Supporting Information

"Dissecting substrate specificities of the mitochondrial AFG3L2 protease"

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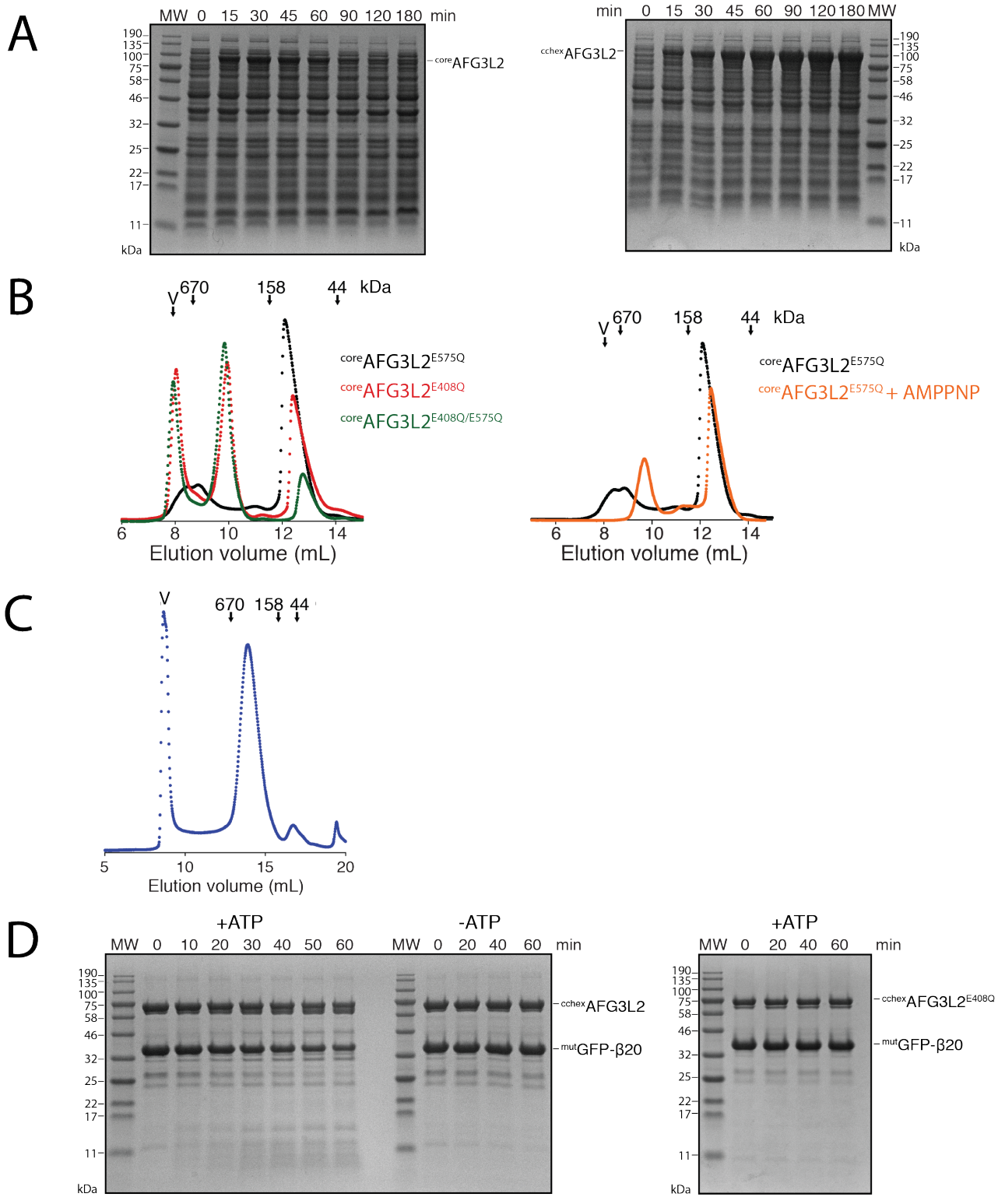

Figure S1. (A) Full uncropped SDS-PAGE gels showing expression of ^{core}AFG3L2 and ^{cchex}AFG3L2. Levels of ^{core}AFG3L2 increase after induction followed by a steady decrease, whereas levels of ^{cchex}AFG3L2 do not decrease after induction. (B) Size exclusion chromatography profiles of ^{core}AFG3L2 constructs. ^{core}AFG3L2^{E408Q} and ^{core}AFG3L2$^{E408Q/E575Q}$ migrate largely as hexamers whereas ^{core}AFG3L2^{E575Q} migrates largely as unassembled protomers. Incubation of ^{core}AFG3L2^{E575Q} with 30 μM AMPPNP shifts the migration profile towards the hexameric species. (C) Size exclusion chromatography profile of ^{cchex}AFG3L2 showing the protein largely migrates as a hexamer (D) Full uncropped representative SDS-PAGE gels from Fig. 1C. showing degradation of ^{mut}GFP-β20 by ^{cchex}AFG3L2 in the presence and absence of ATP and by ^{cchex}AFG3L2^{E408Q} in the presence of ATP.

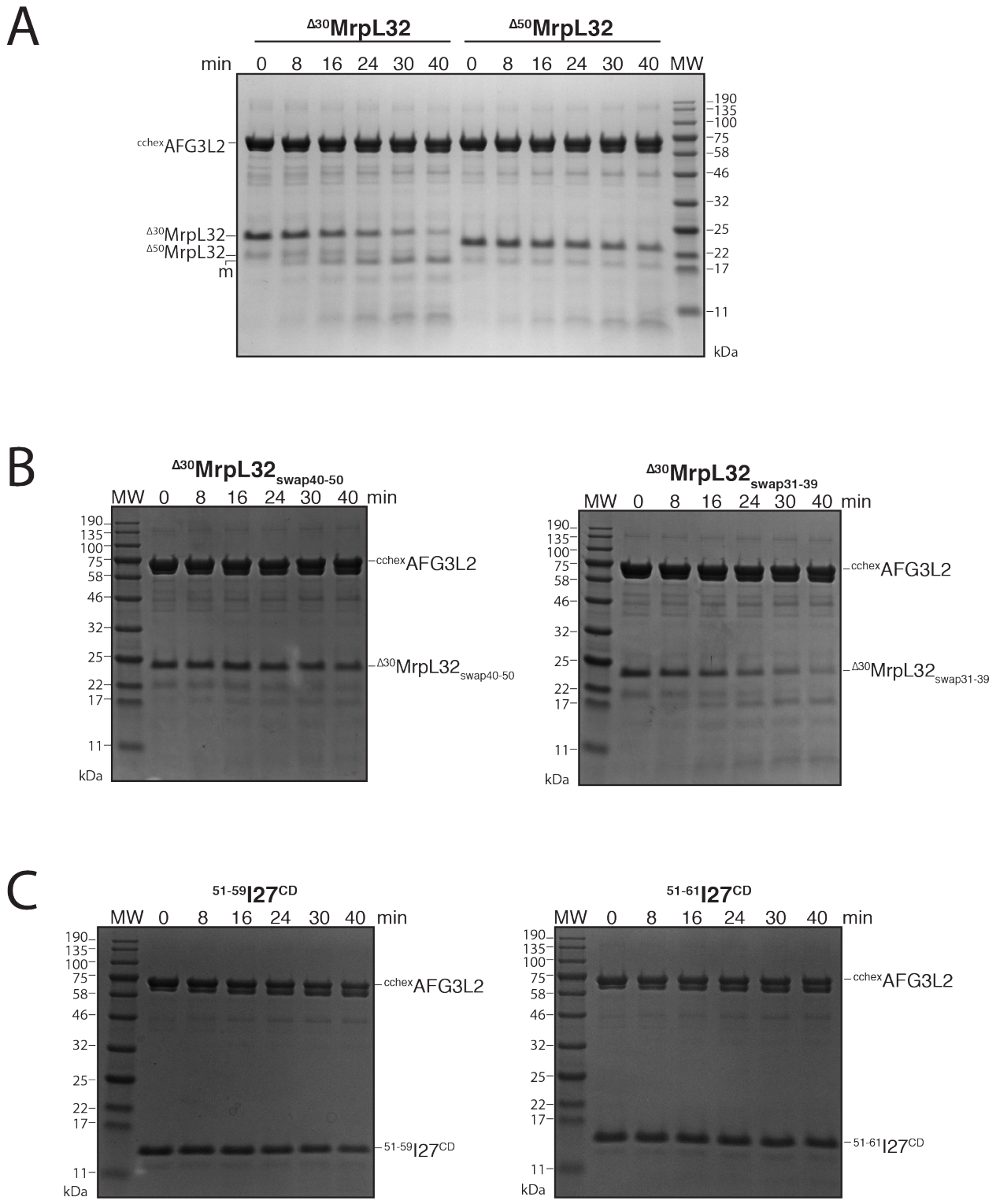

Figure S2. (A) Full uncropped representative SDS-PAGE gel showing proteolysis of $^{\Delta30}$MrpL32 and $^{\Delta50}$MrpL32 by ^{cchex}AFG3L2 in the presence of ATP. Loss of $^{\Delta30}$MrpL32 occurs rapidly and occurs concurrently with accumulation of a smaller molecular weight fragment (m). (B) Full uncropped representative SDS-PAGE gels showing proteolysis of $^{\Delta30}$MrpL32$_{swap40-50}$ and $^{\Delta30}$MrpL32$_{swap31-39}$ by ^{cchex}AFG3L2. (C) Full uncropped representative SDS-PAGE gels showing proteolysis of $^{51-59}$I27^{CD} and $^{51-61}$I27^{CD} by ^{cchex}AFG3L2.

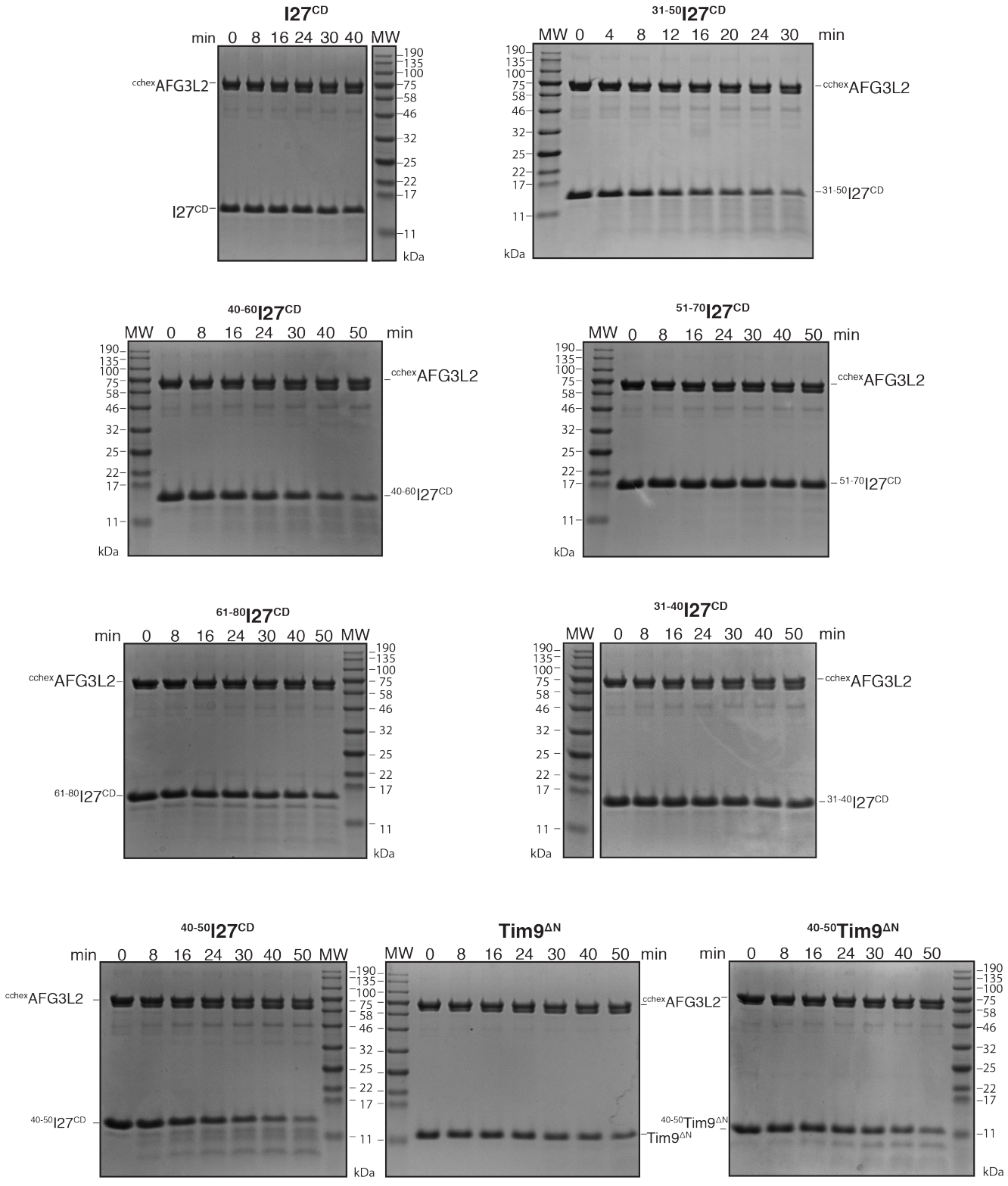

Figure S3. Full uncropped representative SDS-PAGE gels showing degradation of $I27^{CD}$ and $Tim9^{\Delta N}$ constructs bearing sequences derived from MrpL32 at the N-terminus.

A

Substrate	MW (kDa)
^{mut}GFP-β20 (*1*)	33.0
cp7-^{SF}GFP-β20 (*2*)	32.6
I27-β20 (*3*)	14.4
β20-λCI^{N} (*3*)	15.3
Tim10 (*4*)	10.6
Y2853 (*5*)	20.3
HspQ^{Y20} (*5*)	14.9
$^{\Delta 30}$MrpL32	17.9

B

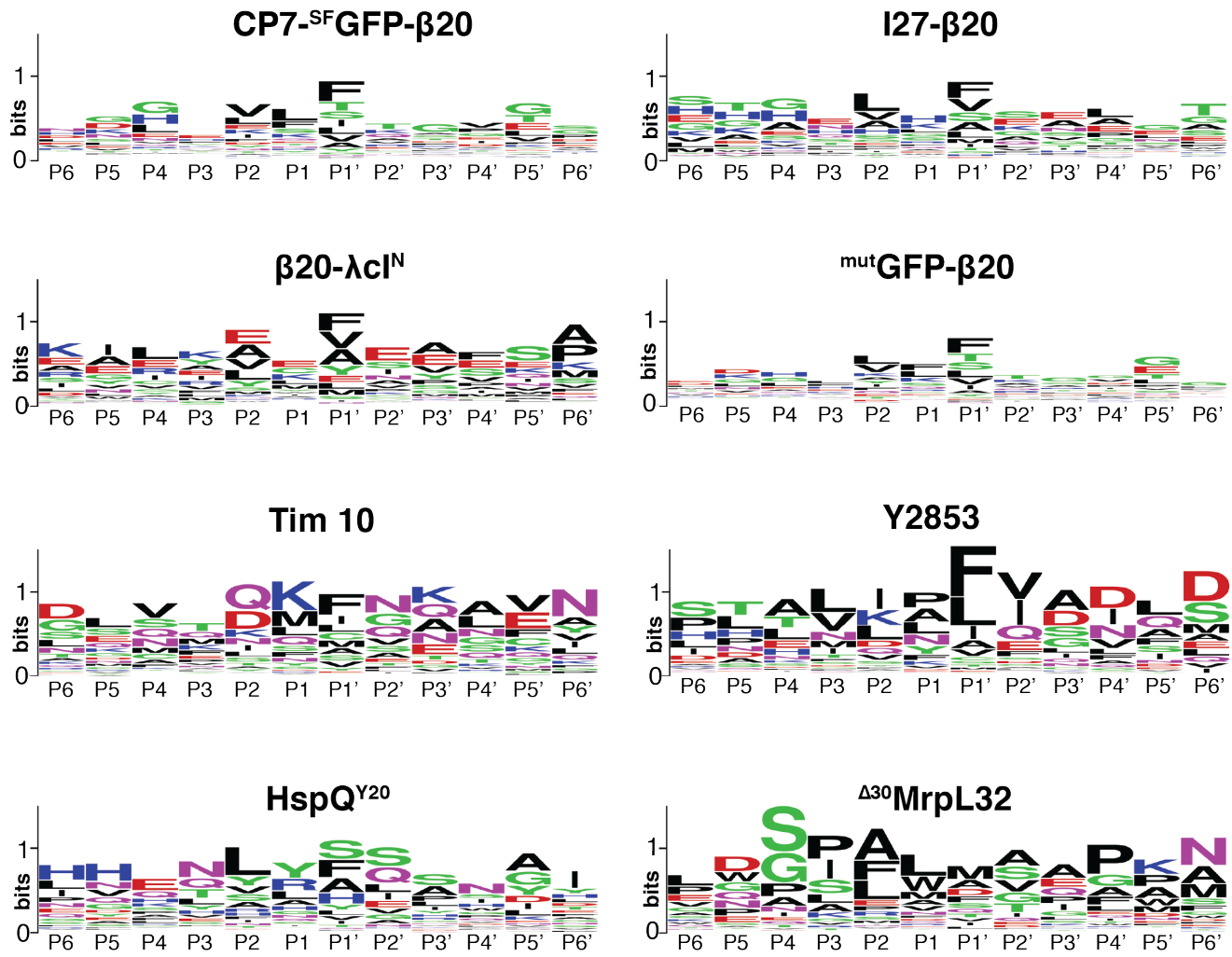

Figure S4. (A) Eight substrates used to generate the AFG3L2 peptidase specificity profile by degradation followed by LC/MS/MS. References for each substrate are given in parantheses. (B) Sequence logos showing individual peptidase specificity profiles from eight protein substrates as determined by LC/MS/MS.

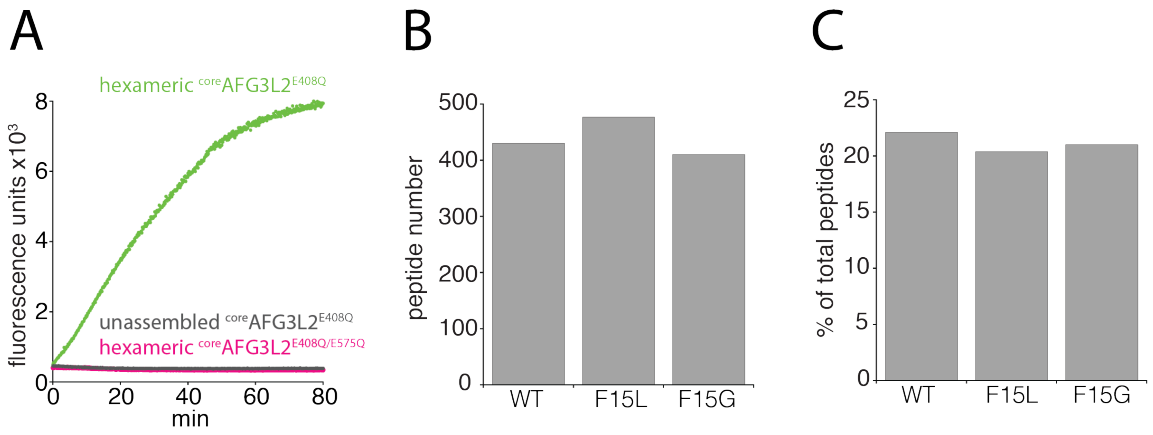

Figure S5. (A) Plot showing rapid cleavage of the reporter peptide Leu-(3-NO2-Tyr)-Phe-Gln-(Lys-Abz) by hexameric $^{core}AFG3L2^{E408Q}$, whereas unassembled coreAFG3L2E408Q does not cleave the peptide comparable to hexameric $^{core}AFG3L2^{E408Q/575Q}$. (B) Plot showing total number of peptides identified by LC/MS/MS resulting from cleavage from $HspQ^{Y20}$ and $HspQ^{Y20}$ variants bearing substitutions of Phe15 to Leu or Gly. (C) Plot showing percentage of total peptides identified by LC/MS/MS resulting from cleavage between residues 65 and 66 of $HspQ^{Y20}$ and $HspQ^{Y20}$ variants bearing substitutions of Phe15 to Leu or Gly.

Protein Substrates	
Substrate	**Initial Degradation Rate (min^{-1} enz_6^{-1})**
$^{\Delta30}$MrpL32	0.79 ± 0.04
$^{\Delta50}$MrpL32	0.38 ± 0.04
$^{\Delta30}$MrpL32$_{swap31-39}$	0.78 ± 0.06
$^{\Delta30}$MrpL32$_{swap40-50}$	0.29 ± 0.02
I27^{CD}	0.14 ± 0.02
$^{31-50}$I27^{CD}	0.76 ± 0.10
$^{40-60}$I27^{CD}	0.43 ± 0.07
$^{51-70}$I27^{CD}	0.18 ± 0.03
$^{61-80}$I27^{CD}	0.32 ± 0.03
$^{31-40}$I27^{CD}	0.24 ± 0.02
$^{40-50}$I27^{CD}	0.65 ± 0.01
$^{51-61}$I27^{CD}	0.18 ± 0.03
$^{51-59}$I27^{CD}	0.33 ± 0.06
Tim9$^{\Delta N}$	0.24 ± 0.04
$^{40-50}$Tim9$^{\Delta N}$	0.41 ± 0.03
Reporter Peptides	
Sequence	**Initial Cleavage Rate (min^{-1} enz_6^{-1})**
LYFQK	0.99 ± 0.05
LYLQK	0.24 ± 0.02
LYSQK	0.098 ± 0.004
LYGQK	- 0.0021 ± 0.002

Table S1. Initial rates for all degradation assays ± s.d.

Supporting Information References

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[2] Wohlever, M. L., Nager, A. R., Baker, T. A., and Sauer, R. T. (2013) Engineering fluorescent protein substrates for the AAA+ Lon protease, *Protein engineering, design & selection : PEDS 26*, 299-305.

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[4] Rampello, A. J., and Glynn, S. E. (2017) Identification of a Degradation Signal Sequence within Substrates of the Mitochondrial i-AAA Protease, *J Mol Biol 429*, 873-885.

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