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Priming and extending: an UbcH5/Cdc34 E2 handoff mechanism for polyubiquitination on a SCF substrate

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Summary

We describe a mechanistic model of polyubiquitination by the $SCF^{\beta TrCP2}$ E3 ubiquitin (Ub) ligase using human IκBα as a substrate. Biochemical reconstitution experiments revealed that the polyubiquitination of IκBα began with the action of the UbcH5 E2 Ub conjugating enzyme, transferring a single Ub to IκBα K21/K22 rapidly and efficiently. Subsequently, the Cdc34 E2 functioned in the formation of polyubiquitin chains. It was determined that an Ub fused at IκBα K21 acts as a receptor, directing Cdc34 for rapid and efficient K48-linked Ub chain synthesis that depends on $SCF^{\beta TrCP2}$ and the substrate's N terminus. The IκBα-linked fusion Ub appears to mediate direct contacts with Cdc34 and the SCF's RING sub-complex. Taken together, these results suggest a role for the multifaceted interactions between the IκBα K21/K22-linked receptor Ub, the SCF's RING complex, and Cdc34~S~Ub in establishing the optimal orientation of the receptor Ub to drive conjugation.

Introduction

In eukaryotes, regulated protein degradation by the ubiquitin (Ub)-proteasome system (UPS) decreases the substrate protein's concentration selectively and drastically, thereby changing cellular programs in an unidirectional manner that profoundly impacts a wide range of biological processes, including cell growth and death, development, signal transduction, transcriptional control, genomic integrity, and tumor suppression (Hershko and Ciechanover, 1998). Central to UPS is the covalent modification of substrates with Ub in the form of polymeric chains, with Ub moieties connected through isopeptide linkage between the ε-amide group of lysine-48 (K48) and the carboxyl end of glysine-76 (G76). It is now widely accepted that the K48-linked polyubiquitin chains are a molecular death code due to their capability of affinity-interactions with the 26S proteasome that drives the degradation of substrate proteins (Pickart, 2001).

Much of the mechanistic insights into substrate polyubiquitination have been built on studies of several model substrates that include IκBα (Karin and Ben-Neriah, 2000), which inhibits the transcription factor NF-κB by sequestering it in the cell cytoplasm. Ub-dependent turnover of human IκBα is governed by a well defined, N-terminally located IκBα degron composed of the DSG-box ($DS^{32}G\varphi XS^{36}$). In response to pro-inflammatory cytokines, the activated IKK

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kinase complex catalyzes the phosphorylation of IκBα Ser32 and Ser36, thereby enabling the phosphorylated DSG-box ($D_pS^{32}G\varphi X_pS^{36}$) to attract and dock the $SCF^{\beta TrCP}$ E3 Ub ligase. The IκBα-E3 interactions nucleate the assembly of mega-protein complexes capable of catalyzing the polyubiquitination of IκBα at either lysine residue K21 or K22, ultimately resulting in its degradation by the proteasome.

The mechanistic framework of $SCF^{\beta TrCP}$–mediated ubiquitination of IκBα has emerged as a result of reconstitution studies (Tan *et al*., 1999; Wu *et al*., 2000) and high-resolution structural analysis (Zheng *et al*., 2002). $SCF^{\beta TrCP}$ is a four-subunit complex composed of Skp1, CUL1, ROC1 (also named Rbx1 or Hrt1), and βTrCP (a substrate-targeting component containing a signature F-box domain that is shared by over 70 protein members in humans). Within the E3 complex, CUL1 functions as a scaffold simultaneously anchoring the Skp1•βTrCP heterodimer and the ROC1 RING finger protein, hence positioning βTrCP within the proximity of ROC1. Upon recruitment of an E2 Ub conjugating enzyme by the ROC1 RING domain, the E3-bound substrate (i.e. IκBα) is placed optimally for chemical reactions with Ub, which is bound to the E2 through thiol ester linkage. SCF-mediated polyubiquitination is activated by conjugation of Nedd8, an Ub like protein, to CUL1 at lysine 720, most likely through conformational regulation of the interactions between ROC1 and CUL1's C-terminal tail (Duda *et al*., 2008; Pan *et al*., 2004; Saha and Deshaies, 2008; Yamoah *et al*., 2008).

At the heart of issues concerning substrate polyubiquitination key questions remain unanswered. Among the most conspicuous of these is the question of how are the E3, the E3-bound substrate, and E2~S~Ub engaged in concerted interactions that commit catalysis and assembly of polyubiquitin chains? Previous studies on SCF-mediated ubiquitination of IκBα have implicated the involvement of two types of E2 enzymes: UbcH5 and Cdc34. The human UbcH5 family consists of three homologues UbcH5a, UbcH5b (also known as Ubc4) and UbcH5c (Gonen *et al*., 1999). Ubc4/5 was initially identified as an E2 enzyme required for IκBα ubiquitination *in vitro* with crude cell extracts (Chen *et al*., 1996), as well as with purified components (Ohta *et al*., 1999; Wu *et al*., 2000). Cdc34 is capable of functioning with the SCF complex for substrate ubiquitination *in vitro* (Feldman *et al*., 1997; Skowyra *et al*., 1997; Tan *et al*., 1999; Wu *et al*., 2000). In budding yeast, Cdc34 is essential for promoting G_1 to S-phase transition by mediating the SCF^{Cdc4}-dependent degradation of the Sic1 CDK inhibitor (Feldman *et al*., 1997; Skowyra *et al*., 1997; and references therein). Human Cdc34 cDNA can functionally substitute for the yeast Cdc34 (Plon *et al*., 1993), underscoring conservation between the two orthologs.

In this work, we explored distinct roles played by UbcH5 and Cdc34 in the $SCF^{\beta TrCP}$ mediated polyubiquitination of IκBα.

Results

Combined actions of UbcH5c and Cdc34 promote rapid and efficient polyubiquitination of IκBα

Previous studies attempting to address the roles of UbcH5 and Cdc34 in TNF-α-induced degradation of IκBα have yielded conflicting reports. Based on transient over-expression experiments with mutant forms of E2, Gonen *et al*. (1999) concluded that the degradation of IκBα required both UbcH5 and Cdc34. However, studies using antisense oligonucleotides by Butz *et al*. (2005) have revealed that down-regulation of Cdc34 caused stabilization of the SCF^{Skp2} substrate p27, but appeared to not affect the stability of the $SCF^{\beta TrCP}$ targets IκBα and β-catenin.

To resolve this conflicting issue, we employed small interference RNA (siRNA) -based approach to evaluate the role for UbcH5 and Cdc34 in the TNF-α-induced degradation of

IκBα in human U2OS cells. As revealed by immunoblot (Fig. 1A), the levels of endogenous UbcH5 or Cdc34 were decreased 80 or 95%, respectively, by the treatment of cells with combined siRNAs that target all three forms of UbcH5 (UbcH5a/b/c) or both Cdc34 species (Cdc34a/b). We observed that the UbcH5- or Cdc34-depledted cells exhibited attenuation in the degradation of IκBα (Fig. 1A), with turnover rates slower than that seen in cells treated with control siRNA (Fig. 1A, graph). These results confirm and extend the observations by Gonen *et al*. (1999) that both UbcH5 and Cdc34 are required for the TNF-α-induced degradation of IκBα. It should be noted that in the reported study by Butz *et al*. (2005), the antisense oligonucleotide used appears to target the Cdc34a form only, thus potentially leaving Cdc34b functional in the treated cells.

To evaluate the contribution of UbcH5 and Cdc34 in catalysis precisely, we employed the reconstituted $SCF^{\beta TrCP2}$-mediated IκBα polyubiquitination system (Tan *et al*., 1999; Wu *et al*., 2000) and monitored the production of IκBα-Ub conjugates as a function of E2 concentration (Figs. 1B and C) and reaction time (Fig. 1D). To facilitate systematic analysis, we categorized reaction products based on apparent molecular masses, with class I to III designating those greater than 250KDa, between 75-250KDa, and below 75KDa, respectively (Figs. 1B-D). Quantification of the class I and class I-III conjugates is presented graphically (Figs. 1B-D, graphs), allowing for direct comparison between UbcH5c and Cdc34 alone or in combination in the enzymatic synthesis of the high molecular weight forms of IκBα-Ub chains, as well as in the overall yield of ubiquitination.

Evidently, co-incubation of UbcH5c and Cdc34 yielded the highest levels of the class I products, with an increased catalytic output of 3 to 4-fold in comparison to single E2-containing reactions (Figs. 1B-D; graph I). Notably, the most pronounced synergistic effects were observed with UbcH5c and Cdc34 at concentrations of 0.05μM and 1μM, respectively (Figs. 1B-C; graph I). Kinetic analysis revealed that the combination of Cdc34 and UbcH5c stimulated synthesis of the class I products in almost linear fashion for a period of 60min (Fig. 1D, graph I).

Intriguingly, analysis of the class I-III products as a whole showed that UbcH5c alone produced the highest catalytic output (Figs. 1B and 1D; graph I-III). Addition of Cdc34 below a concentration of 1μM did not significantly affect the overall yield, but decreased the output at high concentrations (>1μM) (Fig. 1C, graph I-III). These results established UbcH5c as the determining factor for the overall yield in the dual E2 system.

Despite UbcH5c's ability to catalyze IκBα ubiquitination much more rapidly and efficiently than Cdc34, the reactions products formed with UbcH5c alone were dominated by single, di- and tri-ubiquitin moieties (Figs. 1B-D). These short-length conjugates were converted into lengthy products by the addition of Cdc34, which by itself exhibited poor polyubiquitination efficiency (Figs. 1B-D). Similar synergistic effects by UbcH5c and Cdc34 for IκBα polyubiquitination were observed at a range of Ub concentrations (Fig. S1).

Taken together, these observations strongly suggest a collaborative relationship between UbcH5c and human Cdc34 such that UbcH5c functions as an initiator E2 to attach an SCF substrate with single or limited Ub moieties, which is then elongated by Cdc34 to form polyubiquitin chains.

While UbcH5 is highly efficient in converting IκBα into mono-ubiquitinated forms, Cdc34 drives Ub-Ub conjugation

We reasoned that UbcH5c and Cdc34 might each play a distinct role in the initiation and elongation phase of the polyubiquitination reaction. Possibly, these two E2s differ substantially in their capacity to catalyze the ligation of Ub to a substrate, as well as in their ability of Ub-

Ub conjugation. To explore these possibilities we employed single turnover experiments. Initially, we assembled E2~S~Ub^{K0} (Ub^{K0} eliminates polyubiquitination) then performed a chase reaction during which the preformed thiol ester complex was attacked by IκBα K21 or K22 to yield IκBα-Ub^{K0} conjugates (Fig. 2A). In this manner, UbcH5c and Cdc34 could be compared directly for initiating the ligation of Ub to IκBα.

UbcH5c yielded two major reaction products: IκBα (1-54)-Ub^{K0} and IκBα (1-54)-$2Ub^{K0}$ (Fig. 2B). As revealed by site-mutagenesis experiments, these two products formed at IκBα K21 and K22. Replacement of IκBα K21 and K22 with arginine individually produced a single Ub^{K0} conjugate, while double substitution markedly inhibited ubiquitination (Fig. S2A). Thus, the *in vitro* reconstitution system recapitulates *in vivo*, where IκBα K21 and K22 are used for ubiquitination (Scherer *et al*., 1995). Of note, low, but detectable levels of ubiquitination product formed with Ub^{K0} in the K21R/K22R double mutant (Fig. S2A, lane 8). This result explained the observations that at high E2 concentrations (Fig. 2B, lanes 6 and 7), or after a long incubation time (Fig. S2B, lane 4), UbcH5c formed low levels of Ub^{K0} conjugates with sizes larger than that of IκBα (1-54)-$2Ub^{K0}$. This is most likely a result of Ub conjugation to the K38 and K47 residues of IκBα.

UbcH5c was much more effective than Cdc34 in IκBα mono-ubiquitination, with UbcH5c forming substrate-Ub^{K0} conjugates at a rate (Fig. S2B) and an efficiency of (Figs. 2B-C) 10- and 300-times greater than those observed with Cdc34, respectively. Of note, UbcH5c at low levels predominantly formed IκBα (1-54)-Ub^{K0} (Fig. 2B, lanes 2-3; 2C, inset graph), while at higher concentrations it accumulated IκBα (1-54)-$2Ub^{K0}$ as well as conjugates with single Ub moieties attached to non-K21/22 sites (perhaps K38 and K47) (lanes 6 and 7). Finally, UbcH5a and UbcH5b catalyzed the ligation of lysine-less Ub to IκBα with an efficiency similar to that observed with UbcH5c (Fig. S2C, *Top*). Thus, the UbcH5 family E2s are conserved for efficient mono-ubiquitination. In all, UbcH5 but not Cdc34, is capable of catalyzing the ligation of Ub to IκBα rapidly and efficiently.

We next employed single turnover assays to compare the ability of Cdc34 and UbcH5c in driving Ub-Ub ligation. In this system, the E2~S~Ub charging reaction was carried out with high levels of E2 but limiting concentrations of ^{32}P-PK-Ub, a condition preventing ligation between the ^{32}P-PK-Ub molecules. Large excess of non-radioactive bovine Ub (bUb) was added in the chase, thereby favoring the attack of E2~S~^{32}P-PK-Ub by bUb to form a di-Ub species (Fig. 2D). Cdc34 catalyzed di-Ub synthesis with an efficiency of at least 1,000-times greater than that observed with UbcH5c (Figs. 2E-F). Furthermore, Cdc34 formed di-Ub within 1min of incubation, whereas under the same condition UbcH5c yielded barely detectable di-Ub products after 10min (Fig. S2D, *b* and *d*). Finally, neither UbcH5a nor UbcH5b was efficient in supporting di-Ub synthesis (Fig. S2C, *Bottom*), demonstrating that all UbcH5 family E2s are inefficient in catalyzing Ub-Ub ligation. In conclusion, Cdc34 but not UbcH5, catalyzes the ligation of Ub molecules to assemble a substrate-free di-Ub species rapidly and efficiently.

Cdc34 builds Ub chains from the UbcH5c-primed IκBα-linked Ub

We examined the ability of Cdc34 to build Ub chains from the UbcH5c-primed IκBα-linked Ub directly. For this purpose, we prepared an IκBα-Ub substrate having an Ub pre-conjugated by UbcH5c *via* isopeptide bond (generated as diagrammed in Fig. 3A). Incubation of Cdc34 with purified UbcH5c-generated IκBα-Ub produced higher molecular weight forms of IκBα-Ub, concomitant with the disappearance of the mono-ubiquitinated species (Fig. 3B, compare lanes 2 and 3). This reaction depended on initial treatment with UbcH5c (Fig. 3B, lane 4), high levels of Cdc34 (Fig. 3C, lanes 3 and 4), and $SCF^{\beta TrCP2}$ (Fig. 3C, lane 5). Thus, Cdc34 is able to catalyze elongation from the UbcH5c-primed IκBα-anchored Ub to form polyubiquitin chains.

In order to gain insights into the cooperative actions by UbcH5c and Cdc34 in the assembly of Ub chains onto IκBα, we performed E2~S~Ub mixing assays as diagrammed in Fig. 4A. UbcH5c~S~Ub, Cdc34~S~Ub, and E3-substrate complexes were all preformed at low (Figs. 4B and 4D) or high (Fig. 4C) UbcH5c concentration. In addition, Ub^{K48} (Figs. 4B-C) or other forms of single K Ub (Fig. 4D) was utilized in the formation of E2-Ub thiol esters.

The E2~S~Ub mixing experiments revealed four major reaction products designated as GST-IκBα (1-54)-Ub, GST-IκBα (1-54)-2Ub, GST-IκBα (1-54)-Ub_2 and GST-IκBα (1-54)-Ub_n. Irrespective of the forms of Ub (Ub^{K0} to Ub^{K63}) used, UbcH5c formed GST-IκBα (1-54)-Ub and GST-IκBα (1-54)-2Ub with identical mobility (Fig. 4D, lanes 1, 3, 5, 7, 9, and 11). This observation suggests that these two conjugates are mono-ubiquitinated species at IκBα K21 and/or K22 (depicted in Fig. 4A).

Formation of GST-IκBα (1-54)-Ub_2 required UbcH5c and Cdc34 (Fig. 4B, lanes 2-7), as well as Ub^{K48} (Fig. 4D, lane 10). These observations, combined with the well-characterized property of Cdc34 in the utilization of Ub K48 for chain assembly (Chau *et al*., 1989), strongly suggest that GST-IκBα (1-54)-Ub_2 is the product formed by sequential actions of UbcH5c and Cdc34. It follows that UbcH5c initially produces mono-ubiquitinated IκBα at K21 or K22, thereby providing for receptor Ub attack of Cdc34~S~Ub that leads to the formation of a di-Ub chain (depicted in Fig. 4A). In support of this notion, it was observed that co-incubation of UbcH5c and Cdc34 in the presence of native bovine Ub produced GST-IκBα (1-54)-Ub_2 as well (Fig. S3A, compare lane 3 and lane 4). Of note, the use of recombinant PK-Ub precluded the detection of GST-IκBα (1-54)-Ub_2 (Figs. 1B-D and S1), for reasons that remain to be determined.

In addition, combination of UbcH5c~S~Ub^{K48} and Cdc34~S~Ub^{K48} enhanced the production of GST-IκBα (1-54)-Ub_n, the high molecular weight forms of ubiquitination species (Fig. 4B, lanes 6 and 7; Fig. 4C, lanes 2 and 4; Fig. 4D, lane 10; and Fig. S3A, lanes 3-6). It should be noted that the heterogeneity of radioactive materials with molecular weight at the range of 60-150KDa precluded the assessment of ubiquitinated products in this area.

At a low UbcH5c concentration (0.56μM), both GST-IκBα (1-54)-Ub_2 and GST-IκBα (1-54)-Ub_n formed within 3min after mixing the E3-substrate complex with UbcH5c~S~Ub^{K48} and Cdc34~S~Ub^{K48} (Fig. 4B, lane 6), with GST-IκBα (1-54)-Ub_n growing in length with time (Fig. 4B, compare lanes 6 and 7). We observed that Cdc34 decreased the levels of GST-IκBα (1-54)-Ub (Fig. 4B, compare lanes 2 and 3 with lanes 6 and 7). This effect most likely results from Cdc34-mediated inhibition of UbcH5c's mono-ubiquitination activity, rather than conversion of GST-IκBα (1-54)-Ub to high molecular weight forms of ubiquitination products, as Cdc34 seemed to reduce the levels of GST-IκBα (1-54)-Ub in the presence UbcH5c and non-K48 forms of Ub (which cannot be used by Cdc34) (Fig. 4D, compare lanes 1, 3, 5, 7, and 11, with lanes 2, 4, 6, 8, and 12). Moreover, while GST-IκBα (1-54)-2Ub accumulated in the presence of UbcH5c only (Fig. 4B, lanes 2 and 3), it was barely detectable when both UbcH5c and Cdc34 were present (Fig. 4B, lanes 6 and 7). In addition, under conditions where the ubiquitination reaction was initiated first with UbcH5c prior to the addition of Cdc34~S~Ub^{K48}, GST-IκBα (1-54)-2Ub formed (Fig. 4B, compare lane 8 with lanes 6, 7 and 9). Taken together the above findings established that UbcH5c at low levels formed mono-ubiquitinated IκBα at K21 or K22 with which Cdc34~S~Ub interacted rapidly to assemble di-Ub and polyubiquitin chains. In the absence of Cdc34, UbcH5c proceeded to catalyze additional mono-ubiquitination reactions that resulted in the attachment of single Ub moieties to IκBα K21 and K22.

At the high level of UbcH5c (17μM), GST-IκBα (1-54)-2Ub accumulated even in the presence of Cdc34, although GST-IκBα (1-54)-Ub_2 formation was still observed under this condition

(Fig. 4C, lane 8). It should also be noted that abundant UbcH5c catalyzed mono-ubiquitination at other IκBα lysine sites in addition to K21 and K22 (Fig. 2B, lanes 6 and 7). This may explain the formation of Ub^{K48} conjugates by high UbcH5c with apparent molecular weight in the range of 50-75KDa (Fig. 4C, lane 6). UbcH5c alone formed GST-IκBα (1-54)-Ub_n (Fig. 4C, lane 2; Fig. S3A, lanes 3 and 5), utilizing Ub K6, K11, K33, K48 and K63 (Fig. S3B). However, addition of Cdc34~S~Ub yielded GST-IκBα (1-54)-Ub_n forms of higher molecular weight (Fig. 4C, compare lanes 2 and 4; Fig. S3A, compare lanes 3 and 5 with lanes 4 and 6). Collectively, these findings suggest that at high concentrations, UbcH5c is hyperactive in mono-ubiquitination to modify the substrate at lysine sites bearing no physiological significance and mediates polyubiquitination utilizing a range of Ub lysine residues in a non-selective fashion.

Taken together the above reconstitution experiments yielded reactions products that formed as a result of the action of Cdc34 to build Ub chains from the UbcH5c-primed substrate-linked Ub.

Fusion of an Ub at IκBα K21 directs Cdc34 for rapid and efficient Ub chain synthesis in a manner that requires the substrate's N terminus

Once conjugated to IκBα K21/K22 by UbcH5, the substrate-linked Ub functions as a receptor to initiate Ub chain assembly by Cdc34 (Fig. 3). In order to analyze the mode of the action of the receptor Ub in ubiquitination, we engineered models of mono-ubiquitinated IκBα substrate by directly fusing an Ub in the proximity of its degron. Initially, a panel of IκBα-Ub fusion substrates was designed to understand the influence on IκBα ubiquitination by a fused Ub near the end of the substrate's degron (G-I-UbΔGG, shown in Fig. 5A) or at its authentic ubiquitination site K21 (G-I20-Ub-I23, shown in Fig. 5A).

Figs. 5B-E revealed reaction kinetics of IκBα or the IκBα-Ub fusion substrates. It was evident that Ub fusion stimulated ubiquitination by Cdc34 in the absence of UbcH5, with both G-I-UbΔGG and G-I20-Ub-I23 fusions exhibiting higher Ub conjugation activity than the wild type substrate (compare Figs. 5C-D to Fig. 5B; also see Fig. 5E). Notably, substitution of the fusion Ub's K48 in G-I-UbΔGG to arginine inhibited ubiquitination (Fig. S4A), demonstrating that Cdc34 utilized the fusion Ub K48, but not IκBα K21/K22, for Ub chain assembly. Additionally, UbcH5c catalyzed ligation of Ub to GST-IκBα (1-54), G-I-UbΔGG, or G-I-Ub^{K48R}ΔGG with similar efficiency (Fig. S4B), suggesting that Ub fusion with IκBα does not influence UbcH5c's Ub conjugation activity to IκBα. Altogether, these findings revealed an ability of human Cdc34 to catalyze the ligation of the donor Ub to the K48 of IκBα-linked Ub.

However, G-I20-Ub-I23 was more active than G-I-UbΔGG in ubiquitination (compare Fig. 5C to 5D; also see Fig. 5E), suggesting that placement of an Ub at the IκBα K21 position yields a substrate more effective for subsequent Ub chain assembly. This result is in line with a hypothesis that a substrate's physiological Ub receptor lysine residue, such as IκBα K21/K22, provides optimal positioning for the receptor Ub during elongation. Additional mutagenesis experiments revealed that neither fusion Ub's G75G76 nor IκBα K21/K22 was required for the ubiquitination of IκBα-Ub fusion substrates (Fig. S4C). These findings demonstrated that the IκBα-linked fusion Ub acts as a receptor for Ub chain elongation by Cdc34.

It is well documented that the IκBα's DSG-box ($D_pS^{32}GLD_pS^{36}$; see Fig. 6A) binds to βTrCP, thereby initiating ubiquitination (Wu *et al.*, 2003). Using the above ubiquitination system that is directed by the IκBα-linked fusion Ub (Fig. 5), the role of DSG-box-independent IκBα structural elements in Cdc34-mediated Ub chain assembly can be assessed. Thus, a second panel of IκBα-Ub fusion substrates was constructed to compare the ubiquitination of I20-Ub-I23, a substrate composed of IκBα N-terminal 20 amino acids, Ub, IκBα degron and downstream sequence (shown in Fig. 6A), with Ub-I23, which is identical to I20-Ub-I23 except

lacking the IκBα N-terminal sequence (shown in Fig. 6A). At a range of Cdc34 concentrations tested, I20-Ub-I23 exhibited up to 10-fold higher activity than Ub-I23 regarding conversion of substrate to ubiquitination products, as well as accumulation of high molecular weight reaction products (compare Fig. 6B to 6C; also see Figs. 6D-E). These results helped establish a critical role for the IκBα N terminus in Cdc34–mediated Ub chain assembly. Of note, I20-Ub-I23 was significantly more active than G-I20-Ub-I23 (compare Fig. 6B to Fig. 5D), suggesting an inhibitory effect by the placement of a GST moiety at the N-terminus of IκBα-Ub fusion.

In summary, we showed that an Ub engineered at IκBα K21 functions as a receptor that directs Cdc34 for rapid and efficient K48-linked Ub chain assembly. Unexpectedly, this receptor Ub-directed ubiquitination appears to require the IκBα's N terminus.

The IκBα-Ub fusion protein mediates multifaceted interactions with Cdc34, βTrCP and SCF's RING sub-complex to drive Ub-Ub conjugation

We determined the requirement for $SCF^{\beta TrCP2}$ in ubiquitination reactions directed by the IκBα-linked fusion Ub. For this purpose, the ubiquitination of I20-Ub-I23 was assayed with Cdc34 alone, or in the presence of $SCF^{\beta TrCP2}$ or ROC1-$CUL1^{324-776}$ (the SCF's RING sub-complex capable of catalyzing substrate-independent Ub ligation; see Wu *et al*., 2002). The results revealed that Cdc34 alone weakly catalyzed ligation of Ub to the IκBα-Ub fusion substrate (Fig. 7B, compare lanes 2 and 3 to lanes 5 and 6). This reaction was stimulated, albeit at low levels, by ROC1-$CUL1^{324-776}$ (Fig. 7B, compare lanes 5 and 6 to lanes 8 and 9). However, efficient ubiquitination was observed only in the presence of $SCF^{\beta TrCP2}$, which exhibited a 20-fold higher activity than ROC1-$CUL1^{324-776}$ (Fig. 7B, lanes 11 and 12; see graph). Similar results were observed with other Ub fusion substrates (G-I-UbΔGG and Ub-I23; Fig. S5A). Importantly, unlike the Ub fusion substrates displaying low levels of ubiquitination activity with Cdc34 alone and ROC1-$CUL1^{324-776}$, the ubiquitination of the Ub-free IκBα substrate strictly depended on $SCF^{\beta TrCP2}$ (Fig. S5A, compare lanes 1-3 and lanes 4-9). Of note, while neddylation of ROC1-$CUL1^{324-776}$ increased the production of Ub conjugates with I20-Ub-I23 as a substrate, the products formed were dominated by low molecular weight species, in contrast to lengthy products synthesized by $SCF^{\beta TrCP2}$ (Fig. S5B). Taken together, these results established an indispensable role for $SCF^{\beta TrCP2}$ in interacting with the IκBα-linked receptor Ub for chain elongation. The weak, yet detectable activity of Cdc34 and ROC1-$CUL1^{324-776}$ on the Ub fusion substrates suggests that both the E2 and the SCF's RING sub-complex interact with the receptor Ub.

We employed the GST-based pull down assay to measure direct interactions between E3/E2 and IκBα substrates linked with or without a fusion Ub (Fig. 7C). In this case, GST or GST fusions (GST-βTrCP2-Skp1, GST-ROC1-$CUL1^{324-776}$ and GST-Cdc34), in the immobilized forms (onto glutathione beads), were incubated with ^{32}P-labeled substrates that included I20-Ub-I23 (Fig. 7C, lanes 1-5), Ub-I23 (lanes 6-10) or IκBα (1-54) (lanes 11-15). Post washing, elution and separation by SDS-PAGE, the bound radioactive materials were visualized by autoradiography and quantified. In keeping with previous observations that βTrCP2 binds to the IκBα degron (containing the phospho-$D_pS^{32}GLD_pS^{36}$; see Fig. 7A) (Wu *et al*., 2003), we detected specific interactions between GST-βTrCP2-Skp1 and IκBα (1-54) (Fig. 7C, lane 14). Among three substrates tested, the order of the efficiency with which GST-βTrCP2-Skp1 bound was: IκBα (1-54) > I20-Ub-I23 > Ub-I23 (Fig. 7C, compare lane 14 with lanes 4 and 9). Thus, it appears that Ub fusion at IκBα K21 weakens the βTrCP2-IκBα interaction and deletion of the IκBα N terminal amino acids 1-20 further reduces this association. In further support of a role for the IκBα N terminus in stable association with βTrCP2, we observed that the ubiquitination of I20-Ub-I23 was competed effectively by excess GST-IκBα (1-54), but not the N-terminus-lacking, IκBα (22-37) peptide (data not shown).

Consistent with the ability of ROC1-CUL1$^{324-776}$ to activate the limited ubiquitination of I20-Ub-I23, the GST-tagged RING complex was found to bind to this substrate at low, but detectable levels (Fig. 7C, lane 3). GST-ROC1-CUL1$^{324-776}$ bound to IκBα (1-54) at levels comparable to background (GST) (Fig. 7C, lanes 12 and 13). These findings point to a direct interaction between ROC1-CUL1$^{324-776}$ and the IκBα-linked Ub. Unexpectedly, Ub-I23 failed to bind to GST-ROC1-CUL1$^{324-776}$ (Fig. 7C, lane 8), suggesting that the removal of the IκBα N terminal amino acids 1-20 diminishes the RING complex-Ub interaction. In support of this notion, ROC1-CUL1$^{324-776}$ was found to stimulate the yield of ubiquitination products with I20-Ub-I23 at levels higher than with Ub-I23 (7.3% *vs* 3%; compare Fig. 7B, lane 9 and Fig. S5A, lane 9).

GST-Cdc34 was shown to interact with I20-Ub-I23 (Fig. 7C, lane 5) and Ub-I23 (lane 10) specifically, albeit at low levels. By contrast, GST-Cdc34 bound to IκBα (1-54) at background levels (Fig. 7C, lanes 12 and 15). Thus, it appears that Cdc34 is capable of physical association with the IκBα-linked Ub. These findings are in line with the observation that Cdc34 alone activated the ubiquitination of IκBα-Ub fusion substrates (Fig. 7B, lanes 5 and 6; Fig. S5B).

The results of ubiquitination assays and pull down binding experiments are in agreement with a hypothesis that Cdc34 and the SCF's RING sub-complex interact with the IκBα-linked receptor Ub directly and such interactions contribute to the positioning of the receptor Ub for conjugation. In addition, the above findings suggest a dual role for the IκBα N terminus in stabilizing βTrCP2/IκBα, and ROC1-CUL1$^{324-776}$/Ub interactions.

Discussion

Priming and extending: UbcH5 and Cdc34 team up to catalyze the polyubiquitination of the SCF substrate IκBα

Our present work has underscored collaborations between UbcH5 and human Cdc34 in the polyubiquitination of IκBα mediated by the SCF$^{\beta TrCP2}$ E3 ligase. In comparison to single E2-containing reactions, the combined actions of UbcH5c and Cdc34 catalyze the polyubiquitination of IκBα more rapidly and efficiently, with UbcH5c determining the overall yield (Fig. 1B-D). Secondly, the results of single turnover experiments establish that while UbcH5 is highly effective in transforming IκBα into mono-ubiquitinated forms, Cdc34 is the preferred E2 that drives Ub-Ub conjugation (Fig. 2). Moreover, the sequential E2 addition assay yields direct evidence that Cdc34 can assemble chains on the UbcH5c-preconjugated IκBα-anchored Ub (Fig. 3). In further support, E2~S~Ub mixing experiments revealed that Cdc34 builds Ub chains from the UbcH5c-primed IκBα-linked Ub (Fig. 4). Finally, studies with IκBα-Ub fusion substrates demonstrated that direct Ub fusion is sufficient to activate Cdc34 for Ub chain assembly (Figs. 5-6).

Previous studies have revealed that two E2s are required for the polyubiquitination of APC/C substrates (Rodrigo-Brenni and Morgan, 2007) and PCNA (Unk *et al*., 2006), as well as for auto-ubiquitination of BRCA1 (Christensen *et al*., 2007). In agreement with our data on UbcH5 as an initiator E2, Morgan and colleagues have previously observed that Ubc4 (UbcH5 homologue) catalyzed rapid mono-ubiquitination on APC/C substrates, followed by Ubc1-driven extension to form polyubiquitin chains (Rodrigo-Brenni and Morgan, 2007). The key feature of the dual E2-driven ubiquitination pathway appears to be the handoff of the mono-ubiquitinated substrate from an initiator E2, typically robust in mono-ubiquitination but non-processive in Ub chain assembly, to an elongating E2 that is empowered for processive chain synthesis in a linkage-specific manner. However, reconstitution experiments with SCF^{Cdc4} and Sic1 demonstrated that yeast Cdc34 alone is effective in polyubiquitination (Petroski and Deshaies, 2005). Future work is required to determine whether the relatively weak mono-ubiquitination activity associated with human Cdc34 is species and/or substrate specific.

Our reconstitution studies have established that optimal IκBα polyubiquitination, as defined by high yield of substrate-K48-polyubiquitin chains, required low UbcH5 but high Cdc34 concentrations. UbcH5c at high concentrations catalyzes extensive mono-ubiquitination reactions that modify IκBα at non-K21/K22 sites (Figs. 2 and 4), events that seem to bear no physiological significance. In addition, abundant UbcH5 promotes polyubiquitination by using Ub lysine residues in a non-selective fashion (Fig. S3B), which may perturb the production of K48-linked Ub chains necessary to mediate proteasomal degradation. These findings suggest the need for cellular program(s) that restrains UbcH5 activity to prevent potentially disastrous hyper-mono-ubiquitination and/or non-K48-linked polyubiquitination.

The mechanistic basis underlying the biochemical disparity between UbcH5 and Cdc34 in substrate mono-ubiquitination and Ub-Ub conjugation remains to be elucidated. Conceivably, subtle differences between these E2s when complexed with Ub *via* thiol-ester linkage may favor different reaction. Interactions between UbcH5~S~Ub, SCF and the substrate's lysine residue are favored for mono-ubiquitination. On the contrary, Cdc34~S~Ub assimilates much better than UbcH5~S~Ub for interactions with SCF and the receptor Ub, the latter of which either anchors on the substrate, or resides on the distal end of an Ub chain. In support of this premise, studies with the IκBα-Ub fusion substrates showed that pre-positioning an Ub near the IκBα degron activates Cdc34 but not UbcH5c for Ub chain formation (Figs. 5-6; Fig. S4B).

Multifaceted interactions establish the positioning of the receptor Ub for chain elongation

We have reconstituted a Cdc34-mediated ubiquitination system based on an IκBα-linked fusion Ub that functions as a receptor, directing $SCF^{\beta TrCP2}$ dependent, rapid and efficient K48-linked Ub chain assembly (Fig. 6B; Fig. 7B).

In order for the first Ub-Ub conjugation to occur, $SCF^{\beta TrCP}$, Cdc34~S~Ub (donor Ub), and the IκBα-linked receptor Ub must interact in a manner that aligns the donor and receptor Ub molecules optimally for chemical reaction. In this report, we present evidence for direct interactions between the IκBα-linked receptor Ub and Cdc34 or the SCF's RING sub-complex (Fig. 7C). Consistent with physical interactions, Cdc34 alone was able to catalyze limited ligation of an Ub to the IκBα-linked receptor Ub. This reaction was activated by the SCF's RING sub-complex (Fig. 7B). In this context, it is intriguing to note that previous work has established interactions between E3 ligase and Ub/Ubl (Ub like protein) including Nup358/RanBP2 and SUMO-1 (Reverter and Lima, 2005), Rabex-5 and Ub (Mattera *et al*., 2006), as well as RSP5 and Ub (French *et al*., 2009).

This work, combined with previous studies, suggests a requirement for multifaceted interactions to position the receptor Ub for chain elongation. In the IκBα model, the receptor Ub is placed at the substrate's K21/K22 (Fig. 7D). The docking of βTrCP at the IκBα's DSG-box presumably allows the $SCF^{\beta TrCP}$ E3 to position its RING sub-complex near the vicinity of the K21/K22-linked receptor Ub, where Cdc34~S~Ub would be recruited. Such arrangements would effectively increase the local concentrations of the RING complex, Cdc34~S~Ub and the K21/K22-linked receptor Ub, thus allowing for productive interactions among these components that otherwise might not occur efficiently due to the low affinity associations between the IκBα-linked receptor Ub and Cdc34 or the SCF's RING sub-complex (Fig. 7C). As a result, the optimal orientation of the receptor Ub is established, aligning with the donor Ub to drive conjugation.

Our analysis of various IκBα-Ub fusion substrates has defined three elements within IκBα that are critical for the function of the receptor Ub in elongation, with the substrate's DSG-box anchoring βTrCP, K21/K22 docking the receptor Ub, and the N terminus that appears stabilize βTrCP/IκBα, and ROC1-$CUL1^{324\text{-}776}$/Ub interactions (Figs. 6, 7B-C; see Fig. 7D for a diagram). In a recently published work (Jin *et al*, 2008), Rape and colleagues have identified

a TEK-box that is present in several, but not all, APC/C substrates as well as in Ub. The TEK-box appears to play multiple roles in ubiquitination. While the TEK-box in substrates is thought to collaborate with the APC/C-interacting D-box to promote the association of substrates with the APC/C, the Ub's TEK-box appears to be critical for elongation of the K11-linked Ub chains. It remains to be determined whether the IκBα N terminus functions in a manner reminiscent of the TEK-box.

Experimental Procedures

siRNA

Three sets of siRNAs were purchased from Dharmacon including set 1(control; Cat. D-001810-10-20), set 2 (UbcH5a, Cat. L-009387-00-0020; UbcH5b, L-010383-00-0020; and UbcH5c, L-010383-00-0020), as well as set 3 (Cdc34a, Cat. L-003230-00-0020; and Cdc34b, L-009700-00-0020). Human U2OS cells grown in 60mm tissue culture dishes were transfected with siRNAs (40nM) using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocols. At day 3 post-transfection, the cells were treated with TNF-α (5ng/ml) and chased for times as indicated. The resulting cells were harvested and lysed as described previously (Wu *et al*., 2000). The levels of IκBα, UbcH5 or Cdc34 were analyzed by immunoblot analysis after protein separation by 4-20% SDS-PAGE, and quantified by Odyssey Infrared Imaging (Licor). The antibodies were purchased from Santa Cruz (IκBα), Boston Biochem (UbcH5), BD Transduction Lab (Cdc34), and Sigma (α-Tubulin).

Ubiquitination assays

Ubiquitination on matrix—For experiments shown in Figs. 1B-D, S1, S2A, S3A, S4A and S4B, $SCF^{\beta TrCP2}$ (~25nM or in indicated amount), bound to anti-HA beads (10μl), was incubated in a mixture that contained 50mM Tris-HCl, pH7.4, 5mM $MgCl_2$, 2mM NaF, 10nM okadaic acid, 2mM ATP, 0.6mM DTT, 0.1mg/ml BSA, 20nM of ^{32}P-labeled substrate, 8μM of PK-Ub (Figs. 1B-D, S1, S2A, S4A and S4B) or bovine Ub (Fig. S3A), E1 (10nM) and UbcH5c or Cdc34 (in amounts as indicated). The reaction was incubated at 37°C for 60min, or for times as specified, on a thermomixer (1,400rpm; Eppendorf). The reaction products were visualized by autoradiography after separation by 4-12% SDS-PAGE and the products were quantified by phosphoimaging.

Ubiquitination with preformed E2~S~Ub and E3-substrate—For experiments shown in Figs. 2B, S2B and S2C (*Top*), E2~S~Ub^{K0} and the E3-substrate complex were preformed. The E2 charging reaction was assembled in a mixture (5μl) that contained 50mM Tris-HCl, pH7.4, 5mM $MgCl_2$, 2mM NaF, 10nM okadaic acid, 2mM ATP, 0.5mM DTT, 0.1mg/ml BSA, Ub^{K0} (50μM), E1 (0.1μM), and UbcH5c or human Cdc34 (in concentration as specified). The reaction was incubated for 5min at 37°C. EDTA (1μl of 0.5M) was added to a final concentration of 50mM (in 10μl). To assemble the E3-substrate complex, a mixture (4μl), containing $SCF^{\beta TrCP2}$ (0.15μM) and ^{32}P-GST-IκBα (0.16μM) was incubated for 15min at room temperature. Finally, the above two reaction mixtures were combined (in a final volume of 10μl) and incubated at 37°C for times as indicated.

For experiments shown in Fig. 4, UbcH5c~S~Ub or Cdc34~S~Ub was assembled as described above but in a mixture (3μl) containing single K-only Ub (50μM). In Figs. 5, 6, 7B, S3B, S4C and S5A, Cdc34~S~Ub or UbcH5c~S~Ub was preassembled in 5μl as described above with the exception that the pre-charged reaction mixture with PK-Ub (50μM) was combined with the E3-substrate complex without EDTA quenching.

Single turnover di-Ub synthesi—For experiment shown in Figs. 2E, S2C (*Bottom*) and S2D, E2~S~^{32}P-Ub was prepared as described above except with ^{32}P-PK-Ub (1.7μM). The

resulting mixture was then chased (in a final volume of 10μl) with bovine Ub (170μM) at 37° C for times as indicated.

GST pull down

GST or GST fusions (GST-βTrCP2-Skp1, GST-ROC1-CUL1$^{324-776}$ and GST-Cdc34) were absorbed to glutathione beads (10μl) with shaking at 4°C for 2h. After extensive washing, the resulting beads were incubated with purified, ^{32}P-labeled IκBα substrates (I20-Ub-I23, Ub-I23, or IκBα (1-54)), 0.4pmol each, in a mixture containing buffer A (25mM Tris-HCl, pH 7.5, 1mM EDTA, 0.01% NP-40, 10% glycerol, 0.1mM PMSF, 1mM DTT, 0.2 μg/ml of antipain and 0.2 μg/ml of leupeptin) plus 50mM NaCl (20μl). The mixture was incubated at room temperature for 45min, on a thermomixer (1,400rpm; Eppendorf). Following washing twice with buffer A plus 50mM NaCl, the bound radioactive materials were eluted with 20μl of SDS-containing loading buffer. An aliquot (2μl) was subject to 4-20% SDS-PAGE followed by staining with Coomassie blue. The remaining elutes were analyzed by 4-20% SDS-PAGE followed by autoradiography. The bound proteins were quantified by Phosphoimaging and are presented graphically.

Others

Supplemental Data describes procedures for construction of plasmids expressing various IκBα proteins, for preparation of radioactive substrates, SCF$^{\beta TrCP2}$, as well as other enzymes/proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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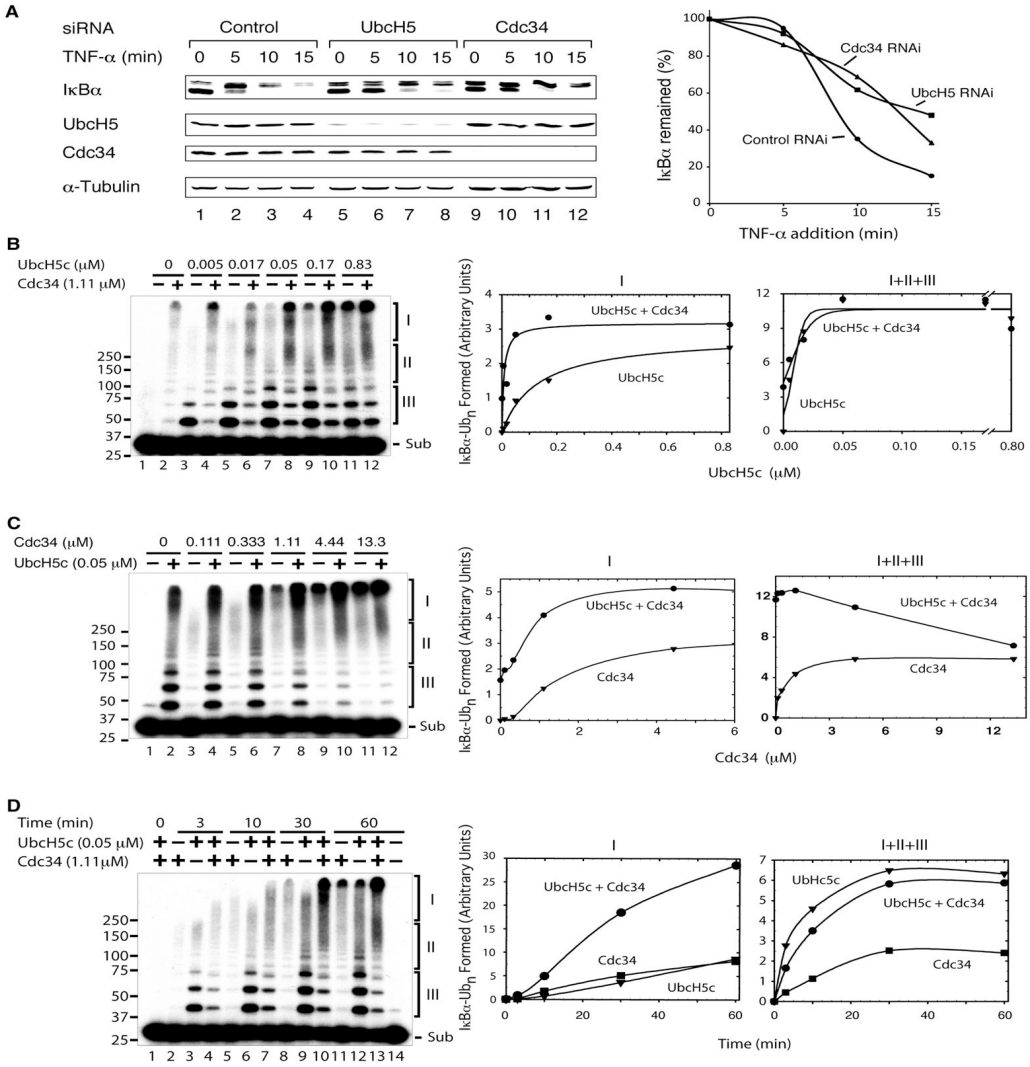

Fig. 1. A) Depletion of UbcH5 or Cdc34 by siRNA in U2OS cells attenuated TNF-α-induced degradation of IκBα

The effects of UbcH5 or Cdc34 siRNA on TNF-α-induced degradation of IκBα were examined. Immunoblots for various indicated proteins are presented, along with the graph showing the reduction of IκBα in TNF-α-exposed cells that had been prior treated with various siRNAs. **B-D) Combined actions of UbcH5c and Cdc34 promote rapid and efficient polyubiquitination of IκBα**. The reconstituted SCF$^{\beta TrCP2}$-mediated IκBα polyubiquitination assay was employed to measure the production of IκBα-Ub conjugates as a function of UbcH5c (panel **B**) or Cdc34 (panel **C**) concentration, as well as of reaction time (panel **D**). The IκBα-Ub conjugates were categorized as classes I-III, representing ubiquitination products with apparent molecular masses greater than 250KDa (class I), between 75-250KDa (class II), and below 75KDa (class III), respectively. Class I and Class I-III products were quantified by Phosphoimaging and are presented graphically.

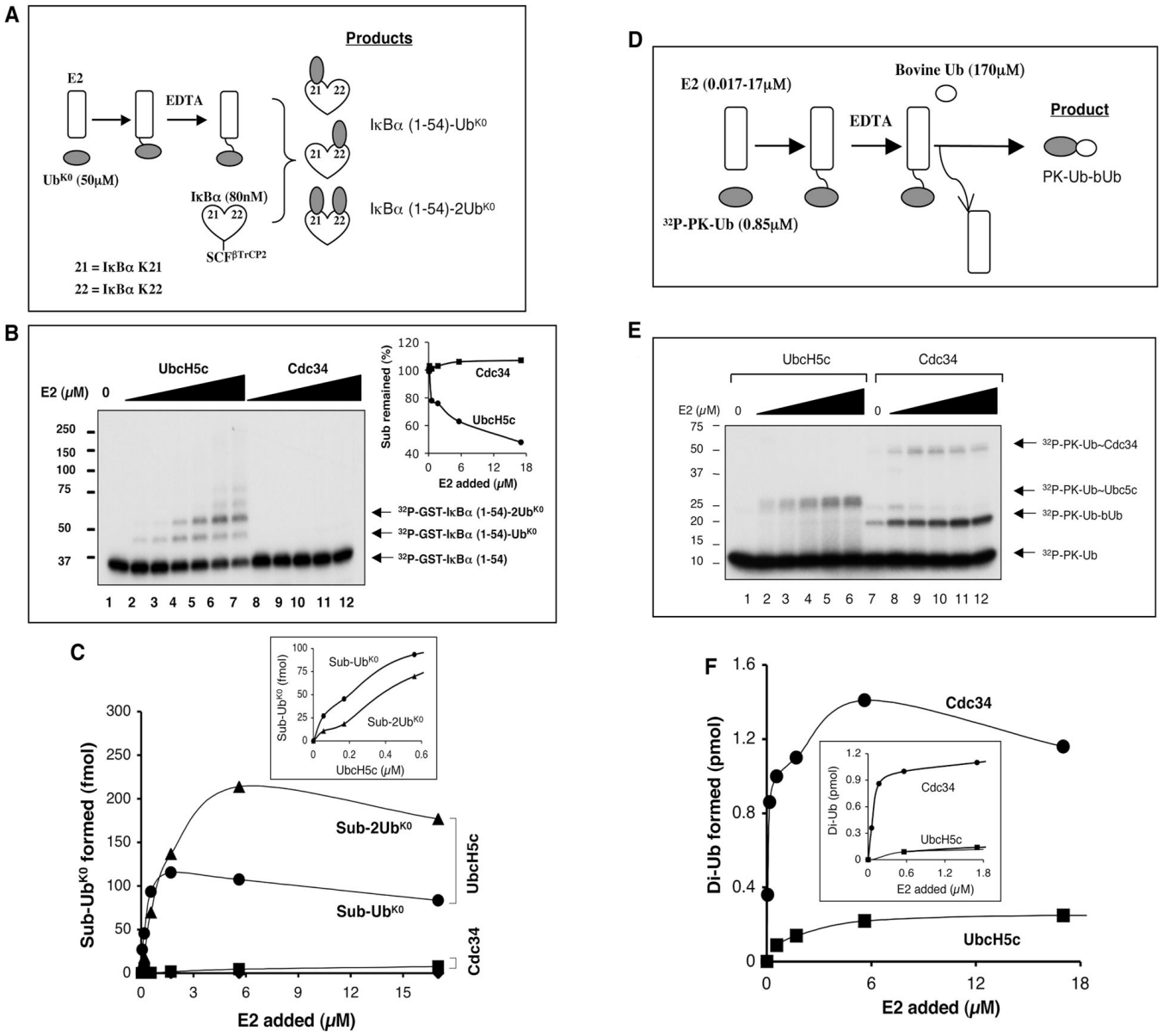

Fig. 2. UbcH5c and Cdc34 catalyze the ligation of Ub to IκBα and Ub-Ub conjugation, respectively

Panel **A** shows the scheme for the single turnover assay that measures the production of mono-ubiquitinated IκBα at K21/K22. In panel **B**, a single turnover assay with Ub^{K0} was carried out as a function of E2 concentration. Substrate utilization is shown graphically. Panel **C** shows the quantification of the ubiquitination products, with the inset graph revealing the production of single and double Ub^{K0} conjugates in the low range of UbcH5c concentrations. Panel **D** shows the scheme for the single turnover assay that measures the production of substrate-free di-Ub. Panels **E** and **F** show auto-radiographic and quantification analysis of di-Ub synthesis with varying E2 concentrations, with the inset graph revealing the production of di-Ub in the low range of Cdc34 and UbcH5c concentrations.

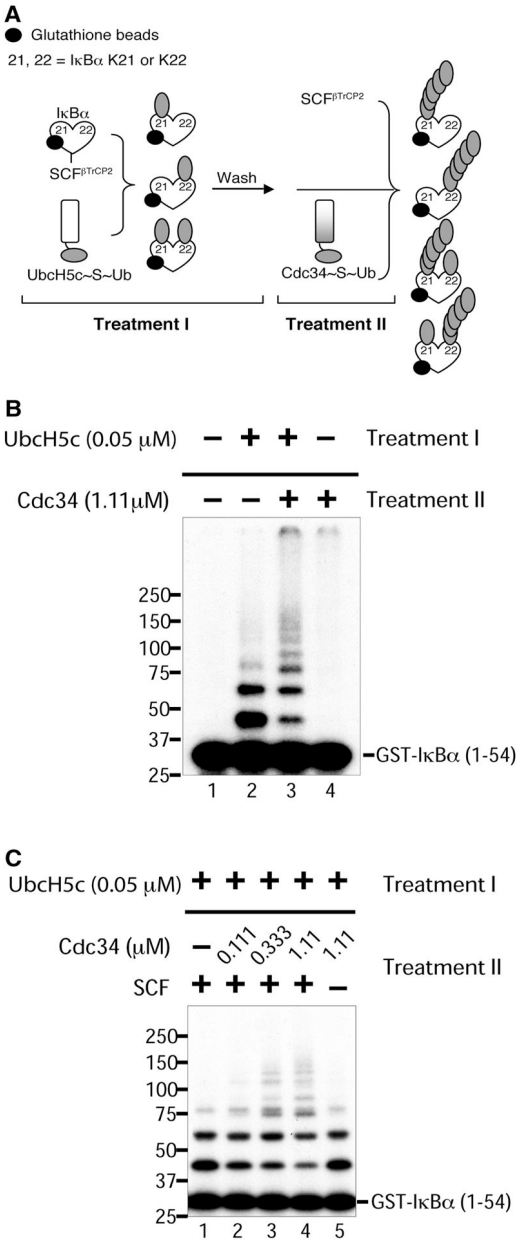

Fig. 3. Cdc34 is able to conjugate the donor Ub to a receptor Ub that is pre-conjugated to IκBα by UbcH5c

Panel **A** shows the reaction scheme. ^{32}P-GST-IκBα (20nM) was bound to glutathione beads (10μl) in a reaction mixture as described for Figs. 1B-D with PK-Ub (8μM), E1 (10nM), UbcH5c (0.05μM) and SCF$^{\beta TrCP2}$ (50nM). The reaction was incubated at 37°C for 60min, on a thermomixer (1,400rpm; Eppendorf). After washing, the beads were incubated with the second reaction mixture containing the same components as described above for another 60min, with the exception that Cdc34 (in amounts as indicated) was added in place of UbcH5c. The reaction products are shown in panels **B** and **C**.

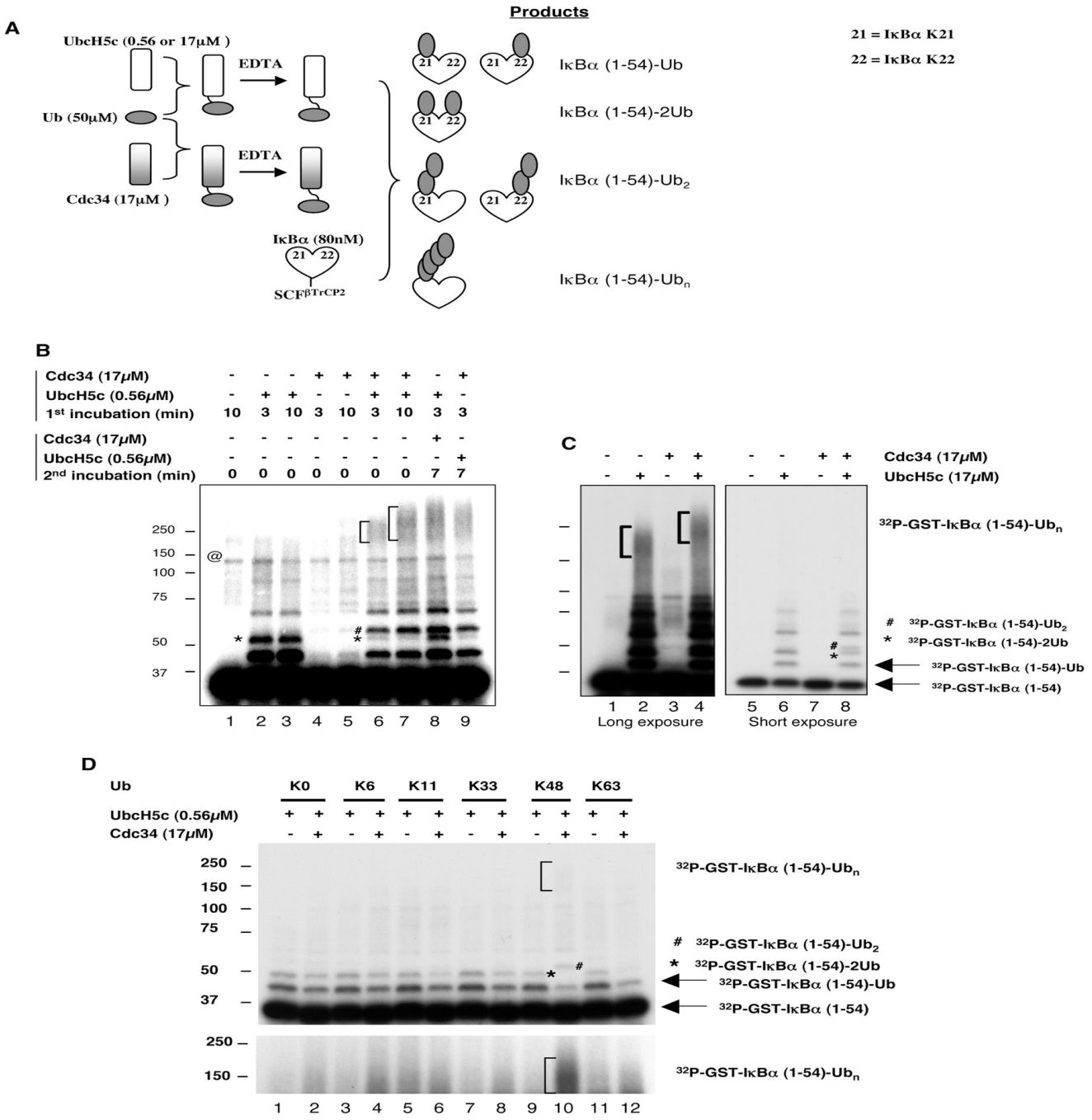

Fig. 4. Cdc34 builds Ub chains from UbcH5c-primed IκBα-linked Ub

Panel **A** shows the scheme for the E2~S~Ub mixing assay that monitors the assembly of Ub chains on IκBα, with various products formed as specified. In panels **B** and **C**, reactions were carried out with Ub^{K48}, Cdc34 (17μM), as well as UbcH5c at 0.56 or 17μM, respectively. @ Denotes background radioactive materials in the range of 100-150KDa that are most likely residual amounts of labeled Flag-IKKβ$^{S177E/S181E}$. In panel **D**, various forms of single K-only Ub variants, as indicated, were used along with Cdc34 (17μM) and UbcH5c (0.56μM). Long exposure of the upper portion of the autoradiogram is also shown to better reveal the increased production of high molecular weight forms of polyubiquitinated species in the presence of Ub K48. Reaction products are marked on the gel.

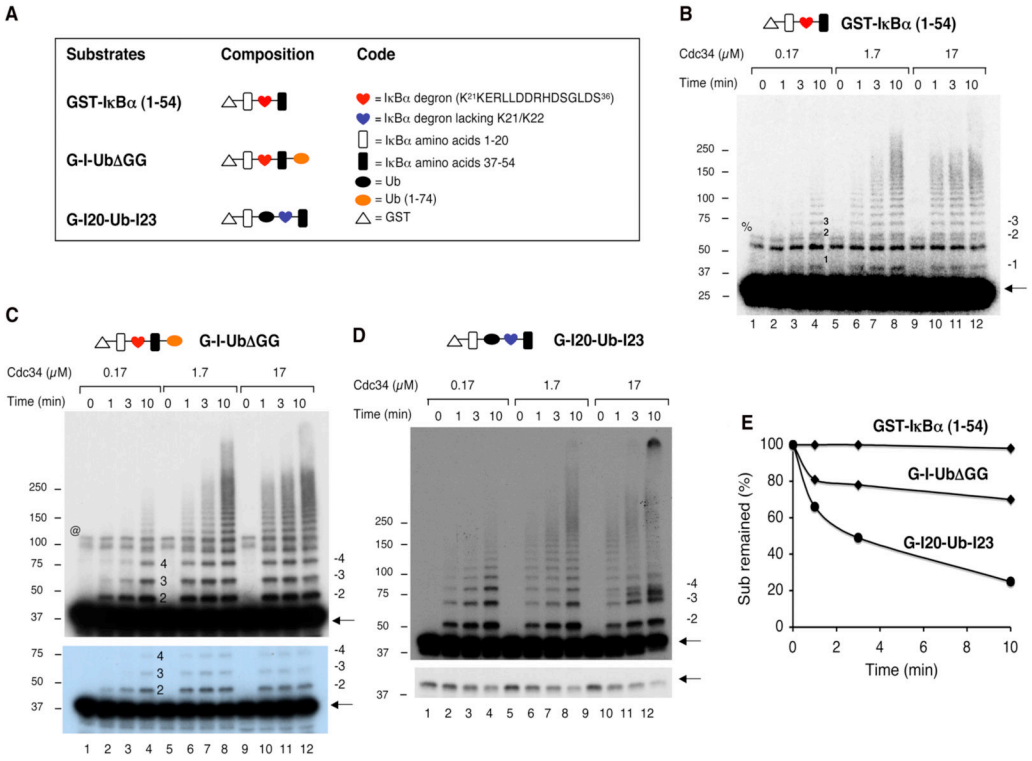

Fig. 5. Placement of an Ub at IκBα K21 directs Cdc34 for efficient chain elongation

A) Composition of IκBα-Ub fusion substrates with symbols denoting IκBα elements, Ub, or GST. B-D) Kinetic analysis of GST-IκBα (1-54), G-I-UbΔGG and G-I20-Ub-I23. $SCF^{\beta TrCP2}$-substrate and Cdc34~S~PK-Ub (in concentrations as indicated) were preformed, then mixed and incubated for times as indicated. Short exposures show the reduction of the input substrates and the production of Ub conjugates of limited length. Marks "1-4" indicate the number of Ub moieties contained in the products. In **B**, % denotes background radioactive materials in the range of 50-60KDa that are most likely residual amounts of labeled cAMP kinase. In **C**, @ marks background radioactive materials at the range of 100KDa that are most likely contaminating, residual amounts of labeled Flag-$IKK\beta^{S177E/S181E}$. **D) Quantification**. Graphs show reduction of each of the three substrates with Cdc34 at 17μM.

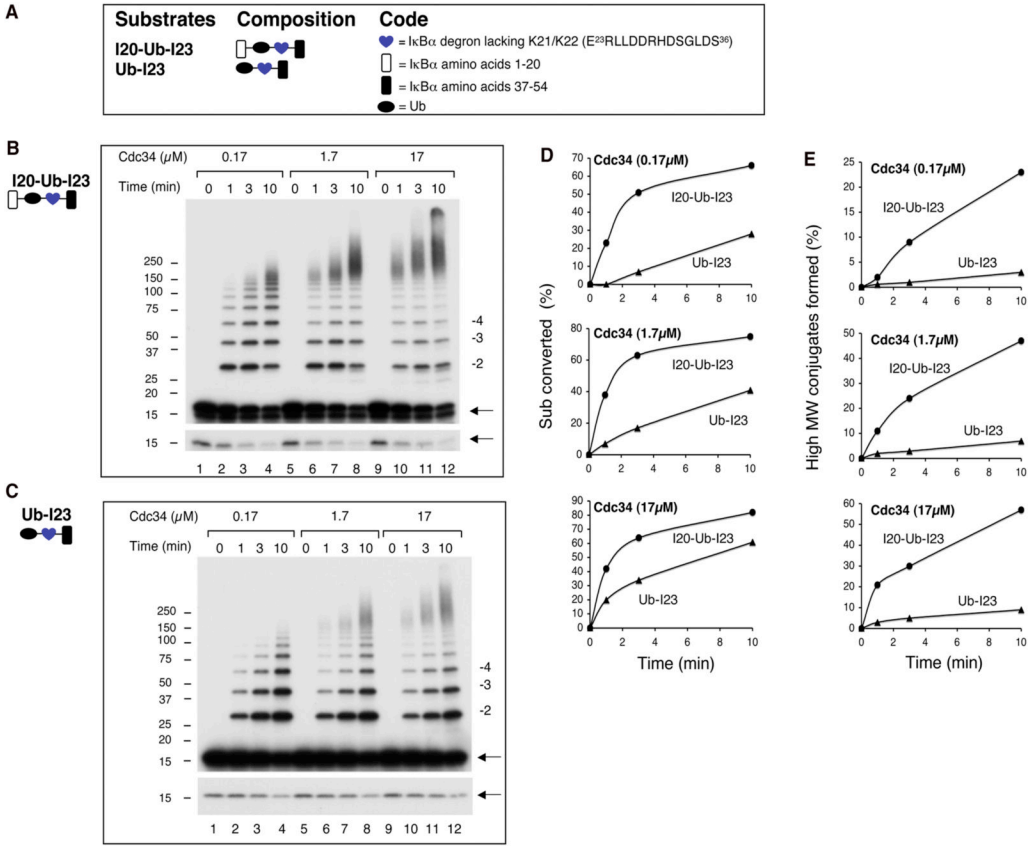

Fig. 6. The IκBα N terminus is required for Ub chain elongation by Cdc34

A) Composition of IκBα-Ub fusion substrates with symbols denoting IκBα elements, Ub, or GST. B-D) Kinetic analysis of I20-Ub-I23 and Ub-I23. $SCF^{\beta TrCP2}$-substrate and Cdc34~S~PK-Ub (in concentrations as indicated) were preformed, then mixed and incubated for times as indicated. A short exposure shows the reduction of the input substrate. Marks "2-4" indicate the number of Ub moieties contained in the products. **D) Quantification**. Graphs show substrate utilization and accumulation of high molecular weight products (containing more than five Ub moieties).

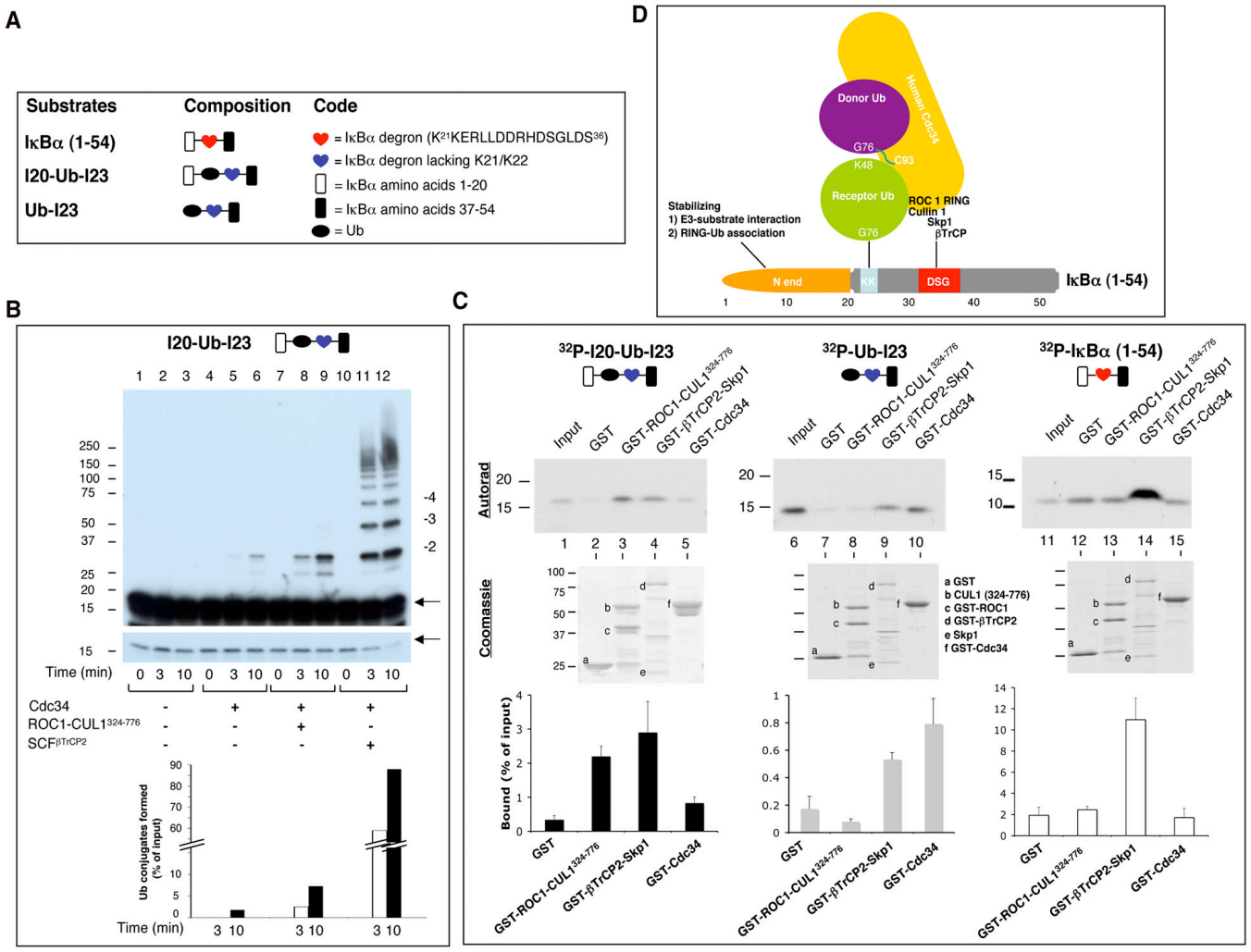

Fig. 7. Analysis of the roles played by Cdc34 and SCF's RING sub-complex in ubiquitination directed by IκBα-linked Ub

A) Composition of IκBα-Ub fusion substrates with symbols denoting IκBα elements, Ub, or GST. B) Ubiquitination analysis of I20-Ub-I23 with Cdc34 alone, or in the presence of $SCF^{\beta TrCP2}$ or ROC1-$CUL1^{324-776}$. The ubiquitination reaction was carried out with $SCF^{\beta TrCP2}$ (0.6pmol; lanes 10-12) or ROC1-$CUL1^{324-776}$ (6pmol; lanes 7-9) during pre-incubation with I20-Ub-I23. A short exposure shows the reduction of the input substrate. Marks "2-4" indicated the number of Ub moieties contained in the products. Bar graph shows the levels of ubiquitination products formed, expressed as a percentage of the input substrate. **C) GST pull-down analysis of interactions between IκBα-linked Ub and Cdc34 or ROC1-$CUL1^{324-776}$**. Input represents 1% of the radioactive substrate used. Note that the graph integrates the results of at least three independent experiments, with error bars for the calculated standard deviation. On the Coomassie blue-stained gel, letters refer to polypeptides as designated. **D) Multifaceted interactions are required to position the receptor Ub for chain elongation on IκBα**. See text for the description of this model.