Pivotal role for the ubiquitin Y59-E51 loop in lysine 48 polyubiquitination

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Lysine 48 (K48)-polyubiquitination is the predominant mechanism for mediating selective protein degradation, but the underlying molecular basis of selecting ubiquitin (Ub) K48 for linkage-specific chain synthesis remains elusive. Here, we present biochemical, structural, and cell-based evidence demonstrating a pivotal role for the Ub Y59-E51 loop in supporting K48-polyubiquitination. This loop is established by a hydrogen bond between Ub Y59's hydroxyl group and the backbone amide of Ub E51, as substantiated by NMR spectroscopic analysis. Loop residues Y59 and R54 are specifically required for the receptor activity enabling K48 to attack the donor Ub-E2 thiol ester in reconstituted ubiquitination catalyzed by Skp1-Cullin1-F-box (SCF)$^{\beta TrCP}$ E3 ligase and Cdc34 E2-conjugating enzyme. When introduced into mammalian cells, loop-disruptive mutant Ub$^{R54A/Y59A}$ diminished the production of K48-polyubiquitin chains. Importantly, conditional replacement of human endogenous Ub by Ub$^{R54A/Y59A}$ or Ub^{K48R} yielded profound apoptosis at a similar extent, underscoring the global impact of the Ub Y59-E51 loop in cellular K48-polyubiquitination. Finally, disulfide cross-linking revealed interactions between the donor Ub-bound Cdc34 acidic loop and the Ub K48 site, as well as residues within the Y59-E51 loop, suggesting a mechanism in which the Ub Y59-E51 loop helps recruit the E2 acidic loop that aligns the receptor Ub K48 to the donor Ub for catalysis.

receptor ubiquitin | E3 ubiquitin ligase | E2 ubiquitin-conjugating enzyme

Central to selective protein turnover by the 26S proteasome is the formation of homotypic lysine 48 (K48)-linked ubiquitin (Ub) chains that tag substrate proteins for degradation (1). Among the most extensively studied systems that produce K48-linked Ub chains is the SCF (Skp1-Cullin1-F-box) E3-directed ubiquitination. SCF is a member of the multisubunit Cullin-RING E3 Ub ligase (CRL) family, the largest of all E3s (2). CRL contains a tandem of a large scaffold protein [Cullin (CUL)] and a RING domain-containing protein (ROC1/Rbx1) that typically associates with an adaptor protein (such as Skp1) in complex with a substrate recognition protein (such as F-box protein). As such, the organization of CRL subunits positions the substrate receptor (such as the F-box protein) within the proximity of ROC1, which recruits an E2-conjugating enzyme that catalyzes the transfer of Ub to a bound substrate. In the SCF reconstitution system, K48-linked polyubiquitin chains on a substrate such as IκBα and β-catenin are produced in a two-step reaction. The E2 UbcH5c deposits the first Ub moiety, forming a substrate–Ub linkage, which is followed by repeated discharge of subsequent Ubs by E2 Cdc34 to form K48-specific Ub chains (3). Human Cdc34 contains a highly conserved charged acidic loop (residues 102–113) that participates in the elongation of K48 chains (4, 5). The current work addresses whether there are determinants on the Ub itself that dictate K48 linkage specificity and, moreover, how Cdc34 might recognize Ub K48.

Results

A Specific Receptor Role for Ub Y59 in Building K48-Linked Ub Chains by Cdc34. Initial screening of 16 positions in Ub for determinants required for SCF$^{\beta TrCP}$/Cdc34's ability to extend a K48-linked Ub chain on IκBα-Ub fusion substrate showed that Y59A substitution had the strongest inhibitory effect (*SI Appendix*, Fig. S1 and Table S1). Several lines of experiments were carried out to characterize further the role of Ub Y59 in ubiquitination. (*i*) In the context of IκBα-Ub fusion, the Ub Y59A mutant led to decreased substrate utilization by 50% and an overall decrease in polyubiquitin product formation (Fig. 1*A*), while maintaining the ability of IκBα-Ub to interact with βTrCP (*SI Appendix*, Fig. S3*A*). In addition, Ub Y59A mutation did not alter the specificity of Cdc34 in catalyzing K48-specific Ub chain elongation, because both the wild-type and Y59A formed polyubiquitin chains only when Ub^{K48} was used as donor, albeit the mutant exhibited lower reaction efficiency (Fig. 1*B*). (*ii*) When used as free Ub, Ub^{Y59A} effectively blocked polyubiquitination of β-catenin by SCF$^{\beta TrCP}$/Cdc34 (Fig. 1*C*), even though both Ub and Ub^{Y59A} formed a Cdc34~Ub thiol ester complex with equal efficiency (*SI Appendix*, Fig. S3*B*). In contrast, Ub^{Y59A} supported SCF$^{\beta TrCP}$/UbcH5c identically as the wild-type Ub (Fig. 1*D*), in a range of Ub concentrations tested (*SI Appendix*, Fig. S3*C*). Thus, Ub Y59A mutation inhibits Cdc34 but not UbcH5c, suggesting a specific requirement of Ub Y59 for supporting Cdc34

Significance

Our identification and characterization of the ubiquitin (Ub) Y59-E51 loop have uncovered a pivotal determinant for lysine 48 (K48) linkage-specific Ub chain synthesis catalyzed by Cdc34 E2 Ub-conjugating enzyme. The Ub Y59-E51 loop appears to anchor a Cdc34 E2-engaging zone, allowing the landing of E2 and enabling it to gain access to the receptor K48. These findings will provide a strong starting point for future biochemical and structural studies aiming to elucidate the detailed interactions between Ub and E2/E3 enzymes that produce the K48 linkage. In addition, the observed global impact of the Ub Y59-E51 loop in cellular K48-polyubiquitination and apoptosis will stimulate investigations for exploring new therapeutic strategies to induce cell killing through pharmacological perturbation of K48-polyubiquitination.

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The authors declare no conflict of interest.

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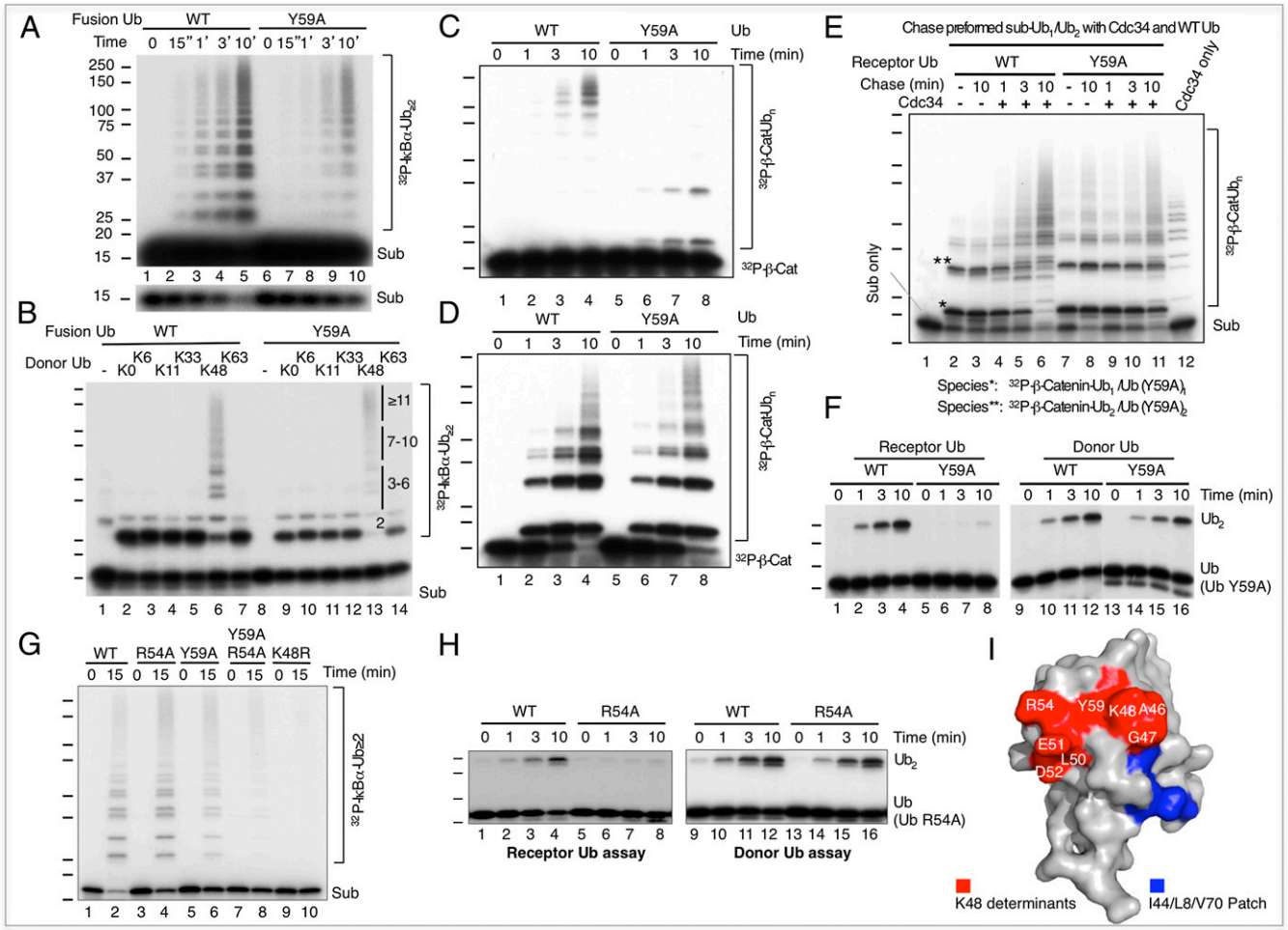

Fig. 1. Biochemical mapping of Ub determinants for K48-ubiquitination by Cdc34. (*A–F*) Analysis of Ub Y59. The effects of Ub^{Y59A} in ubiquitination by $SCF^{\beta TrCP}$/Cdc34 were examined in the form of IκBα-Ub (*A* and *B*), free Ub (*C* and *D*), or isopeptide bonded β-catenin–Ub (*E*). In *B*, reactions contained Ub bearing no K or single K. The effects of Ub^{Y59A} in di-Ub synthesis are shown in *F*. (*G* and *H*) Ub^{R54A} inhibits ubiquitination of IκBα-Ub (*G*) and di-Ub synthesis (*H*). The same protein size markers are used for all gel analysis and position of each marker is specified in *A*. Note that quantification of results in *A–F* is presented in *SI Appendix*, Fig. S2, whereas quantification of *H* is shown in *SI Appendix*, Fig. S4*C*. (*I*) Position of Ub residues critical for K48 function, as well as the I44/V70/L8 hydrophobic patch.

activity. (*iii*) In elongation of the preformed, isopeptide bond-linked β-catenin-Ub_1/Ub_1^{Y59A} (monoubiquitinated) or β-catenin-Ub_2/Ub_2^{Y59A} (diubiquitinated) (Fig. 1*E*, lanes 2 and 7; marked as species* and species**), Cdc34 used both β-catenin-Ub_1 and β-catenin-Ub_2 (with the wild-type Ub) more effectively than β-catenin-Ub_1^{Y59A} and β-catenin-Ub_2^{Y59A} (with Y59A substituted Ub), respectively (Fig. 1*E*). (*iv*) Using a di-Ub synthesis assay that produced exclusively K48-linked conjugates (*SI Appendix*, Fig. S3*D*), Ub^{Y59A} was found to abolish the receptor Ub function, but support the donor Ub activity with efficiency comparable to that of the wild type (Fig. 1*F*). In all, these results establish that Ub Y59 is specifically required for the receptor Ub function, enabling Cdc34 to build K48-linked Ub chains. It is noted that Ub^{Y59A} displayed more pronounced defects in receptor activity when assayed in substrate-independent ubiquitination reactions than in substrate-dependent reactions (*SI Appendix*, Fig. S2), presumably because the reactions by SCF/Cdc34, but not Cdc34 alone, could accommodate an imperfect receptor Ub, such as Ub^{Y59A}, to some extent.

Additional Ub Residues Required for Ub K48 Chain Assembly by Cdc34. Subsequent interrogation of up to 18 residues that span the K48 face of Ub by combining the IκBα-Ub perturbation screen (*SI Appendix*, Fig. S1) and di-Ub synthesis assay (Fig. 1*F*) identified additional residues A46, G47, L50, E51, D52, and R54 that are required for Ub K48 chain assembly by Cdc34 (*SI Appendix*, Table S1). Note that none of these mutations affects Cdc34 charging (*SI Appendix*, Fig. S4*A*). Ub R54, although alone defective (*SI Appendix*, Fig. S4*B*), has synergistic effects with Y59, because $Ub^{R54A/Y59A}$ yielded Ub conjugates (Fig. 1*G*, lane 8) at levels approaching that of IκBα-Ub^{K48R} (lane 10). As with Ub^{Y59A}, Ub^{R54A} failed to function as a receptor to support di-Ub synthesis (Fig. 1*H* and *SI Appendix*, Fig. S4*C*). Both IκBα-Ub^{A46D} and IκBα-Ub^{G47F} showed modest defects in $SCF^{\beta TrCP}$/Cdc34-catalyzed Ub chain assembly (*SI Appendix*, Fig. S4*D*). In di-Ub synthesis, however, both Ub^{A46D} and Ub^{G47F} exhibited pronounced defects as a receptor or donor (*SI Appendix*, Fig. S4 *E* and *F*). Additional experiments revealed defects of Ub L50C in supporting the receptor Ub function and decreased ability of Ub E51R/D52R in mediating either the receptor or donor activity (*SI Appendix*, Fig. S4 *G* and *H*).

Fig. 1*I* shows the position of Ub A46, G47, L50, E51, D52, R54, and Y59 that are proximal to Ub K48. The impact of alteration of this group of residues on Ubc1 was examined because this E2 catalyzes K48-Ub chain formation in vitro in a manner that requires the integrity of Ub Y59 (6-7). The results of *SI Appendix*, Fig. S5 showed that Ub Y59, A46, G47, L50, E51, and D52 are all required for Ubc1-catalyzed di-Ub synthesis. However,

two differences were noted: (*i*) Ub^{R54A} was fully active with Ubc1; and (*ii*) Ub^{A46D} was defective only as a receptor (*SI Appendix*, Fig. S5).

The Ub Y59-E51 Loop. Further di-Ub synthesis experiments revealed the importance of the Ub Y59 hydroxyl group because Y59F, Y59H, and Y59W mutations all inactivated ubiquitination by Cdc34 (Fig. 2*A*). NMR spectroscopic analysis provided first experimental support for the hypothesis that Ub Y59 forms a hydrogen bond with the backbone amide of E51 (8). Comparing the overlaid ^{1}H-^{15}N HSQC spectra for the wild-type and Y59F Ub, it is clear that whereas Ub^{Y59F} maintains the same overall fold as the wild type, the backbone amide for E51 shows a remarkably large chemical shift change in the Y59F spectrum (>1 ppm shift, Fig. 2 *B* and *C*). Amide exchange experiments in 100% $^{2}H_2O$ (*SI Appendix*, Fig. S6 *A–C*) revealed that in the wild-type spectra, the backbone amide of E51 was observable for >30 min. In contrast, the E51 amide was not observable in the Y59F Ub spectra after 5 min, indicating that the E51 amide was no longer protected by the Y59-E51 hydrogen bond in Ub^{Y59F}. Circular dichroism experiments demonstrated that substitution of Ub Y59 to Y59F, Y59A, or Y59W decreased melting temperature from 79.8 °C to 67.4 °C, 58.4 °C, or 57.7 °C, respectively (*SI Appendix*, Fig. S6*D*), which is consistent with the loss of the hydrogen bond between the Y59 hydroxyl to E51 backbone amide. In all, these findings conclude that the Y59-E51 hydrogen bond establishes the Ub Y59-E51 loop critical for the selection of K48 by Cdc34 for linkage specific Ub chain synthesis.

The Y59-E51 Loop and Ub K48 Site Are in Close Proximity to Cdc34 E2. We developed a disulfide cross-linking technique to map Cdc34-Ub transient interactions because it is currently unfeasible to use NMR for measuring direct binding of the Cdc34 catalytic core domain to free Ub because of low affinity with an estimated K_d of >1 mM (9). To this end, $Cdc34^{C93K/E112C}$-Ub was created to mimic donor Ub-E2 (10) (*SI Appendix*, Fig. S7*A*) and to probe for E2 E112, a residue within the acidic loop potently required for catalysis (4, 5). $Cdc34^{C93K/E112C}$–Ub and Ub^{K48C} formed a cross-linked product in a dose- and time-dependent manner (Fig. 3*A*, lanes 1–11), but replacement with $Cdc34^{C93K/T117C}$-Ub showed little product formation (lanes 12–18). Of 17 Ub residues tested, Ub Q49C, K48C, A46C, G47C, E51C, and D52C all displayed significant cross-linking with efficiency around or over 20% (Fig. 3*B*). $Ub^{Y59A/Q49C}$ was found to abolish the cross-linking reaction between Q49C and $Cdc34^{C93K/E112C}$–Ub (*SI Appendix*, Fig. S7*C*), suggesting that the Ub Y59-E51 loop is critical for interactions with Cdc34. However, $Ub^{Y59A/K48C}$ displayed very high levels of self–cross-linking activity, thereby precluding the assessment of the impact of Y59 on Ub K48C cross-linking.

Cdc34 acidic loop residues E108 (Fig. 3*B*) and D103 (*SI Appendix*, Fig. S7*D*) showed cross-linking with multiple Ub residues as well. However, Cdc34 E112C exhibited cross-linking activity with Ub K48C (Fig. 3 *A* and *B*) at levels that are significantly higher than those seen with E108 (Fig. 3*B*) or D103 (*SI Appendix*, Fig. S7*D*). In all, these findings suggest that the Ub K48 site (K48, Q49, A46, and G47), as well as residues within the Y59-E51 loop (E51 and D52), are all in close proximity to the Cdc34 acidic loop. Intriguingly, these cross-linking positive Ub residues appear to structurally arrange as a zone, dubbed as "Cdc34-engaging zone" (Fig. 3*C*). By Y59-E51 hydrogen bonding, the Ub Y59-E51 loop likely helps build and/or stabilize this zone (Fig. 3*C*).

Disruption of the Ub Y59-E51 Loop Inhibits Cellular K48-Polyubiquitination. To assess the role of the Ub Y59-E51 loop in cellular K48-polyubiquitination, we sought to use immunoblot analysis to determine whether expression of a disruptive Ub mutant, such as $Ub^{R54A/Y59A}$, diminishes the production of K48-polyubiquitin chains in cultured cells. For this purpose, we first validated the ability of commercial K48 linkage-specific antibodies for assessing K48-Ub chains made of $Ub^{R54A/Y59A}$ (*SI Appendix*, Fig. S8*A*). Immunoblot analysis revealed that 293T cells expressing HA-Ub produced total K48-Ub chains at levels that were approximately threefold higher than those seen in cells expressing HA-$Ub^{R54A/Y59A}$ (Fig. 4*A*, graph). Immunoprecipitation with anti-HA indicated that the R54A/Y59A mutant decreases chain formation by up to sixfold (*SI Appendix*, Fig. S5*A*, graph). Notably, high levels of Ub conjugates were still observed with the R54A/Y59A mutant (Fig. 4*A*, lane 8), suggesting that the mutant inhibited the production of Ub K48 chains but not those formed by other linkages. Indeed, immunoblot analysis using Ub K63 linkage-specific antibodies revealed that wild-type and R54A/Y59A chains contain the same abundance of K63 chains (*SI Appendix*, Fig. S8*B*). Finally, mass spectrometric analysis of the immune-purified cellular polyubiquitin chains containing HA-Ub or HA-$Ub^{R54A/Y59A}$ revealed an ~2.7-fold

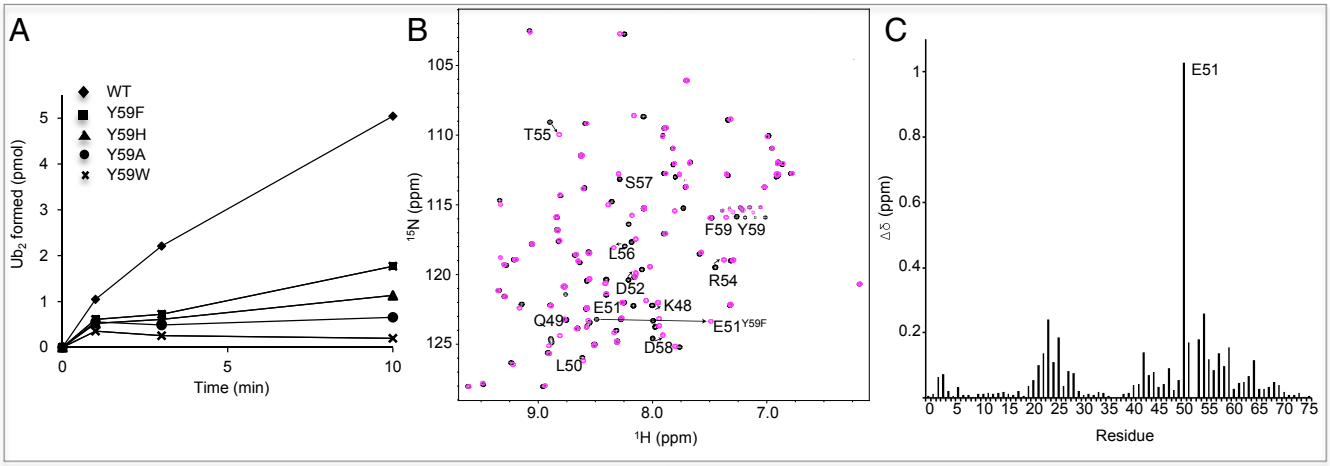

Fig. 2. The Ub Y59-E51 loop. (*A*) The effects of Ub Y59 substitutions on di-Ub synthesis by Cdc34. (*B*) Overlay of ^{1}H-^{15}N HSQC spectra for wild-type Ub (black contours) and Y59F Ub (magenta contours). Amides located in the K48-Y59 loop of Ub are labeled. Arrows indicate where the amide resonances for the wild-type Ub K48-Y59 loop have shifted to in the Y59F spectrum. (*C*) Histogram of amide chemical shift perturbations between wild-type and Y59F Ub. The amide chemical shift changes for each residue in wild-type and Y59F Ub were determined using the spectra shown in *B* and the equation $\Sigma\Delta\delta = |(\Delta\delta^{1}H)| + |(0.2) \times (\Delta\delta^{15}N)|$.

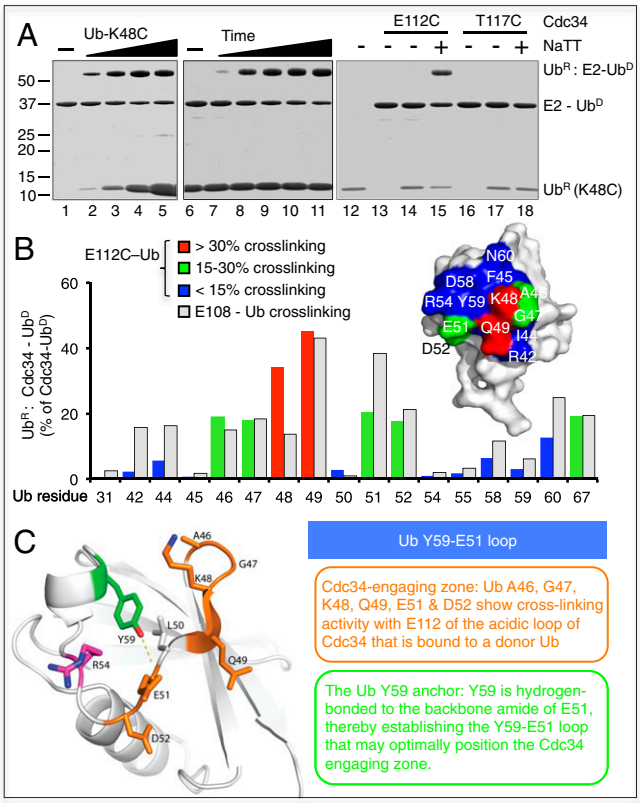

Fig. 3. Mapping Cdc34-Ub interactions by disulfide cross-linking. (*A*) Ub-K48C cross-links to $Cdc34^{C93K/E112C}$-Ub. See *SI Appendix*, Fig. S7*B* for quantification. (*B*) A summary of cross-linking of a panel of Ub variants to $Cdc34^{C93K/E112C}$-Ub or $Cdc34^{C93K/E108C}$-Ub. The positions of Ub residues analyzed for cross-linking with $Cdc34^{C93K/E112C}$-Ub or $Cdc34^{C93K/E108C}$-Ub are shown. Color codes (red, green, and blue) denote the indicated reaction efficiency seen with cross-linking by $Cdc34^{C93K/E112C}$-Ub, shown either in the bar graph or in the Ub structure. Ub^D, donor Ub; Ub^R, receptor Ub. (*C*) Model of the Ub Y59-E51 loop. A dashed yellow line denotes the hydrogen bond between Ub Y59 (green) and the backbone amide of E51, Ub residues positive for cross-linking to $Cdc34^{C93K/E112C}$-Ub are colored gold, and magenta represents Ub R54.

lower K48 chain formation with the R54A/Y59A Ub but a nearly equal level of K63 chain formation (Fig. 4*B* and *SI Appendix*, Fig. S8*C*). Together, these results demonstrate that forced expression of the Ub Y59/R54-disruptive mutant results in a potent and selective loss of Ub K48 chains in vivo, strongly suggesting a requirement for the Ub Y59-E51 loop in cellular synthesis of Ub K48 chains.

Disruption of the Ub Y59-E51 Loop Ignites Apoptosis. To determine the functionality of the Ub Y59-E51 loop in vivo, we developed a conditional Ub-replacement strategy based on modifications of the previously established inducible Ub shRNA system (11) (*SI Appendix*, Fig. S9*A*). Immunoblot analysis revealed that tetracycline induced reexpression of Ub R54A/Y59A or Ub K48R in cells depleted of Ub resulted in a selective loss of K48-linked Ub chains (Fig. 4*C*, red, lanes 5–8), despite yielding total Ub chains at levels comparable to the control (Fig. 4*C*, green, lanes 5–8). Concomitantly, well-characterized proteasomal substrates CDT1 (Fig. 4*C*) and p27 (*SI Appendix*, Fig. S9*B*) accumulated in cells expressing Ub R54A/Y59A or Ub K48R, suggesting that the Ub Y59-E51 loop is critical to optimal maintenance of protein homeostasis.

To evaluate the role of K48-polyubiquitination in cell death, we used Annexin V staining and flow cytometry to detect cells undergoing various forms of cell death, such as apoptosis or necrosis. Kinetic analysis revealed that tetracycline-induced Ub depletion resulted in nearly 100% Annexin V-positive cells within 3 d of the treatment, which was rescued by reexpression of the wild-type Ub (Fig. 4*D*). In contrast, no rescue was observed in cells expressing Ub K48R or Ub R54A/Y59A, each showing Annexin V staining at levels approaching 80% within 4 d of tetracycline treatment (Fig. 4*D*). Treatment of these cells with Z-VAD-FMK (apoptosis inhibitor) markedly attenuated the level of cell death, suggesting apoptosis as a likely mechanism (*SI Appendix*, Fig. S9*C*). In support of these observations, Hoechst 33342 staining also revealed apoptotic nuclear morphology in cells lacking Ub, or expressing $Ub^{R54A/Y59A}$ or Ub^{K48R} (*SI Appendix*, Fig. S9*D*). Finally, cells lacking Ub, or expressing K48R Ub or R54A/Y59A Ub, exhibited a significant sub G_1 population (Fig. 4*E*), indicative of nuclear fragmentation and extensive cell death. Together, these results demonstrate that disruption of the Ub Y59-E51 loop triggers apoptosis.

Discussion

The Ub Y59-E51 Loop: A Structural Determinant for K48-Polyubiquitination. The biogenesis of linkage-specific Ub chains requires the precise alignment of a donor Ub's G76 to a lysine residue of a receptor Ub. In RING E3-dependent ubiquitination reactions, the receptor Ub lysine attacks the donor Ub-E2 thiol ester complex, yielding an isopeptide bond that joins the two Ub molecules. It has become increasingly evident that the donor Ub and receptor Ub each contain determinants that dictate specific interactions with cognate E2s, establishing an optimal orientation of a desired receptor lysine residue to attack the donor Ub~E2 thiol ester complex. As presented in this report, biochemical mapping and characterization of Ub residues (Fig. 1 and *SI Appendix*, Figs. S1–S5 and Table S1), combined with NMR analysis (Fig. 2 and *SI Appendix*, Fig. S6 *A–C*), have uncovered the pivotal role of the Ub Y59-E51 loop in supporting K48-polyubiquitination by Cdc34. The crystal structure of Ub illustrates that Y59 sits at the C terminus of a short α-helix (L56-Y59) that packs against the side chains of K48 and R54 (Fig. 3*C*). In this conformation, Y59 makes a hydrogen bond to the backbone amide of E51 in the center of the loop (8) (Fig. 3*C*). It is possible that substitution of Y59 to alanine (also phenylalanine and tryptophan) causes subtle conformational changes in the Ub Y59-E51 loop because of the loss of the Y59-E51 hydrogen bonding, as demonstrated by differences in amide exchange rates for the backbone amide of E51 using NMR spectroscopy and the measurable decreases in Ub thermal denaturation (Fig. 2 and *SI Appendix*, Fig. S6 *D* and *E*).

The Global Impact of the Ub Y59-E51 Loop in Cellular K48-Polyubiquitination. Immunoblot and mass spectrometric experiments showed that mammalian cellular expression of $Ub^{R54A/Y59A}$ impaired K48-polyubiquitination in vivo (Fig. 4 *A* and *B*). More importantly, replacement of human endogenous Ub by conditional expression of $Ub^{R54A/Y59A}$ yielded cellular phenotypes resembling those observed in cells expressing Ub^{K48R}, including accumulation of CDT1 and p27 substrates and extensive apoptosis (Fig. 4 *C–E* and *SI Appendix*, Fig. S9). Together, these results strongly suggest that the Ub Y59-E51 loop is the predominant determinant governing the production of cellular K48-linked polyubiquitin chains in mammals. Consistent with this, a recent high-throughput screen identified that point mutations to Ub Y59 resulted in an overall reduced fitness of yeast cultures (12). Although the importance of the Ub Y59-E51 loop extends to one other K48-specific E2, Ubc1 (*SI Appendix*, Fig. S5), more extensive E2 survey is required to determine the specificity of this loop.

Role of the Ub Y59-E51 Loop: Anchoring a Cdc34-Engaging Zone to Access Ub K48. The present work provided, to our knowledge, the first evidence locating the Cdc34 acidic loop to Ub in an area

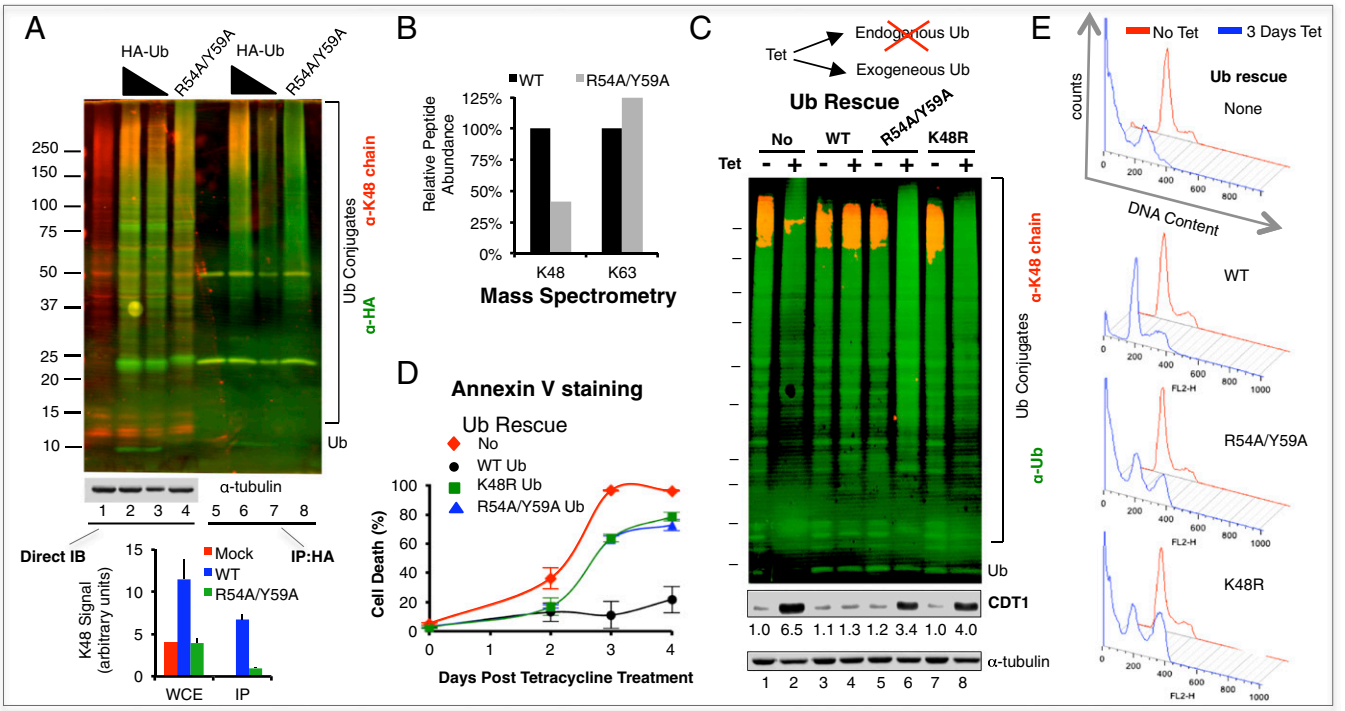

Fig. 4. Disruption of the Ub Y59-E51 loop inhibits cellular K48-polyubiquitination and triggers apoptosis. (*A* and *B*) Analysis of cellular K48-polyubiquitination in cells expressing of HA-Ub or HA-Ub$^{R54A/Y59A}$. (*A*) Immunoblot analysis. Odyssey Infrared Imaging System simultaneously images anti-HA reactive (green) and anti-K48 reactive signals (red). (*B*) Mass spectrometric analysis. See *SI Appendix*, Fig. S8*C* for details. (*C–E*) Ub replacement experiments. (*C*) Immunoblot analysis. CDT1 protein levels are indicated. The same protein size markers are used as in *A*. (*D*) Analysis of cell death by Annexin V staining. Graph represents an average of three independent experiments, with error bars for SD. (*E*) Cell cycle analysis. Quantification of cell cycle profile for each cell line is shown in *SI Appendix*, Fig. S9*E*.

proximal to Ub K48. Three major points were revealed by the cross-linking experiments: (*i*) multiple Ub residues (K48, Q49, A46, G47, E51, and D52) appear to form Cdc34-engaging zone (Fig. 3*C*); (*ii*) at least three Cdc34 acidic loop residues (D103, E108, and E112) could "land" on this "zone" (Fig. 3*B* and *SI Appendix*, Fig. S7*D*); and (*iii*) Cdc34 E112, most potently required for catalysis (4, 5), appears most proximal to Ub K48 (Fig. 3*B* and *SI Appendix*, Fig. S7*D*). A two-phase interaction model could explain such flexibility and specificity displayed by the Cdc34 acidic loop. In the initial phase, multiple Cdc34 acidic loop residues, including D103, E108, and E112, are engaged in contacts with Ub, perhaps in a redundant fashion, to land on the Ub Cdc34-engaging zone (Fig. 3*C*). A second phase then follows, possibly "remodeling" the Ub-E2 acidic loop complex to enable E2 E112 approaching Ub K48. Note that Cdc34 E112 is essential for the transfer of Ub to a substrate or Ub and thus may participate in positioning the donor Ub C-tail (5). In light of the physical association between Cdc34 E112 and the receptor Ub K48 (Fig. 3), it can be further speculated that Cdc34 E112 might help align K48 to the donor Ub G76, a step critical for catalysis.

Blocking Cellular K48-Polyubiquitination and Therapeutic Potential. In this work, we have shown that conditional replacement of human endogenous Ub by Ub$^{R54A/Y59A}$ or Ub^{K48R} resulted in profound cell death (Fig. 4 *D* and *E*). Thus, blocking cellular K48-polyubiquitination may prove to be a new, effective avenue of therapeutics in inducing cell killing. Conceivably, pharmacological perturbation of the receptor Ub Y59-E51 loop would block the production of cellular K48-polyubiquitin chains. An alternative approach is to develop small molecule inhibitors capable of neutralizing major cellular K48-specific E2s, and the recently emerged anti-Cdc34 inhibitor is indicative of such efforts on the horizon (9).

Materials and Methods

SI Appendix, SI Experimental Procedures describes methods for plasmid construction, protein purification, in vitro and in vivo ubiquitination, disulfide cross-linking, NMR, Ub melting temperature, Ub replacement, cell death, and cell cycle. Procedures for screening Ub determinants for K48 Ub chain assembly are detailed in *SI Appendix*, Fig. S1*A*. Validation for this screening is shown in *SI Appendix*, Fig. S1 *B* and *C*.

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