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Examining the Proteome of *Drosophila* Across Organism Lifespan

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A survey of the proteome of *Drosophila melanogaster* at nine time points across the adult lifespan based on several mass-spectrometry-based techniques is presented. In total, there is evidence for 5902 unique peptides corresponding to 1699 different proteins. Of hundreds of relatively abundant components, many appear to be highly dynamic as the adult fly ages. Of those proteins that we observe changing with age, a majority, associated with metabolism, reproduction, and development, are down-regulated. Other biological pathways such as defense response also show variable changes, where some proteins are down-regulated and others are up-regulated. The observed variations are compared with a report of genome-wide changes at the transcriptome level at different ages and the similarities and differences are presented.

Keywords: aging • *Drosophila melanogaster* • proteomics • ion mobility

Introduction

There is tremendous interest in understanding the fundamental processes associated with aging.[1] Morphological changes in humans are well-documented; however, much remains to be understood about processes at the tissue, cellular, and subcellular levels. Studies of model systems, such as *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (worms), *Mus musculus domesticus* (mice), and *Drosophila melanogaster* (fruit flies, hereafter referred to as *Drosophila*) provide much of our insight into the underlying biological pathways associated with aging.[2] Because drafts of the genomes of these model organisms are available as well as a variety of genetic tools, it is possible to systematically introduce changes in order to test hypotheses. In this paper, we survey the proteome of *Drosophila* at nine time points across the lifespan of the adult organism using several mass-spectrometry-based techniques. This report includes evidence for detection of 1699 proteins; a few hundred of the most abundant proteins (those that meet our criteria for change) appear to vary in abundance as the fly ages. These data are compared with previous global studies of transcript abundance. These studies and a comparison of the results of changes in the transcriptome and proteome with age should provide new insights about aging and should be valuable in the design of further experiments that test the ramifications of gene expression as a function of age.

As a model system for studying aging, *Drosophila* is attractive for a number of reasons. These animals have relatively short lifespans, living ~60 days on average;[3,4] they are relatively easy to handle, and it is straightforward to control their environments (diet, temperature, humidity, exposure to light, etc.). It is possible to obtain large populations of animals, and the ages of individuals in the population can be synchronized. Additionally, *Drosophila* is a post-mitotic organism; thus, the adults produce few (if any) new cells in most organs with the exception of the germline.[3,4] Thus, the information that is obtained corresponds directly to how cell types or organs age (without the need to account for contributions from new cells that are formed during the animals' lifetime).

Most important is the well-characterized nature of this model.[4] Many studies have characterized gene expression changes in aging *Drosophila*,[5−15] and the present report benefits from much of this work. Zou et al.[6] have reported a genome-wide study that provided evidence for 132 expressed sequence tags (ESTs) that were regulated with age. The genes identified are involved in metabolism, protein turnover, reproduction, and detoxification. Also recognized were a number of genes that were influenced by oxidative stress when flies are exposed to paraquat, a free radical generator. Landis et al.[13] measured gene expression as a function of age and oxidative stress, and reported that many changes are associated with genes involved in immune response, antioxidant activity, purine biosynthesis, and heat shock. Pletcher et al.[11] obtained transcript profiles for wild-type flies that were fed either *ad libitum* or calorically restricted diets. Genes related to stress response, oogenesis, and response to microbial infection were regulated with age, and genes involved in cell growth, metabolism, and reproduction were regulated in calorically restricted flies. In a more recent study, Kim et al.[15] detected down-regulation of genes involved in energy metabolism, protein turnover, response to light, and synaptic transmission.

The present survey of the changes in protein expression as a function of aging complements the transcriptome information and provides a more complete view of changes that occur. It is common in studies of *Drosophila* to focus on specific regions of the animal (e.g., heads, thorax, abdomen, specific tissues, or cell types).[6,15] As described in more detail below, the

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present work focuses on heads, which are especially interesting. Transcriptome studies indicate that age-related changes in the head differ from those observed in the body.[15] Moreover, studies of heads are valuable in assessing neurological changes.[16] This said, changes at the protein level in these studies must be compared carefully to changes observed in existing transcript studies. This is because of differences in the strains of animals used, sexes of animals (i.e., males or females), rearing conditions (i.e., temperature and diet), body regions harvested (i.e., whole animals vs heads or bodies), and time points and number of time points harvested across organism lifespan.

A number of aging studies have focused on expression of specific proteins;[17−21] however, only two have examined global protein expression for adult *Drosophila* of different ages. In pioneering work, Fleming et al.[22] utilized two-dimensional gel electrophoresis to measure patterns of protein expression in 10-, 28-, and 44-day-old animals (however, no individual or specific proteins were identified). Xun et al.[16] reported proteins expressed at three ages that are associated with different disease states in a *Drosophila* model for Parkinson's disease (transgenic animals expressing the human A30P variant form of α-synuclein); the proteome changes associated with aging were not addressed.

Experimental Procedures

General. Three analyses, which differ in sample workup or the type of instrumentation that is used, have been carried out. In one type of analysis, mixtures of peptides are analyzed by on-line reversed-phase liquid chromatography (LC) combined with mass spectrometry (MS and MS/MS) detection (using a commercial LCQ ion trap). Peptide sequences (and hence proteins) are identified using standard database techniques, as discussed in more detail below. In a second type of analysis, tryptic peptide mixtures are first separated using strong-cation-exchange chromatography (SCX), and individual fractions are analyzed by LC−MS/MS. As reported by others,[23,24] the additional condensed-phase separation substantially increases the number of peptide and protein identifications. In a third type of analysis, we use a prototype gas-phase ion mobility spectrometry (IMS) separation method developed in house.[25,26] This technique differs from the commercially available analyses in that there is no formal mass-to-charge (*m/z*) selection for MS/MS studies. Because of this difference, it is sometimes possible to detect some proteins that were not selected for MS/MS in the commercial approach. Once a peak is identified, it is possible to integrate and normalize ion intensities in order to provide information about relative changes in abundance. At the time of these experiments, the instrumentation and code for interpretation were at an early stage; thus, we used the results from our home-built instruments primarily to complement results from the commercial systems. A more detailed discussion of the IMS instrument is presented below.

Drosophila Husbandry and Harvesting. An initial population of ∼25 000 animals was used to obtain a subgroup of adult wild-type Oregon-R females (Bloomington Stock Center, Indiana University). These animals were age-matched by separating them into cohorts of 100 animals within 12 h of eclosion. These groups were kept together throughout the experiments until a specified age of harvest. Females were transferred to fresh food[27] every 3−4 days. Populations were maintained at 24 ± 1 °C, ∼30%−50% relative humidity, and exposed to an alternating cycle of 12 h of light and 12 h of darkness each day. Animals were harvested daily over the course of the adult lifespan. Briefly, heads are obtained as follows: animals were anesthetized by exposure to CO_2 gas, transferred to Nalgene tubes, and frozen by submerging the tube in liquid nitrogen; tubes were shaken by hand several times to separate body parts, and heads were collected over a bed of dry ice and stored at −80 °C until further use.

Sample Preparation. Twenty-five heads from each daily harvest were pooled to give a total of 175 heads for each experimental time point as follows: 1−7 days for experimental time-point one, 8−14 days (time-point 2), 15−21 days (time-point 3), 22−28 days (time-point 4), 29−35 days (time-point 5), 36−42 days (time-point 6), 43−49 days (time-point 7), 50−56 days (time-point 8), and 57−60 days (time-point 9). This pooling strategy was carried out twice for each experimental time point. This approach (collection in daily increments and pooling of multiple days) provides a means of integrating the proteomic changes across all ages in the lifespan, while minimizing interactions between animals at different ages. For example, 1-day-old animals are not in contact with those that are 7-days old. Another motivation for this pooling strategy is that it provides enough material for replicate experiments. We note that time-point 9 includes only 100 heads due to the lack of living animals that remained at later ages.

Proteins were extracted as follows. The isolated heads were suspended in phosphate-buffer saline solution (containing 4.0 M urea and 0.1 mM α-toluenesulfonyl fluoride) and then ground into a homogeneous slurry using a motorized pestle. The total protein concentration was determined with a Bradford assay to be 1−2 mg. Disulfide bonds in extracted proteins were reduced using a 40-fold molar excess of dithiothreitol at 37 °C for 2 h. The free thiol groups were alkylated with an 80-fold molar excess of iodoacetamide at 0 °C for 2 h in darkness. The reaction was quenched with a 40-fold molar excess of L-cysteine for 30 min at ambient temperature. The final concentration of urea in the protein solution was reduced to 2.0 M with 0.2 M Tris buffer (10 mM $CaCl_2$, pH = 8.0). TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO) was added (2% w/w, mass of enzyme to that of protein) and allowed to incubate for 24 h at 37 °C for digestion. Tryptic peptides were desalted using Oasis HLB cartridges (Waters, Inc., Milford, MA), dried on a centrifugal concentrator, and stored at −80 °C until further use.

SCX Chromatography. One set of the samples was fractionated by SCX, as follows. Mixtures of tryptic peptides (∼1.2 mg) were injected onto a PolySulfoethyl A column (2.1 mm i.d. × 100 mm, 5 μm, 300 Å; PolyLC, Inc., Columbia, MD). Mobile phases consisted of 5 mM potassium phosphate in 75:25 water/acetonitrile at pH = 3.0 (solvent A) and solvent A with the addition of 350 mM KCl (solvent B). The following gradient was delivered at a flow rate of 0.20 mL·min^{-1} using a LC system consisting of a 600 Pump and 2487 Dual Wavelength detector (Waters, Inc., Milford, MA): 0% B for 5 min, 40% B in 40 min, 80% B in 45 min, 100% B in 10 min, and hold 100% B for 10 min. One minute intervals of the eluent (110 total) were collected into 96 well plates, and these were pooled to generate 10 fractions. UV detection was monitored at $\lambda = 214$ nm. Each SCX fraction was desalted using Oasis HLB cartridges, dried with a centrifugal concentrator, and re-suspended in 0.1% formic acid for further analysis.

LC−MS/MS and Off-Line SCX-LC−MS/MS Analyses. A nanoflow LC system (consisting of a FAMOS auto-sampler, Switchos pump, and Ultimate pump, Dionex, Inc., Sunnyvale, CA) was used for sample injection and gradient delivery.

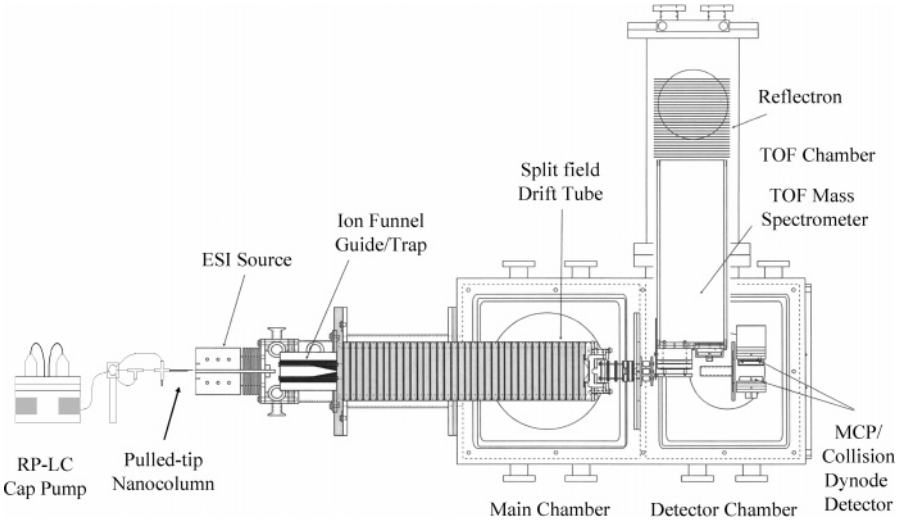

Figure 1. Schematic diagram of the LC−IMS−MS instrument used in this analysis.

Samples were injected onto a trapping column (100 μm i.d. × 1.5 cm; New Objective, Inc., Woburn, MA) packed with 5 μm, 200 Å Magic C18AQ (Microm BioResources, Inc., Auburn, CA) at a flow rate of 4 μL·min^{-1}. Mobile phases were pumped at a flow rate of 250 nL·min^{-1}. Pulled-tip columns were prepared with fused silica (75 μm i.d. × 150 mm; Polymicro Technology LLC, Phoenix, AZ) and packed in-house with a methanol slurry consisting of 5 μm, 100 Å Magic C18AQ (Microm BioResources, Inc., Auburn, CA). Mobile phases were 96.95:2.95:0.1 water/acetonitrile/formic acid (solvent A) and 99.9:0.1 acetonitrile/formic acid (solvent B). The following gradient was utilized for direct LC−MS/MS analysis of tryptic peptide mixtures of each time point sample: 0−5% B in 5 min, 5−20% B in 70 min, 20−40% B in 60 min, 40−80% B in 5 min, 80% B for 10 min, 80−0% B in 5 min, and hold 0% B for 15 min. Individual SCX fractions were separated with gradient conditions (that included a column flush at the end) as follows: 0−5% B in 5 min, 5−20% B in 50 min, 20−40% B in 40 min, 40−80% B in 5 min, 80% B for 10 min, 80−0% B in 5 min, 0% B for 10 min, 40% B in 15 min, 80% B for 5 min, hold 100% B for 7 min, 0% B in 5 min, and hold 0% B for 13 min.

Mass spectra (MS and MS/MS data) were acquired using a quadrupole mass analyzer (LCQ Deca XP MS, Thermo Electron Corporation, Waltham, MA). The following instrument parameters were employed: m/z range, 300−1800; 35% normalized collision energy; an isolation window of 2.0 m/z; and a dynamic exclusion time of 1 min. Samples were analyzed in triplicate for online LC−MS/MS experiments, and each of the 10 SCX fractions were analyzed in duplicate.

LC−IMS−MS. IMS−MS theory and techniques (including the parallel dissociation method used here) have been discussed previously.[28,29] Although this instrumentation is not the focus of the present work, it is worthwhile to include a schematic diagram of the instrument in order to understand the approach. Figure 1 shows such a diagram for the LC−IMS−MS approach used in these studies.[25,26] Briefly, experiments are carried out as follows. Samples are injected onto a trapping column at 4 μL·min^{-1} using a capillary pump (1100 Series, Agilent Technologies, Palo Alto, CA). The flow was split to achieve a flow rate of 250 nL·min^{-1} for analytical separations. Gradient conditions were identical to those described above for the separation of tryptic peptide mixtures. As components elute from the column, they are electrosprayed into the source region of the instrument and accumulated in an ion funnel.[30] Concentrated packets of ions (~100 μs pulses) are injected into the first region (~59.0 cm) of a split-field drift tube that was filled with a mixture of ~2.60 and 0.10 Torr of He and N_2 buffer gases, respectively. Ions separate in this low-field region under the influence of a weak-applied electric field (~11.67 V·cm^{-1}). Ions exit the low-field region into a second shorter drift region (~1.00 cm) that is modulated between either low or high fields. At low fields, precursor ions are focused through the exit orifice of the drift tube. At high fields, ions experience energizing collisions and may fragment.[25,26,31]

The precursor (or fragment) ions are then pulsed into a time-of-flight mass spectrometer for detection. The resulting data sets contain information about retention time (t_R), drift time (t_D), and flight time (t_F), and the positions of peaks are denoted using a standard nomenclature[29,32−34] as values of $t_R[t_D(t_F)]$ in units of min[ms(μs)].

Peptide Assignment and Protein Identification. Data were searched against the National Center for Biotechnology Information *Drosophila* protein database[35] using the MASCOT search algorithm.[36] The parameters for the searches typically allowed for up to two trypsin miscleavages and a fixed carbamidomethyl modification. A parser program, written in-house, was used to filter peptides from the output search with MASCOT scores falling above the homology limit. In the formal MS/MS experiments (conducted on the commercial systems) peptides having scores at or above homology are expected to have less than a 5% chance of occurring at random.[36] The false-positive assignment rate based on our automated data analysis techniques for LC−IMS−MS measurements is substantially greater (~10%), largely because no formal m/z selection of the precursor ion is done in these experiments. Because of this, we first filter all assignments, and then examine all assignments by manual inspection. This procedure leaves only the most reliably assigned features, and few (if any) are expected to be falsely assigned (however, as discussed in more detail below, we note that the number of assignments that are made is much smaller than with the commercially available approaches).

Protein accession numbers for assigned peptides are cross-correlated with FBgn accession numbers in the FlyBase database[37] for final protein identification. Peptide sequences matching to multiple FBgn accessions were removed from the list, and all proteins identified with at least a single unique peptide

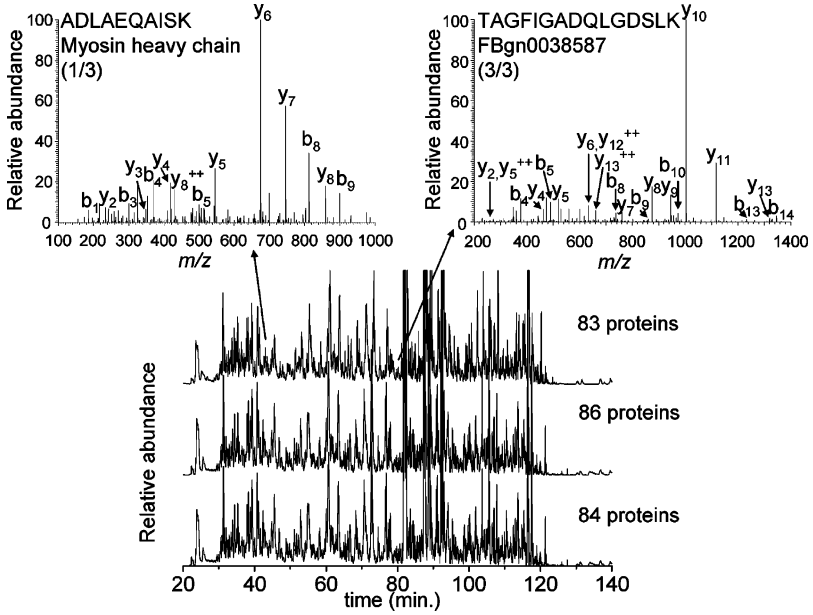

Figure 2. Example data from triplicate LC−MS/MS experiments obtained for a tryptic digest of experimental time-point 1 (i.e., 1−7 days). In the bottom are the base peak chromatograms (BPC) of three back-to-back runs of the tryptic digest mixture. An ion with m/z = 523.31 detected at 45.69 min (indicated by the arrow in the top BPC) and an ion with m/z = 746.99 detected at 80.73 min (indicated by the arrow in the top BPC) were selected for fragmentation in the ion trap. MS/MS spectra have been assigned to $[ADLAEQAISK+2H]^{2+}$ belonging to myosin heavy chain (top left) and $[TAGFIGADQLGDSLK+2H]^{2+}$ belonging to FBgn0038587 (top right). The number of times that the peptides were identified out of the three runs is also shown in parentheses.

were considered. Proteins were categorized based on their Gene Ontology (GO)[38] associations to biological processes.

Semiquantitative Analysis. Two methods for obtaining semiquantitative information about relative changes in abundances for a specific protein across different time points have been employed. Primarily, we rely on a peptide hits (PH) technique that is based on the number of observations of peptides unique to individual proteins and has proven successful in determining initial candidates for biomarker discovery.[39,40] In this approach, we assume that changes in the number of times a protein is identified correlate with changes in abundance. In this analysis, we account for variations in the total signal obtained at different time points by normalizing the total number of hits at each time point relative to the total number of hits obtained for time-point 2 (since this measurement had the largest signal). Overall, this approach is analogous to the normalization techniques employed by Opiteck and coworkers[41] and microarray technology.[42]

One caveat associated with the PH analysis is that all peptide assignments are used in the normalization (even those for a protein that was hit only once across all time points). For the PHT analyses shown below, we use a summation of all hits obtained from the duplicate SCX-LC−MS/MS experiments. This raises the possibility that false assignments would influence the changes that are observed. We have tested this in a few cases by examining profiles that are obtained using normalization factors for only proteins that are assigned with more than three or five hits, respectively. The patterns of change across the time points are essentially indistinguishable from those obtained including the single hit data.

The PHT approach can be corroborated using the LC−IMS−MS data sets. As noted above, because no m/z selection is used in these measurements, changes in the ion signals are valuable for assessing changes in relative abundances. In this analysis, peaks for a specific peptide are integrated, and changes in the relative peak intensities for different time points are used to assess change. Peak intensities are determined by integrating the data sets across an appropriate range of the LC, IMS, and MS parameter space using peak picking algorithms (written in-house). Intensities are normalized to the total ion signal for each time point.[43] When a protein is identified by multiple peptides, the intensities of all appropriate peaks can be used. This approach is not fully automated and, therefore, was done for only a small fraction of the peaks (that appeared to be interesting from the PHT approach).

We note that obtaining accurate intensity information from IMS data is heavily reliant on alignment of peaks in multiple dimensions (i.e., t_R, t_D, m/z). Sometimes, it is the case that multiple peaks match to a single position within the tolerances used to align peaks, in which case, without corroborating information, the correct peak cannot be determined. We are currently working to improve our peak picking algorithms and alignment components of our software. However, we find reasonable agreement in the patterns of change that are observed for some proteins of interest, as discussed below.

Results and Discussion

Example LC−MS/MS Data Sets. Figure 2 shows example data from replicate LC−MS/MS analyses of time-point 1 (1−7-day-old *Drosophila*). The base peak chromatograms (BPCs) in Figure 2 represent three back-to-back runs of this sample on the same analytical column. The BPCs are very similar in appearance, an indication of good reproducibility in the chromatographic separations. Typical MS/MS spectra are shown for the parent ions selected for fragmentation at 45.69 and 80.73 min with m/z = 523.31 and m/z = 746.99, respectively. From MASCOT searches that utilize these data, we obtain homology scores of 47 and 70, leading to assignments of these peaks to $[ADLAEQAISK+2H]^{2+}$, a sequence that cor-

Table 1. Peptide and Protein Coverage from Different Measurements

	LC−MS/MS[b]		SCX−LC−MS/MS[b]		LC−IMS−MS[b]	
age[a]	peptides	proteins	peptides	proteins	peptides	proteins
1−7	310	134	1866	635	207	124
8−14	437	162	2506	829	216	121
15−21	483	165	2222	737	230	124
22−28	461	165	1841	565	172	96
29−35	479	171	1631	522	163	92
36−42	478	171	1852	593	104	54
43−49	430	155	1773	561	77	43
50−56	465	168	1408	506	150	92
57−60	329	129	1860	561	143	88
Total[c]	**1102**	**367**	**5430**	**1437**	**548**	**324**

[a] Age, provided in days since eclosure, represents the pooled samples. [b] The numbers of peptides and proteins reported represent summed results of triplicate LC−MS/MS analyses, duplicate SCX−LC−MS/MS analyses, and an LC−IMS−MS analysis. [c] This is a cumulative total number of unique peptide and protein assignments across all time point samples.

responds to the myosin heavy chain and [TAGFIGADQLGDSLK+2H]$^{2+}$, from an unnamed protein, FBgn0038587, respectively. On the basis of these assignments, we have labeled peaks in the MS/MS spectra of Figure 2 to appropriate fragment ions.

In the present data, ADLAEQAISK and TAGFIGADQLGDSLK tryptic peptides were selected and identified in one of three replicate experiments and in all three analyses, respectively. In some cases, peaks may not be identified due to poor quality of the MS/MS spectra that result in MASCOT scores below homology.[44] In other cases, ions are simply not selected for fragmentation in the ion trap. Overall, we find a similar number of proteins are identified from each of the replicate runs (84, 86, and 83 protein identifications) with 134 different proteins identified in the time-point 1 sample with this approach. Cumulatively, more than 1100 unique peptides and 367 proteins are assigned from all of the experimental time points as summarized in Table 1.

Example Off-Line SCX-LC−MS/MS Data Sets. Figure 3 shows a chromatogram for a single SCX separation of tryptic peptides for time-point 1. A typical distribution of the number of proteins identified (combined from duplicate analyses) in each fraction for this sample is also shown. Cumulatively, 635 proteins were uniquely identified; this is ∼5× more protein identifications than obtained in the LC−MS/MS analyses. For all of the different time points, the number of proteins that are assigned increases by a factor of ∼3−5 upon prefractionation using SCX separation (relative to the LC−MS/MS analysis). A complete comparison of these data sets for individual time points is provided in Table 1. This comparison shows that the increase in peptide hits for SCX relative to LC−MS/MS only is somewhat larger (a factor of ∼4−6).

Example LC−IMS−MS Data Sets. Figure 4 shows example two-dimensional $t_R[t_D]$ base peak contour plots and collision induced dissociation (CID) mass spectra taken for three different time points: time-point 1, corresponding to 1−7-day-old animals; time-point 5, corresponding to 29−35-day-old animals; and, time-point 8, for 50−56-day-old *Drosophila*. The features in the two-dimensional base peak $t_R[t_D]$ plots represent the most intense m/z ions observed at a given LC and drift position under low-field conditions in the drift tube. From the widths of peaks and ranges over which they are observed, we estimate the peak capacity associated with the separations (prior to MS analysis) to be ∼10 000. Inspection of Figure 4 reveals that many ions that would not be separated in a one-

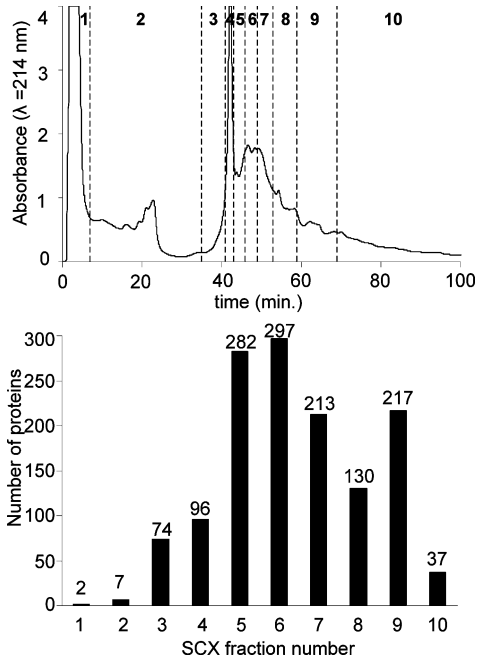

Figure 3. Example data for the SCX-LC−MS/MS experiments. Shown at the top is a typical chromatogram for the SCX separation of tryptic peptides obtained from time-point 1 (1−7 days). Ten fractions were pooled (as indicated by the dashed lines) as follows: first fraction (0−8 min), second fraction (8−36 min), third fraction (36−42 min), fourth fraction (42−44 min), fifth fraction (44−47 min), sixth fraction (47−50 min), seventh fraction (50−54 min), eighth fraction (54−60 min), ninth fraction (60−70 min), and, tenth fraction (70−110 min). The number of unique protein identifications (combined from duplicate LC−MS/MS analyses) for each SCX fraction of time-point 1 is shown in the histogram to the bottom.

dimensional LC separation can be resolved with this approach. Peaks in these data (such as those labeled A−F in the $t_R[t_D]$ plots shown in Figure 4) were aligned with peaks that were detected at similar $t_R[t_D]$ positions under high-field conditions in the drift tube. This allows precursor ions to be matched with their corresponding fragment ions for database searching. Typical CID/MS spectra obtained for the regions labeled A−F, with the corresponding peptide and protein assignments, are also shown in Figure 4 (along with the consistent fragment ion type assignments).

Although many peptide ion peaks are detected using the LC−IMS−MS approach, the assignment rate is relatively low, primarily because without a formal MS selection many different ions contribute to CID−MS spectra. Across each of the nine time points, we make as few as 77 to as many as 230 assignments to peptides for a single time point, corresponding to 43−124 proteins, respectively. The number of assignments for each time point is given in Table 1. We note that, unlike the corresponding triplicate measurements described above for the LC−MS/MS study, these assignments correspond to a single measurement.

Comparison of Assignments from Different Techniques. Figure 5 shows a comprehensive comparison of the protein assignments obtained from the different techniques. The majority of protein identifications (1437 proteins) are from the SCX study; an additional 76 and 182 proteins are identified from the LC−MS/MS and LC−IMS−MS techniques, respectively. Only 106 are in common between the three approaches,

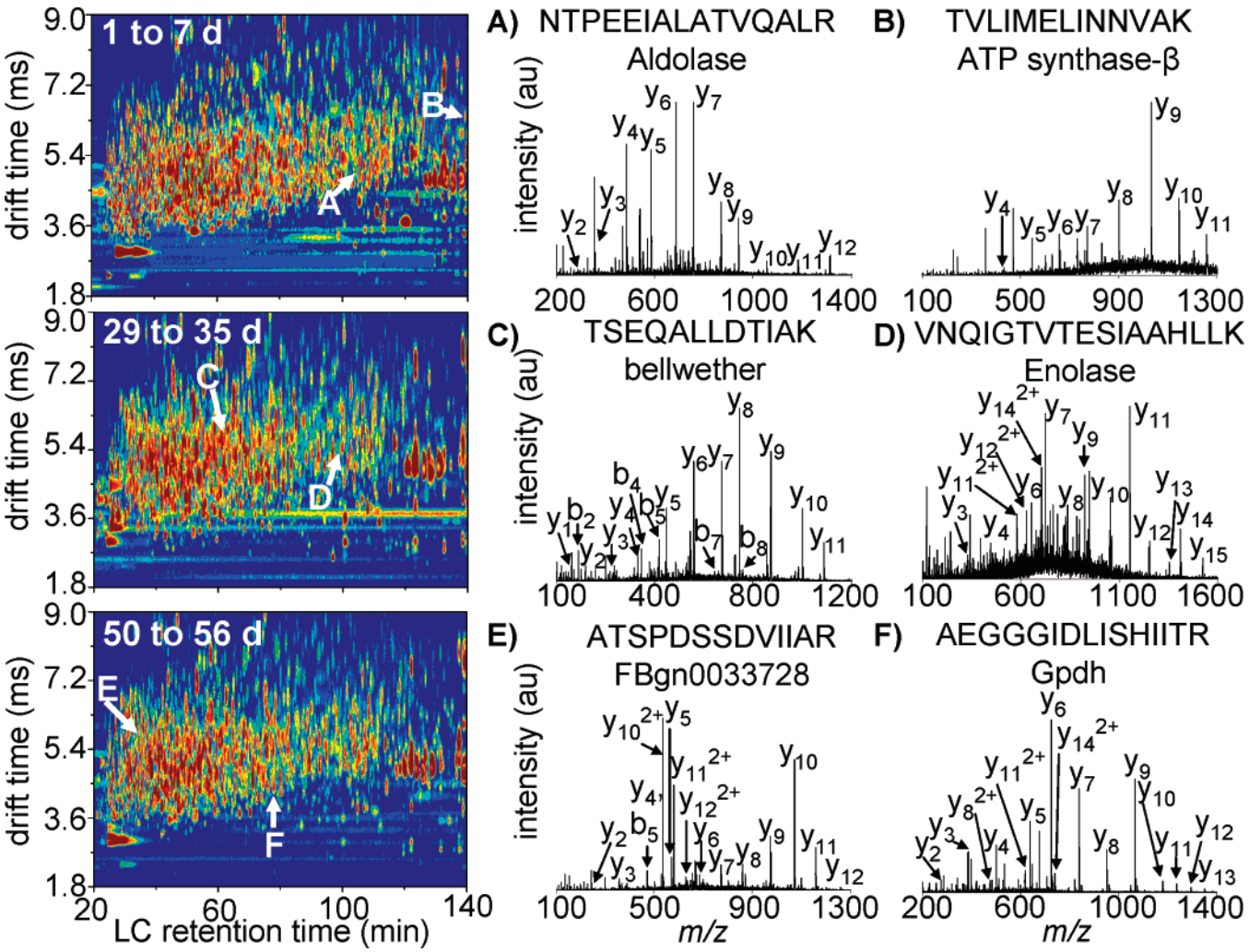

Figure 4. Example LC−IMS−MS data. The three plots to the left (top to bottom) show two-dimensional base peak plots of drift times (ms) vs retention times (min) for the time-point 1 (1−7 days), time-point 5 (29−35 days), and time-point 8 (50−56 days), respectively. Peaks are plotted using a false color scheme, whereby the least intense features are displayed in blue and the most intense *m/z* ion observed at each $t_R[t_D]$ position is shown in red. The mass spectra (A−F) correspond to fragmentation spectra for the peaks located in A−F positions in the two-dimensional data sets (left). Peptide and protein assignments are provided above each spectra.

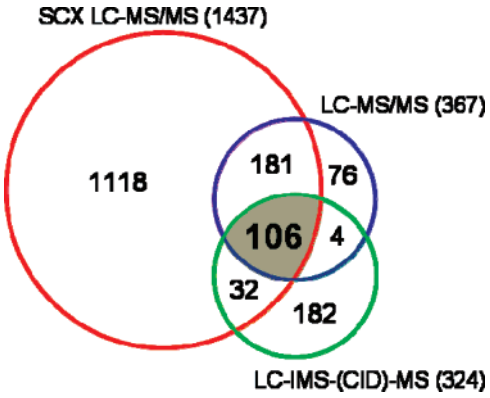

Figure 5. Venn diagram representation of the number of common proteins identified in the LC−MS/MS, SCX−LC−MS/MS, and, LC−IMS-(CID)−MS experiments. In total, there are 1699 unique proteins identified using these three complementary approaches.

primarily because so few proteins are examined in the LC−MS/MS and LC−IMS−MS methods.

Summary of Assignments. In total, the replicate analyses (using all approaches) provide evidence for 5902 unique peptides (identified at least once). These peptides correspond to 1699 different proteins. An example of proteins that are identified with many peptide hits (20 proteins with the most assignments in these experiments) is provided in Table 2. It is likely that many of the heavily hit proteins (probably all of those in Table 2) correspond directly to the dark features in Fleming and collaborators gel studies.[22] On the basis of the number of peptide hits, this analysis indicates that myosin (which is assigned 2891 times across all experiments), is the most abundant protein in the heads of *Drosophila*. Additionally, the number of hits associated with the nine time points (271, 403, 334, 340, 332, 319, 296, 298, and 298, respectively) shows little evidence for any systematic variation with age. Without any normalization, the average myosin hit number is 321 ± 38 (a relative standard deviation of ∼12%).

Other proteins in Table 2 are hit less frequently; for example, the second protein listed in Table 2 is mitochondrial ATP synthase α subunit, identified 755 times across all experiments (∼25% as many identifications as observed for myosin). The

Table 2. List of the 20 Most Abundant Proteins Based on the Number of Peptide Hits[a]

description[b]	FBgn no.[c]	∑ hits[d]	1−7[e]	8−14	15−21	22−28	29−35	36−42	43−49	50−56	57−60
Myosin	FBgn0002741	2891	271	403	334	340	332	319	296	298	298
Mitochondrial ATP synthase α subunit	FBgn0011211	755	84	98	84	91	79	83	66	87	83
H+-ATPase β subunit	FBgn0010217	652	64	63	74	83	66	92	72	69	69
Arginine kinase	FBgn0000116	614	55	74	78	83	62	75	56	71	60
Paramyosin	FBgn0003149	606	67	91	57	77	64	81	55	48	66
Aldolase	FBgn0000064	482	51	81	54	51	47	58	53	45	42
Retinoid-fatty acid binding protein	FBgn0016724	472	58	55	55	75	40	46	55	32	56
Na,K ATPase α subunit	FBgn0002921	466	61	98	51	68	43	43	27	36	39
Alcohol dehydrogenase	FBgn0000055	397	47	58	52	54	39	42	35	26	44
Phosphopyruvate hydratase	FBgn0000579	357	37	54	38	43	32	48	35	40	30
CG7998	FBgn0038587	355	32	47	37	48	43	41	33	38	36
Bangles and beads	FBgn0001090	344	33	58	33	48	43	34	33	33	29
A-Spectrin	FBgn0003470	343	35	76	43	40	20	38	37	26	28
ninac	FBgn0002938	339	35	45	42	39	38	33	38	34	35
Glycerol 3 phosphate dehydrogenase	FBgn0001128	313	42	52	37	41	27	34	28	21	31
Tropomyosin	FBgn0003721	305	29	41	28	41	44	31	24	37	30
Photoreceptor dehydrogenase	FBgn0011693	301	38	41	39	42	45	29	29	20	18
Glutamate dehydrogenase	FBgn0001098	297	20	43	33	39	36	32	26	37	31
Aconitase	FBgn0010100	286	31	40	27	40	27	31	24	37	29
Tropomyosin 2	FBgn0004117	275	31	36	27	31	34	39	30	25	22
	Totals	10850	1121	1554	1223	1374	1161	1229	1052	1060	1076.

[a] A complete list of peptide and protein assignments and peptide hits are provided in Supporting Information Tables SI−SII. [b] The name of the protein, gene, or computed gene (CG) number provided is given as a description of the identified protein. [c] The FlyBase gene number (FBgn no.) was obtained from the FlyBase database. [d] The total number of raw peptide hits observed for each protein across all ages and analyses (LC−MS/MS, SCX-LC−MS/MS, and LC−IMS−MS). [e] The age, in days since eclosure, of each of the pooled samples (see text for details).

number of hits for individual time points suggests that this protein also shows little variation with age.

A complete list of all proteins, peptide hits, and the peptides that are assigned in this study is provided as Supporting Information (Tables SI and SII). It is important to stress that a substantial number (642) of proteins are detected with only one hit across all experiments; 1057 proteins are assigned with at least two peptide hits. Recently, we used a Monte Carlo algorithm to propagate false-positive rates in order to assess the maximum number of times that a protein (from the human plasma proteome) would be hit randomly.[45] From this analysis, it is clear that our threshold of five hits (used below) for comparison of abundances is very conservative for the present data; thus, we expect no false-positive assignments among those proteins that exhibit changes in abundances. We include all assignments in the Supporting Information (Tables SI and SII) for others to use. However, we note that proteins identified with fewer than three assignments should be treated cautiously.

Identifying Changes in Relative Abundances. Examples of the relative abundance profiles (across the nine experimental time points) for four proteins are shown in Figure 6. In these plots, the normalized PH data (from SCX LC−MS/MS data) and relative peak intensities (from LC−IMS−MS data) are shown. Both glutamate dehydrogenase (FBgn0001098, involved in sperm storage and glutamate catabolism) and zeelin 1 (FBgn0038294, involved in muscle thick filament assembly)[37,38] appear to be relatively constant (or possibly undergo a slight increase) as animals age. In these cases, although the relative values obtained from the PHT and integrated intensity approaches are not in absolute agreement, the overall trends in the data sets are similar.

Figure 6 also shows two proteins, prophenoloxidase encoded by the *Black cells* gene (FBgn0000165) and CG4784 (FBgn0036619). Each of these proteins appears to decrease substantially as the animals age. The CG4784 protein is a structural constituent of cuticle.[37,38] The abundance of this protein declines substantially after the first experimental time point and then is relatively constant (or perhaps decreases slightly)

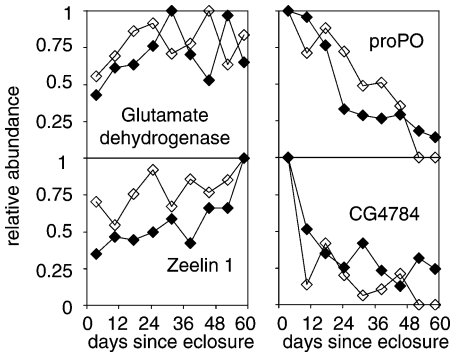

Figure 6. Relative protein abundance as a function of *Drosophila* age, defined here as days since eclosure. The normalized number of peptide hits (closed diamonds) or normalized peak intensities (open diamonds) for individual proteins are displayed on a relative scale, such that a value of 1 represents the largest signal (e.g., PHT or intensity). The two plots on the left are profiles for the proteins, glutamate dehydrogenase (FBgn0001098) and zeelin 1 (FBgn0038294). The plots on the right correspond to profiles for prophenoloxidase (FBgn0000165) and CG4784 (FBgn0036619).

during the remainder of life. The decrease in prophenoloxidase is roughly linear with age; our analysis suggests that the oldest animals exhibit about an order of magnitude less protein than young animals. The *Black cells* gene is so named because prophenoloxidase is an enzyme that catalyzes the synthesis of melanin as a defense response and a part of wound healing.

The substantial decrease in relative abundance of prophenoloxidase with age is correlated with transcriptional changes that are observed in two genome-wide studies of transcript accumulation.[11,13] These data showed evidence for up-regulation of transcripts associated with antimicrobial genes; Pletcher and his collaborators suggested that microbial infection may be responsible for early death (in laboratory animals).[11] A decrease in prophenoloxidase, which leads to a reduction in the rate of wound healing, would be consistent with these ideas. We note that in other genomics studies of 10- and 61-day-old

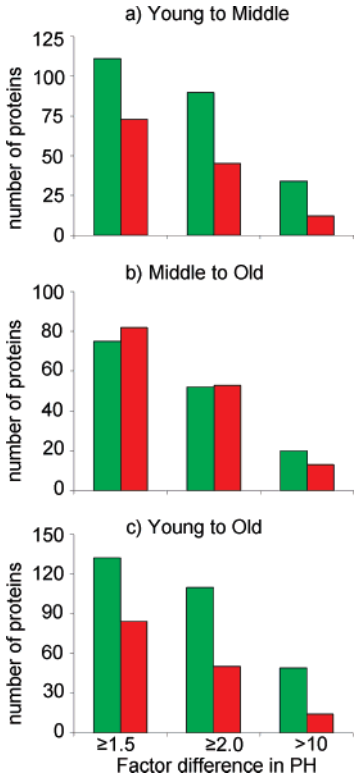

Figure 7. Histogram plot of the total number of proteins that are down- (green) or up- (red) regulated with age as a function of the factor change in peptide hits. Changes in relative protein abundances based on PHT were assessed for (a) young-to-middle, (b) middle-to-old, and (c) young-to-old groups of animals. See text for details.

animals a decrease of a factor of 3 was observed in the transcript for the *Black cells* gene.[13]

Assessing Changes in Large Numbers of Proteins. While Figure 6 shows a few examples of change, the data set in Supporting Information (Table SIII) allows differences to be assessed in many different ways. One attractive feature of such a large data set is that it is possible to set general criteria for change and assess how many different proteins show variations with age. We stress that this comparison for each protein is relative. Additionally, because the PH approach relies on the identification of peptides, it may not always detect changes (that is, the abundance of a peptide may be above the detection limit and therefore lead to a hit, even though the protein concentration changes). Still, with so little information available, the present data set can provide new insight.

As an illustration, consider the PH totals (from the SCX-LC−MS/MS analyses for specific proteins) associated with early (e.g., young, 1−21-day-old), middle (22−42-day-old), and late (old, 43−60-day-old animals) stages of adulthood. To assess change, we consider criteria similar to those reported previously.[16] For example, we require that the difference in total peptide hits between any two sets of animals is at least five and examine PH ratios of 1.5, 2.0, or >10. In this manner, we should identify a large set of proteins that undergo relatively small changes as well as smaller sets that exhibit more substantial changes.

Figure 7 shows the outcome of such an analysis. Between young and middle age, the abundances of 111 proteins decrease while 73 increase (using the 1.5 factor change definition). Of these, some changes are more drastic. Thirty are down-regulated and 12 are up-regulated (when considering a change of at least an order of magnitude; note that many of these large changes are associated with detection in only one of the sample sets, Table SI in Supporting Information). The transition from middle to old age shows 75 down-regulated and 82 up-regulated proteins (using the PH ratio of 1.5 requirement); 52 decrease and 53 increase (for PH ratio greater than 2.0); and 20 are down-regulated and 13 are up-regulated (for >10).

Large Differences between Young- and Old-Aged Animals. It is interesting to consider proteins that undergo large changes in more detail. Table 3 provides a subset of those proteins that fit our >10 criteria (here, we include only six hits and above, and proteins are only identified at early or late ages). Two of the changes that are observed in Table 3 [heat shock protein 22 (hsp22) and larval serum protein 2 (LSP-2)] have been studied in detail by others, using Western blotting techniques. King and Tower show Western analysis data for heat shock protein 22 for 5-, 10-, 15-, 20-, 30-, 40-, and 50-day-old *Drosophila*.[17] They conclude that this protein is substantially up-regulated with aging (and demonstrate a temperature dependent response); from a visual inspection of their data, it appears that no protein is detectable before day 40. *Our detection in the 43−60-day old-animal group, and lack of detection in the early and middle-aged animals, is in agreement with these data.*

The LSP-2 protein is an abundant protein in larva and is down-regulated during the first few days of adult life.[46] We note that, in our early grouping, we observe all six hits in time-point 1. In another study, it has been shown that, in addition to down-regulation early in life, LSP-2 may also accumulate with age (albeit to a much lower level than in young animals), suggesting a temporal differential regulation of this protein.[47] *We stress that the overall lower level with age is consistent with our finding of six hits for LSP-2 in our young group.* However, that we observe no evidence for a later LSP-2 increase caused us to examine four other independent SCX-LC−MS/MS data sets (three for Oregon-R males at varying temperature, and one other control group) that were recorded over several years. We note that in the three sets of Oregon-R males we observe 39 hits for LSP-2 in the youngest adults (2-day-olds). Thirty-seven hits are obtained in the other control group for 1-day-olds. Thus, in total, we have detected LSP-2 with 82 hits, but only for the youngest animals. Our inability to detect LSP-2 in any experiment at later ages, probably reflects its low abundance (i.e., it falls below our detection limit); we note that visual examination of the Western blotting data shows that the primary LSP-2 band is very faint (relative to other bands observed in this gel).[47,48]

Changes in Relative Protein Abundance: Biological Function and Comparison with Genomics Data. Additional insight can be obtained by considering biological function and from comparisons to genomics data. While a protein-to-transcript comparison for all genes is technically possible, such a comparison is beyond the scope of this report. Instead, we focus on the differences between the young and old groups for those proteins that exhibit the most extreme changes in Table 3. Overall, comparison of proteins listed in Table 3 to transcript accumulation studies[15] (that examined the heads of animals) shows very little agreement in the direction of change associated with individual gene products. Most proteins that we observe changing, if detected, were observed with no change at the transcript level. In one case, for imaginal disc growth factor 3, we observe up-regulation at the protein level, whereas

Table 3. List of Proteins That Are Only Detected in Early or Late Life[a]

description[b]	FBgn no.[c]	PH early[d]	PH late.[e]	mRNA[f]	biological process[g]
CG8193	FBgn0033367	20	0	NC	defense response, metabolism, localization
Serine/arginine rich protein 55	FBgn0004587	17	0	NC	metabolism
Tudor-SN	FBgn0035121	15	0	NC	metabolism
CG6781	FBgn0035905	13	0	N/A	defense response, metabolism
myosin II	FBgn0005634	13	0	N/A	development, localization
CG16826	FBgn0032505	11	0	N/A	NS
CG16885	FBgn0032538	10	0	N/A	NS
CG32407	FBgn0052407	9	0	N/A	NS
Decondensation factor 31	FBgn0022893	9	0	NC	metabolism
CG1240	FBgn0035370	8	0	NC	NS
CG4716	FBgn0033820	8	0	NC	NS
CG5653	FBgn0035943	8	0	N/A	localization
dj-1 β	FBgn0039802	8	0	NC	defense response, metabolism
lingerer	FBgn0020279	7	0	N/A	reproduction
CG5867	FBgn0027586	7	0	N/A	NS
raspberry	FBgn0003204	7	0	NC	metabolism, reproduction
lethal (2) 37Cc	FBgn0002031	7	0	NC	development
CG4893	FBgn0036616	7	0	N/A	NS
Heat shock gene 67Bc	FBgn0001229	7	0	N/A	defense response
CG4802	FBgn0034215	7	0	NC	metabolism
nucleoplasmin-1	FBgn0016685	7	0	NC	metabolism
anon-fast-evolving-1A9	FBgn0029676	6	0	NC	defense response
capulet	FBgn0028388	6	0	NC	development, metabolism, reproduction
CG15093	FBgn0034390	6	0	$\downarrow^{1}$	metabolism
larval serum protein-2	FBgn0002565	6	0	N/A	localization
Punch	FBgn0003162	6	0	$\downarrow^{1}$	metabolism
Dek	FBgn0026533	6	0	N/A	metabolism
Chromosomal protein D1	FBgn0000412	6	0	NC	NS
Drosophila dodeca-satellite-binding protein 1	FBgn0027835	6	0	NC	cellular process
CG31075	FBgn0051075	6	0	N/A	metabolism
attacin C	FBgn0041579	0	16	N/A	defense response
PGRP-SB1	FBgn0043578	0	16	N/A	defense response, metabolism
diptericin	FBgn0004240	0	10	N/A	defense response
CG7300	FBgn0032286	0	7	NC	NS
CG9759	FBgn0038160	0	6	$\uparrow^{1,2}$	NS
CG5791	FBgn0040582	0	6	N/A	NS
CG17273	FBgn0027493	0	6	NC	metabolism
Cyp28a5	FBgn0028940	0	6	$\downarrow^{1}$, $\uparrow^{2}$	metabolism
S-Adenosyl-L-homocysteine hydrolase	FBgn0014455	0	6	$\uparrow^{2}$	metabolism
CG4301	FBgn0030747	0	6	N/A	transport
Ribosomal protein L13A	FBgn0037351	0	6	NC	metabolism
Imaginal disc growth factor 3	FBgn0020414	0	6	$\downarrow^{1}$	development, metabolism
heat shock protein 22	FBgn0001223	0	6	$\uparrow^{1,2}$	defense response, metabolism

[a] Proteins in this list appear to be up- or down-regulated based on the number of peptide hits in early to late animals. [b] The name of the protein, gene, or computed gene (CG) number provided is given as a description of the identified protein. [c] The FlyBase gene number (FBgn no.) was obtained from the FlyBase database. [d] The total number of normalized peptide hits across time-points 1−3 corresponding to animals 1−21 days. [e] The total number of normalized peptide hits across time-points 6−9 corresponding to animals 43−60 days. [f] The changes associated with transcripts in studies by Kim et al. (see ref 15). An ↑ or ↓ indicate that the mRNA was up- or down-regulated, respectively, in comparisons of (1) 1- vs 13-day or (2) 13- vs 25-day-old animals. NC represents no change reported in the mRNA, and N/A represents that the mRNA was not detected in the studies. [g] The biological processes associated with proteins utilizing gene ontology (GO) information. Proteins were grouped into broad categories. NS represents those proteins for which a process was not specified.

Kim et al.[15] observe down-regulation at the transcript level. There are several possible reasons for discrepancy, and changes observed at the protein and transcript level may be attributed to differences in the strains and sexes of animals and aging temperatures (i.e., 25 vs 29 °C) used in the two studies. However, in some cases (as discussed below), there is agreement in the direction of change between protein and transcript expression for the same gene.

Metabolism. Examination of Table 3 shows that most proteins that undergo dramatic changes between early and late stages are associated with metabolic processes. Overall, these results indicate that many proteins involved in metabolism are less abundant in old-aged animals. This is consistent with genome-wide studies that also observe a substantial number of down-regulated metabolic-related transcripts.[6,11,15] We note that a few proteins are up-regulated; however, several of these have dual roles and are implicated in defense response (see below).

Protein-to-transcript comparisons show agreement in down-regulation of CG15093 and *Punch*, and up-regulation of Cyp28a5 and *S*-adenosyl-L-homocysteine hydrolase in older animals. Interestingly, we have also observed down-regulation in protein expression of *Punch* in *Drosophila* model studies of Parkinson's disease in 1-, 10-, and 30-day-old animals.[16] These combined results provide evidence for the importance of this protein either in the aging process or its general importance at the end of developmental metabolic processes necessary before adult life.

Development and Reproduction. Down-regulated proteins listed in Table 3 are also involved in development and reproduction. This is expected because, after an adult animal has emerged from the pupal case, a majority of its developmental events are complete. In addition, although adult animals are sexually mature within hours of this emergence, it is well-known that reproduction is down-regulated in aging organisms; this has been observed at the transcript level in aging studies of *Drosophila*.[6,11] Overall, the proteome data support this view. As an example, the Raspberry protein, which is involved in oogenesis, was detected with seven peptide hits in early-age animals but was not detected in late-age animals

(Table 3). Down-regulation of this protein (and other reproduction-related proteins) is consistent with our experimental design in which females were separated from males (and therefore should not have mated after day 1); moreover, it is known that females lay fewer eggs with age.[49]

Defense Response. Defense response mechanisms, such as molecular chaperone pathways and immune response, have been implicated as key factors in the aging process in genome-wide studies of *Drosophila*.[6,11,13,15] As listed in Table 3, Kim et al.[15] detected up-regulation of the molecular chaperone protein hsp22 at the mRNA level in older animals, which is consistent with our findings (see above discussion). Other defense response proteins also exhibit substantial change in early- to late-age animals. For example, Table 3 lists two proteins that are down-regulated with age (CG6781 detected with 13 peptide hits and CG8193 detected with 20 peptide hits in early-age animals). On the other hand, we also observe antibacterial defense proteins that are substantially up-regulated in older animals; diptericin, PGRP-SB1, and attacin C are detected with 10, 16, and 16 peptide hits, respectively. Diptericin was also reported to be up-regulated with age in transcript studies that examined the entire animal.[6,13] Also, transcripts of peptidoglycan recognition proteins were also observed to have abundant changes in older animals.[11]

Conclusions

A summary of the proteome of *Drosophila* at nine points across adult lifespan shows that overall the proteome is highly dynamic with age. Although the most abundant proteins show relatively small differences in relative abundance, the overall analysis provides evidence for hundreds of changes that occur at various stages of adulthood. In the discussion of these results, we provide evidence for a number of proteins that appear to be dramatically down- or up-regulated in comparisons of young- to old-age animals. Most of the proteins in this comparison are known to be associated with metabolism and defense response; such changes are consistent with primary proposed mechanisms for aging. A limited comparison to genomics data (that also examined the heads of animals) shows that the direction of change for several genes agrees at both the protein and transcript level; however, most of the genes do not. Many other differences (revealed by smaller changes across age) are also apparent in these data, but not discussed in this manuscript.

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Supporting Information Available: Tables showing the complete list of sequences and proteins identified in combined experiments; the comparative number of peptide hits from LC−MS/MS, SCX-LC−MS/MS, and LC−IMS−MS; and peptide hits from duplicate SCX-LC−MS/MS analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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