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Graphical Abstract

Highlights
- ABC transporter MRP-5 transports vitamin B12 from mother to offspring in C. elegans
- mrp-5 mutant embryonic lethality is rescued by vitamin B12 injection into gonad
- RNAi of mrp-5 reduces S-adenosylmethionine and methionine content in F1 embryos

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In Brief
How dietary vitamin B12 is transported from the mother to developing offspring is unknown. Na et al. demonstrate that the ABC transporter MRP-5 (multidrug resistance protein 5) transports vitamin B12 from the intestine of C. elegans mothers to the offspring to promote embryonic viability and development.
C. elegans MRP-5 Exports Vitamin B12 from Mother to Offspring to Support Embryonic Development

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SUMMARY

Vitamin B12 functions as a cofactor for methionine synthase to produce the anabolic methyl donor S-adenosylmethionine (SAM) and for methylmalonyl-CoA mutase to catabolize the short-chain fatty acid propionate. In the nematode Caenorhabditis elegans, maternally supplied vitamin B12 is required for the development of offspring. However, the mechanism for exporting vitamin B12 from the mother to the offspring is not yet known. Here, we use RNAi of more than 200 transporters with a vitamin B12-sensor transgene to identify the ABC transporter MRP-5 as a candidate vitamin B12 exporter. We show that the injection of vitamin B12 into the gonad of mrp-5 deficient mothers rescues embryonic lethality in the offspring. Altogether, our findings identify a maternal mechanism for the transit of an essential vitamin to support the development of the next generation.

INTRODUCTION

Maternal micronutrient status during pregnancy greatly affects embryonic development and fetal health (Dror and Allen, 2008; Fall et al., 2003; Owens and Fall, 2008; Skjærven et al., 2016). For instance, the intake of folate supplements by pregnant women reduces the occurrence of neural tube defects in newborns (Czeizel and Dudas, 1992; Viswanathan et al., 2017). Folate functions together with vitamin B12, or cobalamin, in the one carbon cycle to recycle methionine from homocysteine to support the development of offspring. However, the mechanism by which this occurs from the intestine into the gonad to support the development of her offspring is not yet known. Here, we report that the ABC transporter mrp-5 controls vitamin B12 export from the intestine to support C. elegans embryonic development.

RESULTS

An RNAi Screen Identifies the ABC Transporter MRP-5 as a Candidate to Export Vitamin B12 from the C. elegans Intestine

We have previously established the Pdc-1::GFP transgenic C. elegans strain as a reporter of dietary vitamin B12 status: in this strain, GFP is highly expressed when vitamin B12 is low and repressed when levels of this micronutrient are high (Arda et al., 2010; MacNeil et al., 2013; Watson et al., 2014; Watson et al., 2016). GFP expression in this strain is under the control of the promoter of the acyl-coenzyme A (CoA) dehydrogenase acd-1. This gene encodes the first enzyme of an alternate propionate breakdown pathway, or propionate shunt, which does not require vitamin B12 and is transcriptionally activated when this cofactor is in low supply (Watson et al., 2016). We reasoned that a defect in vitamin B12 transport from the mother to her offspring would result in parental generation (P0) animals with low levels of GFP in the presence of vitamin...
Figure 1. A C. elegans Transporter RNAi Screen Identifies mrp-5 as a Potential Vitamin B12 Exporter

(A) Cartoon of the two vitamin-B12-dependent metabolic pathways. Gene names are from C. elegans. 3HP, 3-hydroxypropionate; 5-meTHF, 5-methyltetrahydrofolate; 5,10-meTHF, 5,10-methylenetetrahydrofolate; D-MM-CoA, D-methylmalonyl-CoA; Gly, glycine; HCy, homocysteine; L-MM-CoA, L-methylmalonyl-CoA; Met, methionine; MSA, malonic semialdehyde; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate. The acdh-1 branch in orange text indicates the vitamin-B12-independent propionate shunt.

(B) Diagram of C. elegans transporter RNAi library screen. RNAi-treated animals that displayed GFP fluorescence in the F1, but not the P0, generation in the presence of 64 nM vitamin B12 were considered hits.

(C) Fluorescence and differential interference contrast (DIC) microscopy images of Pacdh-1::GFP animals subjected to RNAi of mrp-5 in E. coli HT115 compared to vector control at P0 and F1 generations in the presence or absence of 64 nM vitamin B12. Scale bars, 50 μm for embryos and 100 μm for adults.

(legend continued on next page)
B12 and F1 embryos with high levels of GFP expression. Further, these embryos would be expected to have developmental defects, because vitamin B12 is required to produce SAM, which is needed to generate biomass and, thus, support development (Watson et al., 2014). We performed RNAi knockdown of 215 predicted C. elegans transporters of the ABC transporter and solute carrier families by feeding animals E. coli HT115 bacteria expressing double-stranded RNA in the presence of 64 nM vitamin B12 (Figure 1B). We identified a single gene, mrp-5, that, when knocked down, resulted in mothers with low levels of GFP expression and dead embryos with high GFP expression (Figure 1C). mrp-5 encodes an ABC transporter belonging to the multi-drug resistance protein (MRP) family (Korolnek et al., 2014). MRP proteins are highly similar, and the C. elegans genome encodes nine family members (http://www.wormbase.org). To test for specificity, we retested eight of the nine members of this family for which RNAi clones were available and found that only mrp-5 RNAi caused activation of GFP expression in the F1 generation (Figure 1D). We then titrated vitamin B12 from 6.4 nM to 6.4 μM and found that GFP expression remains activated in mrp-5 RNAi embryos even under very high vitamin B12 conditions (Figure S1A).

MRP-5 is expressed in the intestine (Korolnek et al., 2014) (Figure S2). If mrp-5, indeed, encodes a transporter that exports vitamin B12 from the C. elegans intestine to support embryonic development, this would lead to the prediction that higher levels of vitamin B12 should be retained in the intestine when the transporter is perturbed. To test this prediction, we devised a highly sensitive experimental setup by using (1) a single-copy Pacdh-1::GFP::H2B strain that expresses GFP in the nucleus, enabling easier monitoring of differences in GFP levels, and (2) an RNAi-compatible strain of E. coli OP50 (Xiao et al., 2015), because animals fed this diet express higher levels of GFP than those fed E. coli HT115 (Figure S1B). Importantly, RNAI of mrp-5 is equally efficient in E. coli HT115 and E. coli OP50, as measured by qRT-PCR (Figure S1C). We found that much lower concentrations of vitamin B12 were sufficient to repress intestinal GFP in mrp-5 RNAi animals compared to vector control animals (Figures 1E and 1F). This finding suggests that vitamin B12 is retained in the intestine when mrp-5 is knocked down. Together, these findings indicate that mrp-5 encodes a transporter that exports vitamin B12 from the intestine of the mother to support embryonic development of her offspring.

Injection of Vitamin B12 into the Gonad Rescues Embryonic Lethality Caused by Loss of mrp-5

We predicted that direct supplementation of vitamin B12 into the gonad should bypass the requirement for vitamin B12 export from the intestine and rescue embryonic lethality caused by perturbation of mrp-5. To test this prediction, we injected either vitamin B12 or water (vehicle) as a control into the gonad of Pacdh-1::GFP animals that were exposed to mrp-5 or vector control RNAi. If the vitamin B12 injection into the gonad bypassed MRp-5-dependent export, we expected to observe live animals with low GFP expression, while injecting water would lead to dead embryos with high GFP expression (Figure 2A). For this experiment, we used the E. coli OP50 RNAi strains we generated (see also Figure 1E), because transgenic Pacdh-1::GFP embryos express high levels of GFP, while they express low GFP levels when fed E. coli HT115 bacteria (Figure S1). Using E. coli OP50 thus enabled us to observe a decrease in GFP expression, while using E. coli HT115 would not.

Injection of vitamin B12 into the gonad of mrp-5 RNAi animals fully and specifically rescued the embryonic viability of their offspring (Figure 2B). In addition, in Pacdh-1::GFP transgenic animals exposed to mrp-5 RNAi, injecting vitamin B12 into the gonad resulted in developed larvae with low levels of GFP, while injection of water resulted in dead embryos with high GFP expression (Figure 2B). We recapitulated these findings with mrp-5(ok2067) deletion mutant animals. Since mrp-5 is an essential gene, the mrp-5 deletion allele is maintained genetically balanced with +/svT1[ron-2(e678)] (Figure 2C). Heterozygous mrp-5 (+/−) mothers injected with vitamin B12 into their gonad produced mrp-5 (−/−) offspring that survived to the adult stage, while animals injected with vehicle control produced mrp-5 (−/−) animals that arrested at the L1–L2 stage (Figure 2D). Importantly, the offspring of the rescued F1 mrp-5 (−/−) animals again exhibited larval arrest, suggesting that, in the absence of mrp-5, vitamin B12 cannot be passed on to the next (F2) generation. Altogether, these findings show that vitamin B12 injected into the gonad of a mrp-5 (+/−) mother can rescue embryonic lethality in her offspring.

Heme Rescues Embryonic Lethality in mrp-5 RNAi Animals

A previous study proposed that mrp-5 encodes a heme transporter (Korolnek et al., 2014). This finding was supported by the observation that embryonic lethality in mrp-5 RNAi animals could be rescued by feeding the animals high doses of heme (Korolnek et al., 2014). How could either feeding of heme or injection of vitamin B12 rescue embryonic lethality of mrp-5 RNAi or mutant animals? ABC transporters are known to be capable of transporting numerous similar types of molecules (Lee and Rosenbaum, 2017; Locher, 2016; Slot et al., 2011). Heme and vitamin B12 share some structural similarities; heme is an iron-containing porphyrin, while vitamin B12 is related to porphyrins and contains cobalt as a metal ion (Figure 3A). Thus, it is possible that MRP-5 transports both molecules. However, if lack of heme and vitamin B12 explains the embryonic lethality of mrp-5 RNAi
animals, one would expect that neither cofactor alone would be sufficient for rescue. Our observation that embryonic lethality can be rescued by injection of vitamin B12 into the gonad indicates that lack of vitamin B12 rather than heme may be the cause of embryonic lethality when MRP-5 function is perturbed. To further examine the effects of heme on animal development, we repeated the experiment published in the previous study (Korolnek et al., 2014) and confirmed that feeding 500 μM heme can rescue embryonic lethality in the offspring of mrp-5 RNAi animals (Figure 3B). Interestingly, however, such a high dose of heme was toxic to developing control animals, arresting most animals at the L1–L2 stage, whereas high doses of vitamin B12 were not toxic (Figure S3). Importantly, one can argue that feeding heme does not actually test for transport, because the
of vitamin-B12-responsive Pacdh-1 intestinal membranes. Therefore, we next asked whether heme feeding heme is that this increases vitamin B12 passage through development in the offspring.

One explanation for the rescue of embryonic development by feeding heme is that this increases vitamin B12 passage through intestinal membranes. Therefore, we next asked whether heme affects the vitamin-B12-responsive Pacdh-1 reporter. We supplemented increasing concentrations of heme and found that increasing concentrations of heme expressed high levels of GFP, indicating low vitamin B12 levels (Figure 3E).

**Methionine and SAM Content Is Reduced in Offspring from mrp-5 RNAi Mothers**

Although vitamin B12 is a cofactor for two different metabolic enzymes (Figure 1A), our previous studies demonstrated that the developmental delay caused by vitamin B12 deficiency is mainly due to its function as a cofactor for the methionine synthase enzyme METR-1, which generates the methyl donor SAM from methionine (Watson et al., 2014). To determine whether the lack of vitamin B12 or the lack of heme in mrp-5-deficient animals results in defects in the SAM cycle, we measured the levels of methionine and SAM in embryos from mothers treated with mrp-5 or vector control RNAi. We reasoned that differences in methionine and SAM content would be most clear in the presence of supplemented vitamin B12, because the E. coli diet is naturally low in this cofactor. Indeed, we detected a reduced methionine and SAM content in embryos from mothers treated with mrp-5 RNAi relative to control animals in the presence of vitamin B12 (Figures 4A and 4B). Further, SAM levels could be restored to wild-type levels by feeding the animals with 500 μM heme. These results suggest that a defect in the SAM...
Loss or reduction of vitamin B12 and heme depends on dietary supplementation of both cofactors. MRP-5 has previously been reported to export heme in C. elegans (Korolnek et al., 2004; Slot et al., 2011). MRP-5 has previously been known to function as exporters of metabolites and drugs (Locher, 2016; Sheps et al., 2004; Slot et al., 2011) and has been identified as a candidate transporter that exports methionine and S-adenosylmethionine from the intestine to surrounding tissues. Wild-type C. elegans can synthesize neither vitamin B12 nor heme by RNAi knockdown or by genetic mutation (Arda et al., 2010). Strain VL1168 (+ unc-119(+)) strain has been described previously (Brenner, 1974), and strain N2 was used as wild-type. MRP-5 Deficient + 500µM Heme (20 µM) (Rao et al., 2005), and high doses of heme are toxic to many systems (Chiabrando et al., 2014). Indeed, supplementing wild-type animals with a dose of 500 µM heme was toxic to C. elegans as well, whereas high doses of vitamin B12 were not (Figures 3B and S3). One mechanism of heme toxicity is by enhanced membrane permeability (Chiabrando et al., 2014). Therefore, we propose that supplementing mrp-5 RNAi animals with high concentrations of heme leads to increased intestinal membrane permeability, enabling both heme and vitamin B12 to pass through without the need for a dedicated transporter (Figure 4C).

More than 15 human genes involved in vitamin B12 processing have been identified (Nielsen et al., 2012), and four of these encode transporters. One of these is the ABC transporter MRP-1 (or ABCC1), which is localized to intestinal epithelial cells. ABC transporters in human and C. elegans are highly similar in sequence and structure. Therefore, we propose that C. elegans MRP-5 may be a functional ortholog of human MRP1 to export vitamin B12 from the intestine to surrounding tissues.

**EXPERIMENTAL PROCEDURES**

**C. elegans Strains**

All C. elegans strains were maintained at standard laboratory conditions as described previously (Brenner, 1974), and strain N2 was used as wild-type. Construction of VL749 (wwls24[Pacdh-1::GFP + unc-119(+)]) strain has been previously described (Verda et al., 2010). Strain VL1168 (wwStI[Pacdh-1::GFP::H2B unc-119(+)] II;avr-14(ad1302) I; unc-119(ed3) III; avr-15(ad1051);
**RNAi Screen**

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to predict C. elegans ABC transporters and solute carrier transporters (Kanehisa et al., 2016). The transporter RNAi mini-library was generated by selecting 215 clones from both the ORFeome and Ahringer RNAi libraries (Karnath et al., 2003; Rual et al., 2004). RNAi screening was carried out on 24-well nematode growth medium (NGM) agar plates containing 2 mM isopropyl-1-thiogalactopyranoside (IPTG), 50 μg/ml ampicillin with or without 64 nM Ado-Cbl (vitamin B12). Pacdrh-1::GFP animals were grown on regular NGM plates with E. coli OP50 without Ado-Cbl supplementation for several generations before harvesting embryos. Embryos were incubated in M9 buffer overnight to obtain synchronized L1 animals. Approximately 15 L1 animals were added to each well containing individual RNAi clones. Animal phenotypes were observed after 80 hr of incubation, when the F1 generation was at the young adult stage.

**Quantitation of Fluorescence**

The animal body was outlined, and fluorescence intensity of the animal was determined using ImageJ (NIH). At least five animals per condition were analyzed, and the mean fluorescence per animals was determined.

**C. elegans qRT-PCR Experiments**

Animals were synchronized by L1 arrest and grown on ampicillin and IPTG plates seeded with either E. coli HT115 (vector RNAi and mpr-5 RNAi) or E. coli OP50 (vector RNAi and mpr-5 RNAi). Approximately 1,000 adult animals were harvested for each condition, in biological duplicate. Animals were washed in M9 buffer, and total RNA was isolated using TRIzol (Invitrogen) followed by DNase I treatment and cleanup using QIAGEN RNeasy columns. cDNA was generated from 1 μg RNA using random primer and M-MuLV reverse transcriptase (New England Biolabs). qPCR was performed in technical duplicates per gene per condition using the Applied Biosystems StepOnePlus Real-Time PCR System and the Fast SYBR Green Master Mix (Thermo Fisher Scientific). Relative transcript abundance was determined using the ∆ΔCt method and normalized to averaged ama-1 mRNA expression levels.

**Vitamin B12 Injections**

Animals were treated with mpr-5 RNAi generated in an E. coli OP50 RNAi compatible strain. Vitamin B12 (3.2 mM) was injected into the gonad of L4 animals using a Narishige microinjection arm attached to the body of a Nikon Eclipse Ti inverted microscope. Sterile water was injected into the control group. After injection, animals were singled and treated with the same RNAi (mpr-5 or vector). Phenotypes were scored after 2 days. mpr-5(ok2067) heterozygotes were singled at the L4 stage. Vitamin B12 was injected as described earlier. After injection, animals were singled, and the phenotype was scored after 5 days.

**SAM Measurement**

SAM was measured using the Bridge-It S-Adenosyl Methionine Fluorescence Assay Kit purchased from Medionics following the manufacturer’s recommendations.

**Heme Solution Preparation**

Hemin is the oxidized form of heme and must be dissolved in acid to make the active form of heme. 10 mM hemin solution was prepared as described previously (Hickman and Winston, 2007). 6.5 mg/ml hemin (Sigma-Aldrich) was dissolved in 0.1 M NaOH and incubated at 37°C for 1 hr. 1 M Tris (pH 7.5) was then added to a final concentration of 0.1 M. The pH was adjusted using HCl, and the hemin solution was stored at 4°C, protected from light, and used within 2 days.

**Relative Quantification of Methionine Using Gas Chromatography-Mass Spectrometry**

Approximately 150,000 embryos were homogenized with 0.5 mL 200- to 300-μm acid-washed glass beads (Sigma-Aldrich) in 1 mL 80% methanol using a FastPrep-24 bead beater (MP Biomedicals), with intermittent cooling in dry ice/ethanol bath. Samples were then extracted on dry ice for 15 min and centrifuged for 10 min at 20,000 g, and 250 μL supernatant was dried under vacuum using a SpeedVac concentrator SPD111V (Thermo Fisher Scientific). Derivatization of dried samples was performed first with 20 μL 20 mg/mL methoxyamine hydrochloride (Sigma-Aldrich) in pyridine at 37°C for 90 min, followed by the addition of 50 μL N-methyl-N-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) and incubation for 3 hr at 37°C. The derivatization reaction was completed by incubation for 5 hr at room temperature. Measurements were performed on an Agilent 7890B/5977B single quadrupole GC-MS (gas chromatography-mass spectrometry) equipped with an HP-5ms Ultra Inert capillary column (30 m × 0.25 mm × 0.25 μm). The inlet temperature was set to 230°C, the transfer line was at 280°C, and the MS source and quadrupole were at 230°C and 150°C, respectively. The trimethylsilyl derivative of methionine was quantified as a 176-m/z ion with two qualifier ions of 128 and 293 m/z. Relative quantification of peak areas was done using samples within a linear response range, after mean normalization to total metabolites and blank subtraction.

**SUPPLEMENTAL INFORMATION**

Supplemental information includes Supplemental Experimental Procedures and three figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.100.

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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