Knockdown of the Small Heat Shock Protein, p26 by RNA Interference Modifies the Diapause Proteome of *Artemia franciscana*

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**Knockdown of the Small Heat Shock Protein, p26 by RNA
Interference Modifies the Diapause Proteome of *Artemia franciscana***

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Abstract

Embryos of the crustacean, *Artemia franciscana* may arrest as gastrulae, forming cysts that enter diapause, a state of reduced metabolism and enhanced stress tolerance. Diapausing cysts survive physiological stresses for years, due in part to molecular chaperones. p26, a small heat shock protein, is an abundant diapause-specific molecular chaperone in cysts and it affects embryo development and stress tolerance. p26 is therefore thought to influence many proteins in cysts and this study was undertaken to determine how the loss of p26 by RNA interference (RNAi) affects the diapause proteome of *A. franciscana*. The proteome was analyzed by shot-gun proteomics coupled to differential isotopic labeling and tandem mass spectrometry. Proteins in the diapause proteome included metabolic enzymes, antioxidants, binding proteins, structural proteins, transporters, translation factors, receptors and signal transducers. Proteins within the diapause proteome either disappeared or were reduced in amount when p26 was knocked down, or conversely, proteins appeared or increased in amount. Those proteins that disappeared may be p26 substrates whereas the synthesis of those proteins that appeared or increased may be regulated by p26. This study provides the first global characterization of the *A. franciscana* diapause proteome and demonstrates that the sHsp p26 influences proteome composition.

Keywords: Diapause, Small heat shock protein, *Artemia franciscana*, RNA interference, Mass spectrometry

Introduction

Diapause, a state of dormancy where metabolism declines, cell division ceases and development stalls, occurs either in response to external signals such as changes in photoperiod and temperature or as an obligatory life history stage (Podrabsky and Hand 1999; MacRae 2010; Clark et al. 2012). Diapause persists even upon exposure to environmental conditions that normally support growth and many organisms show enhanced stress tolerance during diapause (Tauber et al. 1986; Košťál 2006; Podrabsky and Hand 2015; MacRae 2016). When diapause occurs, energy consumption required for the synthesis of macromolecules, transmembrane transport and many other cell processes is reduced and protein degradation is suppressed, the latter extending protein half-life (Clegg 2007).

The brine shrimp *Artemia* is an extremophile crustacean found worldwide in waters of high salinity. During embryogenesis *Artemia* produce either motile nauplii by ovoviviparous development or gastrula stage embryos enclosed in a shell (cysts) by oviparous development (Jackson and Clegg 1996; Liang and MacRae 1999; MacRae 2016). *Artemia* cysts enter diapause and may remain in a state of profound metabolic depression for years during which they exhibit high stress tolerance (Clegg 1997; Clegg and Jackson 1998; Clegg et al. 2000; Toxopeus et al. 2014). Upon diapause termination under favorable conditions including availability of food, suitable temperature, hydration and aeration, development resumes and cysts hatch to yield nauplii. If cysts encounter unfavorable circumstances upon termination of diapause they enter quiescence and remain dormant until environmental conditions permit growth. Resistance to environmental and physiological stressors persists during diapause and quiescence, disappearing as development resumes (MacRae 2016).

Diapause and quiescent cysts of *A. franciscana* possess at least three small heat shock proteins (sHsps), ATP-independent molecular chaperones termed p26, ArHsp21, and ArHsp22 (Liang and MacRae 1999; Qiu and MacRae 2008a, b; King and MacRae 2012; King et al. 2013). These sHsps have similar chaperone activity *in vitro* and they are developmentally regulated, appearing only in diapause-destined embryos. The synthesis of p26, which constitutes 7% of cyst soluble protein, is developmentally regulated but it is not stress inducible (Jackson and Clegg 1996; Liang and MacRae 1999; King et al. 2013). The use of RNA interference (RNAi) has shown that diapause-destined embryos develop more slowly when p26 is knocked down (King and MacRae 2012). Moreover, p26 knockdown cysts terminate diapause more readily than p26 containing cysts and exhibit reduced survival during stress such that upon desiccation and freezing only a small number of cysts remain viable (King and MacRae 2012).

Most sHSPs ameliorate cell stress by preventing the irreversible denaturation of cytoplasmic protein substrates (Haslbeck et al. 2016; Ungelenk et al. 2016; Mymrikov et al. 2017; Marklund et al. 2018; Mogk et al. 2018). Several reports encompassing different biological systems and experimental approaches reveal a wide diversity of sHsp protein substrates (Peschek et al. 2013; Fan et al. 2014; Katsogiannou et al. 2014; Andley et al. 2014; Mymrikov et al. 2017; Dabbaghizadeh et al. 2018). sHsps protect proteins involved in most, if not all, major biological process in cells from metabolism to transcription/translation, movement, transport and signaling, as well as proteins that regulate these activities. To identify proteins influenced by p26 during diapause in *A. franciscana*, the sHsp was knocked down by RNAi and MS-based proteomics was used in concert with differential isotopic labeling to compare cell free protein extracts from cysts with normal and reduced amounts of p26 (Tomanek 2014). Differences between extracts may reveal proteins that contribute to dissimilarities between normal and p26 knockdown cysts such

as lower stress tolerance, slower embryo development and facilitated diapause termination in the latter group. Consequently, the major objectives of this study were to identify proteins in the diapause proteome of *A. franciscana* and determine if these proteins are modified by the knockdown of p26, thus potentially revealing their roles during diapause.

Materials and methods

Culture of *A. franciscana*

A. franciscana cysts obtained from the Great Salt Lake (GSL) (INVE Aquaculture Inc., Ogden, UT, USA), were hydrated for at least 3 h in distilled H₂O on ice, collected by suction filtration and incubated at room temperature in 1 µm filtered, UV treated and autoclaved salt water from Halifax Harbour, NS, Canada, hereafter termed seawater. Unfertilized females possessing a shell gland and thus containing cyst-destined oocytes were maintained in separate wells of 6-well plates and used for cyst production. Animals were fed daily with *Isochrysis galbana* from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine, USA. The research described in this paper was performed in accordance with the ethical guidelines provided by the Canadian Council on Animal Care (CCAC). The University Committee on Laboratory Animals (UCLA) of Dalhousie University approved the research and assigned Protocol Number 117-36.

Synthesis of p26 and GFP double stranded RNA (dsRNA)

Plasmid DNA was obtained from *Escherichia coli* DH5α transformed with the recombinant expression vector pRSET C (Invitrogen, Burlington, Ont., Canada) containing p26 cDNA (Liang and MacRae 1999) by using The GenElute™ Plasmid Miniprep Kit (Sigma-

Aldrich, Oakville, Ont., Canada). The plasmid DNA was employed as template for amplification of *p26* cDNA using previously designed primers (King and MacRae 2012). Green fluorescent protein (GFP) cDNA in the expression vector pEGFP-N1 (Clontech, Mountain View, CA, USA) was used as template for amplification of *GFP* cDNA (Zhao et al. 2012). PCR was carried out with 0.2 mM Platinum® Taq DNA polymerase (Invitrogen) under the following conditions: 5 min at 94°C followed by 35 cycles at 94°C for 30 s, 61°C for 30 s, and 72°C for 60 s, then a final incubation at 72°C for 7 min. The PCR products and GeneRuler 100 bp DNA Ladder (Invitrogen) were resolved in agarose gels, stained with SYBR Safe® (Invitrogen) and visualized with a DNR Bio-imaging Systems MF-Chem/BIS 3.2 Gel Documentation System (Montreal Biotech, Montreal, Quebec, Canada). *p26* and *GFP* dsRNAs were synthesized using the PCR products described above as templates (King & MacRae, 2012; King et al., 2013).

Injection of *A. franciscana* females with *p26* and *GFP* dsRNA

Sixty-eight mature females destined to produce cysts were injected with *p26* dsRNA and 83 with *GFP* dsRNA (King and MacRae 2012) and 24 h post-injection each female exhibiting normal swimming behaviour was incubated with a male for fertilization. Females were observed daily under a dissecting microscope to determine the time of fertilization, marked by the fusion of lateral egg sacs, and to monitor the release of cysts which were harvested 8-days post-release to allow time for entry into diapause, washed 3 times with ice cold distilled water and collected by centrifugation for 30 sec at 3000 x g. Cysts were dried in a fume hood for 1 min and stored at -80°C until used.

130 **Protein extraction for mass spectrometry**

131 Protein extracts for mass spectrometry were prepared from 2090 and 2226 cysts obtained
132 from females injected respectively with either *p26* or *GFP* dsRNA. Cysts were homogenized on
133 ice in 5 ml capped glass grinders (Radnoti LLC 440614, USA) in a laminar flow hood with
134 denaturing buffer (0.8 g urea, 0.4 ml triethylammonium bicarbonate (TEAB) 1 M, H₂O to 1 ml).
135 Supernatants were collected after centrifugation at 3500 x g for 5 min and protein concentration
136 was determined by the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Twenty-five µg
137 of protein was applied to each lane of an SDS-polyacrylamide gel for electrophoresis and
138 western blotting (Liang & MacRae 1999). The membranes were probed with a polyclonal
139 antibody raised in rabbit to p26 (Liang & MacRae 1999) followed by an HRP-conjugated anti-
140 rabbit IgG antibody (Sigma-Aldrich) (King and MacRae 2012).

141

142 **Protein digestion for mass spectrometry**

143 Two hundred µg of cyst protein in 0.5 M dithiothreitol (Sigma D9163) was heated at
144 60°C for 30 min followed by 5 min at room temperature. The samples were sonicated (Branson
145 2800 water bath) at room temperature for 15 min, alkylated in 0.7 M iodoacetamide (Sigma
146 I6125) for 30 min at room temperature, diluted 6 X with H₂O and digested overnight at 37°C
147 with trypsin at a ratio of 100:1 protein to trypsin. Subsequently, trifluoroacetic acid (TFA)
148 (Sigma T6508) was added to 0.1%, and the pH was reduced to less than 3 with 5 µl formic acid
149 (FA) prior to centrifugation for 1 min at 3000 x g. The acidified peptides were desalted using
150 QasisHLB desalting columns (PN WAT094226) conditioned once with 1 ml of 50% acetonitrile
151 (ACN) in 0.1% TFA and twice with 1 ml 0.1% TFA. Peptide samples were loaded into Qasis
152 HLB desalting columns followed by washing five times with 1 ml of 0.1% TFA. Elution was

done twice with 0.5 ml of 50% ACN in 0.1% TFA and once with 0.5 ml of 80% ACN in 0.1% TFA. The eluted samples were dried in a SpeedVac (Thermo Electron Corporation SPD111V) at 20°C and reconstituted in 100 µl of 50 mM TEAB (500 µl 1M TEAB to 4.74 ml of H₂O) before sonication at room temperature for 15 min.

Isotopic labeling of peptides

Two hundred µg of protein digest from cysts containing and lacking p26 were differently tagged by reductive methylation. For light labeling 8 µl of 37% (w/w) formaldehyde (Sigma F8775) was added to the peptide sample prepared from cysts containing p26 whereas for heavy labeling 15 µl of 20% (w/w) deuterated formaldehyde in D₂O (Cambridge Isotope Laboratories, DLM-805-20) was added to the peptide sample from cysts lacking p26. Each sample was mixed and incubated for 5 min at room temperature. Seventeen µl of 6 M sodium cyanoborohydride (NaCNBH₃) was then added to each sample followed by incubation for 1 h at room temperature. Light and heavy samples were combined in a 1:1 ratio (v/v) and the pH of the labeled sample was reduced to less than 3 with 1 µl TFA prior to desalting as described previously and drying in a Speed Vac.

Reversed-phase high performance liquid chromatography (RP-HPLC)

Peptides were initially separated and fractionated by high pH RP-HPLC prior to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. Two hundred µg of the labeled protein digest was suspended in 100 µl of suspension buffer (5% ACN, 10 mM NH₄HCO₂, pH 8.0) and sonicated at room temperature for 15 min. One hundred µl of the labeled sample was then injected at a flow rate of 400 µl/min into a 4.6 x 100 mm monolithic

176 C18 column (Merck, Germany) for separation in an Agilent 1100 HPLC system (G1364C,
177 Agilent Technologies, Santa Clara, California, USA). For chromatographic separation the
178 column initially received buffer A (5% ACN, 10 mM ammonium formate, pH 8.0) for 3 min,
179 then a gradient of 100% to 64% buffer A over 57 min, followed by 100% buffer B (95% ACN,
180 10 mM ammonium formate, pH 8.0) for 5 min. Subsequently, the column was rinsed with 100%
181 buffer B for 14 min followed by 100% buffer A for 11 min. Thirty-seven fractions were
182 collected at 2 min intervals and dried using a Speed Vac. As drying occurred fractions at early,
183 middle and late stages, for example samples 2, 14, 26, were combined into 12 fractions which
184 were suspended in 50 µl of 3% ACN containing 0.1% FA. The samples were centrifuged for 5
185 min at 3000 x g and sonicated at room temperature for 15 min. Samples were further desalted
186 using the stage tip procedure (Rappsilber et al. 2007) after which the columns were centrifuged
187 at 2000 x g for 10 min and washed twice with 200 µl 0.1% TFA by centrifugation for 5 min at
188 3000 x g. Peptides were eluted into fresh tubes by adding 50 µl of 50% ACN in 0.1% TFA to
189 each C18 column followed by centrifugation for 5 min at 3000 x g. Fifty µl of 80% ACN in
190 0.1% TFA was added to each of the 12 samples which were then centrifuged for 5 min at 3000 x
191 g. The eluted samples were dried in a SpeedVac, suspended in 20 µl of suspension buffer (30 µl
192 0.3% ACN, 5 µl 0.5% FA in 970 µl H₂O, pH 2.0), sonicated at room temperature for 15 min and
193 transferred to micro HPLC vials.

194

195 **Liquid chromatography tandem mass spectrometry (LC-MS/MS)**

196 LC-MS/MS was performed using a nano flow liquid chromatography system
197 (Ultimate3000, Thermo Scientific) interfaced to a hybrid ion trap-orbitrap high resolution
198 tandem mass spectrometer (VelosPRO, Thermo Scientific) operated in data dependent

acquisition mode. One μ l of each sample was injected into a C18 packed capillary column (4 μ m C18 Jupiter packing material, 0.0750 x 500 mm capillary column) at a flow rate of 300 nl/min and electro-sprayed at 1.2 kV using a dynamic nanospray probe at 5°C. For chromatographic separation, a linear gradient of 95% buffer A (0.1% FA in MS grade H₂O, pH 2.0) and 5% buffer B (0.1% FA in MS grade ACN, pH 2.0) to 5% buffer A and 95% buffer B over 104 min, with a final wash of 97% buffer A for 15 min, was used. MS/MS spectra were acquired by collision induced dissociation and higher-energy collisional dissociation for the top 15 peaks.

Bioinformatics

Mass spectrometry data files were acquired by Xcalibur (Thermo Fisher) and exported to Proteome Discoverer software, version 2.0 (Thermo Fisher) for protein identification using SequestHT search algorithm. The search was based on a full trypsin digestion with 10 ppm precursor mass tolerance and 0.8 Da fragment mass tolerance for collision-induced dissociation spectra and 0.02 Da for higher energy collisional dissociation spectra. Database searching was carried out against the *Artemia* genome database ORCAE (online resource for community annotation of eukaryotes), a restricted genome from Gent University, Gent, Belgium, and with arthropod and crustacean protein sequences in UniProt. The Common Repository of Adventitious Proteins database was used to identify contaminants. Databases were formatted to FASTA prior to analysis.

For the database search trypsin was used as the digestion enzyme and one missed cleavage site per peptide was allowed. Modifications included in the search were phosphorylation of Thr, Tyr and Ser, oxidation of Met, carbamidomethylation of Cys and mass shifts of +28 Da and +32 Da respectively for light and heavy labeling on peptide amino-terminus

and lysine residues. The results were filtered based on Xcorr vs charge state, 2.2, +2; 3.75, 3+ or greater. Peptide and fragment tolerances were set at 10 ppm and 0.6 m/z, respectively. Acceptance threshold for peptides was $e \leq 0.1$. The resulting LC-MS/MS file which contained identified proteins was transformed into a biologically meaningful dataset as an Excel file. Proteins that could not be identified by the procedures just described were blasted on different web servers and the closest match with threshold $e \leq 0.1$ was chosen.

The resulting data were simplified by using the filtration tool in Proteomic Discover and checked manually to confirm protein identity. Gene ontology (GO) enrichment analysis was utilized wherein the MS/MS detected proteins were mapped to the RefSeq of the equivalent *D. melanogaster* proteins by blasting the protein sequences in the NCBI database and using an e -value of $\leq 10^{-5}$. That is, the NCBI RefSeqs of *D. melanogaster* protein were mapped to UniProt KB using the UniProt Mapping tool. Proteins were identified on the basis of matches to two or more unique peptides, although identities were, as indicated in the tables, also based on a single match. The gene names were then inserted into PANTHER <http://www.pantherdb.org> to functionally characterize the proteins in the diapause proteome. Proteins were assigned functions by use of the UniProt database. Proteins were further organized into functional subgroups by employing KEGG Pathway Maps, <http://www.genome.jp/kegg/pathway.html>, and then displayed in a pie chart. It should be noted that the data are not presented with biological replicates due to the time required to collect sufficient amounts of diapausing cysts and perform a second mass spectrometry analysis, thus the study is qualitative in nature.

RESULTS

Knockdown of p26 in *A. franciscana* cysts

Mass spectrometry revealed that p26 was reduced by approximately 200-fold in cysts from *A. franciscana* females receiving *p26* dsRNA as compared to cysts from females receiving *GFP* dsRNA (Fig. 1). Upon immunoprobing of western blots p26 was not observed in cysts obtained from females injected with *p26* dsRNA but was detected in cysts obtained from females injected with *GFP* dsRNA (Fig. 1, inset).

The diapause proteome of *A. franciscana*

The proteins revealed by MS/MS in *A. franciscana* cysts released from females injected with *GFP* dsRNA represent a partial *A. franciscana* diapause proteome https://dalumy.sharepoint.com/personal/hj269321_dal_ca/_layouts/15/WopiFrame.aspx?docid=03f586a0e9ee54a5ca0afc6690f0be0fb&authkey=AdwcUiu6AKtSweU6NLSaIz0&action=view, the only diapause proteome available for *A. franciscana* at the time of analysis. MS/MS detected 3387 proteins which were reduced to 3212 proteins by removing duplicates and likely contaminants such as trypsin and keratin. Of the remaining proteins 1548 (48.2%) were annotated and 1664 (51.8%) were not. The identification of 639 proteins (29%) was made on the basis of two or more matches of unique peptides to an archived protein and 924 proteins (71%) were identified by a single peptide match. The closest matches were generally to proteins from arthropods.

PANTHER recognized 1406 proteins and functional classification was achieved for 1204 of them (Fig. 2). Metabolic enzymes constituted 44% of the proteins within the diapause proteome followed by binding proteins (35%) and structural proteins (11%). The other groups were composed of transporters (5%), translation factors (3%), receptors (1%), antioxidants (1%), signal transducers (0.2%) and a channel regulator (0.1%), the latter two groups not visible in Fig. 2 due to their small size.

The metabolic enzymes were divided into hydrolases, transferases, oxidoreductases, ligases, isomerases, lyases, enzyme regulators such as kinases, helicases and deaminases (Fig. 3A). The binding proteins were composed of 4 subgroups including those that interacted with nucleic acids, proteins, calcium ions and phospholipids (Fig. 3B). Structural proteins were mainly ribosomal and cytoskeletal (Fig. 3C), whereas the transport proteins were involved in carbohydrate and lipid transport and other transport activities (Fig. 3D). Proteins in the translation group were protein synthesis, elongation and termination factors (Fig. 3E). The last four functional groups, not shown in Fig. 3, consisted of receptors, one of which was a G-protein coupled receptor, antioxidant enzymes of which 7 were peroxidases, signal transducers, a heteromeric G-protein and one channel protein.

Proteins that disappeared from the diapause proteome upon p26 knockdown

Nine proteins, identified on the basis of a match with one peptide, disappeared from the diapause proteome upon knockdown of p26 (Table 1). Six of the proteins participate in genetic information processing, 2 were metabolic enzymes and 1 mediated cellular processing (apoptosis). One protein was unidentified.

Proteins that decreased but did not disappear from the diapause proteome upon p26 knockdown

Eleven proteins in the diapause proteome of *A. franciscana* decreased by 50% or more upon knock down of p26 (Table 2) and of these 6 functioned in genetic information processing, 3 mediated environmental information processes and 2 were not identified.

Proteins that appeared in the diapause proteome upon p26 knockdown

Thirty-five proteins appeared in the proteome of p26 knockdown cysts of which 34 were identified by a match to one peptide (Table 3). Eighteen of the proteins participated in genetic information processing, 10 in metabolism and 6 in environmental information processing. One protein was unidentified.

Proteins normally present in the diapause proteome that increased upon p26 knockdown

Twenty-two proteins increased by at least 2-fold in the diapause proteome when p26 was knocked down (Table 4) of which 12 were implicated in genetic information processing, 7 in metabolism and 3 unidentified.

4. Discussion

The diapause proteome of *A. franciscana*

GFP is widely accepted as a control in RNAi studies using *A. franciscana* because the organism does not synthesize this protein (Liu et al. 2009; Zhao et al. 2012). Thus, injection of females with *GFP dsRNA* is unlikely to affect the proteome of diapausing *Artemia* cysts. The data represent the first use of the 2D-HPLC method coupled with MS/MS to identify proteins in the diapause proteome of an extremophile arthropod and the most comprehensive diapause proteome available to date for diapausing *A. franciscana* cysts. Detected proteins were blasted against several data bases yielding annotation for 48.2% of the proteins which is a reasonable result considering that at the time of analysis the *A. franciscana* genome had not been completely sequenced. A well annotated *A. franciscana* genome is now available and its use in the future will increase protein identification.

Nine functional categories of proteins were identified in the *A. franciscana* diapause proteome with metabolic enzymes accounting for the largest proportion, a result similar to that for *A. sinica* (Zhou et al. 2008) and for diapause eggs of the migratory locust, *Locusta migratoria* (Tu et al. 2015). Because diapausing embryos of *A. franciscana* undergo profound metabolic arrest (Jackson and Clegg 1998; Reynolds and Hand 2004), the abundance of metabolic enzymes seemed unusual, however, low amounts of ATP during diapause is undoubtedly inhibitory and metabolic enzymes may be stored in the cyst for use post-diapause. Antioxidant enzymes were observed in the *A. franciscana* diapause proteome with peroxidases being the only type identified. These enzymes repair diapause-induced oxidative damage in cysts of parthenogenetic *Artemia* exposed to stress (Nunes et al. 2006) and they are up-regulated in the eggs of migratory locust during diapause (Tu et al. 2015).

The second largest group in the diapause proteome of *A. franciscana*, as in *A. sinica* (Zhou et al. 2008), was composed of proteins that bound nucleic acids and other proteins. The protein binders encompass molecular chaperones and these contribute to protein homeostasis during diapause. The nucleic acid binders include proteins that regulate DNA replication, gene expression and RNA metabolism and may be employed during diapause initiation and then saved for use post-diapause. Translation factors, required for protein synthesis during embryogenesis and preparation for diapause, may also be stored in cysts for the post-diapause synthesis of proteins.

Structural proteins formed 11% of the *A. franciscana* diapause proteome and this is similar to the 12% in the proteome of diapause embryos of *A. sinica* (Zhou et al. 2008). Cytoskeletal proteins are an important class of structural proteins that function in cell motility, division, differentiation and intracellular transport. Transport proteins were present in the

diapause proteome and they move along cytoskeletal elements. Transporters may be important for diapause preparation but during diapause such activities cease due to limited ATP.

Receptors, by interacting with environmental and physiological signals, are required for entry into and termination of diapause. In support of this proposal, knocking down receptors in diapause-destined embryos of parthenogenetic *Artemia* inhibits formation of cysts (Ye et al. 2017). Signal transducers, including a heterotrimeric-G protein, resided in the *A. franciscana* diapause proteome and by relaying signals from receptors to intracellular effectors many cellular activities are regulated. A calcium channel regulator, observed earlier in diapause-destined embryos of *A. franciscana* (Qiu et al. 2007), was identified and it may inhibit energy dependent ion transport across membranes.

Proteins that disappeared from the *A. franciscana* diapause proteome or were reduced in amount upon p26 knockdown

Proteins that disappeared from the diapause proteome upon knockdown of p26 included RNA granule protein, mitochondrial isocitrate dehydrogenase [NAD] subunit, glutamine dependent NAD (+) synthase, elongation factor 1 α , ubiquitin conjugation factor E4, U2 snRNP which is a component of the ATP-independent spliceosomal complex and a caspase. Other proteins decreased by more than 50% upon p26 knockdown but did not disappear. Within this group were proteins involved in cyst shell formation, a vacuolar ATPase which mediates proton transport, subunits of Hsp60, Hsp70-4, ubiquitin/ribosomal S27 fusion protein 2 and G11700, the latter two proteins members of the ubiquitin system. These proteins are potentially substrates of p26 and they function before p26 is synthesized. Once p26 appears in the embryo these proteins may bind to it which inhibits their activity because they are no longer required in the diapause-destined embryo. At the same time the proteins are protected from degradation and preserved for

use in post-diapause development. By comparison, knocking down HSP27 in human cancerous cells eliminated three proteins, namely histone deacetylase, transcription factor STAT2 and procaspase-3 and it was proposed that these proteins are substrates of HSP27 (Gibert et al. 2012). The lens of double knockout mice lacking the sHsps α A- and α B-crystallin exhibited reduced amounts of titin, hemoglobin subunit α and the enzymes glucose-6-phosphate isomerase, retinal dehydrogenase 1, glutamate dehydrogenase and ATP synthase involved in resistance to oxidative stress and metabolism (Andley et al. 2013). The knockdown of sHSP26 in maize, *Zea mays* affected 45 proteins most of which were chloroplast proteins (Hu et al. 2015). Three nuclear proteins declined in amount after sHSP26 knockdown and of these only a calmodulin binding protein was identified.

Proteins that appeared in the *A. franciscana* diapause proteome or increased in amount upon p26 knockdown

The loss of p26 also resulted in either the appearance of, or the increase in, fifty-seven proteins, many of which were metabolic enzymes, suggesting that p26 reduces metabolism during embryogenesis and in preparation for diapause by inhibiting their synthesis. Proteins appearing upon the loss of p26 and which regulate the cell cycle, cell growth, gene transcription and protein homeostasis included proteasome 26S subunit non-ATPase 9, E3 binding protein, Rpn1 subunit, Ras-related protein Rab-18, eukaryotic translation initiation factor 3 subunit B and ribosomal protein L6. Should p26 repress their synthesis then development would cease or slow down as occurs in diapause-destined embryos and metabolism would be suppressed as in diapausing cysts.

Small ribonucleoprotein particle U1 subunit 70K, isoform C (smRNP-U1-70K), which increased 7- fold upon the loss of p26, regulates RNA splicing (Krämer 1995), an ATP requiring

process that is unlikely to function during diapause. Importin α , a transporter of proteins into the nucleus, increased three-fold upon the loss of p26, suggesting that its synthesis is inhibited by p26. p26 may suppress the synthesis of snRNP-U1-70K and importin α during diapause because they mediate RNA splicing and protein transport, both energy requiring processes that would be suppressed in dormant cysts.

Considering the functions of the proteins that appeared or increased upon knockdown suggests that p26, perhaps with other regulator(s), inhibits the synthesis of proteins active in promoting growth which halts development. When p26 is eliminated by RNAi the unrestricted synthesis of proteins normally suppressed by p26 occurs. This may happen because a transcriptional enhancer that is inhibited by binding to p26, leading to the cessation of gene expression as cysts enter diapause, continues to function when p26 is eliminated. Other possible explanations for the increase in proteins is that p26 binds and inhibits proteases that degrade these proteins or, of a less interesting nature, cells are compensating in a non-specific way for the loss of such an abundant protein.

Double knockout mice lacking the sHsps α A- and α B-crystallin have age dependent increases in several lens proteins such as β 1-catenin, caspases, histones, vimentin, tau, α -tubulin, proteasome activator complex subunit 2 and ubiquitin-like protei (Andley et al. 2013). Knocking down sHSP26 in maize resulted in the increase of only one non-chloroplast protein, actin-1 (Hu et al. 2015). None of the proteins from either experimental system correspond to the proteins that increased in the p26 knockdown proteome of *A. franciscana* cysts.

Conclusions

Application of differential isotope labeling, 2D-LC and MS/MS yielded the *A. franciscana* diapause proteome and the use of RNAi revealed changes that occurred upon p26 knock down. Twenty-one proteins either disappeared from the diapause proteome or were reduced in amount upon p26 knockdown, suggesting they are p26 substrates. Fifty-seven proteins either appeared in the diapause proteome or increased in amount upon the loss of p26 suggesting that this sHsp, perhaps in cooperation with other regulator(s), inhibits their synthesis during diapause. p26 is therefore important because it affects the proteins found in embryos and thus the development of diapause-destined cysts and their characteristics once formed. Unidentified proteins were observed that changed upon the reduction of p26 and they are apparently specific to *A. franciscana*; their identification in future work is important. The results contribute to our understanding of the cell processes that p26 influences during diapause, information potentially applicable in industries such as aquaculture, agriculture and forestry, as well as in medicine, where the ability of organisms to undergo diapause has important financial and social consequences.

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Draft

Table 1. Proteins that disappeared from the *A. franciscana* diapause proteome upon p26 knockdown

¹ Protein	² Function
Genetic Information Processing	
RNA granule protein, invertebrate	Chaperoning & translation suppressor
*U2 snRNP-associated SURP motif containing protein	RNA processing
Elongation factor 1 α	Protein biosynthesis
Elongation factor-1 α	Protein biosynthesis
Elongation factor-1 α	Protein biosynthesis
*Ubiquitin conjugation factor E4 A	Protein ubiquitination
Metabolism	
Mitochondrial isocitrate dehydrogenase [NAD] subunit	Tricarboxylic acid cycle
*Probable glutamine dependent NAD (+) synthase	NAD biosynthesis
Cellular Process	
* Caspase	Apoptosis
Not Annotated	
artfr59618g00010	Unknown

570

571 ¹Proteins were identified by Proteome Discoverer software. *Proteins were identified manually.
572 ²Proteins were functionally categorized by using the UniProt database. Bold font indicates
573 protein function based on the KEGG PATHWAY.

Draft

Table 2. Proteins that decreased but did not disappear from the *A. franciscana* diapause proteome upon p26 knockdown

¹ Protein	² Function	³ Decrease -p26 : +p26	⁴ Unique Peptides
Genetic Information Processing			
CG11700	Protein ubiquitination	0.5	2
Ubiquitin/ribosomal S27 fusion protein 2	Protein turnover	0.5	2
T-complex protein 1, theta subunit	Chaperoning activity	0.5	3
* T-complex protein 1, beta subunit	Chaperoning activity	0.5	2
*Heat shock 70 kDa protein 4	Chaperoning activity	0.5	2
* Shell gland specific protein	Cyst shell formation	0.2	5
Environmental Information Processing			
*Vacuolar ATPase subunit H	Proton transport regulator	0.4	2
* B0WHJ5	Chitin metabolism	0.2	2
*GL19819	DNA binding	0.5	10
Not Annotated			
artfr5076g00040	Unknown	0.5	2
artfr1800g00080	Unknown	0.4	2

576 ¹Proteins were identified by Proteome Discoverer software. *Proteins were identified manually.
577 ²Proteins were functionally categorized by using the UniProt database. ³-p26:+p26, the decrease
578 in the amount of a protein in cysts lacking p26 as compared to cysts containing p26. ⁴The
579 number of unique peptides sequenced by mass spectrometry that matched the protein of interest.
580 Bold font indicates protein function based on the KEGG PATHWAY.
581

Draft

Table 3. Proteins that appeared in the diapause proteome of *A. franciscana* upon p26 knockdown

¹ Protein	² Function
Genetic Information Processing	
* Coatomer subunit beta	Vesicle mediated protein transport
*Importin-4	Protein transport
*Hypoxia up-regulated protein 1	Cell response to stress
U-box domain-containing protein 35-like	Stress response and ubiquitin system
E3 binding	Ubiquitination
*Heat shock 70 kDa protein 14	Stress response
E9GQY5	DNA and RNA secondary structure unwinding, promotion of transcription
E9GM63	Regulation of transcription initiation from RNA polymerase II promoter
*Mediator of RNA polymerase II transcription subunit 25	Regulation of transcription
RNA recognition motif, spliceosomal PrP8	Pre-mRNA splicing via spliceosome
* CWF19-like protein	mRNA splicing/cell cycle control

E9G6S3	Methionine synthase activity
*tRNA methyltransferase 11-2 homolog	RNA methylation
* Proteasome 26S subunit, non-ATPase 9	Assembly of the 26S proteasome/ transcription co-activator
26S proteasome regulatory complex, non-ATPase sub-complex, Rpn1 subunit	Proteasome regulator
Peptidase S1C	Peptide hydrolase

Metabolism

* 60S ribosomal protein L6	Ribosome large subunit assembly, translation
*Eukaryotic translation initiation factor 3, subunit B	Initiation of translation
Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	Fatty acid metabolism
* Hydroxysteroid dehydrogenase -like protein 2	Steroid biosynthesis
Pyrroline-5-carboxylate reductase	Proline biosynthesis
* Inosine 5' monophosphate dehydrogenase 2	Guanosine monophosphate/purine biosynthesis
Adenosine/AMP deaminase domain	Glycosidase /purine metabolism
*Phospho-triesterase related protein	Glycosidase
*Phospho-glucomutase 2	Carbohydrate/purine metabolism

Phosphoglucomutase	Glucose metabolism
*Pyruvate kinase	Glycolysis
* Glucose dehydrogenase	Fatty acid/ nucleotide and nucleic acids synthesis

Environmental Information Processing

*Transmembrane 9 superfamily member 2	Small molecule transporter
*Chloride intracellular channel protein 2	Regulation of ion channel transmembrane activity
*Ras-related protein Rab-18	GTPase mediated signal transduction
* F1A3Y3	Signal transduction
Nicastrin	Proliferative signaling & cell-cell communication
GD21468	Phosphatidylinositol phosphorylation

Not Annotated

artfr1743g00010	Unknown protein
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¹Proteins were identified by Proteome Discoverer software. *Proteins identified manually.

²Proteins were functionally categorized by using the UniProt database. Bold font indicates protein function based on the KEGG PATHWAY.

Table 4. Proteins normally present in the diapause proteome of *A. franciscana* that increased upon p26 knockdown

¹ Protein	² Function	³ Fold-increase -p26 :+p26	⁴ Unique Peptides
Genetic Information Processing			
Small ribonucleoprotein particle U1 subunit 70K, isoform C	mRNA splicing via spliceosome	6.8	2
*40S ribosomal protein S8	Translation	2.5	3
*40S ribosomal protein S21	Ribosomal/ ribonucleo protein	4.0	3
*Lachesin	Developmental protein/ morphogenesis	2.7	2
*Vesicle-associated membrane protein associated protein A	Protein localization to ER	2.8	2
* Reticulon-1	Regulate ER structure and function	4.9	2
*Similar to lola-like protein	Regulation of transcription	3.3	2
Importin subunit alpha	Protein import into nucleus	2.9	3
*Serine/threonine protein kinase	Protein phosphorylation	3.4	3
Nucleotide-binding α - β plait	Ribosomal/ ribonucleoprotein	5.0	2
* Nucleoprotein TPR	Nucleocytoplasmic transporter activity	2.2	2
*Nuclear pore complex Nup50	Nuclear transport	3.8	3

Metabolism

Male sterility, NAD-binding	Oxidation-reduction, ubiquinone-6 synthesis	2.1	2
Acyl-CoA oxidase/de-hydrogenase, central domain	Lipid metabolism	3.0	2
Aldehyde dehydrogenase C-terminal	One-carbon metabolism	2.2	2
Orotidine 5' phosphate decarboxylase domain	De novo uridine biosynthesis	2.7	2
* 3-Hydroxyanthranilate 3, 4 dioxygenase	Pyridine nucleotide biosynthesis	2.1	2
Dolichyldiphospho oligo saccharide protein glycosyl transferase subunit 1	Protein glycosylation	3.4	2
Glucosamine-6-phosphate isomerase	Carbohydrate metabolism	2.8	2

Not Annotated

Mitochondrial glyco-protein	Unknown	2.3	2
R7W130	Unknown	4.1	5
* E9FU69	Unknown	2.2	2

¹Proteins were identified by Proteome Discoverer software. *Proteins identified manually.

²Proteins were functionally categorized by using the UniProt database. ³-p26/+p26, the increase

in a protein in cysts lacking p26 as compared to cysts containing p26. ⁴The number of unique peptides sequenced by mass spectrometry that matched the protein of interest. Bold font indicates protein function based on the KEGG PATHWAY.

Draft

Figure Legends

Fig. 1. Knockdown of *p26* in diapausing *A. franciscana* cysts. Mass spectrometry yielded the number of spectra for p26 peptides in protein extracts of cysts obtained from females injected with *GFP* dsRNA (blue) and *p26* dsRNA (red). Inset, To confirm knockdown 25 µg of protein extract from cysts produced by females injected with either *GFP* dsRNA (1) or *p26* dsRNA (2) was resolved in SDS-polyacrylamide gels, transferred to nitrocellulose and probed with a polyclonal antibody raised in rabbit to p26, followed by HRP-conjugated goat anti-rabbit IgG antibody.

Fig. 2. Proteins of the *A. franciscana* diapause proteome. The function of proteins in the diapause proteome was determined by PANTHER. The number of proteins in each functional group was divided by the number of proteins in the diapause proteome to give the percentage of proteins in each group and this information was displayed in a pie chart. Two groups consisting of 3 signal transducers and 1 channel regulator were too small to appear in the chart.

Fig. 3. Protein functional subgroups in the diapause proteome of *A. franciscana*. Each functional group of protein in the diapause proteome was divided into subgroups by using PANTHER. A, metabolic enzymes; B, binding; C, structural; D, transporter; E, translation. The last four groups are not shown because they did not divide into subgroups.

Area counts 10^{-3}

700
600
500
400
300
200
100
0

1 2

361

+ p26 - p26

Channel values

- Metabolism (44%)
- Binding (35%)
- Structure (11%)
- Transporters (5%)
- Translation (3%)
- Receptors (1%)
- Antioxidants (1%)
- Signal transducers (0.2%)
- Channel regulator (0.1%)