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Global Proteomic Profiling of *Drosophila* Ovary: A High-resolution, Unbiased, Accurate and Multifaceted Analysis

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Abstract. *Background: Drosophila melanogaster ovary serves as an attractive model system for the investigation of the cell cycle, death, signaling, migration, differentiation, development and stemness. By employing the 3750/+ heterozygote fly strain that carries specific functions in the follicle cell compartment, and a reliable control in GAL4/UAS-based transgenic technology, we herein characterized the protein-expression profiling of D. melanogaster ovary by applying high-resolution proteomic tools and bioinformatics programs. Materials and Methods: Whole-cell total protein extracts derived from 3750/+ fly ovaries were prepared under highly denaturing conditions and after tryptic digestion, their cognate peptides were processed to liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis in a high-resolution LTQ Orbitrap Elite instrument. Obtained protein data were analyzed through use of UniProt, DAVID, KEGG and PANTHER bioinformatics platforms. Results: The 7,583 unique peptides identified show that fly ovary contains at least 2,103 single proteins, which are distributed to all egg chamber compartments, in cytoplasm, membrane and nucleus, compartmentalized into major cellular organelles, and categorized into critical macromolecular assemblies. Among the recognized specific functions, nucleic acid binding, hydrolase, oxidoreductase, transporter and vesicle-mediated trafficking activities were the most prevalent. Determinants implicated in cellular metabolism and gene expression are represented by ~41% and ~17% of the ovarian proteome, respectively. Surprisingly, several proteins were found engaged in aging, immune response and neurogenesis. All major signaling pathways were detected, while apoptotic and non-apoptotic cell death programs were also identified. Remarkably, proteins involved in tumor formation, neurodegenerative and inflammatory diseases were also recognized. The successful remodeling of the proteasome and nearly complete molecular reconstruction of the citrate cycle and fatty acid degradation pathways demonstrate the efficacy, accuracy and fidelity of our combined proteomics/bioinformatics approach. Conclusion: Global proteomic characterization of D. melanogaster ovary allows the discovery of novel regulators and pathways, and provides a systemic view of networks that govern ovarian pathophysiology and embryonic development in fly species as well in humans.*

Drosophila melanogaster has been established as a versatile and dynamic model system for investigating several aspects of cell and developmental biology, mainly due to its short-life cycle and powerful genetics. The high level of structural

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and functional conservation in many fundamental biological processes between Man and fly, and the fact that over 50% of the proteins that are related to human diseases, including cancer, have orthologs in *D. melanogaster* (1-4), render *Drosophila* an ideal model for understanding the molecular mechanisms of human diseases, including malignancies. Hence, *Drosophila* has been effectively used for drug screenings and target discoveries (1, 5), as well as for the study of various pathological conditions, such as aging (6), metabolic disorders and diabetes (7), neurodegenerative diseases (8, 9), inflammatory disorders (10), cardiovascular diseases (11, 12) and cancer (13, 14).

In the same way, *Drosophila* oogenesis represents a valuable developmental platform for genetically and morphologically dissecting a wide range of biological processes, such as stem cell self-renewal (15), axis specification (16), tissue elongation (17), cell migration (18), dorsal appendage morphogenesis (19), cell differentiation, pattern formation (20) and even tumorigenesis with its related signaling circuitry (21). Female *Drosophila* possesses a pair of meroistic polytrophic ovaries, each composed of approximately 15-18 ovarioles (22). A typical ovariole contains a linear succession of progressively developing egg chambers, otherwise known as follicles, divided into several discrete developmental stages [14 stages according to King (22); 20 stages according to Margaritis (23, 24)], based on morphological criteria. Egg chambers are initially formed in the germarium, which also contains the germline and somatic stem cells (21). Each egg chamber is composed of 16 germline cells (15 nurse cells and one oocyte), surrounded by a monolayer of approximately 650 somatic epithelial follicle cells (25). Nurse cells are connected to the developing oocyte through ring canals responsible for supplying it with nutrients, organelles, proteins and maternal RNA transcripts (26). Follicle cells play essential roles in oocyte patterning, yolk protein synthesis and eggshell secretion and construction (27). The eggshell is composed of several protective layers collectively termed as chorion and specialized eggshell structures, such as the dorsal appendages, micropyle, operculum, collar and aeropyle (27).

Drosophila ovary also represents an ideal and outstanding model tissue for studying the genetic determinants, signaling routes and cellular mechanisms that orchestrate programmed cell death in the germline and somatic follicle cells during mid and late oogenesis. Indeed, five distinct cell death programs have, so far, been identified in *Drosophila* ovary, including apoptotic and non-apoptotic ones (28), with at least two of them contributing to the developmentally regulated cell death of nurse cell cluster at stages 12-14, and to the sporadically activated cell death in the germarium and at stages 7-9 of egg chamber oogenesis (29-32).

A unique advantage offered by *Drosophila* is the powerful transgenic technology that enables scientists to drive or silence the expression of a gene in a spatial and temporal manner. In one of the most popular and widely used transgenic schemes, lines expressing the GAL4 yeast transcriptional activator under the control of a cell-/tissue-specific promoter (the driver *gal4*) are crossed with lines carrying a target gene of preference sub-cloned downstream of five GAL4-binding sites (the upstream activation sequence; UAS). In the progeny of such crosses, the transgene of interest (*e.g.* RNAi) is activated only in those cell populations or tissues that synthesize GAL4 (33, 34). Given that heterozygote *Drosophila* strains *gal4*/+ are usually deemed as the most reliable lines of reference (control) for several fly tissues, including ovary, we herein attempted to thoroughly characterize the ovarian proteome of a *gal4*/+ transgenic line exclusively accumulating GAL4 in the follicle cell (over oocyte) compartment.

A total of 2,103 individual proteins were identified and subsequently analyzed using the UniProt, DAVID, KEGG and PANTHER bioinformatics resources, allowing (i) the structural, functional, topological, developmental and disease-related classification of ovarian proteins in *D. melanogaster*, and (ii) the molecular reconstruction of fundamental networks that critically control pathophysiology of ovarian tissue. Interestingly, numerous proteins implicated in tumor formation, and Alzheimer's, Huntington's and Parkinson's diseases in humans were recognized.

Materials and Methods

D. melanogaster stocks and culturing conditions. The *D. melanogaster* fly strains used were the wild-type Oregon-R and P{w[+mW.hs]= GawB}c355, w[1118] (BL: 3750) obtained from Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN, USA. Transgenic line 3750 carries, in a homozygote state, a *gal4* driver that is specifically activated in the follicle cell (over oocyte) compartment of *D. melanogaster* ovary. Fly stocks were maintained at 25˚C and fed on a standard diet (6.4% rice flour, 5% tomato paste, 3.2% sugar, 0.8% yeast, 0.8% agar, 0.4% ethanol and 0.4% propionic acid). Ovarioles carrying egg chambers of all developmental stages of oogenesis were derived from 3-day-old flies. Twelve to 16 h prior to dissection, a small amount of wet yeast was added to stimulate oogenesis.

Protein extraction and peptide generation. Protein samples, derived from 30 ovaries (pairs) of 3750/+ heterozygote flies, occupying a volume of approximately 200 μl, were washed and precipitated with 600 μl of acetone at room temperature overnight. They were then centrifuged at 3,800 × *g* for 20 min and all supernatants were discarded. The pellets were then treated with 8 M urea buffer and 80 mM triethyl ammonium bicarbonate (TEAB) under mild sonication in a water-bath for 30 min. Next, protein quantification took place using the Bradford assay. Protein quantity of 200 ng was isolated from each sample for further processing. Reduction and alkylation steps of the in-solution proteins were carried out using dithiothreitol and iodoacetamide solutions, at concentrations of 10 mM and 55 mM, respectively. The final step of protein treatment included tryptic digestion of proteins for peptide generation and extraction. Trypsin (Roche Diagnostics, Mannheim, Germany) was applied to all samples

at a ratio of 1 μg per 40 μg protein in a humidified atmosphere at room temperature overnight. The next day, peptides were lyophilized in a vacuum concentrator for approximately 2 h and the pellet was dissolved in 0.1% formic acid in double-distilled water for liquid chromatography-mass spectrometric (LC-MS/MS) analysis.

LC-MS/MS analysis and data handling. Extracted peptides (previous steps) were analyzed using the bottom-up approach in an LTQ Orbitrap Elite instrument (Thermo Scientific, Rockford, IL, USA). The MS was coupled to a Dionex Ultimate 3000 HPLC system. For peptide separation, a C18 Acclaim Pepmap 15 cm column was used (Thermo Scientific), coupled to an Aclain Pepmap nano-trap of 2 cm (Thermo Scientific). Phase A was 99.9% H_2O and 0.1% formic acid, and Phase B was 99.9% acetonitrile and 0.1% H_2O. Samples were run at a constant flow rate of 0.3 μl/min in a linear phase B gradient in 4 h runs. Runs were interrupted by 1 h washing steps of the columns with H_2O. The Orbitrap instrument was operated in a positive ion mode, while the 20 most intense spectra, as measured at a 60,000 resolution, were chosen for MS/MS fragmentation, using the higher energy collision dissociation (HCD) function. For HCD of parental ions, a collision energy value of 35% and activation time of 0.1 ms were used. Ions of m/z ≥2 were subjected to MS/MS analysis. The extracted ion chromatogram (raw file) was analyzed using Proteome Discoverer software (Thermo Scientific) and the Sequest search engine (Thermo Scientific). The database used for protein identification searches was the *D. melanogaster* reference proteome, exactly as downloaded from UniProt (version 2.16) without any further modification (35). Identification criteria included a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.05 Da. Trypsin was selected as the cleavage enzyme with a maximum of 0 missed cleavage parameter. A false-discovery rate threshold of 0.5% ensured the quality and reliability of all reported protein identifications.

Bioinformatics subroutines. The obtained Universal Protein Knowledgebase (UniProt v2.16; http://www.uniprot.org) (35) accession numbers were processed through Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources v.6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (36, 37), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps (http://www.genome.jp/kegg) (38, 39) and Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (http://pantherdb.org) (40, 41).

Results

Generation of ovarian peptide library and cognate protein quantification. Protein extracts of 30 manually dissected ovaries (pairs) obtained from the 3750/+ heterozygote *D. melanogaster* strain, usually employed as line of reference (control) in ovarian transgenesis, were prepared in urea-TEAB buffer and, after tryptic digestion, the generated peptides were processed through LC-MS/MS analysis, using an LTQ Orbitrap Elite instrument coupled to a Nano LC. By employing the UniProt protein-sequence search tool, we were able to retrieve 2,103 proteins, and 7,583 cognate unique peptides (Figure 1A; http://users.uoa.gr/~dstravop/Drosophila_Ovary_Proteomics.xlsx). From the 2,103 ovarian proteins found in UniProt, 1,871 were pinpointed in FlyBase (http://flybase.org) and 1,852 in DAVID. Among them, 1,849 were listed with available line stocks, while 1,494 were retrieved with human orthologs, thus unveiling the evolutionary conservation between *D. melanogaster* and humans. The 1,852 respective genes of the ovarian proteome were rather equally distributed in all arms of *Drosophila* female chromosomes (X, 2L, 2R, 3L and 3R), except chromosome 4, at which only 17 genes could be located (Figure 1B).

Distribution of ovarian proteins in cell populations, subcellular organelles and macromolecular structures. Despite the large number of proteins recognized by DAVID (n=1,852), only a small fraction of approximately 12% (n=229) were classified in egg chamber compartments, with 51 of them being associated with follicle cells, 32 with nurse cells, 70 with oocyte, 27 with germarium and 49 with eggshell (Figure 2A), therefore indicating the previously unknown cell-specific expression profile of numerous ovarian proteome components. However, the subcellular topology search proved comparably more informative, with 774 proteins being localized in the cytoplasm, 316 in the membrane and 374 in the nucleus (Figure 3). Among them, 113 were implicated in ribosomal structure, function and biogenesis, and 191 in mitochondrial activity and homeostasis, hence dictating the critical translational and metabolic (bioenergetic) demands of the developing egg chamber (Figure 2B). In the same way, the 47 proteins identified in endoplasmic reticulum, the 39 in Golgi apparatus and the 30 in vesicles undoubtedly corroborate the strong capacity of ovarian cell populations for intense protein synthesis, trafficking and secretion (Figure 2B). Regarding other major organelles, the 8 peroxisomal proteins (Figure 2B), together with the 19 anti-oxidant components (see Figure 4A), directly reflect the effective protective shields developed in the ovarian environments against H_2O_2-driven oxidative load, while the 4 lysosomal proteins (Figure 2B) and the 40 proteasome complex determinants (Figure 2C) indicate the critical contribution of the 'degradome' (collection of biomolecule degradation products) controlling machinery to proteome composition.

Ovarian proteins are architecturally organized into fundamental macromolecular complexes, with the proteasome containing at least 40 structural and functional regulators, the spliceosome 22, the nuclear pore 27, the centrosome 15, the chromosome 77, the cytoskeleton (actin and microtubule) 92, the cell junction 18 and the respiratory chain 40 (Figure 2C).

Functional dissection of proteome content in D. melanogaster ovary. Among the plethora of identified specific functions, 123 proteins appeared to have transporter activity, 68 transcription regulator activity, 320 nucleic acid-binding activity, 326 nucleotide-binding activity, 10 ribonucleoprotein-binding activity, 156 oxidoreductase

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	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	Area	# AAs	MW [kDa]	calc. pI
1												
2	P02844	Vitellogenin-2 OS=Drosophila melanogaster GN=Yp2 PE=1 SV=2 - [VIT2_DROME]	615.20	52.04	5	20	21	306	1.795E10	442	49.6	7.96
3	P02828	Heat shock protein 83 OS=Drosophila melanogaster GN=Hsp83 PE=1 SV=1 - [HSP83_DROME]	521.80	50.49	3	36	39	268	3.166E9	717	81.8	5.02
4	P11147	Heat shock 70 kDa protein cognate 4 OS=Drosophila melanogaster GN=Hsc70-4 PE=1 SV=3 - [HSP7D_DROME]	452.89	63.44	13	37	43	200	4.036E9	651	71.1	5.52
5	P18169	Defective chorion-1 protein, FC125 isoform OS=Drosophila melanogaster GN=dec-1 PE=2 SV=2 - [DEC11_DROME]	414.90	46.03	7	39	39	174	1.910E9	1208	137.4	5.29
6	P02843	Vitellogenin-1 OS=Drosophila melanogaster GN=Yp1 PE=1 SV=1 - [VIT1_DROME]	397.21	65.38	2	24	25	295	2.438E10	439	48.7	7.69
7	M9PF14	Trailer hitch, isoform C OS=Drosophila melanogaster GN=tral PE=4 SV=1 - [M9PF14_DROME]	277.54	56.25	2	0	26	103	2.105E9	656	69.7	9.48
8	Q9VTZ0	GH08269p OS=Drosophila melanogaster GN=tral PE=1 SV=3 - [Q9VTZ0_DROME]	271.15	55.98	2	1	26	102	2.105E9	652	69.3	9.54
9	P29844	Heat shock 70 kDa protein cognate 3 OS=Drosophila melanogaster GN=Hsc70-3 PE=2 SV=2 - [HSP7C_DROME]	256.85	46.19	5	24	28	120	3.056E9	656	72.2	5.36
10	P07186	Chorion protein S19 OS=Drosophila melanogaster GN=Cp19 PE=2 SV=1 - [CH19_DROME]	244.84	79.19	1	9	9	100	8.058E8	173	18.5	9.36
11	M9PFF9	Trailer hitch, isoform D OS=Drosophila melanogaster GN=tral PE=4 SV=1 - [M9PFF9_DROME]	244.65	55.26	2	1	22	90	2.105E9	646	68.7	9.54
12	P07182	Chorion protein S36 OS=Drosophila melanogaster GN=Cp36 PE=3 SV=2 - [CH36_DROME]	229.65	50.70	1	10	10	85	5.389E8	284	29.9	8.69
13	Q05825	ATP synthase subunit beta, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-beta PE=2 SV=3 - [ATPB_DROME]	222.60	42.57	7	17	17	97	1.501E9	505	54.1	5.27
14	P06607	Vitellogenin-3 OS=Drosophila melanogaster GN=Yp3 PE=1 SV=1 - [VIT3_DROME]	208.73	65.24	2	24	24	157	2.239E10	420	46.1	8.50
15	Q7KN62	Transitional endoplasmic reticulum ATPase TER94 OS=Drosophila melanogaster GN=TER94 PE=1 SV=1 - [TERA_DROME]	204.35	45.19	3	29	30	101	5.731E8	801	88.8	5.35
16	Q9V496	Apolipophorins OS=Drosophila melanogaster GN=Rfabg PE=1 SV=2 - [APLP_DROME]	203.53	20.68	3	65	65	118	5.026E8	3351	372.4	7.97
17	P13060	Elongation factor 2 OS=Drosophila melanogaster GN=EF2 PE=1 SV=4 - [EF2_DROME]	199.73	40.52	1	28	28	130	2.794E9	844	94.4	6.60
18	P10987	Actin-5C OS=Drosophila melanogaster GN=Act5C PE=1 SV=4 - [ACT1_DROME]	198.18	55.05	4	1	19	129	6.230E9	376	41.8	5.48
19	P02572	Actin-42A OS=Drosophila melanogaster GN=Act42A PE=1 SV=3 - [ACT2_DROME]	198.18	55.05	4	3	19	128	5.653E9	376	41.8	5.48
20	O02649	60 kDa heat shock protein, mitochondrial OS=Drosophila melanogaster GN=Hsp60 PE=1 SV=3 - [CH60_DROME]	179.41	64.92	3	34	34	87	7.122E8	573	60.8	5.49
21	Q9VMA3	Protein cup OS=Drosophila melanogaster GN=cup PE=1 SV=3 - [CUP_DROME]	171.70	47.90	2	30	30	71	3.596E8	1117	125.6	8.85
2088	Q04135	ORF2 for putative reverse transcriptase OS=Drosophila melanogaster GN=RTase PE=4 SV=1 - [Q04135_DROME]	0.00	2.03	2	1	1	1		888	102.2	9.77
2089	Q960D7	SD06390p OS=Drosophila melanogaster GN=CG5853 PE=2 SV=1 - [Q960D7_DROME]	0.00	4.35	2	1	1	1		689	77.2	7.80
2090	C9QP13	MIP13990p OS=Drosophila melanogaster GN=CG31089-RA PE=2 SV=1 - [C9QP13_DROME]	0.00	35.00	1	1	1	1		60	6.9	9.06
2091	Q4V576	IP14063p (Fragment) OS=Drosophila melanogaster GN=CG31784 PE=2 SV=1 - [Q4V576_DROME]	0.00	5.67	1	1	1	1		617	71.1	8.56
2092	Q8SWV4	LP03070p OS=Drosophila melanogaster GN=Alk PE=2 SV=1 - [Q8SWV4_DROME]	0.00	1.55	2	1	1	1	7.045E8	1158	126.0	6.54
2093	D0IQK7	Lipase OS=Drosophila melanogaster GN=CG18302-RA PE=2 SV=1 - [D0IQK7_DROME]	0.00	4.68	2	1	1	1		406	46.8	8.00
2094	Q8T0F9	LD05471p (Fragment) OS=Drosophila melanogaster GN=SMC2 PE=2 SV=2 - [Q8T0F9_DROME]	0.00	1.52	3	1	1	1		985	112.7	7.08
2095	Q7K501	GH19431p OS=Drosophila melanogaster GN=CG34133 PE=2 SV=1 - [Q7K501_DROME]	0.00	2.36	3	1	1	1		888	97.6	8.51
2096	Q4V4A1	IP03729p (Fragment) OS=Drosophila melanogaster GN=Gr43a PE=2 SV=1 - [Q4V4A1_DROME]	0.00	3.15	3	1	1	1		317	35.1	9.09
2097	Q9GPN8	1-beta dynein (Fragment) OS=Drosophila melanogaster GN=kl-2 PE=2 SV=1 - [Q9GPN8_DROME]	0.00	0.53	2	2	2	3	4.691E6	4167	482.7	6.87
2098	Q6IL06	HDC10905 OS=Drosophila melanogaster GN=HDC10905 PE=4 SV=1 - [Q6IL06_DROME]	0.00	16.67	1	2	2	2		216	25.0	9.72
2099	D2NUL4	MIP15301p OS=Drosophila melanogaster GN=east-RB PE=2 SV=1 - [D2NUL4_DROME]	0.00	1.65	1	1	1	2		1270	136.1	5.29
2100	L0CRQ9	Spindle E (Fragment) OS=Drosophila melanogaster GN=SpnE PE=4 SV=1 - [L0CRQ9_DROME]	0.00	2.33	29	1	1	1		1032	118.1	7.39
2101	Q6IH59	HDC03209 OS=Drosophila melanogaster GN=HDC03209 PE=4 SV=1 - [Q6IH59_DROME]	0.00	24.04	1	1	1	8		104	11.4	6.62
2102	Q6NNZ8	HL01056p (Fragment) OS=Drosophila melanogaster GN=CG32776 PE=2 SV=1 - [Q6NNZ8_DROME]	0.00	5.26	1	1	1	1		780	84.6	6.71
2103	D0IQI1	MIP14933p OS=Drosophila melanogaster GN=CG31149-RA PE=2 SV=1 - [D0IQI1_DROME]	0.00	5.33	2	1	1	1		845	93.3	5.44
2104	B8A3X9	LD18929p OS=Drosophila melanogaster GN=asp-RA PE=2 SV=1 - [B8A3X9_DROME]	0.00	2.05	2	1	1	1	3.075E6	1954	230.1	10.78
2105												

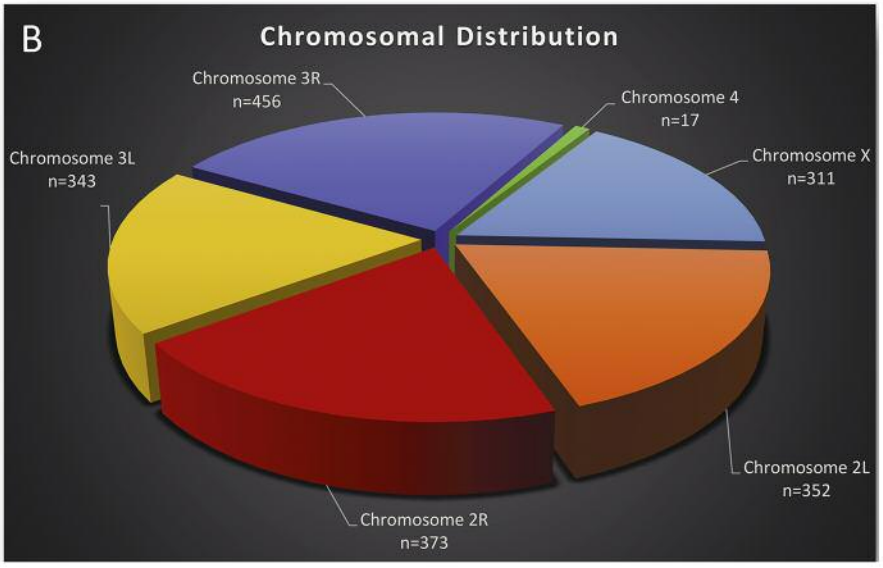

Figure 1. *A: Protein library (n=2,103 members) of 3750/+ (heterozygote) fly ovary, in an Excel file form (an exemplary and reconstructed file-page is given for reasons of convenience and comprehension), indicating each identified protein's accession number (according to UniProt nomenclature), name and description, together with fundamental features of liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) proteomics analysis (as extracted from an LTQ Orbitrap Elite with Nano LC instrument), such as (Mascot) score, sequence coverage, number of unique peptides, number of protein's amino acid residues (AAs), protein's molecular weight (MW) in kDa and protein's (calculated) isoelectric point (pI). Our D. melanogaster ovary protein -entire- content has been suitably uploaded on the http://users.uoa.gr/~dstravop/Drosophila_Ovary_Proteomics.xlsx URL Web address of the Department of Cell Biology and Biophysics, Faculty of Biology, National and Kapodistrian University of Athens (NKUA; Athens, Greece) Server. B: Classification of fly ovary proteome components, according to chromosomal localization of their respective cognate genes. As expected, the members of all six subcategories (X, 2L, 2R, 3L, 3R and 4) add to the number of 1,852 (DAVID) ovarian proteins, therefore demonstrating the accuracy, fidelity and efficiency of our bioinformatics protocols employed herein. The bioinformatics tool utilized was the Chromosome subroutine under General Annotations of DAVID software.*

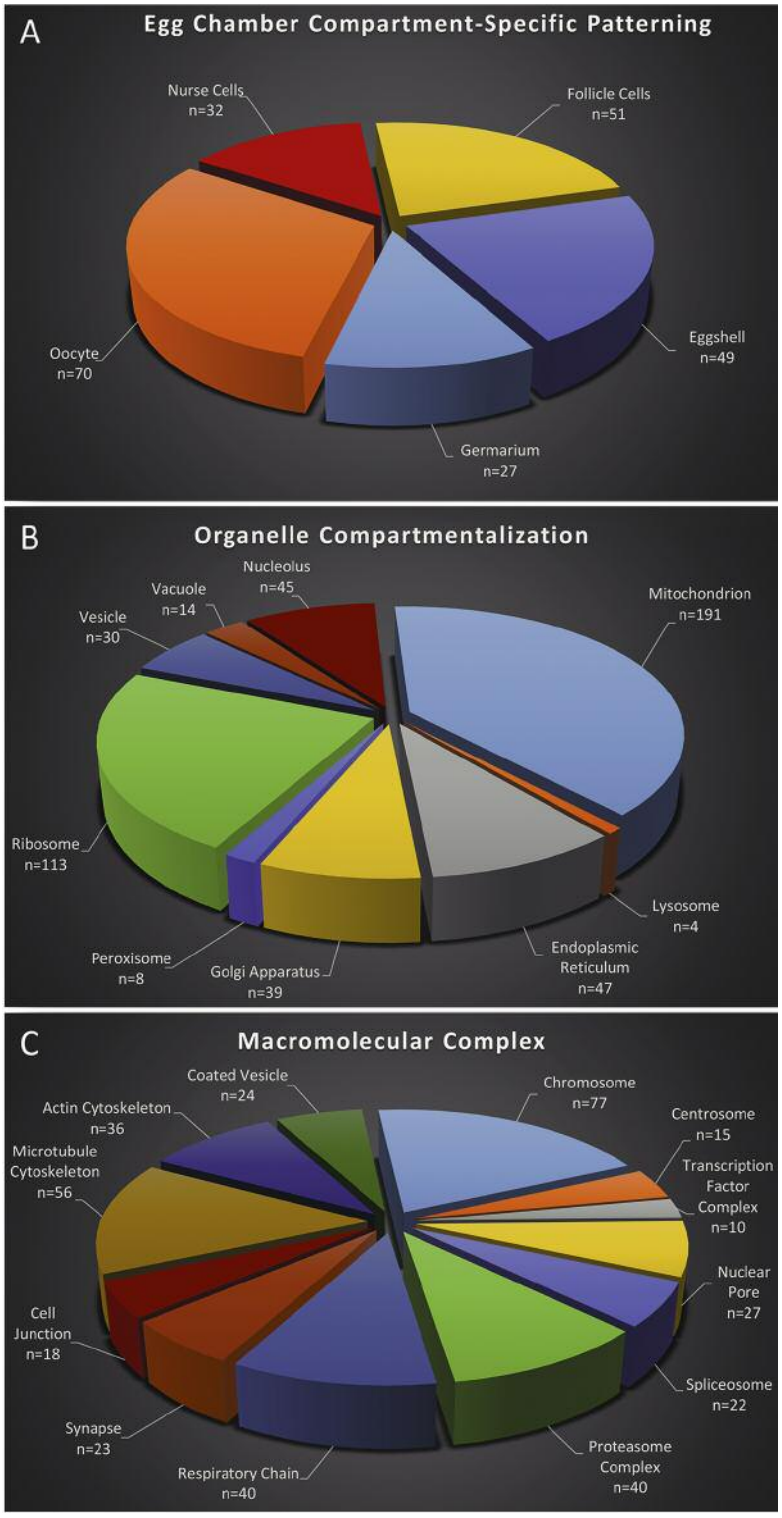

Figure 2. *Classification of ovarian proteome components of Drosophila melanogaster based on their cellular and subcellular positional profiling, and high-order molecular structure formation. A: Egg chamber compartment-specific engagement. B: Compartmentalization in organelles. C: Macromolecular complex assembly. Due to their particular structural properties and functional activities, there are certain proteins being shared between different categories (e.g. Follicle Cells and Eggshell; A). Note the large number of proteins identified in fly ovary Ribosome (B), Mitochondrion (B), Endoplasmic Reticulum (B), Nucleolus (B), Chromosome (C), Proteasome Complex (C), Respiratory Chain (C) and Cytoskeleton (C). The bioinformatics platform utilized was the Gene Ontology (GO) subroutine of DAVID software.*

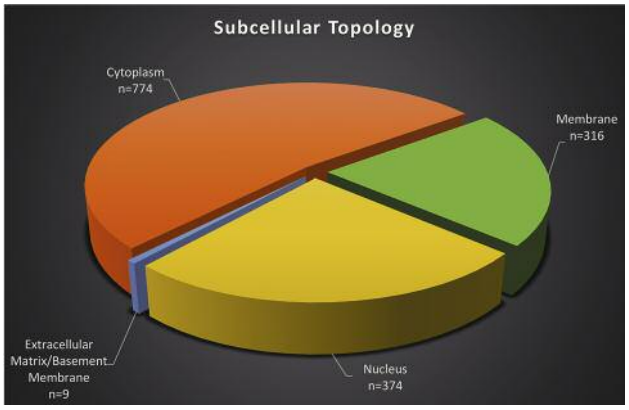

Figure 3. *Fly ovary proteomic profiling, according to each identified protein's topology in the major subcellular compartments Membrane (316), Cytoplasm (774) and Nucleus (374). Note the small number of proteins (9) sorted in the Extracellular Matrix/Basement Membrane subcategory. The bioinformatics platform employed was the Gene Ontology (GO) subroutine of DAVID software.*

activity, 12 peroxidase activity, 71 ligase activity, 30 isomerase activity, 343 hydrolase activity, 27 lipid-binding activity, 48 small ubiquitin-like modifier binding activity, 10 chaperone-binding activity and 35 unfolded protein-binding activity, while 70 proteins were found to be implicated in endocytosis, 31 in secretion and 96 in vesicle-mediated transport (Figure 4A). Closer examination of metabolic circuits unveiled 325 determinants being engaged in nitrogen compound metabolism, 117 in phosphorus metabolism, 11 in sulfur metabolism, 87 in cellular ketone metabolism, 18 in acetyl-CoA metabolism and 39 in cellular carbohydrate metabolism (Figure 5), thus underscoring the major contribution of regulators of the 'metabolome' (collection of small molecule chemicals/metabolites) to proteome configuration (see Figure 2C; 40 respiratory chain proteins).

Regarding the functional classification of ovarian proteome constituents, we can distinguish two types of differently leveled categories; (a) general biological processes inside a representative individual cell and (b) systemic functions of egg chamber compartments as a whole. In terms of the first group, 181 proteins were recognized as participating in the cell cycle, 51 in cell communication, 24 in cell adhesion, 46 in cell motion, 50 in protein folding, 176 in translation, 313 in gene expression, 27 in gene silencing, 80 in RNA processing and 761 in cellular metabolism (Figure 4B). For the second group, 43 proteins were identified as being involved in the regulation of cell differentiation, 75 in embryonic development, 71 in postembryonic development, 73 in pattern specification process, four in stem cell maintenance, 23 in aging and 10 in circadian rhythm (Figure 4C). Most interestingly, in the ovarian proteome, we detected 14 proteins involved in epidermis development, 11 in hair cell differentiation, 17 in regulation of neurogenesis, 25 in neurotransmitter level control and 18 in immune response (Figure 4C), clearly demonstrating the very early genetic predetermination and commitment of egg chamber cell clusters to successfully developing into epithelial, neural and immune systems of the future organism.

Contribution of 'signalome' and 'deathome' to ovarian proteome composition in D. melanogaster. Remarkably, the majority of renowned signaling pathways and networks ('signalome') extensively analyzed in mammalian systems were recognized in *Drosophila* ovary, with the p53 pathway shown to carry 8 proteins, epidermal growth factor (EGF) pathway 17, platelet-derived growth factor (PDGF) pathway 12, fibroblast growth factor (FGF) pathway 16, transforming growth factor-beta (TGFβ) pathway 10, insulin/insulin-like growth factor (IGF)/mitogen-activated protein kinases (MAPK) pathway 3, phosphatidyl-inositol-3-kinase (PI3K) pathway 3, heterotrimeric G-protein pathway 15, integrin pathway 26, cadherin pathway 8, WNT pathway 21, Notch pathway 1 and Hedgehog pathway 7 (Figure 6A). Surprisingly, 15 proteins were identified as being implicated in angiogenesis, 9 in dopamine signaling and 7 in neuronal axon guidance (Figure 6A), hence indicating the occurrence of hidden genetic seeds that orchestrate novel, but still elusive, angiogenic-like sprouting and neuronal wiring mechanisms in the fly ovary, with profound importance in human health.

Distinct cell death sub-routines ('deathome') proved to be clearly involved in the fly ovary, with 37 determinants associated with programmed cell death, 26 with regulation of cell death, six with regulation of caspase activity, seven with FAS apoptotic pathway, 17 with autophagic cell death, 10 with lysosome organization, 49 with phagocytosis, 37 with organelle fission (likely directing a regulated necrosis-like process) and 88 with response to stress (Figure 6B), thus indicating the co-existence and likely synergistic activities of apoptotic and non-apoptotic (*e.g.* autophagy and regulated necrosis-like) cell death programs in the egg chamber compartments, either during development or under stress conditions. The five proteins found to be involved in the regulation of neuronal apoptosis and the 17 in salivary gland histolysis (Figure 6B) imply the early commitment of future non-ovarian cells to death, after completion of their differentiation course.

Decoding the 'fladies': from fly ovary peptides to human maladies. One of the most exciting findings of the present study is the recognition of proteins related to human diseases in fly ovary, as documented by the identification of 23 implicated in tumor formation, 16 in chemokine/cytokine-mediated inflammation, 25 in Parkinson's disease, eight in Alzheimer's disease (related to presenilin and amyloid-

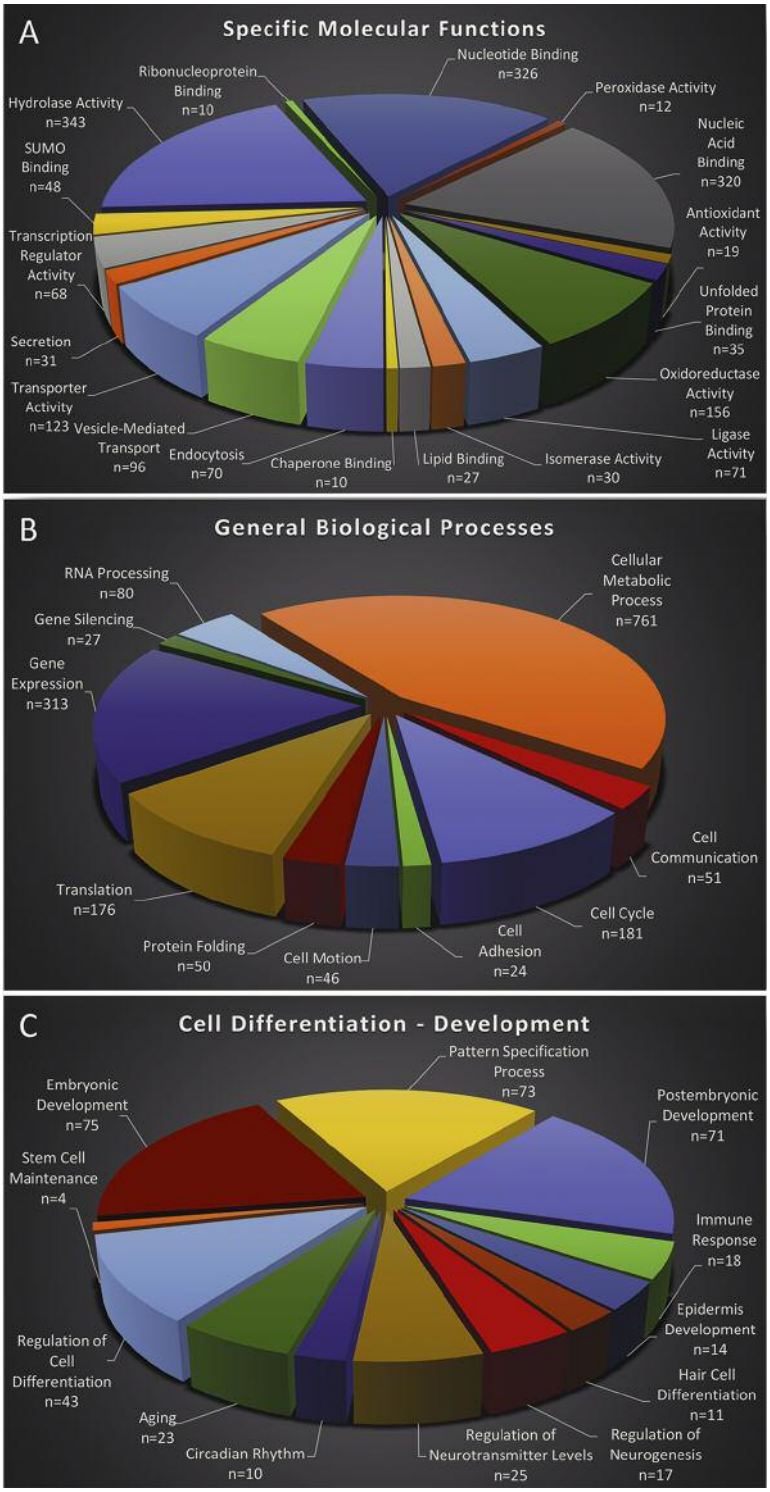

Figure 4. *Categorization of ovarian proteome contents of Drosophila melanogaster according to molecular activities, biological functions and systemic cellular processes. A: Specific molecular functions. B: General biological processes. C: Cell differentiation and development. Note the large number of proteins identified in Nucleic Acid Binding, Nucleotide Binding, Transcription Regulator Activity, Oxidoreductase Activity, Endocytosis, Transporter Activity, Vesicle-Mediated Transport, Hydrolase Activity and Ligase Activity (A); Cellular Metabolic Process, Cell Cycle, Cell Communication, Protein Folding, Translation, RNA Processing and Gene Expression (B); Pattern Specification Process, Postembryonic Development, Regulation of Cell Differentiation and Embryonic Development (C) sub-categories. Due to functional overlaps and shared or common mechanisms, certain proteome members are classified in more than one sub-groups. The bioinformatics process utilized was the Gene Ontology (GO) subroutine of DAVID program.*

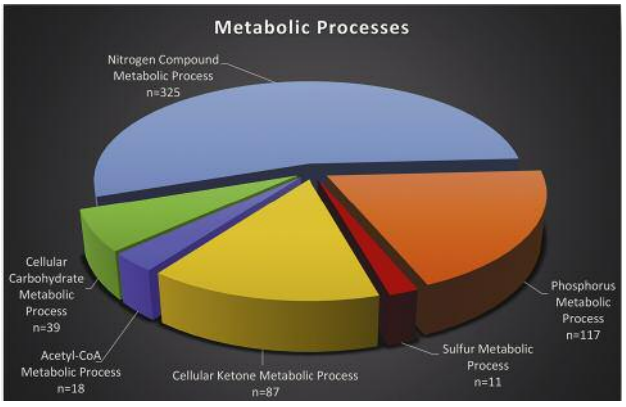

Figure 5. *Categorization of ovarian proteins in Drosophila melanogaster, based on their functional engagement in specific metabolic processes, such as the ones related to Nitrogen (325), Phosphorus (117), Sulfur (11), Ketone (87), Acetyl-CoA (18) and Carbohydrate (39) metabolism. Note the large number of cellular determinants having been recognized in the Nitrogen, Phosphorus and Ketone subclasses. The Gene Ontology (GO) subroutine of DAVID program was the bioinformatics protocol applied.*

secretase pathway) and 23 in Huntington's disease (Figure 6C), unambiguously demonstrating the powerful capacity of *Drosophila* ovarian cell clusters to be successfully employed as valuable model sub-systems for *in vivo* mechanistic and therapeutic studies of human malignancies, inflammatory diseases and neurodegenerative disorders.

Molecular reconstruction of ovarian proteome: from single peptides to complex structures and pathways. The data collection of *D. melanogaster* ovary proteome, obtained through our high-performance and in-depth analysis, allowed the integration of identified single peptides into higher-order assemblies (*e.g.* ribosome and proteasome), and fundamental cellular pathways and circuits (*e.g.* citrate cycle and fatty acid degradation), all synergistically orchestrating egg chamber survival, growth and development. To understand if the lack of (certain) protein detection in each reconstructed molecular map resulted from either technical limitations or represents a developmental stage- or cell type-specific feature of the examined (ovarian) organic system, is an issue of major importance that needs to be further determined. Despite the lack of relatively few components, the ovarian ribosome seems to have been successfully reconstructed (Figure 7A), while in the case of proteasome reconstitution, the vast majority of its structural subunits were undoubtedly recognized (Figure 7B). Remarkably, the metabolic map of the citrate (tricarboxylic acid; TCA) cycle in *D. melanogaster* ovary was almost completely reassembled, with only one determinant being missed (Figure 8A). Similarly, the fatty acid degradation circuit is virtually entirely reconstituted, with, again, only one protein remaining unidentified (Figure 8B), thus demonstrating the important role of fatty acid metabolism in ovarian physiology. Besides the other macromolecular complexes (*e.g.* spliceosome) and metabolic networks (*e.g.* glycolysis/gluconeogenesis and pentose phosphate pathway) analyzed (data not shown), selenocompound metabolism (42) was also examined and, despite its successful reconstruction, it appeared to lack two critical regulators (Figure 9), therefore indicating (without excluding technical reasons) the involvement of alternative biosynthetic routes or pathway silencing specifically in the ovarian tissue. Collectively, the functional reconstitution of the ovarian proteome into molecular pathways provides us with a versatile and powerful tool to pinpoint those protein determinants that control *Drosophila* egg chamber pathophysiology in developmental stage-dependent and cell type-specific manners.

Discussion

D. melanogaster has been proven to overcome mouse-model limitations, and to allow large-scale screening of genes implicated not only in the fly's development and pathophysiology, but also in human diseases, including cancer (43). Genome-wide transgenic RNAi libraries, with significant proteome coverage, have been generated through the binary GAL4/UAS system, targeting gene inactivation to cell types and lifespan stages of our preference (44), with *gal4*/+ serving as the reliable and widely accepted strain of reference (control).

Given its unique cellular composition of somatic (follicle) and germline (oocyte and nurse) cells at the mature stages, and stem (germline, somatic and escort) cells, with their respective niches, at the early stages of development, *Drosophila* ovary offers unrivalled opportunities for identification of genes controlling physiological (*e.g.* differentiation, proliferation, self-renewal, migration and signaling), or pathological (*e.g.* tumor formation, hyperplastic growth and non-regulated death) systemic processes of organ cellular compartments (16, 17, 21, 43, 45). Stem-cell community and tissue invasion by border cells (a follicle cell subpopulation) in fly ovary (16, 18, 43, 45, 46) stand-out as powerful systems for elucidating molecular etiologies and likely for identifying new therapeutics of certain human pathologies, especially ovarian malignancies. Regarding the ovarian signaling network, Hedgehog and WNT pathways (Figure 6A) are required for somatic stem cell maintenance (16, 21, 45, 47, 48), while TGFβ/Dpp and Delta/Notch routes (Figure 6A) are critically involved in germline stem cell formation (21, 45, 49, 50). Perturbation of signaling integrity, through employment of transgenic (including RNAi) technology, amongst others, could cause either elimination or overgrowth of germline and somatic stem cells (4), presumably shedding light on the association between stem and cancer cells. For example, germline stem cells lacking *bam* (a major

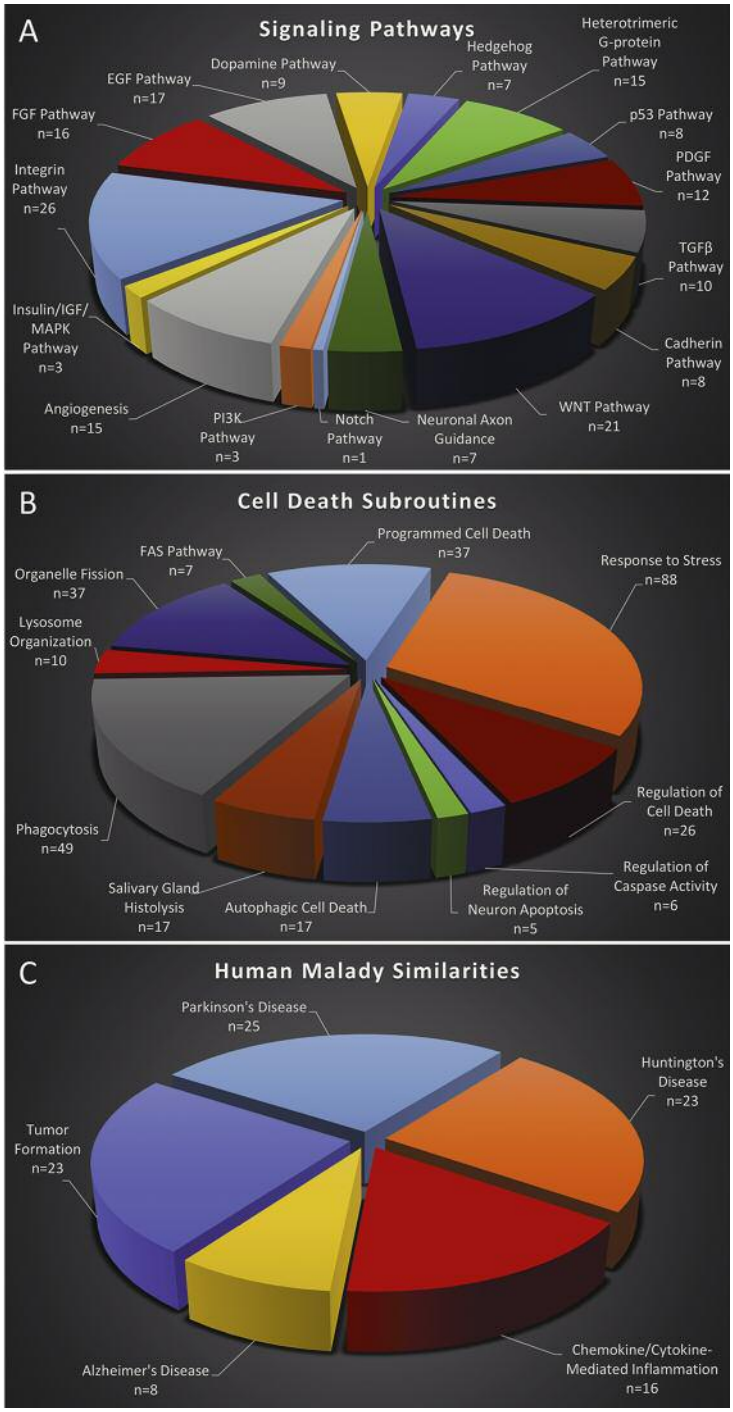

Figure 6. *Cataloging of ovarian 'signalome', 'deathome' and human 'diseasome' in Drosophila melanogaster. A: Signal transduction pathways. B: Cell death and stress subroutines. C: Similarities to human maladies. Note the several proteome determinants in WNT Pathway, Epidermal Growth Factor (EGF) Pathway, Fibroblast Growth Factor (FGF) Pathway, Integrin Pathway, Angiogenesis and Heterotrimeric G-protein Pathway (A); Programmed Cell Death, Regulation of Cell Death, Autophagic Cell Death, Salivary Gland Histolysis, Phagocytosis, Organelle Fission and Response to Stress (B) subcategories. Due to functional crosstalk and inter-relations, certain identified proteins seem to contribute to more than one of the denoted signaling pathways or cell death/stress programs in the fly ovary. C: Sorting of ovarian proteins based on similarities to their evolutionarily conserved counterparts critically implicated in human maladies. Note the ovarian tissue proteomic profiling that is associated with the Tumor Formation, Parkinson's Disease, Huntington's Disease, Alzheimer's Disease and Chemokine/Cytokine-Mediated Inflammation human disease network ('diseasome'). PANTHER canalized via DAVID was the classification system utilized for (A). The bioinformatics protocol utilized was the Gene Ontology (GO) subroutine of DAVID software for (B). PANTHER channeled through DAVID was the categorization system employed for (C).*

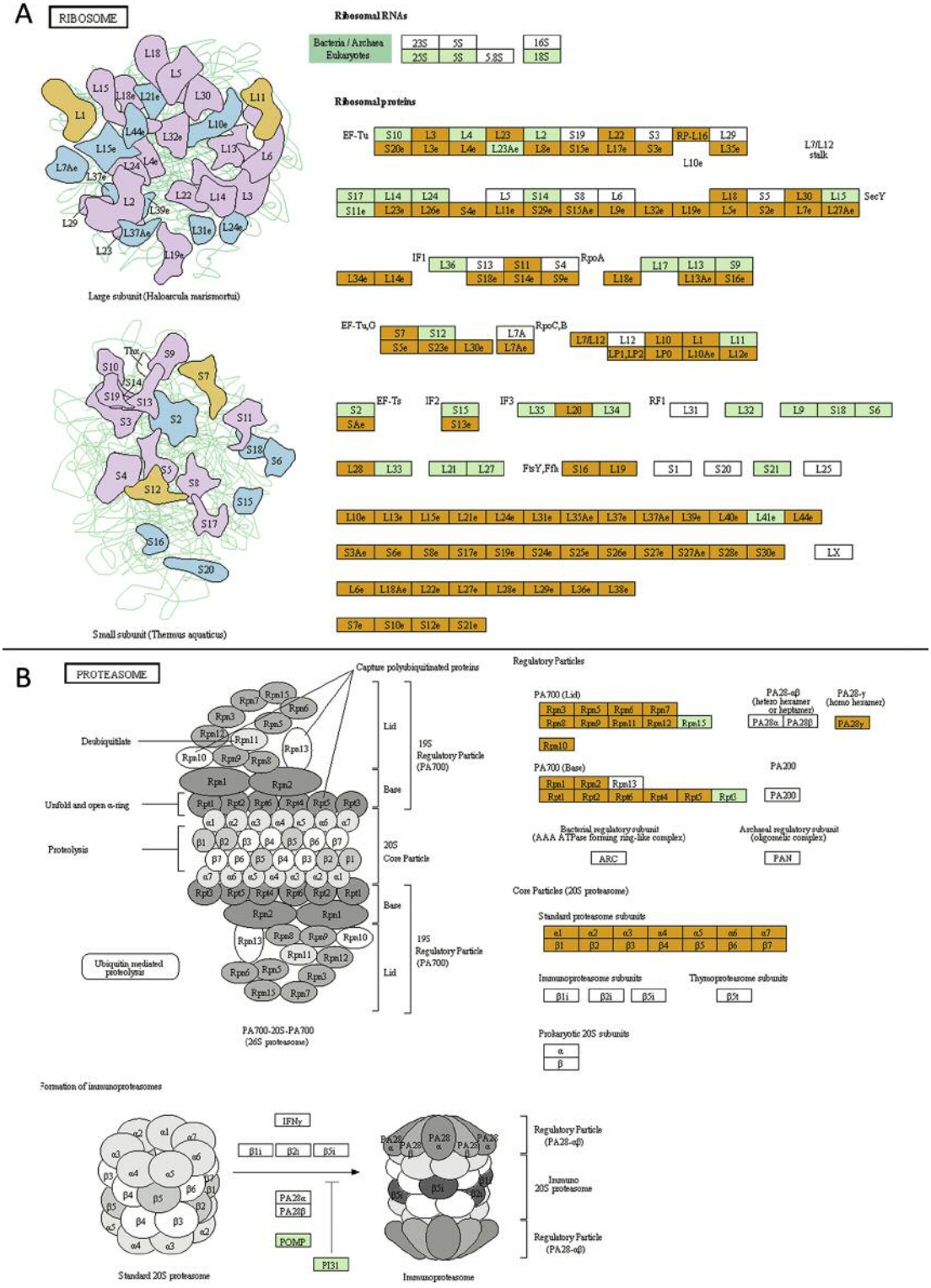

Figure 7. *Molecular reconstruction of subcellular organelles and particles in Drosophila melanogaster ovary: Ribosome (A) and Proteasome (B). By employing the KEGG (pathway maps) bioinformatics tool, most of the ovarian ribosome (organelle) and almost the complete proteasome (particle) structures were successfully remodeled. Brown boxes: proteins identified in the fly ovary in the present study. Green boxes: proteins that were missed in our study (for either technical or biological reasons, e.g. tissue-specific gene silencing). White boxes: proteins that are completely missing from the D. melanogaster proteome (e.g. according to integrative genome annotation analysis).*

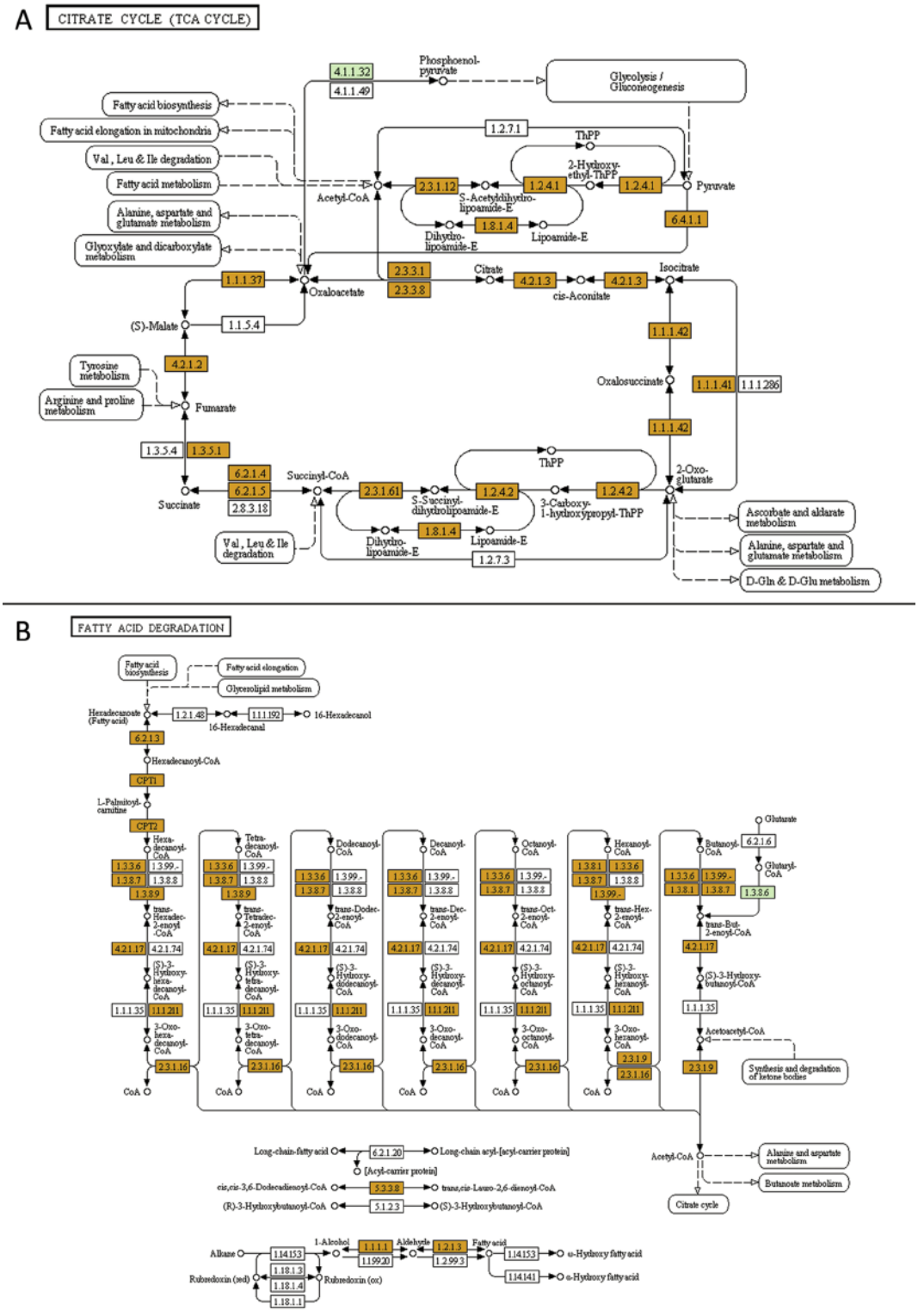

Figure 8. *Wiring diagrams of metabolic pathways in Drosophila melanogaster ovarian tissue. A: Citrate (tricarboxylic acid; TCA) cycle. B: fatty acid degradation. KEGG-mediated molecular reconstitution of both central metabolic circuits proved remarkably effective, with only one component having been missed from each examined network, respectively. Brown boxes indicate the proteins identified in fly ovary in the present study. Green boxes denote the proteins that were missed in our study (for either technical or biological reasons, e.g. tissue-specific transcriptional suppression). White boxes highlight proteins that are completely missing from the D. melanogaster proteome (e.g. according to integrative genome annotation analysis).*

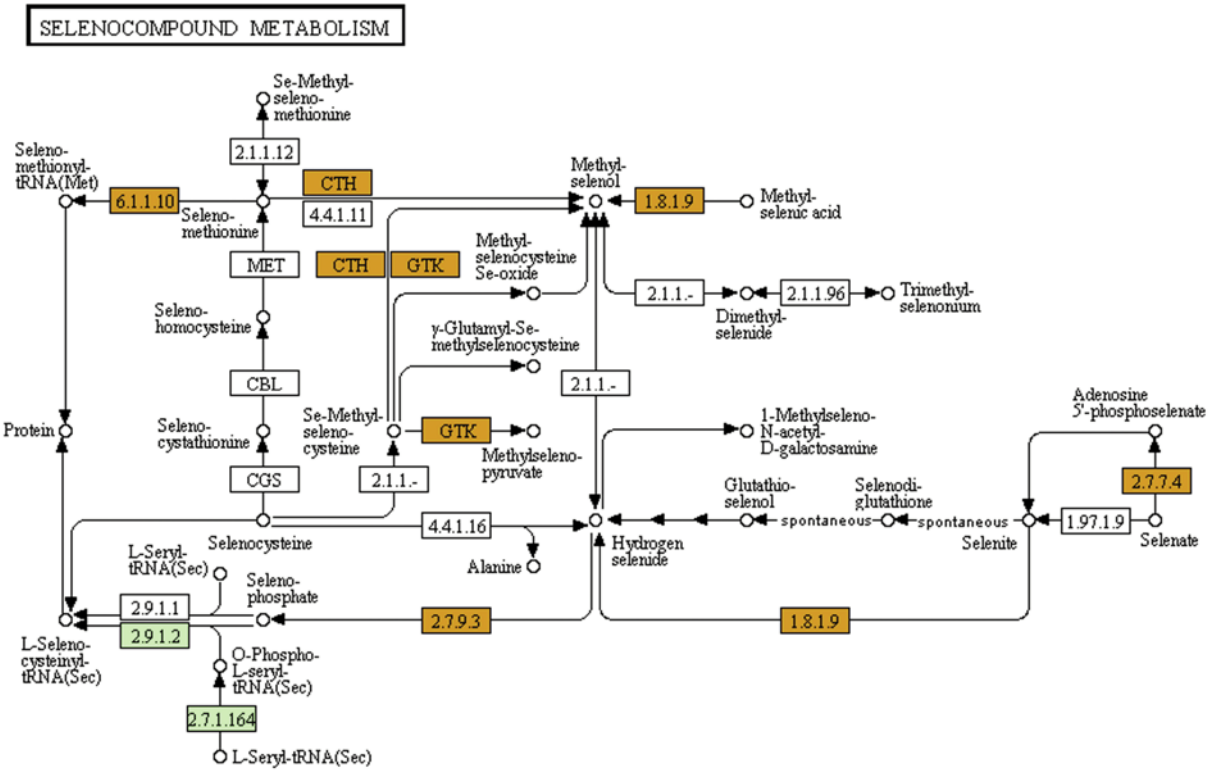

Figure 9. *Reconstitution of the molecular network that controls Selenocompound Metabolism in fly ovary. Through application of the KEGG bioinformatics software, it proved that the ovarian selenocompound biosynthetic wires, albeit their successful reconstruction in an integrated metabolic network, were missing two critical regulators for L-Seleno-Cysteinyl-tRNA (Sec) biosynthesis, likely indicating (without excluding technical reasons) network's functional repression, or engagement of alternative modes of metabolic actions, specifically in the ovarian tissue. Brown boxes: proteins identified in the fly ovary (present study). Green boxes: proteins that were missed in our study (for either technical or biological reasons {e.g. tissue-specific gene regulation}). White boxes: proteins that are completely missing from Drosophila melanogaster proteome (e.g. according to integrative genome annotation analysis).*

Dpp target) fail to differentiate and continue to divide, resulting in ovarian hyperplasia (a pre-neoplastic phenotype) (51). On the other hand, Janus kinase (JAK)/Signal transducer and activator of transcription (STAT), Notch, PDGF/vascular endothelial growth factor (VEGF) receptor and epidermal growth factor receptor (EGFR) signaling pathways (Figure 6A and data not shown) have proven to play essential roles in border-cell migration within fly ovary (21, 52-55). Interestingly, several human homologs of proteins required for border-cell migration are essentially implicated in mechanisms for epithelial-to-mesenchymal transition (a process that compels cells to obtain embryonic features of mesodermic origin), dissemination and propensity for metastasis of human ovarian cancer cells (18, 43, 46, 56). Altogether, it seems that *Drosophila* ovary serves as an excellent model organ for the investigation of stem cell- and epithelium-driven tumors, and their novel targeted therapies (13).

Among the available tools for studying fly oogenesis (*e.g.* screening for female-sterile mutations, mosaic analysis, pole-cell transplantation, RNAi and imaging), RNAi has emerged as a powerful, versatile and multifaceted genetic platform for targeted gene silencing (33, 34, 44, 57). Through employment of the GAL4/UAS transgenic system, each desirable RNAi can be overexpressed in literally any subpopulation of *Drosophila* ovary, therefore providing evidence for the role of its (RNAi) cognate target gene in ovarian pathophysiology. However, given the notable pathology observed in certain lines carrying the *gal4* transgene (58), it is an issue of major importance to always compare the RNAi-mediated phenotypes, and responses, to those of *gal4*/+ (control). Hence, we herein attempted to unveil the ovarian proteomic content of 3750/+ strain of reference that overexpresses GAL4 in the somatic (follicle) cell compartment, aiming not only to reliably characterize the particular transgenic line but to also identify novel targets whose up-regulation or disruption might critically affect ovarian development and function. Hopefully, their human counterparts may likely operate in a similar way in a number of processes, including cellular signaling, death, metabolism and migration, all decisively implicated in epithelial or (somatic) stem cell-directed tumors.

Besides its potential contribution to human (ovarian) malignancies, proteomic profiling of *Drosophila* ovary might also illuminate the function of novel, still elusive, pathogenic mechanisms involved in mammalian infertility. Since multiple forms of cell death (*e.g.* apoptosis, autophagy and necrosis) can be detected in fly ovary during development (Figure 6B) (28), the study of follicular atresia, through RNAi-driven down-regulation (or overexpression) of specific determinants revealed herein, during either aging or stress conditions (*e.g.* targeted drugs), could offer new insights into pathological situations of reduced human fecundity. On the other hand, the involvement of non-apoptotic cell death in human-disease onset and progression has become increasingly appreciated (28, 59, 60). Non-apoptotic death is effective in *Drosophila* germline community, while it also occurs during the developmentally regulated programmed cell death of the nurse cell cluster. Interestingly, isolation and protection of nurse and follicle cells from skillful macrophages dictate the involvement of non-professional phagocytes that, nevertheless, are able to carry out the engulfment process. This 'phagoptotic' form of cell death may significantly contribute to human pathology, including Alzheimer's disease, while successful manipulation of phagocytic cells may strongly benefit cell survival (28, 61). Dissecting cell death programs in *Drosophila* ovary, *via* exploitation of our proteomic platform, presumably opens new windows in understanding the diversity of cell elimination subroutines occurring in nature.

To reliably comprehend and successfully manipulate the molecular mechanisms orchestrating development, differentiation, cancer formation, stem cell maintenance, fertility, cell cycle, signaling, migration and death, in fly ovary, integrated sets of data embracing information regarding gene activity, protein expression, post-translational modification, subcellular localization and complex assembly (interactome networks) are undoubtedly required. Despite recent studies analyzing *Drosophila* transcriptome and its dynamics in the ovary (62, 63), to our knowledge, there has been no report published describing the proteomic content of fly ovary in a high-resolution scale. On the contrary, diverse proteomic technologies have been previously employed for compound eye (64), head (65-67), sperm (67-69), wing imaginal disc (67, 70), immune response (71), embryo (66, 67), larva (72) and cultured cells (72) of *Drosophila*. However, due to technical restrictions, enforced by the extraction protocols and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) protein-resolving platforms (73), in certain cases, the number of proteins identified was rather limited. Membrane proteins and proteins with extreme values of isoelectric point or molecular weight can hardly be resolved, while the restricted separating capacity of 2D-gel technology (a top ~10% of abundant soluble proteins is usually visualized by 2D-PAGE) may result in a compromised resolution and biased profiling (73).

Remarkably, the yield of our technical strategies, using LC-MS/MS analysis, *via* LTQ Orbitrap Elite instrument with Nano LC engagement, showed that they have successfully overcome the difficulties, complications and limitations of the traditional proteomics protocols, as clearly demonstrated by the large number of identified ovarian proteins (n=2,103) and their cognate unique peptides (n=7,583). Nevertheless, a recent report recognized (*via in situ* hybridization and RNA sequencing) 3,475 mRNAs expressed in *Drosophila* ovary (63), likely dictating the operation of mechanisms that can suppress translation and increase transcriptional noise (*i.e.* random fluctuation of gene expression over time), specifically in ovarian tissues of *D. melanogaster*. Interestingly, in mouse embryonic stem cells, genes being regulated by multiple microRNAs are present with significantly reduced noise levels (74), indicating that in certain cellular settings, translation-specific noise might be quantitatively lower compared to transcription-specific noise. In any case, we cannot, of course, exclude the possibility that a number of ovarian proteins were missed for purely technical reasons.

Our proteomic profiling of the 3750/+ transgenic fly ovary, and the subsequent bioinformatics-based mapping and cataloging produced a reliable, accurate, unbiased, multifaceted and versatile systemic platform, of high quality, strong sensitivity and high resolution. For example, fly ovary-specific and unique features, such as the six major proteins of the chorion (a protective layer surrounding the mature oocyte) (75) and the fusome (a membranous cytoplasmic organelle containing skeletal proteins) components (76), can be clearly pinpointed in our collection (Figure 2A and data not shown). In addition, novel issues regarding the contribution of human disease-related determinants to ovarian development, homeostasis and function (Figure 6C), or the tissue-dependent regulation of specific metabolic circuits (Figure 9), are dynamically raised, thus rendering *Drosophila* ovary a valuable and powerful model system for the investigation of human pathophysiology, with special emphasis on cancer, neurodegeneration, inflammation and stem cell management. Even though our proteomic database does not carry ovarian-protein quantification data (albeit, a rough and approximate estimate can be made through comparative evaluation of the obtained Mascot Scores; Figure 1A), we deem that it can still be constructively integrated into the *Drosophila* PeptideAtlas (67, 72, 77, 78), which, hitherto, represents the largest fly proteome catalog described (77, 78).

Since a part of *Drosophila* (ovary) proteome may still be missing, and in an effort to become able to see every protein in the fly (in all tissues and developmental stages), next-generation proteomics platforms must harmonically and complementarily be combined with other advanced technologies of the omics family, such as genomics,

transcriptomics, metabolomics and lipidomics. This combined and integrated strategy will most likely succeed in realizing System Biology's promises, namely the definition of functions, description of topologies and characterization of interactions for every protein in a living organism during lifetime or stress exposure.

Conflicts of Interest

The Authors declare that they have no conflict of interest, whatsoever.

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