Endocytosis of the IFNAR1 chain of Type 1 interferon receptor is regulated by diverse E2 ubiquitin conjugation enzymes

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Ubiquitination of signaling receptors triggers their endocytosis to restrict the extent of cell signaling. Type 1 interferon (IFN1) eliminates its receptor from cell surface via stimulating the ubiquitination of its IFNAR1 chain. While it was suggested that this ubiquitination aids IFNAR1 internalization via relieving a steric hindrance of a linear motif within IFNAR1 from the endocytic machinery, the mechanisms involved remain poorly understood. Here we describe a specific role for two disparate ubiquitin acceptor sites within this receptor. These sites, Lys501 and Lys525/526, exhibit a preference for polyubiquitination via either Lys63- or Lys48-linked chains (K63-Ub and K48-Ub, respectively). Whereas the SCF$^{\beta Trcp}$ E3 ubiquitin ligase controls either type of ubiquitination-dependent IFNAR1 endocytosis, the specificity of these processes is determined by two different E2 ubiquitin conjugating enzymes, Ubc13 and Cdc34. These enzymes can be directly used by SCF^{Trcp} E3 ubiquitin ligase to generate either K63-Ub or K48-Ub in vitro. Ubc13 is involved in IFNAR1 endocytosis driven by the K63-Ub modification of Lys501, whereas the K48-Ub-specific Cdc34 affects receptor endocytosis via ubiquitin conjugation that occurs on Lys525/526. Both types of linkages combine to maximize IFNAR1 endocytosis otherwise suppressed by unfavorable conformation dependent on the presence of a conserved Pro470 within the intracellular domain of IFNAR1. We propose a model where alternate utilization of both E2s to assemble diverse polyubiquitin linkages cooperates to achieve IFNAR1 intracellular domain conformations and spatial arrangements that favor a maximal rate of receptor endocytosis.

Key words: *IFNAR, interferon, receptor, ubiquitin, endocytosis, E2*

Endocytosis of cell surface signaling receptors is a key mechanism that controls the availability of these receptors to interact with the extracellular cognate ligands and, accordingly, restricts the extent and duration of cellular responses to these ligands. Among post-translational modifications that contribute to this regulation is protein ubiquitination. This process of covalent attachment of ubiquitin polypeptides involves the cascade of enzymatic reactions mediated by ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3, reviewed in [1]). Ubiquitination of receptors has emerged as a central mechanism that determines the efficacy of receptor endocytosis and downregulation [2–4].

The type of ubiquitination (mono-ubiquitination versus poly-ubiquitination and, in the latter case, the topology of the polyubiquitin chain) and its requirement for internalization and/or post-internalization sorting varies greatly among different receptors. Numerous studies have highlighted the role of polyubiquitination via the Lys63-linked chains in controlling endocytosis of many cell surface proteins, including uracil permease [5], nerve growth factor receptor TrkA [6], MHC class II proteins [7], prolactin receptor [8], chimeric model endocytic proteins [9], the IFNAR1 chain of the Type 1 interferon (IFN1) receptor [10] and others.

Intriguingly, the rate of endocytosis of IFNAR1, a receptor that plays a key role in IFN1 signaling [11–13], was compromised by overexpression of either K63R or K48R ubiquitin mutants suggesting that polyubiquitination via both Lys63 and Lys48 linkage types (K63-Ub and K48-Ub, respectively) might be required [10]. IFNAR1 is a substrate for the SCF$^{\beta Trcp}$ E3 ubiquitin ligase, which is recruited to the receptor upon its phosphorylation within a specific phospho-degron [14, 15], and is capable of building both K63-Ub and K48-Ub on IFNAR1 *in vitro* [10]. Although ubiquitination can occur on many Lys residues within the intracellular domain of IFNAR1, a cluster of three lysines (Lys501, Lys525 and Lys526) receives a large share of ubiquitin conjugates [14] and the integrity of this cluster is required for efficient IFNAR1 endocytosis [10]. Ubiquitination of this cluster unmasks an adjacent Tyr466-based linear endocytic motif (otherwise blocked by IFNAR1-associated TYK2 Janus kinase), enabling its interaction with the AP2 adaptin complex and subsequent internalization via the clathrin-dependent pathway [10, 16]. While these studies suggest a model where ubiquitination of IFNAR1 leads to either TYK2 dissociation or conformational changes within the intracellular domain of IFNAR1 (reviewed in [17, 18]), many critical questions remain unanswered.

Mass spectrometry analysis revealed the presence of K63-Ub and K48-Ub on wild type IFNAR1 [10], however it remains unclear whether both types of ubiquitin chains (or mixed chains) need to be assembled on IFNAR1 or on a putative endocytic regulator. Furthermore, the determinants of topology specificity (including specific ubiquitin acceptors within the Lys501, 525, 526 cluster and specific E2 conjugation enzymes utilized by SCF$^{\beta Trcp}$ E3 ligase) are yet to be determined. Finally, the mechanism by which either type of ubiquitination contributes to the regulation of IFNAR1 endocytosis remains largely unknown.

Here we focused on specific role of diverse E2 conjugating enzymes in mediating either K63-Ub or K48-Ub on different lysine acceptors within IFNAR1 in internalization of this receptor. Our data indicate that a combined effect of both types of linkages maximizes the rate of IFNAR1 internalization and suggest that IFNAR1 ubiquitination may be conducive for the intracellular domain of IFNAR1 to adopt a spatial conformation favorable for IFNAR1 endocytosis.

Experimental procedures

Plasmids and cells

The constructs for expression of HA-tagged wild type, K48R, and K63R ubiquitin [19] were kindly provided by Yossef Yarden (Weitzmann Institute). Construct for expression of IFN-stimulated response element (ISRE) – driven luciferase [20] was a generous gift from Curt Horvath (Northwestern University). Human IFNAR2 expression vector was kindly provided by John Krolewski (UC Irvine). Construct for expression of dominant negative Ubc13C87A mutant was provided by Zhijian J Chen (UT Southwestern). Vectors for the expression of N-terminal FLAG-tagged human IFNAR1 (wild type and K501, 525,526R mutants) were described previously [10]. These plasmids served as a backbone for generating additional N-FLAG-IFNAR1 constructs for expression of K501R and K525,526 mutants using the site-directed mutagenesis; the results were verified by DNA sequencing. The shRNA constructs for knocking down bTrcp were described previously [8, 21]. The siRNA oligonucleotides for knocking down Cdc34 and Ubc13 were obtained from Sigma.

Human embryo kidney 293T cells, mouse embryo fibroblasts, and HeLa cells (ATCC) were grown in Dulbecco's modified Eagles's medium containing 10 % heat inactivated fetal bovine serum and Penicillin/Streptomycin. The stable U2OS-derivative cells lines for knock-in of ubiquitin or Ubc13 mutants were kindly provided by Zhijian J Chen. These cells were maintained and used as described previously [22]. Briefly, addition of tetracycline (1 μg/mL for 72 h) to these cells silences expression of either endogenous ubiquitin or endogenous Ubc13 concurrent with induction of expression of exogenous RNAi-resistant ubiquitin (wild type or K63R mutant) or Ubc13 (wild type or C87A catalytically inactive mutant). Cells were transfected with Lipofectamine Plus, Lipofectamine 2000, or Oligofectamin (Invitrogen) depending on the cell type and the experiment. For all transfections, empty vectors were included in the mixtures to maintain an equal amount of transfected DNA.

Chemicals, antibodies, reporter assays, and immunotechniques

Murine IFNβ was expressed in Chinese Hamster Ovary cells, and purified to homogeneity from conditioned culture medium using successive Blue Sepharose, copper chelating Sepharose, lentil lectin Sepharose, and Uno S (Biorad) cationic ion exchange chromatographies. The specific activity of the protein was 2×10^8 units/mg in an *in vitro* antiviral cytopathic effect assay using murine L929 cells challenged with encephalomyocarditis virus. The purified protein had an endotoxin level of < 0.05 EU/mg. Human IFNα2 (Roferon) was purchased from Roche. Cycloheximide and other chemicals were purchased from Sigma. Antibodies against FLAG (M2; Sigma), ubiquitin (FLys2; BIOMOL), HA (12CA5, Roche Boehringer Mannheim) were purchased. Secondary antibodies were obtained from Invitrogen and Roche Boehringer Mannheim. Immunoprecipitation and immunoblotting procedures were described previously [23–25].

For reporter assays, mouse embryo fibroblasts were co-transfected with plasmids encoding human IFNAR2, ISRE-firefly luciferase (with a vector for constitutive expression of renilla luciferase), and various human IFNAR1 constructs. 24 h post-transfection, cells were treated with human IFNa or mouse IFNβ (at 2000 U/mL) for 30 min. Then the media was removed and cells were incubated for 24 h without IFN, harvested, and luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega). Data (normalized to renilla luciferase activity) from three independent experiments (each in five replicates) are presented in arbitrary units as Mean ± S.E. P values were calculated using the two-tailed Students' t-test.

IFNAR1 ubiquitination assays

In vitro ubiquitin ligation reactions were carried out as described previously [24] using $SCF^{\beta Trcp}$ purified from 293T cells as outlined elsewhere [26] and recombinant E2s including recombinant Cdc34 ([27], a gift from Zhen-Qiang Pan, Mount Sinai School of Medicine) or 6His-Ubc13/Uve1 ([28, 29], a gift from Ze'ev Ronai, The Burnham Institute). Briefly, 10 μl of packed beads carrying immobilized $SCF^{\beta Trcp\text{-}HA}$ was incubated with E1 (20 pmol), mCdc34 or 6His-Ubc13/Uve1 (1 nmol) in the reaction mixture (total volume 30 μl) that contained 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 0.6 mM DTT, and 1 μg ubiquitin. The mixture was incubated at 37 °C for 60 min, boiled in 20 μl of Laemmli loading buffer, separated on SDS-PAGE and analyzed by immunoblotting.

For assessing *in vivo* ubiquitination of IFNAR1, the FLAG-tagged IFNAR1 species were expressed in 293T cells. Cells were harvested and lysed in 1 % NP40, 50 mM Tris pH 7.6, 150 mM NaCl, 1 mM $NaVO_4$, 1mM PMSF, 2.5 mM EDTA, Protease Inhibitor Cocktail (1:500; Sigma), 1 mM NaF, and 20 mM N-ethyl maleimide. The protein concentration was determined using the Bradford dye-binding assay. Equal amounts of protein lysates were incubated with M2-conjugated beads to immunoprecipitate the FLAG-tagged IFNAR1. The samples were washed three times with 50 mM Tris pH 7.4, 5 % glycerol, 1M NaCl and one time with 50 mM Tris pH 7.4, 5 % glycerol, 150 mM NaCl and after boiling with SDS-containing Laemmli buffer, an aliquot from each sample was analyzed by M2 FLAG immunoblotting to normalize the receptor levels. Based on normalization, amounts of lysates that would yield comparable levels of IFNAR1 were separated

by SDS-PAGE and analyzed by immunoblotting using either anti-ubiquitin antibody (Biomol) or the chain-specific anti-polyubiquitin, K63-Ub or K48-Ub antibodies (Millipore).

IFNAR1 internalization assays

Internalization of endogenous or exogenously expressed IFNAR1 was determined using a high throughput fluorescence-based method described in detail elsewhere [10, 16]. Briefly, cells in 60-mm dishes were transfected with the indicated constructs or induced with tetracycline and plated equally on 24 well poly-D-lysine plates (Becton, Dickinson and Company). Cells were starved in serum-free Dulbecco's Modified Eagle's medium and were either treated with IFNa2a for the indicated time periods, or were kept on ice and not exposed to IFNa2a (time point 0). At given time points, the cells were washed, blocked, and incubated with the anti-IFNAR1 AA3 antibody [30] or anti-FLAG M2 antibody. The primary antibodies were then removed and the cells were washed extensively before adding a goat anti mouse IgG H + L horse radish peroxidase-conjugated antibody. Following another series of washes, the cells were incubated with AmplexRed Ultra Reagent 10-acetyl-3,7-dihydroxyphenoxazine (Molecular Probes). Aliquots were transferred to black 96-well plate (NUNC) and fluorescence was measured by reading with Beckman Coulter DTX880 Mutimode Detector plate reader using a 530 nm filter for excitation and a 590 nm filter for emission. Results were calculated using the following formula:

$$\% \text{ Internalized} = 100 - [(Vs - Vb) \times t_n/(Vs - Vb) \times t_0] \times 100,$$

where: Vs = Value of samples, Vb = Value of background (mock-transfected or probed with irrelevant antibody), t_n = time point n, t_0 = time point 0.

Results

Given that that $SCF^{\beta Trcp}$ E3 ligase was shown to actively utilize Cdc34 E2 ubiquitin conjugating enzyme for K48-Ub of proteasomal substrates such as IκBα [27], we proposed a role for this E2 in IFNAR1 ubiquitination and endocytosis. Conversely, whereas K63-linkage-specific ubiquitination of proteins in vitro can be achieved using a number of E2 enzymes, the ubiquitin-conjugating enzyme 13 (Ubc13, in complex with either Uev1A or Mms[2]) was shown to play a key role in assembling these linkages in vivo and in K63-dependent processes such as DNA recombination [31] and damage repair [32], and activation of signal transduction pathways [33–35].

We used the RNAi approach to determine the role of these E2s in IFNAR1 ubiquitination and internalization. Knockdown of Cdc34 inhibited K48-Ub of IFNAR1 assessed by immunoblot using chain-specific antibody (Figure 1A). Conversely, K63-Ub of IFNAR1 was impaired in cells that received RNAi against Ubc13 (Figure 1A). Given that SCF^{Trcp} was capable of utilizing either of these enzymes for chain-specific ubiquitin ligation *in vitro* (Figure 1B) it is likely that both Cdc34 and Ubc13 may contribute to the regulation of IFNAR1 ubiquitination (via K48-Ub and K63-Ub, respectively) in cells. Accordingly, knockdown of either Cdc34 or Ubc13 noticeably slowed the kinetics of internalization of endogenous IFNAR1 in 293T cells (Figure 1C). In addition, expression of the dominant negative Ubc13 mutant ($Ubc13^{C87A}$, [22]) inhibited the internalization rate of the N-terminally FLAG-tagged wild type IFNAR1 (Figure 1D). Collectively, these data suggest that function of both Cdc34 and Ubc13 is required for maximal rate of IFNAR1 endocytosis.

We then sought to characterize the cluster of ubiquitin acceptor sites within IFNAR1. While the triple K501R/K525R/K526R ($IFNAR1^{3KR}$) mutant was shown to be deficient in overall ubiquitination [14], internalization and trafficking to the lysosomes [10], and degradation [14], the role of specific acceptors remained unclear. Our initial focus was on the putative "canonical" site represented by the Lys525/526 doublet located 9–10 amino acid residues proximal to the βTrcp binding site ($_{534}$DSGNYS). This site is reminiscent of the Lys21/22 ubiquitin acceptor site within IκBα (Figure 2A) and fits the description of preferred ubiquitin acceptors for the $SCF^{\beta Trcp}$ E3 ligase (reviewed in [36]). Consistent with previously reported data [10], the internalization of the $IFNAR1^{3KR}$ mutant was markedly inhibited. Intriguingly, mutation of either the canonical (Lys525/526) or the additional (Lys501) site resulted in an intermediate phenotype; these mutants internalized slower than wild type IFNAR1 but faster that the $IFNAR1^{3KR}$ mutant (Figure 2B). This result indicates that both Lys525/526 and Lys501 sites within the ubiquitin acceptor cluster contribute to the maximal rate of IFNAR1 endocytosis.

Based on these results we next aimed to determine whether ubiquitination of IFNAR1 on either canonical or additional site plays a role in regulating IFNAR1 stability and signaling. Proteolytic turnover of FLAG-IFNAR1 was assessed by a cycloheximide chase assay. $IFNAR1^{3KR}$ mutant lacking both canonical and additional sites displayed a longer half-life compared to the wild type protein (Figure 2C). This result is consistent with previously reported data [14]. An intermediate turnover rates were observed for both K501R and K525/526R mutants (Figure 2C) indicating that ubiquitination of both canonical and additional sites contributes to efficient degradation of IFNAR1.

To test whether these mutants were functional, we co-expressed them together with human IFNAR2 in mouse embryo fibroblasts and assessed activation of the ISRE-driven luciferase. Expression of human IFNAR1 proteins did not affect induction of luciferase by murine IFNβ that functions through the endogenous murine type 1 IFN receptor (Figure 2D, blue bars) indicating that IFN signaling downstream of receptors is not greatly perturbed by the expression of these human proteins. Whereas expression of wild type human IFNAR1 enabled transactivation

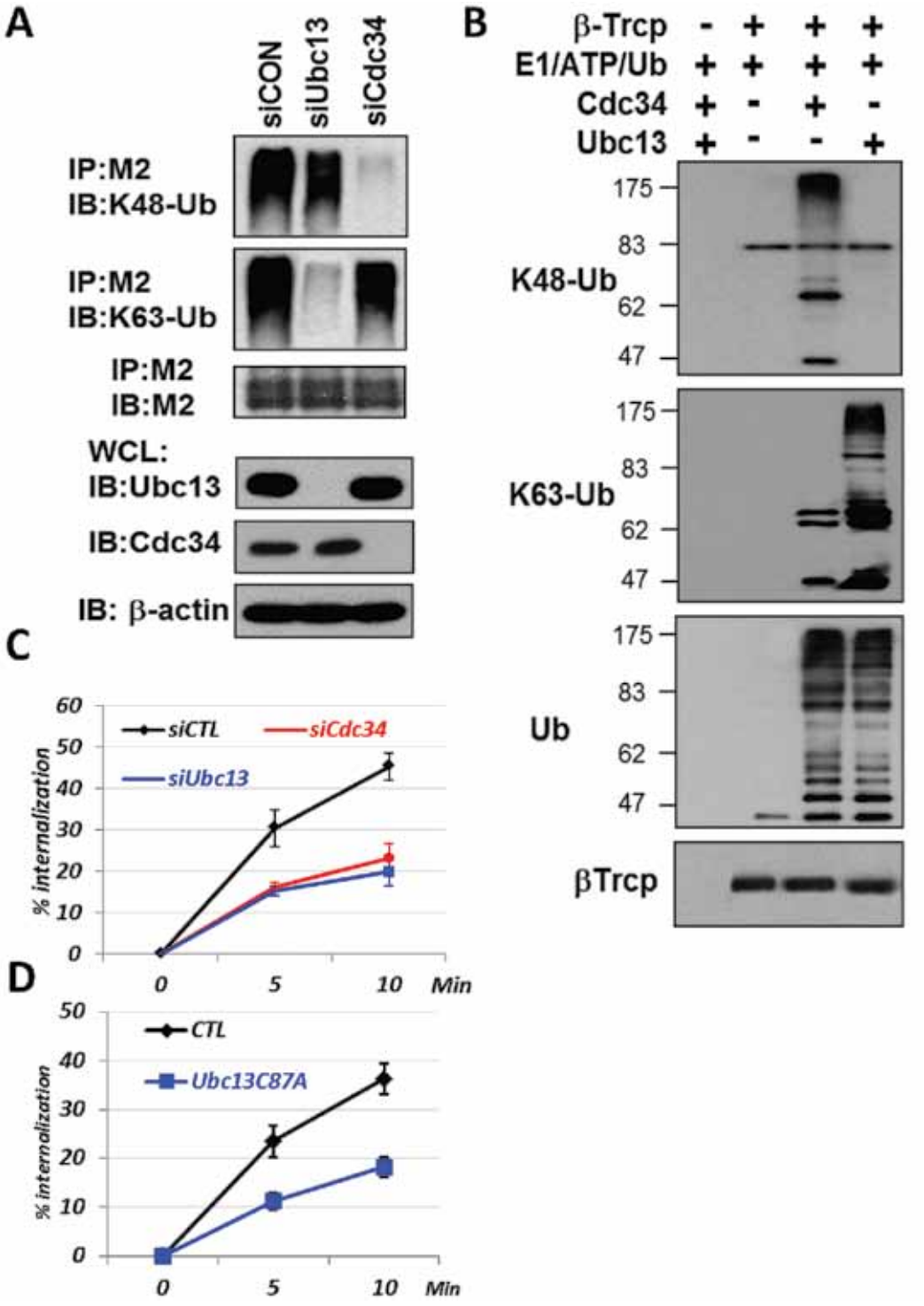


Figure 1. *Role of Cdc34 and Ubc13 in IFNAR1 ubiquitination and endocytosis.*
(A) Chain-specific polyubiquitination of FLAG-IFNAR1 proteins (purified from IFNα-stimulated 293T by immunoprecipitation) was analyzed by immunoblot using the anti-K63-ubiquitin and anti-K48-ubiquitin antibodies as well as antibody against FLAG (M2). Protein levels of endogenous Ubc13 or Cdc34 in the whole cell lysates (WCL) from these cells were measured by immunoblotting using indicated antibodies. An immunoblot for β-actin is included as a loading control.
(B) In vitro ubiquitin ligation reaction catalyzed by the $SCF^{\beta Trcp}$ E3 ubiquitin ligase (omitted as a negative control in the left lane) in the presence of either Cdc34 or complex of Ubc13/Uev1 was analyzed by immunoblot using the anti-ubiquitin Lys63 and anti-ubiquitin Lys48 antibodies as well as non-link-specific anti-ubiquitin antibody (Ub). Levels of βTrcp were also analyzed.
(C) The effect of Cdc34 or Ubc13 knock down on the internalization of endogenous IFNAR1 in 293T cells treated with IFNα (6000 U/mL) for indicated time points was assessed using anti-IFNAR1 AA3 antibody and shown as a percentage of cell surface immunoreactivity loss calculated from three independent experiments (each in five replicates) as average ± S.E. M.
(D) The internalization kinetics of FLAG-IFNAR1 in 293T cells that received control vector (CTL, pCDNA3) or vector for expression of $Ubc13^{C87A}$ mutant treated with IFNα (6000 U/mL) for indicated time points was assessed using anti-FLAG antibody and shown as a percentage of cell surface immunoreactivity loss calculated from three independent experiments (each in five replicates) as average ± S.E.M

of luciferase by a short pulse treatment with human IFNα, the expression of IFNAR1^{3KR} mutant robustly increased sensitivity of mouse cells to human Type 1 IFN. IFNAR1 mutants lacking either the canonical or the additional site modestly yet significantly increased the effects of human IFNα (Figure 2D, black bars). These results suggest that ubiquitination on either canonical or additional sites contributes to attenuating the cellular responses to Type 1 IFN likely through the regulation of IFNAR1 endocytosis and degradation.

Consistent with the previously reported role of βTrcp2 in ubiquitination of endogenous IFNAR1 [10, 15], knockdown of this protein noticeably decreased the level of ubiquitination of FLAG-IFNAR1^{WT} (Figure 3A). Furthermore, endocytosis of wild type IFNAR1 was also impaired in cells that received RNAi against βTrcp^2 (Figures 3B). Importantly, ubiquitination and internalization of IFNAR1 mutants lacking either K525/526 or K501 were also suppressed upon knockdown of βTrcp2 (Figures 3A – B). This result suggests that SCF^{βTrcp} is responsible for ubiquitination of both canonical and additional sites within IFNAR1 and that efficient endocytosis of IFNAR1 relies upon concerted ubiquitination of these sites governed by the same E3 ubiquitin ligase.

Given that mutation of either canonical or additional sites does not dramatically alter overall ubiquitination of IFNAR1 (Figure 3A) yet noticeably affects its internalization (Figures 2B and 3B), degradation (Figure 2C) and signaling (Figure 2D), a qualitative difference between ubiquitination of these sites cannot be ruled out. Indeed, while quantitative mass spectrometry analysis of polyubiquitin linkages revealed that wild type IFNAR1 is decorated with chains of both K63-Ub and K48-Ub types [10], a greater amount of K63-Ub linkages was found on the K525, 526R mutant (65 ± 18 fmole in FLAG-IFNAR1$^{K525, 526R}$ versus 3 ± 0 fmole in FLAG-IFNAR1^{K501R}; C.C. and S.Y.F. unpublished observations). The recent development of chain topology-specific antibodies has enabled us to seek corroboration of these findings. Probing of immunoblots containing immunopurified IFNAR1 proteins with polyubiquitin chain-specific antibodies revealed that both chain types can be present on wild type IFNAR1 (Figures 1A and 3C). Intriguingly, mutation of the canonical site (Lys525, 526) decreased the levels of K48-Ub whereas the IFNAR1 mutant lacking the additional site (Lys501) displayed a deficient K63 linkage-specific signal (Figure 3C). Even considering that some ubiquitination may occur on the four other Lys residues within the

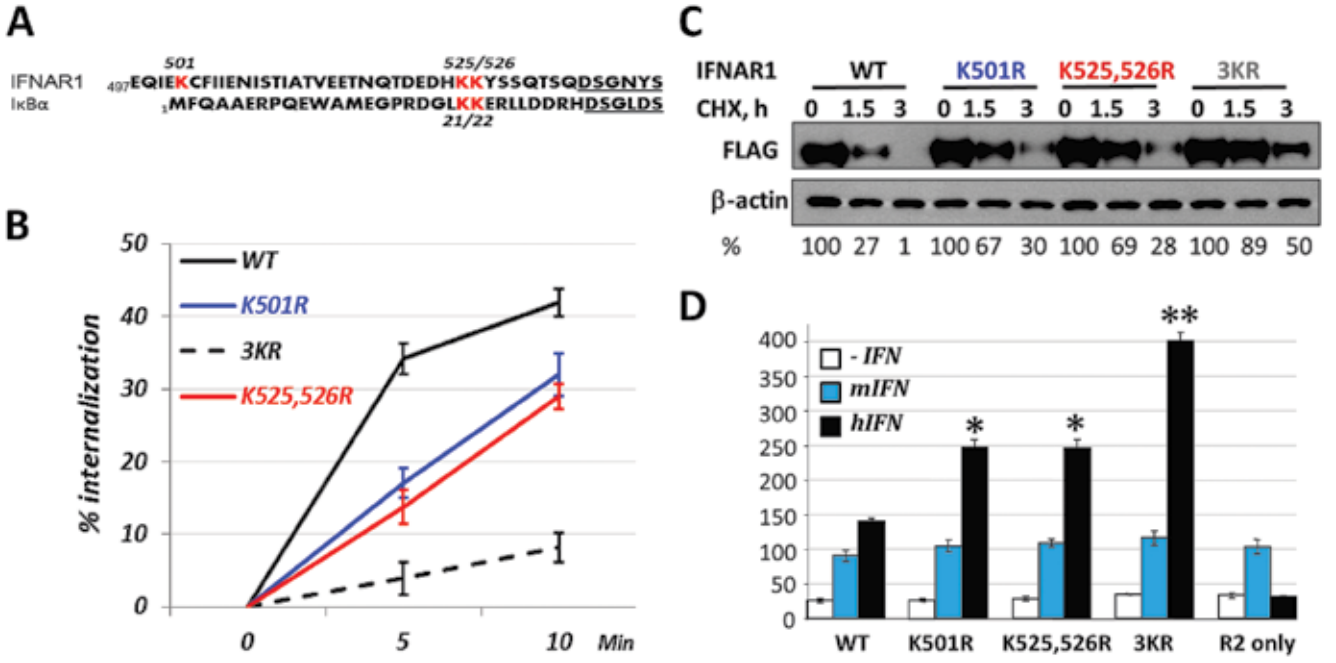
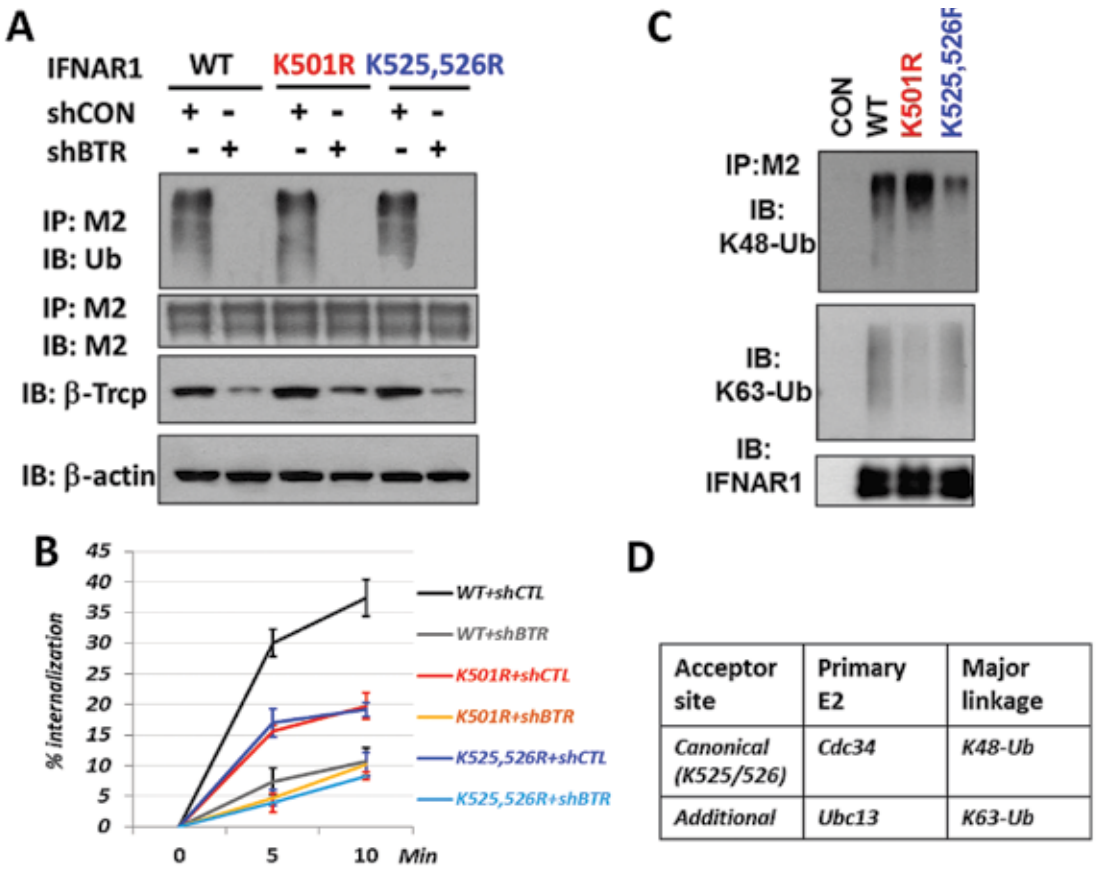


Figure 2. *Role of specific ubiquitin acceptor sites in the internalization and degradation of IFNAR1 and IFNAR1-mediated IFN signaling.*
(A) Similarities in the primary determinants of βTrcp recruitment site and putative ubiquitin acceptor sites between human IFNAR1 and IκBα.
(B) Internalization rate of indicated FLAG-tagged IFNAR1 proteins expressed in 293T cells treated with IFNα (6000 U/mL) for indicated time points was assessed using anti-FLAG antibody and shown as a percentage of cell surface immunoreactivity loss calculated from three independent experiments (each in five replicates) as average ± S.E.M.
(C) Turnover rate of indicated FLAG-IFNAR1 proteins was determined in 293T cells treated with IFNα (6000 IU/mL) and cycloheximide (CHX, 20 μg/mL) for the indicated times. The percentage of IFNAR1 remaining signal (normalized per β-actin signal) relative to time point "0" (assigned a value of 100 %) are depicted below.
(D) Relative activity of ISRE-luciferase reporter expressed in mouse embryo fibroblasts that received human IFNAR2 (R2) and indicated human IFNAR1 expression constructs and that were left untreated (white bars), treated with murine IFNβ (blue bars), or human IFNα (black bars). Normalized (per renilla luciferase activity) values from three independent experiments in arbitrary units are presented. Calculated p value (< 0.002) was obtained in comparison with WT receptor (single asterisk) or with WT, K501R or K525, 526 mutants (double asterisk)



Acceptor site	Primary E2	Major linkage
Canonical (K525/526)	*Cdc34*	*K48-Ub*
Additional	*Ubc13*	*K63-Ub*

Figure 3. *βTrcp-dependent assembly of K63-Ub chains linked to Lys501 and K48-Ub chains conjugated to Lys525, 526 within IFNAR1.*
(A) The ubiquitination of indicated FLAG-tagged IFNAR1 proteins in 293T cells that received either a shRNA control (shCON) or shRNA against βTrcp (shBTR) and were treated or not with IFNα (6000 IU/mL, 5 min) was analyzed by denaturing immunoprecipitation using the M2 antibody followed by immunoblotting with the anti-ubiquitin antibody. Inputs for each immunoprecipated sample were normalized to achieve comparable levels of IFNAR1. Protein levels of endogenous βTrcp and β-actin (included as a loading control) in these cells were also analyzed.
(B) Comparison of internalization rates in cells that received control shRNA against GFP (shCTL) or βTrcp (shBTR) for indicated FLAG-IFNAR1 proteins was performed as in Figure 2B.
(C) K48- and K63-specific polyubiquitination of indicated FLAG-tagged IFNAR1 proteins was analyzed as in panel A. The amount of FLAG tagged IFNAR1 is included as a loading control.
(D) Summary of E2s and types of ubiquitin linkages involved in modification of the canonical and additional acceptor sites within IFNAR1

cytoplasmic domain of IFNAR1 that are outside of the investigated cluster, these data (together with Figure 1A) are suggestive of a scenario where two different ubiquitin acceptor sites may exhibit a preference for diverse types of poly-ubiquitination in a manner dependent on a specific E2 conjugating enzyme (Figure 3D).

We next investigated the importance of these different ubiquitin linkages conjugated to either canonical or additional sites in the regulation of IFNAR1 endocytosis. To this end, we used forced expression of ubiquitin mutants (K63R or K48R) that would prevent elongation of a specific chain in cells. Expression of these specific mutants attenuated the rate of internalization of wild type IFNAR1 receptor by approximately 50 % (reference [10] and data not shown) and noticeably decreased the respective linkage-specific ubiquitination of IFNAR1, protein (Figure 4A). Importantly, the expression of the K48R ubiquitin mutant (but not of K63R) further decreased the efficacy of internalization of IFNAR1^{K501R} (Figure 4B). Conversely, endocytosis of IFNAR1^{K525,526R} was affected by forced expression of K63R but not of the K48R ubiquitin mutant in 293T cells (Figure 4C).

The latter contribution of K63-linked chains in internalization of IFNAR1 is consistent with the reported role of this modification in the endocytosis of a number of other receptors (for example, TrkA [6]). Most of these studies, including our own work, in mammalian cells relied on forced expression of ubiquitin mutants that terminate elongation of chains of specific linkages. Recent development of isogenic U2OS derivatives in which tetracycline treatment confers knockdown of endogenous ubiquitin along with parallel expression of RNAi-insensitive recombinant ubiquitin (wild type or K63R mutant [22, 37]) enabled us to further determine the role of K63 linkage-specific modifications in IFNAR1 internalization. Consistent with results obtained via forced ubiquitin expression, endocytosis of wild type IFNAR1 was compromised in cells expressing ubiquitin^{K63R} (Figure 4D). Remarkably, these cells displayed a decrease in the internalization rate of the IFNAR1^{K525,526R} mutant but not

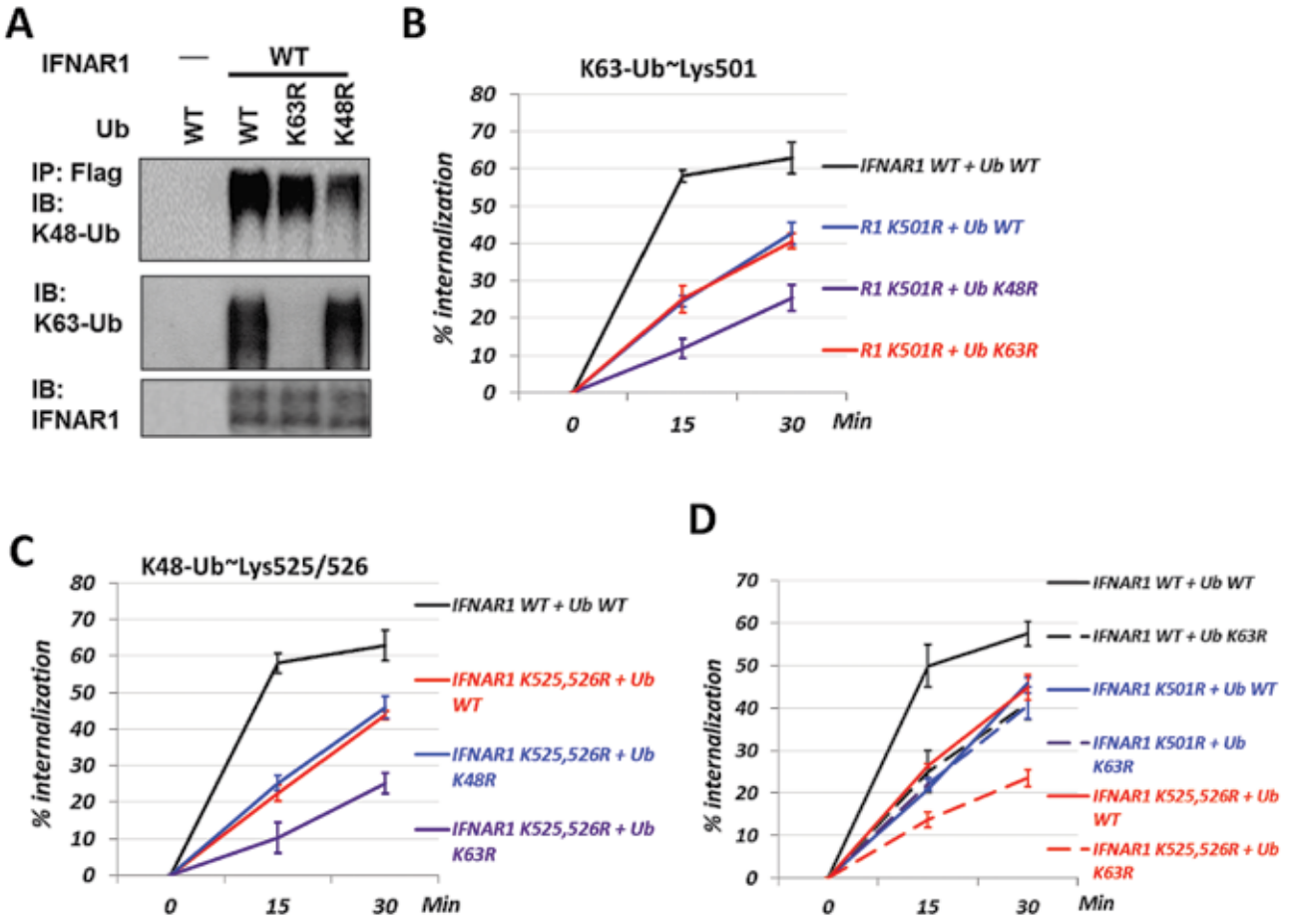


Figure 4. *Disparate effect of ubiquitin mutants on the internalization of IFNAR1 proteins that contain either canonical or additional ubiquitin acceptor sites.*
(A) Chain-specific polyubiquitination of wild type FLAG-IFNAR1 proteins co-expressed with indicated ubiquitin constructs and purified from IFNa-stimulated was analyzed as in panel 3A.
(B) The internalization kinetics of FLAG-IFNAR1^{WT} co-expressed with wild type ubiquitin (WT, black line) was compared with that of FLAG-IFNAR1^{K501R} co-expressed with wild type ubiquitin (blue line), K63R ubiquitin (red line), or K48R ubiquitin (magenta line) in 293T cells. Assays were carried out as in Figure 2B.
(C) The internalization kinetics of FLAG-IFNAR1^{WT} co-expressed with wild type ubiquitin (WT, black line) was compared with that of FLAG-IFNAR1$^{K525, 526R}$ co-expressed with wild type ubiquitin (red line), K63R ubiquitin (magenta line), or K48R ubiquitin (blue line) in 293T cells. Assays were carried out as in Figure 2B.
(D) The internalization kinetics of indicated FLAG-IFNAR1 proteins in U2OS cells induced to knock down endogenous ubiquitin and to re-express either wild type ubiquitin (solid lines) or K63R ubiquitin (dashed lines) were carried out as in Figure 2B

of its K501R counterpart (Figure 4D). These results support the presence of the K63-linked chain on the Lys-501 acceptor site. Taken together with protein ubiquitination data, these results indicate that ubiquitination of the canonical K525/526 site via K48-linked chains together with ubiquitination of K501 via the K63-linked chains enables maximal efficacy of IFNAR1 endocytosis.

To further test the hypothesis that diverse E2s may cooperate in contributing to IFNAR1 regulation via diverse site-specific ubiquitination we tested the effects of modulating E2s activities on IFNAR1 internalization. Knockdown of Cdc34 slowed the kinetics of internalization of endogenous IFNAR1 (Figure 1C) and of wild type FLAG-IFNAR1 expressed in 293T cells (Figure 5A). Intriguingly, whereas cells that received RNAi against Cdc34 also retarded internalization of FLAG-IFNAR1^{K501R} mutant, endocytosis of FLAG-IFNAR1$^{K525, 526R}$ protein was not affected (Figure 5A). These data suggest that the role of K48-Ub in promoting efficient endocytosis of IFNAR1 is facilitated by Cdc34-mediated ubiquitin conjugation to the canonical acceptor site (Lys525/526).

Given that Lys501 of IFNAR1 is ubiquitinated with the K63-Ub that requires Ubc13 (Figures 1A, 3C), we sought to determine whether this E2 was also a regulator of IFNAR1 endocytosis. Knockdown of Ubc13 noticeably inhibited the rate of internalization of wild type IFNAR1 or the IFNAR1$^{K525, 526R}$ mutant but not of the K63-Ub-deficient IFNAR1^{K501R} mutant expressed in 293T cells (Figure 5B). In addition, forced expression of the dominant negative catalytically inactive mutant of Ubc13 (C87A) also decreased the efficacy of internalization of the canonical site-specific mutant and IFNAR1^{WT} but did not alter the endocytosis of IFNAR1^{K501R} (Figure 5C). These results suggest that Ubc13 contributes to the K63-Ub of the additional acceptor site (Lys501) and is important for efficient endocytosis of IFNAR1.

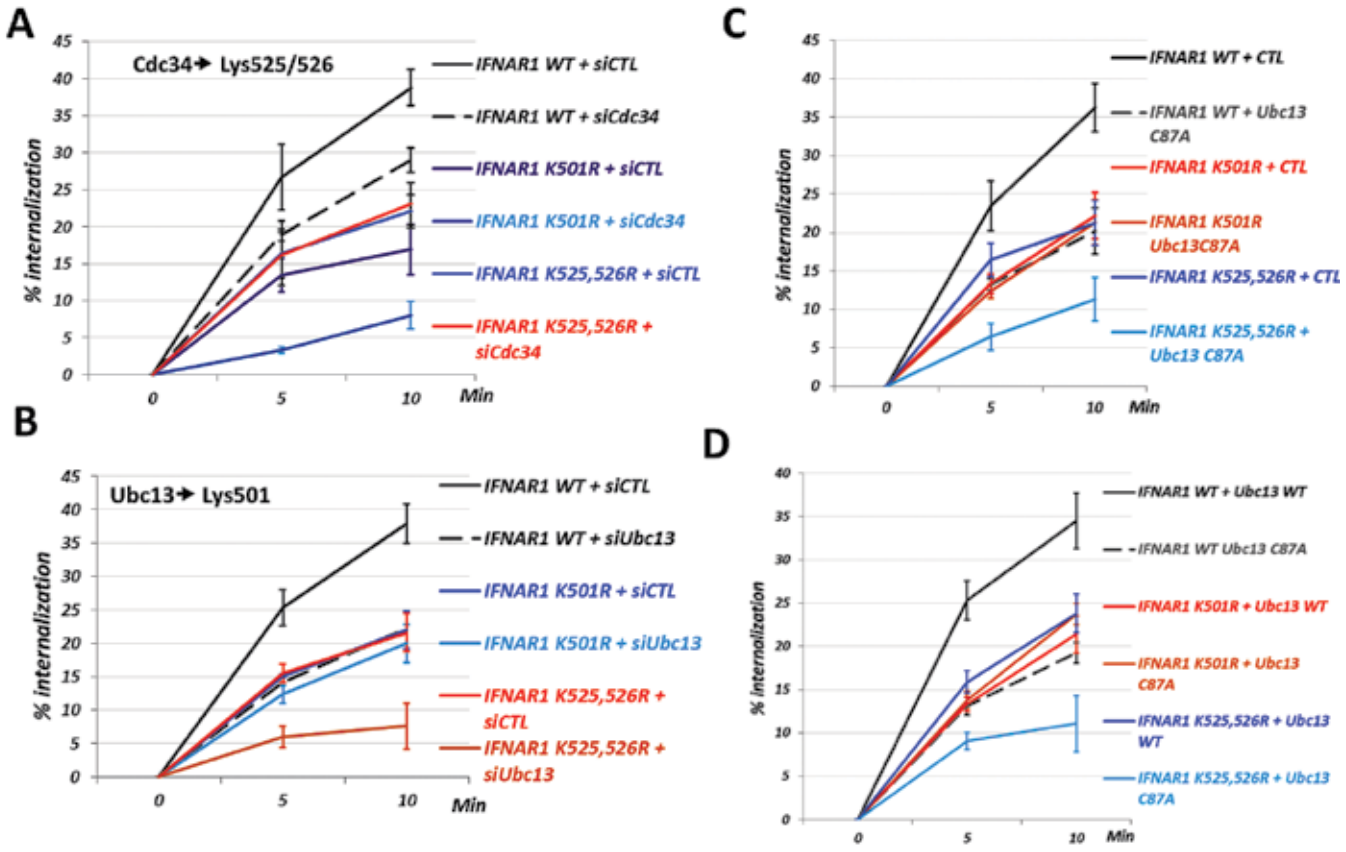


Figure 5. *A combined effect of Cdc34-mediated K48-Ub (assembled on Lys525, 526) and of Ubc13-mediated K63-Ub (assembled on Lys501) is required to achieve maximally efficient internalization of IFNAR1.*
(A) The internalization kinetics of indicated FLAG-IFNAR1 proteins expressed in 293T cells that received control RNAi (siCTL) or RNAi against Cdc34 was analyzed as in Figure 2B.
(B) The internalization kinetics of indicated FLAG-IFNAR1 proteins expressed in 293T cells that received control RNAi (siCTL) or RNAi against Ubc13 was analyzed as in Figure 2B.
(C) The effect of the catalytically inactive mutant (C87A) of Ubc13 on the internalization of indicated FLAG-IFNAR1 proteins in 293T cells. Transfection of cells with pCDNA3 empty vector was used as a control (CTL). Analyses were carried out as in Figure 2B.
(D) The internalization kinetics of indicated FLAG-IFNAR1 proteins expressed in U2OS cells induced to knock down endogenous Ubc13 and re-express either wild type Ubc13 or the Ubc13^{C87A} mutant. Analyses were carried out as in Figure 2B

We further sought to corroborate these conclusions using the U2OS-based cell lines that enable the inducible replacement of endogenous Ubc13 with recombinant (wild type or C87A mutant) proteins [22]. Internalization of FLAG-IFNAR1^{WT} was clearly impaired in cells that re-express catalytically active mutant of Ubc13 (Figure 5D). A similar result was observed on the K48-Ub-dependent IFNAR1 mutant lacking the additional site (Lys501) but not the K63-Ub dependent mutant (IFNAR1$^{K525,\ 526R}$, Figure 5D). Taken together, these results suggest that Ubc13 facilitates an assembly of K63-linked chains on Lys501 of IFNAR1 and this modification cooperates with Cdc34-dependent K48-Ub on Lys525, 526 to contribute to efficient endocytosis of IFNAR1.

It has been previously shown that basal endocytosis of IFNAR1 in naïve human cells is inhibited by its association with TYK2 [38] that serves to shield a specific linear endocytic motif from interacting with the AP2 endocytic machinery complex (Figures 6A – B and Reference [16]). Exposure of cells to Type 1 IFN promotes the recruitment of βTrcp and ensuing ubiquitination of IFNAR1 [14, 15, 39–41]. Overall, IFN-stimulated site-specific (K501, 525, 526) ubiquitination of IFNAR1 was proposed to stimulate its internalization via unmasking this linear endocytic motif [10] in a manner that remains to be understood.

We next investigated putative mechanisms, through which ubiquitination of IFNAR1 may relieve the linear endocytic motif concealment. Given that association of TYK2 with IFNAR1 was similar for wild type receptor and ubiquitination-deficient IFNAR1^{3KR} mutant (Figure 6C), it seems unlikely that IFNAR1 ubiquitination may facilitate its dissociation from TYK2. Furthermore, while IFNAR1 ubiquitination and internalization is stimulated by IFN [14, 15, 39], it was shown that TYK2 remains associated with IFNAR1 throughout early endocytic events and trafficking stages in subcellular fractions from IFN-treated cells [42]. Thus, we considered an alternative model where IFNAR1 ubiquitination may alter either conformation of its intracellular domain or its spatial arrangement with TYK2 and plasma membrane. A highly conserved proline residue

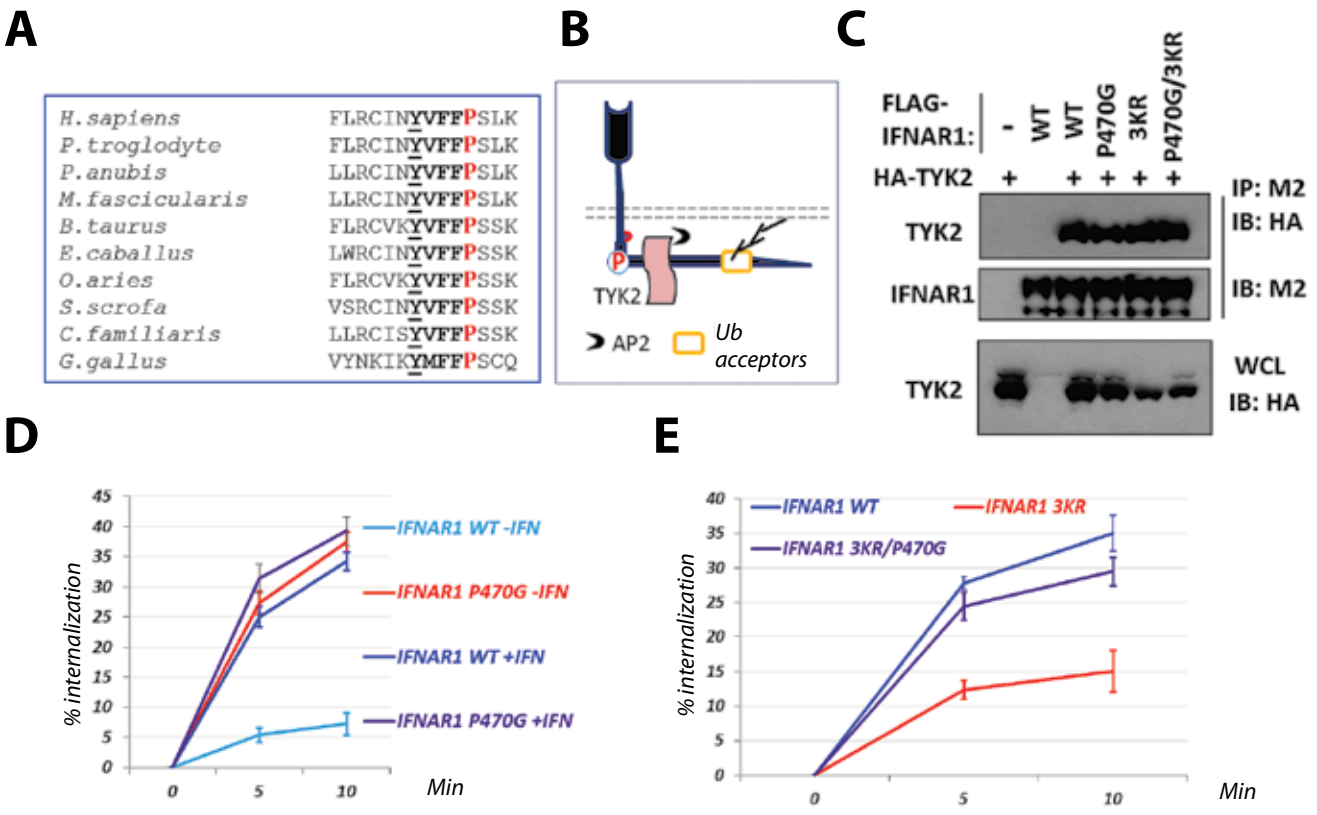


Figure 6. *Combined ubiquitination of canonical and additional sites within IFNAR1 acts upstream of IFNAR1 conformational changes regulated by conserved Pro470.*

(A) Conservation of a specific Pro residue (P470 in human IFNAR1, red letters) adjacent to the endocytic motif (bold letters, critical Tyr residue that interacts with AP2 is underlined) within IFNAR1 from indicated species.

A hypothetical model that proposes the role of proline-mediated IFNAR1 chain bend along with TYK2 association in masking the endocytic motif (red dot) from AP2 endocytic machinery complex. IFNAR1 ubiquitination is proposed to either dislodge TYK2 or affect spatial arrangements between the endocytic motif, plasma membrane and AP2.

(B) Indicated IFNAR1-FLAG (C-terminal tag) proteins with or without HA-tagged TYK2 were co-expressed in 293T cells. Upon IFN treatment (6000U/ml for 10 min), the cells were harvested and lysed and the proteins were immunoprecipitated with anti-FLAG antibody. After a preliminary immunoblot with anti-FLAG antibody (not shown), different amount of reactions were loaded onto another gel to achieve a comparable level of FLAG-tagged IFNAR1 proteins in each lane.

(C) These reactions were analyzed by immunoprecipitation-immunoblotting using the indicated antibodies. Levels of HA-TYK2 in the whole cell lysates (WCL) are also shown.

(D) Internalization rate of indicated FLAG-IFNAR1 expressed in 293T cells was analyzed as described in Figure 2B as well as in the absence of added IFNα (replaced with analogous volumes of PBS).

(E) Internalization rate of indicated FLAG-IFNAR1 expressed in 293T cells was analyzed as described in Figure 2B

distal to the endocytic motif (e. g. Pro470 in human IFNAR1, Figure 6A) may confer a polypeptide chain turn that permits the hindrance of the endocytic motif (Figure 6B). Replacement of this Pro470 with Gly residue to enable chain flexibility generated the IFNAR1^{P470G} protein that efficiently interacted with TYK2 (Figure 6C) yet exhibited a very high rate of internalization even in the absence of IFN treatment (Figure 6D). Strikingly, mutation of this proline residue within the ubiquitination-deficient IFNAR1^{3KR} largely restored the ability of this mutant to undergo efficient internalization (Figure 6E). These results suggest that IFNAR1 ubiquitination functions upstream of conformational/spatial alterations of the intracellular domain of IFNAR1 that ultimately favor efficient endocytosis.

Discussion

Data presented here indicate that the maximal rate of IFNAR1 endocytosis is mediated collectively by the K48-Ub attached to the Lys525, 526 ubiquitin acceptor sites and by the K63-Ub conjugated to the Lys501 site (Figures 2–5). Although SCF$^{\beta Trcp}$ controls the ubiquitin conjugation to the entire Lys cluster important for IFNAR1 endocytosis (Figure 3), these events depend on alternate function of Cdc34 and Ubc13 E2 enzymes for promoting an ubiquitin chain topology-specific internalization (Figure 5).

The Cdc34-dependent conjugation of K48-Ub to the Lys525, 526 sites is consistent with observations reported previously for the proteasomal substrates of bTrcp [36]. Lysines 525 and 526 are located within 9–10 residues proximal from the bTrcp binding site (i. e. degron, 534DSGNYS539) within IFNAR1. In that sense, they represent a canonical acceptor sites for the SCFβTrcp-mediated ubiquitination similar to Lys21/22 within IκBα (Reference (14) and Figure 2A). Preference for such location of ubiquitin acceptor sites was indeed reported for bTrcp proteasomal substrate β-catenin [43] and introduc-

tion of double lysine residues at a similar distance from the bTrcp-binding site converted the latent membrane protein 1 of the Epstein-Barr virus from a pseudosubstrate to a bona fide bTrcp substrate [44]. The findings presented here are consistent with above mentioned similarities in acceptor site location between proteasomal substrates of SCFβTrcp and Lys525/526. These data further suggest that multi-faceted interactions between SCFβTrcp, Cdc34 as an E2 and either Lys21/22 within IκBα [27] or Lys525, 526 within IFNAR1 (this study) as a substrate might be conducive for the elongation of the polyubiquitin chain of K48-linked topology.

However, unlike IκBα which is a cytosolic protein and whose major ubiquitin acceptor sites Lys21/22 are located within a flexible N-terminal region [45], the choice of conjugation sites for IFNAR1 may be affected by the spatial constrains imposed by the anchoring of the transmembrane domain within the plasma membrane. Accordingly, $SCF^{\beta Trcp}$ may lack flexibility in the selection of either substrate lysine residues within IFNAR1 or the types of chains that can be preferentially built on these residues. Conversely, a different type of chain such as K63-Ub might be built by this E3 ligase on the Ly-501 acceptor using an alternative to Cdc34 E2 such as Ubc13. This possibility is consistent with the dependence of the maximal endocytic rate of the $IFNAR1^{K525,\ 526R}$ mutant on integrity of K63 within ubiquitin and on the levels and catalytic activity of Ubc13 (Figures 4–5). Our results are first to demonstrate that $SCF^{\beta Trcp}$ can directly utilize Ubc13/Uev1 complex as an E2 enzyme to catalyze elongation of the K63-linked chain in vitro (Figure 1B). Accordingly, a similar mechanism that may occur in vivo would suffice as the simplest explanation for the βTrcp/Ubc13/K63 chain-dependent IFNAR1 endocytosis. However, it remains plausible that requirements for both βTrcp and Ubc13 for K63-linked ubiquitination on Lys501 within IFNAR1 reflect the indirect regulation and may involve additional E2s/E3s.

Possible roles for other E2 enzymes such as the promiscuous members of the UbcH5 family shown to be capable of ubiquitinating IFNAR1 in vitro [10] and demonstrated to play a role in mono-ubiquitination of substrates followed by elongation of either the K48-linked chain (via Cdc34, [27]) or the K63-linked chain (via Ubc13, [46]), are yet to be clarified. Whereas we did not observe a consistent change in the rate of IFNAR1 endocytosis in cells that received RNAi reagents against the members of UbcH5 family (C.C. and S.Y.F., unpublished data), the role of these E2 species cannot be unequivocally ruled out.

It remains to be determined how these two types of polyubiquitin chains built on two different acceptor substrate lysines with the help of two different E2 enzymes may interact to unmask the linear endocytic motif and to maximize the rate of IFNAR1 internalization. On one hand, it is plausible that the extent of internalization is simply proportional to the overall levels of ubiquitin on the Lys501/525/526 cluster within IFNAR1, and both acceptor sites contribute to the sum by furnishing the type of chain that can be efficiently generated on a given substrate lysine residue because of the constrains of the substrate-ligase-E2 interaction. On the other hand we cannot rule out that these different types of chains may play diverse functions in either recruiting or rearranging the receptor-interacting proteins and that these two functions are complementary in their effects on the IFNAR1 endocytosis rate.

Remarkably, enabling flexibility in the intracellular domain of IFNAR1 via replacing a highly conserved Pro470 with Gly yields a receptor with a very high endocytic rate even in the absence of IFN1 stimulation. Furthermore, introduction of P470G substitution remarkably restored the internalization of a ubiquitination-deficient $IFNAR1^{3KR}$ (Figure 6). This result suggests that ubiquitination of IFNAR1 stimulates its internalization most likely via altering a conformation/spatial arrangement of IFNAR1 intracellular domain. In the context of this model, it is plausible that presence of two types of polyubiquitin chains on IFNAR1 may further promote the adoption of an endocytosis-permissive conformation. Given that the K63-Ub and K48-Ub chains adopt very different conformations [47], interactions between the endocytic motif and endocytic machinery could be augmented and/or stabilized by the presence of both chain types. Future structural studies are warranted to directly investigate this possibility.

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