Supporting Information

Substrate Profiling of Mitochondrial Caseinolytic Protease P via a Site-Specific Photocrosslinking Approach

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Supplementary Figure S1. Screening of different PylRS variants for the incorporation of DiazK in response to a TAG codon introduced into sfGFP at position 149 in *E. coli*. a) Of the tested PylRS variants for the site-specific incorporation of DiazK into position 149 of sfGFP in *E. coli*, usage of RS3, RS4 and RS5 resulted in visible full-length protein synthesis, as judged by SDS-PAGE. Importantly, no misincorporations in absence of DiazK were observed. RS4 (M6 PylRS: Y271M, L274A, C313A, dubbed DiazKRS) exhibited the best incorporation efficiency, as judged from the ratio of full length vs. truncated protein and was subsequently used for all experiments. Expressions of sfGFP-N149DiazK in *E. coli* were similarly performed as described for the hClpP-DiazK-His6 expressions. b) His6-tag purification of sfGFP-N149DiazK followed by ESI-MS analysis confirmed the identity and integrity of DiazK.
**Supplementary Figure S2.** Structural analysis of hClpP to determine suitable positions for the site-specific incorporation of DiazK. 

a) Full catalytic activity of the serine protease hClpP requires assembly of two hClpP heptameric rings (monomer highlighted in dark grey) into a barrel-shaped tetradecamer (top and side view). Highlighted in pink spheres are the catalytic triads of the serine protease (S153, H178, D227), which lie in close proximity to the heptamer-heptamer interaction interface. 

b) Based on the location of the catalytic triad, several positions for the site-specific incorporation of DiazK were elected. Six positions (M88, D92, G123, G124, V125 and S181 (red spheres)) are located close to the catalytic centre (as depicted in the monomer) and within the barrel chamber, as depicted in the top view. M88 (ß1-sheet) and D92 (α2-helix) lie between the substrate entry pore and the catalytic triad, while the G123/G124/V125 junction loop (between β2-sheet and α3-helix) is in close proximity to the catalytic S153. Furthermore, S181 resides on the β7-sheet proximal to H178 of the catalytic triad. K261 resides on the outer side of the barrel. This position was additionally selected to potentially account for interactors.

c) Side view of the selected positions within the tetradecameric structure. All figures are based on PDB: 1TG6, ref. [1]
Supplementary Figure S3. Expression of hClpP-DiazK-H6 variants in *E. coli*. The full SDS-PAGE and western blots of Figure 1b are shown. Expression of hClpP in *E. coli* was performed without the N-terminal 56 amino acid mitochondrial guide-sequence. Full length expression of all hClpP-DiazK variants only occurred in presence of 1 mM DiazK as indicated by SDS-PAGE and western blot (anti H6) analysis and no full-length protein was observed in absence of DiazK.
Supplementary Figure S4. H6-tag affinity purification of hClpP-DiazK variants expressed in *E. coli*. SDS-PAGE of protein fractions after H6-tag purification are shown. Fractions, which were considered pure were pooled, concentrated and rebuffered for subsequent analysis (activity assay, SEC analysis). The two bands, which are visible for the K261 mutant, represent the full length and truncated protein. For the K261-amber variant, the truncated hClpP (K261TAG) appeared to be stable, leading to assembly with the full-length protein into stable heteromeric heptamers. This interaction was likely preserved during H6-tag purification and could only be resolved by denaturing SDS-PAGE. Only fractions, which contained pure full length hClpP-K261DiazK-H6 were pooled and used for subsequent functionality assays.
**Supplementary Figure S5.** Optimization of amber suppression of hClpP in HEK293T cells. Optimal transfection ratios between hClpP-XXTAG:PylRS were determined via WB analysis. Expressions were performed with the ncAA N-tert-butyloxycarbonyl-L-lysine (BocK) (2 mM). hClpP was expressed with its N-terminal 56 amino acid long mitochondrial guiding sequence and a C-terminal HA-tag. The upper running band of the main hClpP band thereby represents unprocessed hClpP, i.e. hClpP were the 56 amino acid sequence has not been cleaved off. As judged by western blot analysis (anti-HA), a ratio of 3:1 hClpP:PylRS was determined as most appropriate.
Supplementary Figure S6. Full blots of Figure 2, i.e. hClpP-DiazK-HA expressions and photocrosslinks in HEK293T cells, are shown. 

a) Full-length of hClpP-DiazK-HA variants only occurred in presence of DiazK. Hsc70 was used as the loading control. The asterisk (*) indicates partially unprocessed hClpP variants, i.e. proteins bearing the 56 amino acid long mitochondrial guiding sequence. 

b) After UV-irradiation of HEK293T cells expressing various hClpP-DiazK-HA mutants, novel, higher running bands occurred (compared to (-) minus UV samples), indicating capture of various hClpP substrates. The crosslinked proteins were subsequently identified by mass spectrometry analyses.
Supplementary Figure S7. Immunofluorescence (IF) microscopy experiments, as exemplarily shown for HEK293T cells overexpressing hClpP-D92DiazK-HA and hClpP-K261DiazK-HA. Fixed cells were incubated with MitoRed (Sigma, 53271) to stain mitochondria and with mouse-anti-HA antibody (1:100, SCBT, sc-7392), followed by an anti-mouse IgG-FITC antibody conjugate (1:100, Sigma, F2057) to stain hClpP-D92DiazK-HA and hClpP-K261DiazK-HA and with DAPI to stain nuclei. Nice overlapping IF signal of FITC with MitoRed was observed, indicating localization of amber-suppressed hClpP in the mitochondria. We observed only very minor, if any, FITC fluorescence outside mitochondria, indicating that only a very little fraction of newly expressed hClpP-DiazK variants resides in the cytosol.
Supplementary Figure S8. Graphical representation of trapping experiments with hClpP-DiazK variants. Enrichment factors are plotted against significance of enrichment. Graphs represent data from three technical replicates for each state. Two sample student’s t-test was conducted by comparison of UV-treated group with UV-untreated as single control group. False discovery rate was determined by Benjamini-Hochberg procedure setting correction at 0.05. Cut-off lines were set at a minimum log₂ change of 1 with a minimum p-value of 0.05. Proteins that are annotated as members of mitochondrial compartment are coloured in blue. Proteins coloured in red indicate known hClpP-interactors.
Supplementary Figure S9. Network analysis for proteins that were enriched by hClpP-D92DiazK variant using String v11 database. The network displays connections between identified proteins based on evidence found in literature as indicated in the legend. Minimum required interaction score was set to 0.7 (high confidence). Mitochondrial proteins are marked with red spheres. Blue spheres indicate members of the regulatory pathway of RNA metabolism. Proteins are further clustered and named for their collective pathway affiliation.
Supplementary Figure S10. Network analysis for proteins that were enriched by hClpP-K261DiazK variant using String v11 database. The network displays connections between identified proteins based on evidence found in literature as indicated in the legend. Minimum required interaction score was set to 0.7 (high confidence). Mitochondrial proteins are marked with red spheres. Proteins are further clustered and named for their collective pathway affiliation.
Supplementary Figure S11. Network analysis for proteins that were enriched by (a) hClpP-M88DiazK, (b) hClpP-G123DiazK, (c) hClpP-G124DiazK, (d) hClpP-V125DiazK, (e) hClpP-S181DiazK using String v11 database. The networks display connections between identified proteins based on evidence found in literature as indicated in the legend. Minimum required interaction score was set to 0.7 (high confidence). Mitochondrial proteins are marked with red spheres. Proteins are further clustered and named for their collective pathway affiliation.
**Supplementary Figure S12.** Substrate comparison between hClpP-DiazK mutants located inside the barrel and outside of the barrel depicted as Venn diagram. The crosslinked mitochondrial substrates of hClpP-DiazK mutants with inner DiazK positions (inside the barrel = M88-, D92-, G123-, G124-, V125- and S181DiazK) were compared with identified mitochondrial substrates of hClpP-K261DiazK (at the surface of the barrel, pointing outside). The partial difference of identified proteins (40 ‘inside’ vs. 50 ‘outside’) indicates that this approach may be suitable to address different compartments of hClpP. Indicative for this observation is the identification of MIPEP only by hClpP-DiazK, which has been previously shown to process pro-hClpP into mature ClpP and thus by virtue requires interaction.[3] The overlap of 23 proteins may hint towards their fate of being both, interactors and substrates of hClpP. Nonetheless, this also exemplifies the challenge of distinguishing between substrates and interactors of hClpP in general. (Venn diagram was generated with: http://bioinformatics.psb.ugent.be/webtools/Venn/)

| hClpP variants with inner DiazK positions | Gene names |
|------------------------------------------|-----------|
| hClpP-DiazK variants with inner DiazK positions (M88, D92, G123, G124, V125, S181) | MRPS39, MRPL3, TOMM6, LACTB, MRPS5, TIMM13, MRPS9, GCSH, MRPS18B, TIMM6B, COX5A, L2HGDH, MRPS16, MRPL4, MRPS11, MRPS6, MRPL15, TOMM5, MRPL59, MRPL21, MRPL17, MRPS22, UQCRC1, MRPS23, MRPS26, MRPL11, PPA2, MRPL51, MRPL45, MRPL13, WBSRCR16, MRPS29, NF51, TOMM40, PARK7, TOMM22, TRUB2, COX4I1, MRPL43, MRPS31 |
| hClpP-K261DiazK | TOMM70A, ACADSB, IARS2, HSPE1, ECHS1, PDHA1, DLAT, MRPS28, OGDH, ACA1, TRAP1, MDH2, MRPS34, DLST, MRPL44, PDHX, GRSF1, SLIRP, LRPRC, DLD, DBT, MRPL1, MRPL39 |
| Overlap | TOMM70A, ACADSB, IARS2, HSPE1, ECHS1, PDHA1, DLAT, MRPS28, OGDH, ACA1, TRAP1, MDH2, MRPS34, DLST, MRPL44, PDHX, GRSF1, SLIRP, LRPRC, DLD, DBT, MRPL1, MRPL39 |
| hClpP-K261DiazK | MRPS27, GOT2, ATP5H, HIBADH, FH, ACAD9, MTHFD1L, ATP5O, TRMT10C, PITRM1, ATRAF2, ATP5F1, ETFA, GLDC, ID3B, DECR1, MMAB, GLS, HSD17B10, TIMM44, GLUD1, ETFB, ME2, HADH, LARS2, HIBCH, ATP5B, YARS2, IDH2, SHMT2, MCCC2, IVD, NDUFA6, ATP5A1, PNPT1, GARS, LRYM7, SSBP1, IDH3A, AC02, ACADM, ALDH2, SUCLG1, HMGCL, OAT, CS, DARS2, ECH1, MIPEP, ATP5L |
**Supplementary Figure S13.** Volcano plot representation of whole proteome analysis with HEK293T hClpP knockout cells (KO) versus wild type HEK293T cells. Enrichment factors are plotted against significance of enrichment. The graph represents data from four technical replicates for each state. Two sample student’s t-test was conducted by comparison of HEK293T hClpP knockout mutant with wild type HEK293T cells as single control group. False discovery rate was determined by Benjamini-Hochberg procedure setting correction at 0.05. Cut-off lines were set at a minimum log2 change of 1 and -1 with a minimum p-value of 0.05. Proteins that are annotated as members of mitochondrial compartment are colored in blue. Filled red circles represent proteins that were previously annotated as ClpP substrates or interactors. Gene names colored in red indicate proteins which were found in trapping experiments with hClpP-DiazK mutant.
Supplementary Figure S14. Volcano plots of rotenone treated HEK293T cells expressing hClpP-D92DiazK and hClpP-K261DiazK after UV-light irradiation in comparison to UV-light untreated samples. Enrichment factors are plotted against significance of enrichment. Graphs represent data from three technical replicates for each state. Two sample student’s t-test was conducted by comparison of UV-treated group with UV-untreated as single control group. False discovery rate was determined by Benjamini-Hochberg procedure setting correction at 0.05. Cut-off lines were set at a minimum log₂ change of 1 with a minimum p-value of 0.05. Proteins that are annotated as members of mitochondrial compartment are coloured in blue. Proteins coloured in red indicate known hClpP-interactors.
Supplementary Tables

Supplementary Table S1.
MS analysis of purified hClpP-DiazK variants expressed in *E. coli*.

| hClpP mutant-H6 | Calculated mass [Da] | Observed mass [Da] |
|-----------------|----------------------|--------------------|
| wt              | 24972.82             | 24972.82           |
| M88DiazK        | 25095.92             | 25095.90           |
| D92DiazK        | 25111.93             | 25111.93           |
| G123DiazK       | 25169.94             | 25169.92           |
| G124DiazK       | 25169.94             | 25169.90           |
| V125DiazK       | 25127.89             | 25128.90           |
| S181DiazK       | 25139.93             | 25140.06           |
| K261DiazK       | 25098.86             | 25098.82           |
Supplementary Table S2.
Mass spectrometry instrument parameters for hClpP-DiazK variants.

| Sample name (hClpP mutant)            | Injection volume [µl] | flow [µl/min] | Spray voltage [kV] |
|---------------------------------------|-----------------------|--------------|-------------------|
| M88DiazK                              | 5                     | 0.3          | 1.87              |
| D92DiazK                              | 5                     | 0.4          | 1.77              |
| G123DiazK                             | 5                     | 0.4          | 1.77              |
| G124DiazK                             | 5                     | 0.4          | 1.77              |
| V125DiazK                             | 5                     | 0.4          | 1.77              |
| S181DiazK                             | 5                     | 0.4          | 1.77              |
| K261DiazK                             | 5                     | 0.4          | 1.77              |
| D92DiazK, rotenone treated            | 1                     | 0.4          | 1.77              |
| K261DiazK, rotenone treated           | 1                     | 0.4          | 1.77              |
**SUPPORTING INFORMATION**

Supplementary Table S3.

Significantly enriched proteins (log2 ratio > 1, -log10 \( p \)-value > 1.30) from trapping experiments in HEK293T cells expressing hClpP-D92DiazK. For annotation of hClpP substrates ref. \([34]\) were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-’.

| Protein names | Gene name | Enrichment factor (log2) | -log10 \( p \)-value | Annotated as substrate |
|---------------|-----------|--------------------------|----------------------|------------------------|
| Heterogeneous nuclear ribonucleoprotein U-like protein 2 | HNRNPUL2 | 6.51294017 | 3.15763196 | |
| Heterogeneous nuclear ribonucleoprotein A/B | HNRNPA8 | 4.53673108 | 3.78749803 | |
| Lups La protein | SSB | 4.23910013 | 3.28167315 | |
| RNA-binding motif, single-stranded-interacting protein 1 | RBMS1 | 4.08901272 | 3.33430836 | |
| Threonine synthase-like 1 | THNSL1 | 4.06954902 | 2.78971573 | |
| Nucleoside-sensitive element-binding protein 1 | YB1X | 3.78115891 | 2.64780991 | |
| 5-3 exoribonuclease 2 | XR2N | 3.78457995 | 1.98764856 | |
| Polyadenylate-binding protein 1 | PABPN1 | 3.68441094 | 5.26251798 | |
| Polyadenylate-binding protein 4 | PABPC4 | 3.62741252 | 3.16526389 | |
| Heterogeneous nuclear ribonucleoprotein D-like | HNRNPDL | 3.58322016 | 2.00047601 | |
| Glycine cleavage system H protein, mitochondrial | GCSh | 3.54482893 | 2.06076792 | |
| Heterogeneous nuclear ribonucleoprotein D-like | HNRNPDL | 3.50246874 | 4.61630576 | |
| Spermidat perinuclear RNA-binding protein | HEL162 | 3.45256433 | 4.48774278 | |
| Heterogeneous nuclear ribonucleoprotein D0 | HNRPD | 3.41360743 | 4.00708115 | |
| Probable RNA pseudouridine synthase 2 | TRUE2 | 3.17982286 | 3.20013949 | |
| THUMP domain-containing protein 1 | THUMPD1 | 3.17442703 | 2.60357959 | |
| Polyadenylate-binding protein | PABPC1 | 3.13070035 | 1.75375947 | |
| Zinc finger CCHC-type antiviral protein 1 | ZC3HAV1 | 2.91996510 | 2.42091828 | |
| Insulin-like growth factor 2 mRNA-binding protein 2 | IGF2BP2 | 2.53435635 | 2.70072355 | |
| ATP-dependent RNA helicase DHX36 | DHX36 | 2.50137463 | 4.09070346 | |
| 28S ribosomal protein S34, mitochondrial | MRPS34 | 2.4945666 | 3.64455329 | |
| 28S ribosomal protein S6, mitochondrial | MRPS6 | 2.38146113 | 2.39327359 | |
| 39S ribosomal protein L1, mitochondrial | MRPL1 | 2.37353792 | 1.61545693 | |
| Heterogeneous nuclear ribonucleoprotein U | HNRNP4U | 2.36706391 | 3.15102073 | |
| Enoyl-CoA hydratase, mitochondrial | ECXS1 | 2.34215643 | 3.04751512 | |
| Probable E3 ubiquitin-protein ligase makorin-2 | MKRN2 | 2.31989415 | 3.55549555 | |
| 39S ribosomal protein L3, mitochondrial | MRPL3 | 2.30510203 | 2.61288642 | |
| 28S ribosomal protein S23, mitochondrial | MRPS23 | 2.28660912 | 1.62554072 | |
| Insulin-like growth factor 2 mRNA-binding protein 3 | IGF2BP3 | 2.22527631 | 2.44375937 | |
| G-rich sequence factor 1 | GRSF1 | 2.19136792 | 2.47773642 | |
| Dihydrolipoyl dehydrogenase; Dihydrolipoyl dehydrogenase, mitochondrial | DLL | 2.15375209 | 2.79680500 | |
| 39S ribosomal protein L15, mitochondrial | MRPL15 | 2.15128517 | 2.90557684 | |
| 28S ribosomal protein S18b, mitochondrial | MRPS18B | 2.09424678 | 2.63309426 | |
| Insulin-like growth factor 2 mRNA-binding protein 1 | IGF2BP1 | 2.06429354 | 2.94118319 | |
| La-related protein 1 | LARP1 | 2.00626309 | 2.20226653 | |
| Dihydrolipoylsine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | DLST | 2.00272941 | 1.87754064 | |
| 39S ribosomal protein L11, mitochondrial | MRPL11 | 2.00228813 | 2.24741739 | |
| 28S ribosomal protein S9, mitochondrial | MRPS9 | 1.97829508 | 2.51746544 | |
| 39S ribosomal protein L4, mitochondrial | MRPL4 | 1.99332411 | 5.59283687 | |
| Heterogeneous nuclear ribonucleoprotein L | HNRNPL | 1.95509582 | 2.67710781 | |
| 39S ribosomal protein L43, mitochondrial | MRPL43 | 1.82365339 | 3.13816028 | |
| 2-oxoglutarate dehydrogenase, mitochondrial | OGHD | 1.81985708 | 2.85632003 | |
| 28S ribosomal protein S5, mitochondrial | MRPS5 | 1.77959124 | 2.43990577 | |
| Lipomamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial | DBT | 1.75809610 | 3.17531859 | |
| Heat shock protein 75 kDa, mitochondrial | TRAP1 | 1.74157142 | 3.62720842 | |
| Pyruvate dehydrogenase protein X component, mitochondrial | PDHX | 1.72785168 | 3.73039999 | |
| 28S ribosomal protein S16, mitochondrial | MRPS16 | 1.67300287 | 3.86417554 | |
| Constitutive coactivator of PPAR-gamma-like protein 1 | FAM120A | 1.66441974 | 2.43200689 | |
| Acetyltransferase component of pyruvate dehydrogenase complex | DLAT | 1.64678128 | 2.61955461 | |
| 28S ribosomal protein S31, mitochondrial | MRPS31 | 1.64251518 | 2.49501168 | |
| NF-kappa-B-repressing factor | NKR | 1.62526107 | 2.19800741 | |
| 39S ribosomal protein L21, mitochondrial | MRPL21 | 1.61435033 | 2.75176284 | |
| 39S ribosomal protein L13, mitochondrial | MRPL13 | 1.53686777 | 1.76171464 | |
| 39S ribosomal protein L45, mitochondrial | MRPL45 | 1.51011912 | 2.81675844 | |
| Acetyl-CoA acetyltransferase, mitochondrial | ACAT1 | 1.49288676 | 4.10884252 | |
| Cytochrome b-24 complex subunit 1, mitochondrial | UCRC1 | 1.43206473 | 2.58944946 | |
| Protein deglycase DJ-1 | PARK7 | 1.40789234 | 1.40334102 | |
| 28S ribosomal protein S22, mitochondrial | MRPS22 | 1.40854136 | 3.1683956 |
| Protein Name                                      | Gene ID   | Fold Change |
|--------------------------------------------------|-----------|-------------|
| Interleukin enhancer-binding factor 3            | ILF3      | 1.39076678  |
| Heterogeneous nuclear ribonucleoprotein K        | HRNPK     | 1.38711028  |
| Leucine-rich PPR motif-containing protein, mitochondrial | LRPPRC     | 1.363213857 |
| Heterogeneous nuclear ribonucleoprotein A0       | HRNPA0    | 1.350752513 |
| SRA stem-loop-interacting RNA-binding protein, mitochondrial | SLURP     | 1.34758695  |
| Heterogeneous nuclear ribonucleoprotein R        | HRNPRR    | 1.34037145  |
| 40S ribosomal protein S17                        | RPS17     | 1.305379232 |
| 39S ribosomal protein L51, mitochondrial         | MRPL51    | 1.294469198 |
| Williams-Beuren syndrome chromosomal region 16 protein | WBSCR16   | 1.292111715 |
| 39S ribosomal protein L39, mitochondrial         | MRPL39    | 1.289264679 |
| Pentatricopeptide repeat domain-containing protein 3, mitochondrial | PTCD3     | 1.275725047 |
| 28S ribosomal protein S26, mitochondrial         | MRPS26    | 1.262624741 |
| RNA-binding motif protein, X chromosome          | RBMX      | 1.254060109 |
| Protein ELYS                                     | AHCTF1    | 1.225773493 |
| Interleukin enhancer-binding factor 2            | ILF2      | 1.18741099  |
| 28S ribosomal protein S11, mitochondrial         | MRPS11    | 1.187133153 |
| 39S ribosomal protein L17, mitochondrial         | MRPL17    | 1.182820002 |
| Far upstream element-binding protein 3           | FUBP3     | 1.172859628 |
| 28S ribosomal protein S23, mitochondrial         | DAP3      | 1.166245143 |
| Cysteine desulfurase, mitochondrial              | NFS1      | 1.087621053 |
| L-2-hydroxyglutarate dehydrogenase, mitochondrial| L2HGDH    | 1.063916524 |
| 39S ribosomal protein L44, mitochondrial         | MRPL44    | 1.001045227 |
Significantly enriched proteins (log₂ ratio > 1, -log₁₀ t-test p-value > 1.30) from trapping experiments in HEK293T cells expressing hClpK-K261DiazK. For annotation of hClpP substrates ref. [3-4] were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-’.

| Protein names | Gene name | Enrichment factor (log2) | p-value t-test (log10) | Annotated as substrate |
|---------------|-----------|--------------------------|------------------------|------------------------|
| Enoyl-CoA hydratase, mitochondrial | ECHS1 | 7.06297412 | 6.14249209 | * |
| 10 kDa heat shock protein, mitochondrial | HSPE1/Hsp10 | 5.22580592 | 1.72892543 | - |
| Isovaleryl-CoA dehydrogenase, mitochondrial | IVD | 5.19725927 | 3.41344426 | + |
| Hydroxycarboxylic Coenzyme A dehydrogenase, mitochondrial | HADH | 4.82912254 | 4.47425768 | + |
| Malate dehydrogenase; Malate dehydrogenase, mitochondrial | MDH2 | 4.56429545 | 5.94513283 | + |
| Sepiapterin reductase | SPR | 4.56307348 | 3.7642302 | - |
| 99.3% Presequence Protease, putatively metalloproteinase | PITR1 | 4.4543369 | 5.58439474 | + |
| Acocitrate hydratase, mitochondrial | ACO2 | 2.46304861 | 4.06724225 | + |
| Isocitrate dehydrogenase [NADP], mitochondrial | IDH2 | 4.13951402 | 3.8569321 | + |
| Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial | ACADSB | 3.87617238 | 2.68320646 | + |
| Acetyl-CoA acetyltransferase, mitochondrial | ACAT1 | 3.68018341 | 4.93768787 | + |
| Citrate synthase; Citrate synthase, mitochondrial | CS | 3.55343119 | 5.48344178 | + |
| Acyltransferase component of pyruvate dehydrogenase complex | DLAT | 3.54185422 | 1.80263618 | + |
| Polyribonucleotide nucleotidyltransferase 1, mitochondrial | PNPT1 | 3.53656324 | 5.08405056 | + |
| Isocitrate dehydrogenase [NAD] subunit, mitochondrial; Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial | IDH3A | 3.56106875 | 3.3788779 | + |
| Single-stranded DNA-binding protein, mitochondrial | SSBP1 | 3.35599755 | 3.85622907 | + |
| Serine hydroxymethyltransferase, mitochondrial | SHMT2 | 3.34584689 | 5.22288862 | + |
| Dihydrioplypyrosine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | DLST | 3.28355551 | 3.95726853 | + |
| Tyrosine-RNA ligase, mitochondrial | YARS2 | 3.21464602 | 2.86106021 | + |
| Dihydrioplyp dehydrogenase, mitochondrial | DLQ | 3.1332798 | 4.18170305 | + |
| 2-oxoglutarate dehydrogenase, mitochondrial | OGDH | 3.05487124 | 3.66429743 | + |
| Acyl-Coenzyme A thiosterase 1; Acyl-Coenzyme A thiosterase 2, mitochondrial | ACOT1 | 2.97547298 | 3.16847496 | + |
| Lipooamide acyl-CoA dehydrogenase complex, mitochondrial | DBT | 2.96526249 | 2.48860334 | - |
| Electron transfer flavoprotein subunit beta | ETFB | 2.90824827 | 2.26341865 | + |
| Fumarate hydratase, mitochondrial | FH | 2.8694989 | 2.44974423 | + |
| Mitochondrial ribonuclease P protein 1 | TRMT10C | 2.7855107 | 1.87222444 | + |
| Mitochondrial import receptor subunit TOM70 | TOMM70A | 2.76650874 | 1.38449283 | - |
| Acyl-CoA dehydrogenase family member 9, mitochondrial | ACAD9 | 2.71775881 | 4.27364214 | + |
| Protein deglycase DJ-1 | PARK7 | 2.71552658 | 1.65359826 | - |
| Heat shock protein 75 kDa, mitochondrial | TRAP1 | 2.70960172 | 3.19950177 | + |
| Co(l)-bicurinic acid a,c-diamide adenylylsulfotransferase, mitochondrial | MAB | 2.69214185 | 2.97033329 | - |
| Pyruvate dehydrogenase protein X component, mitochondrial | PDHX | 2.63960838 | 3.00161336 | + |
| Isoleucine-RNA ligase, mitochondrial | IARS2 | 2.61310514 | 3.81692843 | + |
| Leucine-rich PPR motif-containing protein, mitochondrial | LRPRC | 2.56129265 | 2.89057994 | + |
| ATP synthase subunit d, mitochondrial | ATP5H | 2.55917295 | 1.38680909 | + |
| Glutaminase kidney isofom, mitochondrial | GLS | 2.5211792 | 2.01331491 | + |
| 3-hydroxyacyl-CoA dehydrogenase type-2 | HSD17B10 | 2.46673584 | 4.5363713 | + |
| ATP synthase F(0) complex subunit B1, mitochondrial | ATP5F1 | 2.3569091 | 1.82969105 | + |
| Mitochondrial intermediate peptidase | MIPEP | 2.28592555 | 2.47338517 | + |
| Glycine-RNA ligase | GAR | 2.27018166 | 1.59511486 | + |
| Aldehyde dehydrogenase, mitochondrial | ALDH2 | 2.2700901 | 3.4454489 | - |
| Aspartate–RRA ligase, mitochondrial | DAR52 | 2.26568476 | 2.72366543 | + |
| NAD-dependent malic enzyme, mitochondrial | ME2 | 2.16055562 | 3.58119527 | - |
| Monofunctional C1-tetrahydrofolate synthase, mitochondrial | MTHFD1L | 2.1106542 | 3.40480894 | + |
| Electron transfer flavoprotein subunit alpha, mitochondrial | ETF | 1.99602763 | 4.13475842 | + |
| ATP synthase subunit beta; ATP synthase subunit beta, mitochondrial | ATP5B | 1.99590408 | 3.31934939 | + |
| Hydroxymethylglutaryl-CoA lyase, mitochondrial | HMGL | 1.94890897 | 1.65773869 | + |
| ATP synthase mitochondrial F1 complex assembly factor 2 | ATPAF2 | 1.93302917 | 1.60720171 | - |
| NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 | NDUF6 | 1.90193367 | 1.82497091 | + |
| Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial | PDHA1 | 1.89166832 | 3.68221395 | + |
| Ornithine aminotransferase, mitochondrial | OAT | 1.83684095 | 2.7689186 | + |
| Isocitrate dehydrogenase [NAD] subunit, mitochondrial | IDH3B | 1.83460172 | 3.62711305 | + |
| Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial | SUCLG1 | 1.83005333 | 4.50265362 | + |
| Medium-chain specific acyl-CoA dehydrogenase, mitochondrial | ACADM | 1.81343157 | 4.32905502 | + |
| Complex III assembly factor LYRM7 | LYRM7 | 1.74750392 | 1.82877052 | + |
| Protein Name | Gene Symbol | Normalized p-value | Enrichment p-value | Score |
|--------------|-------------|--------------------|--------------------|-------|
| Glycine dehydrogenase (decarboxylating), mitochondrial | GLDC | 1.65087446 | 1.52630458 | + |
| 2,4-dienoyl-CoA reductase, mitochondrial | DECR1 | 1.62497393 | 1.49161777 | + |
| Mitochondrial import inner membrane translocase subunit TIM44 | TIMM44 | 1.61522675 | 2.26591336 | + |
| G-rich sequence factor 1 | GRSF1 | 1.60489273 | 2.3446218 | + |
| ATP synthase subunit g, mitochondrial | ATP5G | 1.57912763 | 1.53196088 | - |
| Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial | ECH1 | 1.57240359 | 2.11807815 | + |
| ATP synthase subunit O, mitochondrial | ATP5O | 1.54561996 | 2.93276679 | + |
| 39S ribosomal protein L44, mitochondrial | MRPL44 | 1.54289563 | 2.8836699 | + |
| Cytoplasmic dynein 1 light intermediate chain 1 | DYNCL1 | 1.47920227 | 1.35880247 | - |
| 3-hydroxyisobutyrate dehydrogenase, mitochondrial | HIBADH | 1.45746994 | 2.1669445 | - |
| Probable leucine-IRNA ligase, mitochondrial | LARS2 | 1.41318893 | 1.96446322 | + |
| Glutamate dehydrogenase 1, mitochondrial | GLUD1 | 1.39283371 | 3.3153833 | + |
| Aspartate aminotransferase, mitochondrial | GOT2 | 1.38335546 | 1.97231956 | - |
| 28S ribosomal protein S28, mitochondrial | MRPS28 | 1.33673096 | 2.01300666 | + |
| 28S ribosomal protein S27, mitochondrial | MRPS27 | 1.3307972 | 2.73973322 | + |
| 3-hydroxyisobutyryl-CoA hydrolase, mitochondrial | HIBCH | 1.30654263 | 2.89847207 | + |
| Ran GTPase-activating protein 1 | RANGAP1 | 1.25149218 | 1.7788658 | - |
| 28S ribosomal protein S34, mitochondrial | MRPS34 | 1.22402382 | 1.5503205 | - |
| 39S ribosomal protein L1, mitochondrial | MRPL1 | 1.22068787 | 1.7740484 | + |
| Cytosol aminopeptidase | LAP3 | 1.2072506 | 1.48275179 | - |
| Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial | MCCB2 | 1.13666443 | 3.04760163 | + |
| SRA stem-loop-interacting RNA-binding protein, mitochondrial | SLIRP | 1.12037404 | 1.84222529 | + |
| Ubiquitin carboxyl-terminal hydrolase; Probable ubiquitin carboxyl-terminal hydrolase FAP-X | USP9X | 1.05227025 | 1.31671925 | - |
| ATP synthase subunit alpha, mitochondrial | ATP5A1 | 1.00348409 | 3.88912011 | + |
Supplementary Table S5.
Significantly enriched proteins (log₂ ratio > 1, -log₁₀ t-test p-value > 1.30) from trapping experiments in HEK293T cells expressing hClpP-M88Dia2K. For annotation of hClpP substrates ref. [3-4] were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-’.

| Protein names | Gene name | enrichment factor (log₂) | p-value t-test (-log₁₀) | Annotated as substrate |
|---------------|-----------|--------------------------|--------------------------|------------------------|
| 10 kDa heat shock protein, mitochondrial | HSPE1/Hsp10 | 2.520680913 | 2.357710492 | - |
| Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | DLST | 2.465864182 | 2.80002771 | + |
| 39S ribosomal protein L13, mitochondrial | MRPL13 | 2.259292603 | 2.699404838 | + |
| Serine beta-lactamase-like protein LACTB, mitochondrial | LACTB | 2.176890055 | 2.572455941 | - |
| Clathrin light chain B | CLTB | 2.020890554 | 2.431020243 | - |
| Clathrin light chain A | CLTA | 1.966786702 | 2.557270996 | - |
| Acetyltransferase component of pyruvate dehydrogenase complex | DLAT | 1.89736557 | 3.709135937 | + |
| Clathrin heavy chain; Clathrin heavy chain 1 | CLTC | 1.736051559 | 2.961035244 | - |
| Mitochondrial import receptor subunit TOM22 homolog | TOMM22 | 1.710225423 | 1.440738034 | - |
| Hsc70-interacting protein | ST13 | 1.659377416 | 1.952781386 | - |
| Pyruvate dehydrogenase protein X component, mitochondrial | PDHX | 1.573442559 | 2.736885754 | + |
| Unconventional myosin-Id | MYO1D | 1.48491655 | 1.64829459 | - |
| Stress-induced-phosphoprotein 1 | STIP1 | 1.29959933 | 2.367580932 | + |
| Non-specific protein-tyrosine kinase | YES1 | 1.120776494 | 1.381692323 | - |
| Malate dehydrogenase; Malate dehydrogenase, mitochondrial | MDH2 | 1.095053355 | 2.650466362 | + |
| Enoyl-CoA hydratase, mitochondrial | ECHS1 | 1.090738568 | 1.345538671 | + |
| Histone-arginine methyltransferase CARM1 | CARM1 | 1.032886796 | 2.484322024 | - |
Supplementary Table S6.

Significantly enriched proteins (log₂ ratio > 1, -log₁₀ t-test p-value > 1.30) from trapping experiments in HEK293T cells expressing hClpP-G123DiazK. For annotation of hClpP substrates ref. [3-4] were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-’.

| Protein names                                                                 | Gene name          | enrichment factor (log₂) | p-value t-test (-log₁₀) | Annotated as substrate |
|-------------------------------------------------------------------------------|--------------------|--------------------------|-------------------------|------------------------|
| 26S proteasome non-ATPase regulatory subunit 10                              | PSMD10             | 2.599224091              | 3.099309091             |                        |
| Low molecular weight phosphotyrosine protein phosphatase                     | ACP1               | 2.394634883              | 2.20235606              |                        |
| Cytochrome c oxidase subunit 5A, mitochondrial                               | COX5A              | 1.666353861              | 2.481374747             |                        |
| L-2-hydroxylglutarate dehydrogenase, mitochondrial                          | L2HGDH             | 1.296777725              | 2.262133108             |                        |
| Acetyltransferase component of pyruvate dehydrogenase complex                | DLAT               | 1.292052587              | 1.997436201             |                        |
| Dihydrolipoxylysin-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | DLST               | 1.283274333              | 2.210274591             |                        |
| Pyruvate dehydrogenase protein X component, mitochondrial                    | PDHX               | 1.192612966              | 3.005726645             |                        |
| Vimentin                                                                      | HEL113             | 1.184209188              | 2.364866983             |                        |
| SEC23-interacting protein                                                     | SEC23IP            | 1.117851893              | 1.597814887             |                        |
| Tubulin alpha-1A chain; Tubulin alpha-3C/D chain; Tubulin alpha-3E chain     | TUBA1A             | 1.113746643              | 1.816653826             |                        |
| MAP7 domain-containing protein 1                                              | MAP7D1             | 1.087779363              | 1.465578942             |                        |
| Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial | DBT                | 1.006003698              | 3.013900794             |                        |
Supplementary Table S7.
Significantly enriched proteins (log₂ ratio > 1, -log₁₀ t-test p-value > 1.30) from trapping experiments in HEK293T cells expressing hClpP-G124DiazK. For annotation of hClpP substrates ref. [3-4] were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-’.

| Protein names                                                                 | Gene name | enrichment factor (log₂) | p-value t-test (log₁₀) | Annotated as substrate |
|-------------------------------------------------------------------------------|-----------|--------------------------|------------------------|------------------------|
| Glycine cleavage system H protein, mitochondrial                            | GCSH      | 3.83144188               | 3.39578492             | +                      |
| Cytochrome c oxidase subunit 4 isoform 1, mitochondrial                     | COX4I1    | 2.07114728               | 3.26673057             | +                      |
| Low molecular weight phosphotyrosine protein phosphatase                    | ACP1      | 1.80448977               | 1.30789463             | -                      |
| 10 kDa heat shock protein, mitochondrial                                     | HSPE1/Hsp10 | 1.68412463             | 2.08915157             | -                      |
| Inorganic pyrophosphatase 2, mitochondrial                                   | PPA2      | 1.82349447               | 2.91607884             | +                      |
| Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial | DBT       | 1.56262271               | 2.99743635             | -                      |
| Mitochondrial import inner membrane translocase subunit Tim8 B               | TIMM8B    | 1.42010689               | 3.72810838             | -                      |
| Stress-induced-phosphoprotein 1                                             | STIP1     | 1.37785149               | 2.74930439             | +                      |
| Polyadenylate-binding protein 2                                              | PABPN1    | 1.31002935               | 1.34611824             | -                      |
| Growth arrest and DNA damage-inducible proteins-interacting protein 1       | GADD45GIP1 | 1.22106043             | 1.4733748              | -                      |
| Mitochondrial import inner membrane translocase subunit Tim13               | TIMM13    | 1.18352318               | 2.32393757             | -                      |
| Pyruvate dehydrogenase protein X component, mitochondrial                   | PDHX      | 1.05083529               | 3.76064264             | +                      |
| Heat shock protein 75 kDa, mitochondrial                                      | TRAP1     | 1.01244863               | 3.36180087             | +                      |
Supplementary Table S8.

Significantly enriched proteins (log₂ ratio > 1, -log₁₀ t-test p-value > 1.30) from trapping experiments in HEK293T cells expressing hClpP-V125Dia2K. For annotation of hClpP substrates ref. [3-4] were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-‘.

| Protein names                                      | Gene name | enrichment factor (log₂) | p-value t-test (-log₁₀) | Annotated as substrate |
|----------------------------------------------------|-----------|--------------------------|-------------------------|------------------------|
| Protein deglycase DJ-1                             | PARK7     | 2.57212321               | 2.39456514              | -                      |
| Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial | DBT       | 2.52292633               | 3.0810378               | -                      |
| L-2-hydroxyglutarate dehydrogenase, mitochondrial  | L2HGDH    | 2.40566762               | 1.6841459               | -                      |
| Pyruvate dehydrogenase protein X component, mitochondrial | PDHX      | 1.88935089               | 3.0730658               | -                      |
| Proteasome activator complex subunit 3             | PSME3     | 1.80785497               | 2.90815977              | -                      |
| Acetyltransferase component of pyruvate dehydrogenase complex | DLAT      | 1.74389521               | 2.96192323              | +                      |
| Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | DLST      | 1.62713242               | 2.04765556              | +                      |
| 2-oxoglutarate dehydrogenase, mitochondrial         | OGDH      | 1.41948636               | 4.23615689              | +                      |
| Mitochondrial import receptor subunit TOM70        | TOMM70A   | 1.4118385                | 2.55482142              | -                      |
| Dihydrolipoyl dehydrogenase, mitochondrial         | DLD       | 1.3963371                | 2.67590128              | +                      |
| Acetyl-CoA acetyltransferase, mitochondrial         | ACAT1     | 1.3935407                | 3.05525474              | +                      |
| Pyruvate dehydrogenase E1 component subunit alpha  | PDHA1     | 1.36758105               | 2.68051777              | -                      |
| Coiled-coil domain-containing protein 80            | CCDC80    | 1.16726494               | 1.46698247              | -                      |
Supplementary Table S9. Significantly enriched proteins (log$_2$ ratio > 1, -log$_{10}$ t-test p-value > 1.30) from trapping experiments in HEK293T cells expressing hClpP-S181DiazK. For annotation of hClpP substrates ref. [3-4] were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-’.

| Protein names                                                                 | Gene name          | enrichment factor (log$_2$) | p-value t-test (-log$_{10}$) | Annotated as substrate |
|-------------------------------------------------------------------------------|--------------------|-----------------------------|------------------------------|------------------------|
| 39S ribosomal protein L39, mitochondrial                                         | MRPL39             | 3.69523875                  | 2.87025648                  | +                      |
| Mitochondrial import receptor subunit TOM40 homolog                            | TOMM40             | 3.61561521                  | 3.17518351                  | -                      |
| SRA stem-loop-interacting RNA-binding protein, mitochondrial                  | SLIRP              | 2.97568321                  | 1.65137757                  | +                      |
| Cleavage and polyadenylation specificity factor subunit 4                      | CPSF4              | 2.9491717                   | 2.7265779                   | -                      |
| Small nuclear ribonucleoprotein G                                              | SNRPG              | 2.80916087                  | 2.04616458                  | -                      |
| Tripeptidyl-peptidase 1                                                        | TPP1               | 2.78866768                  | 1.32640606                  | -                      |
| Transducin beta-like protein 3                                                 | TBL3               | 2.78135109                  | 1.43187895                  | -                      |
| Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial | DBT                | 2.37619591                  | 3.14234269                  | -                      |
| Septin-2                                                                      | SEPT2              | 2.26404635                  | 2.4847726                   | -                      |
| Mitochondrial import receptor subunit TOM6 homolog                            | TOMM6              | 2.14990807                  | 1.42698883                  | +                      |
| Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial           | ACADS8             | 2.05969874                  | 3.37409556                  | +                      |
| 26S proteasome non-ATPase regulatory subunit 4                                 | PSMD4              | 1.93259303                  | 2.5893379                   | +                      |
| Pyruvate dehydrogenase protein X component, mitochondrial                     | PDHX               | 1.77110227                  | 4.17378336                  | +                      |
| Acetyltransferase component of pyruvate dehydrogenase complex                 | DLAT               | 1.65835698                  | 3.3096671                   | +                      |
| Mitochondrial import receptor subunit TOM5 homolog                            | TOMM5              | 1.62214979                  | 2.86460387                  | -                      |
| Nucleoporin Nup43                                                             | NUP43              | 1.62046878                  | 1.42185773                  | -                      |
| Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | DLST               | 1.518013                    | 3.07556562                  | +                      |
| Plasminogen activator inhibitor 1 RNA-binding protein                          | SERBP1             | 1.43370883                  | 1.71404677                  | -                      |
| Enoyl-CoA hydratase, mitochondrial                                             | ECHS1              | 1.40692139                  | 2.27185593                  | +                      |
| 10 kDa heat shock protein, mitochondrial                                       | HSPE1/Hsp10        | 1.394804                    | 1.50747413                  | -                      |
| rRNA methyltransferase 2, mitochondrial                                        | HEL97              | 1.27911504                  | 2.2129185                   | -                      |
| Cyclin-dependent kinase 4                                                      | CDK4               | 1.20119413                  | 3.4476943                   | -                      |
| Mitochondrial import receptor subunit TOM22 homolog                            | TOMM22             | 1.15445201                  | 2.30656297                  | -                      |
| Heat shock protein 75 kDa, mitochondrial                                       | TRAP1              | 1.08820279                  | 2.82503219                  | +                      |
### Supplementary Table S10.

Significantly enriched proteins (log2 ratio > 1, log10 t-test p-value > 1.30) from whole proteome analysis in HEK293T ClpP knockout cells. For annotation of ClpP substrates ref. [3-4] were considered and proteins marked with ‘+’, if they were previously mentioned in any of the references. If no previous connection to ClpP was identified, proteins were annotated with ‘-’.

| Protein names | Gene name | enrichment factor (log2) | p-value t-test (log10) | Annotated as substrate |
|---------------|-----------|--------------------------|------------------------|------------------------|
| Glutathione S-transferase P | GSTP1;HEL-S-22 | 3.173206667 | 3.827196443 | + |
| Inositol 1,4,5-trisphosphate receptor type 2 | WD repeat domain phosphoinositide-interacting protein 4 | WDR45 | 2.408337593 | 1.499630223 |
| Mitochondrial assembly of ribosomal large subunit protein 1 | MALSU1 | 1.746897221 | 4.405897711 |
| [3-[methyl-2-oxobutanoate dehydrogenase [lipooamid]] kinase, mitochondrial | BCKDK | 1.70224851 | 3.104599943 |
| NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 | NDUF6A | 1.695121288 | 1.619586569 |
| [Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 2, mitochondrial | PDK2 | 1.65921545 | 3.185834772 |
| Decaprenyl-diphosphate synthase subunit 1 | DPS1S | 1.655128956 | 3.248254484 |
| [Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3, mitochondrial | PDK3 | 1.642876625 | 3.857055756 |
| Probable ATP-dependent RNA helicase DDX28 | DDX28 | 1.636013985 | 2.181415042 |
| GATOR complex protein SEC13 | - | 1.624860764 | 1.419270127 |
| Protein VAC14 homolog | VAC14 | 1.595541954 | 1.861170551 |
| Poly(A)-specific ribonuclease PARN | PARN | 1.585329792 | 3.119119302 |
| Arginase-2, mitochondrial;Arginase | ARG2 | 1.556794167 | 2.507832378 |
| Nicotinate-nucleotide pyrophosphorylase [carboxylating] | QPTR;HEL-S-90n | 1.55129528 | 1.930462222 |
| Transducin beta-like protein 3 | TBL3 | 1.541714668 | 2.235732724 |
| Cytochrome c oxidase subunit 1 | MT-CO1;COX1;COX1;COX1;COX1;COX1 | 1.541699409 | 1.546969079 |
| [NADP+] transhydrogenase, mitochondrial | NNT | 1.541247296 | 3.215330366 |
| H(+)/Cl(-) exchange transporter 7;Chloride channel protein | CLCN7 | 1.532600302 | 1.790707262 |
| 39S ribosomal protein L39, mitochondrial | MRPL39 | 1.531505108 | 3.204219098 |
| V-type proton ATPase 116 kDa subunit a isoform 1;V-type proton ATPase subunit a | ATP8V0A1 | 1.520878884 | 2.183670528 |
| Exportin-6 | XPO6 | 1.506718159 | 1.341499584 |
| Large neutral amino acids transporter small subunit 1 | SLC7A5 | 1.477167103 | 1.801000641 |
| Glycine dehydrogenase (decarboxylating), mitochondrial | GLDC | 1.471411228 | 4.680403685 |
| Glutamine synthetase | GLUL;PIG59 | 1.470929623 | 2.622748509 |
| Dihydroyoprimidinase-related protein 1 | CRMP1 | 1.427314758 | 3.325581754 |
| Inactive hydroxyoysteroid dehydrogenase-like protein 1 | HSDL1 | 1.421708584 | 2.478345787 |
| 28S ribosomal protein S27, mitochondrial | MRPS27 | 1.416228924 | 2.885972261 |
| Plasmin-3 | PL53 | 1.402068615 | 3.477388873 |
| F1H/F2H domain-containing protein 1 | FHOD1 | 1.397722721 | 2.081428883 |
| Exocyst complex component 5 | EXOC5 | 1.386257207 | 1.310222222 |
| Enhancer of mRNA-decapping protein 4 | EDC4 | 1.383150578 | 2.37317991 |
| Glutaminase | DKFZp686O15119 | 1.364944935 | 2.862686858 |
| Conserved oligomeric Golgi complex subunit 5 | COG5 | 1.356807709 | 1.492994491 |
| TLD domain-containing protein 1 | TLD1;KIAA1609 | 1.345605855 | 2.940872872 |
| Zinc transporter ZIP10 | ZIC3A910 | 1.33773783 | 2.327281121 |
| Lysine-rich nuclear protein 1 | KNOP1 | 1.332265854 | 2.713394846 |
| DnaJ homolog subfamily A member 3, mitochondrial | DNAJ3 | 1.330582958 | 4.033475277 |
| Multidrug resistance-associated protein 1 | ABC11;DKFZp781G125;MRP | 1.328831196 | 3.598731527 |
| Polymerase delta-interacting protein 2 | POLDIP2 | 1.323642254 | 4.102978472 |
| ADP-ribosylation factor-like protein 2-binding protein | ARL2BP | 1.32509766 | 3.374036421 |
| Carboxymethylisoxazolopyridine homolog | CMX1L | 1.321192741 | 3.347234345 |
| Rab-3A-interacting protein | RAB3IP | 1.317722797 | 1.726163333 |
| Adenodoxin, mitochondrial | FXD1 | 1.317452298 | 1.416827206 |
| Triple functional domain protein | TRIO | 1.308440685 | 1.699227142 |
| Lysocephospholipid acyltransferase 2 | RHODAT2 | 1.303477737 | 2.56935887 |
| Inositol 1,4,5-trisphosphate receptor type 2 | ITPR2 | 1.298715161 | 2.710059145 |
| Phospholipid-transporting ATPase Ig, Phospholipid-transporting ATPase | ATP11C | 1.296540318 | 1.876141105 |
**SUPPORTING INFORMATION**

| Gene Name                          | Accession Number(s) | Description                                      |
|-----------------------------------|---------------------|--------------------------------------------------|
| Mothers against decapentaplegic homolog 3 (Mothers against decapentaplegic homolog) 3 | 1,093614578, 1,496805481 | -                                                 |
| 2-methoxy-6-polyphenyl-1,4-benzoxin methylene, mitochondrial | 1,093120575, 3,29893025 | -                                                 |
| Geranylgeranyl transferase type-1 subunit beta | 1,09295702 | 1,72314902 -                                    |
| Protein TBRG4                      | 1,091749688 | 1,713543545 +                                  |
| General transcription factor 3C polyepitope 1 | 1,091234326, 1,746313592 | -                                                 |
| CDP-diacylglycerol-inositol 3-phosphatidyltransferase | 1,089659691, 2,50502121 | -                                                 |
| Cytoplasmic FMR1-interacting protein 2 | 1,089017868, 1,339675999 | -                                                 |
| Cystathionine beta-synthase         | 1,08834552, 2,383030475 | -                                                 |
| Zinc finger protein 5F              | 1,087993145, 1,83501567 | -                                                 |
| AP-1 complex subunit sigma-2       | 1,085670948, 1,87051971 | -                                                 |
| Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16 | 1,082866428, 2,656648626 | -                                                 |
| Lysophosphatidylcholine acyltransferase 1 | 1,081543037, 1,952192714 | -                                                 |
| Cytochrome c oxidase subunit 2     | 1,08145082, 1,76708417 | -                                                 |
| DNA-dependent protein kinase catalytic subunit | 1,079566159, 1,305483111 | -                                                 |
| WD repeat-containing protein 3      | 1,071441272, 3,203591381 | -                                                 |
| Probable helicase with zinc finger domain | 1,07132673, 1,539244227 | -                                                 |
| Probable histidine--RNA ligase, mitochondrial | 1,067705631, 2,423662229 | -                                                 |
| Proto-oncogene tyrosine-protein kinase S5c | 1,066912651, 2,09461354 | -                                                 |
| Glutamyl-tRNA(Gln) amidotransferase subunit A, mitochondrial | 1,062458992, 2,986145884 | -                                                 |
| NADH-cytochrome b5 reductase 3;NADH-cytochrome b5 reductase 3 membrane-bound form;NADH-cytochrome b5 reductase 3 soluble form;NADH-cytochrome b5 reductase | 1,084012884, 1,81119865 | -                                                 |
| Serine/threonine-protein kinase SMG1 | 1,058713436, 2,171066211 | -                                                 |
| [Pyruvate dehydrogenase [acetyl-transferring]-phosphatase 2, mitochondrial | 1,05544138, 2,075787568 | -                                                 |
| Delta-1-pyrroline-5-carboxylate synthase;Gamma-glutamyl phosphate reductase | 1,054750443, 2,153505089 | +                                                 |
| DNA-3-methyladenine glycosylase | 1,051884174, 2,378683502 | -                                                 |
| Atypical kinase ADC3, mitochondrial | 1,051606178, 1,80577989 | -                                                 |
| Lysine--RNA ligase | 1,051459628, 2,692602429 | -                                                 |
| ADP-ribosylation factor-binding protein GGA2 | 1,051235199, 1,857024014 | -                                                 |
| Nucleoside diphosphate kinase 3;Nucleoside diphosphate kinase | 1,050832272, 2,438801718 | -                                                 |
| Probable 28S RNA (cytosine(4447)-(C)-5')-methyltransferase | 1,049431801, 3,692075435 | -                                                 |
| cDNA FLJ5519 | - | 1,049320221, 1,6928609 | -                                                 |
| Mitochondrial Rho GTPase 2 | 1,048870087, 2,12888372 | -                                                 |
| Methionine synthase reductase | 1,048690796, 1,869042447 | -                                                 |
| 28S ribosomal protein S34, mitochondrial | 1,04804039, 3,261661452 | +                                                 |
| Motile sperm domain-containing protein 2 | 1,044107061, 2,282149228 | +                                                 |
| Cleft lip and palate transmembrane protein 1-like protein | 1,043674469, 1,617666535 | -                                                 |
| Inhibitor of nuclear factor kappa-B kinase-interacting protein | 1,041143894, 3,00977031 | -                                                 |
| Liss1 domain and HEAT repeat-containing protein KIAA1468 | 1,040667534, 1,647590134 | -                                                 |
| Calpin-1 catalytic subunit | 1,037336826, 2,430162479 | -                                                 |
| Ubiquinol-cytochrome-c reductase complex assembly factor 1 | 1,035808088, 2,725401052 | -                                                 |
| Antigen peptide transporter 1 | 1,03324604, 2,072414891 | -                                                 |
| 39S ribosomal protein L28, mitochondrial | 1,032464981, 2,3413517 | -                                                 |
| Catechol-O-methyltransferase | 1,031271935, 1,614735336 | -                                                 |
| Cytochrome b | CYTB, cytB, CYTB, CytB, CytB | -                                                 |
| Motile sperm domain-containing protein 2 | MOSPED2 | -                                                 |
| Phosphorylase b kinase regulatory subunit beta | 1,026652336, 1,578238673 | -                                                 |
| 5-AMP-activated protein kinase catalytic subunit alpha-2 | 1,025482655, 1,428936219 | -                                                 |
| CCR4-NOT transcription complex subunit 10 | 1,023015976, 1,467111191 | -                                                 |
| Endoplasmic reticulum aminopeptidase 1 | 1,020353317, 1,98585378 | -                                                 |
| Alanine--RNA ligase, cytoplasmic | AARS | 1,016955376, 3,110358865 | +                                                 |
| Serine/threonine-protein kinase Ne7 | 1,016818047, 1,85209223 | -                                                 |
| Dehydrogenase/reductase SDR family member 7B | DHRST7 | 1,472475704 | -                                                 |
| Thioredoxin-related transmembrane protein 4 | TMX4 | 1,014892101, 1,944765861 | -                                                 |
| Telomere length regulation protein TEL2 homolog | TEL02 | 1,013275146, 1,626543193 | -                                                 |
| Coronin; Coronin-7 | CORO7-PAM16; CORO7-HC; 1767779 | 1,012895584, 3,790301474 | -                                                 |
| PAB-dependent (A)-specific ribonuclease subunit PAN2 | PAN2 | 1,010728359, 1,651256219 | -                                                 |
| Caspase-8; Caspase-8 subunit p18; Caspase-8 subunit p10 | CASP8; hCASP1 16983 | 1,010406494, 3,055731744 | -                                                 |
| Conserved oligomeric Golgi complex subunit 8 | COG8; hCASP1 2027080 | 1,009614647, 2,357167679 | -                                                 |
| Dehydrogenase/reductase SDR family member 7B | DHRST7; DKFZp564H1664 | 1,007921219, 1,867458483 | -                                                 |
| Diphosphophosphate decarboxylase | MVD | 1,006159566, 2,846140995 | -                                                 |
| E3 ubiquitin-protein ligase 1 | UFL1 | 1,00866876, 1,58208122 | -                                                 |
| Sodium-cell surface antigen heavy chain | SLC3A2 | 1,001928212, 2,484188639 | -                                                 |
| Sodium-coupled neutral amino acid transporter 2 | SLC3A2 | 1,001403332, 1,610205596 | -                                                 |
## Supplementary Table S11

Significantly enriched proteins (log₂ ratio > 1, log₁₀ t-test p-value > 1.30) from trapping experiments of rotenone-treated HEK293T cells expressing hClpP-D92DiaZK. Proteins that were enriched only in rotenone treated samples and in no other hClpP-DiazK variants are marked with '+' and with '-' if they are not uniquely enriched.

| Protein name | Gene name | enrichment factor (log) | p-value t-test (log) | Annotated as substrate |
|--------------|-----------|-------------------------|---------------------|------------------------|
| CDKN2A-interacting protein | CDKN2AIP | 4.040013777 | 5.829336166 | - |
| Glycine cleavage system H protein, mitochondrial | GCSH | 3.547040595 | 6.522687658 | - |
| THUMP domain-containing protein 1 | THUMPD1 | 2.28583187 | 4.467810313 | - |
| L-oxaloacetate dehydrogenase, mitochondrial | L2HG1 | 2.627576481 | 3.54081778 | - |
| RNA-binding protein Musashi homolog 1 | MSI1 | 3.157466777 | 3.508930206 | - |
| RNA-binding protein Musashi homolog 2 | MSI2 | 2.92371033 | 3.00661316 | - |
| Threonine synthase-like 1 | THNSL1 | 2.611644794 | 2.96591338 | - |
| Heterogeneous nuclear ribonucleoprotein U-like protein 2 | HNRNPU2; | 3.97559625 | 2.948937734 | - |
| Polyadenylate-binding protein 2 | PABPN1 | 2.594634424 | 2.659805934 | - |
| Inorganic pyrophosphatase 2, mitochondrial | PP2A | 1.517691785 | 2.637044443 | - |
| Heterogeneous nuclear ribonucleoprotein D-like | HNRNPD1 | 2.11972959 | 2.585085855 | - |
| Protein phosphatase PTC7 homolog | PTPC7 | 2.367260172 | 2.42946468 | - |
| NAD-dependent malic enzyme, mitochondrial | MDMA | 2.70599794 | 2.383645718 | - |
| RNA-binding motif, single-stranded-interacting protein 1 | RBM51 | 4.040859241 | 2.317205155 | - |
| 39S ribosomal protein L53, mitochondrial | MRPL53 | 1.644228898 | 2.278296153 | - |
| 39S ribosomal protein L52, mitochondrial | MRPL52 | 1.684214222 | 2.268756866 | - |
| 39S ribosomal protein L33, mitochondrial | MRPL33 | 3.921019156 | 2.196620941 | - |
| Dihydrolipooyl dehydrogenase, mitochondrial | DLD | 3.568975709 | 2.178907394 | - |
| Pyruvate dehydrogenase protein X component, mitochondrial | PDHX | 3.274047919 | 2.133876801 | - |
| Heterogeneous nuclear ribonucleoprotein D-like | HNRNPD1 | 2.604936259 | 1.989514669 | - |
| Probable E3 ubiquitin-protein ligase makorin-2 | MKRN2 | 3.091680264 | 1.952359517 | - |
| Putative transferase CAF17, mitochondrial | IBA57 | 2.29867109 | 1.927930884 | - |
| 5-3 exoribonuclease 2 | XRN2 | 2.899070362 | 1.894339879 | - |
| RNA-binding motif, single-stranded-interacting protein 2 | RBM52 | 2.731841921 | 1.76350979 | - |
| Scaffold attachment factor B2 | SABF2 | 2.6444863 | 1.705375671 | - |
| Heterogeneous nuclear ribonucleoprotein D0 | HNRPD | 2.8901515 | 1.705329435 | - |
| Enoyl-CoA hydratase, mitochondrial | EC1H1 | 3.209923076 | 1.2562904 | - |
| 3-hydroxyacyl-CoA dehydrogenase type-2 | HSD17B10 | 3.046260469 | 1.627047645 | - |
| Heterogeneous nuclear ribonucleoprotein L-like | HNRNPL1 | 1.78703256 | 1.5285333 | - |
| Dihydrolipopolysaccharide-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | DLST | 2.34655262 | 1.48673972 | - |
| Acetyltransferase component of pyruvate dehydrogenase complex; Dihydrolipopolysaccharide-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial | DLAT | 2.174352815 | 1.389623642 | - |
| Leucine-rich PPR motif-containing protein, mitochondrial | LRPPRC | 3.256026535 | 1.369892574 | - |
| 39S ribosomal protein L11, mitochondrial | MRPL11 | 2.657172419 | 1.554166166 | - |
| Heterogeneous nuclear ribonucleoprotein Q | SYNCRIP | 2.908112696 | 1.348293304 | - |
| 10 kDa heat shock protein, mitochondrial | HSP10/Hsp10 | 2.380939534 | 1.346793493 | - |
| Acetyl-CoA acetyltransferase, mitochondrial | ACA1T | 2.900621023 | 1.346787135 | - |
| Heat shock protein 75 KD, mitochondrial | TRAP1 | 3.443383745 | 1.28523902 | - |
| Hsc70-interacting protein; Putative protein FAM10A4; Putative protein FAM10A5 | ST13 | 2.343873193 | 1.213008881 | - |
| Isocitric–RNA ligase, mitochondrial | IARS2 | 2.172470393 | 1.210529616 | - |
| ATP synthase mitochondrial F1 complex assembly factor 2 | ATPF2 | 2.369971558 | 1.207983975 | - |
| 2-oxoglutarate dehydrogenase, mitochondrial | OGDH | 3.257917422 | 1.207115173 | - |
| ATP-dependent RNA helicase DHX36 | DHX36 | 2.20148615 | 1.187528067 | - |
| Presequence protease, mitochondrial | PITRM1 | 2.58780206 | 1.1651027 | - |
| Lipoamide acetyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial | DBT | 2.845733967 | 1.144769609 | - |
| 39S ribosomal protein L54, mitochondrial | MRPL54 | 1.658340666 | 1.111061732 | - |
| 39S ribosomal protein L52, mitochondrial | MRPL2 | 2.507914069 | 1.11005147 | - |
| 39S ribosomal protein L16, mitochondrial | MRPL16 | 2.99404281 | 1.10972023 | - |
| 2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial | BCOGA | 1.593486921 | 1.100859652 | - |
| 39S ribosomal protein L44, mitochondrial | MRPL44 | 3.076696717 | 1.096454719 | - |
| Lupon Lα protein | SSB | 2.815146607 | 1.095890681 | - |
| Mitochondrial ribonuclease P protein 1 | TRMT10C | 1.63024209 | 1.09022013 | - |
| THUMP domain-containing protein 3 | THUMPD3 | 1.871894572 | 1.08164004 | - |
| Polyadenylate-binding protein; Polyadenylate-binding protein 4 | PABPC4 | 2.757012728 | 1.064928915 | - |
| Zinc finger protein ubi-d4 | PDP2 | 1.477201208 | 1.047100703 | - |
| 39S ribosomal protein L39, mitochondrial | MRPL39 | 3.260246654 | 1.037794749 | - |
### Supplementary Table S12.

Significantly enriched proteins (log₂ ratio > 1, -log₁₀ t-test p-value > 1.30) from trapping experiments of rotenone-treated HEK293T cells expressing hClpP-K261DiazK. Proteins that were enriched only in rotenone treated samples and in no other hClpP-DiazK variants are marked with ‘+’, and with ‘-’, if they are not uniquely enriched.

| Protein names | Gene name | enrichment factor (log₂) | p-value t-test (log₁₀) | Annotated as substrate |
|---------------|-----------|--------------------------|------------------------|------------------------|
| Enoyl-CoA hydratase, mitochondrial | ECHS1 | 2.003698056 | 5.274079005 | - |
| 10 kDa heat shock protein, mitochondrial | HSP1/Hsp10 | 2.416902285 | 3.559659958 | - |
| Tyrosine--tRNA ligase, mitochondrial;Tyrosine--tRNA ligase | YARS2 | 2.676012595 | 3.065404256 | - |
| Malate dehydrogenase;Malate dehydrogenase, mitochondrial | MDH2 | 2.538980576 | 2.600123723 | - |
| Kappa-casein | CSN3 | 3.196187718 | 2.359725952 | + |
| Single-stranded DNA-binding protein;Single-stranded DNA-binding protein, mitochondrial | SSBP1 | 3.129474044 | 2.081043879 | - |
| Polyribonucleotide nucleotidyltransferase 1, mitochondrial | PNPT1 | 1.332356849 | 1.964796431 | - |
| Isovaleryl-CoA dehydrogenase, mitochondrial | IVD | 2.242487823 | 1.945773443 | - |
| Cyclin-dependent kinase 4 | CDK4 | 1.647243029 | 1.94344203 | - |
| Prerelease protease, mitochondrial | PITRM1 | 2.271304681 | 1.935722987 | - |
| Inorganic pyrophosphatase 2, mitochondrial | PPA2 | 2.066109134 | 1.739587784 | - |
| Tubulin beta chain | TUBB | 1.719995809 | 1.61362599 | - |
| Histidine triad nucleotide-binding protein 2, mitochondrial | HINT2 | 2.158128659 | 1.56341949 | - |
| Citrate synthase/Citrate synthase, mitochondrial | CS | 2.614555541 | 1.521754853 | - |
| Putative transferase CAF17, mitochondrial | IBA57 | 1.419573126 | 1.488054911 | - |
| Mitochondrial import receptor subunit TOM40 homolog | TOMM40 | 1.483859403 | 1.405593232 | - |
| Acetyltransferase component of pyruvate dehydrogenase complex | DLAT | 3.254700786 | 3.54489009 | - |
| Hydroxacyl-coenzyme A dehydrogenase, mitochondrial | HADH | 2.39319267 | 1.331132947 | - |
| Acyl-CoA dehydrogenase family member 9, mitochondrial | ACAD9 | 2.455665811 | 1.292857488 | - |
| Mitochondrial ribonucleoside P protein 1 | TRMT10C | 2.355294345 | 1.282247543 | - |
| Aconitate hydratase, mitochondrial | ACO2 | 2.855714637 | 1.259790421 | - |
| Heat shock protein 75 kDa, mitochondrial | TRAP1 | 3.172907231 | 1.242417653 | - |
| Host cell factor 1 | HOFC1 | 1.306021892 | 1.20465155 | - |
| SRA stem-loop-interacting RNA-binding protein, mitochondrial | SLRP | 3.272002454 | 1.12571205 | + |
| NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial | NDUFV2 | 1.84084095 | 1.117319743 | - |
| Glutamate dehydrogenase 1, mitochondrial | GLUD1 | 2.092659677 | 1.045743306 | - |
| Poly [ADP-ribose] polymerase 1 | PARP1 | 1.622107904 | 1.03410085 | - |
| 5-AMP-activated protein kinase catalytic subunit alpha-2;5-AMP-activated protein kinase catalytic subunit alpha-1 | PRKAA1 | 2.443330979 | 1.022978035 | + |
**Experimental Procedures**

**General remarks**

All chemicals and solvents were obtained from commercial suppliers (Carbolution, Sigma Aldrich) and used without further purification unless otherwise stated. Technical grade pentane and DCM were distilled prior use. Thin-layer chromatography (TLC) was performed on Merck Millipore silica gel 60 F-254 plates. The developed silica plates were visualized by UV light (254 nm) and/or staining with ninhydrin or potassium permanganate. Flash column chromatography used for product purification was performed on silica gel 60 (230-400 mesh).

NMR spectra were recorded on Bruker AVHD300 (300 MHz for $^1$H-NMR, 75 MHz for $^{13}$C-NMR). Chemical shifts (δ), reported in ppm, are referenced to the residual proton solvent signals. Coupling constants (J) are reported in Hertz (Hz) while peak multiplicities are described as follows: s (singlet), d (doublet), t (triplet), m (multiplet).

Small molecule liquid chromatography mass spectrometry (LC-MS) was performed on an Agilent Technologies 1260 Infinity LC-MS system with a Phenomenex AerisTM Peptide XB-C18 column (100 x 2.1 mm, 3.6 µm) coupled to a 6310 Quadrupole spectrometer. The solvent system consisted of MQ H$_2$O + 0.1% FA as buffer A and MeCN + 0.1% FA as buffer B. Protein LC-MS was carried out on a Jupiter C4 column (2 x 150 mm, 5µm). Protein mass was calculated by deconvolution within the Chemstation software (Agilent Technologies). Theoretical protein masses were calculated using ProtParam and were manually corrected for the mass of the ncAA. Samples were analysed by UV absorbance at 193, 254 and 280 nm followed by both positive and negative ESI-mode.
Chemical synthesis

Synthesis of 2-(3-methyl-3H-diazirin-3-yl)ethan-1-ol (1)

To 4-Hydroxy-2-butanone (4.0 g, 45.0 mmol, 1.0 eq.) on ice was added NH₃ (7 M in MeOH, 35 ml). After 3 h, hydroxylamine-O-sulfonic acid (5.7 g, 50.0 mmol, 1.1 eq.) was added and the reaction was stirred o.n. at r.t. The mixture was filtered through Celite and the filter cake was washed with MeOH. The solvent was removed and the crude intermediate was taken up in MeOH (40 ml) and Et₃N (8 ml) and stirred on ice. I₂ was added portion-wise until the reaction mixture maintained a dark yellow/brown color. After 3 h, the solvent was removed and taken up in Et₂O (150 ml). The organic phase was washed with 1 M HCl (80 ml). The aqueous phase was extracted again with Et₂O (150 ml). The combined organic phase was washed with 20% w/v Na₂S₂O₃ (100 ml) and brine (100 ml). The organic phase was dried over Na₂SO₄, filtered and removed under reduced pressure to yield a dark yellow oil (1.88 g, 18.8 mmol, 42%). The crude product 1 was used without further purification.

¹H NMR (300 MHz, CDCl₃) δ = 1.07 (s, 3H), 1.63 (t, J = 6.3 Hz, 2H), 3.52 (t, J = 6.3 Hz, 2H).

Synthesis of 2-(3-Methyl-diazirin-3-yl)ethyl (4-nitrophenyl) carbonate (2)

To a solution of 1 (1.9 g, 18.8 mmol, 1.0 eq.) in DCM (100 ml) on ice was added 4-nitrophenyl chloroformate (4.5 g, 22.5 mmol, 1.2 eq.) and pyridine (1.8 ml, 22.5 mmol, 1.2 eq.) and the reaction was stirred o.n. at r.t. The reaction mixture was concentrated under reduced pressure and purified by flash chromatography (10% → 15% EtOAc in pentane) to obtain the final product 2 as a yellow oil (3.1 g, 11.6 mmol, 62%).

¹H NMR (300 MHz, CDCl₃): δ = 1.12 (s, 3H), 1.80 (t, J = 6.4 Hz, 2H), 4.25 (t, J = 6.4 Hz, 2H), 7.40 (d, J = 9.2 Hz, 2H), 8.29 (d, J = 9.2 Hz, 2H).

¹³C NMR (75 MHz, CDCl₃): δ = 20.0, 23.7, 33.9, 64.4, 122.0, 125.5, 145.6, 152.5, 155.6.

Synthesis of N²-(tert-butoxycarbonyl)-N⁶-((2-(3-methyl-3H-diazirin-3-yl)ethoxy)carbonyl)-L-lysine (3)

To a solution of 2 (3.1 g, 11.6 mmol, 1.0 eq.) in dioxane (30 ml) was added Na-Boc-Lysine (3.4 g, 13.9 mmol, 1.2 eq.) and Et₃N (3.2 ml, 23.2 mmol, 2.0 eq.) and the reaction was stirred o.n. at r.t. The reaction mixture was concentrated under reduced and purified by flash chromatography (2% MeOH in DCM → 5% MeOH in DCM +0.5% AcOH) to yield the final product 3 as a colourless oil (2.6 g, 6.9 mmol, 60%).

¹H NMR (300 MHz, DMSO-d₆): δ = 1.02 (s, 3H), 1.23-1.41 (m, 13H), 1.48-1.66 (m, 4H), 2.95 (d, J = 6.4 Hz, 2H), 3.86 (t, J = 6.3 Hz, 2H), 3.91-3.78 (m, 1H), 7.00 (d, J = 7.9 Hz, 1H), 7.15 (t, J = 5.5Hz, 1H).

The chemical shifts are according to literature.[5]

LC-MS (m/z): calcd. for C₁₆H₂₅N₆O₆ [M-H]⁺ 371.2; found: 371.1.
Synthesis of \( N^6-(2-(3\text{-methyl}-3H\text{-diazirin}-3\text{-yl})\text{ethoxy})\text{carbonyl})-L\text{-lysine TFA salt/DiazK (4)\)

To a stirred solution of 3 (4.8 g, 13.0 mmol, 1.0 eq.) in DCM (28 ml) on ice was added TFA (7 ml) and H\( _2\)O (1 ml). After 3 h, the solvent was removed, the crude product precipitated in ice-cold Et\( _2\)O and collected by centrifugation. This was repeated twice and the final product was lyophilised to obtain the final product 4 as a white powder (4.6 g, 13.5 mmol, 96%, TFA salt).

\(^1\text{H NMR\) (300 MHz, D\( _2\)O): \( \delta = 1.06 \) (s, 3H), 1.34-1.48 (m, 2H), 1.50-1.61 (m, 2H), 1.64-1.75 (m, 2H), 1.82-1.95 (m, 2H), 3.16 (t, \( J = 6.5 \) Hz, 2H), 3.74 (t, \( J = 6.1 \) Hz, 1H), 4.04 (t, \( J = 6.0 \) Hz, 2H). The chemical shifts are according to literature.\(^5\)

\text{LC-MS (m/z): calcd. for. C}_{11}\text{H}_{20}\text{N}_{4}\text{O}_{4} [M+H]^+ 273.2; found: 273.2.}

DiazK stock solutions (100 mM) were stored in 100 mM TFA in H\( _2\)O, filtered (0.2 \( \mu \)m filter) and stored at -20°C prior use.

\(^1\text{H NMR of DiazK (4)\}

\[ \text{O} \]
\[ \text{N} \]
\[ \text{N} \]
\[ \text{O} \]
\[ \text{CO} \]
\[ \text{H}_2\text{N} \]
\[ \text{COOH} \]
\[ \text{TFA} \]
Biological Methods

Cloning of hClpP constructs

Plasmids for bacterial expressions: hClpP sequence (lacking the N-terminal 56 amino acid signal sequence and bearing a C-terminal StreptII-tag) was amplified from pET301_hClpP vector (Sieber Lab, TU Munich) and cloned into the pPylt backbone (containing Mb_tRNA_CUA and an arabinose-inducible promoter) with a C-terminal His6-tag using Gibson cloning protocol as recommended (NEB). Respective amber codon (TAG) positions were introduced via SLIM.

Protein sequence of hClpP for bacterial expression:

MPPIPIPVVEQTGRGERAYDIYSRLLRERIVCV
MGPI
DSVASLVIAQLLFLQSESNKKPIHMYINSPPGGVTAGLAIY
DTMQYILNPICTWCVGQAA
SMGSLLLAAGTPGMRHSLPNSRIMIHQPSSGARGQATDIAQAEIMKLKKQLYNIYAKHTKQSLQVIESAMERDRYMSPMEAQEFGILDKVLHPPQDGEDEPTLVQKEPVEAAPAEVPVF

Plasmids for mammalian expression: hClpP sequence (containing the N-terminal 56 amino acid mitochondrial signal recognition motif and a C-terminal FLAG-tag) was amplified from pRK5SV40_hClpP vector (Sieber Lab, TU Munich) and subcloned into a pET17 helper plasmid. FLAG-tag was replaced with an HA-tag via SLIM and respective TAG positions were introduced thereafter via SLIM. hClpP(TAG)-HA constructs were amplified with primers bearing XbaI and BamHI restriction sites prior and after the gene and cloned into pEF1_POI_4xPylt backbone using standard restriction cloning protocols.

Protein sequence of hClpP for mammalian expression:

MWPGILVGARVASCYPALGPRLAHHFPAQRPOQRTLONGLALQCLHATATRALPLIPIVEQTGGERAYDIYSRLLRERIVCV
MGPI
DSVASLVIAQLLFLQSESNKKPIHMYINSPPGGVTAGLAIY
DTMQYILNPICTWCVGQAA
SMGSLLLAAGTPGMRHSLPNSRIMIHQPSSGARGQATDIAQAEIMKLKKQLYNIYAKHTKQSLQVIESAMERDRYMSPMEAQEFGLDKVLHPPQDGEDEPTLVQKEPVEAAPAEVPVF

Expression and purification of recombinant (amber suppressed) hClpP

E. coli DH10β cells were co-transformed with pBK_Mb_PylRS_DiazKRS (Mb mutations: Y271M, L274A, C313A, KanR) and pPylt(-56aa)-hClpP-TAG-His6 (Te6), SOC rescued (1h, 37°C) and cultured in LB medium o.n. at 37°C with 1x antibiotic strength(s). For wt expression, the addition of the PylRS plasmid was omitted. The o.n. culture was diluted into 2xYT to an OD600 of ~0.05 with 1x antibiotic strengths and incubated at 37°C, 200 rpm. At approximately OD600 of ~0.3, DiazK was added to a final concentration of 1 mM (for amber suppressed proteins). At approximately OD600 of ~0.6, protein expression was induced with arabinose (0.02% w/v final concentration). After 3 h (for wt hClpP) or 18 h (for amber suppressed hClpP), expression cultures were harvested via centrifugation (4200 rpm, 15’, 4°C). For SDS-PAGE analysis, 1 ml of culture was pelleted, resuspended in 1x Laemmli loading buffer (normalised according to OD600 values; per OD = 1, 100 µl of 1x loading buffer), boiled at 95°C for 10’, pelleted again (max. speed, 10’) and supernatant was subjected to HisTrap purification on GE Äkta system (hClpP lysis buffer supplemented with imidazole, 20mM (A) and 300 mM (B); gradient A→B). Appropriate fractions were analysed via 15% SDS-PAGE, pooled, concentrated/rebuffered with an Amicon Ultra-4 10K MWCO centrifugal filter units (Millipore) and SEC buffer (20 mM HEPES, pH 7 (6°C), 100 mM NaCl). Samples were aliquoted, flash frozen and stored at -80°C until further use.
**Expression and purification of recombinant *E. coli* ClpX**

*E. coli* ClpX was expressed as described before [9]. In brief, *E. coli* ClpX was overexpressed in *E. coli* (DE3) Rosetta 2 with a N-terminal His6-TEV construct. For this, LB-Medium was inoculated with o.n. cultures, incubated at 37 °C with constant shaking and overexpression was induced at OD<sub>600</sub> = 0.8 by addition of IPTG (1:2000 dilution, 0.5 M stock solution in H<sub>2</sub>O). After incubation for 20 h at 25 °C and 200 rpm bacteria were harvested by centrifugation (6000 g, 10’, 4°C), washed with 30 ml PBS buffer (140 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 1.8 mM KH<sub>2</sub>P<sub>4</sub>, pH 7.6) and again centrifuged at 15,000 g for 10’ at 4°C. Lysis was performed in EcClpX lysis buffer I (50 mM HEPES, 300 mM KCl, 1 mM DTT, 10 mM imidazole, 5 mM MgCl<sub>2</sub>, 15% (v/v) glycerol, pH 7.6) by sonication. Lysate was cleared by centrifugation (38700 g, 30’, 4°C) and processed using a Superdex 200 10/300 (GE Healthcare) with a centrifugal filter with a cut-off of 30 kDa.

**Protein assembly analysis via size-exclusion chromatography**

His-purified protein samples (wt-hClpP-His<sub>6</sub> and all hClpP-DiazK-His<sub>6</sub> amber mutants) were subjected to SEC using a Superdex<sup>TM</sup> 75 10/300 (GE Healthcare) with the SEC buffer. Raw data of the elution profiles were processed using Microsoft Excel and OriginPro16G. Peak intensities (280 nm absorption) from 5-20’ retention time were considered and all signals were normalised to the highest signal intensity.

**hClpP protease activity assay**

*E. coli* ClpX recognizes peptides C-terminally tagged with a short amino acid sequence and forms a functional complex with *H. Sapiens* ClpP. It is therefore suitable as a *H. Sapiens* ClpX substitute for in vitro protease activity assays. To determine residual protease activity of mutated ClpXP, cleavage of ssrA-tagged GFP was monitored by the decrease of fluorescence signal. For this, 59 µl of enzyme buffer mix (0.2 µM hClpP<sub>14</sub>, 0.4 µM eClpX<sub>6</sub>; 10x ATP-regeneration mix: 40 mM ATP, 160 mM creatine phosphate, 200 U/ml creatine phosphokinase in 25 mM HEPES, 200 mM KCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 15% (v/v) glycerol, pH 7.6) protein was eluted with 20 ml elution buffer (50 mM HEPES, 300 mM KCl, 1 mM DTT, 40 mM imidazole, 15% (v/v) glycerol, pH = 7.6) protein was eluted with 20 ml elution buffer (50 mM HEPES, 300 mM KCl, 1 mM DTT, 300 mM imidazole, 15% (v/v) glycerol, pH 7.6). Fractions containing protein were pooled and 1 mM EDTA and 2 mg/ml TEV-protease were added and incubated o.n. at 10 °C under constant agitation. Completion of His-tag cleavage was confirmed by LC-MS and crude protein was purified by size exclusion chromatography in EcClpX lysis buffer I without imidazole. Fractions containing EcClpX were pooled and concentrated using a centrifugal filter with a cut-off of 30 kDa.

**Mammalian cell culturing and photocrosslinking**

HEK293T cell culturing was performed as previously described [10]. Transfections were either performed in 6-well plates or in 10 cm dishes for proteomics experiments (triplicates, +/-UV). 24 h after seeding, media was replaced with fresh DMEM containing 2 mM DiazK (pH was neutralised with 1 M NaOH). Transfection mixtures with plasmids pEF1<sub> (+) </sub>His6aa_hClpP</sub>_TAG_HA_4xPylt and pEF1<sub> Mm </sub>DiazKRS<sub>_4xPylt</sub> (plasmid ratio 3:1) were prepared as depicted in the following table:

| Type of dish | Number of seeded cells/well | Total amount of DNA per well [µg] | OPTIMEM per well [µl] | Polyethyleneimine (1 mg/ml) per well [µl] |
|--------------|----------------------------|-----------------------------------|-----------------------|------------------------------------------|
| 6 well       | 5x10<sup>4</sup>/2ml        | 2                                 | 200                   | 9                                        |
| 10cm dish    | 3.5x10<sup>5</sup>/10ml      | 10                                | 1000                  | 30                                       |

Mixtures were prepared and incubated at r.t. for 15’ prior addition to the wells and cells containing DMEM with the DiazK. 40-44 h post-transfection, cells were washed 3x with 1x PBS (1x well volume). The plates or dishes (in 1x PBS, 0.5x of the usual well volume) were either placed on a cooling pad and irradiated for 15’ with a 15 W, 365 nm...
UV lamp (Vilber, VL-215.L, +UV) prior scraping or harvested (-UV) immediately. Cells were pelleted at 700 g, 4°C, 15', supernatant discarded, flash frozen in liquid nitrogen and stored at -20°C until further use.

**Western Blot**

*bacterial samples:* After SDS-PAGE of expression samples, western blots were performed via standard semidy blot procedure on nitrocellulose membranes (0.2 µm, Amersham™, Protran™, GE Healthcare Life Sciences). Membrane was blocked with 5% skim milk powder in 1x TBS-T (0.1%, 1h, r.t.) followed by incubation with anti-His6-HRP antibody (1:5000, Roche), o.n. at 4°C. Membranes were washed 5x with 1x TBS-T and blots were developed using Amersham ECL™ Prime Western Blotting Detection Reagent (GE Healthcare).

*mammalian samples:* HEK293T cell pellets (-/+ UV) were lysed by freeze/thaw cycles (3x). Lysates were cleared (max speed, 4°C, 15') and supernatant fractions were kept for further analysis. BCA assays (Pierce™ BCA Protein Assay Kit, Thermo Scientific) were performed to ensure equal loading prior SDS-PAGE loading. After transfer onto nitrocellulose membrane, the membrane was blocked with 5% skim milk powder in 1x TBS-T (0.1%, 1h, r.t.), followed by incubation with primary anti-HA antibody (rabbit, 1:5000, provided by Itzen Lab, UKE Hamburg) in 1% skim milk powder in 1x TBS-T (0.1%), o.n. at 4°C. Membranes were washed 5x with 1x TBS-T, followed by incubation with the secondary goat anti-rabbit-IgG-HRP antibody (1:40000, provided by Itzen Lab, UKE Hamburg) for 1 h at r.t. After washing with 1x TBS-T (3x), blots were developed using Amersham ECL™ Prime Western Blotting Detection Reagent (GE Healthcare).

**Rotenone treatment of HEK293T expressing hClpP-DiazK variants**

HEK293T cells were cultivated in 10cm dishes with DMEM containing DiazK and transfected with pEF1_(+)56aa_hClpP_D92TAG_HA_4xPylt or pEF1_(+)56aa_hClpP_K261TAG_HA_4xPylt and pEF1_Mm_DiazKRS_4xPylt as described before. 40 h post-transfection, rotenone (10 mM stock solution in DMSO) was added to the plates to a final concentration of 1 µM. After 6 h, cells were harvested and processed for +/- UV-light treatment and proteomic measurements as described before.

**Immunofluorescence microscopy of HEK293T cells expressing hClpP-DiazK variants**

For each condition, 2.5 x 10⁴ HEK293T cells (25 µl of 10⁶ cells/ml) were incubated with 5 µl transfection mix containing 12 ng plasmid (3:1 POI:RS, POI either hClpP-D92TAG or hClpP-K261TAG) and 0.04 µg PEI (1 mg/ml) per µl OPTIMEM for 15' at r.t. and seeded (V₅ = 30 µl) into the channel of the µ-slide (ibidi µ-slide IV 0.4, cat. No. 80606). After incubation for 3 h at 37°C, 5% CO₂, either 60 µl DMEM or 60 µl DMEM containing 2.5 mM diazK (final concentration in channel: 2 mM) was added to each well of the inlet and incubated at 37°C, 5% CO₂. After 40 h, media in each well of the channel was replaced with DMEM containing 200 nM MitoRed (Sigma 53271, 60 µl each, ~160 nM final MitoRed in each channel) and incubated for 1 h at 37°C. Each channel was washed twice with 1x PBS, the liquid was removed and cells were fixed with 60 µl MeOH for 10' at -20°C. After washing twice with 1x PBS at r.t., the channel was blocked with 60 µl blocking solution (3% BSA and 0.3% Triton-X 100 in 1x PBS) for 1 h at r.t. The liquid was removed and 25 µl of primary anti-HA antibody (mouse, 1:100, Santa Cruz Biotechnology, sc-7392; in 1% BSA and 0.3% Triton-X 100 in 1x PBS) was added and slide was incubated for 2 h at r.t. The channel was washed with 1x PBS (3x), all liquid was removed and subsequently incubated with secondary goat anti-mouse-IgG-FITC antibody solution (Sigma, F0257, 1:100; in 1% BSA and 0.3% Triton-X 100 in 1x PBS) in darkness for 1 h at r.t. The channel was washed once with 1x PBS, liquid was removed and mounting medium containing DAPI (ibidi, cat. No. 50011) was added. Images were acquired on an SP8 Stellaris 8 Falcon confocal microscope (Leica Microsystems) with an HC PL APO CS2 63x/1.4 objective with oil immersion. All fluorophores were imaged sequentially. DAPI was excited at 405 nm, FITC at 495 nm and MitoRed at 569 nm. The laser intensities were kept constant between each channel of the ibidi µ-slide. Images were analyzed with FIJI ImageJ[11] and were presented with the same contrast settings.
Experiments for proteomic analyses of hClpP-crosslinked proteins

In situ trapping and HA-Antibody Enrichment

Cells were thawed on ice, resuspended in 1 ml lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM MgCl$_2$ \cdot 6$\text{H}_2\text{O}$, 1% (v/v) 4-Nonylphenyl-polyethylene glycol (NP-40), 5% (v/v) glycerol, pH = 7.4 at 4°C) and incubated for 30' at 4°C. Afterwards membranes and cell debris were separated by centrifugation (21100 g, 20', 4°C). For protein enrichment, 30 μl monoclonal HA-antibody agarose beads suspension (A2095, isotype IgG1, Merck) per replicate in LoBind Eppendorf tubes were equilibrated with 1 ml wash buffer (50 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) NP-40, 5% (v/v) glycerol, pH = 7.4 at 4°C) and centrifuged at 1000 g for 1' at 4°C. 500 μl cytosolic fraction were incubated with equilibrated beads for 3 h at 4°C on a rotating wheel. Afterwards beads were centrifuged at 1000 g for 1' at 4°C. The supernatant was discarded and beads were washed two times each with 1 ml wash buffer and two times each with 1 ml basic buffer (50 mM Tris/HCl, 150 mM NaCl, pH = 7.4 at 4°C) to remove unspecifically bound proteins.

Digestion, reduction, alkylation

Digestion and reduction of enriched proteins was performed by addition of 25 μl digestion buffer I (5 ng/μl trypsin (in 50 mM acetic acid), 50 mM Tris/HCl, 2 M urea, 1 mM DTT (freshly prepared and diluted 1:1000 from 1 M stock, pH = 8.0) and incubated for 30'. Afterwards, alkylation of free cysteines and further digestion was conducted by addition of 100 μl digestion buffer II (5.5 mM IAA – freshly prepared and diluted 1:100 from 550 mM stock, 50 mM Tris/HCl, 2 M urea, pH = 8.0), following incubation for 16 - 19 h at 25°C with continuous mixing at 650 rpm in a thermostaker (Thermomixer comfort 5355, Eppendorf).

Desalting and sample preparation

Digestion was stopped by adjusting the pH to 2 - 3 via addition of 17.5 μl 10% (v/v) FA in water. Peptide solutions were desalted by stage tips with two-layered C18 material (SDC-XC, 3M) according to a published protocol. In brief, per replicate two layers of C18 material were packed into a 200 μl pipet tip and washed with 70 μl MeOH, 70 μl 80% (v/v) MeCN, 0.5% (v/v) FA and 3x 70 μl 0.5% (v/v) FA (centrifugation: 1000 g, 1-2', r.t.). Samples were loaded and centrifuged at 1000 g for 1-2' at r.t. Beads were washed with 70 μl 0.5% (v/v) FA and the washing solution was also loaded onto the C18 material. Samples were desalted by washing two times with 70 μl 0.5% (v/v) FA. Elution of peptides was conducted with 2x 30 μl 80% (v/v) MeCN, 0.5% (v/v) FA into fresh LoBind Eppendorf tubes followed by speedvac assisted solvent removal. Samples were stored at -80°C until further use. For LC-MS/MS measurement sample peptides were dissolved in 25 μl 1% (v/v) aqueous FA and sonicated 3x 5' with centrifugation for 1' at 17000 g in between. Dissolved peptides were filtered using 0.22 μm Ultrafree-MC® centrifugal filters (UFC30GVNB, Merck) pre-equilibrated with 300 μl 1% (v/v) FA. Filtrates were transferred to MS-vials prior to LC-MS/MS analysis.

Data acquisition on Orbitrap Fusion

Peptide samples from substrate enrichment experiments were analyzed with an UltiMate 3000 nano HPLC system (Dionex) using an Acclaim C18 PepMap100 (75 μm ID x 2 cm) trap and an Acclaim PepMap RSLC C18 (75 μm ID x 50 cm) separation column in EASY-spray setting coupled to an Orbitrap Fusion (Thermo Fisher Scientific Inc.). 1-5 μl peptide sample (see Supplementary table 1) were loaded on the trap and washed with 0.1% (v/v) TFA, then transferred to the analytical column (buffer A: H$_2$O with 0.1% (v/v) FA, buffer B: MeCN with 0.1% (v/v) FA, flow as indicated in the table below, gradient: to 5% buffer B in 7’, from 5% to 22% buffer B in 105’, then to 32% buffer B in 10’, to 90% buffer B in 10’ and hold at 90% buffer B for 10’, then to 5% buffer B in 0.1’ and hold 5% buffer B for 9.9’) and ionized by nanospray ionization (NSI) with capillary temperature of 275 °C. Spray voltage was applied as indicated in the table below. Orbitrap Fusion was operated in a TOP speed data dependent mode. Master scan acquisition was carried out in the orbitrap at a resolution of $R = 120,000$, an AGC target of 2.0e5 in a scan range of 300 - 1500 m/z and a maximum injection time of 50 ms. Monoisotopic Peak Determination was set to “Peptide” and dynamic exclusion was enabled with dynamic exclusion duration set to 60 s with a mass tolerance (low/high) of 10 ppm. Precursors with a charge state of 2 - 7 and intensities greater than 5.0e3 were submitted to fragmentation by higher collisional dissociation (HCD). Isolation of precursors was performed in the Quadrupole with an isolation window of 1.6 m/z. Detection was carried out in the ion trap to an AGC target of 1.0e4 with first
mass set to 120 m/z, Ion Trap Scan Rate set to “Rapid” and “Inject Ions for All Available Parallelizable Time” set to true. Maximum Ion Injection Time was set to 100 ms and peptide fragments were generated by HCD with a collision energy of 30%.

Data processing
MS-data were processed using Andromeda search engine of MaxQuant (MQ) Software (version 1.6.0.1). For identification of peptides, MS/MS spectra were searched against a Uniprot reference proteome (taxon identifier 9606, canonical version, without isoforms, downloaded 2017/07/18). MaxQuant settings were largely set on default with Label-free quantification (LFQ) enabled and Trypsin as digestion enzyme with a maximum of 2 missed cleavages and a minimum peptide length of 7 amino acids. Methionine oxidation and N-terminal acetylation were set as variable modifications with a maximum number of 5 modifications per peptide and carbamidomethylation of cysteines was used as fixed modification. Second peptide search was enabled as well as match between runs with a matching time window of 0.7' and an alignment time window of 20'. For identification false discovery rate on the PSM, protein and site level were set to 0.01.

Statistical MS-data analysis
MaxQuant data were further statistically processed with Perseus software (version 1.6.2.3). A ProteinGroups table was loaded into the program with LFQ intensities of all replicates as main column. Rows were filtered based on categorical columns omitting values that met the criterium of “reverse”, “potential contaminant” and “only identified by site”. After log2 transformation, matrices were further filtered based on valid values. Proteins with less than 50% of valid values in LFQ intensity compared to all replicates were excluded. Remaining missing values were inserted by imputation from a normal distribution (width: 0.3, down shift: 1.2 - 1.8). Categorical annotation of UV-irradiated (+UV) and non-treated (-UV) samples was performed before data were analyzed by two-sided student’s t-test with -UV as single control group with Benjamini-Hochberg false discovery rate correction (FDR 0.05). Additionally, GO-term annotations with GOBP, GOMF and GOCC, downloaded from Uniprot were added. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[12] partner repository with the dataset identifier PXD027954.
Experiment for whole proteome analysis of HEK293T hClpP knockout mutant

For whole proteome analysis HEK293T wild type cells and HEK293T hClpP knockout cells (generously provided by Prof. Aleksandra Trifunovic, CECAD Cologne) were cultivated on 10 cm dishes to a confluency of 30-40%. Experiments were conducted in quadruplicates per state. Medium was removed, and cells were scratched after addition of 500 µl 1x PBS. Cells were centrifuged at 500 g, for 10’ at 4°C and the supernatant was discarded. 500 µl lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, pH = 7.5) was added and cells were incubated for 15’ on ice. Afterwards cell debris was separated by centrifugation at 21100 g for 15’ at 4°C. Protein concentration of cytosolic fractions was adjusted to 100 µg/µl by addition of lysis buffer and proteins were subsequently precipitated by addition of 400 µl acetone (-80°C) and incubation o.n. at -20°C. Protein pellets were centrifuged at 21100 g for 10’ at 4°C and the supernatant was discarded. Washing steps were performed with 1.) 500 µl and 2.) 200 µl methanol (-20°C) with centrifugation steps (21100 g, 10’, 4°C) in between.

Reduction, alkylation and digestion
Sample preparation was conducted according to a modified FASP (Filter aided sample preparation) protocol. For this, filter units (Millipore, Cat. MRCF0R030, NMWL: 30 kDa) were washed once with 500 µl 50 mM aqueous NaOH, centrifuged at 14000 g for 15’ and washed again with 500 µl UA buffer (8 M urea, 0.1 M Tris/HCl, pH = 8.5) with centrifugation at 14000 g for 15’.

Protein pellets were dissolved in 100 µl UA buffer and 1 µl DTT in water (stock solution: 100 mM, final concentration per sample: 1 mM) was added. Samples were incubated at 37°C for 1 h under continuous mixing in a thermostaker. 50 µl of the protein solution was added to the filter units and centrifuged at 14000 g for 15’. 200 µl UA buffer was added and filter units were centrifuged at 14,000 g for 15’.

For alkylation of free cysteines 100 µl iodoacetamide (0.05 M in UA buffer) was added to each filter unit and incubated for 20’ after short mixing for 1’ at 600 rpm. Filter units were centrifuged at 14000 g for 10’ and washed two times with 100 µl UA buffer each (14000 g, 15’). 100 µl ABC buffer (50 mM ammonium bicarbonate) was added and each filter unit was centrifuged at 14000 g for 10’. This step was repeated and afterwards 40 µl ABC buffer containing 2 µl Trypsin (5 ng/µl trypsin (in 50 mM acetic acid)) was added. Digestion was performed by incubation of the filter units in a wet chamber at 37°C for 16 h.

Digested peptides were eluted after transfer of the filter units to new collection tubes by centrifugation at 14000 g for 10’. 40 µl ABC buffer was added to each filter and centrifuged again at 14000 g for 10’.

Desalting and sample preparation
Digestion was quenched by addition of 0.5 µl formic acid and desalting was performed with Sep-Pak® C18 columns (50 mg sorbent per cartridge, 55 – 105 µm particle size, Waters, WAT054955). Columns were washed with 1 ml acetonitrile, 500 µl elution buffer (80% MeCN, 19.5% H₂O, 0.5% FA) and three times with 0.1% FA in H₂O. Samples were loaded onto the columns and washed with 1 ml 0.1% FA and 250 µl 0.5% FA in H₂O. Peptides were eluted by addition of three times 250 µl elution buffer. Samples were dried by speedvac solvent removal and stored at -80°C.

For analysis by LC-MS/MS peptide samples were dissolved in 25 µl 1% FA in H₂O and sonicated three times for 5’. Peptide solutions were filtered with 0.22 µm Ultrafree-MC® centrifugal filters (Merck, UFC30GVNB) after equilibration with 300 µl 1% FA.

Data acquisition on Orbitrap Fusion
Peptide samples from whole proteome experiments were analyzed with an UltiMate 3000 nano HPLC system (Dionex) using an Acclaim C18 PepMap100 (75 µm ID x 2 cm) trap and an Acclaim PepMap RSLC C18 (75 µm ID x 50 cm) separation column in EASY-spray setting coupled to an Orbitrap Fusion (Thermo Fisher Scientific Inc.). 3 µl peptide sample were loaded on the trap and washed with 0.1% (v/v) TFA, then transferred to the analytical column (buffer A: H₂O with 0.1% (v/v) FA, buffer B: MeCN with 0.1% (v/v) FA, 0.4 µl/min, gradient: to 5% buffer B in 7’, from 5% to 22% buffer B in 105’, then to 32% buffer B in 10’, to 90% buffer B in 10’ and hold at 90% buffer B for 10’, then to 5% buffer B in 1.1’ and hold 5% buffer B for 9.9’) and ionized by nanospray ionization (NSI) with capillary temperature of 275 °C. Spray voltage was applied as indicated in the table below. Orbitrap Fusion was
operated in a TOP speed data dependent mode. Master scan acquisition was carried out in the orbitrap at a resolution of $R = 120,000$, an AGC target of $2.0e5$ in a scan range of $300 - 1500 \text{ m/z}$ and a maximum injection time of $50 \text{ ms}$. Monoisotopic Peak Determination was set to "Peptide" and dynamic exclusion was enabled with dynamic exclusion duration set to $60 \text{ s}$ with a mass tolerance (low/high) of $10 \text{ ppm}$. Precursors with a charge state of $2 - 7$ and intensities greater than $5.0e3$ were submitted to fragmentation by higher collisional dissociation (HCD). Isolation of precursors was performed in the Quadrupole with an isolation window of $1.6 \text{ m/z}$. Detection was carried out in the ion trap to an AGC target of $1.0e4$ with first mass set to $120 \text{ m/z}$, Ion Trap Scan Rate set to "Rapid" and "Inject Ions for All Available Parallelizable Time" set to true. Maximum Ion Injection Time was set to $100 \text{ ms}$ and peptide fragments were generated by HCD with a collision energy of $30\%$.

**Data processing**
MS-data were processed using Andromeda search engine of MaxQuant (MQ) Software (version 1.6.17.0). For identification of peptides, MS/MS spectra were searched against a Uniprot reference proteome (taxon identifier 9606, canonical version, without isoforms, downloaded 2017/07/18). MaxQuant settings were largely set on default with Label-free quantification (LFQ) enabled and Trypsin as digestion enzyme with a maximum of $2$ missed cleavages and a minimum peptide length of $7$ amino acids. Methionine oxidation and N-terminal acetylation were set as variable modifications with a maximum number of $5$ modifications per peptide and carbamidomethylation of cysteines was used as fixed modification. Second peptide search was enabled as well as match between runs with a matching time window of $0.7\text{'}$ and an alignment time window of $20\text{'}$. For identification false discovery rate on the PSM, protein and site level were set to $0.01$.

**Statistical MS-data analysis**
MaxQuant data were further statistically processed with Perseus software (version 1.6.2.3). A ProteinGroups table was loaded into the program with LFQ intensities of all replicates as main column. Rows were filtered based on categorical columns omitting values that met the criterium of "reverse", "potential contaminant" and "only identified by site". After log2 transformation, matrices were further filtered based on valid values. Proteins with less than $50\%$ of valid values in LFQ intensity compared to all replicates were excluded. Remaining missing values were inserted by imputation from a normal distribution (width: $0.3$, down shift: $1.5$). Categorical annotation of Hek293T hClpP knockout (KO) and Hek293T wild type (WT) samples was performed before data were analyzed by two-sided student’s t-test with WT as single control group with Benjamini-Hochberg false discovery rate correction (FDR 0.05). Additionally, GO-term annotations with GOCC, downloaded from Uniprot were added.
SUPPORTING INFORMATION

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