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Abstract

The American lobster (*Homarus americanus*) is a commercially important crustacean with an unusual long life span up to 100 years and a comparative animal model of longevity. Therefore, research into its immune system and physiology is of considerable importance both for industry and comparative immunology studies. Peptidylarginine deiminases (PADs) are a phylogenetically conserved enzyme family that catalyses post-translational protein deimination via the conversion of arginine to citrulline. This can lead to structural and functional protein changes, sometimes contributing to protein moonlighting, in health and disease. PADs also regulate the cellular release of extracellular vesicles (EVs), which is an important part of cellular communication, both in normal physiology and in immune responses. Hitherto, studies on EV in Crustacea are limited and neither PADs nor associated protein deimination have been studied in a Crustacean species. The current study assessed EV and deimination signatures in haemolymph of the American lobster. Lobster EVs were found to be a poly-dispersed population in the 10-500 nm size range, with the majority of smaller EVs, which fell within 22-115 nm. In lobster haemolymph, 9 key immune and metabolic proteins were identified to be post-translationally deiminated, while further 41 deiminated protein hits were identified when searching against a Crustacean database. KEGG (Kyoto encyclopedia of genes and genomes) and GO (gene ontology) enrichment analysis of these deiminated proteins revealed KEGG and GO pathways relating to a number of immune, including anti-pathogenic (viral, bacterial, fungal) and host-pathogen interactions, as well as metabolic pathways, regulation of vesicle and exosome release, mitochondrial function, ATP generation, gene regulation, telomerase homeostasis and developmental processes. The characterisation of EVs, and post-translational deimination signatures, reported in lobster in the current study, and the first time in Crustacea, provides insights into protein moonlighting functions of both species-specific and phylogenetically conserved proteins and EV-mediated communication in this long-lived crustacean. The current study furthermore lays foundation for novel biomarker discovery for lobster aquaculture.

Key words: Peptidylarginine deiminases (PADs); protein deimination; American lobster (*Homarus americanus*); extracellular vesicles (EVs); immunity; metabolism; telomerase.

1. Introduction

The American lobster (*Homarus americanus*) is a coldwater crustacean species of major economic importance and is found throughout the coastal and oceanic waters of the Northeastern United States and Canada. The lobster forms a vital part of the marine ecosystem throughout New England, serving both as both predator and prey for a variety of fish and invertebrates. The lobster belongs to the order Decapoda under the phylum Arthropoda, is the heaviest of all Crustacea, as well as all living Arthropoda, and can reach a size span of 64 cm and weight over 20 kilograms, with a putative livespan of 100 years (Holthuis, 1991). The American lobster is susceptible to a number of bacterial, fungal and viral diseases, also due to anthropogenic and environmental factors (Cawthorn, 2011). Therefore, investigations into its immune related mechanisms are of major importance to further current understanding of host-pathogen responses and for the identification of novel biomarkers both for lobster management and aquaculture, and as estimators for environmental pollution.

Due to the unique longevity of the American lobster, including possibly due to their slow senescence and high telomerase activity (Klapper et al., 1998), it is also considered a valuable model for studies in ageing and telomerase function; including with relation to external and epigenetic factors (Louzon et al., 2019). The lobster may therefore be a valuable model organism which can hold information for molecular pathways underlying such unusual longevity traits (Louzon et al., 2019).

Peptidylarginine deiminases (PADs) are a phylogenetically conserved calcium-dependent family of enzymes. In mammals five PAD isozymes are known, while three PAD isozymes have been described in chicken and alligator, but only one PAD form in teleost and cartilaginous fish (Vossenaar et al., 2003; Rebl et al., 2010; Magnadottir 2018a, Magnadottir et al., 2019a; Criscitiello et al., 2019; Criscitiello et al., 2020a,b) Furthermore, PAD homologues, also referred to as arginine deiminases (ADI) (Novák et al., 2016) have been described lower in phylogeny, including in parasites (Gavinho et al., 2019) and bacteria (Bielecka et al., 2014; Kosgodage et al., 2019), as well as in fungi (El-Sayed et al., 2019). In crustaceans, PADs have hitherto not been reported.

PADs convert arginine into citrulline in an irreversible manner, leading to post-translational modification (citrullination/deimination) in numerous target proteins of cytoplasmic, nuclear and mitochondrial origin (Vossenaar, 2003; György et al., 2006; Magnadottir et al., 2018a; Magnadottir et al., 2019a; Criscitiello et al., 2020a; Alghamdi et al., 2019). Deimination causes structural protein changes which can affect protein function and consequently downstream protein-protein interactions. Deimination can amongst other contribute to neo-epitope generation, which results in inflammatory responses, as well as affect gene regulation via deimination of histones (Bicker and Thompson, 2013; Wang and Wang, 2013; Witalison et al., 2015; Yang et al., 2016; Lange et al., 2017;

Mondal and Thompson, 2019). As post-translational changes contribute to protein moonlighting, which allows one protein to exhibit different functions within one polypeptide chain (Henderson and Martin, 2014; Jeffrey, 2018), deimination may facilitate such functional diversity. It has been noted that proteins more susceptible to deimination are proteins rich in beta-sheets and disordered proteins, while the position of the arginine is also important (György et al., 2006; Tarsca et al., 1996). Hitherto, the majority of studies on PADs and downstream deimination have related to human pathological mechanisms, while roles for PADs have recently also focussed on normal physiology in a range of taxa, indicating important roles in metabolic and immunological responses throughout the phylogenetic tree (Magnadottir et al., 2018a, 2019a,b, 2020a,b,c; Phillips et al., 2020; Pamenter et al., 2019; Criscitiello et al., 2019,2020a,b; Bowden et al., 2020). In Crustacea, no data is to date available on PADs or protein deimination and therefore warrants further investigation.

PADs and posttranslational deimination contribute significantly to pathological pathways in inflammatory, autoimmune, neurodegenerative diseases and cancer (Mohanan et al., 2012; Wang and Wang, 2013; Witalison et al., 2015; Lange et al., 2017; Uysal-Onganer et al, 2020; Darrah and Andrade, 2018; Tilvawala et al., 2018; Ruiz-Romero et al., 2019; Fert-Bober et al., 2020; Martinez-Prat et al., 2019; Svärd et al., 2019; Mastronardi et al., 2006; Moscarello et al., 2013; Wei et al., 2013; Yang et al., 2016; Faigle et al., 2019; Méchin et al., 2020; Sancandi et al., 2020). PADs have furthermore been identified to have roles in mucosal, innate and adaptive immunity in a range of taxa (Lange et al., 2019; Magnadottir et al., 2018a and 2018b, 2019a; 2020a,b,c; Criscitiello et al., 2019, 2020a,b,c; Pamenter et al., 2019; Bowden et al., 2020; Phillips et al., 2020). Importantly, PADs have recently been identified as important players in infection and anti-pathogenic responses, including anti-viral (Muraro et al., 2018; Casanova et al., 2020) and anti-parasitic ones (Gavinho et al., 2019), as well as in anti-bacterial mechanisms (Kosgodage et al., 2019), endotoxemia and sepsis (Pan et al., 2017; Biron et al., 2018; Claushuis et al., 2018; Costa et al., 2018; Liang et al., 2018; Muraro et al., 2018; Stobernack et al., 2018; Saha et al., 2019). Important roles for PADs have also been identified in tissue regeneration (Lange et al., 2011; Lange et al., 2014; Lange, 2016), as well as wound healing (Wong et al., 2015; Fadini et al., 2016), while putative roles in ageing have also received attention (Ding et al., 2017; Wong and Wagner, 2018).

Extracellular vesicle (EV) biogenesis, and regulation of EV release from cells, has been found to be regulated by PADs and as this has been identified in a range of taxa, it appears to be a phylogenetically conserved function (Kholia et al., 2015; Gavinho et al., 2019; Kosgodage et al., 2017, 2018, 2019). EVs participate in cellular communication and can be isolated from many body fluids, including serum and plasma. EVs play physiological and pathological roles via transfer of cargo proteins and genetic material, including in inflammatory responses, in infection and host-pathogen

interactions (Inal et al., 2013; Colombo et al., 2014; Lange et al., 2017; Turchinovich et al., 2019; Vagner et al., 2019; Antwi-Baffour et al., 2019; Gavinho et al., 2019). As EVs carry information from their cells of origin, their cargo signatures are usable biomarkers (Hessvik and Llorente, 2018; Ramirez et al., 2018). Currently, very few studies on EVs have been conducted in Crustacea and therefore warrant further exploration for the identification of biomarkers in relation to physiological processes, host-pathogen interactions, for aquaculture as well as environmental effects. Limited studies, hitherto carried out on EVs in Crustacea, include assessment of microRNA cargo in exosomes (small EVs) of red swamp crayfish haemolymph in response to viral infection (Yang et al., 2019) and exosomes in hepatopancreas of woodlouse (Rupp and Ziegler, 2019). While a considerable body of comparative studies has assessed EVs and EV cargo in a range of taxa (Iliev et al., 2018; Gatien et al., 2019; Montaner-Tarbeset al., 2019; Šimundić et al., 2019; Magnadottir et al., 2019b, 2020a, 2020b,c; Criscitello et al., 2019 and 2020a and 2020b; Lange et al., 2019; Pamenter et al., 2019; Bowden et al., 2020), EVs have not been characterised in lobster haemolymph before.

Immune function in decapod crustaceans is relatively well understood (Cerenius et al 2010). The immune system of both extant lobster species has been reviewed (Bowden 2016). Lobster immunity has been studied in relation to disease and climate change (Klymasz-Swartz et al., 2019; Ochs et al., 2020), including a range of pathogens, as well as, metal sequestration and detoxification (Ahearn et al., 2004; Cawthorn, 2011). Conversely, studies on EVs, or roles for post-translational modifications, in relation to their immunity and physiology remain to be investigated. Due to the lobsters unusual longevity and commercial importance, a study on these parameters in lobster is warranted.

The current study characterised EVs and assessed post-translational deiminated protein signatures in haemolymph of the American lobster, reporting for the first time post-translational deimination in a crustacean. This study provides novel insights into lobster immunity and metabolism and may further current understanding of the roles for post-translational modifications in functional diversification of conserved proteins throughout phylogeny.

2. Materials and Methods

2.1 Haemolymph Sampling from Lobster

Live American lobsters (*Homarus americanus*), approximately 600-700g per individual (n=3), were obtained from a local supplier (McLaughlin Seafood, Bangor, Maine, U.S.A.). Haemolymph (approximately 2 ml per animal) was immediately drawn from the open sinus from the ventral side at the base of the tail using a 5 ml syringe and 23G needle. The haemolymph was then frozen at -80°C until further use for the individual experiments.

2.2 Isolation of Extracellular Vesicles and Nanoparticle Tracking Analysis (NTA)

Lobster EVs were prepared from haemolymph (thawed on ice) of three individuals, using sequential centrifugation and ultracentrifugation. Procedures were carried out according to our previously standardised and described protocols (Kosgodage et al., 2018; Criscitiello et al., 2019; Pamenter et al., 2019; Phillips et al., 2020; Criscitiello et al., 2020b; Bowden et al., 2020), also following recommendations of MISEV2018 (the minimal information for studies of extracellular vesicles 2018; Théry et al., 2018). For each individual haemolymp-EV preparation, 100 µl of lobster hemolymph were diluted 1:5 in Dulbecco's PBS (DPBS, ultrafiltered using a 0.22 µm filter, before use). This was then centrifuged for 30 min at 4,000 *g* at 4 °C, to remove of apoptotic bodies and aggregates. Supernatants were then collected and ultra-centrifuged at 100,000 *g* at 4 °C for 1 h. This resulted in EV-enriched pellets, which were resuspended each in 500 µl DPBS and thereafter ultra-centrifuged again for 1 h at 100,000 *g*, at 4 °C. The final resulting EV pellets were resuspended each in 100 µl of DPBS. The EVs were kept frozen at -80 °C until used in the procedures described below (all assessments where performed with EV preparations that had not been frozen for longer than 1 week). Haemolymph-EV size distribution profiles were generated and EVs were quantified using nanoparticle tracking analysis (NTA), based on Brownian motion, and carried out using the NanoSight NS300 system (Malvern, U.K.). Prior to NTA, the EV samples were diluted 1/100 in DPBS (10 µl of EV preparation diluted in 990 µl of DPBS). The diluted EV samples were applied to the NanoSight NS300 (Malvern Panalytical, UK), recording five repetitive reads, 60 sec each. Particle numbers per frame were 40 to 60, camera settings were at level 12 for recording and for post-analysis the threshold was set at 3. Replicate histograms were generated from these videos using the NanoSight software 3.0 (Malvern), representing mean and confidence intervals of the 5 recordings for each sample.

2.3 Transmission Electron Microscopy (TEM)

A pool of lobster haemolymph EVs, from three individual lobsters as described above, was used for morphological TEM assessment, as in previously described protocols (Criscitiello et al., 2020b; Phillips et al., 2020). Following thawing of isolated EV pellets (stored frozen for 1 week before imaging), the EVs were resuspended in 100 mM sodium cacodylate buffer (pH 7.4). One drop (~3-5 µl) of the EV suspension was placed onto a grid which held a carbon support film which had been previously glow discharged. Following partial drying of the EV suspension, the sample was fixed for 1 min at room temperature by placing the grid onto a drop of a fixative solution (2.5 % glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0)). The grid was applied to the surface of three drops of distilled water for washing of the EV sample, removing excess water using a filter paper. The EVs were then stained for 1 min with 2 % aqueous Uranyl Acetate (Sigma-Aldrich), removing excess stain with a filter paper and air drying the grid. TEM Imaging of EVs was carried out with a JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan), which was operated at 80 kV, using a magnification of 30,000x to 60,000x. Recording of digital images was performed with an AMT XR60 CCD camera (Deben, UK).

2.4 Isolation of Deiminated Proteins using F95-enrichment

Total deiminated proteins were isolated from lobster haemolymph using the the F95 pan-deimination antibody (MABN328, Merck) and the Catch and Release®v2.0 immunoprecipitation kit (Merck, U.K.). The F95-antibody specifically detects proteins modified by citrullination and has been developed against a deca-citrullinated peptide (Nicholas and Whitaker, 2002). Lobster hemolymph pools of three individual animals (3 x 50 µl) were used for F95-enrichment, which was performed at 4 °C overnight, using a rotating platform. Elution of deiminated (F95-bound) proteins from the columns was performed according to the manufacturer's instructions (Merck), and the protein eluate was thereafter diluted 1:1 in 2 x Laemmli sample buffer (BioRad, UK). Samples were kept frozen at -20 °C until further use for SDS-PAGE analysis, western blotting and in-gel digestion for LC-MS/MS analysis, as described below.

2.5 Silver Staining

SDS-PAGE (using 4–20 % gradient TGX gels, BioRad, U.K.) was carried out under reducing conditions for the F95-enriched protein eluates from lobster haemolymph, as described in 2.6. The gels were then silver stained according to the manufacturer's instructions, using the BioRad Silver Stain Plus Kit (1610449, BioRad, U.K.).

2.6 Western Blotting Analysis

For western blotting, SDS-PAGE was carried out on lobster haemolymph and haemolymph-EV samples, which had been diluted 1:1 in denaturing 2 x Laemmli sample buffer (containing 5 % beta-mercaptoethanol, BioRad, U.K.) and heated for 5 min at 100 °C. Protein separation was carried out using 4-20 % gradient TGX gels (BioRad U.K.), followed by western blotting at 165V for 1h on a Trans-Blot® SD semi-dry transfer cell (BioRad, U.K.). Membranes were stained with PonceauS (Sigma, U.K.) to assess even protein transfer and then blocked with 5 % bovine serum albumin (BSA, Sigma, U.K.) in Tris buffered saline (TBS) containing 0.1 % Tween20 (BioRad, U.K.; TBS-T) for 1h at room temperature. Primary antibody incubation was carried out overnight at 4 °C on a shaking platform using the following antibodies for lobster haemolymp: F95 pan-deimination antibody (MABN328, Merck; diluted 1/1000 in TBS-T) and anti-human PAD2 antibody (anti-PAD2, ab50257, Abcam; diluted 1/1000), for detection of a lobster PAD protein homologue, due to PAD2 being the most conserved PAD isozyme and the anti-human PAD2 antibody was previously shown to cross-react with PADs across taxa (Lange et al., 2011; Lange et al., 2014; Magnadottir et al., 2018a and 2019a; Criscitiello et al., 2019 and 2020a,b; Pameneter et al., 2019; Phillips et al., 2020; Magnadottir et al., 2020; Bowden et al., 2020). For characterisation of EVs from lobster haemolymph, two phylogenetically conserved EV-markers were assessed using the following primary antibodies: CD63 (ab216130, Abcam, U.K.; diluted 1/1000) and Flotillin-1 (Flot-1, ab41927; diluted 1/2000). The nitrocellulose membranes were washed following primary antibody incubation at RT in TBS-T for 3 x 10 min and therafter incubated with HRP-conjugated secondary antibodies (anti-rabbit IgG (BioRad) or anti-mouse IgM (BioRad) respectively, diluted 1/3000 in TBS-T), for 1h at RT. The membranes were washed for 5x10 min TBS-T and digitally visualised, using enhanced chemiluminescence (ECL, Amersham, U.K.) in conjunction with the UVP BioDoc-ITTM System (Thermo Fisher Scientific, U.K.).

2.7 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analysis of Deiminated Protein Candidates

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was carried out to identify deiminated proteins from *H. americanus* haemolymph (pool of n=3), according to previously described methods in other taxa (Phillips et al., 2020; Criscitiello et al., 2020b). Before LC-MS/MS analysis, the F95-enriched protein preparations (diluted 1:1 in 2x Laemmli buffer and boiled for 5 min at 100°C) were run 0.5 cm into a 12 % TGX gel (BioRad, U.K.). The concentrated protein band (containing the F95 eluate) was excised, trypsin digested and subjected to proteomic analysis using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific Inc, Waltham, MA, U.S.A.) system in conjunction with a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham,

MA, U.S.A.). Peptide seperation was performed using reverse-phase chromatography (flow rate 300 nL/min) and a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2 µm particle size, 100A pore size, 75 µm i.d. x 50 cm length). Peptides were loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5 µm particle size, 100A pore size, 300 µm i.d. x 5 mm length) from the Ultimate 3000 autosampler (0.1 % formic acid for 3 minutes, flow rate 10 µL/min). Thereafter, peptides were eluted from the pre-column onto the analytical column. The linear gradient employed was 2-40 % solvent B (80 % acetonitrile, 20 % water + 0.1 % formic acid) for 30 minutes. An Easy-Spray source (Thermo Fisher Scientific Inc.) was used to spray the LC eluant into the mass spectrometer. An Orbitrap mass analyser (set at a resolution of 70000), was used to measure all m/z values of eluting ions and were scanned between m/z 380-1500. Fragment ions were automatically isolated and generated using data dependent scans (Top 20) by higher energy collisional dissociation (HCD, NCE:25 %) in the HCD collision cell. The resulting fragment ions were measured using the Orbitrap analyser, set at a resolution of 17500. Singly charged ions and ions with unassigned charge states were excluded from selection for MS/MS, employing a dynamic exclusion window of 20 seconds. The data was processed post-run, using Protein Discoverer (version 2.1., Thermo Scientific). All MS/MS data were converted to mgf files. The files were submitted to the Mascot search algorithm (Matrix Science, London, U.K.) to identify deiminated protein hits. Search was conducted against the UniProt database CCP_*Homarus_americanus* *Homarus_americanus*_20200327 (318 sequences; 125441 residues), as well as a common database against crustacea (CCP_Crustacea Crustacea_20200402; 409366 sequences; 123217886 residues). An additional search was conducted against a common contaminant database (cRAP 20190401; 125 sequences; 41129 residues). The fragment and peptide mass tolerances were set to 0.1 Da and 20 ppm, respectively. The significance threshold value was set at of $p < 0.05$ and a peptide cut-off score of 14 for the lobster-specific database and for the common Crustacea database a cut-off score of 40 was applied (carried out by Cambridge Proteomics, Cambridge, U.K.).

2.8 FoldIndex© Analysis of Deiminated Proteins in Lobster Haemolymph

As disordered proteins are more susceptible to deimination, the level of protein disorder for the identified deimination protein hits from LC-MS/MS analysis was assessed using FoldIndex© analysis (Uversky et al., 2000; Prilusky et al., 2005; https://fold.weizmann.ac.il/fldbin/findex). The software was used to identify both the number of disordered regions, as well as the length of individual disordered regions in the identified lobster-specific deimination protein hits. The number of arginines present in the uniprot sequences identified in American lobster were also counted.

2.9 Protein-Protein Interaction Network Analysis

To predict and identify putative protein-protein interaction networks associated to the deiminated proteins from lobster haemolymph, STRING analysis (Search Tool for the Retrieval of Interacting Genes/Proteins; https://string-db.org/) was performed. Protein networks were generated based on protein names and applying the function of "search multiple proteins" in STRING (https://string-db.org/). For a representative choise of database, either "*Drosophila"* or "*Mus musculus*" were selected, as no species-specific protein database is available for lobster in STRING, and it is not possible either to choose a pan-Crustacean database for this type of analysis in STRING. Networks were therefore built representative of the phylum Arthropoda (with *Drosophila* showing most homology protein hits to lobster) and Mammalia (using *Mus musculus*), respectively. Parameters applied in STRING were "basic settings" and "medium confidence". Nodes are connected with colour lines which represent the following evidence-based interactions for the network edges: "known interactions" (these are based on experimentally determined curated databases), "predicted interactions" (these are based on gene neighbourhood, gene co-occurrence, gene fusion, via text mining, protein homology or co-expression). Gene ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways for the deiminated protein networks were furthermore assessed in STRING and are highlighted by colour coding (for each network analysis figure, please see the corresponding colour code key included for the individual nodes and connective lines).

2.10 Statistical Analysis

Generation of NTA curves was carried out using the Nanosight 3.0 software (Malvern, U.K.). The NTA curves show mean (black line) and standard error of mean (SEM), and the confidence intervals are indicated (red line). Protein-protein interaction networks were generating using STRING (https://string-db.org/), applying basic settings and medium confidence. Significance was considered as $p \leq 0.05$.

3. Results

3.1 Characterisation of Lobster Haemolymp-EVs

The NanoSight NS300 was utilised for NTA assessment of particle numbers and size distribution of lobster haemolymph EVs. Haemolymph EVs were found to be poly-dispersed in the size range of 10-500 nm, with main peaks at approximately 56, 115 and 172 nm. The majority of the EVs fell in the size range of 22-115 nm (Figure 1A). Assessment of EVs with the two phylogenetically conserved EV-specific markers CD63 and Flot-1, by western blotting, showed strong positive reaction for CD63, while Flot-1 showed very low positive detectable response (Figure 1B). Transmission electron

microscopy (TEM) revealed a majority of small EVs, approximately 20 nm sized (Figure 1C), and this corresponds to the main peaks observed by NTA analysis. EV yield from lobster haemolymph of the three different individuals ranged from 4.33 - 9.65 x 10^9 particles/ml (SEM:+/- 8.08 x 10^8 particles/ml) and modal EV size was in the range of 56-71 nm.

3.2 PAD Protein Homologue and Deiminated Proteins in Lobster Haemolymph

Anti-human PAD2 specific antibody was used for assessment of a lobster PAD protein homologue by western blotting. A positive protein band at the expected approximate 70 kDa size was identified in lobster haemolymph (Figure 2A) and is similar to the protein size expected for mammalian PAD (70-75 kDa). To assess the presence of total deiminated proteins in lobster haemolymph, western blotting analysis was performed and revealed a prominent band between 50-70 kDa (Figure 2B). Upon further analysis of F95-enriched fractions with silver staining, protein bands between 10-250 kDa were identified, with the most prominent band observed at 50-70 kDa (Figure 2C).

3.3 LC-MS/MS Analysis of Deiminated Proteins in Lobster Haemolymp

Protein identification of deiminated proteins in *H. americanus* haemolymph was carried out following F95-enrichment using LC-MS/MS analysis. Species-specific protein hits with *H. americanus*, as well as hits with other crustaceans were identified using the UniProt Crustacea database (Table 1; see Supplementary Table S1 for full details on all peptide hits). Overall, 10 species-specific *H. americanus* deiminated protein hits were identified. Further 42 hits were identified for other crustaceans, whereof hemocyanin was a common hit with lobster. (Figure 3 and Table 1; see Supplementary Table S1 for full details on peptide hits).

Table 1. Deiminated proteins in haemolymp of American lobster (*H. americanus*), as identified by F95-enrichment and LC-MS/MS analysis. Deiminated proteins were isolated from lobster hemolymph (n=3) by immunoprecipitation using the pan-deimination F95 antibody. The resulting F95-enriched eluate was then analysed by LC-MS/MS and peak list files submitted to Mascot, using both a lobster-specific as well as a common Crustacea database. Both *H. americanus* species-specific peptide sequence hits, as well as hits with other Crustacea are listed, showing number of sequences for protein hits and total score. A full list of protein sequence hits and peptides is further provided in Supplementary Table S1.

Protein name	Symbol	Sequences (Matches)	Total score ($p<0.05$)[‡]
	American Lobster (*Homarus americanus)*		
Hemocyanin alpha-subunit (Fragment)	Q9NFR6_HOMAM	36	2346
Hemocyanin beta subunit (Fragment)	A1YL85_HOMAM	10	701
Cytoplasmic type actin 3	B6EAV2_HOMAM	10	686
Cytoplasmic type actin 1	B6EAV0_HOMAM	10	656
Skeletal muscle actin 3	B6EAU3_HOMAM	10	343
Prophenoloxidase	Q6DN47_HOMAM	6	296
Hemocyanin alpha subunit (Fragment)	A1YL84_HOMAM	5	276
Glyceraldehyde-3-phosphate dehydrogenase	G3P_HOMAM	3	139
Crustin-like protein	A2TEF5_HOMAM	1	75
Hemocyanin subunit 3 (Fragment)	HCY3_HOMAM	1	71
Slow-tonic S2 tropomyosin	Q6E7L4_HOMAM	1	21
PROTEIN NAME (*species*)	**Other Crustacea (common name)**		
Cytoplasmic-type actin 3 (*Penaeus vannamei)*	A0A2H4V3C6_PENVA (Whiteleg shrimp)	12	736
Beta-actin (*Procambarus clarkii)*	A0A0M4J5L0_PROCL (Red swamp crayfish)	11	715
Hemocyanin subunit 1 (*Hirondellea gigas)*	A0A2P2HW39_9CRUS (Hadal amphipod)	9	441
Haemocyanin II (Fragment) (*Nephrops norvegicus)*	Q9U642_NEPNO (Norway lobster, langoustine)	8	443
Hemocyanin subunit 1 (*Procambarus clarkii)*	A0A142BZ27_PROCL (Red swamp crayfish)	8	417
Arginine kinase (*Homarus gammarus)*	KARG_HOMGA (European (common) lobster)	6	377
Hemocyanin subunit 1 (*Scylla paramamosain)*	A0A0U1ZVT9_SCYPA (Green mud crab)	7	365
Hemocyanin subunit L3 (*Penaeus vannamei)*	A0A423SGT1_PENVA (Whiteleg shrimp)	7	365
Hemocyanin subunit Y (*Penaeus japonicas)*	B0L612_PENJP (Kuruma prawn)	6	310
Hemocyanin A chain (*Portunus trituberculatus)*	A0A5B7DUJ6_PORTR (Gazami crab/horse crab)	6	256
Hemocyanin B chain (*Armadillidium nasatum)*	A0A5N5ST07_9CRUS (Pill-woodlouse)	5	243
Hemocyanin (*Macrobrachium nipponense)*	A0A0A0PM26_MACNP (Oriental river prawn)	4	213
Hemocyanin (*Penaeus vannamei)*	A0A3R7PVP7_PENVA (Whiteleg shrimp)	5	197
Hemocyanin A chain (*Armadillidium vulgare)*	A0A444TU07_ARMVU (Carpenter/pill woodlouse)	4	194
Hemocyanin subunit 2 (*Procambarus clarkii)*	A0A142BZ28_PROCL (Red swamp crayfish)	4	180
Hemocyanin subunit 2 (*Portunus trituberculatus)*	A0A5B7CEI8_PORTR (Gazami crab/horse crab)	4	178
Histone H4 (*Tigriopus californicus)*	A0A553NPY7_TIGCA (Splashpool copepod)	4	155
Nucleoside diphosphate kinase B (*Armadillidium nasatum)*	A0A5N5SU49_9CRUS (Pill-woodlouse)	4	153
Beta-actin (*Hirondellea gigas)*	A0A2P2HWK2_9CRUS (Hadal amphipod)	3	125

Histone H2B (*Armadillidium vulgare)*	A0A444SCS5_ARMVU (Carpenter/pill woodlouse)	3	118
Histone H2B (*Lepeophtheirus salmonis)*	A0A0K2U4Y8_LEPSM (Salmon louse)	3	96
Alpha-2-macroglobulin (*Pacifastacus leniusculus)*	G9BIX3_PACLE (Signal crayfish)	4	94
Papilin (*Armadillidium vulgare)*	A0A444SS31_ARMVU (Carpenter/pill woodlouse)	1	82
Ubiquitin (*Penaeus vannamei)*	A0A023H494_PENVA (Whiteleg shrimp)	1	75
Macroglobulin (*Palaemon carinicauda)*	A0A0B4KIG1_PALCI (Ridgetail white prawn)	2	71
Alpha-2-macroglobulin (*Penaeus vannamei)*	A0T1M0_PENVA (Whiteleg shrimp)	2	58
Very-high-density lipoprotein (*Ibacus ciliates)*	Q7M3L1_IBACI (Broad slipper lobster)	1	56
Spindle assembly abnormal protein 6 (*Portunus trituberculatus)*	A0A5B7CML1_PORTR (Gazami crab/horse crab)	2	55
Uncharacterized protein (*Tigriopus californicus)*	A0A553N744_TIGCA (Splashpool copepod)	2	49
Elongation factor 1-alpha (*Neogonodactylus oerstedii)*	Q6JUD7_NEOOE (Rock mantis shrimp)	2	49
Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial (*Armadillidium nasatum)*	A0A5N5T8W0_9CRUS (Pill-woodlouse)	2	47
Zinc finger protein (*Armadillidium nasatum)*	A0A5N5SNF4_9CRUS (Pill-woodlouse)	2	45
Sphingomyelin phosphodiesterase (*Armadillidium vulgare)*	A0A444TUU5_ARMVU (Carpenter/pill woodlouse)	1	45
Hepatocyte growth factor-regulated tyrosine kinase substrate (*Portunus trituberculatus)*	A0A5B7EUX8_PORTR (Gazami crab/ Japanese blue crab/ horse crab)	1	43
Treslin (*Portunus trituberculatus*)	A0A5B7HSZ5_PORTR (Gazami crab / horse crab)	2	43
Putative Ras GTPase-activating protein isoform X1 (*Penaeus vannamei)*	A0A423U3P9_PENVA (Whiteleg shrimp)	1	43
Putative NADPH--cytochrome P450 reductase protein (*Daphnia magna)*	A0A0N8B1M3_9CRUS (Water flea)	1	42
Telomere-associated protein RIF1 (*Armadillidium nasatum)*	A0A5N5TAP5_9CRUS (Pill-woodlouse)	3	42
Uncharacterized protein (*Tigriopus californicus)*	A0A553PA63_TIGCA (Splashpool copepod)	2	41
DNA-binding protein SMUBP-2-like (*Hirondellea gigas)*	A0A2P2I3E3_9CRUS (Hadal amphipod)	1	41
ATP-dependent DNA helicase (*Daphnia magna)*	A0A164L421_9CRUS (Water flea)	2	41
Uncharacterized protein (*Lepeophtheirus salmonis)*	A0A0K2TJX7_LEPSM (Salmon louse)	1	41

[ǂ]Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 20 (for *H. americanus* specific database) or > 40 (for all Crustacea database) indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits.

3.4 Protein-protein Interaction Network Identification of Deiminated Proteins in American lobster Haemolymph

For the prediction of protein-protein interaction networks of the deimination candidate proteins identified in lobster, the protein names were submitted to STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis (https://string-db.org/) (Figure 4). Protein interaction networks were based on known and predicted interactions and represent all deiminated proteins identified in lobster haemolymph and their interaction partners present in the STRING database, based on networks for *Drosophila melanogaster* for a maximum of protein hits within Arthropoda, as protein identifiers for lobster were not available in STRING (Figure 4). The PPI enrichment *p*-value (based on protein name as networks had to be built on *Drosophila* homologous proteins) was found to be $p = 0.076$ for 14 proteins common with *Drosophila*, but using one expansion for the protein network to identify further binding partners, this became $p = 0.0033$, indicating more interactions than expected for a random set of proteins of similar size, drawn from the genome. For protein-protein network analysis based on *Drosophila* homologues the following enrichment was identified: For biological processes, 55 GO-terms were found to be significantly enriched, for molecular function, 1 GO term was significantly enriched (succinate-CoA ligase activity); for cellular component, 11 GO pathways were significantly enriched; for KEGG pathways, 8 pathways were significantly enriched; for reactome pathways, 4 pathways were significantly enriched; 9 UniProt keywords were significantly enriched; 4 PFAM protein domains were significantly enriched; 10 INTERPRO protein domains and features were significantly enriched, while no significant enrichment was found for SMART protein domains. These are represented in Figure 4 for KEGG pathways (Figure 4A), INTERPRO protein domains (Figure 4B), cellular component (Figure 4C), reactome pathways (Figure 4D), UniProt keywords (Figure 4E), PFAM protein domains (Figure 4F), biological processes (Figure 4G-L), respectively, based on *Drosophila* homologues. Annotations for the specific pathways are colour coded within the figures and connective lines for networks indicate whether protein interactions were identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighbourhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology (Figure 4A-L).

When assessing the deiminated crustacean proteins for homologues with *Mus musculus*, PPI enrichment *p*-value (based on protein name as networks had to be built on mouse homologous proteins) was found to be 0.142 for 19 proteins identified as common with mouse, but using one expansion for the protein network to identify further binding partners, this became $p = 0.00219$, indicating more interactions than expected for a random set of proteins of similar size, drawn from the genome. For protein-protein network analysis based on mouse homologues the following enrichment was identified: For biological processes, 98 GO-terms were found to be significantly

enriched, for molecular pathways 35 GO terms were significantly enriched; for cellular component, 34 GO pathways were significantly enriched; for KEGG pathways, 7 pathways were significantly enriched; for reactome pathways, 2 pathways were significantly enriched; 11 UniProt keywords were significantly enriched; 3 PFAM protein domains were significantly enriched; 7 INTERPRO protein domains and features were significantly enriched, while no significant enrichment was found for SMART protein domains. The above listed enrichment analysis for deiminated proteins in lobster, based on homologue proteins identified in mouse using STRING, are represented in Figure 5 for KEGG pathways (Figure 5A), INTERPRO protein domains (Figure 5B), reactome pathways (Figure 5C), UniProt keywords (Figure 5D), PFAM protein domains (Figure 5E), molecular GO functions (Figure 5F-H), cellular GO components (Figure 5I-L), Biological GO processes (Figure 5M-V) respectively, based on *Mus musculus* homologues. Annotations for the specific pathways are colour coded within the figures and connective lines for networks indicate whether protein interactions were identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighbourhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology are also included (Figure 5A-V).

3.5 FoldIndex© Analysis of Deiminated Proteins in Lobster Haemolymph

Deiminated protein hits which were species-specific for *H. americanus* were assessed for number and length of disordered regions using FoldIndex© analysis (https://fold.weizmann.ac.il/fldbin/findex). These are presented, alongside number of arginines present in the uniprot sequences for the identified deimination protein candidates in American lobster (Table 2).

Table 2. FoldIndex© analysis of deiminated proteins identified by F95 enrichment in haemolymph of American lobster (*H. americanus*). The number of disordered regions, residue length of the longest disordered region, total number of disordered residues, as well as number of arginines present in the total number of residues for the individual protein hits is shown.

Protein name (*Homarus americanus*)	**Number disordered regions**	**Longest disordered region**	**Number disordered residues**	**Number of arginines** (out of total residues reported)
Hemocyanin alpha-subunit (Fragment) Q9NFR6_HOMAM	16	76	334	28 (out of 672 residues)
Hemocyanin beta subunit (Fragment) A1YL85_HOMAM	2	60	104	10 (out of 223 residues)
Cytoplasmic type actin 3 B6EAV2_HOMAM	5	17	52	18 (out of 376 residues)
Cytoplasmic type actin 1 B6EAV0_HOMAM	3	15	30	19 (out of 376 residues)
Skeletal muscle actin 3 B6EAU3_HOMAM	3	10	24	19 (out of 377 residues)
Prophenoloxidase Q6DN47_HOMAM	8	41	192	48 (out of 683 residues)
Glyceraldehyde-3-phosphate dehydrogenase G3P_HOMAM	0	0	0	9 (out of 333residues)
Crustin-like protein A2TEF5_HOMAM	0	0	0	5 (out of 112 residues)
Slow-tonic S2 tropomyosin Q6E7L4_HOMAM	5	87	272	21 (out of 284 residues)

4. Discussion

The current study is the first to profile extracellular vesicles (EVs) and deiminated protein signatures in lobster haemolymph, using American lobster (H. *americanus*) as a model species. EV profiles of the lobster, which showed a size range of 10-500 nm, did show a similar size distribution trend as seen for human EVs, which generally mainly fall within 30-300 nm, although some larger peaks above 300 nm were observed and a great majority of lobster EVs fell in a small size range of 22-115. Overall, the lobster haemolymph EVs were strongly positive for CD63, which is a marker of exosomes, and therefore smaller sized EVs (under 150 nm), while Flotillin-1, which generally is a marker for microvesicles (larger EVs above 150 nm), was only detected at low levels. A recent study by our group on horseshoe crab (*Limulus polyphemus*) has revealed similar characteristics of predominantly small CD63-positive EVs in haemolymph (Bowden et al., 2020), indicating that smaller EVs are a common trait in both these Arthropoda. Haemolymph exosomes from a study on whitleg shrimp showed size distribution between 15-200 nm alongside strong CD63 positivity (Yang et al., 2019). Morphological analysis in the current study by TEM confirmed a high proportion of small lobster haemolymph EVs, including with exosome characteristics of “cup-shape”. It can not be excluded that the larger peaks observed by NTA analysis may represent aggregates of some of these smaller EVs (exosomes), although evidence for aggregation was not apparent by TEM analysis.

Following F95-enrichment for identification of deiminated proteins from lobster haemolymph, numberous immunological and metabolic proteins were revealed as deimination candidates. This indicates that such post-translational modification may be a hitherto unrecognized mechanism which allows for protein-moonlighting in lobster immunity and physiology and may modulate

phylogenetically conserved proteins with various roles in immunity and metabolism. A lobster PAD protein homologue was identified in haemolymph using an antibody against human PAD2. This antibody was chosen in the current study as PAD2 is considered to be the most phylogenetically conserved PAD isozyme (Vossenaar et al., 2003; Magnadottir et al., 2018a; 2019a; Criscitiello et al., 2019,2020a,b; Pamenter et al., 2019). It furthermore cross-reacts with PADs and PAD homologues from a range of taxa (Magnadottir et al., 2018a; 2019a; Criscitiello et al., 2019, 2020a,b; Pamenter et al., 2019; Bowden et al., 2020; Phillips et al., 2020) and was also recently verified by our group in horseshoe crab (Bowden et al., 2020). Searching proteomic and genetic databases, no previous report on a PAD or PAD homologue exists for lobster, to our knowledge. Furthermore, our current study is the first to assess the presence of post-translationally deiminated proteins in lobster haemolymph in lobster and any Crustacea to our knowledge. Furthermore, when assessing the lobster-specific protein deimination hits by FoldIndex analysis, many showed a high number of disordered residues and regions. These findings correlate to previous reports that protein structures identified to be most prone to undergo deimination are beta-sheets and intrinsically disordered proteins (Tarsca et al., 1996; György et al., 2006). The position of the arginine is also of importance. For example when arginines are placed next to aspartic acid residues they are more susceptible to undergo deimination/citrullination, whereas arginines that are next to glutamic acid residues are rarely deiminated. When arginines are flanked by proline they are unlikely to be deiminated (Nomura, 1992; György et al., 2006).

Numerous species-specific deiminated protein candidates for lobster (9 hits) and protein hits with other Crustacea (41 hits), were identified in the current study using F95-enrichment in combination with LC-MS/MS analysis. The functions and roles of the individual proteins, including putative effects via post-translational deimination changes, are discussed below:

Hemocyanin was identified as a deimination candidate in lobster haemolymph. This included hemocyanin alpha-subunit (fragment), Hemocyanin beta subunit (fragment) and hemocyanin subunit 3 (fragment) as species-specific for *H. americanus*. In addition, a number of hemocyanins were identified as hits with other Crustacea, including with Hadal amphipod (*Hirondelea gigas*), Norway lobster (*Nephrops norvegicus*), red swamp crayfish (*Procambarus clarkii*), green mud crab (*Scyllas serrata*), whiteleg shrimp (*Litopanaeus vannamei*), Kuruma prawn (*Marsupaneus japonicus*), Gazami crab (*Portunus trituberculatus*), pill-woodlouse (*Armadillidium vulgare*) and Oriental river prawn (*Macrobranchium nipponense*). Hemocyanin is a 75 kDa protein that operates as the oxygen carrier in haemolymph of both Arthropoda and Mollusca, serving a similar function as haemoglobin in human blood. Lobster hemocyanin is composed of 2 x 6 subunits, with alpha and gamma subunits

best described (Kusche and Burmester, 2001). Hemocyanin levels have been linked to molting in lobster as well as environmental pollution (Engel et al., 2001). Furthermore it is linked to immune responses to pathogens (Clark et al., 2013) and its binding to a range of substances has been found to be thermodynamically regulated (Pott et al., 2009). In Arthropoda, hemocyanin is suspended directly into the haemolymph, contrary to being in blood cells as is found in human blood (Burmester, 2002). Some Arthropoda hemocyanins have been found to have antimicrobial, including antibacterial and anti-fungal effects and been suggested as putative novel antimicrobial, including against resistant strains (Jolly et al., 2019). The lobster hemacyanins, identified to be deiminated in the current study, were found to contain a number of disordered regions, as assessed by FoldIndex analysis, with hamocyanin alpha-subunit having higher disorder, with 16 disordered regions, where the longest was 76 disordered residues and a total of 334 disordered residues. In comparison, hemocyanin beta-subunit contained 2 disordered regions, with the longest disordered region containing 60 residues and a total of 104 disordered residues. Furthermore, a large number of arginines was present (˜4.5%). These features contribute to a protein's ability to carry out moonlighting functions, as disordered proteins are also most susceptible to post-translational deimination. While deimination of hemocyanin was recently identified in *L. polyphemus* (Bowden et al., 2020), this is the first description in the subphylum Crustacea, and may contribute to its multifaceted functions in Arthropoda and Mollusca phyla.

Cytoplasmic type actin 1 and 3, as well as **skeletal muscle actin 3**, were here identified to be deiminated in lobster haemolymph. In *H. americanus* twelve actin tissue isoforms have been described, where eight are muscle-related and three are cytoplasmic (Kim et al., 2009). Cytoplasmic actin 1 has been found in all tissues, while cytoplasmic actin 3 is found in hepatopancreas and skeletal muscle actin 3 is a major isoform in cutter claw closer muscle (Kim et al., 2009). In the current study, cytoplasmic actin 1 was identified to contain 3 disordered regions, with the longest disordered region containing 15 disordered residues and a total of 30 disordered residues. In addition, 19 arginines (out of 376 residues) were identified and all could potentially undergo post-translational conversion to citrulline. Cytoplasmic actin 3 was here found to have higher level of disorder, with 5 disordered regions, the longest region containing 17 disordered residues and a total of 52 disordered residues. Furthermore, 18 arginines (out of 376 residues) pose as putative sites for post-translational deimination. Skeletal muscle actin 3 contained 3 disordered region, where the longest contained 10 disordered residues, and the total protein contained 24 disordered residues and 19 arginines (out of 377 residues) which are putative candidates for arginine-citrulline

conversion. The roles for deimination in these different actins and its relevance for function at differing sites warrants further exploration.

Prophenoloxidase (proPO) was identified to be deiminated in lobster haemolymph. It is a type-3 copper-containing protein and forms an important part of both crustacean and insect innate immunity, both in humoral and cellular immune defences (Söderhäll et al., 1994; Lu et al., 2014). In invertebrates, including crustacean, it is activated upon foreign pathogen invasion (Söderhäll et al., 1994) and participates in elimination of pathogens via melanisation (Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004; Tassanakajon et al., 2018). Furthermore, it is involved in pathways for longevity and neuronal activity (Lu et al., 2014). ProPO has been identified in lobster and found to vary between crustacean species (Masuda et al., 2018). Lobster proPO was here found to contain 8 disordered regions, with 41 disordered regions, whereof the longest disordered region contained 192 residues; the number of arginines was found to be 48, out of 683 residues, indicating that this protein is highly prone to post-translational changes via deimination.

Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was identified to be deiminated in lobster haemolymph. It is an evolutionarily conserved enzyme (Martin and Cerff, 2017) with key functions in the glycolytic pathway, as well as having roles in DNA repair, membrane fusion, and nuclear RNA export (Baibai et al., 2010; Nicholls et al., 2012). Lobster GADPH has been assessed for acetylation (Davidson, 1970), but post-translational deimination has not been previously identified in lobster, while GAPDH has previously been identified as deiminated in teleost fish (Magnadottir et al., 2018a). In lobster GAPDH, 9 arginines were identified, out of 333 amino acid residues, but no disordered regions were identified. It must though be noted that disorder is not a requirement for deimination, while disordered proteins will be more prone to this PTM. To what extent deimination contributes to protein folding and function of lobster GAPDH remains to be investigated.

Crustin-like protein was identified to be deiminated in lobster haemolymph. This is an antibacterial protein in American lobster (Christie et al., 2007) as well as in European lobster (Hauton et al., 2006), with activity against Gram-positive bacteria. Crustin-like protein in lobster is similar to a large family of crustacean antimicrobial peptides comprised of four sub-groups, containing a four-disulfide core/whey-acidic-protein (WAP) domain, with activities against Gram-negative and Gram-positive bacteria (Christie et al., 2007; Amparyup et al., 2008; Barreto et al., 2018). Interestingly, in prawn and shrimp, crustin protein was found to act as an antibacterial but to have varying effects on responses to viral infection, ranging from promoting antiviral responses, to having no effects, or

even facilitating viral infection (Antony et al., 2011; Hipolito et al., 2014; Sun et al., 2017; Jin and Zhu, 2019). Furthermore in crab, crustin-like protein has been identified as a candidate bioactive peptide against a range of bacteria and therefore a candidate for measures relating to food preservation (El Menif et al., 2019). In our current study, crustin-like protein was found to contain 5 arginines (out of 112 amino acid residues) that can pose as sites for arginine-citrulline conversion, while no disordered regions were identified. To what extent deimination of crustins plays roles in diverse immune reactions to different bacteria and viral infections may be of interest and needs further exploration.

Slow-tonic S2 tropomyosin was identified to be deiminated in lobster haemolymph. This was the most disordered protein observed, mainly composed of five long disordered regions, with the longest region comprised of 87 disordered residues, with a total of 272 disordered residues (out of a total of 284 amino acids), and 21 arginines. In the American lobster, skeletal muscle fibre polymorphism has been identified, including a gradation between the slow-twitch (S1) and slow-tonic (S2) muscle phenotype in the cutter claw closer muscle (Medler et al., 2004; Kim et al., 2009). In Christmas Island red crab (*Gecarcoidea natalis*), a switch has been observed from the slow phasic (S1) to slow tonic (S2) and fatigue-resistant muscle fibres, as crabs prepare for long distance migration (Postel et al., 2010). Roles for slow and fast fibres have also been investigated in relation to muscle adaption to low habitat temperature in Antarctic isopods (Holmes et al., 2002). Slow-tonic S2 tropomyosin has furthermore been identified to play roles in embryo organogenesis and sex determination in the oriental river prawn (*Macrobrachium nipponense*) (Jin et al., 2014). To what extent post-translational deimination plays roles in moonlighting functions, for phenotypic muscle plasticity and developmental processes, remains to be investigated.

Other proteins identified to be deiminated by assessing hits across the whole Crustacea database furthermore included beta-actin, arginine kinase, histones H2B and H4, nucleoside diphosphate kinase B, alpha-2-macroglobulin, macroglobulin, papilin, ubiquitin, spindle assembly abnormal protein 6, elongation factor 1-alpha, succinate-CoA ligase (mitochondrial), zinc finger protein, sphingomyelin phosphodiesterase, hepatocyte growth factor-regulated tyrosine kinase substrate, treslin, putative Ras GTPase-activating protein isoform X1, putative NADPH-cytochrome P450 reductase protein, telomere-associated protein RIF1, DNA-binding protein SMUBP-2-like and ATP-dependent DNA helicase. These are briefly discussed below.

The deimination of **histones H2B** and **H4** was identified here in Crustacea and has previously been identified in a range of taxa (Pamenter et al., 2019; Criscitiello et al., 2020b) as well as in relation to gene regulation and numerous human pathologies, including cancer (Lange et al., 2017; Fuhrmann and Thompson, 2016; Beato and Sharma, 2020). Histones have been identified as antimicrobial compounds in crustaceans (Smith and Dyrynda, 2015), to be involved in metamorphosis and affected by environmental contaminants (histone H1) (Bauer et al., 2013) and to play role in lobster neurogenesis (histone H3) (Harzsch et al., 1999). Deimination of histones have not been studied in Crustacea or reported before this current study to our knowledge. Interestingly, H2B was in the current study identified as a deimination hit in the carpenter/pill woodlouse (*A. vulgare*) as well as salmon louse (*Lepeophtheius salmonis*), which is a major pathogen in salmon aquaculture and for the salmon industry (McBeath et al. 2006; Igboeli et al., 2014). Histone H4 was identified as a deimination hit in *Tigriopus californicus*, a marine copepod which tolerates high temperatures. This is an invertebrate marine model species for ecotoxicology and environmental genomics (Raisuddin et al., 2007) as well as adaption to rapid changes in environmental temperature (Scheffler et al., 2019), including in relation to mitochondrial function (Harada et al., 2019). Interestingly, in reptiles, extracellular histones H2A and H4 have been identified to act as inhibitors of viral infections *in vitro* (Kozlowski et al., 2016) and in teleost fish, mucosal H2A has been shown to have anti-microbial effects (Fernandes et al., 2002). The multifaceted functions of histones in immunity, including via post-translational regulation, such as deimination identified here, remains to be further investigated throughout phylogeny, in relation to physiological, anti-pathogenic responses or other roles, including in gene regulation related to multiple pathways, such as development.

Alpha-2-macroglobulin was identified as a deiminated hit with signal crayfish (*Pacifastacus leniusculus*) and whiteleg shrimp (*L. vannemei*), while macroglobulin was a hit with ridgetail white prawn. Alpha-2-macroglobulin is a thioester containing protein and a broad-spectrum protease-binding protein, which is a phylogenetically conserved part of the innate immune system (Armstrong and Quigley, 1999; Sottrup-Jensen et al., 1990). Alpha-2-macroglobulin an abundant plasma protein, also present in Arthropoda, including Crustacea (Enghild et al., 1990; Stöcker et al., 1991; Armstrong and Quigley, 1999; Swarnakar et al., 2000). Alpha-2-macroglobulin has previously been identified as a deimination candidate in serum and plasma of a range of taxa (including camelids, birds and alligator, horseshoe crab) (Criscitiello et al., 2020a and b; Phillips et al., 2020; Bowden et al., 2020) and such deimination may contribute to its immunological functions throughout phylogeny.

Papilin was identified as a deimination hit with woodlouse (*A. vulgare*). It is a phylogenetically conserved proteoglycan like sulfated extracellular matrix glycoprotein with homology to the ADAMTS secreted, matrix-associated metalloproteinases (Fessler et al., 2004). Papilin has roles in gastrulation, as well as being associated with the basement membrane and wandering phagocytic haemocytes in *Drosophila* (Kramerova et al., 2000). Papilin has important roles in embryonic development both in *Drosophila* and *Caenorhabditis elegans,* with roles in cell rearrangement, organogenesis and organ growth, with different isozymes expressed according to tissue type, including in the gut (Kramerova et al., 2003; Kawano et al., 2009; Jafari et al., 2010). Papilin has furthermore been identified as a novel wound healing protein in teleost fish mucus (Saleh et al., 2018). Papilin also has roles in nervous system assembly (Ramirez-Suarez et al., 2019), including in spinal cord regeneration (Saunders et al., 2014), and has been identified as a putative biomarker in Alzheimer's disease and major depression (Souslova et al., 2013). The role for post-translational deimination of papilin identified here for the first time to our knowledge in any species therefore provides novel insights into the multifaceted functions of this protein family, and also correlates with association of PADs and deimination in organogenesis and mucosal immunity (Magnadottir et al., 2018a; Magnadottir et al., 2019a; 2019b), wound healing (Wong et al., 2015; Fadini et al., 2016), CNS regeneration (Lange et al., 2011; Lange et al., 2014) and neurodegeneration (Lange et al., 2017; Sancandi et al., 2020)

Ubiquitin was here identified as a deiminated protein hit scoring with whiteleg shrimp (*L. vannamei*). Ubiquitin is phylogenetically conserved and causes post-translational ubiquitination in multiple proteins, contributing to diversity of protein functions and can furthermore undergo post-translational modifications itself (Ohtake and Tsuchiya, 2017), although deimination has not been reported before to our knowledge. Ubiquitin has important roles in homeostasis and contributes to a multitude of cellular functions in physiological and pathological processes (Nakamura, 2018). It is for example linked to autoimmune and neurodegenerative diseases and cancer (Popovic et al., 2014; Zheng et al., 2016; Fujita et al., 2019), as well as playing important roles in cellular homeostasis by regulation of autophagy, cellular stress and damage (Chen et al., 2019). Furthermore, both deimination and ubiquination have been associated with EV biology (Szabó-Taylor et al., 2015; Lange et al., 2017). In Crustacea, ubiquitin mediated pathways have been studied in relation to viral infections (Leu et al., 2013) as well as other immune and anti-pathogenic responses (Götze et al., 2017), metabolism (Mykles, 1997; Hand, 1998) including during molting in lobster (Spees et al., 2003). Deimination of ubiquitin is here described for the first time to our knowledge and may provide insights into its multiple roles in diverse cellular processes.

Spindle assembly abnormal protein 6 (SAS-6) was here identified as a deimination candidate protein through a hit with Gazami crab (*P. trituberculatus*). SAS-6 has important functions in centriole assembly and its oligomerisation is important in this process (Busch et al., 2019). The centriole is a macromolecular structure which is evolutionarily conserved, is fundamental for the organisation of microtubules, and of key importance for formation of cilia, centrosomes and flagella (Gönczy, 2012; Busch et al., 2019; Gönczy and Hatzopoulos, 2019). The deimination of SAS-6 revealed here, may provide novel insights into the control of centriole formation via this post-translational modification.

Elongation factor 1 alpha was here identified as a deimination hit with the rock mantis shrimp. It plays roles in nuclear export of proteins and in cytoskeleton organisation (Khacho et al., 2008) alongside multiple functions relating to metabolism in all multicellular organisms (Wang et al., 2011). Furthermore it has roles in cell growth and apoptosis and is involved in immune responses (Talapatra et al., 2002, Vera et al., 2014). In shrimp, elevated elongation factor 1 alpha levels have been related to stress responses (Wang et al., 2011), while roles in embryonic development brine shrimp (*Artemia salina*) have been identified (Maassen et al., 1985). Previously, it has been identified as a deimination candidate in teleosts (Magnadottir et al., 2018a). The roles for deimination to facilitate its multifaceted functions remains to be further investigated.

Succinate CoA ligase (mitochondrial) was a deimination positive hit with woodlouse (*Armadillidium nasatum*). Succinate CoA ligase is a mitochondrial matix enzyme involved in mitochondrial metabolism and responsible for the conversion of succinyl-CoA and ADP or GDP to succinate and ATP or GTP (Ostergaard, 2008). Succinyl-CoA synthetase has been identified in a range of taxa and indicates conserved functions throughout phylogeny (Johnson et al., 1998; Dacks et al., 2006). Modifications in Succinate CoA ligase have been linked to a number of human pathologies including hypotonia, deafness and Leigh-like syndrome (Ostergaard, 2008) as well as cancers (Chinopoulos and Seyfried, 2018) and infantile mitochondrial disorders (Carrozzo et al., 2007; El-Hattab and Scaglia, 2017). The identification of deimination in succinate CoA ligase identified here in Crustacea may therefore be of importance to understand its multifaceted functions in both physiological and pathological processes throughout phylogeny.

Zinc finger protein was identified as a deimination hit with woodlouse (*A. nasatum*). Orthologues zinc finger proteins are considerably conserved in bilaterians (Seetharam et al., 2010). In Crustacea,

zinc finger protein has been associated to various immunological functions including pathogenic responses (Li et al., 2018; Zuo et al., 2018; Shekar and Venugopal, 2019). Roles in lifespan (Lin et al., 2019) and germ-line development have also been reported (Sagawa et al., 2005). Deimination may add post-translational flexibility to zinc finger protein function and therefore be a contributor to moonlighting functions throughout phylogeny.

Sphingomyelin phosphodiesterase was a F95-enriched protein ID with a hit for a deimination hit with woodlouse (*A. vulgare*). It is a hydrolase with pivotal roles in metabolic processes that involve sphingomyelin and has been associated with several developmental processes in Arthropoda (Han et al., 2018). In mammals, it has been associated with virus-host interactions and viral cell entry (Pastenkos et al., 2019) as well as in inflammatory responses (Traini et al., 2014). It has been identified as a glycoprotein (Traini et al., 2017), while deimination has not been previously reported to our knowledge and may provide novel insights into its functions *in vivo*.

Hepatocyte growth factor-regulated tyrosine kinase substrate was a deimination hit with Gazami crab (*P. trituberculatus*). It is an early endosomal protein with roles in regulation of growth factor-receptor complex trafficking through early endosomes (Rayala et al., 2006), including by forming part of the ESCRT-0 complex (Kojima et al., 2014), which is also related to EV release (Inal et al., 2013; Vilette et al., 2015). It also has roles in endothelial cell polarity and cerebrovascular stability (Yu et al., 2020) as well as roles in signalling in embryonic development (Miura and Mishina, 2011). In Crustacea it has been related to innate immune responses (Sun et al., 2017b). Post-translational phosphorylation and ubiquitination of hepatocyte growth factor-regulated tyrosine kinase substrate has been described (Gasparrini et al., 2012), while deimination has not been previously reported to our knowledge, and may therefore provide further insights into functional diversity via this post-translational modification

Treslin was identified as a deiminated protein hit with Gazami crab (*P. trituberculatus*). It is of pivotal importance in the cell cycle (Charrasse et al., 2015) as it acts as a regulator of the triggering of DNA replication initiation (Kumagai et al., 2010) as well regulating DNA damage response pathways (Boos et al., 2013). Post-translational modifications of treslin identified to date include phosphorylation (Sansam et al., 2015; Mu et al., 2017), which regulates the length of S-phase (Sansam et al., 2015), and epigenetic control of DNA replication via binding of treslin to acetylated chromatin has also been reported (Sansam et al., 2018). The replication function of treslin is broadly conserved from yeast to metazoan (Mueller et al., 2011), while further insights into the specific functions throughout

phylogeny need further investigation (Köhler et al., 2019). The identification of deimination of treslin in the current study therefore provides a novel angle on modulation of DNA replication initiation in diverse taxa throughout this post-translational modification and requires further investigation.

Putative Ras GTPase-activating protein isoform X1 was here identified to be deiminated with a protein hit with whiteleg shrimp (*L. vannamei*). Ras-specific GTPase-activating proteins (RasGAPs) are regulators of Ras proteins, including in cellular growth control, tissue homeostasis, neuronal function and tumourogenesis (Friedman, 1995; King et al., 2013; Zhang and Chen, 2015; Scheffzek and Shivalingaiah, 2019). In Crustacea, GTPase stimulatin has been assessed in shrimp (Chang and Chuang, 2001) and Ras has been identified to have roles during molting in crab (MacLea et al., 2012). A number of post-translational modifications have been identified in Ras GTPases, including farnesylation, methylation, diubiquitylation, nitrosylation, palmitoylation, phosphorylation, peptidyl-prolyl isomerisation, monoubiquitylation, ADP ribosylation and glucosylation (Ahearn et al., 2011). Post-translational deimination has hitherto not been reported to our knowledge and may play hitherto unrecognized roles in Ras GTPase related functions throughout phylogeny

Putative NADPH-cytochrome P450 reductase protein was identified as a deiminated protein hit with water flea (*Daphnia magna*). Cytochromes P450 (CYP) play major roles in the variable response to drugs and this can be affected by regulatory proteins such as NADPH-cytochrome P450 oxidoreductase (Zanger and Schwab, 2013). NADPH-P450 reductase furthermore plays roles in oxidative and ER stress, as well as protein misfolding, also relating to a number of pathologies including ischemia, diabetes, atherosclerosis, neurodegenerative diseases, inflammation and liver and kidney diseases (Zeeshan et al., 2016). In crustaceans, P450-dependent xenobiotic monooxygenation is mainly carried out in the hepatopancreas (James, 1989) and NADPH-cytochrome P450 reductase protein has been identified in a number of invertebrate marine species, including Porifera, Cnidaria, Mollusca, Polychaeta, Crustacea and Echinodermata (Solé and Livingstone, 2005). NADPH cytochrome c reductase has amongst other been found to be an important part in detoxifying responses of prawn, following oil pollution (Arun and Subramanian, 2007). Post-translational deimination of NADPH-cytochrome P450 reductase protein has not been previously described to our knowledge and may play moonlighting roles in drug and xenobiotic metabolism as well as pathologies and inflammatory responses, which will need further exploration.

Telomere-associated protein RIF1 was identified as a deimination candidate scoring with a protein homologue from woodlouse (*A. nasatum*). RIF1 is phylogenetically conserved from yeast to

mammals and has diverse functions in telomere length regulation and the maintenance of genome integrity (Sreesankar et al., 2012). RIF1 is found to be highly expressed in mouse totipotent and pluripotent cells during early development, possibly linked to pluripotency and telomerase length (Adams and McLaren, 2004) and has been found to regulate telomerase length homeostasis in yeast (Yu et al., 2012) as well as in embryonic stem cells, where the mechanism has been linked to heterochromatic silencing (Dan et al., 2014). The identification of deimination in telomere associated proteins is of some interest, as this must be a score with conserved motifs in lobster haemolymph, although RIF1 is not described in lobster, while the slow senescence of lobster is associated with high telomerase activity and related to its unusual longevity (Klapper et al., 1998).

DNA-binding protein SMUBP-2-like, also known as immunoglobulin helicase μ-binding protein 2 (IGHMBP2) and cardiac transcription factor 1 (CATF1), was identified as a deimination protein hit with Hadal amphipod (*H. gigas*). It regulates various nuclear functions and is a transcription regulator that unwinds RNA and DNA duplices in an ATP-dependent reaction (Uchiumi et al., 2010). It has been shown to have negative transcriptional effects on Epstein-Barr virus (Zhang et al., 1999) and mouse mammary tumor virus (Uchiumi et al., 2004), and to regulation transcription and replication of the human neurotropic JCV virus in glial cells (Kerr and Khalili, 1991; Chen et al., 1997). In teleost fish, it is involved in transcriptional activation of anti-freeze liver protein gene in winter flounder (*Pleuronectus americanus*) (Miao et al., 2000). Post-translational deimination has not been described for SMUBP-2 before to our knowledge and may have implications in its transcriptional regulatory functions in diverse taxa.

ATP-dependent DNA helicase was furthermore a deimination protein hit scoring with Hadal amphipod (*H. gigas*). DNA helicases use energy from ATP to unwind double-stranded nucleic acids to access genetic information during cell replication (Castillo-Tandazo et al., 2019). Helicases therefore play multifaceted roles in cellular activities, which relate to DNA and RNA transactions (Limudomporn et al., 2016). Therefore, post-translational modifications in DNA helicases may be of considerable importance as these can modify protein structure and therefore function. Deimination has not been reported before for DNA halicases to our knowledge and provides a novel angle on functional diversity via such post-translational change, requiring further investigation.

Protein network analysis, constructed in the current study using STRING based both on *Drosophila* or mouse homologue proteins, as lobster proteins are not available in the STRING database, correlated with various immunological and metabolic functions of the proteins identified to be deiminated in

lobster and other Crustacea. KEGG and GO enrichment analysis of deiminated Crustacean proteins revealed pathways relating to a number of immune and metabolic pathways. This included phagosome and endocytosis as well as anti-pathogenic (viral, bacterial, fungal) and host-pathogen interactions. Metabolic pathways included carbon metabolism, glycolysis/gluconeogenesis, biosynthesis of amino acids, propanoate metabolism, citrate cycle (TCA cycle), and inositol phosphate metabolism. A number of GO pathways revealed related amongst other to the regulation of vesicle and exosome release, mitochondrial function, ATP generation, metabolism, glycolysis, myoblast fusion, neuronal remodelling, antimicrobial humoral immune responses, regulation of symbiosis, viral life cycle regulation, gene regulation and developmental processes such as biological regulation, morphogenesis, myoblast fusion and somatic muscle development. These GO and KEGG pathways do indeed relate to the various functions of the identified deimination candidates in lobster, which are discussed in detail above. This highlights a novel aspect of post-translational deimination in the regulation of such multiple immune and metabolic pathways in lobster as a model species for Crustacea. Furthermore, post-translational regulation via deimination of these pathways may be present for various conserved and moonlighting protein functions throughout phylogeny and the findings reported here form the basis of future in depth studies.

The characterisation of haemolymph EVs and of post-translational deimination signatures revealed lobster in the current study, and for the first time in Crustacea, contributes to current understanding of deimination and EV-mediated communication in this long-lived Arthropod. Our findings may inform conserved and diverse functions of moonlighting proteins via post-translational deimination throughout phylogeny and aid biomarker discovery for this commercially valuable Crustacean species.

Conclusion

This is the first study to assess PADs, protein deimination and extracellular vesicle profiles in hemolymph of a crustacean, using the American lobster a model species. EV profiles of lobster showed a high proportion for small EVs, which were mainly CD63 positive, indicating a majority of exosomes (small EVs), compared to larger (microvesicles) EVs. Proteomic analysis of deiminated proteins in lobster haemolymph revealed a range of proteins linked to KEGG and GO pathways for key immunological, metabolic, gene regulatory and developmental functions. This study highlights therefore roles for protein moonlighting function in health and disease via post-translational deimination, many of which are phylogenetically conserved. Our study provides novel insights into the immunity and physiology of the American lobster, an unusually long-lived crustacean, as well as

other Crustacea. Comparative studies in lobster may be of translational value for furthering current understanding of mechanisms underlying conserved physiological and pathogenic pathways, also linked to metabolism, immunological responses and longevity, as well as further current understanding of diversification of protein functions, facilitated by post-translational deimination.

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Author Contributions Statement

TB: Resources; Validation; Writing - review & editing.

IK: Methodology; Resources; Visualization.

SL: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Validation; Visualization; Writing -original draft; Writing - review & editing.

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References

Adams, I.R., McLaren, A., 2004. Identification and characterisation of mRif1: a mouse telomere-associated protein highly expressed in germ cells and embryo-derived pluripotent stem cells. Dev. Dyn. 229(4), 733-44.

Ahearn, G.A., Mandal, P.K., Mandal, A., 2004. Mechanisms of heavy-metal sequestration and detoxification in crustaceans: a review. J. Comp. Physiol. B. 174(6), 439-52.

Ahearn, I.M., Haigis, K., Bar-Sagi, D., Philips, M.R., 2011. Regulating the regulator: post-translational modification of RAS. Nat. Rev. Mol. Cell Biol. 13(1), 39-51.

Alghamdi, M., Alasmari, D., Assiri, A., Mattar, E., Aljaddawi, A.A., Alattas, S.G., Redwan, E.M., 2019. An Overview of the Intrinsic Role of Citrullination in Autoimmune Disorders. J. Immunol. Res. 2019, 7592851.

Amparyup, P., Kondo, H., Hirono I., Aoki, T., Tassanakajon, A., 2008. Molecular cloning, genomic organization and recombinant expression of a crustin-like antimicrobial peptide from black tiger shrimp *Penaeus monodon*. Mol. Immunol. 45(4), 1085-93.

Antony, S.P., Singh, I.S., Sudheer, N.S., Vrinda, S., Priyaja, P., Philip, R., 2011. Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, Penaeus monodon, and its expression profile in response to various immunostimulants and challenge with WSSV. Immunobiol. 216(1-2), 184-94.

Antwi-Baffour, S., Malibha-Pinchbeck, M., Stratton, D., Jorfi, S., Lange, S., Inal, J., 2019. Plasma mEV levels in Ghanain malaria patients with low parasitaemia are higher than those of healthy controls, raising the potential for parasite markers in mEVs as diagnostic targets. J. Extracell. Vesicles. 9(1), 1697124.

Armstrong, P.B., Quigley, J.P., 1999. Alpha2-macroglobulin: an evolutionarily conserved arm of the innate immune system. Dev. Comp. Immunol. 23, 375-90.

Baibai, T., Oukhattar, L., Mountassif, D., Assobhei, O., Serrano, A., Soukri, A., 2010. Comparative molecular analysis of evolutionarily distant glyceraldehyde-3-phosphate dehydrogenase from *Sardina pilchardus* and *Octopus vulgaris*. Acta Biochim Biophys Sin (Shanghai). 42(12), 863-72.

Barreto, C., Coelho, J.D.R., Yuan, J., Xiang, J., Perazzolo, L.M., Rosa, R.D., 2018. Specific Molecular Signatures for Type II Crustins in Penaeid Shrimp Uncovered by the Identification of Crustin-Like Antimicrobial Peptides in *Litopenaeus vannamei*. Mar. Drugs. 16(1), pii: E31.

Bauer, M., Greenwood, S.J., Clark, K.F., Jackman, P., Fairchild, W., 2013. Analysis of gene expression in *Homarus americanus* larvae exposed to sublethal concentrations of endosulfan during metamorphosis. Comp. Biochem. Physiol. Part D Genomics Proteomics. 8(4), 300-8.

Bicker, K.L., Thompson, P.R., 2013 The protein arginine deiminases: Structure, function, inhibition, and disease. Biopolymers. 99 (2), 155-63.

Bielecka, E., Scavenius, C., Kantyka, T., Jusko, M., Mizgalska, D., Szmigielski, B., Potempa, B., Enghild, J.J., Prossnitz, E.R., Blom, A.M., Potempa, J., 2014. Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. J. Biol. Chem. 289, 32481–32487.

Biron, B.M., Chung, C.S., Chen, Y., Wilson, Z., Fallon, E.A., Reichner, J.S., Ayala, A., 2018. PAD4 deficiency leads to decreased organ dysfunction and improved survival in a dual insult model of hemorrhagic shock and sepsis. J. Immunol. 200, 1817–1828.

Boos, D., Yekezare, M., Diffley, J.F., 2013. Identification of a heteromeric complex that promotes DNA replication origin firing in human cells. Science. 340(6135), 981-4.

Bowden, T.J., 2017. The humoral immune systems of the American lobster (*Homarus ameri*canus) and the European lobster (*Homarus gammarus*). Fisheries Res. 186 (1), 367-371.

Bowden T.J., Kraev, I., Lange, S., 2020. Post-translational protein deimination signatures and extracellular vesicles (EVs) in the Atlantic horseshoe crab (*Limulus polyphemus*). Dev. Comp. Immunol, in press, https://doi.org/10.1016/j.dci.2020.103714

Burmester, T., 2002. Origin and evolution of arthropod hemocyanins and related proteins. J. Comp. Physiol. B. 172 (2), 95–107.

Busch, J.M.C., Erat, M.C., Blank, I.D., Musgaard, M., Biggin, P.C., Vakonakis, I., 2019. A dynamically interacting flexible loop assists oligomerisation of the *Caenorhabditis elegans* centriolar protein SAS-6. Sci. Rep. 9(1), 3526.

Carrozzo, R., Piemonte, F., Tessa, A., Lucioli, S., Rizza, T., Meschini, M.C., Fattori, F., Santorelli, F.M., 2007. Infantile mitochondrial disorders. Biosci. Rep. 27(1-3), 105-12.

Casanova, V., Sousa, F.H., Shakamuri, P., Svoboda, P., Buch, C., D'Acremont, M., Christophorou, M.A., Pohl, J., Stevens, C., Barlow, P.G. Citrullination Alters the Antiviral and Immunomodulatory Activities of the Human Cathelicidin LL-37 During Rhinovirus Infection. Front, Immunol. 2020, 11, 85.

Castillo-Tandazo, W., Smeets, M.F., Murphy, V., Liu, R., Hodson, C., Heierhorst, J., Deans, A.J., Walkley, C.R., 2019. ATP-dependent helicase activity is dispensable for the physiological functions of Recql4. PLoS Genet. 15(7), e1008266.

Cawthorn, R.J., 2011. Diseases of American lobsters (*Homarus americanus*): a review. J. Invertebr. Pathol. 106(1), 71-8.

Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198, 116-26.

Cerenius, L., Jiravanichpaisal, P., Liu, H., Soderhall, I., 2010. Crustacean Immunity. In: Söderhäll, K. (eds) Invertebrate Immunity. Advances in Experimental Medicine and Biology, vol 708. Springer, Boston, MA.

Chang, K.C., Chuang, N.N., 2001. GTPase stimulation in shrimp Ras(Q(61)K) with geranylgeranyl pyrophosphate but not mammalian GAP. J. Exp. Zool. 290(6), 642-51.

Charrasse, S., Gharbi-Ayachi, A., Burgess, A., Vera, J., Hached, K., Raynaud, P., Schwob, E., Lorca, T., Castro, A., 2017. Ensa controls S-phase length by modulating Treslin levels. Nat. Commun. 8(1), 206.

Chen, N.N., Kerr, D., Chang, C.F., Honjo, T., Khalili, K., 1997. Evidence for regulation of transcription and replication of the human neurotropic virus JCV genome by the human S(mu)bp-2 protein in glial cells. Gene. 185(1), 55-62.

Chen, D., Zhong, Q., 2012. A tethering coherent protein in autophagosome maturation. Autophagy. 8(6), 985-6.

Chen, R.H., Chen, Y.H., Huang, T.Y., 2019. Ubiquitin-mediated regulation of autophagy. J. Biomed. Sci. 26(1), 80.

Chinopoulos, C., Seyfried, T.N., 2018. Mitochondrial Substrate-Level Phosphorylation as Energy Source for Glioblastoma: Review and Hypothesis. ASN Neuro. 10:1759091418818261.

Christie, A.E., Rus, S., Goiney, C.C., Smith, C.M., Towle, D.W., Dickinson, P.S., 2007. Identification and characterization of a cDNA encoding a crustin-like, putative antibacterial protein from the American lobster *Homarus americanus*. Mol. Immunol. 44(13), 3333-7.

Clark, K.F., Acorn, A.R., Greenwood, S.J., 2013. A transcriptomic analysis of American lobster (Homarus americanus) immune response during infection with the bumper car parasite *Anophryoides haemophila*. Dev. Comp. Immunol. 40(2), 112-22.

Claushuis, T.A.M., van der Donk, L.E.H., Luitse, A.L., van Veen, H.A., van der Wel, N.N., van Vught, L.A., Roelofs, J.J.T.H., de Boer, O.J., Lankelma, J.M., Boon, L., de Vos, A.F., van 't Veer, C., van der Poll, T., 2018. Role of peptidylarginine deiminase 4 in neutrophil extracellular trap formation and host defense during *Klebsiella pneumoniae*-induced pneumonia-derived sepsis. J. Immunol. 201(4), 1241-1252.

Coates, C.J., Nairn, J., 2014. Diverse immune functions of hemocyanins. Dev. Comp. Immunol. 45 (1), 43–55.

Colombo, M., Raposo, G., Théry, C., 2014. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell. Dev. Biol. 30, 255–289

Costa, N.A., Gut, A.L., Azevedo, P.S., Polegato, B.F., Magalhães, E.S., Ishikawa, L.L.W., Bruder, R.C.S., Silva, E.A.D., Gonçalves, R.B., Tanni, S.E., Rogero, M.M., Norde, M.M., Cunha, N.B., Zornoff, L.A.M., de Paiva, S.A.R, Minicucci, M.F., 2018. Peptidylarginine deiminase 4 concentration, but not PADI4 polymorphisms, is associated with ICU mortality in septic shock patients. J. Cell. Mol. Med. 22, 4732–4737.

Criscitiello, M.F., Kraev, I., Lange, S., 2019. Deiminated proteins in extracellular vesicles and plasma of nurse shark (*Ginglymostoma cirratum*) - Novel insights into shark immunity. Fish Shellfish Immunol. 92, 249-255.

Criscitiello, M.F., Kraev, I., Lange, S., 2020a. Deiminated proteins in extracellular vesicles and serum of llama (*Lama glama*)-Novel insights into camelid immunity. Mol. Immunol. 117, 37-53.

Criscitiello, M.F., Kraev, I., Petersen L.H., Lange, S., 2020b. Deimination Protein Profiles in *Alligator mississippiensis* Reveal Plasma and Extracellular Vesicle- specific Signatures Relating to Immunity, Metabolic Function and Gene Regulation. Front Immunol. doi: 10.3389/fimmu.2020.00651

Dacks, J.B., Dyal, P.L., Embley, T.M., van der Giezen, M., 2006. Hydrogenosomal succinyl-CoA synthetase from the rumen-dwelling fungus Neocallimastix patriciarum; an energy-producing enzyme of mitochondrial origin. Gene. 373, 75-82.

Dan, J, Liu, Y, Liu, N, Chiourea, M, Okuka, M, Wu, T, Ye, X, Mou, C, Wang, L, Wang, L, et al., 2014. Rif1 maintains telomere length homeostasis of ESCs by mediating heterochromatin silencing. Dev. Cell. 29(1), 7-19.

Darrah, E., Andrade, F., 2018. Rheumatoid arthritis and citrullination. Curr. Opin. Rheumatol. 30(1), 72-78.

Davidson, B.E., 1970. The identification of a reactive lysine residue in lobster glyceraldehyde-3-phosphate dehydrogenase. Eur. J. Biochem. 14(3), 545-8.

Ding, D., Enriquez-Algeciras, M., Bhattacharya, S.K., Bonilha, V.L., 2017. Protein deimination in aging and age-related diseases with ocular manifestations. In: Nicholas A., Bhattacharya S., Thompson P. (eds) Protein Deimination in Human Health and Disease. Springer, Cham.

El-Hattab, A.W., Scaglia, F., 2017. SUCLG1-Related Mitochondrial DNA Depletion Syndrome, Encephalomyopathic Form with Methylmalonic Aciduria. In: Adam, M.P., Ardinger, H.H., Pagon, R.A., Wallace, S.E., Bean, L.J.H., Stephens, K., Amemiya, A., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2020.

El Menif, E., Offret, C., Labrie, S., Beaulieu, L., 2019. Identification of Peptides Implicated in Antibacterial Activity of Snow Crab Hepatopancreas Hydrolysates by a Bioassay-Guided Fractionation Approach Combined with Mass Spectrometry. Probiotics Antimicrob. Proteins. 11(3), 1023-1033.

El-Sayed, A.S.A., Shindia, A.A., AbouZaid, A.A., Yassin, A.M., Ali, G.S., Sitohy, M.Z., 2019. Biochemical characterization of peptidylarginine deiminase-like orthologs from thermotolerant *Emericella dentata* and *Aspergillus nidulans*. Enzyme Microb. Technol. 124, 41-53.

Engel, D.W., Brouwer, M., Mercaldo-Allen, R., 2001. Effects of molting and environmental factors on trace metal body-burdens and hemocyanin concentrations in the American lobster, *Homarus americanus*. Mar. Environ. Res. 52(3), 257-69.

Enghild, J.J., Thøgersen, I.B., Salvesen, G., Fey, G.H., Figler, N.L., Gonias, S.L., Pizzo, S.V., 1990. Alpha-macroglobulin from *Limulus polyphemus* exhibits proteinase inhibitory activity and participates in a hemolytic system. Biochemistry. 29(43), 10070-80.

Fadini, G.P., Menegazzo, L., Rigato, M., Scattolini, V., Poncina, N., Bruttocao, A., Ciciliot, S., Mammano, F., Ciubotaru, C.D., Brocco, E., Marescotti, M.C., Cappellari, R., Arrigoni, G., Millioni, R., Vigili de Kreutzenberg, S., Albiero, M., Avogaro, A., 2016. NETosis Delays Diabetic Wound Healing in Mice and Humans. Diabetes. 65(4), 1061-71.

Faigle, W., Cruciani, C., Wolski, W., Roschitzki, B., Puthenparampil, M., Tomas-Ojer, P., Sellés-Moreno, C., Zeis, T., Jelcic, I., Schaeren-Wiemers, N., Sospedra, M., Martin, R., 2019. Brain citrullination patterns and T cell reactivity of cerebrospinal fluid-derived CD4+ T cells in multiple sclerosis. Front. Immunol. 10, 540.

Fert-Bober, J., Darrah, E., Andrade, F., 2020. Insights into the study and origin of the citrullinome in rheumatoid arthritis. Immunol. Rev. 294 (1), 133-147.

Fessler, J.H., Kramerova, I., Kramerov, A., Chen, Y., Fessler, L.I., 2004. Papilin, a novel component of basement membranes, in relation to ADAMTS metalloproteases and ECM development. Int. J. Biochem Cell Biol. 36(6), 1079-84.

Friedman E., 1995. The role of ras GTPase activating protein in human tumorigenesis. Pathobiology. 63(6), 348-50.

Fujita, Y., Tinoco, R., Li Y., Senft, D., Ronai, Z.A., 2019. Ubiquitin Ligases in Cancer Immunotherapy - Balancing Antitumor and Autoimmunity. Trends Mol. Med. 25(5), 428-443.

Gasparrini, F., Molfetta, R., Quatrini, L., Frati, L., Santoni, A., Paolini, R., 2012. Syk-dependent regulation of Hrs phosphorylation and ubiquitination upon FcεRI engagement: impact on Hrs membrane/cytosol localization. Eur. J. Immunol. 42(10), 2744-53.

Gavinho, B., Rossi, I.V., Evans-Osses, I., Lange, S., Ramirez, M.I., 2019. Peptidylarginine deiminase inhibition abolishes the production of large extracellular vesicles from *Giardia intestinalis*, affecting host-pathogen interactions by hindering adhesion to host cells. bioRxiv 586438. 10.1101/586438.

Gatien, J., Mermillod, P., Tsikis, G., Bernardi, O., Janati Idrissi, S., Uzbekov, R., Le Bourhis, D., Salvetti, P., Almiñana, C., Saint-Dizier, M., 2019. Metabolomic profile of oviductal extracellular vesicles across the estrous cycle in cattle. Int. J. Mol Sci. 20 (24), pii E6339.

Götze, S., Saborowski, R., Martínez-Cruz, O., Muhlia-Almazán, A., Sánchez-Paz, A., 2017. Proteasome properties of hemocytes differ between the whiteleg shrimp Penaeus vannamei and the brown shrimp Crangon crangon (Crustacea, Decapoda). Cell Stress Chaperones. 22(6), 879-891.

György, B., Toth, E., Tarcsa, E., Falus, A., Buzas, E.I., 2006. Citrullination: a posttranslational modification in health and disease. Int. J. Biochem. Cell. Biol. 38, 1662–77.

Gönczy, P., 2012. Towards a molecular architecture of centriole assembly. Nat. Rev. Mol. Cell Biol. 13(7), 425-35.

Gönczy, P., Hatzopoulos, G.N., 2019. Centriole assembly at a glance. J. Cell Sci.132(4), pii: jcs228833.

Hand, S.C., 1998. Quiescence in Artemia franciscana embryos: reversible arrest of metabolism and gene expression at low oxygen levels. J. Exp. Biol. 201(Pt 8), 1233-42

Harada, A.E., Healy, T.M., Burton, R.S., 2019. Variation in Thermal Tolerance and Its Relationship to Mitochondrial Function Across Populations of *Tigriopus californicus*. Front. Physiol. 10, 213.

Harzsch, S., Miller, J., Benton, J., Beltz, B., 1999. From embryo to adult: persistent neurogenesis and apoptotic cell death shape the lobster deutocerebrum. J. Neurosci. 19(9), 3472-85.

Hauton, C., Brockton, V., Smith, V.J., 2006. Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus*, and its response to infection with bacteria. Mol. Immunol. 43(9), 1490-6.

Henderson, B., Martin, A.C., 2014. Protein moonlighting: a new factor in biology and medicine. Biochem. Soc. Trans. 42(6), 1671-8.

Hessvik, N.P., Llorente, A., 2018. Current knowledge on exosome biogenesis and release. Cell. Mol. Life Sci. 75 (2), 193-208.

Hipolito, S.G., Shitara, A., Kondo, H., Hirono, I., 2014. Role of *Marsupenaeus japonicus* crustin-like peptide against *Vibrio penaeicida* and white spot syndrome virus infection. Dev. Comp. Immunol. 46(2), 461-9.

Holmes, J.M., Whiteley, N.M., Magnay, J.L., El Haj, A.J., 2002. Comparison of the variable loop regions of myosin heavy chain genes from Antarctic and temperate isopods. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 131(3), 349-59.

Holthuis, L.B., 1991. *Homarus americanus*. FAO Species Catalogue, Volume 13. Marine Lobsters of the World. FAO Fisheries Synopsis No. 125. Food and Agriculture Organization, p. 58. ISBN 92-5-103027-8.

Iliev, D., Strandskog, G., Nepal, A., Aspar, A., Olsen, R., Jørgensen, J., Wolfson, D., Ahluwalia, B.S., Handzhiyski, J., Mironova, R., 2018. Stimulation of exosome release by extracellular DNA is conserved across multiple cell types. FEBS J. 285 (16), 3114-3133.

Inal, J.M., Ansa-Addo, E.A., Lange, S., 2013. Interplay of host-pathogen microvesicles and their role in infectious disease. Biochem. Soc. Trans. 41 (1), 258-62.

Jafari, G., Burghoorn, J., Kawano, T., Mathew, M., Mörck, C., Axäng, C., Ailion, M., Thomas, J.H., Culotti, J.G., Swoboda, P., Pilon, M., 2010. Genetics of extracellular matrix remodeling during organ growth using the Caenorhabditis elegans pharynx model. Genetics. 186(3), 969-82.

James, M.O., 1989. Cytochrome P450 monooxygenases in crustaceans. Xenobiotica. 19(10), 1063-76.

Jeffrey, C.J., 2018. Protein moonlighting: what is it, and why is it important? Philos. Trans. R. Soc. Lond. B. Biol. Sci. 373 (1738), pii 20160523.

Jin, S., Jiang S., Xiong, Y., Qiao, H., Sun, S., Zhang, W., Li F., Gong, Y., Fu, H., 2014. Molecular cloning of two tropomyosin family genes and expression analysis during development in oriental river prawn, *Macrobrachium nipponense*. Gene.546(2), 390-7.

Jin, Q., Zhu, F., 2019. Differentially expressed genes of the shrimp *Marsupenaeus japonicus* in response to infection by white spot syndrome virus or *Vibrio alginolyticus*. Fish Shellfish Immunol. 92, 348-355.

Johnson, J.D., Mehus, J.G., Tews, K., Milavetz, B.I., Lambeth, D.O., 1998. Genetic evidence for the expression of ATP- and GTP-specific succinyl-CoA synthetases in multicellular eucaryotes. J. Biol. Chem. 273(42),27580-6.

Jolly, J.J., Dzulkiply, S.K., Yusof, M.A., Kamaruding, N.A., Ismail, N., 2019. Broad-spectrum Antimicrobial Activity of Purified Hemocyanin Subunit IIIA Isolated from Asian Horseshoe Crab, *Tachypleus gigas*. Pak J Biol Sci. 22 (9), 427-434.

Kawano, T., Zheng, H., Merz, D.C., Kohara, Y., Tamai, K.K., Nishiwaki, K., Culotti, J.G., 2009. *C. elegans* mig-6 encodes papilin isoforms that affect distinct aspects of DTC migration, and interacts genetically with mig-17 and collagen IV. Development. 136(9), 1433-42.

Kerr, D., Khalili, K., 1991. A recombinant cDNA derived from human brain encodes a DNA binding protein that stimulates transcription of the human neurotropic virus JCV. J. Biol. Chem. 266(24), 15876-81.

Kholia, S., Jorfi, S., Thompson, P.R., Causey, C.P., Nicholas, A.P., Inal, J.M., Lange, S., 2015. A novel role for peptidylarginine deiminases (PADs) in microvesicle release: A therapeutic potential for PAD inhibitors to sensitize prostate cancer cells to chemotherapy. J. Extracell. Vesicles. 4, 26192.

Kim, B.K., Kim, K.S., Oh, C.W., Mykles, D.L., Lee, S.G., Kim, H.J., Kim, H.W., 2009. Twelve actin-encoding cDNAs from the American lobster, *Homarus americanus*: cloning and tissue expression of eight skeletal muscle, one heart, and three cytoplasmic isoforms. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 153(2), 178-84.

Khacho, M., Mekhail, K., Pilon-Larose, K., Pause, A., Côté, J., Lee, S., 2008. eEF1A is a novel component of the mammalian nuclear protein export machinery. Mo.l Biol. Cell. 19(12), 5296-308

King, P.D., Lubeck, B.A., Lapinski, P.E.., 2013. Nonredundant functions for Ras GTPase-activating proteins in tissue homeostasis. Sci. Signal. 6(264), re1.

Klapper, W., Kühne, K., Singh, K.K., Heidorn, K., Parwaresch, R., Krupp, G., 1998. Longevity of lobsters is linked to ubiquitous telomerase expression. FEBS. Lett. 439(1-2), 143-6.

Klymasz-Swartz, A.K., Allen, G.J.P., Treberg, J.R., Yoon, G.R., Tripp, A., Quijada-Rodriguez, A.R., Weihrauch, D., 2019. Impact of climate change on the American lobster (*Homarus americanus*): Physiological responses to combined exposure of elevated temperature and pCO2. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 235, 202-210.

Kojima, K., Amano, Y., Yoshino, K., Tanaka, N., Sugamura, K., Takeshita, T., 2014. ESCRT-0 protein hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is targeted to endosomes independently of signal-transducing adaptor molecule (STAM) and the complex formation with STAM promotes its endosomal dissociation. J. Biol. Chem. 289(48), 33296-310.

Kosgodage, U.S., Trindade, R.P., Thompson, P.T., Inal, J.M., Lange, S., 2017. Chloramidine/Bisindolylmaleimide-I-mediated inhibition of exosome and microvesicle release and enhanced efficacy of cancer chemotherapy. Int J Mol Sci. 18(5), pii E1007.

Kosgodage, U.S., Uysal-Onganer, P., Maclatchy, A., Nicholas, A.P., Inal, J.M., Lange, S., 2018. Peptidylarginine deiminases post-translationally deiminate prohibitin and modulate extracellular vesicle release and miRNAs 21 and 126 in glioblastoma multiforme. Int. J. Mol. Sci. 20(1), pii E103.

Kosgodage, U.S., Matewele, P., Mastroianni, G., Kraev, I., Brotherton, D., Awamaria, B., Nicholas, A.P., Lange, S., Inal, J.M., 2019. Peptidylarginine Deiminase Inhibitors Reduce Bacterial Membrane Vesicle Release and Sensitize Bacteria to Antibiotic Treatment. Front. Cell. Infect. Microbiol. 9, 227.

Köhler, K., Sanchez-Pulido, L., Höfer, V., Marko, A., Ponting, C.P., Snijders, A.P., Feederle, R., Schepers, A., Boos, D., 2019. The Cdk8/19-cyclin C transcription regulator functions in genome replication through metazoan Sld7. PLoS Biol. 17(1), e2006767. d

Kramerova, I.A., Kawaguchi, N., Fessler, L.I., Nelson, R.E., Chen, Y., Kramerov, A.A., Kusche-Gullberg, M., Kramer, J.M., Ackley, B.D., Sieron, A.L., Prockop, D.J., Fessler, J.H., 2000. Papilin in development; a pericellular protein with a homology to the ADAMTS metalloproteinases. Development. 127(24), 5475-85.

Kramerova, I.A., Kramerov, A.A., Fessler, J.H., 2003. Alternative splicing of papilin and the diversity of *Drosophila* extracellular matrix during embryonic morphogenesis. Dev. Dyn. 226(4), 634-42.

Kumagai, A., Shevchenko, A., Shevchenko, A. & Dunphy, W. G., 2010. Treslin collaborates with TopBP1 in triggering the initiation of DNA replication. Cell. 140, 349.

Kusche, K., Burmester, T., 2001. Molecular cloning and evolution of lobster hemocyanin. Biochem. Biophys. Res Commun. 282(4), 887-92.

Lange, S., 2016. Peptidylarginine deiminases as drug targets in neonatal hypoxic-ischemic encephalopathy. Front. Neurol. 7, 22.

Lange, S., Gögel, S., Leung, K.Y., Vernay, B., Nicholas, A.P., Causey, C.P., Thompson, P.R., Greene, N.D., Ferretti, P., 2011. Protein deiminases: new players in the developmentally regulated loss of neural regenerative ability. Dev. Biol. 355(2), 205-14.

Lange, S., Rocha-Ferreira, E., Thei, L., Mawjee, P., Bennett, K., Thompson, P.R., Subramanian, V., Nicholas, A.P., Peebles, D., Hristova, M., Raivich, G., 2014. Peptidylarginine deiminases: novel drug targets for prevention of neuronal damage following hypoxic ischemic insult (HI) in neonates. J. Neurochem. 130 (4), 555-62.

Lange, S., Kraev,I., Magnadóttir, B., Dodds, A.W., 2019. Complement component C4-like protein in Atlantic cod (*Gadus morhua* L.) - Detection in ontogeny and identification of post-translational deimination in serum and extracellular vesicles. Dev. Comp. Immunol. 101, 103437.

Lange, S., Gallagher, M., Kholia, S., Kosgodage, U.S., Hristova, M., Hardy, J., Inal, J.M., 2017. Peptidylarginine deiminases-roles in cancer and neurodegeneration and possible avenues for therapeutic intervention via modulation of exosome and microvesicle (EMV) release? Int. J. Mol. Sci. 18(6), pii E1196.

Leu, J.H., Lin, S.J., Huang, J.Y., Chen, T.C., Lo, C.F., 2013. A model for apoptotic interaction between white spot syndrome virus and shrimp. Fish Shellfish Immunol. 34(4), 1011-7.

Li, Y., Jia, Z., Yi, Q., Song, X., Liu, Y., Jia, Y., Wang, L., Song, L., 2018. A novel GATA-like zinc finger transcription factor involving in hematopoiesis of *Eriocheir sinensis*. Fish Shellfish Immunol. 74, 363-371.

Liang, Y., Pan, B., Alam, H.B., Deng, Q., Wang, Y., Chen, E., Liu, B., Tian, Y., Williams, A.M., Duan, X., Wang, Y., Zhang, J., Li, Y., 2018. Inhibition of peptidylarginine deiminase alleviates LPS-induced pulmonary dysfunction and improves survival in a mouse model of lethal endotoxemia. Eur. J. Pharmacol. 833, 432-440.

Limudomporn, P., Moonsom, S., Leartsakulpanich, U., Suntornthiticharoen, P., Petmitr, S., Weinfeld, M., Chavalitshewinkoon-Petmitr, P., 2016. Characterization of *Plasmodium falciparum* ATP-dependent DNA helicase RuvB3. Malar. J. 15(1), 526.

Lin, C., Liu, M., Zhu, X., Zhang, M., Xu, S., Wang, D., Zhao, Y., 2019. Cloning and expression of the lifespan-associated protein Sir2 from *Daphnia pulex*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 231, 1-10.

Lincet, H., Icard, P., 2015. How do glycolytic enzymes favour cancer cell proliferation by nonmetabolic functions? Oncogene. 34 (29), 3751-9.

Louzon, M., Coeurdassier, M., Gimbert, F., Pauget, B., de Vaufleury, A., 2019. Telomere dynamic in humans and animals: Review and perspectives in environmental toxicology. Environ. Int. 131,105025.

Lu, A., Zhang, Q., Zhang, J., Yang, B., Wu, K., Xie, W., Luan, Y.X., Ling, E., 2014. Insect prophenoloxidase: the view beyond immunity. Front. Physiol. 5, 252.

Lu, Y., Yu, S.S., Zong, M., Fan, S.S., Lu, T.B., Gong, R.H., Sun, L.S., Fan, L.Y., 2017. Glucose-6-Phosphate Isomerase (G6PI) Mediates Hypoxia-Induced Angiogenesis in Rheumatoid Arthritis. Sci.Rep. 7, 40274.

MacLea, K.S., Abuhagr, A.M., Pitts, N.L., Covi, J.A., Bader, B.D., Chang, E.S., Mykles, D.L., 2012. Rheb, an activator of target of rapamycin, in the blackback land crab, Gecarcinus lateralis: cloning and effects of molting and unweighting on expression in skeletal muscle. J. Exp. Biol. 215(Pt 4), 590-604.

McBeath, A.J., Penston, M.J., Snow, M., Cook, P.F., Bricknell, I.R., Cunningham, C.O., 2006. Development and application of real-time PCR for specific detection of *Lepeophtheirus salmonis* and *Caligus elongatus* larvae in Scottish plankton samples. Dis. Aquat. Organ. 73(2), 141-50.

Magnadottir, B., Hayes, P., Hristova, M., Bragason, B.T., Nicholas, A.P., Dodds, A.W., Guðmundsdóttir, S., Lange, S., 2018a. Post-translational protein deimination in cod (*Gadus morhua* L.) ontogeny – novel roles in tissue remodelling and mucosal immune defences? Dev. Comp. Immunol. 87, 157-170.

Magnadottir, B., Hayes, P., Gísladóttir, B., Bragason, B.Þ., Hristova, M., Nicholas, A.P., Guðmundsdóttir, S., Lange, S., 2018b. Pentraxins CRP-I and CRP-II are post-translationally deiminated and differ in tissue specificity in cod (*Gadus morhua* L.) ontogeny. Dev. Comp. Immunol. 87, 1-11.

Magnadottir, B., Bragason, B.T., Bricknell, I.R., Bowden, T., Nicholas, A.P., Hristova, M., Guðmundsdóttir, S., Dodds, A.W., Lange, S., 2019a. Peptidylarginine deiminase and deiminated proteins are detected throughout early halibut ontogeny - complement components C3 and C4 are post-translationally deiminated in halibut (*Hippoglossus hippoglossus* L.). Dev. Comp. Immunol. 92, 1-19.

Magnadottir, B., Kraev, I., Guðmundsdóttir, S., Dodds, A.W., Lange, S., 2019b. Extracellular vesicles from cod (*Gadus morhua* L.) mucus contain innate immune factors and deiminated protein cargo. Dev. Comp. Immunol. 99, 103397.

Magnadottir, B., Uysal-Onganer, P., Kraev, I., Dodds, A.W., Gudmundsdottir, S., Lange, S, 2020a. Extracellular vesicles, deiminated protein cargo and microRNAs are novel serum biomarkers for environmental rearing temperature in Atlantic cod (*Gadus morhua* L.). Aquaculture Rep. 16, 100245.

Magnadottir, B, Uysal-Onganer, P, Kraev, I, Svansson, V, Hayes, P, Lange, S., 2020b. Deiminated proteins and extracellular vesicles - novel serum biomarkers in whales and orca. Comp. Biochem. Physiol. Part D Genomics Proteomics. 34, 100676.

Magnadottir, B., Uysal-Onganer, P., Kraev, I., Svansson, V., Skírnisson, K., Lange, S., 2020c. Deiminated proteins and extracellular vesicles as novel biomarkers in pinnipeds: Grey seal (*Halichoerus gryptus*) and harbour seal (*Phoca vitulina*).Biochimie. 171-172, 79-90.

Martinez-Prat, L., Palterer, B., Vitiello, G., Parronchi, P., Robinson, W.H., Mahler, M., 2019. Autoantibodies to protein-arginine deiminase (PAD) 4 in rheumatoid arthritis: immunological and clinical significance, and potential for precision medicine. Expert Rev. Clin. Immunol. 15 (10), 1073-1087.

Martin, W.F., Cerff, R., 2017. Physiology, phylogeny, early evolution, and GAPDH. Protoplasma. 254(5), 1823-1834. d

Maassen, J.A., Schop, E.N., Brands, J.H., van Hemert, F.J., Lenstra, J.A., Möller, W., 1985. Molecular cloning and analysis of cDNA sequences for two ribosomal proteins from Artemia. The coordinate expression of genes for ribosomal proteins and elongation factor 1 during embryogenesis of Artemia. Eur. J. Biochem. 149(3), 609-16.

Mastronardi, F.G., Wood, D.D., Mei, J., Raijmakers, R., Tseveleki, V., Dosch, H.M., Probert, L., Casaccia-Bonnefil, P., Moscarello, M.A., 2006. Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: a role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation. J. Neurosci. 26 (44), 11387-96.

Méchin, M.C., Takahara, H., Simon, M., 2020. Deimination and peptidylarginine deiminases in skin physiology and diseases. Int. J. Mol. Sci. 21 (2), pii E566.

Medler, S., Lilley, T., Mykles, D.L., 2004. Fiber polymorphism in skeletal muscles of the American lobster, *Homarus americanus*: continuum between slow-twitch (S1) and slow-tonic (S2) fibers. J. Exp. Biol. 207(Pt 16), 2755-67.

Miao, M., Chan, S.L., Fletcher, G.L., Hew, C.L., 2000. The rat ortholog of the presumptive flounder antifreeze enhancer-binding protein is a helicase domain-containing protein. Eur. J. Biochem. 267(24), 7237-46.

Miura, S, Mishina, Y., 2011. Hepatocyte growth factor-regulated tyrosine kinase substrate (Hgs) is involved in BMP signaling through phosphorylation of SMADS and TAK1 in early mouse embryo. Dev. Dyn. 240(11), 2474-81.

Mohanan, S., Cherrington, B.D., Horibata, S., McElwee, J.L., Thompson, P.R., Coonrod, S.A., 2012. Potential role of peptidylarginine deiminase enzymes and protein citrullination in cancer pathogenesis. Biochem. Res. Int. 2012, 895343.

Mondal, S., Thompson, P.R., 2019. Protein arginine deiminases (PADs): Biochemistry and chemical biology of protein citrullination. Acc. Chem. Res. 52 (3), 818-832.

Montaner-Tarbes, S., Pujol, M., Jabbar, T., Hawes, P., Chapman, D., Portillo, H.D., Fraile, L., Sánchez-Cordón, P.J., Dixon, L., Montoya, M., 2019. Serum-derived extracellular vesicles from African swine fever virus-infected pigs selectively recruit viral and porcine proteins. Viruses. 11 (10), pii E882.

Moscarello, M.A., Lei H., Mastronardi, F.G., Winer, S., Tsui, H., Li, Z., Ackerley, C., Zhang, L., Raijmakers, R., Wood, D.D., 2013. Inhibition of peptidyl-arginine deiminases reverses protein-hypercitrullination and disease in mouse models of multiple sclerosis. Dis. Model Mech. 6 (2), 467-78.

Mu, R., Tat, J., Zamudio, R., Zhang, Y., Yates, J.R. 3rd., Kumagai, A., Dunphy, W.G., Reed, S.I., 2017. CKS Proteins Promote Checkpoint Recovery by Stimulating Phosphorylation of Treslin. Mol. Cell Biol. 37(20). pii: e00344-17.

Muraro, S.P., De Souza, G.F., Gallo, S.W., Da Silva, B.K., De Oliveira, S.D., Vinolo, M.A.R., Saraiva, E.M., Porto, B.N., 2018. Respiratory Syncytial Virus induces the classical ROS-dependent NETosis through PAD-4 and necroptosis pathways activation. Sci. Rep. 8 (1), 14166.

Mueller, A.C., Keaton, M.A., Dutta, A., 2011. DNA replication: mammalian Treslin-TopBP1 interaction mirrors yeast Sld3-Dpb11. Curr. Biol. 21(16), R638-40. d

Nakamura N., 2018. Ubiquitin System. Int. J. Mol. Sci. 19(4). pii: E1080.

Nicholas, A.P., Whitaker, J.N., 2002. Preparation of a monoclonal antibody to citrullinated epitopes: its characterization and some applications to immunohistochemistry in human brain. Glia. 37(4), 328-36.

Nicholls, C., Li, H., Liu, J.P., 2012. GAPDH: a common enzyme with uncommon functions. Clin. Exp. Pharmacol. Physiol. 39(8), 674-9.

Nomura, K., 1992. Specificity and mode of action of the muscle-type protein-arginine deiminase. Arch. Biochem. Biophys. 293 (2), 362-9.

Novák, L., Zubáčová, Z., Karnkowska, A., Kolisko, M., Hroudová, M., Stairs, C.W., Simpson, A.G., Keeling, P.J., Roger, A.J., Čepička, I., Hampl, V., 2016. Arginine deiminase pathway enzymes: evolutionary history in metamonads and other eukaryotes. BMC Evol. Biol. 16(1), 197.

Igboeli, O.O., Burka, J.F., Fast, M.D. 2014. *Lepeophtheirus salmonis*: a persisting challenge for salmon aquaculture. Animal Frontiers 4(1), 22–32.

Ochs, A.T., Shields, J.D., Rice, G.W., Unger, M.A., 2020. Acute and long-term manganese exposure and subsequent accumulation in relation to idiopathic blindness in the American lobster, *Homarus americanus*. Aquat. Toxicol. 219, 105379. d

Ohtake, F, Tsuchiya, H., 2017. The emerging complexity of ubiquitin architecture. J. Biochem. 161(2), 125-133.

Ostergaard, E., 2008. Disorders caused by deficiency of succinate-CoA ligase. J. Inherit Metab Dis. 31(2), 226-9.

Pamenter, M.E., Uysal-Onganer, P., Huynh, K.W., Kraev, I., Lange, S., 2019. Post-translational deimination of immunological and metabolic protein markers in plasma and extracellular vesicles of naked mole-Rat (*Heterocephalus glaber*). Int. J Mol. Sci. 20 (21), pii E5378

Pan, B., Alam, H.B., Chong, W., Mobley, J., Liu, B., Deng, Q., Liang, Y., Wang, Y., Chen, E., Wang, T., Tewari, M., Li, Y., 2017. CitH3: a reliable blood biomarker for diagnosis and treatment of endotoxic shock. Sci. Rep. 7.(1), 8972.

Phillips, R.A., Kraev, I., Lange, S., 2020. Protein deimination and extracellular vesicle profiles in Antarctic seabirds. Biology (Basel). 9 (1), pii: E15.

Popovic, D., Vucic, D., Dikic, I., 2014. Ubiquitination in disease pathogenesis and treatment. Nat. Med. 20(11), 1242-53.

Postel, U., Thompson, F., Barker, G., Viney, M., Morris, S., 2010. Migration-related changes in gene expression in leg muscle of the Christmas Island red crab *Gecarcoidea natalis*: seasonal preparation for long-distance walking. J. Exp. Biol. 213(Pt 10), 1740-50.

Pott, A., Menze, M.A., Grieshaber, M.K., 2009. Thermodynamics of effector binding to hemocyanin: influence of temperature. Arch. Biochem. Biophys. 483(1), 37-44.

Prilusky, J., Felder, C.E., Zeev-Ben-Mordehai, T., Rydberg, E.H., Man, O., Beckmann, J.S., Silman, I, Sussman, J.L., 2005. FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics. 21 (16), 3435-8.

Raisuddin, S., Kwok, K.W., Leung, K.M., Schlenk, D., Lee, J.S., 2007. The copepod Tigriopus: a promising marine model organism for ecotoxicology and environmental genomics. Aquat. Toxicol. 83(3), 161-73.

Ramirez, S.H., Andrews, A.M., Paul, D., Pachter, J.S., 2018. Extracellular vesicles: mediators and biomarkers of pathology along CNS barriers. Fluids Barriers CNS. 15(1), 19.

Ramirez-Suarez, N.J., Belalcazar, H.M., Salazar, C.J., Beyaz, B., Raja, B., Nguyen, K.C.Q., Celestrin, K., Fredens, J., Færgeman, N.J., Hall, D.H., Bülow, H.E., 2019. Axon-Dependent Patterning and Maintenance of Somatosensory Dendritic Arbors. Dev. Cell. 48(2), 229-244.e4.

Rayala, S.K., Hollander, Pd., Balasenthil, S., Molli, P.R., Bean, A.J., Vadlamudi, R.K., Wang, R.A., Kumar, R., 2006. Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) interacts with PELP1 and activates MAPK. J. Biol Chem. 281(7), 4395-403.

Rebl, A, Köllner, B, Anders, E, Wimmers, K, Goldammer, T., 2010. Peptidylarginine deiminase gene is differentially expressed in freshwater and brackish water rainbow trout. Mol. Biol. Rep. 37 (5), 2333-9.

Ruiz-Romero, C., Lam, M.P.Y., Nilsson, P., Önnerfjord, P., Utz, P.J., Van Eyk, J.E., Venkatraman, V., Fert-Bober, J., Watt, F.E., Blanco, F.J., 2019. Mining the Proteome Associated with Rheumatic and Autoimmune Diseases. J Proteome Res. 18(12), 4231-4239.

Rupp, U., Ziegler, A., 2019. The effect of exuviae ingestion on lysosomal calcium accumulation and the presence of exosomes in the hepatopancreas of *Porcellio scaber*. J. Struct. Biol. 208(3), 107392.

Sagawa, K., Yamagata, H., Shiga, Y., 2005. Exploring embryonic germ line development in the water flea, *Daphnia magna*, by zinc-finger-containing VASA as a marker. Gene Expr. Patterns. 5(5), 669-78.

Saha, P., Yeoh, B.S., Xiao, X., Golonka, R.M., Singh, V., Wang, Y., Vijay-Kumar, M., 2019. PAD4-dependent NETs generation are indispensable for intestinal clearance of *Citrobacter rodentium*. Mucosal Immunol. 12 (3), 761-771.

Saleh, M., Kumar, G., Abdel-Baki, A.A., Dkhil, M.A., El-Matbouli, M., Al-Quraishy, S., 2018. Quantitative shotgun proteomics distinguishes wound-healing biomarker signatures in common carp skin mucus in response to *Ichthyophthirius multifiliis*. Vet. Res. 49(1), 37.

Sancandi, M., Uysal-Onganer, P., Kraev, I., Mercer, A., Lange, S., 2020. Protein Deimination Signatures in Plasma and Plasma-EVs and Protein Deimination in the Brain Vasculature in a Rat Model of Pre-Motor Parkinson's Disease. Int. J. Mol Sci. 21 (8), 2743.

Sansam, C. G., Goins, D., Siefert, J. C., Clowdus, E. A. & Sansam, C. L., 2015. Cyclin-dependent kinase regulates the length of S phase through TICRR/TRESLIN phosphorylation. Genes Dev. 29, 555.

Sansam, C.G., Pietrzak, K., Majchrzycka, B., Kerlin, M.A., Chen, J., Rankin, S., Sansam, C.L., 2018. A mechanism for epigenetic control of DNA replication. Genes Dev. 32(3-4), 224-229.

Saunders, N.R., Noor, N.M., Dziegielewska, K.M., Wheaton, B.J., Liddelow, S.A., Steer, D.L., Ek, C.J., Habgood, M.D., Wakefield, M.J., Lindsay, H., Truettner, J., Miller, R.D., Smith, A.I., Dietrich, W.D., 2014. Age-dependent transcriptome and proteome following transection of neonatal spinal cord of *Monodelphis domestica* (South American grey short-tailed opossum). PLoS One. 2014 Jun 10;9(6):e99080.

Scheffler, M.L., Barreto, F.S., Mueller, C.A., 2019. Rapid metabolic compensation in response to temperature change in the intertidal copepod, *Tigriopus californicus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 230, 131-137.

Scheffzek, K., Shivalingaiah, G., 2019. Ras-Specific GTPase-Activating Proteins-Structures, Mechanisms, and Interactions. Cold Spring Harb. Perspect .Med. 9(3). pii: a031500.

Seetharam, A., Bai, Y., Stuart, G.W., 2010. A survey of well conserved families of C2H2 zinc-finger genes in Daphnia. BMC Genomics. 11, 276.

Sharma, M., Damgaard, D., Senolt, L., Svensson, B., Bay-Jensen, A.C., Nielsen, C.H., Hägglund, P., 2019. Expanding the citrullinome of synovial fibrinogen from rheumatoid arthritis patients. J. Proteomics. 208, 103484.

Shekar, M., Venugopal, M.N., 2019. Identification and characterization of novel double zinc fingers encoded by putative proteins in genome of white spot syndrome virus. Arch. Virol. 164(4), 961-969.

Šimundić, M., Švara, T., Štukelj, R., Krek, J.L., Gombač, M., Kralj-Iglič, V., Tozon, N., 2019. Concentration of extracellular vesicles isolated from blood relative to the clinical pathological status of dogs with mast cell tumours. Vet. Comp. Oncol. 17 (4), 456-464.

Smith, V.J., Dyrynda, E.A., 2015. Antimicrobial proteins: From old proteins, new tricks. Mol. Immunol. 68(2 Pt B), 383-98.

Söderhäll, K., Cerenius, L., Johansson, M.W., 1994. The prophenoloxidase activating system and its role in invertebrate defence. Ann. NY Acad. Sci. 712, 155-61.

Söderhäll, K., Cerenius, L., 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. 10(1), 23-8.

Solé, M., Livingstone, D.R., 2005. Components of the cytochrome P450-dependent monooxygenase system and 'NADPH-independent benzo[a]pyrene hydroxylase' activity in a wide range of marine invertebrate species. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141(1), 20-31.

Sottrup-Jensen, L., Borth, W., Hall, M., Quigley, J.P., Armstrong, P.B., 1990. Sequence similarity between alpha 2-macroglobulin from the horseshoe crab, *Limulus polyphemus*, and proteins of the alpha 2-macroglobulin family from mammals. Comp. Biochem. Physiol. B. 96 (3), 621-5.

Souslova, T., Marple, T.C., Spiekerman, A.M., Mohammad, A.A., 2013. Personalized medicine in Alzheimer's disease and depression. Contemp. Clin. Trials. 36(2), 616-23. d

Spees, J.L., Chang, S.A., Mykles, D.L., Snyder, M.J., Chang, E.S., 2003. Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster. Cell Stress Chaperones. 8(3), 258-64.

Stobernack, T., du Teil Espina, M., Mulder, L.M., Palma Medina, L.M., Piebenga, D.R. et al., 2018. A secreted bacterial peptidylarginine deiminase can neutralize human innate immune defenses. mBio. 9 (5), pii, e01704-18.

Stöcker, W., Breit, S., Sottrup-Jensen, L., Zwilling, R., 1991. alpha2-Macroglobulin from hemolymph of the freshwater crayfish *Astacus astacus*. *Comp Biochem Physiol B*. 98(4), 501-9.

Szabó-Taylor, K., Ryan, B., Osteikoetxea, X., Szabó, T.G., Sódar, B., Holub, M., Németh, A., Pálóczi, K., Pállinger, É., Winyard, P., Buzás, E.I., 2015. Oxidative and other posttranslational modifications in extracellular vesicle biology. Semin. Cell Dev. Biol. 40, 8-16.

Sreesankar, E., Senthilkumar, R., Bharathi, V., Mishra, R.K., Mishra, K., 2012. Functional diversification of yeast telomere associated protein, Rif1, in higher eukaryotes. BMC Genomics. 13, 255.

Sun, B., Wang, Z., Zhu, F., 2017a. The crustin-like peptide plays opposite role in shrimp immune response to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection. Fish Shellfish Immunol. 66, 487-496.

Sun, Y.X., Tang, L., Gao, J., Feng, Y.Y., Peng, T., Yu, Y.Y., Yang, L.L., Sun, Y., Zhu, B.J., 2017b. A role of tumor susceptibility gene 101 (TSG101) in innate immune response of crayfish *Procambarus clarkii*. Dev. Comp. Immunol. 76, 268-273.

Svärd, A., Renvert, S., Sanmartin Berglund, J., Persson, R.G., Söderlin, M., 2019. Antibodies to citrullinated peptides in serum and saliva in patients with rheumatoid arthritis and their association to periodontitis. Clin. Exp. Rheumatol. 2019 Nov 16. [Epub ahead of print]

Swarnakar, S., Asokan, R., Quigley, J.P., Armstrong, P.B., 2000. Binding of alpha2-macroglobulin and limulin: regulation of the plasma haemolytic system of the American horseshoe crab, Limulus. Biochem J. 347 (3), 679-85.

Talapatra, S., Wagner, J.D., Thompson, C.B., 2020. Elongation factor-1 alpha is a selective regulator of growth factor withdrawal and ER stress-induced apoptosis. Cell Death Differ. 9(8), 856-61.

Tarcsa, E., Marekov, L.N., Mei, G., Melino, G., Lee, S.C., Steinert, P.M., 1996 Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. J. Biol. Chem. 271 (48), 30709-16.

Tassanakajon, A., Rimphanitchayakit, V., Visetnan, S., Amparyup, P., Somboonwiwat, K., Charoensapsri, W., Tang, S., 2018. Shrimp humoral responses against pathogens: antimicrobial peptides and melanization. Dev. Comp. Immunol. 80, 81-93.

Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., et al., 2018. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles. 7, 1535750.

Tilvawala, R., Nguyen, S.H., Maurais, A.J., Nemmara, V.V., Nagar, M., Salinger, A.J., Nagpal, S., Weerapana, E., Thompson, P.R, 2018. The Rheumatoid Arthritis-Associated Citrullinome. Cell. Chem. Biol. 25 (6), 691-704.e6.

Traini, M., Quinn, C.M., Sandoval, C., Johansson, E., Schroder, K., Kockx, M., Meikle, P.J., Jessup, W., Kritharides, L., 2014. Sphingomyelin phosphodiesterase acid-like 3A (SMPDL3A) is a novel nucleotide phosphodiesterase regulated by cholesterol in human macrophages. J. Biol. Chem. 289(47), 32895-913.

Traini, M., Kumaran, R., Thaysen-Andersen, M., Kockx, M., Jessup, W., Kritharides, L., 2017. N-glycosylation of human sphingomyelin phosphodiesterase acid-like 3A (SMPDL3A) is essential for stability, secretion and activity. Biochem. J. 474(7), 1071-1092.

Turchinovich, A., Drapkina, O., Tonevitsky, A., 2019. Transcriptome of extracellular vesicles: State-of-the-art. Front. Immunol. 10, 202.

Uchiumi, F., Komuro, M., Mizuta, R., Tanuma, S., 2004. Characterization of Smubp-2 as a mouse mammary tumor virus promoter-binding protein. Biochem. Biophys. Res. Commun. 321(2), 355-63.

Uchiumi, F., Enokida, K., Shiraishi, T., Masumi, A., Tanuma, S., 2010. Characterization of the promoter region of the human IGHMBP2 (Smubp-2) gene and its response to TPA in HL-60 cells. Gene. 463(1-2), 8-17.

Uversky, V.N., Gillespie, J.R., Fink, A.L., 2000. Why are "natively unfolded" proteins unstructured under physiologic conditions? Proteins. 41 (3), 415-27.

Uysal-Onganer, P., MacLatchy, A., Mahmoud, R., Kraev, I., Thompson, P.R., Inal, J., Lange, S., 2020. Peptidylarginine deiminase isozyme-specific PAD2, PAD3 and PAD4 inhibitors differentially modulate extracellular vesicle signatures and cell invasion in two glioblastoma multiforme cell lines. Int. J. Mol. Sci. 21 (4), pii E1495.

Vagner, T., Chin, A., Mariscal, J., Bannykh, S., Engman, D.M., Di Vizio, D., 2019. Protein composition reflects extracellular vesicle heterogeneity. Proteomics. 19 (8), e1800167.

Vera, M., Pani, B., Griffiths, L.A., Muchardt, C., Abbott, C.M., Singer, R.H., Nudler, E., 2014. The translation elongation factor eEF1A1 couples transcription to translation during heat shock response. Elife. 3, e03164.

Vilette, D., Laulagnier, K., Huor, A., Alais, S., Simoes, S., Maryse, R., Provansal, M., Lehmann, S., Andreoletti, O., Schaeffer, L., Raposo, G., Leblanc, P., 2015. Efficient inhibition of infectious prions multiplication and release by targeting the exosomal pathway. Cell. Mol. Life Sci. 72(22), 4409-27.

Vossenaar, E.R., Zendman, A.J., van Venrooij, W.J., Pruijn, G.J., 2003. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays. 25 (11), 1106-18.

Wang, L., Liu, Y., Wang, W.N., Mai, W.J., Xin, Y., Zhou, J., He, W.Y., Wang, A.L., Sun, R.Y., 2011. Molecular characterization and expression analysis of elongation factors 1A and 2 from the Pacific white shrimp, *Litopenaeus vannamei*. Mol. Biol. Rep. 38(3), 2167-78.

Wang, S., Wang, Y., 2013. Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. Biochim. Biophys. Acta. 1829 (10), 1126-35.

Witalison, E.E., Thompson, P.R., Hofseth, L.J., 2015. Protein arginine deiminases and associated citrullination: physiological functions and diseases associated with dysregulation. Curr. Drug Targets. 16(7), 700-10.

Wong, S.L., Wagner, D.D., 2018. Peptidylarginine deiminase 4: a nuclear button triggering neutrophil extracellular traps in inflammatory diseases and aging. FASEB. J., fj201800691R.

Wong, S.L., Demers, M., Martinod, K., Gallant, M., Wang, Y., Goldfine, A.B., Kahn, C.R., Wagner, D.D., 2015. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. Nat. Med. 21 (7), 815-9.

Yang, L., Tan, D., Piao, H., 2016. Myelin Basic Protein Citrullination in Multiple Sclerosis: A potential therapeutic target for the pathology. Neurochem, Res. 41 (8), 1845-56.

Yang, H., Li, X., Ji, J., Yuan, C., Gao, X., Zhang, Y., Lu, C., Li, F., Zhang, X., 2019. Changes of microRNAs expression profiles from red swamp crayfish (*Procambarus clarkia*) hemolymph exosomes in response to WSSV infection. Fish Shellfish Immunol. 84, 169-177.

Yu, T.Y., Wang, C.Y., Lin, J.J., 2012. Depleting components of the THO complex causes increased telomere length by reducing the expression of the telomere-associated protein Rif1p. PLoS One. 7(3), e33498.

Yu, Z., Zeng, J., Wang, J., Cui, Y., Song, X., Zhang, Y., Cheng, X., Hou, N., Teng, Y., Lan, Y., Chen, Y., Yang, X., 2020. Hepatocyte growth factor-regulated tyrosine kinase substrate is essential for endothelial cell polarity and cerebrovascular stability. Cardiovasc. Res. Feb 11. pii: cvaa016.

Zanger, U.M., Schwab, M., 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol. Ther. 138(1), 103-41.

Zhang, Q., Wang, Y.C., Montalvo, E.A., 1999. Smubp-2 represses the Epstein-Barr virus lytic switch promoter. Virology. 255(1), 160-70.

Zhang, H.K., Chen, J., 2015. Research Advances in the Role of RAS-GTPase-activating Proteins in Tumors. Zhongguo Yi Xue Ke Xue Yuan Xue Bao. 37(3), 364-9.

Zeeshan, H.M., Lee, G.H., Kim, H.R., Chae, H.J., 2016. Endoplasmic Reticulum Stress and Associated ROS. Int. J. Mol. Sci. 17(3), 327.

Zheng, Q., Huang, T., Zhang, L., Zhou, Y., Luo, H., Xu, H., Wang, X., 2016. Dysregulation of ubiquitin-proteasome system in neurodegenerative diseases. Front. Aging Neurosci. 8, 303.

Zuo, H., Yang, L., Zheng, J., Su, Z., Weng, S., He, J., Xu, X., 2018. A single C4 Zinc finger-containing protein from *Litopenaeus vannamei* involved in antibacterial responses. Fish Shellfish Immunol. 81, 493-501.

Figure legends

Figure 1. Extracellular vesicle profiling in lobster haemolymph. A. NTA (Nanoparticle tracking analysis) showing EV size profile from lobster haemolymph showing main peaks at approximately 56, 115 and 172 nm and the majority of EVs in the range of 22-115 nm. **B.** Western blotting analysis shows strongly CD63-positive for lobster haemolymph-EVs, while Flot-1 detection is low, confirming presence of phylogenetically conserved EV-specific markers, and dominant for exosomes (small EVs). **C.** Lobser haemolymph EVs as visualised by transmission electron microscopy (TEM), confirming majority of small EVs; scale bar is 20 nm.

Figure 2. PAD and deiminated proteins in lobster haemolymph. **A.** A lobster PAD homologue, detected via crossreaction with anti-human PAD2 antibody, was identified in lobster haemolymph at the expected size of approx. 70 kDa size, indicative of conserved homologous protein. **B.** F95 positive proteins in lobster haemolymph were dominated by a 50-75 kDa band, when using western blotting and the pan-deimination/citrullination specific F95 antibody. **C.** Silverstaining of F95-enriched protein fractions from lobster haemolymph show protein bands in the size range of 10-250 kDa and these were further analysed by LC-MS/MS (technical replicates of pools of n=3 animals).

Figure 3. F95-enriched (deiminated) protein hits identified in lobster haemolymph. The Venn diagram shows lobster (*Homarus americanus*) specific protein hits, as well as protein hits with other Crustacean, for F95 (deiminated/citrullinated)-enriched proteins, as analysed by LC-MS/MS.

Figure 4. STRING protein-protein interaction networks for F95-enriched protein hits identified in lobster haemolymph – based on Arthropoda (*Drosophila melanogaster*). Protein-protein interaction networks were built for identified deiminated protein hits in lobster haemolymph, based on known and predicted interactions, using *Drosophila* homologue identifiers for STRING analysis. **A. KEGG pathways** relating to the identified deiminated proteins (see colour code for identified pathways highlighted in the figure). **B. INTERPRO protein domains** relating to the identified proteins are highlighted (see colour code included in the figure). **C. GO cellular component pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **D. Reactome pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **E. UniProt keywords** relating to the identified proteins are highlighted (see colour code included in the figure). **F. PFAM protein domains** relating to the identified proteins are highlighted (see colour code included in the figure). **G-L. Biological GO processes** relating to the identified proteins are highlighted; see colour code included in each of figures G-L to highlight the different GO biological

processes. Coloured lines indicate whether protein interactions are identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighbourhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology (see the colour key for connective lines included in the figure); PPI enrichment *p*-value = 0.0033.

Figure 5. STRING protein-protein interaction networks for F95-enriched protein hits identified in lobster haemolymph – based on mammalian homologues (*Mus musculus*). Protein-protein interaction networks were built for identified deiminated protein hits in lobster haemolymph, based on known and predicted interactions, using mouse homologue identifiers for STRING analysis. **A. KEGG pathways** relating to the identified deiminated proteins (see colour code for identified pathways highlighted in the figure). **B. INTERPRO protein domains and features** relating to the identified proteins are highlighted (see colour code included in the figure). **C. Reactome pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **D. UniProt keywords** relating to the identified proteins are highlighted (see colour code included in the figure). **E. PFAM protein domains** relating to the identified proteins are highlighted (see colour code included in the figure). **F-H. GO molecular function pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **I-L. GO cellular component pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **M-V. Biological GO processes** relating to the identified proteins are highlighted (see colour code included in the figure). Coloured lines indicate whether protein interactions are identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighbourhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology (see the colour key for connective lines included in the figure); PPI enrichment *p*-value = 0.00219.

Fig.1

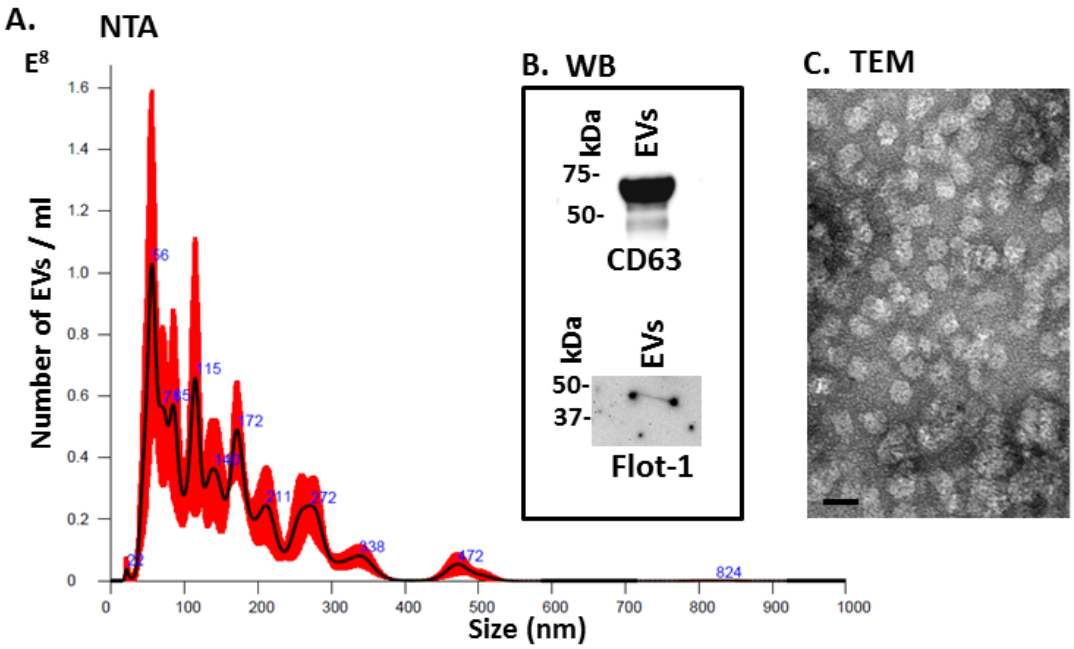

A.
NTA
E8
Number of EVs / ml
Size (nm)
B. WB
kDa
EVs
75-
50-
CD63
kDa
EVs
50-
37-
Flot-1
C. TEM

Fig.2

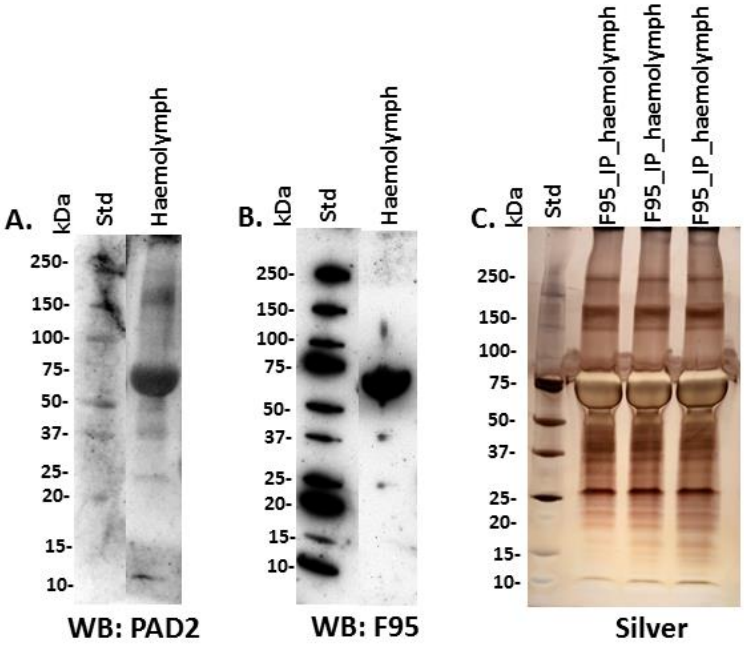

A.
kDa
Std
Haemolymph
250-
150-
100-
75-
50-
37-
25-
20-
15-
10-
WB: PAD2
B.
kDa
Std
Haemolymph
WB: F95
C.
kDa
Std
F95_IP_haemolymph
F95_IP_haemolymph
F95_IP_haemolymph
Silver

Fig.3

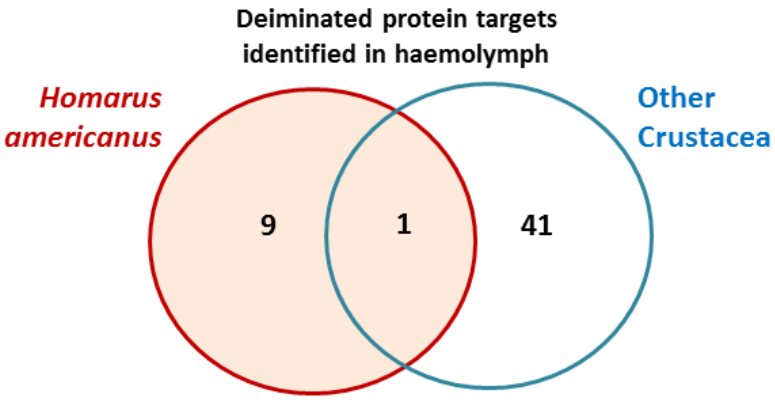
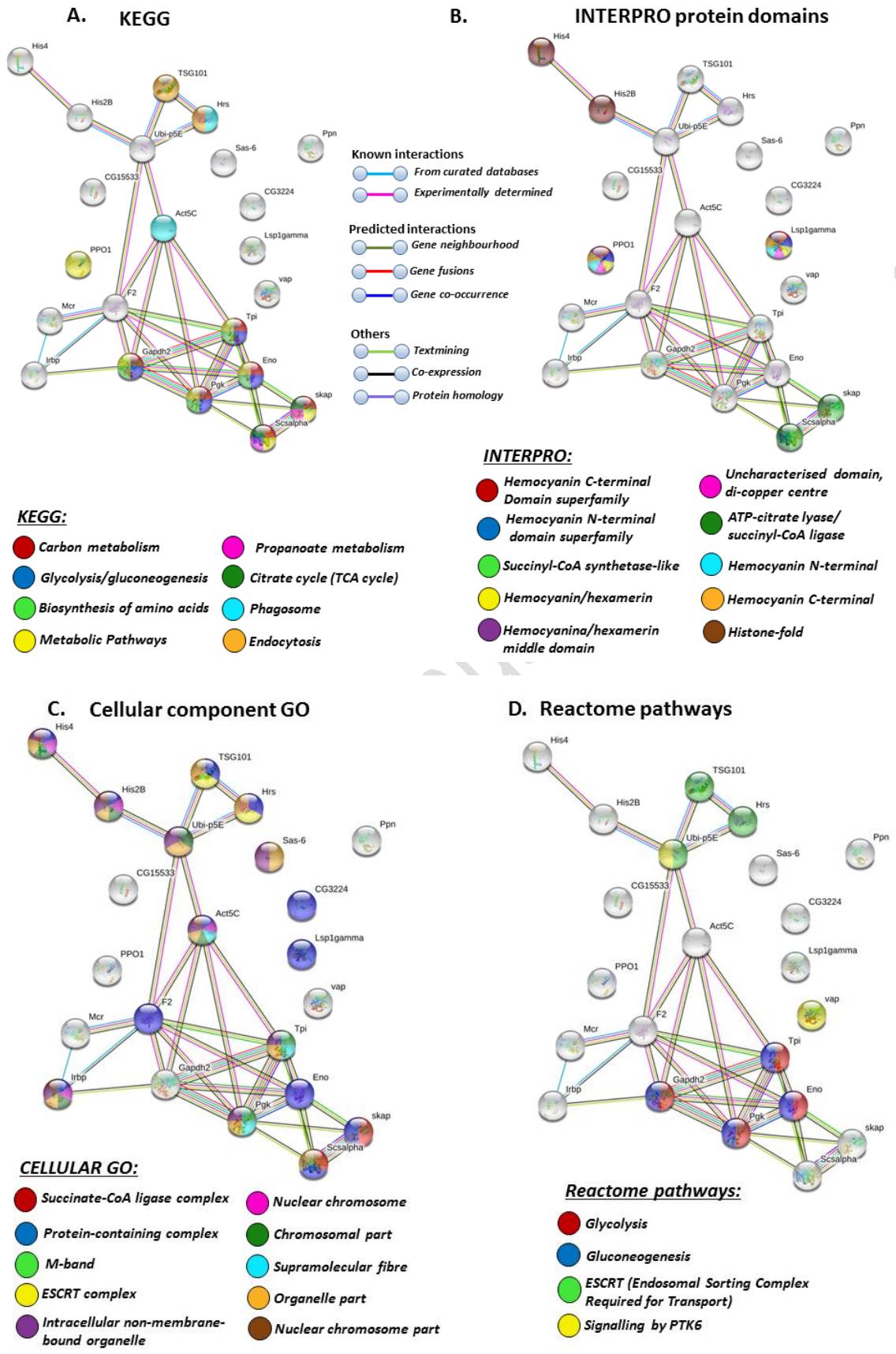
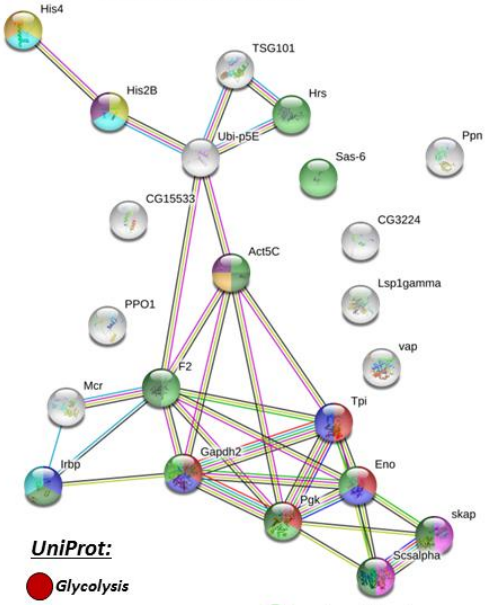


Fig. 4


A. KEGG
B. INTERPRO protein domains
His4
TSG101
His2B
Hrs
Ubi-p5E
Ppn
Sas-6
CG15533
CG3224
Act5C
Lsp1gamma
PPO1
vap
F2
Mcr
Tpi
Irbp
Gapdh2
Eno
Pgk
skap
Scsalpha
Known interactions
From curated databases
Experimentally determined
Predicted interactions
Gene neighbourhood
Gene fusions
Gene co-occurrence
Others
Textmining
Co-expression
Protein homology
KEGG:
Carbon metabolism
Glycolysis/gluconeogenesis
Biosynthesis of amino acids
Metabolic Pathways
Propanoate metabolism
Citrate cycle (TCA cycle)
Phagosome
Endocytosis
INTERPRO:
Hemocyanin C-terminal Domain superfamily
Hemocyanin N-terminal domain superfamily
Succinyl-CoA synthetase-like
Hemocyanin/hexamerin
Hemocyanina/hexamerin middle domain
Uncharacterised domain, di-copper centre
ATP-citrate lyase/ succinyl-CoA ligase
Hemocyanin N-terminal
Hemocyanin C-terminal
Histone-fold
C. Cellular component GO
D. Reactome pathways
CELLULAR GO:
Succinate-CoA ligase complex
Protein-containing complex
M-band
ESCRT complex
Intracellular non-membrane-bound organelle
Nuclear chromosome
Chromosomal part
Supramolecular fibre
Organelle part
Nuclear chromosome part
Reactome pathways:
Glycolysis
Gluconeogenesis
ESCRT (Endosomal Sorting Complex Required for Transport)
Signalling by PTK6

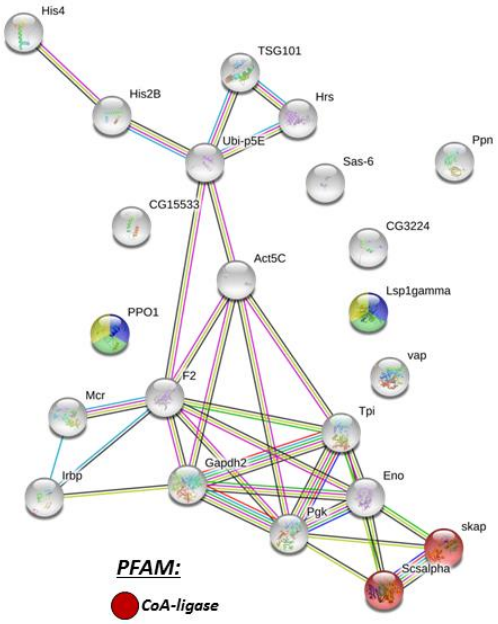
E. UniProt keywords



UniProt:

- *Glycolysis*
- *Polymorphism*
- *Cytoplasm*
- *Nucleosome core*
- *Tricarboxylic acid cycle*
- *Nucleotide binding*
- *Chromosome*
- *Acetylation*
- *Methylation*

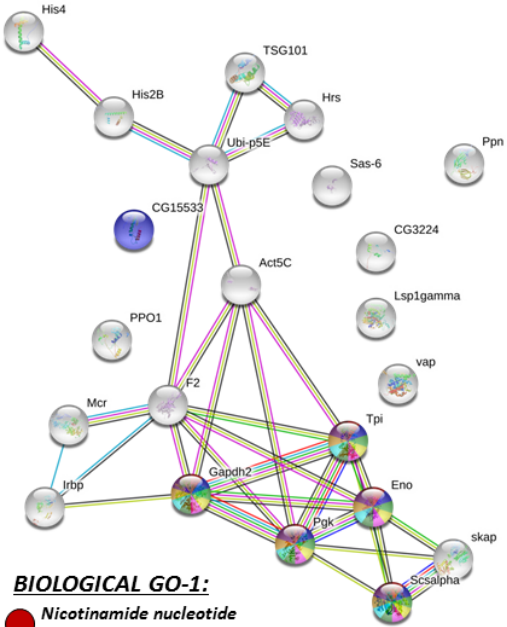
F. PFAM protein domains



PFAM:

- *CoA-ligase*
- *Hemocyanin, Ig-like domain*
- *Hemocyanin, all-alpha domain*
- *Hemocyanin, copper containing domain*

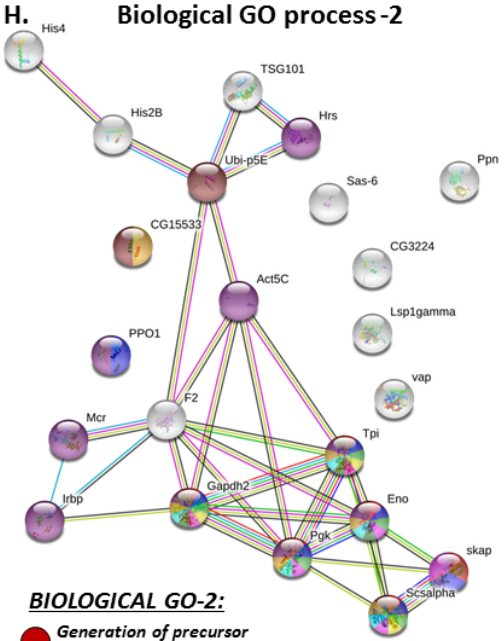
G. Biological GO process -1



BIOLOGICAL GO-1:

- *Nicotinamide nucleotide metabolic process*
- *Organophosphate catabolic process*
- *Pyruvate biosynthetic process*
- *Nicotinamide nucleotide biosynthetic process*
- *ATP biosynthetic process*
- *Nucleotide catabolic process*
- *ATP generation from ADP*
- *Glycolytic process*
- *Pyruvate metabolic process*
- *Purine ribonucleoside monophosphate biosynthetic process*

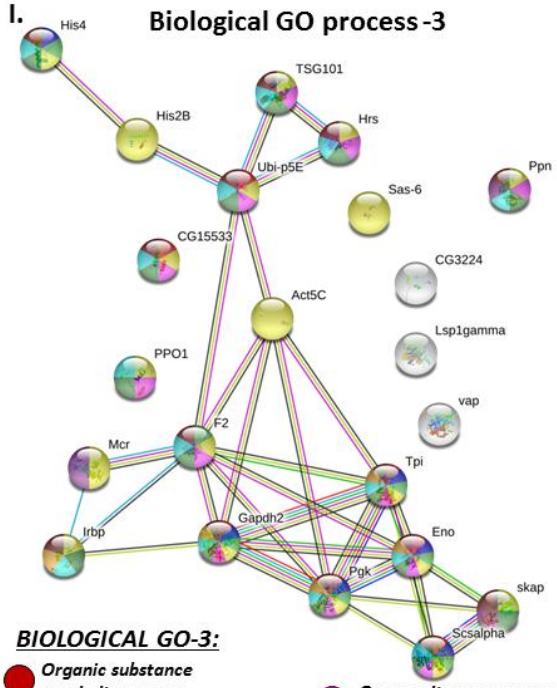
H. Biological GO process -2



BIOLOGICAL GO-2:

- *Generation of precursor metabolites and energy*
- *Drug metabolic process*
- *Purine ribonucleoside mono-phosphate metabolic process*
- *Glucose metabolic process*
- *Regulation of biological quality*
- *Carboxylic acid metabolic process*
- *Glucose homeostasis*
- *Carbohydrate metabolic process*
- *Organophosphate metabolic process*
- *Cellular catabolic process*

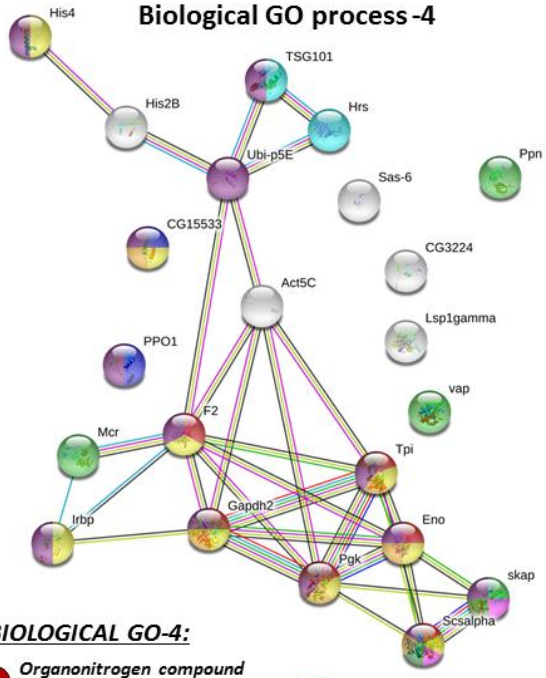
I. **Biological GO process -3**



BIOLOGICAL GO-3:

- *Organic substance catabolic process*
- *Nucleobase-containing compound biosynthetic process*
- *Cellular nitrogen compound biosynthetic process*
- *Cellular process*
- *Regulation of endo-peptidase activity*
- *Organonitrogen compound metabolic process*
- *Organic substance metabolic process*
- *Nitrogen compound metabolic process*
- *Homeostatic process*
- *Primary metabolic process*

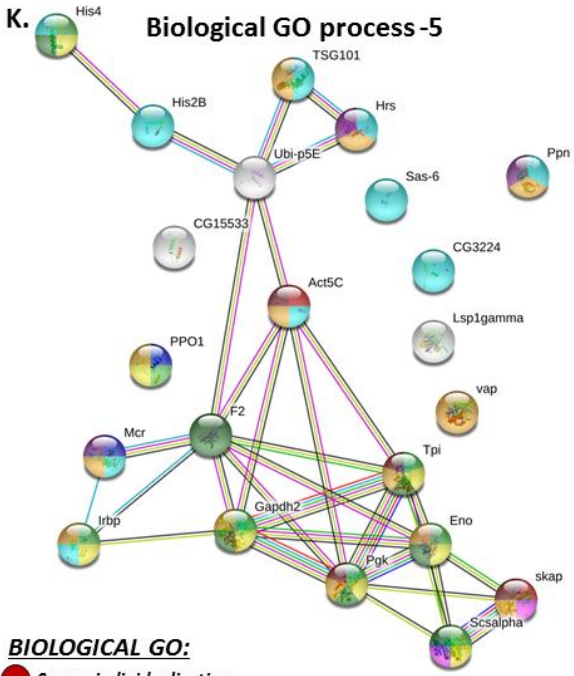
J. **Biological GO process -4**



BIOLOGICAL GO-4:

- *Organonitrogen compound biosynthetic process*
- *Ammonium ion metabolic process*
- *Regulation of hydrolase activity*
- *Cellular nitrogen compound metabolic process*
- *Cellular metabolic process*
- *Tricarboxylic acid cycle*
- *Citrate metabolic process*
- *Neurone remodelling*
- *Phosphate-containing comp. metabolic process*
- *Myoblast fusion*

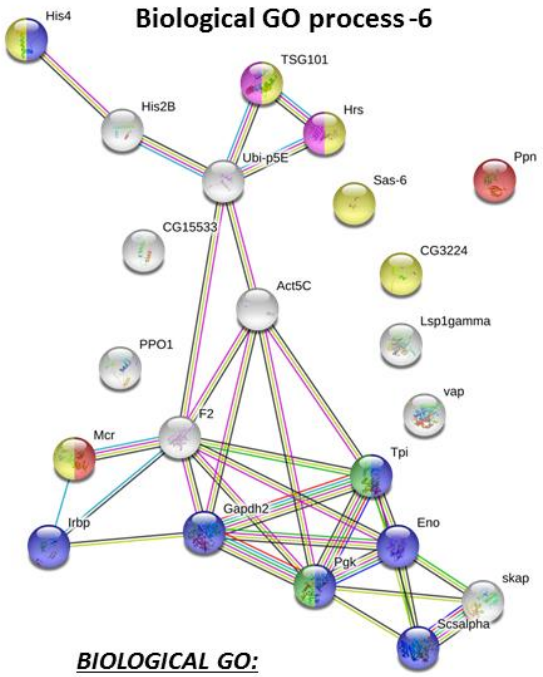
K. **Biological GO process -5**



BIOLOGICAL GO:

- *Sperm individualisation*
- *Defence response to fungus*
- *Cellular aromatic compound metabolic process*
- *Organic cyclic compound metabolic process*
- *Negative regulation of cellular protein metabolic process*
- *Antibiotic metabolic process*
- *Organic substrate biosynthetic process*
- *Cellular component organisation or biogenesis*
- *Biological regulation*
- *Anatomical structure formation involved in morphogenesis*

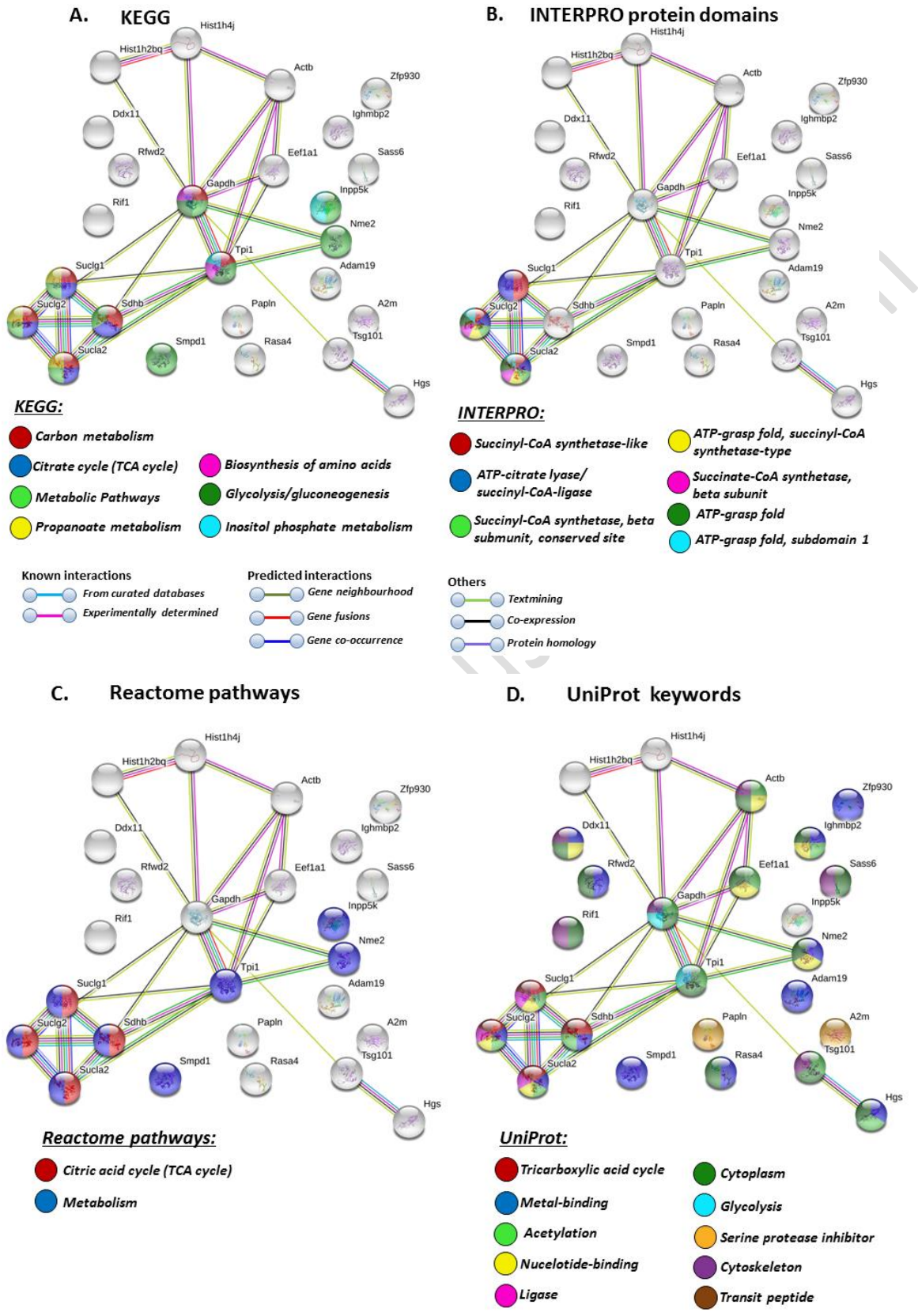
L. **Biological GO process -6**



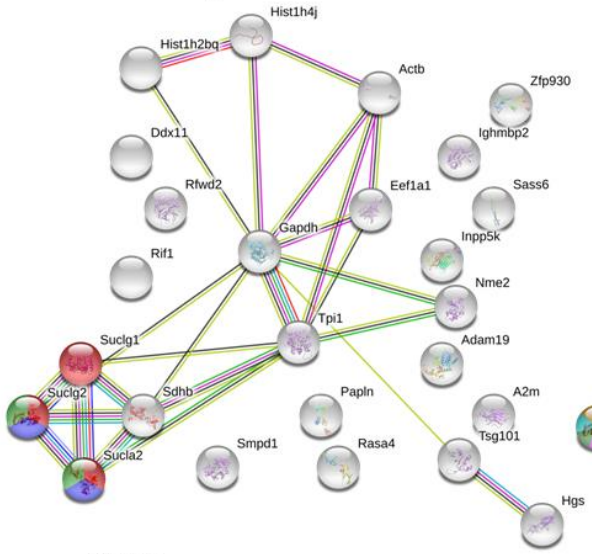
BIOLOGICAL GO:

- *Negative regulation of endopeptidase activity*
- *Nucleobase-containing compound metabolic process*
- *Somatic muscle development*
- *Cellular component biogenesis*
- *Vesicle organisation*

Fig. 5


A. KEGG
B. INTERPRO protein domains
C. Reactome pathways
D. UniProt keywords
Hist1h4j
Hist1h2bq
Actb
Zfp930
Ighmbp2
Ddx11
Rfwd2
Eef1a1
Sass6
Gapdh
Inpp5k
Rif1
Nme2
Tpi1
Adam19
Suclg1
Suclg2
Sdhb
Papln
A2m
Sucla2
Smpd1
Rasa4
Tsg101
Hgs
KEGG:
Carbon metabolism
Citrate cycle (TCA cycle)
Biosynthesis of amino acids
Metabolic Pathways
Glycolysis/gluconeogenesis
Propanoate metabolism
Inositol phosphate metabolism
INTERPRO:
Succinyl-CoA synthetase-like
ATP-grasp fold, succinyl-CoA synthetase-type
ATP-citrate lyase/ succinyl-CoA-ligase
Succinate-CoA synthetase, beta subunit
Succinyl-CoA synthetase, beta submunit, conserved site
ATP-grasp fold
ATP-grasp fold, subdomain 1
Known interactions
From curated databases
Experimentally determined
Predicted interactions
Gene neighbourhood
Gene fusions
Gene co-occurrence
Others
Textmining
Co-expression
Protein homology
Reactome pathways:
Citric acid cycle (TCA cycle)
Metabolism
UniProt:
Tricarboxylic acid cycle
Metal-binding
Acetylation
Nucelotide-binding
Ligase
Cytoplasm
Glycolysis
Serine protease inhibitor
Cytoskeleton
Transit peptide

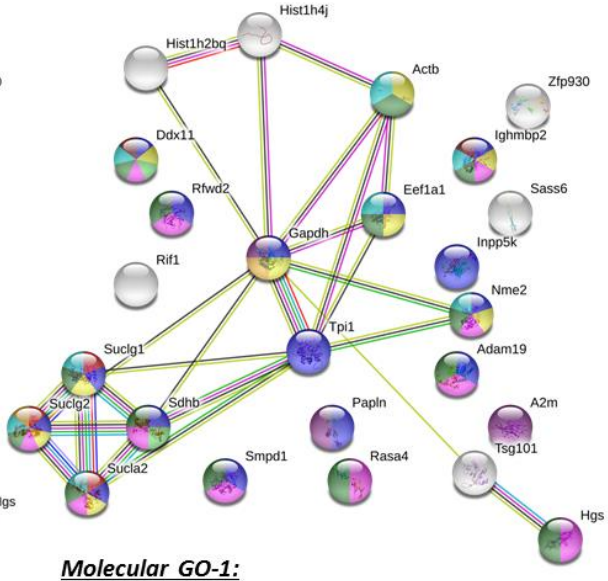
E. PFAM protein domains



PFAM:

- *CoA-ligase*
- *ATP-grasp domain*
- *ATP-grasp domain*

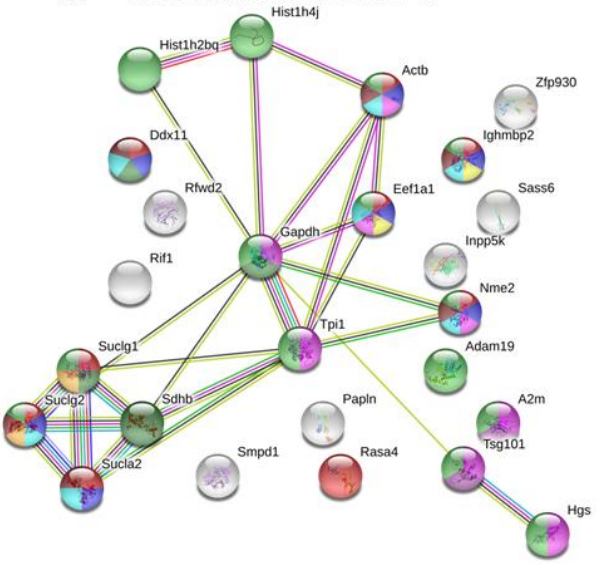
F. Molecular function GO-1



Molecular GO-1:

- *Succinate-CoA ligase activity*
- *Catalytic activity*
- *4 iron, 4 sulfur cluster binding*
- *Nucelotide-binding*
- *Metal ion binding*
- *Ion binding*
- *Purine ribonucleotide binding*
- *Oxidoreductase activity*
- *Endopeptidase inhibitor activity*
- *ATP-dependent DNA helicase activity*

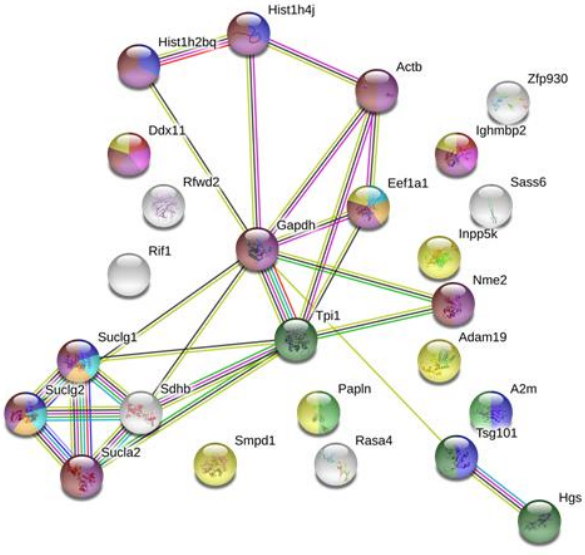
G. Molecular function GO-2



Molecular GO-2:

- *Anion binding*
- *Drug binding*
- *Protein binding*
- *tRNA binding*
- *Enzyme binding*
- *Cofactor binding*
- *Purine ribonucleoside triphosphate binding*
- *GDP binding*
- *Calcium-dependent ATPase activity*
- *ATP-binding*

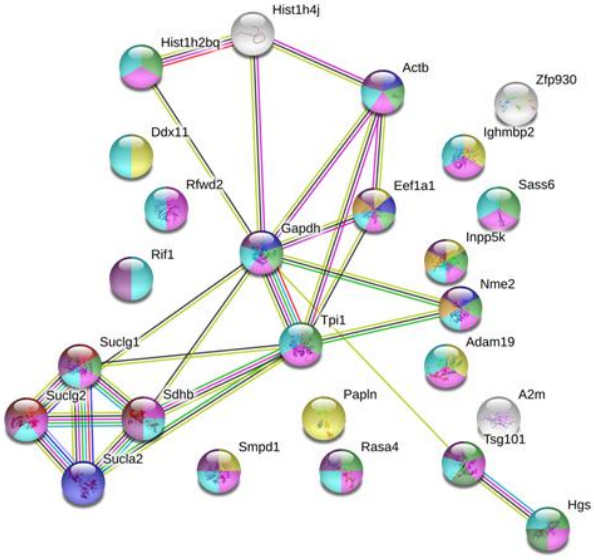
H. Molecular function GO-3



Molecular GO-3:

- *RNA-dependent ATPase activity*
- *Protein dimerisation activity*
- *Serine-type endopeptidase inhibitor activity*
- *Hydrolase activity*
- *Single-stranded DNA binding*
- *Ubiquitin-like protein ligase binding*
- *Purine ribonucleoside binding*
- *Guanyl ribonucletide binding*
- *Heterocyclic compound binding*
- *Organic cyclic compound binding*

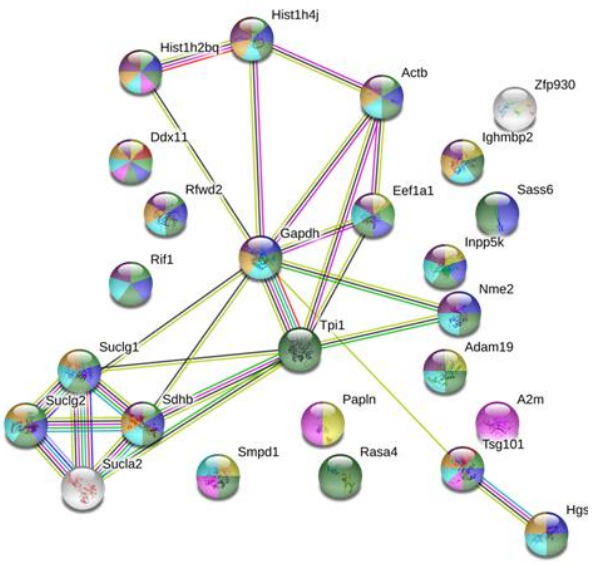
I. Cellular component GO-1



Cellular GO-1:

- *Succinate-CoA ligase complex (GDP-forming)*
- *Myelin sheath*
- *Cytosol*
- *Cytoplasmic part*
- *ESCRT complex*
- *Cytoplasm*
- *Ruffle*
- *Cell periphery*
- *Mitochondrial protein complex*

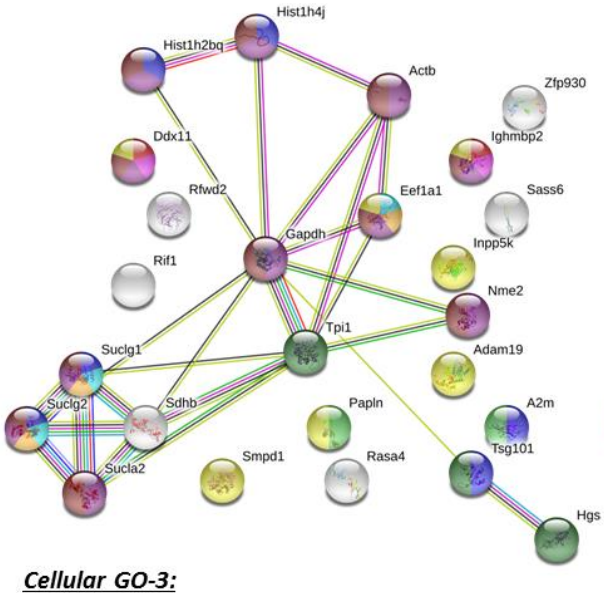
J. Cellular component GO-2



Cellular GO-2:

- *Extracellular exosome*
- *Intracellular organelle part*
- *Intracellular organelle lumen*
- *Extracellular region part*
- *Intracellular part*
- *Intracellular membrane-bounded organelle*
- *Protein-containing complex*
- *Nucleus*
- *Nucleosome*

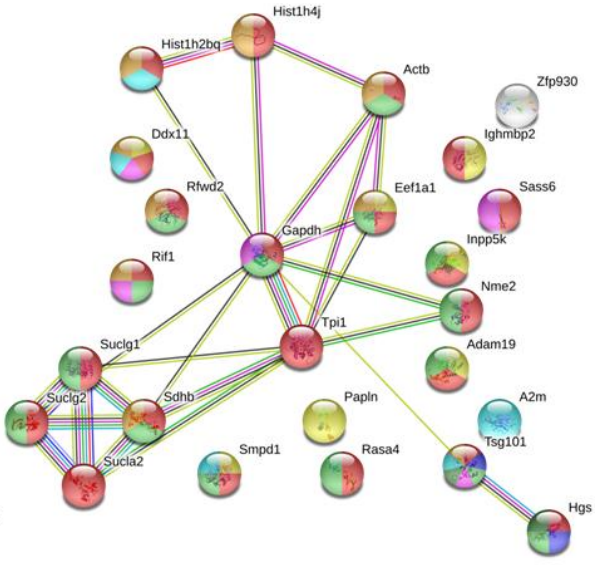
K. Cellular component GO-3



Cellular GO-3:

- *Chromatin*
- *Cytoskeletal part*
- *Intracellular organelle*
- *Ruffle membrane*
- *Cortical cytoskeleton*
- *Plasma membrane*
- *Cytoskeleton*
- *Nuclear chromatin*
- *Nuclear Part*

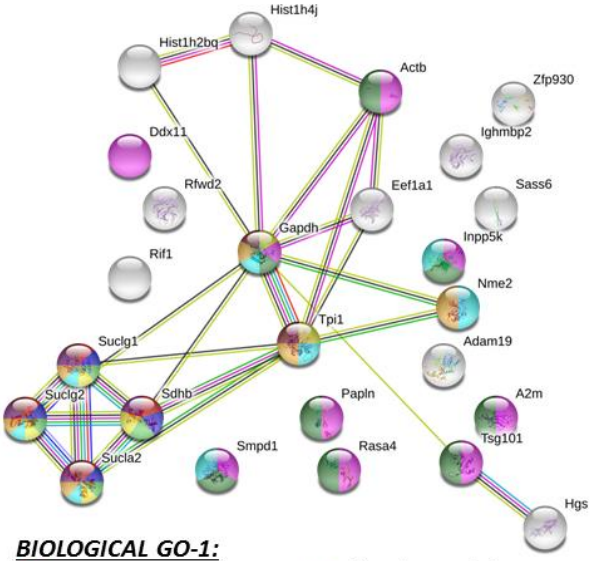
L. Cellular component GO-4



Cellular GO-4:

- *Cell part*
- *Early endosome membrane*
- *Membrane*
- *Microtubule cytoskeleton*
- *Late endosome membrane*
- *Extracellular space*
- *Nuclear lumen*

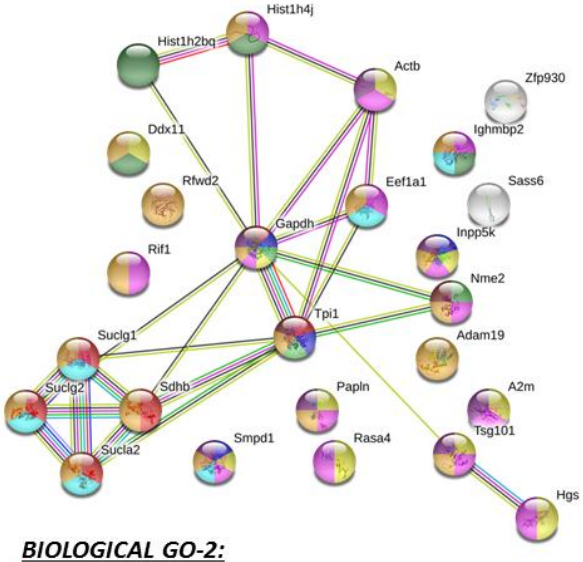
M. Biological GO process -1



BIOLOGICAL GO-1:

- *Succinate metabolic process*
- *Tricarboxylic acid cycle*
- *Citrate metabolic process*
- *Generation of precursor metabolites and energy*
- *Negative regulation of molecular function*
- *Negative regulation of catalytic activity*
- *Organophosphate metabolic process*
- *Purine ribonucleotide metabolic process*
- *Antibiotic metabolic process*
- *Coenzyme metabolic process*

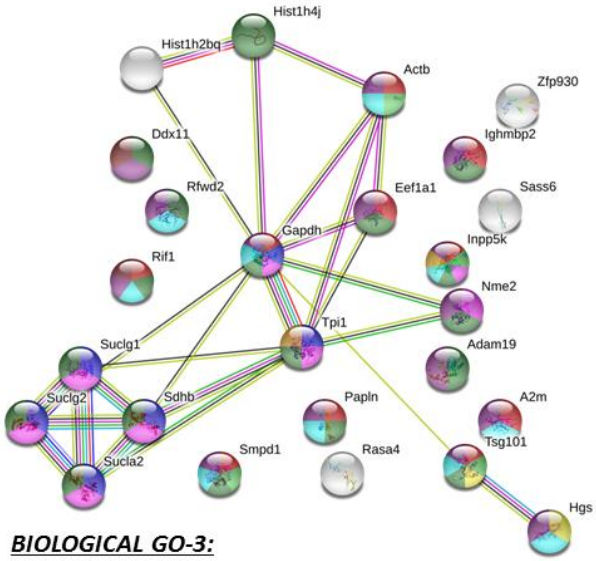
N. Biological GO process -2



BIOLOGICAL GO-2:

- *Drug metabolic process*
- *Organophosphate catabolic process*
- *Nucleoside diphosphate phosphorylation*
- *Regulation of catalytic activity*
- *Negative regulation of cellular process*
- *DNA conformation change*
- *Cellular amide metabolic process*
- *Primary metabolic process*
- *Negative regulation of cellular protein metabolic process*
- *Purine ribonucleoside tri-phosphate biosynthetic process*

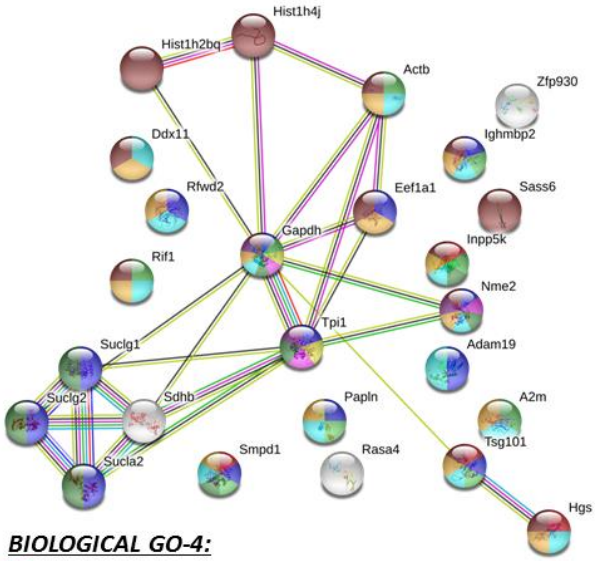
O. Biological GO process -3



BIOLOGICAL GO-3:

- *Negative regulation of cellular metabolic process*
- *Carboxylic acid metabolic process*
- *Negative regulation of protein kinase activity*
- *Positive regulation of exosomal secretion*
- *Small molecule metabolic process*
- *Organic substance metabolic process*
- *Regulation of cellular protein metabolic process*
- *Polyol catabolic process*
- *Regulation of metabolic process*
- *Positive regulation of double-strand break repair*

P. Biological GO process -4

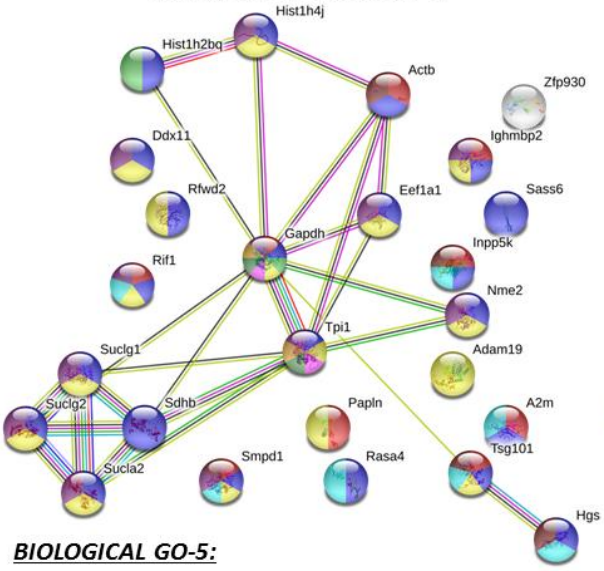


BIOLOGICAL GO-4:

- *Regulation of MAP kinase activity*
- *Organonitrogen compound metabolic process*
- *Negative regulation of nitrogen compound metabolic process*
- *Gluconeogenesis*
- *Nucleobase-containing small molecule biosynthetic process*
- *Phosphate-containing compound metabolic process*
- *Regulation of macromolecule metabolic process*
- *Regulation of cellular metabolic process*
- *Purine ribonucleotide biosynthetic process*
- *Cellular component organisation or biogenesis*

Q.

Biological GO process -5

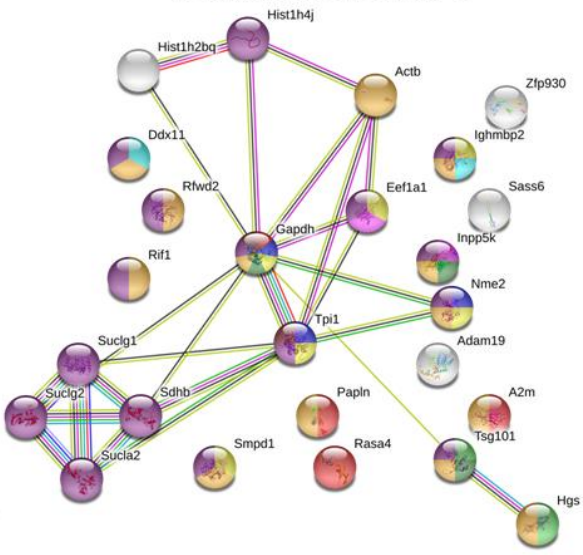


BIOLOGICAL GO-5:

- *Negative regulation of macro-molecule metabolic process*
- *Cellular process*
- *Antimicrobial humoral immune response*
- *Nitrogen compound metabolic process*
- *ATP generation from ADP*
- *Glycolytic process*
- *Negative regulation of response to stimulus*
- *Pyruvate biosynthetic process*
- *Cellular nitrogen compound metabolic process*
- *Regulation of protein kinase activity*

R.

Biological GO process -6

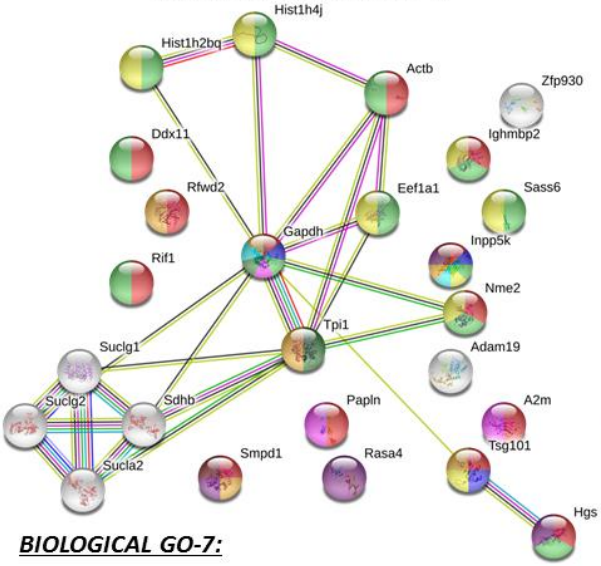


BIOLOGICAL GO-6:

- *Negative regulation of hydrolase activity*
- *Purine ribonucleoside tri-phosphate metabolic process*
- *Endosome to lysosome transport*
- *Organonitrogen compound biosynthetic process*
- *Cellular response to EGF stimulus*
- *Regulation of symbiosis*
- *DNA duplex unwinding*
- *Regulation of nitrogen compound metabolic process*
- *Cellular metabolic process*
- *Nicotinamide nucleotide biosynthetic process*

S.

Biological GO process -7

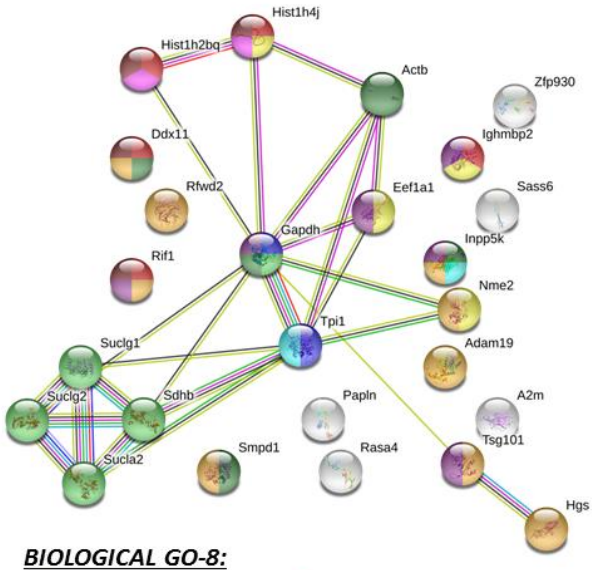


BIOLOGICAL GO-7:

- *Regulation of primary metabolic process*
- *Symbiont process*
- *Cellular component organisation*
- *Cellular component biogenesis*
- *Negative regulation of endopeptidase activity*
- *Pyruvate metabolic process*
- *Modification by host of symbiont morphology/physiology*
- *Organic substance catabolic process*
- *Negative regulation of intracellular signal transduction*
- *Negative regulation of MAP kinase activity*

T.

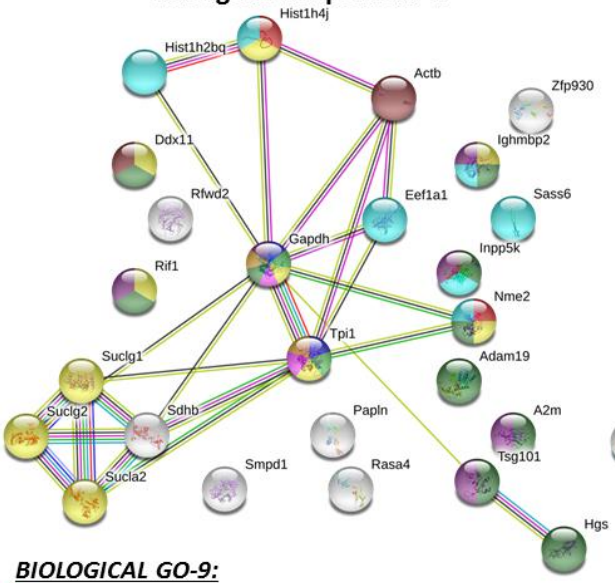
Biological GO process -8



BIOLOGICAL GO-8:

- *Chromosome organisation*
- *ATP biosynthetic process*
- *Oxidation-reduction process*
- *Protein complex oligomerisation*
- *Nucleosome assembly*
- *Response to nitrogen compound*
- *Polyol metabolic process*
- *Positive regulation of macro-molecule metabolic process*
- *Negative regulation of biosynthetic process*
- *Chromatin organisation*

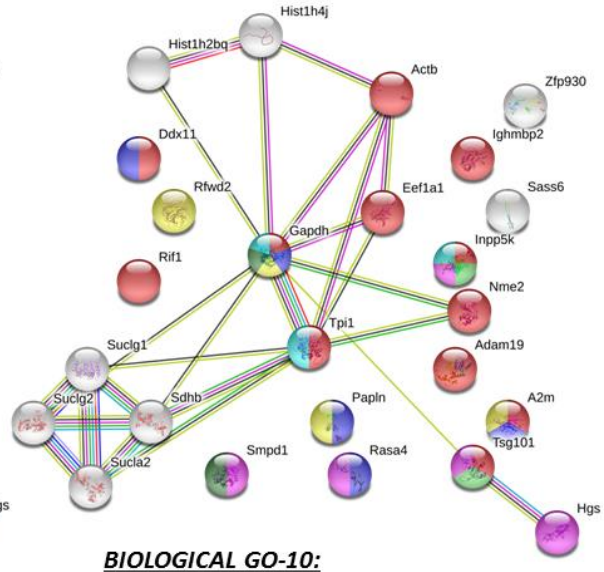
U.



BIOLOGICAL GO-9:

- *Negative regulation of myeloid cell differentiation*
- *Nucleotide catabolic process*
- *Purine ribonucleoside mono-phosphate biosynthetic process*
- *Nucleobase-containing compound metabolic process*
- *Glucose metabolic process*
- *Regulation of gene expression*
- *Cellular component assembly*
- *Nicotinamide nucleotide metabolic process*
- *Negative regulation of gene expression*
- *Negative regulation of protein binding*

V.



BIOLOGICAL GO-10:

- *Developmental process*
- *Regulation of hydrolase activity*
- *Regulation of viral life cycle*
- *Regulation of proteolysis*
- *Negative regulation of signal transduction*
- *Carbohydrate metabolic process*
- *Response to ammonium ion*