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AURKB, in concert with REST, acts as an oxygen-sensitive epigenetic regulator of the hypoxic induction of MDM2

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The acute response to hypoxia is mainly driven by hypoxia-inducible factors, but their effects gradually subside with time. Hypoxia-specific histone modifications may be important for the stable maintenance of long-term adaptation to hypoxia. However, little is known about the molecular mechanisms underlying the dynamic alterations of histones under hypoxic conditions. We found that the phosphorylation of histone H3 at Ser-10 (H3S10) was noticeably attenuated after hypoxic challenge, which was mediated by the inhibition of aurora kinase B (AURKB). To understand the role of AURKB in epigenetic regulation, DNA microarray and transcription factor binding site analyses combined with proteomics analysis were performed. Under normoxia, phosphorylated AURKB, in concert with the repressor element-1 silencing transcription factor (REST), phosphorylates H3S10, which allows the AURKB–REST complex to access the *MDM2* proto-oncogene. REST then acts as a transcriptional repressor of *MDM2* and downregulates its expression. Under hypoxia, AURKB is dephosphorylated and the AURKB–REST complex fails to access *MDM2*, leading to the upregulation of its expression. In this study, we present a case of hypoxia-specific epigenetic regulation of the oxygen-sensitive AURKB signaling pathway. To better understand the cellular adaptation to hypoxia, it is worthwhile to further investigate the epigenetic regulation of genes under hypoxic conditions. [BMB Reports 2022; 55(6): 287-292]

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INTRODUCTION

Acute responses to hypoxia are delicately regulated at the transcriptional level by the hypoxia-inducible factor (HIF)-1/2 and oxygen-sensitive hydroxylases, such as the egl-9 family hypoxia inducible factor (EGLN/PHD)1–3 and factor inhibiting HIF (FIH) (1). The cellular levels of HIF-1/2 increase immediately after hypoxic challenge but gradually subside with time, even under hypoxia (2, 3). Therefore, gene regulation at the epigenetic level is suggested to lead to long-term adaptation to hypoxia. So far, oxygen-sensitive epigenetic regulation has not been extensively studied. In recent years, the methylation of histone H3 at several lysine residues has been induced during hypoxia, leading to the activation or repression of specific gene sets (4, 5). However, histone modifications, except methylation, in response to hypoxia remain largely unexplored.

Histone phosphorylation is involved in diverse cellular functions, such as gene expression, mitosis, and DNA damage response (6). Histone phosphorylation is reciprocally regulated by protein kinases and phosphatases. Kinases catalyze the transfer of a phosphoryl group and confer a negative charge to histones, which weakens the electrostatic interaction between histones and DNA and subsequently provides an open space for transcription factors (7). Aurora kinase B (AURKB) is a protein kinase that phosphorylates the histone H3 at Ser-10 (H3S10). The AURK family includes three serine/threonine kinases: AURK-A, B, and C. They commonly contain a highly conserved kinase domain in the middle. The kinase domain of AURKB contains a conserved phosphorylation site at Thr-232, and autophosphorylation at this residue induces a conformational change that determines its kinase activity towards its substrates, including histone H3 (8).

Repressor element-1 silencing transcription factor (REST), also called the neuron-restrictive silencer factor, is a transcriptional repressor that binds to specific gene regions (9). REST binds to repressor element-1 within its target gene promoters, and can also target distant or non-consensus gene loci (10). On chromatin, REST sequentially recruits several key corepressors, chromatin modifiers, and the chromatin remodeling machinery (11). Although REST was initially considered as a master regulator of

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neurogenesis, it has also been implicated in the regulation of various non-neuronal biological processes (12-14).

In this study, to the best of our knowledge, we report the first evidence of the oxygen-dependent phosphorylation of histone H3S10. We also found that AURKB is activated in an oxygen-dependent manner and phosphorylated H3S10 under normoxic conditions. Furthermore, AURKB interacts with and recruits REST to the *MDM2* promoter, thereby downregulating *MDM2* expression. Under hypoxia, this process is inhibited and *MDM2* expression is upregulated. These results imply that the oxygen-sensitive epigenetic regulation contributes to cellular adaptation to hypoxia independent of the HIF signaling pathway.

RESULTS

Hypoxia inhibits AURKB-dependent histone H3S10 phosphorylation in an HIF-independent manner

In the search for hypoxia-regulated histone modifications, we found that the phosphorylation of histone H3S10 was reduced under hypoxia. First, we incubated various human cells under hypoxic conditions to analyze their phosphorylation status. Because AURKB is known to be the major kinase of H3S10, we checked the cellular levels of AURKB and analyzed its active phospho-Thr-232 form. Cells were harvested and subjected to histone extraction followed by immunoblotting analysis. The total protein levels of H3 and AURKB did not change, but the phosphorylated levels of both proteins were substantially reduced under hypoxic conditions (Fig. 1A). As H3S10 phosphorylation was reduced by AURKB inhibition or knockdown (Supplementary Fig. S1A and B), AURKB was confirmed to be a kinase for H3S10. Both H3S10 and AURKB phosphorylation were reduced in a hypoxic time-dependent manner (Fig. 1B), but were almost completely restored after 8 h of incubation under normoxia (Fig. 1C).

HIFs, including HIF-1 and HIF-2, are master regulators of oxygen homeostasis (1). To examine whether hypoxic inhibition of H3S10 and AURKB phosphorylation is driven by HIF signaling, we overexpressed a stable and constitutively active form of HIF-1α (scHIF-1α). However, the phosphorylated forms of both proteins were unaffected by HIF-1α overexpression (Fig. 1D). Moreover, hypoxic inhibition of H3S10 and AURKB phosphorylation was not affected by the HIF-1/2α knockdown (Fig. 1E). To further evaluate HIF-independent events, we tested various drugs known to stabilize HIF (15-17). Although HIF-1α was profoundly induced by dimethyloxallyl glycine, JNJ-42041935, or VH298, the phosphorylation of H3S10 and AURKB was not reduced (Fig. 1F). As expected, AZD1152, a selective AURKB inhibitor, markedly decreased H3S10 and AURKB phosphorylation, but failed to stabilize HIF-1α. Next, we examined the involvement of the oxygen sensors, PHD1-3 and FIH, in phosphorylation. Oxygen sensors mediate oxygen-dependent regulation of HIFs for protein stability and transcriptional activity (1). Consistently, knockdown of each oxygen sensor did not affect H3S10 and AURKB phosphorylation (Fig. 1G). Collectively, these results suggest that hypoxia blocks H3S10

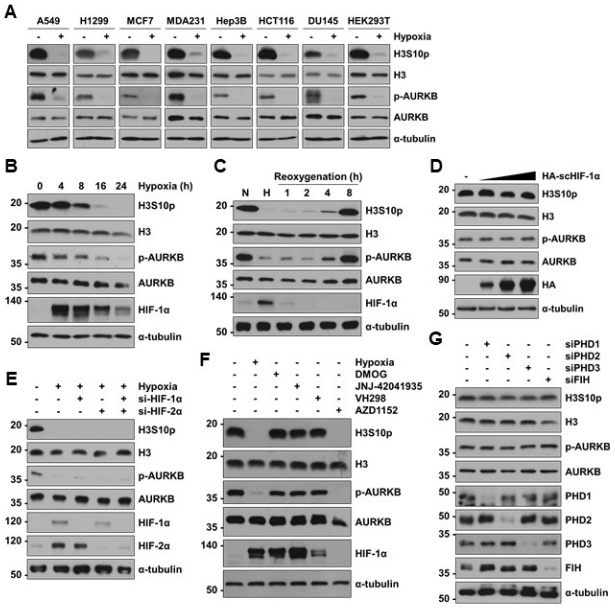

Fig. 1. Hypoxia inhibits AURKB and H3S10 phosphorylation in an HIF-independent manner. (A) Various cell lines were exposed to hypoxia (1% O_2) for 24 h and subjected to histone extraction. Cell lysates and histone extraction samples were analyzed by Western blotting with the indicated antibodies. (B) A549 cells were incubated in hypoxia for the indicated times and analyzed by Western blotting. (C) After cultured in hypoxia for 24 h, cells were reoxygenated (20% O_2) for the indicated times. Protein levels were analyzed by Western blotting. (D) Cells were transfected with increasing amount of the plasmid for HA-scHIF-1α and analyzed by Western blotting. (E) Cells were transfected with HIF-1α or HIF-2α siRNA and then incubated in hypoxia for 24 h. Samples were subjected to Western blotting. (F) Cells were treated for 24 h with hypoxia, DMOG, JNJ-42041935, VH298, or AZD1152. Protein levels were analyzed by Western blotting. (G) Cells were transfected with the indicated siRNAs and subjected to Western blotting.

and AURKB phosphorylation, regardless of previously known oxygen-sensing pathways.

Identification of transcription factors targeting the hypoxia response genes in concert with AURKB

Based on the hypothesis that AURKB-mediated H3 phosphorylation epigenetically regulates hypoxia response genes, we sought to identify the genes regulated in an AURKB-dependent manner. Four experimental groups according to AURKB expression and culture conditions were established (Fig. 2A). In the RNA samples subjected to DNA microarray analyses, the expression levels of AURKB and vascular endothelial growth factor A (a representative index for cellular response to hypoxia) were determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (18). RT-qPCR verified that RNA samples were extracted from cells that responded properly to the four distinct conditions (Fig. 2B). Consequently, DNA microarray analyses identified 61 genes that were differentially expressed

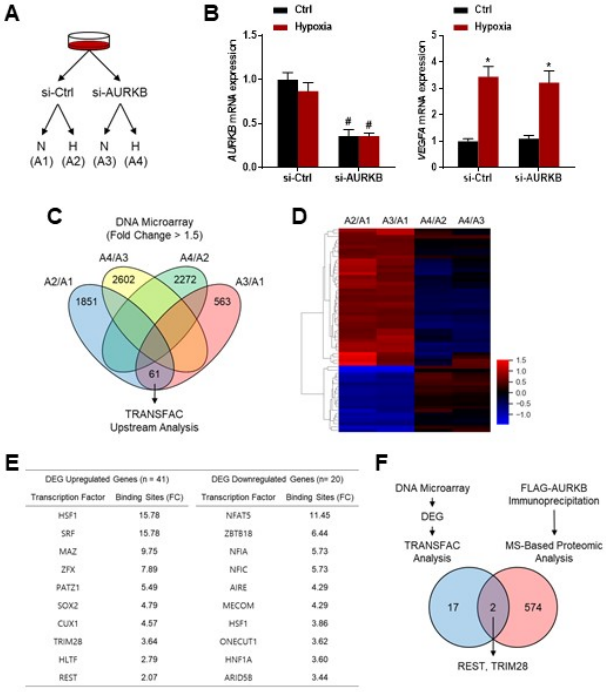

Fig. 2. Identification of REST as potential target regulated by AURKB and hypoxia. (A) A549 cells were transfected with the control or AURKB siRNA and then incubated in normoxia or hypoxia for 24 h. Each group was designated A1, A2, A3, and A4. Cells were harvested and prepared for DNA microarray analysis. (B) *AURKB* and *VEGFA* mRNA expression levels were analyzed by RT-qPCR. (C) DNA microarray results are shown as a Venn diagram. Considering only the genes that were changed more than 1.5-fold in their expression, '61' in the intersection is the number of genes that were repressed AURKB-dependently by hypoxia. (D) Heat map representing color-coded fold-changes of 61 DEGs identified in DNA microarray. (E) TRANSFAC upstream analysis of DEGs categorized into upregulated (n = 41) and downregulated (n = 20) sets. Potential upstream transcription factors were ranked by fold change in numbers of predicted transcription factor binding sites within the gene sets compared to the reference gene sets. (F) Proteomic analysis was performed in immunoprecipitated samples derived from cells overexpressing FLAG-AURKB. Transfected cells were incubated in normoxia or hypoxia for 24 h and subjected to immunoprecipitation with anti-FLAG antibody. Genes belonging to the intersection of DNA microarray and proteomic analysis results were identified and presented as a Venn diagram.

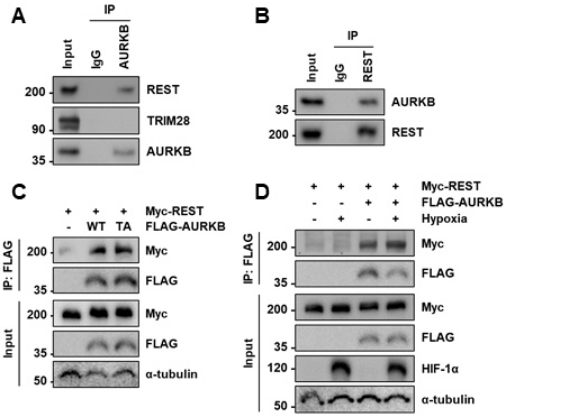

Fig. 3. AURKB associates with REST. (A) A549 cell lysates were immunoprecipitated with anti-AURKB antibody, followed by Western blotting. (B) Cell lysates were immunoprecipitated with anti-REST antibody, followed by Western blotting. (C) Cells were transfected with indicated plasmids (WT, wild type; TA, T232A mutant). Lysates were immunoprecipitated with anti-FLAG antibody, followed by Western blotting. (D) Cells were transfected with indicated plasmids and incubated in hypoxia chamber for 24 h. Lysates were subjected to immunoprecipitation and Western blotting.

in response to hypoxia and AURKB knockdown by small interfering RNA (Fig. 2C). In this study, we hypothesized that AURKB suppression determines the expression of certain genes under hypoxic conditions. If so, the target genes might be regulated either by hypoxia or AURKB suppression under normoxia. In the heatmap clustering analysis, the expression levels of 41 genes were found to be upregulated by hypoxia and AURKB suppression, while those of 20 genes were downregulated under the same conditions (Fig. 2D). Potential target genes are listed in Supplementary Tables S1 and 2. Next, we used the TRANScription FACtor (TRANSFAC) database upstream analysis program to identify potential transcription factors that could regulate these genes. Transcription factors were ranked on the basis of their fold-change values derived by comparing the number of transcription factor-binding sites between our gene sets and the reference gene sets (Fig. 2E). Notably, the number of binding sites does not indicate the probability of DNA binding. Next, we performed proteomic screening analysis to determine which candidate proteins interacted with AURKB. Mass spectrometric analyses, which were performed at least twice in three independent experiments, revealed 576 proteins that potentially interacted with AURKB. Of the interactomes, REST and tripartite motif-containing 28 (TRIM28) were commonly detected in the TRANSFAC and proteomic analyses (Fig. 2F).

AURKB interacts with REST

Results of the immunoprecipitation assay showed that AURKB interacted with REST at endogenous levels, but not substantially with TRIM28 (Fig. 3A). AURKB–REST binding was double-checked by swapping the antibodies used for immunoprecipitation and western blotting (Fig. 3B). To examine whether T232 autophosphorylation of AURKB is essential for this interaction, we expressed a mutated AURKB (T232A) in which T232 was substituted with Ala. There was no difference in the apparent binding affinity for REST between the wild-type and mutant; therefore, autophosphorylation of AURKB was determined to be non-essential for this interaction (Fig. 3C). Furthermore, the AURKB-REST interaction was unaffected by hypoxia (Fig. 3D). These results indicate that the interaction between AURKB and REST occurs regardless of AURKB phosphorylation or ambient oxygen tension.

AURKB is essential for REST recruitment to *MDM2*

Histone phosphorylation relaxes chromatin structure, allowing transcription factors to access the target genes. Given the role of histone phosphorylation, we examined whether phosphorylation of H3S10 facilitated REST to access the target genes. Of the 61 potential target genes shown in Fig. 2D, 25 with high potential REST-binding scores were screened using the chromatin immunoprecipitation (ChIP)-Atlas database (Fig. 4A). To identify the actual target genes of REST, we listed the top 10 genes with high REST-binding scores (Fig. 4B). By overexpressing or knocking down REST, we confirmed that REST robustly controlled the expression of *MDM2* (Fig. 4C and D). In the control cells, *MDM2* mRNA levels were upregulated under hypoxic conditions. This suggests that *MDM2* is repressed under normoxia but upregulated under hypoxia. More importantly, REST knockdown enhanced *MDM2* mRNA expression levels under normoxia, but not under hypoxia (Fig. 4E). This indicates that REST participates in *MDM2* repression under normoxic conditions. Similarly, REST overexpression augmented *MDM2* repression under normoxia, but not under hypoxia (Fig. 4F). In short, REST acts as an *MDM2* repressor in an oxygen-dependent manner. This regulation of MDM2 expression was further confirmed at the protein level (Supplementary Fig. S2A and B). In addition, ChIP analysis showed that REST recruitment to the *MDM2* promoter was significantly reduced under hypoxia (Fig. 4G) and AURKB knockdown under normoxia (Fig. 4H). These findings support our hypothesis that AURKB is essential for REST-driven *MDM2* repression under normoxic conditions. MDM2 is best known for its role in inhibiting the stability and transcriptional activity of the tumor suppressor p53 (19). To further verify its downstream functions, we performed a qPCR screening assay against known transcriptional targets of p53 (20) upon AURKB overexpression. Indeed, in p53 wild-type A549 cells, several p53 target genes (eight out of 91) were found to be activated by AURKB, but this change was not observed in p53 null H1299 cells (Supplementary Fig. S3). Taken together, these results further support our hypothesis that AURKB allows REST to access *MDM2* via phosphorylation of H3S10.

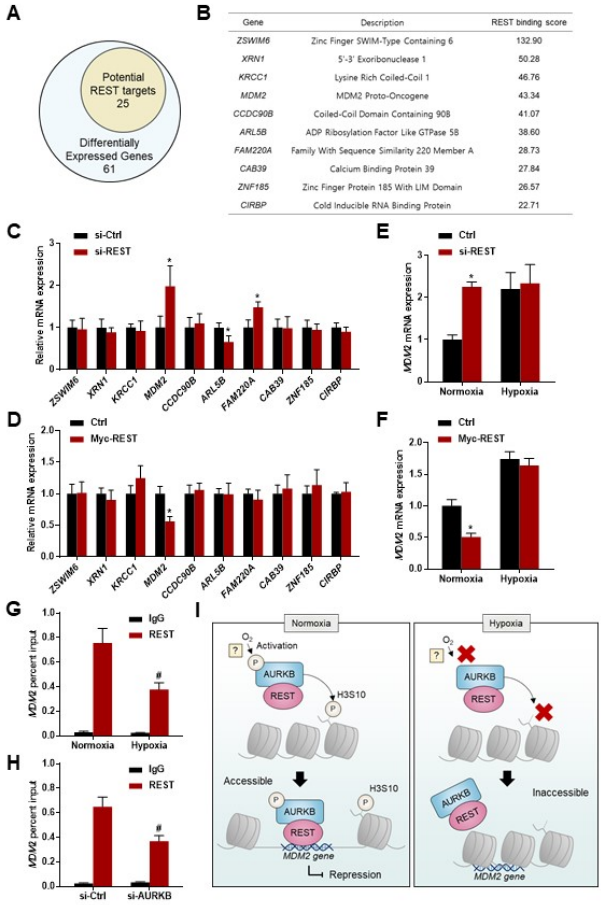

Fig. 4. Hypoxia and AURKB regulate REST-dependent *MDM2* repression. (A) Fraction of potential REST target genes among DEGs detected in DNA microarray analysis. (B) Top 10 potential REST target genes are ranked by REST-binding score according to the ChIP-Atlas database. (C) A549 cells were transfected with si-REST and subjected to RT-qPCR. (D) Cells were transfected with Myc-REST and analyzed by RT-qPCR. (E) Cells were transfected with si-REST and incubated in hypoxia chamber. Samples were analyzed by RT-qPCR. (F) Cells were transfected with Myc-REST and incubated in hypoxia. Samples were analyzed by RT-qPCR. (G) Cells were cultured under hypoxia and REST binding to the *MDM2* gene was analyzed by ChIP assay. (H) Cells were treated with si-AURKB and REST binding to the *MDM2* gene was analyzed by ChIP assay. (I) Proposed mechanism for the oxygen-dependent epigenetic regulation of the *MDM2* gene. In the presence of oxygen, AURKB exists in its phosphorylated active form and phosphorylates H3S10 to unwind tight chromatin, which allows the binding partner REST to access DNA. The phosphorylation of AURKB is inhibited in hypoxia through an unknown mechanism, and consequently REST is unable to access the target gene because chromatin is in a condensed state. P, phosphorylated; X, dephosphorylated; ?, unknown.

DISCUSSION

Epigenetic regulation of hypoxia-responsive genes is an emerging topic in the field of hypoxia biology. Previous studies have documented several histone methyltransferases and demethylases as oxygen-sensitive regulators of gene expression (21). For instance, histone demethylases, lysine demethylase (KDM)-6A and KDM5A, directly sense oxygen and modify the chromatin structure of a subset of hypoxia response genes (5, 22). The histone methyltransferases, G9a and GLP, are also regulated by the oxygen sensor FIH, which determines the oxygen-dependent methylation of H3K9 (4, 23). In contrast, histone phosphorylation in response to hypoxia is relatively unknown. Here, we show that hypoxia inhibits AURKB to control H3S10 phosphorylation for proper gene expression. H3S10 phosphorylation is a marker of mitotic progression that is required for proper chromosome condensation and segregation during cell division. In interphase, H3S10 phosphorylation regulates the transcription of specific genes (24). It has been shown that

AURKB regulates the activity of the cyclin D1 promoter via H3S10 phosphorylation (25).

In hypoxia signaling, AURKB promotes CHIP-dependent degradation of HIF-1α by phosphorylating HIF-1α at multiple serine residues (26). However, little is known about the oxygen-dependent changes in AURKB and H3S10 phosphorylation. H3S10 phosphorylation is generally considered an active marker that promotes the recruitment of transcriptional activators to target genes (24). However, the outcome of H3S10 phosphorylation may vary depending on the target gene. Here, we demonstrated that H3S10 phosphorylation downregulated *MDM2* expression by forming a chromatin structure that favored the recruitment of REST to *MDM2*. Despite many efforts, however, we failed to uncover the mechanism underlying the oxygen-dependent activation of AURKB. It was confirmed that AURKB and H3S10 phosphorylation were not regulated via the HIF-related oxygen-sensing pathway. Therefore, it is plausible that AURKB is regulated by a novel oxygen-sensing system. It is possible that the upstream factor of AURKB is directly regulated by oxygen or oxygen-containing molecules. Alternatively, changes in the cellular context due to hypoxia could indirectly inhibit AURKB autophosphorylation. However, this requires further investigation in future studies.

The E3 ubiquitin ligase, MDM2, ubiquitinates its specific substrates for degradation via the proteasomal system, and its expression is modulated by a variety of stress signals (27). To accomplish this wide range of pleiotropic activities, MDM2 interacts with 100 or more proteins (28). In response to hypoxia, MDM2 regulates two important transcription factors, p53 and HIF-1α. Hypoxia can affect MDM2 at both the mRNA and protein levels, and its expression can be increased or decreased depending on the cellular context (29). MDM2 is overexpressed in several human malignancies due to gene amplification or other yet-to-be-discovered processes. Its oncogenic activity is attributed to the inhibition of the tumor suppressor p53 (19). MDM2 physically blocks the transcriptional activation domain of p53 and simultaneously degrades it via ubiquitination. Therefore, drugs that block the MDM2–p53 interaction have been developed as anticancer agents to induce cell cycle arrest and apoptosis (30). In contrast, MDM2 interacts with and activates HIF-1, which enhances tumor adaptation to hypoxia (31). MDM2 inhibits HIF-1 signaling by promoting the ubiquitination and degradation of HIF-1α (32, 33). In this study, we demonstrated that hypoxia upregulates *MDM2* expression by inhibiting REST-driven gene repression. When the cells are under hypoxia, this epigenetic upregulation of MDM2 may play a role in cell survival by either inhibiting p53-mediated apoptosis or fine-tuning HIF signaling. These findings provide a better understanding of the mechanism by which the cells cope under hypoxic conditions.

In conclusion, our results reveal a novel mechanism of gene regulation that occurs via the hypoxic suppression of histone phosphorylation. AURKB phosphorylates H3S10 under normoxia and guides its binding partner, REST, to the *MDM2* promoter. Under hypoxia, however, AURKB fails to phosphorylate H3S10 due to an uncertain process; hence, REST cannot access the promoter (Fig. 4I).

MATERIALS AND METHODS

Further information is described in Supplementary Materials and Methods.

Plasmids and siRNAs

AURKB cDNA was cloned by RT-PCR and inserted into pcDNA vector (Promega, Madison, WI). REST cDNA clone was obtained from OriGene Technologies (Rockville, MD). HA-scHIF-1α was constructed as previously described (34). Site-directed mutagenesis kit was used to create the mutant constructs (Stratagene, La Jolla, CA). All siRNAs were purchased from Integrated DNA Technologies (Coralville, IA) and listed in Supplementary Table S3.

ChIP, RT-qPCR, and DNA microarray

ChIP assay (Merck, Darmstadt, Germany) and RT-qPCR (Enzynomics, Daejeon, South Korea) kits were used as per the manufacturer's instructions. Supplementary Table S4 contains the primer sequences for PCR. DNA microarray was performed commercially by Ebiogen (Seoul, South Korea).

Immunoprecipitation, Western blot, and mass spectrometric analyses

Immunoprecipitation and Western blot were performed as previously described with minor modifications (35). Anti-HIF-1α antibody used in Western blot was generated as previously described (36). Mass analysis was performed on a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

AUTHOR CONTRIBUTIONS

J.W.P. supervised the study. I.K. designed and performed the experiments with assistance from S.C., S.Y., M.L., and I.K. analyzed proteomics data. I.K. and J.W.P. wrote the manuscript. All authors commented on the manuscript.

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