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## The PeptideAtlas of the Domestic Laying Hen

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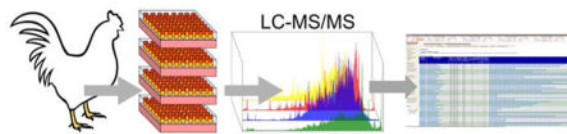
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### Abstract

Proteomics based biological research is greatly expanded by high quality mass spectrometry studies, which are themselves enabled by access to quality mass spectrometry resources, such as high-quality curated proteome data repositories. We present a PeptideAtlas for the domestic chicken, containing an extensive and robust collection of chicken tissue and plasma samples with substantial value for the chicken proteomics community for protein validation and design of downstream targeted proteome quantitation. The Chicken PeptideAtlas contains 6,646 canonical proteins at a protein FDR of 1.3%, derived from ~100,000 peptides at a peptide level FDR of 0.1%. The rich collection of readily accessible data is easily mined for the purposes of data validation and experimental planning, particularly in the realm of developing proteome quantitation workflows. Herein we demonstrate the use of the atlas to mine information on common chicken acute phase proteins and biomarkers for cancer detection research, as well as their localization and polymorphisms. This wealth of information will support future proteome-based research using this highly important agricultural organism in pursuit of both chicken and human health outcomes.

### Graphical Abstract



### Keywords

Biomarkers; Chicken; Ovarian Cancer; PeptideAtlas

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Links to the GUI front-end of the chicken PeptideAtlas are available for reviewers at: <https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/main.cgi?SBEAMSentrycode=G71UmfA>

The related SRMATlas, containing a reference library of SRM transitions derived in these experiments, will be available in 2017.

### Author Contributions

All authors have given approval to the final version of the manuscript.

## INTRODUCTION

The domestic laying hen (*Gallus gallus*) is the most abundant avian species on the planet<sup>1</sup> and a cornerstone species in food production and classical embryological study.<sup>2</sup> This importance was underscored by its selection as the first classical “production” organism to be fully genome sequenced, in 2004.<sup>3</sup> With the establishment of functional genomic tools for the chicken, it has officially entered the post-genomic era dominated by proteomic studies.<sup>4</sup>

Mass spectrometry (MS) based proteomics has reached widespread acceptance as a powerful tool to study organisms and protein products through multiple stages of disease, development, and degradation.<sup>5</sup> While the experimental techniques are well developed and robust, the application of proteomic methods to non-classical model organisms remains nascent. This is particularly true for organisms of predominantly agricultural interest, such as chickens, whose investigators frequently lack access to advanced MS resources available to human health researchers.<sup>6</sup> The result is that only ~10% of the chicken genome is well annotated<sup>7,8</sup> in spite of the enormous potential power of the chicken as a biological model system and its substantive importance as an agricultural product. The chicken was a classic model organism for embryological development in the pre-genomic era and the development of genetic tools have returned it to the fore.<sup>2,9</sup> Further, the hen ovary exhibits many similar genetic and physiological traits to humans and is the only non-human animal known to spontaneously develop ovarian cancer.<sup>10,11</sup>

Clearly there is enormous medical and economic potential in proteomics studies involving *Gallus gallus* that is limited by access to MS resources and the incomplete nature of chicken proteomic/genomic databases. Successful mass spectrometry experiments are greatly enabled by access to publicly available databases of validated data, such as those found in PeptideAtlas<sup>12</sup> or the ProteomeXchange<sup>13</sup> consortium database, PRIDE;<sup>14,15</sup> however, these databases are currently devoid of substantial information regarding the domestic hen. The completion of a reference database for chicken proteomics would substantially advance experimental planning and data validation for global and targeted analysis in this model system.

PeptideAtlas (<http://www.peptideatlas.org>) curates and compiles mass spectrometry data derived from a variety of experiments through the reprocessing of available MS data using the Trans-Proteomic Pipeline (TPP), a freely available open source suite of tools for tandem MS experiments.<sup>16–18</sup> The resulting proteome builds contain high stringency (<1.5% false discovery rate (FDR)) protein-level identifications and peptide observations from numerous experiments in a readily accessible interface, one that is particularly well suited to the evaluation of candidate peptides for targeted proteomics via SRM or data-independent approaches such as DIA.<sup>19</sup> More recently, the TPP has also incorporated the iterative reSpect algorithm to assess spectral chimeras and identify peptides that would otherwise be missed.<sup>20</sup> PeptideAtlas contains numerous well studied proteomes and model organisms such as *Saccharomyces cerevisiae* with > 70% proteome coverage,<sup>21</sup> and includes classical agriculturally important organisms, such as the pig (*Sus scrofa domestica*)<sup>22</sup> and the cow (*Bos Taurus*)<sup>23</sup>.

Here we present an initial build of a chicken PeptideAtlas, containing a variety of structurally complex ovarian tissue types, as well as diagnostically relevant plasma samples. Currently, the chicken PeptideAtlas is directed toward the hen as an ovarian cancer model organism, but data from liver tissue and plasma are included as a useful baseline, and additional tissue types will be incorporated into later builds. We demonstrate the current utility of the atlas through the mining of reported ovarian cancer biomarkers, as well as general inflammatory response proteins, and the selection of candidate peptides for quantitative SRM analysis.

## EXPERIMENTAL PROCEDURES

### Sample Collection and Processing

The biological samples included in the chicken PeptideAtlas were collected from two cohorts of Bovan's white leghorn commercial laying hens in the Poultry Science department at North Carolina State University. Birds were managed in accordance with the Institute for Laboratory Animal Research Guide with all of the husbandry practices being approved by and under the oversight of North Carolina State University Institutional Animal Care and Use Committee (IACUC). Plasma and tissue from the initial cohort of B-strain hens were obtained in a previous study,<sup>24</sup> with samples collected from a second cohort of 300 C-strain hens similarly. In brief, ~2mL of blood was collected from each bird every four months beginning at an age of 26 weeks. The collected blood was centrifuged at  $3000 \times g$  at  $4^\circ\text{C}$  and the plasma was collected in cryogenic tubes for long term storage at  $-80^\circ\text{C}$ . At an age of 136 weeks the birds were sacrificed by cervical dislocation and dissected to ascertain gross internal pathology. Tissue was collected from all birds presenting neoplastic lesions on the ovary, oviduct, duodenum, pancreas, GI tract, and/or liver as well as from a selected number of "healthy" birds with no visible distress. Tissues were preserved for proteomic study by snap freezing in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

Whole tissue samples were lysed using a 1:5 ratio of tissue to buffer containing 50 mM Tris pH = 7.8, 8 M urea, 2 M thiourea, 10 mM EDTA, 10 mM DTT, and 0.001 % sodium azide and homogenized using an OMNI TIP Homogenizing kit, as described previously.<sup>25</sup> A subset of liver and ovary tissues were instead subjected to laser microdissection to isolate yolk, ovarian stroma, and follicular wall tissue, which were subsequently lysed in a buffer of 100 mM Tris-HCl, pH 7.5, 100 mM dithiothreitol (DTT), and 4% sodium dodecyl sulfate.<sup>26</sup> Plasma samples were used as collected.

Cell lysates and plasma were processed for LC-MS/MS analyses using either a 1D SDS-PAGE in-gel digestion protocol<sup>24,25</sup> or filter aided sample preparation (FASP)<sup>27</sup> as described previously<sup>26</sup>. A subset of the FASP prepared samples were de-glycosylated on-filter using peptide-N-glycosidase F (PNGase F) according to a combined glycomics and proteomics protocol<sup>28</sup>.

### LC-MS/MS Analyses

Peptide samples were analyzed by LC-MS/MS using one of three sets of conditions:

1. Separation was performed on a nanoLC-2D (Eksigent Technologies) coupled to an LTQ-FT-Ultra with a 7T FT-ICR Mass Analyzer (Thermo Scientific) in a vented column configuration<sup>29</sup>. The column consisted of a 75  $\mu\text{m} \times 5\text{ cm}$  Integrafrit trap and 75  $\mu\text{m} \times 15\text{ cm}$  Picofrit column (New Objective) self-packed with 5 $\mu\text{m}$  Magic C18AQ stationary phase. Mobile phases A and B consisted of 98/2/0.2 and 2/98/0.2 water/ACN/formic acid, and the separation was performed as a 60 minute gradient from 2–60% B at a flow rate of 350 nL/min. Data was collected in Top-8 data-dependent mode over a mass range of 400 – 1600  $m/z$  with a full scan resolving power of 100,000 FWHM @ 400  $m/z$  using IT-CID and an AGC target of 1e6. Dynamic exclusion windows of 3 minutes were applied to each MS/MS precursor mass.
2. Separation was conducted with an EASY nLC II (Thermo Scientific) was coupled to a Q Exactive benchtop mass spectrometer (Thermo Scientific). The trap and column were arranged in the vented column configuration as in (1), and used a mobile phase gradient of 2%–5% B (2 min), 5%–40% B (200 min) at a flow rate of 300 nL/min. Parameters for the mass spectrometer were set according to optimums derived for proteomics experiments on the Q Exactive.<sup>30</sup> Briefly, data was collected in Top-12 data-dependent MS/MS mode using HCD with a full scan mass range of 400 – 1600  $m/z$ , a full scan resolving power of 70,000 and MS/MS resolving power of 17,500 FWHM @ 200  $m/z$ , and a dynamic exclusion window of 30 s. AGC targets were set at 1e6 and 2e5 for full scan and MS/MS scan respectively, with complementary maximum injections times of 30ms and 250ms.
3. Chromatographic separation was carried out with an EASY nLC-1000 (Thermo Scientific) coupled to a Q Exactive HF (Thermo Scientific). A 75  $\mu\text{m} \times 5\text{ cm}$  trap was prepared with a frit synthesized as described by Meiring *et al.*<sup>31</sup> and coupled to a 75  $\mu\text{m} \times 30\text{ cm}$  Picofrit emitter (New Objective). Both were self-packed with 2.6 mm, 100 Å particle size C<sub>18</sub> stationary phase (Phenomenex) and assembled in the same column setup as in (1) and (2). Peptides were separated by a gradient of 2 – 20% B (100 min), 20 – 32% B (30 min) at 300 nL/min. MS parameters were independently optimized for proteomics experiments on the Q Exactive HF and are reported in Hecht *et al.*<sup>28</sup> The data was collected in Top 20 data-dependent MS/MS mode using HCD with a 15 s dynamic exclusion window, a full scan mass range of 375–1500  $m/z$  and resolving power of 120,000 and 15,000 FWHM @ 200  $m/z$  for the MS<sup>1</sup> and MS<sup>2</sup> scans respectively. The full scan AGC and injection were set to 1e6 and 30ms for the MS<sup>1</sup> scan and 1e5 and 30ms for the MS<sup>2</sup> scans.

### Data Processing and PeptideAtlas Assembly

The processing of collected MS data followed the general workflow of Farrah *et al.*<sup>32</sup> with the Trans-Proteomic Pipeline<sup>18</sup> as the main component (Figure 1).

First, vendor .RAW files were converted to the mzML format<sup>33</sup> using the ProteoWizard<sup>34</sup> msConvert tool version 3.0.794. Next, the mzML files were searched using both Comet<sup>35</sup>

and X!Tandem<sup>36</sup> with the hrk-score plugin. The chicken search database was compiled as a nonredundant union of 24,231 chicken sequences from UniProtKB/TrEMBL, 16,354 sequences from Ensembl release 82,<sup>37</sup> and the cRAP database. For Q Exactive files, parent mass errors were set to  $\pm 20$  ppm with product ion mass tolerances of  $\pm 20$  ppm for X!Tandem and  $\pm 0.03$  Da for Comet. LTQ-FT data used a parent mass error of  $\pm 50$  ppm and product mass tolerance of  $\pm 0.4$  Da for both X!Tandem and Comet. Searching allowed semi-tryptic peptides and a maximum of two missed cleavages. A static modification of carbamidomethyl on C, and a variable modification for oxidation on M, acetylation of protein N-termini, and pyro-glu on Q, E, and C were also used. For samples processed with deglycosylation, a variable modification of deamidation on N was also searched.

The search results were processed with the TPP<sup>18,38</sup> version 4.8.1 tools. The pepXML<sup>17</sup> output for Comet was used directly, and the TPP tool Tandem2XML was used to convert the native X!Tandem output to pepXML. For each experiment, the set of pepXML files from the two search outputs were processed together with PeptideProphet.<sup>39</sup> The two output files were then combined via iProphet.<sup>40</sup>

The iProphet output was further processed using the reSpect algorithm<sup>20</sup> to access spectral chimeras. During reSpect searching the precursor mass tolerance was increased to match the MS isolation window. For LTQ-FT data the parent mass tolerance was increased to 2.0 Da and likewise increased to 1.4 Da for Q Exactive data. The new set of mzML files generated by reSpect were searched and processed using the TPP as for the initial files. For Q Exactive data, this process was repeated an additional time, for two complete rounds of reSpect in total. The spectra used for reSpect were filtered for a minimum iProphet probability  $> 0.0$  during the initial round, and  $> 0.5$  for the second round of analysis.

Using the PeptideAtlas processing pipeline, all the iProphet results from standard and reSpect searches were filtered at a variable probability threshold to maintain a constant FDR threshold for each experiment. The filtered data was assessed with the MAYU software<sup>41</sup> to calculate decoy-based FDRs at the peptide-spectrum match (PSM), distinct peptide, and protein levels. All results were collated in the Chicken PeptideAtlas, available at <http://www.peptideatlas.org>.

## RESULTS & DISCUSSION

### Overview of the PeptideAtlas

The chicken PeptideAtlas represents the most extensive collection of publicly accessible proteomic data for *Gallus gallus*, an important economic organism and resource tool. The database incorporates data from individual birds in various cancerous disease states, and fully samples the reproductive tissue, including differentiated portions of the ovary (i.e. small white follicles, ovarian stroma, etc.), which are nearly unexplored (Table 1).<sup>26</sup> The extensive sampling is enhanced by high resolution MS and MS/MS IT-CID and HCD data collected from both LTQ-FT-ICR and Orbitrap instruments, providing the highest quality mass spectra in order to support further study of reproduction and cancer biology in hen based systems.

Accessing the PeptideAtlas is accomplished through a readily accessible web GUI, providing observation information at both the protein and individual peptide level. Proteins and peptides of interest can be accessed using a sequence and/or list of accession number derived from Uniprot or Genbank This information can be readily used for comparisons between individual proteins contained in a single PeptideAtlas build, across species within the broader PeptideAtlas project, or generated from other sources such as KEGG or Reactome pathways.<sup>42,43</sup>

The chicken PeptideAtlas incorporates mass spectral data from over 800 individual biological experiments containing over 8 million spectra. The current build includes 5 million PSMs with an FDR threshold of 0.01% and over one hundred thousand distinct peptides at a false-discovery rate of 1%. This translates into 6,646 canonical proteins at a protein FDR of 1.3%. A substantial number of additional proteins are at least partially distinguishable, meaning that they possess at least one unique, well detected peptide within the chicken PeptideAtlas; the total number of observed proteins is 11,370, corresponding to 47% of *Gallus gallus*' 23,997 predicted gene products based on the UniProtKB/TrEMBL database. A sizeable fraction of these detected species exist at the single peptide level, which is to be expected based on the reproductive system focus of the current datasets. Future inclusion of additional sample types is expected to increase the overall gene product coverage of the chicken PeptideAtlas.

### Chimeric MS/MS reSpect Analysis

One of the basic assumptions of MS/MS data processing is that the fragment ion spectra obtained are derived from an individual precursor molecule isolated by MS. In spite of continued advancement in MS, precursor isolation windows remain relatively large to the achievable mass accuracy of the analyzers. Further, the complexity of whole proteomes remains well beyond the separation capacity of current chromatographic systems. The end result is that fragment spectra are frequently “chimera” containing peptide fragments from multiple precursors. While this is sometimes an intentional byproduct of the acquisition method, as in data-independent analyses, the collection of chimera can significantly reduce identifications in data-dependent sampling of complex samples due to the algorithmic MS/MS interpretation limitations<sup>44,45</sup>. This build of this initial chicken PeptideAtlas incorporates data processed with the newly developed reSpect tool,<sup>20</sup> which enables multiple processing of complex MS/MS spectra to isolate co-fragmented chimeric peptides and increase overall peptide/protein IDs.

Examining the output of the reSpect reveals the effect of its inclusion on the chicken PeptideAtlas coverage. New peptide identifications derived from the reSpect output account for  $825,135/5,125,800 = 16.1\%$  of the total PSMs included in the chicken PeptideAtlas, only five specific instances of which corresponded to the identification that would originally have been assigned by a standard search. This frequency of spectral chimeras is in concordance with previously reported values.<sup>45</sup> Within the chimeric group, 653,874 (79.2%) of the identifications have precursor masses falling outside the  $m/z$  tolerances of the initial search, and are therefore unintentional precursor inclusions as the result of wide precursor isolation windows. Interestingly, 378,194 (45.8%) of the PSMs were obtained from spectra that were



assigned a 90% probability in the initial search, while a roughly equivalent number, 387,714 (47.0%), were from spectra whose assignment was given a probability of < 50%. Therefore, the chimera spectra seem to be equally likely to be minor components of an otherwise identifiable spectrum or major interferences who impinge spectral assignment.

Regardless of the source or intensity of the chimeras, the reSpect step was able to improve the depth of PSM and peptide identification by an appreciable margin. For the most part, the additional PSMs correspond with peptides that are already included as the primary ID from another spectrum. However, 8,976 unique peptides were identified only as the result of reSpect processing, corresponding to ~ 8% of the total peptide IDs in the chicken PeptideAtlas. The inclusion of additional unique peptide IDs is obviously of significant importance given the goal of complete organism coverage, but additional validation of already identified peptides is likewise important for increasing the confidence of spectral assignment when combining the results of multiple searches in iProphet.<sup>40</sup>

### Ovarian Cancer Biomarkers in the Chicken PeptideAtlas

Ovarian cancer (OVC) is the most lethal of the gynecological cancers, with the single most important prognostic marker being clinical stage at diagnosis.<sup>46,47</sup> Currently, the vast majority of OVC cases present in later stages<sup>47,48</sup> and, as consequence, there is substantial interest in discovering biomarkers for early stages of the disease.<sup>49,50</sup> To this end, the domestic fowl has rapidly emerged as a uniquely suited organism for the study of spontaneous ovarian cancer.<sup>51–54</sup> Throughout the past 75 years, pathological examination of the hen has noted that spontaneous ovarian tumor formation occurs in up to 35% of the population and presents with the same histopathological subtypes seen in human disease.<sup>55–58</sup> An extensive degree of molecular investigation has identified both human similar molecular expression and novel markers using mRNA expression analysis and immunohistochemistry (IHC) based assays.<sup>59–65</sup> One major limitation of the model is the lack of commercially available chicken specific reagents for IHC and Western Blot analysis; the use of cross-reactive human antibodies has yielded inconsistent results, especially in the case of CA125, the primary prognostic marker for human OVC.<sup>66–68</sup> The PeptideAtlas is a powerful resource for mining of proteotypic peptides to develop SRM-based methods<sup>69</sup> that can augment or replace inconsistent mRNA and IHC approaches.<sup>70–72</sup> Further, the current sample set is an empirical examination of the existence of many biomarkers previously unexplored by LC-MS/MS. Several examples of chicken biomarkers will be discussed. An extensive, but by no means complete, list of ovarian cancer associated proteins can be found in Table 2 and in a previous review by Hawkrige *et al.*<sup>53</sup>

### Localization of Serine Protease Inhibitors

Serine protease inhibitors (Serpins) are a superfamily of protease inhibiting proteins ubiquitous to eukaryotic organisms whose regulatory members share a unique mechanism of irreversible “suicide inhibition” of target proteases.<sup>73–75</sup> Because protein homeostasis is controlled by proteinases and their inhibitors, this superfamily has attracted attention in the field of cancer biology, where tumor progression necessarily requires disruption of homeostasis.<sup>75,76</sup> Within the family, specific proteins have been identified as prognostic biomarkers,<sup>77,78</sup> tumor tissue markers,<sup>79</sup> and potential diagnostic markers for a range of

cancers,<sup>80–83</sup> examples of which will be discussed further. Searching the chicken PeptideAtlas for proteins that have a description that contains “SERPIN” yields 18 unique protein groups from multiple subgroups of the serpin family, including many of interest in cancer research.

Serpin class “A” molecules are one of the largest serpin groups in humans, consisting of extracellular molecules.<sup>75</sup> Five chicken orthologs of this class (E1C7T1, F1NPN5, E1BS56, E1C206, and F1NPN4) were identified in the chicken PeptideAtlas with an average coverage of 70%. As expected, excreted species are observed in plasma and liver tissue. Two of the species however, are also observed in hen reproductive tissues. SERPINA1 (E1C7T1, alpha-1-antitrypsin) is the most common serum serpin and its misregulation has been associated with endometrial and cervical cancer in humans.<sup>84</sup> SERPINA3 (F1NPN5, alpha-1-antichymotrypsin) has likewise been associated with the promotion of endometrial cancer cells, as well as numerous other cancer types.<sup>85</sup> The detection of these potential biomarkers in endometrial tissue of the chicken ovary is not unexpected, but cancerous associations derived from human studies have not been confirmed in the chicken. This relationship is worthy of further MS studies in the chicken model system, with particular emphasis on the translation of disease behavior between species.

Serpin class “B” molecules are generally intracellular proteins and similarly to class A are frequently associated with regulation and disease;<sup>76</sup> the current chicken PeptideAtlas build contains three such proteins. SERPINB11 (P01013) is an important regulator of estrogen expression in chicken oviductal tissue; it is implicated in both normal oviduct development in the chicken and the development of ovarian endometrial cancer.<sup>59,79</sup> Peptides from this protein were identified with 100% sequence coverage across healthy and cancerous oviduct tissues, consistent with its specific role in that tissue type. Interestingly, while identifications in plasma or liver tissue are limited to single observations, SERPINB11 peptides can be identified at consistent low levels in ovary samples from some healthy individuals, as well as in cancerous samples. This is consistent with the low levels of expression seen in RT-PCR experiments with developing ovarian follicles<sup>79</sup> but the potential for ovarian tumors to originate from oviduct or mullerian cell incursion is considered quite high.<sup>86</sup> The monitoring of cellular migration in the hen reproductive system offers the potential for understanding the biological origin and progression of ovarian cancer, which will aid in our understanding, detection, and treatment of the disease.

Additional serpins common in specific sample types are associated with multiple disease states and biological stress responses. SERPINC1 (F1NLP7, Antithrombin-III) and SERPINH1 (P13731, Hsp47) are among the most abundant proteins detected in plasma samples within the PeptideAtlas, as befitting their roles in blood. Antithrombin-III is an anticoagulant shown to be effective prognostic markers for some cancer types due to the systemic response to carcinogenesis,<sup>87</sup> but more commonly used as a protein therapeutic.<sup>88</sup> Hsp47 meanwhile, is of interest due to its relationship with thermal regulation and stress.<sup>89</sup> It is therefore apparent that in addition to its use as an ovarian cancer model system, the included samples allow for the creation of experiments addressing additional diseases and biology of interest in chickens.



## Acute Phase Proteins in the Chicken PeptideAtlas

One of the broadest diagnostic tools for the investigation of systemic response to stressors is the acute phase response (APR). The APR is a generalized systematic response of the immune system to numerous events, including trauma, inflammation, infection, stress, or carcinogenesis, with the goal of restoring organism homeostasis and promoting healing.<sup>90</sup> Acute Phase Proteins (APPs) are hepatic proteins produced as the result of pro-inflammatory cytokines during trauma and are a powerful tool for examining immune and stress response.<sup>91</sup> The initial description of APPs involved the changing concentrations of plasma C-reactive protein in response to pneumococcal infection<sup>92</sup> but has since expanded to encompass numerous plasma proteins in both human and non-human animal systems.<sup>93,94</sup> Generally, the APPs are divided into classes based on whether they increase or decrease in concentration in response to the APR, as well as the magnitude of the change. This expression can be highly species specific, requiring the development of unique assays and the selection of unique assay targets for different study organisms.<sup>93</sup>

APP profiles in many species have been used as diagnostic tools and as a mechanism to understand the biological response to system challenge, such as infection.<sup>95</sup> Unsurprisingly, various APPs in the chicken (Table 2) have likewise been thoroughly classified by response and magnitude.<sup>96</sup> Albumin is the only notable negative APP (relative abundance decreasing upon immune challenge) in the chicken, but with plasma concentrations approaching 20 mg/mL it is a trivial target for proteomic analysis.<sup>97</sup> The positive APPs range in concentration from ng/mL – µg /mL quantities and their responses can be categorized as major ( 10-fold abundance increase), moderate (4–10 fold), and minor ( 3 fold).<sup>96–98</sup> Monitoring APPs at these levels has previously relied on enzyme-linked immunoassays (ELISA), often using partially cross-reactive murine or human antibodies.<sup>99</sup> This method is undesirable for the discovery of avian APPs, due to cost, laboriousness, and frequent lack of similarity with more well understood mammalian systems. For example, haptoglobin is a very common APP in a wide range of species,<sup>91</sup> but it was not until the chicken genome was sequenced that it was determined that no homologous protein exists in *G. gallus*.<sup>100</sup> an antioxidant protein from gene PIT54 fills the hemoglobin binding role in chickens instead.<sup>100,101</sup> Invaluable information on novel APP proteins, as well as thorough investigation of existing APPs will be greatly enhanced by the application of quantitative mass spectrometry techniques enabled, in part, by quality peptide and spectral libraries.

The chicken PeptideAtlas contains extensive liver and plasma samples from both healthy and diseased chickens, and contains all well-studied APPs in the organism (Table 3). Due to the underannotated nature of the chicken proteome, many APPs contain multiple potential variants in the global UniProtKB/TrEMBL database that are grouped by the chicken PeptideAtlas under the most likely canonical form. The PeptideAtlas can be used to explore the existence of one or more protein isoforms, as well as a starting point for the development of distinguishing assays.

## Hemopexin Polymorphism

Hemopexin is an acute phase serum glycoprotein with enormous binding affinity for free heme, and is found across multiple animal families.<sup>102</sup> The protein was initially isolated on

the basis of its heme-affinity<sup>103</sup> and has multifunctional properties in iron-metabolism.<sup>104</sup> However, it has been shown to be APP based on regulated expression in response to hormonal stimuli,<sup>105</sup> and infection with various pathogens,<sup>106–108</sup> which is believed to be its primary biological role.<sup>104</sup>

The chicken PeptideAtlas contains multiple entries for chicken Hemopexin proteins derived from multiple sources. The manually annotated UniProtKB/Swiss-Prot database contains chicken protein P20057, which is an N-terminal fragment rather than a complete protein,<sup>109</sup> while the UniProtKB/TrEMBL database contains an entry from the chicken genome (ENSGALG00000022586 / H9L385) as well as a non-genomic sequence fragment from a partial cDNA library (Q90WR3). Mutual alignment of the sequences revealed virtually no similarity between the manually determined N-terminal sequence and either of the other forms, while H9L385 and Q90WR3 possess 99% sequence similarity within the fragment region (Figure S-1 and S-2). The observed peptides within the chicken PeptideAtlas yield over 98% sequence coverage for the genetically derived data, while no portion of the manually determined sequence could be identified, casting doubt on its veracity. Within the two genetic sequences, three polymorphisms can be observed, two of which (p.Arg282Met and p.Lys148Arg) are not addressable because the small peptides constitute the unobserved 2% of the sequence. However, variants of the remaining polymorphism (p.Arg235His) were detected with unique peptides. The peptides CSGEPFQAITSDDSGR from H9L385 and CSGEPFQAITSDDSGHIYAFR from Q90WR3 (polymorphic site noted) were each observed in several hundred quality spectra across experiments, many with nearly full y-ion series, lending credence to the existence of the non-genomic protein sequence. Disaggregation of the samples to examine individual organisms from a published study<sup>24</sup> reveals that specific chickens can present with either one or both of the sequences. Chickens #602, 612, 630, 639, and 666 exhibit peptides from both sequences, while #600, 601, and 650 exhibit only the unique peptide from H9L385 and #620 exhibits the form from Q90WR3, implying both hetero- and homozygosity of the polymorphism occur in the population. The chicken PeptideAtlas thus reveals the existence of multiple forms of a common chicken APP and provides peptides suitable for quantification of either individual polymorphisms or total protein.

### Peptide Deglycosylation

N-linked glycosylation is an extremely common post-translational modification consisting of potentially diverse oligosaccharide structures linked to asparagine residues at a conserved sequence motif (N-X-(S/T), X P) and which affects the majority of gene products.<sup>110</sup> The modification that occurs is a non-template driven process and due to the availability of N-glycan precursor, the accessibility of the glycosylation site, and the variable transit time of proteins, the majority of glycosylation motifs are not modified by sugars; others exhibit partial or complete occupancy, which can have significant implications for their biological function.<sup>111,112</sup>

During sample processing for the chicken PeptideAtlas a representative number of samples were deglycosylated using the enzyme peptide-N-glycosidase F (PNGase F) to cleave N-glycans and generate a detectable asparagine deamidation (mass shift: +0.9848 Da). This

processing has a two-fold implication on the resulting peptides: increased peptide coverage from detection of newly deglycosylated peptides, and identification of potential glycosylation sites for future study based on the co-occurrence of the glycosylation motif and asparagine deamidation. The latter is usually performed with  $^{18}\text{O}$  water to provide unambiguous identification of enzymatic deamidation,<sup>113</sup> but this is unfeasible for a project of this size. Nevertheless, a processing method designed to reduce non-specific deamidation combined with high resolution mass spectrometry enables accurate site determination sufficient for initiating deeper study.<sup>28,114</sup>

The protein prothrombin is an important coagulation precursor with multiple known glycosylation sites in different species.<sup>115</sup> The glycosylation of these sites is important to protein function and processing, but different species exhibit different numbers of active glycosylation sites.<sup>116,117</sup> The protein sequence in the chicken (UniProt Accession: F1NXV6) is 607 amino acids long and contains four potential N-glycosylation motifs at Asn122, Asn144, Asn161, and Asn403 which are homologous with the four sites observed in the human form of the protein (Figure S-3). Specific deamidated versions of each of the four potential glycopeptides (GTInYTK, FnASIYPDLTENYCR, NPDnNSEGPWcYTR, and nLTTNDILVR) were able to be observed, as well as longer fragments containing miss cleavages. Of the tryptic peptides, three of the four were observed only in deglycosylated samples, and were observed only in their deamidated forms; this implies that these sites are wholly glycosylated and the identification of these peptides is entirely related to the PNGase F processing. In contrast, one of the peptides (NPDNNSEGPWCYTR) was observed in both deamidated and non-deamidated forms in the deglycosylated samples, as well as being identified in the PNGase F free preparations, implying only partial site occupancy. Direct comparison of biological samples 6755 and 6798 (Table 1) showed a 70% increase in the number of peptide observations as the result of the inclusion of deamidated, and therefore deglycosylated, peptides. Homology comparison to humans would predict that the three wholly glycosylated sites would be the only occupied sites.<sup>115</sup> However, the rigorous identification of a partially occupied site could point to a different structural or processing role in the chicken than the human, which could be worthy of further study.

## CONCLUSION

The initial build of the chicken PeptideAtlas represents over 100,000 high confidence peptide identifications from MS/MS spectra corresponding to nearly 50% of the translated chicken proteome. The collection is rich in representative proteins of substantial interest in future health research in both chicken and human. Herein we have demonstrated the coverage of the chicken PeptideAtlas for an expansive number of proteins relevant to the chicken as a model for human ovarian cancer, important for investigations of systemic inflammatory responses in a veterinary context, and of general biological and agricultural interest to the study of the chicken organism. The chicken PeptideAtlas provides a readily accessible repository of information on the MS observable chicken proteome, providing a basis for proteomic experiments in the assay of known proteins, useful for discovery of novel biomarkers, and validation of genomic information at the proteomic level. We have demonstrated the use of this information for the isolation of single amino acid polymorphisms in inflammatory proteins as well as localization of tissue specific markers at

the sample level within the chicken PeptideAtlas. This powerful resource is freely available to mine data for efforts such as in biologically driven hypotheses of both human health and agricultural interest and for approaches in biomarker discovery for quantitative MS assays.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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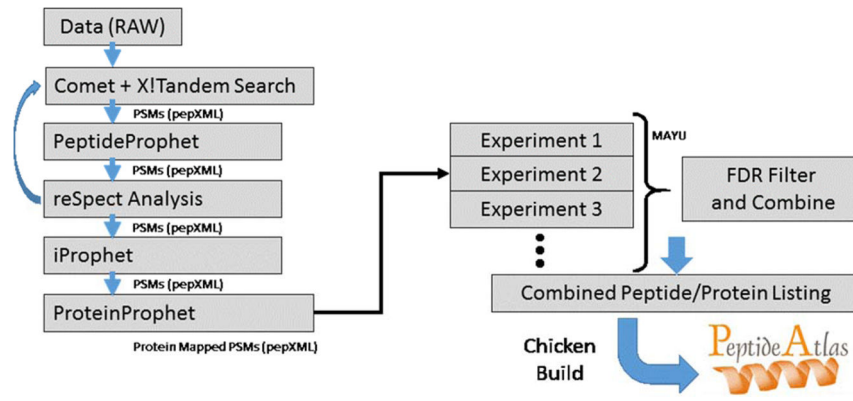
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**Figure 1.** Schematic workflow of the PeptideAtlas build process.



**Table 1**

Summary of sample types included in the chicken PeptideAtlas, including disease status of epithelial ovarian cancer (EOC) and/or oviductal cancer (OVD). A complete list of included files can be found in the Table S-1.

ID	Tissue	Disease State	Instrument Protocol	# Spectra ID'd	# Distinct	# Proteins
6757	Liver	Healthy	2	659875	34645	3616
6773	Ovary	Healthy	1	163185	15048	2725
6758	Ovary	Late Stage EOC	1	170087	17160	3149
6762	Ovary	Early Stage EOC	1	200708	18906	3446
6754	Ovary	Late Stage EOC-OVD	1	1234	671	317
6772	Oviduct	Healthy	1	55924	8888	1232
6761	Oviduct	Early Stage EOC	1	64427	10978	1665
6765	Oviduct	Late Stage EOC	1	43347	10418	1633
6764	Oviduct	Late Stage EOC-OVD	1	24018	8716	1566
6774	Ovarian Granulosa	Healthy	2	69787	16457	2779
6770	Ovarian Stroma	Healthy	2	77159	19709	2915
6756	Small White Follicle	Healthy	2	4938	2329	576
6771	Ovary	Healthy	2	91301	19561	2928
6760	Ovarian Follicle Yolk	Healthy	2	56365	13006	2178
6766	Plasma	Early Stage EOC	1	161840	3594	228
6767	Plasma	Late Stage EOC	1	111422	3849	245
6753	Plasma	Late Stage EOC-OVD	1	57269	3150	206
6763	Plasma	Healthy	1	188913	4277	239
6755	Plasma	Healthy	3	133106	6751	226
6768	Plasma	Late Stage EOC	3	144047	7121	279
6801	Ovary	Healthy	3	269263	29960	4149
6797	Ovary	Late Stage EOC	3	292654	35261	4310
6795	Oviduct	Late Stage EOC	3	331682	36078	4728
6799	Oviduct	Healthy	3	187123	18221	2968
6798	Plasma	Healthy	3	255303	9250	248
6796	Ovary	Healthy	3	482706	32718	3990

ID	Tissue	Disease State	Instrument Protocol	# Spectra ID'd	# Distinct	# Proteins
6800	Ovary	Late Stage EOC	3	507992	33611	4151
6794	Oviduct	Healthy	3	381373	26717	3033
6802	Oviduct	Late Stage EOC	3	379049	36305	4461

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Table 2

A list of ovarian cancer associated protein biomarkers detected in the PeptideAtlas.

Protein	Uniprot Accession	# Peptide Observations	# Distinct Peptides	Protein Group(s)	# Unique Proteins	Sample Type	Ref.
alpha-2-macroglobulin (A2M)	E1C544	48220	440	E1C344	4	P, T	[118]
Angiotensin-converting enzyme	FINYM0	399	45	FINYM0	2	T	[119]
Apolipoprotein A-1	P08250	34918	242	P08250	1	P, T	[120]
Apolipoprotein B	FINV02	103453	1236	FINV02	17	P, T	[121]
B-2 Microglobulin	P21611	525	9	P21611	6	P, T	[122]
Cadherin-1	P08641	791	42	E1C6M9	4	T	[123]
ERBB2/HER2 Analog	Q2EJ72	244	20	E1C0S3	12	P, T	[124]
Fibronectin	P11722	10241	117	FINJT3	6	P, T	[125]
Fibulin-1	FINX60	1613	40	FINX60	3	P, T	[126]
Hras / Kras	P08642	533	12	F5ANT1	4	T	[111]
Metalloproteinase inhibitor 2	R4GIL5	46	4	R4GIL5	2	P, T	[127]
Mucin-6	R9PXQ5	1488	121	R9PXQ5	2	T	[128]
Mucin-5B	Q98UI9	7483	273	Q98UI9	2	T	[128]
Ovalbumin	P01012	117199	528	R9XP5	2	P, T	[129]
Ovostatin	P20740	3570	123	FINU63	4	T	[130]
OVOSTL	FINEW8	1829	69	FINEW8	1	P, T	[130]
SERPINA1 / Alpha-1-antitrypsin	E1C7T1	2158	48	E1C7T1	1	P, T	[84]
SERPINA3 / Alpha-1-antichymotrypsin	FINPN5	1813	44	FINPN5	1	P, T	[85]
SERPINB11	P01013	1357	66	IOJ178	4	T	[59]
SERPINB2 / Plasminogen activator inhibitor 2	E1BTH3	190	15	E1BTH3	1	T	[131]
Transglutaminase	Q01841	16255	102	Q01841	3	T	[129]
Transhyretin	P27731	5864	59	P27731	1	P, T	[132]
Vimentin	P09654	33522	128	FINJ08	22	T	[133]

**Table 3**

Acute phase proteins of the chicken identified in the chicken PeptideAtlas. A highly redundant number of spectra were collected for each peptide, with numerous unique peptide IDs allowing for nearly complete coverage of many APPs.

Protein	PeptideAtlas Protein Name (UniProt Accession Number(s))	PeptideAtlas Group(s)	Spectra	Unique IDs	% Cover	Ref
Ceruloplasmin	FIN9R5, FINPE0, Q5ZIT6, FINY12	FIN9R5	2111	83	68.4	[134]
Fibronectin	P11722, FINIT4, P11722, O57403	FINJT3	10241	117	94.5	[125]
Alpha-1-acid glycoprotein	Q8JIG5, A7UEB0	Q8JIG5	17665	117	87.6	[97]
Ovotransferrin	P02789, E1BQC2, F1P2F0, P02789, P15989, Q4ADI6, Q4ADI7 // FINVN3, Q92062	E1BQC2 // Q92062	159189	668	97.5	[135]
Serum Amyloid A	FINW65, D6P887, Q9PSM7	FINW65	42	5	46.7	[98]
PIT54	Q98TD1, H9KZK6	Q98TD1	13572	144	97.6	[136]
Mannan-binding lectin	G3FGP5, O57451, Q98TA4, FINKG3, U6A6LU9, FINKG3	G3FGP5	628	19	70.1	[137]
Hemopexin	H9L385, Q90WR3 // P20057	H9L385 // P20057	17639	134	98.5	[138]
Serum Albumin	P19121, F2Z4L6, R4GFD0	P19121	194669	819	97.0	[139]
Fibrinogen	F1P4V1, P14448 // F1NUL9, F1P5F9, Q02020 // E1BV78, O93568	F1P4V1 // F1NUL9 // E1BV78	114957	981	87.2	[140]