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E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification

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Summary

Ubiquitin and ubiquitin-like proteins (UBLs) are directed to targets by cascades of E1, E2, and E3 enzymes. The largest ubiquitin E3 subclass consists of cullin-RING ligases (CRLs), which contain one each of several cullins (CUL1, 2, 3, 4, or 5) and RING proteins (RBX1 or 2). CRLs are activated by ligation of the UBL NEDD8 to a conserved cullin Lys. How is cullin NEDD8ylation specificity established? Here we report that like UBE2M (aka UBC12), the previously uncharacterized E2 UBE2F is a NEDD8 conjugating enzyme *in vitro* and *in vivo*. Biochemical and structural analyses indicate how plasticity of hydrophobic E1–E2 interactions and E1 conformational flexibility allow one E1 to charge multiple E2s. The E2s have distinct functions, with UBE2M/RBX1 and UBE2F/RBX2 displaying different target cullin specificities. Together, these studies reveal the molecular basis for and functional importance of hierarchical expansion of the NEDD8 conjugation system in establishing selective CRL activation.

Keywords

Cullin; Cul1; Cul5; Rbx1; Rbx2; Cullin-RING ligase; NEDD8; E2; UBE2M; UBE2F; Ubiquitin

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Introduction

Cullin RING ligases (CRLs) comprise the largest subfamily of E3 ubiquitin ligases. In humans, six cullins (CUL1, 2, 3, 4A, 4B, and 5), two RBX-family RING proteins (RBX1 and 2), and hundreds of substrate receptors assemble into distinct CRLs that mediate ubiquitination of thousands of targets to regulate a vast array of cellular processes (Cardozo and Pagano, 2004; Petroski and Deshaies, 2005; Willems et al., 2004). CRL function is regulated by attachment of the ubiquitin-like protein (UBL) NEDD8 to a conserved Lys in a cullin's C-terminal domain (Pan et al., 2004). NEDD8 both enhances intrinsic CRL ubiquitination activity (Amir et al., 2002; Kawakami et al., 2001; Morimoto et al., 2000; Podust et al., 2000; Read et al., 2000; Wu et al., 2000), and prevents CRL binding to the inhibitor CAND1 (Liu et al., 2002; Zheng et al., 2002). At present, the NEDD8 cascade is known to contain a single E1 (NAE1-UBA3), which activates NEDD8 and ultimately catalyzes transfer of NEDD8's C-terminus to the catalytic cysteine of the only characterized NEDD8 E2, UBE2M (also known as UBC12) (Gong and Yeh, 1999; Liakopoulos et al., 1998; Osaka et al., 1998). The resulting UBE2M~NEDD8 thioester conjugate serves as the direct source of NEDD8 to be covalently attached to a cullin's acceptor Lys. *In vivo* and also in cell lysates, additional factors can also influence a cullin's NEDD8ylation state. As examples, Dcn1 (aka SCCRO) enhances cullin NEDD8ylation (Kim et al., 2008; Kurz et al., 2008; Kurz et al., 2005) and the COP9 Signalosome (CSN) removes ligated NEDD8 from cullins (Lyapina et al., 2001).

Despite the importance of CRL activation by NEDD8, it remains unknown whether there is any specificity in cullin NEDD8ylation, or how such specificity could be established. Clues to selectivity might come from analogy to the ubiquitin (Ub) pathway. A fundamental feature of the Ub pathway is that substrate ubiquitination selectivity is generated by the hierarchical organization of the cascade (Dye and Schulman, 2007; Haas and Siepmann, 1997; Kerscher et al., 2006). In vertebrates, the Ub pathway comprises two E1s (UBA1 and UBA6), tens of E2s and hundreds of E3s promoting Ub transfer to thousands of targets (Chiu et al., 2007; Hershko et al., 2000; Jin et al., 2007; Pelzer et al., 2007; Pickart and Rose, 1985). Within this hierarchy, E1–E2 and E2–E3-substrate interactions are highly specific to ensure precise temporal and spatial regulation of targets by modification with appropriate Ub linkages, which in turn mediate particular downstream functions (Harper and Schulman, 2006; Hicke et al., 2005; Hurley et al., 2006; Pickart and Fushman, 2004; Ravid and Hochstrasser, 2008).

One pivotal feature of a hierarchical E1–E2–E3 cascade is that an E1 needs to charge multiple E2s with Ub, such that E3s can then direct the distinct charged Ub~E2s to downstream targets. Previous studies revealed that an E1's C-terminal ubiquitin-fold domain plays a crucial role in E2 recruitment, and that UBL transfer from E1 to E2 involves rotation of this domain about a flexible linker (Huang et al., 2007; Huang et al., 2005; Lee and Schindelin, 2008; Lois and Lima, 2005; Walden et al., 2003). Nonetheless, it remains poorly understood how any E1 recognizes multiple E2s.

In contrast to the Ub pathway, the NEDD8 conjugation cascade has been reported to contain only a single E2. Given the important and diverse CRL functions in cellular regulation, we wondered whether the NEDD8 cascade is equipped with additional levels of selectivity for cullin NEDD8ylation. Here we describe that like the Ub cascade, the NEDD8 cascade is expanded at the E2 level. We identify UBE2F as a NEDD8 E2. Biochemical studies and a structure of NEDD8 E1's ubiquitin-fold domain in complex with UBE2F's core domain reveal the basis for how an E1 recognizes distinct E2s. We further show that UBE2M-RBX1 and UBE2F-RBX2 pairs specifically regulate NEDD8ylation of Cullins 1–4 and CUL5, respectively, in an E2-RING-dependent manner. Thus, hierarchical expansion of the NEDD8 cascade at the E2-RING level confers selective CRL activation.

Results and Discussion

UBE2F is an E2 for NEDD8 in vitro and in vivo

In characterizing a previously undescribed E2, UBE2F, we discovered that it is a specific NEDD8 conjugating enzyme *in vitro* and *in vivo*. First, using purified components, we tested the ability of UBE2F and several well-characterized E2s to be charged by E1~UBL (~ covalent bond) pathways as follows: NAE1-UBA3~NEDD8 (Fig. 1A); UBA1~Ub (Fig. 1B); SAE1-UBA2~SUMO1 (Fig. 1C); UBA7~ISG15 (Fig. 1D); UBA6~Ub (Fig. 1E); UBA6~FAT10 (Fig. 1F). Control E2s were charged only by their known cognate E1~UBLs (Chiu et al., 2007;Johnson and Blobel, 1997;Johnson et al., 1997;McGrath et al., 1991;Osaka et al., 1998;Pelzer et al., 2007{Jin, 2007 #249; Yuan and Krug, 2001;Zhao et al., 2004). Furthermore, a DTT-sensitive band corresponding to a UBE2F~UBL thioester product was detected only in the presence of NAE1-UBA3 and NEDD8, suggesting that like UBE2M, UBE2F is a NEDD8 conjugating enzyme *in vitro* (Fig. 1A, lanes 8, 9).

We next examined *in vivo* conjugate formation in NIH 3T3 cells between UBE2F with a C-terminal His-Flag tag (UBE2F-HF) and NEDD8. Because thioester bonds are not stable with standard cell lysis procedures, we examined formation of a covalent UBE2F~NEDD8 complex in two ways. First, we used a recently described acid lysis method that preserves thioester conjugates (Jin et al., 2007), and saw a slow migrating band, reactive with anti-Flag and -NEDD8 antibodies, which is sensitive to reduction. This indicates that like UBE2M-HF~NEDD8, a UBE2F-HF~NEDD8 thioester product was formed in cells (Fig. 1G). As a second approach, we generated a UBE2F catalytic Cys to Ser mutant, enabling purification of a stable oxy-ester complex (Wada et al., 2000). Bands corresponding to E2~NEDD8 complexes reacted with antibodies to NEDD8 were observed in cell extracts expressing UBE2M C111S-HF and UBE2F C116S-HF, and after nickel agarose pull-down (Fig. 1H lanes 4 and 7). Together, the results indicate that like UBE2M, UBE2F is an E2 for NEDD8 *in vitro* and *in vivo*.

UBE2F interacts with NEDD8 E1 in a bipartite manner

How does one E1 (NAE1-UBA3, i.e. NEDD8's E1) interact with multiple E2s (UBE2M and UBE2F)? To address this question, we examined UBE2F's E1-binding domains based on previous studies of bipartite interactions between NEDD8's E1 and UBE2M. UBE2M's N-terminal extension binds UBA3's hydrophobic groove; also, UBE2M's catalytic core domain interacts with NEDD8 E1's ubiquitin-fold domain (ufd) (Fig. 2A) (Huang et al., 2007;Huang et al., 2004;Huang et al., 2005). This latter interaction mode is thought to be common among UBLs cascades (Durfee et al., 2008;Huang et al., 2007;Huang et al., 2005;Jin et al., 2007;Lee and Schindelin, 2008;Lois and Lima, 2005;Walden et al., 2003). UBE2F's sequence also includes the two domains, an N-terminal extension and E2 core (Fig. 2B). We tested their binding to NEDD8's E1 by analyzing kinetics of NEDD8 transfer. In comparison to wild-type UBE2F, a mutant (UBE2F^{core}) consisting only of the core domain showed ~18-fold higher K_m (Fig. 2C). Thus, both UBE2F's N-extension and core domains bind NEDD8's E1.

Defining a Φ-Φ-X-Φ UBA3-binding motif common among NEDD8 E2 N-extensions

To define NAE1-UBA3-UBE2F interactions, we performed mutational analysis. Similar K_m values were measured for UBE2F^{core} with wild-type NAE1-UBA3, for wild-type UBE2F and a previously described NAE1-UBA3 docking groove mutant (UBA3 F44, H139, I140, P171, I174, P176, L193, M196, I310A (Huang et al., 2004)), and for UBE2F^{core} with the docking groove mutant (Fig. 2C–E). Thus, UBA3's docking groove binds UBE2F's N-terminal extension. Further Ala mutational analysis of UBE2F's N-terminal sequence showed a similar K_m value for a Met1Ala, Leu2Ala, Leu4Ala triple mutant (Fig. 2F, G). This suggests that UBE2F's N-terminal Met1-Leu2-X-Leu4 sequence mediates the bulk of the N-extension's

interaction with UBA3's docking groove. Comparing UBA3-binding sequences from UBE2F and UBE2M (Leu4-Phe5-X-Leu7 (Huang et al., 2004)) across species revealed a conserved Φ-Φ-X-Φ motif (Φ-hydrophobic; X - any residue) (Fig. 2H, Tables S1–S3).

Structural basis for hierarchical expansion of a UBL cascade at the E2 level

A common feature of ubiquitin and NEDD8 E1s is a critical role for their C-terminal ubiquitin-fold domains (ufds) in E2 binding. Consistent with this notion, we found that the isolated NEDD8 E1 ufd ($NE1^{ufd}$) formed a complex with $UBE2F^{core}$ (Fig. S1), and determined the complex crystal structure at 2.5 Å resolution (Fig. 3A left, S2, Table S4). Overall, $NE1^{ufd}$-$UBE2F^{core}$ adopts a compact, globular ovoid structure similar to $NE1^{ufd}$-$UBE2M^{core}$ (PDB 1Y8X; Fig. 3A) (Huang et al., 2005), with $UBE2F^{core}$ displaying a canonical E2 catalytic core domain fold. Superimposing $UBE2F^{core}$ with prior $UBE2M^{core}$ structures (Huang et al., 2007;Huang et al., 2005) revealed distinct features of $UBE2F^{core}$ including: (1) an offset orientation and a half-turn extension for $UBE2F^{core}$'s N-terminal αa1 helix; (2) a loop insertion immediately following the catalytic Cys116; and (3) an extended C-terminal helix more reminiscent of ubiquitin E2 structures than the 2-stranded β-sheet at the C-terminus of UBE2M (Fig. 3B, S3).

Interestingly, structural comparison shows how NEDD8's E1 binds distinct UBE2F and UBE2M α 1-helix and β 1 β2-loop sequences. For both E2s, interactions are centered around two hydrophobic clusters (Fig. 3C). One cluster involves $NE1^{ufd}$'s Ala424, Ala426, Thr433, and Leu435, and $UBE2F^{core}$'s Val30 and the hydrophobic portion of Lys35 from the α1-helix. These correspond to $UBE2M^{core}$'s Ala27 and Leu32, respectively. The second cluster involves $NE1^{ufd}$'s Thr382, Thr391, SeMet394 (in our structure, Leu394 in wild type $NE1^{ufd}$), Val397, and Ile400 packing against $UBE2F^{core}$'s Val38, Val41, and Leu44 from the α1-helix, and Val54, Phe56, and Leu62 from the β 1 β 2-loop. Although Leu44, Phe56 and Leu62 are conserved between $UBE2F^{core}$ and $UBE2M^{core}$, other residues differ significantly. For example, $UBE2F^{core}$'s Val38 makes hydrophobic interactions, whereas the corresponding $UBE2M^{core}$ Gln35 forms a hydrogen bond with NEDD8 E1's Thr382. Unlike the $NE1^{ufd}$-$UBE2M^{core}$ complex, which is stabilized by numerous ionic interactions, only one stabilizes $NE1^{ufd}$-$UBE2F^{core}$. These structural variations may account for the different K_m values for UBE2Fcore (21.1 ± 1.0 μM) and $UBE2M^{core}$ (6.07 ± 0.36μM; (Huang et al., 2004)) during NEDD8 transfer from E1 to E2.

General implications for hierarchical expansion of UBL cascades

Our data provide general insights into how an E1 can recognize multiple E2s. First, NEDD8's E1 recognizes cognate E2s via two surfaces. The ufd-$E2^{core}$ interaction is common among UBL pathways. The second interaction between E2 N-extensions and UBA3's docking groove both selects for the NEDD8 pathway, and prevents mis-association with a non-cognate E1 (Huang et al., 2008). Notably, a second, unique E2-binding site was recently identified to play a role in specificity for the yeast Ub E1 Uba1p (Huang et al., 2008; Lee and Schindelin, 2008). Thus, multiple E1–E2 interaction surfaces may be a means to increase affinity in a manner that is adaptable to multiple cognate E2 sequences, while selecting against mischarging the wrong E2. Second, both E2 binding sites focus on hydrophobic interactions. Pliability of hydrophobic interactions (Lim and Sauer, 1989) likely accommodates a range of sequences and structures (Fig. 3). Indeed, the corresponding ufd surface from the yeast Ub E1 Uba1p is also hydrophobic, which may facilitate binding to numerous Ub E2s (Table S5). Third, in addition to local structural malleability, E1 conformational flexibility at a more global level is also likely to be important for binding to multiple E2s. This is highlighted by superimposing the $NE1^{ufd}$ portion of $NE1^{ufd}$-$UBE2F^{core}$ with previous structures of $NE1^{ufd}$-$UBE2M^{core}$ and NAE1-UBA3~NEDD8(T)-NEDD8(A)-MgATP-UBE2M (C111A) (Huang et al., 2007; Huang et al., 2005). Due to different UBE2F and UBE2M N-terminal α 1-helix orientations,

the corresponding positions of the two E2s distal from the E1-interface diverge by as much as 6Å (Fig. 3D). In particular, the relative locations for their catalytic Cys thiols differ by ~3.5Å. Given these differences, how might a single E1 be able to transfer a UBL to E2s with different E1-to-E2 Cys geometries and distances? It seems likely that the ability of UBA3's ufd to rotate around a flexible hinge (Huang et al., 2007; Huang et al., 2005) may be important for accommodating multiple E2s. Indeed, ufd rotation has also been observed for the Ub E1 Uba1p (Lee and Schindelin, 2008).

Different global consequences of knocking down Ube2m and Ube2f levels in cells

To begin to address the cellular roles of the multiple NEDD8 E2s, we examined some global properties of UBE2F and UBE2M. First, we compared expression patterns of UBE2M and UBE2F in various tissues and cell lines, and observed similar mRNA and protein expression trends, although UBE2F was generally expressed at lower levels (Fig. S4). Next, we knocked down Ube2f or Ube2m (hereafter, human and mouse genes are designated by capital and small letters, respectively) in NIH 3T3 cells by retroviral shRNA expression (Fig. 4A) and examined effects on proliferation and apoptosis. Ube2m knockdown led to increased cell and nuclear size (Fig. S5). Moreover, FACS profiles revealed accumulation of cells in G2/M phase and increased aneuploidy, and these cells also displayed budding nuclei characteristic of apoptosis (Fig. S5). Accordingly, Ube2m knockdown led to decreased cell numbers and increased Annexin V staining (Fig. 4B, C). By contrast, cells appeared normal when Ube2f was knocked down (Fig. 4B, C).

To examine whether knocking down Ube2f and Ube2m leads to distinct molecular consequences in cells, we performed transcriptome analysis with Affymetrix gene chip microarrays. In both cases, different gene expression profiles were observed in comparison to controls (Tables S6–S9). Furthermore, knockdown of Ube2f and Ube2m led to distinct patterns of gene expression, with only 10% and 5% overlap for the up- and down-regulated genes, respectively (Fig. 4D, S6). Thus, although the molecular pathways leading to the transcriptome differences remain to be elucidated, the differential effects of knocking down the two NEDD8 E2s compared to controls, and compared to each other, likely reflects distinct functions for Ube2m and Ube2f.

Ube2f and Ube2m mediate distinct patterns of Cullin NEDD8ylation

To identify direct downstream functions of UBE2F, we examined effects of Ube2f and Ube2m knockdown on NEDD8ylation of the best-characterized targets, members of the cullin family. In NIH 3T3 cells, knocking down Ube2m led to decreased Nedd8ylation of endogenous Cullins 1–4, and a marginal increase in Nedd8-modified Cul5 (Fig. 5A, S7). By contrast, knocking down Ube2f virtually eliminated the presence of Nedd8ylated Cul5, and had no effect on the levels of Nedd8-modified Cullins 1–4 (Fig. 5A). Similar results were obtained using early passage primary mouse embryonic fibroblasts (MEFs) (data no shown) suggesting that the effects were not exclusive to NIH 3T3 cells. Knocking down Ube2f had no effect on Ube2m levels and vice-versa, so endogenous Ube2f and Ube2m were unable to complement the effects of knocking down the other NEDD8 E2.

Next, we asked whether overexpressing UBE2F could rescue defects caused by Ube2m knockdown,and vice-versa. Human UBE2F and UBE2M, which have 97% and 100% identical protein sequences as their mouse counterparts, are encoded by distinct DNA sequences that escape targeting by shRNAs against the mouse genes. In controls, CMV-mediated UBE2F-HF or UBE2M-HF expression rescued defects from Ube2f or Ube2m knockdowns, respectively (Fig. 5B). UBE2M-HF overexpression did not rescue the Ube2f knockdown-mediated decrease in Nedd8ylated Cul5. However, over-expressing UBE2F-HF partially rescued the Ube2m shRNA-dependent decrease in cullin Nedd8ylation (Fig. 5B) and increase in aneuploidy (Fig.

S8). Thus, *in vivo*, the two NEDD8 E2s have distinct roles in cullin NEDD8ylation, although when overexpressed, UBE2F displayed a broader range of substrates.

NEDD8ylation selectivity in vivo is dictated combinatorially by E2-RBX-Cullin interplay

Cullins interact with RING box proteins (RBX) to form CRLs, and Rbx1 plays a crucial role in CUL1 NEDD8ylation (Gray et al., 2002; Kamura et al., 1999a; Morimoto et al., 2003). In higher eukaryotes, there are two RBX proteins, RBX1 and RBX2, with distinct cullin association preferences. RBX1 preferentially interacts with CULs 1–4, and under some circumstances can bind CUL5 (Kamura et al., 2001; Kamura et al., 1999b; Mahrour et al., 2008; Ohta et al., 1999; Querido et al., 2001; Seol et al., 1999; Skowyra et al., 1999; Yu et al., 2003). By contrast, RBX2 associates exclusively with CUL5 (Kamura et al., 2004; Reynolds et al., 2008). Thus, we tested whether the differential effects of UBE2M and UBE2F on cullin NEDD8ylation might depend on RBX1 and RBX2. Upon knocking down Rbx1 levels in NIH 3T3 cells, the total protein amounts for Cullins 1–4 decreased, while the level of Nedd8ylated Cul5 increased slightly compared to control. By contrast, Rbx2 knockdown only decreased the total levels of Cul5 protein (Fig. 5C). Thus, the cellular levels of distinct cullins are related to the levels of their distinct Rbx partners.

Next we examined the effects of Rbx1 or Rbx2 knockdown in combination with either Ube2m or Ube2f shRNA in NIH 3T3 cells. Rbx1 and Ube2f double knockdown reduced the levels of Nedd8ylated Cul5, while Rbx1 and Ube2m double knockdown had no defect for Cul5 Nedd8ylation. On the other hand, Rbx2 and Ube2m double knockdown reduced the levels of Nedd8ylated Cullins 1–4, while Rbx2 and Ube2f double knockdown had no effect on Nedd8ylation of Cullins 1–4 (Fig. 5C). Together, these data suggested that *in vivo*, NEDD8ylation specificity is established combinatorially: Ube2f pairs with Rbx2 to control Cul5 Nedd8ylation, and Ube2m functions through Rbx1 to mediate Nedd8ylation of Cullins 1–4.

RBX2 is specific for UBE2F in vitro

In order to address the extent to which NEDD8ylation specificity is established by inherent E2-RBX-CUL properties, we turned to *in vitro* assays, using components purified from *E. coli*. As the conserved target Lys is located in the cullin C-terminal domain (CTD), we took advantage of a system to generate highly pure native or non-native complexes between RBX proteins and cullin CTDs by bacterial coexpression (H. Walden, M.S. Lee, D. Duda, and L. Borg, unpublished results). Indeed, RBX1-CUL1^{CTD} and RBX2-CUL5^{CTD} exhibited similar activities as their full-length counterparts in assays testing ^{32}P-NEDD8 transfer from UBE2M or UBE2F to CUL1 and CUL5 (Fig. S9). To extend our analysis, we purified all 10 possible RBX-CUL^{CTD} pairs (RBX 1–2 x CUL 1–5, Fig. S10), and performed pulse-chase assays. First, equivalent amounts of UBE2F~^{32}P-NEDD8 and UBE2M~^{32}P-NEDD8 were generated in pulse reactions. After the pulse was quenched, RBX-CUL^{CTD}s were added, and ^{32}P-NEDD8 transfer from E2 to a given CUL^{CTD} was monitored. RBX2-associated CUL^{CTD}s showed substantially greater NEDD8ylation by UBE2F, whereas RBX1-associated CUL^{CTD}s were NEDD8ylated by both UBE2F and UBE2M (Fig 6A, B). Also, UBE2M is specific for RBX1, whereas UBE2F NEDD8ylates both RBX1- and RBX2-associated CUL^{CTD}s. The cullins themselves further influence NEDD8ylation. UBE2M preferred CUL1^{CTD} over CUL2^{CTD} and CUL5^{CTD}. UBE2F preferred CUL5^{CTD} and CUL1 ^{CTD} over CUL2^{CTD} (Fig. 6A,B). Both NEDD8 E2s exhibited lower activity toward CUL3^{CTD} and CUL4^{CTD}, irrespective of their RBX partner. Taken together, the results indicate that NEDD8ylation specificity is established combinatorially.

To further define RBX-NEDD8 E2 specificity, we examined CUL^{CTD} NEDD8ylation with chimeric RBXs harboring an N-terminal CUL^{CTD}-binding strand from one RBX, and a C-

terminal RING from the other. An RBX's NEDD8 E2 specificity correlates with the identity of the RING domain (Fig. 6C). Thus, the RBX2 RING displays intrinsic specificity for UBE2F, and UBE2M displays intrinsic specificity for RBX1's RING.

An RBX RING's E2-binding surface is critical for cullin NEDD8ylation

In Ub cascades, RING domains recruit E2s via hydrophobic surfaces (Brzovic et al., 2003; Dominguez et al., 2004; Zheng et al., 2000). Indeed, an Ala mutation in place of a critical conserved BRCA1 RING surface Ile decreased E2 binding without perturbing the RING fold (Brzovic et al., 2003) (Fig. S11). Thus, we tested a role for the corresponding Ile in the RBX2 (Ile52) and RBX1 (Ile44) RINGs, and found that their mutation to Ala reduced cullin NEDD8ylation (Fig. 6D, E). We next asked whether a wild-type RBX RING domain could restore NEDD8ylation activity in trans. For these experiments, we generated RBX-CUL mutants with wild-type RING domains, but which cannot be NEDD8ylated due to Arg substitutions replacing the acceptor Lys. Adding these in trans did not rescue defects caused by E2-binding surface RBX2 Ile52Ala or RBX1 Ile44Ala mutations (Fig. 6D,E). Future structural studies of RBX2-UBE2F and RBX1-UBE2M will be required to understand the detailed basis for NEDD8 E2 specificity.

UBE2F-RBX2-CUL5-BC/Cul5box – a metazoan-specific CRL pathway complete with activation mechanism

Phylogenetic analyses identify UBE2F only in metazoans (Fig. S12), appearing late in evolution relative to UBE2M. Interestingly, the evolutionary histories of UBE2F and UBE2M mirror their CRL partners: RBX2 and CUL5 also appear restricted to metazoa, whereas UBE2M, RBX1 and CUL1 are present in all eukaryotes. Thus, our results help to further define the distinct RBX2-CUL5 pathway as also including UBE2F.

At present, it is not known why RBX2-CUL5 would have evolved as a separate branch of the CRL/NEDD8 pathway. CUL5 assembles into CRLs via the ElonginB/C heterodimeric adaptor complex, which recruits substrate-receptors harboring BC(SOCS)/Cul5-boxes. Although potential physiological roles of RBX2-CUL5 are only beginning to become apparent, known functions are associated with signaling pathways consistent with emergence of the pathway in metazoans. Some BC(SOCS)/Cul5-box substrate receptors regulate cytokine signaling (Kile et al., 2002), and CUL5-based CRLs have been implicated in regulating neuronal migration (Feng et al., 2007), chondrocyte differentiation (Dentice et al., 2005), and myogenesis (Nastasi et al., 2004).

The importance of CUL5-based CRLs is highlighted by findings that viral pathogens hijack these ligases. As examples, HIV-1 Vif promotes degradation of the host antiviral factor APOBEC3G (Yu et al., 2003), and human adenoviral E4orf6 and E1B55K together promote degradation of the tumor suppressor protein p53 (Querido et al., 2001), both via CUL5-based CRLs. NEDD8 activation was shown to be critical for these CUL5 degradation pathways through the use of a non-NEDD8ylatable mutant CUL5, or ts41 cells harboring a temperature-sensitive NEDD8 E1. However, CUL5 can bind RBX1, and Vif and E4orf6/E1B55K-based ligases have been found with CUL5-RBX1 (Querido et al., 2001; Yu et al., 2003). Thus, determining which CUL5 pathways depend on RBX2, RBX1, UBE2F, UBE2M, or other as yet unknown cullin NEDD8ylation specificity factors may provide opportunities for therapeutic intervention.

Implications for NEDD8ylation of other targets

In addition to cullins, MDM2, p53, p73, breast-cancer associated protein 3, epidermal growth factor receptor, and ribosomal subunits have been reported as NEDD8 targets (Gao et al., 2006; Jones et al., 2008; Oved et al., 2006; Watson et al., 2006; Xirodimas et al., 2004;

Xirodimas et al., 2008). Little is known about when or how NEDD8 is conjugated to non-cullin targets. Given that many ubiquitin E2s work with a range of RING proteins, it is plausible that NEDD8 E2s may function with other E3s. Indeed, we found that UBE2M and UBE2F can interact with multiple RINGs *in vitro* (Fig. 6, S13), albeit to substantially different extents. Although Ube2f appears to play a more restricted role than Ube2m (Fig. S14), the discovery of UBE2F as a NEDD8 conjugating enzyme may facilitate identifying NEDD8ylation pathways for non-cullin targets.

Implications of hierarchical expansion of the NEDD8 cascade for regulating CRL activation

Our results provide a new view of the cullin NEDD8ylation machinery. Rather than a single NEDD8ylation cascade for all target conjugation, we find that NEDD8ylation is a specific process, with discrete molecular pathways dictating modification of different cullin targets (Fig. 7). Indeed, RBX2 is specific for UBE2F, and UBE2M is specific for RBX1. It appears that RBX proteins establish a primary layer of selectivity as classic RING-type E3s for the NEDD8 pathway, by recruiting both a particular cullin target and a specific NEDD8 E2.

Selectivity may also be influenced by differences in protein levels, as cellular concentrations of UBE2F are generally lower than of UBE2M (Fig. S4). E1-E2 transthiolation efficiency also may affect flux through the different pathways: consistent with findings for the Ub pathway that distinct E2s differ in their charging efficiencies by ~4-orders of magnitude (Haas et al., 1988;Huang et al., 2008), k_{cat}/K_m for NEDD8 transfer to UBE2F (Fig. 2) is ~7-fold lower than for UBE2M (Huang et al., 2004). Taken together, it seems that under normal conditions the larger cellular pool of UBE2M~NEDD8 would preferentially NEDD8ylate RBX1-associated cullins, whereas RBX2 could only work with the more limited amounts of UBE2F~NEDD8.

Our identification of cullin-specific enzymes implies that target NEDD8ylation is regulated. Indeed, we find UBE2F activity restricted toward Rbx2-Cul5 *in vivo*, even though it can cooperate with both RBX1 and RBX2 upon elevated expression in cells (Fig. 5B) and *in vitro* (Fig. 6). Also, different cullins display a range of *in vitro* NEDD8ylation propensities (Fig. 6). Thus, it seems that *in vivo*, additional mechanisms regulate NEDD8ylation of specific cullins.

A complete view of NEDD8ylation will require understanding how UBE2F and UBE2M concentrations are established, and integrating the roles of other factors such as the NEDD8ylation enhancer DCN1, the inhibitor CAND1, and the deNEDD8ylating CSN (Kurz et al., 2008; Kurz et al., 2005; Lyapina et al., 2001). Also, some organisms have additional RBXs and NEDD8s. For example, *D. melanogaster* has three RBXs, each with distinct cullin preferences (Noureddine et al., 2002; Reynolds et al., 2008), and *A. thaliana* has three NEDD8s (Rao-Naik et al., 1998). Thus, we anticipate that many exciting future studies will reveal how these, and perhaps other as yet unknown factors work together to NEDD8ylate specific CRLs under distinct physiological settings to control important ubiquitination pathways.

Experimental Procedures

General methods

Detailed experimental procedures are provided in Supplemental Information. Briefly, constructs, purified proteins, and crystals were prepared by adapting methods described previously (Duda et al., 2008; Huang et al., 2004; Huang et al., 2005; Huang et al., 2008). The structure was determined by molecular replacement using PDB 1Y8X as the search model for PHASER (Storoni et al., 2004). The model was built in O (Jones et al., 1991), refined to 2.5 Å with REFMAC (1994). For mammalian cell expression, UBE2M, UBE2F and variants with a C-terminal His-Flag tag, and NEDD8 with an N-terminal HA-tag were cloned into an MSCV-

IRES-GFP vector (Hawley et al., 1994). Retroviral shRNAs were prepared using the MSCV-LMP shRNA vector (Open Biosystems) carrying a GFP gene. Biochemical assays were performed using methods similar to those described previously (Eletr et al., 2005; Huang et al., 2004; Huang et al., 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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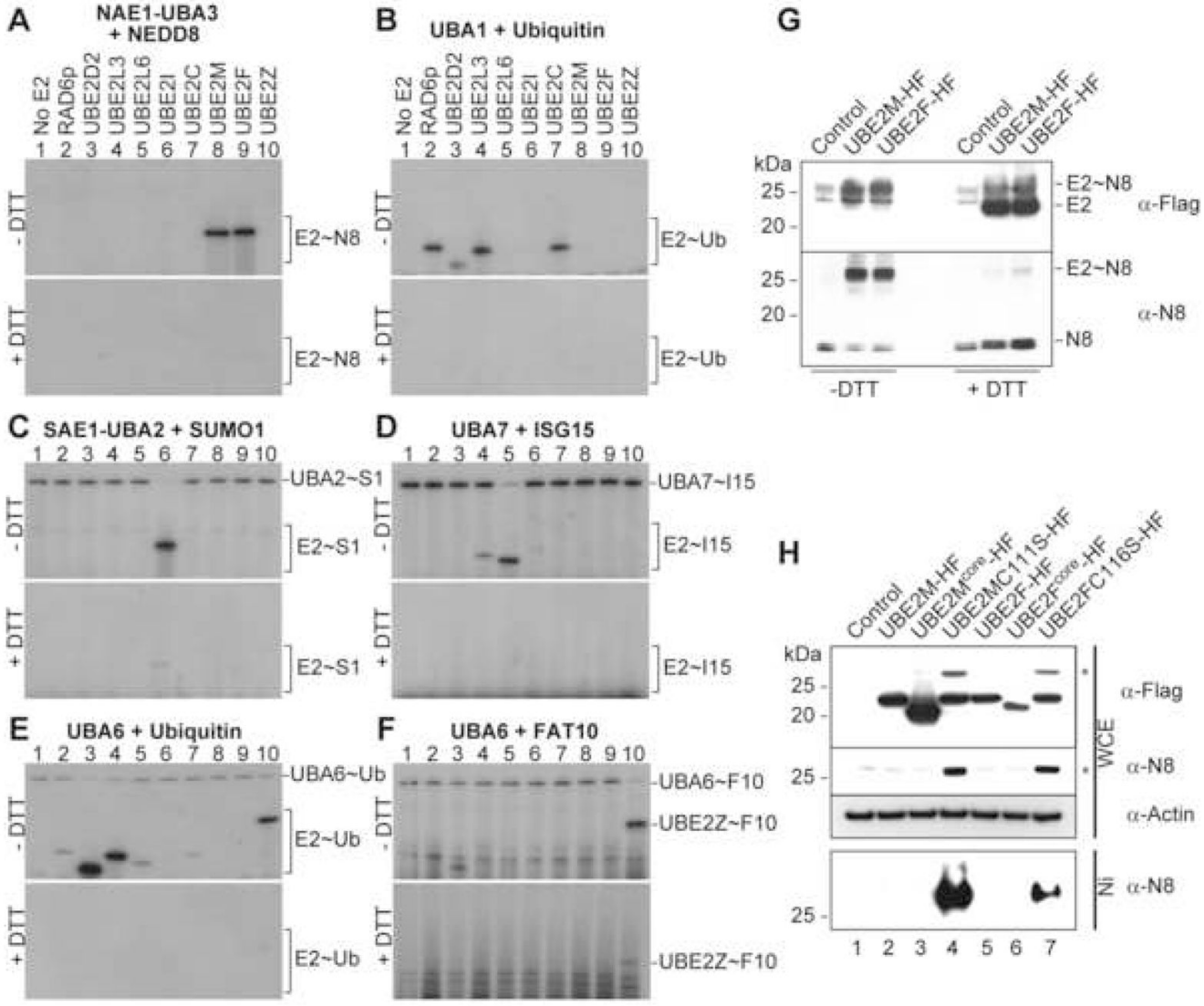

Figure 1. UBE2F forms a NEDD8 thioester conjugate *in vitro* and *in vivo.*

Autoradiograms showing ^{32}P-UBL thioesters after E2 charging by E1~^{32}P-UBLs (top) and reduction with 100 mM DTT (bottom): ***A,*** NAE1-UBA3~NEDD8; ***B,*** UBA1~Ub; ***C,*** SAE1-UBA2~SUMO1; ***D,*** UBA7~ISG15; ***E,*** UBA6~Ub; ***F,*** UBA6~ FAT10. ***G,*** Immunoblot showing 100 mM DTT reduction of E2~NEDD8 complex detected with antibodies against E2 (α-Flag) and NEDD8 (α-N8) from NIH 3T3 cells infected with indicated retroviruses. ***H,*** Immunoblot as in G (top) from whole cell extracts (WCE) of NIH 3T3 cells infected with indicated retroviruses and lysed in urea buffer, and from nickel-agarose pull-downs (His). * expected MW of E2~NEDD8 oxy-ester complex.

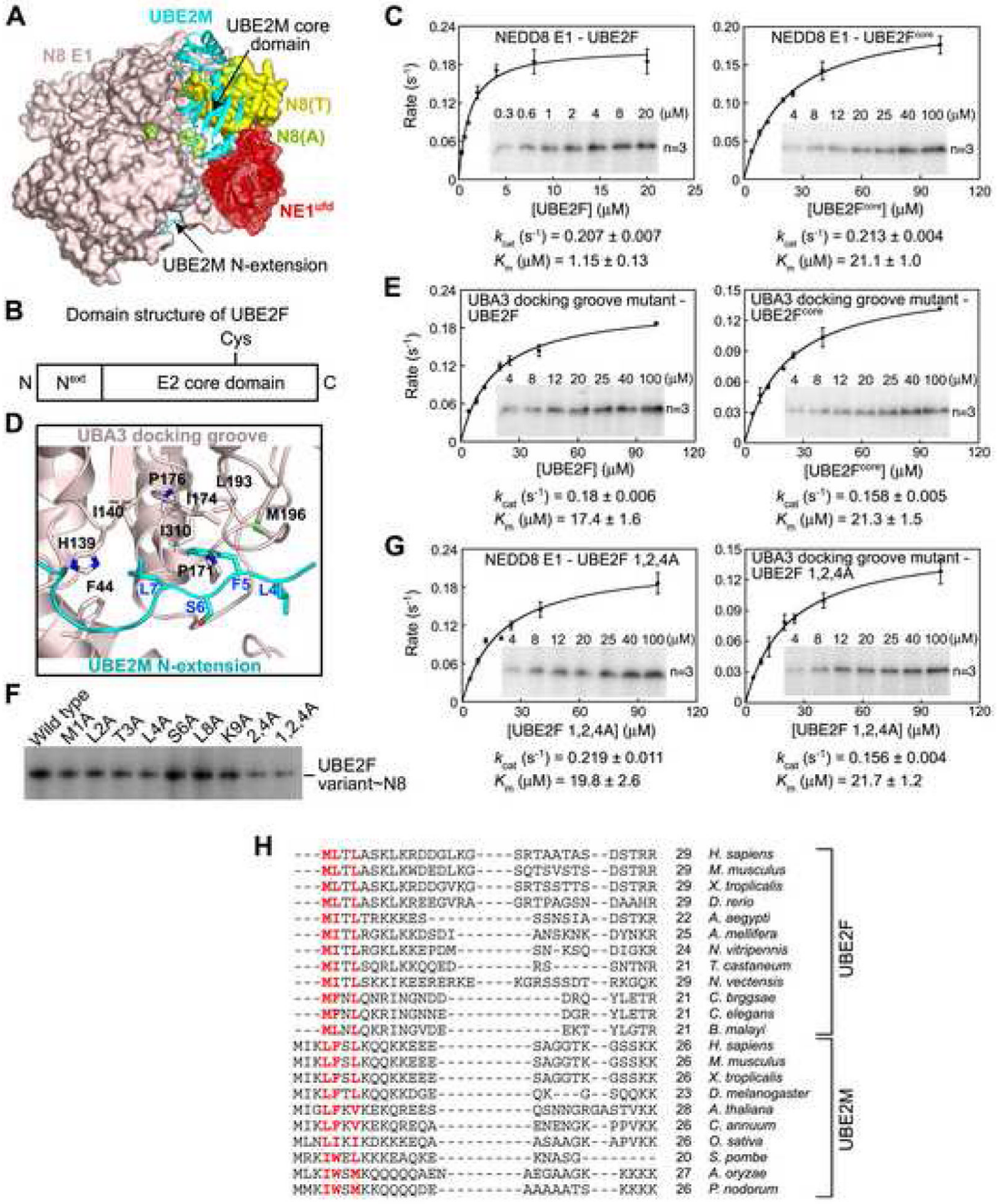

Figure 2. Bipartite UBE2F interaction with UBA3: a conserved Φ-Φ-X-Φ UBA3 in NEDD8 E2 N-extensions

A, Structure of NAE1 UBA3~NEDD8(T)-NEDD8(A)-MgATP-UBE2M(C111A) (Huang et al., 2007) depicting interactions between NEDD8's E1 (NAE1-UBA3, light pink, with ufd in red) and UBE2M (cyan). The thioester-bound NEDD8 (N8(T)) is yellow and adenylation site NEDD8 (N8(A)) is lime. UBE2M's N-extension and core domains are indicated. ***B,*** Domain structure of UBE2F. ***C,*** Michaelis-Menten curves for ^{32}P-NEDD8 thioester conjugate formation by UBE2F and UBE2F^{core}. ***D,*** Detailed interactions between UBE2M's N-extension (blue) and UBA3's docking groove (black) (Huang et al., 2004). ***E,*** Michaelis-Menten curves for UBA3 docking groove mutant-catalyzed ^{32}P-NEDD8 thioester conjugate formation by

UBE2F and UBE2F^{core}. ***F,*** Autoradiogram of ^{32}P-NEDD8 thioester conjugates formed by the indicated Ala mutant of UBE2F. ***G,*** Michaelis-Menten curves for ^{32}P-NEDD8 thioester conjugate formation by UBE2F and NAE1-UBA3 and mutants. ***H,*** Structure-based sequence alignment of UBE2F and UBE2M's N-terminal extension from various species (Tables S2, S3).

Kinetic analyses (C, E, G) show standard errors, insets are representative autoradiograms indicating E2 concentrations and the number of independent replicates, and kinetic constants (k_{cat} and K_m values) are indicated.

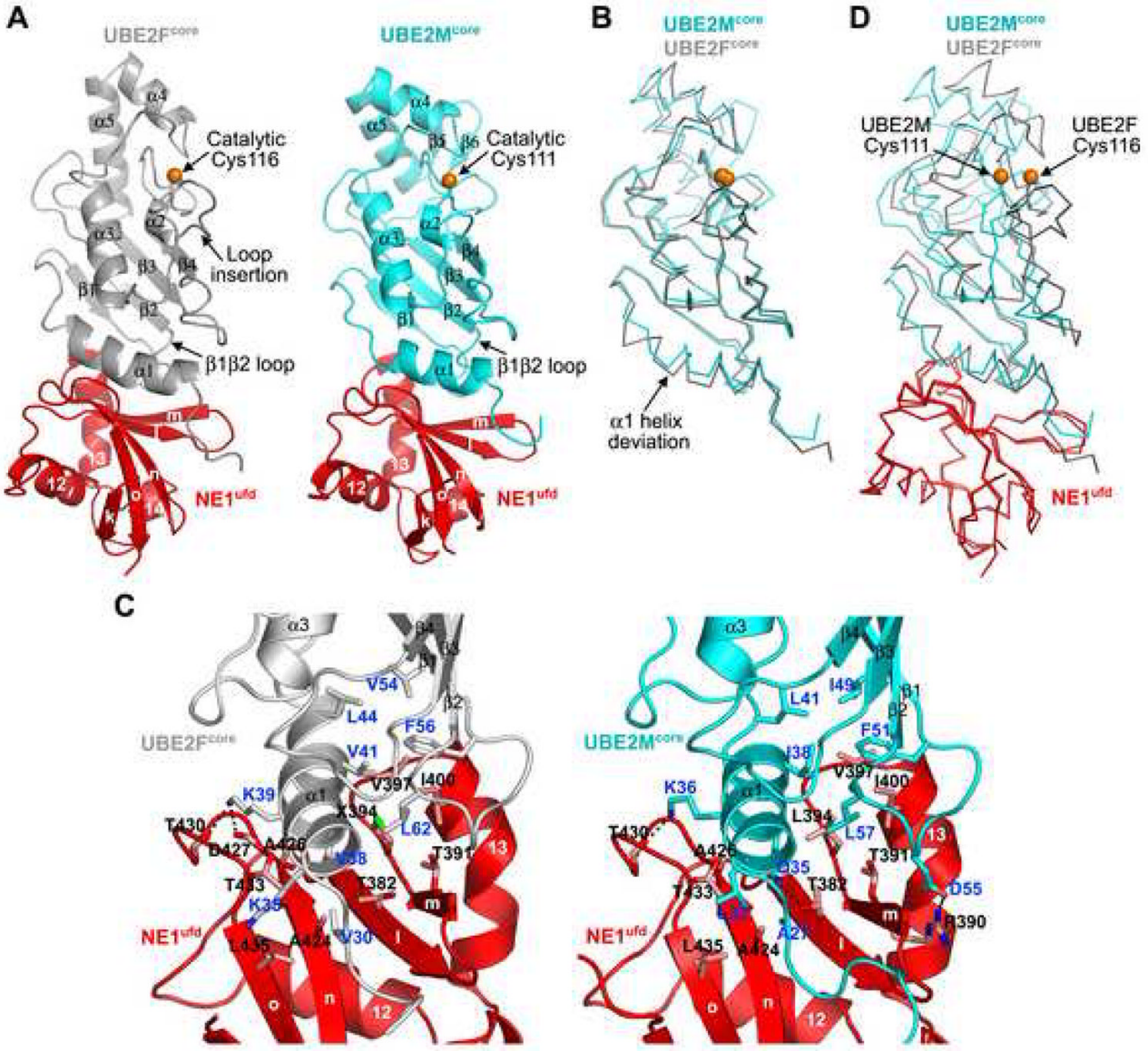

Figure 3. Structural basis for NEDD8 E1 ufd's binding to varying E2 sequences

A, Overall structure of NE1^{ufd}-UBE2F^{core} (left) compared to NE1^{ufd}-UBE2M^{core} (Huang et al., 2005) (right). NE1^{ufd} is red, UBE2F^{core} grey, UBE2M^{core} cyan, and E2 catalytic cysteines orange spheres. ***B,*** UBE2F^{core} and UBE2M^{core} structural superposition. ***C,*** Close-ups of NE1^{ufd}-UBE2F^{core} (left) and NE1^{ufd}-UBE2M^{core} (right) interfaces. Oxygens are red, nitrogens blue, ionic interactions dashed. E2 residues are labeled in blue, and NE1^{ufd}'s in black. X – Leu394SeMet. **D,** Structural superposition of the NE1^{ufd} portions of NE1^{ufd}-UBE2F^{core} and NE1^{ufd}-UBE2M^{core}.

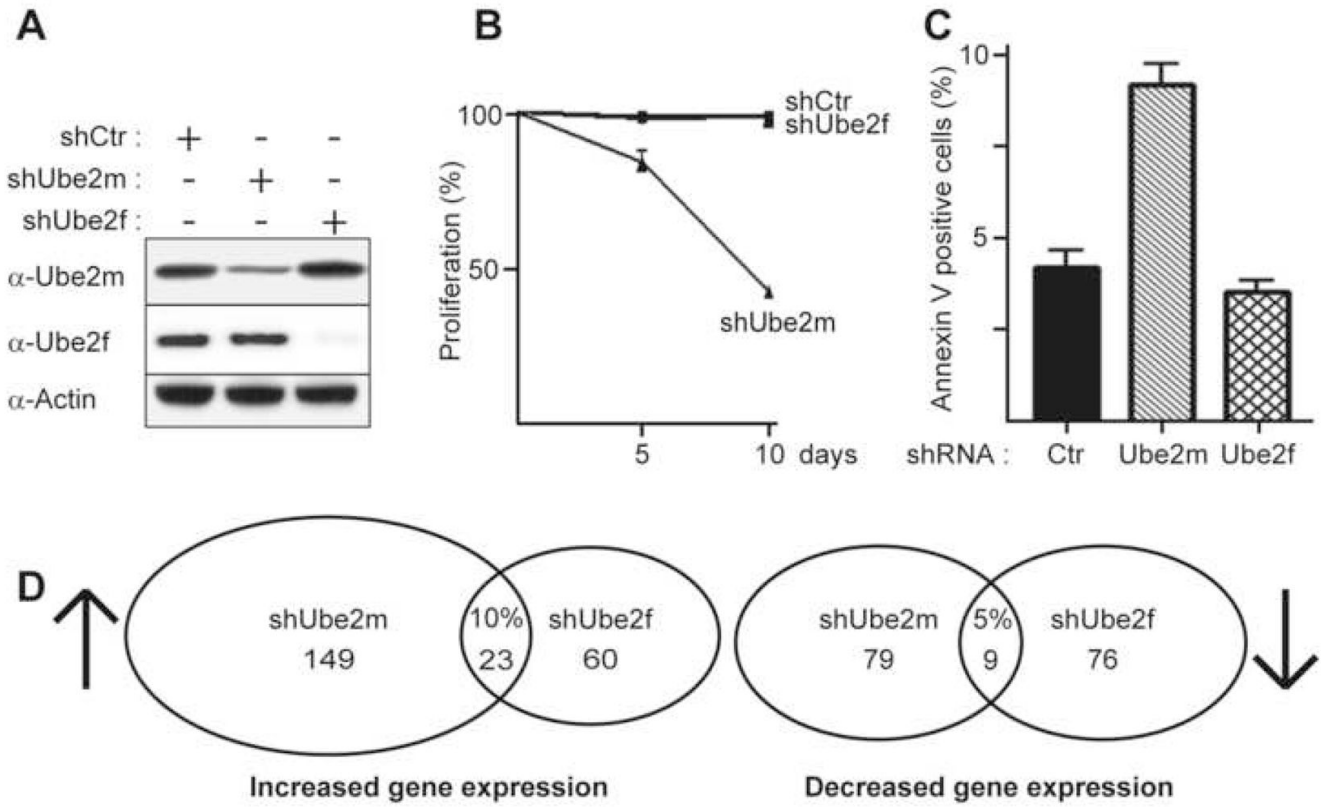

Figure 4. Effects of Ube2f and Ube2m knockdown on global cellular properties

A, Immunoblots of Ube2f and Ube2m proteins after infecting NIH 3T3 cells with retroviruses expressing shRNAs for control (Ctr), Ube2m or Ube2f. ***B,*** Average % of cells in culture (3 independent experiments) relative to control 5 and 10 days after infecting NIH 3T3 cells with the indicated retroviruses. ***C,*** Average % of Annexin V positive cells (3 independent experiments) detected by flow cytometry 8 days after infecting NIH 3T3 cells with the indicated retroviruses. Error bars – standard error. ***D,*** Venn diagram displaying the results of transcriptome analysis from 3 independent experiments. Total numbers of up- and down-regulated genes for NIH 3T3 cells treated with shRNA against Ube2m or Ube2f versus control, displaying % overlap.

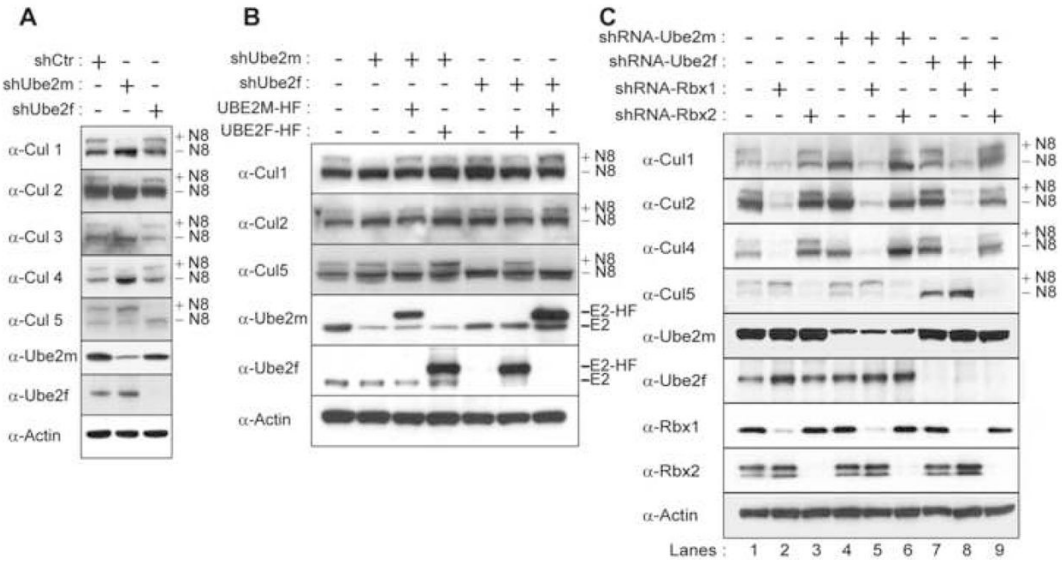

Figure 5. Distinct E2-Rbx pairs regulate cullin NEDD8ylation specificity *in vivo*-N8 indicates unNedd8ylated and +N8 indicates Nedd8ylated cullin

A, Immunoblots of urea lysates from NIH 3T3 cells infected with retroviruses expressing shRNA against control (Ctr), Ube2m or Ube2f, probed with the indicated antisera. ***B,*** Immunoblots of urea lysates from retrovirally infected NIH 3T3 cells as in A, in the absence or presence of retroviruses expressing human UBE2M-HF or human UBE2F-HF, probed with the indicated antisera. ***C,*** Immunoblots of urea lysates from retrovirally infected NIH 3T3 cells as in A, in the absence or presence of retroviruses expressing shRNA against Rbx1 or Rbx2, probed with the indicated antisera.

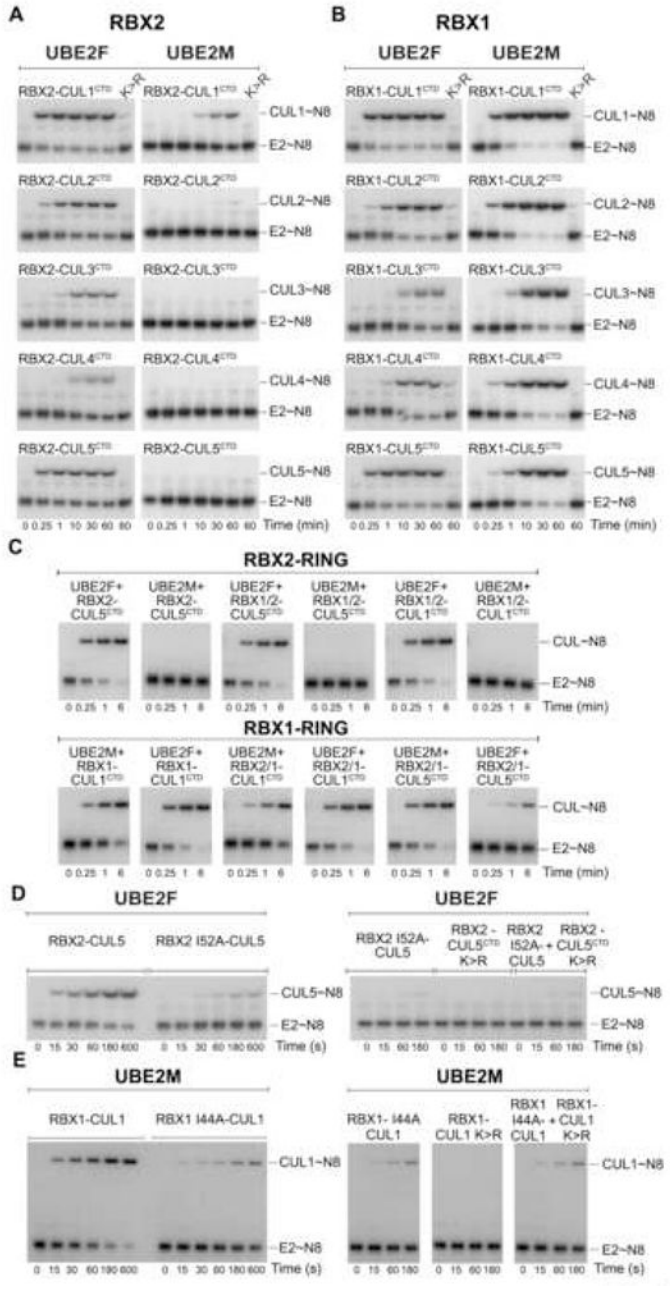

Figure 6. Combinatorial roles of RBXs and E2s establish cullin NEDD8ylation specificity *in vitro*

Autoradiograms of chase reactions monitoring ***A,*** RBX1- or ***B,*** RBX2-mediated ^{32}P-NEDD8 transfer from UBE2F (left) and UBE2M (right) to the indicated CUL^{CTD}. ***C,*** Autoradiograms for chase reactions monitoring ^{32}P-NEDD8 transfer from UBE2F or UBE2M to the indicated CUL^{CTD} mediated by RBX chimeras harboring the indicated N-terminus/RBX2 RING (top panel), and the indicated N-terminus/RBX1 RING (bottom panel). ***D,*** UBE2F reactions as in A and B, except with RBX2(Ile52Ala)-CUL5 (left) and wild-type RBX2 in complex with non-NEDD8ylatable CUL5^{CTD}(K724R) as indicated (right). ***E,*** UBE2M reactions as in A and B, except with RBX2(Ile44Ala)-CUL1 (left) and wild-type RBX1 in complex with non-NEDD8ylatable CUL1^{CTD}(K720R) as indicated (right).

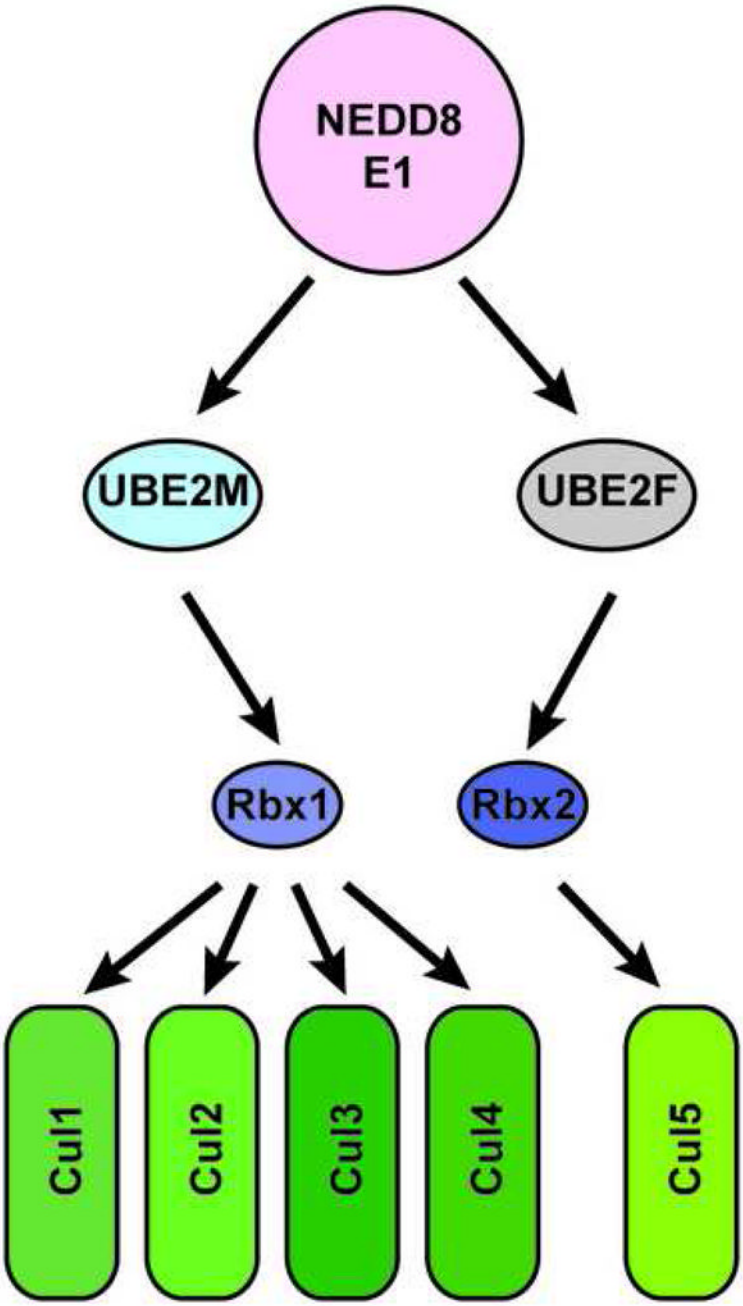

Figure 7. Hierarchical E2-RING expansion of the NEDD8 cascade

Schematic view of distinct molecular pathways dictating specificity of cullin NEDD8ylation.