OXFORD

BIOLOGY Methods & Protocols

Biology Methods and Protocols, 2023, bpac036

https://doi.org/10.1093/biomethods/bpac036

Advance Access Publication Date: 16 December 2022

Innovations

Optimizing simple calreticulin upregulation strategies in *Caenorhabditis elegans*

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Abstract

Calreticulin (CALR) is a multifunctional calcium-binding protein whose expression levels have been correlated with detection, clinical phase of disease, metastasis, and survival of various types of cancer. Therefore, the study of the regulation of the cellular levels of CALR may be important to understand the neoplastic process. *Caenorhabditis elegans*, which has a CALR ortholog (CRT-1), has been used as a model organism for the characterization of CALR, and several conditions promoting the upregulation of *crt-1* have been studied and established to understand the molecular control of *crt-1* transcription and assess the function of the protein. Here, we propose several modifications of previously published *crt-1* upregulation strategies that improve the reproducibility of the assay and allow to achieve higher levels of overexpression. First, the manipulation of synchronized populations of worms instead of mixed-stage animals and the use of solid culture medium in all experimental conditions are proposed. Likewise, we evaluate four new experimental approaches that attempt to promote a higher *crt-1* upregulation [short-term exposure to 30 μg/ml tunicamycin at 25°C, short-term exposure to 7% ethanol (EtOH) at 25°C, short-term exposure to 30°C of worms grown at 25°C, and a long-term exposure to 7% EtOH]. Our results not only validate previously published methods, but also point to a new experimental approach that increases previously achieved levels of *crt-1* upregulation. More specifically, a 6-h exposure of synchronized worms grown at 25°C to 7% EtOH on solid medium promotes almost a 7-fold upregulation of *crt-1*.

Keywords: *Caenorhabditis elegans*; calreticulin; CALR; CRT-1; upregulation; qPCR

Introduction

Calreticulin (CALR) is a multifunctional calcium-binding protein residing mainly in the endoplasmic reticulum (ER). In the ER, it acts as a chaperone that promotes protein folding, assembly, and quality control, and plays a crucial role in calcium homeostasis. This protein also participates in a large variety of processes outside the ER, such as wound healing and immunity [1]. Several studies have demonstrated that CALR expression levels are correlated with detection, clinical phase of disease, metastasis, and survival of various types of cancer [2]. In addition, *CALR* is often upregulated in several tumors, and it has also shown to be a useful biomarker for cancer detection. Thus, the precise regulation of the cellular levels of CALR may play a crucial role [2].

Caenorhabditis elegans has been used as a model organism for the characterization of CALR (CRT-1) functions since, unlike for mice [3], a CRT-1-deficient worm does not show embryonic lethality. In this sense, the description of standard culture conditions that promote *crt-1* upregulation could be a good strategy to understand the molecular control of *crt-1* transcription and to better assess the effects of the protein in the worm. Previous studies have reported that *C. elegans crt-1* is transcriptionally upregulated 2-fold when worms are grown at 25°C instead of 20°C [4]. Additionally, the exposure of animals grown at 20°C to high temperatures (30°C) [5], 7% ethanol (EtOH) [4, 6] or 30 μg/ml tunicamycin [5] for 6 h has also been demonstrated to upregulate *crt-1*. According to these studies, *crt-1* levels increased 3- and ≤4-fold after EtOH and tunicamycin treatment, respectively [4, 5].

The method that we describe here provides several novelties that attempt to enhance assay reproducibility and *crt-1* upregulation levels. To improve reproducibility, this approach eliminates two sources of variability of the original methods. Synchronized worms are used instead of mixed-stage animals so that the exposure of worms to the *crt-1*-upregulating conditions occurs at fixed stages of development and the same type of culture medium is used in all experimental conditions avoiding any difference that could have an impact on developmental timing. Finally, to promote a higher upregulation of *crt-1*, we tested the combination of several experimental approaches that individually are known to stimulate CRT-1 overexpression and compared the expression levels of *crt-1* induced by each of them to facilitate the choice of the most appropriate strategy for each case.

Materials and methods

This assay uses a population of synchronized L1 worms of the N2 Bristol strain. Some of them were grown at 25°C until they reached the early L3 stage and then, they were exposed for 6 h to four short-term experimental conditions that may promote *crt-1* upregulation (25°C, 30 μg/ml tunicamycin at 25°C, 7% EtOH at 25°C, and 30°C) until they reached the late L3 stage. At the same

Received: November 2, 2022. **Revised:** December 1, 2022. **Accepted:** December 6, 2022

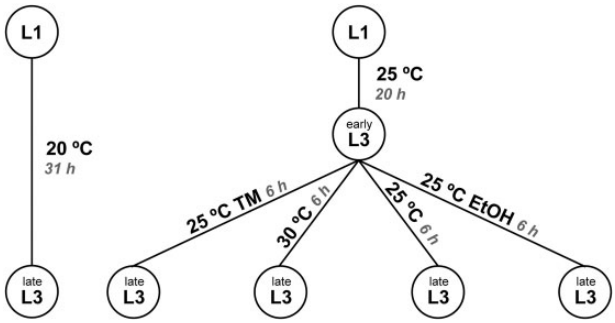

Figure 1: Simplified illustration of the design of the assay. The control group was composed of L1 worms of the *N2* Bristol strain that were grown at 20°C for 31 h until they reached the late L3 stage. Additionally, L1 nematodes of the same strain were grown at 25°C for 20 h until they reached the early L3 stage. At this point, they were exposed for 6 h to conditions that attempt to upregulate *crt-1* (25°C, 30 μg/ml tunicamycin at 25°C, 7% EtOH at 25°C, and 30°C). After that, worms at the late L3 stage were collected and washed from plates to extract their RNA and finally evaluate the expression of *crt-1* transcripts by qPCR.

time, a control group of synchronized L1 worms was grown at 20°C until they reached the late L3 stage (Fig. 1). We also tried to induce a higher upregulation of *crt-1* by a long-term exposure of the worms to 7% EtOH from the L1 stage. In all cases, RNA was extracted from worms at late L3 stage and the expression of *crt-1* transcripts was evaluated by quantitative PCR (qPCR).

The differences between this assay and the previously published ones are: (i) the use of synchronized worms instead of mixed-stage animals; (ii) the use of solid culture media for the exposure of worms to all experimental conditions, including the exposure to 7% EtOH, which in the previous approach was done in liquid medium; and (iii) the evaluation of four new experimental conditions to promote *crt-1* upregulation (short-term exposure to 30 μg/ml tunicamycin at 25°C, 7% EtOH or 30°C of worms grown at 25°C, as well as long-term exposure to 7% EtOH).

C. elegans strain

The wild-type *N2* Bristol strain was acquired from the *Caenorhabditis Genetic Center* (CGC, University of Minnesota, Minneapolis, MN, USA). Nematodes were maintained at 20°C on Nematode Growth Medium (NGM) agar plates seeded with the *Escherichia coli* strain OP50 according to standard protocols.

Age synchronization via bleaching

Animals were age-synchronized by standard hypochlorite treatment of gravid nematodes to obtain eggs that were allowed to hatch in M9 medium at 20°C overnight with gentle agitation [7].

Short-term exposure to high temperatures, tunicamycin, and EtOH

The short-term exposure to high temperatures, tunicamycin, and EtOH was performed in two independent biological replicates of worms grown at a different time point. In each of these biological replicates, a population of approximately 2000 synchronized L1 worms per culture condition was grown on at least two 100-mm NGM plates (technical replicates) at 25°C for 20 h until they reached the early L3 stage. At this stage, they were all transferred to new 35-mm NGM plates seeded with *E. coli* OP50 to be exposed to four different stress conditions (25°C, 30 μg/ml tunicamycin at 25°C, 7% EtOH at 25°C, and 30°C) for 6 h until they reached the late L3 stage. Both tunicamycin and EtOH were added directly to NGM agar prior to solidification. The control group was composed of synchronized L1 worms grown at 20°C for 31 h until they reached the late L3 stage.

Long-term exposure to EtOH

About 250 synchronized L1 worms were transferred to NGM agar plates with 7% EtOH and incubated at 25°C for 4 days. EtOH was added directly to NGM agar prior to solidification.

RNA extraction and cDNA synthesis

Worms were collected and washed after being grown in the described experimental conditions. Total RNA was extracted using TRIzol® Reagent (Thermo Fisher Scientific Inc., Paisley, UK) according to the manufacturer specifications. Concentration and purity of RNA were quantified at 260/280 nm with a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA samples were treated with the DNA-free™ DNA Removal Kit (Invitrogen-Life Technologies, Paisley, UK) and then were reverse transcribed with Invitrogen™ M-MLV Reverse Transcriptase and random primers (Thermo Fisher Scientific Inc., Paisley, UK) according to the standard protocol.

Quantitative real-time PCR (qPCR)

Relative mRNA expression of *crt-1* was validated using qPCR. All reactions were run in 384-well plates using a C100 Touch™ Thermal Cycler CFX384™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). Cycling conditions were: 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. The reaction mix had a final volume of 10 μl per well composed by 5 μl of iTaq™ Universal Probes Supermix (Bio-Rad Laboratories), 0.5 μl of primers and Taqman® probes (×20) (IDT, Integrated DNA Technologies Inc., Coralville, IA, USA), 1 μl of diluted cDNA (12.5 ng/μl), and 3.5 μl of nuclease-free H_2O. Primers and probes (CRT-1 forward primer: 5′-GGGAGAGAACAAGC TCATCAA-3′; CRT-1 reverse primer: 5′-CGTAGGTATTGTCGG AGTTGAG-3′; and CRT-1 probe: 5′-[FAM]-AATCACCTG-[ZEN]-CAAGAGCGACGAACT-3′) were designed using the PrimerQuest® Tool from IDT (http://eu.idtdna.com/pages/tools/primerquest). Each sample was analyzed in triplicate and appropriate negative controls were included. C_T (threshold cycle) values were collected using CFX Manager c3.1 software (Bio-Rad Laboratories). The expression level of each gene was normalized to the expression of the housekeeping gene *tba-1* (Applied Biosystems by Thermo Fisher Scientific Inc., Foster City, CA, USA). Gene expression differences between samples were quantified following the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical calculations were performed using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA). The significance level (α) was set at 0.05. In this sense, differences were considered as non-significant (ns) when $P > 0.05$, significant (*) when $P < 0.05$, very significant (**) when $P < 0.01$, and highly significant (***) when $P < 0.001$. For qPCR, ΔC_T values for each gene were compared between experimental conditions using the parametric one-way ANOVA test followed by multiple comparisons.

Results

Short-term exposure to tunicamycin and EtOH in worms grown at 25°C induces a high upregulation of *crt-1* in worms

As previously published [4], worms grown at 25°C showed a subtle upregulation of *crt-1* (<2-fold) compared with those grown at

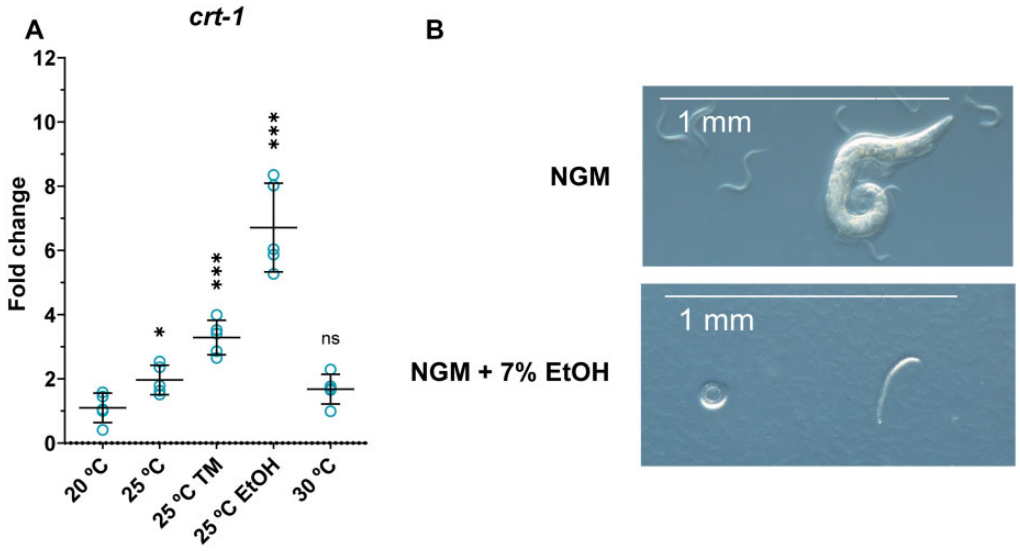

Figure 2: Main effects of the short- and long-term exposure of worms to the experimental conditions tested to upregulate *crt-1*. (**A**) Relative expression levels of *crt-1* when comparing worms grown at 25°C exposed to high temperatures for 6 h, tunicamycin, and EtOH to animals grown at 20°C according to qPCR results. Individual values, means, and SDs are represented. Differences were considered as significant (*) when $P < 0.05$, very significant (**) when $P < 0.01$, and highly significant (***) when $P < 0.001$. TM, tunicamycin. (**B**) Worms after 78 h of incubation at 25°C with or without 7% ethanol. Magnification scale: ×8; scale bar: 1 mm.

Table 1: Comparison of the *crt-1* transcripts levels obtained by previously published *crt-1* upregulation strategies and the strategies shown in this work

Previously published results		Results in this work	
Experimental condition	**Upregulation**	**Experimental condition**	**Upregulation**
25°C (unsynchronized) [4]	2-fold	25°C (synchronized)	<2-fold
20°C + 6 h 30 μg/ml TM (unsynchronized) [5]	≤4-fold	25°C + 6 h 30 μg/ml TM (synchronized)	3-fold
20°C + 6 h 7% ethanol in liquid medium (unsynchronized) [4]	3-fold	25°C + 6 h 7% ethanol in solid medium (synchronized)	7-fold
20°C + 6 h 30°C (unsynchronized) [5]	Not specified	25°C + 6 h 30°C (synchronized)	No upregulation

20°C (Fig. 2A and Table 1) and, as expected, 6-h exposure of worms grown at 25°C to 30 μg/ml tunicamycin and 7% EtOH also significantly increased *crt-1* expression. The increase in expression was about 3-fold in the case of worms exposed to tunicamycin and about 7-fold in those grown with EtOH (Fig. 2A and Table 1). However, we did not observe an increase in *crt-1* expression in worms exposed to 30°C for 6 h after being grown at 25°C when compared with those grown at 20°C (Fig. 2A and Table 1).

Long-term exposure to 7% EtOH is not a good strategy to upregulate *crt-1* in worms

The exposure of L1 animals to 7% EtOH progressively flattened the body-bend amplitudes of the worms until they died at the L1 stage while worms grown without EtOH showed a normal development (Fig. 2B). Therefore, the exposure of L1 worms to 7% EtOH is not a good strategy to upregulate *crt-1* in worms.

Discussion

The purpose of this work was to optimize previously published *crt-1* upregulation strategies in *C. elegans* using synchronized worms, solid culture media, and new experimental conditions.

The use of synchronized worms instead of mixed-stage animals and the use of the same culture media in all experimental conditions may improve assay reproducibility since it eliminates any difference that could have an impact on developmental timing as a source of variability. Furthermore, our results demonstrate that the use of solid culture media is suitable for the exposure of worms to 7% EtOH since it also causes an upregulation of *crt-1* (Fig. 2A).

Some of the experimental approaches that we tested (6-h exposure of worms grown at 25°C to 30 μg/ml tunicamycin or 30°C) resulted in similar or reduced levels of *crt-1* expression compared with previously described assays performed with worms grown at 20°C [5] (Table 1). The increase in expression was about 3-fold in worms exposed to tunicamycin and grown at 25°C, a similar fold-change to that reported for worms exposed to the same concentration of tunicamycin and grown at 20°C (Table 1) [5]. In addition, heat stress (6-h exposure of worms grown at 25 or 30°C) did not cause an increased expression of *crt-1* (Fig. 2A). However, the 6-h exposure of worms to 7% EtOH and grown at 25°C caused almost a 7-fold *crt-1* upregulation (Fig. 2A), the largest fold change described to date. Previous results reported an increase of 3-fold in *crt-1* expression when worms grown at 20°C were exposed to 7% EtOH in liquid medium (Table 1) [4]. In this sense, the use of synchronized worms and the exposure of worms grown at 25°C to this concentration of EtOH in a solid medium considerably improved the upregulation of *crt-1* (Table 1). According to this result, we tested a long-term

exposure of worms to 7% EtOH to ascertain whether it would result in further *crt-1* upregulation or not. However, this long-term exposure to EtOH was lethal for L1 worms.

In summary, here we show an optimization of *crt-1* upregulation strategies in *C. elegans* which increases the upregulation levels achieved by previous methods and improves reproducibility by using synchronized worms and solid culture medium in all experimental conditions.

Data availability

Data available in Supplementary Material. qPCR Results.xlsx: this Excel file contains a table with the original Ct values obtained in the qPCR and the results of their analysis following the $2^{-\Delta\Delta Ct}$ method; qPCR Graph and Statistical Analysis.pzfx: this GraphPad file contains the fold-change values represented in Fig. 2A as well as the numerical results of the statistical analysis performed on dCt values.

Author contributions

Ana Guijarro-Hernández (Conceptualization [supporting], Formal analysis [equal], Investigation [lead], Methodology [lead], Validation [lead], Writing—original draft [lead], Writing—review and editing [supporting]), Cristina Hurtado (Investigation [supporting], Methodology [supporting]), José Luis Vizmanos (Conceptualization [lead], Formal analysis [equal], Funding acquisition [lead], Project administration [lead], Resources [lead], Supervision [lead], Writing—original draft [supporting], Writing—review and editing [lead]).

Funding

This work was supported by the PIUNA 2020 Program of the University of Navarra to J.L.V. (Grant No. 15058203). N2 strain was provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

Conflict of interest statement. None declared.

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