Supporting Information

Mund et al. 10.1073/pnas.1412152111

SI Methods

Plasmids. For mammalian expression, all E3 ligase ORFs (human except Nedd4: African green monkey) were cloned into pcDNA3.1 (Invitrogen) in frame with a triple amino-terminal hemagglutinin (HA) tag or a N-terminal EGFP plus triple HA-tag. The following HECT-only constructs (which include the C terminus in each case) for bacterial and mammalian expression were used: Smurf2 (aa 371–748), Smurf1 (aa 377–757), Nedd4 (aa 521–900), Nedd4L (aa 576–955), WWP1 (aa 546–922), Mule/HUWE1 (aa 3,993–4,374), and Ube3C (aa 664–1,083). In the Mule/Nedd4-HECT chimera, the Mule E2 site (aa 4,152–4,214) was replaced by the Nedd4 E2 binding site (aa 680–740) The following E2 site mutants were used: Smurf2 (WI535,536CT), Nedd4 (EY680,681AH), WWP1 (EF702,703QL), and Mule (E4177A, H4152A, DR4087/8A). Flag-Dvl2 WT and PY motif mutant (PPPY→AAPA) was a generous gift of Michael Graeb and Mariann Bienz (MRC Laboratory of Molecular Biology). His-tagged ubiquitin was expressed from a plasmid encoding a tandem array of eight tagged ubiquitins (1); a plasmid expressing a single HA-tagged ubiquitin was a kind gift from Donna Mallery (MRC Laboratory of Molecular Biology). The ubiquitin fusion degradation pathway substrate Ub-GV-EGFP was generated by cloning ubiquitin G76V in frame with EGFP into pEGFP-N1 (Clontech). Mutations were generated by standard QuikChange mutagenesis and verified by sequencing.

Protein Expression and Purification. For bacterial expression, plasmids were transformed into BL21 (DE3) CodonPlus-competent *Escherichia coli* (Stratagene). Protein expression was induced with 1 mM isopropyl beta-D-thiogalactoside at 30 °C for 3 h or at 18 °C overnight. Cell pellets were resuspended in 50 mM Tris at pH 7.4, 150 mM NaCl [for Nedd4, 500 mM NaCl and 5% (vol/vol) glycerol], lysed by sonication or by passing twice through an EmulsiFlex-C5 homogenizer (Avestin). Fusion proteins were affinity-purified from soluble bacterial lysates by using glutathione-Sepharose (GE Healthcare) or Ni-NTA agarose (Qiagen), according to standard manufacturer's protocols. After elution, recombinant proteins were either further purified by gel filtration on a Sephadex G-75 column (GE Healthcare) (if used for ITC, fluorescence polarization, Alpha screening, or H/D exchange) or directly rebuffered into resuspension buffer including 5–10% (vol/vol) glycerol, using PD-10 desalting columns (GE Healthcare), concentrated with Amicon Ultra centrifugation filter devices (Millipore), and stored at −80 °C until required. Protein stock solution was typically around 10 mg/mL in PBS with 1 mM tris(2-carboxyethyl)phosphine (TCEP).

In Vitro Ubiquitination Assays. In vitro ubiquitination assays were set up as described in ref. 2. Unless otherwise stated, assays contained 150 ng human E1 (BIOMOL, Plymouth Meeting), 150 ng human E2 (UbcH7 or UbcH5c, BIOMOL; 500 ng UbcH7 with Smurf2), 10 μg ubiquitin (BIOMOL), and 20 ng GST-E3 (50 ng for Smurf2 and Mule). For a quantitative readout, untagged ubiquitin (10 μg) was mixed with N-terminally biotinylated ubiquitin (1 μg), and GST-E3 was adsorbed to glutathione-coated (or for some experiments, anti-GST coated) ELISA plates (Pierce; maximum capacity, 10 ng protein) for 45 min, followed by incubation with HRP-coupled Streptavidin for 20 min. Auto-ubiquitinated E3 was quantified after addition of 100 μL tetramethylbenzidine solution (Sigma-Aldrich) on a TECAN Infinite F200 microplate reader. Bicycle or compound inhibitors were pre-incubated with E3 for 10–30 min at the indicated concentrations in half the final volume before the addition of the remaining components of the ubiquitination assay. To assay thioester-bonded intermediates, the ubiquitination reaction (20 μL) was stopped by adding 20 μL sample buffer without β-mercaptoethanol. Each sample was then split into two 20-μL aliquots before adding 20 μL sample buffer with or without β-mercaptoethanol, respectively.

Reversal of Heclin Inhibition. GST-Nedd4-HECT (50 ng in 50 μL) was incubated with various concentrations of heclin in PBS for 20 min at room temperature. Of this, 20 μL was then mixed with 150 μL PBS containing 3% (wt/vol) BSA and 0.1% Triton X-100 and added to the well of a glutathione-coated ELISA plate (10-ng capacity) and incubated for 1 h. Wells were washed 10× with PBS containing 0.1% Triton X-100, and then 30 μL standard ubiquitination reaction mix was added and the plates were incubated for 1 h and processed for ELISA as normal.

Phage Selection. Selections were performed on the phage library in the vector fdD120ss, as described in ref. 3, using a slightly modified protocol. Briefly, 800 mL overnight cultures of infected *E. coli* HB2151 were centrifuged for 30 min at 13,000 × *g*, and phages were precipitated from the supernatant by the addition of PEG 8000/NaCl to 4% (wt/vol) and 500 mM, respectively, and incubation on ice for 30 min. After centrifugation at 4 °C for 30 min at 13,000 × *g*, the pellet was resuspended in 20 mL degassed PBS before treatment with 1 mM TCEP (Thermo Scientific) for 1 h at 42 °C, as described (3). After treatment, phages were again PEG precipitated and redissolved in 50 μM TCEP in PBS before adding Tris-(bromomethyl) benzene (Sigma-Aldrich) and acetonitrile to final concentrations of 60 μM and 10% (vol/vol), respectively, and incubating for 1 h at 30 °C. After further PEG precipitation, phages were resuspended in PBS for selection.

For the initial phage selections, 20 μg His6-tagged HECT domain was typically incubated for 30 min with 40 μL His-tag Dynabeads (Life Technologies) in 0.5 mL PBS at pH 7.4, 300 mM NaCl (Nedd4 only), 5% (vol/vol) glycerol (Nedd4 only), 1% BSA, and 0.05% Tween20. The chemically modified phages (10^{11} transducing units) were rotated in the same buffer for 30 min before mixing with HECT domain and beads and rotating for a further 30 min. After incubation, the beads were isolated magnetically and washed successively six times with 1 mL PBS at pH 7.4, 0.05% Tween20, and twice with PBS alone, changing the tubes on alternate washes. Bound phages were typically eluted with 200 μL of 250 mM imidazole at pH 7.0 and were then used for further infection of HB2151 cells. Cells were plated on 20 × 20 cm 2x tryptone/yeast extract plates containing chloramphenicol and harvested the next day. After three rounds of selection, individual phage clones were grown up and sequenced and, if required, purified and modified as described in ref. 3 and stored at −80 °C until needed.

Screening of Individual Phage Colonies for Target Binding by ELISA. GST-HECT fusions were bound to 96-well glutathione plates (Thermo Scientific) at a concentration of 10 μg/mL in PBS for 1 h, under humidified conditions. Plates were then blocked with 2% (wt/vol) nonfat milk in PBS for 1 h, and the single phage preparations were diluted to OD_{269} 0.5 in PBS/nonfat milk for 30 min. Phage dilutions were aliquoted into the wells and incubated for 1 h before washing five times by immersing the plates in PBS/0.05% Tween 20 and shaking out and then probing with HRP-conjugated anti-M13 antibody (GE Healthcare). Bound HRP antibody was visualized with tetramethylbenzidine substrate and read in a Tecan Infinite F200.

Peptide Synthesis. Bicycle peptides were synthesized by the LMB peptide synthesis group and by Designer Bioscience (St John's Innovation Centre) and were cyclized by the method described in ref. 3.

Cell Transfections and Immunoblotting. HEK-293T cells were maintained in DMEM supplemented with 10% (vol/vol) FBS and 50 U/mL penicillin/streptomycin and were kept at 37 °C in a humidified 10% CO_2 atmosphere. Transient transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, or polyethylenimine (Polysciences). Transfections with polyethylenimine [linear, molecular weight (MW) 25,000] were performed, using the Lipofectamine 2000 transfection protocol, but instead of Lipofectamine 2000, equal volumes of a 1 mg/mL solution of polyethylenimine were used. Typically, cells were harvested 24 h after transfection. Immunoblotting was done using standard procedures, and proteins were detected using antibodies from Sigma-Aldrich (HA, Flag, gamma-tubulin, myc) or Roche (GFP).

Pull-down Experiments. For pull-downs under native conditions, 6×10^6 transfected cells were washed in PBS and subsequently lysed in 800 μL 0.3% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, and a protease inhibitor mixture complete (Roche) for 10 min on ice. Soluble proteins were recovered by centrifugation at 14,000 × *g* for 10 min at 4 °C. Biotinylated bicycle (10 μM) was then incubated with 400 μL lysate for 1 h at 4 °C before adding 10 μL Streptavidin-Dynabeads (Life Technologies) for 30 min. The beads were then washed three times in lysis buffer before elution in 2×SDS/PAGE sample buffer and analyzed by Western blotting. His-Ubiquitin pull-downs were done under denaturing conditions. Typically, 2×10^6 cells were harvested in 0.8 mL 8 M Urea 100 mM Tris (pH 7.4), 2 mM *N*-Ethylmaleimide, and 10 mM iodoacetamide, sonicated, and incubated with 10 μL His-tag Dynabeads (Life Technologies) at room temperature for several hours. The beads were washed three times in 8 M urea and eluted in sample buffer.

Compound Treatment and Toxicity Assay. Compounds I–IV were obtained from Vitas-M Laboratory and stored as 10 mM stock in DMSO. Treatment of transfected cells was in full medium for the indicated times and concentrations. For toxicity assays, cells were seeded into 96-well tissue culture dishes the day before they were incubated with twofold dilutions of compounds or DMSO in full medium for 24 h. One hundred microliters CellTiter-Glo reagent (Promega) were added to 100 μL media, mixed and incubated for several minutes, and then transferred into opaque 96-well ELISA plates to measure the ATP content of cells via a stabilized firefly luciferase activity read-out, which is an established assay for cell viability (4). Note that compound I can inhibit firefly luciferase [see pubchem.ncbi.nlm.nih.gov, compound ZINC00106319 (5)], although controls showed this did not affect CellTiterGlo under the conditions used. Because luciferase inhibitors can also stabilize the protein (6), any effects of compound I or heclin on luciferase-based assays need to be interpreted with great caution.

Alpha Screen. The Alpha screen was done in a final volume of 25 μL. First, 5 μL GST-Smurf2 (15 nM) was preincubated with 1 μL compound (about 10 μM) for 30 min before adding 2.5 μL biotinylated bicycle (50 nM) for a further 30 min. After the addition of 5 μL anti-GST acceptor beads (50 μg/mL) for 60 min, 11.5 μL Streptavidin-coated AlphaLISA donor beads (80 μg/mL) were added for a further incubation for 30 min. The assay was read-out on a PHERAstar FS HTS Microplate Reader with an AlphaLISA module. In total, 17,500 compounds were screened: 14,000 from a peptidomimetic small molecule library and about 3,500 from a natural product library.

ITC. ITC was performed using a MicroCal ITC_{200} calorimeter (GE Healthcare). Titrations were performed at 25 °C in PBS at pH 7.4 and consisted of 20 2 μL injections of 1 mM peptide into 40 μM Smurf2-HECT at 160-s intervals. Binding isotherms and curve fittings were obtained using Origin7.0 (OriginLab Corp).

Fluorescence Polarization Binding Assays. Labeling of the E2 enzyme UbcH5 with BODIPY FL-iodoacetamide (Molecular Probes) was done as described (7). Briefly, the E2 enzyme (100 μM) was re-buffered into 50 mM Tris·Cl at pH 7.5, 250 mM NaCl, and 1 mM TCEP, using PD10 desalting columns. A 20-mM stock solution of BODIPY FL suspended in DMSO was diluted into the E2 solution to a 10-fold molar excess, and the conjugation reaction was allowed to proceed overnight in the dark at 4 °C. To quench the reaction, 5 mM β-mercaptoethanol was added. Any precipitates were removed by centrifugation, and unconjugated free probe was removed using PD10 desalting columns. BODIPY FL-labeled E2 enzyme was then concentrated to 60–100 μM. Concentrations for bodipy-UbcH5 used in the fluorescence polarization (FP) assay were 2 and 1.2 μM for the left and right panels in Fig. S6. Manual titrations were performed using wild-type and mutant HECT stock solutions that varied according to the yield and strength of the interaction. All binding assays were performed at room temperature in PBS containing 5 mM β-mercaptoethanol. Fluorescence polarization assays were carried out on a PHERAstar FS HTS Microplate Reader with a corresponding FP module.

Light Scattering. One milligram per milliliter GST-Nedd4-HECT in PBS was incubated with or without 100 μM heclin for 30 min. Size exclusion chromatography multiangle light-scattering measurements (SEC-MALS) were then performed using a Wyatt Heleos II 18 angle light-scattering instrument coupled to a Wyatt Optilab rEX online refractive index detector. Detector 12 in the Heleos instrument was replaced with Wyatt's Quasi-Elastic Light Scattering detector for dynamic light-scattering measurement. Protein samples (100 μL) were resolved on a Superdex S-200 10/300 analytical gel filtration column (GE Healthcare) running at 0.5 mL/min in PBS buffer with 10 μM TCEP before passing through the light-scattering and refractive index detectors in a standard SEC-MALS format. Protein concentration was determined from the excess differential refractive index based on 0.186 refractive index increment for 1 g/mL protein solution. The concentration and the observed scattered intensity at each point in chromatogram were used to calculate the absolute molecular mass from the intercept of the Debye plot, using Zimm's model as implemented in Wyatt's ASTRA software. Static measurements were also made at room temperature with an AvidNano W130i Dynamic Light Scattering System, with 5 μL BladeCells for GST-Nedd4-HECT [4 mg/mL in PBS, 5% (vol/vol) glycerol, 1 mM TCEP, 250 mM extra NaCl] and for GST-Smurf2-HECT [10 mg/mL in PBS, 5% (vol/vol) glycerol, 1 mM TCEP], with 5% (vol/vol) DMSO alone or with 5% DMSO and 100 μM heclin.

Hydrogen Deuterium Exchange Mass Spectrometry. Smurf2 HECT domain (5 μM) was incubated in PBS for 30 min with or without bicyclic peptide (200 μM), and then deuterium exchange was initiated by diluting the protein solution fivefold with D_2O buffer [99.8% (vol/vol) D_2O ACROS (Sigma) in PBS with 2 mM DTT to give a final percentage D_2O of 95.3]. For the HECT domain (5 μM) with or without heclin (200 μM), the buffer additionally contained 2% (vol/vol) DMSO. For all experiments, deuterium labeling was carried out in triplicate for various times (3 s on ice or 3, 30, or 300 s at 23 °C). The labeling reaction was quenched by the addition of chilled 2.4% (vol/vol) formic acid in 2 M guanidinium hydrochloride to rapidly lower the pH to 2.5. Pro-

tein samples were then snap frozen in liquid nitrogen and stored at −80 °C before analysis.

The quenched protein samples were rapidly thawed and subjected to proteolytic cleavage by pepsin, followed by reversed-phase HPLC separation essentially as previously described (8). The protein was passed through an immobilized pepsin column, 2.1 × 30 mm (Porozyme, ABI), at 230 μL/min for 3 min, and the peptic peptides trapped and desalted on a 2.1 × 5 mm C18 trap column (Acuity BEH C18 Van-guard precolumn, 1.7 μm; Waters). Trapped peptides were subsequently eluted over the course of 12 min, using a 5–36% (vol/vol) gradient of acetonitrile in 0.1% vol/vol formic acid at 40 μL/min. Peptides were separated on a reverse phase column (Aquity UPLC BEH C18 column 1.7 μm, 100 mm × 1 mm; Waters). Peptides were detected on a Xevo G2 Q-TOF (Waters) acquiring over a mass range from 300 to 1,400 *m/z*, with the standard electrospray ionization source and lock mass calibration using [Glu1]-fibrino peptide B (500 fmol/μL). The mass spectrometer was operated at a source temperature of 80 °C, and a spray voltage of 2.6 kV. Spectra were collected in positive ion mode.

Peptide identification was performed by data-dependent acquisition, using a 3–35% (vol/vol) gradient of acetonitrile in 0.1% vol/vol formic acid over 120 min with a Xevo G2 Q-TOF (Waters). This was supplemented with a shorter, 20-min gradient separation to identify and correct the retention time for all peptide samples. The resulting MS/MS data were analyzed using the Mascot database search engine (Matrix Science), using an MS tolerance of 5 ppm and a MS/MS tolerance of 0.15 Da.

Mass analysis of the peptide centroids was performed as described previously, using the software HD-Examiner (Sierra Analytics) (8). Only peptides with a Mascot score >15 were considered. The first round of analysis and identification were performed automatically by the HD-Examiner software; however, all peptides (deuterated and nondeuterated) were manually verified at every state and time for the correct charge state, *m/z* range, presence of overlapping peptides, and expected retention time. Deuterium incorporation was not corrected for back-exchange and represents relative, rather than absolute, changes in deuterium levels. Changes in H/D amide exchange in any peptide may be a result of a single amide or a number of amides within that peptide. All time points in this study were prepared at the same time. Individual time points were analyzed on the mass spectrometer on the same day. The exchange reaction and full analysis were performed on triplicate samples in parallel, and the results were averaged and incorporation of deuterium calculated as a percentage of the maximum theoretically possible for the peptide. See Dataset S1 for the full data.

Because different peptides exchange at very different rates, we calculated the change in percentage deuteration averaged over all times (plotted in Fig. 5 and Fig. S7). This greatly underestimates the maximum size of the change at the optimal point but allows easy comparison. Because heclin in particular has a relatively low affinity for the HECT domain and gave quite subtle changes, we estimated the magnitude of background noise in the results by comparing the data for the different HECT domain controls, in PBS and PBS with 2% (vol/vol) DMSO, which were assayed at different times and completely independently, thus providing a stringent test for reproducibility. The percentage exchange figures with DMSO were divided by 0.98 to correct for dilution of the D_2O. The results showed small changes apparently randomly scattered throughout the sequence (Fig. S7), which were mostly less than 1% and never more than 1.3%, implying that DMSO has little if any effect. In contrast, the changes interpreted in the text involved consistent results from clustered overlapping peptides (with the single exception of peptide 435–440 with heclin) and had magnitude greater than 2%. Smaller effects may well be significant but are hard to distinguish from noise.

Peptide Mass Spectrometry. GST-Smurf2-HECT that had been treated with 100 μM heclin for 30 min was digested with trypsin (Promega) in PBS at an enzyme-to-protein ratio of 1:50. The digests were subsequently analyzed by nano liquid chromatography (LC)-MS/MS on a Q-Exactive plus mass spectrometer (ThermoScientific). LC-MS/MS data were interrogated manually to identify potential disulfide linked peptides, which were confirmed by their absence when the protein was treated with DTT and iodoacetamide (Sigma) before digestion. Apart from the active site peptide AHTCFNR, which could only be detected in digests of nonheclin-treated protein, about 95% of the protein sequence was represented in peptides from the heclin-treated sample, including those containing other cysteines in thiol form.

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2. Mund T, Pelham HR (2009) Control of the activity of WW-HECT domain E3 ubiquitin ligases by NDFIP proteins. *EMBO Rep* 10(5):501–507.
3. Heinis C, Rutherford T, Freund S, Winter G (2009) Phage-encoded combinatorial chemical libraries based on bicyclic peptides. *Nat Chem Biol* 5(7):502–507.
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8. Burke JE, et al. (2011) Dynamics of the phosphoinositide 3-kinase p110δ interaction with p85α and membranes reveals aspects of regulation distinct from p110α. *Structure* 19(8):1127–1137.

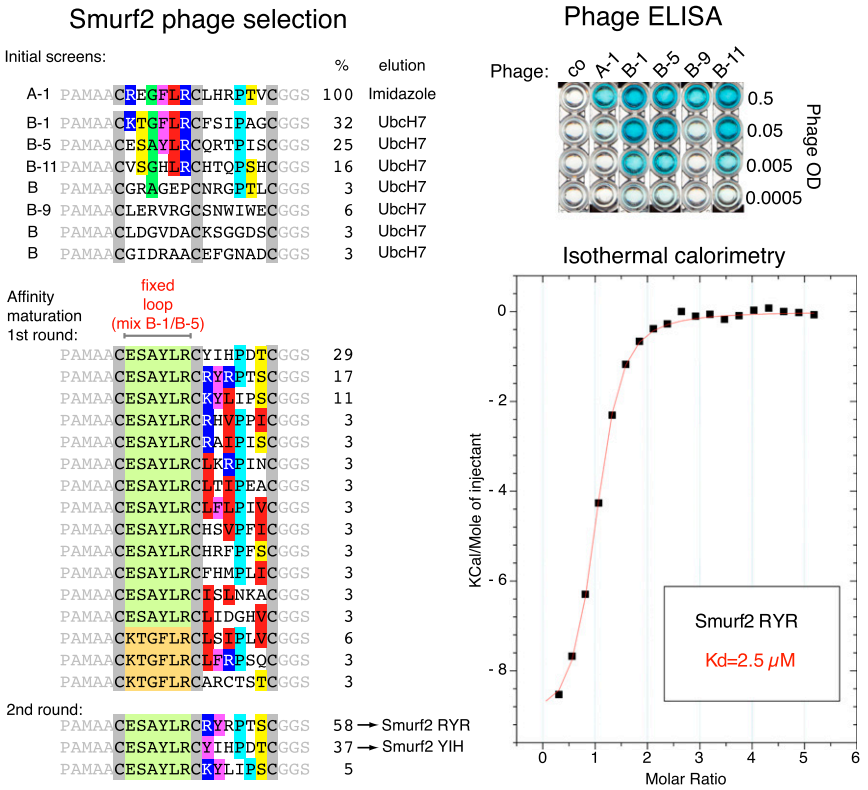

Fig. S1. Isolation of bicycles that bind Smurf2. Shown are sequences of phages isolated at various stages of the selection and an example of phage ELISA to measure binding efficiency. %, abundance of a particular sequence in one batch of phage (because in some cases, multiple batches were sequenced, with differing composition, the % does not necessarily add to 100%). Also shown is isothermal titration calorimetry data for binding of a synthetic bicycle to purified recombinant Smurf2 HECT domain; the dashed line is the fitted curve from which the affinity was derived.

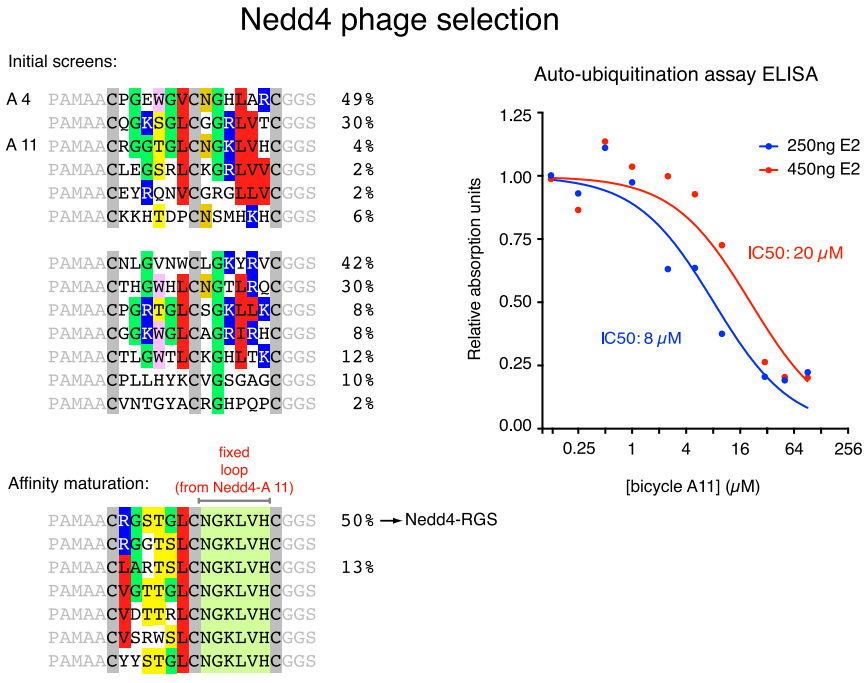

Fig. S2. Isolation of Nedd4 binding bicycles, as in Fig. S1. (*Right*) Titrations of bicycle in the ELISA with two different concentrations of UbcH7. Lines are standard curves fitted to the data to give approximate IC_{50}s.

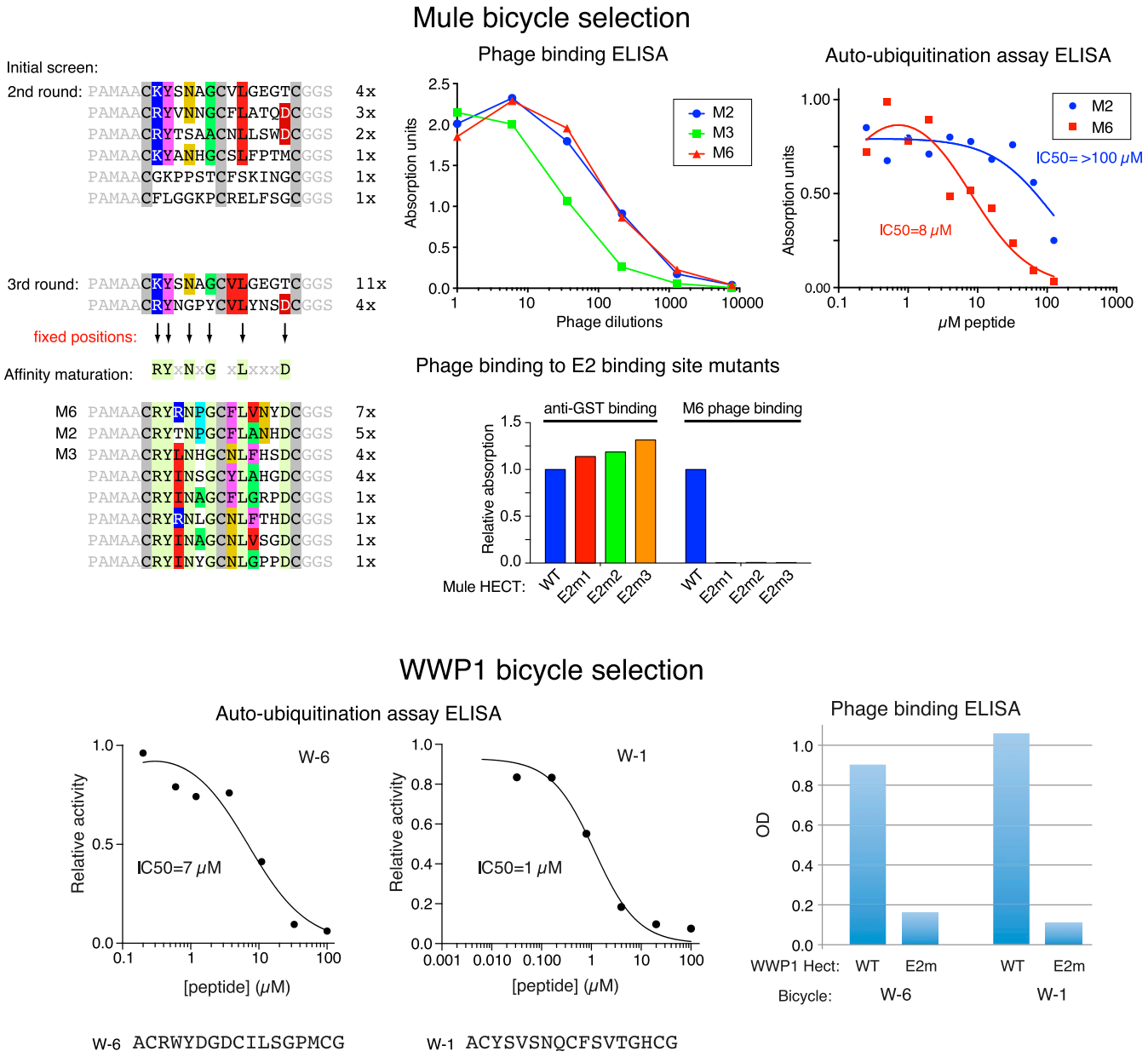

Fig. S3. Isolation and characterization of Mule and WWP1 binding bicycles. The number of times each Mule bicycle sequence was found in a batch of 48 phages sequenced is indicated. The E2 binding site mutations are for Mule: E2m1, E4177A; E2m2, H4152A; and E2m3, DR4087/8AG. For WWP1, EF702,703QL.

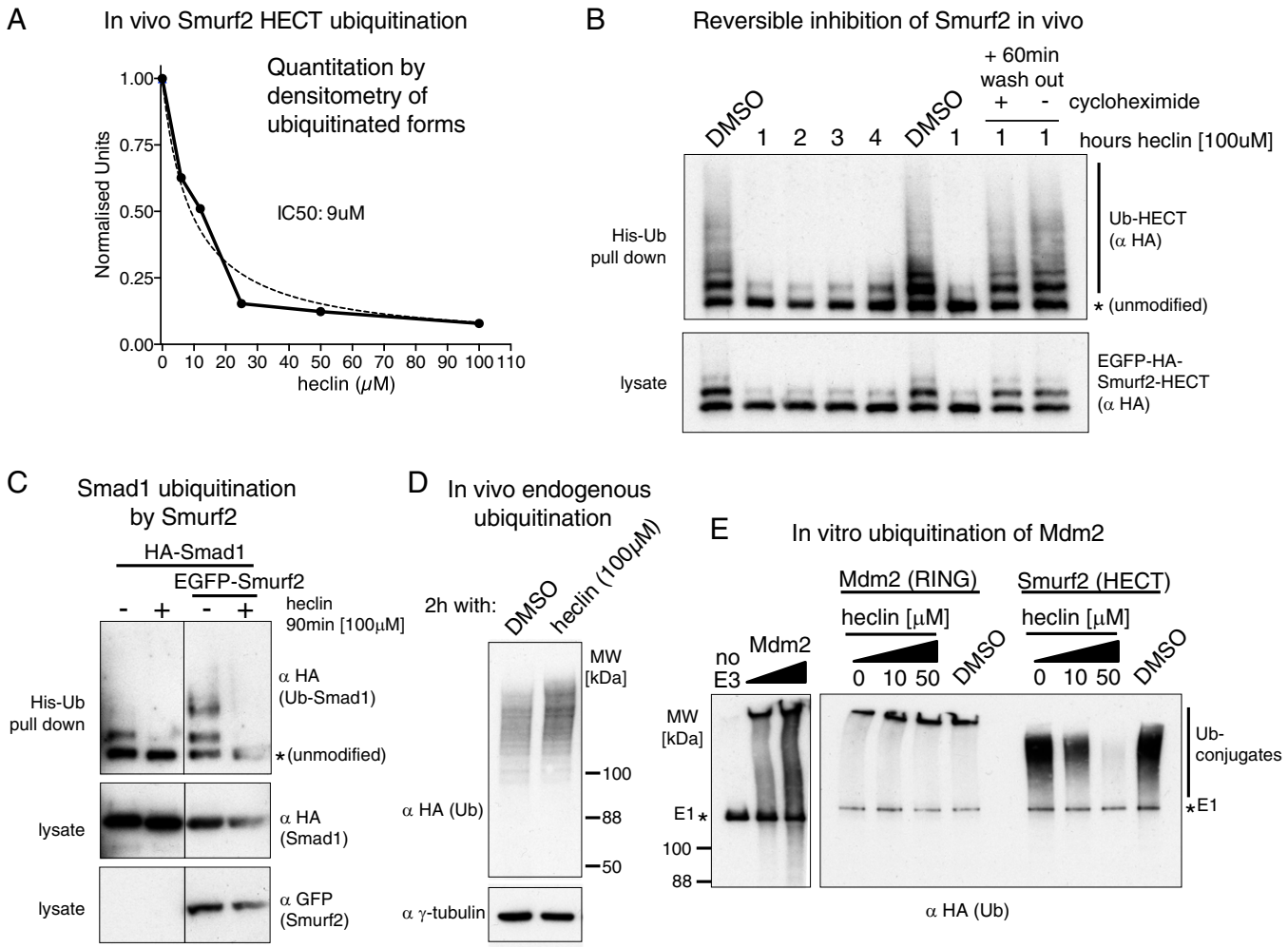

Fig. S4. Further characterization of Smurf2 inhibition by heclin. (*A*) Quantitation of the decrease in ubiquitinated forms of Smurf2 HECT domain, obtained by densitometry of the corresponding bands in Fig. 3*C*. (*B*) Reversible inhibition of Smurf2 in vivo. Inhibition persists for 4 h and can be reversed after 1 h, with reubiquitination occurring even in the presence of cycloheximide to block new protein synthesis. (*C*) Smad1 ubiquitination by Smurf2: HA-Smad1 was expressed with or without EGFP-Smurf2; heclin inhibited both ubiquitination by endogenous ligases and the enhanced ubiquitination induced by coexpressed Smurf2. (*D*) In vivo endogenous ubiquitination: cells were transfected with HA-Ub and immunoblotted after 2 h of treatment with heclin. (*E*) In vitro ubiquitination of Mdm2: Recombinant Mdm2 was assayed as for Smurf2 and generated very large auto-polyubiquitinated forms. This was not inhibited by heclin, whereas Smurf2 auto-ubiquitination performed in parallel was. The E1 enzyme forms a thioester with ubiquitin which is also detected on this blot.

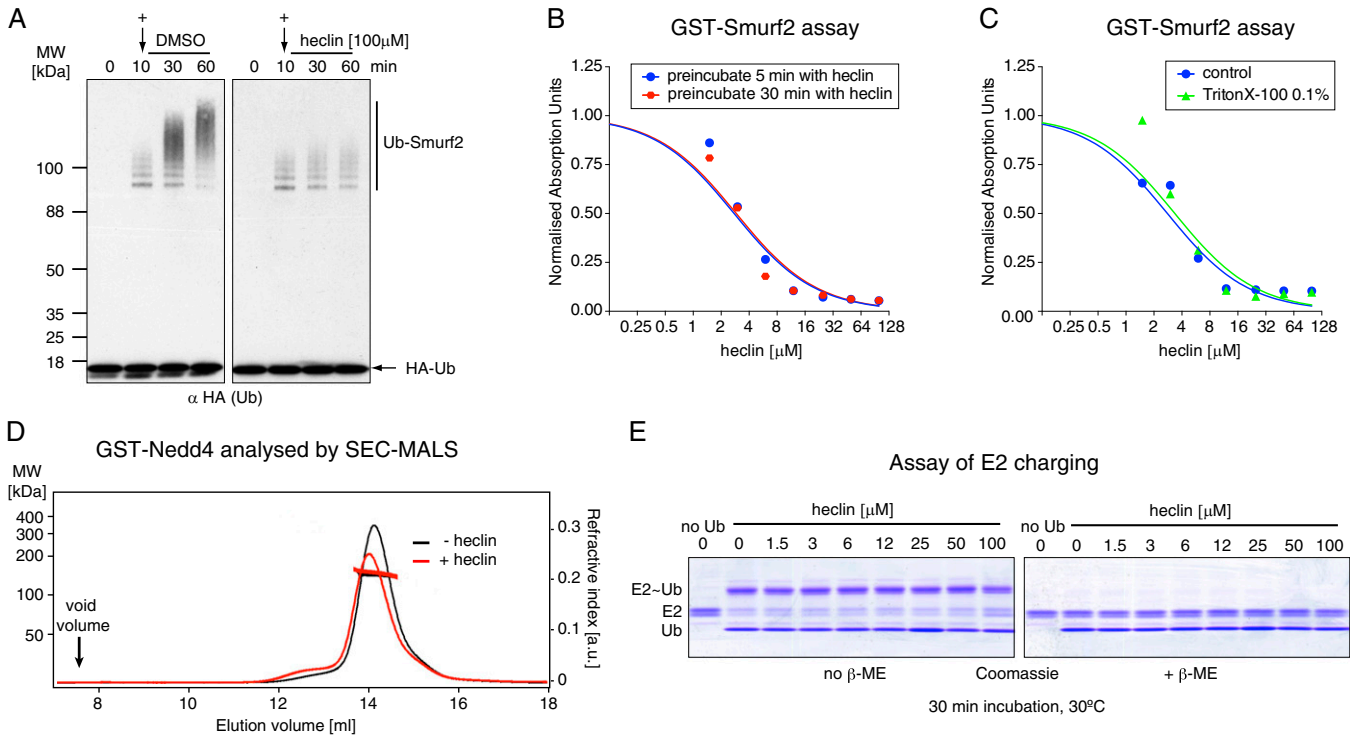

Fig. S5. Heclin inhibition is rapid and specific. (*A*) Either 100 μM heclin or DMSO, as a control, was added to a standard reaction after 10 min incubation, and ubiquitination followed by immunoblotting. (*B*) Preincubation of E3 with heclin for 30 min instead of 5 min had no effect on the inhibition curve. Lines shown are best fits of standard curves. (*C*) Addition of detergent, which can prevent micelle formation and nonspecific inhibition by some compounds, did not affect heclin inhibition. (*D*) GST-Nedd4 does not significantly aggregate on exposure to 100 μM heclin. The protein was analyzed by SEC-MALS (gel filtration coupled to light scattering). The measured size, ~150 kDa, corresponds to a dimer. GST-Nedd4 and GST-Smurf2 were also analyzed in the presence or absence of 100 μM heclin by dynamic light scattering, and all four samples had at least 99% of the mass in a single species with an average hydrodynamic radius of 7 nm. (*E*) Heclin does not inhibit E1-dependent formation of an E2-Ub thioester. Ubiquitination reaction mixes (with 3× normal protein concentrations) lacking E3 and ubiquitin were prepared with indicated concentrations of heclin, the reaction initiated by addition of Ub, and after 30 min, samples were analyzed on nonreducing and reducing gels.

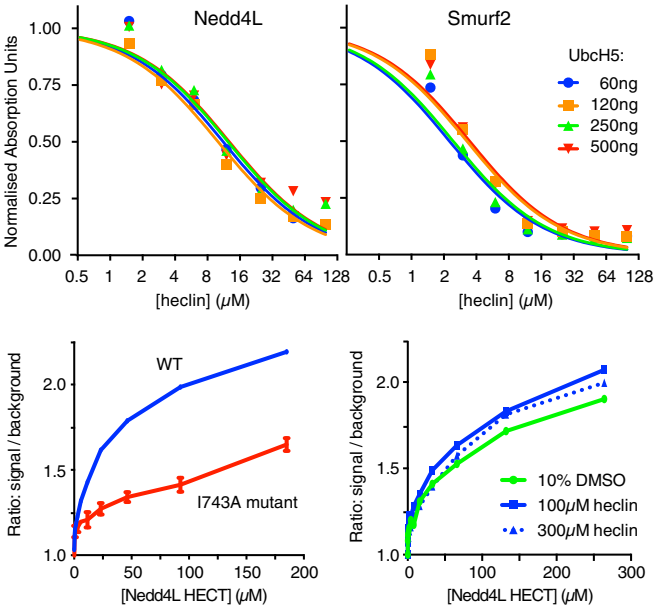

Fig. S6. Heclin does not compete for E2 binding. (*Upper*) Titration of heclin against Nedd4L and Smurf2 in an ELISA with varying levels of UbcH5. (*Lower*) Fluorescence polarization assays with Nedd4L, or Nedd4L with an E2 binding site mutation (7), and heclin as indicated. Nedd4L was used because Smurf2 and Nedd4 bind the E2 too weakly to be detected in this way.

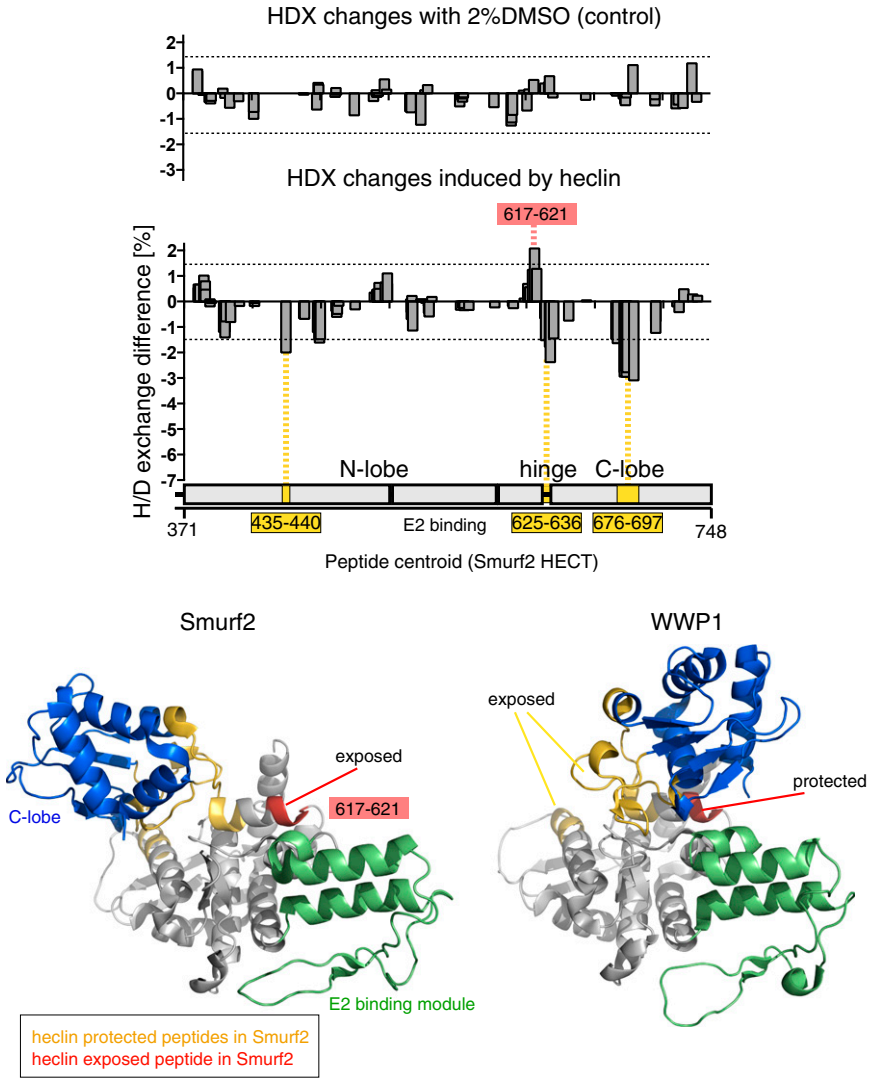

Fig. S7. Heclin-induced changes to Smurf2 conformation, as detected by deuterium exchange. (*Upper*) Deuterium exchange differences, with the value for each peptide plotted as a function of the position of the centroid of the peptide on the protein sequence, as in Fig. 5. The control plot compares samples in PBS plus 2% (vol/vol) DMSO versus PBS alone (see *SI Methods*). The heclin data compare 2% DMSO plus heclin with 2% DMSO. Dotted lines are drawn at ±1.5% as a visual aid. Below, the four significantly varying peptide sequences are mapped onto the crystal structure of Smurf2 (Protein Data Bank ID code 1ZVD), and equivalent sequences, identified by sequence and/or structural homology, are also marked on the crystal structure of WWP1 (Protein Data Bank ID code 1ND7). The C-lobe is in very different positions in the two structures, and it can be seen that the yellow regions are relatively exposed in WWP1, whereas the red region is protected relative to Smurf2. We propose that heclin induces a conformational change in which the C-lobe moves from a position similar to that in WWP1 to one closer to the crystal structure of Smurf2.

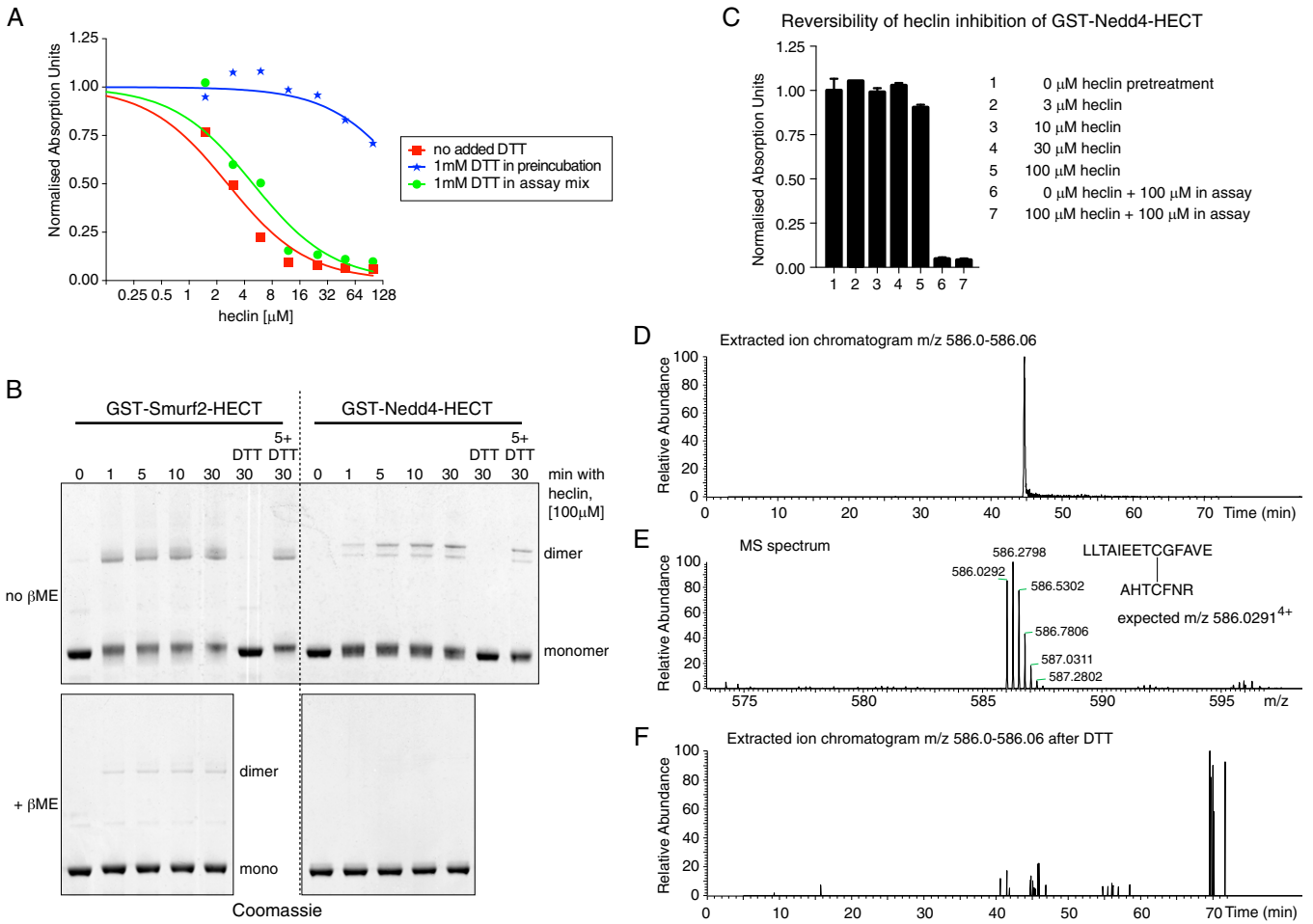

Fig. S8. Heclin induces reversible oxidative changes to HECT ligases. (*A*) DTT reduces heclin inhibition if present during incubation with the E3 but has little effect if added later with other assay components. (*B*) Effects of heclin on GST-HECT fusions. This is a full view of the gels shown in Fig. 7*C*. (*C*) Heclin inhibition can be reversed. After preincubation of GST-Nedd4 with heclin in PBS, samples were adsorbed to glutathione-coated plates for 1 h and assayed in situ (see *SI Methods*). Assays were in duplicate, with range indicated by bars. (*D*) Identification of a potential disulfide bridged peptide induced by heclin in GST-Smurf2-HECT by mass spectrometry. Shown is the extracted ion chromatogram showing the elution position of the peptide at *m/z* 586.0–586.06. (*E*) Mass spectrum of the peptide shown in *D*, showing close correspondence between the predicted and observed mass of this peptide. (*F*) As *D*, but from protein treated with DTT and iodoacetamide before digestion, to destroy disulfide linkages. The scale is amplified 167× compared with *D* to show complete absence of the peptide. AHTCFNR is the active site sequence.

Other Supporting Information Files

Dataset S1 (XLSX)