Published in final edited form as:

Cancer Res. 2019 October 15; 79(20): 5218–5232. doi:10.1158/0008-5472.CAN-19-0198.

Mitochondrial NIX Promotes Tumor Survival in the Hypoxic Niche of Glioblastoma

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Abstract

Cancer cells rely on mitochondrial functions to regulate key survival and death signals. How cancer cells regulate mitochondrial autophagy (mitophagy) in the tumor microenvironment as well as utilize mitophagy as a survival signal is still not well understood. Here we elucidate a key survival mechanism of mitochondrial NIX-mediated mitophagy within the hypoxic region of glioblastoma, the most malignant brain tumor. NIX was overexpressed in the pseudopalisading cells that envelop the hypoxic-necrotic regions, and mitochondrial NIX expression was robust in patient-derived glioblastoma tumor tissues and glioblastoma stem cells (GSC). NIX was required for hypoxia and oxidative stress-induced mitophagy through NFE2L2/NRF2 transactivation. Silencing NIX impaired mitochondrial reactive oxygen species (ROS) clearance, cancer stem cell maintenance, and HIF/mTOR/RHEB signaling pathways under hypoxia, resulting in suppression of glioblastoma survival in vitro and in vivo. Clinical significance of these findings was validated by the compelling association between NIX expression and poor outcome for glioblastoma patients. Taken together, our findings indicate that the NIX-mediated mitophagic pathway may represent a key therapeutic target for solid tumors including glioblastoma.

Keywords

NIX/BNIP3L; mitophagy; pseudopalisading zone; hypoxia; glioblastoma stem cell (GSC)

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Conflict of interest: The authors have declared that no conflict of interest exists.

INTRODUCTION

Glioblastoma is the most aggressive and common primary brain cancer. Multimodality treatment consisting of surgery, external beam radiation therapy, and chemotherapy merely yields a median survival of 12–14 months with a 2-year survival rate between 15–26%; this dismal survival has not significantly changed over the decades despite rapid advances in the clinical sciences and basic research efforts (1).

Macroautophagy (hereafter autophagy) in the tumor microenvironment is a major contributor to tumor recurrence, and is tightly coupled with increased resistance to radiation and chemotherapy. Autophagy process includes the degradation of dysfunctional mitochondria through mitochondrial autophagy (mitophagy), the cytoprotective process that controls the production of reactive oxygen species (ROS) and the elimination of damaged mitochondria (2). It is well established that hypoxia promotes tumor growth and resistance to therapy in cancers (3). The importance of hypoxia-inducible factors 1 and 2 alpha (HIF-1 α and HIF-2 α) signaling for optimal function and survival of GSC have been previously characterized (4, 5). Indeed, we previously showed that hypoxia promotes preferential expansion of CD133-positive GSC through a HIF-1 α -dependent mechanism (6). Hypoxia and subsequent stabilization of HIF-1 α appear to further contribute to cancer pathogenesis through induction of BNIP3 and BNIP3-like (BNIP3L or NIX) pathways resulting in enhanced tumor survival and progression (7). Subsequent reports link autophagy and glioblastoma tumorigenesis in GSC models (8, 9). However, the underlying mechanism by which hypoxia-induced autophagy promotes glioblastoma pathogenesis remains unclear.

Although NIX has been identified as a pro-apoptotic protein with a BCL-2 homology 3 (BH3) domain that mediates p53-dependent apoptosis (10), additional functions of NIX are being discovered. For example, the truncated form of NIX, termed sNIX, prevents NIX-mediated apoptosis by heterodimerization with NIX (11). Also, NIX-mediated mitophagy is required for mitochondrial clearance during maturation of reticulocytes (12), HIF-1 α -induced autophagy (7), and ROS-induced autophagy (13). Yet, how NIX supports tumor growth in hypoxic condition remains murky. Here we investigated a key function of NIX in the glioblastoma microenvironment, and its relationship with low oxygen and oxidative stress. Improved understanding of the relationship between NIX-mediated mitophagy and HIF signaling cascade should contribute to elucidating the mechanisms of glioblastoma treatment resistance.

METHODS

Human glioma patient tissues, cell culture and mycoplasma testing

Human GBM, low-grade gliomas and normal brain tissues were obtained after surgery from patients at the University of Pittsburgh and the University of Virginia. Glioblastoma stem cells (GSCs), XO-1, XO-4, XO-6, XO-8, XO-10 and 1228, were isolated from glioblastoma patient surgical specimens. As previously described (14), GSCs were maintained as tumorsphere cultures in DMEM/F12 (Gibco, #11320-033) supplemented with B27 supplement (Gibco), 20 ng/ml EGF, 20 ng/ml bFGF, and penicillin/streptomycin. Normal human astrocytes (NHA) were from Lonza and cultured according to the manufacturer's

protocol. GBM cell lines were purchased from ATCC and cultured in the following DMEM media (Gibco, #11965–092) including 10 % fetal bovine serum and penicillin/streptomycin. We used low passage cell lines (5–15 cycles). All cell lines are routinely tested for mycoplasma contamination at a core facility of NCI Frederick and were negative. Itemized information of cell lines is available in the supplementary material data.

Intracranial xenograft

All animal procedures were approved by the Animal Care and Use Committee (ACUC) at The University of Virginia. U251 cells (1×10^5) that stably express either sh-Control or sh-Nix were stereotactically injected into the striatum of SCID Balb/c mice. Three weeks after tumor implantation, the tumor formation was evaluated with using of ClinScan animal MRI scanner and the mice survival was further determined.

Chemicals, plasmids and cloning

Chemicals were routinely purchased from chemical supply companies: TBHQ and H_2O_2 (Sigma-Aldrich); DMOG (Tocris). Plasmids were from the following companies: HRE-luciferase (Addgene #26731); pGL2-basic (Promega); and Nix-flag (OriGene). For Nix-1195/+114 promoter construct (P1), the sequence from genomic DNA of U87 cells was amplified with Nix-1195F-SmaI and Nix+114R-BglII (5'-AACCCGGGACAAGTCCATTTTAAAGTTC-3' and 5'-TCGGAGATCTGGCAGGACTGC-3'). Nix-1195/-151 (P2) and Nix-290/+114 (P3) were amplified with Nix-1195F-SmaI and Nix-151R-BglII (5'-AACCCGGGACAAGTCCATTTTAAAGTTC-3' and 5'-AGATCTATGGGATGAGTGATGCCAGT-3'); Nix-290F-NheI and Nix+114R-BglII (5'-GAGTCCTAAGAGCTAGCAAACAGATG-3' and 5'-TCGGAGATCTGGCAGGACTGC-3'), respectively. These sequences (P1~P3) were subcloned into pGL2-basic vector. Nix- ARE mutants (P4~P6) were derived from pGL2-Nix-290/+114 (P3) by substituting the potential ARE sequences. The substitution was performed by Q5 site-directed mutagenesis kit (New England BioLabs) according to manufacturer's instruction using with the following primers: for P4, ARE-B-F and ARE-B-R (5'-CCGGTCATAACAAGAATCACGCAAGAGTTC-3' and 5'-ATGGGATGAGTGATGCCAGTGGCAG-3'); for P5, ARE-C-F and ARE-C-R (5'-GTCAGCCAATCTCGAAAGTCTCCACGTCCG-3' and 5'-GCGCGCGCCGGGAACGAAGTCTTG-3'); and, for P6, ARE-D-F and ARE-D-R (5'-GTGTAAATGCCAATGTGCAAGAGACGGTCTTG-3' and 5'-AACAAAGCCGAGTCCGCCGCCCTTTT C-3').

Imaging of Mito-Timer and mitochondrial superoxide

pMitoTimer was gifted from Dr. Zhen Yan (University of Virginia, VA, US). For comparison of mitochondrial fission and fusion between sh-Control and sh-Nix, cells were fixed and stained with DAPI. The fluorescence was excited with 488 nm laser. For superoxide and mitochondria detection, cells were incubated 2 μ M MitoSOX red mitochondrial superoxide indicator (Invitrogen) and 100 nM MitoTracker green FM (Invitrogen) for 10 minutes at 37°C in the dark. The cells were gently washed three times with warm buffer and observed by fluorescence microscopy.

Transfection of siRNAs, shRNA lentiviral particles and plasmids

All cells were transfected Lipofectamine 2000 (for plasmid, Invitrogen) and Lipofectamine RNAiMAX (for siRNA, Invitrogen) according to the manufacturer's methods. siRNAs against the following genes were purchased from Santa Cruz Biotechnology: NIX, HIF1 α , NFE2L2, and a nontargeting control. HRE-luc and Nix promoter constructs were transiently transfected. For shRNA lentiviral particles against NIX, BNIP3, and a nontargeting control were purchased from Santa Cruz Biotechnology and Sigma-Aldrich. Cells were transduced with using standard manufacturer's method. Stable cell lines were established by transfection with the indicated constructs followed by selection with G418 or puromycin dihydrochloride.

Western blotting

Proteins were extracted by CelLyticTM M (for cell; Sigma) and CelLyticTM MT (for tissue; Sigma) cell lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitors. Protein levels were determined by western blotting using conventional protocols. Proteins were detected using specific primary antibodies from NIX, LC3B, RHEB, p-4EBP1, 4EBP1, p-S6K, S6K, p-AKT, AKT, SIRT3, β -TUBULIN, and hydroxyl-HIF1 α (Cell Signaling); BNIP3, PDGFR β , NFE2L2/NRF2, OCT4, GFAP, and β -ACTIN (Santa Cruz Biotechnology); Hif1 α (Novus); HIF2 α (Abcam); CD133 (EMD Millipore); SOX2 (R&D Systems); and subsequently with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling). Immobilon western chemiluminescent HRP substrate kit (EMD Millipore) was used to visualize protein bands.

Cell fractionation and immunoprecipitation

Cells (5×10^7) were separated by Mitochondria/Cytosol fractionation kit with using the manufacturer's protocol (BioVision). The final products were resuspended in 2 x laemmli buffer (Bio-Rad) for further western blotting. For immunoprecipitation, mitochondria pellets isolated by the Mitochondria/Cytosol fractionation kit were suspended in Mitochondria protein IP lysis buffer (BioVision) including 1 % Triton X-100. After solubilizing the total mitochondrial proteins, anti-NIX or anti-RHEB antibody was incubated to the mitochondrial proteins overnight at 4°C on nutator, and Protein A/G beads were subsequently added to the mixture for 1 hour at 4°C on nutator. After collection and washing process of the beads, samples were eluted by SDS-PAGE gel loading buffer.

Luciferase assay

The luciferase reporter vectors were co-transfected with pSV40-Renilla (Promega) vector, internal control for normalization of the transcriptional activity of the reporter vectors. Forty-eight hours after transfection, the cells were cultured with the indicated treatment conditions, and then luciferase activity was subsequently determined using Dual-luciferase assay reagents (Promega) according to the manufacturer's protocol. Each luciferase activity was normalized to internal control, pSV40-Renilla.

Immunofluorescence

Immunofluorescence assays of paraffin-embedded glioblastoma tissue, glioblastoma frozen section, and U251 slides were performed with conventional protocols. After deparaffinization or fixation, slides were incubated with primary antibodies against RHEB and LC3B (Santa Cruz Biotechnology, 1:200); NIX (Cell Signaling, 1:200); and subsequently with the appropriate AlexaFluor 488 or 555 (Invitrogen, 1:1000). DAPI (1 µg/ml) was stained for nuclei. Samples were photographed with Leica SP5 confocal microscopy.

MTS cell viability assay, ATP level assay and hydrogen peroxide detection assay

Cells were plated in 96-well plates at a density of 1,000 cells per well. Cell viability was assessed by the MTS cell proliferation assay kit (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega). The absorbance of the mixture was measured at 490 nm. ATP level was measured by ATP determination kit (Invitrogen) according to the manufacturer's protocol. Levels of H₂O₂ were measured by Hydrogen peroxide colorimetric detection kit (Enzo Life sciences) with using manufacturer's instructions.

In silico analysis

All gene expression and clinical datasets were downloaded from GlioVis (<http://gliovis.bioinfo.cnio.es/>). Gene signatures analyzed were obtained from the Molecular Signatures Database (15). GO analysis was unbiasedly performed in automatic programs of GlioVis.

Quantification and statistical analysis

For protein bands quantification from western blotting, ImageJ software (National Institutes of Health) was used for converting band intensity to numerable value. All data presented as Tukey's box and whisker plots (the box marked by the median, bounded by the 75th and 25th percentiles) were analyzed with 2-tailed unpaired Student's t-test to analyze differences in the mean between groups. Mantel-Cox log-rank test was used to test significance in survival outcomes. For all analyses, significance was determined at $p < 0.05$ and the p-values for each result were represented on the figures. The statistical analyses, unless specified elsewhere, were measured in GraphPad Prism® (GraphPad software, Inc.) or Excel (Microsoft).

RESULTS

NIX level is enriched at the pseudopalisading zone of glioblastoma and the mitochondrial form of NIX is highly expressed in glioblastoma patient tissues

Tumor cell mitochondria display extensive metabolic reprogramming that makes neoplastic cells more susceptible to mitochondrial perturbations compared to normal cells (16, 17). Mitochondrial biogenesis, an indicator of mitochondrial dynamics and cellular homeostasis, can influence treatment resistance of hypoxic tumors (18), but how gene expression of mitochondrial proteins is maintained in tumors is less known. According to anatomic analyses of glioblastoma, tumor cells located in the pseudopalisading regions that surround

necrotic areas confer a survival advantage in the tumor microenvironment (19, 20). To explore global gene expression changes in the pseudopalisading cells near necrosis (PAN), we analyzed gene expression from the IVY GAP (IVY Glioblastoma Atlas Project) dataset in the PAN compared to the cellular tumor (CT). Many genes associated with glioblastoma growth such as IL8, LOX, VEGFA, CA9, HK2, NAMPT, IL6, MET, ABCA1, and LDHA, were up-regulated in the PAN region, and two mitophagy regulators, BNIP3 and BNIP3L/NIX (hereafter referred to as NIX), were highly expressed in this region (fold change > 3.0, p-value < 0.0001) (Figure 1A). Gene signature enrichment analysis (GSEA) showed that the PAN region in glioblastoma was marked by an increase in the expression level of genes related to hypoxia (Figure 1B) and oxidative stress response (Figure S1A). A panel of hypoxia-related GSEA genesets was positively correlated with up-regulated genes of the PAN region (Figure S1B). Further, three volcano plots demonstrated up-regulation of NIX, a regulator of mitophagy, within the PAN region as analyzed by each hypoxia-related GSEA dataset (Figure S1C–S1E). Therefore, we hypothesized that survival of tumor cells in the hypoxic pseudopalisading region of glioblastoma is in part dependent upon alteration of mitochondrial dynamics leading to expression of NIX (Figure 1C). To further refine this hypothesis, we performed *in silico* analyses with the IVY GAP database. NIX was clearly overexpressed in tumor cells of the PAN region compared to those of other regions, such as CT, IT (infiltrating tumor), LE (leading edge) and MVP (microvascular proliferation) (Figure 1D). Clustered heatmap revealed overexpression of NIX and other members of autophagic response (fold change > 2.0, p-value < 0.05; Mootha mitochondrion and mitochondrion dataset) in the pseudopalisading region compared to CT (Figure S1F and S1G). We also analyzed gene expression within the PAN region with an autophagy geneset because mitochondria-related genes are regulated by selective mitophagy. The four genes, GYS1, STBD1, ATG14, and NIX, were selected as upregulated autophagy-related genes (Figure S1H). The Venn diagram isolated NIX as an up-regulated mitochondrial gene induced by mitophagy and hypoxia (Figure S1I). NIX is a key regulator of mitochondrial dynamics and mitochondria-related disorders (21). Interestingly, the GO analysis profile (grouped by molecular function) of NIX-correlated genes identified some of cytokine and chemokine signatures which suggested immune response-related signaling (Figure S2A), perhaps hinting at a connection between immune response and NIX expression in the tumor microenvironment. Further, another GO analysis by biological function for NIX-correlated genes revealed a connection to genes involved in oxygen response signaling, prompting us to focus on mitochondrial NIX and mitophagy in glioblastoma (Figure S2B). These *in silico* analyses suggest that mitochondrial activity may contribute to anatomical heterogeneity of glioblastoma and NIX expression is particularly robust in the hypoxic regions of glioblastoma.

To investigate the pattern of NIX expression in glioma patients, we analyzed mRNA levels of NIX in Rembrandt, a glioblastoma database. NIX mRNA levels were tumor grade-dependent and higher in glioblastoma compared to normal brain tissue (Figure S2C). Moreover, NIX mRNA expression was higher in the mesenchymal (MES) subtype of glioblastoma, which is characterized by prominent hypoxic-necrotic areas, compared to the proneural (PN) subtype (Figure S2D). To better understand subcellular regulatory mechanisms of NIX in glioblastoma cells, we fractionated U251 cells into cytosolic and

mitochondrial components to compare NIX levels in each. We observed that dimeric forms of NIX and BNIP3 were present only in mitochondria whereas monomers were found in both compartments (Figure 1E). Moreover, exposure to hypoxia increased NIX and BNIP3 dimer levels in the mitochondria. Subcellular expression studies indicate that dimeric forms of NIX and BNIP3 are restricted to the mitochondria and enhanced by hypoxia. To investigate whether NIX-mediated mitophagy plays a pathogenic role in brain cancer, we next examined NIX, BNIP3, and LC-3B by immunoblot in 48 distinct patient-derived glioma specimens (30 glioblastomas and 18 grade II/III gliomas) and 2 normal brain tissues (Figure 1F and S3). In 30/48 cases (62.5 %), NIX dimers were elevated compared to normal brains (Figure 1F and 1G). Furthermore, the expression of BNIP3 dimer and the LC3B-II/I ratio were also elevated in 21/48 (43.8 %) and 37/48 (77.1 %) cases, respectively (Figure 1F and 1H–I). We observed that the frequency of NIX up-regulation and LC3B-II/I ratio was higher in glioblastoma compared to lower grade tumors (Figure 1J and 1K). Heatmap expression analysis showed that NIX expression closely mirrored high LC3B-II/I ratio (Figure 1L). This set of studies suggest that mitochondrial NIX, in its dimeric form, is over-expressed in patient-derived glioblastoma tissues compared to low-grade tumors. Collectively, these observations suggest that NIX-mediated mitophagy and subsequent macroautophagy may function as a coping mechanism for tumor cells to tolerate hypoxic condition.

Mitochondrial NIX is preferentially enriched in glioblastoma stem cells

We sought to determine the expression pattern of NIX in patient-derived glioblastoma specimens. NIX was heterogeneously expressed in tumor tissues (Figure 2A). Also, because glioblastoma stem cells (GSCs) are enriched in hypoxic regions within the tumor (5), we hypothesized that NIX expression pattern might correlate with the distribution of GSCs. We detected elevated basal NIX expression levels in patient-derived GSCs compared to established glioblastoma cell lines and normal human astrocytes (NHA); we also observed higher levels of mitochondrial NIX in GSCs (Figure 2B). Analyses of the expression pattern of NIX dimers suggest that mitochondrial NIX is specific to GSCs within the heterogeneous glioblastoma microenvironment and that NIX-mediated mitophagy may be connected to GSC maintenance. To validate this hypothesis, the expression levels of NIX and PDGFR β were assessed in two types of cell culture conditions under normoxia and hypoxia: differentiation-inducing for GSC (for XO-1 and XO-4) and stemness maintenance (for U87 and U251). We used PDGFR β as a positive control because it is known as a regulator of hypoxia-induced autophagy and a GSC marker (22, 23). GSC conditions significantly elevated only the level of mitochondrial NIX dimer with high PDGFR β expression under hypoxia, but not that of NIX monomer, when compared to differentiated glioma cells (DGCs) (Figure 2C–2E). Expression of BNIP3 dimers and monomers however, was dramatically decreased in GSC condition compared to differentiated condition (Figure S4A). Hypoxia also increased PDGFR β expression in only GSC conditions (Figure 2C and 2D). To test autophagy levels in GSC overexpressing mitochondrial NIX compared to DGC, we compared LC3-II and LC3B-II/I ratio in GSC XO-1 versus the differentiated XO-1 cells in the absence or presence of bafilomycin A. Hypoxia induced autophagy flux and LC3B-II expression in GSC, but not DGC, suggesting that mitochondrial NIX-mediated mitophagy in

GSC rather than DGC is subsequently involved in hypoxia-regulated macroautophagy (Figure 2F).

To determine if NIX directly plays a role in GSC maintenance under hypoxia, we used multiple shRNAs and siRNA approaches to silence NIX expression and examined well-characterized GSC markers (Figure 2G and 2H). NIX disruption suppressed CD133, OCT4 and SOX2 expression under hypoxia as well as normoxia, in spite of that expression of GFAP, a differentiation marker, was not affected by the disruption of NIX (Figure 2G). HIF-1 α has been shown to regulate the self-renewal in human acute myeloid leukemia and glioblastoma (4, 6). HIF-2 α is also an established stem cell marker in GSC (5). NIX disruption suppressed HIF-1 α and HIF-2 α protein levels under hypoxic condition in a time-dependent manner (Figure 2H). However, silencing BNIP3 did not affect HIF-1 α expression level (Figure S4B). The effect was most robust in GSC lines demonstrating high expression levels for NIX dimer. These findings suggest that mitochondrial NIX is enriched in the hypoxic GSC niche.

NIX affects hypoxia-induced macroautophagy and RHEB expression, a regulator of mTOR pathway

NIX is a selective autophagy receptor for mitochondrial quality control or mitochondria number (21). To elucidate the functional interplay between NIX and mitophagy process in tumor cells, we searched for a binding partner of NIX. Predictive functional partner analysis of NIX by STRING identified RHEB (Ras homolog enriched in brain) as a possible binding partner (Figure S5A). A previous study demonstrated RHEB is involved in energetic status dependent mitophagy by binding to NIX to regulate ROS production (24). A separate report showed that BNIP3, an isoform of NIX, also associates with RHEB (25). To determine the relationship and the physical interactions between NIX and RHEB, we investigated endogenous binding by immunoprecipitation, localization by imaging, and compartmental fractionation in both GBM patient-derived tumor tissue and U251 glioblastoma cell line. Indirect epifluorescence microscopy revealed colocalization of NIX and RHEB in GBM tumor tissues (Figure 3A). In the incubation of U251 under hypoxia versus normoxia, their mitochondrial fractions were isolated prior to the IP assay and the endogenous binding between the NIX and RHEB was confirmed under normoxia and hypoxia (Figure 3B). Notably, these cells incubated under hypoxia, respectively, showed a 1.7-fold increase of the interaction level (NIX level immunoprecipitated by RHEB antibody, in the left panel of Figure 3B) and a 1.3-fold increase (RHEB level immunoprecipitated by NIX antibody, in the right panel of Figure 3B), suggesting that the interaction between NIX and RHEB increases upon hypoxia. Silencing NIX negatively regulated RHEB expression under both normoxia and hypoxia (Figure 3C–3E). Unexpectedly, hypoxia induced RHEB puncta formation in U251 glioblastoma cells (Figure 3C and 3D), however, NIX silence under the hypoxia abolished the formation of RHEB puncta (Figure 3D). The silence of NIX down-regulated expression of RHEB in both fractions of cytosol and mitochondria, suggesting that NIX expression is potentially involved in expressional regulation of RHEB transcription (Figure 3E). Hypoxia significantly elevated LC3-II level, a marker of macroautophagy, in U251 glioblastoma cells, whereas silencing NIX abrogated hypoxia-induced LC3-II expression (Figure 3F and S5B) and LC3B-II/I ratio (Figure 3G; immunoblotting results are shown in

Figure S5B - the LC3B ratio, soluble LC3B-I versus lipid-bound LC3B-II, was measured by Image J software). Interestingly, NIX silence altered the LC3B-II/I ratio only under hypoxia, but not normoxia (Figure 3F and 3G). Because LC3B-II level is limited to exactly measure autophagy induction or inhibition, we assessed additional assay by using mCherry-GFP-LC3 reporter to quantify autophagy flux. NIX silence reduced hypoxia-induced autophagy flux, suggesting that NIX is potentially involved in autophagosome formation and probing that NIX-mediated mitophagy is required for hypoxia-regulated macroautophagy in hypoxic glioblastoma cells. (Figure S5C). Profiling of general autophagy markers showed that NIX may be required for ATG3 function, an autophagy regulator of LC3B-II lipidation (Figure S5D). The reduction of RHEB by NIX silence resulted from RHEB mRNA alteration in the transcriptional level (Figure S5E). Considering that mitochondria are the source of bulk ATP via oxidative phosphorylation and that overexpression of RHEB can increase ATP production, we measured ATP level under hypoxia. NIX silencing decreased intracellular ATP levels by ~50 % in glioblastoma cells (Figure 3H). The results of these experiments suggest that mitochondrial NIX directly interacts with RHEB in glioblastoma cells and affects the expression of RHEB and autophagosome formation under hypoxic stress.

NIX contributes to the maintenance of GSC stemness through mTOR pathway

As shown in Figure 2, NIX disruption resulted in down-regulation of stem markers, along with HIF-1 α and HIF-2 α . NIX is a transcriptional target of HIF (7, 26). However, NIX also appears to play a role in HIF expression, because the disruption of NIX expression attenuates HIF level (Figure 2H and S6A). RHEB is an activator of mTOR signaling which in turn positively regulates HIF level (27) and its activity is potentially inhibited by BNIP3 (25). Moreover, according to the cell viability test with mTORC1 inhibitors, the mTOR activity was crucial for determining tumor survival under hypoxia (Figure S6B and S6C). Some types of tumor cells including GSC exploit activated AKT-mTOR pathway to enhance HIF and stem cell marker expression under hypoxia (28, 29), however, in case of most cell lines cultured under regular 10% bovine serum, mTOR pathways were inhibited by hypoxia through REDD1 and TSC1/TSC2, which is well established as a canonical pathway (30). Therefore, we examined the mTOR pathway-related signaling molecules in GSC versus DGC under hypoxia. Hypoxia induced the phosphorylation of S6K, a hallmark of active mTOR pathway, and that of AKT in GSC XO-1, unlike the differentiated cells of XO-1 (Figure 4A). These effects were also validated with U251 cells cultured in stem cell culture with the serum-free condition (Figure S6D). Silencing NIX efficiently suppressed phosphorylation of 4EBP1, p70S6K, and AKT under hypoxia, along with the expression of RHEB in only GSC, but the differentiated XO-1 cells displayed mTOR pathway inhibited by hypoxia and showed less effect on the phosphorylation decrease mediated by NIX silence (Figure 4A). These observations paradoxically suggest which NIX disruption suppressed both mTOR pathway and macroautophagy activity even though each pathway can inhibit the other in certain conditions. Recent studies support this paradoxical observation as activation of p70S6K can activate autophagy (31, 32). To determine the cell-specific effect of NIX silencing, we transfected a sh-NIX construct in GSC and DGC. NIX disruption specifically suppressed hypoxia-induced LC3-II accumulation only in GSC, and not in DGC (Figure 4B). Next, we assessed the stemness ability of GSCs lacking NIX. Silencing NIX reduced tumorsphere size (Figure S6E) and number under hypoxia (Figure 4C–4E). Collectively,

these results support the hypothesis that NIX maintains the RHEB-mTOR-AKT pathway under hypoxia and the self-renewal of GSC. Given that hypoxia specifically maintains GSC via the AKT-mTOR pathway, this further supports the concept that NIX functions as an oncogenic driver for the maintenance of GSC in the hypoxic tumor microenvironment.

Ectopic NIX overexpression into differentiated glioblastoma cell rescues mTOR pathway activity under hypoxia

To confirm whether NIX actually supports mTOR pathway in the hypoxia, along with macroautophagy, we transfected NIX-flag plasmid into U251 differentiated cells, expressing lower NIX than other GSCs, and then, investigated macroautophagy levels and expression or activity of RHEB/S6K/4EBP1 signaling pathway (Figure 5A). Overexpression of NIX monomer and dimer by the transfection in the differentiated cell under hypoxia decreased autophagosome number and LC3-I and -II, suggesting that NIX-mediated mitophagy potentially accelerated autophagy process for removing mitochondria damaged by hypoxic stress (Figure 5B and 5C). And, the ectopic NIX sustained higher phosphorylation levels of 4EBP-1/S6K as well as RHEB expression, which can be down-regulated under hypoxia (Figure 5B and 5C). These results from previous figures led to a potential signaling model for elucidating how GSC adapts to this paradoxical tumor microenvironment, and suggesting that expression balance between NIX and BNIP3 might be important for sensitivity to hypoxic stress (Figure 5D).

NIX is activated by oxidative stress-induced NFE2L2/NRF2 transactivation, and silencing NIX abnormally promotes the production of superoxide under hypoxia

Transcription factors such as HIF-1 α , p53, and SP-1 are known to regulate NIX expression (7, 10). HIF-1 α is a well-known transcriptional promoter of NIX, providing a mechanism by which NIX is activated under hypoxia to mediate mitophagy. As expected, silencing HIF-1 α down-regulated NIX expression (Figure S6A); however, NIX expression was not fully abolished, suggesting that there are additional drivers of NIX expression. It is reasonable then to consider that activation of the HIF-1 α pathway alone may not sufficiently explain the robust presence of NIX in both perinecrotic zone and PAN region of glioblastomas. The PAN region is also characterized by the enrichment of an oxidative stress signature (Figure S1A). We mined expression databases to identify a potential link between oxidative stress and NIX. We observed that KAP1, a positive regulator of NFE2L2/NRF2 (master regulator of oxidative stress; hereafter referred to as NFE2L2), disruption was associated with the striking reduction of NIX expression (33); this suggested that NIX may be involved in cellular oxidative stress management (Figure S7A). NFE2L2 is enriched in vascular and perinecrotic (hypoxic) niche of glioblastoma (3). The transcript of NFE2L2 was overexpressed in glioblastomas in a tumor grade-dependent manner (Figure S7B), and increased expression correlated with poor outcome according to analyses of TCGA and Rembrandt databases (Figure S7C and S7D). The positive correlation between NIX and NFE2L2 in eight glioma databases further strengthened this connection and the potential cooperation in oxidative stress clearance (Figure S7E). These observations led us to suspect that NIX-mediated mitophagy might be activated through interaction with NFE2L2 pathway under oxidative stress conditions.

To determine how NFE2L2 might influence NIX expression, GSCs (XO-1 and XO-4) and GBM cell lines (U87 and U251) were exposed to an NFE2L2 activator, TBHQ. Upregulation of the NFE2L2 pathway with TBHQ led to increased NIX dimer expression, but not NIX monomer, BNIP3 dimer and monomer, in a dose-dependent manner (Figure 6A). Transient transfection of NFE2L2 siRNA construct attenuated either TBHQ- or H₂O₂-induced NIX expression (Figure 6B). Additional support is demonstrated by an experiment with H₂O₂ which demonstrated a dose-dependent induction of NIX and LC3B-II, an autophagy marker (Figure 6C). We then sought to determine if NFE2L2 regulated the transcription of NIX by searching for NFE2L2 binding sites on NIX promoter. Examination of the NIX promoter sequence by UCSC genome browser led to the identification of four putative antioxidant response element (ARE)-binding motifs (Regions A to D, in Figure 6D). As shown in Figure 5D, the full-length NIX promoter (P1; Nix-1195/+114) was activated ~5-fold by TBHQ and ~6-fold by hypoxia, and a truncated promoter construct (P3; Nix-290/+114) was also elevated ~6-fold by TBHQ and ~12-fold by hypoxia. However, activation was not clearly evident with a deletion mutant of the NFE2L2 binding motif (P2; Nix-1195/-151), which is a product of the P1 promoter by deleting the sequence from -150 to +114 (Figure 6D). Destruction of the ARE motif in region B or D by point mutants (P4 and P6), but not in Region C (P5), efficiently abolished the promoter activity upon TBHQ treatment and partially decreased the effect to ~25% when exposed to hypoxia (Figure 6D). NIX promoter activity is briskly induced by the addition of H₂O₂, and such response required regions B and D, as seen with TBHQ treatment (Figure S8A). These data indicate that mitochondrial NIX may function as a mediator of oxidative stress as well as hypoxic stress. Also, the observation that destruction of ARE motifs (region B or D) decreased the promoter activity induced by both oxidative stress and hypoxia suggests that NIX may respond to hypoxia-induced oxidative stress.

Manganese superoxide dismutase (MnSOD/SOD2) is necessary to clear mitochondrial reactive oxygen species (ROS) (34). Based on our experimental observations, we hypothesized that interference of NIX-mediated mitophagy in hypoxia may lead to reduced tumor cell survival in part due to impaired clearance of toxic ROS. Hypoxia can function as a double-edged sword: although a moderate amount of hypoxia-induced ROS positively regulates HIF signaling and GSC self-renewal (35), cancer cells must prevent the accumulation of excessive hypoxia-induced ROS (36). Correlation analysis showed a positive correlation between NIX and mitochondrial SOD2 (R-value: 0.462) (Figure S8B). SOD2 was highly expressed in the PAN region of glioblastoma (Figure S8C) and also present within the mitochondria dataset as a correlated gene of NIX (Figure S1G). Silencing NIX decreased SOD2 expression level under hypoxia along with the reduction of hypoxia-mediated mitophagy signature (Figure S8D). NIX interference also accelerated hypoxia-induced H₂O₂ production in both GSC and established glioblastoma cell lines (Figure 6E).

To determine how NIX might affect mitochondrial dynamics under hypoxic and oxidative stress, we imaged U251-MitoTimer (used to detect mitochondrial mass and status) at varying oxygen tensions. Tumor cells are capable of clearing mitochondria damaged by oxidative stress through mitophagy (18). Live-cell imaging experiments showed that hypoxia decreased mitochondrial mass under hypoxia in a time-dependent manner (Figure S9A). Silencing NIX dramatically prevented the hypoxia-induced reduction of mitochondrial mass,

suggesting obstruction of the mitophagy process resulting in accumulation of damaged mitochondria (Figure 6F). NIX disruption also resulted in higher superoxide levels in tumorspheres (Figure 6G and 6H). This observation was further confirmed in 4 additional cell lines, XO-1, XO-4, U87, and U251, with si-NIX (Figure S9B–S9D). Under normoxia, interfering with NIX expression led to a slight increase of MitoTracker-Green (Figure 6G and 6H) and MitoTimer (Figure S10), implying NIX facilitates mitochondrial clearance (12). These set of experiments suggest that depletion of NIX leads to an imbalance of mitochondrial dynamics and impaired mitochondrial ROS clearance under hypoxia. In summary, in addition to playing a critical role in promoting mitophagy under hypoxic stress, NIX also interacts with NFE2L2 to mount a mitophagic coping response to oxidative stress.

Targeting NIX suppresses glioblastoma survival and extends the lifespan of tumor-bearing mice

Given NIX is a key player in mediating hypoxic and oxidative stress-induced mitophagy, a critical coping mechanism for stressed cells, we investigated the feasibility of therapeutically targeting NIX. We explored the effect of NIX knockdown on *in vitro* tumor growth rate under hypoxic condition. GSCs expressing either sh-NIX or sh-Control were incubated with stem cell- or differentiated-culture condition in a time-dependent hypoxia. NIX disruption strikingly attenuated the cell viability preferentially in the GSCs expressing abundant NIX dimer levels, compared to the DGCs doing less (Figure 7A–7C). Silencing NIX also slowed tumor growth under hypoxia in the differentiated culture conditions in accordance with previous observations (Figure 7A–7C). Next, to test whether silencing NIX affects *in vivo* tumor growth and mice survival, we performed intracranial injection in immune-deficient mice with U251 expressing either sh-Control or sh-NIX. Three weeks after injection, we measured tumor size using an animal MRI scanner. Silencing NIX suppressed tumor growth in the intracranial mice model (Figure 7D) and was associated with longer survival (Figure 7E). The suppression effect by sh-NIX *in vitro* was also confirmed in U251 (Figure S11A). Additionally, we assessed soft agar assay, anchorage-independent proliferation test, and PI/Annexin V staining with NIX knockdown. NIX silencing reduced soft agar colony number (Figure S11B) and induced apoptosis under hypoxia (Figure S11C). Based on the striking biological consequences of NIX disruption, we hypothesized that NIX expression may predict clinical outcome in glioblastoma patients. Furthermore, we used the Rembrandt portal to perform Kaplan-Meier survival analysis based on two NIX expression groups: high versus low group, with different cutoff strategy. High expression of NIX was significantly associated with shorter patient survival (Figure 7F and 7G). Such observations consistently reinforce the notion that higher NIX expression drives glioblastoma tumor growth in both *in vitro* and intracranial mice models, and confers poor clinical outcome.

DISCUSSION

Cancer cells rely on mitochondria to adequately meet cellular energy demands, but emerging evidence suggests new roles for mitochondrial dynamics in cancer. Selective mitophagy plays an important function in evading cell death and maintaining necessary metabolic activity in cancer. Here we report on how increased expression of NIX confers a survival advantage to glioblastoma cells and provide evidence about a potential mechanistic link

between mitochondrial NIX and GSC maintenance in the brain. Our results show that NIX regulates tumor survival in the hypoxic region of glioblastoma, likely including GSC population via two distinct mechanisms: NIX-mediated mitophagy is activated by HIF/hypoxic stress response as well as NFE2L2/oxidative stress; feedback regulation of mitochondrial NIX supports HIF signaling by RHEB/mTOR/AKT pathway (Figure 7H). To our knowledge, this is the first report demonstrating that NIX is overexpressed in the PAN region of glioblastoma and that NIX-mediated mitophagy is activated by hypoxia.

Previous studies of NIX largely relied on routine mRNA-based microarray analysis (8, 11); however, this approach technically limits the ability to detect NIX dimers which are likely functionally relevant to the mitochondria. The expression level of NIX monomers and dimers, by western blotting in 48 glioma patient-derived tissues and GSCs, enabled us to establish a connection between mitochondrial NIX function and dimerization. The results presented in Figure 1 demonstrate that NIX dimers expressed in mitochondria are heterogeneously present in human glioblastoma patient-derived tissues. In addition, dimers were found to be enriched in GSCs, suggesting that NIX-mediated mitophagy in glioblastomas might affect cancer cell hierarchy and tumor initiation. Overexpression of NIX dimers in GSCs suggests a specific function of mitochondrial NIX in GSC maintenance under hypoxia. NIX and mitophagy levels between GSCs and established glioblastoma cell lines suggest that mitochondrial NIX may play a key role in GSC maintenance. Further evidence is provided by the finding that expression of hypoxia-induced NIX dimers and LC3B was abolished upon differentiation of GSCs.

Intracellular ROS homeostasis mediated in mitochondria is essential for maintaining self-renewal capacity in hematopoietic stem cells (37) because excessive ROS contribute to aging, senescence, and apoptotic cell death (38). Previous studies have investigated the various mechanisms linking ROS production in hypoxic setting, such as HIF, NFE2L2, PI3K/AKT, and AMPK signaling cascades (39, 40). We therefore investigated the promoter activity and subsequent expression levels of NIX in low oxygen and oxidative stressful conditions because NIX promoter contains both hypoxia response element (HRE) and antioxidant response element (ARE) for transactivation of HIF and NFE2L2. In consonance, NIX silencing strikingly increased hypoxia-induced H_2O_2 and mitochondrial superoxide production in a hypoxia-dependent manner, resulting in attenuation of tumor cell proliferation. We observed that NIX knockdown under normoxia led to the accumulation of mitochondria indicative of impaired mitophagy, but without abnormal ROS generation. Such observations support the notion that mitochondria accumulation resulting from failure of mitophagy does not affect ROS homeostasis and cell proliferation in normoxia. Thus, our findings indicate that NIX involving in both hypoxic and antioxidant signals regulates mitophagy and ROS clearance to enhance cancer cell survival within the hypoxic regions of glioblastoma.

Yet another mechanism by which NIX may regulate ROS is through interaction with RHEB. Previous studies have shown that RHEB regulates mitochondrial oxidative phosphorylation-induced mitophagy in a NIX dependent pathway (24). Based on this observation we hypothesized that disruption of NIX should further suppress GSC maintenance by negatively regulating mTOR/AKT/HIF signaling. We confirmed the endogenous interaction and the co-

expression of RHEB and NIX. We further observed that NIX knockdown led to the suppression of RHEB, suggesting that NIX/RHEB interaction contributes to mTOR/AKT/HIF signaling in glioblastoma. The mTOR/AKT/HIF pathway has previously been identified as a key regulator of cancer stem cell (CSC) in prostate, pancreatic, breast, and brain tumors (41). In particular, the HIF signaling is critical for hypoxia-induced CSC maintenance and tumor initiation in cancers, including glioblastoma (5, 6, 42). In agreement, suppression of RHEB/AKT/HIF signaling cascade by NIX knockdown strikingly reduced expression of stem cell markers and self-renewal capacity of GSCs.

Mitochondrial dysfunction in the brain is associated with many neurodegenerative diseases, including Alzheimer's and Parkinson's disease, which has been implicated in altered mitochondrial quality control (43, 44). While in degenerative states, impaired mitophagy results in cellular death, in cancer however a proliferative disease, enhanced mitophagy contributes to cell survival even under stressful conditions. In this context, our findings suggest the clinical implication of mitochondrial NIX for glioma patients. First, NIX might offer a novel strategy to therapeutically target hypoxic glioblastomas, known to be refractory to current therapies. Second, targeting NIX may preferentially eliminate GSCs which are in part dependent on mTOR/AKT/HIF signaling network and are often resistant to conventional cytotoxic therapies (14). Finally, assessment of mitochondrial NIX may provide novel insights into the biology and potentially, prognosis of a variety of solid tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Dr. Zhen Yan (University of Virginia, VA, US) for kind donation of plasmid for pMitoTimer. We would like to thank the University of Virginia imaging core and animal core service teams. We also thank the Neuro-Oncology Branch members (NCI, NIH), Dr. Chunxin Wang (NINDS, NIH), Dr. Richard Youle (NINDS, NIH) and lab members of Dr. Jeremy Rich (UCSD) for general discussion and suggestion of this study. This research was supported by the Intramural Research Program of the NIH, NCI, CCR.

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SIGNIFICANCE

NIX-mediated mitophagy regulates tumor survival in the hypoxic niche of glioblastoma microenvironment, providing a potential therapeutic target for GBM.

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Figure 1. NIX mRNA is enriched at the pseudopalisading zone of glioblastoma and dimer of mitochondrial NIX is highly present in glioblastoma patient tissues

(A) Hockey stick graph was generated for comparison of overexpressed genes in pseudopalisading cells around necrosis (PAN) versus cellular tumor (CT). The IVY GAP database was used for the analysis and the gene changes were filtered by $p < 0.0001$. (B) The Gene Set Enrichment Analysis (GSEA) was performed for genes altered in the PAN region. (C) A schematic model was illustrated for main hypothesis showing clinical importance of the PAN region. (D) NIX mRNA levels were investigated in multiple regions of the IVY GAP database. (E) U251 cells with and without 24 hours of hypoxia were fractionated to

cytosol and mitochondria, and levels of NIX and BNIP3 were investigated by Western blotting. SIRT3 and β -TUBULIN were used as markers of mitochondrial and cytosolic fractions, respectively. (F) In 48 human brain tumor tissues, the expression levels of the proteins indicated were determined by Western blot, compared to 2 normal brain tissues. (G-I) Quantification of the bands were calculated for NIX dimer (G), BNIP3 dimer (H) and autophagy flux (I). The protein levels were displayed by tumor grades. (J and K) Levels of NIX dimer and LC3B-II/I ratio in patient tissues were displayed by sub-grouping glioma grade. (L) Log2 fold changes of these proteins were assessed in order of NIX fold change to show the expression correlated among the detected proteins.

Figure 2. Mitochondrial NIX is preferentially enriched in glioblastoma stem cells

(A) NIX were stained by IF in GBM patient tissues. Nuclei were stained with DAPI. Scale bars of image 1 and 2 are 100μm. (B) Western blotting was performed in 12 cell lines and NIX levels were quantified. (C and D) In a hypoxia time-dependent manner, NIX and PDGFRβ levels were determined by Western blotting in 2 GSCs (XO-1, XO-4) and 2 non-stem GBM cell lines (U251, U87). GSCs were cultured under stem cell conditions or differentiated by FBS for 7 days (DGC). (E) Based on the bands of the figure C and D, NIX levels including dimers and monomers were quantified. (F) LC3B levels and LC3B-II/I ratio

were measured in GSC XO-1 cells were cultured under stem cell culture conditions or differentiated by FBS for 7 days (DGC). Western blotting was performed for detecting LC3B levels. Bafilomycin A was treated at 100 nM for 2 hours before cell harvest. (G) Immunoblots in XO-1 lines stably expressing either sh-Control or sh-NIXs. (H) Either si-Control or si-NIX was transiently transfected into the 3 GSCs and 3 non-stem GBM cells as indicated. Cell lines were then cultured under hypoxia in a time-dependent manner followed by Western blotting.

Figure 3. NIX affects hypoxia-induced macroautophagy and RHEB expression, a regulator of mTOR pathway

(A) Immunofluorescence (IF) staining by confocal microscopy in human patient GBM tissue (scale bar = 50 μ m). (B) Co-immunoprecipitation of NIX and RHEB with U251 mitochondria. IgG for each group was used as control antibody. Two milligrams ($\sim 2 \times 10^7$ cells) of lysates were used for mitochondrial fractionation and 50 % of the mitochondrial proteins were used for each IP reaction. Total lysates (10 %) were used as input controls. Protein bands were quantified and the binding levels between NIX and RHEB (H/N ratio) were determined with fold-change normalized by normoxia samples. (C) IF staining of NIX,

RHEB and DNA in sh-Control or sh-NIX #1 stably expressing U251 with 24 hours of hypoxia (scale bar = 50µm). (D) Percent of RHEB puncta positive cells detected in Figure 3C. (E) U251 cell lines stably expressing sh-Control or sh-NIX #1 were fractionated into cytosolic and mitochondrial fractions. SIRT3 and β -TUBULIN served as mitochondrial and cytosolic compartment markers, respectively. (F) U251 cells either stably-expressing sh-Control or sh-NIX #1 were incubated under normoxia or hypoxia (24 hours) followed by the IF staining (scale bar = 50µm). (G) Autophagy flux was calculated by LC3-II versus LC3-I ratio in 4 cell lines (Western blots shown in Supplemental Figure 5B). (H) Intracellular ATP was quantified in U251 stably expressing either sh-Control or sh-NIX #1 cultured for 24 hours under normoxia or hypoxia.

Figure 4. Silencing NIX impairs glioblastoma stem cell maintenance via suppression of canonical RHEB/mTOR/AKT pathway

(A) Western blotting was performed in XO-1 stably expressing either sh-Control or sh-NIXs with normoxia or hypoxia, for the proteins. (B) Western blotting was performed in GSC and DGC with the indicated conditions. The ratio of LCII/LCI for each condition is shown. (D-F) Limiting dilution sphere forming assay was performed with either sh-Control or sh-NIXs for NIX silencing in GSCs under 5 days of hypoxia.[dummy_fig f data observed]

Figure 5. Ectopic NIX overexpression into differentiated glioblastoma cell rescues mTOR pathway activity under hypoxia

(A) Plasmid information of NIX-flag. (B) Immunofluorescence (IF) staining of LC3B, NIX and nuclei by confocal microscopy in differentiated U251 cell with and without NIX-flag transfection under normoxia and hypoxia. scale bars = 25μm. (C) Western blotting was performed in U251 transfected with either Control or NIX-flag in the indicated condition for the proteins shown in the figure. (D) Schematic summary about current observations along with the literature of which BNIP3 inhibits RHEB function.

Figure 6. NIX is activated by oxidative stress-induced NFE2L2/NRF2 transactivation and silencing NIX produces abnormal superoxide under hypoxia

(A) Immunoblots from two GSCs (XO-1, XO-4) and two GBM (U87, U251) cell lines cultured with increasing doses of TBHQ (0, 10, 100 μM) for 24 hrs. (B) U251 were transiently transfected either with si-Control or si-NFE2L2 for 48 hours and then treated with DMSO or 10 μM TBHQ for 24 hours, followed by Western blotting. (C) U251 were treated with increasing amount of H₂O₂ (0, 50, 100, 500 μM) followed by Western blotting. (D) Left panel: schematic diagram of NIX promoter region. Right panel: Nix_{-1195/+114} or its derivatives were transiently transfected into U251 and the promoter activity of each

construct was determined after treatment of either 24 hours of hypoxia or 10 μ M TBHQ. (E) Cellular H₂O₂ levels were measured in XO-1 and U251, expressing either sh-Control or sh-NIX #1, after incubation with normoxia and 24 hours of hypoxia. (F) U251 cells stably co-expressing with MitoTimer and either sh-control or sh-NIX #1 were incubated under normoxia or 24 hours of hypoxia. MitoTimer, a fluorescent protein used to monitor mitochondrial turnover, was imaged by confocal microscopy with DAPI (scale bar = 50 μ m). The quantification of the relative intensity is shown in supplementary Figure S10. (G) si-NIX was transfected into XO-1, followed by imaging of MitoTracker and MitoSOX (scale bar = 10 μ m). Red and Green were calculated for Red/Green ratio. (H) Imaging of MitoTracker and MitoSOX was performed in XO-1 and U251 stably expressing either sh-Control or sh-NIX #1 in the same conditions with Figure 6F (scale bar = 20 μ m).

Figure 7. Targeting NIX suppresses tumor survival and extends lifespan of tumor-bearing mice and glioma patients

(A-C) Cell viability was assessed with either si-Control or sh-NIXs in a time-dependent hypoxia with the indicated cell lines. MTS assay was performed for cell viability in each day (D and E) Intracranial transplantations of 1×10^5 sh-Control or sh-NIX #1 U251 cells were compared by ClinScan MRI animal scanner after 3 weeks of tumor injection, and survival rate was determined. (F and G) Glioma patient survival rates were evaluated according to high versus low NIX expression levels, with two different cutoffs by 25 % quartile (F) and

median value (G) in all tumors of Rembrandt database. (H) A schematic model was illustrated for this study.

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