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Negative Regulation of Hypoxic Responses via Induced Reptin Methylation

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SUMMARY

Lysine methylation within histones is crucial for transcriptional regulation and thus links chromatin states to biological outcomes. Although recent studies have extended lysine methylation to nonhistone proteins, underlying molecular mechanisms such as the upstream signaling cascade that induces lysine methylation and downstream target genes modulated by this modification have not been elucidated. Here, we show that Reptin, a chromatin-remodeling factor, is methylated at lysine 67 in hypoxic conditions by the methyltransferase G9a. Methylated Reptin binds to the promoters of a subset of hypoxia-responsive genes and negatively regulates transcription of these genes to modulate cellular responses to hypoxia.

INTRODUCTION

Hypoxic signaling occurs under several physiological and pathological conditions, such as in solid tumors with low oxygen tension and in ischemia, which is characterized by an

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ACCESSION NUMBERS

The full microarray data set has been deposited in Gene Expression Omnibus (GEO) under submission number GSE15530.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article at doi: 10.1016/j.molcel.2010.06.008.

occlusion of blood supply (Brown and Wilson, 2004; Harris, 2002). Many hypoxic responses are mediated by hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that is comprised of an oxygen-regulated α subunit (HIF-1α orHIF-2α) and a constitutively expressed β subunit (HIF-1β) (Ema et al., 1997; Semenza and Wang, 1992). Under low oxygen concentrations, HIF-1α is stabilized and translocated to the nucleus, leading to specific target gene expression through binding of HIF-1 to a hypoxia response element (HRE, RCGTG in which R is A or G). HIF-1 regulates hundreds of genes involved in many biological processes, including tumor angiogenesis, glycolysis, invasion, metabolism, and survival (Calvani et al., 2008; Pouysségur et al., 2006; Robin et al., 1984; Semenza, 2003; Shweiki et al., 1992). Thus, there is a great deal of interest in targeting HIF-1 and hypoxia signaling for cancer therapy (Dewhirst et al., 2008; Melillo, 2006; Semenza, 2003).

Upon stimulation by upstream signals, the transcription of most genes in the nucleus is regulated by the coordinate action of transcription factors and chromatin-remodeling complexes. DNA is packaged into chromatin by histones that are subject to posttranslational modifications, including methylation, acetylation, ubiquitination, SUMOylation, and phosphorylation, and some of these modifications are directly associated with transcriptional regulation (Jenuwein and Allis, 2001). Among these modifications, histone methylation on lysine residues plays an important role in generating specific molecular marks in chromatin, thus determining a dynamic balance between transcriptionally active and inactive states (Kouzarides, 2007). A number of histone methyltransferases and demethylases that methylate or demethylate histones at specific residues for mono-, di-, and/or trimethylation have been identified and characterized thus far (Klose and Zhang, 2007; Kouzarides, 2007; Martin and Zhang, 2005). However, methylation of nonhistone proteins and the consequent alteration of biological functions have not been well studied. Methyltransferase activity of G9a on nonhistone proteins, including CDYL1, WIZ, ACINUS, and C/EBPβ, has been reported recently (Pless et al., 2008; Rathert et al., 2008). Given that histones are not the sole physiological substrates for these methyltransferases, the investigation of potential nonhistone proteins targeted by histone methyltransferases and the upstream signaling pathway that induces these modifications is emerging as an important area of research.

Reptin and Pontin have been reported to be conserved AAA+ ATPases identified in several ATP-dependent chromatin-remodeling complexes, and they perform a variety of functions, including transcriptional regulation of Wnt/β-catenin signaling pathways and telomerase biogenesis processes (Bauer et al., 2000; Kim et al., 2005, 2006; Shen et al., 2000; Venteicher et al., 2008). Reptin is related to RuvB in bacteria, INO80 ATPase complex in yeast, Polycomb complex PRC1 in *Drosophila*, and a subunit of the Tip60 coactivator complex in mammals (Gallant, 2007; Jha and Dutta, 2009). Reptin has a variety of binding partners, including c-MYC, NF-κB p50, TLE, Hint1, β-catenin, SENP1, and TERT, which have been identified by biochemical purification and binding assays (Olson et al., 2006; Weiske and Huber, 2005; Wood et al., 2000). Recently, we have reported that SUMOylation of Reptin and Pontinisassociated with tumor progression and metastasis (Kim et al., 2006, 2007). Because regulation of the *KAI1* metastasis suppressor gene during cancer metastasis

is mediated by SUMOylated Reptin, we wished to determine what other modifications may be important for functional modulation of Reptin in a signal-dependent manner.

In this manuscript, we report that Reptin is methylated by G9a methyltransferase and that this modification is hypoxia dependent. Biochemical purification of Reptin-binding proteins identified G9a, and its enzymatic activity was required for hypoxia-induced Reptin methylation at lysine residue 67. We have delineated a detailed molecular mechanism by which Reptin methylation participates in the downregulation of a subset of hypoxia target genes involved in metabolism and tumor development using a genome-wide analysis approach.

RESULTS

Reptin Is a Target of G9a Methyltransferase

Purification and characterization of Reptin-interacting proteins by affinity chromatography identified G9a and Suv39h1 histone lysine methyltransferases (Figure 1A). The association of G9a and Suv39h1 with Reptin was confirmed by immunoblot analysis from eluates from purification of Reptin-interacting proteins (Figure 1B). Coimmunoprecipitation assays confirmed that Reptin bound G9a and Suv39h1 at the endogenous expression level (Figures 1C and 1D). To address the functional consequence of Reptin association with G9a or Suv39h1, we explored the possibility that Reptin might be directly methylated by G9a or Suv39h1 as an in vivo nonhistone substrate. Introduction of G9a, but not Suv39h1, resulted in increased Reptin methylation (Figure 1E). Further, in vitro histone methyltransferase (HMTase) assay confirmed that purified G9a was capable of Reptin methylation comparable to histone H3 methylation (Figure 1F). To further determine whether the catalytic activity of G9a is required for Reptin methylation, either wild-type (WT) G9a or R1190Y mutant that has impaired methyltransferase activity was expressed. Immunoprecipitation assay with anti-methyl-lysine antibodies followed by immunoblotting against anti-Reptin antibodies revealed that the G9a mutant failed to methylate Reptin (Figure 1G).

Reptin K67 Methylation Is Induced by Hypoxia

We then attempted to precisely identify the Reptin methylation site. Among the fragments tested, only the N-terminal fragment spanning amino acids 1 to 135 was methylated by G9a (Figure 2A). Next, the four lysine residues within the identified N-terminal region were individually mutated to alanine. Among the mutations, only lysine 67 to alanine (K67A) substitution abrogated G9a-mediated methylation, thereby implicating lysine 67 as the methylation site targeted by G9a (Figure 2B). In order to further characterize Reptin methylation by G9a, Reptin was analyzed by mass spectrometry after incubation with purified G9a. We found >50% of WT Reptin to be monomethylated by G9a, whereas there was no detectable alteration in the methylation state of Reptin K67A (Figure 2C). Further, we confirmed G9a-induced in vivo methylation of Reptin at K67 purified from MCF7 cells by LC-MS/MS (Figure S1A available online). In vitro HMTase assay also showed lack of methylation for Reptin K67A mutant compared to that of WT (Figure 2D). Moreover, performing HMTase assay using either histone H3 or Reptin peptides revealed that G9a was able to methylate Reptin WT peptide comparable to histone H3 methylation, whereas Reptin

K67A mutant showed a lack of methylation by G9a (Figure 2E). Having found that Reptin is methylated at K67 by G9a, we generated a Reptin K67 methyl-specific antibody, as shown by dot blot analysis with no cross-reactivity with the unmethylated Reptin peptide (Figure 2F). Further, we observed a decrease in Reptin methylation in the presence of G9a inhibitor (BIX01294) demonstrating again the involvement of G9a in Reptin methylation, as well as further validating the use of this antibody (Figure 2G).

Although Reptin was readily methylated by G9a, it remained unclear what upstream signal influences G9a-mediated Reptin methylation. Because hypoxic stress was reported to increase both protein levels and enzymatic activity of G9a (Chen et al., 2006), we tested the possibility that hypoxia induces Reptin methylation in a G9a-dependent manner. We exposed MCF7 breast cancer cells, which have been shown to express G9a expression to hypoxic conditions (Kondo et al., 2008). Using anti-Reptin K67 methyl antibodies, we detected an increase in Reptin methylation from 6 hr of hypoxic challenge, which was maintained up to 24 hr, whereas there was no significant change in the total Reptin protein levels (Figure 2H). As expected, G9a protein levels were increased in a hypoxia-dependent manner, which is consistent with the hypothesis that hypoxia-induced Reptin methylation is mediated by G9a. We were able to detect significant increase in methylated Reptin levels upon hypoxic stress, and ~5% to 10% of total Reptin appeared to be methylated (Figures 2I and Figure S1B).

To further investigate whether enzymatic activity of G9a is required for hypoxia-induced Reptin methylation, methylation of Reptin was compared between WT and *G9a*-deficient (*G9a*$^{-/-}$) embryonic stem (ES) cells in the presence or absence of hypoxia (Figure 2J). Intriguingly, Reptin methylation was almost completely abolished in *G9a*$^{-/-}$ ES cells compared to that in WT ES cells. Reconstitution experiments with either WT G9a or enzymatically inactive G9a mutant revealed that Reptin methylation was restored only in WT G9a-reconstituted cells (Figure 2K). We then tested whether Reptin K67A is defective for hypoxia-mediated methylation. Indeed, whereas hypoxia induced methylation of WT Reptin, it had no effect on K67A mutant (Figure 2L).

Reptin-Dependent Target Gene Identification by Microarray Analysis

In order to investigate the role of Reptin methylation on the expression of hypoxia-responsive genes across the whole genome, we performed a microarray analysis from RNAs isolated from MCF7 cells expressing either control shRNA (shNS) or Reptin shRNA (shReptin) in normoxic and hypoxic conditions (Figure 3A). The global analysis investigated the general impact of Reptin on gene expression, and Reptin methylation-dependent target genes were validated by a quantitative RT-PCR analysis using shRNA-resistant Reptin WT or K67A mutant. We used a Gaussian curve fitting to determine the applicable cutoffs in selecting genes that showed significant changes in response to hypoxia (Hwang et al., 2005). Among differentially expressed genes as a response to hypoxia, hierarchical clustering was performed. Hypoxia-responsive genes could be largely categorized into Reptin-dependent (Figure 3B) and Reptin-independent genes (Figure 3C). These two categories could be subdivided into several clusters, including downregulated (green) and upregulated (red) groups and those that lost or gained sensitivity to hypoxic

responsiveness as a result of Reptin knockdown (shReptin/shNS column; see also Table S1). Among hypoxia-responsive genes, 24.6% of genes appeared to be Reptin dependent, whereas the remaining 75.4% of genes were shown to be Reptin independent (Figure 3D).

We examined whether hypoxia and Reptin expression affected genes regulated by several transcription factors such as NF-κB, TCF/LEF, and p53, which have been previously reported to modulate transcription upon hypoxic stress (Cummins and Taylor, 2005). Using a Transcriptional Regulatory Element Database (TRED), the enrichment of target genes for HIF-1, TCF/LEF, NF-κB, and p53 was analyzed on all clusters (Figures 3B and 3C). The enrichment percentage of HIF-1 target genes scored the highest for clusters 1 in the Reptin-dependent group, whereas cluster 2 scored highest in the Reptin-independent group. Cluster 1 contained genes induced by hypoxia and also showed further activation upon Reptin knockdown, whereas cluster 2 represented genes that were activated by hypoxia, but not affected by Reptin knockdown. Other transcription factor target genes within Reptin-dependent clusters (clusters 1, 3, 6, 10, 15, 19, 22, and 24) were relatively low compared to that of HIF-1 targets, indicating that HIF-1 may be a major transcription factor affected by Reptin. We also examined, by systemic approach, Reptin protein interactome in order to gain insight into what other transcription factors and coregulators Reptin may cooperate with (Figure S2). From these analyses, it was clear that Reptin-dependent hypoxia-induced cluster (cluster 1) contained many HIF-1 targets (Figure 3E). We used the *DAVID* Bioinformatics Resource (http://david.abcc.ncigcrf.gov/) to perform functional annotation of Reptin-dependent genes (see Table S2). There were two main functional groups, with 26 genes associated with metabolic processes and 16 genes associated with cell death, apoptosis, and survival, suggesting that these gene sets might be involved in regulating tumor growth properties (Figure 3F).

Reptin Methylation Negatively Regulates a Subset of Hypoxia Target Genes

In order to ascertain the involvement of Reptin methylation on hypoxia target gene regulation, we performed a reporter assay using 3 × HRE-luciferase reporter. Upon knockdown of Reptin, activation of 3 × HRE-luciferase activity by hypoxic challenge was enhanced (Figure 4A). The repression of hypoxia target genes appears to be Reptin specific, as knockdown of Reptin in our experimental system did not affect expression levels of Pontin (Figure S3). Introduction of Reptin WT markedly inhibited hypoxia-dependent activation of 3 × HRE-luciferase activity, whereas Reptin K67A mutant significantly diminished this inhibition (Figure 4B).

Further, to validate Reptin-dependent target genes identified from our microarray analysis as Reptin methylation-dependent genes, we performed a quantitative RT-PCR analysis of Reptin-dependent and -independent genes by reconstitution of shRNA-resistant Reptin WT (WT^R) or K67A mutant ($K67A^R$) following Reptin knockdown (Figures 4C and 4D). Reptin-dependent genes exhibited a significant increase in response to hypoxic challenge following Reptin knockdown. Consistently, introduction of Reptin WT^R following Reptin knockdown had an inhibitory effect on transcript levels of *VEGF*, *PGK1*, and *BNIP3*, whereas reconstitution of Reptin $K67A^R$ appeared to have no effect (Figure 4C). Reptin-independent genes *(IGFBP3*, *KDM3A*, and *CITED2*) were unaltered by reconstitution of

WT^R or $K67A^R$ following Reptin knockdown (Figure 4D). Together, these data provide evidence that Reptin methylation participates in hypoxia-driven negative regulation of a subset of hypoxia target genes involved in metabolism, cell death, and survival.

Methylated Reptin Recruits to Its Target Promoters via Enhanced Binding to HIF-1α

To gain further insight into how Reptin modulates a subset of HIF-1 target genes, we hypothesized that altered interaction between Reptin and HIF-1 α brought about by Reptin methylation may affect HIF-1 α transcriptional activity and thereby specifically modulate downstream hypoxic responses. Coimmunoprecipitation assays revealed that endogenous Reptin associated with HIF-1 α in hypoxic conditions (Figure 5A). In order to determine whether this interaction between Reptin and HIF-1α is methylation dependent, we performed coimmunoprecipitation assays comparing Reptin WT and K67A mutant. The association between Reptin and HIF-1 α appeared to be methylation dependent, as only Reptin WT was able to interact with HIF-1 α in the presence of G9a, whereas Reptin K67A showed lack of interaction (Figure 5B). Reptin failed to exhibit binding to HIF-1 β even in the presence of G9a (Figure 5C), suggesting that Reptin specifically interacts with HIF-1 α in a methylation-dependent manner. Further, upon performing direct interaction assays using in vitro translated ^{35}S-labeled HIF-1 α and nonmethylated (K67me0) and monomethylated (K67me1) Reptin peptides spanning the lysine 67 residue, we found HIF-1 α interacting strongly with K67me1 peptide compared to K67me0 peptide (Figure 5D). These data suggest that G9a-mediated methylation of Reptin at K67 is necessary for the enhanced binding to HIF-1 α.

We then performed a ChIP assay in either WT or HIF-1 α-deficient (*HIF-1* $\alpha^{-/-}$) mouse embryonic fibroblasts (MEFs) to determine whether methylated Reptin can be recruited to HIF-1 target promoter in a HIF-1 α-dependent manner. Both methylated Reptin and HIF-1 α were recruited to the *VEGF* promoter in WT MEFs under hypoxia, whereas we failed to observe recruitment of methylated Reptin in *HIF-1* $\alpha^{-/-}$ MEFs (Figure 5E), suggesting that Reptin requires HIF-1 α binding for its recruitment to the *VEGF* promoter.

We performed quantitative RT-PCR and ChIP assays to determine how transcript levels of Reptin-dependent (Figure 5F) and Reptin-independent (Figure 5G) genes are regulated by the recruitment of methylated Reptin (Figures 5H and 5I). Although HIF-1 α was recruited to *VEGF* and *PGK1* promoters as early as 2 hr, a significant increase in methylated Reptin recruitment could not be detected until time points after 6 hr (Figure 5H). The increase in promoter-associated methylated Reptin with concurrent reduction in RNA polymerase II recruitment suggested that Reptin methylation accounts for a negative feedback loop, thereby fine-tuning the expression of a subset of hypoxia target genes. Consistent with our gene expression data that Reptin imposes a repressive function on these promoters, shRNA-coupled ChIP assays revealed that the recruitment of RNA polymerase II was markedly increased with Reptin knockdown up to 24 hr, whereas methylated Reptin localization on the *VEGF* and *PGK1* promoters was significantly reduced (Figure 5H). Although HIF-1 α recruitment following hypoxia was readily detectable, we failed to detect methylated Reptin to a Reptin-independent gene promoter, *IGFBP3*, along with a lack of change in RNA polymerase II recruitment (Figure 5I).

Our previous studies on Reptin-mediated repression posed a possibility that HDAC1 obtained from purification of Reptin-interacting proteins (Kim et al., 2006; Kim et al., 2005) might be responsible for exerting a negative regulation on *VEGF* promoter during hypoxic stress. Remarkably, the interaction assays revealed a significant increase in Reptin association with HDAC1 in hypoxic condition compared to that in normoxic condition (Figure 5J). Moreover, this increase was not observed for Reptin K67A mutant even in hypoxic condition, further emphasizing the importance of methylation status of Reptin in dictating the repressor complex assembly. Indeed, HDAC1 recruitment to *VEGF* promoter appeared to be Reptin methylation dependent, as reconstitution of Reptin K67A mutant to Reptin knockdown cells showed lack of HDAC1 and RNA polymerase II localization (Figure 5K). Moreover, knockdown of HDAC1 was able to inhibit repressive function of Reptin in hypoxic condition, indicated by a sustained level of RNA polymerase II recruitment to *VEGF* promoter, demonstrating the requirement of HDAC1 for Reptin-mediated transcriptional repression (Figure 5L). Together, these data suggest that hypoxia-G9a-mediated Reptin methylation is crucial for recruiting a repressive complex, including HDAC1, to a subset of hypoxia-responsive target promoters.

Effect of Reptin Methylation on Tumorigenic Properties

As hypoxia-induced expression of HIF-1 α influences the growth and metastases of several cancer types, including those of the breast, prostate, and colon (Harris, 2002; Zhong et al., 1999), and with our finding that G9a-mediated methylation of Reptin under hypoxic condition plays a crucial role in modulating HIF-1 α transcriptional activity, we undertook to determine whether Reptin methylation regulates hypoxia signal-mediated physiology in breast cancer cells and a xenograft mouse model. Quantitative analysis of VEGF levels by ELISA confirmed that hypoxia increases VEGF levels in conditioned media by about 2-fold, and there was a significant further increase in VEGF levels in cells in which Reptin methylation was abrogated either by Reptin knockdown or by overexpressing Reptin K67A mutant (Figure 6A). Together, these data suggest that Reptin methylation occurred in hypoxic condition affecting VEGF protein levels and strongly support our finding that hypoxia-induced Reptin methylation affects both VEGF mRNA and protein levels.

As several cancer cell lines, including those of the breast and colon, have been reported to have VEGF autocrine signaling, which affects tumorigenic and metastatic potential (Calvani et al., 2008; Lee et al., 2007; Mercurio et al., 2005), we examined the effect of Reptin methylation on these processes. First, we performed a cell motility assay in the presence of deferroxamine (DFA), which is an iron chelator that induces HIF-1 activity (Wang and Semenza, 1993b) (Figure 6B). It took ~48 hr for parental MCF7 cells to close the gap, whereas cells expressing Reptin WT showed significantly reduced cell motility. Unlike Reptin WT-expressing cells, cells expressing Reptin K67A migrated over the scratch in a time frame similar to the mock cells, suggesting that Reptin methylation is involved in inhibiting cell migration. In order to determine whether DFA adequately mimics hypoxia, we confirmed Reptin methylation in the presence of DFA treatment in MCF7 cells by immunoprecipitation with anti-Reptin K67 methyl antibody followed by immunoblotting with anti-Reptin antibody (Figure 6C). Reptin was methylated upon DFA treatment in MCF7 cells as expected.

We also performed transwell cell migration assays in hypoxic condition to determine the migratory potential of MCF7 cells and observed similar effects. Reptin knockdown resulted in a 2-fold increase in cells migrating through the filter upon hypoxic challenge (Figure 6D). Consistently, expression of Reptin K67A mutant increased migration in transwell cell migration assays specifically under hypoxic conditions (Figure 6E). We also utilized a quantitative in vitro invasion assay to assess the ability of MCF7 cells to invade through Matrigel in response to hypoxia (Figure 6F). Expression of Reptin WT reduced invasion, whereas knockdown of Reptin or expression of Reptin K67A increased the invasive potential of MCF7 cells over 24 hr compared to control cells. The ability of either Reptin knockdown or K67A overexpression to enhance the invasive potential further illustrates the inhibitory effect of Reptin methylation on metastatic properties of a tumor during hypoxic conditions.

An important question was whether hypoxia-driven Reptin methylation could negatively regulate tumorigenic behavior in vivo. To address this question, we injected MCF7 cells or MDA-MB231 cells stably expressing Reptin WT, Reptin K67A, or shReptin subcutaneously into athymic nude mice (Figures 6G and S4). Reducing endogenous Reptin expression with shReptin resulted in the formation of tumors that were dramatically larger compared to that of control cells expressing shNS. Further, overexpression of Reptin WT significantly reduced tumor growth, whereas tumors derived from cells expressing Reptin K67A were comparable to that of shReptin tumors. Hence, silencing endogenous Reptin expression or ectopically expressing methylation-defective Reptin mutant provides MCF7 cells with a growth advantage, whereas ectopically expressing Reptin WT inhibits tumor growth. HIF-1α and G9a protein expression in the extracted tumors from a mouse xenograft model indicates the recapitulation of hypoxic condition in these tumors (Figure 6H). Indeed, immunoblot data confirmed that the hypoxic microenvironment within the tumor elevated the expression and/or stability of HIF-1α and G9a proteins compared to the expression levels of HIF-1α and G9a proteins in MCF7 cells grown in vitro in normoxia.

To establish the contribution of Reptin-dependent target gene expression to growth regulation, we performed a proliferation assay using MCF7 cells expressing either VEGF or FOS (Figures 6I and 6J). Proliferation was significantly increased by introducing exogenous VEGF, and this increase was inhibited by expressing Reptin WT, whereas Reptin K67A had no effect (Figure 6I). A similar effect on proliferation was observed by modulating the expression of another Reptin-dependent gene, *FOS* (Figure 6J). Collectively, our findings indicate that hypoxia-induced Reptin methylation negatively regulates a subset of HIF-1α target genes and thereby possibly affects tumor growth and invasive properties.

DISCUSSION

Reptin as a G9a Substrate

This is the first report of previously unrecognized evidence that hypoxia-induced G9a methylates Reptin, leading to negative regulation of a subset of hypoxia target genes by inhibiting HIF-1α transcriptional activity (Figure 7). This finding suggests a mechanism for negative regulation of hypoxic signaling pathways. Although purification of Reptin-interacting proteins indicated that both G9a and Suv39h1 bound to Reptin, we did not

observe any changes in Suv39h1 protein expression levels in hypoxia, and only G9a was able to methylate Reptin in a hypoxia-dependent manner, providing evidence that there exists nonhistone substrate specificity for particular methyltransferases in certain signaling pathways.

Functional studies of G9a reported previously involved histone methyltransferase activity whereby G9a methylates histone H3K9 for gene silencing (Chen et al., 2006; Tachibana et al., 2001). Other reports include G9a acting as a coactivator for nuclear receptors such as estrogen receptor α and androgen receptor (Lee et al., 2006). We have extended the role of G9a to include methylation of Reptin. Recently, G9a substrates that contain the ARK peptide sequence have been reported, including CDYL1, WIZ, and ACINUS (Rathert et al., 2008).

Knockout of G9a has been shown to result in a reduction of global H3K9me1 and H3K9me2 levels, and an in vitro G9a HMT assay has been reported to mainly increase H3K9me1 and H3K9me2 levels (Chen et al., 2006). G9a appeared to mainly monomethylate Reptin in vitro, as assessed by mass spectrometry, but we cannot exclude the possibility that, under longer incubation in our assay or under certain physiological conditions, G9a may generate di- and trimethylated Reptin as in the case of H3K9 methylation.

Reptin Methylation Is Mediated by G9a during Hypoxia

Because methylation/demethylation is a dynamic process, the methylation status of nonhistone proteins as well as histones is likely to be determined by the net effect of opposing methyltransferases and demethylases. It is tempting to speculate that demethylation of Reptin might provide a dynamic mechanism for regulating a subset of hypoxia-inducible genes.

We have established a functional link between hypoxia and G9a-mediated Reptin methylation by examining the timing of Reptin methylation, which closely reflected the increase in G9a protein expression by hypoxia. It has been reported that hypoxia increases G9a activity through increasing G9a protein levels, but not through inducing mRNA levels (Chen et al., 2006). We have found that hypoxia and DFA treatment both led to G9a accumulation and also induced Reptin methylation. A significant increase in Reptin methylation was observed starting at 6 hr and was maintained up to 24 hr, which mechanistically supports our negative regulation model. Hypoxia-induced Reptin methylation through increased G9a methyltransferase activity affects Reptin binding to HIF-1α, thereby attenuating HIF-1α transcriptional function. The requirement of Reptin methylation for the enhanced HIF-1α interaction is demonstrated by the observation that interaction of Reptin K67A with HIF-1α is unaltered even when G9a protein levels are elevated under hypoxic conditions.

Selective Negative Regulation of HIF-1 Target Genes by Methylated Reptin

A variety of observations indicate that Reptin functions as a transcriptional corepressor. One such example is in the regulation of Wnt target genes whereby Reptin acts as a corepressor of the β-catenin-TCF4 transcriptional activation complex (Bauer et al., 2000). We have previously reported the ability of Reptin to bring about repression of the metastasis

suppressor gene *KAI1* in a SUMOylation-dependent fashion (Kim et al., 2005, 2006). In this study, we have found that Reptin is also able to confer repressive function in a methylation-dependent manner and that methylation of Reptin is important for maintaining and exerting repression of a subset of HIF-1 target genes that requires HDAC1. It is intriguing that only a subset of hypoxia target genes was affected by Reptin methylation, and there might exist a specificity within hypoxia target gene promoters that determines methylated Reptin recruitment, consequently resulting in a negative regulation. This specificity might be dictated by the temporal dynamics of transcription factors and their coregulators for each target gene or by a common motif in the promoter region in a context-dependent manner.

In vitro analyses of cell motility, invasion, and in vivo tumor growth assays highlight the biological importance of hypoxia-driven Reptin methylation in modulating cell survival and tumor suppressive functions and thus propose another layer of regulation of hypoxia target genes. The finding of a number of genes involved in glucose metabolism as Reptin-dependent targets in hypoxic condition supports the repressive function of Reptin methylation in tumor growth.

It is possible that Reptin may play an important role in regulating its target genes under hypoxia, as Reptin expression levels were found to be much lower in breast and prostate cancer cell lines compared to their normal counterparts (Kim et al., 2006), thus linking homeostasis and disease with Reptin expression. Our finding that G9a-dependent Reptin methylation negatively regulates hypoxia signaling represents a pathway by which HIF-1 transcriptional activity can be repressed, thereby affecting metabolic, cell death, and survival pathways that are important for the development and progression of certain cancers.

EXPERIMENTAL PROCEDURES

Reagents

The following antibodies were purchased from Santa Cruz Biotechnology: anti-GFP, anti-HDAC1, and anti-HIF-1α. The following commercially available antibodies were used: anti-G9a and anti-Suv39h1 (Upstate Biotechnology), anti-methyl lysine (Abcam), anti-FLAG (Sigma), and anti-RNA polymerase II (Berkeley Antibody Company). Anti-methyl Reptin was generated from Abmart.

In Vitro Methyltransferase Assay

All recombinant proteins (GST, GST-Reptin, and His-G9a SET) were purified from *E. coli.* In vitro methyltransferase assays were performed with the reactions assembled in 5 × lysine methyltransferase (KMT) buffer including purified proteins and ^{3}H-S-adenosylmethionine and were incubated 3 hr at 30°C. Laemmli buffer was added to samples, boiled, and loaded on SDS-PAGE for autoradiography.

Chromatin Immunoprecipitation Assay

The ChIP assays were conducted as previously described (Baek et al., 2002; Kim et al., 2006). See Table S3 for sequences of primers used.

Quantitative Real-Time RT-PCR

The abundance of mRNA was detected by an ABI prism 7300 system with SYBR Green (Molecular Probes). Primer pairs were designed to amplify 90–150 bp mRNA specific fragments and were confirmed as unique products by melting curve analysis. The PCR conditions were 95°C (5 min) and 40 cycles of 95°C (30 s), 58°C (30 s), and 72°C (30 s). The quantity of mRNA was calculated using ΔΔCt method and normalized by using primers to detect HPRT. All reactions were performed as triplicates. See Table S3 for sequences of primers used.

In Vitro Cell Migration and Invasion Assay

Cells (5×10^4) were suspended in 6.5 mm 8 μM pore polycarbonate membranes either coated or uncoated with 100 μl matrigel. The lower chamber contained 500 ml of 3T3 24 hr conditioned media used as a chemoattractant. Cell were allowed to either migrate (without matrigel) or invade (with matrigel) for 16–24 hr in normoxic or hypoxic conditions. Membranes were excised from the chamber and fixed in methanol before being stained with DAPI for imaging.

In Vivo Tumorigenesis Assay

For tumor formation in vivo, cells (10^7) with equal volume of matrigel (BD Biosciences, Bedford, MA) were injected subcutaneously at the left flank into 6-week-old athymic *nu/nu* female mice (Orient, Seoul, Korea). Tumors were measured weekly, and the experiment was terminated at week 5. Tumors were excised and weighed. Statistical differences in tumor weights were determined by Student's t test using the Statview package (Abacus Concepts, Inc., Berkeley, CA). These experiments were carried out with the approval of the Institutional Animal Care and Ethics Committee.

Statistical Analysis

Statistical differences in test and control samples were determined by Student's t test or ANOVA using the Statview package (Abacus Concepts, Inc., Berkeley, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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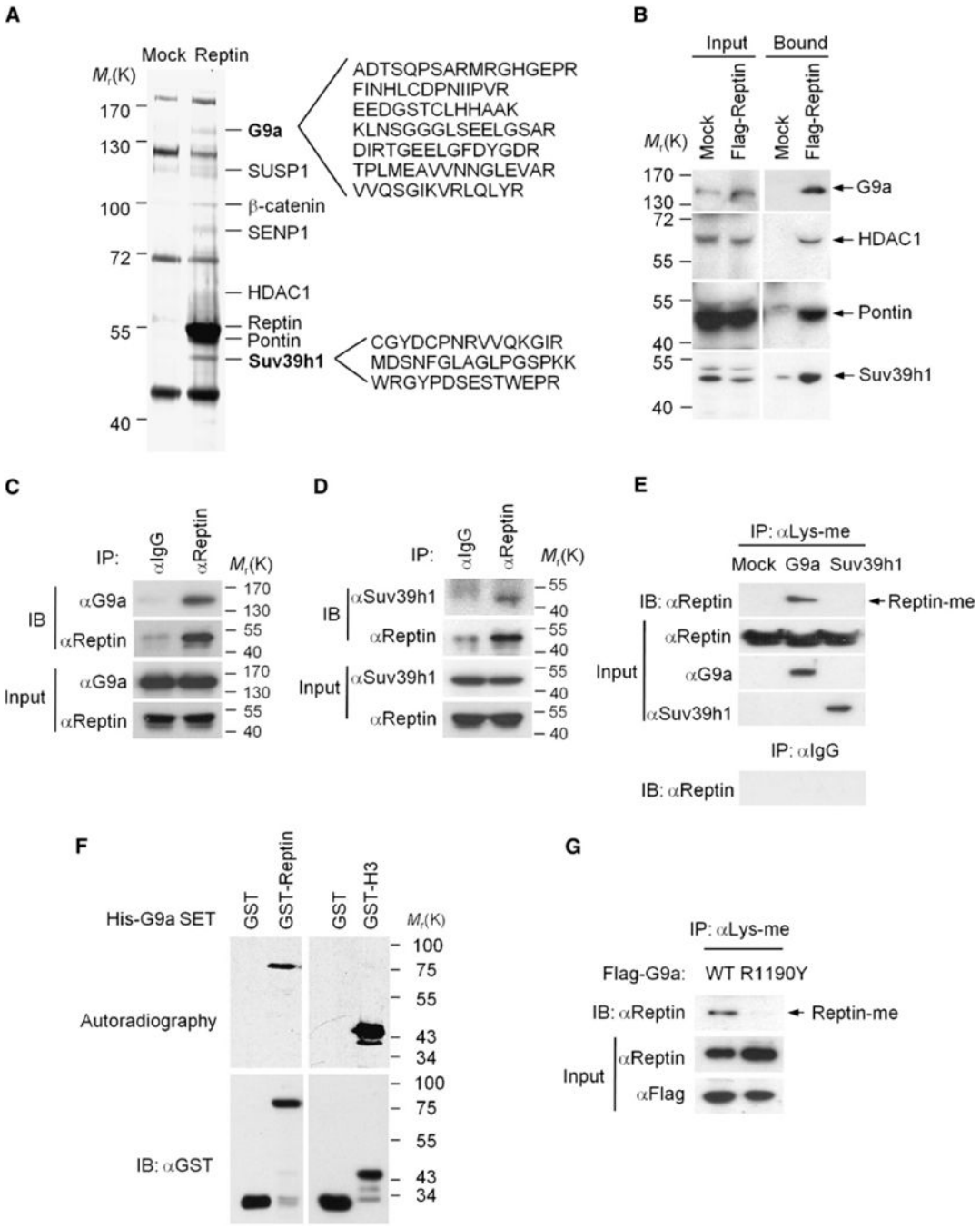

Figure 1. Reptin Methylation by G9a Methyltransferase

(A) Reptin-interacting proteins were purified from HEK293 cells stably expressing Flag-tagged Reptin by coimmunoprecipitation using anti-FLAG antibody. As a negative control, cells expressing empty vector (Mock) were used. The bound proteins were resolved by SDS-PAGE and prepared for LC-MS/MS analysis.

(B) G9a and Suv39h1, along with previously reported Reptin-binding partners such as HDAC1 and Pontin, were detected from the eluates after purification by immunoblot assays.

(C and D) Coimmunoprecipitation of endogenous Reptin with G9a (C) or Suv39h1 (D).

(E) HEK293 cells were transfected with empty vector or with expression vector encoding G9a or Suv39h1. Cell lysates were immunoprecipitated with antibody against methylated lysine followed by immunoblot analysis with anti-Reptin antibody to detect methylated Reptin.

(F)Autoradiogram of in vitro histone methyltransferase assay using recombinant His-tagged G9a SET domain with either GST-tagged Reptin or Histone H3. Levels of GST proteins used in the assay are shown by immunoblotting with anti-GST antibody.

(G) Immunoprecipitation of methylated Reptin with anti-methyl-lysine antibody from HEK293 cell lysates expressing G9a WT or G9a mutant, followed by immunoblot analysis with anti-Reptin antibody to detect methylated Reptin.

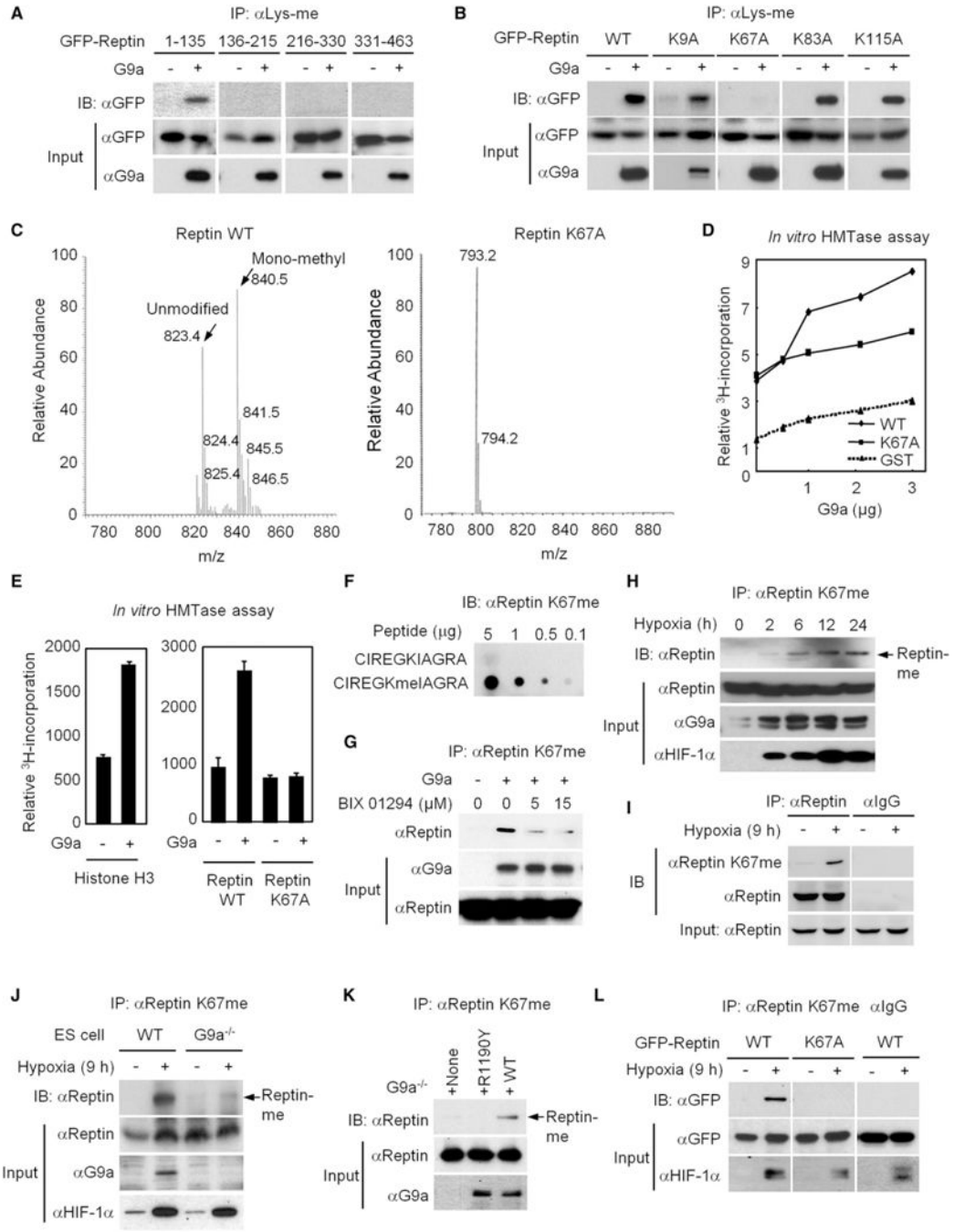

Figure 2. G9a Mediates Hypoxia-Induced Reptin K67 Methylation

(A) HEK293 cells were cotransfected with plasmids encoding each GFP-tagged Reptin deletion construct spanning the indicated amino acid residues with or without plasmid encoding G9a. Whole-cell extracts in the presence or absence of G9a were immunoprecipitated with anti-methyl-lysine antibody followed by immunoblot assay using anti-GFP antibody to detect Reptin.

(B) The four lysine residues (K9, K67, K83, and K115) within the N-terminal 135 amino acids were individually mutated to alanine.

(C) Mass spectrometry analysis of Reptin WT and K67A following methyltransferase assay using G9a.
(D) In vitro methyltransferase assay using purified Reptin and G9a proteins using ^{3}H-SAM as a methyl donor.
(E) In vitro methyltransferase assay using Histone H3, Reptin WT, and K67A peptides with purified GST-G9a using ^{3}H-SAM as a methyl donor. Values are expressed as mean ± SEM of three independent experiments.
(F) Dot blotting of unmodified or methylated Reptin peptides with anti-Reptin K67 methyl antibody.
(G) Immunoprecipitation of methylated Reptin with anti-Reptin K67 methyl antibody from cells treated with BIX 01294, a G9a inhibitor, at doses indicated.
(H) Immunoprecipitation of methylated Reptin with anti-Reptin K67 methyl antibody from MCF7 cells exposed to hypoxia for indicated times followed by immunoblotting with anti-Reptin antibody.
(I) Proportion of methylated Reptin was estimated by immunoblotting with either Reptin K67me antibody or Reptin antibody from Reptin immunoprecipitate exposed to hypoxia as indicated.
(J) Reptin methylation was compared between WT and *G9a*-deficient ES cells in the presence or absence of hypoxic challenge for 9 hr.
(K)*G9a*$^{-/-}$ ES cells were reconstituted with either WT or enzymatically inactive G9a mutant, and changes in Reptin methylation were monitored in cells exposed to hypoxia with anti-Reptin K67 methyl antibody.
(L) Reptin methylation was determined in MCF7 cells expressing either Reptin WT or K67A mutant. Immunoprecipitation of methylated Reptin was performed as in (H).
See also Figure S1.

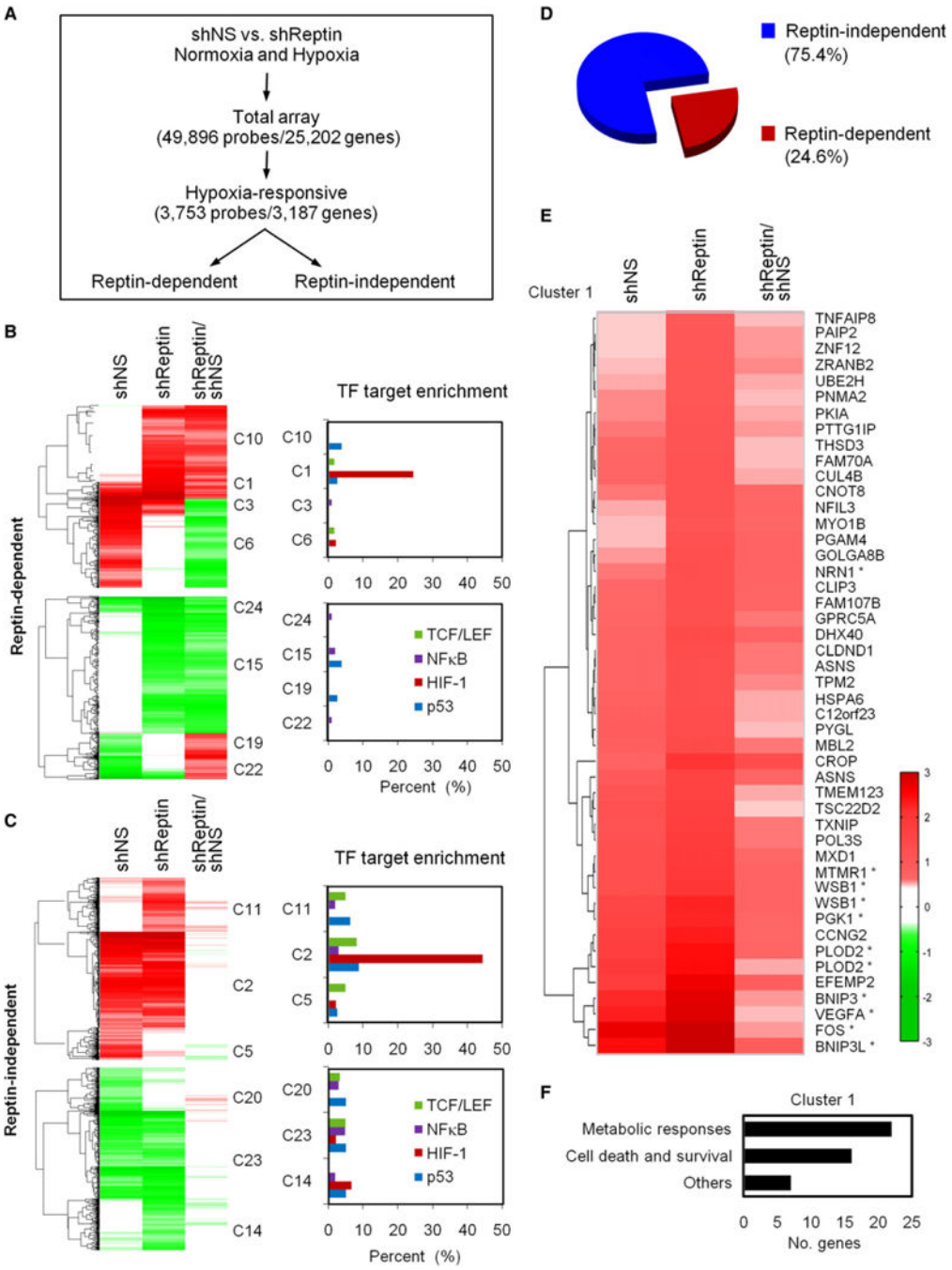

Figure 3. Reptin-Dependent Target Identification by Microarray Analysis

(A) Flow chart showing the strategy of cDNA microarray analysis and Reptin-dependent gene selection process.

(B and C) Identification of Reptin-dependent (B) and Reptin-independent (C) genes by hierarchical clustering and comparing fold change of hypoxia-induced genes from cells expressing shNS and shReptin. Upregulated and downregulated gene clusters are represented as red and green, respectively. Known target genes for HIF-1, NFκB, TCF/LEF,

and p53 were collected, and the enrichment of these genes analyzed for each cluster is shown as a percentage of the total number of genes within each cluster.
(D) Reptin-dependent genes represent about 24.6% of all differentially expressed genes by hypoxia. (E) Heatmap diagram of Reptin-dependent genes (cluster 1) showing gene names with known HIF-1α target genes marked with an asterisk.
(F) Functional classification of Reptin-dependent genes (cluster 1).
See also Figure S2, Table S1, and Table S2.

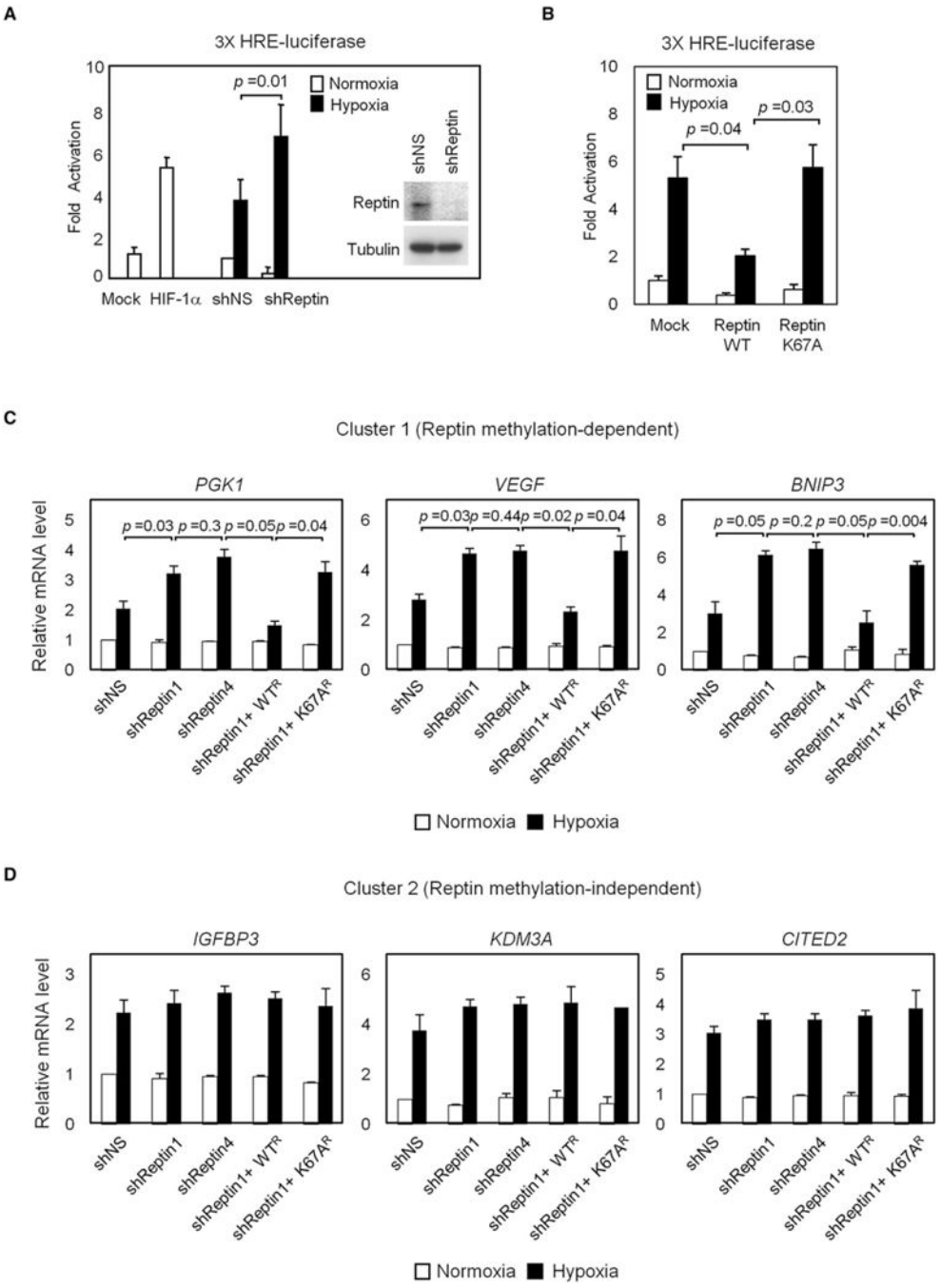

Figure 4. Reptin Methylation Negatively Regulates a Subset of Hypoxia Target Genes

(A) Effect of Reptin knockdown on 3 × HRE-luciferase reporter under normoxia or hypoxia for 9 hr. Results are expressed as fold activation compared to nonspecific shRNA (shNS) under normoxic condition. Efficacy of Reptin knockdown by Reptin shRNA (shReptin) is shown by immunoblot analysis. Values are expressed as mean ± SEM of three independent experiments.

(B) 3 × HRE reporter assay as in (A) with overexpression with Reptin WT and K67A mutant under normoxic and hypoxic conditions. Values are expressed as mean ± SEM of three independent experiments.

(C and D) Quantitative RT-PCR analysis of Reptin-dependent (C) and -independent (D) hypoxia target gene expressions identified following shRNA-mediated knockdown of Reptin and reconstitution of either shRNA-resistant Reptin WT (WT^R) or K67A mutant ($K67A^R$) in MCF7 cells in normoxic and 18 hr of hypoxic conditions. Results are expressed as relative mRNA levels compared to shNS under normoxic condition. Values are expressed as mean± SEM of three independent experiments.

See also Figure S3 and Table S3.

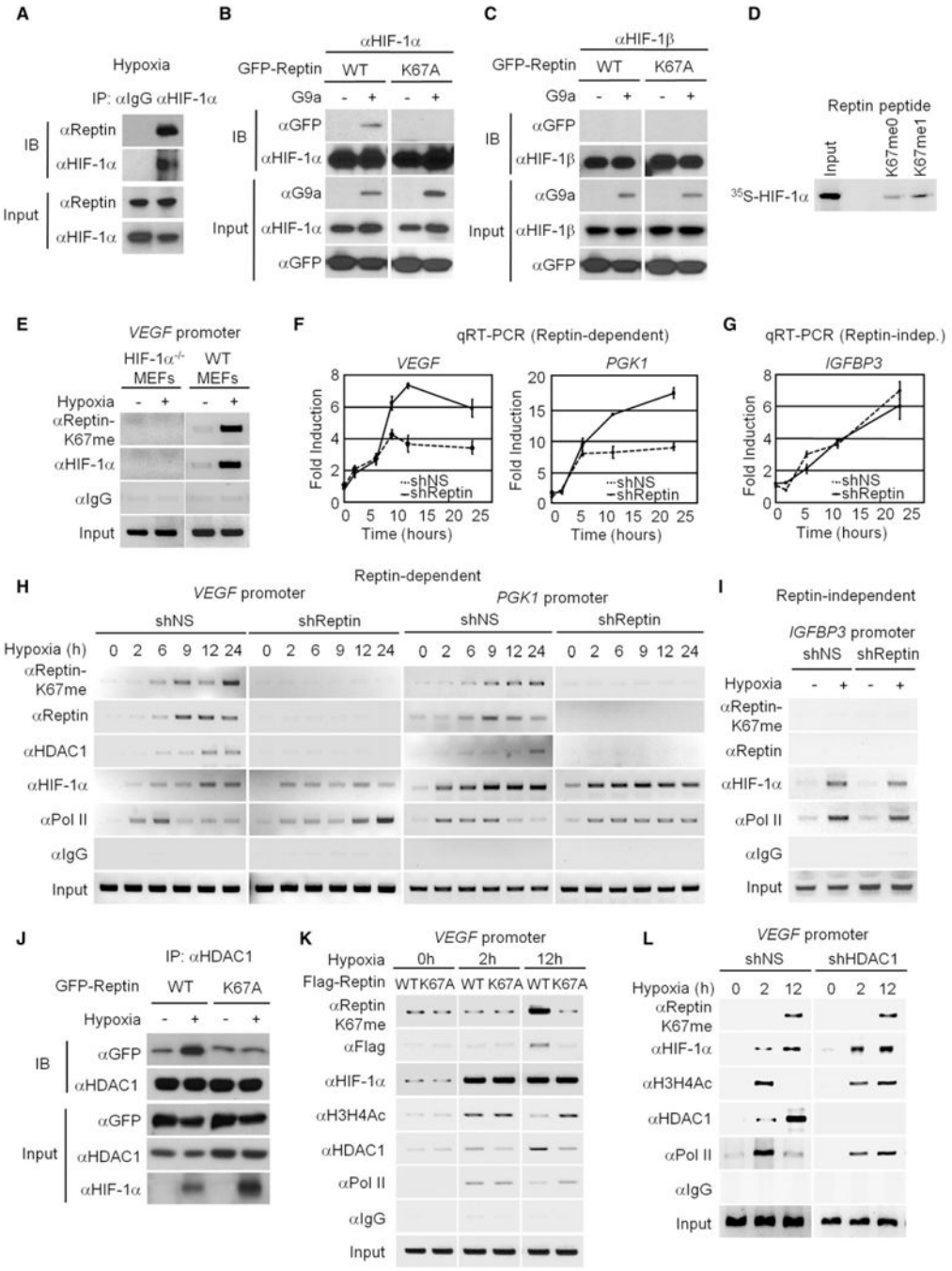

Figure 5. Recruitment of Methylated Reptin to Its Target Promoters via Enhanced Binding to HIF-1α

(A) Endogenous interaction between Reptin and HIF-1 α in MCF7 cells exposed to hypoxia using either anti-HIF-1 α or normal IgG.

(B and C) MCF7 cells were transfected with the indicated expression plasmids, and immunoprecipitation was performed using either anti-HIF-1 α (B) or anti-HIF-1 β (C) and immunoblotted using antibodies indicated.

(D) Interactions between in vitro-translated ^{35}S-HIF-1α and biotinylated Sepharose beads preincubated with nonmethylated (K67me0) and monomethylated (K67me1) Reptin peptides were analyzed by autoradiography.
(E) HIF-1 α-dependent recruitment of methylated Reptin on *VEGF* promoter in WT and HIF-1 α-deficient (*HIF-1* $\alpha^{-/-}$) mouse embryonic fibroblasts (MEFs).
(F and G) Transcript levels of Reptin-dependent (F) and Reptin-independent (G) genes with Reptin knockdown in hypoxic conditions. Values are expressed as mean ± SEM of three independent experiments.
(H) The shRNA-coupled ChIP assay on the *VEGF* and *PGK1* promoters in MCF7 cells with time course of hypoxic stress. Promoter occupancy by methylated Reptin, Reptin, HDAC1, HIF-1 α, and RNA polymerase II was analyzed.
(I) The shRNA-coupled ChIP assay was performed on the *IGFBP3* promoter in MCF7 cells.
(J) MCF7 cells were transfected with either Reptin WT or K67A mutant, and immunoprecipitation was performed using anti-HDAC1 and immunoblotted using antibodies indicated.
(K) MCF7 cells with Reptin knockdown by shReptin reconstituted with exogenous shRNA-resistant Reptin WT^R and $K67A^R$ mutant were exposed for indicated times in hypoxic condition and harvested for ChIP analysis examining the recruitment of various proteins on *VEGF* promoter as indicated.
(L) ChIP assay was performed in MCF7 cells exposed for indicated times in hypoxic condition with HDAC1 knockdown by shHDAC1.
See also Table S3.

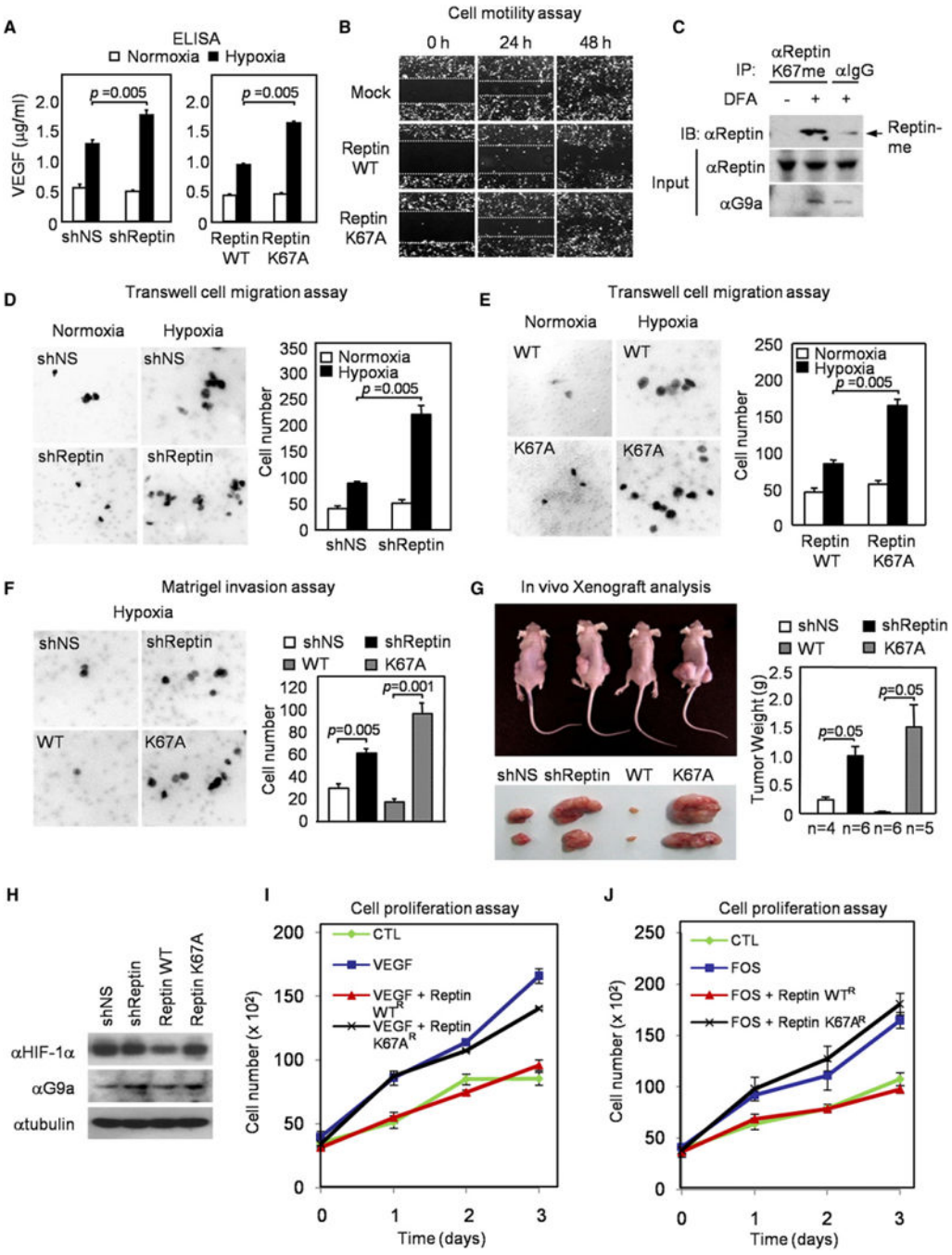

Figure 6. Biological Consequences of Reptin Methylation

(A) Measurement of VEGF by quantitative ELISA from conditioned media collected from MCF7 cells expressing various constructs as indicated. Values are expressed as mean ± SEM of three independent experiments.

(B) Photomicrographs from a scratch-mobility assay of MCF7 cells expressing either Reptin WT or K67A in the presence of DFA (100 μM) for the indicated times.

(C) Immunoprecipitation of methylated Reptin with either anti-Reptin K67 methyl antibody or normal IgG from DFA-treated MCF7 cell lysates followed by immunoblot analysis with anti-Reptin antibody.

(D and E) Photomicrographs (40×) from transwell motility assay of MCF7 cells expressing shNS and shReptin (D) and Reptin WT or Reptin K67A (E) in normoxic and hypoxic conditions for 16 hr. Bar graph shows mean number of cells per filter, and p value is shown from Student's t test analysis. Error bars represent SEM; n = 5 (right).

(F) Matrigel invasion assay using cells expressing the indicated constructs and exposed to hypoxia for 24hr. Bar graph shows mean number of cells per filter, and p value is shown from Student's t test analysis. Error bars represent SEM; n = 5 (right).

(G) In vivo xenograft assay of MCF7 cells expressing the indicated constructs (top). Tumors excised from nude mice (bottom) and statistical analysis of mean tumor weights; p values are shown from Student's t test analysis. Error bars represent SEM from number of tumors indicated.

(H) HIF-1α and G9a protein levels were examined from MCF7 cells expressing various constructs as indicated, as well as xenografts derived from (G) as shown. HIF-1α and G9a protein expression levels in MCF7 cells, grown in vitro in normoxia, were shown as a comparison to the levels observed in the extracted xenografts. Tubulin was used as a loading control.

(I and J) Proliferation was monitored for 3 days in hypoxic condition for MCF7 cells ectopically expressing either VEGF or FOS following shRNA-mediated knockdown of Reptin and reconstitution of either Reptin WT^R or $K67A^R$. Values are expressed as mean ± SEM of triplicate cell counts.

See also Figure S4.

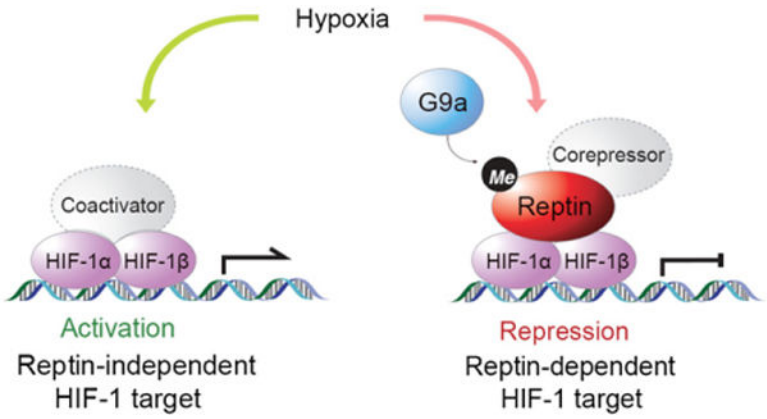

Figure 7. Model for Reptin Methylation-Mediated Negative Regulation of HIF-1 Target Genes

Hypoxia induces Reptin methylation as a result of increased G9a levels. Methylated Reptin is recruited to the promoters of a subset of hypoxia target genes via enhanced binding to HIF-1α, thereby negatively regulating HIF-1 transcriptional activity.