Developmental Biology 429 (2017) 306–320

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Novel functions for the RNA-binding protein ETR-1 in *Caenorhabditis elegans* reproduction and engulfment of germline apoptotic cell corpses

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ARTICLE INFO

Keywords:
ETR-1
C. elegans reproduction
RNA-binding protein
Physiological germline apoptosis
Engulfment
CED-1

ABSTRACT

RNA-binding proteins (RBPs) are essential regulators of gene expression that act through a variety of mechanisms to ensure the proper post-transcriptional regulation of their target RNAs. RBPs in multiple species have been identified as playing crucial roles during development and as having important functions in various adult organ systems, including the heart, nervous, muscle, and reproductive systems. ETR-1, a highly conserved ELAV-Type RNA-binding protein belonging to the CELF/Bruno protein family, has been previously reported to be involved in *C. elegans* muscle development. Animals depleted of ETR-1 have been previously characterized as arresting at the two-fold stage of embryogenesis. In this study, we show that ETR-1 is expressed in the hermaphrodite somatic gonad and germ line, and that reduction of ETR-1 via RNA interference (RNAi) results in reduced hermaphrodite fecundity. Detailed characterization of this fertility defect indicates that ETR-1 is required in both the somatic tissue and the germ line to ensure wild-type reproductive levels. Additionally, the ability of ETR-1 depletion to suppress the published WEE-1.3-depletion infertility phenotype is dependent on ETR-1 being reduced in the soma. Within the germline of *etr-1(RNAi)* hermaphrodite animals, we observe a decrease in average oocyte size and an increase in the number of germline apoptotic cell corpses as evident by an increased number of CED-1::GFP and acridine orange positive apoptotic germ cells. Transmission Electron Microscopy (TEM) studies confirm the significant increase in apoptotic cells in ETR-1-depleted animals, and reveal a failure of the somatic gonadal sheath cells to properly engulf dying germ cells in *etr-1(RNAi)* animals. Through investigation of an established engulfment pathway in *C. elegans*, we demonstrate that co-depletion of CED-1 and ETR-1 suppresses both the reduced fecundity and the increase in the number of apoptotic cell corpses observed in *etr-1(RNAi)* animals. Combined, this data identifies a novel role for ETR-1 in hermaphrodite gametogenesis and in the process of engulfment of germline apoptotic cell corpses.

1. Introduction

RNA-binding proteins (RBPs) play critical roles in controlling gene expression through post-transcriptional regulation of specific target RNAs. Studies in several species have established the essential function of RNA regulation via RBPs in the germ line and throughout early embryonic development (Colegrove-Otero et al., 2005; Detwiler et al., 2001; Lee and Schedl, 2006; Richter and Lasko, 2011). During oogenesis, translational regulation is of the utmost importance, as the oocytes of most animals are transcriptionally quiescent. Therefore, mRNAs must be transcribed by the mother in the early germ line and stored in the oocytes prior to fertilization to be available for translation in the newly formed zygote (reviewed by Li et al., 2010; Robertson and Lin, 2013). In the nematode *Caenorhabditis elegans* at least 20 of the approximately 500 genes annotated to encode RBPs play an essential function in the germ line and early embryonic development (Lee and Schedl, 2006).

ETR-1 (*E*LAV-*T*ype *R*NA-binding protein) in *C. elegans* is one of two members belonging to the highly conserved CELF/Bruno RNA-binding protein family, the other being ETR-1's paralog UNC-75 (Milne and Hodgkin, 1999; WormBase: etr-1, www.wormbase.org). Most species possess multiple (3–10) members of the CELF/Bruno protein family, with individual members typically having distinct roles in the nervous system, muscle, brain, heart, and/or reproductive tissues/organs (Barreau et al., 2006; Dasgupta and Ladd, 2012). ETR-1 has been previously shown to play a developmental role in muscle formation and function, while UNC-75 plays a role in the nervous system (Loria et al., 2003; Milne and Hodgkin, 1999). The *etr-1* locus is complex resulting in 19 coding isoforms and 1 noncoding isoform (Supplementary Fig. S1) (WormBase: etr-1, www.wormbase.

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http://dx.doi.org/10.1016/j.ydbio.2017.06.015
Received 5 December 2016; Received in revised form 1 June 2017; Accepted 8 June 2017
Available online 23 June 2017

org). Notably, in other organisms several members of the CELF/Bruno family are subjected to high levels of alternative splicing, generating multiple protein isoforms that exhibit isoform-specific tissue expression and varying temporal expression (Barreau et al., 2006; Li et al., 2001). Each ETR-1 isoform possesses between one to three highly conserved RNA Recognition Motifs (RRMs) which are domains that are capable of binding single-stranded RNA and enabling the RBP to interact with its target mRNAs (Supplementary Fig. S1) (Cléry et al., 2008; Maris et al., 2005; WormBase: etr-1, www.wormbase.org). A COBALT alignment of all 19 ETR-1 isoforms with their paralog UNC-75 and three predicted homologues (human CUGbp1, *Drosophila* Bruno-2, and *Drosophila* ELAV), shows the highest conservation of amino acids within the RRMs (Supplementary Fig. S2) (Papadopoulos and Agarwala, 2007). Interestingly, there are currently no RNA targets identified for ETR-1, but potential neuronal targets have been recently identified for UNC-75 (Chen et al., 2016; Lee and Schedl, 2006; WormBase: etr-1, www.wormbase.org).

We previously identified ETR-1 in a screen for suppressors of the highly penetrant infertility associated with depletion of the WEE-1.3 inhibitory kinase involved in oocyte meiotic arrest and oocyte maturation (Allen et al., 2014). Others have reported that RNAi depletion of ETR-1 in sensitized strain backgrounds results in fertility defects, including a reduced brood size and sterility (Ceron et al., 2007; Rual et al., 2004). Additionally, it has been previously reported that homologues of ETR-1 in both *Drosophila* and mouse show impaired fertility, exhibiting reproductive defects during both oogenesis and spermatogenesis (Castrillon et al., 1993; Dev et al., 2007; Kress et al., 2007; Schupbach and Wieschaus, 1991). These data taken together suggest a potential reproductive role for ETR-1 within *C. elegans*.

In the *C. elegans* hermaphrodite, the reproductive organ, or gonad, is a U-shaped, bi-lobed structure that consists of both a germline and a somatic component (Greenstein, 2005; Hubbard and Greenstein, 2000). The germ line is capable of producing both sperm and oocytes, with sperm production occurring first and then the hermaphrodite switching to oocyte production (Corsi et al., 2015; Hubbard and Greenstein, 2005). Spermatogenesis begins during the animal's third larval stage, is completed by the fourth larval stage, and all the spermatids are stored in the spermatheca where the first ovulation triggers them to complete spermiogenesis (L'Hernault, 2006). Oogenesis begins in the early young adult developmental stage, with the distal most female germ cells undergoing mitosis to expand the number of germ cells, and then switching to meiosis prior to the bend of the gonad arm (Greenstein, 2005). The germ cell nuclei are initially separated by incomplete plasma membranes, and thus maintain a connection with a common cytoplasmic gonadal core through which mRNAs and proteins can pass (Wolke et al., 2007). During the late stages of oogenesis, in the proximal most region of the gonad, the oocytes complete cellularization and the individualized oocytes await meiotic maturation, ovulation into the spermatheca, and subsequent fertilization (Greenstein, 2005).

The somatic gonad is comprised of five tissues: the distal tip cells (DTCs), five pairs of gonadal sheath cells, the spermatheca, the spermatheca-uterine valves and the uterus (Lints and Hall, 2009a). The gonadal sheath cells (pairs 1–5) cover and closely associate with the germ line of each gonadal arm. Individual sheath cells can be recognized by their location along the gonad proximal-distal axis (Hall et al., 1999). Sheath cell 1 is the distal-most and wedges filopodia between the distal germ cells existing in the syncytial gonad environment (Hall et al., 1999; Lints and Hall, 2009b). Sheath cell 5 is the proximal-most, covering the developing and maturing oocytes (Lints and Hall, 2009b). The sheath cells are instrumental for promoting germline proliferation and exit from meiotic pachytene (pairs 1 and 2); physiological germline apoptosis (pair 3 and a portion of pair 2); and oocyte maturation and ovulation (pairs 4 and 5) (Greenstein, 2005; Hall et al., 1999; Lints and Hall, 2009a).

The goal of the present work is to investigate the function of ETR-1 during *C. elegans* hermaphrodite reproduction. We show that ETR-1 is necessary in both the somatic gonad and the germ line in order to enable an animal to reach full reproductive capacity. Additionally, through antibody staining, we demonstrate that ETR-1 is expressed not only in the muscle, as previously reported, but also in the germ line in both developing oocytes and mature sperm. Finally, our results implicate ETR-1 in physiological germline apoptosis, most likely playing a role in the engulfment of apoptotic germ cell corpses. This study thus demonstrates that ETR-1 is playing a previously unappreciated role during *C. elegans* oogenesis, and may help advance our understanding of the multiple functions that this particular member of the CELF/Bruno protein family is playing.

2. Materials and methods

2.1 Nematode strains and culture conditions

The following *C. elegans* strains were used in this work: Bristol strain N2, NL2098 [*rrf-1(pk1417) I],* NL3511 [*ppw-1(pk1425) I*], *etr-1(tm6221)/egl-26(ku228),* MD701 (*bcIs39[lim-7p::ced-1::GFP + lin-15(+)]*), CB3203 [*ced-1(e1735)*], MT2405 [*ced-3(n717) unc-26(e205) IV*], and MT2551 [*ced-4(n1162) dpy-17(e164) III*]. All strains were grown and maintained under standard conditions at 20° (Brenner, 1974).

2.2 RNA Interference (RNAi)

RNAi experiments were performed via feeding using HT115(DE3) bacterial cells seeded on MYOB plates containing 2 mM IPTG and 25 μg/ml carbenicillin (Allen et al., 2014; Timmons and Fire, 1998). RNAi constructs were obtained from the Open Biosystems ORF-RNAi library (Huntsville, AL) and sequence verified prior to usage. The *etr-1* RNAi construct (Plate 10014C9 in the library) contains the entire *etr-1* coding region (see Supplementary Fig. S1, exons 2–13). L4 hermaphrodites were fed the RNAi constructs for 20–24 h at 24° (Day 1). Animals were then singled to individual, new RNAi plates for another 24 h at 24° (Day 2). The hermaphrodites were then removed from each plate and the plates were incubated for an additional 24 h at 20° prior to the scoring and characterization of the resultant brood. The brood size analysis was conducted by counting all animals on the plate (embryos younger than two-fold; two-fold embryos, dying embryos and larvae). Controls used were dsRNA against: (1) *smd-1(F47G4.7)*, referred to as *control(RNAi)* throughout this report, this construct triggers the RNAi response, but has no reportable phenotypic effect (Andy Golden and Kevin O'Connell, personal communication); and (2) *wee-1.3(Y53C12A.1)* which produces a sterile phenotype and confirms RNAi plates are working. For co-depletions or RNAi using two dsRNAs, bacterial cells were individually grown overnight and then mixed in equal volume before seeding.

2.3 Live Imaging

Animals were placed on a slide with a 3% agarose pad and a drop of 0.2 mM levamisole diluted in M9 buffer. A coverslip was placed onto the slide, sealed with nail polish and allowed to dry. Images were taken using spinning-disk confocal microscopy as described below.

2.4 Microscopy

Fluorescent images of live or fixed samples were captured using a Nikon Ti-E-PFS inverted spinning-disk confocal microscope equipped with a 60× 1.4NA Plan Apo Lambda objective. The system is outfitted with a Yokogawa CSU-X1 spinning disk unit, a self-contained 4-line laser module (excitation at 405, 488, 561, and 640 nm), and Andor iXon 897 EMCCD camera. Confocal fluorescent images and DIC images were acquired and processed using the Nikon NIS-Elements and Adobe Photoshop CS5 software.