brief lifespan, typically ranging from 10 to 30 minutes (60,61), due to KEAP1's tight control over its protein levels (62). KEAP1 is a multi-domain protein in the BTB-Kelch family, essential for regulating NRF2. It consists of 624 amino acids, including 27 critical cysteine residues (63). KEAP1 forms part of the Cullin-RING E3 ubiquitin ligases (CRLs) by assembling with the Cul3 E3 ubiquitin ligase and RING box protein-1 (Rbx1) (64). KEAP1 has three functional domains (64) (**Figure 3**), including:

- The N-terminal BTB (Broad complex, Tramtrack, and Bric à brac) domain senses environmental electrophiles, facilitates KEAP1 homodimerization, and interacts with Cul3.
- The intervening region (IVR), contains a "3-box" motif and reactive cysteine residues (Cys226, Cys257, Cys273, and Cys288) that regulate NRF2 activity.
- The C-terminal Kelch domain (DGR domain): also known as the double glycine repeat (DGR) domain, is crucial for binding to NRF2.

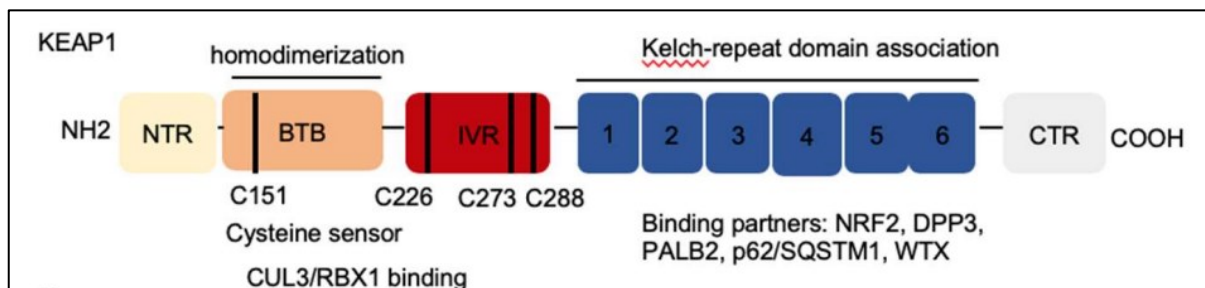


Figure 3. The Architecture of the Kelch-Like-Ech Associated Protein 1 (KEAP1), adapted from (49).

KEAP1 contains five domains, an amino-terminal region (NTR), a broad complex, tramtrack, bric-a-brac (BTB) domain, an intervening region (IVR), six Kelch domains, and the C-terminal region (CTR). The Kelch domain and CTR mediate the interaction with NRF2, p62, DPP3, WTX, and PALB2 that contain ETGE motifs. The BTB domain of KEAP1 facilitates its homodimerization and contributes to the interaction of IVR with the Cul3/RBX1 complex.

Under basal conditions, NRF2-KEAP1 interaction is mainly driven by the ETGE motif, which fits into a pocket within the C-terminal KEAP1's Kelch domain, and the DLG motif in the Neh2 domain acts as a secondary binding site with weaker affinity (64). Hence, NRF2 associates with a KEAP1 homodimer, which subsequently binds to the Cul3-Rbx1 E3 ubiquitin ligase complex (65) (**Figure 4**), promoting the polyubiquitination of NRF2 and degradation. This process ensures that NRF2

levels remain low under non-stress conditions, preventing unwarranted activation of its target genes.

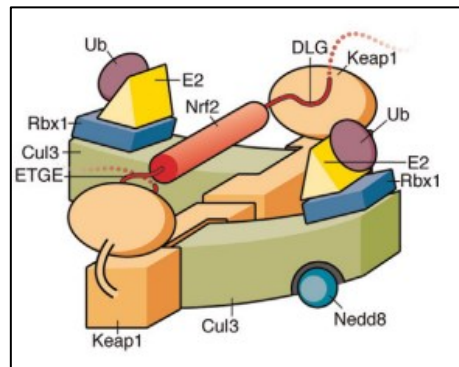


Figure 4. Schematic Representation of KEAP1-NRF2 Interaction and Ubiquitination (64).

This diagram illustrates the interaction between KEAP1 and NRF2 and the subsequent ubiquitination process. KEAP1: Kelch-like ECH-Associated Protein 1 (KEAP1); Nuclear Factor Erythroid 2-Related Factor 2 (NRF2); A conserved motif in the Neh2 domain of NRF2 critical for KEAP1 binding (ETGE); A secondary binding motif in the Neh2 domain of NRF2 (DLG); Ubiquitin (Ub); Cullin 3 (CUL3); RING-box protein 1 (Rbx1); Ubiquitin-conjugating enzyme (E2); Neural precursor cell-expressed developmentally downregulated protein 8 (Nedd8).

Under stressed conditions, KEAP1 undergoes conformational changes (66,67) driven primarily by the "cysteine code" concept. This involves the covalent modification of cysteine residues, which act as sensors for their preferred inducers (67). This concept is supported by the fact that different reactive chemicals (inducers) can modify specific combinations of cysteines to control the KEAP1/NRF2 stress-sensitive response (68). Additionally, KEAP1 features a sophisticated Zn^{2+} sensing mechanism involving specific amino acids such as His-225, Cys-226, and Cys-613. These amino acids can detect liberated Zn^{2+} ions from damaged proteins. Upon Zn^{2+} binding, KEAP1 undergoes structural alterations, disrupting its interaction with the Cul3-RING ubiquitin ligase (CRL) adaptor/scaffold protein (69). Together, these modifications affect the KEAP1-based E3 ubiquitin ligase complex, hindering its proper alignment and interaction with NRF2.

Consequently, the conformational shift in KEAP1 leads to the detachment of the DLG motif from the KEAP1-NRF2 complex, inhibiting NRF2 ubiquitination (**Figure 5**). Subsequently, NRF2 is released, phosphorylated at the Neh2 domain by protein kinase C (PKC), and translocated to the nucleus (70). In the nucleus, NRF2 heterodimerizes with small musculoaponeurotic fibrosarcoma proteins (sMAFs), acting as co-activator dimeric partners (47), and binds to antioxidant response element (ARE) domains. This binding induces the transcription of NRF2 target cytoprotective genes (71). Once the redox equilibrium is restored, NRF2 is targeted for proteasome degradation to maintain basal levels of NRF2 and deactivate the NRF2/KEAP1 signaling pathway (61).

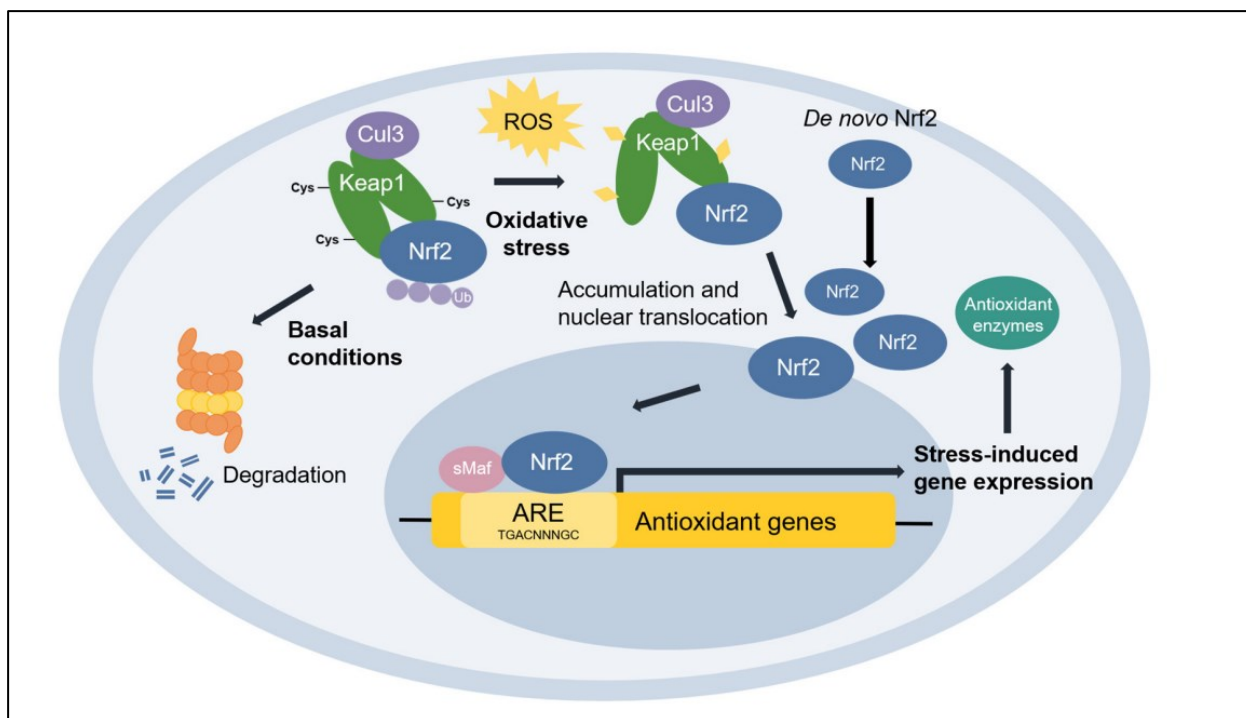


Figure 5. The KEAP1-NRF2 Pathway (1).

Under basal conditions, KEAP1 is bound to NRF2, and NRF2 is ubiquitinated by the Cul3 E3 ubiquitin ligase for degradation by the proteasome. Upon oxidative stress, sensor cysteines in KEAP1 are modified by ROS, leading to NRF2 stabilization, accumulation, and translocation to the nucleus where NRF2 heterodimerizes with sMaf and binds to the ARE to activate the transcription of antioxidant genes. Kelch-like ECH-associated protein 1 (KEAP1); Nuclear factor erythroid 2-related factor 2 (NRF2); Cullin 3 (Cul3); Reactive oxygen species (ROS); Small musculoaponeurotic fibrosarcoma proteins (sMaf); Antioxidant response element (ARE).

Yet other degradation pathways can contribute to NRF2 degradation independent of NRF2-Keap1 interaction (88). For instance, p62-mediated autophagy dysfunction. The p62/SQSTM1 (sequestosome 1) protein is a downstream target of NRF2 that acts as a cargo receptor for the autophagic degradation of ubiquitinated targets. It actively contributes to NRF2 activation through a positive feedback loop, eliciting selective autophagy of KEAP1 (111). Additionally, NRF2 activation could be mediated through KEAP1 inhibition. Electrophiles (72) gonadotrophins, and estrogen, which oxidize KEAP1's cysteine residues (113) also lead to the inhibition of KEAP1 and subsequent activation of NRF2.

(2) *KEAP1 independent or non-canonical NRF2 activation*: Additional regulatory mechanisms are known to be non-canonical NRF2 regulatory pathways (Figure 6). First, there is the β -transducing repeat-containing protein (β -TrCP) axis. β -TrCP recognizes two motifs, DSGIS and DSAPGS, in NRF2's Neh6 domain. Glycogen synthase kinase-3 (GSK3) phosphorylates the DSGIS motif, enhancing β TrCP's affinity for NRF2, thereby promoting NRF2 ubiquitination and degradation (49). Another regulatory axis, established by Lo *et al.*, involves the WDR23-DDB1-CUL4 complex. WDR23 binds to the DIDLID sequence within NRF2's Neh2 domain, regulating NRF2

ubiquitination and degradation (73). The CR6-interacting factor 1 (CRIF1) regulatory axis, also known as growth arrest and DNA damage-inducible proteins-interacting protein 1, shows that CRIF1 interacts with NRF2's Neh2 and C-terminal regions, promoting NRF2 ubiquitination (74). Both pathways mediate NRF2 proteasome degradation both under normal and stressed conditions. Unlike KEAP1-mediated NRF2 degradation that primarily occurs in the cytoplasm, KEAP1-independent regulation affects both cytoplasmic and nuclear NRF2 stability, contributing to the termination of NRF2-mediated transcriptional responses (73–75). Lastly, there is the 3-Hydroxy-3-methylglutaryl reductase degradation (HRD1) axis, which functions as an E3 ubiquitin ligase associated with the endoplasmic reticulum (ER) participating in ER-associated degradation. Under conditions of ER stress, HRD1 interacts with the Neh 4–5 domains of NRF2, leading to the ubiquitylation and subsequent degradation of NRF2, particularly in cirrhotic liver (76). Also, kinases that phosphorylate specific sites on the NRF2 protein, lead to distinct outcomes. For instance, PKC phosphorylates Ser-40 in the Neh2 domain, hindering complex formation with KEAP1 and promoting NRF2 nuclear import (70). Casein kinase 2 (CK-2) phosphorylates serine and threonine residues in the Neh4 and Neh5 domains, elevating nuclear NRF2 levels and enhancing its transcriptional activity (77). MAPKs phosphorylate serine and threonine residues in Neh1, Neh3, and Neh7 domains, facilitating NRF2 nuclear accumulation (78). Additionally, PI3K activation in an oxidative environment disrupts actin polymerization, freeing NRF2 from KEAP1 tethering (79). Finally, 5' AMP-activated protein kinase (AMPK) regulates NRF2 translocation to the nucleus, responding to cellular energy levels for activation (80).

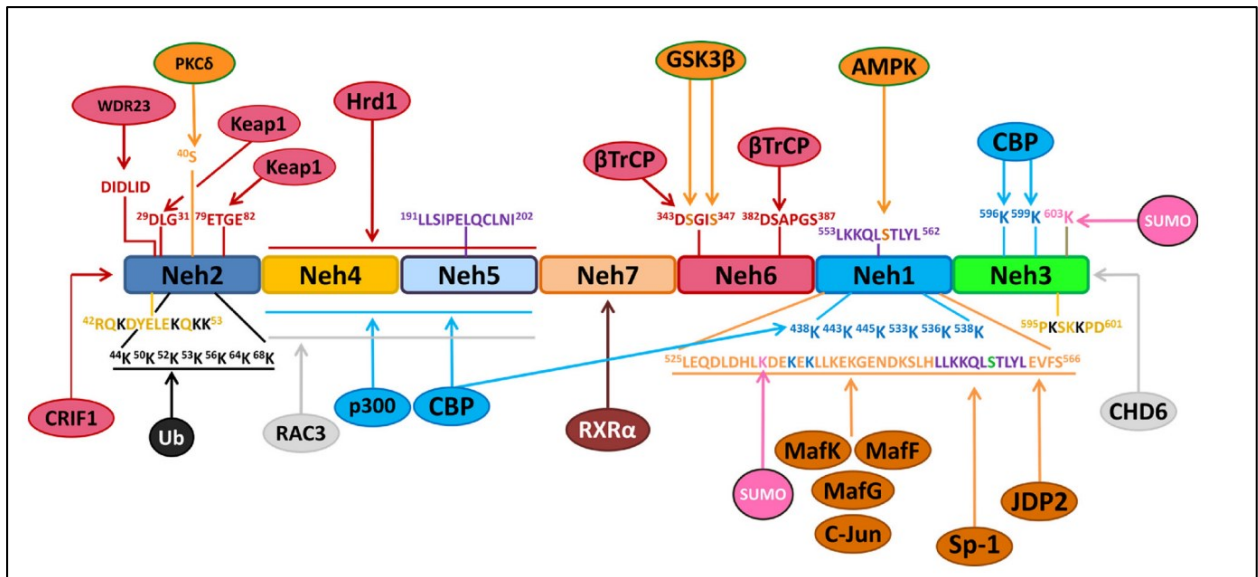


Figure 6. Canonical and Non-Canonical Regulatory Axis Interaction with NRF2 to Modulate its Activation, adapted from (81). Protein Kinase C delta (PKC δ); WD Repeat Domain 23 (WDR23); HMG-CoA reductase degradation protein 1 (Hrd1); Beta-transducin repeat-containing protein (β TrCP); Glycogen Synthase Kinase 3 beta (GSK3 β); AMP-activated protein kinase (AMPK); CREB-binding protein (CBP); Small Ubiquitin-like Modifier (SUMO); E1A-associated protein p300 (p300); Receptor-associated coactivator 3 (RAC3); Retinoid X Receptor alpha (RAC3); CR6-interacting factor 1 (CRIF1); Ubiquitin (Ub); Musculoaponeurotic Fibrosarcoma Oncogene Homolog K (MafK); Musculoaponeurotic Fibrosarcoma Oncogene Homolog F (MafF); Jun Proto-Oncogene (C-Jun); Jun Dimerization Protein 2 (JDP2); Specificity Protein 1 (Sp-1); Chromodomain Helicase DNA Binding Protein 6 (CHD6); NRF2-ECH homology 1-7 (Neh1-7).

A.2.b.ii Other Mechanisms of NRF2 Regulation

Other mechanisms of NRF2 regulation may occur at the transcriptional, post-transcriptional, and post-translational levels. On the transcriptional level, polycyclic aromatic hydrocarbons (82) can activate the *NFE2L2* gene. Oncogene stimulation, mediated by KRAS and BRAF induction of JUN and MYC transcription factors, also activates NRF2 (83). Additionally, transcription factors such as Jun dimerization protein (JDP2), JUN, CREB binding protein (CBP), Brahma-related gene 1 (BRG1), and p21 induce NRF2 activation. In contrast, Fos proto-oncogene, AP-1 transcription factor subunit (cFOS), p53, p65, Fos-related antigen 1 (FRA1), BTB and CNC homology 1 transcription factor (BACH1), CCAAT/enhancer-binding protein (C/EB), activating transcription factor 1 (ATF1), activating transcription factor 3 (ATF3), short-form estrogen-related receptor (SFERR), peroxisome proliferator-activated receptor α (PPAR- α), and retinoic acid receptor (RAR) inhibit NRF2 transcription (84). At the post-transcriptional level, microRNAs (miRNAs) such as miR-507, miR-634, miR-450a, and miR-129-5p suppress NRF2 translation (85). At the post-translational level, hypermethylation of CpG sites in the KEAP1 promoter region in various cancer types leads to reduced KEAP1 expression and subsequent constitutive NRF2 pathway activation (86,87).

A.2.c NRF2 Downstream Targets in Antioxidant Stress Response and Drug Detoxification

The NRF2/ARE pathway regulates numerous ARE-containing cytoprotective genes encoding enzymes involved in essential cell-rescue processes (72) (summarized in **Figure 7**). These enzymes can be categorized into five groups based on the specificity of their functions. First are the phase I enzymes required for mediating oxidation, reduction, and hydrolytic reactions of xenobiotics. This group includes enzymes such as NQO1, carbonyl reductases (CBRs), aldo-keto reductases (AKRs), aldehyde dehydrogenases, and specific cytochrome P450 oxidoreductases, including cytochrome P450s (CYPs) (88). Second, phase II enzymes that catalyze conjugation reactions. This category includes important enzymes like glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), enzymes involved in UDP-glucuronic acid synthesis, and heme oxygenase-1 (HO-1) (72,88). Third, phase III enzymes are responsible for transporting the conjugated metabolites following phase II reactions, primarily functioning as drug efflux transporters. These include multidrug resistance-associated proteins (MDR), breast cancer resistance protein (BCRP), and ATP-binding cassette (ABC) transporters [71]. Fourth, NRF2 triggers antioxidant pathways, including enzymes involved in glutathione (GSH) production, utilization, and regeneration. Key NRF2 targets in GSH synthesis are glutamate-cysteine ligase catalytic (GCLC) and modulator (GCLM) subunits, and glutathione synthetase (GSS) (75). NRF2 also regulates the xCT transporter, which mediates the uptake of cysteine for GSH synthesis (89). Moreover, NRF2 controls several glutathione S-transferases (GST) that eliminate ROS in addition to glutathione reductase (GR) enzymes, involved in GSH synthesis and reduction. These enzymes facilitate GSH regeneration and generate nicotinamide adenine dinucleotide phosphate (NADPH), essential for cellular defense mechanisms (75). Finally, other NRF2 targets include the redox cycling enzymes such as thioredoxin, thioredoxin reductase, sulfiredoxin, peroxiredoxin, superoxide dismutase 1 (SOD1), and catalase (CAT) (49).

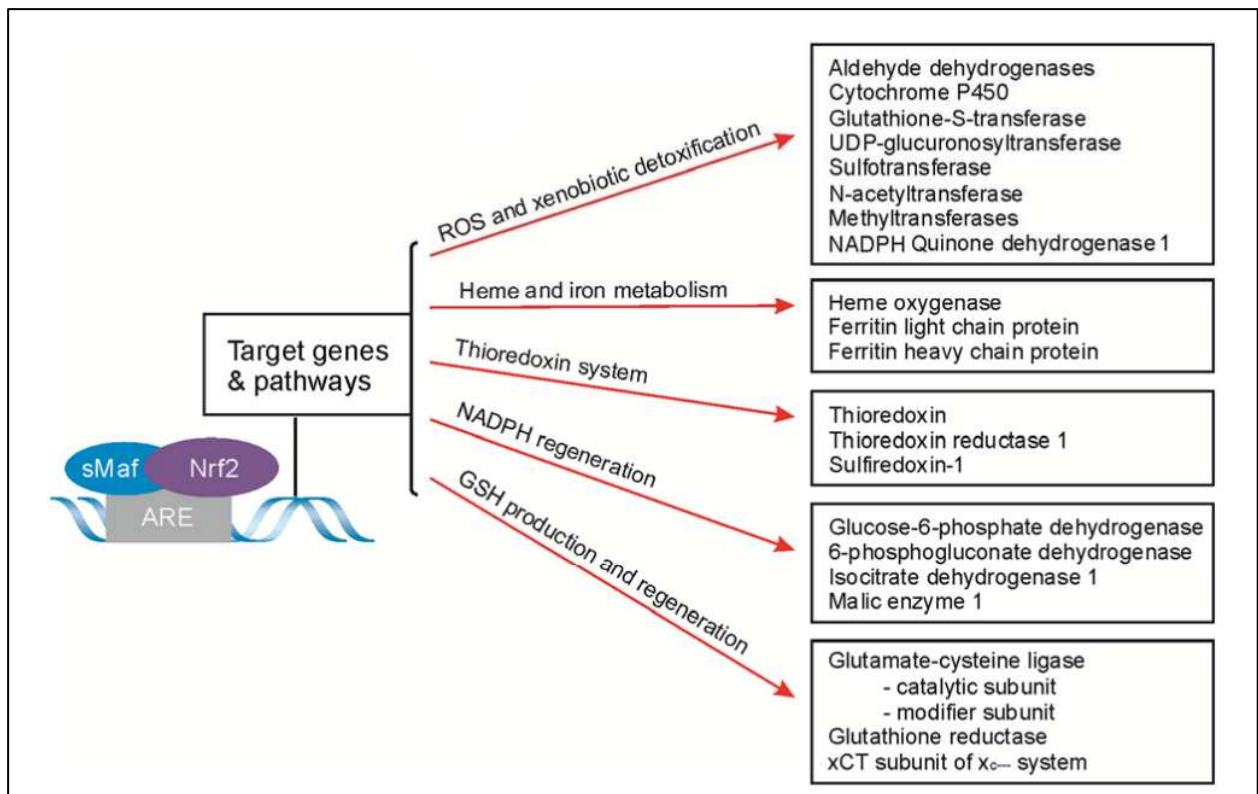


Figure 7. The Principal Cytoprotective Enzymes Encoded by the ARE-driven Genes (1).

Glutathione (GSH); Reduced nicotinamide adenine dinucleotide phosphate (NADPH); Uridine diphosphate (UDP); Cystine/glutamate transporter (xCT).

Key message: NRF2, encoded by the NFE2L2 gene, acts as a master regulator of antioxidant responses, orchestrating cellular adaptations to oxidative stress. Structurally, NRF2 comprises multiple conserved domains, each with distinct functions in its regulation and activity. Notably, the interaction between NRF2 and its negative regulator, KEAP1, governs NRF2 stability and activity. Also, KEAP1-dependent mechanisms may also modulate NRF2 activation and degradation. Various post-translational modifications and signaling pathways further regulate NRF2 activity, ensuring tight control over its transcriptional responses. Additionally, transcriptional, post-transcriptional, and epigenetic mechanisms contribute to the fine-tuning of NRF2 expression and function, highlighting the complexity of NRF2 regulation in maintaining cellular homeostasis.

NRF2 is essential for cellular protection against oxidative stress and detoxification processes. It regulates genes encoding enzymes involved in xenobiotic metabolism (Phase I), conjugation reactions (Phase II), and drug efflux (Phase III). NRF2 also promotes antioxidant pathways by enhancing GSH production and regeneration and controlling redox cycling enzymes, ensuring comprehensive cellular defense and homeostasis. This highlights its pivotal role in maintaining cellular health and resilience against environmental challenges.

B. NRF2 in Brain Physiology

B.1 Brain Composition and Susceptibility to Oxidative Stress

Quantifying the cellular composition of the human brain presents significant challenges due to its large size, complex cell makeup, and limited availability of postmortem brain samples (90). Recent advancements, such as the isotropic fractionator, reveal a glia-to-neuron ratio close to 1:1 and a total glial cell count of fewer than 100 billion, including astrocytes, oligodendrocytes, and microglia (91). The brain's vulnerability to oxidative stress is profound, owing to its high metabolic activity, reliance on oxidative phosphorylation for energy production, absence of energy reserves, abundance of peroxidizable lipids, and elevated iron levels (92,93). To counteract this susceptibility, the NRF2 pathway orchestrates a network of antioxidant defenses, including the glutathione (GSH) system, thioredoxin/peroxiredoxin system, superoxide dismutase (SOD), and catalase, to neutralize excess ROS (94,95). Notably, the expression pattern of the *NFE2L2* gene, encoding NRF2, exhibits regional variations across the brain (**Figure 8A**). Compared to other parts of the brain, high expression levels are observed in vital regulatory centers like the medulla oblongata, responsible for key homeostatic functions, and the basal ganglia, governing motor control, executive functions, and emotions (96). Conversely, the hippocampus displays lower *NFE2L2* expression levels. Furthermore, NRF2 expression varies among different brain cell types, with oligodendrocytes exhibiting the highest levels and neurons the lowest (**Figure 8B**).

B.2 NRF2 in Glial Support and Vulnerability to Oxidative Stress

NRF2 demonstrates diverse activities in the proper physiology of brain cells including its cytoprotective effects (97).

Astrocytes, represent the predominant type of glial cell and play vital roles in brain homeostasis and function (98). Their elaborate branching structure facilitates metabolic exchanges between blood vessels and neurons, enabling nutrient and neurotransmitter transport (99,100). Through the astrocyte-neuron lactate shuttle (ANLS), astrocytes contribute to neuronal energy metabolism by providing lactate derived from glucose (101,102). Moreover, by forming tripartite synapses with pre- and post-synaptic neuronal structures, astrocytes modulate neurotransmitter

release like glutamate, gamma-aminobutyric acid (GABA), dopamine, and serotonin, thereby influencing synaptic transmission and plasticity (103).

Overexpression of NRF2 in astrocytes has shown promise in mitigating cognitive impairment induced by hypoperfusion, attributed to reduced white matter disruption and inflammation (104). Additionally, NRF2 activation suppresses the activation of reactive astrocytes, by counteracting NF- κ B subunit p65 recruitment, attenuating neuroinflammation (105). In spinal cord injury models, astrocytic NRF2 activation has been linked to reduced inflammation and demyelination (106). Beyond neuroprotection, NRF2 activation in astrocytes influences energy metabolism, favoring oxidative phosphorylation over glycolysis under conditions of limited glucose availability (107).

Neurons, the fundamental units of the brain, are electrically excitable cells responsible for processing information and performing various functions (108). They are highly susceptible to oxidative stress due to their reliance on oxidative phosphorylation, enrichment with redox-active metal ions, and the presence of oxidizable polyunsaturated fatty acids (109).

Despite this vulnerability, neurons exhibit low basal expression of NRF2 (**Figure 8B**) (110,111), attributed to epigenetic repression caused by NRF2 promoter hypo-acetylation (111), and rapid Cul3-dependent NRF2 degradation (112). While ectopic expression of NRF2 in neurons can confer protection against oxidative insults (113), it may hinder their maturation (111) and suppress their developmental signaling pathways (114). Hence, reduced NRF2 expression facilitates redox signaling involved in their development (95). Notably, neurons rely on neighboring astrocytes for antioxidant support maintained by cysteine and GSH supply (115). This neuron-astrocyte interaction forms a regulatory loop that sustains NRF2 activation in astrocytes to uphold their neuroprotective function (116).

Microglia, are the resident immune cells of the brain, exhibiting regional diversity (117) and dynamic responses to neuronal activity such as neuronal proliferation and differentiation, as synaptic rebuilding in addition to debris cleaning (118). In pathological conditions, activated microglia can adopt a proinflammatory (M1) state, releasing cytokines like TNF α , IL-6, and IL-1 β (119,120). NRF2 activation promotes the transition of microglia from proinflammatory to neuroprotective M2 state, in response to cues of signaling pathways, including TGF- β /SMAD, TLR/NF- κ B, and JAK/STAT (121). The higher expression levels of NRF2 in astrocytes and microglia compared to neurons suggest their critical roles in protecting neurons from oxidative damage (122).

Oligodendrocytes, another type of glial cells, are responsible for myelination and rapid impulse transmission (123) and are susceptible to oxidative stress during their differentiation and function (124). NRF2 activation in oligodendrocytes plays a crucial role in mitigating oxidative damage (125), suppressing neuroinflammation, and maintaining myelin integrity (126). However, further research is needed to elucidate the implication of NRF2 activation in oligodendrocytes across various neurological disorders.

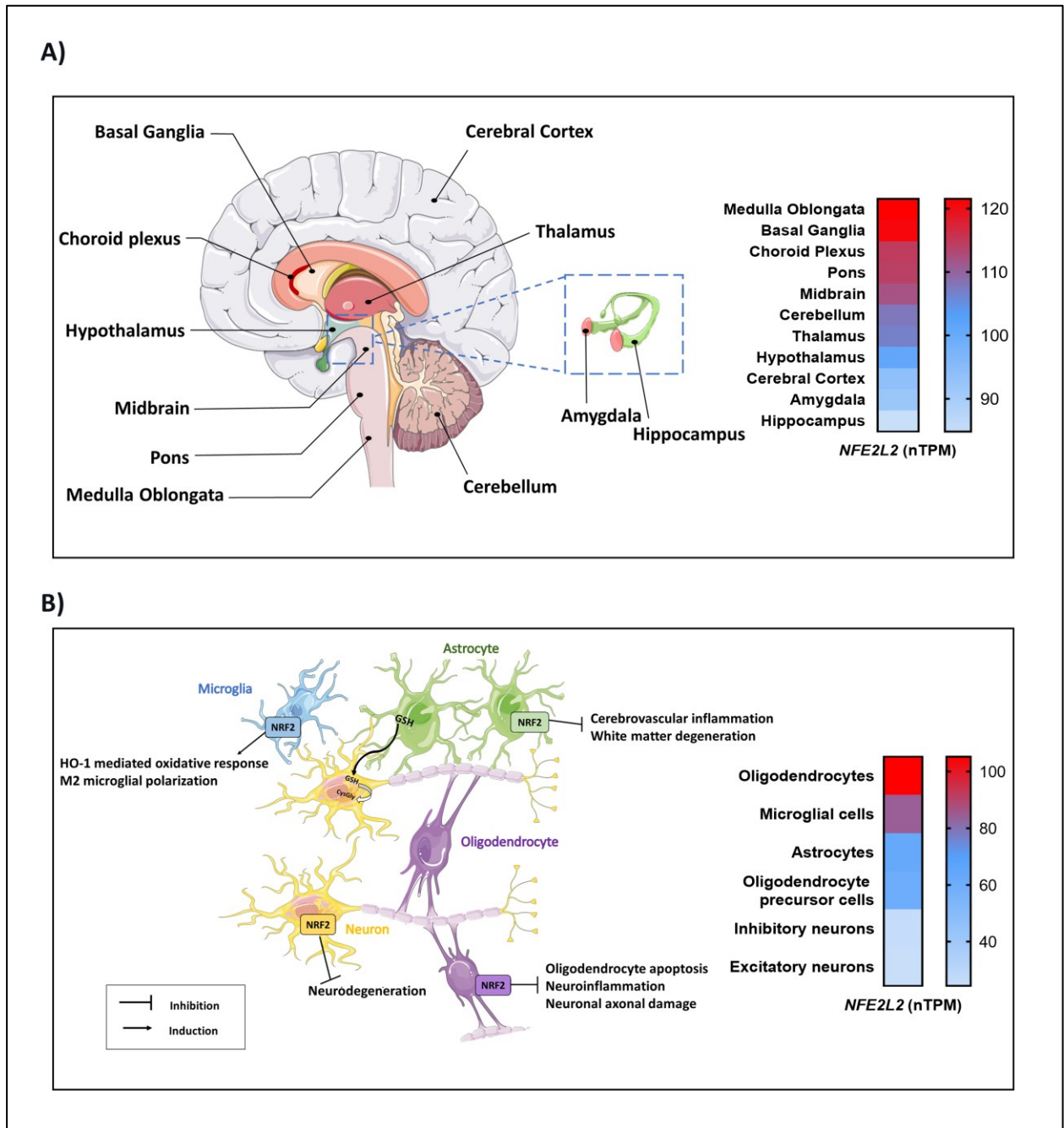


Figure 8. Overview of NFE2L2 Gene Expression and the Role of NRF2 in Brain Physiology (66).

(A) Human brain regions are visually represented on the left side, while the accompanying heat map on the right side displays NFE2L2 gene expression across the various human brain regions. The data were sourced from the human protein atlas (HPA) dataset. (B) Graphical summary of NRF2's role in brain physiology among the different brain cells on the left side, while the accompanying heat map on the right side displays NFE2L2 gene expression across the different brain cells. The data were sourced from the RNA single cell type data from HPA. nTPM, normalized transcript per million; HO-1, heme oxygenase 1; CysGly, cysteinylglycine dipeptide; GSH, glutathione.

B.3 NRF2 in Brain Diseases and Therapy

B.3.a NRF2 in Age-Related Neurodegenerative Disorders

The hallmarks of aging are intricately linked to the dysregulation of NRF2, a master regulator of cellular antioxidant responses, which in turn affects molecular, cellular, and systemic processes (**Figure 9**) (211). This decline in NRF2 activity correlates also with the cognitive decline observed in aging and various neurodegenerative diseases (206).

Alzheimer's disease (AD), is characterized by amyloid plaques, neurofibrillary tangles, and hyperphosphorylated tau proteins (212). Notably, decreased NRF2 expression is observed in brain samples of AD patients (213). NRF2 deletion in early AD pathology leads to elevated beta-amyloid (A β) levels, heightened inflammation, DNA damage, and impaired neuronal function (214–216). Conversely, in AD mice models, overexpression of NRF2 achieved through the injection of a lentiviral vector designed to express NRF2, has shown promising outcomes, including reduced astrocyte reactivity, increased HO-1-mediated neuroprotection, and improved spatial learning (217). Additionally, NRF2 overexpression in the hippocampus of AD mice attenuates amyloid pathology, glial cell reactivity, and cognitive deficits (218).

Parkinson's disease (PD), characterized by dopaminergic neuronal degeneration in the substantia nigra (219), exhibits a reduced NRF2 expression in patient samples that worsens the disease pathology and behavioral dysfunction (220,221). NRF2 knockout in mice models leads to increased neuronal loss, astrogliosis, and microgliosis (206). Moreover, NRF2 downregulation promotes α -synuclein aggregation (222), contributing to neuroinflammation and neuronal dysfunction in PD (223).

Additionally, NRF2's role in neurodegenerative diseases extends to Huntington's disease (HD). HD resulting from the mutant huntingtin gene (HTT), triggers accelerated aging processes within neurons and cellular physiology, accompanied by oxidative stress (224). Evidence has highlighted NRF2 activation as a promising therapeutic approach in HD mouse models. NRF2 activation helped to mitigate HD progression by reducing oxidative stress (225) and attenuating brain atrophy (226).

Moreover, Amyotrophic Lateral Sclerosis (ALS), characterized by progressive motoneuron degeneration, results in muscle paralysis and respiratory failure (227). ALS patient samples reveal

a reduced NRF2 expression in the motor cortex and spinal cord (228). NRF2-mediated antioxidant responses offer neuroprotection against ALS-associated oxidative stress and neuroinflammation by reducing oxidative stress caused by mitochondrial dysfunction and neuroinflammation (227). Furthermore, overexpression of NRF2 prolongs survival in ALS animal models, highlighting its therapeutic potential (229).

In conclusion, NRF2 emerges as a pivotal player in the network of cellular responses, oxidative stress, and inflammation, offering promising therapeutic avenues for mitigating the progression of age-related neurodegenerative diseases.

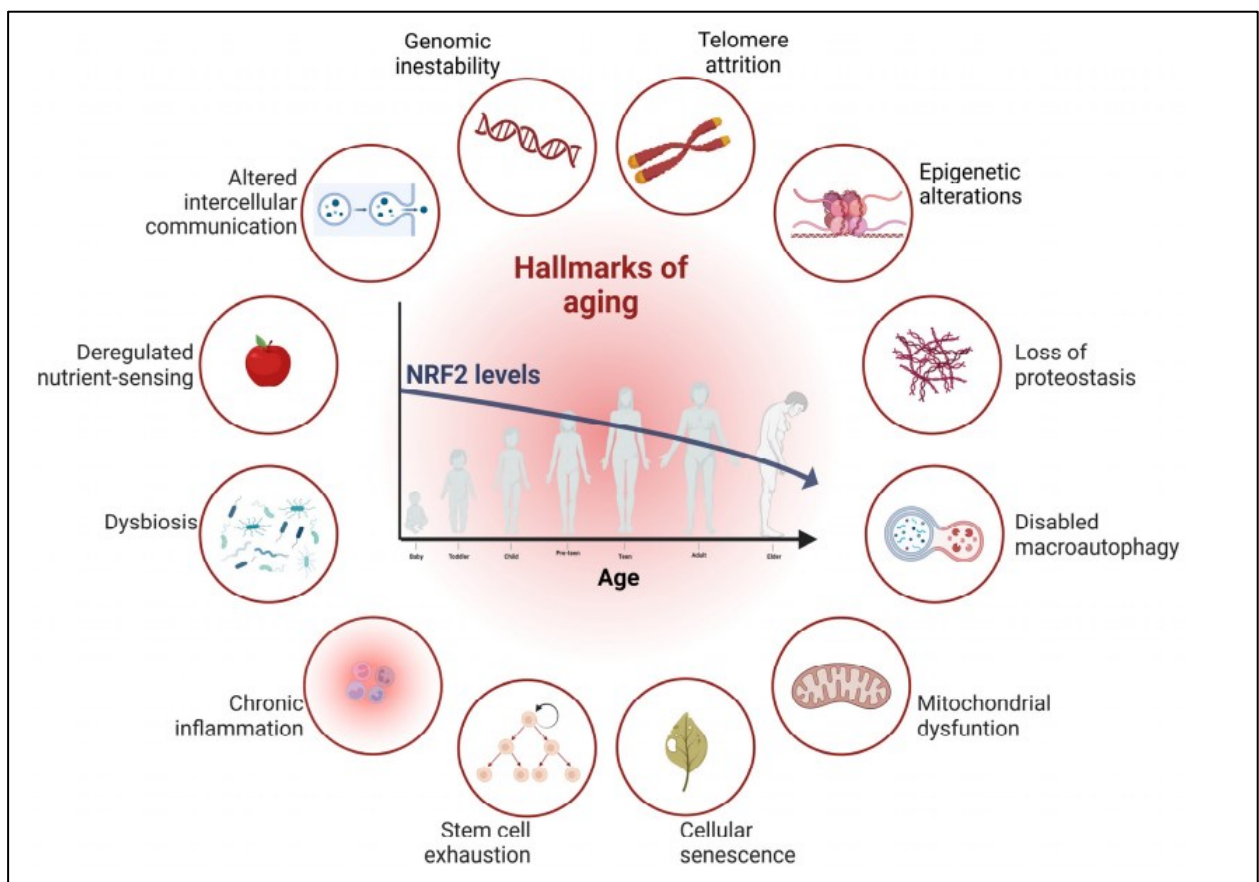


Figure 9. Interconnections Between Aging and NRF2 (127).

The proposed 12 hallmarks of aging and the involvement of the transcription factor NRF2.

B.3.b NRF2 in the Landscape of Primary Brain Tumors

B.3.b.i Genetic and Environmental Risk Factors for Primary Brain Tumors

A variety of risk factors are associated with the onset of brain tumors, including genetic predispositions. Specifically, single-gene inherited disorders such as Li-Fraumeni syndrome and neurofibromatosis markedly increase the risk of developing brain tumors. These conditions involve mutations in critical tumor suppressor genes, which provide cells with growth advantages that can lead to tumor formation in 5 to 10% of all brain tumor cases. Additionally, genome-wide association studies have unveiled inherited risk variants associated with brain tumors, encompassing genes implicated in detoxification, inflammation, DNA repair, and cell cycle regulation (128).

Exposure to moderate-to-high doses of radiation is another risk factor for brain tumors, though less than 5% of these cancers are attributed to radiation exposure. Radiation therapy for childhood cancers, particularly acute lymphoblastic leukemia, increases the risk of developing brain tumors later in life (127). Furthermore, maternal diagnostic radiation during pregnancy is also linked to an increased risk of brain tumor development (129). Moreover, research indicates that exposure to pesticides is associated with more than a 20% increased risk of developing brain cancer (130). Additionally, viral infections can influence tumor progression without directly causing its initial transformation (131). For example, human cytomegalovirus (hCMV) has been detected in nearly 80% of patient brain tumor cases, suggesting a significant role in tumor development or progression. Furthermore, the presence of herpes lymphotropic virus (HHV-6) has been documented in both adult and pediatric brain tumors (131).

Conversely, allergies appear to reduce the risk of brain tumors. Allergic conditions, atopic diseases, and early-life exposure to postnatal infections are associated with a lower risk of CNS tumors. The precise mechanism behind this protective effect remains incompletely understood but is hypothesized to involve enhanced immune surveillance, which impedes abnormal cell growth and diminishes the risk of brain tumor development (132). Moreover, other factors may contribute to brain tumor onset as presented in (**Figure 10**), however, further elucidation of their precise associations and strengths can provide valuable insights into their relative significance in glioma development.

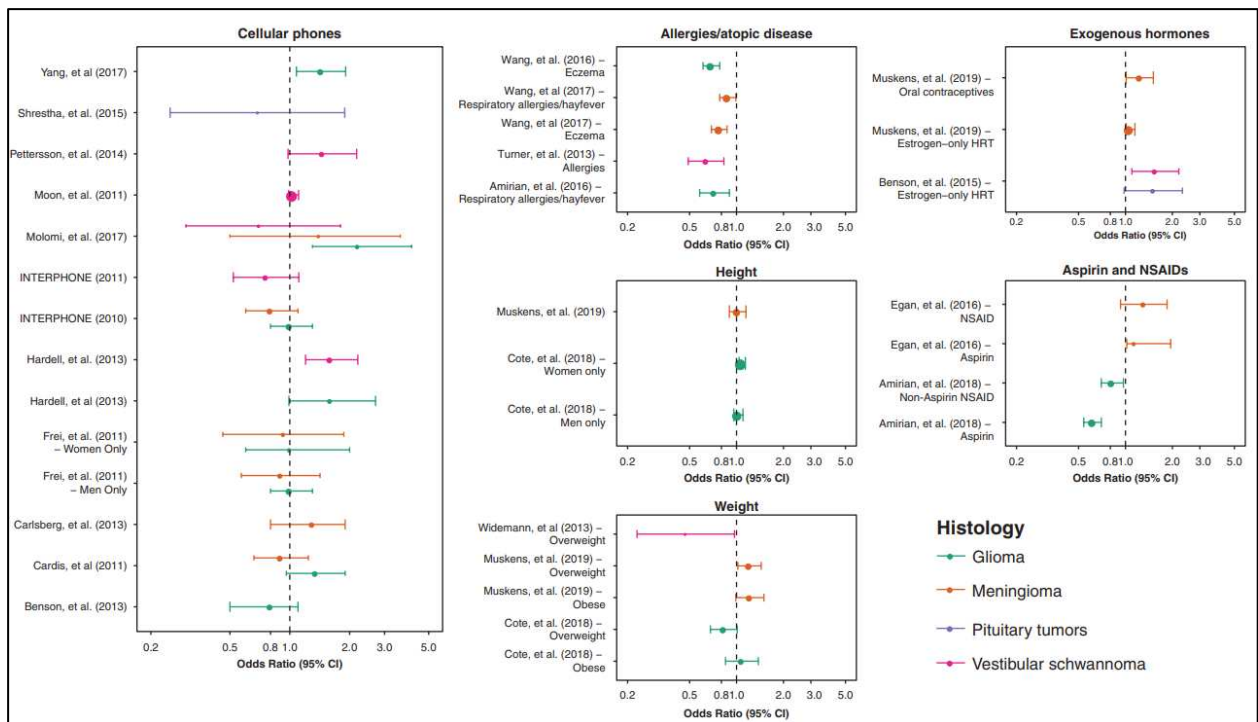


Figure 10. Odds Ratios (ORs) and 95% Confidence Intervals (CIs) of Potential Risk Factors for Primary Brain Tumors (132).

B.3.b.ii Primary brain tumors: Classification and grading system

The World Health Organization (WHO) classifies brain tumors into four grades (I, II, III, and IV) according to their growth rate, with higher grades indicating faster growth (236). Additionally, brain tumors are characterized by their progression stages (Stage 0, 1, 2, 3, and 4). Stage 0 represents abnormal cancerous tumor cells that have not spread to nearby tissues, while stages 1, 2, and 3 signify cancerous cells spreading rapidly. Stage 4 denotes cancer that has metastasized throughout the body (237).

Primary brain tumors are classified based on the type of cells from which they originate and their location within the brain or central nervous system. According to the WHO classification, the main types of primary brain tumors include gliomas, meningiomas, pituitary tumors, schwannomas, medulloblastomas, primary central nervous system lymphomas, craniopharyngiomas, pineal region tumors and choroid plexus tumors (236). Among the most prevalent malignant primary brain tumors are gliomas (235). According to the WHO fifth edition for the classification of tumors of the central nervous system (CNS5) (236), gliomas are divided into six distinct families. These include (1) adult-type diffuse gliomas, which are predominant in adults and encompass tumors like glioblastoma, (2) pediatric-type diffuse low-grade gliomas known for generally favorable prognoses, (3) pediatric-type diffuse high-grade gliomas,

exhibiting aggressive behavior, (4) circumscribed astrocytic gliomas that are characterized by their more solid growth pattern. The classification also includes (5) glioneuronal and neuronal tumors, a diverse group with neuronal differentiation, and (6) ependymomas. Below, **Table 2** outlines glioma types, along with their grades, essential genes, and proteins for diagnostic alterations and classification (236).

Table 2. Key Diagnostic Genes, Molecules, Pathways, and/or Combinations and Grades, adapted from (133).

Tumor Family and Subtypes	Genes/Molecular Characteristically Altered	Profiles	Grade
Adult-type diffuse gliomas			
- Astrocytoma, IDH-mutant	IDH1/2, ATRX, CDKN2A/B		II, III
- Oligodendroglioma, IDH-mutant, and 1p/19q-codeleted	IDH1/2, 1p/19q, promoter CIC, BUBP1, NOTCH1	TERT,	II, III
- Glioblastoma, IDH-wildtype	IDH-wild type, promoter chr7/10, EGFR	TERT,	IV
Pediatric-type diffuse low-grade gliomas			
- Diffuse astrocytoma, MYB- or MYBL1-altered	MYB, MYBL		I
- Angiocentric glioma	MYB		I
- Polymorphous low-grade neuroepithelial tumor of the young	BRAF, FGFR		I
- Diffuse low-grade glioma, MAPK pathway-altered	FGFR1, BRAF		II
Pediatric-type diffuse high gliomas			
- Diffuse midline glioma, H3 K27-altered	H3 K27, TP53, ACVR1, EGFR, EZHIP	PDGFRA,	IV
- Diffuse hemispheric glioma, H3 G34-mutant	H3 G34, TP53, ATRX		IV
- Diffuse pediatric-type high-grade glioma, H3-wildtype, and IDH-wildtype	IDH-wildtype, PDGFRA, MYCN, EGFR	H3-wildtype,	
- Infant-type hemispheric glioma	NTRK family, ALK, ROS, MET		
Circumscribed astrocytic gliomas			
- Pilocytic astrocytoma	KIAA1549-BRAF, BRAF, NF1		I
- High-grade astrocytoma with piloid features	BRAF, NF1, ATRX, CDKN2A/B		
- Pleomorphic xanthoastrocytoma	BRAF, CDKN2A/B		II, III
- Subependymal giant cell astrocytoma	TSC1/2		I
- Chordoid glioma	PRKCA		II
- Astroblastoma, MN1-altered	MNI		
Glioneuronal and neuronal tumors			
- Ganglion cell tumors	BRAF		I
- Dysembryoplastic neuroepithelial tumor	FGFR1		I
- Diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters	Chromosome 14		I
- Papillary glioneuronal tumor	PRKCA		I
- Rosette-forming glioneuronal tumor	FGFR1, PIK3CA, NF1		I
- Myxoid glioneuronal tumor	PDGFRA		
- Diffuse leptomeningeal glioneuronal tumor	KIAA1549-BRAF fusion, 1p		

- Multinodular and vacuolating neuronal tumor	MAPK pathway	I
- Dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease)	PTEN	I
- Extaventricular neurocytoma	FGFR (FGFR1-TACC1 fusion), IDH-wildtype	II
Ependymal tumors		
- Supratentorial ependymomas	ZFTA, RELA, YAP1, MAML2	II, III
- Posterior fossa ependymomas	H3 K27me3, EZHIP	II, III
- Spinal ependymomas	NF2, MYCN	II, III

According to the WHO classification, adult-type diffuse gliomas are now categorized based on the presence or absence of Isocitrate Dehydrogenase 1 and 2 (IDH1 and IDH2) mutations, which significantly impact prognosis. IDH status determination in addition to other molecular parameters, including gene deletions, amplifications, and copy number variations (such as gain of whole chromosome 7, loss of whole chromosome 10, TERT promoter mutations, and EGFR amplification), are essential biomarkers for grading and prognosis estimation across various tumor types (133,134). A summary of the classification and grading of IDH-mutant and IDH-wildtype diffuse gliomas in both adult and pediatric patients is presented below (**Figure 11**), illustrating the increasing complexity of molecular markers in glioma diagnosis.

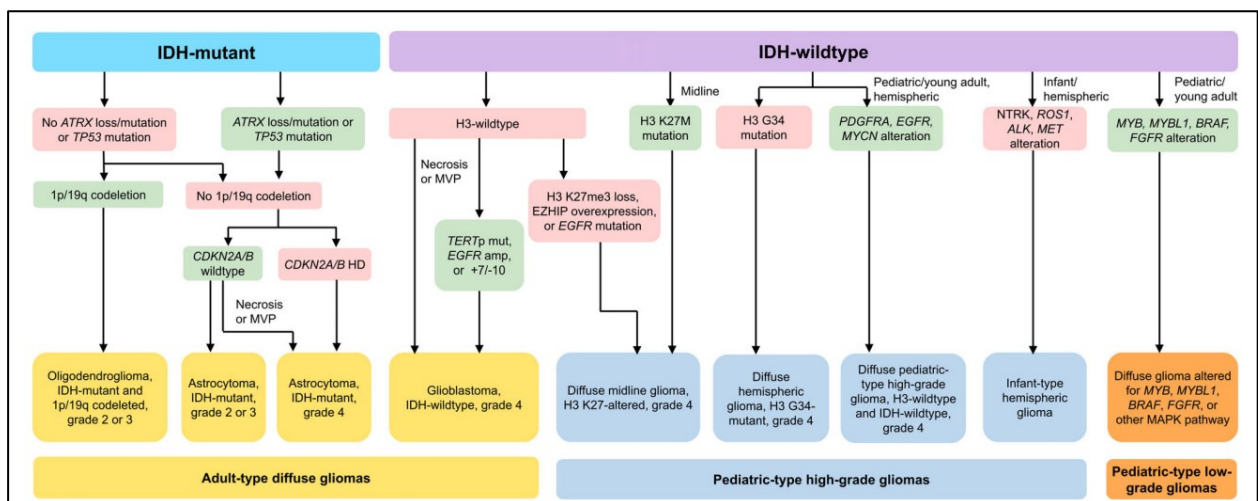


Figure 11. Diagnostic Flowchart for Gliomas in Adults and Pediatrics (135).

amp = amplification; HD = homozygous deletion; mut = mutation; MVP = microvascular proliferation.

B.3.b.iii NRF2 in Brain Cancer and Prognosis

NRF2 expression in primary CNS tumors correlates with more advanced WHO grades. In a study of 72 astrocytic tumors, NRF2 expression scores increased with tumor grading (240). Specifically, this trend was observed in glioblastomas, anaplastic astrocytomas, and other tumors with anaplastic phenotypes, in addition to neuroepithelial tumors like medulloblastoma (MB) and anaplastic ependymoma. However, while higher NRF2 levels tended to correlate with lower overall survival rates in patients with gliomas, this correlation did not reach statistical significance. Although this lack of significance may stem from various factors such as sample size, heterogeneity in patient populations, or other confounding variables, it prompts a critical examination of the relationship between NRF2 expression and clinical outcomes in brain cancer patients.

Although the studies on NRF2 in pediatric cancer are limited, the increased expression of NRF2 in MB (69) suggests its implication in disease progression. MB is a prevalent malignancy among pediatric patients and is classified as grade IV cancer (67). In MB cell lines (D283 and DAOY), induced oxidative stress using a combination of NRF2 activators (nifurtimox and tetrathiomolybdate) upregulated NRF2 target genes and triggered MB cellular death (241). While these findings suggest that the induction of the NRF2 pathway attenuates MB progression, research also warns that elevating NRF2 levels in an attempt to suppress tumor progression might inadvertently promote tumorigenesis due to its protumoral role (242), emphasizing the importance of cautious evaluation in therapeutic strategies.

In terms of adult brain tumors, understanding the intricate interplay between NRF2 and IDH enzymes is essential to understanding tumor progression and prognosis. Despite significant advancements, controversies persist regarding how mutant IDH enzymes, which normally indicate better prognosis, lead to the notable production of D2-hydroxyglutarate (D2-HG) that enhances glioma malignancy (243). The relationship between the NRF2-IDH regulatory axis and its implications in glioma biology is multifaceted and context-dependent. On one hand, the NRF2-IDH regulatory axis may exert protumoral effects. The reprogramming of ROS homeostasis involving the NRF2 antioxidant pathway in response to IDH1 mutations typically occurs within the context of gliomas and other brain tumors (69). This serves as a protective mechanism against oxidative damage caused by D2-HG accumulation (245), promoting tumor survival and

proliferation. Conversely, in patients with anaplastic gliomas harboring IDH1/2 mutations, lower expression levels of NRF2 target genes, including glutathione ligase subunits (GCLC and GCLM), were observed compared to wild-type IDH1/2 tumors (246). This decrease in NRF2-mediated glutathione synthesis favors a better prognosis in IDH mutant gliomas by rendering cancer cells more susceptible to oxidative stress. In contrast, the substantial correlation between IDH mutation and NRF2 expression in high-grade glioma remains controversial in some studies, suggesting that IDH mutation may have a dominant influence on tumor recurrence, independent of NRF2 expression levels (247).

Therefore, the paradoxical nature of NRF2's contribution to glioma prognosis is further underscored by its context-dependent effects, which vary based on tumor type, and its specific molecular contexts.

Key message: *Understanding NRF2's role in both glial support and neuronal vulnerability to oxidative stress is crucial for comprehending brain physiology and diseases. The brain's susceptibility to oxidative damage arises from its high oxygen consumption and reliance on oxidative phosphorylation for energy production. NRF2, a key regulator of antioxidant defenses, demonstrates varied expression across brain regions and cell types. NRF2 activation in the different brain cells exhibits protective effects against the development of age-related neurodegenerative diseases. NRF2 dysregulation contributes to cognitive impairment and pathology progression. Hence, NRF2 modulation holds therapeutic potential in brain physiology and diseases which offers insights into potential therapeutic strategies for neurodegenerative disorders.*

Brain tumors (BTs) present a complex landscape influenced by diverse risk factors, and prognostic markers. BT classification relies on histopathological features and molecular parameters, with the WHO grading system aiding in prognosis estimation. Notably, IDH mutations play a crucial role in glioma classification, reflecting the integration of molecular markers in enhancing diagnostic accuracy and guiding therapeutic strategies.

NRF2 exhibits complex roles in brain cancer. Its upregulation is associated with a reduced progression of some pediatric brain cancers, indicating its therapeutic potential. In contrast, NRF2 expression correlates with more advanced WHO grades in primary brain tumors, indicating its potential as a prognostic marker. Therefore, caution is warranted due to the dual role of NRF2 in cancer, which may have pro-tumoral effects in some BTs and tumor-suppressive activities in others.

C. Glioblastoma

C.1 Exploring Epidemiology to Therapeutic Challenges

C.1.a Epidemiology and Prevalence

Glioblastoma (GB), classified as a grade IV tumor by the World Health Organization (WHO), is among the most aggressive malignancies affecting the brain (248). It represents nearly half of all malignant primary tumors in the central nervous system (CNS), with a median overall survival (OS) of approximately 15 months (249). Despite its rarity, with a global incidence of less than 10 cases per 100,000 people, GB's poor prognosis raises significant public health concerns. It comprises half of all gliomas across age groups, with peak occurrence typically observed between ages 55 and 60 (250).

The incidence of GB between genders is debated, with some studies suggesting a higher occurrence in men. Factors such as female sex hormones, oral hormonal contraception, and hormone replacement therapy may limit GB development in women, though evidence regarding exogenous estrogen levels is inconclusive (249,251,252).

C.1.b Diagnosis and Clinical Examination

GB primarily develops in the supratentorial compartment of the brain [24], particularly in the frontal lobe while occurrences in the brainstem and cerebellum are rare (136). Despite multimodal therapy involving surgery, radiotherapy, and chemotherapy, the 5-year survival rate remains low at 5.5% of patients (137).

The clinical presentation of GB varies depending on tumor location and size at diagnosis. Patients commonly report headaches, nausea, and motor deficits, while intracranial hypertension symptoms, confusion, and visual or speech deficits may also manifest. Epilepsy, present in 15-20% of cases, often correlates with better outcomes due to the cortical location of GBs that trigger seizures. These symptoms, occurring in combination, typically lead to diagnosis weeks or months after onset (138,139).

Magnetic resonance imaging (MRI) is the primary diagnostic tool for GB. Non-contrast scans provide baseline brain imaging, while contrast-enhanced scans using gadolinium enhance tumor

visibility and highlight areas of disrupted blood-brain barrier (138). Additional MRI techniques, such as diffusion/perfusion sequences, offer valuable insights into lesion characteristics, including increased cerebral blood flow indicative of neoangiogenesis. These techniques aid in assessing peritumoral invasion, guiding biopsy, and monitoring disease progression post-treatment (138).

Confirmatory diagnosis of GB involves histopathological and molecular examination. Immunohistochemistry and molecular techniques validate GB diagnosis, focusing on IDH status (135) and mutations in genes like ATRX, EGFR, BRAF, and TERT promoter, along with MGMT promoter methylation status (140,141). MGMT promoter methylation serves as a predictive marker for classical GB and correlates with therapy resistance (142). Magnetic resonance spectroscopy (MRS) complements traditional MRI by providing biochemical information about brain tissues. Elevated choline/*N*-acetyl aspartate and choline/creatinine ratios, along with other metabolic changes detected by MRS, aid in GB diagnosis and differentiation from other pathologies (143,144)

C.1.c Characteristics and Pathogenesis

The current molecular profiling of GB has identified three main molecular subtypes; proneural, classical, and mesenchymal (**Figure 12**).

(1) Proneural Subtype: Derived from lower-grade astrocytomas, proneural GB tumors are characterized by TP53 mutations (145), overexpression of CDK4, CDK6, PDGFRA, MET, PDGFRA (146). Additionally, they often express ASCL1 and OLIG2 markers and tend to occur in young patients (147).

(2) The classical subtype: Marked by the amplification of the EGFR gene (145), mutations in the CDKN2A gene (145,148), chromosome 7 amplification, and the high expression of FGFR3, PDGFA, EGFR, AKT2, and NES (149). Additionally, this subtype often exhibits overexpression of markers associated with precursor or neural stem cells (Nestin Notch, Sonic Hedgehog) (145).

(3) The mesenchymal subtype: Defined by NF1 deletion or mutation, high levels of angiogenic markers (CD31/PECAM-1, VEGF), and inflammatory markers such as fibronectin and COX2 immune-related genes (150). Also, it demonstrates greater necrosis and inflammation, along with activation of the TNF α /NF κ B pathway and the expression of mesenchymal markers (MET, YKL40) (150). This subtype is considered the most aggressive, with the worst prognosis as the

transition to a mesenchymal subtype is often observed during tumor relapse along with treatment resistance (150).

Recent advancements in single-cell RNA sequencing have provided deeper insights into the cellular heterogeneity within GB tumors. As demonstrated by Neftel and colleagues (**Figure 12**), GB tumors comprise multiple cellular states including neural progenitor-like cells (NPC-like), oligodendrocyte progenitor-like cells (OPC-like), astrocyte-like cells (AC-like), and mesenchymal-like cells (MES-like) which can coexist within a single tumor. This coexistence reflects the plasticity of GB cells and emphasizes the complexity of GB pathology, as each cellular state may contribute differently to therapeutic resistance and tumor recurrence.

Ongoing research continues to refine our understanding of GB subtypes, highlighting their diverse molecular profiles and clinical implications. Recent advancements in GB subtyping have introduced functional perspectives, such as the mitochondrial and glycolytic/plurimetabolic subtypes identified by Garofano *et al.* in 2021 (151). The mitochondrial GB subtype, associated with the most favorable clinical outcome, relies exclusively on oxidative phosphorylation for energy production, whereas the glycolytic/plurimetabolic subtype is sustained by aerobic glycolysis and amino acid and lipid metabolism. These classifications, based on transcriptomic profiles and metabolic activities, offer valuable insights into GB heterogeneity and potential treatment strategies.

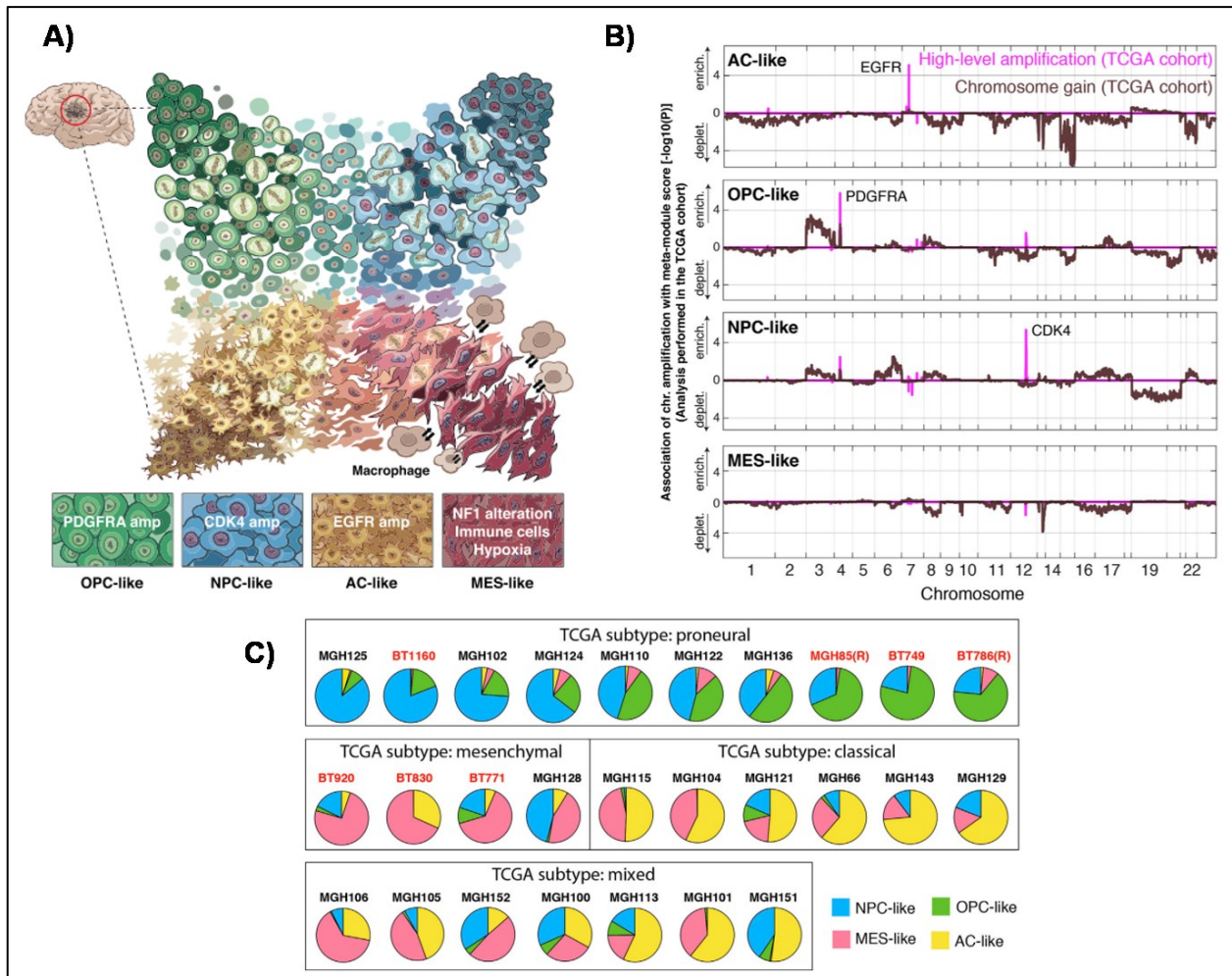


Figure 12. Distribution of Glioblastoma Subtypes and Cellular States with the Associated Chromosomal Amplifications (152).

(A) Model for the cellular states of glioblastoma and their genetic and micro-environmental determinants. Mitotic spindles indicate cycling cells. Lighter or darker tones indicate the strength of each program. Intermediate states are shown in between the four states and indicate transitions. (B) Analysis of the TCGA glioblastoma cohort showing the high-level amplifications of genes relevant to each cellular state (above the zero line, indicating enrichment of the cellular state) or with low bulk scores (shown below the zero line, indicating depletion of the cellular state). (C) Pie charts displaying the fraction of cells in four cellular states in each glioblastoma from the patient cohort. Tumors are grouped by bulk TCGA subtype as labeled. Tumor indices are above each pie chart; pediatric tumors are indicated in red and recurrent tumors with "R."

Phenotypically, GB typically manifests as sizable tumors primarily located in the cerebral hemispheres and white matter of the brain, often exhibiting considerable heterogeneity both within and among patients (153,154). Histologically, the core of GB tumors is characterized by fusiform, atypical, and pleomorphic cells, alongside varying degrees of low-grade neoplastic astrocytes. Central necrosis is a prominent feature within the tumor core, often progressing from a hypoxic central zone. Surrounding these necrotic zones are one or more layers of pseudo-palisading cells arranged radially (153,155). Cytological analysis shows significant pleomorphism in neoplastic cells, characterized by enlarged nuclei with clumped chromatin. Additionally, variations in cellular densities are observed across tumor regions, with the Ki-67 proliferation index ranging from 5% to over 70% at the invasive edge. This variability poses challenges in

obtaining representative samples, particularly in stereotactic needle biopsies where only a small portion of tissue is sampled (153,156).

Vascular anomalies, including endothelial hyperplasia and microvascular proliferation, are additional noteworthy histological features of the tumor (**Figure 13**). Microvascular proliferation, characterized by the formation of multilayered small-caliber blood vessels, indicates rapid growth and is often associated with necrosis and mitotic activity (157).

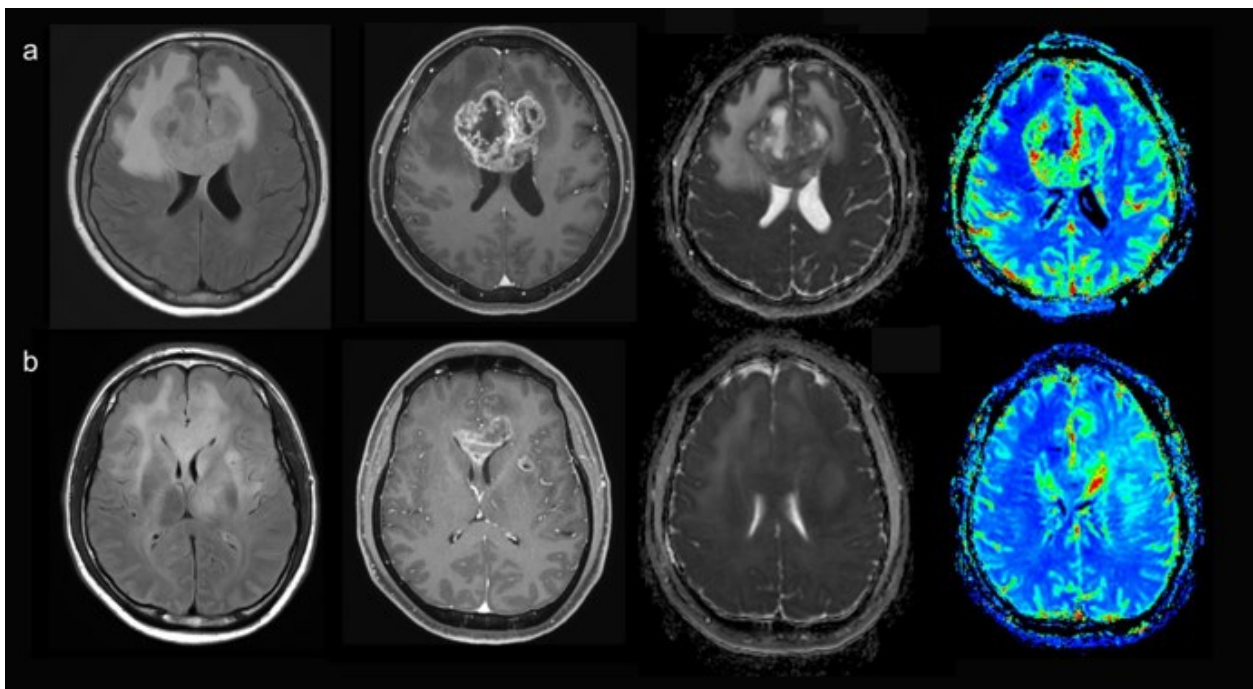


Figure 13. Images in Two Glioblastoma Patients, with Necrosis and Microvascular Proliferation on Histopathology (135).

(a) Images of a 68-year-old female with an enhancing necrotic tumor involving the bifrontal lobes and corpus callosum with edema. The first image (left) is a Fluid-attenuated inversion recovery (FLAIR) sequence showing hyperintense signal areas indicating edema and infiltrative tumor. The second image (middle left) is a contrast-enhanced T1-weighted MRI, demonstrating the enhancing necrotic tumor with clear delineation. The third image (middle right) is the apparent diffusion coefficient (ADC) map, showing areas of increased cellularity (lower ADC values). The fourth image (right) is the cerebral blood volume (CBV) map, highlighting regions of increased relative CBV (rCBV) at the contrast-enhancing portion, indicating higher vascularity. b) Images of a 40-year-old male with enhancing tumors at the corpus callosum and left basal ganglia show strong enhancement with necrosis. The first image (left) is a FLAIR sequence, showing hyperintense signal areas that diffusively involve the bilateral cerebral hemispheres, indicating the presence of a non-enhancing infiltrative tumor component (not edema). The second image (middle left) is a contrast-enhanced T1-weighted MRI, highlighting the enhancing necrotic tumors. The third image (middle right) is the ADC map, showing slightly increased cellularity with lower ADC values in some regions. The fourth image (right) is the CBV map, displaying increased rCBV at the contrast-enhancing portion, indicating higher vascularity.

GB tumors also demonstrate significant invasive capabilities. Invasive GB cells utilize pre-existing anatomical pathways in the brain for invasion, including the perivascular spaces and white matter tract (consisting of myelinated axons) as their preferred routes (158). They infiltrate the perivascular space surrounding blood vessels, in response to chemoattractants produced by endothelial cells, enabling access to essential oxygen and nutrients (159–161). Additionally, the

corpus callosum serves as a significant conduit for tumor cell infiltration to various distant brain regions, facilitating their spread from one hemisphere to the other (158).

Interestingly, GB cells prefer local infiltration within the brain rather than forming distant organ metastases, despite their proximity to blood vessels. They typically invade as individual entities, moving autonomously within brain tissue, evading surgical resection at the primary tumor site and contributing to tumor recurrence by infiltrating surrounding normal brain tissue (**Figure 14**) (158). Their limited metastatic potential is attributed to factors such as the presence of the blood-brain barrier, absence of lymphatic channels preventing lymphatic metastasis, suppression of extracranial growth by the immune system, and speculated lack of ability to invade or degrade extracellular matrix in tissues outside the brain (158).

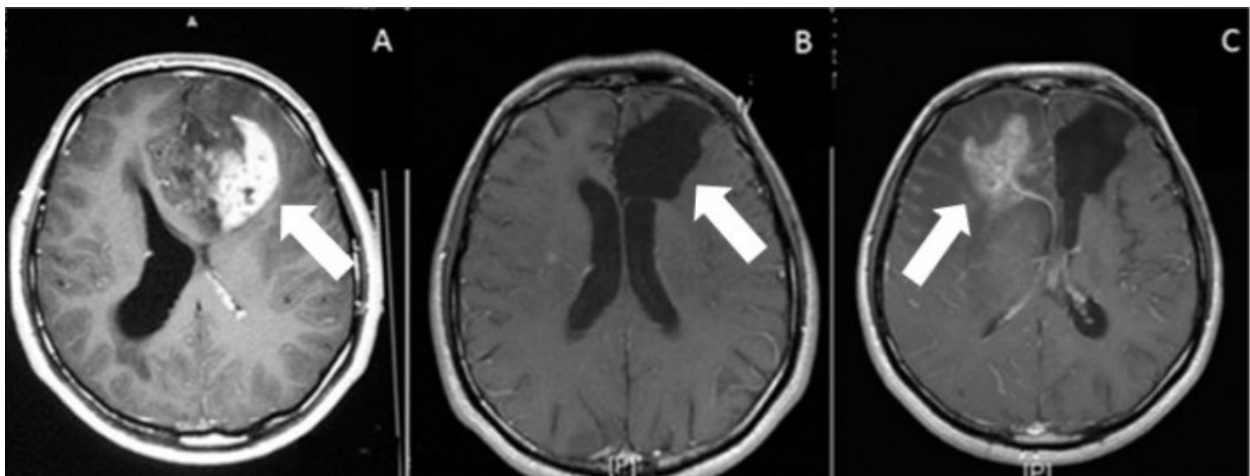


Figure 14. Post Gadolinium Contrast Administration, T1-Weighted Axial Images (158).

(A) Preoperative, heterogeneous irregular enhancement, associated with the left frontal-lobe glioblastoma (arrow). (B) Postoperative (at 1 month) axial weighted image. On the postoperative image, there is no residual enhancement. The arrow shows the operation cavity. (C) Postoperative (at 18 months) axial weighted image shows recurrence of the tumor (white arrow) on the contralateral hemisphere, associated with peripheral edema.

Three distinct forms of GB exist and are primarily identified based on their histological assessments. (1) giant cell glioblastoma, characterized by numerous giant cells with multiple nuclei and atypical mitosis, small fusiform syncytial cells, and a reticulin background; (2) epithelioid glioblastoma, dominated by a relatively uniform population of discohesive rounded epithelioid cells with eccentric nuclei and abundant eosinophilic cytoplasm; and (3) gliosarcoma, characterized by a biphasic component, with one part showing glial morphology and the other manifesting as spindled fibroblast-like sarcoma (153,162).

C.1.d Cell Origin of Glioblastoma: The Role of GSCs

Evidence shows that GB may arise from neural stem cells (NSCs) (163). NSCs, residing in the brain's ventricles, are multipotent and self-renewable cells with proliferation capacity (163). The transformation of NSCs gives rise to glioblastoma stem cells (GSCs) through the inactivation of tumor suppressor genes (TP53, NF1, or PTEN) (164). GSC constitutes a small proportion of the tumor but plays a pivotal role as a reservoir for tumor regeneration and therapy resistance (165). GSCs are characterized by their slow-dividing or quiescent nature and can be found in both the perivascular and central necrotic niches of the tumor (137). Similar to NSCs, they possess remarkable self-renewal, and differentiation capacities (166). While sharing common gene expression patterns and regulatory pathways with NSCs (167), GSCs possess tumorigenic potential, and resistance to therapy rendering them more likely to be the main candidates to explain the origin, tumor reoccurrence, and infiltrative nature of GB (168). GSCs can be recognized and characterized through specific markers and properties. Intracellular proteins such as SOX2, OLIG2, MYC, and NESTIN are prominent markers that help distinguish GSCs from other cell types. Additionally, cell surface markers like CD133, L1CAM, CD44, and A2B5 are widely used to identify and isolate GSCs (276). These markers not only assist in their identification but also provide insights into their stem-like properties, including self-renewal and differentiation capabilities, which contribute to the aggressive nature of GB (169). Several studies have demonstrated bidirectional plasticity between GSCs and more differentiated GB cells in response to environmental factors. Hypoxia (170), and exposure to chemotherapy (171) and radiation (172), have been shown to promote a stem-like phenotype in tumor non-stem cell populations to increase the pool of GSCs over time.

Furthermore, recent single-cell RNA-sequencing studies have suggested a potential neuronal or glial origin. These studies identified cellular profiles resembling neural precursor cells (NPCs), oligodendrocyte precursor cells (OPCs), and astrocytic precursor cells (APCs), which are proposed as potential candidates for initiating GB (137,152). Thus, while NPCs, OPCs, and APCs are implicated, further investigation is necessary to conclusively determine the origins of GB.

C.1.e Current Management and Therapy

The standard treatment regimen for GB is the Stupp protocol, established following the landmark EORTC 26981/22981-NCIC CE3 trial led by Dr. Roger Stupp in 2005 (173). It comprises maximal

safe surgical resection followed by radiotherapy (RT) alongside concurrent and adjuvant TMZ chemotherapy.

- i.* **Surgical resection:** Initial treatment typically involves surgical resection, recommended for patients under 70 in good health (174). Gross total resection (GTR), removing the entire contrast-enhancing tumor, correlates with better survival (175). Advances in surgical techniques, such as awake craniotomy (176) or neuromonitoring (177) and surgical functional MRI navigation (178), aid in maximizing tumor removal while minimizing neurological deficits. However, if complete resection is not possible, a stereotactic or open biopsy is recommended to guide further treatment decisions (46).
- ii.* **Concomitant combination of radiotherapy and temozolomide:** Post-surgical treatment includes involved-field radiotherapy (RT) (60 Gy in 1.8–2.0 Gy fractions/day, 5 days a week) along with concurrent temozolomide (TMZ) (75 mg/m² daily), starting on the first day of radiotherapy and continuing throughout the 6 weeks. Following the completion of radiotherapy, adjuvant chemotherapy with temozolomide is given. This involves 6 cycles of maintenance TMZ given at a higher dose of 150–200 mg/m² for 5 days out of every 28 days (173,179).

In terms of TMZ's mechanism of action, administered orally as a pro-drug, TMZ undergoes rapid chemical conversion at the physiological pH of the stomach to generate its active form, monomethyl triazenoimidazole carboxamide. This active molecule can cross the blood-brain barrier due to its lipophilic nature, allowing it to reach the brain effectively. Subsequently, it induces alkylation of guanine residues in DNA at positions O6 and N7, leading to DNA replication and transcription blockage, resulting in cell death (180). Although TMZ is generally well tolerated, its active form is not confined to the brain, allowing it to impact other organs and potentially cause systemic side effects such as myelosuppression, gastrointestinal disturbances, and hematologic complications (181). Ionizing radiation (IR), on the other hand, exerts its effects by generating free radicals that cause DNA damage, disrupting the cell's ability to replicate and survive, thus contributing to cell death and tumor shrinkage (182). The Stupp protocol, combining TMZ with radiotherapy, increases the median overall survival in GBM patients to 14.6 months. Despite these gains, the overall efficacy remains limited, highlighting the need for

innovative treatments to better combat GBM's aggressive nature while improving patient survival and quality of life.

iii. **Other therapeutic approaches:** In certain cases, for patients with either newly diagnosed or recurrent GB, tumor-treating field (TTF) therapy is combined with standard chemo- or radiation therapy. This approach utilizes low-intensity alternating electric fields generated by electrodes placed on the patient's scalp at the tumor site to disrupt cancer cell division, ultimately leading to cell death (183,184). Other approaches include immunotherapy strategies, such as vaccine therapy, oncolytic viruses, immune checkpoint inhibitors (CPIs), and CAR T cell therapy, which target GB's immune evasion and resistance mechanisms. These approaches aim to minimize organ toxicity while maximizing efficacy (179). However, while these alternatives continue to be explored, the current standard of care remains the Stupp protocol due to its established effectiveness and widespread acceptance in the medical community.

C.1.f Limitations and Challenges in GB Management

Unfortunately, advances in the treatment of GB tumors are hindered by various limitations and resistance mechanisms, as discussed below.

i. **The blood-brain barrier (BBB):** The BBB serves as a critical defense mechanism, regulating the passage of molecules from the blood into the brain (185). In GB, compromised BBB, also known as the blood-tumor barrier (BTB), creates a supportive environment for GB tumor growth by selectively allowing pro-tumoral molecules to pass, including essential nutrients in addition to Epidermal Growth Factor (EGF), Vascular Endothelial Growth Factor (VEGF) needed for tumor growth while impeding the entry of anti-tumoral agents (186–188). Moreover, since the blood-brain barrier operates bidirectionally, it restricts the flow of molecules from the brain to the bloodstream. This characteristic limits the potential of using blood samples for liquid biopsies to detect circulating tumor cells (CTCs), thereby complicating the diagnosis and monitoring of glioblastoma progression (141).

ii. **Normal brain tissue-tumor interactions:** GB's invasiveness depends on its close interaction with normal brain parenchyma to infiltrate into the surrounding normal brain tissue, making it challenging to completely remove them surgically or target them effectively with therapies like radiation or chemotherapy. Moreover, tumor microtubes facilitate DNA repair in tumor cells, by providing a pathway for the exchange of cellular materials, including DNA repair proteins and genetic material, between neighboring cells and support tumor reoccurrence by providing growth signals and the invasion into adjacent brain tissue (189,190).

iii. **Tumor heterogeneity and plasticity:** Tumor heterogeneity in GB includes both inter-tumor heterogeneity and intra-tumor heterogeneity, denoting differences among cells within individual tumors affecting their response to therapy (191).

The inter-tumor mutational landscape of GB is relatively homogeneous compared to many other cancers. For example, GB presents a relatively low rate of somatic mutations (with a median of 2.2 per megabase) in comparison to other solid cancers like lung cancer and melanoma (191). Variations in histopathological and molecular profiles lead to different GB subtypes impacting tumor prognosis and therapy options among patients (191).

The intra-tumor heterogeneity, a striking feature of GB, manifests at different tumor levels (**Figure 15**). **(1) Spatial heterogeneity** refers to the variation in tumor characteristics across different regions within the tumor mass (192). Tumor tissue removed from a single patient often exhibits a heterogeneous architecture (192) that correlates with the three tumor subtypes described previously. For example, five regions could be recognized within the tumor including the regions of hypoxia (HReg), stem cell and resistance regions that correspond to mesenchymal subtype, transformed neuronal regions (TNReg) corresponding to proneural GB-subtype, proliferative regions (PReg) corresponding to classical subtype, and mutation regions (MReg) (192,193). **(2) The existence of tumor clones** represented by genetically distinct populations of cancer cells within a single GB tumor, that present chromosomal alterations, DNA ploidy variations, and EGFR-amplified cells associated with a more aggressive and infiltrative tumor capacity (191). **(3) The varied genomic and transcriptomic profiles in single GB cells** indicate that individual cancer cells within the same tumor can have distinct genetic and RNA expression

patterns. These profiles do not always match those observed when analyzing the bulk tumor, suggesting that different regions or cells within the tumor may behave differently and respond variably to treatment. **(4) Single-cell DNA methylation profiles** which represent the phylogenetic tree of gene mutations among tumor areas and between matched primary and recurrent tumors, enrich the intratumoral heterogeneity (191). **(5) Cellular heterogeneity in the tumor microenvironment (TME)** which represents 50% of the tumor mass and includes various cell populations such as microglia, astrocytes, macrophages, lymphocytes, and endothelial cells (149). In addition, tumor-associated macrophages (TAMs) are also found in different regions within the tumor (194) and contribute to tumor invasion, angiogenesis, and immune suppression (195). Also, myeloid-derived suppressor cells (MDSCs) (196), and tumor-associated neutrophils (TANs) (197) are present to help tumor cells evade the immune system. Furthermore, stromal components like endothelial cells and pericytes/mesenchymal stem cells (MSCs) play critical roles in tumor formation and progression (198,199). The complex interplay of these components within the TME fosters a highly immunosuppressive environment. TAMs contribute by secreting cytokines and growth factors that inhibit T-cell activity and enhance tumor progression. Regulatory T cells (Tregs) further intensify immune suppression by reducing the activation and function of effector T cells, reinforcing the immunosuppressive milieu. MDSCs add to this suppression through various mechanisms that hinder T-cell responses and support tumor growth. Together, these elements create a formidable barrier to effective immune response, highlighting the intricate challenges in treating GB (200).

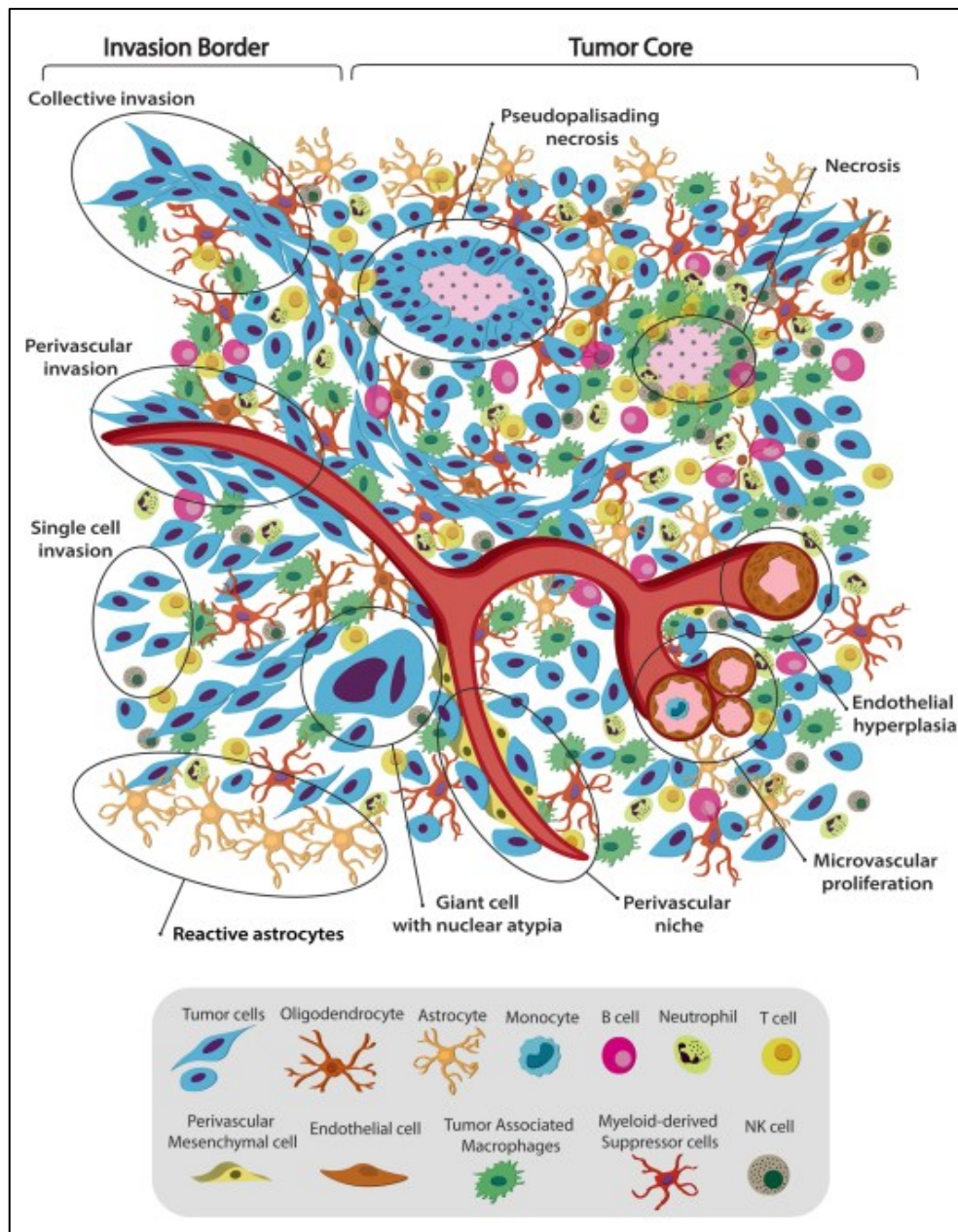


Figure 15. GB Intratumorally Heterogeneity (149).

A schematic representation of the gliomas TME highlighting the spatio-temporal heterogeneity at the histological, dynamic, and cellular level.

- iv. **Therapeutic resistance and recurrence:** GSCs demonstrate robust mechanisms for therapy resistance and tumor recurrence. These cells are capable of surviving chemotherapy (201,202), radiation (203), and the hypoxic niche of the tumor (204,205). GSCs also express high levels of multidrug resistance genes like breast cancer resistance protein 1 (BCRP1) and ATP-binding cassette subfamily G member 2 (ABCG2) (206), as well as anti-apoptotic Bcl-2 family members, making them resilient to cytotoxic drugs (207). Additionally, they demonstrate metabolic flexibility, allowing them to switch between aerobic glycolysis and oxidative phosphorylation in response to therapy (208). Also, GSCs

exhibit cellular plasticity by existing in different states, including proliferative and quiescent states. This cellular plasticity allows the tumor cells to adapt and persist even when exposed to targeted therapies, making the tumor more resistant to treatment. This cellular plasticity enables the tumor that tolerate the failure of targeted therapies (209,210).

Recurrent tumors exhibit molecular differences that justify their poorer prognosis compared to primary tumors, including reduced expression of DNA repair enzymes, altered methylation patterns of proapoptotic genes (211), varied mutation/amplification patterns (212), and changes in extracellular matrix composition (213). Tumor recurrence is not solely mediated by the unique abilities of GSCs for proliferation, self-renewal, and differentiation. It also involves innate and therapy-induced clonal dynamics. GSCs play a significant role in driving the expansion of therapy-resistant subclones (**Figure 16A**). Their plasticity enables them to adapt to the changes in their tumor microenvironment after therapy and repopulate brain tumors with multiple cellular states as shown in **Figure 16B** (214).

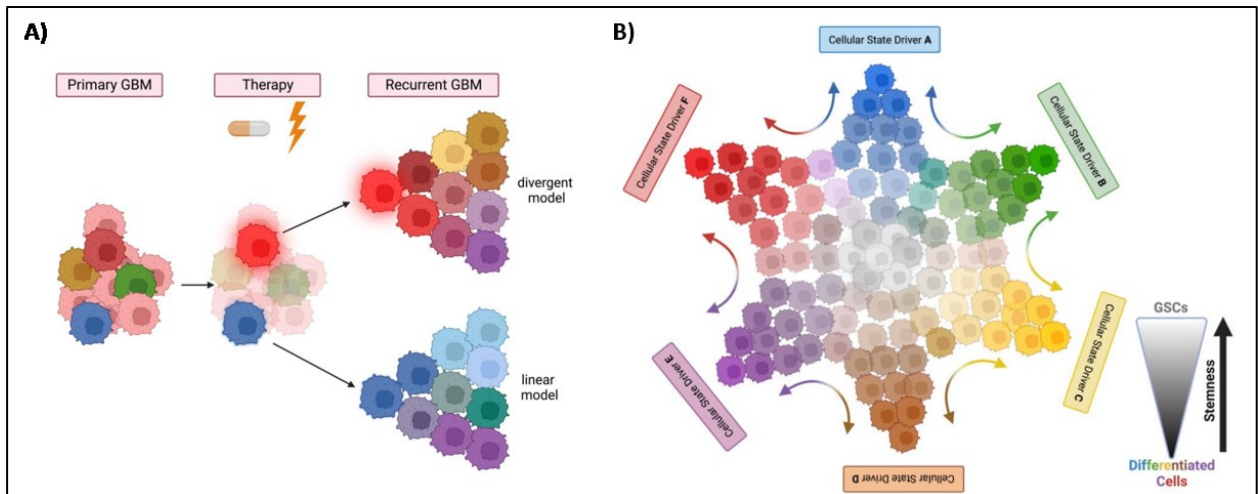


Figure 16. Dynamics of GSCs in Glioblastoma Recurrence and Heterogeneity, adapted from (214).

(A) This model shows the primary GB with a mix of GB cells (light rose) and GSCs (colored). Therapy administration (middle panel) selects for subclonal GSC populations with pre-existing (linear model) or therapy-driven resistance (divergent model), leading to the formation of heterogeneous recurrent GB (right panel). (B) A simplified model of GSC plasticity, where GSCs (gray color) give rise to multiple cellular states, influenced by various intrinsic and extrinsic factors, driving tumor heterogeneity.

C.2 Brain Cellular Energetics and Metabolic Adaptation in GB

C.2.a Fundamentals in Cellular Energetics

Tumor metabolism reprogramming is a hallmark of cancer, crucial for sustaining rapid cell proliferation, growth, and migration (135). GB presents unique metabolic challenges due to the brain's distinct metabolic demands. The brain heavily relies on glucose as its primary energy source, with approximately 20% of the body's daily glucose utilization allocated to meet its metabolic needs (136). Glucose in the brain can be metabolized in various ways, including oxidative phosphorylation and glycolysis, as discussed below.

- i. **Oxidative Phosphorylation:** Under aerobic conditions, pyruvate from glycolysis enters the citric acid cycle as acetyl-CoA and undergoes oxidative phosphorylation in mitochondria (215,216). This process involves electron transfer through protein complexes known as the electron transport chain (ETC), consisting of four complexes (I, II, III, IV) and ATP synthase (complex V) (217,218) (**Figure 17**). Electrons are donated by NADH and FADH₂, generated from the citric acid cycle, and pass through the different complexes. Complex I (NADH dehydrogenase) receives electrons from NADH and transfers them to ubiquinone (CoQ). Complex II (succinate dehydrogenase) directly donates electrons to ubiquinone (CoQ) which transfers them to complex III (cytochrome bc₁ complex). During this process, only complexes I, III, and IV pump protons across the inner mitochondrial membrane, contributing to the formation of a proton gradient. This gradient is used by ATP synthase to convert ADP and inorganic phosphate (Pi) into ATP. The net equation of aerobic respiration is 36 ATP (218).

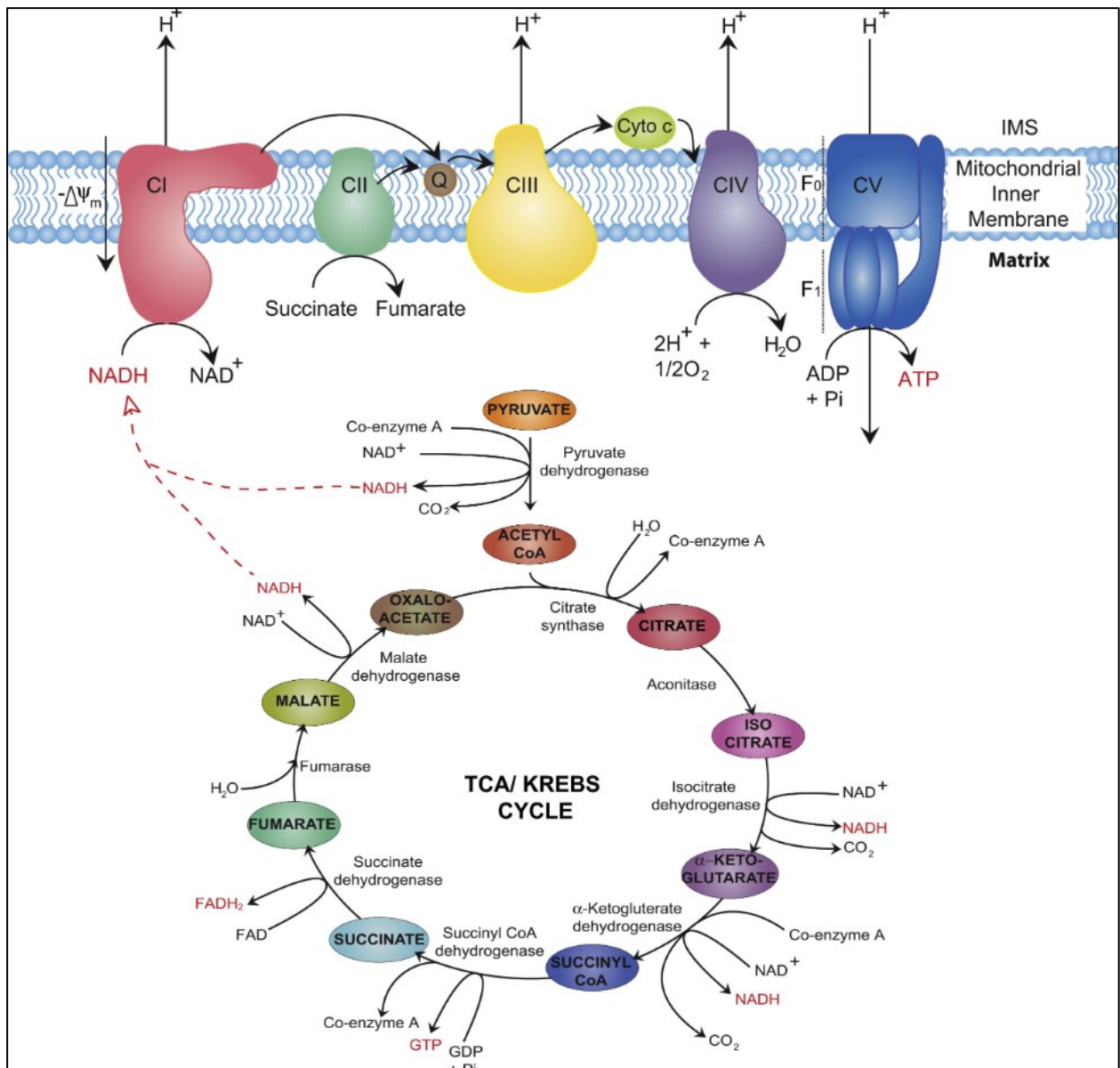


Figure 17. Bioenergetics of the Electron Transport Chain and the TCA/Krebs Cycle (218).

Pyruvate is converted to high-energy molecules like NADH, GTP, and FADH₂ through catalyzation by TCA/Krebs cycle enzymes. NADH generated is shuttled to complex I and is converted to NAD⁺ driving oxidative phosphorylation. Transfer of electrons through the chain maintains the membrane potential via proton pumping into the IMS. In this final step, ADP is phosphorylated to form ATP via complex V (ATP synthase). IMS ; Intermembrane space.

- ii. **Glycolysis and Warburg Effect:** Tumor cells, preferentially convert glucose to lactate, even under aerobic conditions, a phenomenon known as the Warburg effect or aerobic glycolysis (**Figure 18**). This metabolic adaptation allows for faster ATP production, supporting rapid tumor proliferation, particularly in nutrient-restricted TME. Despite being less efficient in ATP production (yielding 2 ATP) compared to oxidative phosphorylation, aerobic glycolysis is favored due to its higher rate of glucose metabolism. Lactate, a byproduct of glycolysis, is produced 10-100 times faster through

glycolysis (219), enhancing various cellular processes like invasion, metastasis, and immune escape, often correlating with poor prognosis (220,221). Also, the Warburg effect facilitates the generation of tumor biomass through nucleotides and fatty acid synthesis, thereby supporting the sustained growth and proliferation of cancer cells (93). The transformation of pyruvate to lactate occurs through the action of lactate dehydrogenases (LDHs) in the cytosol, with LDHA favoring pyruvate conversion to lactate and LDHB exhibiting a greater affinity for lactate conversion back to pyruvate. Lactate transportation across cell membranes is facilitated by proton-linked monocarboxylate transporters (MCTs), with upregulation of MCT1 and MCT4 associated with worsened prognosis of GB (104). MCT1 transports lactate into tumor cells with high affinity to fuel their metabolism, while MCT4 releases lactate, especially in hypoxic conditions. Lactate transportation by MCTs is a bidirectional process influenced by the lactate concentration and H⁺ gradient which is essential for maintaining acid flux (222). MCT4 exports lactate along with H⁺ ions, leading to the acidification of the TME, resulting in suppressed CD8⁺ T cells and immune evasion (223).

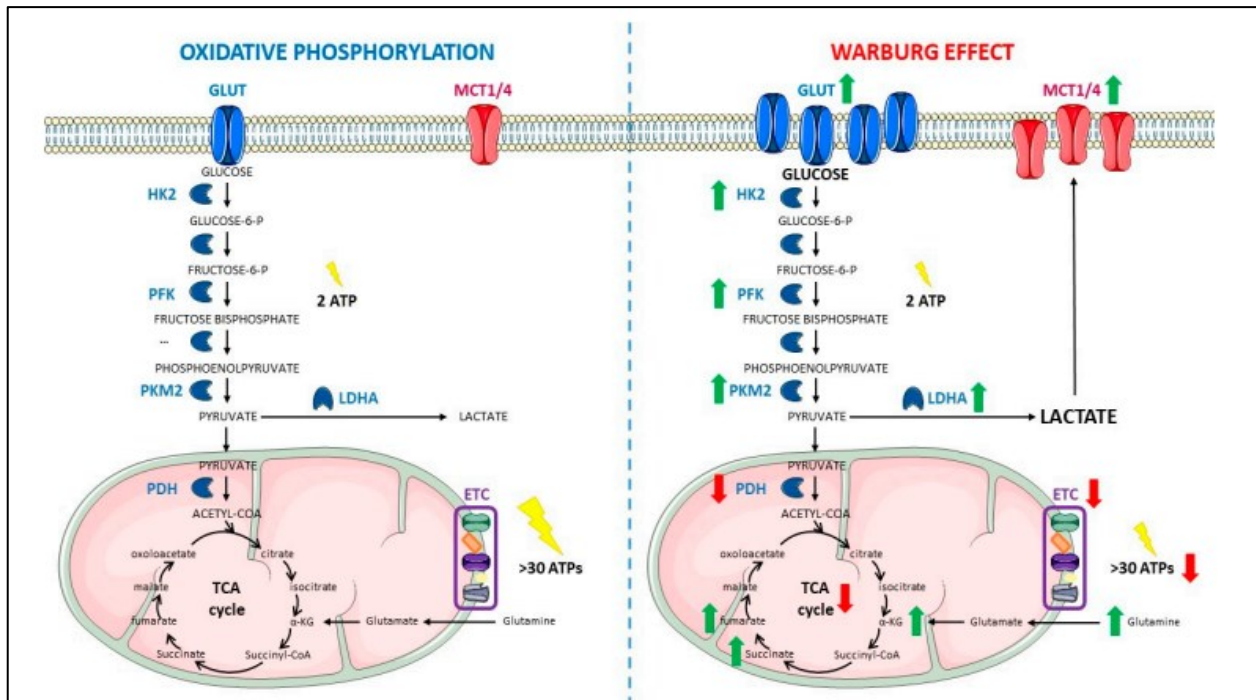


Figure 18. Warburg Effect in Cancer Cells (224).

(Left) Within oxidative metabolism, glucose is introduced inside the cell by GLUTs and degraded through glycolysis to provide pyruvate, which is mainly introduced inside the mitochondrion, with only a small amount being converted into lactate in the cytoplasm. Inside the mitochondrion, acetyl-CoA enters the TCA cycle to generate intermediates for the ETC and ATP. Glutamine is converted to α -KG and incorporated in the TCA cycle. (Right) Under Warburg metabolism, GLUTs are overexpressed, which results in increased glucose uptake. At the same time, there is an upregulation in the glycolytic enzymes HK2, PFK, and PKM2, which leads to an increased glycolytic rate. The glycolytic rate may exceed the mitochondrial rate of pyruvate oxidation, yielding lactate production via the overexpressed LDHA enzyme. Accumulated lactate is pumped out of the cells through MCT1/4 causing microenvironment acidification. In these conditions, PDH and the TCA cycle are also downregulated. Enhanced lactate fermentation yields 2 molecules of ATP per molecule of glucose. Green arrows indicate a net increase whereas red arrows show significant decreases. GLUTs; Glucose Transporters, TCA; Tricarboxylic Acid, ETC; Electron Transport Chain, ATP; Adenosine Triphosphate, HK2; Hexokinase 2, PFK; Phosphofruktokinase, PKM2; Pyruvate Kinase M2, LDHA; Lactate Dehydrogenase A, MCT1/4; Monocarboxylate Transporter 1/4, PDH; Pyruvate Dehydrogenase, α -KG; Alpha; Ketoglutarate.

C.2.b Metabolic Adaptation in GB

Interestingly, spatial metabolic adaptation within GB tumors is evident in different tumor regions (**Figure 19**). Cells in the perivascular niche enhance glycolysis to meet the demands of the TCA cycle based on blood glucose availability. Conversely, cells in hypoxic and nutritionally restricted tumor regions utilize glutamine, lactate, and potentially ketone bodies from the microenvironment as energy substrates, partly supplied by astrocytes (225–227).

- i. Glycolysis in GB Metabolism:** The higher preference for aerobic glycolysis compared to normal brain tissue in GB (228) is orchestrated by various molecular deregulations, such as the activation of oncogenes such as AKT, PTEN loss, or RTK/PI3K pathway activation, occurring in over 80% of GB cases. These signaling pathways upregulate the expression of glucose transporters (GLUTs), thus promoting glucose uptake (229). Moreover, the activation of RAS or MYC, or mutations in tumor suppressor genes like TP53 (230), further stimulates glycolysis and lactate production by inducing the expression of LDHA and pyruvate dehydrogenase kinase 1 (PDK1) which inhibits the entry of pyruvate into the mitochondria, redirecting cellular metabolism towards glycolysis (230–232). Furthermore, hypoxia, a common feature in rapidly growing GB tumors, plays a pivotal role in metabolic reprogramming. Hypoxia activates and stabilizes transcription factors like HIF-1 α and HIF-2 α , promoting a shift toward glycolysis and angiogenesis (233). Specifically, HIF-1 α positively regulates several glycolytic enzymes, including GLUTs, LDHA, and MCT4, thereby enhancing the proliferative phenotype (234).
- ii. Glutamine in GB Metabolism:** Glutamine metabolism provides GB cells with essential metabolites such as glutamate and α -ketoglutarate (α KG), supporting various cellular processes, including nucleotide (235), and fatty acid synthesis (236), as well as mTOR signaling for cell growth (237). Oncogenes such as c-Myc and K-RAS play key roles in regulating glutaminolysis (238,239). Importantly, glutamine synthetase (GS) activity is linked to the response to radiotherapy, as radiation induces metabolic reprogramming, and induction of glutaminogenesis in irradiated cancer cells supports nucleotide synthesis and DNA repair, promoting cell growth under radiation stress (240).

- iii. **Ketone Bodies (KBs) in GB Metabolism:** Regarding ketone bodies (KBs) (Figure 19), their contribution to GB energy production under glucose limitation remains debated. While early studies suggested limited utilization of KBs by GB cells (241), recent findings indicate their ability to sustain growth even under low glucose conditions (242). Further research, especially in clinical settings, is necessary to elucidate the efficacy of ketogenic diets as potential adjuvant therapies alongside standard cancer treatments (243,244).

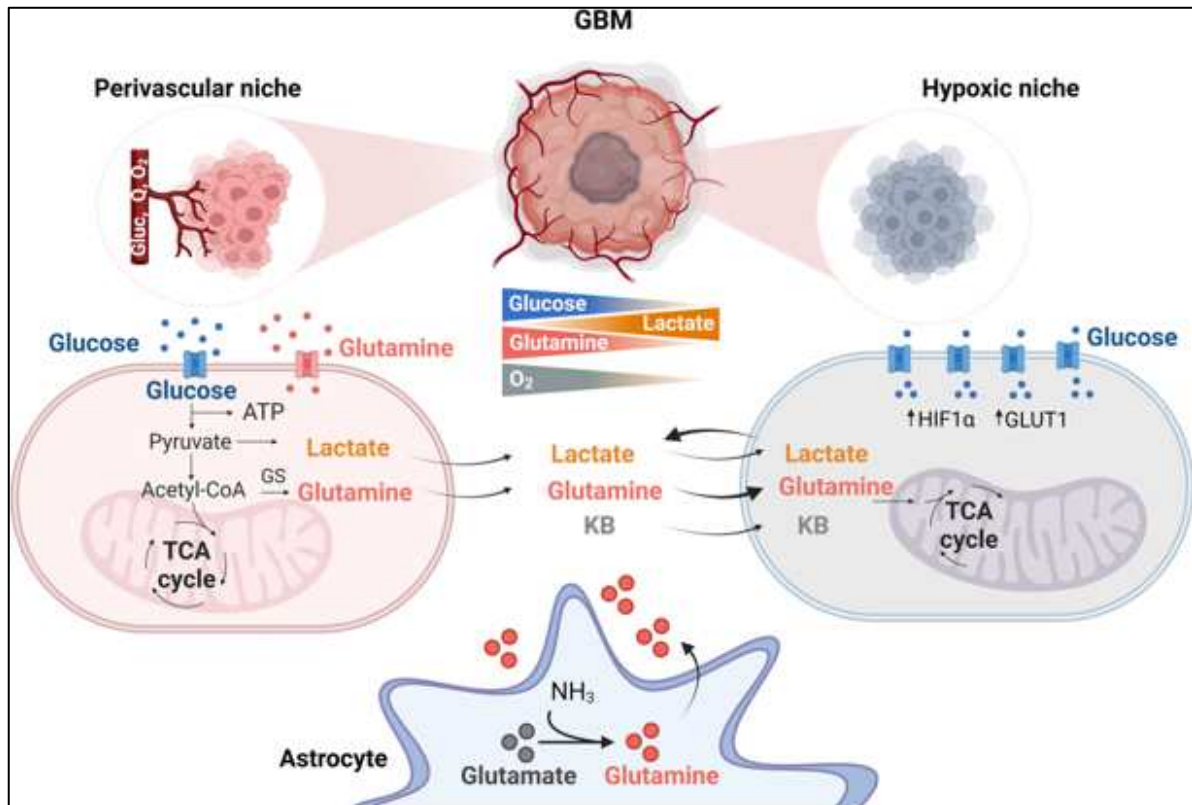


Figure 19. Metabolic Adaptation to Changes in Nutrient and Oxygen Availability Within GB Tumor (215).

The perivascular niche displays an enhanced glycolytic pathway to the TCA cycle based on blood glucose availability. In contrast, cells from hypoxic areas employ glutamine, lactate, and potentially KBs from the microenvironment as energy substrates to supply a truncated TCA cycle. Hypoxia also enhances anaerobic glycolysis optimizing glucose uptake and releasing lactate to be used as a carbon source by neighboring tumor cells. Astrocytes provide glutamine and KB to the tumor microenvironment. TCA; tricarboxylic acid cycle, KBs; ketone bodies.

- v. **Lipids in GB Metabolism:** Lipid metabolism also plays a crucial role in GB tumor growth (245). Lipids are crucial components of the brain, constituting around 50% of its weight. The primary lipid types found in the brain include phospholipids, glycolipids, cholesterol, cholesterol esters (CE), and triglycerides (TAG) (246). GB tumors thriving in such a lipid-rich environment heavily rely on lipids for their rapid progression (245) for lipogenesis and cholesterol synthesis, in addition to lipid catabolism to produce energy through fatty acid oxidation (FAO).

Following the elevation of glucose and glutamine consumption in GB cells, the SREBP/SCAP pathway is activated to enhance lipogenesis (245) (**Figure 20**). Similarly, GB cells, exhibit an elevated dependence on cholesterol, which is essential for tumor proliferation and invasion (247). Although they can synthesize it de novo, GB cells prefer to rely on external sources of cholesterol (248). This dependence is facilitated by the upregulation of low-density lipoprotein receptors (LDLRs) that enhance cholesterol uptake (**Figure 20**). Targeting cholesterol metabolism shows promise as a potential anticancer therapy, offering the advantage of selectively impacting dysregulated cholesterol metabolism in cancer cells while sparing healthy cells (249).

The biosynthesis of lipids is often accompanied by the accumulation of lipid droplets (LDs) (250), particularly overexpressed in GSCs (251). LDs are cellular organelles that store large quantities of neutral lipids to avoid lipid toxicity, including TAG and cholesterol esters CE (245). In response to decreasing glucose levels, LD-triglycerides are released and mobilized for beta-oxidation and energy production within mitochondria (252) to facilitate tumor growth (253). In addition to their role as energy reservoirs, the intrinsic presence of LD contributes to the radioresistance and chemoresistance of cancer stem cells in several cancer types (254,255). Given that the enhanced incorporation of lipids in GSCs maintains their stemness (251), targeting lipid droplets could hold a valuable therapeutic potential in GB.

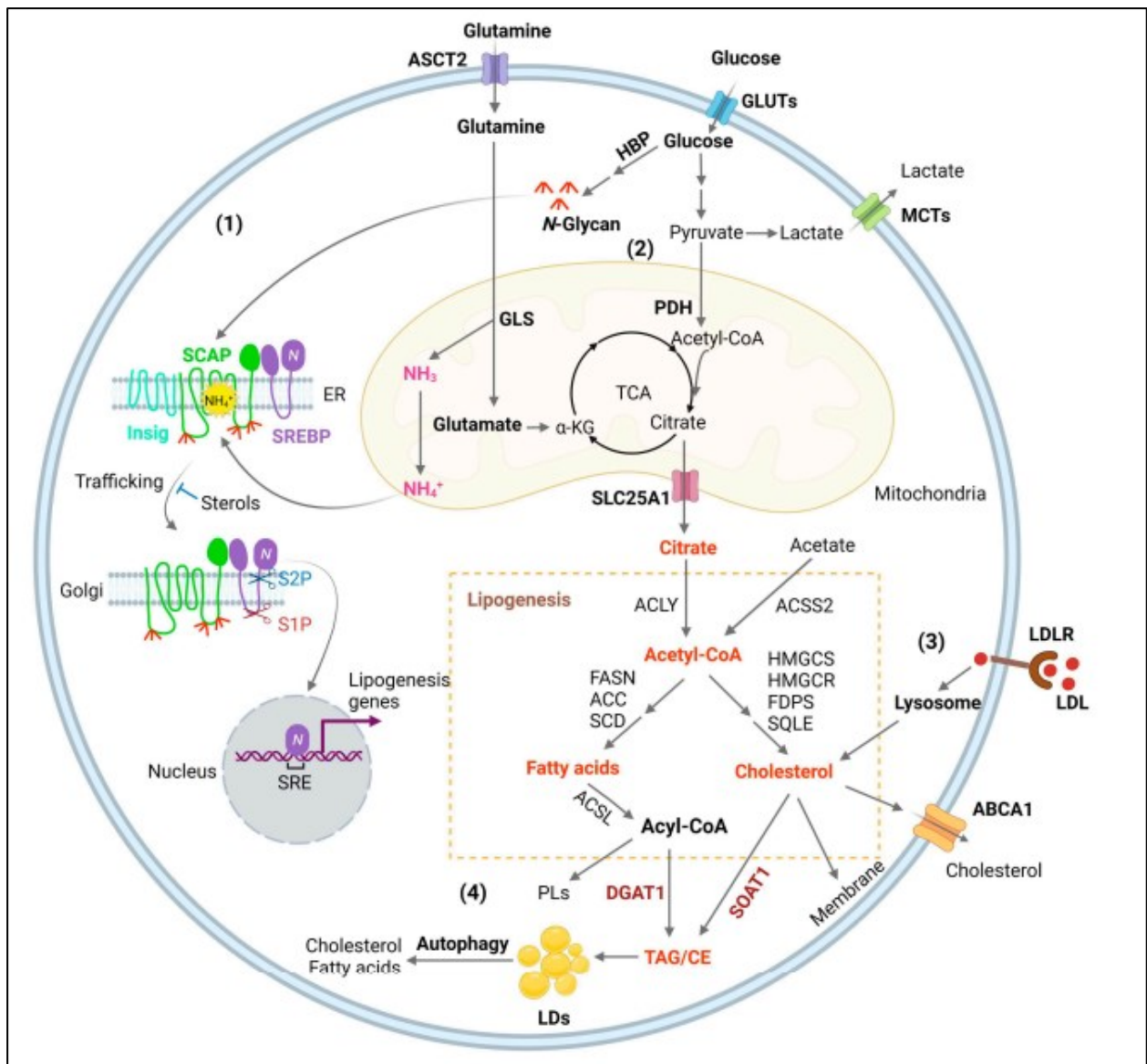


Figure 20. Lipid Metabolism in Glioblastoma; From De Novo Synthesis to Storage (245).

(1) Combined elevation of glucose and glutamine consumption promotes lipogenesis by activating the SREBP/SCAP pathway. Glucose produces N-glycans through HBP, which stabilizes SCAP via N-linked glycosylation. Glutamine enters the mitochondria, and ammonia (NH_3) is released by GLS. NH_3 is protonated and converted to NH_4^+ , which directly binds to the core of SCAP transmembrane domains, leading to its dissociation. Subsequently, SCAP escorts SREBP to the Golgi, where it is cleaved by two enzymes S1P and S2P to release its active N-terminal fragment. Finally, the N-terminal domain goes into the nucleus and binds to the SRE motif located in the promoters of genes involved in lipogenesis to activate their transcription.

(2) Glucose via glycolysis breaks down into pyruvate, which enters the mitochondria and is converted to acetyl-CoA by PDH, followed by condensation with OAA to form citrate to enter the TCA cycle. Citrate is released to the cytosol via SLC25A1 and cleaved by ACLY to acetyl-CoA, which serves as a precursor for fatty acid and cholesterol biosynthesis catalyzed by a series of enzymes that are the main transcriptional targets of SREBPs. In addition, cytosol acetate can be converted to acetyl-CoA for lipid synthesis by ACCS2. Glutamate is converted to α -KG and then enters into the TCA cycle.

(3) SREBPs upregulate the expression of LDLR, which binds to LDL and transports it into cells to be hydrolyzed.

(4) Excess fatty acids and cholesterol are converted to TAG and CE by DGAT1 and SOAT1 to form LDs. Under conditions of nutrient deficiency, LDs are hydrolyzed by autophagy to release free fatty acids and cholesterol for tumor survival. HBP; Hexosamine Biosynthesis Pathway, SCAP; Sterol Cleavage-Activating Protein, SREBP; Sterol Regulatory Element-Binding Protein cleavage activating protein, NH_3 ; Ammonia, GLS; Glutaminase, Insig; Insulin; induced gene, ER; Endoplasmic Reticulum, SREBP; Sterol Regulatory Element; Binding Protein, S1P; Site 1 Protease, S2P; Site 2 Protease, SRE; Sterol Regulatory Element, PDH; Pyruvate Dehydrogenase, OAA; Oxaloacetate, TCA; Tricarboxylic Acid, SLC25A1; Mitochondrial Citrate Transporter, ACLY; ATP Citrate Lyase, ACCS2; Acetyl-CoA Synthetase 2, LDLR; Low-Density Lipoprotein Receptor, LDL; Low-Density Lipoprotein, TAG; Triacylglycerol, CE; Cholesteryl Ester, DGAT1; Diacylglycerol O-acyltransferase 1, SOAT1; Sterol O Acyltransferase 1, LDs; Lipid Droplets.

In terms of lipid catabolism, fatty acid β -oxidation is a vital metabolic process occurring in the mitochondria, where fatty acids undergo sequential oxidation steps, yielding molecules such as FADH₂ and NADH necessary for ATP synthesis during oxidative phosphorylation (215,256). In GB, there is a pronounced upregulation of fatty acid transporters, including carnitine palmitoyltransferase (CPT) transporters like CPT1A and CPT1C. These transporters facilitate the translocation of long-chain fatty acids from the cytoplasm into the mitochondria (**Figure 21**) (257). Additionally, fatty acid binding protein 7 (FABP7) has emerged as a potential marker for glioblastoma stem cells (GSCs) and plays a crucial role in facilitating fatty acid transport to various cell organelles (258). In various GB models, inhibiting CPT1 using etomoxir (259) or blocking FABP7 (260) has shown promising anticancer effects. Moreover, recent studies have associated elevated levels of FAO enzymes, such as CPT1A, CPT2, and ACAD9, with poorer outcomes in recurrent GB patients (258). Furthermore, excessive fatty acids are converted into TAG and stored in lipid LDs by diacylglycerol-acyltransferases (DGATs), specifically DGAT1 and DGAT2 (**Figure 21**). Targeting DGAT1 in GB cells disrupts TAG synthesis, leading to aberrant fatty acid metabolism and mitochondrial damage, ultimately triggering tumor cell apoptosis (261).

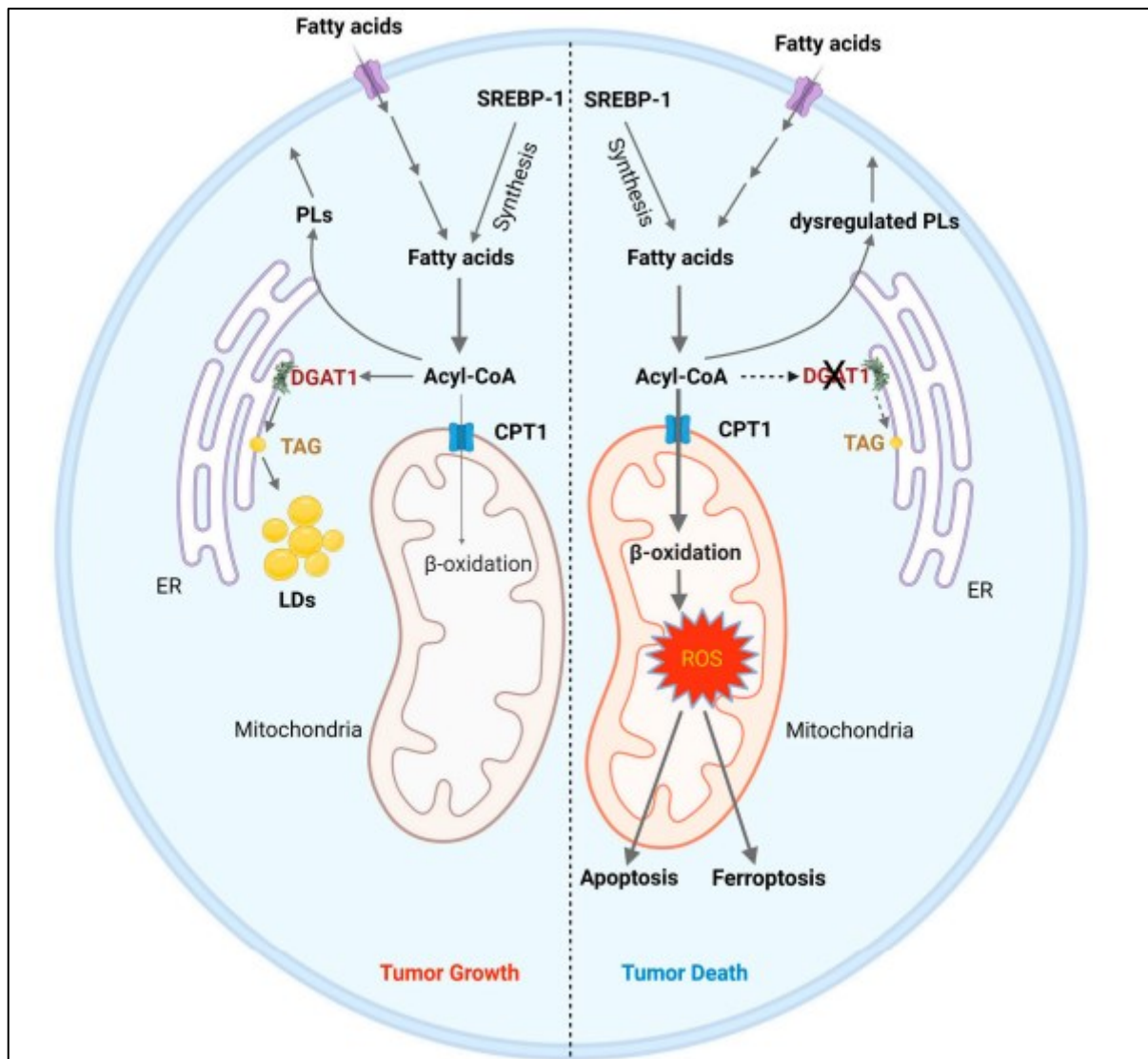


Figure 21. Fatty Acid Oxidation Therapeutic Potential in GB (245).

(Left) Tumor cells acquire fatty acids through uptake or de novo synthesis via the activation of SREBP-1. Fatty acids are converted to acyl-CoAs, which are the substrates for phospholipid (PL) synthesis and produce energy via entering into mitochondria to undergo β-oxidation and oxidative phosphorylation. Excessive acyl-CoA is stored in LDs as catalyzed by DGAT1 in tumor cells. (Right) Inhibiting DGAT1 causes an imbalance of fatty acid catabolism, leading to cell death. Inhibiting DGAT1 forces more acyl-CoAs to enter the mitochondria through CPT1 for β-oxidation, leading to high levels of reactive oxygen species (ROS), which trigger apoptosis and ferroptosis to kill tumor cells. SREBP-1; Sterol Regulatory Element-Binding Protein-1, PL; Phospholipid, LDs; Lipid Droplets, DGAT1; Diacylglycerol O-acyltransferase 1, CPT1; Carnitine Palmitate Transferase 1, β-oxidation; Beta-oxidation, ROS; Reactive Oxygen Species.

Key message: *Glioblastoma presents a challenging landscape characterized by its aggressive nature and resistance to therapy. Epidemiologically, it stands as a significant public health concern, comprising the majority of malignant primary brain tumors in adult and affecting individuals predominantly between 55 to 60 years old, with a median survival rate of around 15 months. Histologically, GB exhibits complexity, including heterogeneity, necrosis, microvascular proliferation, and invasiveness, posing challenges for precise diagnosis and treatment. Molecularly, its origins remain elusive, with neural stem cells (NSCs), glioblastoma stem cells (GSCs), and other progenitor cells implicated, highlighting the tumor's multifaceted nature. Furthermore, molecular subtyping into proneural, classical, and mesenchymal subtypes underscores GB's complexity, each presenting distinct genetic signatures and clinical behaviors. Recent functional subtyping based on transcriptomic profiles and metabolic activities shows promise in understanding and targeting GB's heterogeneity. Diagnosis and management of GB require clinical examination, MRI, and histopathological/molecular analysis guiding personalized treatment plans. Despite the standard Stupp protocol, challenges like the blood-brain barrier, tumor heterogeneity, and therapeutic resistance persist, necessitating novel approaches such as tumor-treating fields therapy and immunotherapy. GB exhibits distinct metabolic characteristics, including a preference for aerobic glycolysis and alterations in lipid metabolism, supporting rapid cell proliferation. Molecular deregulations, hypoxia, and metabolic adaptations further drive metabolic changes in GB cells. Targeting these pathways offers promising avenues for anti-GB therapies, highlighting the importance of understanding cellular energetics and metabolic adaptations in tumor progression.*

D. NRF2 in Glioblastoma

D.1 NRF2/ARE Pathway: A Double-Edged Sword in Cancer Biology and Therapy

The effect of NRF2 activation in the cancer context is stage-dependent. During the very early stages of cancer development, NRF2 activity exerts anti-cancer effects by protecting cells from oxidative stress and genotoxic damage, thereby preventing tumor initiation. It achieves this by upregulating the expression of detoxifying enzymes and antioxidant proteins, which neutralize ROS and repair damaged DNA (75). However, in later stages of cancer, persistent NRF2 activation in cancer cells contributes to tumor progression and resistance to therapy. Cancer cells may exploit the cytoprotective functions of NRF2 to survive in the hostile tumor microenvironment, resist oxidative stress, evade apoptosis, and enhance their proliferation (262). Herein, we discuss this dual role of NRF2 in the main hallmarks of cancer as both a tumor suppressor and a potential oncogene, in the various types of cancer (summarized in **Figure 22**). As indicated in **Figure 22**, certain hallmarks indicate that NRF2 can have complex and context-dependent effects, potentially promoting cancer cell survival and proliferation while also protecting normal cells from damage.

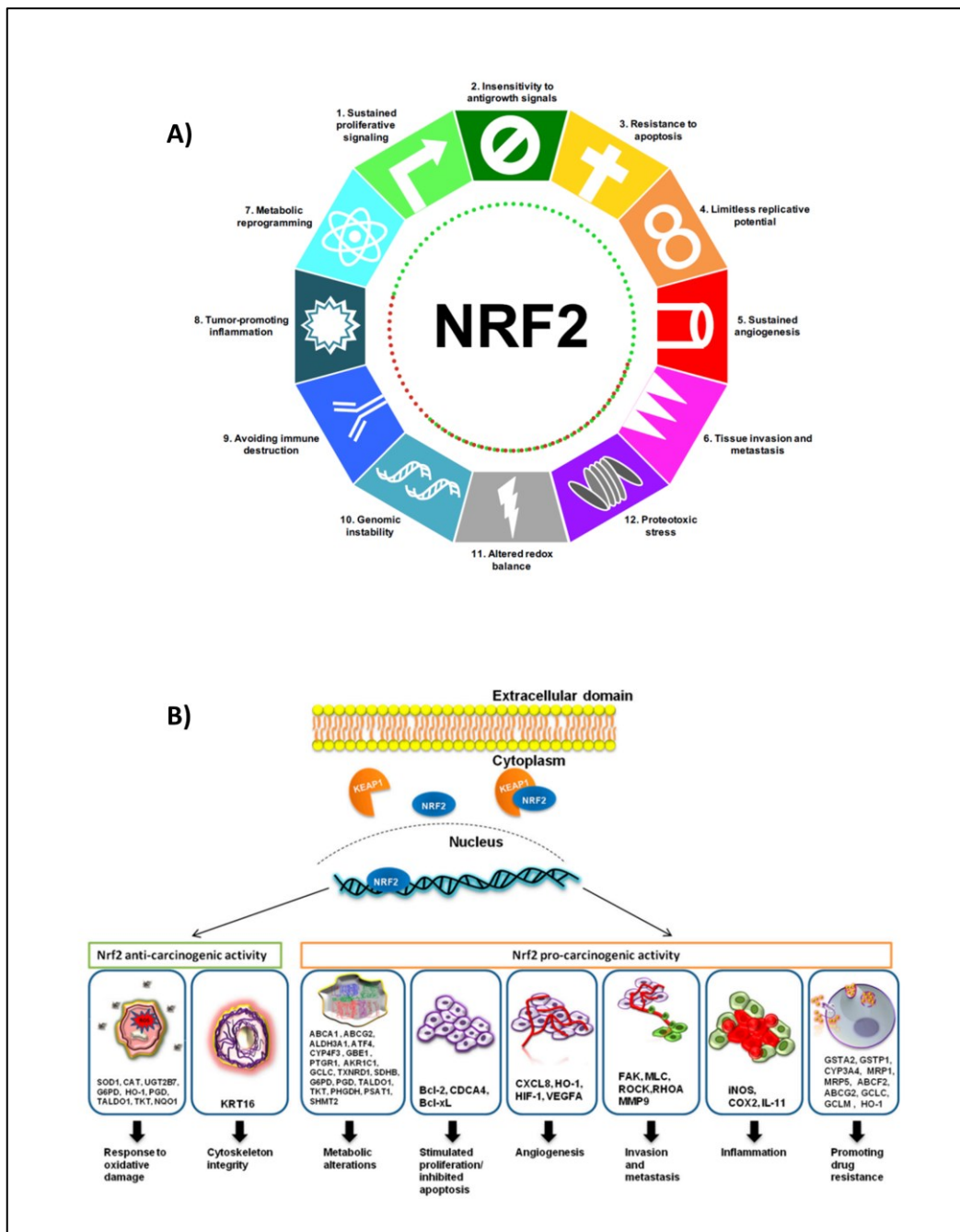


Figure 22. NRF2 Implications and Dual Role in Cancer Development, adapted from (263) and (264).

(A) NRF2 has direct and indirect roles that promote (green dotted lines) or block (red dotted lines) the emergence of the hallmarks of cancer. (B) NRF2, through its targeted genes, has an anti-carcinogenic role in the case of normal cells and a pro-carcinogenic effect in the case of transformed malignant cells. Superoxide Dismutase 1 (SOD1); Catalase (CAT); UDP Glucuronosyltransferase Family 2 Member B7 (UGT2B7); Glucose-6-Phosphate Dehydrogenase (G6PD); Heme Oxygenase 1 (HO-1); Phosphogluconate Dehydrogenase (PGD); Transaldolase 1 (TALDO1); Transketolase (TKT); NAD(P)H Quinone Dehydrogenase 1 (NQO1); ATP-Binding Cassette Subfamily A Member 1 (ABCA1); ATP-Binding Cassette Subfamily G Member 2 (ABCG2); Aldehyde Dehydrogenase 3 Family Member A1 (ALDH3A1); Cytochrome P450 Family 4 Subfamily F Member 3 (CYP4F3); 1,4-alpha-Glucan Branching Enzyme 1 (GBE1); Prostaglandin Reductase 1 (PTGR1); Aldo-Keto Reductase Family 1 Member C1 (AKR1C1); Glutamate-Cysteine Ligase Catalytic Subunit (GCLC); Thioredoxin Reductase 1 (TXNRD1); Succinate Dehydrogenase Complex Iron Sulfur Subunit B (SDHB); Focal Adhesion Kinase (FAK); Myosin Light Chain (MLC); Rho-Associated Protein Kinase (ROCK); Ras Homolog Family Member A (RHOA); Matrix Metalloproteinase 9 (MMP9); C-X-C Motif Chemokine Ligand 8 (CXCL8); Hypoxia-Inducible Factor 1 (HIF-1); Vascular Endothelial Growth Factor A (VEGFA); Inducible Nitric Oxide Synthase (iNOS); Cyclooxygenase 2 (COX2); Interleukin 11 (IL-11); Tumor Necrosis Factor α (TNF α); Interleukin 6 (IL-6); Interleukin 8 (IL-8); Interleukin 1 β (IL-1 β); Glutathione S-Transferase Alpha 2 (GSTA2); Glutathione S-Transferase Pi 1 (GSTP1); Cytochrome P450 Family 3 Subfamily A Member 4 (CYP3A4); Multidrug Resistance Protein 1 (MRP1); ATP-Binding Cassette Subfamily F Member 2 (ABCF2); ATP-Binding Cassette Subfamily G Member 2 (ABCG2); Glutamate-Cysteine Ligase Modifier Subunit (GCLM); Multidrug Resistance Protein 5 (MRP5).

D.1.a NRF2 Anti-tumor Activities

NRF2 mediates diverse mechanisms through which it exerts its protective functions.

(1) NRF2 protects against oxidative damage: Based on the analysis of human cancer microarray data sets (265), it's evident that the absence of NRF2 sets off a harmful chain reaction characterized by decreased GSH expression, heightened ROS levels, and consequent DNA damage, all contributing to tumorigenesis (265). Additionally, NRF2 knockout mice exhibit heightened sensitivity to exogenous chemicals, resulting in the accelerated development of liver cancer (266) in response to fumarylacetoacetate-induced toxicity. These findings highlight the crucial role of NRF2 in protecting against oxidative damage and preventing tumor formation.

(2) NRF2 exerts an anti-inflammatory activity: It is widely accepted that NRF2 exerts an anti-inflammatory effect to protect against carcinogenesis by downregulating the expression of pro-inflammatory genes (262). This is accomplished through the induction of antioxidant genes like heme-oxygenase-1 (*HMOX1*), *NQO1*, and glutamate cysteine ligase catalytic (*GCLC*) and modifier (*GCLM*) subunits (267–269). These antioxidant genes inhibit the activation of the pro-inflammatory transcription factor NF- κ B, consequently preventing the transcription of pro-inflammatory mediators. Additionally, recent research suggests that NRF2 can directly inhibit transcription by binding to the promoter regions of pro-inflammatory genes. In macrophages, this includes genes such as interleukin (IL)-6 and IL-1 β , where NRF2 blocks gene induction and impedes the recruitment of RNA polymerase II (270). Moreover, NRF2 combats inflammation-induced carcinogenesis by regulating the expression of cytoprotective enzymes like *Gpx* and thioredoxin (*Trx*) to suppress inflammatory responses in mice models with gastrointestinal cancer and pancreatic fibrosis, respectively (271,272). Consistently, the notable increase in key inflammatory mediators such as Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF- α) in NRF2-deficient mice underscores NRF2's role in inhibiting pro-inflammatory pathways (273). The overexpression of HO-1, a downstream target of NRF2, reduces oxidative stress and IL-3 levels, further dampening inflammation (274). Additionally, NRF2-dependent induction of *NQO1* downregulates the lipopolysaccharide (LPS)-induced expression of TNF- α and IL-1 β , impairing the inflammatory response (275). In conclusion, NRF2 combats tumor initiation via its anti-inflammatory activity, thus serving as a crucial mechanism in tumor prevention.

(3) *NRF2 inhibits tumorigenesis*: Research using NRF2-knockout mouse models highlights the importance of NRF2 in suppressing cancer initiation. These mice develop more tumors in the forestomach (276), liver (277), and urinary bladder (278) compared to wild-type mice. In the tumor microenvironment, tumor suppressor genes like BRCA1 and protein p21 enhance NRF2 activity. They achieve this by preventing the formation of the KEAP1/NRF2 complex, which otherwise keeps NRF2 in an inactive state (279,280). This protection allows NRF2 to remain active in the nucleus, where it can exert its tumor-suppressive effects.

In humans, specific genetic variations affect NRF2's protective role against cancer. For example, the NRF2 single nucleotide polymorphism (SNP) homozygous allele (-617A7A), has a significant impact on NRF2 gene expression. Individuals who possess this -617A7A allele in a homozygous state produce lower levels of NRF2 protein, resulting in decreased expression of the protective enzymes that NRF2 typically regulates. Consequently, without adequate NRF2-mediated protection, individuals with this SNP are less able to detoxify harmful substances, leading to an increased risk of lung cancer, particularly in smokers (281). Similarly, other genetic variations in NRF2 (11108C>T), NQO1 (609C>T), NOS3 (894G>T), and HO-1 [(GT)(n) dinucleotide length polymorphism] are linked to lower ROS detoxification abilities. Although these genetic variations are not individually linked to a higher risk of breast cancer, postmenopausal women with three or more of these modifications face an increased risk, particularly when combined with high iron intake (282).

These findings underscore the complex and context-dependent role of NRF2 in cancer biology. Specifically, a defect or alteration in NRF2 activity can promote tumor progression, highlighting that NRF2 typically has anti-tumor activities. This emphasizes NRF2's critical role in maintaining cellular homeostasis and preventing malignant transformation.

D.1.b NRF2 Oncogenic Activity

In cancer cells, NRF2 is often constitutively activated due to somatic mutations identified either in the NRF2 or KEAP1 genes. These mutations disrupt the normal regulation of NRF2, leading to its stabilization and continuous activation (51). Hence, NRF2 activation can promote cancer progression, metastasis, and resistance to chemotherapy and radiotherapy (263,283). This phenomenon has been coined the "dark side" of NRF2 referring to its pro-carcinogenic effects in the case of malignant cells (264) (summarized in **Figure 22**).

(1) NRF2 promotes cancer cell growth and proliferation: NRF2 modulates the basal and inducible expression of genes involved in proliferation control, such as *NOTCH1*, *NPNT*, *BMPR1A*, *IFG1*, *ITGB2*, *PDGFC*, *VEGFC*, and *JAG1* (284,285). Cell proliferation rates in *KEAP1^{-/-}* cells are shown to be faster than wild-type cells, and *NRF2^{-/-}* cells proliferate more slowly in hepatocellular carcinoma (286), breast (287), pancreatic (288), and lung cancer (289). Consistently, NRF2 knockdown reduces proliferation and is linked to decreased Ki67 expression and p53-induced senescence (83,290).

Furthermore, cancer cells exhibit higher protein synthesis rates compared to normal non-cancerous cells to support rapid growth. NRF2 contributes to this by upregulating genes in the serine/glycine biosynthetic pathway, including PHGDH, PSAT1, PSPH, SHMT1, and SHMT2. This upregulation occurs through the activation of ATF4, a downstream gene and binding partner of NRF2 (52,291). By enhancing these metabolic pathways, NRF2 supports the increased protein synthesis demands of proliferating cancer cells.

On the other hand, oncogenic proteins like KRASG12D, BRAFV619E, and MYC enhance NRF2 transcription. Cancers driven by KRAS, such as pancreatic and lung cancers, heavily rely on NRF2 to sustain mitogenic signaling (83,292). For instance, NRF2-mediated redox regulation maintains metalloprotease ADAM10 in a reduced state, crucial for shedding EGF and sustaining autocrine growth signaling of pancreatic organoids (293). NRF2 may also promote proliferation independently of growth factor signaling, as lung cancer cells with constitutive NRF2 activation remain resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (294). Moreover, in cancer, the PI3K-AKT pathway is often dysregulated, either through constitutive activation of receptor tyrosine kinases or PTEN inactivation (295). AKT, by inhibiting GSK3, prevents the phosphorylation of the Neh6 degron in NRF2, which is recognized by β -TrCP (296). Consequently, in cells with mutant PTEN, increased PI3K-AKT and NRF2 signaling lead to elevated proliferation rates and enhanced tumorigenicity (291).

(2) NRF2 counteracts antigrowth signals and apoptosis: The insensitivity to antigrowth signals, a hallmark of cancer, is associated with dysregulation of key cellular pathways, including the retinoblastoma protein (RB) pathway and receptor tyrosine kinase (RTK) signaling (297). Interestingly, in cell lines lacking functional RB and in prostate cancer models with RB inactivation, the expression of NRF2 is significantly reduced compared to normal cells or tissues. This decrease in NRF2 levels explains the observed ROS elevation and heightened sensitivity to chemotherapy (265,297).

Additionally, NRF2 induces the expression of cyclin-dependent kinase inhibitors (CDKi) p15 and p21, which arrest the cell cycle in response to moderate oxidative stress. Indeed, this facilitates the restoration of redox homeostasis and prevents excessive damage that could lead to apoptosis (280,285,298). This protective mechanism helps cancer cells survive in the presence of oxidative stress and continue proliferating.

Also, NRF2 directly inhibits apoptosis by upregulating anti-apoptotic proteins such as BCL-2 and BCL-xL, thereby reducing cytochrome c release from mitochondria and suppressing caspase 3/7 activation upon exposure to cytotoxic agents like cisplatin or etoposide (299–301). The homeodomain-interacting protein kinase 2 (HIPK2) is a novel NRF2 target gene with anti-apoptotic functions and involvement in the DNA damage response (302). Intriguingly, it possesses a context-dependent role, as it has been implicated in both apoptosis promotion in normal cells by phosphorylating p53 (303) and inhibition of cancer cell viability and migration through NOTCH1 degradation (304).

Furthermore, NRF2 deletion intensifies cell susceptibility to FAS-induced apoptosis, with this effect partly rescued by supplementing with glutathione (GSH) or its precursor N-acetyl cysteine (NAC) (305,306). Interestingly, elevated ROS levels lead to p53 accumulation and apoptosis; however, p53's influence on the NRF2 pathway is dual-phased: low p53 levels activate NRF2 via p21 upregulation, while higher p53 levels suppress NRF2 (298,307).

In cancer cells, continuous activation of NRF2 prevents ferroptosis, which involves iron-dependent lipid peroxidation leading to regulated cell death (308). This highlights the role of NRF2 in promoting cancer cell survival by inhibiting various forms of cell death, including apoptosis and ferroptosis.

(3) NRF2 sustains tumor angiogenesis: In response to the hypoxic tumor microenvironment, the transcription factor HIF-1 α promotes vasculature formation by inducing the expression of growth factors, cytokines, and extracellular matrix remodelers (309). NRF2 knockdown in xenograft models reduces blood vessel formation and inhibits tumor growth by decreasing HIF-1 α protein levels (263). Interestingly, HIF-1 α signaling can also activate NRF2 via ERK1/2 activation, creating a feedback loop (310). Furthermore, NRF2 and HIF-1 α share common transcriptional targets involved in metabolic reprogramming under hypoxia, such as *HMOX1* and *NQO1* (311,312). However, NRF2's role in sustaining tumor angiogenesis extends beyond its interaction with HIF-1 α . NRF2 independently regulates several angiogenic factors and pathways by directly upregulating VEGF, PDGF, and angiopoietin levels (263) which are crucial for blood vessel

formation. Additionally, NRF2 modulates endothelial cell function angiogenic signaling, which is essential for angiogenesis (313).

(4) NRF2 enhances tumor invasion and metastasis: NRF2 promotes the epithelial-to-mesenchymal transition (EMT) by downregulating E-cadherin expression, thereby facilitating cell migration (314,315). Conversely, NRF2 silencing reduces N-cadherin expression, potentially through the regulation of the NRF2 target gene NOTCH1, a key regulator of EMT (284,316). Interestingly, NRF2 exhibits a contrasting role in normal cells. In these cells, NRF2 inhibits EMT by suppressing the transcription factor Snail expression (317) and upregulating heme oxygenase-1 (318). NRF2 activation correlates with the activation of the RhoA/ROCK pathway, promoting migration and metastasis of breast cancer cells (287). Additionally, NRF2 downregulation correlates with reduced expression or activity of matrix metalloproteinases (MMPs) such as MMP2 and MMP9, required to facilitate ECM remodeling and cell migration (316,319). Furthermore, cancer cells with high NRF2 levels exhibit anchorage-independent growth and enhanced metastatic potential via the induction of osteopontin protein (OPN), an extracellular matrix protein also known as SPP1 (320).

(5) NRF2 regulates cancer metabolism: Cancer cells adapt their metabolism to support rapid growth and proliferation by favoring the Warburg effect. This involves prioritizing aerobic glycolysis given its relatively poor efficiency of generating ATP (2 ATP) to produce anabolic precursors over the more energy-efficient process of glycolysis followed by oxidative phosphorylation (32 ATP) (283). NRF2 plays an essential role in regulating major metabolism pathways in cancer cells (**Figure 23**). Mainly, NRF2 induces the expression of several glycolytic enzymes, such as hexokinase 1 (HK1), HK2, glucose phosphate isomerase 1 (GPI1), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFK2), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFK4), fructose-bisphosphate aldolase A (ALDA), enolase 1 (ENO1), enolase 4 (ENO4), and pyruvate kinase, muscle (PKM). This upregulation enhances glycolytic flow and maintains sufficient levels of glycolytic intermediates needed in biosynthetic pathways for nucleosides, amino acids, and lipids (49). Also, NRF2 induces the pentose phosphate pathway (PPP) by upregulating PPP enzymes including glucose-6-phosphate (G6PD), phosphogluconate dehydrogenase (PGD), transketolase (TKT) and transaldolase 1 (TALDO1), as well as enzymes for de novo nucleotide synthesis enzymes, such as phosphoribosyl pyrophosphate amidotransferase (PPAT) and methylenetetrahydrofolate dehydrogenase 2

(MTHFD2) (75). Also, NRF2 regulates fatty acid metabolism by activating genes encoding key enzymes involved in processes like the elongation of very long chain fatty acids (ELOVL7), fatty acid desaturation (FADS1), and the synthesis and breakdown of acyl-CoA compounds (ACSS2, ACOT7, ACAD10, and ACAD12) [34,35].

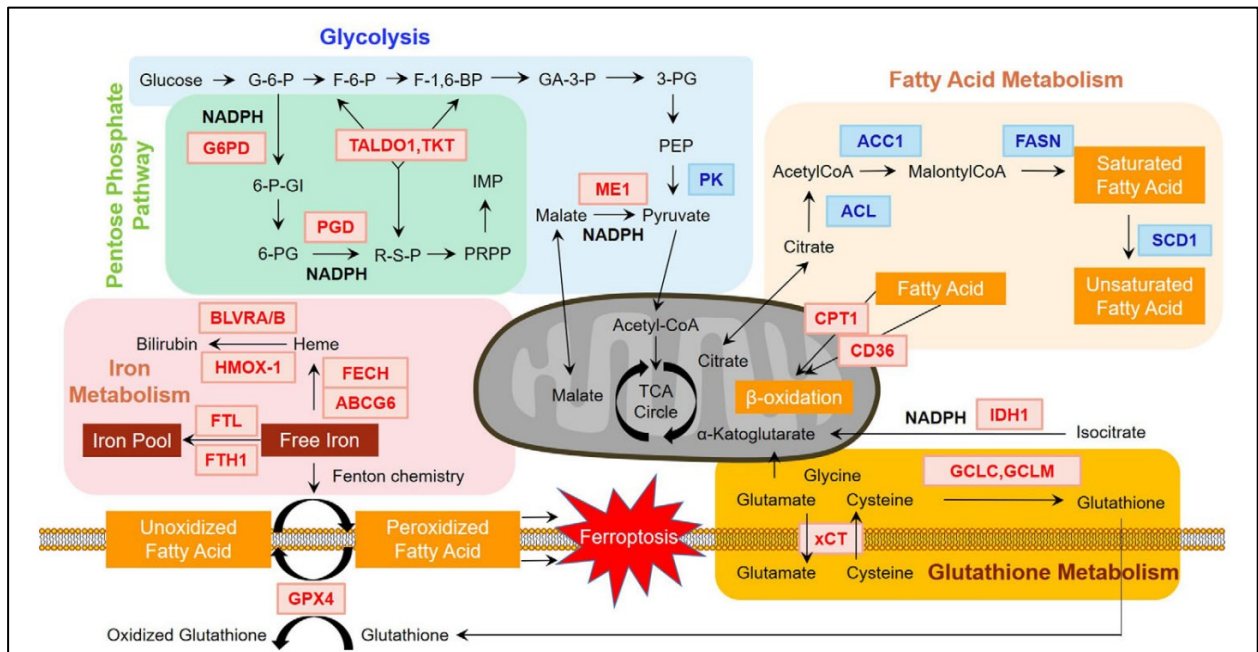


Figure 23. Regulation of Major Metabolic Pathways by NRF2 (321).

This figure illustrates the essential role of NRF2 in regulating major metabolic pathways in cancer cells. Genes positively regulated by NRF2 are shown in red, while those negatively regulated are in blue. Glucose-6-phosphate (G6P); Fructose-6-phosphate (F6P); Fructose-1,6-bisphosphate (F1,6BP); Glyceraldehyde-3-phosphate (GA3P); 3-Phosphoglycerate (3PG); Phosphoenolpyruvate (PEP); Pyruvate kinase (PK); Glucose-6-phosphate dehydrogenase (G6PD); 6-Phosphogluconate dehydrogenase (PGD); Transaldolase 1 (TALDO1); Transketolase (TKT); Malic enzyme 1 (ME1); Isocitrate dehydrogenase 1 (IDH1); Glutamate-cysteine ligase catalytic subunit (GCLC); Glutamate-cysteine ligase modifier subunit (GCLM); Cystine/glutamate transporter (xCT); Glutathione peroxidase 4 (GPX4); Heme oxygenase 1 (HMOX1); ATP-binding cassette subfamily G member 6 (ABCG6); Ferrochelatase (FECH); Biliverdin reductase A/B (BLVRA/B); Ferritin light chain (FTL); Ferritin heavy chain 1 (FTH1); Acetyl-CoA carboxylase 1 (FACC1); Fatty acid synthase (FASN); Stearoyl CoA desaturase 1 (SCD1); Carnitine palmitoyltransferase 1 (CPT1); Cluster of differentiation 36 (CD36); ATP-citrate lyase (ACL); Nicotinamide adenine dinucleotide phosphate (NADPH); Tricarboxylic acid cycle (TCA Cycle); Extracellular matrix (ECM); Osteopontin/Secreted phosphoprotein 1 (OPN/SPP1).

(6) *NRF2 supports cancer stem cells*: Cancer stem cells (CSCs) are a unique subset within tumors capable of self-renewal, and differentiation, enabling tumor survival and resistance to treatments like radiotherapy and chemotherapy (152). The self-renewal capacity of CSCs is largely due to elevated expression of antioxidant enzymes, drug transporters, cell cycle quiescence, and enhanced DNA repair capacity (153). A critical factor contributing to these properties is the elevated expression of NRF2 in CSCs compared to non-CSCs. NRF2 is a transcription factor that enhances CSCs' ability to manage oxidative stress by upregulating antioxidant enzymes, which helps maintain low levels of ROS. This gives CSCs a survival advantage, especially under stress conditions induced by cancer therapies (322). High levels of PERK in dedifferentiated cancer cells activate the NRF2 pathway, further boosting NRF2 activity

and supporting CSCs' stemness by reducing ROS levels through increased expression of antioxidant and drug transport proteins (323). This enhanced capacity allows CSCs to maintain their stem-like properties, contributing to tumor initiation, metastasis, and resistance to treatment. Consequently, NRF2's elevated expression not only bolsters antioxidant defenses but also upregulates glycolytic enzymes and stemness markers, driving the metabolic reprogramming of CSCs and promoting their resilience and aggressiveness in the tumor microenvironment (324). Moreover, NRF2 influences key signaling pathways and stemness-related molecules in various cancers. Here, we summarize evidence of NRF2 signaling's role in conferring CSC properties (**Figure 24**) as reviewed by Hallis and colleagues (154). In head and neck CSCs, loss of KEAP1 leads to persistent NRF2 activation, enhancing CSC self-renewal through NOTCH signaling. In lung cancer, radiation-induced migration relies on NOTCH1, and NRF2 inhibition reduces metastasis by lowering NOTCH1 levels. In breast cancer cells, NRF2-induced heme oxygenase-1 upregulates NOTCH1, promoting mammosphere formation. Similarly, in KEAP1-mutated lung cancer cells, NRF2-CEBPB cooperation remodels the NOTCH3 enhancer, boosting tumor-initiating capacity. Beyond NOTCH signaling, NRF2 upregulates FOXO3 and BMI-1 in breast CSCs, enhancing self-renewal. It also supports colon CSC survival through NRF2/FOXO3-mediated sulfiredoxin-peroxiredoxin upregulation. Persistent NRF2/ β -catenin activation in hepatic stem cells promotes proliferation and tumorigenesis. In liver tumor-initiating cells, NRF2 activates the sonic hedgehog pathway, aiding tumorigenesis. NRF2 stabilizes the stem cell marker Nestin, contributing to oxidative stress resistance and malignancy initiation in non-small cell lung cancer (154).

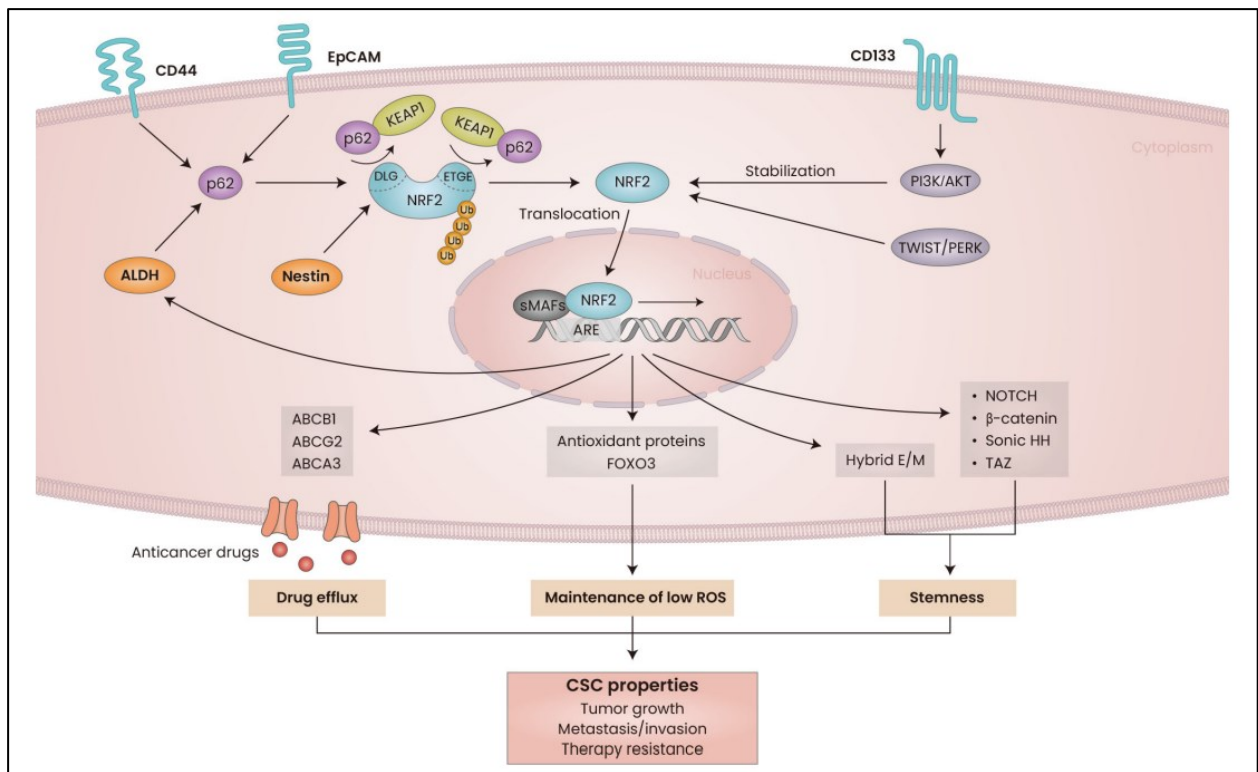


Figure 24. Association of NRF2 with Cancer Stem Cells (CSCs) Properties (322).

NRF2 signaling is activated in CSCs and contributes to CSC properties, such as tumor initiation, metastatic malignancy, and therapy resistance. NRF2 is activated by CSC markers such as CD44, EpCAM, and ALDH, and p62 accumulation is associated with NRF2 activation. CD133 expression leads to NRF2 stabilization through PI3K/AKT pathway activation. TWIST-mediated PERK activation directly induces NRF2 accumulation. Competitive binding of Nestin with KEAP1 induces NRF2 liberation and translocation into the nucleus. NRF2 upregulates multiple antioxidant defense genes and FOXO3 to maintain low ROS levels. ABC transporters, including ABCB1, ABCG2, and ABCA3, are upregulated by NRF2 and contribute to chemotherapy resistance. A high level of NRF2 is also associated with the upregulation of transcription factors, including NOTCH1/3, Sonic Hedgehog, β -catenin, and TAZ to maintain the stemness of cancer cells. NRF2 activation stabilizes cells in a hybrid epithelial/mesenchymal (hybrid E/M) state to support phenotypic conversion to CSCs. Reactive oxygen species (ROS); epithelial cell adhesion molecule (EpCAM); phosphoinositide 3-kinases (PI3K); small MAF proteins (sMAFs); antioxidant response element (ARE); Kelch-like ECH-associated protein 1 (KEAP1); DLG motif (DLG); ETGE motif (ETGE); aldehyde dehydrogenase (ALDH); neurogenic locus notch homolog protein (NOTCH); hedgehog homolog (HH).

(7) NRF2 regulates other hallmarks of cancer: The implication of NRF2 in cancer promotion extends to regulating the limitless replicative potential of cancer cells. For instance, oxidative stress-induced damage to DNA and telomeres promotes senescence, but NRF2 helps mitigate this by decreasing oxidative DNA damage, indirectly preserving telomere length during aging (325). Also, NRF2 activation reduces replication-induced senescence by enhancing proteasomal activity which in turn reduces the accumulation of oxidized and ubiquitylated proteins. This enhancement of proteasomal activity delays the senescence process, thus promoting continued cancer cell proliferation (326,327). In addition, NRF2 prevents DNA damage by reducing the level of ROS (328) and therefore reducing genome instability to enhance cancer cell survival. Furthermore, to cope with oxidative stress, cancer cells utilize various mechanisms such as heat shock proteins, the ubiquitin-proteasome system, and autophagy. Notably, NRF2 is crucial in

regulating these stress-response processes, allowing cancer cells to survive and proliferate under adverse conditions (263).

D.1.c Implications of NRF2 Inhibitors and Activators in Cancer Therapy

Efforts to reduce cancer incidence have led to the concept of chemoprevention, which involves using dietary compounds or synthetic chemicals to prevent cancer, a practice that dates back half a century (114). Considering the anti-tumoral activity of NRF2, numerous NRF2 inducers have been identified for their cancer-preventive effects. Many of these exert their effects through the activation of NRF2. For instance, sulforaphane, abundant in broccoli sprouts, has shown promise in attenuating cancer risk among individuals exposed to aflatoxins and airborne toxins (329). It induces the transcription of phase II enzymes through NRF2 activation and facilitates cancer cell arrest and apoptosis via p53 mechanisms in human colon cancer cells (330). Additionally, it targets NF- κ B (331) as well as both JUN and FOS of the AP-1 complex to perform an anti-inflammatory effect (332). Curcumin, another NRF2 activator found to be safe and well-tolerated, has shown pleiotropic activity (184,185). Other NRF2 activators being evaluated in clinical trials on cancer patients include resveratrol, bardoxolone-methyl (CDDO-Me), oltipraz, and RTA-408 (omaveloxolone). However, translating the observed benefits of NRF2 activators from laboratory studies to clinical practice remains limited (328). Caution is warranted, particularly in cancer patients, as hyperactivation of the NRF2 pathway has been associated with negative effects. For instance, in diabetic patients, certain diabetic drugs have been found to increase the risk of metastatic spread in cancer patients by prolonging NRF2 activation (333). Therefore, the use of NRF2 activators should be carefully evaluated, especially in cancer patients, considering the complex interplay of the NRF2 pathway in cancer development and progression.

In light of the pivotal oncogenic role played by NRF2 in tumor cells, a diverse range of compounds has emerged as potent NRF2 inhibitors, as summarized in **Table 3**. Among these is Brusatol, a triterpene lactone compound extracted from *Brucea javanica*, which sensitizes a broad spectrum of cancer cells by inhibiting the NRF2 signaling pathway, while also enhancing radio-sensitivity and reducing chemoresistance(334). Among the other plant extracts are the flavonoids which constitute a group of natural polyphenolic compounds. For example, apigenin, found in fruits, vegetables, and herbs, reduces the expression of NRF2 and its downstream genes by downregulating the PI3K/Akt pathway (335) and reversing drug-resistant phenotypes apigenin

(336). Luteolin, present in celery, parsley, green pepper, perilla leaf, and chamomile tea (337), sensitizes A549 cells to anticancer drugs like oxaliplatin, bleomycin, and doxorubicin (338). It also sensitizes oxaliplatin-resistant colorectal cancer cells to chemotherapy by inhibiting the NRF2 pathway (339). Wogonin, isolated from the root of *Scutellaria baicalensis*, exhibits anticancer properties, including anti-proliferative, apoptotic, and anti-migration activities (340). It reverses the multi-drug resistance of human myelogenous leukemia by inhibiting MRP1, reducing NRF2 binding to ARE, and lowering NRF2 mRNA levels (341). Chrysin a natural flavonoid derived from blue passionflower, propolis, and honey, inhibits cell proliferation, migration, and invasion, suppressing tumor growth in U87 xenografts by inhibiting NRF2 nuclear localization and suppressing the expression of heme oxygenase-1 (HO-1) and NAD(P)H quinine oxidoreductase-1 (342). In doxorubicin-resistant hepatocellular carcinoma cells, chrysin enhances sensitivity to doxorubicin by inhibiting NRF2 expression and downregulating downstream genes like HO-1, AKR1B10, and MRP5 (343). Although no NRF2-targeted therapy has been approved for cancer treatment, early-phase clinical trials have shown promising results (344). For instance, halofuginone is in Phase I/II trials for AIDS-related and recurrent Kaposi Sarcoma. Other trials are assessing the safety and antitumor activity of Berberine (BBR) in colorectal, lung, and gastric carcinoma. However, the safety of NRF2 inhibitors remains an area of ongoing research, with concerns about potential adverse effects such as liver toxicity and cardiovascular issues.

Table 3. Anticancer Effects of Naturally Occurring NRF2 Inhibitors and Associated Action Mechanisms (344).

Active compounds	Cancer type	Model	Dose	Mechanisms of action
Apigenin	HCC	HCC cells (BEL-7402) and BEL-7402/ADM cells	20 µM; 50 mg/kg	↓Nrf2, ↓HO-1, AKR1B10, MRP5; ↓p-Akt
Luteolin	Lung cancer	A549 cells	1-20 µM;	↓Nrf2, ↓HO-1, NQO1, AKR1C
	CRC	HCT116 and SW620	10 µM; 40 mg/kg	↓Nrf2, ↓HO-1, NQO1, GSTα1/2
	Breast CSCs	MDA-MB-231	1-2 µM	↓Nrf2, ↓ABCG2, Nanog, Oct4, Cripto1, Sirt3, HO-1
Wogonin	CML	K562 cells	40 µM; 40 mg/kg	↓Nrf2, ↓P65, P50, p-Stat3, NF-κB pathway
	CML	K562 cells	40 µM;	↓Nrf2, ↓MRP1, NQO1, HO-1, PI3K, p-Akt,
Chrysin	Glioblastoma	T98, U251, U87 cells	30/60 µM; 40/80 mg/kg	↓Nrf2, ↓HO-1, NQO1, ↓p-JNK, p-P38, p-ERK
	HCC	BEL-7402 cells	10 µM	↓Nrf2, ↓HO-1, AKR1B10, MRP5; ↓p-Akt, p-JNK, p-ERK, p-P38
Halofuginone	Lung cancer	A549, KYSE70 and ABC1 cells	50-100 nM; 0.25 mg/kg	↓Nrf2, ↓NQO1
Trigonelline	Pancreatic cancer	MiaPaca2, Panc1 and Colo357 cells	0.1/0.5 µM; 0.02 mg/kg	↓Nrf2
Berberine	HCC	Huh7, HepG2 and HHL-5 cells	20 µM; 5 mg/kg	↓Nrf2, ↓HO-1, NQO1
	Breast cancer	BT-474 and AU-565 cells	2 µM; 5 mg/kg	↓Nrf2, ↓p-GSK3β, C-Myc
Brusatol	AML	THP1, Molm13, U937, HL60 cells	1 µM	↓Nrf2
	Lung cancer	A549 cells	40 nM; 2 mg/kg	↓Nrf2, ↑Keap-1 ↓MRP1, MRP2, NQO1, γ-GCS,
		A549 cells	40 nM	↓Nrf2, ↓NQO1, GCLC, GCLM
	CRC	HCT116, CT26	300 nM; 2 mg/kg	↓Nrf2, ↑Keap-1
	Hepatoma	Mouse Hepa-1c1c7 cells	300 nM;	↓Nrf2, ↓NQO1
	Melanoma	A357 cells	100 nM; 2 mg/kg	↓Nrf2, ↓HO-1, NQO1, GSTP1
	Breast cancer	MCF-7, MDA-MB-231	40 nM	↓Nrf2, ↑Keap-1, ↓NQO1, GCLM, HO-1
	Glioma	U-251	2 ng/mL	↓Nrf2, ↓NQO1, GCLC
Brucein D	PDAC	Miapaca-2, Capan2 and PANC1 cells	1.5 µM; 2 mg/kg	↓Nrf2, ↓HO-1, NQO1, AKR1B10 γGCSm, MRP1 and MRP5
Cryptotanshinone	Lung cancer	A549 cells	10 µM	↓Nrf2, ↓MRP1, GCLC, GCLM, HO-1, NQO1, ↓p-Akt, p-STAT3, p-JNK, p-ERK
Ginsenoside Rd	NSCLC	A549 cells	80 µM	↓Nrf2, ↓HO-1, NQO1, GCLC, MRP1,
Ascorbic acid	CML	KCL22 cells	0.125 mM	↓Nrf2
Triptolide	NSCLC/liver cancer	A549 cells and HepG2	0.5 µM; 0.25 mg/kg	↓Nrf2
	Heart	H9c2 cells	1.2 mg/kg	↓Nrf2, ↓Bax/Bcl-2, ↑Caspase 3
	Glioblastoma	U251 MG cells	30 nM	↓Nrf2, ↓GCLC, GCLM

D.2 NRF2 in GB Progression

Despite advancements in therapeutic strategies for solid tumors, the prognosis for patients with GB remains minimal (345). NRF2 is significantly overexpressed in GB tissues compared to normal brain tissue. Analysis of clinical samples indicates that NRF2 protein levels and nuclear localization are markedly elevated in human GB tissues (66,346). Additionally, there is a notable increase in NFE2L2 mRNA expression in these tumor tissues compared to the surrounding normal tissues (347) suggesting its implication in the disease prognosis and potential treatment options. Herein, we discuss NRF2-mediated direct mechanisms related to GB progression and indirect interaction with the surrounding microenvironment (**Figure 25**).

a) Cell Proliferation and Survival: NRF2 regulates key processes vital for GB cell growth and viability (347). NRF2's role in enhancing cell proliferation is underscored by its upregulation of various pathways and proteins, including EGFR, Ki-67, Kras, PI3K/Akt (348), and antiapoptotic proteins like Bcl-2 (349). Moreover, the crosstalk between NRF2 and mTOR pathways is pivotal in sustaining GB cell survival, with NRF2 downregulation leading to ATP depletion and impaired AMPK-mTOR activity. This results in reduced U251 cell proliferation

and colony formation (350). Targeting the NRF2 pathway can involve modulating tyrosine kinase-mediated signaling, as illustrated by chrysin, a natural flavonoid with known antioxidant properties and NRF2 inhibitor. Chrysin deactivates the NRF2 signaling pathway by decreasing the translocation of NRF2 into the nucleus. Chrysin has been shown to inhibit T98, U251, and U87 cell proliferation by downregulating the extracellular signal-regulated kinase (ERK)/NRF2 pathway and the two antioxidant enzymes HO-1 and NQO-1. Also, it inhibits the *in vivo* tumorigenicity of U87 xenografts in BALB/c athymic nude mice (351). Additionally, the reciprocal relationship between NRF2 and TERT demonstrates their cooperative role in enhancing GSH synthesis and supporting A172 and U87MG GB cell lines' survival against ROS-induced oxidative damage during proliferation (352).

- b) *Cell Invasion and Malignancy Switch:*** The NRF2/ARE pathway emerges as a critical regulator of matrix metalloproteinases (MMPs), a family of structurally related, zinc-dependent endopeptidases, which are essential for extracellular matrix (ECM) degradation and facilitate tumor cell migration and invasion. MMPs are categorized based on their functions into several distinct groups (353) including collagenases, stromelysins and stromelysin-like MMPs, gelatinases, matrilysins, and membrane-type MMPs (MT-MMPs). These MMPs are responsible for ECM remodeling, and degrading the basement membrane components, processes that are vital for tumor invasion inflammation and angiogenesis. Downregulation of NRF2 expression, using small interfering RNA (siRNA), leads to MMP-9 deactivation and reduced U251 cell invasion, while its upregulation via plasmid transfection enhances MMP9 expression and activity, promoting invasion and migration (319). The involvement of NRF2 in mesenchymal transition is mediated by co-regulatory feedback loops of NRF2 with proteins like autophagy-regulating protein complex Sequestosome1 (SQSTM1/p62) (354) and the promotion of mesenchymal markers like Slug and β -catenin (355). Additionally, suppression of the ERK/NRF2 pathway using chrysin emerges as a promising strategy to mitigate U251 and U87MG cell migration and invasion, further highlighting the intricate role of NRF2 in driving GB malignancy (351).
- c) *GSC maintenance and self-renewal capacity:*** NRF2 proves to be essential for preserving the self-renewal capacity of GSCs both *in vivo* and *in vitro*. Silencing NRF2 expression, using NRF2 shRNA in primary human GSC suppresses GSC proliferation, improves their differentiation, and reduces their expression of key stemness markers such as SRY-box transcription factor 2

(SOX2), B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1), and Cyclin E proteins and promotes their differentiation into neuronal-like cells (356,357). NRF2's function in maintaining the undifferentiated state of GSCs is mediated by inhibiting ROS accumulation through upregulating the expression of target genes including NQO-1 and HO-1 antioxidant enzymes. However, a deeper understanding of the molecular intricacies governing the interplay between ROS and NRF2 in GSC differentiation warrants further investigation (32). Moreover, knocking down NRF2 by CRISPR/Cas9 in U87MG neurospheres enhances GSC sensitivity to low and high-dose rates of gamma irradiation by reducing their antioxidant capacity, stemness, and self-renewal capacity (358). Additionally, lentiviral over-expression of NRF2 induces the expression of the transcriptional coactivator with PDZ-binding motif (TAZ), a key component of the Hippo signaling pathway, which in turn promotes GSC growth and tumorigenicity. The effect was observed across different glioblastoma explants, including GB1 and GB3, as well as in cell lines such as U373 MG and U87 MG. In these experiments, the GSCs were cultured in an appropriate medium to form floating spherical colonies known as neurospheres, which are indicative of their stem cell properties and tumorigenic potential (359).

d) *Metabolic Rewiring:* The NRF2/KEAP1 pathway is a crucial regulator of intracellular metabolic processes, playing a significant role in both anabolic and catabolic metabolism. NRF2 is known to coordinate the PI3K/AKT/mTOR pathway, the p62 pathway, AMPK signaling, the TCA cycle, fatty acid oxidation, and iron metabolism to support cell growth and metabolic reprogramming in various cancer types (360). However, the specific function of NRF2 in GB metabolism remains an area of ongoing exploration. Nonetheless, existing evidence indicates that NRF2 enhances glycolysis. Through the NRF2-TERT loop, NRF2 promotes the activity of enzymes such as glucose-6-phosphate dehydrogenase (G6PD) and transketolase (TKT) in A172 and U87MG GB cells. These enzymes are essential for the pentose phosphate pathway (PPP), which branches from glycolysis and plays a crucial role in cellular metabolism by generating NADPH and ribose-5-phosphate (352). Additionally, NRF2 facilitates the phosphorylation of glycogen synthase (GS), an important regulatory step for glycogen accumulation. Glycogen serves as a glucose reservoir for sustaining glycolysis, particularly in the context of rapidly proliferating tumor cells. In tumors with TERT promoter mutations (C228T and C250T), linked to increased telomerase activity, NRF2's role in

promoting these metabolic processes is crucial (352). This underscores NRF2's intricate role in reshaping the metabolic landscape of GB tumors.

e) *Angiogenesis and Perivascular Microenvironment:* NRF2's overexpression in GB tissues correlates positively with increased microvessel density (MVD), underlining its involvement in angiogenesis regulation. Conversely, RNAi-mediated NRF2-knockdown leads to reduced MVD alongside the accumulation of HIF-1 α and its target genes, including vascular endothelial growth factor (VEGF) in U87 and U251 GB cells. This reduction in NRF2 activity also impairs tumor growth and reduces vessel formation *in vivo* in BALB/c athymic nude mice implanted with these cell lines (361). Together, these NRF2-driven mechanisms foster a supportive microenvironment conducive to tumor growth and colonization.

D.3 NRF2 in GB Resistance and Treatment Strategies

The role of NRF2 in GB extends beyond its involvement in tumor progression to significantly impact resistance to conventional and emerging treatment strategies. Here, we discuss how NRF2 contributes to GB therapy resistance and potential strategies for overcoming treatment challenges in GB (**Figure 25**).

a) Therapy Resistance and Cell Death: NRF2 activation plays a pivotal yet paradoxical role in therapy resistance and cell death in GB. NRF2 activation enhances cell survival mechanisms and confers resistance to chemotherapy agents like temozolomide (TMZ) through GSH induction (362). Moreover, the downregulation of NRF2 using corilagin (a tannin with antioxidant properties) (363), or valproic acid (VPA, a mood stabilizer), and melatonin (MEL, a hormone regulating sleep) (364,365) induces tumor cell apoptosis by inhibiting the NRF2-ARE signaling pathway in TMZ-resistant GB cells.

Conversely, studies, such as the one by Souza and colleagues, have revealed a paradoxical effect of NRF2 in enhancing GB cell vulnerability to chemotherapy. Elevated NRF2 levels were found to increase the susceptibility of TMZ-resistant GB cells to ferroptosis, a form of cell death characterized by iron-dependent lipid peroxide accumulation. This susceptibility is mediated via ATP-binding cassette subfamily C member 1 (ABCC1), which facilitates the export of GSH-drug complexes, leading to reduced drug accumulation and subsequent GSH depletion. This depletion compromises the antioxidant capacity of the cells, rendering them more prone to ferroptosis (366). Moreover, NRF2 affects ferroptosis susceptibility by regulating iron metabolism genes (FTH1, FPN1) and heme degradation genes (HO-1, BLVRA/B), linking its levels to changes in iron balance and oxidative stress (367). The paradoxical role of NRF2 in TMZ treatment highlights its dual impact, contributing to both GB cells' chemotherapy resistance and susceptibility to death. This complexity underscores the intricate interplay of NRF2-mediated responses in GB chemoresistance, necessitating a comprehensive understanding of the development of effective therapeutic strategies.

In terms of radiotherapy, elevated NRF2 levels in GB cells are associated with a poorer response to radiation therapy, as NRF2's protective role against oxidative stress mitigates the therapeutic effects of radiation. Knocking down NRF2 in U87MG neurospheres

significantly increased radiosensitivity by lowering levels of oxidative stress response proteins like SOD1 and APE1, and promoted cell differentiation, leading to increased cell death, reduced neurosphere formation, and impaired cell proliferation (358). Elevated NRF2 levels and its increased nuclear localization have also been observed in recurrent GB tissues from radiotherapy patients, indicating that NRF2 enhances radiation resistance by strengthening antioxidant defenses. Conversely, reducing NRF2 enhances GB cell sensitivity to radiation by increasing oxidative stress and promoting apoptosis in U251 and U87 cells. These findings highlight NRF2 as a promising target for improving the effectiveness of radiotherapy in GB (368).

b) *Tumor Immune microenvironment and Immunotherapy:* The NRF2/ARE pathway is a key regulator of tumor immunosurveillance, impacting cytokine secretion and immune cell function (369). In the context of cancer, the activation of NRF2 correlates with the suppression of interferon- γ (IFN- γ) production, a key cytokine involved in antitumor immunity, and favors the differentiation of CD4(+) T cells towards the Th2 phenotype. This skewing towards the Th2 phenotype undermines the efficacy of antitumor immune responses to establish an immunosuppressive tumor microenvironment conducive to tumor progression (370).

Despite its importance, evidence regarding NRF2's role in the GB tumor microenvironment (TME) remains limited. The activation of the NRF2 pathway, through small molecule activators in cultured microglial cells, has been shown to modulate microglial activity and overall immunological responses in the brain. This modulation includes suppression of excessive inflammation and enhancement of cellular resilience, suggesting potential therapeutic avenues for addressing GB-associated immunoresistance (371). Recent advancements in addressing GB immunotherapy resistance include enhancing T lymphocyte function. One promising approach involves MRI-guided peritumoral administration of mesenchymal stem cells (MSCs) engineered to overexpress CXCL10 and NRF2. This strategy significantly boosts T lymphocyte recruitment and function within the GB TME, improving T-cell activity and resistance to oxidative stress, while remodeling the TME to support anti-tumor immunity. When combined with immune checkpoint blockade (ICB), this cell therapy markedly increases therapeutic efficacy, offering a promising method to overcome GB immunotherapy resistance and enhance treatment outcomes (372). Additionally, NRF2 plays a critical role

in immune escape mechanisms by inhibiting dendritic cell (DC) maturation and suppressing T cell activation. This inhibition leads to reduced T cell proliferation and cytotoxicity against GB cells. Targeting NRF2 in DCs reverses these effects, promoting enhanced DC maturation and improved T-cell activation and cytotoxicity. Therefore, inhibiting NRF2 in DCs presents a valuable strategy for augmenting the effectiveness of immunotherapies by strengthening the anti-tumor immune response (373).

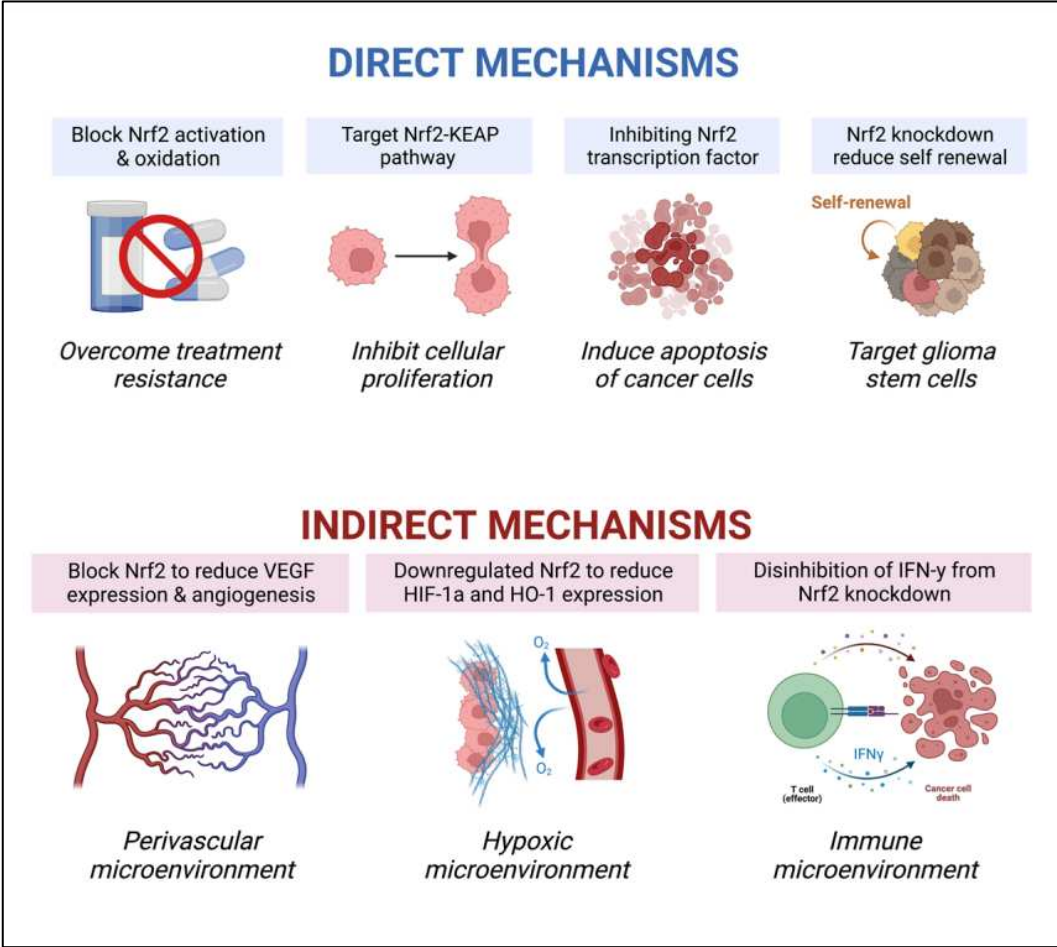


Figure 25. Schematic Showing Potential Targets for GB Therapy Related to NRF2 Signaling and Expression (374).

D.4 NRF2 Prognostic Controversies in GB

Despite that NRF2 is highly expressed in GB tissues compared to normal brain tissue (66), the relationship between NRF2 expression and GB patient survival is still controversial. This is due to conflicting results in published studies, noting that most studies are *in silico* analyses using available databases. On one side, either the Rembrandt database or the SurvExpress tool for GB patient data showed that the upregulation of *NFE2L2* is associated with significantly poorer overall survival rates (66). Consistent with these findings, based on microarray and RNA-seq gene expression analysis, NRF2 overexpression positively correlated with WHO grade in gliomas, and patients whose tumors had high levels of NRF2 survived shorter periods (374).

On the other side, contradictory studies can be highlighted. For example, Haapasalo and colleagues indicate that *NFE2L2* expression was not associated with overall survival in GB patients (375). Similarly, using TCGA GB prognostic clinical data, no difference in the overall survival of patients with high NRF2 activity was reported, but the progression-free survival was strongly decreased (35). To compare with the existing literature, by analyzing the GEPIA2 database, we found that *NFE2L2* gene expression was elevated in GB tumors compared to normal tissue (**Figure 26A**). However, the variation in the overall survival or disease-free survival rates among GB patients with low or high *NFE2L2* gene expression did not achieve statistical significance (**Figure 26B-C**) (66).

At the molecular level, the upregulated expression of NRF2 target genes, including NQO1 and GCLM genes, is evident in GB and correlates with the wild-type IDH1/2 status. However, their expression was not associated with progression-free or overall survival of the GB patients (5).

Regarding GB classification, NRF2, via its co-regulatory positive feedback with p62, mediates the mesenchymal transition of GB (23). This is consistent with the *in-silico* analysis conducted by our team, where we observed that the *NFE2L2* gene is significantly elevated in the mesenchymal subtype. However, we also observe an equally significant increase of the *NFE2L2* gene in the classical subtype of GB (**Figure 26D**), yet the mesenchymal is the most aggressive among the GB subtypes (66,376). This implies that the elevated *NFE2L2* gene levels across different molecular subtypes of GB indicate its potential as a common molecular feature in GB tumorigenesis.

Regarding TMZ resistance, despite the paradoxical role of NRF2 in TMZ resistance as discussed earlier, a robust correlation is evident between *NFE2L2* gene expression and patient response to TMZ, as illustrated by receiver operating characteristic (ROC) analysis of GB patient databases (**Figure 26E-F**) (66). This association suggests the predictive capability and potential clinical

relevance of the *NFE2L2* gene, underscoring the prospect of utilizing NRF2 in therapeutic approaches aimed at addressing GB TMZ resistance.

Therefore, taken together, the contrasting findings on *NFE2L2* or NRF2 expression and GB patient survival likely stem from a combination of methodological differences, tumor heterogeneity, context-dependent effects, sample biases, and variability in data interpretation. However, these observations illustrate the complexity of NRF2's role in GB prognosis, highlighting the necessity for further exploration into NRF2's involvement in GB patient outcomes to elucidate its true prognostic value and inform targeted therapeutic strategies.

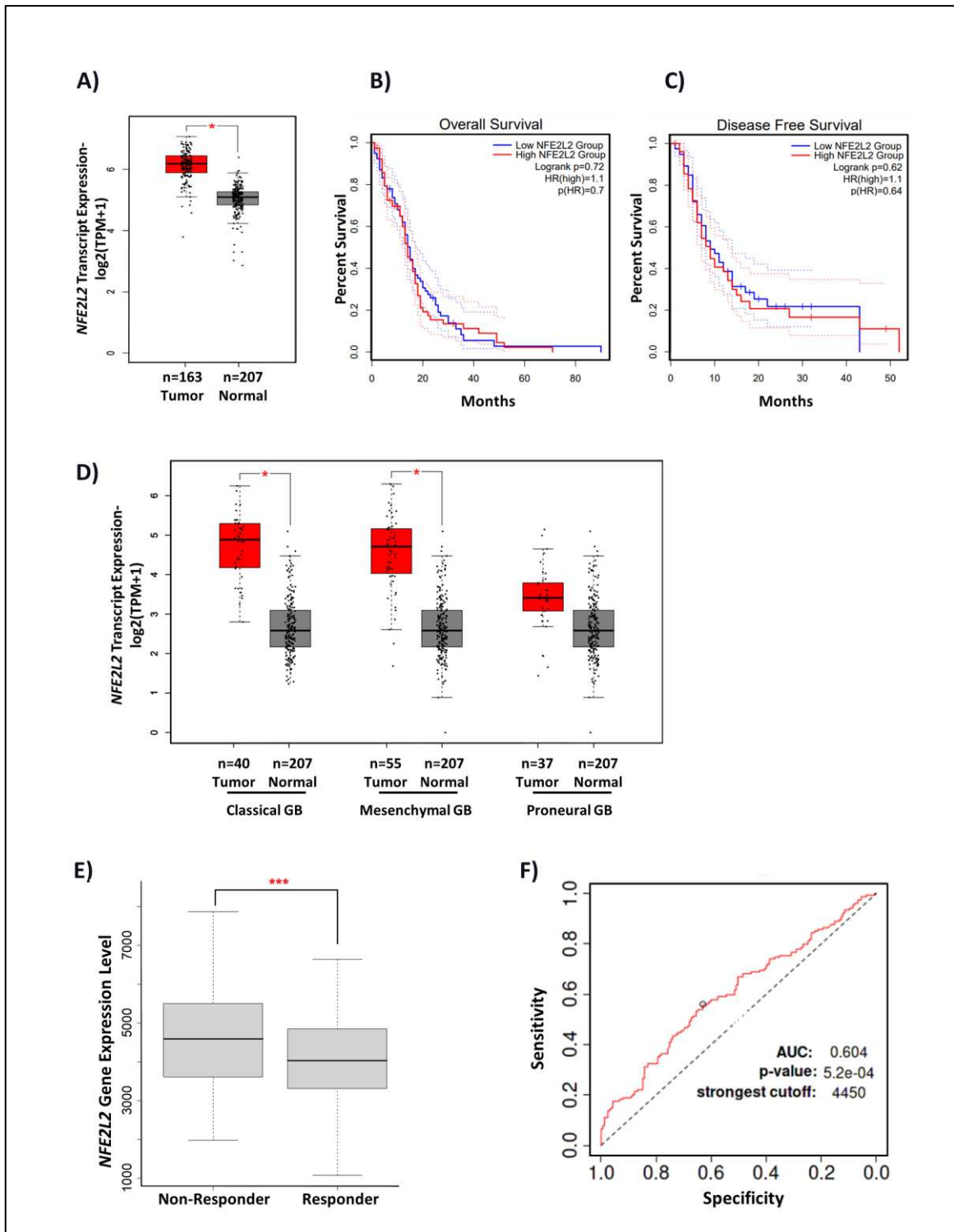


Figure 26. NFE2L2 Gene Expression Levels in GB and the Impact on the Clinical Outcome (66).

(A) Tissue-wise expression profile of the *NFE2L2* gene expression in GB tumors compared to normal tissue. Data is sourced from GEPEIA2 for GB patient databases. (B) Kaplan–Meier survival curves of overall survival and (C) disease-free survival of patients with GB based on the high (red) and low (blue) expression of the *NFE2L2* gene, respectively. (D) Tissue-wise expression profile of *NFE2L2* gene expression in GB subtypes compared to normal tissues. Data is sourced from GEPEIA2 for GB patient databases. (E) ROC plotter showing the *NFE2L2* gene expression in patients classified as responders (165 patients) and non-responders (154 patients) to TMZ treatment (p-value = .0013). (F) ROC curve analysis shows the validity of *NFE2L2* gene expression in discriminating responders and non-responders, with the sensitivity representing the true positive rate and the specificity representing the false positive rate. Data is sourced from ROC Plotter—Online ROC analysis for GB patient data. The red star denotes statistical significance. AUC, the area under the curve; TPM, transcripts per million reads n, number of tissue samples; HR, hazards ratio; TMZ, temozolomide.

Key message: NRF2 regulates the expression of numerous cytoprotective genes involved in antioxidant stress response and drug detoxification. These genes include enzymes categorized into five groups based on their functions: phase I enzymes, phase II enzymes, phase III enzymes, general antioxidant pathways, and redox cycling enzymes. NRF2's regulation of these enzymes helps cells combat oxidative stress, detoxify harmful compounds, and maintain redox homeostasis, thereby promoting cell survival under normal and stressed conditions.

The NRF2/ARE pathway exhibits dual roles in cancer biology and therapy, acting as a double-edged sword. It orchestrates a complex network of downstream targets involved in antioxidant stress response and drug detoxification, encompassing enzymes vital for cellular rescue processes. While NRF2 exerts tumor-preventive effects by opposing oxidative damage, inflammation, and tumorigenesis, it can also promote cancer progression, metastasis, and chemoresistance. Understanding the intricate mechanisms underlying NRF2 activation and its downstream targets is crucial for exploiting its therapeutic potential effectively.

In GB, NRF2 plays a crucial yet nuanced role, affecting both disease progression and treatment response. While it can confer resistance to chemotherapy, it also enhances vulnerability to cell death mechanisms like ferroptosis. Moreover, NRF2 influences essential cellular processes, including cell proliferation and GB stem cell self-renewal, contributing to the tumor's aggressive nature. Its impact extends to metabolic rewiring and fostering an immunosuppressive microenvironment, further complicating treatment strategies. Understanding NRF2's role offers potential avenues for targeted therapies in combating this challenging disease.

Moreover, NRF2's prognostic significance in GB remains a subject of debate, with conflicting data on its association with patient survival. While some studies suggest a link between elevated NFE2L2 levels and poorer survival rates, others propose contrasting findings, hinting at improved survival with increased NRF2 expression. This complexity is compounded by the interplay between NRF2 expression and GB molecular subtypes. Despite its implication in therapy resistance, including resistance to TMZ, and its potential as a predictive marker for treatment response, NRF2's prognostic value appears to be contingent on various contextual factors. Thus, further investigation is warranted to unravel NRF2's complete role in GB pathogenesis and treatment outcomes.

E. Study Objective and Significance

The investigation of NRF2 in cancer has gained significant attention in the last decade due to its multifaceted role in tumorigenesis and treatment resistance. Numerous studies have explored NRF2's involvement in various cancer types, including GB, highlighting its potential as both a therapeutic target.

While research on NRF2's impact on GB patient survival is extensive, conflicting findings in literature databases make it challenging to draw definitive conclusions. Moreover, the scarcity of clinical trials investigating NRF2 inhibitors complicates understanding its therapeutic potential in GB management. NRF2's role has been extensively studied across different experimental *in vitro* and *in vivo* models, using established GB cell lines. These studies have occasionally shown conflicting results regarding NRF2's role in tumor progression and therapeutic resistance while they offered limited evidence about NRF2's involvement in GB metabolism. Additionally, there's increasing awareness of the limitations of using well-established GB cell lines. Consequently, there's been a shift towards utilizing patient-derived GB cell models to explore NRF2's role, although the utilization of such models remains relatively limited in the literature.

In light of these challenges, our study addresses crucial knowledge gaps regarding the role of NRF2 in a clinically relevant glioblastoma (GB) model. Using a 3D GB model that includes both wild-type and CRISPR-Cas9 NRF2 knockout cells, along with pharmacologically induced NRF2 expression via TBHQ, our study aims to elucidate the role of NRF2 in GB progression, stemness, and metabolic profile alterations. With this approach, this study offers several advantages over traditional models and a more faithful representation of clinical outcomes. Ultimately, our findings may provide a better understanding of NRF2's potential as a prognostic tool and its implications for GB development and progression.

II. Experimental Models and Techniques

II. Experimental Models and Techniques

A. Experimental Model

A.1 *In vitro* Modeling: P3 Patient-derived Cell Line and Clinical Relevance

In this study, we employed the P3 cell line derived from a biopsy of a patient with mesenchymal subtype GB at the University Hospital of Bergen (Norway). These cells exhibit an IDH-wild type, EGFR wild-type, and MGMT non-methylated status (377). This GB cell line is a principal model within our laboratory due to its stem-like phenotype, facilitated access to the biopsy data, and the consistent manifestation of a proliferative/angiogenic and invasive phenotype (225,377).

To maintain its stemness phenotype, the P3 cell line is cultured in serum-free neurobasal medium (NBM, without glutamine Gibco® 12348017) supplemented with B27 supplement (Gibco® 17504001), heparin (100 U/mL, Sigma H3149), basic FGF (20 ng/mL, Fisher Scientific 17883153), and penicillin/streptomycin (1000 U/mL, Dutscher P06-07100). Under these culture conditions, P3 cells spontaneously grow in 3D culture, forming multicellular aggregates/spheroids. This culture environment is tailored to maintain the phenotypic and genetic traits of GB tumors while favoring the enrichment of stem cells and promoting their proliferation in the form of non-adherent spheroids (378,379). These stem cells are predominantly implicated in tumor initiation or relapse processes, rendering them an invaluable model for elucidating the mechanisms underlying GB development and exploring potential therapeutic interventions aimed at curtailing tumor progression (169,225,380). To standardize our culture, standard-sized spheroids, referred to as spheres throughout this manuscript, are formed using a well-established sphere-forming protocol (380) and were used in several *in vitro* assays throughout this manuscript (detailed in section B).

Importantly, the P3 spheres employed in our study enhance the transition of pre-clinical glioblastoma models to clinical settings at different levels. First, in comparison to commercially available GB cell lines, patient-derived GB cell lines like P3 offer greater clinical relevance (381,382). Purchased GB cell lines often represent a more limited genetic diversity and may have undergone extensive passaging, which can lead to genetic drift and the loss of original tumor characteristics. In contrast, patient-derived cell lines maintain closer fidelity to the tumor's

heterogeneity and the patient's specific genetic and molecular profile (383,384). This makes them more representative of the actual clinical scenario, allowing for more accurate studies on GB tumor behavior and treatment responses.

Moreover, in comparison to the traditional 2D culture that lacks the spatial organization, genetic modifications, metabolic alterations, and microenvironmental cues present in tumors, the 3D modeling of tumors presents a more clinically relevant tool to evaluate the disease progression and prognosis (385). Mainly, the 3D spheres model serves as an accurate therapeutic predictor where disparities between *in vitro* and *in vivo* results are often less observed (8,10). This is mediated by the ability of the 3D sphere to replicate GB tumor heterogeneity, gene expression, and signaling pathways activation involved in GB tumorigenicity, which is mostly upregulated in 3D cultures compared to 2D (386). Second, 3D culture replicates the intricate three-dimensional structure of tumors, allowing for a more effective evaluation of drugs. This is due to the formation of 3D matrices that influence solute diffusion, creating tissue-scale solute concentration gradients as well as local pericellular gradients (386). Additionally, P3 spheres replicate the oxygen and metabolite gradients (380) (**Figure 27**), closely mimicking the *in vivo* conditions of tumors. Importantly, the retention of the hypoxic core in P3 3D spheres (**Figure 27**) replicates the hypoxic stress and its association with stemness, therapeutic resistance, and GB tumor progression (386).

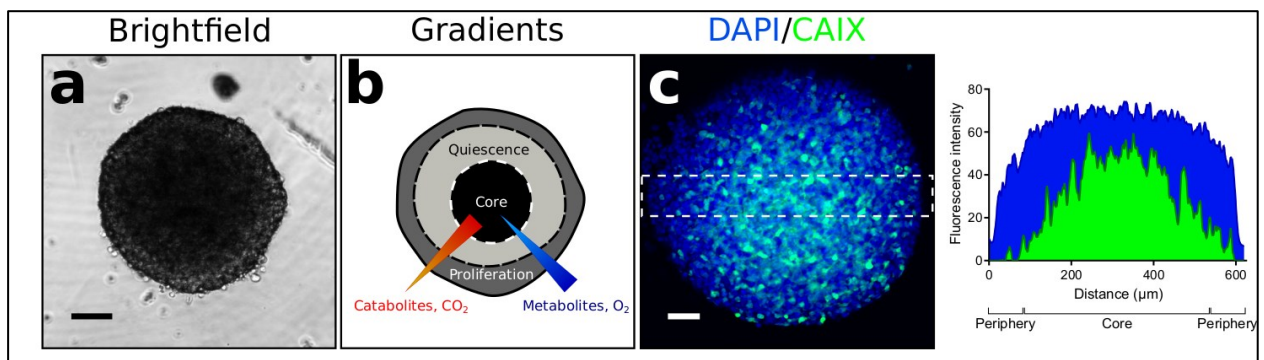


Figure 27. Representation of the P3 Sphere as a Relevant Model to Mimic GB Tumors (380).

A brightfield picture of a P3 sphere shows a round appearance with a dense central area. Scale = 100 μm. (b) Schematic representation showing the O₂, CO₂, metabolite, and catabolite gradients in the sphere. (c) Confocal picture of a sphere stained with DAPI (blue) and with antibodies against carbonic anhydrase IX (green) with the quantification of the fluorescence from the dashed area. Scale = 100 μm.

A.2 Modulation of NRF2 Expression in P3 Cells

In our study, CRISPR/Cas9-mediated gene editing (387) was employed to generate P3-NRF2 knockout cells (KO) thanks to the CRISP'EDIT platform at the TBM core facility, University of Bordeaux. Briefly, cells were transfected with CRISPR/Cas9 plasmids targeting NRF2, followed by genomic DNA extraction after two to three days post-transfection. The efficiency of CRISPR/Cas9-mediated editing was assessed through Sanger sequencing analysis of the targeted locus. The clonal expansion was performed by seeding at a density of 0.5 cells per well in a 96-well plate in complete NBM for three to four weeks to allow for the generation of clones originating from a single edited cell. Genomic DNA was extracted from each clone, and genotyping by sequencing identified NRF2 KO clones (C7 and C16). Control experiments involving cells transfected with non-targeting control guide RNA (gRNA-ctrl-) were maintained in bulk culture without clonal selection to serve as a reference. For further validation, we employed western blotting to confirm the absence of NRF2 protein expression in the KO clones compared to the control P3 cells (**Figure 28**).

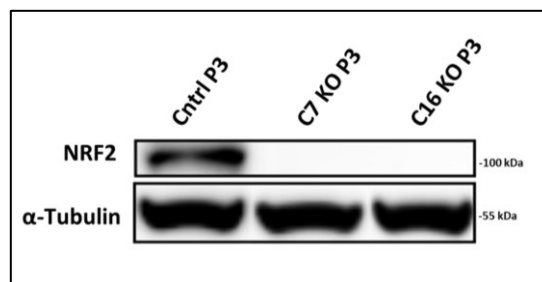


Figure 28. Western Blot Analysis of NRF2 Protein Expression.

Western blot showing the absence of NRF2 protein in C7 KO P3 and C16 KO P3 clones confirming successful CRISPR-Cas9 knockout of the NRF2 gene. α -Tubulin was used as a loading control.

Moreover, to induce NRF2 overexpression, cells were treated with Tertiary butylhydroquinone (TBHQ), a synthetic antioxidant commonly found in processed foods (388), at a relatively non-toxic dose of 25 μ M for 24 hours (**Figure 29A**). Mechanistically, TBHQ modifies critical cysteine residues on KEAP1, disrupting its interaction with NRF2 and preventing NRF2 degradation (389). Consequently, accumulating NRF2 is translocated into the nucleus, where it activates the transcription of genes involved in antioxidant and detoxification pathways, enhancing cellular defense mechanisms against oxidative stress (**Figure 29B**).

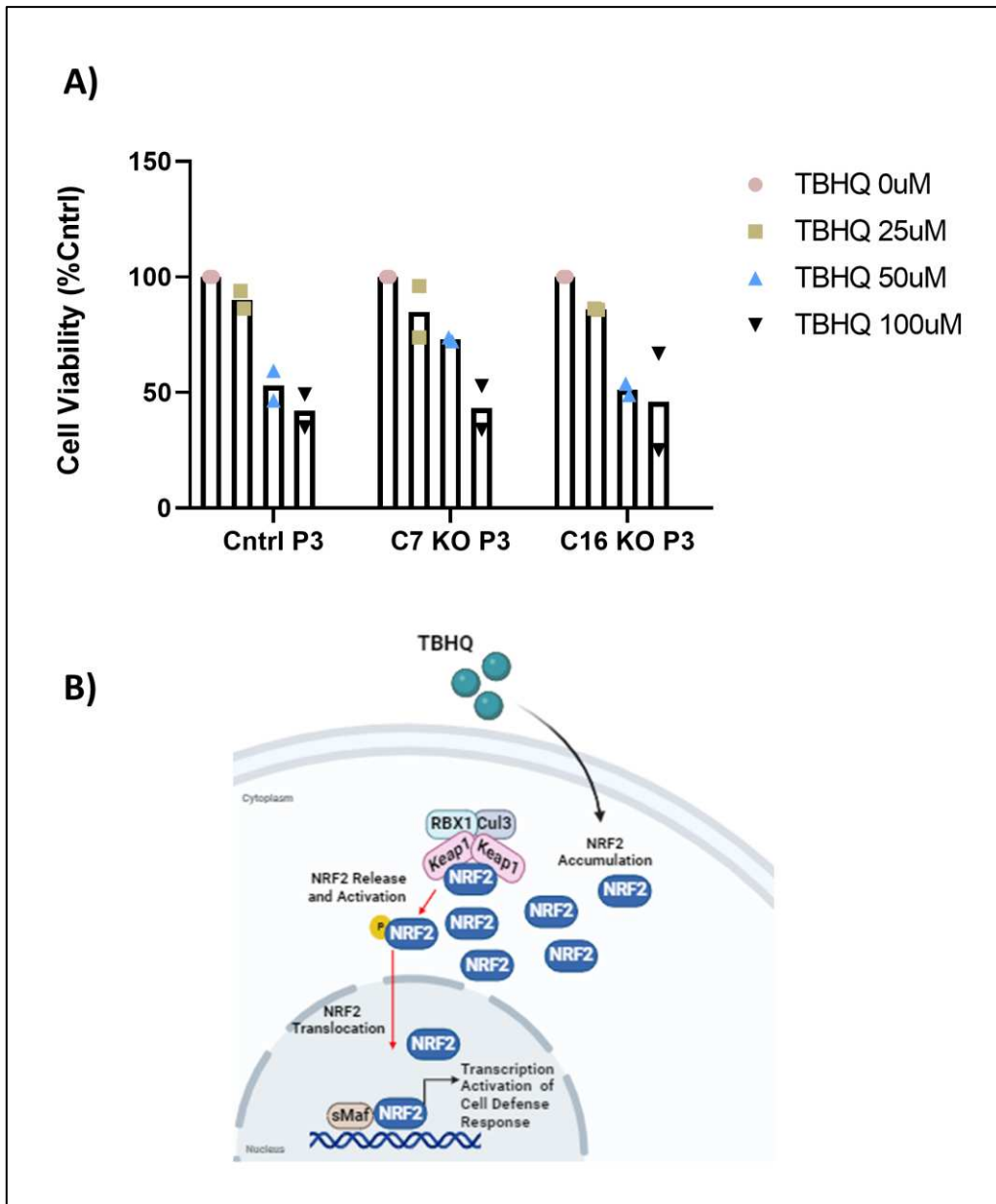


Figure 29. Effect of TBHQ on Cell Viability in P3 Control and NRF2 KO Cells.

Cells were treated with increasing doses of TBHQ (0, 25, 50, and 100 μ M) for 24 hours. Cell viability was assessed using the trypan blue exclusion assay. Data represent the mean \pm SEM per condition across two independent experiments per TBHQ dose used, (3 technical replicates each). (B) Schematic representation of the NRF2 activation pathway under TBHQ treatment, created using BioRender. TBHQ induces the release and activation of NRF2 by disrupting its interaction with the Keap1-Cul3-Rbx1 complex, leading to NRF2 accumulation in the cytoplasm. NRF2 then translocates to the nucleus, where it binds with sMaf proteins to promote the transcriptional activation of cell defense response genes. TBHQ - tert-Butylhydroquinone; NRF2 - Nuclear factor erythroid 2-related factor 2; Keap1 - Kelch-like ECH-associated protein 1; Cul3 - Cullin 3; Rbx1 - RING-box protein 1; sMaf - Small musculoaponeurotic fibrosarcoma oncogene homolog.

A.3 *In vivo* Modeling

To further elucidate the role of NRF2 in GB tumorigenesis and progression *in vivo*, experimentation and mice monitoring were conducted thanks to the Shared Animal Facility of Pessac, University of Bordeaux. All animal experiments were performed according to ethical guidelines and regulations. Male immunodeficient RAG mice, aged 13 weeks, were prepared for intracranial implantation. Each mouse received pre-anesthesia and analgesic administration to ensure comfort and minimize distress. The skulls of the mice were shaved, and disinfected, while their eyes were protected with ocry-gel. Following these preparatory steps, each mouse was carefully positioned in a stereotaxic apparatus to allow precise surgical intervention. A small hole was made in the skull at a specific intracranial coordinate (2.2 mm lateral to the bregma on the left side and 3.5 mm deep) to insert a Hamilton syringe. The syringe was then retracted by 0.1 mm to create a micro-pocket, allowing the spheroids to settle securely within the brain and reducing the risk of cell leakage at the implantation site. Five mice per condition received striatal injections of five luciferase-positive spheres (10,000 cells per sphere) for each group, including P3 NRF2 control, C7, and C16 NRF2 knockout (KO) spheres, establishing a consistent *in vivo* model.

Intracranial implantation was preferred over other implantation methods due to its ability to restore the cerebral microenvironment surrounding the tumor, ensure a precise injection, and accurate tumor volume measurements (421). Following orthotopic injection, mice were regularly monitored for luciferase-positive tumor growth using bioluminescence imaging, daily observations of behavior and appearance, and weekly weight monitoring three times a week. Bioluminescence imaging sessions were conducted once per week to track tumor growth dynamics. All mice were sacrificed via cervical dislocation. Brains from each condition were then removed, frozen in liquid nitrogen, and stored at -80°C. Subsequently, the frozen brains were sectioned using a Cryostat to perform immunohistochemistry. Measures were taken to minimize animal suffering, and protocols were approved by the institutional animal care and use committee.

B. *In vitro* Assays and Techniques

B.1 Assessment of GB Progression

B.1.a Sphere Forming Assay

In a 96-well plate with a curved bottom, 10,000 cells/well in 100µl of complete NBM are seeded from a prepared cell suspension supplemented with 0.4% methylcellulose(380). Methylcellulose, a synthetic derivative of cellulose, promotes cellular aggregation (380,390). The cells are then incubated at 37°C, 5% CO₂, and 95% humidity for 3 days, allowing sphere formation.

B.1.b Sphere Proliferation Assay

In the sphere proliferation assay, we aim to evaluate the proliferation capacity of P3 NRF2 control spheres compared to both TBHQ-induced NRF2 expression and NRF2 KO conditions. Following the 3-day incubation period required for sphere formation, 100µL of complete NBM, pre-warmed to 37°C, is added to each well containing a sphere (**Figure 30**). For specific experimental conditions, treatment such as TBHQ at a concentration of 25µM is added to the medium (applied at 2x concentration to ensure the correct final concentration per well).

Images of each sphere are captured with the Olympus inverted microscope (Olympus IX81 - SS12) at an initial time point (Day 0) and every 2 days for a total duration of 6 days to monitor sphere growth. The areas of the spheres are measured at each time point using semi-automated image analysis with Fiji software using a macro specifically set up in our lab (Fiji macro) (380). The percentage growth of each sphere is determined by comparing its area at the desired time point to its initial area at Day 0.

To investigate the effects of hypoxic stress combined with NRF2 modulation on sphere proliferation, the spheres were incubated at oxygen concentrations of 21%, 1%, and 0.1%. The 0.1% and 1% oxygen conditions were incubated at the Celloxia platform at the TBM core facility, University of Bordeaux. Each experiment was conducted in a minimum of three independent replicates, with eight spheres per experimental condition.

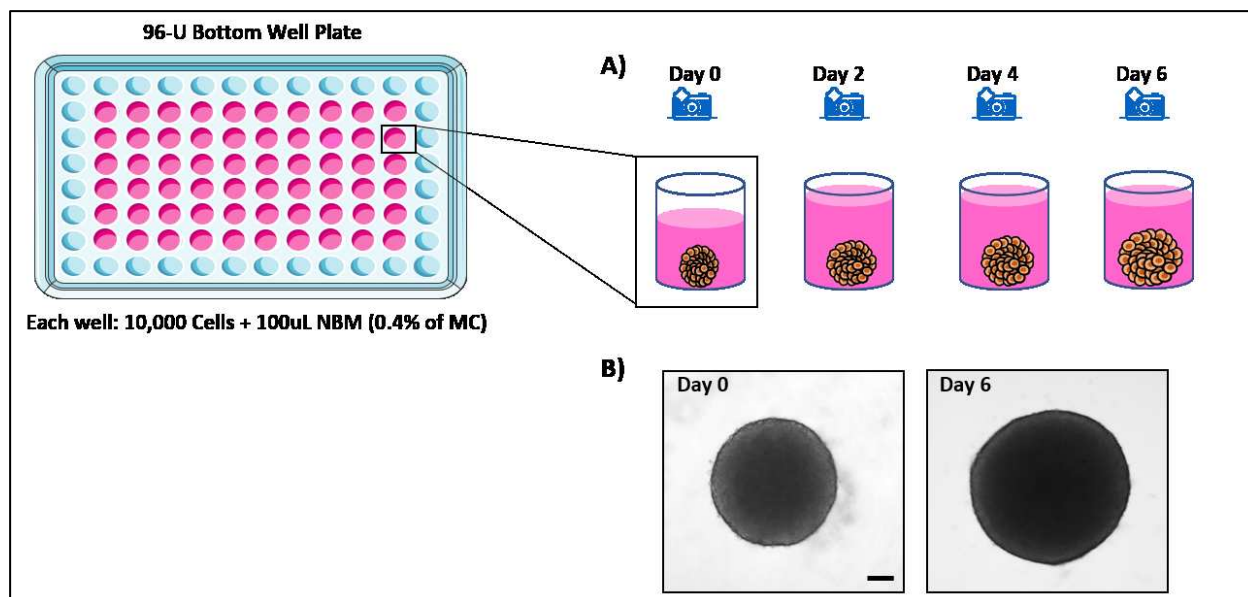


Figure 30. Representation of P3 Sphere Proliferation Assay.

After sphere formation, P3 spheres are kept to proliferate for 6 days in a complete NBM. Images are taken with an inverted microscope to record sphere growth every 2 days. (B) Bright-field images showing the P3 sphere growth across 6 days starting at the initial time point Day 0 until Day 6. Scale bar: 100 μ m.

B.1.c Sphere Invasion Assay

To evaluate the invasive characteristics of P3 cells under conditions of either NRF2 KO or overexpression, pre-prepared spheres were incorporated into an artificial extracellular matrix (ECM). The CNS ECM comprises a complex network of fibrillar proteins, including collagen, laminin, fibronectin, and proteoglycan (386). To replicate the brain ECM environment and facilitate the detachment and invasion of spheroid cells, they were embedded in a 3D gel composed of type I collagen, the primary fibrillar collagen (380).

The collagen matrix (Corning[®] 354236) was prepared on ice using pure collagen I (final concentration 1mg/mL), 1x PBS, NaOH, and sterile water. Spheres were harvested using cut-tip pipette tips, washed with PBS, and then embedded in 100 μ L of the collagen matrix in a flat-bottomed well of a 96-well plate. The plate was incubated at 37 $^{\circ}$ C for 30 minutes to allow the collagen gel to solidify, followed by the addition of 100 μ L of complete NBM on top of the gel. Depending on the experimental requirements, TBHQ treatment at 25 μ M was introduced into either this medium or included in the collagen I mixture (applied at twice the concentration to achieve the desired final concentration per well) or in both ensuring a final concentration of 25 μ M in each condition.

Images of each sphere were captured 24 hours after embedding. The extent of invasion of each sphere was determined using Fiji software using a macro specifically set up in our lab (Fiji macro)

(380) as presented in **Figure 31**. The invasive area was calculated by subtracting the core area from the total area measurement. The invasive area/sphere was then normalized relative to its corresponding sphere core area (Relative Invasive Area = (Total area - Core area) / Core area). A minimum of 8 spheroids were assessed for each experimental condition. The experiment was conducted in a minimum of three independent triplicates, with 8 spheres per condition at a standard oxygen concentration of 21%. Additional conditions were tested at 1% and 0.1% oxygen concentrations to examine the effect of hypoxic stress combined with NRF2 KO on invasion.

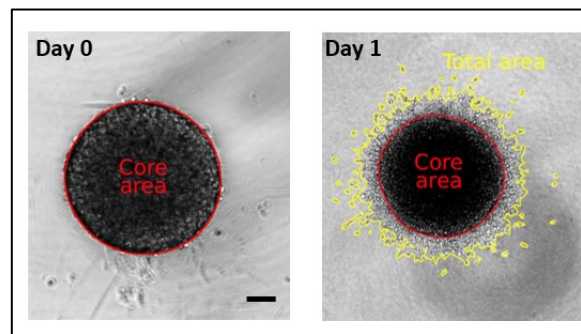


Figure 31. Quantification of P3 Sphere Invasion Using Fiji Software (380).

(A) Brightfield images of the P3 spheres showing the core area in red at the initial time point (T0) while (B) the total area, which contains the core area, is in yellow after 24 hrs. The invasive area corresponds to the subtraction of the total area from the core area after 24 hrs of invasion in the collagen I matrix.

B.2 Functional Analysis of Stemness

To assess P3 stemness in response to NRF2 KO, we performed the extreme limiting dilution assay (ELDA) using P3 control and NRF2 KO clones, following the protocol described by Nguyen and colleagues (391). To ensure a low-detachment surface, we used a 96-well plate precoated with Poly(2-hydroxyethyl methacrylate) (Sigma P3932) at a concentration of 10mg/mL and washed twice with PBS. Decreasing numbers of P3 control and NRF2 KO cells were seeded per well, with doses ranging from 20, 10, 5, to 1 cell(s). The plates were incubated at 37°C, 5% CO₂, and 95% humidity for 7 days. After incubation, wells were examined microscopically for cell growth. The number of positive and negative wells was recorded for each dilution on day 7. Additionally, sphere formation was counted on day 7 for each cell density, with each well-being individually assessed. The experiment was conducted in a minimum of three independent replicates, with fourteen replicates per cell dose.

B.3 Functional Analysis of Mitochondrial Respiration and Activity

B.3.a Resipher Real-Time Bioenergetic Assay

To assess mitochondrial metabolic activity through oxygen consumption rate (OCR), a resipher real-time cell analyzer (Lucid Scientific) was employed. P3 control and NRF2 KO cells were first dissociated using accutase and then plated at a concentration of 1×10^5 cells/well in Nunc 96-well plates (Thermo Fisher Scientific, 10212811) containing complete NBM. The plated cells were subsequently incubated at 37°C in a humidified atmosphere within a 5% CO₂ incubator for 1 hour to allow for proper calibration. Following this incubation period, seeded plates were transferred to the resipher system for real-time OCR monitoring to measure basal respiration. For conditions involving TBHQ treatment, after plating and the initial 1-hour incubation, TBHQ treatment was added at a concentration of 25µM (applied at 2x concentration to ensure the proper final concentration per well), and OCR monitoring was initiated. The experiment was conducted in a minimum of three independent replicates, with a minimum of four replicates per condition.

B.3.b Mitochondrial Membrane Potential Assay

To evaluate the mitochondrial membrane potential ($\Delta\Psi_m$) of cells, we used tetramethylrhodamine (TMRM, Invitrogen), a cell-permeable fluorescent dye, which binds to the mitochondrial membrane. TMRM fluorescence signal is directly proportional to the mitochondrial ETC activity and ATP production (392).

P3 control and NRF2 KO cells were seeded in 6-well plates at a density of 5×10^5 cells, with and without TBHQ treatments at 25µM for 24 hours before the experiment. Following incubation, cells were dissociated using accutase and resuspended in Hanks' Balanced Salt Solution (HBSS) medium (Gibco™, Fisher Scientific 15266355) supplemented with TMRM dye at a final concentration of 20nM. HBSS medium was used due to its balanced electrolyte composition and compatibility with fluorescence assays. A total of 30,000 cells per well were then seeded in a 96-well black/clear bottom plate. After 25 minutes of incubation at 37°C, 5% CO₂, and 95% humidity, Hoechst (0.002 mM) was added to the wells and incubated for an additional 5 minutes to stain the nuclei. Following this, TMRM fluorescence and Hoechst staining were recorded using a Clariostar reader (excitation: 488 nm, emission: 570 nm). Subsequently, oligomycin, an inhibitor of ATP synthase diluted in absolute ethanol, was added to induce potential hyperpolarization at a dose of 2.5mg/mL. TMRM fluorescence intensity was then recorded up to 10 minutes of

incubation at room temperature in the dark. Following this, 50 μ M carbonyl cyanide m-chlorophenyl hydrazine (CCCP), a mitochondrial decoupling agent dissolved in dimethyl sulfoxide (DMSO), was added to wells with TMRM alone or those treated with oligomycin to induce maximal depolarization. TMRM fluorescence was recorded every 10 minutes up to 40 minutes of incubation at room temperature.

Negative controls included untreated cells without TMRM dye and cells treated with dye with the vehicles (ethanol and DMSO) with and without TBHQ treatment (25 μ M). These controls ensured assay specificity. TMRM fluorescence was normalized to the Hoechst signal to ensure consistent cell density across the wells. The experiment was conducted in a minimum of three independent replicates, with at least eight technical replicates per condition.

B.3.c Mitotracker Assay for Mitochondrial Mass

To assess the mitochondrial mass in P3 control and NRF2 KO cells, we used MitoTracker™ Green FM solution (Thermo Fisher Scientific, M7514), a cell-permeable fluorescent dye independent of mitochondrial membrane potential, which accumulates selectively in active mitochondria and interacts with mitochondrial membranes, where it becomes fluorescent. Consequently, the fluorescence intensity of MitoTracker™ is directly proportional to the mitochondrial mass within the cell population (393).

Cells were seeded in 6-well plates for 24 hours at a density of 3×10^5 cells, with and without TBHQ treatment at a dose of 25 μ M. Then cells were dissociated with accutase and diluted in PBS. MitoTracker™ solution was added to the cell suspension at a concentration of 0.1mM, followed by 30 minutes of incubation at 37°C, 5% CO₂, and 95% humidity. Hoechst dye (0.002mM) was introduced to each cell suspension for 5 minutes at 37°C, 5% CO₂, and 95% humidity. Following incubation, cells were washed with PBS and centrifuged. The resulting pellet was resuspended in 800 μ L PBS and transferred to a 96-well black/clear bottom plate, with 100 μ L per well. Fluorescence of Hoechst (excitation: 350nm, emission: 461nm) and MitoTracker (excitation: 490 nm, emission: 516 nm) was measured using the Clariostar reader. The Mitotracker signal was normalized to the Hoechst signal for each condition.

Negative controls, comprising TBHQ 25 μ M treated and untreated cells without MitoTracker, were included to ensure assay specificity. The experiment was conducted in a minimum of three independent replicates, with at least eight technical replicates per condition.

B.4 Assessment of Intra- and Extracellular Lactate Concentrations by Ultra-High-Performance Ionic Chromatography (UHPIC)

To examine the metabolic shifts in the P3 GB model, including extracellular and intracellular lactate levels along with ATP concentrations, we collaborated with the Metabolic Analysis Service of Bordeaux (TBMCore platform), led by Dr. Benoit Pinson.

In brief, P3 control and NRF2 KO spheres were formed in 96 U-bottom well plates. Extracellular metabolites were extracted from the culture media where the spheres were formed using the, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES 20mM pH 7/absolute ethanol (EtOH 2/8) extraction buffer. Following this, spheres were washed twice with ice-cold PBS, and extractions were performed by adding 100 μ L of extraction buffer to fix the spheres, followed by an additional 100 μ L to rinse the well and include any remnants. The collected media and sphere suspension were then transferred to a glass tube and incubated for 3 minutes at 80°C, followed by cooling on ice and evaporation using a rotavapor device for 3 minutes at 65°C. Metabolite separation was conducted by ion chromatography utilizing an AS11-HC-4 μ m column (250 2mm, Thermo Electron) with the Integrion chromatography system (Thermo Electron). This system, equipped with an RFIC eluent generator and suppressor, coupled with a Vanquish UV-Vis diode array detector and conductivity detector, facilitated the separation at a flow rate of 0.38 mL/min and a temperature of 30°C, employing a discontinuous gradient of potassium hydroxide (394). Hydrosoluble metabolites including lactate, AMP (adenosine monophosphate), ADP (adenosine diphosphate), and ATP (adenosine triphosphate), were detected via conductivity and/or absorbance and identified by co-injection with standards and/or their UV spectral signature. For cellular samples, normalization was carried out based on cell number and median volume determined using a Multisizer 4 (Beckman Coulter). Intracellular and extracellular concentrations of lactate were determined using standard curves obtained with pure compounds. Additionally, the sum of adenylate nucleotides ($[AXP] = [AMP] + [ADP] + [ATP]$) and the adenylate energy charge ($AEC = ([ATP] + 1/2 [ADP])/[AXP]$) were calculated. The experiment was conducted in a minimum of three independent replicates, with two technical replicates per condition.

B.5 Assessment Lipid Droplet Formation

Assessment of to investigate the impact of NRF2 on P3 cellular lipid metabolism, we examined lipid droplet formation. P3 control and NRF2 KO cells with and without TBHQ treatment were

seeded in 6-well plates at a density of 3×10^5 cells for 24 hrs (TBHQ was applied at 2x concentration to ensure the proper final concentration per well). Afterward, cells were washed with PBS and resuspended in complete NBM. Subsequently, they were dissociated with accutase and stained with BODIPY 493/503 (Thermo Fisher Scientific), a fluorescent dye known for its ability to stain lipid droplets, at a final concentration of 20 μ l/mL for 30 minutes at 37°C, 5% CO₂, and 95% humidity, protected from light. After staining, cells were carefully washed with PBS to remove excess dye. After centrifugation, the cell pellet was then resuspended in PBS and analyzed using flow cytometry with a BD Accuri™ C6 flow cytometer (BD Biosciences), equipped with an appropriate filter for green fluorescence detection (excitation: 488nm, emission: 530nm). A minimum of 10,000 events were acquired for each sample. A gating strategy was applied based on the negative control (P3 control cells without BODIPY) to select only live cells and define the basal auto-fluorescence value. Data were analyzed using FlowJo software (FlowJo, LLC). The mean fluorescence intensity (MFI) of BODIPY-positive cells was calculated for each experimental condition and compared to the control group. The experiment was conducted in a minimum of three independent replicates, with four technical replicates per condition.

B.6 Western Blot Analysis

Briefly, P3 control and NRF2 KO cells were seeded at a density of 10^6 cells in 6-well plates with and without TBHQ treatment at 25 μ M for 24 hrs in a complete NBM. Then, cells were harvested and lysed using RIPA buffer supplemented with protease and phosphatase inhibitors. Protein concentration in the lysates was determined using a BCA protein assay kit (Thermo Fisher Scientific, 23225), and equal amounts of protein (typically 30 μ g) were mixed with Laemmli-based loading buffer and denatured by heating at 95°C for 5 minutes except for the samples used for MCT1 detection. Samples were loaded onto 12% Bis-Tris-acrylamide gels. Proteins were separated by electrophoresis for 40 minutes at 100V in TG-SDS running buffer (UGAP 3617718) using the Invitrogen™ Mini BlotGreen module and transferred onto 0.2% nitrocellulose membranes for 90 minutes at 20V in NuPAGE transfer buffer (Invitrogen, NP00061) using the Invitrogen™ XCell II™ Blot Module. Membrane blocking is performed with a 5% Bovine Serum Albumin (BSA) solution in 0.01% TBS-T (Tris Buffer Saline, 0.01% Tween, pH 8) for 90min. Membranes were then incubated with primary antibodies against the target protein either for 2 hours at room temperature for α -Tubulin (Abcam 4074, dilution 1:10000) or overnight at 4°C with gentle agitation for MCT1 (Santacruz-365501, dilution 1:1000), LDHA (Santacruz-137243,

dilution 1:1000), LDHB (Santacruz-100775, dilution 1:1000), Tom20 (Santacruz-17764, dilution 1:1000), NRF2 (Abcam 62352, dilution 1:1000), Sox2 (Santacruz-365823, dilution 1:1000) and Olig2 (Abcam 109189, dilution 1:2000). Following primary antibody incubation, membranes were washed three times with TBST, each wash lasting 10 minutes and incubated with appropriate HRP-conjugated secondary antibodies (Anti-mouse, Cell Signaling 7076S, and Anti-rabbit, Cell Signaling 7074S, both at a dilution of 1:2000) for 1 hour. Membranes were then washed three times with 0.01% TBS-T and protein bands were visualized using enhanced chemiluminescence (ECL substrate, Bio-Rad Clarity Western ECL substrate 170-5060). The visualization was performed using the Amersham ImageQuant™ 800 Western blot imaging system. Quantitative analysis of protein bands was performed using Fiji software. The expression of each protein of interest is normalized to control proteins, such as tubulin. The experiments were independently repeated with a minimum of three independent replicates for each target protein.

B.7 Trypan Blue Exclusion Assay

The trypan blue exclusion assay was employed to assess cell viability and determine the percentage of viable cells in response to treatment conditions including TBHQ at 25 μ M dose and TMZ treatment ranging from 0 to 1600 μ M. Initially, P3 control and NRF2 KO cells were seeded into 24-well plates at a density of 10⁵ cells per well and treated for 24 hrs with TBHQ treatment at 25 μ M and 72hrs for TMZ. Following the incubation period, cells were harvested and washed along with the wells with PBS to collect any remaining cells. The collected cells were then centrifuged, and the supernatant was removed. Subsequently, the cells were dissociated using accutase and resuspended in complete NBM. A portion of the cell suspension was mixed with an equal volume of 0.4% Trypan Blue solution (Sigma-Aldrich, T8154). Cell viability, indicated by the percentage of viable and dead cells, was assessed using the Countess 3 Automated Cell Counter (Thermo Scientific Invitrogen). Each experiment was independently replicated two to three times, with at least two technical replicates per condition.

B.8 In vivo Experimentation

B.8.a In vivo Bioluminescence

For *in vivo* bioluminescence analysis, once a week, to assess tumor size, animals received an intraperitoneal injection of 200 μ L of D-luciferin (15 mg/mL; UGAP 3689832). They were then

anesthetized by inhalation of isoflurane (3.5% isoflurane and 2% air/oxygen for induction) and underwent bioluminescence imaging using the PhotonIMAGER *in vivo* imaging system (Biospace Lab). The animals were under anesthesia with 1.5-2.5% isoflurane in a 2% air/oxygen mixture. During the imaging procedure, which lasted approximately 5 to 10 minutes, the animals were placed on a 37°C hot pad in the imaging box. After the imaging session, using M3Vision software, Regions of Interest (ROIs) with the head of the mice are detected. Then a signal intensity curve, in photons per second per steradian (ph/s/sr), providing a quantitative measure of the emitted signal is obtained for each ROI using M3Vision software.

B.8.b In vivo Stainings and Analysis

For immunohistochemistry (IHC), histological sections of 10 µm thickness were prepared using a cryostat from frozen brains embedded in an Optimal Cutting Temperature (OCT) compound. When needed, selected sections were thawed at room temperature for approximately 5 minutes and fixed with 4% paraformaldehyde (PFA) for 15 minutes. Subsequently, they were washed three times in PBS and then incubated in 0.1% PBS-Triton for 15 minutes. After another three washes with PBS, the sections were incubated in a blocking solution (2% SVF, 1% BSA) for 30 minutes at room temperature. Following blocking, sections were incubated with the primary antibodies for Nestin (Invitrogen PA5-11887, dilution 1:250) overnight at 4°C in dark and humid conditions. The next day, the sections were washed three times in PBS before being incubated with the corresponding secondary antibody, Thermo Fisher Scientific Alexa Fluor Green 488nm (A32731) or Far-Red 647nm (A55060) fluorophores, at a concentration of 10µg/ml for 2 hours at room temperature in complete darkness and humidity. Sections were then washed three times with PBS. Subsequently, the sections were mounted between slides and coverslips in a mounting medium to preserve fluorescence while reducing photo-bleaching using ProLong™ Gold Antifade Mountant (Invitrogen P36930). Finally, the slides were stored at 4°C until imaging was conducted using a Hamamatsu NANOZOOMER 2.0HT at the Bordeaux Imaging Center (BIC, TBMcore) platform. Stained brain sections (6 sections/ brain) covering 600µm of tumor/brain were viewed using the NDP.view2 Viewing Software (Hamamatsu Photonics, Massy, France) (395). Tumor and contralateral invasion areas were outlined, and measured across the different brain sections using the software.

Hematoxylin and Eosin (H&E) staining was performed by the Histopathology platform at the TBMCore facility of the University of Bordeaux. Hematoxylin solution was used to stain the nuclei

and Eosin to highlight the cytoplasm. Images of the histological sections were captured by Hamamatsu NANOZOOMER 2.0HT at the Bordeaux Imaging Center platform. The acquired images were then analyzed using NDP.view2 Viewing Software to assess the staining patterns and quantify tumor area.

B.9 Statistical Analysis

GraphPad Prism (version 6.04) was used for analyses. Before conducting these analyses, the normality of the data was assessed. Before performing any comparisons, the normality of the data distribution was tested using the Shapiro-Wilk test. This test determined whether the data were normally distributed, which guided the choice between parametric and non-parametric statistical tests. Outliers were identified and removed if necessary based on the normality test results. An unpaired two-tailed t-test or a Mann-Whitney test made comparisons of two groups. Comparisons of at least three groups were performed using a one-way ANOVA followed by a Dunnett's or Tukey's multiple comparisons test or Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A two-way ANOVA test was used to compare two or more groups across two variables or repeated measures at different times followed by a Tukey's multiple comparisons test. p-values < 0.05 were considered statistically significant.

III. Results

III. Results

A. Exploring the role of NRF2 in GB Progression

A.1 Impact of NRF2 Deletion on P3 Sphere Growth Under Different Oxygen Conditions

A.1.a Effect of NRF2 Knockout on P3 Sphere Growth

We investigated NRF2's role in sphere growth under different oxygen conditions using CRISPR-Cas9 NRF2 knockout cells (C7 and C16 clones). Western blot analysis confirmed the successful knockout of NRF2 in both C7 and C16 cells, as evidenced by the absence of NRF2 protein expression compared to control P3 cells (**Figure 32A**).

We examined P3 sphere growth under normoxic (21% O₂), moderate hypoxic (1% O₂), and severe hypoxic (0.1% O₂) conditions. Brightfield images on day 6 (**Figure 32B**) and quantitative analysis (**Figure 32C**) showed minimal differences in sphere size. Under 21% O₂, quantitative analysis shows that all spheres (control P3, C7, and C16) exhibited steady diameter increases over time. By Day 6, the growth rate of control P3 spheres increased by 115% relative to Day 0. Similarly, C7 KO and C16 KO spheres exhibited 102% and 123% increases in growth, respectively. These findings indicate that NRF2 may not be essential for P3 sphere growth under normoxic conditions.

Under 1% and 0.1% O₂ conditions, we observed a 43% reduction in control P3 sphere growth and a 39-40% reduction in NRF2 KO spheres (C7 and C16) compared to 21% O₂. This indicates that oxygen availability is crucial for proliferation and suggests that NRF2 knockout does not significantly alter hypoxia-induced growth inhibition.

Therefore, these results suggest that while NRF2 knockout does not significantly impact sphere growth under normoxic conditions, it also does not alter the proliferation of these cells under hypoxic stress.

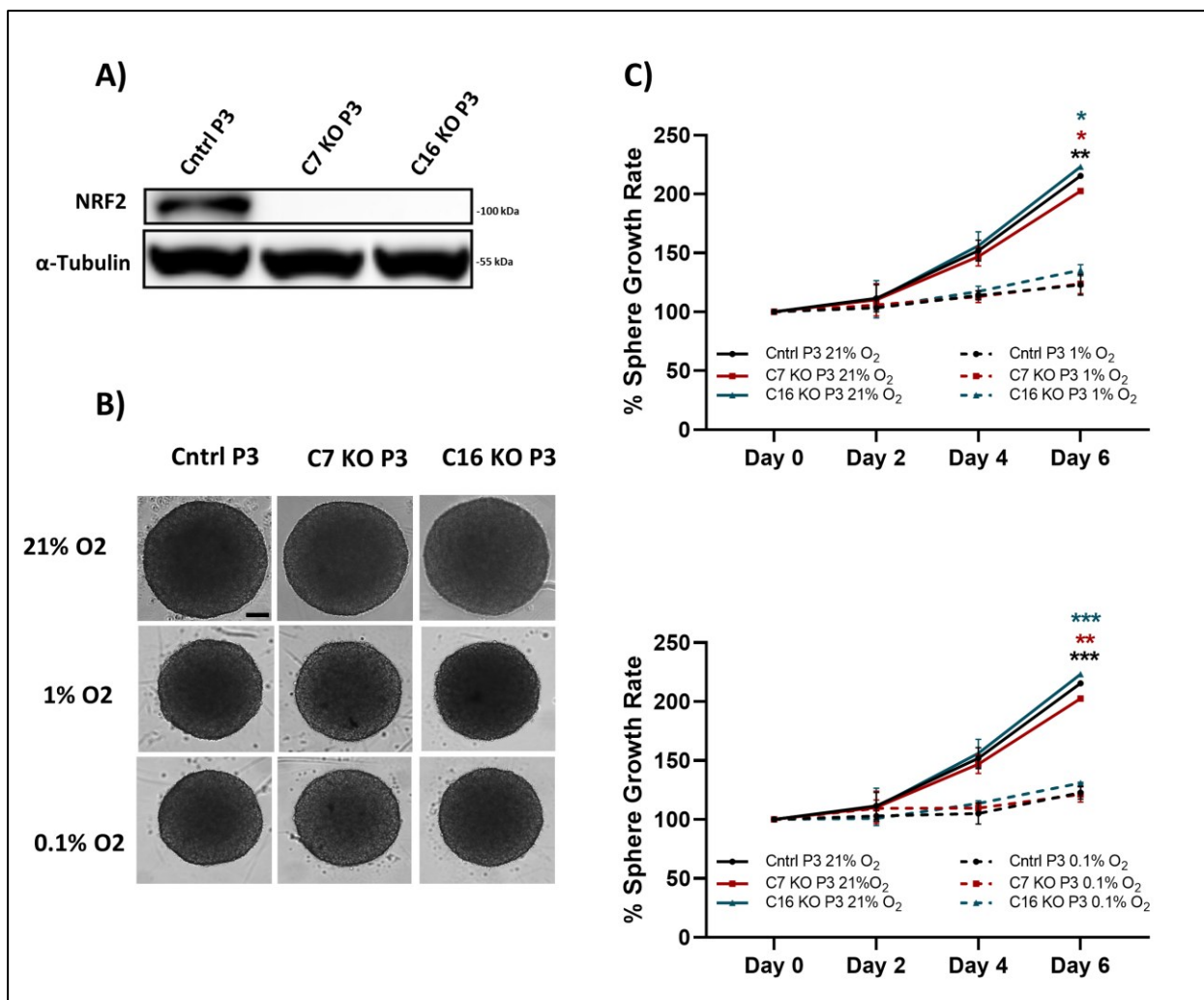


Figure 32. NRF2 Knockout on P3 Sphere Growth.

(A) Western blot analysis of NRF2 protein levels in P3 control (Cntrl), C7 NRF2 KO, and C16 NRF2 KO spheres. α -Tubulin is used as a loading control. (B) Representative brightfield images of P3 control, C7 NRF2 KO, and C16 NRF2 KO spheres cultured under different oxygen conditions (21%, 1%, and 0.1% O_2). Scale bar: 100 μ m. (C) Quantification of sphere growth over time (from day 0 to day 6) under 21%, 1%, and 0.1% O_2 conditions. Data are expressed as the mean \pm SEM from 8-10 spheres per condition across three independent experiments. Statistical analysis was performed using ordinary one-way ANOVA followed by Tukey's multiple comparisons test on Day 6, comparing hypoxic conditions (1% and 0.1% O_2) to the corresponding sphere size under normoxic conditions (21% O_2). Significant differences are indicated as follows: * p <0.05; ** p <0.01; *** p <0.001.

A.1.b Influence of TBHQ-Induced NRF2 Overexpression on P3 Sphere Growth

To examine NRF2 overexpression effects, control P3 spheres were treated with 25 μ M TBHQ, NRF2 inducer, daily for 6 days. NRF2 expression and sphere proliferation were assessed over this period.

Without TBHQ, NRF2 expression in control P3 spheres gradually increased, peaking at days 4 and 6 (**Figure 33A**), suggesting a cumulative accumulation of NRF2 in control P3 spheres over time as they proliferate. TBHQ treatment amplified NRF2 expression by 62-fold after 24 hrs compared to untreated condition, indicating its potency as an inducer upon acute treatment at 25 μ M dose.

Although daily TBHQ treatment sustains high NRF2 expression up till day 4 of incubation, a decrease is observed over day 6, indicating a progressively reduced, induction effect over time (**Figure 33B**). This decline could be attributed to the adaptation of GB cells to the continuous presence of TBHQ, resulting in a reduction in NRF2 levels as part of a cellular adaptation mechanism aimed at maintaining cellular homeostasis. This hypothesis, however, requires further investigation to confirm and elucidate the underlying mechanisms. Importantly, the decline in NRF2 expression is not mediated by P3 cell death, as demonstrated by the trypan blue exclusion assay performed on spheres collected on day 6 (**Figure 33C**). Therefore, TBHQ was deemed safe to use at a 25 μ M dose for acute treatment to induce NRF2 expression. Throughout our subsequent experiments, we consistently used TBHQ at 25 μ M to ensure proper NRF2 induction at 24hrs of treatment.

On the other hand, sphere growth at 21% O₂ (**Figure 33D**) showed a consistent increase from 100% on Day 0 to approximately 250% by Day 6, regardless of TBHQ treatment, suggesting that NRF2 overexpression might not be a key regulator of P3 proliferation. Altogether, these results suggest that while TBHQ successfully enhances NRF2 levels, NRF2's role might not be a key in regulating P3 proliferation.

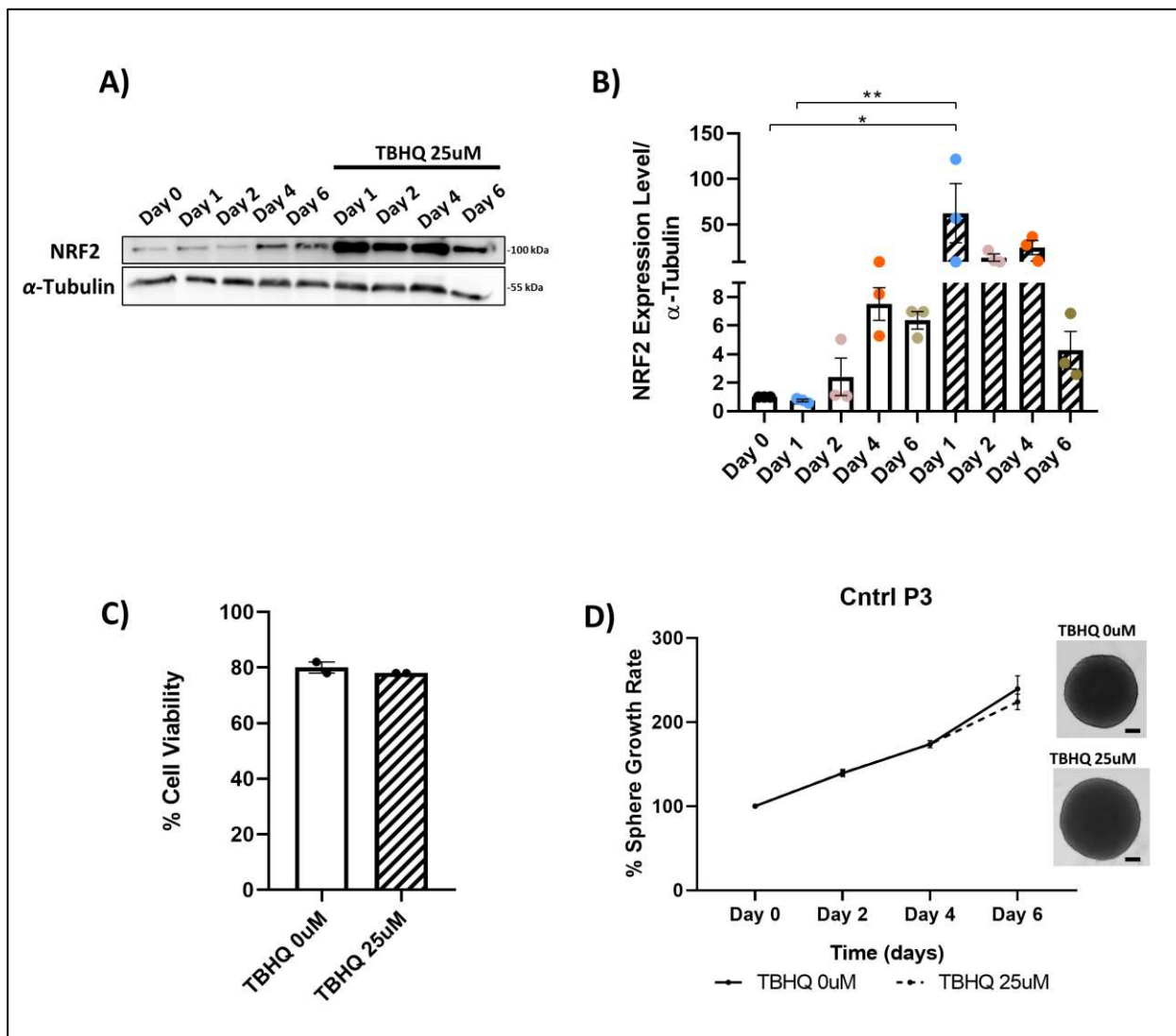


Figure 33. Impact of NRF2 Overexpression on P3 Spheres Proliferation at 21% O₂.

(A) Western blot showing NRF2 protein levels in control P3 spheres with and without TBHQ treatment at 25 μ M over 6 days. α -Tubulin was used as a loading control. (B) Bar graph quantification of NRF2 protein levels from the western blot analysis. The data are normalized to α -Tubulin and presented as fold change relative to the untreated control (Day 0). One-way ANOVA followed by the Kruskal-Wallis test was employed for comparisons between untreated groups and post-24 hrs of treatment. (C) Trypan blue exclusion assay showing cell viability at day 6 in control P3 spheres with and without daily TBHQ treatment at 25 μ M. (D) The growth rate of control P3 spheres with and without daily TBHQ treatment for 6 days. Statistical analysis was performed using a non-parametric *t*-test on day 6. To the right, bright-field images of control P3 spheres on day 6. Scale bar: 100 μ m. Data are expressed as the mean \pm SEM across three independent experiments for western blot and sphere proliferation assays (8 spheres/condition) and two independent experiments for the trypan blue exclusion assay (with 3 technical replicates per condition). Significant differences are indicated as follows: **p*<0.05; ***p*<0.01; ****p*<0.001.

A.2 Role of NRF2 in P3 Sphere Invasion Under Varying Oxygen Conditions

A.2.a Impact of NRF2 Knockout on P3 Sphere Invasion

We assessed NRF2's role in P3 sphere invasion using a collagen I matrix invasion assay. Control P3 and NRF2 KO (C7 and C16) spheres were embedded in the matrix and incubated for 24hrs under different oxygen concentrations (21%, 1%, and 0.1%).

Brightfield images captured after the 24hrs invasion period revealed distinct differences in the invasive properties of the control and NRF2 KO spheres (**Figure 34A**). NRF2 KO spheres exhibited a notable reduction in invasion into the collagen matrix compared to the control spheres. This reduction was characterized by less dispersion of cells from the core spheroid into the surrounding matrix. Quantification of the relative invasion area confirmed a significant reduction in invasion for both NRF2 KO clones at 21% O₂ compared to control spheres (**Figure 34B**), suggesting NRF2 regulates invasive capabilities.

Moreover, the reduction in invasion was consistent across different oxygen concentrations. Specifically, statistical analysis indicated a significant reduction in the invasion area for C16 NRF2 KO spheres at 1% and 0.1% oxygen concentrations compared to the control spheres. In contrast, the C7 NRF2 KO spheres did not show a significant reduction in invasion at the same oxygen levels. This implies that the invasive behavior of C16 NRF2 KO spheres is more sensitive to oxygen concentration changes, particularly under hypoxic conditions. The differential response between C7 and C16 NRF2 KO clones suggests clonal variability in the dependence on NRF2 for invasion under hypoxia.

Therefore, our results underscore NRF2's role in regulating the invasive capabilities of P3 spheres, particularly in C16 KO which shows a higher extent of dependence on NRF2 to mediate invasion. This supports the hypothesis that NRF2 contributes to glioblastoma invasiveness by modulating cellular responses to environmental conditions.

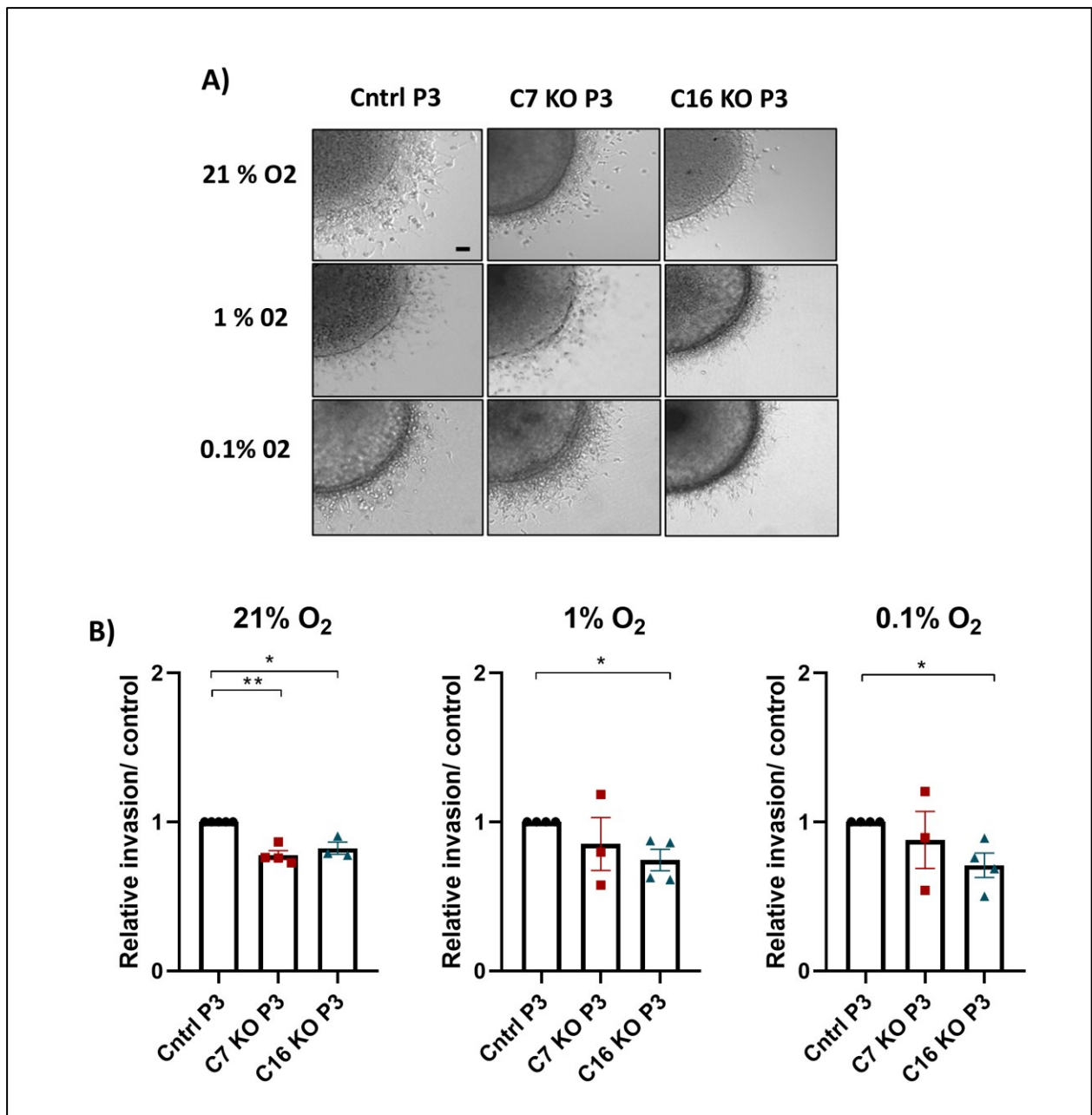


Figure 34. NRF2 Knockout Reduces P3 Sphere Invasion.

(A) Representative brightfield images of P3 spheres in control (Cntrl) or NRF2 KO conditions (C7 and C16 KO), after 24hrs in collagen I at 21%, 1%, and 0.1% O₂. (B) Quantification of P3 sphere invasion relative to the control at the different oxygen concentrations. Data are presented as mean \pm SEM from 8-10 spheres per condition across three to four independent experiments. A one-sample *t*-test was performed for statistical analysis. Significant differences are indicated as follows: **p*<0.05; ***p*<0.01; ****p*<0.001.

A.2.b Effect of NRF2 Overexpression on P3 Sphere Invasion

To determine the effect of NRF2 overexpression on P3 sphere invasion, an invasion assay was performed using Cntrl P3 spheres with and without TBHQ treatment for 24 hrs. TBHQ at a 25 μ M dose was added to the media either directly over the collagen matrix with the embedded spheres or mixed within the collagen itself to ensure thorough diffusion and treatment of the spheres. Bright-field images (**Figure 35A**) indicate similar levels of cell dispersion from the core spheroid into the surrounding matrix across all conditions: no treatment, TBHQ in media, and TBHQ in media and collagen. NRF2 overexpression with and without TBHQ treatment was confirmed in cell lysates of the embedded spheres after 24hrs, as shown in **Figure 35B**, under different conditions. Importantly, increasing NRF2 expression through TBHQ treatment did not lead to any significant change in sphere invasion compared to non-treated conditions (**Figure 35B**). Therefore, our results demonstrate that NRF2 overexpression does not impose any advantage in enhancing the invasive capacity of P3 spheres after 24 hrs of TBHQ treatment, regardless of the administration method.

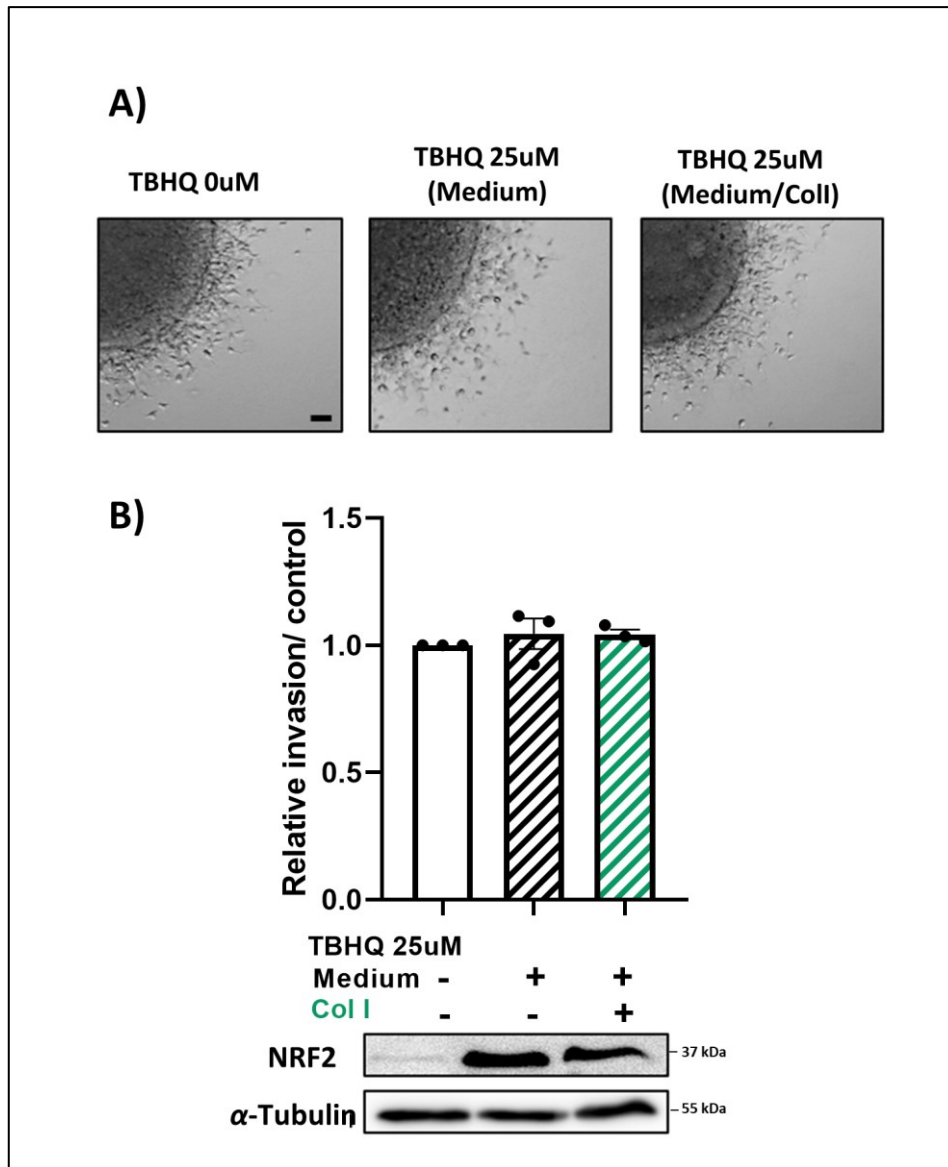


Figure 35. NRF2 Knockout Reduces P3 Sphere Invasion.

(A) Representative brightfield images of P3 spheres in control (Cntrl) or NRF2 KO conditions (C7 and C16 KO), after 24hrs in collagen I at 21%, 1%, and 0.1% O₂. (B) Quantification of P3 sphere invasion relative to the control at the different oxygen concentrations. Data are presented as mean \pm SEM from 8-10 spheres per condition across three to four independent experiments. A one-sample *t*-test was performed for statistical analysis. Significant differences are indicated as follows: **p*<0.05; ***p*<0.01; ****p*<0.001.

A.3 *In vivo* Impact of NRF2 Knockout on Tumor Progression

Next, we evaluated the *in vivo* effect of NRF2 KO on tumor growth by implanting control P3, C7 NRF2 KO, and C16 NRF2 KO luciferase-positive spheres in immunodeficient mouse brains. Bioluminescence imaging was utilized to monitor tumor growth dynamics across time in the different experimental groups (**Figure 36A**). The control group exhibited a steady and gradual increase in bioluminescence over time, reflecting predictable tumor progression. Similarly, the C7 and C16 KO groups demonstrated comparable bioluminescence activity to the control group during the early stages, indicating analogous growth kinetics initially. However, after day 42, the bioluminescence intensity in the C7 KO group began to increase more rapidly, suggesting accelerated tumor growth. By day 60, the bioluminescence signal in the C7 KO tumors was higher than that of the control group, although this difference did not reach statistical significance. In contrast, the C16 KO group displayed a delayed increase in bioluminescence, starting only after day 46, indicating a slower overall progression of tumor growth. By day 60, the bioluminescence in the C16 KO tumors was lower than in the other groups. However, this change was not statistically significant.

Histological analysis using Hematoxylin and Eosin (H&E) staining revealed differential effects of NRF2 knockout on tumor size among the groups. In the C7 KO group, tumor size increased by 20% compared to control (**Figure 36B**); however, this change was not statistically significant. In contrast, the C16 KO group showed a significant reduction of 34% in tumor size compared to the control, supporting the hypothesis that NRF2 may play a pro-tumoral role in GB tumor development.

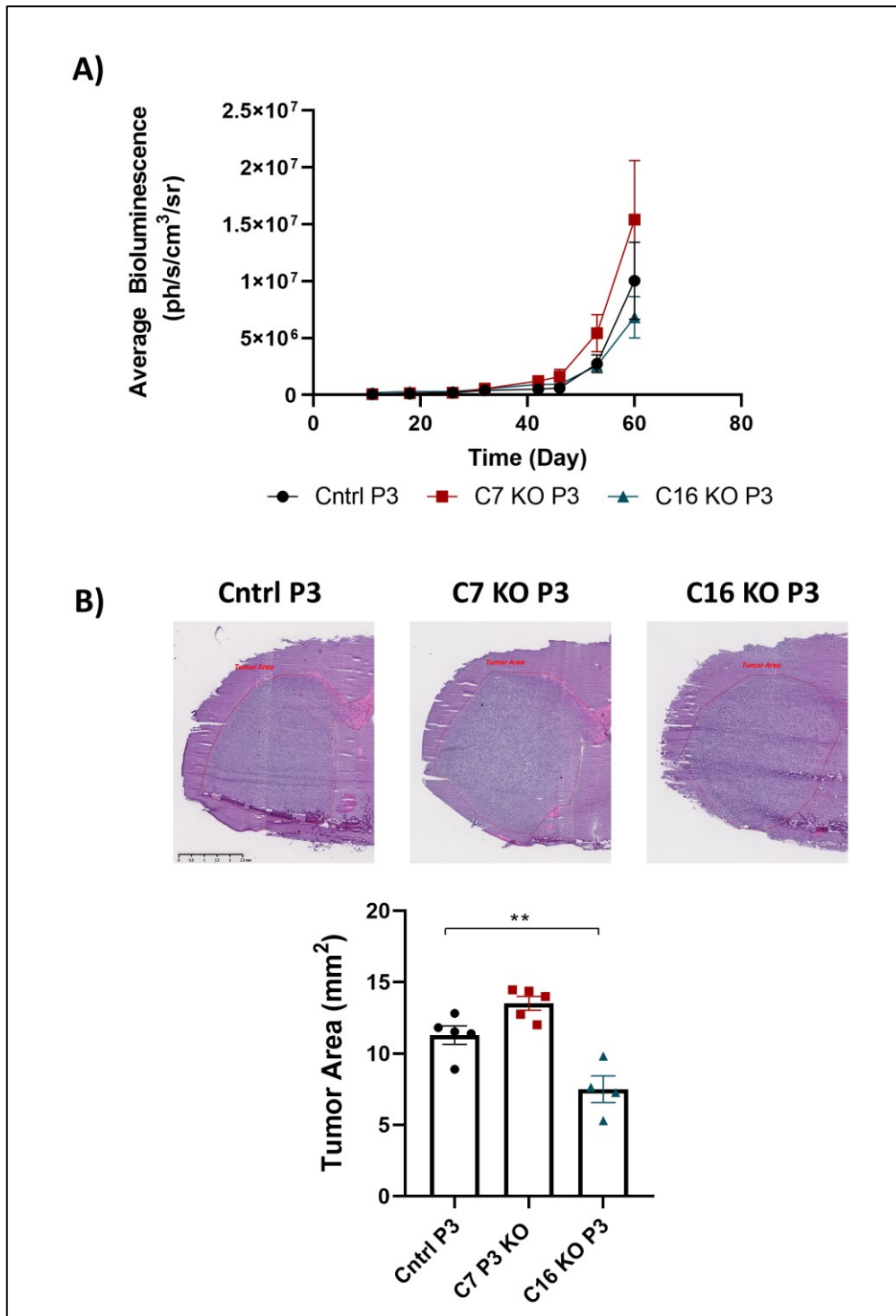


Figure 36. Bioluminescence and Histological Analysis of Brain Tumors in Immunodeficient Mice.

(A) Bioluminescence imaging was used to monitor tumor growth dynamics over time in mice implanted with control P3 (Cntrl P3) tumors and NRF2 KO tumors (C7 and C16 P3). The luciferase activity reflects tumor size and progression. Data represent the mean \pm SEM of 4-5 mice per condition. At day 60, tumor growth differences were statistically analyzed using a one-way ANOVA test to compare tumor sizes across the groups. (B) Representative Hematoxylin and Eosin (H&E) stained sections of brain tumors from the three groups illustrate the histological characteristics of the tumors. Tumor areas are outlined in red and measured using NDP.VIEW 2 software. Data represent the mean \pm SEM of 4-5 mice per condition. Statistical analysis was performed using a one-way ANOVA. Significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

These histological findings are further corroborated by immunohistochemical (IHC) analysis of nestin staining, which demonstrated similar differential effects of NRF2 knockout on GB tumor growth. Specifically, there was a significant 25% increase in tumor area in the C7 KO group compared to the control group, while the C16 KO group exhibited a 22% decrease in tumor area relative to the control (**Figure 37A-B**). Although these findings contrast with our previous *in vitro* observation, where knocking out NRF2 did not significantly impact P3 sphere growth, they underscore the dual impact of NRF2 on *in vivo* tumor progression in the different KO models used. The reduction in tumor size observed in the C16 KO group across both staining methods supports the notion that NRF2 exerts a pro-tumoral effect, contributing to tumor growth. In contrast, the increase in tumor area observed in the C7 KO group suggests that NRF2 may also play an anti-tumoral role. This differential effect highlights that the potential role of NRF2 in GB progression may be influenced by the genetic makeup and specific characteristics of each KO clone.

In terms of invasiveness, the C7 KO group exhibited a slight but statistically insignificant increase in contralateral invasion along the corpus callosum, whereas the invasiveness of tumors in the C16 KO group was similar to that of the control group (**Figures 37A, C**). This suggests that NRF2 knockout does not significantly impact *in vivo* tumor invasiveness. These findings contrast with our *in vitro* results showing a significant reduction in the sphere invasion in both KOs (discussed earlier, **Figure 34B**). This discrepancy highlights the potential influence of the tumor microenvironment *in vivo*, which may modulate the effects of NRF2 absence differently than in controlled *in vitro* conditions.

Taken together, we aimed in our next experiments to elucidate the mechanisms by which NRF2 may influence tumor metabolism and invasiveness, particularly *in vitro*, where more pronounced effects were observed.

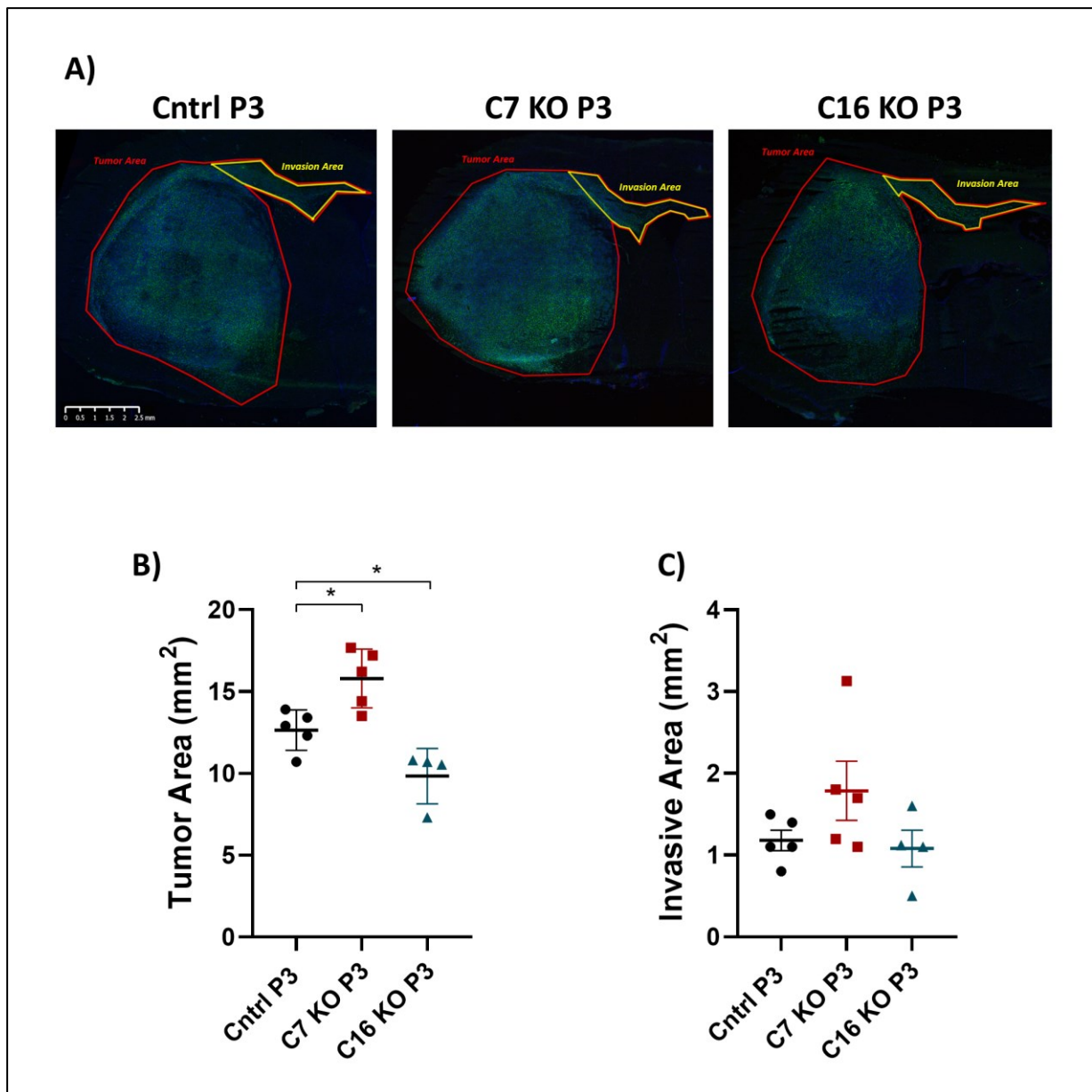


Figure 37. Evaluation of NRF2 Knockout on Tumor Growth and Invasion in vivo.

(A) Immunofluorescent staining of mouse brain slices implanted with control P3 spheres, C7 NRF2 KO spheres, and C16 NRF2 KO spheres. Brain sections are stained with Hoechst (blue) to highlight nuclei and anti-nestin (green) to mark human tumor cells. Tumor areas are delineated with red lines, and regions of tumor invasion into contralateral brain tissue are marked with yellow lines. (B-C) Quantification of the tumor area and invasive area for the control, C7, and C16 NRF2 KO groups. Data represent the mean \pm SEM of 4-5 mice per condition. Statistical analysis was performed using a one-way ANOVA. Significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

B. NRF2 Modulation and Its Effects on Mitochondrial Function

B.1 Differential Impact of NRF2 Modulation on Basal Cellular Respiration

We investigated NRF2 knockout and overexpression on basal cellular respiration in P3 cells by measuring the oxygen consumption rate (OCR) after 24hrs (**Figure 38A**), C7 P3 KO cells showed a significant decrease in OCR compared to the control group, indicating impaired mitochondrial respiration. Conversely, although the OCR of C16 KO P3 cells is slightly reduced, this reduction was not significant compared to the control group, suggesting that the knockout of NRF2 in these cells does not impact mitochondrial respiration to the same extent as in C7 P3 KO cells.

Furthermore, we examined the effect of NRF2 overexpression on basal cell respiration by treating control P3 cells with 25 μ M of TBHQ for 24hrs (**Figure 38B**). The results show a significant decrease in OCR upon TBHQ treatment, implying that NRF2 overactivation can lead to reduced mitochondrial respiration.

These findings suggest that dysregulation of NRF2, whether through deficiency or overactivation, disrupts the balance required for optimal cellular respiration. Although further exploration is needed, these results could be explained by the critical role of NRF2 in maintaining redox balance responsible for efficient mitochondrial function.

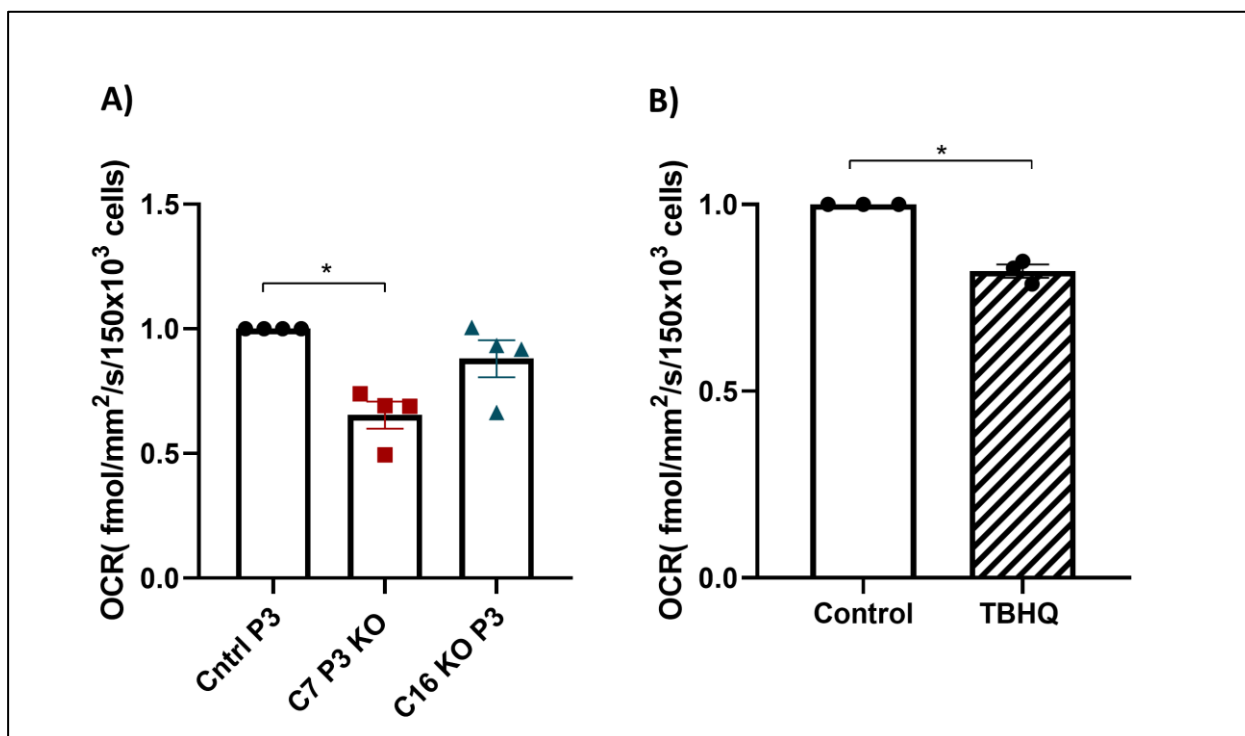


Figure 38. Differential Impact of NRF2 Knockout and Overexpression on P3 Cellular Respiration.

Basal oxygen consumption rate (OCR) was measured after 24hrs in control (Cntrl P3), C7 P3 KO, and C16 KO P3 cells using the Resipher machine. (B) Effect of NRF2 overexpression via TBHQ treatment (25 μ M for 24 hrs) on basal OCR in control P3 cells. Data represent mean \pm SEM from 4-5 technical replicates per condition across three to four independent experiments. Statistical analysis was performed using a one-sample *t*-test. Significant differences are indicated as follows: **p*<0.05; ***p*<0.01; ****p*<0.001.

B.2 Effects of NRF2 Modulation on Mitochondrial Membrane Potential

Next, it was essential to further elucidate the mitochondrial functional status upon NRF2 modulation. We assessed the effects of NRF2 knockout and overexpression on mitochondrial membrane potential ($\Delta\Psi_m$) using the TMRM fluorescence assay. $\Delta\Psi_m$ is a key indicator of mitochondrial health and cellular metabolic state.

The kinetics of TMRM fluorescence is monitored upon the treatment with oligomycin, an ATP synthase inhibitor to induce membrane hyperpolarization, following the treatment with Carbonyl Cyanide m-Chlorophenyl Hydrazone (CCCP), a potent mitochondrial uncoupling agent, to induce mitochondrial depolarization allowing for TMRM signal correction (**Figure 39A**). The results show that the basal $\Delta\Psi_m$ remains stable across control and NRF2 KO cells (**Figure 39B**), indicating no significant differences. These results align with the unchanged OCR in C16 KO cells and suggest that the reduction in OCR in C7 P3 KO cells does not result from a decreased mitochondrial membrane potential (or impairment of proton gradient).

Similarly, upon oligomycin treatment, the hyperpolarization capacity of C7 KO cells was slightly increased but not statistically significant, while that of C16 KO cells remained similar to the control (**Figure 39C**). Maintaining hyperpolarization capacity aligns with the integrity of the mitochondrial membrane, indicating that the essential processes of proton pumping and membrane potential maintenance are still operational despite the absence of NRF2 and the inhibition of ATP synthase.

On the other hand, the basal $\Delta\Psi_m$ remained stable in Cntrl P3 cells upon NRF2 overexpression via TBHQ treatment (**Figure 39D**) while a significant reduction in hyperpolarization capacity compared to untreated controls was observed (**Figure 39E**). These results indicate that while NRF2 overexpression doesn't affect the basal mitochondrial membrane potential in control P3 cells, it significantly impairs the mitochondria's ability to hyperpolarize. This suggests that excess NRF2 may alter the function of the electron transport chain, leading to a reduction in mitochondrial efficiency and adaptive capacity.

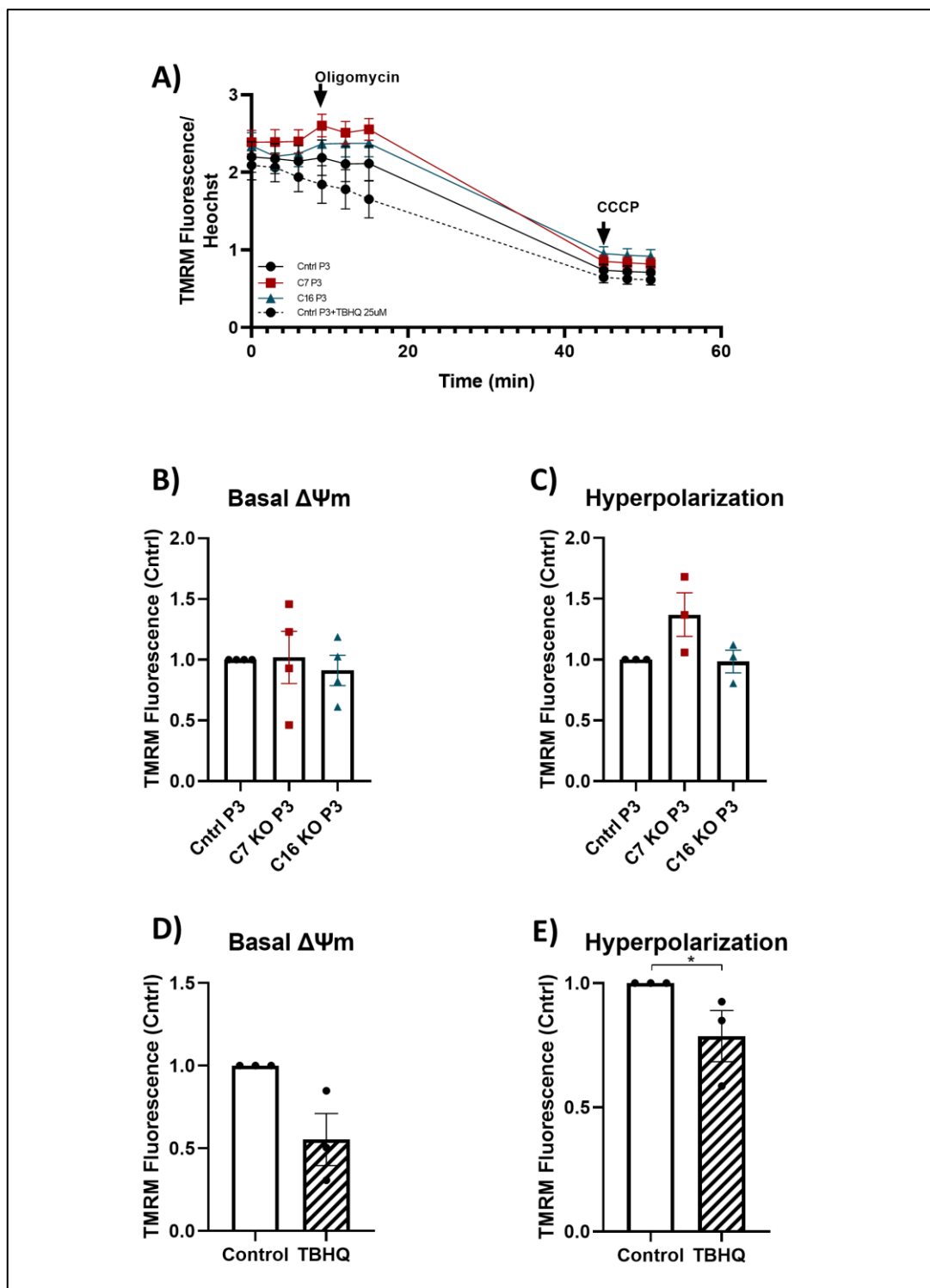


Figure 39. Effects of NRF2 Knockout and Overexpression on Mitochondrial Membrane Potential ($\Delta\psi_m$).

(A) Kinetics of TMRM fluorescence in control (Cntrl P3), C7 KO P3, and C16 KO P3 cells following treatment with 2 μ g/ml oligomycin to induce $\Delta\psi_m$ hyperpolarization and 0.5 mM CCCP to induce $\Delta\psi_m$ depolarization required for TMRM signal baseline correction. (B) Quantification of basal $\Delta\psi_m$ and (C) hyperpolarization capacity in control (Cntrl P3), C7 KO P3, and C16 KO P3 cells after oligomycin treatment. (D) Basal $\Delta\psi_m$ in control P3 cells with and without treatment with 25 μ M TBHQ for 24 hrs. (E) Hyperpolarization capacity in TBHQ-treated control P3 cells compared to untreated controls. Data represent mean \pm SEM from 8 technical replicates per condition across three to four independent experiments. Statistical analysis was performed using a one-sample t-test. Significant differences are indicated as follows: * p <0.05; ** p <0.01; *** p <0.001.

B.3 Impact of NRF2 Modulation on Mitochondrial Biogenesis and Mass

Given these findings, we aimed to better understand mitochondrial dynamics and assess mitochondrial biogenesis in response to NRF2 knockout or overexpression using MitoTracker Green fluorescence.

Our data shows the absence of significant differences in mitochondrial mass among the Cntrl P3 and NRF2 KO groups, as the fluorescence levels remained stable (**Figure 40A**). This shows that NRF2 knockout does not significantly alter the overall mitochondrial biogenesis or turnover in these cells, aligning with the stable $\Delta\Psi_m$ observed earlier and suggesting that the observed decrease in OCR in C7 KO is not a result of reduced mitochondrial mass.

In contrast, a significant reduction in MitoTracker Green fluorescence was observed upon the TBHQ-mediated NRF2 overexpression compared to untreated controls (**Figure 40B**). This reduction indicates a decrease in mitochondrial mass upon NRF2 overactivation. This finding, combined with the previously noted impairment in hyperpolarization capacity, suggests that excessive NRF2 activation can lead to a reduction in mitochondrial content and compromise mitochondrial function.

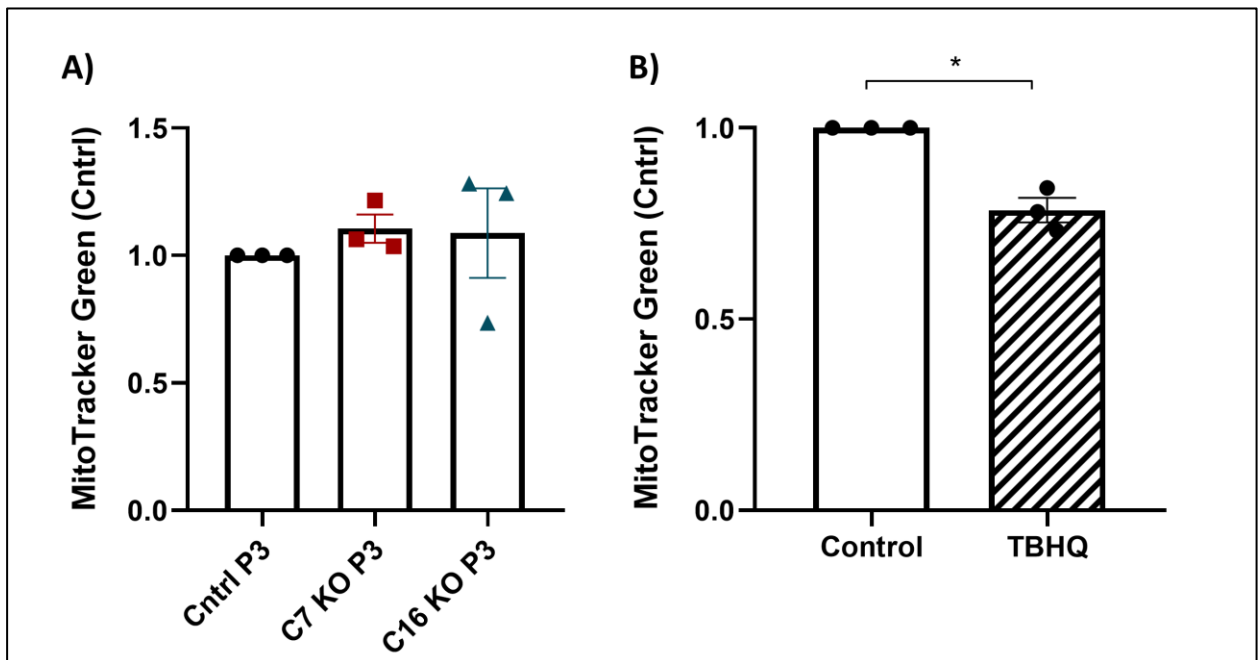


Figure 40. Effects of NRF2 Knockout and Overexpression on Mitochondrial Mass.

Quantification of MitoTracker Green fluorescence measurement of mitochondrial mass in control (Cntrl P3), C7 KO P3, and C16 KO P3 cells. (B) MitoTracker Green fluorescence measurement of mitochondrial mass in control P3 cells with and without treatment with 25 μ M TBHQ for 24hrs. Data represent mean \pm SEM from 8 technical replicates per condition across three independent experiments. Statistical analysis was performed using a one-sample t-test. Significant differences are indicated as follows: * p <0.05; ** p <0.01; *** p <0.001.

B.4 Stability of Mitochondrial Protein Import Machinery in NRF2 KO Cells

Finally, to further understand the impact of NRF2 modulation on mitochondrial protein import and biogenesis, we assessed the expression of Tom20, a key mitochondrial import receptor (**Figure 41A**), in control P3 and NRF2 KO cells. Western blot analysis revealed a moderate reduction in Tom20 expression in NRF2 KO cells; however, this reduction was not statistically significant when compared to the control condition (**Figure 41B**). This observation is consistent with the stable $\Delta\Psi_m$ observed in NRF2 KO cells. These findings suggest that while NRF2 influences some aspects of mitochondrial function, the protein import machinery, as indicated by Tom20 levels, remains sufficiently stable to support mitochondrial membrane potential and overall function in the absence of NRF2.

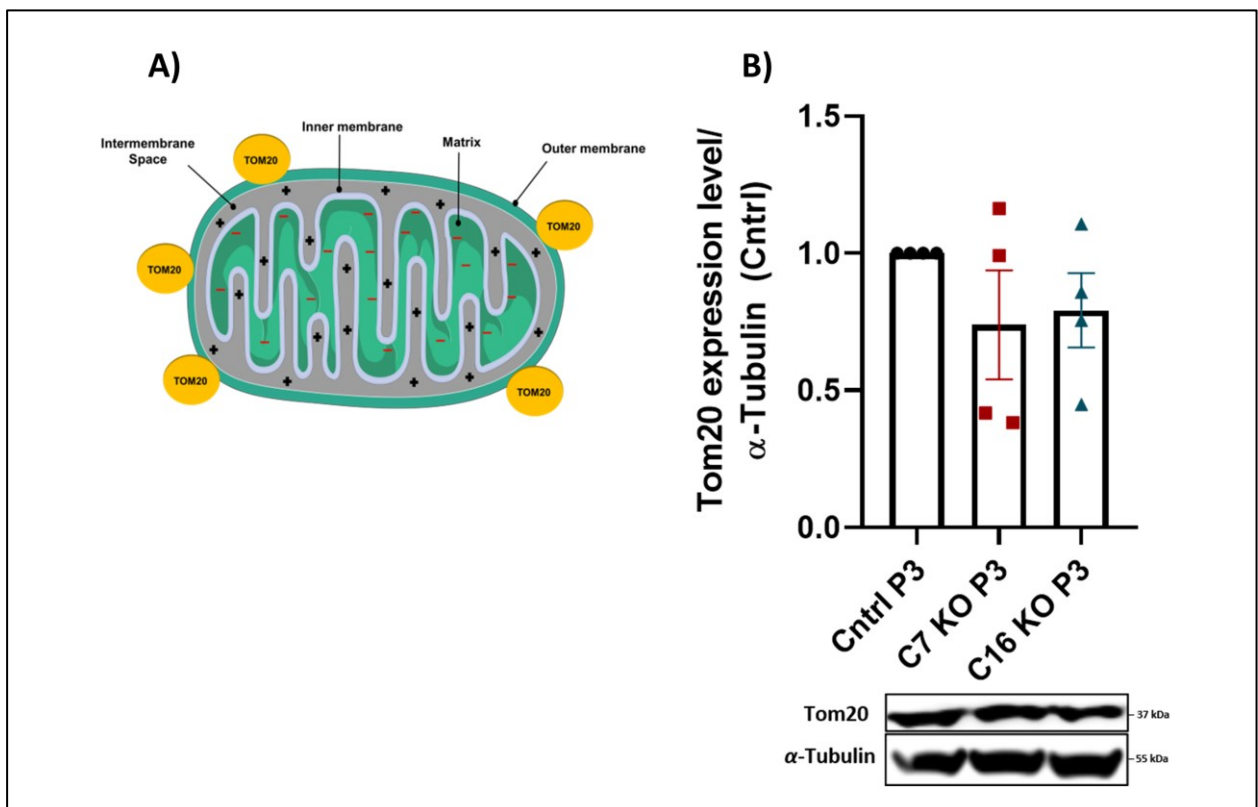


Figure 41. Analysis of TOM20 Protein Expression in Control and NRF2 Knockout Cells.

(A) Schematic representation of mitochondria highlighting the distribution of the TOM20 protein on the outer mitochondrial membrane. (B) Western blot analysis of TOM20 protein expression normalized to α -Tubulin in control (Cntrl P3), C7, and C16 NRF2 KO cells. Data represent the mean \pm SEM per condition across four independent experiments. A one-sample *t*-test was performed for statistical analysis.

C. NRF2 Modulation in Metabolic Regulation of Glioblastoma and Cellular Energy

C.1 NRF2 Knockout Favors Glycolysis

We examined the metabolic impact of NRF2 knockout in P3 spheres by measuring intracellular and extracellular lactate levels in P3 spheres and their surrounding medium, respectively. Extracellular lactate levels did not significantly change across NRF2 KO conditions, but intracellular lactate levels were significantly elevated (2-fold) in C16 KO spheres, but not in C7 KO, compared to the control group (**Figure 42A-B**). This indicates that knocking out NRF2 leads to an intracellular accumulation of lactate in a cell-line-specific response to NRF2 KO leading to a potential shift towards anaerobic glycolysis in C16 KO P3 spheres.

This observation is further supported by the differentially expressed LDHA, the enzyme that converts pyruvate to lactate. LDHA showed a significant 2.3-fold increase in expression in the C16 KO group, while only a modest change was observed in the C7 KO group (**Figure 42C**). This suggests an elevated capacity of lactate production upon knocking out NRF2 in the C16 KO condition. Conversely, LDHB, which is involved in the conversion of lactate to pyruvate, shows a 50 and 40% decrease in C7 and C16 KO groups compared to the control, respectively. Reduction in LDHB indicates a reduced conversion of lactate to pyruvate upon knocking out NRF2 (**Figure 42D**).

To further investigate the effect of NRF2 on lactate transportation, the expression of MCT1, a key transporter for lactate export, was evaluated. In the C7 KO spheres, NRF2 knockout led to a significant 1.3-fold increase in MCT1 expression, whereas there was no significant change in MCT1 expression in the C16 KO spheres (**Figure 42E**). However, despite the enhanced MCT1 expression in the C7 KO condition, lactate export did not improve, suggesting a possible dysfunction in the transporter's activity. Additionally, in the C16 KO condition, the unaltered MCT1 expression alongside increased intracellular lactate accumulation supports that the existing MCT1 is not functioning effectively. Thus, NRF2 knockout impairs MCT1 function, affecting lactate transport differently in C7 and C16 NRF2 KO cells.

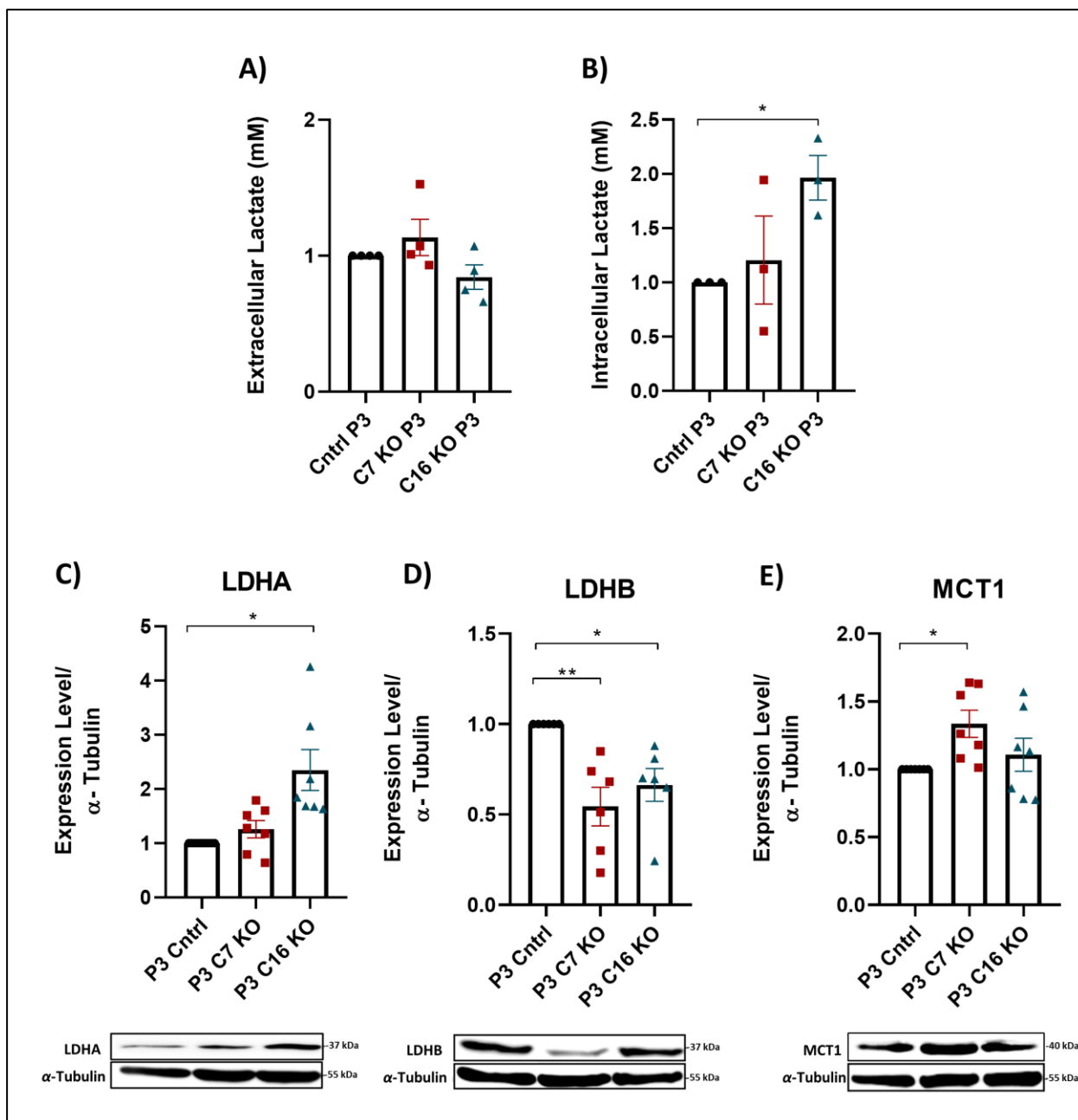


Figure 42. Metabolic Impact of NRF2 Knockout in P3 Sphere Models.

(A-B) Extracellular and intracellular lactate levels were measured by Ultra High-Performance Ionic Chromatography (UHPIC) in P3 sphere media and cells under control (Cntrl), C7, and C16 NRF2 KO conditions. Statistical analysis was performed using a one-sample *t*-test. Data represent the mean \pm SEM from duplicates per condition across three to four independent experiments. (C-E) Western blot analysis of the relative expression levels of LDHA, LDHB, and MCT1 in Cntrl and NRF2 KO P3 cells. Protein expression levels were normalized to α -Tubulin and presented as fold change relative to the control condition. Statistical analysis was performed using a one-sample *t*-test. Data represent the mean \pm SEM per condition across six to seven independent experiments. Significant differences are indicated as follows: **p*<0.05; ***p*<0.01; ****p*<0.001.

C.2 NRF2 Regulates Cellular Energy Dynamics

Also, to further investigate the role of NRF2 in regulating P3 cellular energy, we evaluated ATP levels and the total pool of adenine nucleotides (AXP) in control and NRF2 KO P3 spheres. The results showed higher ATP levels in both C7 and C16 KO cells compared to control cells (**Figure 43A**). However, this increase was statistically significant only in C16 KO cells, indicating enhanced energy availability in the absence of NRF2 in this clone.

Furthermore, we observed a significant increase in the total pool of adenine nucleotides (AXP), which represents the cell's adenine nucleotide content, in both C7 KO and C16 KO cells compared to control cells (**Figure 43B**). This increase in AXP implies that NRF2 plays a significant role in regulating the balance between nucleotide synthesis and degradation. In the absence of NRF2, cells appear to enhance nucleotide availability and maintain sufficient nucleotide levels for ATP production and overall cellular function.

Therefore, NRF2 acts as a key metabolic regulator in glioblastoma cells crucial for cellular energy metabolism, influencing ATP production and nucleotide homeostasis.

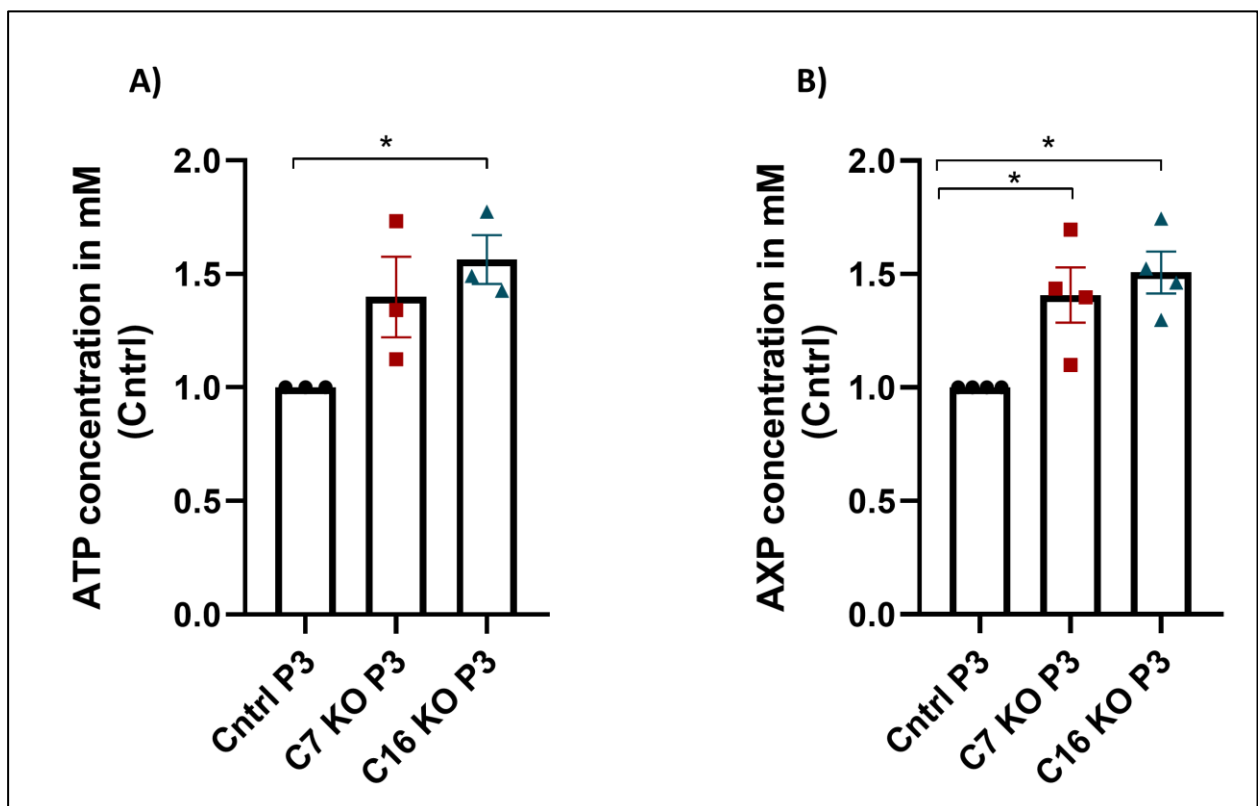


Figure 43. NRF2 Knockout Effects on Cellular Energy Levels in P3 Spheres.

(A) Fold change of adenosine triphosphate (ATP) and (B) total pool of adenine nucleotides (AXP) in C7 KO P3, and C16 KO P3 compared to control P3 (Cntrl P3) spheres, as measured by Ultra-High-Performance Ionic Chromatography (UHPIC). Data are presented as the mean \pm SEM from duplicates per condition across three independent experiments. A one-sample *t*-test was performed for statistical analysis. Significant differences are indicated as follows: **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

C.3 Modest Effect of NRF2 Overexpression on Metabolic Biomarkers

Herein, we aimed to explore the impact of NRF2 overexpression on the regulation of metabolic biomarkers previously examined. Cntrl P3 cells were treated with 25 μ M TBHQ for 24hrs and protein expression was evaluated. Our data shows that although overexpressing NRF2 via TBHQ treatment causes a slight increase in LDHA and MCT1 expression while it slightly decreases that of LDHB compared to non-treated control P3 spheres, non-these changes were statistically significant (**Figure 44**). This indicates that NRF2 upregulation through TBHQ may not have a pronounced regulatory effect on these proteins under the conditions tested.

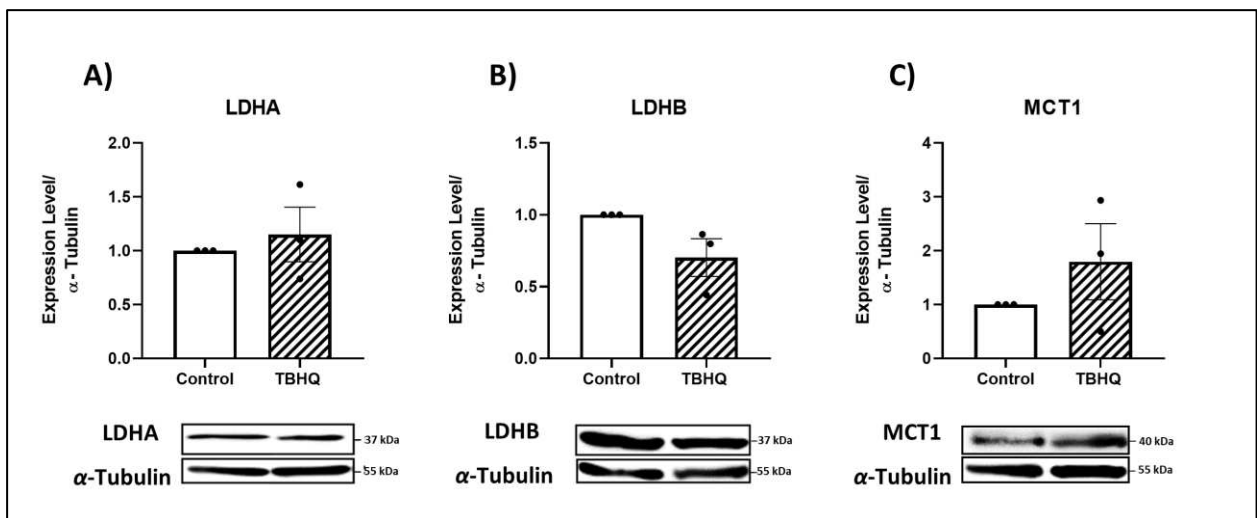


Figure 44. Effect of NRF2 Overexpression on the Expression Levels of LDHA, LDHB, and MCT1 in P3 Control Cells.

Western blot analysis of the relative expression levels of LDHA, LDHB, and MCT1 in control (Cntrl) with and without treatment with 25 μ M TBHQ for 24hrs. Protein expression levels were normalized to α -Tubulin and presented as fold change relative to the control condition. Data represent the mean \pm SEM per condition across three independent experiments. Statistical analysis was performed using a one-sample *t*-test.

C.4 NRF2 Modulation Alters Lipid Metabolism in P3 GB Cells

Finally, we explored whether lipid metabolism is affected by NRF2 modulation in P3 GB cells upon the absence and overexpression of NRF2. Using BODIPY staining to evaluate lipid droplet (LD) formation, NRF2 knockout led to an increase in lipid accumulation in both C7 and C16 P3 KO cells compared to the control condition; however, this elevation was only statistically significant in the C16 KO group indicating disrupted lipid metabolism (**Figure 45A**). To investigate whether NRF2 modulation affects the ability of cells to mobilize lipids for oxidation, we assessed the expression levels of CPT1A, a key enzyme that mobilizes LD-stored triglycerides into free fatty acids and glycerol for β -oxidation in mitochondria. A significant reduction of CPT1A expression is observed in C7 KO P3 cells compared to the control P3, while that of C16 KO P3 cells is similar to that of the control (**Figure 45B**). These results suggest that impaired lipid mobilization due to decreased CPT1A leads to lipid buildup in C7 but not C16 KO P3 cells. This differential impact on CPT1A expression between C7 and C16 KO P3 cells points to distinct regulatory mechanisms of lipid metabolism in response to NRF2 knockout.

Additionally, TBHQ-mediated NRF2 overexpression significantly increased both the lipid accumulation and CPT1A expression in control P3 cells (**Figure 45C-D**). These results demonstrate that while NRF2 activation enhances lipid storage, it also promotes lipid mobilization and oxidation, potentially balancing lipid metabolism.

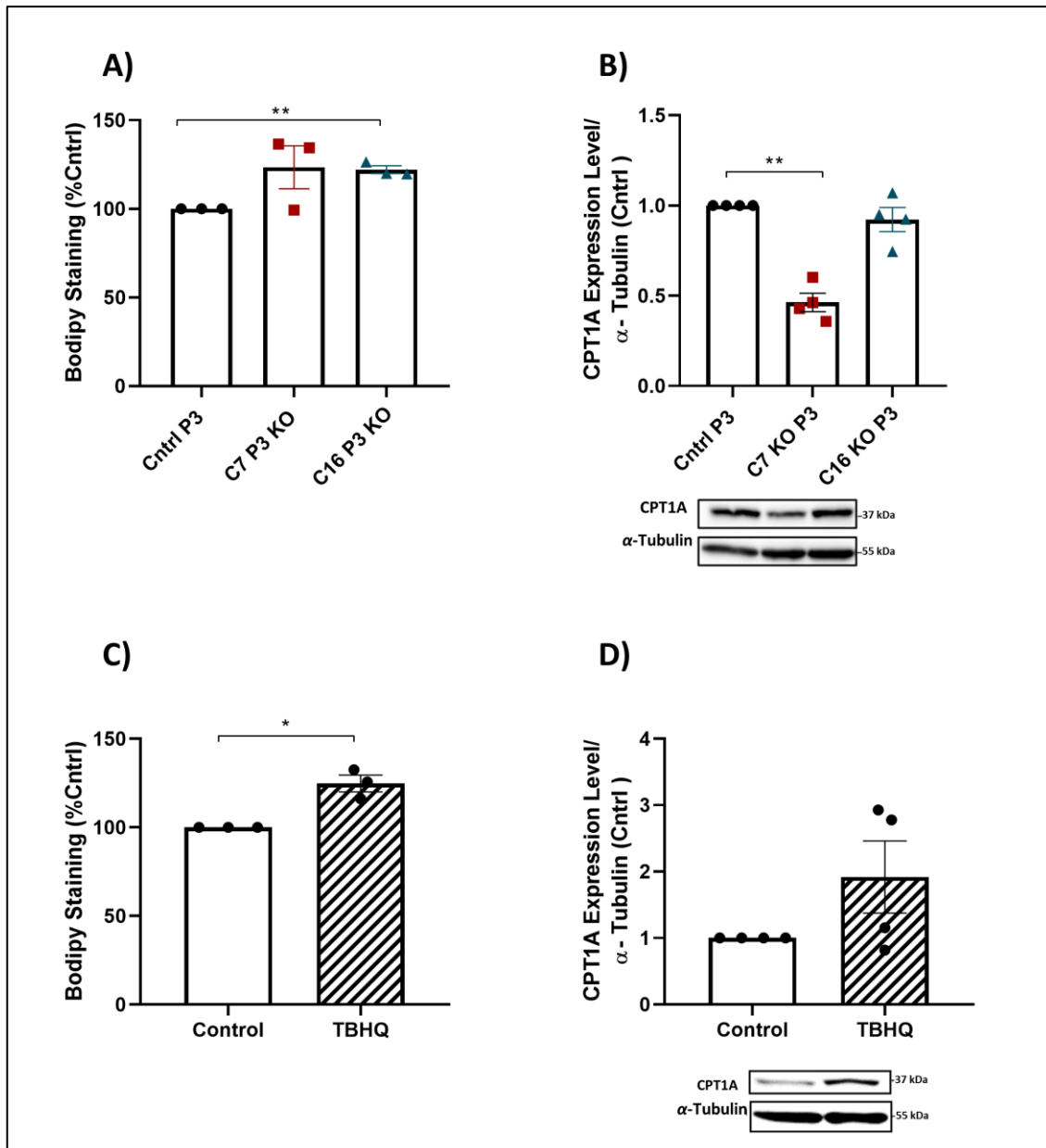


Figure 45. NRF2 Modulation Affects Lipid Metabolism in P3 GB Cells.

(A) Lipid droplet accumulation was assessed using BODIPY staining in control P3 (Cntrl P3), C7 KO P3, and C16 KO P3 cells. The stained cells were analyzed via flow cytometry to quantify the changes in lipid content. Data represent the mean \pm SEM of 4 technical replicates per condition across three independent experiments. (B) Western blot analysis showing the fold change in CPT1A expression levels normalized over α -Tubulin in NRF2 KO cells (C7 KO P3 and C16 KO P3) relative to the control P3 cells (Cntrl P3). (C) BODIPY staining and (D) CPT1A expression in control P3 cells with and without treatment with 25 μ M TBHQ for 24hrs. Data represent the mean \pm SEM per condition across three to four independent experiments. A one-sample *t*-test was performed for statistical analysis. Significant differences are indicated as follows: * p <0.05; ** p <0.01; *** p <0.001.

D. NRF2's Role in Glioblastoma Therapy Resistance and Stemness

D.1 NRF2 KO and P3 Sensitization to TMZ Treatment

We aimed to evaluate the impact of NRF2 KO on the sensitivity of P3 cells to Temozolomide (TMZ) treatment at varying doses using the trypan blue exclusion assay. Cells were treated with increasing concentrations of TMZ (0 to 1600 μ M) for 72hrs, and cell viability was assessed.

Upon treatment with TMZ, cell viability decreased in a dose-dependent manner across all cell lines compared to the initial cell viability of non-treated cells for each group (**Figure 46A**). The sensitivity to TMZ was generally similar between the control (Cntrl P3) and NRF2 KO cells (C7 KO P3 and C16 KO P3) across TMZ doses below 1600 μ M.

However, at 1600 μ M TMZ, the cell viability of the C16 KO P3 cells showed a slightly more pronounced decrease in viability compared to the control (**Figure 46A**). At this dose, the cell viability of C16 KO dropped to approximately 50%, suggesting an IC₅₀ value (half maximal inhibitory concentration) (**Figure 46B**). In contrast, Cntrl P3 and C7 KO P3 cells showed similar levels of toxicity, with cell viability decreasing by 41% and 42%, respectively, at the same TMZ dose (**Figure 46B**). These findings suggest that the IC₅₀ for Cntrl and C7 KO cells might be reached at a slightly higher TMZ dose, estimated to be around 1950 μ M based on the dose-response curves.

Overall, our results show that while P3 cells are generally resistant to TMZ, unless at high doses, NRF2 knockout does not significantly alter their susceptibility to TMZ-induced cell death.

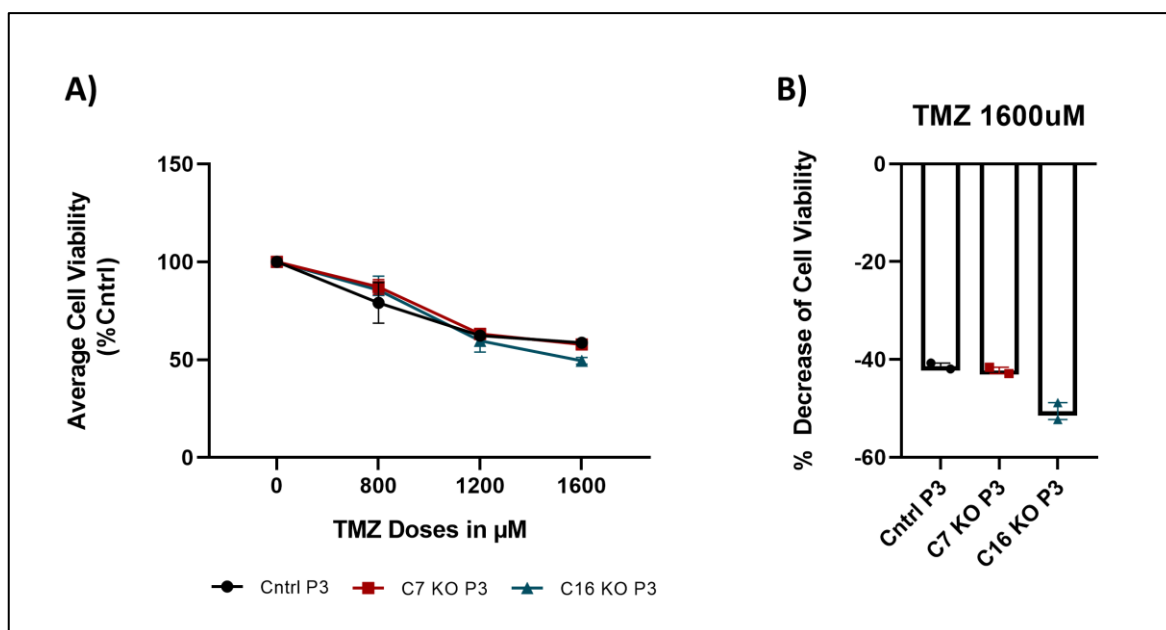


Figure 46. Effect of Temozolomide (TMZ) on the Viability of NRF2 Control and KO P3 Cells.

(A) Trypan blue exclusion assay showing the average cell viability of Control (Cntrl P3), C7 KO P3, and C16 KO P3 cells following treatment with increasing doses of TMZ (0 to 1600 μM) for 72hrs. Cell viability is presented as a percentage of the non-treated control (0 μM). Statistical analysis was performed using a two-way ANOVA to compare cell viability across different TMZ doses within each group and a one-way ANOVA to compare cell viability between groups at 1600 μM of TMZ. Data represent the mean \pm SEM per condition across two independent experiments (3 technical replicates each). (B) Percent decrease in cell viability in response to 1600 μM TMZ treatment for Control (Cntrl P3), C7 KO P3, and C16 KO P3 cells.

D.2 NRF2 KO and Regulation of P3 Stemness

To understand the potential mechanisms influencing the P3 resistance in response to TMZ, we further investigated the expression levels of key stemness markers, Sox2 and Olig2, which are known to be involved in the maintenance of stem-like properties (396) and could influence the response to chemotherapy.

Our results show that Olig2 expression increased by approximately 4-fold and 3-fold in C7 KO and C16 KO, respectively, compared to Cntrl P3 cells (**Figure 47A**). Similarly, Sox2 expression increased by approximately 2-fold and 1.6-fold in C7 KO and C16 KO, respectively (**Figure 47B**). The increased expression of stemness markers Sox2 and Olig2 in NRF2 KO cells suggests that NRF2 may play a role in maintaining stem-like properties in glioblastoma cells.

Furthermore, to assess the effect of NRF2 overexpression on these markers, we induced NRF2 with TBHQ at a concentration of 25 μM for 24hrs. Our results indicate that there were only modest and insignificant changes in the expression levels of Olig2 (**Figure 47C**) and Sox2 (**Figure 47D**) in TBHQ-treated Cntrl P3 cells compared to untreated cells. This suggests that NRF2 overexpression does not significantly alter the expression of these markers, reinforcing the notion that stemness is primarily driven by the absence of NRF2 rather than its overexpression.

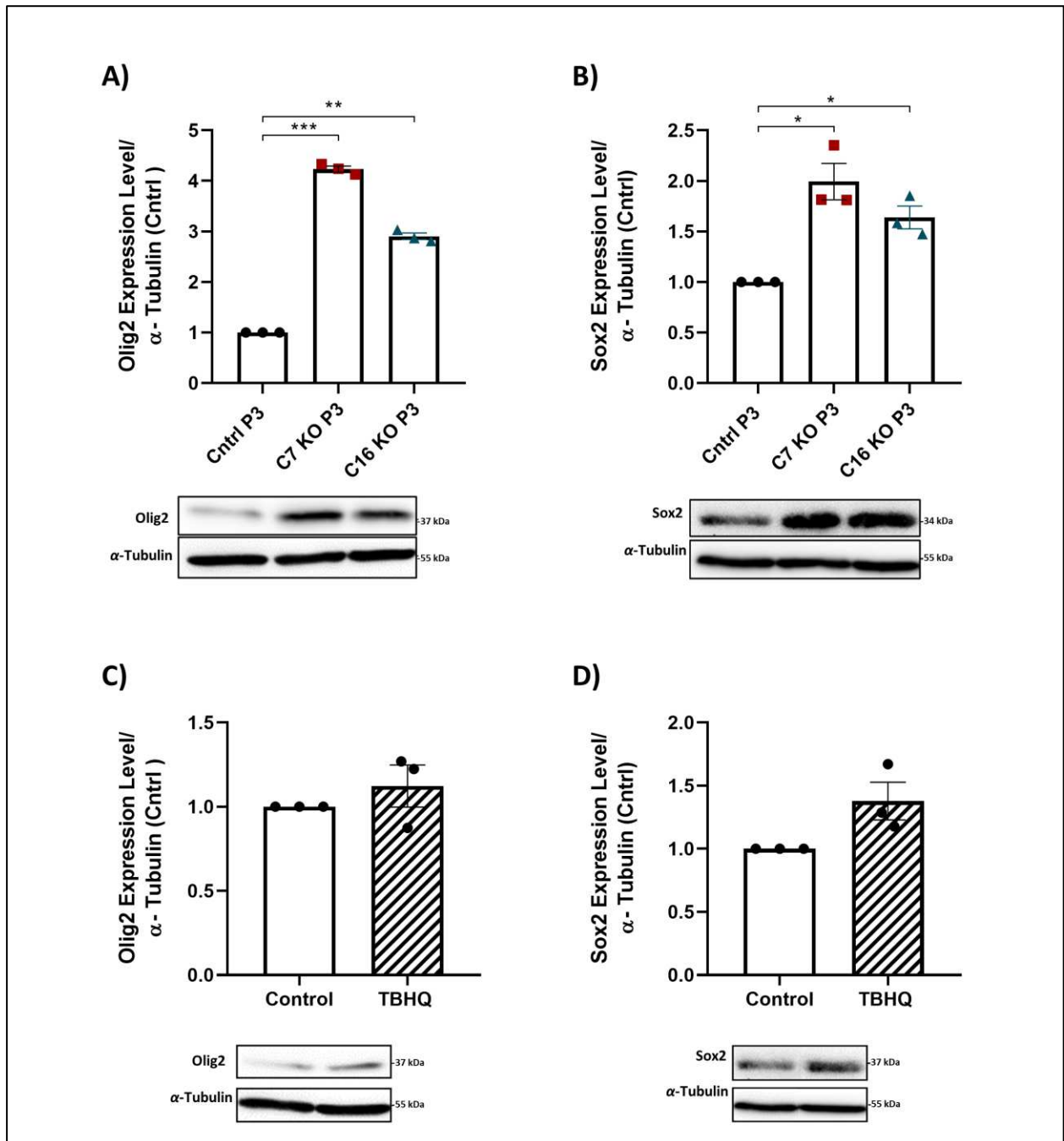


Figure 47. Impact of NRF2 Knockout on the Expression of Stemness Markers in P3 Cells.

(A-B) Quantification of western blot analysis showing the fold change in Olig2 and Sox2 expression levels normalized over α-Tubulin in NRF2 KO cells (C7 KO P3 and C16 KO P3) relative to the control P3 cells (Cntrl P3). (C-D) Western blot analysis shows the fold change in Olig2 and Sox2 expression levels normalized over α-Tubulin in control P3 cells treated with TBHQ (25µM dose for 24hrs) compared to untreated control cells. Data represent the mean ± SEM per condition across three independent experiments. A one-sample *t*-test was performed for statistical analysis. Significant differences are indicated as follows: **p*<0.05; ***p*<0.01; ****p*<0.001.

To further elucidate the role of NRF2 in regulating P3 stem-like properties, we performed an extreme limiting dilution assay (ELDA) to evaluate the sphere-forming efficiency of P3 cells upon knocking out NRF2. Our results reveal no statistically significant differences in sphere-forming capacity for C7 and C16 KO cells compared to control P3 cells (**Figure 48**). This finding suggests that, although NRF2 KO cells exhibit increased expression of stemness markers Sox2 and Olig2, as observed earlier (**Figure 47A-B**), this does not translate into enhanced functional self-renewal capability and sphere formation. Hence, further exploration is needed to provide a more comprehensive understanding of NRF2’s role in GB stemness.

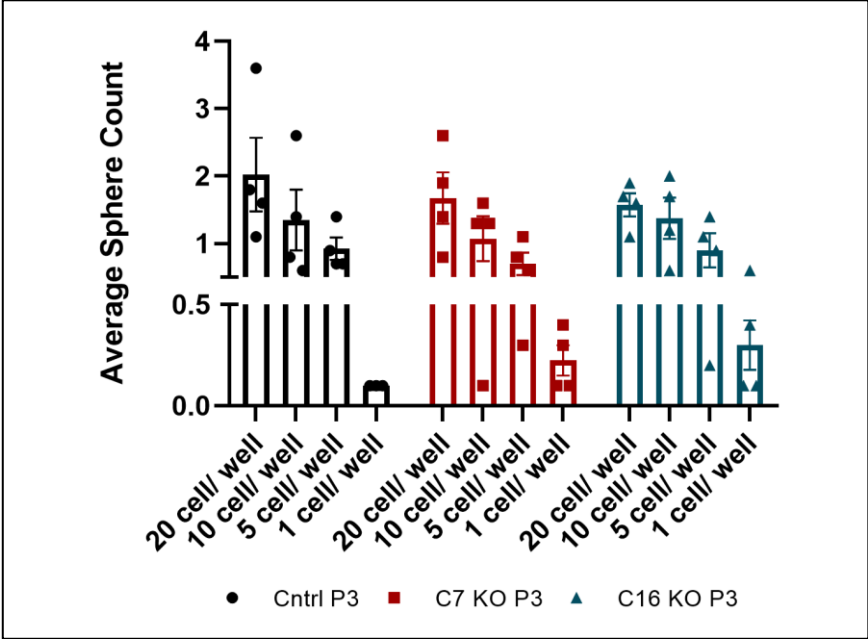


Figure 48. Sphere-Forming Capacity in Control P3 and NRF2 Knockout Cells. Sphere-formation of control P3 (Cntrl P3) and NRF2 KO cell lines (C7 and C16) was assessed by the extreme limiting dilution assay (ELDA). Cells were seeded at varying densities through serial dilution, and sphere formation was evaluated after 7 days. Data represent the mean ± SEM per condition across three to four independent experiments (14 technical replicates each). Statistical analysis was performed using a two-way ANOVA test.

IV. Discussion

IV. Discussion

GB remains one of the most challenging cancers to treat, primarily due to its highly invasive nature and genetic heterogeneity. Traditional studies often rely on established GB cell lines, which fail to capture patient tumors' full complexity and variability while applicable to certain experimental paradigms. Hence, in our study, we specifically employed P3 glioblastoma patient-derived cell lines and 3D spheres to gain clinical relevance and overcome the limitations associated with traditional, purchased GB cell lines. Our study focused on exploring the impact of NRF2 deletion and overexpression on different aspects of the disease, including its progression, metabolism, and therapy resistance. By generating NRF2-deficient models, using CRISPR-Cas9 technology, we were able to observe the resultant changes in cellular behavior and understand how NRF2 contributes to the aggressive nature of GB. Concurrently, we evaluated the effects of NRF2 overexpression through TBHQ treatment, a known activator of NRF2 (388,397), to determine whether increased NRF2 activity influences GB progression.

A. Exploring the role of NRF2 in GB Progression

Our study reveals that knocking out NRF2 does not significantly affect P3 sphere growth *in vitro* in both NRF2 KO clones (C7 and C16). This finding appears to contrast with the existing literature that emphasizes the role of NRF2 in promoting the proliferation and survival of GB cells (347,374). However, it aligns with the broader understanding of cancer cell biology, where cancer cells may often activate redundant signaling pathways independent of NRF2 to ensure survival and growth, thereby circumventing the loss of any single pathway (2,3). Notably, our *in vivo* analysis revealed a differential impact of NRF2 knockout on tumor growth between the two NRF2 KO clones. The C7 KO group exhibited an enhanced tumor growth pattern compared to the control group, suggesting that NRF2 may possess an anti-tumoral role. Knowing that the role of NRF2 in modulating *in vivo* tumor-growth pathways in GB has not been investigated previously, this finding aligns with evidence from other solid cancer models, where NRF2 was shown to upregulate apoptosis and cell cycle regulation (262). Conversely, the C16 KO group displayed reduced tumor growth compared to the control, indicating a pro-tumoral role for NRF2 in this clone. The reduction in tumor size in the C16 KO group aligns with the documented evidence showing that NRF2 can support tumor growth by enhancing cellular antioxidant defenses and metabolic reprogramming in different cancer models (262). These findings demonstrate, for the

first time in a patient-derived GB model, the dual role of NRF2 in GB progression, while it was already seen in other cancer types (398).

Moreover, the differential effects observed in C7 and C16 clones imply that specific genetic or epigenetic alterations may potentially influence NRF2's impact in each clone. Given that, we could hypothesize that the differences between our two clones may be due to distinct genetic backgrounds or epigenetic modifications, potentially reflecting different molecular subtypes of GB. Hence, identifying these alterations through RNA sequencing and gene expression analysis will be crucial to further understanding the underlying mechanisms and the context-dependent role of NRF2 in our GB model.

Also, the observed discrepancies between *in vitro* results where the absence of NRF2 did not affect P3 sphere proliferation, and the *in vivo* findings highlighting a differential impact on tumor growth underscore the complex role of NRF2 in GB proliferation. These differences could be attributed to the potential influence of the tumor microenvironment and its associated heterogeneity compared to *in vitro* settings (191,399), which may modulate NRF2 activity and its downstream effects on tumor growth and progression. Hence, to decipher how the tumor microenvironment interacts with NRF2 signaling pathways, the expression of genes linked to cell proliferation along with the associated signaling pathways should be examined by RNA sequencing using GB patient-derived xenograft (PDX) models.

Moreover, our findings suggest that NRF2's role in cellular proliferation, particularly in the context of the P3 GB model, may not be as straightforward. This notion is further supported by the lack of significant impact on the proliferation of P3 cells upon TBHQ-mediated NRF2 over-expression. TBHQ is primarily utilized as a chemical inducer of NRF2 and a synthetic antioxidant commonly used as a food preservative. TBHQ acts as a pro-oxidant that modifies cysteine residues on KEAP1 through electrophilic attack, resulting in the inhibition of KEAP1's ability to target NRF2 for degradation. This modification disrupts the KEAP1-NRF2 interaction, allowing NRF2 to escape degradation, and allowing for its accumulation and subsequent target gene activation (397). Notably, while some studies have reported that NRF2 activation can enhance GB growth (66), our findings indicate a potential divergence in NRF2 function. One possible explanation for this discrepancy could be due to a specific cellular microenvironment or specific characteristics of the P3 spheres, such as distinct gene expression profiles or epigenetic modifications, compared to other models used in GB disease research with different

experimental setups. Specifically, epigenetic modifications, including DNA methylation and histone modifications (400,401), should be closely examined, as these factors may significantly influence the expression of NRF2 target genes. Such alterations could result in a distinct pattern of gene activation, such as variations in the expression of key NRF2 target genes like *NQO1*, *HO-1*, or *GCLC*, leading to outcomes that differ from those seen in other studies upon NRF2 overexpression.

In terms of invasion, our study elucidates the significant role of NRF2 in regulating the invasive capabilities of P3 GB spheres *in vitro*. This is demonstrated by the substantial reduction in the invasion capacity of NRF2 KO spheres compared to control spheres. This effect was particularly pronounced in the C16 NRF2 KO spheres, indicating a heightened reliance on NRF2 for invasion at varying oxygen concentrations. These findings are consistent with the literature suggesting that NRF2 is a key driver of the invasion in GB (319). Research on human U251 GB cells, reveals that siRNA-mediated downregulation of NRF2 decreases matrix metalloproteinase 9 (MMP9) expression and activity leading to reduced invasion as demonstrated by wound healing and transwell migration assays. The same study shows that plasmid-mediated overexpression of NRF2 enhances MMP9 expression promoting GB cell migration and invasion (319). While our results show that the C16 KO clone is highly dependent on NRF2 to regulate its invasion, especially in low-oxygen environments, C7 exhibits resilience to the knockdown of NRF2 under these conditions. This variability may stem from inherent genetic or epigenetic differences between the clones that may influence how NRF2 impacts their invasive capabilities. This suggests that in the absence of NRF2, different adaptive capacities to manage hypoxic stress may be at play in the two clones. Hence, further exploration is needed to identify the specific genetic and epigenetic alterations responsible for this variability.

However, our observations indicate that NRF2 overexpression does not impact the invasiveness in the P3 spheres within the 24-hour timeframe of our assay, suggesting that the absence of NRF2 is more critical than its overexpression in driving the invasion of GB in the context of our P3 model. This finding is particularly noteworthy, given that NRF2 is known to participate in co-regulatory feedback loops, such as the Sequestosome1 (SQSTM1/p62) complex and the ERK/NRF2 pathway, which have been shown to enhance invasiveness in established GB cell lines like U87, U251, and T98G (351,354). Despite this, the effects of NRF2 overexpression in patient-derived GB models have not been thoroughly investigated throughout the literature.

While there are no specific studies that directly investigate the role of NRF2 in GB tumor invasion *in vivo*, our study demonstrates the lack of a significant effect on invasion in NRF2 KO tumors in mouse models, contrasting with the reduced invasive capacity observed *in vitro* using the P3 spheres. This discrepancy can be attributed to the significantly more complex nature of the *in vivo* tumor microenvironment compared to *in vitro* conditions. The *in vivo* setting involves a more accurate replication of GB tumor heterogeneity (191), including biochemical and biomechanical cues, and interactions between tumor cells, stromal cells, and varying extracellular matrix components, which are not present in controlled *in vitro* settings (399). These interactions that might not be evident *in vitro* can influence GB tumor behavior and progression regulation. To further understand the molecular pathways activated in NRF2 KO tumors *in vivo*, it is essential to investigate how different ECM components interact with GB cells using IHC techniques. Additionally, measuring cytokine levels in the tumor microenvironment and assessing oxidative stress markers, such as ROS and antioxidant enzyme activities within the tumor microenvironment is required in the absence and the presence of NRF2 activation.

B. NRF2 Modulation and Its Effects on Cellular Respiration and Mitochondrial Function

NRF2 is a critical regulator of cellular function and mitochondrial bioenergetics in the brain. It orchestrates the expression of antioxidant and detoxification genes, protecting neural cells from oxidative stress. NRF2 enhances cellular energy production and metabolic stability, supporting overall brain health and resilience against neurodegenerative diseases (66). Mitochondrial function and dynamics, alongside metabolic reprogramming, are closely intertwined with GB progression. Emerging evidence suggests that TMZ treatment disrupts mitochondrial fusion, the critical process by which mitochondria merge to enhance energy synthesis efficiency. This disruption impairs oxidative phosphorylation, the primary pathway for ATP production, and in turn, sensitizes GB cells to TMZ (402). Moreover, altered mitochondrial function, as evidenced by the Warburg effect and metabolic reprogramming in GB, further supports tumor growth by enhancing energy production and biosynthetic pathways (403).

Hence, given the observed changes in the invasive phenotype upon NRF2 knockout *in vitro*, we wanted to understand if NRF2 regulates the invasive capacity of GB tumors by regulating P3 mitochondrial dynamics and cellular function. Evidence on NRF2's role in cellular respiration within brain tissue is still limited, with only a single study demonstrating that the loss of NRF2

impairs cellular respiration in murine neurons and embryonic fibroblasts (404). However, its impact on GB cell respiration remains unclear. To gain a general overview of the metabolic and mitochondrial status, we started by examining the effects of NRF2 expression modulation on basal cellular respiration. Specifically, our results showed impaired cell respiration in C7 KO cells but not in C16 KO cells. This differential impact between the NRF2 KO cell lines suggests intrinsic differences in these NRF2 KO clones that influence their baseline respiration and cellular adaptability to NRF2 absence. Given that NRF2 is a key transcription factor responsible for regulating a broad spectrum of genes essential for cellular function (405), one plausible explanation is that knocking out NRF2 may differentially influence the expression of genes encoding protein subunits of the mitochondrial respiratory chain in both KO clones. This hypothesis is inspired by previous work showing that NRF2 mediated regulation of ETC. Pharmacological activation of NRF2 by sulforaphane upregulates the mitochondrial ETC component NDUFA4 in a human breast epithelial cell line, while genetic upregulation of NRF2 through Keap1 knockdown leads to the downregulation of cytochrome c oxidase subunits COX2 and COX4I1 (406). To test this hypothesis, further investigation is needed to analyze gene expression in isolated mitochondria from control and NRF2 KO P3 cells. Additionally, examining whether the changes in OCR associated with NRF2 deficiency can be rescued by the addition of substrates for mitochondrial respiration will provide further insights into NRF2's role in mitochondrial regulation and its broader impact on cellular metabolism.

Additionally, we show that NRF2 overactivation in control P3 cells can paradoxically impair GB cell respiration. This implies that NRF2's role is not universally beneficial and that its overactivation can be detrimental to cellular respiration. While there is no direct evidence in GB models, the available data supporting this effect in other cancer models is also limited. Nonetheless, this observation is consistent with evidence from lung cancer models, where NRF2 overactivation results in an imbalance in NADH/NAD⁺ ratios, resulting in reductive stress and the blockade of mitochondrial Complex I. This impairs oxidative phosphorylation and cellular respiration, highlighting the role of NRF2 in cellular metabolism (407).

Moreover, to explore if the impacted cell respiration is associated with mitochondrial function, we examined the mitochondrial membrane potential ($\Delta\psi_m$) to assess the functional state of the mitochondria. The mitochondrial membrane potential is a critical component of cellular respiration, reflecting the proton gradient across the inner mitochondrial membrane (408). Given that NRF2 knockdown impairs $\Delta\psi_m$ in cultured primary glioneuronal cells (409), there is no

evidence in the literature regarding NRF2's influence on $\Delta\psi_m$ of GB cells, thus our findings are particularly noteworthy. We observed a stable $\Delta\psi_m$ in both NRF2 KO groups, suggesting that mitochondrial membrane potential maintenance remains intact despite NRF2 deficiency. This demonstrates that the reduction in OCR observed in C7 KO cells is not due to a decreased mitochondrial membrane potential and therefore not due to the impairment of the proton gradient. Given that NRF2 does not appear to directly disrupt the proton gradient across the inner mitochondrial membrane as indicated by the stability of $\Delta\psi_m$, we could hypothesize that NRF2 may instead influence the function or regulation of mitochondrial respiratory chain complexes other than ATP synthase. These complexes (e.g., Complexes I, III, IV) are crucial for maintaining the efficiency of the electron transport chain and cellular respiration, which could explain the reduced OCR in the absence of NRF2 observed earlier, despite the preservation of $\Delta\psi_m$ integrity. To further investigate this hypothesis, we propose conducting gene expression analysis, protein assays, and activity measurements of these complexes and their substrates (e.g., NADH, cytochrome c) in both NRF2 KO and control GB cells. Adding to this, we observed an impaired hyperpolarization capacity in P3 NRF2-overexpressing cells underscoring the detrimental effects of excessive NRF2 activity. Although there is no directly comparable literature addressing the impact of NRF2 overexpression on hyperpolarization capacity in GB or other cancer cells, this observation supports that NRF2's regulatory role requires a delicate balance to ensure optimal mitochondrial function and cellular adaptability.

From a broader aspect, we show that the absence of NRF2 does not significantly affect mitochondrial mass or mitochondrial protein import machinery, indicating that mitochondrial function in maintaining $\Delta\psi_m$ is maintained without compensatory mitochondrial biogenesis. However, excessive NRF2 activation substantially reduces the Mitotracker Green signal, indicating reduced mitochondrial biogenesis. This observation aligns with reports showing that while NRF2 generally supports mitochondrial health, excessive activation can disrupt biogenesis (49,410). Therefore, it is important to identify which specific NRF2 target genes are involved in the regulation of mitochondrial biogenesis and function under conditions of NRF2 modulation. Overall, our investigation underscores the critical role of NRF2 in preserving mitochondrial integrity and sustaining cellular respiration in GB cells. While NRF2 is needed for supporting mitochondrial function, its activity must be precisely regulated. Both NRF2 deficiency and overactivation can disrupt mitochondrial dynamics and impair cellular respiration, likely through the regulation of key complexes involved in these processes.

C. NRF2 Modulation in Metabolic Regulation of Glioblastoma and Cellular Energy

In related contexts, such as lung cancer models, siRNA-mediated knockdown of NRF2 increases glycolytic intermediates, including G6P, F6P, DHAP, pyruvate, and lactate, and is essential for purine nucleotide synthesis from glucose (411). Similarly, in head and neck cancer-initiating cells (HN-CICs) and non-small cell lung cancer (NSCLC) models, NRF2 is not only essential for maintaining glycolysis but also drives this metabolic pathway by promoting the Warburg effect (321,412). Hence, NRF2 plays a significant role in regulating cancer cell metabolism, particularly the glycolysis pathway.

On the other hand, the role of NRF2 in GB metabolism remains significantly underexplored. Preliminary evidence suggests that NRF2 enhances the pentose phosphate pathway (PPP) through the NRF2-TERT regulatory loop. This pathway upregulates the expression of key enzymes such as glucose-6-phosphate dehydrogenase (G6PD) and transketolase (TKT), which are critical control points in the PPP. Additionally, NRF2 may also contribute to glycogen accumulation, further supporting the metabolic needs of GB cells (352). However, its direct involvement in regulating glycolysis in GB remains to be fully elucidated. Our findings add new insights to the limited understanding of NRF2's role in GB metabolic reprogramming. Specifically, our study shows that NRF2 knockout in P3 spheres reveals distinct differences between the C7 and C16 KO groups. A pronounced shift towards glycolysis is observed in the C16 knockout group, characterized by a significant increase in intracellular lactate levels. In contrast, the C7 KO group does not exhibit a similar metabolic shift, indicating a divergent response compared to the C16 KO clone. Hence, further investigations are required to examine intrinsic variations between the NRF2 KO clones influencing their metabolic programming. Importantly, the metabolic shift toward glycolysis in the C16 KO group aligns with the established Warburg effect in GB (219) and previous research indicating that NRF2 loss promotes glycolysis of cancer cells (411). Also, elevated expression of LDHA and reduced expression of LDHB observed in the C16 KO group further support this metabolic shift. Previous studies using GB models have reported that LDHA is upregulated to facilitate glycolysis and lactate production (413), while LDHB, which preferentially converts lactate back to pyruvate, is often downregulated (414). Although the literature does not explore the impact of NRF2 on LDHA in GB models, our findings align with observations in other cancer types. In breast cancer cells, researchers have demonstrated that

the shRNA-mediated knockdown of NRF2 inhibits glycolysis by reducing the expression of genes involved in glucose metabolism, including HK2, PFKFB3, PKM2, and LDHA. Together with our results, this suggests a broader role for NRF2 in regulating key glycolytic pathways across different solid cancers, including GB (415). However, aside from our observation showing that knocking out NRF2 reduces LDHB expression, the role of NRF2 in regulating LDHB remains largely unexplored in the different cancer types including GB highlighting the common need for further investigation into this regulatory relationship.

Previous work in our lab has shown that lactate promotes GB growth and invasion through metabolic interactions in the P3 GB model (225). Lactate is typically exported from cells via the MCT1, along with protons (H^+), which acidifies the extracellular environment and facilitates local invasion by degrading the stromal extracellular matrix (411). Interestingly, in our study, despite increased MCT1 expression in C7 KO cells, we observed no increase in extracellular lactate levels, suggesting impaired transporter function. In contrast, C16 KO cells exhibited unchanged MCT1 expression alongside increased intracellular lactate accumulation, indicating ineffective lactate transport due to MCT1 dysfunction. Based on studies demonstrating how intracellular acidification due to lactate accumulation can hinder cell migration and invasion (416), combined with our current findings, might explain the impaired invasion observed in the P3 model *in vitro*. However, to firmly establish this correlation between intracellular acidification in NRF2 KO cells and their reduced invasion capacity, further investigation is required. This could involve knocking down MCT1 expression or utilizing specific inhibitors to evaluate their effects on P3 invasion in control and NRF2 KO conditions. Nonetheless, our results reveal a novel aspect of NRF2 modulation in GB metabolism and its potential implications for tumor progression.

Moreover, the observed shift toward glycolysis in C16 NRF2 KO cells indicates an increased metabolic activity, as indicated by the elevated ATP and AXP levels. The increase in the AXP pool likely reflects enhanced adenosine nucleotide synthesis, consistent with the upregulation of nucleotide biosynthesis pathways previously reported in GB (23,27). While other studies highlight NRF2's role in enhancing ATP production and purine biosynthesis for nucleotide generation and redox balance in solid cancers like breast cancer (415,417), our study uniquely illustrates how NRF2 loss induces metabolic reprogramming in the P3 GB model. Specifically, NRF2 KO appears to shift the cells toward a greater reliance on glycolysis and adenosine biosynthesis for ATP production, which is vital for supporting cancer cell proliferation (418). This

metabolic adaptation is consistent with the characteristics of GB cells, where ATP hydrolysis is crucial for maintaining mitochondrial $\Delta\psi_m$, thereby necessitating alternative ATP generation through enhanced glycolysis and nucleotide biosynthesis (419). Importantly, this ATP hydrolysis process observed in GB cells is fundamental to explain the maintained $\Delta\psi_m$, as discussed earlier, further underscoring the critical role of glycolysis and nucleotide biosynthesis in compensating for the loss of NRF2 and maintaining cellular energy homeostasis. Given these findings, future investigations should focus on validating ATP hydrolysis in the P3 model by specifically inhibiting ATPase enzymes to observe the consequent effects on cellular ATP levels and mitochondrial function. Additionally, to deepen our understanding of the mechanisms of NRF2-mediated metabolic regulation in GB cells, it would be valuable to assess the expression levels and activity of key glycolytic enzymes (e.g., hexokinase, pyruvate kinase M2) and to quantify the rate of purine biosynthesis in response to NRF2 KO in the P3 GB model.

In addition to these metabolic adaptations, we observed a significant increase in lipid droplet (LDs) formation in NRF2 KO cells. LDs are dynamic, intracellular organelles that primarily serve as storage depots for neutral lipids, such as triglycerides and cholesterol esters. These droplets play crucial roles in energy storage, lipid metabolism, and cellular homeostasis, and are involved in various physiological processes, including lipid trafficking, membrane synthesis, and signaling (420). Based on our observation, we hypothesize that the observed reduction in CPT1A expression, an enzyme primarily involved in the transportation of fatty acids into the mitochondria for β -oxidation, in the C7 KO cells is indicative of lipid accumulation driven by the reduced mobilization of stored triglycerides into free fatty acids for β -oxidation. This suggests that the decrease in β -oxidation may be a direct consequence of impaired CPT1A expression, leading to the observed lipid droplet accumulation. To examine this hypothesis, measuring the levels of free fatty acids and assessing the activity of β -oxidation enzymes in these cells is required. Conversely, in the C16 KO cells, we hypothesize that the enhanced lipid accumulation is linked to increased lactate production, reflecting a lactate-driven shift in lipid metabolism as reported in previous studies (421,422). Lactate reprograms lipid metabolism in GSCs by serving as an alternative carbon source, which is converted into acetyl-CoA, a crucial precursor for fatty acid and phospholipid synthesis. Additionally, lactate metabolism increases NADH production, which is needed for fatty acid synthesis and maintaining redox balance, both of which are essential for efficient lipid metabolism (421). The distinction between these two KO cell lines is critical, as it suggests different metabolic pathways may be altered by NRF2 knockout due to the

genetic makeup of each clone. Therefore, the lipid accumulation observed in our study, whether potentially induced by lactate-induced shifts or reduced lipid mobilization for β -oxidation, suggests a broader metabolic reprogramming that favors lipid storage over utilization for energy upon NRF2 knockout. To test this hypothesis, future investigations should involve quantifying lactate levels both intracellularly and in the extracellular medium while examining the balance between lipid storage and β -oxidation activity in P3 control and NRF2 KO cells.

Collectively, these findings suggest that NRF2 triggers metabolic reprogramming characterized by increased glycolysis, nucleotide reprogramming, and lipid accumulation, favoring cell survival over progression. This metabolic shift likely underpins the reduced invasive capacity observed in NRF2 KO GB cells, potentially driven by lactate-induced intracellular acidification and diminished energy production due to decreased lipid oxidation. These insights suggest the role of NRF2 in modulating the metabolic landscape of GB cells, directly impacting their invasiveness and overall tumor progression.

D. NRF2's Role in Glioblastoma Therapy Resistance and Stemness

GB's poor prognosis is primarily driven by its therapy resistance and the presence of GSCs which significantly contributes to a high recurrence rate (66). Typically, NRF2 is known to confer resistance to chemotherapeutic agents in various cancer types, including TMZ for the treatment of GB due to its regulation of GSH antioxidant response and detoxification pathways (66,362) in addition to upregulating the expression of the *MGMT* gene, which is involved in cancer cell DNA repair (423). Additionally, NRF2 is known to enhance stem cell-like properties in various cancer types, including self-renewal capacity, epithelial-mesenchymal transition, and therapy resistance (322). GSCs, constituting a small portion of GB tumors, are the source of GB reoccurrence (214) and are also inherently resilient to GB chemo and radiotherapy (214). In GB, NRF2 contributes to the pluripotency of these cells (356), being essential for maintaining their self-renewal capacity (357) and proliferation (290).

Our findings reveal that P3 cells exhibit resistance to TMZ treatment across various doses, and knocking out NRF2 does not significantly affect their sensitivity to TMZ. This observation stands in contrast to the existing literature, which predominantly utilizes traditional GB models such as U87 and U251. These models frequently link increased NRF2 expression with TMZ resistance in GB (362,423,424). In comparison, our results suggest a different mechanism at play. It is known

that MGMT, a key DNA repair enzyme, directly counters TMZ's cytotoxic effects by repairing TMZ-induced DNA damage (142,425). The P3 GB cells employed in our study possess an unmethylated MGMT promoter, which likely leads to high MGMT expression levels. This could explain why knocking out NRF2 does not significantly alter TMZ sensitivity in these cells; the high MGMT expression enables efficient DNA repair, contributing to TMZ resistance independently of other pathways, such as NRF2. Moreover, the relatively high TMZ IC50 observed in both control and NRF2 KO P3 cells indicates an inherent resistance to TMZ, consistent with the IC50 value and resistant profile observed in patient-derived GB cultures, as reported by Yuzhakova and colleagues (426).

Moreover, knocking out NRF2 unexpectedly elevates the expression of stemness markers SOX2 and OLIG2, indicating that the absence of NRF2 might paradoxically contribute to the maintenance or enhancement of stem-like characteristics within the tumor cells. This underscores a complex role of NRF2 where it is not the primary factor driving TMZ resistance in these cells, but its absence may unexpectedly upregulate key stemness markers.

Sphere formation is a widely recognized characteristic of GSCs and other cancer stem cells (CSCs). *In vitro*, the ability of cells to form spheres in non-adherent, serum-free conditions is considered indicative of stem-like properties, particularly self-renewal capacity, and tumorigenicity (427,428). Surprisingly, while the elevated expression of SOX2 and OLIG2 in NRF2 KO cells, indicates a shift towards a more stem-like phenotype, this molecular signature does not correlate with enhanced functional self-renewal capability, as evidenced by the lack of increased sphere formation.

This discrepancy highlights that increased expression of stemness markers alone is not sufficient to drive functional stem cell behavior in our GB model. These findings are particularly significant as they offer an alternative perspective to existing reports suggesting that knocked-down NRF2 reduces self-renewal capacity in GB neurospheres (357,358). Consequently, further investigation is warranted to explore whether NRF2 knockout affects the stability or post-translational modifications of stemness markers, which could potentially explain the disconnect between gene expression and functional outcomes. Additionally, gene expression profiling through RNA sequencing should be undertaken to identify stemness-related regulatory pathways or factors that may be altered upon NRF2 loss, with a focus on pathways within the Notch, Wnt/ β -Catenin, and EMT regulatory networks.

V. Conclusion and General Perspectives

V. Conclusion and General Perspectives

Being one of the most aggressive and challenging cancers to treat, GB is characterized by its rapid growth, invasive behavior, and significant genetic heterogeneity. Despite advances in treatment, GB remains largely incurable, necessitating the exploration of new therapeutic targets and strategies. NRF2, a transcription factor pivotal in cellular defense against oxidative stress, has emerged as a key player in cancer progression, including GB. This study aimed to investigate the role of NRF2 in GB using patient-derived P3 GB cell line and 3D spheres, which provide a more accurate representation of the tumor microenvironment compared to traditional cell lines.

Our investigation revealed a complex relationship between NRF2 expression and GB progression (**Figure 49**). Particularly, NRF2 knockout significantly reduced the invasive capabilities of GB cells *in vitro* without impacting their proliferative potential, highlighting NRF2's role as a driver of GB invasiveness, a crucial aspect of the disease that contributes to its poor prognosis. Interestingly, NRF2 overexpression did not enhance either GB cell invasiveness or proliferation, suggesting that the absence of NRF2 is more detrimental to GB progression than its overexpression. This highlights a nuanced role for NRF2, where its absence impacts tumor behavior more profoundly than its overactivity. *In vivo*, the differential effects observed between the two NRF2 knockout clones (C7 and C16) suggest that NRF2's role in tumor growth is influenced by the genetic or epigenetic alterations of each clone. NRF2 could act either as a suppressor or promoter of tumor growth depending on the cellular context in each KO tumor type. This highlights the need for further research to identify the underlying genetic factors that dictate NRF2's function in the different KO models. Moreover, our findings also highlighted the differential impact of knocking out NRF2 on basal cellular respiration of both C7 and C16 KO clones, while preserving mitochondrial function and biogenesis. In contrast, NRF2 overexpression was found to mainly impair basal cell respiration and mitochondrial biogenesis. This suggests a complex role of NRF2 in mitochondrial regulation, where its absence and overexpression produce distinct effects on cellular energy homeostasis. Given these findings, it is imperative to further investigate the expression and activity of mitochondrial complexes to elucidate the precise mechanisms by which NRF2 modulation influences GB mitochondrial function.

Notably, NRF2 knockout-induced metabolic reprogramming, characterized by increased glycolysis and lipid accumulation, offers new insights into how GB cells adapt their metabolic

pathways in response to NRF2 loss. These metabolic changes suggest that NRF2 helps maintain energy production and cellular integrity, which is crucial for the survival of cancer cells under stress. However, the shift towards glycolysis caused via lactate accumulation while favoring lipid storage over oxidation energy production may reduce the cells' invasive potential. This underscores the importance of understanding NRF2's role in regulating both metabolic and invasive properties of GB cells, especially *in vivo* within the complex tumor microenvironment.

In terms of therapy resistance, our study challenges previous reports by demonstrating that NRF2 knockout does not significantly alter the sensitivity of P3 cells to TMZ, a standard chemotherapeutic agent used in GB treatment. This suggests that NRF2 may not be the primary driver of TMZ resistance in these cells. However, the upregulation of stemness markers observed following NRF2 knockout indicates a potential role for NRF2 in modulating the stem-like phenotype of GB cells. Interestingly, this increase in stemness markers does not translate into an enhanced sphere-forming capacity of GB cells, highlighting a complex regulatory mechanism at play. A deeper understanding of the pathways through which NRF2 influences stemness in GB is crucial and could provide valuable insights on how to combat GB recurrence.

Overall, our study has limitations that warrant careful consideration. First, the *in vitro* nature of several experiments may not fully replicate the complexity of NRF2's role within the *in vivo* tumor microenvironment, where interactions with various cellular and molecular components are critical. Additionally, the variability observed between different NRF2 knockout clones indicates a potential variability in the genetic and epigenetic makeup that should be explored along with the underlying mechanisms that may influence NRF2's function in each clone. Despite these challenges, our findings reinforce the critical role of NRF2 in GB, particularly in the regulation of tumor invasiveness and metabolic reprogramming. These insights open promising avenues for further investigation, with the potential to unravel the diverse and intricate roles of NRF2 in GB. Continued research in this direction could significantly advance our understanding and lead to improved outcomes for this aggressive solid cancer.

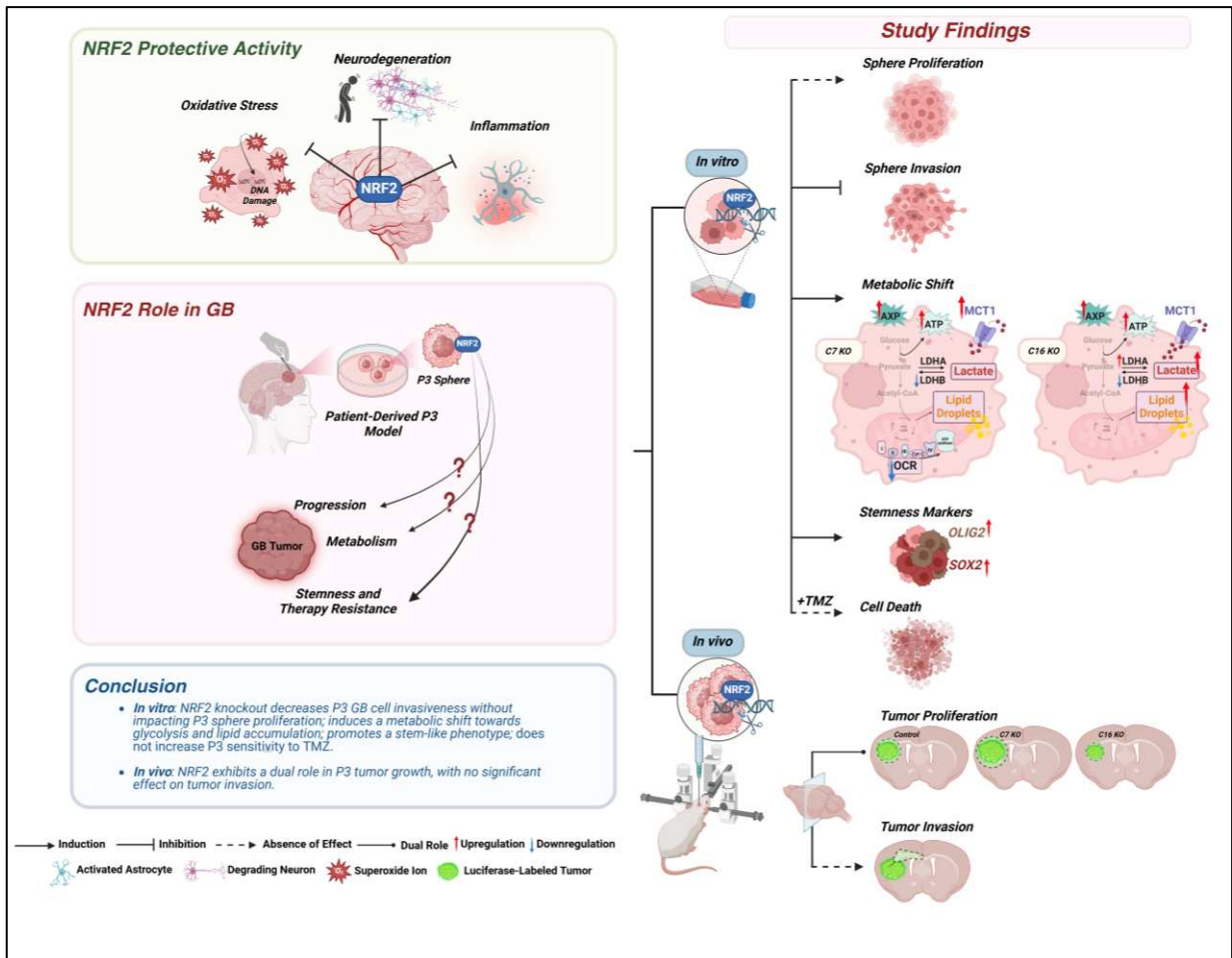


Figure 49. Overview of NRF2 in Brain Physiology and Study Key Finding of NRF2 Role in GB Using P3 Patient-Derived Model. Upper Panel (NRF2 Protective Activity): This panel illustrates the protective roles of NRF2 (nuclear factor erythroid 2-related factor 2) in brain physiology. In the brain, NRF2 is involved in defending against oxidative stress, preventing neurodegeneration, reducing inflammation, and protecting against DNA damage. Middle Panel (NRF2 Role in Glioblastoma - GB): This section focuses on the experimental approach to studying NRF2's role in glioblastoma (GB) using a patient-derived P3 model. It showcases the use of P3 spheres, derived from patient tumor cells, to investigate NRF2's influence on tumor progression, cellular metabolism, stemness, and resistance to therapy. Right Panel (Study Findings): The study findings are displayed, and divided into two parts: *in vitro* and *in vivo* experiments. Bottom panel: Conclusion of key findings from the current study. Figure created with BioRender.com. NRF2: Nuclear Factor Erythroid 2-Related Factor 2; GB: Glioblastoma; P3: Patient-Derived P3 Model; TMZ: Temozolomide; LDHA: Lactate Dehydrogenase A; MCT1: Monocarboxylate Transporter 1; ATP: Adenosine Triphosphate; OCR: Oxygen Consumption Rate; Olig2: Oligodendrocyte Transcription Factor 2; Sox2: SRY-Box Transcription Factor 2.

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Appendix

Appendix I: Exploring the multifaceted role of NRF2 in brain physiology and cancer: A comprehensive review

Moubarak MM, Pagano Zottola AC, Larrieu CM, Cuvelier S, Daubon T, Martin OCB. Exploring the multifaceted role of NRF2 in brain physiology and cancer: A comprehensive review. Neurooncol Adv. 2023 Dec 23;6(1):vdad160. doi: 10.1093/noajnl/vdad160. PMID: 38221979; PMCID: PMC10785770.

Exploring the multifaceted role of NRF2 in brain physiology and cancer: A comprehensive review

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Abstract

Chronic oxidative stress plays a critical role in the development of brain malignancies due to the high rate of brain oxygen utilization and concomitant production of reactive oxygen species. The nuclear factor-erythroid-2-related factor 2 (NRF2), a master regulator of antioxidant signaling, is a key factor in regulating brain physiology and the development of age-related neurodegenerative diseases. Also, NRF2 is known to exert a protective antioxidant effect against the onset of oxidative stress-induced diseases, including cancer, along with its pro-oncogenic activities through regulating various signaling pathways and downstream target genes. In glioblastoma (GB), grade 4 glioma, tumor resistance, and recurrence are caused by the glioblastoma stem cell population constituting a small bulk of the tumor core. The persistence and self-renewal capacity of these cell populations is enhanced by NRF2 expression in GB tissues. This review outlines NRF2's dual involvement in cancer and highlights its regulatory role in human brain physiology and diseases, in addition to the development of primary brain tumors and therapeutic potential, with a focus on GB.

Key Points

- NRF2 is vital for optimal functioning and redox homeostasis in brain cells.
- NRF2 contributes to GSC maintenance, GB development, and metabolic reprogramming.
- Targeting NRF2 offers a potential therapeutic target for GB treatment and therapeutic resistance.

Oxidative Stress and Human Cancer

Cellular Oxidation and Cancer Onset

Cellular redox homeostasis is a state of physiological equilibrium between the intracellular reactive oxygen species (ROS), reactive nitrogen species (RNS), thiol-containing compounds, as well as the antioxidants that control their elimination.¹ Endogenous ROS are mainly produced in the mitochondria as byproducts of oxygen metabolism.^{2,3} Moreover, ROS are also generated in response to exogenous environmental factors, including ultraviolet (UV) and ionizing radiations (gamma-ray/x-ray), some pollutants and chemicals, heavy metals, as well as xenobiotics.⁴ At physiological levels, ROS operate as

second messengers in intracellular Ca²⁺ signaling pathways to govern cell proliferation, differentiation, and apoptosis.^{5,6} However, sustained elevation of free radicals causes damage to cellular DNA, lipids, and proteins in addition to initiating ROS signaling cascades, which in turn amplify the cellular oxidative stress.⁷ Besides, an iron-dependent increase in ROS levels induces p53-dependent cell death,^{8,9} autophagy activation, induction of necrosis, and ferroptosis, causing lipid peroxidation-mediated cell death.¹⁰

Oxidative DNA damage is considered a significant mutagenic and carcinogenic factor by promoting cancer progression through genome instability and chromosomal abnormalities with amplified oncogene activation. In addition, it affects cancer cell metabolism and causes the loss of function in tumor

suppressor genes, leading to DNA damage and altered physiological transcription.¹ Notably, ROS modifies the DNA through guanine to thymine G→T transversions,^{11,12} recognized as the most common mutations in the p53 tumor suppressor gene.^{13–15} Moreover, tandem CCTT substitution was also noted in DNA exposed to free radicals.¹⁶ Cancer progression and survival are improved by ROS-induced phosphorylation of Jun N-terminal kinase (JNK), enhanced expression of cyclin D1, and mitogen-activated Protein Kinase (MAPK) activation. In addition, ROS regulates cellular proliferation by activating the extracellular-regulated kinase 1/2 (ERK1/2) and ligand-independent receptor tyrosine kinase (RTK). They enhance angiogenesis *via* angiopoietin and vascular endothelial growth factor (VEGF) and facilitate tumor invasion and metastasis *via* the release of metalloproteinase (MMP) into the extracellular matrix.¹⁷ Chronic oxidative stress deactivates p53, phosphatase and tensin homolog (*PTEN*) tumor suppressor genes and induces oncogenes expression, including protein kinase B (*AKT*), *ERK*, and *c-MYC* inhibiting apoptosis and promoting cell proliferation, transformation, and metastasis.³ It also impacts cancer cell metabolic reprogramming affecting glycolysis, oxidative phosphorylation, and fatty acid metabolism, to support tumor growth and survival.^{18,19}

Cellular Antioxidant Systems

Endogenous antioxidant systems include enzymatic antioxidants such as superoxide dismutase (SOD) that decomposes superoxide ion (O_2^-),²⁰ catalase (CAT) that neutralizes hydrogen peroxide (H_2O_2),²¹ glutathione peroxidase (GPx) which utilizes glutathione (GSH) to convert H_2O_2 or organic hydroperoxides to water or corresponding alcohols, respectively.²² In addition, the thioredoxin (Trx) system is made up of NADPH, thioredoxin reductase (TrxR), and Trx, which operate on DNA and protein mending by inhibiting ribonucleotide reductase and methionine sulfoxide reductase.²³ Other endogenous antioxidants belong to the hydrophilic and lipophilic radical antioxidants. Besides, phenolics, flavonoids, carotenoids, vitamins A, C, and E, and minerals are classified as exogenous nonenzymatic antioxidants usually derived from diets.²⁴

Increased ROS stimulate the nuclear factor erythroid 2-related factor 2/ Kelch-like ECH-associated protein 1 (NRF2/KEAP1) pathway, which controls an intracellular antioxidant defense by regulating downstream target genes at their antioxidant response elements (ARE) found in the gene promoters of detoxifying enzymes.²⁵ NRF2 regulates the expression of glutathione-S-transferases (GST), NAD(P)H quinone dehydrogenase 1 (NQO1), gamma-glutamylcysteine synthase (γ -GCS), ferritin, and heme oxygenase-1 (HO-1), SOD and catalase along with other cytoprotective processes.^{26,27}

NRF2: A Double-Barreled Aspect

NRF2 Overview: Architecture, Regulation, and Downstream Targets

NRF2, a cap'n'collar (CNC)-basic region-leucine zipper (bZIP) transcription factor encoded by the *NFE2L2* gene, is a soluble protein primarily localized in the cytoplasm,

highly conserved across species, and a major regulator of the cellular antioxidant response.^{28,29} Its structure comprises 7 domains, including a bZIP DNA binding domain at the C terminus and 6 highly conserved NRF2-ECH homologies (Neh) domains.^{28,29} The bZIP domain, located in the Neh1 domain, mediates NRF2 heterodimerization with small musculoaponeurotic fibrosarcoma proteins (sMafs) in the nucleus.³⁰ The Neh2 domain, the main regulatory domain of NRF2 located in the N-terminus, contains 7 lysine residues for ubiquitination, and DLG (Asp-Leu-Gly) and ETGE (Glu-Thr-Gly-Glu) motifs that bind to homologous locations on the KEAP1.^{31,32} Thereby, the Neh2 domain assists NRF2 in attaching to and regulating its inhibitory cytoplasmic chaperone molecule Keap1.³³ Besides, the C-terminal Neh3 domain is needed to maintain protein stability and transcriptional activation,³⁴ while Neh4 and Neh5 engage with the CREB binding protein (CBP) to act as transactivation domains.³³ Although the Neh2 domain is required for NRF2 turnover in homeostatic cells, the redox-insensitive serine-rich Neh6 domain, a newly recognized domain, regulates NRF2 ubiquitination and further degradation in oxidatively stressed cells.^{35,36} Similarly, the other recently discovered Neh7 domain of NRF2 interacts with retinoic X receptor alpha (RXR), a regulator of NRF2, to reduce NRF2's cytoprotective capacity and sensitizing non-small cell lung cancer cells to therapeutic toxicity.³⁷ However, further investigations are required to illustrate the role of these 2 newly discovered domains in the context of oxidative stress.

The KEAP1 repressor protein tightly regulates the NRF2 transcription factor.³⁸ KEAP1, a substrate adaptor protein for the Cul3-Rbx1 E3 ubiquitin ligase complex, primarily localizes in the cytoplasm³⁹ and drives NRF2 proteasome degradation.^{35,40} In response to cellular stress, such as the presence of ROS, disulfide bonds may form on KEAP1 cysteine residues (Cys226, Cys613, Cys622, and Cys624).⁴¹ In addition, when electrophiles are present, KEAP1's cysteine residues bind covalently with these electrophilic compounds through thiol-alkylation.⁴¹ Moreover, KEAP1 has a Zn²⁺ sensor consisting of a group of amino acids, including His-225, Cys-226, and Cys-613, capable of detecting free Zn²⁺ released by damaged proteins. The binding of Zn²⁺ to KEAP1 leads to its structural alteration, disrupting its association with the cullin-3 (Cul3)-RING ubiquitin ligase (CRL) adaptor/scaffold protein.⁴² All the above-described modifications affect the KEAP1-based E3 ubiquitin ligase complex and, therefore, prevent the proper alignment and interaction with NRF2. As a consequence, the resulting conformational shift in KEAP1 induces the detachment of the DLG motif from the KEAP1-NRF2 complex, resulting in the inhibition of NRF2 ubiquitination.^{31,43} NRF2 is then released, phosphorylated at the Neh2 domain by protein kinase C (PKC)⁴⁴ and translocated to the nucleus, where it heterodimerizes sMAFs and binds to antioxidant ARE domains,^{32,43} causing transcription of NRF2 targets cytoprotective genes.⁴⁵ Once the redox equilibrium is restored, NRF2 is released from the ARE sequence. Then, KEAP1, which acts as an adaptor for Cul3-based E3 ligase, transports NRF2 to the cytoplasmic Cul3-E3 ubiquitin ligase machinery to add Lys-48 linked poly-Ub chain, marking it for 26S proteasome degradation.^{46,47} Thereby, a basal level of NRF2 is retained, and the NRF2/KEAP1 signaling pathway is deactivated.²⁹

Other regulatory mechanisms of NRF2 activity and expression have been described. On the transcriptional level, the *NFE2L2* gene could be activated by polycyclic aromatic hydrocarbons.^{48,49} In addition, NRF2 is activated in response to oncogene stimulation and may be mediated via *KRAS* and *BRAF* induction of JUN and MYC transcription factors.⁵⁰ Moreover, transcription factors such as Jun dimerization protein (JDP2), JUN, CREB binding protein (CBP), Brahma-related gene 1 (BRG1), and p21 induce NRF2 activation. In contrast, Fos proto-oncogene, AP-1 transcription factor subunit (cFOS), p53, p65, Fos-related antigen 1 (FRA1), BTB and CNC homology 1 transcription factor (BACH1), CCAAT/enhancer-binding protein (C/EB), activating transcription factor 1 (ATF1), activating transcription factor 3 (ATF3), short-form estrogen-related receptor (SFERR), peroxisome proliferator-activated receptor α (PPAR- α), and retinoic acid receptor (RAR) have been shown to inhibit NRF2 transcription.^{51,52} At the post-transcriptional level, microRNAs (miRNAs), endogenous short noncoding RNAs, can suppress gene expression by interacting with target transcript translation or stability. Among miRNAs, miR-507, miR-634, miR-450a, and miR-129-5p inhibit the translation process of NRF2.⁵³ In addition, it has been documented that hypermethylation of CpG sites in the KEAP1 promoter region occurs in various cancer types,⁵⁴⁻⁵⁶ and such epigenetic changes result in constitutive activation of the NRF2 pathway. Other NRF2 regulation mechanisms involve the p62-mediated dysfunction of autophagy,⁵⁷ electrophilic-mediated inhibition of KEAP1,⁵⁶ and hormone-mediated NRF2 activation by gonadotrophins and estrogen, which inhibits KEAP1 via oxidation of its multiple cysteine residues.⁵⁸

NRF2 is responsible for regulating the transcription of more than 200 genes that play a role in various cellular processes such as cytoprotection, metabolism, and gene transcription.⁵⁹ It activates the transcription of genes involved in the detoxification of reactive species and xenobiotics, such as phase I, II, and III enzymes, including Aldo-keto reductase (*AKR*), NADPH quinone oxidoreductase 1 (*NQO1*), superoxide dismutase (*SOD*), catalase, multidrug resistance-associated protein (*MRP*), and ATP-binding cassette transporters (*ABC*).⁶⁰ In addition, it plays a crucial role in the cellular antioxidant system based on the glutathione molecule. NRF2/KEAP signaling is responsible for regulating the expression of various elements such as the cystine-glutamate antiporter xCT, glutamate cysteine ligase (*GCL*), glutathione peroxidase (*GPX*), and reductase (*GSR*), which are necessary for cysteine import and catalysis of the rate-limiting step in GSH manufacture and ROS detoxification.^{61,62} Similar to this, NRF2 upregulates thioredoxin-1 (*TXN1*),⁶³ thioredoxin reductase 1 (*TRXR1*),⁶⁴ peroxiredoxins (*PRXS*),⁶⁵ and sulfiredoxin-1 (*SRXN1*),⁶⁶ allowing the reduction of oxidized protein thiols and the elimination of peroxides. In addition, NRF2 regulates the transcription of genes involved in metabolism, especially carbohydrate metabolism, and NADPH generation (ie, *G6PD*, glucose-6-phosphate dehydrogenase; *HDK1*, hexokinase domain containing 1; *IDH1*: NADP-dependent isocitrate dehydrogenase), lipid metabolism (ie, *ACOT7*, acetyl-CoA thioesterase 7; *ACOX1*, acetyl-CoA oxidase 1), and heme and iron metabolism (ie *BLVR*, biliverdin reductase; *FTL1*, ferritin, light polypeptide; *HMOX1*, heme

oxygenase 1).⁵⁹ Therefore, NRF2 plays a crucial role in regulating intracellular redox homeostasis.

NRF2's Dual Role in Cancer

NRF2 tumor suppressive activities.—NRF2 exerts an anti-tumor effect, mainly through sustaining cellular redox homeostasis, regulating cell growth, and exerting anti-inflammatory activities.²⁹ For instance, the NRF2 signaling pathway detoxifies ROS and RNS by upregulating the expression of numerous phase II drug-metabolizing enzymes, therefore decreasing the oxidative stress that is strongly associated with cancer development.⁶⁷ Several in vivo studies have emphasized the role of NRF2 in cancer protection using NRF2-deficient mice that expressed reduced levels of phase II enzymes. In addition, NRF2-knockout (KO) mice were found to be more sensitive to chemical toxicants and carcinogens and resistant to the protective effects of chemopreventive drugs, potent NRF2 inducers. These compounds exert NRF2-dependent adaptive responses against carcinogenic insults. They are either natural molecules such as curcumin and resveratrol or synthetic chemicals such as oltipraz, 2-indol-3-yl-methylenequinuclidin-3-ols, and the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9, among others.⁶⁸ Besides limiting original tumor development, another study has shown that NRF2 protects against cancer metastasis by maintaining the redox equilibrium in the hematopoietic and immune systems.⁶⁹ Paradoxically, NRF2 deficiency renders cancer cells more prone to oxidative cell death but more resistant to chemopreventive compounds. Therefore, targeting the NRF2 pathway presents a critical strategy for developing effective chemopreventive medications.

In terms of inflammation, in NRF2-KO animals, cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF) levels are considerably greater compared to control mice, showing that NRF2 inhibits pro-inflammatory mediators.⁷⁰ Besides, NRF2-dependent activation of NQO1 reduces TNF and IL-1 production caused by lipopolysaccharide (LPS), impairing the inflammatory response⁷¹ and subsequent inflammation-induced carcinogenesis. Although ROS elimination is the molecular basis of NRF2-mediated anti-inflammation, NRF2 may also function as an anti-inflammatory mediator in the absence of ROS. This is accomplished by regulating genes encoding for MARCO (macrophage receptor with collagenous structure) and CD36 receptors specific for macrophages, not involved in the oxidative response.⁷² In addition, NRF2 protects against H₂O₂-induced damage via the p38/MAPK pathway.^{73,74} As well, NRF2 inhibits the NF- κ B pathway by stabilizing the NF- κ B inhibitor (IKK)- α and repressing the degradation of (IKK)- β .⁷⁵ On the contrary, the NF- κ B p65 subunit competes with NRF2 for the CH1-KIX domain of the transcriptional coactivator CBP, resulting in the inactivation of the NRF2 pathway.⁷⁶

NRF2 oncogenic activities.—Various factors contribute to the constitutive activation of NRF2 in cancer cells, including somatic mutations in *KEAP1* and *NFE2L2*, exon skipping in *NFE2L2*, methylation of the *KEAP1* promoter, accumulation of p62/Sequestosome-1 (SQSTM1), and

mutation in fumarate hydratase. Constitutive NRF2 activation promotes cancer growth, through metabolic alterations, stimulation of proliferation and inhibition of apoptosis, promotion of angiogenesis, invasion, and metastasis in addition to promoting treatment resistance in various cancer types.^{29,77} On a molecular level, NRF2 overexpression promotes the transcription of the oncogenes *MYC*, *KRAS*, and *BRAF*.⁵⁰ Conversely, the oncogenic activation of NRF2 occurs by inhibiting PTEN/glycogen synthase kinase 3 (GSK-3)/beta-transducin repeat-containing E3 ubiquitin-protein ligase (β -TrCP) activity.⁷⁸ Moreover, NRF2 allows for metabolic reprogramming to enhance cancer cell proliferation by upregulating the expression of glycolytic enzymes such as glucose-6-phosphate dehydrogenase [G6PD], phosphogluconate dehydrogenase [PGD], transketolase [TKT], and transaldolase 1 [TALDO1]⁷⁹; regulating genes implicated in fatty acid and lipid metabolism,⁸⁰ proliferation-associated genes⁸¹ and inhibitory cell-cycle regulators.⁸² Interestingly, NRF2 activation participates, through glucose-regulated protein 78 (GRP78)/ phosphorylated protein kinase RNA-like ER kinase (p-PERK)/NRF2 signaling pathway, to glycolytic gene transcription and simultaneous inhibition of the tricarboxylic acid cycle (TCA), which promotes the Warburg effect.⁸³ Another NRF2-mediated oncogenic activity is the promotion of angiogenesis, mainly by activating heme oxygenase-1 (HO-1),⁸⁴ which in turn regulates VEGF to promote angiogenesis.⁸⁵

Besides, regarding cancer cell apoptosis, siRNA-mediated knockdown of NRF2 results in the down-regulation of HO-1-mediated expression and the sensitization to TNF-induced cell death in a model of acute myeloid leukemia. This suggests that NRF2 inhibits cancer cell apoptosis by regulating the levels of the antioxidant enzyme HO-1.⁸⁶ Also, NRF2 upregulates the expression of anti-apoptotic protein B-cell lymphoma 2 (BCL-2) while it down-regulates the activity of proapoptotic BAX protein and caspases 3/7 to protect against etoposide/radiation-mediated cell apoptosis that leads to drug resistance.⁸⁷ In addition, NRF2 suppresses the activation of proapoptotic c-Jun N-terminal kinases (JNKs)⁸⁸ and induces selective autophagy of KEAP1.^{89,90} Autophagy is a crucial process for cancer cell growth; however, overexpressed NRF2 renders autophagy-dependent cancer cells to overcome the loss of autophagy and allows them to maintain protein homeostasis.⁹¹

Regarding cancer stemness, lower levels of endogenous ROS due to the increased antioxidant capacity mediated by the higher NRF2 expression are reported in cancer stem cells (CSCs) compared to non-CSCs, allowing for the enrichment of their stemness phenotype.^{92–95} This results in reduced mitochondrial-derived ROS and subsequently maintains CSC stemness-associated properties,⁸³ such as the ability to initiate an epithelial-to-mesenchymal transition.⁹⁶ Similarly, persistent NRF2 activation improves the ability of CSC to self-renew, primarily by maintaining cell quiescence and lowering intracellular ROS.^{97,98} In a broader sense, mesenchymal stem cells (MSCs), known to be multipotent stem cells, are present in the tumor niche to encourage cancer cells' ability to spread by promoting their motility and invasiveness.^{99,100} NRF2 is needed to maintain MSCs' stemness and prevent their apoptosis under oxidative stress.¹⁰¹

Moreover, because NRF2 significantly benefits cancer cells, these cells frequently develop NRF2 addiction.^{102,103} Enhanced nuclear accumulation of NRF2 is associated with increased cellular proliferative signals. For instance, phosphoinositide 3-kinase (PI3K)-AKT activation in combination with KEAP1 deficiency in the mouse liver results in a massive accumulation of NRF2 and NRF2-dependent proliferation of hepatocytes and cholangiocytes.^{104,105} However, because simple NRF2 stability and accumulation are insufficient to transform NRF2 from cellular defender to cancer driver, the occurrence of additional oncogenic mutations is required.^{106–108} *KEAP1* mutations paired with activating mutations of *KRAS/HRAS* and *TP53* loss of function are needed to establish NRF2-addicted cancer models.^{109–111} Furthermore, NRF2-dependent malignancies with somatic *KEAP1* or *NFE2L2* mutations differ depending on the specific tissue and species. For example, the mutations of *KRAS/KEAP1* in the human lung tissue induce tumors with aggressive proliferation,¹⁰⁹ whereas *KRAS/KEAP1* mutations in the mice pancreas cause fibrosis rather than malignancy.¹¹² As a result, tissue-specific variables are another factor likely to influence the requirements for developing NRF2-dependent cancer.

In therapy resistance, NRF2-regulated drug efflux transporters are significant predictors of therapy resistance in many tumors. Multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1-5 (MRP1-5), and breast cancer resistance protein (BCRP) are overexpressed as a result of abnormal NRF2 activation leading to widespread chemoresistance.^{113–117}

NRF2 Biology in the Brain

Brain Cellular Composition and NRF2 Expression

Quantifying the cellular makeup of the human brain is highly challenging because of the brain's huge size, cell composition, and limited access to human postmortem brain samples.¹¹⁸ In addition to approximately 100 billion neurons, glial cells (astrocytes, oligodendrocytes, and microglia) are present with a median of 0.85 glia-neuron ratio.^{119,120} In the brain, neutralization of ROS or electrophilic xenobiotics is usually mediated by the glutathione system, thioredoxin/peroxiredoxin system, superoxide dismutases, and catalase.^{121,122} It is interesting to note that the *NFE2L2* gene displays varying expression levels across different brain regions. It exhibits the highest expression primarily in the medulla oblongata, regulating hub of homeostatic functions of the nervous system, and basal ganglia, responsible for motor control, executive functions and emotions.¹²³ On the other hand, the hippocampus shows the lowest level of *NFE2L2* expression (Figure 1A). Similarly, the expression of the *NFE2L2* gene varies among different types of brain cells. It is most highly expressed in oligodendrocytes, while neurons exhibit the lowest level of expression (Figure 1B). Being a master regulator of antioxidant defenses, NRF2 exhibits distinct activities in the brain in addition to its cytoprotective effects.^{124,125} Herein, we will discuss the expression of NRF2 regarding brain biology and the function of different brain cells.

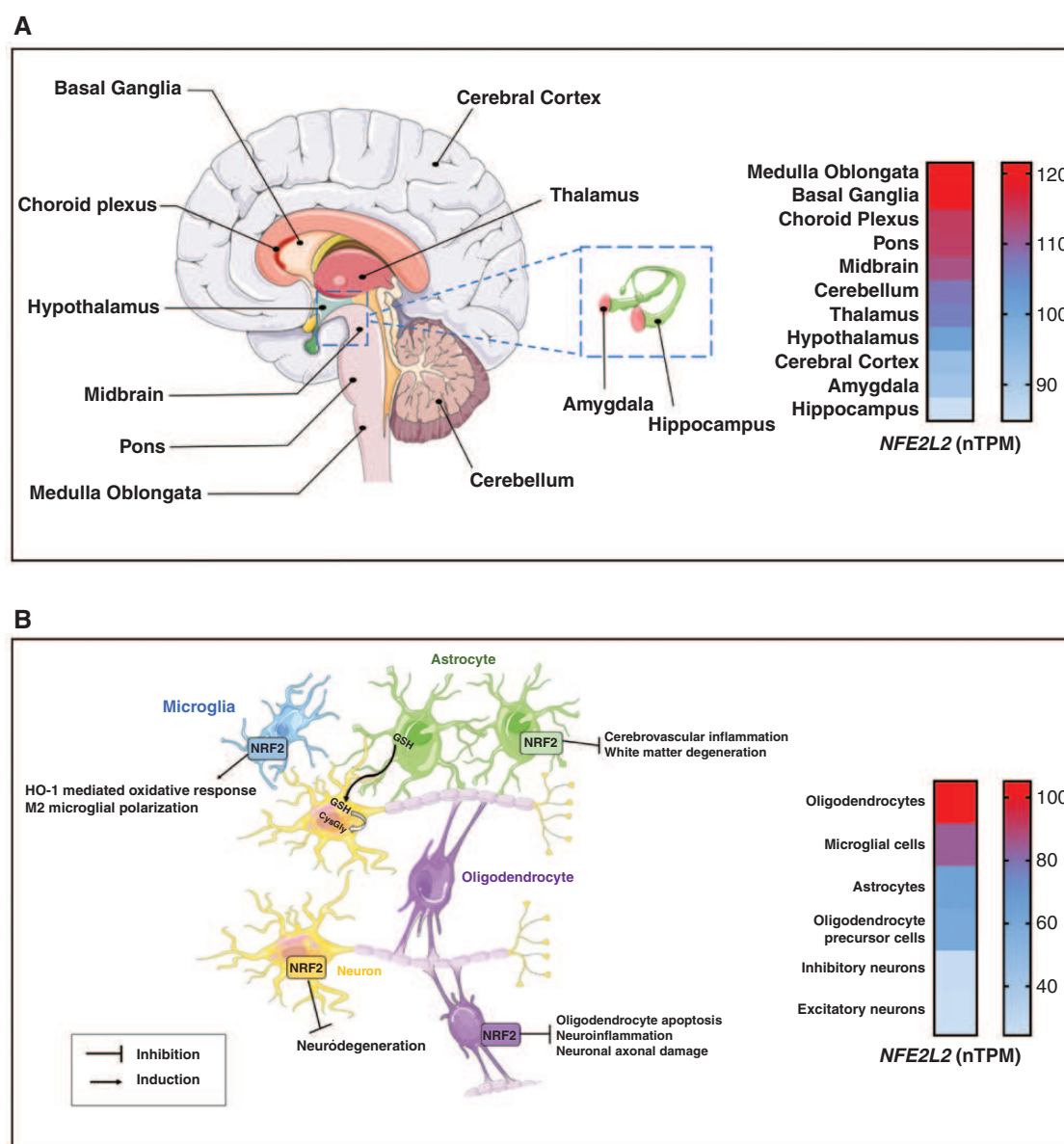


Figure 1. Overview of *NFE2L2* gene expression and the role of NRF2 in brain physiology. (A) Human brain regions are visually represented on the left side, while the accompanying heat map on the right side displays *NFE2L2* gene expression across the various human brain regions. The data were sourced from the human protein atlas (HPA) dataset, available at <https://www.proteinatlas.org/> from version 23.0, accessed from the following URL: <https://www.proteinatlas.org/ENSG00000116044-NFE2L2/brain>. (B) Graphical summary of NRF2's role in brain physiology among the different brain cells on the left side, while the accompanying heat map on the right side displays *NFE2L2* gene expression across the different brain cells. The data were sourced from the RNA single cell type data, available at <https://www.proteinatlas.org/about/download>. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. nTPM, normalized transcript per million; HO-1, heme oxygenase 1; CysGly, cysteinylglycine dipeptide; GSH, glutathione.

In the adult brain, *astrocytes* are the most abundant glial cell type.¹²⁶ Morphologically, protoplasmic astrocytes possess small irregular branching in a globoid distribution and are located in gray matter tissue, whereas fibrous astrocytes have numerous uniform cylindrical fibers and are broadly distributed across white matter tissue.^{127,128} In terms of function, astrocytes facilitate synaptic transmission and information processing, govern the migration

of growing axons and neurons, and connect with blood vessels.^{129,130} In addition, the proportion of astrocytes to neurons differs greatly between species and correlates with cognitive ability.¹³¹

Neurons are fundamental units of the brain and electrically excitable cells responsible for information processing and performing various functions within the brain.¹³² They are highly susceptible to oxidative stress mainly due to

their high reliance on oxidative phosphorylation for energy and enrichment in metal ions (catalyst for oxidative species formation), possess membranes rich in polyunsaturated fatty acids, and exhibit low levels of antioxidants.¹³³ The NRF2-ARE pathway in neurons is noticeably weak both in inhibitory and excitatory neurons (Figure 1B). Stimulation with tert-butylhydroquinone (tBHQ), an NRF2 activator, successfully induces the expression of NRF2 target genes in astrocytes, while no such induction is observed in cerebellar granule neurons.¹³⁴ The lower neuronal NRF2-ARE pathway activation is explained by the fact that basal NRF2 expression is lower in neurons, along with a greater Cul3-dependent NRF2 degradation capability than astrocytes.^{134–136} Also, hypo-expression of NRF2 in neurons results from epigenetic repression caused by NRF2 promoter hypo-acetylation compared to astrocytes.¹³⁴ Furthermore, maturing neurons require fewer antioxidant defenses to facilitate redox signaling involved in their development.^{137,138} Indeed, ectopic expression of NRF2 in neurons exerts a protective role against oxidative insults⁶⁶; however, it retards structural and electrophysiological maturation¹³⁴ and suppresses the activity of c-Jun N-terminal kinase (JNK) and Wnt signaling pathways required for neuronal development.^{139–142} On the other hand, astrocytes usually mature even when they express high amounts of NRF2, indicating that the signaling mechanisms involved in their maturation are less sensitive to the redox state.^{121,143} On the contrary, neurons that present repressed NRF2 expression for their maturation require astrocytic assistance to avoid oxidative damage.¹³⁴ Nearby astrocytes provide cysteine and/or glutathione to neurons, as well as other metabolites, to support neurons' activity.¹⁴⁴

Oligodendrocytes, another type of glial cell, provide structural support and a myelin coating around the neuronal axon to allow for a fast impulse transmission.¹⁴⁵ Evidence suggests that ROS drives the oligodendrocytes differentiation from precursors cells,¹⁴⁶ but oxidative stress is implied in demyelinating diseases.^{147,148} Similarly to neurons, oligodendrocytes receive antioxidant assistance from astrocytes.¹⁴⁹ Conversely, oxidative stress in oligodendrocytes activates an endoplasmic reticulum stress response in an NRF2-dependent manner in response to chemical hypoxia.¹⁵⁰ In the same context, oligodendrocyte apoptosis is more pronounced in addition to neuroinflammation and axonal damage in cuprizone-fed NRF2-deficient mice than in wild-type controls. Also, NRF2-deficient mice exhibited increased vulnerability to cuprizone-induced damage within the commissure anterior white matter tract, a region typically less affected by cuprizone in wild-type animals.¹⁵¹ However, NRF2 activation in oligodendrocytes in the context of other neurological disorders has yet to be thoroughly investigated.¹⁵²

Microglial cells are brain-resident immune cells¹⁵³ found in 5% of the cerebral cortex and up to 12% of the substantia nigra.¹⁵⁴ These cells are responsible for neuronal proliferation and differentiation, as well as removing debris and rebuilding synapses.¹⁵⁵ Microglia exhibit more NRF2 transcripts and ARE promoter activity than neurons in the brain,¹⁵⁶ indicating higher NRF2 expression than neurons. NRF2, which is actively produced by microglia in response to oxidative stress, promotes the activation of the M2-like pro-inflammatory microglial phenotype.¹⁵⁷ However, its

absence increases microgliosis, primarily characterized by the activation and proliferation of microglial cells. This absence also promotes the polarization of microglia towards an M1-like anti-inflammatory phenotype, which contributes to neuronal demise.¹⁵⁸ Knowing that glial activation associated with various neurodegenerative disorders,¹⁵⁹ NRF2-mediated modulation of microglial dynamics regulates neurodegeneration.¹⁶⁰ In contrast, microglia activation in reaction to atrazine-induced neuroinflammation boosts the production of inflammatory factors and inhibits the KEAP1/NRF2-ARE signaling cascade, resulting in increased dopaminergic neuron cell death and neurotoxicity.¹⁶¹ As a result, it appears prudent to conduct further research into the KEAP1/NRF2-ARE signaling pathway in microglia, as it may be a therapeutic target for NRF2 activation in neurodegenerative diseases. It is worth noting that astrocytes induce microglial NRF2 activation and the subsequent microglial HO-1 expression to decrease microglial intracellular ROS levels together with excessive microglial brain inflammation.¹⁶²

NRF2 in Neurological Diseases

Regarding human health, age-related NRF2 system impairment is a significant risk factor for almost all oxidative stress-related neurological diseases. Neurons are non-regenerative and postmitotic; therefore, significant oxidative damage should be avoided or reversed. Neuronal oxidative damage rises with age and is linked to neurodegenerative illnesses.^{151,152} Reduced NRF2 activity is related to both the development of chronic diseases like Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS), as well as increased susceptibility to acute insults like oxidative stress and chronic inflammation in the brain.¹⁶³ In the hippocampus, where neurodegeneration in AD begins, astrocytes from AD patients' brains have lower levels of NRF2.¹⁶³ NRF2 expression is decreased in the motor neurons of the spinal cord and cortex, as shown in the postmortem brains of ALS patients.¹⁶⁴ Supporting the evidence that the NRF2 system is dysfunctional in PD, olfactory neurosphere-derived cells from patients with sporadic PD express low GSH levels, which an NRF2 inducer agent could restore.¹⁶⁵ Hence, age-related reduction in NRF2 contributes to the development of neurodegenerative diseases and other age-related pathologies. Mainly, reduced neural stem cell (NSC) counts due to aging,¹⁶⁶ along with NSCs' clonogenic, proliferative, and differentiating capacities, are associated with NRF2 deficiency.¹⁶⁷ However, the transplantation of NSCs with high expression content of NRF2 lessens age-related declines in dentate gyrus stem cell regeneration.¹⁶⁸ Besides, ROS plays a role in regulating the fate of NSCs by inhibiting self-renewal and promoting differentiation through NRF2-mediated signaling.¹⁶⁹

Moreover, Dang et al. discussed NRF2 expression and its role in oxidative stress-related pathogenesis under acute ischemic stroke-like conditions.¹⁷⁰ Their results show that after the initiation of the stroke, NRF2 was not expressed in the core ischemic zone. However, its expression was elevated in the ischemic penumbra in both glial and neuronal cells. This suggests that NRF2 activation in

the penumbra results from enormous ROS generation owing to reoxygenation, whereas NRF2 activation in the undamaged cortical areas represents a preadaptation to oxidative stress. Surprisingly, compared to other cell types in the unaffected contralateral area, NRF2 expression was elevated in neurons. This phenomenon could also be attributed to the possible ROS independent-NRF2 activation in response to the growth factors, cyto- and chemokines, neurochemical mediators, and cross-hemispherical neural connections. Hence, NRF2 represents a therapeutic target that possesses a cytoprotective role in the brain after the initiation of injury.¹⁷⁰ The activation of endogenous NRF2 has been reported in oligodendrocytes in multiple sclerosis (MS)¹⁷¹; however, it is expressed in actively demyelinating lesions but not in late-stage active lesions.¹⁷² Moreover, in MS, reduced NRF2 expression is reported in oligodendrocytes compared to other central nervous systems (CNS) cell types, suggesting an impaired oxidative stress response.¹⁷³

NRF2 in Brain Metabolic and Mitochondrial Functions

Regarding mitochondrial bioenergetics, it has been shown that KEAP1-knockdown (KD) increases the glucose uptake in neurons and astrocytes compared to NRF2-KO and WT cells. Activation of NRF2 increases cytoplasmic NADPH and NADH levels in neurons and astrocytes; however, it favors energy production over antioxidant defense when glucose availability is limited in astrocytes.¹⁷⁴

In neurodegenerative diseases such as ALS, mutation of SOD1 produces motor neuron injury associated with NRF2 dysregulation coupled with reduced pentose phosphate pathway (PPP) activity and decreased generation of NADPH.¹⁷⁵ In PD, acute and chronic astrocyte exposure to dopamine enhanced PPP activity *via* the KEAP1/NRF2 system.¹⁷⁶ NRF2 eliminates oxidative stress in dopaminergic neurons by supplying NADPH to support the activity of NQO1, which is another target of NRF2.^{177,178} Moreover, NRF2-KO mice were rendered more sensitive to neurotoxicity caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, complex I inhibitor, in animal models of Parkinson's disease.¹⁷⁹

Moreover, knocking out NRF2 negatively affects the mitochondrial NADH redox index, which is the ratio between NADH consumption by complex I and its production in the TCA cycle. Also, a slower NADH and FADH₂ generation is obtained after the inhibition of complex IV in NRF2 mutant neurons.¹⁸⁰ NRF2 is also crucial to maintain mitochondrial integrity, particularly the mitochondria isolated from the brain of rats that were administered a single dose of isothiocyanate sulforaphane, an NRF2 activator, were resistant to the opening of the mitochondrial permeability transition pore.^{181,182}

Regarding mitochondrial biogenesis, treatment with the $\alpha 7$ acetylcholine nicotinic receptor (nAChR) agonist PNU282987 increases the mitochondrial mass and oxygen consumption in primary glial cultures without increasing oxidative stress. However, these results were abolished in the absence of NRF2. This result indicates that NRF2, through the stimulation of HO-1 or binding

with peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PCG-1 α), modulates glial mitochondrial mass.¹⁸³

However, it is important to highlight that NRF2 is an essential player in maintaining mitochondrial homeostasis and structural integrity via various mechanisms that are not exclusive to the brain but extend to various other tissues. Consequently, since oxidative stress, inflammation, and mitochondrial integrity contribute to the development of diseases, the pharmaceutical activation of NRF2 might be a key for both disease prevention and treatment.¹⁸⁴

NRF2 in Brain Cancer

Given the high oxygen consumption of the brain compared to other organs, the implication of oxidative stress in the development of brain tumors is of particular interest.¹⁸⁵ Primary brain tumors (PBT) grow from brain tissue and its surroundings and can be glial or non-glial. In this section, we will discuss the modulation of NRF2 in various types of PBTs and its potential therapeutic applications. We will also focus on glioblastoma, the most common type of glioma in adults, which has a very poor prognosis.

NRF2 in Pediatric Brain Tumors

After hematologic malignancies, CNS tumors are the second most common neoplasm in children.¹⁸⁶ Unfortunately, despite the extensive studies on the dual role of NRF2 in cancer, little is known regarding NRF2's function in most pediatric CNS malignancies. Among pediatric brain tumors, medulloblastomas (MB) are the most prevalent CNS embryonal tumor. MB, classified as a grade 4 cancer, comprises 4 subgroups: WNT, sonic hedgehog (SHH), Group 3, and Group 4; each is associated with different genetic alterations, age at onset, and prognosis.¹⁸⁷ When MB cases are compared to peritumoral control brain tissues, higher expression of NRF2 and HO-1 suggests that the NRF2/HO-1 pathway contributes to the progression of MB and hence might be a therapeutic target for the disease.¹⁸⁸ Others have shown that nifurtimox, an antiprotozoal compound, and tetrathiomolybdate, a copper chelator, act synergistically to induce oxidative stress and subsequent upregulation of NRF2 target genes, including HO-1, GCLM, solute carrier family 7 member 11 (SLC7A11), and SRXN1 in D2 and DAOY MB cell lines.¹⁸⁹ It is worth noting that although the drug combination effectively lowered medulloblastoma cell viability and triggered cellular death,¹⁸⁹ the rise in NRF2, which might exert a protumoral role, should be carefully assessed.

Peroxiredoxins (Prxs) are linked to cell apoptosis,¹⁹⁰ differentiation,¹⁹¹ and resistance to radiation or chemotherapy.^{192,193} In ependymomas, another type of pediatric brain cancer where a tumor arises from ependymal cells,¹⁹⁴ all Prxs (except Prx IV) are upregulated. However, Prx I expression is substantially related to the upregulated cytoplasmic and nuclear NRF2 expression, suggesting that NRF2 plays a role in Prx I production in ependymomas.¹⁹⁵ Additionally, there are no functional studies of NRF2 on

pilocytic astrocytoma, another frequent pediatric CNS cancer. It can likely play a minor role in the development of this tumor, given its low expression compared to higher WHO-grade gliomas. Therefore, the current evidence on the role of the NRF2 pathway in pediatric CNS tumors is limited, necessitating further investigation to enhance our understanding of its significance.

NRF2 in Adult Glioma

In 2021, the World Health Organization (WHO) published a new edition of the classification of tumors of the central nervous system, incorporating molecular and histological pathogenesis, to improve the diagnosis and determination of optimal treatment.¹⁹⁶ This classification separates pediatric and adult gliomas. Gliomas are the most prevalent type of adult brain tumor, comprising approximately 78% of malignant brain tumors. Three types of adult gliomas: oligodendrocytomas and astrocytomas which are isocitrate dehydrogenase (*IDH*) mutated and glioblastomas which are *IDH* wild type were classified.¹⁹⁶ Frequently, brain tumors are also classified according to the WHO grade from grade 1 to grade 4, with grade 1 being the least aggressive and grade 4 being the most aggressive.¹⁹⁷

Overall, in gliomas, the NRF2-KEAP1 pathway acts as a switch for malignancy, mainly through amplifying glutamate secretion and xCT augmentation.¹⁹⁸ Similarly, NRF2 overexpression or KEAP1 knockdown in glioma cells promotes proliferation and oncogenic transformation.¹⁹⁸ However, some discrepancies can be noted according to the type and grade of glioma, particularly regarding prognosis. Indeed, in contrast to other types of cancer, there are relatively few studies that have explored the relationship between NRF2 expression and brain cancer prognosis. NRF2 overexpression is shown to be positively correlated with WHO grades in gliomas.¹⁹⁹ *In silico* analysis, using the Rembrandt glioma dataset, shows that the upregulated *NFE2L2* RNA expression levels are associated with the poor prognosis in grade 2-4 gliomas.²⁰⁰

***IDH*-mutant glioma: oligodendrocytomas and astrocytomas.**—It is well-established that *IDH*-mutated tumors generally have a more favorable disease outcome and give rise to low-grade gliomas.²⁰¹ Somatic mutation in *IDH1*, and less commonly in *IDH2*, are considered as early events. Next, during glioma development, additional subclonal mutations are added leading to higher-grade *IDH*-mutant gliomas. For instance, oligodendrocytoma, arising from oligodendroglial precursors, is classified as grades 2 or 3 while astrocytoma, arising from astrocytic precursors, can be found as grades 2, 3, or 4.¹⁹⁶

Examining the NRF2 pathway in the context of *IDH* mutations, in gliomas with mutated *IDH1/2*, the expression levels of NRF2 target genes, *NQO1* and *GCLM*, were notably elevated and were significantly linked to poorer patient survival, whereas the expression of NRF2 itself did not exhibit such an association.²⁰² However, in primary astrocytomas, an increase in both cytoplasmic and nuclear expression of NRF2, as well as nuclear DJI, a multifunctional protein involved in oxidative stress response,

is associated with *IDH1* mutation.²⁰⁰ These results suggest that the association between NRF2 expression and *IDH* mutation depends on the *IDH*-mutated glioma type but more studies are needed. Interestingly, it has been shown that *IDH1*-mutated cells develop a dependency on the NRF2 antioxidant pathways and, therefore, using NRF2 inhibitors, such as brusatol, suppresses cancer progression.²⁰³

Glioblastomas.—Glioblastoma (GB), classified as grade 4 *IDH1* wild-type glioma, is the most prevalent primary brain tumor with a median survival rate of 15 months^{204–206} and a median age of detection of 65 years.²⁰⁷ GB is detected in the forebrain almost exclusively but may develop in the brain stem, cerebellum, and spinal cord.^{205,208} Despite the therapeutic options, such as surgery with maximal safe resection followed by concurrent radiotherapy and temozolomide (TMZ) and 6-monthly rounds of adjuvant TMZ, recurrent GB management remains a problem with limited treatment options.^{209,210}

NRF2 oncogenic activity has been more studied in GB than in other glial tumors and has recently been reviewed.²¹¹ Evidence shows that knocking down NRF2 attenuates tumor growth by inhibiting cell proliferation, increasing cell apoptosis, and suppressing angiogenesis.^{113,212} Also, the NRF2 pathway is shown to be activated by a positive feedback loop involving p62/SQSTM1, a stress-inducible and multifunctional protein, whereas NRF2 and p62 enhance proliferation, invasion, and mesenchymal transition in GB.²¹³ Finally, NRF2 overexpression partly reversed the ERK and PI3K inhibitor-induced reduction of human GB cell viability,²¹⁴ suggesting that signaling cascades for NRF2 activation may offer new treatments for glioblastoma.

NRF2 Expression in GB Prognosis

It is now widely accepted that NRF2 expression is higher in GB than in normal brain tissue or other types of brain cancer. However, the relationship between NRF2 expression and GB patient survival is still controversial due to conflicting results in published studies, noting that most studies are *in silico* analyses using available databases.

On one side, studies have shown that high NRF2 expression is associated with lowered survival in GB patients. For example, Fan et al. have demonstrated that GB tissues exhibit a significant elevation in *NFE2L2* mRNA expression compared to normal brain tissue samples using the OncoPrint database. Moreover, using the Rembrandt database, they showed that patients with *NFE2L2* expression upregulated by 2-folds or more had significantly poorer overall survival rates compared to those with lower NRF2 expression profiles.¹⁹⁸ Another example is using the SurvExpress tool and the data from 538 GB patients, higher expression of the *NFE2L2* gene and related genes were associated with higher risk for the patient.²¹⁵ In an interesting study, the TCGA GBM prognostic clinical data (520 cases) were stratified by the NRF2 activity status. The authors found no difference in the overall survival of patients with high NRF2 activity but the progression-free survival was strongly decreased.²¹³ However, contradictory studies can be highlighted. For example, *NFE2L2* expression was

not associated with overall survival in GB patients in the Rembrandt database, and an IHC analysis done on 213 GB patients further revealed that nuclear NRF2 expression was a predictor of better survival.²⁰⁰ In another study based on a cohort of 52 GB patients, the expression of 2 NRF2 target genes, *NQO1* and *GCLM*, was not associated with progression-free or overall survival.²⁰² To compare with the existing literature, we analyzed another database, the GEPIA2 database,²¹⁶ and found that *NFE2L2* gene expression was elevated in GB tumors compared to normal tissue (Figure 2A). However, the variation in the overall survival or disease-free survival rates among GB patients with low or high *NFE2L2* gene expression did not achieve statistical significance (Figure 2B-C).

Moreover, GB is classified into subtypes: mesenchymal, classical, proneural, and G-CIMP.^{217,218} The high invasiveness of the mesenchymal subtype is indicated by recurrence and worst survival rates compared to others.^{213,219} The overexpression of *NFE2L2* has been reported in the mesenchymal subtype of GB tumors.²¹³ In our *in silico* analysis, we observed that *NFE2L2* gene expression is significantly elevated not only in the mesenchymal but also in the classical subtype of GB, which is not the case with the proneural subtype compared to normal tissue (Figure 2D).

In light of these findings, it is reasonable to conclude that the divergence observed in the context of NRF2 and GB patients' survival among the different database tools may be attributed to the limitations inherent in the database's methodology, sample size, and selection criteria used. It is important to clarify the link between NRF2 expression and GB prognosis by using cohort patient tissues associated with clinicobiological data. In addition, NRF2 activity is regulated by numerous post-transcriptional and post-translational modifications. Therefore, it is crucial to correlate patients' prognosis with NRF2 protein level expression and its sublocalization since nuclear localization is associated with its activity.

NRF2 in Glioblastoma Stem Cells

The tumorigenic potential of glioblastoma stem cells (GSCs) in GB owes to the progression and therapeutic resistance to chemotherapy and radiation.^{220,221} GSCs constitute a small fraction of the tumor bulk. Yet, they possess high self-renewal capacity, allowing them to sustain tumor growth, neurosphere forming capacity, and therapeutic resistance.²²¹ In GB, under hypoxic conditions, increased necrosis favors the maintenance of GSCs responsible for the tumor's initiation, resistance, and recurrence.^{222,223}

Despite the limited studies conducted on the role of NRF2 in GSC, NRF2 has been shown not only to maintain the self-renewal capacity of GSCs despite the anti-cancer treatment²²⁴ but also to enhance neurosphere proliferation in NSCs.²²⁵ Interestingly, differential NRF2 expression exists between glioma stem cells and non-stem-like cells. For instance, NRF2 is overexpressed in CD133+ GSCs compared to CD133- GB cells,²²⁶ and downregulation of NRF2 improves GSC differentiation as it lowers the number of sphere-like colonies.²²⁷ Also, knocking down NRF2 in GSCs using RNA interference technology resulted in decreased expression of pluripotency-associated transcription factors,

increased expression of markers associated with astrocyte development, caused a significant reduction in S-phase cells, reduced expression of SRY-box transcription factor 2 (SOX2), B-cell-specific moloney murine leukemia virus integration site 1 (BMI-1), and Cyclin E proteins responsible for cell self-renewal.²²⁸ Furthermore, the transcriptional coactivator with PDZ-binding motif (TAZ)-dependent growth, encoded by the gene *WWTR1*, is a crucial element of the Hippo signaling pathway, which regulates the development and stemness in multiple human cancers through the yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) coactivators of the TEA domain (TEAD) transcription factors 1-4.²²⁹ Interestingly, the upregulation of NRF2 induces the expression of TAZ, which acts as an effector of NRF2-induced tumorigenicity in GBs. TAZ ectopic expression also rescues neurosphere growth of NRF2-KD glioma stem cells and, along with NRF2 expression, accelerates GB tumor formation.²³⁰

Cluster of differentiation 90 (CD90), cluster of differentiation 15 (CD15), A2B5, aldehyde dehydrogenase 1 (ALDH1), nestin, and ATP-binding cassette (ABC) transporters are frequently recognized as markers of GSCs.²³¹⁻²³³ These markers help elucidate the tumorigenic process and serve as an effective diagnostic and therapeutic tool for GB. However, the precise mechanisms and functions of these putative markers have not yet been fully clarified. Therefore, identifying various biomarkers rather than just one marker and their correlation with NRF2 expression in the context of GB stem cell self-renewal capacity and maintenance may enable tailored targeting of GSC treatments and further tumor relapse.

NRF2 in GB Metabolism

The role of NRF2 in GB metabolism still needs to be fully elucidated, and a comprehensive understanding of its specific mechanisms and implications in GB metabolism necessitates further investigation. The NRF2-driven human telomerase reverse transcriptase (hTERT) loop mediates the NRF2-PPP regulation. Mainly, hTERT knockdown abrogated the NRF2 level, while overexpression of NRF2 increased hTERT expression. GB patient tumors bearing hTERT promoter mutations associated with increased telomerase activity had an increased NRF2 and transketolase (TKT) expression and decreased glycogen accumulation. Overexpression of NRF2 rescued the Costunolide, a telomerase inhibitor, mediated decrease in G6PD and TKT levels, while the inhibition of hTERT abolished not only the expression of G6PD and TKT but also the phosphorylation of glycogen synthase (GS) and increased glycogen accumulation.²³⁴ The physical interaction of cytochrome B-245 beta chain (CYBB), a major catalytic subunit of NADPH oxidase (NOX) with NRF2, allows for the promotion of a mesenchymal GB phenotype, increased cancer stemness, and the development of resistance in GB.

NRF2 in Therapeutic Resistance

In GB, methylation of the O6-methylguanine-DNA methyltransferase (MGMT) promoter has been demonstrated to predict responsiveness to alkylating drugs such

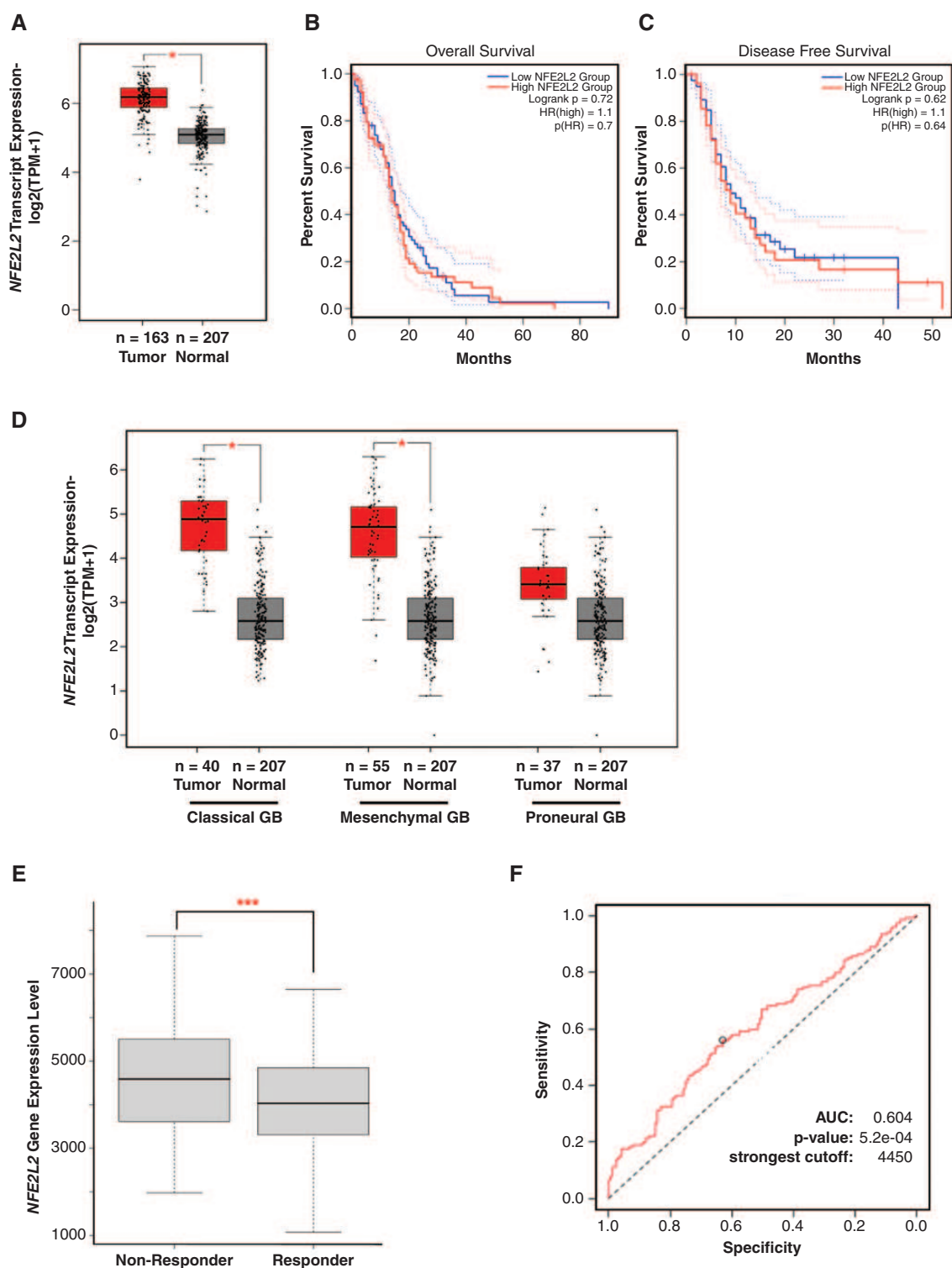


Figure 2. *NFE2L2* gene expression levels in GB and the impact on the clinical outcome. (A) Tissue-wise expression profile of the *NFE2L2* gene expression in GB tumors compared to normal tissue. Data is sourced from GEPIA2 for GB patient databases. (B) Kaplan–Meier survival curves of overall survival and (C) disease-free survival of patients with GB based on the high (red) and low (blue) expression of the *NFE2L2* gene, respectively. (D) Tissue-wise expression profile of *NFE2L2* gene expression in GB subtypes compared to normal tissues. Data is sourced from GEPIA2 for GB patient databases. (E) ROC plotter showing the *NFE2L2* gene expression in patients classified as responders (165 patients) and nonresponders (154 patients) to TMZ treatment (P -value = .0013). (F) ROC curve analysis shows the validity of *NFE2L2* gene expression in discriminating responders and nonresponders, with the sensitivity representing the true positive rate and the specificity representing the false positive rate. Data is sourced from ROC Plotter—Online ROC analysis for GB patient data. The red star denotes statistical significance. AUC, area under the curve; TPM, transcripts per million reads; n, number of tissue samples; HR, hazards ratio; TMZ, temozolomide.

as TMZ, which has become a cornerstone of GB treatment.²³⁵ Mechanistically, at physiological pH, TMZ is activated to produce methyl diazonium ions with methyl groups, which are transported to DNA at the N7 position of guanine, O3 position of adenine, and O6 position of guanine,^{235–237} resulting in numerous DNA adducts and the formation of single- and double-stranded DNA breaks, ultimately causing cell cytotoxicity.²³⁷ However, because of broad TMZ exposure and the very heterogeneous and mutation-prone character of GB, it is quite usual for these deadly tumors to develop TMZ resistance. Unfortunately, over half of GB patients treated with TMZ do not respond to the medication.²³⁷ As a result, TMZ resistance is a significant challenge that must be overcome for the effective treatment of GB.

A recent study has revealed, using a CRISPR activation library, that the NRF2 pathway is involved in TMZ resistance.²³⁸ Moreover, inhibiting the NRF2/ARE pathway sensitizes GB cells to TMZ treatment,²³⁹ implying that targeting NRF2 activation could be a promising strategy to enhance chemoradiation sensitivity in GB. In response to the treatment with TMZ coupled with the suppression of NRF2, the RAS/RAF/MEK signaling pathway was inhibited, leading to a decrease in the proliferation of U251 glioma cells. In addition, the subsequent downregulated HO-1, GSH, TRX, and other oxidative enzymes, along with the elevated Keap1 levels, inhibited the anti-oxidative stress mechanism in glioma cells.²⁴⁰ Three-dimensional tumor models such as spheroid and organoid systems confer an advantage over other culturing methods by mimicking the in vivo characteristics of CNS malignancies.²⁴¹ Knowing that TMZ induces DNA damage, the DNA repair pathways, including O6-methylguanine-DNA methyltransferase (O6-MGMT), base excision repair, and mismatch repair, are implicated in TMZ resistance and other identified mechanisms.^{237,242,243} In an elegant study, Rocha et al. highlighted essential mechanisms involved in TMZ resistance.²⁴³ Briefly, TMZ therapy increases ROS production, which causes NRF2 to be activated, resulting in increased expression of 2 glutathione (GSH) synthesis enzymes, GCLM and glutamate-cysteine ligase, catalytic subunit (GCLC). Consequently, increased GSH availability mediates TMZ resistance by maintaining cancer cells' low ROS content and subsequent reduction of TMZ cytotoxicity.²⁴³ However, GSH depletion mimicked by L-buthionine [S, R]-sulfoximine (BSO) in glioma cells is responsible for overcoming TMZ drug resistance.²⁴³ In a similar context, increased NRF2 expression improves ferroptosis sensitivity in TMZ-resistant GB by increasing the expression of its pro-ferroptosis target ATP-binding cassette sub-family C member 1 (ABCC1), which contributes to GSH depletion. Thus, inducing ferroptosis could be a proper therapeutic method for reversing drug resistance in gliomas with high NRF2 and ABCC1 expression.²⁴⁴ The activation of NRF2 and its downstream target, SOD2, prevented ferroptosis and excessive production of ROS. In contrast, inhibiting SOD2, combined with tolerable ferroptosis-inducing agents like erastin, sensitizes GB cells, overcoming TMZ resistance in mesenchymal GB.²⁴⁵ However, further research is needed to confirm the effectiveness of the disruption of the NRF2/SOD2 antioxidant circuitry approach in developing GB therapeutic strategies.

Moreover, knocking down the *NRF2* gene in glioma neurospheres followed by gamma rays' irradiation resulted in less self-renewal, more differentiated cells, and less proliferative potential.²⁴⁶ Consequently, this suggests that NRF2 suppression enhances cellular sensitivity to radiation-induced oxidative stress. In comparison, a compelling association between *NFE2L2* gene expression and patient response to TMZ is demonstrated using the receiver operating characteristic (ROC) analysis for GB patient database²⁴⁷ (Figure 2E), in addition to the fact that *NFE2L2* gene seems to exhibit a predictive power and of potential clinical utility (Figure 2F). Overall, evidence suggests that NRF2 is a crucial player to be employed in therapeutic strategies involved in GB-TMZ resistance.

Conclusions

This review highlights the pivotal role of cellular redox homeostasis within the intricate landscape of cancer biology. The delicate interplay between reactive oxygen species (ROS), antioxidants, and diverse cellular processes is central to comprehending cancer's genesis, progression, and therapeutic interventions. Moreover, NRF2 emerges as a master regulator, orchestrating an extensive array of cytoprotective genes to maintain redox equilibrium and ensure proper cellular function.

However, NRF2 exhibits a dual role in cancer. It acts as a guardian by preserving redox homeostasis and serving as an anti-inflammatory mediator while simultaneously harboring the potential to fuel cancer growth, drug resistance, metabolic adaptations, and the activation of various oncogenes. Understanding the context-dependent nature of NRF2's actions in cancer is pivotal for developing precise and efficient cancer therapies, thereby shedding light on the intricate landscape of cancer biology.

Within the human brain, NRF2 exhibits diverse expression patterns among different brain cell types, including astrocytes, microglia, oligodendrocytes, and neurons. Its activation is critical in maintaining redox homeostasis, executing distinct functions in neurons and astrocytes, thereby preserving brain health. NRF2's involvement extends to preserving brain mitochondrial function and integrity, offering promising prospects for interventions in brain health maintenance.

Finally, NRF2 in cancer prognosis is a subject of significant interest, yet more studies are needed to explain the intricate relationship between NRF2 expression and brain cancer prognosis, considering various tumor types, grades, and characteristics. In glioblastoma, NRF2 emerges as a prominent player, significantly influencing malignancy, oncogenic transformation, and the development of therapeutic resistance. Noteworthy is NRF2's role in the maintenance of GSCs, which contributes to temozolomide resistance and tumor recurrence. Nonetheless, there remains a need for a comprehensive understanding of the molecular mechanisms underlying NRF2-mediated GSC maintenance and the metabolic pathways implicated in glioblastoma.

Overall, these discoveries highlight how NRF2 is involved in many aspects of cancer and various cell functions. This

knowledge sets a solid basis for further research and the development of precisely targeted therapies, including NRF2 silencing approaches, within the domains of cancer biology and brain health.

Keywords

Brain physiology | NRF2 | glioblastoma stem cells | oxidative stress | therapeutic resistance

Funding

M.M.M. PhD scholarship is financed by the French Research Ministry. Our research is granted by the “Cancéropôle Grand Sud-Ouest,” the “Association pour la Recherche sur les Tumeurs Cérébrales” and the “Ligue contre le Cancer.”

Conflict of interest

None declared.

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Summary of the Study in French

Introduction : Le facteur nucléaire érythroïde 2 lié au facteur 2 (NRF2) est un facteur de transcription crucial régulant les réponses antioxydantes cellulaires. NRF2 joue un rôle double à la fois dans la santé et la maladie, en particulier dans le contexte du stress oxydatif et de la biologie du cancer. Le stress oxydatif, résultant d'un déséquilibre entre la production d'espèces réactives de l'oxygène (ROS) et les défenses antioxydantes, entraîne des dommages cellulaires et contribue à diverses pathologies, dont le cancer, les maladies cardiovasculaires et neurodégénératives. NRF2, encodé par le gène *NFE2L2*, contrôle l'expression des gènes impliqués dans la détoxification, les défenses antioxydantes et l'homéostasie redox cellulaire. La voie NRF2/ARE, bien qu'elle soit protectrice lors des étapes précoces de cancérogenèse, peut paradoxalement promouvoir la progression tumorale et la résistance aux thérapies dans les cancers établis.

Le cerveau humain, avec sa forte activité métabolique et sa dépendance à la phosphorylation oxydative, est particulièrement vulnérable au stress oxydatif. NRF2 joue un rôle vital dans le maintien de l'homéostasie cérébrale, influençant différents types cellulaires comme les astrocytes, les neurones, la microglie et les oligodendrocytes. Lors du vieillissement ou dans le contexte de maladies neurodégénératives telles que la maladie d'Alzheimer, de Parkinson, de Huntington, et la sclérose latérale amyotrophique, la diminution de l'activité de NRF2 est corrélée à la progression de la maladie. L'importance de la voie NRF2 s'étend également aux tumeurs cérébrales primaires, telles que les gliomes, où son expression influence l'agressivité et la progression tumorale. Le double rôle de NRF2 dans les maladies neurodégénératives et les tumeurs cérébrales souligne son potentiel en tant que cible thérapeutique, bien que sa participation complexe nécessite une modulation soignée dans les stratégies de traitement. Le glioblastome (GB), une tumeur cérébrale de grade IV particulièrement agressive, illustre les défis posés par NRF2 dans le cancer. Le GB se caractérise par une expression élevée de NRF2, qui soutient la survie tumorale, la croissance et la résistance aux thérapies conventionnelles telles que la chimiothérapie et la radiothérapie. NRF2 influence des voies clés, y compris celles impliquées dans le métabolisme, l'invasion cellulaire et le maintien des cellules souches du glioblastome (GSCs). Le rôle de cette voie dans la chimiorésistance, notamment contre le témozolomide (TMZ), et son impact sur la survie des patients, mettent en évidence NRF2 comme un facteur critique dans la pathologie du GB. La valeur pronostique mitigée de NRF2

chez les patients atteints de GB, certaines études indiquant des résultats moins favorables associés à des niveaux élevés de NRF2, souligne la nécessité de recherches supplémentaires pour clarifier son rôle et son potentiel thérapeutique.

Objectif : Cette étude comble des lacunes cruciales dans nos connaissances concernant le rôle de NRF2 dans un modèle de GB cliniquement pertinent en utilisant des modèles 3D de GB de type sauvage et avec inactivation de NRF2 via CRISPR-Cas9. Elle vise à élucider le rôle de NRF2 dans la progression du GB, les propriétés souches, et les altérations du profil métabolique, offrant des avantages par rapport aux modèles traditionnels et fournissant une représentation plus fidèle des résultats cliniques. Finalement, ces résultats pourraient améliorer notre compréhension des implications de NRF2 dans le développement et la progression du GB.

Résultats : Notre étude a examiné le rôle de NRF2 dans la progression du GB en utilisant des cellules P3 dérivées de patients atteints de GB et un modèle de sphères 3D, fournissant de nouvelles perspectives sur la manière dont NRF2 influence la dynamique tumorale. Nous avons constaté que l'inactivation de NRF2 n'affectait pas significativement la croissance des sphères *in vitro*, suggérant que NRF2 pourrait ne pas être essentiel pour la prolifération de base dans les conditions contrôlées de notre modèle. Cependant, lorsque évalué *in vivo*, le rôle de NRF2 est devenu plus nuancé, avec des effets distincts sur la croissance tumorale en fonction du clone.

Plus précisément, les tumeurs C7 KO ont montré une croissance accrue, suggérant un rôle pro-tumoral de NRF2, tandis que les tumeurs C16 KO ont montré une croissance réduite, indiquant un rôle anti-tumoral. Ces résultats suggèrent que NRF2 pourrait jouer différents rôles dans la progression du GB. Le profil génétique et épigénétique de chaque clone semble déterminer si NRF2 agit comme promoteur ou suppresseur de la croissance tumorale, soulignant la nécessité d'une compréhension plus nuancée des mécanismes moléculaires spécifiques et des voies par lesquelles NRF2 interagit avec le microenvironnement tumoral et régule les processus cellulaires dans les deux clones. De plus, il a été constaté que NRF2 est crucial pour l'invasion des cellules de GB *in vitro*, son inactivation réduisant significativement la capacité invasive des cellules P3. Cela concorde avec la littérature qui identifie NRF2 comme un moteur clé de l'invasion dans le GB. Cependant, ces effets étaient moins prononcés *in vivo*, soulignant la complexité du microenvironnement tumoral et son influence sur l'activité de NRF2.

Nos résultats ont montré que l'inactivation de NRF2 altérait la respiration cellulaire dans les cellules C7 mais pas dans les cellules C16, indiquant des différences intrinsèques entre les clones dans leur réponse à l'absence de NRF2. Fait intéressant, la surexpression de NRF2 dans les cellules contrôles a altéré la respiration cellulaire, suggérant qu'une activité excessive de NRF2 peut être nuisible à la fonction cellulaire. Ce résultat suggère que le rôle de NRF2 dans la respiration cellulaire est finement équilibré, une activité insuffisante ou excessive de NRF2 pouvant potentiellement entraîner un dysfonctionnement métabolique. Malgré ces effets sur la respiration, l'inactivation de NRF2 n'a pas significativement altéré le potentiel de membrane mitochondrial, ni la masse, ce qui indique que la fonction mitochondriale reste en grande partie intacte en l'absence de NRF2. Cela suggère que bien que NRF2 influence certains aspects de la respiration cellulaire, il pourrait ne pas être essentiel pour maintenir l'intégrité structurelle globale des mitochondries. Cependant, une activation excessive de NRF2 a réduit la biogenèse mitochondriale, soulignant l'importance d'une activité de NRF2 finement régulée pour maintenir la santé mitochondriale. Une suractivation de NRF2 peut perturber l'équilibre délicat nécessaire au maintien de la biogenèse mitochondriale appropriée, pouvant conduire à une santé et une fonction mitochondriale compromises.

En termes de métabolisme, l'inactivation de NRF2 a conduit à un virage significatif vers la glycolyse, plus prononcé dans le clone C16 KO, comme en témoignent l'augmentation des niveaux de lactate intracellulaire et la surexpression de LDHA, une enzyme glycolytique clé responsable de la production de lactate. De plus, l'absence de NRF2 entraîne une réduction de LDHB, indiquant une diminution de la conversion du lactate en pyruvate dans les deux clones KO. Ce changement est en accord avec l'effet Warburg observé dans le GB, où les cellules cancéreuses utilisent préférentiellement la glycolyse pour la production d'énergie, même en présence d'oxygène. En favorisant la glycolyse, la perte de NRF2 pousse les cellules vers un phénotype plus glycolytique, ce qui est associé à une synthèse accrue des nucléotides adénosine et à une disponibilité accrue de l'ATP. En plus de ce virage vers la glycolyse, l'inactivation de NRF2 a également entraîné une accumulation accrue de lipides, potentiellement due à l'accumulation de lactate dans les cellules C16 et à une mobilisation réduite des lipides vers les mitochondries, en particulier par l'expression diminuée de CPT1A dans les cellules C7 KO. L'accumulation de lipides suggère qu'en l'absence de NRF2, les cellules peuvent avoir du mal à utiliser efficacement les lipides pour l'énergie nécessaire au soutien de leur capacité invasive, ce qui entraîne plutôt le stockage de ces lipides. Cependant, des

recherches supplémentaires soient nécessaires pour explorer les gènes cibles de NRF2 et les mécanismes moléculaires spécifiques qui sous-tendent ces changements métaboliques.

Contrairement à la littérature existante, notre étude a révélé que l'inactivation de NRF2 n'affectait pas significativement la sensibilité des cellules P3 au témozolomide (TMZ), un agent chimiothérapeutique standard pour le GB. Cependant, l'inactivation de NRF2 a conduit à une augmentation de l'expression des marqueurs souches SOX2 et OLIG2, suggérant un rôle potentiel de NRF2 dans la régulation des propriétés souches des cellules de GB. Malgré cette augmentation des marqueurs souches, il n'y a pas eu d'augmentation correspondante de la capacité de formation de sphères, ce qui indique que la relation entre NRF2 et les propriétés souches dans le GB est plus complexe qu'une simple association directe. Cela suggère que des mécanismes de régulation supplémentaires, peut-être au niveau post-traductionnel, pourraient être impliqués et devraient être évalués pour mieux comprendre comment NRF2 influence les propriétés souches dans les cellules de GB.

Bien que nos résultats offrent des perspectives précieuses sur le rôle de NRF2 dans le GB, plusieurs limitations doivent être prises en compte. Premièrement, la nature *in vitro* de nombreuses expériences peut ne pas capturer pleinement la complexité du microenvironnement tumoral *in vivo*, où les interactions avec divers composants cellulaires et moléculaires sont essentielles. De plus, la variabilité observée entre les deux clones KO pour NRF2 indique que des différences génétiques et épigénétiques pourraient influencer la fonction de NRF2, nécessitant des recherches supplémentaires permettant la caractérisation de ces clones notamment par séquençage de l'ARN. La dépendance à un seul modèle de GB dérivé de patients pourrait également limiter l'applicabilité de ces résultats à d'autres cas de GB ayant des arrière-plans génétiques différents. Enfin, bien que nous ayons observé des altérations significatives du métabolisme et de l'invasion dues à la modulation de NRF2, les mécanismes moléculaires exacts qui sous-tendent ces changements restent à élucider, en particulier dans les modèles *in vivo* où le rôle du microenvironnement est plus prononcé.

Conclusion : Notre étude met en évidence le rôle de NRF2 dans le GB, en particulier dans la régulation de la progression tumorale, de la reprogrammation métabolique et de la résistance aux thérapies. Bien que l'inactivation de NRF2 n'ait pas affecté significativement la croissance des sphères de GB *in vitro*, son impact s'est révélé plus nuancé *in vivo*, révélant à la fois des effets pro-tumoraux et anti-tumoraux en fonction du clone KO pour NRF2 utilisé. De plus,

NRF2 semble être crucial pour l'invasion des cellules de GB *in vitro*, ainsi que pour la reprogrammation métabolique, où son absence favorise la glycolyse et altère le métabolisme lipidique. De plus, l'étude met en évidence des différences par rapport aux résultats précédents concernant le rôle de NRF2 dans la chimiorésistance, notamment son impact limité sur la sensibilité au TMZ dans les cellules de GB. Cependant, l'augmentation de l'expression des marqueurs de souches dans les cellules déficientes en NRF2 suggère une interaction plus complexe entre NRF2 et le maintien des propriétés souches dans le GB, qui nécessite une exploration plus approfondie. Dans l'ensemble, cette étude fournit une compréhension fondamentale des fonctions diversifiées de NRF2 dans le GB, ouvrant la voie à des recherches futures visant à optimiser les thérapies ciblant NRF2 pour inhiber la progression du GB.