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Supplementary Materials for

Structural insights into ADP-ribosylation of ubiquitin by Deltex family E3 ubiquitin ligases

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Supplementary Materials

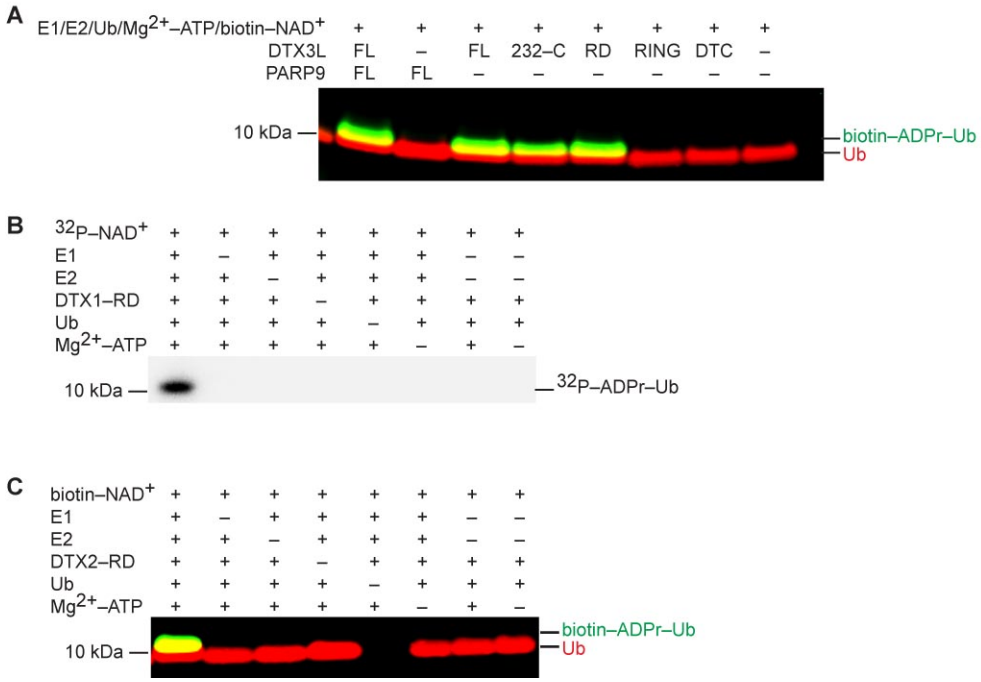

Figure S1. RING-DTC domains of DTX1 and DTX2 catalyze ADP-ribosylation of Ub. (**A**) Western blot showing *in vitro* Ub ADP-ribosylation reactions with DTX3L and PARP9 variants in the presence of E1, E2 UbcH5B, Mg^{2+}-ATP, Ub, and biotin-NAD^+. (**B**) Reduced autoradiogram of ADP-ribosylation reactions with ^{32}P-NAD^+ in which E1, E2 UbcH5B, Ub, Mg^{2+}-ATP or DTX1-RD was omitted as indicated. (**C**) Western blot from *in vitro* reactions with biotin-NAD^+ in which E1, E2 UbcH5B, Ub, Mg^{2+}-ATP or DTX2-RD was omitted as indicated; α-Ub is in red and Neutravidin DyLight is in green. Samples were separated by SDS-PAGE in reducing conditions.

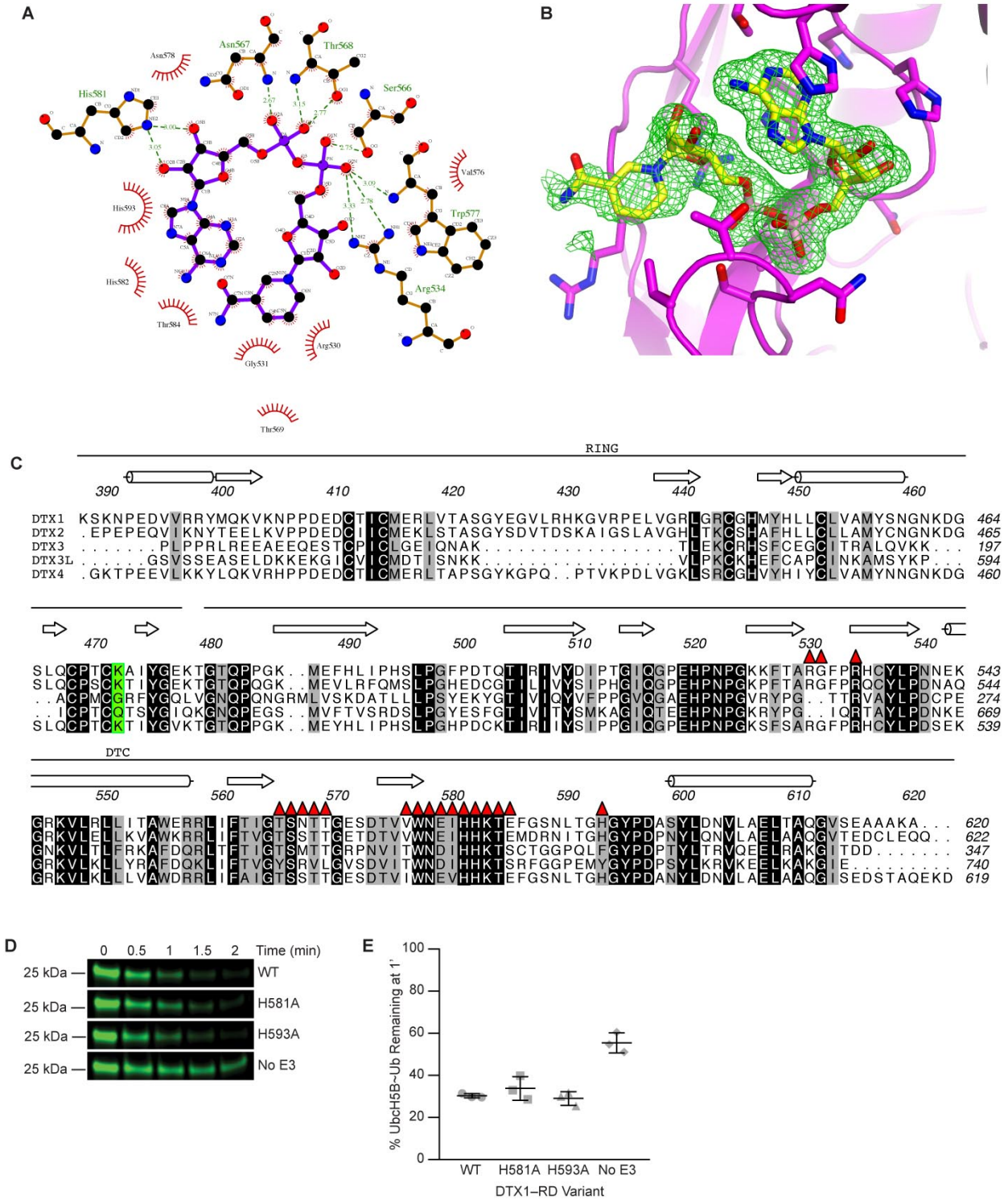

Figure S2. ADP-ribosylation of Ub depends on binding of NAD⁺ to DTC domain.

(**A**) Schematic diagram of interactions between NAD+ and chain A of DTX1-RD generated using LIGPLOT. Dotted green lines represent hydrogen bonds and distances are indicated. Lined red arcs depict residues contributing hydrophobic interactions. (**B**) Close-up of NAD^+-binding site in the DTX1-RD, colored and oriented as in **Fig. 2C**. An unbiased polder Fo-Fc map (2.5 σ) is shown for NAD^+. (**C**) Sequence alignment of the C-terminal RING-DTC

domains from the five members of the Deltex family of E3s. Residues comprising the RING and DTC domains are indicated. Identical residues are highlighted in black and >60% conserved in grey. Surface residues comprising the conserved NAD^+-binding pocket in the DTC domain are indicated by red triangles. The residue corresponding to the linchpin Arg in other E3s is highlighted in green. (**D**) Representative non-reduced SDS-PAGE gels following discharge of UbcH5B~F-Ub over time with DTX1-RD wild-type, H581A, or H593A, or no E3. (**E**) Quantification of single-turnover lysine discharge of UbcH5B~F-Ub with DTX1-RD wild-type (●, WT), H581A (■) or H593A (▲) or no E3 (◆). Data are presented as mean ± SD from three independent experiments (n=3).

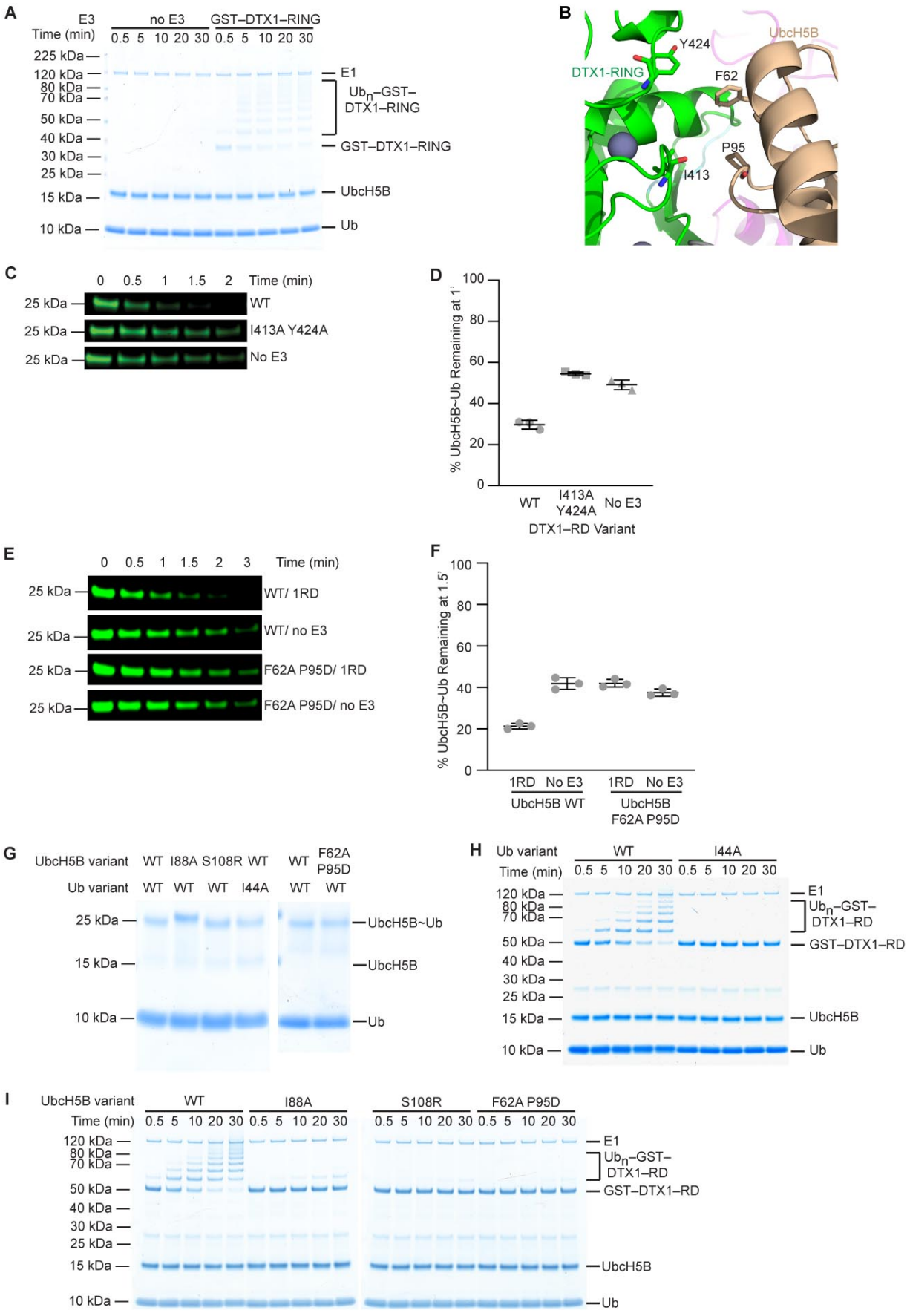

Figure S3. Activity assays with DTX1, UbcH5B and Ub variants.

(**A**) Reduced InstantBlue (Expedeon) stained SDS-PAGE gels following GST-DTX1-RING autoubiquitination over time. Ub_n, ubiquitin of chain length n. (**B**) Predicted interactions between UbcH5B and the RING domain of DTX1 based on a model generated by aligning DTX1-RING with RNF38 bound to E2~Ub (PDB: 4V3L). UbcH5B is colored wheat. DTX1 and atoms are colored as in **Fig. 2** with C atoms colored according to the parent subunit. Key residues are shown as sticks. (**C**) Representative non-reduced SDS-PAGE gel following discharge of UbcH5B~F-Ub over time with DTX1-RD WT or I413A Y424A or no E3. (**D**) Quantification of single-turnover lysine discharge of UbcH5B~F-Ub with DTX1-RD WT (●) or I413A Y424A (■), or no E3 (▲). Data are presented as mean ± SD from three independent experiments (n=3). (**E**) Representative non-reduced SDS-PAGE gel following discharge of UbcH5B variants~Ub over time with DTX1-RD or no E3. (**F**) Quantification of single-turnover lysine discharge of UbcH5B variants~F-Ub with DTX1-RD or no E3. Data are presented as mean ± SD from three independent experiments (n=3). (**G**) Non-reduced InstantBlue (Expedeon) stained SDS-PAGE gel following the formation of UbcH5B~Ub using the indicated UbcH5B and Ub variants. (**H**) Reduced InstantBlue (Expedeon) stained SDS-PAGE gels from following GST-DTX1-RD autoubiquitination with Ub variants as indicated. Ub_n, ubiquitin of chain length n. (**I**) Reduced InstantBlue (Expedeon) stained SDS-PAGE gels following GST-DTX1-RD autoubiquitination with UbcH5B variants as indicated. Ub_n, ubiquitin of chain length n.

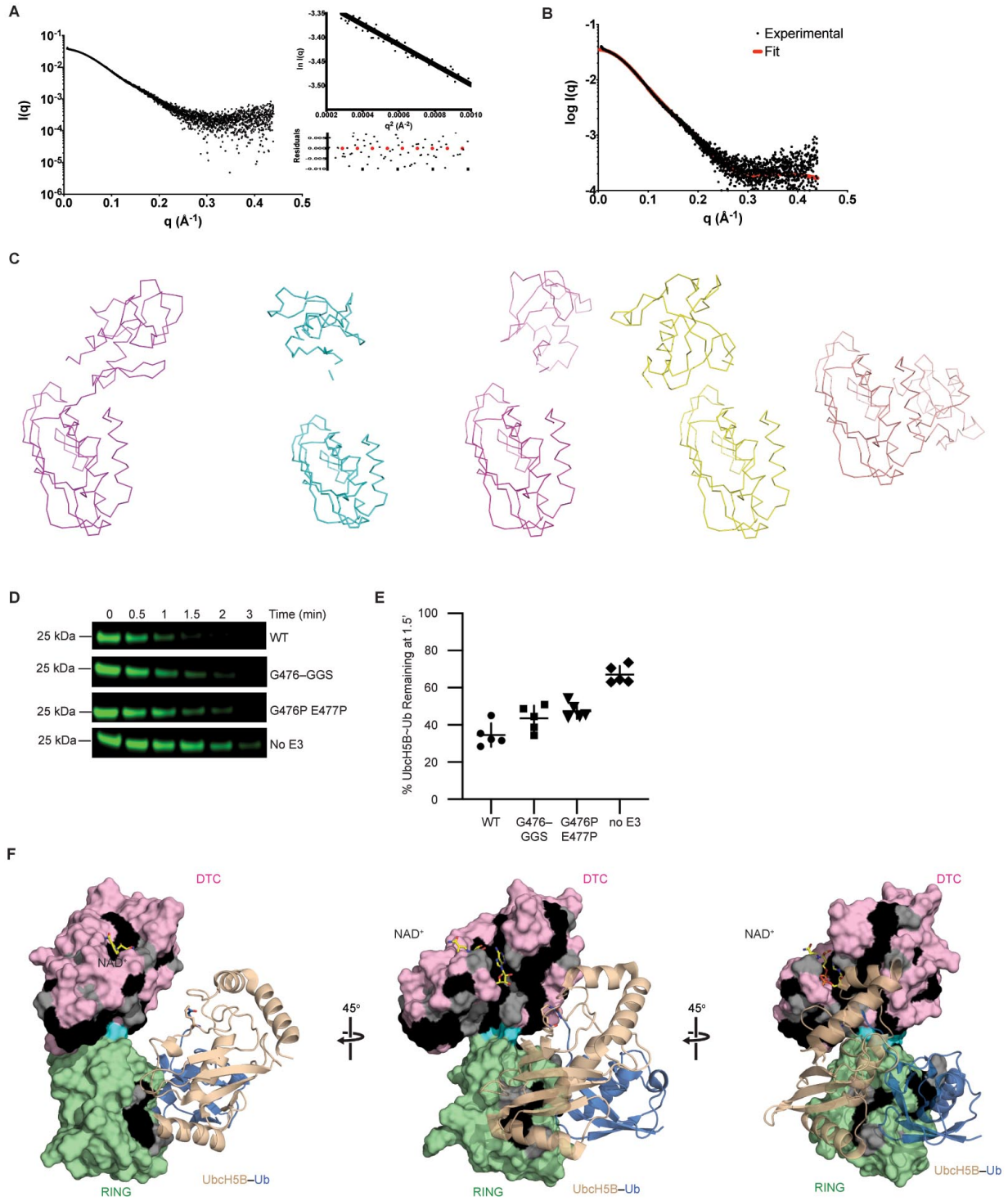

Figure S4. RING and DTC domains are flexible relative to each other.

(**A**) Experimental scattering curve. Inset Guinier region with residuals. (**B**) Experimental scattering curve with the best-fit output from Ensemble Optimization Method. (**C**) From left to right: Crystal structure of DTX1, followed by four of the highest scoring ensembles that fit the experimental data. Representative ensembles from both populations in **Fig. 4C** are shown. (**D**) Representative non-reduced SDS-PAGE gel following discharge of UbcH5B~F-

Ub over time with DTX1-RD WT or G476–GGS or G476P E477P or no E3. (**E**) Quantification of single-turnover lysine discharge of UbcH5B~F-Ub with DTX1-RD WT (●) or G476–GGS (■), or G476P E477P (▲) or no E3 (♦). Data are presented as mean ± SD from five independent experiments (n=5). (**F**) Model of UbcH5B~Ub bound to DTX1-RD. UbcH5B~Ub complex is shown as a cartoon representation and colored as in **Fig. 4B**. DTX1-RD is shown in surface representation and colored as in **Fig. 2E**. Identical and conserved residues identified in fig. S2C are colored in black and red, respectively. The middle and right panels are rotated 45° and 90° about the y-axis, respectively, compared to the panel on the left.

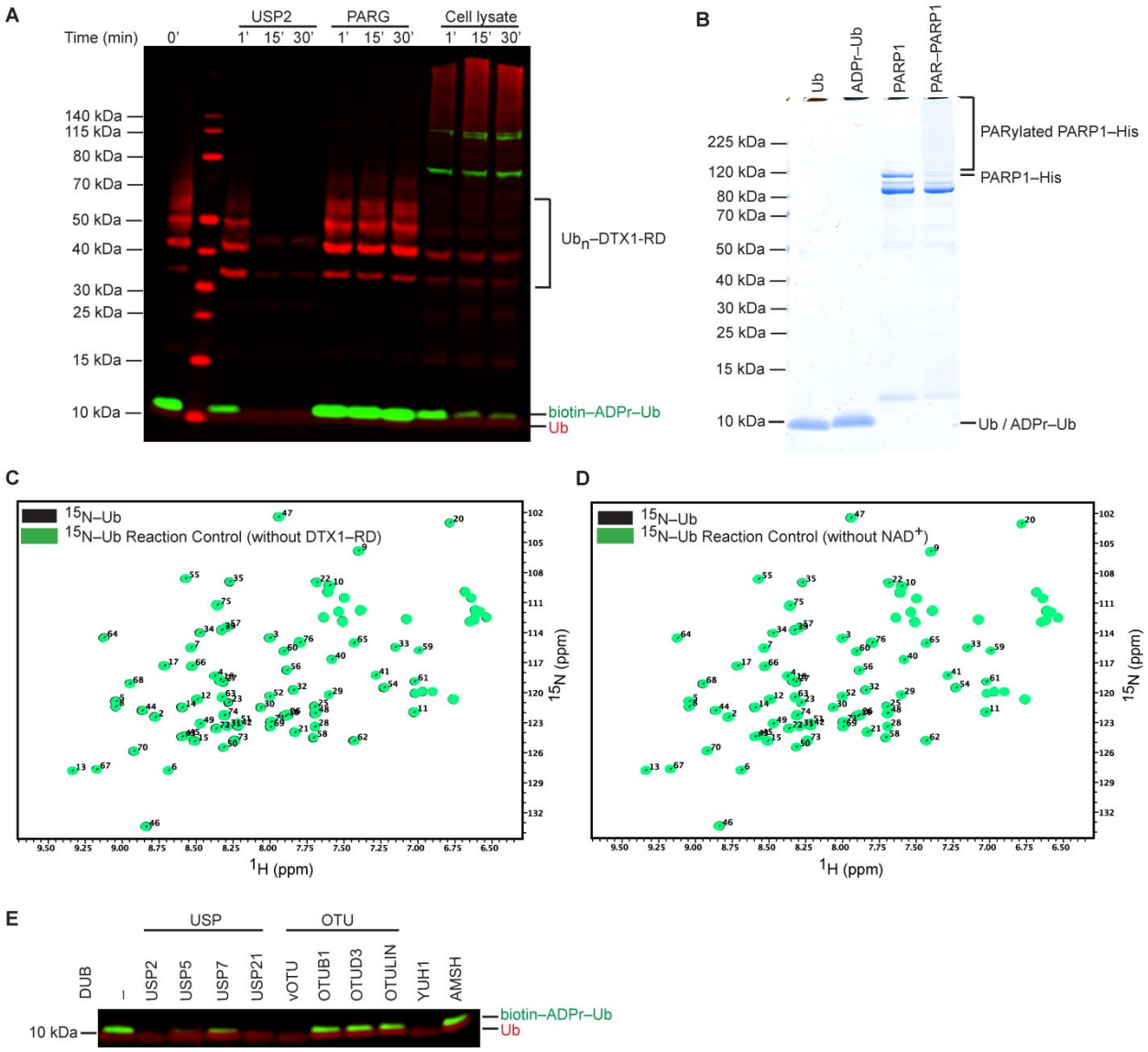

Figure S5. ADP-ribose is attached to Gly76 of Ub by DTX1-RD and removed by Ub-linkage non-specific DUBS.

(**A**) Western blot following *in vitro* hydrolysis of biotin-ADPr-Ub upon treatment with USP2, PARG, or cell lysate; α-Ub is in red and Neutravidin DyLight is in green. (**B**) SDS-PAGE profile of Ub, ADPr-Ub, PARP1, and PARylated PARP1. (**C**) ^{1}H-^{15}N HSQC spectra of ^{15}N-Ub (black) and ^{15}N-Ub purified from control reaction without DTX1-RD (green). (**D**) ^{1}H-^{15}N HSQC spectra of ^{15}N-Ub (black) and ^{15}N-Ub purified from control reaction without NAD^{+} (green). (**E**) Western blot of *in vitro* reactions of biotin-ADPr-Ub treated with various DUBs; α-Ub is in red and Neutravidin DyLight is in green.

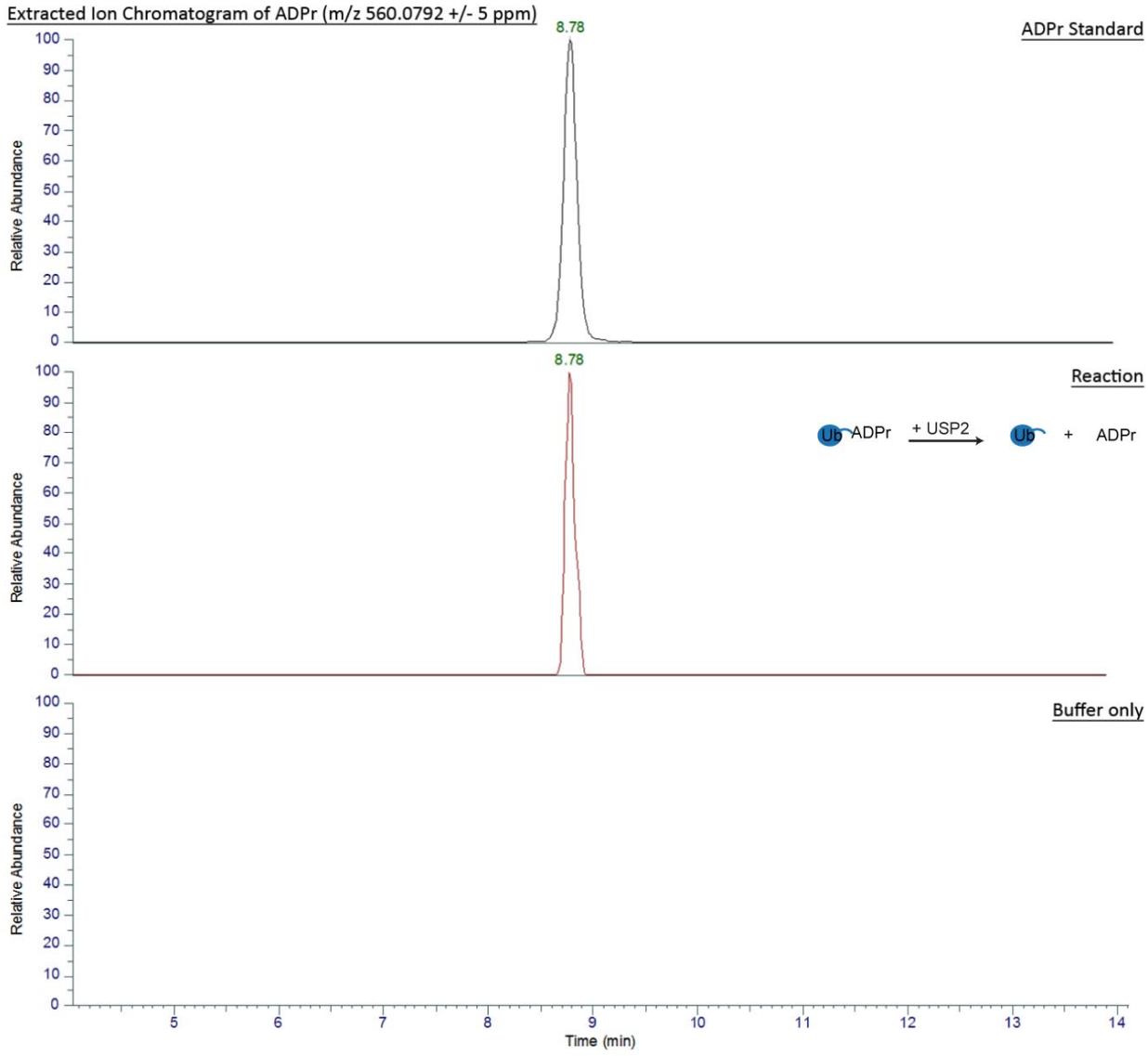

Figure S6. Mass spectrometry analysis of ADPr-Ub treated with USP2.

Extracted ion chromatogram of ADP ribose (m/z 560.0792 +/- 5ppm) for ADP ribose standard (top panel), ADPr-Ub treated with USP2 (middle panel), and reaction buffer only (bottom panel).

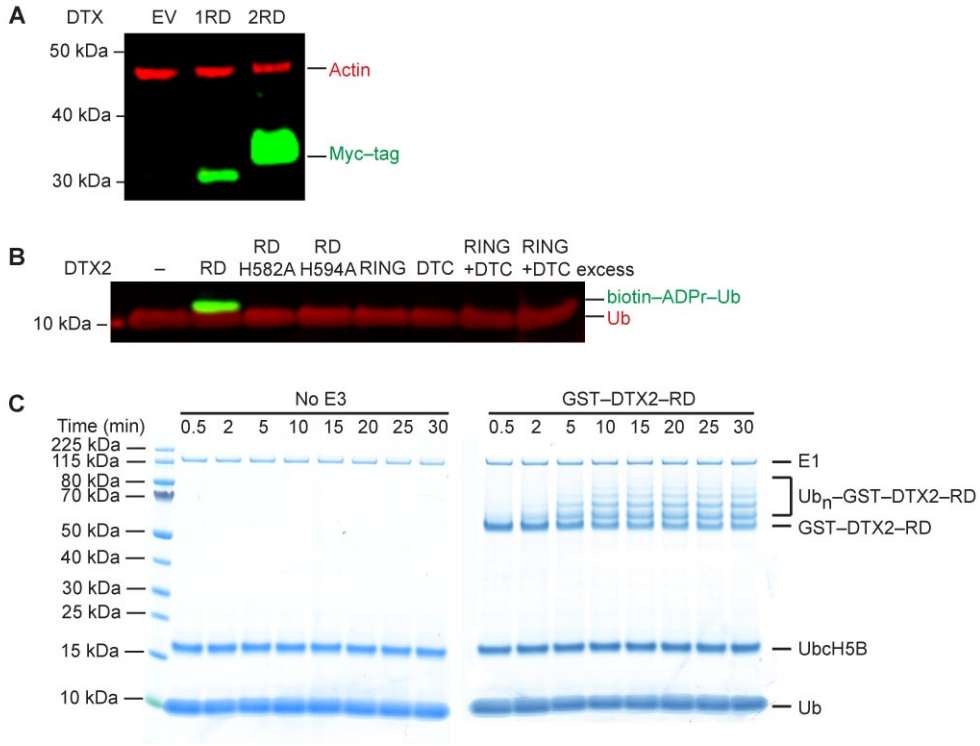

Figure S7. DTX2-RD expression and activity.

(**A**) Western blot of HEK293 whole cell lysate transfected with empty vector (EV), DTX1-RD (1RD), or DTX2-RD (2RD) with an N-terminal Myc tag. (**B**) Western blot from *in vitro* reactions with E1, UbcH5B, Ub, Mg^{2+}-ATP, biotin-labeled NAD^{+} and the indicated DTX2 variant, separated by SDS-PAGE in reducing conditions and visualized with an Odyssey CLx Imaging System; α-Ub is in red and Neutravidin DyLight is in green. (**C**) Reduced InstantBlue (Expedeon) stained SDS-PAGE gels of GST-DTX2-RD autoubiquitination over time. Ub_n, ubiquitin of chain length n.

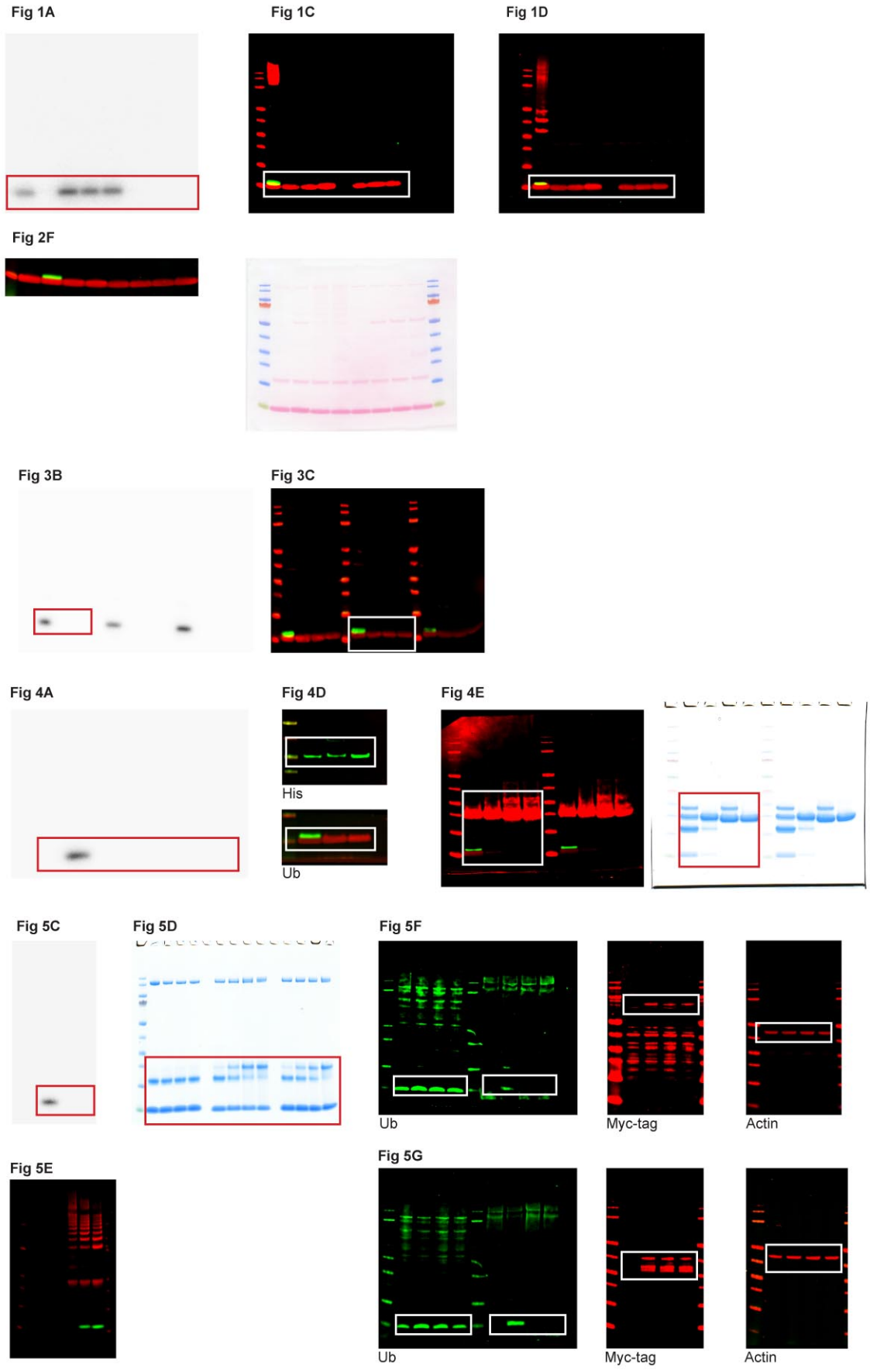

Figure S8. Full gels or blots presented in the main figures

Figure S9. Full gels or blots presented in the supplementary figures