Int. J. Dev. Biol. 62: 641-645 (2018)
https://doi.org/10.1387/ijdb.180023js

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

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* (*C. 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 roles of folic acid 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 mice that knockout the cytoplasmic form of *Shmt* (*Shmt1),* 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

Abbreviations used in this paper: C. elegans ,Caenorhabditis elegans; mel-32, maternal effect lethal-32; MZ, maternal zygotic; Shmt, Serine hydroxymethyltransferase.

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Supplementary Material (one movie) for this paper is available at: https://doi.org/10.1387/ijdb.180023js

Submitted: 11 January, 2018; *Accepted:* 2 August, 2018.

ISSN: Online 1696-3547, Print 0214-6282

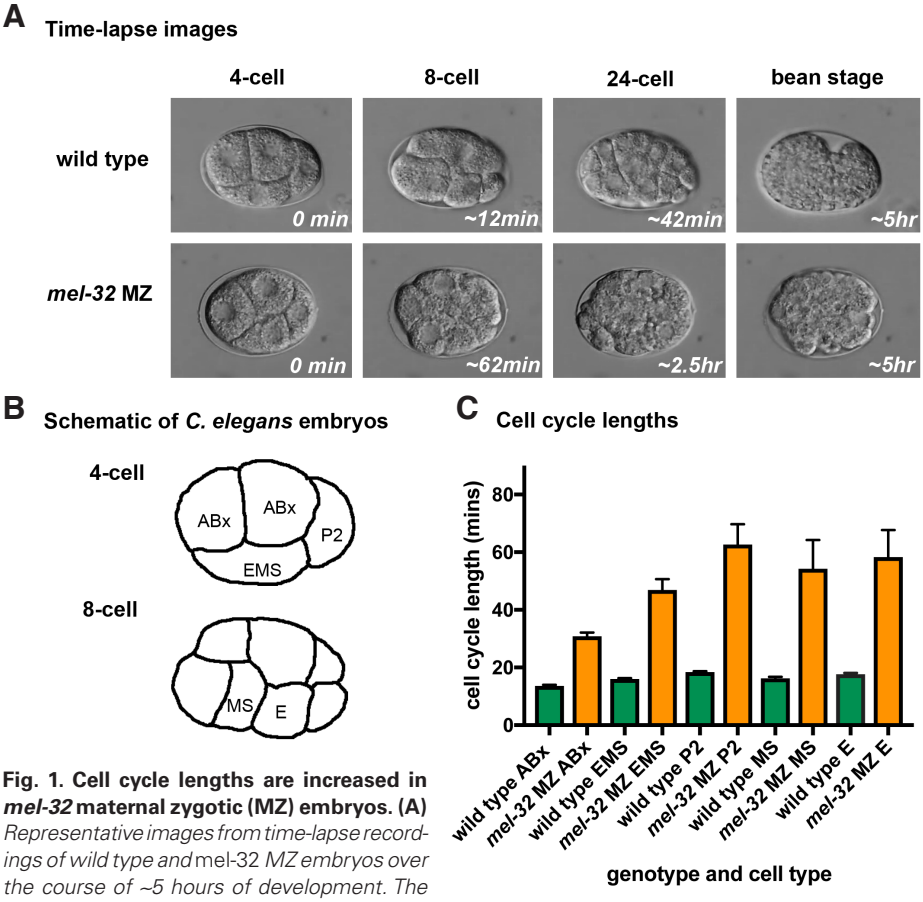

Fig. 1. Cell cycle lengths are increased in *mel-32* maternal zygotic (MZ) embryos. (A) *Representative images from time-lapse recordings of wild type and* mel-32 *MZ embryos over the course of ~5 hours of development. The 4-cell, 8-cell, 24 cell-stages, and bean stages are shown. 0min=beginning of time-lapse, min=minutes, hr=hours.* **(B)** *Schematic of wild type* C. elegans *embryos, illustrating the positions of the ABx, EMS, P2, MS and E cells.* **(C)** *Quantification of cell cycle lengths of early cell divisions for the ABx, EMS, P2, MS and E cells. Green and orange bars represent wild type and* mel-32 *MZ embryos respectively. For each cell division, the length of the cell cycle is significantly increased in* mel-32 *MZ embryos compared to wild type (student's t-test, p<.05); WT(n=4),* mel-32 *MZ ABx/EMS/P2 (n=3),* mel-32 *MZ MS and E (n=4)). Error bars represent standard error of means.*

et al., 2011). Furthermore, if *Shmt1* deficient mice are bred into the splotch mutant (*Pax3*^{sp}), 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 *C. elegans* 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 germline (P2) all showed significant increases in cell cycle lengths. These data suggest that *mel-32/Shmt* is required for normal cell cycle progression in *C. elegans*.

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(sz518)* 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

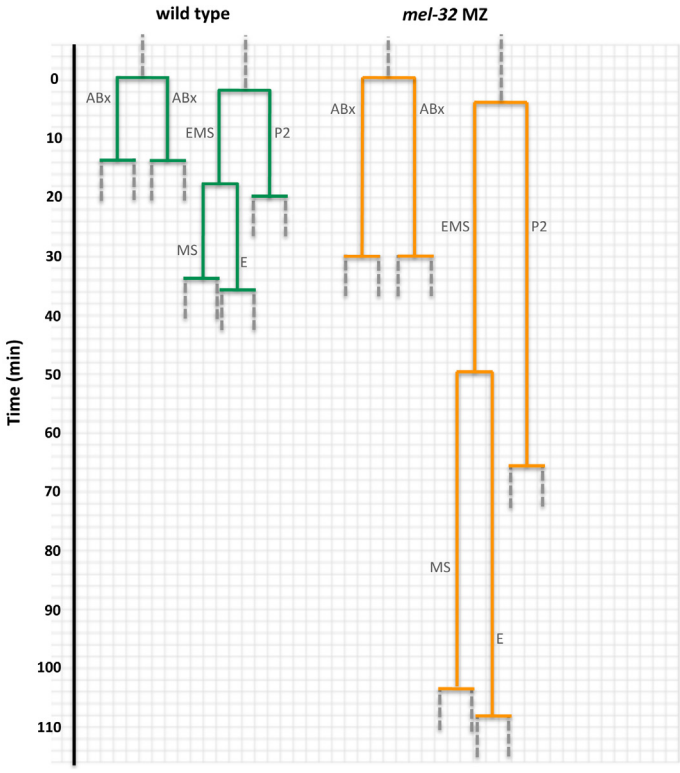

Fig. 2. The order of early cell divisions is not affected in *mel-32* maternal zygotic (MZ) embryos. *Time-lapse microscopy was used to determine the order of cell divisions, represented as an embryonic cell lineage diagram. Green and orange solid lines indicate cell divisions from wild type (n=4) and* mel-32 *MZ embryos (n=4) respectively. Gray dotted lines indicate cells that divided but the length of the cell cycle is not represented. Time in minutes is indicated on the y-axis.*

of *mel-32*; however, we cannot rule out a possible synergistic effect between *mel-32* and *dpy-17* knockouts.

mel-32/Shmt *is not required for the order of cell divisions*

We next asked if the order of cell divisions was affected in *mel-32* MZ embryos. In *C. elegans* the order of each cell division is known and highly invariant (Sulston *et al.*, 1983). To determine the order of cell divisions, we constructed lineage tracings of each cell division, until the division of the MS and E cells. Surprisingly, although the length of each cell cycle is increased significantly, the order in which the cells divide is normal (Fig. 2). These data indicate that while *mel-32/Shmt* has important roles in determining the length of the cell cycle, it is dispensable for determining the order of cell divisions, unlinking these processes.

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.*, 2010). 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;sDp3 (III;f); CB164 *dpy-17(e164).*

TABLE 1

CELL CYCLE LENGTHS

	Abx	EMS	P2	MS	E
	mean±SEM	mean±SEM	mean±SEM	mean±SEM	mean±SEM
WT	13.58±0.44(n=4)	15.98±0.33(n=4)	18.40±0.28(n=4)	16.25±0.55(n=4)	17.58±0.42(n=4)
***mel-32* MZ**	30.89±1.28*(n=3)	46.89±3.77*(n=3)	62.66±7.08*(n=3)	54.25±9.96*(n=4)	58.33±9.37*(n=4)
***mel-32*(RNAi)**	12.55±0.22(n=3)	13.77±0.29 (n=3)	17.31±0.35 (n=3)	16.25±0.21 (n=4)	17.08±0.34 (n=4)
dpy-17(e164)	13.22±0.62 (n=3)	14.55±0.67 (n=3)	17.66±1.20 (n=3)	16.22±1.24 (n=3)	17.88±1.35 (n=3)

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).

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 Carbenicillin and 1mM 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 inference contrast (DIC) imaging was performed on an Olympus BX60 Upright Microscope. All time-lapse recordings were taken on a Celestron Digital Microscope imager HD 5MP 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 (Sulston *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 *mel-32* MZ embryos.

Acknowledgements

We thank our colleagues, especially Jennifer Tenlen, Jennifer Heppert and Jodi Schottenfeld-Roames for their critical reading, suggestions, and comments on this manuscript. We also thank Gerry Misquith for her help in obtaining lab materials. Strains were provided by the Caenorhabditis elegans *Genetics Center, which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). This work was supported by the generosity of West Chester University's Alumni & Foundation Center, the College of the Sciences and Mathematics at West Chester University and the Georgann Cullen Award. JSB would like to thank the many undergraduates at West Chester University that had contributions to this project and the Department of Biology at WCU for support.*

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