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# IDH1, a CHOP and C/EBPβ-responsive gene under ER stress, sensitizes human melanoma cells to hypoxia-induced apoptosis

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ABSTRACT

Isocitrate dehydrogenase1 (IDH1) is of great importance in cell metabolism and energy conversion. However, alterations in IDH1 in response to stress and excise-regulated mechanisms are not well described. Here we investigated gene expression profiles under ER stress in melanoma cells and found that IDH1 was dramatically increased with ER stress induced by tunicamycin. Elevated IDH1 subsequently sensitized human melanoma cells to hypoxia-induced apoptosis and promoted HIF-1α degradation. In addition, we revealed that CHOP and C/EBPβ were involved in hypoxia-induced apoptosis via transcriptional regulation of IDH1 expression. Our data indicate that IDH1, regulated by CHOP and C/EBPβ in response to ER stress treatment, inhibits survival of melanoma cells under hypoxia and promotes HIF-1α degradation. Therefore, we propose that IDH1 may serve as a valuable target for melanoma therapy.


## Introduction

Malignant melanoma is the most aggressive skin cancer with a high mortality rate. Although melanoma only accounts for 10% of all skin cancers, it is responsible for >80% of skin cancer death. The 5 year survival rate for patients with metastatic melanoma is lower than 16% [1–3]. Previous reports have shown that melanoma cells have largely adapted to endoplasmic reticulum (ER) stress, which can render them resistant to ER stress-induced apoptosis [4–6]. However, other studies reported that ER stress caused by tunicamycin or thapsigargin could sensitize human melanoma cells to TRAIL-induced apoptosis [7,8]. Therefore, further investigation is required to understand the impact of ER stress in melanoma cells.

Isocitrate dehydrogenase1 (IDH1) is a well-known $NADP^+$-dependent isocitrate dehydrogenase, which converts isocitrate to α-ketoglutarate (α-KG) [9,10]. IDH1 has been reported to represent the most frequently mutated metabolic genes in human cancers [11–16]. Mutations of IDH1 cause a reduction in the production of α-KG while simultaneously increase the output of 2-hydroxyglutarate, both of which are key biochemical metabolites [17,18]. Numerous studies have reported on the functions of mutant IDH1 in human cancers, including the inhibition of HIF-1α degradation and the influence on histone and DNA demethylation [18–25]. However, the roles of wild type IDH1 under ER stress are not well described.

The C/EBP homology protein (CHOP) is a member of the C/EBP family and has low expression levels during normal cell growth, but is significantly upregulated in response to ER stress [26]. CHOP was reported to regulate energy metabolism, cellular proliferation, and differentiation [27–29]. In addition, CHOP was described to be deficient in its DNA binding domain and thus could not bind to the consensus C/EBP sites. Therefore, CHOP requires heterodimerization with other family members to transcriptionally regulate the expression of responsive genes such as the proapoptotic BH3-only protein (BIM) and the anti-apoptotic protein Bcl-2 [26,30–34].

In this study, we reported increased expression of IDH1 under ER stress in human melanoma cells. Such elevation of IDH1 sensitized melanoma cells to hypoxia-induced apoptosis. Furthermore, we revealed that CHOP and C/EBPβ upregulated IDH1 expression in response to tunicamycin treatment and thus augmented hypoxia-induced apoptosis in human melanoma cells. Therefore, our study proposes a novel therapeutic strategy for melanoma treatment.

## Materials and methods

### *Cell culture and reagents*

Human melanoma cell lines described previously were cultured in DMEM containing 5% FBS. Tunicamycin was purchased from Sigma (T7765). It was dissolved in DMSO to make up stock solutions of 3 mM. The following antibodies were used in this study: antibody against IDH1 (Cell Signaling, Danvers, MA, USA; 3997), CHOP

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(Cell Signaling, Danvers, MA, USA; 2895), PHD-2/Egln1 (Cell Signaling, Danvers, MA, USA; 3293), VHL (Cell Signaling, Danvers, MA, USA; 2738S), PDHK1 (Cell Signaling, Danvers, MA, USA; 3820), C/EBPβ (Santa Cruz Biotechnology, Dallas, TX, USA; SC-150), GRP78 (Santa Cruz Biotechnology, Dallas, TX, USA; SC-13968), GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA; SC-25778), PARP (Santa Cruz Biotechnology, Dallas, TX, USA; SC-8007), LDHA(Santa Cruz Biotechnology, Dallas, TX, USA; SC-33781), HIF-1α (BD Biosciences, 610958).

*RNA interference*

RNA interference was performed as previously described [35]. The shRNA was purchased from Sigma. Sequences targeting IDH1 were 5-CCTATCATCATAGGTCGTCAT-3 and 5-GCTTTGGAAGAAGTCTCTATT-3, sequences targeting CHOP were 5-GCCAA TGATGTGACCCTCAAT-3 and 5-CCTGGAAATGAAGAGGAAGAA-3; sequences targeting C/EBPβ were 5-CCCGTGGTGTTATTTAAAGAA-3 and 5-CCTGCCTTTAAATCCATGGAA-3.

*Real-time RT-PCR*

Total RNA was isolated using Trizol (Ambion). One microgram of total RNA was used to synthesize cDNA using PrimeScript™ RT reagent kit (Takara, RR047A) according to the manufacturer's instruction. The primers were shown in Table S1.

*ChIP assay*

Mel-RM cells treated with or without tunicamycin were crosslinked with 1% formaldehyde for 10 min at room temperature. ChIP assay was performed according to the manufacturer's instructions by using anti-CHOP, anti-C/EBPβ and the ChIP assay kit (Millipore, Merck KGaA, Darmstadt, Germany). Anti-rabbit IgG were used as controls. The bound DNA fragments were eluted and amplified by PCR using the following primer pair: 5-ATGAGCTGTCAGCCTAAGG-3 and 5-TTAGACAGGCAAGCTTGCG-3. PCR products were separated on 2% agarose gel by gel electrophoresis.

*Dual-luciferase reporter assay*

Mel-RM cells with or without CHOP or C/EBPβ were transiently transfected with the indicated luciferase reporter plasmid. Renilla plasmid was also included in each transfection to normalize the transfection efficiency. Firefly and Renilla luciferase activities were analyzed by Dual-Luciferase Reporter Assay system according to the manufacturer's instructions (Promega, Madison, WI, USA). The relative luciferase activities were calculated by normalizing the firefly luciferase activity to Renilla luciferase activity. The represented data were mean ± SD of three independent experiments.

*Measure of cellular α-ketoglutarate (α-KG) concentration*

Cellular α-KG concentration was measured enzymatically by modification of the published method [36]. $10^7$ Mel-RM cells were lysed in 1 ml of 0.1% NP-40 Tris–HCl buffer (pH 7.5) on ice for 1 min and the supernatant was collected by centrifugation at 13,000 × g for 15 min at 4 °C. The supernatant was assayed immediately after centrifugation. To each assay, 10 μl of 0.5 M $NH_4Cl$, 50 μl of 2 mM NADH and 5 units of bovine glutamate dehydrogenase (Sigma-Aldrich) were added into 300 μl of supernatant, the decrease of NADH was monitored on a HITACH F-4600 fluorescence spectrophotometer. A control reaction was carried out identically in parallel with the omission of glutamate dehydrogenase. The α-KG concentration was calculated from changes in fluorescence and was normalized after subtraction of fluorescence value from the control reaction.

*Immunoprecipitation and in vivo HIF-1α ubiquitination assay*

Immunoprecipitation and in vivo HIF-1α ubiquitination assay were performed as previously described.

*Annexin V assay*

Mel-RM and MM200 cells were seeded in 6-well culture plates ($1.5 \times 10^5$ cells/well) and incubated for 24 h at 37 °C. Next, the cells were treated as indicated, followed by incubation in Annexin V (Ax)–FITC and PI (10 μg/mL) at room temperature for 15 min. Finally, fluorescence intensities were determined by fluorescence activated cell sorting (FACS) using a FACSCantoII (BD, Franklin Lakes, NJ, USA).

*Statistical analysis*

All data were presented as means ± SEM. Statistical evaluations were achieved by ANOVA followed by a post-hoc Tukey test. $P < 0.05$ was considered statistically significant.

## Results

*IDH1 is upregulated in melanoma cells under ER stress*

To investigate ER stress-altered gene expression, the melanoma cell line Mel-RM was treated with tunicamycin to induce ER stress by inhibition of glycosylation, and then the cells were processed for cDNA microarray analysis. Among the changes in mRNAs triggered by tunicamycin, we observed a dramatic increase of IDH1 (Fig. 1a), which was confirmed by RT-PCR and Western blot analyses (Fig. 1b and c). To investigate whether IDH1 increase is dependent on tunicamycin concentration, we treated Mel-RM cells with different doses of tunicamycin for 24 h and found that IDH1 expression was augmented at both the mRNA and protein levels following dose elevation of tunicamycin (Fig. 1d and e). Subsequently, ER stress-mediated elevation of IDH1 was further confirmed in a large panel of melanoma cell lines, where IDH1 markedly increased in most melanoma cells after treatment with tunicamycin (Fig. 1f). In contrast, expression levels of other members of the IDH family, IDH2, IDH3A, IDH3B, and IDH3G, were not altered in Mel-RM cells (Fig. 1g).

Previous reports indicate that IDH1 is frequently mutated in human cancers, especially in human gliomas and acute myeloid leukemia. Mutations of IDH1 mostly result in the loss of its catalytic activity that functions in the production of α-KG [11–16]. In melanoma cell lines, about 10% carry mutated IDH1. However, it is not known yet whether IDH1 is mutated in Mel-RM and MM200 cells. To investigate this, whole-exon sequencing was carried out to analyze the sequence of IDH1 in Mel-RM and MM200 cells. As shown in Fig. 1h and Supplementary Fig. S1a, IDH1 was not mutated. We then sought to determine the intracellular levels of α-KG under ER stress. As shown in Fig. 1i and Supplementary Fig. S1b, the levels of α-KG increased by 30% after treatment with tunicamycin. These data suggest that IDH1, but not other members of the IDH family, is elevated in most melanoma cells under ER stress and this elevation of IDH1 promotes production of α-KG in these cells.

*CHOP and C/EBPβ cooperatively regulate IDH1 expression in melanoma cells in response to ER stress*

To investigate the molecular mechanism involved in the IDH1 upregulation in melanoma cells, we analyzed the influence of tunicamycin on transcriptional factors and subsequent effect on IDH1 expression. We detected a positive regulation of CHOP on IDH1 expression upon tunicamycin treatment (Supplementary Fig. S2a–d). Elevated levels of IDH1 protein were correlated with a concomitant increase in the CHOP expression (Fig. 2a). Further, we examined whether CHOP was required for tunicamycin-induced IDH1 expression. In doing this, we performed CHOP knockdown experiments in Mel-RM cells using siRNA. Compared with control cells, CHOP knockdown cells showed a dramatic decrease in IDH1 expression levels (Fig. 2b and c). Similar results were obtained when CHOP was knocked down using a short hairpin RNA (shRNA) lentivirus vector (pLko.1) as shown in Fig. 2d.

Previous reports have shown that transcriptional activation mediated by CHOP involves its heterodimerization with other members of the basic leucine zipper transcription factor family such as C/EBPα and C/EBPβ [28,31,34,37,38]. To ascertain whether CHOP functions alone or in combination with other members in regulating IDH1 expression upon tunicamycin treatment, we depleted the two key C/EBP family members C/EBPα and C/EBPβ using shRNA. In comparison with control cells, C/EBPβ knockdown cells showed a marked decrease in IDH1 expression with tunicamycin treatment, but no effect was observed in C/EBPα knockdown cells (Fig. 2e and f and Supplementary Fig. S2e–g). To confirm these observations, we then overexpressed CHOP together with C/EBPβ or with C/EBPβΔDBD in Mel-RM cells. Compared with control cells, the cells overexpressing