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FOOD & FUNCTION

Folate deficiency and over-supplementation causes impaired folate metabolism: Regulation and adaptation mechanisms in *Caenorhabditis elegans*

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Scope: Impaired folate metabolism increases the risk of birth defects, neurodegenerative and cardiovascular disease, osteoporosis and cancer. We used *Caenorhabditis elegans* to investigate impaired folate metabolism by RNA interference of key enzymes in the methionine synthase (MS) and thymidylate synthase (TS) cycle and by folate deficiency and over-supplementation feeding studies.

Methods and results: Folate status is influenced by genetic variations (polymorphisms), folate deficiency and supplementation. Single RNAi of dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR) and MS revealed that gene regulation is largely affected in both folate cycles. Adaptation requires a close transcriptional connection between TS and MS cycle. Coupled DHFR and MS expression is required to balance both cycles, but seems to reduce the overall rate of folate conversion. Feeding studies showed that folate over-supplementation to functioning metabolism inactivates MS and MTHFR expression and enhances TS activity, which favors DNA synthesis over methylation reactions. Folate deficiency disrupted homeostasis by favoring TS cycle and led to malformation in *C. elegans* offspring. Embryos show aneuploidy and are nonviable lacking DNA repair during meiotic stage of diakinesis.

Conclusion: Single gene silencing alters gene expression in both cycles and disrupts folate homeostasis. Folate over-supplementation and deficiency favors TS over MS cycle and causes prophase DNA damage.

Keywords:
Folate deficiency / Folic acid over-supplementation / Impaired folate metabolism / Polymorphisms / RNAi

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1 Introduction

Folates are essential nutrients that belong to the family of water-soluble B-vitamins. They play a pivotal role in cellular function and biosynthesis via donation of one-carbon units for DNA synthesis in the TS cycle and for methylation reactions in the MS cycle (Supporting Information Fig. 1). It is therefore not surprising that folate deficiency and impaired folate metabolism is associated with abnormalities ranging from chromosome breakage [1], high plasma homocysteine levels [2], cardiovascular disease [3], birth defects [4, 5], neurodegenerative disease [6, 7], osteoporosis and cancer [8–10]. Folate deficiency can disrupt global DNA methylation patterns [11] and hence influence epigenetics [12]. Human folate metabolism is influenced by genetic variations and can be

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Abbreviations: **CBS**, cystathionine beta-synthase; **DHFR**, dihydrofolate reductase; **MS**, methionine synthase; **MTHFR**, methylenetetrahydrofolate reductase; **NTD**, neural tube defect; **PCFT**, proton coupled folate carrier transporter; **RFC**, reduced folate carrier; **RNAi**, RNA interference; **SAH**, S-adenosylhomocysteine; **SAM**, S-adenosylmethionine; **SNPs**, single nucleotide polymorphisms; **TS**, thymidylate synthase

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perturbed by several polymorphisms that reduce the stability and activity of single enzymes [13]. Impaired folate metabolism might promote carcinogenesis by DNA instability or proto-oncogene activation or because of DNA hypomethylation. It is therefore crucial to determine how functional genetic variations modify gene regulation in the two folate cycles and how an imbalanced folate metabolism influences the risk and severity of disease [14,15]. Also benefits and risks of folic acid (over)-supplementations have to be studied and discussed in respect of disease such as an increased risk for cancer [16–18].

Eukaryotes are folate deficient and rely on nutritional uptake [19]. Severe folate deficiency produces embryonic failure in many species. In the genetic model *Caenorhabditis elegans*, complete knock out of the RFC induces hermaphrodite sterility [20]. *C. elegans* is an appropriate model system to study impaired folate metabolism as most enzymes show a high degree of identity to human enzymes (methionine synthase 64%), which is quite unusual for basic enzymes.

Our study aims to unravel regulation and adaptation mechanisms in impaired folate metabolism by the use of the genetic model *C. elegans*. We used siRNA techniques to knock down major genes in folate metabolism mimicking active SNPs. Synthetic folic acid was used for over-supplementation studies on NGM plates and different dilutions of a folic acid/calcium folinate mixture (E. Clegg's medium, http://www.wormbook.org/wli/wbg17.1p33/) was used to study folate deficiency on gene expression, growth and development. Malformation in *C. elegans* offspring was investigated in terms of cell division and DNA repair during embryogenesis by DAPI staining and microscopy.

2 Materials and methods

2.1 Worm strain and cultivation

N2-Bristol wild type strain was obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota Twin Cities, USA). Nematodes were cultivated on nematode growth media (NGM) plates seeded with *E. coli* strain OP50 or in liquid CeHR (*Caenorhabditis elegans* Habitation and Reproduction)-medium, slightly modified from E. Clegg's medium (http://www.wormbook.org/wli/wbg17.1p33/).

2.2 RNAi

For RNAi experiments *E. coli* strain HT115(DE3) was used to produce dsRNA targeting the genes of interest. Plasmids (pL4440_ORFs) were purchased from Thermo Scientific (see Supporting Information). Age synchronized, late L4 staged worms were used for RT-PCR analysis.

2.3 Folate over-supplementation studies

One hundred micromolar synthetic folic acid was dispersed onto NGM plates previously seeded with HT115(DE3) bacteria. Embryos were grown for 48 h at 20°C until late L4 stage. Controls were grown on NGM plates without additional folic acid.

2.4 Feeding studies

One thousand age synchronized L1 worms for each condition were placed into six-well plates containing 3 mL liquid CeHR and either 0, 0.02, 12, 24 or 240 μM of a folic acid/calcium folinate mixture; 0.02 μM is the lowest concentration that enables nematodes to reproduce, 24 μM was applied for controls and 240 μM corresponds to the maximum daily intake in humans. Worms were grown until L4 stage and used for RT-PCR analysis (see Supporting Information).

2.5 RNA extraction, reverse transcription and real-time PCR

For total RNA extraction and reverse transcription, RNeasy MiniKit (Qiagen) and QuantiTect Reverse Transcription Kit (Qiagen) were used. Real-time PCR reactions were performed using the TaqMan assay (Applied Biosystems-Life Technologies) on an Applied Biosystems 7300 Real-time PCR system. mRNA levels were normalized to the level of 3 HKGs and relative to controls as calculated by a ddCt-method (see Supporting Information).

2.6 Statistical analysis

Data were expressed as means of fold change in the specific gene expression as compared to controls. The values shown are representative of duplicate determinations from at least three experiments. Error bars represent SEM. We used unpaired Student's *t*-test to compare each condition (folate concentration, mRNA abundance) with their controls. $p \leq 0.05$ was considered statistically significant ($p <= 0.05^{*}$, $p <= 0.01^{**}$, $p <= 0.001^{***}$).

2.7 Population size, brood size and lifespan

For population studies 100 L1 worms were grown in 1 mL CeHR Medium for 10 days in the presence of 0, 0.01, 0.02, 0.05, 0.24, 2.4, 24 and 240 μM of a folic acid/calcium folinate mixture and for brood size studies, single L1 worms were transferred into 24-well plates containing 1 mL liquid CeHR medium supplemented with 0.02 or 24 μM folate. Nematodes were counted until the end of the egg laying period. In lifespan experiments embryos were grown in liquid CeHR medium

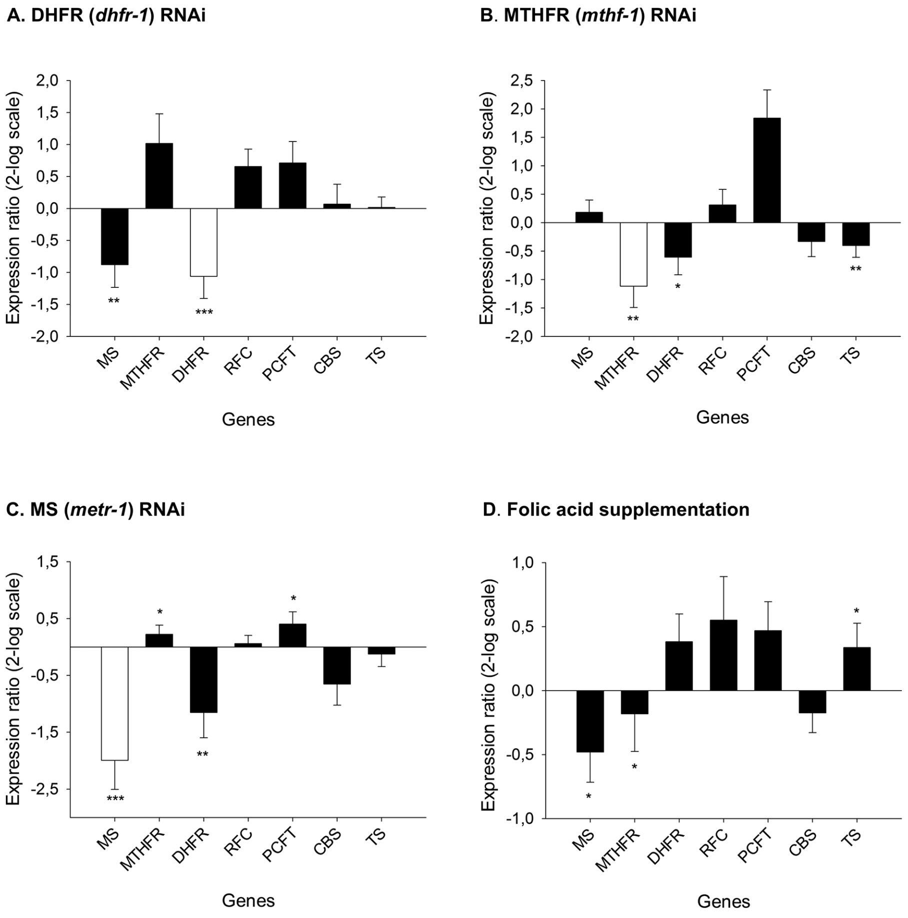


Figure 1. RNAi on NGM plates. Expression-ratio plots of DHFR (A), MTHFR (B), MS (C) and over-supplementation with 100 μM folic acid (D). Abundance of individual genes is calculated over three HKGs and expressed relative to controls (WT), error bars represent means and SEM from fold changes in the specific gene. Bars above represent upregulated, bars below downregulated genes. $p < = 0.05$ was considered statistically significant ($p< = 0.05^{*}$, $p< = 0.01^{**}$, $p< = 0.001$***).

containing either 0.02 or 24 μM folate. The medium was routinely changed every third day.

2.8 DAPI staining of dissected gonads

Dissected gonads (L4 specimen) were fixed in 2% formaldehyde/1× PBS, freeze-cracked and stained with 2 μg/mlg DAPI. 3D stacks (200 nm Z-spacing) were acquired with a CoolsnapHQ2 CCD camera (Photometrics) on a Deltavision deconvolution microscope (SoftWoRx, Applied Precision, Inc).

3 Results

3.1 RNAi alters gene expression in both folate cycles and disrupts homeostasis

Single gene silencing (RNAi) of DHFR, MTHFR or MS altered mRNA expression in both folate cycles. Silencing of *dhfr-1* (DHFR) (Fig. 1A) resulted in severe downregulation of *metr-1* (MS) and led to upregulation of *mthf-1* (MTHFR), *folt-1* (RFC) and *pcft* (PCFT). The conversion through DHFR comprises the first and rate-limiting step in folate conversion. Basically, if DHFR is less active the metabolic rate for the conversion of folic acid into active forms will slow down. Increased mRNA levels of MTHFR due to decreased DHFR expression could reflect a compensation mechanism that promotes the 5-methyl THF pathway. 5-methyl THF is the primary biologically active form of folates that is found in circulation or is transported across membranes. It can enter the methionine synthase cycle independently of folate uptake and conversion through DHFR. Interestingly, silencing of *dhfr-1* led to significant downregulation of MS, which might also decrease the rate of folate overall conversion.

RNAi of MTHFR promoted transporter expression (*pcft* and *folt*-1), but decreased expression levels of *dhfr-1* and *ts* (Fig. 1B). Transcriptional activation of *pcft* increases substrate concentration and thus might enhance enzymatic activity. Knocking down MTHFR leads to adaptive regulation of key enzymes in both cycles. As *metr-1* is slightly increased but *cbs-1* decreased, MTHFR knock down may support accumulation of homocysteine. Because of *ts* downregulation RNAi of MTHFR favors MS cycle over TS cycle.

MS knockdown strongly decreased *dhfr-1* and *cbs-1* mRNA abundances (Fig. 1C), which confirms our observed crosslink between MS and DHFR gene expression. This mechanism might be as important as MTHFR regulation. MS silencing increased *mthf-1*, *folt-1* and *pcft* expression. Increased folate transport and elevated 5-MTHF concentrations might counteract a slower metabolic conversion as a result of DHFR and MS knockdown. MS is also feedback regulated by MTHFR and SAM. Normally SAM activates MTHFR expression when MS levels are low [21, 22]. Therefore, we determined metabolite distribution of Homocysteine, SAM, SAH, Cystathionine in the MS RNAi group over the control by LC-MS (see Supporting Information Fig. 2). Cystathionine, SAH and predominantly SAM levels increased in response to MS silencing, whereas homocysteine levels decreased. Increased levels of SAM with slightly increased levels of SAH in the MS RNAi group reflect lower methylation rates compared to controls which supports our theory that folate metabolism is slowed down in response to lower expression of MS.

3.2 Folic acid over-supplementation inhibits *mthf-1* and *metr-1* expression and favors TS cycle

Folic acid over-supplementation had adverse effects on transcription patterns in both folate cycles. One hundred micromolar synthetic folic acid significantly decreased MTHFR and MS mRNA expression, but increased TS expression (Fig. 1D). Both transporters (PCFT and RCF) and DHFR were stimulated by folic acid over-supplementation, whereas CBS was downregulated. These results show that folic acid over-supplementation to functioning folate metabolism disturbs TS and MS homeostasis by overexpression of TS. Favoring TS cycle might not be beneficial under certain conditions by providing additional building blocks for DNA synthesis.

3.3 mRNA expressions in both folate cycles is adapted to folate concentration: Low and high folate levels promote TS cycle

The exact regulation mechanisms in the two folate cycles under low and high folate concentrations are still poorly understood. We found that expression levels of the folate transporters (PCFT and RFC) were very much dependent on folate concentration. Folate depletion (0 μM) or low folate levels (0.02 μM) strongly increased *pcft* and *folt-1* expression (Fig. 2A and B). High folate feeding (240 μM) promoted RFC activation. Figure 2C shows folate uptake and transport from the intestine to the cell. Both transporters are upregulated and increase substrate concentration under folate deficient conditions. *dhfr-1* and *metr-1* expression was severely reduced under 0 and 0.02 μM folate feeding (Fig. 2D and E), but was very constant under normal to high (12–240 μM) folate concentrations. In contrast, MTHFR, CBS and TS mRNA abundances tend to be high at very low (0, 0.02 μM) folate concentrations (Fig. 2F–H). Overexpression of *mthf-1* and *cbs-1* might be important to balance the two cycles and to control homocysteine levels. Transcription of *ts* was upregulated under folate depletion (0 μM) and under high (240 μM) folate feeding. Taken together, under low (12 μM) and optimal folate (24 μM) levels both cycles are well balanced. Folate depletion (0 μM) or high (240 μM) concentrations favor the TS cycle over the MS cycle.

3.4 Folic acid deficiency reduces brood and population size, but increases lifespan

Folate deficient conditions (0.02 μM) increased lifespan of *C. elegans* as compared to the WT group (Fig. 3A). Increased lifespan seems to be related to an egg-laying defect that was previously observed in liquid CeHR medium [23]. The very low amounts of folate in the medium (skimmed milk) secure survival, but nematodes are not able to reproduce. Population size and brood size were strongly dependent on folate concentration. A folate concentration of more than 0.02 μM was required for nematodes to reproduce and to raise their population size (Fig. 3B and C). We found the highest increase in population growth by feeding nematodes folate concentrations varying from 0.24 to 24 μM folate. Two hundred and forty micromolar did not promote population growth.

3.5 Folate deficiency affects gonad structure

Folate deficiency affects embryonic development in many species and produces nonviable offspring previously shown in *C. briggsae* [24]. In our study, nematodes were able to lay eggs under depletion conditions (0 μM), but hermaphrodites developed a protruding-vulva (Pvl) mutant phenotype. DAPI experiments revealed that folate depletion resulted in smaller and underdeveloped gonads with fewer meiocytes (Fig. 4A). Nuclei in meiotic prophase-diakinesis show DNA fragmentation and strand breakage (Fig. 4B) and DNA aneuploidy was predominant. Embryonic malformation seems to be the result of lacking DNA repair mechanisms and impaired cell division during diakinesis stage of meiosis (Fig. 4C).

4 Discussion

Disturbed folate metabolism is a risk factor for congenital birth defects, congenital heart disease, cleft lip and palate, late pregnancy complications, different kinds of neurodegenerative and psychiatric diseases, osteoporosis and cancer. The complex reactions needed to convert dietary folates to their active forms and the essential function in DNA synthesis and methylation reactions require precise regulation and adaptation mechanisms. A difficulty in the study on diseased

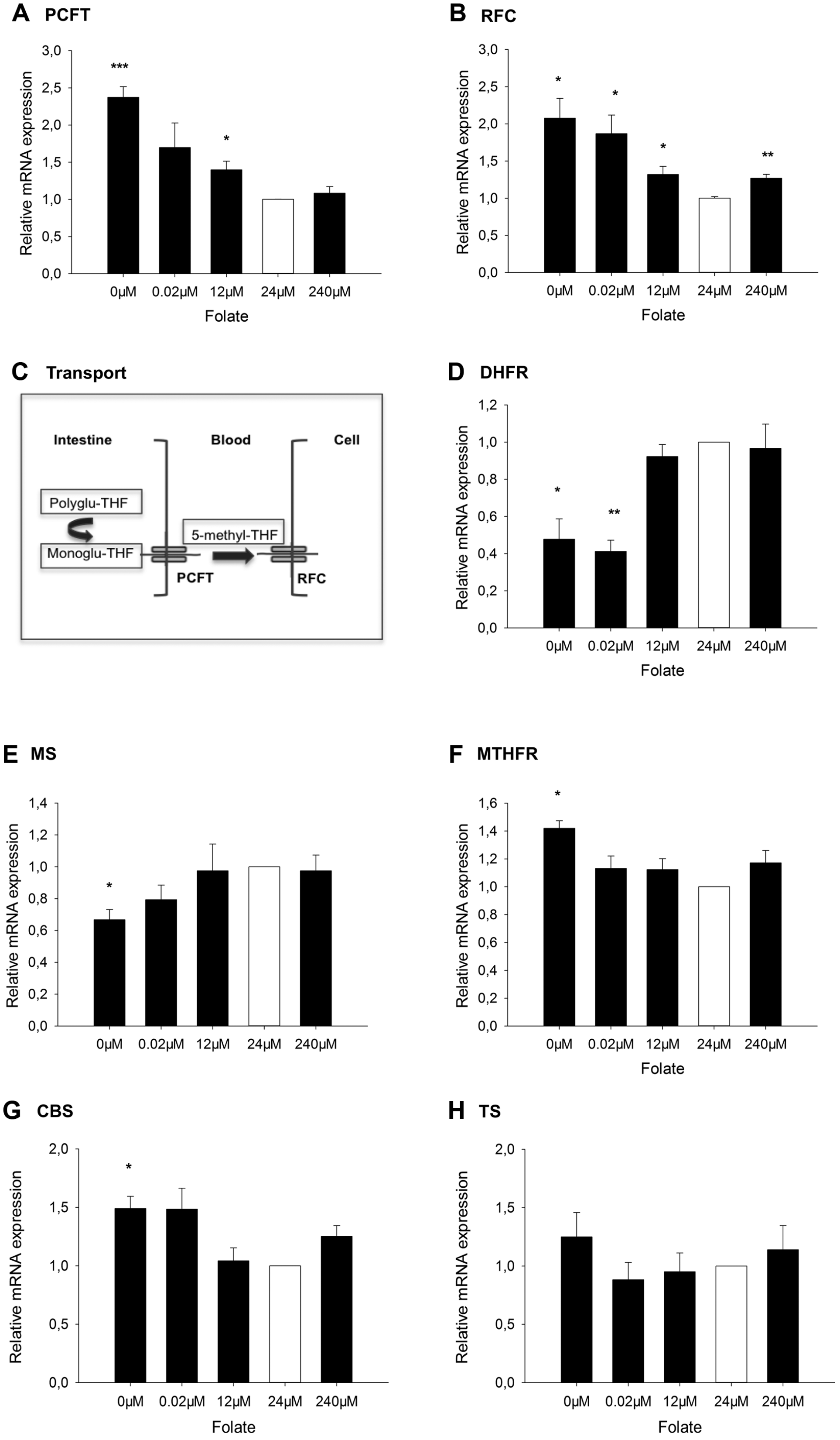
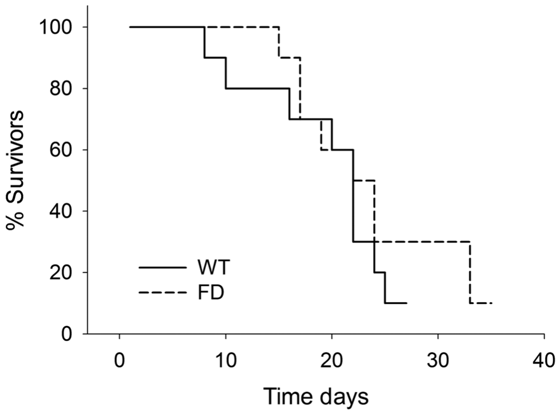
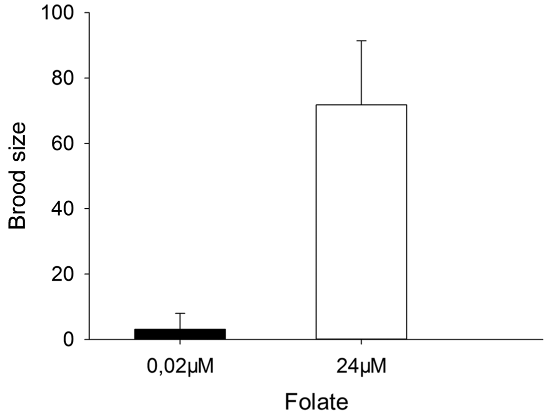


Figure 2. Feeding studies in liquid CeHR medium. 0, 0.02, 12, 24 or 240µM folate (folic acid/calcium folinate) was added to the medium. mRNA levels were expressed relative to controls. PCFT (A), RFC (B) and folate uptake and transport into the cell (C), DHFR (D), MS (E), MTHFR (F), CBS (G), TS (H). All mRNA levels were calculated over three HKGs. Control (24 µM) is set = 1. Error bars represent SEM. $p < = 0.05$ was considered statistically significant ($p< = 0.05^{*}$, $p< = 0.01^{**}$, $p< = 0.001^{***}$).

A Lifespan



B Brood size



C Population size

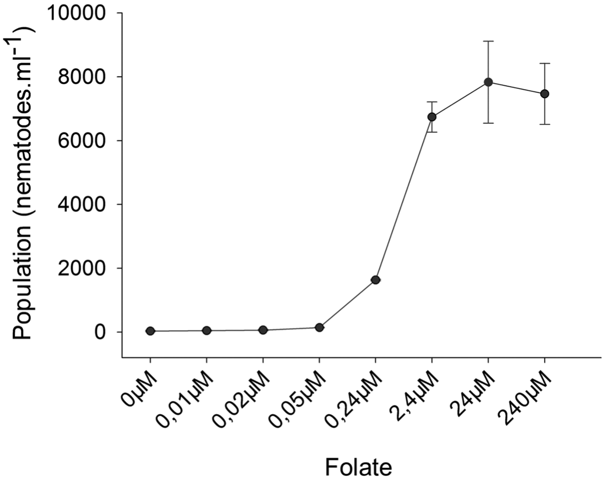


Figure 3. Lifespan under folate depletion conditions (FD = 0 µM folate) compared to WT nematodes (A). B shows brood size under optimal (24 µM) and depletion (0 µM) conditions. Population size in response to given folate concentration is shown in C.

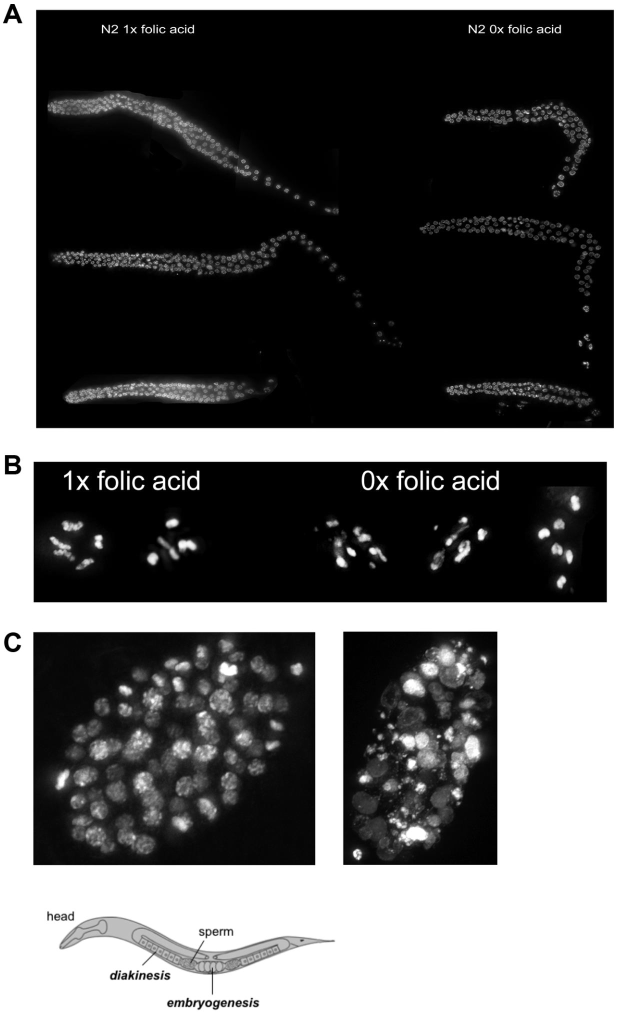


Figure 4. DAPI staining of dissected gonads (A) under control (1× folic acid = 24 µM) conditions (left) and folic acid depletion (0× folic acid = 0 µM) (right). B shows nuclei in meiotic stage of diakinesis. DNA fragmentation under folate depletion shows incomplete DNA repair. Embryos are malformed under folate depletion (0 µM, right) conditions (C).

persons has been the lack of unambiguous biomarkers to prove a connection to genetic variations. Gene regulation and dietary intake strongly effect both folate cycles and causes disease dependent on folate status [25–27].

Here, we modeled impaired folate metabolism by knocking down key enzymes in both folate cycles and by folate deficiency and over-supplementation studies. RNAi of single genes in *C. elegans* showed that both cycles are transcriptionally closely connected. Single RNAi in one cycle resulted in transcriptional regulation of enzymes in the other cycle. We found that DHFR and MS regulation are strongly coupled as silencing of DHFR resulted in downregulation of MS and vice versa. Coupled gene expression of DHFR and MS seems to be an additional mechanism to balance both cycles, particularly important when SNPs are present in the genome. Decreased mRNA expression of both enzymes reduces the metabolic rate of folate overall conversion as DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF). Reduced DHFR expression is associated with a higher risk for neural tube defects (NTD) and with diminished response to cancer medication [28].

Another rate-limiting step that connects TS with MS cycle is the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate by MTHFR. Function of MTHFR is needed for the regulation of available 5-methyl-THF for homocysteine re-methylation at the expense of purines and pyrimidine synthesis. SNPs that decrease MTHFR stability and activity are most widely discussed in the context of human pathologies like cancer and neurological disorders [10,29–33] as it balances TS synthesis and methylation reactions. The functional MTHFR 677TT genotype decreases the availability of 5-methylTHF for methylation reactions [34], reduces global DNA methylation [35, 36] and increases the risk for NTDs [37]. In our experiments MTHFR gene knock down led to decreased DHFR, TS and CBS expression. Reduced activity of DHFR and TS as a result of MTHFR silencing will also slow down DNA synthesis. Increased MS, but decreased CBS expression has shown to be related to hyper-homocysteinemia and abnormal DNA methylation [38].

Our study revealed that all functional SNPs in DHFR, MS, MTHFR might be critical for human metabolism. First because they imbalance both cycles and favor one cycle over the other. Preferring the nucleic acid cycle (TS) at the expense of methylation reactions (MS) facilitates DNA synthesis and rapid cell growth, a condition that is not favorable in carcinogenesis. Second, RNAi induced silencing of a single enzyme was linked to downregulation of other enzymes involving both cycles. This mechanism seems to balance both cycles but might also decrease the metabolic rate for folate overall conversion. Metabolic diseases originate from multifactorial disorders like genetic predisposition, dietary folate intake and illness. Also folic acid over-supplementation might bear a certain risk for humans. Our model organism showed that TS cycle becomes favored over MS cycle. Other side effects of folic acid over-supplementation may also be proliferation and inflammation by providing extra building blocks for DNA and RNA synthesis in rapid cell growth and stress. Yet, over-supplementation to functioning folate metabolism has no demonstrated health benefits in humans.

In our feeding studies, folate deficiency and depletion disrupted folate homeostasis generally at the expense of methylation reactions (MS cycle). Decreased mRNA expression of DHFR significantly decreased MS expression but slightly increased TS expression. Folate restriction transcriptionally increased transporter systems (RFC and PCFT) and MTHFR expression levels. Folate restriction strongly reduced brood and population size in *C. elegans*. Depletion studies showed that hermaphrodite sterility was a result of impaired cell division, DNA strand breakages and a lack of DNA repair during the diakinesis stage of meiosis. Aneuploidy was predominant in embryos and the offspring was nonviable.

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