Supplementary Online Data for Bekes et al. (2014):

SARS hCoV papain-like protease is a unique Lys48 linkage-specific di-distributive deubiquitinating enzyme

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SUPPLEMENTARY EXPERIMENTAL

Cloning, protein expression and purification.

The nucleotide sequence (nts 4451-7179) from MERS HCoV ORF1a (GenBank AGV08402.1) and the nucleotide sequence encoding the SARS PLpro minimal domain (nts 4884-5829, aa 1541-1885, pdb: 3MJ5) were gene synthetized by GenScript; SARS PLpro and MERS PLpro (aa 1480-1511, see Supplementary Fig. 1A) were subcloned into pET28b with a C-terminal 6xHis-tag. All mutations were generated by over-lapping PCR and verified by sequencing. DUBs (MERS and SARS PLpro and mutants, $USP2_{CD}$, USP21) were expressed in *E.coli* BL-21 codon plus RIL cells and purified until homogeneity. Briefly, *E.coli* was grown in 1L shakers at 37ºC until OD_{600}=1.0 and induced with 1mM IPTG and further incubated at 30ºC for 4 hours. Harvested cell pellets were flash frozen in liquid nitrogen and stored at -80ºC until purification. Frozen pellets were thawed on ice and lysed in 20mM Tris, pH=8.0, 350mM NaCl, 1mM PMSF, 1mM Bme, 1% NP-40 and 10mM imidazole by sonication. After centrifugation, the supernatant was applied onto Ni-NTA resin (Quiagen) for batch-binding by rotation for 1 hour at 4ºC. Ni-bound DUBs were eluted with 20mM Tris, pH=8.0, 350mM NaCl, 250mM imidazole and 1mM Bme, concentrated and purified by size-exclusion chromatography on a Supderdex-75 (16/600) column (GE); peak fractions were collected, concentrated to 5-10mg/ml and DUBs were stored at -80ºC until use. $USP2_{CD}$, His-Cdc34 and untagged mono-ubiquitin were produced as previously [1].

Reagents.

Unless indicated otherwise, di-, tri- and tetra-ubiquitin chains, Ub-probes, Ub-AMC and ISG15-AMC were purchased from Boston Biochem. Antibodies used in this study were anti-ISG15 (#2743 from Cell Signaling), anti-ubiquitin (P4D1, sc-8017 from Santa Cruz Biotech), anti-His (in-house, gift of D. Reinberg, NYUMC), anti-FLAG (M2, Sigma), anti-K48-linkage (Apu2, Millipore) and anti-Cdc34 (ab155792, Abcam).

Ub and ISG15 binding assays.
His-tagged catalytic mutant DUBs (1µg) were incubated in 20mM Tris, pH=8.0 and 150mM NaCl with untagged Ubls (3µg, Ub chains and/or ISG15) for 1 hour at 4ºC in 50µl, subjected to a brief Ni-NTA pull-down using 5µl Ni-NTA beads and washed three times in assay buffer. The input and pulled-down Ni-beads were analyzed by SDS-PAGE and SYPRO-staining. Gels were imaged on BioRad Gel-Doc imagers.

Cell culture.
HeLa cells were cultured by standard cell culture technique, in DMEM with penicillin/streptomycin, glutamine and 10% FBS at 37ºC with 5% CO_2. Cells were left untreated or treated with 500units/mL of interferon-beta (human IFNb1a, purchased from PBL Interferon Source, product #1141501) for 48 hours at 37ºC and the proteasome inhibitor MG132 (10µM, Calbiochem) was included for the last 4 hours in the cell culture before harvest. Cells were harvested by trypsin and pellets were frozen at -80ºC. Pellets were lysed on ice for 1 hour in 50mM Tris, pH=7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40 in the presence of a protease inhibitor cocktail (Roche), 1mM 1,10-phenantroline and 20mM N-ethyl-maleimide. The supernatant was cleared by centrifugation, and the lysates were quantified using a BioRad Protein Assay by measuring absorbance at 595nm.

Image quantification.
Quantification of SYPRO-stained gels or western blots was done using ImageJ software, graphs were generated by Prism.

Structural Modeling.
To compare the active site cleft of DUBs, Ub-bound USP2 (PDB:2HD5), Ub-bound SARS PLpro (4MM3), and Ub-bound MERS PLpro (PDB: 4RF0) were aligned based on the last four amino acids of ubiquitin (Leu73-Arg74-Gly75-Gly76) in Pymol.

Generation of poly-Ub-conjugated Cdc34 and subsequent DUB assay
To generate purified poly-Ub-conjugated Cdc34 as a substrate for DUB cleavage, largely devoid of free (unconjugated) Cdc34, His-tagged Cdc34 (1.25µM) was auto-conjugated overnight at 30ºC with Uba1 (0.125µM), ATP and FLAG-tagged ubiquitin (250µM) according to Dong et al. [2]; the following day, the reaction was quenched with 50mM EDTA and 5mM DTT on ice; next the reaction mix was incubated with magnetic anti-FLAG beads (M2 Dyna-beads, Invitrogen) to purify Ub^{FLAG}-conjugates for 1 hour at 4ºC, washed three times with 350mM NaCl buffer, then eluted with 250ng/µl 3X-FLAG-peptide (Sigma); next the elution was incubated with Ni^{2+}-NTA beads (Quiagen) to purify $Cdc34_{His}$-bound chains, washed three times with 350mM NaCl buffer, then eluted with 200mM imidazole in the same buffer. The purified final fraction (eluate #2) was then diluted into DUB cleavage assays; time-course cleavage reactions were performed at 37ºC in the presence of 5mM DTT using 50nM SARS PLpro and 150nM MERS PLpro, USP2 and USP21; cleavage products were analyzed by SDS-PAGE and western blotting for the indicated antibodies.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: MERS PLpro substrate specificity

(A) Sequence alignment of SARS PLpro (PDB: 3MJ5) and ORF1a from MERS hCoV (GenBank AGV08402.1), generated by ClustalW. (B) and (C) Michealis-Menten plots of Ub- and ISG15-AMC cleavage assays for MERS PLpro, assayed at 50nM (B) and SARS PLpro, assayed at 10nM (C). The dotted line for ISG15 indicates extrapolation of the curve, as the final concentration of ISG15-AMC was limiting in the assay. Error bars indicate SEM from minimum two independent experiments. These rates were used to calculate apparent kcat/Km values shown in Figure 1A. (D) Design and setup of the PS-SCL used to assay the cleavage site specificity of MERS PLpro in Figure 1. (E) Heat-map display of cleavage sites specificities for MERS PLpro compared to SARS PLpro and USP5 (from ref. 26). (F) MERS PLpro (at 10 nM) was assayed against a panel of di-ubiquitin chains of all 8 Ub-Ub linkages. See also Figure 1F. Dotted lines indicate cropped images from different gels ran side-by-side. (G) Structures of monoUb bound to USP2 (PDB: 2HD5), to SARS PLpro (PDB: 4MM3), and to MERS PLpro (PDB: 4RF0), highlighting the lack of a defined P4 pocked for SARS and MERS PLpro.

Supplementary Figure 2: SARS PLpro substrate specificity

(A) Time-course cleavage assay of K63-linked tetra-ubiquitin by SARS and MERS PLpro (20nM). (B) Time-course cleavage assay of linear (M1-linked) tetra-ubiquitin by SARS and MERS PLpro (1μM). (C) Serial dilution cleavage assay of SARS and MERS PLpro and their ΔUBL mutants onto K48-linked tetra-Ub. (D) Time-course cleavage assay of SARS WT and its ΔUBL mutant (5nM) on K48-linked tetra-ubiquitin. (E) Lysates prepared from HeLa cells treated with IFN-b and MG132 were incubated with a 1/10-serial dilution of SARS and MERS PLpro (starting at 1μM), the results were visualized by SDS-PAGE and western blotting. Blue box indicates focus on Ub chain cleavage intermediates only observed for SARS PLpro. See also Figure 3D for a time-course assay. (F) Quantification of the loss of higher molecular weight (HMW) Ub and ISG15 signal from Supplementary Figure 2D. Dotted lines are included for clarity, except in (A), where it indicates cropping within the same gel.

Supplementary Figure 3: SARS PLpro is a unique K48-specific di-distributive DUB

(A) Generation of purified $_{His}$Cdc34-poly-Ub^{FLAG}-conjugated substrates for DUB assays. See experimental workflow in Figure 4A, which is described in detail in the Supplementary Experimental section. Purification steps (1% from each step) were visualized by western blotting by FLAG (for Ub) and His (for Cdc34) antibodies; note the enriched poly-Ub-Cdc34 devoid of free Cdc34 in the 2nd (His) elution. (B) Characterization of the purified Cdc34-Ub-conjugates (eluate #2) by a time-course cleavage assay using SARS PLpro. Note that HMW-Ub species are reactive with the anti-K48 antibody, confirming their K48-linkage. Also note the accumulation of cleavage intermediates over time: FLAG blot, Cdc34-Ub accumulation, with loss of HMW-Ub; Cdc34 and His blots, loss of HMW-Cdc34 signal, with accumulation of free and mono-Ub-conjugated Cdc34. (C) Single-time-point assays (1hr at 37ºC) of $_{His}$Cdc34-poly-Ub^{FLAG} cleaved by USP-family DUBs (related to Figure 4C). Dotted lines indicate cropping of different blots and are included for clarity. (D) Mechanism of tri-Ub recognition by SARS PLpro. (E) Differential processing of UBL-conjugates by viral processing proteases: SARS PLpro (in black) recognizes ISGylated substrates

(right panel, top) by contacting both Ubl units within ISG15, which is mediated by a unique surface in SARS PLpro (utilizing both S2 and S1 sub-sites), which results in its remarkable catalytic efficiency in removing ISG15 from substrates. On the other hand, MERS PLpro (in grey) lacks this unique surface and mainly recognizes ISG15 via the proximal Ubl domain in S1 (right panel, bottom). Additionally, ubiquitin recognition by MERS PLpro (left panel, bottom) is also mediated by the S1 sub-site (short black curve). On the other hand, SARS PLpro recognizes poly-Ub substrates modified by K48-linked ubiquitin chains by binding two ubiquitin units in a chain in the S2 and S1 sub-sites (long grey curve) and cleaves the third one in S1', resulting in stabilization of di-UbK48 species as cleaved products (left panel, top), making it a di-distributive DUB. The S2 site for K48-poly-Ub chains is likely somewhat different from and only partially overlapping with the S2 site for ISG15.

SUPPLEMENTARY REFERENCES

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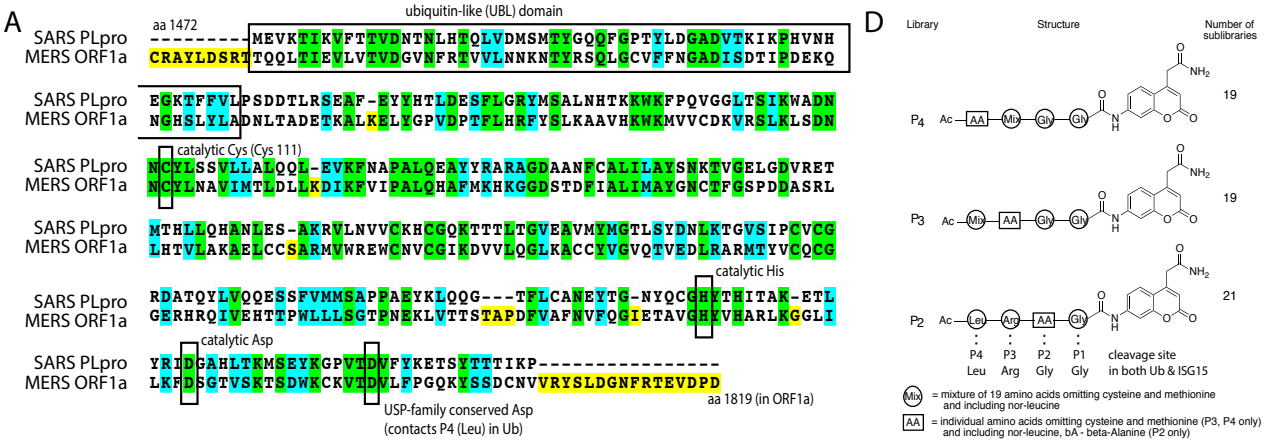
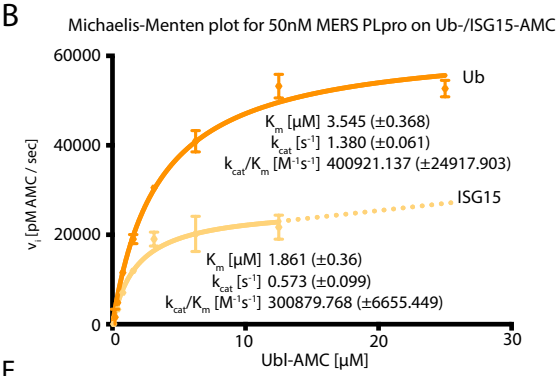
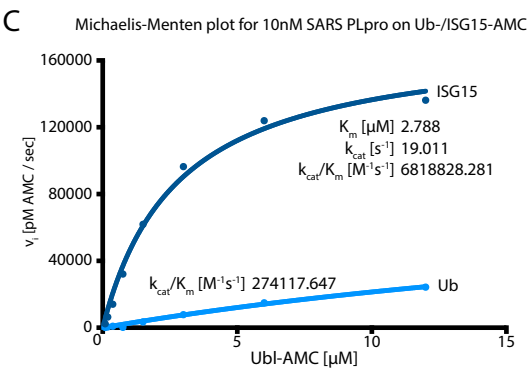
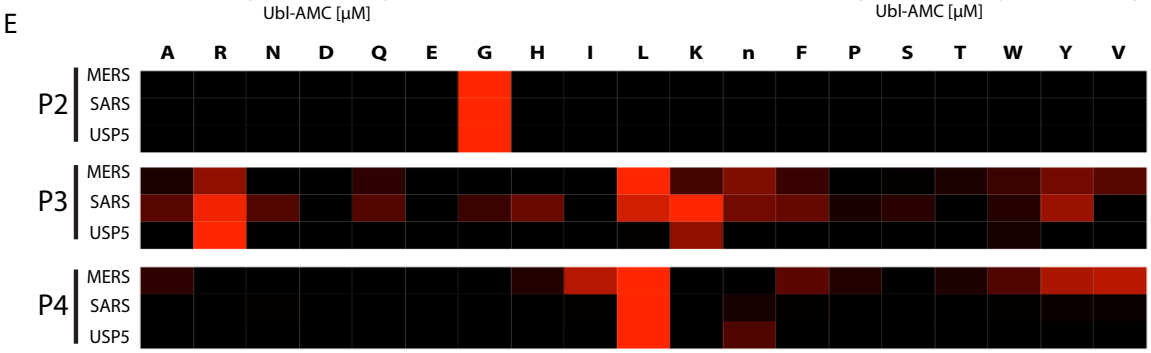
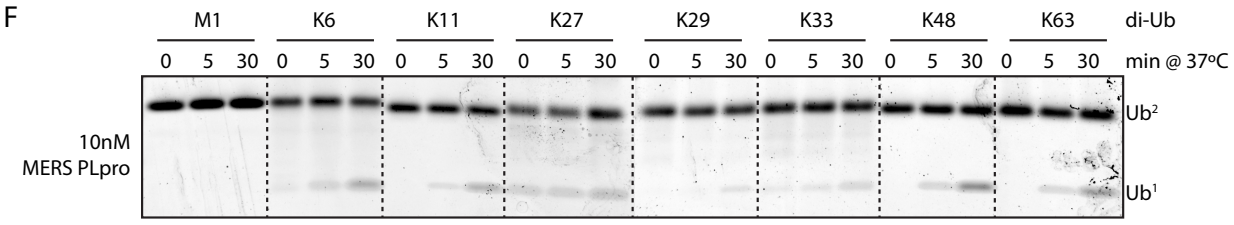
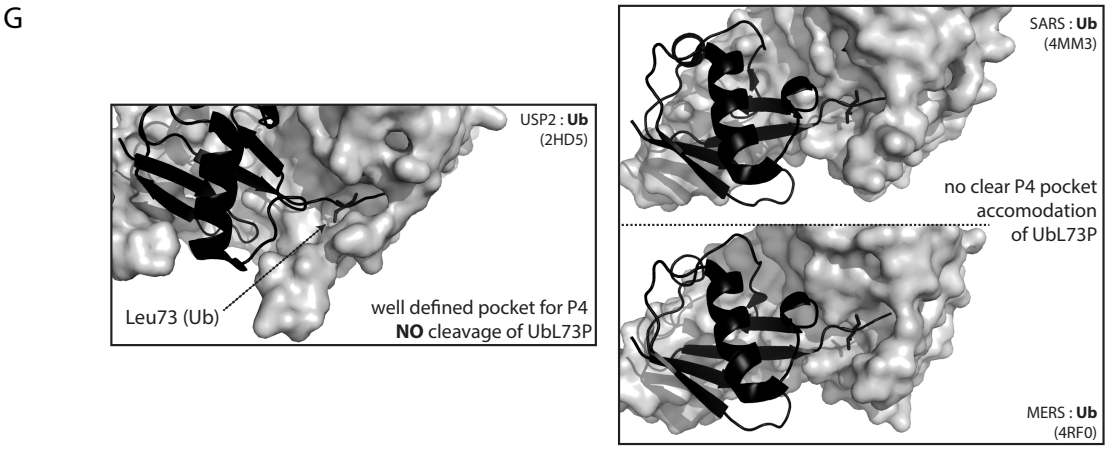




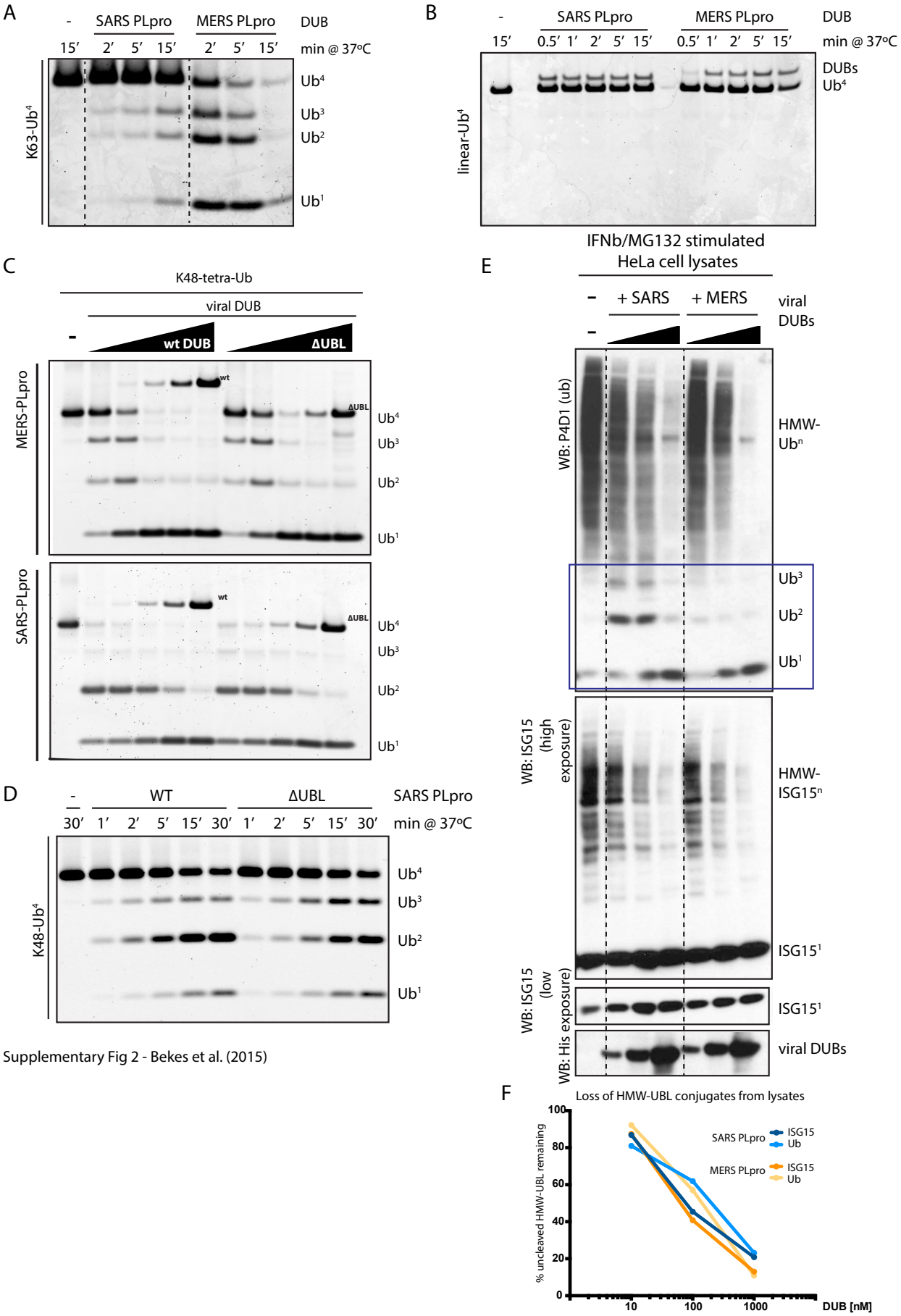




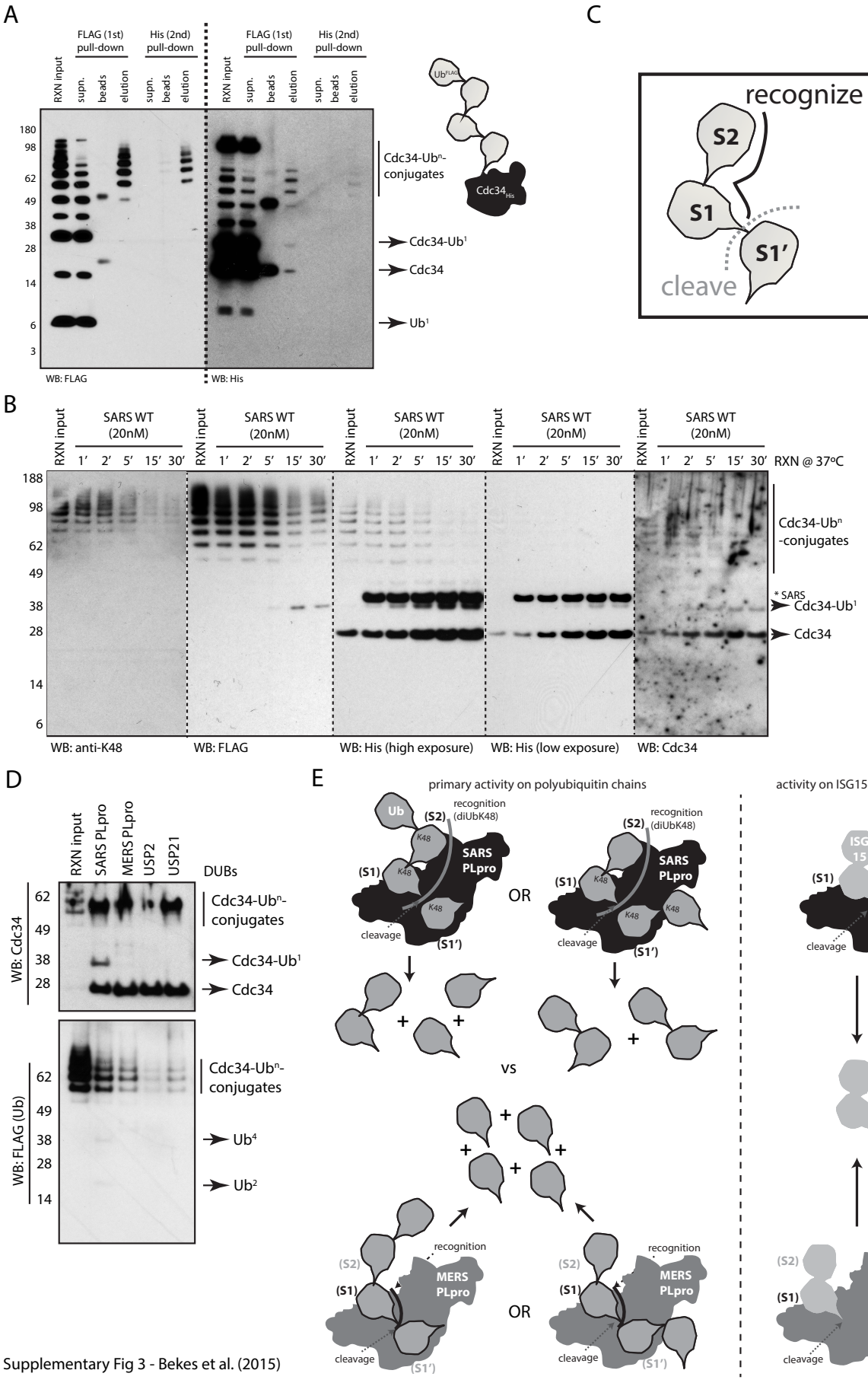




Supplementary Fig 1 - Bekes et al. (2015)



Supplementary Fig 2 - Bekes et al. (2015)



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