JBC RESEARCH ARTICLE

USP38 promotes deubiquitination of K11-linked polyubiquitination of HIF1α at Lys769 to enhance hypoxia signaling

Received for publication, September 14, 2023, and in revised form, November 9, 2023 Published, Papers in Press, December 9, 2023,
https://doi.org/10.1016/j.jbc.2023.105532

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Reviewed by members of the JBC Editorial Board. Edited by Brian D. Strahl

HIF1α is one of the master regulators of the hypoxia signaling pathway and its activation is regulated by multiple post-translational modifications (PTMs). Deubiquitination mediated by deubiquitylating enzymes (DUBs) is an essential PTM that mainly modulates the stability of target proteins. USP38 belongs to the ubiquitin-specific proteases (USPs). However, whether USP38 can affect hypoxia signaling is still unknown. In this study, we used quantitative real-time PCR assays to identify USPs that can influence hypoxia-responsive gene expression. We found that overexpression of USP38 increased hypoxia-responsive gene expression, but knockout of USP38 suppressed hypoxia-responsive gene expression under hypoxia. Mechanistically, USP38 interacts with HIF1α to deubiquitinate K11-linked polyubiquitination of HIF1α at Lys769, resulting in stabilization and subsequent activation of HIF1α. In addition, we show that USP38 attenuates cellular ROS and suppresses cell apoptosis under hypoxia. Thus, we reveal a novel role for USP38 in the regulation of hypoxia signaling.

In nature, organisms are constantly exposed to hypoxia stress (1). HIF, a heterodimer of bHLH-PAS proteins consisting of an unstable alpha subunit (HIF1α and HIF2α) and a stable beta subunit (HIF1β), plays a central role in helping organisms cope with this stress (1, 2). Under normoxic conditions, the HIFα subunit is hydroxylated at two highly conserved prolyl residues by the prolyl hydroxylases (PHD1, PHD2 and PHD3) (also called EglN2, EglN1 and EglN3), whose activity is regulated by the availability of O_2 (1, 3, 4). Hydroxylated HIFα generates a binding site that is recognized by the von Hippel-Lindau (pVHL) tumor suppressor protein complex (2). This complex polyubiquitylates HIFα and targets it for proteasomal degradation. Under hypoxic conditions, the activity of PHDs is inhibited, resulting in the stabilization and accumulation of HIFα proteins. Stabilized HIFα proteins dimerize with HIF1β proteins, translocate to the nucleus, and induce transcription of genes involved in various biological processes (1, 5). Oxygen-dependent hydroxylation is a major regulatory pathway for HIFα activation, and is responsible for the cellular oxygen sensing mechanism (1, 4). In addition to hydroxylation, HIFα is also regulated by other post-translational modifications (PTMs), including ubiquitination/deubiquitination, phosphorylation, acetylation/deacetylation, SUMOylation, methylation, S-nitrosylation, glycosylation and neddylation (6–11). Most of these modifications cause either increase or decrease the stability of HIFα, leading to various biological consequences (12–25).

While deubiquitination is a powerful regulator of protein stability, information on the effect of deubiquitinases (DUBs) on the HIF system is limited (6, 8, 26–30). In addition, DUBs, together with hypoxic conditions, strongly influence many physiological states, such as cancer initiation and progression, cell metabolism, and adaptation and tolerance to hypoxia (1, 4, 6, 31). Therefore, the identification of DUBs that affect hypoxia signaling may provide new insights into the mechanism underlying the regulation of HIF signaling, and oxygen-related pathophysiology.

In this study, we report the identification of ubiquitin-specific protease 38 (USP38) as a positive regulator of hypoxia signaling. USP38 promoted hypoxia-responsive gene expression under hypoxia. USP38 specifically interacted with HIF1α to stabilize HIF1α protein by removing K11-linked polyubiquitin chains of HIF1α depending on its deubiquitinase activity. In addition, USP38 attenuated cellular ROS and suppressed cell apoptosis under hypoxia. Our results revealed a novel function of USP38 in affecting the hypoxia signaling pathway.

Results

USP38 enhances hypoxia signaling

To identify deubiquitinases that affect hypoxia signaling, we cloned 41 members of the ubiquitin-specific proteases (USPs) (30), into expression vectors and examined their effects on the induction of typical hypoxia-inducible gene *LDHA* when overexpressed (Fig. S1) (32). Overexpression of USP38 significantly increased LDHA expression (Fig. S1). We

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next confirmed that overexpression of *USP38* together with HIF1α apparently enhanced the expression of *BNIP3, LDHA, PDK1* and *VEGFA* (Fig. 1*A*). We then treated cells with $CoCl_2$ (200 μM) to mimic hypoxic conditions (33) and examined the effect of USP38 overexpression on the induction of typical hypoxia-inducible genes. Overexpression of *USP38* also enhanced $CoCl_2$-stimulated expression of hypoxia-inducible genes (Fig. 1*B*). Furthermore, overexpression of *USP38* enhanced the expression of hypoxia-inducible genes under hypoxia (Fig. 1*C*). Immunoblotting confirmed that Flag-USP38 was expressed when overexpressed in H1299 cells (Fig. 1*D*). Indeed, the hypoxic conditions were achieved as determined by hypoxyprobe staining (Fig. S2) (34). We then knocked out USP38 in H1299 cells (Fig. 1*E*). In contrast, *USP38* disruption resulted in a reduction of CoCl2-and hypoxia-stimulated expression of hypoxia-inducible genes (Fig. 1, *F* and *G*). To determine whether the screened USP38 specifically enhanced hypoxia signaling, we selected two additional USPs, USP53 and USP25, for validation. These two USPs did not appear to enhance hypoxia signaling based on the initial screening (Fig. S1). As expected, overexpression of USP53 and USP25 had no effect on HIF1α-induced BNIP3 expression (Fig. 1, *H* and *I*). To validate whether the effect of *USP38* on the expression of hypoxia-inducible genes was dependent on the hypoxia signaling, we knocked out of *HIF1β* in H1299 cells and then examined the effect of *USP38* (Fig. 1*J*). In wild-type H1299 cells, overexpression of USP38 significantly enhanced hypoxia-stimulated expression of *BNIP3* and *VEGFA*, but not in HIF1β-deficient H1299 cells (Fig. 1*K*). Notably, USP38 is evolutionarily conserved (Fig. S3). Taken together, these data suggest that *USP38* enhances hypoxia signaling.

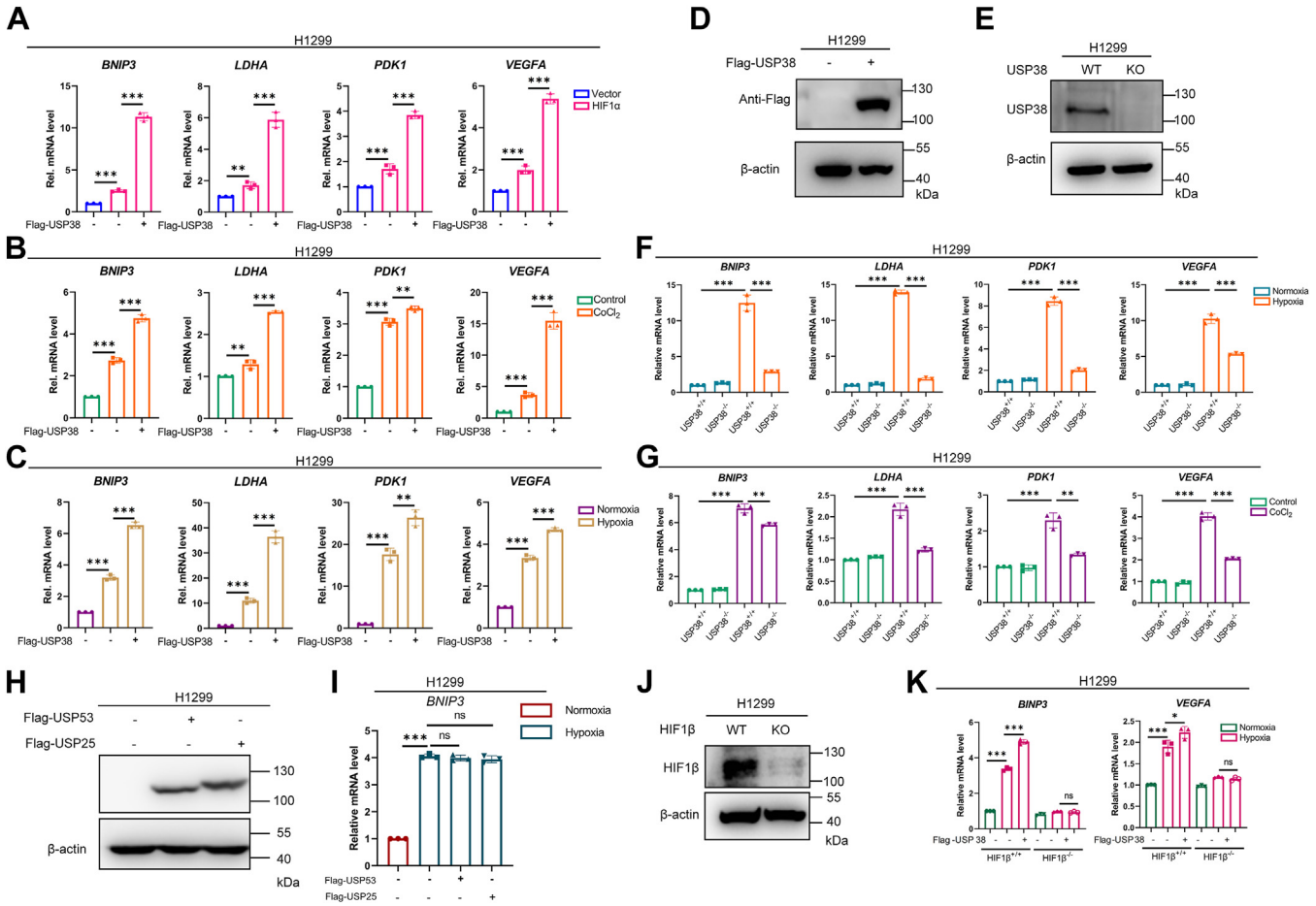

Figure 1. USP38 enhances hypoxia signaling. *A*, qRT-PCR analysis of *BNIP3, LDHA, PDK1* and *VEGFA* mRNA in H1299 cells transfected with Myc empty vector (Vector) and Flag empty vector (–), Myc-HIF1α and Flag empty vector (+), or Myc-HIF1α and Flag-USP38 (+) for 24 h. *B*, qRT-PCR analysis of *BNIP3, LDHA, PDK1* and *VEGFA* mRNA in H1299 cells transfected with Flag empty vector (–) or Flag-USP38 (+) and treated without (Control) or with $CoCl_2$ (200 μM) for 12 h. *C*, qRT-PCR analysis of *BNIP3, LDHA, PDK1* and *VEGFA* mRNA in H1299 cells transfected with Flag empty vector (–) or Flag USP38 (+) and treated under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. *D*, validation of USP38 expression in H1299 cells transfected with empty vector (control) or Flag-USP38. *E*, validation of USP38 expression in wildtype (WT) or USP38-deficient H1299 cells. *F*, qRT-PCR analysis of *BNIP3, LDHA, PDK1* and *VEGFA* mRNA in. USP38-deficient or wildtype H1299 cells ($USP38^{-/-}$ or $USP38^{+/+}$) were cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. *G*, qRT-PCR analysis of *BNIP3, LDHA, PDK1* and *VEGFA* mRNA in USP38-deficient or wildtype H1299 cells ($USP38^{-/-}$ or $USP38^{+/+}$) treated with or without $CoCl_2$ (200 μM) for 12 h. *H*, validation of USP53 or USP25 expression in H1299 cells transected with empty vector (control), Flag-USP53 or Flag-USP25. *I*, qRT-PCR analysis of *BNIP3* in H1299 cells transected with empty vector (control), Flag-USP53 or Flag-USP25. *J*, Validation of HIF1β in HIF1β-deficient or wildtype H1299 cells ($HIF1\beta^{-/-}$ or $HIF1\beta^{+/+}$). *K*, qRT-PCR analysis of *BNIP3* and *VEGFA* mRNA in HIF1β-deficient or wildtype H1299 cells ($HIF1\beta^{-/-}$ or $HIF1\beta^{+/+}$) transfected with Flag empty vector (–) or Flag-USP38 and then cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. ns, not significant; *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data are representative of three independent experiments (mean ± SD of three biological replicates).

USP38 interacts with HIF1α

To elucidate the mechanism of USP38 in the enhancement of hypoxia signaling, we investigated which molecule in the hypoxia signaling pathway is targeted by USP38. We performed co-immunoprecipitation assays to examine whether USP38 interacts with HIF1α, HIF2α, PHD1, PHD2, PHD3, VHL, or FIH, the major components in the hypoxia signaling pathway. USP38 was found to interact with HIFα and VHL (Fig. 2*A*). Further co-immunoprecipitation assays for endogenous protein interaction showed that USP38 could only interact with HIF1α under hypoxia, but not with VHL (Figs. 2, *B–D* and S4). Domain mapping revealed that all domains of HIF1α interacted with the N-terminus and the C-terminus of USP38 (Fig. 2, *E–G*). These data suggest that USP38 may target HIF1α to enhance hypoxia signaling.

USP38 removes K11-linked polyubiquitin chains of HIF1α at lysine 769

Ectopic expression of USP38 caused HIF1α stabilization in a dose-dependent manner (Fig. 3*A*). We further found that ectopic expression of USP38 also stabilized the HIF1α hydroxylation-deficient mutants, HIF1α (in which Pro 402 and 564, and Asp 803 were mutated to alanine) and HIF1α^{DM} (in which Pro402 and 564 were mutated to alanine) (Fig. S5), indicating that USP38-mediated HIF1α stabilization is

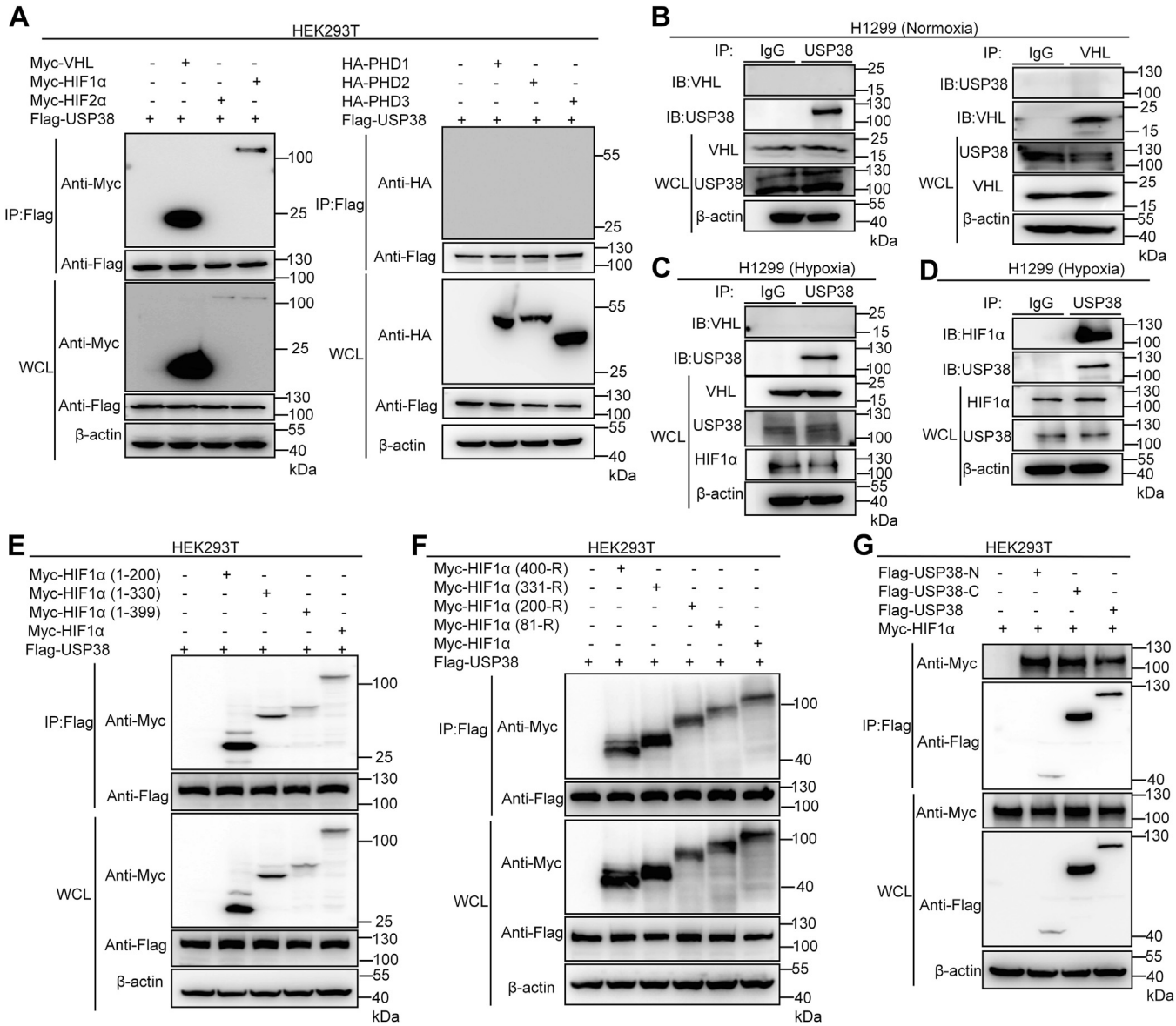

Figure 2. USP38 interacts with HIF1α. *A*, co-Immunoprecipitation (Co-IP) analysis of USP38 interacting with VHL, HIF1α, HIF2α, PHD1, PHD2 or PHD3. HEK293T cells were transfected with the indicated plasmids, anti-Flag conjugated agarose beads were used for immunoprecipitation, and anti-Myc (*left*) or anti-HA antibody was used for immunoblotting (IB). *B*, co-IP analysis of endogenous USP38 interacting with endogenous VHL in H1299 cells under normoxia (21% O_2). *C*, co-IP analysis of endogenous USP38 interacting with endogenous VHL in H1299 cells under hypoxia (1% O_2) for 4 h. *D*, co-IP analysis of endogenous USP38 with endogenous HIF1α in H1299 cells under hypoxia (1% O_2) for 4 h. *E* and *F*, co-IP analysis of USP interacting with full-length or truncated HIF1α in HEK293T cells. *G*, co-IP analysis of HIF1α interacting with full-length or truncated USP38 in HEK293T cells. IB, immunoblotting; IP, immunoblotting; WCL, whole cell lysate.

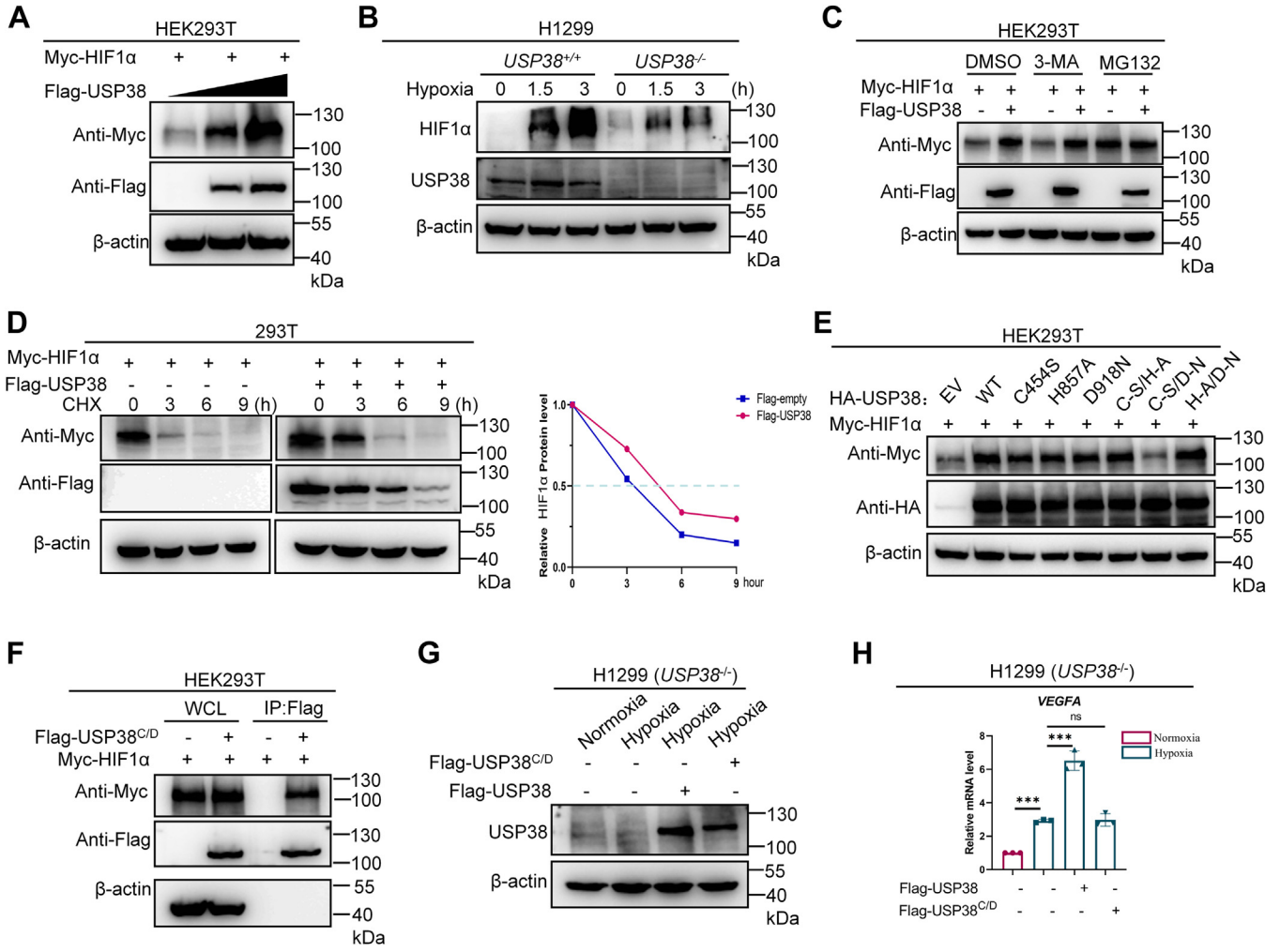

Figure 3. USP38 stabilizes and activates HIF1α depending on its enzymatic activity. *A*, immunoblotting analysis (IB) of exogenous Myc-HIF1α protein in HEK293T cells transfected with an increasing amount of Flag-USP38 expression plasmid. *B*, IB of endogenous HIF1α protein in WT or USP38-deficient H1299 ($USP38^{-/-}$ or $USP38^{+/+}$) cells under hypoxia (1% O_2). *C*, IB of exogenous Myc-HIF1α protein in HEK293T cells transfected with Flag empty vector (–) of Flag-USP38 in the presence of inhibitors, 3-MA and MG132. HEK293T cells were transfected with the indicated plasmids for 6 h and then treated with MG132 (20 μM), or 3-MA (100 ng/ml) for 8 h before IB analysis with the indicated antibodies. *D*, IB of exogenous Myc-HIF1α protein in HEK293T cells transfected with Flag empty vector (–) of Flag-USP in the presence of cycloheximide (CHX, 100 ng/ml) for the indicated times. HEK293T cells were transfected with Myc-HIF1α together with Flag empty vector of Flag-USP38. After 18 h, CHX (100 ng/ml) was added to inhibit new protein synthesis, and the cells were harvested at the indicated timepoints for IB analysis. Quantitation of the protein levels is shown on the *right panel*. *E*, IB of exogenous Myc-HIFα expression in HEK293T cells transfected with HA empty vector (EV), HA-USP38 or its enzymatically deficient mutants including $HA\text{-}USP38^{C454S}$ (in which Cys454 is mutated to Ser454), $HA\text{-}USP38^{H857A}$ (in which His857 is mutated to Ala857), $HA\text{-}USP38^{D918N}$(in which Asp918 is mutated to Asn918), $HA\text{-}USP38^{C/H}$ (in which Cys454 is mutated to Ser454 and His857 is mutated to Ala857), $HA\text{-}USP38^{C/D}$ (in which Cys454 is mutated to Ser454 and Asp918 is mutated to Asn918) or $HA\text{-}USP38^{H/D}$ (in which His857 is mutated to Ala857 and Asp918 is mutated to Asn918). *F*, co-IP analysis of HIF1α interacting with enzymatically deficient mutant of USP38, $USP38^{C/D}$. *G*, IB of exogenous HA-USP38 or its enzymatically deficient mutant expression in USP38-deficient H1299 cells. H1299 ($USP38^{-/-}$) cells that were transfected Flag empty vector (–), Flag-USP38 or its enzymatically deficient ($USP38^{-/-}$) mutant, $Flag\text{-}USP38^{C/D}$, and then cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. *H*, qRT-PCR analysis of *VEGFA* mRNA in *USP38*-deficient H1299 cells transfected Flag empty vector, Flag-USP38, or its enzymatically deficient mutant $Flag\text{-}USP38^{C/D}$, and then cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. ns, not significant; $^{****}p < 0.0001$. Data are representative of three independent experiments (mean ± SD of three biological replicates).

independent of PHD/VHL-mediated oxygen-dependent degradation of HIF1α.

We further confirmed that HIF1α was much higher in wild-type H1299 cells ($USP38^{+/+}$) than in USP38-deficient H1299 cells ($USP38^{-/-}$) under hypoxia (Fig. 3*B*). Addition of MG132 (a proteasome inhibitor) (35) but not of 3-MA (an autophagy inhibitor) (36) counteracted the enhancing role of USP38 on HIF1α stability (Fig. 3*C*), suggesting that USP38 prevents proteasomal degradation of HIF1α. Treatment with cycloheximide (CHX) indicated that USP38 significantly suppressed HIF1α degradation (Fig. 3*D*).

To determine whether USP38-mediated HIF1α stabilization is dependent on the deubiquitinase activity of USP38, we used the enzymatically inactive mutants for subsequent assays (37–39). The double mutant, USP38-C454S/D918N, failed to cause HIF1α stabilization when overexpressed (Fig. 3*E*), but it could interact with HIF1α (Fig. 3*F*). In the *USP38*-deficient H1299 cell, reconstitution of wild-type *USP38* dramatically upregulated *VEGFA* expression, but reconstitution of the mutant, USP38-C454S/D918N, did not do so (Fig. 3, *G* and *H*).

We then determined whether USP38 deubiquitinated HIF1α. Ubiquitination assays showed that overexpression of

USP38 significantly reduced polyubiquitination of HIF1α (Fig. 4*A*). We further confirmed that USP38 removed K11-linked polyubiquitin chains from HIF1α (Fig. 4, *B–D*). *In vivo* ubiquitination assays revealed that polyubiquitination of HIF1α was significantly increased in *USP38*-deficient (*USP38*$^{-/-}$) H1299 cells compared to that in wild-type (*USP38*$^{+/+}$) H1299 cells (Fig. 4*E*). Furthermore, *in vitro* ubiquitination assays showed that the wild-type USP38, but not the enzymatically inactive mutant, catalyzed the deubiquitination of HIF1α with either wild-type ubiquitin or K11-only ubiquitin-linked polyubiquitin chains (Fig. 4, *F* and *G*).

We found that USP38 stabilized the C-terminus (amino acids 331–826) of HIF1α, but not the N-terminus of HIF1α (Fig. 5*A*). Based on a ubiquitination database (https://www.proteomicsdb.org/), six lysine residues (K377, K391, K538, K682, K709 and K769) in the C-terminus of HIF1α are potentially ubiquitinated (Fig. 5*B*). We then examined the effect of USP38 on these six mutants with lysine mutated to arginine of HIF1α (K377R, K391R, K538R, K682R, K709R and K769). Ectopic expression of *USP38* still stabilized the K377R, K391R, K538R, K682R, and K709R mutants, but not the K769R mutant (Figs. 5*C* and S6). We further confirmed that overexpression of *USP38* did not remove either wild-type ubiquitin or K11-only ubiquitin-linked polyubiquitin chains of the HIF1α mutant, HIF1α-K769R, in contrast to that of wildtype HIF1α (Fig. 5, *D* and *E*).

Notably, the lysine 769 is evolutionarily conserved (Fig. 5*F*). In addition, K769 is closed to N803 of HIF1α. However, it appeared that USP38 did not affect hydroxylation of N803-mediated by FIH as FIH interacted with HIF1α^{K769R} similarly to wild-type HIF1α (Fig. S7). Interestingly, the enzymatic activation sites (C454 and D918) of USP38 are conserved in vertebrates, but the deubiquitinated lysine of HIF1α by USP38 seems to be conserved only in mammals (Fig. S8). Thus, USP38 might mainly regulate HIF1α in mammals, but not in lower vertebrates, which is worth further investigation. In addition, USP38 was not regulated by hypoxia (Fig. S9).

Taken together, these data suggest that USP38 acts as a deubiquitinase to remove K11-linked polyubiquitin chains from HIF1α at lysine 769.

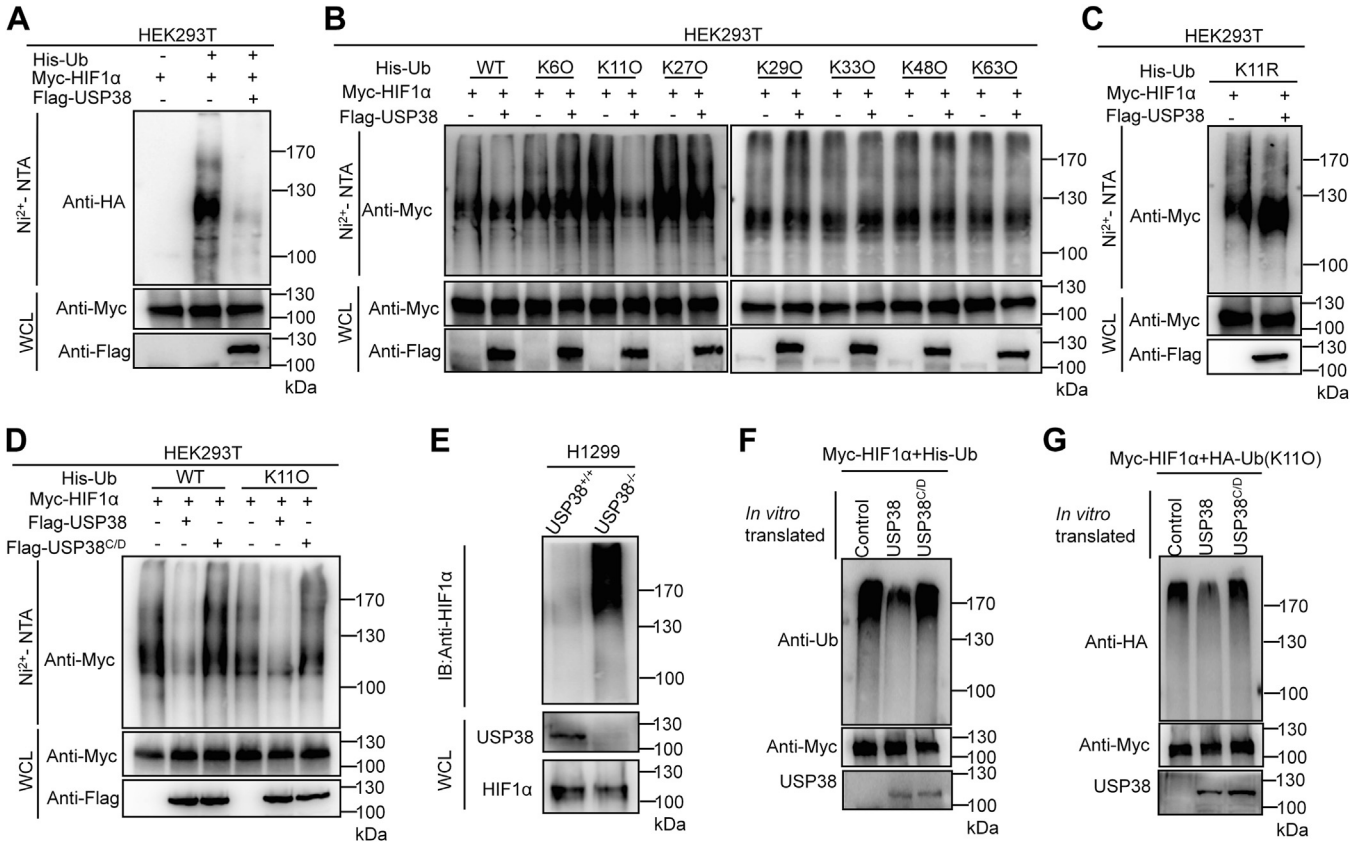

Figure 4. USP38 removes K11-linked polyubiquitin chains of HIF1α. *A*, IB of HIF1α polyubiquitination in HEK293T cells in the absence (–) of presence (+) of USP38. HEK293T cells were transfected with the indicated plasmids for 18 h and then harvested for ubiquitination assays with the indicated antibodies. *B*, USP38 removed K11-linked polyubiquitin chains of HIF1α. HEK293T cells were transfected with the indicated plasmids for 18 h and then harvested for ubiquitination assays with the indicated antibodies. WT, wild-type ubiquitin; K6O, all lysine residues of ubiquitin are mutated to arginine residues except Lys6; K11O, all lysine residues of ubiquitin are mutated to arginine residues except Lys11; K27O, all lysine residues of ubiquitin are mutated to arginine residues except Lys27; K29O, all lysine residues of ubiquitin are mutated to arginine residues except Lys29; K33O, all lysine residues of ubiquitin are mutated to arginine residues except Lys33; K48O, all lysine residues of ubiquitin are mutated to arginine residues except Lys48; K63O, all lysine residues of ubiquitin are mutated to arginine residues except Lys63. *C*, USP38 could not remove K11R-lined polyubiquitin chains of HIF1. K11R, only Lys11 of ubiquitin is mutated to Arg11. *D*, USP38 but not the USP38$^{C/D}$ mutant removed K11-linked polyubiquitin chains of HIF1α. *E*, IB of endogenous HIF1α ubiquitination in wild-type or USP38-deficient H1299 (*USP38*$^{+/+}$ or *USP38*$^{-/-}$) cells cultured under normoxia (1% O_2) for 4 h. *F* and *G*, *in vitro* ubiquitination assay of HIF1α in the presence of wild-type USP38 or the USP38$^{C/D}$ mutant. WCL, whole cell lysate.

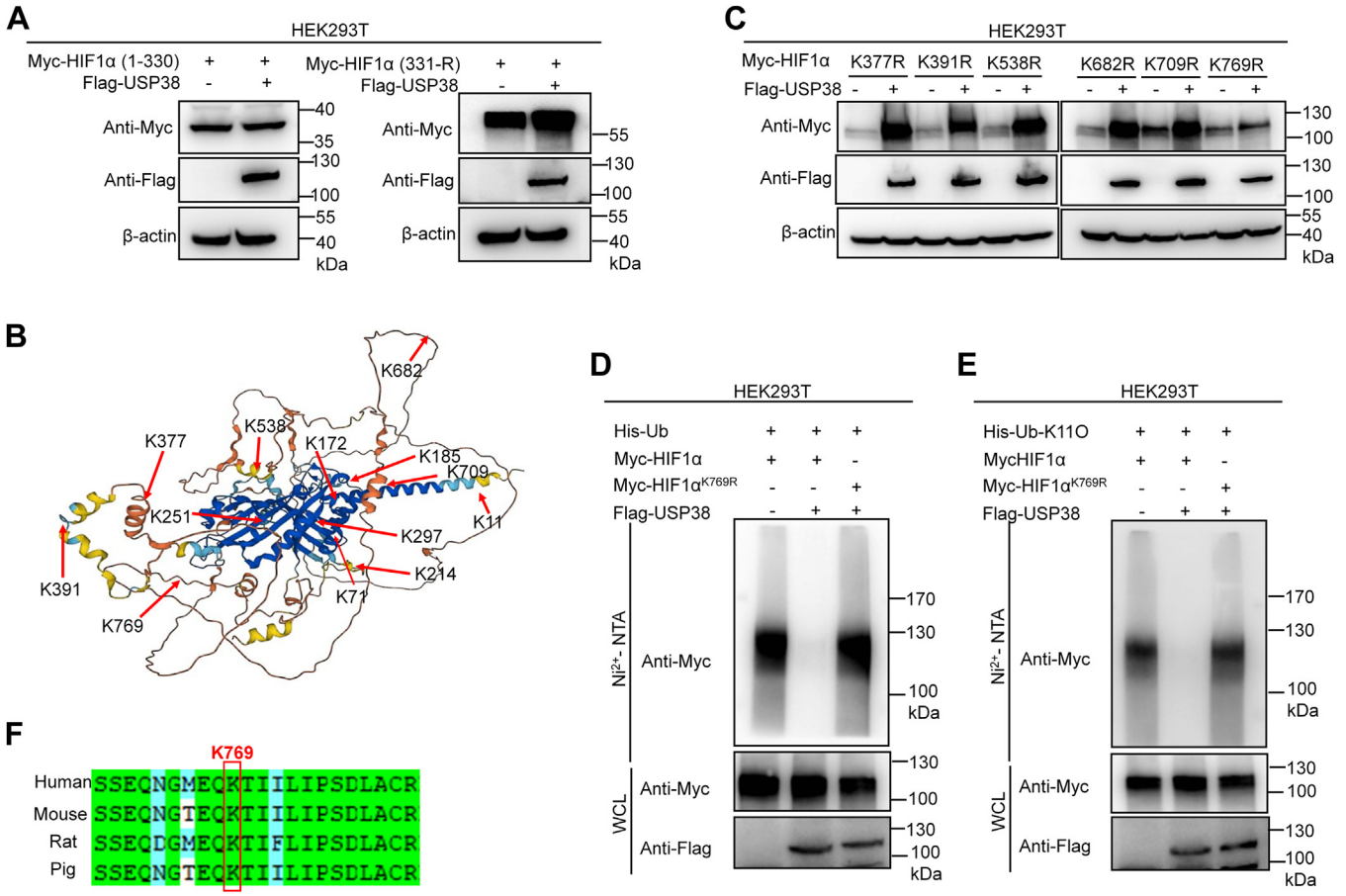

Figure 5. USP38 deubiquitinates HIF1α at Lys769. *A*, IB of exogenous expression of the N-terminus or the C-terminus of HIF1α in HEK293T transfected with Flag empty vector (–) or Flag-USP38 (+). *B*, the potential ubiquitinated lysine residues of HIF1α in the database (https://www.proteomicsdb.org/). *C*, IB of exogenous expression of HIFα mutants in HEK293T transfected with Flag empty vector (–) or Flag-USP38 (+). *D*, IB of ubiquitination of HIF1α in the absence (–) or presence (+) of USP38, or HIF1α^{K769R} in the presence (+) of USP38. *E*, IB of K11-linked ubiquitination of HIF1α in the absence (–) or presence (+) of USP38, or HIF1α^{K769R} in the presence (+) of USP38. HEK293T cells were transfected with the indicated plasmids for 18 h and then harvested for ubiquitination assays (*D* and *E*). *F*, amino acid sequence alignment of partial HIF1α from human (ENSP00000338018), pig (ENSSSCP00000050717), mouse (ENSMUSP00000021530), and rat (ENSRNOP00055025897). (*B*) WCL, whole cell lysate.

Loss of USP38 leads to accumulation of cellular ROS and increased cell apoptosis

We then investigated the biological function of USP38 in influencing hypoxia signaling. HIF1α has been shown to suppress cellular ROS and subsequent ROS-induced consequences (40, 41). Therefore, we investigated whether *USP38* could affect cellular ROS. Under hypoxia, intracellular and mitochondrial ROS levels were significantly higher in *USP38*$^{-/-}$ H1299 cells than in *USP38*$^{+/+}$ H1299 cells (Fig. 6*A*).

Of note, the increased cellular ROS resulted from the knockdown of HIF1α, which causes cell apoptosis (42). We then investigated whether *USP38* could affect cell apoptosis. Under hypoxia, cell apoptosis was much higher in *USP38*$^{-/-}$ H1299 cells than in *USP38*$^{+/+}$ H1299 cells, as revealed by flow cytometric analysis and fluorescence microscopic staining (Fig. 6, *B* and *C*).

To determine whether the effect of *USP38* on cell apoptosis is dependent on HIF1α, we blocked HIF1α activation by adding an inhibitor of HIF1α, PX478 (43). When HIF1α was blocked by PX478, no difference in cell apoptosis was detected between *USP38*$^{+/+}$ and *USP38*$^{-/-}$ H1299 cells under hypoxia (Fig. 6, *D* and *E*). We further knocked down HIF1α in H1299 cells by siRNA (Fig. 6*F*). Similar to that of PX478 addition, when HIF1α was knocked down by siRNA, no difference in cell apoptosis was observed between *USP38*$^{+/+}$ and *USP38*$^{-/-}$ H1299 cells under hypoxia (Fig. 6*G*).

These data suggest that *USP38* may enhance hypoxia signaling to influence cellular ROS and subsequent cell apoptosis dependent on HIF1α.

Discussion

Although the PHD/VHL system represents a major regulatory mechanism for HIFα protein stability under normoxia, other ways to regulate HIFα stability under either normoxia or hypoxia have been defined (2, 6, 7, 44, 45). As an essential post-translational modification of proteins, deubiquitination has been shown to control HIFα subunit abundance, resulting in either enhancement or repression of hypoxia signaling (6, 8, 10, 11, 27–29, 46). Of note, USP37 specifically deubiquitinates HIF2α but not HIF1α (29). Cezanne (OTUD7B)

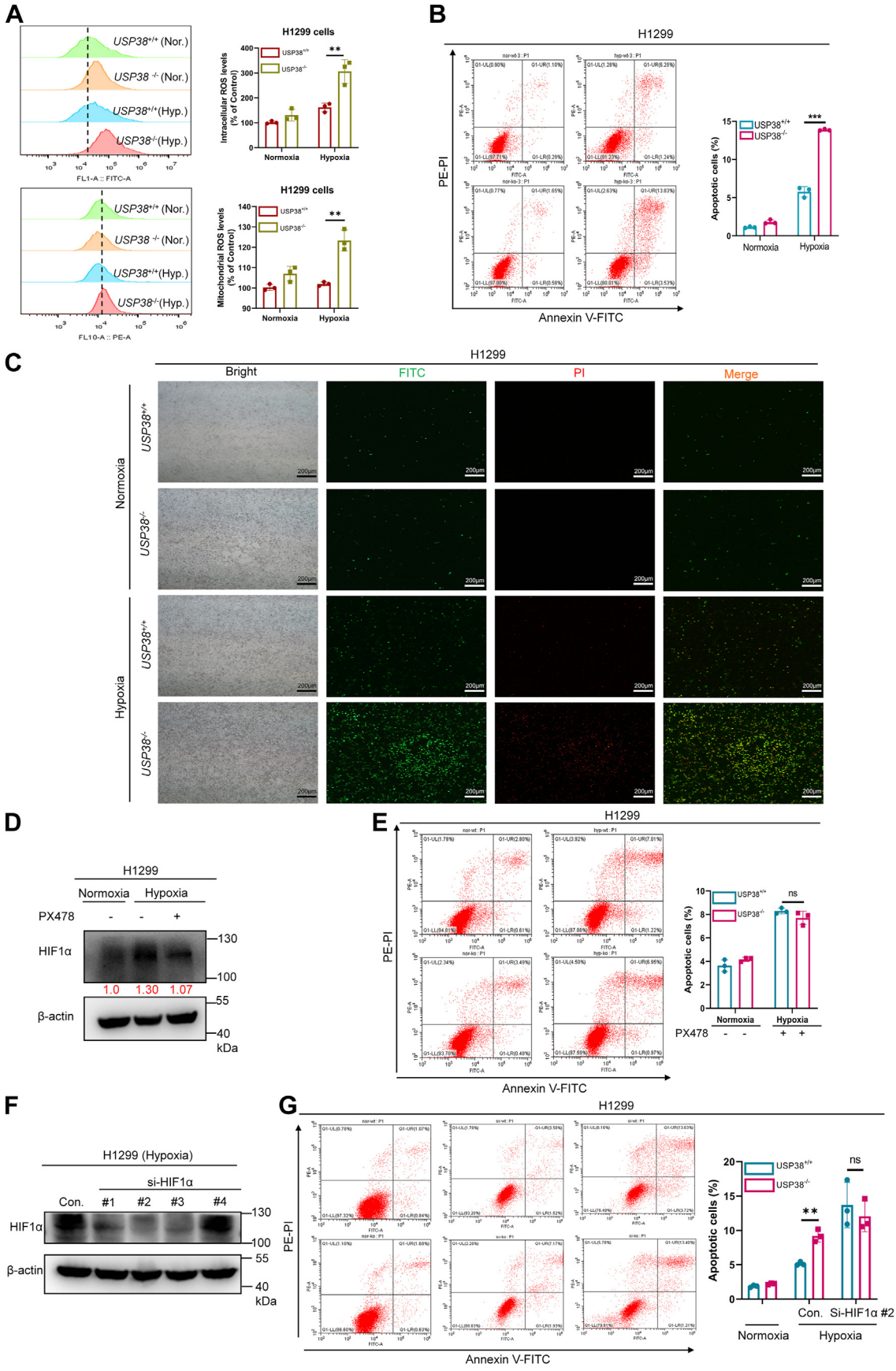

Figure 6. Loss of USP38 leads to accumulation of cellular ROS and increased cell apoptosis. *A*, intercellular ROS (*top panels*) and mitochondrial ROS (*bottom panels*) were accumulated in *USP38* deficient H1299 cells ($USP38^{-/-}$) compared to those in wild-type H1299 cells ($USP38^{+/+}$) under hypoxia. *B*, cell apoptosis was increased in *USP38* deficient H1299 cells ($USP38^{-/-}$) compared to that in wild-type H1299 cells ($USP38^{+/+}$) under hypoxia as revealed by flow

regulates HIF1α in a proteasome-independent manner (46). Here, we found that USP38 removed K11-linked polyubiquitin chains of HIF1α at Lys769, resulting in stabilization of HIF1α and subsequent transactivation under hypoxia. Although the corresponding E3 ligase for catalyzing the K11-linked polyubiquitination of HIF1α at Lys769 is still unknown, our data suggest that this modification may be important for modulating HIF1α function particularly under hypoxia. Of note, the K11-linked polyubiquitination and the modification of HIF1α at Lys769 have hardly been reported for HIF1α. Our results may reveal a unique modulation for HIF1α activation. However, the underlying mechanisms are still poorly understood and need to be further explored.

In this study, we found that knockout of *USP38* resulted in the accumulation of ROS and increased cell apoptosis. However, based on the gene regulation, the hypoxia-induced cell apoptosis-related gene, *BNIP3*, should be downregulated after *USP38* knockout (47, 48), so, these seemed to be contradictory. However, as previously reported, HIF1α-BNIP3-mediated mitophagy can decrease mitochondrial ROS (40), which is consistent with the increase in ROS after *USP38* knockout observed in this study. Indeed, HIF1α-BNIP3-mediated mitophagy has also been shown to attenuate apoptosis by inhibiting the NLPR3 inflammasome (49).

Although we provide evidence to support that *USP38* can enhance hypoxia signaling by stabilizing HIF1α, the physiological relevance of this modulation is still largely unknown due to the lack of animal models. Further investigation of the role of *USP38* in modulating hypoxia signaling *in vivo* will provide insight into the physiological function of *USP38* and the underlying molecular mechanisms.

Experimental procedures

Cell culture

HEK293T and H1299 cells originally purchased from ATCC (www.atcc.org) were cultured in Dulbeccos' modified Eagle's medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS). HEK293T cels and H1299 cells were grown at 37 °C in a humidified incubator containing 5% CO_2.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from cells using TransZol Up (#ET111-01, TransGen Biotech) according to the manufacturer's protocol. cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (AE311-03, TransGen Biotech). Gene expression was examined using MonAmp SYBR Green qPCR Mix (high ROX) (#MQ10301S, Monad Biotech). The value obtained for each gene was normalized to that of the 18s RNA gene. Primers are listed in Table S1.

Plasmids

The expression plasmids: His-ubiquitin, His-ubiquitin mutants (including K6O, K11O, K27O, K29O, K33O, K48O, K63O, and K11R), HIF1α, HIF1α mutants, HIF2α, VHL, PHD1, PHD2 and PHD3, have been described previously. The expression plasmids of USP38, and USP38 mutants were constructed by PCR amplification and subcloned into various expression vectors. Primers are listed in Table S1.

Immunoprecipitation and Western blot analysis

Cells were lysed with 1 ml RIPA buffer (containing 1 mM PMSF, 1 mM Na_3VO_4 and a 1:100 dilution of protease inhibitor mixture) for 30 min at 4 °C. After centrifugation at 12, 000 rpm for 10 min, protein concentrations were measured, and equal amounts of lysates were used for co-immunoprecipitation. Co-immunoprecipitation was performed with the indicated beads and antibodies. The precipitants were then washed three times with RIPA buffer, and the immunocomplexes were eluted with sample buffer containing 1% SDS for 10 min at 95 °C. The immunoprecipitated proteins were separated by SDS-PAGE. Western blot analysis was performed using the indicated antibodies. The Fuji Film LAS4000 mini-luminescence image analyzer was used to photograph the blots.

Ubiquitination assay

HEK293T cells were co-transfected with the plasmids expressing Myc-HIF1α, His-ubiquitin or His-ubiquitin mutants, together with Flag-USP38, Flag-USP38 mutant or Flag empty for 18 h and then lysed with lysis buffer A (6 M guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM imidazole) followed by nickel bead purification and immunoblotting with the indicated antibodies.

In vivo *ubiquitination assay*

For the ubiquitination assay in USP38-deficient or wild-type H1299 cells ($USP38^{-/-}$ or $USP38^{+/+}$), the cells were treated with 1% O_2 for 4 h, then harvested, and lysed with the lysis buffer (50 μl). The supernatants were denatured at 95 °C for 5 min in the presence of 1% SDS. The denatured lysates were diluted with lysis buffer to reduce the concentration of SDS (less than 0.1%). Immunoprecipitation (denature-IP) was performed with anti-HIF1α antibody, followed by immunoblotting with anti-polyubiquitin-specific antibody.

In vitro *deubiquitination assay*

Flag-HIF1α, HA-ubiquitin-K11, and His-ubiquitin were co-transfected into HEK293T cells for 18 h. Denaturation-IP was performed and the precipitates were eluted by the addition of 3× FLAG peptide (#SAE0194, Sigma) to obtain ubiquitinated

cytometry analysis. *C*, cell apoptosis was increased in *USP38* deficient H1299 cells ($USP38^{-/-}$) compared to that in wild-type H1299 cells ($USP38^{+/+}$) under hypoxia as detected by fluorescence microscopy. Scale bar = 500 μm. *D*, IB of HIF1α in H1299 cells treated without (–) or with PX478 (10 μM) under hypoxia. *E*, cell apoptosis was no difference between *USP38* deficient H1299 cells ($USP38^{-/-}$) and wild-type H1299 cells ($USP38^{+/+}$) under hypoxia when HIF1α was inhibited by PX478 as revealed by flow cytometry analysis. *F*, IB of HIF1α in H1299 cells treated without (Con.) or with HIF1α siRNA oligos. *G*, cell apoptosis was no difference between *USP38* deficient H1299 cells ($USP38^{-/-}$) and wild-type H1299 cells ($USP38^{+/+}$) under hypoxia when HIF1α was knocked down by siRNA oligos as revealed by flow cytometry analysis. $^{**}p < 0.01$, $^{***}p < 0.001$. Data are representative of three independent experiments (mean ± SD of three biological replicates).

HIF1α. USP38 was obtained using a coupled *in vitro* transcription/translation kit (#L2080, Promega). The ubiquitinated HIF1α was incubated with *in vitro* synthesized proteins at 37 °C for 2 h followed by an overnight incubation at 16 °C in the presence of 1 μM ATP. The mixture was analyzed by immunoblotting with the indicated antibodies.

Hypoxia treatment

The Ruskinn INVIVO$_2$ 400 workstation was used to treat the cells with hypoxia. Prior to the experiment, the O_2 concentration was adjusted to the appropriate value (1%). H1299 cells were then transfected with plasmids and placed in the workstation for the indicated times.

Detection of apoptotic cells

H1299 cells were cultured under hypoxia for the indicated times. For flow cytometry analysis, the cells were harvested and stained with FITC Annexin V and PI using the FITC Annexin V Apoptosis Detection Kit I (#556547, BD Pharmingen) according to the manufacturer's instructions. Apoptotic cells were detected using Beckman CytoFLEXS, and the data were analyzed using CytExpert software. In addition, the cells were stained with Annexin V-FITC Apoptosis Detection Kit (#C1062, Beyotime) according to the manufacturer's instructions in a 6-well plate and imaged under a Nikon TE2000-U fluorescence microscope.

Measurement of intracellular and mitochondrial ROS levels

H1299 cells were cultured under hypoxia as indicated. After hypoxia treatment, H1299 cells were harvested and washed with PBS. The cells were then incubated in PBS solution containing 5 μM of CM-H2DCFDA (for intracellular ROS) (#C6827. Thermo Fisher Scientific) or 5 μM of 5MitoSOX Red (for mitochondrial ROS) (# M36008, Thermo Fisher Scientific) for 10 min at 37 °C and then gently washed three times with PBS, followed by flow cytometric analysis.

CRISPR-Cas9 knockout cell lines

To generate H1299 knockout cell lines, the double-stranded oligonucleotides corresponding to the target genes were cloned into LentiCRISPRv2 plasmid and then co-transfected with viral packaging plasmids into HEK293T cells grown in 6-well plates. One day after transfection, the cell culture medium was removed and fresh culture medium was added to the plates. Two days later, the viruses were harvested and used to infect H1299 cells in the presence of polybrene (8 μg/ml). Infected cells were selected with puromycin (1 μg/ml) for 2 weeks.

siRNA-mediated gene knockdown

To knockdown of HIF1α in H1299 cells, four siRNA oligos targeting HIF1α were synthesized in GenePharma Company (Shanghai, China). The sense sequences of four siRNA oligos are: #1, 5′- GUUGCCACUUCCACAUAAUTT-3′; #2, 5′-CCG UAUGGAAGACAUUAAATT-3′; #3, 5′-CAGGCCACA UUCACGUAUATT-3′; and #4, 5′- GUCGCUUUGAGU-CAAAGAATT-3′. The RNA oligos were transfected into H1299 cells respectively using normal transfection reagent and HIF1α protein level was detected after transfected for 48 to 72 h.

Antibodies and chemical reagents

Anti-USP38 antibody (#17767-1-AP) was purchased from Proteintech. Antibodies including anti-HIF1α (#36169), anti-VHL (#68547), anti-ARNT (#5537), anti-Ubiquitin (#3936), and normal rabbit IgG (#2729) were purchased from Cell Signaling Technology. Anti-ACTB antibody (#AC026) was purchased from ABclonal. Anti-HA antibody (#901515) was purchased from Covance. Anti-Myc antibody (#SC-40) was purchased from Santa Cruz Biotechnology. Image-iT Green Hypoxia Reagent (#I14834) was purchased from Thermo Fisher Scientific. CoCl2 (#C8661), MG-132 (#474790), anti-Flag antibody (#F1804) and Anti-Flag antibody-conjugated agarose beads (#A2220) were purchased from Sigma. Protein G Sepharose (#17-0618-01) was purchased from GE HealthCare Company. PAX478 (#S7612) was purchased from Selleck.

Validation of cellular hypoxia

Cells were plated in a 6-well plate and incubated overnight at 37 °C. The Image-iT Hypoxia Reagent stock solution was added to the plate at a final concentration of 1 to 10 μM and cells were incubated the cells at 37 °C for 30 min. The medium was replaced with fresh medium. The cells were then transferred to in a regular incubator (21% O_2) or a hypoxia workstation to incubate for 2 to 4 h. Images were observed and photographed using a Nikon TE2000-U fluorescence microscope.

Statistical analysis

Data are presented as the mean ± SD of three technical replicates. Differences between experimental and control groups were determined by Student's t test (when comparing two groups of data) or by two-way ANOVA analysis (when comparing more than two groups of data). Statistical analyses were performed using GraphPad Prism8 software. ns, not significant ($p > 0.05$); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

Data availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by Wuhan Xiao.

Supporting information—This article contains supporting information.

Acknowledgments—We are grateful to Drs Peter J. Ratcliffe, William Kaelin, Amato Giaccia, Eric Huang, Navdeep Chandel and Bo Zhong for the generous gifts of reagents.

Author contributions—R. W., X. C., X. L., and W. X. conceptualization; R. W., X. C., X. L. methodology; R. W., X. C. data curation; R.

W. and W. X. writing–original draft preparation; R. W., X. C., J. L., X. L., J. W. visualization; R. W., X. C., J. L., X. L., J. W. investigation. W. X. supervision, W. X. validation, W. X. writing–reviewing and editing.

Funding and additional information—NSFC [31830101 and 31721005 to W. X]; The Strategic Priority Research Program of the Chinese Academy of Sciences [XDA24010308 to W. X.]; the National Key Research and Development Program of China [2018YFD0900602, to W. X.]. Funding for open access charge: NSFC [31830101 to W. X.].

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: DUB, deubiquitylating enzymes; PTM, post-translational modifications; USP, ubiquitin-specific proteases.

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