Monovalent Pseudo-Natural Product Degraders Supercharge the Native Degradation of IDO1 by KLHDC3

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Abstract

Targeted protein degradation (TPD) modulates protein function beyond inhibition of enzyme activity or protein-protein interactions. Most degraders function by proximity induction, and directly bridge an E3 ligase with the target to be degraded. However, many proteins might not be addressable via proximity-based degraders, and other challenges, such as resistance acquisition, exist. Here, we identified pseudo-natural products derived from (-)-myrtanol, termed iDegs, that inhibit and induce degradation of the immunomodulatory enzyme indoleamine-2,3-dioxygenase 1 (IDO1) by a distinct mechanism. iDegs induce a unique conformational change and, thereby, boost IDO1 ubiquitination and degradation by the cullin-RING E3 ligase $CRL2^{KLHDC3}$, which we identified to also mediate native IDO1 degradation. Therefore, iDegs supercharge the native proteolytic pathway of IDO1, rendering this mechanism of action distinct from traditional degrader approaches involving proteolysis-targeting chimeras (PROTACs) or molecular-glue degraders (MGDs). In contrast to clinically explored IDO1 inhibitors, iDegs reduce formation of kynurenine by both inhibition and induced degradation of the enzyme and should also modulate non-enzymatic functions of IDO1. This unique mechanism of action may open up new therapeutic opportunities for the treatment of cancer beyond classical inhibition of IDO1.

Main

Small-molecule mediated targeted protein degradation (TPD) is a powerful approach to modulate protein functions beyond enzymatic activity and protein-protein interactions.[1] Current strategies for TPD rely on small molecules ("degraders") that induce molecular proximity between the target and an E3 ubiquitin ligase.[2] Degraders can either be heterobifunctional, harboring distinct entities for engaging both target and E3 via dedicated

ligands ("PROTACs") or monovalent, binding to either the target or the ligase to adapt its protein surface and induce a highly cooperative tripartite assembly (Molecular Glue Degraders, MGDs). While both degrader types have been applied successfully for a variety of targets and have entered the clinics, several challenges remain.[3] First, certain protein classes will remain outside the reach of either modality due to difficulties in ligand discovery, or due to a lack of a complementary protein surface with an E3 that can be chemically stabilized. Second, inactivating mutations in E3 ligases may lead to resistance[4-6], and off-target degradation has been observed.[7,8] Therefore, new degrader chemotypes and TPD strategies are in high demand but short supply.

Natural products (NPs) and analogs have yielded E3 ligase ligands and mono- and bivalent inducers of protein degradation[9], raising the question of whether new degrader chemotypes could be derived from NPs. Pseudo-natural products (PNPs) combine natural-product fragments in arrangements and combinations not observed in NPs. They retain the biological relevance of NPs but open new chemical space and therefore may have unexpected and novel targets,[10,11] such that exploration of their bioactivity in particular in unbiased cell-based and phenotypic screens[10,12] may identify novel small-molecule degrader chemotypes and E3 ligases.

The heme-binding enzyme indolamine 2,3-dioxygenase 1 (IDO1) converts tryptophan (Trp) to kynurenine (Kyn). Trp shortage and Kyn elevation have numerous downstream effects including reduced T_{eff} cell proliferation and promotion of T_{reg} cell differentiation, generation of ligands for the aryl-hydrocarbon receptor and ferroptosis-suppressive kynurenine derivatives, all of which are linked to reduced anti-tumor immunity.[13-17] Only recently, the Eppstein Barr virus (EBV) has been reported to induce IDO1 expression, which is linked to EBV-associated lymphoma.[18] In general, clinical exploration of different IDO1 inhibitors has,[19-25] despite encouraging preclinical data,[26,27] met with limited success.[28,29] Plausible

reasons include that IDO1 may have non-enzymatic functions in immunosuppression and in glioblastoma, [30-35]. Nevertheless, there are ten active and three recruiting clinical trials with IDO1 inhibitors[36] underscoring the potential and the continuous interest in IDO1 modulation for disease treatment. The limitations arising from the use of enzyme inhibition only[37] may be overcome by IDO1 degradation, which would eliminate both enzymatic activity and signaling function. Initial investigations of IDO1-directed PROTACs[38,39] support this notion, but small-molecule monovalent degraders or molecular glues targeting IDO1 have yet to be identified.

In the course of a cell-based screen for small molecules that reduce Kyn levels, we identified a class of pseudo-natural products derived from (-)-myrtanol, termed iDegs, that both inhibit IDO1 and induce IDO1 degradation. Rather than "gluing" IDO1 to an E3, binding of iDegs induces structural changes in IDO1 that cause enhanced ubiquitination and augmented degradation by $CRL2^{KLHDC3}$, a ligase we identified to mediate also the ubiquitination and native degradation of IDO1. This cascade of "supercharging" the native degradation defines a unique mechanism of action, a new type of degrader class and a new strategy to induce target protein degradation. Of note, a systematic survey of inhibitor-induced degradation of protein kinases likewise revealed supercharging of native degradation circuit as a frequently occurring mechanism that can be prompted by ligand-induced changes in the activity, localization, and oligomerization of target kinases.[40] Together, these data establish ligand-induced supercharging of native proteolytic mechanisms as generalizable phenomenon and viable alternative to proximity-inducing degraders such as PROTACs or MGDs.

Identification of IDO1 degraders

A library of 157,332 small molecules, obtained from commercial and academic sources and developed in house was screened in a cell-based assay (Kyn assay, see Extended Data Fig. 1a) that monitored IDO1-mediated reduction of cellular Kyn production. IDO1 expression can be

rapidly induced by interferon gamma (IFN-γ).[41] In IFN-γ-stimulated BxPC3 cells,[24] the (-)-myrtanol-derived pseudo-natural product termed iDeg-1 (see Supporting Scheme 1 for the synthesis), inhibited Kyn formation with an IC_{50} of 0.83 ± 0.31 µM in the screening assay (Fig. 1a). This finding was confirmed in a manual Kyn assay in IFN-γ-stimulated BxPC3-, SKOV3- and HeLa cells (IC_{50} values of 1.1 ± 0.1 µM, 1.6 ± 0.3 µM and 1.7 ± 1.2 µM, respectively, Fig. 1b and Extended Data Fig. 1b). Lower Kyn amounts upon iDeg-1 treatment were also detected using LC-MS/MS as an orthogonal readout (Extended Data Fig. 1c). iDeg-1 reduced IDO1-dependent SKOV3 cell death in 2D and 3D cell cultures induced by IFN[42] (Extended Data Fig. 1d-e). iDeg-1 only slightly affected IDO1 enzymatic activity (Extended Data Fig. 1f) and did not impair *IDO1* transcription (Extended Data Fig. 1g-h). Instead, iDeg-1 dose-dependently reduced IDO1 protein levels up to 45 ± 15 % at 10 µM after 24 h (Fig. 1c-d) without inhibiting *in vitro* translation of IDO1 or global protein translation (Extended Data Fig. 1i-n).

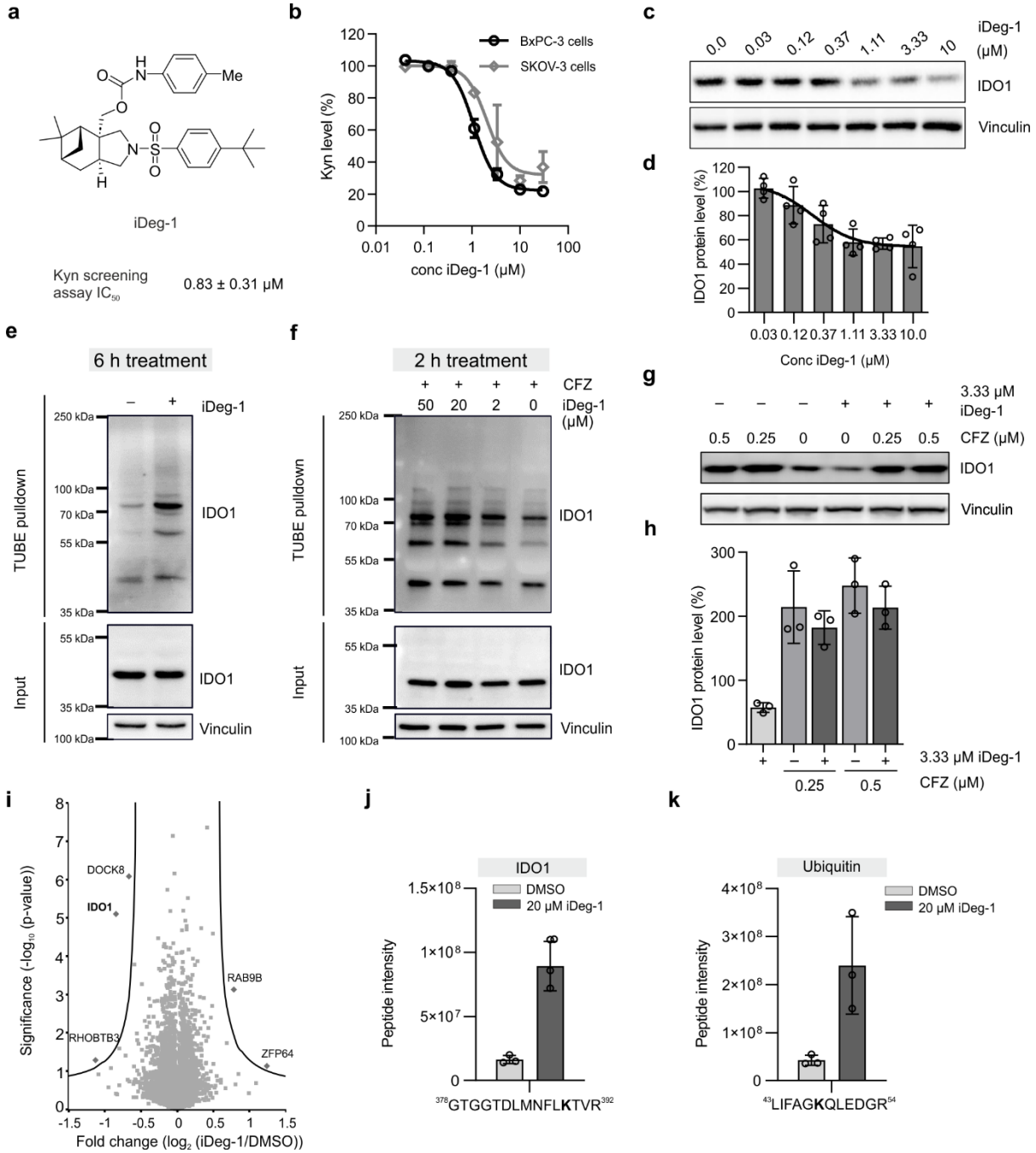


Fig. 1: iDeg-1 reduces IDO1 protein levels via the UPS. a, Structure of screening hit iDeg-1 and IC_{50} value in the Kyn assay in BxPC3 cells. Mean value ± SD, n = 3. **b,** Kyn assay in BxPC3- and SKOV3 cells after treatment with iDeg-1 and 50 or 5 ng/mL IFN-γ, respectively, for 48 h prior to detection of Kyn levels utilizing *p*-DMAB. **c,d,** IDO1 protein levels in BxPC3 cells upon treatment with IFN-γ and iDeg-1 for 24 h. Representative immunoblots (c) and quantified band intensities from c (d). Mean values ± SD, n = 4. **e,f,** TUBE pulldown after treatment of IFN-γ-stimulated BxPC3 cells with iDeg-1 or DMSO. **e,** Cells were treated for 6 h with 50 µM iDeg-1 prior to the TUBE pulldown. Representative immunoblots of n = 3. **f,** Cells were treated with 450 nM carfilzomib (CFZ) 60 min prior to the addition of iDeg-1 or DMSO for 2 h followed by TUBE pulldown. Representative immunoblots of n = 3 for IDO1. **g,h,** HEK239T cells were electroporated with rhIDO1 protein. Cells were treated with CFZ for 30 min prior to the addition of 3.33 µM iDeg-1 and further incubation for 6 h. **g,** Representative immunoblot of n = 3. **h,** Quantified band intensities from g represent samples treated with compound relative to DMSO (set to 100 %). Mean values ± SD, n = 3 or n = 4. **i,** Volcano plot of iDeg-1-induced

changes in the global proteome. HEK239T cells were electroporated with rhIDO1 protein followed by treatment with 10 µM iDeg-1 or DMSO for 6 h and MS analysis. **j,k,** IDO1 immunoprecipitation (IP) and identified peptide of IDO1 (**j**) or ubiquitin (**k**) with diGly modification. IFN-γ-stimulated BxPC3 cells were treated with 20 µM iDeg-1 or DMSO for 6 h prior to the IP. Uncropped blots are shown in Extended Data Fig. 11.

In order to determine whether iDeg-1 induces degradation via the ubiquitin proteasome system (UPS), IFN-γ-stimulated BxPC3 cells were treated with iDeg-1 for 6 h (Fig. 1e), or for 2 and 4 h after pretreatment with the proteasome inhibitor carfilzomib (CFZ; Fig. 1f and Extended Data Fig. 2a). Increased polyubiquitination was detected with a tandem ubiquitin binding entity (TUBE) pulldown from cell lysates.[43] The results showed that iDeg-1 induces IDO1 ubiquitination shortly after compound addition. By contrast, IDO1 protein reduction was only detectable after 24 h in IFN-γ-stimulated BxPC3 cells (Fig 1c-d). Since degradation may be masked by constant IDO1 production in IFN-γ-stimulated cells, we introduced enzymatically active recombinant human IDO1 protein (rhIDO1) in HEK293T cells using electroporation (HEK^{rhIDO1} cells). iDeg-1 dose-dependently reduced Kyn levels with an IC_{50} value of 0.45 ± 0.1 µM (Extended Data Fig. 2b-c) and lowered IDO1 protein levels already after 6 h by 46 % at a concentration of 10 µM (Extended Data Fig. 2d-e). CFZ inhibited degradation (Fig. 1g-h and Extended Data Fig. 2f), indicating involvement of the UPS. iDeg-1 also inhibited Kyn production in HEK293T cells that transiently express IDO1 with an IC_{50} of 0.42 ± 0.2 µM (Extended Data Fig. 2g). These results show that iDeg-1 depletes IDO1 independent of the effects of IFN-γ.

Global proteome analysis of HEK^{rhIDO1}cells after 6 h of treatment with iDeg-1 revealed that in addition to IDO1, out of 7,541 identified proteins only the abundance of dedicator of cytokinesis 8 (DOCK8), a guanine nucleotide exchange factor, and Rho-related BTB domain-containing protein 3 (RHOBTB3), a Rab9-regulated ATPase involved in endosome to Golgi transport were reduced, demonstrating high specificity (Fig. 1i). Analysis of ubiquitinated

proteins for a diglycine (diGly) attached to lysine residues (K-ε-diglycine) which were modified with ubiquitin[44], after iDeg-1 treatment and IDO1 immunoprecipitation uncovered K389 of IDO1 as the site of ubiquitination (Fig. 1j and (Extended Data Fig. 3a). In addition, we observed a 3.5-fold increase in ubiquitin levels after treatment with iDeg-1 confirming iDeg-1-induced IDO1 ubiquitination (Extended Data Fig. 3b). DiGly analysis further revealed K48 linkages in the ubiquitin chains (Fig. 1k and Extended Data Fig. 3c), which are associated with protein degradation via the UPS[45].

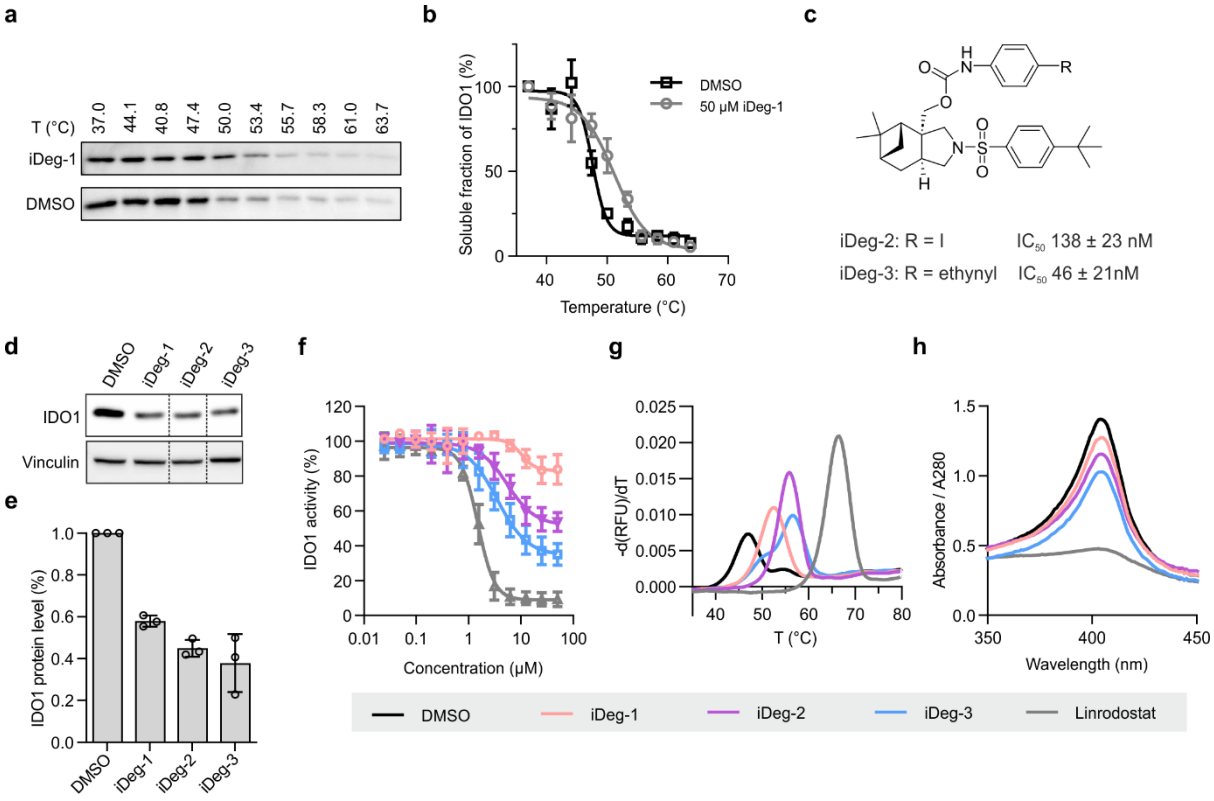


Fig. 2: **iDegs interact with IDO1. a,b,** CETSA in intact SKOV3 cells treated with 50 µM iDeg-1 or DMSO for 1 h followed by heat treatment and immunoblotting. **a,** Representative immunoblot for IDO1. **b,** Quantification of band intensities from a. Mean values ± SD, n = 3. **c,** Structures of iDeg-2 and iDeg-3 and IC_{50} values in the Kyn assay in BxPC3 cells. Mean values ± SD, n = 3. **d,e,** Influence of iDeg-1, 2 and 3 on IDO1 protein levels in BxPC3 cells. Cells were treated with IFN-γ and the compounds (3.33 µM) for 24 h prior to immunoblotting (d). Quantification of band intensities shown in e. Mean values ± SD, n = 3. **f,** Influence on the *in vitro* rhIDO1 activity. rhIDO1 was pre-incubated with the compounds at 37°C for 90 min prior to detection of Kyn levels using *p*-DMAB. Mean values ± SD, n = 3. **g,** rhIDO1 thermal stability in presence of 50 µM iDeg-1, 2 or 3 or DMSO and the apo-IDO1 inhibitor linrodostat (50 µM) using nanoDSF. rhIDO1 and compounds were pre-incubated for 3 h at 37°C prior to the measurement. Representative result (n = 3). **h,** Detection of heme-bound IDO1 by means of UV/Vis spectroscopy in presence of iDeg-1, 2 or 3 (100 µM), DMSO or linrodostat

(100 µM). Incubation at 37°C for 3 h. Representative data for n = 3. Uncropped blots are shown in Extended Data Fig. 12.

Direct engagement of IDO1 in cells was confirmed with a cellular thermal shift assay (CETSA)[46] that revealed thermal stabilization of IDO1 with a shift in the melting temperature (ΔT_m) of 3.5 ± 0.4 °C in the presence of iDeg-1 (Fig. 2a-b). Thermal stabilization of IDO1 by iDeg-1 was dose-dependent as demonstrated by an isothermal CETSA experiment at 50°C (Extended Data Fig. 4a-b).

Initial structure-activity relationship investigations identified two analogs with enhanced activity in the Kyn assay as compared to iDeg-1 that bear an iodine (termed iDeg-2, IC_{50} = 138 ± 23 nM) or an alkyne group (termed iDeg-3, IC_{50} = 46 ± 21 nM) in *para* position of the phenyl carbamate (Fig. 2c, Extended Data Fig. 4c-d). In agreement with the Kyn assay, iDeg-2 and iDeg-3 reduced IDO1 protein levels by 55 ± 3 % and 62 ± 11 % respectively as compared to iDeg-1 with 42 ± 2 % at 3.33 µM (Fig. 2d-e). Although partially, iDeg-2 and 3 inhibited the enzyme *in vitro* (Fig. 2f). Thermal stabilization of rhIDO1 was detected for the three compounds using nanoDSF (Fig. 2g). UV/Vis spectroscopic analysis revealed that in the presence of iDeg-1-3 the specific *Soret* absorbance peak of heme-bound IDO1 (holo-IDO1, Fig. 2h) is reduced indicating that iDegs displace heme and bind to apo-IDO1 with different potency. Accordingly, addition of hemin reduced the potency of iDeg-1, -2 and -3 in the Kyn assay (Extended Data Fig. 4e-g) and dose-dependently elevated Kyn levels in the presence of iDeg-2 (Extended Data Fig. 4h). As we detected both inhibition and degradation of IDO1 by iDeg-3, the compound class was termed iDeg.

iDegs increase structural flexibility of the IDO1 C-terminus

The co-crystal structure of apo-IDO1 in complex with iDeg-2 at 1.6 Å resolution (PDB ID 9FOH, Extended Data Table 1) revealed that iDeg-2 occupies the heme binding site in apo-IDO1 and induces a large conformational rearrangement (Fig. 3a-d). The phenyl carbamate occupies the previously identified lipophilic pocket A[47] in the distal heme site, while the pyrrolidine and the sulfonyl group occupy the heme-binding pocket. The monoterpene scaffold only slightly protrudes into the D-pocket, and the *tert*-butyl phenyl group is located in the solvent-exposed B-pocket (Fig. 3b). Binding to this pocket has been observed before only for holo-IDO1 inhibitors (Fig. 3c and Extended Data Fig. 5a). iDeg-2 binding occurs though a large number of hydrophobic interactions (Y126, C129, F163, V167, E171, F214, I217, D226, G262, S263, A264, Q266, S267, F270, H346 and I349), a water-bridged hydrogen bond between the carbamate nitrogen and the hydroxyl group of S167 and a hydrogen bond between the sulphonyl oxygen of iDeg-2 and H346, which coordinates heme in holo-IDO1 (Fig. 3e-f). In the presence of iDeg-2, there was no detectable electron density for the C-terminal K-helix (Fig. 3d). An overlay of the structures of IDO1 in complex with iDeg-2 and the apo-IDO1 inhibitor linrodostat revealed substantial shifts in helices B, C, H and J and a distortion in the J-helix in the iDeg-2 complex (Fig. 3g and Extended Data Fig. 5b). The J-helix C-terminus moves toward the binding pocket, while the N-terminus shifts away from the K-helix interface, most likely weakening the interaction of the K-helix with the J- and H-helices, and the K-helix, bearing lysine 389, becomes flexible. Previously reported IDO1 inhibitors do not alter the overall structure of IDO1 compared to holo-IDO1 (Extended Data Fig. 5b-c), such that the observed conformational rearrangements represent a novel and unique binding mode of the iDegs.

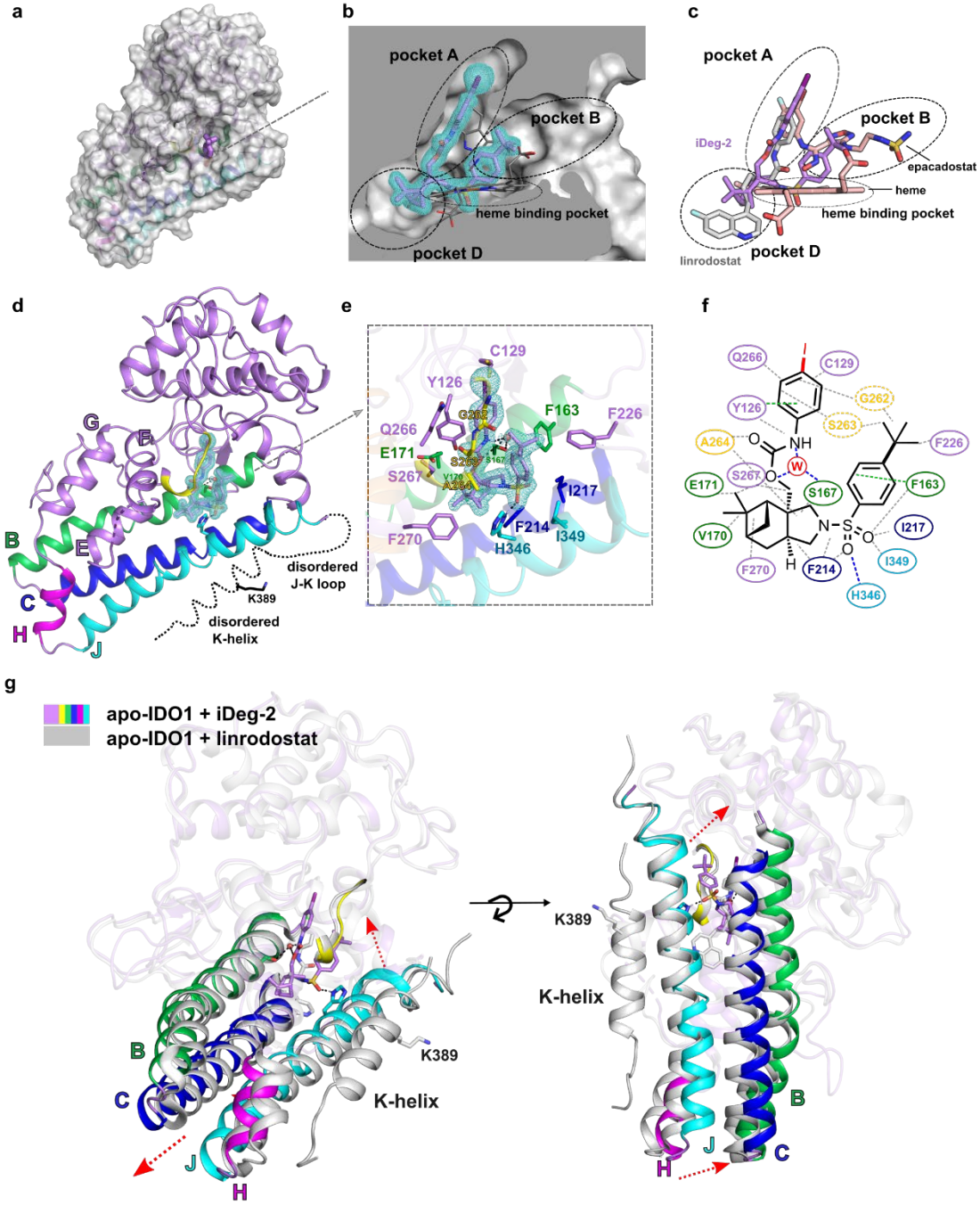


Fig. 3. Co-crystal structure of IDO1 with iDeg-2. **a,** Surface representation of apo-IDO1 with iDeg-2 as sticks bound in the catalytic cleft. **b,** Surface model in the cut-in side view of iDeg-2 in the active site. The electron density (2Fo-Fc) around iDeg-2 in blue contoured at 1.6 σ. Superposition with cyanide- and L-Trp-bound holo-IDO1 (PDB ID: 5wmu). The hydrophobic pockets A, B, D and the heme binding site are indicated. **c,** Overlay of iDeg-2 (violet) with apo-IDO1 inhibitor linrodostat (PDB ID: 6dpr-B) (grey) and holo-IDO1 inhibitor epacadostat in complex with heme (PDB ID: 5wn8) (pink). **d,** Apo-IDO1-iDeg-2 structure shown as cartoon (violet). The J-K loop and the K-helix are disordered. Helices that show conformational changes after iDeg binding are colored. **e,** Zoom-in view of the amino

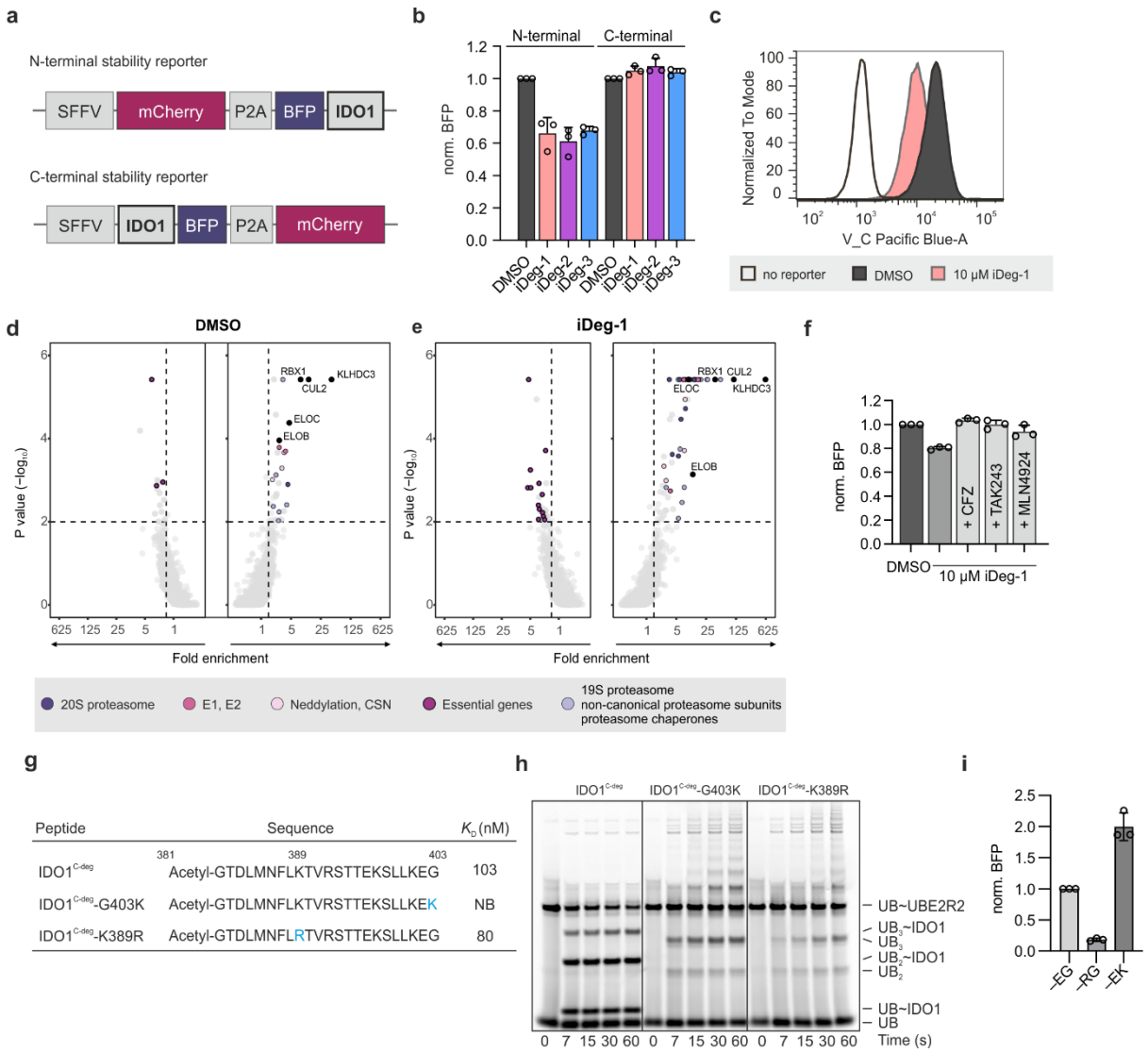
acids involved in iDeg-2 binding. Hydrogen bonds are indicated by black dotted lines. Helices that show a conformational change and amino acids located in these helices are colored like in Fig. 3d. **f,** Schematic illustration of the interactions. Hydrogen bonds, van der Waals interactions and π-π stacking interactions are represented by dotted lines in blue, grey and green, respectively. Side- and main-chain interactions are indicated by solid and dashed circles, respectively. **g,** Overlay of apo-IDO1-iDeg-2 structure (violet and colored helices) with the structure of apo-IDO1-linrodostat (grey, PDB ID: 6dprB). Red arrows indicate the shift of the helices. The position of K389 in the K-helix is indicated. Side view and rotated bottom view of the overlay.

The global conformational rearrangements and shift of the C-terminal K-helix is characteristic for this new class of IDO1 modulators and is probably integral to establishment of their degradation mechanism. Since the compound is deeply buried inside the active site, a direct interaction between iDegs and a potential E3 ligase is unlikely to occur.

iDegs supercharge IDO1 ubiquitination by KLHDC3

To identify the E3 responsible for iDeg-mediated IDO1 degradation, we designed a FACS-based CRISPR-Cas9 screen using a custom sgRNA library targeting 1301 ubiquitin-associated human genes (6 sgRNAs per gene)[48]. We generated an IDO1 stability reporter following our previously reported design in KBM7 cells harboring an inducible Cas9 cassette[49] (Fig 4a, Extended Data Table 2). In brief, IDO1 is expressed as tagBFP fusion followed by the self-cleaving P2A site and mCherry for assay normalization. Supporting a role of the C-terminal helix of IDO1, only N-terminally fused BFP enabled iDeg-induced reporter degradation (Fig 4b). Next, cells were transduced with the custom sgRNA library. After selection for sgRNA positive cells, Cas9 expression and subsequent gene editing was induced via doxycycline for 72 h prior to 14 h compound treatment. Finally, cells were enriched for increased or decreased BFP levels using FACS and the corresponding sgRNAs were quantified by deep sequencing (Fig. 4d-e and Extended Data Fig. 6b and 7), revealing genes functionally required for iDeg-induced degradation.

As expected, knockout of genes coding for proteins of the 19S and 20S proteasome or involved in neddylation counteracted iDeg activity and increased IDO1 levels (Fig. 4d-e and Extended Data Fig. 6b-c), thus phenocopying the IDO1 stability reporter behavior upon chemical perturbation of the proteasome (CFZ), the ubiquitin-activating enzyme (TAK243) or of the neddylation machinery (MLN4924) (Fig 4f). Importantly, we further identified the cullin-RING ligase (CRL) complex including cullin2 (CUL2), RBX1, elongin B/C (EloB and EloC) and the Kelch domain containing protein 3 (KLHDC3) as required for IDO1 degradation. Of note, genetic disruption of the $CRL2^{KLHDC3}$ complex also affected baseline IDO1 turnover under vehicle (DMSO) treatment. In the presence of iDegs, however, knockout of these genes led to an even higher enrichment of the corresponding sgRNAs (Fig. 4e and Extended Data Fig. 6b-c). This indicated that the compounds enhance the efficiency of IDO1 degradation. Contrary to classical degrader modalities such as PROTACs or molecular glue degraders, which typically function by inducing proximity between an E3 and a target that is functionally inconsequential in the absence of the small molecule, iDegs thus appear to supercharge the native route of IDO1 turnover.



Peptide	Sequence	K_D (nM)
IDO1$^{C\text{-}deg}$	381 Acetyl-GTDLMNFLKTVRSTTEKSLLKEG 403	103
IDO1$^{C\text{-}deg}$-G403K	Acetyl-GTDLMNFLKTVRSTTEKSLLKEK	NB
IDO1$^{C\text{-}deg}$-K389R	Acetyl-GTDLMNFLRTVRSTTEKSLLKEG	80

Fig. 4: KLHDC3 is involved in iDeg-mediated IDO1 degradation. a, IDO1 stability reporter design. **b,** Detection of IDO1 levels using the IDO1 stability reporters. KBM7 IDO1 reporter cells were treated with iDeg-1, 2 or 3 (1 μM) for 24 h prior to detection of IDO1 levels using flow cytometry. Normalized BFP to mCherry ratios (norm. BFP) were calculated per genotype respectively. Mean values ± SD, n = 3. **c,** Representative histogram for iDeg-1 mediated depletion of BFP-IDO1 (24h, 10 μM). **d,** Identification of genes required for native IDO1 degradation. **e,** Identification of genes required for iDeg-1-mediated IDO1 degradation. CSN: COP9 signalosome. **f,** IDO1 depletion is rescued by 10 h co-treatment with either CFZ, TAK243 or MLN4924 (1 μM each). Mean values ± SD, n = 3. **g,** Binding affinities of IDO1 C-terminal peptide IDO1$^{C\text{-}deg}$ and peptide mutants (IDO1$^{C\text{-}deg}$-G403K and IDO1$^{C\text{-}deg}$-K389R) to KLHDC3 determined using ITC. NB: no binding. **h,** Fluorescent scan of pulse-chase assay monitoring ubiquitination of C-terminal IDO1 peptides by UBE2R2. UBE2R2 was "pulse" loaded with fluorescent ubiquitin, and the resulting UBE2R2~UB thiolester was added to NEDD8~CRL2^{KLHDC3} incubated with the indicated peptides. Samples were quenched with SDS sample buffer at the indicated timepoints. **i,** IDO1 stability reporter variants in KBM7 cells measured by flow cytometry and depicted normalized to the wt (i.e., –EG) reporter. Mean values ± SD, n = 3.

IDO1 is a natural substrate of CRL2^{KLHDC3}

The CRL substrate receptor KLHDC3 has not yet been employed for small molecule-induced protein degradation. KLHDC3 recognizes degrons with C-terminal glycine[50], which explains why the C-terminally fused IDO1-BFP reporter was not degraded (Fig. 4b). The C-terminal EG-sequence of human IDO1 is consistent with the C degrons (GG, RG, KG, QG, WG, PG, and AG[51]) previously shown to elicit KLHDC3-dependent degradation. In agreement, the peptide IDO1$^{C\text{-}deg}$ corresponding to the C-terminal amino acid sequence 381-403 bound to KLHDC3 with a K_D of 103 nM (Fig. 4g and Extended Data Fig. 8a). Exchanging the C-terminal glycine by a lysine (peptide IDO1$^{C\text{-}deg}$-G403K) completely abolished binding to KLHDC3. Replacement of K389, which is ubiquitinated (peptide IDO1$^{C\text{-}deg}$-K389R), did not affect formation of the E3-degron complex (Fig. 4g and Extended Data Fig. 8a). In order to demonstrate that IDO1 was a substrate for ubiquitination by CRL2^{KLHDC3} we reconstituted biochemical "pulse-chase" ubiquitination assays. Since KLHDC3-EloB/C has been shown to form degron-mimic mediated auto-inhibited tetrameric assemblies, in which the C-degron binding site is occluded by the degron mimic[52], we simplified the assay by using a C-terminal Gly-to-Lys mutant of KLHDC3 that is strictly monomeric. Notably, the wildtype peptide IDO1$^{C\text{-}deg}$, but not the mutant versions, was ubiquitinated *in vitro* in a CRL2^{KLHDC3}- dependent manner (Fig. 4h and Extended Data Fig. 8b). In cells, mutation of the C-terminal glycine increased IDO1 amounts, whereas mutation of the non-optimal –EG degron to an optimal –RG C-terminus reduced IDO1 abundance (Fig. 4i). These findings demonstrate that IDO1 is a substrate of KLHDC3 and that the C-terminal glycine is essential for interaction with the E3 ligase.

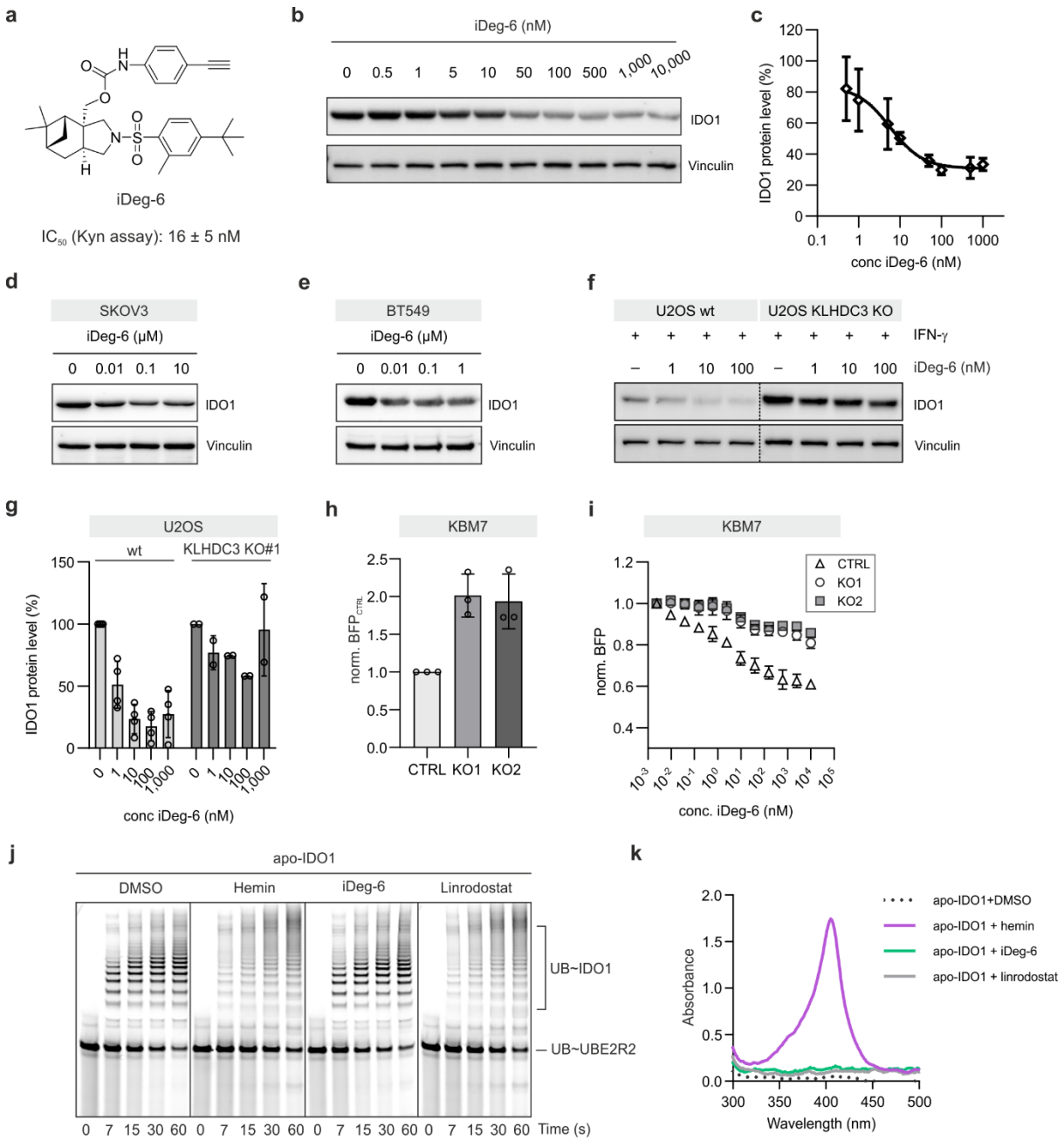


Fig. 5: Validation of KLHDC3 as E3 ligase regulating IDO1. a, Structure of iDeg-6 and IC_{50} value in the Kyn assay in BxPC3 cells. Mean value ± SD, n = 3. **b,c,** Reduction of IDO1 protein levels by iDeg-6. Degradation efficiency was assessed in a modified setup including IFN-γ washout prior to compound addition. BxPC3 cells were treated with 50 ng/mL IFN-γ for 24 h prior to washout, addition of iDeg-6 for 24 h and immunoblotting (b). **c,** Quantification of IDO1 protein levels from b (mean values ± SD, n = 4 except for 50 and 500 nM (n = 3)). **d,e,** IDO1 levels in SKOV3 (d) and BT549 (e) cells. Cells were treated with iDeg-6 for 24 h prior to immunoblotting. **f,g,** IDO1 protein levels in wildtype (wt) U2OS or KLHDC3 knockout (KO) U2OS cells. Cells were stimulated with 5 ng/mL IFN-γ for 24 h prior to washout, treatment with iDeg-6 or DMSO for 24 h and immunoblotting (f). Representative data of n = 4 (U2OS wt) or n = 2 (KO cells). **g,** Quantification of band intensities from f and Extended Data Fig. 9n. Mean values ± SD, n = 3 or n = 2. **h,i,** IDO1 protein levels in KBM7-BFP-IDO1 (CTRL) or KBM7-BFP-IDO1 KLHDC3 KO1 or KO2 cells in absence (h, normalized to CTRL) or presence of iDeg-6 (i, normalized to respective genotype). Mean values ± SD, n = 3. **j,** Fluorescent scan of pulse-chase assay monitoring ubiquitination of apo-IDO1 with or without supplementation with small molecules by $CRL2^{KLHDC3}$. Apo-IDO1 was incubated with the indicated compounds (30 μM) for

90 min at 37°C prior to incubation with $CRL2^{KLHDC3}$ E3 complex. Ubiquitination reactions were initiated by the addition of the pre-formed thioester-linked E2~ubiquitin conjugate (UBE2R2~UB) with the ubiquitin fluorescently-labeled. **k,** Detection of heme load for the conditions in j detected by means of UV/Vis spectroscopy. Representative data for n = 2. Uncropped blots are shown in Extended Data Fig. 13.

Further exploration of the structure-activity relationship (SAR) led to the identification of the inhibitor and degrader iDeg-6 with an IC_{50} of 16 ± 5 nM in the Kyn assay (Fig. 5a and Extended Data Fig. 9a). In a modified setup including washout of IFN-γ followed by compound addition to avoid continuous IDO1 expression, iDeg-6 most efficiently and potently depleted IDO1 in cells with D_{max} of 70 % at 100 nM and DC_{50} for IDO1 degradation of 6.5 ± 3 nM (Fig. 5a-c and Extended Data Fig. 9b-c). *In vitro* iDeg-6 inhibited IDO1 activity nearly completely and more potently than iDeg-1-3 with an IC_{50} of 1.6 ± 0.3 µM. The compound induced thermal stabilization of the protein and heme displacement to a higher extent as compared to iDeg-1, -2 and -3 (Extended Data Fig. 9d-f). We therefore used iDeg-6 for further validation.

iDeg-6 depleted IDO1 protein also in SKOV3 and BT549 cells in the absence of IFN-γ in a dose- and time-dependent manner (Fig. 5d-e and Extended Data Fig. 9g-k). The neddylation inhibitor MLN4924 rescued iDeg-6-induced IDO1 depletion (Extended Data Fig. 9l) and increased IDO1 levels in the absence of iDegs, demonstrating that neddylation is required for both, native and iDeg-induced IDO1 degradation (Extended Data Fig. 9l). Knockout (KO) of KLHDC3 in U2OS or KBM7-BFP-IDO1 cells increased IDO1 amounts and rescued iDeg-6-dependent degradation (Fig. 5f-i and Extended Data Fig. 9m-n, see also Extended Data Fig S6e for iDeg-1, 2 and3).

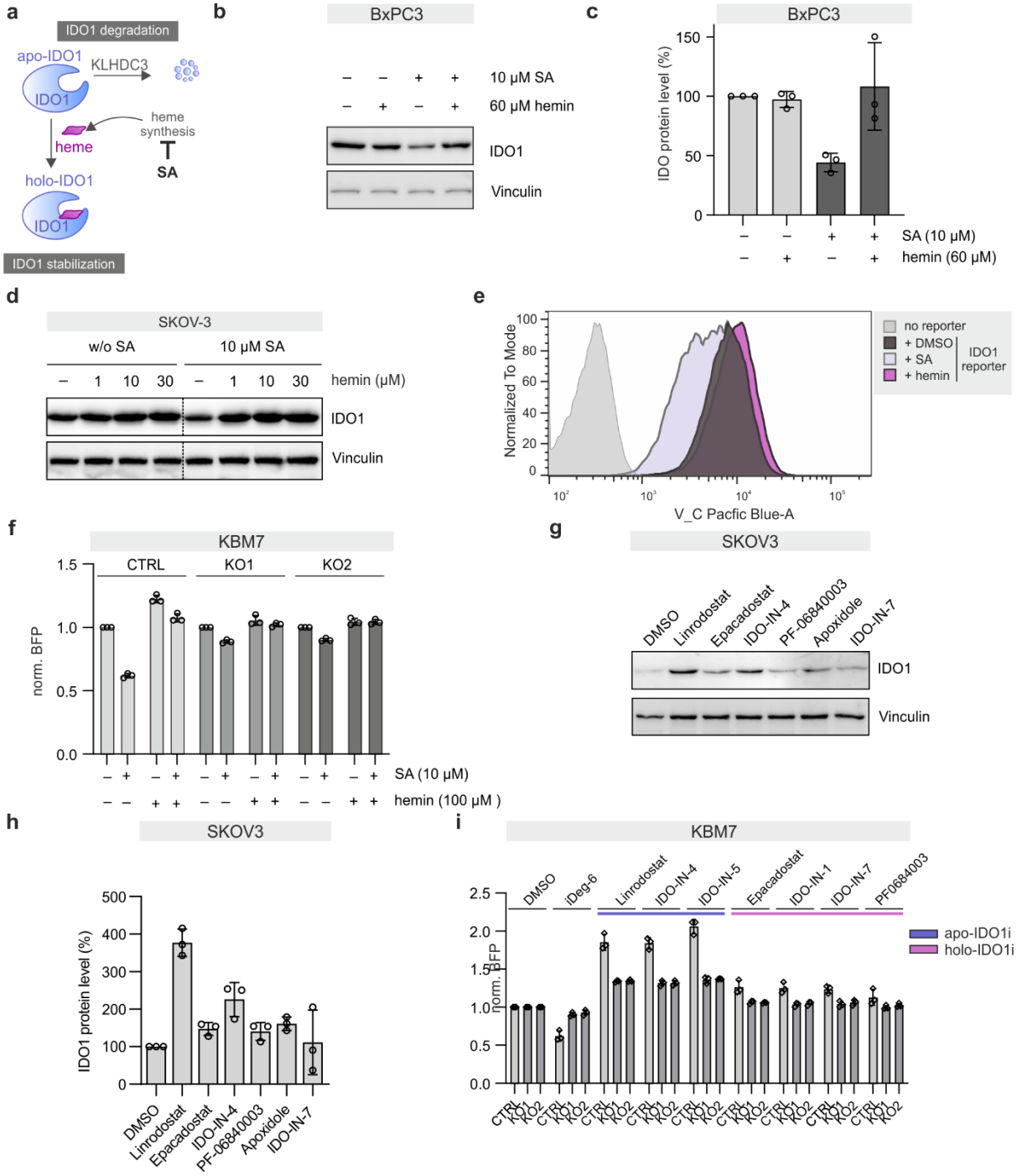


Fig. 6. Apo-IDO and not holo-IDO1 is the preferential substrate for degradation. a, Regulation of IDO1 by heme synthesis and succinyl acetone (SA) as an inhibitor of heme synthesis. **b,c,** Influence of SA and heme on IDO1 protein levels in BxPC3 cells as detected using immunoblotting. Cells were treated with 50 ng/mL IFN-γ with or without 10 μM SA for 24 h followed by the addition of hemin for further 24 h. **c,** Quantification of band intensities from b. Mean values ± SD, n = 3. **d,** Influence of SA and heme on IDO1 levels in SKOV3 cells. Cells were treated with SA for 48 h prior to the addition of hemin for another 24 h and immunoblotting. See Extended Data Fig. 10c for quantified band intensities. **e,f,** Influence of SA and hemin on IDO1 abundance in KBM7-BFP-IDO1 (e) or KBM7-BFP-IDO1 KLHDC3 knockout cells (f). Cells were pre-treated with 10 μM SA for 24 h followed by a treatment for 48 h with 100 μM hemin or DMSO as a control. IDO1 levels were quantified using flow cytometry. Mean values ± SD, n = 3. **g,h,** Influence of known IDO1 inhibitors on IDO1 protein levels in SKOV3 cells. Cells were incubated with the compounds (5 μM) for 48 h prior to immunoblotting (g). **h,**

Quantification of band intensities from g. Mean values ± SD (n = 3). **i,** Influence of IDO1 inhibitors (IDO1i, 1 μM) on IDO1 protein levels in KBM7-BFP-IDO1 or KBM7-BFP-IDO1 KLHDC3 knockout cells. Cells were treated with the compounds for 24 h followed by quantification of IDO1 using flow cytometry. Mean values ± SD, n = 3. Uncropped blots are shown in Extended Data Fig. 14.

Surprisingly, *in vitro*, apo-IDO1 was rapidly ubiquitinated by $CRL2^{KLHDC3}$, which was less pronounced in the presence of heme (Fig. 5j). IDO1 was also ubiquitinated upon treatment of apo-IDO1 with iDeg-6 (Fig. 5j). In contrast, apo-IDO1 inhibitor linrodostat rather suppressed IDO1 ubiquitination under these conditions (Fig. 5j-k). Hence, in a biochemical setting, apo-IDO1 is a better substrate for KLHDC3 than holo-IDO1. These findings suggest a possible model for regulation of IDO1 levels in cells. Freshly translated apo-IDO1 is rapidly cleared in cells by physiological degradation mediated by KLHDC3 in order to keep its concentration low when not needed. In contrast, after an external stimulus, IDO1 expression is upregulated, apo-IDO1 exceeds predominant degradation and is charged with heme to form holo-IDO1. The holo-pool escapes degradation and performs the desired function in response to the stimulus. In contrast to linrodostat and other apo-IDO1-binders, iDeg binding appears to establish a complex conformation that more resembles the structure of the apo-form (for which the structure has not been solved yet) and thereby promotes ubiquitination.

Support for such a mechanism in cells (Fig. 6a) was obtained by treatment with heme synthesis inhibitor succinyl acetone to deplete heme and shift the equilibrium to the apo-IDO1 form. In IFN-γ-stimulated BxPC3 cells or in SKOV3 and KBM7-BFP-IDO1, succinyl acetone reduced IDO1 protein levels and addition of hemin dose-dependently increased the IDO1 amount (Fig. 6b-c and Extended Data Fig. 10a-c). Similar observations were made in SKOV3 cells and the KBM7-BFP-IDO1 stability reporter cells (Fig. 6d-e and Extended Data Fig. 10c). Addition of only hemin dose-dependently increased IDO1 protein levels in SKOV3 cells, which is in agreement with the majority of IDO1 being in the apo-form [22].

Changes in the IDO1 protein level do not result from altered *IDO1* mRNA levels (Extended Data Fig. 10d). Knockout of KLHDC3 counteracted succinyl acetone-mediated IDO1 depletion, indicating that apo-IDO1 degradation is dependent on KLHDC3 (Fig. 6f). These results support involvement of KLHDC3 in the regulation of apo-IDO1 turnover, both under native conditions and in the presence of iDegs. The mechanism of action relies on further pushing IDO1 towards the degradation-sensitive apo-IDO1 state, thus supercharging a physiological degradation circuit.

The binding mode of iDegs and other apo-IDO1 inhibitors to IDO1 are similar, which, in principle, could suggest that apo-inhibitors might generally impact IDO1 abundance. However, IDO1 inhibitors, particularly apo-IDO1 inhibitors like linrodostat, IDO-IN-4 and IDO-IN-5 even increased IDO1 protein in cells, which was partially reverted in KLHDC3 knockout cells (Fig. 6g-i). These findings are in agreement with the biochemical data showing lower ubiquitination of IDO1 in the presence of linrodostat (Fig. 5l). Hence, the IDO1-KLHDC3 degradation circuit can be modified in both directions by liganding the same binding pocket.

Discussion

Clinical investigation of IDO1 inhibition has had limited success[47,53], possibly due to its non-enzymatic signaling function[32,35]. In contrast, IDO1-targeting PROTACs modulate all IDO1-related activities which calls for the development of monovalent or molecular glue degraders for IDO1. We identified monovalent degraders, termed iDegs that function in a manner distinct from molecular glues. iDegs potently induce IDO1 ubiquitination and degradation mediated by the E3 ligase $CRL2^{KLHDC3}$. Notably, KLHDC3 mediates native IDO1 degradation, thus, iDegs exploit and supercharge the native pathway to IDO1 degradation. Under physiological

conditions and *in vitro*, apo-IDO1 but not heme-bound IDO1 was degraded preferentially, whereas heme binding prevented the turnover of IDO1.

Thus far, supercharging of native degradation mechanisms has largely been overlooked and only few examples, i.e., targeting BCL6 or EZH2[54,55], support the notion that monovalent degraders in a more general sense may regulate the native mechanisms of protein homeostasis for the respective targets[2]. Moreover, in a parallel study, Scholes et al. identified supercharging of endogenous degradation pathways as a frequently observed mechanism of action of kinase degradation that is induced by kinase inhibitors.[40] Mechanistically, kinase inhibitors can prompt degradation by changing activity, localization or aggregation state of kinases. Functionally differentiated, iDegs represent a first account of small molecules that act as a switch to induce a conformational state that may resemble the structure of the degradation-sensitive apo-IDO1, thereby channeling the IDO1-iDeg complex to the native degradation mechanism. Collectively, both studies highlight ligand-dependent supercharging of native degradation circuits as a new route to pharmacologic protein degradation that complements degradation strategies that are based on proximity induction, such as PROTACs or MGDs.

iDegs bind in the heme pocket, and, therefore, can be considered apo-IDO1 inhibitors but only the most potent compounds iDeg-2, -3 and -6 inhibited *in vitro* the enzymatic formation of kynurenine, which reflects the efficiency for heme displacement *in vitro*. However, in cells IDO1 is expressed as apo-IDO1, allowing all iDegs to compete with heme for binding, resulting in inhibition of the enzymatic reaction. iDegs induce an unprecedented conformational change of the C-terminal helix favoring K389 ubiquitination by KLHDC3. As degraders, iDegs are exceptional apo-IDO1 inhibitors since other apo-IDO1 modulators rather increased IDO1 protein abundance. Therefore, targeting the same binding pocket can regulate the IDO1-KLHDC3 degradation circuit in both directions.

The dual mechanism of action distinguishes iDegs since protein inhibition and depletion will impair all IDO1 functions, i.e., including the Kyn-independent signaling activity of IDO1. In addition, the increase of IDO1 protein by apo-IDO1 inhibitors is expected to diminish their efficacy. The failure of IDO1 inhibitors in the clinic may be due to its non-enzymatic signaling function that cannot be antagonized by inhibitors only, and to the fact that IDO1 inhibition may upregulate IDO1 protein[35,39]. In contrast, IDO1 degradation would eliminate both, enzymatic activity and signaling function, and may open up new opportunities for the treatment of cancer or, as recent studies imply, the treatment of diseases related to Epstein Barr virus infections[18].

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Author contribution

H.W. and S.Z. designed the project. E.H., B.L., N.S.S., X.-F.C, A.B., D.C.S., A.B., K.R., L-M.P., L.D., and T.T.X. and P.J.M. performed the biological research. X.-F.C., M.B. and K.R.N. synthesized the compounds. E.H., B.L., X.-F.C., N.S.S., analyzed the data. H.I. quantified the CRISPR/Cas9 screen sequencing data. B.L., R.G. and M.L. solved the crystal structure. S.S. adapted and performed the screening assays. K.K., M.G., P.J.M., B.A.S. and G.E.W supervised

project parts. E.H., B.L., S.Z., and H.W. wrote the manuscript. All authors discussed the results and commented on the manuscript.

References

1 Luh, L. M. *et al.* Prey for the Proteasome: Targeted Protein Degradation-A Medicinal Chemist's Perspective. *Angew Chem Int Ed Engl* **59**, 15448-15466 (2020).

2 Hanan, E. J. *et al.* Monomeric Targeted Protein Degraders. *Journal of Medicinal Chemistry* **63**, 11330-11361 (2020).

3 Bekes, M., Langley, D. R. & Crews, C. M. PROTAC targeted protein degraders: the past is prologue. *Nat Rev Drug Discov* **21**, 181-200 (2022).

4 Hanzl, A. *et al.* Functional E3 ligase hotspots and resistance mechanisms to small-molecule degraders. *Nat Chem Biol* **19**, 323-333 (2023).

5 Ottis, P. *et al.* Cellular Resistance Mechanisms to Targeted Protein Degradation Converge Toward Impairment of the Engaged Ubiquitin Transfer Pathway. *ACS Chem Biol* **14**, 2215-2223 (2019).

6 Zhang, L., Riley-Gillis, B., Vijay, P. & Shen, Y. Acquired Resistance to BET-PROTACs (Proteolysis-Targeting Chimeras) Caused by Genomic Alterations in Core Components of E3 Ligase Complexes. *Mol Cancer Ther* **18**, 1302-1311 (2019).

7 Zorba, A. *et al.* Delineating the role of cooperativity in the design of potent PROTACs for BTK. *P Natl Acad Sci USA* **115**, E7285-E7292 (2018).

8 Vetma, V. *et al.* Confounding factors in targeted degradation of short-lived proteins. *bioRxiv*, 2024.2002.2019.581012 (2024).

9 Li, J., Cai, Z., Li, X. W. & Zhuang, C. Natural Product-Inspired Targeted Protein Degraders: Advances and Perspectives. *J Med Chem* **65**, 13533-13560 (2022).

10 Karageorgis, G., Foley, D. J., Laraia, L., Brakmann, S. & Waldmann, H. Pseudo Natural Products-Chemical Evolution of Natural Product Structure. *Angew Chem Int Ed Engl* **60**, 15705-15723 (2021).

11 Grigalunas, M., Brakmann, S. & Waldmann, H. Chemical Evolution of Natural Product Structure. *J Am Chem Soc* **144**, 3314-3329 (2022).

12 Grigalunas, M., Burhop, A., Christoforow, A. & Waldmann, H. Pseudo-natural products and natural product-inspired methods in chemical biology and drug discovery. *Curr Opin Chem Biol* **56**, 111-118 (2020).

13 Gerriets, V. A. & Rathmell, J. C. Metabolic pathways in T cell fate and function. *Trends Immunol* **33**, 168-173 (2012).

14 Munn, D. H. & Mellor, A. L. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends Immunol* **34**, 137-143 (2013).

15 Prendergast, G. C. *et al.* Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer. *Cancer Immunol Immunother* **63**, 721-735 (2014).

16 Fiore, A. *et al.* Kynurenine importation by SLC7A11 propagates anti-ferroptotic signaling. *Mol Cell* **82**, 920-932 e927 (2022).

17 Opitz, C. A. *et al.* An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature* **478**, 197-203 (2011).

18 Müller-Durovic, B. *et al.* A metabolic dependency of EBV can be targeted to hinder B cell transformation. *Science* **0**, eadk4898 (2024).

19 Wang, X. X. *et al.* Recent advances in the discovery of indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors. *Medchemcomm* **10**, 1740-1754 (2019).

20 Rohrig, U. F., Majjigapu, S. R., Vogel, P., Zoete, V. & Michielin, O. Challenges in the Discovery of Indoleamine 2,3-Dioxygenase 1 (IDO1) Inhibitors. *J Med Chem* **58**, 9421-9437 (2015).

21 Pradhan, N. *et al.* Inhibition of immunosuppressive indoleamine 2,3-dioxygenase by targeting the heme and apo-form. *Chem Commun (Camb)* **57**, 395-398 (2021).

22 Nelp, M. T. *et al.* Immune-modulating enzyme indoleamine 2,3-dioxygenase is effectively inhibited by targeting its apo-form. *Proceedings of the National Academy of Sciences* **115**, 3249-3254 (2018).

23 Ortiz-Meoz, R. F. *et al.* Characterization of Apo-Form Selective Inhibition of Indoleamine 2,3-Dioxygenase**. *ChemBioChem* **21**, 1-8 (2020).
24 Hennes, E. *et al.* Cell-Based Identification of New IDO1 Modulator Chemotypes. *Angewandte Chemie (International ed. in English)* **60**, 9869-9874 (2021).
25 Markwalder, J. A. *et al.* Identification and optimization of a novel series of indoleamine 2,3-dioxygenase inhibitors. *Bioorganic & Medicinal Chemistry Letters* **27**, 582-585 (2017).
26 Liu, X. *et al.* Selective inhibition of IDO1 effectively regulates mediators of antitumor immunity. *Blood* **115**, 3520-3530 (2010).
27 Spranger, S. *et al.* Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. *J Immunother Cancer* **2**, 3 (2014).
28 Eynde, B. J. V. d., Baren, N. v. & Baurain, J.-F. Is There a Clinical Future for IDO1 Inhibitors After the Failure of Epacadostat in Melanoma? *Annual Review of Cancer Biology* **4**, 241-256 (2020).
29 Muller, A. J., Manfredi, M. G., Zakharia, Y. & Prendergast, G. C. Inhibiting IDO pathways to treat cancer: lessons from the ECHO-301 trial and beyond. *Semin Immunopathol* **41**, 41-48 (2019).
30 Van den Eynde, B. J., van Baren, N. & Baurain, J. F. Is There a Clinical Future for IDO1 Inhibitors After the Failure of Epacadostat in Melanoma? *Annu Rev Canc Biol* **4**, 241-256 (2020).
31 Zeitler, L. & Murray, P. J. IL4i1 and IDO1: Oxidases that control a tryptophan metabolic nexus in cancer. *J Biol Chem* **299**, 104827 (2023).
32 Zhai, L. *et al.* Tumor Cell IDO Enhances Immune Suppression and Decreases Survival Independent of Tryptophan Metabolism in Glioblastoma. *Clin Cancer Res* **27**, 6514-6528 (2021).
33 Mellor, A. L. & Munn, D. H. Ido expression by dendritic cells: tolerance and tryptophan catabolism. *Nature Reviews Immunology* **4**, 762-774 (2004).
34 Chen, W., Liang, X., Peterson, A. J., Munn, D. H. & Blazar, B. R. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. *J Immunol* **181**, 5396-5404 (2008).
35 Pallotta, M. T. *et al.* Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nat Immunol* **12**, 870-878 (2011).
36 https://clinicaltrials.gov/.
37 Brincks, E. L. *et al.* Indoximod opposes the immunosuppressive effects mediated by IDO and TDO via modulation of AhR function and activation of mTORC1. *Oncotarget* **11**, 2438-2461 (2020).
38 Hu, M. *et al.* Discovery of the first potent proteolysis targeting chimera (PROTAC) degrader of indoleamine 2,3-dioxygenase 1. *Acta Pharm Sin B* **10**, 1943-1953 (2020).
39 Bollu, L. R. *et al.* Identification and Characterization of a Novel Indoleamine 2,3-Dioxygenase 1 Protein Degrader for Glioblastoma. *J Med Chem* **65**, 15642-15662 (2022).
40 Scholes, C. S. *et al.* Inhibitor-induced supercharging of kinase turnover via endogenous proteolytic circuits. *bioRxiv* (2024).
41 Théate, I. *et al.* Extensive profiling of the expression of the indoleamine 2,3-dioxygenase 1 protein in normal and tumoral human tissues. *Cancer Immunol Res* **3**, 161-172 (2015).
42 Adariani, S. R. *et al.* Detection of a Mitochondrial Stress Phenotype using the Cell Painting Assay. *bioRxiv*, 2023.2011.2008.565491 (2023).
43 Hjerpe, R. *et al.* Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep* **10**, 1250-1258 (2009).
44 Kim, W. *et al.* Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* **44**, 325-340 (2011).
45 Chau, V. *et al.* A Multiubiquitin Chain Is Confined to Specific Lysine in a Targeted Short-Lived Protein. *Science* **243**, 1576-1583 (1989).

46 Kawatkar, A. *et al.* CETSA beyond Soluble Targets: a Broad Application to Multipass Transmembrane Proteins. *ACS Chemical Biology* **14**, 1913-1920 (2019).
47 Röhrig, U. F., Michielin, O. & Zoete, V. Structure and Plasticity of Indoleamine 2,3-Dioxygenase 1 (IDO1). *Journal of Medicinal Chemistry* **64**, 17690-17705 (2021).
48 Kagiou, C. *et al.* Alkylamine-tethered molecules recruit FBXO22 for targeted protein degradation. *Nat Commun* **15**, 5409 (2024).
49 Hsia, O. *et al.* Targeted protein degradation via intramolecular bivalent glues. *Nature* **627**, 204-211 (2024).
50 Sherpa, D., Chrustowicz, J. & Schulman, B. A. How the ends signal the end: Regulation by E3 ubiquitin ligases recognizing protein termini. *Molecular Cell* **82**, 1424-1438 (2022).
51 Koren, I. *et al.* The Eukaryotic Proteome Is Shaped by E3 Ubiquitin Ligases Targeting C-Terminal Degrons. *Cell* **173**, 1622-+ (2018).
52 Scott, D. C. *et al.* E3 ligase autoinhibition by C-degron mimicry maintains C-degron substrate fidelity. *Mol Cell* **83**, 770-786 e779 (2023).
53 Peyraud, F. *et al.* Targeting Tryptophan Catabolism in Cancer Immunotherapy Era: Challenges and Perspectives. *Front Immunol* **13** (2022).
54 Wang, X. *et al.* A covalently bound inhibitor triggers EZH2 degradation through CHIP-mediated ubiquitination. *Embo J* **36**, 1243-1260 (2017).
55 Slabicki, M. *et al.* Small-molecule-induced polymerization triggers degradation of BCL6. *Nature* **588**, 164-+ (2020).