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High Levels of Iron Supplementation Prevents Neural Tube Defects in the Fpn1ffe Mouse Model

Bethany A. Stokes1,2, Julia A. Sabatino2, and Irene E. Zohn*2

Introduction

Neural tube defects (NTDs) are among the most common structural birth defects in humans affecting anywhere from 1 in 100 to 6 in 10,000 live births (Li et al., 2006; Parker et al., 2010; Zohn, 2012, 2014; Liu et al., 2016). NTDs such as anencephaly and spina bifida occur when neural tube closure fails in the anterior and posterior ends of the neural tube, respectively. The causes of NTDs are complex and involve both genetic and environmental factors (Zohn and Sarkar, 2008; Zohn, 2012, 2014). Multiple studies implicate periconception maternal nutrition as an important factor influencing the occurrence of NTDs and folic acid has emerged as an important micronutrient (Smithells et al., 1976; Scott et al., 1990; Blom et al., 2006; Czeizel, 2009; Obican et al., 2010). Furthermore, human and animal studies demonstrate a clear benefit of folic acid supplementation for the prevention of NTDs (Czeizel, 2009; Gray and Ross, 2009; Harris, 2009; Obican et al., 2010).

To improve folate levels in women of childbearing age, wheat flour is now fortified with folic acid in many countries and is associated with significant reductions in the incidence of NTDs (Eichholzer et al., 2006; Crider et al., 2011; Obican et al., 2010). However, fortification has only reduced NTD rates to certain levels (Crider et al., 2011). Similarly, NTDs in many mouse models are not prevented by folic acid supplementation (Gray and Ross, 2009; Harris, 2009). Together these observations suggest that not all NTDs can be prevented by folic acid supplementation. Consequently, NTDs still represent a significant proportion of birth defects and there is a pressing need for additional strategies for prevention.

Other nutrients have emerged from retrospective studies as potential factors to influence the incidence of NTDs (Scott et al., 1990; Czeizel, 2009; Czeizel and Banhidy, 2011; Kappen, 2013). Iron deficiency is one of the most common micronutrient deficiencies in women of childbearing age (Lopez et al., 2016). Iron and folate deficiencies often occur simultaneously and iron and folate metabolism are linked in many ways (Herbig and Stover, 2002). However, unlike the wealth of data supporting the importance of folate in prevention of NTDs, only a handful of studies directly investigated the impact of iron and with mixed results (Weekes et al., 1992; Groenen et al., 2004; Felkner et al., 2005; Molloy...
et al., 2014). Mouse models with disruption of iron homeostasis have not provided clarity due to early embryonic lethality or redundancy (De Domenico et al., 2008).

Our previous studies suggested iron might be required for neural tube closure (Zohn et al., 2007; Mao et al., 2010). In the N-ethyl-N-nitroso-urea (ENU)-induced flat iron (ffe) mouse line, we identified a hypomorphic mutation in the iron exporter Fpn1 resulting in NTDs. During neurulation, Fpn1 is expressed in tissues essential for delivery of nutrients to the embryo (Donovan et al., 2000, 2005). Conditional deletion studies demonstrate that Fpn1 expression in the visceral endoderm and visceral endoderm-derived lineages of the yolk sac is critical for neural development (Mao et al., 2010). Multiple transporters are localized to the apical surface of the visceral endoderm to mediate iron uptake from the maternal environment, but Fpn1 is the only transporter on the basal surface responsible for export of iron out of the visceral endoderm to the developing embryo (Donovan et al., 2005). Thus mutation of Fpn1 is expected to result in iron overload in the visceral endoderm along with iron deficiency in the embryo proper.

The visceral endoderm not only provides nutrients to the embryo, but also functions as a specialized signaling center necessary for induction of the anterior neural tube (Srivivas, 2006; Stower and Srivivas, 2014). Mutations that affect formation and/or function of the anterior visceral endoderm (AVE) result in a spectrum of phenotypes ranging from mild anterior truncations to headless embryos (Thomas and Beddington, 1996; Acampora et al., 1998; Kimura et al., 2000, 2005). Thus mutation of Fpn1 is expected to result in iron overload in the visceral endoderm along with iron deficiency in the embryo proper.

MATERIALS AND METHODS

MOUSE LINES AND DIET SUPPLEMENTATION

The Fpn1ffe mouse line was described previously (Zohn et al., 2007) and crossed onto a C3H background (C3H/HeNcr, Charles River Laboratories) for at least 10 generations before analysis. The diets used in this study are based on theAIN-76A rodent diet and were manufactured by Research Diets, Inc (New Brunswick, NJ). High iron diets have added 0.5% carbonyl iron (Sigma) and folic acid supplementation with 10 ppm folic acid compared with 2 ppm in the control diet. A different color dye was added to each diet for ease of identification. Wild-type or Fpn1ffe/+ females from crosses between wild-type females and Fpn1ffe/+ males were switched from standard rodent chow (Tekland Global #2918 with 200 mg/kg iron and 4 mg/kg folate) to the four diets at weaning for approximately 4 weeks before mating.

ANALYSIS OF FERRITIN AND FOLATE IN DAMS AND EMBRYOS

Serum ferritin levels were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturers instructions (Abnova Ferritin [Mouse] ELISA Kit #KA1941). Folate levels were determined by the
microbiological method using *Enterococcus hirae* (ATCC 8043) as described (Horne and Patterson, 1988; Molloy and Scott, 1997). For determination of folate content in embryos, 11.5 dpc embryos were processed as described (Kur et al., 2014) then folate levels determined by the Microbiological assay. Blood samples were sent to Charles River Laboratories, Inc (USA) for CBC analysis.

**STATISTICAL METHODS**

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). All results are reported as mean ± SE. The Fisher's exact test was used to determine significance of reductions in NTD frequency. The significance of the effect of diets on nutrient levels and CBC analyses were determined using two-factor analysis of variance (ANOVA) with posthoc analysis by Sidak’s multiple comparisons or Tukey tests as indicated. Significance of changes in forebrain size, embryo weight, and crown rump length were determined by the unpaired t test.

**Results**

**IRON SUPPLEMENTATION REDUCES THE INCIDENCE OF NTD IN FPN1^{ffe/ffe} MUTANT EMBRYOS**

To determine if iron supplementation could reduce the incidence of NTD in *Fpn1^{ffe/ffe}* mutant embryos, *Fpn1^{ffe/+}* females were fed either a standard synthetic control diet or the identical diet supplemented with 0.5% carbonyl iron for 4 weeks beginning at weaning. Supplemented females were mated to *Fpn1^{ffe/+}* males and timed pregnancies recorded. For these studies, a relatively high dosage of supplemental iron (0.5% carbonyl iron) was used. Previous work demonstrated that the *Fpn1^{ffe}* mutation results in greatly reduced activity of the Fpn1 iron transporter (Zohn et al., 2007). Thus, we reasoned a high dosage of iron would be needed to allow for sufficient iron transport on this hypomorphic mutant background.

While *Fpn1^{ffe/+}* mutants do show both exencephaly and spina bifida (Mao et al., 2010), spina bifida is difficult to assess at earlier stages of development and only exencephaly was scored in this study. At 9.5 dpc, exencephaly was counted in 13 to 24 somite-staged embryos by visual inspection when the neural folds failed to transform from the convex to midline convergent morphology. In embryos dissected at 11.5 dpc, exencephaly was scored when the brain exhibited the “cauliflower like” morphology typical of exencephaly. As shown in Figure 1, the frequency of NTDs in embryos from dams fed the control diet was approximately 75% (n = 48). Supplementation with 0.5% carbonyl iron significantly reduced the incidence of NTDs to 40% (p = 0.0002). No difference was observed in the frequency of NTDs between embryos analyzed at 9.5 and 11.5 dpc (Supporting Figure S1, which is available online). These data demonstrate that NTDs in *Fpn1^{ffe}* mutants can be prevented by periconceptional iron supplementation.

At 9.5 dpc, crown-rump measurements indicate that *Fpn1^{ffe}* mutant embryos were smaller than wild-type littermates on both the control (p = 0.06) and high iron (p ≤ 0.01) diets (Fig. 2A). While smaller, mutant embryos dissected at 9.5 dpc were not developmentally delayed compared with wild-type littermates as indicated by somite numbers (Fig. 2B). Similarly, developmental stage was essentially the same in embryos dissected at 9.5 dpc from dams fed the control versus high iron diets (Fig. 2B). Weights of wild-type versus mutant embryos dissected at 11.5 dpc from dams fed either control or high folic acid diets were similar (p > 0.05). However, weights of mutant and wild-type embryos from dams fed the high iron diet were smaller than embryos from dams fed the control diets (p ≤ 0.005; Fig. 2C).

**NTDS IN THE FPN1^{ffe/ffe} LINE ARE NOT PREVENTED BY FOLATE SUPPLEMENTATION**

To determine if folic acid supplementation can prevent NTDs in the *Fpn1^{ffe}* model, heterozygous females were a fed diet containing 10 or 2 ppm folic acid (high folic acid and control diets, respectively) for 4 weeks before mating. This protocol prevents NTDs in some mouse lines (Carter et al., 1999; Marean et al., 2011), but in others has a negative impact on embryonic development (Marean et al., 2011). While no obvious adverse effects on development were observed on the *Fpn1^{ffe}* background, folic acid supplementation did not reduce the frequency of NTDs in *Fpn1^{ffe/ffe}* mutants (Fig. 1B; 82 vs. 75% p > 0.05). To determine if dual supplementation with folic acid and iron could further reduce the incidence of NTDs, *Fpn1^{ffe/+}* females were supplemented with a diet that contains both 10 ppm folic acid and 0.5% carbonyl iron. Dual supplementation did not reduce the frequency of NTDs beyond the reduction seen with iron supplementation alone (31 vs. 40%; p > 0.05). These results demonstrate that NTDs in the *Fpn1^{ffe/ffe}* mutant line are not prevented by folate supplementation.

Folate supplementation alone had no effect on the size of 9.5 dpc mutant embryos, but dual supplementation improved crown-rump length of mutant embryos (p ≤ 0.05; Fig. 2A). On the other hand, somite/developmental stage was not altered by maternal diet (p > 0.05; Fig. 2B). Weights of embryos dissected at 11.5 dpc were similar between wild-type and mutant embryos from folate or dual supplemented dams; and dual supplementation restored the reduction in embryo weight observed with iron supplementation (Fig. 2C).

**IRON SUPPLEMENTATION INCREASES THE IRON STATUS OF WILD-TYPE AND FPN1^{ffe/+} DAMS**

The effect of supplementation on iron status of wild-type and *Fpn1^{ffe/+}* dams was determined. Measurements of ferritin levels in the serum of pregnant dams served as a proxy of stored iron (Fig. 3). Maternal ferritin levels were increased with iron supplementation in both wild-type
dams (1.51 ± 0.45 vs. 7.85 ± 1.00 µg ferritin/ml; p ≤ 0.001) and to a greater degree in Fpn1ffe/+ dams (2.32 ± 0.46 vs. 15.81 ± 1.94 µg ferritin/ml; p ≤ 0.0001). This enhanced increase in ferritin was expected because the Fpn1ffe is a model of the iron overload disorder hemochromatosis type IV (Zohn et al., 2007). Folic acid supplementation did not alter the iron status of neither wild-type (1.51 ± 0.45 vs. 0.79 ± 0.27 µg ferritin/ml; p > 0.05) nor Fpn1ffe/+ dams (2.32 ± 0.46 vs. 1.81 ± 0.19 µg ferritin/ml; p > 0.05). Dual supplementation with iron and folic acid had no further effect on elevated iron status in wild-type dams (7.87 ± 1.00 vs. 7.29 ± 0.71 µg ferritin/ml; p > 0.05). In Fpn1ffe/+ dams, dual supplementation reduced the iron overload observed with iron supplementation alone (7.63 ± 2.41 vs. 15.81 ± 1.94 µg ferritin/ml; p ≤ 0.001).

**HIGH DOSE IRON SUPPLEMENTATION INFLUENCES FOLATE STATUS**

To determine if folic acid supplementation increased folate status of dams, the folate content of whole maternal blood was compared between wild-type and Fpn1ffe dams (Fig. 4A). Folate levels were lower in blood from pregnant Fpn1ffe/+ females than wild-type dams fed a control diet (34.99 ± 1.48 vs. 42.69 ± 1.55 ng folate/ml; p ≤ 0.05), indicating that Fpn1 deficiency has some impact on folate status. Folate levels increased with folic acid supplementation in both wild-type (42.69 ± 1.55 vs. 50.83 ± 1.82 ng folate/ml; p ≤ 0.05) and Fpn1ffe/+ (34.99 ± 1.48 vs. 47.29 ± 1.95 ng folate/ml; p ≤ 0.001) dams. Surprisingly, supplementation with 0.5% carbonyl iron significantly reduced maternal folate levels in wild-type dams (42.69 ± 1.55 vs. 21.60 ± 2.20 ng folate/ml; p ≤ 0.0001), which was ameliorated by dual supplementation (42.69 ± 1.55 vs. 37.63 ± 3.75 ng folate/ml; p > 0.05). Folate levels were also reduced in Fpn1ffe/+ dams on the high iron diet (42.69 ± 1.55 vs. 26.49 ± 0.95 ng folate/ml; p ≤ 0.001) and improved with dual supplementation (42.69 ± 1.55 vs. 32.14 ± 1.15 ng folate/ml; p ≤ 0.05).

Folate levels were also measured in embryos dissected at 11.5 dpc (Fig. 4B). Fpn1ffe/+ mutant embryos showed reduced folate levels compared with wild-type littermates (35.69 ± 3.17 vs. 13.17 ± 2.62 ng folate/g protein; p ≤ 0.001).
Folate levels did not correlate with NTDs when comparison was made between 
Fpn1 mutants with or without NTDs from dams fed the control diet (16.71 ± 
7.76 vs. 23.08 ± 8.52 ng folate/gm protein; p > 0.05, n = 3, not shown). Folate levels increased with folic acid sup-
plementation in both Fpn1ffe/ffe mutant embryos (13.17 ± 
2.62 vs. 43.97 ± 4.05 ng folate/gm protein; p ≤ 0.001) 
and wild-type littersmates (35.69 ± 3.17 vs. 56.92 ± 8.32 ng folate/gm protein; p ≤ 0.05). Supplementation with 
0.5% carbonyl iron greatly reduced folate levels in wild-
type littersmates (35.69 ± 3.17 vs. 8.37 ± 1.70 ng folate/
Iron supplementation slightly but not significantly reduce folate levels in Fpn1ffe/+ mutant embryos (13.17 ± 2.62 vs. 5.63 ± 3.00 ng folate/gm protein; p = 0.12), which improved to control levels with dual supplementation (13.17 ± 2.62 vs. 15.92 ± 3.14 ng folate/gm protein; p > 0.05).

Discussion

Our previous studies demonstrate that Fpn1ffe/+ mutant embryos show forebrain truncations (Mao et al., 2010). To determine if iron, folate, or combined supplementation can rescue forebrain defects in Fpn1ffe/+ mutants, the size of the forebrain was measured in 9.5 dpc embryos from pregnant dams fed the various diets. Measurements were done on embryos with NTDs. The forebrain was measured in 9.5 dpc embryos from wild-type, mutant, or dual supplementation restored forebrain size to wild-type levels in mutants with NTDs (0.44 ± 0.37 vs. 0.38 ± 0.03 mm; p > 0.05) and (0.44 ± 0.02 vs. 0.32 ± 0.05 mm; p > 0.05), respectively.

**TABLE 1.** Mild Macrocytic Anemia Occurs with High Iron Diet

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Red blood cell count x 10^6 cells/μL</th>
<th>Hemoglobin, g/dL</th>
<th>Hematocrit, %</th>
<th>Mean corpuscular volume, fL</th>
<th>Mean corpuscular hemoglobin, pg</th>
<th>Red cell distribution width, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.43 ± 0.31</td>
<td>15.23 ± 0.45</td>
<td>51.15 ± 0.03</td>
<td>53.65 ± 0.61</td>
<td>13.17 ± 0.37</td>
<td>16.20 ± 0.15</td>
</tr>
<tr>
<td>Fpn1ffe/+</td>
<td>9.54 ± 0.10</td>
<td>15.33 ± 0.40</td>
<td>52.13 ± 0.13</td>
<td>54.83 ± 1.34</td>
<td>15.73 ± 0.09</td>
<td>19.20 ± 0.02</td>
</tr>
<tr>
<td>Wildtype</td>
<td>9.93 ± 0.34</td>
<td>15.25 ± 0.03</td>
<td>51.15 ± 0.03</td>
<td>53.65 ± 0.61</td>
<td>15.33 ± 0.40</td>
<td>16.00 ± 0.17</td>
</tr>
</tbody>
</table>

**TABLE 2.** Effect of Iron and Folic Acid Supplementation on Hematologic Parameters

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Wildtype</th>
<th>Fpn1ffe/+</th>
<th>2-Factor ANOVA P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell count x 10^6 cells/μL</td>
<td>9.43 ± 0.31</td>
<td>9.54 ± 0.10</td>
<td>9.93 ± 0.34</td>
<td>0.001</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>15.23 ± 0.45</td>
<td>15.33 ± 0.40</td>
<td>15.25 ± 0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>51.15 ± 0.03</td>
<td>52.13 ± 0.13</td>
<td>51.15 ± 0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean corpuscular volume, fL</td>
<td>53.65 ± 0.61</td>
<td>54.83 ± 1.34</td>
<td>53.65 ± 0.61</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin, pg</td>
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<td>15.73 ± 0.09</td>
<td>15.33 ± 0.40</td>
<td>0.01</td>
</tr>
<tr>
<td>Red cell distribution width, %</td>
<td>16.20 ± 0.15</td>
<td>19.20 ± 0.02</td>
<td>16.00 ± 0.17</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**FIGURE 5.** A. Forebrain size of Fpn1ffe/+ mutants, which improved to control levels with dual supplementation. B. Forebrain size of Fpn1ffe/+ mutants, which improved to control levels with dual supplementation.
either iron deficiency in the embryo or iron overload in the visceral endoderm (Mao et al., 2010). In this study, we supplemented *Fpn1ffe* pregnancies with a relatively high iron diet and determined the effect on the incidence of NTDs and forebrain truncations. We predicted that iron supplementation would improve iron deficiency but also worsen iron overload in the visceral endoderm. Our data demonstrate that both NTDs and forebrain truncations are prevented by iron supplementation suggesting that these defects are likely due to iron deficiency. However, future experiments to measure iron levels in embryos and the visceral endoderm under these conditions are needed to definitively prove this assumption. Our data also suggest that NTDs in the *Fpn1ffe* model are folate resistant. While the *Fpn1ffe* mutant embryos have lower folate levels than wild-type littermates, folic acid supplementation did not prevent NTDs. These findings are not entirely surprising as folate deficiency alone is not sufficient to cause NTDs in the absence of additional factors (Burgoon et al., 2002; Burren et al., 2008, 2010). Thus our data indicate that NTDs in the *Fpn1* mouse line are iron responsive but folate resistant.

**INTERACTION OF IRON SUPPLEMENTATION AND FOLATE DEFICIENCY**

Our data highlight an important interaction between high levels of iron supplementation and folate status. While supplementation with relatively high levels of iron did prevent NTDs in the *Fpn1* mutant mouse line, it had negative effects on folate status in wild-type dams and embryos. Wild-type dams with high levels of iron supplementation showed signs of macrocytic anemia consistent with folate deficiency. This was further supported by improvement of anemia with the addition of folic acid supplementation. High levels of iron supplementation also resulted in reduced weight of embryo dissected at 11.5 dpc that were restored with dual supplementation. Human data support this negative interaction between high levels or iron supplementation/iron overload and folate status. For example, macrocytic anemia has been reported in individuals with the iron overload disorder hemochromatosis (see Koszewski, 1952; Granville and Dameshek, 1958; Arakawa et al., 1965; Toghill, 1965; for examples).

In our experiments, we used a relatively high level of iron supplementation to overcome the reduced iron transport activity of the Fpn1 transporter in the *Fpn1ffe* model. While these dosages are not likely given to pregnant women, this level of supplementation is within the range of carbonyl iron dosages given in humans with severe anemia. Recommendations for iron supplementation in human populations range from 16 mg iron per day in Canada to 60 mg iron per day by the World Health Organization (Stoltzfus and Dreyfuss, 1998; Cockell et al., 2009). In the United States, the average multivitamin has 18 mg iron and prenatal vitamins contain 30 mg carbonyl iron. However, for severe iron deficiency anemia, dosages of 120 to 360 mg/day carbonyl iron is given (7- to 20-fold increase) and 90 to 150 mg/day (5- to 8.3-fold increase) is typically prescribed during pregnancy.

To compare with guidelines in rodents, the National Research Council recommends 35 mg/kg iron in the average rodent diet and twice this amount during pregnancy (Subcommittee on Laboratory Animal Nutrition, 1995). Thus the addition of 0.5% (500 ppm) carbonyl iron used in this study represents an approximate 15-fold increase.

![Figure 5](image-url)  
**FIGURE 5.** Forebrain truncations in *Fpn1ffe* mutant embryos are rescued by supplementation with a high iron diet. A: In situ hybridization to detect *Six3* expression in 9.5 dpc embryos. The forebrain was measured from the rostral point of the optic vesicle (stained by *Six3*) to the most rostral point of the forebrain as indicated by white line. B: Forebrain measurements in 9.5 dpc wild-type (*Fpn1<sup>+/+</sup>* and *Fpn1ffeffe* embryos from dams fed control (yellow bar), high folic acid (10 ppm, orange bar), high iron (0.5% carbonyl iron, blue bar), or high folic acid and iron (purple bar) diets for 4 weeks before mating. Statistical significance was determined by the unpaired t test. *p*-values: \(<0.0005***\), or nonsignificant (ns). The number of samples represented in each group is indicated.
over the recommended supplementation levels for rodents but is well within the range of dosages recommended for patients with severe anemia. Future studies will determine if iron supplementation with equivalent dosages used during human pregnancy would also have a similar effect on folate status of mouse dams and embryos.

Iron and folate share many commonalities (Herbig and Stover, 2002). Simultaneous deficiencies are common especially during pregnancy and result in complications including increased risk of anemia, low birth weight, premature birth, and mortality. Both iron and folate serve as cofactors for enzymatic reactions involved in a variety of metabolic processes including DNA repair and synthesis. Iron supplementation might influence folate status at multiple levels. For example, Ferritin catabolizes folate into inactive metabolites (Suh et al., 2000, 2001). Another molecular link is the regulation of cytoplasmic serine hydroxymethyltransferase levels by iron (Oppenheim et al., 2000, 2001). Thus the high levels of ferritin in the serum of dams supplemented with iron could potentially cause or otherwise contribute to folate deficiency by catabolism of folate.

On the other hand, sites of iron and folate absorption and hemostasis in the mother and fetus overlap significantly and iron overload in these tissues could potentially interfere with folate absorption and/or metabolism. The primary site of both folate and iron absorption from the diet occurs in the enterocytes of the small intestine with common and distinct transporters (Lipinski et al., 2013; Visentin et al., 2014). Once absorbed, iron and folate are delivered to the liver for storage and/or mobilization to the circulation (Gambling et al., 2011; Lipinski et al., 2013; Visentin et al., 2014). Iron overload occurs in both intestinal enterocytes and liver macrophages with mutation of Fpn1 (Donovan et al., 2005; Zohn et al., 2007). With the relatively high levels of iron given in this study, both sites likely are overloaded with iron potentially interfering with folate absorption and/or metabolism. Similarly, delivery of iron and folate to the embryo during neurulation depends upon the visceral endoderm of the yolk sac (Zohn and Sarkar, 2010) and this tissue also likely becomes overloaded in Fpn1ffe/ffe mutant embryos with high levels of iron supplementation. This could further reduce transport of folate to the embryo.

ROLE OF FPN1 IN TRANSPORT OF OTHER METALS
Fpn1 also transports other metals and Fpn1ffe/+ mice show reduced manganese and zinc levels (Yin et al., 2010; Madejczyk and Ballatori, 2012; Seo et al., 2016). Deficiencies of both of these is implicated in increased NTD risk (Sever and Emanuel, 1973; Cavdar et al., 1980; Soltan and Jenkins, 1982; Buamah et al., 1984; Scott et al., 1990; Velie et al., 1999; Vats et al., 2011; Chandler et al., 2012). However, there is an inverse relationship between iron absorption and absorption of zinc and manganese (Erikson et al., 2002, 2004; Garcia et al., 2007) and iron supplementation competes with Fpn1-mediated transport of these and other metals (Davis et al., 1992; O’Brien et al., 2000; Thompson et al., 2006; Hansen et al., 2009; Zhang et al., 2016). Thus our data that iron supplementation prevents NTDs in this mouse line argue against the possibility that zinc or manganese deficiency are responsible for NTDs in this model. However, additional experiments will be necessary to definitively rule out the involvement of other metals to NTDs in the Fpn1ffe model.

CONCLUSIONS
It is well established that iron deficiency during pregnancy results in increased risk of complications such as premature birth, reduced birth weight, and intellectual disability (Gambling et al., 2011). Because of the increased iron requirement during pregnancy and the difficulty of replenishing stores under these conditions, it is important that sufficient iron stores are present before conception (Bothwell, 2000). Our results presented here and in our previous studies (Mao et al., 2010; Zohn et al., 2007) make a strong case that sufficient iron stores at conception are also important for successful neural tube closure. This study provides additional support for the possibility that iron deficiency could play a role in NTDs in humans and periconception iron supplementation might prevent some folate resistant NTDs.

References
Burren KA, Scott JM, Copp AJ, Greene ND. 2010. The genetic background of the curly tail strain confers susceptibility to


