

# Construction of Human Proteoform Families from 21 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometry Top-Down Proteomic Data

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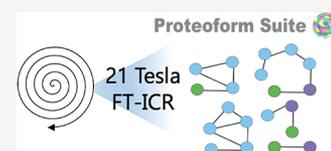
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**ABSTRACT:** Identification of proteoforms, the different forms of a protein, is important to understand biological processes. A proteoform family is the set of different proteoforms from the same gene. We previously developed the software program Proteoform Suite, which constructs proteoform families and identifies proteoforms by intact-mass analysis. Here, we have applied this approach to top-down proteomic data acquired at the National High Magnetic Field Laboratory 21 tesla Fourier transform ion cyclotron resonance mass spectrometer (data available on the MassIVE platform with identifier MSV000085978). We explored the ability to construct proteoform families and identify proteoforms from the high mass accuracy data that this instrument provides for a complex cell lysate sample from the MCF-7 human breast cancer cell line. There were 2830 observed experimental proteoforms, of which 932 were identified, 44 were ambiguous, and 1854 were unidentified. Of the 932 unique identified proteoforms, 766 were identified by top-down MS2 analysis at 1% false discovery rate (FDR) using TDPortal, and 166 were additional intact-mass identifications (~4.7% calculated global FDR) made using Proteoform Suite. We recently published a proteoform level schema to represent ambiguity in proteoform identifications. We implemented this proteoform level classification in Proteoform Suite for intact-mass identifications, which enables users to determine the ambiguity levels and sources of ambiguity for each intact-mass proteoform identification.

**KEYWORDS:** proteoform, proteoform family, 21 tesla, Fourier transform ion cyclotron resonance, top-down proteomics



## INTRODUCTION

Protein diversity plays a central role in the function of biological systems.<sup>1,2</sup> Proteoforms are the different forms of proteins that result from processes such as genetic variation, alternative splicing, and post-translational modification (PTM).<sup>3</sup> A proteoform family is the set of proteoforms derived from the same gene.<sup>4</sup> Proteoforms are identified by top-down mass spectrometry (MS), where intact proteins are analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS), and the intact and fragment ion masses are used to identify and characterize the proteoform.<sup>5–7</sup> However, not all proteoforms observed in the MS1 spectra are subsequently identified by MS2 fragmentation.<sup>8,9</sup> Due to resolution requirements and the low signal-to-noise<sup>10</sup> of intact proteins, which necessitates extensive signal averaging, there is generally insufficient instrument time available to select all observed proteoforms for fragmentation on an LC time scale. Additionally, proteoforms selected for fragmentation may not be identified due to low signal-to-noise (S/N) typically observed in the MS2 spectra of intact proteins (further exacerbated as mass increases<sup>10</sup>), poor fragmentation, or excessively complex fragmentation data.

We have recently developed the open source and freely available software program Proteoform Suite (<https://smithchem-wisc.github.io/ProteoformSuite/>), which is able to identify proteoforms in complex data by intact-mass

alone.<sup>4,11–13</sup> Proteoform Suite compares observed proteoform masses to both a database and to coeluting observed masses, selects frequent mass differences corresponding to modifications, and constructs and visualizes proteoform families from accepted mass differences. Intact-mass analysis and the construction of proteoform families increase the number of proteoform identifications beyond what is identified by top-down alone and enables interesting candidates to be selected for subsequent targeted top-down analysis. In previous work, we have performed intact-mass analysis on isotopically labeled samples from several biological systems, including yeast,<sup>11</sup> *Escherichia coli*,<sup>14</sup> and human Jurkat cell lysate,<sup>15</sup> and label-free samples from systems of reduced biological complexity including yeast<sup>13</sup> and mouse mitochondria.<sup>12</sup> Recently, intact-mass analysis was used in combination with targeted MS2 analysis to identify proteoforms >50 kDa in human heart tissue.<sup>16</sup>

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Here, we extend this strategy to the 21 tesla (T) Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer platform.<sup>17,18</sup> This mass analyzer offers the highest attainable mass accuracy and resolving power. We used Proteoform Suite to construct proteoform families, combining both top-down results and intact-mass observations in size-separated fractions of cell lysate from the MCF-7 breast cancer cell line analyzed by 21 T FT-ICR MS. There were 2830 observed experimental proteoforms, of which 932 were identified, 44 were ambiguous between more than one possible identification, and 1854 were unidentified. Proteoform Suite constructed 520 proteoform families from the data, consisting of 766 unique proteoforms identified by top-down MS2 analysis at 1% FDR using TDPportal and 166 additional proteoforms identified by intact-mass (~4.7% global FDR) using Proteoform Suite. Smith et al. recently published a proteoform level schema to represent ambiguity in proteoform identifications. Here, we have implemented this proteoform level classification in Proteoform Suite for intact-mass identifications, which provides users with the ambiguity levels and sources of ambiguity for each intact-mass proteoform identification. The results in this study demonstrate that the high-quality data obtained on the 21 T FT-ICR MS platform are well-suited for human proteoform identifications and proteoform family construction by intact-mass analysis.

## METHODS

### Data Acquisition

**Sample Preparation.** Two pellets of  $1 \times 10^7$  MCF-7 cells were thawed on ice and resuspended in 10 volumes of lysis buffer consisting of 4% SDS (Sigma-Aldrich), 100 mM Tris pH 7.5 (TekNova), 10 mM DTT (Sigma-Aldrich), 10 mM sodium butyrate (Sigma-Aldrich), and 1X Thermo Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Cell pellets were lysed by heating at 95 °C for 10 min and vortexing every 2 min. Cellular debris was removed by centrifugation at 20,000  $\times$  g for 20 min. Acetone (Sigma-Aldrich) protein precipitation was performed on the supernatant, and the resulting protein pellet was suspended in 150  $\mu$ L of 1% SDS for quantification via the bicinchoninic acid (BCA) assay. Size-based separation of approximately 400  $\mu$ g of each sample into 12 fractions was performed using a GELFrEE 8100 fractionation station with a 10% GELFrEE cartridge (Expedeon) following the manufacturer's recommended procedure. Methanol-chloroform (Honeywell-Burdick & Jackson) extraction was performed on each fraction to remove SDS.<sup>19,20</sup> Each pellet was reconstituted in solvent A: 0.3% formic acid (Thermo Scientific Pierce) and 5% acetonitrile (Honeywell-Burdick & Jackson) in water with % expressed as v/v).

**Liquid Chromatography.** For each injection, 2–4  $\mu$ L of reconstituted intact protein was loaded onto an in-house-fabricated 360 OD  $\times$  150  $\mu$ m ID fused silica microcapillary trap column packed 2.5 cm with PLRP-S resin (5  $\mu$ m particle, 1000 Å pore, Agilent Technologies, Palo Alto, CA, USA). The LC system (Acquity M-Class, Waters, Milford, MA, USA) was operated at a flow rate of 2.5  $\mu$ L/min for loading onto the trap column and washed with 95% solvent A for 10 min. Separation was achieved on an in-house fabricated analytical column packed 17.5 cm with the PLRP-S resin. Samples were eluted at a flow rate of 0.3  $\mu$ L/min over 90 min with the following gradients: (MCF-7 F1&F2) 5–15% B in 5 min, 15–55% B in 80 min, 55–75% B in 5 min; (MCF-7 F3-F6) 5–20% B in 5 min,

20–60% B in 80 min, 60–75% B in 5 min; (MCF-7 F7&F8) 5–25% B in 5 min, 25–60% B in 80 min, 60–75% B in 5 min. The gradients utilized solvent A: 0.3% formic acid and 5% acetonitrile in water; and solvent B: 47.5% acetonitrile, 47.5% 2-propanol, 4.7% water and 0.3% formic acid (% all expressed as v/v). Following separation, proteins were directly ionized by nanoelectrospray ionization (2.5 kV source voltage; 15 V SID) using a 15  $\mu$ m fused-silica PicoTip emitter (New Objective, Woburn, MA) packed 3 mm with PLRP-S resin.

**Mass Spectrometry.** The instrument was operated with Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA), and each fraction was injected for three separate experiments: two data-dependent CID MS/MS runs and one MS1-only run. All spectra were collected in the ion cyclotron resonance (ICR) mass analyzer at 21 tesla (T). For data-dependent experiments, some data acquisition parameters were varied based upon the expected MW range of the proteins contained within each fraction. For MS1 spectra, resolving power (RP) was set to 300 000 at  $m/z$  400; 1E6 automatic gain control (AGC) target; 4–6 microscans per spectrum; 600–2000  $m/z$  range. For MS2 spectra, RP was set to 150 000 or 300 000 at  $m/z$  400; 5E5 AGC target for CID MS2; 1–2 microscans per spectrum; 300–2000  $m/z$  range. CID activation was performed with 10  $m/z$  isolation width, 35% normalized collision energy, 10 ms activation period, 0.25 q, and 3–6 fragment ion fills of the multipole storage device were performed such that cumulative fragment ion targets were 1.5–3.0E6 charges prior to detection in the ICR cell. Data-dependent selection of precursors for MS2 was allowed from 700 to 1400  $m/z$ , and dynamic exclusion was enabled with a repeat count of one and repeat and exclusion durations set to 240 s. Charge state exclusion was enabled for  $[M + H]^+$  and  $[M + 2H]^{2+}$ . For all MS1-only experiments (regardless of fraction/MW), RP was set to 300 000 at 400  $m/z$ ; 1E6 AGC target; six microscans per spectrum; 600–2000  $m/z$  range. All raw data are available on the MassIVE platform with identifier MSV000085978.

### Data Analysis

**Top-Down Data Analysis.** The data (.raw files) derived from data-dependent CID MS/MS experiments (two per fraction for a total of 16 .raw files) were uploaded to the National Resource for Translational and Developmental Proteomics Galaxy<sup>21</sup> web portal for performing top-down proteomics database searches, which is freely available for academic collaborators (<http://nrtdp.northwestern.edu/tdportal-request>). This platform (TDPportal<sup>22</sup>) utilizes two search modes defined for ProSight PTM 2.0:<sup>23,24</sup> a narrow absolute mass search (with precursor mass measurement tolerance of 2.2 Da and 10 ppm fragment mass tolerance), and a biomarker search (similar to traditional “no-enzyme” search with biomarker and fragment mass tolerances set to 10 ppm). Details regarding Xtract deconvolution parameters and other aspects of the data analysis can be found within the TDReport file (available on the MassIVE platform with identifier MSV000085978), which can be viewed with TDViewer software (freely available at <http://topdownviewer.northwestern.edu>). Top-down hits (proteoform spectrum matches) corresponding to 1% protein-level false discovery rate (FDR) were exported to a Microsoft Excel file.

**Deconvolution of MS1-Only Files.** Proteoform Suite performs intact-mass analysis of proteoform masses in the MS1 spectra. To obtain a list of observed proteoform masses, MS1-only raw files were deconvoluted using Thermo Protein

Deconvolution 4.0. We used a fit factor of 70%, minimum S/N of 2, remainder threshold of 10%, minimum detected charge states of 3, and charge range from +5 to +50. A sliding window of 0.5 min and 50% offset was used to deconvolute the retention time range of 0–100 min. A Microsoft Excel file containing the raw experimental components was exported for each raw file.

**Mass and Retention Time Calibration in Proteoform Suite.** Proteoform Suite version 0.3.6 was used for all analysis (<https://github.com/smith-chem-wisc/ProteoformSuite/releases>). Mass calibration of deconvolution and TDPPortal results was performed in Proteoform Suite as previously described.<sup>13</sup> We implemented a retention time calibration algorithm (described in the Retention Time Calibration section of the [Supporting Information](#)) to account for run-to-run variation and the different LC gradients utilized. For both calibrations, well characterized top-down hits (C-score<sup>25</sup>  $\geq$  40) were used as calibration points. Isotopic peaks of different charge states were selected using tolerances of  $\pm 7$  ppm and  $\pm 15$  min in each of the raw data files. These tolerances were selected based on an analysis of top-down precursor mass error and retention time differences ([Supporting Figures S1 and S2](#)). Calibrated files with top-down hits and raw experimental components were exported and used for subsequent Proteoform Suite analyses.

**Proteoform Suite Intact-Mass Analysis.** Intact-mass analysis and construction of proteoform families in Proteoform Suite have been previously described.<sup>4,11–14</sup> Briefly, a theoretical proteoform database was created using a UniProt *Homo sapiens* xml-format database downloaded in March 2017 containing canonical sequences and Uniprot-annotated modifications and truncations, including signal peptides. Theoretical proteoforms were created using combinations of up to two annotated modifications. The theoretical database contained 58 390 theoretical proteoforms (20 218 unique proteins), 28 832 of which had at least one bottom-up peptide (7738 unique proteins).

Raw experimental components were read in from the deconvolution results of the MS1-only raw files, and both monoisotopic mass errors and charge state harmonics were corrected with a 5 ppm tolerance. A list of unique observed experimental proteoforms (intact-mass experimental proteoforms) was created by aggregating the raw experimental components with a mass tolerance of 5 ppm, retention time tolerance of 2.5 min, and a maximum missed monoisotopic mass error of three units. Top-down hits were read in, filtered by applying a C-score cutoff of 3, and aggregated with a retention time tolerance of 5 min to generate a list of top-down proteoforms. A theoretical proteoform for each top-down proteoform was added to the theoretical database if not already present. The lists of observed intact-mass experimental proteoforms and top-down proteoforms were combined, removing any intact-mass experimental proteoforms explained by a top-down proteoform (i.e., already identified) utilizing the same tolerances as used for aggregation. The resulting list of experimental proteoforms contained top-down proteoforms and intact-mass proteoforms corresponding to observed yet proteoforms unidentified by the top-down analysis.

Experimental–theoretical (comparing experimental and theoretical proteoform masses) and experiment–experiment (comparing experimental proteoform masses with one another within 2.5 min retention time) mass comparisons were performed separately. In each comparison, a delta mass histogram was constructed with a bin size of 0.1 Da ([Supporting](#)

[Figure S3](#)), and abundant peaks corresponding to known, common modifications were accepted for proteoform family construction. In order to control the size of the database in the experiment–theoretical comparison, we required each theoretical proteoform to be either identified by top-down analysis or from a protein identified by bottom-up analysis. The bottom-up analysis was performed with the software program MetaMorpheus<sup>26</sup> on a published MCF-7 dataset<sup>27</sup> (described in the Bottom-Up MetaMorpheus Search section of the [Supporting Information](#)).

Proteoform families were constructed from accepted experiment–theoretical and experiment–experiment relations. A mass error tolerance of 1.5 ppm was used for identification to control the FDR. Identification of experimental proteoforms by intact-mass was performed within each proteoform family; beginning with each theoretical proteoform, mass difference connections between proteoforms were followed, and experimental proteoforms were assigned an identification until a dead end was reached or an identification did not meet the mass tolerance (1.5 ppm) or the heuristic criteria (e.g., loss of acetylation when no acetylation is present on the proteoform). Therefore, intact-mass identifications are made in each proteoform family by first identifying experimental proteoforms in experiment–theoretical pairs in each family, and subsequent experiment–experiment connections are used to identify connected experimental proteoforms. Each experiment–experiment pair consists of two experimental proteoforms that are within the 2.5 min retention time tolerance set during the experiment–experiment delta mass comparison. If an intact-mass identification was ambiguous between a top-down identification and another possible identification, the top-down identification was utilized. Relations between proteoforms that did not result in an identification were removed, and proteoform families were reconstructed from accepted relations. Identifications were exported in a tab-delimited text file, and redundant identifications were manually removed. Decoy proteoform families were constructed as previously described,<sup>11,13</sup> and a global false discovery rate was calculated by dividing the average number of proteoforms identified in decoy families by the number of proteoforms identified in target families.

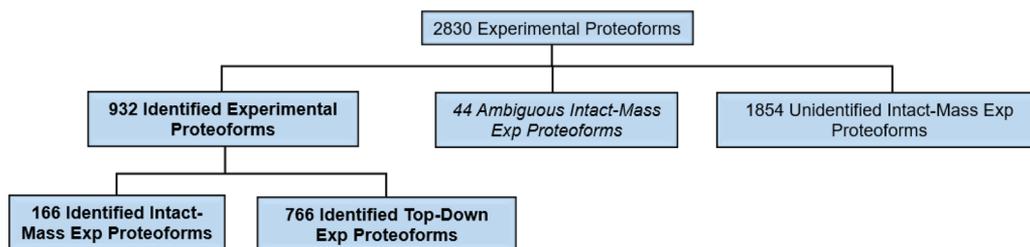
#### Proteoform Suite Top-Down Hit Precursor Analysis.

We evaluated how intact-mass analysis performed on top-down validated identifications. We created a tab-delimited text file from unique top-down identified proteoforms that had a minimum C-score of 40 (well-characterized proteoforms) and input this file into Proteoform Suite. We performed an intact-mass analysis utilizing the same aggregation parameters and database described above. We compared the Proteoform Suite intact-mass identifications to the top-down identifications determined by TDPPortal in Microsoft Excel.

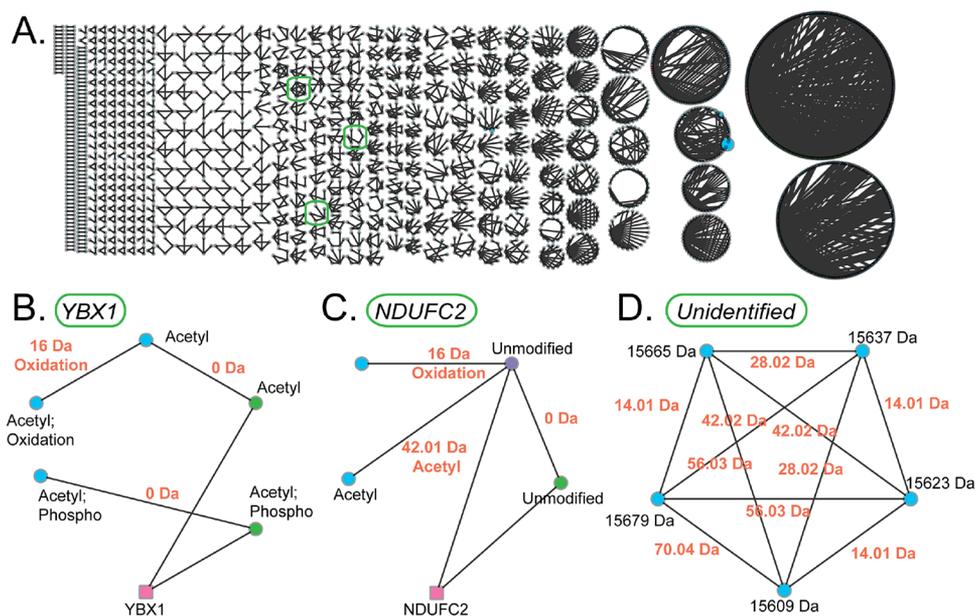
## RESULTS AND DISCUSSION

### Proteoforms and Proteoform Families

From analysis of eight GELFrEE fractions analyzed by 21 T FT-ICR MS, the 16 top-down data-dependent CID MS/MS .raw files were searched against a database of candidate human proteoforms with TDPPortal. This search resulted in the identification of 354 unique proteins (defined by UniProt accession numbers) expressed as 1684 unique proteoforms (defined by Proteoform Record, PFR; Consortium for Top-Down Proteomics Proteoform Repository <http://repository>).



**Figure 1.** Summary of top-down and intact-mass results from analysis of MCF-7 cell lysate using 21 T FT-ICR mass spectrometry.



**Figure 2.** (A) All 542 visualized proteoform families from MCF-7 human lysate. Green squares indicate the proteoform families that are expanded in 2B–D. In these visualized proteoform families, nodes represent proteoform masses, and edges represent mass differences corresponding to a modification (0 Da for exact match). Blue circles are intact-mass experimental proteoforms, purple circles are top-down experimental proteoforms, and green circles are theoretical proteoforms from the database. Pink squares represent genes. (B) Visualized proteoform family from YBX1 gene; intact-mass analysis identified three 35 kDa proteoforms. (C) Visualized proteoform family from the NDUFC2 gene. Top-down MS2 analysis identified the unmodified proteoform, and intact-mass analysis leveraged this identification to identify two additional modified proteoforms. (D) Unidentified proteoform family with multiple methylations present.

[topdownproteomics.org/](http://topdownproteomics.org/)) at 1% FDR. Of these 1684 proteoforms identified by TDPportal, 766 proteoforms from 339 unique proteins exhibited a C-score of 3 or greater (Supporting Table S1), which was used as a threshold when importing top-down results into Proteoform Suite for intact-mass analysis. This is considered the minimum C-score for a proteoform to be identified; proteoforms with a C-score of  $\geq 40$  are considered both identified and well-characterized.<sup>25</sup>

The MS1-only raw files were deconvoluted, revealing additional 2064 intact-mass experimental proteoforms observed but not identified by top-down analysis of which 166 were identified at a calculated global FDR of 4.7% (Supporting Table S2), 44 were ambiguous (Supporting Table S3), and 1854 were unidentified (Supporting Table S4) by Proteoform Suite. The intact-mass FDR is calculated by constructing decoy proteoform families from decoy experiment–theoretical and experiment–experiment relations and determining the ratio of decoy intact-mass identifications made in the decoy families to target identifications made in the target families, as described in detail previously.<sup>11,13</sup> There were an additional 43 protein accessions identified by intact-mass analysis. In the experiment–theoretical comparison, any intact-mass match with a theoretical proteoform required either a top-down proteoform ID or at least one

bottom-up peptide identification to prevent false intact-mass identifications. The summary of the results is shown in Figure 1.

Proteoform Suite constructs proteoform families and provides visualization as a network of nodes (unique proteoform masses) and edges (mass differences between proteoforms). The visualized 520 proteoform families are shown in Figure 2A. There were 336 identified proteoform families (one gene), 14 ambiguous families (more than one gene), and 170 unidentified families (no gene). We selected several examples that exemplify how intact-mass analysis complements top-down analysis. Proteoform Suite identified two 35 kDa proteoforms (acetylated and phosphorylated and acetylated) from the YBX1 Y box-binding protein gene, which were not identified by top-down analysis (Figure 2B). Larger proteoforms are particularly difficult to identify by top-down analysis because of the lower S/N inherent to larger mass and other factors.<sup>10</sup> Top-down analysis identified an unmodified proteoform from the gene NDUFC2. Proteoform Suite was able to identify an acetylated proteoform from this gene by intact-mass experiment–experiment comparison (Figure 2C). Proteoform Suite could not have identified this family by intact-mass alone because the acetylation is not annotated in the database (+42 Da), and the methionine was not cleaved (+131 Da). As a result, the mass

difference between the observed experimental proteoform and the theoretical proteoform in the database was +173 Da, which was not an accepted mass difference in the experiment–theoretical comparison. This example illustrates how MS2 identification was necessary for initial identification, and Proteoform Suite was able to leverage this identification to acquire additional IDs.

Many of the intact-mass identifications were for modified proteoforms. Of the 188 new intact-mass identifications, there were 14 unmodified intact-mass identifications, 114 with at least one acetylation, 27 with at least one phosphorylation, and 62 with at least one methylation. We observed 82 intact-mass proteoforms exhibiting a mass shift of 98.06 Da, which could potentially be an acetone adduct from the acetone precipitation performed.<sup>28</sup> There were 77 intact-mass proteoforms exhibiting a mass shift of 266.15, which corresponds to an SDS adduct, and 343 proteoforms with at least one oxidation. Intact-mass identifications resulting from an oxidation, 98.06 Da shift, or SDS adducts were not counted as additional identifications in number reporting (Figure 1). Although these modifications are likely sample handling artifacts, it is still important to identify these species to prevent misidentification and to potentially include them in quantitative analyses. Due to false discovery constraints, intact-mass analysis is limited to a theoretical database with canonical sequences and a small number of annotated PTMs (in this study, combinations of up to two PTMs). However, the experiment–experiment comparison enables heavily modified proteoforms to be identified; if a proteoform with fewer modifications is identified in the experiment–theoretical comparison or through top-down MS/MS analysis, proteoforms from the same family with additional modifications can be identified through the experiment–experiment delta mass comparison.

At this time, identification of proteoforms by intact-mass alone in Proteoform Suite is also limited to common modifications that are observed at high frequency in the experiment–experiment mass comparison. If an uncommon modification is expected on a proteoform, adding this modification to the database could enable its identification by Proteoform Suite. Intact-mass analysis alone cannot localize modifications, so dynamic modification sites cannot be revealed by intact-mass analysis alone. However, knowledge of proteoform identity gives researchers the opportunity to better gauge whether subsequent targeted top-down experiments should be performed to localize modifications and confirm identifications returned by Proteoform Suite.

### Proteoform Level Classification

Smith et al. recently introduced the five-level proteoform classification system, which indicates the amount of ambiguity in a given proteoform identification, including ambiguity in the gene of origin, modification identification, modification localization, or sequence.<sup>29</sup> Level 1 proteoform identifications have no sources of ambiguity, Level 2's have one source of ambiguity, Level 3's have two different sources of ambiguity, Level 4's have three sources of ambiguity, and a Level 5 indicates that no information other than the observed mass of the proteoform is known. As described above, there were 166 intact-mass identifications and 44 ambiguous identifications. The intact-mass identifications do not have ambiguity in modification identification or sequence, whereas the ambiguous identifications have ambiguity in any of the four possible sources listed.

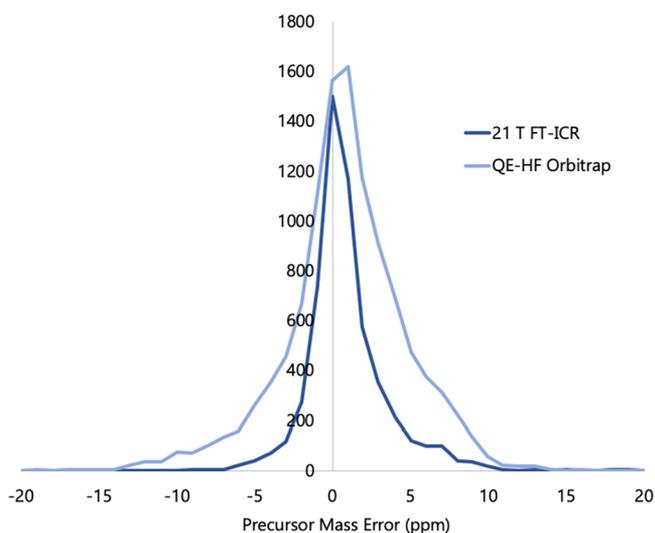
We determined the proteoform level for each of the 166 intact-mass identifications of which 13 were Level 1, 152 were Level 2, and 1 was Level 3. Of the 152 Level 2 identifications, 151 were ambiguous with respect to PTM localization, and one was ambiguous with respect to gene of origin. The one Level 3 identification was ambiguous due to both PTM localization and gene of origin. Of the 44 ambiguous intact-mass experimental proteoforms (Supporting Table S3), 11 were Level 3, 3 were Level 4, and 30 were Level 5. Although these proteoforms are ambiguous, the provided candidates can be helpful when searching for proteins of interest, and a subsequent targeted top-down analysis could determine their identity. The 1854 unidentified experimental proteoforms were also Level 5 assignments (Supporting Table S4).

Modifications are not localized in intact-mass analysis, so any intact-mass identifications of a modified proteoforms must be assigned as Level 2 or higher due to ambiguity in PTM localization. The level of ambiguity is dependent on the size of the theoretical database and search parameters utilized; for example, the number of PTM combinations allowed and the mass tolerances utilized could all affect how many theoretical proteoforms match each experimental mass. However, it is still useful to know for each intact-mass identification the sources and levels of ambiguity within the context of the search space and database utilized. Future analyses could integrate bottom-up modified peptide assignments, modification annotations in repositories, or subsequent targeted top-down analyses to localize PTMs and reduce the ambiguity of such identifications.

### Top-Down Precursor Intact-Mass Analysis

We compared our 21 T FT-ICR top-down results with a previously acquired QE-HF Orbitrap top-down dataset of GELFrEE fractionated MCF-7 cell lysate (data available on the MassIVE platform with identifier MSV000086148). The samples were separated on a 10% GELFrEE cartridge, and fractions 1 to 6 were analyzed (one MS/MS technical replicate). The LC–MS parameters are described in the QE-HF Data Acquisition section of the Supporting Information. We compared the 1% protein-level FDR results from the QE-HF TDPportal MS/MS search (Supporting Table S6) to the results from fractions 1 to 6 (first technical replicate each) of the 21 T FT-ICR TDPportal search to determine the mass accuracy improvements provided by the 21 T FT-ICR platform for human proteoform analysis.

A mass error histogram for all top-down proteoform hits is shown in Figure 3, indicating that the 21 T platform yielded increased mass accuracy for proteoform precursor masses. Missed monoisotopic mass errors were corrected prior to calculating the mass error. Approximately 47% of the 21 T FT-ICR top-down hits had sub-ppm precursor mass accuracy, whereas approximately 20% of the QE-HF top-down hits had sub-ppm precursor mass accuracy. It is important to note that mass error depends significantly on the averagine fits used to determine the monoisotopic mass because mass accuracy is limited to the difference between the averagine and true elemental composition of the analyte.<sup>30</sup> For example, in an analysis of a monoclonal antibody at 21 T, manual examination of light chain fragment monoisotopic masses yielded 0.3 ppm rms mass error based on elemental composition. Following monoisotopic mass assignments via averagine fitting, rms errors of 3.3 ppm were observed for the same data.<sup>31</sup> This issue, combined with difficulties associated with missed monoisotopic mass assignments, was the primary motivation behind the



**Figure 3.** Histogram of precursor mass error for all top-down hits for 21 T FT-ICR and QE-HF Orbitrap top-down analysis of six fractions (one technical replicate each) of MCF-7 cell lysate.

inclusion of an isotope filter algorithm in TDValidator software (Proteinaceous, Inc. Evanston, IL) that can match experimental isotopic peak clusters from the original raw data with calculated isotopic clusters given a specific sequence. Expanding the use of these algorithms to high-throughput top-down proteomics experiments would enable proteoform analysis to more effectively realize the benefits of state-of-the-art high-resolution mass spectrometers like the 21 T.

We performed a separate Proteoform Suite analysis on top-down precursor masses from TDPortal to evaluate the performance of intact-mass identifications returned by Proteoform Suite. We created a deconvolution result input file for Proteoform Suite using the precursor mass, constant intensity (this value is not reported by TDPortal), and retention time for each top-down proteoform that had been identified and well characterized by TDPortal (minimum C-score of 40). We used the same theoretical database and Proteoform Suite parameters comparable to those utilized for the intact-mass analysis described in *Methods*. Of the 489 top-down proteoforms, which met the minimum C-score threshold, 237 were identified in Proteoform Suite, 6 were ambiguous, and 246 were unidentified (Supporting Table S6). For the identified proteoforms, we compared the accession numbers, the unlocalized modifications, and the sequence to the original list of top-down proteoforms to confirm the matches. 221 of the top-down proteoforms matched, and 16 did not (6.8% FDR). However, we noticed that many of the proteoforms that did not match a top-down hit were histone proteoforms, which have high sequence homology and are heavily modified, so they could easily match a different histone identification. When considering only nonhistone proteoforms, 216 matched the top-down proteoform identification, and 10 did not, resulting in a calculated FDR of 4.4%, which is close to the FDR of 4.7% determined in the intact-mass analysis described above. There were 2 ambiguous proteoforms and 201 unidentified proteoforms in the nonhistone intact-mass analysis. There was a corresponding theoretical proteoform in the theoretical database for each top-down identification; therefore, the unidentified proteoforms were unidentified due to either a missed monoisotopic mass error in the deconvolution step or large precursor mass error.

One difference between a typical intact-mass analysis and this top-down precursor intact-mass analysis is that using such a file of true precursor masses means that every mass on the list is a likely real proteoform feature, and therefore the list does not include deconvolution artifacts (apart from missed monoisotopic errors). Deconvolution artifacts are a continuing challenge in top-down proteomics and even more so in intact-mass analysis, which relies heavily on the quality of the deconvolution output.

A challenging remaining problem is how to evaluate the fidelity of family construction for unidentified proteoform families. One possibility for future analysis of unidentified proteoform families is to perform a targeted top-down analysis of intact-mass proteoforms observed in unidentified proteoform families. Once the proteoforms in a family are confidently identified, the mass differences between proteoforms generated by Proteoform Suite could be evaluated for accuracy, i.e., to evaluate whether the proteoforms are grouped into the correct proteoform families.

As discussed in *Methods*, the Proteoform Suite theoretical database was created using sequences from a human canonical database downloaded from UniProt with PTM combinations of up to two annotated PTMs. A theoretical proteoform for each top-down proteoform was then added to the theoretical database if not already present. Of the 489 top-down proteoforms that met the C-score threshold, 277 did not have a corresponding theoretical proteoform mass in the database before supplementation with top-down identifications. This can be due to a larger number of modifications, an amino acid variant, or an unannotated truncation event. The significant proportion of MS2-identified proteoforms that would not be included as a theoretical proteoform in the Proteoform Suite database without a prior top-down analysis shows why intact-mass analysis is highly improved when integrated with top-down analysis; many proteoforms cannot be identified without MS2 data. Intact-mass analysis is, however, able to leverage these top-down MS2 identifications to identify additional, coeluting proteoforms from the same proteoform family. As proteoform identifications are continuously catalogued, databases will be more customized to a given sample and thereby increase the number of intact-mass identifiable proteoforms. Intact-mass analysis offers a simple approach to identify MS2-identified proteoforms in subsequent analyses, which will be particularly useful in quantitative and biological studies. This is one powerful motivation for the construction of deep proteoform catalogs for widely used cell lines, which will facilitate the rapid MS1-based identification and quantification of proteoforms

### Unidentified Proteoform Families

One unique attribute of Proteoform Suite is that proteoform families are constructed and visualized even for families without an identification. Of the 1854 unidentified experimental proteoforms, 463 were in the 170 unidentified families, and 1391 were orphans (no accepted experiment–theoretical or experiment–experiment relations formed). The orphan experimental proteoforms present a greater challenge to identification because no information is known about these experimental proteoforms other than their mass and retention time; additionally, it is difficult to know without manual inspection whether these orphan experimental proteoforms are deconvolution artifacts or real proteoforms observed in the MS1 spectra. A trade-off in deconvolution is using a criteria to prevent as

many artifacts as possible without filtering out low abundance experimental proteoform observations.

Fortunately, the unidentified families present additional information about the experimental proteoforms given the observed mass differences. For example, a family with multiple methylations was observed but remains unidentified (Figure 2D). Construction of families enables organization of observed intact-masses, which facilitates selection of interesting candidates for subsequent targeted analysis, such as the family in Figure 2D. A potential further development of proteoform family analysis could be network analysis to determine whether an experimental or biological condition increases the numbers of certain types of modifications, such as phosphorylation or of certain types of artifacts, such as SDS adduction.

As discussed above, many experimental proteoforms may be unidentified due to the theoretical database utilized, which only contained canonical sequences, combinations of up to two annotated PTMs, and top-down-identified proteoforms. Alternative splicing, amino acid variants, heavily modified proteoforms, and proteoforms with novel modifications all present major challenges for intact-mass analysis. Integration of different types of data, including data from genomic sequencing and bottom-up analyses, could also facilitate identification of more experimental proteoforms by intact mass.<sup>14</sup>

As expected,<sup>18</sup> the number of proteoforms identified decreased as a function of molecular weight (MW). This is largely due to decreased sensitivity inherent to mass spectrometric analysis (on LC timescales) of high MW proteins electrosprayed under denaturing conditions, as well as the need for improved separation of larger proteins by reversed-phase LC.<sup>9,10</sup> For these experiments, a delta mass mode (wide absolute mass search) was not performed in TDPortal, which is particularly detrimental to the identification of high MW proteoforms. Additionally, all MS/MS spectra were taken as a single or the sum of two transients. Sampling rate was prioritized over spectral quality (higher quality is achieved via additional signal averaging, requiring additional time per scan) to the detriment of high MW proteoform identifications. Despite this, over 193 proteins expressed as 428 proteoforms were identified in MCF-7 fractions 7 and 8 at 1% protein-level FDR. These proteoforms ranged in size from 4 to 48 kDa; 34 MCF-7 proteoforms >30 kDa were identified in total by TDPortal.

Increases in the number of proteoform identifications in top-down searches will improve intact-mass analysis. Top-down identification of just one proteoform in a previously unidentified family enables other proteoforms in the family to be identified by intact-mass analysis. One strategy for increasing the number of proteoform identifications in MS/MS search software programs is implementation of an open mass search, where the precursor mass tolerance is widened to enable identification of proteoforms containing unexpected PTMs not annotated in the database.<sup>23,24,32,33</sup> A remaining challenge is interpreting these large delta mass differences between the observed proteoform precursor mass and the match in the theoretical proteoform database. This mass shift interpretation will be necessary for integration of such top-down results in Proteoform Suite. One possibility is to employ the two-pass search strategy global PTM discovery (G-PTM-D)<sup>34</sup> implemented in MetaMorpheus.<sup>26</sup> A first pass open precursor mass search selects for discovered PTMs and adds these to the database; a second search is performed, localizing PTMs and reporting identifications with the novel PTMs localized. Future work will optimize this strategy for top-down proteoform analysis in MetaMorpheus to

identify novel PTM-containing proteoforms that were selected for MS2. These additional identifications will further improve proteoform family identification and intact-mass analysis by increasing the number of identified observed proteoform masses. As more comprehensive top-down analyses are performed and proteoforms are documented in repositories such as the Proteoform Atlas maintained by the Consortium for Top-Down Proteomics, identification by intact-mass will be increasingly useful for identifying larger numbers of proteoforms from complex samples.

## CONCLUSIONS

We used the freely available and open-source software program Proteoform Suite to construct human proteoform families from MCF-7 data acquired on the 21 T FT-ICR MS. TDPortal identified 1694 unique proteoforms at 1% FDR, 766 of which had a C-score of 3 or greater. From these 766 top-down proteoforms and the 2830 intact-mass experimental proteoforms observed, we constructed 520 proteoform families with Proteoform Suite. There were 166 additional proteoforms identified by intact-mass (~4.7% global FDR) using Proteoform Suite. The remaining 1854 were unidentified observed proteoforms, 463 of which contained at least one proteoform relation to another coeluting experimental proteoform. We performed an intact-mass analysis in Proteoform Suite of precursor masses confidently identified by MS2 in TDPortal and found that when histones were excluded from the analysis results, there was a false identification rate of 4.4%. This analysis shows how the 21 T FT-ICR MS platform enables intact-mass identifications in complex biological samples. Construction of proteoform families and intact-mass analysis offer a way to identify additional proteoforms in top-down analyses, visualize results, and provide interesting targeted top-down candidates.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00403>.

Retention time calibration, bottom-up MetaMorpheus search, and QE-HF data acquisition; top-down precursor mass error (Figure S1); top-down precursor retention time differences (Figure S2); experiment–theoretical and experiment–experiment delta mass histograms (Figure S3) (PDF)

Top-down proteoform identifications (Table S1); intact-mass proteoform identifications (Table S2); ambiguous intact-mass proteoforms (Table S3); unidentified intact-mass proteoforms (Table S4); top-down hit results from QE-HF Orbitrap analysis of MCF-7 (Table S5); intact-mass analysis of top-down precursor masses (Table S6) (XLSX)

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## Author Contributions

L.V.S. and M.R.S. developed Proteoform Suite. L.V.S. drafted the manuscript. L.C.A. and D.S.B. performed the mass spectrometry experiments. R.M.M. prepared the samples. L.V.S. and C.P. performed data analysis. L.M.S. provided oversight of the work. All authors edited the manuscript.

## Notes

The authors declare no competing financial interest.

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