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The folic acid metabolism gene mel-32/Shmt is required for normal cell cycle lengths in Caenorhabditis elegans

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ABSTRACT Neural tube defects are common and serious birth defects in which the brain and/or spinal cord are exposed outside the body. Supplementation of foods with folic acid, an essential vitamin, is linked to a lower risk of neural tube defects; however, the mechanisms by which folic acid influence neural tube defect risk are unclear. Our research seeks to identify the basic cellular roles of known folic acid metabolism genes during morphogenesis using the roundworm Caenorhabditis elegans as a simple model system. Here, we used live imaging to characterize defects in embryonic development when mel-32 is depleted. mel-32 is an essential folic acid metabolism gene in C. elegans and a homolog to the mammalian enzyme serine hydroxymethyltransferase (Shmt). Disruption of mel-32 resulted in a doubling or tripling of cell cycle lengths and a lack of directed cell movement during embryogenesis. However, the order of cell divisions, as determined by lineage analysis, is unchanged compared to wild type embryos. These results suggest that mel-32/Shmt is required for normal cell cycle lengths in C. elegans.

KEY WORDS: folic acid, Shmt, mel-32, C. elegans, cell cycle

The neural tube is an embryonic structure that gives rise to the brain and spinal cord. Failure of neural tube closure results in neural tube defects, in which part of the brain and/or spinal cord is exposed outside the skin. Neural tube defects, like spina bifida, are a leading cause of birth defects, which can be fatal or result in lifelong disabilities. Folic acid fortification and supplementation is widely recognized to decrease the risk of neural tube defects (Greene and Copp, 2014). Folic acid fortification of certain grain food products, like breakfast cereals, was mandated by the FDA in the late 1990’s and has decreased the rates of neural tube defects between 10-80%, depending on the location and study (Imbard et al., 2013). Despite this strong correlation between folic acid and neural tube defect risk, the functions of folic acid metabolism genes during neural tube development are poorly understood (Blom et al., 2006; Wallingford et al., 2013).

In this study, we used the roundworm Caenorhabditis elegans (C. elegans) as a simple model system to study how folic acid metabolism genes affect early embryogenesis. Although C. elegans lack a neural tube, cell behaviors that occur during neural tube closure, such as actomyosin dependent apical constriction, are utilized during morphogenesis in C. elegans (Sawyer et al., 2011). C. elegans are advantageous for this study because they are optically transparent, allowing for cells to be easily visualized in vivo, and they are amenable to genetic studies. Importantly, C. elegans have a functional folic acid metabolism pathway (Austin et al., 2010; Balamurugan et al., 2007; Cabreiro et al., 2013; Chaudhari et al., 2016; Ortbauer et al., 2016; Virk et al., 2016) allowing hypotheses to be built concerning the roles of folic acid metabolism genes during morphogenesis across species.

Serine hydroxymethyltransferase (Shmt) is a folic acid metabolism gene that catalyzes the reversible conversion of serine to glycine, generating the one-carbon unit 5,10-methylenetetrahydrofolate (5,10-methylene THF), which is then used in the de novo synthesis of thymidylate (dTMP) from deoxyuridylate (dUMP) (reviewed in Chon et al., 2017). Despite the important roles of Shmt in the folic acid metabolism pathway, mutations in Shmt2a (MacFarlane et al., 2008) result in viable and fertile offspring, presumably due to the redundant functions of Shmt2a (MacFarlane et al., 2008). However, if mice with decreased levels of Shmt1 are fed a folate deficient diet, a neural tube defect known as exencephaly was observed (Beaudin et al., 2011).

Abbreviations used in this paper: C. elegans, Caenorhabditis elegans; mel-32, maternal effect lethal-32; MZ, maternal zygotic; Shmt, Serine hydroxymethyltransferase.
et al., 2011). Furthermore, if Shmt1 deficient mice are bred into the splotch mutant (Pax3<sup>−</sup>), a mutant previously shown to have impaired de novo synthesis of dTMPs, the incidence and severity of neural tube defects increased (Beaudin et al., 2011).

The <i>C. elegans</i> homolog of Shmt was previously identified as mel-32 (maternal effect lethal-32) and shown to be essential during early embryogenesis (Vatcher et al., 1999; Vatcher et al., 1998). We provide evidence that mel-32/Shmt is essential for normal cell-cycle lengths but is not required in determining the order of cell divisions.

Results

**mel-32/Shmt is required for normal cell cycle lengths of early embryonic cell divisions**

mel-32(s2518) is a maternal zygotic (MZ) mutation that results in embryonic arrest at or before the 100 cell stage, a phenotype which can be rescued with genomic SHMT DNA (Vatcher et al., 1998). For simplicity and consistency, we will refer to embryos from mel-32(s2518) homozygous null parents as mel-32 MZ embryos. To better understand the developmental roles of mel-32/Shmt, we used in vivo time-lapse imaging to analyze the developmental defects in mel-32 MZ embryos. In our initial analysis, we noticed that mel-32 MZ embryos required more time to transition from the 4-cell, 8-cell and 24-cell stages. As shown in Fig. 1A, the transition from the 4- to 24-cell stage in wild type embryos normally requires about 40 minutes to complete. However, in mel-32 MZ embryos, these stages took upwards of 2.5 hours to complete. By a little over 5 hrs of development after the 4-cell stage, wild type embryos exhibited extensive cell movements that comprise the gastrulation and epidermal enclosure stages (Chisholm and Hardin, 2005). During gastrulation, endoderm, mesoderm, neural and germ-cell precursors become internalized (Harrell and Goldstein, 2011; Nance et al., 2005) and during epidermal enclosure, epithelial cells surround the embryo (Chisholm and Hardin, 2005). After epidermal enclosure, the wild type embryo takes on a characteristic “bean-shape” as the process of elongation begins (Fig. 1A, Supplementary Movie1). In mel-32 MZ embryos, morphogenesis failed and even after 5 hours of development, the embryo remained amorphous with no evidence of directed cell movements (Fig. 1A, Supplementary Movie1).

To better understand the developmental defects in mel-32 MZ embryos, we analyzed cell cycle lengths in the AB cells (ABx), EMS, P2, MS and E cells (schematic of cell positions shown in Fig. 1B). We observed striking increases in cell cycle lengths in mel-32 MZ embryos, with lengths nearly double or triple the normal cell cycle length (Fig. 1C, Table 1). The cell cycle length defects were not confined to a specific cell lineage, as cells that give rise to the muscle and neurons (MS and AB), endoderm (E), and germine (P2) all showed significant increases in cell cycle lengths. These data suggest that mel-32/Shmt is required for normal cell cycle progression in <i>C. elegans</i>.

To control for the possibility that the dpy-17(e164) mutation, present in the mel-32(s2518) genetic strain as a phenotypic marker, is affecting the cell cycle, we analyzed cell cycle lengths in the BC5078 strain, which carries the dpy-17(e164) mutation only. dpy-17 is a cuticle collagen gene that results in short/stubby worms (Page and Johnstone, 2007), but does not have a known role in the cell cycle. Our data indicate there was no defect in cell cycle length in dpy-17(e164) mutant embryos (Table 1), suggesting that the presence of the dpy-17(e164) mutation in the mel-32(s2518) strain does not cause cell cycle defects. Attempts to disrupt mel-32 using an RNAi-by-feeding approach resulted in a weaker embryonic lethality phenotype compared to mel-32 MZ embryos. Further, early cell divisions in mel-32(RNAi) embryos did not show a cell cycle defect (Table 1). We hypothesize that the RNAi-by-feeding approach resulted in an incomplete knockdown
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Abx</th>
<th>EMS</th>
<th>P2</th>
<th>MS</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>13.58±0.44(n=4)</td>
<td>15.98±0.33(n=4)</td>
<td>18.40±0.28(n=4)</td>
<td>16.25±0.55(n=4)</td>
<td>17.58±0.42(n=4)</td>
</tr>
<tr>
<td>mel-32 MZ</td>
<td>30.89±1.28(n=3)</td>
<td>46.89±3.77(n=3)</td>
<td>62.66±7.08(n=3)</td>
<td>54.25±9.96(n=4)</td>
<td>58.33±9.37(n=4)</td>
</tr>
<tr>
<td>mel-32(RNAi)</td>
<td>12.55±0.22(n=3)</td>
<td>13.77±0.29(n=3)</td>
<td>17.31±0.35(n=3)</td>
<td>16.25±0.21(n=4)</td>
<td>17.08±0.34(n=4)</td>
</tr>
<tr>
<td>dpy-17(e164)</td>
<td>12.22±0.62(n=3)</td>
<td>14.55±0.67(n=3)</td>
<td>17.66±1.20(n=3)</td>
<td>16.22±1.24(n=3)</td>
<td>17.88±1.35(n=3)</td>
</tr>
</tbody>
</table>

Table shows the cell cycle lengths (in minutes) for wild type, mel-32 MZ embryos, mel-32(RNAi) embryos, and dpy-17(e164) embryos. * indicates a statistical difference (one-way ANOVA with Dunnett’s post test, p<.05).

Discussion

In this report, we used the roundworm *C. elegans* as a simple system to better understand how mel-32/Shmt, a folic acid metabolism gene, affects cell behaviors during development. We showed that defects in mel-32 resulted in significantly increased cell cycle times in the early *C. elegans* embryo. Interestingly, mutations in *folt-1*, the *C. elegans* homolog of the folate transporter RFC, may also result in cell proliferation defects suggested by a reduced germline with less sperm and defective oocytes (Austin et al., 2014). The specific phase of the cell cycle affected in *mel-32* MZ mutants is unknown; however, previous data have indicated that folate deficiencies can arrest cells in S phase (Courtemanche et al., 2004). Since the cell divisions analyzed in this study are primarily composed of S and M phases (Bao et al., 2008; Edgar and McGhee, 1988), we predict that S phase is affected in *mel-32* MZ mutants, possibly by decreasing the rate of DNA replication through a reduction of dTMP levels. Alternatively, S phase may be stalled due increased DNA repair. In mammals, reduced levels of nuclear Shmt1 increased the prevalence of DNA nicks and double-stranded breaks resulting in genomic instability (Anderson and Stover, 2009; Blount et al., 1997; MacFarlane et al., 2008; Wilson et al., 2014). In addition, chromosomal breakage has also been observed in humans with folate deficiencies (Blount et al., 1997). Future work will be needed to determine if lack of *mel-32/Shmt* results in DNA damage which may slow the cell cycle in *C. elegans*.

Although the cell cycle was significantly lengthened, the order of early embryonic cell divisions was unaffected in *mel-32* MZ embryos. Conditions that slow the cell cycle in *C. elegans*, such as a temperature decrease, or mutations that cause an overall slowing of development (ie, the *clk-1* gene), also result in a lengthening of the cell cycle while maintaining the relative order of cell divisions (Nair et al., 2013). Although the mechanisms that establish the highly invariant order of cell divisions remain unclear, evidence indicates that cell fate plays an important role (Bao et al., 2008).

It will be important to analyze the expression of cell fate molecular markers to determine if there is evidence of cell differentiation during these early cell divisions in *mel-32* MZ embryos.

Materials and Methods

*C. elegans strains and worm maintenance*

* C. elegans* were cultured and handled as described in (Brenner, 1974). All strains were maintained at 20°C with OP50 *E. coli* as its food source. The following strains were used in this study: WT Bristol N2, BC5078 dpy-17(e164), mel-32(s2518), unc-32(e189)III;Dp3(III);CB164 dpy-17(e164).

* mel-32 is required for normal cell cycle lengths in *C. elegans* 643
RNA interference (RNAi)
RNAi-by-feeding was performed using a protocol outlined previously (Sawyer et al., 2011; Sullivan-Brown et al., 2016) with minor modifications. Three to five L4 larvae from the N2 wild type background were placed on RNAi plates seeded with double-stranded RNA (dsRNA) producing bacterial strains as in (Kamath et al., 2001; Timmons and Fire, 1998). RNAi plates were either made in-house (standard Nematode Growth Media (NGM) plates, with 25 μg/ml of Carbencillin and 1 mM IPTG) or purchased directly from LabExpress (#5003-60). RNAi bacterial feeding strains were obtained from a dsRNA feeding library from the Medical Research Council (MRC) Geneservice (Kamath and Ahringer, 2003) and generously supplied by the Goldstein lab-UNC Chapel Hill.

Microscopy
C. elegans embryos were mounted on poly-L-lysine coated coverslips at the one or four-cell stage and mounted on a 2.5% agarose pad made with M9 buffer (as in Sawyer et al., 2011). Differential interference contrast (DIC) imaging was performed on an Olympus BX60 Upright Microscope. All time-lapse recordings were taken on a Celestron Digital Microscope imager HD SMP and recorded using free time-lapse imaging software by VideoVelocity Time-Lapse Capture Studio. Time-lapse images were taken every 20 seconds, with manual focusing. All images were acquired with a 40x objective. Supplementary Movie 1 is shown at 25 frames per second.

Quantification of cell cycle lengths and lineage analysis
Time-lapse video recordings (as described above) were used to determine the cell cycle lengths by tracking the cell divisions of the ABx, P2, EMS, E, and MS cells as shown in lineage tracings from (Solston et al., 1983). Cell division was measured from the birth of the precursor cell to the birth (end of cytokinesis) of its daughters. Depending on the analysis, a student’s t-test or one-way ANOVA with Dunnett’s post test was used to determine statistical significance (p<.05). Lineages tracings were generated in Microsoft PowerPoint using average cell cycle lengths from wild type and mzl-32 M2Z embryos.

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