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Bioinformatic exploration of RIO protein kinases of parasitic and free-living nematodes

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ABSTRACT

Despite right open reading frame kinases being essential for life, their functions, substrates and cellular pathways remain enigmatic. In the present study, gene structures were characterised for 26 right open reading frame kinase from draft genomes of parasitic and free-living nematodes. RNA-seq transcription profiles of right open reading frame kinase genes were investigated for selected parasitic nematodes and showed that these kinases are transcribed in developmental stages that infect their mammalian host. Three-dimensional structural models of *Caenorhabditis elegans* right open reading frame kinases were predicted, and elucidated functional domains and conserved regions in nematode homologs. These findings provide prospects for functional studies of RIO kinase genes in *C. elegans* and an opportunity for the design and validation of nematode-specific inhibitors of these enzymes in socioeconomic parasitic worms.

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1. Introduction

Parasitic diseases continue to be a substantial burden on human and animal health worldwide. This burden is compounded by food and water shortages as well as poor sanitation (Hotez et al., 2009). The World Health Organization (WHO) estimates that more than 1 billion people are currently affected by parasitic nematodes and that the impact of these worms surpasses diabetes or lung cancer in disability adjusted life years (DALYs) (<http://www.who.int/en/>). Considering the limited number of approved anthelmintic drugs for widespread use in humans or other animals, current treatment programs to control these nematodes can lead to drug resistance (Osei-Atweneboana et al., 2011), requiring continued efforts to search for new interventions. The traditional approach of high-throughput screening for drug discovery is costly and has yielded only a small number of anthelmintic drugs, with no novel classes approved for use in humans in decades (Keiser and Utzinger, 2010). An alternative approach is to understand the molecular and biochemical pathways in parasitic nematodes to guide future discovery efforts.

Major advances in high-throughput sequencing have led to a substantial expansion in the availability of genomic and transcriptomic data for parasitic nematodes in recent years. To date, the draft genomes of *Trichinella spiralis* (clade I) (Blaxter et al., 1998); *Ascaris suum*, *Brugia malayi*, *Loa loa* (clade III); *Strongyloides ratti*, *Bursaphelenchus xylophilus*, *Meloidogyne hapla*, *Panagrellus redivivus* (clade IV); *Haemonchus contortus* and *Pristionchus pacificus* (clade V) have been published (Ghedini et al., 2007; Abad et al., 2008; Dieterich et al., 2008; Opperman et al., 2008; Jex et al., 2011; Kikuchi et al., 2011; Mitreva et al., 2011; Desjardins et al., 2013; Laing et al., 2013; Schwarz et al., 2013; Srinivasan et al., 2013). In addition to the extensive genomic research linked to species of *Caenorhabditis* (e.g., *Caenorhabditis elegans* Sequencing Consortium, 1998; Gupta and Sternberg, 2003), these draft genomes represent a massive resource for the scientific community working on fundamental and applied aspects of parasitic worms.

The benefits of these resources can now be realised through a collective effort of mining and curating smaller datasets such as particular gene families involved in essential biological processes. Protein kinases (PKs) are one of the largest gene families of metazoans and regulate a wide range of cellular processes including cell-cycle progression, transcription, DNA replication and meta-

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bolic processes (Vanrobays et al., 2001; LaRonde-LeBlanc et al., 2005; LaRonde-LeBlanc and Wlodawer, 2005a; Granneman et al., 2010; Campbell et al., 2011; Widmann et al., 2012; Read et al., 2013). PKs can activate/inactivate target proteins by catalysing the transfer of phosphate groups to specific residues (i.e. His/Asp, Ser/Thr/Tyr and Arg) on their target proteins and thus play a regulatory role in nearly all cell signalling pathways (Hanks et al., 1988). Presently, PKs can be divided into eukaryotic protein (ePKs), protein kinase-like (PKL) and atypical protein (aPK) kinases. For instance, of more than 500 human PKs, less than 10% are PKL proteins, many of which were previously classified as aPKs. The PKL kinases represent 19 families, one being the right open reading-frame kinases (RIOKs) (Kannan et al., 2007; Manning et al., 2011).

In the present study, we extend previous studies of RIOKs of selected strongylid nematodes (Hu et al., 2008; Campbell et al., 2011; Ansell et al., 2013), indicating that they (i) are likely to be crucial for development, survival and/or reproduction, (ii) are conserved among nematodes, but (iii) are divergent from related molecules in other ecdysozoans and vertebrates. Multicellular organisms studied to date all have three *riok* genes (*riok-1*, *riok-2* and *riok-3*). Functional studies of these genes in *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (elegant worm), *Drosophila melanogaster* (vinegar fly) and vertebrate cell lines indicate key roles of RIOKs in ribosome maturation, cell-cycle progression and/or chromosome stability (Vanrobays et al., 2001; Angermayr and Bandlow, 2002; Geerlings et al., 2003; Ceron et al., 2007; Simpson et al., 2008; Granneman et al., 2010; Strunk et al., 2011; Widmann et al., 2012; Esser and Siebers, 2013). Recently, another study (Read et al., 2013) has indicated that *D. melanogaster riok-2* (*Dme-riok-2*) is involved in cell cycle progression through the regulation of the Akt signalling pathway, suggesting that *riok-2* might be part of a complex cell-cycle signalling system. In spite of this information, the essential roles of RIOKs have only been assumed for most organisms, including parasitic nematodes (Vanrobays et al., 2001; LaRonde-LeBlanc et al., 2005; LaRonde-LeBlanc and Wlodawer, 2005a; Granneman et al., 2010; Campbell et al., 2011; Widmann et al., 2012; Read et al., 2013). To date, structural information for the RIOK family is based on two crystal structures for *Afu*-RIOK-1 and *Afu*-RIOK-2 of *Archaeoglobus fulgidus* (Archaea) and on *Cth*-RIOK-2 of *Chaetomium thermophilum* (thermophilic fungus) (LaRonde-LeBlanc and Wlodawer, 2004; LaRonde-LeBlanc et al., 2005; Ferreira-Cerca et al., 2012). Remarkably, the latter, fungal kinase acts in vitro as an ATPase rather than a kinase, suggesting that RIOKs might be involved in a process that is distinct from the assumed canonical serine/threonine kinase activity (Ferreira-Cerca et al., 2012). Gaining insights into the structural characteristics and molecular functions of RIOKs, and their involvement in cellular pathways, might provide a basis for the future design of novel drugs against parasitic nematodes. As a first step, we undertook here a detailed investigation of the *riok* gene family in 12 nematode species representing different phylogenetic clades, and for which draft genomes and transcriptomes are presently available.

2. Materials and methods

2.1. Gene prediction and identification

We extracted genomic, transcriptomic and protein datasets for the identification, isolation and curation of full-length *riok* genes from 13 nematode species from WormBase (WS238 www.wormbase.org) for *C. elegans*, *Caenorhabditis briggsae*, *T. spiralis*, *L. loa*, *A. suum*, *B. xylophilus*, *M. hapla*, *Meoidogyne incognita* and from other repositories for *H. contortus* (GenBank Assembly IDs: GCA_000469685.1 and GCA_000442195.1), *P. redivivus* (GenBank Assembly ID: GCA_000341325.1), *P. pacificus* ([\[chus.org\]\(http://www.pristion\)\), *S. ratti* \(GenBank Assembly ID: GCA_000208845.1\) and *B. malayi* \(GenBank Assembly ID: AAQA00000000.1\). Using partial, inferred amino acid \(aa\) sequences of RIOK proteins, *riok* genes were located and extracted using the programs BLAT \(Kent, 2002\) and Exonerate \(Slater and Birney, 2005\). Transcription was established by identifying the corresponding *riok* transcripts in the transcriptomes of individual nematode species, and each gene was manually curated to verify that it was full-length. Using a web-based application \(<http://wormweb.org/exonintrn>\), gene structures and functional protein domains were displayed. The nomenclature of RIOKs followed the guidelines of WormBase \(\[www.wormbase.org\]\(http://www.wormbase.org\)\). Genes/proteins were named according to an abbreviation of the binomial species name, followed by the gene and the gene number. For example, *riok-1* of *C. elegans* is called *Cel-riok-1*, and the encoded protein is *Cel*-RIOK-1.](http://www.pristion</p></div><div data-bbox=)

2.2. Phylogenetic analysis of nematode RIOKs

Phylogenetic analysis of sequence data was conducted using Bayesian inference, employing the program MrBayes 3.1.2 (<http://mrbayes.csit.fsu.edu/index.php>). Posterior probabilities (pp) were calculated for RIOK aa sequences, using 2,000,000 generations, a burnin fraction of 0.25, employing four simultaneous tree-building chains, saving every 100th tree. For each of the three RIOK subfamilies, a phylogenetic tree was constructed based on aa sequence alignments of the minimum kinase domain. RIOK sequences from *T. spiralis* were used as outgroups. Correlations between evolutionary distance (sum of branch lengths between the different RIOKs – through the Python library, BioPhylo [<http://biopython.org/DIST/docs/api/Bio.Phylo-module.html>]) and gene-structural characteristics such as intron length, coding length, gene length, number of exons, as well as species genome size, were calculated using R (linear regression function) for members of each RIOK subfamily.

2.3. Transcription profiling of nematode RIOKs

Transcription profiles of *riok* genes were characterised in different developmental stages of the parasitic nematodes *H. contortus*, *A. suum* and *B. malayi* using extensive RNA-seq data with biological replicates ($n \geq 3$) for *H. contortus* and *B. malayi* (Jex et al., 2011; Laing et al., 2013; Schwarz et al., 2013). RNA-seq data for *A. suum* (SRP010159 and SRP026668) and *H. contortus* (SRP026668) were extracted from the Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/sra>). *Brugia malayi* RNA-seq data were available from the Wellcome Trust Sanger Institute, UK, and were obtained from <http://www.ebi.ac.uk/ena/data/view/PRJEB2709>. The full-length *rioks* were re-predicted for each species genomic sequence. Trimmomatic software was used, as described in Schwarz et al. (2013), to filter the paired-end RNA-seq reads for quality in individual samples (representing different developmental stages and tissues). Each set of the ‘decontaminated’ and quality filtered paired-end RNA-seq data was mapped to each species genomic sequence, using Burrows-Wheeler Aligner (BWA) software (Li and Durbin, 2010). The reads that mapped to each *riok* were extracted using the program SAMtools (Li et al., 2009). For each developmental stage, the $\log_{10}$ value of the resultant read counts was used as input data to produce heat-maps. The same scaling was used for expression levels in parasitic nematodes and *D. melanogaster*; low expression ($\log_{10}$ value 0–2), moderate expression ($\log_{10}$ value 2–4) and high expression ($\log_{10}$ value > 4) were defined. Read counts were used as input data for edgeR (Robinson et al., 2010). Initially, levels of differential transcription were calculated by pairwise comparisons among different developmental stages or sexes of each nematode. Using edgeR-calculated common and gene-wise dispersion factors, genes from *H. contortus* and *B. malayi* with multiple RNA-seq biological replicates ($n = 3$) were considered

differentially transcribed if the fold-change compared with the normalised read count data was ≥ 4 , and the false discovery rate was ≤ 0.05 . For *A. suum*, transcript abundance was assumed to follow a Poisson distribution in edgeR. In this case, genes were considered differentially transcribed if the normalised fold-change between the read counts of pairwise samples was ≥ 4 , and the *P*-value was $\leq 10^{-05}$.

2.4. Structural analysis of nematode RIOKs

Structure-based aa sequence alignments were generated with SBAL (Wang et al., 2012), and conserved and novel domains identified. Three-dimensional (3D) models were built for full length and truncated *Cel*-RIOK-1, -2 and -3 and for their N- and C-terminal domains. The ab initio modeling was based on multiple-threading sequence alignments and iterative template fragment assembly simulations using I-TASSER, by matching individually generated, 3D models with the BioLiP protein function database, which also contains the known crystal structures of *A. fulgidus* (i.e. *Afu*-RIOK-1 and -2) and *C. thermophilum* (*Cth*-RIOK-2) (Roy et al., 2010, 2012; Zhang, 2008). Models with a positive C-score (i.e. confidence score for estimating the quality of the predicted model, ranging between -5 and 2) and the highest TM-score (TeMplate quality-score of the generated 3D model and a known RIOK crystal structure) were selected, and functional predictions, including gene ontology (GO) terms, enzyme classification and putative ligand binding sites, were determined individually for the RIOK proteins and for their N- and C-terminal domains. Finally, consensus sequences of kinase activity-related domains for the *Cel*-RIOKs and RIOKs of eight other nematode species were created using MUSCLE-guided alignments (Edgar, 2004), and nematode-specific consensus sequences were generated and displayed using WebLogo software (Crooks et al., 2004).

3. Results

3.1. Detailed characterisation of riok genes in nematodes

Based on the three characterised *Cel*-rioks (WBGene00019698, WBGene00013688 and WBGene00014012), we identified partial rioks in the draft genomes of 12 other nematode species for which extensive published genomic/transcriptomic data are available. After manual curation, full-length gene sequences were defined for each *riok*-1 to -3 for eight of the 12 nematodes (Table 1). No full-length rioks were found for the three plant-parasitic nematodes, *B. xylophilus*, *M. hapla* and *M. incognita*, or the free-living nematode *P. redivivus* (clade IV). An incomplete sequence of *riok*-1 was identified for *P. pacificus* (*Ppa*-*riok*-1); however, this sequence did encode a putatively functional kinase domain and was therefore retained for subsequent analyses. On average, nematode rioks display a gene length of 4,300 bp, nine exons, an open reading frame (ORF) of ~1,500 bp, and are translated into RIOK proteins with a mean length of 510 aa (Table 1).

Twenty-six full-length *riok* gene structures were defined for nine nematode species representing clades I–V (Supplementary Table S1). The regions recognised to be essential for kinase activity (Angermayr and Bandlow, 2002; LaRonde-LeBlanc and Wlodawer, 2004; LaRonde-LeBlanc et al., 2005; Esser and Siebers, 2013) were derived from multiple sequence alignments, and are displayed together with the gene structures (Fig. 1). The number of exons varies from 1 to 17, depending on the species and *riok* subfamily, with a mean of 10, nine and eight exons for *riok*-1, -2 and -3, respectively (Table 1; Fig. 1). The gene structures of each of the three rioks are relatively conserved between species within clade III and have the same number of exons. In addition, individual *riok*

structures of *B. malayi* and *L. loa* are the same. There is also a degree of conservation in gene structure for the rioks of *C. elegans* and *C. briggsae* (clade V). Compared with the small numbers of exons (varying from three to eight) in the three rioks of these *Caenorhabditis* spp., other members of this clade, including the cosmopolitan nematode *P. pacificus* and the parasitic nematode *H. contortus*, have rioks with more than 10 exons. We did not find a relationship between genome size of the nematodes, lengths of individual rioks or numbers of exons in these genes. For instance, the clade I nematode, *T. spiralis*, has the most complex *riok* gene (*Tsp*-*riok*-1; 17 exons), which is slightly longer than the average of 4,300 bp for all nematode rioks studied here. *Strongyloides ratti* has the smallest genome (52 Mb), the shortest *riok* gene lengths and the smallest numbers of exons. In contrast, *A. suum* and *H. contortus* have the largest genome sizes (273 and 320 Mb, respectively) and have the longest *riok*-1 and *riok*-2 genes that have only 11–12 exons. The *riok*-3 gene of *H. contortus* (3,382 bp) is, however, shorter than those of *B. malayi* and *L. loa* (5,148 and 5,026 bp, respectively), with genome sizes of 91–94 Mb and possessing 11 exons.

We defined the locations of three protein-encoding regions in the gene structures that are linked to kinase activity, and an additional N-terminal domain, most likely adopting a fold of a winged-helix domain (Fig. 1). This domain is encoded in the *riok*-2 genes of all nematode species studied and spans the first 3'-end exons. The ATP binding region (P-loop) encoded in *riok*-2 is located on the same or the following exon, and the catalytic and metal binding regions are located towards the 3'-end of the gene on two neighbouring exons. For *riok*-3, the 5'-end (3–6 exons) codes for a domain of unknown function (DUF), whereas the ATP binding region spans the two following exons present in clade III and V nematodes. For *riok*-1, the 5'-end (1–8 exons) codes for a DUF, followed by the ATP-binding region. The catalytic region of *riok*-1 of *S. ratti* (clade V) and *T. spiralis* (clade I) spans two exons, followed by the metal-binding region on the same exon.

3.2. Transcription profiles of riok genes in different life stages of parasitic nematodes

Transcripts of *riok*-1, *riok*-2 and *riok*-3 were detected in all developmental stages of the three parasitic nematodes studied here, namely *H. contortus*, *B. malayi* and *A. suum*. The transcription profiles of *H. contortus* rioks were very similar for two independent RNA-seq datasets (Laing et al., 2013; Schwarz et al., 2013). In both datasets, the transcription level of *Hco*-*riok*-3 was slightly higher compared with *Hco*-*riok*-1 and *Hco*-*riok*-2, and the transcription levels of *Hco*-*riok*-1 and -2 were similar and consistent throughout the parasite's development (Fig. 2). No major differences in transcription for any of the rioks were detected between the adult females and males of *H. contortus*. Also, in *B. malayi* (Ghedini et al., 2007), transcription levels for the rioks were relatively consistent throughout development, with highest levels in adult females (Fig. 2). In *A. suum*, the transcription of all three genes was highest in the egg stage and, for *Asu*-*riok*-3, it was relatively consistent throughout development. We observed a slight elevation in transcription of *Asu*-*riok*-2 in L4s, but did not detect any difference in transcription for any of the rioks between the two sexes of *A. suum* (Fig. 2). An analysis of available RNA-seq data did not reveal any alternatively spliced transcripts for the three rioks of *A. suum*, *B. malayi* or *H. contortus*. This finding is inconsistent with the two splice variants (*Cel*-*riok*-1a and -1b) of the *riok*-1 in *C. elegans*. The ORFs of both *Cel*-*riok*-1 splice variants were the same across the first six exons. A stop codon at the seventh exon of splice variant *Cel*-*riok*-1b results in a truncated version of the RIOK protein (Fig. 3). This truncated protein does not contain catalytic and metal-binding regions required for kinase activity

Table 1
The right open reading frame kinase genes characterised for nine nematode species. Gene name, number of exons, gene length in bp, codon length (bp), total length of the introns (bp) and the full-length right open reading-frame kinase protein (amino acids, aa) are presented for each *riok* gene and species.

Species	Genome size (Mb)	Gene name	Exons	Gene length (bp)	Coding length (bp)	Intron length (bp)	Protein length (aa)
<i>Strongyloides ratti</i>	52	<i>Sra-riok-1</i>	3	1,709	1,530	179	510
		<i>Sra-riok-2</i>	2	1,621	1,569	52	523
		<i>Sra-riok-3</i>	1	1,696	1,695	0	565
<i>Ascaris suum</i>	309	<i>Asu-riok-1</i>	11	7,425	1,533	5,892	511
		<i>Asu-riok-2</i>	12	12,489	1,587	10,902	529
		<i>Asu-riok-3</i>	11	7,465	1,530	5,935	510
<i>Brugia malayi</i>	94	<i>Bma-riok-1</i>	11	4,281	1,479	2,802	493
		<i>Bma-riok-2</i>	12	3,425	1,470	1,955	490
		<i>Bma-riok-3</i>	11	5,026	1,488	3,538	496
<i>Loa loa</i>	91	<i>Llo-riok-1</i>	11	4,783	1,485	3,298	495
		<i>Llo-riok-2</i>	12	3,521	1,545	1,976	515
		<i>Llo-riok-3</i>	11	5,148	1,518	3,630	506
<i>Caenorhabditis briggsae</i>	104	<i>Cbr-riok-1</i>	6	1,806	1,545	261	515
		<i>Cbr-riok-2</i>	5	3,064	1,596	1,468	532
		<i>Cbr-riok-3</i>	6	1,712	1,461	251	487
<i>Caenorhabditis elegans</i>	100	<i>Cel-riok-1</i>	8	4,922	1,518	3,404	506
		<i>Cel-riok-2</i>	3	4,953	1,587	3,366	529
		<i>Cel-riok-3</i>	5	2,516	1,530	986	510
<i>Pristionchus pacificus</i>	64	<i>Ppa-riok-1</i>	Incomplete	3,842	570	3,272	190
		<i>Ppa-riok-2</i>	14	3,563	1,575	1,988	525
		<i>Ppa-riok-3</i>	10	2,334	1,656	678	552
<i>Haemonchus contortus</i>	320	<i>Hco-riok-1</i>	11	7,865	1,338	6,527	446
		<i>Hco-riok-2</i>	13	6,835	1,587	5,248	529
		<i>Hco-riok-3</i>	14	3,382	1,512	1,870	504
<i>Trichinella spiralis</i>	64	<i>Tsp-riok-1</i>	17	4,904	1,740	3,164	580
		<i>Tsp-riok-2</i>	9	2,954	1,302	1,652	434
		<i>Tsp-riok-3</i>	6	2,071	1,392	679	464

(Angermayr and Bandlow, 2002; LaRonde-LeBlanc and Wlodawer, 2004; LaRonde-LeBlanc et al., 2005; Esser and Siebers, 2013).

3.3. Phylogenetic relationships of RIOK proteins of nematodes

Phylogenetic analyses of inferred protein sequence data by Bayesian inference displayed the relationships of individual RIOKs representing distinct nematode clades (Blaxter et al., 1998) (Supplementary data S1–S3) (Fig. 4). For individual RIOKs, the proteins of *C. elegans* and *C. briggsae* (clade V) grouped more closely together than to those of *H. contortus* (clade V), and all of them grouped together with absolute support (pp = 1.00), to the exclusion of RIOKs representing clade III. However, the RIOK-2 and -3 of *P. pacificus* grouped together (pp = 0.70–0.84) with homologues of the other species representing clade V. The proteins of the filarioids *L. loa* and *B. malayi* (clade III) grouped more closely together than homologues of *A. suum* (clade III), and all of them grouped together with absolute support (pp = 1.00). These two distinct groups of RIOKs representing clades V and III, respectively, grouped to the exclusion of those representing clades I and IV. Therefore, for each of the three RIOKs, the proteins grouped according to their respective evolutionary nematode clade, with those of *T. spiralis* (clade I) being most evolutionarily distant. There was a positive correlation between the evolutionary distance of the RIOKs and two gene-structural characteristics: the number of exons ($P < 0.001$) and coding length ($P < 0.1 \text{ E}^{-06}$).

3.4. Structural analysis and functional predictions of nematode RIOKs

Three-dimensional structural models were predicted ab initio for *Cel*-RIOK-1, -2 and -3, to infer the minimum kinase domains and DUFs. The *Cel*-RIOK models were built based on the crystal structure of *Cth*-RIOK-2 (fungus) (Fig. 5 and Supplementary Figs. S1–S3). For *Cel*-RIOK-2, the full-length protein (winged-helix,

minimal kinase domain and C-DUF) was modelled, while partial proteins were modelled to *Cel*-RIOK-1 (i.e. minimal kinase domain and C-DUF) and *Cel*-RIOK-3 (i.e. minimal kinase domain). The N-lobe, Hinge region and C-lobe that form the minimum kinase domain are predicted to be crucial for kinase activity (LaRonde-LeBlanc and Wlodawer, 2004; LaRonde-LeBlanc et al., 2005), and the number and location of their secondary structure motifs (i.e. α -helices and β -strands) are conserved among the three *Cel*-RIOKs (Fig. 5). The secondary structures that comprise the N-lobe are: three β -strands that form the P-loop (the ATP-binding region), an α -helix and a fourth β -strand. The two β -strands of the Hinge region connect the N-lobe to the C-lobe; the latter lobe contains an α -helix, followed by two β -strands that engulf the catalytic region, which is separated from the metal-binding region (with two β -strands) by a short 8–10 aa tract, replacing the activation loop present in the sequences of other kinases, which binds to the substrate (Fig. 5).

Contrary to the conserved, minimum kinase domain, the N- and C-terminal domains differ considerably in length as well as predicted secondary structure elements (Fig. 5). The N-terminal domain of *Cel*-RIOK-1 is 130 aa in length and is comprised of four α -helices (Fig. 5 and Supplementary Table S2), whereas the C-terminal domain (168 aa) contains a short β -strand and α -helix, followed by a bundle of four α -helices. This assembly is similar to that of the C-terminal domain of *Cel*-RIOK-2 (Fig. 5). The N-terminal domain (92 aa) of *Cel*-RIOK-2 has four α -helices and two β -strands that form a fold, known as the winged-helix structure, plus an additional α -helix. In this context, the longest, extending N-terminal domain is found in *Cel*-RIOK-3 (242 aa) and comprises two α -helices and a β -strand, followed by three α -helices. The C-terminal domain of *Cel*-RIOK-3 is the shortest in this comparison (58 aa) and contains a β -strand and two α -helices. Structural homology models were inferred for the N- and C-terminal domains of *Cel*-RIOK-1 and -3 that were not included in the initial *Cel*-RIOK

Fig. 1. Nematode right open reading-frame kinase (*riok*) gene structures for *Strongyloides ratti* (*Sra*), *Ascaris suum* (*Asu*), *Brugia malayi* (*Bma*), *Loa loa* (*Llo*), *Caenorhabditis briggsae* (*Cbr*), *Caenorhabditis elegans* (*Cel*), *Pristionchus pacificus* (*Ppa*), *Haemonchus contortus* (*Hco*) and *Trichinella spiralis* (*Tsp*). The structures of 26 *riok* genes are displayed (black blocks represent exons and the connecting lines represent introns, using a 100 bp scale). Different functional regions are indicated within the exon blocks by coloured bars and show their location in the mature protein (blue for the winged helix domain, red for the ATP binding region, orange for the catalytic region and green for the metal binding region).

Fig. 2. Transcript profiles of *riok* genes throughout the life cycles of parasitic nematodes. Transcription levels, expressed as log10 of reads per kilo base per million (RPKM), for the three *riok* genes (*riok-1*, -2 and -3) in three parasitic nematodes – *Haemonchus contortus* (*Hco*), *Brugia malayi* (*Bma*) and *Ascaris suum* (*Asu*) – are shown in a three-colour intensity map (from red to green with increasing expression levels between 0 and 5) throughout development in the egg, L1 or microfilaria (Mfil), L2, L3, L4, adult female (Af) and adult male (Am). Infectious/parasitic stages are indicated in red.

models, and GO terms were assigned to *Cel*-RIOK-1-N-DUF (i.e. actin binding, protein localisation, muscle contraction), *Cel*-RIOK-1-C-DUF (kinase regulator activity, transmembrane trans-

porter activity, cell death), *Cel*-RIOK-3-N-DUF (ATPase activity, toxin receptor binding, drug transport) and *Cel*-RIOK-3-C-DUF (ion binding, integral to membrane) (Supplementary Table S2).

Fig. 3. *Caenorhabditis elegans* riok-1 (*Cel-riok-1*) splice variant. The structures of the *Cel-riok* genes (*Cel-riok-1a* and *Cel-riok-1b*) are shown (black blocks represent exons and the connecting lines represent introns, using a 100 bp scale). Different functional regions are indicated within the exon blocks by coloured bars and show their locations in the mature protein (red for the ATP binding region, orange for the catalytic region and green for the metal binding region).

Fig. 4. Phylogenetic relationships of the three right open reading-frame kinase subfamilies (RIOK-1, RIOK-2 and RIOK-3) representing the nematodes studied herein (*Brugia malayi* (Bma), *Loa loa* (Llo), *Ascaris suum* (Asu), *Strongyloides ratti* (Sra), *Trichinella spiralis* (Tsp), *Caenorhabditis briggsae* (Cbr), *Caenorhabditis elegans* (Cel), *Haemonchus contortus* (Hco), and *Pristionchus pacificus* (Ppa)). Posterior probabilities are indicated if <1.00. Coloured boxes represent phylogenetic clades (grey, green, pink and orange boxes represent phylogenetic clades II–V, respectively).

The consensus sequences of the three functional loops (P-loop, catalytic loop and metal-binding loop) of the three RIOKs for nine nematode species showed a higher degree of sequence conservation (52–73%) than the Hinge region (20–25%) (Fig. 5). For the three RIOK consensus sequences of the nine nematodes studied, there was neither variation in the catalytic Asp and Asn residues nor was there variation in the Asp and Gln residues of the metal-binding loop which are crucial for the positioning of the Mn^{2+} ions (LaRonde-LeBlanc and Wlodawer, 2004) (i.e. catalytic loop: Asp 286 to Asn 290 (*Cel*-RIOK-1), Asp 227 to Asn 232 (*Cel*-RIOK-2), Asp 388 to Asn 393 (*Cel*-RIOK-3); metal-binding loop: Asp 302 to Gln 305 (*Cel*-RIOK-1), Asp 245 to Gln 248 (*Cel*-RIOK-2), Asp 406 to Gln 409 (*Cel*-RIOK-3)). The flexible loop is located between the end of the P-loop and the adjacent α -helix, and is characteristic of RIOKs (LaRonde-LeBlanc et al., 2005; Hu et al., 2008). The flexible loop is ~20 aa long, and the conserved sequence pattern for *Cel*-RIOKs is: T-S/L-x-x-x-F/I-K-x-R/K-x-x-Y-V/H-x-x-x-F/K-R/S.

4. Discussion

We combined a bioinformatics approach with manual curation to provide improved insights into functional and structural aspects of the RIOK family of parasitic and free-living nematodes whose draft genomes are publicly available (Ghedini et al., 2007;

Dieterich et al., 2008; Opperman et al., 2008; Jex et al., 2011; Mitreva et al., 2011; Desjardins et al., 2013; Laing et al., 2013; Schwarz et al., 2013; Srinivasan et al., 2013). Due to a lack of reliable culturing systems to propagate parasites in the laboratory and because current gene silencing approaches are unreliable or impractical, functional analyses of genes in parasitic nematodes of animals are challenging (Geldhof et al., 2006; Maule et al., 2011). However, *C. elegans* represents a practical system for comparative analyses of close orthologues from parasitic nematodes.

From a functional perspective, current information shows that the *Cel-riok-1* encodes two splice variants required for viability, endocytosis, fertility and fat storage (Fraser et al., 2000; Ashrafi et al., 2003; Rual et al., 2004; Sönnichsen et al., 2005; Balklava et al., 2007; Ceron et al., 2007). Although the shorter *Cel-riok-1b* variant has not yet been characterised on a protein level, it lacks catalytic and metal binding regions, suggesting that it is a pseudo-kinase that has a regulatory role via its N-DUF. Contrary to *C. elegans*, analyses of RNA-seq data for three parasitic nematodes of socio-economic importance, *A. suum*, *B. malayi* and *H. contortus*, did not have any truncated *riok-1* splice variants. Similar to *Cel-riok-1*, *Cel-riok-2* is required for viability and fertility, whereas *Cel-riok-3* encodes a RIOK that is not essential in *C. elegans* (see Fraser et al., 2000; Ashrafi et al., 2003; Rual et al., 2004; Sönnichsen et al., 2005; Balklava et al., 2007; Ceron et al., 2007). Preliminary experiments have predicted lethal null mutations in

Fig. 5. Secondary and three-dimensional (3D) structural models for *Caenorhabditis elegans* (*Cel-*) right open reading-frame kinases and amino acid residue conservation. The 3D structural models of RIOK-1, -2 and -3 of *C. elegans* are illustrated. The cartoon models are colour-mapped, according to the different domains: N-domain of unknown function (DUF), N-lobe (blue), Hinge region (green), C-lobe (red), C-DUF (pink). For each of the three RIOKs, a linear representation of the predicted secondary structure elements (α -helices and β -strands) is given for important domains and regions (DUFs, N-lobe, Hinge region, C-lobe). A logo representation of the consensus sequence of RIOKs representing nine nematode species is displayed for regions in the N-lobe (P-loop), the Hinge region and the C-lobe (catalytic and metal-binding loops) using the same colour-code as the structural cartoon.

Cel-riok-1 and *Cel-riok-2* (Boag et al., unpublished data). To date, functional studies of *riok* genes in non-nematode species have suggested roles in ribosome maturation, cell cycle progression and chromosome stability (Vanrobays et al., 2001; Angermayr and Bandlow, 2002; Geerlings et al., 2003; Ceron et al., 2007; Simpson et al., 2008; Granneman et al., 2010; Strunk et al., 2011; Widmann et al., 2012; Esser and Siebers, 2013). A recent report investigating differential gene expression in glioblastomas of another important metazoan model organism, *D. melanogaster*, linked over-expression of *Dme-riok-2* in these cancer cells to an escape of apoptosis via an up-regulation of the Akt signalling pathway (Read et al., 2013). Interestingly, decreased expression of *Dme-riok-1* or *Dme-riok-2* resulted in reduced Akt signalling, causing cell cycle exit and apoptosis by inducing p53 activity through the Rpl11-dependent ribosomal stress checkpoint (Read et al., 2013). Another study also showed that, in human cell lines, *Hsa-RIOK-1* and -2 interact with proteins that belong to a large complex involved in ribosomal biogenesis, and that *Hsa-RIOK-2* is released early from this complex during ribosomal maturation (Widmann et al., 2012). The increased cellular level of released RIOK-2 might be an important regulator of the Akt signalling pathway and might thus serve as a ribosomal stress checkpoint to either progress the cell cycle after the completion of ribosomal maturation or activate the apoptosis pathway upon disruption of ribosomal biosynthesis.

The transcription profiles of *rioks* are similar among the different developmental stages of three parasitic nematodes of socio-economic importance: *A. suum* – the large roundworm that infects pigs or humans, *H. contortus* – a parasite of small ruminants, costing the livestock industry hundreds of millions of dollar per annum, and *B. malayi* – a major filarial nematode of humans. No detailed developmental transcription profiles have yet been reported for *Cel-rioks*, but the ModEncode transcription profiles (RNA-seq) of *D. melanogaster rioks* (www.flybase.org for CG11660, CG11859 and CG3008) display a high degree of similarity to the developmental transcription profiles of the parasitic nematode *rioks*. Both essential genes (i.e. *Dme-riok-1* and *Dme-riok-2*) are transcribed at moderate levels in embryonic and larval stages (Graveley et al., 2011). This moderate transcription

is followed by a decrease in transcription levels during pupation and in adult males, whereas a moderate to high transcription is maintained in adult females. The elevated transcription levels of *Dme-riok-1* and *Dme-riok-2* in adult females are likely due to the observed elevated levels in the ovaries, while transcription levels in all other tissues are moderate. The transcription levels of *Dme-riok-3* are also elevated in the ovaries, salivary glands and digestive system of the adult female. Contrary to *Dme-riok-1* and -2, the transcription of *Dme-riok-3* is consistently moderate throughout development, as was observed here for *riok-3* transcription in parasitic nematodes. The constant and moderate transcription levels of *rioks* in the three parasitic nematodes and in *D. melanogaster* suggest that RIOKs perform house-keeping functions, coinciding with roles in essential pathways, such as ribosome maturation, cell cycle progression and chromosome stability.

Gene compactness, functional essentiality and gene expression/transcription are known variables of evolutionary rate (Choi and Hannehalli, 2013). Previous evolutionary studies examined a large number of genes but were restricted to a very small number of complete genomes (*S. cerevisiae*, *C. elegans*, *D. melanogaster*, *Mus musculus* and *Homo sapiens*) (Krylov et al., 2003; Liao and Zhang, 2006; Larracuent et al., 2008; Wang and Zhang, 2009). The variability that we observed in gene structures ($n = 26$) representing nine nematode species is remarkable (with exon numbers ranging from one to 17, and the intron lengths from 52 to 10,000 bp) compared with the very similar ORFs and protein lengths for homologous RIOKs (~1,500 bp relating to ~500 aa). This variability in gene structure is not related to the genome size or the (parasitic versus free-living) life style of species. We did observe a positive relationship between the *riok* gene compactness and the respective evolutionary clade of the nematode species, suggesting conservation in *riok* structure within each phylogenetic clade of nematodes. Functional essentiality of *riok* genes was neither related to gene structure (gene compactness and intron length) nor to transcription level. Remarkably, none of these observations can be explained by current models of evolutionary rates of genes, such as the ‘selection for economy’ model (Urrutia, 2003), which states that transcription levels are positively related to gene

compactness and the ‘genome design’ model (Vinogradov, 2004), which posits that broadly transcribed genes might require a more complex regulatory mechanism and, thus, have longer introns containing regulatory elements.

From a structural biology perspective, RIOKs can be divided into three subfamilies that all share a conserved, minimal kinase domain, but have different N- and C-terminal domains. The structural and functional characteristics of the additional (N- and C-terminal) domains have not yet been studied in detail; previous studies investigated eubacterial RIOK crystal structures, which lack most of these domains (Campbell et al., 2011). Using the structure of *Cth*-RIOK-2 (fungus) (Ferreira-Cerca et al., 2012), we generated ab initio structural models for the *C. elegans* RIOKs and, for the first known time, studied some of the N- and C-terminal regions, which were external to the minimal kinase domain. The 3D structure of this domain is conserved among *C. elegans* RIOKs, as are the secondary structural elements that position the three essential functional kinase loops, namely the P-loop (ATP-binding region), the catalytic-loop (transfer of the phosphate group) and the metal binding loop (binding one or two manganese ions to help catalyse the phosphate transfer). Currently, RIOKs are classified as atypical kinases, because they lack the activation loop which, in other kinases, is located between the catalytic and the metal-binding loops and binds to the substrate (Manning et al., 2002). For this reason, the substrate(s) of RIOKs and the kinase mechanism itself remain unclear. The flexible loop, located in the N-lobe, between the third β -strand and the subsequent α -helix, is characteristic of RIOKs (LaRonde-LeBlanc and Wlodawer, 2005a; Hu et al., 2008), and might substitute the missing activation loop, due to its known involvement in recruiting fungal *Cth*-RIOK-2 to the pre-40S particle during ribosomal biogenesis (Ferreira-Cerca et al., 2012). Sequence conservation between the flexible loop of *Cth*-RIOK-2 and the three *Cel*-RIOKs suggests a similar function of this loop in interacting with the ribosomal complex. The regions external to the minimum kinase domain might also play a role in substrate binding for phosphorylation. Alternatively, RIOKs might not have canonical serine/threonine kinase substrates, but rather have evolved to be ATPases, as suggested by the in vitro ATPase activity of *Cth*-RIOK-2 (Ferreira-Cerca et al., 2012).

To date, no sequence-based homology could be found between the N- and C-terminal domains of RIOK-1 or RIOK-3 of nematodes and any other known proteins. Therefore, we built ab initio models for these DUFs against known structural elements, and predicted possible functions independent from the minimum kinase domains based on allocated GO terms. The *Cel*-RIOK-1-N-DUF is predicted to be involved in actin binding and cellular protein localisation. For *Cel*-RIOK-1-C-DUF, one of the predicted functional annotations was ‘kinase regulator activity’. Deletion of this C-terminal domain (the four C-terminal α -helices) from both RIOK-1 and -2 leads to lethal phenotypes in yeast and fungus (Ferreira-Cerca et al., 2012) and, despite being dispensable for kinase activity, these helices have been suggested to be involved in protein–protein interactions; the C-terminal region of RIOK of the tobacco plant is suggested to interact with a viral molecule, inhibiting the cell-to-cell transport of viral RNA (Yoshioka et al., 2004). Additionally, the recently solved crystal structure of *Cth*-RIOK-2 showed that its C-terminal domain binds between the N- and C-terminal lobes of the kinase domain and inhibits kinase activity (Ferreira-Cerca et al., 2012). This self-inhibiting feature has been identified in other kinases (Xu et al., 1999; Malakhova et al., 2008; Li et al., 2012). The *Cel*-RIOK-1 C-terminal domain is structurally similar to the *Cth*-RIOK-2 C-terminal domain (four- α -helix-bundle) and might, thus, also play a regulatory role by inhibiting kinase activity (Ferreira-Cerca et al., 2012). The *Cel*-RIOK-3-N-DUF domain is a large domain (242 aa) and is predicted to be involved in transmembrane transport (ATP-regulated), while the short C-terminal domain (*Cel*-RIOK-3-C-DUF) is suggested to be involved in ion

binding. Remarkably, the predicted functional annotation of ‘lipid binding’ for RIOK-2 and -3 suggests that the RIOK family represents an evolutionary link between prokaryote lipid kinases and eukaryotic kinases. The N-terminal domain of *Cel*-RIOK-2 has a winged-helix fold (LaRonde-LeBlanc and Wlodawer, 2005b) that allows interactions with nucleic acids (Aravind et al., 2005), which accords with reports of an involvement of RIOK-2 in ribosomal RNA processing (Vanrobays et al., 2001; Schäfer et al., 2003; LaRonde-LeBlanc and Wlodawer, 2004; Granneman et al., 2010; Ferreira-Cerca et al., 2012; Read et al., 2013).

From a drug targeting perspective, the bulk of current protein kinase inhibitors have been designed to target one family of protein kinases, namely the tyrosine kinases, with more than 150 compounds that are currently in clinical trials for cancer treatments, predicted to generate a global annual revenue exceeding \$30 billion (Cohen and Alessi, 2013). Other kinase families are only now starting to receive attention due to their involvement in diseases such as hypertension (Wilson et al., 2001), Parkinson’s disease (Hardy, 2010) and inflammatory and autoimmune diseases (Clark et al., 2012). Initially, inhibitors were designed to target the ATP-binding pocket directly. However, this approach required very potent compounds to compete with ATP, and did not guarantee kinase specificity. A promising strategy is to target neighbouring hydrophobic pockets that are unique to a specific protein kinase, in particular the back pocket of the ATP-binding site (Cohen and Alessi, 2013). However, the most desirable approach for nematocides is to identify compounds that inhibit the activation of a specific kinase, which render the ATP-binding pocket a less preferred target. An example of such rational design is the inhibition of the Akt kinase activation by a highly specific compound, called Akti1/2. The inactivation of Akt is achieved through targeting a region outside of the kinase domain and preventing the conformational change needed for an upstream kinase (PDK1) to phosphorylate and activate Akt (Barnett et al., 2005; Calleja et al., 2009; Green et al., 2008; Hirai et al., 2010).

In the present study, we were guided by results from previous investigations (Hu et al., 2008; Campbell et al., 2011; Ansell et al., 2013) of RIOKs in strongylid nematodes. We showed that these *riok* genes are active during the parasitic stages. In addition, elevated transcription of *rioks* in the ovaries of female vinegar flies (Graveley et al., 2011) indicate a link between these kinases and the reproductive process. A similar function for *rioks* in the reproductive system of parasitic nematodes might represent an ‘Achilles heel’, enabling drug development. Previous work (Campbell et al., 2011) focused on targeting the minimum kinase domain of *Hco*-RIOK-1, in particular the ATP-binding pocket. However, our current evidence from the present functional and structural investigation indicates that RIOK-2 might also be a drug target candidate. The crystal structure of the fungal *Cth*-RIOK-2 allows the study of the back pocket of the ATP-binding region and the design of specific compounds to bind these hydrophobic residues. The functional importance of the C-terminal domains of RIOK-1 and RIOK-2 (with kinase-inhibiting activity) (Angermayr and Bandlow, 2002; Ferreira-Cerca et al., 2012) and their flexible loop (interacting with the ribosomal complex) (Ferreira-Cerca et al., 2012) appear to make these regions ideal for the design of specific compounds that can inhibit RIOK activation or interactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2014.06.005>.

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