Supplemental Material and Methods

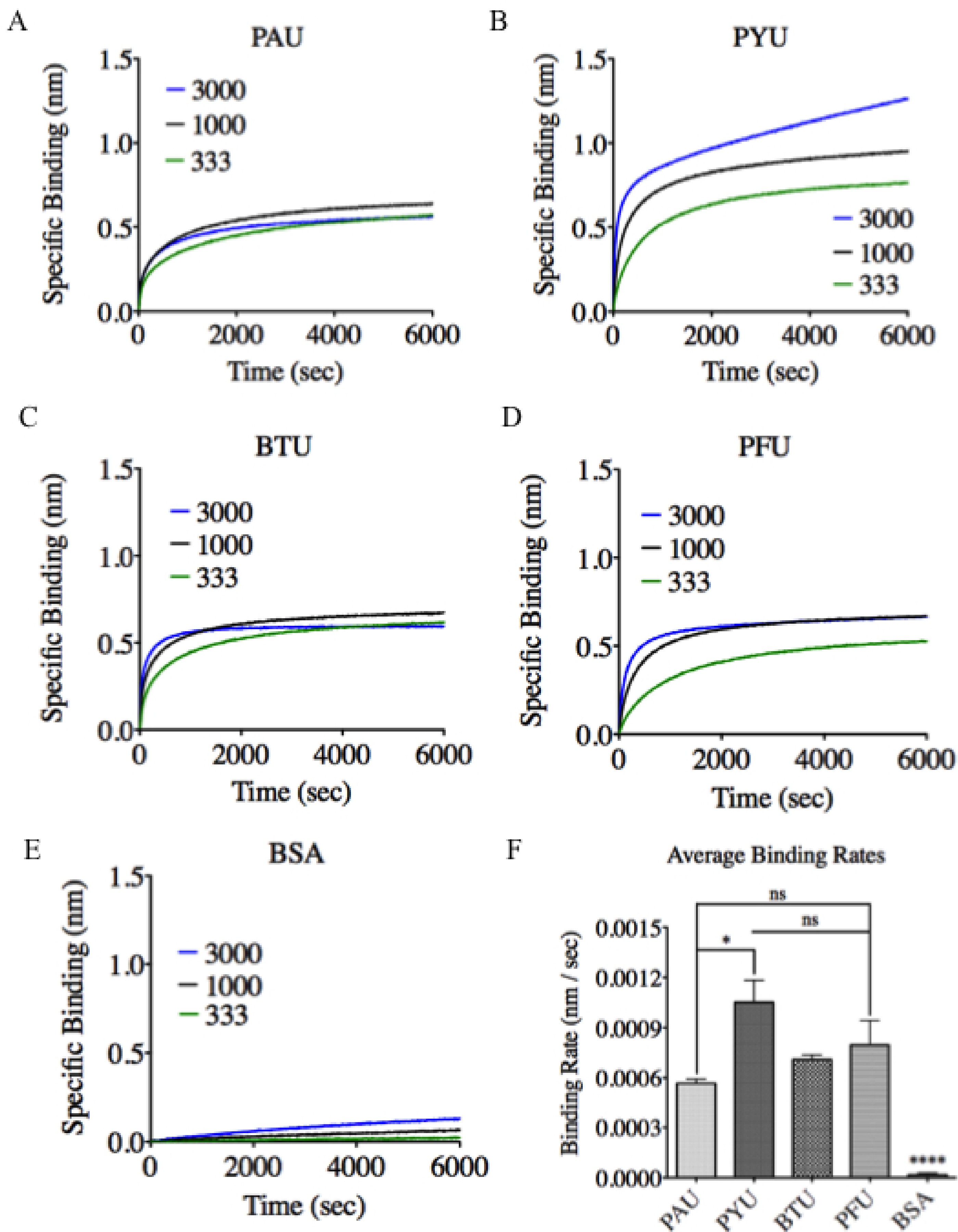
Supplemental bacterial strains and cloning. Strain AR58 (gift from Dr. Arthur Haas) was lysogenized with lambda DE3 phages (Millipore) according to manufactures recommendations with the exception that the phage incubation step was conducted at 42°C. Plasmid pET15b was digested with enzymes NdeI and BamHI for ligation with two complementary primers encoding 5` - NdeI-NotI-KpnI-SacI-SpeI-BamHI-3` restriction sites in a contiguous array. This plasmid was then digested with NcoI and NdeI to insert an N-terminal histidine and AviTag (underlined) sequence prior the the ATG start codon (primer translation: MGSSHHHHHSSGLVPRGSSGLNDIFEAQKIEWHEH) or with SpeI-BamHI to insert a C-terminal AviTag (primer translation: SGLNDIFEAQKIEWHEH**). The gene corresponding to PcrV was PCR amplified and cloned into the N-terminal AviTag plasmid with SpeI-BamHI restriction sites using standard procedures. Monoubiquitin sequences were modified with R72K-R74K mutations using the site-directed mutagenesis kit and procedures similar to the Materials and Methods section. This mutation significantly reduced degradation of the linear ubiquitin chain into monomers during expression and purification (data not shown). Ubiquitin_{KK} sequences were amplified with NdeI-KpnI, KpnI-SacI or SacI-SpeI terminal restriction sites for cloning into the modified pET vector containing a C-terminal AviTag. In summary, the coding sequence of this construct includes an N-terminal histidine tag and thrombin recognition site, followed by three ubiquitin monomers in a linear sequence and a C-terminal AviTag.

Supplemental protein expression and purification. Strain BL21 (DE3) pLyS was transformed with pET21a *birA* (addgene: 20857). This strain was grown in LB broth with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol at 37°C until $OD_{600} = 0.5$ before a 2 h induction at 30°C with 0.25 mM IPTG. Cells were harvested, lysed by French pressure cell and purified by cobalt metal affinity chromatography as in the Materials and Methods section. Affinity resin was eluted in buffer containing 100 mM EDTA prior to purification by size exclusion chromatography as described. Triubiquitin_{KK} and PcrV proteins were expressed and purified in similar fashion from strains AR58 (DE3) pJY2 and BL21 (DES) pLysS, respectively. Purified elution fractions were buffer exchanged into 50 mM sodium phosphate, 150 mM sodium choloride, pH 7.4 (Buffer A), prior to removal of the his-tag via thrombin cleavage. Samples were passed through benzamidine sepharose and cobalt affinity resin prior to concentration and size exclusion chromatography. Biotinylation was conducted for 2 h, 30°C in 50 mM Tris-HCl, pH 8.3, 10 mM ATP, 10 mM $MgCl_2$ and 50 mM D-biotin using a 1:100 enzyme to AviTag ratio. BirA was removed by affinity chromatography and excess biotin was removed by buffer exchange into Buffer A using a PD-10 column. Concentrated samples were measured by A_{280} before storage at -80°C until use. In vitro catalytic constants (k_{cat}) measured for triubiquitin_{KK}-AviTag were 1.8, 0.91, 0.33 and 0.67 sec^{-1} for PAU, PYU, BTU and PFU, respectively.

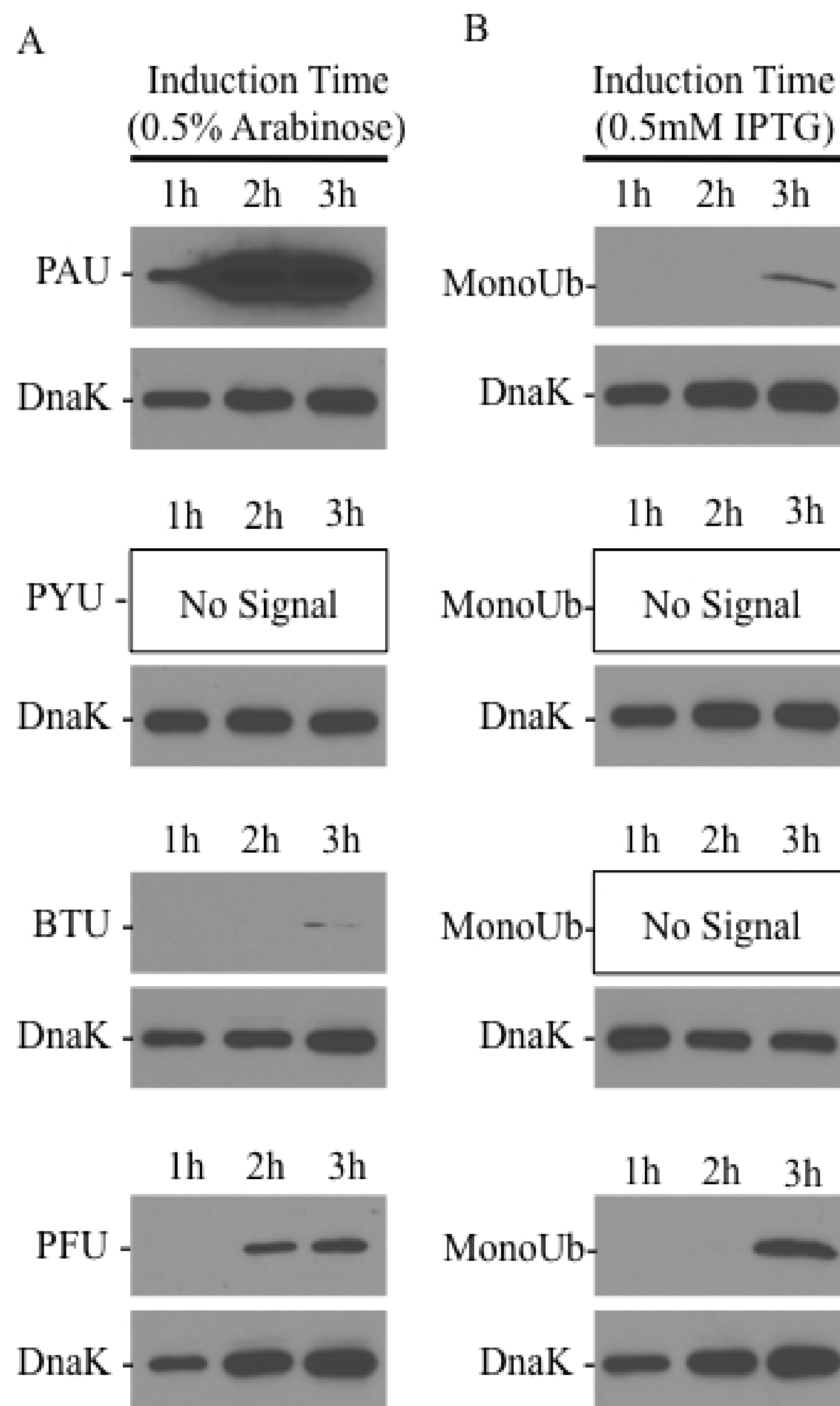
Bio-layer interferometry. Streptavidin sensor probe tips (ForteBio) were pre-wet for at least 15 min in Buffer A prior to each experiment. Assays were initialized on an Octet RED96 machine (ForteBio) by a 60 sec baseline step before loading for 10 min with biotinylated ligand (100 nM concentration per well). A second baseline was acquired for 60 sec in a fresh buffer well followed by a 10 min association step with either 333, 1000 or 3000 nM soluble toxin and a 10 min dissociation step in fresh buffer. Binding was normalized to the second baseline step and all observed background binding signal to PcrV-bound probe tips were subtracted from ubiquitin-bound probe tips. All experiments were performed in Buffer A.

TABLE S1 List of plasmids used in this work

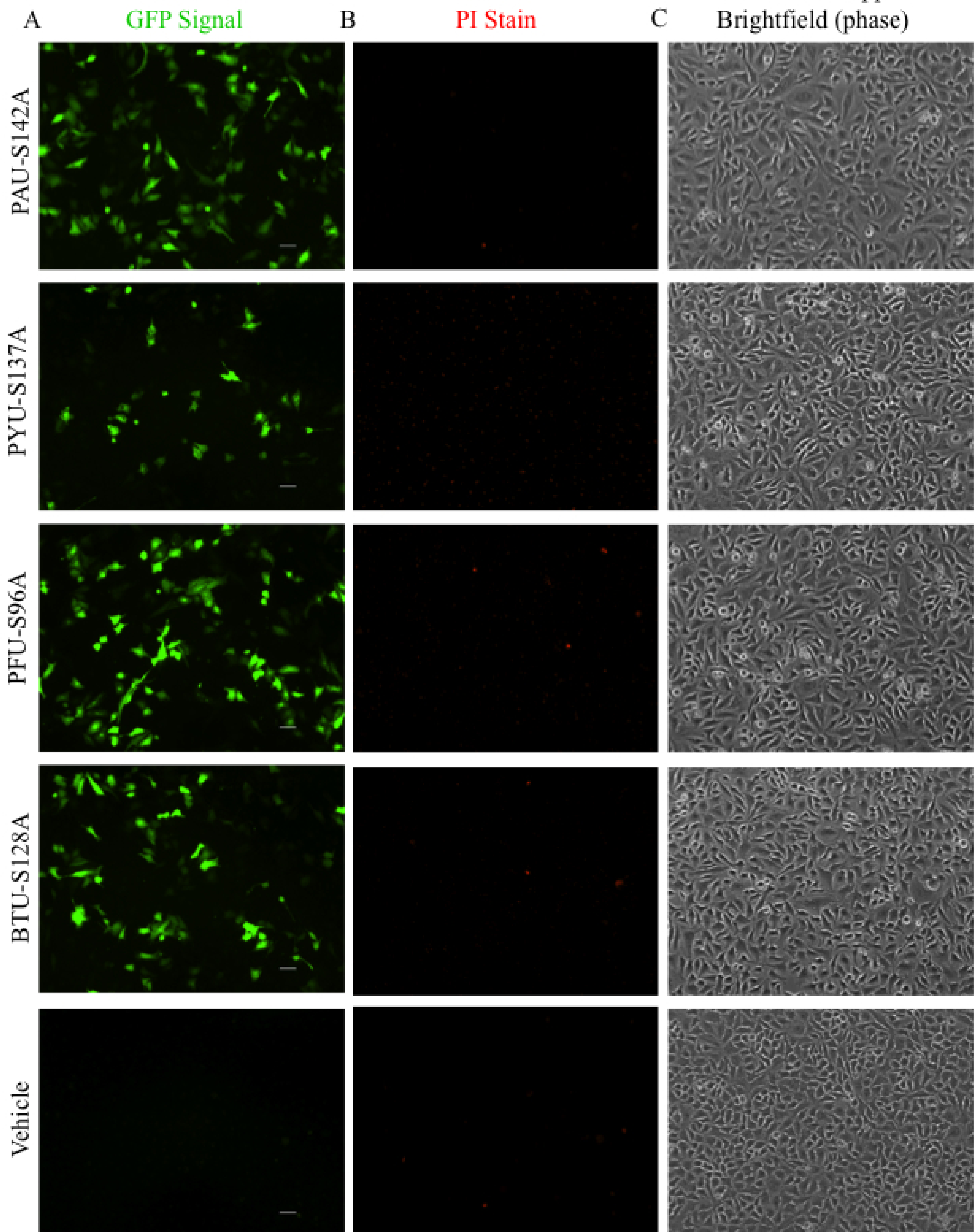
Plasmid	Description	Reference
pET15b	Prokaryotic IPTG inducible expression plasmid with T7 promoter, N-terminal thrombin cleavage site and N-terminal hexahistidine tag; Ampicillin resistance marker	Novagen
pET15b	Prokaryotic expression vector *his-PAU*	Benson et al., 2006
pET15b	Prokaryotic expression vector *his-PFU*	This work
pET15b	Prokaryotic expression vector *his-BTU*	This work
pET15b	Prokaryotic expression vector *his-PAU S142A*	This work
pET15b	Prokaryotic expression vector *his-PFU S96A*	This work
pET15b	Prokaryotic expression vector *his-BTU S128A*	This work
pJN105	Prokaryotic L-arabinose inducible plasmid with pBAD promoter; Gentamycin resistance marker	Newman and Fuqua, 1999
pJN105	Prokaryotic expression vector *his-PAU*	This work
pJN105	Prokaryotic expression vector *his-PAU S142A*	This work
pJN105	Prokaryotic expression vector *his-PYU*	This work
pJN105	Prokaryotic expression vector *his-PYU S137A*	This work
pJN105	Prokaryotic expression vector *his-PFU*	This work
pJN105	Prokaryotic expression vector *his-PFU S96A*	This work
pJN105	Prokaryotic expression vector *his-BTU*	This work
pJN105	Prokaryotic expression vector *his-BTU S128A*	This work
pLysS	Plasmid expressing T7 lysozyme; Chloramphenical resistance marker	This work
pJY2	Plasmid expressing T7 lysozyme and select rare codons	Enzo Life Sciences
pG-KJE8	Plasmid expressing tetracycline-inducible groEL-groES and L-arabinose-inducible dnaK, dnaJ and grpE	Takara Bio Inc.
pCOLADuet-1	Prokaryotic IPTG inducible expression plasmid with T7 promoter and N-terminal hexahistidine tag proceeding 5` expression site followed by a T7 promoter, rbs and second expression site; Kanamycin resistance marker	Novagen
pCOLADuet-1	Prokaryotic expression vector for native human ubiquitin	This work
pET21a	Prokaryotic expression vector for the BirA biotin ligase	Addgene 20857
pET15b-AviTag	Prokaryotic expression vector for the expression of PcrV with a N-terminal AviTag	This work
pET15b-AviTag	Prokaryotic expression vector for the expression of triubiquitin with a C-terminal AviTag	This work
pEGFP-N1	Eukaryotic expression vector with CMV IE promoter and eGFP at the 3` end of an insert; Kanamycin/Neomycin resistance markers	Takara Bio Inc.
pEGFP-N1	Eukaryotic expression vector for *his-PAU-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PYU-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PFU-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-BTU-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PAU S142A-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PYU S137A-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PFU S96A-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-BTU S128A-eGFP*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PAU-2x STOP Codon*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PYU-2x STOP Codon*	This work
pEGFP-N1	Eukaryotic expression vector for *his-PFU-2x STOP Codon*	This work
pEGFP-N1	Eukaryotic expression vector for *his-BTU-2x STOP Codon*	This work



Supplemental Figure 1. Bio-layer interferometry binding analysis of PLA_2 homologs to ubiquitin. A) Association curves of PAU (333, 1000, or 3000nM concentrations) binding to AviTag triubiquitin. Background binding to an AviTag PcrV control protein is subtracted from the above data. B-E) Association curves similar to A measuring specific binding of PYU, BTU, PFU or bovine serum albumin (BSA), respectively. F) Average binding rates from 100-200 seconds after initiation of the association step over the three concentrations tested. P-values are (*, P=0.02) and (****, P<0.0001).

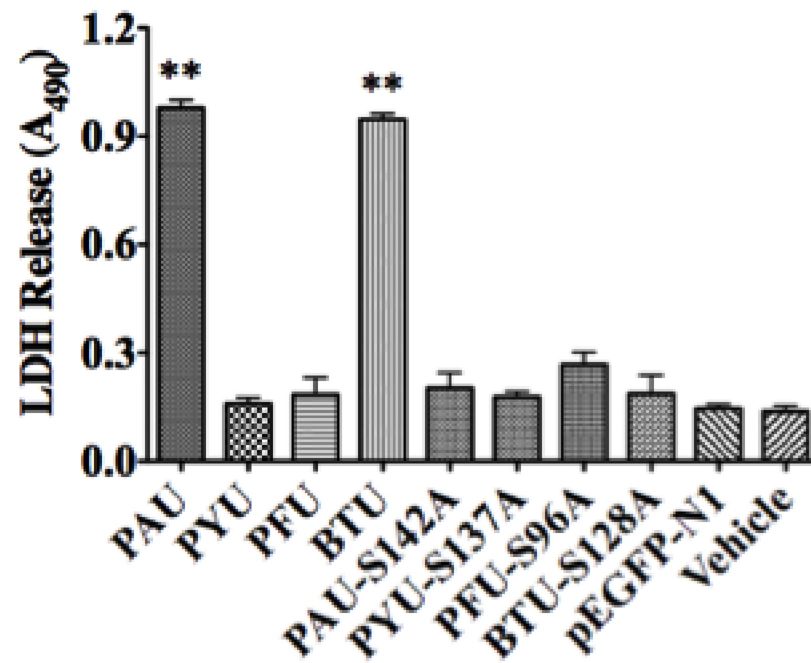


Supplemental Figure 2. Western blot analysis of bacterial lysates expressing either recombinant enzyme or monoubiquitin in strains containing both constructs. A) His-tag signal from lysates of *E. coli* BL21(DE3) pJY2 pJN105 His_6-enzyme pCOLADuet monoubiquitin induced in medium containing 0.5% arabinose to exclusively express the enzyme. IPTG was not included during the induction step. B) His-tag signal from lysates of *E. coli* BL21(DE3) pJY2 pJN105 His_6-enzyme pCOLADuet monoubiquitin induced in medium containing 0.5mM IPTG to exclusively express monoubiquitin. Arabinose was not included during the induction step.



Supplemental Figure 3. Microscopic analysis of HeLa cell cultures transfected with S→A enzyme-eGFP fusions expressed from the CMV promoter. A) Fluorescence signal from eGFP fused to serine-to-alanine catalytic point mutant sequences of each enzyme or an eGPF control at 24 h post transfection. B) Corresponding propridium iodide staining of images shown in A. C) Brightfield images of the corresponding fluorescence images in A. Scale bars represent 50 μm.

A



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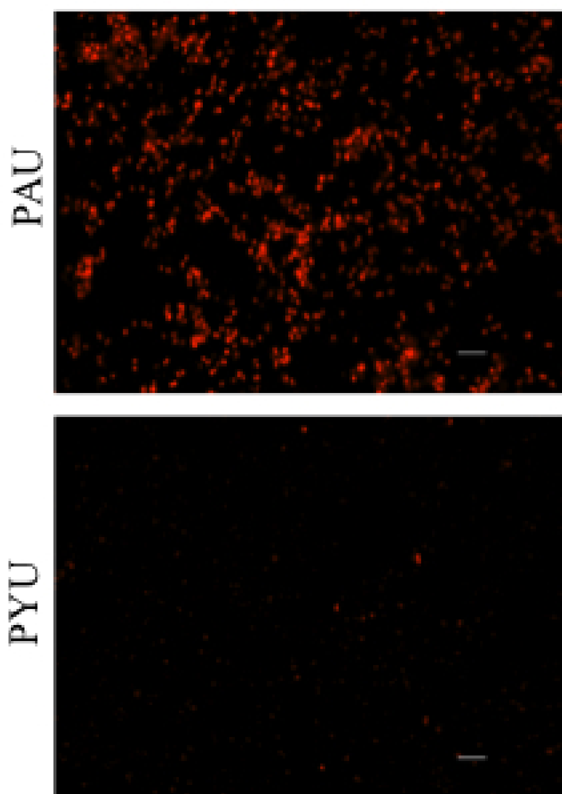

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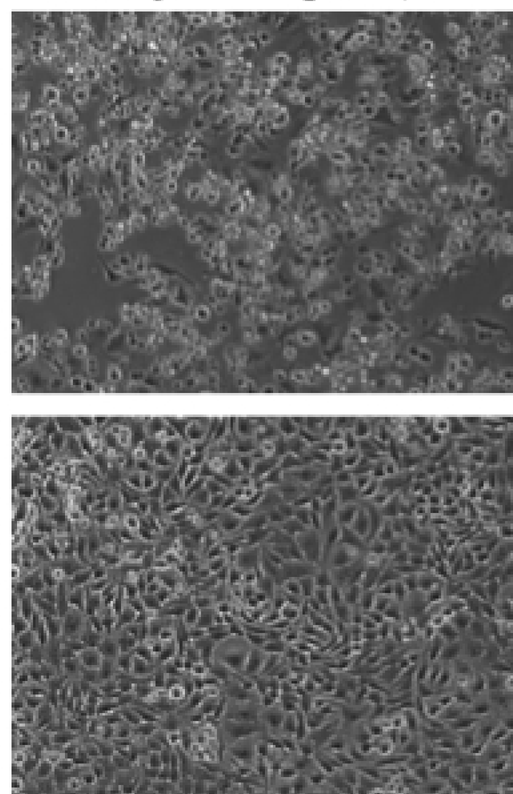

Supplemental Figure 4. Toxicity analysis of non-eGFP tagged ExoU homologs. A) LDH assay as described in the Materials and Methods for non-eGFP tagged versions of each homolog or control at 24 h post transfection. Results are the mean of 4 independent experiments B) Propridium iodide staining of HeLa cells transfected with equal amounts of either PAU or PYU not encoding the eGFP tag. C) Brightfield image of the corresponding image in B. The scale bar represents 50 µm.