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
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% H2O and 0.1% formic acid, and Phase B was 99.9% acetonitrile and 0.1% H2O. 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 H2O. 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 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 centrosome, 15 with chromosome 77, the cytoskeleton (actin and microtubule) 92, the cell junction 18 and the respiratory chain 40 (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 H2O2-driven oxidative load, while the 41 peroxisomal 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...
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). B: Classification of fly ovary proteome components, according to chromosomal localization of their respective cognate genes. As expected, the numbers of all six gene categories (X, 2L, 2R, 3L, 3R and 4) add to the number of 1,852 (DAVID ovarian) proteins 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.
process, four in stem cell maintenance, 23 in aging and 10 postembryonic development, 73 in pattern specification ovarian proteome, we detected 14 proteins involved in differentiation, 75 in embryonic development, 71 in circadian rhythm (Figure 4C). Most interestingly, in the identified as being involved in the regulation of cell (Figure 4B). For the second group, 43 proteins were 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, 374 in systemic functions of egg chamber compartments as a whole. In terms of the first group, 181 proteins were recognized as systemic functions 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, heterotrimetric 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 closely 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.
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 malignanties. 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).
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* germine 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 skilful 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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