Target protein localization and its impact on PROTAC-mediated degradation

Graphical abstract

Highlights

- Protein subcellular context can affect the efficacy of PROTAC-mediated degradation
- Halo and FKBP12\textsuperscript{F36V} degradation assessed in different subcellular compartments
- Halo- or FKBP12\textsuperscript{F36V}-directed PROTACs recruiting VHL and/or CRBN tested
- Differentially localized proteins showed varying levels of degradation with PROTACs

Authors
Luke M. Simpson, Lorraine Glennie, Abigail Brewer, Jin-Feng Zhao, Jennifer Crooks, Natalia Shpiro, Gopal P. Sapkota

Correspondence
g.sapkota@dundee.ac.uk

In brief
Simpson et al. interrogate how the subcellular context of a target protein influences its amenability to PROTAC-mediated degradation. Differentially localized proteins displayed varying levels of degradation using the same respective PROTACs, suggesting that the subcellular context of the target protein can influence the efficacy of PROTAC-mediated POI degradation.
Target protein localization and its impact on PROTAC-mediated degradation

Luke M. Simpson,1,2 Lorraine Glennie,1,2 Abigail Brewer,1 Jin-Feng Zhao,1 Jennifer Crooks,1 Natalia Shpiro,1 and Gopal P. Sapkota1,3,*
1Medical Research Council (MRC) Protein Phosphorylation & Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK
2These authors contributed equally
3Lead contact
*Correspondence: g.sapkota@dundee.ac.uk
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SUMMARY

Proteolysis-targeting chimeras (PROTACs) bring a protein of interest (POI) into spatial proximity of an E3 ubiquitin ligase, promoting POI ubiquitylation and proteasomal degradation. PROTACs rely on endogenous cellular machinery to mediate POI degradation, therefore the subcellular location of the POI and access to the E3 ligase being recruited potentially impacts PROTAC efficacy. To interrogate whether the subcellular context of the POI influences PROTAC-mediated degradation, we expressed either Halo or FKBP12F36V (dTAG) constructs consisting of varying localization signals and tested the efficacy of their degradation by von Hippel-Lindau (VHL)- or cereblon (CRBN)-recruiting PROTACs targeting either Halo or dTAG. POIs were localized to the nucleus, cytoplasm, outer mitochondrial membrane, endoplasmic reticulum, Golgi, peroxisome or lysosome. Differentially localized Halo or FKBP12F36V proteins displayed varying levels of degradation using the same respective PROTACs, suggesting therefore that the subcellular context of the POI can influence the efficacy of PROTAC-mediated POI degradation.

INTRODUCTION

Targeted protein degradation (TPD) is a promising strategy for both therapeutics and research, providing new opportunities to overcome some of the current limitations associated with traditional small-molecule therapeutics and gene silencing approaches (Chamberlain and Hamann, 2019; Sun et al., 2019). Various technologies using TPD strategies have thus been developed, such as proteolysis-targeting chimeras (PROTACs) (Bondeson et al., 2015; Bondeson and Crews, 2017). PROTACs are heterobifunctional molecules consisting of two distinct target-binding moieties, connected by an optimized linker (Nalawansha and Crews, 2020). Typically, PROTACs bind a target protein of interest (POI) with one moiety, while simultaneously binding an E3 ubiquitin (Ub) ligase of interest with the other, bringing the POI into spatial proximity of the E3, triggering POI ubiquitylation and subsequent proteasomal degradation (Sakamoto et al., 2001; Lucas and Ciulli, 2017; Toure and Crews, 2016). PROTACs offer several benefits over conventional small-molecule inhibitors that often have target enzymatic activity. By removing the POI, PROTACs abolish both enzymatic and non-enzymatic functions, such as scaffolding, chaperoning, and trans-activating roles (Burslem et al., 2018; Cromm et al., 2018; Popow et al., 2019). In addition, although PROTACs are typically larger in size than most conventional small molecules, evidence suggests promising oral bioavailability, tissue penetration and in vivo activity for some PROTACs (Xiang et al., 2021; Neklesa et al., 2019). Excitingly, multiple orally bioavailable PROTAC candidates have progressed to human clinical trials (Mullard, 2019; Petrylak et al., 2020).

The human genome encodes more than 600 E3 ubiquitin ligases, some of which exist as larger complexes with regulatory and substrate-recruiting subunits that serve to direct E3 catalytic activity to specific substrates (Li et al., 2008). Yet only a handful of E3 ubiquitin ligases or substrate-recruiting subunits have been successfully hijacked and used for PROTAC development, including the von Hippel-Lindau (VHL) and cereblon (CRBN) substrate receptors (Jevtic et al., 2021; Kannt and Dikic, 2021; Belcher et al., 2021; Bond and Crews, 2021). VHL is the substrate receptor in a complex with Elongin B and C adaptors, Cullin 2 (CUL2) and the RING box protein 1 (RBX1) E3 ubiquitin ligase, collectively referred to as the CRL2VHL E3 ligase complex (Cardote et al., 2017). Under oxygen-rich conditions, VHL recognizes and binds to hydroxyproline-modified hypoxia-inducible factor 1α (HIF1α), bringing HIF1α into proximity of CRL2VHL, mediating its ubiquitylation and degradation through the ubiquitin-proteasome system (UPS) (Ohh et al., 2000; Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). PROTACs that use hydroxyproline mimetics have been developed to recruit VHL and bring CRL2VHL into proximity of specific POIs, including the estrogen-related receptor α (ERRα) and the receptor-interacting
Figure 1. PROTAC-mediated degradation of Halo and dTAG proteins with respective PROTACs

(A) The structures of PROTACs used in this study to mediate degradation of Halo and FKBP12\(^{F36V}\) proteins: (i) dTAG-VHL, (ii) dTAG\(^2\)-1, (iii) dTAG-13, and (iv) HaloPROTAC-E.
serine/threonine protein kinase 2 (RIPK2), to induce their ubiquitylation and degradation through the UPS (Bondeson et al., 2015). Additional VHL ligands, including the VHL/HIF-1α interaction inhibitors VH032 (Galdeano et al., 2014) and VH298 (Frost et al., 2016), have also been used to develop efficient PROTAC degraders (Tovell et al., 2019a, 2019b; Zengerle et al., 2015; Chan et al., 2015). Similarly, efficient CRBN-recruiting degraders have been developed (Belcher et al., 2021). CRBN is the substrate receptor for the Cullin 4A (CUL4A) E3 ligase and is referred to as CRL4ACRBN (Petzold et al., 2016). The endogenous substrates of CRBN have remained largely elusive, although a few, including MEIS2 (Fischer et al., 2014), glutamine synthase (Van Nguyen et al., 2016), c-jun (Yang et al., 2018) and casein kinase 1α (Shen et al., 2021), have been reported to be degraded through the UPS. Immunomodulatory imide drug (IMiD) derivatives, such as thalidomide, lenalidomide and pomalidomide, act as molecular glues between CRBN and various neosubstrates, mediating their CRL4ACRBN-dependent ubiquitylation and proteasomal degradation (Kronke et al., 2014, 2015; An et al., 2017; Dunbar et al., 2021). PROTACs which use IMiD derivatives have been developed to recruit CRBN to specific POIs, including the bromodomain (BRD) and extra-terminal domain protein BRD4 and the cytoplasmic signaling FK506-binding protein FKB12 (Winter et al., 2015; Lu et al., 2015), to promote their CRL4ACRBN-dependent ubiquitylation and proteasomal degradation.

Protein tag-based PROTAC technologies have been developed to determine the effectiveness of UPS-mediated POI degradation and its effect on POI function (Roth et al., 2019). Halo/VHL-based PROTACs that bind the HaloTag (Los et al., 2008; Chana et al., 2009) using a chloroalkane group, and VHL through either a hydroxyproline derivative (HaloPROTAC3) (Buckley et al., 2015) or VH298 (HaloPROTAC-E) (Tovell et al., 2019a), have been described for the degradation of various Halo-tagged POIs. The degradation tag (dTAG) system uses an AP1867 ligand, which selectively binds an FKB12 mutant protein (FKBP12F36V) and not wild-type (WT) FKB12 (Clackson et al., 1998; Nabet et al., 2018, 2020). dTAG-based PROTACs that recruit either CRBN (dTAG-13) (Erb et al., 2017; Nabet et al., 2018) or VHL (dTAGV-1) (Nabet et al., 2018, 2020) have been developed to mediate robust degradation of FKB12F36V-tagged POIs. Interestingly, differences between the efficiency and range of targets that dTAGV-1 and dTAG-13 could degrade have been observed (Nabet et al., 2020). For example, only dTAGV-1 was able to degrade the FKB12F36V-tagged oncogenic transcription factor EWS/FLI and K-RAS GTPase, whereas dTAG-13 was not (Zeng et al., 2020; Bond et al., 2020; Nabet et al., 2020). In contrast, efficient degradation with CRBN-recruiting PROTACs was observed when targeting the oncogenic tyrosine kinase BCR-ABL, whereas VHL-recruiting PROTACs were less successful (Lai et al., 2016). Because PROTACs redirect an endogenous E3 ligase to a neosubstrate and consequently rely on endogenous cellular machinery, differences in efficacy of degradation might arise from several factors, including abundance and activity of the E3 ligase being recruited, and the subcellular distribution of the E3 ligase or POI (Zhang et al., 2019).

To systematically investigate the impact of POI localization on PROTAC-mediated degradation, we designed and expressed either Halo or FKB12F36V (dTAG) protein tags with or without varying localization signals (nucleus, cytoplasm, outer mitochondrial membrane [OMM], endoplasmic reticulum [ER], Golgi [facing either lumen or cytoplasm], peroxisome, or lysosome) and sought to test the efficacy of their degradation by leveraging on VHL- or CRBN-recruiting PROTACs targeting either Halo or dTAG (Figures 1A and 1B). We included a FLAG reporter on these for detection and a HiBiT tag to allow quantification of degradation (Schwinn et al., 2018, 2020; Riching et al., 2018, 2020, 2021; Imaide et al., 2021; Le Boulch et al., 2020; Caine et al., 2020; Larson et al., 2021; Martinez et al., 2021; Uchida et al., 2021). For Halo, the Halo/VHL-recruiter HaloPROTAC-E, which uses the VHL ligand VH298 (Tovell et al., 2019a; Frost et al., 2016) was used. For dTAG, the FKB12F36V/CRBN recruiter dTAG-13 was tested (Nabet et al., 2018), along with a FKB12F36V/VHL-recruiting PROTAC consisting of the VHL warhead VH032 (Chan et al., 2018; Tovell et al., 2019a; Frost et al., 2016) and the dTAG binder AP1867 (Nabet et al., 2018, 2020; Clackson et al., 1998), which we synthesized and termed dTAGV-VHL (Figure 1A). We also compared dTAG-VHL and dTAGV-1 (Nabet et al., 2020), when the latter became available.

Two independent tag-based systems, Halo and dTAG, and two independent E3s, VHL and/or CRBN, were tested to provide a comprehensive analysis of the impact of target POI localization on the efficacy of PROTAC-mediated target degradation.

**RESULTS**

**Degradation of Halo and FKB12F36V proteins with respective PROTACs**

We first expressed Halo (FLAG-Halo-HiBiT) or FKB12F36V (FLAG-FKB12F36V-HiBiT) proteins without added subcellular localization signals in U2OS cells by retroviral transduction. Anti-FLAG immunofluorescence (IF) microscopy showed a pan-cellular distribution of both Halo and FKB12F36V proteins,
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whereas no signal was observed in U2OS wild-type control cells (Figure 1C). When cells expressing Halo were treated with increasing concentrations of HaloPROTAC-E (5–5,000 nM) for 24 h, a concentration-dependent reduction of Halo was observed, with an optimal degradation seen with 250 and 500 nM relative to DMSO-treated controls (Figure 1D). When cells were treated with 500 nM HaloPROTAC-E over a course of 24 h, time-dependent Halo degradation was observed, with maximal degradation observed at 24 h (Figure 1E). Similarly, when cells expressing FKBP12F36V were treated with increasing concentrations of either dTAG-VHL (5–5,000 nM) or dTAG-13 (5–5,000 nM) for 24 h, a concentration-dependent reduction of FKBP12F36V with both compounds was observed. For dTAG-VHL, optimal degradation was observed with 500 nM, while with dTAG-13 the maximal degradation was observed with 250 and 500 nM (Figure 1F). When cells were treated with 500 nM dTAG-VHL or dTAG-13 over a course of 24 h, we observed an optimal degradation of FKBP12F36V by dTAG-VHL within 1 h, whereas slower degradation kinetics were observed for dTAG-13, with optimal degradation observed at 6 h (Figure 1G). Given robust degradation of both Halo and FKBP12F36V proteins with their respective PROTACs, we sought to direct these protein tags to different subcellular compartments through established localization signals (Figure 1B) to assess the impact of subcellular distribution on PROTAC-induced degradation.

Nuclear-localized Halo and dTAG proteins are efficiently degraded by corresponding PROTACs

We expressed either Halo (FLAG-NLS-Halo-HiBiT) or FKBP12F36V (FLAG-NLS-FKBP12F36V-HiBiT) proteins consisting of the SV40 T-antigen nuclear localization sequence (NLS) (Kalderon et al., 1984) in U2OS cells by retroviral transduction. Both NLS-tagged Halo and FKBP12F36V proteins localized to the nucleus, as observed by anti-FLAG IF microscopy staining, which showed predominantly overlapping staining with DAPI nuclear staining, whereas the signal was absent from non-transduced U2OS WT control cells (Figure 2A).

When cells expressing FLAG-NLS-Halo-HiBiT were treated with increasing concentrations of HaloPROTAC-E (5–5,000 nM) for 24 h, dose-dependent degradation of FLAG-NLS-Halo-HiBiT was observed by both HiBiT lytic assay (Figure 2B) and western blot (Figure 2C), with optimal degradation seen with 500 nM. A time course treatment with 500 nM HaloPROTAC-E over 24 h showed time-dependent degradation of FLAG-NLS-Halo-HiBiT, with optimal degradation observed at 24 h (Figures 2D and 2E). Similarly, when cells expressing FLAG-NLS-FKBP12F36V-HiBiT were treated with increasing concentrations of either dTAG-VHL or dTAG-13 (5–5,000 nM) for 24 h, dose-dependent degradation of FLAG-NLS-FKBP12F36V-HiBiT was observed with both dTAG-VHL and dTAG-13 (Figures 2F and 2G), whereas no changes in endogenous FKBP12 protein levels were observed with either PROTAC (Figure 2G). Interestingly, a higher level of FLAG-NLS-FKBP12F36V-HiBiT degradation was seen with lower concentrations of dTAG-13 compared with dTAG-VHL (Figures 2F and 2G). Optimal degradation was observed with 500 nM of either dTAG-VHL or dTAG-13 (Figures 2F and 2G). A time course treatment with 500 nM dTAG-VHL or dTAG-13 over 24 h showed time-dependent degradation of FLAG-NLS-FKBP12F36V-HiBiT protein, with optimum degradation observed at 24 h with both compounds (Figures 2H and 2I). However, quicker and more efficient degradation was observed with dTAG-13 compared with dTAG-VHL (Figures 2H and 2I).

Characterization of the mode of action for the degradation of nuclear Halo and dTAG proteins by PROTACs

Because we describe the synthesis of a dTAG-VHL PROTAC and demonstrate degradation of FLAG-FKBP12F36V-HiBiT and FLAG-NLS-FKBP12F36V-HiBiT proteins in cells, we sought to validate its mode of action and tolerability in cells. In parallel, we generated a dTAG-VHL-inactive PROTAC by using a hydroxyl epimer of the VH032 that is incapable of binding to and recruiting VHL (Tovell et al., 2019a) as a negative control for the active dTAG-VHL. In cells expressing FLAG-NLS-FKBP12F36V-HiBiT, dTAG-VHL PROTAC was able to degrade the FKBP12F36V protein in a time- and dose-dependent manner, whereas the dTAG-VHL-inactive compound was unable to degrade the FKBP12F36V protein at any concentration tested over 24 h (Figures 3A and 3B). Furthermore, neither compound affected the levels of endogenous FKBP12 protein, which does not bind to the dTAG warhead (Figures 3A and 3B). We also tested the cytotoxicity of both dTAG-VHL-active and dTAG-VHL-inactive molecules at concentrations ranging from 125 nM to 16 μM and found no measurable cytotoxicity at these

Figure 2. Nuclear-localized Halo and dTAG proteins are efficiently degraded by corresponding PROTACs

NLS, SV40 T-antigen nuclear localization sequence (A) Wild-type (WT) U2OS cells or those expressing FLAG-NLS-Halo-HiBiT or FLAG-NLS-FKBP12F36V-HiBiT were fixed and subjected to anti-FLAG IF microscopy. DNA is stained with DAPI. Scale bars, 10 μm. (B) WT U2OS cells or those expressing FLAG-NLS-Halo-HiBiT were treated with indicated concentrations of HaloPROTAC-E for 24 h. HiBiT protein levels were then quantified using the lytic detection system. Corresponding WT relative light unit (RLU) values were subtracted from FLAG-NLS-Halo-HiBiT values and the percentage (%) of HiBiT RLU values were then calculated against DMSO-treated controls ± SD of n = 3 independent experiments. (C) Immunoblot analysis of (B). (D) As in (B), except WT U2OS cells or those expressing FLAG-NLS-Halo-HiBiT were treated with 500 nM HaloPROTAC-E for indicated times. (E) Immunoblot analysis of (D). (F) As in (B), except WT U2OS cells or those expressing FLAG-NLS-FKBP12F36V-HiBiT were treated with indicated concentrations of dTAG-VHL or dTAG-13 for 24 h. (G) Immunoblot analysis of (F). (H) As in (F), except WT U2OS cells or those expressing FLAG-NLS-FKBP12F36V-HiBiT were treated with 500 nM dTAG-VHL or dTAG-13 for indicated times. (I) Immunoblot analysis of (H). For (C), (E), (G), and (I), extracts were resolved by SDS-PAGE and transferred on to PVDF membranes, which were subjected to immunoblotting with indicated antibodies. Solid circles denote endogenous FKBP12, and open circles denote FLAG-NLS-FKBP12F36V-HiBiT. S.E., short exposure; L.E., long exposure. Blots are representative of 3 independent experiments.
Figure 3. Characterization of the mode of action for the degradation of nuclear Halo and dTAG proteins by PROTACs

(A) U2OS cells expressing FLAG-NLS-FKBP12(36V)-HiBiT were treated with active or inactive dTAG-VHL at the indicated concentrations for 24 h.

(B) U2OS cells expressing FLAG-NLS-FKBP12(36V)-HiBiT were treated with either active (500 nM) or inactive (500 nM) dTAG-VHL for the indicated time points.

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concentrations, while MG132, which was used as a positive control, led to profound cell death (Figure 3C). To confirm whether dTAG-VHL/dTAG-13-mediated FLAG-NLS-FKB12\textsuperscript{F36V}-HiBiT degradation requires the binding of either VHL or CRBN, respectively, and is facilitated by the CRL machinery and the proteasome, FLAG-NLS-FKB12\textsuperscript{F36V}-HiBiT-expressing cells were pre-treated with VH298 (50 \text{\mu M}, 2 h; Figure 3D), which occupies the same binding pocket in VHL as VH302, the pan-CUL NEDDylation inhibitor MLN4924 (Soucy et al., 2009) (1 \text{\mu M}, 2 h; Figure 3D), the CRBN-binding imidazoline (Lu et al., 2014; Kronke et al., 2014) (10 \text{\mu M}, 24 h; Figure 3E), and proteasome inhibitors MG132 (20 \text{\mu M}, 2 h; Figure 3F) or bortezomib (5 \text{\mu M}, 2 h; Figure 3F) and then treated with 500 \text{nM} dTAG-VHL or dTAG-13 for 24 h. In the presence of VH298, dTAG-VHL-mediated, but not dTAG-13-mediated, nuclear FKBP12\textsuperscript{F36V} degradation was inhibited (Figure 3D), suggesting that dTAG-VHL requires the binding to VHL, whereas dTAG-13 does not. In lenalidomide-treated cells, a reduction in ZFP91 protein levels, a previously reported lenalidomide neosubstrate (An et al., 2017), was observed (Figure 3E), suggesting successful CRBN engagement. In the presence of lenalidomide, dTAG-13-mediated, but not dTAG-VHL-mediated, FLAG-NLS-FKB12\textsuperscript{F36V}-HiBiT degradation was inhibited, suggesting that dTAG-13 requires the binding to CRBN, whereas dTAG-VHL does not. In the presence of MLN4924 (Figure 3D), MG132 (Figure 3F) or bortezomib (Figure 3F), both dTAG-VHL- and dTAG-13-mediated nuclear FKBP12\textsuperscript{F36V} degradation was inhibited, suggesting the requirement of the CRL machinery and the proteasome for both PROTACs. An increase in HIF1\textalpha protein levels was observed with both VH298 and MLN4924, while a collapse in the higher molecular weight band corresponding to NEDDylated CUL2 was seen only with MLN4924, suggesting successful inhibition of VHL and CUL NEDDylation, respectively (Figure 3D). An increase in protein polyubiquitylation was observed with both MG132 and bortezomib compared with DMSO-treated controls, suggesting successful inhibition of the proteasome (Figure 3F).

To confirm that HaloPROTAC-E-mediated nuclear Halo protein degradation requires the binding of HaloPROTAC-E to VHL and is facilitated by the CRL machinery and the proteasome, similar treatments as above were undertaken. In the presence of VH298 (Figure 3G), MLN4924 (Figure 3G), MG132 (Figure 3H), or bortezomib (Figure 3H), HaloPROTAC-E-mediated FLAG-NLS-Halo-HiBiT degradation was inhibited, suggesting the requirement of VHL, the CRL machinery, and the proteasome. An upward electrophoretic band shift for FLAG was observed in the presence of HaloPROTAC-E following inhibition of VHL, CUL NEDDylation, or proteasome, suggesting modification of nuclear Halo, potentially due to the covalent nature of Halo binding with the chloroalkane moiety of HaloPROTAC-E (Figures 3G and 3H).

**Efficient degradation of cytoplasmic-localized Halo and dTAG proteins with PROTACs**

We expressed Halo (FLAG-Halo-nes-HiBiT) or FKB12\textsuperscript{F36V} (FLAG-FKB12\textsuperscript{F36V}-NES-HiBiT) proteins consisting of the HIV Rev protein leucine-rich nuclear export signal (NES) (Fischer et al., 1995; Meyer et al., 1996; Wen et al., 1995) in U2OS cells and confirmed their cytoplasmic localization with anti-FLAG immunofluorescence microscopy, which showed diffused and predominantly cytoplasmic staining that did not overlap with DAPI nuclear staining (Figure S1A). Treatment of cells expressing FLAG-Halo-nes-HiBiT with increasing concentrations of HaloPROTAC-E (5–5,000 nM) for 24 h showed dose-dependent degradation of cytoplasmic Halo protein, with optimal degradation seen with 500 nM by both HiBiT lytic assay (Figure S1B) and western blot (Figure S1C). A time course treatment of cells with 500 nM HaloPROTAC-E over 24 h revealed time-dependent degradation of cytoplasmic Halo protein, with optimal degradation observed at 24 h (Figures S1D and S1E). HaloPROTAC-E-mediated degradation of cytoplasmic Halo protein was inhibited by pre-treatment of cells with VH298 (Figure 3G), MLN4924 (Figure 3G), MG132 (Figure 3H), or bortezomib (Figure 3H), suggesting the requirement of VHL, the CRL machinery, and the proteasome. Similar to that seen with FLAG-NLS-Halo-HiBiT (Figures 3G and 3H), an upward electrophoretic band shift for FLAG was observed in the presence of HaloPROTAC-E following VHL, CUL, or proteasomal inhibition, suggesting modification of Halo-NLS-HiBiT potentially due to the covalent nature of binding with HaloPROTAC-E. Next, when cells expressing FLAG-FKB12\textsuperscript{F36V}-NES-HiBiT were treated with increasing concentrations of either dTAG-VHL or dTAG-13 (5–5,000 nM) for 24 h, dose-dependent degradation of cytoplasmic FKB12\textsuperscript{F36V} was observed with both dTAG-VHL and dTAG-13, with optimal degradation for both occurring at 500 nM (Figures S1F and S1G). A higher level of degradation was observed with lower concentrations of dTAG-13 compared with dTAG-VHL (Figures S1F and S1G). However, a high-dose hook effect was observed with 5,000 nM of either dTAG-VHL or dTAG-13, potentially due to binary complex formation out-competing that of the active ternary complex (Douglass et al., 2013). A time course treatment of cells expressing cytoplasmic FKB12\textsuperscript{F36V} with 500 nM dTAG-VHL or dTAG-13 over 24 h showed time-dependent degradation of FKB12\textsuperscript{F36V} with both dTAG-VHL and dTAG-13 (Figures S1H and S1I). However,
quicker and more efficient degradation was observed with dTAG-13 compared with dTAG-VHL (Figures S1H and S1I).

Degradation of outer mitochondrial membrane-localized Halo and dTAG proteins with PROTACs

We expressed Halo (HiBiT-FLAG-Halo-FIS1101–152) or FKBP12/FKBP12(F36V)-FIS1101–152 proteins containing the C-terminal OMM localization signal of the mitochondrial fission 1 (FIS1) protein (residues 101–152 [FIS1101–152]) (Allen et al., 2013) in U2OS cells by retroviral transduction. Both HiBiT-FLAG-Halo/FKBP12(F36V)-FIS1101–152 proteins showed overlapping mitochondrial signal with mitochondrial marker ATPb (Allen et al., 2013; Zhao et al., 2020; Singh et al., 2021) when cells were subjected to IF microscopy with anti-FLAG and anti-ATPb antibodies, respectively (Figure 4A).

When we treated cells expressing HiBiT-FLAG-Halo-FIS1101–152 with increasing concentrations of HaloPROTAC-E (5–5,000 nM) for 24 h, dose-dependent degradation of HiBiT-FLAG-Halo-FIS1101–152 was observed, with optimal degradation seen with 500 nM (Figures 4B and 4C). A time course treatment of these cells with 500 nM HaloPROTAC-E over 24 h resulted in time-dependent degradation of HiBiT-FLAG-Halo-FIS1101–152, with optimal degradation seen at 24 h (Figures 4D and 4E). When cells expressing HiBiT-FLAG-FKBP12(F36V)-FIS1101–152 were treated with increasing concentrations of either dTAG-VHL or dTAG-13 (5–5,000 nM) for 24 h, dose-dependent degradation of HiBiT-FLAG-FKBP12(F36V)-FIS1101–152 was observed only with dTAG-13, but not with dTAG-VHL (Figures 4F and 4G). Optimal degradation, albeit partial, was seen with 250 nM dTAG-13, while a high-dose hook effect was observed with 5,000 nM of both dTAG-VHL and dTAG-13 (Figures 4F and 4G). When cells expressing HiBiT-FLAG-FKBP12(F36V)-FIS1101–152 were subjected to a time course treatment with 250 nM dTAG-VHL or dTAG-13 over 24 h, time-dependent degradation was observed only with dTAG-13, with optimal degradation seen after 24 h but not with dTAG-VHL (Figures 4H and 4I).

Degradation of endoplasmic reticulum-localized Halo and dTAG proteins with PROTACs

To investigate whether ER-localized proteins can be degraded with PROTACs, cells expressing Halo (HiBiT-FLAG-Halo-KDEL) or FKBP12(F36V) (HiBiT-FLAG-FKBP12(F36V)-KDEL) proteins consisting of the C-terminal KDEL ER retention sequence (Munro and Pelham, 1987) were generated. ER localization of these proteins was confirmed by IF microscopy, which showed overlapping staining with anti-FLAG and anti-calnexin (an ER marker) (Gödel et al., 2013) antibodies, although partial nuclear staining was also observed (Figure 5A). Next, when cells expressing HiBiT-FLAG-Halo-KDEL were treated with increasing concentrations of HaloPROTAC-E (5–5,000 nM) for 24 h, dose-dependent degradation of ER-localized Halo was observed with both HiBiT lytic assay and western blotting, with optimal degradation seen with 50 nM (Figures 5B and 5C). A time course treatment with 50 nM HaloPROTAC-E over 24 h showed time-dependent degradation of ER-localized Halo, with optimal degradation seen at 24 h (Figures 5D and 5E). Similarly, when HiBiT-FLAG-FKBP12(F36V)-KDEL expressing cells were treated with increasing concentrations of either dTAG-VHL or dTAG-13 (5–5,000 nM) for 24 h, dose-dependent degradation of HiBiT-FLAG-FKBP12(F36V)-KDEL was observed with both compounds, although higher levels of degradation was seen with lower concentrations of dTAG-VHL compared with dTAG-13, with optimal degradation for both seen with 500 nM (Figures 5F and 5G). This was opposite to what was observed for nuclear-localized (Figures 2F–2I), cytoplasmic-localized (Figures 4F–4I), and OMM-localized (Figures 4F–4I) FKBP12(F36V) protein, where dTAG-13 was the superior degrader to dTAG-VHL. A high-dose hook effect was seen with 5,000 nM of both dTAG-VHL and dTAG-13 (Figures 5F and 5G). A time course treatment of HiBiT-FLAG-FKBP12(F36V)-KDEL expressing cells with either 500 nM dTAG-VHL or dTAG-13 over 24 h resulted in time-dependent degradation of FKBP12(F36V) with dTAG-VHL, with optimal degradation seen at 24 h, whereas only partial degradation was observed with dTAG-13 (Figures 5H and 5I). To confirm whether PROTAC-mediated degradation of ER-localized Halo and FKBP12(F36V) proteins was facilitated by the CRL machinery and the proteasome, cells expressing HiBiT-FLAG-Halo/FKBP12(F36V)-KDEL were pre-treated with Vh298, MLN4924, or the proteasome inhibitor MG132 or bortezomib 2 h prior to administering the respective PROTACs for 24 h. In the presence of Vh298 and MLN4924, dTAG-VHL-mediated ER-localized FKBP12(F36V) protein degradation was completely rescued (Figure S2A), suggesting that dTAG-VHL requires the
Figure 5. Degradation of endoplasmic reticulum (ER)-localized Halo and dTAG proteins with PROTACs

KDEL, ER lumen retention signal.

(A) Wild-type (WT) U2OS cells or those expressing HiBiT-FLAG-Halo-KDEL or HiBiT-FLAG-FKBP12<sup>36V</sup>-KDEL were fixed and subjected to anti-FLAG and anti-calnexin immunofluorescence microscopy. DNA is stained with DAPI. Scale bars, 10 μm.

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binding to VHL and the VHL-CUL2 CRL. Similarly, in the presence of VHH28 and MLN4924, HaloPROTAC-E-mediated degradation of ER-localized Halo protein was partially rescued, but the observation was confounded by the HaloPROTAC-E-induced mobility shift of the HiBiT-FLAG-Halo-KDEL (Figure S2B). Both MG132 and bortezomib rescued dTAG-VHL-, dTAG-13-, and HaloPROTAC-E-induced degradation of ER-localized FKBPI2/F36V and Halo proteins (Figures S2C–S2E), suggesting the involvement of the proteasome for degradation. These results imply that the degradation of ER-localized Halo and FKBPI2/F36V proteins by PROTACs is carried out by the respective E3 ligases and the proteasome.

**Degradation of Golgi-localized Halo and dTAG proteins with PROTACs**

We used the N-terminal transmembrane domain (residues 1–117) of human mannosidase II (MAN2A1) (Velasco et al., 1993) to mediate Golgi-lumen localization of Halo (MAN2A1–117-FLAG-Halo-HiBiT) or FKBPI2/F36V (MAN2A1–117-FLAG-FKBPI2/F36V-HiBiT) proteins in U2OS cells. To confirm that MAN2A1–117-FLAG-Halo/FKBPI2/F36V-HiBiT localized at the Golgi, WT U2OS cells or those expressing MAN2A1–117-FLAG-Halo-HiBiT or MAN2A1–117-FLAG-FKBPI2/F36V-HiBiT were subjected to anti-FLAG and anti-GM130 (Golgi marker) (Kowada et al., 2020; Zhang et al., 2021) immunofluorescence microscopy (Figure 6A). Both MAN2A1–117-FLAG-Halo-HiBiT and MAN2A1–117-FLAG-FKBPI2/F36V-HiBiT staining overlapped with GM130 staining, suggesting that both localize predominantly at the Golgi. When MAN2A1–117-FLAG-Halo-HiBiT-expressing cells were treated with increasing concentrations of HaloPROTAC-E (5–5,000 nM) for 24 h, no robust changes in MAN2A1–117-FLAG-Halo-HiBiT protein levels were observed by HiBiT lytic assay (Figure 6B), but a reduction was observed with 5,000 nM by western blot (Figure 6C). A time course treatment of cells with 500 nM HaloPROTAC-E over 24 h (Figures 6D and 6E) did not result in any changes in MAN2A1–117-FLAG-Halo-HiBiT levels at any time points relative to DMSO-treated controls. Similarly, when MAN2A1–117-FLAG-FKBPI2/F36V-HiBiT-expressing cells were treated with increasing concentrations of either dTAG-VHL or dTAG-13 (5–5,000 nM) for 24 h, no reduction in MAN2A1–117-FLAG-FKBPI2/F36V-HiBiT levels was observed with either dTAG-VHL or dTAG-13 by HiBiT lytic assay (Figure 6F). However, a high-dose hook effect was observed with 5,000 nM of dTAG-13 by HiBiT lytic assay (Figure 6F), whereas a reduction in MAN2A1–117-FLAG-FKBPI2/F36V-HiBiT levels was observed only with 5,000 nM dTAG-VHL by western blot (Figure 6G). A time course treatment of cells with 500 nM dTAG-VHL or dTAG-13 over 24 h showed no robust changes in MAN2A1–117-FLAG-FKBPI2/F36V-HiBiT protein levels with either dTAG-VHL or dTAG-13 (Figures 6H and 6I). To determine if the reduction in levels of Golgi-lumen localized Halo or FKBPI2/F36V proteins by high concentrations (5,000 nM) of HaloPROTAC-E and dTAG-VHL respectively was due to degradation through the proteasome, we used the proteasomal inhibitors MG132 and bortezomib. Neither MG132 nor bortezomib treatment was able to rescue the reduction in Golgi-lumen localized Halo or FKBPI2/F36V proteins caused by HaloPROTAC-E or dTAG-VHL, respectively (Figures S3A and S3B). Instead, both MG132 and bortezomib led to unusual mobility patterns of Golgi-lumen localized Halo or FKBPI2/F36V proteins shown by western blot, suggesting potential effects of these inhibitors on Golgi homeostasis. Treating cells expressing MAN2A1–117-FLAG-Halo-HiBiT protein with the lysosomal inhibitor bafilomycin A1 also failed to rescue the reduction in levels of Halo protein caused by HaloPROTAC-E (Figure S3C).

We sought to test whether placing Halo on the cytoplasmic face instead of the lumen of the Golgi affected HaloPROTAC-E-mediated degradation. U2OS cells expressing HiBiT-FLAG-Halo-MAN2A1–117 were generated and by IF microscopy, anti-FLAG staining showed speckled localization pattern that is typical of Golgi which also partially overlapped with GM130 Golgi staining (Figure S3D). Treating these cells with increasing concentrations of HaloPROTAC-E (5–5,000 nM) for 24 h resulted in dose-dependent degradation of the Halo protein, with robust degradation observed at 250 nM (Figure S3E). A time course treatment of cells with 250 nM HaloPROTAC-E over 24 h showed time-dependent degradation of the Halo protein, with optimal degradation seen at 24 h (Figure S3F).

**Degradation of peroxisome-localized Halo and dTAG proteins with PROTACs**

To investigate whether peroxisome-localized proteins can be degraded with PROTACs, U2OS cells expressing Halo (HiBiT-FLAG-Halo-PEX26237–305) or FKBPI2/F36V (HiBiT-FLAG-FKBPI2/F36V-PEX26237–305) proteins consisting of the C-terminal peroxisome localization signal of PEX26 (residues 237–305 [PEX26237–305] (Hosoi et al., 2017; Ray et al., 2020) were generated. Both HiBiT-FLAG-Halo-PEX26237–305 and HiBiT-FLAG-FKBPI2/F36V-PEX26237–305 immunostaining overlapped with the peroxisome marker catalase (Zhang et al., 2021) immunostaining, suggesting that both localize predominantly at peroxisomes.
Figure 6. Degradation of Golgi-localized Halo and dTAG proteins with PROTACs
MAN2A1_{1,117}, trans-membrane domain (residues 1–117) of human mannosidase II, Golgi localization signal.

(A) Wild-type (WT) U2OS cells or those expressing MAN2A1_{1,117}-FLAG-Halo-HiBiT or MAN2A1_{1,117}-FLAG-FKBP12^{F36V}-HiBiT were fixed and subjected to anti-FLAG and anti-GM130 immunofluorescence microscopy. DNA is stained with DAPI. Scale bars, 10 μm.

(legend continued on next page)
As in (B), except WT U2OS cells or those expressing MAN2A1-117-FLAG-Halo-HiBiT were treated with 500 nM HaloPROTAC-E for 24 h, time-dependent TMEM192-FLAG-Halo-HiBiT degradation was observed, with optimal degradation seen at 500 nM, whilst a high-dose hook effect was observed at 5,000 nM (Figures 7B and 7C). When cells were subjected to a time course treatment with 500 nM HaloPROTAC-E over 24 h, time-dependent HiBiT-FLAG-Halo-PEX26237–305 degradation was observed, with optimal degradation seen at 24 h (Figures 7D and 7E). We also treated cells expressing HiBiT-FLAG-FKBP12\(^{396V}\)-PEX26237–305 with increasing concentrations of either dTAG-VHL or dTAG-13 (5–5,000 nM) for 24 h. Although a substantial reduction in TMEM192-FLAG-FKBP12\(^{396V}\)-HiBiT signal was not observed by HiBiT lytic assay with either dTAG-VHL or dTAG-13 (Figure 8F), a high-dose hook effect was seen with 5,000 nM of both dTAG-VHL and dTAG-13. By western blot, a reduction in TMEM192-FLAG-FKBP12\(^{396V}\)-HiBiT protein level was observed when cells were treated with 250 and 500 nM dTAG-VHL, but not with dTAG-13 (Figure 8G). A time course treatment of TMEM192-FLAG-FKBP12\(^{396V}\)-HiBiT-expressing cells with 500 nM dTAG-VHL or dTAG-13 over 24 h resulted in no reduction in TMEM192-FLAG-FKBP12\(^{396V}\)-HiBiT levels with either dTAG-VHL or dTAG-13 at any time point by HiBiT lytic assay (Figure 8H), whereas a partial reduction with dTAG-VHL after 24 h was evident by western blot (Figure 8I).

**Evaluation of additional factors that potentially influence PROTAC-mediated target protein degradation**

We sought to evaluate whether the target degradation observed happened at the intended subcellular location or in the cytoplasm at the translation sites. We pre-treated U2OS cells expressing nuclear or ER localized FKBP12\(^{396V}\) with cycloheximide to block new protein synthesis prior to a time course treatment with 500 nM dTAG-VHL over 24 h. Used as a control, cycloheximide led to rapid and complete loss in levels of cMYC compared with controls (Figures 9A and 9B). Cycloheximide also resulted in substantial reduction in levels of both nuclear- and ER-localized FKBP12\(^{396V}\) after 6 h and almost complete reduction after 24 h. Under these conditions, dTAG-VHL caused a faster rate of reduction in levels of both nuclear- and ER-localized FKBP12\(^{396V}\), resulting in substantial reduction within 1–3 h, with a slightly better rate of reduction seen for nuclear localized FKBP12\(^{396V}\) (Figure 9A). These data suggest that PROTAC-mediated degradation can occur at the site where protein is localized to. In the absence of cycloheximide, the rate of dTAG-VHL-mediated reduction in levels of FKBP12\(^{396V}\) was slightly slower (Figures 9A and 9B), suggesting that the levels of FKBP12\(^{396V}\) detected in PROTAC-treated cells reflects the difference in degradation versus synthesis of the target protein, which is a dynamic process.
Figure 7. Degradation of peroxisome-localized Halo and dTAG proteins with PROTACs

PEX26237–305, peroxisome localization signal.

(A) Wild-type (WT) U2OS cells or those expressing HiBiT-FLAG-Halo-PEX26237–305 or HiBiT-FLAG-FKBP12F36V-PEX26237–305 were fixed and subjected to anti-FLAG and anti-catalase immunofluorescence microscopy. DNA is stained with DAPI. Scale bars, 10 μm.

See legend continued on next page.
Other factors that might influence PROTAC efficiency include the subcellular distribution of the E3 ligases that the PROTACs bind to and recruit and the cell type. Previous studies have demonstrated that CRBN localizes mainly to the cytoplasm, although they also note that many proteins identified as CRBN substrates localize to different subcellular compartments such as nucleus (Ikaros), plasma membrane (CK1ε and FAM83F) and ER (BK channels) (Tateno et al., 2020; Dunbar et al., 2021; Liu et al., 2014). We also explored the subcellular distribution of VHL and CRBN in U2OS cells by both IF microscopy and subcellular fractionation. We included WT and CRBN−/− DLD1 colorectal adenocarcinoma cells (Dunbar et al., 2021) as controls for anti-CRBN immunostaining, whereas no VHL-knockout or knockdown controls were available. Under these conditions, CRBN immunostaining signals in WT DLD1 cells and U2OS cells were observed mainly in the cytoplasm, whereas only trace background signal was evident in CRBN−/− DLD1 cells (Figure 9C). VHL immunostaining signal was evident in both cytoplasm and nucleus, as observed by non-overlapping and overlapping signals with the nuclear DAPI staining, in U2OS cells as well as both WT and CRBN−/− DLD1 cells (Figure 9D). The resolution of the IF signals was not conclusive enough for us to establish localization of CRBN or VHL at every subcellular compartment that we localized Halo or FKBP12F36V to. Therefore, we resolved the cells into cytoplasmic, nuclear and membrane fractions and analyzed VHL and CRBN abundance in each fraction. CRBN was detected predominantly in the cytoplasm but also in membrane fractions in U2OS and WT DLD1 cells, while some was also detected in nuclear fraction of DLD1 cells. VHL was also detected predominantly in cytoplasmic fractions and some in membrane fractions in all the cells (Figure 9E), which has also been reported previously (Lewis and Roberts, 2003; Shiao et al., 2003). Western blots with antibodies targeting GAPDH (cytoplasmic), Na+/K+ ATPase (membrane), and lamin A/C (nuclear) served as controls for fractionation (Figure 9E). Clearly, establishing the precise subcellular distribution of endogenous E3 ligases is quite challenging, as it relies on the sensitivity and specificity of antibodies, and from our observations, it is not possible to infer a causal link between the efficiency of PROTAC-mediated degradation of target proteins to subcellular distribution of the target E3 ligase.

To investigate whether PROTAC-mediated patterns of degradation of differentially localized Halo or FKBP12F36V proteins observed in U2OS cells were also evident in other cell types, we included one other routinely used cell line, namely, cervical carcinoma HeLa cells, in our tests. First, we generated HeLa cells expressing nuclear-localized (FLAG-NLS-FKBP12F36V-HiBiT), ER-localized (HiBiT-FLAG-FKBP12F36V-KDEL), or Golgi-localized (MAN2A11–117-FLAG-FKBP12F36V-HiBiT) FKBP12F36V, and when these cells were treated with either dTAG-VHL (Figure S4A) or dTAG-13 (Figure S4B) for 24 h, robust degradation of nuclear- and ER-localized, but not Golgi-localized, FKBP12F36V was evident compared with DMSO-treated controls, just as we observed in U2OS cells (Figures 2G, 2I, 5G, 5I, 6G, and 6I). Next, we generated HeLa cells expressing nuclear-localized (FLAG-NLS-Halo-HiBiT), ER-localized (HiBiT-FLAG-Halo-KDEL), or peroxisome-localized (HiBiT-FLAG-Halo-PEX26237–305) Halo proteins. When these cells were subjected to treatment with HaloPROTAC-E for 24 h, robust degradation of nuclear-, ER-, and peroxisome-localized Halo proteins was observed (Figure S4C), which was comparable with the degradation seen in U2OS cells previously (Figures 2C, 2E, 5C, 5E, 7C, and 7E). These observations suggest that at least in the two cell lines tested, similar degradation patterns were evident with the PROTACs that we used.

Finally, over the course of this study, a VHL-recruiting FKBP12F36V-directed PROTAC, dTAGV-1, was reported (Nabet et al., 2020). It is different from dTAG-VHL in that it uses a distinct linker and a slightly different VHL ligand (see Figure 1A for comparison of compounds). This allowed us to test both PROTACs in parallel for their ability to degrade differentially localized FKBP12F36V protein, especially in locations where the degradation by dTAG-VHL was not complete. First, when U2OS cells expressing nuclear-, cytoplasmic-, ER-, peroxisome-, lysosome-, or Golgi-localized FKBP12F36V protein were treated with increasing concentrations of dTAGV-1 (5–5000 nM) for 24 h, concentration-dependent degradation of FKBP12F36V protein was observed for nuclear-, cytoplasmic-, ER-, and peroxisome-localized FKBP12F36V proteins, with optimal degradation observed at 50 nM (Figures S5A and S5B). The optimal degradation by dTAG-VHL in contrast required 500 nM (Figures 2F, 2G, 2I, 5F, 5G, 7F, and 7I). Slight degradation of lysosome-localized FKBP12F36V protein was observed at higher concentrations of dTAGV-1 but no degradation was evident for Golgi-localized FKBP12F36V protein (Figure S5C). A time course treatment of U2OS cells expressing ER-, peroxisome-, or lysosome-localized FKBP12F36V protein with dTAG-VHL (500 nM) or dTAG-1 (500 nM) over 24 h resulted in time-dependent degradation of the target proteins.
degradation of ER- and peroxisome-localized FKBP12<sup>F36V</sup> protein with optimal degradation at 24 h (Figures S5D and SSE), while lysosome-localized FKBP12<sup>F36V</sup> protein was degraded only slightly by both PROTACs (Figure S5F). Although ER-localized FKBP12<sup>F36V</sup> protein was degraded more robustly and rapidly with dTAG<sup>V</sup>-1, peroxisome-localized FKBP12<sup>F36V</sup> protein was degraded more robustly and rapidly with dTAG-VHL (Figures S5D and SSE).

**DISCUSSION**

In this study, using established Halo and FKBP12<sup>F36V</sup> proteins, we explored the ability of VHL- and/or CRBN-recruiting PROTACs to degrade these proteins when the proteins were localized to different subcellular compartments, including the nucleus, cytoplasm, OMM, ER, Golgi, peroxisome, or lysosome. Table S1 summarizes how subcellular distribution of Halo or FKBP12<sup>F36V</sup> proteins affects their degradation by VHL- or CRBN-recruiting PROTACs. Halo proteins localized to all subcellular compartments above except the Golgi lumen, were degraded with the Halo/VHL-recruiting PROTAC, HaloPROTAC-E (Figure S6A). In contrast, only nuclear-, cytoplasmic-, and ER-localized FKBP12<sup>F36V</sup> protein were degraded efficiently with the FKBP12<sup>F36V</sup>/VHL-recruiting PROTAC dTAG-VHL (Figure S6B). With the FKBP12<sup>F36V</sup>/CRBN-recruiting PROTAC, dTAG-13, FKBP12<sup>F36V</sup> protein localized to the nucleus, cytoplasm, ER, and OMM were degraded (Figure S6C). When we compared dTAG-VHL with dTAG<sup>V</sup>-1, a structurally different PROTAC that also recruits dTAG to VHL, the optimal degradation of cytoplasmic-, nuclear-, and ER-localized FKBP12<sup>F36V</sup> by dTAG<sup>V</sup>-1 occurred at a lower concentration (50 nM) than dTAG-VHL (500 nM). Interestingly, however, there was a difference in the kinetics of degradation for ER- and peroxisome-localized FKBP12<sup>F36V</sup> protein, as ER-localized protein was degraded more robustly and rapidly with dTAG<sup>V</sup>-1, while peroxisome-localized FKBP12<sup>F36V</sup> protein was degraded more robustly and rapidly with dTAG-VHL, highlighting the contribution of the local context to PROTAC-mediated degradation even when the PROTACs recruit the same E3 ubiquitin ligase and otherwise lead to similarly efficient degradation of targets in other subcellular compartments. Given that differentially localized Halo and FKBP12<sup>F36V</sup> proteins display different levels of degradation by the same respective PROTACs, it is evident that the subcellular context of the target protein can influence the efficacy of PROTAC-mediated degradation. Furthermore, the differences observed between the degradation of Halo and FKBP12<sup>F36V</sup> proteins in some of the same cellular compartments by PROTACs recruiting VHL suggests that the nature of the target protein itself in a particular subcellular location, how it engages with the PROTAC and is presented to the E3 will likely determine whether it is amenable to PROTAC-mediated degradation.

More efficient degradation of nuclear- and cytoplasm-localized FKBP12<sup>F36V</sup> protein by CRBN-recruiting dTAG-13 over dTAG-VHL and dTAG<sup>V</sup>-1 was observed, while ER-localized FKBP12<sup>F36V</sup> protein was more efficiently degraded with dTAG-VHL and dTAG<sup>V</sup>-1 compared with dTAG-13, suggesting that the choice of the E3 ligase recruited by the PROTAC also determines whether a target protein localized to a specific subcellular compartment can be degraded by the PROTAC. In line with this, slight degradation of peroxisome- and lysosome-localized FKBP12<sup>F36V</sup> protein by dTAG-VHL and dTAG<sup>V</sup>-1, but not dTAG-13, was also observed by western blot. However, as there were differences in the efficiency of degradation caused by dTAG-VHL and dTAG<sup>V</sup>-1 for peroxisome- and ER-localized FKBP12<sup>F36V</sup> protein, the structure of PROTACs that recruit the same target and E3 ligase also influences the formation of productive ternary complexes differently in different subcellular compartments. Furthermore, it has been previously reported that differentially localized FKBP12<sup>F36V</sup>-tagged solute carrier (SLC) transporters were degrade to varying extents by CRBN-recruiting PROTACs (Bensimon et al., 2020). For example, SLC transporters located at the plasma membrane, lysosome, ER, and Golgi were degraded with CRBN-recruiting PROTACs, whereas those located at the inner mitochondrial membrane were not (Bensimon et al., 2020). In this study, lack of effective tools to determine the subcellular distribution of endogenous VHL and CRBN at all the subcellular locations that we placed the Halo and FKBP12<sup>F36V</sup> proteins precluded us from establishing a causal link between location of the E3 ligases and the degradation efficiency.

**Figure 8. Degradation of lysosome-localized Halo and dTAG proteins with PROTACs**

TMEM192, lysosome localization signal.

(A) Wild-type (WT) U2OS cells or those expressing TMEM192-FLAG-Halo/HiBiT or TMEM192-FLAG-FKB12<sup>F36V</sup>-HiBiT were fixed and subjected to anti-FLAG and anti-LAMPI immunofluorescence microscopy. DNA is stained with DAPI. Scale bars, 10 μm.

(B) WT U2OS cells or those expressing TMEM192-FLAG-Halo/HiBiT were treated with indicated concentrations of HaloPROTAC-E for 24 h. HiBiT protein levels were then quantified using the lytic detection system. Corresponding WT relative light unit (RLU) values were subtracted from TMEM192-FLAG-Halo/HiBiT and anti-LAMP1 immunofluorescence microscopy. DNA is stained with DAPI. Scale bars, 10 μm.

(C) Immunoblot analysis of (B).

(D) As in (B), except WT U2OS cells or those expressing TMEM192-FLAG-Halo/HiBiT were treated with 500 nM HaloPROTAC-E for indicated times.

(E) Immunoblot analysis of (D).

(F) As in (B), except WT U2OS cells or those expressing TMEM192-FLAG-FKBP12<sup>F36V</sup>-HiBiT were treated with indicated concentrations of dTAG-VHL or dTAG-13 for 24 h.

(G) Immunoblot analysis of (F).

(H) As in (F), except WT U2OS cells or those expressing TMEM192-FLAG-FKBP12<sup>F36V</sup>-HiBiT were treated with 500 nM dTAG-VHL or dTAG-13 for indicated times.

(I) Immunoblot analysis of (H).

For (C), (E), (G), and (I), extracts were resolved by SDS-PAGE and transferred on to PVDF membranes, which were subjected to immunoblotting with indicated antibodies. Solid circles denote endogenous FKBP12, and open circles denote TMEM192-FLAG-FKBP12<sup>F36V</sup>-HiBiT. Blots are representative of 3 independent experiments.
Figure 9. Evaluation of additional factors that might influence PROTAC-mediated target protein degradation

(A and B) U2OS cells expressing either FLAG-NLS-FKBP12<sup>36V</sup>-HiBiT or HiBiT-FLAG-FKBP12<sup>36V</sup>-KDEL were treated with cycloheximide (CHX; 20 μg/mL) only, dTAG-VHL (500 nM) only, or pre-treated with CHX (20 μg/mL) for 1 h before dTAG-VHL (500 nM) treatment for 24 h. Cells were lysed at indicated time points and
It is also evident that efficient PROTAC-mediated target protein degradation at a specific subcellular location is not governed solely by the E3 used, as a VHL-recruiting HaloPROTAC-E was able to efficiently degrade an OMM-localized Halo protein, whereas a corresponding OMM-localized FKBP12$^{\text{F36V}}$ protein was not efficiently degraded by dTAG-VHL. Similarly, as mentioned above, differences in the efficiency of degradation were observed for either ER- or peroxisome-localized FKBP12$^{\text{F36V}}$ protein between dTAG-VHL and dTAG$^\text{ΔC}$-1, although both PROTACs efficiently degrade nuclear and cytoplasmic-localized FKBP12$^{\text{F36V}}$ protein. Furthermore, a lysosome-localized Halo protein was successfully degraded with a VHL-recruiting Halo-based PROTAC, whereas a corresponding lysosome-localized FKBP12$^{\text{F36V}}$ protein was less amenable to degradation with either VHL- or CRBN-recruiting dTAG-based PROTACs. Meanwhile, the lysosome-localized SLC38A9 has been reported previously to be robustly degraded with the same CRBN-recruiting dTAG-based PROTAC, dTAG-13 (Bensimon et al., 2020). These results indicate that presentation of the target protein itself to the PROTAC in a particular subcellular context is an important consideration for PROTAC-mediated degradation.

Interestingly, it was evident that Halo or FKBP12$^{\text{F36V}}$ proteins localized to the inside of the Golgi were less amenable to degradation by their corresponding PROTACs. Given that VHL- or CRBN-based recruiters were less successful in degrading Golgi-lumen localized Halo or FKBP12$^{\text{F36V}}$ proteins, especially at concentrations where other non-Golgi-localized proteins were degraded, it may be worthwhile to use Golgi-localized E3 ligases such as PISH (plenty of SH3 domains) (Alroy et al., 2005; Kim et al., 2006) for their targeted degradation, which may be more successful. The presence of functional proteasomes may also be required for successful Golgi-localized target protein degradation using PROTACs (Eisenberg-Lemer et al., 2020). However, given the proteasome inhibitors appear to cause unusual mobility patterns and a slight loss in levels of Golgi-lumen-localized Halo and FKBP12$^{\text{F36V}}$ proteins, it is likely that there are possible side effects of proteasome inhibition on Golgi homeostasis. Previously, it has been reported that the Golgi-localized SLC33A1, for which the dTAG tag was designed to face the cytoplasm, was robustly degraded by dTAG-13 (Bensimon et al., 2020). Similarly, we have shown that Halo protein localized to the cytoplasmic surface of Golgi was efficiently degraded by HaloPROTAC-E. These results suggest that PROTAC accessibility to target proteins, and perhaps access to the proteasome following target protein ubiquitylation, are important factors to consider when targeting Golgi-localized proteins for PROTAC-mediated degradation. The disappearance of the native molecular weight species of Golgi-lumen localized Halo and FKBP12$^{\text{F36V}}$ proteins by western blot, but not HiBIT lytic assay, at high concentrations of PROTACs, with no rescue in levels evident by proteasomal inhibition, implies potential post-translational modifications. These could very well be off-target effects of high compound concentrations causing Golgi toxicity but this needs to be investigated further.

The HiBIT lytic system was used in parallel with the gold standard western blotting to quantify the degradation of HiBIT-tagged proteins. For the degradation of Halo-tagged proteins with HaloPROTAC-E, the HiBIT lytic system generally reflected the trends and amount of degradation that was observed with western blots (Figure S6D), albeit the levels of degradation observed by western blot detection of proteins at their native molecular weight were more profound compared with those shown by HiBIT lytic signals. In contrast, larger variation was evident between western blot and HiBIT lytic assays when analyzing the degradation of FKBP12$^{\text{F36V}}$-tagged proteins with dTAG-VHL and dTAG-13 PROTACs (Figures S6E and S6F). Whether this variation is an accurate representation of the PROTAC-mediated degradation of FKBP12$^{\text{F36V}}$ or a limitation of the HiBIT lytic system needs to be further addressed. When analyzing target proteins at their native molecular weights by western blotting, proteins that are polyubiquitylated but not yet degraded upon PROTAC treatment may not be detected, but the more sensitive HiBIT lytic system would be expected to detect both non-ubiquitylated and ubiquitylated pools of the target protein. This could explain some of the discrepancies between the two systems seen above.

Recently, a systematic computational approach was used to assess the PROTAC tractability (PROTACtability) of the human proteome (Schneider et al., 2021). This analysis was performed using a series of criteria based on data from a diverse range of publicly available resources. Using this approach, more than 1,000 proteins were identified that had not yet been described in the literature as PROTAC targets, providing potential opportunities for future PROTAC-based therapies. One parameter used to predict the PROTACtability of a protein was the cellular localization of the protein. Interestingly, the PROTACtability of proteins located in the nucleus or cytoplasm was determined as "good," whereas a lower "gray" score was assigned to membrane-localized proteins, and all other subcellular localizations were assigned an even lower score and determined as "unfavorable." Using a Halo/VHL-recruiting PROTAC, we have demonstrated that Halo-tagged proteins localized to the OMM, the ER, the peroxisome and the lysosome can all be degraded by PROTACs. These data therefore suggest that substantially more than the 1,067 nuclear- and cytoplasmic-localized proteins reported by this computational analysis (Schneider et al., 2021) could potentially be targeted for degradation using PROTAC-based technologies.

Presently, a lot of emphasis on PROTAC design and development is placed on compounds with the highest binding potencies and the strength of in vitro ternary complexes. Our findings indicate that even for PROTAC compounds optimized for these parameters, simply placing the same target protein to
different subcellular compartments affects the efficiency with which the PROTACs degrade the target. A recent study which evaluated the effects of chemical and cellular variables on target protein degradation using promiscuous kinase inhibitors as warheads found that the formation of a stable ternary complex or the evidence of cellular target engagement by themselves did not predict degradation efficiency, while varying the recruited E3 ligase did influence degradation of some targets (Donovan et al., 2020). Similarly, molecular glue degraders lenalidomide and pomalidomide were shown to degrade the plasma membrane-localized FAM83F-CIK1 complex but do not degrade other pools of cellular CK1α in complex with other FAM83 proteins (Dunbar et al., 2021), even though FAM83G was identified as one of the top interactors of CRBN upon lenalidomide treatment (Kronke et al., 2014). Although strong in vitro ternary complexes could help explain the efficiency of certain PROTACs in cells over others (Casement et al., 2021), it is increasingly evident that cell-based PROTAC assays that take into account the target protein subcellular context, including localization and the target protein macromolecular complexes, are better predictors of how useful and applicable a PROTAC will be.

**SIGNIFICANCE**

Proteolysis-targeting chimeras recruit a protein of interest into spatial proximity of an E3 ubiquitin ligase, promoting POI ubiquitylation and degradation through the ubiquitin-proteasome system. Understanding how the subcellular context of the POI and the nature of the E3 ligase being recruited affects PROTAC-mediated degradation has profound implications for pairing POIs and E3 ligases for PROTAC development. Our findings suggest that the subcellular distribution of the POI and the nature of the E3 ligases recruited by PROTACs are important factors that affect the ability of PROTACs to degrade the POI through the UPS. Moreover, the nature of the POI within a specific location, and not just the nature of the E3 ligase being recruited there, also determines whether a PROTAC can degrade the POI. We have shown here that proteins localized to the nucleus, cytoplasm, outer mitochondrial membrane, endoplasmic reticulum, peroxisome and lysosome are degraded differently by different PROTACs but those localized to the inner lumen of the Golgi appear to be resistant to degradation by PROTACs.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

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**AUTHOR CONTRIBUTIONS**

L.M.S. and L.G. performed experiments, collected and analyzed data, and contributed to the writing of the manuscript. J.-F.Z. and A.B. performed some experiments and analyzed data. J.C. generated constructs used in this study. N.S. developed the strategy for and performed the synthesis of dTAG-VHL (active) and dTAG-VHL-inactive compounds. G.P.S. conceived the project, analyzed data, and contributed to the writing of the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Sheep monoclonal anti-dTAG | MRC PPU Reagents and Services | DA179 |
| Mouse monoclonal anti-HaloTag® | Promega | G921A |
| Rabbit monoclonal anti-vinculin | Abcam | ab129002; RRID:AB_11144129 |
| Mouse monoclonal anti-β-actin | Abcam | ab8226; RRID:AB_306371 |
| Rabbit polyclonal anti-CUL2 | Invitrogen | Cat#51-1800; RRID:AB_87649 |
| Rabbit polyclonal anti-FKBP12 | Abcam | ab24373; RRID:AB_732383 |
| Mouse monoclonal anti-FLAG® M2- Peroxidase (HRP) | Sigma-Aldrich | A8592; RRID:AB_439702 |
| Rabbit monoclonal anti-GAPDH | Cell Signaling Technology | Cat#2118S |
| Mouse monoclonal anti-HIF1α | BD Biosciences | Cat#610959; RRID:AB_398272 |
| Mouse monoclonal mono- and poly- ubiquitinated conjugates (Ub) | Enzo Life Sciences | BML-PW8810; RRID:AB_10541840 |
| Rat monoclonal anti-α-tubulin | Thermo Fisher Scientific | Cat#MA1-80189; RRID:AB_2210200 |
| Rabbit polyclonal anti-ZFP91 | Bethyl Laboratories | Cat##A303-245A; RRID:AB_10953803 |
| Rabbit monoclonal anti-c-Myc | Cell Signaling Technology | Cat#5605S |
| Rabbit monoclonal anti Na,K-ATPase | Cell Signaling technology | Cat#23565; RRID:AB_2798866 |
| Rabbit polyclonal anti-Lamin A/C | Cell Signaling Technology | Cat#2032; RRID:AB_2136278 |
| Mouse monoclonal anti-LC3 | MBL Life Science | Cat#M152-3; RRID:AB_1279144 |
| Rabbit polyclonal anti-VHL | Cell Signaling technology | Cat#68547; RRID:AB_2716279 |
| Rabbit monoclonal anti-CRBN | Cell Signaling Technology | Cat#71810; RRID:AB_2799810 |
| Mouse monoclonal anti-ATPβb | Abcam | ab14730; RRID:AB_301438 |
| Rabbit monoclonal anti-calnexin | Cell Signaling technology | Cat#2679; RRID:AB_2228381 |
| Rabbit polyclonal anti-catalase | Sigma-Aldrich | Cat#219010; RRID:AB_2071738 |
| Mouse monoclonal anti-FLAG | Sigma-Aldrich | Cat#F1804; RRID:AB_262044 |
| Rabbit monoclonal anti-FLAG | Cell Signaling technology | Cat#14793; RRID:AB_2572291 |
| Rabbit monoclonal anti-GM130 | Cell Signaling technology | Cat#12480; RRID:AB_2797933 |
| Rat monoclonal anti-LAMP1 | Santa Cruz Biotechnology | Cat#sc-19992; RRID:AB_2134495 |
| Goat anti-rabbit IgG, HRP-linked | Cell Signaling technology | Cat#7074S; RRID:AB_2099233 |
| Goat anti-rat IgG (H+L), HRP-linked | Thermo Fisher Scientific | Cat#62-9520; RRID:AB_87993 |
| Goat anti-mouse IgG (H+L), HRP-linked | Thermo Fisher Scientific | Cat#31430; RRID:AB_228307 |
| Donkey anti-mouse IgG Alexa Fluor™ 488 | Thermo Fisher Scientific | Cat#A1202; RRID:AB_141607 |
| Donkey anti-rabbit IgG Alexa Fluor™ 488 | Thermo Fisher Scientific | Cat#A1206; RRID:AB_2535792 |
| Goit anti-mouse IgG Alexa Fluor™ 594 | Thermo Fisher Scientific | Cat#A11005; RRID:AB_2534073 |
| Goat anti-rabbit IgG Alexa Fluor™ 594 | Thermo Fisher Scientific | Cat#A11012; RRID:AB_2534079 |
| Donkey anti-rat IgG Alexa Fluor™ 594 | Thermo Fisher Scientific | Cat#A21209; RRID:AB_2535795 |

| **Chemicals, peptides, and recombinant proteins** |        |            |
|-----------------------------------------------|--------|------------|
| HaloPROTAC-E | Tovell et al., 2019a | N/A |
| dTAG-13 | Tocris Bioscience | Cat#6605 |
| dTAG-VHL (Active) | This paper | N/A |
| dTAG-VHL (Inactive) | This paper | N/A |
| dTAG-V1 | Tocris Bioscience | Cat#6914 |
| Cycloheximide (CHX) | Sigma-Aldrich | Cat#C7698 |
| VH298 | Tocris Bioscience | Cat#6156 |
| NAE inhibitor, MLN4924 | Sigma-Aldrich | Cat#5054770001 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| MG-132, proteasome inhibitor | Abcam | ab141003 |
| Bortezomib | LC Laboratories | CatB-1408 |
| Bafilomycin A1 | Enzo Life Sciences | BML-CM110 |
| Lenalidomide | Cayman Chemicals | Cat#14643 |
| Immobilon Western Chemiluminescent HRP Substrate | Merck | Cat#WBKLS0500 |
| ProLong™ Gold Antifade Mountant with DAPI | Life Technologies | Cat#P36935 |

**Critical commercial assays**

- **CellTox™ Green Cytotoxicity Assay**
  - Promega
  - Cat #G8742

- **Nano-Glo® HiBiT Lytic Detection System**
  - Promega
  - Cat #N3040

- **ProteoExtract® Subcellular Proteome Extraction Kit**
  - Merck
  - Cat #539790

**Deposited data**

- **Data obtained in this study**
  - This paper
  - [https://data.mendeley.com/datasets/fcfztkcmyp/draft?a=44000ca7-85d8-4ee4-8da3-324b537f82d6](https://data.mendeley.com/datasets/fcfztkcmyp/draft?a=44000ca7-85d8-4ee4-8da3-324b537f82d6)

**Experimental models: Cell lines**

| Human: HEK293-FT | Invitrogen | Cat#R7007 |
| Human: U2OS | ATCC | HTB-96 |
| Human: DLD-1 | ATCC | CCL-221 |
| Human: DLD-1 CRBN<sup>-/-</sup> | Dunbar et al. (2021) | N/A |
| Human: HeLa | ATCC | CCL-2 |

**Recombinant DNA**

- **pCMV5-gag-pol**
  - Cell Biolabs
  - Cat#RV-111

- **pCMV5-VSV-G**
  - Cell Biolabs
  - Cat#RV-110

- **pBabeD-puromycin FLAG-Halo-HiBiT**
  - MRC PPU Reagents and Services
  - DU61380

- **pBabeD-puromycin FLAG-FKBP12<sup>36V</sup>-Halo-HiBiT**
  - MRC PPU Reagents and Services
  - DU61379

- **pBabeD-puromycin FLAG-NLS-Halo-HiBiT**
  - MRC PPU Reagents and Services
  - DU61387

- **pBabeD-puromycin FLAGS-NLS-Halo-HiBiT**
  - MRC PPU Reagents and Services
  - DU61385

- **pBabeD-puromycin FLAGS-Halo-NES-HiBiT**
  - MRC PPU Reagents and Services
  - DU61388

- **pBabeD-puromycin FLAGS-FKBP12<sup>36V</sup>-NES-HiBiT**
  - MRC PPU Reagents and Services
  - DU61394

- **pBabeD-puromycin HIBIT-FLAG-Halo-F1S101-152**
  - MRC PPU Reagents and Services
  - DU61396

- **pBabeD-puromycin HIBIT-FLAG-FKBP12<sup>36V</sup>-F1S101-152**
  - MRC PPU Reagents and Services
  - DU61395

- **pBabeD-puromycin HIBIT-FLAG-Halo-KDEL**
  - MRC PPU Reagents and Services
  - DU61565

- **pBabeD-puromycin HIBIT-FLAG-FKBP12<sup>36V</sup>-KDEL**
  - MRC PPU Reagents and Services
  - DU61566

- **pBabeD-puromycin MAN2A1<sub>1-117</sub>-FLAG-Halo-HiBiT**
  - MRC PPU Reagents and Services
  - DU61578

- **pBabeD-puromycin HIBIT-FLAG-Halo-MAN2A1<sub>1-117</sub>**
  - MRC PPU Reagents and Services
  - DU61894

- **pBabeD-puromycin MAN2A1<sub>1-117</sub>-FLAG-FKBP12<sup>36V</sup>-HIBIT**
  - MRC PPU Reagents and Services
  - DU61577

- **pBabeD-puromycin HIBIT-FLAG-Halo-PEX26<sub>237-305</sub>**
  - MRC PPU Reagents and Services
  - DU61580

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gopal Sapkota (g.sapkota@dundee.ac.uk).

Materials availability
All constructs used in this study are available to request from the MRC PPU Reagents & Services webpage (http://mrcppureagents.dundee.ac.uk) and the unique identifier (DU) numbers provide direct links to the cloning strategies and sequence details. DU numbers can also be found in the Key resources table. All constructs were sequence-verified by the DNA Sequencing Service, University of Dundee (http://www.dnaseq.co.uk).

Data and code availability
- Unprocessed western blot and HiBiT data have been deposited at Mendeley Data https://data.mendeley.com/datasets/fcfzkcmyp/draft?a=44000ca7-85d8-4ee4-8da3-324b537f82d6 and are publicly available as of the date of publication. These datasets can also be accessed from the Key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
All procedures were carried out under aseptic conditions meeting biological safety requirements. HEK293-FT cells (Invitrogen, Cat# R70007) which are a clonal isolate of HEK293 cells transformed with the SV40 large T antigen, U2OS cells (ATCC, Cat# HTB-96) that are human epithelial bone osteosarcoma cells derived from a 15-year-old Caucasian female, DLD-1 cells (ATCC, CCL-221), cereblon knock-out DLD-1 cells (CRBN<sup>-/-</sup>) (generated by Dr. Karen Dunbar) and HeLa cervical carcinoma cells, originally derived from a 31-year old female in 1951, were maintained in DMEM (Life Technologies) containing 10% (v/v) foetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM L-glutamine (Lonza), 100 U/mL penicillin (Lonza) and 0.1 mg/mL streptomycin (Lonza). Cells were grown at 37°C with 5% CO<sub>2</sub> in a water-saturated incubator. For passaging, cells were incubated with trypsin/EDTA at 37°C to detach cells.

METHODS DETAILS

Plasmids
For production of retroviral vectors, the following were cloned into pBabeD-puromycin plasmids: FLAG-Halo-HiBiT (DU61380), FLAG-FKBP12<sup>F36V</sup>-HiBiT (DU61379), FLAG-NLS-Halo-HiBiT (DU61387), FLAG-NLS-FKBP12<sup>F36V</sup>-HiBiT (DU61385), FLAG-Halo-NES-HiBiT (DU61388), FLAG-FKBP12<sup>F36V</sup>-NES-HiBiT (DU61394), HIBI-FLAG-Halo-FIS1<sub>101-152</sub> (DU61396), HIBI-FLAG-FKBP12<sup>F36V</sup>-FIS1<sub>101-152</sub> (DU61395), HIBI-FLAG-Halo-KDEL (DU61565), HIBI-FLAG-FKBP12<sup>F36V</sup>-KDEL (DU61566), MAN2A1<sub>1-117</sub>-FLAG-Halo-HiBiT (DU61578), HIBI-FLAG-Halo-MAN2A1<sub>1-117</sub> (DU61894), MAN2A1<sub>1-117</sub>-FLAG-FKBP12<sup>F36V</sup>-HiBiT (DU61577), HIBI-FLAG-Halo-PEX2<sub>237-305</sub> (DU61580), HIBI-FLAG-FKBP12<sup>F36V</sup>-PEX2<sub>237-305</sub> (DU61567), TEMEM192-FLAG-Halo-HiBiT (DU61579), TEMEM192-FLAG-FKBP12<sup>F36V</sup>-HiBiT (DU61593). All constructs were sequence-verified by the DNA Sequencing Service, University of Dundee (http://www.dnaseq.co.uk). These constructs are available to request from the MRC PPU Reagents and Services webpage (http://mrcppureagents.dundee.ac.uk).
Retroviral generation of stable cell lines

Retroviral pBabeD-puromycin vectors encoding the desired construct (6 µg) were co-transfected with pCMV5-gag-pol (3.2 µg) and pCMV5-VSV-G (2.8 µg) (Cell Biolabs) into a 10 cm diameter dish of ~70% confluent HEK293-FT cells. Briefly, plasmids were added to 1 mL Opti-MEM medium to which 24 µL of 1 mg/mL PEI was added. Following a gentle mix and incubation at room temperature for 20 min, the transfection mix was added dropwise to HEK293-FT cells. 16 h post-transfection, fresh medium was added to the cells. 24 h later, the retroviral medium was collected and passed through 0.45 µm sterile syringe filters. Target cells (~60% confluent) were transduced with the optimised titre of the retroviral medium diluted in fresh medium (typically 1:1–1:10) containing 8 µg/mL polybrene (Sigma-Aldrich) for 24 h. The retroviral medium was then replaced with fresh medium, and 24 h later, the medium was again replaced with fresh medium containing 2 µg/mL puromycin for selection of cells which had integrated the constructs. A pool of transduced cells were utilised for subsequent experiments following complete death of non-transduced cells placed under selection in parallel.

Synthesis of dTAG-VHL-Active and dTAG-VHL-Inactive

The following abbreviations apply to the PROTAC synthesis methodology:
- DCM: Dichloromethane
- DIC: N,N-Diisopropylcarbodiimide
- DIPEA: N,N-Diisopropylethylamine
- DMAP: 4-(Dimethylamino)pyridine
- DMF: N,N-Dimethylformamide
- EtOAc: Ethyl acetate
- Et2O: Diethyl ether
- EDCI: N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
- HOBt: 1-Hydroxybenzotriazole
- MeCN: Acetonitrile
- MeOH: Methanol
- TFE: 2,2,2-Trifluoroethanol
- THF: Tetrahydrofuran

dTAG-VHL-Active and dTAG-VHL-Inactive were synthesised in line with previous studies describing FKBP12- and VHL-recruiting ligands (Testa et al., 2018; Jørgensen and Bols, 2018; Erb et al., 2017; Keenan et al., 1998). All chemicals were purchased from commercial vendors and used without further purification. (2R,3S,4S)-1-((S)-14-amino-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-3-fluoro-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (7) and (2R,3R,4S)-1-((S)-14-amino-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-3-fluoro-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (9) were prepared as described (Testa et al., 2018). Flash column chromatography and Prep HPLC were performed by using Buchi PrepChrom C-700, prepacked Buchi Sepacore Flash Cartridges and HPLC C18 column Gemini NY(RP)C18 110, 21.2 × 150 mm, 10 µm particle size. NMR spectra were recorded on a Bruker Ascend 500 MHz (Data S1). Low resolution mass spectra and analytical HPLC traces were recorded on an Agilent Technologies 1200 series HPLC connected to an Agilent Technologies 6130 quadrupole LC/MS, connected to an Agilent diode array detector. The column used was a Waters XBridge column (50 mm × 2.1 mm, 3.5 µm particle size), with a gradient from 5% to 95% of acetonitrile in water (with 0.1% of formic acid or aqueous ammonia solution) over 3 or 7 min. The flow rate was 0.7 mL/min.

For synthesis of (S)-1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylic acid (4) (Keenan et al., 1998), to a solution of (S)-2-(3,4,5-trimethoxyphenyl)butanoic acid (1 g, 3.93 mmol) in anhydrous DCM (20 mL), thionyl chloride (2 mL, 27.41 mmol) was added at 0°C. The mixture was stirred at RT for 16 h, the volatiles were removed in vacuo to yield 2 as a yellow oil (1.06 g, 3.93 mmol). The crude (S)-2-(3,4,5-trimethoxyphenyl)butanoyl chloride was used in the next step without further purification. Suspension of D(-)-tartaric salt of L(-)-piperocic acid (1.97 g, 7.07 mmol) and NaHCO3 (2.64 g, 31.44 mmol) in dioxane (100 mL) was refluxed for 1 h and a solution of freshly prepared 2 (1.06 g, 3.93 mmol) in dioxane (5 mL) was added dropwise. The reaction mixture was refluxed for 24 h
and cooled to 0°C. The cold reaction mixture was diluted with 1N HCl aq (60 mL) and extracted with EtOAc (2 × 100 mL). The combined organic layer was washed with brine and concentrated. The crude residue was purified by RP C18 flash column chromatography (SVP D40-RP18 25–40 μm 90 g), in a gradient of MeOH/H2O (5%–95%) to afford 4 (1 g, 2.73 mmol, 69%) as white solid.

1H NMR (500 MHz, CDCl3, mixture of rotamers 1:4): δ = 6.47 (s, 0.4H), 6.45 (s, 1.6H); 5.37 (d, J = 5.1 Hz, 0.8H), 4.72 (d, J = 5.1 Hz, 0.2H); 4.67 (d, J = 13.7 Hz, 0.2H), 3.80-3.78 (bs, 1.8H); 3.86 (s, 1.8H), 3.84 (s, 2.4H), 3.83 (s, 4.8H); 3.60 (t, J = 7.1 Hz, 0.8H), 3.39 (t, J = 7.1 Hz, 0.2H); 2.90-2.83 (m, 0.8H), 2.71-2.64 (m, 0.2H); 2.29 (d, J = 13.6 Hz, 0.8H), 2.02 (d, J = 13.6 Hz, 0.2H); 2.17-2.06 (m, 1H); 1.81-1.58 (m, 3.8H), 1.53-1.33 (m, 1.8H), 1.29-1.23 (m, 0.2H), 1.10-1.02 (m, 0.2H); 0.94 (t, J = 7.3 Hz, 2.4H), 0.90 (t, J = 7.3 Hz, 0.6H). (Data S1).

For the synthesis of 2-(2-((R)-3-(3,4-dimethoxyphenyl)-1-hydroxypropyl)phenoxy)acetic acid (ortho-AP1867) (Erb et al., 2017), (R)-2-(2-((3,4-dimethoxyphenyl)-1-hydroxypropy)phenoxylacetic acid 5 was prepared according to Keenan et al. (1998) in 5 steps starting from 3,4-dimethoxybenzaldehyde and 2-hydroxycetophenone.

1H NMR (500 MHz, CDCl3): δ = 7.31-7.24 (m, 2H); 7.03 (t, J = 7.4 Hz, 1H); 6.87 (d, J = 8.2 Hz, 1H); 6.82-6.73 (m, 3H); 4.88 (dd, J = 5.4 Hz, J = 8.2 Hz, 1H); 4.73 (ABq, J = 16.0 Hz, 2H); 3.87 (d, 6H); 2.82-2.71 (m, 1H), 2.70-2.61 (m, 1H); 2.36-2.26 (m, 1H), 2.23-2.13 (m, 1H). (Data S1).

2-Chlorotrityl chloride resin 4 g (Activetec, 100–200 mesh, 0.84 mmol/g) was suspended in a solid phase reactor in DCM (40 mL) and stirred at RT for 1 h. The liquid was expelled with argon (slight positive overpressure). A solution of the acid 5 (0.62 g, 1.78 mmol) and DIPEA (1.25 mL, 7.17 mmol) in DCM (30 mL) was added portion-wise to the swollen resin, gently stirred at RT for 16 h. The liquid was expelled with argon and the resin was rinsed with DCM (15 mL). A mixture of DIPEA/MeOH/DCM (1:2:17, 20 mL) was added to the resin, stirred for 15 min, and filtered. This operation was repeated twice. Finally, the resin was washed successively with DCM (20 mL), MeOH (20 mL), Et2O (20 mL) and dried.

To the loaded resin 6 a solution of the acid 4 (0.44 g, 1.20 mmol) and DMAP (0.29 g, 2.4 mmol) in DCM (30 mL) was added followed by a solution of DIC (0.20 mL, 1.29 mmol) in DCM (5 mL). The reaction mixture was stirred at RT for 16 h and the liquid was expelled with argon. The resin was washed with DCM (4 × 20 mL) and dried. A solution of 20% TFE in DCM (20 mL) was added to the dry resin, stirred at RT for 16 h and filtered. The filtrate was collected and concentrated. Crude residue was purified by RP C18 flash column chromatography (SVP D40-RP18 25–40 μm 90 g), in a gradient of MeOH/H2O (5%–95%) to afford ortho-AP1867 (0.162 g, 0.233 mmol, 13%) as white amorphous solid.

1H NMR (500 MHz, CDCl3, mixture of rotamers, only peaks of the major rotamer are reported): δ = 7.25 (t, J = 7.7 Hz, 1H); 7.08 (d, J = 8.0 Hz, 1H); 6.97 (t, J = 7.7 Hz, 1H); 6.83 (d, J = 8.0 Hz, 1H); 6.79 (d, J = 8.0 Hz, 1H); 6.70 (s, 1H); 6.68 (s, 1H), 6.44 (bs, 2H); 6.18 (t, J = 6.8 Hz, 1H); 5.41-5.35 (m, 1H); 4.65 (ABq, J = 16.0 Hz, 2H); 3.86 (s, 6H); 3.85 (s, 3H); 3.82 (s, 3H); 3.74 (s, 3H); 3.35-3.80 (m, 1H), 3.58 (t, J = 7.0 Hz, 1H); 2.83 (dt, J = 2.7 Hz, J = 13.0 Hz, 1H); 2.63-2.47 (m, 2H); 2.32-2.18 (m, 2H), 2.11-2.03 (m, 3H); 1.78-1.65 (m, 3H); 1.62-1.23 (m, 2H); 0.90 (t, J = 6.0 Hz, 3H). (Data S1).

For the synthesis of (R)-3-(3,4-dimethoxyphenyl)-1-(((S)-1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carbonyl)-17,17-dimethyl-2,14-dioxo-6,9,12-trioxa-3,15-diazoctadecyl)oxy)phenylpropyl(S)-1-((S)-2((3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylic acid ( ortho-AP1867) to a solution of acid ortho-AP1867 (0.035 g, 0.050 mmol) in DMF (1 mL), HOBT (0.008 g, 0.060 mmol), EDCI (0.012 g, 0.060 mmol), 7 (0.045 g, 0.07 mmol), and DIPEA (0.035 mL, 0.2 mmol) were added at RT. The mixture was stirred at RT for 16 h, diluted with water (2 mL) and extracted with EtOAc (20 mL). The organic layer was washed with brine (5 mL) and concentrated. The crude residue was purified by column chromatography on silica (8 g), gradient from petroleum spirit to 100% EtOAc, then to 20% of MeOH in EtOAc. Thus, obtained crude material (0.06 g) was further purified on HPLC RP C18 column with gradient MeCN/H2O (5%–95%) to give 8 (dTAG-VHL binder, inactive) (0.041 g, 0.031 mmol, 62%) as amorphous solid.

1H NMR (500 MHz, CDCl3, mixture of rotamers, only peaks of the major rotamer are reported): δ = 8.67 (s, 1H), 7.41-7.34 (m, 4H), 7.33-7.25 (m, 1H); 7.20 (dt, J = 1.5 Hz, J = 7.8 Hz, 1H); 7.10-6.99 (m, 1H); 6.91-6.84 (m, 1H); 6.83-6.72 (m, 3H); 6.69-6.65 (m, 2H); 6.57-6.31 (m, 2H); 6.15 (t, J = 6.8 Hz, 1H); 5.49 (d, J = 4 Hz, 1H); 5.20 (bs, 0.5H); 5.10 (bs, 0.5H); 4.90 (bs, 0.5H); 4.85 (bs, 0.5H); 4.68-4.39 (m, 4H), 4.30-4.17 (m, 1H); 3.99 (ABq, J = 16.0 Hz, 2H); 3.95-3.87 (m, 1H); 3.86 (s, 3H); 3.85 (bs, 3H); 3.79 (s, 3H); 3.69 (s, 3H); 3.59-3.55 (m, 1H); 3.65-3.32 (m, 1H); 3.59-3.55 (m, 0.2H), 1.10-1.02 (m, 0.2H); 0.94 (t, J = 7.3 Hz, 2.4H), 0.90 (t, J = 7.3 Hz, 0.6H). (Data S1).
For the synthesis of (R)-3-(3,4-dimethoxyphenyl)-1-(2-(((S)-16-((2R,3R,4S)-3-fluoro-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamooyl)pyrrolidine-1-carbonyl)-17,17-dimethyl-2,14-dioxo-6,9,12-triazaoctadecyl)oxy)phenyl)propyl (S)-1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)pyrrolidine-2-carboxylate

\[ \text{dTAG-VHL, active} \], to a solution of acid ortho-AP1867 (0.162 g, 0.233 mmol) in DMF (2 mL), HOBt (0.038 g, 0.28 mmol), EDCI (0.054 g, 0.28 mmol), 9 (0.177 g, 0.277 mmol) and DIPEA (0.17 mL, 0.93 mmol) were added at RT. The mixture was stirred at RT for 16 h, diluted with water (5 mL) and extracted with EtOAc (25 mL). The organic layer was washed with brine (5 mL) and concentrated. The crude residue was purified by column chromatography on silica (40 g), gradient from petroleum spirit to 100% EtOAc, then to 20% of MeOH in EtOAc. Thus, obtained crude material (0.27 g) was further purified on HPLC RP C18 column with gradient MeCN/H2O (5 % – 95 %) to give \( \text{dTAG-VHL, active} \) (0.15 g, 0.114 mmol, 49%) as amorphous solid.

\[ \text{1H NMR (500 MHz, CDCl3, mixture of rotamers, only peaks of the major rotamer are reported):} \]
\[ \delta = 8.69 \text{ (s, 1H)}; 7.73-7.66 \text{ (m, 1H)}; 7.41-7.31 \text{ (m, 4H)}; 7.24-7.16 \text{ (m, 3H)}; 6.90-6.83 \text{ (m, 1H)}; 6.81-6.72 \text{ (m, 2H)}; 6.71-6.64 \text{ (m, 2H)}; 6.49 \text{ (bs, 1H)}; 6.15 \text{ (t, J = 6.4 Hz, 1H)}; 5.50 \text{ (bs, 1H)}; 5.31 \text{ (s, 0.5H)}; 5.20 \text{ (s, 0.5H)}; 4.83-4.75 \text{ (m, 1H)}; 4.65-4.30 \text{ (m, 6H)}; 4.03-3.96 \text{ (m, 3H)}; 3.95-3.75 \text{ (m, 2H)}; 3.86 \text{ (s, 3H)}; 3.84 \text{ (bs, 6H)}; 3.79 \text{ (s, 3H)}; 3.69-3.38 \text{ (m, 14H)}; 2.68-2.41 \text{ (m, 1H)}; 2.59-2.42 \text{ (m, 2H)}; 2.52 \text{ (s, 3H)}; 2.29-1.17 \text{ (m, 12H)}; 0.96 \text{ (s, 9H)}; 0.89 \text{ (t, J = 7.0 Hz, 3H)}.\]

\[ \text{19F NMR:} \]
\[ \delta = -204.65; \text{C68H89FN6O17S, expected for [M + H]+ 1313.61, found [M + H]+ 1313.59. (Data S1).} \]

\[ \text{Treatment of cells with compounds} \]
The following chemicals were added to cell media at stated concentrations and times: HaloPROTAC-E (synthesised as previously described (Tovell et al., 2019a; Simpson et al., 2020)), dTAG-13 (Tocris Bioscience), dTAG-VHL (active and inactive), dTAG\(_{24-1}\) (Tocris Bioscience), cycloheximide (CHX, Sigma-Aldrich), VH298 (Tocris Bioscience), MLN4924 (Sigma-Aldrich), MG132 (Abcam), bortezomib (LC Laboratories), bafilomycin A1 (Enzo Life Sciences), lenalidomide (Cayman Chemicals).

\[ \text{HiBiT protein quantification} \]
HiBiT protein levels were quantified using the Nano-Glo HiBiT lytic detection system (Promega, N3040) as described by manufacturer’s instructions (Schwinn et al., 2018). WT cells and those stably expressing HiBiT-tagged proteins were plated 1,000 cells per well in 96-well format. Following seeding of cells, cells were treated with indicated compounds for indicated times in duplicate. Nano-Glo HiBiT lytic reagent, containing lytic buffer, lytic substrate and LgBiT, was added to each well, incubated for 10 min on an orbital shaker (at 600 RPM) and luminescence was read using a PHERAstar FS plate-reader (BMG Labtech). Corresponding WT RLU values were subtracted from HiBiT-positive values and the percentage (%) of HiBiT RLU values were then calculated against DMSO-treated controls ± SD of \( n = 3 \) independent experiments. Data was analysed using Excel (Microsoft) and Prism\(_{\text{C210}}\) software (v9.4.0).

\[ \text{Cytotoxicity assay} \]
CellTox\(_{\text{C212}}\) Green Assay (Promega, Cat. #G8742, (Kupcho et al., 2014)) was used to assess the cytotoxicity of dTAG-VHL-Active and dTAG-VHL-Inactive compounds (24 h, 0–16 \( \mu \)M) in U2OS cells. The fluorescent signal produced by the CellTox\(_{\text{C212}}\) Green dye, upon selective binding to the DNA of cells with impaired membrane integrity, is proportional to cytotoxicity. Fluorescence was measured using ex: 480 nm em: 530 nm by a PHERAstar FS plate reader before normalisation to DMSO treatment. MG132 treatment (24 h, 40 \( \mu \)M) was included as a positive control. Data was analysed using Excel (Microsoft) and Prism\(_{\text{C210}}\) software (v9.4.0).

\[ \text{Cell lysis} \]
Cells were harvested by washing twice with phosphate-buffered saline (PBS) and scraping into ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 0.27 M sucrose, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium \( \beta \)-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate and 1% NP-40) supplemented with 1 \( \times \) cOmplete\(_{\text{C212}}\) protease inhibitor cocktail (Roche). After incubation for 10 min on ice, lysates were clarified by centrifugation at 17,000 xg for 20 min at 4°C. Protein concentration was determined according to the Bradford assay to enable normalisation between samples.
**Subcellular fractionation**

Wild-type U2OS, wild-type DLD1 and CRBN−/− DLD1 cells were scraped, pelleted, and resuspended in PBS; cytoplasmic, nuclear and membrane fractions from these cells were collected using the ProteoExtract® Subcellular Proteome Extraction Kit (MerckMillipore). Buffer volumes used to collect fractions from each cell pellet were altered as follows: 200 μL of Extraction Buffer I was used to collect cytoplasmic fractions, 200 μL Extraction Buffer II was used to collect membrane fractions and 100 μL of Extraction Buffer III was used to collect nuclear fractions. Initially, cell pellets were resuspended twice in 1 mL wash buffer and an additional wash step using PBS was introduced between each extraction step.

**SDS-PAGE and western blotting**

Cell lysates containing equal amounts of protein (10–20 μg) were resolved by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked in 5% (w/v) non-fat milk (Marvel) in TBS-T (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% Tween 20) and incubated overnight at 4°C in 5% (w/v) BSA/TBS-T or 5% (w/v) milk/TBS-T with the appropriate primary antibodies. Primary antibodies used at indicated dilutions include: anti-dTAG (DA179, MRC PPU Reagents and Services, 1:1000), anti-HaloTag (G921A, Promega, 1:5000), anti-β-actin (ab8226, Abcam, 1:1000), anti-CUL2 (51–1800, Invitrogen, 1:1000), anti-FKBP12 (ab24373, Abcam, 1:1000), anti-FLAG (A8592, Sigma-Aldrich, 1:2,500), anti-GAPDH (2118, CST, 1:5,000), anti-HIF1α (610959, BD, 1:1,000), anti-mono- and poly-ubiquitylated conjugates (Ub) (BML-PW8810, Enzo, 1:2,000), anti-α-tubulin (MA1-80189, Thermo Fisher Scientific, 1:5,000), anti-ZFP91 (A303-245A, Bethyl, 1:1,000), anti-cMYC (5605, CST, 1:1,000), anti-Na+/K+-ATPase (23565, CST, 1:1000), anti-Lamin A/C (2032, CST, 1:1000), anti-LC3 (M152-3, MBL, 1:1000), anti-VHL (68547, CST, 1:1000), anti-HCRBN (71810, CST, 1:1000).

Membranes were subsequently washed with TBS-T and, unless using primary antibody directly conjugated to HRP, incubated with HRP-conjugated secondary antibody for 1 h at room temperature. HRP-coupled secondary antibodies used at indicated dilutions include: goat anti-rabbit-IgG (7074, CST, 1:2,500), goat anti-rat IgG (62–9520, Thermo Fisher Scientific, 1:5,000), goat anti-mouse-IgG (31430, Thermo Fisher Scientific, 1:5,000). After further washing, signal detection was performed using ECL (Merck) and ChemiDoc MP System (Bio-Rad).

**Immunofluorescence microscopy**

Cells were seeded onto sterile glass coverslips in 6-well dishes. Coverslips were washed twice with PBS and either (i) fixed with 4% (w/v) paraformaldehyde (Thermo Fisher Scientific) for 10 min, washed twice with and incubated for 10 min in DMEM/10 mM HEPES pH 7.4 followed by one wash in PBS before cell permeabilisation was carried out using 0.2% NP-40 in PBS for 4 min or (ii) incubated immediately after washing in ice-cold methanol for 20 min for fixation and permeabilisation. Samples were blocked by washing twice and incubating for 15 min in blocking buffer (1% (w/v) BSA/PBS). Coverslips were incubated for 1 h at 37°C with primary antibodies in blocking buffer and washed three times in blocking buffer. The following primary antibodies were used at the following concentrations: mouse anti-ATPb (Ab14730, Abcam, 1:1,000), rabbit anti-calnexin (2679, CST, 1:200), rabbit anti-catalase (219010, Sigma-Aldrich, 1:1,000), mouse anti-FLAG (F1804, Sigma-Aldrich, 1:200), rabbit anti-FLAG (14793, CST, 1:200), rabbit anti-GM130 (12480, CST, 1:1,000), rat anti-LAMP1 (sc-19992, Santa Cruz, 1:200), rabbit anti-VHL (68547, CST, 1:200), rabbit anti-CRBN (71810, CST, 1:200). Coverslips were then incubated for 30 min at room temperature with Alexa fluor coupled secondary antibodies in blocking buffer and washed an additional three times in blocking buffer. Donkey anti-mouse IgG Alexa Fluor 488 (A21202, Thermo Fisher Scientific), donkey anti-rabbit IgG Alexa Fluor 488 (A21206, Thermo Fisher Scientific), goat anti-mouse IgG Alexa-Fluor 594 (A11055, Thermo Fisher Scientific), goat anti-rabbit IgG Alexa-Fluor 594 (A11012, Thermo Fisher Scientific) and donkey anti-rat IgG Alexa-Fluor 594 (A21209, Thermo Fisher Scientific) secondary antibodies were used at a 1:500 dilution. After submerging in ddH2O, cells were mounted onto glass slides using ProLong gold antifade mountant with DAPI (Life Technologies) and visualised using a DeltaVision system (Applied Precision) and deconvolved using SoftWoRx (Applied Precision). Images were processed using ImageJ (Schneider et al., 2012) and OMERO 5.4.10 software (Allan et al., 2012).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Western blot densitometry was measured using ImageJ (Schneider et al., 2012) and adjusted relative densities were calculated using Excel (Microsoft). All statistical analyses were performed, and graphs were generated using Prism software (v9.4.0). Statistical details including the exact value of n and any statistical tests performed are stated in the figure legends. All graphs display the mean ± standard deviation (SD) of 3 biological replicates.