Expanding the Global Map of Protein Post-Translational Modifications With Immunoaffinity Enrichment and nDIA Analysis on the Orbitrap Astral Mass Spectrometer

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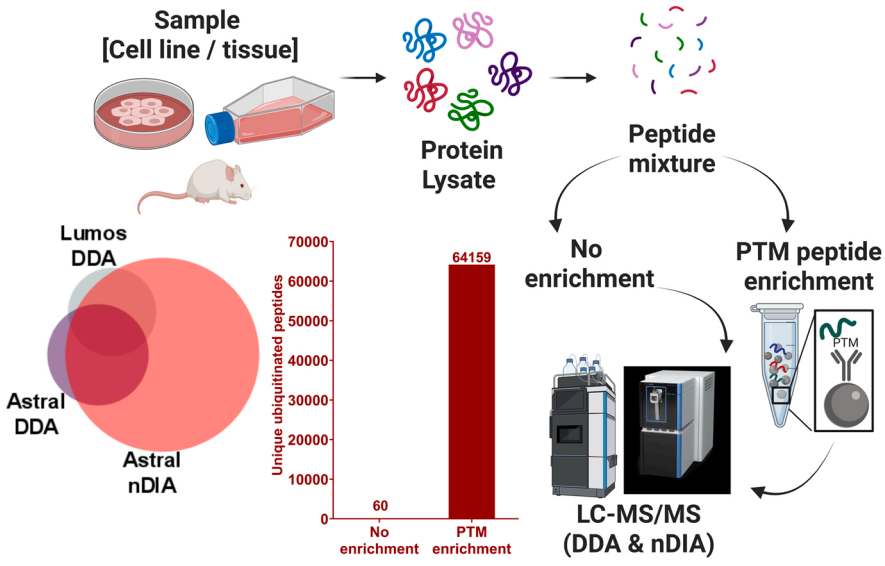
In Brief

nDIA analysis of PTM enriched peptides enables identification of thousands of phosphorylated (STY), ubiquitinated, acetylated, succinylated and mono-methylated peptides from cells and tissue at different input amounts of peptides used for enrichment. In half the acquisition time, nDIA analysis on Orbitrap Astral MS provided much greater depth of coverage for all PTMs compared to DDA analysis. The study highlights the need for enrichment of PTM peptides prior to LC-MS/MS analysis to enable the detection of a relatively minor population of PTM peptides (ranging from 0.005% to 0.1%) compared to unmodified peptides.

Graphical Abstract



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Highlights

- Identified >106k ubiquitination, 64k phosphorylation, 14k acetylation, 5k succinylation, and 1k methylation sites.
- nDIA analysis provided much greater depth of post-translational modification (PTM) coverage compared to data-dependent acquisition analysis.
- Enrichment enabled the detection of low abundance PTM peptides.
- Mass spectrometer data can be used for the generation of PTM specific libraries for data-independent acquisition analysis.
- PTM datasets may be used for the training and development of data-independent acquisition software tools.

Expanding the Global Map of Protein Post-Translational Modifications With Immunoaffinity Enrichment and nDIA Analysis on the Orbitrap Astral Mass Spectrometer

Mukesh Kumar[1,*], Anthony P. Possemato[1], Barry M. Zee[1], Srikanth Subramanian[1], Jian Min Ren[1], Alissa J. Nelson[1], Bin Zhang[1], Sean D. Landry[1], Jeffrey C. Silva[1], Brett Larsen[2], Tonya Pekar Hart[3], Matthew P. Stokes[1], and Sean A. Beausoleil[1,*]

Post-translational modifications (PTMs) contribute greatly to the diversity of the human proteome by affecting protein structure, function, interactions, stability, localization, and more. The study of PTMs is essential to understand various cellular functions, disease mechanisms, and aid in the development of biomarkers and design of therapeutic targets. Owing to their diversity, dynamic nature, and low stoichiometry compared to unmodified proteome counterparts, the analysis of PTMs remains challenging. In this study, immunoaffinity enrichment of PTM peptides was combined with analysis using data dependent acquisition and narrow window Data Independent Acquisition (nDIA) on the Orbitrap Astral Mass Spectrometer (Orbitrap Astral MS) as well as comparative analysis using the Orbitrap Fusion Lumos Mass Spectrometer (Orbitrap Fusion Lumos MS) for ubiquitination, phosphorylation, acetylation, succinylation, and methylation. Human cell lines and mouse tissue samples at various input peptide amounts were immunoenriched and mass spectrometry data was acquired on both instruments to assess depth of coverage and number of novel sites identified. The study identified a total of 106152 unique ubiquitin sites, 64397 phosphorylation sites (43721 phosphoserine, 8414 phosphothreonine and 12262 phosphotyrosine), 14245 acetylation, 5272 succinylation and 1461 mono-methylation sites. In half the acquisition time, nDIA analysis of immuno-enriched samples on Orbitrap Astral MS provided much greater depth of coverage for all PTMs compared to data dependent acquisition analysis on Orbitrap Fusion Lumos MS, with up to 33-fold more PTM peptides identified and quantified. Overall, the data presented in this study demonstrates the need for enrichment for PTM detection and the utility of combining antibody-based peptide capture and nDIA analysis on the Orbitrap Astral MS as powerful tools for discovery and profiling of protein post-translational modifications in cells and tissues.

The complexity of the human proteome far surpasses the approximately 20,000 genes that encode it, primarily due to the essential creation of diverse proteoforms. Starting with a limited genomic blueprint, the cellular machinery employs alternative splicing to produce multiple mRNA transcripts from a single gene, each potentially yielding a distinct protein isoform with a unique amino acid sequence. This inherent variability is then massively amplified by a vast array of post-translational modifications (PTMs), such as phosphorylation, acylation, ubiquitination, and methylation, which derivatize these isoforms, altering their structure, localization, interactions, and ultimately their biological roles. The combinatorial effect of alternative splicing and the multitude of possible PTMs results in hundreds of thousands to potentially millions of unique proteoforms within cells and tissues, enabling a dynamic and intricate network of molecular machines that allows for fine-tuned regulation of biological processes and precise responses to a wide range of internal and external cues, far exceeding the information that is directly encoded in the genome (1, 2).

Liquid Chromatography Electrospray Ionization Tandem Mass Spectromtry (LC-ESI-MS/MS) are crucial for addressing the challenge of analyzing the proteome, carrying out the identification of endogenously expressed proteins and the detection and localization of a diverse array of PTMs that exist across a wide dynamic range, including those at very low abundance (3–7). To address the challenge of low abundance PTMs, specialized enrichment strategies targeting specific modifications (*e.g.,* Immobilized Metal Affinity Chromatography (IMAC)-based phosphopeptide enrichment, immunoaffinity-based PTM peptide capture) are often employed prior to LC-MS/MS analysis to increase their detectability (8, 9). Furthermore, advancements in sample

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preparation methods that are amenable to automation, robust LC interfaces, mass spectrometry sensitivity as well as data analysis algorithms are continuously improving the ability to identify and quantify more of the proteome, including transient and sub-stoichiometric PTMs within the complex proteomic landscape (10–15).

Immunoaffinity enrichment methods such as PTMScan have aided in overcoming the challenges of monitoring low abundance post-translationally modified peptides and have been used in hundreds of previous studies for the enrichment of PTM peptides prior to LC-MS/MS analysis (9, 16–19). Instead of relying solely on mass spectrometry to detect a minor fraction of PTM peptides or potentially rare, modified peptides within a complex digest, PTMScan utilizes highly specific antibodies that selectively bind to peptides containing a particular PTM or a conserved sequence motif surrounding a modification site (20–23). These unique antibodies are conjugated to beads, allowing for efficient pull-down and isolation of the modified peptides of interest, effectively increasing their concentration and detectability for downstream LC-MS/MS analysis. This targeted enrichment dramatically reduces the complexity of the sample presented to the mass spectrometer, enabling the identification and quantification of PTMs that might be present at very low stoichiometry and would otherwise be masked by the overwhelming presence of more abundant unmodified peptides in global proteomic approaches.

Traditional data dependent acquisition (DDA) methods of bottom-up proteomics face inherent challenges in comprehensively identifying and quantifying low abundance proteins and low-level post-translational modifications. DDA typically selects the most abundant peptide ions in each mass spectrum for fragmentation and analysis, leading to a sampling bias where less abundant species are often missed. This stochastic selection limits the reproducibility and depth of proteome coverage, particularly for PTMs that are sub-stoichiometric and thus underrepresented. In contrast, next-generation proteomics leverages data independent acquisition (DIA) methods on more advanced mass spectrometers with greater sensitivity and acquisition speed as well as high mass accuracy and resolution to achieve a more complete picture of the sample being analyzed (24–28). DIA systematically fragments all ions within defined mass-to-charge windows, generating a comprehensive digital map of the biological specimen. This unbiased approach, coupled with the enhanced sensitivity and rapid scanning capabilities of modern instruments, allows for more consistent and deeper proteome coverage, significantly improving the detection and quantification of low abundance proteins and challenging PTMs, even those present at very low levels across a wide dynamic range. The complexity of the resulting DIA data requires sophisticated bioinformatics tools for deconvolution and analysis, but it offers a more thorough perspective of the proteome compared to DDA.

The Thermo Fisher Scientific Orbitrap Astral MS represents a significant leap forward in mass spectrometry, enabling superior depth and coverage of the proteome in nDIA experiments compared to earlier generation Orbitrap instruments through several key innovations (29, 30). Firstly, it incorporates a novel mass analyzer using a compact, high-speed design with a 30-m asymmetric ion track, allowing significantly faster acquisition rates (up to 200 Hz) of high-resolution accurate mass MS/MS spectra with enhanced sensitivity. Secondly, the synchronized parallel acquisition is crucial for nDIA, allowing high dynamic range, high resolution full scan (MS1) acquisition in the Orbitrap analyzer and sensitive high-resolution accurate mass MS/MS data across the entire mass range within the nDIA windows in the Astral analyzer. Thirdly, the improved sensitivity of the Astral analyzer allows for the detection and quantification of lower abundance peptides such as those containing PTMs. This faster acquisition speed, enhanced sensitivity, and parallel analyzer operation on the Orbitrap Astral MS overcome the limitations of earlier Orbitrap instruments in nDIA, providing a much more detailed and quantitative view of the proteome (28–30).

This study illustrates how the combination of immunoaffinity enrichment with Orbitrap Astral MS high sensitivity, speed, and nDIA capabilities represents a leap forward in the ability to comprehensively profile PTMs in cells and tissues. Antibody based affinity enrichment overcomes the challenge of low abundance PTMs by increasing their relative concentration, while the Orbitrap Astral MS nDIA platform provides the depth, sensitivity, and quantitative rigor needed for their comprehensive identification and quantification. nDIA analysis of immuno-enriched samples compared to unenriched samples identified 1069-fold more ubiquitinated peptides (60 *versus* 64159), 219-fold more phosphotyrosine peptides (43 *versus* 9428), 88-fold more acetylated peptides (127 *versus* 11211), 125-fold more succinylated peptides (46 *versus* 5781) and 19-fold more mono-methylated peptides (74 *versus* 1411). The number of PTM modified peptides identified on the Orbitrap Astral MS using nDIA significantly outpaced the depth of coverage achieved on older generation instruments. For low abundance PTMs like phosphotyrosine, nDIA-Hybrid Search (nDIA-HBS) analysis on Orbitrap Astral MS identified up to 33-fold more modified peptides compared to DDA analysis on Thermo Fisher Scientific Orbitrap Fusion Lumos MS. For high complexity PTMs like ubiquitination, nDIA-HBS on Orbitrap Astral MS identified up to 28-fold more modified peptides compared to DDA on an Orbitrap Fusion Lumos MS.

Integrating this detailed PTM information with global proteome profiling data generated on the same advanced instrument will provide a more complete understanding of the dynamic proteome and the intricate regulatory mechanisms governing cellular processes in response to various stimuli.

EXPERIMENTAL PROCEDURES

Ethical Statement

The protocol and procedures involving mice were approved by Cell Signaling Technology's Institutional Animal Care and Use Committee (IACUC), under approval number C112624-04, ensuring compliance with ethical standards throughout the study.

Cell Culture

HCT116 human colorectal carcinoma cells (ATCC CCL-247) were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and were passaged when reaching 80 to 90% confluence using a 0.25% trypsin-EDTA solution. For proteasome inhibition studies, HCT116 cells were seeded in appropriate culture vessels at a density of 5×10^5 cells/ml and allowed to adhere for 24 h under standard culture conditions. MG132 (Z-Leu-Leu-Leu-al; Sigma-Aldrich # 474790) was dissolved in DMSO to prepare a 10 mM stock solution, which was stored in aliquots at −20 °C. Twenty-four hours after seeding, the culture medium was replaced with fresh complete medium containing 10 μM MG132 (final DMSO concentration <0.1%). Control cultures received an equivalent volume of DMSO. Cells were incubated for 18 h at 37 °C, 5% CO_2, under standard culture conditions.

Jurkat T cells (ATCC TIB-152) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were passaged every 2 to 3 days to maintain a density between 0.5 and 1.5×10^6 cells/ml. For pervanadate treatment, cells in logarithmic growth phase were harvested by centrifugation at 300*g* for 5 min at room temperature and resuspended in fresh complete medium at a density of 1×10^6 cells/ml. A 20 mM sodium pervanadate stock solution was freshly prepared immediately before use by combining 1 ml 100 mM sodium orthovanadate with 3.4 ml of deionized H_2O and 12 μl hydrogen peroxide (30% solution) and incubated at room temperature for 10 min. Next, 100 μl of 1 M HEPES was added, and the solution was gently mixed. The solution was added to the cell suspension to achieve a final concentration of 1 mM pervanadate. Cells were incubated with pervanadate for 30 min at 37 °C, 5% CO_2, under standard culture conditions.

Following treatment, cells were washed three times with ice-cold PBS, pelleted by centrifugation at 300*g* for 5 min at 4 °C to remove residual impurities, and flash frozen in dry ice/ethanol slurry that was prepared by gradually adding 95% laboratory-grade ethanol to crushed dry ice until a semi-fluid mixture was achieved. After the final centrifugation, all supernatant was removed from the cell pellets, and the tubes containing cell pellets were immediately immersed in the dry ice/ethanol slurry for 10 min, ensuring tube caps remained above the slurry level. Frozen cell pellets were stored at −80 °C until lysis.

Western Blot Analysis

Media was aspirated from culture plates after incubation. Jurkat cells untreated or treated with pervanadate and HCT116 cells untreated or treated with MG132 were lysed directly in the culture plates by addition of 1 × SDS sample DTT. Cell lysates were scraped from the plates and collected into 1.5 ml microcentrifuge tubes. Samples were sonicated three times for 5 s on ice, with 10 s rest between each using a probe sonicator at 30% power (Thermo Fisher Scientific) to disrupt cellular debris and reduce viscosity, then heated at 95 °C for 5 min to ensure complete protein denaturation and reduction. Equal volumes of lysates were loaded onto 4 to 20% gradient polyacrylamide gels (Bio-Rad) and separated by SDS-PAGE at 200V for 40 min. Proteins were transferred to nitrocellulose membranes (Bio-Rad) using the Bio-Rad Trans-Blot Turbo System with the mixed molecular weight transfer protocol (2.5A, 25V) for 7 min. Transfer efficiency was verified by Ponceau S staining. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), shaking for 1 h at room temperature. Primary antibodies, Phospho-Tyrosine (P-Tyr-1000) MultiMab Rabbit mAb mix (CST #8954), Ubiquitin (E4I2J) Rabbit mAb (CST #43124), were diluted 1:1000 in 5% bovine serum albumin in TBS-T and incubated overnight with gentle rocking at 4 °C. After three washes with TBS-T (5 min each), membranes were incubated with horseradish peroxidase-conjugated secondary antibody, Anti-rabbit IgG, horseradish peroxidase -linked antibody (CST #7074) at a 1:2000 dilution in 5% milk/TBS-T, shaking for 1 h at room temperature. Following three additional TBS-T washes, proteins were detected using enhanced chemiluminescence reagent SignalFire ECL Reagent (CST #6883) and visualized with a ChemiDoc imaging system (Bio-Rad).

Cell/Tissue Lysis, Protein Extraction and Digestion

Human cultured cells and mouse tissue samples were resuspended in 8M urea, 20 mM HEPES pH 8.5, with 2X Phosphatase Inhibitor Cocktail (CST #5870). Resuspended samples were homogenized with a polytron probe homogenizer and sonicated 2 times for 30 s at 8 W output power using a microtip with samples placed on ice between each pulse, followed by reduction using DTT (5 mM, 30 min) and alkylation using IAA (10 mM, 30 min). Samples were diluted to 2 M urea with 20 mM HEPES pH 8.5 with 1 mM $CaCl_2$ and digested overnight at 37 °C with LysC (Wako-Chem). Samples were further diluted to 1 M urea with 20 mM HEPES pH 8.5 with 1 mM $CaCl_2$ and digested for 6 h with Trypsin (Pierce #90058). Samples were acidified with 1:10 volume of 20% TFA, and peptides were purified over Waters Sep-Pak C18 columns (Waters #WAT023590). An aliquot of the purified peptides was used to determine concentration using a Quantitative Colorimetric Peptide Assay kit (Pierce #23275) and the remainder of the peptide was lyophilized and stored at −80 °C.

Immunoaffinity Enrichment

Immunoaffinity enrichment was carried out using Cell Signaling Technology post-translational modification scan high specificity (PTMScan HS) kits. The following PTMScan HS kits were used: Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit (CST #59322); Phospho-Tyrosine (P-Tyr-1000) Kit (CST #38572); Acetyl-Lysine Motif (Ac-K) Kit (CST # 46784); Succinyl-Lysine Motif (Succ-K) Kit (CST # 60724); Mono-Methyl Arginine Motif (mme-RG) Kit (CST # 98567), for the enrichment of ubiquitinated, phosphotyrosine, acetylated, succinylated and mono-methylated peptides. The enrichment was performed according to the protocol provided by the manufacturer. Different input amounts of peptides (0.1, 0.3, 1 and 3 mg) were resuspended in 1.5 ml of 1X HS Immuno Affinity Purification (IAP) bind buffer. Resuspended peptides were sonicated briefly in a sonicator bath to ensure complete solubilization. The appropriate amount of antibody bead slurry was then added to the peptides followed by end-over-end rotation for 2 h at 4 °C. After binding, the supernatant was transferred to a new Eppendorf tube to allow for sequential enrichment. The antibody bead resin was washed 4 times with 1 ml HS IAP wash buffer and two times with 1 ml water, and peptides were eluted with 50 μl (2x) of 0.15% TFA. Eluted peptides were desalted using C18 tips. The same protocol was repeated for all IAP experiments.

Immobilized Metal Affinity Chromatography (IMAC) Enrichment

Different peptide inputs were used for IMAC enrichment (0.1 mg, 0.25 mg and 0.5 mg). Peptides were resuspended in 80% acetonitrile

(MeCN) in 0.1% TFA and transferred to a 96 well plate for IMAC enrichment on the AssayMap Bravo (Agilent, CA). Using the provided Phosphopeptide Enrichment application on the Bravo, phosphopeptides were enriched using Fe-NTA cartridges (Agilent, #G5496-60085). IMAC enriched peptides were dried using vaccumm centrifuge. The dried peptides were resuspended in 0.1% TFA and desalted following the provided Peptide Cleanup 2.0 program on AssayMap Bravo, using 5 µl C18 cartridges (Agilent #5190-6532). Clean peptides were dried and resuspended for LC-MS/MS analysis.

LC-MS/MS Analysis

Prior to LC-MS/MS analysis, peptides were reconstituted in 0.1% trifluoroacetic acid (TFA) in 5% MeCN. Peptide mixtures were analyzed by nano LC coupled to either the Orbitrap Astral MS or Orbitrap Fusion Lumos MS. On the Orbitrap Astral MS a Thermo Fisher Scientific Vanquish Neo UHPLC system was used to load samples directly onto an IonOptics C18 Aurora Ultimate column (25 cm × 75 µm ID, 1.7 µm (AUR3-25075C18-TS)). For nDIA analysis a 45-min gradient using Mobile phase A (5% MeCN in 0.1% FA) starting at 2% A and mixing to 30% Mobile phase B (93% MeCN in 0.1% FA) was used at a flowrate of 350 nl/min. For DDA analysis on the Orbitrap Astral MS a 90-min gradient using the same buffer start and end points along with the same flowrate was used. For DDA analysis on the Orbitrap Fusion Lumos MS a Thermo Fisher Scientific EASY-nLC 1200 system was used combined with a 50 cm analytical column packed in-house with 1.8 µm C18 (Sepax, # 101181-0000). The same solvents were used as described above to run a 90-min gradient with the same start and endpoints.

Mass Spec Parameters

Orbitrap Astral MS DDA Parameters—MS1 spectra were collected in the Orbitrap every 1.2 s at a resolving power of 120,000 at *m/z* 200 over *m/z* 375 to 1500 with a standard automatic gain control (AGC) target and a maximum injection time of 25 ms. The MIPS filter was applied with Peptide mode and "Relax Restrictions when too few Precursors are Found" set to True. Precursors were filtered to charges states 2 to 6. A Dynamic Exclusion filter was applied with 25 s duration and 10 ppm low and high mass tolerance and exclude isotopes set to True. An intensity filter was applied with a minimum precursor intensity of 5000 required for selection. MS2 scans were collected in the Astral mass analyzer with an isolation window of 1.6 *m/z*, normalized collision energy of 27, a scan range of 110 to 2000 *m/z*, a standard AGC target of 100% (1e4 charges), and a maximum injection time of 15 ms.

Orbitrap Astral MS nDIA Parameters—MS1 spectra were collected in the Orbitrap every 0.6 s at a resolving power of 240,000 at *m/z* 200 over *m/z* 380 to 980. The MS1 normalized AGC target was set to 200% with a maximum injection time of 5 ms. DIA MS2 scans were acquired in the Astral analyzer with the Precursor Mass Range set to 380 to 980 *m/z* range with the DIA window set to Auto and the Isolation Window set to 2 *m/z.* The Window Placement Optimization was set to off, and the DIA window Mode was set to *m/z* range. The HCD Collision Energy was set to 25% with the Scan Range in the Astral analyzer set to 150 to 2000 *m/z* with a Standard AGC Target and an Ion Injection Time set to 3.5 ms.

Orbitrap Fusion Lumos MS DDA Parameters—MS1 spectra were collected at a resolving power of 120,000 at m/z 200, over a range from *m/z* 300 to 1500 with a standard AGC target and a maximum injection time of 50 ms. The MIPS filter was applied with Peptide mode selected. Precursors were filtered to charge states of 2 to 7 and a Dynamic Exclusion filter was applied with 30 s duration and 10 ppm low and high mass tolerance. MS2 scans were collected in the Orbitrap analyzer at a resolution of 30,000 at *m/z* 200 with a defined first mass of 110 *m/z* and an isolation window of 1.6 *m/z.* A fixed Collision Energy of 27 was used with a Standard AGC and a maximum injection time of 50 ms.

Data Processing

The *Homo sapiens* (Human) and *Mus musculus* (mouse) proteome databases were downloaded from Uniprot.org. The *homo sapiens* database was downloaded on March, 13, 2024 and contains 42,505 entries including reviewed, canonical, and isoform sequences. The *M. musculus* database was downloaded on March, 09, 2024 and contains 25,572 entries, including reviewed, canonical, and isoform sequences. Decoys were generated by Spectronaut and MSFragger for nDIA and DDA searches, respectively using the default methods of the respective search engines.

nDIA mass spectra were processed using Spectronaut v20.4 (Biognosys AG, Switzerland) (31). DirectDIA module was used for searching using the default parameters with the following changes: Fixed modification was set to Carbamidomethyl (C) and specific variable modifications were added for different enrichments: GlyGly (K) for ubiquitination, Acetyl (K) for Acetylation, Methyl (R) for Mono-Methylation, Phospho (STY) for phosphorylation, and Succinyl (K) for Succinylation. Minimum peptide length 6, Missed Cleavage three and Max Variable Modification was set to 5. For Identification, the mass tolerance was selected as Dynamic for both calibration and main search and correction factor of one for both MS1 and MS2, PSM, Peptide and Protein Group FDR were set to −0.01, PTM Localization Filter was Checked with Min Localization Threshold of 0.75. In Result Filters, Best N Fragments per peptide Max 20 and Min 3. Peptide charge (Max 4, and Min 2). For nDIA-HBS analysis, all parameters remain the same as nDIA Library Free Search (nDIA-LFS) except for checking the box Hybrid (DDA + DIA) Library in the workflow section. After searching, the standard peptide report as well as a PTM site report was exported and further processed. To determine the number of modified peptides, data were filtered by "EG.ModifiedSequence", and then sorted by "PEP.RunEvidenceCount" (modified peptides were only retained with minimum of one for PEP.RunEvidenceCount). Next, among the retained modified peptides, duplicate entries representing multiple precursors were deleted and only unique modified sequences were retained. Next, the retained unique modified sequence was sorted according to "EG.TotalQuantity" and any peptide with a zero value was omitted from the list of identified peptides.

DDA data was processed using MSFragger v4.1 (32). LFQ phospho workflow was used for searching IMAC and phosphotyrosine PTMScan data. LFQ ubiquitin workflow was used for ubiquitin search. For acetylation, succinylation and methylation search the monoisotopic mass of 42.010565 Da on lysine, 100.016044 Da on lysine, and 14.015650 Da on the arginine residue was added, respectively. For phosphorylation searches, up to two missed cleavages were allowed, and for ubiquitination, acetylation, succinylation and methylation up to three missed cleavages were allowed. Precursor mass tolerance of ±10 ppm and fragment mass tolerance of ±20 ppm was used for all searches and 1% of FDR was applied at both peptide and protein levels. As with DIA, search specific modifications were added. For the analysis of modified peptides, the "combined_modified_peptide.tsv" file was used. First the peptide was filtered based on "modified sequence" column containing the specific modifications. Next the modified peptides were sorted according to "Intensity" and peptides with zero intensity not included in the analysis.

Experimental Design and Statistical Rationale

The study aimed to evaluate the combined use of immuno affinity enrichment, and the nDIA analysis on the Orbitrap Astral MS for the identification and quantification of PTM peptides. The experiment was designed to enrich PTM peptides from four different amounts of input peptide (0.1 mg, 0.3 mg, 1.0 mg and 3.0 mg) and samples from each input amount were analyzed by DDA and nDIA analysis on Orbitrap Astral MS as well as by DDA on Orbitrap Fusion Lumos MS. Analyses of different PTMs including phosphorylation, ubiquitination, acetylation, succinylation and mono-methylation are presented in the study. The need for enrichment was tested by running unenriched samples in parallel on the Orbitrap Astral MS with nDIA and searching for each PTM.

RESULTS

Overview of the Workflow Used for the Enrichment and Analysis of PTM Peptides

To evaluate the efficacy of immunoaffinity enrichment and the Orbitrap Astral MS for the analysis of PTMs, proteins from untreated and treated human cell lines (HCT116, Jurkat) and mouse liver tissue were digested to peptides and enriched with the appropriate PTM antibody (ubiquitin, tyrosine phosphorylation, acetylation, methylation, or succinylation) or Fe-IMAC beads (serine/threonine phosphorylation). Enriched peptides were analyzed using DDA on the Orbitrap Fusion Lumos MS and by DDA and nDIA on the Orbitrap Astral MS. Acquired mass spectrometric DDA and nDIA data were processed using MSFragger and Spectronaut, respectively. A schematic of the workflow used in this study is shown in Figure 1.

Immunoaffinity Enrichment and Analysis of Ubiquitinated Peptides

To evaluate the performance of the Orbitrap Astral MS for the analysis of ubiquitination, cellular proteins from the human colorectal carcinoma HCT116 cell line were extracted and digested to peptides. Different amounts of total peptide input (0.1 mg, 0.3 mg, 1 mg and 3 mg) were used for immuno-enrichment of ubiquitin remnant-containing peptides using the PTMScan HS Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit (CST # 59322). A ubiquitin K-ε-GG containing peptide retains the diglycine remnant from ubiquitin after proteolytic digestion by trypsin (33). This remnant, a modified lysine residue (K-ε-GG), serves as a signature of ubiquitination, allowing identification of specific ubiquitinated sites on proteins. Enriched peptides were analyzed by DDA on Orbitrap Fusion Lumos MS and by DDA and nDIA on Orbitrap Astral MS. From the 3 mg input amount, DDA analysis on Orbitrap Fusion Lumos MS and Orbitrap Astral MS identified 8389 and 10788 unique ubiquitinated peptides (K-ε-GG), compared to 31928 and 49194 unique ubiquitinated peptides (K-ε-GG) by Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS analysis respectively (Fig. 2*A*). These unique peptides mapped to 2791, 3566, 6184 and 7009 unique protein groups, respectively. In half the mass spec acquisition time (45-min *versus* 90-min), nDIA analysis on Orbitrap Astral MS identified up to 4.9- and 3.6-fold more unique ubiquitinated peptides compared to DDA analysis on Orbitrap Fusion Lumos MS and DDA analysis on Orbitrap Astral MS, respectively. From the lowest input amount (0.1 mg) 222, 1322, 2236, and 6594 unique ubiquitinated peptides were identified using Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS, respectively. The Orbitrap Astral MS nDIA-HBS analysis identified up to 29.0-, 4.0- and 3.0-fold more ubiquitinated peptide compared to Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA and Orbitrap Astral MS nDIA-LFS analysis, respectively (Fig. 2*A*). When using the HBS option in Spectronaut, nDIA and DDA data from all input amounts were added in the search as library extension runs. When comparing with nDIA-LFS, the benefit of nDIA-HBS was more pronounced at the lower input amount and the benefit was minimized at the highest input amount as shown by the 3.3-, 2.4-, 1.8- and 1.1-fold increase in the identification of ubiquitinated peptides from 0.1 mg, 0.3 mg, 1 mg and 3 mg, respectively. nDIA analysis on Orbitrap Astral MS yielded >25000 additional unique peptides when compared with DDA analysis on Orbitrap Fusion Lumos MS and Orbitrap Astral MS (Fig. 2*B*). To further validate and test the extent of the number of ubiquitinated peptides that can be identified by Orbitrap Astral MS, HCT116 cells were treated with the proteasome inhibitor MG132 to maximize the presence of ubiquitinated proteins in the sample. Western blotting of whole cell lysates from untreated and MG132 treated cells using anti-ubiquitin antibody confirmed the increased level of ubiquitinated proteins in MG132 treated cells compared to untreated cells (Fig. 2*C*).

1 mg and 3 mg input peptide from untreated and MG132 treated HCT116 cell line samples were used for immunoaffinity enrichment for ubiquitinated peptides and analyzed by DDA and nDIA analysis on Orbitrap Astral MS. From the 3 mg input amount more than 80000 unique ubiquitinated peptides were identified from MG132 treated cells, two-fold more than in untreated cells (Fig. 2*D*). When comparing the number of ubiquitinated peptides identified by DDA and nDIA analysis, a total of 27871 were identified in both DDA and nDIA runs, with 3200 peptides identified uniquely in DDA, and 41406 peptides uniquely identified in nDIA (Fig. 2*E*). These numbers were achieved using a shorter, 45-min gradient for nDIA and a longer, 90-min gradient for DDA. Quantification of PTM sites confirmed the increased level of ubiquitination in MG132 treated cells compared to untreated cells (Fig. 2*F*). Out of 106152 unique ubiquitin sites identified in this study, a total of 60815 sites matched to known sites when mapped against the PhosphoSitePlus database (34) and a total of 45337 sites are uniquely identified in this study (Fig. 2*G*). Identification and quantification of all ubiquitinated peptides by DDA and nDIA-LFS and nDIA-HBS are provided in Data S1, *A*, *B1*, and *B2* respectively.

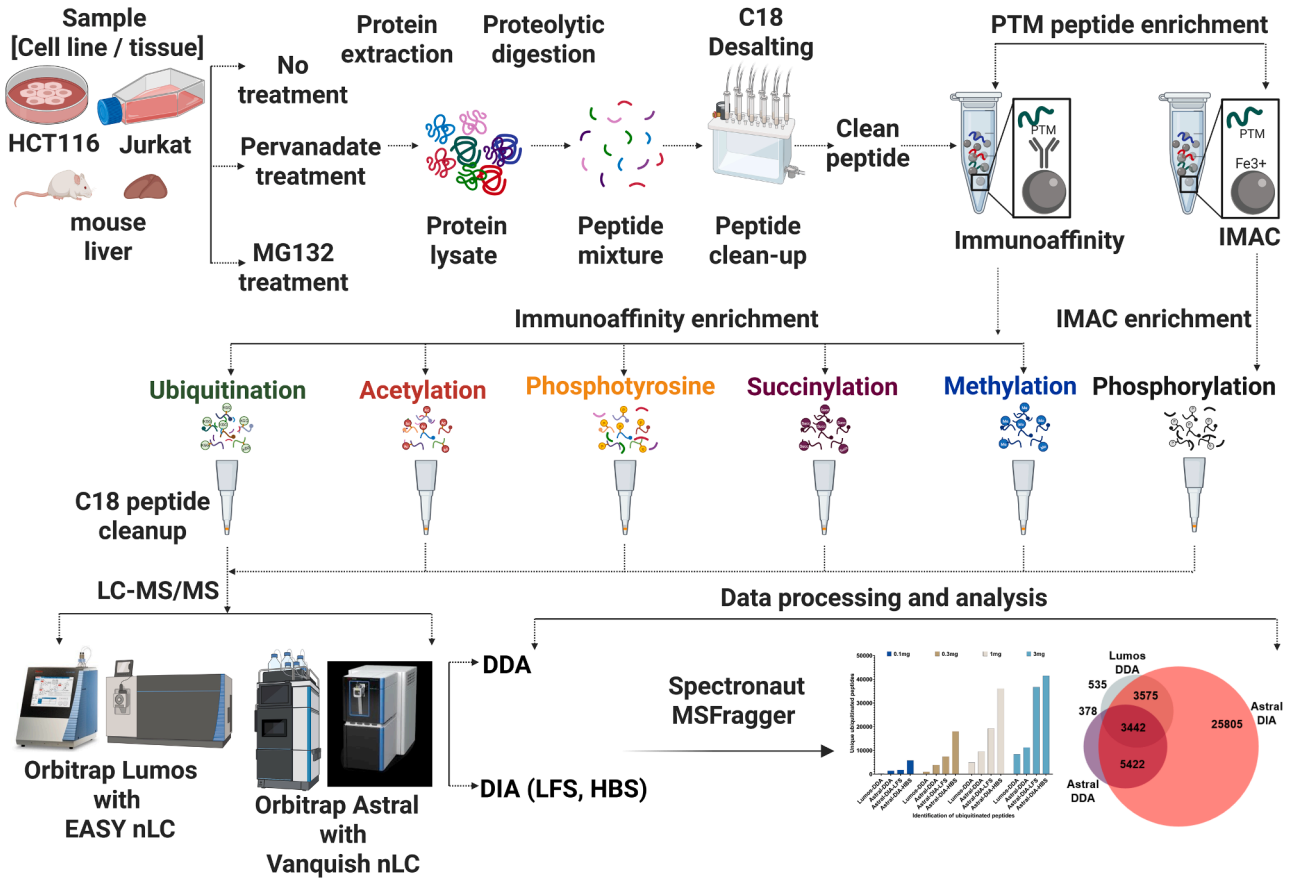

FIG. 1. **Schematic of the workflow used for the enrichment and analysis of PTM peptides.** Total proteins were extracted from untreated or MG132 treated human colorectal carcinoma (HCT116) cells, untreated or pervanadate treated T-cell acute lymphoblastic leukemia (Jurkat) cells, and from untreated mouse liver tissue. Protein extracts were reduced, alkylated and digested to peptides using trypsin and Lys-C. Digested peptides were desalted using Sep-Pak C18 cartridges. Peptides were enriched with Fe-IMAC for serine/threonine/tyrosine phosphopeptides. PTMScan HS kits were used to enrich lysine ubiquitin remnant (K-ε-GG), lysine-acetylation, tyrosine-phosphorylation, lysine-succinylation, and mono-methylation of arginine. Prior to LC-MS/MS analysis, enriched peptides were desalted using C18 tips, loaded onto a reverse phase C18 column, and separated at a nanoflow rate using EASY-nLC system or Vanquish Neo UHPLC system connected to Orbitrap Fusion Lumos MS and Orbitrap Astral MS, respectively. Data was acquired using DDA and nDIA, and processing of the data was carried out using Spectronaut and MSFragger. Figure was created with BioRender.com. DDA, data dependent acquisition; nDIA, narrow window Data Independent Acquisition.

Enrichment and Analysis of Phosphorylated Peptides

IMAC was used as a general enrichment tool for phosphorylated peptides. 0.1 mg, 0.25 mg, and 0.5 mg of HCT116 peptides were used for IMAC enrichment and analyzed by LC-MS/MS using DDA acquisition on Orbitrap Fusion Lumos MS, and DDA and nDIA analysis on Orbitrap Astral MS. From the 0.1 mg input amount, a total of 13314, 21642, 19441 and 28199 unique phosphorylated peptides were identified by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS, respectively (Fig. 3*A*). A similar number of phosphorylated peptides were identified in both the 0.25 mg and 0.5 mg peptide input amount for the IMAC enrichment 16766 (0.25 mg), 16731 (0.5 mg); 23745 (0.25 mg), 23906 (0.5 mg); 23230 (0.25 mg), 23166 (0.5 mg), 32009 (0.25 mg), and 31762 (0.5 mg) were identified by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS, respectively (Fig. 3*A*).

nDIA analysis of IMAC enriched samples on the Orbitrap Astral MS identified up to 6-fold more phosphorylated peptides per minute compared to the Orbitrap Fusion Lumos MS at each of the input amounts tested (Fig. 3*A*). Among all phosphorylated peptides detected, approx. 87% were serine phosphorylated, 11% threonine phosphorylated, and approx. 2% tyrosine phosphorylated (Fig. 3*B*). To overcome the limitation of IMAC for the enrichment of phosphotyrosine, immunoaffinity was used to enrich phosphotyrosine-containing peptides. 0.1 mg, 0.3 mg, 1 mg, and 3 mg of input peptides from HCT116 cells were used to evaluate enrichment efficacy of immunoaffinity capture and the analytical performance of Orbitrap Astral MS for detection of low abundance phosphotyrosine peptides. From the lowest

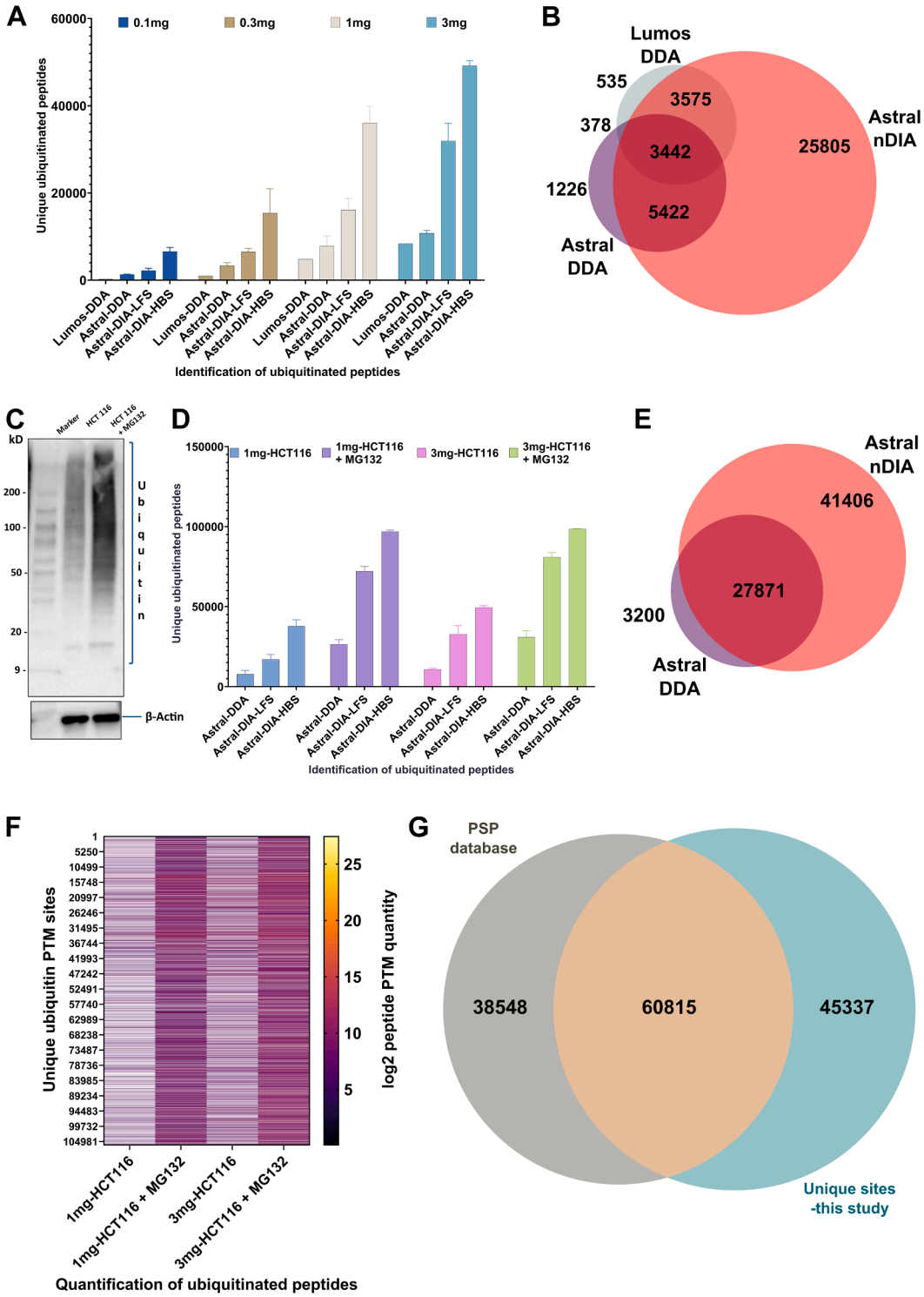

FIG. 2. **Enrichment and analysis of ubiquitinated peptides.** *A,* the number of unique ubiquitinated peptides identified and quantified in cells by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS, and Orbitrap Astral MS nDIA-HBS, from different amounts of input peptide used for immunoaffinity enrichment. The *error bars* represent mean with SD (n = 3 for Orbitrap Astral MS nDIA-LFS, Orbitrap Astral MS nDIA-HBS and n = 2 for Orbitrap Astral MS DDA). *B,* the overlap of ubiquitinated peptides identified and quantified from 3 mg input across different acquisition methods. *C,* anti-ubiquitin Western blot of untreated and MG132 treated HCT116 cells. *D,* the number of unique ubiquitinated peptides identified and quantified by Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS, and Orbitrap Astral MS nDIA-HBS from untreated and MG132 treated HCT116 cells at different input peptide amounts used for immunoaffinity

input amount used for enrichment (0.1 mg), Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS identified 6x, 7x and 32x more phosphotyrosine peptides respectively compared to Orbitrap Fusion Lumos MS DDA (Fig. 3*C*). From the 3 mg input amount nDIA on Orbitrap Astral MS identified 2119 phosphotyrosine peptides compared to 294 phosphotyrosine peptides by Orbitrap Fusion Lumos MS DDA (Fig. 3*C*). In contrast to IMAC, 97% of all phosphorylated peptides enriched by immunoaffinity were tyrosine phosphorylated, with the remaining approx. 2% serine phosphorylation, and approx. 1% threonine phosphorylation (Fig. 3*D*). To further validate, Jurkat cells were treated with pervanadate - a potent protein tyrosine phosphatase inhibitor commonly used to increase tyrosine phosphorylation in cells. Western blotting of whole cell lysate from untreated or pervanadate treated Jurkat cells using a pan anti-phosphotyrosine antibody confirmed the increased level of protein tyrosine phosphorylation with pervanadate treatment (Fig. 3*E*). Peptides from untreated and pervanadate treated Jurkat cells were used for immunoaffinity based phosphotyrosine enrichment followed by LC-MS/MS analysis. 8690, 9269, and 10330 unique peptides containing tyrosine phosphorylation were detected by DDA, nDIA-LFS and nDIA-HBS analysis on Orbitrap Astral MS, compared to 5019 phosphotyrosine peptides from DDA analysis on the Orbitrap Fusion Lumos MS (Fig. 3*F*). Pervanadate treatment of samples increased the number of phosphotyrosine peptides identified 31-fold (161 vs. 5019), 30-fold (289 vs. 8690), 36-fold (255 vs. 9269) and 29-fold (349 vs. 10330) with Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS respectively (Fig. 3*F*), in line with the analysis by Western blot (Fig. 3*E*). Identification and quantification of all phosphorylated peptides by DDA, nDIA-LFS and nDIA-HBS are provided in Data S2, *A, B1* and *B2*, respectively. When mapped against the PhosphoSitePlus database, out of 12262 phosphotyrosine sites identified, 8231 sites matched to previously identified sites, and 4031 phosphotyrosine sites were uniquely identified in this study (Fig. 3*G*).

Enrichment and Analysis of Acetylated, Succinylated and Mono-Methylated Peptides

To further test the application of immunoaffinity enrichment and nDIA on the Orbitrap Astral MS, the analysis was extended to other biologically important PTMs such as acetylation and succinylation of lysine and mono-methylation of arginine. For acetylation analysis, 0.1 mg, 0.3 mg, 1 mg and 3 mg of total peptide from HCT116 cells were used for immunoaffinity enrichment followed by LC-MS/MS analysis. From the lowest input amount of 0.1 mg peptide, a total of 1927, 1667, 2560 and 6923 unique acetylated peptides were identified by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS respectively (Fig. 4*A*). From the 3 mg input amount, Orbitrap Astral MS nDIA-HBS identified a total of 14858 unique acetylated peptides, 3.6-fold more than the 4072 acetylated peptides identified by Orbitrap Fusion Lumos MS DDA (Fig. 4*A*). nDIA-LFS analysis on Orbitrap Astral MS identified 1.5-fold more number of unique acetylated peptides as DDA analysis in half the run time (45-min *versus* 90-min gradient) (Fig. 4*A*). Out of the 14245 unique acetylated sites identified in this study, 6919 sites were previously identified in the PhosphoSitePlus database, and a total of 7326 sites were uniquely identified in this study (Fig. 4*B*). Tryptic peptides from mouse liver were used for the enrichment of succinylated and arginine mono-methylated peptides. 1 mg and 3 mg input peptide were used for the immunoaffinity enrichment followed by LC-MS/MS analysis.

nDIAHBS analysis on Orbitrap Astral MS identified 5719 and 6674 unique succinylated peptides from 1 mg and 3 mg input amount, compared to 3193 and 4640 succinylated peptides, respectively, by Orbitrap Fusion Lumos MS DDA (Fig. 4*C*). When compared to nDIA-LFS, DDA analysis on Orbitrap Astral MS using a 2x longer gradient (90-min *versus* 45-min), identified 20% and 11% more succinylated peptides from 1 mg and 3 mg respectively. When the same nDIA data are searched in hybrid mode (nDIA-HBS) using DDA runs to extend the library, nDIA-HBS identified up to 30% more succinylated peptides compared to DDA in half the analysis time. When mapped against the PhosphoSitePlus database, 1933 succinylated sites were previously known and 3339 sites are uniquely identified in this study (Fig. 4*D*). Immunoaffinity analysis of arginine mono-methylation from mouse liver resulted in the identification of 1179, 1456, 1395, and 1439 unique arginine mono-methylated peptides by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS, respectively from 3 mg input peptide (Fig. 4*E*). From the 1 mg input enrichment, Orbitrap Astral MS nDIA-HBS identified 44%, 24% and 40% more arginine monomethylated peptide compared to Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA and Orbitrap Astral MS nDIA-LFS, respectively (Fig. 4*E*). Comparing to the PhosphoSitePlus database, 1052 arginine mono-methylation sites were previously identified in other studies, and 409 sites were uniquely identified in this study (Fig. 4*F*). Identification and quantification of all acetylated, succinylated and mono-methylated

enrichment. The *error bars* represent mean with SD (n = 2). *E,* the overlap of ubiquitinated peptides identified and quantified by Orbitrap Astral MS DDA and Orbitrap Astral MS nDIA acquisition methods. *F,* the quantification of ubiquitinated PTM peptides identified by Orbitrap Astral MS nDIA from untreated and MG132 treated HCT116 cells. *G,* the overlap of ubiquitin sites identified in this study when compared to all ubiquitin sites reported in the PhosphoSitePlus database (34). DDA, data dependent acquisition; nDIA, narrow window Data Independent Acquisition.

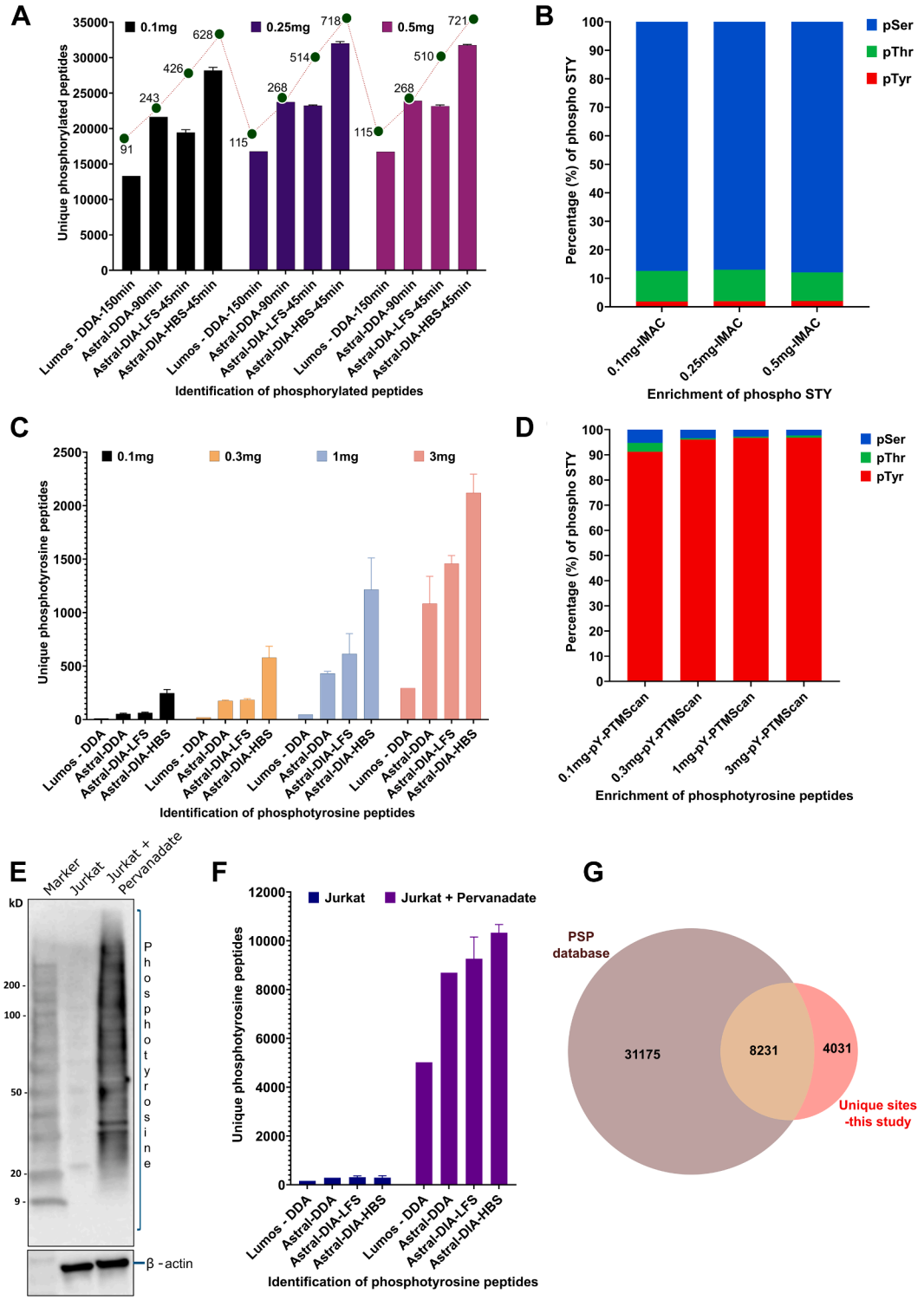

FIG. 3. **Enrichment and analysis of phosphorylated peptides.** *A,* the number of unique phosphorylated peptides identified and quantified by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS, and Orbitrap Astral MS nDIA-HBS, from different input peptide amounts used for IMAC enrichment. The number on top of each bar represents the number of quantified phosphopeptides per minute by the respective methods. The *error bars* represent mean with SD (n = 3 for Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS). *B,* the percentage of phosphoserine, phosphothreonine and phosphotyrosine peptides identified and quantified by IMAC enrichment at different input amounts. *C,* the number of unique phosphotyrosine peptides identified and quantified by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS, and Orbitrap Astral MS nDIA-HBS from different peptide input amounts used for

peptides by DDA, nDIA-LFS and nDIA-HBS are provided in Data S3, *A*, *B1* and *B2*, respectively.

Need for Enrichment of PTM Peptides Prior to LC-MS/MS Analysis

Technological advancements like the Orbitrap Astral MS have provided unprecedented speed and sensitivity and have enabled the detection of tens to hundreds of thousands of peptides in a short analysis time, providing in-depth analysis of thousands of protein groups in a typical bottom-up proteomics analysis. Even with this increased speed and sensitivity, the detection of PTM peptides in unenriched HCT116 cells from a 45-min Orbitrap Astral MS nDIA-LFS method quantified a total of 112440 unique peptides from 8,440 protein groups but only 24 (0.02%) ubiquitinated, 127 (0.1%) acetylated, and six phosphotyrosine (0.005%), peptides were identified (Data S4). However, when immunoaffinity enrichment methods such as PTMScan HS were employed on HCT116 cells prior to Orbitrap Astral MS nDIA-LFS analysis, a total of 36691 unique ubiquitinated sites were detected (Fig. S1*A*). Even in MG132-treated HCT116 cells, only 60 ubiquitinated peptides were identified in unenriched samples compared to 64159 ubiquitinated peptides with immunoaffinity enrichment (Fig. S1*B*). A total of 1,374 phosphotyrosine peptides were identified with immunoaffinity enrichment *versus* 6 phosphotyrosine peptides in unenriched HCT116 samples (Fig. S1*C*).

In Jurkat cells treated with pervanadate, where the phosphotyrosine level is approx. 28-fold higher than untreated cells (Fig. 3, *E* and *F*), only 43 phosphotyrosine peptides were identified without enrichment compared to 9,428 phosphotyrosine peptides with immunoaffinity enrichment (Fig. S1*D*). Similarly, the number of acetylated, succinylated and monomethylated peptides showed a similar pattern of low identification rate in unenriched samples (Data S4) *versus* more comprehensive coverage in immunoaffinity enriched samples 127 *versus* 11211 for acetylation, 46 *versus* 5,781 for succinylation, and 74 *versus* 1,411 for arginine mono-methylation Figure S1, *E–G*, respectively.

DISCUSSION

The study of protein post-translational modifications is critical to understanding the signaling and protein dynamics underlying cellular processes and disease states. IMAC and antibody based enrichment methods such as PTMScan have long been used to enrich for post-translationally modified peptides prior to LC-MS/MS analysis, simplifying the mixture of peptides delivered to the instrument and allowing identification and quantification of thousands of sites of modification across a variety of PTMs (9, 16–19). Recent advancements in instrumentation and software have allowed unprecedented coverage of the proteome, nearing a comprehensive view of protein expression (27, 29, 30), however we wanted to understand how new instruments like the Orbitrap Astral MS could further improve the coverage of PTMs with or without conventional enrichment methods typically employed in LC-MS based PTM studies. In this study, the performance of the Orbitrap Astral MS instrument using DDA, nDIA-LFS, and nDIA-HBS was compared to an older generation Orbitrap Fusion Lumos MS instrument using unenriched and a variety of enrichment samples to assess depth of coverage on multiple PTM types and the ability to uncover novel sites (Fig. 1).

Lysine ubiquitination plays a central role in protein degradation as well as regulating cell signaling events (35–39). The PTMScan ubiquitin remnant enrichment kit recognizes the diglycine motif left behind after trypsin digestion of ubiquitinated sites (K-ε-GG). Using this antibody to enrich samples previously allowed identification of thousands of sites of ubiquitination on older generation instruments such as the Orbitrap Fusion Lumos MS. In this study, the number of identified and quantified ubiquitin sites increased across all sample amounts (0.1 mg, 0.3 mg, 1 mg, 3 mg), with nDIA analysis on Orbitrap Astral MS enabling much larger gains in number of identified peptides, up to a 29-fold increase compared to Orbitrap Fusion Lumos MS DDA (Fig. 2, *A* and *B*). The proteasome inhibitor MG132 was used to provide an even richer sample from which to enrich ubiquitinated peptides, and at both 1 mg and 3 mg input peptide the number of ubiquitin K-ε-GG peptides identified increased compared to untreated control, up to nearly 98000 unique peptides in the 3 mg sample on Orbitrap Astral MS with nDIA-HBS (Fig. 2, *C–F*). This demonstrates the capacity of the K-ε-GG antibody beads to enrich high amounts of modified peptide, and is also, to our knowledge, the highest number of K-ε-GG peptides ever identified in a single shot LC-MS/MS analysis. nDIA analysis of both untreated and MG132 treated samples returned much higher numbers of modified peptides than DDA analysis on the same instrument, demonstrating the value of nDIA in analysis of post-translationally modified peptides. This study also yielded over 45000 novel sites of ubiquitin modification compared to known sites in the PhosphoSitePlus database, massively expanding the number of

immunoaffinity phosphotyrosine enrichment. The *error bars* represent mean with SD (n = 3 for Orbitrap Astral MS nDIA-LFS, Orbitrap Astral MS nDIA-HBS and n = 2 for Orbitrap Astral MS DDA). *D,* the percentage of phosphoserine, phosphothreonine and phosphotyrosine peptides identified and quantified by immunoaffinity phosphotyrosine enrichment at different input amounts. *E,* an anti-phosphotyrosine Western blot of untreated and pervanadate (a protein tyrosine phosphatase inhibitor) treated Jurkat cells. *F,* the number of unique phosphotyrosine peptides enriched by immunoaffinity and identified and quantified by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS, and Orbitrap Astral MS nDIA-HBS from untreated and pervanadate treated Jurkat cells. The *error bars* represent mean with SD (n = 2 for Orbitrap Astral MS nDIA-LFS and Orbitrap Astral MS nDIA-HBS). *G,* the overlap of phosphotyrosine sites identified in this study when compared to all phosphotyrosine sites reported in the PhosphoSitePlus database. DDA, data dependent acquisition; nDIA, narrow window Data Independent Acquisition.

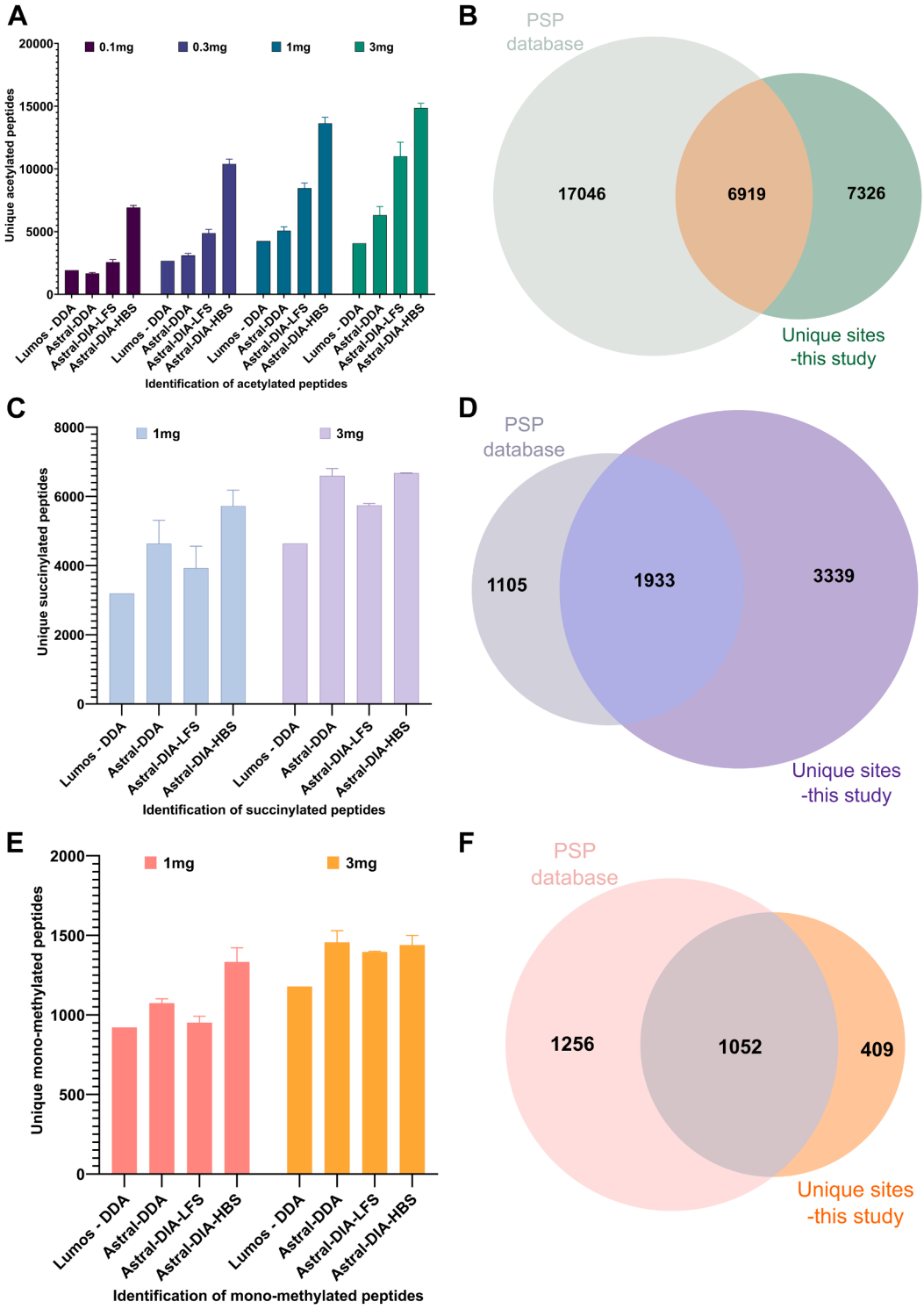

FIG. 4. **Enrichment and analysis of acetylation, succinylation and arginine methylation.** *A*, *C* and *E, Panels A*, *C* and *E*, respectively show the number of unique acetylated, succinylated and arginine mono-methylated peptides identified and quantified by Orbitrap Fusion Lumos MS DDA, Orbitrap Astral MS DDA, Orbitrap Astral MS nDIA-LFS, and Orbitrap Astral MS nDIA-HBS from different amounts of input peptide used for immunoaffinity enrichment. The *error bars* represent mean with SD: for acetylation n = 3 for Orbitrap Astral MS nDIA-LFS, Orbitrap Astral MS nDIA-HBS and Orbitrap Astral MS DDA; for succinylation and monomethylation n = 2 for Orbitrap Astral MS nDIA-LFS, Orbitrap Astral MS nDIA-HBS and Orbitrap Astral MS DDA). *B*, *D* and *F, Panels B*, *D* and *F* show the overlap of acetylation, succinylation and arginine mono-methylation sites identified in this study when compared to all acetylation, succinylation and arginine mono-methylation sites reported in the PhosphositePlus database, respectively. DDA, data dependent acquisition.

known ubiquitination sites and providing a rich resource for future studies of protein ubiquitination (Fig. 2*G*).

Previous work has demonstrated the utility of the Orbitrap Astral MS instrument in phosphoproteomics experiments (40). This study confirmed those earlier findings that DIA on Orbitrap Astral MS allows significant gains in depth of phosphopeptide coverage using Fe-IMAC enrichment (Fig. 3, *A* and *B*). The vast majority of sites identified from IMAC enriched samples were phosphoserine and phosphothreonine (~98%) as described previously (19). To achieve more comprehensive coverage of the tyrosine phosphoproteome a phosphotyrosine (pY) specific enrichment is needed, such as the PTMScan phosphotyrosine pY-1000 kit. Indeed, this enrichment allowed identification of hundreds to thousands of phosphotyrosine sites across samples (Fig. 2, *C* and *D*), and the use of Orbitrap Astral MS provided a large increase in pY peptides identified compared to the older generation Orbitrap Fusion Lumos MS, with up to 33-fold more pY peptides identified with Orbitrap Astral MS nDIA than Orbitrap Fusion Lumos MS DDA. As with MG132 for ubiquitination analysis, the tyrosine phosphatase inhibitor pervanadate was used to treat Jurkat cells and create a maximally phosphorylated sample (Fig. 3*E*). Pervanadate treatment allowed identification and quantification of over 10000 phosphotyrosine sites with Orbitrap Astral MS nDIA, compared to less than 1000 sites with untreated Jurkat cells (Fig. 3*F*). This again demonstrates the capacity for peptide enrichment of the beads, as well as the gains in depth of coverage that can be made by employing nDIA on the Orbitrap Astral MS. 10000 phosphotyrosine sites represent one of the larger single shot datasets collected to date, and overall, the phosphotyrosine data in this study contributed more than 10% new sites to the PhosphoSitePlus database (Fig. 3*G*), even though pY data has been curated into that database for over 20 years.

Acetylation profiling was similarly improved with nDIA on Orbitrap Astral MS, with nearly 3-fold more AcK peptides identified and quantified with Orbitrap Astral MS nDIA-HBS compared to Orbitrap Fusion Lumos MS DDA, and nearly 26% additional sites not previously in the PhosphoSitePlus database (Fig. 4, *A* and *B*). The gains for succinyl lysine and mono-methyl arginine were more subtle, however in both cases nDIA analysis on Orbitrap Astral MS outperformed DDA on Orbitrap Fusion Lumos MS despite a 2X longer gradient used for the Orbitrap Fusion Lumos MS DDA analysis (Fig. 4, *C* and *E*). Again, as with other modifications, the nDIA profiling on Orbitrap Astral MS contributed new sites to the PhosphoSitePlus database, with more than 2X new sites for succinylation (110% novel, Figs. 4*D*), and 18% new sites for mono-methyl arginine (Fig. 4*F*).

For each PTM profiled in the study, an unenriched sample was run in parallel using nDIA on Orbitrap Astral MS. This served two purposes: To evaluate the ability of the Orbitrap Astral MS, given its speed and sensitivity, to identify PTM peptides without the need for enrichment, and to verify that nDIA searching is not providing spurious identification of PTM peptides (Fig. S1, *A–G*). In all cases, there were very few modified peptide identifications in the unenriched samples, while enrichment allowed identification and quantification of thousands to tens of thousands of modified peptides, highlighting the need to enrich samples to effectively profile PTMs. Likewise, the low number of PTM peptide identifications in the unenriched samples demonstrates that the DIA software is not providing artificial identification of PTM peptides in a sample where they have not been specifically enriched.

Enrichment methods such as the PTMScan HS circumvent the challenge of identifying low abundance post-translationally modified peptides through immunoaffinity enrichment of peptides harboring specific class of PTMs including but not limited to lysine ubiquitination, tyrosine phosphorylation, lysine acetylation, lysine succinylation, and arginine monomethylation. The Orbitrap Astral MS represents a major leap forward in the speed, sensitivity, and depth of coverage achievable for bottom-up proteomics, owing to its nearly lossless ion transfer ability, combined with ~200-Hz MS/MS acquisition rates at high resolving power (27, 29, 30). Combining PTMScan sample preparation with the Orbitrap Astral MS operated under nDIA allows unprecedented access to the PTM landscape in cells and tissues. For all PTMs tested in this study, identification of PTM-containing peptides with Orbitrap Astral MS nDIA far outpaced the previous generation Orbitrap Fusion Lumos MS. These data also represent a major improvement in the catalog of known PTMs in human cells and mouse tissue, with thousands of unique PTM sites identified that are not currently in the PhosphoSitePlus database. The protocol detailed in this study will provide a framework for future analysis of protein post-translational modifications in the study of cellular signaling, disease biology, and target and biomarker discovery.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (41) partner repository with the dataset identifier PXD065579.

Supplemental Data—This article contains supplemental data.

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conceptualization; M. P. S. and S. A. B. supervision; S. A. B. project administration.

Conflict of Interest—M. K., A. P. P., B. M. Z., S. S., J. M. R., A. J. N., B. Z., S. L., J. C. S., M. P. S., and S. A. B. are employees of Cell Signaling Technology. B. L., and T. P. H., are employees of Thermo Fisher Scientific.

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REFERENCES

1. Harper, J. W., and Bennett, E. J. (2016) Proteome complexity and the forces that drive proteome imbalance. *Nature* **537**, 328–338
2. Jensen, O. N. (2006) Interpreting the protein language using proteomics. *Nat. Rev. Mol. Cell Biol.* **7**, 391–403
3. McDonald, W. H., and Yates, J. R., 3rd (2002) Shotgun proteomics and biomarker discovery. *Dis. Markers* **18**, 99–105
4. Yates, J. R., 3rd (2013) The revolution and evolution of shotgun proteomics for large-scale proteome analysis. *J. Am. Chem. Soc.* **135**, 1629–1640
5. Eng, J. K., McCormack, A. L., and Yates, J. R. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**, 976–989
6. Clauser, K. R., Baker, P., and Burlingame, A. L. (1999) Role of accurate mass measurement (+/– 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal. Chem.* **71**, 2871–2882
7. Fenyo, D., Qin, J., and Chait, B. T. (1998) Protein identification using mass spectrometric information. *Electrophoresis* **19**, 998–1005
8. Block, H., Maertens, B., Spriestersbach, A., Brinker, N., Kubicek, J., Fabis, R., *et al*. (2009) Immobilized-metal affinity chromatography (IMAC): a review. *Methods Enzymol.* **463**, 439–473
9. Rush, J., Moritz, A., Lee, K. A., Guo, A., Goss, V. L., Spek, E. J., *et al*. (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* **23**, 94–101
10. Qian, W. J., Jacobs, J. M., Liu, T., Camp, D. G., and Smith, R. D. (2006) Advances and challenges in liquid chromatography-mass spectrometry-based proteomics profiling for clinical applications. *Mol. Cell Proteomics* **5**, 1727–1744
11. Guo, T., Steen, J. A., and Mann, M. (2025) Mass-spectrometry-based proteomics: from single cells to clinical applications. *Nature* **638**, 901–911
12. Ye, Z., Sabatier, P., van der Hoeven, L., Lechner, M. Y., Phlairaharn, T., Guzman, U. H., *et al*. (2025) Enhanced sensitivity and scalability with a chip-tip workflow enables deep single-cell proteomics. *Nat. Methods* **22**, 499–509
13. Serrano, L. R., Mellors, J. S., Thompson, J. W., Lancaster, N. M., Robinson, M. L., Overmyer, K. A., *et al*. (2025) SPE-CZE-MS quantifies zeptomole amounts of phosphorylated peptides. *J. Proteome Res.* **24**, 3049–3061
14. Glykofridis, I. E., Henneman, A. A., Balk, J. A., Goeij-de Haas, R., Westland, D., Piersma, S. R., *et al*. (2022) Phosphoproteomic analysis of FLCN inactivation highlights differential kinase pathways and regulatory TFEB phosphoserines. *Mol. Cell Proteomics* **21**, 100263
15. Rivera, K. D., Olive, M. E., Bergstrom, E. J., Nelson, A. J., Lee, K. A., Satpathy, S., *et al*. (2021) Automating UbiFast for high-throughput and multiplexed ubiquitin enrichment. *Mol. Cell Proteomics* **20**, 100154
16. Rikova, K., Guo, A., Zeng, Q., Possemato, A., Yu, J., Haack, H., *et al*. (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* **131**, 1190–1203
17. Moritz, A., Li, Y., Guo, A., Villén, J., Wang, Y., MacNeill, J., *et al*. (2010) Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. *Sci. Signal.* **3**, ra64
18. Stokes, M. P., Farnsworth, C. L., Moritz, A., Silva, J. C., Jia, X., Lee, K. A., *et al*. (2012) PTMScan direct: identification and quantification of peptides from critical signaling proteins by immunoaffinity enrichment coupled with LC-MS/MS. *Mol. Cell Proteomics* **11**, 187–201
19. Stokes, M. P., Farnsworth, C. L., Gu, H., Jia, X., Worsfold, C. R., Yang, V., *et al*. (2015) Complementary PTM profiling of drug response in human gastric carcinoma by immunoaffinity and IMAC methods with total proteome analysis. *Proteomes* **3**, 160–183
20. Zhang, H., Zha, X., Tan, Y., Hornbeck, P. V., Mastrangelo, A. J., Alessi, D. R., *et al*. (2002) Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *J. Biol. Chem.* **277**, 39379–39387
21. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., *et al*. (2003) A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* **21**, 921–926
22. Svinkina, T., Gu, H., Silva, J. C., Mertins, P., Qiao, J., Fereshetian, S., *et al*. (2015) Deep, quantitative coverage of the lysine acetylome using novel anti-acetyl-lysine antibodies and an optimized proteomic workflow. *Mol. Cell Proteomics* **14**, 2429–2440
23. Guo, A., Gu, H., Zhou, J., Mulhern, D., Wang, Y., Lee, K. A., *et al*. (2014) Immunoaffinity enrichment and mass spectrometry analysis of protein methylation. *Mol. Cell Proteomics* **13**, 372–387
24. Purvine, S., Eppel, J. T., Yi, E. C., and Goodlett, D. R. (2003) Shotgun collision-induced dissociation of peptides using a time of flight mass analyzer. *Proteomics* **3**, 847–850
25. Venable, J. D., Dong, M. Q., Wohlschlegel, J., Dillin, A., and Yates, J. R. (2004) Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. *Nat. Methods* **1**, 39–45
26. Gillet, L. C., Navarro, P., Tate, S., Röst, H., Selevsek, N., Reiter, L., *et al*. (2012) Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol. Cell Proteomics* **11**, O111.016717
27. Guzman, U. H., Martinez-Val, A., Ye, Z., Damoc, E., Arrey, T. N., Pashkova, A., *et al*. (2024) Ultra-fast label-free quantification and comprehensive proteome coverage with narrow-window data-independent acquisition. *Nat. Biotechnol.* **42**, 1855–1866
28. Kitata, R. B., Yang, J. C., and Chen, Y. J. (2023) Advances in data-independent acquisition mass spectrometry towards comprehensive digital proteome landscape. *Mass Spectrom. Rev.* **42**, 2324–2348
29. Stewart, H. I., Grinfeld, D., Giannakopulos, A., Petzoldt, J., Shanley, T., Garland, M., *et al*. (2023) Parallelized acquisition of orbitrap and astral analyzers enables high-throughput quantitative analysis. *Anal. Chem.* **95**, 15656–15664
30. Heil, L. R., Damoc, E., Arrey, T. N., Pashkova, A., Denisov, E., Petzoldt, J., *et al*. (2023) Evaluating the performance of the astral mass analyzer for quantitative proteomics using data independent acquisition. *bioRxiv*. https://doi.org/10.1101/2023.06.03.543570
31. Bruderer, R., Bernhardt, O. M., Gandhi, T., Miladinović, S. M., Cheng, L. Y., Messner, S., *et al*. (2015) Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. *Mol. Cell Proteomics* **14**, 1400–1410
32. Kong, A. T., Leprevost, F. V., Avtonomov, D. M., Mellacheruvu, D., and Nesvizhskii, A. I. (2017) MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. *Nat. Methods* **14**, 513–520
33. Sahu, I., Zhu, H., Buhrlage, S. J., and Marto, J. A. (2023) Proteomic approaches to study ubiquitinomics. *Biochim. Biophys. Acta Gene Regul. Mech.* **1866**, 194940
34. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res.* **43**, D512–D520
35. Ravid, T., and Hochstrasser, M. (2008) Diversity of degradation signals in the ubiquitin-proteasome system. *Nat. Rev. Mol. Cell Biol.* **9**, 679–690
36. Clague, M. J., and Urbe, S. (2010) Ubiquitin: same molecule, different degradation pathways. *Cell* **143**, 682–685
37. Zhao, L., Zhao, J., Zhong, K., Tong, A., and Jia, D. (2022) Targeted protein degradation: mechanisms, strategies and application. *Signal. Transduct. Target. Ther.* **7**, 113
38. Yang, W. L., Zhang, X., and Lin, H. K. (2010) Emerging role of Lys-63 ubiquitination in protein kinase and phosphatase activation and cancer development. *Oncogene* **29**, 4493–4503
39. Sun, L., and Chen, Z. J. (2004) The novel functions of ubiquitination in signaling. *Curr. Opin. Cell Biol.* **16**, 119–126
40. Lancaster, N. M., Sinitcyn, P., Forny, P., Peters-Clarke, T. M., Fecher, C., Smith, A. J., *et al*. (2024) Fast and deep phosphoproteome analysis with the orbitrap astral mass spectrometer. *bioRxiv*. https://doi.org/10.1101/2023.11.21.568149
41. Perez-Riverol, Y., Bandla, C., Kundu, D. J., Kamatchinathan, S., Bai, J., Hewapathirana, S., *et al*. (2025) The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res.* **53**, D543–D553