

A cyclic peptide toolkit reveals mechanistic principles of peptidylarginine deiminase IV regulation

M. Teresa Bertran ^{a#}, Robert Walmsley ^{b#}, Thomas Cummings ^b, Iker Valle Aramburu ^c, Donald J. Benton ^d, Rocio Mora Molina ^b, Jayalini Assalaarachchi ^b, Maria Chasampalioti ^b, Tessa Swanton ^c, Dhira Joshi ^e, Stefania Federico ^e, Hanneke Okkenhaug ^f, Lu Yu ^g, David Oxley ^g, Simon Walker ^f, Venizelos Papayannopoulos ^c, Hiroaki Suga ^h, Maria A. Christophorou ^{b*}, Louise J. Walport ^{a,h,i*}

^a Protein-Protein Interaction Laboratory, The Francis Crick Institute, London, NW1 1AT, UK

^b Epigenetics, The Babraham Institute, Cambridge, CB22 3AT, UK

^c Antimicrobial Defense Laboratory, The Francis Crick Institute, London, NW1 1AT, UK

^d Structural Biology, The Francis Crick Institute, London, NW1 1AT, UK

^e Chemical Biology, The Francis Crick Institute, London, NW1 1AT, UK

^f Imaging, The Babraham Institute, Cambridge, CB22 3AT, UK

^g Proteomics, The Babraham Institute, Cambridge, CB22 3AT, UK

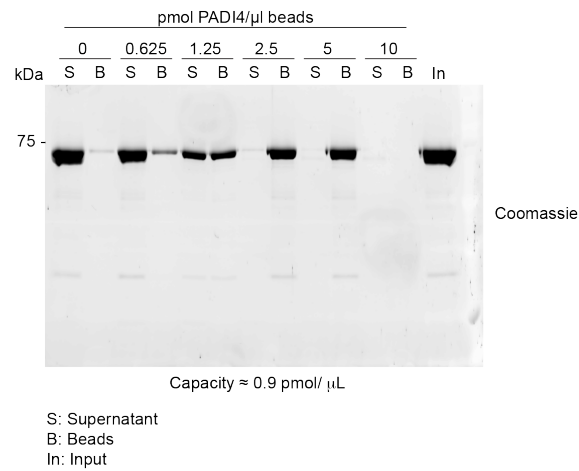
^h The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan

ⁱ Imperial College London, Department of Chemistry, London, W12 0BZ, UK

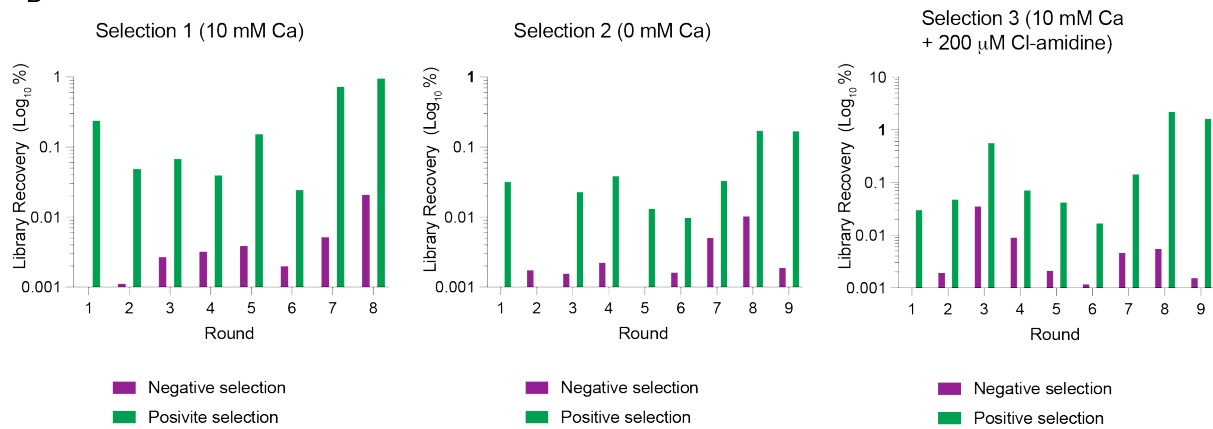
#These authors contributed equally to this study

*To whom correspondence should be addressed: l.walport@imperial.ac.uk or maria.christophorou@babraham.ac.uk

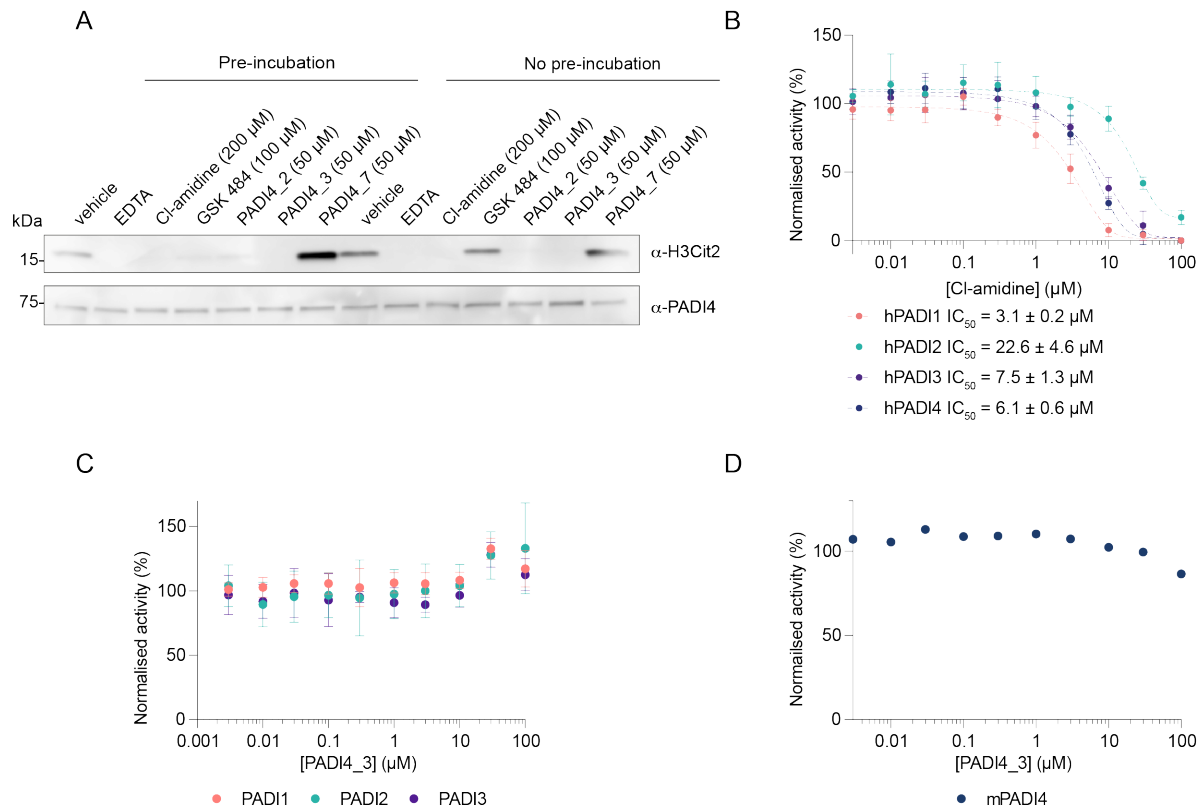
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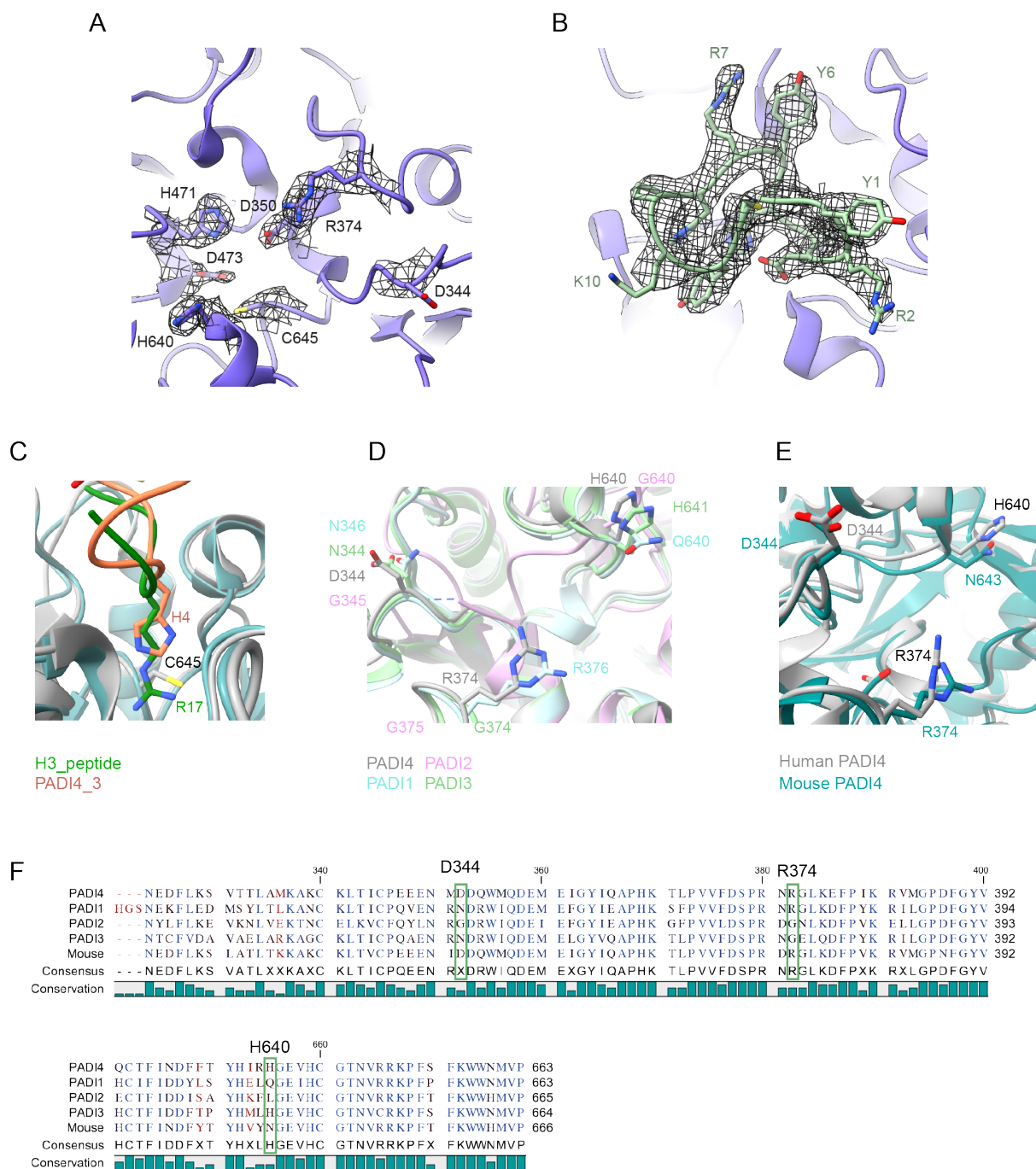
B



Supplementary Figure 1. RaPID selections with hPADI4. A. Determination of Avi-His-PADI4 loading level on streptavidin beads. **B.** RaPID selection recoveries. Violet bars and green bars represent the percentage of the input RaPID library recovered after affinity panning against streptavidin beads or streptavidin beads coated with biotinylated PADI4 respectively.

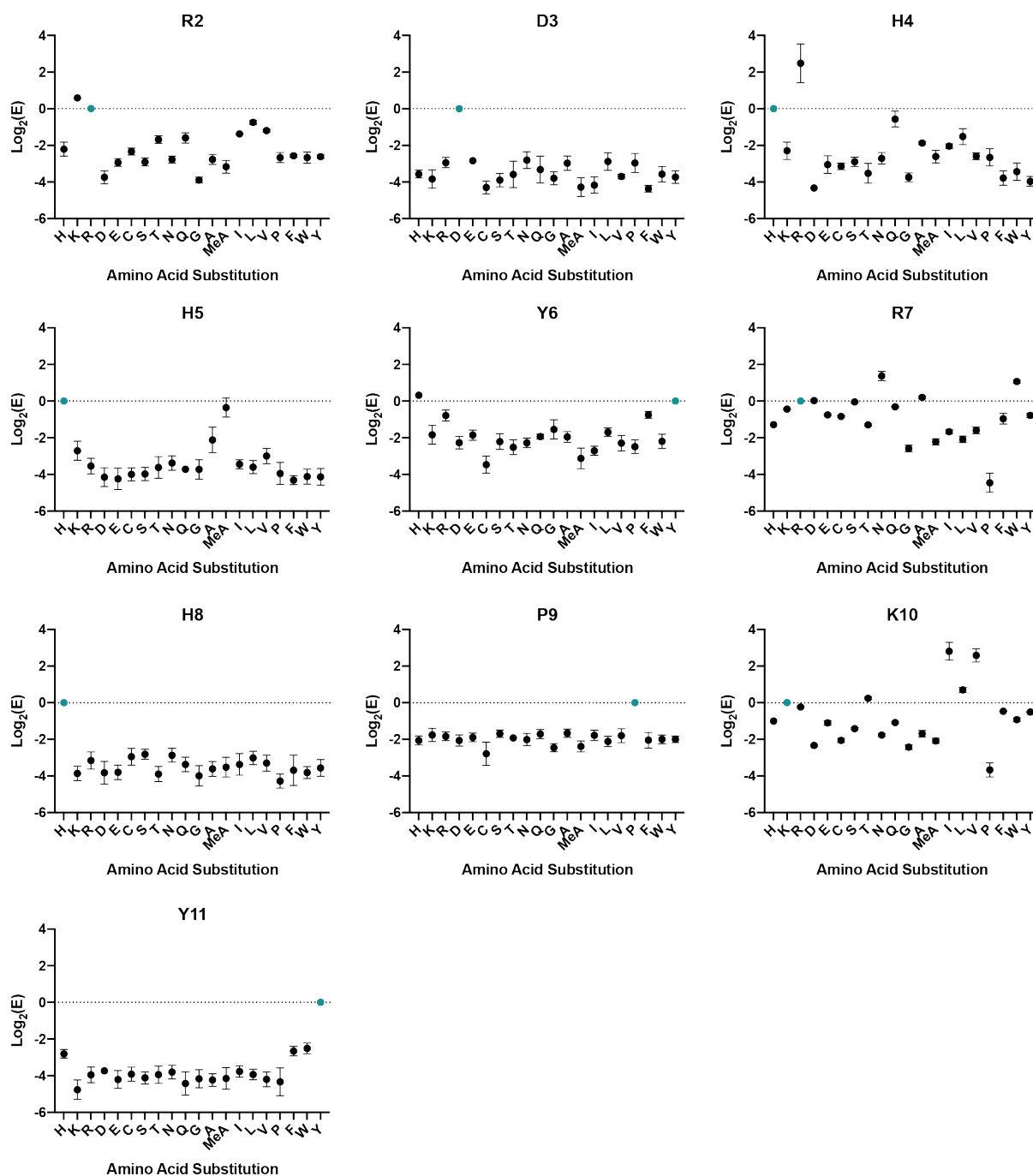


Supplementary Figure 2. PADI4_3 is a potent and selective hPADI4 inhibitor. A. Immunoblot analysis for citrullinated R2 on histone H3 (H3Cit2), as a measure of PADI4 activity, in hPADI4-stable mES cells. Cell extracts were incubated with indicated PADI inhibitors either 20 mins prior (pre-incubation) or concurrent with the addition of calcium (no pre-incubation). Cellular PADI4 serves as a loading control. **B.** Inhibition of hPADIs by Cl-amidine. COLDER assays of PADI family members with different concentrations of Cl-amidine and 10 mM $CaCl_2$. Data is normalised to activity of each PADI in the presence of 0.1% DMSO vehicle. Data shows mean $\pm$ SEM of three independent replicates. Each independent replicate was performed in triplicate. **C.** PADI4_3 does not inhibit PADI1, PADI2 or PADI3. COLDER assays with PADI family members with different concentrations of PADI4_3 and 10 mM $CaCl_2$. Data is normalised to activity of each PADI in the presence of 0.1% DMSO vehicle. Data shows mean $\pm$ SEM of three independent replicates. Each independent replicate was performed in triplicate. **D.** COLDER assay of mPADI4 with different concentrations of PADI4_3 and 10 mM $CaCl_2$. Data shows mean $\pm$ SEM of three independent replicates. Each independent replicate was performed in triplicate.

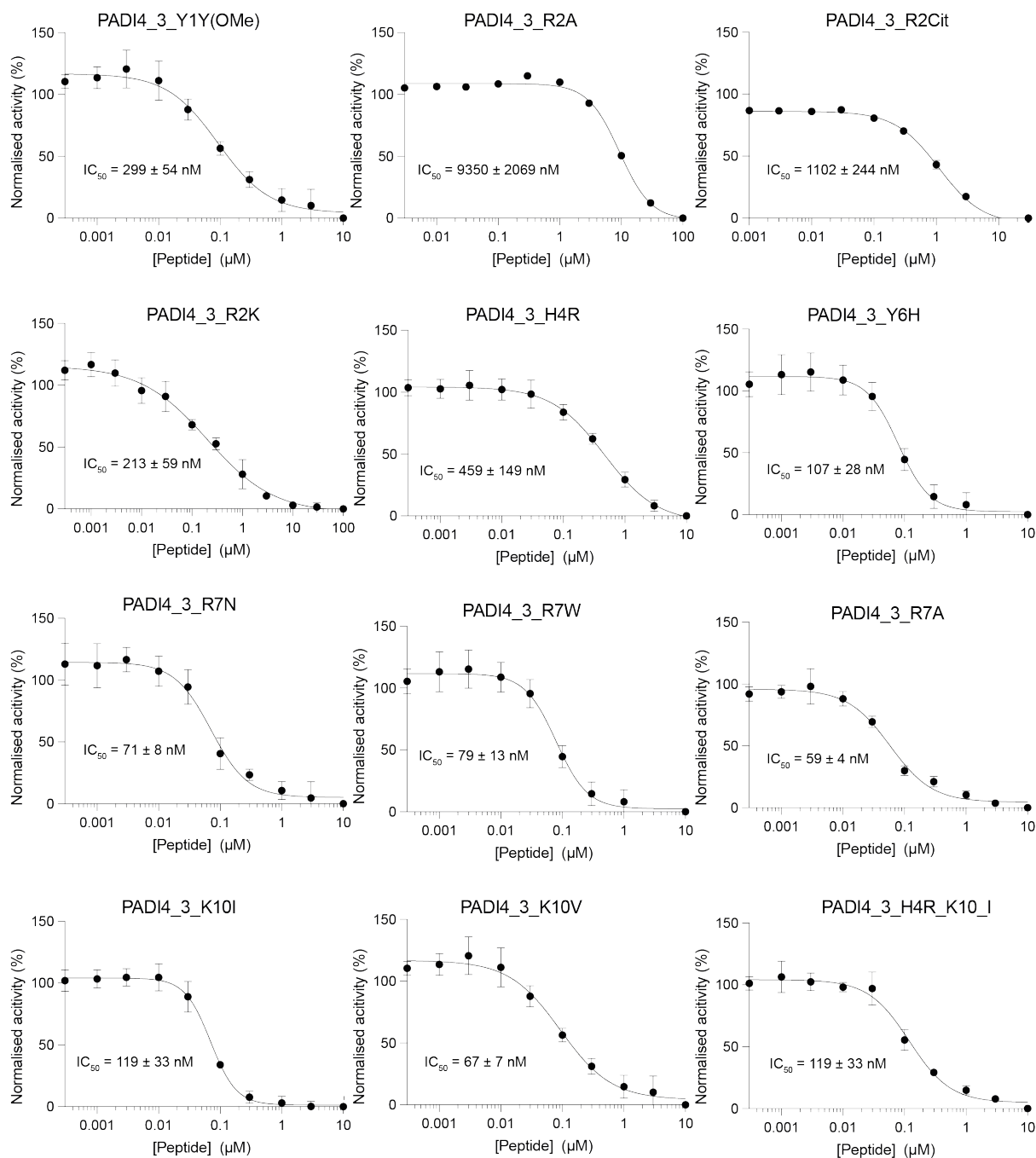


Supplementary Figure 3. PADI4_3 binds to the active site of PADI4 and is selective for human PADI4. **A.** PADI4 map showing electron density of significant amino acids involved in PADI4_3 binding. **B.** Electron density of PADI4_3. **C.** Alignment of cryoEM structure of PADI4 bound to PADI4_3 with X-ray crystal structure of PADI4 bound to an H3 peptide with R17 occupying the active site of PADI4 (PDB: 2DEX). PADI4_3 H4 binds in the same pocket as R17. **D.** Alignment of human PADI4 (grey) with other human PADI isozymes. PADI1: AlphaFold model (cyan), PADI2 PDB: 4N2B (pink), PADI3: AlphaFold model (green). Side chains of residues important for interactions with PADI4_3 are shown as sticks. **E.** Alignment

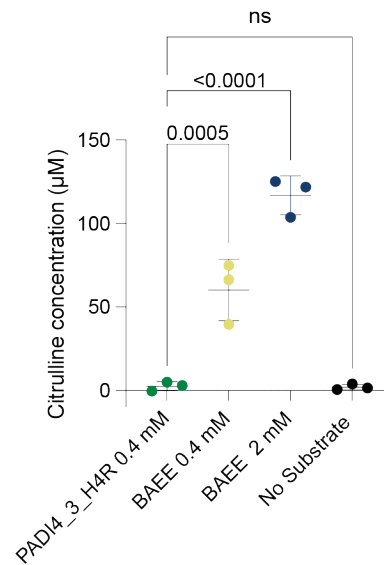
of human PADI4 (grey) with its mouse ortholog (teal) (mouse PADI4: AlphaFold model). Side chains of residues important for interactions with PADI4_3 are shown as sticks. **F.** Sequence alignment of human PADI4 with other human PADI enzymes and mouse PADI4. Residues highlighted in green boxes are involved in the binding of PADI4_3 peptide and are not conserved.



Supplementary Figure 4. Deep mutational scanning of PADI4_3. $\text{Log}_2(E)$ scores are plotted for each amino acid substitution, for every position varied in the PADI4_3 sequence. Data are plotted as the mean $\pm$ standard deviation of three independent single round selections against immobilised PADI4.

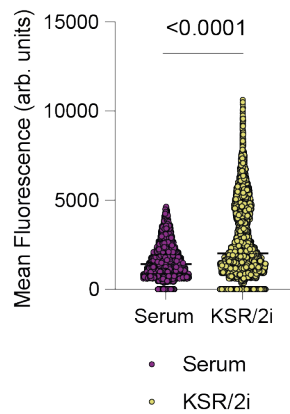


Supplementary Figure 5. Inhibition of hPADI4 by PADI4_3 analogues. hPADI4 inhibition by different concentrations of peptides (10-0.0003 μM) and 10 mM CaCl₂ measured by COLDER assay. Data is normalised to activity of PADI4 in the presence of 0.1% DMSO vehicle. Data shows mean ± SEM of three independent replicates. Each independent replicate was performed in triplicate.

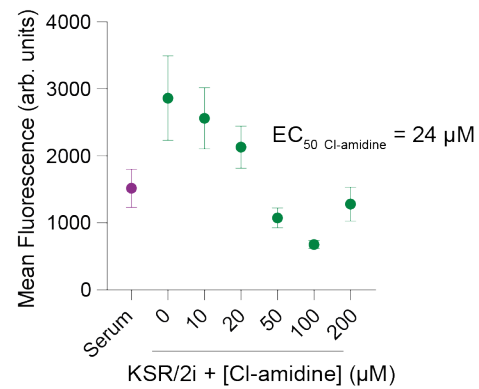


Supplementary Figure 6. PADI4_3_H4R is not a PADI4 substrate. COLDER assay performed with 0.4 mM PADI4_3_H4R, BAEE was used as positive control. Data represents mean $\pm$ SEM of three independent replicates. Each replicate was done in triplicate. A two-way ANOVA was carried out to determine if the differences among means were significant.

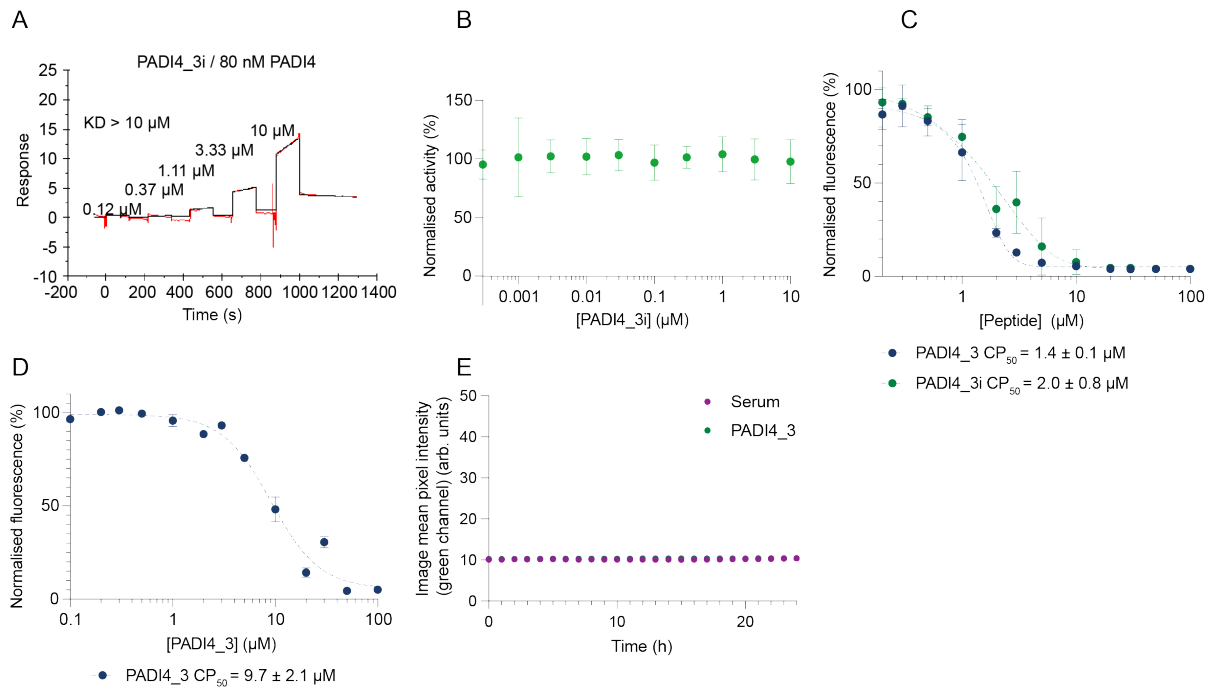
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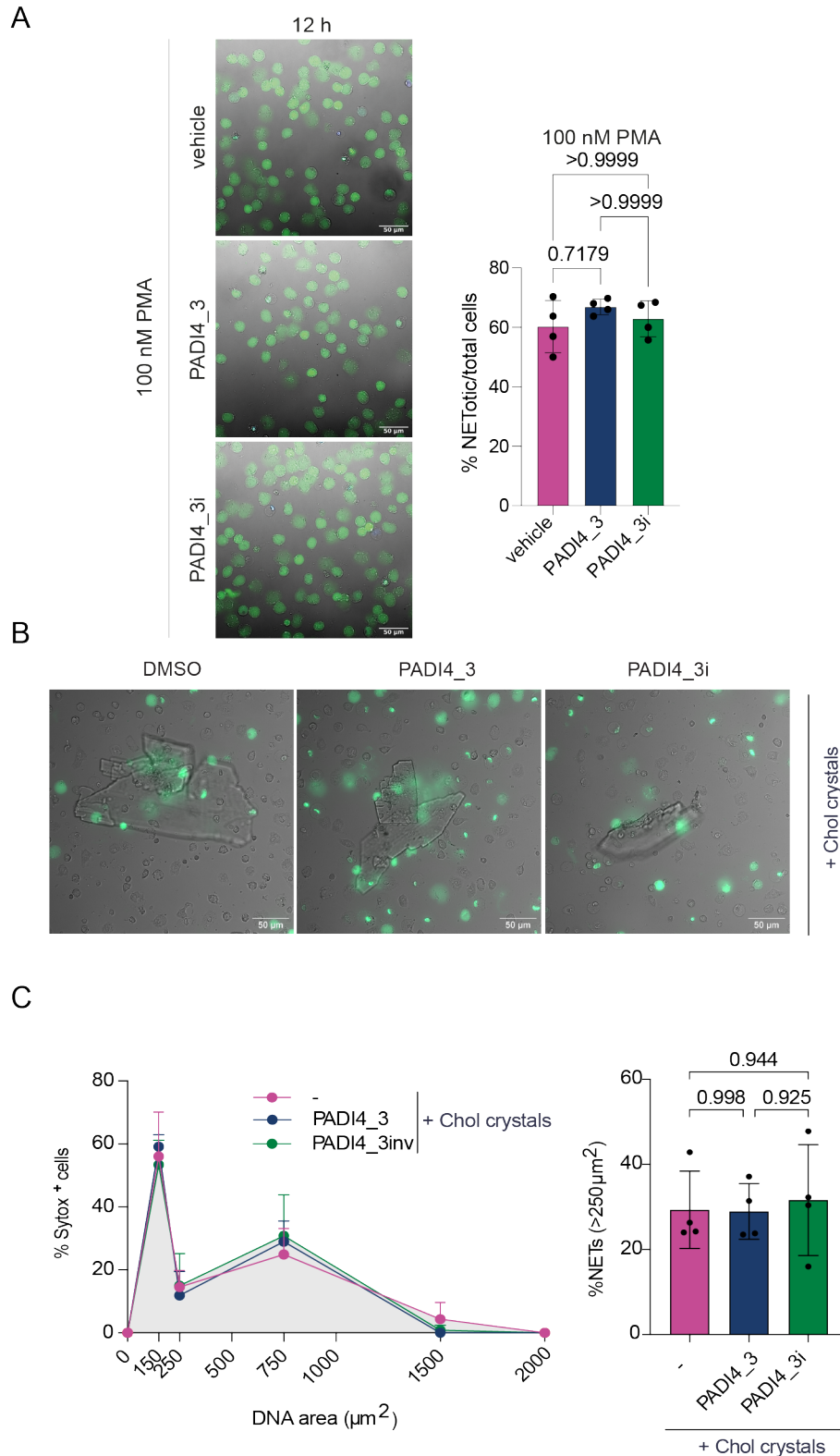
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Supplementary Figure 7. Validation of high content microscopy method. **A.** High content imaging-based quantification of mean H3Cit immunofluorescence intensity in hPADI4-stable mES cells grown in Serum or KSR/2i for 3 h. Each data point represents the mean H3Cit intensity per cell. Cells from three technical replicates and three biological replicates are included. A two-tailed t-test was carried out to determine if the differences among means were significantly different. **B.** High content imaging-based quantification of mean H3Cit immunofluorescence intensity in hPADI4-stable mES cells stimulated with KSR/2i for 3 h, in the presence of increasing concentrations of Cl-amidine. Each data point represents the mean H3Cit intensity per conditions from three technical replicates.

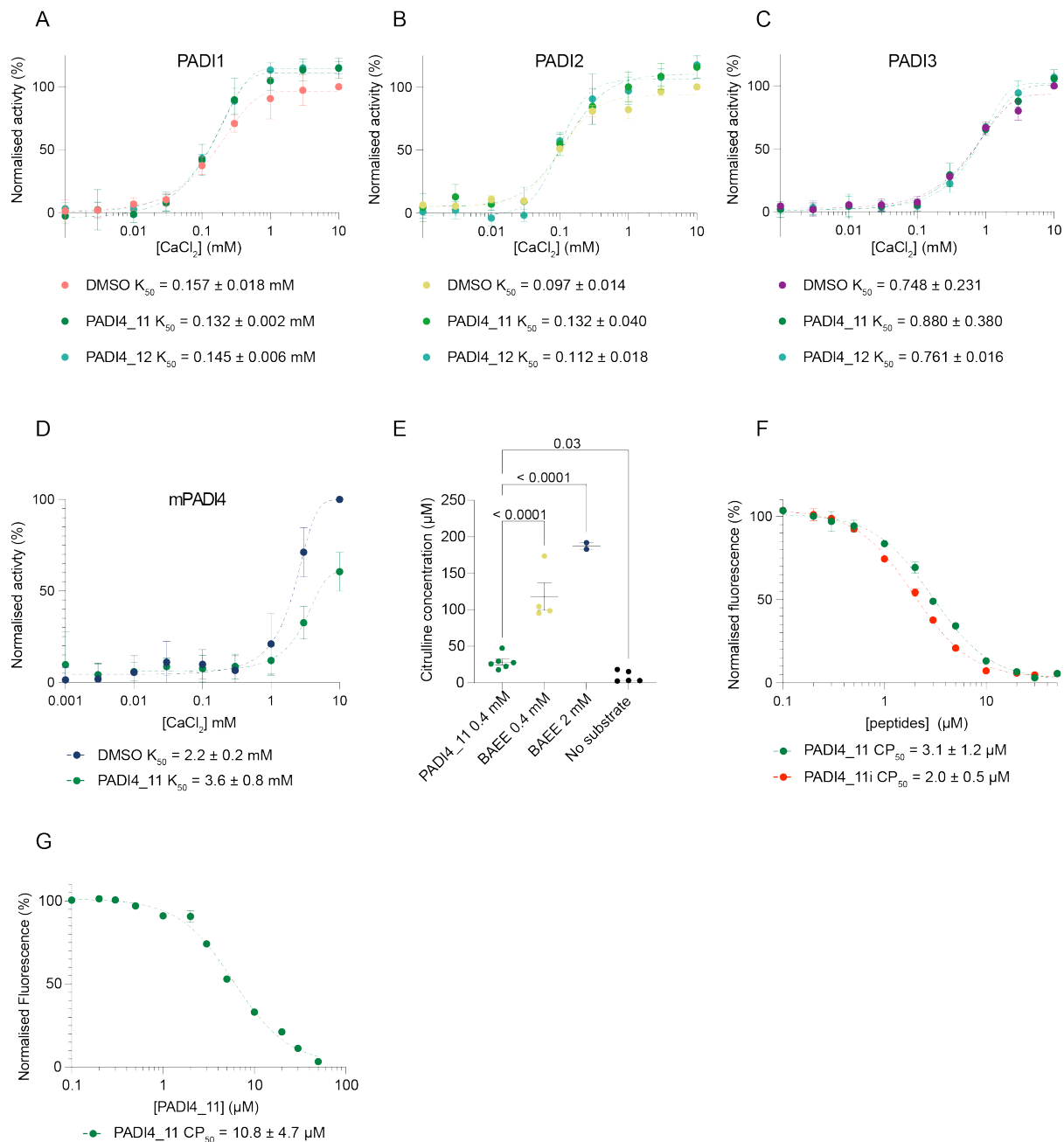


Supplementary Figure 8. PADI4_3i does not inhibit PADI4 and PADI4_3 and PADI4_3i are cell permeable and non-toxic. **A.** PADI4_3i does not bind to PADI4. Binding kinetics between PADI4 and PADI4_3i measured by SPR. A representative experiment is shown. This experiment was performed three times with similar results. **B.** PADI4_3i does not inhibit PADI4. COLDER assay with PADI4_3i at different concentrations (10 - 0.0003 μM) and 10 mM CaCl_2 . Data are normalised to activity of PADI4 in the presence of 0.1% DMSO vehicle. Data show mean $\pm$ SEM of three independent replicates. Each replicate was performed in triplicate. **C.** PADI4_3 and PADI4_3i enter cells. CAPA assay with PADI4_3 and PADI4_3i. Data show mean $\pm$ SEM of three different experiments. Each replicate was performed in triplicate. Data are normalised to cells with no peptide treated with TMR dye (Promega) (100 %) and cells with no peptide and no dye (0 %). **D.** CAPA assay with PADI4_3 at 4 °C demonstrates that PADI4_3 enters the cells via active cellular transport. Data show mean $\pm$ SEM of two independent experiments. Each replicate was performed in triplicate. Data are normalised as in **C.** **E.** Assessment of cytotoxicity by live cell imaging with Incucyte® Cytotox Green Dye, as a measure for cell death. hPADI4 expressing mES cells treated with 1 μM PADI4_3 or DMSO vehicle (0.1%), and imaged for 24h.



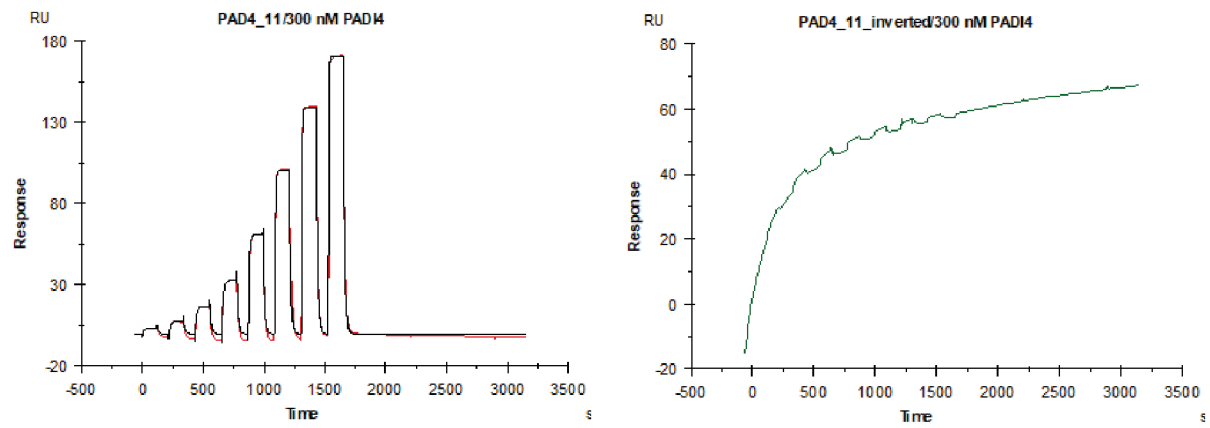
Supplementary Figure 9. PADI4_3 does not affect NETosis in human neutrophils stimulated with PMA or cholesterol crystals. A. Bright field micrograph of human neutrophils pre-incubated with vehicle, PAD4_3 or PAD4_3i peptides (50 μM), imaged 12h post-stimulation with PMA (left) and the corresponding quantification of the percentage of

NETotic cells over total cells (right). Data represents mean $\pm$ SD of four replicates. A Kruskal-Wallis test was carried out to determine if the differences in means are significantly different. p-values were adjusted using the Dunn's correction **B**. Human neutrophils were pre-incubated with DMSO vehicle (0.5%) or 50 μ M PAD4_3 or PAD4_3i and the membrane-impermeable dye Sytox-green before stimulation with 0.1 mg/ml cholesterol crystals. Images were acquired by time-lapse fluorescence microscopy, with images at 8h shown. **C**. Quantification of (B). (left) DNA area was measured by Sytox-green signal and areas were distributed into bins of increasing area sizes and plotted as a percentage of Sytox-green positive (dead) cells. Data shown are means $\pm$ SD from one representative experiment. (right) Percentage of NETotic cells in each treatment condition.

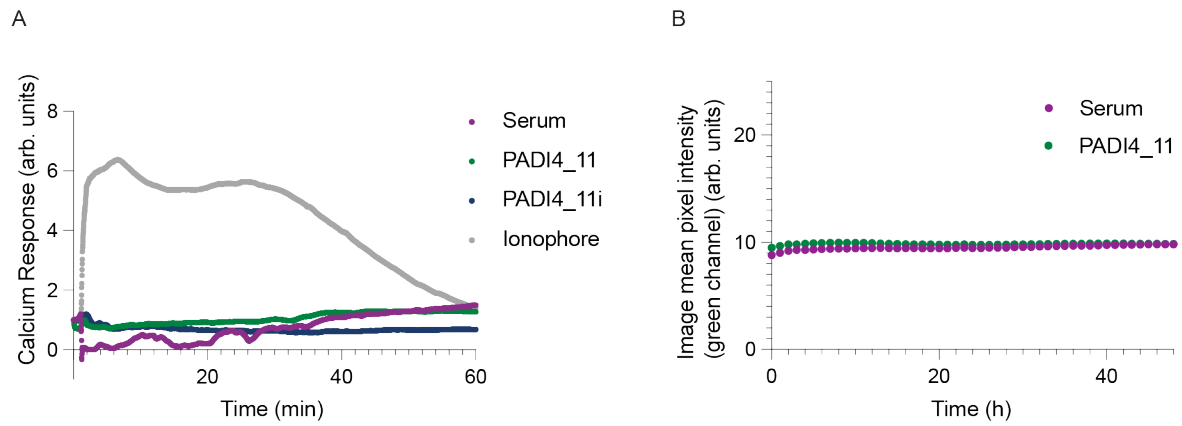


Supplementary Figure 10. PADI4_11 is selective for hPADI4, may be a very poor PADI4 substrate and is cell permeable. A-D. PADI4_11 and PADI4_12 do not activate hPADI1 (A), hPADI2 (B), hPADI3 (C) or mPADI4 (D). COLDER assays were performed in presence or absence of PADI4_11 or PADI4_12 at 30 μM and different concentrations of CaCl₂. $K_{50Ca^{2+}}$ is the concentration of CaCl₂ that yields half maximal PADI activity. Data represent mean ± SEM of three independent replicates. Each replicate was performed in triplicate. Data were normalised against the activity of each PADI in the presence of 0.1% DMSO vehicle and 10 mM CaCl₂. **E.** PADI4_11 may be a very poor PADI4 substrate. COLDER assay performed with 0.4 mM PADI4_11 and BAEE was used as positive control. Data represents mean ± SEM of three independent replicates. . A two-way ANOVA was carried out to determine if the

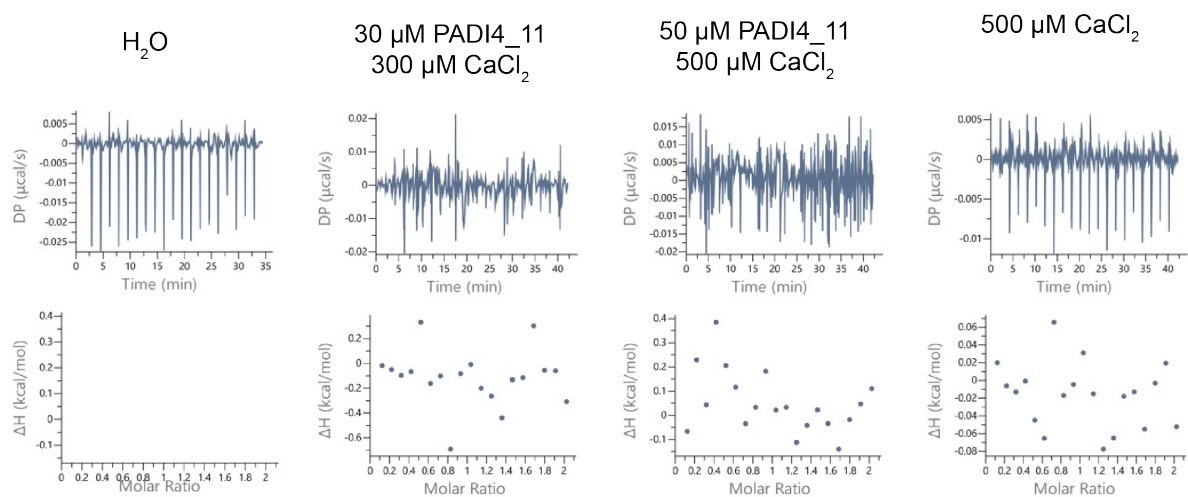
differences among means were significant. Each replicate was done in triplicate. **F.** PADI4_11 and PADI4_11i are cell permeable. CAPA assay with PADI4_11 and PADI4_11i. Data show mean $\pm$ SEM of three independent experiments performed in triplicate. Data are normalised with cells with no peptide treated with TMR dye (Promega) (100 %) and cells with no peptide and no dye (0 %). **G.** CAPA assay with PADI4_11 at 4 °C demonstrates that PADI4_11 enters the cells via active cellular transport. Data show mean $\pm$ SEM of three independent experiments performed in triplicate. Data was normalised as in **F.**



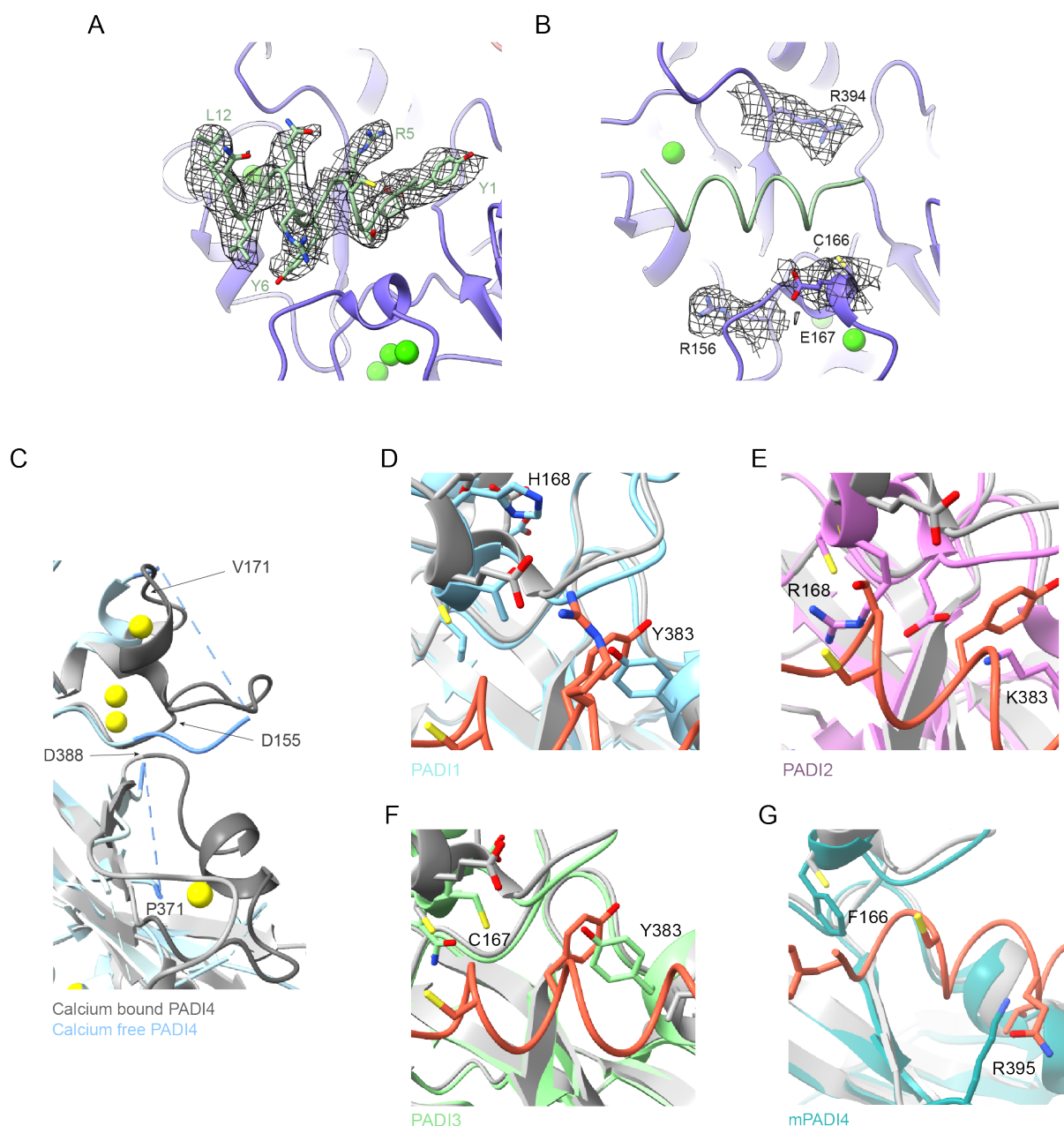
Supplementary 11. PADI4_11i does not bind to PADI4. SPR analysis of hPADI4 with either PADI4_11 or PADI4_11i peptides. Experiments were performed in triplicate and a representative experiment is shown.



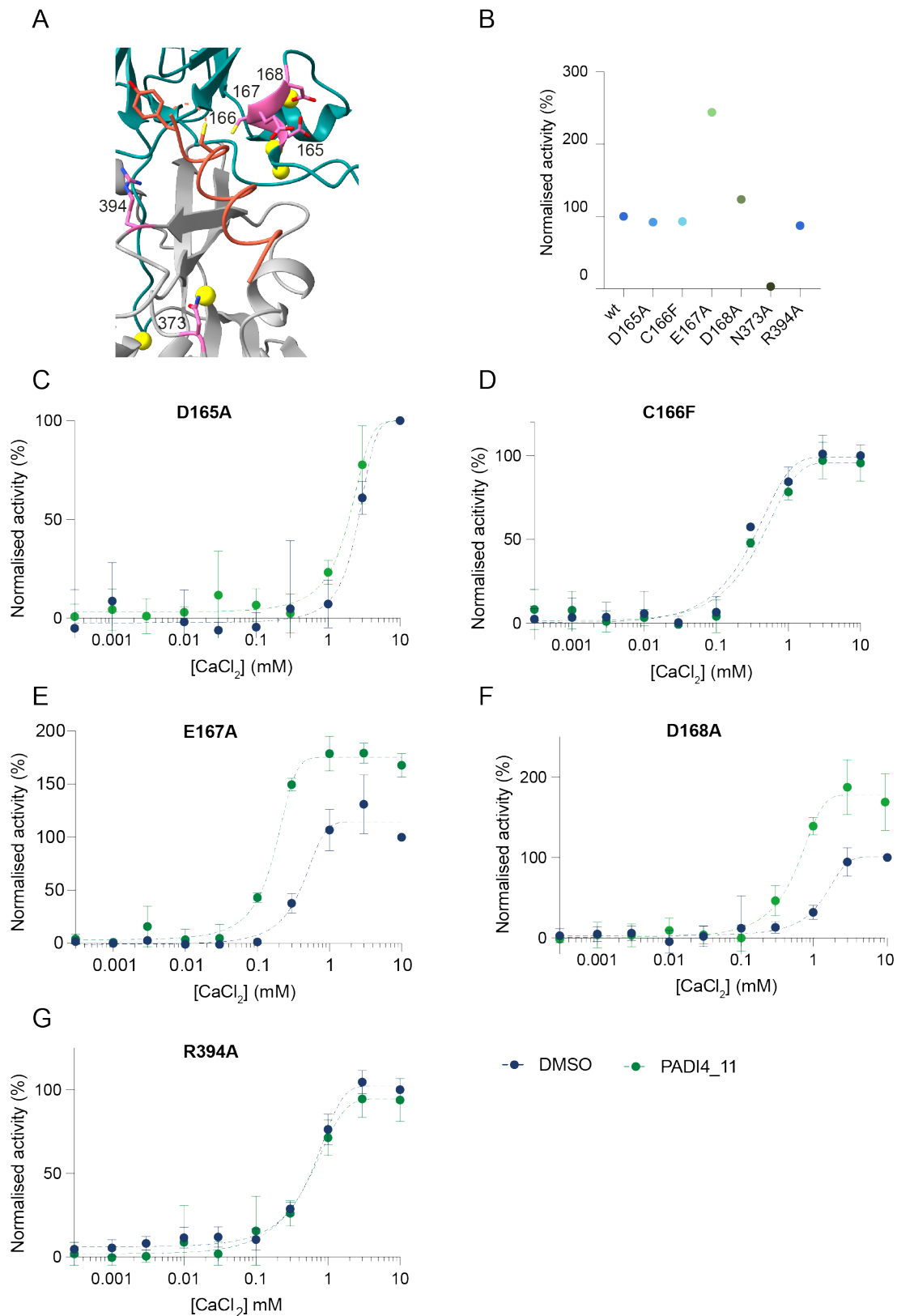
Supplementary 12. Time course of calcium influx. A. Calcium influx into cells over the course of 60 minutes, as measured by Calbryte intensity, after treatment with 25 μ M PADI4_11 or PADI4_11i. Calcium ionophore (10 μ M) used as a positive control for calcium influx. **B.** Assessment of cytotoxicity by live cell imaging with Incucyte® Cytotox Green Dye, as a read-out for cell death. hPADI4 expressing mES cells treated with 25 μ M PADI4_11 or DMSO vehicle (0.1%), and imaged for 48 h.



Supplementary Figure 13. PADI4_11 does not bind to calcium. Isothermal Titration Calorimetry between PADI4_11 peptide (30 and 50 μ M) and CaCl₂ (300 and 500 μ M).



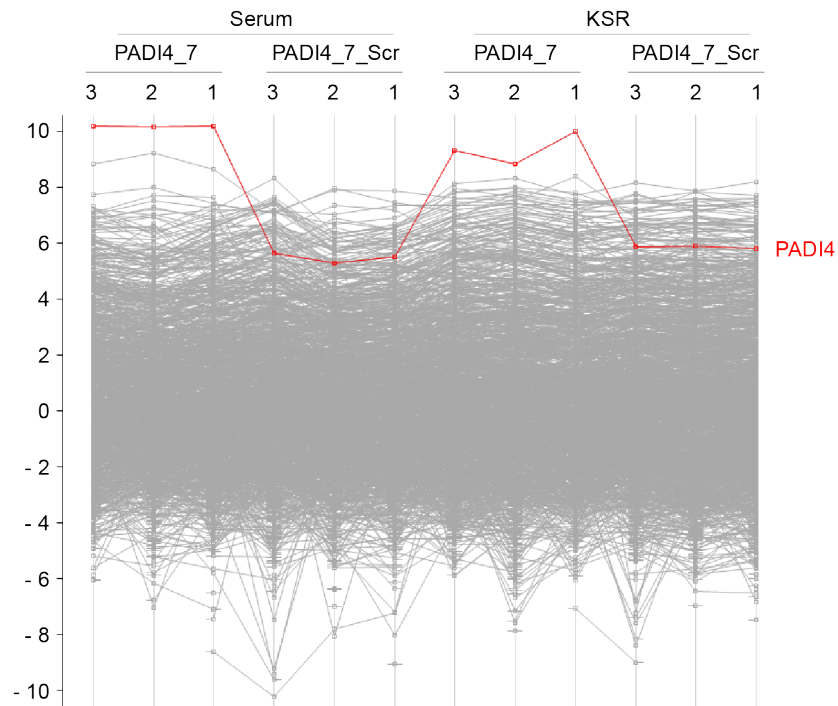
Supplementary Figure 14. PADI4_11 binds selectively to hPADI4. **A.** Electron density of PADI4_11. **B.** Electron density of significant PADI4 amino acids involved in the binding with PADI4_11. **C.** Alignment of PADI4 bound to calcium (grey) and calcium free PADI4 (PBD ID: 1WD8) (blue). Loops involved in the binding of PADI4_11 peptide are shown in dark grey. **D.** Structure alignment of PADI1 AlphaFold model (cyan) with hPADI4 in complex with peptide PADI4_11. **E.** Structure alignment of PADI2 (PDB: 4N2B, magenta) with hPADI4 in complex with peptide PADI4_11. **F.** Structure alignment of PADI3 AlphaFold model (light green) with hPADI4 in complex with peptide PADI4_11. **G.** Structure alignment of mouse PADI4 AlphaFold model (teal) with hPADI4 in complex with peptide PADI4_11. Protein residues predicted to clash with PADI4_11 are highlighted as sticks.



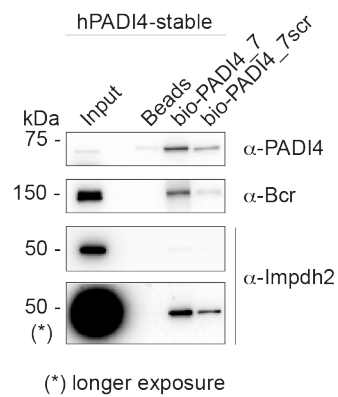
Supplementary Figure 15. PADI4_11 cannot activate some hPADI4 variants. A. View from a PADI4 cryoEM structure with PADI4_11 (orange). Residues involved in the binding that were mutated for following experiments are shown in pink. **B.** Different PADI4 variants have

different activities. Activity of each PADI4 variant was measured by COLDER assay at 10 mM CaCl_2 following 30 min incubation at RT. Activity was normalised against the activity of wild-type PADI4. **C-G.** PADI4_11 is not able to activate some PADI4 variants. COLDER assays were performed in presence or absence of PADI4_11 at 30 μM and different concentrations of CaCl_2 . Activity was normalised against the activity of each variant at 10 mM CaCl_2 in absence of peptide. Data represents mean $\pm$ SEM of two independent replicates. Each replicate was performed in triplicate.

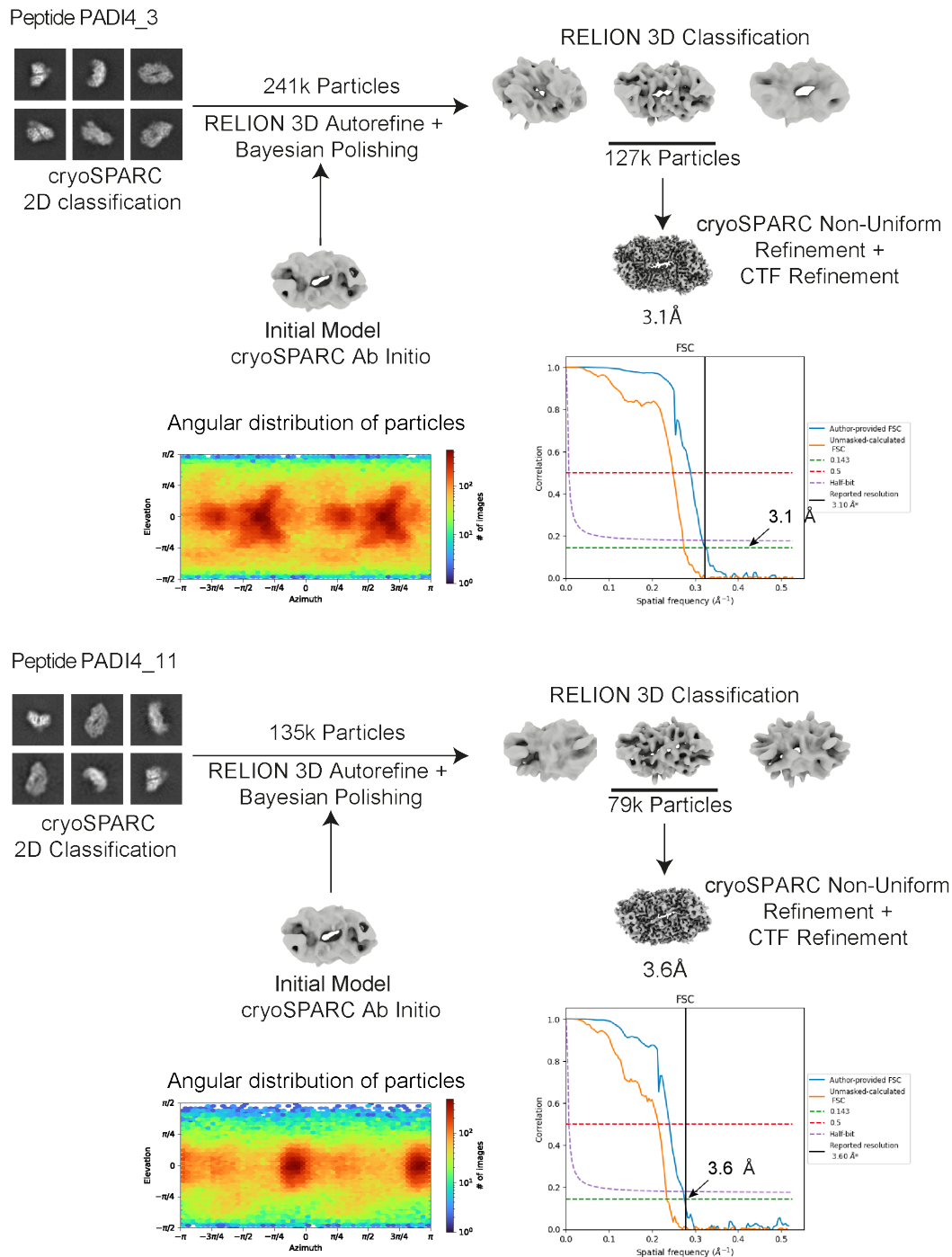
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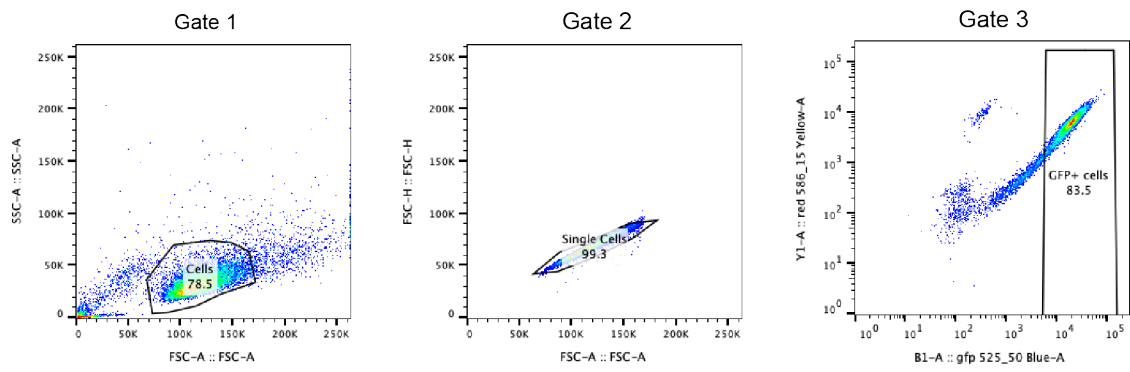
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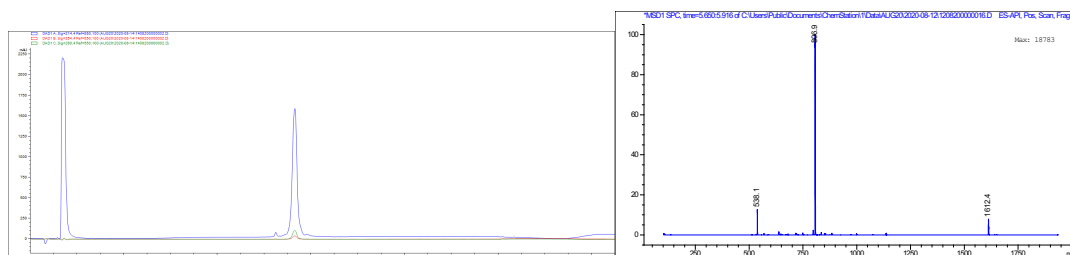
Supplementary Figure 16. Specific enrichment of PADI4 by bio-PADI4_7 and confirmation of interactors by immunoblot. A. Enrichment profile for PADI4 compared to all other identified proteins across all pull-down samples, as determined by Mass Spectrometric analysis. **B.** Immunoblot analysis of bio-PADI4_7 and bio-PADI4_7scr pull-downs for PADI4, Bcr and Impdh2.



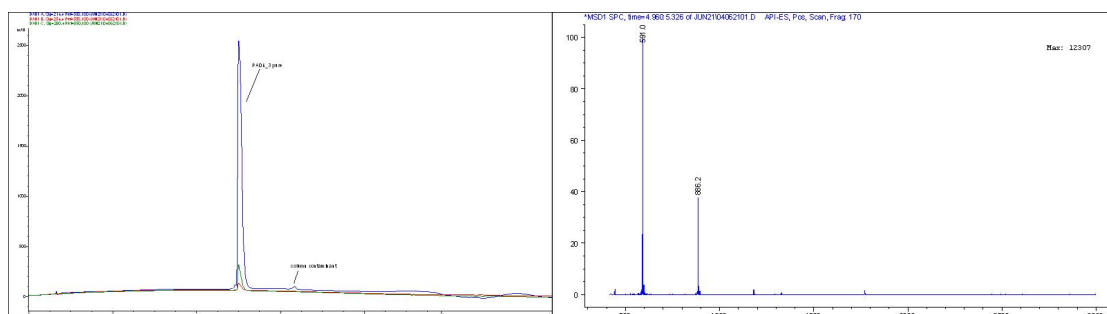
Supplementary Figure 17. Cryo-electron microscopy data processing pipeline for the structures of PADI4 in complex with PADI4_3 (top) and PADI4 in complex with PADI4_11 (bottom).



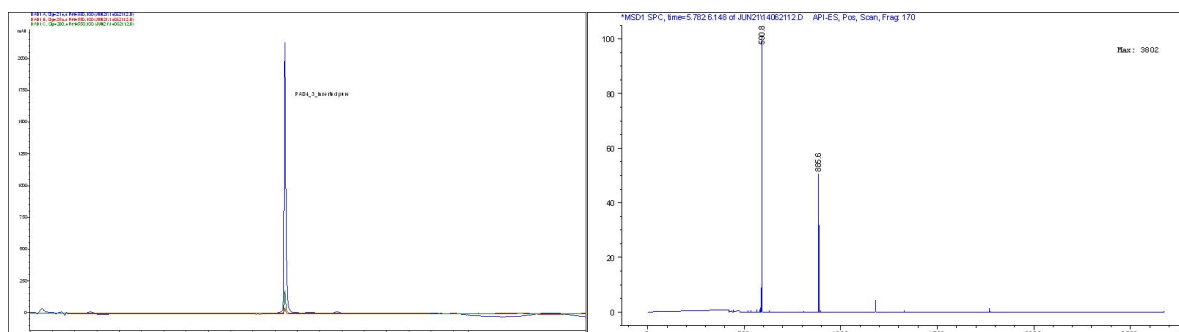
Supplementary Figure 18. Gating workflow followed to analyse Chloroalkane Penetration Assay (CAPA).



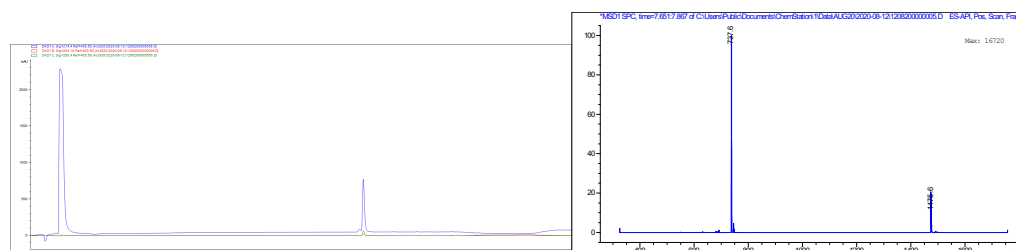
Supplementary Figure 19. PADI4_2 Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



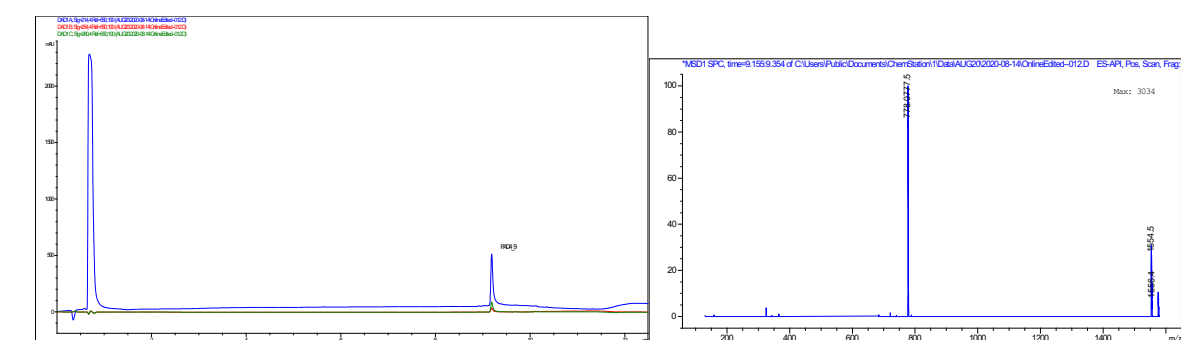
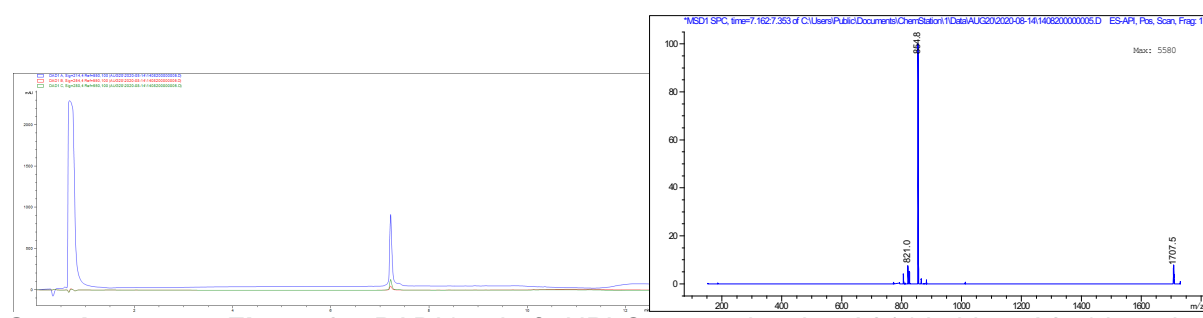
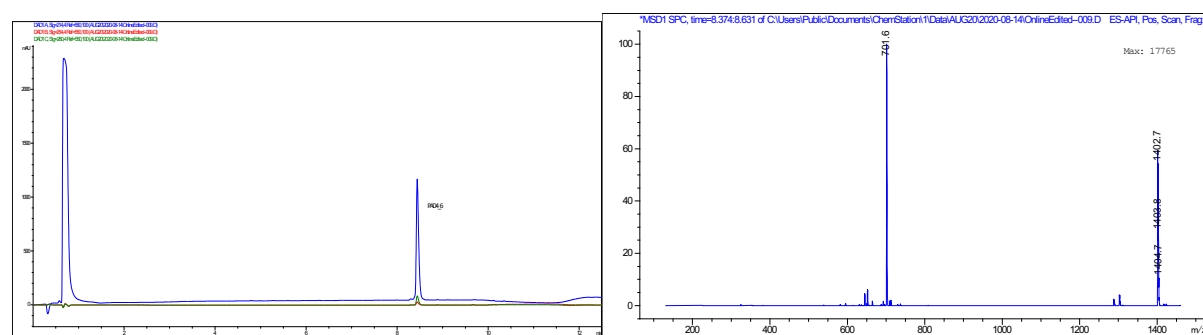
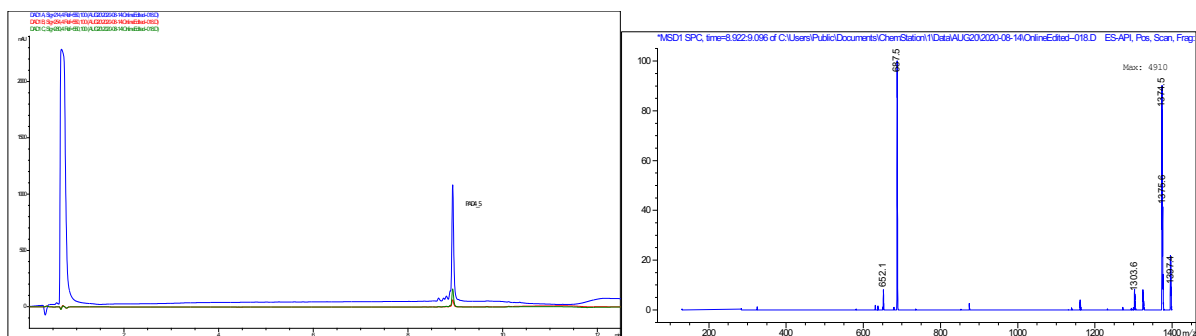
Supplementary Figure 20. PADI4_3 Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace

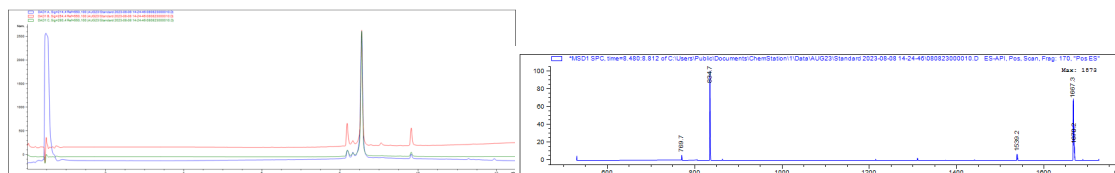


Supplementary Figure 21. PADI4_3i Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace

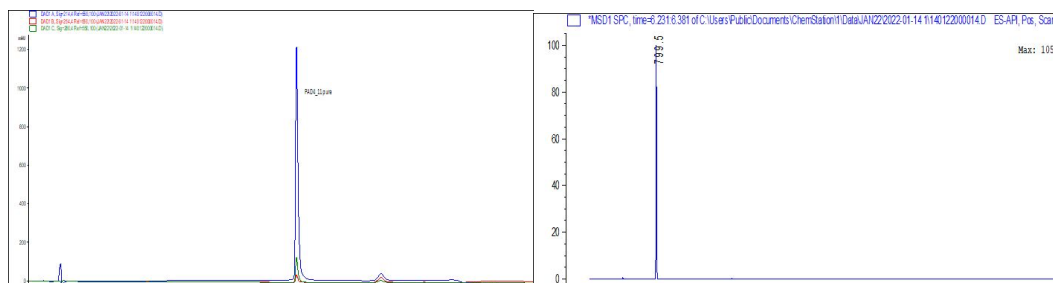


Supplementary Figure 22. PADI4_4 Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace

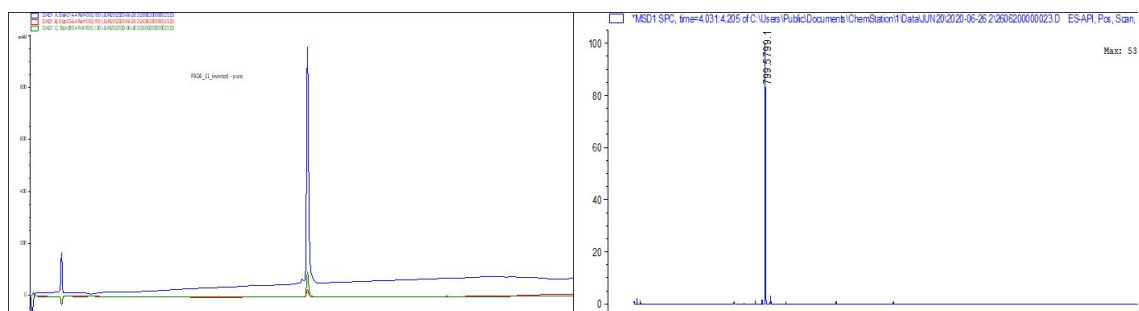




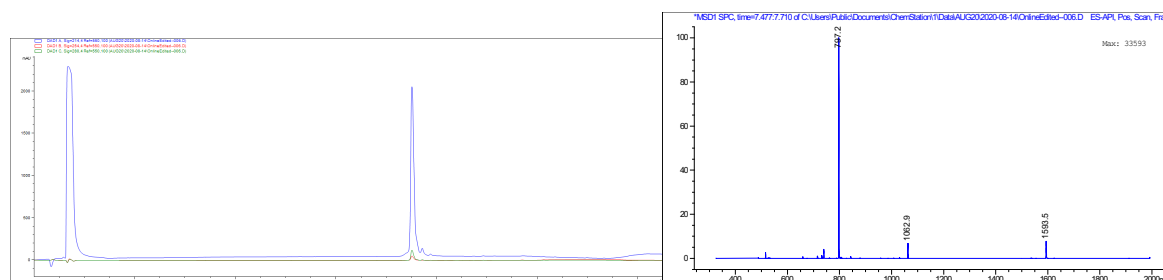
Supplementary Figure 27. PADI4_10 Top: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Bottom: ESI-MS trace



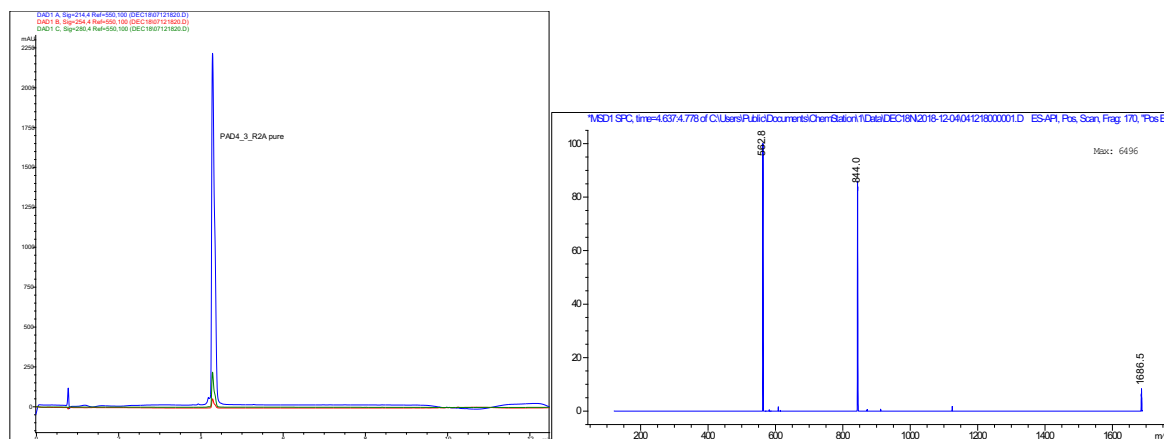
Supplementary Figure 28. PADI4_11 Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



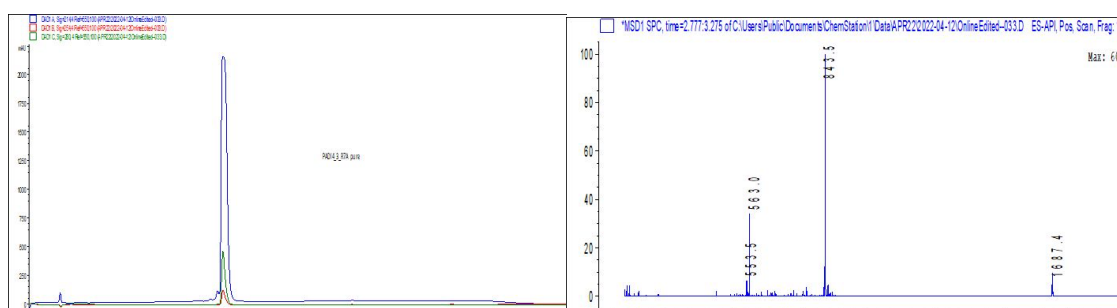
Supplementary Figure 29. PADI4_11i Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



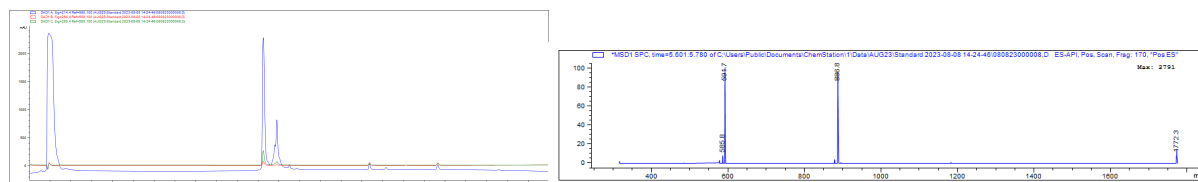
Supplementary Figure 30. PADI4_12 Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



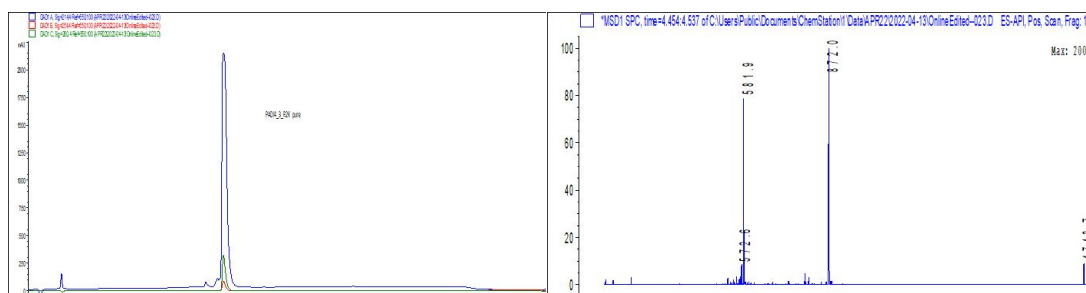
Supplementary Figure 31. PADI4_3_R2A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace of crude peptide



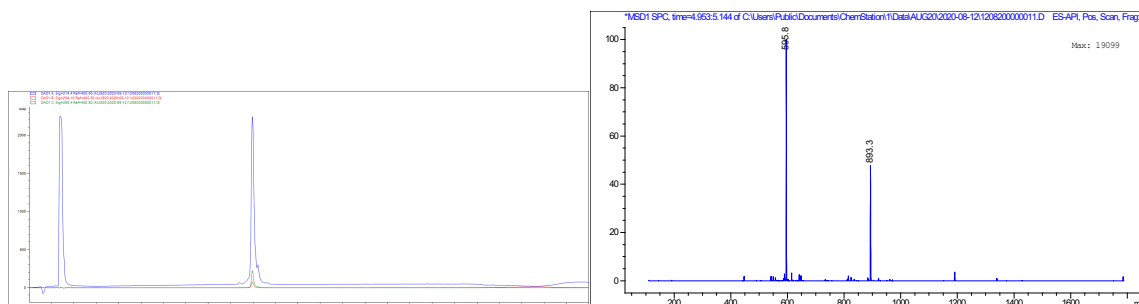
Supplementary Figure 32. PADI4_3_R7A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



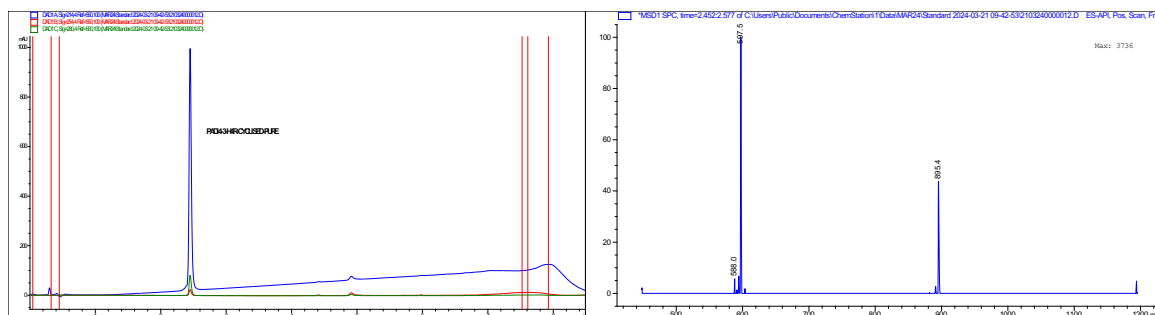
Supplementary Figure 33. PADI4_3_R2cit Top: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Bottom: ESI-MS trace



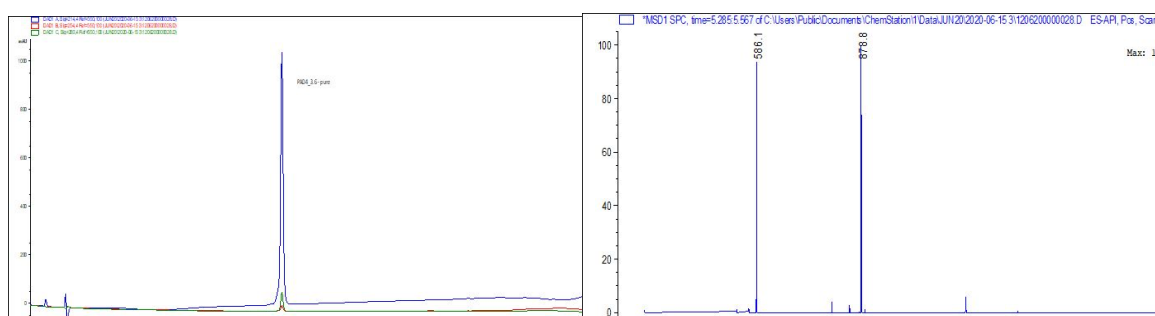
Supplementary Figure 34. PADI4_3_R2K Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



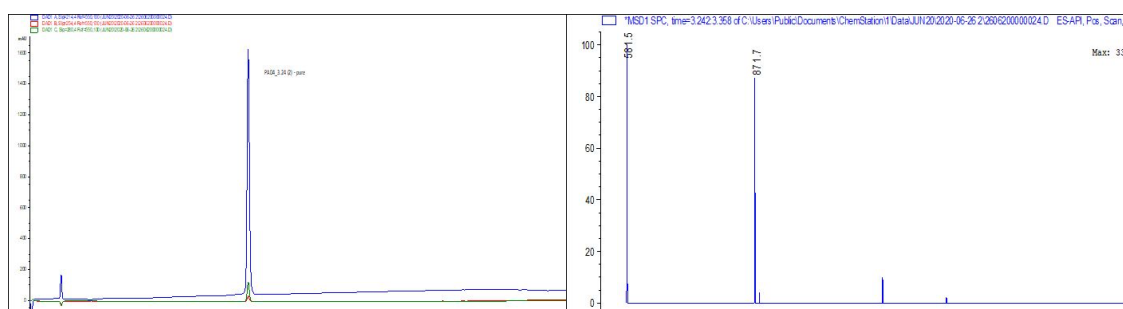
Supplementary Figure 35. PADI4_3_y1(OMe)Y Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



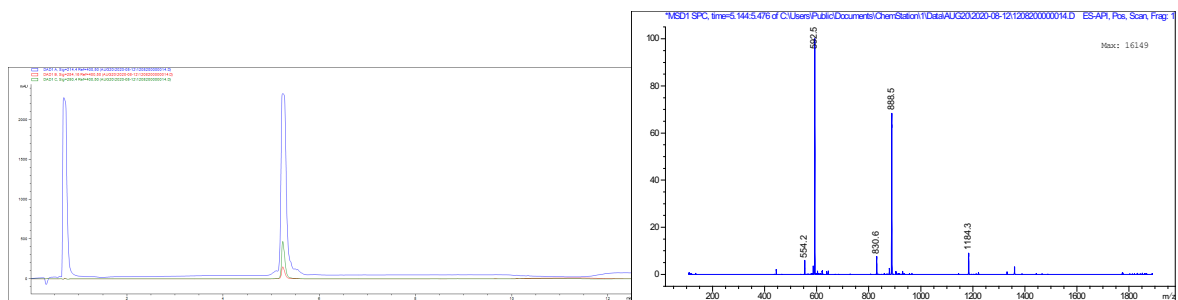
Supplementary Figure 36. PADI4_3_H4R Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



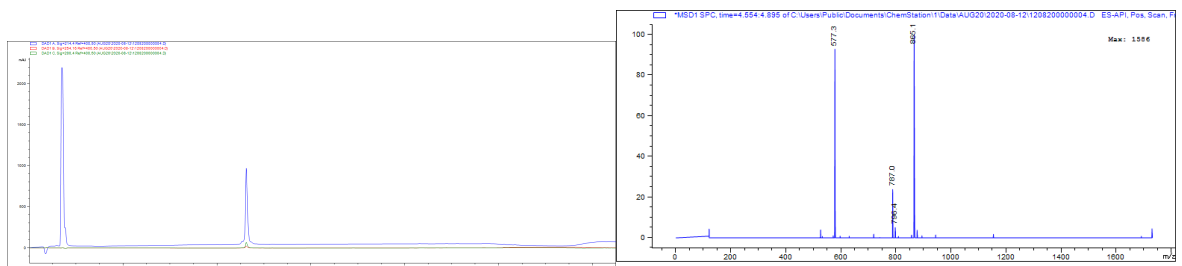
Supplementary Figure 37. PADI4_3_K10I Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



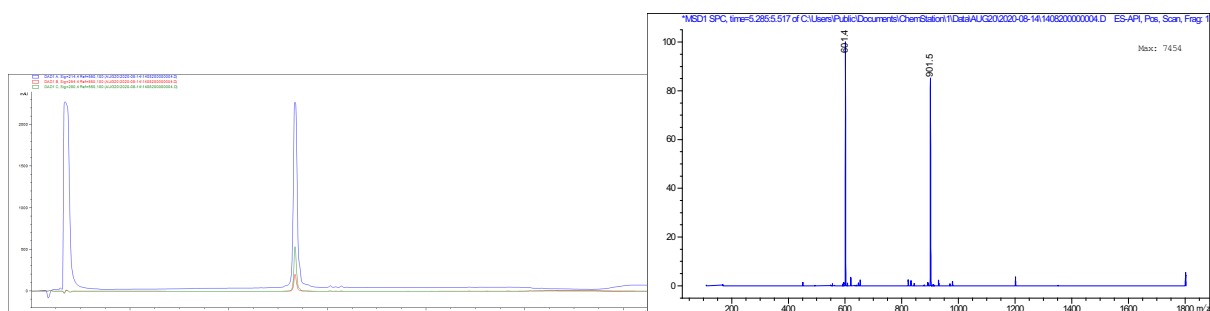
Supplementary Figure 38. PADI4_3_K10V Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



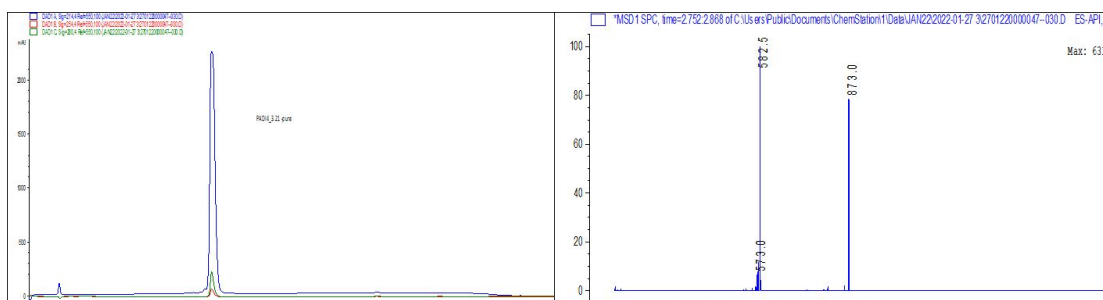
Supplementary Figure 39. PADI4_3_H4R_K10I Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



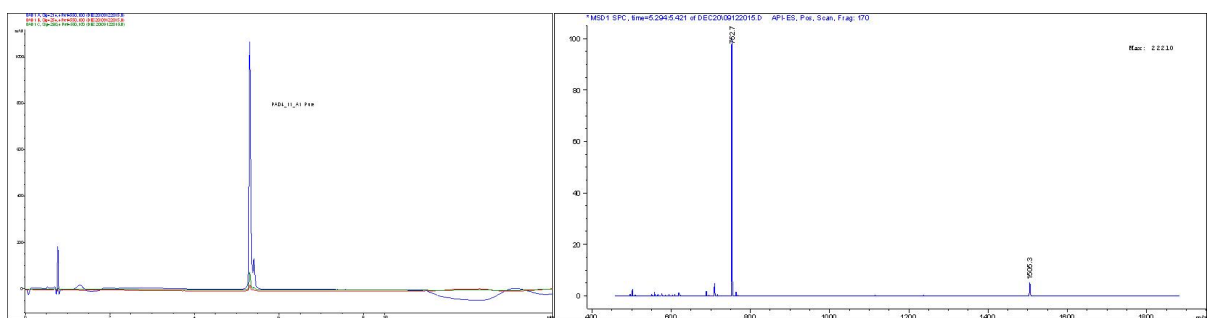
Supplementary Figure 40. PADI4_3_R7N Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



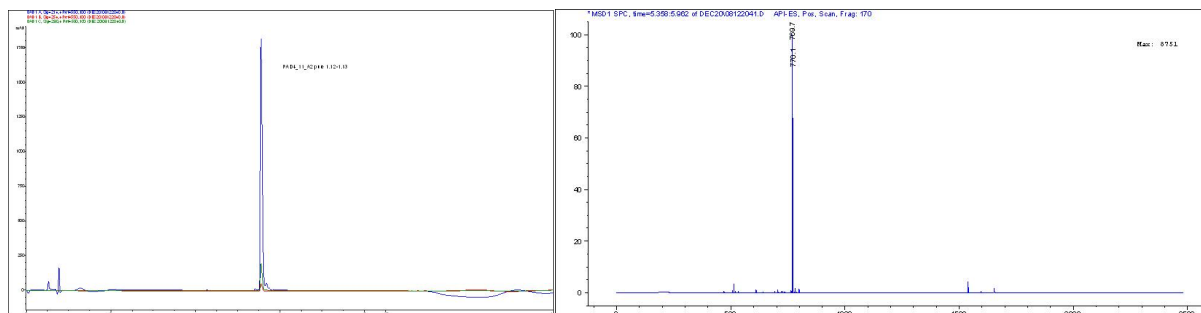
Supplementary Figure 41. PADI4_3_R7W Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



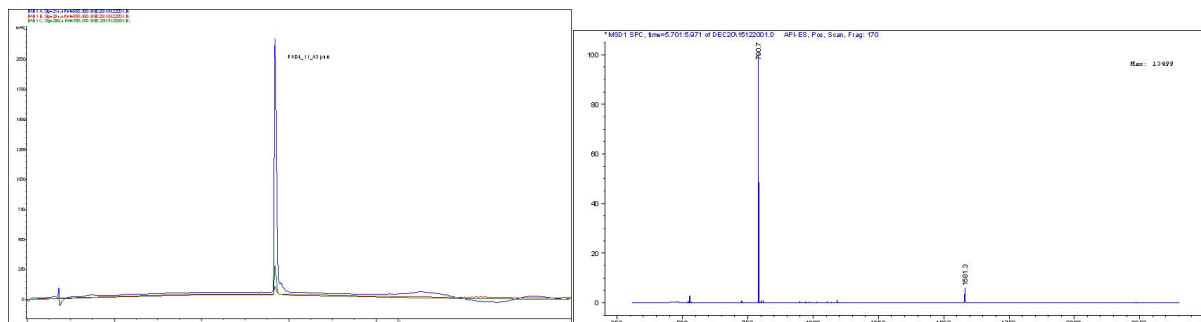
Supplementary Figure 42. PADI4_3_Y6H Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



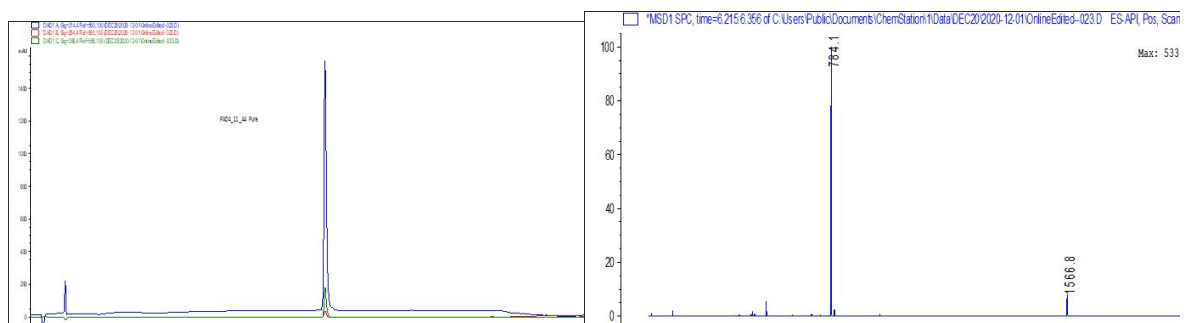
Supplementary Figure 43. PADI4_11_Y1A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



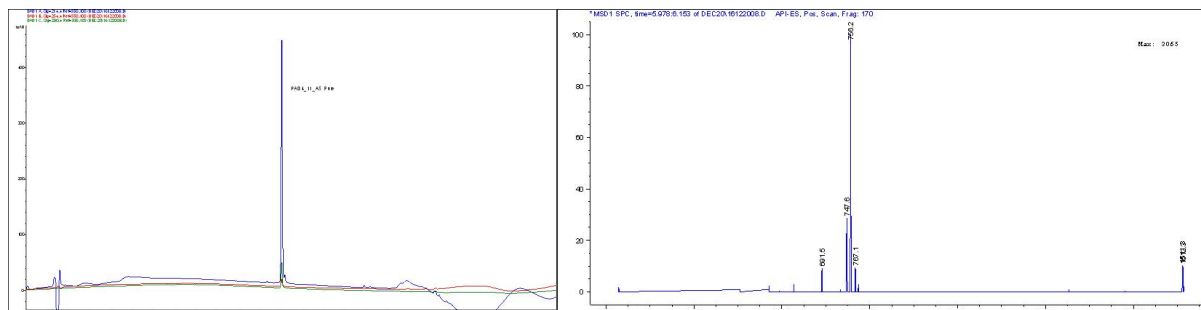
Supplementary Figure 44. PADI4_11_E2A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



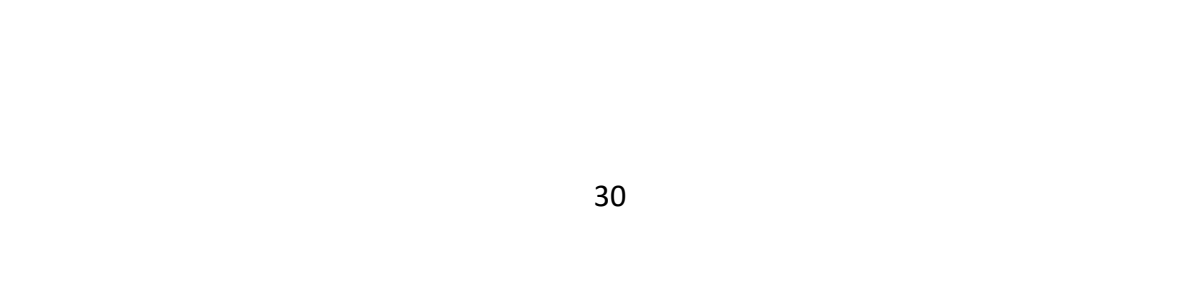
Supplementary Figure 45. PADI4_11_S3A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace

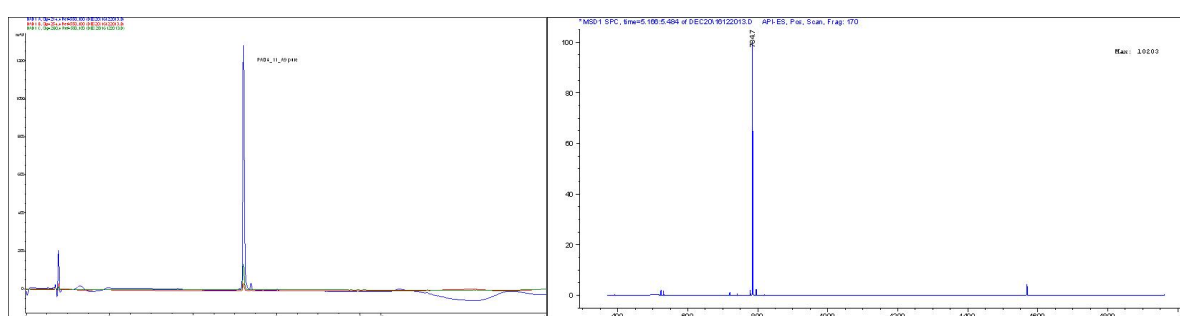
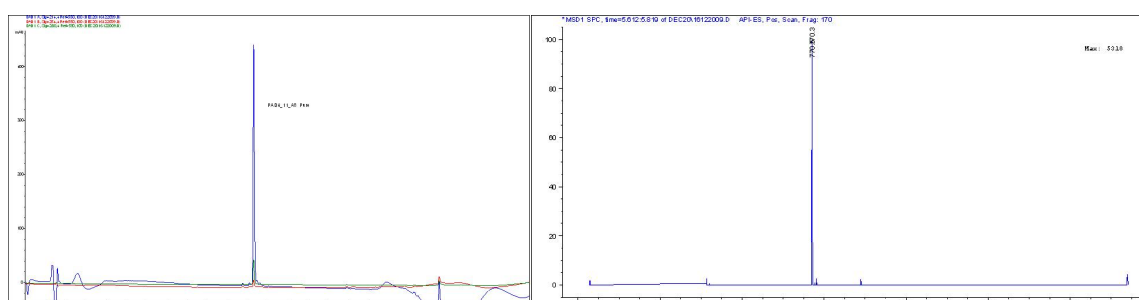
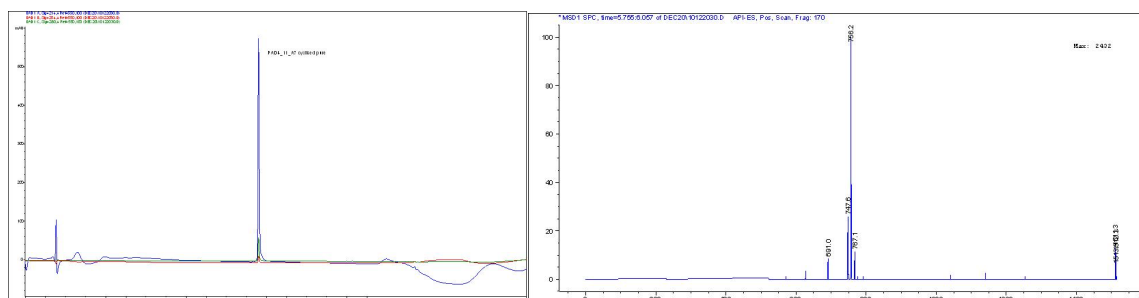
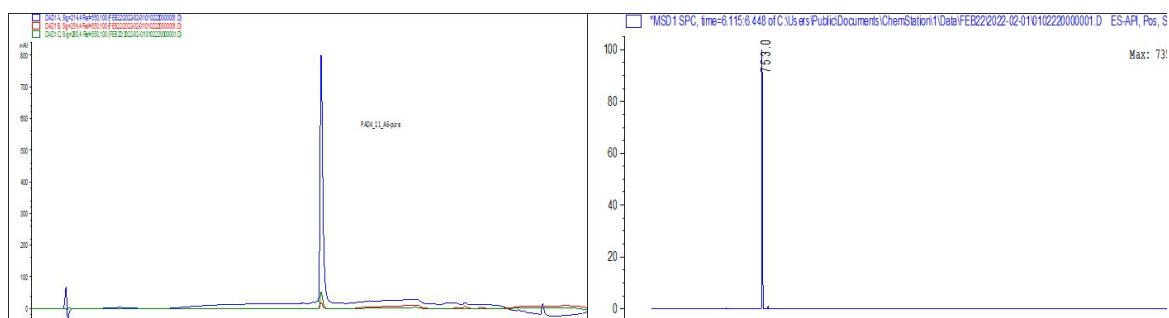


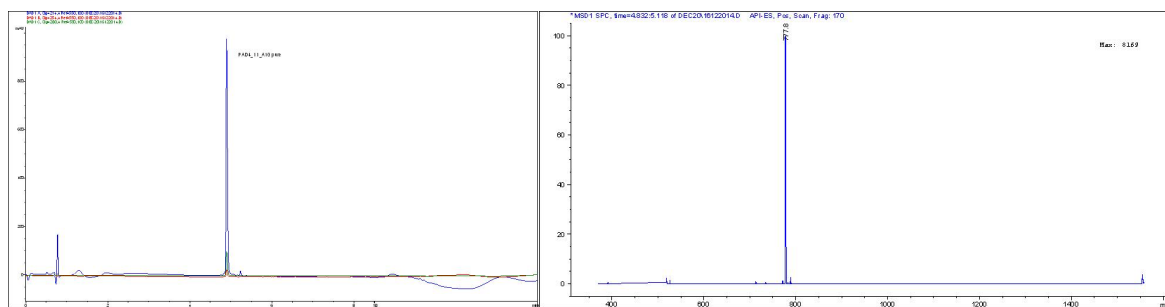
Supplementary Figure 46. PADI4_11_C4A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



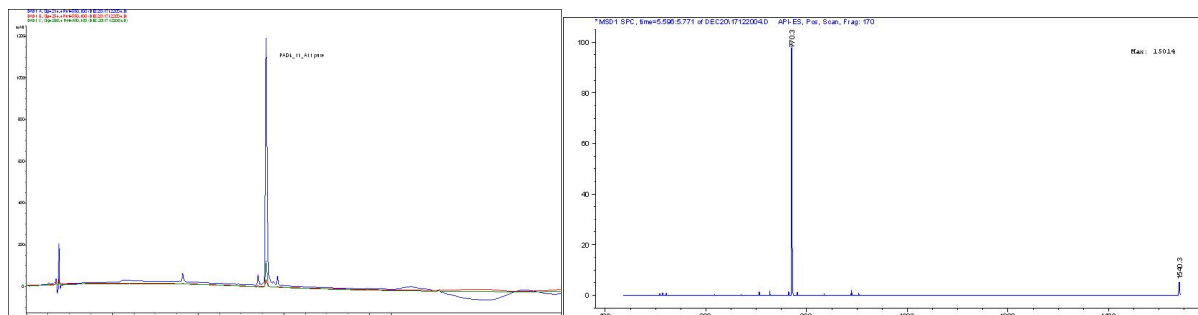
Supplementary Figure 47. PADI4_11_R5A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



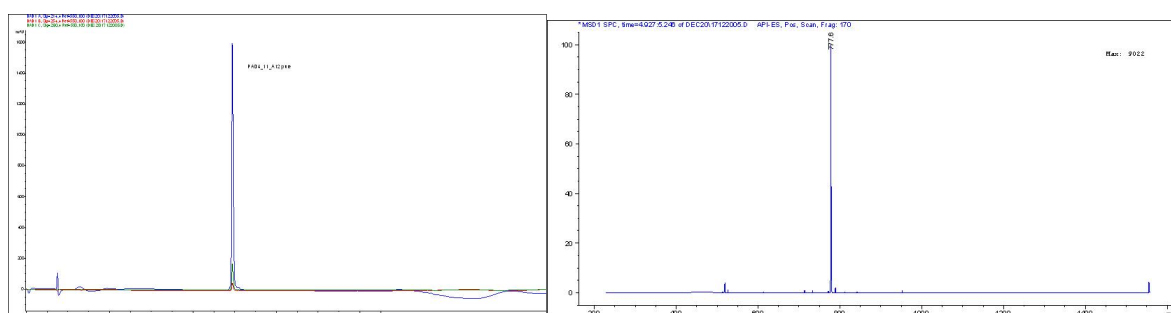




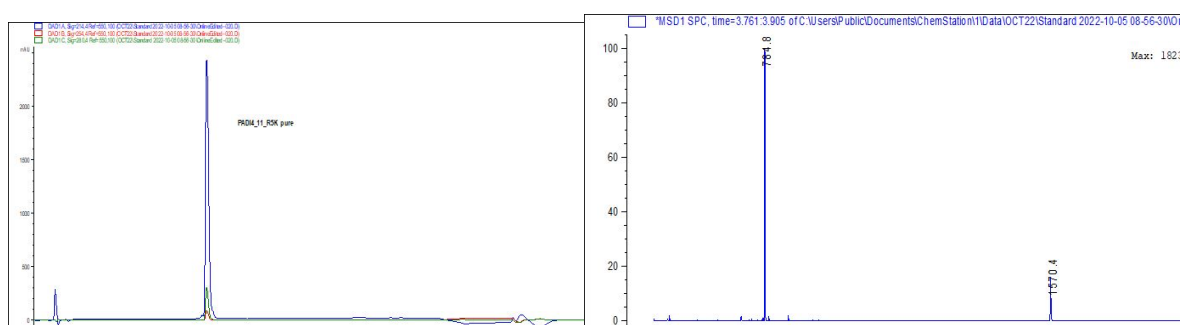
Supplementary Figure 52. PADI4_11_L10A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



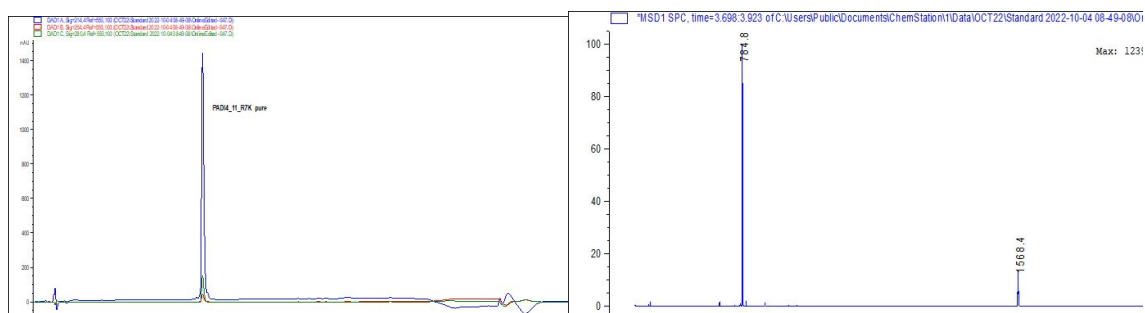
Supplementary Figure 53. PADI4_11_Q11A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



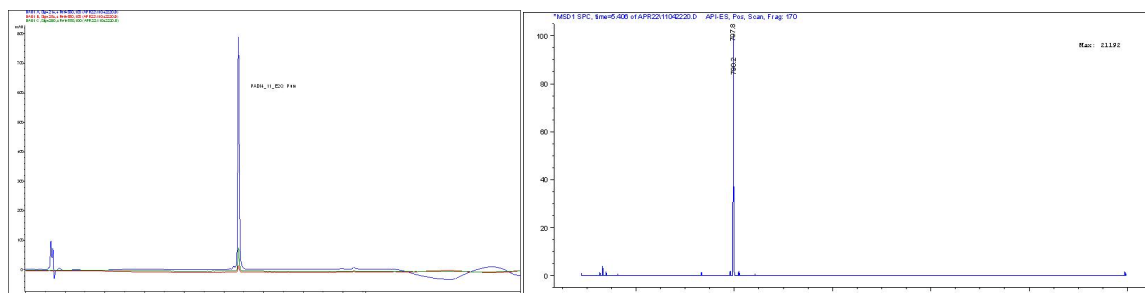
Supplementary Figure 54. PADI4_11_L12A Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



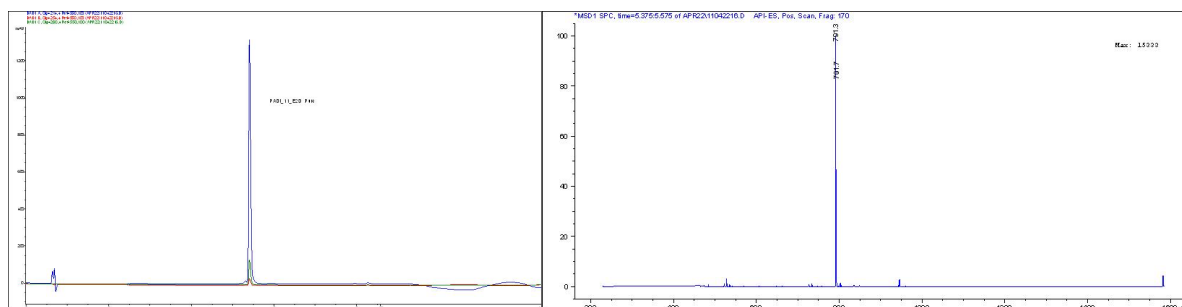
Supplementary Figure 55. PADI4_11_R5K Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



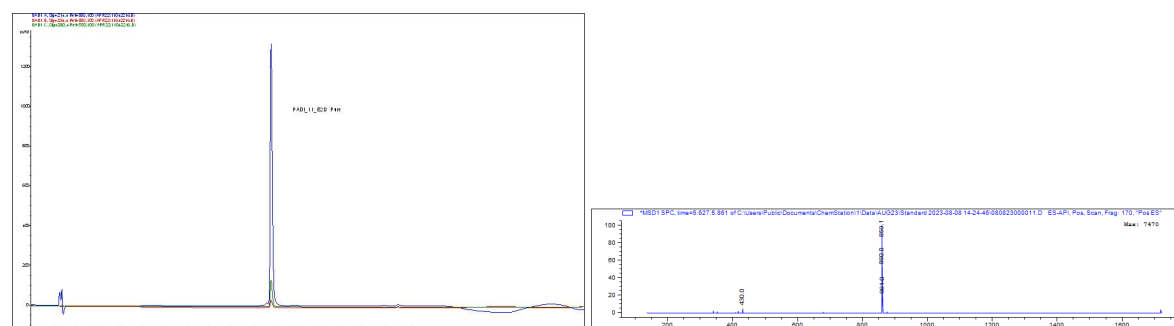
Supplementary Figure 56. PADI4_11_R7K Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green.



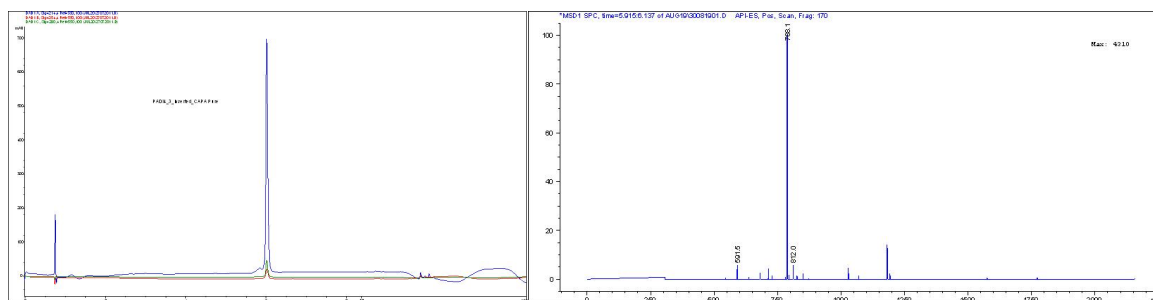
Supplementary Figure 57. PADI4_11_E2Q Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



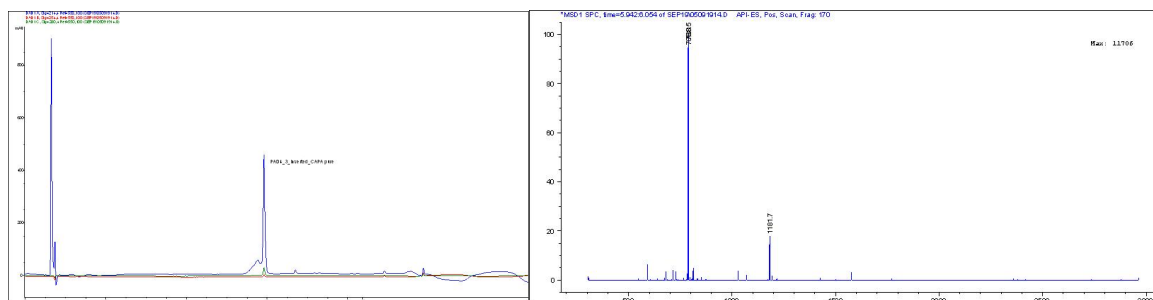
Supplementary Figure 58. PADI4_11_E2D Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



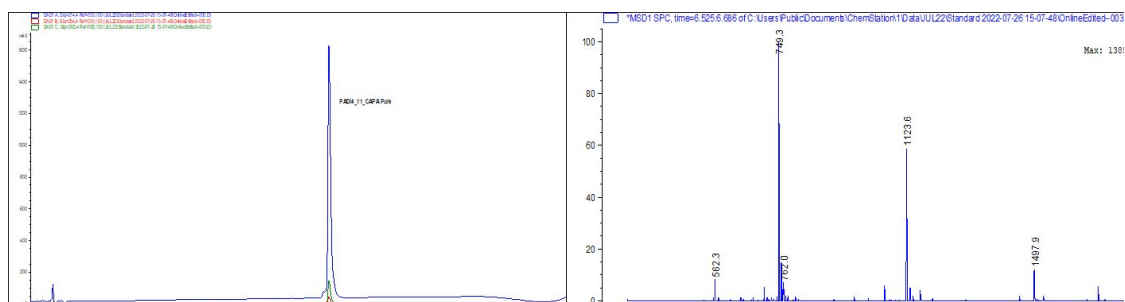
Supplementary Figure 59. PADI4_11B Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



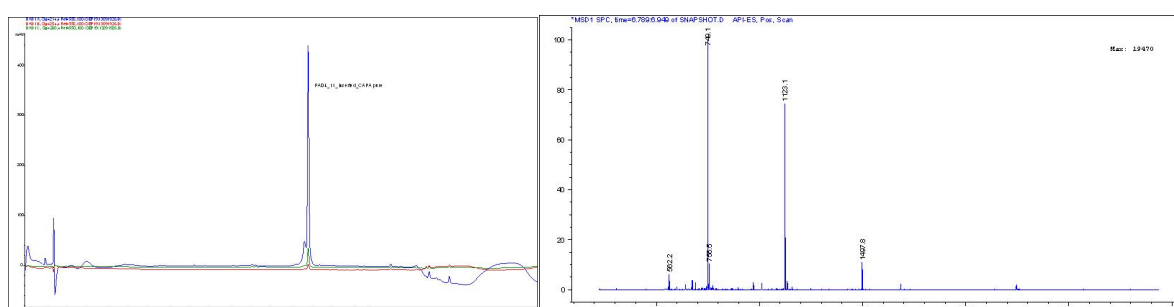
Supplementary Figure 60. PADI4_3i_CAPA Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



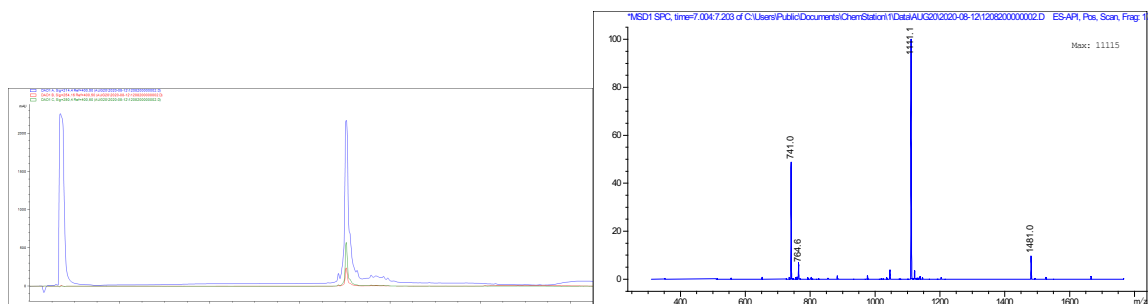
Supplementary Figure 61. PADI4_3i_CAPA Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



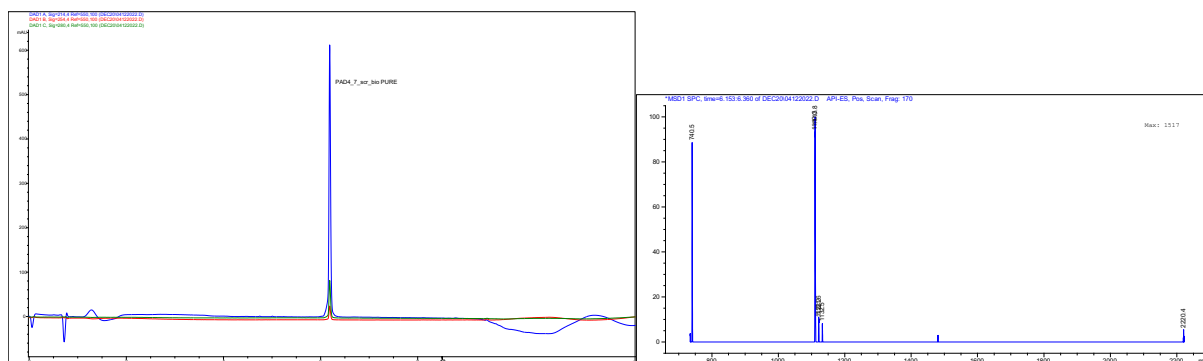
Supplementary Figure 62. PADI4_11i_CAPA Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



Supplementary Figure 63. PADI4_11i_CAPA Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



Supplementary Figure 64. PADI4_7_bio Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace



Supplementary Figure 65. PADI4_7scr_bio Left: HPLC trace, showing A214 in blue, A254 in red and A280 in green. Right: ESI-MS trace

Supplementary Table 1. SPR data of selected macrocyclic peptides enriched in the RaPID selections. Data shows average $\pm$ standard deviation of three independent experiments.

	10 mM Ca^{2+}			0 mM Ca^{2+}			200 μM Cl-amidine + 10 mM Ca^{2+}		
	Kd (nM)	Kon (1/Ms)	Koff (1/s)	Kd (nM)	Kon (1/Ms)	Koff (1/s)	Kd (nM)	Kon (1/Ms)	Koff (1/s)
PAD4_1	205 $\pm$ 52	$7.6 \cdot 10^4 \pm 0.8 \cdot 10^4$	$2 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	105 $\pm$ 17	$9.5 \cdot 10^4 \pm 0.6 \cdot 10^4$	$1 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	728 $\pm$ 175	$4 \cdot 10^4 \pm 3 \cdot 10^3$	$3 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$
PAD4_2	13 $\pm$ 4	$8 \cdot 10^4 \pm 2 \cdot 10^4$	$1.0 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$	>5 μM	-	-	>5 μM	-	-
PAD4_3	2.7 $\pm$ 0.5	$2.5 \cdot 10^5 \pm 0.2 \cdot 10^5$	$7 \cdot 10^{-4} \pm 1 \cdot 10^{-4}$	>5 μM	-	-	>5 μM	-	-
PAD4_7	39 $\pm$ 24	$5 \cdot 10^6 \pm 5 \cdot 10^6$	$2 \cdot 10^{-1} \pm 2 \cdot 10^{-1}$	9 $\pm$ 1	$1 \cdot 10^7 \pm 1 \cdot 10^7$	$1 \cdot 10^{-1} \pm 1 \cdot 10^{-1}$	36 $\pm$ 12	$9 \cdot 10^6 \pm 6 \cdot 10^6$	$4 \cdot 10^{-1} \pm 2 \cdot 10^{-1}$
PAD4_11	457 $\pm$ 109	$2 \cdot 10^5 \pm 7 \cdot 10^5$	$1 \cdot 10^{-1} \pm 2 \cdot 10^{-2}$	>5 μM	-	-	382 $\pm$ 27	$3 \cdot 10^5 \pm 5 \cdot 10^4$	$1 \cdot 10^{-1} \pm 1 \cdot 10^{-2}$
PAD4_12	679 $\pm$ 83	$2 \cdot 10^5 \pm 5 \cdot 10^4$	$1 \cdot 10^{-1} \pm 3 \cdot 10^{-2}$	>5 μM	-	-	674 $\pm$ 191	$3 \cdot 10^5 \pm 8 \cdot 10^4$	$2 \cdot 10^{-1} \pm 2 \cdot 10^{-2}$

Supplementary Table 2. Cryo-EM data collection, refinement, and validation statistics.

	PADI4_3 (EMDB-19011) (PDB 8R8U)	PADI4_11 (EMDB-19012) (PDB 8R8V)
Data collection and processing		
Voltage (kV)	300	300
Electron exposure (e ⁻ /Å ²)	28.0	28.0
Defocus range (μm)	-1 – -3	-1 – -3
Pixel size (Å)	0.95	0.95
Symmetry imposed	C2	C2
Final particle images (no.)	127k	79k
Map resolution (Å)	3.1	3.6
FSC threshold 0.143		
Refinement		
Initial model used (PDB code)	1WD9	1WD9
Model resolution (Å)	3.2	3.7
FSC threshold 0.5		
Map sharpening <i>B</i> factor (Å ²)	-146.3	-187.6
Model composition		
Non-hydrogen atoms	10140	10198
Protein residues	1282	1292
Ligands	10	10
<i>B</i> factors (Å ²)		
Protein	46.7	63.1
Ligand	29.6	49.2
R.m.s. deviations		
Bond lengths (Å)	0.002	0.005
Bond angles (°)	0.456	0.708
Validation		
MolProbity score	1.20	1.64
Clashscore	4.12	7.29
Poor rotamers (%)	0.18	0.26
Ramachandran plot		
Favored (%)	98.01	96.38
Allowed (%)	1.99	3.62
Disallowed (%)	0.00	0.00

Supplementary Table 3. SPR binding affinities for the different PADI4_3 analogues. Data represents average $\pm$ standard deviation of three independent replicates.

	Sequence	K _D (nM)	K _A (1/Ms)	K _D (1/s)
PADI4_3_Y1Y(Ome)	dY(Ome)RDHHYRHPKYCG	23 $\pm$ 2	$1 \cdot 10^{05} \pm 1 \cdot 10^{04}$	$3 \cdot 10^{-03} \pm 2 \cdot 10^{-05}$
PAD4_3_R2A	dYADHHYRHPKYCG	239 $\pm$ 8	$2 \cdot 10^{05} \pm 5 \cdot 10^{04}$	$3 \cdot 10^{-02} \pm 2 \cdot 10^{-02}$
PAD4_3_R2cit	dYcitDHHYRHPKYCG	20 $\pm$ 11	$5 \cdot 10^{05} \pm 6 \cdot 10^{05}$	$2 \cdot 10^{-03} \pm 2 \cdot 10^{-04}$
PADI4_3_R2K	dYKDHHYRHPKYCG	9 $\pm$ 2	$2 \cdot 10^{05} \pm 2 \cdot 10^{04}$	$2 \cdot 10^{-03} \pm 4 \cdot 10^{-04}$
PADI4_3_H4R	dYRDRHYRHPKYCG	14 $\pm$ 6	$1 \cdot 10^{06} \pm 1 \cdot 10^{06}$	$1 \cdot 10^{-02} \pm 7 \cdot 10^{-03}$
PADI4_3_Y6H	dYRDHHHRHPKYCG	30 $\pm$ 15	$6 \cdot 10^{04} \pm 3 \cdot 10^{04}$	$1 \cdot 10^{-03} \pm 2 \cdot 10^{-04}$
PADI4_3_R7N	dYRDHHYNHPKYCG	41 $\pm$ 18	$1 \cdot 10^{04} \pm 6 \cdot 10^{03}$	$4 \cdot 10^{-04} \pm 1 \cdot 10^{-05}$
PADI4_3_R7W	dYRDHHYWHPKYCG	6 $\pm$ 3	$2 \cdot 10^{05} \pm 1 \cdot 10^{05}$	$2 \cdot 10^{-03} \pm 2 \cdot 10^{-03}$
PAD4_3_R7A	dYRDHHYAHPKYCG	7 $\pm$ 1	$7 \cdot 10^{04} \pm 2 \cdot 10^{04}$	$5 \cdot 10^{-04} \pm 7 \cdot 10^{-05}$
PADI4_3_K10I	dYRDHHYRHPIYCG	4 $\pm$ 2	$1 \cdot 10^{05} \pm 4 \cdot 10^{04}$	$4 \cdot 10^{-04} \pm 2 \cdot 10^{-04}$
PADI4_3_K10V	dYRDHHYRHPVYCG	5 $\pm$ 2	$8 \cdot 10^{04} \pm 2 \cdot 10^{04}$	$4 \cdot 10^{-04} \pm 4 \cdot 10^{-05}$
PADI4_3H4R_K10_I	dYRDRHYRHPIYCG	7 $\pm$ 3	$7 \cdot 10^{05} \pm 4 \cdot 10^{05}$	$2 \cdot 10^{-03} \pm 2 \cdot 10^{-03}$

Supplementary Table 4. Primers used in this study. SP18 is an 18-atom hexa-ethyleneglycol spacer.

Primer Name	Sequence
<i>mPadi4_fw</i>	CATATGATGGCCCAGGGTGCGGTGATC
<i>mPadi4_rev</i>	CTCGAGTCAGGGCACCATGTGCCACCAC
hPADI4_D165A_fw	ATCTTCTGCCATGGCCTGCGAGGATGATGAAG
hPADI4_D165A_rev	CATCATCCTCGCAGGCCATGGCAGAAGATTCTG
hPADI4_D168A_fw	CTGCCATGGACTGCGAGGCTGATGAAGTGCTTGAC
hPADI4_D168A_rev	GTCAAGCACTTCATCAGCCTCGCAGTCCATGGCAG
hPADI4_D167A_fw	CTTCTGCCATGGACTGCGCGGATGATGAAGTGCTTGAC
hPADI4_D167A_rev	GTCAAGCACTTCATCATCCGCGCAGTCCATGGCAGAAG
hPADI4_N373A_fw	TTCGACTCTCCAAGGGCCAGAGGCCTGAAGG
hPADI4_N373A_rev	CTCCTTCAGGCCTCTGGCCCTTGAGAGTCTG
hPADI4_R394A_fw	GATTTTGGCTATGTAAGTGCAGGGCCCCAAACAGGGGG
hPADI4_R394A_rev	CCCCCTGTTTGGGGCCCTGCAGTTACATAGCCAAAATC
hPADI4_C166F_fw	CTCGAATCTTCTGCCATGGACTCCGAGGATGATGAAGTGCTTG
hPADI4_C166F_rev	CAAGCACTTCATCATCCTCGAAGTCCATGGCAGAAGATTCTG
T7g10M.F48	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG
long_HA_reverse	TTTCCGCCCCCGTCCTAAGAACCAGAACCAGAACCTGCATAGTCGGGCACGTCGTATGGGTAGCTGCCGCTGCC
short_HA_reverse	TTTCCGCCCCCGTCCTAAGAACCAGAACCAGAACC
PAD4_3_F_NNK1	CTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKGATCATCATTATAGGCATCCGAAGTATTGC
PAD4_3_F_NNK2	CTATAGGGTTAACTTTAAGAAGGAGATATACATATGAGGNNKCATCATTATAGGCATCCGAAGTATTGC
PAD4_3_F_NNK3	CTATAGGGTTAACTTTAAGAAGGAGATATACATATGAGGGATNNKCATCATTATAGGCATCCGAAGTATTGC
PAD4_3_F_NNK4	CTATAGGGTTAACTTTAAGAAGGAGATATACATATGAGGGATCATNNKTATAGGCATCCGAAGTATTGC
PAD4_3_F_NNK5	CTATAGGGTTAACTTTAAGAAGGAGATATACATATGAGGGATCATCATNNKAGGCATCCGAAGTATTGC
PAD4_3_R_NNK6	TATGGGTAGCTGCCGCTGCCGCAATACTTCGGATGMNNATAATGATGATCCCTCATAT
PAD4_3_R_NNK7	TATGGGTAGCTGCCGCTGCCGCAATACTTCGGMNNCCTATAATGATGATCCCTCATAT

PAD4_3_R_NNK8	TATGGGTAGCTGCCGCTGCCGCAATACTTMNNATGCCTATAA TGATGATCCCTCATAT
PAD4_3_R_NNK9	TATGGGTAGCTGCCGCTGCCGCAATAMNNCGGATGCCTATA ATGATGATCCCTCATAT
PAD4_3_R_NNK10	TATGGGTAGCTGCCGCTGCCGCAMNNCTTCGGATGCCTATA ATGATGATCCCTCATAT
PAD4_AttB1_F	GGGGACAAGTTTGT CAAAAAAGCAGGCTTCACCATGGCCCAGGGGACATTGATCC G
PAD4_AttB2_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGGGCACCA TGTTCCACC
Pu_linker	[5'Phos]CTCCCGCCCCCGTCC[SP18][SP18][SP18][SP18][SP18] 8]CC[Puromycin]

Supplementary Table 5. Macrocytic peptides used in this study. All peptides are cyclic (thioether bond between the Acetylated N-terminus and the cysteine side chain) unless otherwise specified. Abbreviations: Ac – N-terminal acetylation; y – D-Tyrosine; Cit – citrulline; Y(OMe) – O-Methyl-Tyrosine; B – β -Ala; K(Z) – Z attached via an amide bond to the lysine side chain where Z could be: bio (biotin), Cl (cl-alkane). Residues modified from the parent sequences are highlighted in bold. *Mass determined by MALDI.

Peptide Name	Peptide Sequence	Mass Calculated /Da	Deconvoluted Mass Observed /Da
PAD4_1	yFYRIGFWYPNYQC(S-)G-NH ₂	2016.3	2015.3*
PAD4_2	yRDHRSPFDGYC(S-)G-NH ₂	1611.7	1611.5
PAD4_3	yRDHHYRHPKYC(S-)G-NH ₂	1771.0	1770.1
PAD4_3i	y DR HHYRHPKYC(S-)G-NH ₂	1771.0	1769.8
PAD4_4	yHRLIVVIYVC(S-)G-NH ₂	1473.8	1473.6
PAD4_5	yATPWLIVVLC(S-)G-NH ₂	1373.7	1372.8
PAD4_6	yLVLITRLVLC(S-)G-NH ₂	1401.8	1400.9
PAD4_7	yYPKGSWGYKLFC(S-)G-NH ₂	1708.0	1707.5
PAD4_8	yTLWTVLVVIC(S-)G-NH ₂	1406.7	1427.5* (+Na)
PAD4_9	yAQWYIWVLLC(S-)G-NH ₂	1553.9	1553.4
PAD4_10	yPWEISVWLLYC(S-)G-NH ₂	1668.0	1666.3
PAD4_11	YESC(S-)RYRQVLQL-NH ₂	1596.8	1596.0
PAD4_11_i	YSEC (S-)RYRQVLQL-NH ₂	1596.8	1597.6
PAD4_12	YEPC(S-)RFREILDL-NH ₂	1592.8	1593.5
PAD4_3_R2A	y AD HHYRHPKYC(S-)G-NH ₂	1685.8	1685.8
PAD4_3_R7A	yRDHHY A HPKYC(S-)G-NH ₂	1685.8	1684.1
PAD4_3_R2cit	y Cit DHHYRHPKYC(S-)G-NH ₂	1771.9	1772.0
PAD4_3_R2K	y KD HHYRHPKYC(S-)G-NH ₂	1742.3	1742.8
PAD4_3_y1(OMe)Y	Y(OMe)RDHHYRHPKYC(S-)G-NH ₂	1785.0	1784.9
P4_3_H4R	yRDR H YRHPKYC(S-)G-NH ₂	1790.0	1789.4
P4_3_K10I	yRDHHYRHP I YC(S-)G-NH ₂	1755.9	1755.6
P4_3_K10V	yRDHHYRHP V YC(S-)G-NH ₂	1741.9	1741.6
P4_3_H4R_K10I	yRDR H YRHP I YC(S-)G-NH ₂	1775.0	1774.9
P4_3_R7N	yRDHHY N HPKYC(S-)G-NH ₂	1728.9	1728.6
P4_3_R7W	yRDHHY W HPKYC(S-)G-NH ₂	1801.0	1800.9
P4_3_Y6H	yRDHH H RHPKYC(S-)G-NH ₂	1744.9	1744.7
PAD4_11_Y1A	AESC (S-)RYRQVLQL-NH ₂	1504.7	1503.8
PAD4_11_E2A	YASC (S-)RYRQVLQL-NH ₂	1538.8	1539.4
PAD4_11_S3A	YEAC (S-)RYRQVLQL-NH ₂	1580.8	1581.3
PAD4_11_C4A	Ac-YES A RYRQVLQL-NH ₂	1524.7	1565.5
PAD4_11_R5A	YESC(S-) A YRQVLQL-NH ₂	1511.7	1510.7
PAD4_11_Y6A	YESC(S-) R ARQVLQL-NH ₂	1504.7	1505.8
PAD4_11_R7A	YESC(S-) R Y A QVLQL-NH ₂	1511.7	1512.2
PAD4_11_Q8A	YESC(S-) R Y R AVLQL-NH ₂	1539.8	1538.8
PAD4_11_V9A	YESC(S-) R Y R Q A LQL-NH ₂	1568.8	1569.4
PAD4_11_L10A	YESC(S-) R Y R Q V AQL-NH ₂	1554.8	1555.6
PAD4_11_Q11A	YESC(S-) R Y R QVL A L-NH ₂	1539.8	1540.3
PAD4_11_L12A	YESC(S-) R Y R QVL Q A-NH ₂	1554.8	1555.2
PADI4_11_R5K	YESC(S-) K YRQVLQL-NH ₂	1568.3	1568.0
PADI4_11_R7K	YESC(S-) K Y K QVLQL-NH ₂	1568.3	1568.4

PADI4_11_E2Q	YQSC(S-)RYSRQVLQL-NH ₂	1593.8	see 2+ ion 797.8
PADI4_11_E2D	YDSC(S-)RYSRQVLQL-NH ₂	1580.8	1581.0
PADI4_11B	YESC(S-)Y-NH ₂	858.9	858.1
PAD4_3_CAPA	yRDHHYRHPKYC(S-)GSBK(Cl)-NH ₂	2364.4	2362.4
PAD4_3i_CAPA	y DR HHRHPKYC(S-)GSBK(Cl)-NH ₂	2364.4	2364.3
PAD4_11_CAPA	YESC(S-)RYSRQVLQLGSBK(Cl)-NH ₂	2247.5	2245.5
PAD4_11i_CAPA	y SEC (S-)RYSRQVLQLGSBK(Cl)-NH ₂	2246.9	2244.8
P4_7_bio	yYPKGSWGYKLFC(S-)GSBK(Bio)-NH ₂	2220.6	2220.1
P4_7scr_bio	yYPKGSWGYKLFC(S-)GSBK(Bio)-NH ₂	2220.6	2220.4

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hPADI2 plasmid

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His Tag
Avi Tag
PADI2

hPADI3 plasmid

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His Tag
TEV protease cleavage site
PADI3

[illegible]

46

mPADI4 plasmid

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His Tag
Avi Tag
mPADI4

48

hPADI4_C166F plasmid

GCTAACAGCGCGATTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCAGTCGCGTACCCTTTCATGGGAGAAAAATAACTGTTGATGGGTGCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCCACTGACCGGTTGGCGGAGAAGATTGTCACCGCGCGTTTACAGGCTTCGACGCCGCTTCGTTTACCATCGACACACCGCATCCGCTGATCGCGCGGAGATTAACTCGCGCGGACATAATTTGCGACGCGCGTGACAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCGCTTCCACTTTTTTCCGCGCTTTTCGCGAAGACGTGGCTGGCCCTGGTTTACCACGCGCGGGAACCGGTCTGATAAGAGACACCGGCATATCTCTCGACATCGTATAACGTTACTGGTTTACATTCACCACCTGAATTAATGACTCTCTCCGCGCGTATCATGCCATACCGCGAAAGGTTTTCGCCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCAGTAGTAGGTTGAGCCGTTGAGCACCGCCGCCCAAGGAATGGTGATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCTGCCACCATAACCCAGCGCGAAACAAGCGCTCATGAGCCGAAGTGGCGAGCCGATCTTCCCCCATCGGTGATGTCTGGCGGATAGGGCCAGCAACCCGCACTGTGGCGCCGTTGATGCCGCCACGATGCGTCCGGCTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTTGTAGCGGATAACAATCCCCCTCTAGAAATAATTTGTTTAACTTTAAGAAGGAGATATACCATGAGCGCCCTGAACGATATTTTGAAGCCGAGAAAAATGAATGGCATGAAGGACAGCGCTGGAGGTTACAGTATCACCACCACCATCATCTGATGACGAAGCTTTCGCGCCGCACTCGAGCACCAACCACCACCACCCTGAGATCCGGCTGCTAAACAAAGCGGAAAGGAAAGTGAAGTGGCTGCTGCCACCTGAGCAATAACAGCAATAACCCCTTGGGGCCTCTAAACGGGTCTTGAAGGGTTTTTTCGCGTGAAGAGAACTATATCCGATTTCGCGAATGGCAATGGGACGCGCCCTGTAGCGCGCATTAAGCGCGCGGTGTGGTGGTTACGCGCAGCGTGACCCTACACTTGGCAGCGCCCTAGCGCTCCTTTCGCTTTCCTTTCCTTTCCTTTCCTCGCCACGTTTCGCGCGCTTCCCCGCTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTATAGTGCTTTACGGACCTTCGACCCCAAAAACTTGATTAGGGTGATGTTTACAGTGGGCGCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGGTTGGAGTCCACGTTCTTTAATAGTGACTCTTGTTCCAACTGGAACAACACTCAACCTTACCTCGGTCTCGTTTTCGATTATAAGGATTTTCGCCATTCGCGCTCGTCCGCTTTCGCTAAATAAGTGAAGTATTAATTTCCCTTCGTCAAAAAATAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTCCAGACTTGTTCACAGGCCGATTACGCTCGTCATCAAAATCACTCGCATCAACAAACCGTTATTATTCTCGTGATTGCGCCTGAGCGAGACGAAATACGCGACTGTGTTAAAGGACAATTACAAACAGGAATCGAATGCAACGGCGCAGGGTCGGAACAGGAGAGCGCACGAGGAGCTTCCAGGGGGAACCGCTGGTATCTTTATAGTGTATACCTGGAATGCTGTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAAATCCGTGAGCCAGTTAGTGTGACCATCTGTAACATCAATTCACCAAAATTTAACGCAATTTTAACAAAATATTAACGCTTACAATTTAGTGGCACTTTTCGGGGAATGTGCGCGGAACCCCTATTGTGTTATTTTTCTAAATACATTCAAAATATGATCCGCTCATGAATTAATTTCTAGAAAACTCATTCGAGCATCAAAATGAAATGCAATTTATTCATATACGATTATCAATATTTTGAAGGAGCGCTTTCTCTAATGAAGGAGAAAACTCACCGAGGAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATCCGACTCGTCCCAACATCAATACAACCTATTAATTTCCCTTCGTCAAAAAATAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTCCAGACTTGTTCACAGGCCGATTACGCTCGTCATCAAAATCACTCGCATCAACAAACCGTTATTATTCTCGTGATTGCGCCTGAGCGAGACGAAATACGCGACTGTGTTAAAGGACAATTACCAACAGGAATCGAATGCAACGGCGCAGGGTCGGAACAGGAGAGCGCACGAGGAGCTTCCAGGGGGAACCGCTGGTATCTTTATAGTGTCTATAACACCCCTTGATTTACTGTTTATGTAAGCAGACAGTTTTATTGTTTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTTCCTGAGCGTCAGACCCCGTAAAAAGATCAAGGATCTTCTTGAGATCTTTTTTTCGTGCGGTATCTGCTGCTTGCAAAACAAAAAACCCCGCTACAGCGGTGGTTGTTTGGCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAATACTGTCCTTCTAGTGATAGCCGTAGTTAGGCCACCCTTCAAGAACTCTGTAGCACCGCTACATACCTCGCTGCTTAATCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTCTTACCGGTTGGACTCAAGACGATAGTTACCAGGATAAGCGCAGCGGTGGGCTGAACGGGGGTTTCGTGCACACAGCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGTTCCCGAAGGAGAAAGCGGACAGGTATCCGGTAAGCGCGCAGGGTCGGAACAGGAGAGCGCACGAGGAGCTTCCAGGGGGAACCGCTGGTATCTTTATAGTCTGTCGGGTTTCGCCACCTTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGCGGAGCCCTATGGAAAAACGCCAGCAACCGCGGCTTTTACGGTTCCCTGGCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCTGATTCTGTGGATAACCGTATTACCAGCTTTGAGTGAGCTGATACCGCTCGCCGACGCGAACGACCGAGCGCAGCGATGAGTGAGCGAGGAAGCGGATTCACAGATGTCTGCCGTATTTCTCCTTACGCATCTGTGCGGTATTTACACCCGCAATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCTAGGCTGCGCCCGACACCCCGCAACCCCGCTGACGCGCCCTGACGGGCTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTGAGAGGTTTTACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCTGTAAGCGGATTACAGATGTCTGCCGTGTTTCATCCGCTCCAGCTCGTTGAGTTTCTCCGAAGCGTTAATGGTGTCTGGCTCTGATAAAGCGGCCATGTTAAGGGCGGTTTTTCTCTGTTTGGTCACTGATGCCCTCCGTGAAGGGGATTCTGTTTCATGGGGTAATGATACCGATGAAACGAGAGGATGCTCAGGATACGGGTACTGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGCGGGACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGTGCGGGCGCTGACTCCGCGTTTCCAGACTTTACGAAACAGGAAACCGAAGACCATTCATGTTGTTGTCAGGTCGCAGACGTTTTGACGACGACGTTCGTTTACGTTCTGCTCGCGTATCGGTGATTCTGCTAACCAGTAAGGCAACCCCGCAGCCTAGCCGGGTCTCAACGACAGGAGCAGCATCATGCGCACCCGTGGGGCGCCATGCCGGCGATAAATGGCTTTCGCGGAAACGTTTGGTGCGGACAGTGACGAAGGCTTGCTGCGCAGCTGTCATTAATGAGCTGTCTGCGGAAACGTTTGGTGCGGAAACCTGTCTGCCAGTGCATTAATGAGCTGTCTTCGGTATCGTTCGATATCCCACTACCGAGATATCCGACCAACGCGCAGCCCGGACTCGGTAAATGGCGCGCATATGCGCTGCGCTTGGCAACCAAGCATCGAGTGGGAACGATGCCCTCATTCAGATTTCGATGGTTTTGTTGAAACCGGCATGGCACTCCA

His Tag
Avi Tag
hPADI4
C166F variant

50

hPADI4_D168A plasmid

TCCAC TTTTCCCGCG TTTTCG CAGAAACG TG GGTGGCCTGG TTACCACGCGGGAAACGGGTCTGATAAGAGACACCGGCATAC TCTCGC GACATCGTATAACGTTAC
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ATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGAGC **GGCCTGAACGATATTTTGAAGCCGAGAAAATTAATGGCATGAAG**
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GCCATGGACTCGGAG **CGT** **GATGAAGTGCTTGACAGCGAAGACCTGCAGGACATGTCTGCTGATGACCCCTGAGCACGAAGACCCCAAGGACTTCTTCACAAACCATAC**
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His Tag
Avi Tag
hPADI4
D168A variant

52

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