frontiers

Supplementary Material

Oxidation is an underappreciated post-translational modification in the regulation of immune responses associated with changes in phosphorylation

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1 Supplementary Figures

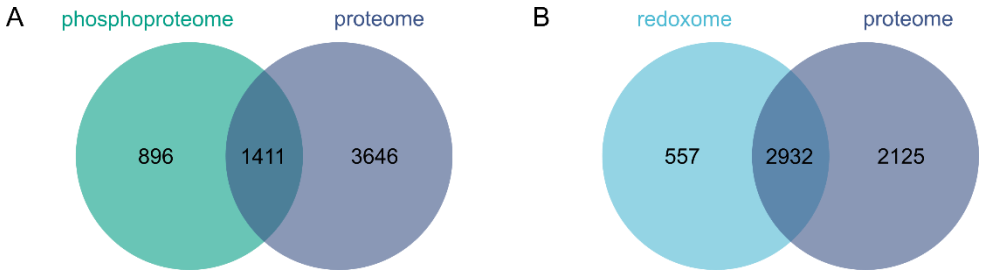

Figure S1: Overlaps in proteome proteins and proteins found phosphorylated or oxidized. Shown are the overlaps of proteins found in the proteome and such for which phosphorylation sites (A) or oxidation sites (B) were identified. Since site intensities were normalized to changes on the protein level, only modified proteins with the protein intensity available in the proteome were investigated further.

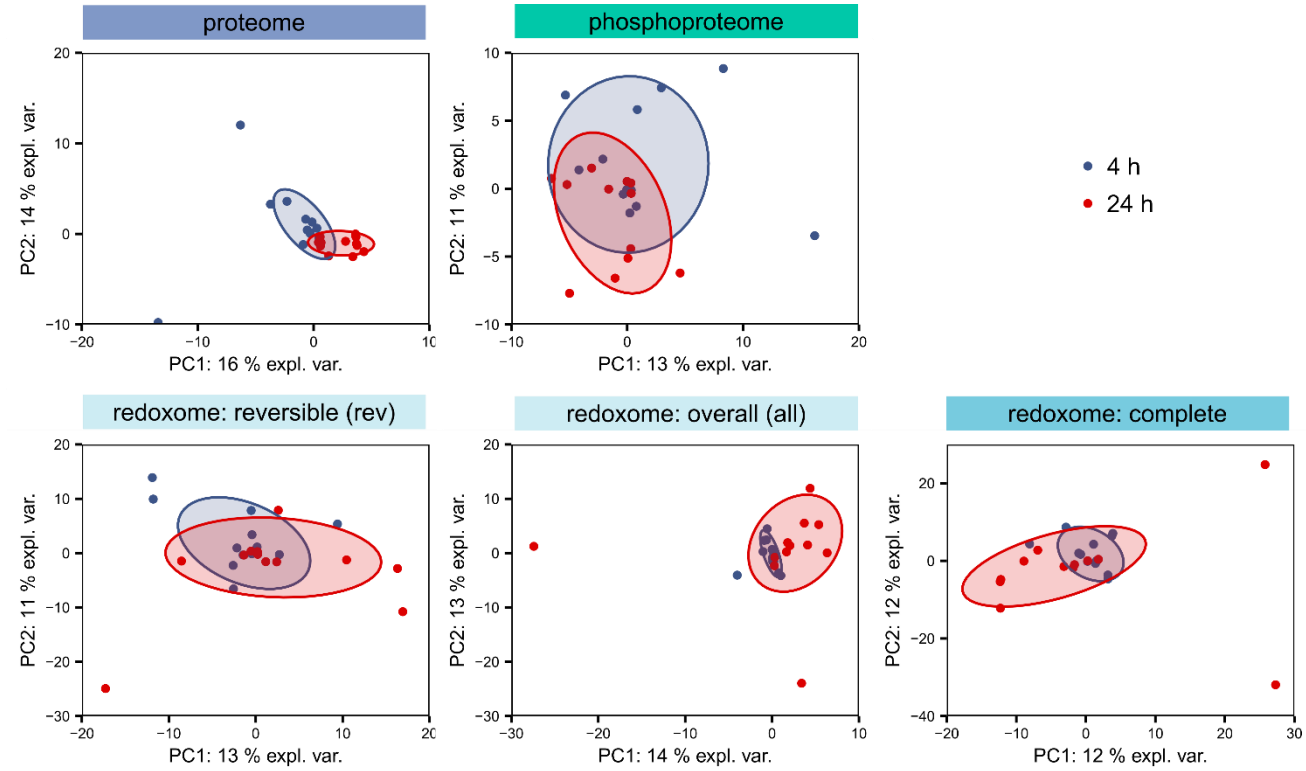

Figure S2: Principal component analyses (PCAs) of reliably identified proteins and sites. PCAs were performed with reliably quantified proteins (proteome), phosphorylation sites (phosphoproteome), reversible oxidation sites (redoxome: reversible), overall oxidation sites (redoxome: overall), and the combination of both (redoxome: complete) to assess the data reproducibility after 4 h and 24 h LPS treatment. The very low explained variances in the first two principal components suggest a good reproducibility.

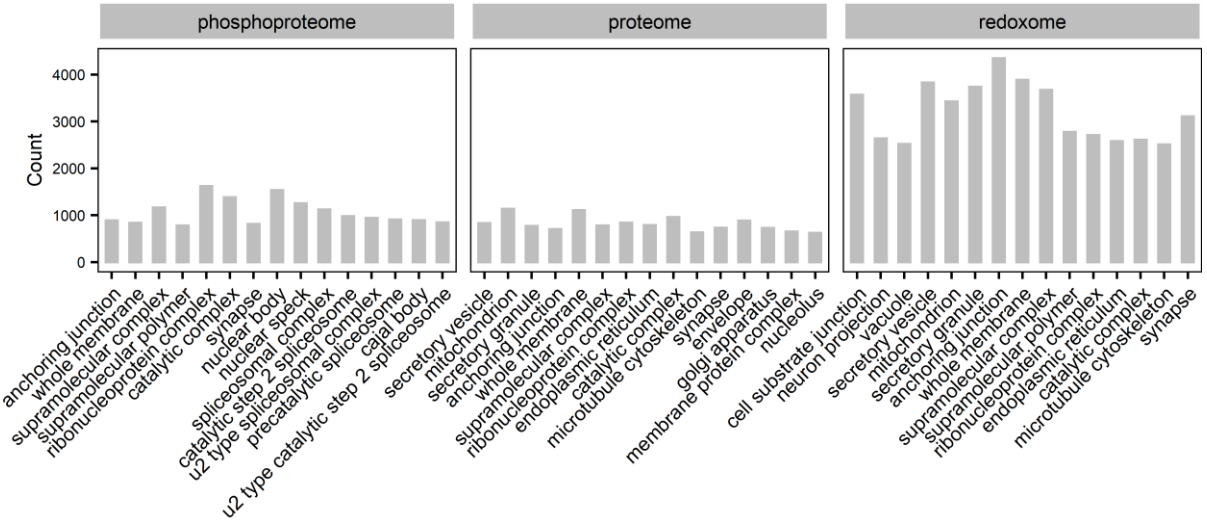

Figure S3: Assignment of identified proteins to cellular compartments.
Shown are the compartments assigned to most phosphorylation sites, proteins, or oxidation sites quantified here.

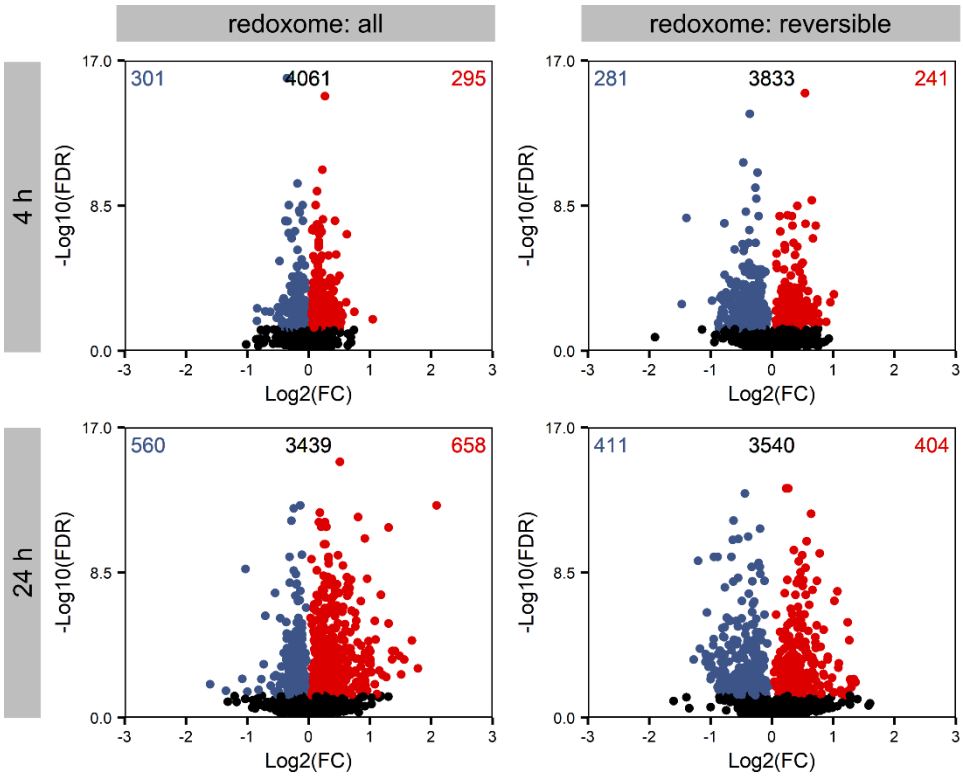

Figure S4: Affected oxidation sites.
Shown are significantly (FDR≤0.05) altered oxidation sites after LPS treatment of THP-1 macrophages for 4 h and 24 h, respectively. Overall oxidation (redoxome: all) and reversible oxidation (redoxome: reversible) are distinguished. Log2(FCs) and -Log10(FDRs) are depicted, highlighting the numbers of significantly increased (FDR≤0.05, Log2(FC)>0) or decreased (FDR≤0.05, Log2(FC)<0) sites in the corners.

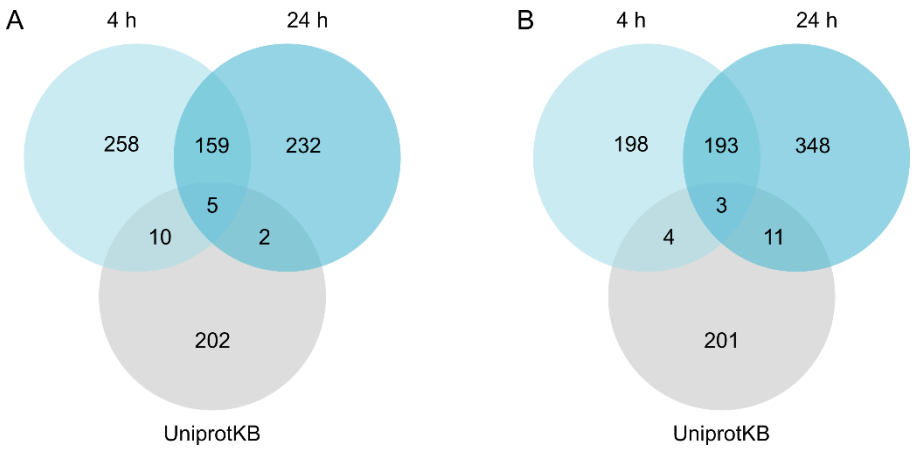

Figure S5: Overlap of affected oxidized proteins with proteins known to be modified on cysteine. Proteins showing significantly (FDR≤0.05) altered overall (A) or reversible (B) oxidation after 4 h or 24 h, respectively, were compared to proteins known for cysteine modification in general or the presence of S-nitrosocysteine in particular. For this comparison, information available in the UniProtKB was used.

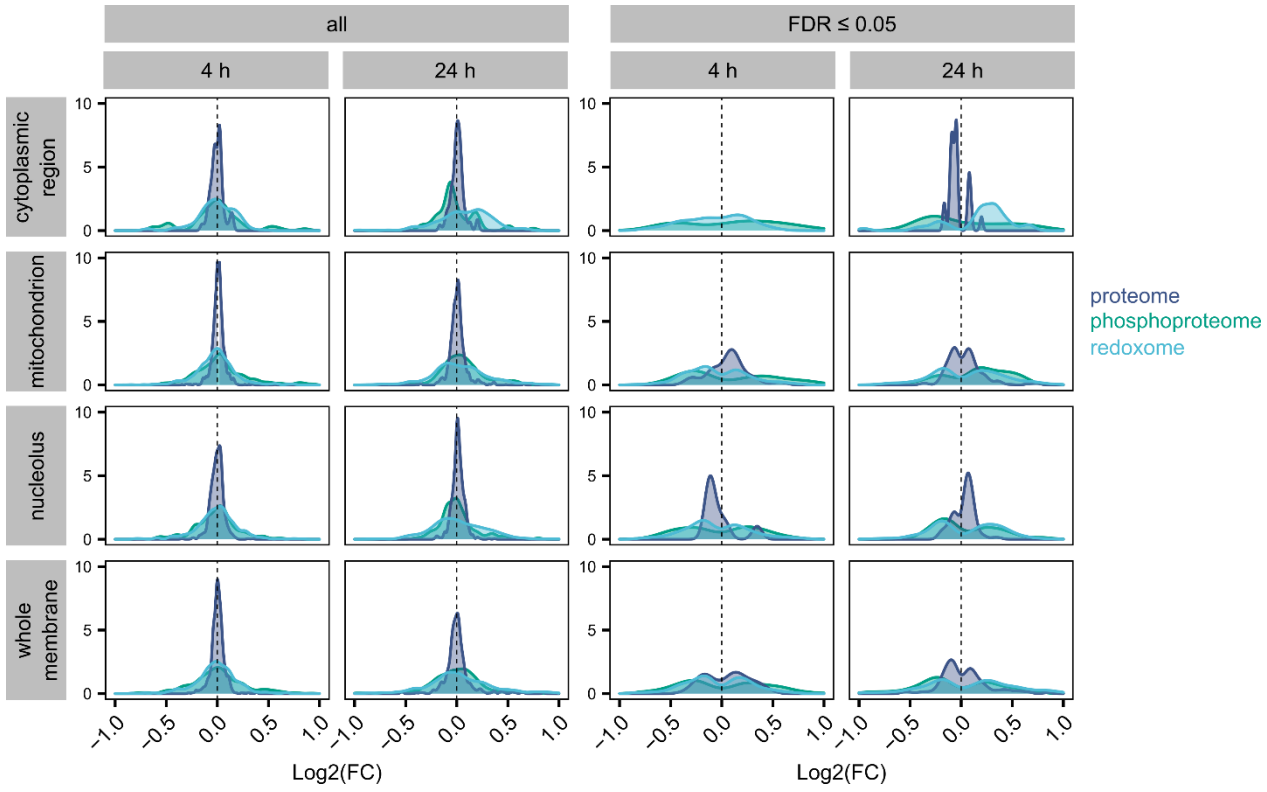

Figure S6: Compartment densities.
Shown are compartment-specific Log2(FC) distributions for all identified proteins/sites and significantly (FDR≤0.05) altered proteins/sites.

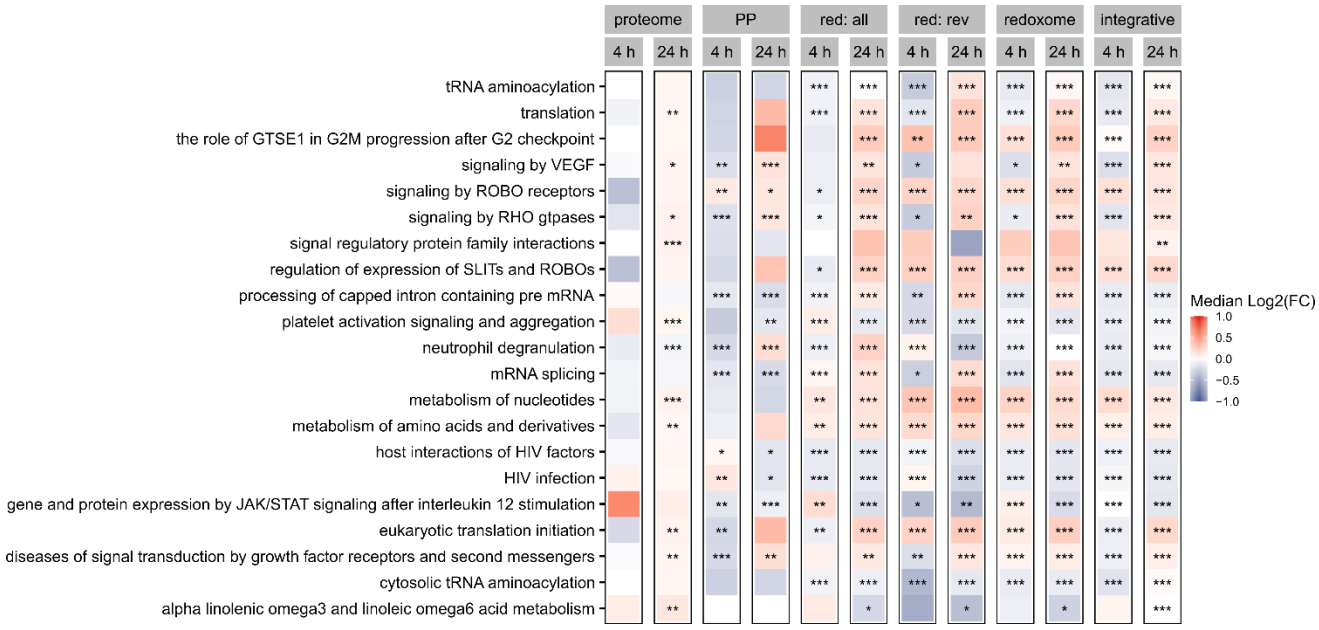

Figure S7: Top 5 significantly enriched Reactome pathways.
Combination of the top 5 Reactome pathways, significantly enriched (FDR≤0.05) either in one of the investigated omics layers or based on the integrative pathway enrichment. The significance of enrichment is provided with asterisks: * - FDR≤0.05, ** - FDR≤0.01, *** - FDR≤0.001. The color reflects the median Log2(FC) of the proteins/sites assigned to the pathway. The enrichment analysis was performed based on significantly (FDR≤0.05) altered proteins (proteome), phosphorylation sites (PP), oxidation sites in general (red: all), reversibly oxidized sites (red: rev), the combination of both types of oxidation sites (redoxome), or the combination of all three omics layers (integrative).

2 Supplementary Methods

2.1 Proteome

20 µg protein per sample was prepared for untargeted proteomics using a paramagnetic bead approach (1-3) in combination with enzymatic cleavage using trypsin and tandem mass tag (TMT, Thermo Scientific, USA) labeling as described before (4). After preparation, samples were analyzed using liquid chromatography (LC), i.e. nano-UPLC system (Ultimate 3000, Dionex, USA), coupled to a mass spectrometer (MS, QExactive HF, Thermo Scientific, USA) via a chip-based ESI source (Nanomate, Advion, USA).

2.1.1 Sample preparation on paramagnetic beads

2.1.1.1 Protein clean-up and enzymatic cleavage

20 µg per sample was used, and the volume was adjusted to 50 µl with 100 mM TEAB (Tetraethylammonium tetrahydroborate, Sigma-Aldrich, USA) first. Afterwards, samples were reduced with 5 µl 200 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride, Sigma-Aldrich, USA) in 100 mM TEAB for 1 h at 55 °C. Subsequently, 5 µl 375 mM iodoacetamide (Merck KGaA, Germany) in 100 mM TEAB was added and incubated for 30 min at room temperature in the dark. Next, 70 µl pure acetonitrile (ACN) was added to the samples to reach a final organic content higher than 50 % (v/v), facilitating protein binding to SP3 paramagnetic beads. 2 µl SP3 carboxylated beads (SpeedBeads™ magnetic carboxylate modified particles, Cytiva, Merck KGaA, Germany) per sample were washed with water three times. After the third washing step, water was removed, and samples were added to the beads. After 8 min of protein binding to the beads, the supernatant was discarded. Then, the beads were washed twice with 200 µl 70 % (v/v) ethanol and once with 200 µl pure ACN. Finally, the proteins were digested with trypsin (1:50) for 16 h at 37 °C. Since trypsin is added in aqueous solution to the samples, the proteins are not bound to the beads during enzymatic cleavage.

2.1.1.2 TMT labeling

For TMT labeling, approximately 60 µg TMT labeling reagent (TMT 6-plex, Thermo Fisher Scientific, USA) in 5 µl pure ACN was added to the obtained peptide samples, followed by 1 h incubation at room temperature. The reaction was quenched for 15 min at room temperature by adding 1 µL 5 % (v/v) hydroxylamine in 100 mM TEAB.

2.1.1.3 Peptide clean-up

To facilitate the subsequent peptide clean-up, 140 µl pure ACN was added to each sample, resulting in an organic content higher than 95 % (v/v), enabling peptide binding to the beads. During this step, samples bearing the different TMT labels were combined. The supernatant was removed after 8 min of incubation, allowing for peptide binding to the beads. Afterwards, the samples were washed with 200 µl pure ACN. Finally, the peptides were eluted in two steps. First, with 200 µL 87 % (v/v) ACN containing 10 mM ammonium formate (pH 10), and next with two times adding 50 µL water containing 2 % (v/v) DMSO and combination of the two aqueous supernatants. Thus, two fractions of peptides were generated, evaporated and re-dissolved in water containing 0.1 % (v/v) formic acid.

2.1.2 LC-MS data acquisition

Table S1: LC gradient proteome.
Ultimate 3000, Dionex, USA
Flow rate: 0.3 µl/min, A: 0.1 % (v/v) formic acid, B: 80 % (v/v) ACN, 0.08 % (v/v) formic acid.

Time [min]	%B
0	4
5	4
100	30
140	55
155	99
160	99
165	4
180	4

Table S2: MS parameters proteome.
QExactive HF, Thermo Scientific, USA
Data-dependent acquisition

Parameter	Setting
Polarity	positive
Default charge state	2
MS1 resolution	120K
MS1 AGC target	3e6
MS1 max IT	120 ms
MS1 scan range	350 – 1550 *m/z*
MS1 data type	profile
MS2 resolution	60K
MS2 AGC target	1e5
MS2 max IT	120 ms
TopN	15
Isolation window	0.7 *m/z*
MS2 fixed first mass	120 *m/z*
(N)CE	34
MS2 data type	profile
Min AGC target	2e3
Charge exclusion	Unassigned, 1, >6
Dynamic exclusion	45 s

2.1.3 Data processing using Proteome Discoverer 2.5

Table S3: Processing workflow parameters proteome.

Parameter	Setting
Mass analyzer	FTMS
MS order	2
Activation type	HCD
Precursor selection	Use MS1 precursor
Min precursor mass	350 Da
Max precursor mass	5000 Da
S/N threshold	1.5
Enzyme name	Trypsin (Full)
Max missed cleavages	2
Min peptide length	6
Max peptide length	150
Precursor mass tolerance	10 ppm
Fragment mass tolerance	0.6 Da
Dynamic modifications	Oxidation (M), TMT6plex (peptide N-terminus), Acetyl (protein N-terminus)
Statis modifications	TMT6plex (K), Carbamidomethyl (C)
FDR (strict)	0.01
FDR (relaxed)	0.05

Table S4: Consensus workflow general parameters proteome.

Parameter	Setting
Peptide confidence at least	High
Minimum number of peptide sequences	2
FDR (strict)	0.01
FDR (relaxed)	0.05
Apply strict parsimony principle	True

Table S5: Consensus workflow quantification parameters proteome.

Parameter	Setting
Peptides to use	Unique + Razor
Apply quan value corrections	True
Co-isolation threshold	50
Average reporter S/N threshold	10
Normalization mode	None
Scaling mode	None
For Protein Roll-Up	Use all peptides
Maximum allowed fold change	100

2.2 Redoxome

20 µg protein per sample was prepared for untargeted proteomics using sequential iodoTMT labeling and the described peptide clean-up on paramagnetic beads (paragraph 2.1.1.3). After preparation, samples were analyzed using liquid chromatography (LC), i.e. nano-UPLC system (Ultimate 3000, Dionex, USA), coupled to a mass spectrometer (MS, QExactive HF, Thermo Scientific, USA) via a chip-based ESI source (Nanomate, Advion, USA).

2.2.1 Sample preparation

For the redoxome, the mixes of iodoTMT-labeled peptides were subjected to peptide clean-up on paramagnetic beads (paragraph 2.1.1.3). Only the volume had to be scaled up as described in the manuscript.

2.2.2 LC-MS data acquisition

To measure the redoxome samples, the same LC and MS parameters described for the proteome have been used (paragraph 2.1.2).

2.2.3 Data processing using Proteome Discoverer 2.5

Table S6: Processing workflow parameters redoxome.
Compared to processing proteomics samples, the IMP-ptmRS node has to be added to the workflow.

Parameter	Setting
Mass analyzer	FTMS
MS order	2
Activation type	HCD
Precursor selection	Use MS1 precursor
Min precursor mass	350 Da
Max precursor mass	5000 Da
S/N threshold	1.5
Enzyme name	Trypsin (Full)
Max missed cleavages	2
Min peptide length	6
Max peptide length	150
Precursor mass tolerance	10 ppm
Fragment mass tolerance	0.6 Da
Dynamic modifications	Oxidation (M), Acetyl (protein N-terminus)
Statis modifications	iodoTMT6plex (C)
FDR (strict)	0.01
FDR (relaxed)	0.05

Table S7: Consensus workflow general parameters redoxome.
Compared to processing proteomics samples, the Peptide Isoform Grouper node has to be added to the workflow. The minimum number of peptide sequences per protein has to be decreased to 1 to keep all identified peptides. Notably, analysis is performed on the peptide level, not the protein level.

Parameter	Setting
Peptide confidence at least	High
Minimum number of peptide sequences	1
FDR (strict)	0.01
FDR (relaxed)	0.05
Apply strict parsimony principle	True

Table S8: Consensus workflow quantification parameters redoxome.

Parameter	Setting
Peptides to use	Unique + Razor
Apply quan value corrections	True
Co-isolation threshold	50
Average reporter S/N threshold	10
Normalization mode	None
Scaling mode	None
For Protein Roll-Up	Use all peptides
Maximum allowed fold change	100

2.3 Phosphoproteome

70-100 µg protein per sample was prepared for untargeted proteomics using TMT labeling on paramagnetic beads (paragraph 2.1.1). Phosphorylated peptides were enriched using a two-step enrichment workflow. After preparation, samples were analyzed using liquid chromatography (LC), i.e. nano-UPLC system (Ultimate 3000, Dionex, USA), coupled to a mass spectrometer (MS, QExactive HF, Thermo Scientific, USA) via a chip-based ESI source (Nanomate, Advion, USA).

2.3.1 Sample preparation

For the phosphoproteome, the same paramagnetic bead approach described for the proteome (paragraph 2.1.1) has been applied. Only the elution after the peptide clean-up was done differently than for proteome and redoxome, not in two fractions but only with water. Afterwards, a two-step enrichment using a workflow based on the HighSelect™ TiO2 Phosphopeptide Enrichment Kit (Thermo Scientific, USA) and the High-Select™ Fe-NTA Phosphopeptide Enrichment Kit (Thermo Scientific, USA) was performed as described before (5).

2.3.2 LC-MS data acquisition

Table S9: LC gradient phosphoproteome.
Ultimate 3000, Dionex, USA
Flow rate: 0.3 µl/min, A: 0.1 % (v/v) formic acid, B: 80 % (v/v) ACN, 0.08 % (v/v) formic acid.

Time [min]	%B
0	4
5	4
82.5	18
120	30
150	55
155	99
165	99
170	4
180	4

Table S10: MS parameters phosphoproteome.
QExactive HF, Thermo Scientific, USA
Data-dependent acquisition

Parameter	Setting
Polarity	positive
Default charge state	2
MS1 resolution	120K
MS1 AGC target	3e6
MS1 max IT	150 ms
MS1 scan range	350 – 1550 *m/z*
MS1 data type	profile
MS2 resolution	60K
MS2 AGC target	2e5
MS2 max IT	150 ms
TopN	15
Isolation window	0.7 *m/z*
MS2 fixed first mass	120 *m/z*
(N)CE	34
MS2 data type	profile
Min AGC target	2e3
Charge exclusion	Unassigned, 1, >6
Dynamic exclusion	45 s

2.3.3 Data processing using Proteome Discoverer 2.5

Table S11: Processing workflow parameters phosphoproteome.
Compared to processing proteomics samples, the IMP-ptmRS node has to be added to the workflow.

Parameter	Setting
Mass analyzer	FTMS
MS order	2
Activation type	HCD
Precursor selection	Use MS1 precursor
Min precursor mass	350 Da
Max precursor mass	5000 Da
S/N threshold	1.5
Enzyme name	Trypsin (Full)
Max missed cleavages	2
Min peptide length	6
Max peptide length	150
Precursor mass tolerance	10 ppm
Fragment mass tolerance	0.6 Da
Dynamic modifications	Oxidation (M), TMTpro (peptide N-terminus), Acetyl (protein N-terminus), Phospho (S, T, Y)
Statis modifications	TMTpro (K), Carbamidomethyl (C)
FDR (strict)	0.01
FDR (relaxed)	0.05

Table S12: Consensus workflow general parameters phosphoproteome.
Compared to processing proteomics samples, the Peptide Isoform Grouper node has to be added to the workflow. The minimum number of peptide sequences per protein has to be decreased to 1 to keep all identified peptides. Notably, analysis is performed on the peptide level and not on the protein level .

Parameter	Setting
Peptide confidence at least	High
Minimum number of peptide sequences	1
FDR (strict)	0.01
FDR (relaxed)	0.05
Apply strict parsimony principle	True

Table S13: Consensus workflow quantification parameters phosphoproteome.

Parameter	Setting
Peptides to use	Unique + Razor
Apply quan value corrections	True
Co-isolation threshold	50
Average reporter S/N threshold	10
Normalization mode	None
Scaling mode	None
For Protein Roll-Up	Use all peptides
Maximum allowed fold change	100

3 References

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