What we know currently about the Metalloproteins in the protozoa *Tetrahymena pyriformis* and *thermophila.*

Christos T. Chasapis*[1], Maria Stefanidou[2]

[1]Institute of Chemical Engineering Sciences (ICE-HT), Foundation for Research and Technology, Hellas (FORTH), GR-26504, Patras, Greece

[2]Department of Forensic Medicine and Toxicology, School of Medicine, National and Kapodistrian University of Athens, 75, Mikras Asias street, 11527, Goudi, Athens, Greece

*Corresponding Author: Email: cchasapis@iceht.forth.gr

***Abstract*—** In recent years, the protozoan *Tetrahymena* is used as a model to detect aquatic toxicity and eco-toxicological effects with its application as a "whole-cell biosensor" (WCB) to be the mostly known for the environmental monitoring of heavy metal pollution. This review attempts to summarize the current state of knowledge of identified metalloprotein coding genes in *Tetrahymena pyriformis* and *thermophile* species..

***Keywords*— metalloproteins, protozoan, Tetrahymena, whole-cell biosensor (WCB).**

I. INTRODUCTION

Metals are fundamental for the correct functioning of cells, playing an integral role in metabolic pathways and processes. Although, their excess is toxic and the metal availability should be tightly controlled [1-3]. Moreover, metalloproteins are widespread in living organisms. In particular, the average constitution of zinc proteins in prokaryotic organisms is (6.0% and 4.9% in archaea and bacteria respectively) which is lower in eukaryotic organisms (8.8%) [4,5]. Furthermore, proteome level analyses of the occurrence of nonheme iron proteins have shown that they constitute on average 7.1% of archaeal, 3,9% of bacterial and only of 1.1% of eukaryotic proteomes [4]. Copper proteins are less pervasive than zinc and nonheme iron proteins and typically account for less than 1% of an organism's proteome [6].

Tetrahymena is a non-pathogenic unicellular, free-living mobile ciliate protozoan that responds rapidly with great sensitivity to the presence of pollutants in nature, special metal toxicity [7]. This has resulted in them being used as test systems for assessing ecological risk [8]. Recently, the concept of "whole-cell biosensor" (WCB) has been introduced by several authors [9,10], as a very useful alternative to classical biosensors. Both prokaryotic and eukaryotic microorganisms have been used to design WCBs for metals [11,12]. Among eukaryotic microorganisms, ciliates offer specific advantages as environmental sensors: they do not have a cell wall in their vegetative stage, minimizing the sensitivity to environmental pollutants as well as delay the cell response [13]. Recently, *Tetrahymena pyriformis* and *thermophila* have been used to design WCBs to detect heavy metals in aquatic or soil samples [14-18]. In these WCB modules, a quantifiable molecular reporter is fused to specific gene promoter of metalloprotein, known to be activated by metals inducing their overexpression.

This article attempt to summarize the current state of knowledge of identified metalloproteins (binders of essential and not essential metals) in *Tetrahymena pyriformis* and *thermophila*, that are reviewed and manually annotated in the protein database of UniProtKB/Swiss-Prot. Furthermore, the new role of *Tetrahymena* as a potential whole-cell biosensor for monitoring heavy metal pollution, through the overexpression of metalloprotein targets, is discussed.

II. METALLOPROTEINS IN *TETRAHYMENA PYRIFORMIS*

The complete nucleotide sequence of its mitochondrial (mt) genome [19], revealed a linear molecule of 47,172 bp (78.7% A. T) excluding telomeric sequences. Based on the Uniprot catalog on proteins, there are 18 IDs for *T. pyriformis* proteome with "metal-binding" annotation and only 6 are reviewed (manually annotated in the protein database of UniProtKB/Swiss-Prot, Table 1) and the rest are unreviewed (automatically annotated in UniProtKB/TrEMBL). Since there are many difficulties involved in identifying metalloproteins found in even the most basic of life forms, according to recent studies in archaea *Pyrococcus furiosus* [20], it is expected to exist encoded metalloproteins in the *Tetrahymena pyriformis* genome that are not yet uncovered.

TABLE 1
THE REVIEWED METAL-BINDING PROTEINS ENCODED BY *TETRAHYMENA PYRIFORMIS* GENOME PROVIDED FROM UNIPROT CATALOG

Uniprot Entry	Gene name	Entry name	EC number	Protein name	Metal-binding	Essential metal	Status	PubMed ID
P11947	COI	COX1_TETPY	1.9.3.1	Cytochrome c oxidase subunit 1	Fe/Cu	Yes	reviewed	2833363
P00079	-	CYC_TETPY	-	Cytochrome c	Fe	Yes	reviewed	187170
P17724	-	TRHBN_TETPY	-	Group 1 truncated hemoglobin	Fe	Yes	reviewed	8485156; 2111321
P19666	-	SODF_TETPY	1.15.1.1	Superoxide dismutase [Fe]	Fe	Yes	reviewed	2170391
O97388	-	MT1_TETPY	-	Metallothionein-1 (MT-1)	Cd	No	reviewed	10393238; 7813475
P02598	-	CALM_TETPY	-	Calmodulin (CaM)	Ca	Yes	reviewed	1339295; 1703538; 6114734

2.1 Iron and copper binding proteins

2.1.1 *Cytochrome C Oxidase Subunit I*

Complete nucleotide sequence of the *Tetrahymena pyriformis* mitochondrial (mt) genome released one iron/copper binding protein-coding gene: Cytochrome *c* Oxidase Subunit I (cox1 gene) [19, 21]. In *T. pyriformis*, mtDNA-encoded proteins display unusual sequence characteristics that are not seen in the same proteins in other eukaryotes. In the case of cox1 gene, the predicted protein has 698 amino acids, including an N-terminal 48 amino acid extension and a 109 amino acid insert, in comparison with human COX1. These extension and insert segments, originally found in eukaryotic *Paramecium Aurelia* [22], are not highly hydrophobic but are relatively rich in lysine, arginine and serine. *Tetrahymen*a COX1 shows a 64% amino acid identity with *Paramecium* but less than a 38% amino acid conservation with human (Fig 1) [22]. Furthermore, *Tetrahymen*a COX1 has the typical bimetallic center that is conserved in human COX1 and it is formed by heme A3 iron and copper B. The predicted metal binding sites, curated by Uniprot, of iron are His111, 636 and 538 and of Copper B center are His401, Tyr405, His450 and His451.

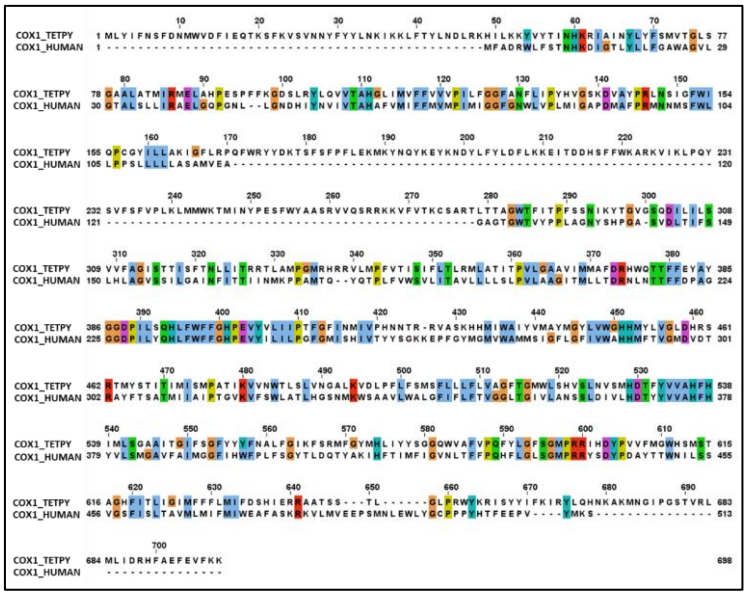

FIGURE 1: SEQUENCE ALIGNMENT OF *T. PYRIFORMIS* (P11947) WITH HUMAN (P00395) COX1. ACCESSION NUMBERS IN PARENTHESES ARE THOSE OF THE UNIPROT DATABASE.

2.2 Iron binding proteins

2.2.1 *Cytochrome C*

The amino acid sequence of the *T. pyriformis* Cytochrome C has reported a long time ago and it consists of 109 residues [23]. The two highly conserved cysteines in all prokaryotic and eukaryotic Cytochromes, which are responsible for the heme attachment, are located in the postilion 25 and 28. Furthermore, the His29 and Met88 are the ligands for the axially coordination of heme iron. Although, there are considerable differences from amino acid sequences of other known eukaryotic and human mitochondrial cytochrome c, such as lower isoelectric point (which interpret as an affinity with bacterial cytochromes), an extra-long N-terminal portion and short C-terminal portion and absence of a basic residue and of glutamine in the positions (Gln24 and Ala27) just before the first and second heme attachment respectively (Fig 2).

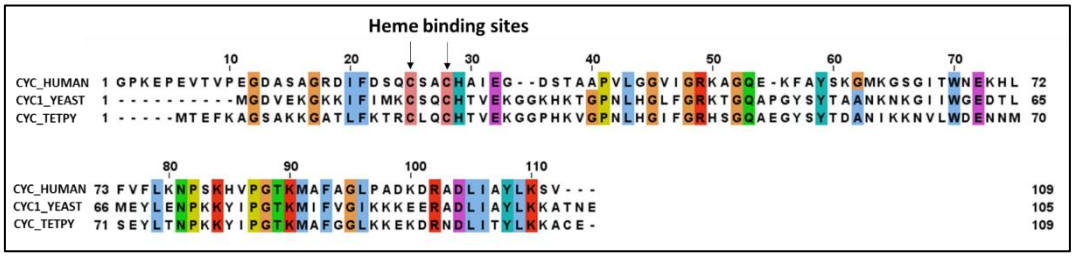

FIGURE 2: MULTIPLE SEQUENCE ALIGNMENT OF *T. PYRIFORMIS* (P00079) WITH *SACCHAROMYCES CEREVISIAE* (P00044) AND HUMAN CYTOCHROME C (P99999). ACCESSION NUMBERS ARE THOSE OF THE UNIPROT DATABASE.

2.2.2 *Hemoglobin*

Comparison between *T. pyriformis* and *T. thermophila* reveal that their sequences were 83.5% identical to each other and homologous to other protozoan and cyanobacteria hemoglobins, but not to proteins of the human globin family (shows an 18%, 16% and 13% amino acid identity with human hemoglobin subunit alpha, beta and gamma respectively) [24]. So, protozoan and cyanobacteria hemoglobins make a unique group in the globin family. (Fig. 3A). The crystal structure of the Fe(II)–O2 complex of *T. pyriformis* trHb was determined at 1.73-Å resolution (pdb id: 3AQ5) and O2-binding properties were measured [25]. The X-ray structure consists of two molecules of *T. pyriformis* which are present in an asymmetric unit (Fig3 B). *T. pyriformis* trHb showed a typical two-over-two a-helical sandwich fold. Tyr25 and Gln46 were hydrogen-bonded to a heme-bound O(2) molecule. Tyr25 donated a hydrogen bond to the terminal oxygen atom, whereas Gln46 hydrogen-bonded to the proximal oxygen atom. Site-directed mutations of hydrogen-bonding donor or acceptor residues greatly enhanced autooxidation from the Fe(II)–O2 to the Fe(III) forms of *T. pyriformis* trHb. Structural studies of mutant Fe(III) complexes of *T. pyriformis* trHbs showed that loss of hydrogen bonding destabilized the bound O2 molecule, resulting in fast O2 dissociation and autooxidation. The reaction between NO and a Fe(II)–O2 complex of *T. pyriformis* trHb, in a crystal state, resulted in the formation of a Fe(III)–H2O complex. These data indicate that *T. pyriformis* trHb may play a role in NO detoxification and that the primary function of the protein may not involve O2 storage [25].

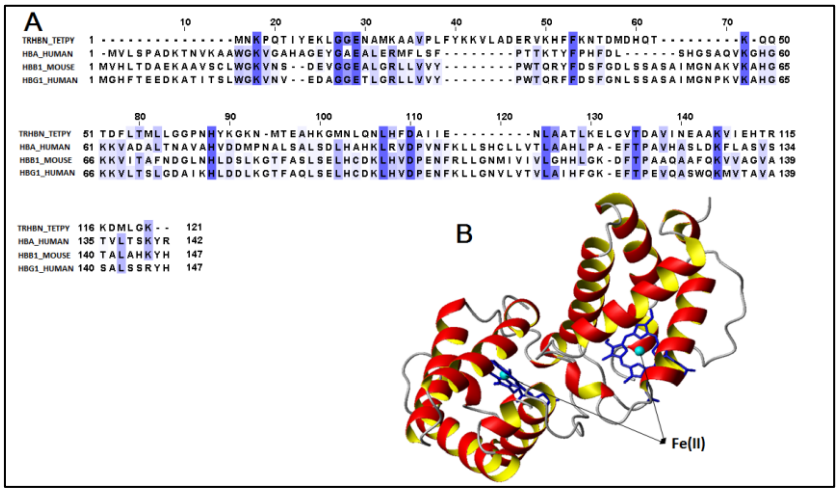

FIGURE 3: A: MULTIPLE SEQUENCE ALIGNMENT OF *T. PYRIFORMIS* (P17724) WITH MOUSE (P02088) AND HUMAN (P69905, P69891) HEMOGLOBINS; B: CRYSTAL STRUCTURE OF *TETRAHYMEN*A TRHB, FE(II)-O_2 FORM (PDB ID: 3AQ5). ACCESSION NUMBERS ARE THOSE OF THE UNIPROT DATABASE.

2.2.3 *Superoxide dismutase [Fe]*

An iron containing superoxide dismutase has been isolated from *T. pyriformis* in the tetrameric form [26]. It has a molecular weight of 85,000 and is composed of four identical subunits.Comparison of sequences of other iron-containing superoxide dismutases reveals a relatively low degree of identity (33-34%). However, a higher percentage identity is found with mammalian manganese-containing superoxide dismutases (41-42%). Crystallographic data available for a number of both Fe and Mn-SODS have revealed extensive structural similarities of the two classes of isoenzymes. In particular, residues (Gly92, Gly93, Gln167, Gln168) occurring within a sphere of 10 Å radius from the metal cofactor in human and mouse Mn-SODs are also conserved in T. *pyriformis* but no in prokaryotic Fe-SOD (Fig. 4) [26-29].

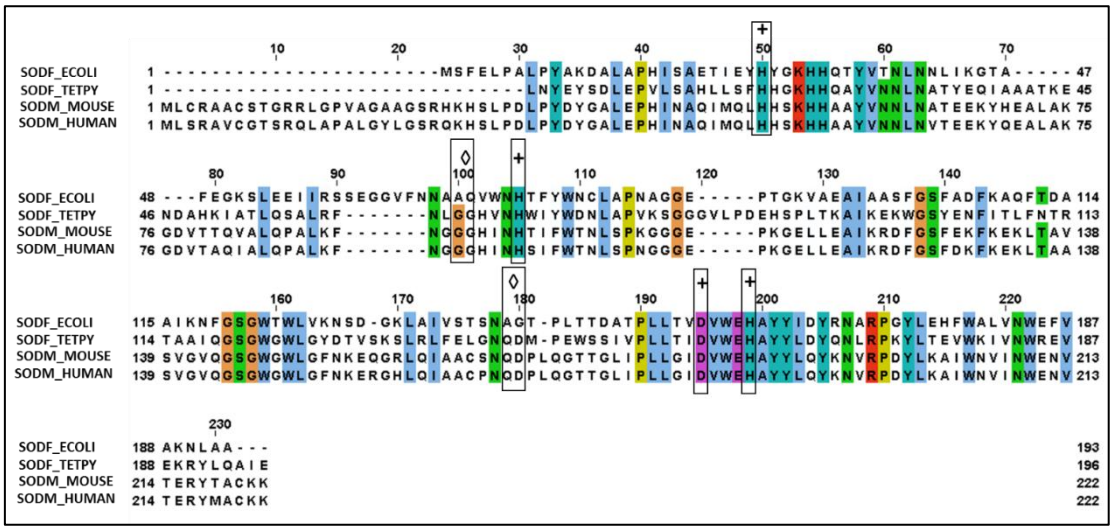

FIGURE 4: COMPARISON OF AMINO ACID SEQUENCE OF Mn-SOD FROM HUMAN (P09671), MOUSE (P04179) AND Fe-SOD FROM *T. PYRIFORMIS* (P19666) AND *E.COLI* (P0AGD3). METAL LIGANDS ARE INDICATED WITH SMALL CROSSES AND RESIDUES OCCURRING WITHIN A SPHERE OF 10 Å RADIUS FROM THE METAL COFACTOR IN SOD_S WITH RHOMBUS. ACCESSION NUMBERS IN PARENTHESES ARE THOSE OF THE UNIPROT DATABASE.

2.3 Cadmium binding proteins

2.3.1 *T. pyrimorfis Metallothioneins (TpMTs)*

Metallothioneins are rich in cysteines, which participate in metal binding. MTs of *Tetrahymena* not only play a role in heavy metal homeostasis and detoxification but also respond to a wide variety of stimulants [30-31]. TpMTs are been considered to fall into two groups: Member of the first group is the TpMT-1 metalloprotein, that is the first ciliate Metallothionein that was isolated from *T.pyriformis*. The mRNA and protein levels of TpMT-1 increase significantly with Cd concentration in the medium [32]. Member of the second group is TpMT-2, where the mRNA expression pattern did not fluctuate as much higher than that of TpMT-1 under the Cd effects, instead of exhibiting higher lever under the exposure of Cu [32].

2.4 Calcium binding proteins

2.4.1 *Calmodulin (CaM)*

The cDNA encoding *T. pyriformis* Calmodulin was isolated and characterized, revealing that CaM consists of 149 residues. It has four putative calcium-binding loops (D21-E32, D57-E68, D94-E105, D130-E141) homologous to EF-hand calcium-binding sites which are highly conserved in CaM proteins [33,34] (Fig 5). It is reported that CaM interact with adenylyl cyclase (AC) and influence and regulate AC activity of ciliate T. pyriformis [35,36].

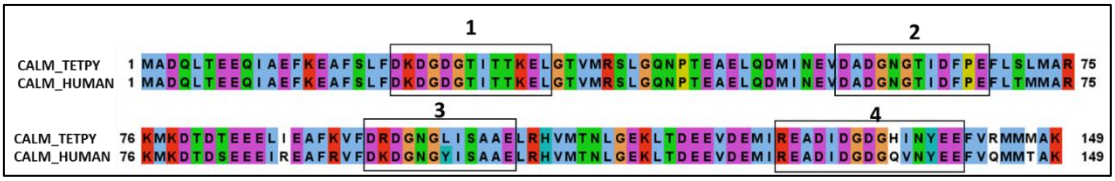

FIGURE 5: COMPARISON OF AMINO ACID SEQUENCE OF CAM FROM *T. PYRIFORMIS* (P02598) AND HUMAN (P62158). METAL BINDING SITES ARE INDICATED WITH NUMBERS. ACCESSION NUMBERS IN PARENTHESES ARE THOSE OF THE UNIPROT DATABASE.

III. METALLOPROTEINS IN *TETRAHYMENA THERMOPHILA*

Sequencing and analysis of *T. thermophila* genome (strain SB210), revealed 104 Mb length and composition of approximately 225 chromosomes with more than 27,000 predicted protein-coding genes. It is remarkable, that 15,000 genes out of them are well conserved in genomes of other organisms [37]. Based on the UniProt catalog, there are 431 entries for *T. thermophila* proteome with "metal-binding" annotation and from these only 3 are reviewed (manually annotated in the protein database of UniProtKB/Swiss-Prot, Table 2) and the rest are unreviewed (automatically annotated in UniProtKB/TrEMBL).

TABLE 2
THE REVIEWED METAL-BINDING PROTEINS ENCODED BY *TETRAHYMENA THERMOPHILA* GENOME PROVIDED FROM UNIPROT CATALOG.

Uniprot Entry	Gene name	Entry name	EC number	Protein name	Metal-binding	Essential metal	Status	PubMed ID
A4VDN2	FEN1,	FEN1_TETTS	3.1.-.-	Flap endonuclease 1	Mg	Yes	reviewed	16933976
Q230X8	HEN1	HENMT_TETTS	2.1.2.n8	Small RNA 2'-O-methyltransferase	Mg	Yes	reviewed	19240163 16933976
P0DJ24	RPL37	RL37_TETTS	-	60S ribosomal protein L37	Zn	Yes	reviewed	16933976;

3.1 Magnesium binding proteins

3.1.1 *Flap endonuclease , FEN1*

Flap endonucleases are a class of nucleolytic enzymes that act as both 5'-3' exonucleases and structure-specific endonucleases on specialized DNA structures during DNA replication, repair, and recombination processes. Flap endonucleases have been identified in eukaryotes, prokaryotes, archaea, and some viruses. Divalent metal ions, such as Mg^{+2}, are essential cofactors in the FEN's biological functions and structural studies of several flap endonucleases reveal that they have two conserved metal-binding sites with conserved aspartate and glutamate residues [37]. The FEN1 *T.thermpophila* protein has 384 amino acids length and its two binding sites are occupied by aspartates and glutamates: (Asp34, Asp90, Glu162, Glu164) and (Asp183, Asp185, Asp234) respectively.

3.1.2 *HEN1, Small RNA 2'-O-methyltransferase*

Methyltransferase adds a 2'-O-methyl group at the 3'-end of piRNAs. In the ciliated protozoan *Tetrahymena* thermophila, two classes of small RNAs have been identified: RNAs approximately 28-29 nt long (scnRNAs) and constitutively expressed approximately 23-24 nt siRNAs. It is reported that scnRNAs, but not siRNAs, are 2'-O-methylated at their 3' ends. The *Tetrahymena* HEN1 homolog Hen1p is responsible for scnRNA 2'-O-methylation. Loss of Hen1p causes a reduction in the length of scnRNAs, defects in programmed DNA elimination, and inefficient production of sexual progeny. The reported 3.1 Å crystal structure of full-length HEN1 from Arabidopsis in complex with a 22-nucleotide small RNA duplex and cofactor product S-adenosyl-L-homocysteine describe a Mg^{2+}-dependent 2´-O-methylation mechanism. Treatment with increasing concentrations of EDTA that chelates Mg in the reaction eventually eliminates HEN1 activity suggesting that HEN1 is indeed a Mg^{2+}- dependent small RNA methyltransferase. Mutation of any of coordinated residues to alanines completely abolished HEN1 activity [38]. HEN1 protein from *T.thermophila* consists of 423 amino acids and four metal binding sites which are highly conserved in homologs HEN1 (Glu124, Glu127, His128, His177) [38].

3.2 Zinc binding proteins

3.2.1 *60S ribosomal protein L37, RPL37*

Eukaryotic ribosomes are considerably larger than their bacterial counterparts. As a consequence, the eukaryotic 60S subunit in yeast or *T. thermophila* has a total molecular weight of about 2 million daltons, whereas that of the 50S subunit in *E. coli* is 1.3 million daltons. The increased level of structural complexity of eukaryotic ribosomes reflects functional differences between prokaryotes and eukaryotes. In the past, two crystal structures of *T.thermophila* ribosomal subunits were reported : the 3.5 Å X-ray structure of 60S subunit in complex with initiation factor 6 (eIF6), cocrystallized with the antibiotic cycloheximide [39] and the 3.7 Å X-ray structure of 40S subunit in complex with eIF1 and eIF1A initiation factors

[40,41,42]. The structure of the 60S ribosomal subunit contains 42 proteins of which 16 are present in all domains of life, 20 are shared between eukarya and archaea, and 6 are eukaryotic-specific. Most RPs (ribosomal proteins) contain eukaryotic-specific extensions which are critical for establishing an intricate protein- RNA network. One of this type protein is the RPL37, that adopts a C4-type zinc finger fold where the metal binding residues are four cysteines : C19, C22, C34, C37 (Fig 6).

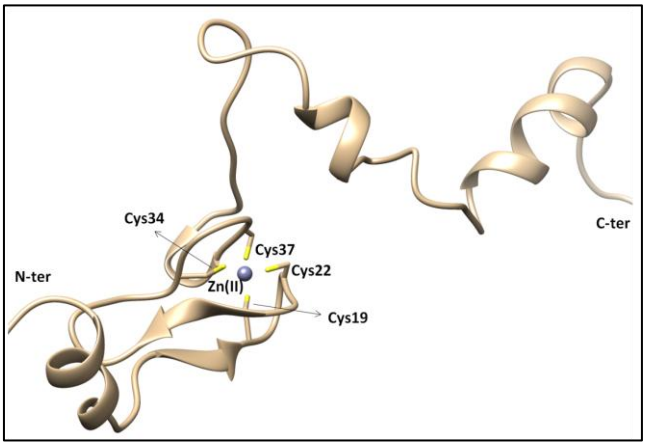

FIGURE 6: METAL BINDING SITE OF THE X-RAY STRUCTURE OF *T.THERMOPHILA* RIBOSOMAL PROTEIN L37 (PDB ID: 4V8P).

3.3 Cadmium binding proteins

3.3.1 *T.thermophila Metallothioneins (MTTs)*

The metallothioneins genes that were isolated from *T.thermophila* are reported as MTT1, MTT2, MTT3, MTT4 and MTT5 [44-48]. Sequence comparisons have revealed that MTT3 and MTT5 share a regular, conserved and hierarchical modular structure with MTT1 and *T.pyriformis* TpMT1 and TpMT2. MTT1 MTT3 and MTT5 are more efficiently induced by Cd than Cu and behave as multi-stress response proteins [45]. All the known metallothioneins from the ciliate genus *Tetrahymena* were subdivided into two well-defined subfamilies, 7a and 7b based on four criteria: phylogenetic analysis *Tetrahymena* MTs, their cysteine clusters position, the location of Lys relative to Cys residues, and the differential induction of gene expression by the heavy metals Cd and Cu. Subfamily 7a includes *T. thermophila* MTT1, MTT3 and MTT5 and *T. Pyriformis* MT-1 and MT-2. Subfamily 7b consists of *T. thermophila* MTT2 and MTT4, as well as *T. pigmentosa* MT-2 [45].

IV. *IN SILICO* INTERACTOME NETWORKS OF *T.THERMOPHILA* METALLOPROTEINS THAT ARE ANNOTATED IN UNIPROT/SWISS-PROT

Applying STRING web tool (http://string-db.org/), we provide the predicted protein-protein interaction (PPI) network (Figure 7) of the three *T. thermophila* proteins FEN1, HEN1, RPL37 that are reviewed and manually annotated in the protein database of UniProtKB/Swiss-Prot. The PPI information reported in STRING is derived from the following four sources: genomic context, high-throughput experiments, (conserved) coexpression, and previous knowledge, indicating that they can widely measure the associations between proteins, including direct (physical) and indirect (functional) associations. The interaction score indicates the strength of the interaction [49].

The top 10 interactors (with highest confidence score: 0,98) from *T. thermophila* proteome that are interconnected with FEN1 are listed below: ATP-dependent DNA helicase, RecQ family protein (Q23ED9); Proliferating cell nuclear antigen (Q22BB4); DNA repair protein RAD50, putative (I7MFI1); DNA ligase I, ATP-dependent proteins (I7LWR8, Q24FD9); BRCT domain protein (Q22DN2); DNA polymerase (Q22HL1); Ser/thr phosphatase family protein (Q22G12); DNA replication helicase Dna2, putative (I7MF71) and ATP-dependent DNA helicase and RecQ family protein (Q22AI7). The 10 most confident interactions (score: 0,77) for the HEN1 network link the protein with Piwi-like proteins (A4VE05, A4VE06, A8VSS4, A8VSR3, I7M7N0, Q0GM58, Q0MRE2, Q23ND1), Twi12p (A4ZYY6) and programmed DNA degradation 2 protein (O15645). Finally, RPL37 is interconnected with 60S ribosomal proteins (with confidence score 0,99) : L18a (P0DJ18), L35a (P0DJ22), L34 (P0DJ23), L6 (P0DJ56), L13 (P0DJ58), L30 (P0DJ59), L36a (Q22X38), L22 (Q23BV5), L14 (Q24C27) and L36 **(**Q24F59).

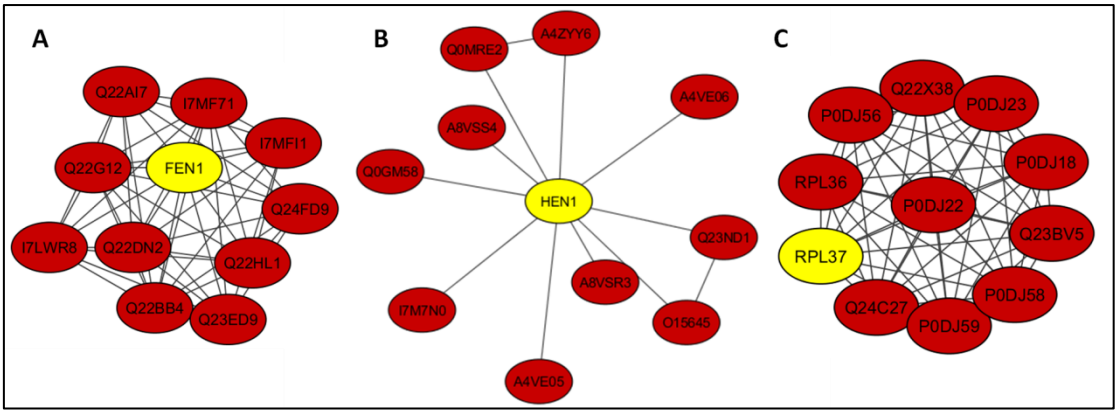

FIGURE 7: INTERACTION NETWORKS OF FEN1 (A), HEN1 (B), RPL37 (C) PROTEINS FROM *T. THERMOPHILA* PROTEOME. ACCESSION NUMBERS ARE THOSE OF THE UNIPROT DATABASE.

V. APLICATIONS OF TETRAHYMENA METALLOPROTEIN CODING GENES IN HEAVY METAL POLLUTION

Tetrahymena is a genus of free-living ciliated protozoa which is ubiquitous in freshwater environments. Due to its convenience for lab cultivation and sensitivity toward environmental contamination, *Tetrahymena* has been utilized as a model to detect aquatic toxicity and eco-toxicological effects for many years. A novel role of *T thermophila* ciliates is that could be used as whole-cell biosensors (WCBs) or as a potential cellular source of molecular biomarkers/biosensors to detect pollutants (such as heavy metals) in environmental samples [13]. *T. thermophila* MT promoters might be used to design metal biosensors and especially the *MTT1* or *MTT5* genes due to their rapid and strong induction by metals [44,50]. The first example of ciliate-based WCBs were the MT promoters from *T. thermophila* linked with the luciferase reporter gene, to detect heavy metals in aquatic or soil samples [15]. These *T thermophila* WCBs (strains MTT1Luc and MTT5Luc), which have been validated using natural samples, exclusively detect bioavailable metals and demonstrate a high and differential sensitivity in both artificial and natural samples [15]. Recently, it was introduced an alternative WCB using the green fluorescent protein (GFP) as a reporter gene and the CdMT promoter from the *T. thermophila MTT1* gene [14]. In this case, the metal exposure in the novel strains induces overexpression of metallothionein genes themselves. The use of *gfp* as a reporter gene has the advantage that the reporter signal (fluorescence) can be detected in vivo while it can be easily detected by fluorescence microscopy and quantified by flow cytometry.

VI. CONCLUSION

This critical review attempts to provide a detailed characterization of the metalloproteins from the *T. pyriformis* and *T. thermophila* proteomes that are reviewed and manually annotated in the protein database of UniProtKB/Swiss-Prot. We don't give any description of the unreviewed Uniprot entries that are deposited and automatically annotated in UniProtKB/TrEMBL since proteins predicted by the TrEMBL database could be hypothetical. In the most cases presented in this review, metals work as cofactors of enzymes related to the biology of *Tetrahymena* ciliate protozoa. Apart from *Tetrahymena* metalloenzymes, both *T. pyriformis* and *T. thermophila* proteomes contain (non essential-metal) binding proteins (like Cd-metallothioneins that are involved in the cellular metal-detoxification process). In conclusion, the most of *Tetrahymena* metalloproteins have not yet been identified (in *T. thermophila* proteome only 3 proteins out of 27000 Uniprot IDs are annotated as metalloproteins) indicating that the roles of nanny metals in protozoan's biological systems are still unknown. Thus, further computational or/and experimental studies are required to identify and annotate more metalloproteins in these *Tetrahymena* species.

REFERENCES

[1] C Andreini, I Bertini, G Cavallaro, GL Holliday, JM Thornton (2008) Metal ions in biological catalysis: from enzyme databases to general principles. J.Biol.Inorg.Chem.13: 1205-1218
[2] M Valko, H Morris, M T Cronin (2005) Metals, toxicity and oxidative stress.Curr. Med. Chem.12: 1161–1208
[3] M R Bleackley, R T Macgillivray (2011) Transition metal homeostasis: from yeast to human disease Biometals 24: 785–809
[4] C Andreini, I Bertini, A Rosato (2009) Metalloproteomes: a bioinformatic approach. Acc Chem Res.:42(10):1471-9
[5] C Andreini, L Banci, I Bertini, A Rosato (2006) Zinc through the three domains of life. J Proteome Res. 5(11):3173-8.

[6] TV O'Halloran, VC Culotta, (2000) Metallochaperones, an intracellular shuttle service for metal ions. J Biol Chem. 18;275 33:25057-60.
[7] M Stefanidou, G Alevizopoulos, C Spiliopoulou (2008) DNA content of Tetrahymena pyriformis as a biomarker for different toxic agents. Chemosphere. 74(1):178-80
[8] MP Sauvant, D Pepin, E Piccinni (1999) Tetrahymena pyriformis: a tool for toxicological studies. A review. Chemosphere. 38(7):1631-69.
[9] S Belkin, (2003) Microbial whole-cell sensing systems of environmental pollutants. Curr. Opin Microbiol 6:206-212
[10] M Park, SL Tsai, W Chen (2013) Microbial biosensors: engineered microorganisms as the sensing machinery. Sensors 13(5):5777-95
[11] S Magrisso, Y Erel, S Belkin (2008) Microbial reporters of metal bioavailability. Microb Biotechnol 1:320–330
[12] JR van der Meer, S Belkin (2010) Where microbiology meets microengineering: design and applications of reporter bacteria. Nat Rev Microb 8:511–522
[13] JC Gutierrez, A Martin-Gonzalez, S Diaz, F Amaro, R Ortega, A Gallego, MP Lucas Ciliates as cellular tools to study the eukaryotic cell – heavy metal interactions In: SE Brown, WC Welton. Nova Science Publishers, New York, pp 1-44
[14] F Amaro, AP Turkewitz, A Martín-González, JC Gutiérrez, (2014) Functional GFP-metallothionein fusion protein from Tetrahymena thermophila: a potential whole-cell biosensor for monitoring heavy metal pollution and a cell model to study metallothionein overproduction effects. Biometals 27(1):195-205
[15] F Amaro, AP Turkewitz, A Martín-González, JC Gutiérrez. (2011) Whole-cell biosensors for detection of heavy metal ions in environmental samples based on metallothionein promoters from Tetrahymena thermophila. Microb Biotechnol. (4):513-22
[16] JN Park, MJ Sohn, DB Oh, O Kwon, SK Rhee, CG Hur (2007) Identification of the cadmium-inducible Hansenula polymorpha SEO1 gene promoter by transcriptome analysis and its application to whole-cell heavy-metal detection systems.Appl Environ Microbiol. 73:5990–6000
[17] SF D' Souza, (2001) Microbial biosensors.Biosens Bioelectron.;49:337–353
[18] A. La Terza, S. Barchetta, F. Buonanno, C. (2008) The protozoan ciliate *Tetrahymena thermophila* as biosensor of sublethal levels of toxicants in the soil.Miceli Fresenius Environmental Bulletin Vol.17 (n 8b):1144-1150
[19] G Burger, Y Zhu, TG Littlejohn, SJ Greenwood, MN Schnare, BF Lang, MW Gray, (2000) Complete sequence of the mitochondrial genome of Tetrahymena pyriformis and comparison with Paramecium aurelia mitochondrial DNA J Mol Biol., 297(2):365-80
[20] J Edqvist, G Burger and MW Gray (2000) Expression of mitochondrial protein-coding genes in Tetrahymena pyriformis J Mol Biol. 297: 381-393
[21] A Cvetkovic, AL Menon, MP Thorgersen, JW Scott, FLII Poole, FE Jenney et al. (2010) Microbial metalloproteomes are largely uncharacterized. Nature 466: 779–782
[22] Z Ziaie, Y Suyama (1987) The cytochrome oxidase subunit I gene of Tetrahymena: a 57 amino acid NH2-terminal extension and a 108 amino acid insert. Curr Genet. 12(5):357-68
[23] GE Tarr, WM Fitch (1976) Amino acid sequence of cytochrome c from Tetrahymena pyriformis Phenoset A. Biochem J. 159(2):193-9
[24] T Takagi, H Iwaasa, H Yuasa, K Shikama, T Takemasa, Y Watanabe, (1993) Primary structure of Tetrahymena hemoglobins. Biochim Biophys Acta. Apr 29;1173(1):75-8.
[25] J Igarashi, K Kobayashi, A Matsuoka A hydrogen-bonding network formed by the B10-E7-E11 residues of a truncated hemoglobin from *Tetrahymena pyriformis* is critical for stability of bound oxygen and nitric oxide detoxification J Biol Inorg Chem. (2011) 16(4):599-609
[26] D Barra., ME Schinina., F Bossa, K Puget, P Durosay, A Guissani, AM Michelson, (1990) A tetrameric iron superoxide dismutase from the eucaryote *Tetrahymena pyriformis*. J. Biol. Chem. 265:17680-17687
[27] MW Parker and CCF Blake (1988) Crystal structure of manganese superoxide dismutase from Bacillus stearothermophilus at 2.4 A resolution. J. Mol. Biol. 199: 649- 661
[28] D Ringe, GA Petsko, F Yamakura, K Suzuki, D Ohmori (1983) Structure of iron superoxide dismutase from Pseudomonas ovalis at 2.9-A resolution. Proc. N&l. Acud. Sci. U. S. A. 80, 3879-3883
[29] A Carlioz, ML Ludwig, WC Stallings, JA Fee, HM Steinman, D Touati (1988) Iron superoxide dismutase. Nucleotide sequence of the gene from Escherichia coli K12 and correlations with crystal structures. J. Biol. Chem. 263,1555-1562
[30] F Boldrin, G Santovito, P Irato, E Piccinni, (2002) Metal interaction and regulation of Tetrahymena pigmentosa metallothionein genes. Protist 153: 283–291
[31] F Dondero, M Cavaletto, AR Ghezzi, A LaTerza, M Banni, A Viarengo, (2004) Biochemical characterization and quantitative gene expression analysis of the multi-stress inducible metallothionein from Tetrahymena thermophila Protist 155: 157–68.
[32] C Fu, W Miao (2006) Cloning and characterization of a new multi-stress inducible metallothionein gene in Tetrahymena pyriformis. Protist 157: 193–203
[33] T Takemasa , T Takagi, M Edamatsu, Y Watanabe (1992) Calmodulin cDNAs from two species of Tetrahymena. Biochim Biophys Acta., 1132(2):219-21
[34] M Yazawa, K Yagi, H Toda, K Kondo, K Narita, R Yamazaki, K Sobue, S Kakiuchi, S Nagao, Y Nozawa (1981) The amino acid sequence of the Tetrahymena calmodulin which specifically interacts with guanylate cyclase. Biochem Biophys Res Commun. 99(4):1051-7.

[35] S Nagao, Y Nozawa (1985) Calmodulin-binding proteins of Tetrahymena microsomal membranes. Biochem. Physiol. B. 82: 689–693 103

[36] JE Schultz, S Klumpp, (1984) Calcium/calmodulin-regulated guanylate cyclases in the ciliary membranes from Paramecium and tetrahymena. Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17: 275–283

[37] Eisen JA, Coyne RS, Wu M, Wu D, Thiagarajan M, Wortman JR, et. Al. Macronuclear genome sequence of the ciliate Tetrahymena thermophila, a model eukaryote. PLoS Biol. 2006, (9):e286

[38] Y Huang, LJi, Q Huang, DG Vassylyev, X Chen & JB Ma, (2009) Structural insights into mechanisms of the small RNA methyltransferase HEN1.Nature 461(7265):823-7,

[39] VG Panse, AW Johnson, (2010) Maturation of eukaryotic ribosomes: acquisition of functionality.Trends Biochem. Sci. 35: 260-6

[40] S Klinge, F Voigts-Hoffmann, M Leibundgut, S Arpagaus, N Ban (2011) Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6. Science. 334 (6058):941-8.

[41] J Rabl, M Leibundgut, SF Ataide, A Haag, N Ban (2011) Crystal structure of the eukaryotic 40S ribosomal subunit in complex with initiation factor 1. Science 331(6018) 730-6

[42] M Weisser, F Voigts-Hoffmann, J Rabl, M Leibundgut, N Ban The crystal structure of the eukaryotic 40S ribosomal subunit in complex with eIF1 and eIF1A Nat Struct Mol Biol. (2013) (8):1015-7

[43] CD Klaassen, J Liu, S Choudhuri (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. Ann Rev Pharmacol Toxicol 39: 267–294

[44] F Boldrin, G Santovito, J Gaertig, D Wloga, D Cassidy-Hanley, et al. (2006) Metallothionein gene from Tetrahymena thermophila with a copper-inducible-repressible promoter. Eukaryot Cell 5: 422–425

[45] S Díaz, F Amaro, D Rico, V Campos, L Benítez, A Martín-González, EP Hamilton, E Orias, JC Gutiérrez (2007) Tetrahymena metallothioneins fall into two discrete subfamilies PLoS One. 2(3):e291.

[46] F Boldrin, G Santovito, A Formigari, Y Bisharyan, D Cassidy-Hanley, TG Clark, E Piccinni MTT2, a copper-inducible metallothionein gene from Tetrahymena thermophila. (2008) Comp Biochem Physiol C Toxicol Pharmacol. 147(2):232-40

[47] Gutiérrez JC, Amaro F, Díaz S, de Francisco P, Cubas LL, Martín-González A. Ciliate metallothioneins: unique microbial eukaryotic heavy-metal-binder molecules. (2011) J Biol Inorg Chem Oct;16(7):1025-34.

[48] Gutiérrez JC, Amaro F, Martín-González A. (2009) From heavy metal-binders to biosensors: ciliate metallothioneins discussed Bioessays. Jul;31(7):805-16

[49] A. Franceschini, D. Szklarczyk, S. Frankild, M. Kuhn, M. Simonovic, A. Roth, J. Lin, P. Minguez, P. Bork, C. von Mering (2013) STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res., 41 pp. D808–15

[50] Shang Y, Song X, Bowen J, Corstanje R, Gao Y, Gaertig J, Gorovsky MA. A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression and overexpression of homologous and heterologous genes in *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. USA.2002;97:3734–3739