Using the frozen extracted pellets from homogenization described in NMR sample preparation, we further delipidated by resuspending the pellets in chloroform/methanol/water (4:8:3, v/v/v) as described previously (1). Insoluble proteins were pelleted by centrifugation, and protein pellets were washed twice with ice-cold acetone. Finally, protein power was dried under a nitrogen evaporator.

Preparation of glycopeptides and release of N-linked glycans was performed as described previously (1). Briefly, approximately 5 mg of protein powder from each sample was resuspended in 500 µL of 40 mM NH_4HCO_3, 1 M urea, 20 µg/mL trypsin, and 20 µg/mL chymotrypsin and incubated overnight (16-18 h) at 37 °C. The glycopeptide mixture was boiled for 5 min and adjusted to 5% AcOH (acetic acid) prior to a Sep-Pak C18 cartridge column clean up. Glycopeptides were eluted stepwise in 20% isopropanol in 5% AcOH, 40% isopropanol in 5% AcOH, and 100% isopropanol. The eluates were pooled and evaporated to dryness. Dried glycopeptides were resuspended in 50 mM citrate phosphate buffer (pH 5.0) for digestion with peptide:N-glycosidase A (PNGase A) and incubated for 18 h at 37 °C. We chose to utilize PNGase A for the release of N-linked glycans since its substrate-specificity is less stringent than the commonly used PNGase F. Specifically, PNGase A is capable of releasing N-glycan species containing α1-3-linked fucose on the chitobiose core, known to be synthesized by C. elegans (2, 3). PNGase A-released oligosaccharides were separated from residual peptides by another round of Sep-Pak C18 cartridge clean-up, and the glycan flow-through was collected. Released N-glycans were dried down using a SpeedVac.

Since there is currently no available enzyme for the comprehensive release of O-linked glycans, we employed a commonly used chemical release strategy via reductive β-elimination using NaOH and $NaBH_4$ (4). Approximately 5 mg of protein powder from each sample was processed for reductive β-elimination to release O-linked glycan alditols as described previously (5). Briefly, protein powder was resuspended in 100 mM NaOH containing 1 M $NaBH_4$ and incubated for 18 h at 45 °C in a glass tube sealed with a teflon-lined screw top. Following incubation, the protein concentration of the reaction mixture was determined via absorbance at 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For normalization of all samples, 2 mg of the reaction mix was neutralized with 10% acetic acid on ice and desalted using a AG-50W-X8 (H^+ form) column (1 mL bed volume) prior to borate removal and Sep-pack C18 cartridge clean-up. Released O-glycans were dried down using a SpeedVac.

Both N- and O-glycans were permethylated to introduce hydrophobicity, fragmentation, and facilitate in-line separation by reverse-phase (C18) chromatography prior to detection by mass spectrometry (MS) (6,7). Furthermore, permethylation of glycans greatly improves MS ionization efficiency resulting in improved quantification. All released N- and O-linked glycans were permethylated prior to MS analysis according to the method by Anumula and Taylor (8).

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