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Quantitative analyses for effects of neddylation on CRL2$^{\text{VHL}}$ substrate ubiquitination and degradation

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Abstract

Through catalyzing the ubiquitination of key regulatory proteins, cullin-RING ubiquitin ligases (CRLs) play essential biological roles and their activities are controlled by multiple mechanisms including neddylation, the conjugation of NEDD8 to cullins. Upon neddylation, a CRL, such as the CUL1-based CRL1, undergoes conformational changes that accelerate substrate ubiquitination. Given the structural diversity across subfamilies of CRLs and their substrates, to what extent neddylation modulates the activity of individual CRLs remains to be evaluated. Here, through reconstituting the CRL2 ubiquitination reaction in vitro, we showed that neddylation promotes CRL2$^{\text{VHL}}$-dependent degradation of both full-length HIF1α and the degron peptide of HIF1α, resulting in more than 10-fold increase in the rate of substrate ubiquitination. Consistently, pevonedistat (also known as MLN4924), an inhibitor of neddylation, inhibits the degradation of HIF1α in RCC4 cells stably expressing VHL in cycloheximide chase assays. However, such inhibitory effect of pevonedistat on HIF1α degradation was not observed in HEK293 cells, which was further found to be due to CRL2$^{\text{VHL}}$-independent degradation that was active in HEK293 but not RCC4 cells. After truncating HIF1α to its Carboxy-terminal Oxygen-Dependent Degradation (CODD) domain, we showed that pevonedistat inhibited the degradation of CODD and increased its half-life by six-fold in HEK293 cells. Our results demonstrate that neddylation plays a significant role in activating CRL2, and the cellular activity of CRL2$^{\text{VHL}}$ is better reflected by the degradation of CODD than that of HIF1α, especially under conditions where CRL2-independent degradation of HIF1α is active.

KEYWORDS

CODD, CRL2 ubiquitin ligase, neddylation, protein degradation, ubiquitination

1 | INTRODUCTION

Ubiquitination, one of the most important posttranslational modifications, is catalyzed by sequential enzyme reactions involving E1 ubiquitin (Ub) activating enzymes, E2 conjugating enzymes, and E3 ligases.[1,2] As the largest family of E3 ligases, cullin-RING ligases (CRLs) ubiquitinate ~20% of cellular proteins degraded by the

ubiquitin-proteasome system,[3] and aberrant regulations of CRLs have been found in various diseases, which provides potential therapeutic targets.[4] A CRL complex typically comprises four subunits, including a cullin, a RING protein, an adaptor protein, and an interchangeable substrate receptor.[5] Specifically, CUL2 forms a functional E3 ligase, referred to as CRL2, with the RING protein RBX1, the Elongin B/C adaptor complex, and a substrate receptor.[6,7] VHL is the most well-known substrate receptor for CRL2, and its mutation has been implicated in cancers.[8] One of the canonical substrates of CRL2^{VHL} is the hypoxia-inducible factor 1α subunit (HIF1α), a key transcription factor responsible for cellular oxygen sensing.[9] Under normoxic conditions, HIF1α is hydroxylated by prolyl-hydroxylase (PHD) and then recognized by CRL2^{VHL} for ubiquitination and degradation. Under hypoxia, PHD is inhibited so that HIF1α accumulates and translocates to the nucleus.

To properly maintain protein homeostasis in cells, CRLs are tightly regulated by orchestrated pathways.[10–15] Posttranslational modification of cullin proteins by NEDD8, termed cullin neddylation, represents one of the most important mechanisms. Similar to the process of ubiquitin conjugation, NEDD8 is covalently conjugated to the C-terminal WHB domain of cullin proteins through a cascade of enzymes comprising E1 NEDD8-activating enzyme (NAE), E2 NEDD8-conjugating enzyme, and E3 NEDD8 ligase.[16] Biochemical and structural studies have demonstrated that cullin neddylation promotes the activity of CRLs, leading to increased rates of substrate ubiquitination.[17–23] Interestingly, a recent CRISPR/Cas9 screen for mutations that reduce the small molecule-induced degradation of CRL neo-substrates identified COP9 signalosome, an enzyme that reverses NEDD8 modification, as being required for CRL4-based but not CRL2-based degraders.[24] This finding suggests that neddylation may exert a different role in CRL2 regulation. We thus decided to establish quantitative assays to evaluate the effect of cullin neddylation on CRL2 activities.

2 | RESULTS

We firstly developed a cycloheximide (CHX) chase assay to study the degradation of HIF1α, the well-known natural substrate of CRL2^{VHL}. We treated HEK293 cells with desferrioxamine (DFX), an inhibitor of prolyl hydroxylase,[25] to increase the basal level of HIF1α so it could be robustly detected by Western blot (WB). After washing off DFX, cells were treated with CHX for various periods of time and were harvested in SDS sample buffer. To access the effect of cullin neddylation on HIF1α degradation, we performed the CHX chase assay in the

absence and presence of pevonedistat, a small molecule inhibitor of NAE.[26] As expected, the pevonedistat treatment eliminated neddylated CUL2, but surprisingly, the degradation of HIF1α was marginally affected in the absence of cullin neddylation (Figure 1a). We repeated the CHX chase assay using HEK293 Flp-In T-REX cells that express exogenous ^{FLAG}HIF1α, and similarly, the degradation of ^{FLAG}HIF1α was almost unaffected by the pevonedistat treatment (Figure 1b).

We rationalized two potential explanations for these unexpected results. First, the ubiquitination of HIF1α may not heavily rely on CUL2 neddylation. Because neddylation induces a conformational change in the cullin CTD and RBX1,[20,21] which potentially closes a gap between the CRL substrate binding site and the E2 ∼ Ub bound to RBX1[5] and thus promotes substrate ubiquitination,[20,21] it is possible that a large substrate like HIF1α could undergo efficient ubiquitination by bridging the gap without the help of neddylation. Alternatively, it is possible that the degradation of HIF1α in HEK293 cells in the CHX chase assay was primarily cullin independent.

We tested the first possibility by comparing the rate of HIF1α ubiquitination with or without CUL2 neddylation in vitro. To obtain full-length HIF1α protein, we expressed ^{FLAG}HIF1α in human RCC4 cells that naturally lack VHL and accumulate HIF1α in normoxia (Figure S1a,b),[9] extracted the ^{FLAG}HIF1α through immunoprecipitation and quantified its concentration by WB using recombinant ^{FLAG}IκBα as a standard (Figure S1c). The purified ^{FLAG}HIF1α was then mixed with recombinant CRL2^{VHL} for in vitro ubiquitination. As a control, we also included a peptide substrate derived from the amino-terminal oxygen-dependent degradation (NODD) motif of HIF1α[27] that was used in our previous study.[28] As shown in Figure 2a,b, ubiquitination of full-length ^{FLAG}HIF1α (∼110 kDa) and the degron peptide (∼4 kDa) was observed while reactions proceeded over time, only in the presence of CUL2. Importantly, the ubiquitination of both ^{FLAG}HIF1α and the degron peptide was accelerated by CUL2 neddylation, with an average 13- and 11-fold increase in modification rate, respectively (Figure 2a–c). These results clearly show that, at least in vitro, neddylation promotes the CRL2^{VHL}-dependent substrate ubiquitination regardless of the substrate sizes. Following this experiment, we repeated the CHX chase assay for HIF1α degradation in RCC4 cells lacking VHL (VHL null) and RCC4 cells stably expressing VHL (+VHL) (Figure S1a). We firstly found that HIF1α disappeared only in RCC4 (+VHL) cells but not RCC4 (VHL null) cells (Figure 2d, upper left), suggesting that CRL2^{VHL}-dependent ubiquitination is the primary, if not only, mechanism for HIF1α degradation in these cells. We then asked if pevonedistat affects the degradation of
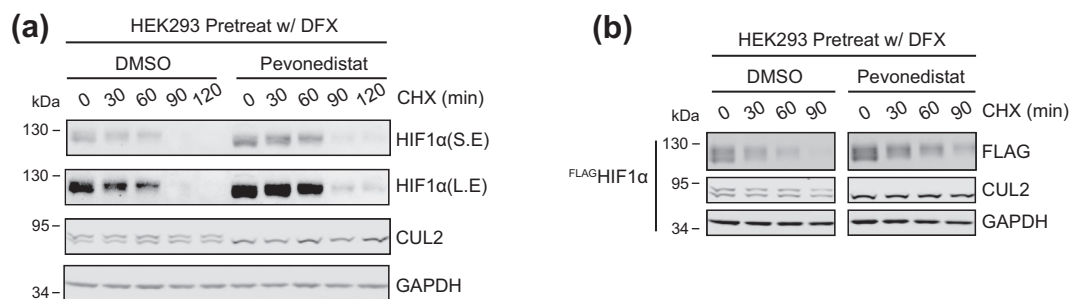
FIGURE 1 Eliminating cullin neddylation failed to inhibit the degradation of HIF1α in HEK293 cells. (a) HEK293 cells were pretreated with DFX for 5 h and pevonedistat or equivalent volume of DMSO for 1 h. After washout of inhibitors, cells were treated by CHX with or without pevonedistat for indicated time and were collected for WB analyses. (b) same as in (a) except that HEK293 cells containing tetracycline induced ^{FLAG}HIF1α were used

HIF1α in RCC4 cells in the CHX chase assay. In contrast to results from HEK293 cells, pevonedistat inhibited the degradation of HIF1α in RCC4 (+VHL) cells and exhibited no effects on HIF1α levels in RCC4 (VHL null) cells (Figure 2d, right panel). Consistently, the degradation of exogenous ^{FLAG}HIF1α in RCC4 (+VHL) cells was also inhibited by pevonedistat (Figure 2e). These results, together with results from the in vitro assays, demonstrate that cullin neddylation is required for CRL2^{VHL}-dependent degradation of HIF1α.

The above findings also point out that the degradation of HIF1α in HEK293 cells observed in Figure 1 was likely due to cullin-independent activities. To test this possibility, we treated HEK293 cells with VH-298, a potent inhibitor for the VHL-HIF1α interaction.[29] The VH-298 treatment efficiently stabilized ^{FLAG}HIF1α, suggesting successful inhibition of the VHL-HIF1α interaction (Figure 3a). However, in the CHX chase assay, ^{FLAG}HIF1α disappeared both in the absence and presence of VH-298 (Figure 3b), indicating that HIF1α was primarily degraded through CRL2^{VHL}-independent pathways. As a result, the degradation of HIF1α in this assay did not reflect the activity of CRL2. We then hypothesized that, through truncating HIF1α, it is possible to remove motifs for cullin-independent degradation and generate a substrate that depends on CRL2^{VHL} for ubiquitination and degradation in HEK293 cells.

Essential to the binding of HIF1α to VHL is the hydroxylation of Pro402 and Pro564 in the ODD domain[30] (Figure 3c). We expressed the carboxy-terminal ODD (CODD) domain (Figure 3c, S2) in HEK293 Flp-In T-REx cells and monitored its degradation in the CHX chase assay. Like ^{FLAG}HIF1α, ^{FLAG}CODD disappeared rapidly after the addition of CHX (Figure 3d, left panel). The pevonedistat treatment increased the half-life ($t_{1/2}$) of ^{FLAG}HIF1α by ~50% (Figure 3d, e). In contrast and consistent with results in Figure 2, inhibition of neddylation led to much slower degradation of

^{FLAG}CODD (Figure 3d), with an average 5.8-fold increase in $t_{1/2}$ (Figure 3e). Notably, only a lower molecular weight band, representing the hydroxylated ^{FLAG}CODD[31] (Figure 3f), persisted in pevonedistat-treated cells, while the upper band representing the nonhydroxylated ^{FLAG}CODD (Figure 3F) disappeared at a rate similar to that in cells with no pevonedistat (Figure 3d). These results suggest that the pevonedistat treatment does not change the rate at which CODD is hydroxylated, and once hydroxylated, CODD is quickly ubiquitinated and degraded in the presence of ongoing CUL2 neddylation. Taken together, we conclude that the degradation of CODD serves as a better reporter for CRL2^{VHL} activity than that of full length HIF1α in HEK293 cells, one of the most heavily used cell lines in life sciences.

3 | DISCUSSION

Biochemical and structural studies have provided important insights into the impact of neddylation on the activity and conformation of CRLs such as CRL1 and CRL5.[17–20,32–34] Given that various substrate receptor modules and substrates are engaged by CRLs,[3,35,36] considerable structural diversity across CRLs exists, and therefore, to what extent neddylation modulates the activity of individual CRLs remains an open question. Here, we developed quantitative assays to study the effect of neddylation on CRL2^{VHL}-mediated ubiquitination and degradation. Using RCC4 (+VHL) cells as the experimental model, we found that cullin neddylation promoted the CRL2^{VHL}-dependent degradation of HIF1α. Using in vitro assays, we showed that regardless of significant difference in substrate sizes, neddylation resulted in >10-fold increase in rates of CRL2^{VHL} substrate ubiquitination, an effect consistent with previous findings using CRL1.[33,34]

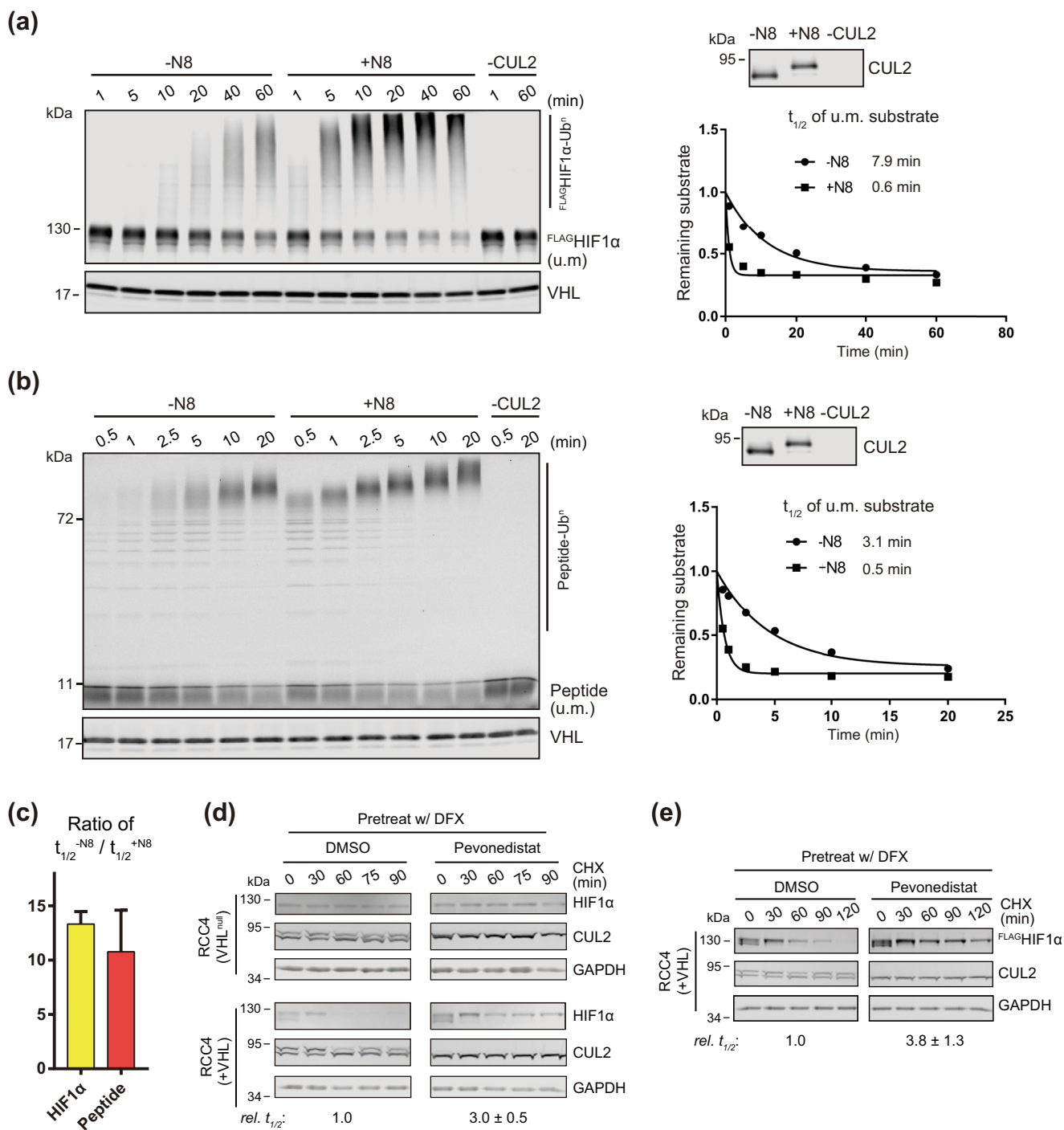Unexpectedly, our CHX chase assay revealed little effect of neddylation inhibition on the degradation rate of HIF1α

FIGURE 2 Neddylation is required for efficient CRL2^{VHL}-dependent degradation of HIF1α in vitro and in RCC4 (+VHL) cells. (a,b) Full-length ^{FLAG}HIF1α purified from RCC4 (VHL null) cells stably expressing ^{FLAG}HIF1α (a) or HIF1α degron peptide (b) were ubiquitinated in vitro for indicated time with or without CUL2 neddylation. Samples without CUL2 served as negative controls. Intensities of unmodified (u.m.) ^{FLAG}HIF1α or degron peptide bands were normalized to intensities of VHL bands for regression analyses. WB of CUL2 from each group and regression curves of remaining unmodified substrates were shown on the right. (c) Average relative changes of substrate $t_{1/2}$ by neddylation from experiments in (a,b). Error bars: range of values, $n = 2$. (D) RCC4 (VHL null) and RCC4 (+VHL) cells were pretreated with DFX for 3 h and pevonedistat or equivalent volume of DMSO for 1 h. After washout of inhibitors, cells were treated by CHX with or without pevonedistat for indicated time and were collected for WB analyses. (e) Same assays as performed in (d) with ^{FLAG}HIF1α stably expressed in RCC4 (+VHL) cells. In (d,e), relative $t_{1/2}$ was shown as average ± range of values ($n = 2$)
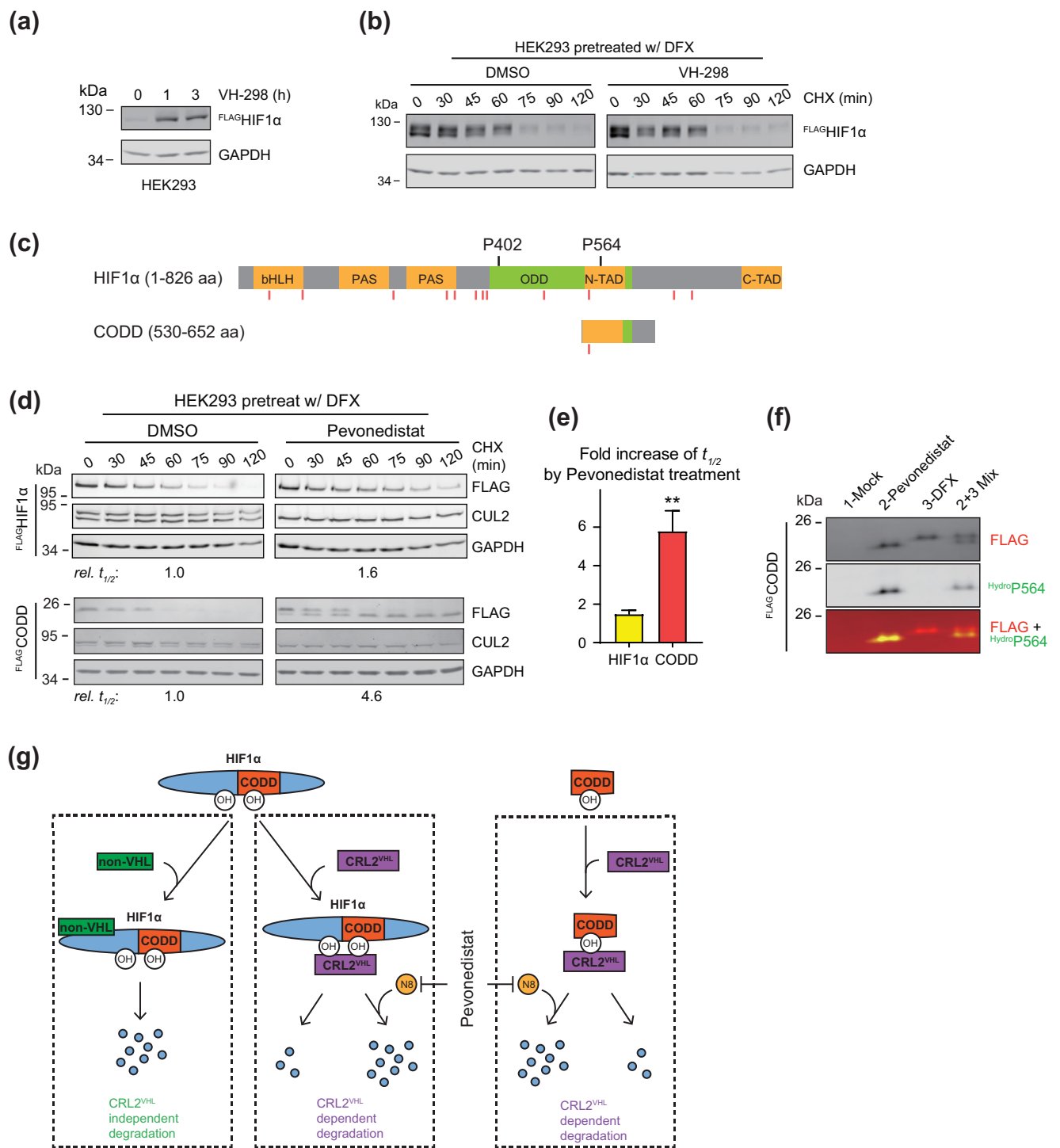
FIGURE 3 The degradation of CODD reports CRL2[VHL] activity in HEK293 cells in which VHL-independent degradation of full-length HIF1α is active. (a) HEK293 cells containing tetracycline induced [FLAG]HIF1α were treated by 100 μM VH-298 for indicated time and were collected for WB analyses. (b) The same cells as in (a) were pretreated with DFX for 5 h and VH-298 or equivalent volume of DMSO for 1 h. After washout of inhibitors, cells were treated by CHX with or without VH-298 for indicated time and were collected for WB analyses. (c) Schematic illustration of the CODD truncation of HIF1α. Red vertical lines denote ubiquitination sites reported in GGBase. (d) Same assays as in Figure 1b with HEK293 cells containing tetracycline induced [FLAG]HIF1α or [FLAG]CODD. Relative $t_{1/2}$ of [FLAG]HIF1α or [FLAG]CODD elimination are shown below the corresponding blot. (e) Average changes in relative $t_{1/2}$ from experiments in (d). Error bars: SEM, $n = 3$, $p < 0.01$. (f) Unmodified (upper band) and hydroxylated (lower band) [FLAG]CODD separated on a 12.5% SDS-PAGE gel. HEK293 cells with tetracycline induced expression of [FLAG]CODD were treated for 4 h with DMSO (Lane 1), or pevonedistat (Lane 2), or DFX (Lane 3), and were collected WB analyses. Equal volumes of samples in Lanes 2 and 3 were mixed and included in Lane 4. The bottom blot shows overlay of signals from FLAG and hydroxy-HIF-1α (Pro564) antibodies. (g) Summary of HIF1α degradation machineries. CRL2[VHL] binds the hydroxylated ODD domain of HIF1α or the hydroxylated CODD truncation to catalyze their ubiquitination and degradation. This CRL2[VHL]-dependent degradation is accelerated by neddylation and can be inhibited by pevonedistat. CRL2[VHL]-independent degradation acts through regions other than the HIF1α CODD domain and cannot be inhibited by pevonedistat

in HEK293 cells. We found this was because the CRL-independent pathway predominated in HIF1α degradation in HEK293 when HIF1α accumulated to a higher level (Figure 3). Consistent with this explanation, VHL-independent degradation has been reported previously,[30,37–41] and pevonedistat failed to increase HIF1α levels in several gastric cancer cell lines that contain higher basal levels of HIF1α.[42] The activity of this CRL-independent HIF1α degradation varies in different cell lines, as it was not detected in RCC4 cells (Figure 2d). In light of these findings, we suggest that when quantifying rates of HIF1α degradation using a CHX chase assay, pevonedistat treatment should be included as a control to access if HIF1α is primarily degraded by the CRL-dependent pathway in the cell line and the experimental condition used. If pevonedistat fails to inhibit HIF1α degradation, indicating CRL-independent degradation predominates, then we suggest using the CODD truncation of HIF1α as an alternative reporter for CRL2^{VHL} activity (Figure 3g). The $t_{1/2}$ of CODD was significantly increased upon neddylation inhibition (Figure 3e), and furthermore, hydroxylated CODD migrated faster than unmodified CODD on a 12.5% SDS-PAGE gel (Figure 3f). These features not only make CODD a relevant model for studying CRL2-dependent protein degradation, but also provide the advantage of monitoring the CODD hydroxylation rate in the same assay.

4 | MATERIALS AND METHODS

4.1 | Generation of stable cell lines

HEK293 Flp-In T-REx cells (Thermo Fisher Scientific) stably integrating ^{FLAG}HIF1α and ^{FLAG}CODD were generated as described previously.[15] The expression of integrated genes were induced by 2 µg/ml tetracycline for 24 h. RCC4 (VHL null) and RCC4 (+VHL) cells (Sigma-Aldrich) overexpressing ^{FLAG}HIF1α were generated via lentivirus-mediated integration as described previously.[15]

4.2 | Antibodies

Antibodies for HIF1 α (Novus, NB100-449), CUL2 (Thermo Fisher, 51–1800), GAPDH (Santa Cruz, sc-47724), FLAG (Sigma-Aldrich, F1804), VHL (Cell Signaling Technology, 68547S), and hydroxy-HIF-1α (Pro564) (Cell Signaling Technology, 3434S) were used in this study. Primary antibodies used were shown next to each blot. All ^{FLAG}HIF1α and ^{FLAG}CODD were blot with FLAG antibody, except that hydroxy-HIF-1α (Pro564) antibody was also used to detect hydroxylated ^{FLAG}CODD (Figure 3f).

4.3 | HIF1α and CODD degradation assay

Cells were grown in 6-well plates and treated with 200 µM DFX for indicated time to stabilize HIF1α, ^{FLAG}HIF1α, or ^{FLAG}CODD. Pevonedistat (10 µM, MedChemExpress), or VH-298 (100 µM, Tocris Bioscience), or DMSO was added to the medium for 1 h and was then washed out. To monitor protein degradation, 60 µg/ml CHX along with Pevonedistat (1 µM) or VH-298 (100 µM) or DMSO was added to the medium. At different time points post the start of the CHX treatment, cells were washed with PBS and lysed in 2× SDS sample buffer. Cell lysates were collected, sonicated, and fractionated by SDS-PAGE for WB analyses. FLAG or HIF1α signals were measured using the Image Studio software (LI-COR Biosciences), normalized to GAPDH signals from the same sample, and fit to a single exponential in Prism to calculate half-lives.

4.4 | Preparation of ^{FLAG}HIF1α protein for in vitro ubiquitination

RCC4 (VHL null) cells stably expressing ^{FLAG}HIF1α were lysed by sonication in Pierce IP lysis buffer (ThermoFisher Scientific) containing 1 mM DTT and 1× protease inhibitor cocktail (PIC, Roche). The cell lysates were cleared by centrifugation at 15,000 g for 15 min, and the resulting supernatant was incubated with anti-FLAG beads (Sigma-Aldrich) for 2 h at 4°C. After washing the beads three times, bound ^{FLAG}HIF1α was eluted with 3× FLAG peptide (Sigma-Aldrich) and was concentrated using an Amicon Ultra-4 Centrifugal Filter (Millipore). To determine the concentration of the purified ^{FLAG}HIF1α, serial dilutions of recombinant ^{FLAG}IκBα with a known concentration, along with diluted aliquots of the purified ^{FLAG}HIF1α, were analyzed using WB with the FLAG antibody. The concentration of ^{FLAG}HIF1α was then calculated by fitting the WB signal to the standard curve of ^{FLAG}IκBα. Recombinant ^{FLAG}IκBα was generated by expressing ^{FLAG}IκBα on a pGEX vector in E. coli cells, followed by purification with glutathione agarose beads, thrombin cleavage, and gel filtration chromatography.[28,43,44]

4.5 | In vitro neddylation and ubiquitination

Neddylation reactions were performed for 1 h at room temperature in 1× reaction buffer [30 mM Tris (pH 7.5), 5 mM MgCl$_2$, 2 mM DTT, 2 mM ATP and 1× PIC (Roche)] containing 0.25 µM NAE, 3 µM UBC12, 0.2 µM CUL2•RBX1 (R&D Systems) and 0.2 µM NEDD8 (R&D Systems).

NEDD8 was opted out for the no neddylation group. CUL2•RBX1 was opted out for the negative control. In vitro ubiquitination was performed at room temperature in $1\times$ reaction buffer containing 0.5 μM Ub-E1, 0.2 μM Cdc34, 30 μM ubiquitin, 0.1 μM CUL2•RBX1, 0.1 μM VHL•ElonginB/C, and 0.1 μM purified [FLAG]HIF1α or 0.1 μM TAMRA labeled HIF1α degron peptide [Ac-KLRREPDALTLLA-(hydroxylated-P)-AAGDTIISLDFGSNGRRASYK(TAMRA)-amide].[28] At indicated time points, aliquots of the reaction mixtures were withdrawn and mixed with $4\times$ SDS sample buffer. Samples were fractionated with SDS-PAGE and analyzed by WB on an Odyssey Imager (LI-COR Biosciences), or by fluorescence scan (Typhoon scanner). Levels of unmodified [FLAG]HIF1α or peptide were normalized to levels of VHL and then fit to a single exponential in Prism to calculate half-lives.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Kankan Wang: Data curation (equal); formal analysis (lead); investigation (lead); methodology (lead); validation (lead); writing – original draft (lead); writing – review and editing (supporting). **Kurt M. Reichermeier:** Conceptualization (supporting); data curation (supporting); investigation (supporting); methodology (supporting); writing – original draft (supporting). **Xing Liu:** Conceptualization (lead); data curation (equal); formal analysis (supporting); funding acquisition (lead); methodology (supporting); project administration (lead); supervision (lead); writing – review and editing (lead).

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SUPPORTING INFORMATION

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