FZR-1 knockdown in *C. elegans* to test the role of APC/C<sup>Cdh1</sup> in the abbreviated cell cycle

Cassandra Rigor

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FZR-1 knockdown in *C. elegans* to test the role of APC/C\(^{Cdh1}\) in the abbreviated cell cycle

**Abstract**
Cancer is characterized by defects in the cell cycle, the program cells use to replicate and divide. One important factor controlling the cell cycle is a protein complex known as the anaphase promoting complex (APC/C\(^{Cdh1}\)). This complex controls the canonical cell cycle, but whether it functions similarly in the abbreviated cell cycle, a non-canonical form of the cell cycle, is unknown. Neural stem cells and embryonic stem cells are examples of cell types that undergo this alternative form of the cell cycle, which is characterized by a shortened G1 phase. We hypothesized that APC/C\(^{Cdh1}\) is not required in the abbreviated cell cycle. To test this hypothesis, RNAi was used to knock down expression of the *C. elegans* homolog of Cdh1 and epifluorescence microscopy was used to visualize the adult hermaphrodite gonads to count the number of germline stem cells (GSCs) that are in active cell division. We found that RNAi-treated animals had significantly fewer GSCs in active cell division than the control; however, the difference was only slight. We cannot say for certain whether our hypothesis was supported or not, but our findings suggest that there may be a potential minor role of APC/C\(^{Cdh1}\) in the abbreviated cell cycle.

**Degree Type**
Open Access Senior Honors Thesis

**Department**
Biology

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**Subject Categories**
Biology

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FZR-1 KNOCKDOWN IN C. ELEGANS TO TEST THE ROLE OF APC/C^{CDH1} IN THE

ABBREVIATED CELL CYCLE

By

Cassandra Rigor

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in Biology

Approved at Ypsilanti, Michigan, on this date April 17th, 2020

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Abstract
Cancer is characterized by defects in the cell cycle, the program cells use to replicate and divide. One important factor controlling the cell cycle is a protein complex known as the anaphase promoting complex (APC/C$^{C_{Dh1}}$). This complex controls the canonical cell cycle, but whether it functions similarly in the abbreviated cell cycle, a non-canonical form of the cell cycle, is unknown. Neural stem cells and embryonic stem cells are examples of cell types that undergo this alternative form of the cell cycle, which is characterized by a shortened G1 phase. We hypothesized that APC/C$^{C_{Dh1}}$ is not required in the abbreviated cell cycle. To test this hypothesis, RNAi was used to knock down expression of the $C.~elegans$ homolog of Cdh1 and epifluorescence microscopy was used to visualize the adult hermaphrodite gonads to count the number of germline stem cells (GSCs) that are in active cell division. We found that RNAi-treated animals had significantly fewer GSCs in active cell division than the control; however, the difference was only slight. We cannot say for certain whether our hypothesis was supported or not, but our findings suggest that there may be a potential minor role of APC/C$^{C_{Dh1}}$ in the abbreviated cell cycle.

Introduction
The cell cycle is a series of events that takes place in a cell where DNA replication occurs and a cell divides into two new daughter cells (Tyson and Novak, 2008). The cell cycle allows an organism to grow, reproduce, and maintain and repair its tissues throughout life (Cooper 2000). It is critical that progression through the cell cycle is tightly regulated in controlling DNA replication and cell division so that the organism maintains the appropriate number of cells. One type of inappropriate cell proliferation – having too many cells – is a fundamental characteristic underlying all cancers, one of the leading causes of death worldwide (Collins et al., 1997). Most
types of tumors found in humans have errors in particular transition checkpoints of the cell cycle (Diaz-Moralli et al., 2013). By understanding more of the cell cycle’s mechanisms, we may eventually be able to figure out ways to manipulate it for devising potential cancer treatments and therapies.

**The canonical cell cycle**

The canonical cell cycle consists of four phases: G1, S, G2 and M. DNA replication occurs during S phase, nuclear division occurs during M phase, and cell growth occurs during gap phases called G1 and G2 (White and Dalton, 2005). In normal cells, certain requirements must be satisfied in order for a cell to transition into the next phase of the cell cycle at the right time and remain in each phase for the right length of time. Signals from the cell’s internal and external environment determine if and when a cell will proceed into the next phase of the cell cycle (Tyson and Novak, 2008). These internal and external signals affect the activity of key cell-cycle regulators that influence a cell’s decision to continue its progression through the cycle. This activity is called checkpoint control and there are three main checkpoints in the cell cycle: (1) DNA-damaged induced (G1/S) checkpoint, (2) DNA-replication induced (G2/M) checkpoint, (3) spindle-assembly checkpoint (Heuvel, 2005) (Figure 1).
The main molecules that regulate progression through the cell cycle are cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitor proteins (CKIs), and two families of E3 ubiquitin ligases (Heuvel, 2005). At each phase, certain regulators are up-regulated while others are down-regulated in order to move to the next phase of the cycle. CDKs, with their corresponding cyclins, drive the progression through the cell cycle. CDK activity is regulated by the presence of cyclins and the activity of CKIs and ubiquitin ligases. In particular, ubiquitin ligases, enzymes that tag other proteins for degradation, are important molecules needed for cell-cycle progression (Neganova and Lako, 2008). The anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase composed of at least 11 core subunits, is one of the two important ubiquitin ligases that regulate cell cycle transitions and is conserved from yeast to humans (Li and Zhang, 2009). Ubiquitin-mediated proteolysis, the breakdown of proteins by attaching ubiquitin molecules to a target protein for degradation, is the main mechanism responsible for maintaining directionality of the cell cycle (Rizzardi and Cook, 2012). The degradation of proteins that were required at a previous phase helps to promote an irreversible...
commitment to the next phase of the cycle. All these components are working together and coordinated in a specific manner in order to go through the cell cycle successfully (Figure 2).

Figure 2. Important regulators of the cell cycle. Figure from Pearson Education (2012).

In addition to being one of the two important ubiquitin ligases that regulate cell cycle transitions, APC/C specifically coordinates the progression through and exit of mitosis into the G1 phase and the transition from G1 to S phase (Castro et al., 2005). The activity of APC/C is dependent on its association with an adaptor protein, cell-division cycle protein 20 (Cdc20) or Cdc20 homologue-1 (Cdh1). Cdc20 and Cdh1 activate APC/C during early mitosis and during late mitosis/early G1, respectively, via phosphorylation (Qiao et al., 2010). During early mitosis, cyclin-Cdk activity is high, causing the phosphorylation of APC/C subunits, which allows the association of Cdc20 with APC/C, denoted as APC/C_{Cdc20} (Castro et al., 2005). As the cell
continues into late mitosis, cyclin-Cdk activity decreases, resulting in the dephosphorylation of APC/C subunits resulting in the dissociation of Cdc20 from APC/C. This dissociation of Cdc20 allows Cdh1 to associate with APC/C, denoted as APC/C\textsuperscript{Cdh1} (Castro et al., 2005). Cdh1 association targets Cdc20 for degradation, marking the sharp and irreversible transition from mitosis to G1 (Qiao et al., 2010). APC/C\textsuperscript{Cdh1} is responsible for degrading certain mitotic cyclins that will then decrease Cdk activity in early G1. This decrease creates proper conditions for the assembly of pre-replicative complexes (pre-RCs) so that the cell can progress to S phase (White and Dalton, 2005). Right before the onset of DNA replication, a certain level of early mitotic inhibitor-1 (Emi1) protein accumulates that triggers the rapid and irreversible inactivation of APC/C\textsuperscript{Cdh1} so that other proteins needed by pre-RCs can accumulate (Cappell et al., 2016). Thus, APC/C\textsuperscript{Cdh1} is active during early G1, but must be inactivated near the end of G1 so that the cell can carry onto S phase (Figure 3).

Figure 3. Schematic of the canonical cell cycle indicating when APC/C\textsuperscript{Cdh1} is on and off. Figure from Cappell (2016).
The abbreviated cell cycle

The canonical cell cycle is well studied (Heuvel, 2005; Neganova and Lako, 2008; Tyson and Novak, 2008) but knowledge on variations of this cell cycle is limited. One form of the cell cycle of particular interest is the abbreviated cell cycle, which is characterized by a short G1 phase (Dalton, 2015). The same sequence of phases of the canonical cell cycle comprise the abbreviated cell cycle; however, there is a difference in the duration of certain phases. In a typical eukaryotic somatic cell undergoing the canonical cell cycle, the G1 phase may last about 11 hours whereas in embryonic stem cells undergoing the abbreviated cell cycle, the G1 phase is shorter and lasts about 2 hours (Cooper, 2000; Coronado et al., 2013) (Figure 4). Cells undergoing the abbreviated cell cycle spend most (about 65%) of their time in S phase (Ballabeni et al., 2011; Abdelalim, 2013). Another difference between the canonical and abbreviated cell cycle is in how the cell receives signals in order to transition onto the next phase. A cell with the canonical cell cycle (i.e., a somatic cell) monitors its environment for mitogens and other growth factors through a G1/S transition checkpoint during the G1 phase. The cell must overcome the G1/S restriction checkpoint in order to continue onto DNA replication and cell division. However, cells with the abbreviated cell cycle advance through the G1/S transition in a mitogen-independent manner, which could account for a much shorter G1 period in these types of cells (Abdelalim, 2013).

Alternative forms of the canonical cell cycle are specific to certain cell types. Induced pluripotent stem cells, neural stem cells, and embryonic stem cells are cell types that undergo the abbreviated cell cycle. Unearthing the components and mechanisms of the abbreviated cell cycle could provide potential avenues for therapies and treatment of certain diseases like, for example, cancer.
Does APC/C\textsuperscript{Cdh1} play a role in the abbreviate cell cycle?

While APC/C\textsuperscript{Cdh1} plays an essential role in managing the cell cycle’s proper transition into S phase within the canonical cell cycle, it is unknown whether APC/C\textsuperscript{Cdh1} functions similarly in the abbreviated cell cycle. Knocking down Cdh1 in the canonical cell cycle prompts early onset of DNA replication and therefore decreases the duration of the G1 phase, but also results in genome instability (Yuan et al. 2014). The G1 phase in the abbreviated cell cycle in stem cells is significantly shorter than the G1 phase in the canonical cell cycle in somatic cells. This difference raises the question as to how stem cells are able to proliferate and maintain genome stability with such a short G1 phase because when a short G1 phase is artificially induced in somatic cells, the outcome is genome instability via DNA replication stress (Macheret and Halazonetis, 2018). This finding led us to propose the following general questions:

- How are cell cycle transitions controlled in the abbreviated cell cycle?
- How is the cell cycle program modified to enable the rapid passage through G1?
Caenorhabditis elegans, an excellent model for investigating APC/C\(^{\text{Cdh1}}\) function

An excellent system for investigating the function of APC/C\(^{\text{Cdh1}}\) is the model organism, *Caenorhabditis elegans*. The transparency of the body of *C. elegans* allows for easy examination, using microscopy, of the effects of genetic manipulations on development (Joshi et al., 2010). Furthermore, the relatively simple system of the germline of *C. elegans* makes for an excellent model for investigating the role of APC/C\(^{\text{Cdh1}}\) in the abbreviated cell cycle. The *C. elegans* germline has a linear organization in which mitotically dividing germ cells, germline stem cells (GSCs), are located at the distal end of the gonad. As the cells migrate away from the distal region toward the proximal region, they begin the course of differentiation into gametes, undergoing meiosis (Yuan et al., 2014). The distal region of *C. elegans* gonad is known as the progenitor zone and a pool of GSCs are maintained by Notch signaling at the mesenchymal niche comprised of a single cell called the distal tip cell (Kimble and Seidel, 2013). The cells in this area will be the focus of our analysis in this study (Figure 5). The GSCs share many similar features to mammalian adult stem cells and cell proliferation of the GSCs in the distal region is maintained throughout the organism’s lifetime (Joshi et al., 2010).
The *C. elegans* homolog of the human APC/C co-activator Cdh1 is called fuzzy and cell division cycle 20 related 1 (*fzr-1*) (The et al., 2015; Medley et al., 2017). Functional disruptions in most *C. elegans* genes failed to display an obvious phenotype which poses a difficulty for scientists to assign a specific function for each gene (Hodgkin, 2001). This is the case for the *fzr-1* gene; however, more information is being gathered by examining *fzr-1* and its interactions with other genes. An example of one of those genes is *lin-35/Rb*, which encodes the *C. elegans* ortholog of the Retinoblastoma pocket protein (pRb), which serves a redundant function as *fzr-1* (Fay et al., 2002; Fay and Yochem, 2007). Given the highly conserved C-domain region and regions of homology dispersed throughout the large N-terminal domain of APC/C(FZR1/Cdh1) in *Drosophila* and murine models (Fay et al., 2002), findings from the organisms in those studies could help characterize the function of that protein in *C. elegans*. Studies using murine models and human cell lines have found that *fzr-1* is required for proper regulation of cyclin levels and cell cycle progression during the canonical cell cycle, specifically during late mitosis and G1 phase (Sigl et al., 2009). In *C. elegans*, when *fzr-1* was inactivated via RNAi microinjection, the result was sterility suggesting a role in reproduction (Fay et al., 2002). The *fzr-1* co-activator
seems to also play a role in centrosome assembly when complexed with APC/C by acting as a negative regulator (Medley et al., 2017). Research on the effects of \textit{fzr-1} knockdown on \textit{C. elegans} is still limited.

As previously described, in the canonical cell cycle APC/C\textsuperscript{Cdh1} is essential in the proper transition from M to G1 and G1 to S phase, but knowledge in its role in the abbreviated cell cycle is still poorly understood. When Cdh1 was depleted in HeLa cells via siRNA, the G1 phase was shortened. This reduction was due to the premature onset of DNA replication occurring nearly 40\% earlier compared to the control which also resulted in a prolonged S phase (Yuan et al., 2014). This early onset and prolonged duration of S phase can make way for replication errors and subsequent genome instability. How this information pertains to stem cells is still a question under investigation. Stem cells showcase similar characteristics of a shortened G1 and prolonged S phase as in Cdh1-depleted HeLa cells. Despite these similar characteristics, stem cells don’t exhibit any errors in replication.

We hypothesize that APC/C\textsuperscript{Cdh1} is not required for normal progression in the abbreviated cell cycle. The observation that stem cells exhibit a shortened G1 phase could suggest that APC/C\textsuperscript{Cdh1} is never active, that is, not expressed or always in an inactive state. Our study will examine the germline stem cells located in the progenitor zone of hermaphrodite \textit{C. elegans} gonads and the effects the \textit{fzr-1} knockdown has on those cells. We will achieve knockdown of \textit{fzr-1} by the RNA interference (RNAi) method and utilize a standard M-phase marker to visualize and score the number of germline stem cells that are undergoing active mitosis. Therefore, if APC/C\textsuperscript{Cdh1} is not required for normal progression in the abbreviated cell cycle, we hypothesize that \textit{fzr-1} RNAi-treated animals will display germline stem cells undergoing active mitosis in the progenitor zone.
Materials & Methods

We performed gene knockdown of our gene of interest, *fzr-1*, through a process known as RNA interference (RNAi). To carry out RNAi, we used the ‘feeding’ method with *Escherichia coli* HT115 bacteria that contained our RNAi construct and subsequently introduced double-stranded RNA to target *fzr-1* for downregulation. We used T444T, a more highly efficient RNAi vector compared to the traditional L4440 (Sturm et al., 2018), as the backbone of our vector and inserted the *fzr-1* gene into T444T to produce our RNAi construct.

C. elegans strains and maintenance

The N2 strain of *Caenorhabditis elegans* was used. The animals were maintained on nematode growth media (NGM) at 15°C with *E. coli* OP50 as the food source. NGM contained 3 g/L NaCl, 2.5 g/L peptone, 20 g/L agar, 25 ml/L 1 M potassium phosphate buffer (1 M K₂HPO₄ mixed with 1 M KH₂PO₄ to reach a pH of 6.0), 1 mM CaCl₂, 1 mM MgSO₄, and 5 μg/ml cholesterol (Seidel et al., 2018).

Construction of plasmid with the *fzr-1* gene and transformation into *E. coli* HT115

The overall cloning strategy we performed included the following: nucleic acid purification to isolate the vector and the *fzr-1* insert, polymerase chain reaction (PCR) to amplify the vector and *fzr-1* insert, and Gibson assembly reaction to combine the vector and insert, and lastly, transformation into bacterial cells.
(A) Purification and PCR amplification of T444T and fzr-1

T444T was the RNAi feeding vector used for the fzr-1 gene. T444T was a gift from Tibor Vellai (Addgene plasmid #113081; http://n2t.net/addgene:113081; RRID:Addgene_113081). The gene of interest, fzr-1, was isolated from complementary DNA (cDNA) from the pMS9-1 plasmid purchased from M. Song (Oakland University, Michigan). We followed the High-Yield Supplementary Protocol in the Qiagen miniprep kit to purify the T444T vector and fzr-1 cDNA. We amplified three fragments of DNA using PCR: the T444T vector, a long fragment of fzr-1 (1675 bp), and a short fragment of fzr-1 (575 bp). The sequences of the forward (F) and reverse (R) primers are listed in Table 1. For the T444T vector, we used L4440 forward and reverse primers. For the long fzr-1 fragment, we used fzr-1_exon2_F as the forward primer and fzr-1_exon4_R as the reverse primer. For the short fzr-1 fragment, we used fzr-1_exon4_Fa as the forward primer and fzr-1_exon4_R as the reverse primer (Figure 6).

Table 1. Primer sequences used for PCR amplification of the T444T vector, the long fzr-1 fragment, and the short fzr-1 fragment.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligo Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T444T</td>
<td>Forward</td>
<td>GCGGCGCCTCTAGAAGACTAGTGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGGCGCCACCGCGTGGAGCTCGAATTCAT</td>
</tr>
<tr>
<td>Long fzr-1</td>
<td>Forward</td>
<td>CGAGCTCCACCAGCGTGCGGCGCCGATACATCGCCACGAGTCACA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATCCACTAGTTCTAGACGCGCCGCGGCTGCCGAGTCAACGTAT</td>
</tr>
<tr>
<td>Short fzr-1</td>
<td>Forward</td>
<td>CGAGCTCCACCAGCGTGCGGCGCCGATAAAGCTTTTGCGATTTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATCCACTAGTTCTAGACGCGCCGCGGCTGCCGAGTCAACGTAT</td>
</tr>
</tbody>
</table>
Figure 6. Representation of the primers used during PCR and the resulting amplified regions of \textit{fzr-1} cDNA.

The PCR mix for T444T consisted of Q5 High-Fidelity 1X Master Mix, 0.5\( \mu \)M L4440 forward primer, 0.5\( \mu \)M L4440 reverse primer, T444T (40 ng), and sterile water for a total volume of 55 \( \mu \)l. The PCR conditions for T444T were as follows: initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 1.5 minutes. The final extension was performed at 72°C for 2 minutes. The T444T PCR product was stored at 10°C until further use. The PCR mix for each the long and short \textit{fzr-1} fragment consisted of 1X OneTaq DNA Polymerase Master Mix, plasmid containing \textit{fzr-1} cDNA (pMS9-1, 1 ng), 0.2 \( \mu \)M forward primer, 0.2 \( \mu \)M reverse primer, and sterile water to bring up to a total volume of 50 \( \mu \)l. The PCR conditions for each the long and short \textit{fzr-1} fragment were as follows: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of: denaturation at 94°C for 30 seconds, annealing at 59°C for 45 seconds, and extension at 68°C for 1 minute, with a final extension at 68°C for 5 minutes. The \textit{fzr-1} PCR products were
stored at 10°C until further use. For the analysis of all three PCR products, 1.5% agarose gels were prepared and run for 30 minutes at constant voltage of 120V.

(B) PCR sample preparation for Gibson Assembly to generate RNAi construct

We prepared the T444T and fzr-1 PCR products by digestion and purification, respectively. The long and short fzr-1 PCR products were each joined together with the T444T PCR product by Gibson Assembly in order to create our RNAi vector. Prior to the Gibson Assembly, the T444T PCR sample was digested with DpnI (20 units) in 1X CutSmart buffer. DpnI is a restriction enzyme that only cleaves methylated DNA (NEB). This digest reduces carryover of the original plasmid from being transformed into bacteria that could result in colonies that have the original plasmid rather than the Gibson assembly product. We purified both fzr-1 PCR products, the long and short fragment, by using the instructions from QIAquick PCR Purification Kit.

The Gibson reaction with the long fzr-1 fragment contained 1X Gibson Master Mix, DpnI-treated T444T PCR product (95.2 ng), purified fzr-1 PCR product (124.02 ng), and sterile water for a total volume of 10 μl. The Gibson reaction with the short fzr-1 fragment contained 1X Gibson Master Mix, DpnI-treated T444T PCR product (95.2 ng), purified fzr-1 PCR product (42.96 ng), and sterile water for a total volume of 10 μl. The Gibson reactions were incubated at 50°C for 1 hour and then stored at -20°C until further use.

(C) Transformation into NEB DH5α competent E. coli Cells and purification of RNAi construct

We transformed our Gibson assembly reactions into NEB DH5α competent E. coli cells to screen for the cells that have the recombinant DNA and to subsequently obtain a culture of them. 2 μl of Gibson reaction was added to 50 μl of NEB DH5α competent cells and incubated for 30
minutes on ice. The cells were incubated in a 42°C water bath for 30 seconds and then immediately placed on ice for 2 minutes. 500 μl of LB media was added to the sample and then incubated with shaking (~230 rpm) at 37°C for 1 hour. The cells were spread onto LB plates supplemented with ampicillin and were allowed to grow overnight at 37°C.

Single colonies were inoculated into liquid LB media supplemented with ampicillin (100 μg/ml) and grown for 16-17 hours at 37°C with shaking. We followed the High-Yield Supplementary Protocol in the Qiagen miniprep kit to purify the RNAi construct.

(D) Digestion of RNAi construct to confirm proper assembly

We allocated a portion of our purified sample and digested it with restriction enzyme NotI-HF to subsequently confirm proper assembly of the construct via gel electrophoresis. The digestion reaction consisted of 1X CutSmart buffer, our RNAi construct (~500 ng), NotI-HF (2.5 units), and sterile water for a total volume of 30 μl. NotI-HF was expected to cut twice in our RNAi construct (Figure 7); therefore, proper assembly is verified by the display of two bands on the gel, one band for the T444T vector backbone and another band for the fzr-1 insert. The gel (1.5% agarose) was prepared in 1X TAE and run at a constant voltage of 120 V for 20 minutes.

![NotI-HF restriction sites on RNAi construct](image)

Figure 7. Visual representation of NotI-HF restriction sites on our RNAi construct.
(E) Transformation of RNAi construct into *E. coli* HT115 (DE3) strain

After we verified proper construction, we transformed the remaining purified sample into the *E. coli* HT115 strain to produce the bacteria that *C. elegans* will feed on for the RNAi assay. CaCl$_2$ was used to induce competency in the HT115 strain. An overnight culture of HT115 in liquid LB media supplemented with tetracycline (10 μg/ml) was prepared. On the following day, the overnight culture was diluted 1:100 in LB supplemented with tetracycline (10 μg/ml) then incubated at 37°C with shaking (~210 rpm) until OD$_{600}$ nm = 0.4. The sample was centrifuged (4,800 x G) for 10 minutes at 4°C then resuspended to 0.5X original volume (OD$_{600}$ nm = 0.8) with cold, sterile 50 mM CaCl$_2$ followed by a 30-minute incubation on ice. Our RNAi construct (~350 ng) was added to 200 μl of CaCl$_2$ competent HT115 and incubated on ice for 30 minutes. The cells were incubated in 37°C water bath for 1 minute then immediately placed on ice for 2 minutes. 1 ml of LB media was added to each sample followed by an incubation period with shaking (~210 rpm) at 37°C for 1 hour. The cells were spread onto LB plates supplemented with ampicillin and tetracycline and were allowed to grow overnight at 37°C. The plates were stored at 4°C until further use (Figure 8).
A

Purification of T444T vector and fzr-1 cDNA

- PCR

Linear T444T PCR product

- PCR

fzr-1 fragment PCR product

B

DpnI Digest

PCR Purification

Gibson Assembly

RNAi construct
C

Transformation into NEB DH5α

LB plate + antibiotics

Culture of transformed NEB DH5α

Miniprep

RNAi Construct

D

NotI-HF Digest

+ Gel Electrophoresis

RNAi Construct Confirmed!

E

Transformation into HT115

RNAi
Figure 8. General schematic of RNAi construct assembly and transformation into CaCl$_2$ competent *E. coli* HT115 (DE3) strain. (A) Purification and PCR amplification of T444T and *fzr-1* (B) PCR sample preparation for Gibson assembly to generate RNAi construct (C) Transformation into NEB DH5α competent *E. coli* cells and purification of RNAi construct (D) Digestion of RNAi construct to confirm proper assembly (E) Transformation of RNAi construct into *E. coli* HT115 (DE3) strain

**Phenotype assay screening for RNAi efficacy**

We performed a phenotype assay to test the efficiency of our RNAi construct on embryo lethality. RNAi worm plates were prepared with 3 g/L NaCl, 2.5 g/L peptone, 3 g/L KH$_2$PO$_4$, 0.55 g/L K$_2$HPO$_4$, 20 g/L agar, 0.001 M MgSO$_4$, 0.001 M CaCl$_2$, 5 mg/ml cholesterol, 25 mg/ml carbenicillin, and 1 M IPTG. RNAi feeder plates were seeded with the transformed RNAi bacteria following the protocol from Anderson et al. (2008). The empty RNAi vector, L4440, served as the negative control. The egg-5 RNAi strain served as the positive control. L4 larvae exposure to RNAi of egg-5 inhibits the formation of the eggshell in *C. elegans* by blocking the oocyte-to-embryo transition which results in embryo lethality (Zou et al., 2019). L4s were allowed to feed for 48 hours and then a single animal was placed onto its own individual RNAi plate seeded with the same RNAi strain as the plate they came from. The single adults were allowed to feed for 6-8 hours then removed from each plate. The number of eggs laid was counted. The plates were incubated overnight at 20°C, then the number of unhatched eggs was counted (Figure 9).
Figure 9. Schematic of phenotype assay to test the efficiency of our RNAi construct on embryo lethality.
**Antibody staining**

We dissected out *C. elegans* gonads and stained them with an antibody that specifically binds to phospho-histone H3, a standard M-phase marker (Hans and Dimitrov, 2001). L4s were picked onto NGM plates seeded with OP50, washed with M9, and transferred onto NGM plates seeded with HT115 bacteria containing one of the RNAi constructs. The worms underwent a 48-hour feeding period at 20°C and were then washed off the plate with 1X PBS + 0.1% Tween-20 (PBST). The worms were transferred to a glass lid containing 1X PBS + 0.1% Tween-20. The worms were anesthetized in 0.25mM levamisole causing the animals to be paralyzed. Gonads were dissected by decapitating the animal with a scalpel, which causes the gonads to spill out of the body. Dissection of as many gonads as possible (~200) was completed within an 8-minute time frame. The gonads were fixed with 3% paraformaldehyde in PBST and incubated with rocking at room temperature for 20 minutes. Paraformaldehyde was removed and the gonads were washed with PBST. 300 ul of -20°C methanol was added to the sample. Gonads were incubated at -20°C for 10 minutes and washed 3 times with PBST. 3% Bovine Serum Albumin (BSA) in PBST was added and the sample was incubated with rocking at room temperature for 20 minutes. BSA is used as a blocking agent to prohibit any non-specific binding when the primary and secondary antibodies are added. The primary antibody, mouse anti-phospho-histone H3 (Cell Signaling Technology, Danvers, MA, #9706), was prepared with 3% BSA in PBST at a 1:200 dilution. Incubation with the primary antibody was performed overnight with rotation at 4°C. The secondary antibody, Cy-3 donkey anti-mouse (Jackson ImmunoResearch, Westgrove, PA, #715-165-151), was prepared with 3% BSA in PBST at a 1:100 dilution. Incubation with the secondary antibody was performed overnight with rotation at 4°C. Gonads were mounted in Vectashield with DAPI and stored at 4°C until use.
**Scoring of phospho-histone H3-positive (PH3+) cells**

The focus of our analysis is on the cells located in the progenitor zone of the gonads of hermaphrodite *C. elegans*. The primary antibody recognizes and binds to phosphorylated histone H3 (Hans and Dimitrov, 2001). Only those cells that contain phosphorylated histone H3 are the cells undergoing active mitosis at the time of data collection. These cells in active M-phase are designated as PH3+ cells. We used a Nikon Y-FL Epi-Fluorescence microscope at 60x magnification to count the number of PH3+ cells in the progenitor zone using the Texas Red filter. DAPI staining was done to verify phospho-histone H3+ cells and to assess overall cell morphology.

**Statistical Analysis**

For the RNAi phenotype assay, a $X^2$ goodness of fit test was performed to determine if a significant relationship existed between the different RNAi strains (control, Egg-5, long $fzr$-1, short $fzr$-1) and the embryonic lethality phenotype (i.e. the number of eggs unhatched).

The PH3+ data were analyzed using a two-tailed T-test to determine if there was a significant increase or decrease in the number of PH3+ cells between the control and the two RNAi strains.
**Results**

To knock down expression of *fzr-1* in *C. elegans*, we created an RNAi feeding vector by amplifying T444T and *fzr-1* cDNA via PCR and performing a Gibson assembly reaction. T444T was used as the vector backbone and we used two sets of primers to create two *fzr-1* inserts of different sizes, one long and one short *fzr-1* fragment. To analyze our PCR products (T444T, long *fzr-1* fragment, and short *fzr-1* fragment), we performed gel electrophoresis. We verified the proper sizes of the T444T, long *fzr-1* fragment, and short *fzr-1* fragment PCR products to be 2469 bp, 1675 bp, and 575 bp, respectively, (Figure 10A). We performed a miniprep to isolate the Gibson assembly of our two RNAi constructs (one containing the long *fzr-1* fragment and one with the short *fzr-1* fragment) from NEB DH5α competent *E. coli* cells. We took a subset of each sample and digested it with NotI-HF restriction enzyme to confirm proper assembly of the constructs via gel electrophoresis. Each construct displayed two bands, one band for the T444T vector backbone and the other band for the corresponding *fzr-1* insert (Figure 10B). After we verified proper construction, we transformed each of the remaining purified sample into the *E. coli* HT115 strain to produce the bacteria that *C. elegans* will feed on for the RNAi assay. This procedure generated two RNAi feeding vectors to knock down *fzr-1*, one containing a longer fragment of the *fzr-1* gene and one containing a shorter fragment of this gene.
Figure 10. (A) Agarose gel electrophoresis of PCR products of the T444T vector (2469 bp), long $fzr$-1 fragment (1675 bp), and short $fzr$-1 fragment (575 bp). (B) NotI-HF digestion of each RNAi construct (lane labeled 1 containing the long $fzr$-1 fragment and lane labeled 2 with the short $fzr$-1 fragment) to verify proper construction by displaying a two-band pattern.
To examine the efficacy of RNAi knockdown of *fzr-1*, we fed L4 hermaphrodites an RNAi strain for 48 hours and then assessed the induction of the embryo lethal phenotype by examining the egg hatching rate. The *egg-5* RNAi strain was used as the positive control and the empty RNAi vector, L4440, served as the negative control. The expectation that the knockdown of *fzr-1* and knockdown of *egg-5* would cause embryo lethality is based on previous reports (Fay et al., 2002; Zou et al., 2019). The average percentage hatch rate of *egg-5*, L4440, long *fzr-1* fragment, and short *fzr-1* fragment were 7.7%, 99.4%, 91.4%, and 97.7%, respectively (Figure 11). We observed that treatment with the long *fzr-1* RNAi feeding vector had a slight but significantly lower percentage of hatched embryos compared to treatment with the empty vector control. Worms treated with the short *fzr-1* RNAi feeding vector showed no significant difference of the percentage of hatched embryos compared to the control worms. RNAi treatment with the short *fzr-1* had a significantly higher percentage of hatched embryos compared to the long *fzr-1* RNAi treatment however, the difference was only slight. These results showed that the *egg-5* RNAi induced significant embryo lethality as expected, but lethality induced by knockdown of the long *fzr-1* fragment was only slight.
Figure 11. The average percentage hatch rate of the control and the different RNAi treatments. RNAi treatment with the long fzr-1 fragment had a slight but significantly lower percentage of hatched embryos compared to treatment with the empty vector control. There was no significant difference between RNAi treatment with the short fzr-1 fragment and treatment with the empty vector control. RNAi treatment with the short fzr-1 had a significantly higher percentage of hatched embryos compared to the long fzr-1 RNAi treatment however, the difference was only slight. (* p < 0.05, Chi square test)
To test whether knockdown of *fzr-1* causes a cell-cycle defect in germline stem cells (GSCs), we counted the number of GSCs in active mitosis at the distal region of gonad in *C. elegans* hermaphrodites. We used an antibody that binds to a standard M-phase marker, phospho-histone H3, to identify the cells undergoing active mitosis at the time that the animals were dissected – designated as PH3+ cells. We could not include the images of our samples when visualized under the fluorescent microscope in this paper. However, our images would have closely resembled the image taken in the study from Seidel and Kimble (2015) (Figure 12A). The average number of PH3+ cells in the control (L4440), *fzr-1* (long fragment) RNAi treatment, and *fzr-1* (short fragment) RNAi treatment were 5.93, 5.01, and 5.42, respectively (Figure 12B). There was no significant difference in the number of PH3+ cells between the control and the short *fzr-1* fragment or between the two *fzr-1* fragments. Animals treated with the long *fzr-1* fragment RNAi feeding vector had slightly but significantly fewer PH3+ cells than animals treated with the empty vector control. These results showed that treatment with the *fzr-1* RNAi feeding vector had only a slight effect on the cell cycle.
Figure 12. Effect of RNAi knockdown of \textit{fzr-1} on mitosis in the germline stem cells in the \textit{C. elegans} gonad. (A) Image from Seidel and Kimble (2015) that would have resembled our images of the distal region of an adult hermaphrodite stained with DAPI and phospho-histone H3 to visualize M-phase GSCs. (B) The average number of PH3+ cells in the control (L4440), \textit{fzr-1} (long fragment) RNAi treatment, and \textit{fzr-1} (short fragment) RNAi treatment. There was no significant difference in the number of PH3+ cells between the control and the short \textit{fzr-1} fragment or between the two \textit{fzr-1} fragments. Animals treated with the long \textit{fzr-1} fragment RNAi feeding vector had slightly but significantly fewer PH3+ cells than animals treated with the empty vector control (* \textit{p} < 0.05, Chi square test).
**Discussion**

APC/C\textsuperscript{Cdh1} is an important regulatory molecule in the canonical cell cycle responsible for degrading certain mitotic cyclins via ubiquitination to coordinate the transition from G1 to S phase. The purpose of this study was to investigate the role of APC/C\textsuperscript{Cdh1} in the abbreviated cell cycle. To do this, we knocked down \textit{fzr-1}, the \textit{C. elegans} homolog of the human APC/C co-activator Cdh1, via RNAi and assessed the effects of knockdown on the cell cycle in germline stem cells located at the progenitor zone of adult hermaphrodites. Germline stem cells were analyzed because they undergo the abbreviated cell cycle. We used immunohistochemistry and epifluorescence microscopy to visualize the number of germline stem cells undergoing active mitosis. Our main finding was that treatment with the \textit{fzr-1} RNAi feeding vector had minimal effect on the cell cycle as evident from the significant but only slightly fewer number of PH3\textsuperscript{+} cells in the RNAi-treated animals. Ideally, with this result, we would conclude that our hypothesis is supported that the \textit{C. elegans} homolog of APC/C\textsuperscript{Cdh1} is not required for the normal progression in the abbreviated cell cycle. However, it is impossible for us to conclusively know this because the RNAi phenotype with respect to embryo lethality did not give us the expected phenotype suggesting that the RNAi knockdown may have been incomplete.

We performed an experiment to assess how effective the \textit{fzr-1} RNAi treatment was in inducing embryo lethality, a known phenotype (Fay et al., 2002). In contrast to our expectation that RNAi treatment of \textit{fzr-1} would induce significant embryo lethality like our positive control \textit{egg-5} (Zou et al., 2019), we found that lethality induced by RNAi knockdown of the long \textit{fzr-1} fragment was only slight. The weak phenotype that we observed suggests that our RNAi knockdown of \textit{fzr-1} was incomplete, RNAi knockdown of \textit{fzr-1} alone could perhaps not have
been enough to produce a strong embryo lethal phenotype, or that other genes are involved that could compensate for the loss of function as a result from \textit{fzr-1} RNAi knockdown.

This compensation could be explained by genetic redundancy, where two or more genes carry out the same function (Nowak et al., 1997). Therefore, if only one of the two or more genes was inactivated, there could be little to no effect on the phenotype. However, when redundant genes are inactivated in certain combinations, severe defects could result in what is called a synthetic phenotype (Fay et al., 2002). A previous study found \textit{fzr-1} to function redundantly with a gene called \textit{lin-35}/Rb, which encodes the \textit{C. elegans} ortholog of the Retinoblastoma pocket protein (pRb) (Fay et al., 2002). In that study, when \textit{fzr-1} was inactivated via RNAi injection in a \textit{lin-35} mutant, the double mutants displayed embryo lethality and tissue hyperproliferation whereas only low-penetration phenotypes were seen in single mutants of \textit{fzr-1} and \textit{lin-35} (Fay et al., 2002). There is also some evidence that \textit{fzr-1} has synthetic interactions with other genes, specifically with a subset of class B synthetic multivulval (SynMuv) genes, which are the set of genes that exhibit the best-known case of redundancy (Ferguson and Horvitz, 1989). The exact mechanisms of the synthetic interactions between \textit{fzr-1} and other regulators are still largely in question. A potential experiment in the future is to utilize combinatorial RNAi where multiple genes can be targeted for knockdown (Tischler et al., 2006). Through this high throughput method, we can learn more about the genetic interactions and effects of \textit{fzr-1} knockdown in combination with knockdown of other genes in order to potentially provide a clearer understanding on the specific functions of \textit{fzr-1}.

In addition to genetic redundancy, perhaps the method of RNAi by feeding was not sufficiently effective in silencing \textit{fzr-1}. Some genes are more effectively silenced by RNAi feeding compared to microinjection and vice versa. Other genes are equally susceptible to both
the feeding and microinjection method (Kamath et al., 2001). Due to time constraints, RNAi by microinjection could not be performed in this study but is a good candidate in executing future studies to uncover more information on *fzr-1* function.

Despite our inability to establish whether or not our hypothesis was supported, our results suggest a potential minor role of APC/C<sup>Cdh1</sup> in the abbreviated cell cycle. We hope future studies could implement alternative methods to hopefully achieve a more definitive result. RNAi by microinjection may be more efficient in silencing *fzr-1* than RNAi by feeding. Another potential avenue in revealing APC/C<sup>Cdh1</sup> function in the abbreviated cell cycle is to see *fzr-1* knockdown in combination with knockdown of other genes via combinatorial RNAi. Our study is a step headed in the right direction in determining the role of APC/C<sup>Cdh1</sup> in the abbreviated cell cycle. The more we discover about the main mediators of the abbreviated cell cycle, the more likely we can develop techniques to control it. Those techniques may eventually be useful in treating various diseases involved with cell cycle aberrancies, like cancer.
Works Cited


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