Supplementary information

SITE DIRECTED MUTAGENESIS OF INSULIN-DEGRADING ENZYME ALLOWS TO SINGLE OUT THE MOLECULAR BASIS OF PEPTIDASE VERSUS E1-LIKE ACTIVITY: THE ROLE OF METAL IONS

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EXPERIMENTAL PROCEDURES

Materials. Ub from bovine red cells was obtained from Sigma Aldrich and purified by extensive dialysis against pure water for 24 h at 48 °C, as reported elsewhere.[1] Protein concentration in pure water was routinely measured by UV (ε 280 = 1280 M^{-1} cm^{-1}). Dithiothreitol (DTT) was purchased from Fluka. IDE was purchased either from GIOTTO Biotech s.r.l .(Italy). Human recombinant Ub activating Enzyme (UBA1), E2-25K (E2) were purchased from Boston Biochem. Ammonium molybdate heptahydrate and sodium pyrophosphate were obtained from Aldrich and used without further purification. The solution containing 400 mM EDTA and 1,4 M NaOH was prepared and used for terminate the enzimatic reactions. For SDS PAGE NuPAGE® Novex® Bis-Tris gels and 2-(N-morpholino) ethanesulfonic acid (MES) buffer were obtained from Invitrogen. For Western blotting, mouse anti mono- and poly-ubiquitinylated conjugates, mAb (clone FK2) was obtained from ENZO Life Science. All solutions were prepared with ultrapure Milli Q water. Aβ1-40 (GenScript) was treated with trifluoroacetic acid (TFA) and hexafluoro-isopropanol (HFIP), as previously reported,[2] in order to enrich the protein sample of the monomeric form.

Circular Dichroism spectroscopy. Far-UV CD spectra of IDE mutants were recorded using a Jasco J-810 spectropolarimeter equipped with a Peltier thermally controlled cuvette holder (JASCO PTC-348). Protein samples (1.8 - 2 μM) were dissolved in pH 7.4 10 mM phosphate buffer. CD spectra were recorded in the far UV region (260-200 nM) using a 0.1 cm path length quartz cuvette, with the following settings: 0.1 nm data pitch, 50 nm/min scan rate, band width 1 nm, response time of 2 s. All spectra, corresponding to an average of 3 scans, were base-line-corrected by subtracting the signal of the protein-free buffer from the sample. CD melting curves were collected at 222 nm using the following settings: 1 K /min heating rate, range 20-90 °C, 1 nm bandwidth, and 0.2 °C steps. CD melting curves are reported as normalized fraction of unfolded vs folded protein for an easy comparison.

Mass spectrometric measurements of IDE proteolytic activity

All the enzymatic reactions were performed in conditions of steady-state kinetics. Moreover, the stock solutions of IDE (both the wild type and the mutant forms) (1-2 μM) were appropriately treated with zinc(II) (100 μM) in order to ensure saturation of the enzyme. After 30 min at 37°C, the metal ion, the samples were desalted by using a PD-10 column (GE Healthcare).

$AcGlyGluArgGlyPhePheTyrThrProLysAlaNH_2$ (B20–30) peptide was synthesized as described previously.[3] IDE enzymatic digestions were carried out by incubating at 37°C a solution containing B20-30 50 μM, IDE 8 ng· ml^{-1} in PBS 10 mM and metal ions 50 μM (as indicated). B20-30 Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) analysis was performed

using a Thermo-Finnigan LCQ Deca XP instrument. Capillary temperature was 300°C. The chromatographic analysis, coupled with ESI-MS detection, was performed with solvents A (0.05% TFA in water) and B (0.05% TFA in acetonitrile) on a Jupiter C4 PHENOMENEX® (4.6x150 mm, 5-μm particle size) column, at flow rate of 0.2 mL min^{-1}. The temperature of the column was 45°C, as a better separation of the B20-30 fragments on the chromatogram was recorded at this temperature and the overall time of the analysis was 32 min. The XCALIBUR software was used to analyse the data. Other peptides which might have worked as internal standards for quantitative measurements have not been used to avoid possible interactions with the enzyme leading to alterations of IDE activity. For this reason, IDE activity was estimated by calculating the following ratio:

$$\text{Activity} = I_{754.3}/(I_{1313.7}+I_{657.3}) \quad (1)$$

Where $I_{754.3}$ is the area of the peak at m/z 754.3 (main fragment produced by IDE enzymatic digestion), whereas $I_{1313.7}$ and $I_{657.3}$ are the areas of the mono and doubly charged molecular peak of the B20-30 observed in the chromatogram. In order to compare the activity of the various mutants towards the B20-30 substrate at the different experimental conditions, we have chosen a unique digestion time (48 h) and we have compared the activity of each mutant to the activity of the *wt* IDE calculated from Eq. (1) (see Table 1S).

Enzymatic reactions of Aβ1-40 (10 μM) and IDE (5 nM) were carried out at 37 °C for 60 minutes. copper(II) and zinc(II) ions were equimolar to the concentration of Aβ1-40. Hydrolytic patterns of the Aβ degradation catalysed by *wt* IDE and all the mutants were analyzed by MALDI TOF/TOF 5800 Analyzer (AB SCIEX, Foster City, CA). The instrument is equipped with a nitrogen UV laser (λ=337 nm) pulsed at a 20 Hz frequency. An accelerating potential of 20 kV and a grid percentage equal to 70% were used for the ion reflector mode. Mass spectra were recorded with the laser intensity set just above the ionization threshold (2800 in arbitrary units) to avoid fragmentation and labile group losses, to maximize the resolution and to result in a strong analyte signal with minimal matrix interference. Time delay between laser pulse and ion extraction was set to 450 ns. Typically, mass spectra were obtained by accumulation of 200 and 800 - 1000 laser shots in reflector and linear mode, respectively, and processed using Data Explorer 4.0 software (Applied Biosystems). Saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in water:acetonitrile = 2.3:1 with 0.3% TFA was used as matrix. N-terminal amidated Aβ1-16 (0.5μM) was used as an internal standard and was dissolved in the matrix solution. Therefore, the intensities of all peaks assigned to the Aβ1-40 hydrolytic peptides were reported as relative percentage of the amidated Aβ1-16 peak.

The Aβ1-40 hydrolytic pattern produced by the action of <u>*wt*</u> IDE and F530A was also analysed using a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific) coupled to an Ultimate 3000 HPLC RSLCnano system (Dionex Thermo Scientific) through an EASY-Spray source (Thermo Scientific). Capillary temperature and voltage were 300 °C and 2 kV, respectively. The MS instrument acquisition was performed in Full scan mode (70,000 resolution, scan range 500 to 2000 m/z, maximum injection time 50 ms, AGC target 1·106) and MS/MS mode (17,500 resolution, scan range 200 to 2000 m/z, maximum injection time 50 ms, AGC target 1·105). The chromatographic separation of peptides was performed on EASY-Spray PepMap® C18 column (75 μm × 150 mm, 3 μm particle size, 100 Å pore size) at a flow rate of 0.3 μL/min with solvent A (water with 0.1% formic acid) and solvent B (80% acetonitrile, 0.1% FA in water). All the protein adducts and the peptide fragments were eluted by using a linear gradient from 5% to 40% of eluent B. Peak detection for quantitative evaluation of the peptide fragments was carried out using the extracted ion chromatogram (XIC) related to the most abundant charged species detected for each peptide fragment. MS and MS/MS (HCD) spectra were used for the identification and the unambiguous assignment of the peptide fragments

Lys48 Ub-chain elongation reactions in tube tests

Lys48 Ub-chain elongation reactions were performed at pH 7.4 (T = 25°C) in small volumes (40 µL) of ligation buffer (50 mM MOPS, 5 mM $MgCl_2$, 30 µM DTT and 2 mM ATP) containing Ub (10 µM), UBA1 (500 nM) or IDE native or IDE mutant (2 µM) and E2-25K (1 µM). The reactions were quenched after 3 hours incubation and size-fractioned by SDS-polyacrylamide gel electrophoresis. They were then electro-transferred onto a nitrocellulose membrane (GE Healtcare, Lifescience). The membranes were blocked with Odyssey blocking buffer for 1 hour and then incubated overnight at 4°C with polyubiquitin antibody. The membrane was washed thrice for 5 minutes with PBS-T (PBS,0.05% Tween 20) and then incubated with IRDye 800–labeled secondary antibody (1:12000) from Molecular Probes (Eugene, OR) for 30 minutes. Visualization of membrane was done using the LI-COR Odyssey IR Imaging System (LI-COR Biosciences, Lincoln.

Spectrophotometric determination of $[P_2O_7]^{2-}$ anion in Ub activation

The Ub chain elongation reactions were carried out for one hour as described in the previous paragraph. The amounts of reactions (10 µL) were collected in a 96 well plate and analysed by a Varioskan plate reader. In each well, 10 µL of a 400 mM EDTA solution were used to quench all reactions. Then, solutions were incubated for 10 min with 200 µl of 3 mM ammonium molibdate in 0,6 M HCl (60% AcN/W). The colorimetric reaction was started by adding 80 µL of a solution of ascorbic acid (500 mM) in 2M HCl (60%AcN/W). After 10 min the optical absorbance was measured at 790 nm. Calibration curves for accurate pyrophosphate measurements were obtained by using standard solutions containing different pyrophosphate concentrations (0-70 µM). Data from three independent experiments are represented as means ± standard deviation. The chi-square test was used for statistical analysis. A P value below 0.05 was considered as significant.

Figures

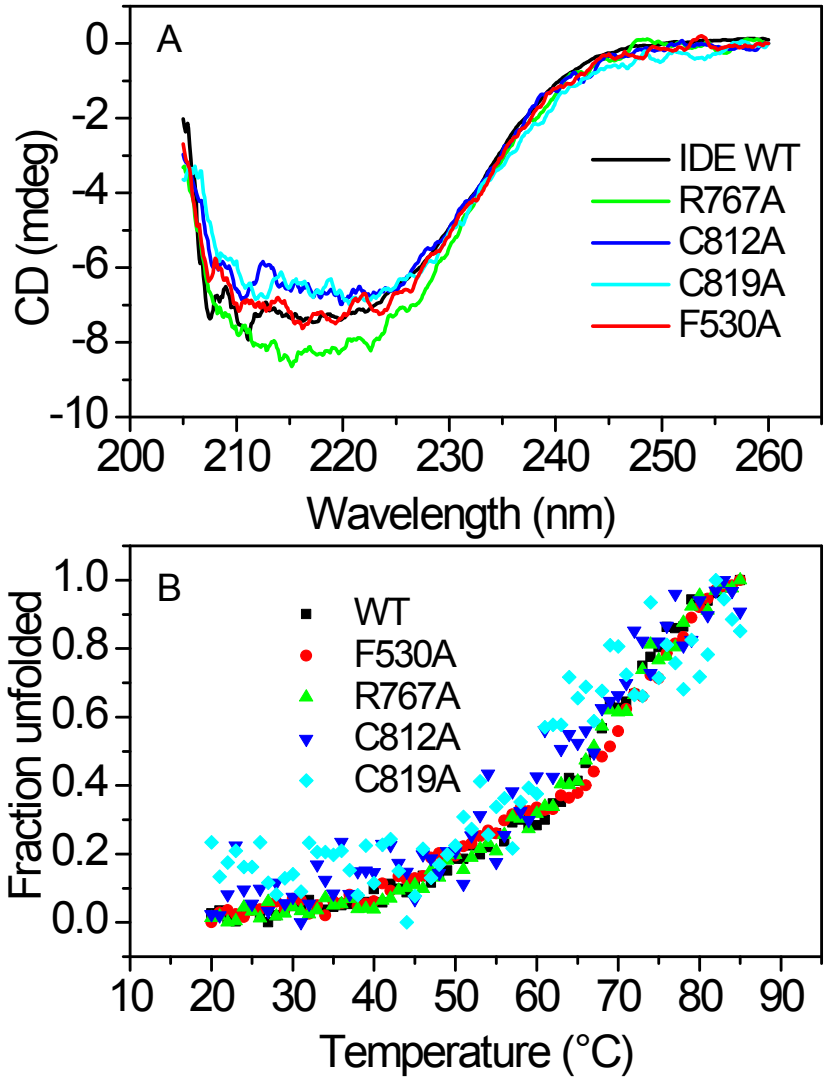

Figure 1S. Panel A. CD spectra of *wt* IDE and its F530A, R767A C812A and C819A mutants in the far UV region. CD spectra were collected at 25°C, 10 mM phosphate buffer pH 7.4 and protein concentration ranging from 1.8 to 2.0 μM. Panel B. CD melting profiles of *wt* IDE and its mutants obtained by monitoring the decrease of the CD signal at a 220 nm as a function of temperature (heating rate 1 K/min). The experimental conditions were the same reported in panel A.

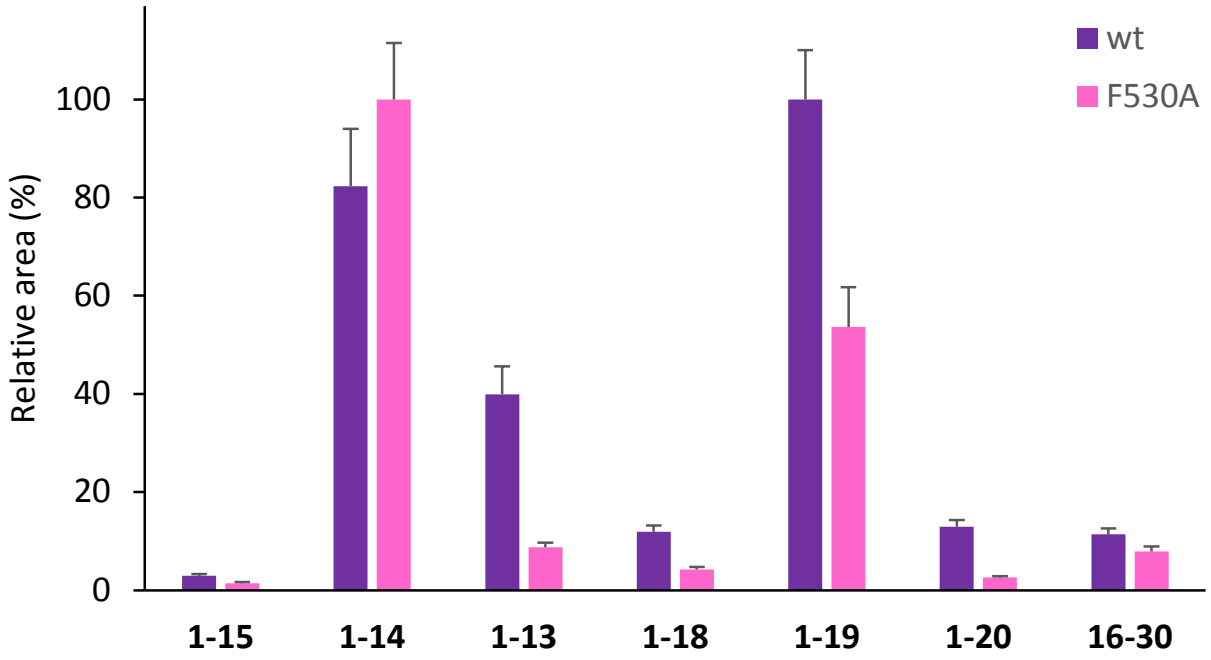

Figure 2S. Comparison between the hydrolytic peptide pattern of Aβ1-40 in the presence of *wt* IDE and F530A mutant.

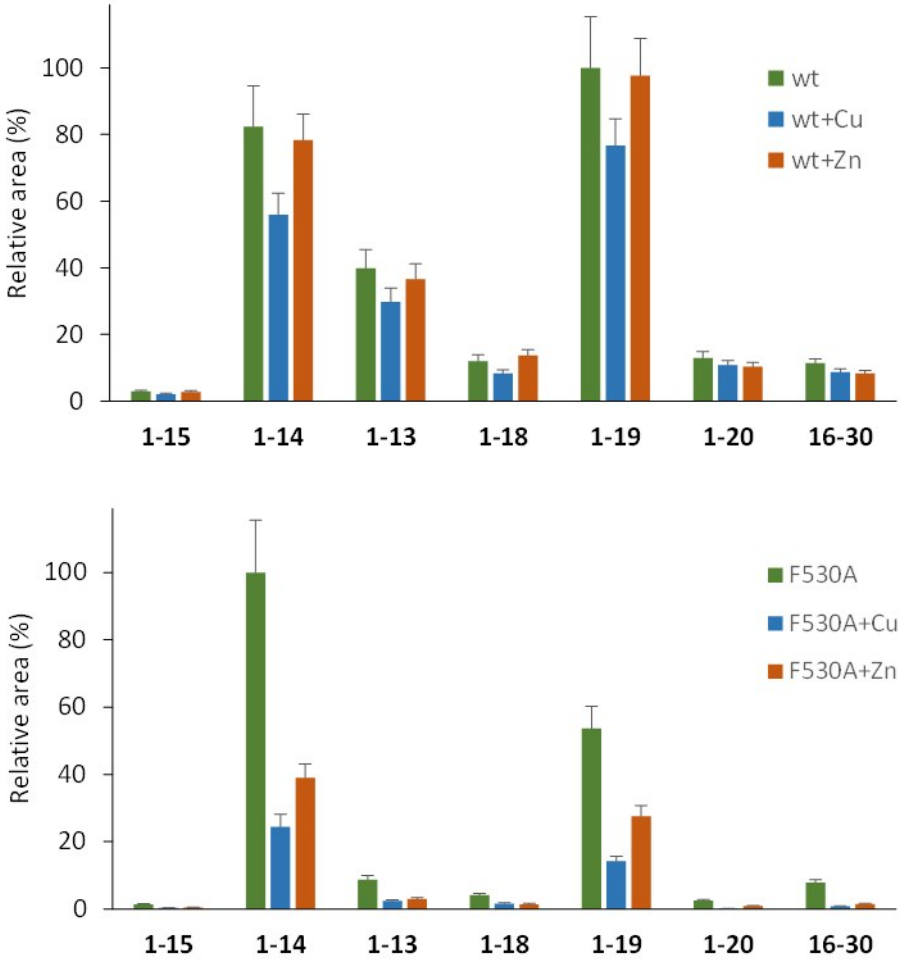

Figure 3S. *Wt* IDE (top) and F530A (bottom) mediated hydrolytic peptide patterns of Aβ1-40 compared to those in the presence of copper(II) and zinc(II).

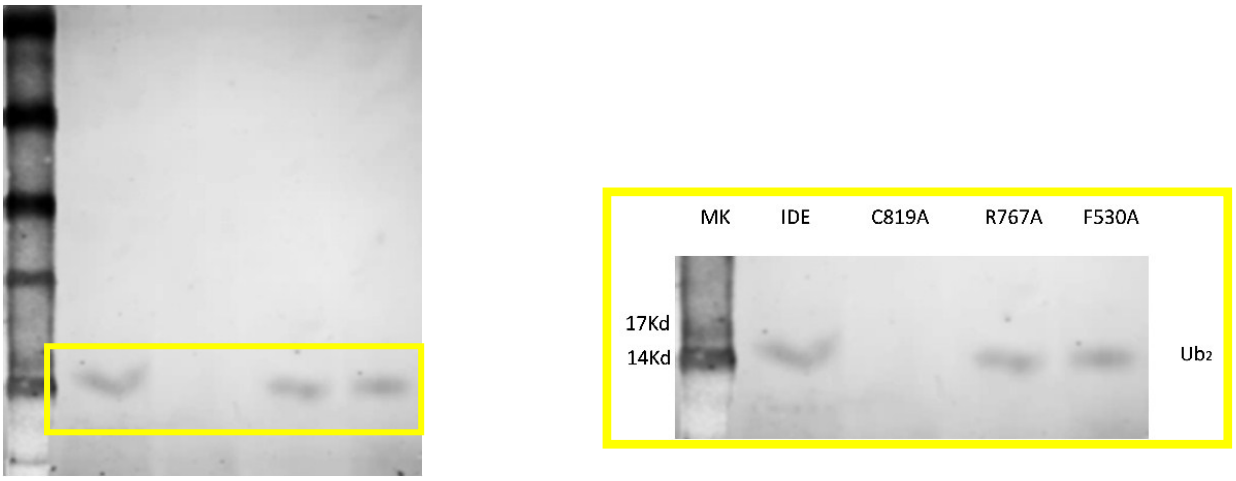

Figure 4S. Western blots of polyubiquitination reactions performed in presence of IDE or IDE mutants C819A, R767A, F530A. Reaction mixture: Ub (10µM), IDE (or IDE mutant) 2 µM, DTT (500µM), MgATP (5 mM) E225K (1µM) in ubiquitination buffer (Tris-HCl 50 Mm pH 7,2).

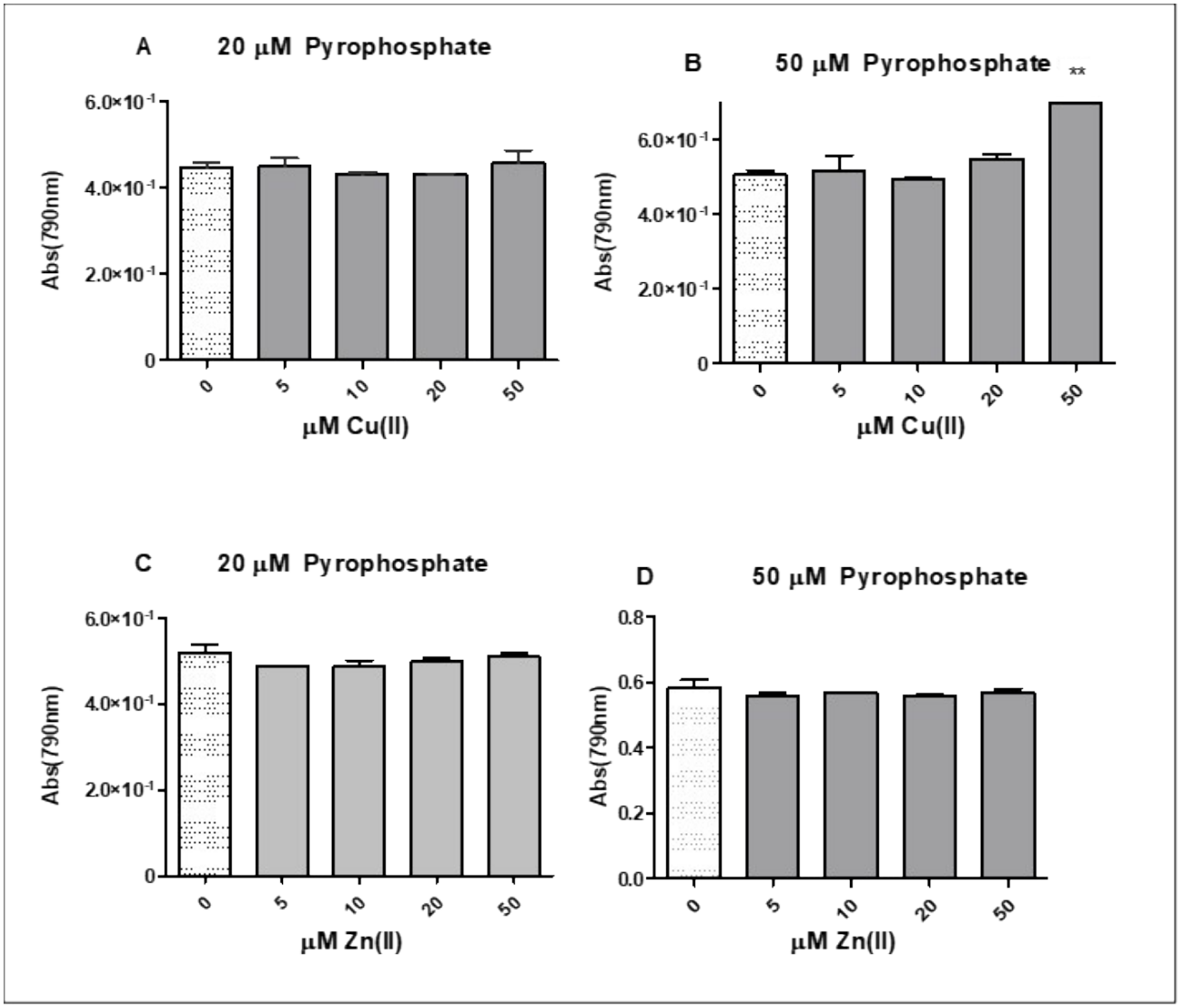

Figure 5S. Colorimetric determination of pyrophosphate in standard solutions of sodium pyrophosphate, respectively 20 and 50 μM in absence and in presence of Cu(II) and Zn(II) in increasing concentration (range 0-50 μM) to evaluate possible interferences with molybdenum assay.

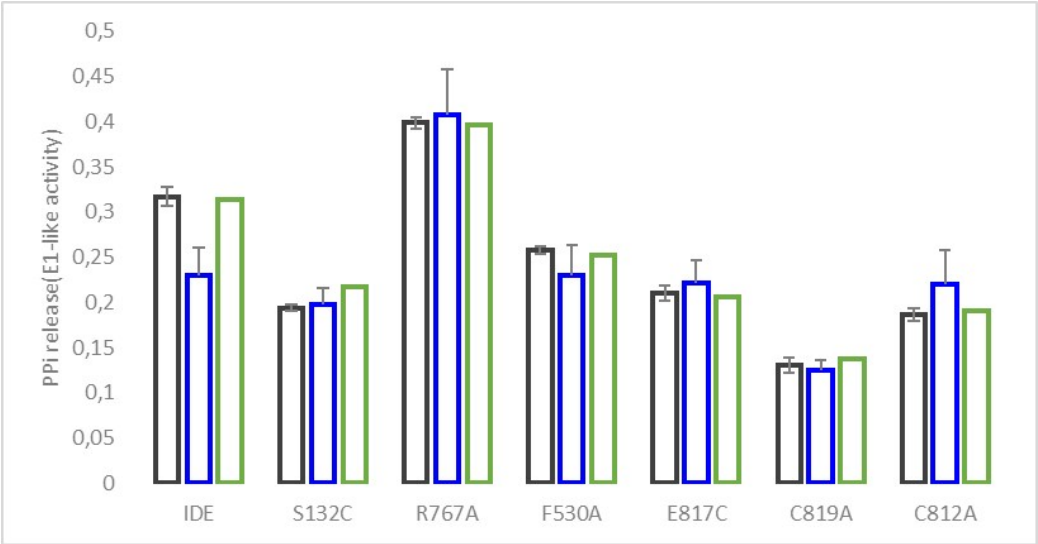

Figure 6S. Pyrophosphate formation during Ub chain elongation reactions by different IDE mutants, in presence of 20 μM copper(II) (blue bars) and zinc(II) (green bars) solutions.

Tables

IDE	$\frac{I_{754.3}}{I_{1313.7} + I_{657.3}}$	Percentage of activity (%)	Relative activity (%)
Wt	0.096	100	100
Wt **+ Cu^{2+}**	0.0087	9.06	9.06
Wt **+ Zn^{2+}**	0.123	128.1	128.1
C812A	0.011	11.45	100
C812A + Cu^{2+}	0.0024	2.5	21.8
C812A + Zn^{2+}	0.0079	8.23	71.9
C819A	0.0085	8.85	100
C819A + Cu^{2+}	0	0	0
C819A + Zn^{2+}	0.0052	5.41	61.1
F530A	0.018	18.75	100
F530A + Cu^{2+}	0.005	5.20	27.7
F530A + Zn^{2+}	0.013	13.75	73.3
R767A	0.0056	5.83	100
R767A + Cu^{2+}	0.0032	3.07	52.6
R767A + Zn^{2+}	0.0042	4.38	75.1

Table 1S. Relative activity of IDE mutants towards the B20-30 peptide. The third column indicates the percentage of activity respect to the *wt* enzyme; the last column on the right describes the relative activity of the various mutants in the presence of the indicated metal ions (1:1 with B20-30) referred to the activity of each mutant without metal ion addition.

Aβ fragment	MW	wt	F530A	R767A	C812A	C819A
1-13	1561.77	8.6	-	-	-	-
16-30	1581.88	24.1	7.1	-	-	-
1-14	1698.80	31.0	11.7	-	-	-
21-40	1886.09	7.2	-	-	-	-
15-33	1993.19	1.4	-	-	-	-
20-40	2033.16	58.0	3.9	-	-	-
14-33	2130.20	0.5	-	-	-	-
19-40	2180.22	3.3	0.9	0.7	-	-
1-19	2314.21	276.6	43.7	-	-	-
1-20	2461.27	27.9	5.8	-	-	-
16-40	2520.31	7.6	-	-	-	-
1-21	2532.30	-	-	-	0.3	-
15-40	2648.56	11.5	2.9	-	-	-
14-40	2785.60	0.6	-	-	-	-
1-29	3318.71	0.4	-	0.9	-	-
1-33	3672.88	2.0	-	-	-	-

Table 2S. List of the peptide amyloid fragments formed, along with the relative activity of IDE wild type (*wt*) and mutants towards $A\beta_{1\text{-}40}$, using Aβ1-16 (0.5μM) as internal standard.

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