Communication

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A Small Molecule That Switches a Ubiquitin Ligase From a Processive to a Distributive Enzymatic Mechanism

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Supporting Information Placeholder

ABSTRACT: E3 ligases are genetically implicated in many human diseases, yet E3 enzyme mechanisms are not fully understood and there is a strong need for pharmacological probes of E3s. We report the discovery that the HECT E3 Nedd4-1 is a processive enzyme, and that disruption of its processivity by biochemical mutations or small molecules switch Nedd4-1 from a processive to a distributive mechanism of polyubiquitin chain synthesis. Furthermore, we discovered and structurally characterized the first covalent inhibitor of Nedd4-1, which switches Nedd4-1 from a processive to a distributive mechanism. To visualize the binding mode of the Nedd4-1 inhibitor we used X-ray crystallography and solved the first structure of a Nedd4-1 family ligase bound to an inhibitor. Importantly, our study shows that processive Nedd4-1 but not the distributive Nedd4-1:inhibitor complex is able to synthesize polyubiquitin chains on the substrate in the presence of the deubiquitinating enzyme USP8. Therefore inhibition of E3 ligase processivity is a viable strategy to design E3 inhibitors. Our study provides fundamental insights into the HECT E3 mechanism, and uncovers a novel class of HECT E3 inhibitors.

E3 ligases regulate all aspects of biology and there is a strong need for E3 ligase inhibitors and probes.[1-3] Nedd4-1 is a HECT E3 ubiquitin ligase (~28 known) and regulates mammalian metabolism, growth, and development.[4] Furthermore it is a promising drug target to treat cancers,[5] obesity,[6] Parkinson's disease,[7] and viral infections.[8] HECT E3s form an obligatory HECT E3~Ub thioester during the catalytic cycle for the subsequent ligation of the ubiquitin onto the substrate lysine. Current biochemical studies of HECT E3s suggest a mode of chain elongation, which may occur by either a processive or a distributive mechanism (Figures 1, S1).[9-13] In this model, the last ubiquitin (Ub) of the growing polyubiquitin chain binds the N-lobe of the catalytic HECT domain proximal to the C-lobe, which positions

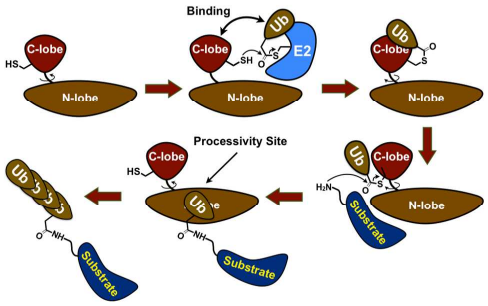

Figure 1. Simplified model of HECT E3 mediated protein ubiquitination. E2~Ub thioester binds HECT E3 domain, followed by transthiolation reaction producing HECT E3~Ub thioester. Subsequently, HECT E3~Ub ligates Ub onto the lysine of the substrate, followed by polyubiquitin chain growth. C- and N-lobe of HECT domain rotate relative to each other during the catalytic cycle.

this polyUb chain for the addition of another Ub molecule for polyubiquitin chain growth. *However, whether HECT E3 ligases are processive or distributive enzymes, and how this process might be targeted for inhibition, had not been completely investigated.* Herein, we present the first rigorous proof that Nedd4-1 is a processive enzyme, and describe the discovery of a first-in-class Nedd4-1 inhibitor. The discovered Nedd4-1 inhibitor is the first example of an E3 inhibitor that switches the enzyme from a processive to a distributive mechanism of polyubiquitin chain synthesis. Furthermore, when Nedd4-1 becomes distributive, substrate ubiquitination can be efficiently antagonized by the deubiquitinating enzyme USP8 *in vitro*.

The crystal structure of the Nedd4-1 HECT domain with Ub bound to the N-lobe[10-11] has two solvent-exposed Cys residues: the catalytic Cys^{867} and the non-catalytic Cys^{627}. Cys^{627} is located in the N-lobe of Nedd4-1 near Phe^{707} of Nedd4-1 and Ile^{44} of Ub, two residues that form critical hydrophobic contacts (Figure 2A). Mutation of Phe^{707} to Ala in Nedd4-1 disrupts Nedd4-1:Ub binding and affects the kinetics of

polyubiquitin chain growth.[11] The equivalent F618A mutation

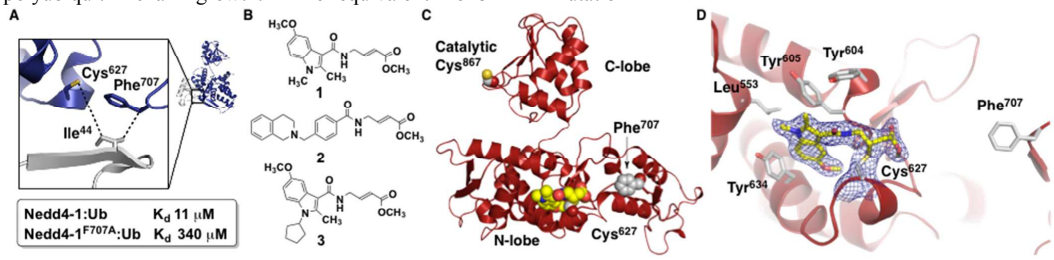

Figure 2. A) The hotspot interface of Nedd4-1 (blue) and ubiquitin (gray) in the Nedd4-1:Ub complex (PDB ID 2XBB) responsible for polyubiquitin chain elongation, with the key side chains depicted as sticks. B) Electrophilic compounds **1-3** used in this work. Compounds **1** and **2** are specific hits, and compound **3** is an optimized covalent inhibitor of Nedd4-1. C) Cartoon depiction of the crystal structure of Nedd4-1 bound to fragment hit **1** with key side chains and the fragment shown as spheres. D) Close-up view of the small molecule-binding site with the key side chains depicted as sticks and colored by atom type. The $2F_O - F_C$ electron density map (blue mesh, contoured at 1.0 σ) is presented for Cys^{627} and **1**.

in Rsp5, the *S. cerevisiae* homolog of Nedd4-1, also disrupts its binding to Ub and results in temperature-sensitive growth defects, suggesting an essential function of this site *in vivo*.[12] Therefore, inhibitors that target these cysteines would either inhibit Nedd4-1 completely (Cys^{867}), or inhibit the ability of Nedd4-1 to elongatepolyubiquitin chains (Cys^{627}). To search for Nedd4-1 inhibitors, we used our previously reported covalent tethering method.[14] The HECT domain of Nedd4-1 was treated with a mixture of electrophilic fragments, and compounds **1** and **2** were identified as weak covalent modifiers of cysteine residues in Nedd4-1 using mass spectrometry (Figure 2B, S2). Point mutation studies revealed that compounds **1** and **2** selectively reacted with the non-catalytic Cys^{627} of Nedd4-1 (Figure S3) in a time- and concentration-dependent manner (Figure S4). The catalytic Cys^{867} of Nedd4-1 was more reactive than Cys^{627} with the N-acetyl electrophile **4** that has only CH_3 as a directing group (Figure S5), suggesting that compounds **1** and **2** are specific hits, since they modify the less reactive non-catalytic cysteine in the presence of the more reactive catalytic cysteine. Furthermore, compound **1** demonstrated an initial structure-activity relationship, a further indication that covalent labeling of Nedd4-1 by **1** was specific (Figure S6).

To visualize the binding mode of compound **1**, we crystallized the Nedd4-1:**1** complex and solved the structure to 2.44 Å resolution (PDB ID: 5C91) (Figure 2C, S7). Notably, this structure is the first of a HECT E3 ubiquitin ligase bound to a small molecule. The overall conformation is virtually identical to the previously reported structure of Nedd4-1[10-11] (root-mean-square deviation (rmsd): 0.316 Å; Figure S7). Our structure confirms that **1** forms a stable covalent bond with Cys^{627} and reveals that the hydrophobic indole core of **1** is oriented towards a pocket of the N-lobe formed by residues Leu^{553}, Glu^{554}, Asn^{602}, Tyr^{604}, Tyr^{605}, Leu^{607}, and Tyr^{634} (Figure 2D, S7). This ligand orientation explains why compound **6**, which contains an EtO- group on the indole core, did not label Nedd4-1, since the ligand binding pocket cannot accommodate this sterically bulkier group (Figure S6). The aromatic edge-to-face interactions of Tyr^{605} and Tyr^{634} with the indole moiety of **1** provide further stabilization of the ligand conformation, while a hydrogen bond between the backbone carbonyl oxygen of Tyr^{605} and the amide NH of **1** positions the connecting region between Cys^{627} and the indole group.

The ester methoxy group points inward and towards the cavity formed by $Gly^{606}O$, $Asn^{621}N$, $Asn^{623}O$, and $Asn^{623}C\beta$, which are within 3.4 – 4.4 Å of the methyl group. However, since the methyl ester group is freely rotatable around the C-C bond, our crystallographic data cannot exclude a partial conformation in which the methoxy and the carbonyl groups are exchanged. Interestingly, it was shown that Tyr^{605} of Nedd4-1 is also important for non-covalent Ub binding and polyubiquitin chain elongation by Nedd4-1.[11] Since **1** forms an edge-to-face binding interaction with Tyr^{605}, the ligand should block the interaction between Leu^{73} of Ub and Tyr^{605} of Nedd4-1 observed in the Nedd4-1 HECT:Ub complex structure.

Our initial experiments showed that labeling of Nedd4-1 with **1** could be completely inhibited by 60 μM of Ub (Figure S8), which is the approximate concentration of Ub in cells.[15] Therefore further improvements in the potency of **1** were necessary. Since the N-methyl group in **1** was shown to tolerate substitutions, we prepared a series of N-substituted analogues **3, 11-13** (Figure S9). Of these, the N-cyclopentyl analogue **3** was the most potent. Indicative of its selectivity, compound **3** did not label the HECT domains of the related ligases WWP1 or E6-AP that don't have cysteine at this position (Figure S10, S11B), but it was effective at labeling the highly homologous HECT ligase Nedd4-2, which has an almost identical non-covalent Ub-binding site and a cysteine at that position (Figure S11). Furthermore, compound **3** did not react with the deubiquitinase USP8, Human Rhinovirus 3C protease, the E1 enzyme Ube1, or the E2 enzyme UbcH5a, all of which have reactive catalytic cysteines (Figure S12).

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Comment [S2]: Added at reviewer 2's request

Fluorescence polarization (FP) experiments using fluorescein-Ub were used to confirm that **3** inhibits the

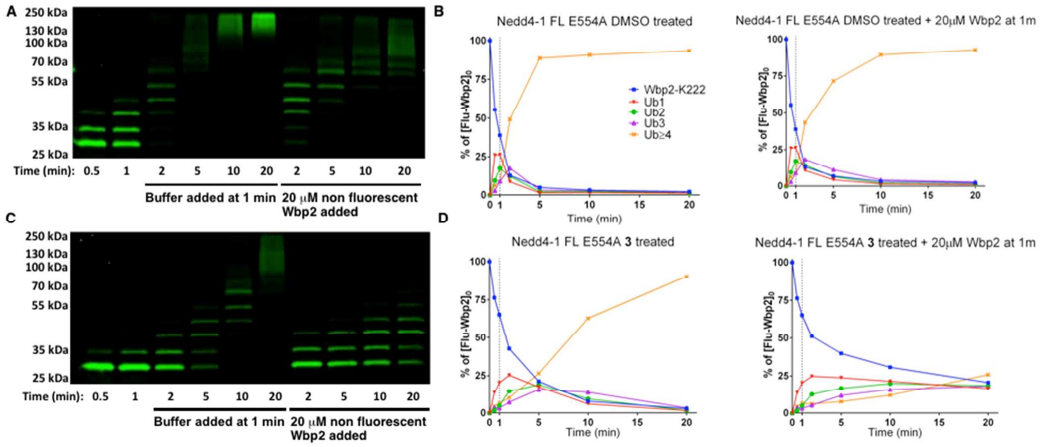

Figure 3. Covalent inhibitor **3** switches Nedd4-1 from a processive to a distributive enzymatic mechanism. A) Full length Nedd4-1 with the activating E554A mutation (150 nM) was incubated with fluorescent Flu-Wbp2 substrate (100 nM) in the presence of ATP, Ub, E1 and E2 enzymes. After 1 min, a 200-fold excess of non-fluorescent Wbp2 substrate or empty buffer was added to the reaction mixture, and further ubiquitination of Flu-Wbp2 was monitored. B) The amount of ubiquitinated Flu-Wbp2 was plotted as a function of time. C) The same experiment was conducted with full-length Nedd4-1 E554A covalently modified with compound **3**. D) The amount of ubiquitinated Flu-Wbp2 in C) was plotted as a function of time.

Nedd4-1:Ub interaction. Compound **3** disrupted Nedd4-1:Ub binding with second-order inhibition kinetics (K_I 29.3 μM, k_{inact} 5.8×10^{-5} s^{-1}; k_{inact}/K_I = 1.98 M^{-1} s^{-1}) and was 22.2-fold more potent than compound **1**. (Figure S13). Notably, the FP assay requires a high concentration of Nedd4-1 (8 μM); so with a K_I of 29.3 μM we achieve half-maximal covalent inhibition at only a 3.7-fold excess of inhibitor relative to Nedd4-1. Furthermore, in contrast to indole **1**, compound **3** was able to label Nedd4-1 even in the presence of 60 μM Ub, indicative of its increased potency (Figure S14). To investigate if compound **3** affects the ability of Nedd4-1 to elongate polyUb chains we used Wbp2-C-K222 as a substrate.[13] Wbp2-C-K222 has only one acceptor lysine residue (Lys^{222}) and a single cysteine residue (Cys^{73}), which we modified with 5-iodo-acetamidofluorescein for quantification purposes (abbreviated Flu-Wbp2; Figure S15). Remarkably, we observed that the catalytic HECT domain of Nedd4-1 covalently modified with compound **3** was not able to build long polyUb chains on Flu-Wbp2, as compared to Nedd4-1 treated with DMSO or the negative control electrophile **14** (Figure S16). Importantly, this inhibition does not occur because **3** inhibits the formation of the Nedd4-1~Ub thioester (Figure S17). However, since the Nedd4-1:**3** complex could still build short polyUb chains on Flu-Wbp2, we asked if these chains are built with a processive or a distributive mechanism.

We hypothesized that since compound **3** disrupts Ub binding to the non-covalent Ub-binding site of Nedd4-1, the ubiquitinated substrate would be more likely to dissociate from the Nedd4-1:inhibitor complex in between rounds of addition of Ub to the growing chain. Therefore, inhibitor-bound Nedd4-1 should assemble polyUb chains via a distributive mechanism. To distinguish between processive and distributive mechanisms, we used an assay wherein full length Nedd4-1 with or without inhibitor pretreatment is allowed to ubiquitinate Flu-Wbp2 for 1 min, followed by addition of a 200-fold excess of non-fluorescent Wbp2. If Nedd4-1 is processive, it should remain bound to Flu-Wbp2-Ub_x and continue to elongate the polyUb chain on Flu-Wbp2 even in the presence of the large excess of non-fluorescent Wbp2. If Nedd4-1 is distributive, it should dissociate from Flu-Wbp2-Ub_x between rounds of ubiquitination. In this case, Flu-Wbp2-Ub_x will be outcompeted by non-fluorescent Wbp2 and polyUb chain growth on Flu-Wbp2 will be inhibited.

For these experiments, we used full-length Nedd4-1 with the activating E554A mutation, which disrupts the autoinhibitory conformation of wild type full length Nedd4-1.[17] We found that E554A Nedd4-1 was processive and efficiently converted Flu-Wbp2 into ≥Ub_4 modified Flu-Wbp2 even after addition of a 200-fold excess of non-fluorescent Wbp2 (Figure 3A-B). However, in the case of the Nedd4-1:**3** complex (Figure S19), we found that ubiquitination of Flu-Wbp2 was significantly inhibited upon addition of a 200-fold excess of non-fluorescent Wbp2. Furthermore, consumption of monoubiquitinated Flu-Wbp2 and the formation of Ub_2/Ub_3 and ≥Ub_4 modified Flu-Wbp2 were also inhibited (Figure 3C-D). This observation indicates that inhibitor-bound Nedd4-1 dissociates from Flu-Wbp2-Ub_x before adding Ub_{x+1}, and is therefore distributive. Similar re-

Comment [AS3]: Added in response to reviewer 1 request.

sults were observed for the Nedd4-1 E554A F707A mutant (Figure S20). *These experiments prove for the first time that Nedd4-1 is processive, and when the non-covalent interaction between the N-lobe and Ub is disrupted by compound **3** or the F707A mutation the enzyme becomes distributive. Previously, it was assumed, but not rigorously proven, that HECT E3s are processive and not distributive enzymes.*

Since endogenous intracellular deubiquitinating enzymes (DUBs) reverse protein ubiquitination, we hypothesized that distributive Nedd4-1 would be more susceptible to antagonism by DUBs than processive Nedd4-1. To test this hypothesis, full length Nedd4-1 E554A with or without compound **3** bound and the Nedd4-1 E554A F707A mutant were allowed to ubiquitinate Flu-Wbp2 in the presence of the DUB USP8. *In vitro*, USP8 can disassemble K48- and K63-linked polyUb chains,[18] while Nedd4-1 predominantly makes K63-linked chains.[11] Remarkably, we observed that untreated Nedd4-1 robustly polyubiquitinated Flu-Wbp2 in the presence of USP8 after 30 min. However, neither compound **3**-treated Nedd4-1 nor the F707A mutant were able to consume Flu-Wbp2 or build $\geq Ub_4$ chains on Flu-Wbp2 in the presence of USP8, even though they consumed Flu-Wbp2 in the absence of USP8 (Figure 4, S21). Since distributive Nedd4-1 dissociates from the substrate in between rounds of Ub addition to the growing polyUb chain, this provides an opportunity for the DUB to hydrolyze the Ub chain before another Ub can be added to it by Nedd4-1. Processive catalysis appears to be a necessary condition for the formation of $\geq Ub_4$ chains on the substrate in the presence of DUB.

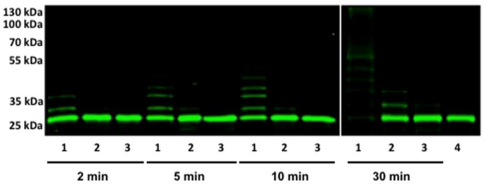

Figure 4. Ubiquitination of Flu-Wbp2 (100 nM) by a full length Nedd4-1 (150 nM) at different time points in the presence of the deubiquitinase USP8 (200 nM) shows that distributive Nedd4-1 is inhibited by USP8, but processive Nedd4-1 is not. Lane 1: DMSO treated Nedd4-1 full length E554A, lane 2: compound **3** treated Nedd4-1 full length E554A, lane 3: Nedd4-1 full length E554A F707A, and lane 4: no ATP control.

In summary, we report the first rigorous proof that the HECT E3 Nedd4-1 is a processive enzyme, and that disrupting non-covalent binding of Ub to the N-lobe of Nedd4-1 switches Nedd4-1 to a distributive enzyme. Furthermore, we report the discovery and structural characterization of a covalent Nedd4-1 inhibitor that targets this processive site and switches Nedd4-1 from a processive to a distributive mechanism. We also show that introducing a DUB antagonist into the assay augments the inhibitory effect of compound **3** on Nedd4-1 while the untreated enzyme is still able to build long polyUb chains. E3 ligase inhibitors with this mechanism of action were not previously known and this work outlines a conceptually new design strategy for E3 ligase inhibitors. Taken together, our studies provide fundamental insights into the HECT E3 enzymatic mechanism and represent an important case study in the emerging area of E3 ligase inhibitor discovery.[19] Further studies of Nedd4-1 inhibitors will be reported in due course.

Comment [S4]: Added in response to reviewer 3's comments.

ASSOCIATED CONTENT

Supporting information available free of charge via the Internet at http:/pubs.acs.org.

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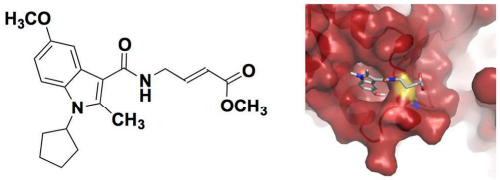

Covalent Inhibitor of Nedd4-1 Ubiquitin Ligase