SUPPORTING INFORMATION

Super-Heavy TMTpro labeling reagent: An alternative and higher charge state-amenable stable isotope-labeled TMTpro variant

Alban Ordureau[1], Qing Yu [1], Ryan D. Bomgarden [2], John C. Rogers [2], J. Wade Harper[1], Steven P. Gygi [1] and Joao A. Paulo [1, *]

[1] Department of Cell Biology, Blavatnik Institute of Harvard Medical School, Boston, MA, USA
[2] Thermo Fisher Scientific, Rockford, IL, USA.

Supplemental Methods. Methods highlighted include: Sample preparation, liquid chromatography and tandem mass spectrometry and data analysis.

Figure S1: Showcasing interference of co-eluting, non-isobaric TMTpro variants using a phosphopeptide.

Figure S2: TMTpro variant-labeled proteomes in a TMTpro16 background.

SUPPLEMENTAL METHODS

Sample preparation. Reference peptides for Parkin (phosphorylated or not at Ser65) were from JPT Peptides Technologies (SpikeTides™_TQL Proteotypic Peptides; internally labeled at R72* = Arg U-13C6;U-15N4) [1]. Dried peptides (1 nmol each) were resuspended in 100 mM EPPS, pH 8.5 and digested at 37°C for 6 h with 0.2 µg Trypsin protease. Peptides were then subjected to disulfide bond reduction with 5 mM TCEP (room temperature, 10 min) and alkylation with 25 mM chloroacetamide (room temperature, 20 min) followed by addition of equal volume of 5% formic acid, combined 1:1 and subjected to C18 StageTip desalting. Samples were resuspended in 100 mM EPPS, pH 8.5 containing 30% (v/v) acetonitrile and aliquoted to achieve the indicated ratio. Tandem Mass Tag labeling of each sample was performed by adding TMTpro, TMTproZero, or Super Heavy TMTpro reagent. Following incubation at room temperature for 1 h, labeling efficiency of a small aliquot was tested, and the reaction was then quenched with hydroxylamine to a final concentration of 0.5% (v/v) for 15 min. The TMT-labeled samples were pooled together, vacuum centrifuged to near dryness, resuspended in 5% formic acid and C18 StageTip desalting [2] was performed.

Liquid chromatography and tandem mass spectrometry. Mass spectrometry data were collected using an Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific, San Jose, CA), combined with a high-field asymmetric waveform ion mobility spectrometry (FAIMS) Pro interface, coupled to a Proxeon EASY-nLC1200 liquid chromatograph (Thermo Fisher Scientific). Peptides were separated on a 100 µm inner diameter microcapillary column packed in house with ~35 cm of Accucore150 resin (2.6 µm, 150 Å, Thermo Fisher Scientific, San Jose, CA) with a gradient consisting of 10-20% (0-22min) (ACN, 0.1% FA) over a total 30 min run at ~630 nL/min. For analysis, we loaded 50 pmol peptides per injection of each peptide onto the column. This corresponded to: ~41.66 pmol of TMTproZero- or shTMTpro-labeled peptide and 8.33 pmol of TMTpro16 total (more specifically, 520 fmol per reporter ion channel). Data were collected in Profile mode for each scan events and we used Advance Peak Determination (APD) algorithm. The scan sequence began with an MS1 spectrum (Orbitrap analysis; resolution 120,000 at 200 Th; mass range 500−1400 m/z; automatic gain control (AGC) target 4×10^5; maximum injection time 50 ms). Precursors for MS2 analysis were selected using a cycle type of 1.25 sec/CV method (FAIMS CV=-40 as optimized previously for these two peptides). MS2 analysis consisted of

higher-energy collisional dissociation (Orbitrap analysis; resolution 50,000 at 200 Th; AGC 2.0×10^5; isolation window 2 Th; collision energy (%) 37; First mass (m/z) 110; maximum injection time 300 ms). Dependent scan was performed based on the following precursor targeted inclusion list (831.1741, z=4 and 851.1657, z=4; mass tolerance ±15ppm).

Data analysis. LC-MS/MS data analysis was performed using Skyline (v19.1) software [3] with manual validation of precursors and fragment ion masses.

Supplemental references.

1. Ordureau, A.; Sarraf, S. A.; Duda, D. M.; Heo, J. M.; Jedrychowski, M. P.; Sviderskiy, V. O.; Olszewski, J. L.; Koerber, J. T.; Xie, T.; Beausoleil, S. A.; Wells, J. A.; Gygi, S. P.; Schulman, B. A.; Harper, J. W., Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. Mol Cell 2014, 56 (3), 360-75.
2. Rappsilber, J.; Ishihama, Y.; Mann, M., Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem 2003, 75 (3), 663-70.
3. MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J., Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 2010, 26 (7), 966-8.

Figure S1:

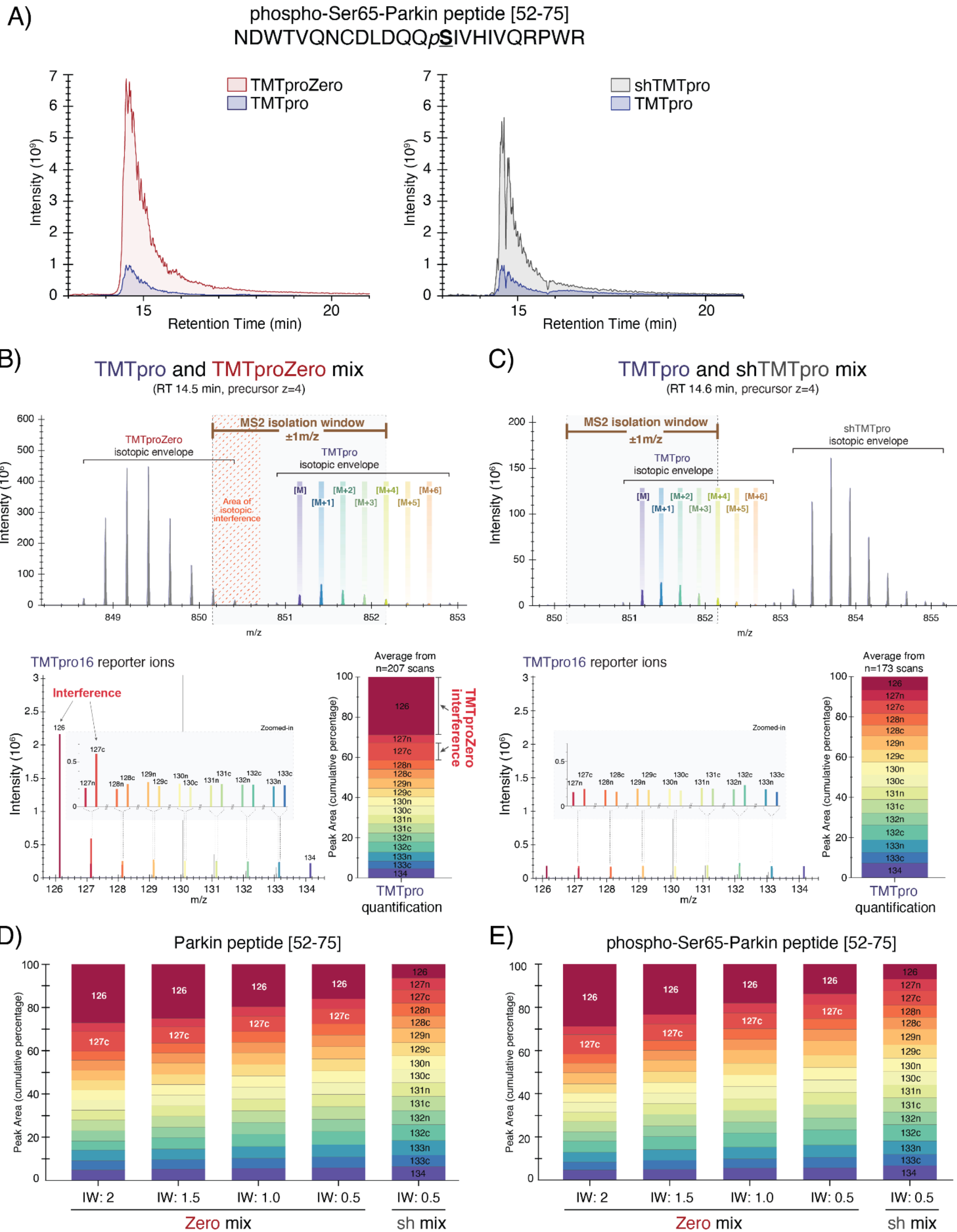

Figure S1: Showcasing interference of co-eluting, non-isobaric TMTpro variants using a phosphopeptide. A) Chromatogram showing co-elution of the phospho-Ser65-Parkin peptide (residues 52-75), NDWTVQNCDLDQQpSIVHIVQRPWR, labeled with TMTproZero (left) and shTMTpro (right) reagents spiked into a TMTpro16-labeled sample. The peptide is alkylated at the cysteine residue, phosphorylated at the serine residue, and the first arginine residue is heavy isotope labeled (+10.0082 Da). **B)** MS1-stage spectrum depicting TMTproZero-labeled phospho-Ser65-Parkin peptide and showing overlapping isotopic envelopes (top). TMTpro16 reporter ion profile shows interference due to TMTproZero-labeled peptides (bottom left). The relative peak areas of the TMTpro reporter ions across the 16 channels illustrate interference (bottom right). **C)** MS1-stage spectrum depicts shTMTpro-labeled phospho-Ser65-Parkin peptide and shows no overlap of isotopic envelopes (top). TMTpro16 reporter ion profile indicates no measurable interference when utilizing shTMTpro-labeled peptides (bottom left). The relative peak areas of TMTpro reporter ions across the 16 channels show equal peak areas for each reporter ion (bottom right). **D)** Relative peak areas of the TMTpro reporter ions across the 16 channels illustrating interference for data acquired with variable isolation windows (Th). **E)** same as in **D)** but for the Serine 65 phosphorylated Parkin peptide.

Figure S2:

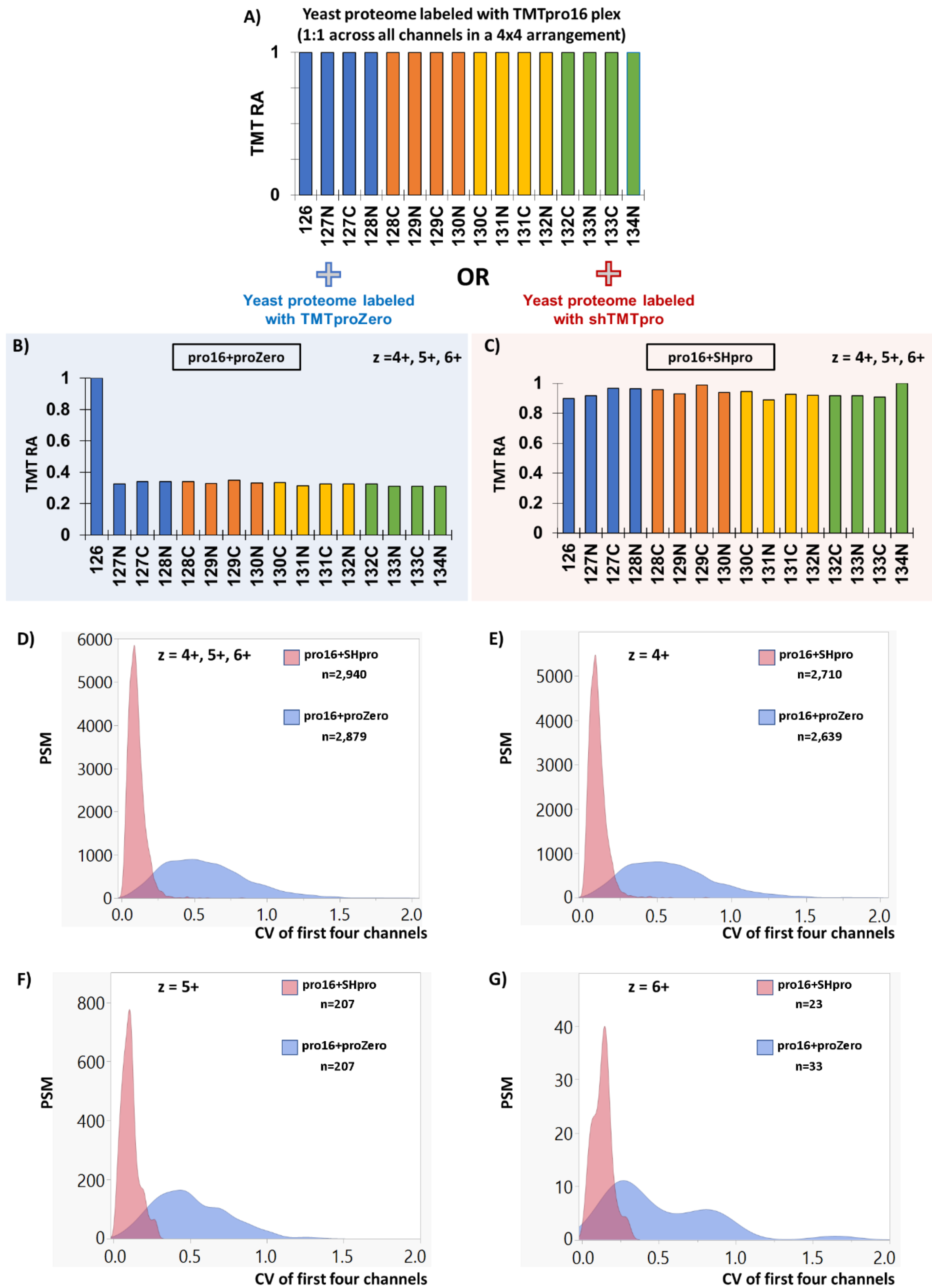

Figure S2: TMTpro variant-labeled proteomes in a TMTpro16 background. A) Yeast *(S. cerevisiae*) was labeled at a 1:1 ratio across all channels using TMTpro16 (four strains in quadruplicate). Mixtures of these strains were also labeled with shTMTpro and TMTproZero. Two samples were constructed combining TMTproZero with TMTpro16, as well as shTMTpro with TMTpro16 at a 1:1 ratio. hrMS2 data were acquired only for ions with charge states of 4+ or above for each of these two samples. We performed database matching using TMTpro16 as the only isobaric tag modification. **B-C)** Bar graph illustrating the TMT relative abundance (RA) values across the 16 channels for all PSMs for **B)** TMTproZero with TMTpro16 and **C)** shTMTpro with TMTpro16. We note the prominent 126 reporter ion signal measured in the samples with TMTproZero-labeled peptides is absent in the samples with shTMTpro-labeled peptides. Kernel density distribution plots of the coefficient of variation (CV) values for the first four channels (reporter ions 126-128N) of the TMTpro16-plex samples as plotted for **D)** all peptide-spectral matches (PSMs), **E)** z = 4+ only, **F)** z = 5+ only, and **G)** z = 6+ only.