



# C/EBP $\beta$ mediates NQO1 and GSTP1 anti-oxidative reductases expression in glioblastoma, promoting brain tumor proliferation

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## ABSTRACT

Glioblastoma (GBM) is the most common and most aggressive brain tumor, associated with high levels of reactive oxidative species (ROS) due to metabolic and signaling aberrations. High ROS levels are detrimental to cells, but it remains incompletely understood how cancer cells cope with the adverse effects. Here we show that C/EBP $\beta$ , a ROS responsive transcription factor, regulates the transcription of NQO1 and GSTP1, two anti-oxidative reductases, which neutralize ROS in the GBM and mediates their proliferation. C/EBP $\beta$  is upregulated in EGFR overexpressed GBM cells, inversely correlated with the survival rates of brain tumor patients. Interestingly, C/EBP $\beta$  binds the promoters of NQO1 and GSTP1 and escalates their expression. Overexpression of C/EBP $\beta$  selectively decreases the ROS in EGFR-overexpressed U87MG cells and promotes cell proliferation via upregulating NQO1 and GSTP1; whereas knocking down C/EBP $\beta$  elevates the ROS and reduces proliferation by repressing the reductases. Accordingly, C/EBP $\beta$  mediates the brain tumor growth *in vivo*, coupling with NQO1 and GSTP1 expression and ROS levels. Hence, C/EBP $\beta$  regulates the expression of antioxidative reductases and balances the ROS, promoting brain tumor proliferation.

## 1. Introduction

Cancer cells are characterized by increased aerobic glycolysis (termed the Warburg effect) and high levels of oxidative stress [1]. This oxidative stress is exerted by reactive oxygen species (ROS) that accumulate as a result of an imbalance between ROS generation and elimination. The high ROS levels in cancer cells are a consequence of alterations in several signaling pathways that affect cellular metabolism. Many signaling pathways that are linked to tumorigenesis can also regulate the metabolism of ROS through direct or indirect mechanisms [2]. Glioblastoma (GBM) is the most malignant human brain tumor, which is highly aggressive, infiltrative, and destructive. Approximately 40% of glioblastomas show amplification of the EGFR gene locus [3] and about half of these tumors express a mutant receptor (EGFRvIII) that is constitutively active due to an in-frame deletion of exons 2 to 7 within the extracellular ligand-binding domain [4,5]. Despite tremendous efforts in recent years, effective therapies based on GBM common mutations and alterations have been disappointing [6,7]. The EGFR is a receptor tyrosine kinase that regulates fundamental processes

of cell growth and differentiation. In response to ligation, EGFR forms homodimers and heterodimers activating several intracellular signal pathways, such as phosphatidylinositol 3'-kinase (PI3K)/Akt and Ras/mitogen-activated protein kinase (MAPK), leading to increase in DNA synthesis [8]. Primary GBMs usually possess EGFR amplification, PTEN mutation, and loss of chromosome 10, while TP53 mutations are common in secondary GBM, unlike the primary types [9,10]. These mutations affect the redox balance in the tumor environment. For instance, ligation of EGFR by EGF induces endogenous production of intracellular reactive oxygen species (ROS) and H<sub>2</sub>O<sub>2</sub> in cancer cell lines [8,11]. Also, high levels of H<sub>2</sub>O<sub>2</sub> (200 pM) significantly increase the Tyr autophosphorylation by EGFR, resulting in the generation of ROS [8]. EGFRvIII overexpression in glioblastoma cells causes increased levels of ROS, DNA strand break accumulation, and genome instability [12,13].

Cancer cells adapt to the imbalanced redox status created by their rapid growth and other conditions, such as oxygen and limited availability of nutrients, by developing alternative metabolic reactions that render them insensitive to further stress inducers such as chemotherapy

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## Abbreviations

GBM	Glioblastoma
ROS	Reactive oxidative species
EGFR	Epidermal growth factor receptor
PTEN	Phosphatase and tensin homolog
PI3K	Phosphoinositide 3-kinase
MAPK	Mitogen-activated protein kinase
Nrf2	Nuclear erythroid 2-related factor 2

AREs	Antioxidant response elements
NQO1	NAD(P)H quinone oxidoreductase 1
GSTP1	Glutathione S-Transferase Pi 1
C/EBP $\beta$	CCAAT/enhancer-binding protein $\beta$
ER	Endoplasmic reticulum
EMSA	Electrophoretic Mobility Shift Assay
CHIP	Chromatin immunoprecipitation
LDH	Lactate Dehydrogenase

and radiation [14]. These ROS levels are counteracted by elevated antioxidant defense mechanisms in cancer cells [15]. Nuclear erythroid 2-related factor 2 (Nrf2), a redox-sensitive transcription factor, is involved in the regulation of the antioxidant response element (ARE)-mediated expression of phase II detoxifying antioxidant enzymes [16]. In response to oxidative stress, Nrf2 is activated and form heterodimers with other bZIP proteins, bind to cis-acting element(s) termed antioxidant response elements (AREs) in the promoters of target genes [17], inducing transcriptional responses. ARE-dependent genes encode various enzymes, including detoxification enzymes such as glutathione S-transferases (GSTs), NAD(P)H quinone oxidoreductase 1 (NQO1), and heme oxygenase 1 (HO-1), as well as antioxidant enzymes such as catalase (CAT), sulfiredoxin (SRX),  $\gamma$ -glutamate-cysteine ligase catalytic subunit (GCLC) and regulatory subunit (GCLM). Nrf2, therefore, plays a central role in the cellular adaptive response to oxidative stress [18]. In addition to these reductases, active Nrf2 also triggers an immediate induction of CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ). Cebp $\beta$  promoter associates with Nrf2 transcription factor via ARE binding site in the promoter of the Cebp $\beta$  gene during adipogenesis and upregulates its expression [18]. Most recently, we reported that NQO1 is regulated by PTEN in glioblastoma, mediating cell proliferation and oxidative stress [19].

CCAAT/enhancer-binding proteins (C/EBP) are a family of transcription factors of the basic-leucine zipper (bZIP) class. C/EBPs are a six-member family of transcription factors that bind to DNA as homodimers and heterodimers. They are involved in the regulation and expression of numerous genes. C/EBPs affect gene expression by binding to a DNA binding site (consensus sequence "CCAAT"), which is present in many gene promoters and enhancer regions [20]. C/EBP $\beta$  is highly expressed in the intestine, liver, kidney, lungs, spleen, adipose tissue, pancreatic cells [21]. Notably, C/EBP $\beta$  is involved in the regulation of pro-inflammatory gene expression in glial activation and plays a key role in the induction of neurotoxic effects by activated microglia [22]. Although C/EBP $\beta$  increases the expression of a wide variety of target genes that regulate numerous metabolic processes, C/EBP $\beta$  binding sites are particularly found in regulatory sequences of genes that are associated with the inflammatory response [23] or the ER stress pathway [24]. When the transactivation domain of C/EBP $\beta$  becomes phosphorylated by the inflammatory stimuli, transcription of the C/EBP $\beta$  gene increases [25], which subsequently elevates the expression of various proinflammatory genes including IL-6 [26]. Interestingly, increased C/EBP $\beta$  expression has been detected in breast cancer, ovarian tumors and colorectal tumors [27–30]; by contrast, C/EBP $\beta$   $-/-$  mice are refractory to tumorigenesis [31], supporting that C/EBP $\beta$  plays a critical role in mediating cancer progression.

In the current study, we show that C/EBP $\beta$  is highly increased in GBM, and EGFR overexpression escalates its expression, associated with elevated ROS levels. Remarkably, C/EBP $\beta$  mediates both NQO1 and GSTP1 transcriptional expression. Manipulation of C/EBP $\beta$  levels tightly dictates NQO1 and GSTP1 expression, regulating ROS and GBM cell proliferation. Notably, the depletion of NQO1 and GSTP1 abolishes the oncogenic activities of C/EBP $\beta$ . Therefore, these findings provide an innovative mechanism shedding light on how GBM tumors balance the redox and modulate cell proliferation.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

The human glioblastoma cell lines were gifts from Dr. Paul S. Mischel's lab [32]. LN229/EGFR cell line was a gift from Dr. William Weiss lab at UCSF. In brief, the human glioblastoma cell line U87MG were stably transfected with vector control, PTEN, EGFR, and EGFRvIII, with various selection antibiotics. For wild-type EGFR, EGFRvIII, PTEN, 0.7  $\mu$ g/mL of puromycin, 150  $\mu$ g/mL of hygromycin and 400  $\mu$ g/mL of G418 was added individually. The cells were supplemented with 10% fetal bovine serum (Hyclone, USA), penicillin (100 U/ml) and streptomycin (100 U/ml) (ABAM Life Technologies, USA) in a humidified incubator with 5% CO<sub>2</sub> at 37 °C.

### 2.2. Luciferase assay

The cells were seeded in 24-well plates and cultured overnight. Cells were then transfected with C/EBP $\beta$  overexpression or knockdown together with NQO1 and GSTP1 luciferase reporter LightSwitch™ Promoter GoClone® (Active Motif). Forty-eight hours post-transfection, the cells were harvested in passive lysis buffer and analyzed using a LightSwitch Assay Reagent according to the manufacturer's protocol (Active Motif) on a microplate reader. Relative light units of NQO1 and GSTP1 luciferase were normalized to RenSP luciferase light units to control for transfection efficiency. The experiments were performed in triplicate.

### 2.3. EMSA (Electrophoretic Mobility Shift Assay; DNA: protein)

Nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Life Technologies). Protein concentrations were determined using a Coomassie Brilliant Blue protein assay kit (Bio-Rad). Double-stranded oligonucleotide probe for NQO1 and GSTP1 promoter or its mutation were labeled with biotin. Unlabeled probes were used as competitors. EMSA assay was performed as described in the LightShift Chemiluminescent EMSA Kit (Life Technologies). EMSA assay was recruited to detect the C/EBP $\beta$  binding ability on site -723~ -705 of NQO1 promoter (AGGGGTGGTGCAGTGCAT), Mutation probe 1 (AGGGAAAATGCAGTGGCAT) or Mutation probe 2 (AGGGGTGGTGCAGTGAAAA) and supershift; EMSA detect the C/EBP $\beta$  binding ability on site -729~ -715 of GSTP1 promoter (CAACATGGTGAACCCCGT), Mutation probe 1 (CAACAAAATGAAACCCCGT) or Mutation probe 2 (CAACATGGTGAACCAAAA).

### 2.4. Chromatin immunoprecipitation (ChIP)

Briefly, the cells were fixed with 1% formaldehyde for 10 min and crosslinking was quenched with 2 M glycine for 5 min. Samples were rinsed 3 times with cold PBS. After nuclei isolation, the chromatin was sonicated using a Covaris S220 sonicator to obtain the desired DNA fragment size (major band at 500 bp). The sonicated chromatin was pre-cleaned by two rounds of centrifugation with maximum speed at 4 °C. In total, 5  $\mu$ l of anti-C/EBP $\beta$  (C19, Santa Cruz) or 5  $\mu$ l of anti-IgG

(ab133470, Abcam) were used to precipitate the chromatin fragments that contain DNA-protein crosslinking ChIP samples, which was collected with 50  $\mu$ l Dynabeads protein A (Invitrogen, 10001D). The chromatin was sheared with a sonicator. The enrichment of specific DNA sequences was examined by PCR using primer pairs of the human NQO1 promoter (CCC GCTAGC GCTTCCCTATAACTGCTATCT and CTT CTCGAG GCACTTGGGGGCCATA) and GSTP1 promoter (CTT GCTAGC AGGAGTCGAGACCAGCC and CTT CTCGAG AGGCTGGAGT GCAGTGG). PCR assay also detected each input sample and internal reference  $\beta$ -actin.

## 2.5. Cell proliferation assays

The cell survival was analyzed with the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously. The cells were seeded ( $1 \times 10^4$ ) in the regular growth medium, in 5 days, 20  $\mu$ l of MTT (5 mg/mL in PBS, Sigma, USA) was added to each well and the plates were incubated for 4 h at 37 °C. The resulting formazan product was dissolved with DMSO. The absorbance at a wavelength of 490 nm was recorded using a microplate reader (BioTek Instruments Inc., USA).

## 2.6. Measurement of reactive oxygen species formation

The DCFH-DA method was used to detect the levels of intracellular reactive oxygen species (ROS). After different treatments, the cells were collected and then incubated with 5  $\mu$ M DCFH-DA (ROS dye, #C6827, Invitrogen, USA) for 2 h at 37 °C. The fluorescence intensity was measured by the microplate reader (BioTek Instruments Inc., USA) with settings at excitation and emission equal to 485/535 nm. The Confocal Microscope (Olympus, FV1000, Japan) was also employed to obtain the images.

## 2.7. LDH measurements

The cytokine concentrations were measured using LDH assay kits (Promega Corporation, USA). Briefly, after collecting the supernatants, 50  $\mu$ l of LDH detection reagent was added to each well. After 1 h of incubation at room temperature, 50  $\mu$ l of Stop Solution was added to each well. Finally, the absorbance at 490 nm was recorded (BioTek Instruments Inc., USA).

## 2.8. GSH/GSSG ratio measurements and protein carbonyl assay

GSH/GSSG ratio and Protein carbonyl levels were measured from cell homogenates using GSH/GSSG-Glo™ (Promega Corporation, USA) and Protein Carbonyl Assay Kit (#ab126287, Abcam, Cambridge MA, USA), respectively.

## 2.9. Quantitative real-time PCR

The total RNA extraction was performed using Trizol reagent (Invitrogen, California, USA) which following the manufacturer's instructions. Reverse transcription was performed with the SuperScriptIII reverse transcriptase (Life Technologies). All real-time PCR reactions were performed using the ABI 7500-Fast Real-Time PCR System and Taqman Universal Master Mix Kit (Life Technologies). Gene-specific primers and probes were designed from Taqman (Life Technologies). The relative quantification of gene expression was calculated as  $2^{-\Delta\Delta Ct}$  method. All tests were conducted in triplicates.

## 2.10. Western blot analysis

After various treatments, the cells were harvested and the total proteins were extracted. Western blot assays were performed as described in our previous study. Equal amounts of the proteins were

loaded on SDS-PAGE gels and were then transferred to a PVDF membrane. Primary antibodies to the following targets were used: p-EGFR (#2236, CST, USA); EGFR (#2232, CST, USA); PTEN (#9559S, CST, USA); p-C/EBP $\beta$  (#3084S, CST, USA); C/EBP $\beta$  (#7964, Santacruz, USA); NQO1 (#3187, CST, USA); GSTP1 (#3369, CST, USA);  $\beta$ -actin (#3700, CST, USA).

## 2.11. Transfection and infection of the cells

The overexpressing plasmids were purchased from Addgene. The siRNAs were obtained from Santa Cruz. The cells were transfected with 20 nM siRNA or 2  $\mu$ g plasmid using the Lipofectamine 3000 and P3000 (#L3000075, Invitrogen, USA) using the manufacturer's protocol.

## 2.12. In vivo mouse model experiments

Animals were housed, maintained, and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. For xenograft animal models, different groups of cells ( $2 \times 10^6$ ) in 100  $\mu$ l of PBS were inoculated subcutaneously into 6-week-old nude mice obtained from The Jackson Laboratory. The body weight and the tumor growth were assessed every 3 days. The total tumor volume (TV) was calculated according to the following formula: TV (mm<sup>3</sup>) =  $a \times b^2/2$ , where "a" is the minimum diameter and "b" denotes the maximum diameter. The mice were euthanized after 28 days.

## 2.13. Hematoxylin-eosin (H&E) staining and immunohistochemistry

The tumors and primary organs from the nude mice of the above models were fixed in 10% formalin overnight and were then embedded in paraffin. Different sections were prepared and H&E staining was conducted to detect any histological changes of the tumors and organs. The paraffin-embedded samples were stained using Ki67 (#550609, BD, USA) and 4-HNE (#46545, Abcam, USA) antibodies for immunohistochemistry using a technique that has been reported previously. Photographs were taken using a microscope (Olympus, Japan).

## 2.14. Bioinformatic analysis

Bioinformatic data analysis was obtained from the TCGA data portal (<http://cancergenome.nih.gov/dataportal/data/about>), UALCAN (<http://ualcan.path.uab.edu/index.html>) [33] and GlioVis (<http://gliovis.bioinfo.cnio.es>) respectively [34].

## 2.15. Statistical analysis

Data visualization and analysis were performed with GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed using either Student's t-test or one-way ANOVA. Significant Difference among groups was assessed as \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## 3. Results

### 3.1. C/EBP $\beta$ is highly expressed in brain tumors, correlating with poor survival rates

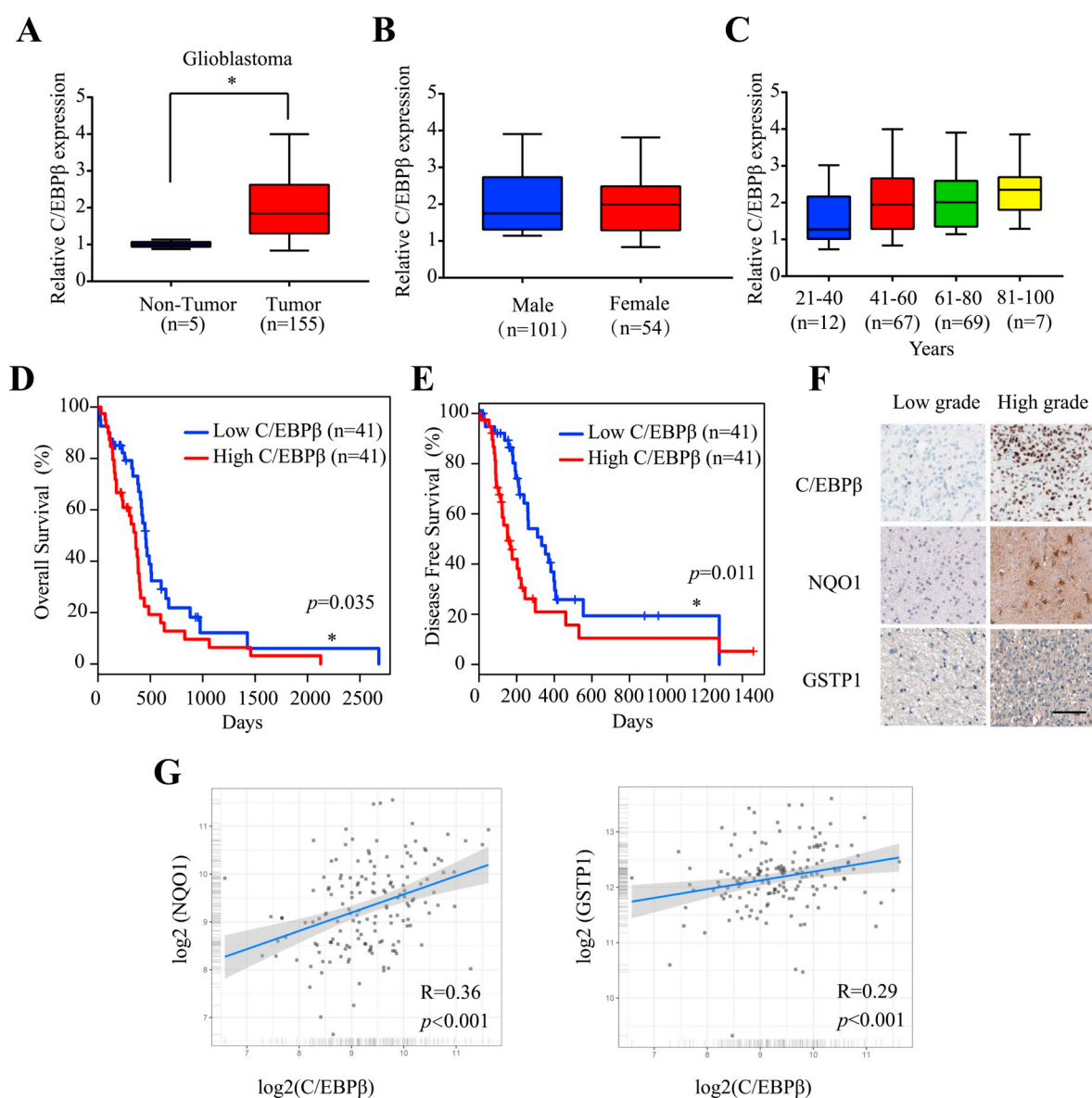
Cancers are tightly associated with extensive inflammation and ROS. C/EBP $\beta$  is transcriptionally activated by inflammatory cytokines such as IL-6, IL-1 $\beta$ , and TNF- $\alpha$ , and bacterial LPS [35]. Moreover, its upstream transcription factor Nrf2 is highly active in gliomas [36]. Hence, we hypothesized that C/EBP $\beta$  might be escalated and activated in GBM. To test this possibility, we explored whether C/EBP $\beta$  is implicated glioma tumorigenesis by searching the TCGA (The Cancer Genome Atlas) database. Remarkably, we found that C/EBP $\beta$  was

selectively upregulated in GBM versus neighboring non-tumor tissues (Fig. 1A). However, its expression was independent of sex or age in the malignant GBM (Fig. 1B & C). Interestingly, C/EBP $\beta$  levels were inversely correlated with overall survival rates and disease-free survival (Fig. 1D & E). Since Nrf2 mediates C/EBP $\beta$  mRNA transcription, in addition to both NQO1 and GSTP1, we also analyzed the correlation between C/EBP $\beta$ , NQO1 and GSTP1, respectively. Consistent with our findings in GBM patients samples, a positive correlation was observed among the expression of C/EBP $\beta$ , NQO1 and GSTP1 (Fig. 1F&G). Hence, these findings suggested that C/EBP $\beta$  was upregulated in the tumors tissues of GBM patients, with high C/EBP $\beta$  expression correlating to a low patient survival rate.

### 3.2. C/EBP $\beta$ expression couples with ROS concentrations, NQO1 and GSTP1 levels in U87MG cells

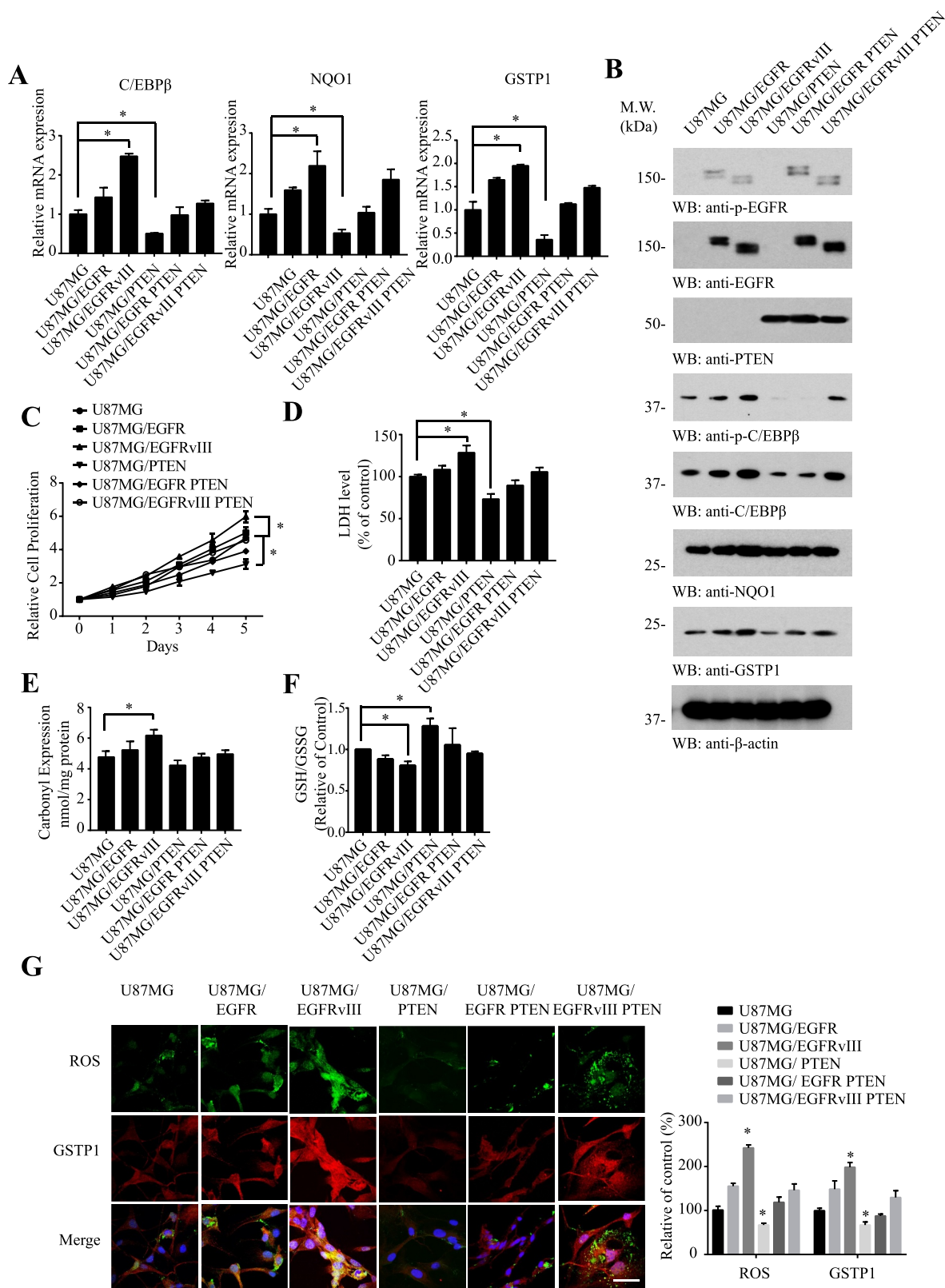
Since EGFR is frequently amplified in GBM that is associated with

PTEN deletion. These mutations on EGFR affect the redox balance in the cancer cells. To assess whether C/EBP $\beta$  is indeed escalated in GBM, we analyzed its mRNA expression levels in PTEN-deficient U87MG glioblastoma cell lines stably transfected with EGFR or EGFRvIII [37]. qRT-PCR (Quantitative RT-PCR) showed C/EBP $\beta$  mRNA levels gradually escalated from U87MG to U87MG/EGFR to U87MG/EGFRvIII cells. Giving PTEN back to U87MG cells decreased C/EBP $\beta$  levels. Quantification of NQO1 and GSTP1 mRNA displayed a similar pattern to C/EBP $\beta$  (Fig. 2A). Immunoblotting revealed C/EBP $\beta$  protein concentrations echoed its mRNA levels in different cell lines. It is worth noting that p-C/EBP $\beta$  T235, a marker for C/EBP $\beta$  activation, was substantially reduced in the presence of PTEN. Consistently, NQO1 and GSTP1 mirrored the C/EBP $\beta$  expression patterns (Fig. 2B). Immunofluorescent co-staining demonstrated that as ROS escalated, both NQO1 and GSTP1 fluorescent signals augmented (Fig. 2G and Supplementary Fig. 1A). As expected, U87MG/EGFRvIII exhibited the fastest cell proliferation rates with U87MG/PTEN the slowest (Fig. 2C). LDH assay for the



**Fig. 1.** C/EBP $\beta$  is the prognostic biomarker in glioblastoma patients.

(A) C/EBP $\beta$  expression in TCGA (The Cancer Genome Atlas) GBM samples compared with normal tissues. C/EBP $\beta$  expression compared between (B) gender and (C) age in the TCGA data set. (D) Overall survival in TCGA GBM patients stratified according to C/EBP $\beta$  expression. (E) Disease-Free Survival in TCGA GBM patients stratified according to C/EBP $\beta$  expression. (F) Immunohistochemistry analyses of C/EBP $\beta$ , NQO1 and GSTP1 expression in the human tissues. Bar: 100  $\mu$ m. (G) Correlation between C/EBP $\beta$  with NQO1 expression and C/EBP $\beta$  with GSTP1 expression.



**Fig. 2.** C/EBPβ expression couples with ROS levels in U87MG cells, coupled with NQO1 and GSTP1 levels.

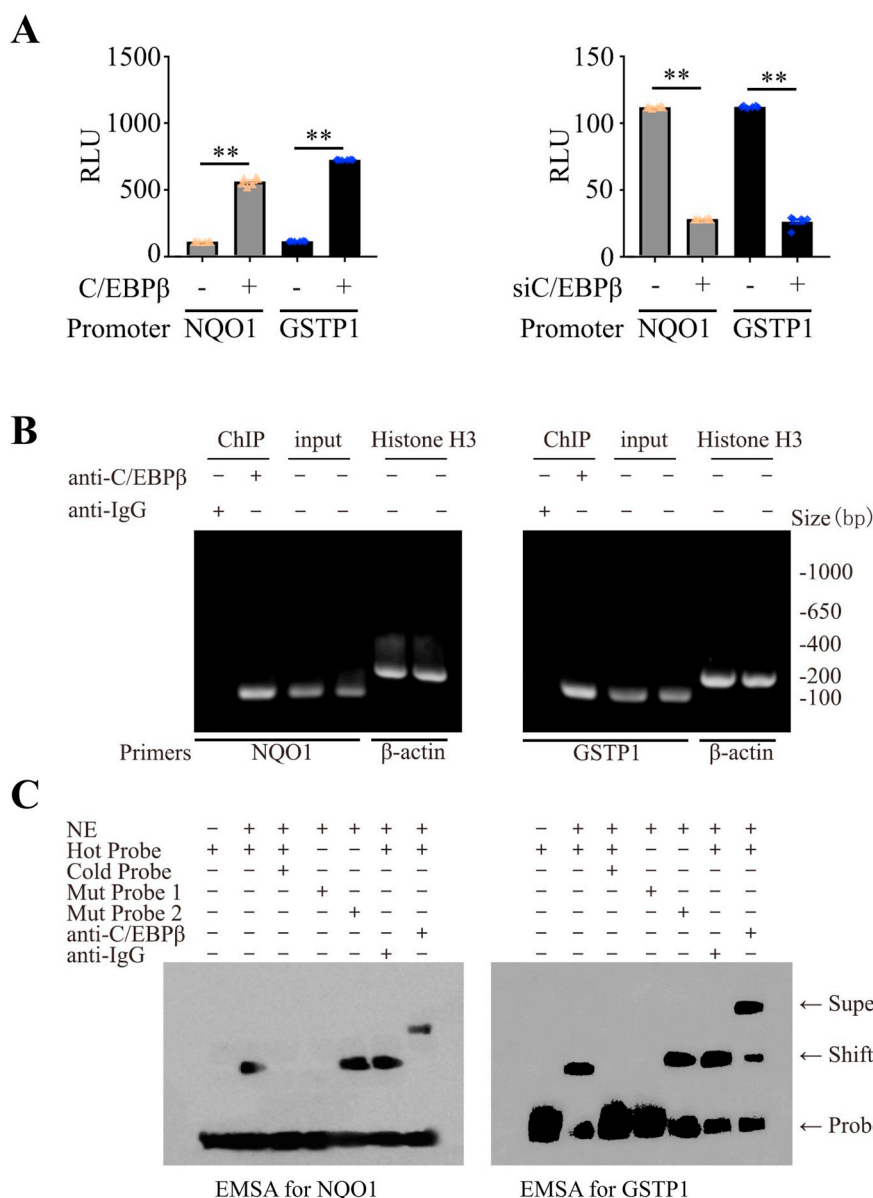
C/EBPβ expression levels correlate with NQO1 and GSTP1 in (A) mRNA and (B) protein levels. Different U87MG cell lines were analyzed by qRT-PCR and immunoblotting. (C) Cell proliferation over 5 days was dramatically increased in EGFRvIII-expressing cells compared with parental U87MG cells. (D) LDH levels, (E) Carbonyl Expression and (F) GSH/GSSG levels of different U87MG cell lines. (G) Co-staining of ROS (Green) with GSTP1 (Red) in 6 cell lines. Bar: 20 μm. Data are means ± SEM (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001, one way ANOVA, *n* = 3).

spontaneous cell death also unveiled a similar format (Fig. 2D), indicating that escalated ROS stress somehow increases cell death. To further examine the oxidative stress in these cells, we conducted protein carbonyl assay and GSG/GSSG ratio assay. We corroborated that carbonyl expression was significantly elevated in U87MG/EGFRvIII cells, fitting with ROS levels (Fig. 2E). By contrast, GSH/GSSG ratios were only augmented in U87MG/PTEN cells, inversely correlated with the ROS levels in the cells (Fig. 2F). Hence, C/EBPβ and NQO1 and GSTP1 exhibit the same expression format in U87MG cells and EGFRvIII highly escalates their expression levels.

To further interrogate the potential link among the ROS, C/EBPβ and cell proliferation in U87MG/EGFRvIII cells, we treated the cells with various small molecular ROS inhibitors including Ascorbic acid, YCG063, and Acetylcysteine. As a result, these inhibitors strongly repressed ROS levels in U87MG/EGFRvIII cells, associated with robust cell proliferation escalation (Supplementary Fig. 2A & B). Interestingly, immunoblotting showed that these inhibitors evidently decreased p-C/EBPβ and its total levels. Concomitantly, NQO1 and GSTP1 levels were also reduced by these inhibitors as well (Supplementary Fig. 2C). Together these findings suggest that ROS activates p-C/EBPβ, resulting in NQO1 and GSTP1 upregulation.

### 3.3. C/EBPβ is a transcription factor for both NQO1 and GSTP1 reductases

The tight correlation between C/EBPβ and NQO1/GSTP1 makes us wonder whether C/EBPβ acts as a transcription factor for both NQO1 and GSTP1 as well. To address this possibility, we performed the promoter activity assay with luciferase-conjugated NQO1 and GSTP promoter plasmids, respectively, which were co-transfected with C/EBPβ into the cells. Overexpression of C/EBPβ significantly elevated their luciferase activities compared to the control vector. In contrast, the knocking down of endogenous C/EBPβ in the cells greatly diminished the luciferase activities (Fig. 3A). To determine whether C/EBPβ indeed binds to the DNA on their promoters, we performed ChIP (chromatin immunoprecipitation) assay using C/EBPβ antibody and control IgG. C/EBPβ antibody specifically pulled down NQO1 or GSTP1 genomic DNAs as compared to control IgG, though an equal amount of inputs were employed; Genomic DNA samples were pulled down with anti-Histone H3 and normalized by β-actin primers as the positive control. from both samples (Fig. 3B). Next, we also conducted EMSA (Electrophoretic Mobility Shift Assay) and Supershift Assay to further validate whether C/EBPβ binds to genomic DNAs from these two gene targets. C/EBPβ-containing nuclear extract potentially interacted with the hot probe,



**Fig. 3.** C/EBPβ acts as a transcription factor for NQO1 and GSTP1.

(A) Human NQO1 and GSTP1 promoters were constructed within a LightSwitch™ Promoter Reporter Vector. These plasmids were co-transfected into cells with C/EBPβ-GFP overexpression plasmids or siRNA for 48 h. Luciferase activities were calculated by using the Luciferase Reporter Assay System, which indicated the promoter activities. (Mean ± SEM, \*\*P < 0.01 vs. vector, n = 3, one-way ANOVA). (B) ChIP assay was performed to detect the binding sites of C/EBPβ on the NQO1 and GSTP1 promoter. The DNA-protein crosslinking ChIP samples were immunoprecipitated with anti-C/EBPβ antibody or IgG. After reversing crosslinks, PCR assay also detected each input sample. Genomic DNA samples were pulled down with anti-Histone H3 and normalized by β-actin primers as a positive control. The equal amount of input was confirmed with NQO1 and GSTP1. (C) Nuclear extract proteins (NE) were isolated from cells transfected with C/EBPβ-GFP for 48 h. EMSA assay was recruited to detect the C/EBPβ binding ability on site -723~ -705 of NQO1 promoter (AGGGGTGGT GCAGTGGCAT), Mutation probe 1 (AGGGAAAATG CAGTGGCAT) or Mutation probe 2 (AGGGGTGGT GCAGTGAAAA) and supershift; EMSA detect the C/EBPβ binding ability on site -729~ -715 of GSTP1 promoter (CAACATGGTGAAACCCCGT), Mutation probe 1 (CAACAAAATGAAACCCCGT) or Mutation probe 2 (CAACATGGTGAAACCAAAA) and supershift. Data are representative of three independent experiments.

which was completely competed away from the genomic DNAs by overwhelming cold probe or mut 1 probe; in contrast, mut 2 probe failed to compete C/EBP $\beta$  from the hot probe. Remarkably, anti-C/EBP $\beta$  but not control IgG incurred a super-shift on the gel, supporting that C/EBP $\beta$  specifically associated with the hot probe (Fig. 3C). Together, these studies support that C/EBP $\beta$  specifically binds to the promoter DNAs from both NQO1 and GSTP1 genes.

### 3.4. Knocking down of C/EBP $\beta$ in U87MG/EGFRvIII cells increases ROS and diminishes NQO1 and GSTP1 expression

To examine whether indeed C/EBP $\beta$  mediates NQO1 and GSTP1 mRNA expression, we chose the parental U87MG and EGFRvIII stably transfected cells. Compared to control siRNA or si-HSF, another transcription factor as control, knocking down of C/EBP $\beta$  significantly reduced NQO1 and GSTP1 from U87MG/EGFRvIII cells but not U87MG cells, though C/EBP $\beta$  itself was substantially eradicated from both cell lines (Fig. 4A), fitting with the observation that C/EBP $\beta$  was selectively upregulated with phosphorylated active status in U87MG/EGFRvIII cells (Fig. 2B). Immunoblotting validated that the deletion of C/EBP $\beta$  selectively decreased both NQO1 and GSTP1 expression levels in U87MG/EGFRvIII cells but not U87MG cells (Fig. 4B), in alignment with their reduced mRNAs by C/EBP $\beta$  knockdown. Remarkably, depletion of C/EBP $\beta$  from U87MG/EGFRvIII cells significantly reduced the cell proliferation as compared to control siRNA or si-HSF. By contrast, these siRNAs displayed no effect on U87MG cell proliferation (Fig. 4C). ROS, carbonyl expression, and GSH/GSSG ratio analysis demonstrated that knocking down of C/EBP $\beta$  selectively increased ROS levels and oxidative stresses in U87MG/EGFRvIII cell but not in U87MG cells, whereas si-control or si-HSF had no any effect in ROS levels (Fig. 4D, F & G). LDH assay revealed that the deletion of C/EBP $\beta$  specifically enhanced cell death in U87MG/EGFRvIII but not U87MG cells, while si-control or si-HSF had no effect (Fig. 4E). Hence, these data support that C/EBP $\beta$  selectively mediates NQO1 and GSTP1 mRNA transcription and protein expression in U87MG/EGFRvIII GBM cells.

We also extended the study into another GBM cell line LN229 that contains intact PTEN. We found that knocking down C/EBP $\beta$  led to selective reduction of NQO1 and GSTP1 protein levels in EGFR-overexpressed LN229 cells but not the parental cells. In contrast, si-HSF or control siRNA revealed no effect in either cell line (Supplementary Fig. 3A). In alignment with U87MG/EGFRvIII cell line, the depletion of C/EBP $\beta$  selectively decreased LN229/EGFR cell proliferation without affecting LN229 cell growth (Supplementary Fig. 3B). Markedly, ROS and cell death were significantly elevated in LN229/EGFR but not LN229 cells, when C/EBP $\beta$  was deleted. By contrast, the deletion of HSF exhibited no demonstrable effect in either cell line (Supplementary Fig. 3C & D).

### 3.5. Overexpression of C/EBP $\beta$ in U87MG/EGFRvIII cells reduces ROS and escalates NQO1 and GSTP1 levels

To further investigate the roles of C/EBP $\beta$  on NQO1 and GSTP1 expression, we overexpressed C/EBP $\beta$ , HSF or control vector into U87MG and U87MG/EGFRvIII cells. qRT-PCR showed that C/EBP $\beta$  overexpression greatly escalated both NQO1 and GSTP1 mRNAs in U87MG/EGFRvIII cells but not in U87MG cells, though C/EBP $\beta$  was greatly expressed in both cell lines. By contrast, HSF or control vector displayed no effect (Fig. 5A). Western blotting confirmed that overexpression of C/EBP $\beta$  selectively augmented NQO1 and GSTP1 in U87MG/EGFRvIII cells but not in U87MG cells, albeit C/EBP $\beta$  was highly overexpressed in both cells (Fig. 5B). As expected, C/EBP $\beta$  overexpression significantly enhanced U87MG/EGFRvIII cell proliferation compared with the control vector or HSF. In contrast, it failed to stimulate U87MG cell proliferation as compared to control or HSF plasmid (Fig. 5C). The deletion of C/EBP $\beta$  also selectively reduced ROS levels and carbonyl expression and increased ratio of GSH/GSSG in

U87MG/EGFRvIII cells as compared to U87MG cells. Again, control vector or HSF overexpression exhibited no effect in the oxidative stresses (Fig. 5D, F & G). Consistently, overexpression of C/EBP $\beta$  significantly decreased cell death in U87MG/EGFRvIII cell versus U87MG cells; by contrast, HSF overexpression did not affect LDH levels in any of the cell lines (Fig. 5E).

Next, we also extended our study into LN229 and LN229/EGFR cells. Once more, overexpression of C/EBP $\beta$  selectively increased NQO1 and GSTP1 in LN229/EGFR but not LN229 cells. In contrast, HSF exhibited no detectable effect (Supplementary Fig. 4A). Consequently, C/EBP $\beta$  but not HSF overexpression significantly stimulated LN229/EGFR cell proliferation. However, neither of them augmented LN229 cell proliferation (Supplementary Fig. 4B). Fitting with NQO1 and GSTP1 reductases elevation by C/EBP $\beta$  overexpression, ROS levels in LN229/EGFR cells were reduced as compared to LN229 cells, and the cell death also displayed the same effect as revealed by LDH assay (Supplementary Fig. 4C & D). Hence, C/EBP $\beta$  selectively affects ROS and cell proliferation via mediating NQO1 and GSTP1 expression in the context of EGFR overexpression.

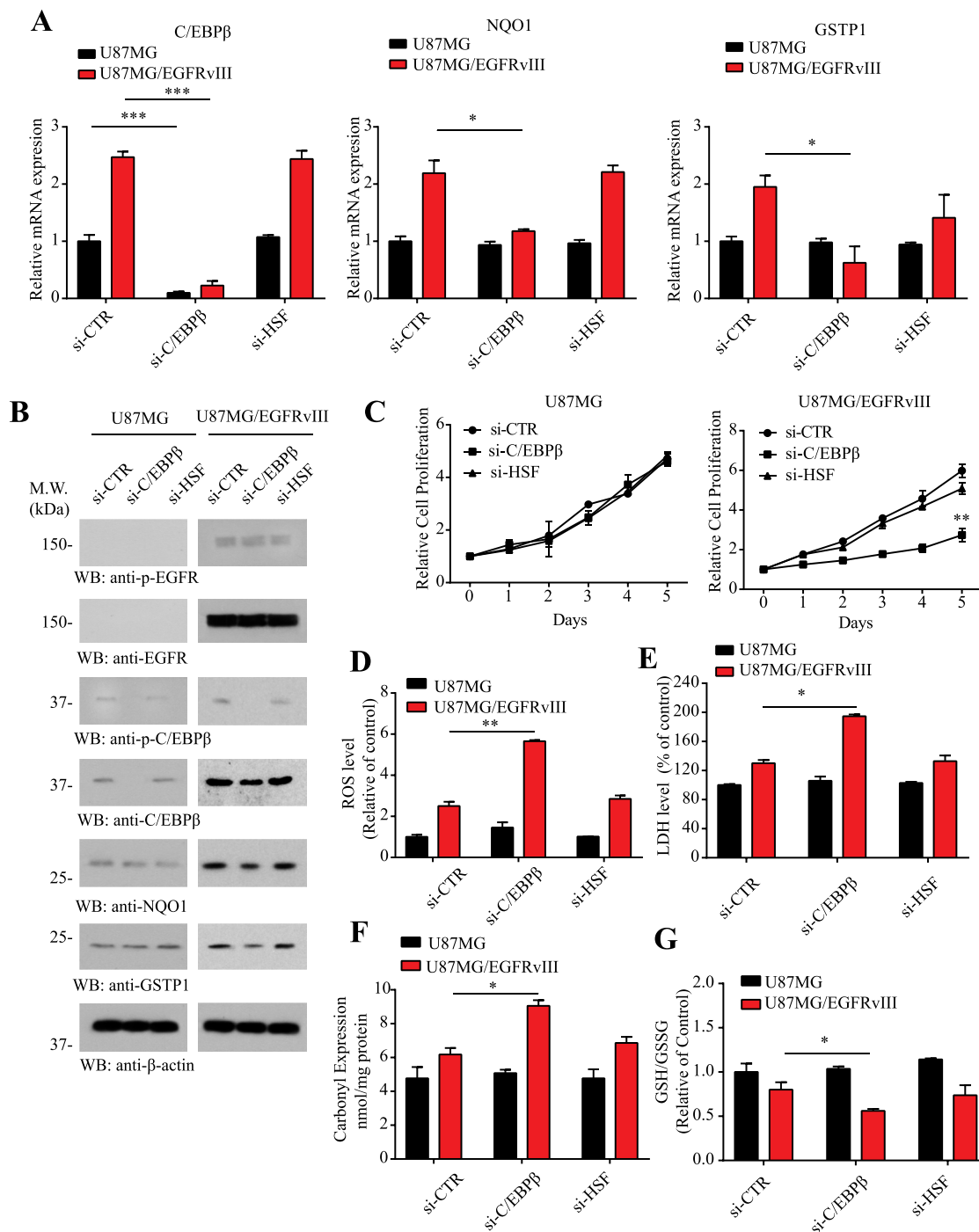
### 3.6. Deletion of NQO1 and GSTP1 abolishes C/EBP $\beta$ overexpression-regulated ROS and cell proliferation

To interrogate whether NQO1 and GSTP1 indeed act downstream of C/EBP $\beta$  and mediate its biological functions in U87MG/EGFRvIII cells, we knocked down NQO1 or GSTP1 separately or simultaneously in the presence of C/EBP $\beta$  overexpression. Cell proliferation assay demonstrated that the deletion of NQO1 or GSTP1 significantly reduced cell proliferation, and the maximal effect occurred when both of them were deleted in control vector-transfected cells. C/EBP $\beta$  overexpression strongly stimulated cell proliferation. However, when NQO1 and GSTP1 both were deleted, the stimulatory effect was significantly abrogated (Fig. 6A). Immunoblotting validated the overexpression of C/EBP $\beta$  and knockdown of NQO1 and GSTP1 in the transfected cells (Fig. 6B). LDH assay revealed that deleting both of NQO1 and GSTP1 increased cell death in control vector or C/EBP $\beta$  overexpressed U87MG/EGFRvIII cells (Fig. 6C), inversely correlated with cell proliferation results. As expected, overexpression of C/EBP $\beta$  strongly repressed ROS levels in U87MG/EGFRvIII cells due to the high expression of the reductases of NQO1 and GSTP1. Accordingly, deleting either of NQO1 or GSTP1 or both strongly escalated ROS levels (Fig. 6D). Thus, C/EBP $\beta$  mediates U87MG/EGFRvIII cell proliferation and decreases the ROS via regulating NQO1 and GSTP1 reductases' expression.

To further assess whether NQO1 and GSTP1 are required for mediating C/EBP $\beta$ 's biological effects, we treated C/EBP $\beta$ -overexpressed U87MG/EGFRvIII cells with vehicle control or the inhibitor MNPC that simultaneously blocks both NQO1 and GSTP1 reductase activities. MNPC is a derivative of FDA-approved nitazoxanide (NTZ) and tizoxanide (TIZ) for treating antiparasitic and cryptosporidium-infection that inhibits reductases including NQO1 and GSTP1 [38,39]. Interestingly, MNPC significantly inhibited U87MG/EGFRvIII cell growth as compared to vehicle control. It displayed a similar antagonistic effect in C/EBP $\beta$  overexpressed U87MG/EGFRvIII cells as well (Supplementary Fig. 5A & B). Interestingly, MNPC considerably elevated LDH activities and ROS levels in U87MG/EGFRvIII cells. The stimulatory effect was pronouncedly decreased when C/EBP $\beta$  was transfected (Supplementary Fig. 5C & D). Therefore, blocking NQO1 and GSTP1 substantially diminishes C/EBP $\beta$  overexpression-elicited effects, supporting that these reductases upregulation mediates the biological events triggered by C/EBP $\beta$ .

### 3.7. C/EBP $\beta$ modulates NQO1 and GSTP1 and ROS levels, mediating GBM proliferation *in vivo*

To explore whether C/EBP $\beta$  also mediates U87MG/EGFRvIII proliferation *in vivo*, we established the stably transfected cells by



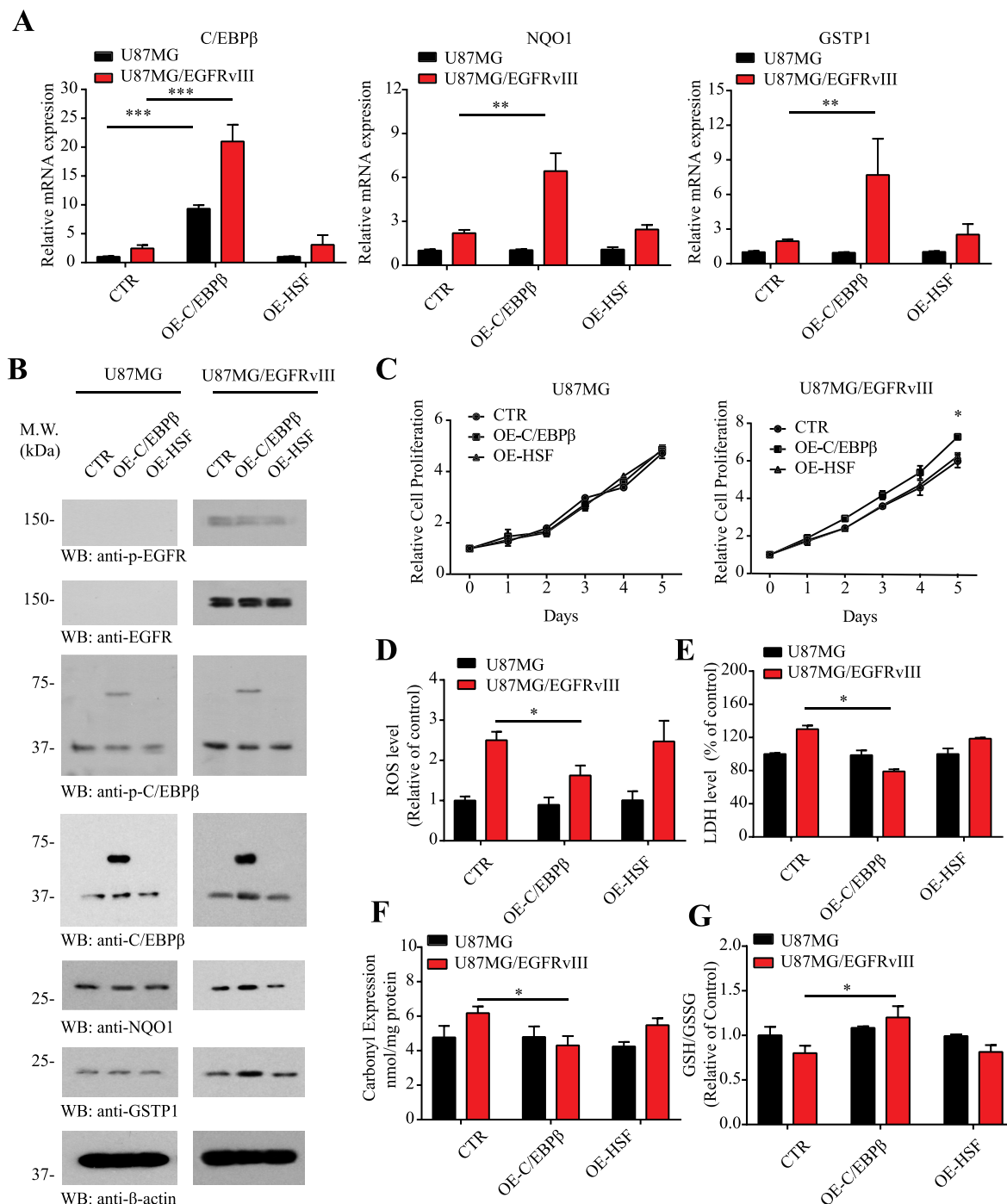
**Fig. 4. The knocking down of C/EBPβ in U87MG/EGFRvIII cells increases ROS and diminishes NQO1 and GSTP1 expression.**

Knockdown of C/EBPβ reduces NQO1 and GSTP1 expression in (A) mRNA and (B) protein levels in U87MG and U87MG/EGFRvIII cell lines. The cells were analyzed by qRT-PCR and immunoblotting. (C) Cell proliferation over 5 days in U87MG and U87MG/EGFRvIII-expressing cells after knocking down C/EBPβ. (D) The ROS levels, (E) LDH levels, (F) Carbonyl Expression and (G) GSH/GSSG levels in U87MG and U87MG/EGFRvIII cell lines after knocking down C/EBPβ. Data are means ± SEM (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001, one way ANOVA, n = 3).



overexpressing or knocking down of C/EBP $\beta$ . The GFP-C/EBP $\beta$  or GFP-shRNA-C/EBP $\beta$  plasmid transfected cells were validated by immunofluorescent staining and immunoblotting (Supplementary Fig. 6A & B). The stable cells were inoculated in nude mice subcutaneously, and the tumor volumes were quantitatively measured at different time points. The tumor growth did not significantly affect the nude mice body weight (Fig. 7A). Noticeably, overexpression of C/EBP $\beta$  strongly stimulated tumor growth as compared to control cells; in contrast, the deletion of C/EBP $\beta$  robustly reduced tumor growth (Fig. 7B&D). After

the termination of the experiment, we monitored tumor weights and found that C/EBP $\beta$  overexpression significantly elevated tumor weights, whereas depletion of C/EBP $\beta$  mitigated tumor weights compared to control (Fig. 7C). H&E staining showed the major organs appeared normal (Fig. 7E). Ki67 staining supported that cell proliferation was strongly escalated in C/EBP $\beta$  overexpressed cells, while the deletion of C/EBP $\beta$  prominently attenuated cell proliferation in the tumors as compared to control (Fig. 7F, middle panels). ROS staining assay with 4-HNE (4-hydroxynonenal), a marker for ROS level, showed that



**Fig. 5.** Overexpression of C/EBP $\beta$  in U87MG/EGFRvIII cells reduces ROS and escalates NQO1 and GSTP1 levels.

Overexpression of C/EBP $\beta$  increases NQO1 and GSTP1 expression in (A) mRNA and (B) protein levels in U87MG and U87MG/EGFRvIII cell line. The cells were analyzed by qRT-PCR and immunoblotting. (C) Cell proliferation over 5 days in U87MG and U87MG/EGFRvIII-expressing cells after overexpression C/EBP $\beta$ . (D) The ROS levels, (E) LDH levels, (F) Carbonyl Expression and (G) GSH/GSSG levels in U87MG and U87MG/EGFRvIII cell lines after overexpression C/EBP $\beta$ . Data are means  $\pm$  SEM (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001, one way ANOVA,  $n$  = 3).

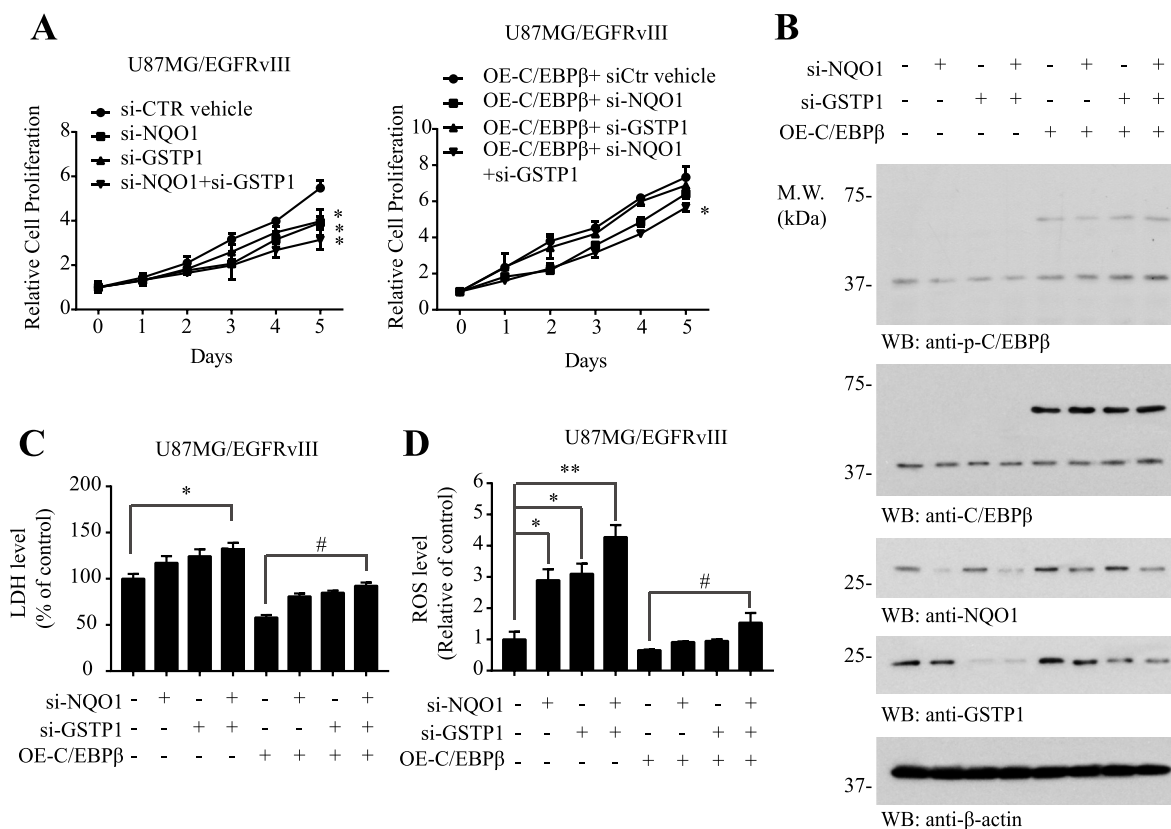
C/EBP $\beta$  overexpression evidently ameliorated ROS. By contrast, the deletion of C/EBP $\beta$  strongly augmented ROS levels, inversely coupled with NQO1 and GSTP1 immunohistochemical staining (Fig. 7F & G).

#### 4. Discussion

Most of the cancer cells rely on highly progressed glycolytic activity to substantially acquire energy (i.e. the Warburg effect) and are prone to be induced into the oxidative stress state due to the elevation of ROS [40]. The regulation of oxidative stress is an important factor in both tumor development and responses to anticancer therapies. In the current report, we show that C/EBP $\beta$ , which is mediated by the ROS sensitive transcription factor Nrf2, regulates the reductases NQO1 and GSTP1 mRNA transcription and protein expression in GBM cell lines with EGFR amplification. Overexpression of C/EBP $\beta$  increases both of the reductases, leading to repression of ROS and increased cell proliferation. On the other hand, depletion of C/EBP $\beta$  significantly attenuates both reductases' expression, resulting in the escalation of ROS and decreased cell proliferation in EGFR overexpressed GBM cells. Employing siRNA to knock down both of them, we found that C/EBP $\beta$ -triggered cell proliferation and ROS reduction are primarily mediated by these two reductases. In alignment with these findings, the tumor volumes from C/EBP $\beta$  stably expressed U87MG/EGFRvIII cells are much larger than those from C/EBP $\beta$ -depleted cells. Concurrently, the GBM cell proliferation in C/EBP $\beta$ -overexpressed U87MG/EGFRvIII cells, validated with Ki67 staining, is highly augmented as compared to C/EBP $\beta$  knocked down cells. These effects are associated with increased NQO1 and GSTP1 levels and reduced ROS levels. Hence, our findings demonstrated that C/EBP $\beta$  plays a key role in mediating redox status in EGFR-amplified malignant GBM cells via modulating the reductases NQO1 and GSTP1 expression. Interestingly, deletion or inhibition of

these two reductases significantly represses cell proliferation and increases cell death (Fig. 6 & Fig. S5), and these events are tightly coupled with ROS escalation due to reductase inactivation. Hence, disruption of the fragile balance between the elevated ROS and reductase activities in EGFR-amplified GBM effectively triggers GBM cell death, resulting in suppression of cell proliferation.

Mammalian cells have an ingenious antioxidant system that serves to protect them from excessive intracellular ROS and extracellular oxidants. Superoxide dismutases (SODs), catalases, and glutathionylation-related enzymes are representative antioxidant enzymes that balance the ROS levels and protect cellular components from oxidative harmfulness. Of the glutathionylation-related enzymes, the glutathione S-transferases (GSTs) superfamily is classically identified as phase II detoxifying enzymes that contribute to neutralizing exogenous as well as endogenous electrophiles, including ROS, through the conjugation of glutathione (GSH) [41]. In humans, GST superfamily consists of numerous isozymes, including pi (GSTP), etc. [42]. Of the seven GST isotypes, GSTP1 acts as a predominant member, and GSTP-silencing using siRNA results in a remarkable hampering of cancer cell growth and promoted excessive ROS generation [43]. GSTP intervenes in various cellular events via its non-enzymatic activity and plays a pivotal role in cellular proliferation. GSTP binds to c-Jun N-terminal kinase (JNK) as a natural inhibitor of JNK in cancer cells, leading to resistance to apoptosis [44]. Accordingly, many GSTP inhibitors have been developed [45]. For instance, GSH analog TLK117, an esterified ezatiostat (TLK199) that binds to GSTP and induces JNK activation, is in a clinical trial for the treatment of myelodysplastic syndrome [46]. The SNP in the GSTP1 gene (rs1695) is a substitution of A [Iso] to G [Val] at position 105 leads to miscoded GSTP1 protein with decreased enzymatic activity and less effective detoxification. A Val variant of GSTP1 has been reported as a risk factor in esophageal cancer [47] and in Asian-

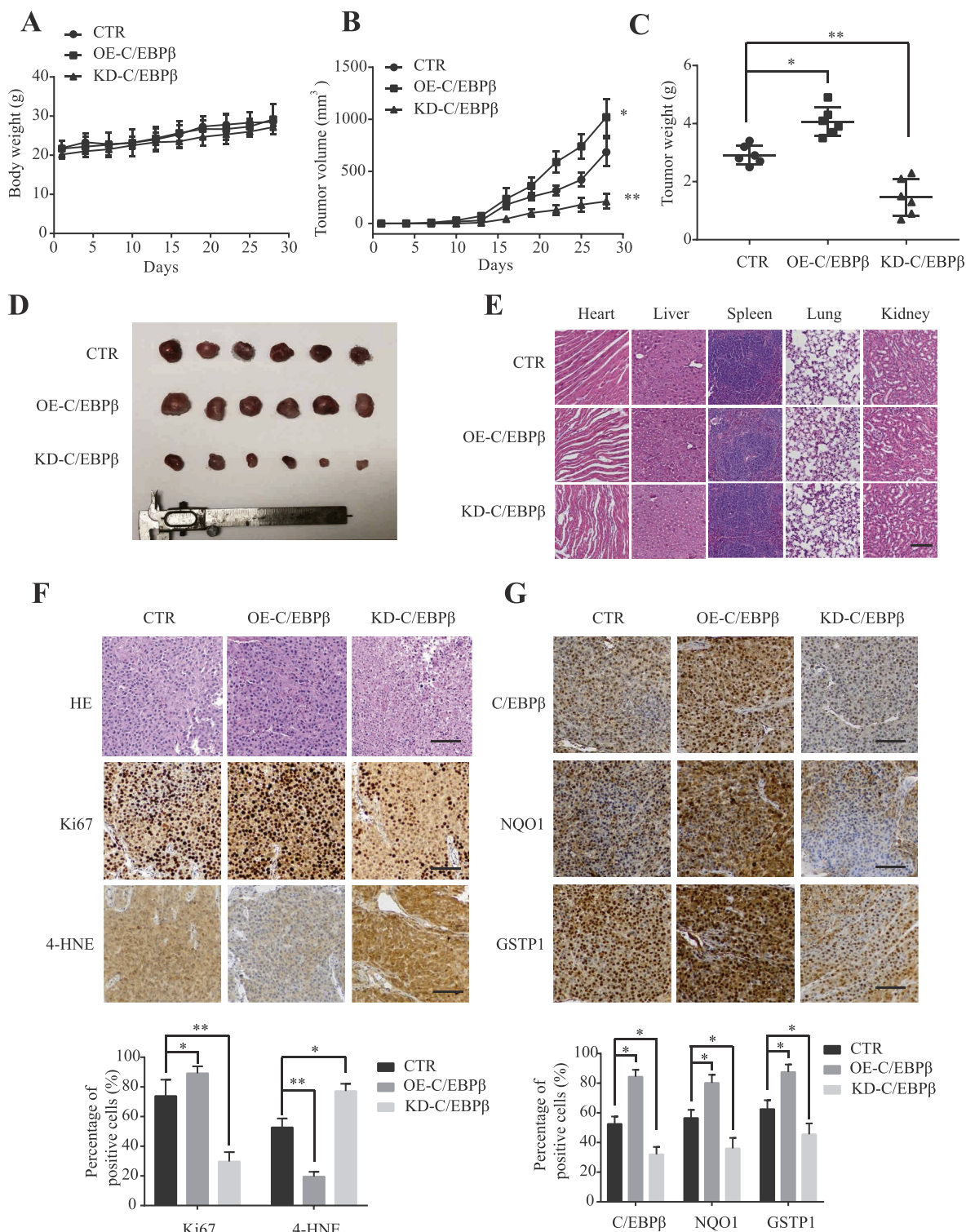


**Fig. 6. Deletion of NQO1 and GSTP1 abolishes C/EBP $\beta$  overexpression regulated ROS and cell proliferation.**

(A) Cell proliferation over 5 days in U87MG/EGFRvIII-expressing cells after overexpressing C/EBP $\beta$  and knocking down NQO1 and GSTP1. (B) Western blot analysis of U87MG/EGFRvIII cells C/EBP $\beta$  and knocking down NQO1 and GSTP1. (C) The LDH levels and (D) The ROS levels in U87MG and U87MG/EGFRvIII cell lines after overexpressing C/EBP $\beta$  and knocking down NQO1 and GSTP1. Data are means  $\pm$  SEM (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001, one way ANOVA,  $n$  = 3).

associated breast cancer [48,49]. NQO1 is also a phase II detoxifying enzyme that may be involved in cancer prevention. NQO1 genetic C609T polymorphism is associated with the risk of various cancers [50–52]. A polymorphic form of NQO1 (p.P187S) is associated with increased cancer risk and certain neurological disorders (such as

multiple sclerosis and Alzheimer's disease), possibly due to its roles in the antioxidant defense. p.P187S has greatly reduced FAD affinity and stability, due to destabilization of the flavin binding site and the C-terminal domain, which leads to reduced activity and enhanced degradation [53].



**Fig. 7. The effect of C/EBPβ in the nude mice model.**

(A) The body weight of mice during the experiment. (B) Tumor growth suppression caused by different groups. (C) Tumor weight of the mice at the endpoint. Significant differences are considered when \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . (D) Tumor growth suppression caused by different groups. (E) Histological analysis of hematoxylin-eosin stained heart, liver, spleen, lung, and kidney of different mice groups. Bar: 100  $\mu\text{m}$ . (F) H&E, Ki67 and 4-HNE staining of tumor slices in various groups. Bar: 100  $\mu\text{m}$ . (G) IHC staining of C/EBPβ, NQO1, and GSTP1 in tumor slices in various groups. Bar: 100  $\mu\text{m}$ . Data are means  $\pm$  SEM (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , one way ANOVA,  $n = 3$ ).

High ROS levels are generally detrimental to cells, and the redox status of cancer cells usually differs from that of normal cells. Because of metabolic and signaling aberrations, cancer cells exhibit elevated ROS levels. For instance, EGFR amplification and mutation are detected in more than 40% of malignant GBM. Accordingly, we show that ROS levels and cell proliferation and C/EBP $\beta$  are all coordinately elevated in U87MG/EGFR and LN229/EGFR as compared to their parental cells, associated with enhanced levels of reductase NQO1 and GSTP1 to counteract the harmful ROS (Fig. 2, Fig. S1). The NQO1 and ROS levels in U87MG cells discrepancy in our previous report and the current studies might be due to the different treatments and time windows. For instance, the cellular ROS and NQO1 levels were immediately analyzed after EGF treatment previously, whereas the cells in the current study are not treated with EGF [19]. Stimulation of EGFR by EGF induces endogenous production of intracellular reactive oxygen species (ROS) and H<sub>2</sub>O<sub>2</sub> in cancer cell lines [8,11] that subsequently alter NQO1 temporal expression. To ascertain that these reductases indeed are accountable for C/EBP $\beta$ -mediated cell proliferation and balanced oxidative stress, we manipulate C/EBP $\beta$  levels and find that both cell proliferation and ROS tightly oscillate upstream C/EBP $\beta$  and downstream NQO1 and GSTP1 (Figs. 4, 5, Fig. S3 and Fig. S4). Notably, depletion of NQO1/GSTP1 or inhibition of these reductases robustly blunts C/EBP $\beta$ 's stimulatory activities (Fig. 6, Fig. S5). It is worth noting that these effects are only observed in EGFR-amplified GBM cells but not in normal EGFR GBM cells, indicating that C/EBP $\beta$  activity is somehow mediated by EGFR signaling. Interestingly, C/EBP $\beta$  T235 is greatly phosphorylated and activated by p-MAPK [54], one of the key downstream effectors of the EGFR signaling pathway, supporting this conjecture. Furthermore, p-C/EBP $\beta$  signals in EGFR or EGFRvIII GBM cells are much stronger than parent cells (Figs. 2, & 4, Fig. S3).

The observation that this is balanced by an increased antioxidant capacity suggests that high ROS levels may constitute a barrier to tumorigenesis. However, ROS can also promote tumor formation by inducing DNA mutations and pro-oncogenic signaling pathways. These contradictory effects have important implications for potential anticancer strategies that aim to modulate levels of ROS [2]. Nrf2 might be the most important regulator of the expression of molecules that possess antioxidant functions. Under resting conditions, Nrf2 is constitutively degraded by the Kelch-like ECH-associated protein 1 (KEAP1)-Cullin 3 (CUL3) E3 ligase complex [55]. Under the oxidative stress, KEAP1 is oxidized and modified so that it cannot bind to Nrf2, thereby leading to the stabilization of Nrf2 and its translocation to the nucleus [56]. Transcriptional activation of antioxidant genes through an antioxidant response element (ARE) is largely dependent upon Nrf2. The genes that contain a functional ARE include those encoding GSTA1, GSTA2, NQO1, and gamma-glutamylcysteine synthetase heavy and light subunits that play a role in defense against oxidative stress. Besides these reductases, C/EBP $\beta$  has been reported to be the downstream target gene of Nrf2 [18]. C/EBP $\beta$  plays multiple physiological roles. By regulating transcription of specific sets of genes, it participates in a cell-specific manner in cellular programs such as cell differentiation, proliferation, cell death and survival, energy metabolism as well as in more global responses such as inflammation. The C/EBP $\beta$  mRNA expression is elevated in 100% of mammary tumors isolated from these ErbB2 overexpressing mice and, interestingly, C/EBP $\alpha$  mRNA is detected at low levels in 30% of tumors, C/EBP $\delta$  mRNA at variable levels in 50% of the tumors, and CHOP mRNA is detectable in most of the tumors examined [57]. Fitting with our observations that C/EBP $\beta$  is elevated in malignant brain tumors (Fig. 1), C/EBP $\beta$  expression is increased breast cancer, ovarian tumors and colorectal tumors [27–30]. Noticeably, C/EBP $\beta$   $-/-$  mice are refractory to tumorigenesis [31]. This finding is consistent with our observation that C/EBP $\beta$  depletion decreases NQO1 and GSTP1 expression and reduces U87MG/EGFRvIII and LN229/EGFR cell proliferation, inversely coupled with increased ROS levels (Figs. 4 & 7).

It is well understood that the deregulation of tumor-suppressor genes and oncogenes frequently affects intracellular ROS levels [58]. Cancer cells are constantly challenged by the need to balance oxidative stress and

control ROS levels as ROS exerts both pro- and anti-growth effects in cancer [2]. As high ROS levels are harmful to cells, oxidative stress can have a tumor-suppressive effect. The induction of oxidative stress can lead to the preferential killing of cancer cells. Numerous drugs with direct or indirect effects on ROS have been used for effective cancer therapies. Drugs that directly affect ROS metabolism target two of the three major antioxidant pathways, such as those involving reduced glutathione and thioredoxin [2]. Currently, a number of anticancer agents are being developed based on the modulation of cellular oxidative stress with strategies to regulate cellular ROS levels [2]. Accordingly, we employed the dual inhibitor MNPC that simultaneously binds and blocks both NQO1 and GSTP1, and found that MNPC significantly reduces C/EBP $\beta$  overexpressed U87MG/EGFRvIII cell proliferation, associated with increased ROS and cell death validated by LDH assay (Fig. S5). The efficacy of EGFR-targeted small-molecule inhibitors or monoclonal antibodies in glioblastomas that also have mutation or deletion of the gene encoding phosphatase and tensin homolog (PTEN) has been modest [10,59]. Conceivably, crushing the tenuous redox balance in EGFR-amplified gliomas using small molecular inhibitors blocking NQO1 and GSTP1 reductases might provide an innovative pharmacological intervention strategy for treating these lethal diseases.

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### Declaration of competing interest

No potential conflicts of interest were disclosed.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101578>.

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