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SIRT6 regulated nucleosomal occupancy affects Hexokinase 2 expression

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ABSTRACT

To understand the molecular association between inflammation and dysregulated metabolism in glioblastoma, the effect of IL-1β on Hexokinase 2 (HK2) expression was investigated. IL-1β induced HK2 expression was accompanied by heightened SIRT6 and MZF1 levels. IL-1β mediated overall decrease in chromatin compactness on HK2 promoter involved diminished nucleosomal occupancy around the most labile region bearing MZF1 sites. Importantly, SIRT6 and MZF1 served as negative regulators of HK2. Ectopic SIRT6 induced formation and recruitment of MZF1-SIRT6 complex to MZF1 site was concomitant with increased nucleosomal occupancy. The function of SIRT6 as co-repressor of MZF1 was inconspicuous in cells treated with IL-1β alone, as IL-1β-induced HIF-1α prevented SIRT6 availability for interaction with MZF1. Taken together, SIRT6 over-expression establishes a condition whereby reconfiguration of the HK2 promoter chromatin structure makes it receptive to interaction with MZF1/SIRT6 complex, thereby favouring a regulatory state conducive to diminished transcription.

1. Introduction

Aerobic glycolysis or the Warburg effect is a core hallmark of cancer cells [1]. In glioblastoma multiforme (GBM) high dependency on glycolysis [2] is accompanied by elevated expression of Hexokinase 2 (HK2) - the enzyme that catalyzes the first committed step of glycolysis. The ability of elevated HK2 levels to maintain high glucose catabolic rates in GBM makes it an attractive anti-glioma therapeutic strategy [3]. Besides dysregulated metabolism, the involvement of inflammation in promoting tumor growth through regulation of mitochondrial bioenergetics is known [4]. Also, emerging evidences are linking metabolism and activation of inflammatory pathways in the progression of metabolic disorders [5]. Despite the recognized interplay between metabolism and inflammation, the molecular link connecting the two crucial drivers of tumor progression still remains largely elusive.

SIRT6 - an NAD^+ dependent class III Histone deacetylase (HDAC) not only serves as a tumor suppressor by regulating aerobic glycolysis in cancer cells [6] but also exhibits context dependent pro- and anti-inflammatory responses [7]. SIRT6 occupancy on several promoters in response to pro-inflammatory TNFα has been found to be highly dynamic [8]. Nucleosome association promotes the deacetylase activity of SIRT6 [9], and expression of several genes associated with glucose metabolism are suppressed by SIRT6 mediated deacetylation at their promoters [10]. Nucleosome dynamics affects access of transcriptional machinery to specific sites crucial for orchestrating transcriptional changes [11–13]. IL-1β induces HIF-1α activation in glioma [14], and SIRT6 regulates glucose homeostasis via HIF-1α [15]. Upregulated HK2 contributes to hypoxia tolerance mediated by activation of HIF-1α [16], and SIRT6 affects glucose homeostasis via HIF-1α [15]. As interplay of nucleosome and transcription factors determines chromatin landscape to affect gene expression and since there is a considerable overlap between transcriptional circuitries in metabolic adaptation and inflammation, we investigated whether IL-1β affects HK2 gene by reorganization of nucleosomal landscape.

2. Results

2.1. IL-1β increases hexokinase 2 transcript and protein levels

IL-1β present in abundance in glioblastoma plays a critical role in its progression [14]. As inflammation is linked with regulation of a number of metabolic genes [5], we checked the transcript levels of genes involved in glucose metabolism under inflammatory conditions using human metabolic profiler qRT-PCR array. Treatment of U87MG glioma cells with IL-1β for 24hr increased HK2 mRNA levels ~2 folds as compared to untreated control (Supplementary Table 1). To validate the metabolic PCR array results, Western blot and qRT-PCR analysis was performed. IL-1β treatment increased HK2 mRNA transcript (Fig. 1a) and protein levels (Fig. 1b) in a time dependent manner. The 24hr treatment regime was selected for subsequent experiments because all cell lines showed maximal increase in HK2 expression at 24hr.

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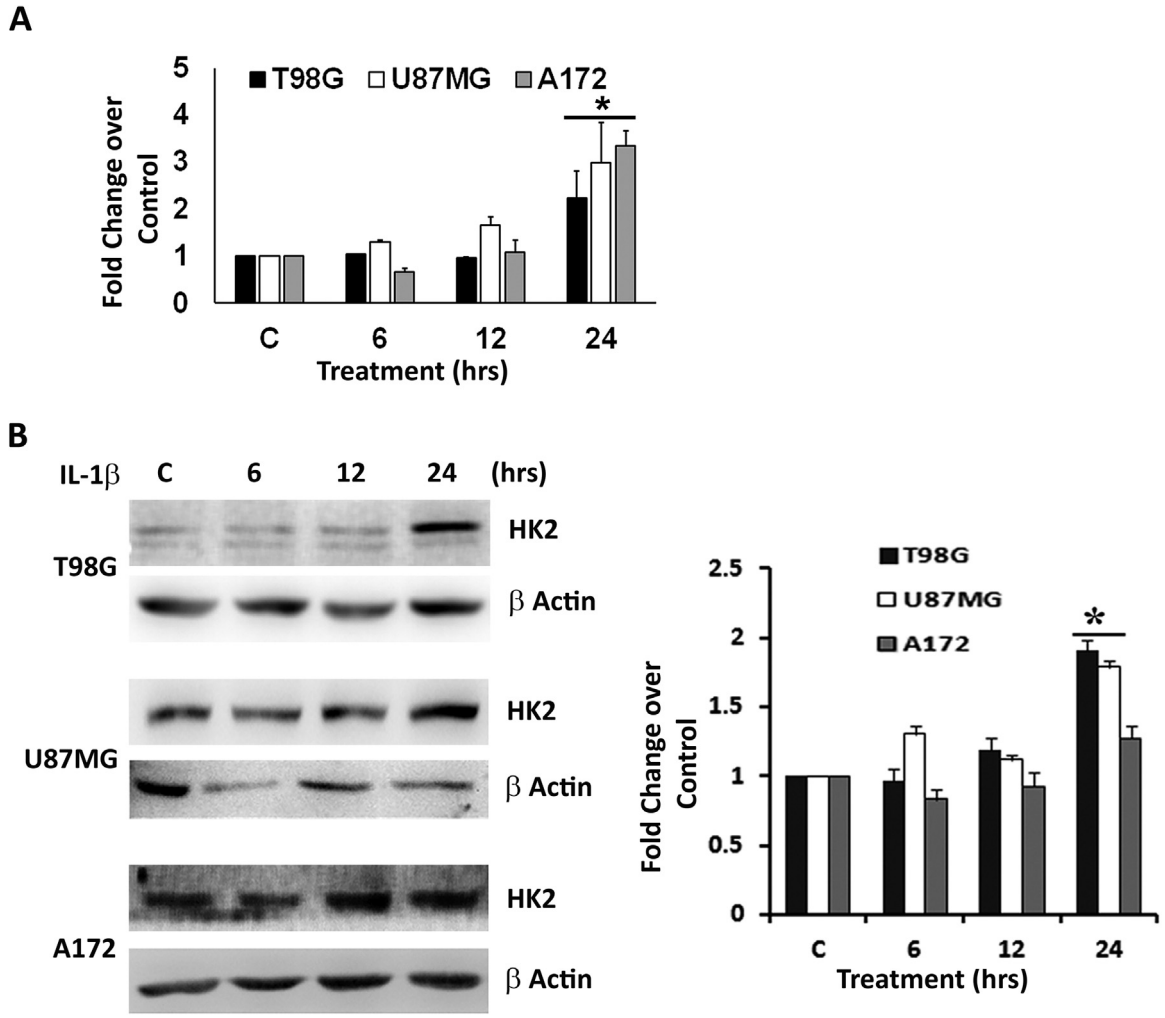

Fig. 1. IL-1β induces HK2 expression in glioma cells. (a) The qPCR graph shows relative fold change in HK2 mRNA levels over control upon IL-1β treatment. qPCR data bars indicate mean relative fold change values ± SEM of two independent sets. * Significant change from control ($P < 0.05$). (b) HK2 expression in glioma cells treated with IL-1β for different time points as demonstrated by Western blot. A representative blot is shown from three independent experiments with identical results. Blots were re-probed for β-actin to establish equivalent loading. Densitometric measurements were performed on the immunoblots and values indicate fold change over control. Bands were normalized to their corresponding β-actin levels.

2.2. Nucleosome positioning along HK2 promoter identifies a highly labile region

Nucleosome occupancy and positioning regulate gene expression [17]. To address the nucleosome signature of HK2 promoter, we employed nucleosome-scanning assay (NuSA) to provide an idea about nucleosome position on DNA [17,18]. The positional stability of nucleosomes on HK2 promoter was determined as described [19]. To resolve the location of nucleosomes and achieve a high resolution map, the position, size and overlap of amplicons was selected as described (Fig. 2a and Supplementary Table S1). On the HK2 promoter region, we identified a highly labile nucleosome around −456 bp to −693 bp region that could only be detected under cross-linked conditions (Fig. 2b).

2.3. IL-1β induces chromatin re-organisation at HK2 promoter

Changes in gene expression correlate with alterations in nucleosome positioning [20]. We performed NuSA to determine whether IL-1β affects the nucleosomal landscape of HK2 promoter to regulate its expression. IL-1β treatment resulted in low enrichment of overall nucleosomal DNA comprising the promoter region of HK2 gene, as depicted by NuSA (Fig. 2c). Decreased PCR amplification for the region around −687 bp was observed across all glioma cell lines investigated. Thus, HK2 promoter becomes more accessible to MNase digestion following IL-1β treatment and displayed chromatin in a more open and partially nucleosome-free structure.

2.4. IL-1β induced HK2 is negatively regulated by SIRT6

Sirtuins and other HDACs play a role in chromatin compaction [21] and SIRT6 suppresses expression of glucose metabolic genes [10]. On investigating the status of SIRT6 in IL-1β treated cells with elevated HK2 levels and decreased chromatin compaction, an increase in nuclear SIRT6 levels was observed (Fig. 3a). SIRT6 served as a negative regulator of HK2 expression, as siRNA mediated knock-down of SIRT6 either alone or in presence of IL-1β increased HK2 expression (Fig. 3b). Conversely, SIRT6 over-expression abrogated IL-1β induced increase in HK2 levels (Fig. 3c). Under normal conditions, HK2 is bound to the mitochondrial outer membrane via its association with voltage dependent anionic channel (VDAC) which promotes the glycolytic function of HK2 while preventing the intrinsic cell death pathway [22]. Increased association of HK2 with mitochondrial membrane upon siRNA mediated knock-down of SIRT6 (Fig. 3d), indicated the role of SIRT6 as negative regulator of HK2 function in addition to its expression.

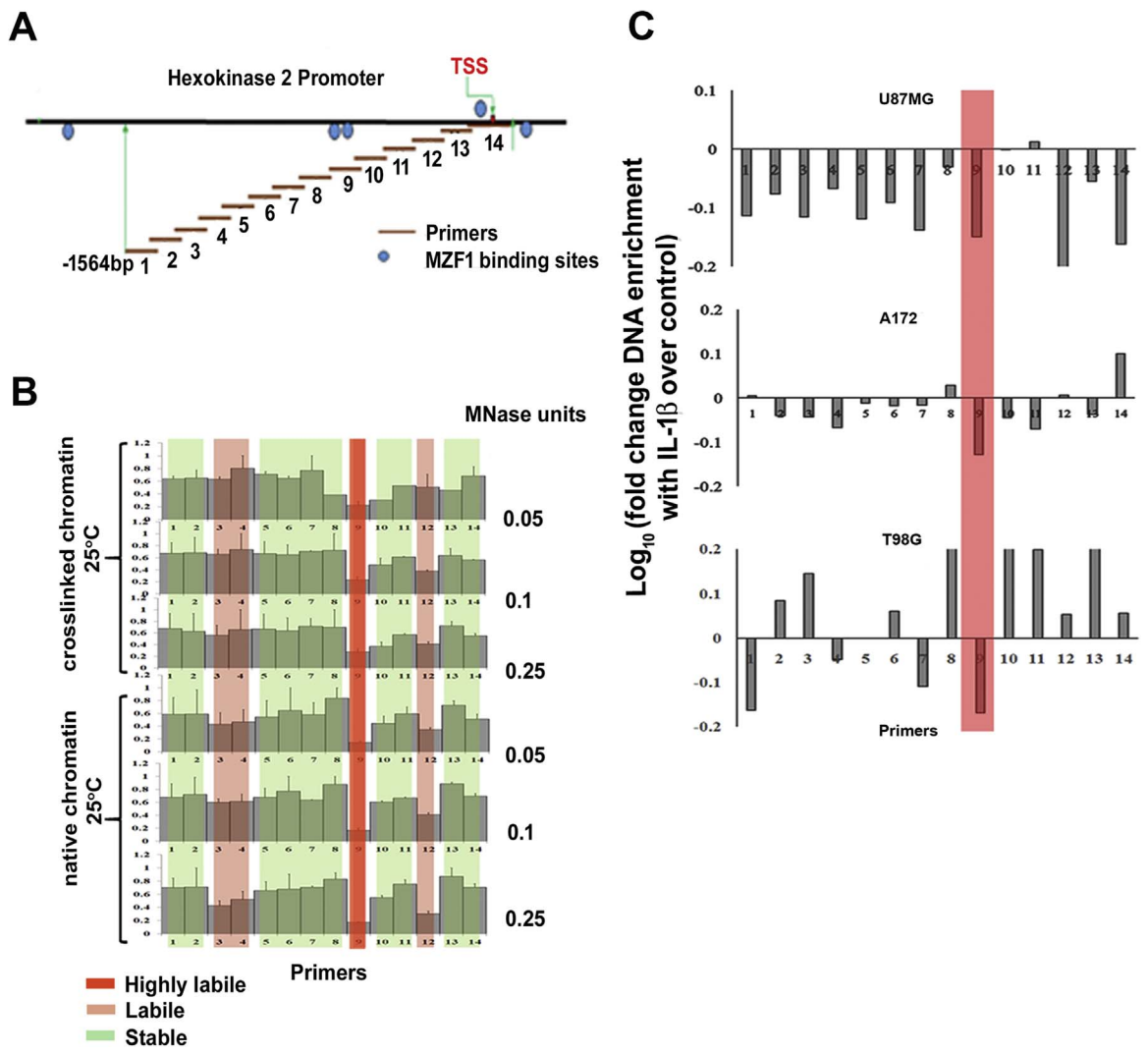

Fig. 2. IL-1β affects nucleosomal landscape of HK2 promoter. (a) Schematic showing HK2 promoter region. The region under study is depicted here (−1823 bp to ~ +100 bp) with NuSA and ChIP primers, MZF1 binding sites and Transcription Start Site (TSS). (b) NuSA on mononucleosomal native and cross-linked chromatin reveals a map of nucleosome position and stability. Bars represent nucleosomal DNA enrichments relative to undigested DNA. qPCR data bars indicate mean values ± SEM of two independent sets. Highly labile, labile and stable regions (depicted by colour codes) on HK2 promoter are indicated by vertical overlays. (c) NuSA graphs for glioma cells depicting $\log_{10}$ (fold change DNA enrichment with IL-1β over control) across different regions. The results are average of two independent mononucleosomal preparations. qPCR values are corrected using appropriate genomic DNA controls. Vertical red overlay depicts region of interest.

2.5. Ectopic SIRT6 expression promotes chromatin compaction at HK2 promoter

To ascertain the mechanism regulating the inhibitory effect of SIRT6 on HK2 expression, we looked into alterations in chromatin architecture upon ectopic SIRT6 expression. NuSA experiments performed in SIRT6 over-expressing glioma cells revealed higher enrichment of DNA along the HK2 promoter (Fig. 4), indicating increased number of nucleosomes or other DNA binding proteins. This result was in conjunction with the previous findings that sirtuins and other HDACs play a role in chromatin compaction and heterochromatin formation [21]. Following SIRT6 over-expression, HK2 promoter remained less accessible to MNase digestion, suggesting that decreased local chromatin accessibility is associated with attenuation of HK2 expression (Fig. 4). The extent of compaction was cell line dependent (Fig. 4).

2.6. MZF1 negatively regulates HK2 expression

NuSA indicated that IL-1β increases the opening up of chromatin at HK2 promoter which could facilitate binding of transcriptional machinery and other regulatory proteins to effect HK2 transcription. Interestingly, a number of Myeloid Zinc Finger-1 (MZF1) binding sites are located on the promoter as well as within HK2 gene. As DNA binding protein MZF1 is known to play both activating [23,24] and repressive roles [25] in transcription of genes, we investigated its role in IL-1β-induced HK2 expression. IL-1β increased MZF1 expression in a time dependent manner (Fig. 5a). MZF1 exerted a negative role on HK2 expression as siRNA mediated MZF1 knock-down increased HK2 levels (Fig. 5b). Since MZF1 and SIRT6 served as negative regulators of HK2, the correlation between SIRT6 and MZF1 was investigated. Decreased MZF1 levels observed upon siRNA mediated SIRT6 knock-down (Fig. 5c), suggested SIRT6 to be positive regulator of IL-1β induced MZF1 expression.

2.7. SIRT6-MZF1 complex regulates HK2

Since IL-1β induced SIRT6 and MZF1 served as negative regulators of HK2, we investigated the interaction between these two nuclear proteins and its combined effect upon HK2 expression. We based our hypothesis on the fact that MZF1, being a transcription factor and containing a DNA binding domain, could bind to HK2 promoter

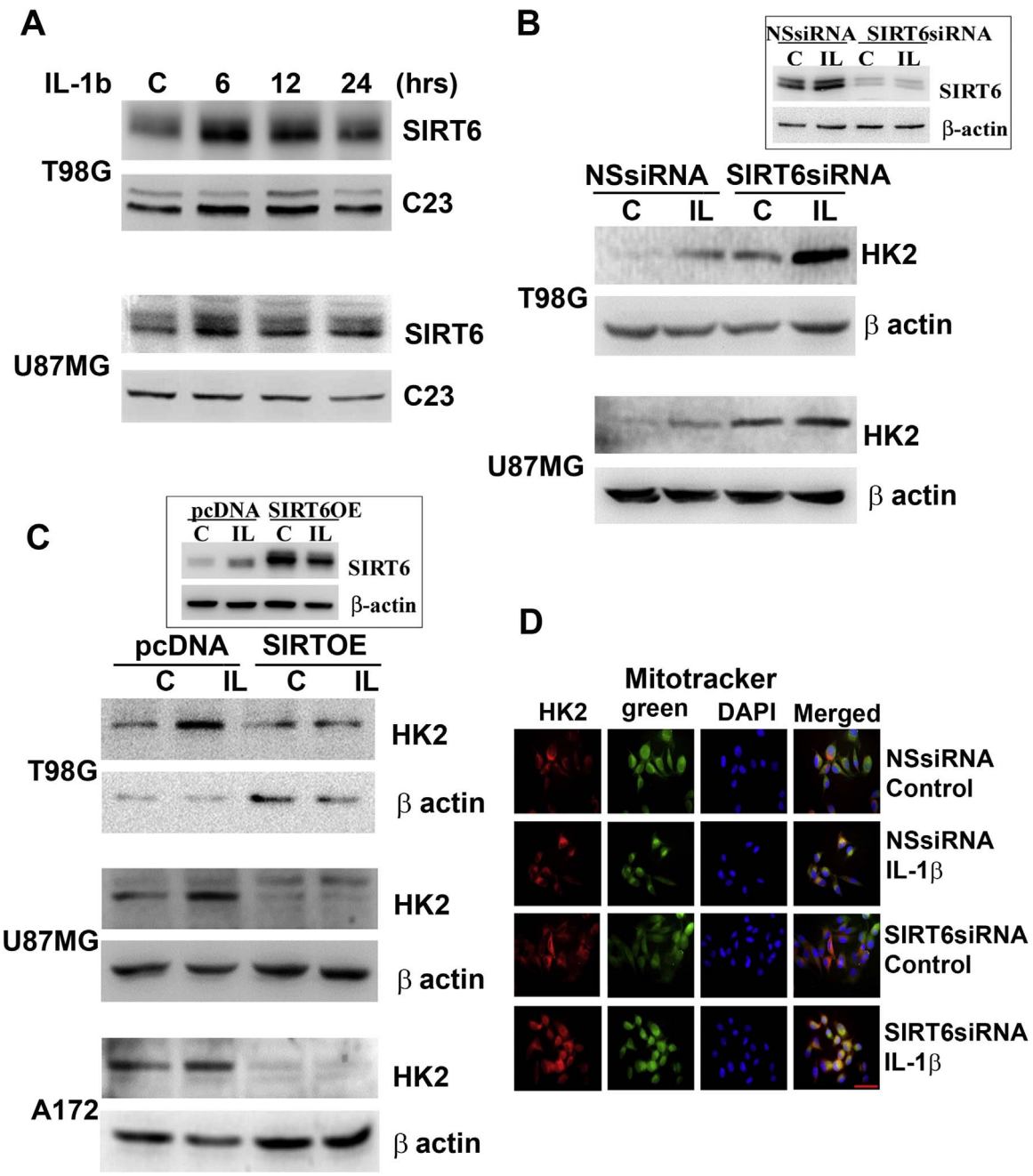

Fig. 3. IL-1β induced SIRT6 negatively regulates HK2 expression. (a) Western blot analysis depicting IL-1β induced SIRT6 levels in glioma cells. (b) siRNA mediated knock-down of SIRT6 increases HK2 protein expression in presence and absence of IL-1β. Inset shows knockdown efficiency of SIRT6 siRNA. (c) SIRT6 over-expression decreases HK2 protein levels in glioma cells. Inset shows increased SIRT6 expression upon transfection with SIRT6-OE construct. Representative blots (*a*-c) are shown from three independent experiments with identical results. Blots were re-probed for c23 or β-actin to establish equivalent loading. (d) siRNA mediated SIRT6 knock-down increases co-localisation of HK2 and mitochondria upon IL-1β treatment. Immunostaining with anti-HK2 antibody (red), mitotracker green dye (green) and DAPI (blue) at 40x magnification is depicted.

directly; whereas, SIRT6 - a HDAC could interact with MZF1 to subsequently affect HK2 gene transcription. Co-immunoprecipitation revealed a decreased interaction between SIRT6 and MZF1 in IL-1β treated glioma cells (Fig. 6a). Interestingly, ectopic SIRT6 expression increased SIRT6- MZF1 interaction in the presence of IL-1β (Fig. 6a). The inconspicuous interaction observed between SIRT6-MZF1 in the presence of IL-1β; was restored upon SIRT6 over-expression (Fig. 6a). This could be explained by the fact that SIRT6 over-expression further elevates IL-1β induced increase in MZF1 levels (Fig. 6b). Thus, SIRT6 over-expression induced SIRT6-MZF1 complex formation is concomitant with ability of these negative regulators to attenuate HK2 expression.

2.8. SIRT6 over-expression induces MZF1 binding to MZF1 site

Since MZF1-SIRT6 complex is associated with negative regulation of HK2 expression upon SIRT6 over-expression, ChIP was performed to inquire MZF1 binding at these sites. While treatment with IL-1β alone had no significant effect on MZF1 recruitment to MZF1 sites, SIRT6 over-expression increased enrichment of MZF1 to this site both in the presence and absence of IL-1β (Fig. 6c). Together with immune-precipitation, ChIP and NuSA results clearly indicated that abundant SIRT6 facilitates formation of MZF1-SIRT6 complex and its recruitment to MZF1 cognate sites (region spanning amplicon 9) on HK2 promoter. The assembly of MZF1-SIRT6 complex and its recruitment

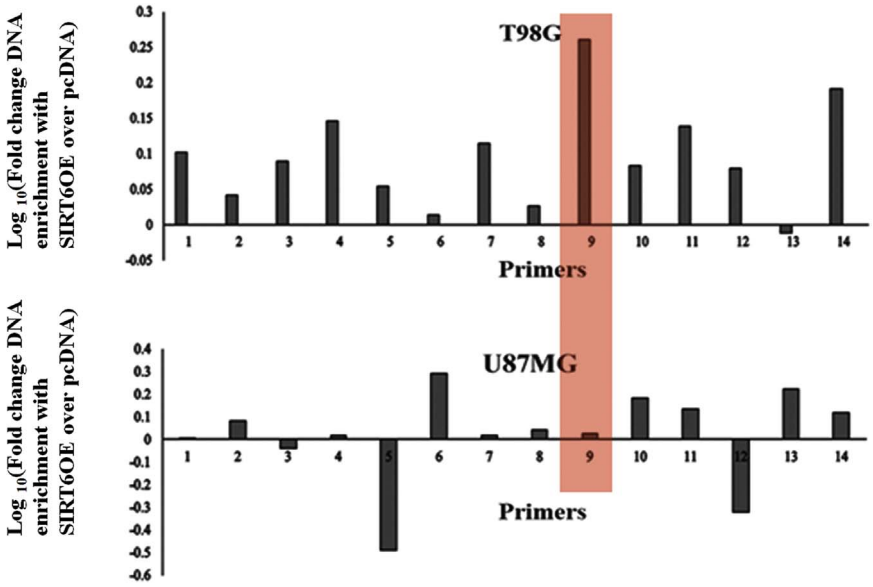

Fig. 4. IL-1β induced SIRT6 affects chromatin architecture of HK2 promoter. SIRT6 over-expression results in chromatin re-organisation at HK2 promoter. NuSA on mononucleosomal DNA and subsequent qPCR shows increased DNA enrichment upon SIRT6 over-expression as compared to pcDNA control cells. The results are average of two independent mononucleosomal preparations. qPCR values are corrected using appropriate genomic DNA controls. Vertical red overlay depicts region of interest.

to HK2 promoter, coupled with site specific alteration of nucleosomal architecture, leads to SIRT6 dependent inhibition of HK2 transcription.

2.9. IL-1β induced HIF-1α regulates SIRT6 expression

SIRT6 acts as a co-repressor of HIF-1α transcriptional activity [10]. As we have shown that IL-1β induces HIF-1α activation [14], we questioned whether elevated level of HIF-1α in IL-1β treated cells regulates induction of SIRT6 to consequently affect HK2 expression. A dramatic increase in SIRT6 expression was observed upon siRNA mediated knock-down of HIF-1α in IL-1β treated cells (Fig. 6d). Interestingly, HIF-1α was found to regulate SIRT6 and MZF1 interaction, as knock-down of HIF-1α increased SIRT6-MZF1complex formation (Fig. 6e). These results demonstrate that HIF-1α restricts the ability of SIRT6 to bind MZF1 under conditions of relatively low SIRT6 levels.

3. Discussion

Our observation that SIRT6 negatively regulates HK2 is in agreement with recent findings that suppression of SIRT6 enhances glycolysis [26]. The transcriptional repression of HK2 is dependent on the *'relative abundance'* of SIRT6, as elevated SIRT6 levels are translated into decreased HK2 gene transcription. SIRT6 dependent formation of MZF1-SIRT6 complex, and its recruitment to MZF1 sites of the HK2 promoter showing greatest nucleosomal instability; is absolutely essential for dampening HK2 gene expression under conditions of SIRT6 abundance. The inconspicuous formation of MZF1-SIRT6 complex and subsequently their decreased binding to MZF1 site in IL-1β treated glioma cell is HIF-1α regulated. The involvement of MZF1 in transcriptional regulation [27,28], is reflected by the inability of SIRT6 to suppress HK2 expression in the absence of MZF1. Also, this role of MZF1 as a negative regulator of HK2 expression correlates with its tumor suppressive role [29]. Nucleosome association promotes the deacetylase activity of SIRT6 [9], and expression of several genes associated with glucose metabolism are suppressed by SIRT6 mediated deacetylation at their promoters. Complexity of nucleosome positioning not only contributes to the flexibility of gene expression [20], but usage of transcription factor binding site correlates with nucleosomal occupancy [30]. This could account for increased nucleosomal occupancy upon engagement of MZF1 site under conditions of relative SIRT6 abundance.

Inflammation driven HIF-1α stands out as an important player in glioma biology [14,31–33]. The ability of HIF-1α knock-down to rescue SIRT6 dependent changes in glycolytic gene expression is known [10]. Moreover, SIRT6 over-expression is known to decrease HK2- a HIF-1α target [34]. The increase in SIRT6 levels upon HIF-1α knock-down points towards this ability of HIF-1α to affect SIRT6 dependent changes in expression of glycolytic gene such as HK2. As repression of HK2 is dependent on relative abundance of SIRT6, it is likely that low SIRT6 levels in IL-1β treated cells with elevated HIF-1α levels are insufficient to repress HK2. Thus, a negative feedback loop regulates IL-1β induced increase in HK2 to override SIRT6 repressive function. Thus, HIF-1α dependent SIRT6 availability establishes a condition whereby reconfiguration of the HK2 promoter chromatin structure makes it receptive to interaction with MZF1/SIRT6 complex, to favour a regulatory state conducive to diminished transcription (Graphical Abstract).

Our findings implicate SIRT6 as the node in integrating metabolic signals under inflammatory conditions. Given that inflammation rewires energy metabolism in the tumor microenvironment [35], the prospect of targeting altered metabolism in inflammation has been suggested as a substantial therapeutic promise [36]. The ability of elevated HK2 levels to maintain high glucose catabolic rates in GBM makes it an attractive anti-glioma therapeutic strategy [3]. Since HK2 inhibition would restrict glucose utilisation at the very initial step, endeavours to target HK2 expression and functional activation by enhancing SIRT6, possibly through HIF-1α inhibition would serve as effective therapeutic strategy. Thus, therapies targeted towards elevating SIRT6 levels in tumor cells through HIF-1α inhibition, could convert a pro-tumor IL-1β inflammatory milieu into an anti-tumor metabolically challenged system.

4. Materials and methods

4.1. Cell Culture and Treatment

Glioblastoma cell lines U87MG, T98G and A172 purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were grown in Dulbecco's modified eagle medium (DMEM from Life Technologies, Carlsbad, CA, USA) substituted with 10% fetal bovine

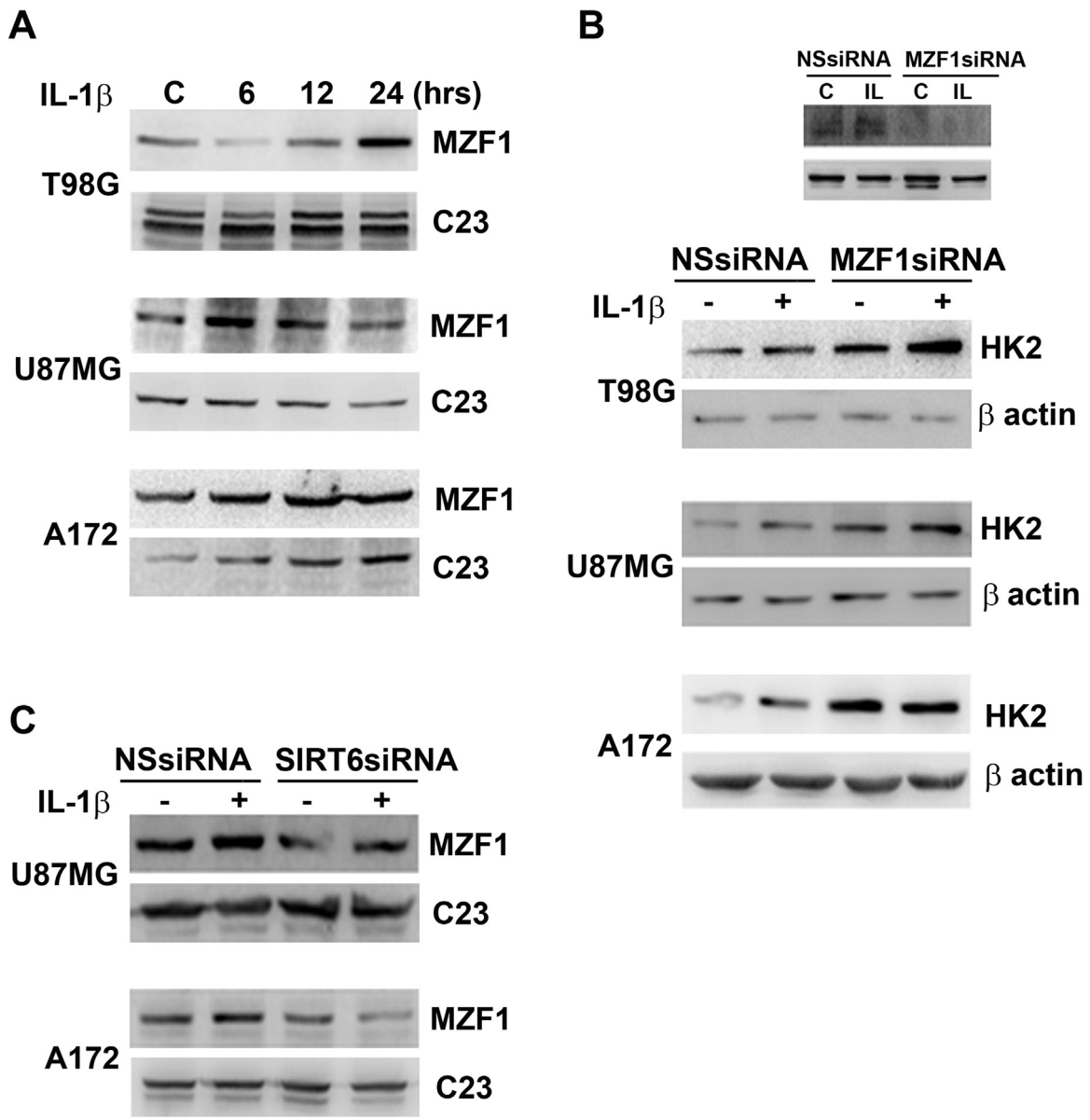

Fig. 5. SIRT6 regulated MZF1 is necessary for SIRT6 dependent abrogation of HK2 expression. (a) IL-1β increases MZF1 levels in a time dependent manner as depicted by Western blot. (b) siRNA mediated MZF1 knock-down increases HK2 levels as depicted by Western blot analysis. Inset shows the transfection efficiency of MZF1 siRNA. (c) SIRT6 positively regulates MZF1 expression. siRNA mediated knock-down of SIRT6 decreases IL-1β induced MZF1 expression as shown by Western blot analysis. Representative blots (*a*-c) are shown from three independent experiments with identical results. Blots were re-probed for c23 or β-actin to establish equivalent loading. NSsiRNA Non-specific siRNA; SIRT6OE SIRT6 over-expression.

serum (FBS). After attaining about 60–70% confluency, cell media was replaced with serum free DMEM. After 6hr of serum starvation, cells were treated with 10 ng/ml IL-1β (R & D Systems). All the reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

4.2. Human Metabolism qRT-PCR array

qRT-PCR was performed using The Human Glucose Metabolism RT2 Profiler containing 84 metabolism-related genes (Qiagen, Hilden, Germany) as described previously [37]. Five housekeeping genes were included on the array (B2M, HPRT1, RPL13A, GAPDH and ACTB) to normalize the transcript levels. U87MG cells were treated with IL-1β for 24hr and results were analysed as per user manual guidelines using integrated web-based software package for the PCR Array System (RT2 Profiler PCR Array Human Glucose Metabolism PAHS-006Z).

4.3. Western blot analysis

Protein from whole cell lysates or nuclear lysates were isolated from control and treated glioma cells, and Western blot was performed as described [14]. The following antibodies were used- HK2 (Cell Signaling, Danvers, MA, USA), SIRT6, HIF-1α, MZF1 (Novus Biological, Cambridge Science Park, Cambridge, UK) β-actin (Sigma), c23 (Santa Cruz Biotechnology, CA, USA). Secondary antibodies were purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Images were photographed using ECL (Millipore) on Syngene G: Box system (Cambridge, UK) using Gene-Sys software.

4.4. Nucleosomal Scanning Assay

$6x10^6$ glioma cells treated with or without IL-1β were collected 24 h post treatment. Nuclei purification and MNase digestion (0.1U, 5 min, 25 °C) was performed according to manufacturer's protocol using EZ

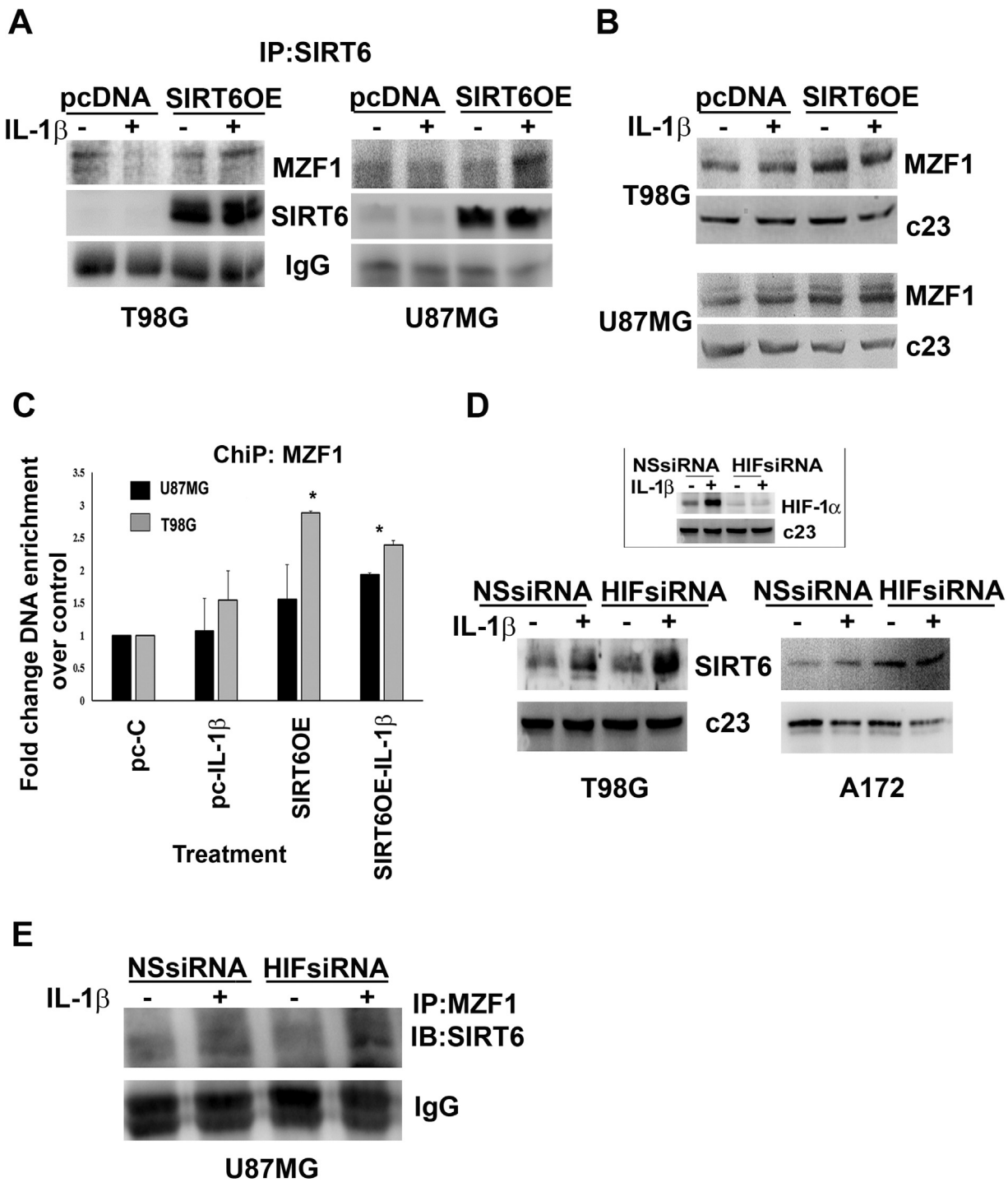

Fig. 6. HIF-1α dependent SIRT6 abundance mediates association and recruitment of SIRT6-MZF1 complex on HK2 promoter. (a) Co-immunoprecipitation assay with anti-SIRT6 antibody shows that ectopic SIRT6 expression either alone or in combination with IL-1β causes increased nuclear interaction of SIRT6 and MZF1. IgG levels are shown to establish equivalent loading. (b) SIRT6 positively regulates MZF1 expression as depicted by Western blot. SIRT6-OE increases MZF1 expression in the presence or absence of IL-1β (c) ChIP was performed on the region having two MZF1 binding sites on HK2 promoter in T98G and U87MG glioma cells. Immunoprecipitation with MZF1 antibody and subsequent qPCR analysis shows enhanced DNA enrichment upon SIRT6 over-expression both in the presence or absence of IL-1β. Diluted input (5%) was used as a positive control. Relative enrichment was calculated with respect to pcDNA control levels after correction for background signals. Graph depicts representative ChIP-qPCR results averaged from three independent experiments. *Significant change from pcDNA control ($P < 0.05$). (d) Western blot depicts enhanced SIRT6 levels in glioma cells upon siRNA mediated HIF-1α knock-down. The inset shows transfection efficiency of HIF-1α siRNA. Representative blots are shown from three independent experiments with identical results. Blots were re-probed for c23 to establish equivalent loading. (e) HIF-1α regulates SIRT6-Mzf1 interaction. Co-immunoprecipitation assay with anti-SIRT6 antibody shows that siRNA mediated knock-down of HIF-1α increases nuclear interaction of SIRT6 with MZF1. IgG levels are shown to establish equivalent loading. Blots are representative images of two independent experiments showing similar results.

Nucleosomal DNA Prep kit (Zymo Research, Irvine, CA, USA). Mononucleosomal DNA obtained was run on 3% agarose gel to check proper shearing. Mononucleosomal DNA fraction was purified from the gel using QIAquick Gel Extraction kit (Qiagen). qRT-PCR was performed using a set of 14 overlapping primers (Supplementary Table 2) scanning the promoter of HK2 gene (~ −1600 bp from transcription start site). All the primers were purchased from Sigma Aldrich. Undigested genomic DNA was used as control and results were

normalized accordingly. Non-template controls were run for each primer.

4.5. Nucleosomal Stability Assay

Approximately, $9x10^6$ U87MG cells were seeded in 6×90 mm petriplates. After 24hr of serum starvation, cells in 3 of the plates were fixed with 1% formaldehyde. Fixed (F) and unfixed (UF) cells were administered three different doses of MNase −0.05, 0.1 and 0.25U for 5 min. NuSA and qRT-PCR analysis of the HK2 promoter yielded six sets of data (F-1, F-2, F-3, UF-1, UF-2, UF-3) for each sample. Fixed samples were reverse cross-linked, RNase A and proteinase K treated. and the DNA was purified by phenol chloroform method. qRT-PCR was performed using the same primer sets as in NuSA (Supplementary Table 2). Results were corrected using appropriate native, undigested genomic DNA samples.

4.6. Transfection

Glioma cell lines were seeded in antibiotic-free DMEM, and the media was replaced with OptiMEM (GIBCO, Life Technologies, Carlsbad, CA, USA) 2hr prior to transfection. Cells were transfected either with Flag-tagged SIRT6 plasmid, or empty vector (pcDNA) obtained from Addgene, as described previously [31]. Similarly, cells were transfected with 50 nmol/l duplex SIRT6 siRNA (cat#L-013306-00-0005), MZF1siRNA (cat#L-006578-00-0005), HIF-1α siRNA (cat# H-00765-00-0023), HK2 siRNA (cat# L-006735-00-0005), or non-targeting siRNA (cat#D-001210-03-20) (GE Healthcare Dharmacon Inc., Lafayette, CO, USA). Lipofectamine2000 or RNAi Max reagents (Life Technologies) were used for all transfections.

4.7. Quantitative Real Time PCR

To analyse the endogenous HK2 mRNA levels in IL-1β treated cells, RNA was isolated using RNeasy kit (Qiagen) and cDNA was synthesised using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) on Veriti Thermal cycler (Applied Biosystems). Real time PCR was performed as described previously [14] using ViiA7 Real Time thermocycler (Applied Biosystems Inc.) and results were plotted as fold change over control for HK2 mRNA transcript. All samples were normalized with their respective 18SrRNA *CT* values.

The qPCR primers used are listed as follows: HK2 forward and reverse, 5'-TCGCATCTGCTTGCCTACTTC −3′ and 5′-CTTCTGGAGCCCATTGTCCGT −3′, respectively; 18SrRNA forward and reverse, 5'-CAGCCACCCGAGATTGAGCA −3′ and 5′-TAGTAGCGACGGGCGGTGTG-3′, respectively.

4.8. Co-immunoprecipitation

Immunoprecipitation was performed with nuclear extracts (100 μg) obtained from glioma cells transfected with Flag-SIRT6 construct treated with or without IL-1β. Protein extracts were incubated with 2 μg of anti-SIRT6 (Abcam) overnight as described previously [38]. The lysates were then incubated with 30 μl of protein G Plus-sepharose (Amersham, GE Healthcare Bio-Sciences) at 25 °C for 2-4hr. Beads were pelleted, washed six times in immunoprecipitation buffer, and resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Western blots were performed to determine the recruitment of MZF1. Similarly, nuclear extracts from cells transfected with HIF-1α siRNA and treated with or without IL-1β were incubated with anti-Mzf1 and Western blots were performed to determine the recruitment of SIRT6.

4.9. Chromatin immuno-precipitation (ChIP) and ChIP qPCR Assay

ChIP was performed by enzymatic DNA shearing (Chip-IT Enzymatic; Active Motif) as previously described [31]. Cells transfected with pcDNA or Flag-SIRT6 were treated with or without IL-1β for 24hr and fixed in 1% formaldehyde at room temperature for 8 mins. Isolated nuclei were lysed and then enzymatically sheared with the Enzymatic Shearing kit (Active Motif). Antibodies against SIRT6 and MZF1were used for IP, and a non-specific IgG antibody (Abcam) was used as a control. Following reverse cross-linking and DNA purification, DNA from input (1:10 diluted) or immunoprecipitated samples were assayed by qRT-PCR. The MZF1 binding sites of the HK2 promoter were PCR amplified. qRT-PCR was performed on DNA recovered by ChIP with Power SYBR green PCR Master Mix (Applied BiosystemsInc.), with a ViiA7 real-time thermocycler (Applied Biosystems Inc.) for 40 cycles. The cycle thresholds (*CT*s) of immunoprecipitated material were normalized to their corresponding input DNA (5% input) and corrected by using non-specific IgG *CT*s to allow direct comparison of different conditions as described [31]. Relative (*n*-fold) enrichment was calculated with respect to the control levels. Non-template controls were run for the primers. The sequences of the primers used for qPCR analysis of the amplified regions were as follows: HK2 ChIP primer F- 5′-ACCACACGTCCCATCTCAG −3′ and R- 5′-ATGTGGCTTATTGAGGGGCT −3′.

4.10. Immunofluorescence

Cells were seeded in 4-well chamber slides (Nunc, Nalgene) and after attaining 50% confluence, transfection with SIRT6 siRNA was carried out. Eighteen hours post transfection, cells were treated with IL-1β for 24 h, stained with 500 nM MitoTracker Green FM (Invitrogen) as described previously [37], and fixed with 4% paraformaldehyde for 20 min. Cells were incubated overnight at 4 °C with HK2 antibody followed by Alexa Fluor 594 goat secondary antibody (Invitrogen) incubation for 1hr at room temperature. Images were captured and analysed using Zeiss ApoTome Imager. Z1 at 40× magnification.

4.11. Statistical analysis

All comparisons between groups were performed with the two-tailed paired Student *t*-test. All *P* values of less than 0.05 were taken as significant.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.yexcr.2017.05.005.

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