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## DATASET BRIEFS

**Towards the molecular deciphering of *Pomacea canaliculata* immunity: first proteomic analysis of circulating hemocytes**

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**Abbreviations:** GH, granular hemocytes; AH, agranular hemocytes; *Pc*, *Pomacea canaliculata*

**Keywords:** apple snail; hemocytes; immunity; mollusks; proteomics

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Abstract

*Pomacea canaliculata* is a freshwater snail of interest to the scientific community with biological features, that include invasiveness, human parasite hosting and adult regeneration. Knowledge about the immune system of *P. canaliculata* is of particular interest as it may represent the target for strategies aimed at controlling the spread of the snail population and its hosting of the human parasite *Angiostrongylus cantonensis*. Moreover, the immune functions likely have a role in the snail's ability to regenerate as an adult. Despite its importance in multiple processes, very little is known about the molecular basis of *P. canaliculata* immunity. Aiming to contribute to filling this gap, we have studied the ultrastructure and performed the first proteomic analysis of circulating hemocytes in control snails, evidencing 83 unique proteins, 96% of which have identifiable homologs in other species. Fifteen proteins were retrieved as potentially involved in immune-related signaling pathways, including hemocyanin, a C1q-like protein and the chaperonin, HSP90 together with cytoskeleton and cytoskeleton-related proteins involved in cell motility and membrane dynamism. This first proteome study on non-stimulated hemocytes, provides a valid reference for future investigations on the molecular changes in these cells under stressful circumstances, like pathogen exposure, wounding or environmental changes.

*Pomacea canaliculata* (*Pc*) is a freshwater snail of increasing interest to the scientific community, due to its versatility as a research model. Its biology is under study from many different point of view and, among others, an outstanding regeneration capability has been demonstrated in adults.<sup>[1], [2]</sup> This snail fecundity and adaptability allow it to reproduce in wide ranging environments and lead *Pc* to be indexed among the 100 most invasive species of the world.<sup>[3]</sup> Unfortunately, golden apple snails are also the intermediate host of the human parasitic worm *Angiostrongylus cantonensis*. This has created considerable concerns among sanitation authorities, especially in Asia, where attempts to develop efficacious strategies to eradicate this pest are being studied.<sup>[4]</sup> Although permissive towards *A. cantonensis*, the immune system of *Pc* is highly efficient and based on cellular, *i.e.*, hemocytes, and soluble components.<sup>[5]</sup> Past studies on the immune system of *Pc* have provided information about its hemocyte morphology<sup>[6]</sup> and replication<sup>[7]</sup> via flow cytometry, optical microscopy<sup>[6], [8]</sup> and ultrastructural studies<sup>[6], [8], [9]</sup> but no molecular information is currently available. Here we present, beside information on hemocyte ultrastructure, the first proteomic data set on *Pc* hemocytes.

Previous optical microscopy observations indicated that circulating hemocytes of *Pc* can be divided into the small blast-like and the larger hemocytes.<sup>[6]</sup> The latter display a low nucleus to cytoplasm ratio and can present either agranular or granular cytoplasm.<sup>[6]</sup> Consistently, present electron microscopy results evidenced two different morphotypes of circulating hemocytes: granular (GH) and agranular (AH) hemocytes. More than 80% of GH were characterized by an electron-dense cytoplasm containing different types of granules. Changes in granule electron-density could be due to differences in their enzyme content (**Figure 1A-C**). In line with previous phagocytic tests and TEM observations<sup>[6]</sup>, the presence of numerous pseudopodia suggests that GH may have phagocytic capabilities (**Figure 1A**). The intracellular digestion of phagocytosed material can determine the isolation of carbohydrate constituents

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3 and their conversion in glycogen deposits<sup>[10]</sup> that were clearly visible in most GH (**Figure 1A,**  
4 **B**). Cueto and colleagues observed glycogen deposits also in hemocytes with agranular  
5 cytoplasm in the kidney of adult *Pc* and this has been related with a potential role of hemocytes  
6  
7 in the mobilization of these deposits.<sup>[8]</sup> In our experiments, AH showed no pseudopods and  
8 cytoplasm without granules (**Figure 1D-E**). Some of these AH are likely related with Group I  
9 hemocytes, *i.e.*, hemocytes with a blast-like morphology.<sup>[6]</sup>  
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11 SEM observations were also performed to integrate the information collected by TEM, and  
12 evidenced round or ovoid hemocytes (Figure 1F-H). Rare biconcave cells, whose shape could  
13 resemble that of mammalian erythrocytes, were also observed (Fig. 1H). To our knowledge, this  
14 is the first time these infrequent elements have been observed in *Pc*. Scant information is  
15 available in other mollusks, but in the gastropods *Lymnaea stagnalis* and *Biomphalaria*  
16 *glabrata*, cells with analogous morphology have been reported and listed as hemocytes,  
17 although their role in immunity has not yet been clarified.<sup>[11]</sup>  
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19 To obtain the first proteomic database of the *Pc* hemocytes, proteins were extracted from all  
20 the circulating hemocytes described above and analysed by LC-MS/MS. For each replicate, 100  
21 µg of proteins were incorporated into a polyacrylamide gel matrix and without performing an  
22 electrophoresis<sup>[12]</sup> the tube-gel was cut in small pieces. Subsequently, proteins underwent a  
23 standard in-gel digestion protocol<sup>[13]</sup> and were analysed by LC-MS/MS (experimental details  
24 are provided in Supporting Information).  
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26 MS raw data were batch using X!Tandem search engine integrated in Trans-Proteomic Pipeline  
27 version 5.1.0<sup>[14]</sup> and identified using a mRNA-seq reference database (21,373 sequences)  
28 created by Sánchez Laboratory at the Stowers Institute for Medical Research (Kansas City, MO,  
29 USA. Searching parameters included: i) static modification of carbamidomethyl on cystein  
30 (+57.021 Da) and dynamic modification of methionin oxidation (+15.995 Da) and  
31 propionamide cysteine (+71.037 Da); ii) precursor ions and fragment ion tolerances were set  
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to 40 ppm and 0.5 Da, respectively; iii) enzyme specificity was set to trypsin with maximum one missed cleavage; iv) no restriction was set on protein mass; v) peptide charge was set to 2+, 3+ and 4+. PeptideProphet and ProteinProphet algorithms were used to estimate the significance of peptides and proteins matched.<sup>[15]</sup> Peptide identifications with a PeptideProphet probability of a minimum of 95% were submitted to ProteinProphet and the derived proteins were filtered at a maximum 0,9% error rate. The search was also performed against a decoy database allowing a false discovery rate (FDR) <1% at both peptide and protein levels. Peptides, containing fewer than 7 amino acids, were not considered since short peptides could too easily match decoy sequences. Proteins identified having at least one unique peptide were kept.

A total number of 7806 MS/MS spectra were acquired and, from these, 1519 spectra were used to assign unique peptides (254). These 254 unique peptides were assigned to 83 unique proteins (**Table 1**) and 96% proteins presented a homologue sequence in the NCBI nr-protein sequences database (**Table 1**; **Table S1**).

Computational analysis of identified proteins was conducted using BLASTp version 2.4.0 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>), the search homology strategy was obtained setting the *Protostomia* taxid (33317) at an expect threshold 1e-05 and filtering low complexity regions.

Identified proteins were processed by AgBase version 2.00 (<http://agbase.msstate.edu>) and the Gene Ontology annotations (GO) were generated by sequence similarity using GOanna tool.<sup>[16]</sup> Searching parameters were: i) UniProt database; ii) expect threshold 1e-05; iii) low complexity filtering. Afterwards, proteins were classified with PANTHER tool version 13.1 (<http://www.pantherdb.org/>).

Among the identified proteins, 76 had a matched GO annotation (**Figure S1**; **Table S1**). GO-enrichment analysis revealed that most proteins appeared to be involved in cellular process (40 proteins), metabolic process (27 proteins) within the biological process (**Figure S1**). As far

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3 as the cellular component, 36 and 27 proteins were in cell part and in organelle, respectively  
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5 **(Figure S1)**. Finally, on the basis of molecular function, 27 and 23 proteins presented catalytic  
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7 and binding activity, respectively **(Figure S1)**.  
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9 Moreover, 86% of proteins were annotated in the KEGG database  
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11 (<http://www.genome.jp/kegg/>)<sup>[17]</sup> used to classify pathways in circulating hemocytes from *Pc*.  
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13 Proteins were grouped into 29 levels **(Figure 2)**. The Cellular process set was the most  
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15 abundant, followed by the Organismal systems set. In particular, we identified several  
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17 cytoskeleton proteins (*i.e.*, different actin forms, actin-related proteins, myosin heavy chain,  
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19 tubulin beta, clathrin heavy chain) that are crucial for cell division and migration, cell shape,  
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21 aggregation and dispersion of granules within the cytoplasm as well as for phagocytosis, a  
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23 process essential for eliminating pathogen infection and tissue remodelling.<sup>[18]-[20]</sup>  
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25 Fifteen different proteins were grouped in the Organismal systems category, immune-system  
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27 sub-category. These proteins were retrieved as potentially involved in immune-related  
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29 signaling pathways **(Figure 2)**. These annotations are largely derived from vertebrate  
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31 homology and this require further analyses before we can definitely state the function of each  
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33 protein we have identified. This notwithstanding, we have identified two different types of  
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35 hemocyanins involved in oxygen and carbon dioxide transport: hemocyanin alpha D-subunit  
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37 and hemocyanin G-type units Oda to Odg-like **(Table 1)**. *In vitro* and *in vivo* studies in  
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39 arthropods highlighted that hemocyanin may present phenoloxidase activity.<sup>[21]</sup> Interestingly,  
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41 hemocyanins themselves could represent the precursor for antimicrobial peptides in shrimps  
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43 and antiviral factors in arthropods and mollusks, as well.<sup>[21]</sup> Another interesting hit is a  
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45 Complement C1q-like protein 4 **(Table 1)**. C1q-like protein have already been observed by  
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47 proteomic analysis of the fluid component of the hemolymph of the bivalve *Crassostrea gigas*  
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49 and it has been related to anti-viral response.<sup>[22]</sup> In the perivitellin fluid of *Pc*, proteomic  
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51 analysis also evidenced immune defense proteins, including a C1q domain-containing  
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protein.<sup>[23]</sup> A gene ontology search after proteomic analysis has been recently performed also on the hemolymph (cells and plasma) of the freshwater snail *Bithynia siamensis goniomphalos* infected with the flatworm *Opisthorchis viverrini*. Several proteins were observed to undergo significant changes after the infection, including three different HSPs (HSP-70, HSP-83 and HSP-90).<sup>[24]</sup> This could support a potential involvement in immunity also for the HSP90 we have observed in our control snails (**Table 1**). In these respects, HSP90, a molecular chaperon, has been proposed as a possible tool for biomonitoring, being highly sensitive towards different stress conditions (thermal shock, starvation, hypoxia, heavy metals).<sup>[25]</sup>

In summary, we present for the first time a proteomic profile of circulating hemocytes of *Pc*. Being one of the few cell-specific proteomic study performed in mollusks, this study represents a reference for detecting and analysing the molecular changes intervening in *Pc* hemocytes under stressful circumstances, like pathogen exposure, wounding or sudden environmental changes. With the aims of controlling the spread of apple snails using environmentally friendly products, and to understand the cellular and molecular basis of its regenerative ability, it is important we gain as much information as possible about its immune system. As of today very little is known about the processes regulating hemocyte maturation in mollusks, and the effect of environmental conditions on this maturation. Gaining information on the proteome of the mature cells is an important step helping to set the basis for further and more detailed studies on the maturation of a very resistant and extremely reactive innate immune system.<sup>[26]</sup>



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**Conflict of interest statement**

The authors have declared no conflict of interest

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## Legends

**Figure 1.** Circulating granular (GH) and agranular (AH) hemocytes observed by TEM (A-E) and SEM (F-H). (A) GH with electron-dense cytoplasm, pseudopodia (arrows) and glycogen deposits (\*). All GH showed abundant cytoplasm and round or ovoid nuclei (N) in eccentric positions (A) with large chromatin clumps, smooth (SER) and rough (RER) endoplasmic reticulum, mitochondria (M), lysosomes, and vacuoles (V). The minority of GH presented electron-lucent cytoplasm either with electron-lucent (B) or electron-dense (C) granules (Gr). GH with electron-lucent granules may also exhibit very short pseudopodia (B) while no pseudopodia have been observed in GH with electron-dense granules (C). AH are characterized by absence of granules and by a different nucleus/cytoplasm ratio (D, E). Rarely, AH were characterized by large nuclei and scant and poorly organized cytoplasm only containing few rough endoplasmic cisternae and mitochondria (E). SEM observations evidenced either ovoid or round hemocytes (F-H). The ovoid cells generally exhibited rough surface projections and marginal spherule bulges (F), whereas the round cells showed either a spongy (G) or a smooth surface (H). In (H) a small and biconcave cell is seen in contact with the round and smooth hemocyte. Bar= 2  $\mu$ m.

**Figure 2.** Pathway assignment based on KEGG PATHWAY Database. The histogram details the categories (Metabolism, Genetic information processing, Environmental information processing, Cellular process and Organismal systems) presented in the pie chart, and the table lists the proteins assigned to the Cell motility and Immune system sub-categories.

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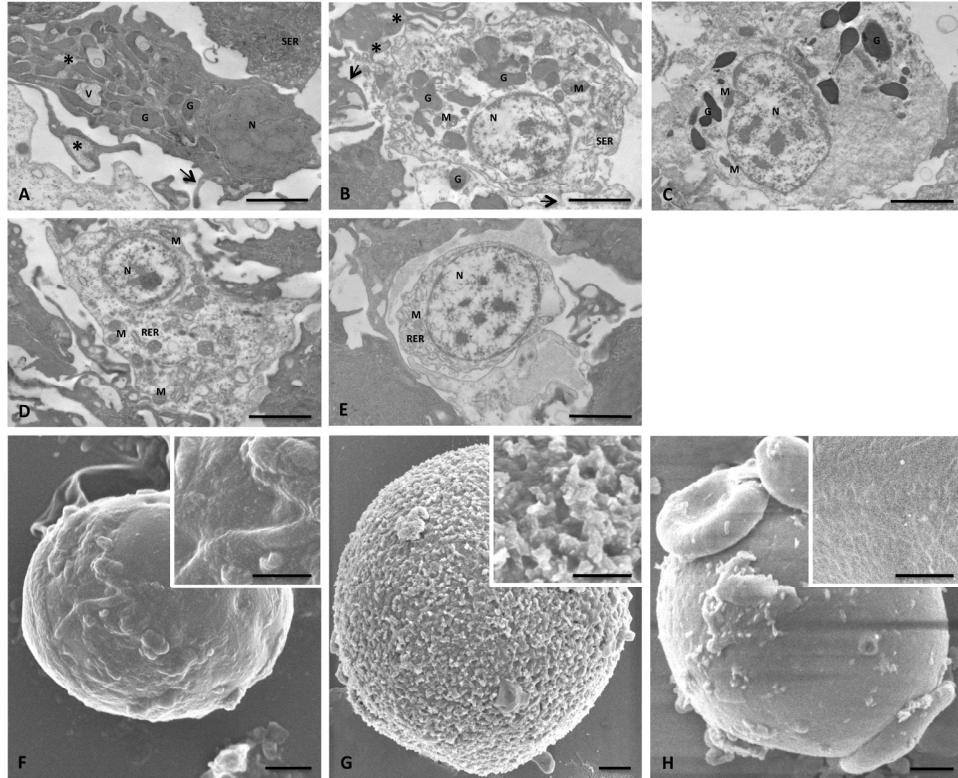


Figure 1. Circulating granular (GH) and agranular (AH) hemocytes observed by TEM (A-E) and SEM (F-H). (A) GH with electron-dense cytoplasm, pseudopodia (arrows) and glycogen deposits (\*). All GH showed abundant cytoplasm and round or ovoid nuclei (N) in eccentric positions (A) with large chromatin clumps, smooth (SER) and rough (RER) endoplasmic reticulum, mitochondria (M), lysosomes, and vacuoles (V). The minority of GH presented electron-lucent cytoplasm either with electron-lucent (B) or electron-dense (C) granules (Gr). GH with electron-lucent granules may also exhibit very short pseudopodia (B) while no pseudopodia have been observed in GH with electron-dense granules (C). AH are characterized by absence of granules and by a different nucleus/cytoplasm ratio (D, E). Rarely, AH were characterized by large nuclei and scant and poorly organized cytoplasm only containing few rough endoplasmic cisternae and mitochondria (E). SEM observations evidenced either ovoid or round hemocytes (F-H). The ovoid cells generally exhibited rough surface projections and marginal spherule bulges (F), whereas the round cells showed either a spongy (G) or a smooth surface (H). In (H) a small and biconcave cell is seen in contact with the round and smooth hemocyte. Bar= 2  $\mu$ m.

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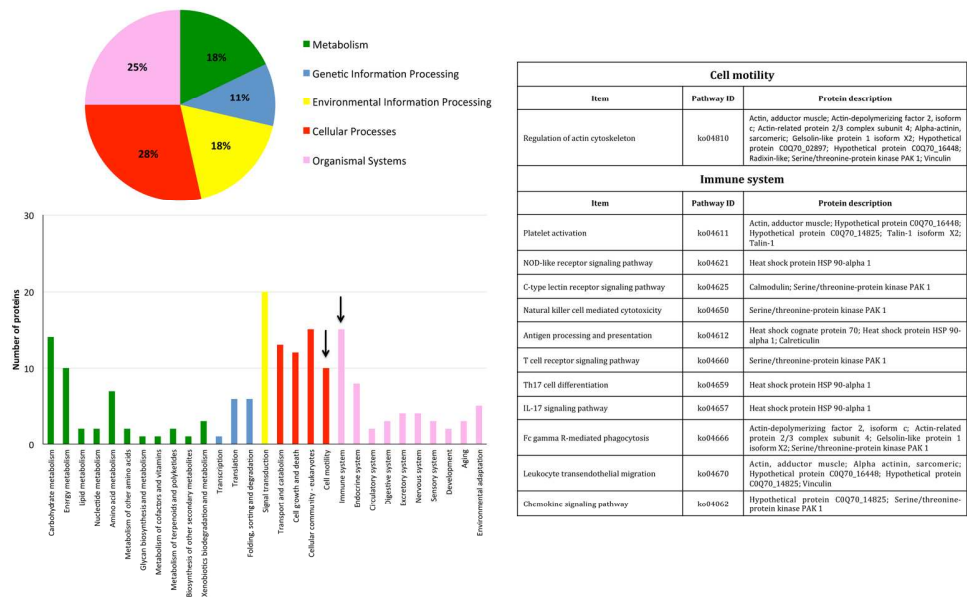


Figure 2. Pathway assignment based on KEGG PATHWAY Database. The histogram details the categories (Metabolism, Genetic information processing, Environmental information processing, Cellular process and Organismal systems) presented in the pie chart, and the table lists the proteins assigned to the Cell motility and Immune system sub-categories.

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**Table 1.** Proteins identified from circulating hemocytes of *Pc* by LC-MS/MS. Proteins were annotated by BLASTp using *Protostomia* taxid (33317). Proteins with no homologue in database are reported as N/D.

	Protein ID	Protein description
1	Pcan_011630.p1	14-3-3 like protein
2	Pcan_001946.p1	14-3-3 protein epsilon
3	Pcan_032071.p1	14-3-3 protein zeta-like
4	Pcan_016727.p1	40S ribosomal protein S4
5	Pcan_036700.p1	60S ribosomal protein L4-like
6	Pcan_025816.p1	60S ribosomal protein L7
7	Pcan_015580.p1	78 kDa glucose-regulated protein
8	Pcan_015636.p1	Actin, adductor muscle
9	Pcan_044355.p1	Actin-depolymerizing factor 2, isoform c
10	Pcan_048742.p1	Actin-related protein 2/3 complex subunit 4
11	Pcan_005018.p1	Actin-related protein 3
12	Pcan_003007.p1	Adenylyl cyclase-associated protein 1
13	Pcan_013762.p1	ADP, ATP carrier protein
14	Pcan_008108.p1	Aldehyde dehydrogenase, mitochondrial-like
15	Pcan_001462.p1	Aldo-keto reductase family 1 member B10
16	Pcan_006174.p1	Alpha-actinin, sarcomeric
17	Pcan_013012.p1	Alpha-L-fucosidase-like
18	Pcan_008078.p1	Annexin A7
19	Pcan_012990.p1	ATP synthase subunit alpha, mitochondrial
20	Pcan_010701.p1	ATP synthase subunit beta, mitochondrial
21	Pcan_026515.p1	Beta-tubulin
22	Pcan_036674.p1	Beta-tubulin
23	Pcan_001273.p2	Calmodulin
24	Pcan_013039.p1	Calreticulin
25	Pcan_000560.p1	Clathrin heavy chain 1 isoform X3
26	Pcan_006103.p1	Complement C1q-like protein 4
27	Pcan_022360.p1	Coronin-1C
28	Pcan_023019.p1	Cytosolic malate dehydrogenase
29	Pcan_010300.p1	Elongation factor 1 alpha



30	Pcan_011330.p1	Enolase
31	Pcan_026361.p1	Enolase-phosphatase E1
32	Pcan_005748.p1	F-actin-capping protein subunit beta-like
33	Pcan_009413.p1	Protein singed
34	Pcan_030747.p1	Fructose-bisphosphate aldolase-like
35	Pcan_014279.p1	Gelsolin-like protein 1 isoform X2
36	Pcan_008329.p1	Glyceraldehyde 3-phosphate dehydrogenase
37	Pcan_000340.p2	Glyoxylate reductase/hydroxypyruvate reductase-like
38	Pcan_002319.p1	Glyoxylate reductase/hydroxypyruvate reductase-like
39	Pcan_005422.p1	Heat shock cognate protein 70 (pomacea canaliculata)
40	Pcan_007487.p1	Heat shock protein 90-alpha1
41	Pcan_032712.p1	Hemocyanin alpha D-subunit
42	Pcan_002695.p1	Hemocyanin G-type, units Oda to Odg-like
43	Pcan_038843.p1	Heterogeneous nuclear ribonucleoprotein D-like isoform X2
44	Pcan_000429.p1	Hillarin-like
45	Pcan_011994.p1	Hypothetical protein C0Q70_00113
46	Pcan_020348.p1	Hypothetical protein C0Q70_02897
47	Pcan_001385.p1	Hypothetical protein C0Q70_04853
48	Pcan_009824.p1	Hypothetical protein C0Q70_14825
49	Pcan_017756.p1	Hypothetical protein C0Q70_15704 (Pomacea canaliculata)
50	Pcan_001758.p1	Hypothetical protein C0Q70_16448
51	Pcan_007591.p1	Integrin beta 7
52	Pcan_013426.p1	Malate dehydrogenase mitochondrial
53	Pcan_013092.p1	Mu-class gst glutathione S-transferase
54	Pcan_014242.p1	Myosin heavy chain non-muscle isoform X7
55	Pcan_002353.p1	Myosin 10
56	Pcan_027318.p1	Mammalian ependymin-related protein 1
57	Pcan_002346.p1	NADP-dependent malic enzyme-like
58	Pcan_004967.p1	NADPH-dependent aldehyde reductase ARI1-like isoform X1
59	Pcan_004290.p1	Phosphoenolpyruvate carboxykinase
60	Pcan_014575.p1	2,3- bisphosphoglycerate-dependent phosphoglycerate mutase
61	Pcan_021812.p1	Peptidyl-prolyl cis-trans isomerase
62	Pcan_008040.p1	Plastin-1
63	Pcan_008193.p1	Pyruvate kinase PKM-like isoform X9
64	Pcan_004776.p1	Rab gdp-dissociation inhibitor

65	Pcan_001402.p1	Radixin-like
66	Pcan_044354.p1	Ras-related protein Rab-1A
67	Pcan_005786.p1	Ras-related protein Rab-10
68	Pcan_004335.p1	Ras-related protein Rab-11B
69	Pcan_006308.p1	Ras-related protein Rab-14
70	Pcan_008452.p1	Septin-11-like isoform X1
71	Pcan_014369.p1	Serine/threonine-protein kinase PAK 1
72	Pcan_010806.p1	Synaptic vesicle membrane protein VAT-1 homolog-like isoform X2
73	Pcan_000720.p1	Talin-1
74	Pcan_021691.p1	Talin-1 isoform X2
75	Pcan_008028.p1	Transitional endoplasmic reticulum ATPase
76	Pcan_010483.p1	Transketolase
77	Pcan_012794.p1	Tropomyosin
78	Pcan_007984.p1	Tubulin alpha-1 chain
79	Pcan_038556.p1	Ubiquitin-40S ribosomal protein S27a
80	Pcan_010435.p1	Vinculin
81	Pcan_007859.p1	N/D
82	Pcan_017297.p1	N/D
83	Pcan_033193.p1	N/D