



Original Articles

Proteolysis of a histone acetyl reader, ATAD2, induces chemoresistance of cancer cells under severe hypoxia by inhibiting cell cycle progression in S phase

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ABSTRACT

Cancer cells acquire chemoresistance in hypoxic regions of solid tumors, which is suggested to be at least partly due to reduction of their proliferative activity. However, molecular mechanisms behind it have not been fully elucidated. Here, we revealed the importance of active proteolysis of a histone acetylation reader, ATPase family AAA domain containing 2 (ATAD2), under hypoxia. We found that inactivation of an O₂/Fe²⁺/α-ketoglutarate-dependent dioxygenase triggered ATAD2 proteolysis by the proteasome system upon severe hypoxia in a hypoxia-inducible factors (HIFs)-independent manner. Consistently, ATAD2 expression levels were markedly lower in perinecrotic hypoxic regions in both xenografted and clinical tumor tissues. The ATAD2 proteolysis was accompanied by a decrease in the amount of acetylated histone H3 lysine 27 and inhibited cell cycle progression from the early to late S phase under severe hypoxia. The retardation of S phase progression induced chemoresistance, which was blocked by overexpression of ATAD2. Together, these results indicate that ATAD2 proteolysis upon severe hypoxia induces chemoresistance of cancer cells through heterochromatinization and the subsequent retardation of S phase progression; therefore, inhibition of ATAD2 proteolysis is expected to be a strategy to overcome chemoresistance of hypoxic tumor cells.

1. Introduction

Oxygen consumption by cancer cells is known to markedly exceed oxygen supply to them because of their high metabolic demands and aberrant proliferation. In addition to such an imbalance, high interstitial pressure resulting from the fragility and leakiness of the tumor vasculature causes hypoxic regions in most malignant solid tumors [1–3]. It is now well-recognized that cancer cells acquire resistance to anticancer

therapies, such as chemotherapies and radiotherapy, under hypoxic conditions [4,5]. Clinical studies also repeatedly reported a negative aspect of tumor hypoxia: the intratumoral hypoxic fraction positively correlates with the poor prognosis of cancer patients [6,7]. Under these circumstances, tumor hypoxia is recognized as one of the most important issues to be overcome for the development of better cancer treatment strategies.

How do cancer cells acquire chemoresistance in hypoxic regions?

Abbreviations: ATAD2, ATPase family AAA domain containing 2; HIFs, hypoxia-inducible factors; HDAC, histone deacetylase; HAT, histone acetyl transferase; BD, bromodomain; RCC, renal cell carcinoma; CHX, cycloheximide; α-KG, α-ketoglutarate; DFO, deferoxamine; DMOG, dimethylxalylglycine; PHD, prolyl 4-hydroxylases; VHL, von-Hippel Lindau; ccRCC, clear cell renal cell carcinoma.

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One of the key factors is hypoxia-inducible factor 1 (HIF-1). Since HIF-1 was firstly identified as a transcription factor for cellular hypoxia responses in 1993 [8], it has been demonstrated that it functions not only in angiogenesis, invasion, metastasis, and metabolic reprogramming of cancer cells [9–13], but also in the induction of chemoresistance of hypoxic tumor cells [14,15]. Under hypoxic conditions, HIF-1 induces the expression of an energy-dependent membrane efflux pump, ATP-dependent translocase (ABCB1)/multidrug resistance protein (MDR1), that transports a variety of structurally unrelated xenobiotics to maintain their intracellular concentrations at subtoxic levels [16]. HIF-1 inhibits Caspase-9 by inducing transcription of an inhibition of apoptosis (IAP) family gene, Survivin, and eventually promotes chemoresistance under hypoxic conditions [17,18].

In addition to these mechanisms, cell cycle retardation upon hypoxia has been associated with cancer chemoresistance regardless of its dependency on HIF-1; this is because most conventional chemotherapeutic agents, such as alkylating agents, platinum agents, and taxane-based agents, damage cancer cells that proliferate rapidly. Therefore, molecular mechanisms that potentially delay cell cycle progression upon hypoxia, such as activation of p53, p21, and p27, or suppression of cyclin D1, induce chemoresistance of hypoxic cancers [19–21].

Not only genetic regulation as above but also epigenetic control, which potentially alters global chromatin condensation, is also known to be a factor influencing the rate of cell cycle progression and eventually triggering the chemoresistance of cancer cells under hypoxia. A representative mode of epigenetic regulation for that is histone acetylation. In fact, histone deacetylases (HDACs) that catalyze deacetylation of histone proteins and cause more condensed chromatin, referred to as heterochromatin [22], are known to induce chemoresistance, which supports the development of HDAC inhibitors as anticancer agents [23]. HDAC inhibitors have been confirmed to exhibit expected therapeutic effects, to some extent, in both pre-clinical and clinical studies [23]; however, the further identification of a key protein among epigenetic regulators is expected in order to develop new anticancer agents with more marked therapeutic effects.

The histone acetylation status is determined by both histone acetyltransferases (HATs) and HDACs. These two play pivotal roles, but histone acetylation reader protein harboring bromodomain (BRD) for the recognition of acetylated lysin is also critical [24]. ATPase family AAA domain containing 2 (ATAD2) has one bromodomain and two ATPase associated with diverse cellular activities (AAA) domains, all of which bind acetylated lysin residues of histone H4 [25]. ATAD2 has been suggested to be required for the maintenance of histone hyperacetylation through protecting the acetylation from removal by HDAC2 [26]. In addition, it has been reported that ATAD2 works as a co-regulator of several oncogenic transcriptional factors, such as estrogen receptor α (ER α) [27], androgen receptor (AR) [28], E2F1 transcription factor [29], and Myc proto-oncogene protein [30], and thus has a positive impact on the cell cycle progression and proliferation of various types of cancer cells.

As for ATAD2, we recently acquired preliminary data showing that its protein levels were markedly reduced under hypoxic conditions. Given the accumulating knowledge on the positive impact of ATAD2 on cell cycle progression and proliferation of cancer cells, as above, we formulated a hypothesis that the decrease in ATAD2 expression levels causes chemoresistance of cancer cells under hypoxia through the retardation of cell cycle progression. In the present study, we investigated how ATAD2 expression levels are decreased upon hypoxia, and found that ATAD2 is actively proteolyzed through the proteasome system when an $O_2/Fe^{2+}/\alpha$ -KG-dependent dioxygenase is inactivated under hypoxia. Moreover, we revealed the functional importance of active proteolysis of ATAD2 in chemoresistance of cancer cells under hypoxia through the retardation of cell cycle progression from the early to late S phase.

2. Materials and methods

2.1. Cell culture and reagents

Human renal cell carcinomas (RCCs) [31], ACHN, 786-O, and RCC4, a human cervical epithelial adenocarcinoma cell line, HeLa, and an immortalized human embryonic kidney cell line, HEK293 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μ g/mL). Cells were incubated in a well-humidified incubator with 5% CO_2 and 95% air at 37 °C for normoxic conditions. Cells were incubated in INVIVO2 400 (BAKER Ruskinn, Sanford, ME, USA) for hypoxic conditions at 1% O_2 or in INVIVO2 500 for severer hypoxic conditions at < 0.1% O_2 . Small interfering RNA (siRNA) against ATAD2 was purchased from Thermo Fisher Scientific (Waltham, MA, USA) (Cat# 1299001, Stealth siRNAs #HSS120808 and #HSS120810). PolyFect Transfection Reagent (Cat# 301107, Qiagen, Hilden, Germany) was used for transient transfection. Deferoxamine (Cat# D9533-1G) and dimethylxylglycine (Cat# D3695-10 MG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Camptothecin (Cat# 038-18191), Carboplatin (Cat# 033-25231), and Cycloheximide (Cat# 037-20991) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Docetaxel (Cat# CDX-D0371) was purchased from AdipoGen (San Diego, CA, USA).

2.2. Plasmid DNA

To construct pcDNA4/ATAD2, the coding sequence of the human *atad2* gene was amplified from the cDNA of HeLa cells by PCR using the following two primers: 5'-AAAGGATCCGCCACCATGGTGGTCTCCGC-3' and 5'-CCCGATATCTCATCTGGAACAACCTGAAG-3', and inserted between the *Bam*HI and *Eco*RV sites of pcDNA4/myc-His B (Thermo Fisher Scientific). To construct two kinds of plasmids, pX330/sgATAD2-#1 and -#2, each of which expresses a different kind of guide RNA (gRNA), for ATAD2 knockout, the following two sets of oligo DNAs were annealed, and the resultant double-stranded DNA was inserted into the *Bbs*I site of pX330. The nucleotide sequences of the two sets of oligo DNAs were as follows: #1 forward: 5'-AAACGTCGCGCAGCCGAGGCCAGC-3'; #1 reverse: 5'-CACCGCTGGCCTCGCGGTGCGCGAC-3'; #2 forward: 5'-CACCGGACCTCCATTAACGATCCAG-3'; #2 reverse: 5-AAACCTGGATCGTTAATGGAGTCC.

2.3. In vivo experiments

Mice were bred and maintained at 23 ± 2 °C with 50–70% humidity in the Animal Research Center, Graduate School of Medicine, Kyoto University. Cancer cell suspensions (HeLa cells: 50 μ L of 1.0×10^6 cells in PBS, ACHN cells: 100 μ L of 5.0×10^6 cells in PBS) were subcutaneously transplanted into right hind legs of 2 athymic nude mice (BALB/c Slc nu/nu: SLC, Japan), respectively, under anesthesia with 2.5% of isoflurane gas in oxygen flow (1.5 L/min). A hypoxia marker, pimonidazole hydrochloride (Cat. # HP2-200Kit, Hypoxyprobe Inc., Burlington, MA, USA), was intraperitoneally administered into every tumor-bearing mouse at a dose of 60 mg/kg 60 min before harvesting the tumor xenograft from mice sacrificed by fracture dislocation of the cervical spine under anesthesia 21 days after the transplantation. Every 4 xenografted tumors were subjected to immunohistochemical analysis.

2.4. Immunohistochemical analyses

Formalin-fixed and paraffin-embedded sections of tumors were deparaffinized in xylene, rehydrated in 100, 90, 80, and 70% ethanol sequentially, retrieved in Histofine™ deparaffinizing antigen retrieval buffer, pH 6.0 (Cat# 415281, Nichirei, Tokyo, Japan) at 95 °C for 40 min, blocked with Protein Block Serum-Free Ready-To-Use (Cat.

#X0909, Dako, Santa Clare, CA, USA) for 30 min at room temperature, and stained with the indicated combination of primary antibodies, anti-human ATAD2 rabbit polyclonal antibody (Cat# HPA029424, Sigma-Aldrich, St. Louis, MO, USA), anti-human CD31 mouse monoclonal antibody (Cat. # ab9498, Abcam), anti-human CA9 mouse monoclonal antibody (Cat. # ab107257, Abcam), and/or anti-pimonidazole FITC-conjugated mouse monoclonal antibody (4.3.11.3 mouse FITC-MAb: Cat. # HP2-200Kit, Hypoxyprobe Inc.). The slides were incubated with Alexa Fluor 594 secondary antibody (Cat. #A-11072, Thermo Fisher Scientific) Alexa Fluor 488 secondary antibody (Cat. #A-11070, Thermo Fisher Scientific), or Alexa Fluor 546 secondary antibody (Cat. #A-11060, Thermo Fisher Scientific), and with Hoechst 33342 (Cat. #H3570, Thermo Fisher Scientific) for counterstaining. Fluorescence images were obtained with a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

2.5. Western blotting

HEK293, ACHN, 786-O, and RCC4 cells (3×10^5 cells/well and 1×10^5 cells/well in 6-well plates for HEK293 cells and other cells, respectively) were incubated under normoxic (20% O₂) or hypoxic (1% or < 0.1% O₂) conditions for the periods mentioned in each figure legend, and lysed in 80 µL CellLytic M Buffer (Cat. #C2978, Sigma-Aldrich). Anti-ATAD2 antibody (Cat. #HPA029424, Sigma-Aldrich), anti-Histone H3 (acetyl K27) antibody (Cat. #ab4729, Abcam), anti-HIF-1α antibody (Cat. #610959, BD Biosciences, Franklin lakes, NJ, USA), and anti-β-actin antibody (Cat. #sc-69879, Santa Cruz, Heidelberg, Germany) were used as primary antibodies. Anti-mouse and anti-rabbit IgG horseradish peroxidase-linked whole antibodies (GE Healthcare, Chicago, IL, USA) were used as secondary antibodies. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used to detect chemiluminescent signals according to the manufacturer's instructions. The image was obtained by Amersham Imager 680 (GE Healthcare).

2.6. qRT-PCR

Cells were treated under normoxic or hypoxic conditions for 24 h. Total RNA was extracted using Sepasol RNA I Super (Cat. #09379-84, Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Total RNA (0.5 µg) was subjected to reverse transcription using PrimeScript RT reagent Kit (Perfect Real Time, Takara Bio, Kusatsu, Japan). The indicated genes' mRNA levels were quantified using the qRT-PCR technique with the following primers: ATAD2 forward primer: 5'-CCGAGTACTCTGTGGCTTG-3'; ATAD2 reverse primer: 5'-TTGACTTTTTGCGAATTTTCC-3'; β-actin forward primer, 5'-TGGCACCAGCACAATGAA-3'; β-actin reverse primer, 5'-CTAAGTCA-TAGTCCGCCTAG-3'. β-actin mRNA levels were used as an internal control.

2.7. FACS analysis

Forty-eight hours after HEK293 cells (3×10^5 cells/well in 6-well plates) were transiently transfected with the indicated plasmids, the cells were incubated under normoxic (20% O₂) or severe hypoxic (<0.1% O₂) conditions for an additional 24 h and harvested in culture medium. The cells were pulse-labeled with 10 µM BrdU for 30 min before the harvest, and subjected to fixation, permeabilization, immunofluorescent staining, and DNA staining with 7-Amino-Actinomycin D (7AAD) using the BD APC BrdU Flow Kit (Cat# 552598, BD Biosciences, Franklin lakes, NJ, USA) according to the manufacturer's instructions. Immunofluorescent staining was performed with anti-BrdU antibody (Cat# 552598, BD Biosciences) according to the manufacturer's instructions for BrdU staining, and with anti-human ATAD2 rabbit polyclonal antibody (Cat. #HPA029424, Sigma-Aldrich) as the primary antibody and with F(ab')₂-Goat anti-Rabbit IgG (H + L) Cross-Adsorbed

Secondary Antibody, Alexa Fluor 488 (Cat. #A-11070, Thermo Fisher Scientific) for ATAD2 staining. Intensities of 7AAD, BrdU, and ATAD2 were analyzed using BD FACSCanto II Flow Cytometer (BD Biosciences) and DIVA software (BD Biosciences).

2.8. CRISPR/Cas9-mediated gene knockout (KO)

Twenty-four hours after HEK293 cells (6×10^5 cells/well in 6-well plates) were transfected with pX330/sgATAD2-#1 and -#2, they were reseeded in a φ100-mm dish and cultured for 2 weeks. The resultant colonies were isolated and established as clones. An ATAD2 KO clone, HEK293 ATAD2-KO, was selected by genotyping using the following primers: forward primer: 5'-CCTGTCAAAGGCCAAGTGCT-3'; reverse primer: 5'-CTGCAGCATGACGGCATAGA-3'. ATAD2 KO was further confirmed by Western blotting.

2.9. Colorimetric cell viability assay

In the case of HEK293 cells, 24 h after the cells (6×10^5 cells/well in a 6-well plate) were transiently transfected with the indicated plasmids, they were reseeded on a 96-well plate (5×10^3 cells/well), pre-incubated under normoxic (20% O₂) or severe hypoxic (<0.1% O₂) conditions for 24 h, and treated with Camptothecin under the same oxygen conditions as the pre-incubation for 48 h. In the case of cancer cells, 24 h after the cells (3×10^3 cells/well in a 96-well plate) were transfected with siRNA against ATAD2, they were treated with each anticancer drug under the same oxygen conditions as the pre-incubation for 48 h. The viability of cells was monitored with Cell Count Reagent SF (Cat. #07553-44, Nacalai Tesque) according to the manufacturer's instructions.

2.10. Patient and tissue samples

The clinical study was carried out in accordance with the Helsinki Declaration. The protocol of this study using human renal and lung cancer samples was approved by the Ethics Committee of Kyoto University Hospital. Written-informed consent was obtained from every patient. Every sample was surgically obtained from patients who were pathologically diagnosed with clear cell renal cell carcinoma at Kyoto University Hospital.

2.11. TCGA proteome analysis

A data set of TCGA Breast Cancer Proteome (Study ID: b8da9eeb-57b8-11e8-b07a-00a098d917f8, Project ID: CPTAC2 Retrospective) was obtained from The CPTAC Data Portal. Of the 27 samples in which both HIF-1α and ATAD2 were detected, those with a relative standard deviation over 2.0 were omitted. The normality of the remaining data was confirmed by the Shapiro-Wilk test: p-values were 0.66 and 0.967 for HIF-1α and ATAD2, respectively. For analysis of the correlation between HIF-1α and ATAD2 levels, Pearson's correlation coefficient and the P-value were calculated.

2.12. Statistical analyses

The significance of differences was determined using Student's t-test. A P-value < 0.05 was considered significant.

3. Results

3.1. A histone acetyl reader, ATAD2, is degraded at the protein level upon severe hypoxia

ATAD2 has been suggested to be required for histone hyperacetylation and promote both replication of genomic DNA and proliferation of various types of cancer cells [25,29,32]. Therefore, we

hypothesized that ATAD2 activity might be downregulated under hypoxic conditions, which may account for the chemoresistance of hypoxic tumor cells by retarding cell proliferation. To examine this possibility, we first performed immunohistochemical analyses and investigated the ATAD2 expression pattern in tumor xenografts with a human renal cell carcinoma (RCC) cell line, ACHN [31]. We found that the tumor tissues exhibited a heterogeneous expression pattern of ATAD2 proteins. ATAD2 levels were found to be low in a part of pimonidazole-positive hypoxic regions, particularly beside areas of necrosis (Fig. 1A). The same pattern was confirmed in HeLa tumor xenografts as well; ATAD2 expression was markedly decreased in hypoxic regions (Fig. 1B). These data clearly show that the expression of ATAD2 protein is markedly reduced in hypoxic regions of xenografted tumors.

Cancer cells are exposed to various stresses/stimuli in hypoxic regions, such as hypoxia and low nutrients. To test whether a hypoxic stimulus is sufficient to decrease the ATAD2 expression levels, we cultured cells under hypoxic conditions *in vitro* and performed Western blotting for ATAD2. When a renal cell carcinoma (RCC) cell line, ACHN, was cultured under severe hypoxic conditions at $< 0.1\%$ O_2 , expression levels of ATAD2 were markedly decreased in a hypoxic treatment period-dependent manner but hardly reduced under milder hypoxic conditions at 1% O_2 (Fig. 1C). The severe hypoxia-dependent decrease was also confirmed in a non-cancerous human embryonic kidney cell line, HEK293 cells, suggesting the generality of the phenomenon (Fig. 1D).

To identify the regulatory step responsible for the decrease in ATAD2 protein levels upon severe hypoxia, we next carried out qRT-PCR for ATAD2 mRNA. Severe hypoxic treatment increased rather than

decreased ATAD2 mRNA levels, meaning that changes in the ATAD2 mRNA levels cannot account for the decrease in ATAD2 protein upon severe hypoxia (Fig. 1E). To further narrow down the regulatory step, we next analyzed the possible involvement of the proteasome system using a proteasome inhibitor, MG132. The decrease in the ATAD2 protein levels was almost completely blocked when treated with MG132 under severe hypoxic conditions, showing that ATAD2 protein is actively degraded by the proteasome pathway upon severe hypoxia. (Fig. 1F). Consistently, when we analyzed the stability of ATAD2 protein under normoxia and severe hypoxia by monitoring the rate of ATAD2 decay after inhibiting neo-synthesis of ATAD2 with a translation inhibitor, cycloheximide (CHX), ATAD2 levels were rapidly decreased under severe hypoxia compared with under normoxia (Fig. 1G).

As ATAD2 protein has been reported to be highly expressed in the S phase [30], we next examined whether the ATAD2 proteolysis upon severe hypoxia was S phase-specific. FACS analysis to quantify ATAD2 protein levels in each cell cycle phase demonstrated that ATAD2 levels were decreased regardless of the cell cycle status, indicating that the proteasome system works for ATAD2 in every cell cycle phase upon hypoxia (Fig. 1H).

3.2. Proteolysis of ATAD upon severe hypoxia is regulated by an O_2 / Fe^{2+} / α -KG-dependent dioxygenase but independent of the VHL-HIF-1 axis

α -Ketoglutarate (α -KG)-dependent dioxygenases are recognized as oxygen sensors of various biological species because they use molecular oxygen as a substrate [33]. Because the α -KG-dependent dioxygenases contain ferrous iron (Fe^{2+}) as a prosthetic group, they need not only

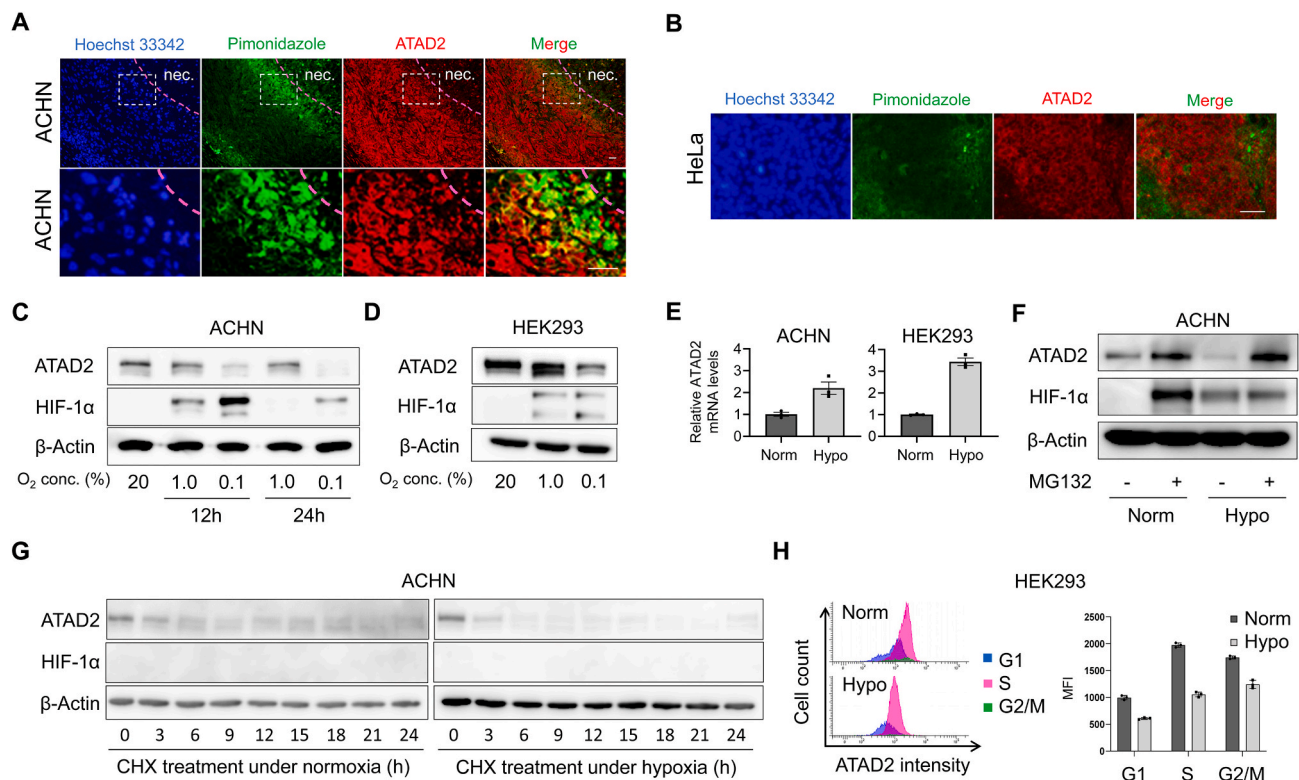


Fig. 1. A histone acetyl reader, ATAD2, is degraded at the protein level upon severe hypoxia. **A** and **B**, Immunohistochemical analyses of the indicated tumor xenografts for ATAD2 (red) and pimonidazole (green). Counter staining with Hoechst 33342 (blue). Scale bars, 50 μ m. Bottom images of the ACHN xenografts are high-magnification images of the dotted boxes in the top images. Dotted pink curves represent the border between hypoxic and necrotic (nec.) regions. Four tumor-bearing mice were subjected to the analysis, and representative images are shown. **C–H**, Lysates and total RNA were harvested after the indicated cells were cultured under normoxic (Norm: 20% in **E–H**), severe hypoxic (Hypo: $< 0.1\%$ in **E–H**), or the indicated oxygen (in **C** and **D**) conditions for the indicated time period (**C** and **G**) or for 24 h (**D–F** and **H**), and subjected to Western blotting for the indicated antibodies (**C**, **D**, **F**, and **G**), qRT-PCR for ATAD2 mRNA (**E**) ($n = 3$), and FACS analysis (**H**). Cells were treated with MG132 (10 μ M) for 6 h before the harvest (**F**) or with cycloheximide (CHX, 50 μ g/mL) under normoxia (Norm: 20%) or severe hypoxia (Hypo: $< 0.1\%$) for the indicated time period (**G**). FACS analysis was conducted to quantify the ATAD2 expression levels in G1, S, and G2/M phases after 24 h of severe hypoxic treatment using 7AAD, BrdU, and anti-ATAD2 antibody (**H**).

oxygen and α -KG but also Fe^{2+} for their enzymatic activity [33]. To examine whether the ATAD2 proteolysis upon severe hypoxia is triggered by inactivation of an α -KG-dependent dioxygenase, we employed an Fe^{2+} chelator, deferroxamine (DFO), and an inhibitory analog of α -KG, dimethylxalylglycine (DMOG). We found that ATAD2 protein levels were markedly reduced by chelating Fe^{2+} even under normoxic conditions, and that the reduction was blocked by inhibiting the proteasome with MG132 (Fig. 2A). Moreover, ATAD2 protein levels were gradually decreased by DMOG as well in a dose-dependent manner even under normoxia (Fig. 2B). These data collectively showed that the proteasome system-dependent ATAD2 degradation was mediated by inactivation of an $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase.

Hypoxia-inducible factors (HIFs) are recognized as master transcription factors responsible for an adaptive response to hypoxia. There are three types of HIFs, HIF-1 to HIF-3, all of which are heterodimers composed of one α subunit (HIF-1 α , HIF-2 α , or HIF-3 α) and one β subunit (HIF-1 β ; also known as aryl hydrocarbon receptor nuclear translocator). HIFs activity is mainly regulated through the proteolysis of HIF- α s, which is triggered through $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent prolyl-hydroxylation by prolyl 4-hydroxylases (PHDs) and completed by the von-Hippel Lindau (VHL)-dependent ubiquitination and subsequent proteasomal degradation. Therefore, we next tested whether ATAD2 proteolysis is under the control of the PHD-VHL system or dependent on HIFs or their downstream genes. To directly approach these questions, we employed clear cell renal cell carcinoma (ccRCC) cell lines, 786-O and RCC4, both of which are functional VHL-deficient [31]. Western blotting demonstrated that the expression of ATAD2 was reduced under severe hypoxic conditions in these VHL-deficient cells, just the same as in VHL-proficient ACHN and HEK293 cell lines (Fig. 2C). Again, qRT-PCR showed that the ATAD2 mRNA levels were not reduced under severe hypoxia, meaning that the changes in mRNA levels cannot account for the decrease in ATAD2 protein upon severe hypoxia (Fig. 2D). The involvement of both the $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase and proteasome system, observed using VHL-proficient cells, could be confirmed in the VHL-deficient cells as well; reduction of ATAD2 protein was promoted by an inhibitory analog of α -KG, DMOG, (Fig. 2E) and an Fe^{2+} chelator, DFO, (Fig. 2F) under normoxia, but blocked by a proteasome inhibitor, MG132, under severe hypoxia (Fig. 2G). These data indicate that proteolysis of ATAD2 upon severe hypoxia is regulated by an $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase, but is not under the control of the same PHD-VHL-dependent system as that of HIF- α s and is not dependent on HIFs or their downstream genes.

3.3. ATAD2 protein levels were markedly decreased in hypoxic regions of clinical tumor tissues

To validate our findings about ATAD2 proteolysis in human tumor tissues, we carried out immunohistochemical analyses using clinical samples. We intentionally utilized ccRCC tissues, up to 90% of which harbor VHL mutations [31], in order to directly examine independence of the ATAD2 proteolysis on the VHL-HIF system. We confirmed a heterogeneous ATAD2 expression pattern in clinical ccRCC tissues as well (Fig. 3A), consistent with the pattern in the xenografted tumors (Fig. 1A and B). ATAD2 expression was high in proximal areas of CD31-positive blood vessels (Fig. 3A). On the other hand, it was lower in regions approximately 100- μm distal to the vessels, which were considered to be hypoxic [1].

In order to directly test whether ATAD2 protein levels are reduced in hypoxic regions of clinical tumor tissues, we next used squamous cell lung tumor tissues, whose VHL-HIF system is intact, and performed immunohistochemical analyses for ATAD2 and an intrinsic hypoxia marker, carbonic anhydrase 9 (CA9), whose expression is under the control of HIF-1 (Fig. 3B). ATAD2 expression was not detected in CA9-positive hypoxic regions. Consistent with our *in vitro* and *in vivo* findings, these immunohistochemical data collectively indicate that ATAD2 expression was markedly decreased in hypoxic areas of human tumor tissues independent of the VHL-HIFs axis.

In addition, the analysis of TCGA proteome data from human breast cancer showed that the protein expression level of ATAD2 was negatively correlated with that of HIF-1 α , further supporting our finding that ATAD2 protein is decreased in hypoxic regions (Fig. 3C).

3.4. ATAD2 proteolysis delays progression of cell cycle from early to late S phase under severe hypoxia

As ATAD2 has been reported to be involved in the regulation of histone acetylation levels on nascent chromatin during DNA replication [25,26], we next investigated the impact of the decrease in ATAD2 protein under severe hypoxia on both the histone acetylation status and cell cycle distribution, particularly on the S phase proportion. Western blotting revealed the reduction of acetylated histone H3 lysine 27 (H3K27ac) protein under severe hypoxic conditions, as expected (Fig. 4A). The reduction was rescued by forced expression of ATAD2 (Fig. 4A), suggesting the involvement of ATAD2 proteolysis in global heterochromatinization under severe hypoxic conditions.

FACS-based cell cycle analysis with the use of both 7AAD for DNA

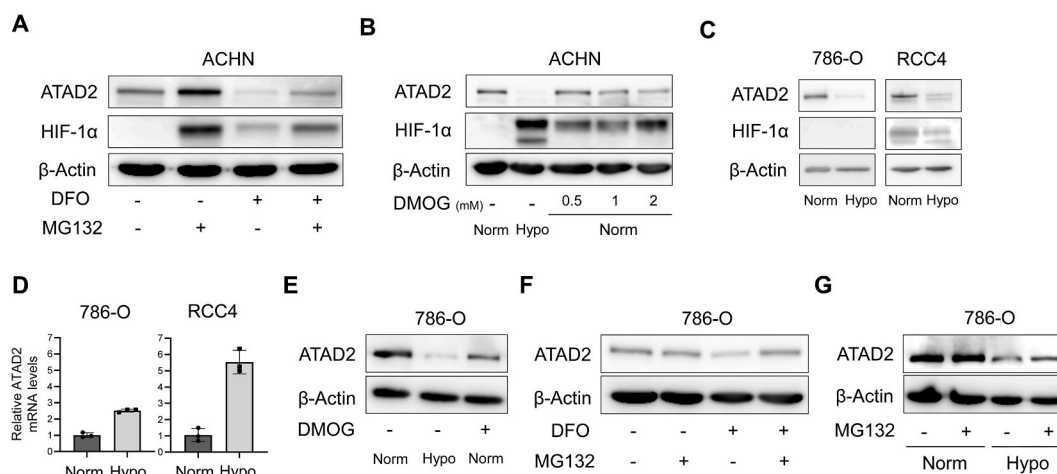


Fig. 2. Proteolysis of ATAD2 upon severe hypoxia is regulated by an $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase but independent of the VHL-HIF-1 axis. The indicated cells were cultured under normoxia (Norm: 20%) or severe hypoxia (Hypo: < 0.1%) for 24 h in the presence or absence of DFO (100 μM for 24 h), MG132 (10 and 20 μM for ACHN and 786-O, respectively, for 6 h), and/or DMOG (0.5 mM for 24 h) as indicated. Cell lysates and total mRNA were harvested for Western blotting for the indicated proteins (A-C, and E-G) and qRT-PCR (D), respectively. Results are the means $\pm$ SD, $n = 3$.

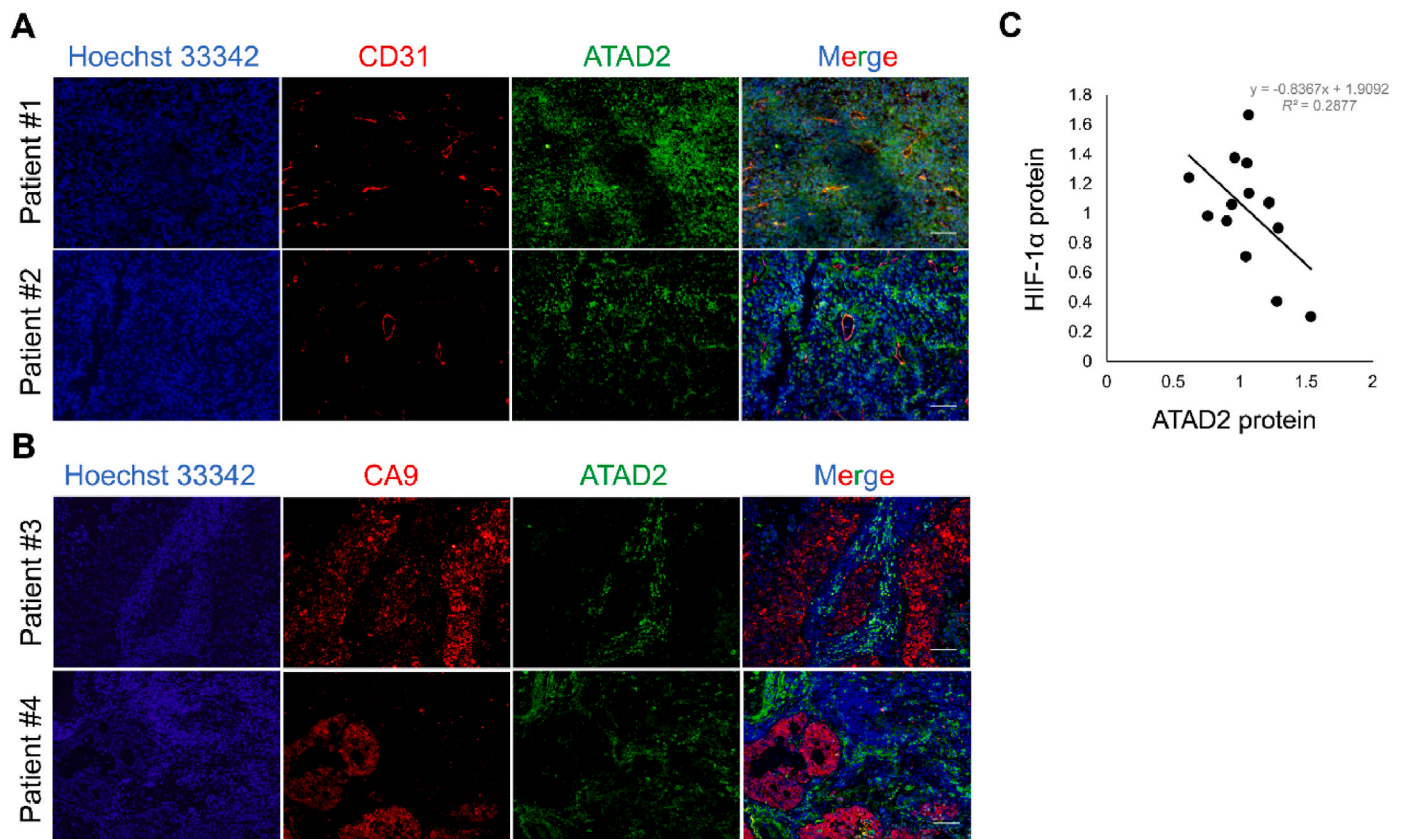


Fig. 3. ATAD2 protein levels were markedly decreased in hypoxic regions of clinical tumor tissues. **A** and **B**, Immunohistochemical analyses of clinical ccRCC tumors (**A**) and squamous cell lung tumor (**B**) from two independent patients co-stained with ATAD2 (green in **A** and **B**), CD31 (red in **A**), CA9 (red in **B**), and Hoechst 33342 (blue in **A** and **B**). Scale bars, 100 μ m. **C**, Correlation diagram between protein expression levels of HIF-1 α and ATAD2.

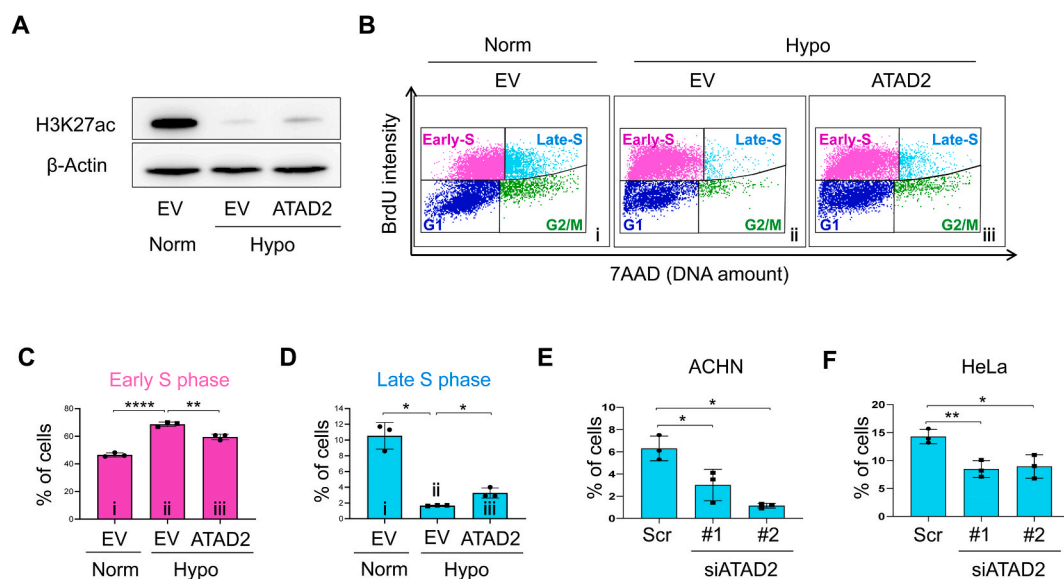


Fig. 4. ATAD2 proteolysis delays progression of cell cycle from early to late S phase under severe hypoxia. **A–D**, Forty-eight hours after transfection with pcDNA4/myc-His (EV) or pcDNA4/ATAD2 (ATAD2), HEK293 cells were cultured under normoxia (Norm: 20%) or severe hypoxia (Hypo: < 0.1%) for 24 h, and subjected to Western blotting for the indicated proteins (**A**) and to cell cycle analyses using FACS (**B–D**). **B**, Representative scatter plot data. Dark blue dots, G1 phase; green dots, G2/M phase; pink dots, early S phase; cyan dots, late S phase. **C** and **D**, The proportions of cells in the early (**C**) and late (**D**) S phase based on the cell cycle distribution in **B**. **E** and **F**, Seventy-two hours after the indicated cells were treated with scramble siRNA (Scr) or siRNA against ATAD2, cells were subjected to FACS analysis to quantify the proportion of cells in the late S phase. Results are the mean $\pm$ SD. $n = 3$. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$, unpaired two-tailed t -test.

contents and BrdU for DNA synthesis revealed that severe hypoxic treatment significantly decreased the proportion of cells in the late S phase, but significantly increased that in the early S phase (i and ii in Fig. 4B, C, and D). Interestingly, forced expression of ATAD2 slightly but significantly recovered S phase progression under severe hypoxic conditions (iii in Fig. 4B, C, and D; Supplementary Fig. 1A). All of these data collectively indicate that ATAD2 proteolysis causes the retardation of S phase progression from the early to late S phase, through the deacetylation of histone protein and resultant heterochromatinization of chromatin under severe hypoxia. Consistently, loss-of-function studies demonstrated that silencing of ATAD2 significantly decreased the proportion of cells in the late S phase even under normoxic conditions (Fig. 4E and F; Supplementary Figs. 1B and 1C).

3.5. ATAD2 proteolysis causes chemoresistance of cancer cells under severe hypoxia

Resistance to chemotherapy is induced under hypoxic conditions in general. As we could clearly reproduce it with the use of an inhibitor of DNA topoisomerase I, Camptothecin (Fig. 5A), we decided to apply this experimental setting to examine whether the severe hypoxia-mediated proteolysis of ATAD2 was associated with chemoresistance of hypoxic cells. A colorimetric cell viability assay demonstrated that forced expression of ATAD2 almost completely abrogated the severe hypoxia-mediated chemoresistance, supporting our hypothesis that the chemoresistance was induced by ATAD2 proteolysis under severe hypoxic conditions (Fig. 5B; Supplementary Fig. 1A). Consistently, a loss-of-function study using siRNA against ATAD2 demonstrated that the ATAD2-silencing significantly increased resistance to Camptothecin even under normoxic conditions (Fig. 5C; Supplementary Fig. 1D). ATAD2 KO cells also exhibited resistance to Camptothecin compared with their parental cells, which could be partially but significantly abrogated by the overexpression of ATAD2 (Fig. 5D; Supplementary Fig. 1E).

We then examined whether the decrease in ATAD2 expression caused resistance to not only Camptothecin but also other conventional chemotherapeutic agents, such as a platinum-based drug, Carboplatin,

and a tubulin-stabilizing agent, Docetaxel. As expected, ATAD2 silencing slightly but significantly induced the resistance of cancer cells to each of these drugs (Fig. 5E and F; Supplementary Fig. 1D). Taken together, these data indicate that ATAD2 proteolysis upon severe hypoxia causes chemoresistance of cancer cells. Of note, chemoresistance by ATAD2 silencing was confirmed with the use of a VHL-deficient ccRCC cell line, 786-O cells, in Fig. 5C, E, and F, supporting our conclusion in Fig. 2 that proteolysis of ATAD2 upon severe hypoxia is not under the control of the same PHD-VHL-dependent system as that of HIF- α .

4. Discussion

Here, we demonstrated that a histone acetyl reader, ATAD2, was actively degraded by the proteasome system when an $O_2/Fe^{2+}/\alpha$ -KG-dependent dioxygenase was inactivated under severe hypoxia. We additionally found that the proteolysis caused chemoresistance of cancer cells by inhibiting cell cycle progression from the early to late S phase with deacetylation of histone H3K27 upon severe hypoxia.

We, for the first time, revealed that ATAD2 protein levels are significantly decreased upon severe hypoxia. We concluded that it resulted from proteolysis, but not from death of cells for the following reasons. First, the decrease of ATAD2 protein levels was rescued by the proteasome inhibitor, MG132, *in vitro*. Second, we could detect β -actin in Western blotting even under severe hypoxia *in vitro*. Third, since the hypoxia-marker pimonidazole detects hypoxic regions through its reduction by reductases in living cells, the existence of pimonidazole-positive but ATAD2-negative cells means that they still survive in perinecrotic regions.

This is the first report to show that ATAD2 protein is actively degraded by the proteasome system under severe hypoxic conditions. Although we revealed its importance from the viewpoint of cancer biology (particularly chemoresistance of hypoxic cancer cells), its physiological significance remains unknown. However, it might be reasonable to hypothesize that cell cycle retardation works to reduce wasted consumption of energy under hypoxic conditions, which is markedly beneficial for the maintenance of energy homeostasis. To test this hypothesis and investigate the physiological significance of ATAD2

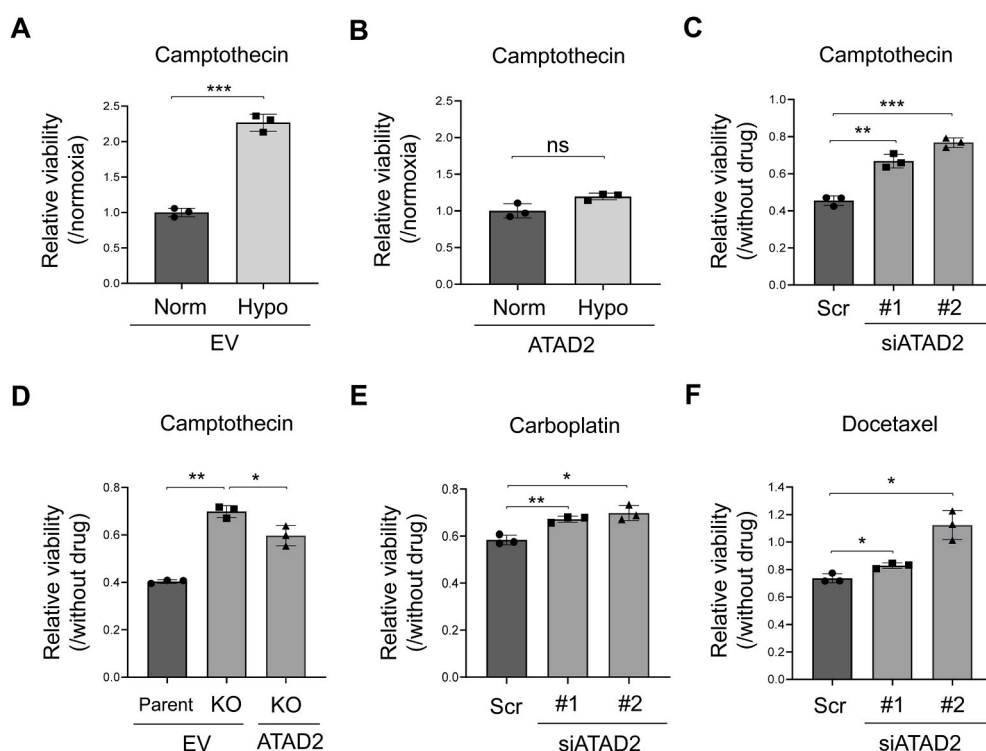


Fig. 5. ATAD2 proteolysis causes chemoresistance of cancer cells under severe hypoxia. HEK293 cells (A, B, and D), 786-O cells (C, E, and F), and HEK293 ATAD2-KO cells (D) were transfected with or without the indicated plasmid or siRNA, cultured under normoxic (Norm: 20%) or severe hypoxic (Hypo: < 0.1%) conditions in the presence of Camptothecin (0.2 μ M in A and B, 3 μ M in C, and 2 μ M in D), Carboplatin (100 μ M in E), or Docetaxel (10 nM in F), and subjected to the colorimetric cell viability assay. Results are the mean $\pm$ SD. $n = 3$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant, unpaired two-tailed t -test.

proteolysis in the presence of hypoxic stimuli, it is important to generate an ATAD2 knockout mouse and analyze its phenotypes.

ATAD2 proteins were found to be degraded when cells were treated with an inhibitory analog of α -KG, DMOG, or an Fe^{2+} chelator, DFO, even under normoxic conditions. These data suggest that ATAD2 is stabilized through the activation of an $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase under normoxia but destabilized through its inactivation under hypoxia. We have not identified the dioxygenase participating in the regulation of ATAD2 stability, but our *in vitro* and *in vivo* data gave us some hints. Our immunohistochemical analyses using both a hypoxia marker, pimonidazole, and anti-ATAD2 antibody demonstrated that ATAD2 expression levels were markedly decreased in a part of hypoxic regions of xenografted tumors. Particularly, ATAD2 was undetectable in cellular layers adjacent to areas of necrosis. In addition, according to the results of our *in vitro* studies, hypoxic stimuli may be sufficient to account for the active proteolysis even without other stimuli, such as the depletion of nutrients. Moreover, ATAD2 was more efficiently degraded under severer hypoxic conditions, such as $< 0.1\%$ O_2 . Taken together, the unidentified $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase can be considered to lose activity under such severe oxygen conditions. This information helps us to narrow down and identify candidate dioxygenases.

There are some putative and possible mechanisms underlying ATAD2 proteolysis upon severe hypoxia. First, an unidentified $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase might directly modify ATAD2 for its stabilization under normoxia. Second, an unidentified dioxygenase might modify an unknown factor and indirectly stabilize ATAD2 under normoxia or destabilize it under severe hypoxia. In addition to identification of the unknown dioxygenase itself as above, that of its modification target is also important. Moreover, it is necessary to explore a ubiquitinating enzyme for ATAD2 proteolysis, which is different from VHL. We may be able to accomplish this with the use of the previously reported information that three lysine residues at 63, 175, and 931 in ATAD2 proteins are ubiquitinated [34–36].

Inconsistent with our main finding about ATAD2 proteolysis upon severe hypoxia, ATAD2 expression was reported to be induced at the transcriptional initiation level in a HIF-1 α -dependent manner in stomach cancer cells [37]. There is a possibility that whether ATAD2 expression is downregulated at the protein stability level or upregulated at the transcriptional initiation level might differ in a cell context-dependent manner because we mainly used kidney-derived cells here, and not stomach cancers. However, in fact, we also confirmed the same kind of tendency, suggesting the importance of regulation at the transcriptional initiation level; ATAD2 mRNA levels are upregulated under hypoxia, to some extent, although we have not tested the HIF-1-dependency.

Here, we could reveal that ATAD2 proteolysis inhibited cell cycle progression from the early to late S phase under severe hypoxia. However, it remains unclear whether ATAD2's function as a histone acetylation reader is critical for the retardation because we have not analyzed the role of ATPase activity of ATAD2 in it. It would be interesting to examine the importance of the ATPase activity by overexpressing a point mutant of ATAD2 lacking the ATPase activity in the HEK293-derived ATAD2 KO cells we generated here.

It has been repeatedly reported that ATAD2 had positive impacts on both proliferation and cell cycle progression of various types of cancer cells [30,38]. Clinical studies have consistently shown that ATAD2 expression levels are associated with poor prognoses of various cancer patients [39]. These data justify the rationale of a novel therapeutic strategy inhibiting ATAD2. However, our findings additionally provide novel insight into the utilization of ATAD2 for the development of an antitumor strategy from a different viewpoint. The extent of the decrease in ATAD2 expression in hypoxic regions of tumor tissues is expected to provide valuable information on how much cancers exhibit resistance to a chemotherapeutic agent and show recurrence after treatment.

Our colorimetric cell viability assays demonstrated that the forced

expression of ATAD2 abrogated the hypoxia-mediated chemoresistance of renal cell carcinoma cells. There are few effective anticancer drugs for the treatment of kidney cancers because of their chemoresistant characteristics, and surgery is the standard treatment at present. However, our data suggest the possibility that inhibition of ATAD2 proteolysis upon severe hypoxia may be a novel way to improve the efficacy of chemotherapy and the combination of surgery and chemotherapy.

The contribution of ATAD2 proteolysis to the chemoresistance of hypoxic cancer cells was not necessarily marked, and it could not fully account for hypoxic cell chemoresistance. However, it is also true that silencing of ATAD2 partially but significantly enhanced chemoresistance of cancer cells, indicating the function of ATAD2 proteolysis in chemoresistance. Moreover, we demonstrated that artificial activation of ATAD2 significantly enhanced the therapeutic effect of chemotherapy. Because many types of conventional chemotherapeutic agents are given to cancer patients daily for some weeks, we could expect that the chemoenhancing effect would accumulate during the treatment period and eventually exhibits big outcome even if the effect of each administration is not so big.

The present study revealed a novel mechanism underlying chemoresistance of hypoxic tumor cells and provides a rational basis for inhibiting the hypoxia-dependent ATAD2 proteolysis to overcome this. In order to realize this concept, we need to fully elucidate the molecular mechanisms behind ATAD2 proteolysis upon severe hypoxia and identify regulatory factors, such as an $\text{O}_2/\text{Fe}^{2+}/\alpha$ -KG-dependent dioxygenase and a ubiquitinating enzyme for ATAD2.

Authors' contributions

T. Haitani performed the experiments mainly under the guidance of M. Kobayashi, S. Koyasu, and H. Harada, analyzed the data, and co-wrote the manuscript. M. Kobayashi and S. Koyasu contributed to data analysis and critical discussion. S. Akamatsu, T. Menju, H. Date, and O. Ogawa contributed to the research using human cancer samples and critical discussion. T. Suwa, J.-M. Nam, and P. T. L. Nguyen helped with *in vitro* data collection. Y. Onodera contributed to the TCGA analysis. H. Harada designed and supervised the study, analyzed the data, and co-wrote the manuscript.

Ethics approval and consent to participate

All animal experiments were performed in the Animal Research Center, Graduate School of Medicine, Kyoto University according to a protocol approved by the Animal Research Committee of Kyoto University prior to the study. The clinical study was carried out in accordance with the Helsinki Declaration. The protocol of this study using human renal and lung cancer samples was approved by the Ethics Committee of Kyoto University Hospital. Written-informed consent was obtained from each patient. Every sample was surgically obtained from patients who were pathologically diagnosed with clear cell renal cell carcinoma at Kyoto University Hospital.

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Declaration of competing interest

The authors declare that they have no financial conflict of interests or personal relationships that could inappropriately influence and bias the work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2021.12.028>.

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