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**Simmonds**(10) **Pub. No.: US 2016/0053281 A1**(43) **Pub. Date: Feb. 25, 2016**(54) **ENHANCED EXPRESSION OF RNA  
VECTORS****Publication Classification**(71) Applicant: **BOARD OF REGENTS, THE  
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**C12N 15/86** (2006.01)(72) Inventor: **Peter Simmonds**, Edinburgh (GB)(52) **U.S. Cl.**  
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*2760/00051* (2013.01); *C12N 2770/00051*  
(2013.01)(73) Assignee: **The University Court Of The  
University Of Edinburgh**, Edinburgh  
Midlothian (GB)(21) Appl. No.: **14/779,069**(57) **ABSTRACT**(22) PCT Filed: **Mar. 24, 2014**(86) PCT No.: **PCT/US2014/050917**

§ 371 (c)(1),

(2) Date: **Sep. 22, 2015****Related U.S. Application Data**(60) Provisional application No. 61/866,331, filed on Aug.  
15, 2013.

Compositions and method for treating a folate-resistant disease in a subject are disclosed. The methods involve administering to the subject an effective amount of a composition containing a formate. For example, the method can be used to reducing the risk of neural tube defects during pregnancy. The method can also be used to treat other conditions normally treatable by folate supplementation.

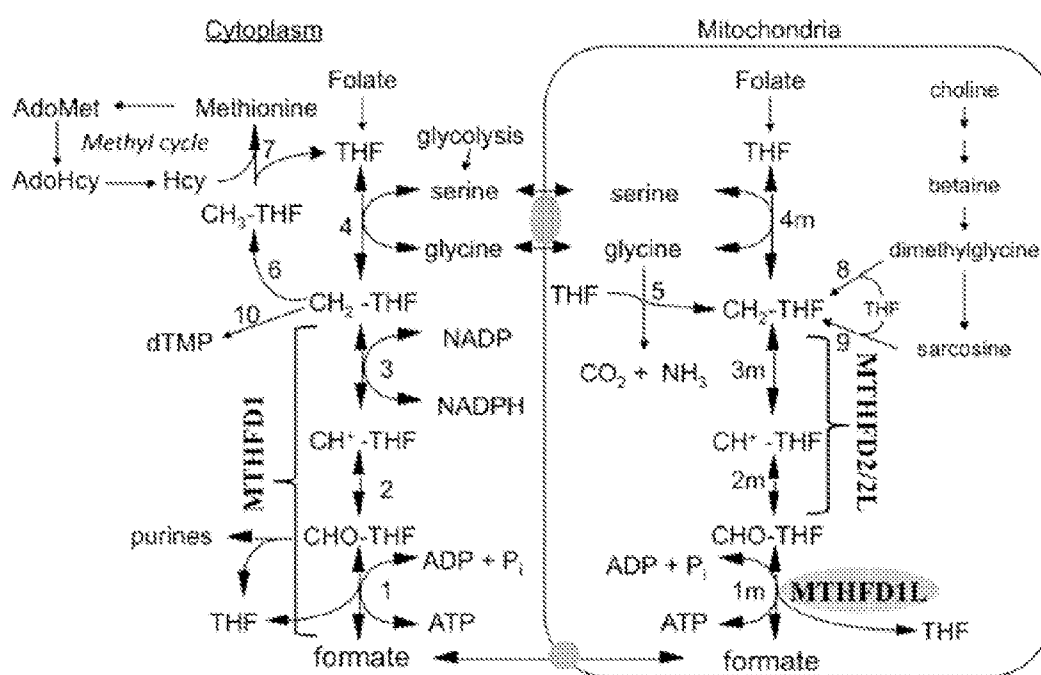
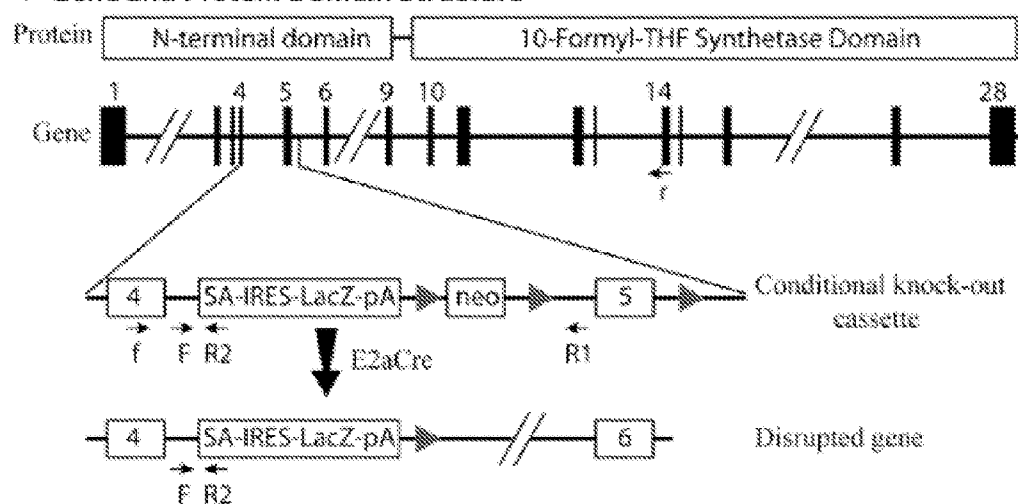


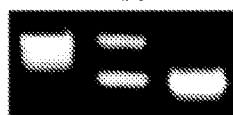
FIGURE 1

# **A Gene and Protein Domain Structure**



## **B**

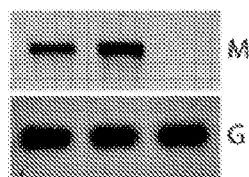
### **Genotype**



+/+ z/+ z/z

## **C**

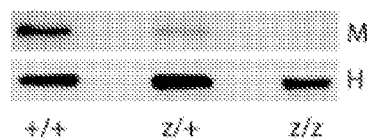
### **mRNA (RT-PCR)**



+/+ z/+ z/z

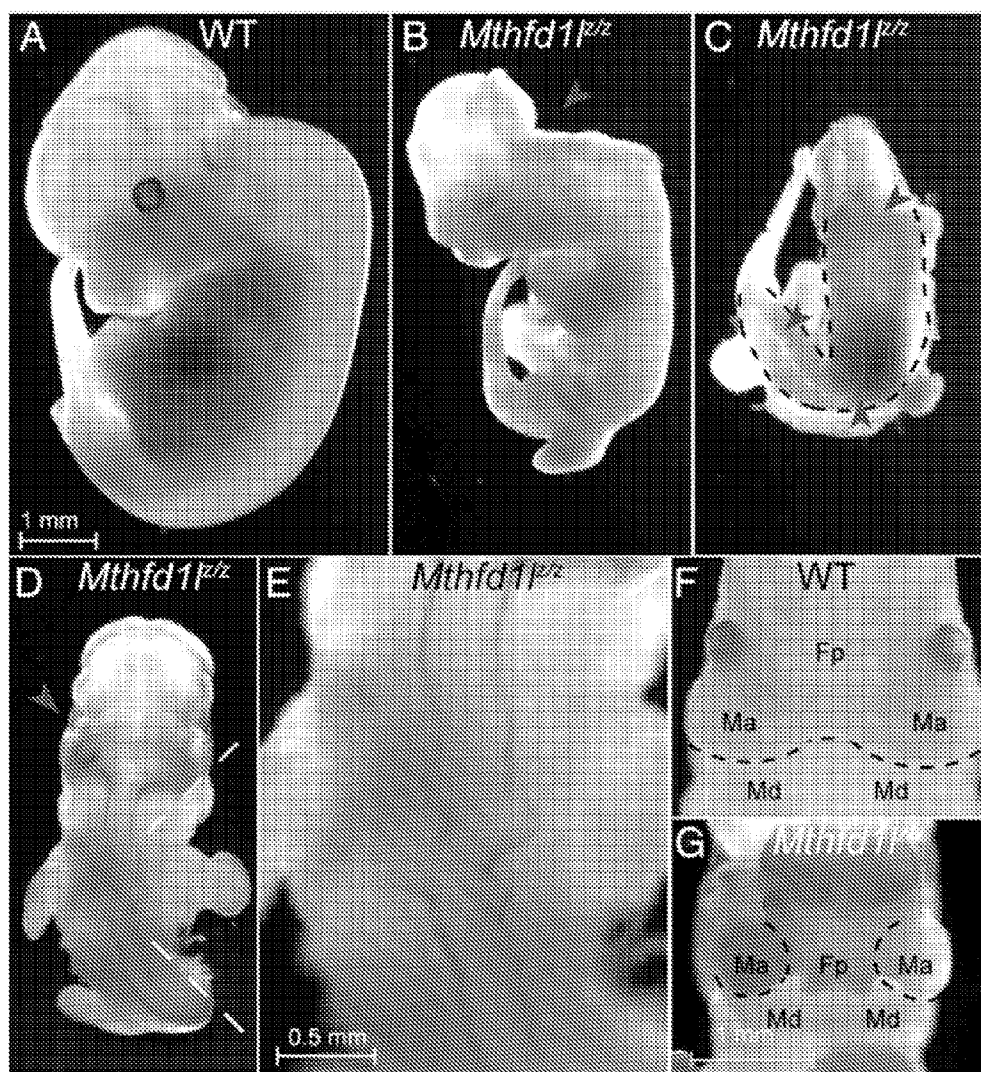
## **D**

### **Protein (Immunoblot)**

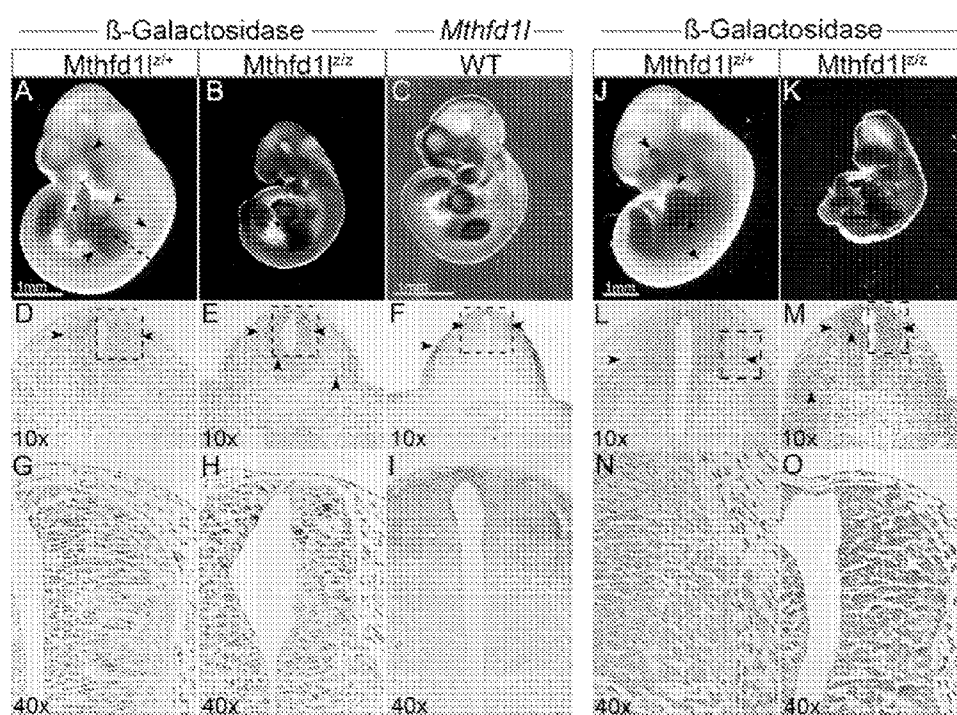


+/+ z/+ z/z

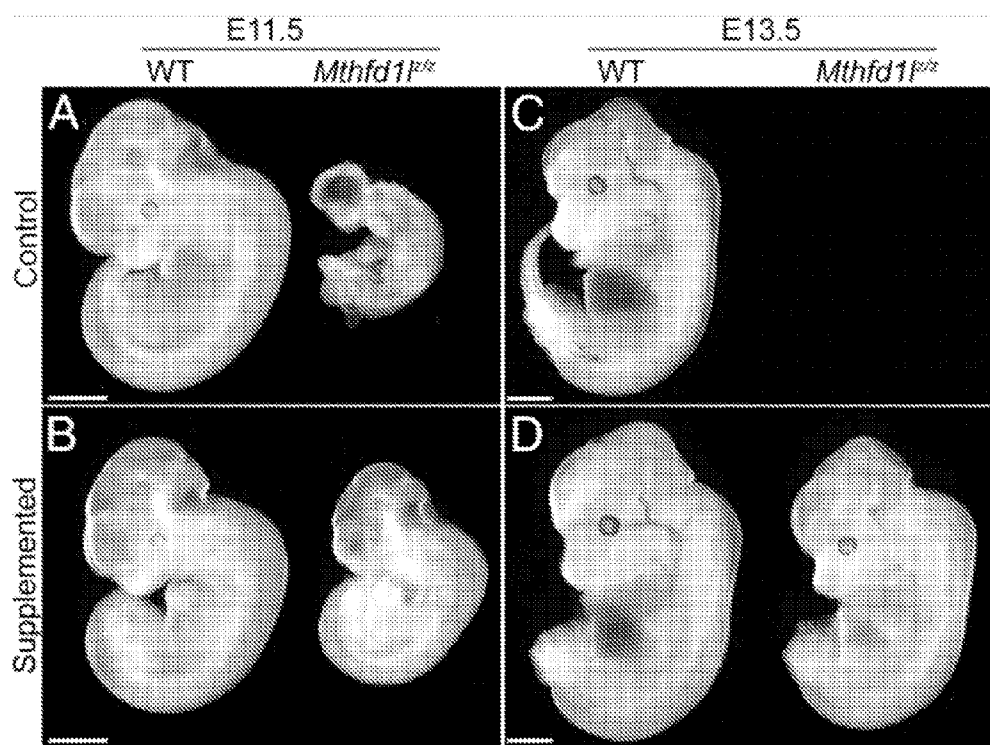
**FIGURES 2A-2D**



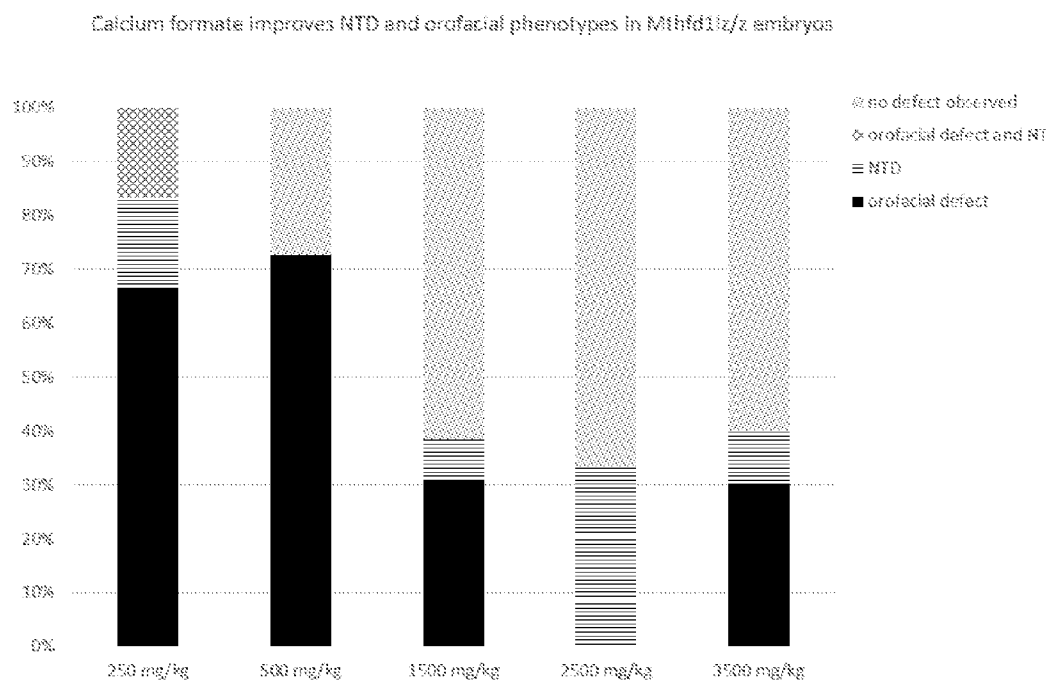
**FIGURES 3A-3G**



**FIGURE 4A-4O**



**FIGURES 5A-5D**

**FIGURES 6**

## ENHANCED EXPRESSION OF RNA VECTORS

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 61/866,331, filed Aug. 15, 2013, which is hereby incorporated herein by reference in its entirety.

### STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with Government Support under Grant Nos. GM086856 and HD074428 awarded by the National Institutes of Health. The Government has certain rights in the invention.

### BACKGROUND

[0003] Closure of the neural tube during development is a highly complex but poorly understood process. Not surprisingly, neural tube defects (NTDs) have a multifactorial etiology, including both genetic and environmental factors. The importance of maternal folate status to NTD risk was first suggested more than forty years ago (Hibbard E D & Smithells R W (1965) *Lancet* 1:1254). Many human studies show that periconceptional intake of supplemental folic acid can reduce the incidence of NTDs by as much as 70% in some populations (Ross M E (2010) *Wiley Interdiscip Rev Syst Biol Med* 2:471-480). These results led to mandated fortification of all enriched cereal grain products with folic acid in the U.S. beginning in 1996 to ensure that women of child-bearing age would consume adequate quantities of the vitamin. While folic acid fortification has decreased NTD incidence in some subpopulations, fortification has not completely eliminated NTDs (Hobbs C A, et al. (2010) *Folate in Health and Disease*, ed Bailey L B (CRC Press, Taylor & Francis Group, Boca Raton, Fla.), 2nd Ed, pp 133-153). Despite the strong clinical link between folate and NTDs, the biochemical mechanisms through which folic acid acts during neural tube development remain undefined.

### SUMMARY

[0004] A method for treating a folate-resistant disease in a subject is disclosed that involves administering to the subject an effective amount of a composition containing a formate. For example, the method can be used to reduce the risk of neural tube defects during pregnancy. The method can also be used to treat other conditions normally treatable by folate supplementation. For example, the method can be used to treat hyperhomocysteinemia. In some cases, the subject being treated has a family history of neural tube defects. In some embodiments, the subject has been diagnosed with a folate deficiency, e.g., by a blood test. In some embodiments, the subject has been diagnosed with high plasma homocysteine, e.g., by a blood test. In some embodiments, the method involves administering to the subject an effective amount of a first composition containing a formate and a second composition containing a folate. In other embodiments, the method involves administering to the subject an effective amount of a composition containing both a formate and a folate. Therefore, also disclosed is a composition containing a formate, a folate, and a pharmaceutically or neutraceutically acceptable carrier. Also disclosed is a composition containing formate in a unit dose for prenatal supplementation.

[0005] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

### DESCRIPTION OF DRAWINGS

[0006] FIG. 1 is a schematic showing mammalian one-carbon metabolism. Reactions 1-4 are in both the cytoplasmic and mitochondrial (m) compartments. Reactions 1, 2, and 3, 10-formyl-THF synthetase, 5,10-methenyl-THF cyclohydrolase, and 5,10-methylene-THF dehydrogenase, respectively, are catalyzed by trifunctional C<sub>1</sub>-THF synthase (MTHFD1) in the cytoplasm. In mammalian mitochondria, reaction 1m is catalyzed by monofunctional MTHFD1L and reactions 2m and 3m by bifunctional MTHFD2 or MTHFD2L. The other reactions are catalyzed by the following: 4 and 4 m, serine hydroxymethyltransferase (SHMT); 5, glycine cleavage system (GCS); 6,5,10-methylene-THF reductase; 7, methionine synthase; 8, dimethylglycine dehydrogenase; 9, sarcosine dehydrogenase; 10, thymidylate synthase. All reactions from choline to sarcosine are mitochondrial except the betaine to dimethylglycine conversion, which is cytoplasmic. Hcy, homocysteine; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine.

[0007] FIGS. 2A to 2D demonstrate that *Mthfd11*<sup>+/z</sup> embryos do not produce detectable protein or full length transcripts. FIG. 2A is an illustration of the *Mthfd11* gene, comprising 28 exons, and encoding a protein with two domains. The catalytic domain begins in exon 10. Mice in which exon 5 was initially flanked by LoxP sites were crossed with the E2a-Cre line, producing offspring with complete recombination at the LoxP sites (grey arrowheads) and the resulting deletion of exon 5 and the neomycin phosphotransferase gene (Neo). Transcription from the endogenous *Mthfd11* promoter produces a transcript containing exons 1-4 of *Mthfd11* fused to an internal ribosome entry site (IRES) and the  $\beta$ -galactosidase gene (LacZ) followed by a polyadenylation site (pA). SA, splice acceptor site. FIG. 2B shows mouse genotyping by allele specific amplification. Mouse genomic DNA was subjected to allele specific amplification using a mixture containing F, R1 and R2 primers. A genomic fragment of 444 bp was amplified from the wild-type allele and a 324 bp fragment was amplified from the *Mthfd11*<sup>Δ</sup> allele. FIG. 2C shows RT-PCR analysis of mRNA expressed in whole E11.5 embryos using primers f in exon 4 and r in exon 14, yielding a 1087 bp product. M, *Mthfd11*L; G, glyceraldehyde-3-phosphate dehydrogenase. FIG. 2D is an immunoblot of mitochondria isolated from whole E11.5 embryos. Each lane was loaded with 7  $\mu$ g of total mitochondrial protein. Blots were probed with antibodies against MTHFD1L (M, 100 KDa) or the mitochondrial matrix marker, Hsp60 (H, 60 KDa).

[0008] FIGS. 3A to 3G are images showing that *Mthfd11*<sup>+/z</sup> embryos have neural tube and facial defects. Compared to wild-type E12.5 embryos (FIG. 3A), E12.5 *Mthfd11*<sup>+/z</sup> embryos (FIGS. 3B-3E) exhibit a spectrum of neural tube defects including exencephaly (FIG. 3B, arrowhead). FIG. 3C shows an embryo with completely open neural folds (craniorachischisis), indicated by the dashed lines. Note that the embryo curves to the left so the entire open neural tube is visible (arrowheads). FIG. 3D shows an embryo with exencephaly (arrowhead) and a wavy neural tube (magnified in FIG. 3E). FIG. 3F shows wild-type, 51 somite embryo show-



ing normal facial development. FIG. 3G shows the same *Mthfd11<sup>z/z</sup>* embryo (51 somites) imaged in 3D and 3E displaying facial defects. The maxillary processes (Ma) are globular, and are broadly separated from the midline (indicated by the frontonasal prominence, Fp). The mandibular processes (Md) in the *Mthfd11<sup>z/z</sup>* embryo appears undergrown. Embryos in FIGS. 3D, 3E, and 3G are stained for  $\beta$ -galactosidase activity. Embryos in FIGS. 3A, 3B, 3C and 3D are all imaged at the same magnification (1.25 $\times$ ). Embryos in FIGS. 3F and 3G are at the same magnification (2 $\times$ ). The embryo in 3E is at 3.2 $\times$  magnification.

[0009] FIGS. 4A-4O are images showing aberrant neural tube morphology in *Mthfd11<sup>z/z</sup>* embryos. FIGS. 4A and 4J show that *Mthfd11<sup>z/+</sup>* embryos at approximately E10.5 and E11.5, respectively, exhibit regionalized  $\beta$ -galactosidase staining that is highest in the eyes, heart, limb and dorsal midline region. Dashed line in FIG. 4A indicates the level of all sections below, at 10 $\times$  and 40 $\times$  magnification. FIGS. 4B and 4K show that *Mthfd11<sup>z/z</sup>* littermates of embryos in 4A and 4J, respectively, exhibit  $\beta$ -galactosidase staining that appears superficially ubiquitous. FIGS. 4D, 4G, 4H, and 4N show that sections through *Mthfd11<sup>z/+</sup>* embryos have  $\beta$ -galactosidase activity in the basal area of the dorsal neuroepithelium while *Mthfd11<sup>z/z</sup>* embryos (FIGS. 4E, 4F, 4M, 4O) have a smaller neural tube, kinks in the lumen, and a broader dorsal lumen. FIGS. 4C, 4F, and 4I show *Mthfd11* gene expression visualized by in situ hybridization in a wholemount embryo (FIG. 4C) and a section through the same embryo (FIGS. 4F and 4I). Precise age of embryos: 4A=37 somites; 4B=34 somites; 4C=33 somites; 4J=44 somites; 4K=42 somites. The regions outlined by dashed boxes are magnified in the panels immediately below them. Embryos in FIGS. 4A, 4B, and 4C are imaged at 1.6 $\times$ , and embryos 4J and 4K are imaged at 1.25 $\times$ . Black arrowheads highlight areas of  $\beta$ -galactosidase staining or in situ hybridization.

[0010] FIGS. 5A-5D show that maternal supplementation with sodium formate improves development and growth in *Mthfd11<sup>z/z</sup>* embryos. Pregnant dams were administered a calculated dose of 7500 mg sodium formate/kg/day. *Mthfd11<sup>z/z</sup>* embryos dissected at E11.5 (FIG. 5B) show improved growth compared to control embryos from unsupplemented dams (FIG. 5A). E13.5 embryos from unsupplemented (FIG. 5C) and supplemented (FIG. 5D) dams. Precise age of embryos: A=47 somites; B=36 somites; C=45 somites; D=41 somites. Scale bars correspond to 1.0 mm.

[0011] FIG. 6 is a bar graph showing calcium formate improves NTD and orofacial phenotypes in *Mthfd11<sup>z/z</sup>* embryos.

#### DETAILED DESCRIPTION

[0012] Disclosed are compositions and method based on the discovery that formate supplementation can protect against folate-resistant neural tube defects. Therefore, formate may be used to supplement or replace folic acid in subjects for whom folic acid treatment has proved insufficient or ineffective.

[0013] The term “folate-resistant” refers to a disease, such as a neural tube defect, that results from deficient or subop-

timal folate one-carbon metabolism. Folic acid supplementation is not sufficient to treat or prevent these diseases.

[0014] The term “one-carbon metabolism” refers to the body’s use of tetrahydrofolate (THF) as a ‘carrier’ of one-carbon units (i.e., CO<sub>2</sub>, HCOOH, HCHO, H<sub>3</sub>COH, CH<sub>4</sub>) that are created by and needed for many crucial reactions that take place in the body. When folate is ingested, it is first converted to dihydrofolate, and then converted to THF. THF-mediated one-carbon metabolism is a metabolic network of interdependent biosynthetic pathways that is compartmentalized in the cytoplasm, mitochondria, and nucleus. One-carbon metabolism in the cytoplasm is required for the synthesis of purines and thymidylate and the remethylation of homocysteine to methionine. One-carbon metabolism in the mitochondria is required for the synthesis of formylated methionyl-tRNA; the catabolism of choline, purines, and histidine; and the interconversion of serine and glycine. Mitochondria are also the primary source of one-carbon units for cytoplasmic metabolism.

[0015] The term “folate deficiency” refers to inadequate levels of folate or one or more of its metabolites during one-carbon metabolism. Thus, this term refers to both a dietary deficiency and a metabolic deficiency.

[0016] The term “subject” refers to any individual who is the target of administration or treatment. The subject can be a vertebrate, for example, a mammal. Thus, the subject can be a human or veterinary patient. The term “patient” refers to a subject under the treatment of a clinician, e.g., physician.

[0017] The term “therapeutically effective” refers to the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

[0018] The term “treatment” refers to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

[0019] The term “prevent” refers to a treatment that forestalls or slows the onset of a disease or condition or reduced the severity of the disease or condition. Thus, if a treatment can treat a disease in a subject having symptoms of the disease, it can also prevent that disease in a subject who has yet to suffer some or all of the symptoms.

**[0020]** In some embodiments, the disclosed compositions and methods can be used to treat any disease characterized by folate deficiency, particularly those that are folate-resistant. Folate deficiency can occur when the body's need for folate is increased, when dietary intake of folate is inadequate, when the body excretes (or loses) more folate than usual, or when the body has deficient or suboptimal folate one-carbon metabolism. Patients with folate deficiencies can have formamino glutamate in their urine. This is an intermediate in the conversion of histadine to glutamate, which is THF dependent.

**[0021]** Situations that increase the need for folate include certain anemias, kidney dialysis, liver disease, malabsorption, including celiac disease, pregnancy and lactation (breastfeeding), tobacco smoking, and alcohol consumption. Medications that can interfere with folate utilization include anticonvulsant medications (such as phenytoin, and primidone), metformin (sometimes prescribed to control blood sugar in type 2 diabetes), methotrexate, an anti-cancer drug also used to control inflammation associated with Crohn's disease, ulcerative colitis and rheumatoid arthritis, sulfasalazine (used to control inflammation associated with Crohn's disease, ulcerative colitis and rheumatoid arthritis), triamterene (a diuretic), and oral contraceptives.

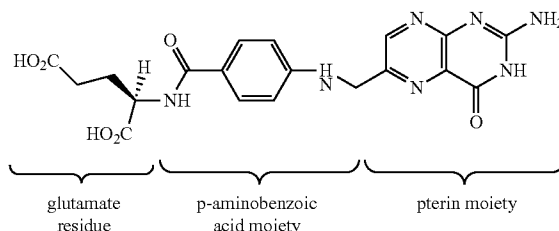
**[0022]** The disclosed methods can therefore be used to treat any folate deficiency condition treatable by folate supplementation. The disclosed methods can also be used to treat subject who has a limited ability to absorb or metabolize folic acid. Folate deficiencies (along with deficiencies in vitamin B6 and vitamin B 12) can lead to many medical conditions, including glossitis (chronic inflammation of the tongue), diarrhea, depression, confusion, anemia, fetal neural tube defects and brain defects (during pregnancy), cardiovascular disease, and hyperhomocysteinemia. Hyperhomocysteinemia is characterized by abnormally high levels of homocysteine in the blood (e.g., above 15  $\mu\text{mol/L}$ ). Hyperhomocysteinemia is typically managed with vitamin B6, folic acid, and vitamin B12 supplementation. Therefore, in some embodiments, the disclosed compositions comprising formate may be used to treat a subject with hyperhomocysteinemia.

**[0023]** The formate of the disclosed compositions and methods can be formic acid (methanoic acid) or a pharmaceutically acceptable salt or ester thereof. Pharmaceutically acceptable salts are known in the art, and can be prepared using standard methods. See, for example, Remington's Pharmaceutical Sciences, 20th ed., Lippincott Williams & Wilkins, Baltimore, Md., 2000, p. 704; and "Handbook of Pharmaceutical Salts: Properties, Selection, and Use," P. Heinrich Stahl and Camille G. Wermuth, Eds., Wiley-VCH, Weinheim, 2002. Pharmaceutically acceptable salt can include alkali metal salts, including sodium or potassium salts; alkaline earth metal salts, e.g., calcium or magnesium salts; and salts formed with suitable organic ligands, e.g., quaternary ammonium salts. Examples of suitable formate salts include calcium formate, sodium formate, ammonium formate, potassium formate, magnesium formate, and combinations thereof. In a preferred embodiment, the formate is calcium formate.

**[0024]** The folate of the disclosed compositions and methods can be folic acid (pteroyl-L-glutamic acid), a bioactive derivative thereof (e.g., a reduced form of folic acid), or a pharmaceutically acceptable salt or ester thereof. Folic acid (also referred to as folate, vitamin M, vitamin B9, vitamin Bc (or folacin), and pteroyl-L-glutamate) is a form of the water-

soluble vitamin B9. Chemically, folic acid is a tripartite molecule containing a pterin moiety, a para-aminobenzoic acid moiety, and a  $\gamma$ -linked glutamate residue, as shown below.

Folic Acid



**[0025]** Upon administration, folic acid is enzymatically converted to a biologically active folate in vivo. Therefore, formulations containing, for example, both a reduced folate and folic acid, may have the benefit of providing both a readily available biologically active folate as well as a longer term source of a biologically active folate (e.g., folic acid).

**[0026]** The folate can be a natural folate, such as levomefolic acid (also known as 5-MTHF, methylfolate, or 5-methyltetrahydrofolate), or a pharmaceutically acceptable salt or ester thereof. In some embodiments, the folate is L-methylfolate (also known as L-5-methyltetrahydrofolate (L-5-MTHF), which is a metabolically active form of folate).

**[0027]** In some embodiments, the folate is a reduced folate (e.g., folinic acid), or a natural isomer of a reduced folate. Suitable reduced folates are known in the art, and described, for example, in U.S. Pat. Nos. 5,350,851, 5,997,915, and 6,808,725 to Bailey et al.; U.S. Pat. Nos. 6,011,040 and 6,441,168 to Muller et al.; and U.S. Pat. No. 6,921,754 to Hahnlein et al.

**[0028]** The folate can be one or more natural isomers of reduced folate, one or more synthetic isomers of reduced folate, or combinations thereof. In formulations containing both natural and synthetic isomers of reduced folate, the natural and synthetic folates may be incorporated in equal molar ratios, or different molar ratios.

**[0029]** Natural isomers of reduced folates are described in WO 1997027764 A1, which is hereby incorporated by reference for the teaching of these natural isomers of reduced folates. Natural isomers of suitable reduced folate include, but are not limited to, (6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5-formyl-(6S)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, and 5-formimino-(6S)-tetrahydrofolic acid. Other natural isomers of reduced folate include the polyglutamyls, such as the diglutamyl, triglutamyl, tetraglutamyl, pentaglutamyl, and hexaglutamyl, derivatives of (6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5-formyl-(6S)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, and 5-formimino-(6S)-tetrahydrofolic acid.

**[0030]** Synthetic isomers of reduced folate include, but are not limited to, (6R)-tetrahydrofolic acid, 5-methyl-(6R)-tetrahydrofolic acid, 5-formyl-(6S)-tetrahydrofolic acid, 10-formyl-(6S)-tetrahydrofolic acid, 5,10-methylene-(6S)-

tetrahydrofolic acid, 5,10-methenyl-(6S)-tetrahydrofolic acid, 5-formimino-(6R)-tetrahydrofolic acid, and polyglutamyl derivatives thereof.

**[0031]** Any or all of the natural and synthetic isomers of reduced folate can be present as a single enantiomer or a mixture of enantiomers and/or diastereomers. The reduced folates can be either amorphous or crystalline. In certain embodiments, the reduced folate is in an amorphous state. In other embodiments, the reduced folate is crystalline. In still other embodiments, the one or more reduced folates are a mixture of amorphous and crystalline materials.

**[0032]** The folate can be a pharmaceutically acceptable salt of any of the folates described above. Pharmaceutically acceptable salts of folates include derivatives of folates wherein the parent folate is modified by making the acid-addition or base-addition salt thereof. Example of pharmaceutically acceptable salts include but are not limited to mineral or organic acid salts of basic residues such as amines; and alkali or organic salts of acidic residues such as carboxylic acids.

**[0033]** The pharmaceutically acceptable salts of the compounds can be synthesized from the parent compound, which contains a basic or acidic moiety, by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, non-aqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 20th ed., Lippincott Williams & Wilkins, Baltimore, Md., 2000, p. 704.

**[0034]** In certain embodiments, the folate salt is a glucosamine salt of a folate (e.g., a glucosamine salt of a reduced folate), a galactosamine salt of a folate (e.g., a galactosamine salt of a reduced folate), or combinations thereof. Examples of suitable glucosamine and galactosamine salts include D-glucosamine dihydrofolate and D-glucosamine tetrahydrofolate, unsubstituted or substituted with a 5-methyl-, 5-formyl-, 10-formyl-, 5,10-methylene-, 5,10-methenyl moiety; and D-galactosamine dihydrofolate and D-galactosamine tetrahydrofolate, unsubstituted or substituted with a 5-methyl-, 5-formyl-, 10-formyl-, 5,10-methylene-, 5,10-methenyl moiety, wherein the reduced folates exhibit a (6R, S), (6S), or (6R) configuration. Suitable glucosamine and galactosamine salts of reduced folates are known in the art, and described in U.S. Pat. No. 7,947,662 to Valoti, et al., which is incorporated herein by reference.

**[0035]** In certain embodiments, the folate comprises D-glucosamine (6R,S)-tetrahydrofolate, D-glucosamine (6S)-tetrahydrofolate, D-glucosamine (6R)-tetrahydrofolate; D-galactosamine (6R,S)-tetrahydrofolate, D-galactosamine (6S)-tetrahydrofolate, D-galactosamine (6R)-tetrahydrofolate; D-glucosamine 5-methyl-(6R,S)-tetrahydrofolate, D-glucosamine 5-methyl-(6S)-tetrahydrofolate, D-glucosamine 5-methyl-(6R)-tetrahydrofolate; D-galactosamine 5-methyl (6R,S)-tetrahydrofolate, D-galactosamine 5-methyl-(6S)-tetrahydrofolate, D-galactosamine 5-methyl-(6R)-tetrahydrofolate, or combinations thereof. In a preferred embodiment, the folate comprises D-glucosamine 5-methyl-(6S)-tetrahydrofolate (also known as N-[4-[[[(6S)-2-amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridinyl]methyl]amino]benzoyl]-L-glutamic acid, glucosamine salt).

**[0036]** The disclosed compositions can optionally contain one or more additional vitamins, minerals, other nutraceuti-

cals, or combinations thereof. The disclosed compositions can optionally include vitamin A. Vitamin A can be provided in any suitable form for administration, including as a retinyl ester (e.g., retinyl acetate or palmitate), alpha-carotene, beta-carotene, gamma-carotene, beta-cryptoxanthin, and combinations thereof. The disclosed compositions can optionally contain vitamin B<sub>1</sub> (thiamine) or a derivative thereof. Derivatives of vitamin B<sub>1</sub> include compounds formed from vitamin B<sub>1</sub> that are structurally distinct from vitamin B<sub>1</sub>, but retain the active function of vitamin B<sub>1</sub>, such as allithiamine, prosultiamine, fursultiamine, benfotiamine, sulbutiamine, and combinations thereof. The disclosed compositions can optionally contain vitamin B<sub>2</sub> (riboflavin) or a derivative thereof. Derivatives of vitamin B<sub>2</sub> include compounds formed from vitamin B<sub>2</sub> that are structurally distinct from vitamin B<sub>2</sub>, but retain the active function of vitamin B<sub>2</sub>. The disclosed compositions can optionally contain vitamin B<sub>6</sub> (pyridoxine) or a derivative thereof. Derivatives of vitamin B<sub>6</sub> include compounds formed from vitamin B<sub>6</sub> that are structurally distinct from vitamin B<sub>6</sub>, but retain the active function of vitamin B<sub>6</sub>. Examples of suitable forms of B<sub>6</sub> that can be incorporated into the pharmaceutical formulations include pyridoxine, pyridoxine 5'-phosphate, pyridoxal, pyridoxal 5'-phosphate, pyridoxamine, pyridoxamine 5'-phosphate, and combinations thereof. The disclosed compositions can optionally contain vitamin B<sub>12</sub> (cobalamin) or a derivative thereof. Examples of suitable forms of vitamin B<sub>12</sub> include, but are not limited to, cyanocobalamin, hydroxocobalamin, nitrocobalamin, methylcobalamin, deoxyadenosylcobalamin, adenosylcobalamin, and combinations thereof. The disclosed compositions can optionally contain pantothenic acid (also known as vitamin B<sub>5</sub>) or a derivative or salt thereof (such as calcium pantothenate). Derivatives of pantothenic acid include compounds formed from pantothenic acid that are structurally distinct from pantothenic acid, but retain the active function of pantothenic acid. The disclosed compositions can optionally contain biotin (also known as vitamin B<sub>7</sub> and vitamin H) or a derivative thereof. Derivatives of biotin include compounds formed from biotin that are structurally distinct from biotin, but retain the active function of biotin. The disclosed compositions can optionally contain vitamin B<sub>3</sub> (niacin) or derivatives thereof, such as niacinamide. Derivatives of vitamin B<sub>3</sub> include compounds formed from vitamin B<sub>3</sub> that are structurally distinct from vitamin B<sub>3</sub>, but retain the active function of vitamin B<sub>3</sub>. The disclosed compositions can optionally contain vitamin C (ascorbic acid) or a salt or derivative thereof (such as sodium ascorbate). Derivatives of vitamin C include compounds formed from vitamin C that are structurally distinct from vitamin C, but retain the active function of vitamin C. The disclosed compositions can optionally contain vitamin D<sub>3</sub> (cholecalciferol) or a derivative thereof. Derivatives of vitamin D<sub>3</sub> include compounds formed from vitamin D<sub>3</sub> that are structurally distinct from vitamin D<sub>3</sub>, but retain the active function of vitamin D<sub>3</sub>. The disclosed compositions can optionally contain vitamin E (dl- $\alpha$ -tocopheryl acetate) or a derivative thereof. Derivatives of vitamin E include compounds formed from vitamin E that are structurally distinct from vitamin E, but retain the active function of vitamin E. The disclosed compositions can optionally contain vitamin K (phyloquinone) or a derivative thereof. Derivatives of vitamin K include compounds formed from vitamin K that are structurally distinct from vitamin K, but retain the active function of vitamin K.

[0037] The disclosed compositions can optionally contain one or more additional minerals. For example, the disclosed compositions can optionally contain a suitable source of zinc, such as a biocompatible zinc salt (e.g., zinc sulfate). The disclosed compositions can optionally contain an iron source, such as a biocompatible iron salt. Examples of suitable iron salts include, but are not limited to, ferrous sulfate, ferrous fumarate, ferrous succinate, ferrous gluconate, ferrous lactate, ferrous glutamate, ferrous glycinate, and combinations thereof. The disclosed compositions can optionally contain a suitable source of copper, such as a biocompatible copper salt (e.g., cupric oxide or cupric sulfate). The disclosed compositions can optionally contain a magnesium source, such as a biocompatible magnesium salt. Examples of suitable magnesium salts include, but are not limited to, magnesium stearate, magnesium carbonate, magnesium oxide, magnesium hydroxide, magnesium sulfate, and combinations thereof. The disclosed compositions can optionally contain a calcium source, such as a biocompatible calcium salt. Biologically-acceptable calcium compounds include, but are not limited to, calcium carbonate, calcium sulfate, calcium oxide, calcium hydroxide, calcium apatite, calcium citrate-malate, bone meal, oyster shell, calcium gluconate, calcium lactate, calcium phosphate, calcium levulinate, and combinations thereof.

[0038] The disclosed compositions can optionally contain one or more additional nutraceutical agents, such as proteins, carbohydrates, amino acids, fatty acids, antioxidants, plant or animal extracts, or combinations thereof. Exemplary nutraceutical agents and dietary supplements are disclosed, for example, in Roberts et al., (*Nutraceuticals: The Complete Encyclopedia of Supplements, Herbs, Vitamins, and Healing Foods*, American Nutraceutical Association, 2001). Nutraceutical agents and dietary supplements are also disclosed in *Physicians' Desk Reference for Nutritional Supplements*, 1st Ed. (2001) and *The Physicians' Desk Reference for Herbal Medicines*, 1st Ed. (2001). In some cases, the disclosed compositions may contain one or more amino acids. Examples of suitable amino acids which can be incorporated into the pharmaceutical formulations include phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, histidine, arginine, cysteine, glycine, glutamine, proline, serine, tyrosine, and combinations thereof. In some cases, the disclosed compositions may contain one or more botanic extracts. Examples of suitable botanic extracts include leucocyanidins, ginkgo biloba, ginseng, green tea, valerian, passion flower, chamomile, aloe vera, green tea, guggul, and combinations thereof. In some embodiments, the disclosed compositions may contain one or more fatty acids, omega-3 fatty acids, omega-6 fatty acids, omega-9 fatty acids, phospholipids, or combinations thereof. Alpha-linolenic (ALA), docosahexaenoic (DHA), and eicosapentaenoic (EPA) acids are examples of omega-3 fatty acids. Linoleic acid (LA) and arachidonic acid (AA) are examples of omega-6 fatty acids. Oleic (OA) and erucic acid (EA) are examples of omega-9 fatty acids. Other suitable nutraceuticals that can be optionally incorporated into the pharmaceutical/nutraceutical formulations described herein include lutein, phosphatidylserine, lipoic acid, melatonin, glucosamine, chondroitin, lycopene, whole foods, food additives, herbs, phytonutrients, antioxidants, flavonoid constituents of fruits, fish and marine animal oils (e.g. cod liver oil), probiotics (e.g., lactobacilli, spores, yeasts, and combinations

thereof), S-Adenosyl methionine (S-AdoMet), ubiquinone, choline, and combinations thereof.

[0039] In some embodiments, the method involves administering to the subject an effective amount of a first composition containing a formate and a second composition containing a folate. For example, the first composition and the second composition can be administered within about 0 to 12 hours of each other, including about 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 hours of each other. In other embodiments, the method involves administering to the subject an effective amount of a composition containing both a formate and a folate. Therefore, also disclosed is a composition containing a formate, a folate, and a pharmaceutically or nutraceutically acceptable carrier.

[0040] The formate is preferably present in the composition, and/or administered to the subject, in an amount that is about 100-100,000 times higher than the folate, including about 1,000 to about 50,000 times higher, or about 10,000 to about 50,000 higher. In some embodiments, the formate is administered in a daily dosage of about 1 mg/kg to about 1 g/kg, such as about 10 mg/kg to about 500 mg/kg, including about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, or 500 mg/kg.

[0041] Also disclosed is a composition containing formate in a unit dose for prenatal supplementation. In some embodiments, the unit dose is a daily unit dose. In other embodiments, the dose is administered two to three times per day. Therefore, in some embodiments, the unit dose contains about 0.5 mg to about 100 g of the formate, such as about 100 mg to about 10 g, including about 100, 200, 300, 400, 500, 600, 700, 800, 900 mg, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 g of the formate.

[0042] The unit dose can also contain about 100 µg to about 1.0 mg of folic acid, such as about 400 µg to about 800 µg of the folic acid. The unit dose can alternatively contain about 1 mg to about 100 mg of L-methylfolate, including about 15 mg L-methylfolate, or of a pharmaceutically acceptable salt thereof (e.g., L-methylfolate calcium (levomefolate calcium)).

[0043] Disclosed are pharmaceutical/nutraceutical compositions containing therapeutically (or diagnostically) effective amounts of one or more folates and/or formates and a one or more pharmaceutically acceptable excipients. The term "pharmaceutically acceptable" and "nutraceutically acceptable" refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

[0044] Pharmaceutical excipients suitable for administration of the folates and/or formates provided herein include any such excipients known to those skilled in the art to be suitable for the particular mode of administration. Representative excipients include solvents, diluents, pH modifying agents, preservatives, antioxidants, suspending agents, wetting agents, viscosity modifiers, tonicity agents, stabilizing agents, and combinations thereof. Suitable pharmaceutically acceptable excipients are preferably selected from materials which are generally recognized as safe (GRAS), and may be administered to an individual without causing undesirable biological side effects or unwanted interactions.

[0045] Pharmaceutical formulations can be formulated for any suitable mode of administration. In certain embodiments,

the pharmaceutical formulation is formulated for enteral delivery. Examples of compositions for enteral consumption (enteral including oral, intragastric, or transpyloric), include oral solid dosage forms, food preparations, food supplements, essential nutrient preparations, and vitamin preparations. Oral solid dosage forms are known in the art, and described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton Pa. 18042) at Chapter 89. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets, pellets, powders, or granules. A description of possible solid dosage forms for is given by Marshall, K. In: Modern Pharmaceutics Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979.

**[0046]** Examples of suitable food or vitamin preparations include those to which folic acid is currently added for use by either humans or other animals. More specifically, these compositions are, but are not limited to, multivitamin (with or without minerals and other nutrients) preparations (such preparations can be in solid, liquid or suspension forms); breakfast foods such as prepared cereals, breakfast drink mixes, toaster pastries and breakfast bars; infant formulas; dietary supplements and complete diet and weight-loss formulas and bars; animal feed or animal feed supplements (for example, for poultry), and pet foods. The disclosed compositions can also be used in a vitamin formulation containing at least one other vitamin (for example another vitamin such as a B vitamin) other than ascorbic acid (vitamin C), although ascorbic acid can be an additional component. Such nutrients or vitamins can be those intended for either human or animal use. Other ingredients may also be present, such as fillers, binding agents, stabilizers, sweeteners, including nutritive sweeteners (e.g. sucrose, sorbitol and other polyols) and non-nutritive sweeteners (e.g. saccharin, aspartame, and acesulfame K), colorants, flavors, buffers, salts, coatings, and the like that are known to those skilled in the art of vitamin formulation.

**[0047]** Formulations containing folates and/or formates can be administered as a medicament or a nutritional supplement. In certain embodiments, the formulations are administered to a female who is pregnant, a female who is attempting to become pregnant, a female who has had a miscarriage, or a female who has carried a fetus having a neural tube defect, a cleft lip defect, and/or a cleft palate defect.

**[0048]** In some cases, the formulations are administered to a subject who has a limited ability to absorb or metabolize folic acid. In some cases, the formulations are administered to a subject who possesses one or more mutations in 10-methylenetetrahydrofolate reductase (MTHFR), the enzyme that is responsible for conversion of folic acid to 5-methyltetrahydrofolate (5-MTHF). In particular embodiments, the subject is homozygous or heterozygous for a 677C→T polymorphism of methylenetetrahydrofolate reductase.

**[0049]** In some embodiments, the formulations are administered as a medicament, a food additive or a nutritional supplement, for the prevention and/or the treatment of neurological affliction such as, for instance, subacute encephalitis associated with dementia and vacuolar myelopathies; psychopathological, vascular and cardiovascular such as, for instance premature occlusive arterial disease, severe vascular disease in infancy and childhood, progressive arterial stenosis, intermittent claudication, renovascular hypertension, ischemic cerebrovascular disease, premature retinal artery and retinal vein occlusion, cerebral occlusive arterial disease, occlusive peripheral arterial disease, premature death due to

thromboembolic disease and/or ischemic heart disease; cancer; autoimmune diseases, such as, for instance, psoriasis, celiac disease, arthritic and inflammation conditions; megaloblastic anaemia due to folate deficiency, intestinal malabsorption, for maintaining and/or normalizing the homocysteine level and/or metabolism; alterations of the synthesis and/or the functioning and/or the changes of DNA and RNA and the alterations of cell synthesis; depressive illnesses; and combinations thereof.

**[0050]** A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

## EXAMPLES

### Example 1

#### Deletion of Mthfd11 Causes Embryonic Lethality and Neural Tube and Craniofacial Defects in Mice

**[0051]** Folate-dependent one-carbon (1C) metabolism is highly compartmentalized in eukaryotes, and mitochondria play a critical role in cellular 1C metabolism. The cytoplasmic and mitochondrial compartments are metabolically connected by transport of 1C donors such as serine, glycine, and formate across the mitochondrial membranes, supporting a mostly unidirectional flow (clockwise in FIG. 1) of 1C units from serine to formate, and onto purines, thymidylate (dTMP), and methionine. It appears that under most conditions, the majority of 1C units for cytoplasmic processes are derived from mitochondrial formate. This formate is exported to the cytoplasm where it is reattached to tetrahydrofolate (THF) for use in de novo purine biosynthesis, or further reduced for either thymidylate synthesis or remethylation of homocysteine to methionine. The 1C unit interconverting activities represented in FIG. 1 by reactions 1-3 (and 1m-3m in mitochondria) are the central players in this intercompartmental pathway. These crucial reactions are catalyzed by members of the methylenetetrahydrofolate dehydrogenase (MTHFD) family in eukaryotes. The first member of this family to be characterized was the cytoplasmic MTHFD1 protein, a trifunctional enzyme possessing 10-formyl-THF synthetase, 5,10-methenyl-THF cyclohydrolase, and 5,10-methylene-THF dehydrogenase activities (reactions 1-3). This enzyme incorporates formate, released from mitochondria, into the cytoplasmic 1C THF pool as 10-formyl-THF (CHO-THF), which is required for de novo purine biosynthesis. MTHFD1 can also catalyze reduction of the 1C unit to 5,10-methylene-THF (CH<sub>2</sub>-THF) for dTMP synthesis (reaction 10), or for methyl group biogenesis via 5-methyl-THF (CH<sub>3</sub>-THF) (reaction 6). The MTHFD2 protein is a mitochondrial bifunctional CH<sub>2</sub>-THF dehydrogenase/methenyl-THF cyclohydrolase (reactions 3m, 2m). Mitochondrial CH<sub>2</sub>-THF dehydrogenase isozyme, encoded by the Mthfd2l gene, is bifunctional, possessing both CH<sub>2</sub>-THF dehydrogenase and methenyl-THF cyclohydrolase activities (reactions 3m, 2m). The final step in the mammalian mitochondrial pathway to formate (reaction 1m) is catalyzed by mitochondrial 10-formyl-THF synthetase, encoded by the Mthfd11 gene. Despite sharing 61% amino acid similarity with the cytoplasmic trifunctional MTHFD1, MTHFD1L is a monofunctional enzyme, possessing only the 10-formyl-THF synthetase activity (reaction 1m). The Mthfd11 gene is expressed in most

adult tissues, but at higher levels in spleen, thymus, brain, and placenta. The *Mthfd11* gene is also expressed at all stages of mammalian embryogenesis and ubiquitously throughout the embryo but with localized regions of higher expression along the neural tube, the brain, craniofacial structures, limb buds, and the tail bud. Moreover, metabolic tracer experiments in mouse embryonic fibroblasts (MEFs) showed that more than 75% of 1C units that enter the cytoplasmic methyl cycle are mitochondrially derived. Thus, in both embryos and adults, MTHFD1L catalyzes production of formate from 10-formyl-THF, the last step in the flow of 1C units from mitochondria to cytoplasm.

**[0052]** To investigate the role of mitochondrial formate production during development, *Mthfd11* knockout mice were analyzed. As disclosed herein, loss of MTHFD1L is lethal to developing embryos, causing fetal growth restriction and aberrant neural tube closure with 100% penetrance in embryos that develop past the point of neural tube closure. Although there are other folate-related mouse models that exhibit NTDs, the *Mthfd11* knockout mouse is the first fully penetrant model that does not require feeding a folate-deficient diet to cause this phenotype. Moreover, maternal supplementation with sodium formate is shown herein to decrease the incidence of NTDs and partially rescue the growth defect in embryos lacking *Mthfd11*. These results reveal the critical role of mitochondrial formate in mammalian development, providing a mechanistic link between folic acid and neural tube defects.

#### **[0053] Methods**

**[0054]** Mice. All protocols used within this study were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice were maintained on a C57B1/6 genetic background. Mice harboring a floxed conditional knockout cassette between exons 4 and 6 of *Mthfd11* were obtained from the Wellcome Trust Sanger Institute (EU-COMM ID 37226). Mice carrying the floxed *Mthfd11* allele were mated to mice expressing Cre recombinase under control of the E2a promoter (E2a-Cre) (Lakso M, et al. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93:5860-5865) to generate heterozygous *Mthfd11*<sup>+/−</sup> embryos lacking exon 5 and the neomycin resistance cassette (FIG. 2A). All mice were given ad libitum access to water and standard mouse chow (LabDiet 5K67).

**[0055]** Genotyping. Genotyping was carried out by a modified PCR method (Stratman J L, et al. (2003) *Transgenic Res.* 12:521-522). A mixture of three primers was used to detect the wild-type and/or recombined allele (FIG. 2A). To detect the wild-type allele, a forward primer (F) binding in the 5' region outside of the conditional cassette (5'-GAGTAT GTGAT TGCTT GGACC CCCAG GTTCC-3' (SEQ ID NO:1)) and a reverse primer (R1) binding 5' to exon 5 (5'-TGGCT CCCGA GGTG TCTTC TGGCT ATGAT-3' (SEQ ID NO:2)) were employed. Amplification using these primers results in a 444 bp amplicon from the wild type allele. Amplification of the mutant allele uses the forward primer F, and a reverse primer (R2) complementary to a region only found in the gene-targeting cassette (5'-CGGCG CCAGC CTGCT TTTT GTACA AACTT G-3' (SEQ ID NO:3)). Amplification using these primers results in a 324 bp amplicon in the presence of the mutant allele. See FIG. 2A for a schematic of primer binding sites used to detect *Mthfd11* and *Mthfd11*<sup>Δ</sup>. Cre recombinase was detected using 5'-GCATT ACCGG TCGAT

GCAAC GAGTG ATGAG-3' (SEQ ID NO:4) and 5'-GAGTG AACGA ACCTG GTCGA AATCA GTGCG-3' (SEQ ID NO:5) to produce a 408 bp amplicon, and SRY was detected using 5'-TTGTC TAGAG AGCAT GGAGG GCCAT GTCAA-3' (SEQ ID NO:6) and 5'-CCACT CCTCT GTGAC ACTTT AGCCC TCCGA-3' (SEQ ID NO:7) to detect a 273 bp amplicon.

**[0056]** Reverse transcription-PCR (RT-PCR). Total RNA was prepared from *Mthfd11*<sup>+/+</sup>, *Mthfd11*<sup>+/−</sup>, and *Mthfd11*<sup>Δ/Δ</sup> mouse embryos dissected at E11.5. First strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamers. PCR was performed using a forward primer f (5'-CTCAC ATCTG CTTGC CTCCA-3' (SEQ ID NO:8)) binding in exon 4 and a reverse primer r (5'-ATGTC CCCAG TCAGG TGAAG-3' (SEQ ID NO:9)) binding in exon 14 to amplify a 1087 bp amplicon from the wild-type transcript (see FIG. 2A for a schematic of primer binding sites). Primers amplifying a 115 bp amplicon (Forward: 5'-AGAGA CGGCC GCATC TTC-3' (SEQ ID NO:10), Reverse: 5'-CAAT GGCAG CCCTG GTGA3' (SEQ ID NO:11)) from GAPDH were used as a positive control for cDNA quality.

**[0057]** Mitochondrial Isolation and Immunoblotting. Mitochondria were isolated from one embryo (*Mthfd11*<sup>+/+</sup> and *Mthfd11*<sup>+/−</sup>) or three embryos (*Mthfd11*<sup>Δ/Δ</sup>) as previously described (Pike ST, et al (2010) *J Biol Chem* 285:4612-4620), except embryos were homogenized by pipetting. Protein concentration was determined by BCA assay (Thermo Fisher Scientific, Rockford, Ill.). Proteins were separated by SDS-PAGE and immunoblotted using rabbit polyclonal anti-MTHFD1L (1:1000) (Prasanna P & Appling D R (2009) *Arch. Biochem. Biophys.* 481:86-93). After incubation with HRP-conjugated goat anti-rabbit IgG (1:5000) (Invitrogen), reacting bands were detected using ECL Plus (GE Healthcare Life Sciences, Piscataway, N.J.). After stripping, blots were re-probed with rabbit polyclonal anti-Hsp60 (1:1000) (Enzo Life Sciences, Ann Arbor, Mich.).

**[0058]** Histology. Embryos were stained for β-galactosidase activity overnight as described previously (Whiting J, et al. (1991) *Genes Dev.* 5:2048-2059). Stained embryos were embedded in paraffin, sectioned at the level of the forelimb (four micrometer thickness) and counterstained with nuclear fast red. *Mthfd11* wholemount in situ hybridization was performed using a riboprobe against the 3'UTR as previously described (Pike S T, et al (2010) *J Biol Chem* 285:4612-4620). Embryos were then embedded in OCT medium and cryosectioned at the level of the forelimb (12 μm thickness).

**[0059]** Maternal supplementation with sodium formate. *Mthfd11*<sup>+/−</sup> matings were set up in a cage equipped with a water bottle containing either 0.37 M or 0.55 M sodium formate. The females had access to the supplemented water at least one day before observation of the plug. These concentrations were calculated to deliver either 5000 or 7500 mg sodium formate/kg/day, respectively, based on an average water intake of 5 mL per day for a 25 g C57BL/6 mouse (Green E L ed (1966) *Biology of the Laboratory Mouse* (Dover Publications, Inc., New York), 2nd Ed, p 706). The effect of formate supplementation was analyzed by a two-sided chi square test for NTD incidence and two-way ANOVA with Bonferroni post-test for crown-rump length.

**[0060]** Maternal supplementation with calcium formate. *Mthfd11*<sup>+/−</sup> matings were set up in a cage equipped with a water bottle containing 0.0096 M, 0.019 M, 0.056 M or 0.096

M calcium formate. The females had access to the supplemented water at least one day before observation of the plug. These concentrations were calculated to deliver 250, 500, 1500 or 2500 mg calcium formate/kg/day, respectively, based on an average water intake of 5 mL per day for a 25 g C57BL/6 mouse (Green E L ed (1966) Biology of the Laboratory Mouse (Dover Publications, Inc., New York), 2nd Ed,

The ratio of Mthfd11<sup>+/+</sup> to Mthfd11<sup>+/+</sup> to Mthfd11<sup>z/z</sup> was 55:117:0, indicating that the Mthfd11<sup>z/z</sup> genotype causes embryonic lethality ( $p=2.0\times10^{-11}$ ). If it is assumed that the Mthfd11<sup>z/z</sup> genotype is lethal, Mthfd11<sup>+/+</sup> and Mthfd11<sup>+/+</sup> genotypes were observed in the expected frequency ( $p=0.75$ ). Males and females were found at the expected frequencies, and Mthfd11<sup>+/+</sup> mice appear healthy and breed normally.

TABLE 1

Mthfd11 nullizygous mice are not viable						
Genotype	Observed genotype distribution			Expected genotype distribution		
	Male	Female	Total	Male	Female	Total
Mthfd11 <sup>+/+</sup>	24	31	55	21.5	21.5	43
Mthfd11 <sup>+/+</sup>	56	61	117	43	43	86
Mthfd11 <sup>z/z</sup>	0	0	0	21.5	21.5	43
Total embryos	80	92	172	86	86	172
Total litters observed			31			
Mean litter size (mean ± SD)			5.5 ± 2.0			
P value, observed vs. expected genotype distribution (1:2:1)*			1.9994 × 10 <sup>-11</sup>			
P value, observed vs. expected genotype distribution assuming embryonic lethality*			0.7494			

\*Analyzed by  $\chi^2$  test.

p 706). The effect of formate supplementation was analyzed by a two-tailed T-test for crown-rump length

[0061] Results

[0062] Mthfd11 is Essential in Mice

[0063] A strain of conditional knockout ready Mthfd11 mice was obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM). In this strain, the Mthfd11 locus is modified by the insertion of a cassette, containing a splice acceptor, internal ribosome entry site, the  $\beta$ -galactosidase gene (LacZ) followed by a polyadenylation signal, and the gene for neomycin phosphotransferase (Neo), between exons 4 and 6 of Mthfd11 (FIG. 2A). This allele has three LoxP sites: one between the polyadenylation signal and Neo, and two flanking exon 5. To generate a null allele, the mice were crossed to a Cre deleter strain, E2a-Cre (Lakso M, et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:5860-5865). Recombination at the LoxP sites removes Neo and exon 5 to produce a disrupted allele containing LacZ followed by a polyadenylation signal (FIG. 2A). Transcription of the disrupted allele is expected to produce a transcript containing exons 1-4 spliced to LacZ. The disrupted Mthfd11 allele is designated as Mthfd11<sup>f</sup>. The genotype was confirmed by PCR (FIG. 2B) and RT-PCR analysis indicated that the wild-type Mthfd11 transcript is absent in Mthfd11<sup>z/z</sup> embryos (FIG. 2C). Full-length MTHFD1L protein is undetectable in Mthfd11<sup>z/z</sup> embryos (FIG. 2D), indicating that this is a likely null allele. No difference in growth from weaning to 5 weeks of age was observed between Mthfd11<sup>+/+</sup> and Mthfd11<sup>z/1</sup> mice. To determine the viability of homozygous null (Mthfd11<sup>z/z</sup>) mice, Mthfd11<sup>z/1</sup> mice were intercrossed and the genotype distribution was determined (Table 1). A total of 172 weanlings from 31 litters were examined. The average litter size was 5.5 pups. The Mthfd11 genotypes were not distributed as expected for Mendelian inheritance of the nonfunctional Mthfd11<sup>f</sup> allele.

[0064] Homozygous deletion of Mthfd11 results in delayed embryonic growth and defective neural tube closure. Since no Mthfd11<sup>z/z</sup> pups were recovered at birth, the embryonic phenotype was investigated. Embryos were dissected from pregnant dams at E8.5-E15.5, genotyped using yolk sac tissue, and their gross morphology was examined. All observed Mthfd11<sup>z/z</sup> embryos exhibited a growth delay compared to wild-type and Mthfd11<sup>+/+</sup> littermates. The severity of the developmental delay was variable, but on average the null embryos appeared to lag approximately 0.75 days behind their littermates. Some of the Mthfd11<sup>z/z</sup> embryos died early during the gestational period, but all that survived past the point of neural tube closure (E9.5) exhibited aberrant neural tube phenotypes. Out of 152 embryos dissected at E11.5-E12.5, 52 Mthfd11<sup>1/1</sup> embryos, 74 Mthfd11<sup>z/1</sup> embryos, and 26 Mthfd11<sup>z/z</sup> embryos were obtained, and 28 resorptions were observed. Of the 26 Mthfd11<sup>z/z</sup> embryos, 15 exhibited a clear NTD phenotype (exencephaly or craniorachischisis) and 9 displayed a wavy neural tube phenotype (FIGS. 3B-3D). The remaining two Mthfd11<sup>z/z</sup> embryos had completely open neural tubes but were not scored as having craniorachischisis because these embryos had failed to turn, suggesting that they may have been in the process of resorption. The most common NTD phenotype was exencephaly with a wavy neural tube (FIG. 3D; n=11), or exencephaly alone (FIG. 3B, n=3). The most severe NTD observed was craniorachischisis (FIG. 3C; n=1). The nine Mthfd11<sup>z/z</sup> embryos whose neural tubes had closed all displayed a wavy neural tube with a small, aberrantly formed head (FIG. 4B). In all, 20/24 Mthfd11<sup>z/z</sup> embryos exhibited a wavy neural tube, and the earliest observation of this phenotype was at E9.5. The location of the waviness in the neural tube was variable, but most embryos exhibited a wavy neural tube beginning at approximately the same axis as the forelimb and extending caudally past the

forelimb, as depicted in FIGS. 3D and 3E. Because many studies have noted an increased incidence of NTDs in females (Harris M J & Juriloff D M (2007) Birth Defects Res A Clin Mol Teratol 79:187-210), E11.5-E12.5 *Mthfd11<sup>z/z</sup>* embryos were genotyped for presence of the sex-receptor Y (SRY) locus. No bias was found for either sex [females n=6 (40%), males n=9 (60%), p=0.44].

**[0065]** In addition to aberrations in neural tube closure, facial deformities were also noted in *Mthfd11<sup>z/z</sup>* embryos that were most apparent at the later stages. When compared to somite-matched wild-type or heterozygous embryos, E12.5 *Mthfd11<sup>z/z</sup>* embryos display immature maxillary and mandibular processes (FIG. 3G). In *Mthfd11<sup>z/z</sup>* embryos the maxillary processes of the first branchial arch appear globular and more widely separated than in somite-matched control embryos. In addition, the mandibular processes were undergrown (FIGS. 3F,G; 7/7). No surviving *Mthfd11<sup>z/z</sup>* embryos were observed after E12.5, preventing a later analysis of the phenotype.

**[0066]** Histological Analysis of Neural Tube Phenotypes

**[0067]** Control (*Mthfd11<sup>z/+</sup>*) and *Mthfd11<sup>z/z</sup>* embryos were sectioned and stained for  $\beta$ -galactosidase at E10.5 and E11.5. This allowed visualization of regionalized  $\beta$ -galactosidase activity, which should act as a reporter for *Mthfd11* transcription (FIG. 2A). To confirm that the LacZ reporter recapitulated endogenous *Mthfd11* gene expression, the patterns detected by  $\beta$ -galactosidase staining in *Mthfd11<sup>z/+</sup>* sections were compared with endogenous *Mthfd11* gene expression. Using in situ hybridization, transcript expression was observed in the ectoderm, underlying mesenchyme, and dorsal neural tube (FIGS. 4C, 4F, 4I). In the neural tube, the highest expression was detected in the basal surface of the dorsal neuroepithelium (FIG. 4F, arrowheads).  $\beta$ -galactosidase activity was more restricted in *Mthfd11<sup>z/+</sup>* embryos (FIG. 4D), but was still seen within the same region of the neural tube as the gene expression pattern. It was concluded that the LacZ reporter partially recapitulates endogenous *Mthfd11* gene expression with the difference most likely being due to reduced sensitivity.

**[0068]** *Mthfd11<sup>z/+</sup>* wholemount embryos stained for  $\beta$ -galactosidase activity have the highest levels in the eyes, heart, limb, and dorsal midline region (FIGS. 4A and 4J). In sectioned *Mthfd11<sup>z/+</sup>* embryos,  $\beta$ -galactosidase activity was predominantly detected at the basal surface of the dorsal neuroepithelium, and was rarely detected in cells within and outside of the neural tube (FIG. 4D, 4G, 4L, 4N). In sectioned *Mthfd11<sup>z/z</sup>* embryos,  $\beta$ -galactosidase activity was robustly detected both inside and outside the neural tube (FIGS. 4E, 4H, 4M, 4O). Similar to *Mthfd11<sup>z/+</sup>* embryos,  $\beta$ -galactosidase was detected in the neuroepithelium; however, expression was less restricted dorsally in the nulls. In *Mthfd11<sup>z/z</sup>* embryos with closed neural tubes, the morphology was abnormal throughout the neural tube in all embryos sectioned (E10.5

and E11.5, n=6). Neural tubes had abnormally shaped lumens, including asymmetric dorsal-lateral bulges as well as a broader dorsal lumen that were not seen in controls (FIGS. 4D, 4G, 4E, 4H, 4L-4O).

**[0069]** Dietary Supplementation with Sodium Formate

**[0070]** Because deletion of *Mthfd11* is expected to result in loss of mitochondrial formate production, experiments were conducted to determine if maternal formate supplementation would improve development of *Mthfd11<sup>z/z</sup>* embryos. Pregnant dams were given ad libitum access to water containing sodium formate to achieve a calculated dose of 5000 or 7500 mg sodium formate/kg/day; controls were given water without formate. As before, *Mthfd11<sup>z/z</sup>* embryos were not recovered from unsupplemented dams (17 *Mthfd11<sup>+/+</sup>*, 34 *Mthfd11<sup>z/+</sup>*, 0 *Mthfd11<sup>z/z</sup>* embryos, deviating significantly from the expected Mendelian ratio; p=0.0002). When dams were supplemented with 5000 mg/kg/day sodium formate, 4 *Mthfd11<sup>+/+</sup>*, 14 *Mthfd11<sup>z/+</sup>*, and 8 *Mthfd11<sup>z/z</sup>* embryos were obtained from 3 litters between E15.5-18.5. This genotype distribution does not differ significantly from the expected Mendelian ratio (p=0.50), suggesting at least a partial rescue by formate. Next, the morphology of E10.5-E15.5 embryos from dams supplemented with 7500 mg/kg/day sodium formate was examined, obtaining 10 *Mthfd11<sup>+/+</sup>* embryos, 31 *Mthfd11<sup>z/+</sup>* embryos and 14 *Mthfd11<sup>z/z</sup>* embryos from 6 litters, again conforming to the expected Mendelian ratio (p=0.48). Of the 14 *Mthfd11<sup>z/z</sup>* embryos, 11 displayed normal neural tube closure and 3 had exencephaly (FIG. 5). Thus, compared to nulls from unsupplemented dams, formate supplementation at 7500 mg/kg/day gives a significantly higher frequency of nulls with normal neural tube closure (p=0.028). Importantly, 6 of the 14 *Mthfd11<sup>z/z</sup>* embryos were dissected from dams at E13.5 or E15.5, whereas no surviving *Mthfd11<sup>z/z</sup>* embryos were observed after E12.5 from unsupplemented dams. While supplemented versus non-supplemented embryos were not compared after E12.5 due to lethality of the unsupplemented embryos, formate supplementation partially rescued the growth defect in *Mthfd11<sup>z/z</sup>* embryos (FIGS. 5A, 5B). The crown-rump length of formate supplemented *Mthfd11<sup>z/z</sup>* E11.5 embryos was significantly greater than in unsupplemented *Mthfd11<sup>z/z</sup>* embryos (5.0±0.1 vs. 3.6±0.3 mm respectively, p<0.01). Supplementation had no significant effect on crown-rump length of wild-type embryos (5.9±0.2 mm).

**[0071]** Dietary Supplementation with Calcium Formate

**[0072]** Table 2 shows that maternal supplementation with calcium formate improves development and growth in *Mthfd11<sup>z/z</sup>* embryos. Pregnant dams were administered calculated doses of 250-2500 mg/kg/day. While *Mthfd11<sup>z/z</sup>* embryos dissected at E15.5 are significantly smaller than wild-type and *Mthfd11<sup>z/+</sup>* littermates, there is a dose-response effect, where an increase in growth is observed in *Mthfd11<sup>z/z</sup>* embryos with increasing maternal calcium formate intake (Table 2).

TABLE 2

Maternal supplementation with calcium formate improves development and growth in <i>Mthfd11<sup>z/z</sup></i> embryos					
Genotype	Crown-rump length (cm)				
	250 mg/kg	500 mg/kg	1500 mg/kg	2500 mg/kg	3500 mg/kg
<i>Mthfd11<sup>+/+</sup></i>	1.39 ± 0.1	1.32 ± 0.17	1.46 ± 0.2	1.43 ± 0.07	1.34 ± 0.2
<i>Mthfd11<sup>z/+</sup></i>	1.38 ± 0.1	1.36 ± 0.21	1.46 ± 0.1	1.39 ± 0.09	1.33 ± 0.1



TABLE 2-continued

Maternal supplementation with calcium formate improves development and growth in <i>Mthfd11</i> <sup>+/z</sup> embryos					
Genotype	Crown-rump length (cm)				
	250 mg/kg	500 mg/kg	1500 mg/kg	2500 mg/kg	3500 mg/kg
<i>Mthfd11</i> <sup>+/z</sup>	1.05 ± 0.1	1.03 ± 0.06	1.17 ± 0.13	1.20 ± 0.04	1.1 ± 0.2
total <i>Mthfd11</i> <sup>+/z</sup> embryos	6	11	13	15	10
number with facial defect	4	8	4	0	3
number with NTD	2	0	1	5	1
number of litters	7	5	5	5	7
CR length ttest, wt vs null	6.30E-06	9.10E-005	0.00016	4.40E-010	0.013
CR length ttest, wt vs het	0.89	0.63	0.88	0.22	0.89
null vs. null at 2500 mg/kg	4.17E-06	2.46923E-08	0.544314488	—	0.064369727

**[0073]** As shown in Table 3 and FIG. 6, calcium formate improves NTD and orofacial defect phenotypes in a dose-dependent manner in the mouse model.

TABLE 3

Maternal supplementation with calcium formate improves NTD and orofacial phenotypes in <i>Mthfd11</i> <sup>+/z</sup> embryos					
	250 mg/kg	500 mg/kg	1500 mg/kg	2500 mg/kg	3500 mg/kg
orofacial defect	4	8	4	0	3
NTD	1	0	1	5	1
orofacial defect and NTD	1	0	0	0	0
no defect observed	0	3	8	10	6
total <i>Mthfd11</i> <sup>+/z</sup> embryos	6	11	13	15	10
	Orofacial/NTD Phenotype as a percentage				
orofacial defect	0.66666667	0.72727273	0.30769231	0	0.3
NTD	0.16666667	0	0.07692308	0.33333333	0.1
orofacial defect and NTD	0.16666667	0	0	0	0
no defect observed	0	0.27272727	0.61538462	0.66666667	0.6

#### **[0074]** Discussion

**[0075]** This study shows that all embryos lacking *Mthfd11* exhibit aberrant neural tube closure including craniorachischisis and exencephaly and/or a wavy neural tube. The NTD phenotype (exencephaly and craniorachischisis) is accompanied by abnormal neural tube morphology characterized by asymmetric bulges in the neuroepithelium and a wider lumen in wavy areas of the neural tube. In addition to the NTD phenotype, *Mthfd11*<sup>+/z</sup> embryos show immature maxillary and mandibular process development. Finally, maternal formate supplementation is shown to significantly reduce the incidence of NTDs, partially rescue the growth defect, and allow survival past the point of lethality seen in unsupplemented *Mthfd11*<sup>+/z</sup> embryos. This knockout mouse is the first fully penetrant folate-pathway mouse model that does not require feeding a folate-deficient diet to cause these phenotypes. More than ten folate-related mouse mutants have been characterized thus far (Harris MJ & Juriloff DM (2010) Birth Defects Res A Clin Mol Teratol 88:653-669), but NTDs are observed in only three: *Folr1*, *Shmt1* and *Amt*. *Folr1* encodes folate receptor 1, one of the major folate transport systems, and homozygous knockout of *Folr1* produces a severe folate deficiency in the embryo that can be rescued with maternal 5-formyl-THF supplementation (Spiegelstein O, et al. (2004)

Dev. Dyn. 231:221-231). This rescue is “tunable”, and depending on the dose of 5-formyl-THF administered to mothers during gestation, *Folr1*<sup>-/-</sup> embryos develop NTDs and orofacial deformities, or can be rescued to birth. Homozygous knockout of *Shmt1*, which encodes a cytoplasmic folate-metabolizing enzyme (FIG. 1, reaction 4), gives rise to a low frequency of NTDs in embryos from *Shmt1*<sup>-/-</sup> dams fed a folate-deficient diet (Beaudin A E, et al. (2011) Am. J. Clin. Nutr. 93:789-798; Beaudin A E, et al. (2012) Am. J. Clin. Nutr. 95:109-114). *Amt* encodes an aminomethyl-transferase that is a subunit of the mitochondrially localized glycine cleavage system (GCS) which processes glycine to donate 1C units to THF, forming CH<sub>2</sub>-THF (FIG. 1, reaction 5). Homozygous deletion of *Amt* is embryonic lethal, and *Amt*<sup>-/-</sup> embryos develop NTDs with 87% penetrance (Narisawa A, et al. (2012) Hum Mol Gen 21:1496-1503). The phenotype exhibited by the *Amt* knockout mouse suggests that the demand for 1C units is high during neurulation. This is consistent with the observation that maternal supplementation with methionine, which provides 1C units via the methyl cycle (FIG. 1), significantly improves neurulation in *Amt* null embryos while folic acid has no effect (Narisawa A, et al. (2012) Hum Mol Gen 21:1496-1503). The importance of the GCS to neural tube development is also supported by

the occurrence of NTDs in the *nehe* mouse (Zhou X & Anderson K V (2010) *Dev. Biol.* 344:185-195). The *nehe* mouse carries a hypomorphic allele of lipoic acid synthetase, the mitochondrial enzyme that catalyzes the synthesis of lipoic acid, an essential cofactor for GCS and several other mitochondrial enzymes. One other folate pathway mouse model, targeting *Mthfd1* (FIG. 1, cytoplasmic reactions 1-3), also displays disorganized neural tube closure in heterozygous *Mthfd1<sup>g+/+</sup>* embryos from *Mthfd1<sup>g/-</sup>* dams fed a folate-deficient diet (Beaudin A E, et al (2012) *Am. J. Clin. Nutr.* 95:882-891). Although the defects in this model are not classic NTDs, they are reminiscent of the wavy neural tube phenotype observed in *Mthfd11<sup>+/z</sup>* embryos.

**[0076]** The fact that *Mthfd11<sup>+/z</sup>* embryos develop NTDs confirms that integrity of the mitochondrial 1C pathway is essential for normal neural tube development. As illustrated in FIG. 1, mitochondria possess multiple enzymes that produce CH<sub>2</sub>-THF from various 1C donors (reactions 4m, 5, 8, and 9) (Tibbetts A S & Appling D R (2010) *Annu. Rev. Nutr.* 30:57-81). Embryonic mitochondria also possess redundant dehydrogenase/cyclohydrolase enzymes that can oxidize CH<sub>2</sub>-THF to 10-CHO-THF (MTHFD2 and MTHFD2L; reactions 2m and 3m). MTHFD2 is expressed in transformed cells and embryonic or nondifferentiated tissues, but not in adult differentiated tissues (Christensen K E & Mackenzie R E (2008) *Vitam. Horm.* 79:393-410). Homozygous knockout of *Mthfd2* is embryonic lethal, but does not cause NTDs (Di Pietro E, et al (2002) *Mol. Cell Biol.* 22:4158-4166). *Mthfd2* nullizygous embryos develop to about E15.5 and display no gross developmental abnormalities, but they are noticeably smaller and paler than wild-type and heterozygous littermates. The lack of NTDs in *Mthfd2* nullizygous embryos is likely due to the existence of MTHFD2L, a second mitochondrial dehydrogenase/cyclohydrolase. MTHFD2L is expressed in embryos and adults (Bulusani S, et al. (2011) *J. Biol. Chem.* 286:5166-5174) and can presumably support mitochondrial 1C flux to the level of 10-formyl-THF in *Mthfd2* nullizygous embryos.

**[0077]** On the other hand, only one enzyme with 10-formyl-THF synthetase activity (MTHFD1L) is known to exist in mitochondria, and this activity is required to produce formate and THF from 10-CHO-THF (FIG. 1, reaction 1m). Thus, loss of MTHFD1L activity is expected to completely abolish mitochondrial formate production. This metabolic block would then starve the cytoplasm for formate, creating a "formyl trap", analogous to the methyl trap seen when 5-methyl-THF accumulates in vitamin B<sub>12</sub>-deficient cells (Herbert V & Zalusky R (1962) *J. Clin. Invest.* 41:1263-1276; Noronha J M & Silverman M (1962) *Vitamin B12 and Intrinsic Factor*, 2nd European Symposium, ed Heinrich H C (Verlag, Stuttgart), pp 728-736). Although the *Mthfd11* knockout mouse is replete of folate, it has no way to catalyze the conversion of 10-formyl-THF to THF plus formate, leading to a trapping of mitochondrial 1C units as 10-formyl-THF, which cannot exit the mitochondrion. This in turn would cause a deficiency in cytoplasmic 1C units, which are needed in stoichiometric amounts for purine and thymidylate production as well as the methyl cycle. The phenotypes of the *Shmt1* and *Amt* knockout mice are consistent with this model of mammalian 1C metabolism. The *Shmt1* gene, which encodes cytoplasmic serine hydroxymethyltransferase (FIG. 1, cytoplasmic reaction 4), is not essential in mice (Macfarlane A J, et al. (2008) *J. Biol. Chem.* 283:25846-25853), indicating that mitochondrial SHMT (mitochondrial reaction 4m) is fully capable of providing all the 1C units needed in both the embryo and the adult. The neural tube phenotype in *Amt* nullizygous embryos lacking GCS activity (mitochondrial reaction 5) (Narisawa A, et al. (2012) *Hum. Mol. Gen.* 21:1496-

1503) is less severe than that in *Mthfd11* knockout embryos. Presumably, the existence of alternative mitochondrial 1C donors (e.g. serine) allows some of the *Amt<sup>-/-</sup>* embryos to develop normally. On the other hand, all mitochondrial 1C units, whatever their source, must pass through the MTHFD1L reaction to supply the cytoplasm with formate, and any defect in this step would be expected to cause a more severe phenotype. The importance of mitochondrially-derived formate is demonstrated by the disclosed results showing a significant reduction in the incidence of NTDs and partial rescue of *Mthfd11<sup>+/z</sup>* embryonic growth with maternal formate supplementation.

**[0078]** MTHFD1L thus controls the flux of 1C units from mitochondria into cytoplasmic processes such as purine and thymidylate biosynthesis and the methyl cycle (FIG. 1). De novo purine biosynthesis is essential for cell division, and S-adenosylmethionine (AdoMet) synthesis is critical for chromatin and DNA methylation, which play essential roles during cell differentiation (Bai S, et al. (2005) *Mol. Cell Biol.* 25:751-766; Kobayakawa S, et al. (2007) *Genes Cells* 12:447-460) and cell migration (Horswill M A, et al. (2008) *Exp. Eye Res.* 86:586-600). Epigenetic modifications are particularly dynamic, with extensive reprogramming of DNA methylation during early embryogenesis (Borgel J, et al. (2010) *Nat. Genet.* 42:1093-1100). Disruption of the methyl cycle is known to induce NTDs in cultured mouse embryos (Dunlevy L P, et al. (2006) *Birth Defects Res. A Clin. Mol. Teratol.* 76:544-552) and cranial defects are observed in DNA methyltransferase 3b nullizygous embryos (Okano M, et al. (1999) *Cell* 99:247-257). Recent studies in humans have linked maternal folate status and occurrence of NTDs to tissue-specific DNA hypo- and hypermethylation patterns, with decreased DNA methylation observed in NTD brain tissue compared to normal embryos (Chang H, et al. (2011) *J. Nutr. Biochem.* 22:1172-1177). Protein methylation is also involved in neural tube development, and hypomethylation of neural tube proteins is accompanied by a failure of the neural tube to close in rat embryos cultured in methionine-deficient conditions (Coelho C N & Klein N W (1990) *Teratology* 42:437-451). The cytoskeletal proteins  $\beta$ -actin and tubulin are known to be methylated during neural tube closure (Moe-phuli S R, et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:543-548), and proper function of the cytoskeleton is required for cranial neural tube closure (Copp A J, et al. (2003) *Nat. Rev. Genet.* 4:784-793).

**[0079]** A common polymorphism in *Mthfd11* has been shown to be strongly associated with NTD risk in an Irish population (Parle-McDermott A, et al. (2009) *Hum. Mutat.* 30:1650-1656), suggesting that MTHFD1L also plays an important role in human neural tube development. Importantly, disruption of MTHFD1L function does not cause cellular folate deficiency (like a transport defect), rather it blocks a specific metabolic step the production and release of formate from mitochondria into the cytoplasm. This metabolic defect causes aberrant neural tube closure including craniorachischisis and exencephaly and/or a wavy neural tube phenotype in 100% of *Mthfd11<sup>+/z</sup>* embryos (FIG. 3).

**[0080]** Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

**[0081]** Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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gcacugggua ucaagaagag agacauccuc ucgaagaaga ccaaggaccu gaccaagcug	960
aaagagugca uggauaagua uggccugaau cuaccaaugg ugacauacgu gaaagaugaa	1020
cucagaucu	1029

<210> SEQ ID NO 3  
 <211> LENGTH: 1245  
 <212> TYPE: RNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: CpG-low Region 1

<400> SEQUENCE: 3

gucgacucag uggugccagu caacaauauc aaagucaacc ugcaaagcau ggaugcuuau	60
cauuuaggag ucaauacagg gaaccaccag ggggaaaaga uuuuugcuu ccaauugcag	120
ccuggguuag agucuguuuu caagagaacc cuuauggggg agauucuuua uuauuugca	180
cacugguacg ggagcauuua gcugacaauuc acauuuugug gaucugccau ggcaacugga	240
aaacucuugu uagcuuauuc accaccaggu gcugaugugc cugcaaccag gaaacaggcu	300
auguuaggca cacacaugau uugggauuaa gggcuucagu ccagcugugu uuugugcauc	360
ccauggauaa gucagacaca cuacagguua gugcaacaag augaaucac aagugcaggc	420
aaugugacau guugguacca aacaggaaua guggugcccc cuggcacucc aaauaagugu	480
guagugcuuu guuuugcauc agcuuguaau gauuucucag uuaggauugu uagggacacc	540
ccuuucauag gacaaacagc acugcugcaa ggagacacag aaacagcuau ugaccaugca	600
auugccaggg uagcagauac uguggcaagu gguccuagua auucaaccag uaucccagca	660
cucacagcag uugagacagg ucacaccuca caaguggagc ccagugauac aaugcagacu	720
agacauguca aaaacuacca cucuaggucu gagucaacug uggaaaacuu ucuaguagg	780
ucagcuugug uguacauaga agaguacuac accaaggacc aagacaugu uauuagguac	840
auguccugga caauaaaugc cagaagaauu gugcaauuga ggagaaaguu uagcuguuu	900
acauacauga gauuugauau ggaaauacc uuuguauuca caaguagaca acuaccuggg	960
acuagcauag cacaagauau gccaccacuc acccaccaga ucauguacau accaccaggu	1020
ggcccaguac caaacagugu aacagauuuu gccuggcaga caucaacaaa cccagcauu	1080
uuucggacag aaggaaaugc cccaccuagg augucuauuc cauucaucag uauuggcaau	1140
gcuuuuagca acuuuuauga ugggugguca cacuuuuccc aaauggugu guauggauac	1200
aaugcccuga acaacauggg caagcuguau gcaagacaug uuaac	1245

<210> SEQ ID NO 4  
 <211> LENGTH: 1029  
 <212> TYPE: RNA

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&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: CpG-low Region 2

&lt;400&gt; SEQUENCE: 4

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gaauucgcug uugcuaugau gaagagaaac ucaaguacag ugaagacuga guauggugag      60
uuuacuaugc ugggcaucua ugacaagugg gcaguuuugc caaggcaugc uaaaccugga      120
ccaaccaucc ugaugaauga ccaagagggu gggguguuag augccaagga acuaguggac      180
aaggauaggc cuaaccugga gcugacacua cucaaguuaa acagaaauga gaaguucaga      240
gacauacagag gcuucuuagg uaaggaggaa guggaaguca augaggcugu gcuggcaaua      300
aacacuagca aguuuuccua cauguacauu ccaguagggc agguuacaga uuauggcuuc      360
cuaaaccugg gugguacacc caccaaaaga augcuuaugu auaacuucc cacaagagca      420
ggccagugug gagggguacu auguccacu ggcaaaguuu ugggaaucca uguuggugga      480
aauggccauc aaggcuucuc agcagcacuu cucaaacacu acuuuaauga ugaacaagga      540
gagauugagu ucauugagag uucaaaggaa gcaggguucc caaucauuua ugcaccaggu      600
aaaaccaagc uggagccaag ugucuccac caaguauuug aaggcaacaa agagccagca      660
guccucagga acagugaccc aaggcucaaa gcuaauuuug aggaggccau cuuuuccaaa      720
uacauuggga augucaacac acacauagau gaauacaugu uggaggcugu ugaccuuau      780
gcaggacaau uggccacccu agauaucagc acugaaccaa ugaaguugga ggaugcugug      840
uauugguacug aaggccuuga agcucugac uuaacaacaa gugcaggcu ccccuauug      900
gcacugggua ucaagaagag agacauccuc ucaaagaaga ccaaggaccu gaccaagcug      960
aaagagugca uggauaagua uggccugaau cuaccaaugg ugacauaugu gaaagaugaa     1020
cucagacu      1029

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&lt;210&gt; SEQ ID NO 5

&lt;211&gt; LENGTH: 1245

&lt;212&gt; TYPE: RNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: UpA-low Region 1

&lt;400&gt; SEQUENCE: 5

```

gucgacuccg uggugcccgu caacaacauc aaagucaacc ugcaaagcau ggaugcgau      60
cacauugagg ucaacaccgg gaaccaccag ggggaaaaga uuuuugcguu ccaaauagcag     120
ccggguugg agucguuuu caagagaacc cucauggggg agauucuaa uuauuugca      180
cacuggucag ggagcaucaa gcugacaauu acuuuuugug gaucggcgau ggcaacugga      240
aaacucuugu uggcgauuuc accaccaggu gcugaugugc ccgcgaccag gaaacaggcg      300
auguugggca cacacaugau ugggacauc gggcuucagu cgagcugugu uuugugcauc      360
ccauggauca gucagacaca cuaccguug gugcaacaag augaaucac gagugcaggc      420
aaugugacgu guugguacca aacaggaaui guggugcccc cuggcacucc aaacaagugu      480
gucgugcuuu guuuugcauc agcuugcaau gauuucucag uucgaugcu gagggacacc      540
ccuuucaucg gacaaacagc acugcugcaa ggcgacaccg aaacggcgau ugacaaugca      600
aucgccaggg ugcagacac gguggcgagc gguccgagca auucgaccag cauccagca      660
cucacagcag uugagacagg ucacacguca caagucgagc ccagcgacac aaugcagacc      720

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agacauguca aaaacuacca cucgcguucu gagucaaccg uggaaaacuu ucucagucgc	780
uccgcguugug uguacaucga agaguacuac accaaggacc aagacaaugu caacagguac	840
augucgugga caaucaaugc cagaagaug gugcaauuga ggagaaaguu ugagcuguuc	900
acauacauga gauuugacau ggaaauacg uuugucauca caagcagaca acuuuccuggg	960
acgagcaucg cacaagacau gccgccacuc acccaccaga ucauguacau cccaccaggu	1020
ggcccggucc caaacagcgu cacagauuuu gcguggcaga caucaacaaa cccagcauu	1080
uucuggacag aaggaaacgc gccaccucgc auguccauuc cauucaucag cauuggcaau	1140
gcuaucagca acuuucauga cgggugguca cacuuuuccc aaaacggugu guacggauac	1200
aacgcccuga acaacauggg caagcuguac gcacgucaug uuaac	1245

<210> SEQ ID NO 6  
 <211> LENGTH: 1029  
 <212> TYPE: RNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: UpA-low Region 2

<400> SEQUENCE: 6

gaauucgccg uugccaugau gaagagaaac ucaagcacag ugaagacuga gnauggugag	60
uucacgaucg ugggcaucua ugacaagugg gccguuuugc cagccaugc caaaccugga	120
ccaaccaucc ugaugaauga ccaagagguc ggcguguugg acgccaagga acugguggac	180
aaggauugca caaaccugga gcugacacuc cucaaguuga accggauga gaaguucaga	240
gacaucagag gcuucuuggc gaaggaggaa guggaaguca acgaggcugu gcuggcaauc	300
aacaccagca aguuccaaa cauguacauu ccaguugggc aggucacaga uuacggcuuc	360
cugaaccugg gugggacacc caccaaaaga augcucaugu acaacuuccc cacaagagca	420
ggccagugug gcggggugcu cauguccacu ggcaaaguuu ugggaaucca uguuggugga	480
aauggccauc aaggcuucuc agcagcacuu cucaaacacu acuucaauga ugaacaagga	540
gagauugagu ucauugagag uucaaaggaa gcaggguucc caaucauca cgcaccagc	600
aaaaccaagc uggagccaag ugucuuccac caaguguuug aaggcaaca agagccagca	660
guccucagga acagugacc cgcucuaaa gccaaauucg aggaggccau cuuuuccaaa	720
uacauuggga augucaacac acacaucgau gaauacaugu uggaggcugu ugaccauuau	780
gccggacaa uggccaccu ugacaucagc acugaaccaa ugaaguugga ggaugcugug	840
uacggcacug aaggccuuga agcucuugac uugacaacaa gugcaggcua ccccuuguc	900
gcacugggga ucaagaagag agacauccuc ucgaagaaga ccaaggaccu gaccaagcug	960
aaagagugca uggacaagua uggccugaau cuuccaagg ugacauacgu gaaagaugaa	1020
cucagaucu	1029

<210> SEQ ID NO 7  
 <211> LENGTH: 1245  
 <212> TYPE: RNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: CpG and UpA-low Region 1

<400> SEQUENCE: 7

gucgacucag uggugccagu caacaacauc aaagucaacc ugcaaagcau ggaugcuuau	60
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cacauugagg ucaacacagg gaaccaccag ggggaaaaga uuuuugcuuu ccaaaugcag	120
ccuggguugg agucuguuuu caagagaacc cugauggggg agauucugaa uuauuugca	180
cacuggucag ggagcaucaa gcugacauuc acuuuuugug gaucugccau ggcaacugga	240
aaacucuugu ugguuuauuc accaccaggu gcugaugugc cugcaaccag gaaacaggcc	300
auguugggca cacacaugau uugggacauu gggcuucagu ccagcugugu uuugugcauc	360
ccauggauca gucagacaca cuacagguug gugcaacaag augaaucac aaugcaggc	420
aaugugacau guugguacca aacaggaauu guggugcccc cuggcacucc aaacaagugu	480
guugugcuu guuuugcauc agcuugcaau gauuucucag ucaggauucu cagggaacc	540
ccuuucauug gacaaacagc acugcugcaa ggagacacag aaacagccau ugacaaugca	600
auugccaggg uugcagacac uguggcaagu gguccaagca auucaaccag cauccagca	660
cucacagcag uugagacagg ucacaccuca caaguggagc ccagugacac aaugcagaca	720
agacauguca aaaacuacca cuccaggucu gagucaacug uggaauuu ucucagcagg	780
ucagcuugug uguacauuga agaguacuac accaaggacc aagacaauu caacagguac	840
auguccugga caaucauugc cagaagaauu gugcaauuga ggagaaugu ugagcuguuc	900
acauacauga gauuugacau ggaaauacc uuugugauca caagcagaca acucccuggg	960
acaagcauug cacaagacau gccaccacuc acccaccaga ucauguacau uccaccaggu	1020
ggcccagugc caaacagugu cacagauuu gccuggcaga caucaacaaa cccagcauu	1080
uucuggacag aaggaaugc cccaccaagg auguccauuc cauucaucag cauuggcaau	1140
gcuaucagca acuuucauga ugggugguca cacuuuuccc aaaauggugu guauggauac	1200
aaugcccuga acaacauggg caagcuguau gcaagacau uuaac	1245

&lt;210&gt; SEQ ID NO 8

&lt;211&gt; LENGTH: 1029

&lt;212&gt; TYPE: RNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: CpG and UpA-low Region 2

&lt;400&gt; SEQUENCE: 8

gaauucgucg uugccauau gaagagaac ucaagcacag ugaagacuga guauggugag	60
uucaccaugc ugggcaucua ugacaagugg gcaguuuugc caaggcaugc caaaccugga	120
ccaaccuucc ugaugauga ccaagagguu gggguguugg augccaagga acugguggac	180
aaggauugga ccaaccugga gcugacacuu cucaaguuga acagaauga gaaguucaga	240
gacaucagag gcuucuggc caaggaggaa guggaaguca augaggcugu gcuggcauuc	300
aacaccagca aguucccaa cauguacauu ccaguggggc aggugacaga uuauggcuuc	360
cugaaccugg guggaacacc caccaaaaga augcucaug acaacuucc cacaagagca	420
ggccagugug gagggguuuc cauguccacu ggcaaaguu uggaauuca uguuggugga	480
aauggccauc aaggcuucuc agcagcacuu cucaaacacu acuucaauga ugaacaagga	540
gagaauagau ucauugagag uucaaaggaa gcaggguucc caaucauca ugcaccagc	600
aaaaccaagc ugagaccaag ugucuuccac caaguguuug aaggcaaca agagccagca	660
guccucagga acagugaccc aaggcucaaa gccaaauuug aggaggccau cuuuuccaaa	720
uacauuggga augucaacac acacauugau gaauacaugu ugagggcugu ugaccauuu	780

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```
gcaggacaa uggccacccu ggacauacagc acugaaccaa ugaaguugga ggaugcugug 840
uaugggcacug aaggccuuga agcucuugac uugacaacaa gugcaggcua ccccuauugug 900
gcacuggggga ucaagaagag agacauccuc ucaaagaaga ccaaggaccu gaccaagcug 960
aaagagugca uggacaagua ugccugaa cucccaaugg ugacauaugu gaaagaugaa 1020
cucagaucu 1029
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<210> SEQ ID NO 9
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: E7 qRT-PCR sense primer
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<400> SEQUENCE: 9
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```
tccggcccct gaatcgggct aa 22
```

```
<210> SEQ ID NO 10
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: E7 qRT-PCR antisense primer
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<400> SEQUENCE: 10
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```
cacccaaagt agtcggttcc gc 22
```

```
<210> SEQ ID NO 11
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: outer, sense sequencing primer
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<400> SEQUENCE: 11
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```
cccaatttga tgtaacacca cacatgg 27
```

```
<210> SEQ ID NO 12
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: inner, sense sequencing primer
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<400> SEQUENCE: 12
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```
gatattccag gcgaagtaca caacc 25
```

```
<210> SEQ ID NO 13
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: EV7 WT, outer, antisense sequencing primer
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<400> SEQUENCE: 13
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```
caaagcacta cacacttatt tggag 25
```

```
<210> SEQ ID NO 14
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<223> OTHER INFORMATION: R1/R2 CpG-high, outer, antisense sequencing primer

<400> SEQUENCE: 14

attcgaacgg agaaatcggt ac 22

<210> SEQ ID NO 15

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: R1/R2 UpA-high, outer, antisense sequencing primer

<400> SEQUENCE: 15

tcccttagca tacgtactga gaaat 25

<210> SEQ ID NO 16

<211> LENGTH: 21

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: EV7 WT, inner, antisense sequencing primer

<400> SEQUENCE: 16

gcaccactat tcctgtttgg t 21

<210> SEQ ID NO 17

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: R1/R2 CpG-high, inner, antisense sequencing primer

<400> SEQUENCE: 17

aacaaagcac gacgcactta tt 22

<210> SEQ ID NO 18

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: R1/R2 UpA-high, inner, antisense sequencing primer

<400> SEQUENCE: 18

cattacaagc tgatgcaaaa catag 25

<210> SEQ ID NO 19

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: outer, sense sequencing primer

<400> SEQUENCE: 19

tgagcccgtat catcaaatca 20

<210> SEQ ID NO 20

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: inner, sense sequencing primer

<400> SEQUENCE: 20

ttttaacccc acgaacctga 20

<210> SEQ ID NO 21

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: outer, antisense sequencing primer

<400> SEQUENCE: 21

ttgccgagtt gttcgacata 20

<210> SEQ ID NO 22

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: inner, antisense sequencing primer

<400> SEQUENCE: 22

caagtcacgg atgtctgcaa 20

<210> SEQ ID NO 23

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: region 1 sense (outer) PCR primer

<400> SEQUENCE: 23

cccaatttga tgtaacacca cacatgg 27

<210> SEQ ID NO 24

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: region 1 sense (inner) PCR primer

<400> SEQUENCE: 24

gatattccag gcgaagtaca caacc 25

<210> SEQ ID NO 25

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: region 1 antisense (outer) PCR primer

<400> SEQUENCE: 25

cccatactcg gatgtgcttg gg 22

<210> SEQ ID NO 26

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: region 1 antisense (inner) PCR primer

<400> SEQUENCE: 26

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cactcggatt gtgcttgaca tctg 24

<210> SEQ ID NO 27  
<211> LENGTH: 23  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: region 2 sense (outer) PCR primer

<400> SEQUENCE: 27

caaggagcat acacaggaat acc 23

<210> SEQ ID NO 28  
<211> LENGTH: 22  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: region 2 sense (inner) PCR primer

<400> SEQUENCE: 28

ggtacctact cttaggcaag ca 22

<210> SEQ ID NO 29  
<211> LENGTH: 23  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: region 2 antisense (outer) PCR primer

<400> SEQUENCE: 29

gaatgtctgc ctcacgcga act 23

<210> SEQ ID NO 30  
<211> LENGTH: 23  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: region 2 antisense (inner) PCR primer

<400> SEQUENCE: 30

aagctggacg cttcaatgag cct 23

<210> SEQ ID NO 31  
<211> LENGTH: 24  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: GAPDH qRT-PCR sense primer

<400> SEQUENCE: 31

gaaatcccat caccatcttc cagg 24

<210> SEQ ID NO 32  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: GAPDH qRT-PCR antisense primer

<400> SEQUENCE: 32

gagccccagc cttctccatg 20

<210> SEQ ID NO 33

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```

<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: IFN-beta qRT-PCR sense primer

```

```

<400> SEQUENCE: 33

```

```

gaccaacaag tgtctcctcc aaa

```

23

```

<210> SEQ ID NO 34
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: IFN-beta qRT-PCR antisense primer

```

```

<400> SEQUENCE: 34

```

```

gaactgctgc agctgcttaa tc

```

22

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1. A synthetic RNA expression vector comprising a sequence encoding an expression product, the nucleic acid comprising at least one region in which the nucleotide composition has been modified such that the frequency of at least one of CpG and UpA dinucleotides is reduced relative to normal frequency.

2.-3. (canceled)

4. The synthetic RNA expression vector of claim 1 which is a recombinant RNA viral vector.

5. The synthetic RNA expression vector of claim 1 in which the frequency of both CpG and UpA dinucleotides is reduced relative to normal frequency.

6. The synthetic RNA expression vector of claim 1 in which the frequency of CpG dinucleotides in the at least one region in which the nucleotide composition has been modified is reduced by at least 50%.

7. The synthetic RNA expression vector of claim 1 in which the frequency of UpA dinucleotides in the at least one region in which the nucleotide composition has been modified is reduced by at least 50%.

8. (canceled)

9. The synthetic RNA expression vector of claim 1 in which the frequency of at least one of CpG and UpA dinucleotides in the at least one region in which the nucleotide composition has been modified such that it contains no CpG and/or UpA dinucleotides.

10. The synthetic RNA expression vector of claim 1 in which, considering the synthetic RNA expression vector as a whole, the frequency of at least one of CpG and UpA dinucleotides is reduced by at least 20%.

11. (canceled)

12. The synthetic RNA expression vector of claim 1 in which the frequency ratio of the relevant dinucleotide is 0.4 or lower.

13. The synthetic RNA expression vector of claim 1 in which the region of nucleic acid with reduced frequency of CpG or UpA dinucleotides is in a sequence which encodes an expression product.

14. (canceled)

15. The synthetic RNA expression vector of claim 1 in which substantially all non-constrained coding sequences of the vector have been modified to reduce the frequency of at least one of CpG and UpA dinucleotides.

16. The synthetic RNA expression vector of claim 1 in which substantially all of the coding regions of the synthetic RNA expression vector have been modified to have a reduced at least one of CpG and UpA dinucleotide frequency.

17. The synthetic RNA expression vector of claim 1 in which regions totaling at least 50% of the total length of the synthetic RNA expression vector have been modified to reduce the at least one of CpG and UpA dinucleotide frequency.

18.-22. (canceled)

23. The synthetic RNA expression vector of claim 1 wherein the synthetic RNA expression vector comprises a recombinant single stranded RNA (ssRNA) virus genome.

24. (canceled)

25. The synthetic RNA expression vector of claim 1 which comprises a RNA virus adapted for expression in a suitable expression system for the production of a virus vaccine.

26.-28. (canceled)

29. A virion comprising a synthetic RNA expression vector according to claim 1.

30. (canceled)

31. A host cell comprising a synthetic RNA expression vector according to claim 1.

32.-34. (canceled)

35. An RNA virus vaccine composition, the composition comprising a virion according to claim 29, which comprises a sequence encoding one or more antigens.

36. A method of producing an expression product, the method comprising:

providing a host cell comprising a synthetic RNA expression vector according to claim 1;  
incubating said cell under suitable conditions to induce expression from the vector; and  
recovering the expression product.

37. (canceled)

38. The method of claim 36 wherein the expression product is a virion.

39. The method of claim 36 wherein the method includes the following steps:

providing an RNA sequence encoding an expression product;

altering the nucleotide composition of said sequence to reduce the frequency of at least one of CpG and UpA dinucleotides; and  
introducing a synthetic RNA expression vector comprising said nucleic acid into a host cell.

**40.** (canceled)

**41.** The method of claim **36** which is a method for production of a viral vaccine.

**42.-44.** (canceled)

**45.** A method of producing a synthetic RNA expression vector, the method comprising:

providing a primary nucleotide sequence of interest;  
identifying at least one region of said primary nucleotide sequence susceptible to modification to reduce the frequency of at least one of CpG and UpA dinucleotides;  
identifying one or more sequence modifications in said at least one region which will reduce the frequency of at least one of CpG and UpA dinucleotides;  
providing a modified nucleotide sequence comprising some or all of said sequence modifications;  
producing a synthetic RNA expression vector comprising said modified sequence which has a reduced frequency of at least one of CpG and UpA dinucleotides compared to a corresponding synthetic RNA expression vector which comprises the primary nucleotide sequence.

**46.-49.** (canceled)

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