SUPPLEMENTARY MATERIALS

DETAILED EXPERIMENTAL PROCEDURES

Sample lysis

The lysis procedure below is an example of *Xenopus laevis* egg and embryo sample preparations, which are complicated by the presence of sacs containing large amounts of yolk proteins such as the vitellogenins. The iFASP protocol, however, is compatible with (almost) any sample preparation protocol and we give our method below for completeness.

Embryo lysis:

Xenopus laevis eggs or embryos (approx. 20) were collected into an Eppendorf tube, as much excess buffer as possible removed and snap-frozen in liquid nitrogen. To lyse embryos without disrupting yolk sacs, 10 µl *Xenopus* Lysis Buffer (XLB: 20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% Igepal, 1 Protease Inhibitor Cocktail Tablet (Roche) per 10 ml) was added per embryo and mixed by pipetting up and down until all cells were disrupted. Lysates were spun for 10 minutes at 14k rpm 4 ˚C. The supernatant was removed and stored on ice. Protein concentrations in each sample were determined using BCA assay (Thermo kit #23225).

FASP

Six 100 µg protein samples (< 30 µl lysate) were reduced using 1 µl 200 mM tris(2-carboxyethyl)phosphine (TCEP) at 55 °C for 1 hour. TCEP was used but the reduction protocol used, like sample lysis, can be determined by the individual user. The protocol then followed the

manufacturer's protocol for the FASP™ Protein Digestion Kit (#44250, Protein Discovery) with exceptions as described. Samples were mixed with 200 µl Urea Sample Solution (8 M urea in 0.1 M Tris/HCl pH 8.5) in a 10kDa MWCO spin column (Millipore) and spun 14k x g for 15 minutes at room temperature (care should be taken to carry out the urea washes at room temperature to prevent urea precipitation). A further wash with 200 µl 8 M urea solution was carried out in a similar fashion. Alkylation was carried out with 100 µl 0.05 M iodoacetamide solution in 8M urea, shaking at 600 rpm (taking care to close the Eppendorf lids over the spin columns) for one minute and incubating in the dark at room temperature (23 ˚C) for 20 minutes before centrifugation at 14k x g for 15 minutes. Two further washes with 8 M urea solution were carried out. Subsequently, three washes with 100 µl 50 mM ammonium bicarbonate solution were performed (alternatively, if there are concerns about ammonium bicarbonate quenching the labels, 100 mM triethylammonium bicarbonate (TEAB) can be substituted and this was performed in some cases as described in the text and Supplementary Tables 1 and 2). Trypsin digestion was carried out using 1:100 enzyme-to-protein ratio with a stock concentration of 0.2 µg/µl Sequencing Grade Modified Trypsin (V5111, Promega) incubated at 37 ˚C for 16 hours with shaking at 600 rpm. The shaking at 600 rpm is necessary to ensure efficient trypsin digestion on the filter. Also, to prevent the column drying out overnight, seal the lid and spin column to the Eppendorf with a small piece of parafilm.

iFASP – TMT labeling

TMTsixplex™ Isobaric Label Reagent Set (Thermo #90062) labels were equilibrated to room temperature. To each tube was added 41 µl anhydrous acetonitrile and the reagent was dissolved

for 5 minutes with occasional vortexing, the TMT label solutions were briefly centrifuged to collect the liquid at the bottom of the tube and the full amount of each label was transferred to its respective sample before vortexing for 5 minutes (for the TMT Mock iFASP experiment, acetonitrile alone was added to each sample). The reactions were incubated at room temperature for one hour with shaking at 600 rpm (again, keeping the samples agitated on the spin filter is important to assist the labeling reaction). To quench the reaction, 8 µl of 5% hydroxylamine was added to the samples and incubated at room temperature for a further 30 minutes with shaking at 600 rpm. Peptides were eluted from the spin columns using two washes with 40 µl 50 mM ammonium bicarbonate solution and one wash with 50 µl 0.5 M NaCl solution. Samples were then combined and partially dried in a Speedvac to a volume of around 200 µl to remove acetonitrile before acidification with 1 ml 1 % trifluoroacetic acid and 10 µl 10 % trifluoroacetic acid (to bring the pH to 2-3) for 30 minutes at 37 ˚C with shaking at 900 rpm.

iFASP – iTRAQ labeling

iTRAQ® 8-plex (Sciex #4390812) labels were equilibrated to room temperature then spun briefly to collect the solution. To each tube was added 50 µl isopropanol and mixed for 5 minutes with occasional vortexing. The labels were briefly centrifuged to collect the liquid at the bottom of each tube and the full amount of each label was transferred to its respective sample before vortexing for 5 minutes. The reactions were incubated at room temperature for two hours with shaking at 600 rpm (as with the TMT labels, agitation of the samples is important to ensure proper labeling). Peptides were eluted from the spin columns using two washes with 40 µl 50 mM ammonium bicarbonate solution and one wash with 50 µl 0.5 M NaCl solution. Samples were then combined and partially dried in a Speedvac to a volume of around 200 µl to remove

acetonitrile before acidification with 1 ml 1 % trifluoroacetic acid and 10 µl 10 % trifluoroacetic acid (to bring the pH to 2-3) for 30 minutes at 37 ˚C with shaking at 600 rpm. For iTRAQ experiments, samples were washed with either ammonium bicarbonate or triethylammonium bicarbonate prior to trypsin digestion as described in the text.

In-solution digest and TMT-labeling

Six 100 µg protein samples were precipitated by methanol/chloroform precipitation and the pellets resuspended using 0.1% Rapigest in 100 mM TEAB by vortexing and sonication followed by incubation at 37 °C for 15 minutes while mixing at 900 rpm. 0.5 µg Sequencing Grade Modified Trypsin (V5111, Promega) was then added and the samples incubated at 37 °C for a further 45 minutes whilst mixing at 900 rpm. Samples were reduced using 50 µl 200 mM TCEP at 55 °C for 1 hour. Alkylation with 50 µl 375 mM iodoacetamide solution was performed at room temperature for 30 minutes in the dark. Digestion was performed in 1 µg of trypsin dissolved in 50 mM ammonium bicarbonate solution with incubation at 37 °C for 16 hours whilst mixing at 900 rpm. TMTsixplex™ Isobaric Label Reagent Set labels were equilibrated to room temperature. To each tube was added 41 µl anhydrous acetonitrile and the reagent was dissolved for 5 minutes with occasional vortexing. The full amount of each label was transferred to its respective sample and reactions were incubated at room temperature for one hour. To quench the reaction, 8 µl of 5% hydroxylamine was added to the samples and incubated at room temperature for a further 30 minutes. Samples were combined and partially dried to remove acetonitrile before acidification with formic acid was carried out for 30 minutes

at 37 ˚C. The sample was centrifuged at 21k x g for 15 minutes at 4 ˚C and the supernatant removed from the insoluble Rapigest pellet.

Sample cleanup and electrophoresis

Peptides were desalted on an OASIS HLB column (Waters) by equilibrating the column with 4 washes with 1 ml 0.1% formic acid, 70% acetonitrile (in H_2O); washing the column 4 times with 1 ml 0.1% formic acid; passing the sample through the column twice; washing the sample 10 times with 0.1% formic acid; and eluting the peptides with 500 µl 0.1% formic acid 30% acetonitrile, followed by 300 µl 0.1% formic acid 50% acetonitrile, followed by 300 µl 0.1% formic acid 70% acetonitrile. Samples were evaporated to dryness.

Samples were resuspended in 3.6 ml of rehydration buffer (0.2% IPG buffer pH 3-10 (GE Healthcare, 17-6000-87) and peptides were separated into 24 fractions according to isoelectric point using Immobiline™ DryStrip pH 3-10, 24cm (GE Healthcare, 17-6002-44) dry polyacrylamide gel strips with the 3100 OFFGEL fractionator (Agilent, G3100AA). Buffer from each well was collected and 150 µl of 0.1 % TFA added to each well. Peptides eluted after 15 minutes were also collected and added to previous fractions. Peptide fractions were cleaned using TARGA C18 Microspin Columns (The Nest Group, SS18R) by equilibrating the column with 2 washes with 1 ml 0.1% formic acid, 70% acetonitrile (in H_2O); washing the column 2 times with 1 ml 0.1% formic acid; passing the sample through the column twice; washing the sample 4 times with 0.1% formic acid; and eluting the peptides with 100 µl 0.1% formic acid 30% acetonitrile, followed by 100 µl 0.1% formic acid 50% acetonitrile, followed by 100 µl

0.1% formic acid 70% acetonitrile. Samples were evaporated to dryness and resuspended in MS loading buffer (5% acetonitrile, 0.1% formic acid in H_2O).

Chromatography and mass spectrometry (LC-MS/MS)

Samples were placed in a micro-autosampler linked to a nanoflow HPLC pump (Eksigent). Reverse phase columns were packed in-house using Magic C18 particles (5 μm, 200 Å; Michrom) and PicoTip Emitters (New Objective). Peptides were eluted with a 60 minute linear gradient from 95% buffer A (water with 0.2% formic acid) and 5% buffer B (acetonitrile with 0.2% formic acid) to 65% buffer A.

LTQ-Orbitrap: The LTQ-Orbitrap was run in positive ion mode. Full scans were carried out at a resolution of 30k using a scan range of 395 to 1200 m/z. A normalized collision energy of 35 (using Pulsed Q-Dissociation (PQD)) was used to activate both the reporter ions and parent ions for fragmentation. Scans were carried out with an activation time of 30 ms. The isolation window was set to 1.0 m/z.

Q Exactive: The Q Exactive was run in positive ion mode. Full scans were carried out at a resolution of 70k with an automatic gain control (AGC) target of 3×10^6 ions and a maximum injection time of 20 ms, using a scan range of 300 to 1500 m/z. Using a Top 10 method to select precursor ions for MS/MS, a normalized collision energy of 30 (using High-energy Collision Dissociation (HCD)) was used to activate both the reporter ions and parent ions for fragmentation. Scans were carried out at a resolution of 17.5k with an AGC target of 5×10^4 ions and a maximum injection time of 80 ms. The isolation window was set to 1.6 m/z and the

fixed first mass to 100.0 m/z to ensure reporter ions were detected. An underfill ratio of 1.0% was set and a dynamic exclusion of 20 s applied.

Data analysis

Searches were performed against the *Xenopus laevis* protein database downloaded from Xenbase (www.xenbase.org, with protein entries containing 'J' residues removed) supplemented with protein entries for common lab contaminants. A decoy database was generated using reversed sequences for searches on Mascot.

Proteome Discoverer: Thermo .RAW files were loaded directly into the "Spectrum Files" node of Proteome Discoverer. Spectra were filtered in the "Spectrum Selector" node using the following criteria: 'use MS1 precursor' for precursor selection; filter spectrum properties only using precursor mass range 600-5000 Da and a minimum peak count of 1; filter scan events using 'any' for all settings except use MS2 for MS order and selecting only full scans as scan type; filter peaks using a signal-to-noise ratio threshold of 1.5 for Fourier Transform (FT); and for unrecognised properties, 'automatic' for charge replacement, 'ITMS' for mass analyzer, 'MS2' for order, 'CID' for activation type and 'positive' for polarity. In the "Mascot" node, searches were performed with the enzyme set to 'trypsin'; number of missed cleavages set to '0', instrument set to 'FT-ICR'; precursor mass tolerance set to 10 ppm and fragment mass tolerance set to 20 mmu. No taxonomy was fixed and 'don't use average precursor mass' was selected. TMT 6-plex (K and N-term) modifications were set as dynamic modifications to calculate labeling efficiency. Glutamine-pyro-glutamic acid on N-terminal glutamines and methionine oxidation were set as dynamic modifications whilst carbamidomethylation on cysteines was set

as a static modification. False Discovery Rate (FDR) was finally determined by using the "Percolator" node with a maximum ΔC_n of 0.05, a strict FDR of 0.01 and a relaxed FDR of 0.05 with validation based on q-value. The "Reporter Ion Quantifier" node was used for TMT 6plex quantification using isotopic label correction for kit #NC171871. Only unique peptides were used for quantification and values were normalized on the protein median using a minimum protein count of 20. The integration window tolerance was set to 0.1 Da and integration was based on the most confident centroid. Only peptides identified with a high rate of confidence (1% FDR) were used for labeling efficiency analysis. Labeling efficiency was calculated using high confidence peptides and taking the number of TMT-labeled unique peptide sequences as a percentage of the total number of unique sequences according to the method of Nogueira et al., 2012.

Supplementary Figures

TMT label	126	127	128	129*	130	131
Protein used (µg)	100	30	10	100	30	10
TMT-labeled PSMs	27326	27340	20121	26096	19711	21068

Supplementary Table 1: Comparison of digestion and labeling of different amounts of protein. The amounts of protein used in each channel are given as well as the number of TMT-labeled PSMs observed (27800 TMT-labeled PSMs total; labeling efficiency was 99.0%). *The 129 channel containing 100 µg was prepared using TEAB and not ABC.

Label	113	114	115	116	117	118	119	121
Solution	ABC	TEAB	ABC	TEAB	ABC	TEAB	ABC	TEAB
iTRAQ-labeled PSMs	36080	34039	33598	34559	35614	33557	35389	35024

Supplementary Table 2: iTRAQ-labeled samples. 100 µg of *Xenopus laevis* egg lysate was used for iFASP and labeled using iTRAQ reagents (42516 iTRAQ-labeled PSMs total; labeling efficiency was 99.3%). Samples were prepared using either TEAB or ABC as designated.