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Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes?

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Although cellular proteins conjugated to K48-linked Ub chains are targeted to proteasomes, proteins conjugated to K63-ubiquitin chains are directed to lysosomes. However, pure 26S proteasomes bind and degrade K48- and K63-ubiquitinated substrates similarly. Therefore, we investigated why K63-ubiquitinated proteins are not degraded by proteasomes. We show that mammalian cells contain soluble factors that selectively bind to K63 chains and inhibit or prevent their association with proteasomes. Using ubiquitinated proteins as affinity ligands, we found that the main cellular proteins that associate selectively with K63 chains and block their binding to proteasomes are ESCRT0 (Endosomal Sorting Complex Required for Transport) and its components, STAM and Hrs. *In vivo*, knockdown of ESCRT0 confirmed that it is required to block binding of K63-ubiquitinated molecules to the proteasome. In addition, the Rad23 proteins, especially hHR23B, were found to bind specifically to K48-ubiquitinated proteins and to stimulate proteasome binding. The specificities of these proteins for K48- or K63-ubiquitin chains determine whether a ubiquitinated protein is targeted for proteasomal degradation or delivered instead to the endosomal-lysosomal pathway.

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Introduction

Degradation of intracellular proteins by the 26S proteasome is mediated primarily by the conjugation of polyubiquitin (polyUb) chains to substrates (Finley, 2009). The formation of these chains requires three types of cellular enzymes (E1, E2, and E3), which catalyse the covalent attachment of Ub molecules to lysine residues in the target protein (Hershko and Ciechanover, 1998; Pickart, 2001). As a result, at least seven different types of Ub linkages can be formed, depending on whether the Ubs are attached to K6, K11, K27, K29, K33, K48, or K63 on the proximal Ub (Ikeda and Dikic, 2008; Kim *et al*, 2009). In addition to homogeneous chains composed of a single type of specific linkage, chains composed of mixed linkages are also formed (Kim *et al*, 2007).

K48-Ub chains appear to be the primary signal for proteasomal degradation, and attachment of four or more Ub molecules to the protein is sufficient to target proteins to the proteasome (Thrower *et al*, 2000). *In vivo*, all types of Ub chains, except K63-chains, accumulate when proteasome function is blocked (Jacobson *et al*, 2009; Xu *et al*, 2009). However, formation of K63 chains on cell proteins directs them to other fates, especially endosomal trafficking to the lysosome, intracellular signalling, and DNA repair (Ikeda and Dikic, 2008). Nevertheless, isolated K48- and K63-ubiquitinated proteins bind to purified 26S proteasomes with similar affinities (Kim *et al*, 2007; Peth *et al*, 2010) and support substrate degradation at comparable rates (Hofmann and Pickart, 2001; Kim *et al*, 2007; Saeki *et al*, 2009). This surprising lack of chain preference *in vitro* is clearly opposite to observations *in vivo*, where treatment with proteasome inhibitors does not affect the stability of proteins conjugated to K63 chains (Xu *et al*, 2009). Furthermore, only K48 but not K63-polyubiquitinated proteins have been observed to co-localize with proteasomes in cells (Newton *et al*, 2008).

The eukaryotic 26S proteasome is a 2.5 megadalton, ATP-dependent complex composed of the hollow cylindrical 20S core particle, which contains the proteolytic sites, and the 19S regulatory particle, which binds ubiquitinated substrates. This particle contains two subunits that function as high affinity receptors for ubiquitinated proteins Rpn10 (S5a) and Rpn13 (Husnjak *et al*, 2008; Schreiner *et al*, 2008; Peth *et al*, 2010), which do not distinguish K48 or K63 chains *in vitro* (Peth *et al*, 2010).

The present studies were undertaken to learn why proteins linked to K63 chains in cells do not become bound to proteasomes, as they do with purified 26S particles. Three types of mechanisms can explain why K63-ubiquitinated proteins do not undergo proteasomal degradation *in vivo*. (1) Factors may exist in cells that either prevent their binding to the 19S complex or (2) promote their deubiquitination by one or more of the 26S-associated deubiquitinating enzymes (DUBs), Usp14, Uch37, or Rpn11 (Finley, 2009), and release from the 19S without proteolysis. Alternatively, (3) cytosolic factors may exist that selectively enhance the binding of K48-ubiquitinated substrates to the 26S.

To determine how K63-ubiquitinated proteins are protected from proteasomal degradation, we examined in mammalian cell extracts the binding of K48- and K63-ubiquitinated proteins to the 26S complex. Using more physiological approaches than

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in prior studies of Ub-binding proteins (UBPs), we have identified cell proteins that bind preferentially to K63-ubiquitinated proteins and block their binding to proteasomes. We show here that components of the ESCRT (Endosomal Sorting Complex Required for Transport) pathway (Williams and Urbe, 2007) associate strongly with K63 chains, provided they contain more than four Ubs, and can prevent binding of purified as well as endogenous K63-ubiquitinated proteins to the 26S, thus targeting them to the endosomal-lysosomal pathway. In addition, the Rad23 proteins (human homologues of yeast Rad23 (hHR23)), which have been proposed to 'shuttle' proteins to the proteasome (Elsasser *et al*, 2004), associate specifically with K48 conjugates and promote their binding to the 26S complex. Together, these chain-specific UBPs seem to determine whether Ub conjugates are degraded by lysosomes or proteasomes *in vivo*.

Results

In cell extracts proteasomes bind preferentially to K48-polyUb chains

To determine whether cells contain factors that influence the binding of K63-polyUb chains to the 26S, we first compared the binding of purified 26S to resin-bound K63- and K48-polyubiquitinated proteins, using the assay described by Peth *et al* (2010). Ub conjugate-affinity columns were formed by incubating the ligases, E6AP or Nedd4 (bound to a GST resin), with E1, E2, Ub and ATP. E6AP forms homogenous K48-polyUb chains, and Nedd4 homogenous K63-polyUb chains (Supplementary Figure S2A; Kim *et al*, 2007). Kim *et al* showed that both these HECT E3 ligases form Ub chains on a single lysine and not through multiple short monoUb chains. The washed resin-bound ubiquitinated proteins were incubated with pure 26S proteasomes at 4°C, and the amounts of bound proteasomes were measured by assaying the cleavage of LLVY-AMC at 37°C (Peth *et al*, 2010). This assay of activity was shown to accurately reflect the amount of 26S proteasomes bound to the Ub conjugates (Peth *et al*, 2010), as measured by immunoblot (see below), but the activity assay was faster and easier to quantitate.

Using this method, we confirmed that purified 26S bind both types of chains with similar high affinities (Figure 1A, right panel), as reported previously (Peth *et al*, 2010). To learn whether mammalian cell extracts contain factors that might inhibit the binding of the Nedd4-K63 conjugates to proteasomes, rat muscle extracts were incubated with the resin-bound conjugates at 4°C (Figure 1A; Supplementary Figure S1A). To ensure that only proteasome activity was being measured with this assay, the control lysate was treated with the proteasome inhibitor, Bortezomib/Velcade, and the very low amounts of Bortezomib-insensitive activity were subtracted. After washing the resin, we found that in these tissue lysates, proteasomes bound efficiently to the K48 conjugates, but not to the K63 chains (Figure 1A, left panel). Thus, in the presence of cell extracts, proteasomes and Ub chains behave as they do *in vivo*.

To ensure that this failure of the ubiquitinated Nedd4 to bind to the proteasome was due to the K63 chain and not the Nedd4 protein, we forced Nedd4 to attach to itself shorter K63 or K48 chains by incubating resin-bound Nedd4 with E1, E2, ATP, and K48 or K63-tetraUb (Ub_4) in place of monoUb. With time, each type of tetraUb chain became covalently bound to Nedd4 (Supplementary Figure S2B). These conjugates were then incubated with the muscle extract. As expected, many more proteasomes became bound to the Nedd4-K48 chains than to the Nedd4-K63 conjugates (Supplementary Figure S2C). Thus, factors within the lysate must block 26S binding to the K63 conjugates.

Lysate proteins bind to K63 conjugates and prevent proteasomal binding

We therefore examined whether proteins in the cell lysate could prevent the binding of K63 chains to purified 26S particles. The muscle extract was first ultra-centrifuged for 6 h to remove the endogenous proteasomes (Supplementary Figure S2F and G; Gaczynska *et al*, 1993). This lysate and a set amount of purified 26S proteasomes were incubated with the resin-bound conjugates at 4°C, and the amount of bound proteasomes measured (Figure 1B; Supplementary Figure S1B). In contrast to the untreated K48- and K63-polyUb chains, which bound the proteasomes similarly, incubation with the lysate prevented K63 conjugates from binding to the purified 26S, but did not reduce K48-chain binding (Figure 1B). A similar selective inhibition of K63-chain binding was observed with lysates of rabbit and rat muscle, as well as HEK293 cells (Supplementary Figure S2E). Thus, soluble factors present in many (presumably all) mammalian cells inhibit K63-conjugate binding to 26S proteasomes.

One possible explanation of these results could be that enzymes in the cell lysate caused the K63 conjugates to be deubiquitinated much more rapidly than the K48 conjugates. Although there should be little or no deubiquitination occurring since binding was assayed at 4°C, we examined whether there might be some deubiquitination during incubation with the extract. Immunoblots of the polyubiquitinated E6AP and Nedd4 did not show any significant difference in the levels of ubiquitination before or after incubation (Supplementary Figure S2H). To confirm that destruction of the K63 chains was not responsible for their failure to bind proteasomes, the resin-bound substrates and lysate were treated with N-ethylmaleimide (NEM) and 1,10-o-phenanthroline (oPT) to inactivate the two classes of DUBs, cysteine and metalloproteinases. Despite this treatment, the cell extracts still blocked only K63-chain binding to the proteasomes (Figure 1C; Supplementary Figure S2I).

Although polyubiquitinated Nedd4 and E6AP bind strongly to purified proteasomes, they are not rapidly degraded *in vitro* (Peth *et al*, 2010). We therefore examined whether the cell extracts also prevent binding and degradation of K63-polyubiquitinated Sic1, a short-lived protein *in vitro* and *in vivo* (Saeki *et al*, 2009). This substrate was preincubated with pure 26S proteasomes, the proteasome-depleted cell extract, or both together for 15 min at 4°C, before incubation at 37°C, during which we assayed the degradation of polyubiquitinated Sic1 by immunoblot. As expected, the polyubiquitinated Sic1 was rapidly degraded by the 26S proteasomes (Supplementary Figure S3A, left). Although the Sic1-Ub conjugates were not deubiquitinated in the cell extract at 4°C, at 37°C the Ub chains were completely removed within 20 min. This rapid disassembly of K63 chains in mammalian extracts is consistent with prior reports (Cooper *et al*, 2009). Treatment of these samples with NEM and oPT prevented this deubiquitination, and also prevented the efficient degradation of the polyubiquitinated Sic1 by the

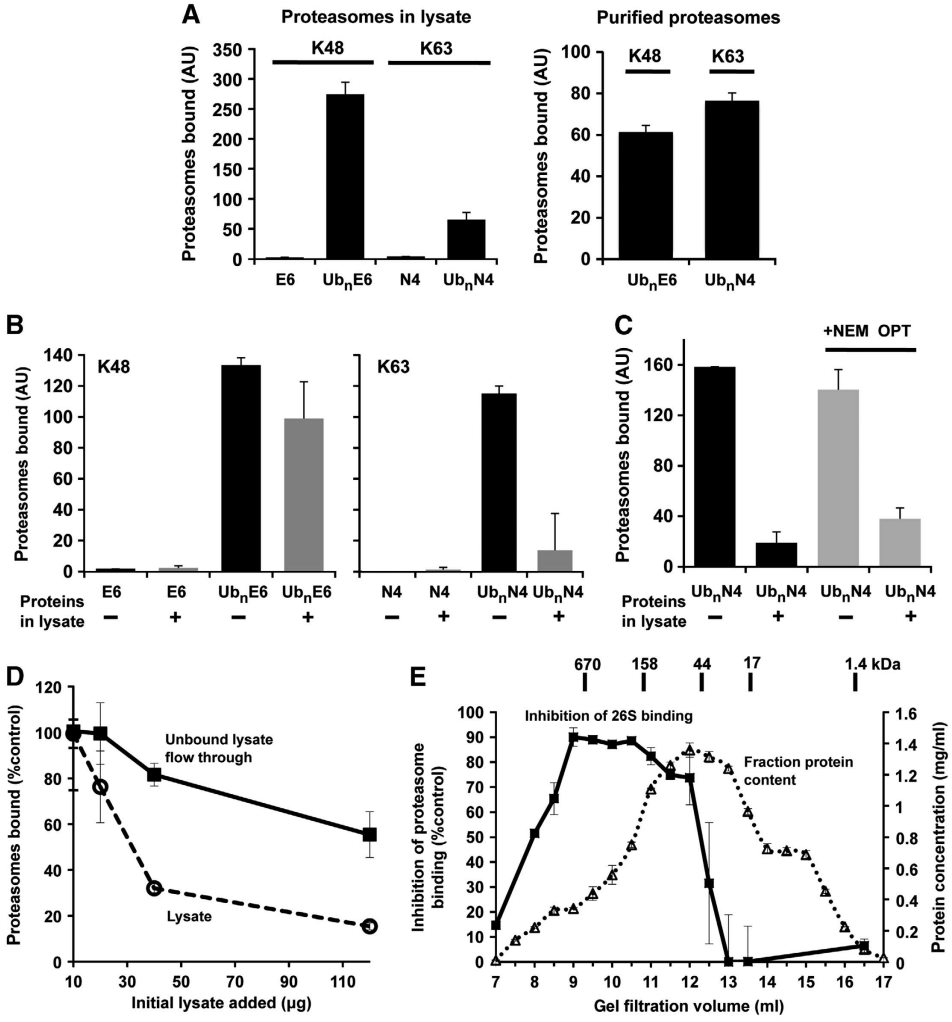

Figure 1 Proteins in lysate prevent proteasome binding to K63-polyUb chains. (**A**) Although pure proteasomes bind to K48 and K63 chains, proteasomes in the muscle lysate bind efficiently only to K48 conjugates. Ubiquitinated E6AP and Nedd4 were incubated with purified 26S particles and the bound proteasomes were measured by LLVY-AMC cleavage (right panel). These same ubiquitinated conjugates were incubated with a rat muscle extract (120 μg) (left panel), with or without the addition of Bortezomib (1 μM) and the proteasomes from the lysate that bound to the conjugates were measured by LLVY-AMC cleavage (Bortezomib residual activity was subtracted). (**B**) The cell lysate contains factors that reduce binding of K63, but not K48-polyUb conjugates to the proteasome. The proteasome depleted of rabbit muscle extract (200 μg) was incubated with the ubiquitinated substrates and proteasomes as shown in Supplementary Figure S1B. (**C**) DUB inhibitors do not influence the inhibition of K63-conjugate binding to proteasomes by cell extracts. Proteasome binding to the Ub conjugates was measured as in (**B**), with or without 4 mM N-ethylmaleimide (NEM) and 1 mM 1,10-o-phenanthroline (oPT). (**D**) Factors within the cell extract that inhibit the binding of K63 conjugates to the proteasomes, bind tightly to K63 chains and can be depleted from cell extracts. The rat muscle extract, ubiquitinated Nedd4 and 26S proteasomes were incubated as in (**B**). The unbound fraction of the lysate (flow-through) was then assayed for its capacity to block proteasome binding. The flow-through was incubated with a fresh column of ubiquitinated Nedd4 and proteasomes, and the conjugate bound proteasome fraction was then measured. (**E**) Multiple cellular proteins or complexes can prevent K63 conjugates binding to the proteasome. Proteins in the rat muscle lysate (4 mg/ml) were separated according to their molecular weight using a Sephacryl S300HR column. After a void volume of 7 ml, 0.5 ml lysate fractions were collected and incubated with the ubiquitinated Nedd4 and proteasomes, to assay their ability to block K63-conjugate binding. All values are the means ± s.e.m. See also Supplementary Figure S2. E6, E6AP; N4, Nedd4.

proteasome (Supplementary Figure S3A, right). To differentiate the initial binding of the Ub conjugates to the proteasome from the subsequent deubiquitination, we measured the binding of the polyubiquitinated Sic1 to the 26S at 4°C. While K63-polyubiquitinated Sic1 bound to the pure proteasomes (Supplementary Figure S3B), incubation of the cell extract with the polyubiquitinated Sic1 prevented conjugate binding to the 26S (Supplementary Figure S3B). Thus, the capacity of the extract to block K63-chain binding to the proteasome was observed with multiple K63-polyubiquitinated substrates (Sic1 and Nedd4) and caused an inhibition of degradation.

To determine whether the cell factors that prevent conjugate binding to the 26S did so by binding with higher affinity to the K63 chains, the cell extract was first depleted of these K63-binding proteins by incubation with the ubiquitinated Nedd4 column. To ensure depletion of K63-binding proteins, the resin-bound ubiquitinated Nedd4 was first incubated with different concentrations of the extract. The unbound (flow-through) fraction was removed and incubated with the fresh resin-bound ubiquitinated Nedd4. The conjugates were then incubated with pure 26S proteasomes, and the 26S bound fraction measured. Pretreatment with increasing amounts of cell extract caused greater inhibition of K63-conjugate

binding to proteasomes (Figure 1D). The extract proteins reduced K63 chain binding up to 80% below the levels found with no extract. By contrast, after exposure to the K63 chain, the unbound fraction (flow-through) lost most of its capacity to prevent K63 conjugates binding to proteasomes. By contrast, pretreatment of the cell extract with the K48-Ub conjugates (which should deplete it of K48-binding proteins) had no effect on the ability of the extract to prevent K63-Ub chains binding to the 26S (Supplementary Figure S2J). Therefore, the cell proteins that bound specifically to K63 chains were also the ones that blocked proteasomal binding to K63 chains.

To define the size distribution of the cell proteins that can block K63 conjugates from binding to proteasomes, we used gel filtration on a Sephacryl S300HR column to fractionate lysate proteins according to size. These fractions were then incubated with the resin-bound K63 chains to determine which fractions prevented K63 binding to the 26S (Figure 1E). Inhibition of Ub-conjugate binding was observed over a wide range of molecular weights, with maximal inhibition observed from 150 to 670 kDa. This wide range suggests that multiple proteins, and/or multimeric complexes inhibit K63 chains from binding to the 26S (see below).

To define the nature of this association between these cellular proteins and the K63 conjugates, we examined whether increasingly stringent washes could release the K63-associated proteins and restore the ability of the conjugates to bind the 26S. After incubating the ubiquitinated Nedd4 with cell extracts, the resin-bound conjugates were washed with increasing sodium chloride concentrations (up to 900 mM) or detergents (1% Triton X-100 or 0.1% SDS), and then they were incubated with purified proteasomes and the bound 26S species measured (Supplementary Table S1). Neither the high salt concentrations nor the detergent reduced the inhibition of conjugate binding to proteasomes. Thus, a number of cellular proteins can block proteasome binding by associating tightly with K63 chains through non-ionic, presumably hydrophobic, interactions.

Identification by mass spectrometry of K63- and K48-specific binding proteins

These findings suggested that the proteins responsible for blocking proteasome binding contain one or more Ub binding domains (UBDs). If so, a large excess of a UBD should elute the UBD-containing cell proteins from the resin-bound conjugates and allow us to identify them by mass spectrometry (MS). After the resin-bound K48- and K63-polyUb conjugates were incubated with the HEK293 lysate and extensively washed, the proteins remaining bound to the Ub chains were eluted with a large excess of the Ub interacting motif (UIM) of S5a/Rpn10. The His-tagged UIM was then removed using NiNTA, and the eluted proteins separated by SDS–PAGE (Figure 2A; Supplementary Figure S1C). A few dominant protein bands were observed only in the Ub-conjugate samples (Figure 2A). The major protein migrating at 90 kDa that bound to both types of Ub chains was identified as USP5 by immunoblot (Supplementary Figure S4A). (This association of USP5 with conjugates was also used to measure the efficiency of the elution of UBPs from the resin; Supplementary Figure S4A, right.) MS analysis of the dominant bands in K48-polyUb sample identified them as hHR23B, hHR23A, and S5a/Rpn10 (a UIM-containing subunit of the 26S proteasome that is also found free in the cytosol; Kim *et al*, 2009). The Rad23 proteins contain a Ub-like (Ubl) domain that can bind to the proteasome as well as a UBA (Ub-associated) domain, which binds directly to Ub chains (Chen and Madura, 2002). Such UBA-Ubl proteins appear to help shuttle ubiquitinated substrates to the 26S (Elsasser *et al*, 2004; Verma *et al*, 2004).

In contrast surprisingly, few discrete gel bands were observed for proteins bound to the K63 conjugates (Figure 2A, right panel). Therefore, we concentrated the K63-eluted fractions by precipitation with TCA and analysed the precipitate by LS-MS/MS. In total, 80 proteins were identified in the K63-chain analysis and 106 in the K48-chain MS analysis (Table I; Supplementary MS data file). Among the K63-specific proteins were several components of the endosomal trafficking system, namely, Hrs, STAM, Vps37, and Epsin (Table I), none of which were found in the K48-bound fraction. Interestingly, other components of the endosomal pathway were found to bind to both K48 and K63 chains, including TOM (target of Myb)1, an endosomal trafficking protein (Yamakami *et al*, 2003; Seet *et al*, 2004), although more TOM1 peptides were identified in the K63 sample. Similarly, TOM1L2, a protein closely related to TOM1, and Tollip, a TOM1 interacting protein, were detected in both K48- and K63-bound fractions.

In addition to hHR23A and B, the other predominant proteins identified as binding to K48 conjugates are also known to be involved in proteasomal degradation. FAF1 and UFD1 form a complex with p97/VCP (homologue of yeast cdc48) (Meyer *et al*, 2000; Song *et al*, 2005), an AAA ATPase involved in targeting of ubiquitinated proteins by the proteasome for degradation. Also, HUWE1 is a Ub ligase that can associate with the 26S (Besche *et al*, 2009). All the proteins that were identified with confidence by MS (>1 peptide or manually verified), which encode UBDs and bound to the Ub conjugates, are listed in Table I. Two DUBs were identified as binding selectively to the K48 chains, USP25 and Ataxin 3, and two that bound to both types of conjugates, USP5 and USP13.

Characterization of K48- and K63-binding proteins

To learn which proteins influence proteasome binding to Ub conjugates, we focused on those proteins that demonstrated clear specificity for Ub linkages: the Rad23 proteins for K48 chains and the ESCRT0 complex (composed of Hrs and STAM) for K63 conjugates, as well as TOM1, which associated with both types of chains. Hrs, STAM, and TOM1 were all found by western blot in the gel filtration fractions of the lysates that showed the greatest inhibition of 26S binding (Supplementary Figure S4B). We also confirmed by immunoblotting the association of these UBD-containing proteins with the Ub conjugates identified by MS. When the resin-bound conjugates and non-ubiquitinated E3s were incubated with the cell extract, and the bound cellular proteins separated by SDS–PAGE, Hrs and STAM were bound only to the K63-ubiquitinated substrate, whereas hHR23B became bound only to K48 chains (Figure 2B). Again, TOM1 was found to associate with both K48 and K63 conjugates, but it showed greater binding to K63 chains. In addition, these UBD proteins did not bind to the non-ubiquitinated proteins; in particular, we did not detect any hHR23B binding to E6AP, as had been reported previously (Kumar *et al*, 1999).

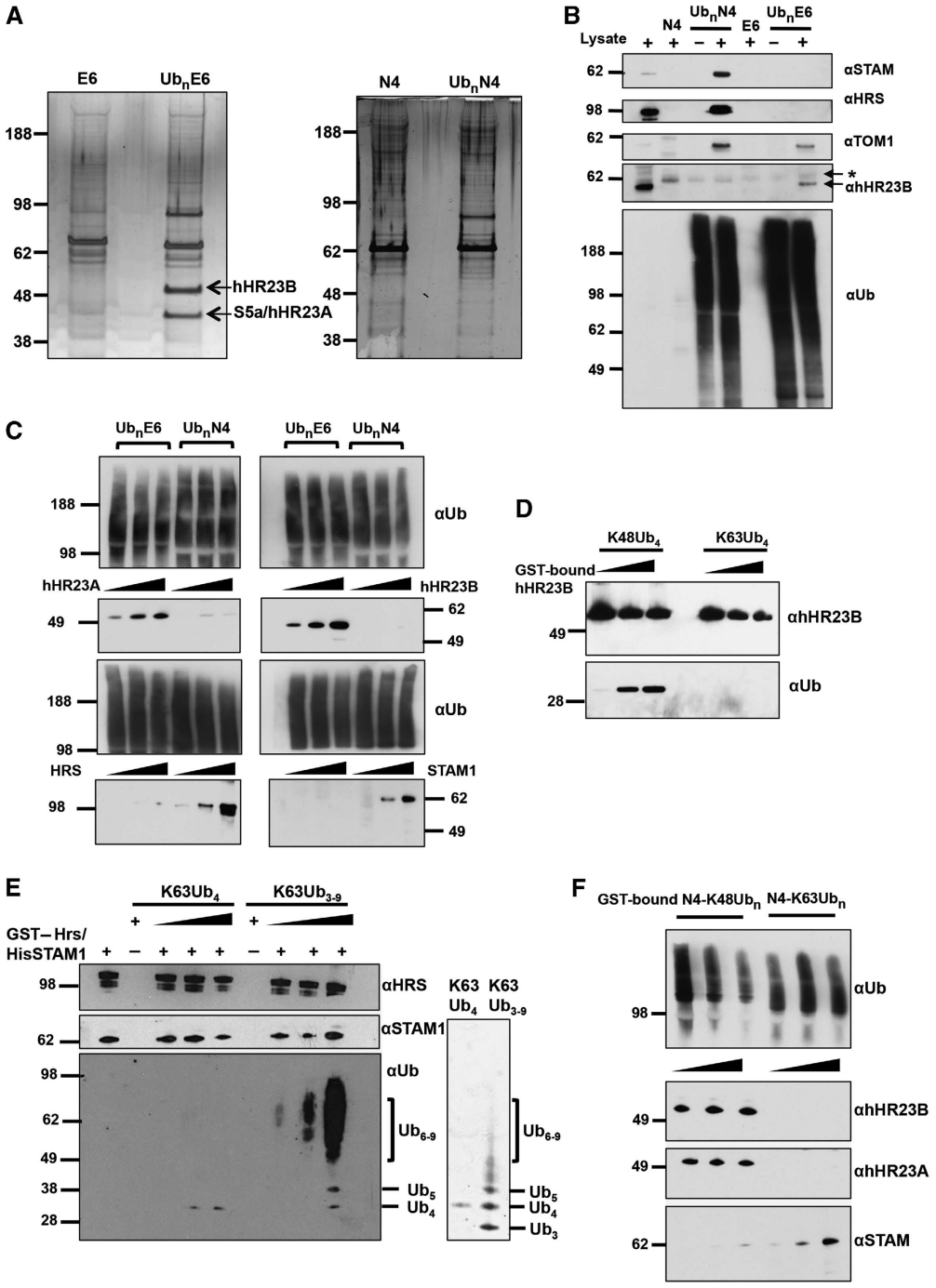

Figure 2 Identification of K63- and K48-specific Ub-binding proteins. (**A**) Representative silver-stained gel of proteins eluted from K48 or K63 conjugates. After washing columns, proteins bound to the control or ubiquitinated substrates (E6AP and Nedd4) were eluted with His_{10}-UIM, and 10% of eluted volume was separated by SDS–PAGE. (**B**) Identified UBD-containing proteins demonstrate specificity for K48 or K63 chains. Control or ubiquitinated substrates (E6AP and Nedd4) were incubated with an HEK293 lysate. Following washing, proteins were eluted from the resin in SDS-loading buffer and immunoblotted for the Rad23 proteins, TOM1, Hrs, and STAM. The size range of ubiquitinated E6AP and Nedd4 is shown with the anti-Ub antibody. *Non-specific band with anti-hHR23B antibody. (**C**) The ESCRT0 components, Hrs and STAM, bind specifically to K63 chains, while hHR23A and hHR23B are selective for K48 conjugates. Purified recombinant hHR23A, hHR23B, Hrs, and STAM at concentrations of 10, 50, and 100 nM were incubated with the ubiquitinated E6AP or Nedd4 resins. (**D**) HHR23A and B bind specifically to K48 chains. Resin bound GST-hHR23B (100 nM) was incubated with increasing concentrations (10, 50, and 100 nM) of K48- or K63-Ub tetramers in TBSG with 0.1% Triton X-100. Following washing, proteins were eluted from the resin and immunoblotted for hHR23B or Ub. (**E**) ESCRT0 preferentially binds to longer K63 chains. GST–Hrs and His–STAM were incubated with increasing concentrations of K63-Ub tetramers or K63 chains of mixed lengths (3–9mers), and immunoblotted for Ub (left panel). A Coomassie stained gel of the Ub-chain inputs is also shown (right panel). (**F**) K48 or K63 tetramers were forced onto GST–Nedd4 as described in Supplementary Figure S2. These conjugates were then incubated with hHR23A, hHR23B, and STAM (10, 50, and 100 nM) and the bound protein fraction visualized by immunoblotting.

Table I Proteins identified by mass spectrometry that bound to K48- or K63-polyubiquitin conjugates

	K48 specific	K63 specific	Both K48 and K63
Deubiquitinating enzymes	Ataxin 3[a]		USP13[a]
	USP25[a]		USP5[a]
Ubiquitin interacting proteins	hHR23B	HRS	TOM1
	hHR23A	STAM1	TOM1L2
	S5a	Epsin1	Tollip
	FAF1	P62 (SQSTM1)	WRNIP1
	UFD1L		
Ubiquitin ligases	HUWE1[a]		
Endosomal proteins		VPS37C	

[a]These proteins also contain known UBDs.

To confirm that these UBD-containing proteins interacted directly with the Ub conjugates and not through an association with another UBP in the extract, we expressed and purified recombinant forms of these proteins (Supplementary Figure S4C) and examined their ability to associate with the ubiquitinated substrates. The resin-bound polyubiquitinated E6AP and Nedd4 were incubated with increasing concentrations of the purified UBD-containing proteins, hHR23A, hHR23B, Hrs and STAM, and the fraction of each that bound to the resin was determined by immunoblotting. HHR23B, at concentrations up to 100 nM, bound only to the K48 conjugates (Figure 2C). Furthermore, we also found that GST-hHR23B bound selectively K48-linked tetraUb chains and showed no binding of K63 tetramers (Figure 2D). HHR23A also showed a clear preference for K48 conjugates, but some binding to K63 chains was observed at concentrations of 50 and 100 nM hHR23A (Figure 2C). Conversely, at all concentrations tested, the ESCRT0 proteins Hrs and STAM bound only to K63 conjugates (Figure 2C).

Interestingly, using resin-bound GST–Hrs and His–STAM, we could barely detect any interaction between these ESCRT0 proteins with pure K63 tetramers (Figure 2E), suggesting a preference for longer chains. Accordingly, when we used a mixture of chains containing 3–9 Ub monomers, we observed selective binding of Hrs and STAM to chains composed of 6–9 Ub molecules, with minimal binding to K63 tetramers and no binding to Ub trimers (Figure 2E) or a K48-linked mixture of chains (Supplementary Figure S4D). Thus, the ESCRT0 proteins preferentially bind to K63 conjugates longer than 4 Ub molecules. It is noteworthy that nearly all prior *in vitro* studies of UBPs have utilized much shorter constructs (monoUb or di-Ub chains), whose behaviour clearly differs from that of these long Ub chains.

Finally, to confirm that the UBD-containing proteins bound to these specific Ub chains and not to the E6AP or Nedd4 molecules, we incubated Nedd4 with E1, E2, ATP and either K63- or K48-tetraUb to force the attachment of K63 or K48 chains to Nedd4 (Figure 2F). HHR23B bound only to these Nedd4-K48 conjugates, whereas STAM predominantly associated with the Nedd4-K63 chains (Figure 2F). Thus, the Rad23 proteins show clear specificity for K48 conjugates, and the ESCRT0 proteins for the K63 chains.

ESCRT0 prevents K63 conjugates from binding to the 26S proteasome

To determine if the K63-specific proteins could in fact prevent the binding of proteasomes to the K63-conjugated E3, we incubated the resin-bound ubiquitinated Nedd4 or E6AP with increasing concentrations of pure Hrs or STAM and purified proteasomes. After 30 min, the resins were washed and the binding of 26S measured. Both Hrs and STAM inhibited the binding of K63-polyUb conjugates to the 26S in a concentration-dependent manner. Similarly, low concentrations (80 nM) of Hrs or STAM decreased conjugate binding to proteasomes by 80% (Figure 3A and B). A very similar decrease in Ub-conjugate binding was also observed with the K63-polyubiquitinated Sic1; for example, Hrs (50 nM) decreased K63-Sic1 Ub-conjugate binding (30 nM) to proteasomes by 80% (Supplementary Figure S5A). By contrast, these ESCRT0 components had little effect on the ability of K48 conjugates to bind the 26S particles. At 80 nM, Hrs or STAM decreased K48-polyUb binding to proteasomes by only about 10% (Figure 3A and B). The half maximal inhibitory concentrations (IC_{50}) of both Hrs and STAM for proteasome binding were 50 nM for K63 chains and 310 nM for K48 chains. Thus, the preferential binding of Hrs and STAM to K63 chains in cell extracts is an inherent property of each.

In the cytosol, Hrs and STAM associate to form a heterodimer, the ESCRT0 complex (Ren *et al*, 2009). We therefore examined the ability of ESCRT0 to reduce conjugate binding to proteasomes by adding equimolar ratios of Hrs and STAM to the Ub conjugates and 26S particles. ESCRT0 at 20 nM decreased K63-conjugate binding to proteasomes by over 80% without affecting the ability of K48 chains to bind (Figure 3C). The IC_{50} of ESCRT0 for inhibition was <10 nM for K63 conjugates and $\sim$250 nM for K48 chains. Thus, the ESCRT0 complex clearly shows greater selectivity for K63 chains and stronger inhibition of K63 conjugates binding to 26S particles than Hrs or STAM by themselves.

To confirm that the decrease in peptidase activity by ESCRT0 was due to fewer proteasomes binding to the K63 chains, we measured the levels of 20S α-subunits bound to the K63 conjugates after incubation with ESCRT0 and the purified 26S molecules (Figure 3E and F). An identical decrease in binding to the K63-Ub conjugates was demonstrated by western blotting for proteasome subunits and assays of peptidase activity (Figure 3E and F). Thus, ESCRT0 clearly prevents the K63 chains from binding to the proteasomes.

Previous studies have shown that the UBDs of ESCRT0 can bind Ub but without much specificity for K63 or K48-tetraUb chains (Ren and Hurley, 2010). The high specificity of the STAM and Hrs proteins for K63 conjugates that we observed suggests that this selectivity is not due to the individual UBDs, but results from their correct positioning in the protein. We therefore examined whether the VHS (*V*ps27, *H*rs, and *S*TAM) domain, a UBD common to both ESCRT0 proteins, could prevent K63 conjugates from binding to the proteasome. At high concentrations of STAM-VHS (10 μM), there was a reduction in the levels of 26S bound to the K63 conjugates. However, even at these very high concentrations, the STAM-VHS domain blocked both types of chains binding to the proteasome, with only a modest two-fold preference for the K63 conjugates (Supplementary Figure S5B). Therefore, as predicted, isolated UBDs do not show the same strong linkage selectivity as the entire ESCRT0 complex.

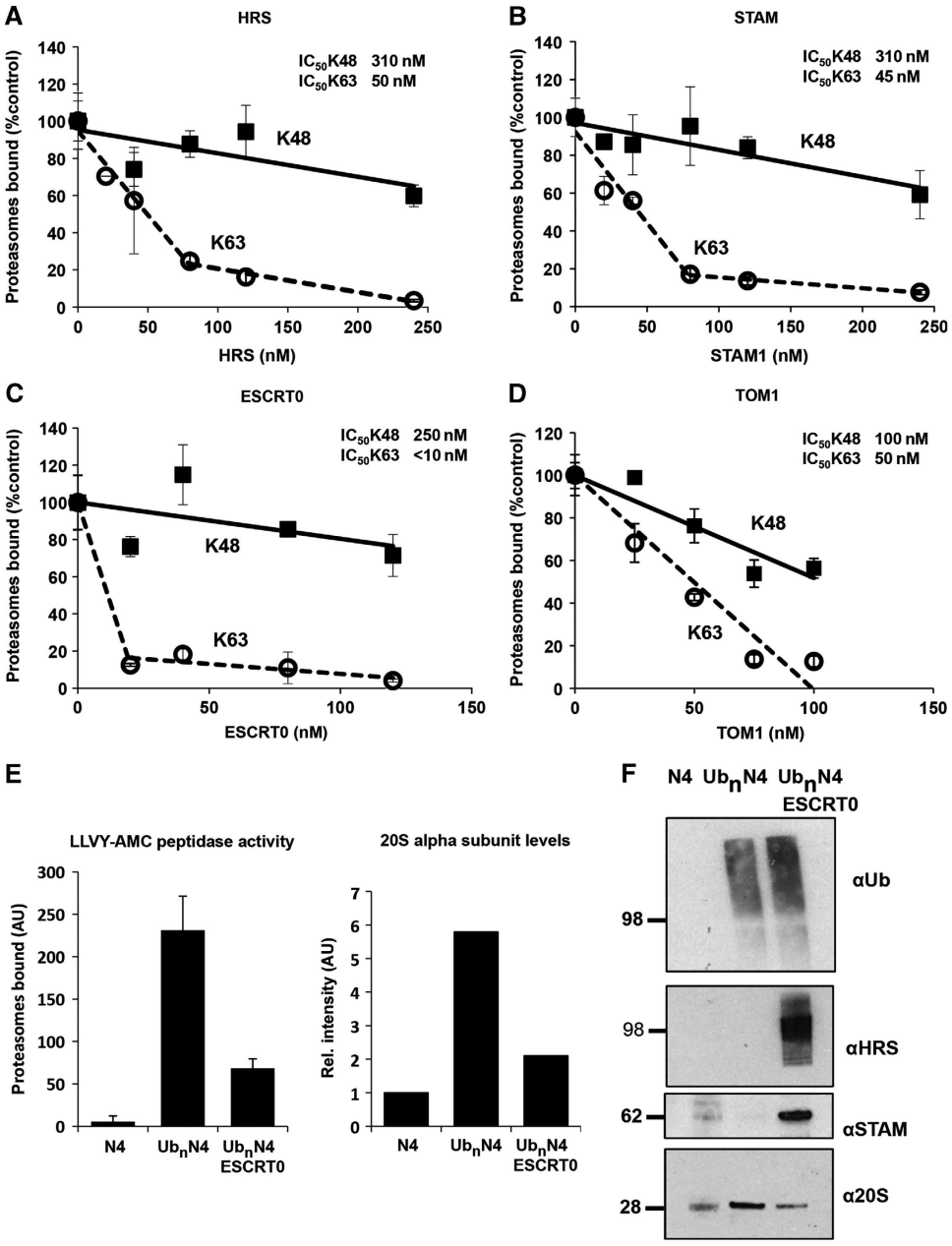

Figure 3 ESCRT0 and its components selectively block K63-polyUb conjugate binding to the proteasome. (**A**–**D**) Hrs, STAM, and the ESCRT0 complex inhibit K63 chains binding the 26S. Increasing concentrations of pure Hrs, STAM, TOM1, or ESCRT0 (Hrs and STAM used in equal amounts to allow formation of 1:1 complex) were incubated with the polyubiquitinated conjugates and 10 nM 26S particles. All values are the means ± s.e.m. (**E**, **F**) Hrs and STAM (50 nM) were incubated with the 26S particles as described. Proteasome activity in these samples was measured by peptidase activity (**E**, left graph), and immunoblotting with densitometric quantification (ImageJ) of the 20S α-subunits (**E**, right graph and **F**).

In addition, we examined whether TOM1, which bound to both K48 and K63 conjugates but with a preference for the K63 chains (Figure 2B; Table I) could also reduce conjugate binding to the proteasome (Figure 3D). TOM1 did inhibit the binding of the polyUb chains to the proteasome, but in contrast to the ESCRT0 proteins, TOM1 showed little or no selectivity for chain type. The IC_{50} of TOM1 for inhibition of binding to K63 chains was 50 nM versus 100 nM for K48. Thus, the inherent specificity of UBD proteins (e.g., of Hrs and STAM) or the lack of specificity (of TOM1) for Ub-chain type correlated exactly with their ability to inhibit proteasome binding to K63 or K48 conjugates.

HHR23A and B increase proteasome binding to K48-polyUb chains

As hHR23B and hHR23A in cell extracts selectively bind to K48 chains, we studied their ability to influence proteasome binding. The resin-bound Ub conjugates were incubated with pure recombinant hHR23A or hHR23B, washed, and then incubated with 26S proteasomes. Both hHR23A and hHR23B stimulated 26S binding to K48 conjugates in a concentration-dependent manner (Figure 4A). (This effect was due to greater proteasome binding and not due to an increase in the peptidase activity of the 26S; in fact, hHR23B did not enhance the rate of GGL-AMC cleavage beyond that observed

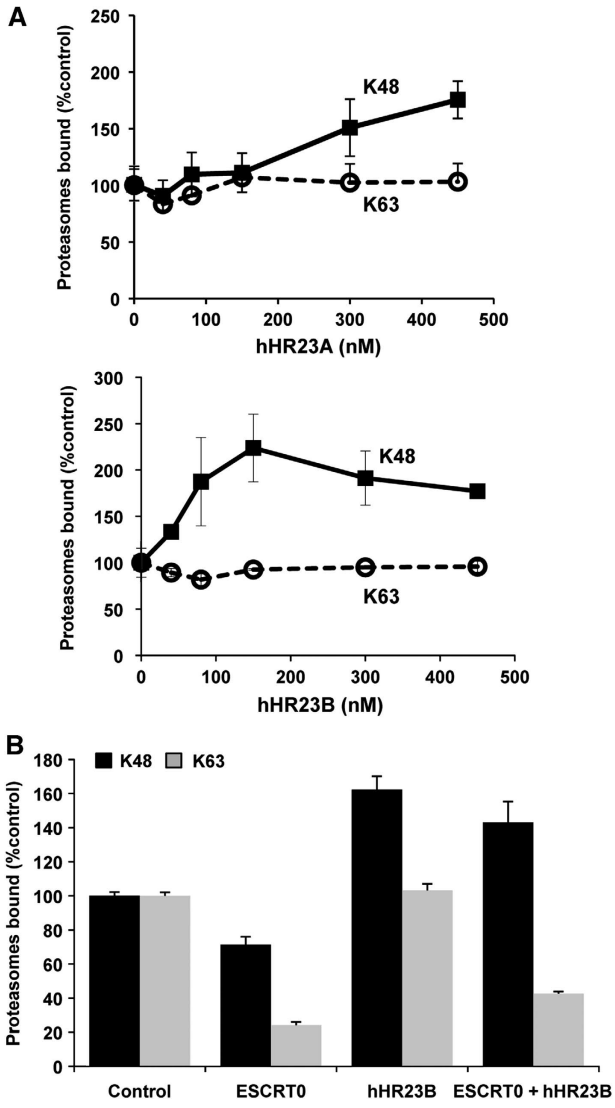

Figure 4 HHR23A and B, by associating with K48 chains, increase proteasome binding to the K48 conjugates. (**A**) HHR23A and hHR23B stimulate K48-Ub conjugate binding to the 26S proteasome. The pure proteins were first incubated with ubiquitinated Nedd4 or E6AP for 30 min at 4°C. After washing, the resin was incubated with 10 nM 26S proteasomes for 30 min at 4°C, and their binding measured. (**B**) The combined presence of ESCRT0 and hHR23B together enhance the selectivity and also amount of binding of proteasomes to K48 chains. Polyubiquitinated Nedd4 or E6AP was incubated with 10 nM 26S proteasomes, and 50 nM ESCRT0, hHR23B or both together at 4°C for 30 min. All values are the means ± s.e.m. See also Supplementary Figure S4.

with K48 conjugates alone; Peth *et al*, 2009; Supplementary Figure S6.) In addition, the Rad23 proteins had no effect on the binding of K63 chains to the 26S particles. Furthermore, there were clear differences between the abilities of hHR23A and hHR23B to stimulate conjugate binding. HHR23B was stimulatory at lower concentrations (Ka 40–70 nM), while hHR23A showed no enhancement of binding below 150–200 nM (Figure 4A). This greater ability of hHR23B to stimulate conjugate binding implies that hHR23B is more likely than hHR23A to fulfill a role as a 'shuttling factor' that delivers K48-ubiquitinated substrates to the proteasome.

Physiological concentrations of hHR23B and ESCRT0 together determine selectivity of proteasome binding

To learn whether hHR23B and ESCRT0 together could account for the selective binding of proteasomes to K48 chains in cell extracts, we studied their combined effects on 26S binding (Figure 4B). The ubiquitinated proteins were incubated with low concentrations (50 nM) of hHR23B, ESCRT0, or both together, and proteasome binding measured. As expected, ESCRT0 primarily inhibited 26S binding to K63 chains, and hHR23B stimulated K48-conjugate binding (Figure 4B). The combination of ESCRT0 and hHR23B both blocked the association of the K63 chains with the proteasome, and stimulated the binding of K48 conjugates to the 26S (Figure 4B). Thus, the opposite chain specificities of these two UBD proteins had additive effects in enhancing the net discrimination between the K48 and K63 chains in determining which conjugates bind to proteasomes.

To determine if these observations with ESCRT0 and hHR23B can account for the differences in proteasome targeting in crude extracts and *in vivo*, we measured the approximate content of these UBD-containing proteins and of proteasomes in the muscle extracts. (It was not possible to measure the concentrations of Ub conjugates, since they are heterogenous in types of Ub linkages and in length, making any estimation of *in vivo* concentrations meaningless.) To generate a standard curve, increasing amounts of the pure Rad23 and ESCRT0 proteins were immunoblotted, and the intensities of the bands measured (Supplementary Figure S7A–C). The amounts of these UBD proteins in the lysate were then determined by comparing the intensities of the specific bands with the standard curve (Supplementary Figure S7A–C). The 26S content was measured by calculating the specific proteasomal peptidase activity compared to the known activity of isolated 26S particles (Supplementary Figure S7D). STAM, at 30 nM, was the most abundant UBD protein in the muscle lysate and was significantly more abundant than Hrs (3 nM) (Supplementary Figure S7C). Importantly, these concentrations of the ESCRT0 components are comparable to the amounts that block 26S binding to the K63 conjugates (Figure 3). The amounts of hHR23B present in the lysate appeared about two-fold higher than that of hHR23A (11 nM versus 5 nM) (Supplementary Figure S7C). This concentration of hRad23B appeared somewhat lower than that allowing half-maximal stimulation of proteasome binding to K48 chains (40 nM), and the concentration of hHR23A in the lysate was much below its Ka (150 nM) (Figure 4A). These findings are consistent with our observations that the lysate could inhibit proteasome binding to K63 conjugates but did not stimulate binding to K48 conjugates (Figure 1B).

However, within the cell, concentrations of these UBD proteins must be many fold higher. The total protein concentration in mammalian cells has been estimated to be between 200 and 300 mg/ml (Ellis, 2001) (i.e., 60-fold higher than in our crude lysates), which would imply that the intracellular concentrations of hHRad23B are about 600 and 300 nM for hHR23A (Supplementary Figure S7C). Thus, *in vivo*, both Rad23 proteins, but especially hHR23B, are very likely to stimulate K48-conjugate binding to the proteasome, while the ESCRTO proteins at intracellular concentrations (STAM at 1.4 μM and Hrs at 200 nM) far exceed those necessary to block almost completely proteasome association with K63 chains.

Decreasing ESCRT0 levels in cells allows K63 conjugates to bind to proteasomes

To confirm that the ESCRT0 complex is of primary importance in preventing K63-chain binding to 26S in cells, we measured the effect of reducing ESCRT0 in HEK293T cells by siRNA (Figure 5). Three days after transfection with siRNA for STAM and Hrs, the content of these proteins was reduced by about 50% below those in control cells (Figure 5B). Lysates were then prepared from ESCRT0-difficient and control cells, depleted of proteasomes, and incubated with the K63 or K48 conjugates in the usual manner (Figure 5A and C). The lysates of cells with decreased ESCRT0 content had a reduced capacity to block K63-conjugate binding to 26S. After downregulation of ESCRT0, the binding of K63 conjugates to the proteasome increased about two-fold (19–35%, $P = 0.01$) (Figure 5C), even though these cells contain many other proteins capable of binding to K63 chains (Figure 1E; Table I). Therefore, endogenous ESCRT0 is a key factor in capturing K63 conjugates and preventing their binding to the proteasome.

ESCRT0 prevents polyubiquitinated MHC Class I molecules from binding to proteasomes in vivo

The ESCRT0 complex is required for the efficient endocytosis and lysosomal targeting of cell surface proteins that are modified by attachment of K63 Ub chains (Shields and Piper, 2011). To determine if ESCRT0 prevents the binding of such ubiquitinated proteins to the proteasome *in vivo*, we examined whether decreasing ESCRTO content might cause a K63-ubiquitinated surface protein, MHC Class I, to associate with the proteasome.

Viruses have evolved several Ub-dependent mechanisms to downregulate surface MHC Class I molecules (Randow and Lehner, 2009). The Karposi's sarcoma associated Herpes Virus (HHV8) encodes several Ub ligases that act on surface MHC Class I to avoid detection by T cells (Nathan and Lehner, 2009). The HHV8 ligase, K3, ubiquitinates Class I at the cell surface by forming K63 chains, which signal internalization and degradation of the Class I via the endosomal-lysosomal pathway (Duncan *et al*, 2006). Since ESCRT1 components are required for the efficient internalization of ubiquitinated Class I (Hewitt *et al*, 2002), we could examine whether proteasomes might bind to ubiquitinated MHC Class I molecules after partial depletion of ESCRT0.

To confirm that ESCRT0 was also required for efficient MHC Class I downregulation by the K3 ligase, wild-type HeLa cells and cells stably expressing the viral ligase K3 (HeLa-K3) were transfected with or without the siRNA to STAM and Hrs, and the levels of cell surface Class I molecules were measured by flow cytometry, and by immunoprecipitation followed by immunoblot (Figure 6A and B). K3 expression caused a dramatic downregulation in surface Class I, in accord with prior reports. However, in the HeLa-K3 with reduced ESCRT0, there were two-fold more Class I molecules in the cells and on the cell surface (Figure 6A). In addition, a similar ladder of polyubiquitinated Class I molecules was found in the HeLa-K3 cells with or without siRNA to the ESCRT0 proteins (Figure 6B, top panel). Thus, when ESCRT0 levels are reduced, the ubiquitinated MHC Class I molecules are not rapidly deubiquitinated.

To label MHC Class I at the plasma membrane, HeLa and HeLa-K3 cells transfected with or without siRNA to the ESCRT0 proteins were incubated with a conformation-specific Class I antibody (W6/32) for 3 h at 37°C. These Class I proteins were immunoprecipitated, and the amount of proteasomes associated with Class I molecules was measured by assaying peptidase activity using the specific substrate, LLVY-AMC (Figure 6C–E). Following ESCRT0 knockdown, there was a rescue of Class I molecules that had been on the plasma membrane (Figure 6D). Furthermore, there was a clear two-fold increase ($P < 0.05$) in the amount of 26S proteasomes isolated with the MHC Class I following ESCRT0 knockdown over the amount found in the HeLa-K3 control (which resembled the non-specific binding of proteasomes seen in the unlabelled HeLa cells) (Figure 6E).

This increase in proteasome binding of MHC class was not due to a non-specific disruption of the endocytic machinery, since treatment of the cells with Bafilomycin A (an inhibitor of lysosomal acidification) or knockdown of TSG101 (an ESCRT1 component) increased the total cellular levels of Class I but had no effect on the amount of 26S bound to the MHC Class I molecules (Figure 6D and E). Interestingly, the addition of the proteasome inhibitor, MG132, to these cells did not affect the total levels of mature Class I (Supplementary Figure S8A). This lack of degradation of mature Class 1 was in clear contrast to the degradation of immature Class I by the ERAD pathway, which was induced by knockdown of β2microglobulin (β2m) in HeLa cells (Burr *et al*, 2011), where there was an association with proteasomes and addition of MG132 did cause an increase in cellular Class I levels (Supplementary Figure S8B). Thus, the K63-ubiquitinated Class I bound 26S but was not degraded, which is not surprising as the proteasome by itself would not be expected to extract Class I from the membrane.

To test further if MHC Class I molecules and proteasomes interact following knockdown of ESCRT0, we examined the subcellular localization of these proteins. HeLa-K3 cells were incubated with the MHC Class I conformational antibody and a fluorescently tagged transferrin for 15 min at 37°C. The uptake of the transferrin served as a marker to identify newly formed endosomes from the plasma membrane. The subcellular localization of 26S proteasomes and MHC Class I was visualized by confocal microscopy. As expected, following ESCRT0 knockdown, the amount of Class I molecules increased at the plasma membrane as well as in the endosomes (Supplementary Figure S8C and D). Furthermore, even though proteasomes, as expected, were observed throughout the cell, there was some accumulation of 26S complexes in the same cellular compartment as the MHC Class I molecules (Supplementary Figure S8D). (However, because of the large pool of proteasomes in cells, relative to the likely number of K63-bound MHC molecules, a large redistribution of proteasomes in this experiment would not have been anticipated). Together, these several observations are further evidence that ESCRT0 is important in preventing the binding of K63 chains to the proteasome *in vivo*.

Discussion

These findings can help account for a major unexplained feature regarding the fate of Ub conjugates in cells. K63-conjugated proteins are not degraded by proteasomes *in vivo*

because cytosolic UBD-containing proteins, especially those comprising the ESCRT0 complex, bind to them selectively and block their binding to 26S proteasomes. It had been reported that K63 chains are degraded more rapidly in the cytosol than K48 chains (Cooper *et al*, 2009), and that K63 conjugates are preferentially deubiquitinated by the 26S-associated DUBs (Jacobson *et al*, 2009; Lee *et al*, 2010). Although we also observed rapid deubiquitination of K63-ubiquitinated Sic1 in cell extracts at 37°C, this deubiquitination was not due to the 26S-associated DUBs, as proteasomes had been depleted from the lysate. By studying conjugate binding to proteasomes at 4°C, we could specifically monitor the initial binding steps (to Rpn10 and Rpn13), which precedes conjugate digestion by the 26S-associated DUBs, USP14, Uch37, and Rpn11 (Peth *et al*, 2009). Under these conditions, we did not observe deubiquitination of the K63 or K48 conjugates nor any difference in 26S binding when DUB inhibitors were added. Also, since K63 and K48 conjugates bound similarly to pure proteasomes and were degraded similarly (Kim *et al*, 2007), we found no evidence for selective deubiquitination of K63 chains. Thus, the K63-specific UBD-containing proteins seem to be the only clearly documented mechanism to ensure that K63 conjugates are not degraded by proteasomes. Even if K63 chains are preferentially deubiquitinated *in vivo* by cytosolic DUBs, it is difficult to see how such a mechanism would not also prevent their delivery to lysosomes via the endosomal (ESCRT) pathway.

UBD proteins are more selective for K48 or K63-polyubiquitinated proteins than for short Ub chains

Unlike most prior studies of UBPs, we have utilized long chains linked to proteins, instead of free, di-, or tetra-Ubs, and have thus been able to uncover important new aspects of binding specificity that had been missed previously. Among the diverse types of UBDs, the most prevalent class are the ones containing α-helical domains, which interact with the I44 hydrophobic region in the Ub molecule. These α-helical UBDs include the UBA domain, UIM, DIUM (*d*ouble sided-UIM), GAT (*G*GA *a*nd *T*OM) domain and VHS domain. All the proteins shown here to bind selectively to K48 or K63 chains contain multiple UBDs of the α-helical class. For example, hHR23A and hHR23B encode two UBA domains within their C-termini, while both Hrs and STAM encode N-terminal VHS domains and an additional α-helical UBD, a UIM in STAM, and a DUIM in Hrs.

Although isolated α-helical UBDs have a greater affinity for Ub tetramers than for mono-Ub (Varadan *et al*, 2005; Hurley *et al*, 2006), they show little or no preference for K48 or K63 tetramers. For example, the second UBA domain of hHR23A has a slightly higher affinity for $K48Ub_4$ ($K_d = 8\,\mu M$) than for $K63Ub_4$ ($K_d = 28\,\mu M$) (Raasi *et al*, 2004), but the VHS and DIUM domains of Hrs bind these different Ub tetramers similarly (Ren and Hurley, 2010). Even when the entire UBD-containing proteins or ESCRT0 complexes were studied, only small differences in binding to K48 ($K_d = 43\,\mu M$) and K63 tetramers ($K_d = 8\,\mu M$) were observed (Ren and Hurley, 2010) in contrast to our finding of strong specificity for K63-polyubiquitinated proteins.

We therefore chose not to examine the UBDs in isolation, but instead to study the binding of the entire proteins to K48 and K63 chains linked to proteins. This approach enabled us to demonstrate a clear specificity of mammalian Rad23 proteins for K48-ubiquitinated substrates and of ESCRT0 for K63 conjugates that had not been recognized. In fact, when we did study an isolated UBD, the VHS domain of STAM, it only prevented conjugate binding at very high concentrations (10 μM) and showed only a modest preference for K63 chains. It is noteworthy that the affinities of the ESCRT0 and Rad23 proteins for Ub chains ranged between 30 and 150 nM, which is much higher than those we observed for the VHS domain of STAM and at least 100-fold greater than those of isolated domains described previously. Presumably, this greater affinity for the polyUb chains is due to avidity effects. It is also possible that the structural differences between the tightly packed K48 chains and the looser K63 chains are more easily recognized when the number of Ub moieties is greater. Either mechanism can account for our finding that the UBD-containing proteins determine which Ub conjugates bind to the proteasome in cells.

Our findings of linkage specificities for UBD proteins and the higher affinities of the UBD-containing proteins for long Ub chains resulted from our use of more physiological conditions, specifically our use of full-length UBD-containing proteins, and poly-ubiquitinated proteins rather than free mono, di, or tetra-Ub chains. Using conjugates containing eight or more Ub molecules, we found that STAM binds selectively to K63 chains, and we also show that ESCRT0 binds predominantly to chains 6–9 Ubs long. Thus, Ub-chain length is clearly an important determinant of the avidity of UBD proteins for Ub conjugates and consequently the fate of ubiquitinated proteins in cells.

The present findings probably apply to most cell types since both ESCRT0 and Rad23 are highly conserved throughout eukaryotes (although lower eukaryotes, like *S. cerevisiae*, encode only a single ancestral Rad23 protein). However, there are presently no quantitative data available for meaningful cell-to-cell comparisons of these processes. The intracellular concentrations of proteasomes (Lightcap *et al*, 2000) and presumably of UBD-containing proteins vary widely between cell types. In addition, the concentration of Ub conjugates, as well as Ub, certainly differ between cell types and vary under deficient physiological conditions. Although K63 linkages are abundant in cells (Dammer *et al*, 2011), it is not possible by available methods to differentiate between the content of K63 chains or of mixed chains containing K63 linkages. Our most extensive studies here were performed on skeletal muscle extracts whose rates of proteolysis, endocytosis, and content of proteasomes are lower than in most mammalian tissues. Thus, it remains possible that in other cell types, the relative concentrations of K63-specific binding proteins and K63-Ub chains are quite different, and that other UBD-containing proteins may block the binding of K63 conjugates to proteasomes.

Stimulation of K48-conjugate binding to proteasomes by Rad23 proteins

The Rad23 proteins are believed to function as 'shuttling factors' that facilitate the delivery of Ub conjugates to the 26S (Elsasser *et al*, 2004; Verma *et al*, 2004). However, there are conflicting reports on the effects of hHR23A on conjugate binding and degradation by proteasomes, and the effects of hHR23B have received less attention. Although over-expression of Rad23 in yeast was observed to increase the

number of ubiquitinated proteins at the proteasome (Chen and Madura, 2002), Rad23 is only required for the degradation of certain ubiquitinated substrates, and many proteins are efficiently degraded in ΔRad23 strains (Verma *et al*, 2004). In addition, pure hHR23A was reported to inhibit the binding and degradation of certain substrates by the 26S (Raasi and Pickart, 2003), perhaps due to their use of much higher concentrations of hRad23A (2.5 μM) than those studied here or found *in vivo*. In the muscle lysates, we found that the concentration of hHR23A to be ~5 nM (Supplementary Figure S4; Figure 1), which implies an intracellular concentration of about 300 nM.

It is surprising that hHR23A has been the main focus of research on the 'shuttling' of Ub conjugates rather than hHR23B. Although hHR23A is more closely related to the yeast Rad23, the levels of hHR23B in mammalian cells are higher than those of hHR23A (about 2-fold higher in rat muscles (Supplementary Figure S4) and 10-fold higher in mouse fibroblasts) (Okuda *et al*, 2004). Furthermore, hHR23B stimulated proteasome binding to K48 chains at significantly lower concentrations than hHR23A (Figure 4A), and no other Ubl-UbA protein was identified in the present analysis. This greater efficacy of hHR23B could reflect a greater affinity for K48 chains or for proteasomes, and the Ubl domain of hHR23B has been reported to have a higher affinity for the 26S than that of hHR23A (Chen and Madura, 2006). Gene deletion studies also indicate a greater importance of hHR23B *in vivo*. While a hHR23A null mouse has only a mild phenotype (Friedberg and Meira, 2004), a hHR23B knockout shows severely impaired embryonic development and a very high rate of neonatal death (Ng *et al*, 2002). Thus, hHR23B seems to be the primary 'shuttling factor' for K48-Ub conjugates in mammalian cells.

ESCRT0 proteins prevent the binding of K63-Ub conjugates to proteasomes

In the ESCRT pathway, K63-ubiquitinated substrates, generally membrane proteins, are first bound by the ESCRT0 complex, which targets them to the endosomal-lysosomal system (Shields and Piper, 2011). As shown in Figure 3, this high affinity binding of ESCRT0 (at concentrations <20 nM) can also protect the K63-ubiquitinated proteins from proteasomal degradation by preventing their binding to the 26S complex. Because intracellular concentrations of the ESCRT0 proteins must be much higher (STAM 1400 nM and Hrs 200 nM in muscle; Supplementary Figure S5), they should be of major importance in blocking K63 binding to the 26S *in vivo*, as demonstrated in Figures 5 and 6. Even though polyUb chains by themselves have a very high affinity for the 26S (Ka ≈35 nM) (Thrower *et al*, 2000; Peth *et al*, 2010), the ESCRT0 components at 20 nM were effective inhibitors of proteasome binding, while Rad23B at 50 nM, able to enhance K48-conjugate binding.

Direct evidence of ESCRT0's physiological importance in blocking K63-conjugate binding to proteasomes was the appearance of 26S bound to K63-ubiquitinated MHC Class I molecules following partial depletion of STAM and Hrs by siRNA. We chose to study surface Class I because it is well established that after infection with Herpes virus (HHV8), the viral ligase K3 dramatically downregulates surface Class I by forming a K63-polyUb chain on a single lysine residue in its cytosolic tail (Duncan *et al*, 2006; Cadwell and Coscoy, 2008;

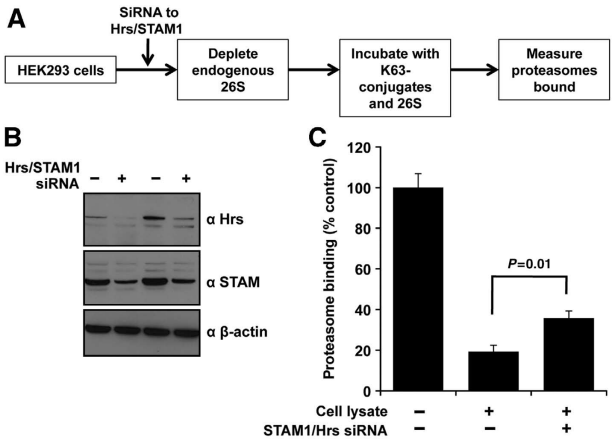

Figure 5 Decreasing ESCRT0 levels in cells allows K63 conjugates to bind to the proteasome. (**A**–**C**) HEK293T cells were transfected with or without siRNA targeting Hrs and STAM. After 72 h, the cells were lysed and depleted of endogenous 26S proteasomes in the usual manner. The lysates were then incubated with the pure ubiquitinated Nedd4 resins, and the amount of proteasomes bound to the resins measured. Schematic of the experimental method (**A**), representative immunoblots of STAM and Hrs knockdowns (**B**), and measurements of proteasomes bound to the K63 conjugates (**C**) are shown. β-actin served as a loading control (**B**, bottom panel). All values are the means ± s.e.m.

Randow and Lehner, 2009). Also, the ESCRT pathway (i.e., the ESCRT1 protein, TSG101) had been implicated in downregulation of Class I from the cell surface (Hewitt *et al*, 2002), and we confirmed here, using siRNA, that the ESCRT0 proteins STAM and Hrs are also required for efficient downregulation of Class I by K3. Although surface Class I molecules were not found in association with proteasomes, K63-ubiquitinated Class I molecules that had been at the cell surface became associated with 26S proteasomes. Furthermore, pharmacological blockage of lysosomal function or downregulation of ESCRT1 did not cause ubiquitinated MHC molecules to associate with proteasomes. This association with the 26S was a specific consequence of the downregulation of ESCRT0. Although there was a clear association of Class I molecules with 26S, we did not observe proteasomal degradation of Class I, presumably because the Class I molecules could not be extracted from the surface membrane by the 26S. By contrast, when the MHC Class I molecules failed to fold properly in the ER, due to the lack of β-microglobulin, they became bound to 26S and were efficiently degraded by proteasomes. It is also noteworthy that MHC Class I molecules were not rapidly deubiquitinated when ESCRT0 components were reduced (Figure 6; Hewitt *et al*, 2002). This supports our finding that preferential deubiquitination is not the mechanism by which K63 conjugates are prevented from binding to the proteasomes in cells. Together, these findings provide further evidence that, in cells, K63-ubiquitinated Class I molecules associate with ESCRT0, which prevents their binding to the proteasome and targets the conjugates to lysosomes.

Potential roles of other K63-specific UBD proteins in preventing binding to proteasomes

While these studies indicate a key role of ESCRT0 in determining the fate of K63 conjugates, other UBD-containing

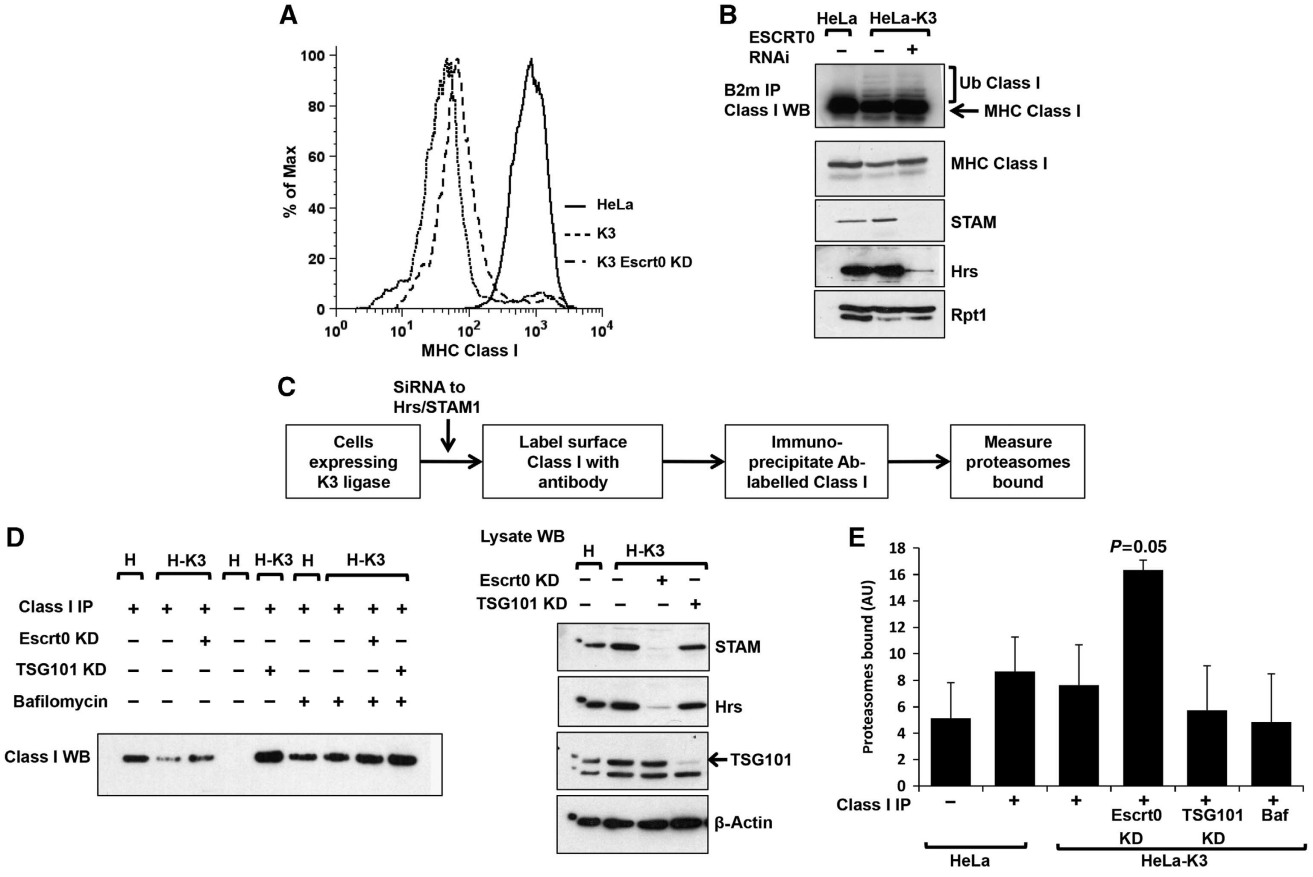

Figure 6 ESCRT0 prevents polyubiquitinated MHC Class I molecules from binding to the proteasome *in vivo*. (**A**) The viral ligase K3 downregulates cell surface MHC Class I, which is partially rescued by knockdown of ESCRT0. HeLa and HeLa-K3 cells were transfected with siRNA targeted to STAM and Hrs. After 72 h, the levels of surface Class I were measured by flow cytometry. (**B**) ESCRT0 knockdown in HeLa-K3 cells partially rescues total MHC Class levels, but does not result in deubiquitination of Class I. HeLa and HeLa-K3 cells, transfected with siRNA targeting Hrs and STAM, were immunoprecipitated for Class I with a β2m antibody, and immunblotted for total Class I levels (HC10 antibody), STAM and Hrs. Polyubiqutinated Class I molecules were visualized on longer exposures (top panel), while shorter exposures show the partial rescue of Class I following Hrs and STAM knockdowns (middle and lower panels). The proteasome subunit, Rpt1, served as a loading control (bottom panel). (**C**–**E**) Ubiquitinated cell surface MHC Class I associates with the 26S proteasome following a reduction in endogenous ESCRT0. HeLa and HeLa-K3 cells were transfected with siRNA against Hrs, STAM, or TSG101 in the usual manner. The cells were then labelled for 3 h with the conformational Class I antibody (W6/32) with or without 0.6 μM Bafilomycin A (Baf). These Class I molecules were immunoprecipitated, and the amount of proteasomes bound measured by LLVY-AMC peptidase activity. (**C**) Schematic diagram of the experiment. (**D**) Immunoblots of immunoprecipitated Class I (left), and lysate immunoblots of STAM, Hrs, TSG101, and β-actin (right). (**E**) Measurements of proteasomes bound. All values are the means ± s.e.m.

proteins probably also serve this function in cells. For example, TOM1 is closely related to Hrs and STAM and can form a similar complex to ESCRT0 through its association with Tollip and Endolfin (Yamakami *et al*, 2003; Seet *et al*, 2004). This complex contains multiple UBDs, yet surprisingly, TOM1, unlike STAM and Hrs, bound to both K63 and K48 conjugates. However, it is unclear whether TOM1 or the TOM1-like (TOM1L1 and TOM1L2) complexes interact directly with components of the ESCRT machinery. Unlike the ESCRT pathway, which is conserved from yeast to vertebrates, TOM1 is found only in higher eukaryotes, and thus it may have additional functions aside from lysosomal trafficking. Possibly TOM1, when in complex with other UBD-containing proteins, may exhibit greater selectivity for K63 chains. Gel filtration analyses of the cell extracts showed that proteins ranging from 150 to 670 kDa caused maximal inhibition of K63-conjugate binding to proteasomes. However, the ESCRT0 proteins and TOM1 were only identified in fractions up to ~300 kDa. Thus, other still-unidentified multimeric complexes probably also contribute to the inhibition of K63-conjugate binding to the 26S *in vivo*, and they remain to be identified.

These studies have utilized cell lysates that contain both nuclear and cytosolic proteins. Although hHR23A and hHR23B are present within both the nucleus and cytosol (Katiyar and Lennarz, 2005), ESCRT0 and the TOM1 complexes are limited to the cytosol as are lysosomes (Shields and Piper, 2011). Ubiquitination of nuclear proteins by K63 chains is required for DNA repair mechanisms (Bergink and Jentsch, 2009). Because these nuclear K63 conjugates cannot interact with cytosolic ESCRT0, other UBD-containing proteins in the nucleus may function like ESCRT0 to bind selectively to K63-polyUb chains and protect them from proteasomal degradation. Alternatively, in the nucleus, K63 conjugates may be preferentially deubiquitinated by the 26S-associated DUBs, as recently reported to occur in the DNA-damage response (Butler *et al*, 2012), or they may eventually be degraded by proteasomes, unlike K63 conjugates in the cytosol. Further studies will be essential to define the fates of nuclear K63 chains, and whether similar mechanisms

involving specific UBD proteins protect these conjugates from proteasomal degradation as function in the cytosol.

Materials and methods

Plasmids, purification of UBD-containing proteins antibodies

A complete list of plasmids and antibodies are available in Supplementary data. The UBD-containing proteins (GST or His tagged) were expressed in *E. coli* and purified using standard techniques (full details in Supplementary data). Immunofluorescence is described in Supplementary data.

Preparation of cell and muscle lysates

Mammalian muscles (3 g, rat or rabbit muscle) were homogenized in 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT and 10% glycerol (TBSG), and were centrifuged at 15 000 *g* for 15 min to remove the myofibrils and nuclei. The cell lysate was collected following ultracentrifugation for 1 h at 100 000 *g*. To remove proteasomes, the lysates were spun for an additional 6 h at 100 000 *g* (Gaczynska *et al*, 1993). Lysates were also purified from HEK293 cells as described, except a HEPES buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 10% glycerol) was used with or without 0.5% Triton X-100.

For the ESCRT0 knockdown experiments, HEK293T cells were transfected with siRNA targeting Hrs and STAM1 (Dharmacon, ON-TARGET plus SMARTpool reagents) using oligofectamine in 6 cm dishes. After 72 h, the cells were lysed by sonication and depleted of endogenous proteasomes in the usual manner.

Purification of 26S proteasomes and Ub conjugates

Resin-bound Ub conjugates were obtained by allowing GST-tagged Nedd4 or E6AP to autoubiquitinate as described before (Peth *et al*, 2010). To form K48 or K63 chains on Nedd4, the resin-bound E3 was incubated with E1 (50 nM), UbcH5 (400 nM), 4 mM ATP and 5 μM K48 or $K63Ub_4$ (Boston Biochem) at 37°C overnight. The E1, E2, and unbound Ub tetramers were removed by extensive washing. K63-polyubiquitinated Sic1 was generated as reported previously (Saeki *et al*, 2005; Lee *et al*, 2010).

26S particles were purified from rabbit muscle by the Ubl-affinity method in the presence of 150 mM NaCl as previously described (Besche *et al*, 2009).

Proteasome binding of Ub conjugates and measurement of 26S activity

Rabbit 26S proteasomes (10 nM) were incubated with polyUb conjugates (20 nM), and proteasome activity was measured by cleavage of suc-LLVY-AMC, as described by Peth *et al* (2010) with some modifications. To study their effects on proteasome binding, the muscle extracts were incubated with the control or ubiquitinated E3s in 20 mM Tris, 150 mM NaCl, 1 mM DTT and 10% glycerol for 30 min at 4°C. The resins were washed three times, followed by a 1-ml wash in 50 mM Tris, 1 mM DTT, before incubation with the 26S particles. The UBD-containing proteins were either incubated together with 26S proteasomes and Ub conjugates at specified concentrations, or preincubated (hHR23A, hHR23B) for 30 min at 4°C and washed as described above.

Identification of UBD-containing proteins that bound to the Ub conjugates

The rabbit muscle extracts or HEK293 lysates (4 mg) were incubated with 0.4 mg untreated or ubiquitinated Nedd4 or E6AP for 30 min at 4°C. The resins were loaded onto a 20-ml empty column (Bio-Rad), washed three times with 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 10% glycerol and 0.5% Triton-X 100, before a final wash without detergent. Proteins bound to the Ub conjugates were eluted from the resin following incubation with a one-bed volume of 2 mg/ml His_{10}-UIM (from S5a/Rpn10). The eluted proteins were then incubated with equilibrated NiNTA (100 μl/mg His_{10}-UIM) (Qiagen) for 20 min to remove the UIM. The samples were then separated by SDS–PAGE, or the whole sample TCA precipitated before analysis by LC-MS/MS. The samples were then separated by SDS–PAGE, or the whole sample TCA precipitated before analysis by MS (see Supplementary data).

Measurements of MHC Class I and proteasome binding

HeLa and HeLa-K3 cells (a kind gift from Paul Lehner) were transfected with siRNA targeting Hrs and STAM, or β2m, as described. After 72 h, the cells were treated with 20 μM MG132 for 3 h, and lysed in TBS containing 0.5% Triton-X 100, 10% glycerol, 1 mM ATP, 5 mM $MgCl_2$ and 1 mM DTT. The 26S proteasomes were then isolated using the Ubl-affinity method and immunblotted for MHC Class I. Alternatively, to immunoprecipitate MHC Class I, the cells were lysed with 1% digitonin, precleared using protein A sepharose, and Class I was immunoprecipitated with a β2m antibody.

To isolate the cell surface MHC Class I, the HeLa cells were incubated for 3 h with an excess of the conformational specific antibody, W6/32. The cells were then lysed with 1% digitonin in TBS containing 10% glycerol, 1 mM ATP, 5 mM $MgCl_2$ and 1 mM DTT. The MHC Class I molecules were then immunoprecipitated using protein A sepharose, and the amount of 26S bound measured by LLVY-AMC cleavage.

Supplementary Data

Supplementary Data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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