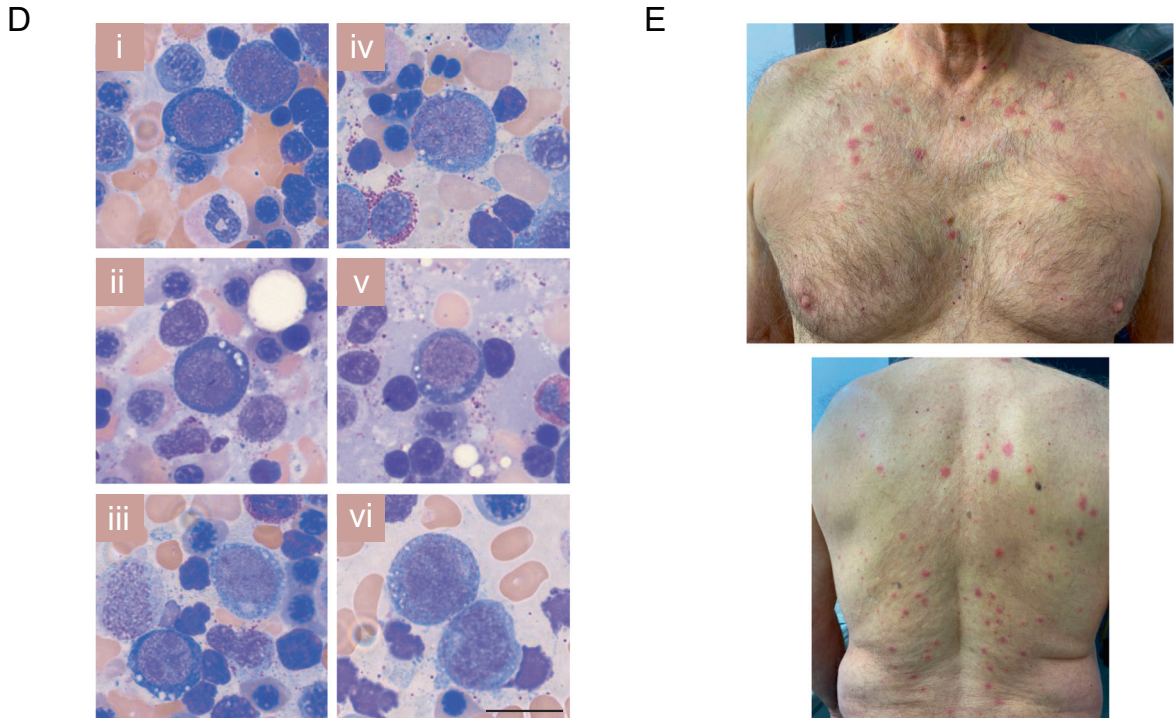
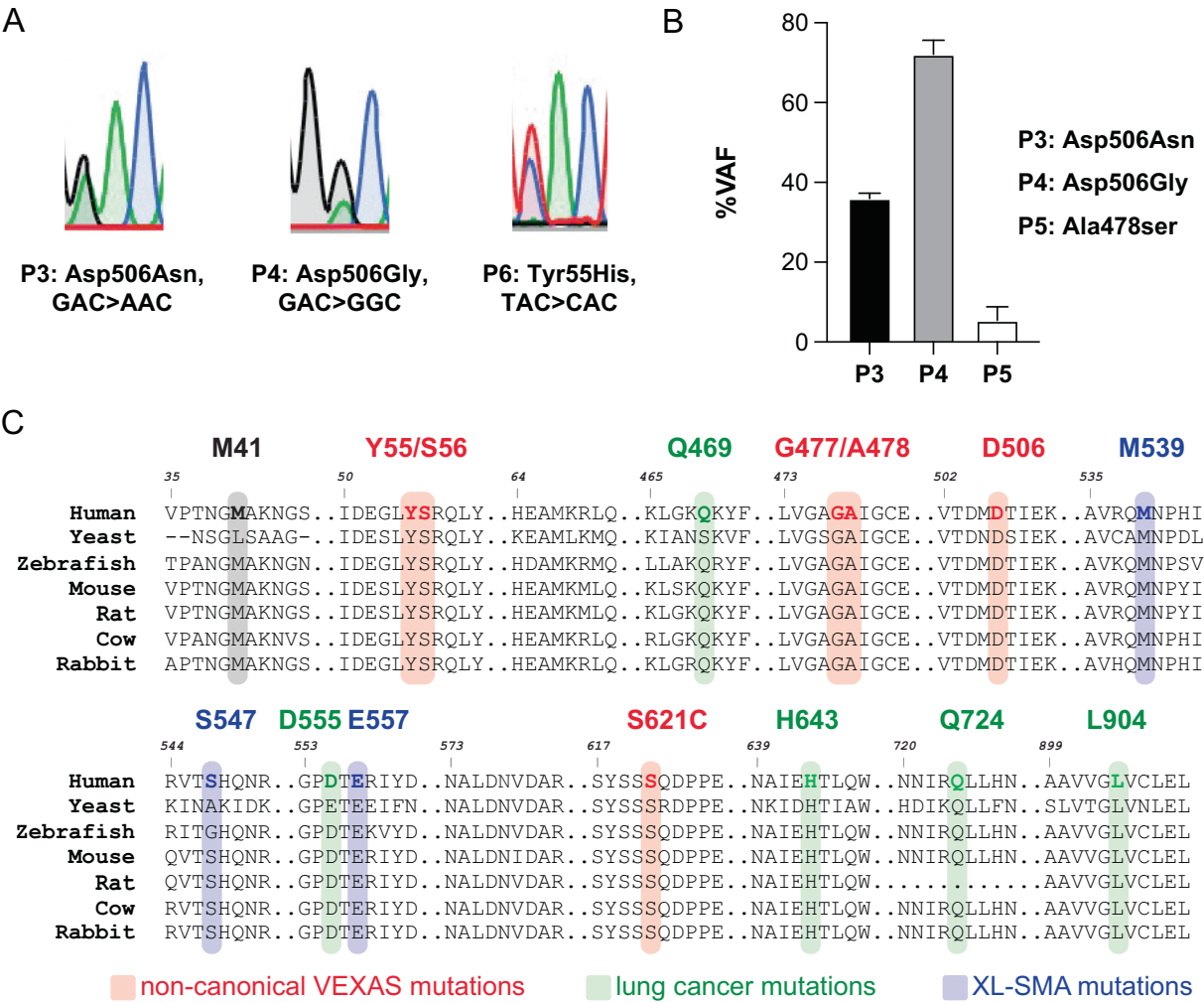


Expanded View Figures

Figure EV1. Identification and clinical phenotypes of novel non-pMet41 VEXAS mutations.

(A) Sanger sequencing confirming novel variants. (B) Digital droplet PCR (ddPCR) confirmation for novel variants in P3, P4, P5. $n = 3$ technical replicates, error bars = s.d. (C) Conservation of protein sequence for UBA1. (D) Cytoplasmic vacuoles were seen in a subset of the proerythroblasts (Panel i, ii, iii) and promyelocytes (Panel iii, iv, v, vi) from P6. Scale bar = 20 μm . (E) Sweets syndrome in P6.



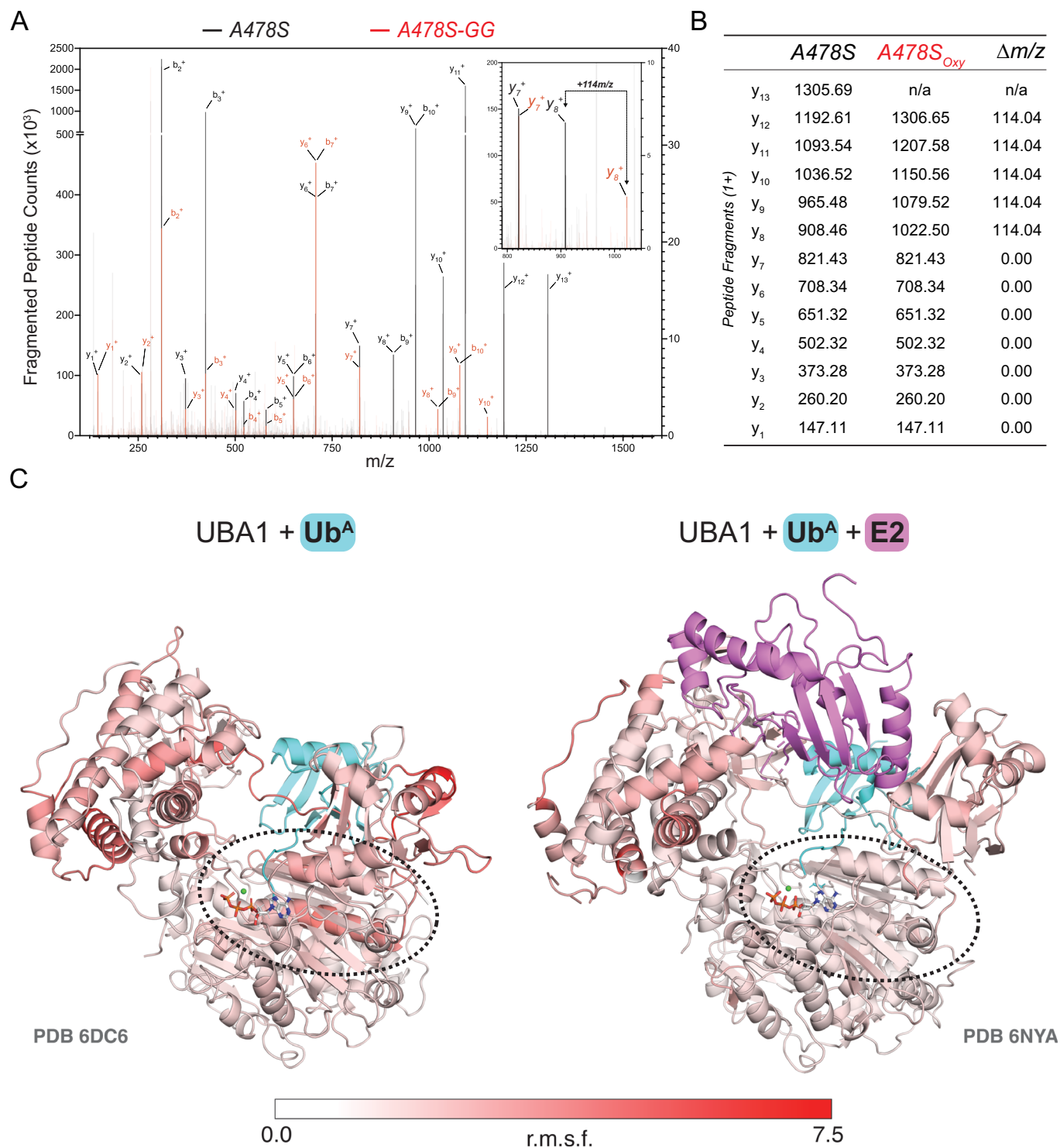


Figure EV2. UBA1 p.A478S forms an aberrant oxyester at the mutation site.

(A) Annotated MS/MS spectrum of A478S peptide with (red) and without (black) diGlycine remnant. Insert highlights the difference of 114 Da, indicative of the diGlycine remnant. (B) Table summarizing the masses of fragments of the unmodified and ubiquitylated A478S peptide, pinpointing the diGlycine remnant ($\Delta m/z$ 114.04 Da) on S478. (C) Side-by-side ribbon diagrams of UBA1 bound to ubiquitin (cyan) and ATP (indicated as a stick diagram with carbon, oxygen, nitrogen, and phosphorus in gray, red, blue, and orange) following 100 ns of molecular dynamics in the absence (left, PDB: 6DC6) or presence (right, PDB: 6NYA) of E2 enzyme (Ubc3, purple). The per residue r.m.s.f. (root mean square fluctuation) value is indicated as a red gradient (scale bar). A black dashed circle highlights the region where ubiquitin, ATP, and Mg²⁺ (green sphere) bind.

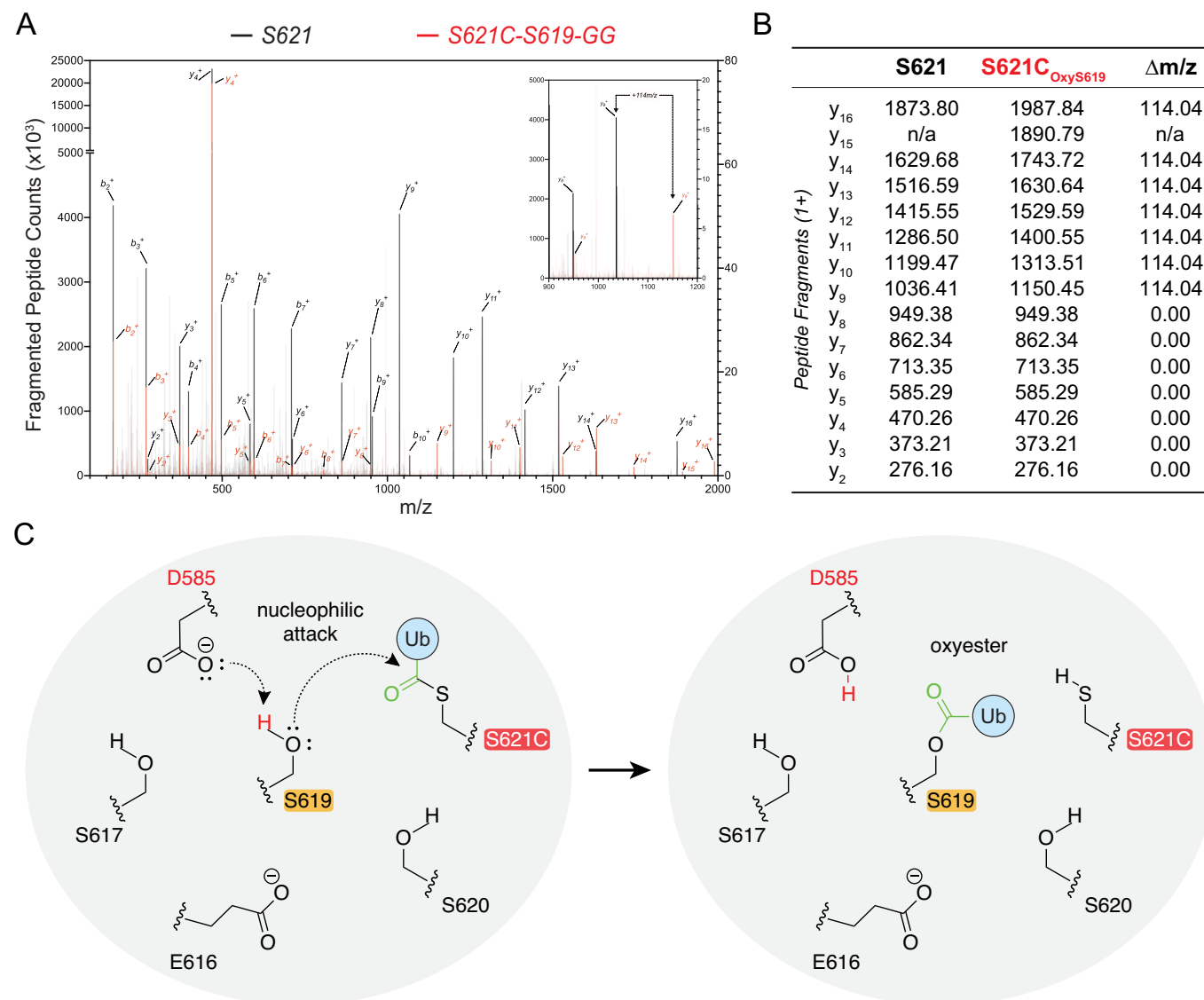


Figure EV3. UBA1 p.S621C forms an aberrant oxyester at S619 via a thioester intermediate at S621C.

(A) Annotated MS/MS spectrum of S621C peptide with (red) and without (black) diGly remnant. Insert highlights the difference of 114 Da, indicative of the diGly remnant. (B) Table summarizing the masses of fragments of the unmodified and ubiquitylated S621C peptide, pinpointing the diGly remnant ($\Delta m/z$ 114.04 Da) on S619. (C) Proposed catalytic mechanism for oxyester formation through deprotonation of S619 via the putative catalytic base D585 forming an oxyanion that promotes a nucleophilic attack of the thioester at S621C.

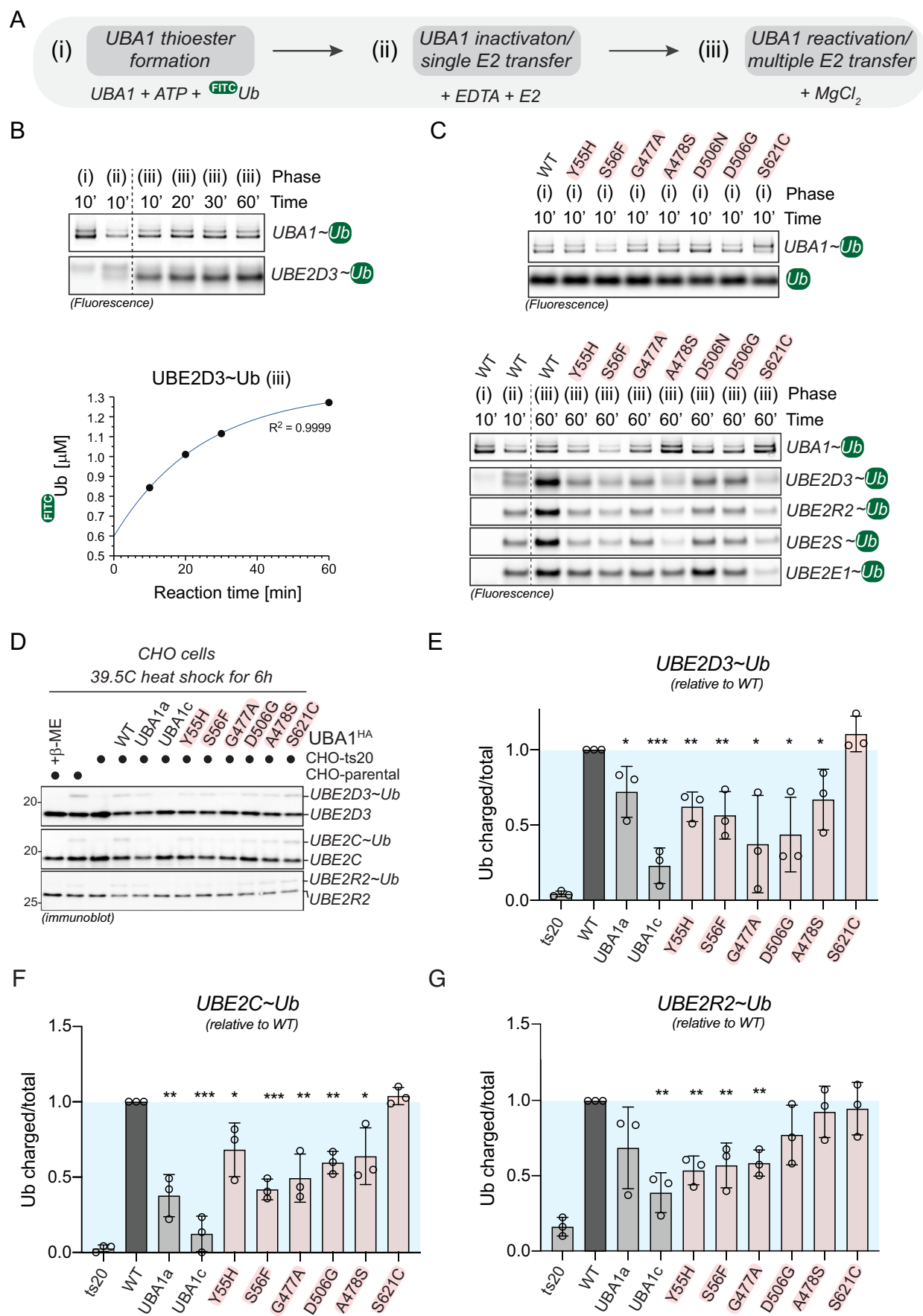


Figure EV4. Non-canonical VEXAS mutations are deficient in transferring ubiquitin to diverse E2 enzymes in vitro and exhibit defects in cells.

(A) Schematic overview of the sequential, three-phase in vitro assay used to measure UBA1 transthiolation. (i) Complete charging of UBA1 by incubation of 250 nM UBA1b with 10 μ M FITC-ubiquitin and 5 mM ATP (ii) Quenching of UBA1 charging and single transfer to E2 enzyme by addition of 100 mM EDTA and 1 μ M E2 enzyme (iii) Reactivation of UBA1 charging and multi-transfer to E2 enzyme by addition of 100 mM $MgCl_2$ (B) UBA1 WT was subjected to the experiment described in panel (A) using UBE2D3. Reactions were subjected to SDS page and analyzed by fluorescence imaging. *Upper panel:* Fluorescence scan showing UBA1 and UBE2D3 ubiquitin thioester levels after each reaction phase. *Lower panel:* UBE2D3 ubiquitin thioester levels in reaction phase (iii) were quantified and plotted against the reaction time, revealing that UBE2D3 is maximally charged after 60 min. (C) Non-canonical VEXAS mutations (red) are deficient in E2 transthiolation in vitro. Indicated UBA1 proteins were subjected to the experiment described in panel (A) using either UBE2D3, UBE2R2, or UBE2S. Reactions were subjected to SDS page and analyzed by fluorescence imaging. *Upper panel:* Fluorescence scan showing UBA1 charging after reaction phase (i) as control. *Lower panel:* Fluorescence scan showing UBA1 re-charging and E2 transfer after reaction phase (iii). Quantifications of 3 biological replicates are shown in Fig. 5F. (D) Non-canonical VEXAS mutations (red) are impaired in supporting E2 ubiquitin thioester levels in cells. CHO ts20 cells were reconstituted with indicated UBA1 variants and incubated at the permissive temperature for 6 h, followed by immunoblotting using antibodies against indicated E2 enzymes. (E-G) Quantification of ubiquitin charging levels of indicated E2s (charged/total) shown in panel (D). $n = 3$ biological replicates, error bars = s.d., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA.