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Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation

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Summary

Conjugation of ubiquitin-like protein Nedd8 to cullin (i.e. neddylation) is essential for the function of cullin-RING ubiquitin ligases (CRLs). Here we show that neddylation stimulates recruitment of ubiquitin-conjugating enzyme (E2) esterified with ubiquitin (E2~Ub), helps bridge the ~50 Å gap between E2 and substrate bound to SCF to enable their reaction, and facilitates formation of amide bonds in E2's active site. Together, these effects potently stimulate transfer of ubiquitin to substrate. We propose that the initiator ubiquitin spans the gap, and the impact of neddylation on transfer of subsequent ubiquitins by the E2 Cdc34 arises from improved E2 recruitment and enhanced amide bond formation in the E2 active site. The combined effects of neddylation greatly enhance the probability that a substrate molecule acquires ≥4 ubiquitins in a single encounter with a CRL. The surprisingly diverse effects of Nedd8 conjugation underscore the complexity of CRL regulation and suggest that modification of other ubiquitin ligases with ubiquitin or ubiquitin-like proteins may likewise have major functional consequences.

Introduction

Protein modification by the attachment of ubiquitin to cellular proteins is a key mechanism in regulating many cellular and organismal processes. Ubiquitin is covalently attached to target proteins via an isopeptide bond between the C-terminus of ubiquitin and a lysine residue of the acceptor substrate (Pickart, 2004). Additional ubiquitins can be conjugated to any of the seven lysine residues of ubiquitin to form a polyubiquitin chain on the substrate. Assembly of a chain of ≥4 ubiquitins linked together via Lys48 marks cellular proteins for degradation by the 26S proteasome (Chau et al., 1989; Thrower et al., 2000). In contrast, monoubiquitination serves as a non proteolytic signal in intracellular trafficking, DNA repair and signal transduction pathways (Hicke et al., 2005).

Ubiquitination of proteins is achieved through an enzymatic cascade involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes (Dye and Schulman, 2007). Ubiquitination occurs when an E3 binds to both substrate and an E2 thioesterified with ubiquitin (E2~Ub), bringing them in proximity so that the ubiquitin is transferred from E2 to substrate, either directly or via a covalent E3~ubiquitin thioester intermediate. The pairing of E2s and substrates by E3s determines specificity in ubiquitination. There are two major types of E3s in eukaryotes, defined by the presence of either a HECT

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domain or a RING fold (Pickart, 2001). HECT-domain E3s form a thioester intermediate with ubiquitin whereas RING ligases facilitate direct transfer of ubiquitin from E2~Ub to the substrate. RING ligases are conserved from yeast to human, with more than 500 different RING ubiquitin ligases being potentially expressed in human cells. However, the mechanism of ubiquitin transfer by these enzymes remains unknown.

The most intensively studied RING E3s are members of the cullin-RING ligase (CRL) superfamily (Cardozo and Pagano, 2004; Petroski and Deshaies, 2005a; Willems et al., 2004). CRLs are modular multisubunit complexes that contain a cullin scaffold and a zinc-binding RING domain subunit. The C-terminal region of the cullin binds to the RING protein, which recruits the E2 to form the enzymatic core, whereas the N-terminal region of cullin recruits substrate receptors via adapter proteins. SCF, the prototype of the CRLs, consists of the cullin Cul1, the RING subunit Rbx1/Roc1/Hrt1, the adapter protein Skp1, and an F-box protein such as Skp2 or β-TrCP that binds substrate. Substrates recruited to SCF for ubiquitination are usually covalently modified by phosphorylation (Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997). Yeast SCF complexes specifically employ Cdc34 as the E2 (Feldman et al., 1997; Skowyra et al., 1997), whereas human SCF utilizes either Cdc34 or UbcH5c (Butz et al., 2005), although the basis for differentiating between E2s is not known. Cdc34 predominantly forms Lys48- linked polyubiquitin chains, whereas UbcH5c forms polyubiquitin chains linked through multiple lysine residues (Kim et al., 2007).

Whereas substrate recruitment to SCF is now understood for some complexes, the actual ubiquitination reaction has resisted detailed description. The ubiquitination reaction catalyzed by E2~Ub–SCF can be subdivided into two steps: transfer of the first ubiquitin to substrate (chain initiation) and polymerization of ubiquitin chains by formation of ubiquitin-ubiquitin linkages (chain elongation) (Petroski and Deshaies, 2005b). Perplexingly, structural studies of SCF subcomplexes suggest that there is a ~50 Å gap between bound substrate and the active site cysteine of E2 docked on SCF (Orlicky et al., 2003; Wu et al., 2003; Zheng et al., 2002). Hence it is unclear how SCF facilitates chain initiation, since for ubiquitin transfer to occur substrate must contact the thioester bond that joins ubiquitin to E2.

CRL enzymes are regulated by a reversible covalent modification of the cullin with the ubiquitin-like protein, Nedd8 (Pan et al., 2004). The neddylation pathway is essential among eukaryotes, with the exception of budding yeast (Osaka et al., 2000; Tateishi et al., 2001). The conjugation of Nedd8 requires a ubiquitin-like enzyme cascade involving the Nedd8-activating enzyme AppBp1-Uba3, the Nedd8-conjugating enzyme Ubc12, the RING protein Rbx1, and the activator Dcn1, resulting in neddylation of Cul1 at lysine 720 (Kamura et al., 1999; Kurz et al., 2005; Pan et al., 2004).

Nedd8 conjugation coupled with F-box–Skp1 binding modulates the assembly of CRLs by displacing the sequestration factor CAND1 from Cul1, resulting in the assembly of an intact functional SCF complex (Goldenberg et al., 2004). However, the Nedd8 conjugation pathway remains essential even in the absence of CAND1 suggesting that Nedd8 regulates CRLs primarily by other mechanisms (Chuang et al., 2004). Previous studies have reported that Nedd8 modification of Cul1 stimulates ubiquitination of the substrates p27 and IκB by SCF^{Skp2} and $SCF^{\beta\text{-}TrCP}$ respectively (Furukawa et al., 2000; Morimoto et al., 2000; Podust et al., 2000; Read et al., 2000; Wu et al., 2000). Neddylation is thought to activate SCF by stabilizing its association with Ubc4 (a close relative of UbcH5), apparently via a Nedd8 binding site on Ubc4 (Kawakami et al., 2001; Sakata et al., 2007). However, the same site on UbcH5 binds ubiquitin and promotes ubiquitination by the E3 BRCA1, which is not modified by Nedd8 (Brzovic et al., 2006). Moreover, Cdc34 lacks the equivalent binding site. These observations raise the question of whether enhanced recruitment of E2~Ub is the primary mechanism by which neddylation activates SCF.

In this work, we performed detailed enzymological analysis of SCF in a reconstituted system and devised new assays to explore the impact of Nedd8 conjugation on SCF activity. Our work shows that Nedd8 has a profound effect on SCF, stimulating every functional parameter that was evaluated. Importantly, neddylation enhances chain initiation on substrates by 'bridging the gap' that exists between the substrate and the E2 in the structure of SCF. Overall, neddylation greatly increase the probability that a substrate acquires a tetraubiquitin chain in a single encounter with SCF.

Results

Neddylation enhances Cdc34 binding to SCF

To measure the impact of Nedd8 conjugation on the transient association between E2 and SCF in solution we developed an assay based on fluorescence resonance energy transfer (FRET). The human UbcH5c and Cdc34 genes were fused to a hexapeptide sequence that binds covalently to the dye Lumio Green (LG) (Adams et al., 2002). Incorporation of LG was efficient (data not shown) and did not compromise E2 activity (Figure S1). To mimic thioesterification of E2 with ubiquitin, we generated E2~Ub species containing a stable oxyester bond (Figure S1). SCF was labeled by fusing cyan fluorescent protein (CFP) to the C-terminus of Cul1. CFP had little effect on the E3 activity (Figure S1). To test the effect of neddylation, Cul1 (CFP)–Rbx1 was quantitatively neddylated and purified (Figure S1). For all experiments we used the human 'split Cul1' developed by Zheng et al. (2002). Both unmodified and neddylated 'split Cul1' behave indistinguishably from intact Cul1 in both protease hypersensitivity and ubiquitin ligase assays (B. Schulman, personal communication).

We observed efficient FRET with Cul1 (CFP)–Rbx1 and LG-labeled Cdc34 (Figure 1A). For equilibrium binding, Cdc34-LG titration yielded a dissociation constant (K_d) of 82 ± 10 nM, with neddylation lowering the K_d by 3-fold (Figure 1B). A similar effect of neddylation was seen with Cdc34~Ub, but K_d values were ~2-fold lower (Figure 1C and S2). This indicates that both neddylation of Cul1 and esterification of Cdc34 made positive but relatively minor contributions to E2–E3 interaction (Figure 1D). The maximum FRET efficiencies for all pairs were within the experimental error, suggesting that there were no major changes in average distance between the donor (C-terminus of Cul1) and the acceptor (C-terminus of Cdc34) molecules upon neddylation of Cul1 or charging of Cdc34. We were not able to carry out binding experiments with LG-labeled UbcH5c and Cul1 (CFP)–Rbx1, due to poor binding affinity (Figure S2).

Neddylation enhances both E2 recruitment and β-catenin turnover by $SCF^{β\text{-}TrCP}$

The relatively modest effects of neddylation observed by FRET is difficult to reconcile with the >10-fold stimulation of Cdc34-dependent SCF activity upon neddylation (Podust et al., 2000; Wu et al., 2002). Therefore, to address in detail how neddylation enhances SCF activity, we undertook a kinetic analysis of substrate ubiquitination using recombinant SCF.

SCF can be regarded as a bi-substrate enzyme upon whose surface E2~Ub and substrate react to yield an ubiquitinated substrate and a discharged E2 (Petroski et al., 2006). To evaluate the impact of neddylation on binding of E2 and transfer of ubiquitin to substrate, we carried out ubiquitination reactions with saturating amounts of a chemically synthesized β-catenin phosphopeptide substrate bearing a single lysine (Wu et al., 2003) using unmodified or quantitatively neddylated $SCF^{β\text{-}TrCP}$ (Figure S3) in the presence of varying concentrations of Cdc34. Although we have not evaluated the K_m for substrate binding, neddylation has no effect on this parameter (Kawakami et al., 2001; Read et al., 2000).

Kinetic data from the assay described above highlighted three major points. First, in agreement with FRET, neddylation lowered the K_m for Cdc34 by 4-old (Figure 2A and Table 1). Second, $K_m \gg K_d$, suggesting that the Michaelis complex (Cdc34~Ub–$SCF^{\beta\text{-}TrCP}$–β-catenin) was not in rapid equilibrium with free Cdc34~Ub. Third, neddylation stimulated the maximal rate of ubiquitination observed under our reaction conditions (k_{cat}) by 3.6-fold at saturating Cdc34 (Figure S4 and Table 1). Thus, neddylation not only enhanced binding of Cdc34~Ub, but also stimulated the transfer of ubiquitin from SCF-bound Cdc34~Ub to the substrate lysine. Overall, neddylation improved k_{cat}/K_m by nearly 18-fold. Notably, modified substrates acquired long ubiquitin chains despite the presence of a large excess of unmodified substrate, regardless of the neddylation status of SCF. This implies that ubiquitination by Cdc34–SCF is processive (which is shown rigorously in Figure 5), but that Nedd8 has only a modest effect on processivity.

Whereas these results pointed to multiple effects of neddylation, prior work with Ubc4 emphasized Nedd8's effect on stabilizing E2–E3 association (Kawakami et al., 2001). To address whether Nedd8 acts differently on Cdc34 and UbcH5c, we performed kinetic analyses with the latter. Unlike Cdc34, UbcH5c can ubiquitinate Lys720 of Cul1, mimicking the effects of neddylation (B. Schulman, personal communication and Figure S5). The significance of this is not understood, and was not explored here. To circumvent this potential complication, all UbcH5c reactions with unmodified SCF employed K720R Cul1 which did not affect basal SCF activity (Figure S5).

Three main points emerge from the kinetic data obtained with UbcH5c. First, neddylation had a larger effect on reducing the K_m for UbcH5c (Figure 2B and Table 1) than it did for Cdc34 (15- vs. 4-fold). This is consistent with previous reports that neddylation greatly enhances coimmunoprecipitation of Ubc4 with $SCF^{\beta\text{-}TrCP}$ (Kawakami et al., 2001) and Nedd8 binds Ubc4 (Sakata et al., 2007). Second, Nedd8 stimulated the rate of β-catenin ubiquitination at saturating UbcH5c by 2.5-fold (Figure S4 and Table 1). Overall, neddylation improved k_{cat}/K_m by 38-fold. Third, neddylation considerably enhanced the length of ubiquitin chains conjugated to substrate by UbcH5c (Figure 2B, compare lanes 9 and 20).

The maximal rates of substrate ubiquitination reported above for Cdc34 and UbcH5c were estimated based on the number of substrates modified per minute. However, the total number of ubiquitins transferred is also dependent on the number of ubiquitins added per molecule of modified substrate. When this is taken into account, neddylation increased the maximal rate of total ubiquitination by 7-fold for Cdc34 and 5-fold for UbcH5c.

Neddylation improves E2 recruitment and rate of ubiquitin transfer by SCF^{Skp2}

To address the generality of the results we obtained with the $SCF^{\beta\text{-}TrCP}$-β-catenin E3−substrate pair, we examined the effect of neddylation on both K_m and k_{cat} for ubiquitination of the native protein substrate p27 by SCF^{Skp2}. Ubiquitination and subsequent degradation of p27 is initiated by phosphorylation on Thr187 and is stimulated by p27's association with Cdk2−CycE (Montagnoli et al., 1999). In agreement with previous results (Shirane et al., 1999), the use of chain-terminating K0 ubiquitin revealed that at least 3 residues within p27 were ubiquitinated (Figure S6). We then compared the rate of ubiquitination of p27−Cdk2−CycE heterotrimeric complex with both Cdc34 and UbcH5c. Neddylation of SCF^{Skp2} reduced the K_m for Cdc34 by 2-fold (Figure S7 and Table 1) and enhanced k_{cat} by 6-fold (Figure S8 and Table 1). Similar results were seen with UbcH5c: Nedd8 lowered K_m by 3.5-fold (Figure S7 and Table 1) and increased k_{cat} by 2.4-fold (Figure S8 and Table 1). Thus, for two different E2s, neddylation of SCF^{Skp2} caused reduction in K_m and increase in k_{cat} that together translated into 8 to 12-fold enhancements in k_{cat}/K_m. These results combined with the $SCF^{\beta\text{-}TrCP}$–β-catenin data indicate that Nedd8 did at least two things: it increased recruitment of E2~Ub to SCF and increased the rate of ubiquitination of bound substrates.

Neddylation increases the rate of chain elongation of an ubiquitinated substrate

Ubiquitination of Sic1 by yeast Cdc34–SCF^{Cdc4} is a two step process involving a slow initiation step followed by rapid Lys48 specific chain elongation (Petroski and Deshaies, 2005b). The data in Table 1 show that Nedd8 attachment stimulated the initiation step, since it enhanced the rate of conversion of substrate from an unmodified form to an ubiquitinated species.

Visual inspection of autoradiograms suggests that neddylation also enhanced chain elongation by both E2s. To directly address this point in a quantitative manner we generated a monoubiquitinated β-catenin phosphopeptide substrate (Ub−β-catenin; Figure S9), and measured the kinetics of its ubiquitination by $SCF^{\beta\text{-}TrCP}$ using Cdc34 or UbcH5c as E2. Three notable observations emerged from these experiments (Figure 2C, 2D, S10 and Table 1). First, neddylation improved k_{cat} and lowered K_m resulting in an overall increase in k_{cat}/K_m for both Cdc34 and UbcH5c. Thus, neddylation stimulates chain elongation, although the magnitude of this effect varied considerably. Second, with Cdc34, Ub−β-catenin substrates were ubiquitinated 15–30 fold more rapidly than β-catenin substrates. This is consistent with Cdc34 attaching the first ubiquitin more slowly than it builds polyubiquitin chains (Petroski and Deshaies, 2005b). Third, k_{cat}/K_m for UbcH5c remained essentially unchanged (Table 1). Hence, unlike Cdc34, UbcH5c shows little preference for chain elongation over chain initiation. Given that Ubc4 modifies 3 sites on ubiquitin in roughly equivalent stoichiometry (Kirkpatrick et al., 2006), it appears that UbcH5c modifies any one of these sites about 3-fold more slowly than it modifies the lysine on the β-catenin phosphopeptide. This resonates with the observation that Ubc4 plus APC monoubiquitinate multiple lysines of cyclin B before forming ubiquitin-ubiquitin linkages (Kirkpatrick et al., 2006). Remarkably, reactions with UbcH5c and unmodified $SCF^{\beta\text{-}TrCP}$, ubiquitination actually slowed down by 6-fold when Ub −β-catenin was used instead of β-catenin. This was accompanied by a 6-fold decrease in K_m for UbcH5c, which accounts for the lack of change in k_{cat}/K_m (Table 1). It appears that UbcH5c~Ub–$SCF^{\beta\text{-}TrCP}$–Ub−β-catenin is trapped in a high affinity albeit non-productive complex, and neddylation alters the complex to a productive conformation that accelerates the reaction of the bound species by over 16-fold.

Ubiquitination is limited by the dynamics of substrate binding and product dissociation

In multi-turnover reactions such as those shown in Figures 2, the k_{cat} for substrate consumption is the combined rate of the chemical step as well as the rate at which substrates bind and products dissociate from the enzyme. If substrates and products are in rapid equilibrium with the enzyme, the k_{cat} approaches the rate of the chemical step. Alternatively, if the chemical step is fast and either substrate binding or product dissociation is slow, k_{cat} is constrained by the slow step and underestimates the rate of the chemical step. In the specific case of SCF, the pattern of products that are formed suggests that the dynamics of substrate binding and product dissociation restrain reaction rates.

To accurately estimate the impact of neddylation on the rate of ubiquitin transfer (k_{obs}), we carried out single-turnover reactions with both E2s and both SCF-substrate complexes (Figure 3 and S11). In these experiments, SCF was present in stoichiometric excess of substrate and E2~Ub was saturating SCF. The rate of ubiquitin transfer was estimated based on the rate of consumption of unmodified substrate (Table 2). We highlight three salient observations from these experiments. First, neddylation enhanced the rate of ubiquitin transfer in all four pair-wise combinations. This supports a role of neddylation in stimulating the transfer of ubiquitin from E2~Ub to substrate lysine. Second, in every case k_{obs} was greater than k_{cat} measured in multi-turnover reactions. This suggests that the substrate and ubiquitinated product are not in rapid equilibrium with SCF; rather, product dissociation is a slow step, as expected for a processive reaction. Third, the rates of ubiquitin transfer for a given E2 converge – i.e. the rates of β-catenin and p27 modification by unmodified Cdc34~Ub–SCF complexes were nearly

identical, as were the magnitude of rate enhancement upon neddylation. This is notable considering the major differences in these two different SCF–substrate pairs. Our favored explanation is that under conditions where the impacts of E2 and substrate binding dynamics are neutralized, the principal effect of neddylation is on the rate-limiting chemistry that occurs at the E2 active site, which is characteristic for a particular E2, but largely independent of substrate. Interestingly, even for the fastest rates that we measured, k_{obs}/K_m values were in the range of 10^4–10^5 M^{-1} E2 sec^{-1} which is consistent with the idea that isopeptide bond formation was rate-limiting.

Neddylation has two distinct effects on the chemical step

Single-turnover experiments revealed that Nedd8 conjugation promotes β-catenin ubiquitination with either E2 by ~10 fold. This enhancement could be due to global effects on the positioning of the E2 and the substrate that are held ~50 Å apart in the crystal structure of unmodified SCF (Orlicky et al., 2003; Wu et al., 2003; Zheng et al., 2002) or to local effects on the chemical environment of the E2 active site or both.

To explore whether neddylation accelerates ubiquitination by bridging the ~50 Å gap between the bound substrate and E2, we performed a crosslinking experiment utilizing a heterobifunctional crosslinker (BMPS) with NHS ester and maleimide groups. BMPS was first attached to the β-catenin peptide via the lysine residue and then mixed with unmodified or neddylated $SCF^{β\text{-}TrCP}$ in the presence of saturating UbcH5c. With unmodified $SCF^{β\text{-}TrCP}$, the peptide was crosslinked only to β-TrCP (Figure 4A, lane 7). However, in the presence of Nedd8 modification, the peptide primarily crosslinked to UbcH5c (Figure 4A, lane 8). Importantly, crosslinking of β-catenin to UbcH5c was abolished by mutation of the active site cysteine (Figure 4A, lane 10). Moreover, crosslinking of β-catenin to UbcH5c occurred only in the context of the Nedd8-modified $SCFis^{β\text{-}TrCP}$ holoenzyme; β-catenin did not crosslink to UbcH5c in the absence of SCF (Figure 4A, lane 5) or in the presence of Cul1-Rbx1 (Figure 4A, lanes 3 and 4). Unfortunately we were unable to perform similar test with Cdc34 due to its non-specific crosslinking with the peptide in the absence of SCF.

To further explore the effect of Nedd8 conjugation, we devised an assay to evaluate whether neddylation accelerates catalysis that is independent of juxtaposition of E2~Ub and substrate. The assay involves ubiquitin discharge from Cdc34~Ub to a nucleophile, hydroxylamine, in the presence of unmodified or neddylated SCF. The idea underlying this experiment is that freely diffusing hydroxylamine can collide with the thioester to form Ub-NHOH (confirmed by mass spectrometry; data not shown), and the rate at which this occurs is a measure of the ability of Cdc34's active site to catalyze the formation of an amide linkage. Whereas both unmodified and neddylated SCF activated the discharge of Cdc34~Ub, the reaction was 2.8-fold faster with neddylated enzyme (Figure 4B). In a related experiment, we saw a similar ~2.2-fold rate enhancement for the formation of diubiquitin upon neddylation (Figure S12). Together, these results imply that Nedd8 can modestly stimulate isopeptide bond formation independent of any conformational effects it may have in promoting juxtaposition of substrate and E2~Ub bound to SCF.

Neddylation allows substrates to acquire longer ubiquitin chains in a single round of SCF binding

Neddylation influenced every measurable parameter in ubiquitination by SCF tested in our assays. The key issue from a physiological perspective is to what degree the catalytic effects, when combined influence the likelihood that a substrate acquires a degradation-competent ubiquitin chain in a single binding event with SCF. It is generally assumed that the chain must be acquired in a single encounter with SCF, because if a partially ubiquitinated substrate dissociates from SCF it might interact with numerous monoubiquitin-binding domains or be

subject to deubiquitination. In light of this, we sought to address two questions: what is the maximal amount of ubiquitination that can occur each time a substrate binds SCF and how does neddylation influence the outcome? Accordingly, we devised a chase protocol to monitor substrate ubiquitination under conditions that prevent rebinding to SCF. We refer to this as the 'single encounter' assay.

A single encounter assay has 3 sets of reactions. In reaction 1 (Figure 5, lanes 2–4), ubiquitination was initiated by mixing one tube containing ^{32}P-labeled β-catenin substrate and SCF$^{\beta\text{-TrCP}}$ with a second tube containing E1, E2, ATP, and ubiquitin. This reaction documents the progress of a single-turnover reaction as described earlier. Reaction 2 (Figure 5, lanes 5–7) was the same, except that the first tube contained additional unlabeled substrate in a large excess. This control documents the efficacy of the competitor. In reaction 3 (Figure 5, lanes 8–10), competitor was present in tube 2. This measures the progress of ubiquitination for ^{32}P-labeled β-catenin substrate that was pre-bound to SCF$^{\beta\text{-TrCP}}$ prior to mixing tubes 1 and 2.

The data in Figures 5A and 5B confirm that labeled β-catenin peptide was rapidly ubiquitinated by Cdc34~Ub-SCF$^{\beta\text{-TrCP}}$, but that this reaction was largely blocked by cold peptide. When the reaction was carried out with unmodified Cdc34~Ub–SCF$^{\beta\text{-TrCP}}$ under single encounter conditions (Fig. 5A lanes 8–10), 2% of input substrate was converted to ubiquitinated products. By contrast, in a single encounter reaction carried out with neddylated Cdc34~Ub–SCF$^{\beta\text{-TrCP}}$, 13% of substrate was modified (Fig. 5B lanes 8–10). Further, it is readily apparent that neddylation increased the average number of ubiquitin molecules conjugated to modified substrate, although we were unable to accurately quantify the difference due to the reaction's low efficiency (Figure 5C and Table 2). To facilitate quantification, we repeated the experiment with Ub−β-catenin substrate (Figure S13). A higher fraction of Ub−β-catenin was consumed in a single encounter with both unmodified and neddylated SCF$^{\beta\text{-TrCP}}$ (Table 2), and neddylation increased the number of ubiquitin molecules transferred per modified product from 3.5 to 6.5. With UbcH5c, single encounter experiments with β-catenin substrate yielded qualitatively similar results (Figure 5D to 5F). Neddylation enhanced the fraction of substrate molecules that were modified and increased the number of ubiquitins attached to the substrate (Table 2). Taken together, these experiments show that neddylation accelerates both chain initiation as well as chain elongation, such that a higher fraction of substrates are modified, and those that are modified acquire longer ubiquitin chains.

Discussion

The sequence of events in a ubiquitination reaction

We propose the following sequence of events for ubiquitination of a substrate by SCF. Both substrate and E2~Ub bind SCF, most likely in a random order (Swinney et al., 2005). Since the E2~Ub and the substrate binding sites on SCF are at a considerable distance (Zheng et al., 2002), occupancy of these sites does not automatically place reactants in sufficient proximity for the transfer reaction to occur. Rather, substrate lysines sample the local environment until one productively collides with bound E2~Ub. The lysine–E2~Ub complex then proceeds to the transition state and a lysine−Ub isopeptide linkage is formed. The spent E2 dissociates, allowing a fresh E2~Ub to bind and resulting in the transfer of a second ubiquitin to form a chain.

This scenario identifies 6 steps that neddylation might stimulate: binding of substrate (1) or E2~Ub (2); juxtaposition of a substrate lysine and E2~Ub (3); stabilization of the oxyanion transition state (4); or dissociation of E2 (5) or Ub−substrate (6). Because chain initiation and elongation occur upon different substrates, the properties of these reactions and how they respond to neddylation may differ. Prior data exclude an effect of Nedd8 on step 1 (Kawakami et al., 2001; Read et al., 2000). We found that single-turnover reactions are substantially more

responsive to neddylation than multi-turnover reactions, which implies that the major impact is on steps 2–4. Indeed, we provide evidence that neddylation stimulates E2 binding (step 2), and promotes juxtaposition of substrate and E2~Ub (step 3) during chain initiation and stabilization of the transition state (step 4) during chain elongation, as discussed below. Our observations reveal the minimum effects that Nedd8 conjugation has upon ubiquitination of a substrate, and there may be additional effects that remain undetected. The broad scope of Nedd8's impact on SCF function hints at the complexity of target modulation by conjugation of ubiquitin-like proteins.

Neddylation enhances E2 recruitment

Neddylation enhances the recruitment of E2~Ub to SCF. Improved binding was manifested as a reduction in K_d for Cdc34~Ub, and a reduced K_m for E2 in all combinations tested. Esterification with ubiquitin also modestly improves the affinity of Cdc34 for SCF by ~2-fold. Consistent with our observation, Ubc2~Ub binds E3α 6-fold more avidly than Ubc2 (Siepmann et al., 2003). It may not be necessary to have a large difference in relative affinity of E2 and E2~Ub for E3 because in vivo, most E2 appears to be charged with ubiquitin (Jin et al., 2007).

Neddylation bridges the gap

The crystal structure of SCF suggested the existence of a ~50 Å gap between the substrate docking site and the active site of the E2. This has posed a conundrum for thinking about how SCF promotes substrate ubiquitination. To evaluate whether Nedd8 conjugation stimulates chain initiation by bringing substrate and E2~Ub into proximity (step 3), we tested whether neddylation enhances a crosslinking reaction between substrate and E2 that is completely orthogonal to the chemistry of isopeptide bond formation. The logic underlying this experiment is that 'global' effects of neddylation on juxtaposing reactants on the surface of SCF should translate to a distinct chemical reaction, whereas 'local' effects of Nedd8 on transition-state chemistry should not. Remarkably, β-catenin peptide was exclusively crosslinked to β-TrCP in the presence of unmodified SCF, but became primarily crosslinked to UbcH5c when assayed with neddylated SCF. This result suggests that neddylation potently stimulates chain initiation via a conformational change in SCF that allows substrate and E2~Ub to productively encounter each other.

Neddylation stabilizes the transition state

Neddylation of SCF stimulates the ability of hydroxylamine and free ubiqutin to attack Cdc34~Ub to yield Ub-NHOH and diubiquitin, respectively. Since these reactions involve acceptors that are not known to bind SCF, we propose that neddylation increases the k_{cat} for these reactions by altering the chemical environment of Cdc34's active site, rather than through a positioning effect. We assume that this k_{cat} effect applies to both chain initiation and chain elongation, which are modeled by formation of Ub-NHOH and diubiquitin, respectively. Though small, this effect is important as it substantially increases the fraction of substrate that acquires a chain of ≥4 ubiquitins in a single encounter of substrate with SCF. We speculate that neddylation provokes conformational changes within Rbx1 that are propagated to the active site of docked E2~Ub. Evidence for allosteric communication between the RING docking site and the active site of E2 has been reported for another E2–E3 pair (Ozkan et al., 2005).

Putting it together

The model developed above rationalizes numerous experimental observations. First, for Cdc34 reactions, neddylation stimulates chain initiation more than chain elongation. We propose that the 50Å gap is most difficult to bridge for transfer of the first ubiquitin to substrates whose

acceptor lysine(s) are positioned close to the ligase-binding epitope (e.g. IκB). Once ubiquitin is attached, the new Lys48 acceptor projects 25–30 Å from the isopeptide bond, bringing the nucleophile closer to the E2 active site. Thus, for chain initiation on substrates like IκB, both the positioning and active site effects of neddylation stimulate the reaction rate with the former making the greater contribution, whereas for chain elongation the impact of the positioning effect declines dramatically and consequently the active site effect dominates. Some substrates may have an extended structure and display lysines close to the E2 active site. The effect of neddylation on such substrates would be predicted to be modest – in line with the effects of neddylation on chain elongation by Cdc34 reported here.

In contrast to Cdc34, neddylation has a potent stimulatory effect on both chain initiation and elongation by UbcH5c. Once ubiquitin is conjugated to substrate the rate of chain elongation with unmodified SCF slows down even as the K_m for UbcH5c improves. Thus, whereas ubiquitin conjugated to substrate stabilizes formation of a productive complex with Cdc34~Ub-SCF, the same modification stabilizes formation of a non-productive ternary complex with UbcH5c. Neddylation relieves the inhibitory constraint imposed by the substrate-conjugated ubiquitin, and thereby restores activity to the same level seen with unmodified substrate.

The pervasive effects of neddylation on human SCF are most consistent with Nedd8 evoking a conformational change that impacts on multiple aspects of ubiquitination. Given the proximity of the Nedd8 conjugation site to the E2 docking site, the postulated conformational change may reconfigure the topology of the E2–E3 interface resulting in (i) enhanced affinity, (ii) allosteric changes in the E2 active site (Ozkan et al., 2005), and (iii) altered relative positioning of docked E2~Ub and substrate. Given the depth and breadth of Nedd8's effects on human SCF, it is difficult to understand why budding yeast SCF is so indifferent to this modification. In yeast SCF, sequence variation in either Cdc34 or SCF subunits might mimic the effect of neddylation or bound Sic1 may position its lysines near E2~Ub, thereby neutralizing much of the stimulatory effect of Nedd8 conjugation.

Why are UbcH5c and Cdc34 so different?

UbcH5c and Cdc34 employ different strategies to ubiquitinate substrate. In the presence of saturating UbcH5c, substrate in pre-formed complexes with SCF is almost quantitatively ubiquitinated before it dissociates (Figure 5), but on average is not conjugated with sufficient ubiquitins to serve as a substrate for the proteasome. By contrast, Cdc34 ubiquitinates only a small fraction of pre-bound substrate, but the substrates that are modified receive long chains. The net effect is that the fraction of pre-bound substrates that receive chains ≥4 ubiquitins is very similar for both UbcH5c and Cdc34. Perhaps the two E2s exploit their complementary strengths by collaborating in vivo, with UbcH5c promoting chain initiation and Cdc34 promoting chain elongation, as has been postulated for APC (Rodrigo-Brenni and Morgan, 2007). However, we have been unable to produce compelling biochemical data to support this model. Although mammalian SCF has been subject to considerable scrutiny, it remains unknown to what extent it depends directly on Cdc34 versus UbcH5 within cells.

The surprisingly diverse effects of Nedd8 conjugation underscore the complexity of ligase mechanism and regulation. Ubiquitin ligases remain among the most enigmatic of enzymes. If we are to understand how different patterns of ubiquitination are generated to effect different biological outcomes, how these reactions are regulated, and how they might be modulated by drugs, it is essential that we acquire a deeper understanding of how E2–E3 complexes work.

Experimental procedures

Expression and purification of recombinant proteins

All proteins were recombinantly expressed in either E. coli or Hi5 insect cells and purified using standard procedures as outlined in Table S1 and Supplemental methods.

FRET measurement

Cdc34 labeling reactions were performed using Lumio Green (Invitrogen) as described previously (Adams et al., 2002). Labeling efficiency was verified by mass spectrometry and was greater than 90%. Fluorescence measurements were carried out on a FluoroLog-3 spectrofluorometer (Jobin Yvon). Labeled proteins were incubated for 10 min in a buffer containing 20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 5% glycerol (v/v), and 0.5 mM DTT at 23 °C before all measurements. Samples were excited at 430 nm, and the emission spectra were acquired from 450 to 570 nm. FRET efficiency (E) was calculated by relative fluorescence intensities (at 475 nm) of the donor in the presence (F_{DA}) and absence (F_D) of acceptor and is represented by $E = 1 - (F_{DA} / F_D)$.

Ubiquitination assay

β-catenin peptide substrate (Ac-KAWQQQSYLD(pS)GIH(pS)GATTTAPRRASY-OH) had a PKA site (RRAS) at the C-terminus, and was labeled with 5 kU of cAMP-dendent protein kinase (NEB) in the presence of [γ-^{32}P]ATP. p27 (10 μM) was phosphorylated by Cdk6−Cyc K (100 nM) in the presence of [γ-^{32}P]ATP for 1 hr at 30°C in a reaction buffer containing 50 mM Tris-Cl (pH 7.5), 60 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT (Hao et al., 2005).

All ubiquitination reactions (20 μl) were performed at 23°C (except when indicated) in a buffer containing 30 mM Tris-Cl (pH 7.3), 100 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT and typically contained 1 μM E1, E2 (variable), E3 (variable), and ubiquitin (75 μM). All reactions were quenched in reducing SDS sample buffer, resolved by SDS-PAGE, phosphor imaged, and quantified using Image Quant (G. E. Healthcare). All values reported are the average of at least two independent experiments.

E2−substrate crosslinking

β-catenin peptide was coupled to an amine-sulfhydryl crosslinker (BMPS; Pierce) via the amine group of the attacking lysine in PBS (pH 7.4) for 1 hr at 23°C. Excess crosslinker was separated from the peptide using a spin column. The reactive peptide was incubated with UbcH5c in the presence of $SCF^{\beta\text{-}TrCP}$ in buffer containing 30 mM Tris-Cl (pH 7.3), 100 mM NaCl, 5 mM $MgCl_2$, 2 mM TCEP for 30 min at 23°C. Reactions were quenched with β-mercaptoethanol, resolved by SDS-PAGE and analyzed by immunoblotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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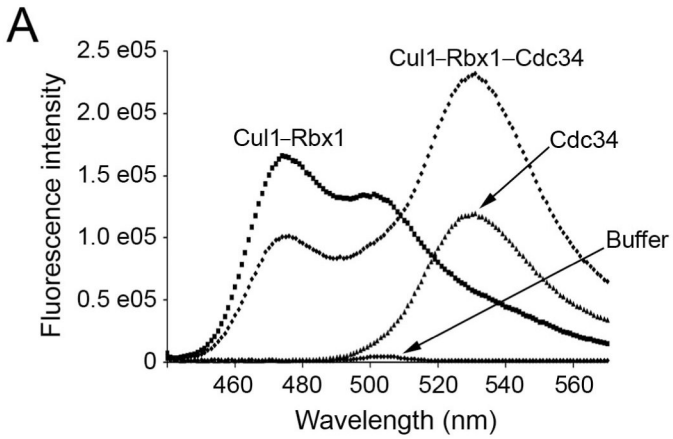

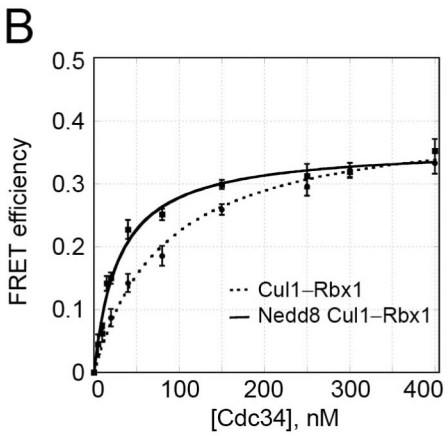

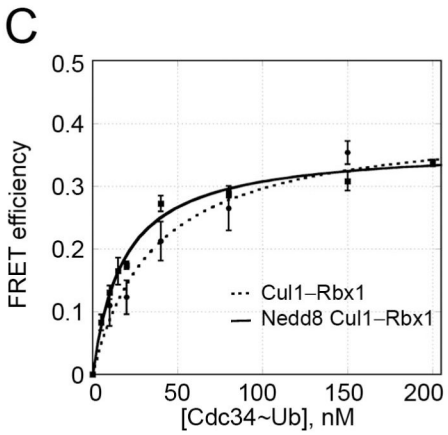

D

E2–E3 combination	Dissociation constant (nM)
Cdc34 – Cul1–Rbx1	82 ± 10
Cdc34 – Nedd8 Cul1–Rbx1	29 ± 4
Cdc34~Ub – Cul1–Rbx1	36 ± 6
Cdc34~Ub – Nedd8 Cul1–Rbx1	18 ± 2

Figure 1. Neddylation enhances Cdc34 binding to Cul1-Rbx1

(A) Fluorescence emission spectra of buffer, 40 nM Cul1–Rbx1, 300 nM Cdc34, and Cdc34–Cul1–Rbx1 complex following excitation at 430 nm.

(B, C) Equilibrium binding titrations of Cdc34 (B) and Cdc34~Ub (C) with Cul1–Rbx1 and Nedd8 Cul1–Rbx1. FRET efficiency was plotted as a function of Cdc34 concentration.

(D) Quantification of dissociation constant (mean ± SD).

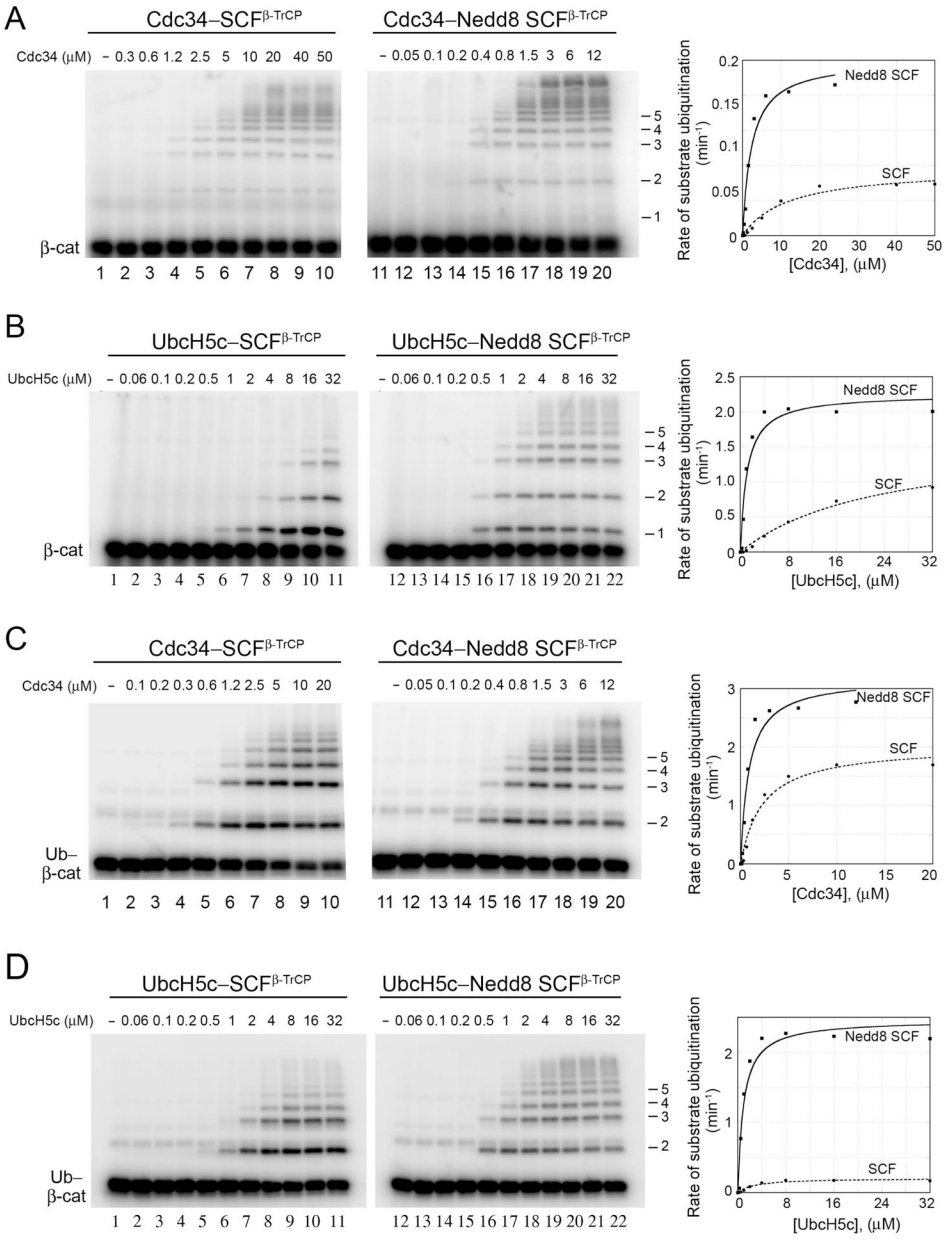

Figure 2. Cul1 neddylation enhances E2– SCF$^{\beta\text{-TrCP}}$ interaction and the rate of ubiquitination of β-catenin and monoubiquitinated β-catenin substrates

(A) ^{32}P-labeled β-catenin (5 μM) was incubated with E1, ATP, ubiquitin, Cdc34 (indicated amounts), and 300 nM SCF$^{\beta\text{-TrCP}}$ for 75 min (lanes 1 through 10) or 300 nM Nedd8 SCF$^{\beta\text{-TrCP}}$ for 60 min (lanes 11 through 20). Substrate ubiquitination per min was normalized to the amount of SCF and rate of formation of modified β-catenin was plotted as a function of Cdc34 concentration.

(B) Same as in panel (A) but using UbcH5c as the E2 and 300 nM SCF$^{\beta\text{-TrCP}}$ for 10 min (lanes 1 through 11) or 120 nM Nedd8 SCF$^{\beta\text{-TrCP}}$ for 6 min (lanes 12 through 22).

(C) ^{32}P-labeled monoubiquitinated β-catenin (5 μM) was incubated with E1, ATP, ubiquitin, Cdc34 (indicated amounts), and 120 nM SCF$^{\beta\text{-TrCP}}$ for 12 min (lanes 1 through 10) or

neddylated $SCF^{\beta\text{-}TrCP}$ for 6 min (lanes 11 through 20). Rate of substrate ubiquitination was plotted as a function of Cdc34 concentration.
(D) Same as in panel (A) but using UbcH5c as the E2, and 300 nM $SCF^{\beta\text{-}TrCP}$ for 40 min (lanes 1 through 11) or 120 nM neddylated $SCF^{\beta\text{-}TrCP}$ for 6 min (lanes 12 through 22). Shown is a representative experiment.

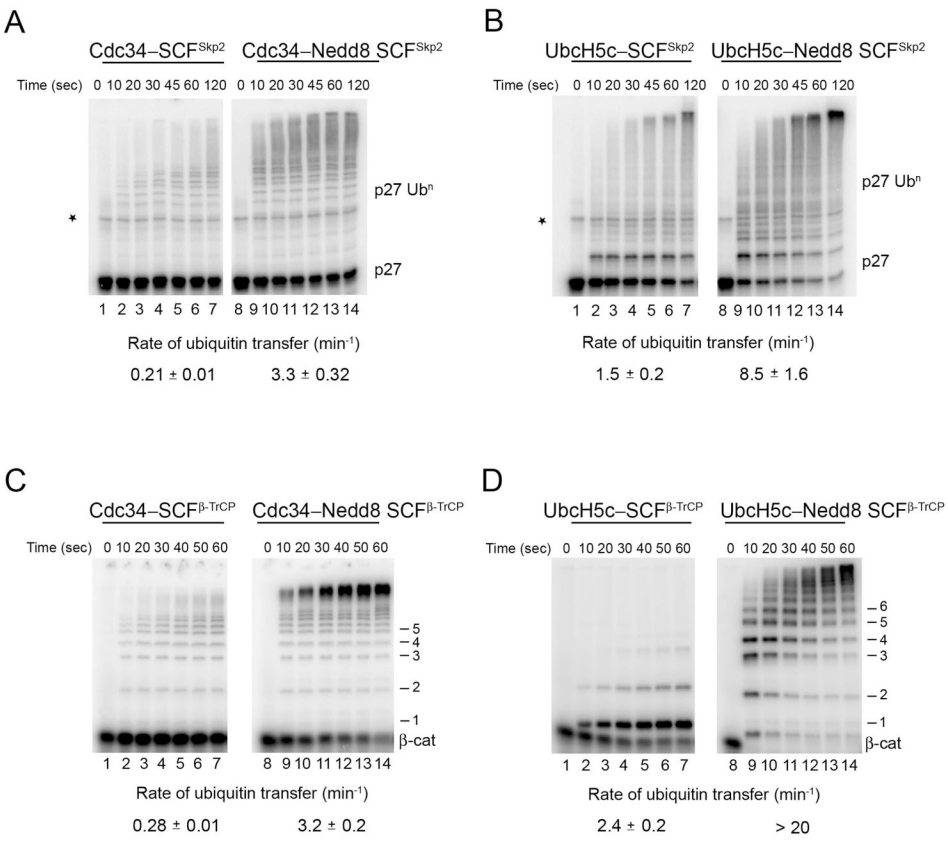

Figure 3. Neddylation increases the rate of ubiquitin transfer

(A) Phosphorylated p27 (200 nM) in complex with Cdk2–Cyc E was ubiquitinated under single-turnover conditions in the presence of E1, ATP, ubiquitin, 40 μM Cdc34, and 600 nM SCF^{Skp2} (lanes 1 through 7) or Nedd8 SCF^{Skp2} (lanes 8 through 14). These reactions were carried out at both 23°C (shown above) and at 5°C to accurately estimate substrate turnover (Figure S11). The ubiquitin transfer rates at 23°C are reported.

(B) Same as in panel (A) but using 40 μM UbcH5c as the E2.

(C) ^{32}P-labeled β-catenin (100 nM) was ubiquitinated under single-turnover conditions in the presence of E1, ATP, ubiquitin, 40 μM Cdc34, and 300 nM $SCF^{β\text{-}TrCP}$ (lanes 1 through 7) or Nedd8 $SCF^{β\text{-}TrCP}$ (lanes 8 through 14). The ubiquitin transfer rates at 23°C are reported.

(D) Same as in panel (C) but using 40 μM UbcH5c as the E2. Shown is a representative experiment.

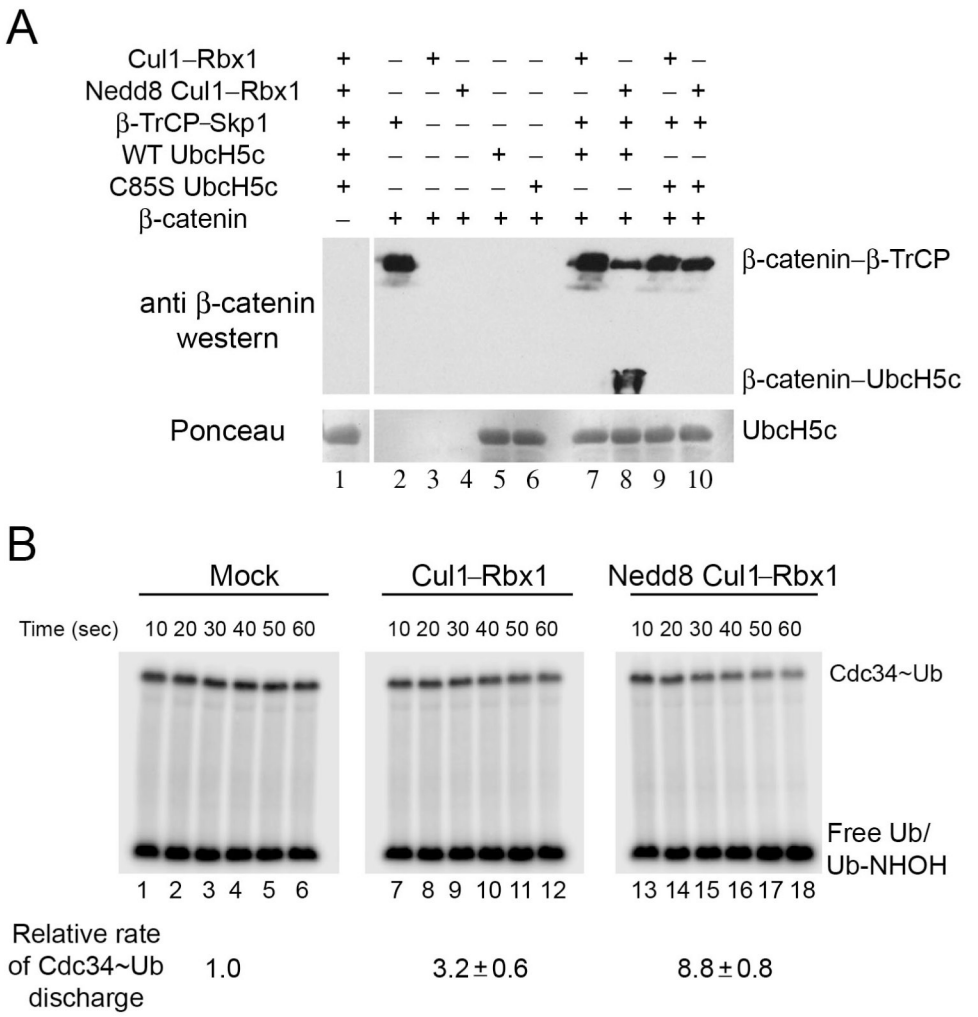

Figure 4. Neddylation enables crosslinking of β-catenin substrate to the UbcH5c active site cysteine and increases the rate of Cdc34~Ub decay to form Ub-NHOH

(A) β-catenin peptide (100 nM) coupled to a crosslinker containing maleimide group was incubated with premixed UbcH5c (30 μM) and 300 nM $SCF^{\beta\text{-}TrCP}$ or subcomplexes thereof. Reaction products were analyzed by immunobloting with β-catenin antibody. Blots were also stained with Ponceau S to detect UbcH5c (bottom panel) or probed with Cul1 and Skp1 antibodies to confirm equal loading (data not shown).

(B) Cdc34 (40 μM) was pre-incubated with 80 μM ^{32}P-labeled K48R ubiquitin in the presence of 2 μM E1 and ATP for 10 min at 23°C, followed by 5 min incubation with apyrase and no Cul1–Rbx1 (lanes 1 through 6), 400 nM Cul1–Rbx1 (lanes 7 through 12), or 400 nM Nedd8 Cul1–Rbx1 (lanes 13 through 18). Discharge of Cdc34~Ub was initiated by adding 5 mM hydroxylamine, aliquots were removed at indicated times and quenched with non-reducing SDS-PAGE sample buffer with 5 mM NEM. Relative discharge rate of Cdc34~Ub to Ub-NHOH was estimated (Figure S12) and reported (mean ± SD).

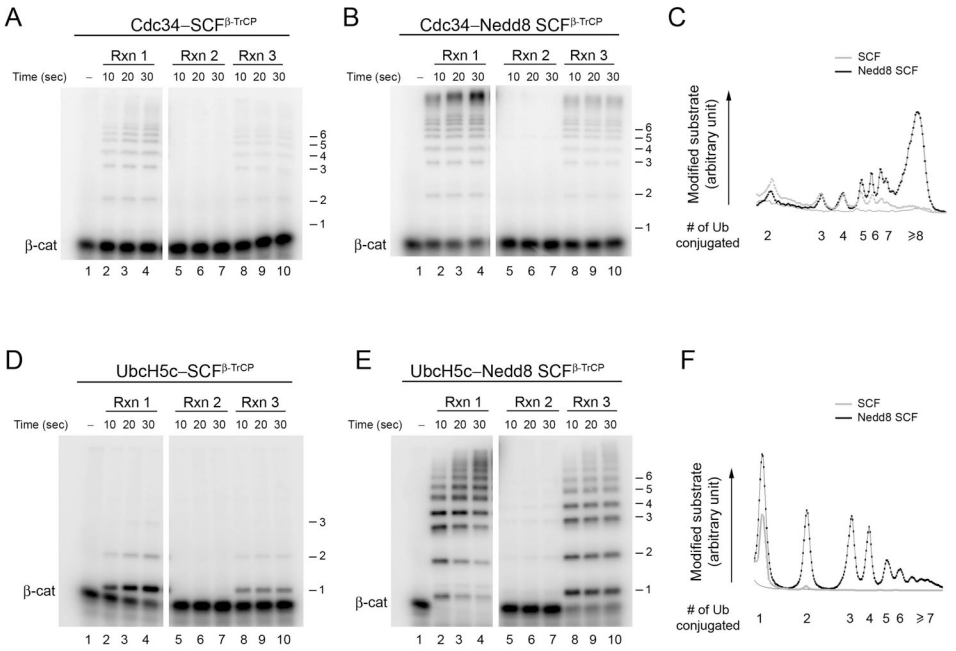

Figure 5. Neddylation increases the fraction of substrate that acquires a long ubiquitin chain in a single E3 binding event

(A) ^{32}P-labeled β-catenin (100 nM) was incubated under single-turnover conditions with 40 μM Cdc34 and 300 nM SCF$^{\beta\text{-TrCP}}$ using three different order-of-addition schemes. In scheme 1 (lanes 2–4) reaction was initiated by mixing E1, E2 and Ub with E3 and labeled substrate. In scheme 2 (lanes 5–7) reaction was initiated by mixing E1, E2 and Ub with E3, labeled substrate and 100 μM cold substrate. In scheme 3 (lanes 8–10) reaction was initiated by mixing E1, E2, Ub and cold substrate with E3 and labeled substrate.

(B) Same as in panel (A) but using 300 nM Nedd8 SCF$^{\beta\text{-TrCP}}$.

(C) Phosphoimager analysis of the results shown in (A) and (B). The number of ubiquitins conjugated is shown only for the modified substrates in a single binding event.

(D) Same as in panel (A) but using 40 μM UbcH5c.

(E) Same as in panel (D) but using 300 nM Nedd8 SCF$^{\beta\text{-TrCP}}$.

(F) Phosphoimager analysis of the results shown in (D) and (E).

Table 1

Estimates of K_m and k_{cat} for substrate ubiquitination

	E2	E3	K_m (10^{-6} M)*	k_{cat} (min^{-1})¶	k_{cat}/K_m (10^4 min^{-1} M^{-1})
β-catenin					
	Cdc34	$SCF^{β\text{-}TrCP}$	12 ± 2.8	0.054 ± 0.003	0.46
	Cdc34	Nedd8 $SCF^{β\text{-}TrCP}$	2.4 ± 0.5	0.19 ± 0.02	8.0
	UbcH5c	$SCF^{β\text{-}TrCP}$	17 ± 3.8	0.93 ± 0.03	5.5
	UbcH5c	Nedd8 $SCF^{β\text{-}TrCP}$	1.1 ± 0.3	2.3 ± 0.19	210
p27-Cdk2-CycE					
	Cdc34	SCF^{Skp2}	6.1 ± 1.8	0.047 ± 0.006	0.77
	Cdc34	Nedd8 SCF^{Skp2}	3.0 ± 1.2	0.28 ± 0.05	9.4
	UbcH5c	SCF^{Skp2}	9.1 ± 2.1	0.45 ± 0.05	5.0
	UbcH5c	Nedd8 SCF^{Skp2}	2.6 ± 1.0	1.1 ± 0.18	41
Ub−β-catenin					
	Cdc34	$SCF^{β\text{-}TrCP}$	2.3 ± 0.48	1.8 ± 0.09	76
	Cdc34	Nedd8 $SCF^{β\text{-}TrCP}$	0.86 ± 0.25	2.8 ± 0.17	330
	UbcH5c	$SCF^{β\text{-}TrCP}$	2.8 ± 0.8	0.15 ± 0.02	5.2
	UbcH5c	Nedd8 $SCF^{β\text{-}TrCP}$	0.92 ± 0.25	2.3 ± 0.16	250

*K_m was estimated by fitting 2 independent data sets to Michaelis-Menten kinetics.

¶k_{cat} was estimated based on the formation of product such that at most 35% of the substrate was consumed.

Table 2

Substrate	E2	E3	Rate of Ubiquitin transfer [*] k_{obs} (min^{-1})	% of Substrate modified	Average number of ubiquitin conjugated [1]	Rate of Substrate dissociation [‡] k_{off} (min^{-1})	$t_{1/2}$ for substrate dissociation [¶] (sec)
β-catenin	Cdc34	$SCF^{β\text{-}TrCP}$	0.28	2.1	3.3 (.087)	13	3.1
β-catenin	Cdc34	Nedd8 $SCF^{β\text{-}TrCP}$	3.2	13	7.2 (1.2)	21	2.0
β-catenin	UbcH5c	$SCF^{β\text{-}TrCP}$	2.4	12	1.1 (0.16)	17	2.4
β-catenin	UbcH5c	Nedd8 $SCF^{β\text{-}TrCP}$	> 20 [§]	80	3.1 (3.1)	-	-
Ub−β-catenin	Cdc34	$SCF^{β\text{-}TrCP}$	> 20 [§]	50	3.5 (2.2)	-	-
Ub−β-catenin	Cdc34	Nedd8 $SCF^{β\text{-}TrCP}$	> 20 [§]	64	6.5 (5.2)	-	-

[*] Estimated from Figure 3. Values reported are average of 2 independent experiments.

[1] The first number was calculated based on modified substrates as shown in Figure 5C and 5F. The number in parentheses is a maximal estimate of average # of ubiquitins transferred per bound substrate, and assumes (based on reactions with neddylated SCF and UbcH5c) that at least 80% of input substrate was pre-bound to SCF.

[‡] Rates calculated using partitioning of chemical reaction and substrate dissociation.

[¶] $t_{1/2} = 0.693/k_{off}$

[§] Actual estimates could not be made since bulk of the reaction was over before the first time point.