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Proteomics of purified lamellocytes from *Drosophila melanogaster* Hop^{tum-l} identifies new membrane proteins and networks involved in their functions.

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Abstract

In healthy *Drosophila melanogaster* larvae, plasmatocytes and crystal cells account for 95% and 5% of the hemocytes, respectively. A third type of hemocytes, lamellocytes, are rare, but their number increases after oviposition by parasitoid wasps. The lamellocytes form successive layers around the parasitoid egg, leading to its encapsulation and melanization, and finally the death of this intruder. However, the total number of lamellocytes per larva remains quite low even after parasitoid infestation, making direct biochemical studies difficult. Here, we used the Hop$^{Tum\text{-}l}$ mutant strain that constitutively produces large numbers of lamellocytes to set up a purification method and analyzed their major proteins by 2D gel electrophoresis and their plasma membrane surface proteins by 1D SDS-PAGE after affinity purification. Mass spectrometry identified 430 proteins from 2D spots and 344 affinity-purified proteins from 1D bands, for a total of 639 unique proteins. Known lamellocyte markers such as PPO3 and the myospheroid integrin were among the components identified with specific chaperone proteins. Affinity purification detected other integrins, as well as a wide range of integrin-associated proteins involved in the formation and function of cell-cell junctions. Overall, the newly identified proteins indicate that these cells are highly adapted to the encapsulation process (recognition, motility, adhesion, signaling), but may also have several other physiological functions (such as secretion and internalization of vesicles) under different signaling pathways. These results provide the basis for further *in vivo* and *in vitro* studies of lamellocytes, including the development of new markers to identify coexisting populations and their respective origins and functions in *Drosophila* immunity.

Key words: *Drosophila melanogaster*, lamellocytes, proteomics, hemocytes purification, protein purification, Q-orbitrap spectrometry.

Introduction

The innate immune system is an evolutionarily conserved host defense system with key features shared between invertebrates and mammals (1, 2). *Drosophila melanogaster* has emerged as a powerful model for studying hematopoietic cell development in normal and pathogenic contexts (3-5). During development, cell fate specification and differentiation of *D. melanogaster* blood cells depend on signaling pathways and transcription factors that are well-conserved in vertebrate systems, including humans (3-5). In *D. melanogaster* larvae, three main types of circulating hemocytes have been described, involved in many physiological processes and in responses to injury and infection by pathogenic microorganisms and parasites (1-6). Under normal conditions, plasmatocytes account for more than 95% of circulating hemocytes; they are responsible for phagocytosis of apoptotic debris during development and of pathogens during infection (7-9). In addition, they synthesize and secrete antimicrobial peptides (AMPs) (10) and several structural proteins of the extracellular matrix such as Tiggrin (11), Peroxidasin (12), Papilin (13) and two Collagen IV molecules, Viking and Cg25C (14, 15). This extracellular matrix synthesis activity is essential during development for the remodeling of certain tissues. Crystal cells represent the remaining 5% of hemocytes; these non-phagocytic cells contain tyrosine-rich crystalline inclusions and two prophenoloxidases (PPO1 and PPO2) involved in various melanization processes (16-18). Under stress, crystal cells lyse and release the two pro-enzymes that are activated by proteolysis to produce different quinone derivatives from tyrosine that ultimately generate the black melanin involved in clot formation during wound healing or nodulation (19, 20). Under healthy conditions, very few lamellocytes circulate but their numbers increase sharply in the fly larva after infestation by Hymenoptera endoparasitoid wasps (5, 21-23). The main role of this cell type is the formation, with the help of plasmatocytes, of a cell capsule around the wasp egg. The plasmatocytes recognize the egg and form the first cell layer to which the lamellocytes will adhere to form several additional layers (24). Degradation of the lamellocytes leads to melanization of the cell capsule by the release of their PPO3-specific phenoloxidase (18, 23) and causes the death of the parasitoid mainly by the effect of reactive oxygen and nitrogen species produced locally during melanin formation (25). To be successful, Drosophila parasitoid wasps must inhibit this immune response, with one of the primary strategy being venom injection during egg-laying. Interestingly, the venom of wasps from the genus Leptopilina contains a vesicular material that targets lamellocytes and probably impairs their function (26-28). However, their mechanism of action on lamellocytes remains unknown.

A question that is still being debated is the origin of the first lamellocytes that adhere to the plasmatocytes around the wasp egg. Plasmatocytes and crystal cells are derived from *Drosophila* embryonic prohemocytes that still circulate at the larval stage and are also present in subcuticular clusters, called the sessile hemocyte compartment (5, 29-32). During the larval stage, a second phase of hematopoiesis also begins in a specialized organ, the lymph gland, in which hematopoietic progenitors give rise to mature hemocytes (5, 33-34). During this hematopoietic phase, crystal cells, plasmatocytes and lamellocytes are produced and released into the late larval / early pupal circulation as a result of the bursting of the lymph gland, a bursting that occurs earlier in case of parasitism (34, 35).

The composition of *Drosophila* hemocytes has been inferred indirectly from studies of fly mutants, either by targeting one or more proteins or based on a global transcriptomic analysis of flies producing only a subset of the three hemocyte types (35, 36). Most information has been obtained by differential comparison between normal and pathogenic conditions caused by bacteria or LPS that do not induce significant lamellocyte production (5, 33, 36). More recently, several studies of Drosophila wild-type hemocytes using single cell mRNA transcriptomics under normal and pathogenic conditions have been published, and after parasitism, up to five interacting lamellocytes clusters have been described based on the expression level of specific genes (37-40). However, as the level of gene expression does not always reflect the presence or amount of protein due to unknowns related to post-transcriptional, translational and protein degradation regulation, we decided to perform a proteomic analysis of lamellocytes in order to gain new insights into these cells.

Proteomic studies of whole hemolymph from several insect species including *D. melanogaster* have been performed (41-46) but few are available on purified hemocytes and most of the species used for this had a high number of circulating cells and/or a large hemolymph volume (44-46). Under healthy conditions, from a few hundred hemocytes at larval stage 1 to a few thousand at stage 3 circulate in a wild-type *Drosophila* larva, and these cells are primarily plasmatocytes (21, 47). This predominance of one cell type poses a problem for the study of other types of hemocytes, particularly lamellocytes, whose numbers remain low even after stimulation by parasitoid infestation (48). One way to address this is to use a mutant strain such as Hop$^{Tum\text{-}l}$ that has a dominant gain-of-function mutation in the JAK/STAT pathway, which results in higher cell proliferation and a large increase in the number of circulating hemocytes, including lamellocytes (47-50). However, even under these conditions, other problems remain, such as the few hundred nanoliters of hemolymph available per larva and the means of separating lamellocytes from others cells (48, 51). Recent studies have shown that flow cytometry can distinguish different types of hemocytes expressing fluorescent proteins or labeled with surface markers and has been used for single cell transcriptome analysis and to purify lymph gland plasmatocytes from the Hop^{Tum-l} strain (52). Although they can be adapted to small numbers of cells, these technics require specific equipment and technical skills, and can be time-consuming to obtain the number of live cells needed for certain biochemical analyses. Because the morphology and size of *D. melanogaster* plasmatocytes and lamellocytes are very different, we used a density gradient method to purify lamellocytes from Hop^{Tum-l} hemolymph, a simpler and more affordable method commonly used to separate immune cells in the human clinic and that has already used successfully in some insects (53-55). Here, we set up a workflow to purify lamellocytes and performed a proteomic study. We first analyzed the major protein spots after whole-cell solubilization and separation by two-dimensional gel electrophoresis (2D), followed by 1D-SDS PAGE bands of affinity-purified biotinylated cell surface proteins. Our results confirmed cell type purification by the high level of known lamellocyte markers (e.g., Prophenoloxidase 3, Myospheroid, Atilla) and recent potential markers described in single cell transcriptomics. We also described new surface components and a large number of proteins involved in the different functions and putative networks of these cells (cell adhesion and junction, cytoskeleton rearrangement, specific chaperones, cell signaling, secretion, endocytosis, etc.). This study complements the various transcriptomic studies and pave the way to a better understanding of their differentiation mechanisms and their roles in the immune response and physiology. This purification method will also be a valuable tool to further study these different functions under *in vitro* conditions.

Results and Discussion

Lamellocytes purification

In *D. melanogaster* Hop^{Tum-l} hemolymph, the number of circulating plasmatocytes and lamellocytes is increased compared with wild-type *Drosophila* larvae, and lamellocytes account for more than 30 % of the hemocytes at larval stage 2/3 used here (30, 50). The presence of lamellocytes in Hop^{Tum-l} hemolymph was verified by microscopic observation (Fig. 1A), which also showed that cells accumulate at two interfaces corresponding to fractions 4 (20-40% interface) and 6 (40-50% Interface) (Fig 1B and 1C, respectively) of the seven fractions collected after separation on discontinuous Percoll density gradient. In the hemolymph, cells of different sizes were present, the majority (~70%) certainly representing plasmatocytes, had a small diameter between 5 to 10 µm corresponding to a surface distribution of between 100 to 400 µm^2 (Fig. 1D). The remaining cells, with larger diameter and surface areas >400 µm^2, were assimilated to lamellocytes. In fraction 4 of Percoll gradient, the majority of cells (>80%) were large and flat with a surface area >400 µm2, whereas fraction 6 contained a majority of small cells with few large cells (Fig. 1C). Both cell fractions showed a similar protein profile on SDS-PAGE and were immunoreactive for Atilla (L1 antibody), a well-defined marker of lamellocytes (56) (Fig 1F). To further demonstrate that the large cells enriched in fraction 4 were indeed lamellocytes, we immunolabeled the cells with monoclonal antibodies directed against Atilla and myospheroid, another described marker of this cell type (57) (Fig. 2). In hemolymph, only the largest cells were strongly labeled by Atilla, whereas myospheroid labeled almost all cells, but more strongly the largest ones (Fig. 2A, 2C) (see also below). In fraction 4 (Fig. 2B, 2D), almost all cells were strongly reactive with both antibodies confirming enrichment in lamellocytes.

Proteomics of whole lamellocytes enriched fraction

Fraction 4 was highly enriched in lamellocytes and was therefore used to perform 2D gel separation of their whole-cell proteins (see Mat. and Meth.). Several hundred spots were observed and the 70 most intense were processed for protein identification by Q-orbitrap mass spectrometry (shown in Fig. 3). The most abundant proteins in each of the 70 spots and some of their characteristics and functions are shown in Table 1. Almost all of these major proteins were found at the expected molecular weight (MW). In some cases, the same protein was identified in several neighboring spots, certainly due to post-translational modifications, and sometimes, the presence of degradation products was observed in spots of lower MW. For better understanding, proteins identified by proteomics in this study will be highlighted in bold throughout the text.

As expected for whole-cell proteomics, the major proteins in the most intense spots were cytoskeletal components (Table 1) including several forms of **actin** and **tubulin** and their associated proteins (**flare**, **capulet**, **twinfilin**…), proteins involved in cell trafficking (**Rab11**, **RhoGDI**…), metabolism (**enolase**, **alcohol dehydrogenase…**) and signaling (**translationally controlled tumor protein (TCTP)**, **calmodulin**…). Different types of chaperones (**calreticulin**, **HSPs**…) were also found, including six of the eight **CCT** components of the polypeptide 1 of the chaperonin-containing tailless complex (TCP-1) (also named TCP-1-ring complex (TRiC)) (58,59). Interestingly, only 10% of cellular proteins depend on TCP-1 for their biogenesis, including the cytoskeleton proteins α- and β-actin and ɣ -tubulin (59). Among the most intense spots were also several proteins involved in cellular protection against oxidative damage and cellular detoxification (**protein disulfide isomerase**, **peroxiredoxin**, **glutathione S transferase D1**...) as well as some mitochondrial components (**porin**, **ATPases**…). In this table, the only protein reported to be specific to the lamellocytes is **prophenoloxidase 3**

(**PPO3**) found in spots 4, 5 and 30, the latter certainly corresponding to a degradation product.

With the sensitivity of Q-orbitrap LC-MS-MS, the 70 spots led to the identification of a total of 430 proteins using the *D. melanogaster* NCBI database (Table S1), indicating that several proteins were identified in each spot, especially those of high molecular weights for which the resolving power of the gel is lower. In Table S1, all identified proteins were ranked according to their Mascot score, which gives an idea of the relative abundance of each protein in this protein set (see Mat. and Meth.). Three hits (post-translational modification ubiquitin, viral proteins Polyprotein and Gypsy) absent from the *D. melanogaster* genome were therefore not retained as *D. melanogaster* proteins, and two proteins (**HDC13314**, **HDC12021**), although described previously (60), did not have corresponding annotated genes in Flybase. Among the 10 most abundant proteins, various cytoskeletal components (such as different **actin** and **tubulin**) and chaperones (**Hsc-70**) were predominant, but **PPO3** had the second highest score confirming its high abundance in these cells. A second PPO identification of (rank 17) corresponded to a chimeric cDNA sequence between the PPO3 and PPO2 ones in the database (61) (Figure S1) and therefore had no corresponding gene in the *D. melanogaster* genome. However, some of the identified peptides were specific to **PPO2** (Figure S1), indicating its presence in lamellocytes, although in a lower amount than PPO3 (see also below). The third *D. melanogaster* PPO, **PPO1,** was also identified but with a very low score (score 50, rank 219). Other known markers of lamellocytes were identified: the beta-PS **myospheroid** integrin and two alpha integrins, **alphaPS4** (*ItgaPS4*) and **alphaPS5** (*ItgaPS5*), whose genes are overexpressed in Hop$^{Tum\text{-}l}$ lamellocytes (34, 36, 62). This list also contains many known integrin-associated Proteins (IAPs) such as **GP93**, **talin**, **vinculin**, and **fermitin** (63, 64). It should be noted that the major larval hemolymph proteins, larval serum proteins **LSP1-gamma** and **LSP1-beta** (65) were found with very low Mascot scores (24 and 20 respectively; Table S1), suggesting that our procedure efficiently removed most of the soluble proteins in the hemolymph.

Proteomics of affinity purified surface membrane proteins from lamellocytes

Lamellocytes are involved in the formation of the capsule around parasitoid eggs. To do so, they must be attracted to and recognize the egg and/or the plasmatocytes that form the first layer around it, adhere to it, expand and stack to form the multicellular capsule that will also be melanized (24, 51, 66). These events indicate that cell motility, cell-cell adhesion, and cell shape change are essential components of the response of lamellocytes to parasitoid wasp eggs. These cells must also possess specific receptors/components at the plasma membrane (PM) to perform these functions. Although some of the proteins found in the 2D analysis were integral membrane proteins (i.e. integrins) or proteins that bind to them (such as IAPs), the direct solubilization method used is not the best for many membrane proteins (67). We therefore attempted to enrich these types of protein using N-octyl-glucopyranoside and tween-20, two mild non-ionic detergents that are effective in extracting a wide range of membrane proteins (67, 68). We coupled this extraction method with biotin labeling of cell surface proteins to further purify them (see Mat. and Meth.). Purification separated a subset of the total extract of cellular proteins, of which very few proteins bound nonspecifically to avidin (Fig. 4). From 1D SDS-PAGE of the affinity-purified proteins, 25 bands were cut (Fig. 4) and analyzed. The major protein identified in each band is listed in Table 2 with its roles/functions and putative or demonstrated cell localization. Among them, cytoskeleton proteins and their associated proteins were in the majority and only the two integrins **alphaPS4** and **alphaPS5** were integral PM proteins. **Rab1,** which is involved in vesicle-mediated transport and recycling, may be indirectly connected to PM, as are **Filamin-**

A, **14-3-3-epsilon,** and some other cytoskeletal components. The **beta subunit of ATP synthase**, the **voltage dependent anion-selective channel** (**porin**), and some other mitochondrial components may be enriched because they are endogenously biotinylated or complexed with endogenously biotinylated proteins (69, 70). Finally, **PPO3** was in band 4, either because it is highly enriched in these cells, or because it is associated with membranes. We then analyzed the 344 proteins identified by MS-MS in these 25 bands (see Table S2, proteins listed based on their mascot score). A good indication that we have extracted/enriched a subset of proteins from the plasma membrane was the high score for integrins, membrane ATPases, membrane receptors and transporters, and the presence of GPI-anchored proteins. Based on demonstrated or predicted cellular localization from the Uniprot database (http://www.uniprot.org), Flybase comments or the literature, ~34% (116/344) of proteins were tagged for PM or secretion (Table 3). It is noteworthy that many of the identified proteins may also be present transiently at the membrane level, such as proteins involved in membrane proteins or vesicular trafficking/recycling, or forming complexes with integral membrane proteins such as PM-associated cytoskeletal proteins like the different forms of actin involved in cell spreading (filopodia/lamellipodia) (71). Among the proteins, **PPO2** (score 82, rank 109) and **PPO1** (score 46, rank 207) were also unambiguously identified, confirming their presence and medium and low abundance in these cells, respectively. Since several studies have reported that crystal cells (the major producer of PPO1 and PPO2) are rarely found in Hop$^{tum\text{-}l}$ larvae (30, 36), contamination by these cells seems unlikely. Thus, both PPO3 and PPO2 required for the completion of capsule melanization (18) are present in lamellocytes and therefore no other source of PPO may be needed. Furthermore, while many of the proteins found in our analysis are ubiquitous and could originate from any cell type, we did not find plasmatocyte-specific markers such as

Nimrod-C1, Draper and Eater or Croquemort, nor did we find plasmatocytes-secreted proteins such as hemolectin or extracellular matrix proteins (Tiggrin, peroxidasin, etc.) (10-15). This suggests that, if present, their number/amount of proteins was very low. We therefore considered the proteomics dataset (Tables S1 and S2) as lamellocyte proteins and used them to analyze more specific networks and functional pathways that may be important in these cell functions.

Integrins and Integrins-associated Proteins

Our two different approaches demonstrated the presence of integrins and many IAPs. The *Drosophila* genome encodes five α-subunits of integrins, αPS1 (*mew*), 2 (*if*), 3 (*scb*), 4 (*ItgaPS4*), and 5 (*ItgaPS5*), and two β-subunits, βPS (*mys*) and β-nu (*Itgbn*) (72,73). These subunits form different alpha-beta dimers that play diverse roles in structural and signaling aspects of cell migration and cell-cell interaction. The two main heterodimer combinations of integrins reported in *Drosophila* are αPS1/βPS and αPS2/βPS. For lamellocytes, we found 5 integrin subunits, 3 alpha (**αPS1, 4, 5**) and the two beta (**βPS** (**myospheroid), β-nu**), which therefore possibly form a large number of dimers. However, the high and nearly identical mascot score of **βPS**, **αPS4,** and **αPS5** suggests that these may form the major integrin complexes on the surface of lamellocytes. It has been shown that **βPS** is essential for normal hemocyte motility and migration to wounds (74), as well as for successful encapsulation of wasp eggs (57). Loss of ECM or core integrin complex components (**talin** and **fermitin 1**, the orthologs of mammalian Kindlin 1) also negatively affects hemocyte migration (74) since **talin** and **vinculin** bind integrins to the cytoskeleton and are required for mechano-transduction. In addition, integrin recruitment to integrin-mediated cell adhesion sites and integrin activation both require a direct interaction between **Talin** and the **Rap1 (roughened)** GTPase (75). In addition to **vinculin** and **actin**, **talin** recruits other IAPs, such as

parvin, **paxillin**, the **adapter protein crk** (*crk*) and myosin II non-muscle **zipper** (*zip*) providing scaffolds for the assembly of the protein complex. The **Crk** adaptor protein plays a role in hemocyte migration during embryogenesis (15). It is a platelet-derived growth factor receptor (PDGFR)/vascular endothelial growth factor receptor (VEGFR) related-protein that binds to tyrosine-phosphorylated proteins transducing signals from a wide variety of sources including growth factors, extracellular molecules, bacterial pathogens and apoptotic cells, and acts on cell adhesion, spreading, and cell migration in a Rac-dependent manner (76, 77). The non-muscle myosin II **zipper** is involved in multiple functions, including cell proliferation and migration. **Zipper** aggregation function depends on the Epidermal Growth Factor Receptor (EGFR) signaling, and the c-Jun N-terminal kinase (JNK) pathway is essential for its involvement in epithelial cell shape changes during cuticular wound closure(78). EGFR signaling in lymph gland progenitors and circulating hemocytes controls their multiplication and differentiation into lamellocytes in response to parasitism (79, 80). Interestingly, we found **rhomboid-5**, a protease-like protein that regulates the secretion of several EGFR ligands and may indirectly activates its downstream signaling pathway, as well as **Arouser** and **Aveugle,** proteins also involved in EGFR signal transduction (81). **Filamin** (**cheerio**) is a large actin-binding protein that stabilizes three-dimensional actin networks and links them to cell membranes. It binds to the tails of integrin and competes for binding with **talin**, impacting the activation of **talin**-dependent integrins (82, 83). **Filamin** also interacts with **vinculin**. Interestingly, a *Drosophila* filamin gene product (*filamin-240*) is restricted to lamellocytes among blood cells and may be involved in their development through its interaction with the Toll receptor (84). Over 20 other different cellular proteins, including membrane receptors and intracellular signaling macromolecules, bind to filamin (83). This may explain the presence of γ-secretase components, **presenilin** and **nicastrin**, with presenilin interacting with the C-terminal domain of filamin (83, 85). The γ-secretase is an intramembrane-cleaving multi-subunit protease (I-CLiP) that has more than 90 reported substrates and is required for proper notch signaling.

Basigin/EMMPRIN/CD147 is a membrane protein that interacts with integrins (Figure S2) in vertebrates and invertebrates (86, 87). It is a cell surface IgG family glycoprotein that stimulates the secretion of matrix metalloproteases (MMPs) involved in tissue remodeling, cell-cell junctions, cell motility etc. (88). Drosophila MMPs can be membrane-tethered and secreted, and **Mmp1** appears in our list of membrane proteins. **Basigin** also associates closely with membrane transporters such as **CD98hc**, the heavy chain of a family of amino acid transporters (89). By regulating integrin adhesive signaling and amino acid transport, the expression level of **CD98hc** controls cell proliferation and plays a crucial role in vertebrate lymphocyte clonal expansion, epithelium turnover and tumorigenesis (90). **Basigin** interacts with many other membrane proteins (Figure S2; see also below), and is important for proteins movement and scaffolding at the membrane (87). Finally, it binds to secreted proteins such as cyclophilins A and B (here **CG2852,** the homolog of the human CypB) (91), a binding required for leukocyte recruitment and migration in response to extracellular cyclophilin signaling (92).

Septate-like junction components (SJ)

During capsule formation, electron microscopic observations indicated that cell-cell junctions morphologically resembling to septate-like junctions (SJ) were formed between adjacent hemocytes (24). Septate junctions are similar to the vertebrate tight junctions and function as permeability barriers. They may play a role in protecting the Drosophila larva from local production of reactive species during capsule melanization (23). In larval sessile islets, hemocytes are also connected to each other by septate junctions and they sometimes contact other cells such as neurons (28-31). To date, more than 24 proteins are known to be involved in SJ formation (93, 94) and here we have identified several key components such as **Neuroglian**, **Gliotactin**, **Contactin**, **Discs large 5** and the **Na+/K+ ATPase** (both **Atpα** and **Nrv1**), macroglobulin complement-related **Mcr** proteins, and GPI-anchored proteins. Both **basigin** and **integrins** interact with some of these (Figure S2). Inhibition of **Neuroglian** expression in hemocytes (L1-type Cell Adhesion Molecules (CAM) Neuroglian, L1-Cam), a member of the immunoglobulin superfamily, prevented encapsulation of parasitoid eggs: no lamellocytes, but plasmatocytes, were observed on the egg surface 48h after oviposition (95). The cell surface **Neuroglian** may be involved in direct hemocyte interactions, while intracellularly it may regulate the localization of the nucleokinesis complex protein Lissencephaly 1 (95). Interestingly, the mammalian homolog of **Neuroglian** is also required for platelet-platelet interactions (96). **Gliotactin** is a cholinesterase-like transmembrane protein required for the integrity of the blood-nerve barrier, and its loss results in SJ degradation and permeability (97). **Discs large 5** (**Dlg5**) is a protein of the membrane-associated guanylate kinase adaptor (MAGUK) family, which typically serve as molecular scaffolds and mediate the formation and localization of signaling complexes (98). **Dlg5** localizes to the apical membrane and adherens junction of the *Drosophila* follicular epithelium and its loss also results in an abnormal distribution of SJ components such as **Fasciclin-III** and **Neuroglian** (99, 100). **Contactin**, **Neuroglian, Mcr** and Neurexin-IV (Nrx-IV) have also been reported to form a tripartite complex and the organization of the SJ depends on the interactions between these highly conserved cell-adhesion molecules (101). We did not find Nrx-IV, suggesting t either it was not purified by our methods or in low amounts, or that in this cell type it is absent or not required for the type of junctions formed. It has also been shown that the **Gα(o)** protein cooperate with other G proteins to maintain the correct localization of SJ proteins (102).

Proteins that are members of the GPI-membrane anchored Lymphocyte Antigen 6 (Ly-6) protein family are also required for SJ assembly (Boudin, Crooked and Coiled) (103-105), as well as proteins involved in endocytosis and recycling, such as the clathrin light and heavy chains (**Clc** and **Chc**), **Rab5** and **Rab11** (106) (see also below). Members of the Ly-6 superfamily are cysteine-rich cell surface proteins, usually GPI-anchored, that play immune-related related roles in mammals. In *Drosophila*, the Ly-6 family is divided into two chromosomal groups comprising nine genes (Cluster III and V) (103-105). Cluster III contains three contiguous genes (***atilla**, crok* and *CG6583*) while Cluster V contains six genes: *CG31675*, *twit*, ***CG9336***, *CG9338*, *CG31675* and *CG14401*. **Atilla** and ***CG9336,*** as well as the predicted Ly-6 family protein encoded by ***CG15347,*** are among the membrane proteins of lamellocytes (Table 3; Figure S3). ***CG9336*** has been localized in glial cells, trachea, heart and lymph gland during embryogenesis (103), whereas little information is available on ***CG15347***. Thus, the presence of at least three Ly-6 family proteins on the lamellocytes membrane suggests possible functional redundancy. Recently, another GPI plasma membrane-anchored protein, the 71-kDa protein **undicht** (***udt***; **CG10217**), found here on lamellocytes, has been shown to be essential for septal junction integrity in *Drosophila* epithelia (107).

Internalization/exocytose complex

Clathrins (**Clc** and **Chc**) are major components of clathrin-mediated Endocytosis (CME) (108). Activated receptors in the extracellular membrane bind to clathrin via the AP-2 adaptor protein complex, consisting of an obligate heterotetramer of α (**AP-2α**), β2, μ2 and σ2. The interaction of the receptor-AP-2 complex with clathrin induces pit formation on the cytoplasmic side (108). **Endophilin** contributes to the pit-vesicle transition by increasing membrane curvature and final vesicle formation. Release of the attached vesicle from the inner side of the membrane requires the subsequent action of dynamin (shibire) and cytoskeleton components (108). The **homologous** protein **AP180 (LAP)** may be required to determine the amount of membrane to be recycled, perhaps by regulating the size of the clathrin cage (109). Once the vesicle is released in the cytosol, the clathrin scaffold is removed by the action of auxilin and the chaperone **Hsc-70** (110).

Several other proteins may be involved with AP-2 in the recruitment of clathrin to form pits. **Synaptotagmin 1**, a membrane protein that mediates calcium-dependent exocytosis of synaptic vesicles (111), also has all of the properties of the AP-2 receptor and AP-2 binds to it with high affinity (112). In this study, **synaptotagmin-like protein 2** (**ESyt2**), a member of the synaptotagmin family, was also found to score higher (Table S2). **ESyt2** plays a role in the rapid internalization of activated Fibroblast Growth Factor Receptor 1 (FGFR), most likely via the AP-2 complex, and signaling for this receptor functions via the ERK pathway (113). **Scamp** is one of the Secretory Carrier Membrane Proteins (SCAMPs), which play a role in clathrin-mediated vesicle budding (114). SCAMPs are evolutionarily conserved integral membrane proteins from insects to mammals that regulate membrane depolarization and Ca2+-induced regulated secretion. The *Drosophila* genome contains a single **Scamp** gene (115). It is also well established that soluble NSF attachment protein receptors (SNAREs) such as syntaxin, SNAP-25, and synaptobrevin-2/VAMP-2, form the core of the membrane fusion machinery that regulates calcium-triggered exocytosis in neurons (116). Several family members involved in vesicular trafficking and in different SNARE complexes such as **Synaptobrevin** (**Syb**), **Syntaxin 13**, **Ykt6 v-SNARE**, **VAMP7 Golgi SNAP receptor complex member 1** (**Gos28)** have been found (117-121). The **CG1572** membrane protein, which has a

MARVEL (MAL and Related proteins for VEsicle trafficking and membrane Link) domain, might also be involved in vesicle transporters or in the regulation of junctions (122).

The presence in our analysis of proteins from these intracellular compartments could result from the extraction of membranes from the ER, Golgi, Lysosomes or their derived vesicles which may also explain the presence of many proteins from these different origins (**Alix**, Annexins (**AnxB9, AnxB10, AnxB11**), **Arfaptin**, **ArfGAP**, **ODR4 homolog**, **Pdi, Rush hour**, **Ral**, **Reticulon-like1 protein**, etc.). However, their presence after the affinity column suggests that they were either biotinylated and thus at the plasma membrane or associated with biotinylated surface proteins. Since many of these proteins may also be involved in exocytosis, endocytosis, chaperoning of plasma membrane proteins, vesicular transport and plasma membrane recycling, they could be found at the PM during these processes. This may also explain the presence of the different Rab proteins (**Rab1**, **Rab2**, **Rab5**, **Rab7**, **Rab8**, **Rab10**, **Rab11**, **Rab35**) and their regulatory proteins such as the **RabGDP dissociation inhibitor** (*Gdi*) that regulate endo- and exocytosis and transport of intracellular vesicles (123, 124). It should also be remembered that lamellocytes are large flat cells with a small amount of cytoplasm, suggesting that the intracellular compartments may be close to or directly in contact with the plasma membrane (125).

Among other endocytosis related proteins, we found in the 2D whole cell analysis (Table S1) the proteins **Flotillin 1** and **Flotillin 2** that have been shown to be involved in clathrin-independent endocytosis (CIE) and in various cellular processes such as cell adhesion, signal transduction through receptor tyrosine kinases, and vesicular cell trafficking (126). Flotillins participate in the formation of specific membrane microdomains (suggested to be lipid raft domains) that can form uncoated membrane pits that can be invaginated and internalized into the cell. To date, it has been suggested that several cargo molecules, such as **basigin**

(CD147), the GPI-anchored protein CD59 (a mammalian Ly-6-like protein), the cholera toxin B subunit (CTxB), proteoglycans and proteoglycan-bound ligands, as well as cholesterol transport protein, are internalized by the CIE pathway (127, 128). In addition, flotillins can participated in the pre-classification of several receptors (such as EGFR) and other cargo molecules prior to their endocytosis by the CME-dependent pathway (126). Recently, we demonstrated that venosomes, vesicles present in Figitid wasp venom that alter lamellocyte function, enter these cells through a flotillin/lipid raft-dependent endocytic pathway (129). In this previous study, we immunolocalized clathrin, flotillin and several of the Rab proteins (Rab-5, -7, -11) in Hop^{tuml} lamellocytes, confirming their presence.

Membrane receptors and associated proteins

SR-CI, is a multiform recognition receptor that binds to both Gram- and Gram+ bacteria and has high binding affinity to low-density lipoproteins (130-132). Our 1D analysis also showed the presence of **SR-CIV**. Members of the *Drosophila* scavenger C receptor class are SR-CI and CII, two membrane-bound receptors, and CIII and CIV, which lack a transmembrane domain and can be secreted (130).

Two Gram Negative Binding Proteins, **GNBP3** (133, 134) and **CG30148/GNBPLike4** ((135); whose producing tissue in the larva is unknown), were also on our list of membrane-associated proteins. GNBPs are pattern recognition receptors that activate a broadly specific inflammatory response. GNBP3 can be membrane-bound or free in the hemolymph. It is required for activation of the Toll pathway in response to fungal infections and is overexpressed during nemato-bacterial infections (136). There is no available information on GNBPLike4.

The *Drosophila* fat body produces two main types of lipoprotein particles: Lipophorin (LPP), the major lipid carrier in hemolymph, and Lipid Transfer Particle (LTP). Lipophorin and lipoprotein receptors (LpRs and LRPs, respectively) belong to the Low-Density Lipoprotein Receptor (LDLR) family and play a key role in the uptake of lipoprotein particles (137) and in the endocytosis of different receptor complexes (138). Lipophorin receptors (LpRs) are also involved in the *Drosophila* immune response (139, 140). The lipoprotein receptor **LRP1** recognizes apolipoproteins and a wide variety of extracellular proteins or protein complexes, including many protease inhibitor complexes (141). In *Drosophila* **LRP1** binds the thrombospondin/Notch complex and stimulates Notch activity by driving endocytosis of the Notch ectodomain (142). LRP-dependent endocytosis in vertebrates is also a major pathway for the uptake of transferrin, an iron binding protein. In our list (Table S2), we found the **Transferrin 2** (*tsf2*) protein that can bind to LPR1 for uptake into lamellocytes and is required for septal junction assembly in epithelial cells (106). The presence of **Ferritin** (*Fer1HCH*), the major cellular protein that stores transferrin-supplied iron (143,144), supports a role for transferrin iron transport in lamellocytes. Because of oxygen generation, the amount of iron must be carefully regulated in the cell, however, an increase in radical intermediates occurs during encapsulation (145).

Sema1b is a transmembrane protein member of the Semaphorin family. Semaphorins associate with plexins and other cell surface receptors (including integrins) to mediate cell-cell contact, migration, and activation of cell signaling (146, 147). It is also known that membrane-associated semaphorins can interact with signaling proteins through their cytoplasmic domains, suggesting that they mediate reverse signaling events (147). **Lectin-24A** transcription (found in 2D analysis) is strongly and rapidly induced after wasp parasitism (148-150). **Sema1b** and **Lectin-24A** may participate in cell-cell recognition/adhesion during capsule formation.

In mammals, the TGF-β signaling pathway controls several aspects of hematopoiesis and is involved in inflammation and tissue repair (151). In *D. melanogaster,* the TGF-β pathway is involved in the immune response to injury and infection by bacteria and nematodes (152, 153). TGF-β receptors are single-pass transmembrane proteins classified into Type I and Type II receptors. **Punt** (*Put*) is a large type II receptor that is involved in both Dpp/BMP and Activin signaling (154). Downstream signaling from Punt depends on its heterodimeric form with other receptors and the activation state of other pathways such as EGFR. In Drosophila, the cytosolic protein **CtBP** exerts a repressive role in the Dpp pathway (155). Because activin-β is expressed by the neurons in the peripheral nervous system and regulates the proliferation and adhesion of hemocytes in the subcuticular sessile compartment, it may control the release and formation of pro-lamellocyte cells (156). BMP signaling is also important for maintaining the number of cells in the posterior signaling center of the lymph gland (157).

Drosophila encodes more than 200 membrane G-protein-coupled receptors (GPCRs) involved in many different biological functions (158). **Methuselah-like 4** was the only GPCR we found (Table S1). The Methuselah/Methuselah-like gene family is involved in development, lifespan and multiple stress response. One putative ligand of these receptors is Stunted (Sun), a circulating insulinotropic peptide produced by fat cells (159), a second, with a role in immunity has been described more recently, growth-blocking peptide (GBP) (160). Signal transduction of GPCRs depends on heterotrimeric G-proteins Gα, Gβ and Gγ. Gα proteins are membrane- anchored GTPases that catalyze the hydrolysis of GTP to GDP: **Gα(o)** regulates adenylate cyclases while **Gα(q/11)** targets phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate (IP3) and diacylglycerol (DAG) (158). **Gα(o)** is also involved in the regulation of vesicular trafficking

(102). GPRCs are inactivated by arrestins that have also been implicated in receptors endocytosis and cross talk with other signaling pathways (161). **kurtz** β-arrestin (krz) and arrestin-like **CG1105** may play this role in lamellocytes.

DAG stimulates the translocation of **protein kinase C-delta** (*PKCdelta*) from the cytosol to membranes, including plasma membranes (162). In mammals, PKC-delta is a substrate for caspase-3. Its proteolytic activation has been directly linked to apoptosis (163) and negatively regulated collagen-induced platelet aggregation (164). In *Drosophila*, PKC activity is required to mediate 20E-induced protein expression (165). Finally, cell surface expression of certain GPCRs, ion channels, G-alpha protein, and even other types of receptors, is dependent on an **odr-4 (*CG10616*)-**dependent pathway (166,167).

Transmembrane transports

Activation of **Trp** transient potential channels and Trp-Like receptors is mediated via the phosphoinositide cascade, with Ca^{2+} and diacylglycerol (DAG) being essential for generating the response (168). Trp are polymodal cellular sensors involved in a wide variety of cellular processes, including Ca^{2+} and Mg^{2+} homeostasis and lysosomal function (169,170). Trp channels are opened by the activated **Gαq** subunit, which in turn activates phospholipase C, ultimately leading to cell depolarization. *Drosophila* Trp channels are also regulated by binding of the immunophilin **FKBP59** and **Calmodulin** (168,171,172).

The Drosophila vacuolar H^+-ATPase (V-ATPase) multigene family includes 33 genes (173), and we have found eight of these V-ATPase-complex proteins (**Vha100-2**; **Vha100-1**; **Vha68-2**; **Vha55**; **Vha44**; **Vha36-1**; **Vha26**; **VhaSFD**) among which six are considered PM proteins (Vha68-2, Vha55, Vha44, Vha36-1, Vha26, VhaSFD) (173). V-ATPase functions as an electrogenic H^+ pump regulating the pH of intracellular compartments, which in turn governs the dissociation receptor ligands, promotes coupled transport of substrates across membranes, and also participates in the recycling of receptors (i.e. LPRs) and various cotransporters (174,175). In tissues where the V-ATPase is expressed, it may serve to acidify the extracellular microenvironment. The latter function may be important for lamellocytes during capsule formation because a decrease in pH around the egg may promote the melanization reaction (176) or directly kill the egg. Interestingly, activated neutrophils and macrophages use V-ATPases to maintain neutral cytoplasmic pH, i.e. proton extrusion, during a metabolic respiratory burst (177).

A cotransporter present on lamellocytes is the **major facilitator of the transporter superfamily 3** (*MFS3*), an inorganic phosphate-sodium symporter (178). This type of transporter, coupled to H^+ or Na^+ gradients, maintains the intracellular concentration of inorganic phosphate against the electrochemical gradient. Phosphate-sodium symporters are involved in some of the cellular effects of phosphate in fly cells, such as activation of MAP Kinases (178). A second membrane transporter is the **CG1208** sugar transporter**,** a member of the solute carrier 2 family (SLC2) responsible for basal glucose uptake.

Aquaporins (AQPs) are integral membrane proteins that transport water and, in some cases, small solutes such as urea. The *Drosophila* genome contains eight genes encoding aquaporins, including the *Drosophila* integral protein (**DRIP**), a highly selective water-specific channel with high sequence similarity to vertebrate AQP4 (179). The integral protein **PRIP** is the other aquaporin we found in the analyses. Insect PRIPs have heterogeneous solute preferences and can transport both water and urea (180). Aquaporins have been implicated in cell migration events by facilitating the rapid changes in cell volume that accompany changes in cell shape. This effect may be particularly pronounced at the leading edge of migrating cells, where changes in local cytoplasmic osmolality may produce actin polymerization/depolymerization and transmembrane ion fluxes (181).

Cell surface and secreted enzymes and inhibitors

ADAMs (a disintegrin and metalloproteinases) are membrane-anchored metalloproteases involved in cell-cell and cell-matrix interactions. Their protease activity is also important in the shedding of ectodomains from surface proteins (182). The functions of *Drosophila* ADAMs are less known than their mammalians counterpart (183). Here we found ADAM **meltrin** involved in the embryonic central nervous system (184). *Drosophila* meltrin is the ortholog of human MELTRIN alpha (ADAM 12) involved in cell fusion that leads to the formation of multinucleated cells such as macrophage-derived giant cells (185). ADAMs are also activated by several GPCRs to produce a mature ligand for EGFR leading to EGFR transactivation. ADAM-10 was the first to be implicated in EGFR transactivation and other studies have identified ADAM-12 as a player in this process (186).

In *Drosophila,* there are only 2 non-redundant Mmps (Mmp1 and Mmp2) that cleave different substrates and are expressed in a time- and tissue-dependent manner (187). **Mmp1** expression was found to be upregulated after injury under the control of the JNK pathway (188). The substrates of Mmp1 are not known, but it may play a role in capsule formation as it participates in ECM remodeling and cell migration.

Dipeptidyl-peptidase III (DppIII) cleaves the N-terminal dipeptides of various bioactive peptides. In insects, DppIII is thought to be involved in the degradation of the neuropeptide proctolin (189), a pentapeptide that modulates multiple physiological processes such as muscle and cardiac contraction, circulation, stomach and gut motility, etc. (190). In mammals, *DPP3* (the DppIII ortholog) is expressed in many immune cell types, although its function in these contexts has not been investigated (191).

CG17337 is a dipeptidase that belongs to the M20 family of metallopeptidases expressed ubiquitously in *Drosophila*. It has a high sequence identity with mammalian CNDP2 (carnosine dipeptidase II) (192). In mouse and human cells, CNDP2 localizes in the cytosol and nucleoplasm, whereas **CG17337** has been shown to be an extracellular component of larval hemolymph (39, 193). In some cancers, CNDP2 plays a role in signaling; its increase activates the p38 and JNK MAPK pathways to induce cell apoptosis and its decrease the ERK MAPK pathway to promote cell proliferation (194).

CG8945 is a zinc metallo-carboxypeptidase of the non-peptidase M14A homolog subfamily. Carboxypeptidases are soluble, secreted enzymes (such as carboxypeptidase A1 from the human pancreas) that hydrolyze the single C-terminal amino acids of polypeptide chains. **CG8945** has a signal peptide and can be exported from the cell, its exact role being unknown. Another protease whose exact function is unknown is **CG5390,** a Trypsin-like serine protease, which is suggested to be secreted rapidly into the hemolymph after fungal infection (193).

Along with these different proteases, two major classes of secreted inhibitors have been discovered, serine proteases inhibitors (SERPIN) and endopeptidase inhibitors. The *D. melanogaster* genome contains 29 serpin genes (195-197) and four of these gene products were identified: **Spn38F** (Serpin 3), **Spn43Ab**, **Spn55B** (Serpin 6) and **Spn88Ea** (Serpin 5). Three of them have a recognized inhibitory function (**Spn38F**, **55B** and **88Ea**), whereas **Spn43Ab** is considered a non-inhibitory serpin (198). **Spn43Ab**, **Spn55B** and **Spn88Ea** have been found in larval hemolymph (199) and **Spn38F** is present in male accessory glands and has an antimicrobial effect (200). The role of **Spn43Ab** is still unclear, it is highly expressed in larval stages 1 and 2, late pupae, and adults, and its deletion is not associated to any phenotype (201). **Spn55B** is involved in wound repair and final tissue regeneration after wound healing (202-204). **Spn88Ea (**Spn5) negatively regulates the Toll pathway: when overexpressed, the level of the active C-terminal form of Toll ligand Spaetzle (Spz) is downregulated, whereas in **Spn88Ea**-deficient larvae, Toll receptor expression if upregulated (205). **Spn88Ea** secreted by normal epithelial cells also acts as a component of the extracellular surveillance system that facilitates the removal of premalignant cells from the epithelium. The exact role that serpins may play in lamellocytes and/or encapsulation will require further work, but some are clearly involved in the activation of the PPO1 and PPO2 cascades (18, 206).

The two endopeptidase inhibitors present, the Thioester-containing protein 4 (**TEP4**) and at a low level macroglobulin complement-related protein (**Mcr**; also TEP6), both belong to the Thioester-containing proteins (TEP) superfamily, which are secreted immune-related effector proteins (207, 208). In *Drosophila,* the family is composed of six genes (Tep1–Tep6). TEP2, **TEP4** and **Mcr** promote *in vitro* phagocytosis of some Gram-negative bacteria and fungal pathogens (209) probably by activating the Toll pathway (210). The latter study also shows that TEPs may participate in defense against parasitoids but without explaining the underlying mechanism. Apart from its role in septal junctions described above, **Mcr** (whose thioester-binding site appears to be nonfunctional) is essential in the epithelium for macrophage migration to epithelial wounds (211). These different inhibitors may also control the activity of some of the peptidases described above**.**

There are four γ-glutamyl transpeptidase genes in the *D. melanogaster* genome (GGT1, CG17636, CG1492 and **CG4829**). The γ-glutamyl transpeptidase **CG4829** is a glycosylated membrane protein expressed in various tissues (212) and has been used in embryos to visualize hemocyte precursors and their spreading pattern during late embryogenesis (213, 214). GGTs play a key role in glutathione metabolism, amino acid uptake, and redox homeostasis. These functions may also involve the various Glutathione S-transferases (**GST-**

D1, -D3, -D9, -E12, -E6) and thioredoxins (**Jafrac1, Sh3beta, Clot, CG5554, CG6888, CG9911, CG12547**) (215, 216).

Secreted factors

We found members of two families of secreted growth factors: the imaginal disk growth factors **Idgf4** and **Idgf5** (217), and the adenosine deaminase-related growth factors (**ADGF-A**; (218,219)). The IDGF family is composed of six secreted glycoproteins belonging to the glycosyl-hydrolase family, but their catalytic domain is inactive and can serve as a chitinase-like protein binding module (Chitinase-like protein). IDGFs cooperate with insulin to stimulate cell proliferation, polarization and motility (220). They are produced by the fat body of larvae (217) but *idgf-1*, *2* and *3* have been detected in hemocytes transcripts and *in situ* hybridization has shown that *idgf-1* mRNA is present in a majority of lymph gland cells (221). Whether Idgf4 and Idgf5 are synthesized or uptaken from the hemolymph by lamellocytes remains to be determined.

ADGF-A (Adenosine deaminase-related growth factor A) is one of the most abundantly expressed ADGFs in the *Drosophila* larva, particularly in the gut and lymph gland, and hemocytes are the primary regulator of adenosine levels in the larval hemolymph. Modulation of extracellular adenosine during the inflammatory response is an evolutionary mechanism conserved from insects to vertebrates (222). ADGF-A is specifically expressed in aggregating melanotic capsules-forming hemocytes and at sites of inflammation and mutation of the adenosine receptor (AdoR) significantly reduces the number of lamellocytes and thus resistance to wasp infection (223).

Chd64 (**Transgelin**) is an actin-binding protein containing a calponin (CH) homology domain, it is the homolog of human Transgelin-2 that regulates T cell activation and stabilization of

T–B cell conjugates in human (224). In *Drosophila*, **Chd64** is suggested to play a role in cross-talk between 20-hydroxyecdysone (20E) and juvenile hormone (JH) signaling pathways (225).

GO analysis.

The gene annotation symbol in Flybase was retrieved for each identified protein and the two proteomic lists were compared (Fig S4): only 134 genes were common (Fig S4, list 1D 2D), 296 being specific to the 2D whole cell approach (including two proteins without an associated gene) (Fig S4, list 2D) and 209 to the purification approach (including one protein without an associated gene) (Fig S4, list 1D). We generated a set of unique gene tags and used it for gene ontology (GO) analyses. The most enriched cell components compared to all *Drosophila* GOs are involved in organelles, lipid particles and the non-membrane bound cytoskeleton. The biological processes are related to binding (anion, nucleotides, small molecules…), while the most enriched biological processes are cytoskeleton and organelle organizations. The most enriched pathways are related to endocytosis, phagosomes, amino acid biosynthesis and metabolic pathways (see Table S3). Note that most of the proteins in the phagosome pathway are also involved in endoplasmic and cytoskeletal functions (226, 227). The list of unique tags can also be used in the String program (https://string-db.org/) to construct interaction networks. Considering only the experimental evidence in the String program, we observed several clusters (Figure S5): the most intense ones are formed by ribosomal proteins and cytoskeletal proteins, then a fuzzier cluster includes TCP proteins linked to proteins in a Rab cluster, the integrin cluster, and some IAPS, which are themselves linked to the Vacuolar ATPases cluster. Other protein clusters described above are also visible (such as Ykt6, Gos28, Vamp7, etc.) in addition to new ones formed by unknown proteins in the same metabolic pathway (i.e., Kdn, CG5261, CG7430, etc.).

Comparison with hemocytes/lamellocytes transcriptomics

Several proteomic analyses have been performed on *Drosophila* hemolymph under different physiological conditions and on the wound response clot (40, 193, 199), but to our knowledge, none on hemocytes. The proteome of mbn-2 cells (plasmatocyte-like cells derived from embryos maintained in culture) was analyzed after stimulation with bacterial LPS, leading to the identification of 24 intracellular proteins with increased or decreased amounts, involved in Ca^{2+} signaling, nuclear transport, phagocytosis, and cytoskeletal remodeling (228). Previous work has also analyzed the global transcriptome of wildtype and mutants *D. melanogaster* to explore the response to immune challenge (36, 140, 149, 229-231). In their study, Irving et al. (36) addressed this question using different mutants for hemocyte types including Hop$^{Tum\text{-}l}$; 2517 genes were identified that were at least twice overexpressed in hemocytes compared to the whole larva, including 406 genes for Hop$^{Tum\text{-}l}$. From their data, we compiled a partial list of 69 differentially expressed genes for Hop$^{Tum\text{-}l}$ hemocytes that were "tagged" immunity-related genes (Table S4) and compared it with our list of unique genes. 12 genes were in common: the three PPOs, the five integrins, Thioester-containing protein 4, scavenger receptor SR-CI, vinculin and viking.

Recent publications have used single-cell RNA sequencing to define different populations of *Drosophila* hemocytes from the different hematopoietic origins (37-40). These studies identified in the hemolymph and lymph glands of wild-type *Drosophila* larvae more than 10 groups of prohemocytes and plasmatocytes, one or two groups of crystal cells, and, in wasp-parasitized larvae one (39), two (37, 38) or even five different groups of lamellocytes (40), some of which represent different states of lamellocytes maturation following parasitism. Indeed, after parasitism, circulating and resident plasmatocytes transdifferentiate into lamellocytes (during the first 24 h after infestation), at the same time as hemocytes proliferate in the lymph glands that begin to lyse 48 h after infestation, releasing new lamellocytes into the circulation. There may be differences in gene expression in lamellocyte groups from the two origins (40). These studies showed that most hemocyte clusters expressed myospheroid and atilla (although at higher level in those of lamellocytes) explaining why we observed a plasmatocyte cell labelling in our immunoassay (Fig 2).

Fu et al., (39) defined ten genes whose expression is restricted to the lamellocyte group and of these four, *cheerio* (***Cher***), the *nuclear protein lamin C* (***LamC***), *short stop* (***shot***) and methuselah-like 4 (***mthl4***) were found here by proteomics. Cattenoz et al., (37) defined two lamellocytes cluster (LM1 and LM2) with LM-2 intermediate between plasmatocytes and LM-1, and described novel shared and specific markers for these two populations (see Table S5); LM1 markers were expressed at low levels in LM2 and in other hemocytes, whereas markers enriched in LM2 were also expressed in other hemocytes. We found that 67% (31/46) of LM1, LM2 markers were present in our proteomics, whereas 37% (19/51) of LM1 and 38% (5/13) of LM2 markers were recovered, respectively (Table S5). Cho et al., (38) also described two lamellocytes populations (also named LM1 and LM2, LM1 being suggested to be proLM2 lamellocytes) and we compared our data with the top 35 gene markers obtained for these two clusters (see Table S6). Most of the common LM1-LM2 markers (6/7), 43 % (12/28) of LM1s and 25 % of LM2s (7/28) were present in our data. Finally, Tattikota et al., (40) described five distinct groups of lamellocytes, that were named LM1-4 and CC based on the expression of most enriched genes (see Table S7A and S7B). CC and LM1 cells had low *atilla* expression and were enriched in crystal cell marker genes such as *PPO1,* and LM2, LM3, and LM4 could be subtypes of mature lamellocytes. Four out of the 10 top markers of CC-LM1 clusters (including *PPO1* and *PPO2*) and 7/13 of the LM2-LM3-LM4 clusters were found here by proteomics (see Table S7A) as well as 50% (9/18) of the most expressed LM2 genes over all conditions tested by the authors (see Table S7B).

Overall, all of these comparisons suggest that several types of pro-lamellocytes and mature lamellocytes are produced in Hop$^{tum\text{-}l}$ larvae and circulate in the larval hemolymph and that our procedure did not appear to specifically enrich any of the categories.

Conclusion

This work demonstrates the feasibility of purifying lamellocytes from the Hop$^{Tum\text{-}l}$ mutant of *D. melanogaster*, thus enabling proteomic and biochemical approaches such as the enrichment method and analysis of their membrane proteins. Our proteomic data confirm previous studies and observations but also provide new information about these cells. As expected, these highly mobile and plastic cells possess abundant cytoskeletal mechanisms. They are metabolically active, and the high content of different and specific chaperones suggests that they are also transcriptionally active and actively produce new proteins. This is also confirmed by the high number of endo- and exocytosis pathways, suggesting that these cells are involved in the active uptake and secretion of hemolymph components or components involved in the encapsulation process. Among the large number of membrane proteins identified, integrins and IAPs form a clear network involved in cell adhesive interactions and in the formation of cell-cell junctions that should play a key role during the encapsulation process. All of these components are inherent in the bidirectional relationships between the cell and its extracellular matrix, in the context of 'mechanoreciprocity' that allows cells to change position, define movement trajectory, cell-cell association, and cell fate decisions in a temporal manner (232). This network also includes members of different signaling pathways such as EGFR, BMP etc., and we also found downstream components of these pathways such as many kinases and phosphatases as well as regulators of transcription factors such as SAM-domain containing proteins that may be involved in lamellocyte development and differentiation (27, 29, 232, 233). Many of the identified proteins do not yet have a clear specific function(s); however, their structure or sequence homology to known proteins provide information about their potential role. Others have known function(s) and were also found in transcriptomic screens such as **mthl4**, **Chd64**, **CG14610**, **Drip**, **shot,** etc. For these proteins, it may be interesting to test in the future their specificity as lamellocyte markers and to explore their role in lamellocyte functions. Therefore, although much work remains to be done to fully exploit all this information, this work will open new perspectives for a better understanding of the different tasks performed by lamellocytes in *Drosophila* physiology and immune response. They will also be essential for a better understanding of the interaction with the components of the venom injected by parasitoid wasps that lead to the alteration of the function of these cells and to parasitic success (26-28, 127).

Materials and methods

Biological material and hemolymph collection

D. melanogaster Hop$^{Tum\text{-}l}$ flies (N° 8492, Bloomington Drosophila Center) were maintained on standard Drosophila medium (10% corn meal, 10% yeast, agar, and nipagin) at 25°C with a 12h light/dark cycle and 50% humidity. This temperature allowed the production of a large amount of hemocytes without melanotic tumor formation as previously reported (30). Hemolymph was collected from late L2 or young of L3 larvae (5 day old larvae). Larvae were washed three times in PBS, and then the anterior cuticle was gently torn with forceps in insect Ringer solution (IR).

Lamellocytes enrichment by density gradient

A fresh 90% Percoll® stock solution (GE Healthcare) was prepared by 9:1 (v/v) dilution with 10x IR before each purification. This stock solution was then diluted with IR to prepare solutions with final concentrations of 50%, 40%, 20% and 10%. A discontinuous gradient was formed in 750 µl tubes by ascending layers of 150µl of 50% solution, 200µl at 40%, 150µl at 20% and 100µl at 10%. Hemolymph from 100 larvae was collected in 100µl of IR, loaded at the top of the gradient, and centrifuged at 500g for 30 min (4°C) using a swinging rotor (rotor 1154L; Mikro 220R; Hettich). Seven 100µl fractions were gently collected from the top (fraction 1) to the bottom (fraction 7) of the tube using a slow-suction syringe to preserve the cells. In a preliminary study, to determine the position and type of cells in the gradient, each fraction was divided in half: 50µl were used for microscopic observation and 50µl were centrifuged at 500g to pellet the cells. The pellet was resuspended in 200µl of IR, centrifuged again, and the cells were resuspended in 50µl of 2x reducing Laemmli buffer (234) before separation on 12.5% SDS-PAGE (25µl for silver staining and 25µl for western blot).

The two-dimensional gel separation was performed as previously described (235): for isoelectric focusing (IEF), fraction 4 containing lamellocytes was centrifuged at 500g and the pellet was resuspended in 35µl of IR, mixed with 5µl of denaturing solubilization solution (0.15M dithioerythritol, 10% SDS) and heated to 95°C, 5min. After cooling, the sample was mixed (1/1, v/v) with a 9.2M solution of urea, 0.1M dithioerythritol and 2% CHAPS. IEF was performed using a 4% acrylamide gel in 15cm tube containing 9.2M urea, 2% ampholytes [1% pH 3-10 (Pharmacia) and 1% pH 2-11 (Servalytes)] and 2% of CHAPS. After migration, the IEF gel was incubated with Laemmli 4x reducing buffer and laid on top of a 12.5% SDS-PAGE. After separation, the proteins were silver stained according to Morrissey (236). Molecular weights were estimated using predefined protein markers (Thermo Scientific).

Biotin labeling, extraction and purification of membrane proteins.

One hundred µl of lamellocytes-enriched fraction 4 was mixed gently with 2mM NHS-SS-PEO4-biotin (Interchim), a cleavable and non-permeable coupling compound, and incubated for 30min (4°C) under gentle agitation. Labeled cells were harvested by centrifugation (500g, 10 min) and resuspended with 100µl IR with 3% BSA to inactivate residual NHS-biotin. After a second wash with 200µl IR, the cell pellet was suspended in 200µl of extraction solution (Sol1: IR supplemented with 30mM Octyl β-D-glucopyranoside (Sigma), 0.5% Tween (Sigma) and a cocktail of protease inhibitors (SigmaFAST Protease Inhibitor Cocktail EDTA free; Sigma) and incubated 30min at 4°C. Solubilized proteins were recovered in the supernatant after 10min centrifugation at 5000g (4°C) to remove cell debris and nuclei. The supernatant was then mixed with pretreated streptavidin magnetic beads (GE Healthcare) and incubated for 1h at 4°C with gentle agitation. After incubation with the biotinylated proteins, the beads were collected, washed once with 100µl of Sol1 and again with 10x IR to remove nonspecifically bound proteins. Avidin-bound proteins were released by heating at 96°C for 10min in the presence of 30µl of Laemmli buffer containing 2.5% β-mercaptoethanol to cleave the S-S bond from the NHS-biotin arm. The samples were run directly on a 12.5% SDS-PAGE or stored for a short time at -20°C. Streptavidin beads were pretreated before use as follows: 25µl of the beads were washed twice in 500µl of IR, incubated with 500µl of 3% IR-BSA for 30min, rinsed with IR and incubated with Sol1 for 1h. As a control for nonspecific binding, an equivalent amount of unlabeled extracted membrane proteins was treated similarly to the labeled extract.

Immunohistochemistry.

A 50µl drop of hemolymph (or gradient fractions) was placed in the center of a sitting coverslip in a 12-well culture plate to form a wet chamber. Cells were allowed to adhere for

1h, then fixed for 6min with 100% acetone and washed 3 times with PBS, then incubated with PBS 0.3% BSA for 30min. After fixation, the cells were incubated with the indicated primary antibody. After 1h of incubation at RT, cells were washed three times with PBS and incubated for 1h with the secondary antibody (1/500th; goat anti-rabbit IgG Fluoprobes 488; Interchim). Actin was then labeled with phalloidin (7nM, Alexa-phalloidin 490; Interchim). After three washes with PBS and one with deionized water, the coverslip was mounted using an anti-fading medium containing DAPI (Interchim) to label the nuclei. Photographs were taken with an LSM 880 laser scanning confocal microscope (Zeiss).

Cell surface measurements

The surface distributions of hemocytes from hemolymph and Percoll gradient fractions were estimated using the Image J software (https://imagej.nih.gov/ij/). Digital images of actin-labeled cells were taken with the axioplan Z1 microscope (Zeiss), contrasted, and the cells transformed into ROI to measure their surface area. Cells were grouped into 6 classes according to their surface area (25 <100 μm^2; 100 <250 μm^2; 250 <400 μm^2; 400 <625 μm^2; 625 <900 μm^2; > 900 μm^2).

Immunoblotting

Proteins separated on a 12.5 % SDS-PAGE were transferred to a nitrocellulose membrane. The membrane was blocked with 2% skim milk in 0.1% TBS-Tween (TBS-T) and incubated overnight at 4°C with the indicated antibody. After three 10min washes in TBS-T, the membrane was incubated with the peroxidase-coupled goat antirabbit secondary antibody (1/2000; Sigma) for 2 hours in TBS-T-2% skim milk at RT. After three more washes with TBS-T, the membrane was revealed using a chemiluminescent substrate (Substrat HRP Immobilon Western, Merck-Millipore) and imaged with a digital analyzer (ChemiGenius2; Syngene).

Mass spectrometry

Protein identification was performed by LC-MS/MS (Q-orbitrap mass spectrometer, Q-Exactive, Thermo-Fischer Scientific). Gel spots and excised bands were treated with trypsin (10ng/µl; Promega) to generate the protein fragments. Peaklists were generated with Proteome Discoverer 2.0 in mgf format. Data analysis was performed with Mascot 2.3 software (http://www.matrixscience.com) using the NCB *D. melanogaster* nonredundant database (https://www.ncbi.nlm.nih.gov; March 27th, 2017). Mascot Mudpit scoring parameters were used (sum of score above threshold of significant peptide matches plus the average threshold of these matches). The significance threshold was set to $p<0.05$, a maximum number of hits fixed to AUTO (to display all of the hits that have a protein score exceeding the average identity threshold score for an individual peptide match). An automatic search of the decoy database was done. The analysis was performed with a mass tolerance of 20 mDa for fragment ions and 10 ppm for parent ions. The carbamidomethylation of cysteines and the oxidation of methionines shown as variable modification for calculation of peptide masses. The maximum number of missed cuts by trypsin was set at 5 in case of biotinylated proteins and to 2 otherwise. Residual modification of peptides by cleavable biotin was considered a described (237).

Additional data with details on the identified peptide (m/z, charge, error, amino acid sequence, modifications, identity and homology scores, expectation value, unique peptide) as well as details on the identified proteins (protein family number, coverage, misscleavages, number of peptides, number of significant peptides, proteins matching the same set of peptides) are given in Table S1 and S2 (see raw data sheet). A brief analysis of the proteins identified with confidence is also provided (analysis sheet).

GO analysis.

For each identified protein, its name, gene number and information were retrieved from Flybase (http://flybase.org) or from the literature. The list of gene identifications was transformed into protein IDs (https://david.ncifcrf.gov/content.jsp?file=conversion.html) for use in GO analysis software (Panther GO; http://pantherdb.org), String v11 (https://string-db.org) and ShinyGO v0.51 (http://bioinformatics.sdstate.edu/go/; with the different available options). The Venn diagram was made online (http://bioinformatics.psb.ugent.be).

Data availability

The complete proteomics raw data for this paper are deposited in the PRIDE Archive (https://www.ebi.ac.uk/pride/archive/). The project name is : *Drosophila melanogaster* lamellocytes proteome; the project accession number: PXD016876 and the Project DOI: 10.6019/PXD016876.

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Author Contributions Statement

B.W. performed the purifications and fluorescence microscopy, as well as gel electrophoresis and Western blotting with the help of S.F. M.B. performed the proteomics analysis. B.W., J.L.G. and M.P. analyzed the data and wrote the manuscript. J.L.G. and M.P. obtained the funding and designed and coordinated the work. All authors read and approved the final manuscript.

Competing financial interests:

The authors declare no competing financial interests.

Figures legend

Figure 1: Purification of lamellocytes by Percoll. A-C, Microscopic images of hemocytes present in total hemolymph (A) and in layers 4 (B) and 6 (C) of the Percoll gradient (green phalloidin-labeled actin). D, Surface distribution of hemocytes in hemolymph (black column) and in fraction 4 (gray) showing enrichment in large cells (489 cells analyzed for hemolymph; 444 cells for fraction 4; average of 2 separate experiments). E, Silver-stained 12.5% SDS-PAGE of the different gradient fractions (1-top, 7-bottom) and total hemolymph (He). F, Western blot of the same samples revealed with the polyclonal antibody against the lamellocyte marker Atilla (1/2000) showing that fractions 4 and 6 may be enriched in lamellocytes. Molecular weights (MW) in kiloDaltons (kDa).

Figure 2: Histological identification of Percoll-purified lamellocytes. Confocal microscopy images of hemocytes present in hemolymph and Percoll layer 4 after labeling with anti-Atilla/L1 (1/100; left panels) or myospheroid (Mys) (1/100; right panels) antibody and revealed with the Alexa green fluorescent secondary antibody. In blue, nuclei labeled with DAPI. In the hemolymph (upper panels), many cells are not labeled with the antibodies (as shown by the cell nuclei in blue), whereas in fraction 4 (lower panels) almost all cells are labeled.

Figure 3: Two-dimensional gel of whole cell proteins from lamellocytes. Solubilized whole-cell proteins were first separated according to their isoelectric point (Ac, acidic side, about pH 4; Bas, basic side, about pH 9), and then according to their apparent molecular weight on a 12.5% SDS-PAGE. Among the hundreds of visible spots, the most intense, numbered from 1 to 70, were cut out and analyzed by mass spectrometry. Gel silver-stained. Molecular weights (MW) in kiloDaltons (kDa).

Figure 4: Avidin affinity purification of lamellocyte membrane proteins. Proteins from the various purification and control steps were followed by 12.5% SDS-PAGE. Membrane cell extract without (odd lanes) or with Biotin labeling (even lanes) was incubated with magnetic avidin beads; supernatants containing unretained proteins are shown in lanes 1 and 2, respectively. Lanes 3 and 4, supernatants from the first insect ringer (IR) wash of the corresponding beads; lanes 5 and 6, supernatants from the 10x IR wash; lanes 7 and 8, supernatants from proteins removed from the beads by β-mercaptoethanol treatment. In lane 9, the beads alone were treated with β-mercaptoethanol. The bands from lane 8 were cut out for mass spectrometry analysis (on the right, lane 8 enlarged with the excised bands numbered). Molecular weights (MW) in kiloDaltons (kDa).

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Table I: Major proteins identified in each of the 2D electrophoresis spots.

Features and functions were obtained either from the gene description in flybase (http://flybase.org) or from the cited literature.

Spot N°	Protein name	*Gene symbol*	Highlights on functions	Theoretical MW
1	Glycoprotein 93	*Gp93*	Gp93, a member of HSP90 family, is a resident ER luminal chaperone required for the functional expression of protein domains that display adhesion activity such as integrins.	90182
2	Heat shock protein 83	*Hsp83*	Hsp83 a member of HSP90 family, is the Drosophila homologue of Hsp90.	81814
3	Heat shock 70-kDa protein cognate 3 (GRP78) (BIP)	*Hsc70-3*	Hsc70-3 is one of the six *D. melanogaster* Hsp70. This protein is a key indicator of ER stress.	72190
4 - 5	Phenoloxidase 3; DoxA3; PPO3	*PPO3*	PO catalyzes the oxidation of mono- and di-phenols to ortho-quinones, which subsequently polymerize into melanin.	79264
6	Heat shock protein 70 kDa cognate 4; Hsc70-4; Hsc4	*Hsc70-4*	The 70 kDa heat-shock cognate protein (Hsc70) catalyzes in vitro and in vivo the uncoating of clathrin-coated vesicles, the final step of receptor-mediated endocytosis.	71087
7	V-type proton ATPase catalytic subunit A isoform 2	*Vha68-2*	V-ATPases are proton pumps which transport H+ across eukaryotic membranes, play a role in cotransport processes and have been implicated in functions such as ion transport and fluid secretion across plasma membranes.	68259
8	Flare, isoform A; Actin interacting protein 1 (AIP1)	*flr*	flare (AIP1) is a protein promoting cofilin-mediated F-actin disassembly.	66509
9 - 10	Chaperonin containing TCP1 subunit 6; T-cp1zeta	*CCT6*	CCTs form a chaperonin complex involved in folding of many cytoskeletal components and cell cycle regulators.	58210
11	Chaperonin containing TCP1 subunit 1; T-cp1alpha	*CCT1*		59519
12	Chaperonin containing TCP1 subunit 5; T-cp1epsilon	*CCT5*		59241
13	Chaperonin containing TCP1 subunit 2; T-cp1beta	*CCT2*		58027
14	Stress induced phosphoprotein 1; Hsp70/Hsp90 organizing protein; HOP	*Stip1*	Overexpression of stress-induced phosphoprotein 1 (STIP1) is a co-chaperone of heat shock protein (HSP) 70/HSP90.	55663
15-16	Chaperonin containing TCP1 subunit 7; T-cp1eta	*CCT7*		59349
17	Heat shock protein 60A	*Hsp60A*	Hsp60A is a mitochondrial chaperone.	60758
18	Chaperonin containing TCP1 subunit 8; T-cp1theta	*CCT8*		59396
19	Glutamate dehydrogenase	*Gdh*	Glutamate dehydrogenase (GDH) is the main enzyme for glutamate metabolism that catalyzes the reversible oxidative deamination of l-glutamate to α-keto-glutarate using NAD+ and/or NADP+ as coenzymes.	60941
20	Protein disulfide isomerase	*Pdi*	Protein disulfide isomerase (PDI) is a member of the thioredoxin superfamily of redox proteins with three catalytic activities: thiol-disulfide oxireductase, disulfide isomerase and redox-dependent chaperone. As a chaperone, PDI resides normally in the lumen of the ER but is been also detected at the cell surface, the cytosol and as circulating secreted enzyme.	55746
21	β-Tubulin at 60D; Tubulin-beta-3	*βTub60D*	The drosophila genome possesses 4 *β-tubulin* genes, each encoding a specific isoform.	50745
22-23	α-Tubulin at 85E; Tubulin alpha-2 chain	*αTub85E*	In adult flies the Tubulin alpha-2 chain gene is expressed only in males, where it may be testes-specific.	49935
24	β-Tubulin at 56D, isoform B	*βTub56D*		50115
25	Mitochondrial ATP synthase alpha subunit precursor; bellwether	*blw*	Mitochondrial ATP synthase synthesizes ATP from ADP and inorganic phosphate using the energy provided by the proton electrochemical gradient across the inner mitochondrial membrane.	59384

26	Calreticulin	*Calr*	Calreticulin is a lectin-type calcium-binding chaperone, promoting folding, assembly and quality control in the ER.	46779
27	ATP synthase beta subunit	*ATPsynβ*	A subunit of mitochondrial ATP synthase.	53487
28	GDP dissociation inhibitor, isoform A	*Gdi*	Rab GDP dissociation inhibitor is a down-regulator of Rab-GTPases such as Rab11 involved in the exocytic transport of lipids, receptors and transporters. Gdi binds to GDP-Rabs allowing the transfer of these essential proteins for membrane trafficking from donor to acceptor membranes or to form a cytoplasmic pool.	49871
29	Eukaryotic translation initiation factor 4A	*eIF-4A*	In eukaryotes, translation initiation is facilitated by multiple protein factors collectively called eiFs (for eukaryotic translation initiation factors). The complex consisting of the eiF4 group factors including the mRNA cap-binding eiF4e protein, large scaffolding protein eiF4G and RNA helicase eiF4A is assisted by the eiF4B co-factor to unwind local secondary structures and create a ribosome site on mRNA. eIF4A is required for cell survival during starvation.	45750
30	Prophenoloxidase 3	*PPO3*		79264
31	Enolase	*Eno*	Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during both glycolysis and gluconeogenesis and is an important enzyme in various biological processes.	54276
32	Capulet, isoform A	*capt*	Drosophila homologue of the cyclase-associated proteins (CAPs); may have a conserved role in linking signal transduction to reorganization of the actin cytoskeleton.	45576
33	Eukaryotic translation elongation factor 1 gamma	*eEF1γ*	Eukaryotic Elongation Factor 1 (eEF1) complex is responsible for aminoacyl-tRNA transfer on the ribosome. The primary role attributed to eEF1γ in translation elongation is as a structural scaffold for eEF1β..	48953
34	Actin 5C, isoform A	*Act5C*	The actin cytoskeleton provides structural support for cells and mechanical forces to drive membrane protrusion, cell migration and vesicle trafficking. Many different signaling pathways contribute to the reorganization of specific actin structures. There are six actins genes in Drosophila, given rise to multiple isoforms. Actin 5C is a cytoplasmic actin expressed mainly in undifferentiated cells.	41795
35-37	Actin 42A	*Act42A*	Actin 42A is a cytoplasmic actin expressed mainly in undifferentiated cells.	41797
38-39	Actin 5C, isoform A	*Act5C*		41795
40	Alcohol dehydrogenase	*Adh*	Alcohol dehydrogenase (ADH) is a key enzyme involved in the oxidation of ethanol to acetaldehyde, but seems also to catalyze the conversion of this highly toxic product into acetate.	40364
41	Twinfilin	*twf*	twinfilin (twf) encodes a protein that sequesters actin monomers and promotes actin turnover in multiple cellular processes.	39046
42	Stubarista; P40; laminin receptor	*sta*	Ribosomal protein S2 (RPS2)	30209
43	60S acidic ribosomal protein P0; Ribosomal protein LP0	*RpLP0*	A structural component of ribosome	34181
44	Eb1; Calponin-like	*Eb1*	The +TIP end-binding protein 1 (EB1) accumulates at the growing plus ends of microtubule. EB1 controls microtubule plus-end tracking, dynamics at microtubule plus ends, microtubule and α/β-tubulin binding, and microtubule polymerization.	32555
45-46	Annexin IX; Annexin B9	*AnxB9*	Annexin IX is a drosophila member of a family of soluble, hydrophilic proteins that bind to negatively charged phospholipids in a Ca2-dependent manner. These proteins are implicated in the regulation of phagocytosis, cell signaling, and membrane-associated cytoskeleton.	36017
47	14-3-3epsilon	*14-3-3ε*	The drosophila 14-3-3epsillon protein has a critical role in cellular metabolism and in concert with the 14-3-3zeta isoform is also a regulator of multiple signaling pathways, for example the Ras/Mapk signaling. 14-3-3 proteins also control the Tctp-Rheb GTPase interaction involved in tissue growth via modulation of the Tor signaling pathway.	29780
48	Receptor of activated protein kinase C 1	*Rack1*	RACK1, a member of the WD-repeat family of proteins that shares homology to the β subunit of G-proteins (Gβ), is a key mediator of various pathways and cellular function. It has a role in shuttling proteins around the cell, anchoring proteins at particular locations and in stabilizing protein activity. It interacts with the ribosomal machinery, with several cell surface receptors and with proteins in the nucleus.	35695
49-50	Actin 42A	*Act42A*		41797

51	Lethal(2)37Cc, isoform B; Prohibitin like	*l(2)37Cc*	lethal(2)37Cc is a member of the prohibitin family. Prohibitin is a lipid raft-associated integral membrane protein. Prohibitins seems mainly localized at the mitochondrial membrane and have potential roles such as a tumor suppressor, an anti-proliferative protein, a regulator of cell-cycle progression and in apoptosis. Prohibitins have been also identified as a membrane-associated protein in different mammalian immune cells and play a role in inflammation.	30365
52	Voltage dependent anion-selective channel; porin	*porin*	Voltage-dependent anion channel (VDAC) has been suggested to be a mediator of mitochondrial-dependent cell death induced by Ca2+ overload, oxidative stress and Bax-Bid activation. Porin is a housekeeping gene transcribed in every fly developmental stage, its loss - resulted in locomotive defects and male sterility link to mitochondrial morphological defects.	30531
53	Actin 5C, isoform A	*Act5C*		41795
54	GH22994p; Calcyphosin-like protein	*CG10126*	GH22994p is a protein of unknown function with a EF-hand domain and a Ca++ binding site suggesting function related to calcium signaling response. It is a target of both the cyclic AMP and the Ca+2-phophatidylinositol cascades.	24379
55	Rab11	*rab11*	Rab11 is a small GTPase that, by cycling from an active to an inactive state, controls key events in vesicular transport and the exocytic and recycling pathway. Rab11 regulation and compartmentalization function through its interaction with phosphoinositides.	24230
56	Rho protein GDP-dissociation inhibitor	*RhoGDI*	RhoGDI is a down-regulator of Rho family GTPases including Rhoa, Rhoc, Rac1, Rac2 and Cdc42 that regulate many aspects of intracellular actin dynamics and cell migration. RhoGDI prevents nucleotide exchange and membrane association of Rho-GTPases.	23204
57	Thioredoxin peroxidase 1; Peroxiredoxin	*Jafrac1*	Thioredoxin peroxidase 1 is a thiol-specific cytoplasmic peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides and participates also in cadherin-mediated cell adhesion.	21724
58-60	Glutathione S transferase D1	*GstD1*	Glutathione S transferase D1 (GST) is a cytosolic enzyme involved in the conjugation of glutathione with a wide range of endogenous compounds and xenobiotic alkylating agents. The S-Glutathionylation is an important cell mechanism to maintain the reduced/oxidized glutathione ratio at the optimal level but also for oxidation of proteins cysteine residues for their normal functioning and their ability to participate in signal transduction cascades.	23851
61	Translationally controlled tumor protein	*Tctp*	Tctp is a family of evolutionarily conserved proteins involved in a number of fundamental processes, including cell proliferation, apoptosis and DNA damage control. In Drosophila, Tctp is required for organ growth by promoting Rheb function for Tor signaling as a guanine nucleotide exchange factor.	19625
62	Eukaryotic translation elongation factor 5	*eIF-5A*	eIF-5A is a mRNA-binding protein involved in translation elongation of proteins particularly with consecutive prolines.	17580
63	Cofilin/actin depolymerizing factor homolog; twinstar	*tsr*	Cofilin is a ubiquitous actin-binding factor required for the reorganization of actin filament. Its dephosphorylation enables its actin severing and depolymerizing activity	17142
64	Calmodulin, isoform A	*cam*	Calmodulin (CAM) participates in a variety of intracellular transduction processes by modulating signaling molecules in response to calcium changes.	16800
65	Ribosomal protein S12	*RpS12*	A structural component of ribosome.	16571
66	Unknown (product of CG14610)	*CG14610*	*CG14610* expression increases in *D. melanogaster* after parasitization by the *Asobara tabida* wasp.	17084
67	Abnormal wing discs, isoform C	*awd*	Abnormal wing discs (awd) belongs to a family of genes implicated in metastasis suppression, metabolic homeostasis and epithelial morphogenesis. awd is necessary for Rab5 function and is essential for Notch signaling via its endocytic role.	17159
68	Actin 42A	*Act42A*		41797
69-70	Profilin; Protein chickadee	*chic*	Profilin is a small actin binding protein that at high concentrations prevents the polymerization of actin, whereas it enhances it at low concentrations. It is involved in endocytic uptake, actin filament recycling and cell migration during development.	13715

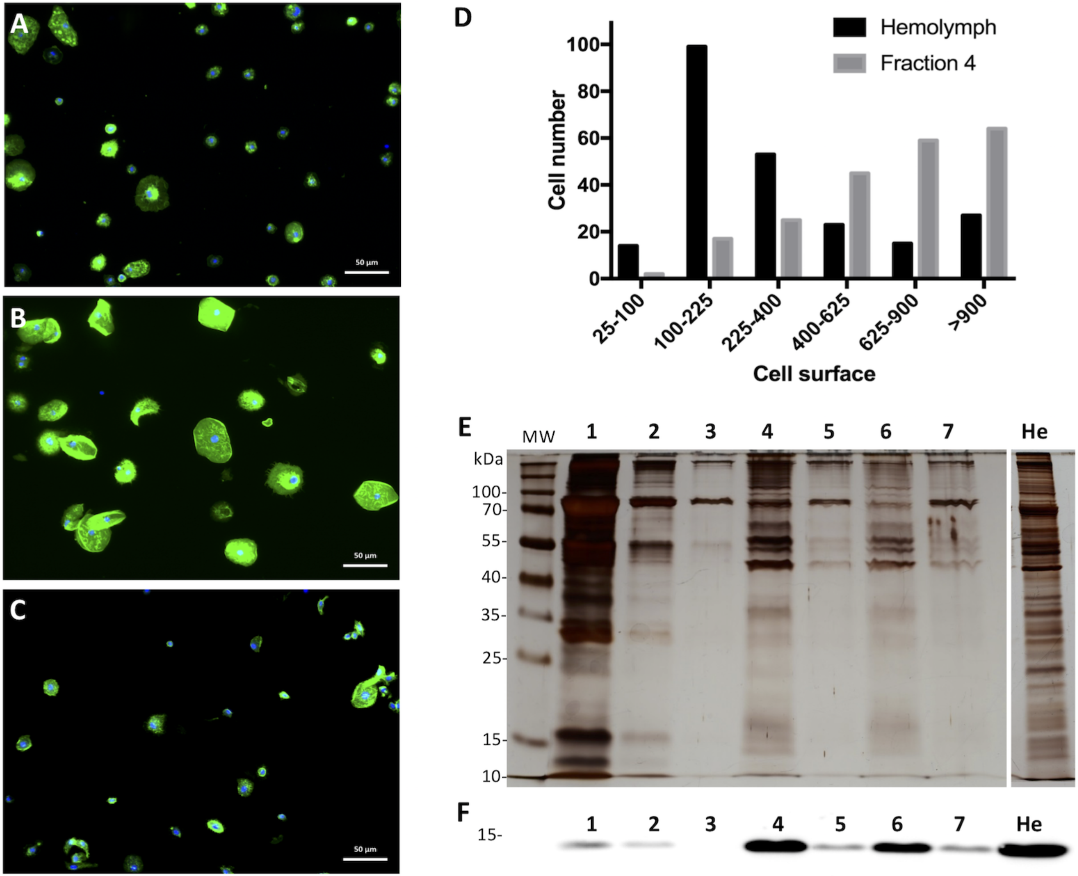
Table II: The major protein in each of the 1D SDS-PAGE Band

Highlighted in grey, plasma membrane proteins or plasma membrane associated proteins; In bold, mitochondrial proteins.

Bande N°	Protein	Protein name	*Gene*	Roles/Cellular localization
0	gi|440217525	Filamin-A (cheerio, isoform N)	*cher (sko)*	Actin binding / Plasma membrane, cytoskeleton
1	gi|157400337	Integrin alphaPS4 subunit	*ItgaPS4*	Cell-Cell Adhesion/ Plasma Membrane
2	gi|7291606	Integrin alphaPS5 subunit	*ItgaPS5*	Cell-Cell Adhesion / Plasma Membrane
3	gi|22945621	Reticulon-like1, isoform F	*Rtnl1*	Endoplasmic Reticulum organization / ER Membrane
4	gi|7291521	Prophenoloxidase 3	*PPO3*	Immunity / Cytoplasm, Secreted
5	gi|157661	Heat shock protein cognate 4	*Hsc70-4*	Chaperone binding / cytoplasm, nucleus, mitochondria
6	gi|2981227	Fimbrin	*Fim*	Actin binding / Apical cortex, cytoplasm
7	**gi|33636453**	**Heat shock protein 60A, isoform B**	***Hsp60A***	**Chaperone / Mitochondrion matrix**
8	gi|622993	Protein disulfide isomerase	*Pdi*	Protein Folding / Endoplasmic Reticulum
9	gi|135396	Tubulin alpha-1 chain	*αTub84B*	Structural constituent of microtubules / Cytoskeleton
10	**gi|287945**	**ATP synthase beta subunit, partial**	***ATPsynβ***	**ATP Production / Mitochondrial Membrane**
11	gi|7946	Enolase, isoform A	*Eno*	Glycolysis / cytoplasm
12	gi|156750	Actin	*Act42A*	Structural constituent of cytoskeleton / Cytoskeleton
13	gi|114794360	Actin	*Act87E*	Endocytosis / Cytoskeleton
13-1	gi|7301073	RH40150p (CG5854)	*CG5854*	Epimerase dehydratase, NAD(P) binding protein / ?
14	gi|156750	Actin	*Act5C*	Structural constituent of microtubules / Cytoskeleton
15	gi|157478	Glyceraldehyde-3-phosphate dehydrogenase (Gadph-2)	*Gadph-2*	Pyruvate Metabolism / Cytoplasm
16	gi|25012828	RH01338p (Annexin B9)	*AnxB9*	Actin binding / Cell cortex and endomembrane system
17	gi|1814377	14-3-3 epsilon	*14-3-3ε*	Ras-mediated pathways / Cytoplasm, nucleus, plasma membrane
18	**gi|1568662**	**Voltage dependent anion-selective channel**	***porin***	**Anion Channel / Mitochondrial Membrane**
19	gi|2313033	rab1	*rab1*	Vesicle transport and secretion / Golgi, vesicles and plasma membranes
20	gi|7263022	Transgelin (calponin-like protein Chd64)	*Chd64*	Actin binding / Cytoskeleton-exosomes secreted
21	gi|473593	Cofilin/actin depolymerizing factor homolog (Twinstar)	*tsr*	Actin cytoskeleton dynamics / Cytoskeleton, nucleus, cell junction
22	gi|473593	Cofilin/actin depolymerizing factor homolog (Twinstar)	*tsr*	Actin cytoskeleton dynamics / Cytoskeleton, nucleus, cell junction
23	gi|8482	Ribosomal protein	*RpLP2*	Protein synthesis / Cytoplasm

Table III: Plasma membrane proteins or associated with, identified after affinity purification.
Mascot score, protein name, gene ID and putative or demonstrated multi-localizations are indicated. PM: plasma membrane/ Csk: cytoskeleton/ Cy: cytoplasm/ ER: endoplasmic reticulum/ mi: mitochondria/ Nu: nucleus/ Go: Golgi/Mb: membranes/ Es: endomembrane system/mi: mitochondria.

Score	Protein name	Gene id	Localization
1202	integrin alphaPS4	*ItgaPS4*	PM
742	Integrin beta-PS; myospheroid protein	*mys*	PM
693	integrin alphaPS5	*ItgaPS5*	PM
636	Filamin-A; cheerio, isoform N	*cher*	Csk;PM
467	fimbrin	*Fim*	Csk;Cy;PM
390	GH09052p (glucose transmembrane transporter activity)	*CG1208*	PM
366	capulet, isoform B	*capt*	Csk;PM;Cy
361	Sodium/potassium-transporting ATPase subunit alpha	*Atpalpha*	PM
320	rab1	*rab1*	PM;ER;Go
287	annexin B9a	*AnxB9*	PM;Es
279	Drab5	*Rab5*	PM;Es
270	14-3-3 protein zeta; Protein Leonardo	*14-3-3zeta*	ER;Cy;PM
262	Drab11	*rab11*	PM;Es;Nu;Go
259	V-type proton ATPase catalytic subunit A isoform 2	*Vha68-2*	PM
230	GH01619p	*rab2*	PM;Go
208	RH03540p; Ly-6 family	*CG15347*	PM
166	GH24511p; Ubiquitin activating enzyme 1, isoform A	*Uba1*	PM;Cy
159	neuroglian	*Nrg*	PM
155	CD98 heavy chain, isoform A	*CD98hc*	PM
154	Vacuolar H+-ATPase 55kD subunit	*Vha55*	PM;Es;Cy
145	Annexin B10	*AnxB10*	Mb
136	Protein rush hour	*rush*	PM
131	Integrin alpha-PS1	*mew*	PM
121	Dipeptidyl peptidase 3	*DppIII*	PM;Cy;Mb
119	Rab7, isoform A	*Rab7*	PM;Es;Mb
116	Vacuolar H+ ATPase 44kD subunit, isoform C	*Vha44*	PM
112	gliotactin, isoform A	*Gli*	PM
87	Scavenger receptor class C, type I; SR-CI	*Sr-CI*	PM
85	LD04844p (MARVEL protein)	*CG1572*	PM
78	V-type proton ATPase subunit E	*Vha26*	PM
77	AT18611p (beta-1,3-glucan recognition protein)	*CG30148*	S;PM
77	AP-2 complex subunit alpha	*AP-2alpha*	PM
75	V-type proton ATPase subunit a	*Vha100-2*	PM
74	Vinculin	*Vinc*	Csk;PM
74	synaptobrevin, isoform A	*Syb*	PM
74	CG4829, isoform A (Gamma-glutamyltranspeptidase)	*CG4829*	PM
70	FI19011p1 (Angiotensin II, type I receptor-associated protein)	*CG32638*	PM
69	NTPase, isoform E	*NTPase*	PM;ER;Go;Mb
69	LP10861p (B-cell receptor-associated protein 29/31)	*CG13887*	ER;PM;Es
63	Des-1 protein	*ifc*	PM;mi
62	matrix metalloproteinase 1	*Mmp1*	PM;S
60	rhea	*rhea*	Csk;PM
60	contactin	*Cont*	PM
59	Basigin	*Bsg*	PM;Csk
59	ADAM metalloprotease	*Meltrin*	PM;S
59	RE67340p; Ly-6 family	*CG9336*	PM ;Mb
58	atilla; Ly-6 family	*atilla*	PM
55	like-AP180, isoform C	*lap*	Go;PM
54	CG1532, isoform A	*CG1532*	Nu;PM;Cy
54	Swiprosin-1	*Swip-1*	PM
52	Ras-related protein Rap1	*Rap1*	PM
50	G protein α q subunit	*galphaq*	PM
49	secretory carrier membrane protein	*Scamp*	PM;Mb
47	extended synaptotagmin-like protein 2, isoform A	*Esyt2*	ER;PM;Mb
48	Ras-like protein 2	*Ras64B*	PM;Mb
46	Coatomer subunit delta	*deltaCOP*	PM;ER;Cy
44	transferrin 2	*Tsf2*	Cy;PM;Mb;S
42	CG10217, isoform A; undicht	*udt*	PM
42	Ras-related protein Ral-a	*Rala*	PM;Mb
42	calcium-binding protein 1, isoform A	*CaBP1*	ER;PM
41	Vacuolar H+ ATPase 36kD subunit 1	*Vha36-1*	PM
36	G protein alpha o subunit	*Galphao*	PM
36	parvin, isoform A	*parvin*	Csk;Cy;PM
36	prip, isoform A	*prip*	PM
36	Dras1	*Ras85D*	PM;Cy
35	nervana 1	*nrv1*	PM
34	ALG-2 interacting protein X	*ALiX*	Csk;Es;S;PM
34	nicastrin	*Nct*	Mb;PM
33	fasciclin 3, isoform D	*Fas3*	Mb;PM
32	fermitin 1, isoform A	*Fit1*	Cy;PM
32	Grasp65	*Grasp65*	PM;Er;Go;Es
31	Vacuolar H+ ATPase 100kD subunit 1	*Vha100-1*	PM;Es
31	Protein odr-4 homolog	*CG10616*	Es;PM
31	Integrin beta-nu	*Itgbn*	PM
30	Lipophorin receptor 2; putative alpha2M-receptor-like	*LpR2*	PM
29	LDL receptor protein 1, isoform G	*LRP1*	PM;Cy
29	Protein aveugle	*ave*	PM;Cy
26	major facilitator superfamily transporter 3	*MFS3*	PM;Cy
26	Protein ROP	*ROP*	PM;Cy
26	MIP05539p	*CG31195*	PM
24	ArfGAP with SH3 domain, ankyrin repeat and PH domain	*Asap*	PM;Nu;Cy
24	syntaxin 13, isoform A	*Syx13*	PM;Es
24	similar to semaphorin-I	*Sema1b*	S;Cy;Mb
22	CG9917 (27 kDa hemolymph protein?)	*CG9917*	S;Mb
21	LD44267p (Arrestin-like)	*CG1105*	PM
21	ADP-ribosylation factor 1	*Arf79F*	PM;Go;Mb
21	synaptotagmin 1, isoform G	*Syt1*	PM;Mb;Es
20	Presenilin homolog	*Psn*	Es;Go;PM


A
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B
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C
50 µm
D
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80
100
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100-225
225-400
400-625
625-900
>900
Cell surface
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3
4
5
6
7
He
kDa
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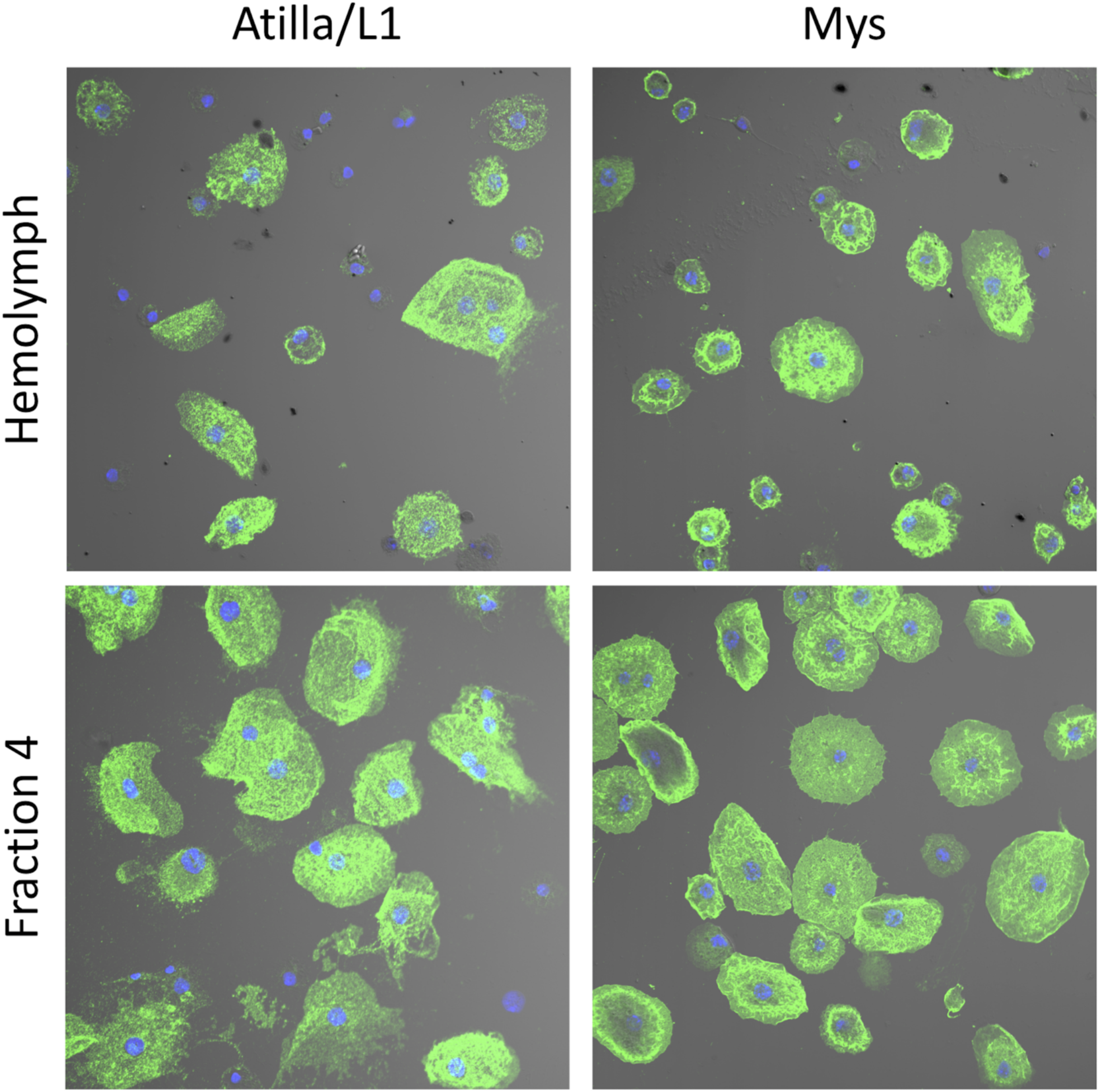
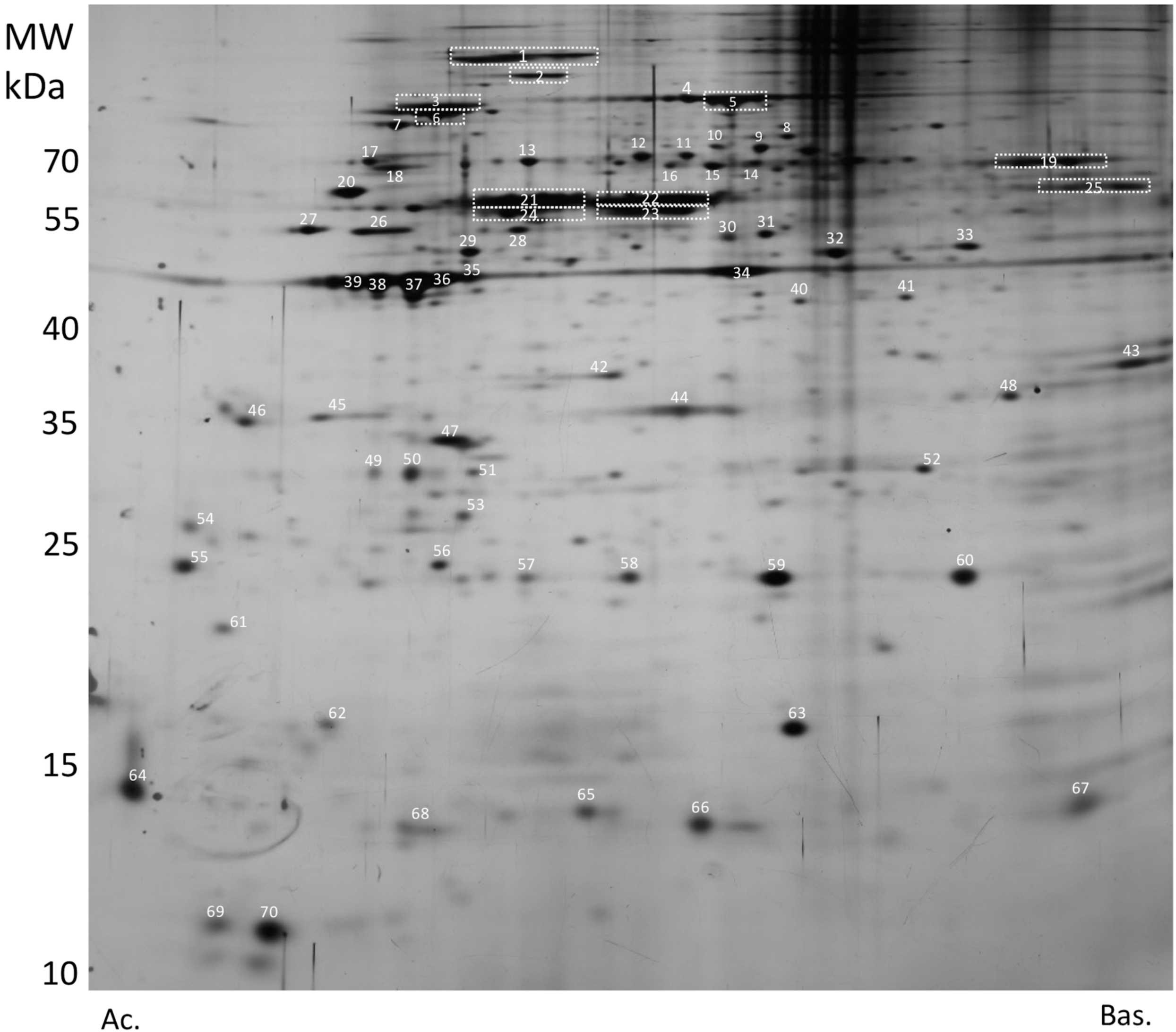
He


Atilla/L1
Mys
Hemolymph
Fraction 4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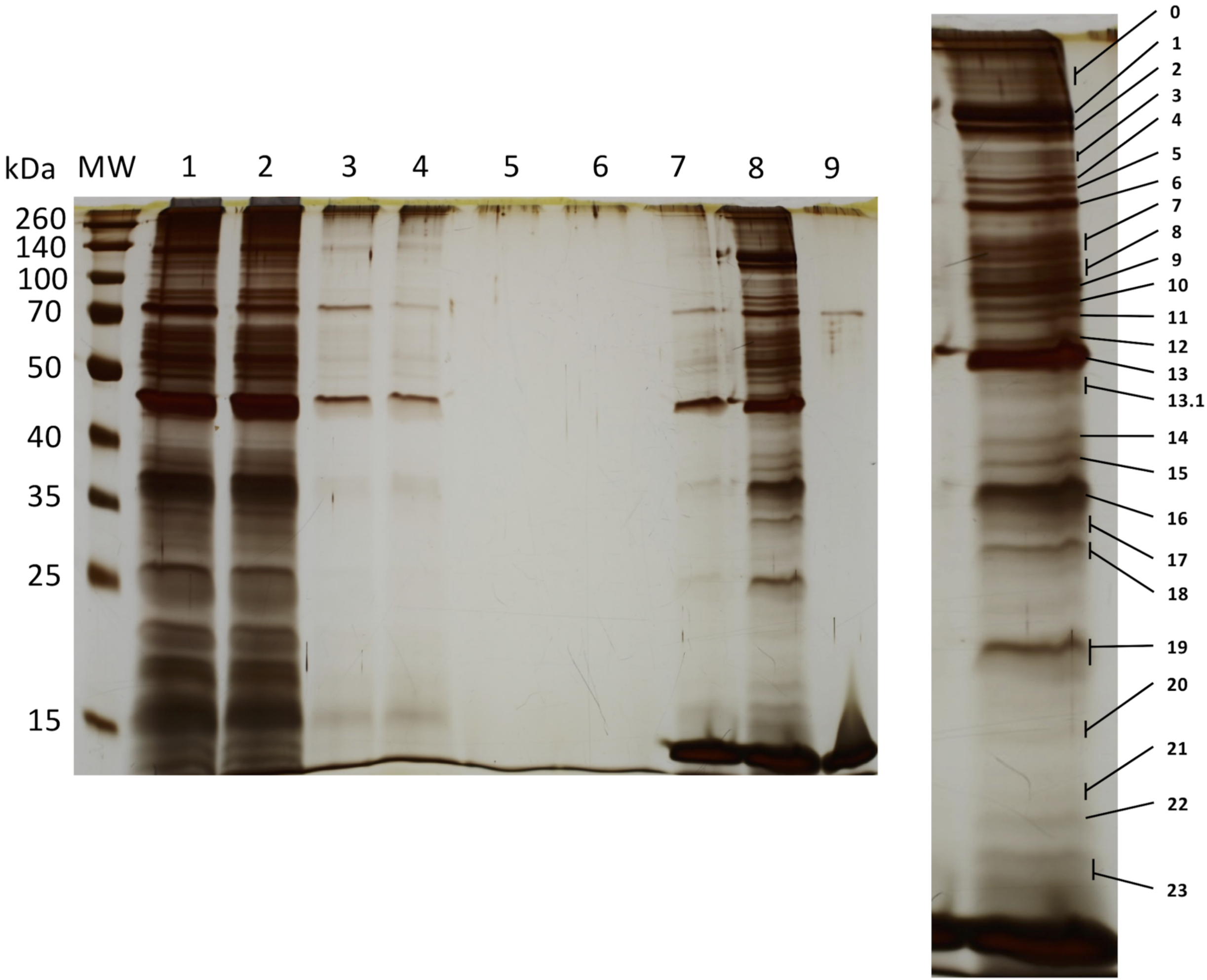
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140
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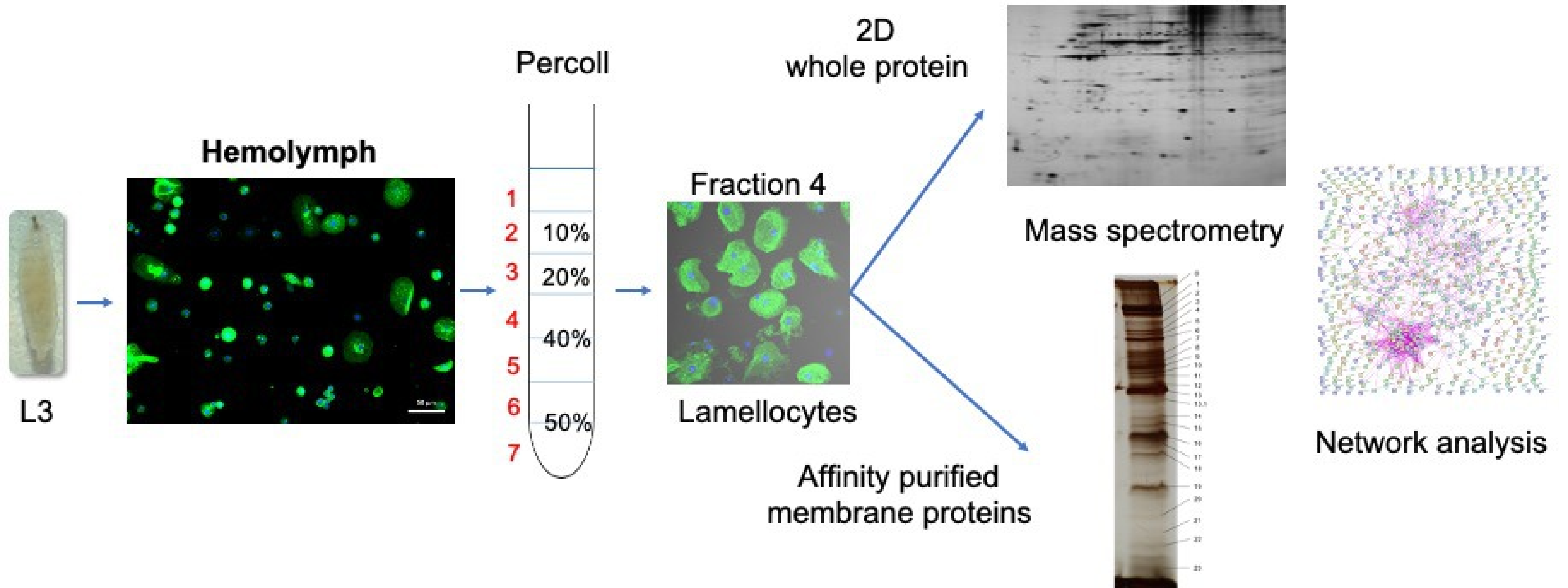
L3
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Percoll
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Fraction 4
Lamellocytes
2D
whole protein
Mass spectrometry
Affinity purified
membrane proteins
Network analysis