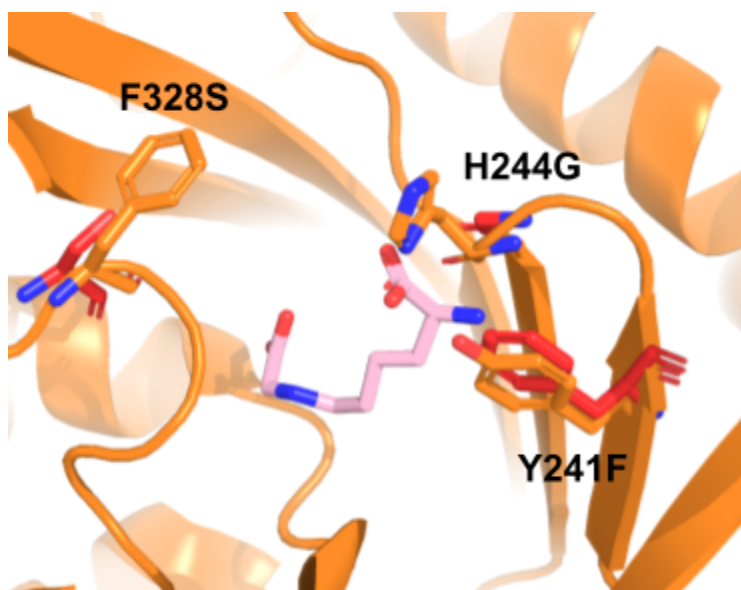


	Glycine		Free CML		AA[CML]AA	
	K_M (mM)	k_{cat} (1/s)	K_M (mM)	k_{cat} (1/s)	K_M (mM)	k_{cat} (1/s)
BsGO	0.73 ± 0.06	0.17 ± 0.002	8.2 ± 3.3	$7.1 \pm 1.7 \times 10^{-2}$	n.d.	n.d.
Bs1GO	0.011 ± 0.001	$4.0 \pm 0.3 \times 10^{-3}$	2.6 ± 0.3	$1.2 \pm 0.1 \times 10^{-2}$	n.d.	n.d.
CrGO	0.36 ± 0.04	$4.6 \pm 0.1 \times 10^{-3}$	1.7 ± 1.3	$1.3 \pm 0.3 \times 10^{-2}$	0.64 ± 0.04	$7.5 \pm 0.2 \times 10^{-4}$

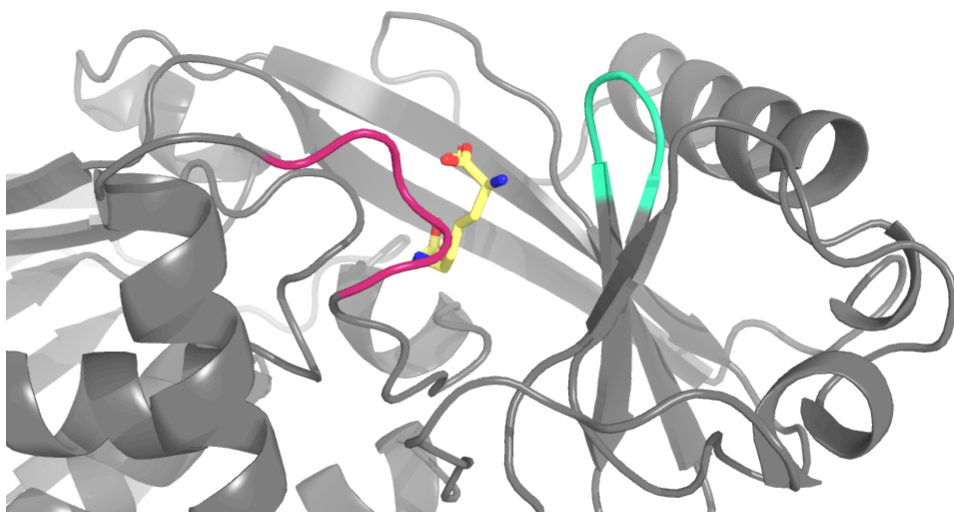
Supplementary Table 1. Comparison of the kinetics of BsGO, Bs1GO, and CrGO. BsGO and Bs1GO have no detectable activity on CML peptide.



Supplementary Figure 1. Key differences in the active sites of BsGO (orange) and Bs1GO (red). CML (pink) is modelled in the active site.

	Mutations (from previous generation)
CrGO-764	A35T, P87S, V129A, R141C, M194I, F195C, G243R
CrGO-785	H49W (A50+E51 Deleted)
CrGO-794	A59G, A196S, Q212K
CrGO-865	L83I, S85T
CrGO-897	H226A, E80Q, H230R

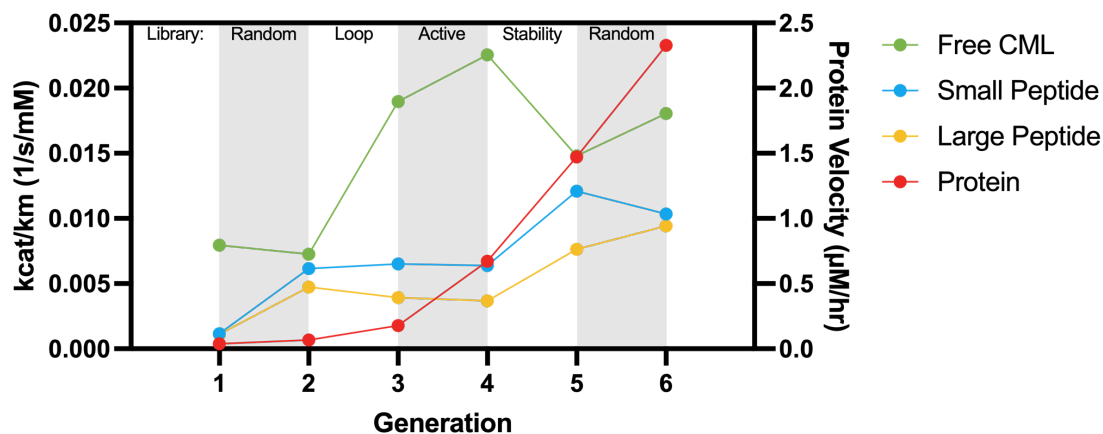
Supplementary Table 2. Summary of mutations across five rounds of engineering.



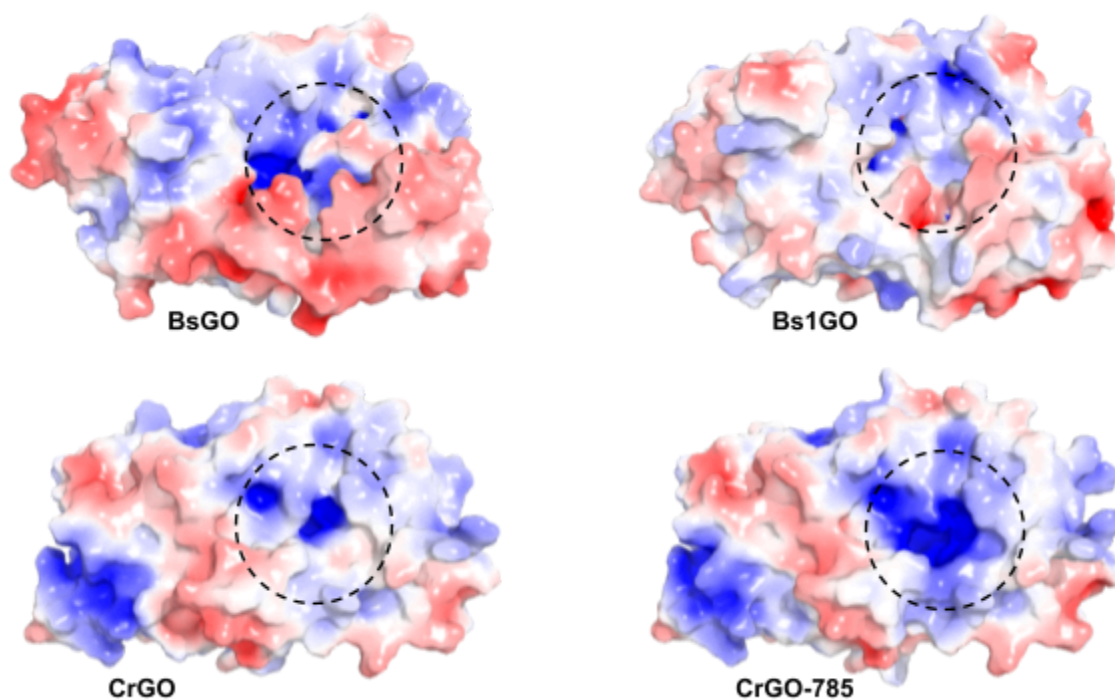
Supplementary Figure 2. Loop 1 (magenta) and loop 2 (cyan) in WT CrGO may obstruct peptides from binding in the active site. Free CML (yellow) is modelled in the active site.

	Glycine		Free CML		AA[CML]AA		AAA[CML]AAA	
	K_m (mM)	k_{cat} (1/s)	K_m (mM)	k_{cat} (1/s)	K_m (mM)	k_{cat} (1/s)	K_m (mM)	k_{cat} (1/s)
WT CrGO	0.36 ± 0.04	$4.6 \pm 0.1 \times 10^{-3}$	1.7 ± 1.3	$1.3 \pm 0.3 \times 10^{-2}$	0.64 ± 0.04	$7.5 \pm 0.2 \times 10^{-4}$	0.44 ± 0.11	$4.3 \pm 0.2 \times 10^{-4}$
CrGO-764	1.5 ± 0.3	$7.3 \pm 0.5 \times 10^{-3}$	3.8 ± 2.5	$2.7 \pm 0.7 \times 10^{-2}$	0.36 ± 0.04	$2.2 \pm 0 \times 10^{-3}$	0.44 ± 0.04	$2.1 \pm 0 \times 10^{-3}$
CrGO-785	3.6 ± 1.9	$4.2 \pm 1 \times 10^{-3}$	0.94 ± 0.15	$1.8 \pm 0.1 \times 10^{-2}$	0.21 ± 0.02	$1.4 \pm 0 \times 10^{-3}$	0.29 ± 0.02	$1.1 \pm 0 \times 10^{-3}$
CrGO-794	14 ± 1	$6.7 \pm 0.7 \times 10^{-3}$	2.3 ± 0.7	$5.1 \pm 0.5 \times 10^{-2}$	0.47 ± 0.09	$3.0 \pm 0.1 \times 10^{-3}$	0.48 ± 0.06	$1.8 \pm 0.1 \times 10^{-3}$
CrGO-865	11 ± 2	$6.9 \pm 0.6 \times 10^{-3}$	2.9 ± 1.0	$4.4 \pm 0.8 \times 10^{-2}$	0.44 ± 0.15	$5.3 \pm 0.4 \times 10^{-3}$	0.20 ± 0.12	$1.5 \pm 0.3 \times 10^{-3}$
CrGO-897	10 ± 8	$7.4 \pm 2.2 \times 10^{-3}$	2.2 ± 1.0	$4.0 \pm 0.6 \times 10^{-2}$	0.51 ± 0.10	$5.3 \pm 0.4 \times 10^{-3}$	0.14 ± 0.07	$1.3 \pm 0.2 \times 10^{-3}$

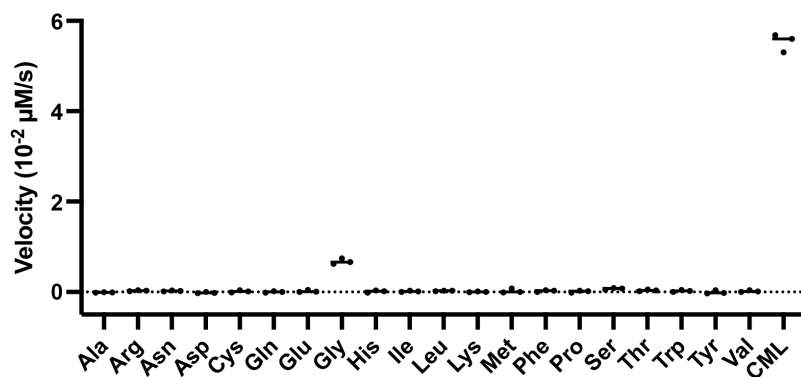
Supplementary Table 3. Summary of engineering improvements.



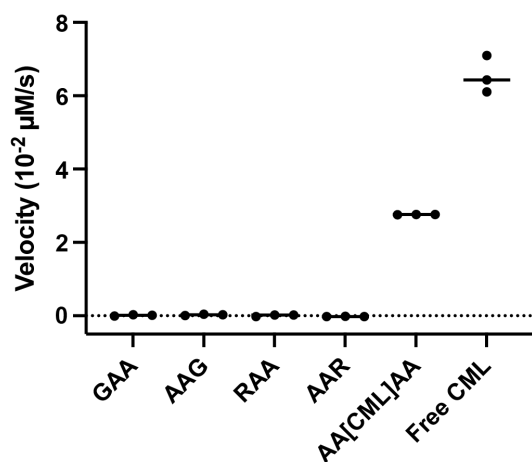
Supplementary Figure 3. Summary of changes in activity on a variety of substrates. Free CML and CML peptide activity is measured in catalytic efficiency (left axis) and protein activity is measured in initial velocity on CML-BSA (right axis). Small peptide is AA[CML]AA; large peptide is AAA[CML]AAA.



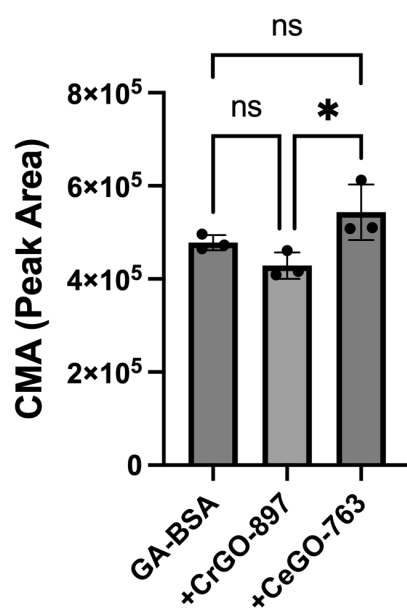
Supplementary Figure 4. Surface charge map of various enzymes. Red indicates a negative charge and blue indicates a positive charge. The active site opening is circled in black. CrGO greatly reduces the steric interference present in BsGO and Bs1GO. CrGO-785 further opens the positively charged active site.



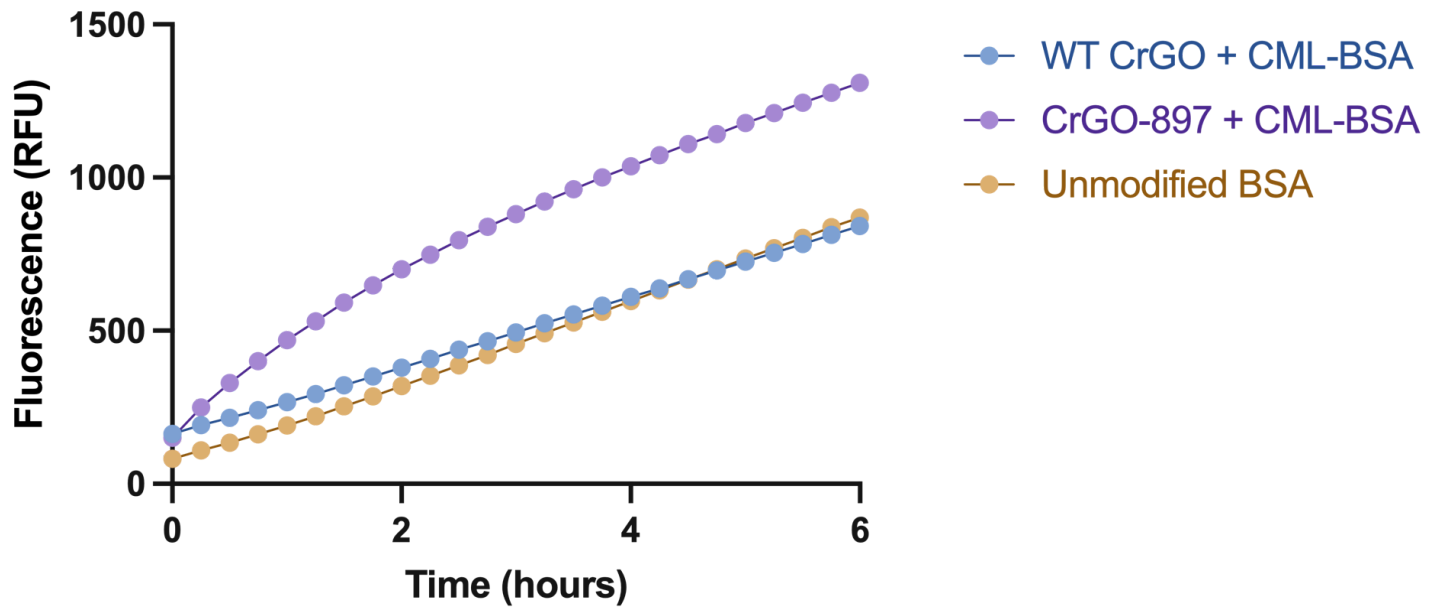
Supplementary Figure 5. CrGO-897 has no detectable activity on free amino acids other than glycine. CrGO-897 (0.5 μM) was incubated with the indicated substrate at 1 mM for 20 min.



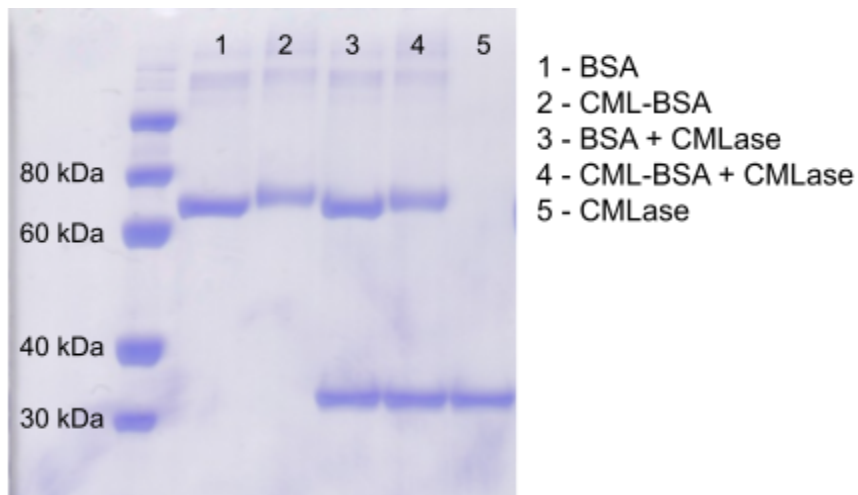
Supplementary Figure 6. CrGO-897 has no detectable activity on terminal glycine or arginine tripeptides. CrGO-897 (0.5 μM) was incubated with the indicated substrate at 1 mM for 20 min.



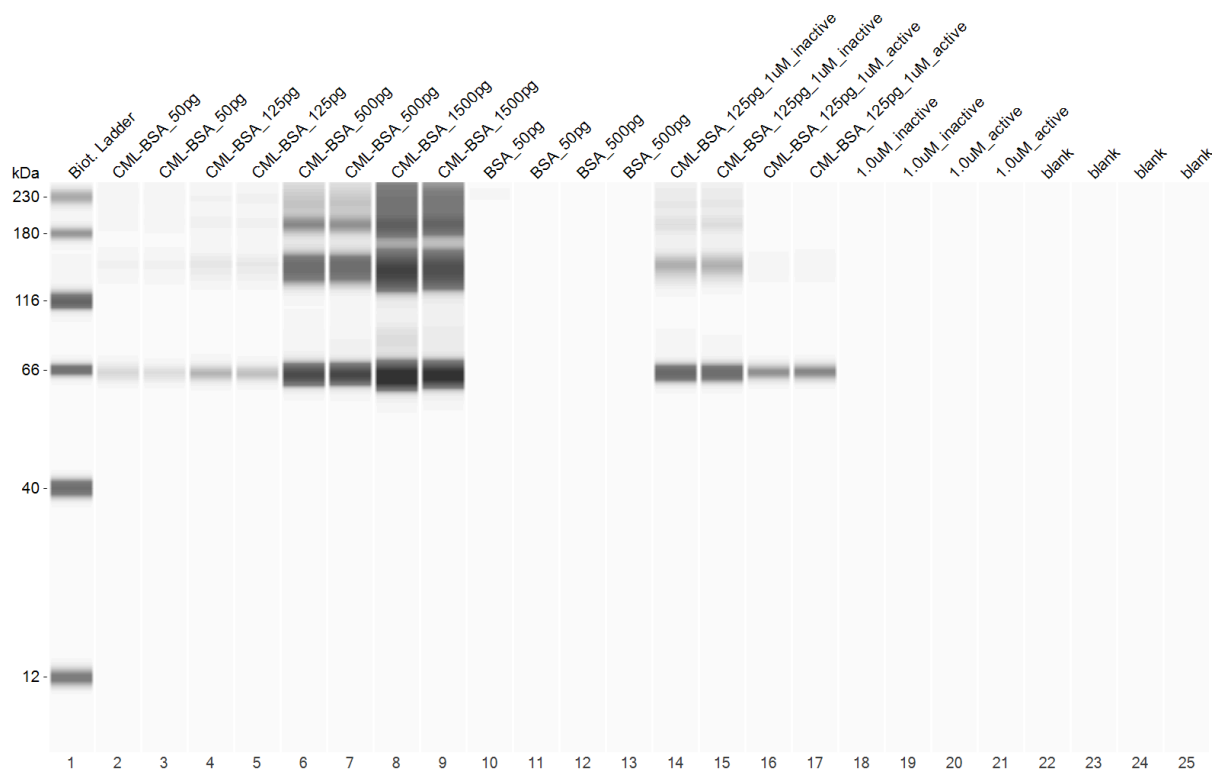
Supplementary Figure 7. CrGO-897 has no detectable activity on carboxymethyl arginine (CMA). Activity on CMA modified BSA as accessed by LC-MS/MS following enzymatic hydrolysis as described in the methods.



Supplementary Figure 8. CML-BSA with WT CrGO and unmodified BSA show no activity in the continuous assay. The AmplexRed substrate shows high levels of background fluorescence due to the prolonged time frame of the assay. This background was removed for subsequent data processing.



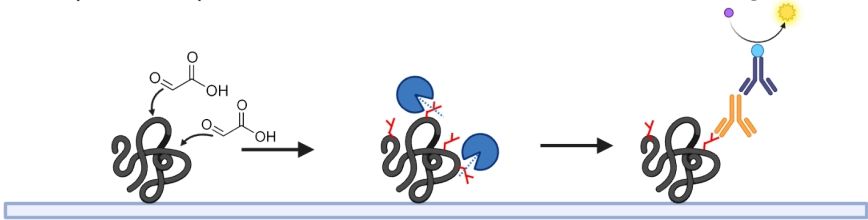
Supplementary Figure 9. Overnight incubation of CML-BSA and CMLase shows no degradation of protein substrates. SDS-PAGE gel shows overnight products at identical conditions to reactions processed by LC-MS.



Supplementary Figure 10. Anti-CML capillary-based Western confirms enzymatic removal of CML from CML-BSA by CMLase. Reactions were analyzed by automated capillary electrophoresis (Jess Simple Western, Bio-Techne) using an anti-CML primary antibody (Abcam, ab27684) and HRP-conjugated secondary antibody. Lane 1, biotinylated molecular weight ladder; lanes 2–9, CML-BSA standard curve at 50, 125, 500, and 1500 pg loaded in duplicate; lanes 10–13, unmodified BSA at 50 and 500 pg loaded in duplicate, confirming antibody specificity for the CML adduct; lanes 14–15, CML-BSA (125 pg loaded) treated overnight with 1 μ M catalytically inactive control enzyme (CeGO-763); lanes 16–17, CML-BSA (125 pg loaded) treated overnight with 1 μ M active CMLase (CrGO-897); lanes 18–19, 1 μ M inactive enzyme alone; lanes 20–21, 1 μ M active enzyme alone; lanes 22–25, blank. The CML-BSA standard curve was included to confirm linearity of detection across the assay's dynamic range; direct quantitative comparison between standard curve and treatment lanes is not intended, as the two sample sets were prepared and diluted via independent routes prior to loading. The controlled comparison is between active CMLase- and inactive-enzyme-treated samples, which were processed in parallel under identical conditions. Anti-CML signal is detected predominantly at the expected ~66 kDa molecular weight corresponding to the BSA monomer, with additional higher-molecular-weight bands at ~120 and ~180 kDa consistent with glycation-induced BSA dimers and higher-order aggregates characteristic of CML-BSA preparations. Active CMLase treatment produces a reproducible reduction in 66 kDa signal and loss of the higher-molecular-weight species relative to inactive-enzyme-treated controls, indicating enzymatic removal of CML rather than steric masking or proteolytic degradation of the substrate. Reactions were performed in duplicate.

CML Detection Assay

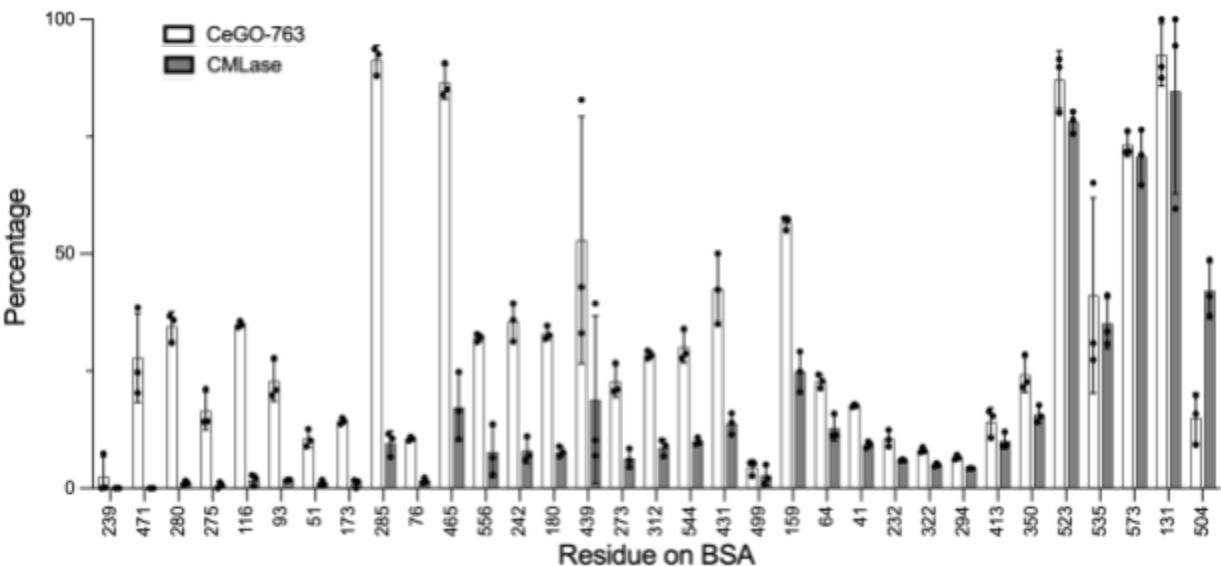
- 1 Chemically modify
plate bound protein
- 2 Enzymatically repair
modifications
- 3 Detect remaining
CML damage



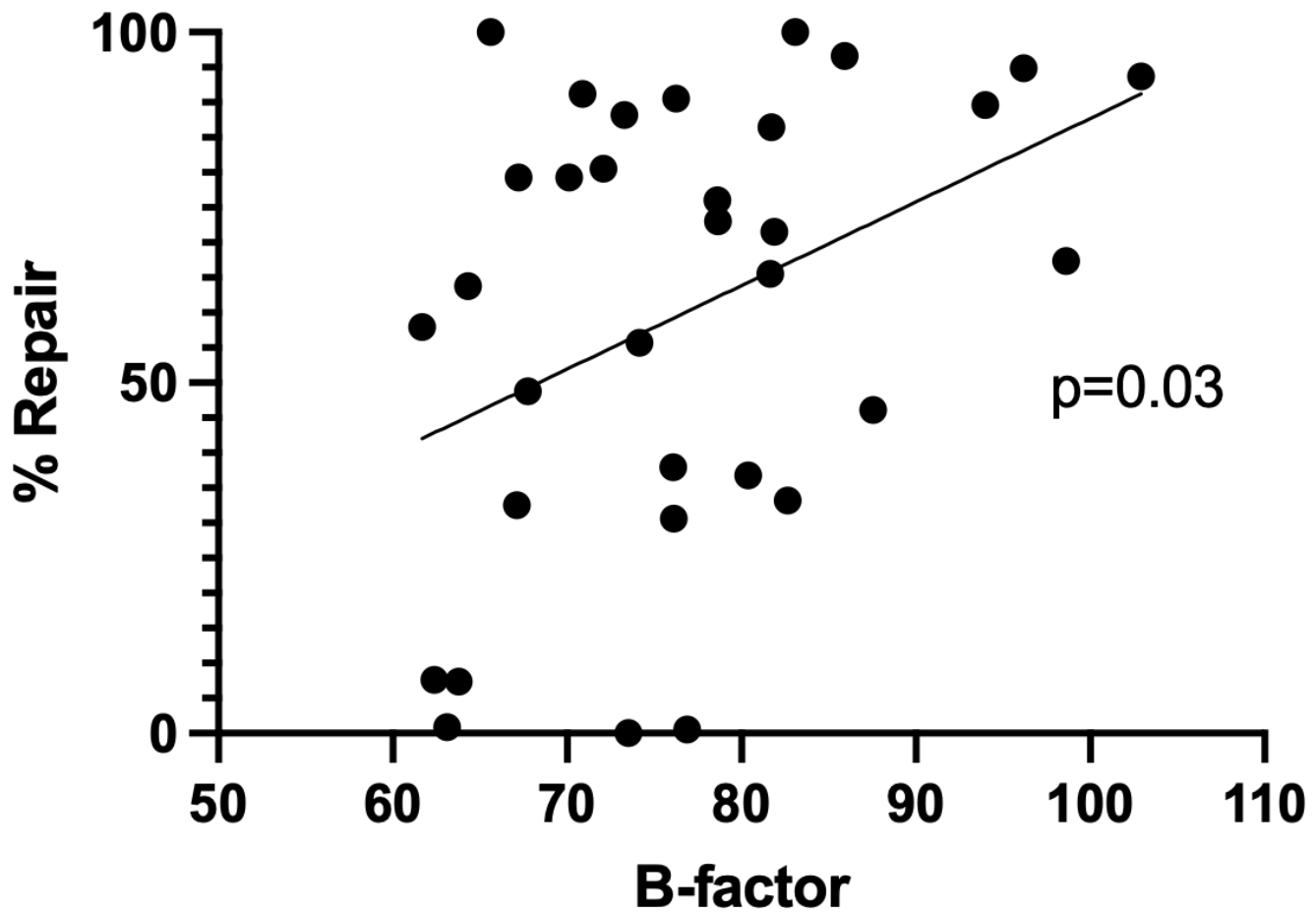
Supplementary Figure 11. ELISA protocol schematic.

BSA		Casein		Collagen		Eye TPE		Hemoglobin	
Modification Level (A370)	Repair (%)	Modification Level (A370)	Repair (%)	Modification Level (A370)	Repair (%)	Modification Level (A370)	Repair (%)	Modification Level (A370)	Repair (%)
0.44 ± 0.02	37 ± 10	0.85 ± 0.04	92 ± 1	1.12 ± 0.02	60 ± 5	1.3 ± 0.06	88 ± 1	1.46 ± 0.04	82 ± 2
0.21 ± 0.03	53 ± 2	0.48 ± 0.02	97 ± 6	0.61 ± 0.03	65 ± 5	0.73 ± 0.01	90 ± 2	0.66 ± 0.04	87 ± 6
0.03 ± 0.004	47 ± 17	0.07 ± 0.02	134 ± 61	0.12 ± 0.03	80 ± 6	0.13 ± 0.01	91 ± 5	0.25 ± 0.01	89 ± 7

Supplementary Table 4. CML repair (%) measured by ELISA on different levels of modification. Various model proteins demonstrate different levels of modification when following the protocol described in methods. Levels of repair vary from 36-100% across all conditions.



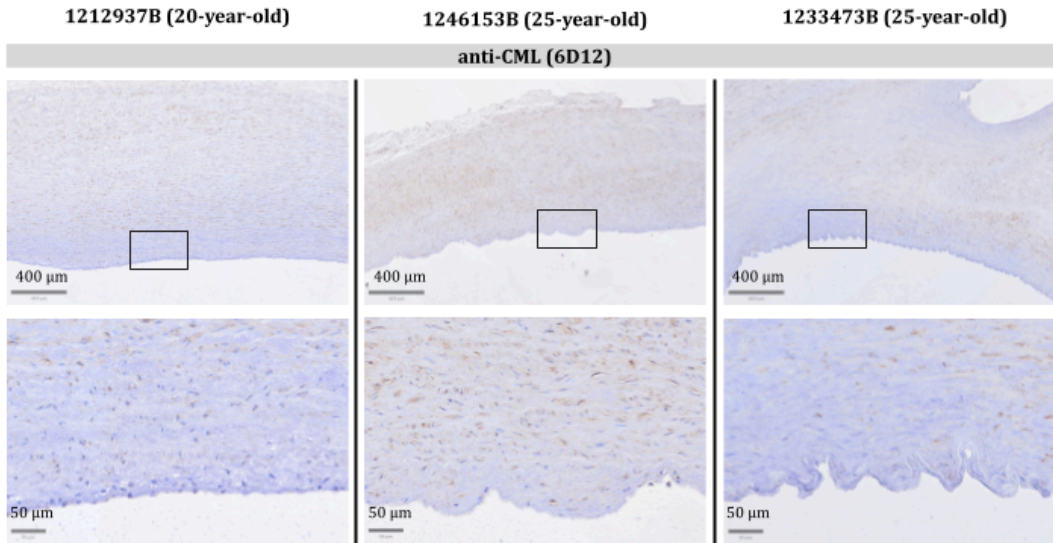
Supplementary Figure 12. CML modification percentages at individual sites, ranked by decreasing repair efficiency as determined by LC-MS/MS proteomics.



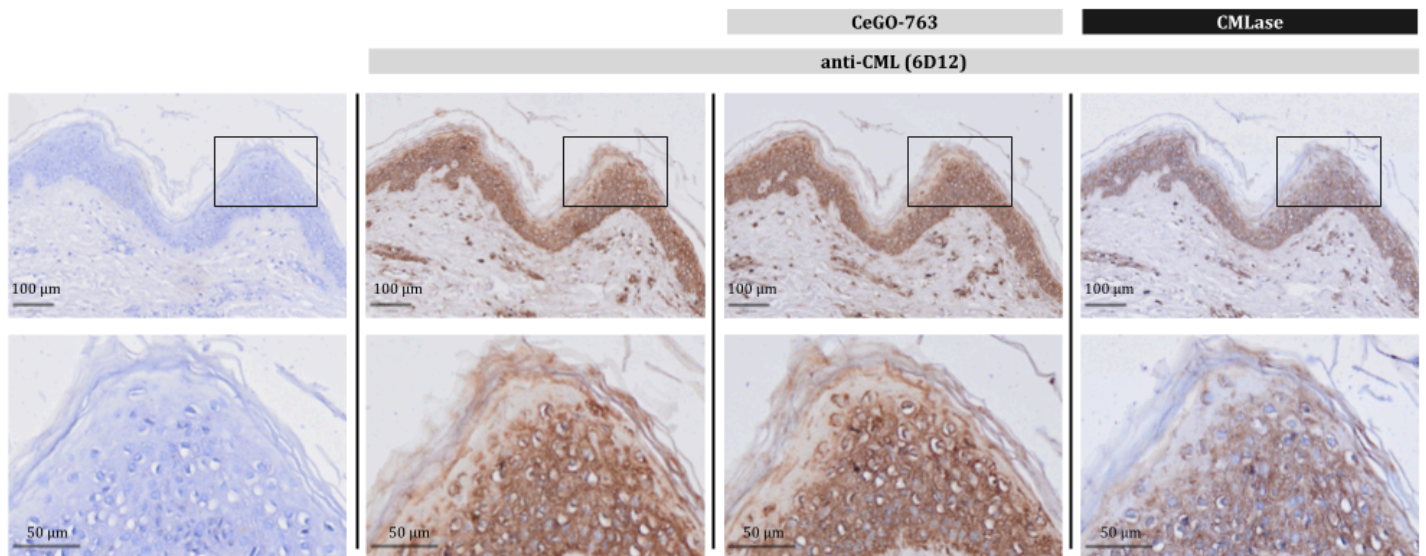
Supplementary Figure 13. Percent repair is correlated with b-factor (PDB 4F5S). Spearman nonparametric correlation two tailed p-value of 0.034.



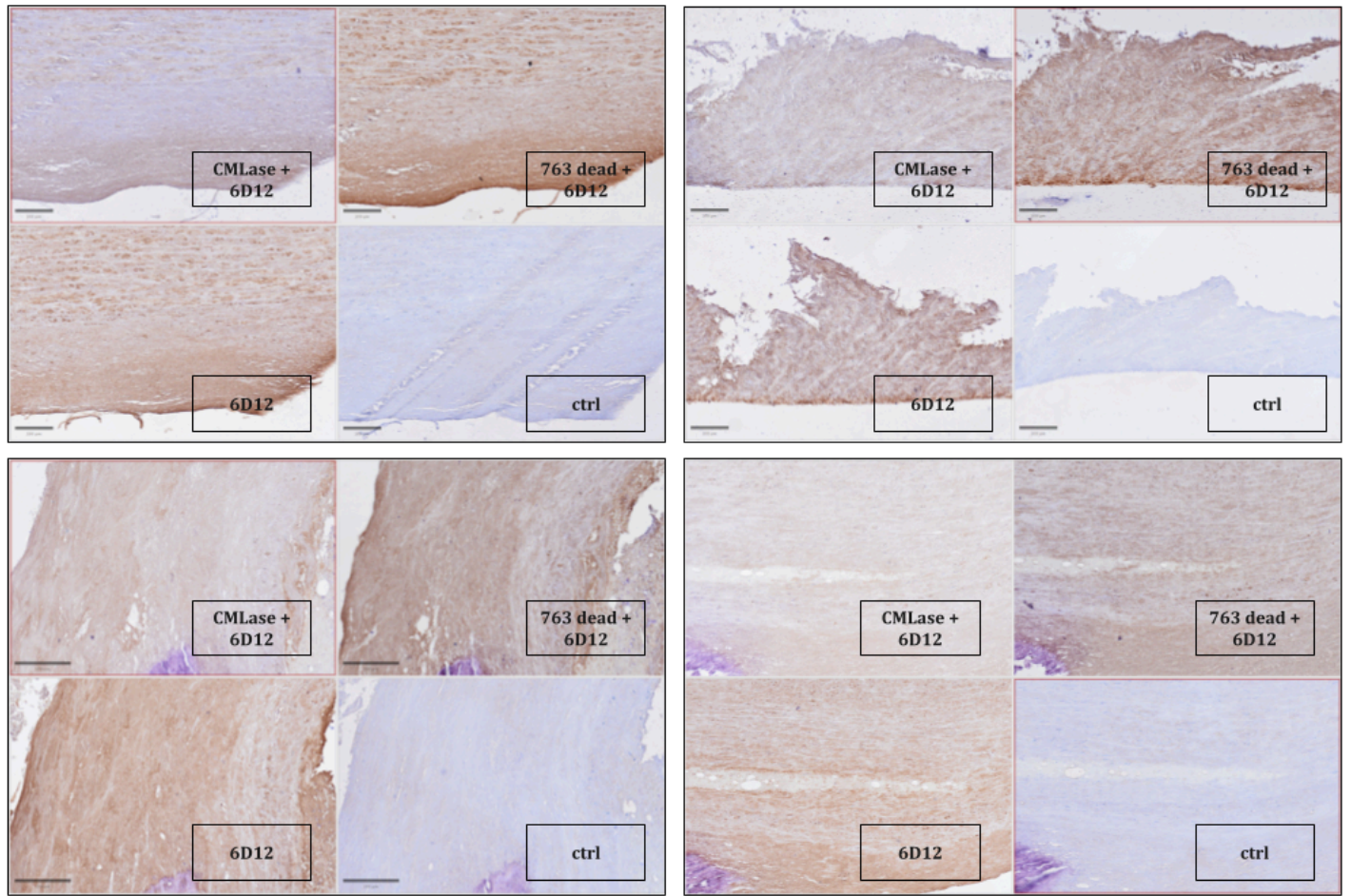
Supplementary Figure 14. Sequence logos showing the local environment of CML-K sites on BSA. Sequence logos represent the local amino acid environment (5 residues) surrounding (CML-K) sites, identified across three stratified cohorts: Left panel with all CML-K sites (n=33) detected on BSA; Middle panel with Top 10 sites exhibiting the largest CML removal; and right panel with Bottom 10 sites exhibiting the least CML removal. The height of each amino acid letter represents its relative probability at that specific position. Amino acids are color-coded by chemical properties.



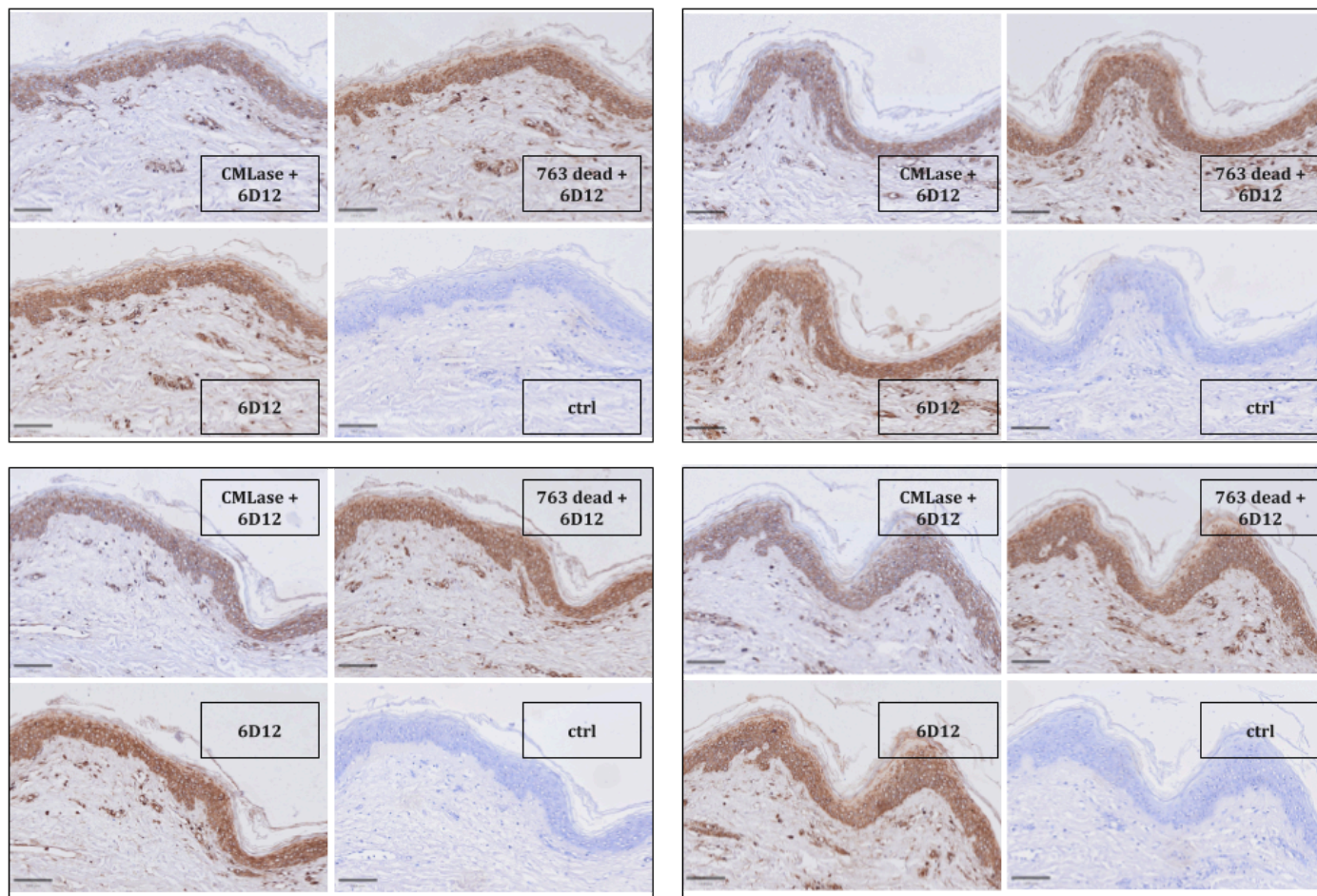
Supplementary Figure 15. Arterial tissue from young donors does not stain for CML. Sections of abdominal aorta from young donors stained with H&E and by immunohistochemistry (IHC) using anti-CML antibody 6D12.



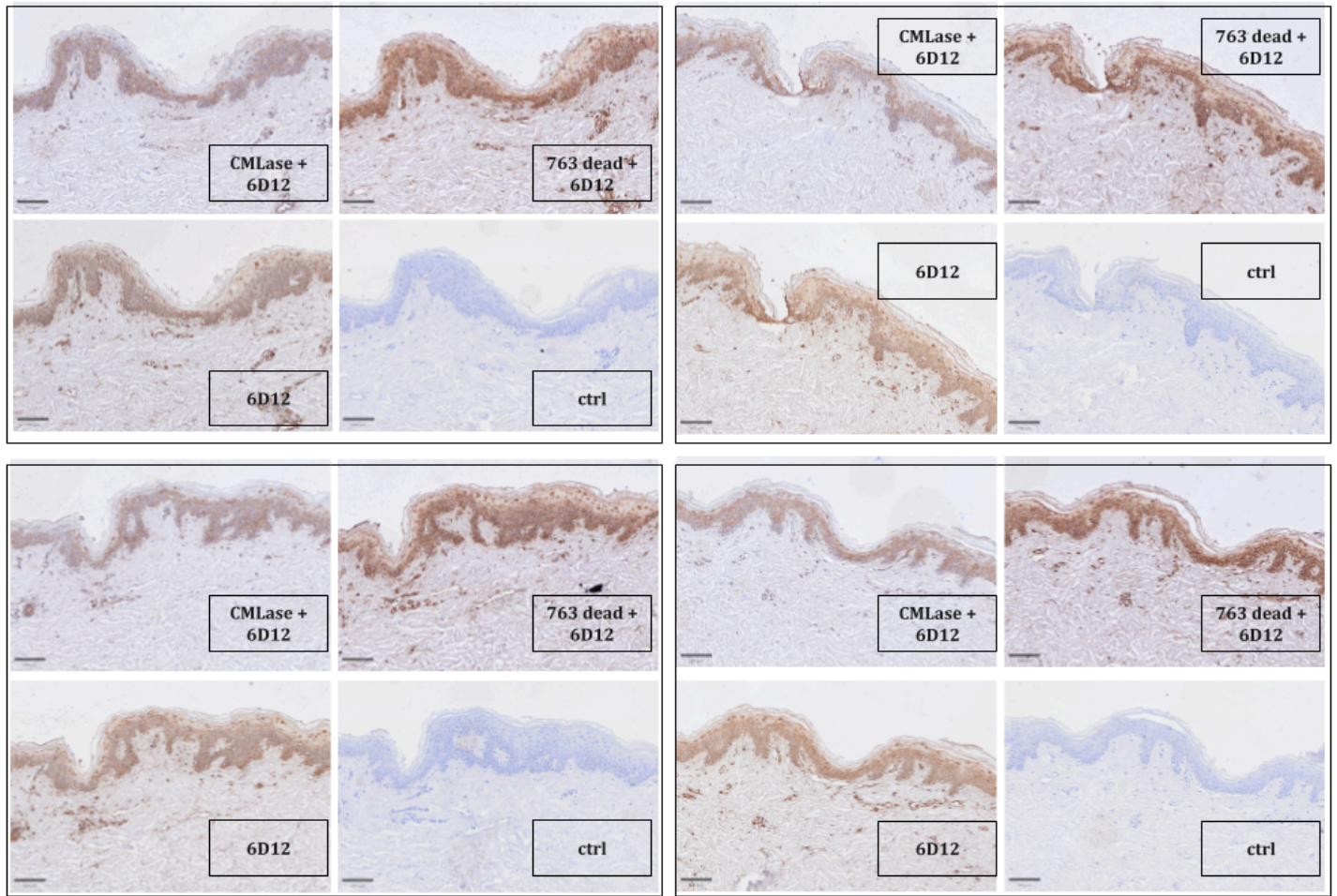
Supplementary Figure 16. CMLase treatment reduces CML staining in human skin. Consecutive sections of abdominal skin biopsies from a 74 year old human donor incubated overnight with no enzyme (panels 1-2), with an inactivated control (panel 3) or CMLase (panel 4). Following overnight incubation, sections were washed and stained with H&E and by immunohistochemistry (IHC) using anti-CML antibody 6D12 (panels 2-4).



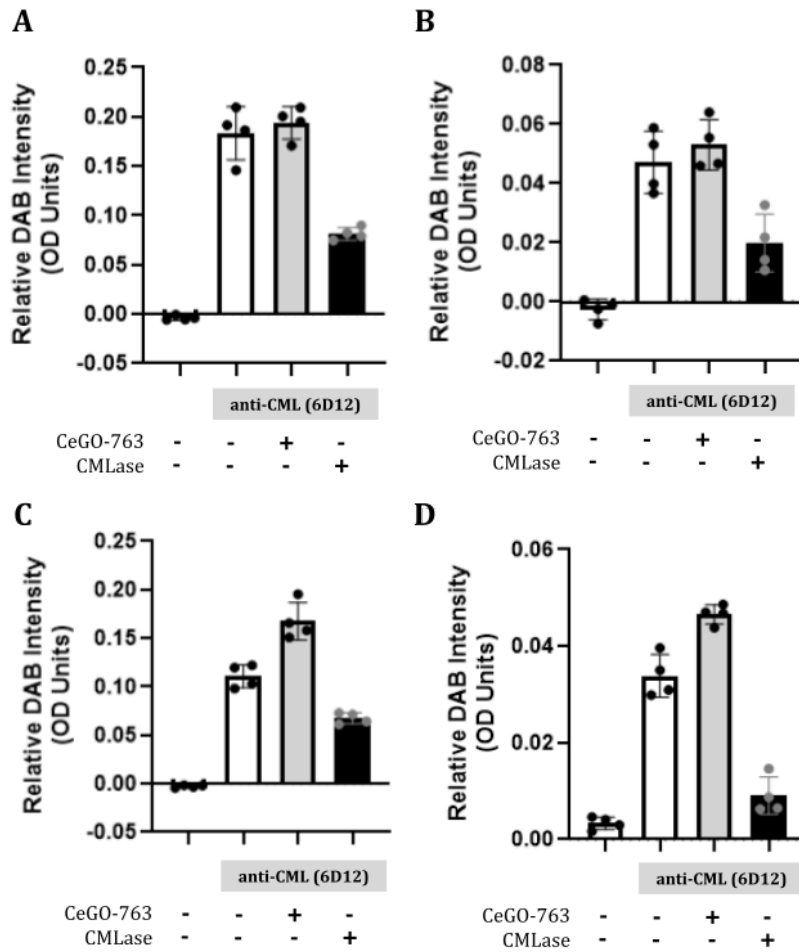
Supplementary Figure 17. Arterial tissue sections from 75-year-old donor used for DAB quantification in Fig. 4E. Full size histology image sections were processed into smaller images (4 frames shown above) for quantitative analysis of DAB staining. Local features of consecutive tissue sections were aligned between enzyme treated and control images. DAB intensity quantified as described in methods. All images are at the same resolution with the scale bar set at 100 μ m.



Supplementary Figure 18. Skin tissue sections from 74 year-old-donor used for DAB quantification in Supplementary Fig. 20. Full size histology image sections were processed into smaller images (4 frames shown above) for quantitative analysis of DAB staining. Local features of consecutive tissue sections were aligned between enzyme treated and control images. DAB intensity quantified as described in methods. All images are at the same resolution with the scale bar set at 100 μ m.



Supplementary Figure 19. Skin tissue sections from 31 year-old-donor used for DAB quantification in Supplementary Fig. 20. Full size histology image sections were processed into smaller images (4 frames shown above) for quantitative analysis of DAB staining. Local features of consecutive tissue sections were aligned between enzyme treated and control images. DAB intensity quantified as described in methods. All images are at the same resolution with the scale bar set at 100 μm .



Supplementary Figure 20. CMLase reverses CML modifications in human abdominal skin biopsies. Relative DAB Intensity was calculated as optical density (OD) from DAB-stained regions (n=4 regions) using color deconvolution in ImageJ. OD values reflect DAB intensity with higher values indicating stronger staining. (A) Epidermis from 74-year-old. (B) Dermis from 74-year-old. (C) Epidermis from 31-year-old. (D) Dermis from 31-year-old.