Multicenter Evaluation of Label-Free Quantification in Human Plasma: Benchmarking with a High Dynamic Range Multispecies Sample Set
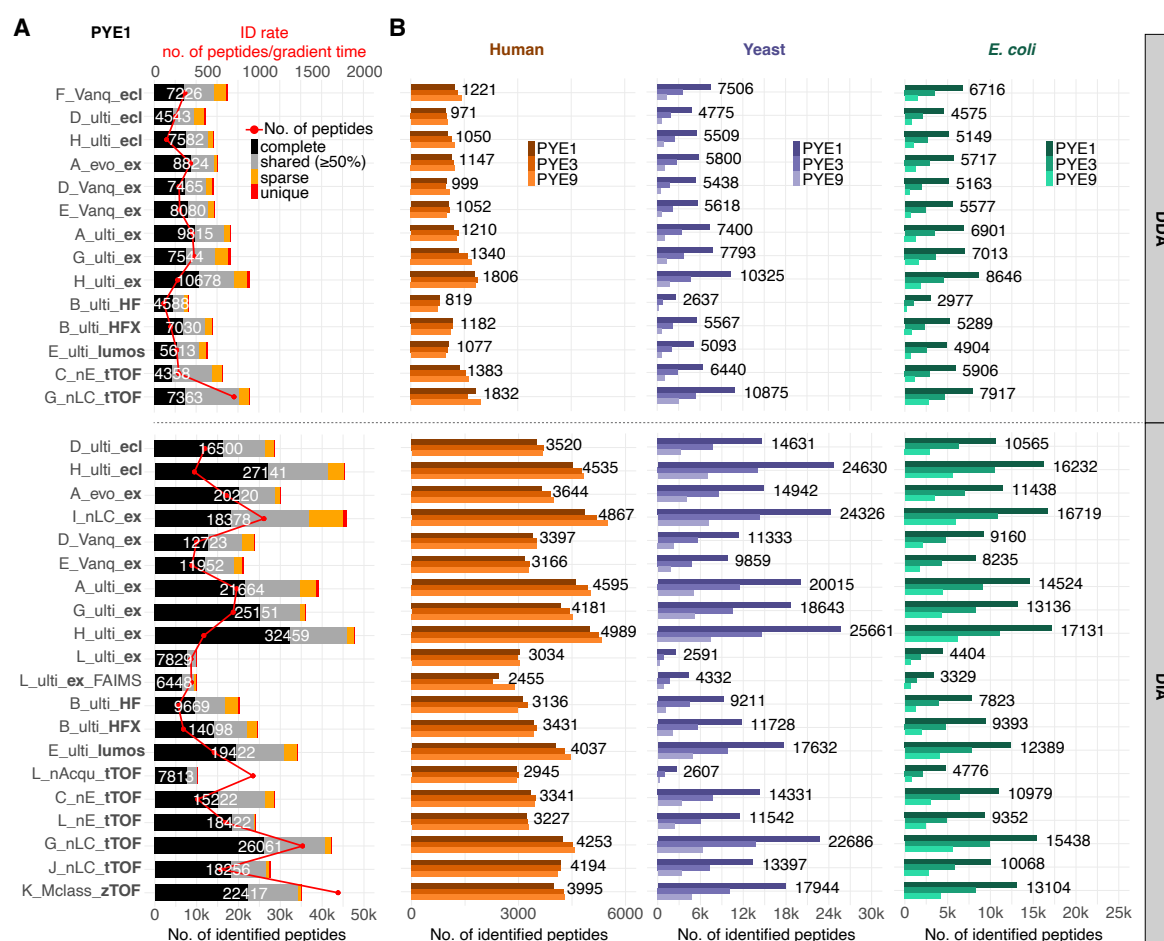
Ute Distler[1,2,*], Han Byul Yoo[1,2], Oliver Kardell[3], Dana Hein[1,2], Malte Sielaff[1,2], Christian Leps[1,2], Marian Scherer[1,2], David Gomez-Zepeda[5], Christine von Toerne[3], Juliane Merl-Pham[3], Teresa K. Barth[6], Johanna Tüshaus[7], Pieter Giesbertz[8,9], Torsten Müller[4,10], Georg Kliewer[4,10], Karim Aljakouch[4,10], Barbara Helm[11,12], Henry Unger[11,13], Dario L. Frey[11,12], Dominic Helm[13,14], Luisa Schwarzmüller[14], Oliver Popp[15], Di Qin[16], Susanne Wudy[17], Ludwig Roman Sinn[18,19], Julia Mergner[20], Christina Ludwig[17], Axel Imhof[6], Bernhard Kuster[7,17], Stefan F. Lichtenthaler[8,9,21], Jeroen Krijgsveld[4,10], Ursula Klingmüller[11,12,13,22], Philipp Mertins[15], Fabian Coscia[16], Markus Ralser[18], Michael Mülleder[19], Stefanie M. Hauck[3], Stefan Tenzer[1,2,4,5,*]

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1 Supplementary Data

Supplementary Fig. 1: Number of identified peptides in the PYE sample set for different LC-MS setups and sites. (A) Number of identified peptides in the PYE1 sample set for the different setups. Colours indicate the number of peptides identified in all replicate runs per setup (complete, black), more than 50% of runs (grey) as well as sparse (orange) and unique peptide identifications (red). White numbers indicate peptides identified in all replicate runs and red dots the number of identified peptides in relation to the programmed gradient length, i.e. peptide IDs per min. Letters refer to the laboratories. (B) Number of identified peptides across the whole PYE dataset (PYE1, PYE3, PYE9) for each setup split by species (human: orange, yeast: violet, *E. coli*: green). Numbers refer to peptides identified in PYE1.

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DDA PYE1
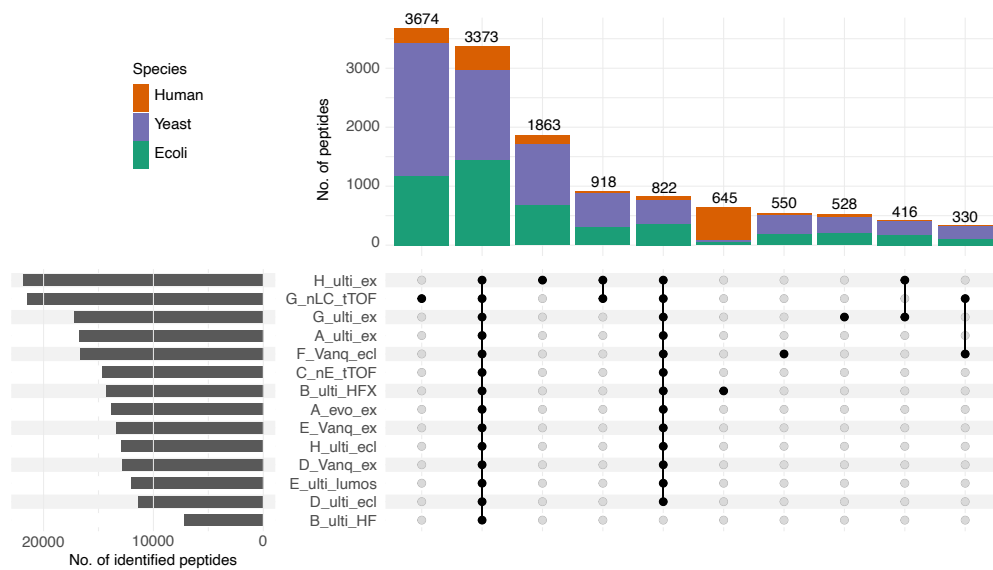
B

DIA PYE1

Supplementary Fig. 2: Peptides identified in the PYE1 sample set across multiple sites and LC-MS platforms. Overlap of identified peptides in the P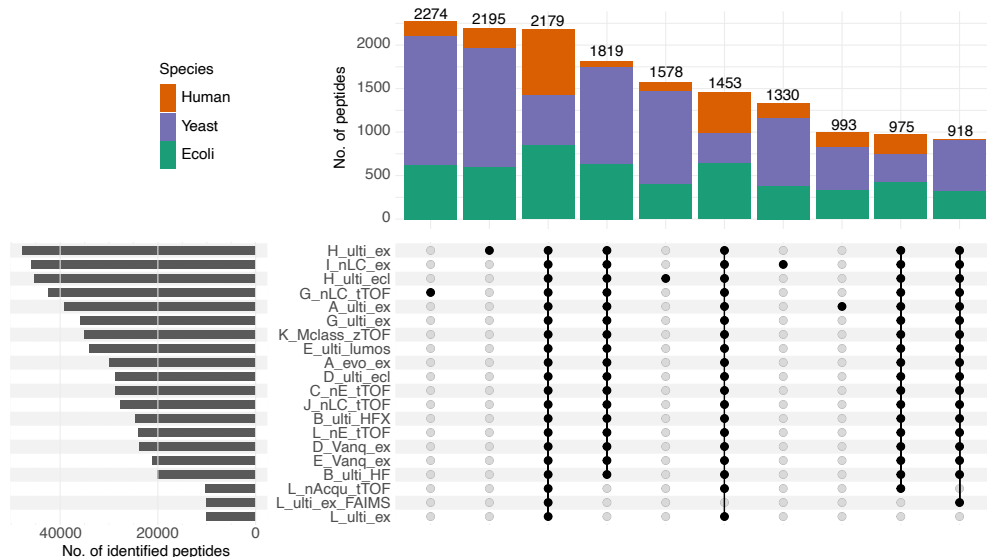YE1 sample set by (A) DDA-based and (B) DIA-based approaches. Proportions of detected human (orange), yeast (violet) and *E. coli* (green) peptides are indicated in the bars.
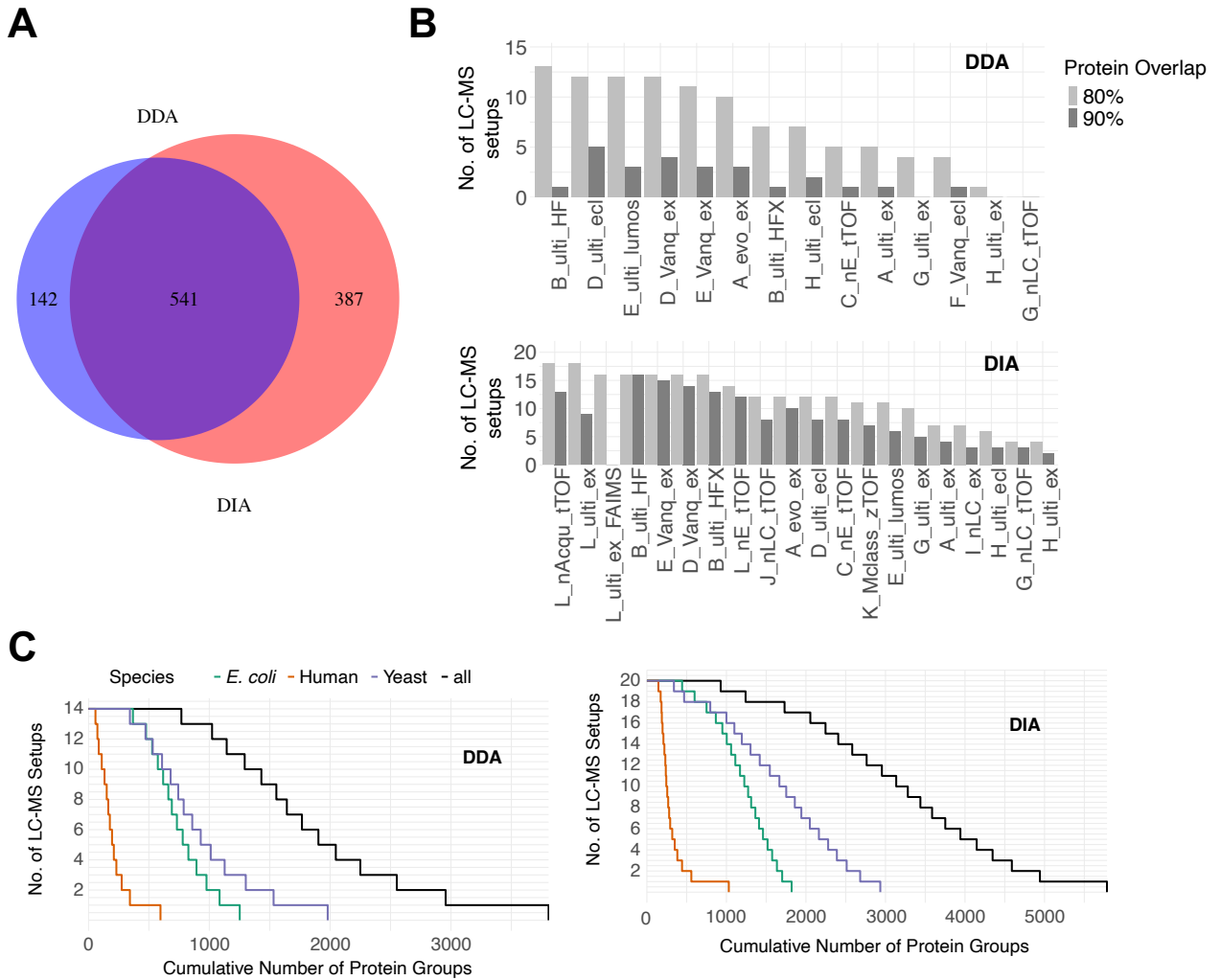
Supplementary Fig. 3: Summary statistics on the numbers of proteins identified across all 34 LC-MS setups. (A) In total, 541 proteins were shared and commonly identified in all 34 LC-MS setups. To generate the Venn diagram, only those proteins were selected that were identified across all 14 DDA as well as all 20 DIA LC-MS setups (see also Figure 2 C,D). (B) Count of LC-MS setups that share 80% (light gray) and 90% (dark gray) of proteins with the respective setup listed on the x-axis. Particularly, for DIA-based approaches the number of setups with e.g. similar proteome coverage or setups with deep coverage that identify >80 % or even >90 % of proteins from setups with lower IDs is high indicating good identification reproducibility across multiple platforms and sites. (C) Number of proteins that are identified across multiple LC-MS setups split by species (human: orange, *E. coli*: green, yeast: violet, black: sum of all proteins).

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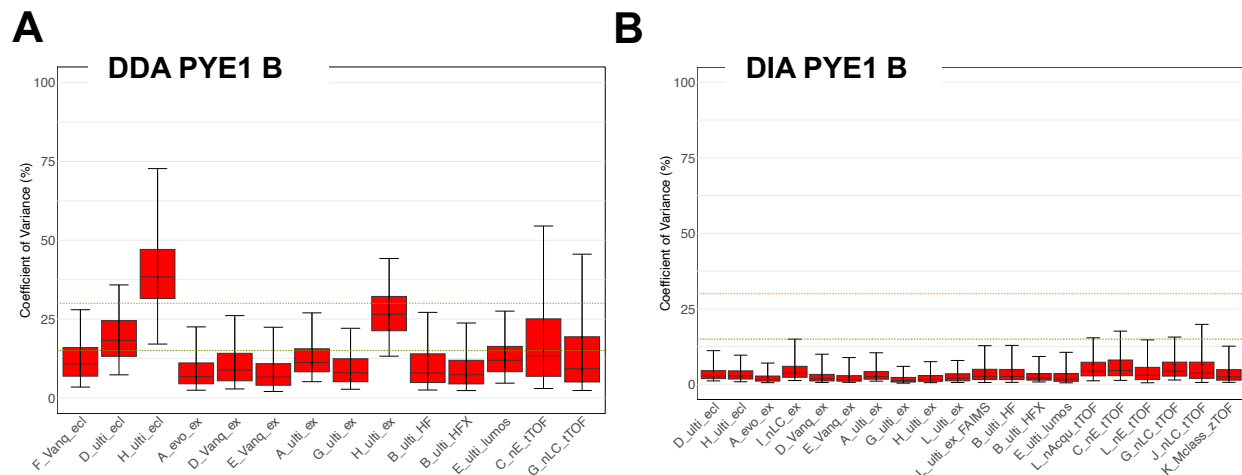
Supplementary Fig. 4: Reproducibility of LC-MS analyses. Coefficients of variation (CVs) of protein abundances for replicate analyses of sample PYE1 B were calculated for each LC-MS setup revealing lower quantitative reproducibility for (A) DDA as compared to (B) DIA approaches.

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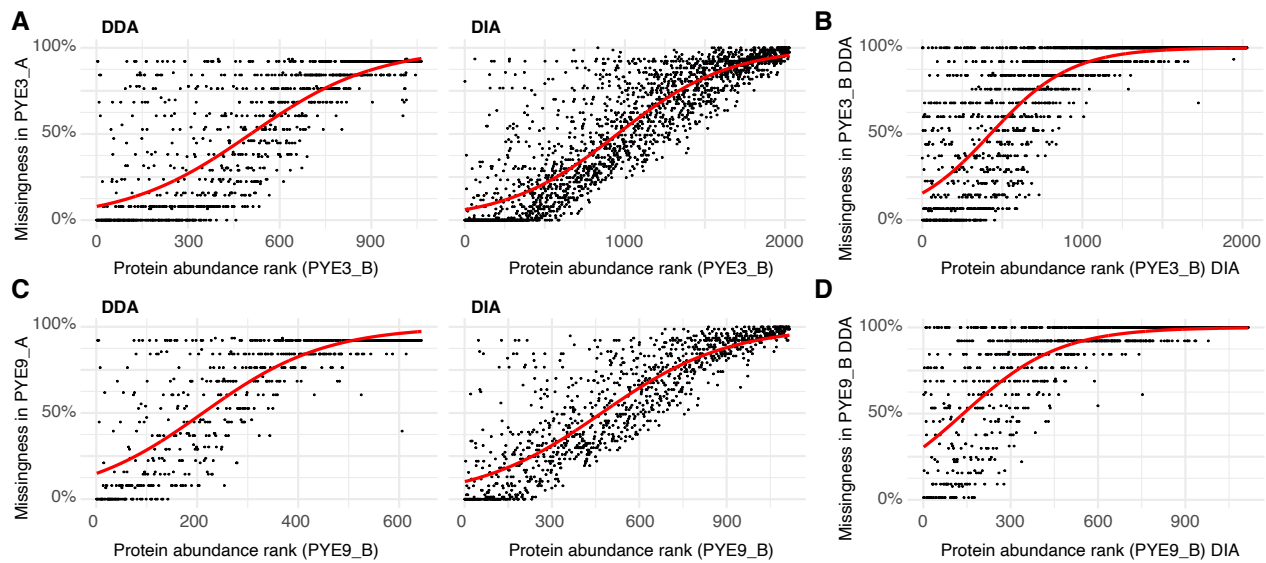
Supplementary Fig. 5: Comparison of missing values in the DDA versus the DIA dataset for PYE3 and PYE9 demonstrate higher proteome coverage and less missingness in the DIA dataset. (A) Percent of missing values for yeast proteins in PYE3 A as compared to PYE3 B (ranked by protein abundance) for the DDA and DIA dataset. (B) Percent of missing values for yeast proteins in the PYE3 B DDA dataset as compared to the PYE3 B DIA dataset dependent on protein abundance. (C) Percent of missing values for yeast proteins in PYE9 A as compared to PYE9 B (ranked by protein abundance) for the DDA and DIA dataset. (D) Percent of missing values for yeast proteins in the PYE9 B DDA dataset as compared to the PYE9 B DIA dataset dependent on protein abundance.

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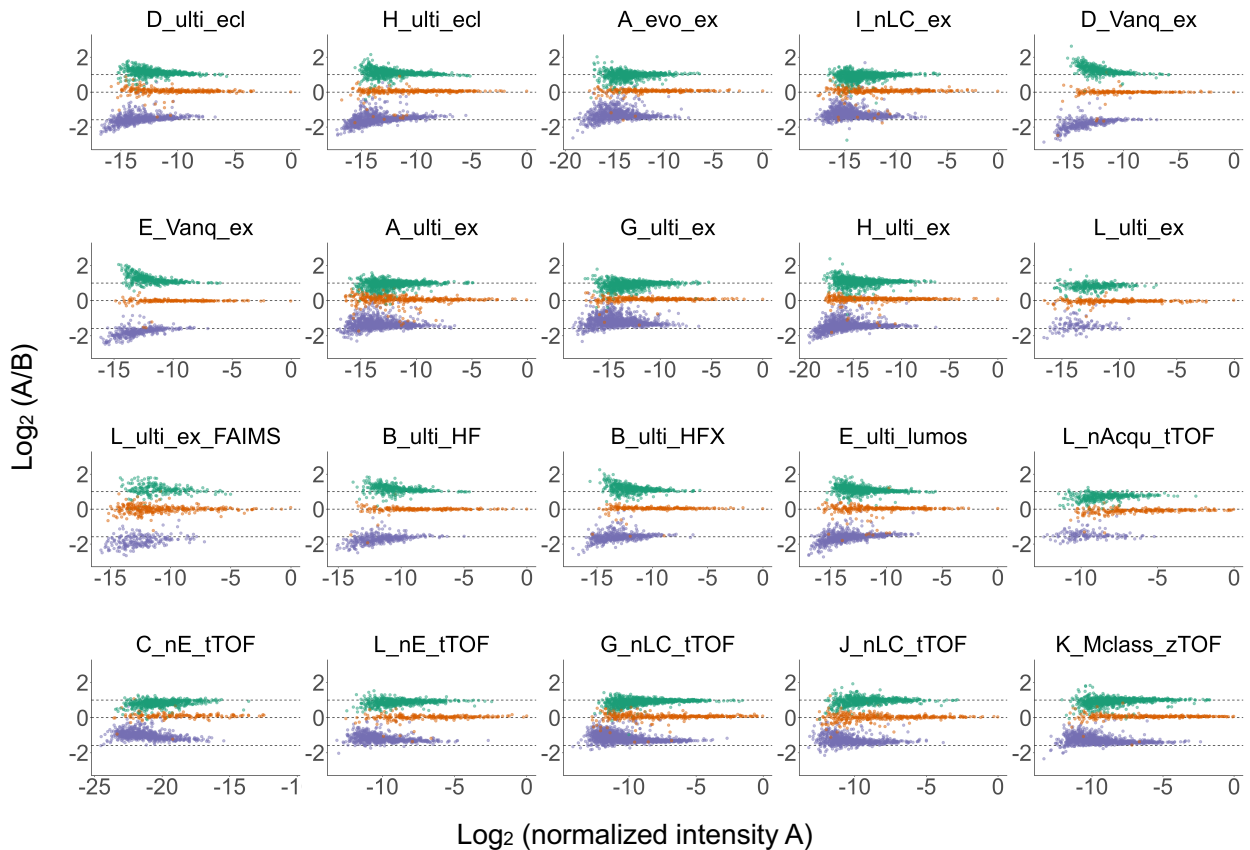
Supplementary Fig. 6. Protein level label-free quantification results for the PYE3 set analysed with different LC-MS setups across multiple sites in DIA mode. Log-transformed ratios (log$_2$(A/B)) of proteins were plotted over the log-transformed intensity of sample A for the PYE3 set summarizing the quantitative results for the DIA data acquired with 20 different LC-MS setups.

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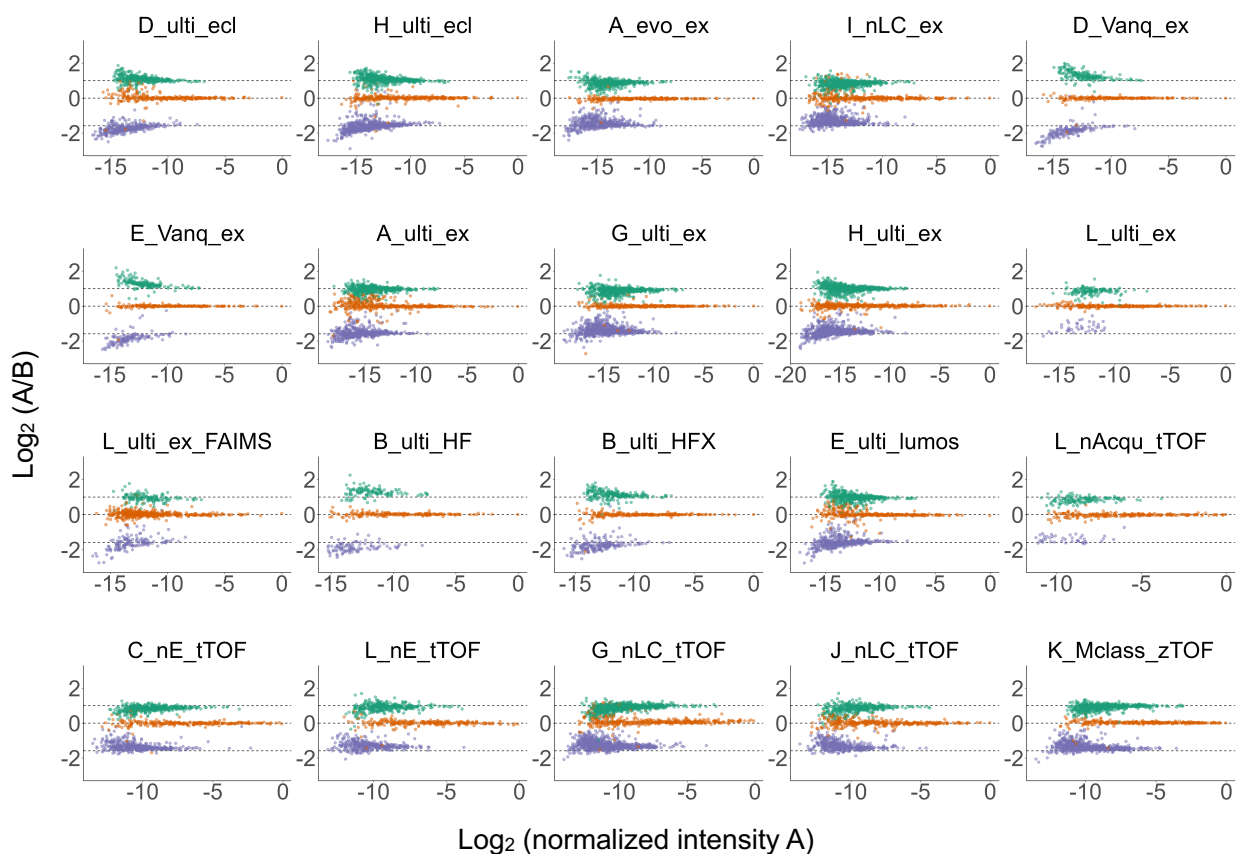
Supplementary Fig. 7. Protein level label-free quantification results for the PYE9 set analysed with different LC-MS setups across multiple sites in DIA mode. Log-transformed ratios ($\log_2(A/B)$) of proteins were plotted over the log-transformed intensity of sample A for the PYE9 set summarizing the quantitative results for the DIA data acquired with 20 different LC-MS setups.
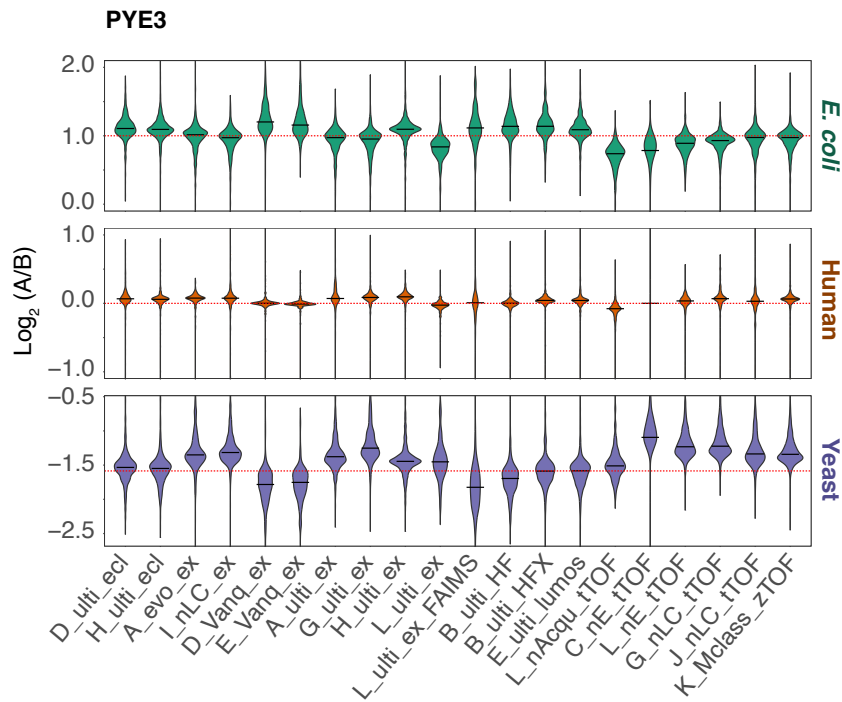
Supplementary Fig. 8. Quantitative metrics of the DIA dataset. Violin plots of log-transformed ratios (log$_2$(A/B)) of protein abundances for the PYE3 set acquired in DIA mode with 20 different LC-MS setups. Solid lines within the violin plot indicate the median log$_2$(A/B) value for each setup and dashed lines the expected log$_2$(A/B) values for human (orange), yeast (violet), and *E. coli* (green) proteins.

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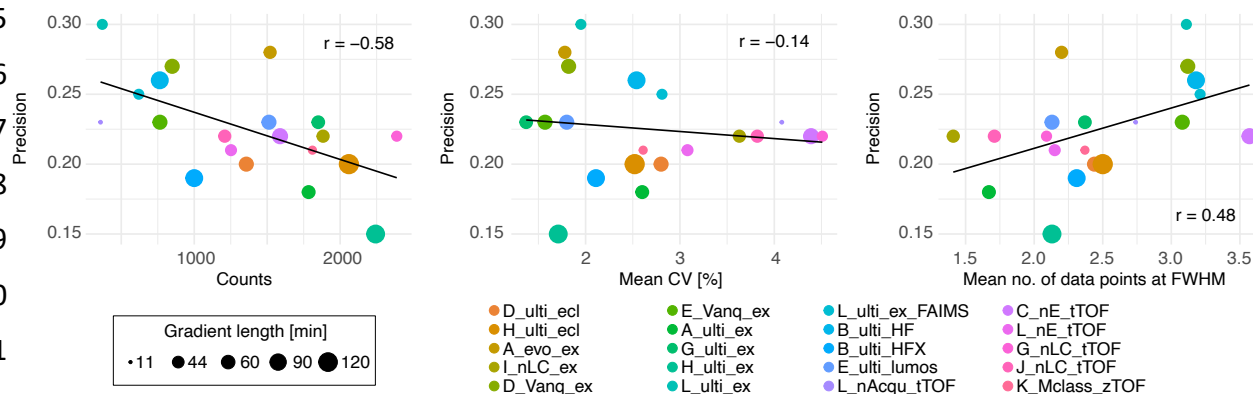
254 **Supplementary Fig. 9. Number of quantified proteins is one of the main factors**

255 **affecting precision.** Correlation of precision (defined as interquartile range Q075 –

256 Q025, calculated for yeast proteins in the PYE1 set) with other metrics such as number

257 of identified proteins (left), mean CV [%] of protein abundances (middle) and number

258 of datapoints acquired across the chromatographic peak (right panel).

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2   Extended Material and Methods: LC-MS settings

2.1   Laboratory A

<u>DDA - UltiMate 3000 RSLC & Exploris 480:</u>

300 ng (1.5 µL) of PYE peptide mixture was injected to an UltiMate 3000 RSLC nano-HPLC (Dionex, Germering, Germany) coupled to an Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were separated on an IonOpticks Odyssey column (25 cm x 75 µm, C18 1.6 µm) at 40°C by a 50 minutes non-linear gradient at a flow rate of 300 nL/min. Data were acquired in a data-dependent mode with a Top20 method. MS1 spectra were acquired with a resolution of 60,000 in a mass range from *m/z* 350-1,200 with 300% AGC target and a maximum injection time of 25 ms. The resolution for MS2 was 15,000, AGC target set to standard, and maximum injection time to 22 ms. Fragment ion data were collected in centroid mode. Normalized HCD collision energy was 30%. First mass was defined at *m/z* 110. Isolation width was set to 1.4 Th.

<u>DDA - Evosep One & Exploris 480:</u>

The Evotips for each run were loaded with 20 µL peptide solution containing around 500 ng of sample peptides. They were placed in the Evosep One (Evosep, Odense, Denmark) autosampler until analysis. The 30 samples per day method employing a 44 minutes gradient was chosen and a 15 cm column (Dr. Maisch C18 AQ, 1.5 µm beads, 150 µm ID) used for separation of peptides. The LC was coupled to an Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Data were acquired in a data-dependent mode with a Top20 method. MS1 spectra were acquired with a resolution of 120,000 in a mass range from *m/z* 375-1,500. The resolution for MS2 was 15,000, AGC target 100% and maximum injection time 40 ms. Fragment ion data were recorded in profile mode. Isolation width was set to 1.4 Th.

<u>DIA - UltiMate 3000 RSLC & Exploris 480:</u>

300 ng (1.5 µL) of PYE peptide mixture was injected to an UltiMate 3000 RSLC nano-HPLC (Dionex, Germering, Germany) coupled to an Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were separated on an IonOpticks Odyssey column (25 cm x 75 µm, C18 1.6 µm) at 40°C by a 50 minutes

297   non-linear gradient at a flow rate of 300 nL/min. Data-independent acquisition was

298   performed at a full scan resolution of 120,000. MS1 spectra were recorded over a

299   mass range from *m/z* 380-980, maximum injection time was set to 100 ms and the

300   normalized AGC target to 300%. The MS1 scan was followed by DIA scans covering

301   a precursor mass range between *m/z* 380-980 at an isolation window width of 20 Th

302   with 1 Th overlap. Thus, the number of scan events was 30. The resolution was set to

303   30,000 and the AGC target to 3,000%.

304

305   <u>DIA - Evosep One & Exploris 480:</u>

306   The Evotips for each run were loaded with 20 µL peptide solution containing around

307   500 ng of sample peptides. They were placed in the Evosep One (Evosep, Odense,

308   Denmark) autosampler until analysis. The 30 samples per day method employing a

309   44 minutes gradient was chosen and a 15 cm column (Dr. Maisch C18 AQ, 1.5 µm

310   beads, 150 µm ID) used for separation of peptides. The LC was coupled to an Exploris

311   480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Data-

312   independent acquisition was performed at a full scan resolution of 120,000. MS1

313   spectra were recorded over a mass range from *m/z* 380-980, maximum injection time

314   was set to 100 ms and the normalized AGC target to 300%. The MS1 scan was

315   followed by DIA scans covering a precursor mass range between *m/z* 380-980 at an

316   isolation window width of 20 Th with 1 Th overlap. Thus, the number of scan events

317   was 30. The resolution was set to 30,000 and the AGC target to 3,000%.

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319   2.2   Laboratory B

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321   <u>DDA - UltiMate 3000 RSLC & Q Exactive HF:</u>

322   The peptide mixtures, prepared as stated in the instruction, were injected into an

323   UltiMate 3000 RSLC nano-HPLC (Thermo Fisher Scientific, Bremen, Germany)

324   coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Peptides

325   were separated on a nanoEase M/Z HSS T3 column (25 cm x 75 µm, C18 1.8 µm,

326   100 Å, Waters, Eschborn, Germany) at 40°C by a 90 minutes non-linear gradient at a

327   flow rate of 250 nL/min. Data were acquired in a data-dependent mode. MS1 spectra

328   were recorded at a resolution of 60,000 in a mass range from *m/z* 300 to 1,500. From

329   the MS1 scan, the 10 most abundant ions were selected for HCD fragmentation with

330   a normalized collision energy of 27, an isolation window width of 1.6 Th, and a dynamic

331   exclusion of 30 s. The resolution for MS2 was 15,000, the AGC target was set to 1e5

332   and maximum injection time to 50 ms. Unassigned charges, and charges of "1 plus"

333   and ">8 plus" were excluded. Spectra were recorded in profile mode.

334

335   DDA - UltiMate 3000 RSLC & Q Exactive HFX:

336   The peptide mixtures, prepared as stated in the instruction, were injected to an

337   UltiMate 3000 RSLC nano-HPLC (Dionex, Germering, Germany) coupled to a Q

338   Exactive HFX mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

339   Tryptic peptides were automatically loaded on a C18 trap column (300 µm inner

340   diameter (ID) × 5 mm, Acclaim PepMap100 C18, 5 µm, 100 Å, LC Packings) at

341   30 µL/min flow rate, followed by separation on a C18 reversed phase analytical column

342   (nanoEase M/Z HSS T3 column; 25 cm x 75 µm, C18 1.8 µm, 100 Å, Waters,

343   Eschborn, Germany) at 40°C by a 90 minutes non-linear gradient at a flow rate of

344   250 nL/min. Data were acquired in a data-dependent mode with a Top15 method if

345   peptide ions were at least doubly charged. The high-resolution (60,000 full width at

346   half-maximum) MS spectrum was acquired with a mass range from m/z 300 to 1,500

347   with automatic gain control target (AGC) set to 3 x 10^6 and a maximum of 30 ms

348   injection time. The resolution for MS2 was 15,000, the AGC target was set to 1e5 and

349   the maximum injection time to 50 ms. Isolation window width was 1.6 Th. The

350   normalized collision energy was 28, and the spectra were recorded in profile mode.

351

352   DIA - UltiMate 3000 RSLC & Q Exactive HF:

353   The peptide mixtures, prepared as stated in the instruction, were injected to an

354   UltiMate 3000 RSLC nano-HPLC (Dionex, Germering, Germany) coupled to a Q

355   Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

356   Peptides were separated on a nanoEase M/Z HSS T3 column (25 cm x 75 µm, C18

357   1.8 µm, 100 Å) at 40°C by a 90 minutes non-linear gradient at a flow rate of 250 nL/min.

358   The DIA method consisted of a survey scan from m/z 300 to 1,650 at 120,000

359   resolution and an automatic gain control (AGC) target of 3e6 or 120 ms maximum

360   injection time. Fragmentation was performed via higher energy collisional dissociation

361   with a target value of 3e6 ions determined with predictive AGC. Precursor peptides

362   were isolated with 37 variable windows spanning a precursor mass range from m/z

363   300 to 1650 at 30,000 resolution with an AGC target of 3e6 and automatic injection

364 time. The normalized collision energy was 28, and the spectra were recorded in profile
365 mode.
366
367 <u>DIA - UltiMate 3000 RSLC & Q Exactive HFX:</u>
368 The peptide mixture, prepared as stated in the instruction, were injected to an UltiMate
369 3000 RSLC nano-HPLC (Dionex, Germering, Germany) coupled to a Q Exactive HFX
370 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were
371 separated on a nanoEase M/Z HSS T3 column (25 cm x 75 µm, C18 1.8 µm, 100 Å)
372 at 40°C by a 90 minutes non-linear gradient at a flow rate of 250 nL/min. The DIA
373 method consisted of a survey scan from m/z 300 to 1,650 at 120,000 resolution and
374 an automatic gain control (AGC) target of 3e6 or 120 ms maximum injection time.
375 Fragmentation was performed via higher energy collisional dissociation with a target
376 value of 3e6 ions determined with predictive AGC. Precursor peptides were isolated
377 with 37 variable windows spanning from m/z 300 to 1,650 at 30,000 resolution with an
378 AGC target of 3e6 and automatic injection time. The normalized collision energy was
379 28, and the spectra were recorded in profile mode.
380
381 2.3   Laboratory C
382
383 <u>DDA - nanoElute & timsTOF Pro:</u>
384 Samples were analyzed on a nanoElute LC coupled to a timsTOF Pro mass
385 spectrometer with a CaptiveSpray ion source (Bruker Corporation, Billerica, MA, USA).
386 Samples were injected on a 15 cm self-packed C18 column (75 µm internal diameter)
387 with 1.9 µm ReproSil-Pur 120 C18-AQ resin (Dr Maisch, Germany). A gradient of water
388 (A) and acetonitrile (B) supplemented with 0.1% formic acid was applied at a flow rate
389 of 300 nL/min and a column temperature of 50°C. The following gradient was applied:
390 0 min, 2%B; 2 min, 5%B; 62 min, 24%B; 72 min, 35%B; 75 min, 60%B; 78 min, 85%B.
391 The MS was operated in data-dependent acquisition parallel accumulation-serial
392 fragmentation (ddaPASEF) mode. Ion accumulation and separation using trapped ion
393 mobility spectrometry (TIMS) was set to a ramp time of 100 ms with an ion mobility
394 range $(1/K_0)$ from 0.6 to 1.6 Vs/cm^2. One MS cycle included one TIMS full MS scan
395 and 10 PASEF scans with up to 12 precursors targeted per scan.
396
397 <u>DIA - nanoElute & timsTOF Pro:</u>

Samples were analyzed on a nanoElute LC coupled to a timsTOF Pro mass spectrometer with a CaptiveSpray ion source (Bruker Corporation, Billerica, MA, USA). Samples were injected on a 15 cm self-packed C18 column (75 µm internal diameter) with 1.9 µm ReproSil-Pur 120 C18-AQ resin (Dr Maisch, Germany). A gradient of water (A) and acetonitrile (B) supplemented with 0.1% formic acid was applied at a flow rate of 300 nL/min and a column temperature of 50°C. The following gradient was applied: 0 min, 2%B; 2 min, 5%B; 62 min, 24%B; 72 min, 35%B; 75 min, 60%B; 78 min, 85%B. The MS was operated in a data-independent acquisition parallel accumulation-serial fragmentation (diaPASEF) mode. Ion accumulation and separation using trapped ion mobility spectrometry (TIMS) was set to a ramp time of 100 ms with an ion mobility range ($1/K_0$) from 0.6 to 1.45 Vs/cm^2. One MS cycle included one TIMS full MS scan followed by multiple MS/MS scans comprising in total 26 windows with a width of 27 Th covering a *m/z*-range of 350 to 1,002. Two windows were recorded per PASEF scan. This resulted in a total cycle time of 1.4 s.

2.4 Laboratory D

DDA – Vanquish Neo & Exploris 480:

Mixed proteome samples (2 µg/µL protein, prepared in the Tenzer lab, based on the concept initially described in Navarro *et al.*, 2016[1]) containing tryptic digest of human plasma with variable spike-ins of tryptically digested yeast and *E.coli*, respectively, were transferred undiluted to MS vials. Samples were analysed in five replicate injections according to the following measurement scheme: blank-500 ng in-house Hela QC-2x blank-six samples (A9,B9,A3,B3,A1,B1)-blank.

Peptides were analyzed on a Vanquish Neo (micro-flow configuration) coupled on-line to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 10 µg of peptides were applied onto a commercially available Acclaim PepMap 100 C18 column (2 µm particle size, 1 mm ID × 150 mm, 100 Å pore size; Thermo Fisher Scientific), and separated using a two-stepped gradient: In the first step, a 50 minutes linear gradient ranging from 3% to 24% solvent B (0.1% FA, 3% DMSO in ACN) in solvent A (0.1% FA, 3% DMSO in HPLC grade water) at a flow rate of 50 µL/min was applied. In the second step, solvent B was further increased from 24% to 31% over a 10 minute linear gradient. The mass spectrometer was operated in data-dependent acquisition (DDA) and positive ionization mode. MS1 full scans (*m/z* 360 –

432 1,300) were acquired with a resolution of 60,000, a normalized AGC value of 100%

433 and a maximum injection time of 25 ms. Peptide precursor selection for fragmentation

434 was carried out using a fixed cycle time of 1.2 s. Only precursors with charge states

435 from 2 to 6 were selected and dynamic exclusion of 30 s was enabled. Peptide

436 fragmentation was performed using higher energy collision induced dissociation (HCD)

437 and a normalized collision energy of 28%. The precursor isolation window width of the

438 quadrupole was set to 1.3 Th. MS2 spectra were acquired with a resolution of 15,000,

439 a fixed first mass of m/z 100, a normalized automatic gain control (AGC) target value

440 of 100% and maximum injection time of 22 ms.

441

442 <u>DDA – UltiMate 3000 RSLC & Orbitrap Eclipse:</u>

443 Mixed proteome samples (2 µg/µL protein, prepared in the Tenzer lab, based on the

444 concept initially described in Navarro et al., 2016[1]) containing tryptic digest of human

445 plasma with variable spike-ins of tryptically digested yeast and *E.coli*, respectively,

446 were diluted 33.3-fold with 0.1% formic acid and transferred to MS vials. Samples were

447 analysed in six replicate injections according to the following measurement scheme:

448 blank-100 ng in-house Hela QC-2x blank-six samples (A9,B9,A3,B3,A1,B1)-blank.

449 LC-MS/MS measurements were performed on a Dionex Ultimate 3000 RSLC nano

450 system coupled to an Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific,

451 Bremen, Germany). 300 ng of peptides were delivered to a trap column (ReproSil-pur

452 C18-AQ, 5 µm, Dr. Maisch, 20 mm × 75 µm, self-packed) at a flow rate of 5 µL/min in

453 100% solvent A (0.1% formic acid in HPLC grade water). After 10 minutes of loading,

454 peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, Dr.

455 Maisch, 400 mm × 75 µm, self-packed) at 50°C and separated using a 60 min gradient

456 from 4% to 32% of solvent B (0.1% FA, 5% DMSO in ACN) in solvent A (0.1% FA, 5%

457 DMSO in HPLC grade water) at 300 nL/min flow rate. The mass spectrometer was

458 operated in data-dependent acquisition and positive ionization mode. MS1 full scans

459 (m/z 360–1,300) were acquired with a resolution of 60,000, a normalized AGC value

460 of 100% and maximum injection time of 50 ms. Peptide precursor selection for

461 fragmentation was carried out using a fixed cycle time of 2 s. Only precursors with

462 charge states ranging from 2 to 6 were selected and dynamic exclusion of 30 s was

463 enabled. Peptide fragmentation was performed using higher energy collision induced

464 dissociation (HCD) and a normalized collision energy of 30%. The precursor isolation

465 window width of the quadrupole was set to 1.3 Th. MS2 spectra were acquired with a

466 resolution of 15,000, a fixed first mass of *m/z* 100, a normalized automatic gain control
467 (AGC) target value of 200% and maximum injection time of 22 ms.
468
469 DIA – Vanquish Neo & Exploris 480:
470 Mixed proteome samples (2 µg/µL protein, prepared in the Tenzer lab, based on the
471 concept initially described in Navarro et al., 2016[1]) containing tryptic digest of human
472 plasma with variable spike-ins of tryptically digested yeast and *E.coli*, respectively,
473 were transferred undiluted to MS vials. Samples were analysed in five replicate
474 injections according to the following measurement scheme: blank-500 ng in-house
475 Hela QC-2x blank-six samples (A9,B9,A3,B3,A1,B1)-blank.
476 Peptides were analyzed on a Vanquish Neo (micro-flow configuration) coupled on-line
477 to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen,
478 Germany). 10 µg of peptides were applied onto a commercially available Acclaim
479 PepMap 100 C18 column (2 µm particle size, 1 mm ID × 150 mm, 100 Å pore size;
480 Thermo Fisher Scientific), and separated using a two-stepped gradient: In the first step,
481 a 50 minutes linear gradient ranging from 3% to 24% solvent B (0.1% FA, 3% DMSO
482 in ACN) in solvent A (0.1% FA, 3% DMSO in HPLC grade water) at a flow rate of
483 50 µL/min was applied. In the second step, solvent B was further increased from 24%
484 to 31% over a 10 minutes linear gradient. The mass spectrometer was operated in
485 data-independent acquisition (DIA) mode. MS1 full scans (*m/z* 360–1,300) were
486 acquired with a resolution of 120,000, a normalized AGC target value of 100% and a
487 maximum injection time of 50 ms. MS2 scans (*m/z* 200–1,800) were acquired from
488 *m/z* 360 to *m/z* 1,260 over a total 40 DIA segments with isolation window widths of
489 22.5 Th using 1 Th overlap. The scan resolution in the Orbitrap was set to 15,000 with
490 the AGC target set to "standard" and a maximum injection time of 22 ms. The HCD
491 collision energy was set to 28%.
492
493 DIA – UltiMate 3000 RSLC & Orbitrap Eclipse:
494 Mixed proteome samples (2 µg/µL protein, prepared in the Tenzer lab, based on the
495 concept initially described in Navarro et al., 2016[1]) containing tryptic digest of human
496 plasma with variable spike-ins of tryptically digested yeast and *E.coli*, respectively,
497 were diluted 33.3-fold with 0.1% formic acid and transferred to MS vials. Samples were
498 analysed in six replicate injections according to the following measurement scheme:
499 blank-100 ng in-house Hela QC-2x blank-six samples (A9,B9,A3,B3,A1,B1)-blank.

500 LC-MS/MS measurements were performed on a Dionex Ultimate 3000 RSLC nano
501 system coupled to an Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific,
502 Bremen, Germany). 300 ng of peptides were delivered to a trap column (ReproSil-pur
503 C18-AQ, 5 µm, Dr. Maisch, 20 mm × 75 µm, self-packed) at a flow rate of 5 µL/min in
504 100% solvent A (0.1% formic acid in HPLC grade water). After 10 minutes of loading,
505 peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, Dr.
506 Maisch, 400 mm × 75 µm, self-packed) at 50°C and separated using a 60 min gradient
507 from 4% to 32% of solvent B (0.1% FA, 5% DMSO in ACN) in solvent A (0.1% FA, 5%
508 DMSO in HPLC grade water) at 300 nL/min flow rate. The mass spectrometer was
509 operated in data-independent acquisition (DIA) mode. MS1 full scans (*m/z* 360–1,300)
510 were acquired with a resolution of 120,000, a normalized AGC target value of 100%
511 and a maximum injection time of 50 ms. MS2 scans (*m/z* 200–1,800) were acquired
512 over a total of 40 DIA segments with variable isolation window widths (see Table 1)
513 using 1 Th overlap. The scan resolution in the Orbitrap was set to 30,000 with the AGC
514 target value of 1000% and a maximum injection time of 54 ms. The HCD collision
515 energy was set to 30%.

516

517 **Table 1:** *DIA window scheme on the Orbitrap Eclipse applied by laboratory D.*

m/z	z	isolation window (m/z)	m/z	z	isolation window (m/z)
368	2	16	669	2	18
383	2	16	686	2	18
398	2	16	703	2	18
413	2	16	720	2	18
428	2	16	737	2	18
443	2	16	754	2	18
458	2	16	771	2	18
473	2	16	788	2	18
488	2	16	805	2	18
503	2	16	823	2	20
518	2	16	842	2	20
533	2	16	861	2	20
548	2	16	880	2	20
563	2	16	899	2	20
578	2	16	918	2	20
593	2	16	939,5	2	20
608	2	16	963,5	2	25
623	2	16	992,5	2	35
638	2	16	1034	2	55
653	2	16	1179	2	242

518

519

2.5   Laboratory E

DDA – Ultimate 3000 RSLC & Orbitrap Fusion Lumos:

Mixed proteome samples (2 µg/µL protein, prepared in the Tenzer lab, based on the concept initially described in Navarro et al., 2016[1]) containing tryptic digest of human plasma with variable spike-ins of tryptically digested yeast and *E.coli*, respectively, were diluted 33.3-fold (60 ng/µL protein) with 2% ACN, 0.1% FA in H_2O and transferred to MS vials. Samples for nanoflow measurements were analysed in six replicate injections according to the following measurement scheme: 1. blank, 2. 50 ng Pierce Hela QC, 3. 2x blank, 4. six samples (A9,B9,A3,B3,A1,B1), 5. blank.

LC-MS/MS measurements were performed on a Dionex Ultimate 3000 RSLCnano system coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 300 ng of peptides were delivered to a trap column (ReproSil-pur C18-AQ, 5 µm, Dr. Maisch, 20 mm × 75 µm, self-packed) at a flow rate of 5 µL/min in 100% solvent A (0.1% formic acid in HPLC grade water). After 10 minutes of loading, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, Dr. Maisch, 400 mm × 75 µm, self-packed) at 50°C and separated using a 60 min gradient from 4% to 32% of solvent B (0.1% FA, 5% DMSO in ACN) in solvent A (0.1% FA, 5% DMSO in HPLC grade water) at 300 nL/min flow rate. The mass spectrometer was operated in data-dependent acquisition and positive ionization mode. MS1 spectra (*m/z* 360–1,300) were recorded at a resolution of 60,000 using an automatic gain control (AGC) value of 4e5 and maximum injection time (maxIT) of 50 ms. MS2 spectra of up to 20 precursor peptides were acquired at a resolution of 15,000 with an automatic gain control (AGC) target value of 5e4 and maximum injection time (maxIT) of 22 ms. The precursor isolation window width was set to 1.3 Th and fragmentation was performed using higher energy collision induced dissociation (HCD) and a normalized collision energy of 30%. Dynamic exclusion was enabled with 20 s exclusion time (mass tolerance +/-10 ppm). Precursors that were singly-charged, unassigned or with charge states >6+ were excluded for fragmentation.

DDA – Vanquish Neo & Exploris 480:

Mixed proteome samples (2 µg/µL protein) were transferred directly into MS vials. Samples were analysed in six replicate injections according to the following

measurement scheme: 1. blank, 2. Pierce Hela QC, 3. 2x blank, 4. six samples (A9,B9,A3,B3,A1,B1), 5. blank.

Peptides were analyzed on a Vanquish Neo (microflow configuration) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 10 µg of peptides were applied onto a commercially available Acclaim PepMap 100 C18 column (2 µm particle size, 1 mm ID × 150 mm, 100 Å pore size; Thermo Fisher Scientific), and separated using a two-stepped gradient: In the first step, a 50 minutes linear gradient ranging from 3% to 24% solvent B (0.1% FA, 3% DMSO in ACN) in solvent A (0.1% FA, 3% DMSO in HPLC grade water) at a flow rate of 50 µL/min was applied. In the second step, solvent B was further increased from 24% to 31% over a 10 minutes linear gradient. The mass spectrometer was operated in data-dependent acquisition (DDA) and positive ionization mode. MS1 full scans (*m/z* 360 – 1,300) were acquired with a resolution of 60,000, a normalized AGC target value of 100% and a maximum injection time of 50 ms. Peptide precursor selection for fragmentation was carried out using a fixed cycle time of 1.2 s. Only precursors with charge states ranging from 2 to 6 were selected and dynamic exclusion of 30 s was enabled. Peptide fragmentation was performed using higher energy collision induced dissociation (HCD) and a normalized collision energy of 28%. The precursor isolation window width of the quadrupole was set to 1.1 Th. MS2 spectra were acquired with a resolution of 15,000, a fixed first mass of *m/z* 100, a normalized automatic gain control (AGC) value of 100% and maximum injection time of 40 ms.

DIA – Ultimate 3000 RSLC & Orbitrap Fusion Lumos:

Mixed proteome samples (2 µg/µL protein, prepared in the Tenzer lab, based on the concept initially described in Navarro et al., 2016[1]) containing tryptic digest of human plasma with variable spike-ins of tryptically digested yeast and *E.coli*, respectively, were diluted 33.3-fold (60 ng/µL protein) with 2% ACN, 0.1% FA in H_2O and transferred to MS vials. Samples for nanoflow measurements were analysed in six replicate injections according to the following measurement scheme: 1. blank, 2. 50 ng Pierce Hela QC, 3. 2x blank, 4. six samples (A9,B9,A3,B3,A1,B1), 5. blank.

LC-MS/MS data were acquired on a Dionex Ultimate 3000 RSLCnano system coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 300 ng of peptides were delivered to a trap column (ReproSil-pur

587   C18-AQ, 5 µm, Dr. Maisch, 20 mm × 75 µm, self-packed) at a flow rate of 5 µL/min in

588   100% solvent A (0.1% formic acid in HPLC grade water). After 10 minutes of loading,

589   peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, Dr.

590   Maisch, 400 mm × 75 µm, self-packed) at 50°C and separated using a 60 min gradient

591   from 4% to 32% of solvent B (0.1% FA, 5% DMSO in ACN) in solvent A (0.1% FA, 5%

592   DMSO in HPLC grade water) at 300 nL/min flow rate. The mass spectrometer was

593   operated in data-independent acquisition (DIA) mode. MS1 full scans (*m/z* 360 – 1,300)

594   were acquired with a resolution of 120,000, a normalized AGC target value of 1E6 and

595   a maximum injection time of 50 ms. The MS1 scan range covered with DIA windows

596   was set from *m/z* 350 to 1,150 (inclusion list with 41 DIA windows starting at *m/z* 358

597   to *m/z* 1,111 with variable isolation window widths from 16 Th to 78 Th and 1 Th

598   overlap between scans, see Table 2). The MS2 scan range was set from *m/z* 200 to

599   *m/z* 1800 at 30,000 resolution with an automatic gain control (AGC) value of 5e5 and

600   a maximum injection time of 54 ms. Fragmentation was performed using higher energy

601   collision induced dissociation (HCD) and a normalized collision energy (NCE) of 30%.

602

603   **Table 2:** *DIA window scheme on the Orbitrap Fusion Lumos applied by laboratory E.*

m/z	z	isolation window (m/z)	m/z	z	isolation window (m/z)
358	2	16	659	2	18
373	2	16	676	2	18
388	2	16	693	2	18
403	2	16	710	2	18
418	2	16	727	2	18
433	2	16	744	2	18
448	2	16	761	2	18
463	2	16	778	2	18
478	2	16	795	2	18
493	2	16	813	2	20
508	2	16	832	2	20
523	2	16	851	2	20
538	2	16	870	2	20
553	2	16	889	2	20
568	2	16	908	2	20
583	2	16	929,5	2	20
598	2	16	953,5	2	25
613	2	16	977,5	2	25
628	2	16	1006,5	2	35
643	2	16	1048	2	50
			1111	2	78

604

605   <u>DIA – Vanquish Neo & Exploris 480:</u>

606   Mixed proteome samples (2 µg/µL protein) were transferred directly into MS vials.

607   Samples were analysed in six replicate injections according to the following

measurement scheme: 1. blank, 2. Pierce Hela QC, 3. 2x blank, 4. six samples (A9,B9,A3,B3,A1,B1), 5. blank.

Peptides were analysed on a Vanquish Neo (microflow configuration) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 10 µg of peptides were applied directly onto a commercially available Acclaim PepMap 100 C18 column (2 µm particle size, 1 mm ID × 150 mm, 100 Å pore size; Thermo Fisher Scientific), and separated using two-stepped gradient: In the first step, a 50 minutes linear gradient ranging from 3% to 24% solvent B (0.1% FA, 3% DMSO in ACN) in solvent A (0.1% FA, 3% DMSO in HPLC grade water) at a flow rate of 50 µL/min was applied. In the second step, solvent B was further increased from 24% to 31% over a 10 minutes linear gradient. The mass spectrometer was operated in data-independent acquisition (DIA) mode. MS1 full scans (m/z 360 – 1,300) were acquired with a resolution of 120,000, a normalized AGC target value of 100% and a maximum injection time of 50 ms. The MS1 scan range covered with DIA windows was set from *m/z* 360 to 1,260 using an inclusion list of 40 DIA windows (from *m/z* 371.25 to *m/z* 1248.7) with a fixed isolation window width of 22.5 Th and 1 Th overlap between scans. The MS2 scan range was set from *m/z* 200 to *m/z* 1,800 at 15,000 resolution. Normalized automatic gain control (AGC) target value was used at 100% and a maximum injection time of 22 ms was used. Fragmentation was performed using a normalized collision energy (NCE) of 28%.

2.6   Laboratory F

<u>DDA – Vanquish Neo & Orbitrap Eclipse:</u>

From each sample, 9 µg PYE-peptide mix was injected per run (4.5 µL each). Peptides were separated on a Thermo Scientific™ PepMap™ 100 C18 HPLC Column (1 mm ID x 15 cm lengths) using a Thermo Scientific™ Vanquish Neo UHPLC System. PYE samples were separated using a non-linear 60 min acetonitrile gradient at a flow rate of 50 µL/min (Buffer A: 0.1% FA in H_2O with 3% DMSO, Buffer B: 0.1% FA in ACN with 3% DMSO). The column oven temperature was set to 55°C. Samples were exclusively analysed in data-dependent acquisition (DDA) mode on a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer. MS1 scans were acquired using a resolution of 120,000, a scan range of 360-1,300 and a maximum injection time of 50 ms. The cycle time was set to 1.5 s. Charges states from 2-6 were included and the dynamic

642   exclusion was set to 30 s. MS2 spectra were acquired with the ion trap using a HCD

643   collision energy of 28%. The ion trap scan rate was set to rapid, the maximum injection

644   time was 32 ms, normalized AGC target to 100% and the isolation window to 0.4 Th.

645

646   2.7   Laboratory G

647

648   <u>DDA – EASY-nLC & timsTOF Pro:</u>

649   Mixed proteome samples were analyzed on an EASY-nLC 1200 system (Thermo

650   Fisher Scientific, Bremen, Germany) coupled to a timsTOF Pro mass spectrometer

651   with a Captive Spray ion source (Bruker, Bremen, Germany). PYE peptide mixtures

652   (corresponding to 200 ng) were separated on an IonOpticks Aurora column (25 cm x

653   75 µm, C18 1.6 µm) at a flow rate of 300 nL/min. Column was heated to 50°C. Mobile

654   phase A was ddH$_2$O (Biosolve Chimie), 0.1% (v/v) FA (Biosolve Chimie), and mobile

655   phase B was 80% ACN in dH$_2$O, 0.1% (v/v) FA. During the elution, the percentage of

656   solvent B was increased in a linear fashion from 4 to 35% in 30 min, then from 35 to

657   100% for 10 min. The MS was operated in data-dependent acquisition parallel

658   accumulation-serial fragmentation (ddaPASEF) mode. Full scan MS spectra with mass

659   range m/z 100 to 1700 were acquired. Ion accumulation and separation using trapped

660   ion mobility spectrometry (TIMS) was set to a ramp time of 100 ms with an ion mobility

661   range (1/K$_0$) from 0.85 to 1.30 Vs/cm^2. Number of PASEF ramps was set to 10 with

662   total cycle time of 1.16 s.

663

664   <u>DDA - UltiMate 3000 RSLC & Exploris 480:</u>

665   PYE peptide mixtures (corresponding to 1 µg) were injected to an UltiMate 3000 RSLC

666   nano-HPLC (Dionex, Germering, Germany) coupled to an Exploris 480 mass

667   spectrometer (Thermo Fisher Scientific, Bremen, Germany). Mobile phase A was

668   ddH$_2$O (Biosolve Chimie), 0.1% (v/v) FA (Biosolve Chimie), and mobile phase B was

669   80% ACN in dH$_2$O, 0.1% (v/v) FA. Peptides were separated on a Waters BEH column

670   (75 µm × 250 mm, C18, 1.7 µm, 130 Å) at 35°C running a 48 min linear gradient. The

671   percentage of solvent B was increased in a linear fashion from 2 to 30% in 47 min,

672   then from 30 to 80% for 1 min. After washing the column with 80% B, the column was

673   re-equilibrated to initial conditions resulting in an overall analysis time of 60 min. Data

674   were acquired in a data-dependent mode at a fixed cycle time of 1 s. MS1 spectra

675   were acquired with a resolution of 60,000 covering a mass range from m/z 380-1,400

676   with 300% AGC target and a maximum injection time of 45 ms. The MS2 resolution

677   was set to 15,000 with an AGC target of 100%, and a maximum injection time of 22 ms.

678   Fragment ion data were collected in centroid mode. Normalized HCD collision energy

679   was 26%. First mass was defined at *m/z* 110. Isolation width was set to 1.4 Th.

680

681   <u>DIA – EASY-nLC & timsTOF Pro:</u>

682   Mixed proteome samples were analyzed on an EASY-nLC 1200 system (Thermo

683   Fisher Scientific, Bremen, Germany) coupled to a timsTOF Pro mass spectrometer

684   with a CaptiveSpray ion source (Bruker, Bremen, Germany). PYE peptide mixtures

685   (corresponding to 200 ng) were separated on an IonOpticks Aurora column (25 cm x

686   75 µm, C18 1.6 µm) at a flow rate of 300 nL/min. Column was heated to 50°C. Mobile

687   phase A was ddH$_2$O (Biosolve Chimie), 0.1% (v/v) FA (Biosolve Chimie), and mobile

688   phase B was 80% ACN in dH$_2$O, 0.1% (v/v) FA. During the elution, the percentage of

689   solvent B was increased in a linear fashion from 4 to 35% in 30 min, then from 35 to

690   100% for 10 min. The MS was operated in data-independent acquisition parallel

691   accumulation-serial fragmentation (diaPASEF) mode. Full scan MS spectra with mass

692   range m/z 100 to 1700 were acquired. Ion accumulation and separation using trapped

693   ion mobility spectrometry (TIMS) was set to a ramp time of 100 ms with an ion mobility

694   range (1/K$_0$) from 0.7 to 1.4 Vs/cm^2. One MS cycle included one TIMS full MS scan

695   followed by multiple MS/MS scans comprising in total 26 windows with a fixed mass

696   width of 30 Da and 0.10 Da overlap covering a *m/z*-range of 374 to 1,152 Da resulting

697   in a cycle time estimate of 1.26 s.

698

699   <u>DIA - UltiMate 3000 RSLC & Exploris 480:</u>

700   PYE peptide mixtures (corresponding to 1 µg) were injected to an UltiMate 3000 RSLC

701   nano-HPLC (Dionex, Germering, Germany) coupled to an Exploris 480 mass

702   spectrometer (Thermo Fisher Scientific, Bremen, Germany). Mobile phase A was

703   ddH$_2$O (Biosolve Chimie), 0.1% (v/v) FA (Biosolve Chimie), and mobile phase B was

704   80% ACN in dH$_2$O, 0.1% (v/v) FA. Peptides were separated on Waters BEH column

705   (75 µm × 250 mm, C18, 1.7 µm, 130 Å) at 35°C running a 48 min linear gradient. The

706   percentage of solvent B was increased in a linear fashion from 2 to 30% in 47 min,

707   then from 30 to 80% for 1 min. After washing the column with 80% B, the column was

708   re-equilibrated to initial conditions resulting in an overall analysis time of 60 min. Data-

709   independent acquisition was performed at a full scan resolution of 120,000. MS1

spectra were recorded over a mass range from *m/z* 350-1,400, maximum injection time was set to 45 ms and the normalized AGC target to 300%. The MS1 scan was followed by 31 DIA scans covering a precursor mass range between *m/z* 410-980 using a variable isolation window widths. The resolution was set to 30,000 and the AGC target to 1,000%. Normalized HCD collision energy was 28% and fragment ion data were recorded in profile mode.

Table 3: DIA window scheme on the Orbitrap Exploris 480 by laboratory G.

m/z	z	isolation window (*m/z*)	m/z	z	isolation window (*m/z*)
410.4365	2	21	663.0513	2	16
429.9453	2	20	678.5584	2	17
447.9535	2	18	694.5656	2	17
464.9612	2	18	711.0731	2	18
481.4688	2	17	729.0813	2	20
496.9758	2	16	747.5897	2	19
512.4828	2	17	766.5984	2	21
527.9899	2	16	786.6074	2	21
542.9967	2	16	807.617	2	23
558.0035	2	16	830.1272	2	24
572.5101	2	15	854.1381	2	26
587.0167	2	16	880.6502	2	29
602.0236	2	16	910.1636	2	32
617.0303	2	16	942.6783	2	35
632.0372	2	16	980.1954	2	42
647.5442	2	17			

2.8   Laboratory H

<u>DDA – Ultimate 3000 RSLCnano & Exploris 480:</u>

PYE peptide mixtures (corresponding to 660 ng) were separated on an Ultimate 3000 RSLCnano LC system (Thermo Fisher Scientific, Bremen, Germany) using a Waters BEH reversed-phase column (75 µm × 250 mm, C18, 1.7 µm, 130 Å). Mobile phase A was water containing 0.1% (v/v) formic acid. Peptides were separated running a gradient of 2 to 30% mobile phase B (0.1% (v/v) formic acid in ACN) in 103 min at a flow rate of 300 nL/min. Total analysis time was 120 min including wash and column re-equilibration steps. Column temperature was set to 35°C. Mass spectrometric analysis of eluting peptides was conducted on an Orbitrap Exploris 480 instrument platform (Thermo Fisher Scientific). The funnel RF level was set to 40, and heated capillary temperature was at 275°C.

733   Data were acquired in data-dependent mode at a fixed cycle time of 2 s. Full MS (MS1)
734   resolution was set to 60,000 at *m/z* 200, full MS automated gain control (AGC) target
735   to 300%, and maximum injection time to 45 ms. Mass range was set to *m/z* 380 –
736   1,400. The MS2 resolution was set to 15,000 with an AGC target of 100%, and a
737   maximum injection time of 22 ms. Fragment ion data were collected in centroid mode.
738   Normalized HCD collision energy was 26%. Isolation width was set to 1.4 Th. Data
739   were acquired in positive ion mode.

740

741   DDA – Ultimate 3000 RSLCnano & Eclipse:
742   PYE peptide mixtures (corresponding 660 ng) were separated on an Ultimate 3000
743   RSLCnano LC system (Thermo Fisher Scientific, Bremen, Germany) using a self-
744   packed 30 cm x 100 µm, 1.9 µm reversed-phase column (Reprosil-Pur 120 C18-AQ
745   resin, Dr Maisch). Mobile phase A was water containing 0.1% (v/v) formic acid.
746   Peptides were separated running a gradient of 2 to 38% mobile phase B (0.1% (v/v)
747   formic acid in ACN) in 121 min at a flow rate of 300 nL/min. Total analysis time was
748   141 min including wash and column re-equilibration steps. Mass spectrometric
749   analysis of eluting peptides was conducted on an Orbitrap Eclipse (Thermo Fisher
750   Scientific).
751   Data were acquired in data-dependent mode at a fixed cycle time of 3 s. Full MS (MS1)
752   resolution was set to 120,000 at *m/z* 200. Ion transfer time was determined
753   automatically ("auto mode"). Mass range was set to *m/z* 380 – 1,400. The MS2
754   resolution was set to 15,000 with a maximum injection time of 22 ms. Fragment ion
755   data were collected in centroid mode. Normalized HCD collision energy was 30%.
756   Isolation width was set to 1.2 Th. Data were acquired in positive ion mode.

757

758   DIA – Ultimate 3000 RSLCnano & Exploris 480:
759   PYE peptide mixtures (corresponding to 660 ng) were separated on an Ultimate 3000
760   RSLCnano LC system (Thermo Fisher Scientific, Bremen, Germany) using a Waters
761   BEH reversed-phase column (75 µm × 250 mm, C18, 1.7 µm, 130 Å). Mobile phase A
762   was water containing 0.1% (v/v) formic acid. Peptides were separated running a
763   gradient of 2 to 30% mobile phase B (0.1% (v/v) formic acid in ACN) in 103 min at a
764   flow rate of 300 nL/min. Total analysis time was 120 min including wash and column
765   re-equilibration steps. Column temperature was set to 35°C. Mass spectrometric
766   analysis of eluting peptides was conducted on an Orbitrap Exploris 480 instrument

platform (Thermo Fisher Scientific). The funnel RF level was set to 40, and heated capillary temperature was at 275°C.

Data were acquired in DIA mode, full MS (MS1) resolution was set to 120,000 at *m/z* 200 and full MS automated gain control (AGC) target to 300% with a maximum injection time of 45 ms. Mass range was set to *m/z* 350–1,400. Fragment ion spectra were acquired with an AGC target value of 1000%, applying a DIA scheme consisting of 47 windows with variable window widths and 1 Th overlap. MS2 resolution was set to 30,000 and maximum injection time was set to 54 ms. Normalized collision energy was fixed at 28% and fragment ion data collected in centroid mode. Data were acquired in positive ion mode.

DIA – Ultimate 3000 RSLCnano & Eclipse:

PYE peptide mixtures (corresponding 660 ng) were separated on an Ultimate 3000 RSLCnano LC system (Thermo Fisher Scientific, Bremen, Germany) using a self-packed 30 cm x 100 µm, 1.9 µm reversed-phase column (Reprosil-Pur 120 C18-AQ resin, Dr Maisch). Mobile phase A was water containing 0.1% (v/v) formic acid. Peptides were separated running a gradient of 2 to 38% mobile phase B (0.1% (v/v) formic acid in ACN) in 121 min at a flow rate of 300 nL/min. Total analysis time was 141 min including wash and column re-equilibration steps. Column temperature was set to 35°C. Mass spectrometric analysis of eluting peptides was conducted on an Orbitrap Eclipse (Thermo Fisher Scientific).

Data were acquired in DIA mode, full MS (MS1) resolution was set to 120,000 at *m/z* 200 and full MS automated gain control (AGC) target to 300% with a maximum injection time of 45 ms. Mass range was set to *m/z* 350–1,400. Fragment ion spectra were acquired applying a DIA scheme consisting of 55 windows with variable window widths and 1 Th overlap. MS2 resolution was set to 30,000 and maximum injection time was set to 54 ms. Normalized collision energy was fixed at 28% and fragment ion data collected in profile mode. Data were acquired in positive ion mode.

2.9 Laboratory I

DIA – EASY-nLC & Exploris 480:

HeLa QC standard samples as well as blanks were acquired in data-dependent mode. An EASY-nLC 1200 (Thermo Fisher Scientific, Bremen, Germany) was coupled to an

801 Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). Samples were

802 separated using a 44 min gradient and loaded directly onto the analytical column

803 (75 µm x 20 cm column packed in-house with C18-AQ 1.9 µm beads, Dr. Maisch

804 Reprosil-Pur 120) at a flow rate of 250 nL/min. A total amount of 100 ng HeLa Protein

805 Digest Standard (Pierce) was injected on column to perform a system QC. MS1 was

806 recorded at a resolution of 60,000 and MS2 at 15,000. The top20 abundant peaks

807 were selected for fragmentation using a 1.3 Th isolation window and 22 ms maximum

808 injection time with an AGC target set to 100% corresponding to 100,000 ions.

809 DIA acquisition of the main sample cohort (PYE peptide mixtures) was done using the

810 same LC-MS setup as described above. MS1 spectra were acquired at a resolution of

811 120,000 and MS2 at a resolution of 30,000. A number of 40 isolation windows with

812 variable isolation widths were used for data-independent acquisition mode. The AGC

813 target was set to 3,000% corresponding to a number of 3e6 ions and the maximum

814 injection time was specified to 54 ms. For fragmentation, a stepped collision energy

815 approach was used.

816

817 ## 2.10 Laboratory J

818

819 <u>DIA – EASY-nLC & timsTOF Pro2:</u>

820 The samples were measured on an EASY-nLC 1200 system (Thermo Fisher Scientific,

821 Bremen, Germany) coupled to a TIMS quadrupole TOF mass spectrometer (Bruker

822 timsTOF Pro2). Peptides were separated using a 44 min gradient at a flow rate of 250

823 nL/min on a home-packed column (20 cm × 75 µm i.d., 1.9 µm ReproSil-Pur C18-AQ

824 particles from Dr. Maisch) at 50℃.

825 To perform analyses in diaPASEF mode, the default method from Bruker was used

826 ('diaPASEF – long gradient') with a scan range of *m/z* 100 to 1,700, ion mobility range

827 of 0.6-1.6 $1/K_0$ and a dia window scheme comprising 32 windows with a fixed window

828 width of 26 Th and 1 Th overlap covering a precursor *m/z*-range from 400-1,201. The

829 tims ramp time was set to 100 ms resulting in an overall cycle time of approx. 1.7 s.

830

831 ## 2.11 Laboratory K

832

833 <u>DIA – Acquity M-Class & ZenoTOF 7600:</u>

834 Samples were acquired in data-independent acquisition (DIA)/Zeno-SWATH mode, on

835 a ZenoTOF 7600 mass spectrometer (SCIEX, Toronto, Canada) coupled online to an

836 Acquity M-Class UPLC system (Waters, Milford, MA, USA). Each measurement used

837 0.5 µg sample which was separated by reversed-phase chromatography (on a

838 nanoEase M/Z HSS T3 column; 100 Å, 1.8 µm, 0.3 x 150 mm) with buffers A (0.1%

839 formic acid) and B (acetonitrile with 0.1% formic acid) ramping from 3 to 40% buffer B

840 at a flow rate of 5 µL/min and at 35°C using a 30 min gradient as described by Wang

841 *et al.*[2].

842 The mass spectrometric method was identical to the one employed by Wang *et al.*[2]

843 with the following exceptions: The spray voltage was set to 5,000 V and a scheduled

844 ionization between minutes 2.5 and 22 was used.

845

846 2.12 Laboratory L

847

848 <u>DIA - nanoAcquity & timsTOF Pro:</u>

849 Samples (corresponding to 250 ng) were analyzed on a timsTOF-Pro (Bruker

850 Corporation, Billerica, MA, USA) coupled online to a nanoAcquity LC system (Waters).

851 Peptides were directly injected onto an Aurora 5 cm x 150 µm analytical emitter

852 column (1.6 µm C18, IonOpticks, Australia) at a flow rate of 2 µL/min for loading,

853 washing and equilibration, and 1 µL/min for the separation of peptides running a linear

854 gradient from 0 to 34% B (mobile phase A = 0.1% FA in water, mobile phase B = 0.1%

855 FA in ACN). Eluting peptides were analyzed in positive ion mode using parallel

856 accumulation serial fragmentation (PASEF) enhanced DIA. The dual TIMS (trapped

857 ion mobility spectrometer) was operated at a fixed duty cycle close to 100% using

858 equal accumulation and ramp times of 50 ms each spanning a mobility range from

859 $1/K_0 = 0.6$ Vs cm^{-2} to 1.4 Vs cm^{-2}. We defined 36 × 25 Th isolation windows with 1 Th

860 overlap covering a precursor mass range from *m/z* 300 to 1,165 resulting in 2-3

861 diaPASEF scans per TIMS cycle and an overall cycle time of 0.84 s. The collision

862 energy was ramped linearly as a function of the mobility from 59 eV at

863 $1/K_0 = 1.3$ Vs cm^{-2} to 20 eV at $1/K_0 = 0.85$ Vs cm^{-2}. MS1 and fragment ion spectra

864 were recorded with a mass range spanning from *m/z* 100-1,700.

865

866 <u>DIA - nanoElute & timsTOF Pro2:</u>

867   PYE peptide mixtures (corresponding to 250 ng) were injected and separated on a
868   nanoElute LC system (Bruker Corporation, Billerica, MA, USA) at a flow rate of
869   850 nL/min using a reversed phase C18 column (PepSep^{TM}, 25 cm x 150 µm 1.5 µm)
870   which was heated to 40°C. Peptides were loaded onto the column in direct injection
871   mode at 800 bar. Mobile phase A was 0.1% FA (v/v) in water and mobile phase B 0.1%
872   FA (v/v) in ACN. Peptides were separated running a non-linear gradient from 2% to
873   38% mobile phase B over 35.5 min. Afterwards, column was rinsed for 5.5 min at 95%
874   B. Eluting peptides were analyzed in positive mode ESI-MS using parallel
875   accumulation serial fragmentation (PASEF) enhanced DIA mode on a timsTOF Pro2
876   mass spectrometer (Bruker Corporation). The dual TIMS was operated at a fixed duty
877   cycle close to 100% using equal accumulation and ramp times of 100 ms each
878   spanning a mobility range from $1/K_0 = 0.6$ Vs cm^{-2} to 1.6 Vs cm^{-2}. We defined
879   36 × 25 Th isolation windows from *m/z* 300 to 1,165 resulting in 2-3 diaPASEF scans
880   per TIMS cycle and an overall cycle time of 1.7 s. The collision energy was ramped
881   linearly as a function of the mobility from 59 eV at $1/K_0 = 1.3$ Vs cm^{-2} to 20 eV at
882   $1/K_0 = 0.85$ Vs cm^{-2}. MS1 and fragment ion spectra were recorded with a mass range
883   spanning from *m/z* 100-1,700.

884

885   DIA – Ultimate 3000 RSLCnano & Exploris 480:
886   PYE peptide mixtures (corresponding to 150 ng) were separated on an Ultimate 3000
887   RSLCnano LC system (Thermo Fisher Scientific, Bremen, Germany) using a
888   PEPMAP100 C18 5 µm 0.3 x 5 mm trap (Thermo Fisher Scientific) and an HSS-T3
889   C18 1.8 µm, 100 µm x 100 mm analytical reversed-phase column (Waters
890   Corporation). Mobile phase A was water containing 0.1% (v/v) formic acid and 3% (v/v)
891   DMSO. Peptides were separated running a gradient of 2 to 35% mobile phase B (0.1%
892   (v/v) formic acid, 3% (v/v) DMSO in ACN) in 29 min at a flow rate of 800 nL/min. Total
893   analysis time was 40 min including wash and column re-equilibration steps. Column
894   temperature was set to 55°C. Mass spectrometric analysis of eluting peptides was
895   conducted on an Orbitrap Exploris 480 instrument platform (Thermo Fisher Scientific).
896   The funnel RF level was set to 40, and heated capillary temperature was at 250°C.
897   Data were acquired in DIA mode, full MS (MS1) resolution was set to 120,000 at *m/z*
898   200 and full MS automated gain control (AGC) target to 300%. Mass range was set to
899   *m/z* 345–1,250. Fragment ion spectra were acquired with an AGC target value of
900   1000%, applying a DIA scheme consisting of 21 windows (with a variable window width

901 and a 0.5 Th window overlap). Resolution was set to 30,000 and ion transfer time was

902 determined automatically ("auto mode"). Normalized collision energy was fixed at 27%.

903 Data were acquired in positive ion mode.

904

905 <u>DIA – Ultimate 3000 RSLCnano & Exploris 480 with FAIMS:</u>

906 PYE peptide mixtures (corresponding to 150 ng) were separated on an Ultimate 3000

907 RSLCnano LC system (Thermo Fisher Scientific, Bremen, Germany) using a

908 PEPMAP100 C18 5 µm 0.3 x 5 mm trap (Thermo Fisher Scientific) and an HSS-T3

909 C18 1.8 µm, 100 µm x 100 mm analytical reversed-phase column (Waters

910 Corporation). Mobile phase A was water containing 0.1% (v/v) formic acid and 3% (v/v)

911 DMSO. Peptides were separated running a gradient of 2 to 35% mobile phase B (0.1%

912 (v/v) formic acid, 3% (v/v) DMSO in ACN) in 29 min at a flow rate of 800 nL/min. Total

913 analysis time was 40 min including wash and column re-equilibration steps. Column

914 temperature was set to 55°C. Mass spectrometric analysis of eluting peptides was

915 conducted on an Orbitrap Exploris 480 instrument platform coupled to a FAIMS Pro

916 interface (Thermo Fisher Scientific). The funnel RF level was set to 40, and heated

917 capillary temperature was at 275 °C.

918 Data were acquired in DIA mode, full MS (MS1) resolution was set to 120,000 at *m/z*

919 200 and full MS automated gain control (AGC) target to 300%. Mass range was set to

920 *m/z* 345–1,250. Fragment ion spectra were acquired with an AGC target value of

921 1000%, applying a DIA scheme consisting of 20 windows (window width: 45 Th with a

922 0.5 Th overlap). Resolution was set to 30,000 and ion transfer time was determined

923 automatically ("auto mode"). Normalized collision energy was fixed at 27%. Data were

924 acquired in positive ion mode. FAIMS voltage was set to -36 V.

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