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Unravelling stress granules in the deep cold: Characterisation of TIA-1 gene sequence in Antarctic fish species

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ABSTRACT

Stress granules (SGs) are cytoplasmic foci lacking membranes, comprising non-translating messenger ribonucleoproteins, translational initiation factors, and additional proteins. Their formation is crucial for rapidly modulating gene expression in response to adverse environmental conditions, such as pollution and infections. Limited research has focused on investigating the molecular components of SGs in fish, with minimal exploration in Antarctic fish. This study characterises for the first time the transcript sequences of one key protein component of SGs, TIA-1 (T-cell intracellular antigen 1), in two Antarctic endemic fish species, i.e. *Trematomus bernacchii* and *Chionodraco hamatus*. The mRNA-binding protein TIA-1 acts as a post-transcriptional regulator of gene expression and its aggregation leads to the formation of SGs in response to cellular damage. The *in vitro* and bioinformatic analyses of the TIA-1 gene sequences of these two species highlighted interesting peculiarities, which include the transcription of alternatively spliced isoforms unique to the notothenioid lineage, potentially unlocking further insights into their unique adaptations to extreme environmental conditions. This is the first study to analyze *tia-1* expression levels in different tissues of Antarctic fish species. Our key findings indicate that the TIA-1 gene is expressed at particularly high levels in the liver and spleen of *C. hamatus*, as well as in the heart and skeletal muscle of *T. bernacchii*. This suggests that those tissues play a significant role in the stress response mechanisms of the studied species. This study provides novel insights into the molecular adaptations of Antarctic fish, highlighting the potential importance of TIA-1 in their response to environmental stressors. The unique features of TIA-1 identified in these species may offer broader implications for understanding how Antarctic fish regulate gene transcriptions in their extreme environments.

1. Introduction

Stress granules (SGs) are cytoplasmic foci forming in eukaryotic cells, lacking membranes, with a size ranging from 0.1 to 2.0 μm [1–3]. Typically, SGs contain mRNAs stalled in translational initiation, 40 S ribosomal subunits, essential translation initiation factors (eIF4E, eIF4G, eIF4A, eIF4B, eIF3, eIF2), mRNA binding proteins (e.g. TIA-1, TIAR, PABP), acting as translational repressors, and additional proteins (e.g. post-translation modification enzymes, metabolic enzymes, proteins for RNA remodelling), which can affect SG assembly and disassembly [1,2,4–8]. These granules are dynamic structures that allow the storage of selected mRNAs during periods of stress and subsequent recovery [9], thus serving as a fundamental mechanism for rapidly adjusting gene expression in response to stress, such as oxidative stress, heat shock and exposure to toxins [10]. Previous studies have shown that the development of SGs has been observed in response to viral infections [11], genetic diseases [12], tumours [13] and other diseases. The eukaryotic initiation factor 2 alpha (eIF2α) phosphorylation can trigger SG aggregation [1,3]. This process inhibits or delays translation initiation by reducing the availability of the ternary complex eIF2α-GTP-tRNAi Met, leading to a stall in translation [4,14].

T-cell restricted antigen (TIA) proteins, particularly TIA-1 and TIA-1 related nucleolysin (TIAR), are DNA/RNA binding proteins that exhibit dynamic shuttling between the nucleus and the cytoplasm. This shuttling enables them to modulate transcriptional and post-transcriptional

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Abbreviations:

SG	Stress granule
TIA-1	T-cell intracellular antigen 1
eIF	eukaryotic initiation factor
TIAR	TIA-related nucleolysin
RRM	RNA recognition motifs
PrD	Prion-related domain
UTR	Untranslated region
A	adenine
U	uridine
C	cysteine
ROS	Reactive oxygen species
LiCl	Litium chloride
qRT-PCR	quantitative real time PCR
AIC	Akaike information criterion
cAIC	corrected AIC
BIC	Bayesian information criterion
BI	Bayesian inference
ML:	maximum likelihood
MCMC	Markov Chain Monte Carlo
SLAC	Single-Likelihood Ancestor Counting
FEL:	Fixed Effects Likelihood
aBSREL:	adaptive Branch Site random effect likelihood
MEME	Mixed Effects Model of Evolution
Q	glutamine
L:	long
I	intermediate
S	short
H_2O_2	hydrogen peroxide
Prdx2	peroxiredoxin 2

processes within the cell [15]. They are well-established components in the formation of SGs and serve as reliable markers of these structures [9]. The ability of TIA proteins to modulate gene expression confers a crucial functional role in antiviral, inflammatory, immune, and possibly oncogenic and ageing-associated responses, among others [16]. TIA-1 and TIAR proteins are characterised by three RNA recognition motifs (RRMs) located at their -NH_2 termini and a prion-related domain (PrD), glutamine-rich, at their -COOH termini. One of the main differences between TIA-1 and TIAR is at the level of PrD, which between the two proteins shares only 51 % of identity [9,17,18]. PrD is crucial for SG formation through the self-association of TIA proteins [9]. *In vitro* studies have shown that RRM1 lacks RNA-binding activity, RRM2 exhibits a high affinity for adenine (A)/uridine (U)-rich elements present in the 3′ or 5′ untranslated regions (UTRs) of specific mRNAs, RRM3 seems to bind mRNAs without sequence specificity, even if recent studies indicate that this domain enhances RRM2 activity by binding to cysteine (C)-rich RNA sequences [15,19]. Our understanding of these proteins, including their amino acid composition and gene expression, is severely limited in aquatic animals [20,21] and almost absent for Antarctic fish [22]. Despite being situated at the Earth's southernmost extreme and relatively isolated from other continents, the Antarctic continent is still vulnerable to the impact of human activities. The anthropogenic actions manifest directly through the uncontrolled dispersion of xenobiotics and indirectly as a driving factor of climate change [23–25]. Despite its distance from pollution sources, emerging contaminants have been detected in Antarctica over the past few decades [26,27]. These emerging pollutants include endocrine-disrupting chemicals, perfluorinated compounds, pharmaceuticals, and personal care products [27–30].

Moreover, the stress induced by temperature variations due to climate change adds to the environmental challenges faced by Antarctic ecosystems [31–33]. While the environmental fate of these xenobiotics and their biological effects remain not fully understood, their potential harm to wildlife, even at relatively low concentrations, is widely acknowledged. Additional exposure to these external factors can induce stress conditions, typically reflected at the cellular level by an elevated production of reactive oxygen species (ROS). Excessive ROS production can result in oxidative stress, adversely affecting cell viability [20].

Considering the pivotal role that TIA proteins play in the stress response in gene regulation through the formation of SGs, the present study aims to elucidate the unique structural characteristics of TIA-1 acquired during the evolution of Antarctic fish in response to specific selective pressures. First, the gene sequences TIA-1 encoding were characterised in *Chionodraco hamatus* and *Trematomus bernacchii*. Subsequently, phylogenetic and evolutionary analyses were performed using *tia-1* sequences obtained in silico from a few representative Antarctic nototheniods and sub-Antarctic relatives.

2. Materials and methods

2.1. Ethical procedures

The sample collection and animal research conducted in this study comply with the Italian Ministry of Education, University and Research regulations concerning activities and environmental protection in Antarctica and with the Protocol on Environmental Protection to the Antarctic Treaty, Annex II, Art. 3. All the activities on animals performed during the Italian Antarctic Expedition were under the control of a PNRA Ethics Referent, which acts on behalf of the Italian Ministry of Foreign Affairs. In particular, the required data for the project identification code PNRA16_00099 are as follows. Name of the ethics committee or institutional review board: Italian Ministry of Foreign Affairs. Name of PNRA Ethics Referent: Dr. Carla Ubaldi, ENEA Antarctica, Technical Unit (UTA). All experiments were performed under the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines; EU Directive 2010/63/EU; and Italian DL 2014/26 for animal experiments.

2.2. Fish sampling

Adult specimens of *T. bernacchii* (Boulenger, 1902) and *C. hamatus* (Lönnberg, 1905) were collected during November 2022 using rod and line in the Ross Sea at Baia Terra Nova (74°42′S, 164°7′E) at depths ranging from approximately 30 to 100 m. After sampling, the organisms ($n = 10$) were placed in thermostated and aerated tanks filled with natural seawater (sampled at 5 m depth) at the aquarium facility of the Italian research station Mario Zucchelli (pH 8.03 ± 0.01, oxygen 6.82 ± 0.04 mL/L and salinity 34.79 ± 0.01 PSU). Temperature was constantly monitored and maintained at −2 °C. The fish were acclimated for 8 days to mitigate the stress that resulted from the sampling. During this period, the fish were not fed, and all the possible external sources of stress (light pollution and noise) were minimised to simulate their natural environment. At the end of this period, the fish were euthanised with an overdose of ethyl 3-aminobenzoate methanesulfonate salt (0.065 g/L; Sigma; St. Louis, MO, United States) and dissected. Tissue samples were taken from the liver, heart, spleen, kidney, and white muscle, frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

2.3. Primer design, total RNA extraction, cDNA synthesis, PCR amplification and sequencing

Homologous TIA-1 coding sequences of teleost of the Perciformes order were collected from the NCBI database (https://www.ncbi.nlm.

nih.gov/) and multi-aligned with the MUSCLE program [34]. Primers for the characterization of *tia-1* in *T. bernachii* and *C. hamatus* (Table S1) were designed on the most conserved regions and checked with the IDT Oligo Analyzer tool (https://eu.idtdna.com/calc/analyzer).

For total RNA extraction from tissue samples (95 mg ± 2), we used Biozol buffer (BioFlux™) according to the manufacturer's protocol. Further purification was needed to remove carbohydrate contaminants by using 8 M Litium Chloride (LiCl). The concentration and purity of total RNAs were evaluated using the Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific) by examining the A_{260}/A_{280} and A_{260}/A_{230} ratios. Additionally, its integrity was confirmed by visualising RNAs on a 1 % agarose gel.

cDNA synthesis was carried out in two steps. In the first reaction, a mix containing 2 μl Oligo (dT) Anchor primer (10 μM) (Table S1), 1.0 μl total RNA [1 μg/μl], and 2.0 μl H_2O RNAse-free (EURxR) was incubated at 70 °C for 5 min and then at 4 °C for 5 min to select the mRNA. In the second reaction, a mix containing 2.5 μl ImProm-II™ 5x reaction buffer (Promega), 2.5 μl $MgCl_2$ [25 mM] (Promega), 1.0 μl dNTPs mix [10 mM] (EURxR), 0.5 μl RNasin Ribonuclease Inhibitor [40 U/μl] (Promega), 1.0 μl Reverse Transcriptase 160u/μl (Promega), and 7.5 μl H_2O RNAse-free (EURxR) was used to obtain the double-stranded cDNA.

PCR reactions were performed on 50 ng of cDNA with the 2x YourTaq™ PCR Master Mix and using primers reported in (Table S1). The PCR program was set as follows: 95 °C for 5 min and 40 cycles at 95 °C for 30 s, annealing temperature for 30 s, 72 °C for 1 min, with a final elongation step at 72 °C for 5 min.

Following the electrophoresis of PCR products on a 1.5 % agarose gel, we gel-purified the amplicons using the Wizard® SV Gel and PCR Clean-Up System (Promega) kit. We sequenced them at Eurofins Genomics (Ebersberg, Germany) by the Sanger method on an ABI 3730XL 96-capillary model sequencer. The obtained *tia-1* sequences of our two target species were validated by nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and translated with the Expasy tool (https://web.expasy.org/translate/) to verify, with the SMART tool (http://smart.embl-heidelberg.de/index2.cgi), the domain organisation typical of TIA-1. *Ad hoc* primers (Table S2) were designed on the nucleotide sequences obtained by sequencing to quantify *tia*-1 by quantitative real-time PCR (qRT-PCR).

2.4. Quantitative real-time PCR (qRT-PCR)

Transcript levels of *tia-1* were quantified by qRT-PCR, using the qPCRBIO SyGreen Mix Separate-ROX (PCR Biosystems) kit, in 10 μL volume containing 50 ng of cDNA, on the Applied Biosystems 7500 Real-Time PCR System. The qRT-PCR cycling parameters were the following: 95 °C for 2 min, 38 cycles at 95 °C for 20 s and 60 °C for 1 min, and finally 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s for the melting profile analysis (detection of genomic contamination). Each sample was run three times (technical triplicate). Primer (Table S2) amplification efficiency was verified at first (absolute quantification). Relative quantification values were determined using the Pfaffl mathematical model ($2^{-\Delta\Delta Ct}$ calculation) [35], and the transcription levels were normalised to *β-actin* used as a housekeeping gene to compensate for variations in the amounts of cDNA.

2.5. Sequence alignment and phylogenetic reconstructions

The sequences used in this paper are summarised in Table S3, together with their GeneBank accession numbers. MUSCLE was used to obtain multiple sequence alignment [34].

The jModelTest2 [36] was used to conduct a statistical selection of best-fit nucleotide substitution models to analyze TIA-1 molecular evolution in organisms. Analyses were performed using 88 candidate models and three types of criteria: Akaike information criterion (AIC), corrected AIC (cAIC) and Bayesian information criterion (BIC). ProtTest3 was used to select the best-fit model of TIA-1 protein evolution [37]. This statistical analysis used one hundred and twenty-two candidate models and the three criteria mentioned above.

Phylogenetic trees were built using the Bayesian inference (BI) method implemented in Mr. Bayes 3.2 [38]. Four independent runs, each with four simultaneous Markov Chain Monte Carlo (MCMC) chains, were performed for 1,000,000 generations sampled every 1000 generations. Furthermore, we also used the maximum likelihood (ML) method implemented in PhyML 3.0 [39], while the relative bootstrap analyses were performed on 100,000 trees. FigTree v1.3 software was used to display the annotated phylogenetic trees.

2.6. Positive selection analysis

Statistical methods available in the Hy-Phy package were used to detect the presence of positive or negative selection in the molecular evolution of TIA-1 [40]. These methods were accessed via the Datamonkey 2.0 web application [41]. Single-Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), and adaptive Branch Site random effect likelihood (aBSREL) software were used to identify potential positive selection. The Mixed Effects Model of Evolution (MEME) program was also used to detect sites under episodic diversifying selection.

2.7. Antarctic fish TIA-1 molecular evolution

The genome assemblies of *T. bernacchii*, *C. hamatus*, *Notothenia rossii*, *Chaenocephalus aceratus*, *Histiodraco velifer*, *Dissostichus mawsoni*, *Eleginops maclovinus*, and *Cottoperca gobio* were retrieved from NCBI Datasets, specifically from the individual species sheets (*T. bernacchii* GCF_902827165.1, *C. hamatus* GCA_009756495.1, *N. rossii* GCA_949606895.1, *C. aceratus* GCA_023974075.1, *H. velifer* (GCA_943590885.1), *D. mawsoni* (GCA_011823955.1), *E. maclovinus* GCF_036324505.1, and *C. gobio* GCF_900634415.1). These species were selected as key representatives of the main notothenioid lineages, previously highlighted by molecular phylogey [42]. For each species, the gene positions were identified using a combined BLASTn and tBLASTn approach based on the mRNA and protein sequences obtained from *T. bernacchii*. Gene annotations were corrected if present or manually added if absent, ensuring the presence of canonical splicing sites.

We selected available transcriptomic datasets from Illumina paired-end libraries for each species only if they were linked to samples in physiological conditions and not exposed to any treatment (Table S4). The relative contribution of different isoforms to total TIA-1 expression were evaluated by assessing the number of reads mapped to specific exon-exon junctions, allowing us to unequivocally attribute them to a single isoform. Relative expression levels were calculated only for samples where at least 30 reads could be mapped. The predicted subcellular localisation of the different TIA-1 proteoforms was investigated with DeepLoc 2.0 [43].

2.8. Statistical analysis

qRT-PCR data are presented for the two target species as the mean of the analysed samples ($n = 10$) ± standard deviation. Statistical analyses were conducted using the PRIMER.exe program (Version 1.0, Stanton A. Glantz, Italy). A one-way ANOVA was initially performed, and significant differences were further assessed using the t-Student Newman-Keuls test, with a significance level set at $p < 0.05$.

3. Results

3.1. C. hamatus and T. bernacchii tia-1 transcript organisation

A preliminary comparative analysis of the gene sequences homologous to *tia-1* associated with genomes deposited in Ensembl clearly revealed the presence of multiple (in most cases two) paralogous genes

in the overwhelming majority of teleost species (59 out of the 65, 91 %), with the exception of a few early-branching fish lineages. This is in stark contrast with tetrapods, where only 2 % species have multiple *tia-1* paralogs (Ensembl, data retrieved in July 2024). The presence of shared sequence features and the association with sinteny blocks allow a clear-cut discrimination between two distinct group of fish *tia-1* sequences, suggesting that these may be the product of a fish-specific duplication event, similar to the one that independently led to the origins of *tia-1* and its paralog *tiar* in mammals. Although both fish paralogous genes can be considered as *tia-1* related, for the sake of clarity all the analyses reported in this manuscript will be focused on only one of these, corresponding to the sequence that could be successfully amplified with the approach outlined in section 2.3.

In *C. hamatus*, the sequencing of the amplicons generated with the PCR amplifications from cDNA (Table S1) resulted in a partial coding sequence of 885 nt correspondent to 295 aa. The nucleotide sequence was later completed through data from transcriptomic analyses (Fig. S1). The *in-silico* analysis indicated that in *C. hamatus tia-1,* the completed coding region is 1164 nt long and encodes a putative protein of 387 aa.

Among species reported in Table S3, the *tia-1* sequence of *C. hamatus* showed the highest identity (97.78 %) with the *tia-1* homologous sequence of *C. aceratus* [44], according to Clustal Omega analysis (Table S4) (https://www.ebi.ac.uk/jdispatcher/msa/clustalo).

The analysis of the amplicon obtained with primer listed in Table S1 on a cDNA from *T. bernacchii* resulted in a partial coding sequence of 729 nt, which corresponded to 243 aa, that was later completed thanks to the combined analysis of transcriptomic data generated in previous publication [45] and the genome sequence of this species [46] (Fig. S1).

The *in-silico* analysis indicated that in *T.bernacchii tia-1,* the completed coding region is 1167 nt long and encodes a putative protein of 388 aa. Furthermore, in the 3′-UTR, 4 putative rapid degradation signals (ATTTA). Differently from *C. hamatus*, the *tia-1* sequence of *T. bernacchii* showed the highest identity (99.74 %) with the orthologous *tia-1* sequence of *D. mawsoni* (Table S3).

3.2. Protein organisation

From the multi-alignment of all thirty-one amino acid sequences of teleosts considered in the present study, we can confirm the general conservation of the characteristic domains of TIA-1. Fig. 1 below is a frame of the multi-alignment showing only Antarctic fish homologous sequences of TIA-1. The region from amino acid 6 to amino acid 80 corresponds to the RRM1 domain, exhibiting a conservation of 100 % across the four Antarctic sequences. Within this domain, two characteristic motifs, RNP1 (DPYCFVEF) and RNP2 (LYVGNL), are fully conserved. From amino acids, 95 to 170 is present in the RRM2 domain, which maintains a conservation rate of 100 %. Similarly, this domain encompasses two characteristic motifs fully conserved, which are RNP1 (KGYGFVSF) and RNP2 (VFVGDL). The third conserved domain, RRM3, spanning from amino acid 192 to 271, exhibits a conservation rate of 88.60 %. Once again, this domain harbours the RNP1 (KGYSFVRF) and RNP2 (VYCGGV) motifs, the second one fully conserved. Interestingly, the RNP1 sequence of the third domain, typically fully conserved, displays in *C. hamatus* a Glu^{239} and a Met^{246} instead of Lys^{239} and a Phe^{246}.

It is precisely *C. hamatus* that exhibits the highest degree of amino acid substitution, not only with respect to non-Antarctic species but also among the other three Antarctic fish. Its sequence exhibits a Tyr^{181} instead of a Thr^{181}.

Additionally, *C. hamatus* shows Asn^{185} instead of Asp^{185}, Thr^{205} instead of Ser^{205}, Ile^{234} replaces Arg^{234}, Val^{256} instead of Ile^{256}, an Ala^{258} instead of Ser^{258}, a Ser^{259} instead of Val^{259}, and Ser^{264} instead of Ile^{264}. The Tyr^{273} and Trp^{274} are substituted by Trp^{273} and Gly^{274} respectively. Lastly Leu^{288} is replaced by Pro^{288}. Interestingly, all the Gln (Q) residues in the PrD shown in the multi-alignment are fully conserved in Antarctic fish species and generally in all the teleost sequences analysed.

3.3. Molecular phylogeny and evolution

All cDNA coding region sequences were aligned using T-Coffee in combined libraries of local and multiple alignments, which are known to induce high accuracy and performance in sequence alignments. jModelTest 2 software determined the TIM2+I + G model as being the best-fit model of all analysed coding region sequences evolution with a γ shape value (four rate categories) of 0.933 using all statistical criteria: AIC, cAIC and BIC (–lnL = 11170.94). ProtTest3 was used for amino acid sequence evolution best-fit model determination; its statistical results determined the JTT + G model was the best one to apply for the phylogenetic analysis of TIA-1 amino acid sequences, with a gamma shape value (four rate categories) of 0.401 using all statistical criteria: AIC, cAIC and BIC (–lnL = 3516.79).

The phylogenetic cladogram constructed using nucleotide sequences of TIA-1 (Fig. 2) from the same species is also well-resolved, with an average posterior probability support of 92.1 % for all nodes in the BI analysis, and an average bootstrap support value of 65.7 % in the ML analysis.

The topology of this cladogram largely mirrors the aminoacidic sequence-based one (Fig. S2), with a strongly supported cluster (100 % Bayesian probability and ML bootstrap value) grouping fish sequences. TIA-1 nucleotidic sequences of species in the order Perciformes group together (98 % BI and 68 % ML), with the exception of *Maylandia zebra* and *Lates calcarifer*. This branch also shows more politomic nodes. Within the teleosts cluster, the nucleotidic sequences of the four Antarctic fishes exhibit a clear phylogenetic relationship to each other and with *E. maclovinus*, strongly distinct from TIA-1s of other teleosts (100 % posterior probability and 80 % ML bootstrap value). On the other hand, the relationships among the four analysed Antarctic notothenioid sequences were not well resolved, leading to different branch topologies in the cladograms obtained using both nucleotide and amino acid sequence data.

3.4. Positive selection analysis

The selection analyses carried out on teleost TIA-1 coding sequences with SLAC, FEL and aBSREL algorithms detected the presence of both sites evolving under purifying and diversifying selection, even though the former were much more abundant than the latter. The results referring to all the compared teleosts sequences are presented in Table S5 and are graphycally represented in Fig. 3 below.

In detail, most of the 43 identified negatively selected codons were located between the RRM1 and the RRM3 domains. On the other hand, only two codons were detected as subjected to episodic positive/diversifying selection. The first one was located within the RRM1 domain at the amino acid level in position 28 (numbers are referred to *T.bernacchii,* Fig. 3), which resulted in a change from Thr to Gly in three non-Antarctic fish species. The second one, which as well did not target Antarctic fish species, was characterised by the nonsynonymous mutation from Ala^{124} to Cys^{124} in RRM2.

3.5. Antarctic fish TIA-1 molecular evolution

As briefly mentioned above, the analysis of genome data available in Ensembl revealed that albeit *tia-1* is an evolutionarily conserved gene present as a single-copy ortholog in most vertebrates, nearly all fish species have two distinct paralogs, only one of which was characterized in detail in this study.

The fish TIA-1 genes we characterized from *C. hamatus* and *T. bernacchii* encodes a protein with a high level of sequence identity (80–85 %) to its mammalian orthologs, with most polymorphisms located in the C-terminal Q-rich domain. The TIA-1 gene resides in a synteny block conserved across species in the notothenioid clade,

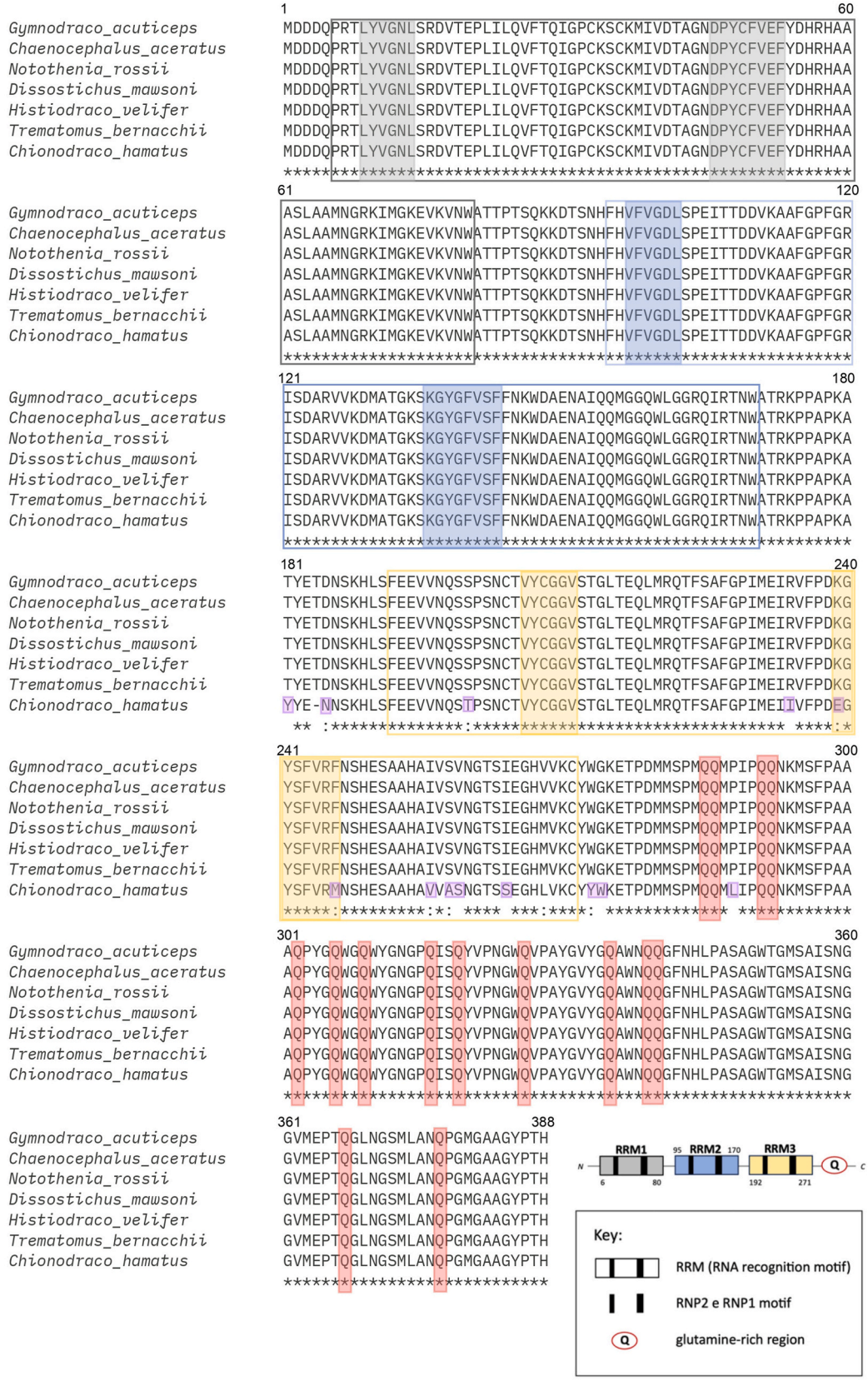

Fig. 1. TIA-1 domain organisation in Antarctic fish. Boxes of different colours refer to the three domains: RMM1 in light grey, RMM2 in blue and RMM3 in light yellow. Within each domain, the RNP1 and RNP2 motifs are highlighted. Amino acid substitutions for *C. hamatus* are highlighted in dark blue, amino acid substitutions specific to Antarctic fish are highlighted in light green, and glutamine (Q) residues are highlighted in light red; numerals refer to character counts.

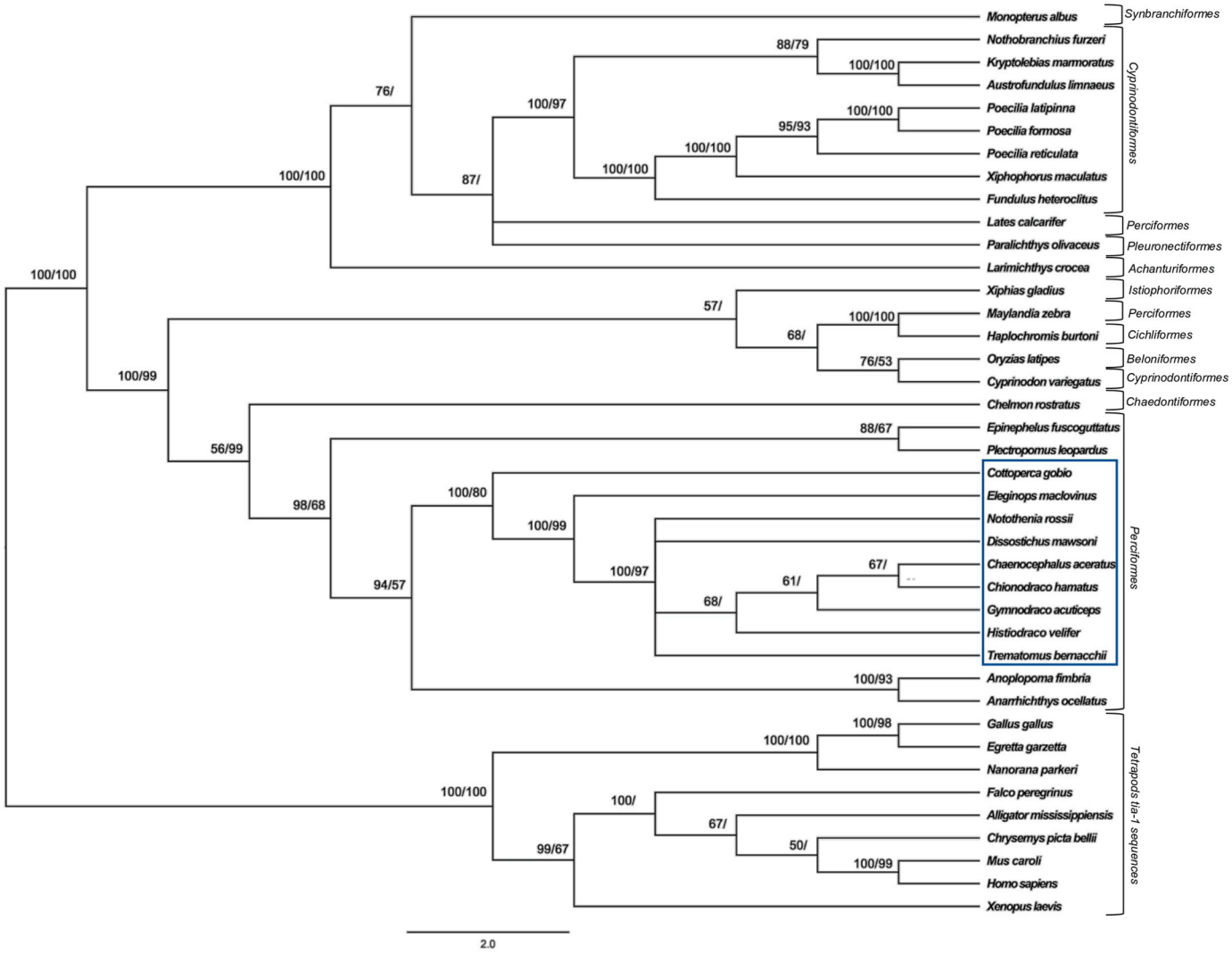

Fig. 2. Phylogenetic relationships among TIA of various organisms reconstructed on the basis of nucleotide sequences of the coding region and using both BI (arithmetic mean = - 11401.63; harmonic mean = - 11438.61) and ML (arithmetic mean = - 11367.39) methods. Posterior probability (first number) and bootstrap values (second number, if present) higher than 50 % are indicated on each node. The scale for branch length (2.0 substitution/site) is shown below the tree.

flanked by the evolutionarily conserved TTC37 and ZNF703 genes. Although a few alternatively spliced isoforms are occasionally annotated in available genomes, in all fishes, the isoform that most closely matches the primary mammalian transcript has a highly conserved architecture consisting of 12 exons and 11 introns. Our analysis available of Notothenioidei genomes confirmed the presence of TIA-1 genes with the expected architecture in all chromosome-scale assemblies, with partial gene sequences being often detected in fragmented genomes generated in earlier sequencing efforts. However, an in-depth investigation of available transcriptomic datasets revealed the expression of two additional splicing isoforms with alternative 3' ends. As illustrated in Fig. S3, using *T. bernacchii* as an example, the canonical splicing isoform retains the expected splicing of intron 11, producing a 388 amino acid proteoform, referred to as "long" (L). In the two alternatively spliced variants, intron 11 is either entirely skipped or shortened to 506 nucleotides (compared to 1439 in the canonical isoform) due to the use of an alternative splicing acceptor site. The complete skipping of intron 11 results in a truncated polypeptide due to an in-frame STOP codon, producing a 343 amino acid proteoform named "short" (S), lacking the C-terminal portion of the Q-rich domain. Using the alternative splicing acceptor site, the isoform encodes a 351 amino acid proteoform, named "intermediate" (I), with an anionic C-terminal end highly divergent from the canonical splicing isoform. Despite their shorter length, both the S and I proteoforms were predicted to have a nuclear localisation with high confidence, thanks to the preservation of nuclear localisation signals. Similarly, all three isoforms preserve a functional nuclear export signal.

Comparative analysis of Notothenioid genomes revealed that both the in-frame stop codon found in the S isoform and the alternative splicing acceptor site and stop codon in the I isoform were conserved in all Antarctic species. The extension of these analyses to sub-Antarctic notothenioid species revealed that the TIA-1 gene of *E. maclovinus* retained these features, allowing transcription of both the S and I isoforms. On the other hand, the orthologous gene in the more divergent species *C. gobio* did not conserve the alternative splicing acceptor site, thus supporting only the transcription of the S and L isoforms.

While investigating the relative abundance of the three alternatively spliced TIA-1 isoforms in *T. bernacchii*, *N. rossii*, *C. hamatus*, *D. mawsoni*, *C. aceratus*, *E. maclovinus*, and *C. gobio*, we found that the I isoform was largely dominant in the overwhelming majority of investigated tissues in all Antarctic species. I was generally followed by S, whereas the L isoform usually accounted for less than 100 % of total TIA-1 transcription (Fig. 4). Interestingly, this situation was largely different in *E. maclovinus*, where the expression of the I isoform was marginal in all

1	**M**	D	D	D	Q	P	R	T	L	Y	V	G	N	L	S	R	D	V	T	E
21	P	L	I	L	Q	V	F	T	Q	I	G	P	C	K	S	C	K	M	I	V
41	D	T	A	G	N	D	P	Y	C	F	V	E	F	Y	D	H	R	H	A	A
61	A	S	L	A	A	M	N	G	R	K	I	M	G	K	E	V	K	V	N	W
81	A	T	T	P	T	S	Q	K	K	D	T	S	N	H	F	H	V	F	V	G
101	D	L	S	P	E	I	T	T	D	D	V	K	A	A	F	G	P	F	G	R
121	I	S	D	A	R	V	V	K	D	M	A	T	G	K	S	K	G	Y	G	F
141	V	S	F	F	N	K	W	D	A	E	N	A	I	Q	Q	M	G	G	Q	W
161	L	G	G	R	Q	I	R	T	N	W	A	T	R	K	P	P	A	P	K	A
181	T	Y	E	T	D	N	S	K	H	L	S	F	E	E	V	V	N	Q	S	S
201	P	S	N	C	T	V	Y	C	G	G	V	S	T	G	L	T	E	Q	L	M
221	R	Q	T	F	S	A	F	G	P	I	M	E	I	R	V	F	P	D	K	G
241	Y	S	F	V	R	F	N	S	H	E	S	A	A	H	A	I	V	S	V	N
261	G	T	S	I	E	G	H	M	V	K	C	Y	W	G	K	E	T	P	D	M
281	M	S	P	M	Q	Q	M	P	I	P	Q	Q	N	K	M	S	F	P	A	A
301	A	Q	P	Y	G	Q	W	G	Q	W	Y	G	N	G	P	Q	I	S	Q	Y
321	V	P	N	G	W	Q	V	P	A	Y	G	V	Y	G	Q	A	W	N	Q	Q
341	G	F	N	H	L	P	A	S	A	G	W	T	G	M	S	A	I	S	N	G
361	G	V	M	E	P	T	Q	G	L	N	G	S	M	L	A	N	Q	P	G	M
381	G	A	A	G	Y	P	T	H	–											

Fig. 3. Graphical representation that highlights negatively selected codons (in pink) and positively selected codons (in yellow), using the *T. bernacchii tia-1* sequence as a reference. As discussed in the text, the two episodes of positive selection did not target Antarctic fish species.

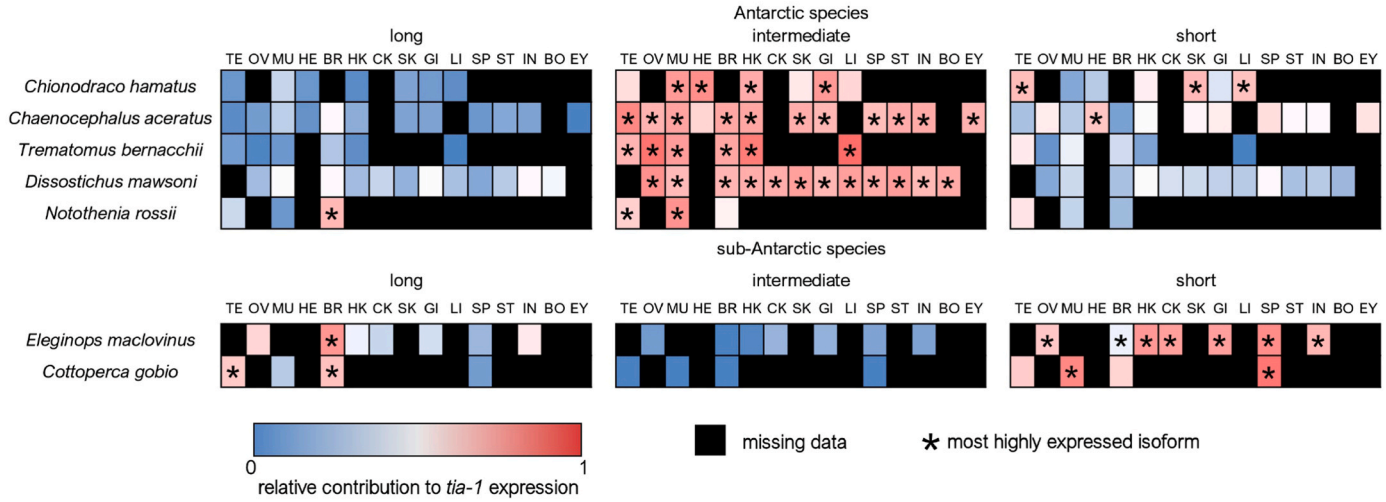

Fig. 4. Heat map summarizing the relative contribution to TIA-1 gene expression of the S, I and L splicing isoforms in five Antarctic and two sub-Antarctic notothenioid fish species, inferrened from available RNA-seq datasets (black squares indicate tissues with no available information or an insufficient number of mapped reads). TE: testis; OV: ovary; MU: skeletal muscle; HE: heart; BR: brain; HK: head kidney; CK: caudal kidney; SK: skin; GI: gills; LI: liver; SP: spleen; ST: stomach; IN: intestine; BO: bone; EY: eye. The most highly expressed splicing isoform for each tissue and species is marked with an asterisk.

analysed tissues, and the L and S isoforms were on the other hand generally expressed at similar levels. Consistent with the absence of a suitable splicing acceptor site at the genome level, no expression of the I isoform was detected in *C. gobio*.

3.6. qRT-PCR

The TIA-1 gene expression was investigated in the liver, spleen, gills, heart, and skeletal muscle of *T. bernacchii* and *C. hamatus*. The primers were designed to amplify simultaneously all the isoforms. Transcript levels of *tia-1* are reported in Fig. 5. In *C. hamatus* we observed high *tia-1* expression levels in the liver and spleen, similar in these two tissues and about five times higher than those measured in gills, heart, and skeletal muscle ($p < 0.05$). In *T. bernacchii, tia-1* expression levels were higher in the heart and skeletal muscle than in the other tissues.

Expression levels were similar in heart and skeletal muscle and about five times higher than those measured in the liver, spleen, and gills ($p < 0.05$).

4. Discussion

Antarctic fish species have undergone various morphological and physiological adaptations throughout their evolutionary history, largely shaped by their unique environment [47–49]. They exhibit remarkable

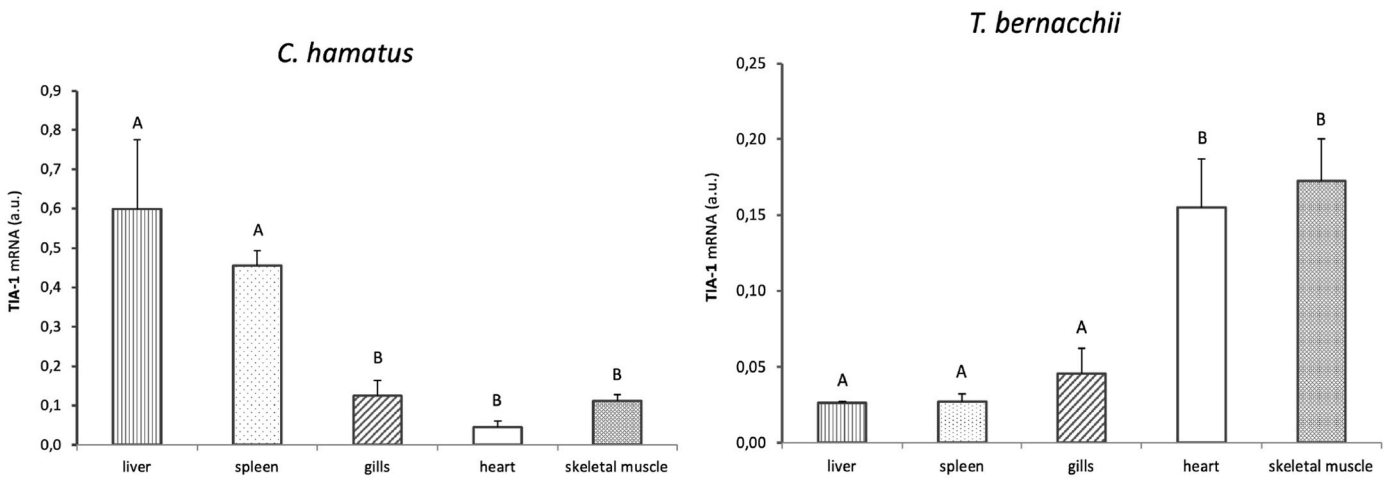

Fig. 5. TIA-1 mRNA expression levels in liver, spleen, gills, heart, and skeletal muscle of *C. hamatus (A)* and *T. bernacchii* (B) in physiological conditions. Different letters correspond to significant statistical differences ($p < 0.05$) among various tissues, with ten specimens per species ($N = 10$).

capabilities in implementing physiological responses to mitigate stress conditions, particularly oxidative stress, which can threaten their survival [50–52]. The unique capabilities of Antarctic fish could reasonably involve regulation processes, such as those performed by SGs, with their assembly and disassembly, given that the formation of these foci can rapidly change when organisms are experiencing stressful conditions.

Recent studies suggest that the observed discrepancies between mRNA and protein expressions of antioxidant enzymes in Antarctic fish cannot be fully explained by molecular half-lives, implying a significant role for post-transcriptional mechanisms, possibly involving SGs, in gene regulation [22,53,54]. In the present study, we have characterised for the first time a gene encoding TIA-1 in two Antarctic teleost species, *T. bernacchii* and *C. hamatus.* Our analysis of the obtained sequences confirms a very high degree of identity (more than 90 %) at the RNA-recognition motifs (RRMs) level, consistent with previous findings in the literature. The remarkable conservation of the RRM2 domain across Antarctic fish, highlighted by the lack of nonsynonymous mutations, underscores its essential role in mRNA recognition and nuclear accumulation of TIA-1 [55]. In contrast, the RRM3 domain exhibits a notable number of amino acid substitutions, especially in *C. hamatus,* compared to other teleosts. These changes predominantly involve polar or non-polar amino acids, aligning with the trend of cold-adapted proteins to favour polar residues [56–58]. Such adaptations, documented in Antarctic fish proteins [59,60], are strategically located near functional motifs, suggesting a potential link to protein activity. In the context of TIA-1, these modifications are primarily observed in the RRM3 domain, hinting at its significant influence on mRNA interactions and gene expression regulation.

Previous studies have shown that the complex formed between TIA-1 and mRNA exhibits increased compactness when RRM2 is combined with the presence of the RRM3 domain [19]. The high number of nonsynonymous mutations present in the third domain of *C. hamatus* TIA-1 compared with its notothenioid relatives may be associated with the necessity of higher efficiency of this domain in recognising mRNAs. Indeed, these mutations contribute to the insertion of amino acids into RMM3 that can implement hydrophobic and π-interactions RNA-protein [61], such as Glu^{239} e Met^{246}, both of which are present in the RNP2 motif that recognises RNAs rich in C and U [9]. Given that Channichthyidae, the family to which *C. hamatus* belongs, are the only vertebrates incapable of synthesising functional haemoglobin, the hypothesis suggesting a correlation between the structural peculiarities of TIA-1 and the hemoglobin-deficient condition of this species [62] is particularly intriguing. The absence of haemoglobin in *C. hamatus* is compensated by the unique physicochemical conditions of the Southern Ocean, where low temperatures enhance the solubility of oxygen in the environment and the body fluids. This adaptation and other unique morphological and physiological adjustments enable Channichthyidae to survive exclusively in Antarctic waters. However, the lack of haemoglobin has drawbacks, as these organisms constantly live under hypoxic conditions, which intensify during locomotor activities that require increased oxygen consumption [63]. Consequently, it can be asserted that they are exposed, more than the Antarctic red-blooded fish, to stressogenic conditions, necessitating a more plastic gene regulatory system at the cellular level [64].

Therefore, TIA-1 may emerge as a crucial component of such regulatory mechanisms in this context. Nevertheless, since the sequence of *C. aceratus*, another member of the family Channichthyidae, does not share the same distinctive features found in *C. hamatus*, such adaptations may be specific to the *Chionodraco* lineage and not extanded to all haemoglobin-less notothenioids.

The phylogenetic analysis confirmed the clustering of *tia-1* with orthologues from other teleosts. The substantial amino acid conservation contributes to the uniform distribution of fish TIA-1s in the phylogenetic cladograms, demonstrating well-defined sequence relationships within distinct orders. The observation mentioned earlier suggests that the molecular evolution of the TIA-1 protein is synchronised with the evolutionary paths of various fish orders and families. This result is in line with the phylogeny of other anti-stress proteins [20,53,54,60, 65–73]. In particular, all Antarctic teleost TIA-1 sequences clustered within the Perciformes order and formed a distinct cluster, a result that is closely related to the unique evolutionary history of Antarctic fishes, which, over the past 11.6 million years, underwent radiation in isolation within the geographic barrier of the Antarctic circumpolar current, subjected to robust environmental selective pressures [42].

Overall, the four Antarctic TIA-1 sequences displayed a high level of pairwise sequence homology at the amino acid level, with the overwhelming majority of the polymorphisms being associated with the RMM3 domain of *C. hamatus* that, as mentioned above, displayed unique features compared with its red-blooded counterparts. Not surprisingly, the very low amount of phylogenetically informative mutations detectable in notothenioid proteins led to discrepancies between the topologies of the trees obtained from the multiple alignment of amino acid and nucleotide sequences, with the latter displaying a higher resolution. This observation was consistent with the hypothesis that the TIA-1 gene was subjected to strong purifying selection. This was supported by detecting a strong prevalence of codons evolving under purifying selection over those subjected to episodic diversifying selection in the multiple alignment of fish TIA-1 sequences. The action of purifying selection plays a crucial role in the evolution of gene families by ensuring protein function and/or structure preservation [74]. Our

analysis further indicated that the codons evolving under adverse selection predominantly encode amino acids within the first and second RNA-recognition motifs. This observation suggests the existence of evolutionary constraints, which minimise their variability to maintain proper function in recognising mRNA.

On the other hand, positive selection is an essential source of evolutionary innovation, and it has often been advocated as a major force underlying the adaptation of species to new environments [74]. Many protein families have experienced significant positive selection along their evolution, including those involved in immunity, reproduction, and cell signalling [75,76]. Our analyses detected two amino acid residues that have been positively selected during the evolution of TIA-1, i.e. Thr^{28} and Ala^{124}, which are placed in the RRM1 and RRM2 domains, respectively. However, amino acid substitutions at these sites were only detected in non-Antarctic species. Therefore, the selection at this site is unlikely to bear any biological significance in the context of cold adaptation.

Our evolutionary analysis of the TIA1 gene has revealed intriguing insights into the uniqueness of Antarctic fish sequences. Although nearly all fish species have two well-recognizable distict TIA-1-like paralogous genes, our cloning approach only targeted one of these, which was found to be present in a single copy with the expected architecture in all available genomes of Notothenioidei [47]. Extending our investigation to transcriptomic datasets from previous studies [46,77] uncovered two additional splicing isoforms, characterised by alternative 3' ends, significantly impacting the C-terminal end of the encoded proteins. These three isoforms are named "long" (L), which encodes the canonical proteoform analysed in section 3.2, "intermediate" (I), and "short" (S).

Our analysis showed that the S and I isoforms are conserved across all Antarctic fish species, indicating their biological significance. To trace the evolutionary origins of these unusual TIA-1 splicing variants and explore their potential link to cold adaptation, we investigated their transcription in two species occupying key positions in notothenioid fish evolution: *Eleginops maclovinus* [78] and *Cottoperca gobio* [79]. These species represent two basally divergent non-Antarctic notothenioid families, Eleginopidae (the sister lineage of Cryonotothenioidea) and Bovichtidae, respectively [80].

Our findings suggest that the acquisition of an alternative splicing acceptor site within intron 11, leading to the production of the I isoform, occurred in the last common ancestor of the Cryonotothenioidea + Eleginopidae lineage (estimated to have lived in the Eocene) [81]. This event likely happened before colonising the polar environment but after the divergence from the lineage, of extant Bovichtidae species (i.e. in the late Cretaceous) [81]. In contrast, the origins of the S isoform are more ancient, preluding the divergence between notothenioids and other percomorph lineages. Supporting this view, preliminary investigations into TIA-1 splicing patterns in other non-Antarctic species revealed that the S isoform is produced in multiple species.

Interestingly, the analysis of the relative abundance of the three alternatively spliced TIA-1 isoforms in Antarctic and sub-Antarctic notothenioids showed that the L isoform was expressed at poor levels in *T. bernacchii C. hamatus*, and all other tested Antarctic species, whereas isoform I was the most abundant one, followed by isoform S. Although the expression of isoform I was detectable at non-negligible levels also in *E. maclovinus*, its biological relevance was much lower in this sub-Antarctic species, where the L isoform was much more abundant and nearly as much as expressed as isoform S.

The lack of suitable splicing sites consistent with the production of the I isoform in *C. gobio* determined the transcription of two isoforms only, i.e. S and L, in this early diverging sub-Antarctic notothenioid species. Overall, these observations suggest an increased prominence of the I isoform, compared with the canonical L isoform, in the Antarctic environment, even though the origins of this unusual variant encoding a protein with a shorter C-terminal end can be traced back to a non-Antarctic notothenioid ancestor.

Although alternative splicing leads to the production of proteins with significantly shorter C-terminal ends by the S and I isoforms, these alterations are unlikely to have an impact on the nuclear accumulation of TIA-1, which is determined by RRM2 and by the first half of the C-terminal region [55], which are not affected by alternative splicing. Similarly, the nuclear export of TIA-1 to the cytoplasm should also not be affected by the relative abundance of the three splicing isoforms since the nuclear export signal is placed within the RRM3 domain [55]. The bioinformatics analysis of the encoded proteoforms confirmed this interpretation, strongly supporting the preservation of nuclear localisation and export signals in all Antarctic notothenioids. On the other hand, we can hypothesise that the evolutionary advantage provided by the I isoform may be due to the increased functionality of the protein since a low-complexity Q-rich domain seems to favour protein-protein interactions [82], compensating for the lower affinity due to the lower temperatures of the Antarctic environment.

The real-time PCR results revealed transcription of TIA-1 mRNA in all examined tissues of both *T. bernacchii* and *C. hamatus*. However, remarkable variations in expression levels were observed among tissues within each species and between the two species. Notably, in *C. hamatus*, the highest expression of the TIA-1 mRNA was observed in the spleen and liver.

The spleen in fish plays a crucial role in immune defence as a significant site for B lymphocyte accumulation and thrombocyte differentiation [83]. Reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2), can be generated in large quantities during inflammatory processes and phagocytic cell activity [84]. The coordinated response likely plays a role in mitigating the adverse effects of heightened H_2O_2, thus preserving immune function. It is plausible that this delicate balance in gene expression involves the participation of TIA-1 and SGs. The Antarctic marine environment is rich in microorganisms and parasitic organisms, affecting a wide range of vertebrates. Parasitism is widespread among Antarctic teleosts, primarily by nematodes [85], with species from the Channichthyidae family notably affected [86].

The significant gene expression observed in the liver of *C. hamatus* may be attributed to the liver's remarkable capacity to express antioxidant enzymes, including superoxide dismutases, peroxiredoxins, catalase, and selenium-dependent glutathione peroxidases, as well as metallothioneins. This facilitates the detoxification of ROS generated by its high metabolic activity [87]. It is important to note that the liver's substantial metabolic activity is more closely associated with its detoxifying function than its digestive role, especially considering this organism's limited and intermittent digestive activity. This detoxification is particularly targeted towards metals such as cadmium and copper, which are prevalent in relatively high concentrations in the Antarctic coastal marine environment due to the geographical nature of the seabed [88,89]. The biomagnification of these metals could pose a significant concern for *C. hamatus* and other fish, given their prominent position in the trophic network [90].

In contrast, *T. bernacchii* exhibits a higher expression of TIA-1 mRNA in skeletal muscle and heart. The increased mRNA accumulation in skeletal muscle may be linked to *T. bernacchii*'s relatively higher activity levels among benthic teleosts in Antarctic waters. The activity of *Trematomus bernacchii* can be attributed to its limited feeding behavior, a hallmark of this benthic species. Known for targeting nearly stationary, sedentary prey, *T. bernacchii* exhibits a hunt-and-peck predation style, complemented by a lie-in-wait strategy. It likely relies on swift, short bursts of movement to capture its prey effectively [91]. This enhanced locomotor activity results in a higher oxidative metabolism of skeletal muscle, crucial for species maintaining some locomotor activity even in extremely cold conditions. Antarctic fish, including *Trematomus bernacchii*, have been shown to possess a higher density of mitochondria in their muscle tissue compared to temperate species. This higher mitochondrial density is hypothesized to partially counteract the inhibitory effects of low temperatures on enzymatic reactions, as well as on oxygen and nutrient diffusion rates [92,93]. It is important to note that this higher mitochondrial density is common among various Antarctic fish

species and is not exclusive to *T. bernacchii*.

The high mitochondrial density in skeletal muscle, combined with the predominant use of lipids as the primary energy source in Antarctic fish, creates conditions that favour ROS formation and increase susceptibility to oxidative stress [94]. Consequently, there is an upregulation in the expression of antioxidant enzymes [53,58,59,95], necessitating precise regulation of their expression.

Since skeletal muscle is a metabolically active tissue, it experiences increased ROS production due to its high energy consumption [96]. This underscores the necessity for efficient anti-stress responses to mitigate the impact of oxidative stress and cellular integrity. The comparable levels for TIA-1 mRNA in the heart, which also has high energy demands, suggest a need for effective regulation of stress-responsive genes. The elevated oxygen consumption in the heart leads to increased production of reactive oxygen species, necessitating rapid activation of anti-stress responses to counteract potential cellular damage from normal cardiac physiology [97].

Real-time PCR analysis provides insights into messenger accumulation in various organs and supports the previously proposed hypothesis of post-transcriptional control involving SGs. This theory suggests that a portion of transcribed mRNA could be stored in intracellular structures like SGs, where it may undergo storage for future translation in the cytoplasm [3]. This condition is characteristic of organisms under stressogenic conditions, enabling cells to adjust the translation of molecules that counteract stress rapidly [98].

Supporting this hypothesis, elevated TIA-1 mRNA expression in both species analysed corresponds to low levels of peroxiredoxin 2 (*prdx2)* mRNA translation. This is evident in the liver and spleen of *C. hamatus* and the heart and skeletal muscle of *T. bernacchii.*

In contrast, in icefish, the translation of the *prdx2* mRNA is nearly complete in the gills and skeletal muscle despite low *tia-1* expression levels [22]. Similarly, in the liver and spleen of *T. bernacchii*, where TIA-1 messenger gene expression is low, there is substantial translation of *prdx2* mRNA. These observations support the post-transcriptional control hypothesis mediated by SGs. However, it is important to note that in the heart of *C. hamatus* and the gills of *T. bernacchii,* low levels of PRDx2 biosynthesis are observed despite minimal TIA-1 mRNA expression. This raises the possibility that other SG-forming proteins, like TIAR, may be at play, emphasising the need to explore their gene expression in these organisms further.

With all the caution, we are confident that these results were minimally affected by housing conditions during the acclimatisation period, including food deprivation. It is known that Antarctic fish, due to low temperatures, are characterised by extremely low metabolic rates compared to other fish [99]. In relation to this, they evolved infrequent feeding during the summer period and even absent during the winter period. Recent experiments have shown that starvation periods of up to 50 days brought no change in the weight and body chemical composition of two Antarctic species, *Notothenia coriiceps* and *N. rossii* [100]. Regarding cortisol, literature data indicate that in non-Antarctic fish during fasting there is an increase in the blood concentration of cortisol, not related to a stress condition but only to activate gluconeogenesis [101]. Similar experiments have not been done with Antarctic fish, but *N. rossii* exposed to heat stress for 10 days showed an increase in blood cortisol values between 1 and 4 h that returned to control levels within the next 24–48 h [102]. We did not sample blood and therefore cannot measure cortisol levels, but we have data on antioxidant defences. These data tell us that in *Trematomus bernacchii,* the levels of enzymes such as superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxins do not vary during the 8-day enclosure. This finding differs from the findings of other authors studying a non-Antarctic species (*Paramisgurnus dabryanus*), in which the activities of glutathione peroxidase (but not superoxide dismutase and catalase) increased significantly in the liver after 30 days of fasting. [103]. Therefore, we believe that 8 days of fasting does not represent a stressful condition or one characterised by elevated blood cortisol levels.

5. Conclusions

In conclusion, the findings presented in this paper provide initial insights into the molecular characteristics of genes encoding the nucleation proteins of SGs in Antarctic fish. This serves as an initial step for investigating the structural and evolutionary study of these proteins within specific ecological contexts, such as Antarctica. The gene expression data illustrate how the two analysed species can coordinate efficient physiological responses from a regulatory standpoint to counteract the heightened risk of cellular stress prevalent in the Antarctic marine environment. Additionally, our expression analyses provide valuable insights into characterizing different species that have developed unique physiological adaptations to their environments. In the case of icefish, these adaptations include their distinct physiology related to the absence of haemoglobin.

CRediT authorship contribution statement

E. Piva: Formal analysis, Writing – original draft, Visualization. **E. Nicorelli:** Formal analysis, Writing – original draft. **S. Pacchini:** Investigation, Data curation, Writing – review & editing. **S. Schumann:** Investigation, Writing – review & editing. **L. Drago:** Investigation, Writing – review & editing. **G. Vanzan:** Data curation, Writing – review & editing, S. Kholdihaghighi: Data curation, Writing - review and editing. **A.M. Tolomeo:** Investigation, Writing – review & editing. **P. Irato:** Resources, Writing – review & editing. **R. Bakiu:** Methodology, Formal analysis, Writing – original draft. **M. Gerdol:** Methodology, Validation, Formal analysis, Data curation, Writing – original draft. **G. Santovito:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109903.

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