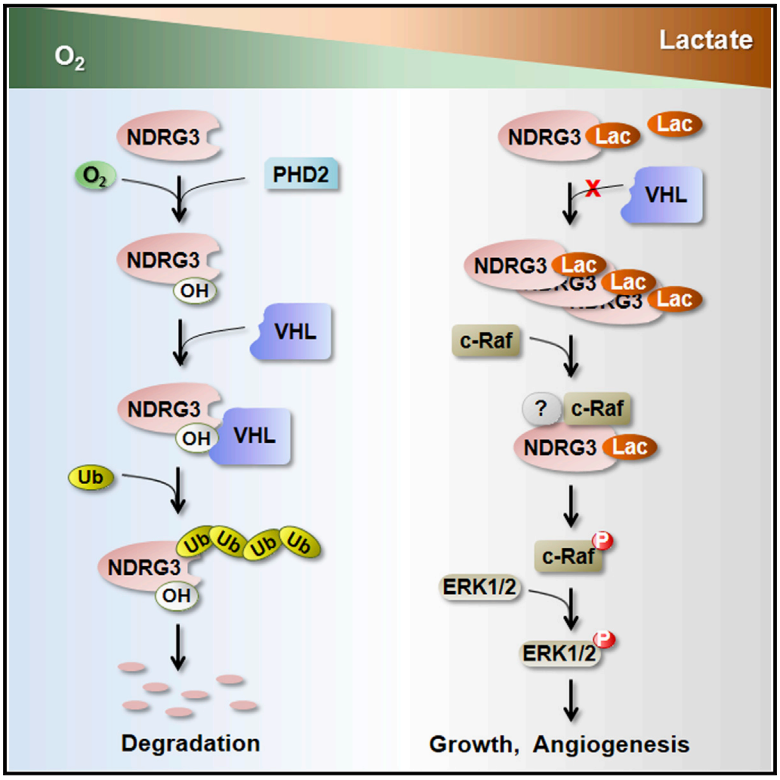
Article

Cell

# A Lactate-Induced Response to Hypoxia

## Graphical Abstract




## Highlights

- NDRG3 is an oxygen-regulated substrate of PHD2/VHL pathway
- Lactate binds to NDRG3, boosting its levels in hypoxia
- NDRG3 activates Raf-ERK signaling to mediate lactate-triggered hypoxia responses

## Authors

Dong Chul Lee, Hyun Ahm Sohn, ..., Kyung Chan Park, Young Il Yeom

## Correspondence

kpark@kribb.re.kr (K.C.P.), yeomyi@kribb.re.kr (Y.I.Y.)

## In Brief

Lactate, a common product of anaerobic metabolism, can promote a hypoxic response independent of HIF. It does so by binding as stabilizing the NDRG3 protein that, in turn, triggers signals for cell growth and angiogenesis.

## Accession Numbers

GSE55214

Lee et al., 2015, Cell *161*, 595–609
April 23, 2015 
http://dx.doi.org/10.1016/j.cell.2015.03.011

CellPress

Article

Cell

# A Lactate-Induced Response to Hypoxia

Dong Chul Lee,[1] Hyun Ahm Sohn,[1] Zee-Yong Park,[5] Sangho Oh,[3] Yun Kyung Kang,[6] Kyoung-min Lee,[5] Minho Kang,[1] Ye Jin Jang,[1] Suk-Jin Yang,[1] Young Ki Hong,[1] Hanmi Noh,[1,7] Jung-Ae Kim,[1,7] Dong Joon Kim,[1] Kwang-Hee Bae,[4,7] Dong Min Kim,[1] Sang J. Chung,[1] Hyang Sook Yoo,[1] Dae-Yeul Yu,[1] Kyung Chan Park,[1,*] and Young Il Yeom[1,2,7,*]

[1]Medical Genomics Research Center

[2]Ochang Branch Institute

[3]Korean Bioinformation Center

[4]Research Center for Integrative Cellulomics

Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea

[5]Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea

[6]Department of Pathology, Inje University Seoul Paik Hospital, Seoul 100-032, Korea

[7]Department of Functional Genomics, Korea University of Science and Technology, Daejeon 305-350, Korea

*Correspondence: kpark@kribb.re.kr (K.C.P.), yeomyi@kribb.re.kr (Y.I.Y.)

http://dx.doi.org/10.1016/j.cell.2015.03.011

## SUMMARY

**Organisms must be able to respond to low oxygen in a number of homeostatic and pathological contexts. Regulation of hypoxic responses via the hypoxia-inducible factor (HIF) is well established, but evidence indicates that other, HIF-independent mechanisms are also involved. Here, we report a hypoxic response that depends on the accumulation of lactate, a metabolite whose production increases in hypoxic conditions. We find that the NDRG3 protein is degraded in a PHD2/VHL-dependent manner in normoxia but is protected from destruction by binding to lactate that accumulates under hypoxia. The stabilized NDRG3 protein binds c-Raf to mediate hypoxia-induced activation of Raf-ERK pathway, promoting angiogenesis and cell growth. Inhibiting cellular lactate production abolishes the NDRG3-mediated hypoxia responses. Our study, therefore, elucidates the molecular basis for lactate-induced hypoxia signaling, which can be exploited for the development of therapies targeting hypoxia-induced diseases.**

## INTRODUCTION

Oxygen homeostasis is essential for metazoan physiology. Under low oxygen conditions, cells resort to hypoxia-induced responses to adapt to and survive harsh environments (Cassavaugh and Lounsbury, 2011). Hypoxia responses are an integral part of normal physiology during embryonic development and postnatal life. They are also pathophysiologic components of many disorders, including cancer, inflammation, and cardiovascular diseases.

Hypoxia inducible factors (HIFs) play central roles in hypoxia responses by controlling the expression of a host of hypoxia-responsive genes functioning in diverse processes, including metabolism, oxygen delivery, pH regulation, angiogenesis, cell proliferation, and survival (Harris, 2002; Cassavaugh and Lounsbury, 2011). In particular, the HIF-mediated upregulation of glycolysis and suppression of the citric acid (TCA) cycle is a crucial adaptive response at the early stage of hypoxia (Cassavaugh and Lounsbury, 2011). The expression and activity of HIFs are tightly regulated by oxygen-dependent hydroxylation of their $\alpha$ subunits (Semenza, 2003).

Growing evidence indicates that hypoxia has many aspects that are not explained by HIF-mediated mechanisms alone. For example, the inhibition of HIF-mediated pathways does not always prevent tumor growth; tumors derived from HIF-1$\alpha$-deficient embryonic stem (ES) cells have growth advantages owing to decreased hypoxia-induced apoptosis and increased stress-induced proliferation (Carmeliet et al., 1998). A number of reports suggest that tumor angiogenesis constitutes the major pathway of HIF-independent tumorigenesis. Thus, angiogenesis was preserved when *HIF1A* was knocked-out in ES cells (Höpfl et al., 2002). Several lines of evidence indicate that the pro-angiogenic factor, vascular endothelial growth factor (VEGF), can be induced via both HIF-dependent and HIF-independent pathways (Mizukami et al., 2004). Induction of other pro-angiogenic factors such as IL-8 preserves the angiogenic response in HIF-1$\alpha$-deficient colon cancer cells (Mizukami et al., 2005). Moreover, multiple pathways and transcription factors (TFs) other than HIFs are known to respond to hypoxia to induce biological responses in a HIF-independent manner. Among those oxygen-regulatable TFs are NF-$\kappa$B, AP-1, and CEBP, which are activated in hypoxia (Cummins and Taylor, 2005). Consequently, several reports demonstrated that some of the genes regulated by hypoxia were not regulated by HIFs, suggesting a role for other oxygen-regulated pathways that are, similar to HIF pathways, controlled by prolyl hydroxylase domain (PHD) enzymes (Elvidge et al., 2006). Also, a number of protein kinases such as PKA, PKC, PI3K, AKT, JNK, PTK2B (Pyk2), SRC, MAPK14 (p38), and ERK1/2 are reported to be activated in hypoxia (Seta et al., 2002). However, despite all of these studies, key elements and mechanisms responsible for oxygen-dependent regulation of the HIF-independent branch of hypoxia responses remain elusive.

In this study, we identified an oxygen-regulated protein, NDRG3 (*NDRG* family member 3; NM_032013), as a bona fide substrate of the PHD2/VHL system. NDRG3 was highly induced

under oxygen-limited conditions in diverse cell types, although its mRNA expression was independent of HIF levels under hypoxia. Interestingly, NDRG3 required binding by the glycolytic end-product lactate for its hypoxic accumulation, rendering its expression indirectly dependent on HIF expression as HIF-1α regulates the hypoxic expression of lactate dehydrogenase A (*LDHA*). We found that NDRG3 plays critical roles in lactate-induced hypoxia signaling by mediating the activation of the Raf-ERK pathway to promote angiogenesis and cell growth during prolonged hypoxia. Thus, *NDRG3* provides a critical genetic element for the oxygen- and lactate-dependent regulation of prolonged hypoxia responses.

## RESULTS

### Identification of NDRG3 as the Substrate of PHD2

To identify the regulators of hypoxia responses, we searched for PHD2-binding proteins in MCF-7 cells expressing Flag-tagged PHD2 via Flag-mediated immunoprecipitation coupled to mass spectrometry. Among the candidates enriched in the protein bands reproducibly exhibiting differential immunoprecipitation patterns between mock and PHD2-Flag fractions, we chose *NDRG3* for further studies since it belongs to a gene family implicated in cell proliferation, migration, and invasion as well as in differentiation and development (Melotte et al., 2010), which are biological features closely associated with hypoxia (Harris, 2002; Cassavaugh and Lounsbury, 2011) (Figure S1A).

To characterize NDRG3 in detail, we developed an affinity-purified polyclonal antibody specific to NDRG3 among the human *NDRG* family members (Figure S1B). This antibody detected NDRG3 as a 42-KDa band in the PHD2-Flag immunoprecipitation fraction (Figure 1A). We verified the NDRG3-PHD2 interaction by immunoprecipitating endogenous NDRG3 with PHD2-Flag from HeLa cells grown under hypoxia (Figure 1B) and directly by a pull-down assay using recombinant PHD2-His and NDRG3-GST proteins (Figure S1C). Thus, we concluded that NDRG3 is a bona fide PHD2-binding protein.

We then examined possible functional relationships between PHD2 and NDRG3 using a PHD inhibitor, desferrioxamine (DFX). Although the basal-level expression of NDRG3 was negligible, PHD inhibition caused its dose-dependent accumulation in HeLa (Figure 1C) and MCF-7 cells (Figure S1D). These results were reproducible with two other PHD inhibitors, dimethyloxaloylglycine (DMOG) and $CoCl_2$ (Figure S1E), suggesting that the NDRG3 protein expression might be under PHD-mediated posttranslational control. We then examined different PHD family members for their involvement in the regulation of NDRG3 by silencing their expression under normoxia using small interfering RNAs (siRNAs). The analysis results revealed that, as in the case of HIF-1α, PHD2 is the major regulator of NDRG3 expression among the PHD family members (Figure 1D, left). This was supported by the identification of differential interactions between NDRG3 and PHD2 in a co-immunoprecipitation assay (Figure S1F). Depletion of *VHL*, the targeting element of E3 ubiquitin ligase complex, also caused NDRG3 accumulation under normoxia (Figure 1D, right), suggesting that NDRG3 is likely a target of PHD2/VHL-mediated posttranslational modification. To address this point more thoroughly, we prepared several variants of the NDRG3 protein carrying single amino acid changes in their putative PHD2-docking site, predicted from a docking model between a putative NDRG3 structure and the published PHD2 structure (Chowdhury et al., 2009) (Figure S1G). A co-immunoprecipitation assay showed that the NDRG3 mutants could be ranked according to their PHD2-binding strengths in the following order: V296D > Q97E > R47D ≈ N66D, which, interestingly, appeared to be inversely correlated with their protein expression levels in normoxia (Figure 1E). Moreover, NDRG3 variants retaining higher affinity for PHD2 co-immunoprecipitated higher amount of HA-tagged VHL protein (Figure 1E), indicating that the interaction of NDRG3 with PHD2 and VHL is a critical determinant of its protein expression. Next, in an in vivo ubiquitination assay, the amount of ubiquitin immunoprecipitated with NDRG3 was increased by overexpression of NDRG3, while it was decreased by silencing of its expression by different short hairpin RNAs (shRNAs) (Figures 1F and S1H). In addition, proteasome inhibition with MG132 dramatically increased the detected levels of NDRG3 in HeLa cells (Figure S1I). Collectively, these results demonstrate that NDRG3 is a PHD2-interacting protein whose expression is negatively regulated by PHD2/VHL-mediated proteasomal pathways.

### Oxygen-Dependent Regulation of NDRG3 Protein Expression

Since PHD2 critically depends on $O_2$ availability for its activity, we examined whether the NDRG3 protein expression is regulated in an oxygen-dependent manner. NDRG3 accumulated in MCF-7 cells at rates that were inversely correlated with $O_2$ concentrations (Figures 2A and S2A). Consistent with this, NDRG3 ubiquitination was significantly suppressed in HeLa cells under hypoxia (Figure 2B). The hypoxic induction of NDRG3 was demonstrated in cancer cells of diverse tissue origins as well as in non-transformed cells (Figure S2B), suggesting a universality of the phenomenon. However, in contrast to HIF-1α protein showing a sort of bell-shaped induction pattern at the early stage of hypoxia, NDRG3 exhibited a sigmoidal expression pattern, starting when HIF-1α levels began to decline and lasting until later stages of hypoxia (Figure 2C). The hypoxic expression of NDRG3 slowly diminished as cells were reoxygenated (Figure S2C). These results strongly suggest that NDRG3 protein expression is negatively regulated by oxygen.

Next, we investigated the molecular basis of the oxygen-dependent regulation of NDRG3 protein expression. Mass spectrometric analysis revealed that NDRG3 is specifically hydroxylated at proline 294, suggesting that it might be the residue modified by PHD2 (Figure 2D). Site-directed mutagenesis of proline 294 to alanine (P294A) resulted in pronounced accumulation of the variant protein in normoxia (Figure 2E, left). Moreover, a co-immunoprecipitation assay showed that the P294A mutant protein possessed a significantly reduced binding affinity for PHD2 and VHL proteins compared to wild-type (Figure 2E, right), indicating that proline 294 is the critical target site of PHD2-mediated hydroxylation that determines NDRG3 protein stability in normoxia.

Since the expression of HIF-1α immediately preceded that of NDRG3 (Figure 2C), we investigated the possibility of HIF-1α transcriptionally regulating *NDRG3* expression during hypoxia.

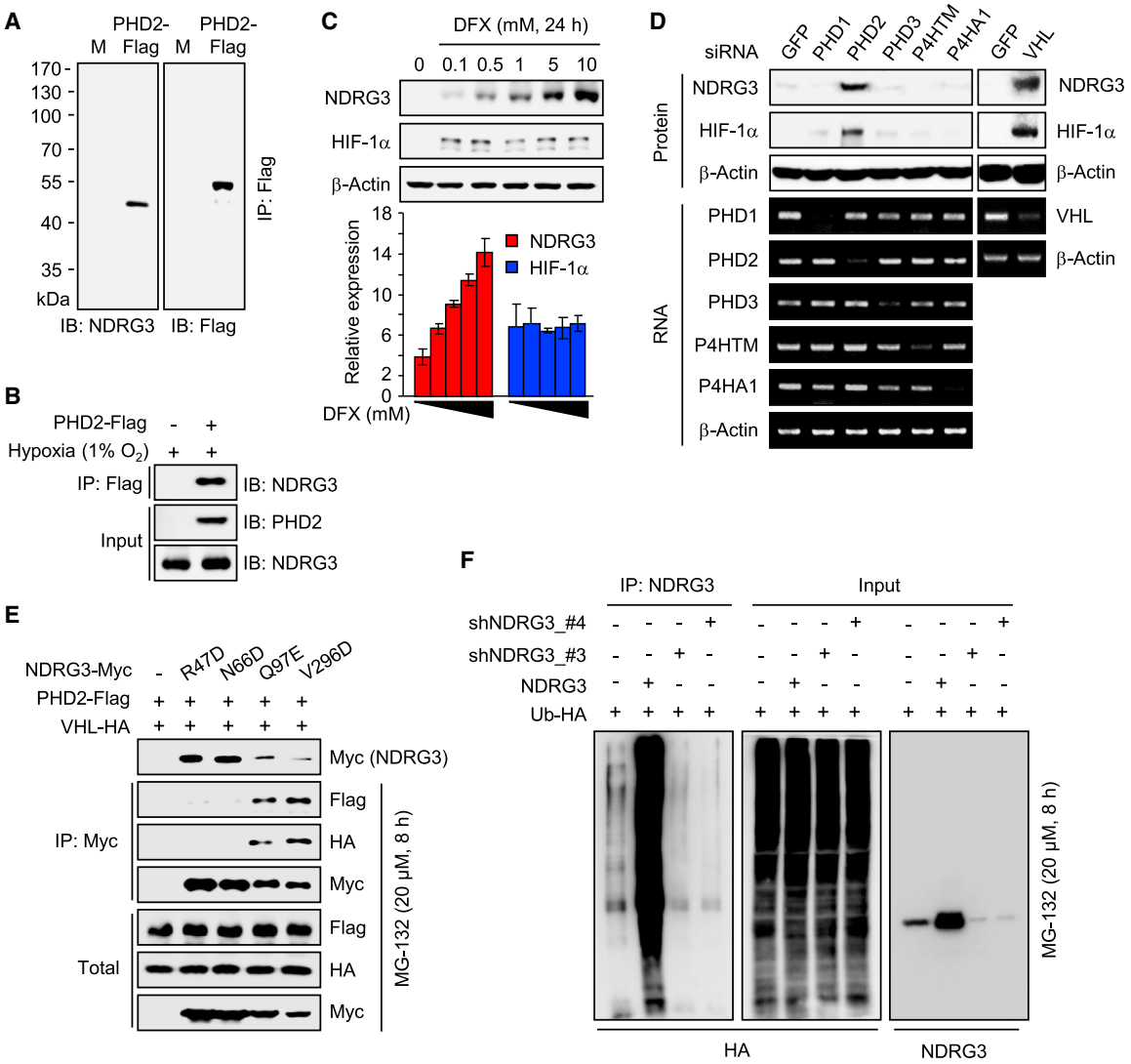



**Figure 1. NDRG3 Protein Is Regulated by PHD2/VHL-Mediated Proteasomal Pathway**

(A) Identification of NDRG3 as a PHD2-binding protein in MCF-7 cells.

(B) Validation of PHD2-NDRG3 interaction in HeLa cells under hypoxia.

(C) Induction of NDRG3 protein by inhibiting PHDs with desferrioxamine (DFX) in HeLa cells at normoxia. Results are mean ± SD of three experiments.

(D) Effects of depleting different PHD family members (left) or VHL (right) on NDRG3 protein expression in HeLa cells in normoxia.

(E) Expression pattern of NDRG3 variants mutated in putative PHD2-binding sites and their interaction with PHD2 and VHL in HEK293T cells.

(F) Ubiquitination assay of NDRG3 protein in HeLa cells at normoxia.

See also Figure S1.

RT-PCR analysis showed that *NDRG3* mRNA level remained virtually unchanged during hypoxia, even when HIF proteins reached their peak levels (Figure S2D). This result indicates the HIF independence of *NDRG3* transcription and confirms the posttranslational nature of *NDRG3* expression during hypoxia. Depletion of different subunits of HIF had no effects on *NDRG3* mRNA levels, confirming the HIF independence of its transcription (Figure 2F). It is noteworthy that although NDRG3 protein expression in hypoxia was clearly detectable in HIF-silenced cells, it was significantly reduced compared to control by HIF-1β knockdown and, to a much lesser extent, by HIF-1α knockdown, suggesting a potential non-transcriptional effect of the HIF pathway on NDRG3 protein expression. Meanwhile, we could show that HIF played a role as a transcriptional activator for the hypoxic expression of another *NDRG* family member, *NDRG1* (Figure S2E). These results collectively indicate that HIF activity is not required for the transcriptional regulation of *NDRG3* expression during hypoxia.

## Role of NDRG3 in the Regulation of Hypoxia Responses

We investigated the potential functions of NDRG3 in hypoxia by correlating its protein expression profile with the genomic

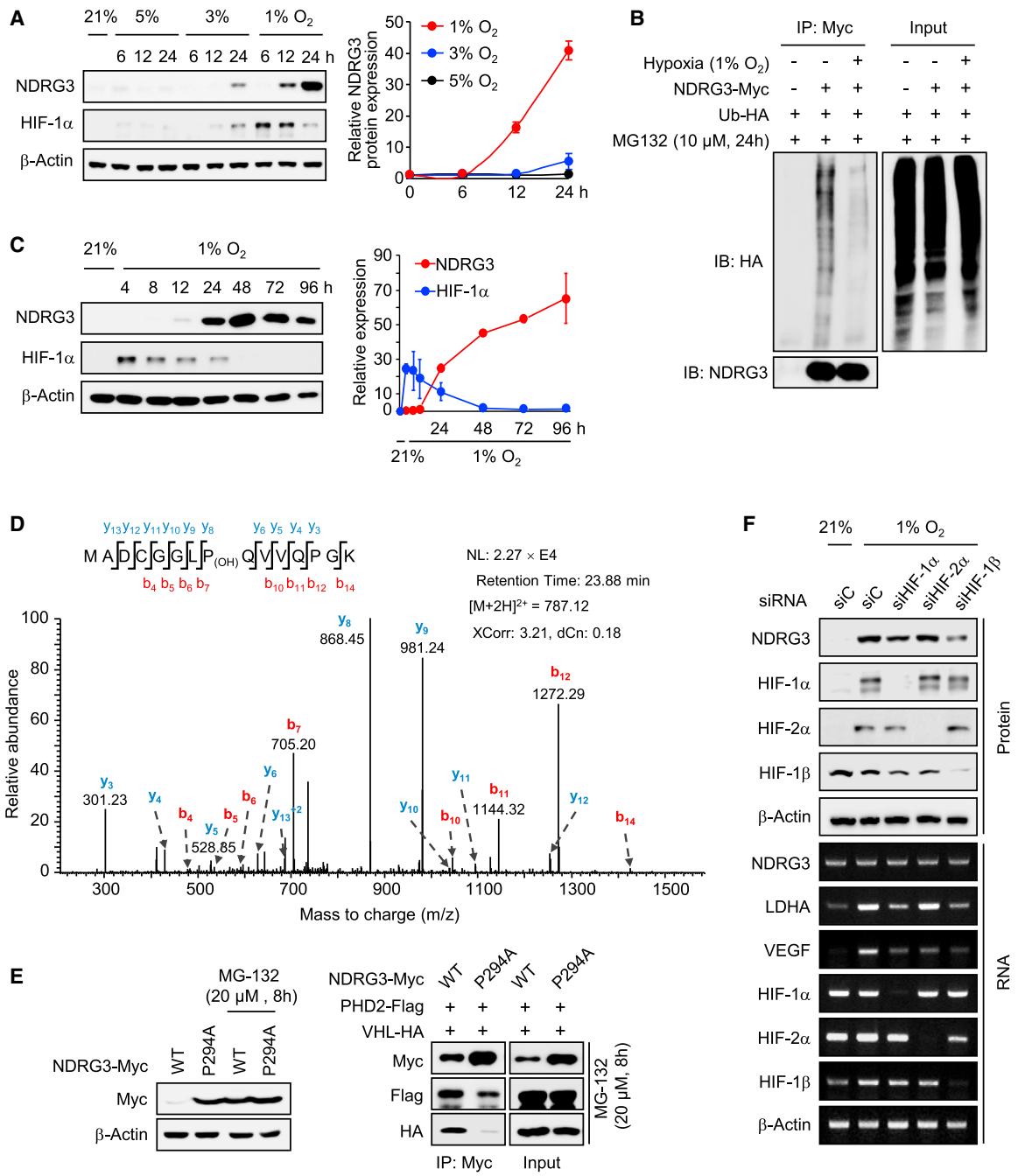



**Figure 2. NDRG3 Expression Is Regulated in an Oxygen-Dependent Manner**

(A) NDRG3 protein expression at different oxygen concentrations in MCF-7 cells. Quantified values for western blot images are shown on the right. Results are mean ± SD of three experiments.

(B) Oxygen dependency of NDRG3 protein ubiquitination in vivo.

(C) NDRG3 protein expression in MCF-7 cells during prolonged hypoxia. Quantified values for western blot images are shown on the right. Results are mean ± SD of three experiments.

(D) Site of prolyl hydroxylation in NDRG3 identified by micro-LC-MS/MS analysis.

(E) Expression pattern of an NDRG3 variant mutated in putative prolyl hydroxylation site (P294A) (left) and its interaction with PHD2 and VHL proteins (right) in HEK293T cells. WT, wild-type.

(F) Effects of silencing HIF proteins on hypoxic expression of NDRG3 in Huh-1 cells.

See also Figure S2.

activity profile of five gene ontology categories representative of hypoxia responses (Figure S3A). The genomic activity of a gene ontology was estimated via the gene set enrichment analysis, whereby a standardized difference score (*Z* score) was calculated from transcriptome expression data of Huh-7 cells at a particular time point during hypoxia. The results showed that NDRG3 protein expression was highly correlated with the activity of ''angiogenesis,'' ''anti-apoptosis,'' ''proliferation (positive),'' and ''motility'' functions but not with ''glycolysis'' (Figure 3A). On the other hand, depletion of NDRG3 at 24 hr under hypoxia, when cellular NDRG3 protein expression should have otherwise reached a significant level, caused significant changes in the activity of ''angiogenesis,'' ''anti-apoptosis,'' ''proliferation (positive),'' and ''motility'' categories but not that of ''glycolysis'' (Figure 3B). In contrast, ''glycolysis'' was significantly targeted by HIF-1$\alpha$ depletion at 6 hr under hypoxia, when HIF-1$\alpha$ protein expression is expected to have reached its peak level (Figure S3B). Consistently, the ectopic expression of a normoxia-stable variant of NDRG3 (N66D in Figure 1E) caused the upregulation (>1.5-fold) of genes having primary functions in angiogenesis > proliferation ≈ growth ≈ apoptosis ≈ migration > glycolysis (Figure S3C).

We then experimentally evaluated the roles of NDRG3 in ''angiogenesis,'' ''anti-apoptosis,'' and ''proliferation,'' the functions often implicated in tumor growth and significantly targeted by NDRG3 depletion (Figure 3B). In tube forming assays using HUVEC cells, NDRG3 depletion caused significant suppression of the angiogenic activity induced by hypoxia in Huh-7 cells (Figure S3D). In parallel, the Matrigel plug assay showed that *NDRG3* knockdown inhibited the angiogenic activity of Huh-7 cells in BALB/c-nu mice (Figure 3C). At the molecular level, hypoxia-induced expression of pro-angiogenic markers was abolished by NDRG3 depletion, while it was upregulated in normoxia by NDRG3(N66D) (Figure 3D). Next, examination of the anti-apoptotic activity of NDRG3 via caspase-3/7 and PARP cleavage assays indicated that NDRG3 depletion significantly promotes apoptosis in hypoxia (Figure 3E). Accordingly, the hypoxia-induced expression of anti-apoptotic genes, notably members of the IAP (inhibitor of apoptosis proteins) family, was abolished by NDRG3 depletion in Huh-7 cells (Figure 3F). Moreover, the depletion of *NDRG3* using an shRNA targeting its 3′-UTR (Figure S1H, #5) significantly inhibited the growth of Huh-7 cells under mild hypoxia (3% $O_2$; Figure 3G), but this phenotype was effectively rescued by a recombinant NDRG3(N66D) expression vector lacking the natural 3′-UTR sequences of *NDRG3* (Figures 3G and S3E). In addition, *NDRG3* knockdown strongly suppressed the tumorous growth of Huh-7 cells in BALB/c-nu mice (Figures 3H and S3F). Interestingly, simultaneous depletion of NDRG3 and either of the HIFs completely abrogated tumor growth, suggesting complementary roles for NDRG3 and HIFs in hypoxic cell growth (Figure 3H). Immunofluorescence microscopy of resected tumors revealed that NDRG3 depletion effectively suppressed the expression of markers of tumor angiogenesis (IL8 and CD31) and cell proliferation (Ki-67), while their levels in HIF-depleted tumors were comparable to those in controls (Figure S3G). In contrast, the ectopic expression of NDRG3(N66D) highly promoted colony formation of Huh-1 cells in soft agar (Figure S4J) as well as their tumorigenic activity in BALB/c-nu mice (Figures 3I and S3H). These results demonstrate that NDRG3 plays crucial roles in promoting angiogenesis, anti-apoptosis, and cell proliferation during hypoxia.

## L-Lactate Triggers the NDRG3-Mediated Hypoxia Responses

Compared to HIF-1$\alpha$, which showed an early induction pattern during hypoxia and rapidly disappeared upon reoxygenation of cells, NDRG3 started accumulating relatively later in hypoxia and its levels slowly declined upon reoxygenation (Figures 2C and S2C). The long lag periods observed for the accumulation and degradation of NDRG3 suggested that multiple layers of regulation might be involved in its hypoxic expression. Therefore, we explored biochemical features relevant to ''prolonged hypoxia'' other than low oxygen levels and found that NDRG3 protein expression is highly correlated with cellular lactate production; NDRG3 protein expression began at ~6 hr under hypoxia, closely following the lactate production pattern (Figure 4A). On the other hand, suppression of lactate production with a LDHA inhibitor, sodium oxamate, specifically inhibited the NDRG3 protein accumulation in a dose-dependent manner (Figure 4B). Similarly, inhibition of lactate production via siRNA-mediated depletion of *LDHA* (Figure 4C) or disruption of glycolysis with 2-deoxyglucose (Figure S4A) suppressed the hypoxic NDRG3 protein expression. Depriving cells of glucose and/or glutamine, the input substrates for glycolysis and glutaminolysis, respectively—two major metabolic pathways leading to intracellular lactate production—also reduced the NDRG3 protein accumulation with a parallel reduction in lactate production but without affecting the transcription of *NDRG3* (Figure 4D). However, compared to the significant consequences of glucose deprivation, the glutamine effect seemed relatively minor. In contrast, the facilitation of lactate production (via LDHA overexpression and/or pyruvate overfeeding; Figure S4B) or its intracellular accumulation (by blocking export through MCT4; Figure 4C) augmented the hypoxic accumulation of NDRG3 protein. These results indicate that, unlike HIF proteins, oxygen deprivation per se is not enough to cause the accumulation of NDRG3 protein, but glycolytic production of lactate is additionally required.

We then verified the effects of lactate on NDRG3 protein dynamics more directly by providing exogenous lactate to the cells whose intracellular lactate production had been compromised by genetic or pharmacological means. Lactate exogenously added to Huh-1 cells dose dependently restored the hypoxic NDRG3 protein expression that had been reduced by *LDHA* silencing, without affecting the level of *NDRG3* mRNA or HIF-1$\alpha$ protein (Figure 4E). Similar results were obtained when lactate production was suppressed by glucose deprivation (Figure S4C) or oxamate treatment (Figure S4D). However, the lactate-mediated restoration of NDRG3 protein expression was abrogated by siRNA targeting *MCT1*, a monocarboxylate transporter responsible for importing extracellular lactate into the cell, both in normoxic and hypoxic conditions (Figure 4F). We also observed similar effects of *MCT1* knockdown in Huh-1 cells subjected to oxamate treatment or glucose deprivation (Figures S4E and S4F). Collectively, these results indicate that NDRG3 requires lactate build-up for its protein accumulation under hypoxia, pointing to the possibility that NDRG3 might function as a

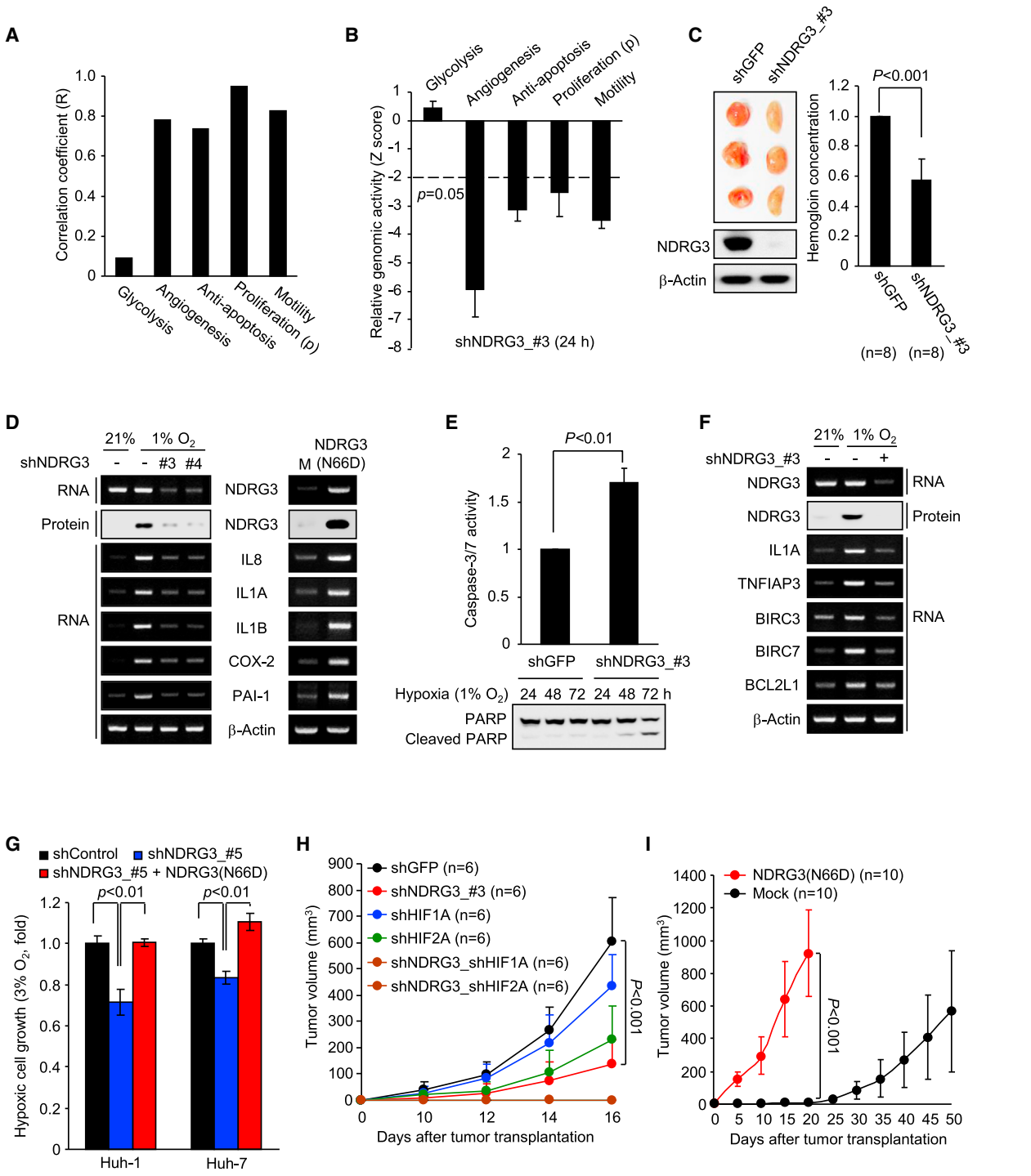



**Figure 3. *NDRG3* Is a Critical Regulator of Prolonged Hypoxia Responses**

(A) Correlation analyses between the NDRG3 protein expression during hypoxia and the activity of five representative hypoxia-responsive gene sets.

(B) Changes in the activity of hypoxia-responsive gene sets upon *NDRG3* silencing in Huh-7 cells at 24 hr under hypoxia (1% $O_2$).

(C) Matrigel plug assay of NDRG3-mediated angiogenic activity. The p value was assessed by Student's t test.

(D) Regulation of pro-angiogenic gene expression by *NDRG3*. Gene expression in *NDRG3*-silenced Huh-7 cells under hypoxia (1% oxygen, left) or in NDRG3(N66D)-overexpressing HeLa cells at normoxia (right) was examined by RT-PCR.

(E) Effects of *NDRG3* knockdown on hypoxia-induced apoptosis in Huh-7 cells. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.

(F) Regulation of hypoxia-induced anti-apoptotic gene expression by *NDRG3*.

*(legend continued on next page)*

hypoxia-inducible lactate sensor, triggering HIF-independent biologic responses in the cell.

We, therefore, examined the functional significance of hypoxic NDRG3 expression in the context of lactate metabolism. Inhibition of lactate production with oxamate caused a dose-dependent suppression of Huh-1 cell growth under mild hypoxia (Figure S4G). However, this effect was effectively rescued by ectopically expressing NDRG3(N66D) (Figure S4G), suggesting that NDRG3 may play a critical role in lactate-induced hypoxic cell growth. Apparently, NDRG3(N66D) exerted no direct effects on lactate production irrespective of oxamate treatment (Figure S4H) nor was its expression affected by oxamate (Figure S4I), indicating that the rescuing effect is truly inherent in NDRG3(N66D) itself. The effect of oxamate on cell growth and its rescue by NDRG3(N66D) were further corroborated by colony forming assays using Huh-1 cells (Figure S4J). We then examined the role of *NDRG3* in the growth of cells whose *LDHA* expression was ablated by RNAi. Depletion of *LDHA* by shRNA suppressed the growth of Huh-1 cells under mild hypoxia (Figure S4K) as well as their tumorous growth in BALB/c-nu mice (Figures 4G and S4L). Again, NDRG3(N66D) effectively compensated for the *LDHA* deficit both in vitro and in vivo. Moreover, in tube forming assays using HUVEC cells, oxamate suppressed the angiogenic activity induced in Huh-1 cells under hypoxia (Figure 4H). However, the ectopic expression of NDRG3(N66D) restored the angiogenic activity in these cells in spite of the oxamate treatment. Thus, lactate appears to be a crucial signal for hypoxic cell growth and angiogenesis, and NDRG3 functions as a key mediator of the lactate-induced hypoxia responses.

## Molecular Mechanism of the Lactate Regulation of NDRG3 Protein Expression

We investigated the molecular mechanism for the lactate-induced NDRG3 protein accumulation by examining the effect of lactate on the ubiquitination of NDRG3. In vitro, lactate inhibited NDRG3 ubiquitination, catalyzed by the PHD2/VHL complex immunoprecipitated from HEK293T cells (Figure 5A), indicating that lactate can block the modification of NDRG3 protein by PHD2/VHL. It seems clear that lactate does not affect the HIF-1$\alpha$ protein expression under hypoxia (Figures 4 and S4). We, therefore, examined the possibility of lactate directly modulating NDRG3 by investigating interactions between the two molecules. An in vitro binding experiment using GST-tagged recombinant NDRG3 protein and [$^{14}$C]-labeled L-lactate indicated that NDRG3 physically and directly binds lactate (Figures 5B and S5B). To verify the NDRG3-lactate interaction further, we predicted the putative lactate-binding domain of NDRG3 by a docking simulation (not shown). Site-directed mutagenesis of the predicted lactate-binding domain showed that mutations in some of its amino acid residues can impair the hypoxic accumulation of the mutant proteins (Figure S5A). One of the variants whose glycine-138 was mutated to tryptophan (N3(G138W) in Figure S5A) hardly accumulated under hypoxia, but accumulated in the presence of MG132, suggesting that it may have lost the lactate-binding capability necessary for escaping PHD2/VHL-mediated proteasomal degradation. Indeed, we observed that recombinant N3(G138W)-GST protein has a severely impaired lactate-binding capability in an in vitro binding assay (Figures 5C and S5B). These results suggest that binding by lactate inhibits proteasomal degradation of NDRG3 by blocking its modification by PHD2/VHL. Moreover, once formed, the NDRG3-lactate complex seems to remain quite resistant to the PHD2/VHL-mediated modification since the hypoxically accumulated NDRG3 protein was maintained for a while after culturing the cells in fresh medium under normoxia (Figure S2C). In contrast, HIF-1$\alpha$ rapidly disappeared upon reoxygenation, demonstrating the exquisite oxygen dependency of its post-translational regulation.

We further investigated the mechanisms behind the lactate-induced changes in NDRG3 protein dynamics through protein binding analyses in HEK293T cells expressing epitope-tagged NDRG3, PHD2, and VHL. Binding between NDRG3 and PHD2 during early (6 hr) or late (24 hr) hypoxia did not significantly differ from that in normoxia, suggesting that neither low oxygen nor high lactate levels affects the NDRG3-PHD2 interaction (Figure 5D). By contrast, binding between NDRG3 and VHL was significantly reduced at 24 hr under hypoxia, while it was maintained at normoxic levels at 6 hr under hypoxia, indicating that high lactate levels but not low oxygen levels might affect the NDRG3-VHL interaction. We then verified these observations using NDRG3 variants having defects in prolyl hydroxylation by PHD2 (P294A) or lactate binding (G138W). None of the wild-type or variant NDRG3 species showed significant differences in their PHD2-binding capacity between normoxia and hypoxia (24 hr) (Figure 5E). On the other hand, the VHL-binding capacity of wild-type NDRG3 was significantly reduced under hypoxia compared to that in normoxia, while those of P294A and G138W were barely changed by hypoxia. Notably, the interaction of P294A with VHL was negligible whereas the G138W-VHL interaction was strongly maintained, regardless of oxygen level. Consistently, ubiquitination of wild-type NDRG3 was significantly reduced under hypoxia, while P294A and G138W were negligibly and strongly ubiquitinated, respectively, in both normoxia and hypoxia (Figure 5F). Conversely, oxamate treatment specifically augmented the hypoxic interaction of wild-type NDRG3 with VHL as well as its ubiquitination (Figures 5G and S5C). Inhibition of hypoxic lactate production by glucose deprivation also resulted in the augmentation of the NDRG3-VHL interaction (Figure S5D). Addition of exogenous lactate to oxamate-treated cells specifically inhibited the NDRG3-VHL interaction in both normoxia and hypoxia (Figure S5E). Thus, we conclude that the NDRG3-PHD2 interaction is not affected by cellular oxygen or lactate levels, while the NDRG3-VHL

(G) Inhibition of hypoxic cell growth by *NDRG3* knockdown and its rescue by NDRG3(N66D) overexpression. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.

(H) Effects of the knockdown of *NDRG3* and/or HIF-$\alpha$ on the tumorigenic activity of Huh-7 cells in vivo. The p value was assessed by Student's t test.

(I) Tumorigenic activity of Huh-1 cells overexpressing NDRG3(N66D). The p value was assessed by Student's t test.

See also Figure S3.

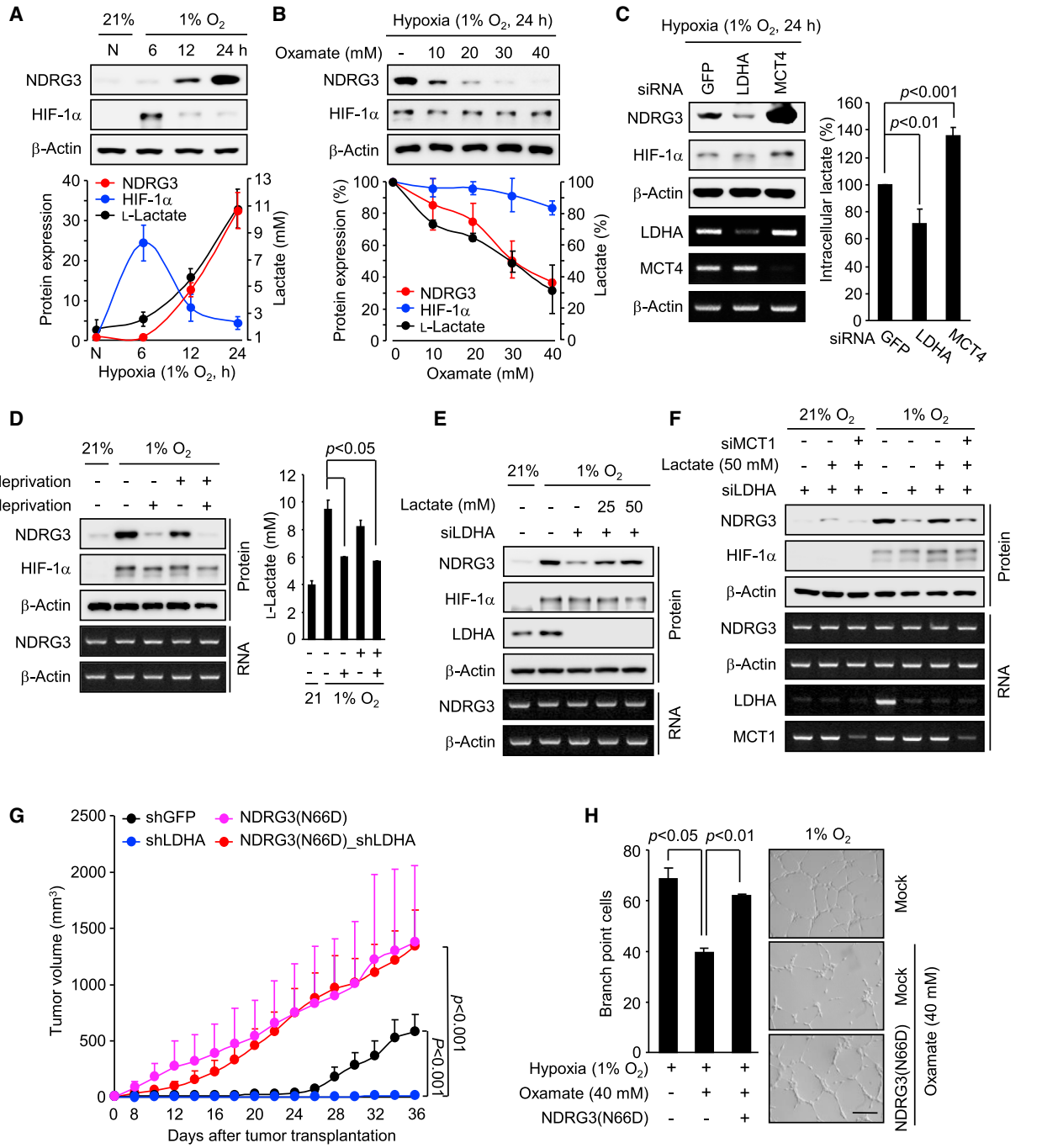



**Figure 4. Lactate Signals for the NDRG3-Mediated Hypoxia Responses**

(A) Intracellular NDRG3 protein accumulation and gross lactate production by MCF-7 cells during hypoxia. N, normoxia. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.

(B) Effects of the pharmacological inhibition of lactate dehydrogenase on hypoxic lactate production and NDRG3 protein expression by MCF-7 cells. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.

(C) Effects of depleting the genes of lactate metabolism on the hypoxic expression of NDRG3 protein and intracellular lactate levels in HeLa cells. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.

(D) Hypoxic expression of NDRG3 and lactate production in Huh-1 cells deprived of glucose (Glc) or glutamine (Gln). Results are mean ± SD of three experiments. The p values were assessed by Student's t test.

(E) Effects of exogenous lactate on the hypoxic expression of NDRG3 in *LDHA*-silenced Huh-1 cells.

(F) Effects of *MCT1* depletion on the NDRG3 protein expression induced by exogenous lactate in *LDHA*-silenced Huh-1 cells.

*(legend continued on next page)*

interaction is significantly inhibited by lactate but not by low oxygen levels.

In summary, excess lactate built up during hypoxia directly binds to NDRG3 and inhibits its ubiquitination and proteasomal degradation by disrupting the NDRG3-VHL interaction. However, the question remains whether failure of NDRG3 ubiquitination in high lactate conditions is due to the inhibition of PHD2-mediated hydroxylation of NDRG3 that is required for VHL binding and awaits further studies.

## Activation of Raf-ERK Signaling by NDRG3 during Hypoxia

To understand the molecular mechanisms of NDRG3′s function in hypoxia, we searched for possible NDRG3-regulated kinases via a phosphoarray analysis using PLC/PRF/5 cells stably expressing shRNA for *NDRG3* or *GFP* (Figure S6A). *NDRG3* depletion selectively suppressed hypoxia-induced ERK1/2 phosphorylation (Figures 6A and S6A). We then examined whether the kinases upstream of ERK1/2 could be regulated by NDRG3 and found that hypoxia-induced phosphorylation of c-Raf (at Ser338) and B-RAF1 (at Ser445) is abrogated by *NDRG3* depletion in SK-Hep-1 cells (Figure 6B). These results suggest that NDRG3 might play an essential role in the activation of the RAF-ERK signaling pathway. We, therefore, examined the effect of manipulating NDRG3 expression on c-Raf phosphorylation and found that ectopically expressed c-Raf was significantly phosphorylated in normoxia, with a concomitant phosphorylation of ERK1/2 (Figure 6C). However, depletion of basal-level NDRG3 expression by siRNA abrogated this response. On the other hand, the ectopic expression of NDRG3(N66D) highly induced the phosphorylation of c-Raf and ERK1/2 (Figure 6C). Also, the hypoxia-induced phosphorylation of endogenous c-Raf and ERK1/2, which was suppressed by the 3′-UTR-targeting shRNA of *NDRG3*, could be rescued by the recombinant NDRG3(N66D) expression vector (Figure 6D). Reciprocal in vitro pull-down assays indicated that NDRG3 can physically and directly interact with c-Raf (Figure S6B). Consistently, ectopically expressed c-Raf immunoprecipitated endogenous NDRG3 protein specifically under hypoxia (Figure S6C). Moreover, an NDRG3(N66D)-containing complex immunoprecipitated from HEK293T cells mediated the phosphorylation of recombinant c-Raf in an in vitro kinase assay (Figure 6E). These results indicate that NDRG3 is directly involved in the phosphorylation of c-Raf.

We then examined the biological implications of the NDRG3-mediated c-Raf-ERK1/2 phosphorylation. We observed, from the immunoprecipitation analysis of endogenous proteins, increasing amounts of c-Raf-NDRG3 complexes during the progression of hypoxia, in parallel with a temporal increase in the phosphorylation levels of c-Raf and ERK1/2 (Figure 6F). This result suggests a potential role of NDRG3-mediated c-Raf-ERK1/2 phosphorylation in hypoxia response regulation. Ablation of *LDHA* to inhibit lactate production effectively suppressed the hypoxia-induced phosphorylation of c-Raf and ERK1/2 as well as NDRG3 protein expression (Figure 6G). Exogenously provided lactate rescued the siLDHA-mediated suppression of c-Raf and ERK1/2 phosphorylation, but this rescue was blocked by silencing *MCT1* expression. In addition, disruption of glycolysis via glucose deprivation effectively suppressed the hypoxic phosphorylation of c-Raf and ERK1/2 as well as NDRG3 expression, which could be rescued by NDRG3(N66D) (Figure 6H). In contrast, glutamine deprivation exhibited negligible effects. These results indicate that hypoxia-induced phosphorylation of c-Raf and ERK1/2 is dependent on lactate production, mainly from glycolysis, and NDRG3 functions as an essential mediator of the lactate-induced activation of Raf-ERK pathway.

## Dependence of Lactate-Induced Hypoxic Cell Growth and Angiogenesis on NDRG3-Mediated ERK1/2 Activity

Finally, we examined the biologic relevance of the NDRG3-mediated activation of the Raf-ERK pathway to lactate-triggered hypoxia responses. Exogenously provided lactate significantly compensated for the growth deficit of Huh-1 cells under mild hypoxia, caused by *LDHA* silencing (Figure 7A). However, the lactate-mediated rescue was abrogated by depletion of *NDRG3* or pharmacological blockade of ERK signaling. Similarly, exogenous lactate restored the hypoxia-induced angiogenic capacity of *LDHA*-knockdown Huh-1 cells in tube-forming assays, which was again abolished by *NDRG3* depletion or ERK inhibition (Figure 7B). In parallel, the hypoxic expression of angiogenic marker genes, disrupted by *LDHA* knockdown, was recovered by exogenous lactate but disrupted again by *NDRG3* depletion or ERK inhibition (Figure S7A). We then examined the relevance of NDRG3-mediated Raf-ERK pathway activation to the growth of tumors in vivo. Western blot analysis of the tumors formed by Huh-1 cells engineered for *LDHA* and/or *NDRG3* expression in the in vivo tumorigenesis analysis (Figures 4G and S4L) indicated that phosphorylation of c-Raf and ERK was clearly upregulated in tumors expressing NDRG3(N66D) compared to mock controls (Figures 7C and S7B). Consistently, we observed a predominant expression of angiogenic marker genes in NDRG3(N66D)-expressing tumors. These results, together with those in Figures 4 and 6, demonstrate that lactate plays essential roles in promoting cell growth and angiogenesis under hypoxia, depending on the NDRG3-mediated activation of the c-Raf-ERK1/2 pathway.

We then examined the clinical relevance of NDRG3 expression and ERK1/2 activity by immunohistochemical analysis of human hepatocellular carcinoma (HCC). NDRG3 was barely expressed in the normal liver, while moderate to strong levels were detectable in HCC tissues in the cytoplasm and the plasma membrane (Figure 7D). Among 103 HCC cases examined using antibodies for NDRG3 and phospho-ERK1/2, 25 cases (24.3%) were positive for NDRG3 protein expression in a manner that was significantly associated with ERK1/2 activation (Figure 7D). In summary, these results indicate that aberrant NDRG3

(G) Dependence of the tumorous growth of Huh-1 cells on *LDHA* and its rescue by *NDRG3*. The p values were assessed by Student's t test. n = 5/group.
(H) Inhibition of hypoxia-induced angiogenesis by oxamate and its rescue by *NDRG3*. Results are mean ± SD of two experiments. The p values were assessed by Student's t test. Scale bar, 200 μm.
See also Figure S4.

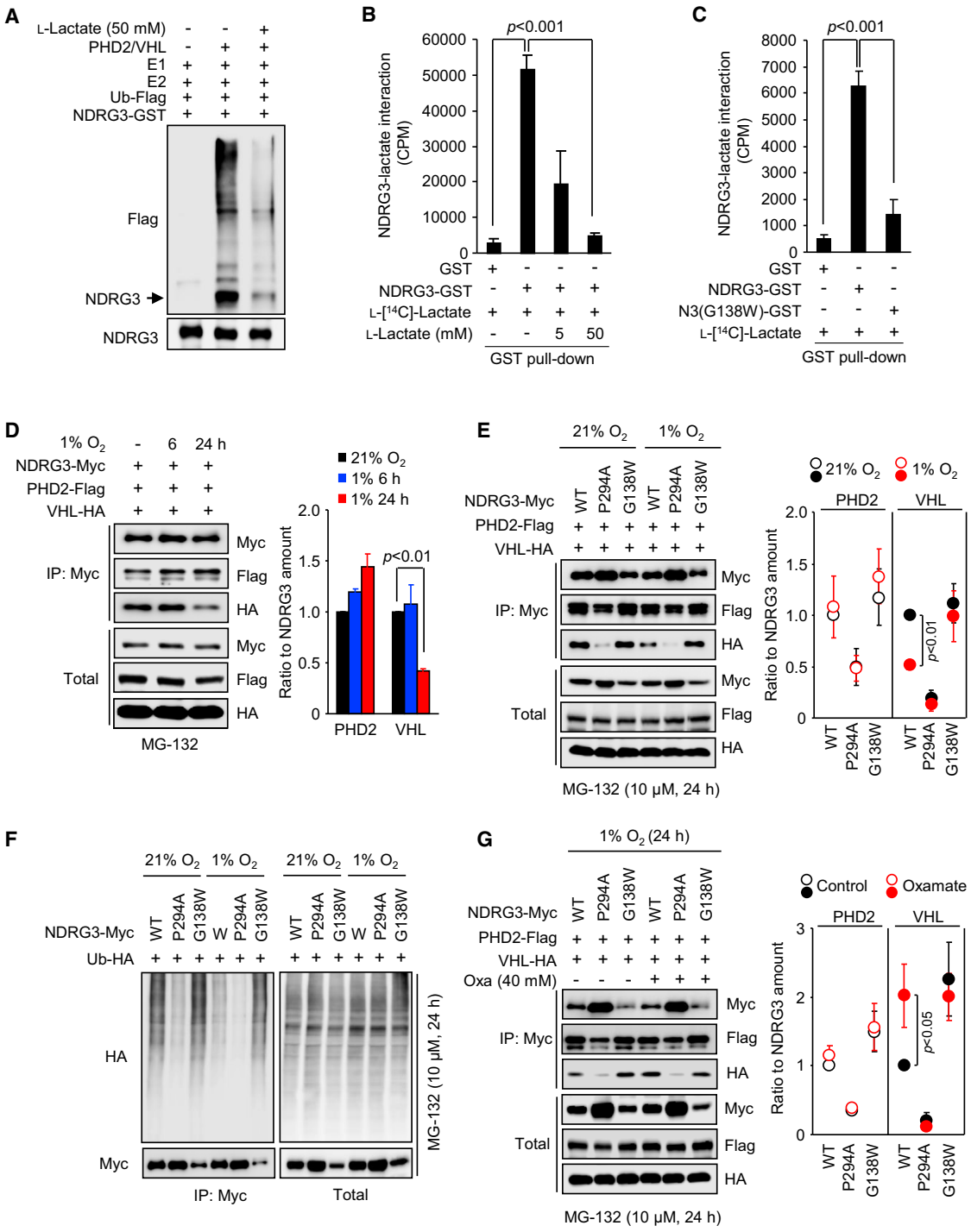



**Figure 5. Lactate Binds to NDRG3 and Blocks Its Ubiquitination by VHL**

(A) Effects of lactate on in vitro ubiquitination of NDRG3 by recombinant PHD2/VHL complex immunoprecipitated from HEK293T cells expressing PHD2-Flag and VHL-HA.

(B) Molecular interaction between L-lactate and NDRG3 protein in vitro. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.

(C) Molecular interaction between L-lactate and a variant NDRG3 protein (N3(G138W)) mutated in the putative L-lactate binding site. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.

(D) Interaction profile of NDRG3 protein with PHD2 or VHL in HEK293T cells during the progression of hypoxia. Quantified values for western blot images are shown on the right. Results are mean ± SD of two experiments. The p value was assessed by Student's t test.

*(legend continued on next page)*

expression is closely associated with tumor development in vivo as well as the pathological activation of the ERK pathway.

## DISCUSSION

Lactate has been regarded as a dead-end product of glycolysis and glutaminolysis until it recently emerged as an alternative energy source and an inducer of tumor angiogenesis (Doherty and Cleveland, 2013). Knockdown of *LDHA* expression or inhibition of its activity suppressed tumor cell growth in vitro and in vivo (Fantin et al., 2006; Le et al., 2010). However, the key elements and mechanisms of lactate-induced biological responses remained unknown. In this study, we showed the existence of NDRG3-mediated lactate signaling and its roles in hypoxia responses. During hypoxia, low oxygen concentrations and elevated lactate levels highly induced NDRG3 protein expression, leading to the activation of the Raf-ERK pathway to promote angiogenesis and hypoxic cell growth. Thus, NDRG3 acts as a lactate sensor that triggers downstream kinase signaling in a hypoxia-dependent manner, and the NDRG3-Raf-ERK axis provides the genetic basis for the lactate-induced hypoxia responses.

We showed that *NDRG3* expression is genetically independent of HIFs and rather determined at the protein level by lactate. Lactate accumulates at the later phase of hypoxia, promoted by the upregulation of glycolysis and *LDHA* expression during the earlier stages of hypoxia where HIF-1α plays a critical role as a part of metabolic adaptation (Cassavaugh and Lounsbury, 2011). Therefore, the lactate signaling and subsequent biologic responses appear to be functionally coupled to the HIF-1α-induced metabolic reprogramming, by employing NDRG3 as the critical link. In this regard, it is suggested that portions of the hypoxia responses, especially those occurring at the later phase of hypoxia, that have been so far attributed to HIF-1α might, in fact, be under the direct control of NDRG3-mediated lactate signaling. The results of gene set enrichment analysis for the functions of NDRG3 and HIF-1α during hypoxia support this possibility (Figures 3 and S3). Therefore, our study suggests that HIF-1α and NDRG3 might form an oxygen-dependent regulatory chain for hypoxia responses, which is broadly divided into two chronological phases (Figure 7E); at the early phase, low $O_2$ levels signal for the accumulation of HIF-1α, which then regulates the gene expression necessary for early adaptive responses including metabolic reprogramming, while at the later phase, upregulated lactate production signals for the accumulation of NDRG3, which subsequently activates the Raf-ERK pathway to induce responses necessary for coping with prolonged hypoxia.

The lactate-NDRG3-Raf-ERK axis of hypoxia signaling suggests that hypoxic lactate production might be an integral part of normal physiology, playing active roles in promoting angiogenesis and cell growth under prolonged hypoxia. It stands to reason that the functional coupling between HIF-1α-induced metabolic reprogramming and NDRG3-mediated lactate signaling ensures that cells facing prolonged hypoxia achieve the maximal possible growth in a hypoxic environment. This can be achieved by, first, generating biosynthetic building blocks and energy via the HIF-1α-mediated upregulation of glycolysis, and subsequently, by providing cues for cell growth and angiogenesis via the NDRG3-mediated c-Raf-ERK signaling. Therefore, NDRG3-mediated lactate signaling may provide a self-sufficient mechanism for the cells in local tissues to recover from hypoxia without the need for additional extracellular signals, for example, during development. Moreover, NDRG3-mediated signaling provides an extra layer of biological security for the cells escaping prolonged hypoxia since the NDRG3 protein, once stabilized by lactate binding, remains quite stable even when cells are reoxygenated.

Growing evidence suggests that lactate may play active roles in cancer progression, as it mediates cancer-cell intrinsic effects on metabolism as an oxidative metabolite and non-cancer-cell autonomous effects on several cell types in the tumor microenvironment (Doherty and Cleveland, 2013). Our results indicate that glycolysis is the main source of lactate production that is responsible for the hypoxic induction of NDRG3 protein expression and Raf-ERK activation. Cancer cells frequently exhibit an increased dependence on glycolysis, and therefore, the discovery of the lactate-NDRG3-Raf-ERK axis and its role in angiogenesis and hypoxic cell growth may provide an important explanation for the growth advantage offered by a glycolytic phenotype to cancers. In this regard, lactate might be considered an oncometabolite that drives the progression of solid tumors as an alternative fuel, an agent modulating the tumor microenvironment, and a signaling molecule.

Many characteristics of hypoxia responses are also exploited by diseased cells (Cassavaugh and Lounsbury, 2011). The presence of hypoxia is correlated with poor patient prognosis and poor treatment outcome in cancers (Jubb et al., 2010; Semenza, 2004), and therefore, hypoxia has been an important target for cancer therapy. Although HIF is the prime target in this regard, concerns have been raised that the simple inhibition of HIF may not be enough to prevent the progression of hypoxia-induced diseases, since many studies indicated that compensatory, HIF-independent pathways can be induced when a single factor is inhibited (Mizukami et al., 2005, 2007; Carmeliet et al., 1998; Rapisarda et al., 2009; see Introduction for supporting examples). These observations collectively led to the suggestion that the most successful anti-hypoxia strategy may require a combination of agents inhibiting HIF-independent as well as HIF-dependent pathways (Mizukami et al., 2007; Fong, 2008). Despite the likelihood of functional coupling with HIF-1α, *NDRG3* seems to have distinct functions in hypoxia response regulation as indicated by gene set enrichment analysis of the transcriptome data for *NDRG3*- and *HIF1A*-depleted cells during

(E) Interaction of different forms of NDRG3 proteins with PHD2 or VHL in HEK293T cells at different oxygen conditions. WT, wild-type. Quantified values for western blot images are shown on the right. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.
(F) Ubiquitination assay of variant NDRG3 proteins from HEK293T cells grown in normoxia or under hypoxia.
(G) Effects of inhibiting hypoxic lactate production by oxamate on the interaction of different NDRG3 proteins with PHD2 or VHL in HEK293T cells. Quantified values for western blot images are shown on the right. Results are mean ± SD of three experiments. The p value was assessed by Student's t test.
See also Figure S5.

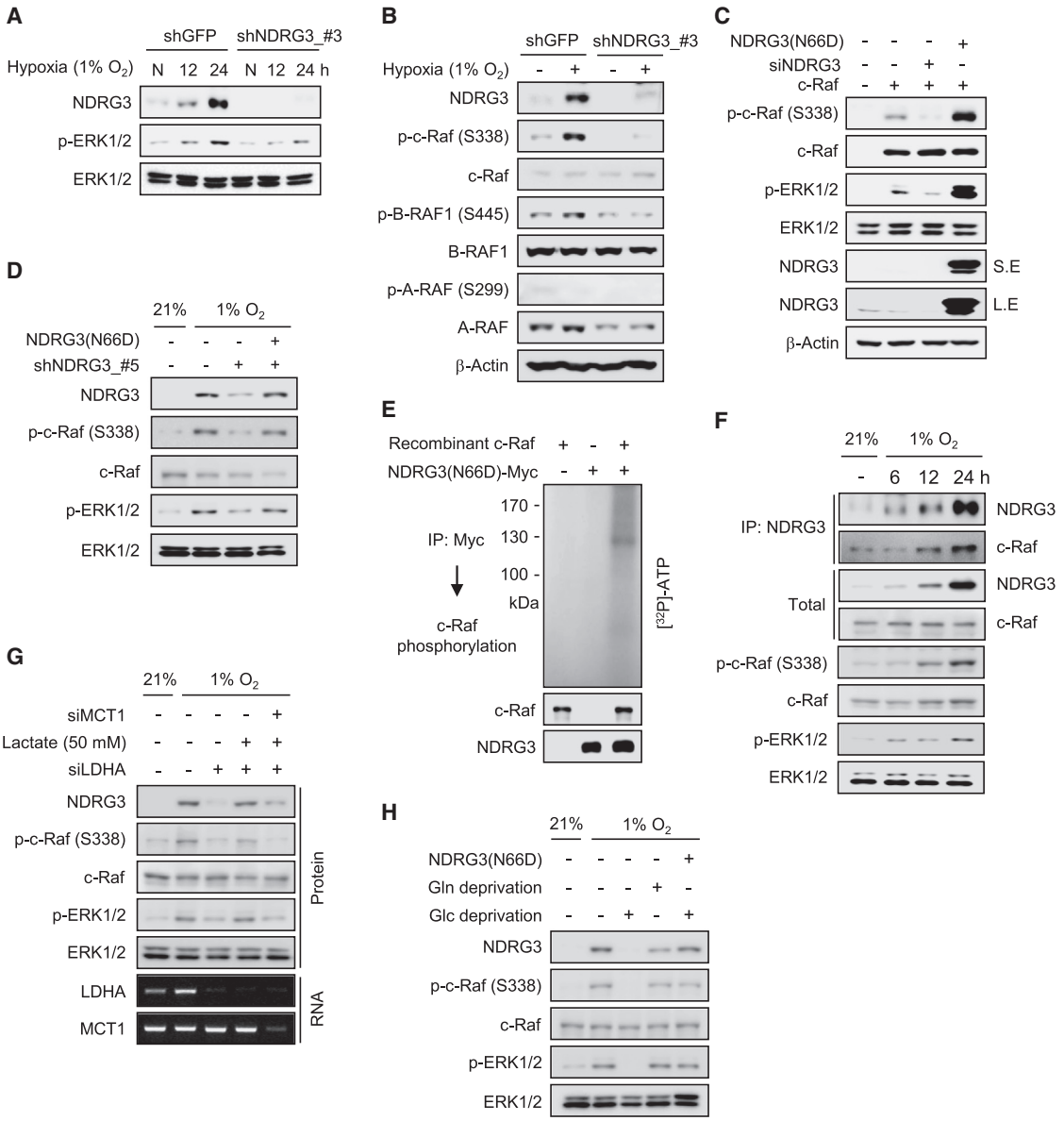



**Figure 6. NDRG3 Is Required for Hypoxia-Induced Raf-ERK1/2 Activation**

(A) Effects of *NDRG3* knockdown on hypoxia-induced ERK1/2 activation in SK-Hep-1 cells.

(B) Effects of *NDRG3* knockdown on hypoxia-induced RAF activation in SK-Hep-1 cells.

(C) Activation of ERK1/2 and c-Raf by NDRG3 in normoxia in HEK293T cells.

(D) Suppression of the hypoxic phosphorylation of c-Raf and ERK1/2 by *NDRG3* knockdown and its rescue by ectopic expression of NDRG3.

(E) In vitro phosphorylation of recombinant c-Raf protein by the NDRG3-containing complex immunoprecipitated from HEK293T cells.

(F) Interaction profile between endogenous NDRG3 and c-Raf proteins during progression of hypoxia. Phosphorylation profiles of c-Raf and ERK are also shown.

(G) Lactate dependence of the NDRG3-mediated c-Raf-ERK activation during hypoxia in Huh-1 cells.

(H) Suppression of the hypoxic phosphorylation of c-Raf and ERK1/2 by glucose deprivation and its rescue by NDRG3 overexpression.

See also Figure S6.

hypoxia. Therefore, these observations, along with the roles of *NDRG3* in hypoxia responses as shown in this study, suggest that combinatorial targeting of *HIF* and *NDRG3* might prove highly effective in cancer therapy. Abrogation of tumor growth when *NDRG3* was depleted in combination with either HIFs supports the feasibility of this strategy (Figure 3H).

In conclusion, *NDRG3* provides a crucial genetic evidence for the oxygen-dependent regulation of HIF-independent hypoxia signaling. The regulation and functions of NDRG3 in hypoxia imply that the PHD2/VHL system can control both HIF-dependent and HIF-independent hypoxia responses in an oxygen-dependent manner. Therefore, the lactate-NDRG3-Raf-ERK signaling

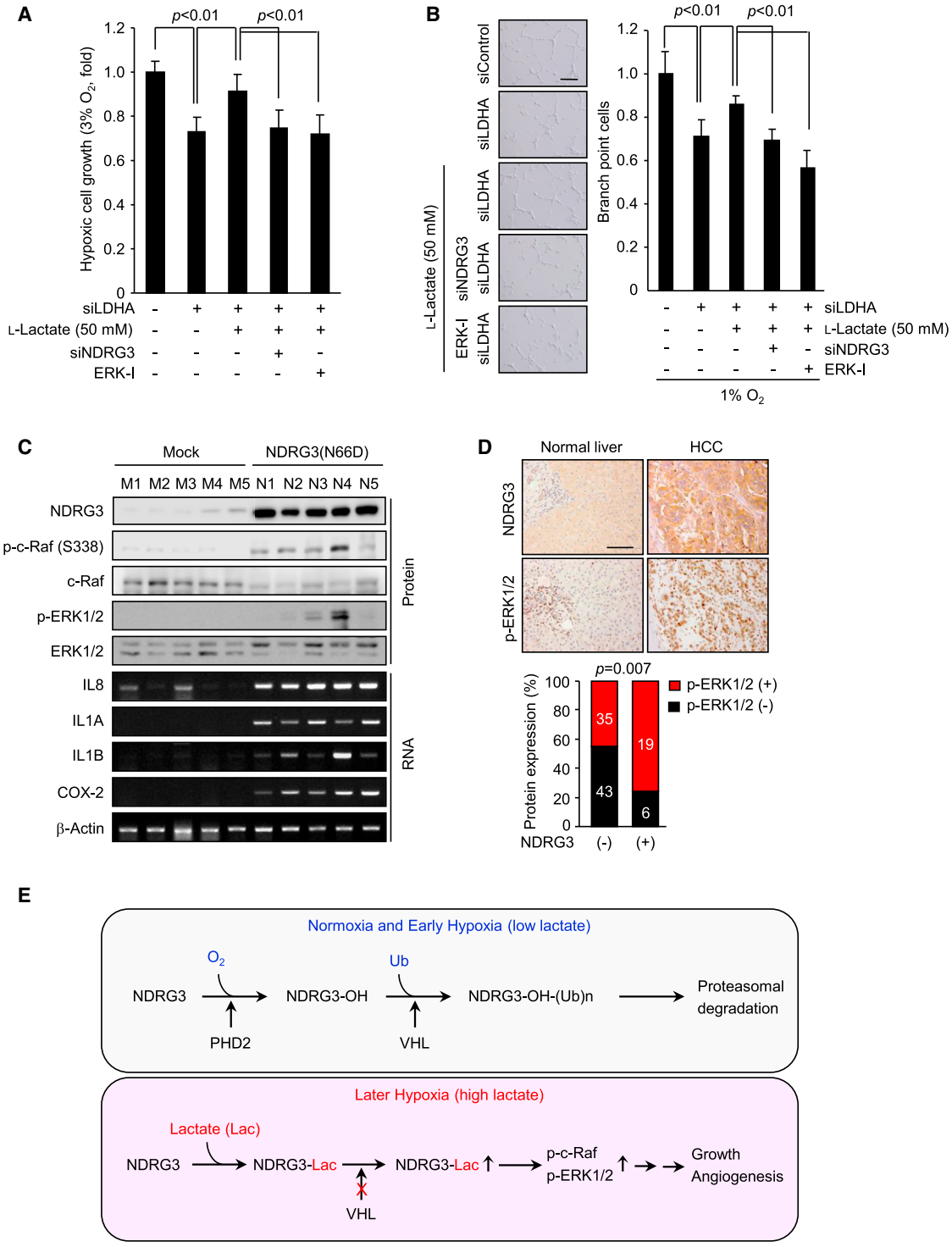



**Figure 7. Lactate-Induced Cell Growth and Angiogenesis Depend on NDRG3 Expression and ERK1/2 Activity**

(A) Dependence of the hypoxic growth of Huh-1 cells on lactate, NDRG3, and ERK1/2 activity.

(B) Dependence of the hypoxia-induced angiogenic activity of Huh-1 cells on lactate, NDRG3, and ERK1/2 activity.

(C) Upregulation of c-Raf-ERK1/2 phosphorylation and pro-angiogenic gene expression in tumor xenografts formed by Huh-1 cells overexpressing NDRG3.

(D) Immunohistochemical analysis of NDRG3 and phospho-ERK1/2 expression in human liver cancers. Relationship between NDRG3 protein and phospho-ERK1/2 expression was assessed by $\chi^2$ test.

(E) A scheme outlining the regulatory mechanism for prolonged hypoxia responses involving lactate and NDRG3.

See also Figure S7.

pathway may provide an extended mechanistic clue to the understanding of disorders caused by mutations in *VHL* (hemangioblastoma, renal cell carcinoma, pheochromocytoma, etc.) (Maher et al., 2011) or *PHD2* (familial erythrocytosis-3) (Percy et al., 2006) as well as the hypoxia-related physiological and pathophysiological responses (Cassavaugh and Lounsbury, 2011).

## EXPERIMENTAL PROCEDURES

### Cell Lines

Human cell lines, PLC/PRF/5, SK-HEP-1, MCF-10A, MCF-7, IMR-90, HeLa, SW480, and HEK293T were purchased from American Type Culture Collection. Two human hepatoma cell lines, Huh-1 and Huh-7, were obtained from Japanese Cancer Research Resources Bank. Cells were cultured under standard conditions (see Extended Experimental Procedures).

### Identification of PHD2-Binding Proteins

In order to identify PHD2-binding proteins, we carried out mass spectrometric analysis of the proteins immunoprecipitated from MCF-7 cells cultured under hypoxia for 24 hr in the presence of the proteasome inhibitor, MG132. We avoided the yeast two-hybrid screen as it has known technical limitations for some types of proteins. A detailed method is described in Extended Experimental Procedures under the subtitle Micro-LC-MS/MS Analysis and Protein Database Search.

### RNA Interference

We used commercial pooled siRNA products (SMARTpool, Dharmacon) for transient knockdown of *NDRG3*, *NDRG1*, *HIF-1A*, *EPAS1* (*HIF-2A*), *ARNT* (*HIF-1B*), *VHL*, *LDHA*, *MCT1*, and *MCT4*. Otherwise, siRNAs were synthesized from Samchullypharm (Korea). The sequences of siRNAs are listed in Table S1.

### Protein Structure Modeling and Docking Simulation

Prediction of NDRG3 protein structure was achieved using Modeler 9v10 (Eswar et al., 2008). Protein-protein and protein-ligand docking simulations were performed using the HEX6.3 program (Ritchie and Kemp, 2000) and the Autodock Vina software (http://vina.scripps.edu/index.html), respectively. Detailed methods are described in Extended Experimental Procedures.

### L-Lactate Measurement and Binding Assay

L-Lactate production was measured using the EnzyChrom L-Lactate Assay kit (BioAssay Systems). The protocol for the analysis of interaction between recombinant NDRG3-GST protein and L-[$^{14}$C]-lactate (PerkinElmer) is described in Extended Experimental Procedures.

### Statistical Analysis

Statistical significance of the data was mostly assessed by using the Student's t test except for the tissue microarray data for which the $\chi^2$ test was used.

### Miscellaneous Methods

Virus-mediated gene expression, immunoprecipitation and western blotting, RT-PCR, site-directed mutagenesis, expression and purification of recombinant proteins, production of anti-NDRG3 antibody, ubiquitination assays, gene expression profiling, cell growth assays, apoptosis assay, in vitro kinase assay, tumorigenesis in a mouse model, in vivo angiogenesis assay, immunofluorescence microscopy, and tissue microarray analysis are described in Extended Experimental Procedures. Contents dealing with human and animal subjects were approved by the Institutional Review Board of Inje University Seoul Paik Hospital (Seoul, Korea) and KRIBB, respectively. Antibodies and primer sequences used for RT-PCR analyses and site-directed mutagenesis are listed in Tables S2, S3, and S4.

## ACCESSION NUMBERS

The GEO accession number for the microarray data reported in this paper is GSE55214.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.03.011.

## AUTHOR CONTRIBUTIONS

All experiments were conceived by Y.I.Y., D.C.L., K.C.P., and H.S.Y. and mainly carried out by D.C.L. H.A.S. and D.-Y.Y. carried out the animal studies. Y.K.K. performed tissue microarray analysis. S.O. performed protein structure modeling and docking simulation. Z.-Y.P., K.L., and K.-H.B. carried out proteomic analyses. M.K., Y.J.J., and S.-J.Y. performed transcriptomic analyses. Y.K.H. and S.J.C. worked on recombinant proteins. H.N., D.M.K., and D.J.K. performed cell-based assays. Y.I.Y., K.C.P., and D.C.L. wrote the manuscript. Y.I.Y., K.C.P., and J.-A.K. supervised the project.

## ACKNOWLEDGMENTS

We thank Chul-Ho Lee (KRIBB) and MoonKyung Kang (Inje University) for the helps with animal studies and viral particles preparation, respectively. This work was supported by the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2010-0030020, NRF-2011-0028171, NRF-2013M3A9B5076422, NRF-2006-2006799) and by KRIBB Research Initiative Program.

Received: August 6, 2014
Revised: December 15, 2014
Accepted: February 20, 2015
Published: April 16, 2015

## REFERENCES

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature *394*, 485–490.

Cassavaugh, J., and Lounsbury, K.M. (2011). Hypoxia-mediated biological control. J. Cell. Biochem. *112*, 735–744.

Chowdhury, R., McDonough, M.A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K.S., Domene, C., and Schofield, C.J. (2009). Structural basis for binding of hypoxia-inducible factor to the oxygen-sensing prolyl hydroxylases. Structure *17*, 981–989.

Cummins, E.P., and Taylor, C.T. (2005). Hypoxia-responsive transcription factors. Pflugers Arch. *450*, 363–371.

Doherty, J.R., and Cleveland, J.L. (2013). Targeting lactate metabolism for cancer therapeutics. J. Clin. Invest. *123*, 3685–3692.

Elvidge, G.P., Glenny, L., Appelhoff, R.J., Ratcliffe, P.J., Ragoussis, J., and Gleadle, J.M. (2006). Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. J. Biol. Chem. *281*, 15215–15226.

Fantin, V.R., St-Pierre, J., and Leder, P. (2006). Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell *9*, 425–434.

Fong, G.H. (2008). Mechanisms of adaptive angiogenesis to tissue hypoxia. Angiogenesis *11*, 121–140.

Harris, A.L. (2002). Hypoxia—a key regulatory factor in tumour growth. Nat. Rev. Cancer *2*, 38–47.

Höpfl, G., Wenger, R.H., Ziegler, U., Stallmach, T., Gardelle, O., Achermann, R., Wergin, M., Kaser-Hotz, B., Saunders, H.M., Williams, K.J., et al. (2002). Rescue of hypoxia-inducible factor-1alpha-deficient tumor growth by wild-type cells is independent of vascular endothelial growth factor. Cancer Res. *62*, 2962–2970.

Jubb, A.M., Buffa, F.M., and Harris, A.L. (2010). Assessment of tumour hypoxia for prediction of response to therapy and cancer prognosis. J. Cell. Mol. Med. *14*, 18–29.

Le, A., Cooper, C.R., Gouw, A.M., Dinavahi, R., Maitra, A., Deck, L.M., Royer, R.E., Vander Jagt, D.L., Semenza, G.L., and Dang, C.V. (2010). Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc. Natl. Acad. Sci. USA *107*, 2037–2042.

Maher, E.R., Neumann, H.P., and Richard, S. (2011). von Hippel-Lindau disease: a clinical and scientific review. Eur. J. Hum. Genet. *19*, 617–623.

Melotte, V., Qu, X., Ongenaert, M., van Criekinge, W., de Bruïne, A.P., Baldwin, H.S., and van Engeland, M. (2010). The N-myc downstream regulated gene (NDRG) family: diverse functions, multiple applications. FASEB J. *24*, 4153–4166.

Mizukami, Y., Li, J., Zhang, X., Zimmer, M.A., Iliopoulos, O., and Chung, D.C. (2004). Hypoxia-inducible factor-1-independent regulation of vascular endothelial growth factor by hypoxia in colon cancer. Cancer Res. *64*, 1765–1772.

Mizukami, Y., Jo, W.S., Duerr, E.M., Gala, M., Li, J., Zhang, X., Zimmer, M.A., Iliopoulos, O., Zukerberg, L.R., Kohgo, Y., et al. (2005). Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. Nat. Med. *11*, 992–997.

Mizukami, Y., Kohgo, Y., and Chung, D.C. (2007). Hypoxia inducible factor-1 independent pathways in tumor angiogenesis. Clin. Cancer Res. *13*, 5670–5674.

Percy, M.J., Zhao, Q., Flores, A., Harrison, C., Lappin, T.R., Maxwell, P.H., McMullin, M.F., and Lee, F.S. (2006). A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. Proc. Natl. Acad. Sci. USA *103*, 654–659.

Rapisarda, A., Shoemaker, R.H., and Melillo, G. (2009). Antiangiogenic agents and HIF-1 inhibitors meet at the crossroads. Cell Cycle *8*, 4040–4043.

Ritchie, D.W., and Kemp, G.J. (2000). Protein docking using spherical polar Fourier correlations. Proteins *39*, 178–194.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer *3*, 721–732.

Semenza, G.L. (2004). Intratumoral hypoxia, radiation resistance, and HIF-1. Cancer Cell *5*, 405–406.

Seta, K.A., Spicer, Z., Yuan, Y., Lu, G., and Millhorn, D.E. (2002). Responding to hypoxia: lessons from a model cell line. Sci. STKE *2002*, re11.