K63 ubiquitylation triggers proteasomal degradation by seeding branched ubiquitin chains

Fumiaki Ohtake, Hikaru Tsuchiya, Yasushi Saeki, and Keiji Tanaka

*Laboratory of Protein Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan

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Different polyubiquitin chain linkages direct substrates toward distinct cellular pathways. K63-linked ubiquitylation is known to regulate proteasome-independent events such as signal transduction, but its function in the context of heterogeneous ubiquitin chains remains unclear. Here, we report that K63 ubiquitylation plays a critical role in proteasome-mediated substrate degradation by serving as a “seed” for K48/K63 branched ubiquitin chains. Quantitative analysis revealed that K48/K63 branched linkages preferentially associate with proteasomes in cells. We found that ITCH-dependent K63 ubiquitylation of the proapoptotic regulator TXNIP triggered subsequent assembly of K48/K63 branched chains by recruiting ubiquitin-interacting ligases such as UBR5, leading to TXNIP degradation. These results reveal a role for K63 chains as a substrate-specific mark for proteasomal degradation involved in regulating cell fate. Our findings provide insight into how cellular interpretation of the ubiquitin code is altered by combinations of ubiquitin linkages.

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K48/K63 branched chains because the protein level of TRAF6, a substrate of the K48/K63 branched chains, was unchanged by HUWE1 knockdown. This is presumably because activation of IL-1β signaling is a transient event; therefore, inactivation of TRAF6 must be regulated at the level of deubiquitylation rather than degradation (19). Accordingly, we speculated that the effect of K48/K63 branched chains on proteasomal degradation varies depending on substrates and contexts.

To begin investigating the possible role of K48/K63 branched chains in the proteasomal degradation, we quantified cellular ubiquitin chains in cells treated with inhibitors of the proteasome (MG132) or lysosome (bafilomycin A). We used the ubiquitin-replacement strategy and coprecipitated proteasome-associated ubiquitin chains with antibody against the 20S-α6 antibody to purify the proteasome and associated proteins. (D and E) Coprecipitated ubiquitin chains in C were analyzed by Ub-AQUA/PRM. Data were normalized to the value of the input (whole-cell lysate) lane for each linkage. In D, total K48 or K63 linkages are the sum of branched, unbranched, and endogenous (unreplaced) ubiquitin-derived linkages (means ± SEM; n = 4). Uncropped gel images are provided in Fig. S4. Cont, control; IP, immunoprecipitation.

K48/K63 branched linkages [sum of branched, unbranched, and unreplaced (endogenous) ubiquitin-derived linkages] were not significantly affected by MG132, consistent with previous studies (8-10).

The data presented here imply that K48/K63 branched linkages are involved in proteasomal degradation. Previous studies suggested that K63 chains are less enriched in the proteasome than K48 chains in mammalian cells (11, 12) and in yeast (16). Moreover, ubiquitin chains mixed/branched at multiple linkages inhibit recognition by the proteasome (21). Hence, we asked whether K48/K63 branched linkages associate with the proteasome. We used the ubiquitin-replacement strategy and coprecipitated proteasome-associated ubiquitin chains with antibody against the 20S-α6 subunit (Fig. 1B). We confirmed the presence of the 26S proteasome by analyzing the 19S subunit Rpt6 (Fig. 1C). Ubiquitylated substrates associated with the proteasome only after cells were treated with MG132 for 1 h (Fig. 1C).

Ubiquitin linkage quantification with Ub-AQUA/PRM revealed that the total abundance of K48 linkages is elevated in proteasome precipitants in the presence of MG132 (Fig. 1D). In contrast, the total abundance of K63 linkages is only slightly enhanced (Fig. 1D).
ITCH modifies TXNIP with K48/K63 branched ubiquitin chains to promote its proteasomal degradation. (A) Strategy for screening of candidate E3s related to K48/K63 branched ubiquitin chains. (B) Cells treated with or without MG132 were subjected to affinity purification using K63TUBE, and the interactants were identified by shotgun analysis. The number of peptide spectrum matches (PSM) from MG132-treated or untreated samples were plotted by averaging values from two biological replicates. Ubiquitin ligases related to K63 chains are shown in red, and other ligases are shown in black (Dataset S1). (C) In vitro ubiquitylation assay with purified ITCH. ITCH (0.15 μg) was incubated with E1 (50 ng), UBCH7 (50 ng), and ubiquitin (2 μg) for 2 h at 37 °C in an in vitro ubiquitylation assay. The data were normalized to the level of total ubiquitin chains (means ± SEM, n = 2). (D and E) U2OS cells were transfected with the indicated siRNAs (D) or treated with the indicated inhibitors for 8 h (E). Total cell lysates were subjected to immunoblotting to detect endogenous TXNIP. (F) FLAG-TXNIP was immunopurified from TXNIP/ITCH-expressing 293T cells and subjected to UbiCrest analysis with the indicated DUBs. The number of peptide spectrum matches (PSM) from MG132-treated or untreated samples were plotted by averaging values from two biological replicates. (G) Precipitated ubiquitin chains were subjected to Ub-AQUA/PRM. The data were normalized against the total abundance of R54A-derived chains (means ± SEM, n = 3). (H) ITCH-dependent increase in the abundance of ubiquitin chains in G was calculated by subtracting amounts of ubiquitin linkages in ITCH(WT)-transfected and untransfected cells. (I) U2OS cells were transfected with the indicated siRNAs and incubated with doxycycline for 4 d to allow ubiquitin replacement. The cells were treated with 20 μM MG132 for 3 h, and cell lysates were subjected to immunoprecipitation with anti-TXNIP antibody or normal IgG as indicated. Precipitated ubiquitin chains were subjected to AQUA/PRM (means ± SEM, n = 3). P values were calculated with one-way ANOVA. (J) FLAG-TXNIP was immunopurified from TXNIP/ITCH-expressing 293T cells and subjected to UbiCrest analysis with the indicated DUBs. K48 chains (Upper), K63 chains (Middle), and total ubiquitin (Lower) were stained with specific antibodies. IP, immunoprecipitation.
ITCH Modifies TXNIP with K48/K63 Branched Ubiquitin Chains to Promote Its Proteasomal Degradation. As the roles of K48/K63 branched chains seem to be substrate-dependent, we next tried to identify an E3 substrate pair that could serve as a model for K48/K63 branched chain-mediated proteasomal degradation. To this end, we performed a proteomic screen in which E3s/substrates associated with K63 chains or K48/K63 branched chains were isolated by using K63TUBE (K63-specific tandem ubiquitin-binding domain), a K63 chain-specific binder (22) (Fig. 2A). We confirmed that K63TUBE enriched unbranched K63 chains and K48/K63 branched chains (Fig. S2A and B). As candidates related to proteasomal degradation should accumulate after brief proteasome inhibition, we compared spectrum counts for each interactant in the presence or absence of MG132 (Fig. 2B). From this screen, we identified several ubiquitin ligases related to K63-linked chains (Fig. 2B, red) as well as other ubiquitin ligases (Fig. 2B, black).

Among several ubiquitin ligases enriched in the presence of MG132 (Fig. 2B), we focused on ITCH, a HECT-type E3 ligase belonging to the NEDD4 family, closely related to WWP1 and WWP2 (23, 24). ITCH has various biological functions such as inhibition of inflammation, and also acts as a tumor-promoting factor (25, 26). Interestingly, even though biochemical studies indicate that ITCH, as well as other NEDD4 family ligases, assemble mainly K63 chains (27), other studies report that ITCH facilitates proteasomal degradation of substrates including LAT51, p63, and TXNIP (26, 28). Similarly, yeast Rsp5 induces processing of Mga2-p120, possibly through K63 chains (29). From these results, we hypothesized that ITCH-dependent K63 chain formation may induce proteasomal degradation through a mechanism involving subsequent formation of K48/K63 branched chains. Consistent with previous studies, we confirmed that ITCH preferentially assembles K63 chains in vitro and forms K48 chains only to a much lesser extent (Fig. 2C).

TXNIP, an ITCH substrate, is an important tumor suppressor (30) that promotes apoptosis by regulating multiple factors, including thioredoxin, p53, and inflammasomes (31, 32). We selected TXNIP as a model substrate for characterizing the cellular function of ITCH because of its robust accumulation after ITCH knockdown (Fig. 2D). Because ITCH and WWP1 share several substrates (33), we also knocked down WWP1. Double knockdown of ITCH and WWP1 resulted in greater accumulation of endogenous TXNIP (Fig. 2D). Degradation of endogenous TXNIP was mediated through the proteasome, but not the lysosomal pathway (Fig. 2E).

We next asked whether TXNIP is modified by K48/K63 branched chains. Ubiquitylation of FLAG-TXNIP immunopurified from Ub(R54A)-expressing cells was enhanced by coexpression of WT, but not catalytically inactive (CS) ITCH (Fig. 2F). Subsequent Ub-AQUA/PRM analysis revealed that TXNIP is modified by K48/K63 branched linkages, as well as unbranched K63 linkages, in an ITCH-dependent manner (Fig. 2G). Unbranched K48 linkages were also more abundant (Fig. 2G). When the ITCH(WT)-dependent increase in each ubiquitin linkage was calculated by subtracting the linkage abundance in ITCH(WT)-transfected and nontransfected cells (Fig. 2G), K63 and K48 linkages accounted for most of the ITCH-mediated ubiquitylation of TXNIP (Fig. 2H). Because the ITCH-dependent ubiquitin linkages in cells (Fig. 2H) do not reflect the linkage specificity of ITCH in vitro (Fig. 2C), we assumed that unknown K48-specific ubiquitin ligase(s) cooperate with ITCH in cells.

To investigate whether endogenous TXNIP is modified with K48/K63 branched ubiquitin chains, we analyzed TXNIP immunopurified from U2OS_shUb^*-Ub(R54A) cells by Ub-AQUA/PRM. The results revealed that endogenous TXNIP is indeed modified with K48/K63 branched ubiquitin chains, and that this modification is significantly inhibited by knockdown of ITCH/WWP1 (Fig. 2F and Fig. S2 C and D).

To acquire information about higher-order ubiquitin chain architectures, we conducted a ubiquitin chain restriction assay using DUBs (UbiCrest) (34) on TXNIP-conjugated ubiquitin chains purified from ITCH-expressing cells (Fig. 2F). We found that the level of K48 linkages was massively decreased by the K63-specific DUB AMSH (Fig. 2I, Upper). On the contrary, K63 linkages were retained after treatment with the K48-specific DUB OTUB1, but the molecular weights of the chains decreased somewhat (Fig. 2I, Middle). These results indicated that K48 linkages are incorporated onto preformed K63 linkages conjugated to TXNIP, and suggested that K63 chains assembled by ITCH serve as seeds for subsequent assembly of K48 branches by unknown ligase(s).

UBR5/HUWE1/UBR4 Cooperate with ITCH to Assemble K48/K63 Branched Chains and Are Required for TXNIP Degradation. To identify the K48 branching factor(s) that cooperate with ITCH, we screened for TXNIP-interacting proteins (Fig. 3A). Among the interactants, we identified ITCH itself, along with the ITCH-related ubiquitin ligases WWP1 and WWP2, which presumably act redundantly. We then focused on three other ubiquitin ligases, UBR5, HUWE1, and UBR4 (Fig. 3A, Right). UBR5 is a HECT-type ligase that targets a variety of substrates for proteasomal degradation mediated by K48 chains (23). HUWE1, another HECT-type ligase, targets various substrates (23, 35) and serves as the chain branching factor for TRAF6 during NF-κB activation (5). UBR5 and HUWE1 have ubiquitin-binding domains and are therefore considered to be ubiquitin-interacting ubiquitin ligases (5, 36). UBR4 is less well-characterized because of its high molecular weight, but has been reported to target substrates for proteasomal degradation (37).

We validated the interactions of these three ligases with TXNIP by coimmunoprecipitation analysis of FLAG-tagged TXNIP (Fig. 3B) and endogenous TXNIP (Fig. 3C).

We next asked whether the identified ligases were involved in ITCH-dependent branched chain assembly. Simultaneous knockdown of UBR5, HUWE1, and UBR4 resulted in a significant decrease in ITCH-dependent K48/K63 branched linkages on TXNIP (Fig. 3 D and E). The ITCH-dependent increase of unbranched K48 linkages was also abolished by the knockdown, suggesting that K48 branched ubiquitin is further elongated through K48 chains by these ligases. Unbranched K63 linkages were slightly decreased by the knockdown. The precise mechanism remains unknown, but, given that K63 chains adopt open architectures, we assume that unknown K48-specific ubiquitin ligase(s) cooperate with ITCH in cells. Various DUBs, it is possible that branched linkages protect K63 chains from DUBs.

Cycloheximide chase experiments revealed that simultaneous knockdown of UBR5, HUWE1, and UBR4 abolished ITCH-dependent accelerated turnover of TXNIP (Fig. 3F). Moreover, endogenous TXNIP accumulated to high levels in the triple knockdown, as in the case of the ITCH/WWP1 double knockdown (Fig. 3G). Knockdown of single components resulted in slight accumulation of TXNIP, with UBR5 knockdown exhibiting the clearest effect (Fig. 3G, lanes 2–4), suggesting that UBR5, HUWE1, and UBR4 play overlapping roles in TXNIP degradation, and that UBR5 is the most important of the three. ITCH and WWP1 were expressed at normal levels in the UBR5/HUWE1/UBR4 knockdown cells (Fig. 3G, lane 5), suggesting that the
K63 ligases (ITCH and WWP1) alone are not sufficient for TXNIP degradation, and that the K48 ligases (UBR5, HUWE1, and UBR4) are also required. We then examined turnover of endogenous TXNIP. Consistent with the results described here earlier, turnover of endogenous TXNIP was delayed by simultaneous knockdown of either ITCH and WWP1 or UBR5, HUWE1, and UBR4 (Fig. 3H). Together, these results indicated that K63 and K48 ubiquitination play nonredundant roles in TXNIP degradation.

**ITCH-Dependent K63 Ubiquitination Triggers Recruitment of UBR5 and Assembly of K48/K63 Branched Chains.** The knockdown experiments described earlier suggested that ITCH and UBR5 are major contributors to TXNIP degradation. Hence, we further explored the molecular mechanism of branched chain assembly by ITCH/UBR5 and the role of K63 linkages in this process. In an in vitro ubiquitination reaction, incubation of Ub(R54A) with a mixture of purified ITCH and UBR5 proteins (Fig. 4A) resulted in generation of polyubiquitin chains positive for K48 and K63 chain-specific

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**Fig. 3.** UBR5/HUWE1/UBR4 cooperate with ITCH in formation of K48/K63 branched chains and are required for TXNIP degradation. (A) FLAG-TXNIP–interacting proteins were communoprecipitated from 293T cells and subjected to shotgun analysis. The number of peptide spectrum matches (PSM) for each identified protein are shown as a heat map. Known E3 ubiquitin ligases within the list are indicated to the right of the map (Dataset S2). (B) FLAG-TXNIP–interacting ubiquitin ligases identified in A were validated by communoprecipitation and immunoblotting as indicated. (C) Endogenous TXNIP interacts with UBR5, HUWE1, and UBR4. Lysates from 293T cells were subjected to immunoprecipitation with anti-TXNIP antibody or normal IgG as indicated. (D) Lysates from 293T cells transfected with the indicated siRNAs were subjected to shotgun analysis. The number of peptide spectrum matches (PSM) for each identified protein are shown as a heat map. Known E3 ubiquitin ligases within the list are indicated to the right of the map (Dataset S2). (E) U2OS cells transfected with the indicated siRNAs were subjected to cycloheximide chasing analysis (means ± SEM; n = 3). (F) 293T cells transfected with the indicated siRNAs and FLAG-TXNIP/Ub(R54A) with or without ITCH were subjected to Ub-AQUA/PRM as in Fig. 2D. The data were normalized against the total abundance of R54A-derived chains (means ± SEM; n = 3). (G) U2OS cells transfected with the indicated siRNAs and expression vectors for FLAG-TXNIP and ITCH were subjected to cycloheximide chasing analysis (means ± SEM; n = 3). (H) 293T cells transfected with the indicated siRNAs were subjected to cycloheximide chase analysis. (Right) Band intensities from two biological replicates. CHX, cycloheximide; Cont, control; IP, immunoprecipitation.

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antibodies (Fig. 4B, lane 3 of each panel). Subsequent Ub-AQUA/PRM analysis revealed assembly of K48/K63 branched linkages by the mixture of ITCH and UBR5 (Fig. 4C).

UBR5 contains a UBA domain, which we speculated might recognize preformed K63 chains. We conducted an in vitro ubiquitin pulldown assay by using Halo-tagged UBA domain of UBR5 as bait (Fig. S34). Although ubiquitin monomer or K48-linked diubiquitin associated with UBR5(UBA) only weakly, K63-linked diubiquitin strongly associated with UBR5(UBA) (Fig. 4D). Our data are in agreement with a previous report (36) and consistent with the notion that certain UBAs have higher affinity for K63 chains as a result of increased avidity to the open conformation of K63 chains (38).

To clarify the role of preformed K63 linkages in branched chain formation, we performed a two-step ubiquitylation assay by using recombinant TXNIP as a substrate. GST-tagged TXNIP immobilized onto beads was sequentially reacted with or without ITCH and UBR5 (Fig. 4E). Consistent with the notion that ITCH recognizes TXNIP through a direct interaction between the ITCH WW domain and the TXNIP PPxY motif (39), ITCH alone readily ubiquitylated TXNIP, whereas UBR5 alone did so only subtly (Fig. 4E, Upper, lanes 2 and 3). However, reaction with UBR5 after preformation of K63 chains by ITCH resulted in a massive increase in polyubiquitin chains conjugated to TXNIP (Fig. 4E, Upper, lane 4). We note that the migration pattern of ubiquitin chains and reactivity with pan-ubiquitin antibodies can be affected by chain type, length, and branching. Therefore, the molecular weight distribution of anti-ubiquitin signals (Fig. 4E, Upper) might not be quantitatively accurate. Subsequent Ub-AQUA/PRM analysis revealed that preformed K63 chains facilitate K48 chain modification of TXNIP by UBR5 (Fig. 4E, Lower). To quantify branched linkage formation,

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**Fig. 4.** ICH-dependent K63 ubiquitylation triggers recruitment of UBR5 and assembly of K48/K63 branched chains. (A–C) Ub(R54A) (1 μg) was reacted with FLAG-ITCH (0.15 μg, 3 h) and/or FLAG-UBR5 (0.2 μg, 2 h) in the presence of E1 (50 ng) and UBCH7 (50 ng) at 30 °C in an in vitro ubiquitylation reaction. (A) Coomassie staining of the purified ITCH and UBR5 proteins. (B) Reactants were analyzed by immunoblotting. (C) Linkage types of ubiquitin chains assembled by a mixture of ITCH and UBR5 were analyzed by Ub-AQUA/PRM (n = 3). (D) Ubiquitin monomer, K48-linked diubiquitin, and K63-linked diubiquitin (1 μg) were subjected to an in vitro pull-down assay with the Halo-tagged UBA domain of UBR5 [UBR5(UBA)]. (E) In vitro ubiquitylation assay of TXNIP-GST-TXNIP (0.4 μg) bound to glutathione beads was reacted with or without ITCH (0.15 μg in the presence of Ub(WT) (2 μg), E1 (50 ng), and UBCH7 (50 ng) at 37 °C for 0.5 h. After the other components were washed out, TXNIP was further reacted with UBR5 (0.2 μg), Ub(WT) (2 μg), E1 (50 ng), and UBCH7 (50 ng) at 37 °C for 2 h. After the beads were washed, TXNIP-conjugated ubiquitin chains were analyzed by immunoblotting (Upper) or Ub-AQUA/PRM (Lower; means ± SD; n = 2). (F) Two-step in vitro ubiquitylation assay of TXNIP was performed as in E by using Ub(R54A) (means ± SEM; n = 3).
we performed the same analysis using Ub(R54A) (Fig. 4F). UBR5 assembled K48/K63 branched linkages on TXNIP previously modified with K63 chains by ITCH. UBR5-dependent assembly of unbranched K48 linkages was also potentiated by preassembly of K63 chains (Fig. 4F). These results suggested the importance of K63 chains as seeds for assembly and elongation of K48/K63 branched ubiquitin chains.

**ITCH Counteracts TXNIP During Apoptotic Cellular Response.** Finally, we assessed the functional significance of K63 chains in the regulation of proteasomal degradation. To this end, we asked whether ITCH counteracts TXNIP function. TXNIP is a proapoptotic factor, regulating signaling factors related to apoptosis and cell-cycle arrest such as p53. TXNIP is also a tumor suppressor, and accordingly its expression is repressed in malignant tumors (30). On the contrary, ITCH is a tumor-promoting and antiapoptotic factor (25). Indeed, based on a database of patients with breast cancer (40), low TXNIP expression or high ITCH expression is positively correlated with poor prognosis (Fig. 5A).

To determine whether ITCH antagonizes TXNIP, we analyzed the expression of apoptotic markers after treating cells with camptothecin (Fig. S3B). Simultaneous knockdown of ITCH and WWP1 induced abnormal accumulation of TXNIP (Fig. 5B). Concomitantly with this, we observed increased levels of cleaved caspase-3 and cleaved PARP1 in ITCH/WWP1-knockdown cells after camptothecin treatment (Fig. 5B, lanes 5 and 6). Simultaneous knockdown of TXNIP canceled the increased levels of apoptotic markers caused by ITCH/WWP1 knockdown (Fig. 5B, lanes 7 and 8). Moreover, knockdown of either ITCH and WWP1 or UBR5, HUWE1, and UBR4 caused accumulation of TXNIP in camptothecin-treated cells (Fig. S3C), suggesting that K48/K63 branched chains are involved in TXNIP regulation. Together, these results suggested that ITCH counteracts TXNIP during the apoptotic response by accelerating its degradation.

**Discussion**

K63-linked ubiquitin chains are the second most abundant linkage type in cells, and homogeneous K63 chains are well known to regulate proteasome-independent processes (4). However, the cellular role of the K63 linkage within heterogenous ubiquitin chains remains largely uncharacterized. In this study, we found that K63 ubiquitylation by ITCH regulates proteasomal degradation of TXNIP by triggering formation of K48/K63 branched chains (Fig. 5C). In this context, K63 chains conjugated to TXNIP act as a substrate-specific mark to recruit K48 ligases in a manner like in which phosphorylation of substrates recruits ubiquitin ligases for degradation. These results show that a nondegradative ubiquitin code can be converted to a degradation signal by branching of ubiquitin chains. Together with previous reports (5, 13, 14), our data provide insight into how cellular interpretation of the ubiquitin code depends on the context formed by the combination of ubiquitin linkages.

To date, no other substrate whose turnover is specifically regulated by K48/K63 branched chains has been identified to our knowledge. However, given that a substantial percentage of K63 linkages in cells appear in K48/K63 branched linkages (5), we speculate that the contribution of the K48/K63 branched chains to proteasomal degradation is considerable. Notably in this regard, NEDD4 family ligases appear to regulate substrate turnover through multiple mechanisms. Rsp5 facilitates substrates turnover in which DUBs trim K63 chains to enable assembly of K48 chains (42, 43), whereas we observed that K63 linkages conjugated to TXNIP are utilized for subsequent assembly of K48 chains (42, 43), whereas we observed that K63 linkages conjugated to TXNIP are utilized for subsequent elongation of K63 chains in vitro (44). Considered together, these observations suggest that certain other NEDD4 family ubiquitin ligases may also utilize the K48/K63 branched chains for proteasomal degradation of substrates by cooperating with other ligases that assemble K48 ligases.

Moreover, an independent study recently reported that UBR5 and UBR4 mediate quality control of misfolded proteins by assembling K11/K48 heterotypic chains (41). Therefore, UBR5 and UBR4 appear to regulate heterotypic/branched chains in different cellular contexts, presumably in collaboration with different E3s. Accordingly, the identification of other substrates
and cellular pathways regulated by UBR5, HUWE1, and UBR4 through branched chains merits further effort.

The mechanism that directs K48/K63 branched chains toward signaling (in the case of TRAF6) or degradation (in the case of TXNIP) remains unknown. The decision may in part depend on substrates, as transient activation of TRAF6 is regulated at the level of deubiquitylation rather than degradation through interaction with DUBs such as A20 and CYLD (19). Alternatively, higher-order chain structures, which we are currently unable to distinguish, may be different.

Our findings reveal a ubiquitin-dependent pathway leading to the proteasome. We speculate that one advantage of utilizing K63 chains for degradation is the specific targeting of substrates by K63 ligases like ITCH. Because substrate-specific and context-dependent protein degradation is crucial for cells, separation of substrate targeting by ITCH and subsequent addition of degradative ubiquitin chains by UBR5 may enable cells to differentially regulate these two steps. In addition, it is likely that multiply branched polyanionic ubiquitin chains promote efficient proteasomal targeting, as proposed previously (13). It is also possible that K48 branching from K63 chains ensures rapid degradation of K63 chain-activated substrates, similar to the role of the ubiquitin chain editing mechanism, i.e., removal of K63 chains and subsequent addition of K48 chains (45). After combination of the K48-branching with K63 chains, the K48 linkages elongated from K63 chains are likely to be recognized by shutting factors such as Rad23 and delivered to the proteasome (11). Consistent with this, a previous study showed that the K48 linkage within the K48/K63 branched ubiquitin trimer can be recognized by Rad23 in vitro (46). TXNIP is a critical regulator of apoptosis, and, consequently, the TXNIP–ITCH–UBR5 axis is an important pathway regulating cell fate decision through targeted degradation. In summary, our results demonstrate that K63 ubiquitylation promotes proteasomal degradation by serving as a seed for K48/K63 branched chains, revealing an additional aspect of the ubiquitin code.

Materials and Methods

For shotgun MS analysis, an Easy nLC 1000 system (Thermo Fisher Scientific) was connected online to a Q Exactive mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source. The TriEasy instrument was operated in a data-dependent MS/MS mode by using the Xcalibur software (Thermo Fisher Scientific). For quantification of peptides by PRM, AQUA peptides (10–50 fmol) per injection were added to samples. The Q Exactive instrument was operated in the targeted MS/MS mode by Xcalibur software. Data were processed using by PinPoint software 1.3 (Thermo Fisher Scientific). Typical fragment ions used for quantification were previously described (5). Details of materials and methods are described in SI Materials and Methods.

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