

Deubiquitinases Sharpen Substrate Discrimination during Membrane Protein Degradation from the ER

Zai-Rong Zhang,^{1,3,*} Juan S. Bonifacino,¹ and Ramanujan S. Hegde^{2,*}

¹Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 18 Library Drive, Bethesda, MD 20892, USA

²MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge CB2 0QH, UK

³Present address: Nanobiology Institute, Department of Cell Biology, Yale University School of Medicine, Orange, CT 06477, USA

*Correspondence: zairong.zhang@gmail.com (Z.-R.Z.), rhegde@mrc-lmb.cam.ac.uk (R.S.H.)

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SUMMARY

Newly synthesized membrane proteins are queried by ubiquitin ligase complexes and triaged between degradative and nondegradative fates. The mechanisms that convert modest differences in substrate-ligase interactions into decisive outcomes of ubiquitination are not well understood. Here, we reconstitute membrane protein recognition and ubiquitination in liposomes using purified components from a viral-mediated degradation pathway. We find that substrate-ligase interactions in the membrane directly influence processivity of ubiquitin attachment to modulate polyubiquitination. Unexpectedly, differential processivity alone could not explain the differential fates in cultured cells of degraded and nondegraded clients. Both computational and experimental analyses identified continuous deubiquitination as a prerequisite for maximal substrate discrimination. Deubiquitinases reduce polyubiquitin dwell times preferentially on clients that dissociate more rapidly from the ligase. This explains how small differences in substrate-ligase interaction can be amplified into larger differences in net degradation. These results provide a conceptual framework for substrate discrimination during membrane protein quality control.

INTRODUCTION

Eukaryotic integral membrane proteins insert, assemble, and mature at the endoplasmic reticulum (ER) (Skach, 2009). Membrane proteins that fail to mature are ubiquitinated by ER resident ubiquitin ligases and degraded by the proteasome in a process termed ER-associated degradation (ERAD) (Vembar and Brodsky, 2008). ERAD pathways are also utilized for regulatory control of membrane proteins on the basis of cellular demand

(Hampton, 2002; DeBose-Boyd, 2008). Thus, all newly synthesized membrane proteins are triaged between degradative and nondegradative fates at the ER.

Proper triage is critical to cellular homeostasis and physiology. Failure to efficiently target misfolded proteins for degradation can lead to proteotoxicity, cell death, and disease (Chiti and Dobson, 2006). Conversely, overaggressive surveillance would result in degradation of functional products and loss-of-function consequences (Grove et al., 2011). Inappropriate triage of regulated factors can also lead to imbalanced expression levels with pathologic effects (DeBose-Boyd, 2008). Thus, achieving the correct balance in membrane protein triage is vital, but the mechanisms that regulate client discrimination are not well understood.

A key step in triage between degradative and nondegradative fates is client ubiquitination by an ER-resident ubiquitin ligase (Hirsch et al., 2009). A polyubiquitin tag serves as a signal for downstream factors to extract the membrane protein and deliver it to the proteasome for degradation (Vembar and Brodsky, 2008). The acquisition or not of polyubiquitin is therefore a deciding factor for client triage. The simplest model is one where clients destined for degradation are recruited to the ligase, whereas other proteins are not. This paradigm of discrimination, based solely on ligase access, is common in regulated degradation. For example, Sic1 is targeted for degradation only after phosphorylation generates a mark for ubiquitin ligase recruitment (Feldman et al., 1997). Although attractively simple, there are several reasons to suspect that ligase access alone cannot provide sufficient discriminatory power in quality control.

First, quality control is usually thought to involve broad “surveillance” of many potential clients, with commitment for degradation of only a minor subset. Second, most quality-control ligases must recognize a highly diverse range of clients; not only are there numerous individual proteins, but each one can be misfolded in myriad ways. This means that a single “recognition motif” or degron is unlikely, placing constraints on the level of selectivity that can be achieved by simple interaction. And third, essentially all newly synthesized proteins are potential clients early in their biosynthesis before they have folded, assembled, and matured. Thus, the issue may be less about

whether a client interacts with a ligase, but rather more subtle aspects of the nature of the interaction.

Indeed, coimmunoprecipitation (co-IP) and crosslinking studies show qualitatively similar ligase interactions for degraded and nondegraded versions of potential clients. For example, the client Hmg2p interacts with the ligase Hrd1p regardless of whether Hmg2p is subsequently degraded or not (Gardner et al., 2001; Sato et al., 2009). Similarly, the ligase-client interactions for degraded and nondegraded forms of TCR α are also remarkably similar (Ishikura et al., 2010). Thus, ligase access per se does not obligate client degradation; rather, a more nuanced model is needed to explain how modest differences in ligase interaction are converted to large differences in client fate.

Efforts to study this process in mechanistic depth have been hampered by various challenges. In vitro systems to date are typically composed of microsomes (or semipermeabilized cells) combined with cytosol (Shamu et al., 1999; Nakatsukasa et al., 2008; Garza et al., 2009). Although the cytosol is experimentally accessible, the membrane components remain a challenge. Full control of reactions in the membrane requires the capacity to independently reconstitute purified client and ligase complexes. Unfortunately, a misfolded protein client poses considerable obstacles to expression, purification, and reconstitution. Furthermore, the multiple components and complex topology of ER-resident ligases (Hirsch et al., 2009) has thus far precluded their functional membrane reconstitution. Thus, it has not been possible to rigorously examine how the nature of client-ligase interactions in the membrane influences ubiquitination and commitment for degradation.

To circumvent these issues, we turned to virus-mediated degradation of host membrane protein from the ER. Several viruses encode membrane proteins that exploit host ubiquitin ligases for regulated degradation of key cellular proteins (Isaacson and Ploegh, 2009). Such systems have yielded key insights into ERAD previously and provide several attractive features for reconstitution studies. First, the client is not recognized on the basis of misfolding, meaning that a native protein amenable to recombinant production can be used. Second, the system is typically orthogonal to endogenous pathways and utilizes a single defined ligase. Third, viral systems are usually rapid, efficient, and robust. Thus, viral systems of regulated degradation often encapsulate the salient features of a physiologic process in a simplified format.

In this study, we exploit these advantages to reconstitute with purified components the ubiquitination of CD4 by the HIV-encoded protein Vpu. The reconstituted system permitted quantitative mechanistic analysis to reveal key aspects of substrate discrimination and commitment for degradation. We discovered that modest differences in client-ligase interaction are converted into clear differences in polyubiquitination by a combination of processivity differences and deubiquitinases (DUBs) that impact the dwell time of a degradation mark on potential clients. These findings have implications for the mechanism underlying membrane protein triage and quality control at the ER.

RESULTS AND DISCUSSION

Experimental System

The HIV-encoded membrane protein Vpu interacts with CD4 at the ER and routes it for proteasomal degradation (reviewed by

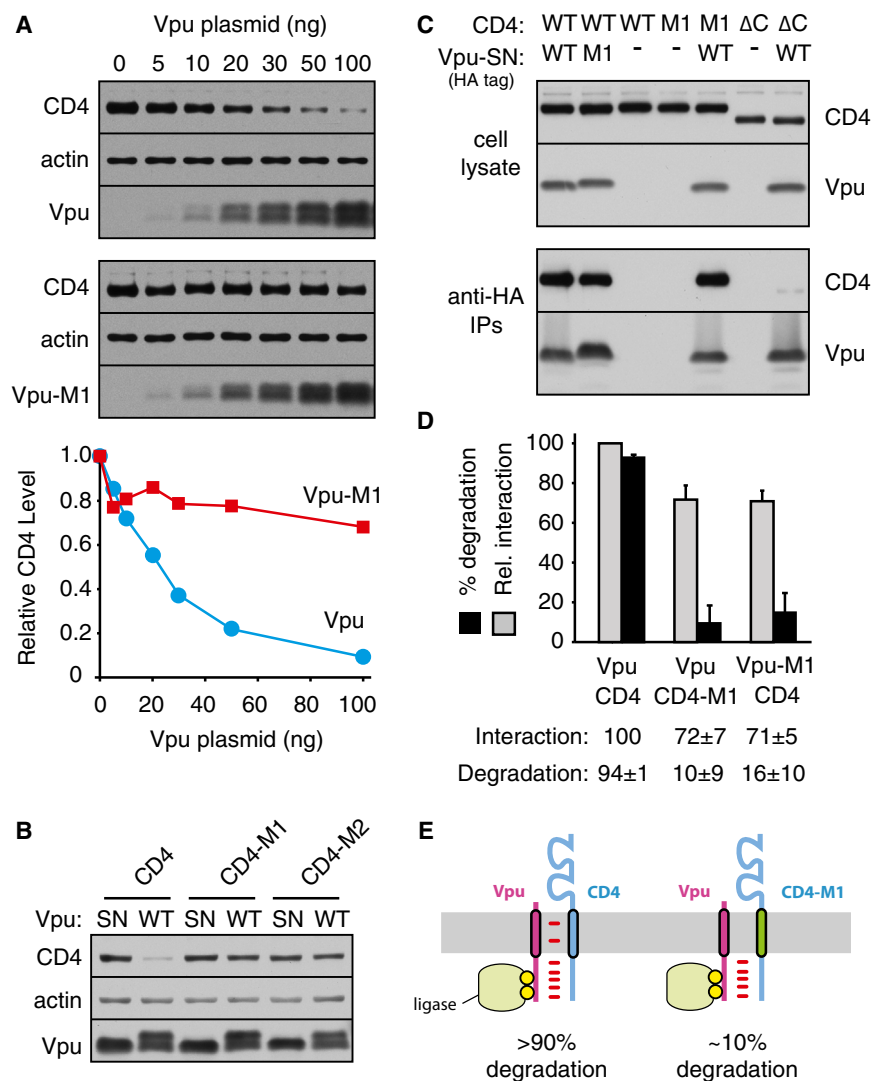
Nomaguchi et al., 2008; Figure S1A available online). Although Vpu is not a ubiquitin ligase, its phosphorylation at two sites by casein kinase 2 (CK2) permits a high-affinity interaction with the SCF ^{β TrCP} ubiquitin ligase complex via its F-box protein β TrCP (Margottin et al., 1998). Ligase recruitment to the ER leads to ubiquitination of the unstructured cytosolic tail of CD4. Phospho-Vpu therefore converts the nucleocytoplasmic SCF ^{β TrCP} complex into an ER ubiquitin ligase that mediates selective degradation of its CD4 client. Importantly, the basic features of this system work in various heterologous systems (Chen et al., 1993; Schubert et al., 1998; Meusser and Sommer, 2004), arguing against cell-type-specific features in CD4 degradation. The Vpu-mediated CD4 degradation pathway therefore affords a simplified model of membrane protein degradation whose mechanistic details may be amenable to dissection by in vitro reconstitution. Our strategy was to first define versions of CD4 and Vpu that differ in their membrane interactions and degradation and then use these model clients and nonclients to investigate the basis of substrate discrimination during membrane protein triage.

Transmembrane-Domain-Dependent Interactions Modulate CD4 Degradation by Vpu

CD4 expression in cultured cells was downregulated in a dose-dependent manner by coexpression of Vpu (Figure 1A). A phosphorylation site Vpu mutant (Vpu-SN; see Table S1 for construct details) was completely inactive (Figure S1B), as characterized in earlier work (Schubert and Strelbel, 1994; Magadán et al., 2010). Changing three residues in the transmembrane domain (TMD) of Vpu (I17F/V21F/V25L, termed Vpu-M1) largely abolished its ability to downregulate CD4 (Figure 1A). Similar results were seen when the Vpu TMD was replaced with heterologous TMDs (Vpu-M2, and Vpu-M3; Figure S1C). Replacing the TMD of CD4 with heterologous TMDs (CD4-M1 and CD4-M2) also rendered it refractory to Vpu downregulation (Figure 1B) unless the heterologous TMD (CD4-M3) could interact with Vpu (Figure S1D). Importantly, the various CD4 and Vpu TMD mutant proteins were verified by fractionation experiments to be membrane inserted (data not shown). Results from the steady-state experiments with the M1 mutants were confirmed by pulse-chase analysis (Figure S1E). Thus, Phospho-Vpu-mediated CD4 degradation from the ER depends on features of the TMDs of both proteins (Magadán and Bonifacino, 2012).

To examine the contribution of TMDs to the CD4-Vpu interaction, we performed co-IP experiments. The Vpu-SN phosphorylation mutant permitted interactions to be assessed in the absence of downstream degradation. As expected (Bour et al., 1995; Magadán et al., 2010), Vpu-SN coimmunoprecipitated CD4 (Figure 1C) and vice versa (Figure S1F). However, a qualitatively similar interaction by co-IP was also observed with TMD mutants of either Vpu or CD4 (Figures 1C, S1F, and S1G). Deleting the cytosolic domain of CD4 showed a marked (>80%), but not complete, loss in co-IP with Vpu (Figure 1C), consistent with an interaction between their cytosolic tails (Bour et al., 1995). Combining the CD4 cytosolic deletion with a Vpu TMD mutant abolished the interaction entirely (data not shown). Thus, the CD4-Vpu interaction is bipartite via both the cytosolic and TMD regions.

The contribution of the TMD was judged to be relatively minor based on the modest ~30% reduction in co-IP with the CD4 or



Vpu M1 mutants (Figure 1D). This indicates that the TMD mutant proteins are engaged in CD4-Vpu complexes to at least 70% the level as the wild-type pair. Yet, phospho-Vpu-dependent CD4 degradation is almost completely lost (quantified in Figure 1D), suggesting that even small decreases in interaction are sufficient to effect a large change in degradation. This mirrors earlier observations with ER ubiquitin ligases, where degraded versus nondegraded versions of clients displayed surprisingly minor differences in ligase interaction (Gardner et al., 2001; Meacham et al., 2001; Sato et al., 2009; Ishikura et al., 2010). Thus, the Vpu-CD4 system and their TMD mutants provide a simple model system to dissect the mechanistic basis of selective degradation for subtly different client-ligase interactions in the membrane (Figure 1E).

In Vitro Reconstitution of CD4 Ubiquitination by a Vpu-Bound Ubiquitin Ligase

To facilitate in vitro reconstitution of Vpu-mediated CD4 ubiquitination, we isolated ER microsomes from cells expressing HA-

Figure 1. TMD-Dependent CD4 Degradation by Vpu

(A) Increasing amounts of Vpu (top) or Vpu-M1 (panel) plasmids were cotransfected with a fixed amount (100 ng) of CD4 and analyzed 24 hr later by immunoblotting. The two Vpu bands correspond to phosphorylated and unphosphorylated forms. Actin is a loading control. Quantification of CD4 is shown below the blots.

(B) Wild-type (WT) Vpu or the Vpu-SN phosphorylation mutant (SN) was coexpressed with the indicated CD4 constructs and analyzed by immunoblotting.

(C) HA-tagged Vpu-SN constructs containing the wild-type or M1 mutant transmembrane domain were coexpressed with the indicated CD4 constructs and analyzed by immunoblotting directly (5% lysate; top) or after IP with anti-HA antibodies (bottom).

(D) Quantification of CD4 degradation and interaction (by co-IP) for the indicated Vpu-CD4 pairs. Degradation was analyzed as in (B), and interaction was analyzed as in (C). Mean \pm SD from at least three experiments.

(E) Disrupting TMD-TMD interactions between Vpu and CD4 markedly reduces CD4 degradation despite minimal effects on their overall interaction. See also Figure S1 and Tables S1 and S2.

tagged Vpu or phosphorylation-deficient Vpu-SN. Coimmunoprecipitation experiments showed that Vpu, but not Vpu-SN, was associated with the SCF ^{β TrCP} complex (Figure 2A). The Vpu-SCF ^{β TrCP} complex was completely stable to 0.8 M salt wash (Figure S2A), illustrating that this complex essentially behaves as an integral membrane ubiquitin ligase. Importantly, the microsomes were functional for protein translocation (Figure 2B),

allowing ³⁵S-labeled proteins to be introduced into them by in vitro translation.

Given that Vpu has at most one or two residues in the ER lumen, we reasoned that the only relevant portion of CD4 for its selective ubiquitination was the TMD and cytosolic tail. A “mini-CD4” (mCD4) containing only these domains was constructed (Table S2) and verified to insert efficiently and correctly into ER microsomes by in vitro translation (Figure 2C). Although this construct does not contain a signal peptide, the TMD, now being positioned near the N terminus, acts as a type I signal anchor to mediate targeting and insertion (Higy et al., 2004). Protease protection assays combined with IPs verified that mCD4 acquires the correct type I topology (Figure 2C). Appending an N-terminal glycosylation site to mCD4 resulted in mCD4 glycosylation (Figure 2C), corroborating its insertion by an independent means.

Following mCD4 in vitro translation and insertion, microsomes were isolated and washed with 0.8 M salt to remove the cytosol and any noninserted mCD4. Next, ubiquitination reactions were

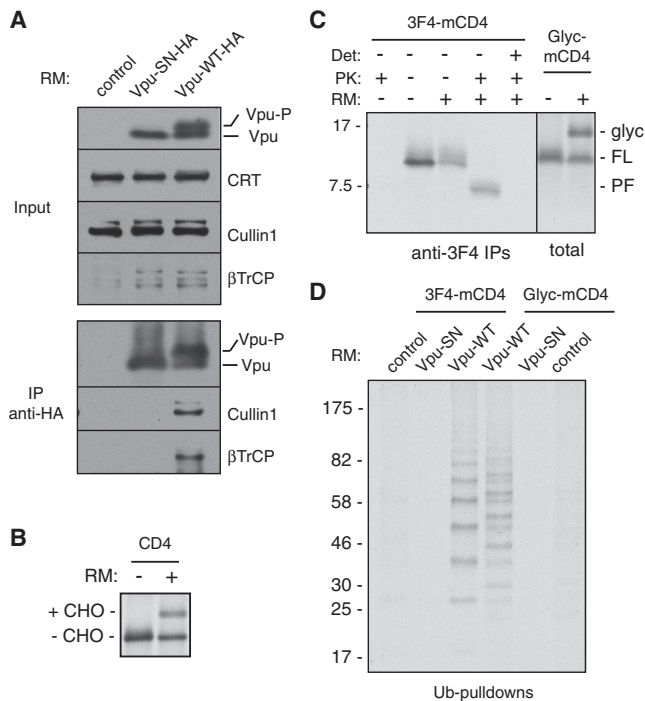


Figure 2. Reconstitution of Vpu-Mediated CD4 Ubiquitination on Microsomes

(A) HEK293-derived rough microsomes (RM) from cells expressing nothing (control), Vpu-HA, or Vpu-SN-HA were analyzed directly or after anti-HA IPs. The positions of phosphorylated and unphosphorylated Vpu are indicated. CRT is the ER resident chaperone calreticulin.

(B) CD4 in vitro translated in the presence of HEK293 RMs is translocated as evidenced by its glycosylation.

(C) 3F4-mCD4 was in vitro translated with ^{35}S -methionine in the absence or presence of RMs and subjected to digestion with proteinase K (PK) in the absence or presence of detergent (Det). A matched construct containing a consensus glycosylation site (Glyc-mCD4) was also analyzed. The products were immunoprecipitated using antibodies against the N-terminal 3F4 tag and analyzed by SDS-PAGE and autoradiography. The bands are as follows: FL, full-length mCD4; glyc, glycosylated mCD4; PF, protease-protected fragment.

(D) 3F4-mCD4 or Glyc-mCD4 was in vitro translated and translocated into the indicated RMs from HEK293 cells, and the isolated, high-salt washed RMs were subjected to a ubiquitination reaction by adding E1 and E2 enzymes, His-tagged ubiquitin, and ATP. Ubiquitinated products were pulled down via the His tag and the mCD4 (or Glyc-mCD4) visualized by autoradiography.

See also Figure S2 and Tables S1 and S2.

initiated by adding purified E1 and E2 enzymes, together with tagged-ubiquitin and ATP. Ubiquitin pull-downs followed by visualization of the radiolabeled mCD4 revealed a ladder of ubiquitinated mCD4 (Figure 2D). Glycosylated mCD4 was also ubiquitinated (Figure 2D), verifying that membrane-inserted mCD4 was being modified. Ubiquitination was dependent on SCF ^{βTrCP} because a parallel reaction with phosphorylation-deficient Vpu-SN failed to be ubiquitinated (Figure 2D), consistent with its inability to coimmunoprecipitate SCF ^{βTrCP} (Figures 2A and S2A). Furthermore, the absolute dependence on phospho-Vpu illustrates that endogenous ligases in this system do not perceive mCD4 as a misfolded protein for ubiquitination. Optimal polyubiquitination was achieved using a mixture of Ubch3 and

Ubch5 (Figure S2B), consistent with different E2 enzymes being optimal for initial ubiquitination versus chain elongation (Rodrigo-Brenni and Morgan, 2007; Wu et al., 2010). Use of a K48R mutant ubiquitin showed deficient chain elongation (Figure S2C). Ubiquitination was not observed when the four lysines in the tail of CD4 were mutated to arginines (data not shown). Thus, the Vpu-SCF ^{βTrCP} ligase complex ubiquitinates mCD4 on isolated membranes in vitro, setting the stage for reconstitution with purified factors.

Reconstitution of Membrane Protein Ubiquitination with Recombinant Components

The modularity of the Vpu-CD4 system afforded the opportunity to assemble it from individual recombinant factors in liposomes (Figure 3A). We expressed and purified mCD4 and Vpu from *E. coli* (Figure 3B), confirmed that purified Vpu can be phosphorylated efficiently by purified Casein Kinase 2 (CK2; data not shown), and that the cytosolic domain interacted efficiently with βTrCP in a phosphorylation-dependent manner (Figure S3A). We then optimized conditions for reconstitution of mCD4 and Vpu into synthetic liposomes such that most of it was incorporated in the correct orientation as determined by protease protection assays (Figure S3B). Co-IP experiments showed that when mCD4 and Vpu were coinorporated into the same liposomes, they interacted with each other (Figure 3C). By contrast, mixing separate liposomes each containing mCD4 or Vpu, followed by solubilization and IP, showed minimal interaction (Figure 3C).

Incubation of liposomes containing mCD4 and Vpu with purified recombinant SCF ^{βTrCP} complex (Figure 3B), E1 and E2 enzymes, ubiquitin, and ATP resulted in polyubiquitination of mCD4 (Figure 3D). Ubiquitination was strictly dependent on Vpu phosphorylation because neither unphosphorylated wild-type Vpu nor CK2-treated Vpu-SN supported mCD4 ubiquitination (Figure 3D). As expected from earlier work (Duda et al., 2008; Saha and Deshaies, 2008), the speed and efficiency of ubiquitination was improved by Nedd8 modification of the SCF ^{βTrCP} complex (Figures S3C and S3D).

The cytosolic domains of CD4 and phospho-Vpu, despite the capability to interact weakly (Singh et al., 2012), failed to produce substantial CD4 ubiquitination (Figures 3E and 3F). By contrast, tethering these His-tagged cytosolic domains to the surface of membranes via Ni²⁺-NTA lipid permitted ubiquitination (Figures 3E and 3F). However, the ubiquitination was substantially less processive than membrane-inserted mCD4 and Vpu as evidenced by fewer ubiquitins on the substrate (around three to seven, compared to over ten). This effect was not due to membrane insertion per se because replacing the TMDs of Vpu and mCD4 with oppositely charged interacting coiled coils permitted highly processive ubiquitination in the absence of any membrane (Figures 3E and 3F).

These results rigorously illustrate that Vpu-mediated CD4 ubiquitination requires no additional factors beyond the SCF ^{βTrCP} complex, its associated E2 enzymes, E1, and ubiquitin. Moreover, the data suggest that the cytosolic domains of CD4 and Vpu interact too weakly to mediate efficient ubiquitination in solution. However, constraining them to a membrane surface allows some ubiquitination, presumably by limiting their diffusion

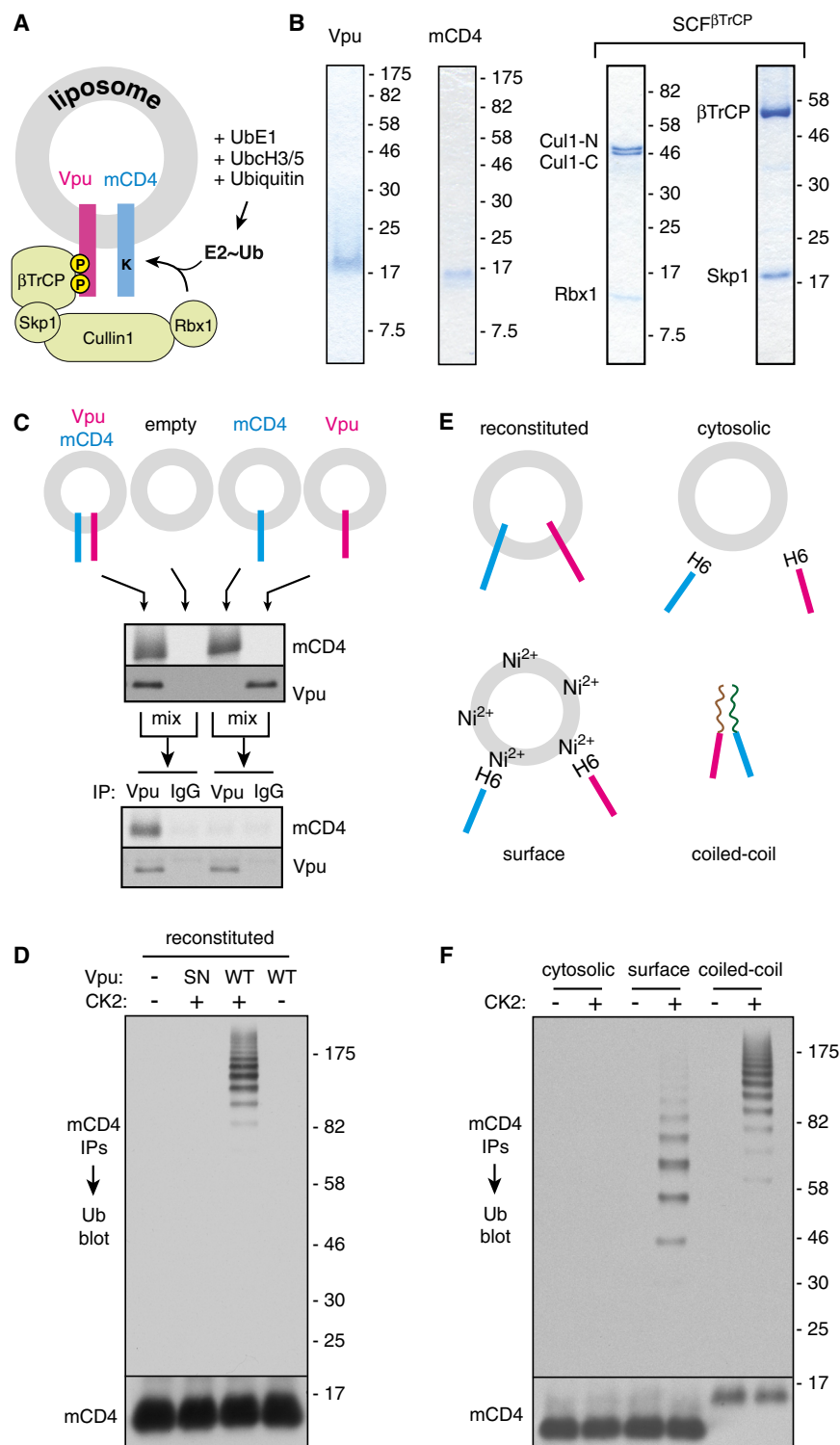


Figure 3. TMD-Dependent mCD4 Ubiquitination with Purified Factors

(A) Diagram depicting the purified system. (B) Coomassie-stained gels of recombinant purified proteins.

(C) Co-IP analysis of recombinant Vpu and mCD4 in reconstituted proteoliposomes. Radiolabeled mCD4 and recombinant Vpu were incorporated either separately or together into liposomes, and the indicated samples were mixed, solubilized, and subjected to IP with anti-Vpu or control IgG. The samples before and after IP were analyzed by immunoblotting for Vpu and autoradiography for mCD4, respectively.

(D) Liposomes reconstituted with mCD4 alone, with Vpu (WT), or with Vpu-SN were incubated with CK2 (as indicated) and ubiquitination factors (E1, UbcH3, UbcH5c, Myc-ubiquitin, ATP). mCD4 was immunoprecipitated and the samples blotted for ubiquitin or mCD4.

(E and F) Vpu and mCD4 combinations as depicted in (E) were subjected to ubiquitination and analyzed as in (D).

See also Figure S3 and Tables S1 and S2.

permits the ligase time to sequentially build long ubiquitin chains. This establishes a membrane protein ubiquitination reaction that relies on TMD-dependent substrate-ligase interaction to maximize processivity.

Substrate-Ligase Interactions Modulate Ubiquitination Processivity

We next asked whether the discrimination of correct from incorrect substrates observed in cultured cells could be recapitulated in vitro. To facilitate quantitative analysis, we modified the recombinant system to contain radiolabeled substrate generated by in vitro translation (Figure S4A). The translated and microsome-inserted substrate was separated from the translation extract and bulk microsomal proteins and mixed with purified lipids and recombinant Vpu prior to reconstitution into proteoliposomes. The final proteoliposomes in this radiolabeled recombinant system contained Vpu and radiolabeled mCD4, but undetectable levels of proteins from the original in vitro translation reaction and more than 95% removal of microsomal proteins (data not shown). The remainder

and degrees of freedom to enhance their interaction. The TMDs add to this interaction, presumably by orienting the cytosolic domains and by interacting within the membrane to further stabilize the Vpu-CD4 complex. Prolonged interaction

of the components (for ubiquitination) was from recombinant sources. As expected, mCD4 ubiquitination in the radiolabeled recombinant system displayed a strict dependence on phospho-Vpu, showed high processivity and was easily quantifiable

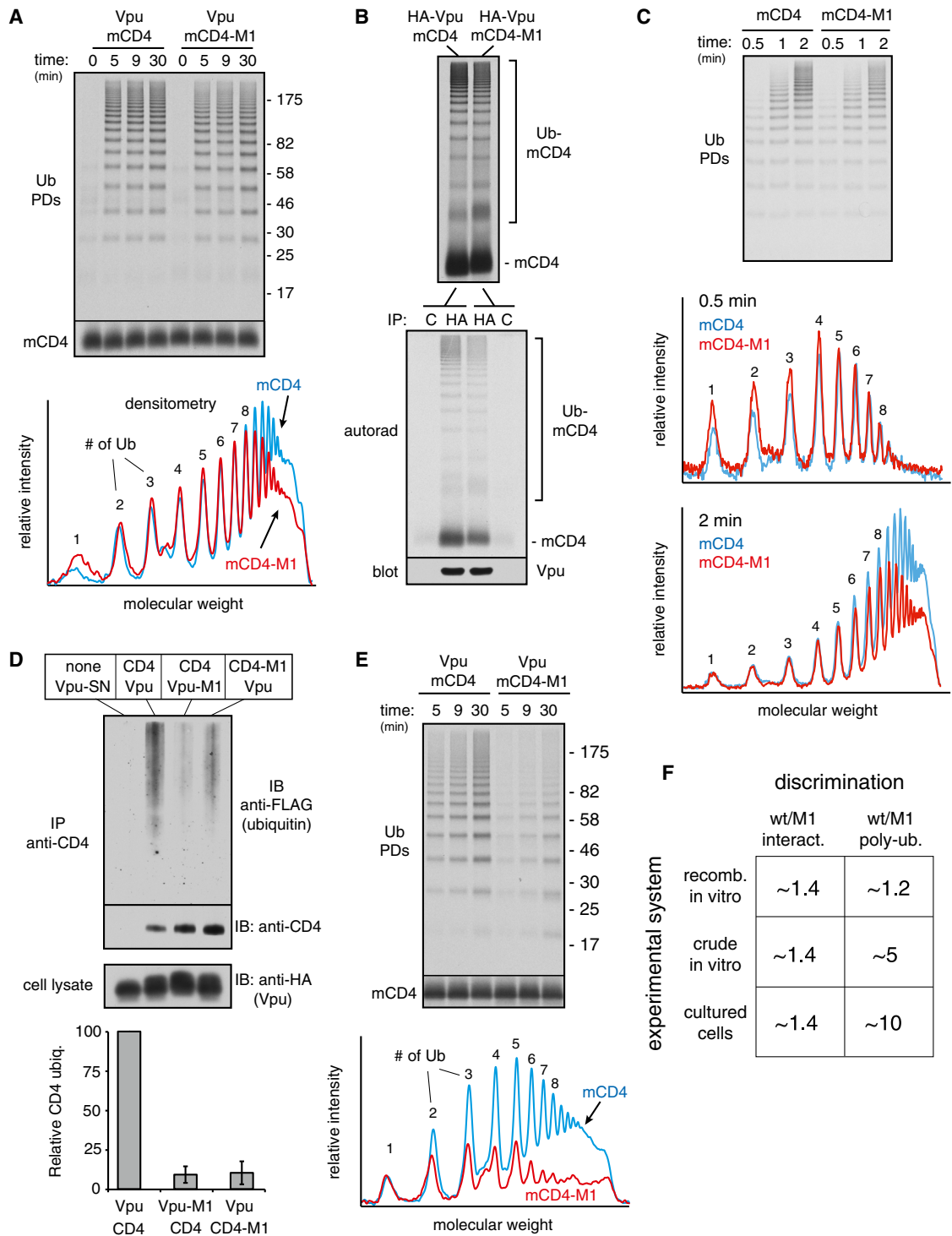


Figure 4. Substrate Discrimination in Cells, In Vitro, and in Purified Systems

(A) Analysis of discrimination in a radiolabeled reconstituted ubiquitination system. Radiolabeled mCD4 (or mCD4-M1) isolated from in vitro translation reactions (see Figure S4A) was coreconstituted with recombinant Vpu into liposomes. The purified proteoliposomes were subjected to ubiquitination with recombinant SCF^{βTrCP}, purified E1 and E2 enzymes, His6-tagged ubiquitin, and ATP. The reaction at different time points was stopped and analyzed for ubiquitinated mCD4 via pull-downs of the tagged ubiquitin (Ub PDs). Total mCD4 in the reaction is shown in the bottom panel. The graph below the autoradiograph depicts the densitometry profiles of the 5 min samples, with the individual ubiquitinated species indicated.

(B) Ubiquitination reactions as in (A) were either analyzed directly (top) or after solubilization and native IP using anti-HA or control antibodies (bottom). The HA-tagged Vpu recovered in the IP was analyzed by blotting, and the mCD4 was analyzed by autoradiography.

(legend continued on next page)

given that only the substrate was radiolabeled (Figures S4B and S4C).

Unexpectedly, the Vpu-mediated ubiquitination of mCD4 and mCD4-M1 was nearly identical (Figure 4A). Co-IP experiments showed that the Vpu-mCD4-M1 interaction *in vitro* was reduced modestly relative to the Vpu-mCD4 interaction (Figure 4B), very similar to co-IP experiments in cells (Figure 1D). Furthermore, crosslinking analysis in proteoliposomes showed that β TrCP crosslinks almost equally well to mCD4 versus mCD4-M1 (Figure S4D). Importantly, ubiquitination was strictly dependent on the Vpu-SCF complex (Figures S4B and S4E), arguing against any other contaminating activities in the system that could explain the lack of difference. Thus, discrimination of correct from incorrect clients in the minimal *in vitro* system was incongruent with degradation efficiencies observed in cultured cells.

Similar results were observed when Vpu-M1 was used for mCD4 ubiquitination (Figures S4C and S4F). The lack of discrimination was observed over a wide range of concentrations (Figure S5A) and could not be easily explained by excessively high (or low) substrate or ligase levels. However, careful quantification of the ubiquitination profiles from the *in vitro* reactions revealed a subtle but highly reproducible difference in the high molecular weight species (see densitometry profiles, Figures 4A, S4F, and S5B). This reduction reflects a slight deficiency in building particularly long chains for the mutant mCD4-Vpu pairs relative to the wild-type pair, suggesting reduced processivity of ubiquitin addition.

Indeed, analysis of ubiquitination profiles with finer temporal resolution showed that at very short time points (30 s), mCD4-M1 was indistinguishable from mCD4 (Figure 4C). Only at later times is a difference seen preferentially for longer ubiquitin chain lengths. Thus, ubiquitins are initially added identically to mCD4 and mCD4-M1, consistent with the crosslinking results showing that Vpu-bound β TrCP “sees” these two proteins almost equally well (Figure S4D). However, mCD4-M1 presumably dissociates slightly faster from the Vpu-ligase complex than mCD4. This would give mCD4-M1 slightly less time than mCD4 to acquire ubiquitins. Although this may not be significant for a single encounter, the additive effect of multiple encounters would explain the difference preferentially seen for long ubiquitin chains. We therefore conclude that modest differences in interaction translate to differences in processivity of ubiquitin addition that translate to differences in long-chain modified substrates.

Discrepant Substrate Discrimination in Reconstituted versus Native Systems

Although the processivity differences in ubiquitination *in vitro* are consistent with similarly subtle differences in interaction by co-

IP, neither result matches the substantially greater difference in degradation for wild-type versus mutant TMDs. Indeed, Vpu-mediated CD4 ubiquitination in cultured cells under conditions of proteasome inhibition showed a clear ~5- to 10-fold difference between CD4 and CD4-M1 (Figure 4D). This corresponded not only to decreased degradation (e.g., Figure 1), but also to decreased dislocation as judged by the absence of a deglycosylated product upon proteasome inhibition (Figure S5C). The cultured cell system therefore converts modest differences in CD4-Vpu interaction into substantial differences in CD4 polyubiquitination, dislocation, and degradation. This feature of discrimination is only partially recapitulated in the radiolabeled recombinant system as a slight difference in processivity (Figure 4A). By contrast, use of ER microsomes instead of reconstituted proteoliposomes displayed clear discrimination between wild-type and mutant mCD4-Vpu pairs (Figures 4E and S5D). Thus, the radiolabeled recombinant *in vitro* system seems to lack some feature(s) that imparts substrate discrimination to the crude *in vitro* and cellular systems (summarized in Figure 4F).

Kinetic Modeling Suggests a Role for Deubiquitination in Substrate Discrimination

A clue to the missing feature came from the observation that enhanced discrimination in the crude *in vitro* system was accompanied by shorter ubiquitin chains (Figure 5A). This suggested that the crude system contained deubiquitination activity, which has been speculated to influence the timing of degradation from the ER (Blount et al., 2012; Lederkremer and Glickman, 2005; Feldman and van der Goot, 2009). Indeed, ubiquitinated mCD4 produced in the radiolabeled recombinant system was deubiquitinated when incubated in a crude cell lysate (Figure S6A). Ubiquitin removal occurred progressively from the distal end of the chain (Figure S6B), illustrating that mCD4 is subject to exodeubiquitination.

To understand how deubiquitination can influence substrate discrimination, we turned to kinetic modeling. Pioneering studies by Pierce et al. (2009) established a kinetic description of SCF $^{\beta$ TrCP-mediated ubiquitination of β -catenin peptide. We expanded and revised the kinetic model in a few minor ways (Figures 5B and S6C) and verified that it produced a ubiquitination profile roughly comparable to that observed experimentally (Figure 5C, green bars, compare to Figure 5A).

We then incorporated DUBs into the model such that they can remove the terminal ubiquitin from the chain. Using a DUB concentration of 0.4 μ M, and a K_{cat} of 0.5 s^{-1} (Hassiepen et al., 2007), the model produces a ubiquitination profile similar to that observed experimentally in the crude *in vitro* Vpu-mCD4 system (Figure 5C, orange bars, compare to Figure 5A). Using

(C) Analysis of ubiquitination reactions as in (A) at short time points.

(D) CD4 ubiquitination in cells. The indicated combinations of Vpu and CD4 were cotransfected with FLAG-ubiquitin, treated with proteasome inhibitor (40 μ M MG132) for 5 hr, subjected to IP with anti-CD4, and analyzed by immunoblotting for FLAG-ubiquitin and CD4. Vpu levels in the lysate are also shown. The graph shows quantification (mean \pm SD; $n = 3$) of relative CD4 ubiquitination normalized to total CD4.

(E) Ubiquitination reactions of *in vitro* translated mCD4 or mCD4-M1 in Vpu-containing HEK293 microsomes. Quantification of the 30 min time point is shown below the autoradiograph.

(F) Summary of the results of interaction and ubiquitination analysis from cell culture, crude *in vitro*, and recombinant *in vitro* systems. The approximate ratios of wild-type (wt) to M1 mutant recovered by co-IP (interaction) and observed to be polyubiquitinated are indicated. The recombinant *in vitro* system shows poor discrimination.

See also Figures S4 and S5 and Tables S1 and S2.

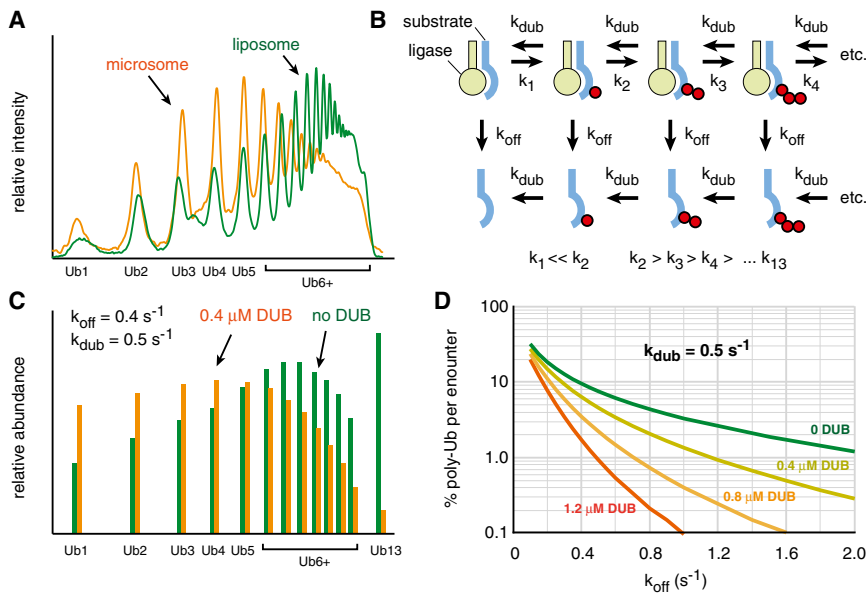


Figure 5. Kinetic Modeling of Ubiquitination without DUBs versus with DUBs

(A) Normalized ubiquitination profiles of reactions performed in liposomes (green) versus microsomes (orange).

(B) Kinetic model of substrate ubiquitination, substrate dissociation, and deubiquitination. See Figure S6C for additional details.

(C) Output of the kinetic model at 20 s using the indicated parameters without (green) or with (orange) DUBs. The relative amounts of the individual ubiquitinated species are plotted.

(D) Relationship between polyubiquitination (defined as four or more ubiquitins), k_{off} , and DUB activity predicted by the model analyzed at 20 s. See also Figure S6.

this as a starting point, we used the model to examine the role of DUB activity in the relationship between client-ligase interaction and polyubiquitination. In the absence of DUB activity, we observed that substrate polyubiquitination (defined here as four or more ubiquitins) in a single encounter decreased progressively with increasing K_{off} values for the substrate-ligase interaction (Figure 5D). This is easily rationalized because rapid substrate dissociation from the ligase provides less time to build ubiquitin chains. In the presence of DUB activity, a qualitatively similar relationship between K_{off} and polyubiquitination was observed (Figure 5D). However, overall polyubiquitination was less than without DUBs (particularly at higher K_{off} values), and the curve was progressively steeper with increasing DUB concentration.

The net effect of this is to increase discrimination (as defined by fold differences in polyubiquitination) between two substrate-ligase pairs of differing rates of dissociation (K_{off}). For example, in the absence of DUBs (Figure 5D, green curve), a 2-fold increase in K_{off} (from 0.4 to 0.8) results in an ~ 2.2 -fold decrease in polyubiquitination (from 9.4% to 4.3%). In the presence of high DUB activity (Figure 5D, red curve), that same 2-fold increase in K_{off} results in an ~ 8.3 -fold decrease in polyubiquitination (from 1.66% to 0.2%). Thus, DUB activity can noticeably improve substrate discrimination in this model, albeit at the expense of overall ubiquitination efficiency. This point is also revealed by inspecting the kinetic model output at different time points (Figures S6D and S6E).

DUB Activity Enhances Substrate Discrimination by Vpu

Adding cytosol to the radiolabeled recombinant system during the ubiquitination reaction decreased overall polyubiquitination and ubiquitin chain length and increased discrimination between mCD4 and mCD4-M1 (Figure 6A). Pretreatment of the cytosol with Ubiquitin-aldehyde partially reversed each of these effects, illustrating that their origin was likely to involve DUBs (Figures 6A and 6B). The same results, albeit somewhat muted, were ob-

erved when the mCD4-Vpu-M1 pair was analyzed (Figure S7A). The incomplete reversal with ubiquitin-aldehyde can be explained by incomplete inhibition of DUB activity (Figure S7B), whereas the slightly reduced discrimination over time seems to be due to DUBs losing activity sooner than the SCF (Figure S7C). These technical quirks notwithstanding, at least one contributing factor to discrimination imparted by cytosol can be attributed to DUB activity.

Use of a purified DUB (the catalytic domain of USP2) in lieu of cytosol also imparted discrimination to the radiolabeled recombinant mCD4-Vpu system in a concentration-dependent manner (Figures 6C–6E). Consistent with predictions from the kinetic modeling, discrimination came at the expense of ubiquitination efficiency (Figure 6F). Other purified DUBs (USP7, Ataxin3) could also impart discrimination in this assay, albeit to different extents that correlated with their relative catalytic activities (data not shown). It therefore appears that DUB activity, rather than a specific DUB, is the crucial determinant in imparting discrimination in this system.

Evidence for a Role of DUBs in Substrate Discrimination in Cultured Cells

An important insight from our *in vitro* and modeling studies is that a nondegraded client (i.e., CD4-M1) is nevertheless ubiquitinated, but that the ubiquitin chains are rapidly counteracted and rarely long enough to trigger degradation. Thus, lack of degradation is not due to a lack of ligase interaction or ubiquitination, but to rapid deubiquitination. This conclusion makes two testable predictions. First, nondegraded clients should be transiently modified with ubiquitin chains that do not usually reach a sufficient length to trigger degradation. Second, reducing cellular DUB activity should mute substrate discrimination by preferentially reducing the levels of normally nondegraded substrates. Each of these predictions was examined to evaluate the role of DUBs in cultured cells.

We analyzed ubiquitination of clients without proteasome inhibitor pretreatment and attempted to detect individual ubiquitinated species rather than the smear typically seen with prolonged inhibitor-treated samples. Strikingly, ubiquitinated

products were observed at similar levels for CD4 in the presence of Vpu, CD4-M1 in the presence of Vpu, and CD4 in the presence of Vpu-M1 (Figure 7A). However, the ubiquitin chains were clearly different: whereas CD4 intended for degradation contained a substantial amount of polyubiquitin, CD4-M1 contained primarily one to three ubiquitins. This difference is readily apparent when the ubiquitination profiles are plotted and normalized to the amount of substrate (Figure 7A, graph). The same was observed for CD4 coexpressed with Vpu-M1.

With proteasome inhibitor pretreatment for 3 hr, polyubiquitination of CD4 was increased, consistent with its eventual destiny of degradation (Figure 7B). By contrast, the oligoubiquitination of CD4-M1 decreased modestly (Figure 7B). With even longer proteasome inhibition, the oligoubiquitin species become undetectable relative to the polyubiquitin smear, which preferentially accumulates on CD4 and not CD4-M1 (e.g., see Figure 4D). This can be explained by the observation that proteasome inhibition prevents ubiquitin recycling, leading to depletion of free ubiquitin within 30 min to 3 hr (Melikova et al., 2006). Under these conditions, substrates that are transiently ubiquitinated and deubiquitinated will diminish in their ubiquitination in the face of limiting ubiquitin, whereas polyubiquitinated substrates that would have been degraded by the proteasome will accumulate.

These observations suggest that in cultured cells, CD4 and CD4-M1, which interact similarly with the Vpu-ligase complex, are both ubiquitinated. However, the slightly weaker-interacting CD4-M1 contains fewer ubiquitins and is not degraded. This is presumably due to constant DUB activity that limits chain growth. Inhibiting DUB activity should therefore preferentially reduce the levels of CD4-M1, thereby reducing its discrimination from CD4. Indeed, treatment of cells with the broadly acting DUB inhibitor PR-619 (Tian et al., 2011) reduced Vpu-mediated discrimination of CD4 from CD4-M1 by selectively reducing the levels of the latter (Figure 7C). Similar effects were seen with the CD4-Vpu-M1 pair. Prolonged treatment, although toxic to cells, led to nearly complete elimination of discrimination in the CD4-Vpu system (data not shown). Thus, as seen *in vitro*, optimal discrimination in cultured cells relies on DUB activity.

Conclusions and Perspective

Our *in vitro*, cellular, and *in silico* analysis of Vpu-mediated CD4 degradation shed light on general mechanisms underlying client discrimination, an event of critical importance in membrane protein quality and quantity control. We propose that discrimination with respect to eventual degradation is imparted by a combination of four parameters. First is the encounter of a potential client with the ligase complex, a prerequisite for ubiquitination. Second is the length of time this interaction is maintained, which would directly influence the number of ubiquitins that are added in each encounter (i.e., processivity). Third is the accessibility and activity of counteracting DUBs, which influences the length and dwell time of a ubiquitin chain. And fourth is downstream commitment events that determine client fate such as polyubiquitin-dependent engagement of the p97 complex for extraction (Wolf and Stolz, 2012), incorporation into COPII vesicles for ER export (Gillon et al., 2012), or achievement a configuration that markedly reduces ligase interaction. Uncommitted clients

would re-enter the above cycle, with any delay in re-engaging the ligase resulting in rapid, unopposed deubiquitination to the ground state.

In the absence of DUB activity, our modeling and reconstitution studies suggest that discrimination would rely completely on large differences in client-ligase interaction. This is likely to be the case for degradation of soluble proteins from the lumen of ER, where access to the cytosolically disposed ligase activity is controlled by various upstream factors such as chaperones, adaptors, and glycosylation enzymes (Vembar and Brodsky, 2008). Discrimination for luminal clients is therefore enforced by a chaperone network to triage clients between repeated folding attempts and delivery to a ligase, similar to reactions occurring during quality control in the cytosol (Buchberger et al., 2010).

By contrast, ligase access in the plane of the membrane is qualitatively different because the client and ligase are constrained to only two dimensions within a continuous compartment, and the catalytic activity of the ligase is accessible to the client without any topological rearrangement. Ligase-client interactions for membrane proteins may therefore be rather promiscuous and frequent, albeit relatively brief in most instances. It is in this regimen that discrimination relies on DUB activity to convert modest differences in interaction into larger differences in polyubiquitin acquisition and degradation. Our modeling and purified DUB experiments suggest that DUB activity, rather than a particular DUB, is sufficient for effecting discrimination. Nevertheless, it is plausible that individual quality-control pathways utilize different DUBs that are recruited to the site of ubiquitination (Blount et al., 2012). Indeed, many DUBs have been observed to interact with ligases for reasons that are unclear (Sowa et al., 2009).

The role for DUBs proposed here in controlling the generation of a degradation signal is worth distinguishing from downstream roles for DUBs. Once a degradation signal is generated and recruits ubiquitin binding proteins (such as the p97 complex; Wolf and Stolz, 2012), the substrate is committed for degradation. From this point, DUBs are thought to be involved in removing ubiquitins for threading through p97 (Ernst et al., 2009) and the proteasome (Ventii and Wilkinson, 2008). Given these multiple sites of action, it is not surprising that modulating deubiquitination in slightly different ways can have different outcomes for substrate degradation. For example, expression of a heterologous DUB free in the cytosol versus tethered to p97 affects different types of clients at different stages of the degradation process (Ernst et al., 2011). Similarly, a single client can be both stimulated and inhibited in its degradation depending on which DUB is knocked down (Sowa et al., 2009). These observations underscore the advantage of biochemically resolving a complex multistep process into individual reactions that can be mechanistically analyzed.

The appreciation of a role for DUBs in discrimination helps to explain otherwise puzzling aspects of quality control. For example, Vpu interacts sufficiently well with CD4 and β TrCP to not only coimmunoprecipitate with them, but also to retain them in the ER (Magadán et al., 2010; Magadán and Bonifacino, 2012). Despite these robust interactions and the fact that ubiquitination in the CD4-Vpu-SCF ^{β TrCP} complex occurs in mere

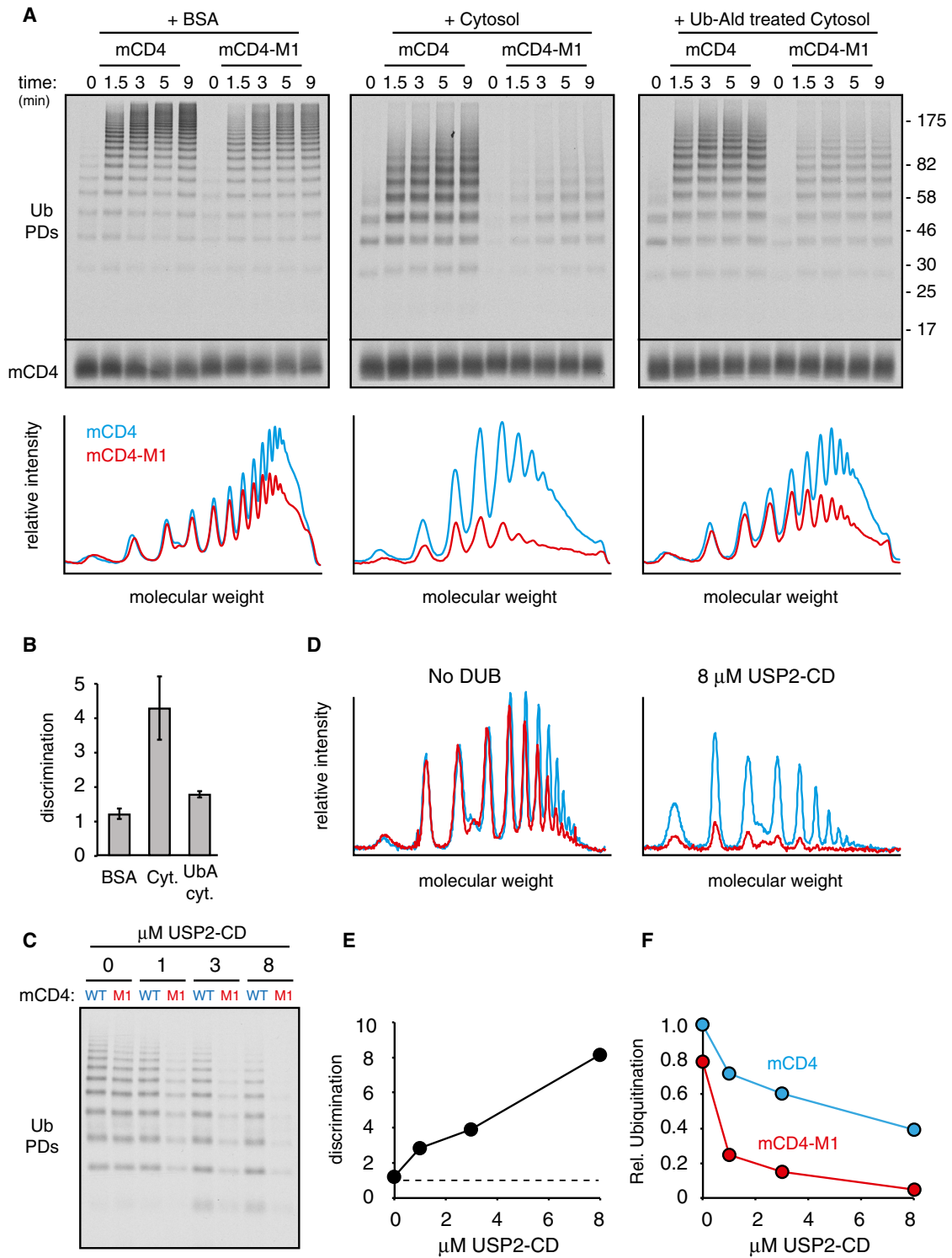


Figure 6. DUB Activity Contributes to Substrate Discrimination In Vitro

(A) The radiolabeled recombinant system was used to analyze mCD4 versus mCD4-M1 ubiquitination in the presence of BSA, HEK293 cell cytosol, or cytosol pretreated with ubiquitin-aldehyde. Note that ubiquitin-aldehyde only partially inhibits DUB activity (Figure S7B). The graph below the autoradiograph depicts the densitometry profiles of the 5 min samples.

(B) The ratio of polyubiquitination acquired on mCD4 divided by that acquired on mCD4-M1 in a parallel reaction is defined as discrimination. This value was determined for three experiments performed as in (A), quantified, and shown in the graph (mean ± SD).

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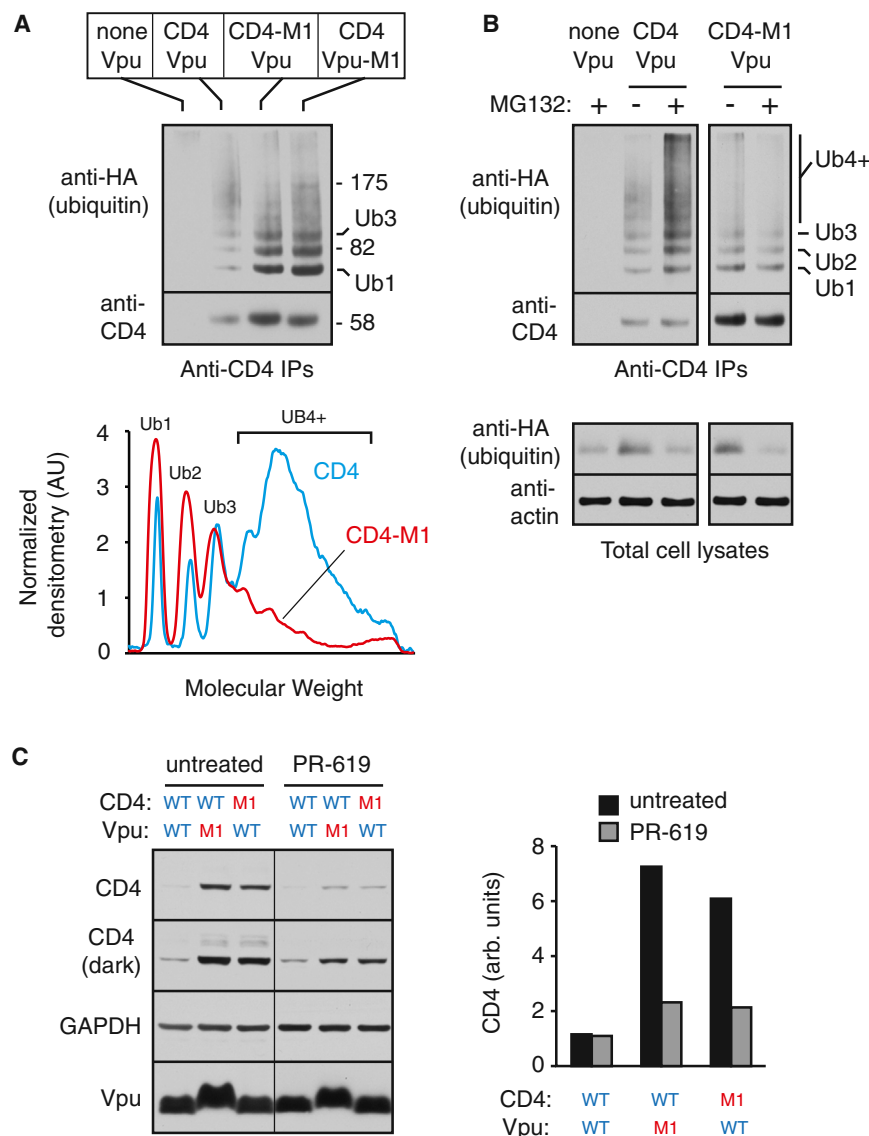


Figure 7. Evidence of a Role for DUBs in Substrate Discrimination in Cultured Cells

(A) Analysis of CD4 ubiquitination as in Figure 4D, but without proteasome inhibitor pretreatment. The positions of CD4 containing one and three ubiquitins are indicated. Below the blot is shown the ubiquitination profiles, normalized for the relative amount of total CD4, for the reactions containing CD4 versus CD4-M1.

(B) Analysis similar to (A) on cells pretreated without or with proteasome inhibitor (40 μ M MG132 for 3 hr). The bottom panel shows immunoblots of total lysates, confirming that unconjugated ubiquitin levels are depleted several-fold after proteasome inhibition.

(C) Vpu-mediated degradation of CD4 in the absence and presence of the deubiquitinase inhibitor PR-619. Seven hours following transfection of the indicated plasmids, the cells were incubated for 13 hr without or with 25 μ M PR-619 before harvesting and analysis by immunoblotting. Two exposures of the CD4 blot are shown. The results are quantified in the graph. See also Tables S1 and S2.

For substrates whose ligase interaction is marginally less stable, ubiquitins would be initially added at the same rate. However, the ligase would dissociate slightly earlier, allowing unopposed DUBs to rapidly deubiquitinate the substrate. The combination of less polyubiquitin production and shorter dwell time necessarily reduces opportunities for downstream steps in degradation, resulting instead in regeneration of the nonubiquitinated substrate. This provides a mechanism for achieving markedly different substrate half-lives for two clients with very similar ligase interactions. Although seemingly wasteful, the energy utilized for repeated de- and reubiquitination is employed to

seconds, CD4 degradation is remarkably slow ($t_{1/2}$ of 30 min or more). This can now be rationalized by the observation that very high DUB activity markedly reduces the proportion of substrates that acquire a polyubiquitin degradation signal with each ligase encounter, and the length of time that signal persists. This means that a large number of encounters are needed to get substantial degradation, explaining the relatively slow degradation of CD4 in cultured cells relative to its rapid polyubiquitination *in vitro*.

achieve substantial discrimination by essentially summing the outputs of numerous encounters.

This concept, a variation on kinetic proofreading, has been proposed to explain the order of degradation of key cell-cycle regulators (Rape et al., 2006). In that system, the APC ligase was shown to display differential processivity toward its clients due to differences in their interactions. The processivity differences were inversely related to their rate of degradation, providing an explanation for how proteins with modestly different

(C) Ubiquitination reactions of mCD4 versus mCD4-M1 in the radiolabeled recombinant system supplemented with the indicated concentrations of the recombinant USP2-CD deubiquitinase. Reaction time was 30 s, although similar results were seen at later time points as well.

(D) Densitometry analysis of the reactions from (C) without or with 8 μ M USP2-CD.

(E) Discrimination plotted as a function of USP2-CD concentration derived from (C).

(F) Relative polyubiquitination efficiency of mCD4 and mCD4-M1 as a function of USP2-CD concentration. Values were normalized to the value observed for mCD4 in the absence of USP2-CD.

See also Figure S7 and Tables S1 and S2.

APC interaction are nevertheless discriminated effectively. Similar principles are used in signaling to convert modest differences in an input stimulus into large differences in output by exploiting multisite phosphorylation with competing dephosphorylation (Malleshaiah et al., 2010; Trunnell et al., 2011). By linking the output to a maximally phosphorylated state, competing dephosphorylation can effectively dampen output until a certain threshold input is reached.

Analogous principles utilizing multistep ubiquitination and competing deubiquitination may explain how two membrane proteins of very similar structure, such as Hmg2 in the presence of low versus high sterol, are decisively discriminated despite similar interactions with quality-control ligases. Reconstitution of such quality-control events in a tractable biochemical system represents an important future goal. We anticipate that many ligase interactions with membrane proteins will display modest differences between folded and misfolded versions. This is because ligases need to recognize a wide range of unrelated clients that are unlikely to share any uniform “degron,” necessitating a rather broad sampling of potential clients using relatively weak interactions. Our findings suggest that achieving a wide dynamic range of degradation within a relatively narrow range of interactions involves exploiting the dynamic interplay between ubiquitination and deubiquitination, adding a previously unappreciated dimension to the mechanism of protein quality control.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

Vpu- and CD4-derived constructs were modifications of published plasmids (Magadán et al., 2010) and described in Tables S1 and S2. The interacting coiled-coil segments used in Figure 3F have been described (Wang et al., 2011). Expression plasmids for SCF^{βTrCP} and UbcH3 were from R. Deshaies and B. Schulman (Duda et al., 2008; Saha and Deshaies, 2008). Antibodies were from the following sources: anti-CD4 (Leica Microsystems), anti-Vpu (Magadán et al., 2010), anti-FLAG (Sigma-Aldrich), anti-Myc (Cell Signaling), anti-HA (Roche), anti-Actin (Abcam), anti-Cul1 (Invitrogen), anti-βTrCP (Invitrogen), anti-3F4 (Signet), anti-His6 (QIAGEN), anti-GAPDH (Sigma), and anti-CRT (Abcam).

Recombinant Proteins

Vpu and mCD4 were expressed in BL21 (DE3) pLysS cells and purified from inclusion bodies under denaturing conditions (with 6 M Urea) and the His tag removed by TEV protease. The Cul1/Rbx1 and βTrCP138/Skp1 subcomplexes were expressed, purified, and assembled as before (Li et al., 2005; Saha and Deshaies, 2008). Unmodified SCF^{βTrCP} complex was used in Figures 3D, 3F, 4B, S4B, S4C, and S4E, whereas Nedd8-modified complex was used in all other experiments. UbcH3 was expressed and purified as before (Saha and Deshaies, 2008). Other proteins (Ube1, UbcH5c, various tagged ubiquitins, APPBP1/Uba3, UbcH12, Nedd8, and USP2 catalytic domain) were purchased from Boston Biochem.

Cell Culture Studies

HeLa, HEK293, and Flp-In 293 T-Rex cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal bovine serum, 2 mM glutamine, and antibiotics. Transfections utilized Lipofectamine 2000 (Invitrogen). Pulse-chase analysis, ubiquitination analysis, IPs, SDS-PAGE, immunoblotting, and microsome isolation were as before (Furman et al., 2002; Magadán et al., 2010). Native IPs were performed on samples solubilized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% digitonin plus protease inhibitor cocktail (Roche).

In Vitro Translation and Reconstitution

Translation in rabbit reticulocyte lysate and protease protection assays has been described (Sharma et al., 2010; Fons et al., 2003). Reconstitutions were as before (Mariappan et al., 2011) and typically contained 5–1,000 ng recombinant Vpu, mCD4, 1% (w/v) DeoxyBigCHAP, and 200 μg unilamellar liposomes in a total volume of 100 μl. Detergent was removed with 40 mg Bio-beads and the proteoliposomes collected by centrifugation for use in ubiquitination assays. Lipid composition was typically 16:3.8:0.2 ratio of phosphatidyl-choline, phosphatidyl-ethanolamine, and rhodamine-PE. Ni-NTA lipid was included at 0.5 mol percent in Figure 3F.

In Vitro Ubiquitination

Ubiquitination reactions typically contained 2 mM ATP, 500 nM SCF^{βTrCP} complex, and ubiquitin-charged E2s (5 μM UbcH3 and 0.2 μM UbcH5c) in the following buffer: 30 mM Tris-HCl (pH 7.5), 120 mM NaCl, 5 mM MgCl₂, and 2 mM DTT. The reaction was carried out at 25°C for various times. Samples were denatured in 2% SDS and boiled for 3 min prior to downstream analysis. His6-ubiquitin conjugates were purified using immobilized Co²⁺ resin.

Miscellaneous

SDS-PAGE used Tris-Tricine gels. Radioactive signals were detected on film for figures and phosphor screen for quantitation. Kinetic modeling employed KinTek explorer as described (Pierce et al., 2009) with details given in Figure S6C. For further details, please refer to Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.06.038>.

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