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Modeling Anterior Development in Mice: Diet as Modulator of Risk for Neural Tube Defects

Claudia Kappen

Abstract

Head morphogenesis is a complex process that is controlled by multiple signaling centers. The most common defects of cranial development are craniofacial defects, such as cleft lip and cleft palate, and neural tube defects, such as anencephaly and encephalocele in humans. More than 400 genes that contribute to proper neural tube closure have been identified in experimental animals, but only very few causative gene mutations have been identified in humans, supporting the notion that environmental influences are critical. The intrauterine environment is influenced by maternal nutrition, and hence, maternal diet can modulate the risk for cranial and neural tube defects. This article reviews recent progress toward a better understanding of nutrients during pregnancy, with particular focus on mouse models for defective neural tube closure. At least four major patterns of nutrient responses are apparent, suggesting that multiple pathways are involved in the response, and likely in the underlying pathogenesis of the defects. Folic acid has been the most widely studied nutrient, and the diverse responses of the mouse models to folic acid supplementation indicate that folic acid is not universally beneficial, but that the effect is dependent on genetic configuration. If this is the case for other nutrients as well, efforts to prevent neural tube defects with nutritional supplementation may need to become more specifically targeted than previously appreciated. Mouse models are indispensable for a better understanding of nutrient–gene interactions in normal pregnancies, as well as in those affected by metabolic diseases, such as diabetes and obesity.

Keywords

anencephaly; craniofacial defect; diabetes; exencephaly; folic acid; high-fat diet; knockout; macronutrient; maternal diet; metabolism; micronutrient; mouse model; mutant; neural fold; neural tube defect; nutritional supplementation; obesity; orofacial cleft; pregnancy in diabetics; spina bifida; targeted disruption

INTRODUCTION

Anterior structures of the mammalian body, such as the brain and the precursor structures for the craniofacial region are among the first organs formed during embryonic development. They arise as the neural tube forms from neural plate ectoderm by elevation of the lateral edges. These meet at the dorsal midline and fuse to close the neural tube. The

future craniofacial region is populated by cells that migrate from the neural crest, the dorsal-most aspects of the neural folds. Neural crest-derived cells eventually differentiate to form the craniofacial bones [Richtsmeier and Flaherty, 2013].

Induction of neural crest cell migration temporally overlaps with closure of the neural tube, but is not dependent on it. Closure of the cranial neural tube initiates at closure initiation sites 2 and 3, which are in the vicinity of two key signaling centers in cranial development, the midbrain–forebrain junction and the nasal prominence, respectively. This is illustrated in Figure 1A [from Fleming et al., 1997]. When the neural tube fails to close in the cranial region, severe defects arise that affect both the brain and craniofacial structures. Defects in closure initiation site 2 cause cranial neural tube defects, such as anencephaly in humans and exencephaly in rodents, while defects at site 1 lead to spinal neural tube defects, and failure to close at all three sites is responsible for craniorachischisis. Example of mouse embryos with a cranial and a spinal neural tube defect, respectively are shown in Figure 1C,D. The figure also illustrates that, as observed in different rodent strains, the risk for neural tube defects is associated with position of the closure site 2 [Harris and Juriloff, 2007, 2010], so that a delay in neural tube closure has the greatest detrimental effect in strains with the greatest distance between closure sites 1 and 2, if the latter is even present. Multisite closure has also been confirmed for human cranial development [Seller, 1995].

Neural tube closure defects are among the most common human birth defects, but their etiology is still poorly understood. It is generally believed that genetic and environmental factors contribute to the risk for defective neural tube closure in multifactorial fashion, but the largely disappointing results from extensive genetic screens [Greene et al., 2009b; Pangilinan et al., 2012] would argue that environmental influences provide major risk factors. The contribution of environmental contaminants and medications to risk for neural tube and heart defects and oral clefts has recently been reviewed [Zhu et al., 2009]. The objective in this article is to highlight the role of nutrient and maternal diet as major environmental factors [Cetin et al., 2010] for normal and abnormal cranial development. A specific focus is to illustrate how mouse models have been used to uncover interactions between nutritional status and molecular mechanisms that underlie birth defect risk.

In recent years, evidence has emerged that the risk for neural tube defects can be modulated by maternal nutrition, providing the basis for strategies that aim to prevent neural tube defects by manipulation of maternal diet [Czeizel et al., 2011]. The success of supplementation and food fortification with the micronutrient folic acid [Obican et al., 2010] attests that dietary approaches can be effective: after mandatory fortification of the grain and flour supply in the United States, the incidence of neural tube defects was reduced by 30% [Mosley et al., 2009]. The reduction was greater in areas with higher baseline rates, which was also observed in other countries with flour fortification [De Wals et al., 2007; Orioli et al., 2011; Cortes et al., 2012]. Typically, spina bifida incidence was reduced to a greater extent than anen-cephaly [Lopez-Camelo et al., 2010]. Similarly, folic acid can reduce orofacial clefts, although the effect of fortification was moderate [Czeizel et al., 1999; Yazdy et al., 2007]. These findings imply that a large fraction of human neural tube defects have been unresponsive to folic acid. Attempts at identification of genetic risk factors have been largely disappointing [Greene et al., 2009b; Pangilinan et al., 2012], with causative

mutations identified only for very rare types of neural tube defects, for example, craniorachischisis, the failure to close the neural tube along the entire anterior–posterior axis. A recent review highlights progress on identification of genes that contribute to craniorachischisis [Juriloff and Harris, 2012a]. But for the more common NTDs, further investigation into contributing factors and molecular mechanisms for susceptibility is needed to develop more effective prevention strategies. We here review the emerging evidence that nutrient composition of the maternal diet plays key role in neural tube defects risk.

NEURAL TUBE DEFECTS, METABOLISM, AND NUTRITION

Strong indication that abnormal maternal metabolism contributes to elevated risk for developmental defects came from the observation of a greater incidence of congenital defects in infants born to mothers who were diabetic during pregnancy [Kucera, 1971; Mills, 1982]. Diabetic embryopathy in humans denotes a spectrum of congenital defects [Goto and Goldman, 1994; Kousseff, 1999a; Correa et al., 2008; Banhidy et al., 2010]: neural tube defects, such as anencephaly, encephalocele and spina bifida, and cleft palate with or without cleft lip, eye anomalies, heart defects, and caudal growth defects are all found with increased incidence in women with prepregnancy diabetes compared to non-diabetic women. Generally, there is an at least 3-fold higher risk for all congenital anomalies (rate of 7%) in diabetic pregnancies, but odds are higher for specific defects: NTDs occur 5.4-fold more frequently, at a rate of 10%, heart defects are 3.6-fold more common and found in about one out of four infants born to diabetic mothers (26% rate), and the most specific association was found for the relatively rare caudal dysplasias (3% rate), which occurred with 170-fold higher frequency in pregnancies affected by maternal diabetes [Bell et al., 2012]. A recent study from Europe reported slightly lower odds ratios (3.23 for encephalocele, 1.9 for anencephaly), heart defects (between 2.0 and 2.59 for selected heart defects, 1.43 for ventricular septal defects) and caudal regression (odds ratio of 22.06), but found no specific association of spina bifida and several other defects to maternal diabetes [Garne et al., 2012]. The authors speculate that diabetes may have a more specific effect than previously thought. The most important predictor for birth defects in women with diabetes was hyperglycemia, followed by preexisting nephropathy, while maternal smoking was not predictive [Bell et al., 2012]. It has been suggested [Buchanan and Kitzmiller, 1994] that embryopathy and fetopathy due to maternal diabetes may arise from different pathogenic mechanisms, as maternal metabolic adaptations change between early and late pregnancy trimesters. In this regard it is interesting that a higher rate of defects was also found with gestational diabetes [Kousseff, 1999b]. Given this type of diabetes is diagnosed late in pregnancy while most major congenital defects arise in the first trimester, it is possible that this group encompasses undiagnosed diabetes that existed prior to pregnancy [Kousseff, 1999b]. Similarly, congenital defects also occur with higher frequency in women with type II diabetes, but obesity is often a confounder in such pregnancies [Anderson et al., 2005].

Only recently has maternal prepregnancy obesity been established as an independent risk factor for neural tube defects. Blomberg and Kallen [2010] reported from a study of more than 1 million pregnancies in Sweden that risk for neural tube defects was 4-fold greater and

for orofacial clefts 1.9-fold greater in pregnancies of women with morbid obesity (BMI > 40), and BMI greater than 30 was associated with increased risks for hydrocephaly. A small research study in Australia [Oddy et al., 2009] also found greater risk for neural tube, craniofacial, and heart defects when BMI was greater than 30. In a population-based study of more than 1.5 million births in New York State, a significantly elevated risk for heart defects was found for obese women, but not for overweight women [Mills et al., 2010]. A recent report [Waller et al., 2007] from the National Birth Defects Prevention Study documents that neural tube and heart defects were significantly more common when mothers were obese prior to pregnancy. Odds ratios of 3.8 and 2, for neural tube and heart defects, respectively, were found in women with prepregnancy BMI greater than 30 [Watkins et al., 2003], with elevated tendency for heart defects and multiple anomalies even in overweight women. A Texas study [Anderson et al., 2005] confirmed higher risk for anencephaly, spina bifida, and hydrocephaly in mothers with BMI greater than 30, but found no effect on holoprosencephaly. Holoprosencephaly has been reported in children born to diabetic mothers [Barr et al., 1983; Miller et al., 2010; Bell et al., 2012]. The pathogenesis of holoprosence-phaly [Pan et al., 2013; Richtsmeier and Flaherty, 2013] and its relationship to neural tube defects [Murdoch and Copp, 2010] have recently been reviewed and will therefore not be covered here.

Another line of evidence links elevated risk for NTDs to poor diet quality, particularly the percentage of calories derived from fat and sweets [Carmichael et al., 2003]. High dietary glycemic load was associated with increased risk for craniosynostosis (OR 3.2) and for orofacial clefts (OR 2.2) [Yazdy et al., 2011] and neural tube defects (at least 1.5-fold higher risk) [Yazdy et al., 2010]. Thus, metabolic abnormalities, glycemic load, and poor diet quality (despite adequate caloric intake) in the mother are all associated with greater susceptibility to abnormal development of the embryo. Evidence of successful prevention of birth defects in metabolically abnormal pregnancies through vitamin supplementation is limited to date, but encouraging [Correa et al., 2003, 2008, 2012; Murphy et al., 2010; Banhidy et al., 2011].

MATERNAL DIABETES AND NEURAL TUBE DEFECTS IN ANIMAL MODELS

The association of diabetes and hyperglycemia with birth defects has been unequivocally established in experimental animal models, predominantly rats and mice [reviewed in Jawerbaum and White, 2010 and by Eriksson, 2009]. We will here focus primarily on neural tube defects, although heart defects [Morishima et al., 1996; Morgan et al., 2008], caudal growth defects [Chan et al., 2002], and craniofacial abnormalities [Nordquist et al., 2012] have also been described in rodent diabetic pregnancies. In mice, neural tube defects are generally more frequent in pregnancies affected by diabetes and hyperglycemia, whether in chemically induced or naturally occurring diabetes. In the non-obese diabetic (NOD) inbred strain of mice, which become diabetic spontaneously [Leiter, 1989], neural tube defect frequency can be as high as 40% [Otani et al., 1991] (and Salbaum and Kappen, unpublished results). When the dam is hyperglycemic, defective closure is found both anteriorly and posteriorly in NOD embryos at gestation day E10.5, at a time when neural tube closure is complete in embryos from non-diabetic dams of the same mouse strain. In the FVB inbred strain of mice, which were made diabetic by injection of Strepto-zotocin, NTD incidence

can be as high as 21% in hyperglycemic dams [Pavlinkova et al., 2009; Kappen et al., 2011]. For the C57BL/6 strain, rates of 55% have been reported in the STZ diabetes model *in vivo* [Li et al., 2012], and ~15% for the Alloxan-induced diabetes model [Machado et al., 2001], although we have not been able to reproduce these high frequencies in either model (Kappen, unpublished results), and another report also found the C57BL/6 strain refractory to neural tube defects under conditions of hyperglycemia [Fine et al., 1999]. Outbred strains, such as ICR, appear to be susceptible to diabetes-induced neural tube defects [Oyama et al., 2009; Wu et al., 2012], with NTD incidence as high as 75% in ICR mice, although non-diabetic controls also showed a high rate of defects (24%) in one study [Phelan et al., 1997]. In the rat, genetic background also appears to modulate the susceptibility to craniofacial defects in diabetic pregnancies [Ejdesjo et al., 2011; Nordquist et al., 2012], although the molecular mechanisms underlying differential susceptibility are unknown at present. In the mouse, strain-specific differences in the position of the rostral closure site [Juriloff et al., 1991] have been suggested to influence the susceptibility to anterior neural tube defects. In rat embryos cultured in serum from hyperglycemic dams, or in increasing concentrations of glucose, a delay of rostral neural tube closure has been documented [Peng et al., 1994].

The prevailing theory for pathogenesis of diabetes-induced developmental defects follows the concept of “fuel-mediated teratogenesis” [Freinkel, 1980] whereby altered fuel supply and altered fuel metabolism cause immediate and long-lasting effects, such as birth defects and metabolic syndrome, respectively. Excess glucose also inhibits uptake of critical nutrients, such as inositol [Weigensberg et al., 1990] and arachidonic acid. Consequently, supplementation of myo-inositol and arachidonic acid have been linked to lower incidence of defects in diabetic pregnancies and in embryos cultured under hyperglycemic conditions [Goldman et al., 1985; Baker et al., 1990; Goto and Goldman, 1994]. It has been suggested that turnover of phosphatidyl inositol is related to prostaglandin production from arachidonic acid, so that reduced inositol availability would lead to deficiency of prostaglandins, particularly prostaglandin E2 [Eriksson, 2009]. This functional deficiency can be rescued by arachidonic acid in diabetic pregnancies [Reece and Wu, 1997], and addition of PGE2 at intermediate concentrations was able to prevent glucose-induced defects in cultured embryos; however, high PGE2 concentrations were teratogenic [Baker et al., 1990].

That glucose *per se* is teratogenic was demonstrated by culture of normal embryos *in vitro* in media containing high concentrations of glucose [Sadler et al., 1988] and by injecting glucose into otherwise normal pregnant dams [Fine et al., 1999; Li et al., 2007], which also caused embryonic defects in mice. In the rat, high glucose concentrations were less teratogenic, at least in the strains tested [Sadler, 1980; Buchanan et al., 1985], leading to the postulate that additional serum factors contribute to teratogenesis [Sadler et al., 1988]: serum from insulin-treated diabetic rats was teratogenic despite normal glucose levels [Sadler and Horton, 1983], which was traced back to “somatomedin-inhibitors” [Balkan et al., 1988], now known as insulin-growth factor binding proteins.

Extensive evidence implicates oxidative stress in the pathogenesis of hyperglycemia-induced defects [Yang et al., 1997; Cederberg et al., 2001; King and Loeken, 2004; reviewed in Eriksson, 2009; Zabihi and Loeken, 2010; Eriksson and Wentzel, 2012]. Consequently, supplementation of anti-oxidants is able to lower the incidence of defects in

diabetic pregnancies [Reece and Wu, 1997; Siman and Eriksson, 1997; Wiznitzer et al., 1999a; Cederberg et al., 2001; Sugimura et al., 2009]. It has been suggested that in this context, folic acid also acts as an anti-oxidant, as it is able to reduce neural tube defect rates in pregnancies affected by hyperglycemia [Gareskog et al., 2006; Oyama et al., 2009]. What is currently unclear is how these factors affect apoptosis, which has been proposed as a cellular mechanism underlying neural tube closure defects [Harris and Juriloff, 1999], although there is conflicting evidence [Massa et al., 2009]. Thus, there are potentially multiple cellular and molecular pathways at which nutrition and diet modulate how the teratogenic insult is translated into the final outcome, neural tube defects in the cranial region.

While the studies referred to above highlight the role of metabolic factors for proper neural tube closure and point a critical role for some micronutrients, the role of macronutrients and diet composition is less well investigated. It has been suggested that hyperlipidemia in diabetic pregnancies may also contribute to adverse outcomes [Herrera and Ortega-Senovilla, 2010], but this has not been studied relative to the first trimester in human pregnancies, or in animal models. There is evidence from the rat model that safflower oil or olive oil could be beneficial in diabetic pregnancies [Higa et al., 2010], but molecular mechanisms are far from understood. Our own work in the STZ mouse model has demonstrated that diet composition has a critical role in modulating the frequency of neural tube defects in diabetic pregnancies [Kappen et al., 2011]. A diet with greater content of fat (the diet recommended for pregnancy and lactation, LabDiet Purina 5015) was associated with ~21% incidence of neural tube defects in diabetic pregnancies, while a diet with greater content of protein (the rodent maintenance chow LabDiet Purina 5001) produced about ~6–12% neural tube defects. The diet that produced more neural tube defects also had a greater effect on fetal growth retardation in these diabetic dams [Kappen et al., 2011]. While there are also micronutrient differences between these diets, they are replete for rodent nutritional requirements. Thus, our results showed that embryonic, and placental [Kappen et al., 2012] development respond to nutrient composition of nutritionally sufficient diets. In a comparison of two commercial diets in rat diabetic pregnancies, the carbohydrate-rich diet was associated with a greater risk of malformations [Giavini et al., 1991]. To identify the specific components and concentrations that affect cranial development will require modeling in mouse models that have a higher incidence of neural tube defects, or deficiencies in pathways that respond to nutrient stimulation or metabolism. In the following sections, we review the limited literature available to date on nutritional modulation of cranial development; where informative, we include evidence from developmental defects in other tissues, such as the heart.

MECHANISMS AND MOUSE MODELS: NUTRIENT MODULATION OF NEURAL TUBE DEFECTS

The most detailed evidence of the impact of diet composition on cranial neural tube development comes from the extensive work of Harris and Juriloff with the SELH/Bc mouse strain [Juriloff et al., 1989; Macdonald et al., 1989]. This strain is genetically prone to neural tube defects, especially exencephaly [Gunn et al., 1995; Juriloff et al., 2001]. Intriguingly,

when the pregnant dams were fed the same commercial diets as mentioned above (Purina 5001 and 5015, respectively), the lower-fat-higher-protein diet was associated with a lower incidence of neural tube defects than the higher-fat-lower-protein diet [Harris and Juriloff, 2005]. Because both diets vary in other components as well, Harris and Juriloff tested many diet formulations, by supplementing to the 5015 diet various micronutrients, such as folic acid, methionine, niacin, Brewers' yeast added, riboflavin, Vitamin B12, methyl-donors choline/betaine/folic acid/Vitamin 12 combined, and inositol, either in drinking water or administered intraperitoneally at embryonic days 7.75 and 8.5. All these formulations, as well as high levels of zinc in the drinking water, produced significantly higher rates of neural tube defects than the Purina 5001 diet. Synthetic diets formulated to resemble the fat and protein compositions of 5001 and 5015, respectively, while containing the same micronutrient complement, produced the same high NTD rate. These results left open which ingredient in the Purina 5001 was effective in lowering NTD incidence, and hence could be considered preventive [Harris and Juriloff, 2005]. The authors also showed that short-term exposure to this diet just from mating onwards was effective in lowering NTD incidence. Thus, differences between diets become effective in the short time window between copulation and neural tube closure. Harris and Juriloff showed that during this time, Purina 5015 appeared to accelerate embryo development relative to the Purina 5001 diet, but combined with the delay of midbrain closure in the SELH/Bc strain, this presumably creates asynchrony between tissues in the same embryo [Stoate et al., 2008]; this asynchrony then would lead to failure of neural tube closure. Thus, according to this model, the composition of the maternal diet modulates embryonic growth and, at least under conditions of a genetic predisposition, can promote developmental asynchronies that derail morphogenesis.

Macronutrients

Evidence to support a role for lipid metabolism in neural tube defects comes from mouse models in which the *ApoB* gene was ablated. In embryos homozygous for mutant *ApoB* alleles, exencephaly and hydrocephalus were found [Farese et al., 1995; Homanics et al., 1995] with penetrance on a mixed genetic background around 32% for a hypomorphic allele. Greater penetrance was observed in homozygotes with a null-allele, and heterozygotes for this *ApoB* deficiency also exhibited exencephaly [Huang et al., 1995]. Since ApoB is involved in lipid transport, this suggests that NTDs can be caused by inadequate lipid supply to the embryo. Indeed, Terasawa et al. [1999] showed that ApoB is expressed in the visceral endoderm, which transports nutrients to the embryo, and that it is required there for lipid transport. Homanics et al. [1995] speculated that Vitamin E deficiency secondary to ApoB deficiency might be the cause of the NTDs and supplemented α -tocopherol (Vitamin E). While supplementation of Vitamin E restored low Vitamin E levels in the dams to normal, only a non-significant trend toward fewer NTDs was observed. Thus, other lipid deficiencies may be present.

Interestingly, maternal diet may affect different developmental systems differently. This was revealed by a recent study on mutants with disruption of the *Cited2* gene: mice homozygous for the deficiency exhibit neural tube defects, heart defects, and craniofacial defects [Bentham et al., 2010]. The incidence of heart defects in homozygous mutant embryos was higher when the dam was fed a high fat diet for 8 weeks before mating. The cleft palate

phenotype was also more frequent with this diet and appeared in homozygotes and heterozygotes for the mutant allele. The detrimental diet also increased the severity of the heart defects [Bentham et al., 2010]. This has important relevance to human heart defects, as 2% of humans with congenital heart defects were found to have mutations in the *Cited2* sequence [Crider and Bailey, 2011]. Bamforth et al. [2001] attribute the heart defects to abnormal neural crest cell migration, which is consistent with the cleft palate phenotype. However, not all phenotypes in the *Cited2* mutants are explained by neural crest deficiencies.

Neural tube defects occur at high frequency in *Cited2* homozygous mutant embryos, and at low frequency even in heterozygotes. *Cited2* is a negative regulator of Hif1 α [Yin et al., 2002], so that Hif1 α and target genes are upregulated in *Cited2* knockout embryos. *Vegf* is one of the prominent Hif1 α target genes and is induced by hypoxia [Semenza et al., 1999], prompting the speculation that the defects could be caused by Hif1 α -mediated VEGF elevation in the absence of *Cited2*. The authors also observed greater proliferation in spinal ependymal layer, while there was cell death in the cranial mesenchymal compartment. Intriguingly, the high fat diet had no effect on the incidence of neural tube defects in *Cited2* mutants, indicating that the response to diet is not solely mediated by Hif1 α , and that additional pathways may be contributing to the pathogenesis of each type of defect observed [Dunwoodie, 2009]. Thus, diet may differentiate between neural crest-mediated defects and defects due to deficiencies in other tissues, such as the neuroepithelium proper, or there could be temporal phases in which cells giving rise to different structures in the embryo are differentially sensitive to diet components.

Folic Acid Deficiency and Neural Tube Defect Risk

The most extensively studied nutrient relative to defects in cranial development is the micronutrient folic acid, following the observation that human neural tube defects were associated with low maternal folic acid levels [Smithells et al., 1976]. Subsequently, periconceptional supplementation of folic acid [Schorah et al., 1983] was shown in many studies to reduce the incidence of neural tube defects [Schorah and Smithells, 1993], heart defects and orofacial clefts [Czeizel, 2009]. In mice, removing folate from the diet will compromise implantation [Heid et al., 1992], but when additional care was taken to eliminate the supply of folate produced by gut bacteria, and to prevent the reuptake of folate from consumption of feces, this was not sufficient to cause neural tube defects in the embryos that survive past the neural tube closure stage [Burgoon et al., 2002].

When all folate supply to embryonic cells is inhibited, through targeted deletion of reduced folate carrier (RFC, encoded by the *Slc19a1* gene) [Zhao et al., 2001; Gelineau-van Waes et al., 2008] in the mouse, embryos die by E9.5, demonstrating that folic acid is required for embryonic development. Similarly, folate binding protein 1 (encoded by the *Folr1* gene) deficiency [Piedrahita et al., 1999] is associated with multiple embryonic defects, including heart and neural tube defects, and in pregnancies with suboptimal supplementation of folic acid, craniofacial defects [Tang and Finnell, 2003]. Due to the presence of these alternative transport mechanisms in the embryo, supplementation of folic acid extends survival of *Slc19a1* mutant embryos [Zhao et al., 2001; Gelineau-van Waes et al., 2008], and is able to

prevent developmental defects in *Folr1* homozygous mutants [Spiegelstein et al., 2004], promoting survival to birth when folic acid is supplied through lactation and nutrition. Thus, low folate availability impairs embryonic development and survival, but the manifestation of neural tube defects likely requires other factors predisposing to susceptibility, such as genetic liabilities.

Several groups have shown that folate deficiency can exacerbate NTD occurrence in certain genetic backgrounds. Burren et al. [2008] created folate deficiency by combining a no-folate diet with antibiotic, which reduces folate in the mother, and subsequently folate (and protein content) content in embryo. In both homo- and hetero-zygotes for the *Pax3/Spotch^H* allele, folate deficiency increased NTDs: *Spotch^H/Spotch^H* already have 61% cranial NTDs on standard diet, with folate deficiency this went up to 88%; among heterozygotes that are normally asymptomatic with replete diet, 15% had exencephaly. Delayed development was also observed. In this strain, folate supplementation reduced NTDs, but did not ameliorate the delay. Attributing the cranial NTDs to reduced cell proliferation, the authors speculate that the differential effect of supplementation probably involves a local cranial rather than general stimulation of cell proliferation by folate. Another allele of the *Pax3* gene, as present in the *Spotch* mutant, did not exacerbate NTD risk with folate deficiency [Li et al., 2006], although it is possible that folate deficiency in that study was not as severe as before [Greene et al., 2009a].

In a later report, Burren et al. [2010] used the same folate-deficient diet as before to study its effect on *curly tail* mutants [van Straaten and Copp, 2001], which carry a hypomorphic allele of the *Grainy head like 3 (Grhl3)* gene. In this model, the folate-deficient diet is associated with smaller litter size and increased rate of resorptions in pregnancies of wild-type mice as well as mutants. In homozygous *curly tail* mutants, the incidence of cranial NTDs increased with folate deficiency, from ~10% on standard diet to over 50% on folate-deficient diet; wild-type embryos with the same genetic background as the *curly tail* strain had no NTDs on standard diet, but ~30% on the folate-deficient regimen. The authors also observed a significant reduction in embryo growth and delay in development in folate-deficient dams, so that at E10.5, embryos resembled size and somite count of E9.5 embryos. This effect was present regardless of embryonic genotype at the *Grhl3* locus. Thus, the major factor conferring risk in this study was the genetic background, which did not raise risk for NTDs when dietary conditions were replete for folate. The conclusion from these results is that risk for cranial defects can be modified by diet even when the underlying genetic susceptibility is asymptomatic in nutritionally replete conditions.

In both genetic paradigms, embryonic folate status more than maternal status appeared to be critical for NTD risk. Consistent with this conclusion are observations that homozygous *Spotch^H* embryos have a deficiency in thymidine synthesis [Fleming and Copp, 2000] that can be overcome by supplying folic acid or thymidine. *Curly tail* homozygous mice [Tran et al., 2002] and embryos [De Castro et al., 2010] also display alterations in one-carbon metabolism [De Castro et al., 2010]. Specifically the ratio of S-adenosyl methionine (SAM) to S-adenosyl homocysteine (SAH) was reduced, implying that less SAM may be available for methylation reactions. In folate-deficient conditions, this ratio changed in favor of SAM, yet NTDs occurred more frequently, arguing against a causal role of diminished

methylation in NTD etiology [De Castro et al., 2010]. In *curly tail* mutants, thymidylate synthesis was normal, indicating that in these two models, different folate metabolic pathways may be involved. Since these mutant models have intrinsic alterations in folate metabolism, it is perhaps not surprising that the susceptible genotypes exacerbate NTD risk under conditions of folate deficiency. What is currently unclear is how the transcription factors encoded by the *Pax3* and *Grhl3* genes cause the respective metabolic alterations, and to what extent the non-metabolic roles of these proteins contribute to neural tube defects.

A non-metabolic response to folate-deficiency involves the *Alx3* homeobox transcription factor. Embryos homozygous for a targeted *Alx3* null allele [Beverdam et al., 2001] display defective neural tube closure [Lakhwani et al., 2010]. Expression of *Alx3* in wild-type embryos is diminished when the dam is fed a folate-free diet; in embryos placed into culture at E8.5 and examined one day later, *Alx3* expression is dependent on folate content of the culture media. These findings suggest that *Alx3* is induced by folic acid and that folic acid is required for *Alx3* expression. In pregnancies of homozygous *Alx3* mutant dams, homozygous mutant embryos were selectively underrepresented, and there was greater variation of developmental progression at E9.5, implying that some embryos had delayed development. In about half of the embryos that formed more than 14 somites, the neural tube stayed open in the midbrain and hindbrain regions, and NTDs were observed even in embryos up to 22 somites, again arguing that defective neural tube closure is not simply due to developmental delay in this model. This conclusion is further supported by the absence of any spinal defects. The *LacZ* reporter that was knocked into the *Alx3* locus was expressed in the normal *Alx3* expression domain, and expression of other genes involved in cranial neural tube defects, such as *Cart1*, *Cited2*, or *Twist* (which is an upstream regulator of *Alx3* in cranial mesenchyme [GarciaSanz et al., 2013]), was unaffected. When a folate-free diet was administered, *Alx3* homozygous mutant embryos were severely retarded, precluding assessment of neural tube closure. Thus, while *Alx3* is dependent on folate status, *Alx3* is not the only factor affected by folate deficiency. In *Alx3* mutants, the cranial mesenchyme does not expand properly, and facial clefts are found with incomplete penetrance. In humans, *ALX3* mutations are associated with isolated cleft palate [Jugessur et al., 2009] and frontonasal dysplasia [Twigg et al., 2009]. Taken together, these studies have identified *Alx3* as a nutrient-responsive candidate gene for defects in cranial development. They also suggest that regulation of transcription factor expression is a mechanism by which optimal nutrition can prevent developmental defects.

Nutrient Supplementation as an Approach to Prevent Cranial Defects

Although the success of folic acid in preventing neural tube defects is well documented [Obican et al., 2010], the molecular mechanisms involved in the beneficial effect are still poorly understood. Even less is known about the ability of folate to affect developmental defects in metabolically abnormal pregnancies, such as those affected by maternal diabetes or obesity [Czeizel, 2009; Banhidy et al., 2011]. In rodent models of diabetic pregnancy, supplementation of folate [Wentzel and Eriksson, 2005; Oyama et al., 2009], myo-inositol [Baker et al., 1990], Vitamins C and E [Cederberg and Eriksson, 2005; Wentzel and Eriksson, 2005], and arachidonic acid [Goldman et al., 1985] have all been shown to reduce the incidence of diabetes-induced developmental defects. Diet composition was also shown

to affect the incidence of cleft palate induced by the teratogens Phenytoin [High and Kubow, 1994], triamcinolone [Zhou and Walker, 1993], or cortisone [Miller, 1977], but molecular sequelae are unknown in these models. Recently published reviews by Harris [2009] and Stover [2009] cover studies to understand the roles of folate metabolic pathway genes in cranial and spinal neural tube defects; therefore our focus here will be specifically on nutritional modulation of cranial defects in non-folate-pathway models.

Folic Acid Supplementation Can Reduce NTD Risk: Responsive and Unresponsive Models

A protective effect of folic acid supplementation in the *Pax3/Spotch^H* mutant is, perhaps, not so surprising, considering that these mutants have a defect in thymidine synthesis, which folate supplementation is able to correct [Fleming and Copp, 1998]. Daily administration of folate at between embryonic days E7.5 and E9.5 was able to reduce the incidence of spinal defects at E10.5, but exencephaly and resorptions were more frequent in litters derived from crosses of *Pax3/Spotch* heterozygotes [Stottmann et al., 2006]. Gefrides et al. [2002] reported no effect of folic or folinic acid supplementation on NTD rate at E18.5 in *Pax3/Spotch* mutants. Wlodarczyk et al. [2006] studied the *Pax3/Spotch* allele on a new genetic background (CXL), supplementing heterozygous mutant females with a variety of compounds: inositol, thiamine, thymidine, and methionine were administered by gavage from 2 weeks before mating, 5-MTHF (5-methyltetrahydrofolate) and α -tocopherol by intraperitoneal injection on gestational days 8.5 and 9.5, 5-FTHF (5-formyltetrahydrofolate; folinic acid) (by gavage and intraperitoneal injection), and folic acid (by gavage and high-content diet). Unexpectedly, they found a highly elevated rate of resorptions with 5-MTHF and the folic acid fortified diet. The fortified diet also led to deviation from the expected Mendelian ratio of genotypes, with a selective loss of homozygous mutants. Overall, only the fortified diet, folic acid at 25 mg/kg given by gavage, and 5-MTHF at 20mg/kg injected caused a reduction of NTD incidence among the homozygous mutant embryos; lower doses showed a trend but no statistically significant effect. Perplexingly, while the rate of spina bifida was reduced with fortified diet, the relative incidence of exencephaly was increased among the NTDs, suggesting that the diet has the potential to raise the severity of those defects that do occur. Furthermore, the appearance of embryos that had only exencephaly but not spina bifida was modulated by several compounds, suggesting that the two defects may respond differently to diet.

Tead2 was proposed as a regulator of *Pax3* based upon its ability to bind to a DNA motif in the *Pax3* gene [Milewski et al., 2004]. However, in *Tead2* homozygous null embryos, *Pax3* expression is normal. Exencephaly was observed in 19% of embryos when the pregnant dam was homozygous for the *Tead2* deletion and mated to a heterozygous *Tead2* mutant male [Kaneko et al., 2007]. In homozygous mutant embryos, exencephaly incidence was 30%, and of the *Tead2* heterozygotes, 7% displayed exencephaly. These results argue for a maternal effect of the *Tead2* deficiency, since opposite crosses (homozygous mutant male to heterozygous mutant female) had an overall lower incidence of defects. Of particular note, folic acid supplementation produced a trend toward reduction of NTDs, but the effect was not statistically significant. Thus, the role of Tead2 in NTDs is independent of *Pax3*, and the resulting NTDs are folate resistant [Kaneko et al., 2007]. On the other hand, not all *Pax3* alleles are folate responsive [Gefrides et al., 2002; Greene et al., 2009a; Marean et al.,

2011], indicating that other factors, such as strain background or duration and mode of delivery affect folate's effectiveness in preventing neural tube defects.

Folate supplementation also had no effect on NTD incidence in the *Alx3* mutant [Lakhwani et al., 2010]. The expression of other cranial neural tube defect genes, such as *Cited2* or *Cart1/Alx1* was not altered in cranial mesenchyme of *Alx3* homozygotes, neither was expression of the third *Alx* gene, *Alx4*. These results indicate that *Alx3* acts independently in cranial mesenchyme survival and expansion. *Alx4* mutants do not develop cranial defects, unless the gene dosage of *Cart1/Alx1* is reduced to heterozygosity [Qu et al., 1999]: then, facial clefts are present. When *Alx4* mutants are also homozygous for the mutant *Cart1/Alx1* allele, midline clefts are even more severe, and are found in combination with exencephaly, as expected for homozygous *Cart1/Alx1* mutants [Zhao et al., 1996]. In addition to exencephaly, *Cart1/Alx1* mutants have other severe craniofacial defects, including deformed cranial bones and absence of eyes. Thus, like *Alx3*, *Cart1* is required for survival of forebrain mesenchyme. Supplementation of folic acid was able to rescue neural tube closure and cranial bone development [Zhao et al., 1996], although about half of the embryos had shortened faces, indicating that forebrain mesenchyme may not have been fully restored in all individuals, probably due to increased apoptosis of cranial mesenchyme. In the mutants with a closed neural tube, the authors still found the cranial neuroepithelium thicker than in wild type, and speculate that unchecked cell proliferation in the absence of folate supplementation leads to extrusion of the midbrain, hence the phenotype of exencephaly. Then, in this model, folic acid may act either to inhibit early mesenchymal apoptosis, or to limit neuroepithelial cell proliferation. Double mutants for any of the *Alx* genes have not been tested for their response to nutritional supplementation. This is highly relevant since mutations in the *ALX3* and *ALX4* genes have been found in humans with craniofacial bone abnormalities [Wu et al., 2000; Wuyts et al., 2000; Mavrogiannis et al., 2001; Jugessur et al., 2009; Kayserili et al., 2009, 2012; Twigg et al., 2009].

On the other hand, neural tube defects can arise despite normal *Twist* and *Cart1* expression, as demonstrated in a model homozygous for a hypomorphic *Gcn5* allele [Lin et al., 2008], which exhibits exencephaly. In these mice, head mesenchyme appeared normal, and there were no abnormalities in proliferation or apoptosis detected. Neural crest cell production and migration were also apparently normal. Supplementation of folic acid was able to reduce, but not completely suppress, the occurrence of NTDs in this strain.

Intriguingly, the high incidence of 80% exencephalic embryos among the *Cited2* homozygotes was reduced to ~13% when the dams' diet was supplemented with folic acid [Barbera et al., 2002]. Barbera et al. attribute the neural tube defects in this strain to increased apoptosis at the forebrain—midbrain junction and in the hindbrain. In folic acid supplemented embryos, however, they detected apparently similar levels of apoptosis, suggesting that folic acid affects neurulation in this model by mechanisms other than preventing cell death. Considering the differences in pathogenesis and in folate responses between these models, it is conceivable that the nutrient affects only particular cell types or specific cellular pathways, suggesting that the assumption of a generally preventive effect may have to be re-evaluated even for folate-responsive models.

Crooked tail mutants carry a gain of function in *Lrp6*, the Wnt co-receptor in the canonical signaling pathway. In homozygous mutants, exencephaly can be rescued by supplementation of folic acid [Ernest et al., 2006]. It is known that a low folate diet raises homocysteine levels, while lowering glutathione and cysteine levels in wild-type mice. Interestingly, *crooked tail* mutants have lower glutathione and cysteine levels (homocysteine levels were normal), suggesting that the defects arise, at least in part, from low folate levels, which the supplementation corrects. The authors therefore typed the response to folate levels in livers by gene expression profiling. The expression profiles from wild type and *crooked tail* on folate-containing diets clustered together, whereas *crooked tail* mutants and wild types on folate-deficient diet clustered together [Ernest et al., 2006]. By comparing liver expression profiles from other mouse strains/mutants, the authors attempted to predict folate-responsiveness of unrelated mouse strains. The prediction for *Ski* mutants was that they would be resistant.

Ski null mutants exhibit exencephaly and abnormal facial morphology with flat and short snout and abnormal jaw in 80% of homozygous mutants; embryos without neural tube defects had mild to complete facial clefts [Berk et al., 1997; Colmenares et al., 2002]. On the mixed 129×Swiss and 129×C57BL/6 backgrounds, a small proportion (depending on day of assessment) of heterozygotes also have exencephaly or clefts; when the mutant allele was on the C57BL/6 genetic background, however, exencephaly was reduced to 5% incidence [Colmenares et al., 2002]. When the exencephaly prone *Ski* mutants were supplemented with folate, the developmental defects indeed proved to be folate resistant [Ernest et al., 2006]. The authors speculate that defective neural tube closure in this model is secondary to impaired cell survival, since the mutants have increased apoptosis in the cranial mesenchyme [Berk et al., 1997]. Thus, as in *Alx3* and *Cart1* mutants, folic acid may not be able to rescue early loss of cranial mesenchymal cells. On the other hand, considering that the *Lrp6/crooked tail* mutants may have a defect in folate utilization, and their responsiveness to supplementation would be expected.

Folic Acid Can Be Detrimental

More complicated, however, was the response in mutants with an *Lrp6* loss-of-function allele [Gray et al., 2010]. High folic acid diet reduced incidence of developmental defects in homozygous mutant embryos when the count was normalized to all conceptions, including resorptions. But this was only an apparent “rescue,” since fewer than the expected 25% homozygotes were recovered live, indicating selective loss of homozygotes before the time of pheno-type assessment at E12.5 to E13. Selective loss of homozygotes was found regardless of folic acid content in the diet, but the loss was greater with high folate content. When viable *Lrp6* homozygous mutants were examined for phenotype at E10, 68% displayed NTDs with low folate diet, 100% had neural tube defects on the high folate diet. Thus, a diet with the same level of folate that rescued *Lrp6/crooked tail* mutants failed to rescue the *Lrp6* null mutants; instead, high folic acid concentration impaired embryonic survival, and among surviving embryos, neural tube defects were not prevented. In contrast to the *Lrp6/crooked tail* mutants with constitutively activated Wnt signaling, the *Lrp6* null mutants had reduced Wnt signaling activity. The authors speculate that folate blunts the cellular response to Wnt signaling, rectifying a hyperactive pathway in *Lrp6/crooked tail*

mutants and further decreasing Wnt signaling in the *Lrp6* null mutant. These results imply that, at least in certain genotypes, folic acid supplementation can have detrimental effects. These studies are complemented by reports that high folic acid supplementation can be associated with increased embryonic loss and growth delay, and increased incidence of ventricular septal heart defects [Mikael et al., 2013], and that high dose folinic acid supplementation was associated with delay in cartilage maturation [Kappen et al., 2004].

One recent study in several mouse strains was conducted by Marean et al. [2011] who employed mutants for six different genes in the same feeding scheme with control and high folic acid-containing diets. The defective neural tube closure in these mouse models was considered to be due to different cellular and molecular defects, such that in *Zic2* mutants, dorsolateral hinge point formation was affected (100% spina bifida, 20% exencephaly on original Teklad diet), while in *Frem2* (30% midbrain exencephaly) and in *Grhl2* (100% penetrant exencephaly) mutants, neural tube fusion was perturbed. In *L3P* mutants (30% full exencephaly), neural patterning is hypothesized to be defective, in *Pax3* (*Sploch*; 100% spina bifida, 73% exencephaly) mutants, progenitor cell survival and differentiation is impaired, and in mutants for *Shroom3* (100% exencephaly), actin regulation is abnormal. Common to all these mutants was also that they are not complete null mutants. No effect of high folic acid (long term) diet was seen in *Frem2* and *Grhl2* mutants. A marginal beneficial effect was seen in *Pax3* mutants, with reduction of NTD incidence from 100% to 97% (either with feeding fortified diet or with injection of folic acid, exencephaly as well as spina bifida were reduced), and in *Zic2* mutants, with reduction from 100% to 95%. In *L3P* mutants short-term feeding of the high folic acid diet led to increased survival of homozygous mutant embryos but did not protect against NTDs, and longer-term feeding of the high folic acid diet was even detrimental, resulting in a statistically significant increase in NTDs in this strain. In *Shroom3* mutant embryos, NTDs were reduced in the short-term feeding scheme, but with long-term feeding, survival of homozygotes decreased substantially. Surprisingly, there was a higher incidence of NTDs in *Grhl2* heterozygous mutants with short-term feeding, and loss of heterozygous and homozygous mutant embryos. No loss of heterozygotes was found upon long-term feeding, but homozygous mutants were still lost, albeit at a smaller rate. The fact that long-term high folic acid diet can induce loss of mutants (in the *Shroom3* and *L3P* strains) further suggests that folic acid is not universally beneficial [Strickland et al., 2013]. Whether these genes may have unexpected roles in nutrient metabolism is currently unknown. Considering the different response patterns in the different strains, the authors speculate that folic acid could be working through multiple pathways.

Mutations in Folate Metabolic Enzymes and Mitochondrial Folate Metabolism

Mutants with deficiencies in folate metabolic enzymes shed some light on this notion. 5,10-Methylenetetrahydro-folate is the carbon donor for synthesis of thymidine monophosphate, the precursor to the thymidine nucleotide in DNA [Stover, 2009]. Deficiency of SHMT1 (cytosolic serine-hydroxymethyl trans-ferase 1), the enzyme producing 5,10-methylenetetrahydrofolate, in *Shmt1* null homozygous mutant embryos, was associated with reduced growth but did not cause neural tube defects under normal conditions [Beaudin et al., 2011]. Yet, when dams were on a diet deficient in folate and choline, embryonic growth

was further reduced and 13% of homozygotes, and even some heterozygotes, had neural tube defects at E11.5; intriguingly, only exencephaly was observed. A follow-up study showed that folate deficiency was the cause, and dietary choline deficiency had no effect [Beaudin et al., 2012]. When *Shmt1* heterozygotes were made homozygous for the *Spotch* allele, 46% of embryos with the compound genotype had NTDs, including exencephaly and spina bifida. Double homozygotes for *Shmt1* and *Spotch* exhibited 66% NTDs, a significantly higher frequency than in mutants for *Spotch* alone on this genetic background (22%) [Beaudin et al., 2011]. Thus, *Shmt1* mutation sensitizes mice to NTDs, by reducing de novo thymidylate synthesis, but this is insufficient to cause neural tube defects in otherwise normal mice.

Other single gene mutations in genes encoding folate metabolizing enzymes are also unlikely to account for NTD pathogenesis: Under normal dietary conditions, mutant alleles for the *Cbs* (cystathionine beta synthase), *Mthfr* (methylenetetrahydrofolate reductase) and *Shmt1* genes are asymptomatic for NTDs, and homozygous *Mthfd2* (bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mito-chondrial) null mutants can complete neural tube closure but die later (Mouse Genome Database [Eppig et al., 2012]). Knockouts for *Mtr* (encoding methionine synthase), *Mtrr* (methionine synthase reductase), *Mthfd1* (methylenetetrahydrofolate dehydroge-nase NADP+ dependent), and *Mthfs* (5,10-methenyltetrahydrofolate synthe-tase) genes all die before E9.5, precluding a functional analysis with respect to NTDs.

The only folate pathway mutants that exhibit neural tube defects are embryos homozygous for disruption of genes encoding mitochondrial folate metabolizing enzymes. In mutants for MTHFD1like [Momb et al., 2013], the enzyme that converts 10-formyl-tetrahydrofolate to formate, craniorachi-schisis or exencephaly with wavy neural tube were found with 100% penetrance. On a normal diet, there was also growth restriction and reduced growth of maxillary and mandibular processes evident by E12.5, after which there was no survival. Supplementation of sodium formate to the drinking water partially rescued the homozygous mutant embryos to longer survival: of 14 embryos assessed between E10.5 and E15.5, only 3 had exencephaly, the others had closed tubes, and growth defects were also reversed. Thus, mitochondrially derived methyl groups, in the form of formate, are critical for proper craniofacial development and neural tube closure.

Mitochondrial formate is made from 5,10-methylene-tetrahydrofolate as the precursor, and the production of 5,10-methylene-tetrahydrofolate is catalyzed by the glycine cleavage system, of which aminomethyltransferase (AMT) is a major component. Interestingly, non-synonymous sequence variants in the *AMT* gene and missense mutations have been reported in spina bifida patients [Narisawa et al., 2012]. Mice homozygous for a gene trap allele at the *Amt* locus lack all glycine cleavage enzyme activity. While heterozygotes were viable, 87% of homozygous mutant embryos exhibited NTDs at E17.5, predominantly anterior NTDs and exencephaly [Narisawa et al., 2012]. Craniorachischisis was found at low frequency (5%). The authors attempted to rescue the defects by supplementation of folate, thymidine monophosphate, methionine, or the latter two combined. Folate was unable to rescue the defects. There was a trend for methionine-based supplementation to yield fewer malformed embryos, but sample numbers were small and potential deviation from

Mendelian distribution of genotypes at the time of inspection was not investigated. It is currently unclear whether the NTDs arise from lack of glycine-derived methyl groups or whether accumulation of glycine itself is to blame [Narisawa et al., 2012]. Taken together, the latter two mouse models provide strong evidence that mitochondrial folate metabolism is crucial for cranial development, at least in part due to the cellular requirement of methyl donors.

Other Methyl Donors

Several methyl donor diets were tested for their ability to rescue craniofacial defects in *twisted gastrulation* mutants [Billington et al., 2013]: these mutants have jaw defects (micrognathia and agnathia) with or without midline defects characteristic of holoprosencephaly. The defects occur with incomplete penetrance (40% rate for all defects combined) on the C57BL/6 background when dams are fed a control diet. With folate, B12, betaine, and choline supplemented to the diet, the incidence of branchial arch defects, which cause facial clefts, was reduced to 20%. However, unaffected were the midline defects, which arise from defective signaling from prechordal plate to forebrain and to frontonasal ectoderm.

Intriguingly, the authors observed higher defect frequencies in second pregnancies, when complete anterior truncations were also observed, irrespective of diet. Since neural crest cells are the source of cranial mesenchyme, protective effect of diet on branchial arch development was interpreted as a beneficial action particularly on neural crest cells. Because the midline defects were unresponsive to methyl donor diet, the authors speculate that they arise from an independent pathological process [Billington et al., 2013]. Their model suggests that different structures are differentially sensitive to folic acid and methyl donor supplementation, with greater response in craniofacial and less in neural structures.

Inositol

In studies with cultured embryos, inositol was found to be required in the medium for normal neural tube closure [reviewed in Cockroft, 1991]. Consequently, inositol deficiency from the medium increased the frequency of neural tube defects in cultured *curly tail* mutant embryos [Cockroft et al., 1992]. A role for inositol metabolism in cranial neurulation was also reported for rat embryos from diabetic pregnancies [Reece et al., 1997; Wentzel et al., 2001]. Preliminary evidence suggests that inositol supplementation could also be beneficial in humans [Cavalli et al., 2011]. In mouse genetic models, myo-inositol has been reported to be beneficial in models that are folate resistant [Greene and Copp, 1997], such as the *curly tail* mouse. On the other hand, in homozygous *Spotch* mutants on the CXL background, inositol supplementation significantly reduced survival of homozygotes and increased, among the survivors, the frequency of individuals with both exencephaly and spina bifida [Wlodarczyk et al., 2006]. In offspring from *Noggin* mutants, there was a nonsignificant trend for reduction of exencephaly but not spinal defects, although resorptions also increased [Stottmann et al., 2006]. Embryos carrying homozygous deficiency alleles for *Grhl3* were completely unresponsive to inositol supplementation [Ting et al., 2003]. Thus, as with folic acid, genetic background has a strong modifying role on the response to inositol supplementation.

Genetic ablation of the production of specific inositol phosphate species, by targeted disruption of the upstream kinases, also causes neural tube defects. Reduced levels of inositol 1,3,4-triphosphate 5/6 kinase (*Itpk1*) were found in mice carrying an *Itpk1* gene trap allele [Wilson et al., 2009]. Homozygous embryos of this strain had exencephaly and/or spina bifida (and skeletal defects) in ~15% of cases, on a mixed C57BL/6 × 129(P2)Ola genetic background. There was also some embryo loss and growth restriction before E12.5. Inositol phosphate multikinase (*Ipk2*) null mutants die after E9.5 with growth restriction and multiple defects, including abnormal folding of the neural tube [Frederick et al., 2005]. Embryos that were developmentally delayed by E8.5 exhibited no evidence of somite formation, although mesoderm was present. The anterior neural tube remained open at E9.5 and embryos did not turn. These results demonstrate that inositol phosphates are crucial messengers in mammalian development. Null mutants for the phosphatidylinositol-4-phosphate 5-kinase type I γ (encoded by the *Pip5k1c* gene), which catalyzes the synthesis of phosphatidylinositol-4,5-bisphosphates (PIP₂), also exhibit open neural tubes and exencephaly by E11.5 in the anterior region. They also have heart defects [Wang et al., 2007]. Homozygous mutants for Type III phosphatidylinositol phosphate kinase (PIPKIII, encoded by the *Pikfyve* gene) die by E8.5 with visceral endoderm defects [Takasuga et al., 2013]. The authors interpret the demise of embryos as caused by insufficient nutrient transport. Taken together, these results strongly implicate phosphoinositol deficiency in neural tube defect etiology. Whether supplementation of specific forms of inositol phosphates, or any other nutrient, can rescue the defects has not been determined. Intriguingly, the *Pip5k1c* mutants respond to folic acid supplementation [Wang et al., 2007]. It therefore appears that, depending on genetic, metabolic and environmental context, NTDs may be preventable by either FA, or inositol or both. On the other hand, there are several mutant strains that are non-responsive to either nutrient, including SELH/Bc, the *Grhl3* null mutant, mutants for *Zic2* and *Zic3* [Franke et al., 2003], and MEKK4/Map3k4-deficient mice [Chi et al., 2005].

Emerging evidence suggests a connection of inositol phosphates to developmental signaling pathways, particularly Wnt signaling [Qin et al., 2009]. Upon binding to Wnt3a, Lrp6 is phosphorylated through an interaction of disheveled (Dvl) with phosphatidylinositol-4,5-bisphosphate [Pan et al., 2008]. Thus, formation of phosphatidylinositol is required for phosphorylation of Lrp6, which then stimulates Wnt/ β -catenin signaling [Tanneberger et al., 2011]. In the gut, Wnt signaling is also stimulated under conditions of a low folate diet, causing a higher incidence of APC-dependent tumors [Ciappio et al., 2011]. Folate and Vitamin B supplementation to pregnant dams lowered tumor incidence in the offspring. The evidence from the *Lrp6* mutants also supports the notion that folate supplementation dampens Wnt signaling [Gray et al., 2010]. Thus, it appears that inositol and folate may have opposite effects on Wnt signaling, providing an explanation why they may only be beneficial/ effective in specific genetic configurations. Resistance to both folic acid and inositol would then be suggestive of non-Wnt pathways in the pathogenesis of neural tube defects. Interestingly, intestinal Wnt signaling is also stimulated by high fat diet [Liu et al., 2012] and by high glucose [Chocarro-Calvo et al., 2013]. It has been proposed that in diabetic pregnancies, inositol supplementation is able to restore the levels of inositol phosphates that are required for prostaglandin synthesis [Baker et al., 1990], particularly of prostaglandin E₂. Low prostaglandin E₂ levels have been documented in embryos exposed

to hyperglycemic conditions [Baker et al., 1990; Wentzel and Eriksson, 2005]. Arachidonic acid supplementation is also thought to restore PGE₂ production in diabetic pregnancies [Wiznitzer et al., 1999b]. Interestingly, interactions between prostaglandin and Wnt signaling have been described in multiple cell types [Liu et al., 2010], and modulate cell migration during gastrulation [Speirs et al., 2010], possibly by controlling stem cell dynamics and cell specification [Goessling et al., 2009]. The combined evidence provides strong support for the proposition that the Wnt signaling pathway is a major target for dietary manipulation, and in pregnancies affected by diabetes [Pavlinkova et al., 2008] and obesity.

Multiple Pathways in Neural Tube Defect Pathogenesis

It is generally accepted that neural tube defects in humans arise from combination of genetic predisposition and environmental factors, but until recently, this had only been investigated in a few mouse models. By now, more than 400 genes have been identified that cause neural tube defects when disrupted in mice, or contribute as modifiers [Harris and Juriloff, 2010; Salbaum and Kappen, 2010] (and annotations in the Mouse Genome Database [Eppig et al., 2012]). This review highlights recent progress to characterize and type these models according to their responses to nutritional supplementation and maternal diet. Not unsurprisingly, supplementation can rescue defects in models with underlying nutrient deficiencies, but the mechanisms involved in the responses in models without known deficiencies are less clear. Strain responses to various nutrients/diets may be predictable from gene expression signatures that not only serve to identify nutritional targets [Ernest et al., 2006], but possibly also allow classification of pathogenic pathways in neural tube defect etiology based upon diet/nutritional status. Harris and Juriloff [2007, 2010] offered such a classification considering genetically controlled pathways; it should eventually be possible to develop parallel insight into the environmentally controlled pathways. The ultimate goal would be to identify the diet manipulable factors in the multifactorial origin of cranial defects.

Diverse Patterns of Responses to Nutrient Supplementation

Collectively, the studies reviewed above have shown that susceptibility to anterior neural tube defects can be modulated by maternal diet. The responses to diet may involve multiple molecular pathways, and different cellular targets. This is underscored by the different response patterns to dietary supplementation: beneficial and detrimental responses to nutrients have been observed, as well as absence of a response, or “resistance” (see Table I). Since responsiveness and unresponsiveness were to different nutrients, it is likely that, in addition to nutrient transport deficiencies, at least three or four different pathways can be targeted in the embryo proper. These include deficiencies of nutrients for normal metabolism, such as of arachidonic acid or inositol, oxidative stress, such as with Vitamin E supplementation, and de novo thymidine synthesis and mitochondrial formate production, as highlighted by the folate pathway mutants SHMT and MTHFD1like. Possible interactions of these pathways have not been studied in detail, although findings in the diabetic models would be consistent with a convergence of pathways, since except for high carbohydrate or Purina 5015 diet, all tested nutrients had a beneficial effect. In some mutant models, supplementation ameliorates high levels of apoptosis or promotes cell survival, but this is

inconsistent between models. Similarly, while some neural crest defects respond to dietary manipulation, not all do. Furthermore, neural crest defects and neuroepithelium defects in a supplementation-sensitive individual may respond differently to nutrient supplementation, possibly in different temporal windows. It has been shown in the SELH/Bc mutant that developmental asynchronies will increase the risk for neural tube defects [Stoate et al., 2008]. In many models, it was not investigated whether the genetically manipulated locus itself is a target of diet, such as in the case of the *Alx3* gene, whose expression is dependent on folic acid status. In some models that exhibit both anterior and posterior NTDs, the two defects seem to be differentially modulated by maternal diet. Collectively, these insights can be taken as evidence that different mechanisms underlie the pathogenesis of NTDs along the anterior–posterior axis and that there is heterogeneity in causation and response to supplementation even within cranial neural tube defects.

Prevention of Cranial Defects by Folic Acid: Emerging Doubts About A Universally Beneficial Role

Although not as extensively studied, it is conceivable that the response to a specific nutrient shows a similar variation in different genetic contexts as observed for folic acid supplementation. Without considering differences in route and duration of administration, folic acid has a beneficial action in decreasing neural tube defect incidence in multiple models: in mice with mutations in the *Cart1/Alx1*, *Cited2*, *Gcn5*, *Pip5k1c*, *L3P*, *Shroom3*, and *Zic2* genes, the *Lrp6/crooked tail* and *Pax3/Spotch* alleles, and diabetes-exposed rat and mouse embryos. The genes targeted by these mutations code for various types of molecules, from transcription factors (*Cart1*, *Pax3*) to enzymes (*PIP5K1 γ*), and they are not known to participate in shared signaling pathways, apart from the fact that they all cause defects when disrupted. Similarly, mutations for different types of molecules are represented among the folate resistant and folate and inositol resistant models. Particularly surprising are the findings of detrimental action of folic acid in the CXL *Spotch*, *Grhl2*, *L3P*, *Shroom3*, and *Lrp6* null mutants, some of which experience increased resorption and excessive loss of the mutant genotype, as well as increasing NTD rates. These studies are complemented by reports that high folic acid supplementation can be associated with increased embryonic loss and growth delay, and ventricular septal heart defects [Mikael et al., 2013] even in wild-type pregnancies, and that high dose folinic acid supplementation was associated with delay in cartilage maturation [Kappen et al., 2004]. These findings are highly concerning from a prevention standpoint [Obican et al., 2010; Oakley, 2012], since duration of nutrient exposure was important for the effect in some of the models, raising the possibility that long-term exposure and high levels of folic acid in humans could also be detrimental. A recent review [Strickland et al., 2013] addresses possible mechanisms underlying detrimental effects, with emphasis on cancer risk. Epidemiological evidence suggests that folic acid supplementation also had detrimental effects on certain growth parameters and wheezing or asthma in infants [Burdge and Lillycrop, 2012]. Given that folic acid can affect multiple pathways in the cell (see above), and affects DNA methylation and epigenetic programming [Waterland and Jirtle, 2003, 2004], it is particularly important to consider that different genetic backgrounds can affect the biological outcome. In this regard it is interesting that apoptosis could be prevented by folic acid supplementation in *Cited2* and *Cart1* mutants, suggesting a role in cell survival and proliferation. On the other hand, from

the *Alx3* and the *Lrp6/crooked tail* mutants, it has been postulated that folic acid may counteract cell proliferation, and Wnt signaling, respectively. These unresolved questions and concerns highlight the need to continue studies on this and other nutrients, and to identify the molecular basis for their biological actions under different genetic and pathogenic conditions, such as maternal diabetes and obesity.

Mouse Models, Despite Limitations, Are Indispensable Tools in NTD and Nutrition Research

Mouse models have been instrumental for the progress in our understanding of nutrient–gene interactions. Yet, it is important to recognize some limitations of the studies performed to date: In some studies, diets were made from commercially available chow components; commercial diets can vary in composition over time, and depending on the natural ingredients used, may contain unrecognized compounds with activity in female reproduction [Wang et al., 2005]. Furthermore, the base diets are not always comparable between laboratories, and nutrient supplementation was administered in various forms, limiting comparability between the different models. A second concern regarding diets is related to the finding that carbohydrate and fat content of the maternal diet [Rosenfeld et al., 2003] and type of polyunsaturated fatty acids [Fountain et al., 2008] can affect the sex ratio in progeny. Because there is a female excess of NTDs [Juriloff and Harris, 2012b] in many, although not all, mouse models, skewing of sex ratio would be expected to influence the interpretation of diet effects as detrimental or beneficial. Another important parameter in mouse modeling studies is the time point of investigation. While it has been argued that delays in neural tube closure can be compensated for and therefore early assessment might overestimate the incidence of defects [Kalter, 1996], one also has to keep in mind that late assessment misses early resorptions. Early lethality could be due to implantation defects, defective nutrient supply, turning defects, lethal defects not associated with cranial development (such as, e.g., heart defects), or severe delay of embryonic development. The example of the *Lrp6* null mutants [Gray et al., 2010] shows that estimates of beneficial action can be misleading when certain genotypes are selectively eliminated prior to assessment of NTDs and when defect rate is calculated relative to surviving progeny only. Although re-sorptions typically go unnoticed in human pregnancies, any diet that increases resorption rate in mice raises concern about the potential adverse effects of that diet on human fertility. A few studies suggest a beneficial effect of micronutrient supplementation on female fertility, but the available evidence is limited [Grajecki et al., 2012]. Another caveat is highlighted by the finding in the *curly tail* mouse model [Burren et al., 2010] that genetic background rather than the specific mutation at the *Grhl3* locus confers predisposition to developmental defects under particular nutritional conditions. The chance for accumulation of such background polymorphisms is higher with mutant models—as compared to commercially available strains—since they are often bred in small colonies in individual laboratories where genetic drift may not be tightly controlled. Finally, as demonstrated for the *Pax3/Spotch* [Fleming and Copp, 1998], *Lrp6/crooked tail* [Ernest et al., 2006] and *Grhl3/curly tail* [Tran et al., 2002] strains, mutant alleles in non-metabolic genes may be associated with metabolic alterations. Although the molecular bases for the metabolic changes are unknown at present, it is conceivable that other neural tube defect models also have metabolic deficiencies that could profoundly affect the response to nutritional supplementation; also unknown is how

these responses may be modulated by maternal metabolic disease, such as obesity or diabetes.

In this regard it is also noteworthy that inositol supplementation can exacerbate valproic acid teratogenicity [Massa et al., 2006], further highlighting the complexities of teratogenic nutrient–gene interactions.

Open Questions

How normal is mother's metabolism?—Metabolic disease of the mother and nutrient deficiencies are well-known risk factors for neural tube defects in humans. Consequently, adequate intake of deficient nutrients can lower NTD incidence, as demonstrated for folic acid [Obican et al., 2010]. In animal models of neural tube defects, however, maternal metabolism has received much less attention, probably because many of the genes involved in mouse NTDs have no apparent role in metabolism. For example, Pax3 is considered a developmental transcription factor with roles in neural crest derivatives and myogenesis. Therefore, it was unexpected that *Pax3* mutant mice exhibit deficiency in thymidine synthesis [Fleming and Copp, 2000]. Similarly, from the role of Lrp6 as a Wnt co-receptor, it was not readily obvious that the *crooked tail* mutants would have a folate utilization defect [Ernest et al., 2006]. In *curly tail* homozygous mice, in which the transcription factor Grhl3 is disrupted, one carbon metabolism is altered [De Castro et al., 2010]. Many other mouse NTD models have not been investigated in this regard. It is also unknown to what extent such metabolic perturbations are present in heterozygotes, or whether they might be exaggerated during pregnancy. Thus, unrecognized metabolic perturbations may be present in more models than currently appreciated. A prominent example is the Alx3 transcription factor, which, independent of its role in cranial mesenchyme, is required for insulin secretion and glucose homeostasis in the adult [Mirasierra et al., 2011; Vallejo, 2011]. Studying NTD models for metabolic defects would not only have implications for a better understanding of the cause of neural tube defects and their prevention, but could also reveal novel roles for developmental genes in adult physiology.

Does the embryo respond directly to the nutrient?—An important open question for understanding nutrient modulation of cranial development is whether the nutrient effects are direct, correcting abnormal metabolic or signaling pathways in the embryo, or indirect, correcting defective nutrient transport and supply to the embryo. Cranial development, and anterior neural tube closure, initiate before the placenta is formed; the major route of nutrient transport to the embryo is provided by visceral endoderm that surrounds the embryo [Zohn and Sarkar, 2010]. Supply of lipoproteins to the developing embryo is regulated by ApoB, as demonstrated by the visceral endoderm abnormalities in the *ApoB* mutants [Farese et al., 1995]. Iron transport is also dependent on the visceral endoderm as demonstrated by accumulation of iron outside of the embryo in mutants deficient in Ferro-portin1 [Narisawa et al., 2012]. Intriguingly, FolR1 is highly expressed in the visceral endoderm, and undetectable in the embryo as the neural tube develops [Salbaum et al., 2009], implicating the visceral endoderm as a major target for folate supplementation, and suggesting that the various developmental defects in the *Folr1* mutants could be secondary to a transport defect in extraembryonic cells [Salbaum et al., 2009]. Notably, the *Glut2* gene, which encodes a

high capacity glucose transporter, is expressed predominantly in visceral endoderm prior to neural tube closure, and only at very low levels in the embryo proper [Smith and Gridley, 1992]. Genetic removal of GLUT2 reduced the incidence of embryos with malformed neural tubes in diabetic pregnancies [Li et al., 2007], but it also reduced overall embryonic survival to E10.5, even of heterozygous mutants. This suggests that not only embryonic glucose uptake but also glucose transport to the embryo by the visceral endoderm could be deficient in *Glut2*-deficient mutants. Culture of embryos and yolk sac from diabetic pregnancy models also provided evidence for an important role of the visceral endoderm [Sadler and Horton, 1983; Reece et al., 1985; Pinter et al., 1986; Sadler et al., 1988], in that ketone bodies and somatomedin inhibitors impaired pinocytosis and phagocytosis [Hunter and Sadler, 1992]. Given that visceral endoderm originates from the blastocyst, the genetic composition of visceral endoderm cells is identical to the embryo in any mutant model. The important implication of this is that in mutants that have altered metabolism due to cell-autonomous defects, the visceral endoderm cells will have those same defects, and thus aberrant visceral endoderm sensing and transport of nutrients may be contributing to neural tube defects. In addition, visceral endo-derm also plays an important role in providing developmental signals to the embryo [Bielinska et al., 1999] that are required for successful gastrulation, axial extension, left-right asymmetry, early organogenesis and neurulation. It is thus conceivable that metabolic influences on visceral endoderm affect key developmental signals for morphogenesis of the embryo. Although not studied in many neural tube defect mutants, the visceral endoderm may therefore hold clues specifically to the etiology of diet-responsive cranial defects.

How do embryonic cells sense nutrients?—A second unresolved question is how nutrient composition is sensed by embryonic cells, or by the visceral endo-derm. In analogy to the adult organism [Yuan et al., 2013], the ATP/ADP level sensing AMPK pathway [Oakhill et al., 2011] and the amino acid sensing mTOR pathways [Dennis et al., 2001] could play a role. Mutants with deficiencies in mTOR do not develop past the early implantation stage [Gangloff et al., 2004], indicating an essential requirement before gastrulation. Mutant embryos in which the rictor or mLst8 components of the mTOR2 complex were ablated, developed with apparently normal neural tube closure and cardiogenesis; their growth was delayed and they died later, possibly from defects in vascular development (*mLst8* mutants) [Guertin et al., 2006] and deficits in Akt/ PKB signaling (*rictor* mutants) [Shiota et al., 2006]. Although a *flat-top* phenotype has been reported for mutants with an ENU-induced null allele for the *Frap* gene (which encodes TOR in the mouse) [Hentges et al., 2001], the impaired brain growth did not prevent closure of the neural tube in *flat-top* mutants. Taken together, these results indicate that the post-implantation embryo critically requires the mTOR1 complex, and that the mTOR2 complex is not essential during gastrulation and neural tube closure. To study mTOR1-mediated nutrient sensing specifically during neural tube closure would require the generation of conditional mouse mutants in which the timing of ablation can be tightly controlled.

What is the role of mitochondria in neural tube defects?—Mouse mutants with deficiency of any of the subunits of AMPK do not exhibit embryonic defects, although they exhibit metabolic defects as adults (Mouse Genome Informatics Database [Eppig et al.,

2012]). This indicates that under nutritionally replete conditions, AMPK activity is not essential for neural tube closure or cranial development. In addition to ATP sensing, AMPK also has a role in suppressing lipid synthesis and stimulating mitochondrial uptake and oxidation of fatty acids [Yuan et al., 2013]. The importance of mitochondrial metabolism for cranial development is highlighted by the phenotype of the ENU-induced mutant *nearly headless, nehe* [Zhou and Anderson, 2010]. In these mutants, the gene encoding the enzyme catalyzing synthesis of Lipoic acid (*Lias*) is disrupted. Lipoic acid needs to be endogenously synthesized during embryonic development [Yi and Maeda, 2005], and is a cofactor for several mitochondrial complexes. Thus, the *nehe* mutants likely have mitochondrial defects, such as of ATP production, as reflected in elevated AMPK levels. Since upregulation of AMPK can inhibit the cell cycle, the mutants exhibit reduced cell proliferation, which would account for their smaller size, but not the cranially restricted phenotype. Interestingly, cellular morphology was perturbed in the anterior visceral endoderm and the primitive streak [Zhou and Anderson, 2010], leading the authors to hypothesize that the pre-chordal plate has a particularly high requirement for mitochondrial metabolism. Another line of evidence for the role of mitochondria comes from the high penetrance of exencephaly and craniorachischisis in mutants with deficiency of mitochondrial MTHFD1like [Momb et al., 2013]. Thus, specific regions or cell types in the developing embryo may be exquisitely sensitive to nutrients due to energetic requirements. Yet, it remains unresolved how nutrient and metabolic pathways are linked to the developmental mechanisms that control development of cranial structures and neural tube closure. Lastly, mechanisms that underlie the positioning of cranial closure sites and developmental asynchronisms are still enigmatic.

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Biography

Claudia Kappen holds the Peggy M. Pennington Cole Endowed Chair in Maternal Biology at the Pennington Biomedical Research Center/Louisiana State University System in Baton Rouge, LA. After receiving her Ph.D. degree in Molecular Genetics from the University of Cologne, Germany, she trained in Developmental Genetics at Yale University. She previously held faculty appointments at Mayo Clinic Scottsdale, AZ and the University of Nebraska Medical Center in Omaha, NE. Her research interests include the role of Hox genes in skeletal patterning, mechanisms of gene regulation, and the effects of maternal nutrition and metabolic disease in embryonic development.

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Figure 1. Neural tube defects in mouse models. A: Schematic depiction of cranial neural tube closure. Closure initiation sites 1, 2, and 3 are indicated (from Fleming and Copp [2000], with permission from Oxford University Press). B: Image of a normal embryo from a normal pregnancy. C: Image of an embryo from a diabetic mouse pregnancy, with anterior neural tube defect. D: Image of an embryo with posterior neural tube defect.

TABLE I

Nutrient Responses in Models of Defective Cranial Development

Model	Responsive to supplementation		Unresponsive to supplementation	Refs.
	Beneficial	Detrimental		
diabetic rat, STZ		high carbohydrates		[Giavini et al., 1991]
		high sucrose		[Zusman and Ornoy, 1986]
	safflower oil			[Higa et al., 2010; Reece et al., 1996]
	myo-inositol			[Khandelwal et al., 1998]
	folate			[Higa et al., 2010]
	folate/safflower oil combined			[Higa et al., 2010]
	folate/Vitamin E combined			[Gareskog et al., 2006]
	arachidonic acid			[Goldman et al., 1985]
	lipoic acid			[Wiznitzer et al., 1999a]
	butylated hydroxytoluene			[Eriksson and Siman, 1996]
	vitamin E ^a			[Siman and Eriksson, 1997; Viana et al., 2003]
	vitamin E/folate			[Siman and Eriksson, 1997]
	Vitamin E/Vitamin C			[Cederberg et al., 2001]
	high dose Vitamin C			[Cederberg and Eriksson, 2005]
diabetic mouse, STZ	folic acid			[Oyama et al., 2009]
	Lipoic acid			[Sugimura et al., 2009]
	Purina 5001	Purina 5015		[Kappen et al., 2011]
SELH/Bc	Purina 5001	Purina 5015		[Harris and Juriloff, 2005]
		retinoic acid		[Tom et al., 1991]
			folic acid	[Harris and Juriloff, 2005]
			methionine	[Harris and Juriloff, 2005]
			niacin	[Harris and Juriloff, 2005]
			Brewer's yeast	[Harris and Juriloff, 2005]
			riboflavin	[Harris and Juriloff, 2005]
			Vitamin B12	[Harris and Juriloff, 2005]
			Methyl donors choline/betaine/folic acid/Vit12	[Harris and Juriloff, 2005]
			inositol	[Harris and Juriloff, 2005]
MTHFD1like	formate ^b			[Momb et al., 2013]
twisted gastrulation	methyl donors: folate/B12/betaine/choline ^c			[Billington et al., 2013]
AMT	marginal response to methionine		methionine	[Narisawa et al., 2012]
			thymidine monophosphate	[Narisawa et al., 2012]
			thymidine/methionine	[Narisawa et al., 2012]
			folate	[Narisawa et al., 2012]

Model	Responsive to supplementation		Unresponsive to supplementation	Refs.
	Beneficial	Detrimental		
Cited 2		high fat ^d		[Bentham et al., 2010]
	folic acid ^d			[Barbera et al., 2002]
Alx3		folate deficiency	folate resistant	[Lakhwani et al., 2010]
Shmt		folate deficiency ^e	choline deficiency had no effect	[Beaudin et al., 2012]
<i>Pax3/SpotchH</i>		folate deficiency	methionine	[Burren et al., 2008]
	folic acid			[Fleming and Copp, 1998]
	thymidine			[Fleming and Copp, 1998]
<i>Pax3/Spotch</i>			folic acid	[Gefrides et al., 2002; Li et al., 2006]
			marginal effect of high folic acid	[Marean et al., 2011]
	folic acid			[Stottmann et al., 2006]
CXL Spotch		folate-deficient diet ^f	thymidine	[Wlodarczyk et al., 2006]
		myo-inositol ^g	thiamine	[Wlodarczyk et al., 2006]
		methionine	α-tocopherol (Vitamin E)	[Wlodarczyk et al., 2006]
	folate fortified diet ^h			[Wlodarczyk et al., 2006]
	folic acid ⁱ			[Wlodarczyk et al., 2006]
	folinic acid ^j			[Wlodarczyk et al., 2006]
	5 methyltetrahydrofolate ^k			[Wlodarczyk et al., 2006]
L3P	short-term high folic acid ^l	long term high folic acid ^m	short-term folic acid ^l	[Marean et al., 2011]
Shroom3	short-term high folic acid ⁿ	long-term high folic acid ^o	long-term high folic acid ^o	[Marean et al., 2011]
Zic2	long term high folic acid ^p			[Marean et al., 2011]
Cart1/Alx1	folic acid			[Zhao et al., 1996]
Gcn5	folic acid			[Lin et al., 2008]
PIP5K1γ	folic acid			[Wang et al., 2007]
Lrp6/ <i>crooked tail</i>	folic acid			[Carter et al., 1999]
Lrp6 null		folic acid ^q		[Gray et al., 2010]
Grhl2		folic acid ^r	folic acid ^r	[Marean et al., 2011]
Frem2			folic acid ^s	[Marean et al., 2011]
Tead2			folic acid resistant	[Kaneko et al., 2007]
Ski			folic acid resistant	[Ernest et al., 2006]
Grhl3/ <i>curly tail</i>		inositol-deficiency ^t	folate resistant	[Cockroft et al., 1992; Tran et al., 2002]
	retinoic acid ^t			[Chen et al., 1994]
	inositol ^t			[Greene and Copp, 1997]
Grhl3 null			folic acid and inositol resistant	[Ting et al., 2003]
Map3k4/MEKK4			folic acid and inositol	[Chi et al., 2005]

Model	Responsive to supplementation		Unresponsive to supplementation	Refs.
	Beneficial	Detrimental		
Zic3/bent tail			resistant folic acid and inositol resistant	[Franke et al., 2003]

^a High concentrations promote resorptions.

^b Supplementation also prolongs embryo survival.

^c Supplementation reduced branchial arch defects but not midline defects.

^d Folic acid reduces NTD incidence; high fat diet causes higher rate of cleft palate and heart defects but has no effect on NTDs.

^e Folate deficiency causes exencephaly in otherwise non-symptomatic mutants.

^f Increased resorptions and rate of neural tube defects, no effect on survival of particular genotypes.

^g Inositol supplementation significantly reduced survival of homozygotes and increased, among the survivors, the frequency of individuals with both exencephaly and spina bifida.

^h Significantly higher rate of resorptions, decreased survival of homozygous mutants, and a higher fraction of embryos had both exencephaly and spina bifida.

ⁱ Reduced rate of exencephaly and spina bifida combined, greater incidence of exencephaly only.

^j Trend, reduction of neural tube defects was not significant.

^k Increased resorptions, decreased survival of homozygotes and decreased NTD incidence.

^l Short-term high folic acid promotes survival of homozygous mutants but has no effect on NTD incidence.

^m Long-term high folic acid detrimental to homozygote survival, and higher rate of NTDs.

ⁿ Short-term high folic acid reduced NTD frequency; no effect on survival rates.

^o Long-term high folic acid reduced survival of homozygous mutants, but had no effect on NTD frequency.

^p Long-term high folic acid increased homozygote survival, and had marginal beneficial effect on NTD incidence.

^q Earlier lethality of homozygotes, higher rate of NTDs.

^r Appearance of NTDs in heterozygotes with short-term and long-term exposure to high folic acid; selective loss of homozygous mutants regardless of diet, and without effect on NTD incidence.

^s Non-significant trend toward higher incidence of NTD with long-term high folic acid diet.

^t Was reported before it was discovered that the genetic background alone (not Grhl3 genotype [Burren et al., 2010]) confers sensitivity to folate deficiency; responses to other nutrients were not specifically evaluated again.