



# Identification of essential genes for *Acanthamoeba castellanii* excystation during encystation and excystation



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## Abstract

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*Acanthamoeba* is an opportunistic pathogen that causes *Acanthamoeba* keratitis, granulomatous amoebic encephalitis, and other cutaneous diseases. The life cycle of *Acanthamoeba* consists of 2 stages of trophozoites and cysts. Under adverse environmental conditions, *Acanthamoeba* encysts, while the conditions become favorable for growth, it reverts to the trophozoite form. *Acanthamoeba* excystation is crucial for its proliferation and can lead to recurrent infections after incomplete treatment. To identify the factors involved in excystation, *A. castellanii* was subjected to either encystation- or excystation-inducing conditions, and gene expression profiles were compared using mRNA sequencing. *A. castellanii* samples were collected at 8 h intervals for analysis under both conditions. Differentially expressed gene analysis revealed that 1,214 and 1,163 genes were upregulated and downregulated, respectively, by more than 2-fold during early excystation. Five genes markedly upregulated in early excystation (*ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, *ACA1\_275740*, and *ACA1\_112650*) were selected, and their expression levels were confirmed via real-time PCR. Small interfering RNA (siRNA) targeting these 5 genes was transfected into *Acanthamoeba* and gene knockdown was validated through real-time PCR. The silencing of *ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, and *ACA1\_112650* inhibited excystation and suggested that these genes might be essential for excystation. Our findings provide valuable insights for suppressing *Acanthamoeba* proliferation and recurrence.

**Keywords:** *Acanthamoeba*, encystation, excystation, gene knockdown

## Introduction

*Acanthamoeba* spp. are opportunistic pathogens and causative agents of severe infections, including *Acanthamoeba* keratitis and granulomatous amoebic encephalitis [1]. The life cycle of *Acanthamoeba* spp. included such 2 stages as the vegetative trophozoite and resilient cyst form [2]. Under favorable growth conditions, the trophozoite stage predominates, but when faced with adverse conditions, such as nutrient deprivation, pH fluctuations, or drug exposure, the trophozoite transitions into the cyst form [3]. *Acanthamoeba* cyst form has garnered attention owe to its resistance to various biocides and disinfectants [4]. Additionally, cysts can remain viable and pathogenic for several years, play a key role in transmitting *Acanthamoeba* infections [5]. Consequently, advancements in antimicrobial agents

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**Conflict of interest**

The authors declare no conflict of interest related to this study.

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have targeted the cyst stage or aimed to prevent encystation. Despite these advances, recurrent *Acanthamoeba* infections caused by the excystation of drug-resistant cysts remain a persistent challenge [6].

Excystation, where cysts revert to the metabolically active trophozoite form, is critical to both the onset and recurrence of *Acanthamoeba* infections [7]. Although encystation and amebic drug resistance have been extensively researched, there remains a lack of studies on amebic excystation. *Acanthamoeba* cysts possess pores known as ostioles, through which they monitor environmental changes. Under favorable conditions, trophozoites emerge from the cysts through these ostioles, initiating excystation. Previous studies have elucidated the morphological changes that occur during excystation as well as the effects of various environmental and physiological conditions on *A. castellanii* excystation [7,8]. Nonetheless, genetic studies on excystation are scarce. Specifically, no comprehensive analysis of gene expression changes during excystation has been reported. Given that the transitions between trophozoites and cysts involve different gene expression patterns, understanding how these mechanisms are genetically regulated could improve *Acanthamoeba* keratitis treatment.

To address this knowledge gap, we investigated the total transcriptional changes of *A. castellanii* during excystation (8, 16, 24, and 48 h) using RNA sequencing, comparing them to the encystation process (8, 16, 24, and 48 h). Furthermore, several highly expressed genes during early excystation were identified, and their potential roles in this process were explored. Our findings may provide a foundation for developing more effective treatments for *Acanthamoeba* infections.

## Materials and Methods

### Ethics statement

Not applicable.

### Cultivation, encystation, and excystation of *Acanthamoeba*

*Acanthamoeba castellanii* (ATCC 30868) was obtained from the American Type Culture Collection and cultured axenically in PYG medium (20 g/L proteose peptone, 1 g/L yeast extract, 0.1 M glucose, 4 mM MgSO<sub>4</sub>, 0.4 mM CaCl<sub>2</sub>, 3.4 mM sodium citrate, 0.05 mM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 2.5 mM Na<sub>2</sub>HPO<sub>4</sub>, and 2.5 mM KH<sub>2</sub>PO<sub>4</sub> (pH 6.5)) at 25°C. Encystation was induced using encystment medium (95 mM NaCl, 5 mM KCl, 8 mM MgSO<sub>4</sub>, 0.4 mM CaCl<sub>2</sub>, 1 mM NaHCO<sub>3</sub>, and 20 mM Tris-HCl (pH 9.0)) at 25°C. Excystation was induced on a 1.5% non-nutrient agar (Thermo Fisher, Waltham, MA, USA) plate coated with heat-treated *Escherichia coli*. Morphological changes during encystation and excystation were observed using a light microscope.

### RNA isolation, library preparation, and sequencing data analysis

Total RNA was isolated from *Acanthamoeba* trophozoites, encysting *Acanthamoeba* (8, 16, 24, and 48 h), and excysting *Acanthamoeba* (8, 16, 24, and 48 h) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). All subsequent RNA sequencing steps were performed by eBiogen (Seoul, Korea). Libraries were prepared from 2 µg of total RNA using the SMART-

er Stranded RNA-Seq Kit (Clontech Laboratories, Mountain View, CA, USA). mRNA was isolated using the Poly(A) RNA Selection Kit (Lexogen, Vienna, Austria). Isolated mRNA was used for cDNA synthesis and shearing, following the manufacturer's instructions. Indexing was performed using Illumina indexes 1–12, and PCR was used for enrichment. Library quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) to evaluate mean fragment size, and quantification was performed using a library quantification kit on a StepOne Real-Time PCR System (Thermo Fisher Scientific, Branford, CT, USA). High-throughput sequencing was performed as paired-end 100 sequencing using the HiSeq 2500 system (Illumina, San Diego, CA, USA). For sequencing analysis, mRNA sequencing reads were mapped using TopHat to obtain alignment files. Differentially expressed genes (DEGs) were identified based on counts from unique and multiple alignments using coverage in Bedtools. Read count data were normalized using the quantile normalization method in EdgeR within R using Bioconductor. The alignment files were also employed to assemble transcripts, estimate abundances, and detect differential gene or isoform expression using Cufflinks. Gene expression levels were calculated using the fragments per kilobase of exon per million fragments method. Genes were classified based on DAVID searches (<http://david.abcc.ncifcrf.gov/>).

#### **Real-time PCR analysis**

Total RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Hanover, IN, USA). Real-time PCR was performed on a Magnetic Induction Cyclor PCR system (PhileKorea, Seoul, Korea) using the following default thermocycler program for all genes: preincubation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Reaction mixtures were prepared using Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) with different sense and antisense primers (Supplementary Table S1). The reference gene was 18S ribosomal DNA.

#### **Gene silencing**

Small interfering RNAs (siRNAs) targeting 5 *Acanthamoeba* genes (*ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, *ACA1\_275740*, and *ACA1\_112650*) were synthesized by Bioneer Inc. (Bioneer, Daejeon, Korea) based on their cDNA sequences (Supplementary Table S2). The siRNA (final concentration: 100 nM) was transfected into live *Acanthamoeba* trophozoites at a cell density of  $4 \times 10^5$  cells using Effectene transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol. Transfection efficiency was determined by observing fluorescing cells under a fluorescent microscope (Leica, Wetzlar, Germany). siRNA-transfected *Acanthamoeba* underwent encystation for 48 h, followed immediately by excystation for 8 h. Real-time PCR was then performed as described above. The Effectene transfection reagent was used as a negative control.

#### **Statistical analysis**

Data are presented as the means  $\pm$  standard deviations (SD) from 3 independent experiments. Statistical analyses were performed using Student's *t*-test. *P*-values  $>0.05$  were considered statistically significant.

## Results

### DEGs of *Acanthamoeba* during encystation and excystation

To identify genes involved in *Acanthamoeba* encystation, DEGs during encystation (8, 16, 24, and 48 h) and excystation (8, 16, 24, and 48 h) were compared using mRNA sequencing. A total of 15,655 genes in *Acanthamoeba* exhibited more than a 2-fold change in expression during these processes (Table 1). A Venn diagram revealed that some DEGs overlapped between these processes (8, 16, 24, and 48 h) groups (Fig. 1). The expression of numerous genes was confirmed to either increase or decrease

**Table 1.** Number of genes showing significant increase or decrease (more than 2-fold) during encystation and excystation

	No. of increase	No. of decrease	Total
En 8 <sup>a</sup> /Tro <sup>l</sup>	1,947	1,874	3,821
En 16 <sup>b</sup> /Tro	2,365	2,191	4,556
En 24 <sup>c</sup> /Tro	2,523	2,399	4,922
En 48 <sup>d</sup> /Tro	2,009	1,861	3,870
Ex 8 <sup>e</sup> /Tro	1,842	1,492	3,334
Ex 16 <sup>f</sup> /Tro	2,182	1,718	3,900
Ex 24 <sup>g</sup> /Tro	2,113	1,800	3,913
Ex 48 <sup>h</sup> /Tro	3,682	3,123	6,805
Ex 8/En 48	1,214	1,163	2,377
Ex 16/En 48	1,414	1,219	2,633
Ex 24/En 48	1,045	986	2,031
Ex 48/En 48	3,403	2,526	5,929

<sup>a</sup>En 8, Encystation 8 h.

<sup>b</sup>En 16, Encystation 16 h.

<sup>c</sup>En 24, Encystation 24 h.

<sup>d</sup>En 48, Encystation 48 h.

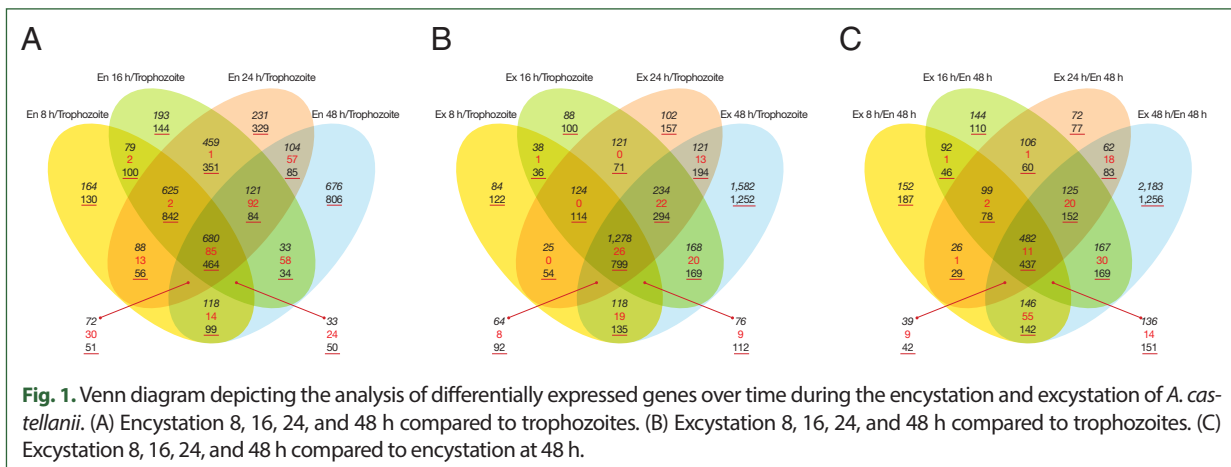
<sup>e</sup>Ex 8, Excystation 8 h.

<sup>f</sup>Ex 16, Excystation 16 h.

<sup>g</sup>Ex 24, Excystation 24 h.

<sup>h</sup>Ex 48, Excystation 48 h.

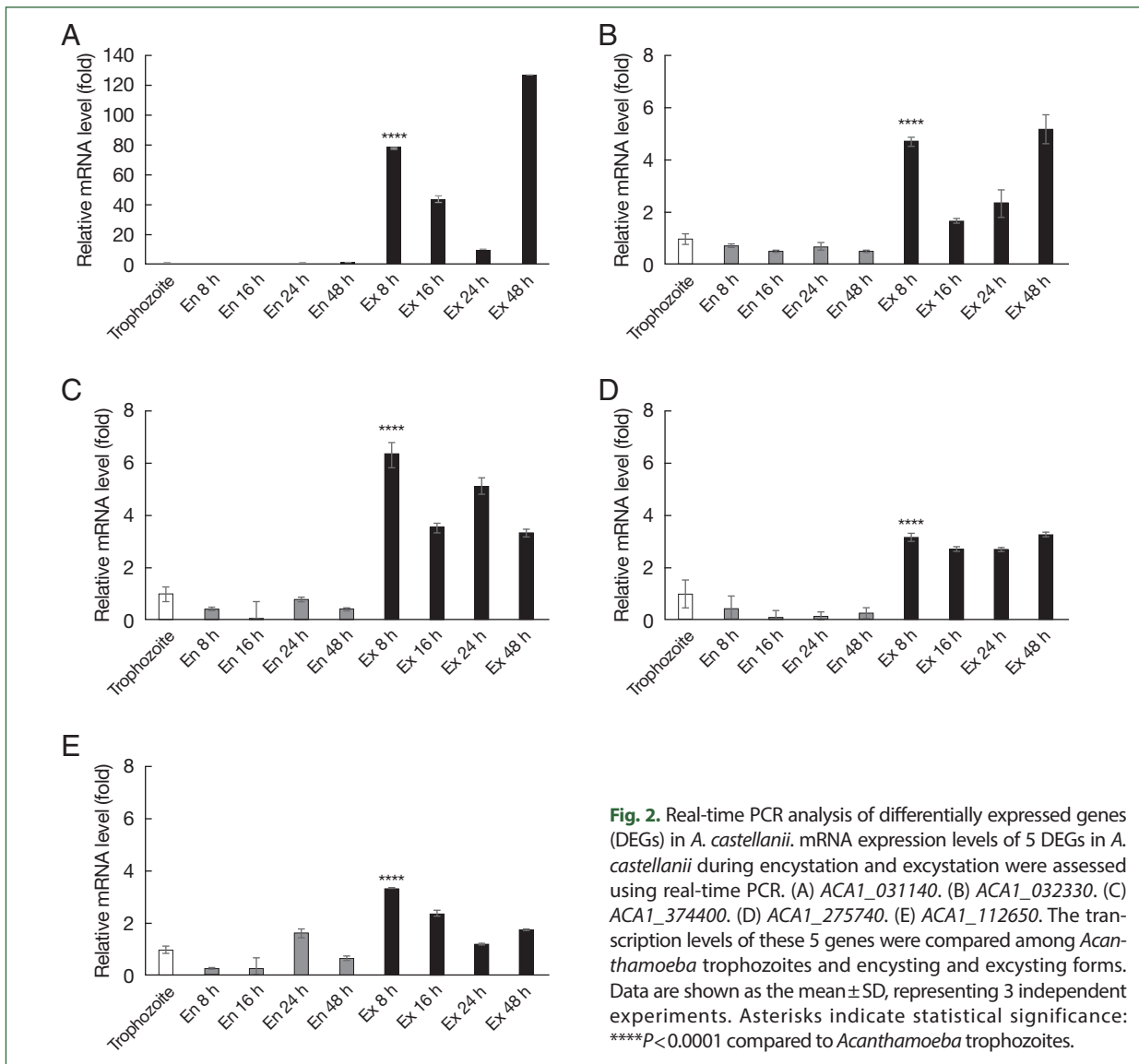
<sup>l</sup>Tro, Trophozoite.

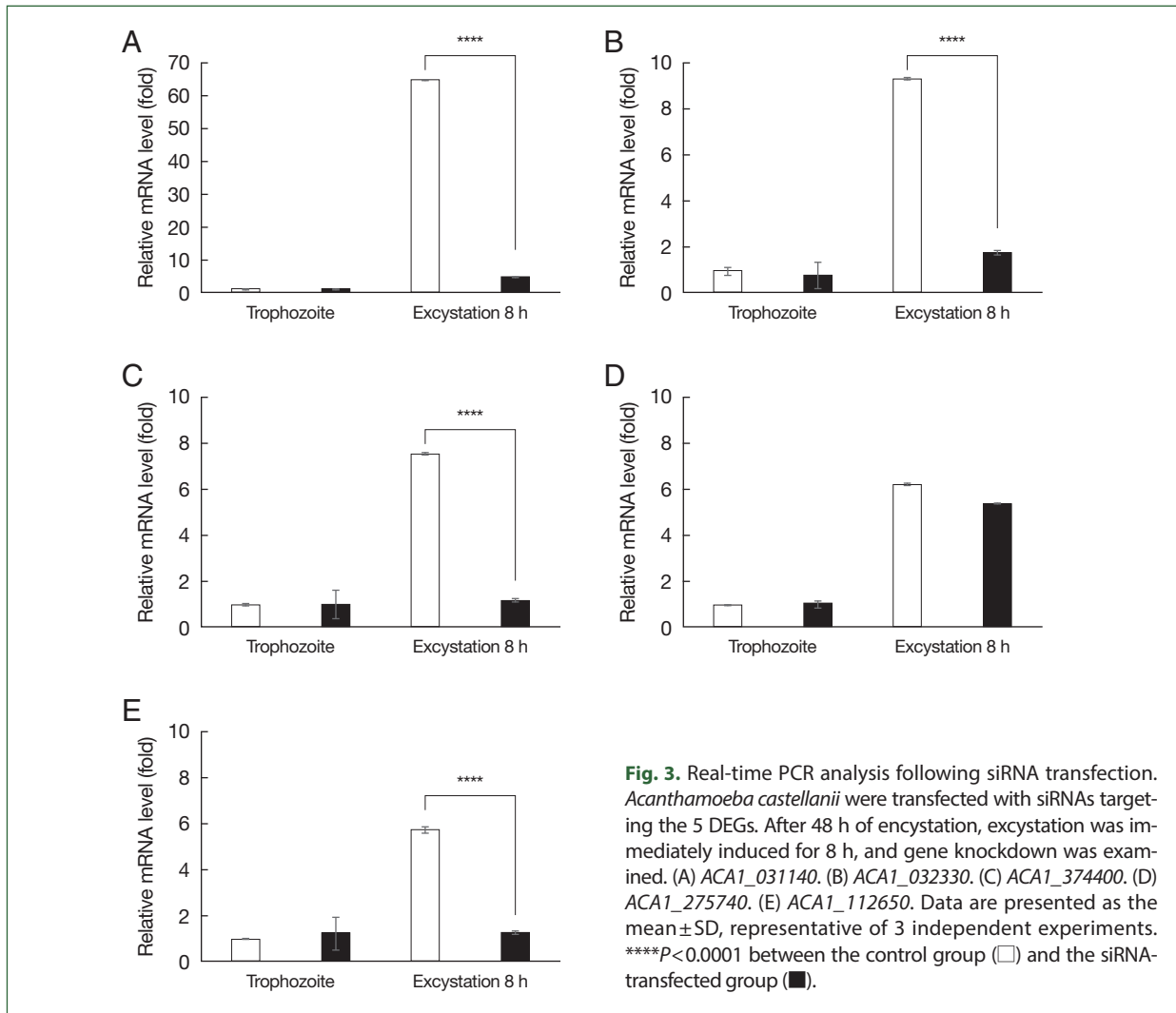


during both stages. We focused on genes highly expressed during the early stages of excystation (after 8 h of excystation) in mature *Acanthamoeba* cysts (after 48 h of encystation).

### Verification of gene expression changes via real-time PCR analysis

Based on mRNA sequencing analysis, 5 DEGs (*ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, *ACA1\_275740*, and *ACA1\_112650*) highly expressed during the early stages of excystation compared to encystation (Ex 8/En 48) were selected for validation via real-time quantitative PCR. The genes and their primers are listed in Supplementary Table S1. As shown in Fig. 2, none of the 5 genes showed increased expression during encystation, but their expression increased during excystation. Specifically, *ACA1\_031140* showed an approximately 80-fold increase in expression 8 h into excystation (Fig. 2A). At 48 h after excystation, many trophozoites (the active feeding stage of amoebae) were already present, indicating



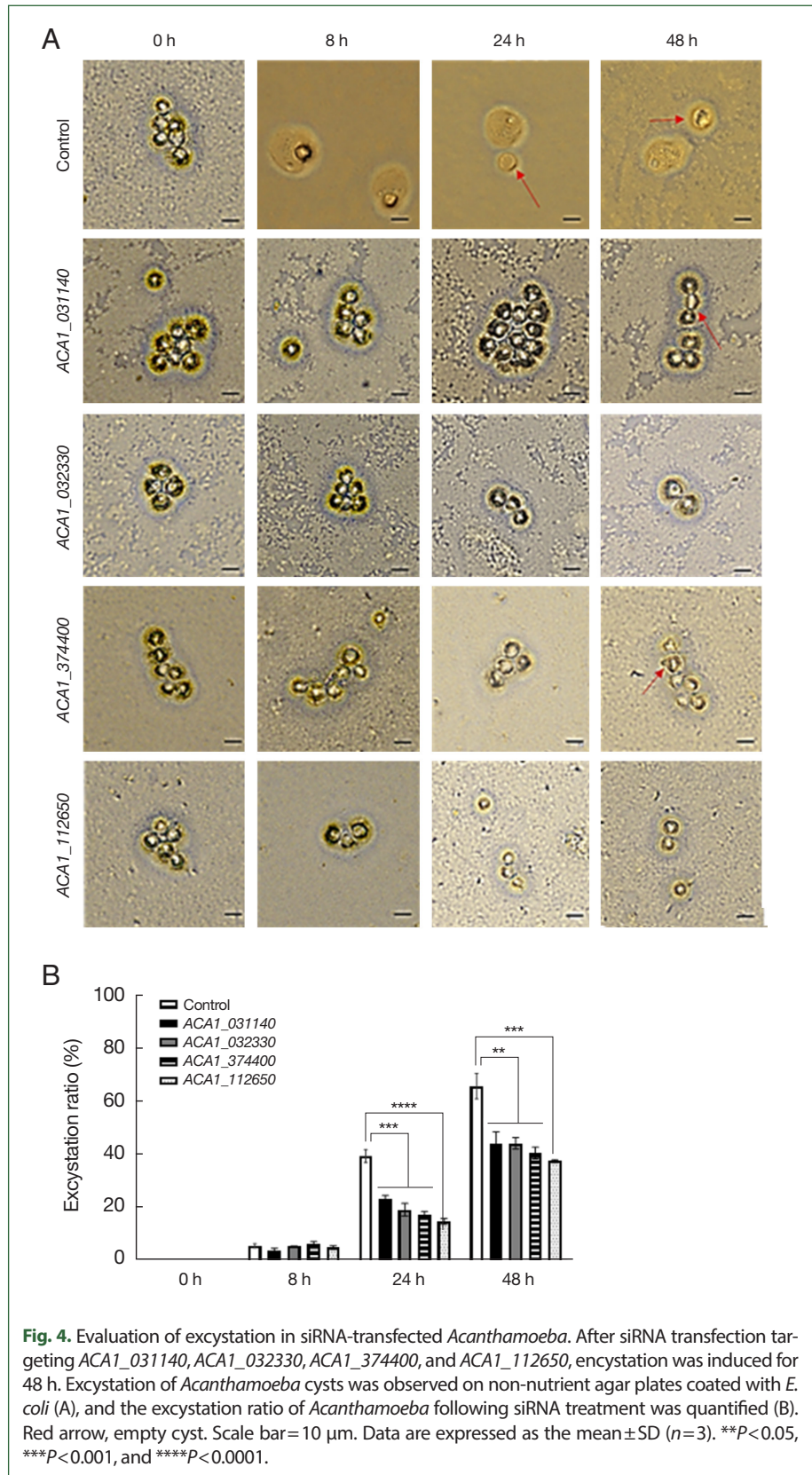


that excystation was likely complete; therefore, this stage may not form part of the ongoing excystation process. Thus, the significant increase in gene expression at 48 h after excystation was not considered meaningful (Fig. 2A).

#### siRNA-mediated gene silencing

To observe the effects of gene knockdown in vitro, 5 siRNAs specific to *ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, *ACA1\_275740*, and *ACA1\_112650* were prepared (Supplementary Table S2). After transfecting *Acanthamoeba* with these siRNAs, real-time PCR was performed to evaluate gene silencing efficiency (Fig. 3). In the trophozoite control groups, siRNA transfection did not affect mRNA levels (Fig. 2A-E). However, in the 8 h excystation group, siRNA transfection significantly reduced mRNA expression of *ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, and *ACA1\_112650* to basal levels (Fig. 2A-C and E). Transfecting siRNA specific to *ACA1\_275740* had a negligible impact on the 8 h excystation group (Fig. 2D).





### Effects of gene silencing on *Acanthamoeba* excystation

To investigate the effects of *ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, and *ACA1\_112650* gene knockdown in *Acanthamoeba*, the excystation ratio was examined (Fig. 4). The excystation rate was calculated by counting trophozoites and cyst shells that emerged after inducing encystation of siRNA-transfected amoebae and culturing them on an agar plate coated with heat-treated *E. coli* (Fig. 4A, red arrows indicate empty cysts). The excystation rate shown in Fig. 4A was quantified and presented in Fig. 4B. After inducing excystation in *Acanthamoeba* mature cysts, excystation rates were  $6.3 \pm 0.9\%$ ,  $40.0 \pm 2.7\%$ , and  $65.9 \pm 5.2\%$  at 8, 24, and 48 h, respectively. In siRNA-treated *Acanthamoeba*, no significant differences in excystation rates were observed at 8 h compared to the control group. However, as time progressed, significant differences appeared, with excystation rates reaching  $24.0 \pm 0.7\%$ ,  $17.6 \pm 1.3\%$ ,  $18.5 \pm 1.2\%$ , and  $14.9 \pm 1.1\%$  at 24 h for *ACA1\_031140*, *ACA1\_032330*, *ACA1\_374400*, and *ACA1\_112650*, respectively (Fig. 4B). By 48 h, excystation rates were  $43.6 \pm 4.9\%$ ,  $43.6 \pm 1.5\%$ ,  $41.0 \pm 2.3\%$ , and  $37.4 \pm 0.5\%$  for the 4 aforementioned genes, respectively, indicating a 20% reduction in excystation rates. Although not all time points showed significant differences in excystation rates for siRNA-treated groups, the lowest excystation rate was observed with *ACA1\_112650* inhibition.

## Discussion

Encystation and excystation are critical processes in the life cycle of *Acanthamoeba* spp., playing key roles in survival, drug resistance, and infection. Understanding the molecular mechanisms underlying these processes could reveal potential targets for disrupting *Acanthamoeba*'s life cycle. In this study, we identified 15,655 DEGs involved in encystation and excystation in *A. castellanii*, finding 4 genes that suppressed excystation by 20% during the process' early stages. BLAST analysis revealed that DEGs *ACA1\_031140*, *ACA1\_032330*, and *ACA1\_374400* encode hypothetical proteins with unknown functions, whereas *ACA1\_362260* encodes nicotinamide N-methyltransferase (NNMT; Supplementary Table S1), a metabolic enzyme involved in regulating methylation potential, which in turn affects DNA and histone epigenetic modifications [9]. NNMT plays a major role in the development of various cancers and shows promise as a biomarker for tumor diagnosis and treatment [10]. Although NNMT's role in *Acanthamoeba* spp. remains unreported, its increased expression during early excystation suggests that it may regulate the expression of genes critical for excystation.

During encystation in *Acanthamoeba* spp., rounded trophozoites appear approximately 12 h after induction of encystation [11]. Immature precysts with a continuous cell wall form around 24 h later, with the number of precysts increasing such that by 72 h, 70% of the trophozoites develop into mature cysts with a characteristic double-layered cyst wall. Time-lapse, phase-contrast studies on excystation have shown that the amoeba detaches from the inner cyst wall within 4–8 h after excystation induction [12]. Between 8 and 24 h, the detachment becomes more pronounced, and cytoplasmic buds penetrate the cyst wall, eventually leading to the emergence of a trophozoite, leaving behind an empty cyst wall. Based on these observations, gene expression changes were monitored at 8, 16, 24, and 48 h during encystation and excystation (Table 1; Fig. 1). We posited that genes showing increased



expression during early excystation in *Acanthamoeba* play pivotal roles in this process. Therefore, we focused on 5 genes for which expression increased 8 h after excystation began: ACA1\_031140, ACA1\_032330, ACA1\_374400, ACA1\_275740, and ACA1\_112650.

In conclusion, this study identified numerous genes, that exhibit expression changes during encystation and excystation (Table 1). However, owing to the limited genomic information available for *Acanthamoeba* spp., many DEGs were found to encode hypothetical proteins, complicating efforts to characterize their functions. Further research is required to explore and characterize these DEGs across different stages of *Acanthamoeba*'s life cycle. In particular, focusing on the 4 excystation-related genes identified in this study could provide insights into their function and shared mechanisms among pathogenic amoebae. Such findings could contribute to developing improved treatment and prevention strategies for *Acanthamoeba* infections and combating other cyst-forming protozoa.

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## References

- Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. *Parasit Vectors* 2012;5:6. <http://doi.org/10.1186/1756-3305-5-6>
- Wang Y, Jiang L, Zhao Y, Ju X, Wang L, et al. Biological characteristics and pathogenicity of *Acanthamoeba*. *Front Microbiol* 2023;14:1147077. <http://doi.org/10.3389/fmicb.2023.1147077>
- Khan NA. *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol Rev* 2006;30(4):564-595. <http://doi.org/10.1111/j.1574-6976.2006.00023.x>
- Coulon C, Collignon A, McDonnell G, Thomas V. Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. *J Clin Microbiol* 2010;48(8):2689-2697. <http://doi.org/10.1128/JCM.00309-10>
- Mazur T, Hadaś E, Iwanicka I. The duration of the cyst stage and the viability and virulence of *Acanthamoeba* isolates. *Trop Med Parasitol* 1995;46(2):106-108
- Anwar A, Khan NA, Siddiqui R. Combating *Acanthamoeba* spp. cysts: what are the options? *Parasit Vectors* 2018;11(1):26. <http://doi.org/10.1186/s13071-017-2572-z>
- Lakhundi S, Khan NA, Siddiqui R. The effect of environmental and physiological conditions on excystation of *Acanthamoeba castellanii* belonging to the T4 genotype. *Parasitol Res* 2014;113(8):2809-2816. <http://doi.org/10.1007/s00436-014-3941-6>
- Chambers JA, Thompson JE. A scanning electron microscopic study of the excystment process of *Acanthamoeba castellanii*. *Exp Cell Res* 1972;73(2):415-421. [http://doi.org/10.1016/0014-4827\(72\)90066-3](http://doi.org/10.1016/0014-4827(72)90066-3)
- Li XY, Pi YN, Chen Y, Zhu Q, Xia BR. Nicotinamide N-Methyltransferase: a promising biomarker and target for human cancer therapy. *Front Oncol* 2022;12:894744. <http://doi.org/10.3389/fonc.2022.894744>
- Wang W, Yang C, Wang T, Deng H. Complex roles of nicotinamide N-methyltransferase in cancer progression. *Cell Death Dis* 2022;13(3):267. <http://doi.org/10.1038/s41419-022-04713-z>
- Lorenzo-Morales J, Kliescikova J, Martinez-Carretero E, De Pablos LM, Profotova B, et al. Glycogen phosphorylase in *Acanthamoeba* spp.: determining the role of the enzyme during the encystment process using RNA interference. *Eukaryot Cell* 2008;7(3):509-517. <http://doi.org/10.1128/EC.00316-07>
- Mattar FE, Byers TJ. Morphological changes and the requirements for macromolecule synthesis during excystment of *Acanthamoeba castellanii*. *J Cell Biol* 1971;49(2):507-519. <http://doi.org/10.1083/jcb.49.2.507>