Methods

Sample Preparation. Pierce™ HeLa protein digest standard and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA). K562 and yeast digest standards were from Promega (Madison, WI). Mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) were respectively prepared from LC-MS grade water and acetonitrile purchased from Honeywell (Charlotte, NC). The HeLa and K562 digest standards were reconstituted to a final concentration of 200 ng/µL in 100 µL of mobile phase A to form stock solutions. For these experiments, the stock solutions were further diluted in mobile phase A to 10 ng/µL and 0.2 ng/µL. To create a mixed-species proteome, yeast and HeLa digests were combined at 8:2 HeLa:yeast at a total concentration of 10 ng/µL. HeLa and K562 cells (ATCC, Manassas, VA) were cultured and harvested as described previously.[1] Following pelleting and removal of cell media, cells were resuspended in phosphate-buffered saline (PBS) to reach a concentration of ~300,000 cells/mL. The cellenONE X1 (Cellenion, Lyon, France) was used for single-cell isolation and reagent dispensing for nanoPOTS sample processing using a recently developed one-step protocol[2] and dried on-chip prior to analysis.

Cultured cells of human origin used in this study are exempt from human subjects research regulations according to US federal regulation 45 CFR 46, Exemption X4.

Separations. Analytical and micro-solid-phase-extraction (SPE or trap) columns were prepared in-house[3] using Dr. Maisch (Ammerbuch, Germany) ReproSil-Pur C18 media having 1.9 µm diameter and 120 Å pore size. Columns were packed in 20-µm-i.d. × 30-cm-long fused silica capillary (Polymicro, Phoenix, AZ). Trap columns were 50-µm-i.d. × 5-cm-long. Both columns were fritted using Kasil Frit Kit (Next Advance, Troy, NY). Trap columns were fritted on both ends to enable bidirectional flow. Capillary ends were polished using the Capillary Polishing Station (ESI Source Solutions, Woburn, MA). A 10-µm-i.d. chemically etched nanoelectrospray emitter from MicrOmics Technologies (Spanish Fork, UT) were connected to the analytical column via a PicoClear union (New Objective, Woburn, MA).

Aliquots of bulk-prepared protein digest were analyzed from glass vials using an Ultimate 3000 nanoLC system (Thermo Fisher) modified with a 10-port valve as described previously.[4] Samples were loaded onto the SPE at ~0.3 µL/min for 10 min with 1% mobile phase B before the valve was switched to deliver the sample onto the analytical column. The flowrate through the analytical column was ~15 nL/min. Electrospray potential (2.2 kV) was applied to the Nanovolume union (VICI, Houston, TX) upstream of the analytical column. Single cell samples were analyzed directly from nanowell chips via a custom autosampler as described previously.[5] For 40-min active gradients, mobile phase B was increased from 1 to 2% in 1 min, 2 to 8% in 5 min, 8 to 15% in 15 min, 15 to 20% in in 9 min, 20 to 25% in 6 min, and 25 to 45% in 10 min. For column washing and regeneration, mobile phase B was increased from 45 to 80% B in 5 min, stepped to 90% for 5 min, stepped to 1% and held for 25 min. For 20-min active elution gradients, the following steps were modified: mobile phase B was ramped from 8 to 15% in 7.5 min, then to 20% in 4.5 min and to 25% in 3 min.

MS Acquisition. The LC column/emitter assembly was interfaced with an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher) via the Nanospray Flex Ion Source. The temperature of the ion transfer tube was set to 200 °C and the RF lens setting was 50%. For MS1, the Orbitrap resolution was set to 120,000 (m/z 200) with the normalized AGC target set to 300%. The scan range was between 375-1575 m/z and the maximum injection time was set to 118 ms. To trigger MS2 for all experiments, the precursor intensity

threshold was set to 5.0E3, charge state was 2 to 4, and dynamic exclusion was 20s for 20 min LC gradients and 25s 40 min gradients. The cycle time was 1.5 s. For standard DDA, the isolation window was 1.6 Th, the HCD collision energy was 30%, and the MS2 resolution was set to 15,000 and 60,000 for 10 and 0.2ng HeLa/K562 protein digest standard, respectively. The maximum MS2 injection time for 10 and 0.2 ng was 22 and 118ms, respectively, and the AGC target was 200%. For WWA, a combination of settings for the isolation windows, resolution, and injection time were evaluated. The AGC target was set to the maximum 1000% for all experiments. The isolation widths were 2, 4, 8, 12, 18, 24 and 48 Th. The MS2 resolution was 30,000, 45,000, 60,000 and 120,000 with corresponding 54 ms, 86 ms,118 ms and 246 ms injection times. For single cell samples, we selected an isolation window of 8 Th for WWA with 60,000 MS2 resolution and 118 ms injection time.

For DIA experiments, the precursor scanning range was from 400 to 800 m/z. A fixed window of 50 m/z increment as well as SWATH acquisitions are detailed in Supplemental Table 1. Window overlap was 1. HCD collision energy was 30%. Resolution of 60000, injection time of 118ms and AGC target of 1000% for 0.2 ng standard and single cell experiments. For 10 ng DIA library, 30000 resolution, 54ms injection time and AGC target of 1000% were set.

Data Analysis. For DDA and WWA experiments, raw files were searched using Proteome Discoverer (PD) (Thermo Scientific, version 3.0.1.13) with the CHIMERYS identification node using prediction model inferys_2.1_fragmentation as default settings. Database search included human (Uniprot version 2022-8-11) and yeast (Uniprot, version 2022-8-11) as well as common contaminants (PD, version 2018-10-26). Enzyme was set as Trypsin with maximum 2 missed cleavages. Other parameters included peptide lengths of 7-30 amino acids, a maximum of 3 modifications, and charges between 2 and 4. Fragment mass tolerance was 20 ppm. Carbamidomethyl (C) was fixed as a static modification by the CHIMERYS software. Results were filtered with the Percolator node at 1% FDR. For MBR, retention time tolerance was set to 0.25 min and mass tolerance was 5 ppm.

For DIA experiments, FrapPipe (version 1.8) and DIA-NN (version 1.8.1) were used with the default DIA_Speclib_Quant workflow. The spectral libraries were generated by FragPipe with RT calibration set to ciRT. Quantification was performed with DIA-NN with Robust LC (high precision) as quantification strategy and MBR enabled. Database search was also human (version 2022-8-11) with added decoys and common contaminants. FDR was set to 1%.

For CV calculations, Pearson r calculations, and quantifiable proteins, all protein data were imported into the R programming language from the "…_Proteins.txt" files exported from PD or the "…_pg_matrix.tsv" exported from DIA-NN. Medium and low confidence proteins were removed, as were contaminants, and for PD, any proteins with <2 unique peptides. Next, these proteins were sorted according to gradient lengths and sample size, normalized to the median in the separate groups, and filtered for <33% missing values. For MBR missing values, this meant excluding any without "High" or "Peak Found" proteins in PD files, and just those without "High" for MS2-quantifiable proteins. DIA-NN used only valid values for data analyzed with or without a 10-ng spectral library. Pearson correlations used log-10 transformed mean abundances of quantifiable proteins. For PCA, k nearest neighbors was used to impute missing values with a k = 5, and the prcomp function was used in R. All figures were exported to Microsoft PowerPoint for further formatting.

Throughout the manuscript, when significant differences in proteome coverage are asserted to result from different MS acquisition conditions, p-values were calculated based on a two-tailed t test and found in each case to be <0.01.

CRISPR/Cas9 and Immunoblotting. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, 11965-092) supplemented with 10% fetal bovine serum (FBS; Genesee Scientific, 25-514) at 37°C in a 5% CO2 incubator. ATG9A knockout HeLa cells (ATCC) were generated using single-guide RNA 5′-CTGTTGGTGCACGTCGCCGAG-3′ against human ATG9A (ENSG00000198925). CRISPR design tools available at www.atum.bio and crispr.mit.edu were used. The gRNAs were cloned into the pSpCas9(BB)-2A-Puro (PX459) plasmid. PX459 was a gift from Feng Zhang (Addgene, 48139). Cells expressing the gRNA constructs were separated by serial dilution and monoclonal lines were isolated manually under puromycin selection. Knockout efficiency was measured by Western blotting with the following antibodies: ATG9A rabbit monoclonal (Abcam, ab108338), p62/SQSTM1 mouse monoclonal (Abcam, ab56416), Actin (Cell Signaling Technology, 4970S).

String analysis. Proteins showing significant differences in HeLa WT vs ATG9A KO cells were imported into the web-based STRING (v11.5) tool for assembly of functional networks allowing a minimum interaction score cutoff of 0.4 and with the text-mining option for active interaction sources disabled. Interaction networks built in STRING were imported into Cytoscape (v3.9.1) to allow mapping of protein abundance data onto individual nodes. *P*-Values for gene ontology (GO) and pathway enrichments were calculated using a hypergeometric test (statistical background = whole genome) followed by Benjamini–Hochberg correction for multiple hypothesis testing using the STRING enrichment analysis widget.

References

[1] K. G. I. Webber, T. Truong, S. M. Johnston, S. E. Zapata, Y. Liang, J. M. Davis, A. D. Buttars, F. B. Smith, H. E. Jones, A. C. Mahoney, R. H. Carson, A. J. Nwosu, J. L. Heninger, A. V. Liyu, G. P. Nordin, Y. Zhu, R. T. Kelly, *Anal. Chem.* **2022**, *94*, 6017-6025.

[2] S. M. Johnston, K. G. I. Webber, X. Xie, T. Truong, A. Nydegger, H.-J. L. Lin, A. Nwosu, Y. Zhu, R. T. Kelly, *J. Am. Soc. Mass Spectrom.* **2023,** submitted.

[3] Y. Cong, Y. Liang, K. Motamedchaboki, R. Huguet, T. Truong, R. Zhao, Y. Shen, D. Lopez-Ferrer, Y. Zhu, R. T. Kelly, *Anal. Chem.* **2020**, *92*, 2665-2671.

[4] Y. Liang, H. Acor, M. A. McCown, A. J. Nwosu, H. Boekweg, N. B. Axtell, T. Truong, Y. Cong, S. H. Payne, R. T. Kelly, *Anal. Chem.* **2021**, *93*, 1658-1666.

[5] S. M. Williams, A. V. Liyu, C.-F. Tsai, R. J. Moore, D. J. Orton, W. B. Chrisler, M. J. Gaffrey, T. Liu, R. D. Smith, R. T. Kelly, L. Paša-Tolić, Y. Zhu, *Anal. Chem.* **2020**.

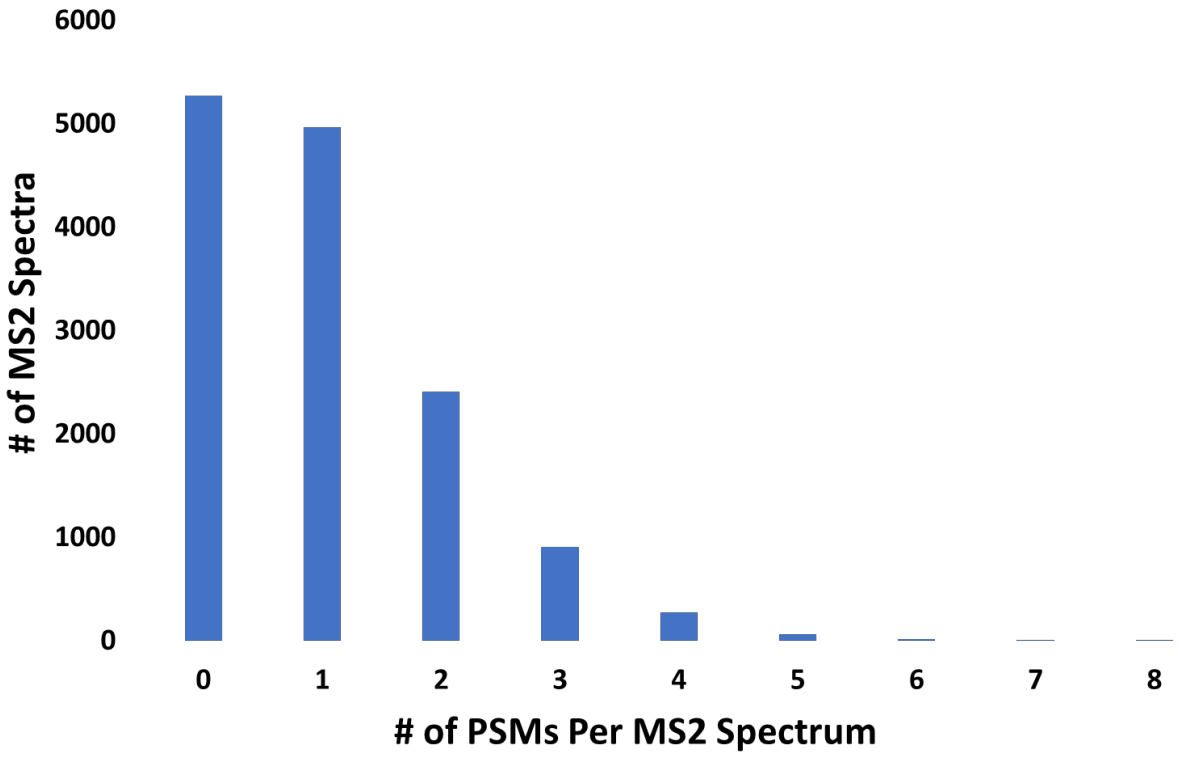

Figure S1. Number of PSMs Per MS2 Spectrum. Data shown are for the analysis of 0.2 ng HeLa digest by WWA using 40 min gradients and 12 Th isolation windows.

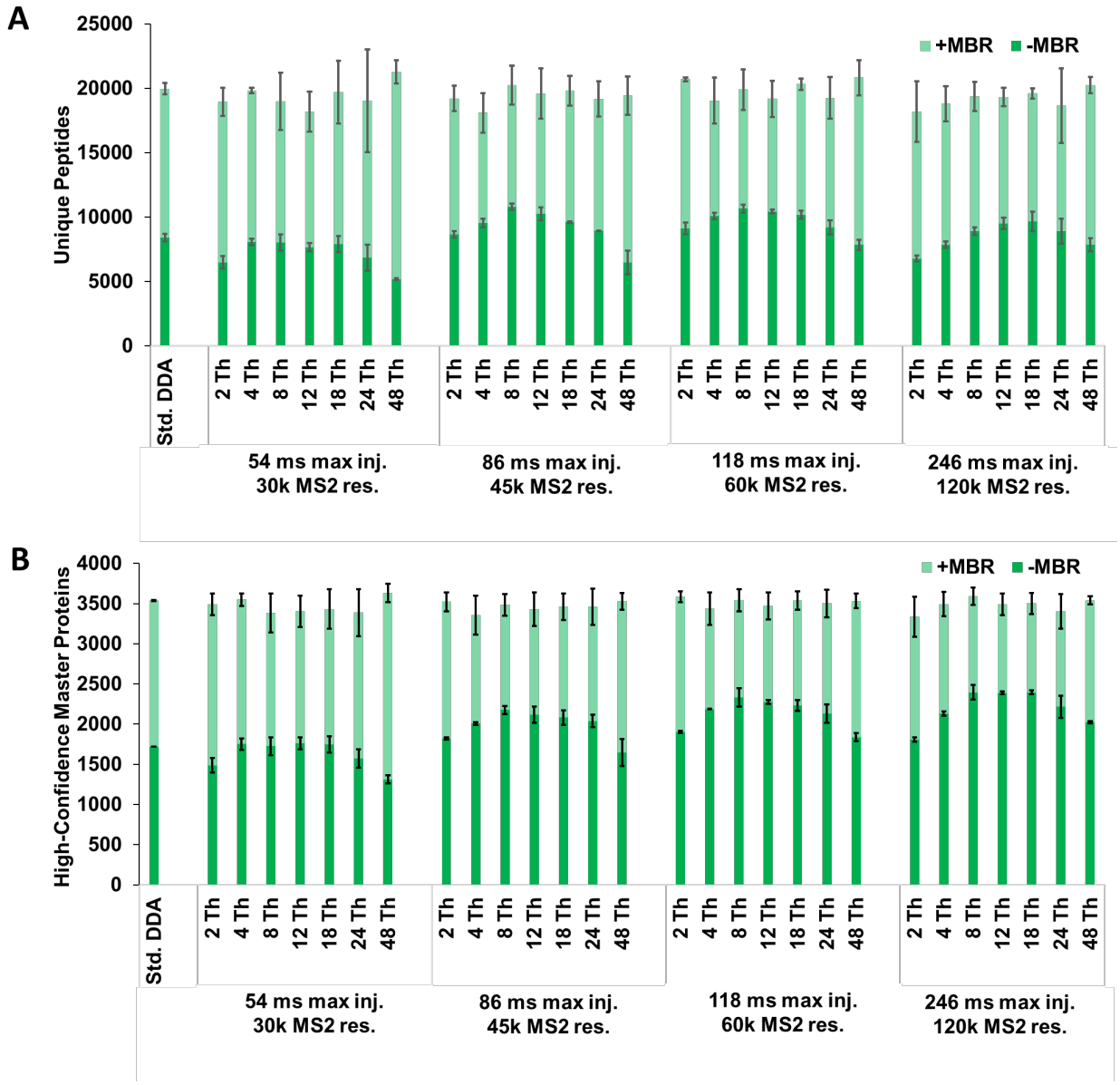

Figure S2. Parameter optimization experiments for 0.2 ng aliquots of HeLa digest with MBR using 40-min gradients. (A) Number of unique peptides identified as a function of MS acquisition settings. (B) Number of identified high-confidence master proteins (1% FDR). All identifications are based on MS2 identification and MBR. Std. DDA conditions are listed in Methods. Error bars indicate ± 1 std. dev., and n=2 for all conditions.

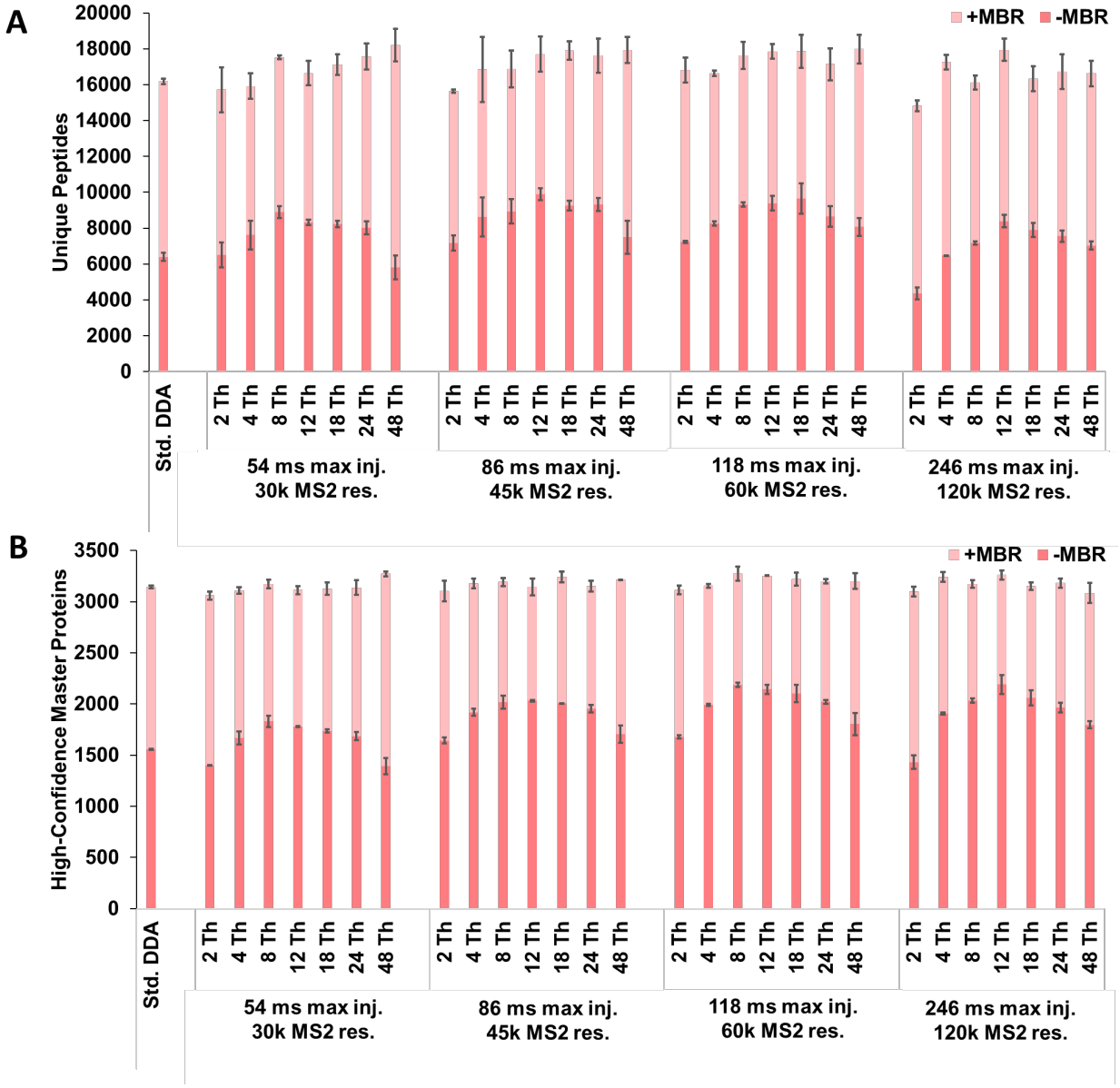

Figure S3. Parameter optimization experiments for 0.2 ng aliquots of HeLa digest with MBR using 20-min gradients. (A) Number of unique peptides identified as a function of MS acquisition settings. (B) Number of high-confidence master proteins (1% FDR). All identifications are based on MS2 identification and MBR. Std. DDA conditions are listed in Methods. Error bars indicate ± 1 std. dev., and n=2 for all conditions.

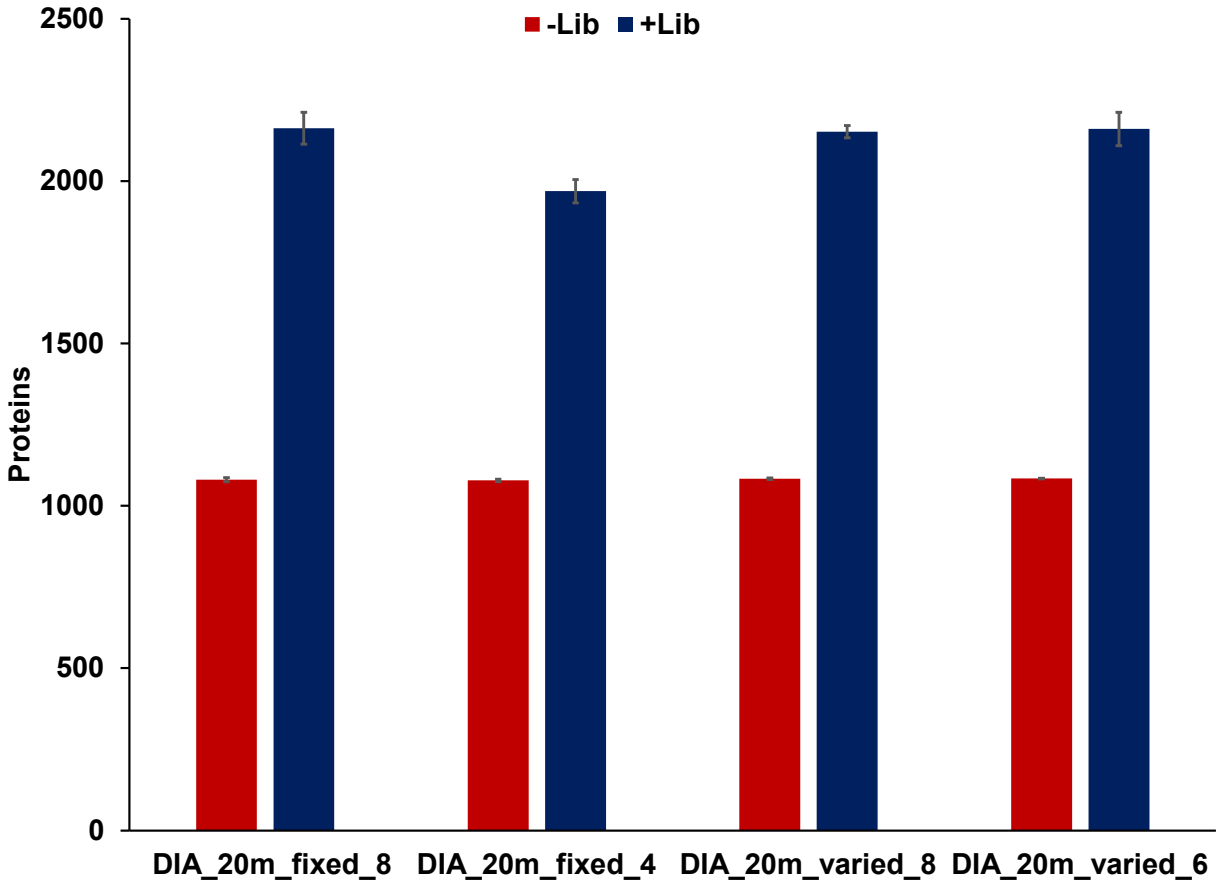

Figure S4. Comparison between different scanning windows for 20-min gradient DIA using 0.2ng aliquots Hela digest. Number of proteins identified as a function of scanning method with and without a spectral library. Scan windows are detailed in Supplemental Table 1.

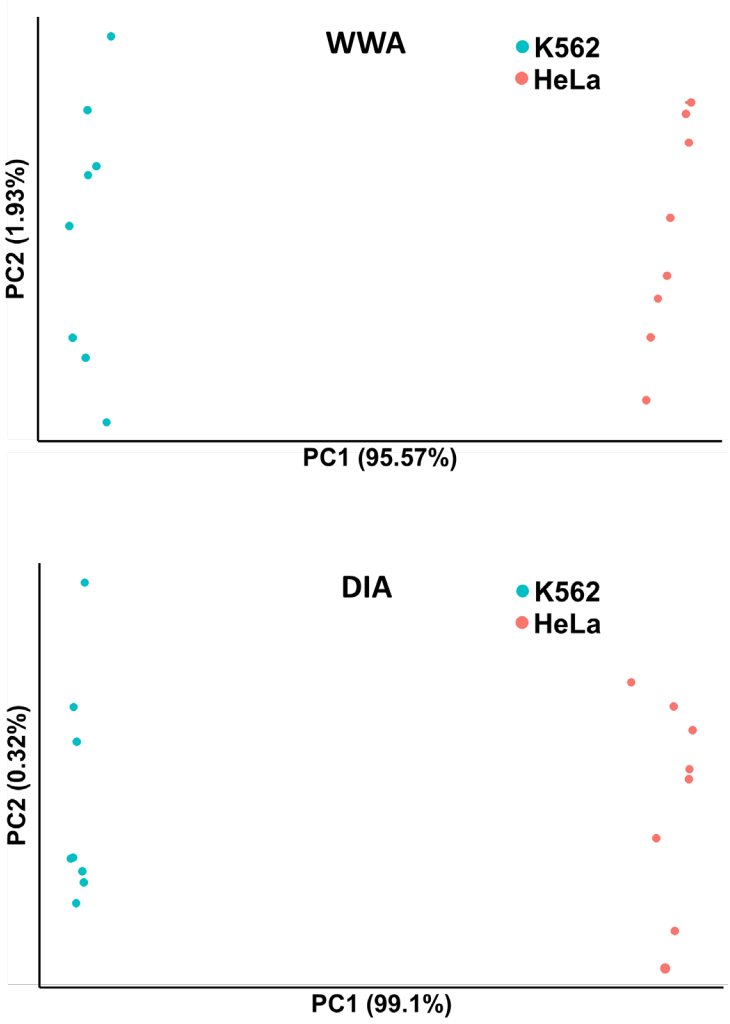

Figure S5. PCA plot of 0.2 ng HeLa and K562 digest for WWA and DIA analyses. Data shown are for 40 min gradients and include match between runs (WWA) and spectral library matching (DIA). After filtering for <33% missing values, intensity values were log2 transformed, median normalized and KNN imputation was applied (k = 5).

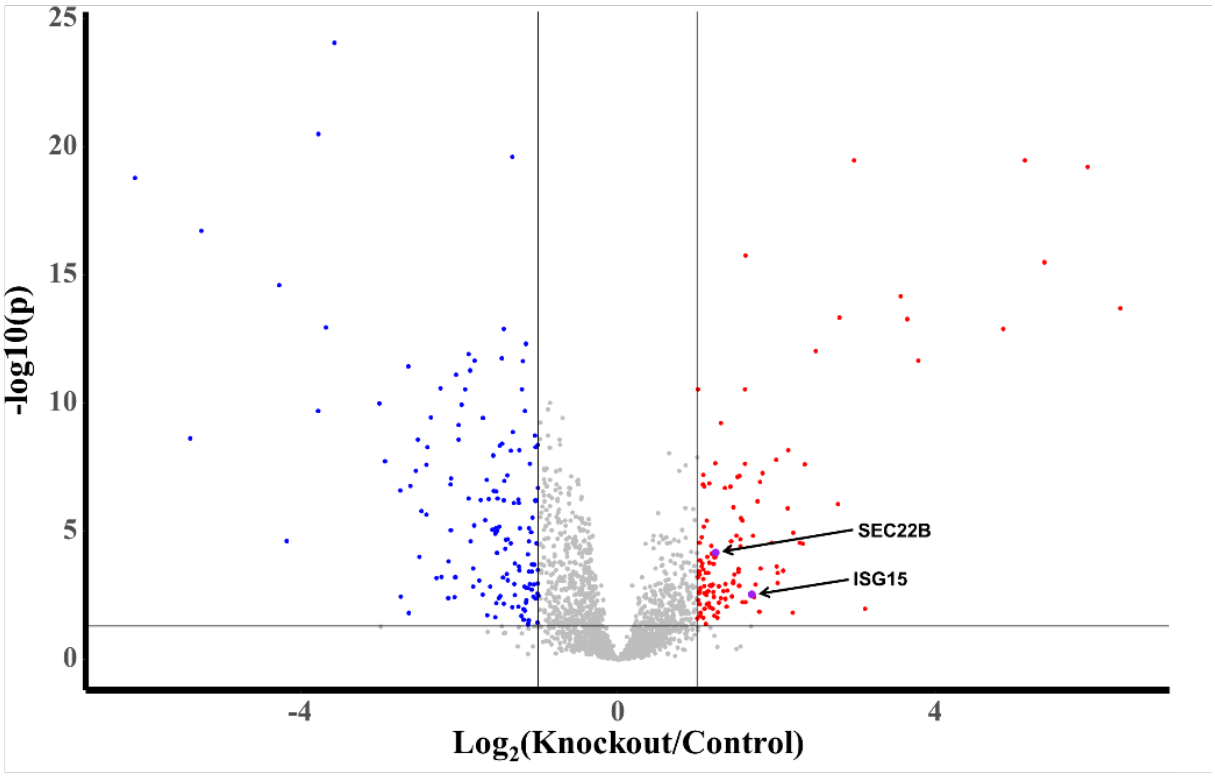

Figure S6. Volcano plot for *atg9a* knockout vs control HeLa cells. Significantly upregulated proteins mentioned in the text (SEC22B and ISG15) are indicated.

Table S1. Detailed scanning windows for DIA experiments.

	40-min gradient		20-min gradient			
	DIA_fixed_8	**DIA_varied_8**	**DIA_fixed_8**	**DIA_fixed_4**	**DIA_varied_8**	**DIA_varied_6**
Scan Windows	400-450	400-420	400-450	400-500	400-420	400-440
	450-500	420-445	450-500	500-600	420-445	440-490
	500-550	445-475	500-550	600-700	445-475	490-550
	550-600	475-510	550-600	700-800	475-510	550-620
	600-650	510-555	600-650		510-555	620-710
	650-700	555-605	650-700		555-605	710-800
	700-750	605-680	700-750		605-680	
	750-800	680-800	750-800		680-800	