Supplementary Methods

Pulsed SILAC-TMT data extraction and normalization

Database searching of SILAC and, at the same time, TMT labeled samples was performed using MaxQuant specifying SILAC amino acids as variable modifications and TMT10plex as a label. As a consequence, TMT intensities provided in MaxQuant's proteinGroup.txt output file are derived from the sum of both heavy and light peptides, rendering these quantitative information unfeasible for the assessment of the decay or increase of either of both labels. Hence, TMT data extracted from the evidence.txt output file which discriminated K0/R0 and the K8/R10 labeled peptides was utilized for quantitative analyses. Reverse hits, non-human contaminants and missed cleavage peptides that contained both a light and a heavy version of lysine or arginine were removed.

Data normalization was conducted under the premise that the total protein amount (i.e. light plus heavy labeled protein) was equal across TMT channels since identical protein amounts were digested and TMT labeled for all pulse time-points. As a result, the principle underlying total sum normalization procedures should also be applicable to pulsed SILAC-TMT samples obtained under steady-state conditions. However, in this regard, two additional factors must be considered: (i) Depending on the time-points chosen, intensities of light and heavy SILAC peptides can exhibit globally differential distributions leading to a preferential picking of the overall more intense of both labels for fragmentation in a DDA type of experiment; (ii) TMT intensities do correlate with the MS1 intensity of the peptide and are further highly dependent on at which moment in the elution profile the peptide was fragmented. Consequently, a normalization based on a simple summation of TMT intensities could be biased towards synthesis or degradation curves. On account of this, we introduced a so-called row wise normalization (1) before the total sum normalization procedure. This equalizes TMT reporter intensities representing completely labeled proteins, i.e. the first channel of decreasing and the last channel of increasing curves, irrespective of the MS1 intensities and time of fragmentation, thus allowing for a total sum normalization. In detail,

the following steps were performed for each cell culture replicate separately: First, for identical peptide sequences, intensities of the same TMT channel derived from different evidence entries were summed up. Subsequently, sequences containing channels with zero intensity in between channels with non-zero intensities were removed. Then, sequence entries were filtered for those for which information on synthesis and degradation behavior was available, i.e. which were quantified in both SILAC labeling states. Accordingly, a row-wise normalization factor ($\mathrm{N_{row}}$), which compensates for differences in overall TMT intensity levels due to different MS1 intensities and differing times of peak picking for the corresponding light and heavy peptides, was calculated for all remaining peptide sequences and SILAC labeling states. The calculation was based on the TMT reporter intensities (RI) in the first ($0\ \mathrm{h}$) and last ($\mathrm{inf.\ h}$) channel of peptides representing degradation (deg) and synthesis (syn) behavior, respectively:

$$\mathrm{N_{row}}\left(\mathrm{pep_{deg}}\right) = \frac{\mathrm{RI_{deg}}(0\ \mathrm{h}) + \mathrm{RI_{syn}}(\mathrm{inf.\ h})}{2 \cdot \mathrm{RI_{deg}}(0\ \mathrm{h})} \qquad (1)$$

$$\mathrm{N_{row}}\left(\mathrm{pep_{syn}}\right) = \frac{\mathrm{RI_{deg}}(0\ \mathrm{h}) + \mathrm{RI_{syn}}(\mathrm{inf.\ h})}{2 \cdot \mathrm{RI_{syn}}(\mathrm{inf.\ h})} \qquad (2)$$

This factor was multiplied with all TMT channel intensities of the particular peptide sequence in the respective labeling state. Thereafter, all intensities belonging to the same TMT channel were summed up for all sequences in both labeling states and a total sum normalization factor ($\mathrm{N_{sum}}$), which normalizes for TMT sample mixing differences, was computed for each TMT channel ($\mathrm{RI(x)}$):

$$\mathrm{N_{sum}}(\mathrm{RI(x)}) = \frac{\text{median of all } \sum_{\text{all sequences}} \mathrm{RI(x)}}{\sum_{\text{all sequences}} \mathrm{RI(x)}} \qquad (3)$$

Finally, these normalization factors were applied to respective TMT channels of all entries of MaxQuant's evidence output table.

Curve fitting and underlying kinetic model

Before curve fitting, TMT intensity ratios were computed to enable the implementation of global, intensity independent curve fitting constraints. For peptides illustrating synthesis, increasing TMT intensity were

normalized to the intensity in last (inf. h) TMT channel, whereas for peptides displaying degradation, ratios were calculated relative to the intensity of the first (0 h) TMT channel. The kinetic model which was subsequently applied for curve fitting has previously been described by Boisvert *et al.* (2) and Welle *et al.* (3). Briefly, it was based on the assumptions that (i) the probability of a protein being degraded is the same for "old" and newly synthesized proteins and stays constant over the life-time of these proteins (i.e. the degradation rate is constant), (ii) protein synthesis occurs at a constant rate, and (iii) cells are in steady-state implying that the average abundance of a protein per cell doesn't change during the course of the experiment (i.e. the synthesis rate of a protein equals its degradation rate). Hence, first order labeling kinetics were adopted for curve fitting, thus, TMT reporter intensity ratios (RIR) were fitted to exponential equations:

$$\mathrm{RIR_{syn}(t)} = \left(\mathrm{B_{syn}} - \mathrm{A_{syn}}\right) \cdot \mathrm{e}^{-\mathrm{K_{syn}} \cdot \mathrm{t}} + \mathrm{A_{syn}} \qquad (4)$$

$$\mathrm{RIR_{deg}(t)} = \left(\mathrm{A_{deg}} - \mathrm{B_{deg}}\right) \cdot \mathrm{e}^{-\mathrm{K_{deg}} \cdot \cdot \mathrm{t}} + \mathrm{B_{deg}} \qquad (5)$$

where $\mathrm{RIR_{syn}}$ is the proportion of proteins which have incorporated the newly provided label and $\mathrm{RIR_{deg}}$ is the fraction of proteins still bearing the old label at each time point t in hours. $\mathrm{K_{syn}}$ and $\mathrm{K_{deg}}$ are the rate constants of labeling incorporation and loss, respectively. As they should be the same by definition of steady-state conditions, they are referred to as turnover rate K in the following. *A* refers to the maximum of the curve (i.e. the normalized total protein amount) and should be 1 in an ideal case. *B* accounts for a potential curve offset which ideally should be 0. Offsets bigger than 0 could either be attributed to the recycling of amino acids or ratio compression. Consequently, $(A - B)$ represents the amplitude of the fitted curve.

The turnover rates, curve maxima and offsets were obtained for each evidence entry via applying a nonlinear least square (NLS) algorithm in R (version 3.3.3, function "nls") (4). To remove poor quality quantitative data, different filter criteria for K, R^2, A, and B were applied, and curves that were at the border of passing these filter criteria were manually inspected. The final filtering criteria are based on the

goal to filter out spectra which show a high variation of data points along the fitted curve (A and R^2), a high ratio compression (B), or resulted in turnover rates (K) which simply could not be determined accurately considering the pulse time-points we have chosen in the experimental design. Eventually, only entries were kept that met following filter criteria: K: 0-5; B: 0-0.3; A: 0.67-1.5; coefficient of determination R^2 of the curve fit ≥0.8.

Subsequently, 50 % turnover times were derived from equations *(4)*/*(5)* as time at which half of the amplitude is reached:

$$\mathrm{T50\%} = \frac{\ln(2)}{\mathrm{K}} \qquad (6)$$

In order to estimate protein and peptide degradation (or synthesis) rates (k) and half-lives ($\mathrm{T1/2}$), one needs to account for the influence of sheer cell doubling on labeling kinetics. Cell doubling parameter were determined via fitting an exponential growth equation to cell counts monitored over time during the pulse experiment. Subsequently, labeling rates were corrected for cell doubling rates ($\mathrm{k_{cd}}$) to obtain degradation rates:

$$\mathrm{k} = \mathrm{K} - \mathrm{k_{cd}} \qquad (7)$$

Accordingly, half-lives were calculated using equations *(4)*/*(5)* and *(7)*:

$$\mathrm{T1/2} = \frac{\ln(2)}{\mathrm{K} - \mathrm{k_{cd}}} \qquad (8)$$

Peptide and proteins rates were obtained from a combined fit including all evidence entries belonging to the respective peptide or protein sequence. In addition, only protein group unique peptides were allowed for determination of whole protein (group) turnover.

Estimation of protein copy numbers from TMT intensities

In principle, copies of a protein can be determined from its mass, molecular weight (MW) and the Avogadro constant ($\mathrm{N_A}$):

$$\text{copies (protein x)} = \frac{\text{mass (protein x)}}{\text{MW (protein x)}} \cdot \mathrm{N_A} \qquad (9)$$

Usually the mass of a protein in a sample or a single cell is not known. However, based on the assumption that, for in depth proteome analyses, a protein's MS signal intensity as a fraction of the total MS signal is an appropriate measure for the proportion of its mass to the total protein mass (5), it can be approximated as:

$$\text{mass (protein x)} = \frac{\text{MS intensity (protein x)}}{\text{total MS intensity}} \cdot \text{total protein mass} \qquad (10)$$

Accordingly, protein copy numbers per cell can be derived from equations *(9)* and *(10)* as:

$$\text{copies/cell (protein x)} = \frac{MS\ intensity\ (protein\ x)}{total\ MS\ intensity} \cdot \frac{\text{cell protein mass}}{\text{MW (protein x)}} \cdot \mathrm{N_A} \qquad (11)$$

For determination of total protein mass per cell, protein amounts were quantified with the Bradford method (Coomassie (Bradford) Protein Assay Kit, Thermo Fisher Scientific) for increasing cell numbers (3e4, 6e4, 1.2e5,2.5e5, 5e5, 7.5e5, 1e6) from three different HeLa cell batches in three replicates each. Linear correlation of cell numbers with corresponding protein amounts resulted in 251.4 pg protein per single HeLa cell.

As already stated above, TMT intensities are critically dependent on the time in the elution profile at which the precursor was picked for fragmentation rendering the direct use of TMT intensities inappropriate for the calculation of intensities as needed in equation *(11)*. However, due to the experimental design, the first or the last TMT channel always represented the completely light or heavy labeled peptide which could be utilized to obtain meaningful single protein to total protein intensity ratios. To do so, the fraction of MS1 intensity (MS1 intensity fraction) corresponding to completely labeled peptides were calculated:

$$\text{MS intensity fraction}_{deg} = \frac{\mathrm{RI_{deg}(0\ h)}}{\sum_{\text{all time-points}} \mathrm{RI_{deg}}} \cdot \text{MS1 intensity}\left(\mathrm{pep_{deg}}\right) \qquad (12)$$

$$\text{MS intensity fraction}_{syn} = \frac{\mathrm{RI_{syn}(inf.\ h)}}{\sum_{\text{all time-points}} \mathrm{RI_{syn}}} \cdot \text{MS1 intensity}\left(\mathrm{pep_{syn}}\right) \qquad (13)$$

Accordingly, protein copies per cell were determined by using the estimated protein amount per cell and by deriving the protein intensity from the sum of intensities of peptides assigned exclusively to the respective protein group:

$$\text{copies/cell (protein x)} = \frac{\sum_{\text{all unique peptides for protein x}} \text{MS intensity fraction}}{\sum_{\text{all peptides}} \text{MS intensity fraction}} \cdot \frac{2.514 \cdot 10^{-7}}{\text{MW (protein x)}} \cdot \text{N}_{\text{A}}$$

Since the MS intensity fraction can be calculated for light and heavy labeled peptides, protein copies per cell could be computed twice for each cell culture replicate. Median values were taken for correlation analysis with protein half-lives.

Comparison of MS1 and MS3 based turnover estimation

For the comparison of pulsed SILAC-TMT and classical pulsed SILAC samples derived from identical protein digests, raw files of the two approaches were searched together in separate experimental groups in the MaxQuant software. For the MS3 data, TMT intensities were normalized as described above. Then, they were summed up for all peptides with the same labeling state belonging to the same protein group followed by calculation of TMT ratios for label incorporation and loss as described above. Curves were fitted to resulting protein TMT ratios. For the classical approach, respective ratios for synthesis and degradation curves were calculated by dividing the light or heavy SILAC intensities by the sum of both channels. For the curve fitting of SILAC data, missing quantitative data for up to 3 out of 6 time-points were allowed, meanwhile counting zero intensities resulting from quantification of only 1 SILAC channel as valid (i.e. non-missing) values.

Preparation of single time-point pulsed samples following induction of oxidative stress by rotenone

For the investigation of the effects of rotenone induced, oxidative stress on turnover of respiratory chain complex I (NADH dehydrogenase) proteins, HeLa cells were seeded in light medium at 2e6 cells/10 cm dish in three replicates per condition. Following 40 h of cultivation, 1 uM rotenone (≥ 95 %) in DMSO and

5 mM L-(-)-malic acid and L-glutamic acid (Sigma) were added to the cells. Cells treated with DMSO and 5 mM L-(-)-malic acid and L-glutamic acid or DMSO only served as control. After 0.5 h, light medium was removed, cells were washed twice using PBS with Mg^{2+}/Ca^{2+}, and heavy medium containing the above stated treatment or respective control supplements was added to cells. Cells were lysed in urea lysis buffer (8 M urea, 40 mM Tris-HCl (pH 7.6), 1 x EDTA-free protease inhibitor (cOmplete™, Mini, Roche) and 1x phosphatase inhibitor cocktail) 3 or 8 hours after medium exchange. Lysates were cleared by centrifugation for 20 min at 20,000 g and 4 °C and protein concentration was determined by the Bradford method (Coomassie (Bradford) Protein Assay Kit, Thermo Fisher Scientific). After reduction (10 mM DTT, 30 °C, 30 min) and alkylation (50 mM chloroacetamide, room temperature, 30 min, in the dark), lysates were diluted to 1.6 M urea using 40 mM Tris-HCl (pH 7.6). Digestion was performed by adding trypsin (Promega, 1:50 enzyme-to-substrate ratio) and incubating overnight at 37 °C at 700 rpm. Digests were acidified by addition of neat FA to 1 % and desalted using self-packed StageTips (five disks, Ø 1.5 mm, C18 material, 3M Empore™; wash solvent: 0.1 % FA; elution solvent: 0.1 % FA in 50 % ACN). Eluted samples were frozen, dried down in a SpeedVac and stored at -20 °C until LC-MS analysis.

Setup of a parallel reaction monitoring assay (Tier 3 category) for respiratory chain complex I members

For generation of a spectral library for a PRM assay of NADH dehydrogenase proteins, nanoflow LC-ESI-MS measurements of single time-point pulse samples upon rotenone and control treatment and of a non-treated, non-pulsed HeLa sample were performed in a DDA mode as described in the main method section with following modifications: A scheduled mass to charge inclusion list with a dynamic exclusion of 30 s was set up for peptides of respiratory chain complex I members utilizing information on charge states and iRTs from the ProteomeTools project (6). MS2 spectra of peptides from the targeted mass-to-charge (m/z) list were recorded with scan priority 1 in the orbitrap at 15K using an AGC target value of 2e5 and a maxIT of 100 ms. Non targeted m/z values were recorded with scan priority 2 using an AGC target value of 1e5, a maxIT of 22 ms and a dynamic exclusion of 60 s.

The spectral library for peptides of respiratory chain complex I members was constructed using the Skyline 3.7.0 software (7) and a MaxQuant derived msms.txt and a Mascot derived dat file. To generate the msms.txt file, a MaxQuant search was set up for treated, pulsed SILAC samples as described in the main methods for solely SILAC labeled samples. The non-treated, non-pulsed HeLa sample was searched using Mascot Distiller (v2.6.1.0) specifying Trypsin/P as enzyme with up to 2 missed cleavages allowed, Carbamidomethyl (C) as fixed modification, Acetyl (N-term) and Oxidation (M) as variable modifications, 10 ppm peptide tolerance and 0.05 Da MS/MS tolerance. Allowed peptide charge states included 2+, 3+, and 4+precursors and the de-charging option was disabled. Uniqueness of NADH dehydrogenase peptides was checked against the canonical SwissProt database as background proteome in Skyline. Precursor charge states and transitions were automatically chosen from the spectral library resulting in 38 and 93 peptide entries for the MaxQuant and the Mascot derived library. A scheduled inclusion list containing mass and charge information for light and heavy labeled peptides with 6 min monitoring windows was exported for the PRM LC-MS method (see supplementary table S4).

PRM LC-MS measurements were performed as described in the main methods with following modifications: 100 fmol of PRTC retention time calibration mixture (Pierce) per sample injection were spiked into samples. The MS acquisition method was set up to switch between two separate experiments after one duty-cycle. The first experiment consisted of a full scan MS1 spectrum recorded in the Orbitrap from 360 to 1300 m/z at a resolution of 15K using an AGC target value of 4e5 and a maximum injection time of 50 ms. The second experiment consisted of a tMS^2 PRM scan triggering peptide isolation (isolation window 1.3 m/z) and fragmentation (HCD, 28 % NCE) based on the scheduled list containing m/z and charge information. MS2 spectra were recorded in the Orbitrap mass analyzer from 100 to 2000 m/z at a resolution of 15K using an AGC target value of 2e5 and a maximum injection time of 200 ms.

The generated RAW files were imported into Skyline for data filtering and analysis. The transitions were extracted allowing precursor charges 2, 3, 4 and ion types y and specifying orbitrap as mass analyzer with

15K resolution. Peaks were integrated using the automatic peak finding function followed by manual curation of all peak boundaries and transitions to remove fragment ions exhibiting interferences. At least 3 transitions that showed a robust elution profile and a dot product larger than 0.8 were required in at least one of the two SILAC channels in at least one condition. The summed area under the fragment ion traces and heavy-to-light (H/L) ratios were exported for every peptide to Microsoft Excel for data normalization and analysis (see supplementary table S4).

Normalization and analysis of PRM data

Since cell treatments can lead to a change in growth behavior of cells and this would globally influence SILAC ratios in a pulse experiment, H/L ratios derived from the PRM experiment were normalized based on a median centering of total H/L ratios across all measured samples of each pulse time-point. For this, the DDA MS analyses were utilized to calculate a normalization factor which shifted the median of log transformed H/L ratios of all samples of one pulse time-point to the same value. This normalization factor was then applied to log transformed H/L ratios of the PRM experiment. After normalization, the Perseus software suite (v.1.5.6.0) (8) was employed to perform two-sided t-tests corrected for multiple testing at 5 % permutation based FDR. S0 was calculated in R (version 3.4.1, package “samr”) based on the significance analysis of microarrays (9).

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