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Figures and figure supplements

Mechanism of ubiquitin ligation and lysine prioritization by a HECT E3

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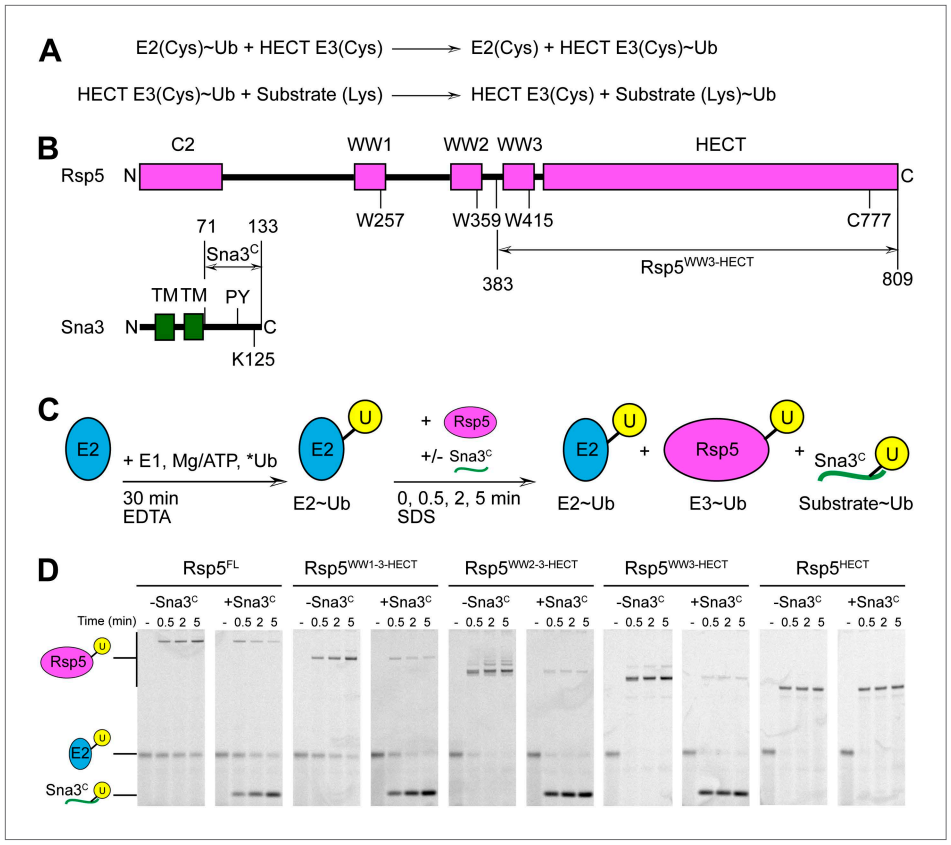

Figure 1. Rsp5$^{WW3\text{-}HECT}$-Sna3^{C} as a minimal model HECT E3-substrate system to study Ub ligation. (**A**) Schematic view of two-step HECT E3 ubiquitination mechanism. First, Ub is transferred from an E2~Ub intermediate to the HECT E3 catalytic cysteine (Cys) to form a labile, thioester-linked E3~Ub intermediate. Second, Ub is transferred from the E3 Cys to a substrate's primary amino group, which is typically from a lysine side-chain. Ub can also be a substrate for the second reaction during polyubiquitination. (**B**) Schematic views of Rsp5 and Sna3 sequences. (**C**) Schematic description of pulse-chase assay. A thioester-bonded E2~Ub intermediate was enzymatically generated by mixing E1, the E2 UbcH5B, and a fluorescently labeled version of Ub for 30 min. This 'pulse' reaction was quenched by addition of EDTA. In the 'chase', wild-type or deletion mutant versions of Rsp5 were added alongside a synthetic peptide corresponding to Sna3^{C}. Formation of the Rsp5~Ub intermediate and the Sna3^{C}~Ub product were monitored at the indicated time-points. (**D**) Imaging of nonreducing SDS-PAGE gels monitoring pulse-chase fluorescent Ub transfer from E2 to Rsp5 to substrate for the indicated versions of Rsp5, in the absence or presence of Sna3^{C}. Rsp5$^{WW3\text{-}HECT}$ is the minimal version mediating Ub ligation to Sna3^{C}.

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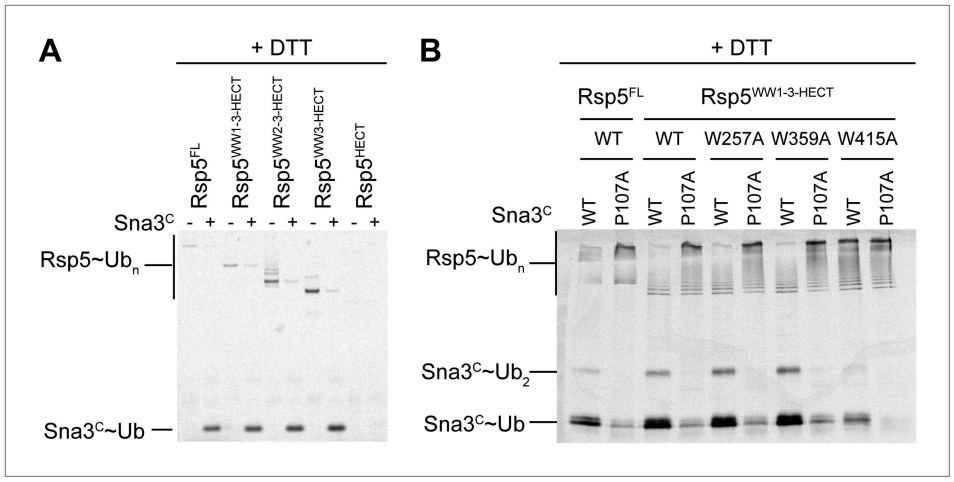

Figure 1—figure supplement 1. Mutational data defining $Rsp5^{WW3\text{-}HECT}$ as a minimal E3 mediating Ub ligation to $Sna3^{C}$ and autoubiquitination. (**A**) DTT-treated controls of the end points of reactions shown in ***Figure 1B***. (**B**) Fluorescent imaging of reducing SDS-PAGE gels monitoring multiple turnover fluorescent Ub ligation by full-length Rsp5 ($Rsp5^{FL}$), or a construct containing all three WW domains plus the HECT domain ($Rsp5^{WW1\text{-}3\text{-}HECT}$), in the presence of wild-type or a P107A PPXY motif mutant version of $Sna3^{C}$.

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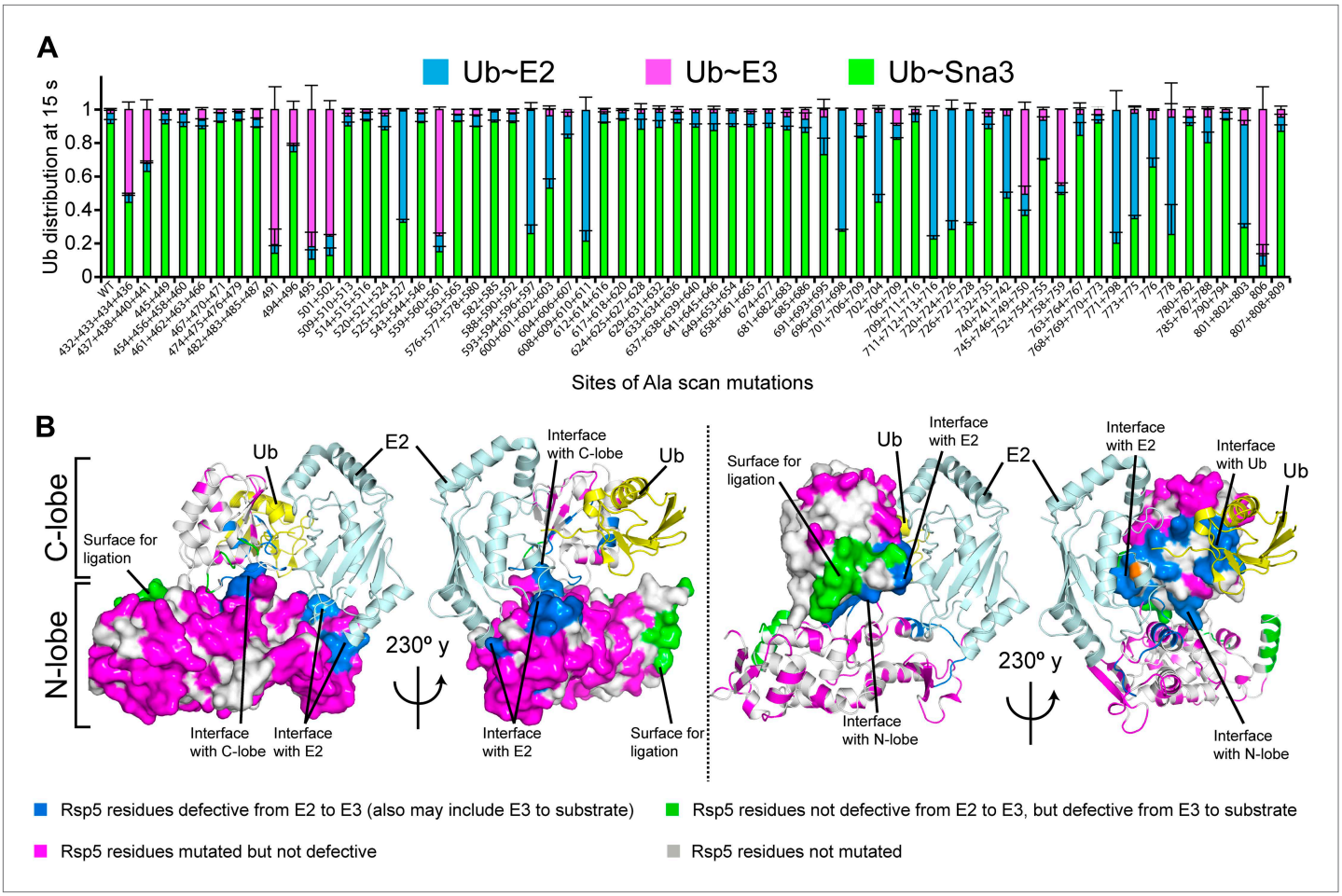

Figure 2. Alanine scanning mutagenesis suggests distinct specific HECT domain architectures to receive and ligate ubiquitin. (**A**) Summary of effects of indicated HECT domain Ala mutations on pulse-chase fluorescent Ub transfer from E2 to Rsp5$^{WW3\text{-}HECT}$ and then from Rsp5$^{WW3\text{-}HECT}$ to Sna3^{C}. The assay scheme is shown in ***Figure 1C***, except a 15 s chase was used. Cyan bars—accumulation of thioester-linked E2~Ub intermediate, reflecting a defect in Ub transfer from E2 to E3. Purple bars—accumulation of thioester-linked E3~Ub intermediate, reflecting a defect in Ub transfer from E3 to substrate. Green bars—ratio of Sna3^{C}~Ub product formed. Standard deviations are calculated from three independent replications. (**B**) Locations of mutations hindering E2-to-Rsp5$^{WW3\text{-}HECT}$ (blue) or Rsp5$^{WW3\text{-}HECT}$-to-substrate (green) Ub transfer mapped on prior E2~Ub-HECT domain structure (***Kamadurai et al., 2009***). Magenta surfaces represent residues mutated and found not essential for activity.

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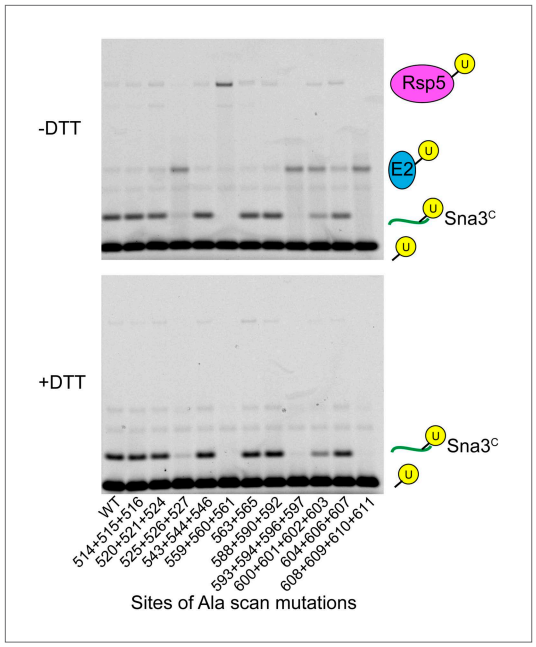

Figure 2—figure supplement 1. Representative raw data from Ala scan. Fluorescent imaging of representative nonreducing (top) and reducing (bottom) gels from assays used for data in ***Figure 2A***, monitoring pulse-chase fluorescent Ub transfer from E2 to the indicated versions of $Rsp5^{WW3\text{-}HECT}$ to $Sna3^{C}$. The chase was 15 s.

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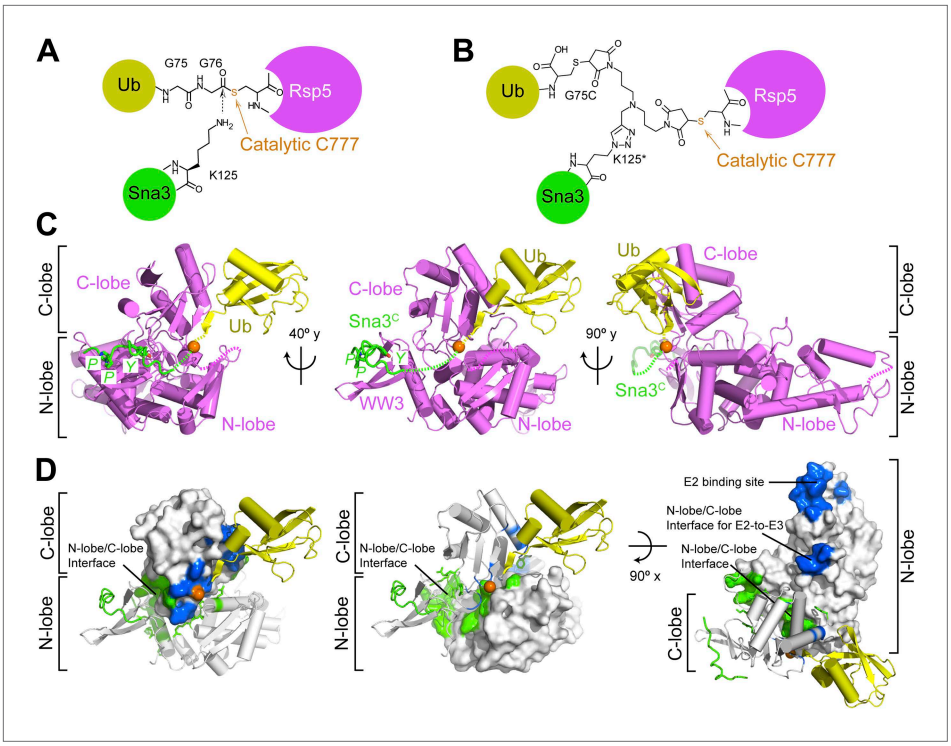

Figure 3. Structure of a trapped proxy for an Rsp5~Ub-Sna3^{C} intermediate. (**A**) Chemical representation of substrate lysine attack of an Rsp5~Ub thioester bond. (**B**) Chemical representation of the trapped proxy, depicting covalent bonds linking Rsp5 Cys777, a Ub Cys75 (in place of Gly75-Gly76) and Sna3^{C} Cys125 (in place of Lys125) in Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} complex. (**C**) Three views of Rsp5$^{WW3\text{-}HECT}$ (violet) xUb (yellow) xSna3^{C} (green), with catalytic Cys shown as orange sphere. Regions not observed in electron density are indicated with dashed lines. (**D**) Locations of Ala scan mutations hindering E2-to-Rsp5$^{WW3\text{-}HECT}$ (blue) or Rsp5$^{WW3\text{-}HECT}$-to-substrate (green) Ub transfer mapped on Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} structure.

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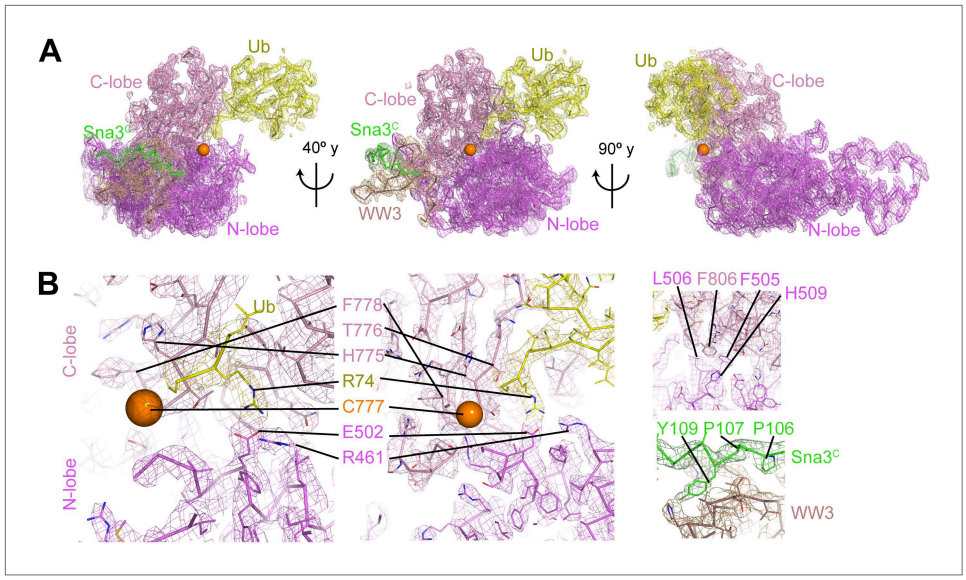

Figure 3—figure supplement 1. Electron density for Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} structure. (**A**) Final 2Fo−Fc electron density and Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} crystal structure, colored by domain: WW3 domain—beige, N-lobe—violet, C-lobe—pink, Ub—yellow, Sna3^{C}—green. The maps are displayed at 1σ contour level, the structures as Cα traces for one copy in the asymmetric unit. Overall, the complex is supported by good electron density, except for the crosslinker, associated C-terminal portion of Sna3^{C}, and the N-lobe catalytic loop, which are not visible or too patchy for building. (**B**) Close-up views highlighting active site region, N-lobe (violet)/C-lobe (pink)/Ub (yellow) interfaces, and PPXY motif (green) from Sna3^{C} (beige) bound to Rsp5's WW3 domain.

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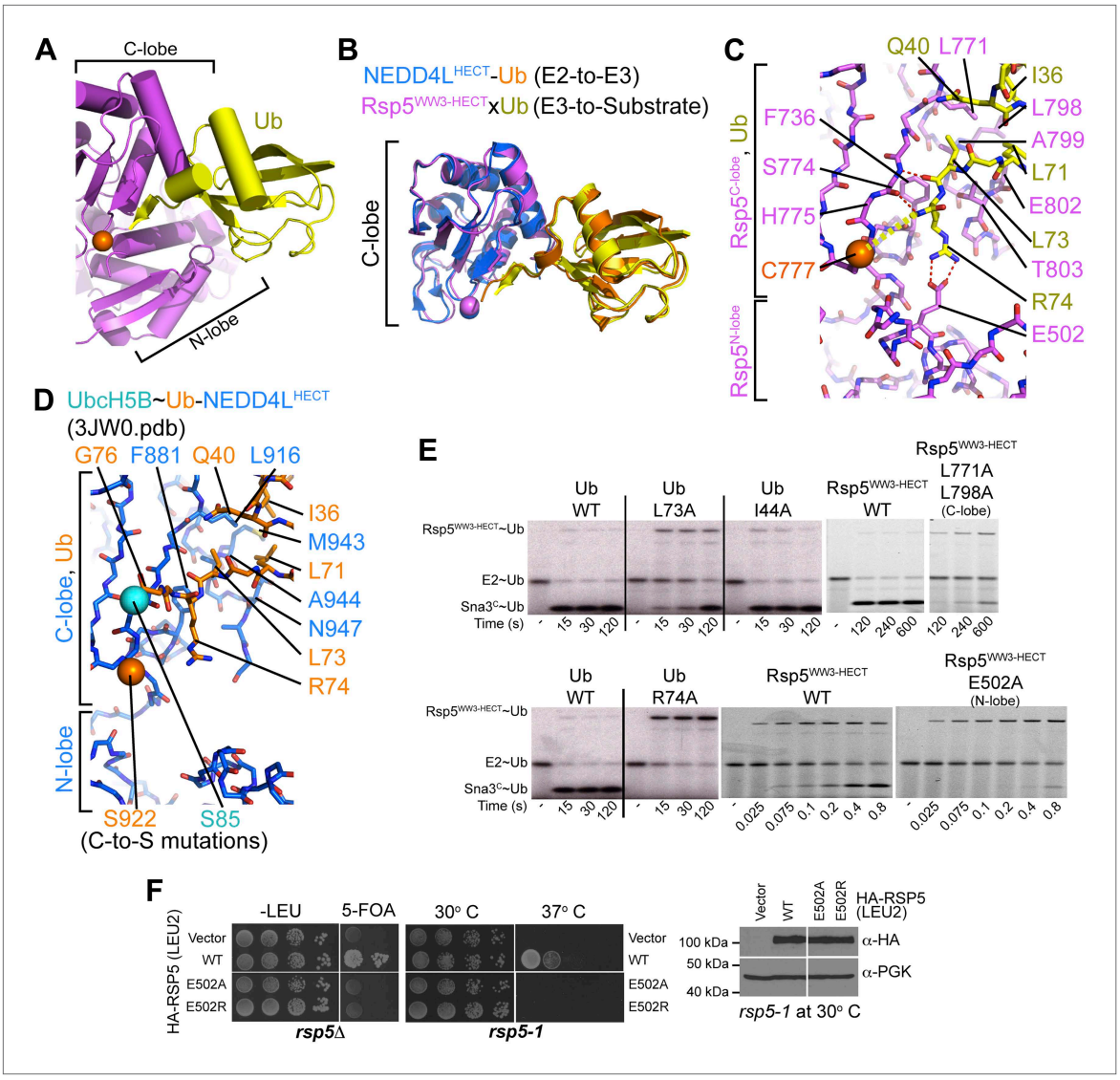

Figure 4. HECT domain–Ub interactions for ligation. (**A**) Ub's C-terminal tail and its covalent linkage to the Rsp5 catalytic Cys are shown sandwiched between the Rsp5 N- and C-lobes in the crystal structure of Rsp5$^{WW3\text{-}HECT}$ (violet) xUb (yellow) xSna3^{C} (not shown). (**B**) C-lobe-Ub portions superimposed for Rsp5$^{WW3\text{-}HECT}$ (violet) xUb (yellow) xSna3^{C} (not shown) and E2 (not shown)~Ub (orange)-NEDD4L^{HECT} (blue) (***Kamadurai et al., 2009***). (**C**) Close-up view of interactions between Rsp5 C-lobe and the C-terminal tail of covalently linked Ub in the crystal structure of Rsp5$^{WW3\text{-}HECT}$ (violet) xUb (yellow) xSna3^{C} (not shown). (**D**) Close-up view of interactions between NEDD4L C-lobe and the C-terminal tail of the E2-linked Ub in the crystal structure of E2 (only the Cys-to-Ser mutation at the active site is shown) ~Ub (orange)-NEDD4L^{HECT} (blue) (***Kamadurai et al., 2009***). (**E**) Nonreducing gels from pulse-chase E2-to-Rsp5-to-Sna3^{C} Ub transfer assay, using indicated versions of Rsp5$^{WW3\text{-}HECT}$ and of fluorescent or radioactive Ub. Bands corresponding to thioester-linked E2~Ub and Rsp5$^{WW3\text{-}HECT}$~Ub intermediates and isopeptide-bonded Sna3^{C}~Ub product are indicated. (**F**) Yeast complementation assays for the indicated HA-Rsp5 mutants.

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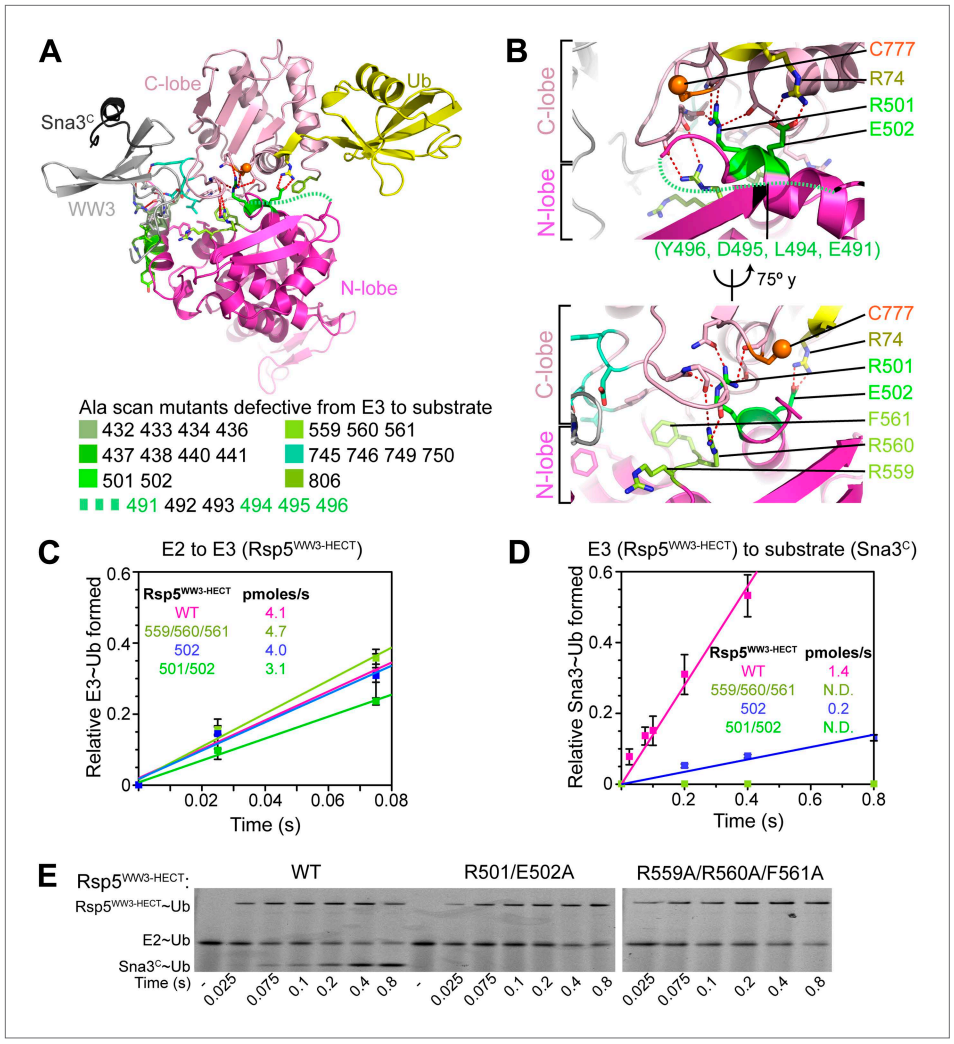

Figure 5. Ala mutations selectively impaired for ligation map to N-lobe/C-lobe interface in Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} crystal structure. (**A**) Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} crystal structure highlighting locations of Ala mutants (sticks, colored by mutant as indicated) selectively impaired for ligation. Dotted line indicates approximate locations of mutations in the N-lobe not visible in electron density. (**B**) Close-up views highlighting N-lobe/C-lobe interface locations of Ala mutants selectively impaired for ligation. Dotted line indicates approximate locations of mutations in the N-lobe not visible in electron density. (**C**) Initial rates of fluorescent Ub transfer from E2 to the indicated versions of Rsp5$^{WW3\text{-}HECT}$ measured in rapid quench-flow pulse-chase assays. (**D**) Initial rates of fluorescent Ub transfer from the indicated versions of Rsp5$^{WW3\text{-}HECT}$ to Sna3^{C} measured in rapid quench-flow pulse-chase assays. (**E**) Fluorescent images of nonreducing SDS-PAGE gels showing rapid-quench flow pulse-chase fluorescent Ub transfer from E2 to Rsp5$^{WW3\text{-}HECT}$ to Sna3^{C}, for the indicated versions of Rsp5$^{WW3\text{-}HECT}$. Bands corresponding to thioester-linked E2~Ub and Rsp5$^{WW3\text{-}HECT}$~Ub intermediates and isopeptide-bonded Sna3^{C}~Ub product are indicated.

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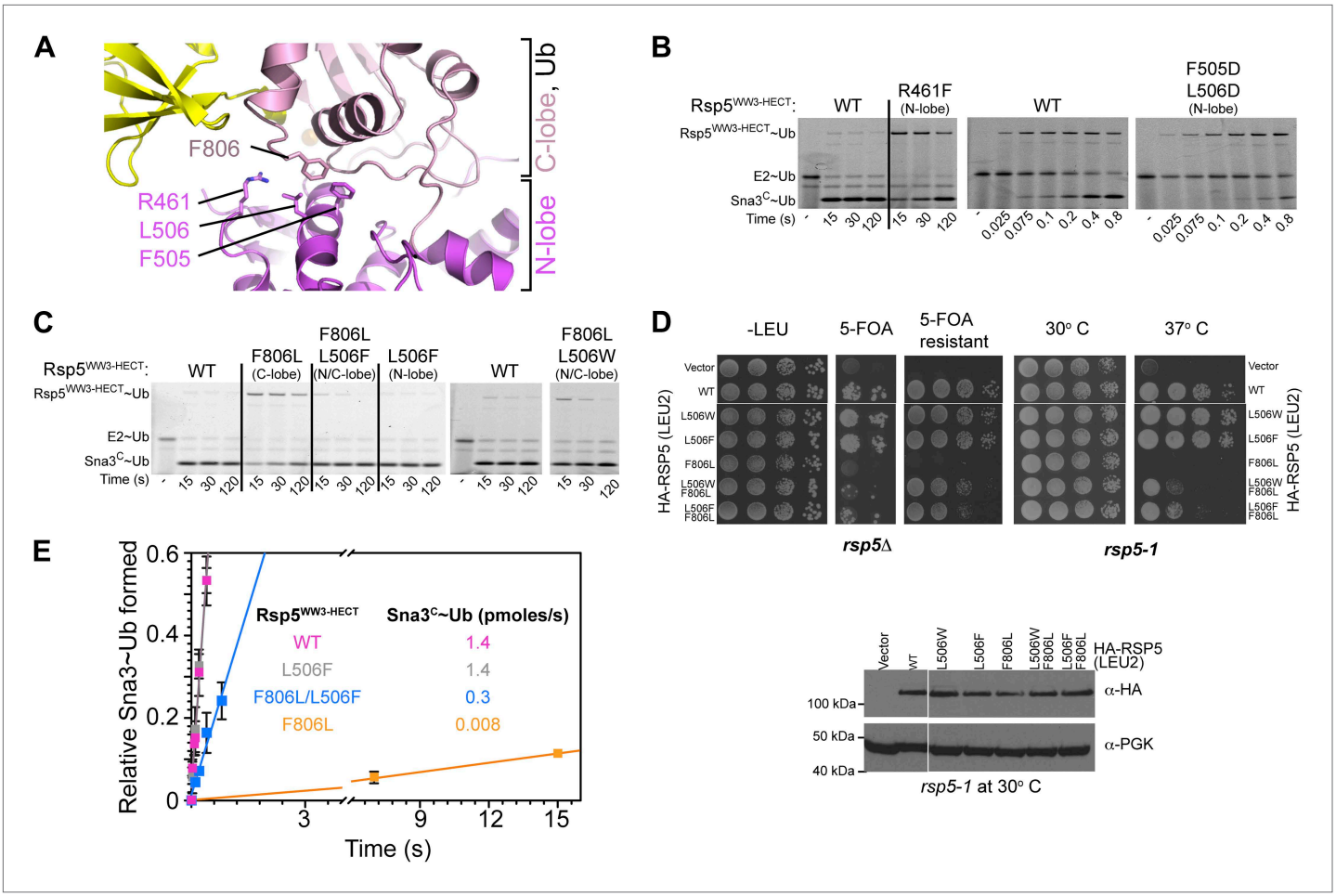

Figure 6. HECT domain C-lobe/N-lobe interactions anchoring architecture for ligation. (**A**) Close-up view of a portion of the C-lobe/N-lobe interface in Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} structure. (**B,C**) Nonreducing gels from pulse-chase transfer assay of fluorescent Ub from E2-to-Rsp5-to-Sna3^{C} using indicated mutants of Rsp5. Bands corresponding to thioester-linked E2~Ub and Rsp5$^{WW3\text{-}HECT}$~Ub intermediates and isopeptide-bonded Sna3^{C}~Ub product are indicated. (**D**) Left: HA-tagged WT and mutant *rsp5* alleles housed on low copy plasmids were assessed for their ability to complement the essential function of *RSP5* in either serially diluted *rsp5-1* temperature-sensitive cells grown at restrictive 37°C or in *rsp5Δ* null cells after eviction of wild-type *RSP5* plasmid on 5-FOA. Below: whole cell lysates of *rsp5-1* transformants immunoblotted for HA and PGK. (**E**) Rates of pulse-chase fluorescent Ub ligation from the indicated versions of Rsp5$^{WW3\text{-}HECT}$ to Sna3^{C}.

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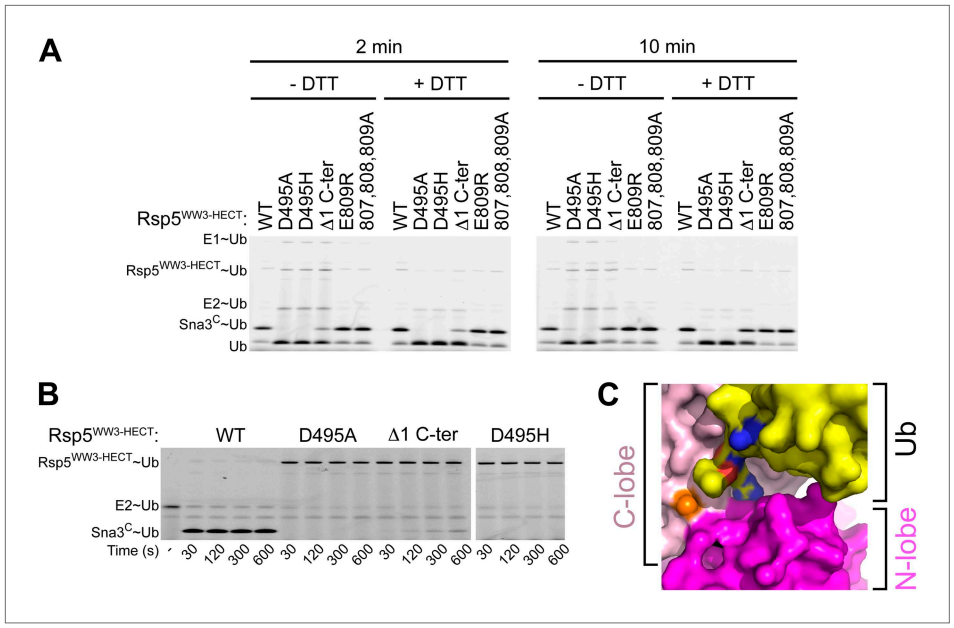

Figure 6—figure supplement 1. A role for HECT domain C-terminus in ligation. (**A**) Multiple-turnover assays showing ligation of fluorescent methylated Ub to Sna3^{C} for 2 or 10 min, by the indicated versions of Rsp5$^{WW3\text{-}HECT}$. Δ1 C-ter refers to deletion of the C-terminal residue Glu809. Reactions are shown in the absence of DTT (left) to confirm the abilities of mutant proteins to form E2~Ub and E3~Ub intermediates, and with DTT (right) to show isopeptide-bonded products. (**B**) Nonreducing gels from pulse-chase transfer assay of fluorescent Ub from E2-to-Rsp5-to-Sna3^{C} using indicated mutants of Rsp5. Bands corresponding to thioester-linked E2~Ub and Rsp5$^{WW3\text{-}HECT}$~Ub intermediates and isopeptide-bonded Sna3^{C}~Ub product are indicated. (**C**) Surface view of Rsp5$^{WW3\text{-}HECT}$xUbxSna3^{C} structure, with the HECT domain N-lobe in magenta and C-lobe in pink, and Ub in yellow. Ub's residues 72, 73, and 74 are shown with nitrogens in blue and oxygens in red to highlight exposed basic patches.

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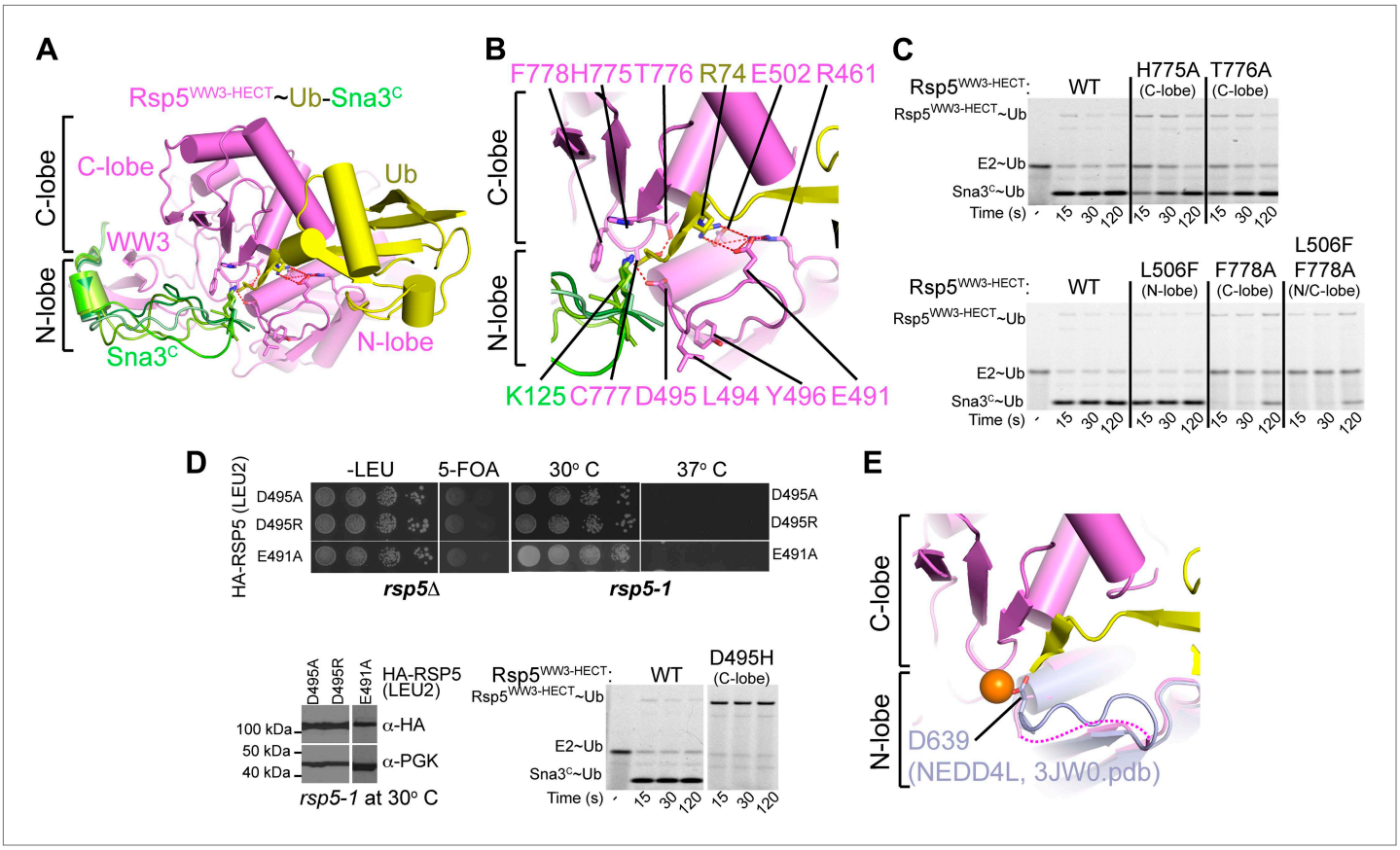

Figure 7. A distinctive active site for HECT E3-mediated ligation. (**A**) Rosetta-generated models for Sna3^{C} (different models in different shades of green) target lysine approaching Rsp5$^{WW3\text{-}HECT}$ (violet) active site Cys777 thioester-linked to Ub (yellow). (**B**) Model for residues aligning thioester-bound Ub and Sna3^{C} acceptor lysine approaching the active site. (**C**) Nonreducing gels from pulse-chase assay for transfer of fluorescent Ub from E2-to-Rsp5-to-Sna3^{C} using indicated versions of Rsp5$^{WW3\text{-}HECT}$. Bands corresponding to thioester-linked E2~Ub and Rsp5$^{WW3\text{-}HECT}$~Ub intermediates and isopeptide-bonded Sna3^{C}~Ub product are indicated. (**D**) Yeast complementation assays for the indicated HA-Rsp5 mutants. (**E**) Close-up of structural superposition of N-lobes showing catalytic loop from NEDD4L^{HECT} (***Kamadurai et al., 2009***) not visible in Rsp5$^{WW3\text{-}HECT}$ (violet) structure.

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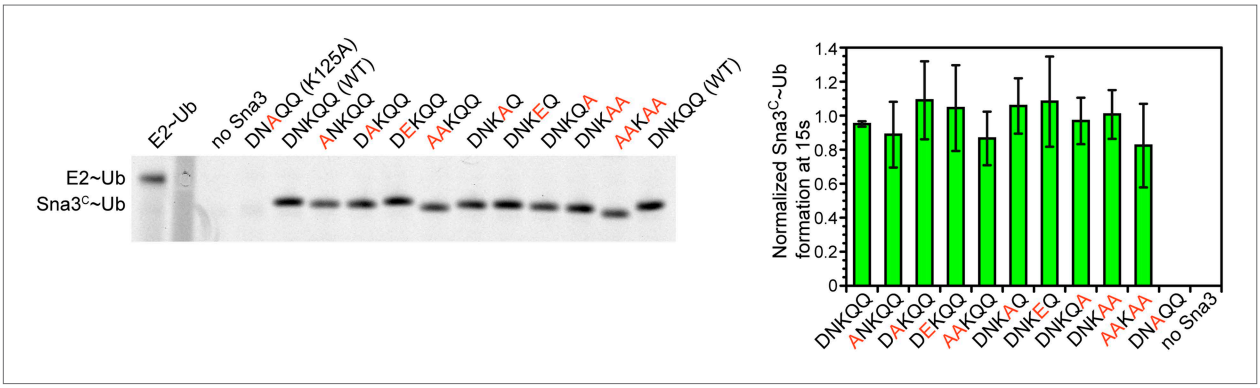

Figure 7—figure supplement 1. Residues proximal to the acceptor lysine in Sna3 play insignificant roles in ubiquitination. The residues around K125 (DNKQQ) are individually or collectively mutated to Ala or Glu and product formation at 15 s in pulse-chase fluorescent Ub transfer assay with Rsp5$^{WW1\text{-}3\text{-}HECT}$ is shown. Neither the Ala or Glu mutations significantly affect Sna3^{C} ubiquitination.

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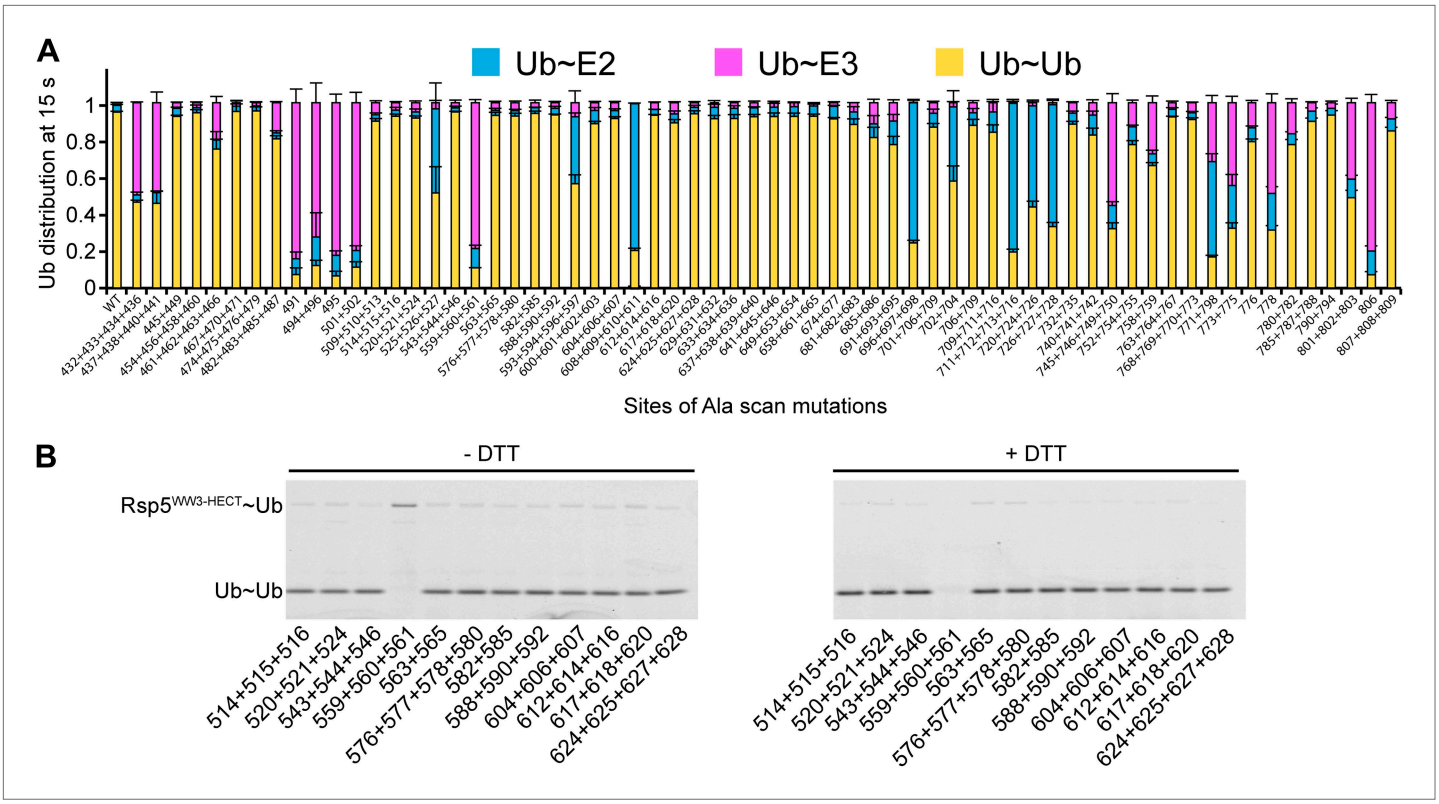

Figure 8. Ala scan for HECT domain surfaces required for di-Ub synthesis. (**A**) Summary of the effects of indicated Rsp5$^{WW3\text{-}HECT}$ HECT domain Ala mutations on pulse-chase fluorescent Ub transfer from E2 to Rsp5$^{WW3\text{-}HECT}$ and then to Ub. Total activity within each gel lane was determined by adding all intensities for all three species (E2~Ub, Rsp5$^{WW3\text{-}HECT}$~Ub, and Ub~Ub) and was used to estimate the relative yield of each with respect to wild-type proteins. The error bar shows the standard deviation (SD) for three independent replicates. (**B**) Fluorescent imaging of representative nonreducing (left) and reducing (right) gels from assays used for data in **A**, monitoring pulse-chase fluorescent Ub transfer from E2 to the indicated versions of Rsp5$^{WW3\text{-}HECT}$ to Ub. The chase was 15 s.

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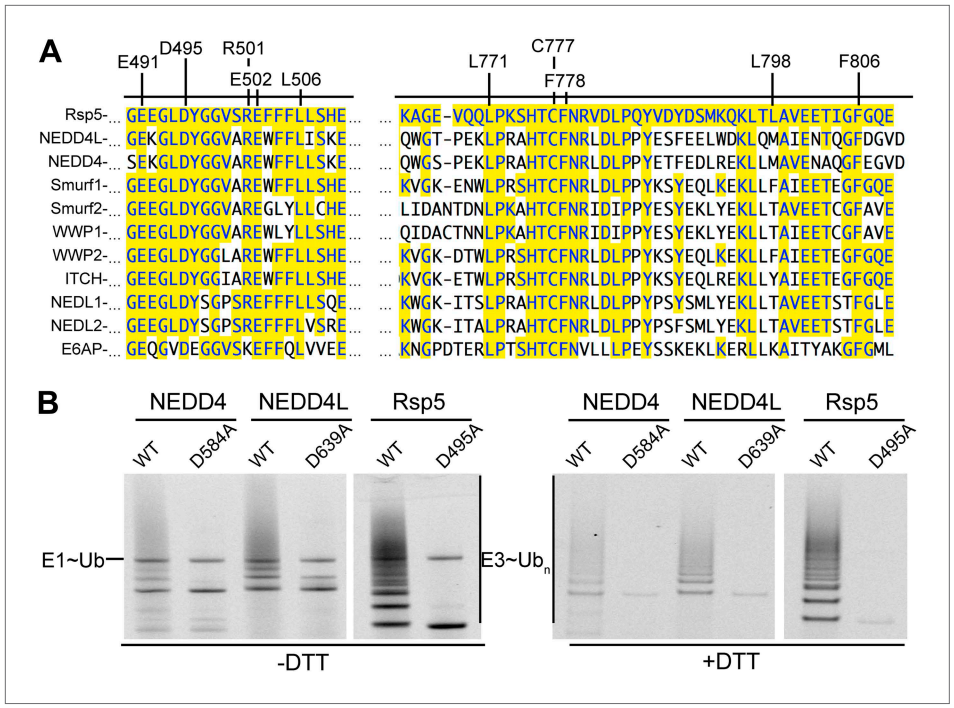

Figure 9. Key residues for ligation are conserved across HECT E3s. (**A**) Alignment of portions of sequence from Rsp5 with corresponding regions of human NEDD4L, NEDD4, Smurf1, Smurf2, WWP1, WWP2, ITCH, NEDL1, NEDL2, and E6AP showing conservation of key residues establishing the ligation mechanism. Identity to Rsp5 sequence is highlighted in yellow. (**B**) Fluorescent images of nonreducing (left) and reducing (right) gels of multiple turnover autoubiquitination assays for wild-type and the indicated Asp-to-Ala mutant versions of NEDD4$^{WW1\text{-}4\text{-}HECT}$, NEDD4L$^{1\text{-}3\text{-}4\text{-}HECT}$, and Rsp5$^{WW1\text{-}3\text{-}HECT}$.

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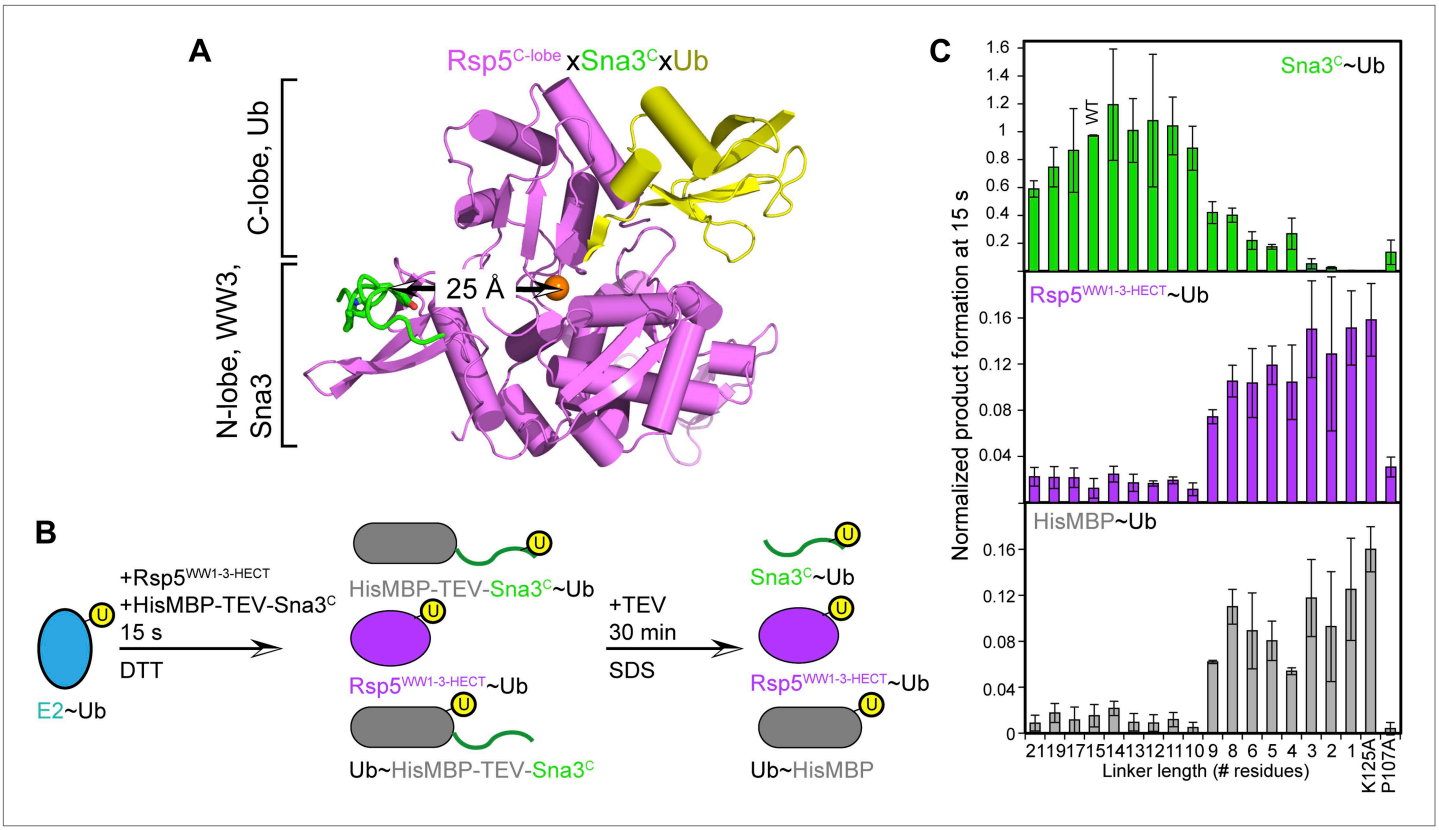

Figure 10. HECT domain architecture for ubiquitin ligation suggests mechanism for substrate lysine prioritization. (**A**) Structure of Rsp5$^{WW3\text{-}HECT}$ (violet) xUb (yellow) xSna3^{C} (green), highlighting 25 Å distance between the alpha carbon of Sna3^{C} Tyr109 in the PPXY motif and the sulfur of Rsp5$^{WW3\text{-}HECT}$ Cys777. (**B**) Schematic view of substrate selection assay. (**C**) Rsp5$^{WW1\text{-}3\text{-}HECT}$ selection between substrates with relative distributions of non-reducible Sna3^{C}~Ub (green bar graph), autoubiquitinated Rsp5$^{WW1\text{-}3\text{-}HECT}$~Ub (violet bar graph), and HisMBP~Ub (gray bar graph) products of reactions with substrates bearing the indicated number of residues between the Sna3^{C} PPXY motif and lysine. Effects of Ala mutations in place of the Sna3^{C} acceptor Lys125 and the WW3-binding Pro107 are shown as controls.

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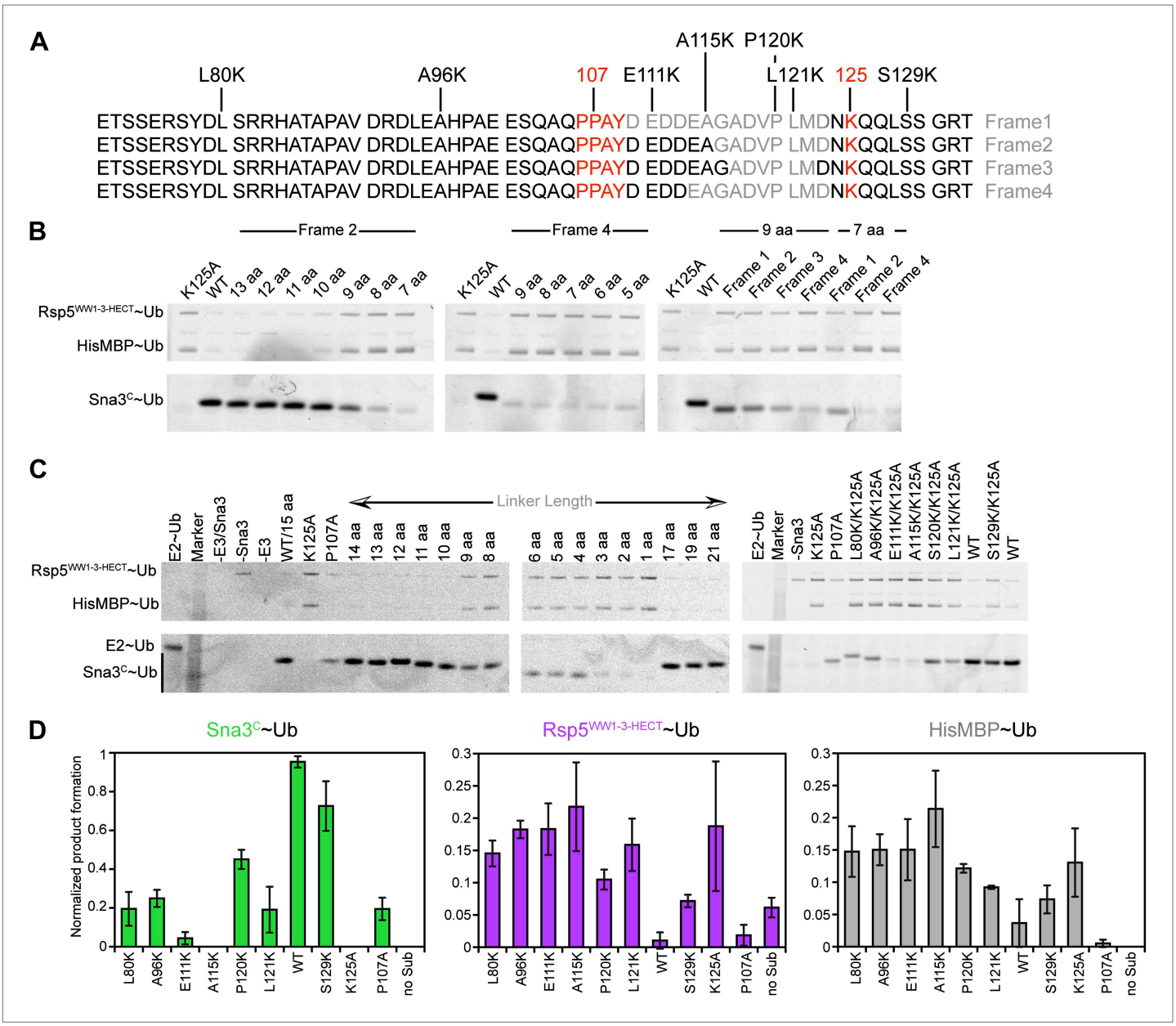

Figure 10—figure supplement 1. Data supporting Rsp5$^{WW3\text{-}HECT}$ target Lys prioritization. (**A**) Wild-type Sna3^{C} has 15 residues between the PPXY motif and acceptor Lys125. Deletion mutants were made starting at different positions after the PPXY motif, referred to as Frame1 for deletions immediately following Tyr109, as Frame2 for deletions immediately following Ala115, as Frame3 for deletions immediately following Gly116, and as Frame4 for deletions immediately following Asp113. The maximum deletion sequence for each frame, including for data shown in **B**, is represented in gray. Positions of Lys substitution (tested in **C**) in the K125A background are indicated above the sequences. (**B**) Pulse-chase assays performed with fluorescent Ub~E2 added to a mixture of Rsp5$^{WW1\text{-}3\text{-}HECT}$ and HisMBP-TEV-Sna3^{C}, and the reaction terminated by adding DTT to reduce any remaining intermediates and identify only isopeptide-bonded reaction products. Shown are reaction products for mutants with the indicated number of linker residues after treatment with TEV protease, allowing comparison of the levels of fluorescent Ub-ligated Sna3^{C}, HisMBP, and autoubiquitinated Rsp5$^{WW1\text{-}3\text{-}HECT}$ (see also ***Figure 10C***). (**C**) Representative raw data from pulse-chase assays with substrates containing different linker lengths in Frame2 or Lys in different positions in the sequence in the K125A background. All lanes but E2~Ub alone were treated with DTT to examine ligation products. (**D**) Plots showing the yield for each species from **C** for the lysine mutants, normalized with respect to total activity with wild-type proteins.

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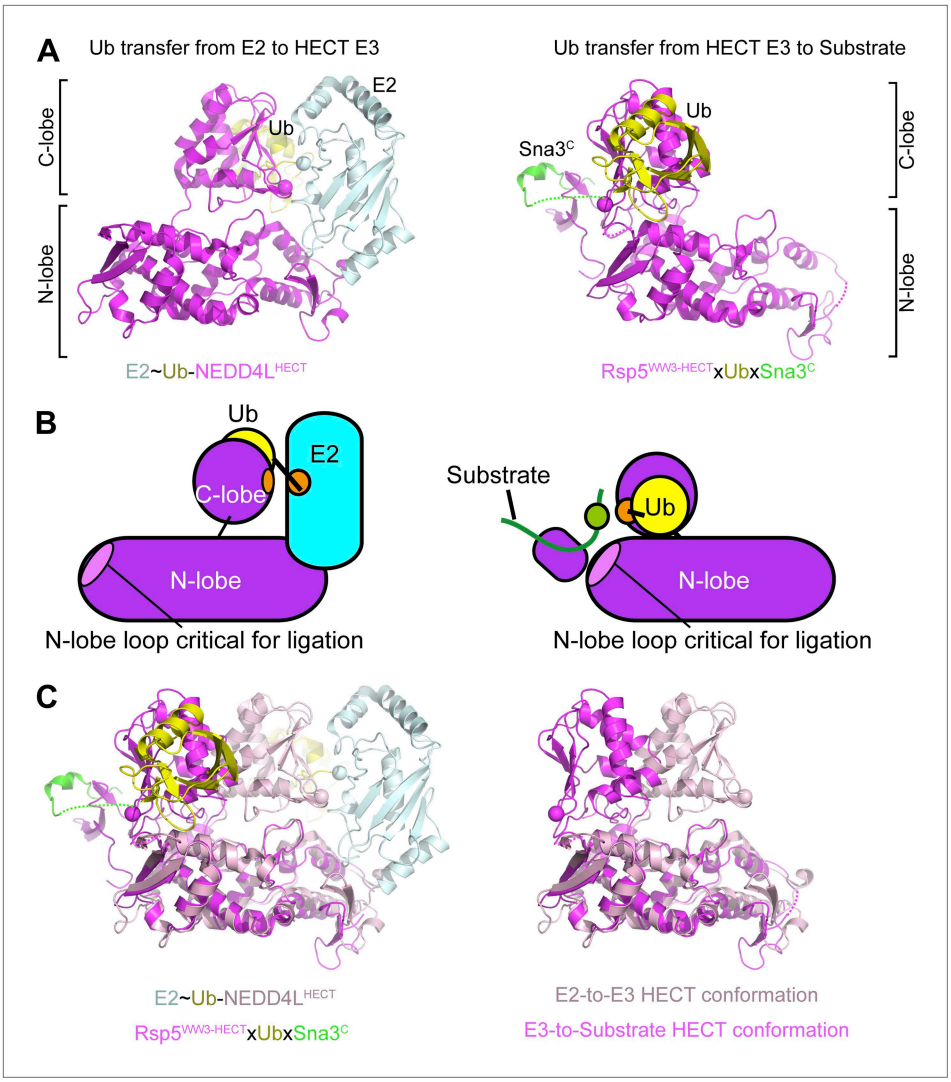

Figure 11. Structural view of the ubiquitin transfer cascade for a HECT E3. (**A**) Top left: Prior structure of E2 (pale cyan, with active site as sphere)~Ub (yellow)-NEDD4L^{HECT} (violet, with active site as sphere) (***Kamadurai et al., 2009***). Right: new structure of Rsp5$^{WW3\text{-}HECT}$ (violet, with active site as sphere) xUb (yellow) xSna3^{C} (green) aligned over the N-lobes of the NEDD4L and Rsp5 HECT domains. (**B**) Schematic views of E2-to-E3 Ub transfer and E3-to-substrate Ub ligation. (**C**) The different relative orientations of the HECT domain N-lobe with respect to the HECT domain C-lobe~Ub are highlighted by superimposing the N-lobes of the E2 (pale cyan, with active site as sphere)~Ub (yellow)-NEDD4L^{HECT} (pink, with active site as sphere) (***Kamadurai et al., 2009***) and Rsp5$^{WW3\text{-}HECT}$ (violet, with active site as sphere) xUb (yellow) xSna3^{C} (green) structures. The HECT domain portions of the two structures are shown on the right.

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