A germline-targeted genetic screen for xrn-2 suppressors identifies a novel gene C34C12.2 in Caenorhabditis elegans

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Abstract

XRN2 is an evolutionarily conserved 5'-to-3' exoribonuclease, which degrades or trims various types of RNA in the nucleus. Although XRN-2 is essential for embryogenesis, larval development and reproduction in Caenorhabditis elegans, relevant molecular pathways remain unidentified. Here we create a germline-specific xrn-2 conditional mutant and perform a genetic screen for suppressors of sterility. Loss-of-function alleles of dpy-10, osr-1, ptr-6 and C34C12.2 genes are identified. Depletion of DPY-10, OSR-1 or PTR-6 increases expression of gpdh-1 that encodes a glycerol-3-phosphate dehydrogenase, thereby elevates glycerol accumulation to suppress sterility of the mutant. The C34C12.2 protein is predominantly localized in the nucleolus of germ cells and shows a similarity to Saccharomyces cerevisiae Net1, which is involved in rDNA silencing. Depletion of NRDE-2, a putative interacting partner of C34C12.2 and a component of the nuclear RNAi machinery, restores fertility to the xrn-2 conditional mutant. These results may help to identify an essential role of XRN-2 in germline development.

Keywords: XRN2, C34C12.2, glycerol regulation, mutagenesis screen, germline development.

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Introduction

XRN2 is an evolutionarily conserved 5'-to-3' exoribonuclease. Predominantly localized in the nucleus, it degrades or trims various types of RNA for their maturation, level control or surveillance (Miki and Großhans, 2013; Nagarajan et al., 2013). Targets of XRN2 include the precursor, mature or aberrant forms of rRNA, tRNA, mRNA and microRNA (miRNA). Among multiple functions of XRN2, its role in rRNA maturation has been well-characterized in many species including yeast (Amberg et al., 1992; Petfalski et al., 1998; Fang et al., 2005), ciliates (Couvillion et al., 2012), kinetoplastids (Sakyaïma et al., 2013), plants (Zakrzewska-Placzek et al., 2010) and mammals (Wang and Pestov, 2011). Precursor rRNA (pre-rRNA) is transcribed as a single molecule by RNA polymerase I in the nucleolus. During its processing into three rRNA species, XRN2 plays crucial roles in maturation of 5.8S and 25S/28S (in yeast/mammals, respectively) rRNAs by trimming the 5' ends of their precursors.

We have previously shown that xrn-2 is ubiquitously expressed throughout the development of Caenorhabditis elegans (C. elegans) and that its activity is required for embryogenesis, larval development and reproduction (Miki et al., 2014a). Although XRN-2 has been reported to degrade miRNA (Chatterjee and Grosshans, 2009; Miki et al., 2014a,b) and pre-mRNA (Miki et al., 2016) and to terminate transcription by RNA polymerase II on a subset of genes (Miki et al., 2017) in C. elegans, it remains unclear whether these functions are required for development. Ubiquitous expression and the multifunctional nature of XRN-2 make it difficult to relate one molecular pathway to one developmental process. Genetic screens for enhancers or suppressors of each developmental phenotype are expected to address the issue.

Here we create a germline specific xrn-2 conditional mutant and perform a genetic screen for suppressors of sterility. Loss- or reduced-function alleles of dpy-10, osr-1, ptr-6 and C34C12.2 genes are recovered. Depletion of DPY-10, OSR-1 or PTR-6, but not of C34C12.2, increases accumulation of glycerol, leading to restoration of fertility in the mutant animals. C34C12.2 is predominantly localized in the nucleolus of germ cells and partially homologous to Saccharomyces cerevisiae Net1, which has a role in rDNA silencing. Depletion of NRDE-2, a putative interacting partner of C34C12.2 and an effector protein in the nuclear RNAi machinery, restores fertility to the xrn-2 conditional mutant, indicating that the two proteins might function together to counteract the role of XRN-2 in germline development.

Material and Methods

Worm strains

The Bristol N2 strain was used as wild type. The mutant strains used are listed in Table S1.
Worm culture

Worms were cultured on Nematode Growth Medium (NGM) agar seeded with Escherichia coli OP50 according to the standard methods described previously (Brenner, 1974).

Single copy transgene insertion

Mos1-mediated single-copy transgene insertion (MosSCI) was performed as described previously (Frøkjaer-Jensen et al., 2008). Insertion loci are shown in Table S1.

Mutagenesis screen

About 4,000 xrn-2ts<sup>emo</sup> animals at the fourth larval stage were harvested, washed and incubated with 50 mM ethyl methanesulfonate (EMS) in 6 ml of M9 buffer for 4 hours at 20 °C. The worms were washed three times with M9 buffer and incubated at 20 °C. Once many eggs appeared on the plate, P0 animals were moved to fresh plates. This process was repeated for 2 days. Once many larvae of the F2 generation appeared, the animals were harvested, from which F1 animals were removed by brief centrifugation. The mutated F2 larvae were incubated at 25.5 °C and screened for fertility. Gravid animals were isolated and backcrossed 5 times with the parental strain. Mutations were mapped by single nucleotide variant analysis using the MiModD software (Baumeister Lab., University of Freiburg, Germany) according to the guideline.

Microscopy

Stereoscopic images were obtained with an M205A stereo microscope (Leica, Solms, Germany) or an SMZ25 stereo microscope (Nikon, Tokyo, Japan). DIC and fluorescent images were obtained using an Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany).

RNAi

RNAi clones were obtained from the Ahringer library (Kamath and Ahringer, 2003). RNAi was performed by the feeding method (Timmons and Fire, 1998) with bacteria carrying the insertless L4440 RNAi vector as a negative control.

Quantitative reverse transcription PCR (RT-qPCR)

Worms were harvested, washed three times with M9 medium, resuspended in 700 μl of TRIzol Reagent (Thermo Fischer Scientific, Waltham, MA, USA) and frozen in liquid nitrogen. They were broken open by five repeats of freeze and thaw using liquid nitrogen using 42 °C heating block, before RNA was extracted and purified by the Direct-zol RNA MiniPrep Kit (Zymo Research, CA, USA) according to the supplier’s protocol. cDNA was generated from total RNA by the High-Capacity cDNA Reverse Transcription Kit with oligo(dT)18 Primer (Thermo Fischer Scientific) according to the supplier’s protocol. RT-qPCR was performed by QuantStudio 3 Real-Time PCR system (Thermo Fischer Scientific) using PowerUp SYBR Green Real-Time PCR Master Mix (Thermo Fischer Scientific) and specific primers for gpdh-1 (sense: GCAATTGTGGCGGTGAAACTGG, antisense: CCTGTGTTCCTGGATCTGCAC) and act-1 (sense: AAATACCGCTTGGCCCATCAA, antisense: GCACCTTGCGGTGAACGATGGAT).

Results

Creation of a germline-specific xrn-2 conditional mutant

In order to gain more insight into roles of XRN-2 in C. elegans development, we decided to perform genetic screens for suppressors of developmental defects caused by inactivation of XRN-2. A conditional mutant is a powerful tool, which enables screening for genetic modifiers of an essential gene. We have previously reported temperature-sensitive alleles of xrn-2 in C. elegans (Miki et al., 2014a, 2016, 2017). The mutant animals showed defects in many developmental processes including embryogenesis, larval development and fertility at restrictive temperatures. Genetic suppressor screens using these mutants had failed to identify any mutations other than those in xrn-2 itself. Since xrn-2 is expressed ubiquitously throughout development (Miki et al., 2014a), we reasoned that a single allele might not be able to suppress all developmental defects in different tissues or cells of xrn-2 mutant animals. Therefore, to narrow down the target of screening, we focused on germline development. In order to create a germline specific xrn-2 temperature-sensitive mutant (xrn-2<sup>ts<sup>emo</sup></sub>), we expressed a green fluorescent protein (GFP)-fused functional xrn-2 transgene (xrn-2::gfp) in somatic cells of xrn-2<sup>(xe31)</sup>, a temperature-sensitive mutant of xrn-2<sup>(xe31)</sup> (Miki et al., 2017), by MosSCI (Frøkjaer-Jensen et al., 2008). For rescue of somatic cells, we expressed xrn-2::gfp using the promoter of dpy-18. XRN2-GFP signal was detected in hypodermal and other somatic cells, but not in the intestine or the gonad (Figure 1A). For rescue of the embryo, we expressed xrn-2::gfp using the promoter of pes-2.1, which is active specifically in the embryo (Figure 1B). When incubated at a permissive temperature (20 °C) from the first larval (L1) stage, the xrn-2<sup>ts<sup>emo</sup></sub> animals developed to adult and reproduced. At a restrictive temperature (26 °C), on the other hand, xrn-2<sup>ts<sup>emo</sup></sub> animals developed to adult but were sterile, while xrn-2<sup>ts</sup> animals ceased development as larvae (Figure 1C), as previously reported (Miki et al., 2017). Thus, the xrn-2<sup>ts<sup>emo</sup></sub> strain can function as a tool to screen for genetic suppressors of sterility caused by XRN-2 inactivation in the germline. In order to identify developmental stages that require XRN-2 for fertility, we applied different timings of temperature shifts to xrn-2<sup>ts<sup>emo</sup></sub> animals. The animals were fertile, when temperature was elevated from the middle of L4 stage, though not of L2 stage (Figure 1D). These results indicate that XRN-2 plays a crucial role in germline development before the mid-L4 stage.
Suppressors of xrn-2 sterility

Figure 1 - Creation of a germline-specific xrn-2 temperature-sensitive mutant. (A) Animals with a dpy-18 promoter-driven xrn-2::gfp transgene (Pdpy-18::xrn-2::gfp) were incubated at 20 °C and observed. GFP signal was detected in hypodermal cells (top) but not in the intestine or the gonad (bottom). Corresponding DIC images are shown (right). (B) Animals with a pes-2.1 promoter-driven xrn-2::gfp transgene (Ppes-2.1::xrn-2::gfp) were incubated at 20 °C and observed. GFP signal was detected in embryos. Corresponding DIC images are shown right. (C) Wild-type, xrn-2ts and xrn-2ts<sup>tsm</sup> animals were incubated at 20 °C or 26 °C from L1 stage for 72 hours and observed by stereomicroscopy at the same magnification. An inset shows an adult animal without embryos at higher magnification. (D) xrn-2ts<sup>tsm</sup> animals were incubated at 20 °C from L1 stage until the middle of L2 (left) or L4 (right) stage, then at 26 °C until the adult stage. The animals were observed by stereomicroscopy at the same magnification. An inset shows embryos and a hatched larva.
dpy-10, osr-1, ptr-6 and C34C12.2 genes are identified as genetic suppressors of xrn-2ts\textsuperscript{germ}.

We mutagenized xrn-2ts\textsuperscript{germ} animals with EMS and isolated four strains that were able to reproduce at 25.5 °C (Figure 2A). Genomic DNA sequencing of these strains followed by mutation mapping identified recessive alleles of dpy-10, osr-1, ptr-6 and C34C12.2 genes (Table 1). dpy-10 encodes a collagen protein in the cuticle (Levy et al., 1993). The dpy-10(kid6) allele has a missense mutation, and the mutant animals showed a dumpy phenotype. osr-1 was identified as a gene whose loss conferred resistance to osmotic stress on animals (Solomon et al., 2004), and the osr-1(kid1) allele has a nonsense mutation. ptr-6 encodes a member of patched family proteins, and the ptr-6(kid4) allele changes an amino acid in an evolutionarily conserved extracellular ligand binding site (Kuwabara and Labouesse, 2002; Zugasti et al., 2005; Daggubati et al., 2022). Although we recovered another allele of ptr-6 with a missense mutation that substituted glycine at amino acid position 635 to glutamic acid, the strain carrying the allele was permanently lost due to an extreme difficulty in cryopreservation as previously reported (Choi et al., 2016). C34C12.2 encodes a protein of unknown function, and the C34C12.2(kid2) allele changed the guanine at the 5' splice site of the fourth intron to alanine, abrogating splicing. Thus, these alleles were expected to reduce or abolish the gene functions. Consistently, RNAi-mediated knockdown of each of the genes restored fertility to xrn-2ts\textsuperscript{germ} animals at a restrictive temperature (Figure 2B). These results suggest that the four genes counteract the function of xrn-2 in germline development directly or indirectly.

![Figure 2 - dpy-10, osr-1, ptr-6 and C34C12.2 genes were identified as genetic suppressors of xrn-2ts\textsuperscript{germ}. (A) Animals of indicated genotypes were incubated at 25.5 °C from the L1 stage for 72 hours and observed. Oocytes and embryos were found in all strains except xrn-2ts\textsuperscript{germ}. (B) xrn-2ts\textsuperscript{germ} animals were exposed to mock RNAi or RNAi for indicated genes from L1 to adult at 25.5 °C and observed by stereomicroscopy at the same magnification. Embryos were found in all conditions except mock RNAi.](image-url)
Table 1 - Alleles recovered from the screen.

| Gene(allele)       | Type of mutation | Nucleotide change | Codon change | Amino acid change |
|-------------------|------------------|-------------------|--------------|------------------|
| dpy-10(kid6)      | Missense         | G → A             | GGA → AGA    | G131R            |
| osr-1(kid1)       | Nonsense         | C → T             | CAA → TAA    | Q239Stop         |
| ptr-6(kid4)       | Missense         | G → A             | GGA → GAA    | G223E            |
| C34C12.2(kid2)    | Splice site      | G → A*            | n/a          | n/a              |

*n*’ splice site of intron 4
n/a: not applicable

**DPY-10, OSR-1 and PTR-6 control glycerol accumulation**

We became aware from literature searches that *dpv-10*, *osr-1* and *ptr-6* were among positive genes in a genome-wide RNAi screen for activation of the promoter of *gpdh-1*, a glycerol-3-phosphate dehydrogenase that mediates glycerol synthesis (Lamitina et al., 2006). Consistently, RNAi-mediated knockdown of *dpv-10*, *osr-1* or *ptr-6* increased *gpdh-1* mRNA levels (Figure 3A), which correlated roughly with the rates of animals that restored fertility (Figure 3B). Thus, DPY-10, OSR-1 and PTR-6 negatively regulate *gpdh-1* expression, and depletion of each of them de-represses *gpdh-1* to elevate glycerol levels, leading to restoration of fertility to *xrn-2*ts* germ* animals. Our attempt to restore fertility to *xrn-2*ts* germ* animals by providing glycerol externally from culture plates failed, possibly because the animals were reluctant to take exogenously provided glycerol (Figure S1).

The *xrn-2ts* and *xrn-2*ts* germ* strains have the *xrn-2*(se31) allele that has a missense mutation to destabilize XRN-2 at elevated temperatures (Miki et al., 2017). Since glycerol is known to stabilize proteins (Vagenende et al., 2017), we suspected that the *dpv-10*, *osr-1* and *ptr-6* alleles stabilized mutant XRN-2 in the germ cells of *xrn-2*ts* germ* animals at the elevated temperature, leading to restoration of fertility. Indeed, *dpv-10* alleles have been reported to suppress phenotypes of several temperature-sensitive mutants (Maine and Kimble, 1989; Goh and Bogaert, 1991; Nishiwaki and Miwa, 1998; O’Rourke et al., 2007). If this is the case, elevated accumulation of glycerol, as it diffuses throughout the animal body, should restore fertility to *xrn-2*ts* germ* animals by other means than *gpdh-1* upregulation (Figure 3C). These results indicate that *gpdh-1* upregulation restored fertility to *xrn-2*ts* germ* animals by other means than stabilizing mutant XRN-2 at the elevated temperature.

**NRDE-2 is not required for the RNAi response**

Knockdown of *nrde-2* restored fertility to *xrn-2*ts* germ* animals (Figure 3B). Thus, the C34C12.2 allele is likely to restore fertility to *xrn-2*ts* germ* animals through a mechanism that is different from the *dpv-10*, *osr-1* and *ptr-6* alleles. No cellular functions or developmental roles of C34C12.2 have been reported until now. Protein homology search by Position-Specific Iterated BLAST (National Center for Biotechnology Information, Bethesda, MA, USA) found a similarity between C34C12.2 and *Saccharomyces cerevisiae* Net1 (Figure S3). Net1 is a core subunit of the regulator of nucleolar silencing and telophase exit (RENT) complex (Straight et al., 1999). It tethers the RENT complex to RNAi for silencing by NAD-dependent deacetylase Sir2, another component of the complex, in the nucleolus. In order to examine whether C34C12.2 is localized in the nucleolus of germ cells, we created a transgenic strain that expressed GFP-fused C34C12.2 by MosSCI. As shown in Figure 4, C34C12.2 was predominantly localized in the nucleolus. Consistent to its potential role in germline development, C34C12.2 was detected in germ cells, oocytes and sperm, in addition to the hypodermis and the intestine.

**Knockdown of nrde-2 restores fertility to xrn-2*ts* germ* animals**

Our previous study failed to find C34C12.2 in XRN-2-containing complexes purified from whole-worm lysates (Miki et al., 2014b), raising the possibility that the two proteins function without stable physical interaction in germ cell nuclei. Wan and colleagues identified C34C12.2 in NRDE-2-containing complexes by immunoprecipitation mass spectrometry, although its function was not addressed in the study (Wan et al., 2020). NRDE-2 is an effector protein in the nuclear RNAi pathway. An Argonaute protein, NRDE-3 in the soma or HRDE-1 in the germline, bound by an endogenous siRNA species 22G RNA, translocates from the cytoplasm to the nucleus, where it recruits NRDE-1, -2 and -4 to counteract the role of XRN-2 directly or indirectly in germ cells, knockdown of *nrde-2* should restore fertility to *xrn-2*ts* germ* animals. Indeed, *xrn-2*ts* germ* animals depleted of NRDE-2 regained fertility (Figure 2B).
Figure 3 - Knockdown of dpy-10, osr-1, ptr-6 or C34C12.2 gene increases gpdh-1 expression. (A) Wild-type animals were exposed to mock RNAi or RNAi for indicated genes from L1 to adult at 25 °C. Levels of gpdh-1 mRNA were quantified by RT-qPCR and normalized to act-1 mRNA levels with values of mock-treated animals defined as 1 (n = 5, means ± SEM). p-values were calculated according to the two-sided paired t-test and marked: *p < 0.05, **p < 0.01, ***p < 0.001, n.s. not significant. Values are shown in Table S2. (B) xrn-2ts germ animals were exposed to mock RNAi or RNAi for indicated genes from L1 to adult at 26 °C, and rates of fertile animals were examined (n = 100 in each condition from two independent experiments). (C) Animals of indicated genotypes were incubated at 25 °C from L1 stage for 72 hours and observed by stereomicroscopy at the same magnification.

Discussion

XRN-2 is a multifunctional protein that is involved in various RNA-processing pathways. Therefore, a mutagenesis screen is unlikely to isolate an allele that suppress all developmental defects caused by XRN-2 inactivation. An allele that can suppress sterility may not be sufficient for survival and maintenance of xrn-2 mutant animals, if it fails to suppress the larval arrest phenotype, for example. We overcame this issue by restricting a target of screening to a single developmental process, namely germline development. The xrn-2<sup>ts</sup> conditional mutant was created by expressing a functional xrn-2 transgene in the somatic cells and the embryo of the xrn-2<sup>ts</sup> mutant. A promoter of dpy-18 was used to drive expression of xrn-2::gfp in somatic cells of larvae. Consistent to the previous report of dpy-18 expression (Hill et al., 2000), XRN2-GFP was detected in the hypodermis of the larvae, while missing in some somatic tissues such as the intestine. Nevertheless, the mutant animals were able to develop to adult at an elevated temperature without showing somatic phenotypes such as larval arrest, a molting defect and vulval bursting, which had been reported for xrn-2 (Frand et al., 2005; Miki et al., 2014a). Although xrn-2 is expressed ubiquitously (Miki et al., 2014a), its activity in the hypodermis might be sufficient for somatic development of larvae. If so, a soma specific xrn-2 conditional mutant could be created by expressing functional xrn-2 in the embryo and the germline of the xrn-2<sup>ts</sup> mutant using appropriate promoters, which would function as a useful tool to dissect the roles of XRN-2 in somatic development of larvae. Our approach to create a spatially restricted conditional mutant would be useful to dissect a gene that has multiple essential functions in different tissues or cell-types. Although this method requires a conditional allele and has limited versatility as compared to the auxin-inducible degron system (Nishimura et al., 2009; Zhang et al., 2015; Negishi et al., 2022), it has advantages of low-cost and the ease of control, particularly in long-run experiments such as a genetic screen in this study.
Suppressors of \textit{xrn-2} sterility

Figure 4 - \textit{C34C12.2} is predominantly localized in the nucleolus. Animals with a \textit{gfp::C34C12.2} transgene were incubated at 20 °C and observed. Insets show cell nuclei at higher magnification. GFP signal was detected in the nucleolus of germ cells, oocytes, sperm, hypodermal cells and intestinal cells. Corresponding DIC images are shown right. Punctate signal in the intestine is the autofluorescence of gut granules (Coburn and Gems, 2013)
Our results indicate that elevated accumulation of glycerol restores fertility to \textit{xrn-2ts} animals by other means than stabilizing the mutant XRN-2 protein. A previous study suggests that increase in glycerol accumulation may function as an adaptive response to osmotic stress in the \textit{C. elegans} germ line to maintain the quality of germ cells and oocytes (Davis et al., 2017). This is consistent with the observation that animals that lack \textit{gpdh-1} and \textit{gpdh-2} genes showed reduced brood size as compared to wild-type animals under osmotic stress (Lamitina et al., 2006). Perhaps XRN-2 functions to maintain proper osmolality in the germ line through an unknown mechanism. If so, high levels of glycerol could function as a chemical chaperon to protect XRN-2-inactivated germ cells from osmotic stress by stabilizing proteins and other structures. However, note that we cannot formally exclude the possibility that stabilization of mutant XRN-2 by glycerol is responsible for the phenotypic rescue. If somatic development of \textit{C. elegans} larvae requires higher activity of XRN-2 than germ line development, for instance, stabilization of mutant XRN-2 by glycerol might be merely insufficient for rescue of \textit{xrn-2ts} animals from larval arrest.

C34C12.2 is unique among the genes identified in our screen on the point that it does not affect \textit{gpdh-1} expression. C34C12.2 is partially homologous to \textit{S. cerevisiae} Net1, which tethers the RENT complex to rDNA for silencing (Straight et al., 1999). We found that depletion of NRDE-2, a putative interacting partner of C34C12.2, restored fertility to \textit{xrn-2ts} animals. NRDE-2 functions an effector of the nuclear RNAi machinery, which negatively regulates expression of pre-mRNA and pre-rRNA (Guang et al., 2010; Liao et al., 2021). From these results and its predominant localization in the nucleolus, we speculate that C34C12.2, by interacting with the nuclear RNAi machinery through NRDE-2, represses expression of pre-mRNA and pre-rRNA that loss of its function results in elevation of \textit{rRNA} levels. Thus, the \textit{C34C12.2} loss-of-function allele may compensate for a decrease in \textit{rRNA} accumulation that is caused by XRN-2 inactivation. If so, \textit{rRNA} maturation might be an essential function of XRN-2 in germ line development. Further study on interaction between C34C12.2 and NRDE-2 and the impact of C34C12.2 dysregulation on \textit{rRNA} accumulation will shed light on the roles of C34C12.2 and XRN-2 in germ line development.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

TSM conceived the study; IA, AR and TSM conducted the experiments; IA, AR and TSM analyzed and interpreted the data; TSM wrote the manuscript. All authors read and approved the final version. IA and AR contributed equally to this work.

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Internet Resources
MiModD software, https://mimodd.readthedocs.io (accessed 21 June 2016).
PSI-BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed 22 September 2022).

Supplementary material
The following online material is available for this article:
Table S1 – Worm strains.
Table S2 – gpdh-1 RT-qPCR data.
Figure S1 – Incubation with glycerol did not restore fertility to xrn-2(900) animals.
Figure S2 – Knockdown of dpy-10, osr-1 or ptr-6 did not rescue xrn-2ts animals from larval arrest.
Figure S3 – C34C12.2 shows homology to Trypanosoma brucei.

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