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Alvaro de Obeso Fernández del Valle (Corresponding autor)

Affiliation: Departamento de Bioingeniería, Escuela de Ingeniería y Ciencias, Tecnologico de Monterrey, Ave. Eugenio Garza Sada 2501, Monterrey 64849

e-mail: adeobeso@tec.mx

ORCID: 0000-0001-8387-0957

Jesús Gómez-Montalvo

Affiliation: Departamento de Bioingeniería, Escuela de Ingeniería y Ciencias, Tecnologico de Monterrey, Ave. Eugenio Garza Sada 2501, Monterrey 64849

ORCID: 0000-0001-7811-6415

Sutherland K. Maciver

Affiliation: Centre for Discovery Brain Sciences, Edinburgh Medical School, Biomedical Sciences, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland, UK.

ORCID: 0000-0001-8234-6061

Abstract

Intron retention (IR) refers to the mechanism of alternative splicing in which an intron is not excised from the mature transcript. IR in the cosmopolitan free-living amoeba *Acanthamoeba castellanii* has not been studied. We performed an analysis of RNA sequencing data during encystment to identify genes that presented differentially retained introns during this process. We show that IR increases during cyst formation, indicating a potential mechanism of gene regulation that could help downregulate metabolism. We identify 69 introns from 67 genes that are differentially retained comparing the trophozoite stage and encystment after 24 and 48 hours. These genes include several hypothetical proteins. We show different patterns of IR during encystment taking as examples a lipase, a peroxin-3 protein, an Fbox domain containing protein, a proteasome subunit, a polynucleotide adenylyltransferase and a tetratricopeptide domain containing protein. A better understanding of IR in *Acanthamoeba*, and even other protists, could help elucidate changes in life cycle and combat disease such as *Acanthamoeba* keratitis in which the cyst is key for its persistence.

Keywords. *Acanthamoeba*, intron retention, encystment, alternative splicing

Introduction

Alternative splicing (AS) is the process through which primary transcripts can be modified in different arrangements to produce functionally different mature mRNA. AS is responsible for most of the complexity of the eukaryotic proteome (Blencowe 2006). Intron retention (IR) refers to the form of AS where an intron remains in the mature mRNA instead of being spliced out (Grabski et al. 2021). IR is a complex and evolutionarily conserved mechanism of gene regulation, and genes that go through this process are highly regulated (Jacob and Smith 2017; Schmitz et al. 2017). In many cases, the study of IR has been neglected or misinterpreted as noise. However, next generation sequencing techniques have highlighted the importance of such mechanisms in physiological and pathological processes of eukaryotic organisms (Jacob and Smith 2017).

IR is a mechanism of controlling and enhancing gene expression in eukaryotes (Vanichkina et al. 2018). It is the most prevalent mode of alternative splicing in non-animal eukaryotes (McGuire et al. 2008; Tapial et al. 2017; Grau-Bové et al. 2018). IR is also linked to the down-regulation of gene expression via the nonsense-mediated decay (NMD) pathway (Lykke-Andersen and Jensen 2015; Brogna et al. 2016; Wong et al. 2016).

IR has not been extensively studied in the cosmopolitan free-living amoeba *Acanthamoeba castellanii* or other organisms of the genus. However, one study compared exon skipping and intron retention in 65 eukaryotic transcriptomes including *Acanthamoeba*. The goal was to observe the relationship between genome architecture and alternative splicing events. This study showed that IR is more prevalent in 64 of the species than exon skipping, including *Acanthamoeba* (Grau-Bové et al. 2018). It also included other amoebae such as *Naegleria gruberi* and *Dictyostelium discoideum* (Grau-Bové et al. 2018).

A. castellanii has a highly variable genome size. For example, the genome for *A. castellanii* Neff strain is 41 Mb, while *A. castellanii* ATCC 50370 genome is 115.3 Mb (Clarke et al. 2013; Chelkha et al. 2018). The genome has at least 56,920 annotated ORF and 15,655 genes reported in AmoebaDB (Amos et al. 2022). *Acanthamoeba* genes have an average of 6.2 introns (Roy 2006; Clarke et al. 2013). Other forms of AS in *Acanthamoeba* have been reported. The SBDS gene (for the Shwachman-Bodian-Diamond syndrome protein) is upregulated during encystation and phagocytosis where it presented two diverse patterns of expression (Wang et al. 2021). Additionally, the cytochrome P450 monooxygenase provides resistance to polyhexamethylene biguanide and other drugs thanks in part to AS processes (Huang et al. 2021).

Acanthamoeba is capable of forming an extremely resilient cyst when conditions are not favorable. In this article we present the first study related to IR in *Acanthamoeba* encystment. We show that IR is an important component in gene regulation during encystment. We compared retained introns of *Acanthamoeba* trophozoites and encysting organisms after 24 and 48 hours. We demonstrate that the number of retained introns increases during encystment related to a decrease in metabolism related to the dormant cyst.

Materials and methods

Acanthamoeba cultures

Acanthamoeba strain SB-53 (de Obeso Fernandez del Valle 2018), which is closely related to the Neff strain (ATCC 30010), was used for this study. *Acanthamoeba* cultures were grown in axenic medium (Bacto tryptone 14.3 g l^{-1}, yeast extract 7.15 g l^{-1}, glucose 15.4 g l^{-1}, Na_2HPO_4 0.51 g l^{-1} and KH_2PO_4 0.486 g l^{-1} pH 6.5). To stimulate encystment, Neff encystment media (NEM) containing of 0.1 M KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$, 10mM Tris, pH 8.8. was used (Neff et al. 1964).

RNA sequencing

Encystment was induced in confluent cultures, changing axenic media to NEM. Samples were taken at 0 (also referred to as trophozoite stage), 24 and 48 hours after inducing encystment. RNA extraction was performed using RNeasy Mini Kit (QIAGEN) following the manufacturer´s instructions. RNA quality and purity were assessed using the QUBIT RNA BR Assay Kit (Thermo Fischer Scientific). cDNA libraries were prepared for an automated TruSeq mRNAseq (next-generation shotgun sequencing) from total RNA. Sequencing was performed with a HiSeq-4000 75PE by Edinburgh Genomics.

A. castellanii Neff strain reference genome (FASTA and GTF) was obtained from ENSEMBL Protists (Clarke et al. 2013; Kersey et al. 2017). Quality control was assessed using FASTQC (Andrews 2010). STAR was used to index the genome and align the reads (Dobin et al. 2013). Differential expression analysis was performed using EdgeR (Robinson et al. 2009).

Intron Retention Analysis

Six samples in total were analyzed, two belonging for each timepoint: 0, 24 and 48 hours. iREAD and IRFinder software were used to identify IR events (Middleton et al. 2017; Li et al. 2020). The intersection of the results from both software tools was used to identify retained introns. The previously mapped RNAseq analysis was compared with the intron annotation file created using GTFtools (Li 2018). In both programs, default settings were used to identify and classify an IR event by a minimum retention of the intron in 10% of the transcripts (IR ratio ≥ 0.1). Differential expression of introns was calculated using EdgeR (Robinson et al. 2009). A direct comparison of differential expression of genes and retained introns was performed to assess if the increase of IR events was not caused by the increase of the gene. The comparison is expressed as a Log2IRratio (encystment vs trophozoite) obtained by dividing the IR ratio mean during encystment (either at 24h or 48h) and the IR ratio mean at 0h. AmoebaDB was used when required to identify specific genes (Amos et al. 2022).

Results

The expression of three genes related to the encystment process were compared at the different timepoints (Hirukawa et al. 1998; Dudley et al. 2008; Moon et al. 2009). This was used to prove that encystment was under way at a molecular level and can be observed in (de Obeso Fernandez del Valle 2018). Differential expression of the cyst specific protein 21, encystation mediating serine proteinase and mitogen-activated protein kinas can be o0bserved in figure 1.

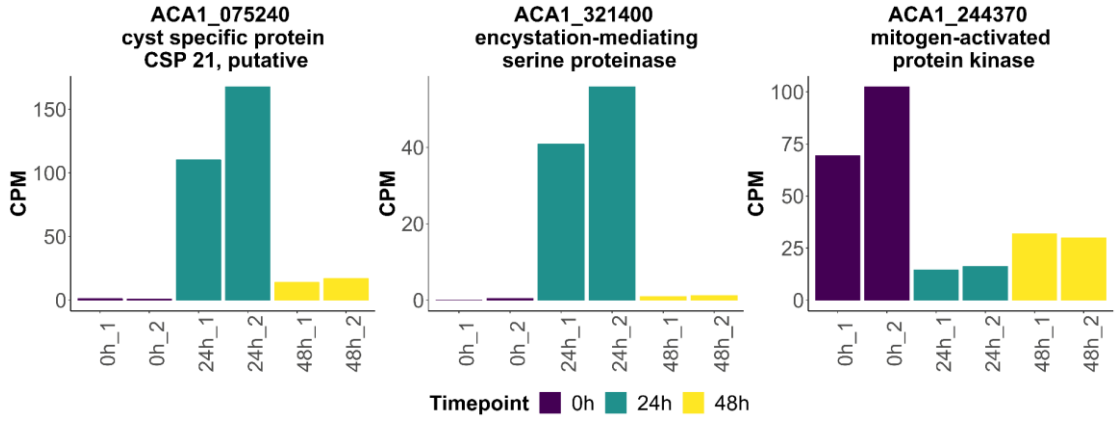

Figure 1. Differential expression of three genes reported to be involved during *Acanthamoeba* encystment. Expression of each gene is represented in counts per million (CPM) and both replicates are shown in each graph.

IR increased during encystment of *Acanthamoeba* as shown after 24 and 48 hours in Table 1. The total number of retained introns identified across the three treatments was 987 and included 891 genes or slightly over 5% of the genes in the genome. The number of retained introns had an increase of over 30% after 48 hours of encystment (633 to 825). However, some of the retained introns are shared in the three samples. There are 226 unique IR events after 24 hours and 282 events after 48 hours in contrast to the original timepoint of 0 hours. There are also 169 introns being differentially retained from 24 to 48 hours. We found 110 IR events that are lost during the encystment process after 24 hours.

Table 1. Retained introns of *Acanthamoeba* during encystment

	0 hours	24 hours	48 hours
Retained introns	633	749	825
IR not seen at 0 hours		226	282

After identifying the retained introns, the expression of genes and retained introns during encystment was compared to verify that the overrepresentation of IR events was not caused by an increase of gene overexpression since overexpression of a gene would lead to overrepresentation of a retained intron. We considered a Log2IRratio (encystment vs trophozoite) above 1 or below -1 as the threshold for differentially retained introns. After 24 hours, we found 33 instances in which the IR event had a Log2IRratio (encystment vs trophozoite) above 1, and 9 below -1. After 48 hours, but not at 24 hours, there were 23 IR events that were overrepresented in relation to the gene and only 4 that were underrepresented. A heatmap of the differentially retained introns is shown infigure 2. These results are also shown in figure 2**Error! Reference source not found.** as scatterplots comparing proportionality of genes and retained introns. Note that genes showing the lowest change in expression were associated with higher changes in IR during encystment, compared to genes showing greater log2FC, most of which showed minimum changes in IR levels. Of the overrepresented IR events after 24 hours two of them (ACA1_070640 and ACA1_315050) had two introns retained.

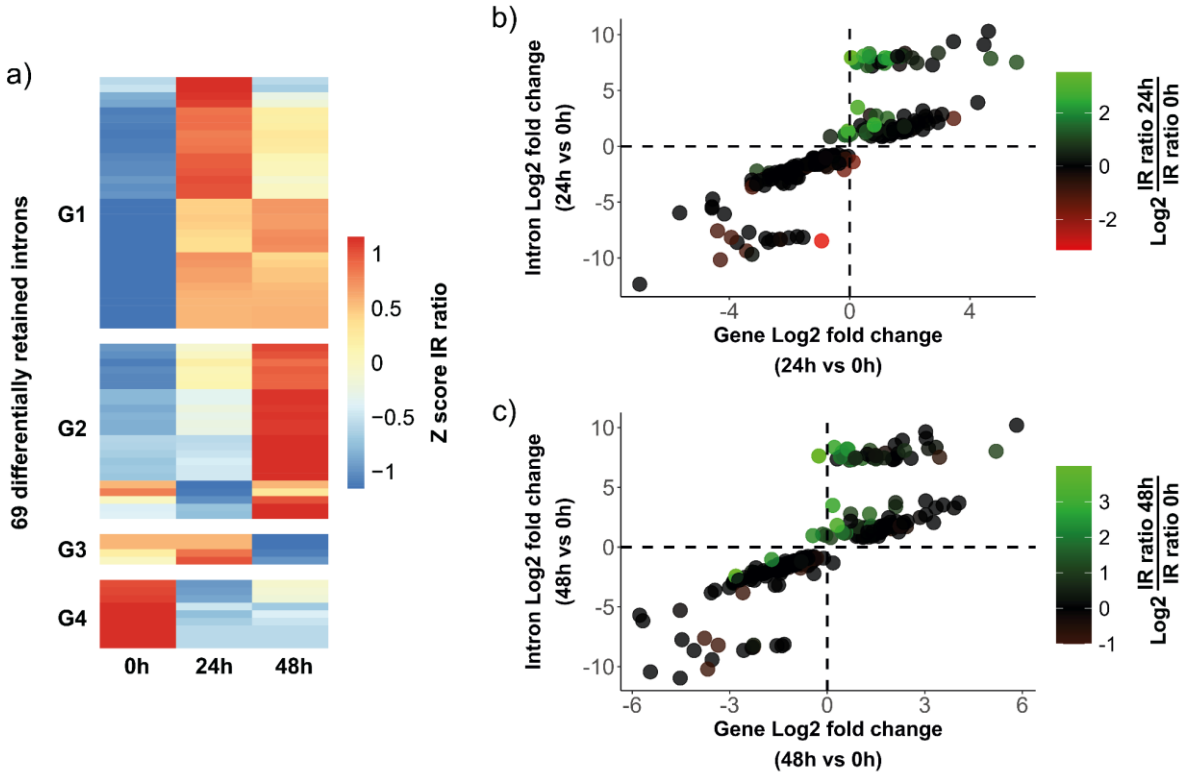

Fig. 2 Differentially retained introns/genes ratio encystment vs trophozoite). a) Heatmap of differentially retained introns where: G1 are upregulated retained introns after 24h; G2 after 48 hours; G3 decreased after 48 hours; and G4 decreased after 24 hours. b) and c) Scatterplots contrasting differential expression of genes and retained introns. Each dot represents one intron. Introns that are positively retained in contrast to their genes are shown in green, while introns that are negatively retained are shown in red. Some introns are not represented as the division between the IR ratio encystment vs trophozoite resulted in an undefined state (divided by zero)

Several introns are retained across the organism. In figure 3we show the retention of introns in six selected genes with some of the highest overrepresentation of retained introns. To select these genes, hypothetical proteins were ignored. These examples include a lipase, a peroxin-3 protein, an Fbox domain containing protein, a proteasome subunit, a polynucleotide adenylyltransferase and a tetratricopeptide domain containing protein.

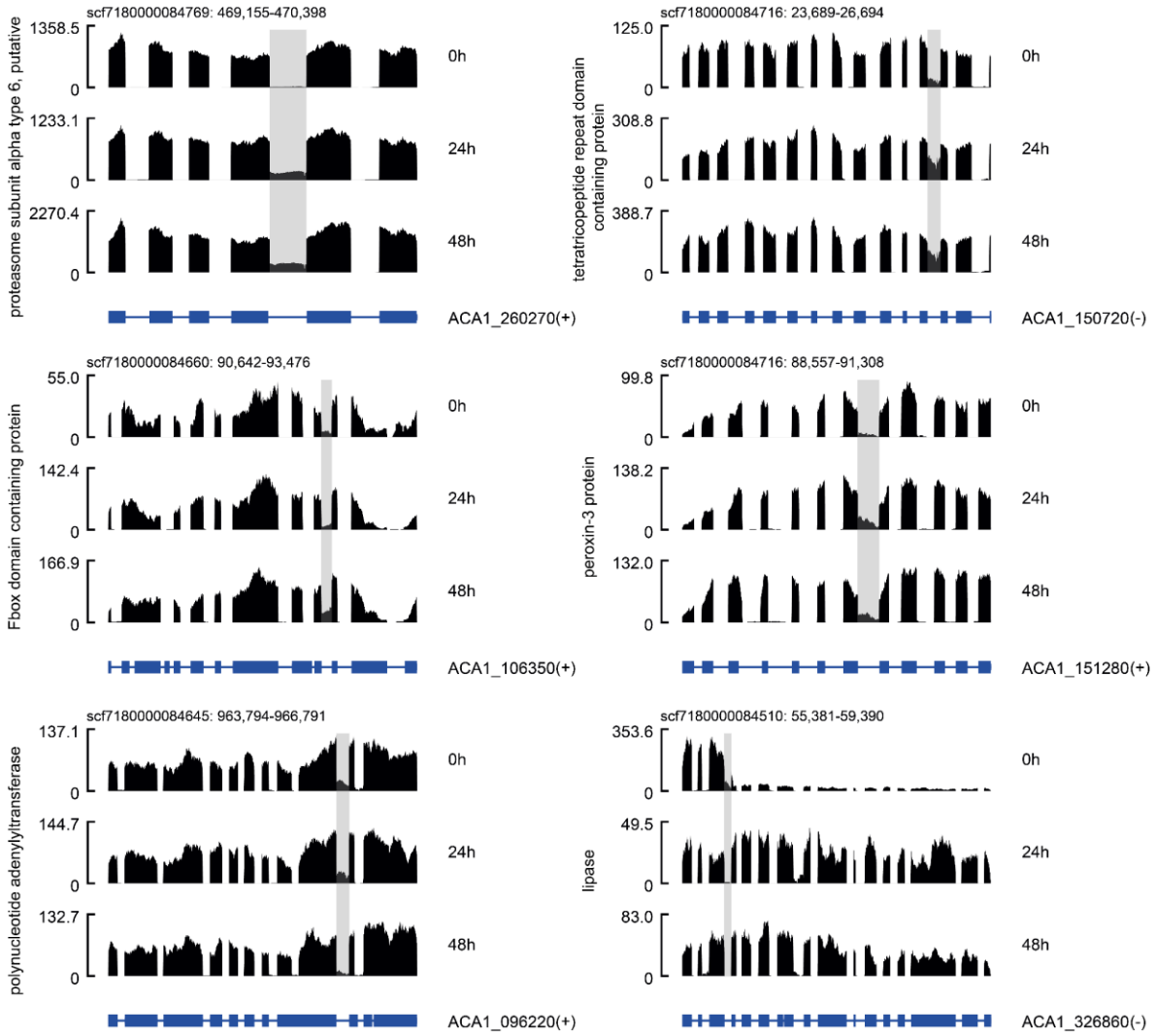

Fig. 3 Six genes with retained introns during encystment. In the bottom part of each RNA-seq density plot, in blue, you can observe normal architecture of the gene representing exons with thicker lines. The differentially retained introns are highlighted inn gray. A plus sign (+) next to the gene_ID represent 5' to 3' direction from left to right, while a minus sign (-) represents the opposite direction. To the left, you can see the name of the gene as obtained from AmoebaDB. RNA seq density plots were generated using Spark (Kurtenbach and William Harbour 2019).

A full list of the 67 genes with differentially retained introns alongside the corresponding differential expression data can be found in the supplementary materials. **Discussion**

Effect of IR in specific proteins

Gene expression patterns have been studied in life cycle events of different protozoa and in *Acanthamoeba* for under certain conditions (Moon et al. 2011; Maciver et al. 2019). IR events during *Acanthamoeba* encystment were overrepresented in 67 genes. Of these, we focused on six genes with some of highest changes in IRratio that were not annotated as hypothetical proteins. We found that three of those genes code for proteins related to degradation of proteins and lipids. The encystment process consists of a first stage characterized by degradation and autophagy (Moon et al. 2008a, b; Leitsch et al. 2010). A second stage follows with the production of cyst specific proteins and structures (Leitsch et al. 2010). The transcript for an FBox domain containing protein (ACA1_106350) retained the tenth intron of the gene. FBox machinery is important in defense and protection, such as cyst formation. *Legionella pneumophila* is capable of surviving inside *Acanthamoeba* with the assistance of the high jacking of the ubiquination machinery (Price et al. 2009). The

Fbox machinery participates in the mediation of ubiquination of proteins to be degraded by the 26S proteasome. The proteasome subunit alpha type6 (ACA1_260270) which is part of the 26S proteasome, also presented IR events, although the inclusion of the intron in the mRNA did not generate a premature stop codon. This shows a potential source for potential variability as it generated two different amino acid sequences between mRNA with and without the intron.

A lipase (ACA1_326860) presented retained introns during the trophozoite stage interestingly presented a particular expression of transcripts (figure 3). The lipase has 17 exons, of which 14 were underrepresented in transcripts during trophozoite stage after the retained intron. This shows a possible upregulation of the mature transcript during encystment even with a small overrepresentation of the retained intron, and a clear example of IR as gene regulation mechanism during encystment. In contrast, the peroxin-3 protein (ACA1_151280) has a retained intron during encystment and potentially affects peroxisome biogenesis and therefore might alter oxidative process such as the ones related to lipids. Peroxisomes, and therefore peroxin-3, have been identified in several species of *Acanthamoeba* and other free-living amoebae and are also involved in protein import, which could be affected during encystment (González-Robles et al. 2020).

A polynucleotide adenylyltransferase (ACA1_096220) presented one retained intron which could probably affect the polyadenylation of other mRNAs. In this case, the downregulation of the intron occurred after 48 hours. Additionally, the tetratricopeptide repeat domain containing protein (ACA1_150720) helps modulate interactions with different proteins, which in this case is near impossible to establish. However, this can be related to the downregulation and decrease in metabolic function during the latent stage. Moreover, an ankyrin repeat protein (ACA1_315050; seen in the supplementary materials) presented two differentially retained introns and reinforces the hypothesis as this protein family is the most abundant protein-protein interaction motif (Al-Khodor et al. 2010). These discoveries highlight the lengths we have to cover to understand the AS events, transcriptome variation and their effect on the proteome during *Acanthamoeba* encystment, and almost certainly similar organisms with life cycles that include drastic life cycle events such as encystment.

Effect of IR at the species level

Several early developed protists or unicellular eukaryotes have very few introns and include the genera *Giardia*, *Entamoeba* and *Trichomonas* (McGuire et al. 2008). Apicomplexans, such as *Toxoplasma gondii* and *Plasmodium falciparum,* have shown that IR might be a widespread mechanism for differentiation and could partly explain the increase in IR in *Acanthamoeba* (Lunghi et al. 2016). Some eukaryotic species have a high retention of introns (Ner-Gaon et al. 2004). For example, IR can affect around 80% of protein coding genes in humans (Middleton et al. 2017). Other mammals have shown the presence of such events between 50-75% of their genes (Braunschweig et al. 2014). Although IR is not the main route of alternative splicing in most animals, microscopic animal parasites such as *Echinococcus* present high volumes of IR retention (33-36% of genes presented some sort of alternative splicing), highlighting the diversity that exists in IR and alternative splicing across organisms (Liu et al. 2017). Additionally, the potato cyst nematode *Globodera rostochiensis* uses IR to regulate chorismate mutase which plays an important role in pathogenesis (Lu et al. 2008). IR has even been linked to multicellularity through *Creolimax fragantissima* (de Mendoza et al. 2015). The phenomenon has been shown to happen in all kingdoms and viruses that present introns in their genome (McGuire et al. 2008). *Acanthamoeba* showed small IR at 5% in comparison to other organisms. Nevertheless, several of these genes might play a crucial role in encystment, survival and differentiation.

Encystment in *Acanthamoeba* leads to a vegetative cyst stage that helps protect against stressors, leading to a decrease in metabolic pathways and functions. In S*accharomyces cerevisiae*, IR has been linked to starvation and stress response, where spliced introns “clutter” the spliceosome inhibiting its function and playing a role in transcripts regulation and yeast growth (Morgan et al. 2019; Parenteau et al. 2019).

Studies in other protists or potentially infectious organisms have been recorded. For example, IR has been studied in *Trypanosoma evansi*, the agent of “surra”. IR was the main form of alternative splicing in *T. evansii*, where 819 of 820 genes analyzed presented it. These genes mainly have functions related to RNA processing and cellular components. This occurs mainly during the parasitic stage (Zheng et al. 2019). *Entamoeba*

198 *histolytica*, the causative agent of amoebiasis, has shown that its main route of alternative splicing is IR (Valdés
199 et al. 2014). IR has also been linked to virulence of *E. histolytica* through the splicing factor U2AF84 (Gonzalez
200 Blanco et al. 2021).

201 IR studies are a novel way of analyzing RNAseq data that could provide new insights in *Acanthamoeba* and
202 other microorganisms of interest (Zheng et al. 2020). The study of IR provides important insight in regulating
203 gene expression that could increase the understanding of the biology of different organisms and the development
204 and persistence of infection (Lunghi et al. 2016). For example, IR events are regulated by methylation processes
205 creating a link between the comprehension of epigenetic regulation and IR (Wong et al. 2017). IR has been
206 demonstrated to play an important role in several diseases, therefore it should not be overlooked (Wong et al.
207 2016). IR studies and the insight provided in gene expression might open the possibility for new therapies
208 against *Acanthamoeba* diseases, since encystment has been identified as the key to persistence (Lorenzo-
209 Morales et al. 2015). Understanding IR and other ways of alternative splicing offer insight into the
210 transcriptional landscape of these organisms (Monteuuis et al. 2019). As new data is being generated through
211 the increased use of next generation sequencing and other high-throughput techniques, processes like IR and
212 alternative splicing are valuable resources for understanding the biology of organisms such as *Acanthamoeba*.

213 Further research is required to better understand this process and the impact in general of alternative splicing in
214 *Acanthamoeba* and other pathogenic protists. This should help combat different diseases caused by amoebae
215 such as *Acanthamoeba* keratitis. Additionally, a more in-depth study of IR events in different conditions and
216 microorganisms might help comprehend the process.

217 **Declarations**

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225 **Competing interests**

226 Sutherland K. Maciver is part of the editorial board of Parasitology Research.

227 **Availability of data and material**

228 The information of the discussed genes can be found in the supplementary materials. Everything else can be
229 provided upon request.

230 **Ethics approval**

231 Not applicable

232 **Consent to participate**

233 Not applicable

234 **Consent for publication**

235 Not applicable

236 **Author contributions**

A.d.O.F.d.V conceived the study, isolated the strain, performed RNA-Seq, analyzed RNA expression and IR data and wrote the first draft of the manuscript. J.G.M. planned and analyzed IR data and contributed intellectually to the paper. S.K.M. supervised the project, contributed intellectually and edited the manuscript. All authors read and approved the final manuscript.

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