Hexokinase 2 and nuclear factor erythroid 2-related factor 2 transcriptionally coactivate xanthine oxidoreductase expression in stressed glioma cells

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Running title: HK2 as transcriptional co-activator of Nrf2

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Abstract
A dynamic network of metabolic adaptations, inflammatory responses and redox homeostasis is known to drive tumor progression. A considerable overlap between these processes exists, but several of their key regulators remain unknown. To this end, here we investigated the role of the proinflammatory cytokine IL-1β in connecting these processes in glioma cells. We found that glucose starvation sensitizes glioma cells to IL-1β-induced apoptosis in a manner that depended on reactive oxygen species (ROS). Although IL-1β-induced JNK had no effect on cell viability under glucose deprivation, it mediated nuclear translocation of hexokinase 2 (HK2). This event was accompanied by increases in the levels of sirtuin 6 (SIRT6), nuclear factor erythroid 2-related factor 2 (Nrf2), and xanthine oxidoreductase (XOR). SIRT6 not only induced (i) ROS-mediated cell death, but also facilitated (ii) a nuclear Nrf2–HK2 interaction, and (iii) recruitment of the Nrf2–HK2 complex to the ARE site on the XOR promoter. Importantly, HK2 served as transcriptional coactivator of Nrf2 to regulate XOR expression, indicated by decreased XOR levels in siRNA-mediated Nrf2 and HK2 knockdown experiments. Our results highlight a non-metabolic role of HK2 as transcriptional coactivator of Nrf2 to regulate XOR expression under conditions of proinflammatory and metabolic stresses. Our insights also underscore the importance of nuclear activities of HK2 in the regulation of genes involved in redox homeostasis.

Introduction
Emerging data indicate that interplay between inflammation and metabolism plays a critical role in tumor progression. In addition to its ability to extensively metabolise glucose for aiding increased energy demands, cancer cells are also under oxidative stress associated with increased production of ROS (1). The rapid glycolytic rate in glioblastoma (2) is concomitant with elevated levels of hexokinase-2 (HK2), that catalyzes the first step of the glycolytic pathway (3). While HK1 is the predominant isoform in low-grade gliomas, highly upregulated HK2 levels in GBM correlates with poor prognosis (3). The subcellular localization of HK2 is sensitive to extracellular glucose, with the distribution of HK2 between cytoplasm and mitochondria being dynamically regulated by glucose availability (4). HK2 regulates ROS levels (5), and glucose withdrawal increases ROS production in glioma cells (6). Also, the ability of diverse chemotherapeutic agents to induce glioma cell apoptosis through increased intracellular ROS generation is known (7-9).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor which provides cytoprotection against oxidative stress. Oxidative stress mediates activation of Nrf2 (10) which is known to regulate ROS production by mitochondria and NADPH oxidase (11). In addition to contributing towards the maintenance of redox homeostasis, Nrf2 affects the expression of metabolic genes (12,13). Interestingly, SIRT6 not only regulates redox homeostasis by serving as an Nrf2 coactivator (14) but also affects glucose
homeostasis via HIF-1α (15). Nrf2 regulates HIF-1α accumulation (16), and the latter serves as a regulator of HK2 (17). Moreover, IL-1β induced HK2 in glioma is dependent on relative abundance of HIF-1α-dependent SIRT6 levels (18). Also, HIF-1α-dependent subcellular localization of HK2 regulates cytoskeletal organization to consequently affect MHC-I clustering under inflammatory conditions in glioma cells (19).

Disrupting glycolytic flux serves as a trigger for inflammation and cell death (20). Interestingly, glycolytic inhibitors and metabolic conditions that affect hexokinase function and localization induce inflammasome activation involved in IL-1β secretion (21). Xanthine oxidoreductase (XOR), involved in catalyzing purines to uric acid, regulates IL-1β secretion upon NLRP3 inflammasome activation (22). Moreover, XOR is also known to regulate HIF-1α through ROS in glioma cells (23). Given that inflammation rewires energy metabolism in the tumor microenvironment (24), the prospect of targeting altered metabolism in inflammation has been suggested as a substantial therapeutic promise (25). As there is a link between inflammation, metabolic status, and redox homeostasis, we investigated the effect of inflammation and glucose deprivation on oxidative stress in glioma cells.

Results
Increased death upon glucose deprivation in IL-1β-treated glioma cells is ROS-dependent
While treatment with IL-1β or glucose deprivation alone had no effect on glioma cell viability, IL-1β triggered a significant decrease in cell viability under low glucose conditions within 3 hours (Fig. 1a). Death was accompanied by alteration in the expression of molecules associated with cell cycle progression (Supplementary Fig. 1a), and increase in cytochrome c and Bcl2 levels (Supplementary Fig. 1b). Cancer cells exhibit glucose metabolism-dependent inhibition of cytochrome c-mediated apoptosis (26), and glucose deprivation promotes ROS generation and death (6). An increase in ROS generation was observed in glucose-deprived cells treated with IL-1β, as compared to those exposed to low glucose condition (Fig 1b). As elevated ROS level induces glioma cell apoptosis upon inhibition of glucose metabolism (7), we evaluated the role of elevated ROS in affecting glioma cell viability. The ability of ROS inhibitor NAc to rescue glucose deprived cells from IL-1β-induced death suggested that apoptosis triggered by IL-1β under glucose starvation is ROS-mediated (Fig. 1c). However, pretreatment of cells with PEGylated Catalase (an H2O2 decomposing enzyme) was unable to rescue death, indicating that H2O2 is not the major ROS species contributing to cell death (Supplementary Fig. 1c). This was concomitant with no change in H2O2 levels in cells treated with IL-1β under glucose deprivation (Supplementary Fig. 1d).

IL-1β-induced death upon glucose deprivation is JNK independent
We have previously demonstrated the importance of ROS-induced JNK activation in triggering glioma cell apoptosis (7). On investigating the status of JNK in IL-1β-treated glioma cells in the presence and absence of
glucose, an increase in JNK phosphorylation was observed only in cells treated with IL-1β in the absence of glucose (Fig. 1d). However, JNK inhibitor SP600125 failed to rescue death in cells treated with IL-1β under conditions of glucose deprivation (Fig. 1e), suggesting non-involvement of JNK in the process. The induction of cell death in both U87MG (p53 wild type) and T98G (p53 mutant) cell lines in response to inflammation and glucose deprivation, suggested that induction of death under this condition is independent of the p53 status.

**Increased SIRT6 expression regulates cell death**

In addition to its function as a regulator of ROS generation (27), SIRT6 also plays a dominant role in affecting energy balance through control of glucose homeostasis (15). Besides, IL-1β increases SIRT6 levels in glioma cells (18). An increase in SIRT6 levels was observed upon IL-1β treatment under glucose deprivation (Fig. 2a). On evaluating the contribution of elevated SIRT6 levels in IL-1β-induced death under glucose-deprived condition, viability of cells upon SIRT6 overexpression and depletion was determined. While siRNA-mediated SIRT6 knockdown increased viability of IL-1β treated cells under glucose deprivation (Fig. 2b), ectopic SIRT6 expression increased cell death (Fig. 2c).

Given the known function of SIRT6 as a regulator of ROS generation (27) and the ability of SIRT6 to affect viability of IL-1β-treated glucose deprived cells, ROS levels upon SIRT6 knockdown as well as overexpression was investigated. A decrease in ROS levels upon SIRT6 knockdown (Fig. 2d) and an increase upon ectopic SIRT6 expression (Fig. 2e) was observed. Increase in ROS levels was concomitant with decrease in the expression of mitochondrial superoxide dismutase (SOD2), a critical detoxifier of mitochondrial ROS (Fig. 2f). This decrease in SOD2 levels in glucose-deprived cells treated with IL-1β was further diminished upon SIRT6 overexpression (Fig. 2f).

**IL-1β increases nuclear localization of HK2 upon glucose deprivation**

Inhibiting HK2 activity by 2-Deoxyglucose (2DG) which competitively inhibits cellular uptake and utilization of glucose, increases its nuclear localization (28). Since both JNK (7) and SIRT6 (18) regulate HK2 expression in glioma cells, we investigated the status of HK2 in IL-1β-treated glucose-deprived cells expressing elevated pJNK and SIRT6 levels. Western blot analysis revealed a decrease in cytosolic (Fig. 3a) and an increase in nuclear (Fig. 3b) HK2 levels in IL-1β-treated cells upon glucose deprivation. SIRT6 enhances the expression of pro-inflammatory cytokine-chemokines (29), and HK2 dissociation from mitochondria triggers NLRP3 inflammasome activation and IL-1β production (21). On investigating the status of NLRP3 and IL-1β in cells exhibiting heightened SIRT6 and nuclear HK2 levels, an increase in the mRNA expression of both was observed upon glucose deprivation only in the presence of IL-1β (Fig. 3c). As we have previously reported JNK-dependent regulation of HK2 in glioma cells (7), and since JNK activation was dramatically elevated under conditions of increased nuclear
HK2 translocation, the role of JNK in this shuttling was investigated. IL-1β-induced increase in nuclear HK2 localization upon glucose deprivation was JNK dependent, as SP600125 prevented IL-1β-mediated HK2 accumulation in the nucleus under this condition (Fig. 3d, 3e).

**HK2 has no role in cell death but negatively regulates HIF-1α activation**

HK2 determines cellular fate by affecting both cytoprotection and apoptosis induction based on the metabolic state (30). To investigate the involvement of altered HK2 localization in affecting cell death, the viability of cells upon siRNA-mediated HK2 knockdown was determined. HK2 knockdown failed to rescue cell death (Supplementary Fig. 2a), suggesting the non-involvement of HK2 in IL-1β-triggered cell death under glucose starvation. While HK2 induces apoptosis upon glucose depletion, it is also known to protect cells from death during hypoxia (30). Given the non-involvement of HK2 in death induction, we investigated whether its inability to rescue death could have stemmed from changes in HIF-1α levels. Interestingly, HK2 knockdown further heightened HIF-1α activation in IL-1β treated cells deprived of glucose (Supplementary Fig. 2b). This further confirmed our previous observation that a negative correlation exists between HK2 and HIF-1α levels in glioma cells (18).

**SIRT6 regulates Nrf2**

SIRT6 regulates oxidative stress responses by serving as an Nrf2 coactivator (14) and Nrf2 accumulation in cancer cells offers protection against oxidative stress (31). Importantly, Nrf2 is translocated to the nucleus in response to pro-oxidant stimuli (32). On determining Nrf2 levels in glucose deprived IL-1β-treated cells exhibiting heightened ROS, an increase in nuclear Nrf2 levels was observed (Fig. 4a). As increased nuclear Nrf2 level was concomitant with elevated SIRT6, the role of SIRT6 in regulating Nrf2 levels was investigated. The ability of siRNA-mediated SIRT6 knockdown to decrease nuclear Nrf2 level in glucose deprived IL-1β treated cells (Fig. 4b) further indicated the role of SIRT6 as a regulator of Nrf2.

**HK2 interacts with Nrf2 in a SIRT6-dependent manner**

As increased nuclear Nrf2 level was concomitant with increased nuclear HK2 accumulation, we investigated the association between the two. Immunoprecipitation revealed increased association between Nrf2 and HK2 under glucose deprived conditions only in the presence of IL-1β (Fig. 4c). Moreover, not only did siRNA-mediated SIRT6 knockdown decrease nuclear Nrf2 level in glucose deprived IL-1β treated cells, but it also abrogated Nrf2-HK2 interaction (Fig. 4c). These results provide strong evidence that Nrf2 is an interacting partner of HK2, and that SIRT6 is crucial in facilitating the interaction.

**XOR is a target of HK2 and Nrf2**

Nuclear translocation of Nrf2 in response to oxidative stress triggers transcriptional program through its binding to antioxidant response element (ARE) of antioxidant genes associated with maintenance of cellular redox balance (33). HK2 serves as an intracellular glucose sensor of yeast cells to affect gene regulation (34),...
whereby it functions through a variety of structurally unrelated factors to sustain transcriptional repression at the SUC2 gene (34). Besides, dissociation of HK2 from mitochondria activates NLRP3 inflammasome (21), and XOR-dependent IL-1β secretion upon NLRP3 inflammasome activation is known (21). Given the involvement of XOR in regulating cellular redox homeostasis through ROS generation (23), the status of XOR in glucose deprived IL-1β-treated cells was determined. An increase in XOR expression was observed in IL-1β-treated glucose-deprived cells, exhibiting elevated ROS, IL-1β and NLRP3 levels, as compared to cells treated with IL-1β or glucose-deprived media alone (Fig. 5a). This increase was accompanied by elevated XOR mRNA levels (Supplementary Fig. 2e) as well as its enzyme activity (Supplementary Fig. 2c). As Nrf2 was found to interact with HK2, the role of HK2 and Nrf2 in regulating XOR expression was determined. Increase in XOR expression was found to be dependent on both HK2 and Nrf2, as siRNA-mediated knockdown of Nrf2 (Fig. 5b) and HK2 (Fig. 5c) decreased IL-1β induced XOR expression under glucose deprivation. Also, RNA polymerase II (Pol II) was found to be part of this nuclear Nrf2/HK2 complex (Supplementary 2d).

To investigate whether other known targets of Nrf2 require HK2 in their regulation, the expression of known Nrf2 target HMOX1 (Heme oxygenase 1) (35) was determined under HK2 knockdown condition in cells treated with IL-1β under glucose deprivation. While siRNA-mediated knockdown of HK2 abrogated IL-1β and glucose deprivation stress-induced increase in XOR mRNA levels, HK2 had no effect on HMOX1 expression (Supplementary Fig. 2e). Thus, the ability of HK2-Nrf2 complex to regulate Nrf2-dependent genes was found to be target specific and not all Nrf2 target genes follow HK2-dependent regulation.

**Nrf2 knockdown abolishes binding of HK2-NRF2 complex to ARE site on XOR promoter**

Several studies have reported nuclear shuttling of HK2 (36,37), and in the context of SUC2 promoter HK2 functions as a transcriptional repressor (34). In view of our observations that XOR expression is regulated by both Nrf2 and HK2, the occupancy of Nrf2-HK2 complex at ARE site of XOR promoter to affect its expression was investigated. ChIP assay revealed increased enrichment of Nrf2 (Fig. 5d) as well as HK2 (Fig. 5e) on the ARE site of XOR promoter in glucose-deprived IL-1β-treated cells. This raises the possibility that HK2 could directly bind DNA or may bind to ARE through its interaction with Nrf2. However, enhanced binding of HK2 in glucose-deprived IL-1β-treated cells was abrogated upon Nrf2 knockdown (Fig. 5f). Taken together, the results suggest that abundantly available nuclear HK2 in glucose-deprived IL-1β-treated cells serve as a coactivator of Nrf2 in XOR transcriptional regulation, by facilitating increased binding of HK2-Nrf2 complex to ARE site on XOR promoter (Fig. 5g).

**Discussion**

Non-canonical functions of glycolytic enzymes in gene regulation are just beginning to be
understood, with nuclear shuttling of such enzyme being involved in the regulation of transcriptional events in glioma (38). Several studies have reported nuclear shuttling of HK2 in yeast as well as in cancer cells (36,39-41), with nuclear localization of HK2 being regulated by glucose (39). Though presence of glucose reduces HK2 nuclear translocation (28), glucose deprivation in itself was not sufficient to affect nuclear HK2 translocation in glioma cells. An inflammatory stimulus is necessary for prompting HK2 cytoplasmic-nuclear shuttle under glucose-deprived condition. As IL-1β is known to induce a prolonged hypoglycaemia in the brain (42), it is possible that nuclear HK2 level becomes pronounced under glucose deprivation only in presence of IL-1β. While HK2 had no involvement in cell death, it served as a negative regulator of HIF-1α. This is of interest as HIF-1α-dependent subcellular localization of HK2 is known to regulate inflammation-mediated cytoskeletal organization that influences immune-related outcome (19).

Importantly, SIRT6 served as a negative regulator of cell death under metabolically compromised inflammatory condition. This ability of SIRT6 to affect death could be attributed to its ability to regulate ROS levels (27). As HIF-1α-dependent SIRT6 abundance regulates HK2 levels in IL-1β-treated glioma cells (18), this study underscores SIRT6 as a crucial factor in the orchestration of redox regulatory responses under inflammatory conditions. In addition to regulating cell death, SIRT6-dependent nuclear Nrf2 accumulation and subsequent formation of Nrf2-HK2 complex was found to be indispensable for XOR expression. Disruption of glycolytic flux-mediated induction of mitochondrial ROS accumulation (43,44), as well as dissociation of hexokinase from mitochondria is known to activate NLRP3 inflammasome (21). As XOR inhibition attenuates NLRP3 inflammasome activation by impairing IL-1β secretion (22), it is possible that increased XOR is crucial for regulating inflammatory cell responses under diminished glycolytic flux. Our findings indicate that inhibiting glucose flux alone is not sufficient to induce apoptosis in absence of inflammatory trigger. It is a unique state of the cell exhibiting limited glycolytic flux, inflammation and oxidative stress that sets the stage for SIRT6-mediated death.

Compared to the relatively well-known metabolic functions of HK2 in tumor progression, the present study highlights a previously unknown non-metabolic role of HK2 in regulating transcription of genes associated with redox regulation by serving as a coactivator of Nrf2. While the nuclear translocation of HK2 and its ability to serve as a coactivator of Nrf2 is a prerequisite for facilitating transcription of XOR, the expression of Nrf2 target HMOX1 was found to be independent of HK2. Thus, Nrf2-HK2 complex appears to be specific for regulation of XOR and is not a general feature for regulating other known Nrf2 targets. However, it is possible that the coactivator function of HK2 may not be limited only to transactivation of Nrf2. It will therefore be interesting to identify other HK2-binding partners to reveal the non-canonical function of
HK2 in a broad area of cellular functions. As there is considerable overlap between metabolic and inflammatory responses in glioma cells, understanding mechanisms through which metabolic genes subserve non-canonical functions to affect growth in metabolically stressed inflammatory conditions atypical of tumor micro-environment would provide new insights into glioma biology.

**Materials and Methods**

**Cell culture and treatment**

Glioblastoma cell lines A172, U87MG and T98G obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in DMEM (Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies). On attaining semi-confluence, cells were switched to serum free media (SFM) and after 6 hours, cells were glucose starved in glucose-free DMEM (Life Technologies) in the presence or absence of 10 ng/ml IL-1β (R&D Systems). Treatment with ROS inhibitor N-Acetyl Cystine (NAC) (2.5 mM), JNK inhibitor SP600125 (10 μM), H₂O₂ decomposing enzyme PEGylated catalase (20 μM) was given according to the experimental requirements.

**Determination of cell viability**

Viability of cells treated with IL-1β in the presence or absence of glucose-free DMEM was assessed using the MTS assay (Promega, Madison, WI, USA) as described (8). Similarly, the viability of cells treated with ROS inhibitor N-Acetyl Cystine (NAC), or PEGylated catalase (H₂O₂ inhibitor) or transfected with HK2 siRNA, SIRT6 siRNA or SIRT6 overexpression construct and treated with IL-1β in the presence or absence of glucose was determined. Values were expressed as average absorbance of technical replicates for every treatment condition.

**Measurement of ROS**

Intracellular ROS generation in cells treated with IL-1β in the presence and absence of glucose and ROS inhibitor or upon siRNA-mediated SIRT6 knockdown as well as SIRT6 overexpression was assessed using fluorescent dye Dihydro ethidium (DHE) as described previously (8). Briefly, cells were stained with 1 μM DHE in SFM for 45 minutes at 37°C and then washed twice with 1xPBS. Fluorescence intensity was measured at 535 nm wavelength using Infinite M200PRO (Tecan) microplate plate reader.

**Measurement of hydrogen peroxide**

Amplex™ Red Hydrogen Peroxide/Peroxidase Assay was used to determine the relative levels of H₂O₂ in cells deprived of glucose in the presence or absence of IL-1β. Briefly, cells were collected in 1x reaction buffer and 50 μl of Amplex Red/HRP working solution was added to the 50 μl cell suspension as per the manufacturer’s instructions. The samples were incubated in dark at room temperature for 30 minutes in a black 96-well plate. Fluorescence intensity was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm using Infinite M200PRO (Tecan) microplate plate reader.

**Western blot analysis**

Western blot analysis was performed on protein lysates isolated from control or cells treated with IL-1β in the presence or absence of glucose,
and/or transfected with different constructs or siRNAs as described previously (45) using antibodies against Nrf2 (cat#ab31163), SIRT6 (cat#ab62739), p27 (cat#ab32034), SOD2 (cat#ab13533) (Abcam), pJNK (cat#ab4668), JNK (cat#ab9252), HK2 (cat#ab2867) (Cell Signaling), RNA Pol II (cat#ab39097) (Active Motif), p21 (cat#ab05-345) (Upstate), Cyclin E (cat#ab247), Cyclin A (cat#ab239), Bcl2 (cat#ab7382), Cytochrome c (cat#ab13560), XOR (cat#ab20991), (Santa Cruz Biotechnology). Secondary antibodies were purchased from Vector Laboratories Inc. (Burlingame, CA, USA). The blots were stripped and re-probed with anti-β-actin (cat#A3854), (Sigma), anti-β-tubulin (cat#ab9104), or c23 (cat#ab55486), (Santa Cruz Biotechnology) to determine equivalent loading (46). Images were photographed using ECL (Millipore) on Syngene G: Box system (Cambridge, UK) using GeneSys software.

**Transfection**

5x10³ cells were seeded onto 96-well plates and two hours prior to transfection, cell media was replaced with OptiMEM (GIBCO, Life Technologies). Transfection with 70 nM duplex HK2, 50nM SIRT6 and Nrf2 or non-specific (NS) siRNA (Thermo Fischer Scientific) was carried out using Lipofectamine RNAi Max reagent (Life Technologies-Invitrogen) as described previously (45). Similarly, transfection with either 10 ng Renilla luciferase expression vector (pRL-TK) or 0.3 μg HIF-1α luciferase construct was performed using Lipofectamine 2000 (Life Technologies) and luciferase activity was measured using Dual Luciferase assay kit according to the manufacturer’s protocol (Promega) using GloMax 96 microplate luminometer (Promega), as described previously (45).

**Confocal microscopy**

For immunofluorescence staining, cells were grown in 4 well chamber glass slide system (Nunc Lab Tek) and treated with JNK inhibitor (SP6001215) before depriving cells of glucose and treating with IL-1β. After washing with 1x PBS the cells were fixed in 4% PFA (paraformaldehyde) for 20 minutes and subsequently permeabilized by 0.1% triton-X and 0.1% BSA in 1x PBS for 10 minutes at room temperature. Cells were blocked with 2% BSA and 3% normal donkey serum in 1x PBS, incubated with anti-HK2 antibody at a dilution of 1:500, washed 3 times with 1x PBS, followed by Alexa 594 conjugated anti-rabbit antiserum (Invitrogen cat#ab21207) at a dilution of 1:500 for 1 hour at room temperature. Mounting was done with DAPI and the slides were visualised using Spinning Disk Confocal Microscope (ZEISS Observer.Z1). Multiple images were taken from different fields at 63x magnification. Image analysis was carried using ZEN 2.3 lite (Carl Zeiss) software to generate a line profile of the mean fluorescence intensity.

**Co-immunoprecipitation**

Endogenous HK2 was immunoprecipitated with anti-HK2 antibody from nuclear extracts obtained from treated and/or transfected cells. Briefly, nuclear lysates were incubated with 3 μg of the indicated antibody for 16 hours and subsequently incubated for 4 hours with a mixture of protein G/A-Sepharose beads (GE Health Care). The immunoprecipitated samples were resolved on 8 to 10% SDS-PAGE after
washing the beads five times in IP buffer. 10% input was also resolved. Western blot analysis was performed with the immunoprecipitates and inputs with specific antibodies.

**Xanthine oxidoreductase activity assay**
The Amplex® Red Xanthine/Xanthine Oxidase Assay Kit (cat# a22182) was used for detecting xanthine oxidase activity in the cell lysates according to the manufacturer’s instructions.

**Chromatin immunoprecipitation (ChIP) and ChIP-qPCR assay**
ChIP was performed by enzymatic DNA shearing (Chip-IT Enzymatic; Active Motif) to investigate Nrf2 binding on ARE site (-593 bp to -582 bp) of XOR promoter, as previously described (45). Cells were deprived of glucose in the presence or absence of IL-1β for 2-4 hours and fixed in 1% formaldehyde at room temperature for 8 minutes. Isolated nuclei were lysed and then enzymatically sheared with the Enzymatic Shearing kit (Active Motif). Antibodies against Nrf2 or HK2 were used for IP. Following reverse cross-linking and DNA purification, DNA from input (1:10 diluted) or immunoprecipitated samples were assayed by qRT-PCR. The primers spanning the ARE site on XOR promoter were as follows: XOR ChIP primer F- 5’-TTTACAAGCACTCCAAAAA - 3’ and R- 5’- TGAACCTGACTCAAATCTTG - 3’.

**Quantitative real time PCR**
To analyse mRNA levels of different genes in cells treated with IL-1β in the presence or absence of glucose or HK2 knockdown. Real time PCR was performed as described previously (13) using ViiA7 Real Time thermocycler (Applied Biosystems Inc.) and results were plotted as fold change over control. All samples were normalised with their respective 18S rRNA CT values. qRT-PCR primers used were as follows:

| Gene   | Primer          |
|--------|-----------------|
| XOR    | Forward         |
|        | 5’-GGACACAGTTGGCTTGTGAAGGT-3’ |
|        | Reverse         |
|        | 5’-GGAAGGTGGTTTTGCAACAGC-3’ |
| IL-1β  | Forward         |
|        | 5’-GACCTTCCAGGAGAATGACC-3’ |
|        | Reverse         |
|        | 5’-GGCTTATCCTTTCAACACG-3’ |
| NLRP3  | Forward         |
|        | 5’-CTTCTCTGATGACCGCCAAG-3’ |
|        | Reverse         |
|        | 5’-GCGACAAAACCTGAAAGGAAG-3’ |
| HMOX1  | Forward         |
|        | 5’-GGGTGTATAGAGAAGCCAAGA-3’ |
|        | Reverse         |
|        | 5’-AGCTCTGTCAGACTTCTCAA-3’ |
| HK2    | Forward         |
|        | 5’-TCGCCATCTGCTTGGCTACTTCC-3’ |
|        | Reverse         |
|        | 5’-CTTCTGAGGCCCATGGTCCGT-3’ |
| 18SrRNA| Forward         |
|        | 5’-CAGCCACCCCGAGATTGAGCA-3’ |
|        | Reverse         |
|        | 5’-TACGGGACGGCGGTGTG-3’ |

**Statistical Analysis**
All comparisons between groups were performed either by two-tailed paired Students t-test or one-way ANOVA (Bonferroni’s Multiple Comparison Test) for multiple comparisons between more than two groups. All p values of less than 0.05 were taken as significant.
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Conflict of interest

The authors declare no conflict of interest.

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Abbreviations: IL-1β, interleukin 1 beta; ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; HK2, hexokinase 2; SIRT6, sirtuin 6; Nrf2, nuclear factor erythroid 2-related factor 2; XOR, xanthine oxidoreductase; NAc, N-acetyl-L-cysteine; NLRP3, NOD-like receptor family pyrin domain-containing protein 3.
**LEGENDS**

**Figure 1. Glucose starvation sensitizes glioma cells to IL-1β-induced apoptosis in a ROS dependent manner.**

(a) IL-1β induces glioma cell death under glucose deprivation (b) Increase in DHE fluorescence intensity depicting heightened ROS generation in glucose-deprived IL-1β-treated cells. (c) Increase in absorbance representing rescue of cell death by ROS inhibitor NAc. (d) Western blots demonstrating increased pJNK levels in glioma cells treated with IL-1β in the presence or absence of glucose. (e) MTS assay showing JNK-independent cell death. Inset shows the efficacy of JNK inhibitor. The graphs represent scatter plots with each data point representing (a, c, and e) average absorbance values depicting glioma cell viability and (b) average fluorescence intensities depicting ROS levels. (-)G denotes glucose-free DMEM. SP600125 (JNK inhibitor). One-way ANOVA (Bonferroni's Multiple Comparison Test) was used for statistical analysis. (n=4 in a, c, and e), (n=3 in b). p<0.05*, p<0.01**, p<0.001***

**Figure 2. SIRT6 affects cell viability through regulation of redox homeostasis**

(a) Western blot demonstrating SIRT6 levels in IL-1β-treated glioma cells in the presence or absence of glucose. Blots are representative images of three independent experiments showing similar results. Blots were re-probed for β-actin to establish equivalent loading. Densitometry data depicting fold change in SIRT6 expression over control under indicated treatment conditions normalized to corresponding loading controls are shown. (b) siRNA mediated knockdown of SIRT6 increases and (c) SIRT6 over-expression decreases viability of glioma cells deprived of glucose and treated with IL-1β, as determined by MTS assay. Insets (b and c) show knockdown efficiency of SIRT6 siRNA and increased SIRT6 expression upon transfection with SIRT6-OE construct. (d, e) DHE fluorescence intensity depicting ROS
generation in glucose-deprived IL-1β-treated cells is SIRT6 dependent. (f) SIRT6 regulates SOD2 expression under combination of inflammatory and metabolic stress. Densitometry data depict fold change in SOD2 expression over control under indicated treatment conditions normalized to corresponding loading controls. Each data point in the scatter plots represents (b and c) average absorbance values depicting glioma cell viability (n=4) and (d and e) fluorescence values depicting ROS levels under indicated treatment conditions from independent experiments (n=3). (-)G denotes glucose-free DMEM. NS siRNA (non-specific siRNA); SIRT6OE (SIRT6 over-expression). Two-tailed paired students t-test (b and c) and one-way ANOVA (Bonferroni’s Multiple Comparison Test) (a, d, e and f) were used for statistics analysis. p<0.05*, p<0.01**, p<0.001***

**Figure 3. IL-1β induces JNK-dependent nuclear localization of HK2.** Western blot demonstrating (a) cytosolic and (b) nuclear HK2 levels in glioma cells treated with IL-1β in the presence or absence of glucose. (c) qRT-PCR analysis shows increased IL-1β and NLRP3 mRNA levels in cells treated with IL-1β under glucose deprivation. Each data point in the scatter plots represents fold change with respect to (-G) from independent experiments (n=4). (d) Western blot demonstrating nuclear HK2 levels in cells treated with or without IL-1β or SP600125 in the presence or absence of glucose. Western blots are representative images of three independent experiments showing similar results. Blots were re-probed for β-actin or c23 to establish equivalent loading. (e) JNK regulates nuclear localization of HK2. Immunofluorescence microscopy revealed nuclear HK2 localization in glucose-deprived cells in the presence of IL-1β. Treatment with JNK inhibitor (SP600125) prevented HK2 localization to nucleus. Cells were immune-stained with anti-HK2 (HK2, red). The nucleus is marked with DAPI (blue). Merged images (Merge) are shown. Representative images of 63x magnification from three independent experiments are shown for indicated conditions. SP denotes JNK inhibitor (SP600125). Adjacent line profiles show mean fluorescence intensities
of HK2 and DAPI measured by ZEN lite 2.3 (Carl Zeiss) software. (Scale bars, 10μm), p<0.05*

**Figure 4. SIRT6 regulates Nrf2-HK2 association.**

(a) Western blot depicts elevated nuclear Nrf2 expression in IL-1β–treated glioma cells under glucose starvation. (b) siRNA mediated knockdown of SIRT6 prevents nuclear Nrf2 accumulation in cells treated with IL-1β under glucose starvation, as demonstrated by Western blot analysis. Blots were re-probed for c23 to establish equivalent loading. (c) SIRT6 positively regulates nuclear HK2-Nrf2 interaction in the presence of IL-1β in glucose-starved glioma cells. Co-immunoprecipitation shows decreased HK2-Nrf2 association in IL-1β–treated glucose-deprived cells upon siRNA mediated knockdown of SIRT6. Confounding effect of unequal precipitation on the interaction studies was ruled out by almost equal IgG levels in each condition. Blots are representative images of three independent experiments showing similar results.

**Figure 5. HK2 serves as a coactivator of Nrf2 in regulating XOR expression.** (a) IL-1β increases XOR levels under conditions of glucose deprivation. siRNA mediated knockdown of either (b) Nrf2 or (c) HK2 prevents IL-1β-induced XOR expression in glucose-deprived cells, as depicted by Western blot analysis. Insets show knockdown efficiency of NRF2 and HK2 siRNA. Western blot images are representation of three independent experiments showing similar results. Blots were re-probed for β-actin to establish equivalent loading. Densitometry data of fold change in XOR expression over control under different treatment conditions normalized to corresponding loading controls are shown. Each data point in the scatter plot denotes fold change with respect to control from independent experiments (n=3). (d) ChIP performed on the region having ARE binding site on XOR promoter in T98G and U87MG glioma cells, indicate increased Nrf2 and HK2 binding at ARE site in IL-1β-treated glucose-deprived cells. (e) ChIP assay depicts decreased HK2 binding at XOR promoter upon
siRNA-mediated Nrf2 knockdown in IL-1β-treated glucose-deprived cells. Diluted input (5%) was used as a positive control. Relative enrichment was calculated with respect to control levels after correction for background signals. Graphs are representative data of three independent experiments. (-)G denotes glucose deprived DMEM. One-way ANOVA (Bonferroni’s Multiple Comparison Test) was used for statistical analysis. p<0.05*, p<0.01**, p<0.001***. (f) Schematic depiction of the importance of HK2 as a coactivator of Nrf2 in regulation of XOR under inflammatory and metabolic stress.
Hexokinase 2 and nuclear factor erythroid 2-related factor 2 transcriptionally coactivate xanthine oxidoreductase expression in stressed glioma cells
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