

# The hypoxia-responsive lncRNA *NDRG1-OT1* promotes *NDRG1* degradation via ubiquitin-mediated proteolysis in breast cancer cells

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## ABSTRACT

**Hypoxia can lead to solid tumor aggressiveness by driving multiple signaling pathways. Long non-coding RNAs respond to several extrinsic stimuli, causing changes in cancer cells by participating in multiple steps of gene expression. However, genomic profiling of long non-coding RNAs regulated by oxygen in breast cancer remained unclear. Therefore, the aims of this study were to identify oxygen-responsive long non-coding RNAs in breast cancer cells, and to delineate their regulatory mechanisms. The expression profiling of long non-coding RNAs in breast cancer cells growing under normoxic, hypoxic, and re-oxygenated conditions was examined using next-generation sequencing technology. Four hundred and seventy-two lncRNAs oxygen-responsive lncRNAs were identified. After examining the top three differentially expressed lncRNAs in hypoxia, we selected N-Myc Downstream Regulated Gene 1-Overlapping 1 (*NDRG1-OT1*) for further study, especially the most responsive isoform, *NDRG1-OT1\_v4*. We overexpressed *NDRG1-OT1\_v4* under normoxia and performed microarray analysis to identify 108 *NDRG1-OT1\_v4* regulated genes and their functions. Among these genes, we found that both *NDRG1* mRNA expression and *NDRG1* protein levels were inhibited by *NDRG1-OT1\_v4*. Finally, we used co-immunoprecipitation to show that *NDRG1-OT1\_v4* destabilizes *NDRG1* by promoting ubiquitin-mediated proteolysis. Our findings reveal a new type of epigenetic regulation of *NDRG1* by *NDRG1-OT1\_v4* in breast cancer cells.**

## INTRODUCTION

Hypoxia is a crucial determinant of the aggressiveness of solid tumors [1]. Because of the rapid growth of tumor cells and inadequate vascular distribution in the tumor microenvironment, an imbalance in oxygen delivery and consumption results in hypoxia [2, 3]. Previous studies have shown that hypoxia can affect the proliferation, invasion, and metastasis of tumors [4, 5], the

induction of apoptosis [6], and angiogenesis [7]. Tumors become more aggressive as a result.

N-myc downstream-regulated gene 1 (*NDRG1*) has various effects on tumor cell function. It has been reported to respond to many stressful environments, such as DNA damage [8] and hypoxia [9, 10]. In hypoxia, up-regulation of *NDRG1* leads to cellular differentiation, proliferation, re-distribution of the cell cycle, and metastasis [11–13]. The expression of *NDRG1* depends on both transcriptional

and epigenetic regulation. In transcriptional regulation, many transcription factors, such as HIF-1 [13], aryl hydrocarbon receptor (AHR) [14], and activating protein 1 (AP-1) [15], have been reported to bind to *NDRG1*'s promoter and activate its expression. Regarding epigenetic regulation, *NDRG1* was reported to be regulated epigenetically by histone acetylation [16] and microRNA-769-3p [17]. However, no studies have indicated that *NDRG1* is regulated by DNA methylation or non-coding RNA.

Long non-coding RNAs (lncRNAs) comprise a subgroup of non-coding RNAs whose lengths are greater than 200 nucleotides. Recent studies have indicated that many lncRNAs respond to cellular stresses, such as drugs [18], hormones [19, 20], radiation [21, 22], and hypoxia [23–25]. lncRNAs respond to these stimuli by participating in several steps of gene expression, leading to changes in cellular function. For example, lncRNAs epigenetically regulate gene expression [26, 27], inhibit target gene degradation [28], block the effect of microRNA (miRNA) by forming a sponge structure to absorb miRNA [29, 30], and regulate synthesis and degradation of target proteins [31, 32]. Although some oxygen-responsive lncRNAs have been identified and play a role in the onset of several cancers [27, 32], genomic profiling of lncRNAs regulated by oxygen in breast cancer remained to be explored.

In this study, next-generation sequencing was used to identify oxygen-responsive lncRNAs in MCF-7 breast cancer cells. Among these differentially expressed lncRNAs, we chose a hypoxia-induced lncRNA, *NDRG1-OT1*, for further analysis, particularly the isoform *NDRG1-OT1\_v4*, which had the highest expression levels in hypoxia. In order to identify genes regulated by *NDRG1-OT1\_v4*, the genomic expression profiling of MCF-7 cells overexpressing *NDRG1-OT1\_v4* in normoxia was examined. Among the *NDRG1-OT1\_v4*-regulated genes, we focused on *NDRG1* and found that *NDRG1-OT1\_v4* inhibited *NDRG1* expression by promoting *NDRG1* degradation via the ubiquitin-proteasome pathway.

## RESULTS

### Identification of lncRNA expression profiles in different O<sub>2</sub> conditions

In order to examine all lncRNAs regulated by O<sub>2</sub> in MCF-7 cells, cells were harvested under normoxia (O<sub>2</sub>), hypoxia (N<sub>2</sub>), and re-oxygenation (Re-O<sub>2</sub>) conditions in triplicate. The lncRNA expression profiles were analyzed using a next-generation sequencing (NGS) system. A Student's t-test was conducted to examine the difference in FPKMs between each condition. The criteria for selecting O<sub>2</sub>-responsive lncRNAs consisted of fold change > 3 and significant differences ( $P < 0.001$ ) between the N<sub>2</sub> and O<sub>2</sub> conditions (Figure 1A) as well as Re-O<sub>2</sub> and N<sub>2</sub> (Figure 1B). In addition, lncRNAs were further filtered to include

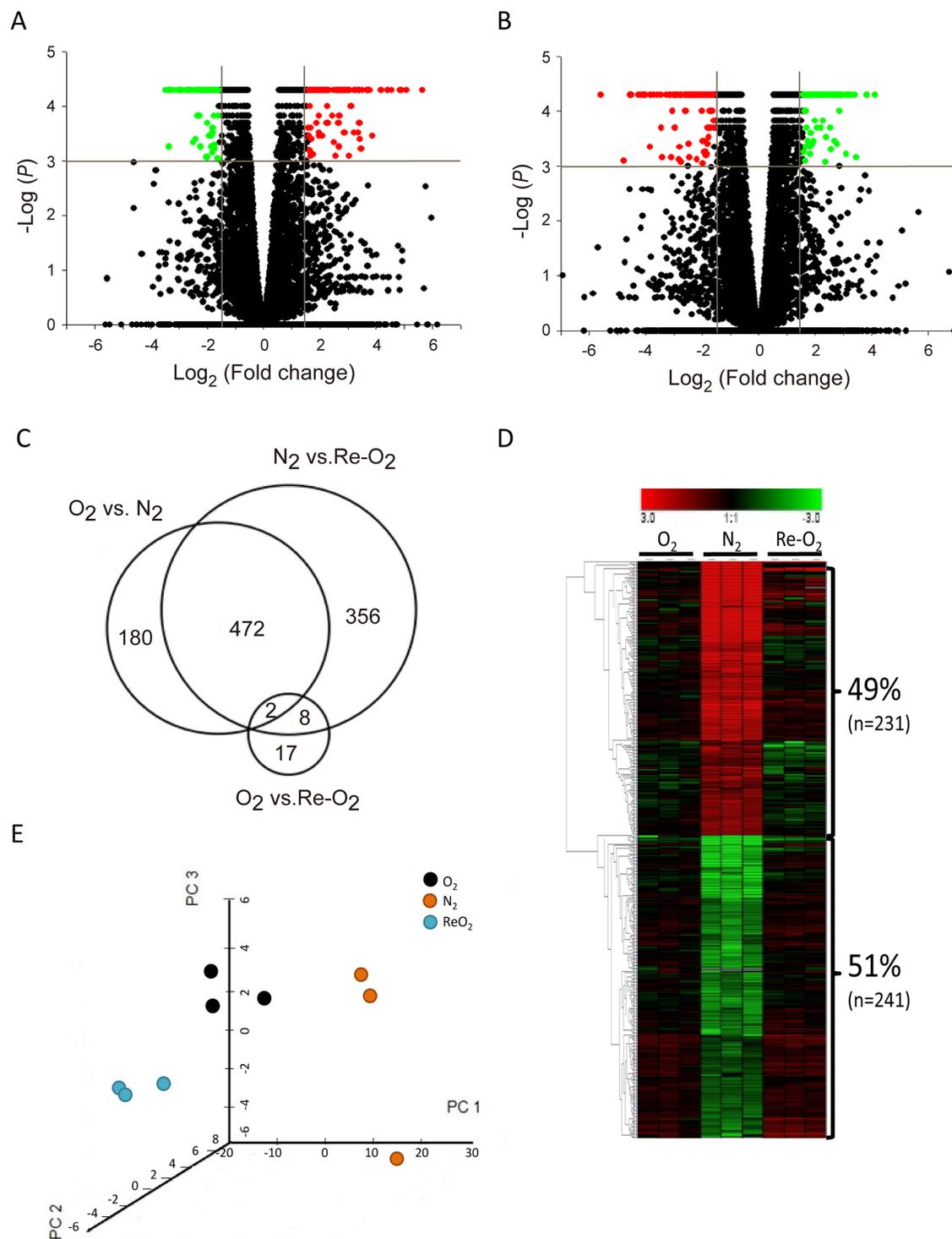
only those with an opposite response between shifting from normoxia to hypoxia and from hypoxia to re-oxygenation, but similar expression levels between normoxia and re-oxygenation (Figure 1C). In total, 472 lncRNAs met these criteria (Figure 1C). Among them, 49% (n = 231) were up-regulated in N<sub>2</sub> and down-regulated in Re-O<sub>2</sub>, while 51% (n = 241) were down-regulated in N<sub>2</sub> and up-regulated in Re-O<sub>2</sub> (Figure 1D). Lastly, principal component analysis (PCA) using expression levels of these 472 O<sub>2</sub>-responsive lncRNAs also showed distinct clusters of transcriptional responses for each condition (Figure 1E).

### *NDRG1-OT1* was hypoxia responsive lncRNA

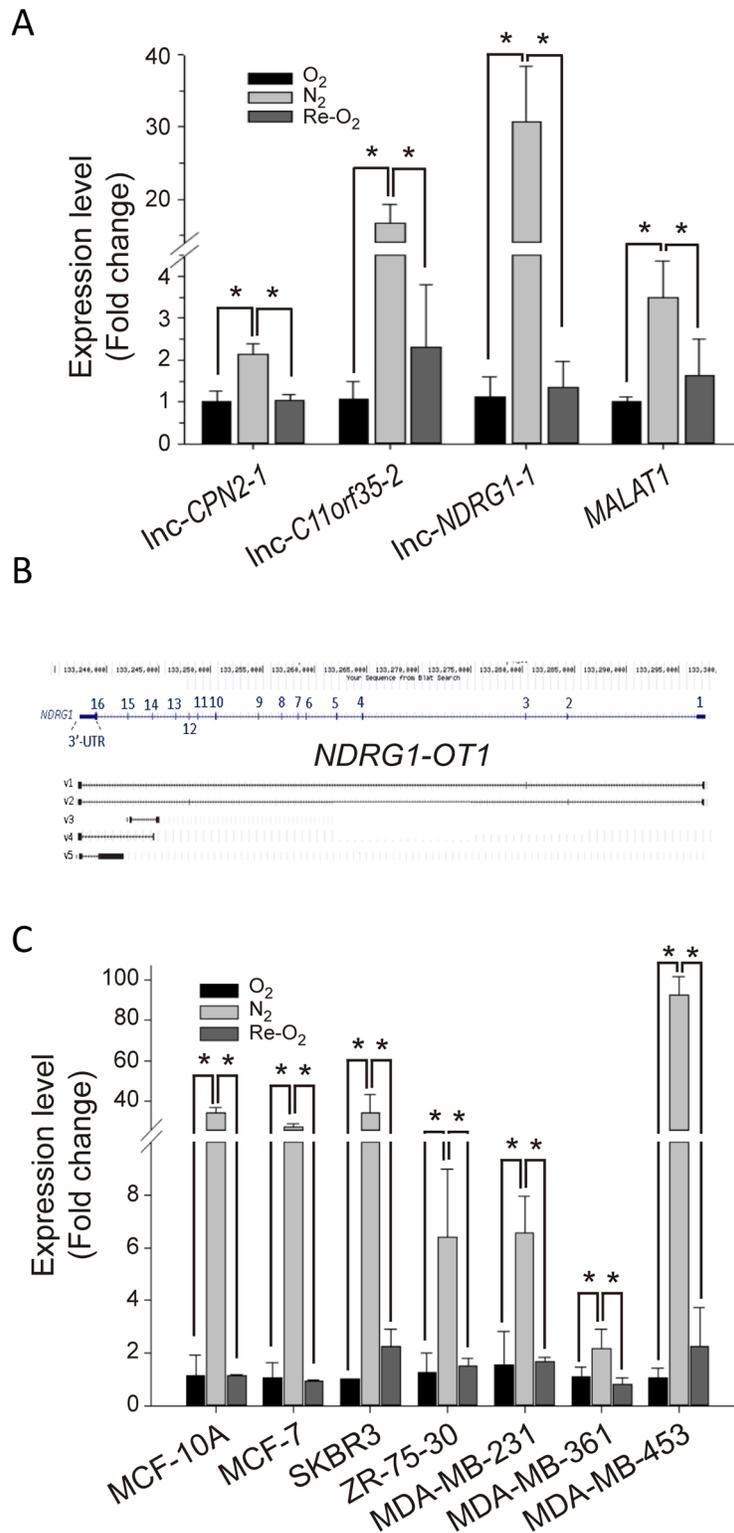
Among these differentially expressed lncRNAs, we used quantitative RT-PCR to validate the expression profiles of the top three hypoxia-inducible lncRNAs: *lnc-CPN2-1*, *lnc-C11orf35-2* and *lnc-NDRG1-1*. lncRNA *MALAT1* was used as a positive control for hypoxia [33]. Similar to the expression pattern in NGS, all three lncRNAs were significantly up-regulated under N<sub>2</sub> and down-regulated under Re-O<sub>2</sub> (Figure 2A). Especially, we were particularly interested in *lnc-NDRG1-1*. The main reason was that *lnc-NDRG1-1* was located in *NDRG1* (Figure 2B), which has been systemically investigated in our lab [14, 17, 34]. Therefore, we decided to investigate the relationship between this lncRNA and *NDRG1* in the following experiments. In addition, following the guidelines on gene nomenclature of the Human Genome Organization (HUGO), we renamed *lnc-NDRG1-1* as *NDRG1-OT1*, which it is called hereafter. As shown in Figure 2B, *NDRG1-OT1* has five isoforms. The expression levels of these isoforms in MCF-7 cells under different O<sub>2</sub> conditions were different (Table 1). Among them, excluding v1 and v3 transcripts that did not express in normoxia, the v4 transcript had the highest expression level in N<sub>2</sub> and the largest fold changes among different O<sub>2</sub> environments. The phenomenon were observed in other breast cancer cell lines (Figure 2C). Therefore, we investigated the role of this isoform in the regulatory mechanisms of *NDRG1-OT1* on its target genes under different O<sub>2</sub> conditions.

### Gene expression profiling in MCF-7 cells overexpressing *NDRG1-OT1\_v4*

In order to identify genes regulated by *NDRG1-OT1\_v4*, the gene expression of wild type MCF-7 cells and MCF-7 cells overexpressing *NDRG1-OT1\_v4* in O<sub>2</sub> was compared using Illumina Human HT-12 v4 BeadChips. Student's t-test was used to examine the difference in gene expression intensities. The criteria for defining *NDRG1-OT1\_v4*-regulated genes included fold change > 2 and significant differences ( $P < 0.05$ ) (Figure 3A). In total, 108 genes met the criteria, with 47 induced by *NDRG1-OT1\_v4* and 61 inhibited by *NDRG1-OT1\_v4* (Figure 3B). To



**Figure 1: Identification of oxygen-responsive lncRNAs in MCF-7 cells using next generation sequencing analysis.** (A) Volcano plots of differentially expressed lncRNAs responding to hypoxia ( $N_2$ ). Criteria for selecting  $O_2$ -responsive lncRNAs: fold change  $>3X$  and  $P < 0.001$ . Red points: up-regulated lncRNAs in  $N_2$ ; green points: down-regulated lncRNAs in  $N_2$ . (B) Volcano plots of differentially expressed lncRNAs responding to reoxygenation ( $ReO_2$ ). Criteria for selection were as in panel (A). Red points: down-regulated lncRNAs in  $ReO_2$ ; green points: up-regulated lncRNAs in  $ReO_2$ . (C) Venn diagram of differentially expressed lncRNAs in different  $O_2$  conditions.  $O_2$ : normoxia. (D) Heat map and hierarchical cluster analysis of  $O_2$ -responsive lncRNAs. Red: up-regulated lncRNAs in  $N_2$  and down-regulated in  $ReO_2$ ; green: down-regulated lncRNAs in  $N_2$  and up-regulated in  $ReO_2$ . (E) Principal component analysis (PCA) of  $O_2$ -responsive lncRNAs. PCA was plotted using expression of differentially expressed probes after quantile normalization. Each dot represents each sample. Three independent experiments were done in normoxia (black), hypoxia (orange), and re-oxygenation (cyan) respectively.

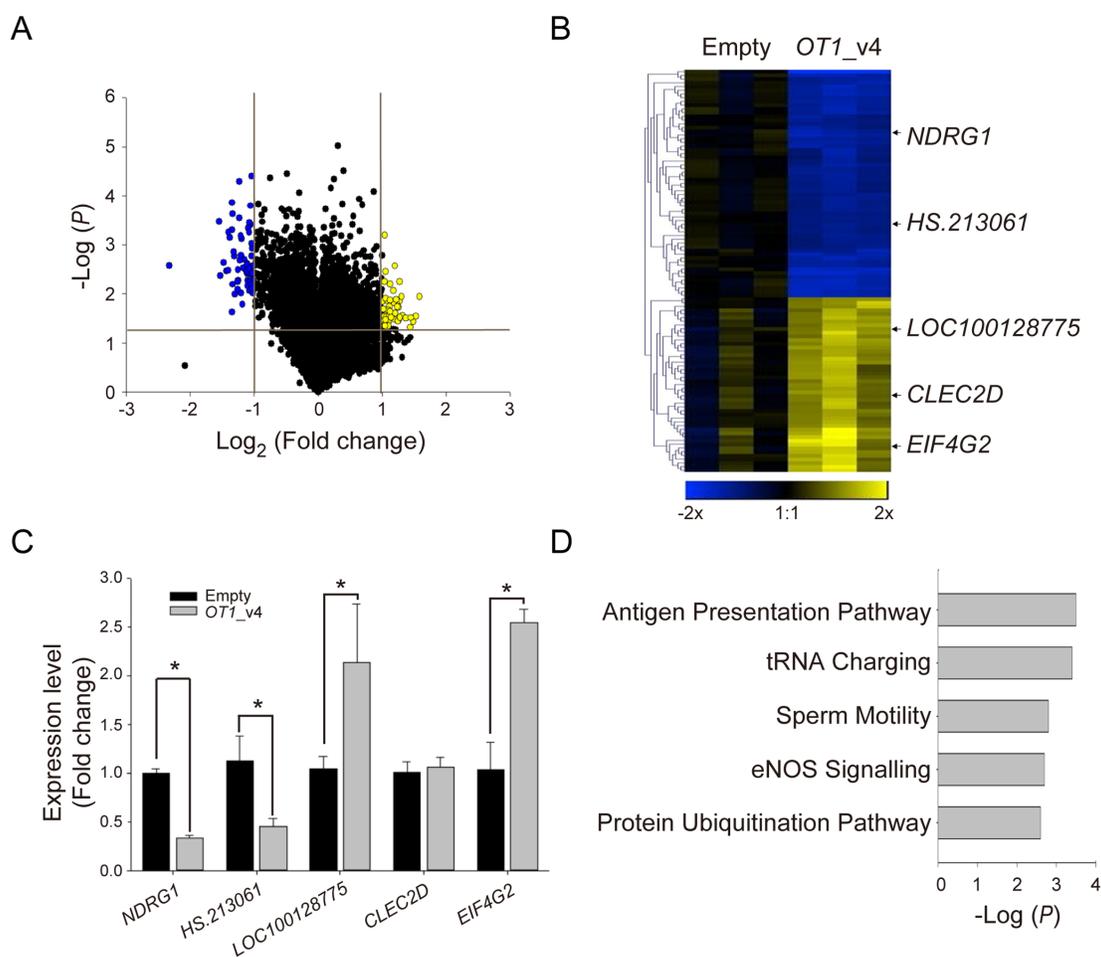


**Figure 2: *NDRG1-OT1\_v4* is up-regulated in hypoxia and down-regulated in re-oxygenation. (A)** Validation of *lnc-CPN2-1*, *lnc-C11orf35-2*, *lnc-NDRG1-1* and *MALAT1* expression levels among different O<sub>2</sub> conditions by quantitative RT-PCR. Internal control: 18S. Positive control for hypoxia: *MALAT1* [33]. The relative expression level of each condition was normalized to O<sub>2</sub>. Data were repeated at least 3 times, and the results are means ± SDs. \*, P < 0.05. **(B)** Schematic diagram of *NDRG1-OT1* isoforms. Transcription of *NDRG1-OT1* was overlapped with *NDRG1*, located in chromosome 8. The exon number and position are shown on the sequence of *NDRG1*. v1-v5 indicate the different isoforms. **(C)** Expression profiling of *NDRG1-OT1\_v4* in six breast cancer cells and one breast normal cell line (MCF-10A) under different O<sub>2</sub> conditions.

**Table 1: The amount of *NDRG1-OT1* isoforms among different O<sub>2</sub> conditions**

| <i>NDRG1-OT1</i> isoform | O <sub>2</sub> |            | N <sub>2</sub> |            | ReO <sub>2</sub> |            |
|--------------------------|----------------|------------|----------------|------------|------------------|------------|
|                          | FPKM (%)       | Read count | FPKM (%)       | Read count | FPKM (%)         | Read count |
| <i>NDRG1-OT1_v1</i>      | 0.0<br>(0.0)   | 0.0        | 0.0<br>(0.0)   | 0.0        | 0.0<br>(0.0)     | 0.0        |
| <i>NDRG1-OT1_v2</i>      | 4.6<br>(53.4)  | 16.3       | 119.7 (38.1)   | 441.0      | 2.1<br>(22.7)    | 6.9        |
| <i>NDRG1-OT1_v3</i>      | 0.0<br>(0.0)   | 0.0        | 4.0<br>(1.3)   | 7.7        | 0.0<br>(0.0)     | 0.0        |
| <i>NDRG1-OT1_v4</i>      | 3.6<br>(42.3)  | 9.3        | 182.4 (58.0)   | 490.17     | 6.8<br>(74.3)    | 16.7       |
| <i>NDRG1-OT1_v5</i>      | 0.4<br>(4.3)   | 7.6        | 8.2<br>(2.6)   | 175.17     | 0.3<br>(3.0)     | 5.5        |

FPKM, fragments per kilobase of transcript per million mapped reads.



**Figure 3: Identification of *NDRG1-OT1\_v4*-regulated genes using microarray analysis. (A)** Volcano plot of differentially expressed genes in MCF-7 cells over-expressing *NDRG1-OT1\_v4* in O<sub>2</sub>. Criteria for selecting *NDRG1-OT1\_v4* regulated genes: fold change >2X and *P* < 0.05. Yellow points: up-regulated genes in cells over-expressing *NDRG1-OT1\_v4*; blue points: down-regulated genes. **(B)** Heat map and hierarchical cluster analysis of *NDRG1-OT1\_v4*-regulated genes. Yellow: up-regulated genes in cells over-expressing *NDRG1-OT1\_v4*; blue: down-regulated genes. Top five differentially expressed genes were indicated. **(C)** Relative transcriptional levels of the top five differentially expressed genes in MCF-7 cells overexpressing *NDRG1-OT1\_v4* by quantitative RT-PCR. Internal control: 18s. The relative expression level was normalized to empty vector. **(D)** Top five pathways in which *NDRG1-OT1\_v4* regulated genes were enriched using Ingenuity Pathway Analysis (IPA). The significance of pathways was determined by IPA's default threshold (-log(*P*-value) < 1.3).

**Table 2: Top five differentially expressed genes responding to ectopic *NDRG1-OT1\_v4***

| Gene                | Fold change (log <sub>2</sub> ) | P-value               | Rank ( Fold change ) |
|---------------------|---------------------------------|-----------------------|----------------------|
| <i>NDRG1</i>        | -2.32                           | 3.0x10 <sup>-3*</sup> | 1                    |
| <i>HS.213061</i>    | -1.58                           | 1.2x10 <sup>-2*</sup> | 2                    |
| <i>LOC100128775</i> | 1.53                            | 4.0x10 <sup>-3*</sup> | 3                    |
| <i>EIF4G2</i>       | 1.53                            | 2.9x10 <sup>-2*</sup> | 4                    |
| <i>CLEC2D</i>       | 1.49                            | 3.7x10 <sup>-2*</sup> | 5                    |

\**P* <0.05 was considered significant.

validate the *NDRG1-OT1\_v4*-regulated genes identified by microarray, we used RT-PCR to examine the top five differentially expressed genes responding to ectopic *NDRG1-OT1\_v4* (Table 2). As shown in Figure 3C, the results of RT-PCR, except *CLEC2D*, were similar to those of microarray. Furthermore, pathway analysis showed that these genes were involved in the antigen presentation pathway, tRNA charging, sperm motility, eNOS signaling, and protein ubiquitination (Figure 3D).

#### ***NDRG1* mRNA and protein levels are down-regulated by *NDRG1-OT1\_v4***

Among the top five differentially expressed genes, we were particularly interested in *NDRG1* because it is a hypoxia-responsive gene with the highest fold change in response to ectopic expression of *NDRG1-OT1\_v4*. We used quantitative RT-PCR and western blotting to validate *NDRG1* mRNA (Figure 4A&4B) and protein levels (Figure 4C–4F) in MCF-7 and SKBR3 breast cancer cells. Both transcript and protein levels of *NDRG1* were significantly down-regulated in cells overexpressing *NDRG1-OT1\_v4*.

#### ***NDRG1-OT1\_v4* promotes *NDRG1* ubiquitination and degradation**

Since the protein ubiquitination pathway was one of the pathways that *NDRG1-OT1\_v4*-regulated genes were involved in (Figure 3D), we next investigated whether *NDRG1-OT1\_v4* down-regulated *NDRG1* by affecting the stability of *NDRG1*. We first examined the stability of *NDRG1* mRNA by treating cells with a transcriptional inhibitor, actinomycin D (10 µg/mL). MCF-7 & SKBR3 cells were harvested at the indicated time points (0, 2, 4, 8 h) after treatment. As shown in Figure 5A&5B, the degradation of *NDRG1* mRNA was not affected by *NDRG1-OT1\_v4*.

Next, in order to examine the effects of *NDRG1-OT1\_v4* on stability of *NDRG1* protein, cells overexpressing *NDRG1-OT1\_v4* were examined by treating with a protein synthesis inhibitor, cycloheximide (10 µg/mL). Within 8 h after cycloheximide treatment, the protein levels of *NDRG1* were significantly diminished

in cells overexpressing *NDRG1-OT1\_v4* compared to control cells, indicating *NDRG1-OT1\_v4* promoted *NDRG1* degradation (Figure 5C–5F). When MCF-7 & SKBR3 cells were treated with both cycloheximide and MG132, a proteasome inhibitor, *NDRG1-OT1\_v4* no longer promoted *NDRG1* degradation (Figure 5G&5H). In addition, to examine whether down-regulation of *NDRG1* by *NDRG1-OT1\_v4* was via the ubiquitin-proteasome system, we performed immunoprecipitation with an anti-*NDRG1* antibody, followed by immunoblotting using an anti-ubiquitin antibody. The results showed that *NDRG1-OT1\_v4* increased ubiquitin binding to *NDRG1* (Figure 5I&5J). These results suggest that *NDRG1-OT1\_v4* inhibits *NDRG1* expression through the ubiquitin-proteasome pathway.

## **DISCUSSION**

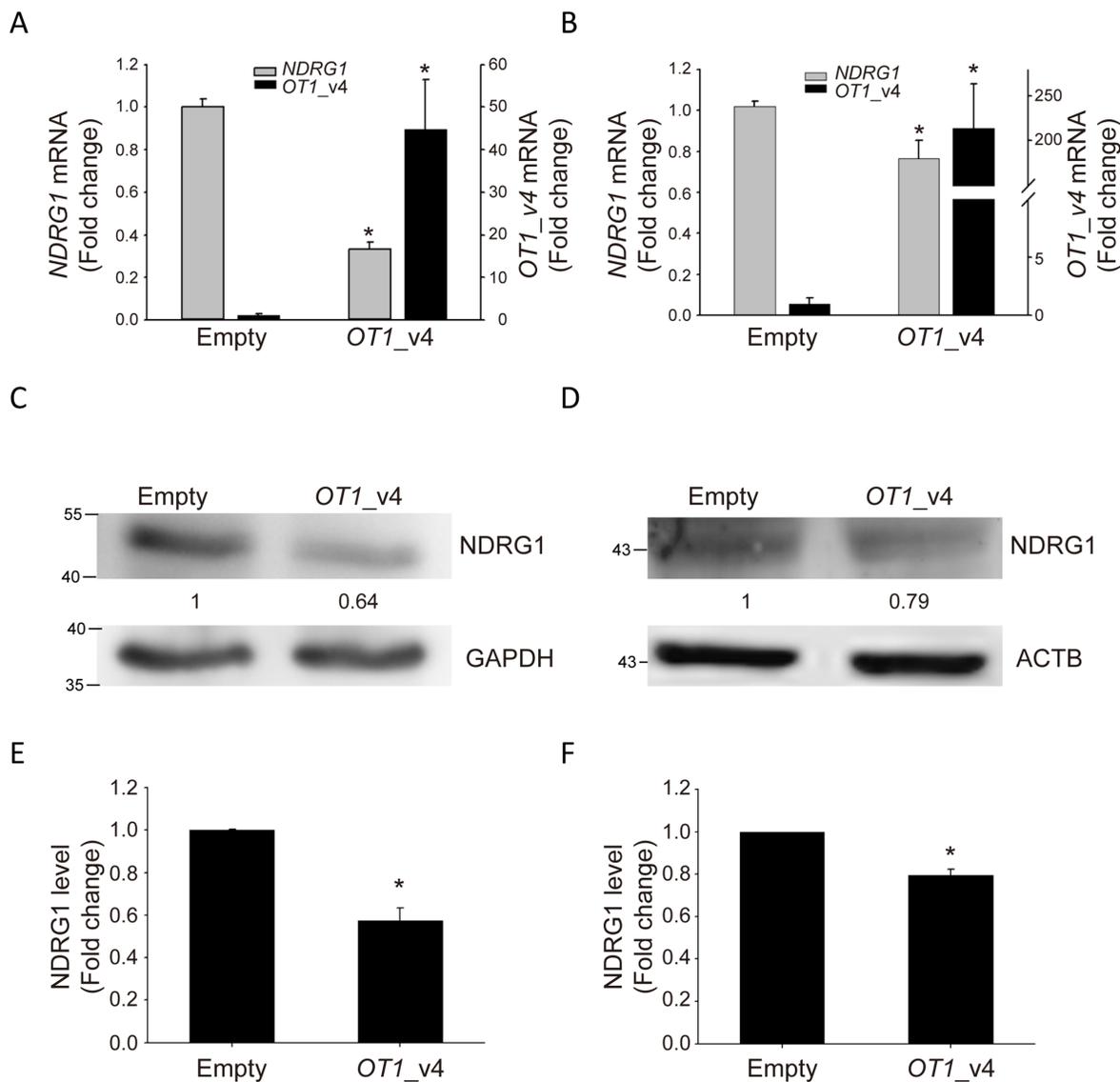
To identify oxygen-responsive lncRNAs in breast cancer, we identified 472 lncRNAs responding to changes in oxygen concentration. Among these lncRNAs, *NDRG1-OT1*, especially *NDRG1-OT1\_v4* isoform, was the most responsive to oxygen changes. Next, *NDRG1-OT1\_v4* was overexpressed in MCF-7 cells under normoxia and *NDRG1* was chosen for further analysis because it had the greatest fold change in expression. Lastly, we identified the mechanism by which *NDRG1-OT1\_v4* inhibits *NDRG1* via ubiquitin-mediated proteolysis.

In this study, NGS was used to identify oxygen-responsive lncRNAs in breast cancer MCF-7 cells (Figure 1). Among the oxygen-responsive lncRNAs, *NDRG1-OT1* was chosen for further analysis based on several reasons. First, *NDRG1-OT1* was in the top three lncRNAs that were strongly up-regulated under hypoxia and down-regulated under re-oxygenation. Second, since lncRNA usually up/down-regulates its neighboring genes [27, 35], we found that *NDRG1-OT1* was transcribed within the hypoxia-inducible gene *NDRG1* (Figure 2B). Therefore, we hypothesized that *NDRG1-OT1* may participate in breast cancers acclimatizing to hypoxia and hence chose *NDRG1-OT1* for further experiments.

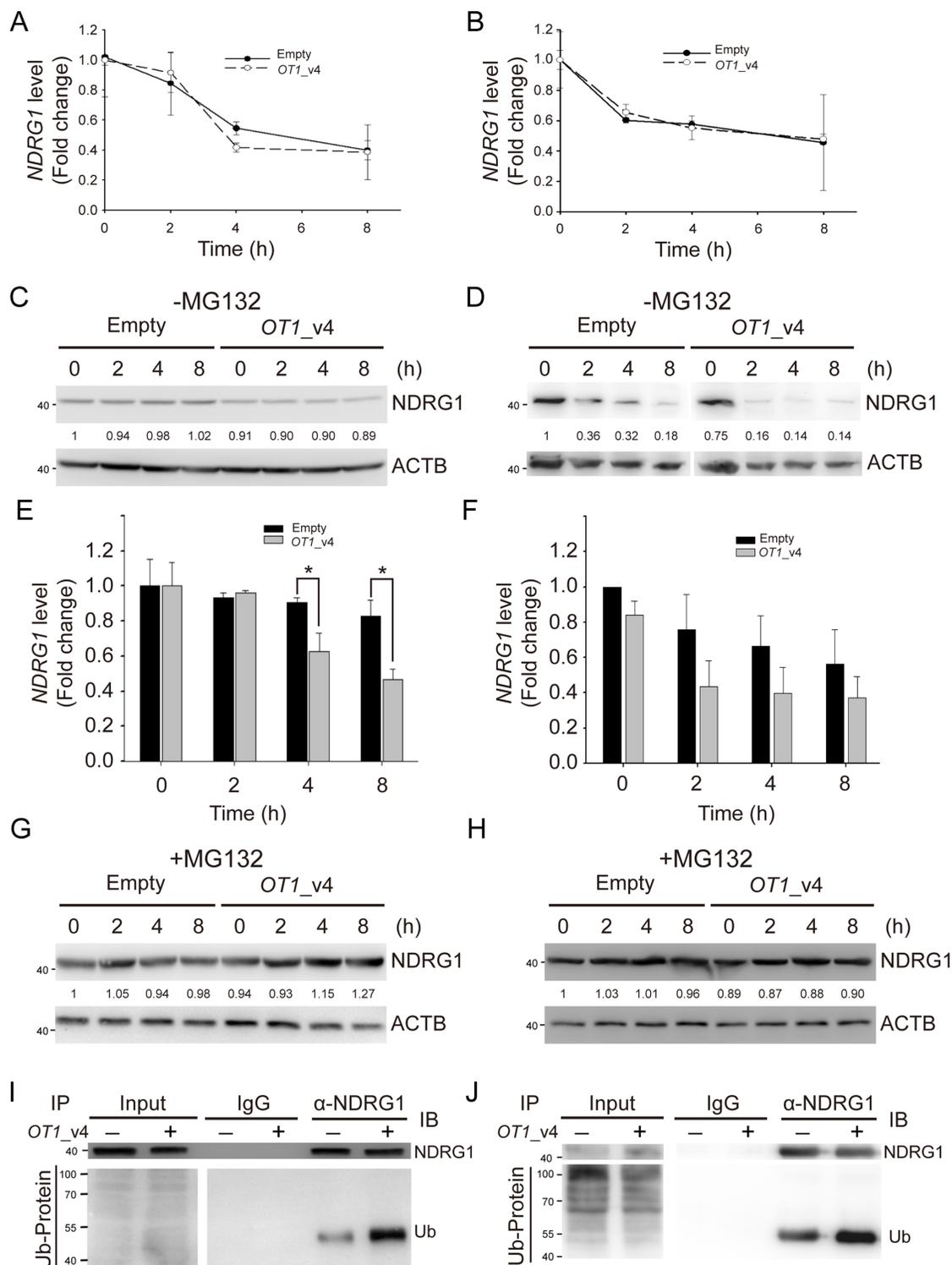
lncRNAs usually have more than one transcript form, and different isoforms have different biological

functions. For example, *UCA1* has three isoforms, but only the 1.4 kb (v1) isoform promotes bladder cancer proliferation and metastasis [31]. Similarly, *NDRG1-OT1* has five isoforms. Four of the five *NDRG1-OT1* isoforms, including v4, contained the *NDRG1* 3'-UTR, but only *NDRG1-OT1\_v4* contained parts of the *NDRG1* 14<sup>th</sup> exon and 14<sup>th</sup> intron. Since *NDRG1-OT1\_v4* had the highest expression level and proportion under hypoxia, we speculated that the 14<sup>th</sup> exon and 14<sup>th</sup> intron of *NDRG1-OT1\_v4* may play a pivotal role in regulating cell functions under hypoxia.

To explore the function of *NDRG1-OT1\_v4*, we used microarray to identify genes regulated by *NDRG1-OT1\_v4*. Pathway analyses showed that these *NDRG1-OT1\_v4* regulated genes were involved in the antigen presentation pathway, tRNA charging, sperm motility, endothelial nitric oxide synthase (eNOS) signaling, and the proteasome-ubiquitination pathway (Figure 3D). Since *NDRG1-OT1\_v4* was a hypoxia-inducible lncRNA, these pathways should be associated with hypoxia too. Indeed, previous studies have demonstrated that hypoxia increased T cell stimulation in dendrite cells [36], cardiac and



**Figure 4: *NDRG1-OT1\_v4* inhibits *NDRG1* mRNA and protein levels.** (A and B) Relative transcriptional level of *NDRG1* (gray, left axis) and *NDRG1-OT1\_v4* (black, right axis) in MCF-7 (A) and SKBR3 (B) cells overexpressing *NDRG1-OT1\_v4*. Two  $\mu$ g of plasmid were transfected into MCF-7 cells. *NDRG1* mRNA was measured by quantitative RT-PCR. Internal control: 18s. The relative expression levels were normalized to empty vector. (C and D) Western blot of *NDRG1* in *NDRG1-OT1\_v4* overexpressed MCF-7 (C) and SKBR3 (D) cells. Relative protein amount of *NDRG1* in MCF-7 cells overexpressing *NDRG1-OT1\_v4*. Internal control: GAPDH (C) and ACTB (D). (E and F) Quantification of *NDRG1* in MCF-7 (E) and SKBR3 (F) cells transfected with *NDRG1-OT1\_v4* plasmid. The relative expression levels were normalized to empty vector. Data were repeated at least 3 times, and the results are the means  $\pm$  SDs \*,  $P < 0.05$ .



**Figure 5: *NDRG1-OT1\_v4* promotes *NDRG1* degradation via ubiquitination.** (A and B) Temporal profile of *NDRG1* in *NDRG1-OT1\_v4*-overexpressed MCF-7 (A) and SKBR3 (B) cells treated with actinomycin D (10  $\mu$ g/mL). *NDRG1* mRNA was measured by quantitative RT-PCR. Internal control: 18s. (C and D) Western blot of *NDRG1* in *NDRG1-OT1\_v4*-overexpressed MCF-7 (C) and SKBR3 (D) cells treated with cycloheximide (10  $\mu$ g/mL). (E and F) Quantification of *NDRG1* in (C&D). The results are the means  $\pm$  SDs. \*,  $P < 0.05$ . (G and H) Western blot of *NDRG1* in *NDRG1-OT1\_v4*-overexpressed MCF-7 (G) and SKBR3 (H) cells treated with cycloheximide (10  $\mu$ g/mL) and MG132 (20  $\mu$ g/mL). (I and J) Co-immunoprecipitation of *NDRG1* and ubiquitin in MCF-7 (I) and SKBR3 (J) cells overexpressing *NDRG1-OT1\_v4* in the presence of MG132 (20  $\mu$ g/mL). Cells were immunoprecipitated with *NDRG1* antibody, followed by western blotting. Data were repeated at least 3 times.

**Table 3: The primers for quantitative RT-PCR**

| Gene/lncRNA         | Sequence (5' to 3')   |
|---------------------|---|
| <i>NDRG1-OT1_v4</i> | (F) CTCCAGGTTCTGTACTACTG<br>(R) GCGGCAGGTAACGAGTCATTG         |
| <i>NDRG1</i>        | (F) GGCAACCTGCACCTGTTTCATCAAT<br>(R) TGAGGAGAGTGGTCTTTGTTGGGT |
| 18s rRNA            | (F) TCAACTTTCGATGGTAGTCGCCGT<br>(R) TCCTTGGATGTGGTAGCCGTTTCT  |

F, forward; R, reverse.

hepatic eNOS production [37], and protein ubiquitination [38]. Hypoxia also negatively regulated the motility of sperm [39]. Moreover, some studies have discovered that hypoxia influences ubiquitin-mediated proteolysis via lncRNAs [23, 32].

In this study, we found that *NDRG1-OT1\_v4* inhibited its overlapped gene *NDRG1* at both mRNA and protein levels in at least two breast cancer cell lines, MCF-7 & SKBR3 (Figure 4). According to previous findings, expression levels of several lncRNAs were mainly correlated with those of their nearest genes, resulting in either a positive [27] or negative effect [26]. Although our findings indicated that *NDRG1-OT1\_v4* had a negative effect on *NDRG1*, *NDRG1-OT1\_v4* does not seem to play a major role. Previous studies have demonstrated that *NDRG1* expression under different oxygen concentrations was regulated by both transcriptional [14, 40] and epigenetic factors [17]. Therefore, *NDRG1-OT1\_v4* is likely one of several regulatory mechanisms that fine tunes the expression of *NDRG1* in response to changes in oxygen concentrations.

In addition, mRNA and protein levels of *NDRG1* were both decreased by overexpressing *NDRG1-OT1\_v4*. Regarding mRNA stability, upon treatment with the transcriptional inhibitor actinomycin D, we found that *NDRG1-OT1\_v4* did not affect *NDRG1* mRNA stability (Figure 5A & 5B). Unlike other lncRNA, such as *TINCR*, it has been found to regulate mRNA stability [28]. Therefore, we expected that *NDRG1-OT1\_v4* might inhibit the transcription of *NDRG1*. Deletion analysis of lncRNA followed by luciferase reporter assays or dot-blot assays [41] could be used to identify the pivotal region of lncRNA regulating its target genes.

On the other hand, when MCF-7 cells were treated with the protein synthesis inhibitor cycloheximide, *NDRG1-OT1\_v4* promoted NDRG1 degradation via ubiquitin-mediated proteolysis (Figure 5C–5J). Although previous studies have indicated that lncRNAs can promote protein stability by blocking protein from interacting with ubiquitin [19, 23, 32], we observed an opposite effect of lncRNA on ubiquitin-mediated proteolysis. Namely, we

first reported that *NDRG1-OT1\_v4* destabilized NDRG1 by promoting ubiquitination (Figure 5C–5J).

There were some limitations in this study. First, in order to investigate *NDRG1-OT1\_v4*-regulated genes, using both overexpression and silencing of *NDRG1-OT1\_v4* would be optimal to reduce the false positives. However, when we tried to knock down *NDRG1-OT1\_v4* under hypoxia by transducing several shRNAs against *NDRG1-OT1\_v4*, not only was the *NDRG1-OT1\_v4* down-regulated but also its downstream gene *NDRG1*. Because of this off-target effect, we only conducted the experiments on ectopically expressed *NDRG1-OT1\_v4*. Second, to examine whether this novel regulatory mechanism of *NDRG1-OT1\_v4* exists in other cancer types, similar experiments should be performed in different cancer cell lines and clinical samples.

In conclusion, we identified a group of oxygen-responsive lncRNAs in breast cancer cells and a novel regulatory mechanism of *NDRG1-OT1\_v4* by enhancing the degradation of its down-stream target NDRG1 protein.

## MATERIALS AND METHODS

### Cell culture and treatments

Breast cancer cell lines, MCF-7, SKBR3, and MDAMB-231, were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Carlsbad, USA), which was supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin-streptomycin-amphotericin solution (Biological Industries, Beit-Haemek, Israel). Breast cancer cell line ZR75-30 was maintained in Roswell Park Memorial Institute (RPMI). Breast cancer cell lines, MDAMB-361 and MDAMB-453, were cultured in Leibovitz's L-15 (L15) culture medium (GIBCO) with 20% FBS and 1% antibiotics. Normal cell line MCF-10A were maintained in DMEM and Ham's Nutrient Mixture F12 (F12) mixed buffer (GIBCO, Carlsbad, CA, USA) with 5% horse serum, 20 ng epidermal growth factor, 0.5 mg hydrocortisone, 100 ng cholera toxin, 10 µg insulin, and 1% penicillin-

streptomycin-amphotericin solution (Biological Industries, Beit-Haemek, Israel).

Except MDAMB-361 and MDAMB-453 growing in a humidified atmosphere without CO<sub>2</sub>, other cells were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, or in an *In Vivo* 2-200 hypoxia chamber (Ruskin Technology, Leeds, UK) filled with a gas mixture of 0.5% O<sub>2</sub>, 5% CO<sub>2</sub> and 94.5% N<sub>2</sub>, for 24 h. For the drug treatment experiment, MCF-7 cells were mixed with 10 µg/mL cycloheximide (Merck, Darmstadt, Germany) or 10 µg/mL actinomycin D (Sigma, St. Louis, USA).

### Next-generation sequencing and analysis of oxygen-responsive lncRNA

Total RNA extracted from MCF-7 was digested to 180 bp fragments after removing rRNAs with a Ribo-Zero Gold kit (Illumina, San Diego, USA). The RNA fragments were reverse-transcribed to cDNA using a TruSeq Stranded total RNA kit (Illumina), and were ligated to specific adapters at both ends of these cDNA fragments to produce a sequencing library. Next, the sequencing libraries were hybridized with adapter's oligonucleotide sequence on the surface of flow cells (Illumina). Each bound fragment was amplified into a clonal cluster through bridge amplification. All of the clusters on the flow cell were finally sequenced by HiScan SQ (Illumina).

The FastQC program (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to examine the quality control of sequencing reads, and TopHat 2 (<https://ccb.jhu.edu/software/tophat/indshe.html>) was used to align sequencing reads to human genome references (GRCh37/hg19). Then, these mapped reads were matched to lncRNAs from the Lncipedia database (<http://www.lncipedia.org/>) and were normalized to Fragments Per Kilobase of transcript per Million mapped reads (FPKM) as the expression level of a given lncRNA using Cufflink (<http://cole-trapnell-lab.github.io/cufflinks/>). Finally, Cuffdiff (<http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/>) was utilized to compare the different FPKMs for each condition. All of the data have been deposited in Gene Expression Omnibus (GEO, GSE84167).

### Plasmid DNA construction and transfection

The pUC57 plasmid containing *NDRG1-OT1\_v4* was purchased from GenScript (GenScript, Piscataway, USA), digested by *Bam*H1 (BioLabs, New England, UK) and *Eco*R1 (BioLabs), and inserted into the pcDNA3.1+ plasmid (Thermo Fisher Scientific Taiwan). MCF-7 cells were transfected with pcDNA3.1+*-NDRG1-OT1\_v4* or empty vector using jetPRIME transfection reagent (Polyplus-transfection, New York, USA) according to the manufacturer's instructions. After 4 h, the transfection medium was replaced with fresh medium-containing serum. After 24 h, cells were checked for RNA expression by quantitative RT-PCR.

### RNA extraction and quantitative RT-PCR

Total RNA was extracted by TRIpure reagent (Roche Diagnostics, Branchburg, USA) according to the manufacturer's protocol. One µg of total RNA was reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, USA). Ten percent of each cDNA was used as the template for quantitative real-time PCR with FastStart Universal SYBR Green Master reaction mixture (Roche) and custom primers (Table 3). Finally, the reaction was carried out on an ABI Step One plus system (Applied Biosystems). Each reaction was done in triplicate, and data were normalized to loading control 18s rRNA.

### Human genome microarray analysis

The total RNA was primed with T7 Oligo(dT) and amplified by an Illumina TotalPre RNA Amplification Kit (Ambion, Austin, USA) to synthesize the cDNA containing a T7 promoter sequence. Following the first strand of cDNA synthesis, the second strand of cDNA was synthesized by converting the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. The reaction employed DNA polymerase and RNAase H to simultaneously degrade the RNA and synthesize the second strand of cDNA. After cleanup, *in vitro* transcription was conducted using the double-stranded cDNA as a template and T7 RNA polymerase to synthesize multiple copies of biotinylated complementary RNA (cRNA). After amplification, the cRNA was mixed with an equal volume of hybridization buffer and hybridized to Illumina Human HT-12 v4 BeadChips (Illumina) at 58°C for 16 h. After hybridization, the BeadChip was washed and stained with streptavidin-Cy3 dye. The intensity of the beads' fluorescence was detected by HiScan SQ (Illumina), and the results were analyzed using BeadStudio software.

After scanning, the intensity data of Illumina Human HT-12 v4 BeadChips were analyzed using Partek software (Partek, St. Charles, USA) for mRNA analysis. Background-adjusted signals were normalized by a quantile normalization algorithm. All data have been uploaded to GEO (GSE72881). Furthermore, Ingenuity Pathway Analysis (Ingenuity Systems Inc. Redwood City, USA) was applied to comprehend the biological functions and signaling pathways of differentially expressed genes.

### Protein extraction and western blotting

MCF-7 cells were lysed in RIPA lysis buffer (Sigma), and protein concentration was determined using Bio-Rad Protein assay reagent (Bio-Rad Laboratories, Hercules, USA). Then, the protein lysate was separated by 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories). The membranes were blocked by TBST Blocking buffer (Arrowtec, Taiwan) for 10 min and hybridized to a primary antibody consisting of NDRG1

(Abcam Inc., Cambridge, USA) and ubiquitin (Abcam). After immunoblotting, the membranes were washed by TBS (Omics Bio, Taiwan) with Tween20 and reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG (GeneTex, Irvine, USA). The protein bands were visualized using an enhanced chemiluminescence system (Millipore, Billerica, USA).

### Co-immunoprecipitation

The immunoprecipitation antibodies, 5  $\mu$ g NDRG1 Ab (Abcam) and 5  $\mu$ g normal mouse IgG (Millipore), were reacted with 50  $\mu$ L G beads (Life Technology, Gaithersburg, USA) in 200  $\mu$ L Ab binding & washing buffer (Life Technology), followed by overnight shaking at 4°C. MCF-7 cells were harvested and lysed in RIPA buffer (Sigma) after reacting with 20  $\mu$ M MG132 (Abmol, Houston, USA) for 4 h. Then, protein concentration was determined using Bio-Rad Protein assay reagent (Bio-Rad Laboratories). One thousand  $\mu$ g of protein lysate were reacted with Ab-G beads during overnight shaking at 4°C. After immunoprecipitation, protein-Ab-G bead complexes were separated and washed with washing buffer (Life Technology). Finally, protein-Ab-G bead complexes were boiled for 10 min, and western blotting was performed.

### Statistical analysis

All data are reported as means  $\pm$  SDs for at least 3 independent experiments. The difference between each group was analyzed using Student's t test.  $P < 0.05$  was considered significant, if no other  $\alpha$  value was specified.

### Author contributions

LCL conceived and designed the experiments. HCL, JCY, and LYC performed the experiments. HCL, HHC, and LCL analyzed the data. MHT, EYC, and LCL contributed reagents, materials, and/or analysis tools. HCL and LCL wrote the paper. All authors reviewed the manuscript.

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### CONFLICTS OF INTEREST

The authors declare that they have no competing financial interests.

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