PNAS
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Main manuscript for

NAD deficiency due to environmental factors or gene-environment interactions causes congenital malformations and miscarriage in mice

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Classification
Major: Biological Sciences
Minor: Medical Sciences

Keywords
congenital malformation, miscarriage, NAD, metabolism, embryonic development

Abstract

Causes for miscarriages and congenital malformations can be genetic, environmental, or a combination of both. Genetic variants, hypoxia, malnutrition or other factors individually may not affect embryo development but collectively may do so. Biallelic loss-of-function variants in *HAAO* or *KYNU*, two genes of the NAD synthesis pathway, are causative of congenital malformation and miscarriage in humans and mice. The variants affect normal embryonic development by disrupting the synthesis of NAD, a key factor in multiple biological processes, from its dietary precursor tryptophan, resulting in NAD deficiency. This study demonstrates that congenital malformations caused by NAD deficiency can occur independent of genetic disruption of NAD biosynthesis. C57BL/6J wildtype mice had offspring exhibiting similar malformations when their supply of the NAD precursors tryptophan and vitamin B3 in the diet was restricted during pregnancy. When the dietary undersupply was combined with a maternal heterozygous variant in *Haao*, which alone does not cause NAD deficiency or malformations, the incidence of embryo loss and malformations was significantly higher, suggesting a gene-environment interaction. Maternal and embryonic NAD levels were found to be deficient. Mild hypoxia as an additional factor exacerbated the embryo outcome. Our data show that NAD deficiency, as a cause of embryo loss and congenital malformation, is not restricted to the rare cases of biallelic mutations in NAD synthesis pathway genes. Instead, monoallelic genetic variants and environmental factors can result in similar outcomes. The results expand our understanding of the causes of congenital malformations and the importance of sufficient NAD precursor consumption during pregnancy.

Significance Statement

Causes for congenital malformations and miscarriages can be genetic, environmental, or a combination of multiple factors, however, in most cases the underlying reasons are unknown. This study shows that a dietary undersupply of tryptophan and vitamin B3, the metabolic precursors of nicotinamide adenine dinucleotide (NAD), during pregnancy is a cause for frequent multiple birth defects and miscarriages in wildtype mice. When the maternal malnutrition coincided with a genetic variant that impaired NAD synthesis, or low oxygen levels as an environmental factor, embryos were more severely affected. Our findings in mice suggest NAD deficiency, due to environmental factors or gene-environment interactions, as a possible cause of congenital malformations in human cases in which no causative gene defect is present.

Main Text

Introduction

Congenital malformations are a leading cause of death, morbidity, and disability, affecting 3-6% of all live births (1). Known causes include chromosomal aneuploidies, pathogenic genetic variants, and various environmental factors. In many cases no obvious singular cause for the birth defect is identified, because the etiologies are predominantly multifactorial and likely result from complex, yet unidentified interactions between genes and the environment. Similarly, spontaneous miscarriages are very common, constituting about 15% of all clinically recognized pregnancies, and 1% of women trying to conceive experience recurrent miscarriage, defined as three consecutive miscarriages (2). Similar to congenital malformations, causes for recurrent miscarriage are varied and complex, and 40-75% of cases are classified as idiopathic (2, 3).

Gene-environment interactions leading to miscarriage or birth defects are generally difficult to identify, because implicated factors individually may not be overtly damaging, but in combination with

other specific factors will disrupt the highly regulated processes of embryonic development (1, 4, 5). Aside from congenital malformations, gene environment interactions are also involved in other processes and constitute, for example, the central concept of pharmacogenetics, which studies the effects of genetic heterogeneity on drug response (6). Similarly, diet is an environmental factor that has been implicated in gene environment interactions and congenital malformations (7). The composition of the maternal diet has, for example, been linked to the causation of neural tube defects. In 1991, a randomised double-blind trial revealed that these defects are primarily a vitamin deficiency disorder, which can be prevented in 80% of cases by daily dietary supplementation with folic acid before and during the first trimester of pregnancy (8). Furthermore, neural tube defects are influenced by genetic factors, and single nucleotide polymorphisms in genes involved in folate metabolism have been identified as potential risk factors (9).

Deficiency of nicotinamide adenine dinucleotide (NAD) during pregnancy, due to a genetic disruption of its synthesis pathway, was identified as a cause of multiple congenital malformations in families that also had recurrent miscarriages. In four individuals who exhibited a spectrum of similar congenital defects in multiple organs including the heart, vertebrae, kidneys, and limbs, biallelic loss-of-function variants in *HAAO* or *KYNU* were found. *HAAO* and *KYNU* encode 3-hydroxyanthraninlic acid oxygenase (HAAO) and kynureninase (KYNU), essential enzymes of the kynurenine pathway of NAD synthesis. The four affected individuals had strikingly lower serum NAD levels as a consequence of these loss-of-function variants, compared to heterozygous family members who were asymptomatic (10). Their malformation phenotypes have been classified as Congenital NAD Deficiency Disorder (also called vertebral, cardiac, renal, and limb defects syndrome; Phenotype MIM numbers 617660 and 617661, respectively). The findings were reproduced in mice, showing that if maternal dietary NAD precursors were sufficiently limited, all embryos died regardless of whether they were homozygous null, heterozygous or wildtype for a loss-of-function allele. Further, a level of NAD precursors could be identified where only homozygous null embryos were affected and developed similar combinations of malformations. NAD deficiency, embryo loss (miscarriage) and embryo abnormalities were completely prevented in these mouse models by supplementation with nicotinic acid (niacin) during pregnancy, presumably boosting NAD synthesis via the Preiss-Handler pathway (10).

The biomolecule NAD (where NAD refers to its oxidised and reduced form, NAD^+ and NADH) is crucial to cellular energy metabolism, constituting an essential co-enzyme of the cellular ATP production system. NAD is also involved in cell signalling and numerous cellular processes, such as cell division, DNA damage repair, chromatin remodelling, and mitochondrial function (11, 12). NAD is synthesised *de novo* from tryptophan via the kynurenine pathway, which occurs primarily in the liver, and from vitamin B3. Vitamin B3 is a collective term for the NAD precursors nicotinic acid (NA), nicotinamide (NAM), and nicotinamide riboside (NR). NA and NAM are converted to NAD in the Preiss-Handler and salvage pathway, respectively. Cellular NAD content is regulated by its synthesis and consumption. Sirtuins, poly(ADP-ribose) polymerases, and cADP-ribose synthetases consume NAD, and the NAM generated in these processes can be recycled back to produce NAD (11-13).

Human carriers of loss-of-function variants in 17 NAD synthesis genes are present in the Genome Aggregation Database (gnomAD), an aggregate of human exome and genome sequencing data from 141,456 individuals without severe pediatric diseases (14). Within these genomes, there are 341 alleles with predicted loss-of-function variants across the 17 genes. Because congenital malformations frequently have multifactorial etiologies, individuals carrying these variants might be predisposed to developing NAD deficiency if they are exposed to other factors affecting NAD levels. Furthermore, over 3000 alleles with missense variants in these genes are present in gnomAD, some of which might contribute to multifactorial scenarios. The actual proportion of individuals carrying such variants might be even higher given that the gnomAD cohort is enriched for healthy individuals.

Here, we aimed to show that NAD deficiency defects, as seen with homozygous loss-of-function variants in *Haao* or *Kynu*, can be induced by environmental factors and gene environment interactions. We investigated whether NAD deficiency, embryo loss, and similar congenital malformations occur in wildtype mice, or those with a heterozygous loss-of-function variant in an NAD synthesis gene. In the tested scenario a limited dietary supply of NAD precursors during pregnancy represented the environmental factor, which was combined with either a heterozygous variant in *Haao* as a genetic factor, or with hypoxia as another environmental factor, presumed to affect NAD synthesis. Our findings support the hypothesis that some unsolved human cases of congenital malformations are due to NAD deficiency.

Results

Restricted maternal intake of NAD precursors leads to embryo loss and multiple congenital malformations in wildtype mice

Pregnant mice that genetically lack the ability to convert tryptophan to NAD, produce offspring that die *in utero* or have multiple congenital malformations due to NAD deficiency, when NAD precursor vitamins (vitamin B3) are limited (10). We addressed whether C57BL/6J mice without such genetic defects similarly have miscarriages and malformed embryos when their dietary intake of tryptophan and vitamin B3 is limited during pregnancy. From the start of pregnancy, mice were fed NAD precursor vitamin-depleted and tryptophan-free feed (NTF), and the drinking water was supplemented with defined concentrations of tryptophan.

First, we determined the amount of NAD precursors that pregnant mice require to sustain embryogenesis. Female mice that were put on NTF with 400 mg/L tryptophan in the drinking water (NTF+TW400, Table 1) from conception were unable to sustain pregnancy. When dissected at E18.5, embryos appeared as necrotic conceptuses 2-5 mm in size, indicative of death during early embryogenesis. Increasing the concentration of tryptophan in the water to 500 mg/L (NTF-TW500) resulted in a large proportion (66%) of affected (dead or malformed) embryos. Of the live embryos, 62% were malformed (Table 2), most of which exhibited multiple malformations in various organs (Fig. 1, *SI Appendix* Fig. S1). Digit malformations occurred most frequently (55%). Skeletal malformations were similarly common (52%), and included rib and vertebral anomalies, of which vertebral body fusions were predominant. Congenital heart defects (CHD) were identified in 25% of embryos. Other malformations included underdeveloped eyes (46%), hypoplastic kidneys (29%), caudal agenesis (42%), and cleft palate (23%). Abdominal wall and skull defects were present at lower frequencies (Fig. 1B, *SI Appendix* Table S1). Some of the malformations occurred in isolation in a subset of embryos. A further increase in tryptophan content to 600 mg/L (NTF+TW600) saw only 29% of embryos affected, and among the 91% of embryos that were alive, 22% were malformed (Fig. 1A, Table 2). The incidence of affected embryos significantly increased when comparing the data for Standard, NTF+TW600, NTF+TW500, and NTF+TW400 diets with decreasing NAD precursor content (Table 2). Also, with reduction of NAD precursors in the diet, the frequency and diversity of malformations increased (Fig. 1B, *SI Appendix* Table S1). The E18.5 embryos were significantly reduced in weight on NTF-TW500 and NTF-TW600 treatment compared to those on the Standard diet (*SI Appendix* Fig. S2). Generally, the embryo outcome was variable among litters, but litter sizes were comparable between dietary treatment groups, and the severity of embryo outcome appeared not to depend on litter size (*SI Appendix* Fig. S3).

These data demonstrate that a maternal dietary deficit of NAD precursors during pregnancy constitutes an independent environmental factor causative of embryo loss as well as multiple congenital malformations reminiscent of genetic NAD-deficiency models.

Mild hypoxia during gestation as an additional environmental factor increases the likelihood of embryo malformations in wildtype mice

Aforementioned results show that a diet restricting NAD precursor intake is one way of inducing embryo loss and malformation. Given NAD synthesis from tryptophan requires activity of the oxygenases tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO), kynurenine-3-mono-oxygenase (KMO), and HAAO, we then investigated whether limiting the activity of those oxygenases by hypoxia during pregnancy could also lead to NAD deficiency and the same embryo outcomes. NTF+TW600 diet was deemed a suitable treatment to test other environmental factors, because it only mildly increased the malformation rate compared to Standard diet (Table 2), therefore a potential worsening due to other factors could be readily identified. Exposure of pregnant mice to 8% oxygen at E9.5 for 8 h has previously been shown to cause no embryo loss and only infrequent malformations when the diet is rich in NAD precursors (15, 16). Exposure to severe hypoxia (5.5% oxygen for 8 h) has been shown to cause the highest incidence of malformations when done at E9.5 (17).

Pregnant mice on NTF+TW600 diet exposed to 8% oxygen at E9.5 for 8 h, had a more than 2-fold increase in the incidence of affected offspring compared to mice on NTF+TW600 without hypoxia, largely through a significant increase in the proportion of malformed embryos (Fig. 1A, Table 2). Interestingly, 50 of the 74 malformed embryos had isolated malformations. Hypoxia-exposed embryos exhibited significantly more vertebral and kidney malformations, but less eye defects (Fig. 1B). These experiments show that hypoxic exposure during a key period in embryonic organogenesis can exacerbate the effect of the NAD precursor-restricted diets on early embryonic development.

A maternal *Haao* loss-of-function mutation exacerbates the effect of dietary NAD precursor restriction on embryonic development

Next, we investigated potential gene-environment interactions between a parental heterozygous loss-of-function variant in *Haao* and the dietary restriction of tryptophan. *Haao*$^{+/-}$ females were mated with *Haao*$^{+/-}$ males. Female mice on Standard diet throughout pregnancy had no higher incidence of embryo loss or malformations than wildtype mice (Table 2, Fig. 2). This shows that provided a mother consumes an adequate amount of NAD precursors during pregnancy, a maternal *Haao*$^{+/-}$ genotype alone does not induce embryo loss or malformations, independent of embryo genotype. With pregnant *Haao*$^{+/-}$ females maintained on NTF+TW600 diet until E18.5, we observed a significant increase in the incidence of affected embryos from 29% to 61% when compared to offspring of wildtype parents on the same diet. This is mainly attributed to a more than 4-fold increase in embryo deaths from 9% to 42%, frequently of entire litters (Table 2, *SI Appendix* Fig. S4).

Categorising embryos whose mothers were on NTF+TW600 diet by their *Haao* genotype revealed a significantly higher incidence of malformations in *Haao*$^{-/-}$ embryos compared to all the other genotypes (*SI Appendix* Fig. S5A). It was not possible to genotype dead embryos, due to death early in development and complete necrosis of conceptuses by E18.5. The genotypic distribution of surviving embryos at E18.5 conformed to the expected Mendelian ratio (*SI Appendix* Fig. S5C), indicating that the parental *Haao*$^{+/-}$ genotype increased the mortality of all three embryo genotypes. Therefore, embryonic deaths appeared to occur independent of embryo genotype and dependent upon the maternal genotype.

To strengthen the hypothesis that the observed embryo loss and malformations are due to deficiency of NAD and not of tryptophan, we performed experiments in which the NTF+TW600 diet was supplemented with 15 mg/L NA in the drinking water (NTF+TW600+NW15). The percentage of affected embryos of all genotypes was considerably lower with NTF+TW600+NW15 than with the non-supplemented NTF+TW600 diet (15% to 61%) and similar to the baseline rate observed with Standard

diet (Table 2). Embryo loss and malformation were therefore not caused by restriction of dietary tryptophan *per se*, but rather by general limitation of NAD precursors.

As a positive control we mated *Haao*$^{-/-}$ females with *Haao*$^{+/-}$ males, as done previously (10), and as in our experiments with wildtype C57BL/6J mice, we kept pregnant females on a precursor-restricted diet (NF+NW6) throughout pregnancy. At E18.5, *Haao*$^{-/-}$ embryos from this treatment exhibited very similar types of malformations to those seen in embryos from wildtype parents treated with NTF+TW500 or NTF+TW600 diets (*SI Appendix* Table S1, *SI Appendix* Fig. S6). Complete genetic disruption of the NAD *de novo* pathway resulted in the same malformations as a restriction of dietary tryptophan and vitamin B3 supply.

We also examined the impact of combining two environmental factors, maternal diet and oxygen supply during pregnancy, with the genetic component of maternal heterozygosity for *Haao*. Pregnant *Haao*$^{+/-}$ females that had been mated with wildtype males were fed NTF+TW600 diet until embryo collection at E18.5. They were either exposed to 8% oxygen at E9.5 for 8 h (NTF+TW600+HYP) or remained exclusively in a normoxic environment. Similar to the *Haao*$^{+/-}$ intercrosses, embryos from matings with only a maternal *Haao*$^{+/-}$ mutation exhibited a higher rate of affected embryos and reduced embryo survival compared to embryos from wildtype matings with NTF+TW600 diet (Table 2). This gene-environment effect was irrespective of embryo genotype (*SI Appendix* Fig S7). The added hypoxia treatment resulted in a non-significant increase in proportion of embryos with congenital malformations compared to the normoxic controls on the same diet (Table 2, Fig. 2A).

In summary, these results evidence a strong gene-environment interaction between maternal heterozygosity for NAD synthesis genes and NAD precursor restricted diets. Although the maternal NTF+TW600 diet affected embryos only mildly, and the maternal *Haao*$^{+/-}$ mutation had no overt effect under adequate NAD precursor supply, with both factors combined the proportion of dead and affected embryos was significantly elevated (Table 2).

Maternal and embryonic NAD levels are lowered under maternal treatment conditions that cause embryo loss and congenital malformations in mouse embryos

We collected embryos at E9.5 and E11.5 to assess survival rates during embryonic organogenesis. With a restriction of maternal NAD precursor intake, we observed both higher rates of embryo death, as well as developmental delay at E9.5 whereby embryos morphologically resembled E8.5 embryos (*SI Appendix* Fig. S8). At E11.5, the mortality of embryos was similarly elevated with a reduction of maternal dietary NAD precursors (*SI Appendix* Table S2, *SI Appendix* Fig. S9).

We then aimed to directly measure NAD levels in pregnant mice and their offspring. We collected maternal livers and corresponding embryos at E11.5, as at this timepoint structures in which we observed malformations, specifically the heart (18) and kidney (19), are in a key stage of organogenesis. Firstly, we compared maternal NAD stores. Liver NAD levels in non-pregnant control females maintained on Standard diet were 673 ± 86 nmol/g. By contrast, pregnant females had significantly lower NAD levels (561 ± 65 nmol/g), and all other cases were compared with this group (Fig. 3, *SI Appendix* Table S3). *Haao*$^{-/-}$ females maintained on the NF+NW6 diet, which provides ample supply of tryptophan but restricts supply of NAD precursor vitamins, produce a large proportion of *Haao*$^{-/-}$ embryos with malformations at E18.5 (*SI Appendix* Fig. S6). At E11.5, pregnant *Haao*$^{-/-}$ mothers on NF+NW6 had significantly lower liver NAD levels than pregnant wildtypes on Standard diet (Fig. 3, *SI Appendix* Table S3). Pregnant wildtype mice on NTF+TW500 diet, which frequently had offspring with multiple malformations, had liver NAD levels similar to those of *Haao*$^{-/-}$ mice on NF+NW6 diet. By contrast, liver NAD levels of wildtypes on the comparatively more tryptophan-rich NTF+TW600 diet were only insignificantly lower than those of the Standard diet group. These data indicate that lowered maternal NAD levels cause embryo demise irrespective of the NAD deficiency being caused by limited

NAD precursors in the diet, or genetic blockage of NAD synthesis. They also show that pregnancy alone leads to a reduction in maternal NAD liver stores.

Next, we compared NAD levels in whole E11.5 embryos. Embryo NAD levels were significantly lower compared to the Standard diet group when wildtype mothers were fed NTF+TW600 diet, even lower with NTF+TW500 and further reduced when NTF+TW600 was combined with hypoxia (Fig. 3, Table 3). In experiments involving *Haao*$^{+/-}$ mothers on NTF+TW600 diet, maternal liver NAD levels were not significantly affected by the genetic variant, but the embryos had significantly lower NAD levels. Comparing the embryo NAD levels of the three embryo genotypes between Standard and NTF+TW600 diet revealed that the diet effect is significant whereas the embryo genotype effect is not (Table 3). These data indicate the NAD precursor restricted diet has a general impact on all embryos at this stage, independent of their genotype (Fig. 3). The NA-supplemented NTF+TW600+NW15 diet restored the embryo NAD levels, which was consistent with the lack of malformations at E18.5.

We measured embryo NAD levels at E9.5 to further evaluate the impact of the 8 h hypoxia treatment. Similar to the observations at E11.5, the NTF+TW600 diet resulted in a significant reduction in embryo NAD levels at E9.5 compared to Standard diet. Embryos exposed to hypoxia (NTF+TW600+HYP) and collected directly after the treatment had NAD levels that were lowered compared to NTF+TW600, without reaching statistical significance, concordant with the results obtained with E11.5 embryos (*SI Appendix* Fig. S10, *SI Appendix* Table S4).

Overall, the embryo NAD levels at E9.5 and E11.5 correlate with observed embryo outcomes at E18.5 and confirm the hypothesised gene-environment effect. Severe NAD deficiency, defined as <1.39 nmol NAD per mg protein in the embryos (Fig. 4) and disrupted embryonic development could be induced by modifying the gestational environment, solely by restricting the maternal intake of NAD precursors using NTF+TW500 (providing approximately 57.1 µg NAD precursors per day). In the presence of a maternal *Haao*$^{+/-}$ variant, the less restrictive NTF+TW600 diet (providing 67.5 µg/day of NAD precursors) caused very severe NAD deficiency (<1.2 nmol/mg protein). Pregnant mice that genetically lack the ability to convert tryptophan to NAD, in our case due to a homozygous *Haao*$^{-/-}$ variant, have severely NAD deficient embryos with a high incidence of malformations under a diet comparatively more abundant in NAD precursors, namely NF+NW6 providing 159.7 µg of NAD precursors per day (Fig. 4).

Discussion

Biallelic loss-of-function variants in either *Haao* or *Kynu* (*Haao*$^{-/-}$ or *Kynu*$^{-/-}$) in mice, which genetically block the synthesis pathway from tryptophan to NAD, cause congenital malformations and embryo loss as a consequence of NAD deficiency (10). Similar to mice, a *HAAO*$^{-/-}$ or *KYNU*$^{-/-}$ genotype in humans causes reduced NAD levels and congenital malformations in multiple organs, classified as Congenital NAD Deficiency Disorder. To date, four unrelated human cases of this disorder have been described, with each of them displaying heart, vertebral, and renal defects. Additional clinical features were reported for these individuals, including talipes, syndactyly, and cleft palate. Miscarriage was also a feature in three of the families (10).

This study demonstrates that NAD deficiency during pregnancy results in embryo loss and congenital malformations, irrespective of it being caused by environmental factors, genetic variants, or a combination of both. Firstly, we showed that an NAD precursor-restricted diet fed to mice during pregnancy is a causative environmental factor for congenital malformations. Reduction of dietary tryptophan and NAD precursor vitamins throughout pregnancy caused NAD deficiency in wildtype mice and resulted in similar malformations and embryo loss as seen with the biallelic variants. The severity of embryo outcome correlated with the extent of NAD precursor-restriction in the diet (Fig. 1A, Fig. 4). In

addition to combinations of malformations, embryos also had isolated abnormalities in various organs such as the heart, skeleton, and kidney (Fig. 1B), indicating that NAD deficiency during embryonic development results in a variable spectrum of phenotypes. When dissected at E9.5, embryos from NAD precursor restricted treatment groups frequently appeared developmentally delayed and resembled E8.5 embryos, indicating a perturbation of normal embryonic development (*SI Appendix* Fig. S8).

The NAD *de novo* pathway involves oxygenases requiring oxygen as co-substrate (20). Exposure of pregnant mice to mild hypoxia for 8 h at E9.5 has been shown to only insignificantly affect embryonic development when the diet is rich in NAD precursors (15, 16). Here, we show that when the hypoxia coincides with dietary NAD precursor deficiency, the malformation incidence is increased, with a distinct rise in frequency of isolated defects (Fig. 1A, Table 2). Similarly, while a maternal heterozygous loss-of-function variant in *Haao* alone confers no overt phenotypic effect in the offspring, embryos were significantly more likely to become malformed or die when mothers were given NAD precursor-restricted diets throughout pregnancy, indicating a gene-environment interaction (Fig. 2A, Table 2, Fig. 4). These data demonstrate that genetic and environmental factors which individually are benign with respect to NAD synthesis and embryonic development can disrupt the highly regulated embryo development processes and adversely affect pregnancy when occurring in combination. Essentially, the stronger the genetic component, the lesser the degree of a dietary/environment component required to cause NAD deficiency and impair embryonic development.

The essential amino acid tryptophan is not only catabolised for NAD synthesis in the kynurenine pathway but is also used for protein synthesis and biotransformations leading to serotonin and other chemical messengers. Quantitatively, the kynurenine pathway is the most important metabolic pathway and accounts for over 90% of tryptophan catabolism (21). Two of our findings suggest that the embryonic malformations and deaths caused by the tryptophan- and vitamin B3 restricted diets are a result of NAD deficiency and not due to tryptophan deficiency *per se*. The NA-supplemented NTF+TW600+NW15 diet contains the same limited amount of tryptophan as NTF+TW600 but significantly reduced the proportion of dead and malformed embryos to values resembling the Standard diet with adequate tryptophan content (Fig. 2). Furthermore, there is a striking similarity to the types of malformations seen in embryos of *Haao*$^{-/-}$ mothers maintained on NF+NW6 diet, which is vitamin B3 restricted but contains 1.8 g/kg tryptophan (*SI Appendix* Table S1).

A relatively narrow margin was discovered between sufficient NAD precursors and levels that cause complete loss of embryos. With NTF+TW400 diet all embryos died early in embryogenesis, whereas with a moderately higher maternal NAD precursor supply (NTF+TW600), the majority survived and less than 50% of embryos were malformed (Fig. 1, Table 2). To put the dietary treatments used in this study into a context of human requirements, the daily NAD precursor dosage that an average human would consume, should they follow diets equivalent to those fed to our pregnant mice (Table 1), can be calculated (22) and related to the recommended daily intake (RDI) for a healthy individual of a particular life stage and gender group. The RDI for a pregnant woman of 14-50 years of age is 18 mg/day of NAD precursors (23, 24). Such calculation shows that diets which caused malformations in the offspring of wildtype and *Haao*$^{+/-}$ female mice, would be below the RDI when converted to the human equivalents, i.e. they potentially also represent a nutritional deficiency in humans (*SI Appendix* Table S5). Conversely, the amount of NAD precursors a mouse receives by consuming Standard and NTF+TW600+NW15 diets theoretically would be sufficient (above the RDI), when converted to the human equivalents. According to data of a survey conducted in the USA in 2015-2016, the usual NAD precursor intake of females between ages 12-49 is in the range of 20.9 (±0.63) to 24.2 (±0.87) mg/day (25), and data from a previous survey indicate that only 1% of adults had intakes of vitamin B3 from foods and beverages below the estimated average requirement (EAR), the daily nutrient level estimated to meet the requirements of half the healthy individuals in a particular life stage and gender group (26).

While these population-based statistical data indicate that the majority of the US population receive sufficient supply of NAD precursors with their diet, the gene-environment interactions observed in mice likely also apply to humans and pregnant women with a deleterious variant in a gene required for the absorption or conversion of NAD precursors to NAD might have an actual dietary requirement that is considerably higher than the EAR.

We investigated the relationship between maternal liver and whole embryo total NAD levels, embryo phenotype, and embryo survival. Pregnant mice with a *Haao*$^{-/-}$ genotype maintained on NF+NW6 diet had significantly lower liver NAD levels than wildtype (*Haao*$^{+/+}$) mice on Standard diet (Fig. 3, Fig. 4). Pregnant wildtype mice on NTF+TW500 diet had similarly low liver NAD levels, indicating that dietary restriction of NAD precursors including tryptophan has similar consequences on maternal NAD stores as a genetic disruption of NAD *de novo* synthesis. With NTF+TW500, both maternal liver and embryos were NAD deficient at E11.5, consistent with the very high incidence of multiple malformations observed in embryos at E18.5 (Fig. 1, Fig. 4).

A maternal *Haao*$^{+/-}$ mutation combined with the NTF+TW600 diet resulted in embryo NAD levels that were lower than those of offspring from wildtype matings on the same diet (Fig. 3, Fig. 4). This gene-environment effect was also reflected at E18.5, at which stage pregnant mice of the *Haao*$^{+/-}$ genotype had a significantly higher rate of affected embryos than wildtype mice (Fig. 4, Table 2). Embryos of all three *Haao* genotypes had similarly low NAD levels at E11.5, suggesting that at this embryonic stage the embryos were not reliant on their own NAD *de novo* pathway, and the maternal genotype was the main determinant for the embryo outcome. This is likely because the mother predominantly provides a circulating intermediate that feeds into the salvage pathway, NAM or NMN, to the embryos (12). Also, the *de novo* pathway is primarily active in the liver and kidney (12, 13), and the embryos are already affected (malformed/developmentally delayed) before liver and kidney tissues have developed this metabolic activity (19, 27).

Whole blood NAD levels of *Haao*$^{-/-}$ mice maintained on NF without any NAD precursors added to the water (Table 1) remained unchanged for at least four days and then started to decline to about 50% of initial levels within two weeks (*SI Appendix* Fig. S11). Conversely, the amount of NAD available to the conceptus appears to be reduced very quickly, because embryos will not survive if their *Haao*$^{-/-}$ mothers were given the NAD precursor vitamin depleted NF in the first five days of pregnancy (from E0.5-5.5) (10). This implies the diet has a greater effect on embryogenesis than the NAD store in the body.

Energy and nutrient requirements are generally higher during pregnancy to allow for fetal growth, and studies in humans and rats suggest that the production of NAM from tryptophan gradually becomes more efficient, peaking late in pregnancy (28, 29). At the later stage of E18.5, when the mother generated NAD more efficiently from tryptophan, the *Haao*$^{-/-}$ embryos of the NAD precursor restricted NTF+TW600 group had a significantly higher malformation incidence compared to the other genotypes, but the embryo mortality was similar across all genotypes (*SI Appendix* Fig. S5A, C). In late pregnancy the embryos likely also rely on their own NAD *de novo* synthesis for growth and development, because they require more NAD and their liver and kidneys have formed. But the insufficient NAD precursor supply during early stages of embryogenesis (prior to E9.5) presumably has the biggest impact, given the rates of embryo death at this stage.

Individual embryo NAD levels were variable among dietary treatment groups (Fig. 3), consistent with the variability of embryo outcomes at E18.5. The number of embryos per litter did not have an influence on litter outcome (*SI Appendix* Fig. S3, *SI Appendix* Fig. S4). Inconsistent rates of mouse feed and water consumption could have contributed to this variability, but the relatively consistent maternal liver NAD levels suggest a relatively consistent NAD precursor supply from the diet. Despite this, the amount of NAD precursors the embryos receive may still differ between litters. Larger mice with bigger livers might buffer their circulating NAD precursor levels more effectively than lower weight mice. We

tried to minimise this variable by using female mice of a similar age range and giving them Standard diet with defined and standardised contents for 3 weeks before mating.

An interesting finding from the liver NAD measurements is the distinct reduction of NAD levels due to pregnancy (Fig. 3). Similar observations have been reported for humans, and reduced levels of NAD precursors in the blood during all trimesters of pregnancy appear to be common, even with non-deficient diets and ample dietary vitamin intake (30). These findings indicate that the risk of developing NAD deficiency is elevated during pregnancy, the time when sufficient NAD supply is required to ensure normal embryonic development.

In humans, a variety of factors can impair NAD synthesis and potentially lead to NAD deficiency. While insufficient dietary supply of vitamin B3 is rare today, NAD synthesis has also been shown to be impaired by pathophysiological factors such as inflammation, type 2 diabetes, and obesity (31-33). Kynurenine aminotransferases (KATI/II/III/IV) and kynureninase (KYNU), enzymes of the kynurenine pathway, require pyridoxal 5'-phosphate, a form of vitamin B6, as a co-factor, and its deficiency affects tryptophan metabolism (34). Vitamin B6 deficiency is rare in developed countries, but low plasma vitamin B6 status has been reported in cases of obesity and diabetes, and linked to oral contraceptive use, smoking, excessive alcohol consumption, and certain drugs (35, 36). Pregnant women are also predisposed to having depressed plasma vitamin B6 levels (37). Deficiency of vitamin B6 can therefore be a confounding factor on NAD synthesis, especially in the context of pregnancy.

Our data show that a genetic factor, i.e. a heterozygous variant of a gene related to NAD synthesis, can cause severe NAD deficiency when combined with other factors, even if the variant alone does not lead to an overt phenotype. Numerous genes are involved with NAD synthesis, including those encoding amino acid transporters, NA transporters, and enzymes of the *de novo* and salvage pathways, and mutations in these genes are potentially implicated in NAD deficiency. Gene variant databases such as gnomAD indicate that there are numerous individuals carrying heterozygous variants in these genes. Together with the many environmental factors that affect vitamin B3, vitamin B6, and NAD levels, NAD deficiency might occur in more pregnancies than expected from dietary intake survey data.

In summary, our data show that NAD deficiency leading to miscarriage and congenital malformation is not restricted to rare cases of biallelic loss-of-function mutations in NAD synthesis pathway genes, but can also be provoked by combinations of other, presumably more common factors. Also, depending on the cause, NAD deficiency can result in isolated congenital defects which are more common than syndrome-like combinations of malformations. This study supports the notion that NAD deficiency is clinically relevant. It would be interesting to determine the range of serum NAD levels before and during pregnancy in families with a history of miscarriage or congenital malformations and investigate the prevalence of NAD deficiency. In patients with non-syndromic malformations for which no causative genetic variant or obvious other factor is detectable, NAD deficiency is a possible cause for considering. Therefore, the findings of this study expand our understanding of the causes of congenital malformations and the importance of adequate maternal intake of NAD precursors during pregnancy.

Materials and Methods

Animal experiments

All animal experiments were performed in accordance with protocols approved by the Garvan Institute of Medical Research/St Vincent's Animal Experimentation Ethics Committee, Sydney, Australia (approvals 15/27 and 18/27). The *Haao* loss-of-function mouse line (allele *Haao*^{em1Dunw}) has been described previously (10).

Female mice to be used in timed matings were fed a "Standard" feed with defined composition (Table 1) (AIN93G, Specialty Feeds, Glen Forrest, Australia) for at least 3 weeks prior to mating. From the

start of pregnancy their food was replaced with a feed depleted in NA, NAM, and NR, as well as tryptophan (NAD precursor vitamin-depleted and tryptophan-free feed, NTF) (SF16-097, Specialty Feeds), and drinking water that contained defined concentrations of tryptophan (Tryptophan-supplemented Water, TW) or NA (NA-supplemented Water, NW) (Table 1). Pregnant mice were maintained on these diets until embryo collection at E11.5 for NAD quantification or E18.5 for embryo phenotyping.

For gestational hypoxia experiments (HYP), pregnant mice at E9.5 were exposed to 8% oxygen at normal atmospheric pressure for 8 h, as described (15). After exposure, the mice were either dissected immediately or returned to normoxia for embryo harvest at E11.5 or E18.5.

For a detailed description of mouse genotyping, dietary treatments, and embryo phenotyping, see *SI Appendix*, Supplementary Materials and Methods.

NAD quantification

Total NAD levels (NAD^+ and NADH) of whole embryo lysates were measured with an enzymatic cycling assay that utilises diaphorase-catalysed conversion of resazurin to the fluorescent resorufin.
For a detailed description of the protocol for NAD measurements, see *SI Appendix*, Supplementary Materials and Methods.

Statistical analysis

Two-sided Fisher's exact test was used to compare numbers of normal and affected embryos, dead and alive embryos, and normal and malformed embryos between two treatment groups. Chi-squared test was used for comparing multiple groups. In experiments involving mouse matings with *Haao* loss-of-function mutations, malformation incidence among the embryo genotypes was compared using Fisher's Exact test with Freeman-Halton extension (2×3 contingency table). This test was also used to compare observed embryo numbers for each genotype with expected Mendelian genotype distribution. One-way ANOVA with Dunnett's multiple comparisons was used to compare NAD levels and embryo weights between different treatment groups (using wildtypes on Standard diet as control group), and unpaired two-tailed t-test to compare two groups. In experiments involving mice with *Haao* loss-of-function mutations, the main effects of embryo genotype and diet and their interaction were analysed using a 2-way ANOVA followed by Tukey's multiple comparison test to assess effects of genotype within the diet groups. NAD levels are displayed as mean ± SD. P values < 0.05 were considered significant. All statistical analyses were performed with Prism (version 8, GraphPad Software), except for Fisher's Exact test with Freeman-Halton extension, for which an online tool was used (38).

Data availability

All data are available in the manuscript and *SI Appendix*.

Acknowledgments

The authors would like to thank Joelene Greasby and Kavitha Iyer for technical assistance, Eleni Giannoulatou and Loïc Thibaut for statistical genetics assistance, and Gavin Chapman and Paul Mark for criticially assessing the manuscript. This work was supported by National Health and Medical Research Council (NHMRC) [Project Grant ID 1162878, Fellowship ID 1042002] to S.L.D.; Office of Health and Medical Research NSW Government to S.L.D.; The Key Foundation to S.L.D; St Vincent's Clinic Foundation to S.L.D.

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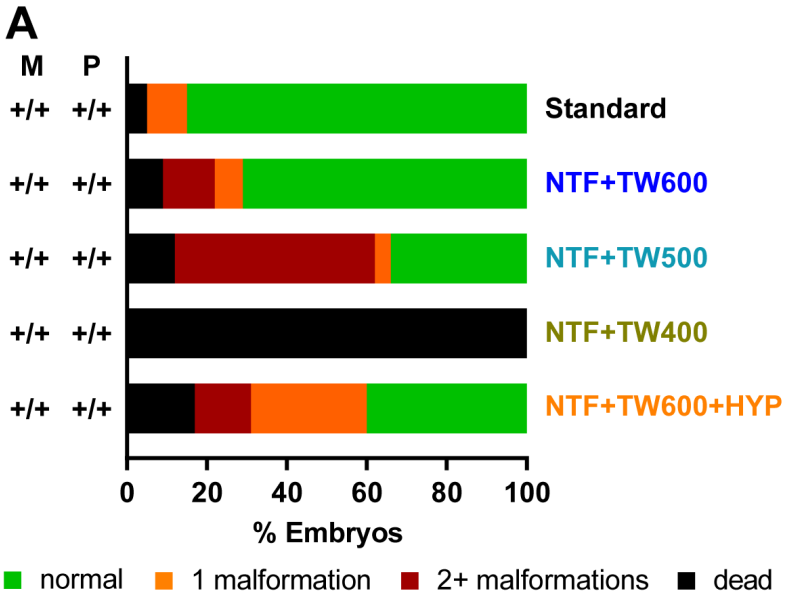
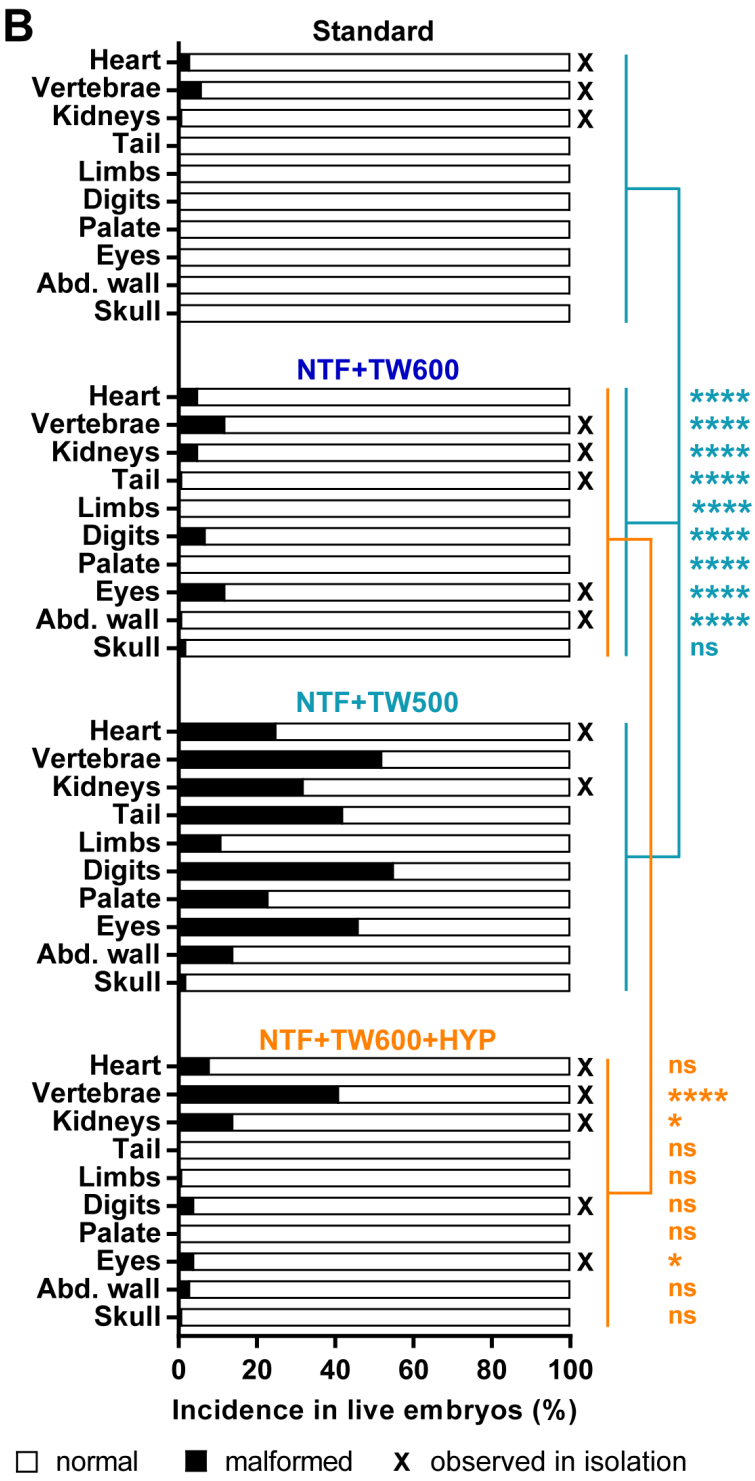
Fig. 1. Phenotypic embryo outcomes at E18.5 in offspring from C57BL/6J wildtype mice show that with a reduction of NAD precursors throughout pregnancy, the incidence of dead and malformed embryos increases. Mild hypoxia as an additional environmental factor leads to a further increase in the proportion of malformed embryos. **A**: Percentage of normal embryos (green), embryos with isolated malformations (orange), embryos with more than one malformation (red), and dead embryos (black), within maternal diet treatment groups, as indicated on the right. M, maternal *Haao* genotype; P, paternal *Haao* genotype. In this set of experiments all mice were $Haao^{+/+}$. See Table 2 for statistics and embryo numbers. **B:** Incidence of specific organ defects. Black bars represent percentage of embryos among the respective treatment group that exhibited the indicated defect. Lines and asterisks indicate statistical comparison of malformation incidence between Standard, NTF+TW600, and NTF+TW500 groups (light blue) by Fisher's Exact test with Freeman-Halton extension, and NTF+TW600 and NTF+TW600+HYP groups (orange) by two-sided Fisher's exact test, with *P<0.05; ****P<0.0001; ns, not significant. The vertebrae category includes rib malformations. Abd. wall, abdominal wall; X, malformation types observed in isolation in one or more embryos.

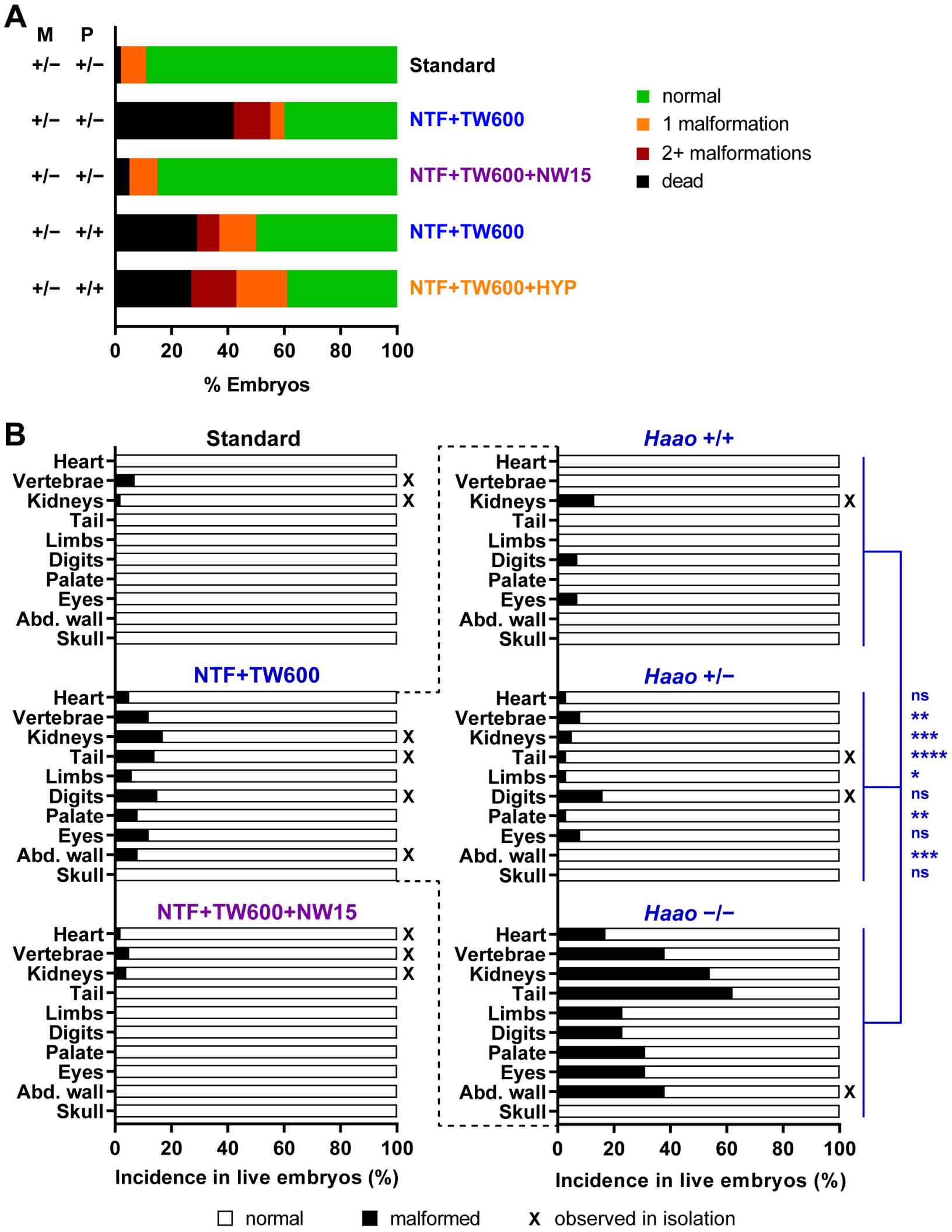
Fig. 2. Phenotypic embryo outcomes at E18.5 in offspring from mice with a $Haao^{+/-}$ mutation show a high incidence of embryo mortality with NTF+TW600 diet and indicate a gene-environment effect. With additional supply of NA in the water (NTF+TW600+NW15) embryo mortality is reduced. **A:** Summarised embryo outcomes. The dietary treatments throughout pregnancy are indicated on the right. M, maternal *Haao* genotype; P, paternal *Haao* genotype. See Table 2 for statistics and embryo numbers. **B:** Incidence of specific organ defects occurring in the respective treatment condition (left column), and of the different embryo *Haao* genotypes within the NTF+TW600 group (right column). Asterisks indicate significant differences between the three embryo *Haao* genotypes by Fisher's Exact test with Freeman-Halton extension, with *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, not significant. The vertebrae category includes rib malformations. Abd. wall, abdominal wall; X, malformation types observed in isolation in one or more embryos.

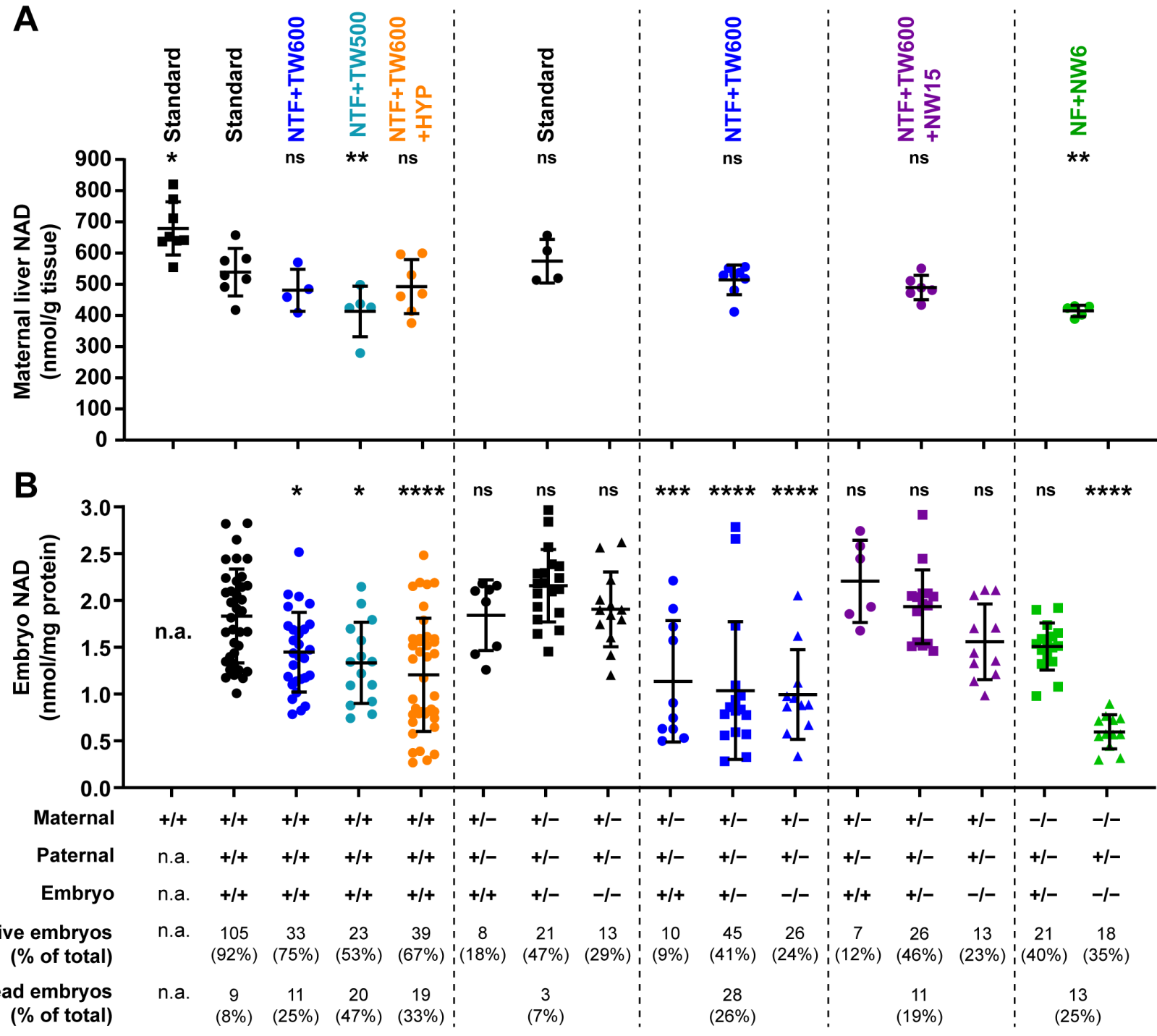
Fig. 3. Maternal liver and embryo total NAD levels (NAD^+ and NADH), and phenotypic embryo outcomes at E11.5. Dots represent NAD levels and bars indicate the mean ± SD. **A:** Maternal liver NAD levels. Treatment groups are indicated on top. The parental and embryo *Haao* genotypes are indicated at the bottom. Liver tissue was collected at E11.5 along with the embryos. The first column represents liver NAD levels of non-pregnant females of similar age to the pregnant females, maintained on Standard diet for at least 3 weeks prior to dissection. **B:** Whole embryo NAD levels. Analysed embryos were offspring of the mothers whose liver NAD levels were measured. The total numbers and percentages of live and dead embryos observed with each treatment condition are indicated at the bottom. Note that not every collected embryo underwent NAD measurement. Asterisks indicate NAD levels that are significantly different to those of the pregnant C57BL/6J wildtype Standard diet group (second column) by one-way ANOVA with Dunnett's multiple comparisons test. n.a., not applicable (no embryos in non-pregnant females). For a summary of NAD level values, see Table 3, *SI Appendix* Table S3.

Fig. 4. Summary of NAD levels and phenotypic outcomes of all treatment conditions tested in this study. Four thresholds for maternal liver and embryo NAD levels were set to specify normal, mild, severe, and very severe NAD deficiency. Similarly, four levels for the incidence of affected embryos at E18.5 were set. For the classification of embryo outcomes at E18.5 involving *Haao* genotypes, the assumption was made that the genotypes of dead (resorbed) embryos, which could not be genotyped, were distributed in a normal Mendelian ratio (25:50:25), based on the finding that the genotypes of surviving embryos did not significantly deviate from a normal Mendelian ratio.

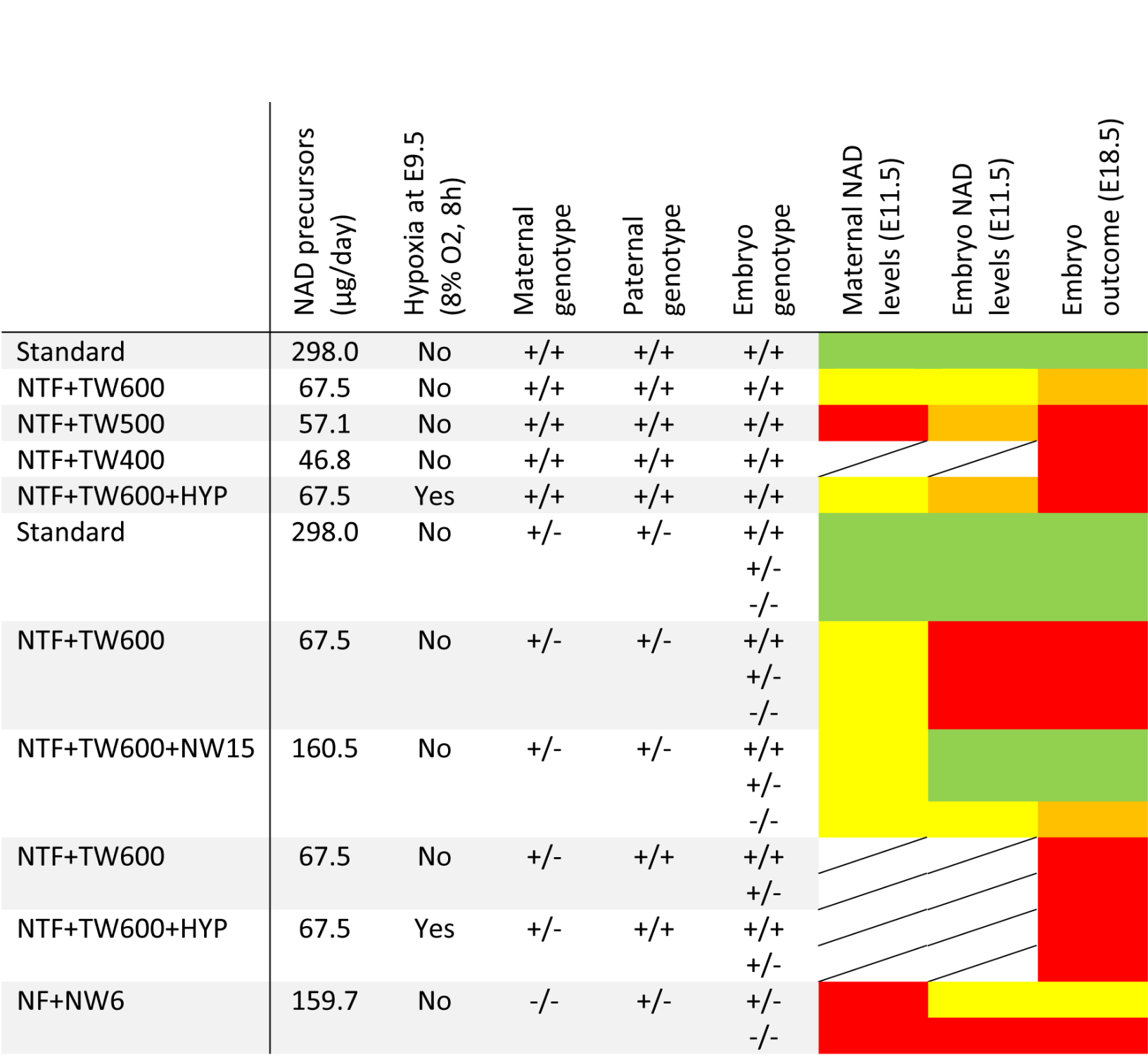

A
M P
+/+ +/+
Standard
NTF+TW600
NTF+TW500
NTF+TW400
NTF+TW600+HYP
0 20 40 60 80 100
% Embryos
normal
1 malformation
2+ malformations
dead


B
Standard
Heart
Vertebrae
Kidneys
Tail
Limbs
Digits
Palate
Eyes
Abd. wall
Skull
NTF+TW600
NTF+TW500
NTF+TW600+HYP
X
****
ns
*
0 20 40 60 80 100
Incidence in live embryos (%)
normal
malformed
X observed in isolation


A
M
P
+/−
+/+
Standard
NTF+TW600
NTF+TW600+NW15
NTF+TW600
NTF+TW600+HYP
normal
1 malformation
2+ malformations
dead
0
20
40
60
80
100
% Embryos
B
Standard
NTF+TW600
NTF+TW600+NW15
Haao +/+
Haao +/−
Haao −/−
Heart
Vertebrae
Kidneys
Tail
Limbs
Digits
Palate
Eyes
Abd. wall
Skull
X
ns
**
***
****
*
Incidence in live embryos (%)
normal
malformed
observed in isolation


A
Maternal liver NAD (nmol/g tissue)
900
800
700
600
500
400
300
200
100
0
* Standard
Standard
NTF+TW600
NTF+TW500
NTF+TW600 +HYP
Standard
NTF+TW600
NTF+TW600 +NW15
NF+NW6
ns
**
ns
ns
ns
ns
**
B
Embryo NAD (nmol/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
n.a.
*
*
****
ns
ns
ns
***
****
****
ns
ns
ns
ns
****
Maternal +/+ +/+ +/+ +/+ +/+ +/− +/− +/− +/− +/− +/− +/− +/− +/− −/− −/−
Paternal n.a. +/+ +/+ +/+ +/+ +/− +/− +/− +/− +/− +/− +/− +/− +/− +/− +/−
Embryo n.a. +/+ +/+ +/+ +/+ +/+ +/− −/− +/+ +/− −/− +/+ +/− −/− +/− −/−
Live embryos (% of total) n.a. 105 (92%) 33 (75%) 23 (53%) 39 (67%) 8 (18%) 21 (47%) 13 (29%) 10 (9%) 45 (41%) 26 (24%) 7 (12%) 26 (46%) 13 (23%) 21 (40%) 18 (35%)
Dead embryos (% of total) n.a. 9 (8%) 11 (25%) 20 (47%) 19 (33%) 3 (7%) 28 (26%) 11 (19%) 13 (25%)

	NAD precursors (µg/day)	Hypoxia at E9.5 (8% O2, 8h)	Maternal genotype	Paternal genotype	Embryo genotype	Maternal NAD levels (E11.5)	Embryo NAD levels (E11.5)	Embryo outcome (E18.5)
Standard	298.0	No	+/+	+/+	+/+			
NTF+TW600	67.5	No	+/+	+/+	+/+			
NTF+TW500	57.1	No	+/+	+/+	+/+			
NTF+TW400	46.8	No	+/+	+/+	+/+			
NTF+TW600+HYP	67.5	Yes	+/+	+/+	+/+			
Standard	298.0	No	+/-	+/-	+/+ +/- -/-			
NTF+TW600	67.5	No	+/-	+/-	+/+ +/- -/-			
NTF+TW600+NW15	160.5	No	+/-	+/-	+/+ +/- -/-			
NTF+TW600	67.5	No	+/-	+/+	+/+ +/-			
NTF+TW600+HYP	67.5	Yes	+/-	+/+	+/+ +/-			
NF+NW6	159.7	No	-/-	+/-	+/- -/-			



	Maternal NAD (nmol/g tissue)	Embryo NAD (nmol/mg protein)	Embryos affected at E18.5 (%)
Normal	>525	>1.6	0-15
Mild	525-475	1.6-1.4	16-25
Severe	474-425	1.39-1.2	26-50
Very severe	<425	<1.2	>50

Table 1. Overview of the mouse treatments and their abbreviations used throughout the text

Diet/Treatment	NA in feed (mg/kg)	Tryptophan in feed (mg/kg)	NA in water (mg/L)	Tryptophan in water (mg/L)	NAD precursors (µg/day)[1]	Hypoxia at E9.5 (8% O_2, 8 h)
Standard	31.4	2700	0	0	298.0	-
NTF+TW400	1.4	0	0	400	46.8	-
NTF+TW500	1.4	0	0	500	57.1	-
NTF+TW600	1.4	0	0	600	67.5	-
NTF+TW600+HYP	1.4	0	0	600	67.5	Yes
NTF+TW600+NW15	1.4	0	15.0	600	160.5	-
NF+NW6	1.4	1800	6.0	0	159.7	-

[1]NAD precursors (=niacin equivalents) are based on an average consumption of 3.9 g food and 6.2 mL water per day and on the approximation that 60 mg of dietary tryptophan is equivalent to 1 mg of NA for the conversion to NAD (39, 40).

NA, nicotinic acid; NF, NAD precursor vitamin-depleted feed; NTF, NAD precursor vitamin-depleted and tryptophan-free feed; NW, NA-supplemented water; TW, tryptophan-supplemented water; HYP, pregnant mouse exposed to hypoxia (8 h of 8% oxygen at E9.5).

Table 2. Summary of the embryo counts and phenotypic outcomes at E18.5 with the tested treatments

	Genotype			Of all embryos				Of live embryos	
	M	**P**	**Treatment**	**Normal**	**Affected**	**Alive**	**Dead**	**Normal**	**Malformed**
a	+/+	+/+	Standard	81 (85%)	14 (15%)	90 (95%)	5 (5%)	81 (90%)	9 (10%)
b	+/+	+/+	NTF+TW600	72 (71%)	29 (29%)	92 (91%)	9 (9%)	72 (78%)	20 (22%)
c	+/+	+/+	NTF+TW500	25 (34%)	49 (66%)	65 (88%)	9 (12%)	25 (38%)	40 (62%)
d	+/+	+/+	NTF+TW400	0 (0%)	36 (100%)	0 (0%)	36 (100%)	-	-
e	+/+	+/+	NTF+TW600 +HYP	68 (40%)	103 (60%)	142 (83%)	29 (17%)	68 (48%)	74 (52%)
f	+/−	+/−	Standard	39 (89%)	5 (11%)	43 (98%)	1 (2%)	39 (91%)	4 (9%)
g	+/−	+/−	NTF+TW600	44 (39%)	68 (61%)	65 (58%)	47 (42%)	44 (68%)	21 (32%)
h	+/−	+/−	NTF+TW600 +NW15	51 (85%)	9 (15%)	57 (95%)	3 (5%)	51 (89%)	6 (11%)
i	+/−	+/+	NTF+TW600	36 (50%)	36 (50%)	51 (71%)	21 (29%)	36 (71%)	15 (29%)
j	+/−	+/+	NTF+TW600 +HYP	40 (39%)	62 (61%)	74 (73%)	28 (27%)	40 (54%)	34 (46%)
k	−/−	+/−	NF+NW6	24 (42%)	33 (58%)	50 (88%)	7 (12%)	24 (48%)	26 (52%)
	P (a, b, c, d): Diet effect			< 0.0001		< 0.0001		< 0.0001	
	P (a, f): Parental genotype effect with sufficient NAD precursors			ns		ns		ns	
	P (b, g): Parental genotype effect under NAD precursor restriction			< 0.0001		< 0.0001		ns	
	P (b, e): Hypoxia effect under NAD precursor restriction			< 0.0001		ns		< 0.0001	
	P (g, h): Effect of nicotinic acid supplementation			< 0.0001		< 0.0001		0.0044	
	P (i, j): Hypoxia effect under NAD precursor restriction and maternal heterozygosity			ns		ns		ns	
	P (b,i): Maternal genotype effect under NAD precursor restriction			0.0066		0.0009		ns	

P values were calculated by two-sided Fisher's exact test when comparing two groups or Chi-square test when comparing multiple groups.
M, maternal *Haao* genotype; P, paternal *Haao* genotype; ns, not significant.

Table 3. Embryo NAD levels at E11.5 with different maternal treatments during pregnancy

	Maternal genotype	Paternal genotype	Embryo genotype	Treatment	NAD (nmol/mg protein ± SD)	n	*P*
a	+/+	+/+	+/+	Standard	1.84 ± 0.501	36	
b	+/+	+/+	+/+	NTF+TW600	1.46 ± 0.424	28	0.0219
c	+/+	+/+	+/+	NTF+TW500	1.34 ± 0.433	15	0.0116
d	+/+	+/+	+/+	NTF+TW600+HYP	1.21 ± 0.604	39	<0.0001
e	+/−	+/−	+/+	Standard	1.85 ± 0.377	8	>0.9999
f			+/−		2.16 ± 0.387	19	0.2049
g			−/−		1.91 ± 0.400	13	0.9995
h	+/−	+/−	+/+	NTF+TW600	1.14 ± 0.648	10	0.0010
i			+/−		1.04 ± 0.737	16	<0.0001
j			−/−		1.00 ± 0.478	11	<0.0001
k	+/−	+/−	+/+	NTF+TW600+NW15	2.21 ± 0.440	6	0.6201
l			+/−		1.94 ± 0.395	15	0.9991
m			−/−		1.57 ± 0.405	11	0.6894
n	−/−	+/−	+/−	NF+NW6	1.52 ± 0.252	16	0.2596
o			−/−		0.60 ± 0.181	12	<0.0001
1	*P* (a, b, c): Diet effect				0.0004		
2	*P* (b, d): Hypoxia effect under NAD precursor restriction				0.0423		
3	*P* (e, f, g / h, I, j): Diet and genotype effects				0.3977 (interaction) 0.5489 (genotype effect) <0.0001 (diet effect)		
4	*P* (h, i, j / k, l, m): Diet and genotype effects with nicotinic acid supplementation				0.3530 (interaction) 0.1037 (genotype effect) <0.0001 (diet effect)		

P values were calculated by ANOVA followed by Dunnett's multiple comparisons test, comparing all treatment groups to the pregnant wildtype on Standard diet control group (first row).
Additional ANOVA between selected groups are shown at the bottom. Tests 3 and 4 represent 2-way ANOVA followed by Tukey's multiple comparison test to evaluate genotype and diet effects, as well as potential interactions.

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Supplementary Information for

NAD deficiency due to environmental factors or gene-environment interactions causes congenital malformations and miscarriage in mice

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This PDF file includes:

Supplementary Materials and Methods

Genotyping

To genotype mice with the allele *Haao*^{em1Dunw} (MGI:6285800), ear clippings (adult mice), lung tissue (E18.5 embryos), or yolk sacs (E11.5 embryos) were collected and DNA extracted by digesting the tissue samples with proteinase K. DNA fragments specific for the wildtype and null alleles were amplified by PCR using primers as described previously (1). PCR products were size fractioned by agarose gel electrophoresis.

Dietary treatments

Female mice to be used in timed matings were fed a “Standard” feed with defined composition (containing 31.4 mg/kg nicotinic acid (NA) and 2.7 g/kg tryptophan; totalling 298 µg NAD precursors/day) (AIN93G, Specialty Feeds, Glen Forrest, Australia) for at least three weeks prior to mating. This step overall reduced and standardized the NAD precursor intake compared to the diet used for maintaining the mouse colonies, which was less defined and extremely rich in NAD precursors (~90 mg/kg NA, 3.7 g/kg tryptophan, totalling ~592 µg of NAD precursors/day) (Rat and Mouse Premium Breeder Diet, Gordons Specialty Feeds, Bargo, Australia). At the time of mating, female mice were between 58 and 120 days old. Females were checked for vaginal copulation plugs every morning during timed matings, and if present, their food was replaced with a feed lacking nicotinamide (NAM), nicotinamide riboside (NR), as well as tryptophan, and containing 1.4 mg/kg of NA (NAD precursor vitamin-depleted and tryptophan-free feed, NTF) (SF16-097, Specialty Feeds), and drinking water that contained defined concentrations of tryptophan (Tryptophan-supplemented water, TW) or NA (NA-supplemented water, NW) (Table 1). In a subset of experiments, a feed similarly depleted in NAD precursor vitamins, but containing tryptophan (NAD precursor vitamin-depleted feed, NF) (SF16-049, Specialty Feeds) and NW were provided to mice throughout pregnancy. Control females were maintained on Standard feed. The time point of plug detection was set to gestational day (E) 0.5. Mice remained on the special diets until embryo collection at E9.5 or E11.5 for NAD quantification, or E18.5 for embryo phenotyping.

Phenotyping

Pregnant mice were sacrificed at E18.5 by cervical dislocation. The number of live and dead embryos in each litter was noted. Live embryos were weighed and sacrificed by decapitation. General morphology was assessed with light microscopy. Hearts were removed and assessed for structural malformations using optical projection tomography as described previously (2). Lengths of dissected kidneys were measured with the grid of a hemocytometer. Skeletal morphology was examined following an alcian blue/alizarin red double staining protocol modified from Wallin et al.

(3), as described (1). Embryos were photographed using a Leica M125 microscope (Leica Microsystems, Wetzlar, Germany). For the purpose of quantifying embryo outcomes, we grouped malformation types according to the body part or organ they affected and counted the number of affected organs. Embryos with one or more malformations were classified as malformed.

NAD quantification

E9.5 and E11.5 embryos were collected between 2 and 5 pm to minimize circadian differences between litters, put into 1.5 mL tubes, snap frozen in liquid nitrogen, and stored at -80°C. Ice cold embryo lysis buffer (1% w/v dodecyltrimethylammonium bromide (DTAB) in tris-buffered saline pH 7.4) was added (100 uL for E9.5 embryos, 300 µL for E11.5 embryos), and embryos disintegrated using a pipette followed by sonication. The lysate was diluted in lysis buffer (1:3 for E9.5 and 1:4 for E11.5 embryos). NAD standards and embryo lysates were loaded in duplicate to a black-walled 96-well microplate (10 µL per sample). 200 µL of reaction mix (100 mM Tris pH 8, 5% (v/v) ethanol, 32 µM resazurin, 10 µM riboflavin mononucleotide, 15 units/mL alcohol dehydrogenase, 0.5 mg/mL bovine serum albumin, 0.1 mg/mL diaphorase, 0.5% (v/v) nonyl phenoxypolyethoxylethanol) was added to the wells and the solution mixed. Total NAD concentration was determined by measuring fluorescence at excitation 540 nm, emission 590 nm over 12 min using a microplate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany). Protein content was quantified by BCA protein assay. The lower limit of detection of the assay was <0.5 µM, as determined with NAD standard curves that were included in every assay, whereby the limit was defined as a fluorescence signal increase over the 12 min duration that is 3x greater than the signal increase of a blank sample. All embryo NAD assays included a control lysate aliquot made from a large batch of embryos to assess the assay reproducibility. The coefficient of variation of NAD levels in these aliquots was 18.5% (mean ± SD = 2.35 ± 0.43 nmol NAD per mg protein, n = 42). The coefficient of variation for repeated measurement of 0.125 to 2 µM NAD standards (each n = 4), which excludes biological variability, was between 8.8% and 14.7%.

Maternal liver tissue was taken at the time of embryo collection, snap frozen in liquid nitrogen and stored at -80°C. For measuring NAD, a small aliquot of frozen liver (~10 mg) was weighed, and 50 µL of liver lysis buffer (0.2 M NaOH with 1% w/v DTAB) added per mg of tissue. Samples were sonicated and neutralized with an equal volume of 0.2 M HCl/0.25 M Tris, followed by centrifugation for 10 min at 4˚C and 15000 rpm to remove debris. Lysate supernatants were diluted 1:4 to 1:10 and their NAD levels measured as described for the embryos.

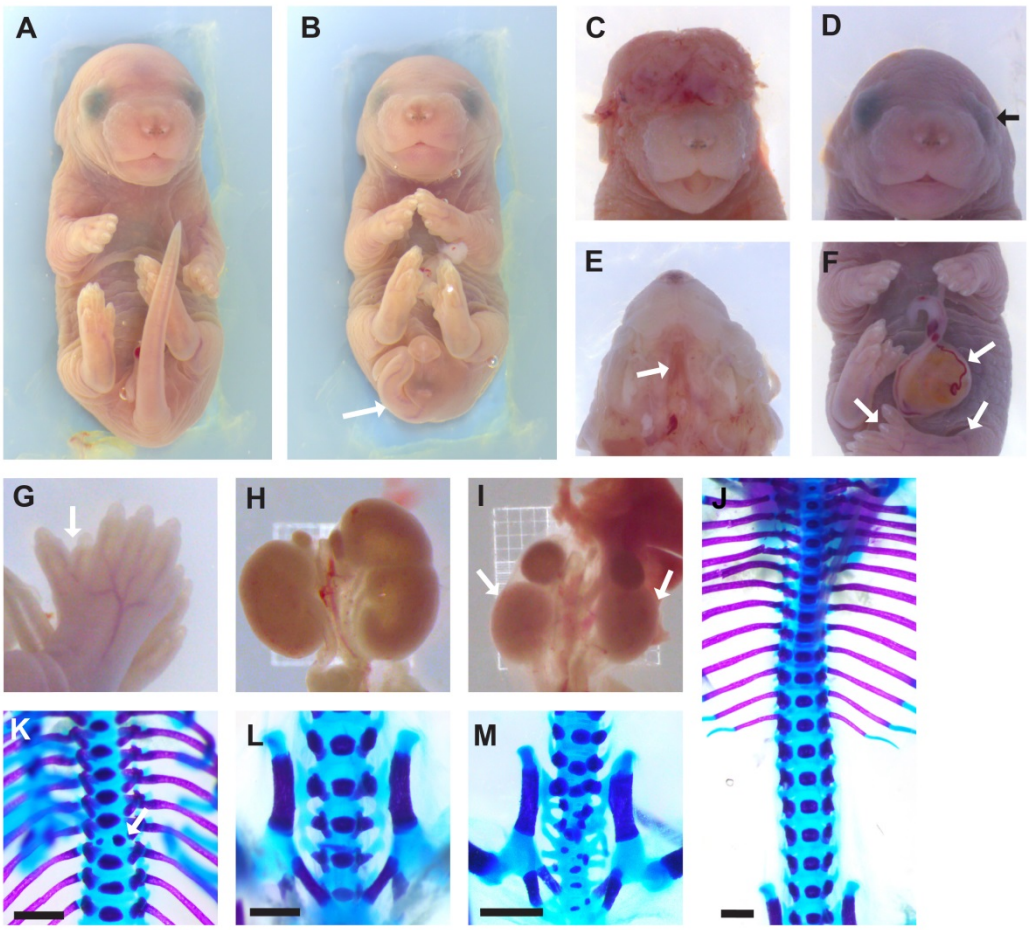

Fig. S1. Representative images of some of the observed embryo phenotypes at E18.5. **A:** Normal E18.5 embryo. **B:** Embryo with caudal agenesis (arrow). **C:** Exencephaly (skull defect). **D:** One-sided microphthalmia (arrow). **E:** Cleft palate (arrow). **F:** Omphalocele (abdominal wall defect), polydactyly, and hind limb talipes (arrows). **G:** Polydactyly. **H:** Dissected urogenital tract showing normal kidney (left) and duplex kidney (right). Note the transparent appearance of the medulla region of the duplex kidney indicating hydronephrosis. White grid in the background is 3 mm x 3 mm in size. **I:** Two hypoplastic kidneys. **J:** Normal embryo skeleton from thoracic to lumbar region stained by alcian blue/alizarin red. **K:** Hemivertebra (arrow) in the thoracic skeleton. **L:** Normal sacral region of the skeleton. **M:** Sacral region with multiple abnormal vertebrae and agenesis. Scale bars are 1 mm.

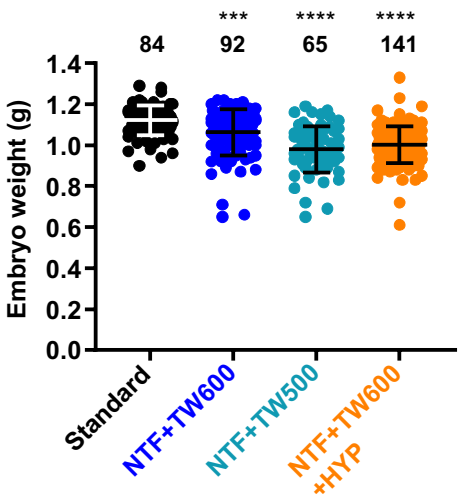

Fig. S2. E18.5 embryos from litters of C57BL/6J wildtype mice that were fed NAD precursor restricted diets throughout pregnancy were significantly lighter compared to pregnant mice fed the Standard diet. Dissected embryos were weighed prior to assessment for congenital malformations. Bars indicate mean ± SD. Numbers of embryos are indicated above graph. ***P<0.001; ****P<0.0001 by one-way ANOVA with Dunnett's multiple comparisons using wildtypes on Standard diet as the control group.

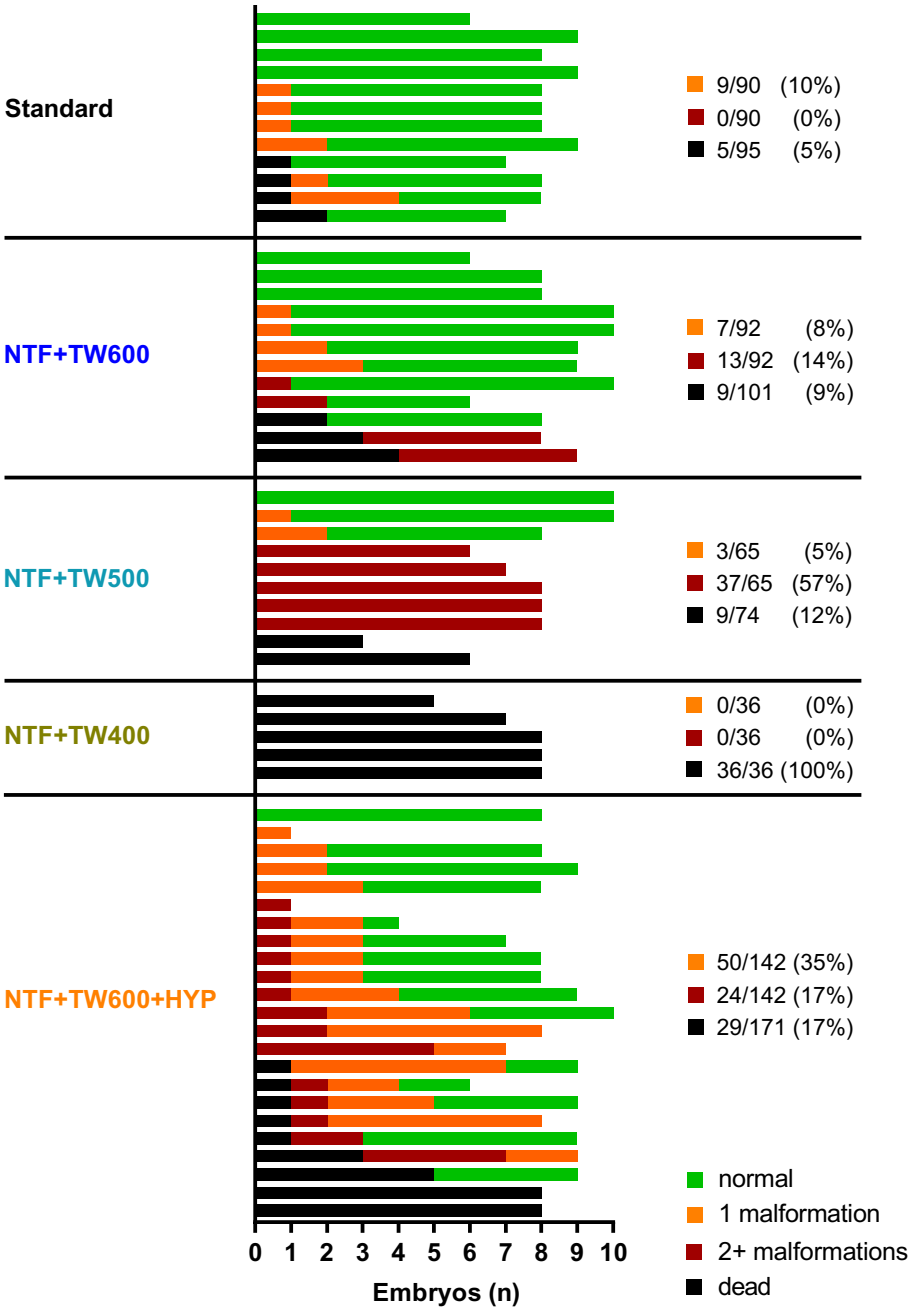

Fig. S3. Phenotypes of C57BL/6J wildtype mouse embryos at E18.5, within the maternal diet treatment groups, as indicated on the left (see Table 1 for a detailed description of diets). Each horizontal bar represents a litter and length of the bars indicates the total number of embryos per litter. All dead embryos were found to be early resorptions. Total counts and percentages of embryos within each treatment group are summarized on the right.

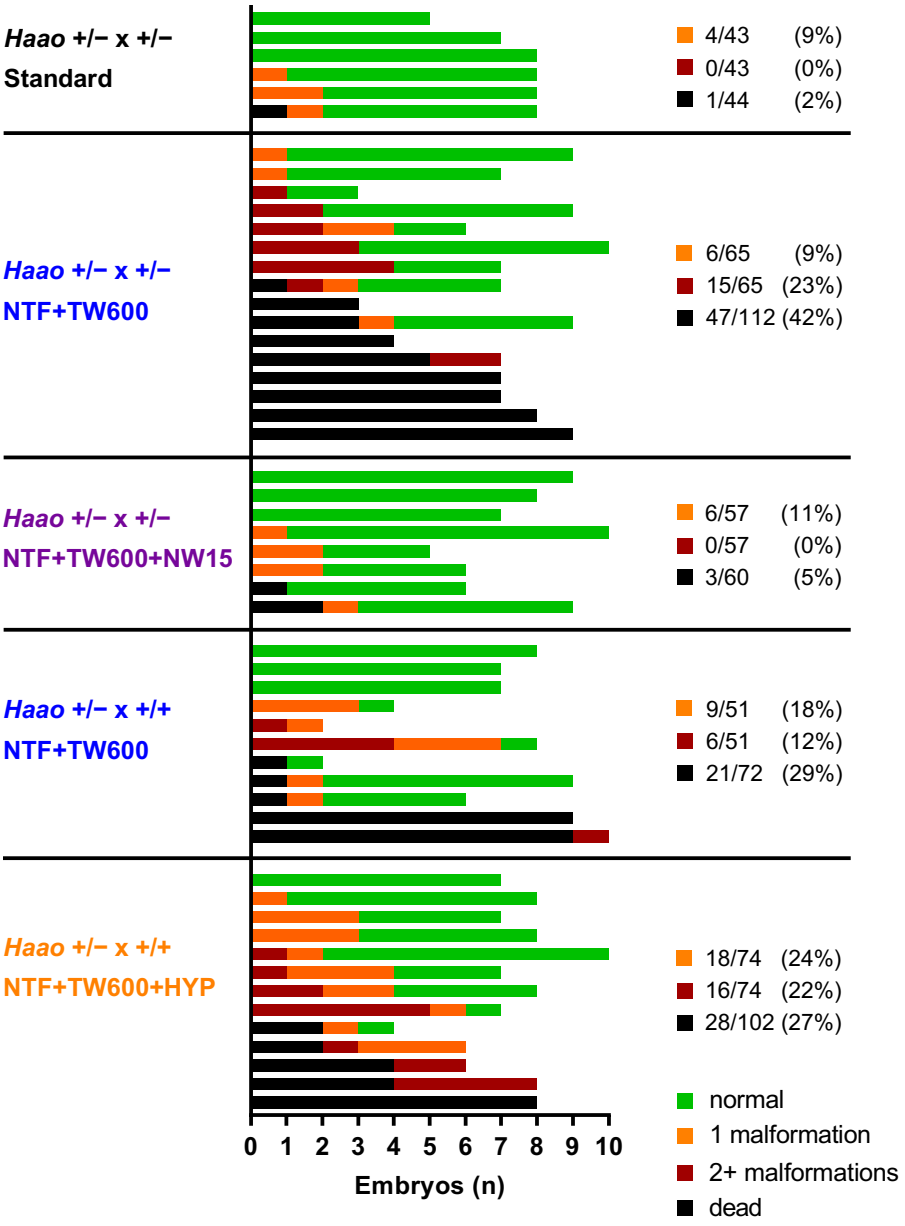

Fig. S4. Phenotypes of embryos at E18.5 within the maternal diet treatment groups that involve mothers with a *Haao*$^{+/-}$ loss-of-function variant. Treatment conditions and the mating scheme (maternal x paternal) are indicated on the left. Each horizontal bar represents a litter and length of the bars indicates the total number of embryos per litter. All dead embryos were found to be early resorptions. Total counts and percentages of embryos within each treatment group are summarized on the right.

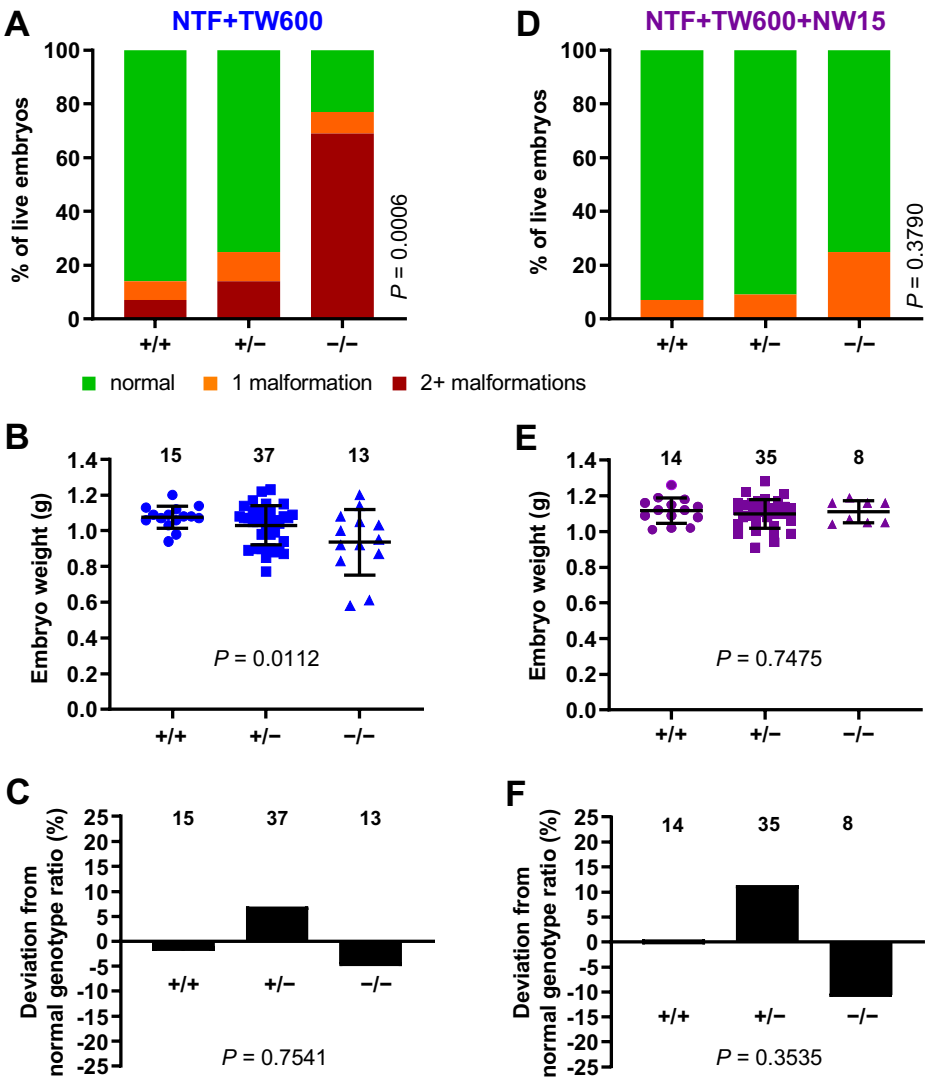

Fig. S5. Embryo genotype influences phenotype of offspring of mice with a *Haao*$^{+/-}$ loss-of-function variant and maternal NAD precursor intake restriction throughout pregnancy. **A:** Summarised live embryo outcomes within the NTF+TW600 treatment group, sorted by *Haao* genotype, as indicated on the x axis. There is a correlation between the proportion of live embryos with malformations (orange and red) and the embryo genotype (*P*=0.0006, Fisher's Exact test with Freeman-Halton extension). Dead embryos were excluded, as they could not be genotyped due to complete resorption early in gestation. **B:** Comparison of embryo weights. Concordant with the malformation prevalence, there is a correlation between embryo genotype and weight at E18.5 (*P*=0.0112, ordinary one-way ANOVA). Bars indicate mean ± SD. Numbers of embryos are indicated above graph. **C:** The embryo genotype distribution does not significantly deviate from the normal Mendelian ratio. Bars indicate percentage deviation from the expected (25:50:25) genotype distribution. *P*=0.7541 by Fisher's Exact test with Freeman-Halton extension. **D:** Summarized embryo outcome of the NTF+TW600+NW15 group sorted by genotype. The proportion of malformed embryos is strongly reduced with the NTF+TW600+NW15 diet among all three embryo genotypes compared to NTF+TW600. **E, F:** Embryo weights and percentage deviation from the expected genotype distribution. There is no correlation between embryo genotype and embryo weight, and embryo genotypes do not deviate significantly from the normal Mendelian ratio.

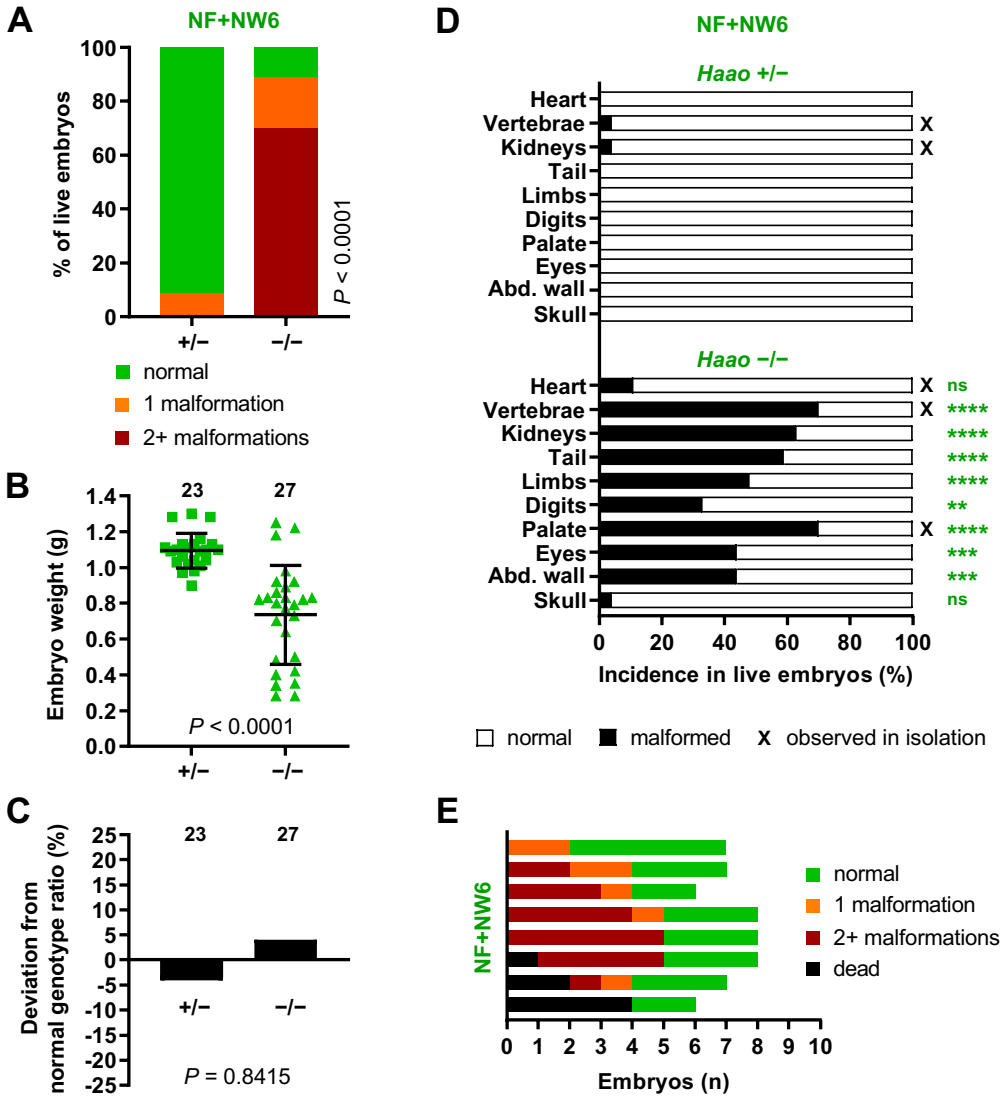

Fig. S6. Embryo genotype strongly influences phenotype of offspring of mice with maternal *Haao*$^{-/-}$ and paternal *Haao*$^{+/-}$ loss-of-function variant, and maternal NAD precursor intake restriction throughout pregnancy with NF+NW6 diet. **A:** Summarised live embryo outcomes of the NF+NW6 treatment group sorted by genotype, as indicated on the x axis. *Haao*$^{-/-}$ embryos have a high incidence of congenital malformations, whereas *Haao*$^{+/-}$ embryos are mostly unaffected (P<0.0001, Fisher's Exact test). Dead embryos were excluded, as they could not be genotyped due to complete resorption early in gestation. **B:** Comparison of embryo weights. *Haao*$^{-/-}$ embryos are significantly lighter (P<0.0001, unpaired two-tailed t-test). Bars indicate mean ± SD. Numbers of embryos are indicated above graph. **C:** The embryo genotype distribution does not significantly deviate from the normal Mendelian ratio. Bars indicate percentage deviation from the expected (50:50) genotype distribution (P=0.8415, Fisher's Exact test). **D:** Incidence of malformations in the indicated organs among live embryos of the respective *Haao* genotypes. The vertebrae category includes rib malformations. Abd. wall, abdominal wall; X, malformation types observed in isolation in one or more embryos. Asterisks indicate significantly different malformation incidence (Fisher's Exact test). **E:** Embryo outcomes in individual litters. Each horizontal bar represents a litter and the length of bars represents the number of embryos per litter.

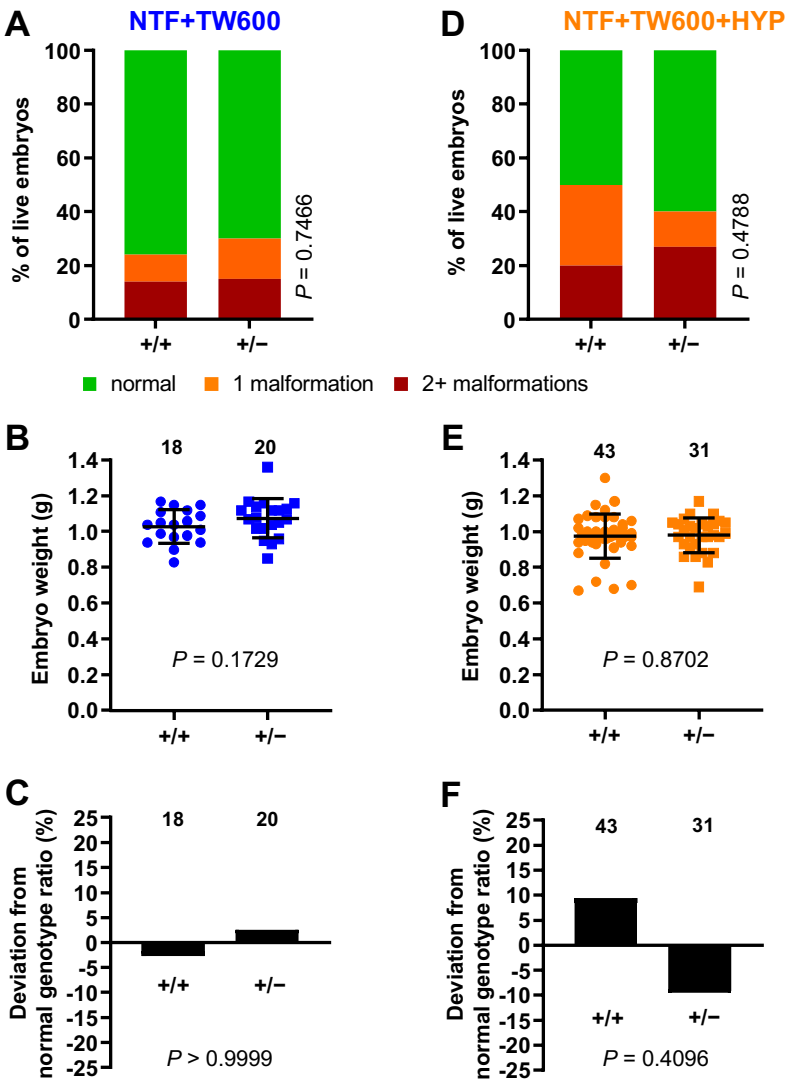

Fig. S7. The embryo genotype has no significant influence on embryo phenotype at E18.5 in embryos from matings that involved a maternal *Haao*$^{+/-}$ loss-of-function variant and maternal NAD precursor restriction throughout pregnancy, with exposure to hypoxia. **A:** Summarised live embryo outcomes of the NTF+TW600 treatment group sorted by *Haao* genotype, as indicated on the x axis. There is no correlation between the proportion of embryos with malformations and the embryo genotype (*P*=0.7466, Fisher's Exact test). **B:** Comparison of embryo weights. The embryo genotype has no influence on embryo weight (*P*=0.1729, unpaired two-tailed t-test). Bars indicate mean ± SD. Numbers of embryos are indicated above graph. **C:** The embryo genotype distribution does not significantly deviate from the normal Mendelian ratio. Bars indicate percentage deviation from the expected (50:50) genotype distribution (*P*>0.9999, Fisher's Exact test). **D:** Summarised embryo outcome of the NTF+TW600+HYP group with maternal gestational hypoxia (8 h of 8% oxygen at E9.5). The proportion of malformed embryos of both genotypes is increased compared to the normoxic NTF+TW600 group. Both embryo genotypes are affected equally (*P*=0.4788). **E, F:** There is no correlation between embryo genotype and embryo weight in the NTF+TW600+HYP group, and embryo genotypes do not deviate significantly from the normal Mendelian ratio.

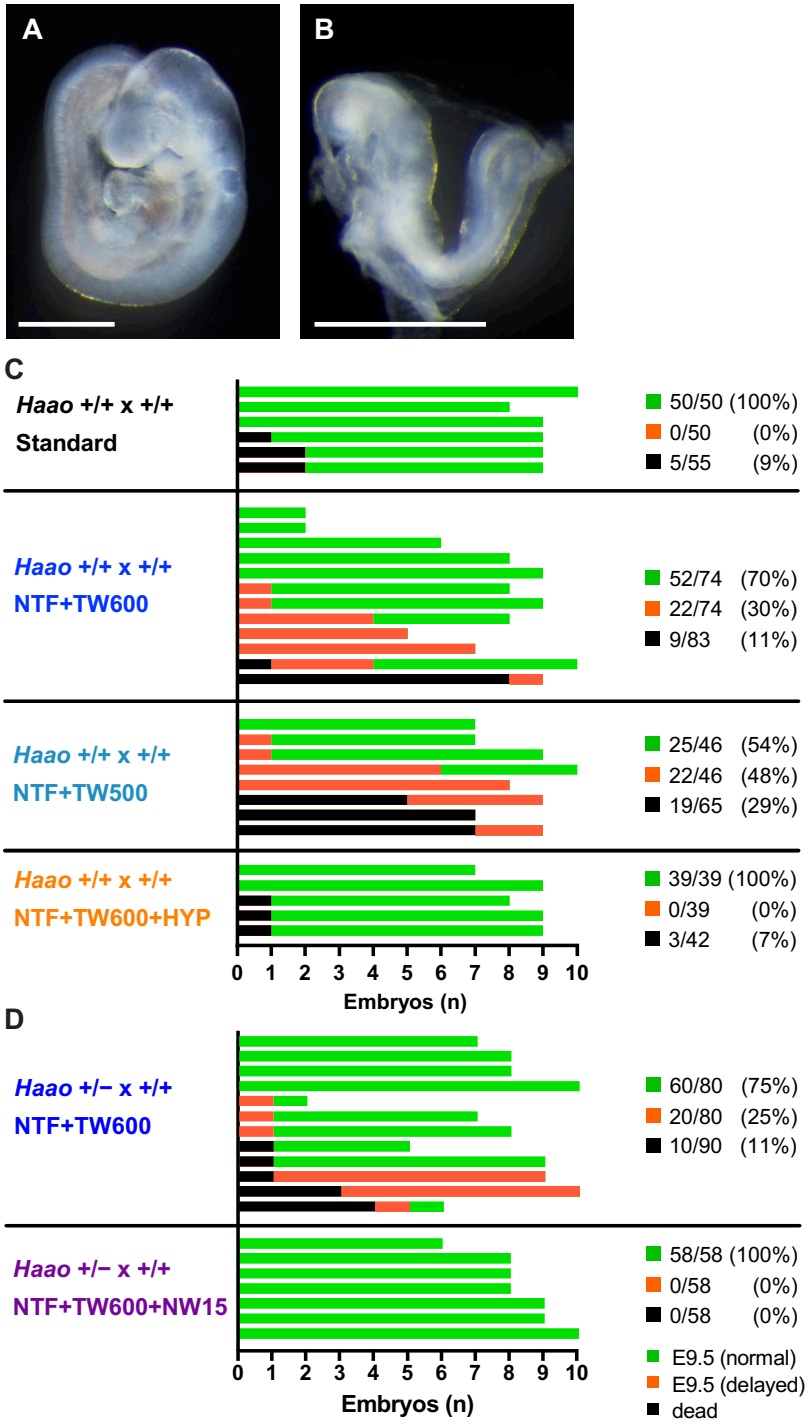

Fig. S8. E9.5 embryos in litters of C57BL/6J wildtype mothers and those involving a parental *Haao*$^{+/-}$ loss-of-function variant frequently exhibited developmental delay under maternal dietary NAD precursor restriction throughout pregnancy. **A:** normal embryo at E9.5. **B:** Embryo at E9.5 that morphologically resembles an E8.5 embryo. Scale bars 1 mm. **C, D:** Summarised embryo outcomes at E9.5 for each of the mating schemes and maternal diet treatments, as indicated on the left. Each horizontal bar represents a litter and length of the bars indicates the number of embryos per litter. All dead embryos were found to be early resorptions. Total counts and percentages of embryos within each treatment group are summarized on the right.

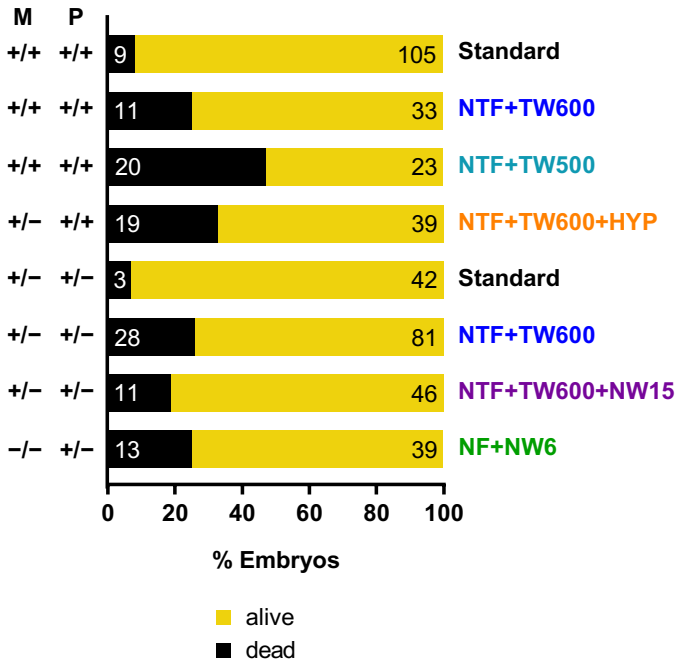

Fig. S9. Summarized embryo outcomes at E11.5 show that dietary NAD precursor restriction during pregnancy is associated with higher rates of embryo death, similar to the observations at E18.5. The maternal dietary treatments throughout pregnancy are indicated on the right. M, maternal *Haao* genotype; P, paternal *Haao* genotype. See *SI Appendix* Table S2 for statistical comparisons between treatment groups.

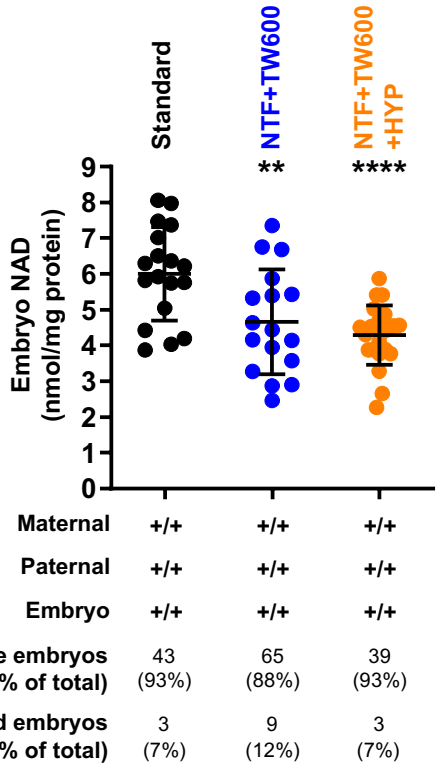

Fig. S10. Whole embryo total NAD levels (NAD^+ and NADH) and embryo outcomes at E9.5. Dots represent NAD levels and bars indicate the mean ± SD. The parental and embryo *Haao* genotypes are indicated at the bottom (all mice were wildtype). The total numbers and percentages of live and dead embryos observed with each treatment condition are indicated at the bottom. Note that not every collected embryo underwent NAD measurement. Asterisks indicate NAD levels that are significantly different to those of the pregnant C57BL/6J wildtype Standard diet group by one-way ANOVA with Dunnett's multiple comparisons test. For a summary of NAD level values, see *SI Appendix* Table S4.

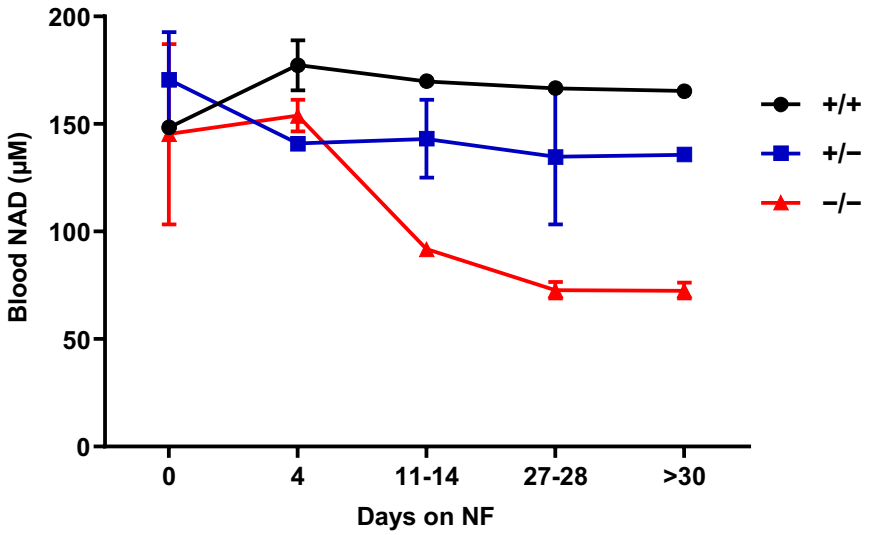

Fig. S11. Feeding mice an NAD precursor restricted diet results in depletion of whole blood NAD levels in *Haao*$^{-/-}$ mice over time, but does not affect blood NAD levels in *Haao*$^{+/+}$ and *Haao*$^{+/-}$ mice. Non-pregnant mice were maintained on NF (containing 1.4 mg/kg NA and 1800 mg/kg tryptophan, but no NAD precursors added to the water, Table 1), culled at indicated days after the start of treatment and their blood taken. Blood NAD levels were measured by an enzymatic assay, as done for liver and embryo NAD measurements. Values are mean ± SD, *n* = 1-3). Blood NAD levels in *Haao*$^{+/+}$ and *Haao*$^{+/-}$ mice remain stable over 4 weeks, indicating that the mice receive sufficient NAD precursors. Conversely, *Haao*$^{-/-}$ mice cannot utilise the tryptophan for NAD synthesis and their blood NAD levels start to decline after ~4 days and reach ~50% of initial levels within 2-3 weeks, indicating a depletion of NAD stores in the body.

Table S1. Summary of all types and incidence of congenital malformations observed at E18.5

Location	Malformation		Standard	NTF+TW600	NTF+TW500	NTF+TW600 +HYP	Standard	NTF+TW600	NTF+TW600+ NW15	NTF+TW600	NTF+TW600+ HYP	NF+NW6
		M	+/+	+/+	+/+	+/+	+/-	+/-	+/-	+/-	+/-	-/-
		P	+/+	+/+	+/+	+/+	+/-	+/-	+/-	+/+	+/+	-/+
Heart	*Total*		*3/90 (3%)*	*5/92 (5%)*	*16/65 (25%)*	*11/142 (8%)*	-	*3/65 (5%)*	*1/57 (2%)*	*3/51 (6%)*	*9/74 (12%)*	*3/50 (6%)*
	Bicuspid aortic valve		2 (2%)	1 (1%)	5 (8%)	4 (3%)	-	-	-	1 (2%)	3 (4%)	1 (2%)
	Membranous ventricular septal defect		1 (1%)	2 (1%)	9 (14%)	2 (1%)	-	2 (3%)	1 (2%)	1 (2%)	5 (3%)	2 (4%)
	Muscular ventricular septal defect		-	2 (2%)	4 (6%)	5 (4%)	-	1 (2%)	-	1 (2%)	1 (1%)	-
	Overriding aorta		-	1 (1%)	5 (8%)	1 (1%)	-	2 (3%)	-	-	3 (4%)	-
	Patent truncus arteriosus		-	-	2 (3%)	-	-	-	-	-	-	2 (4%)
	Double outlet right ventricle		-	-	2 (3%)	1 (1%)	-	-	-	-	-	-
Vertebrae and ribs*	*Total*		*5/90 (6%)*	*11/92 (12%)*	*34/65 (52%)*	*58/142 (41%)*	*3/43 (7%)*	*8/65 (12%)*	*3/57 (5%)*	*7/51 (14%)*	*25/74 (34%)*	*20/50 (40%)*
	Cervical vertebrae		4 (4%)	10 (11%)	27 (42%)	7 (5%)	3 (7%)	6 (9%)	3 (5%)	6 (12%)	6 (8%)	7 (14%)
	Thoracic vertebrae		1 (1%)	1 (1%)	9 (14%)	18 (13%)	-	4 (6%)	-	1 (2%)	12 (16%)	3 (6%)
	Lumbar vertebrae		-	2 (2%)	6 (9%)	42 (30%)	-	-	-	1 (2%)	21 (28%)	-
	Sacral vertebrae		-	3 (3%)	4 (6%)	5 (4%)	-	1 (2%)	-	1 (2%)	2 (3%)	-
	Rib fusions		-	-	4 (6%)	4 (3%)	-	-	-	-	-	-
	Extra pair of ribs		-	-	-	3 (2%)	-	-	-	-	-	-
	Missing or under-developed ribs		-	-	1 (2%)	6 (4%)	-	2 (3%)	-	1 (2%)	4 (5%)	14 (28%)
Kidneys†	*Total*		*1/90 (1%)*	*5/92 (5%)*	*21/65 (32%)*	*20/142 (14%)*	*1/43 (2%)*	*11/65 (17%)*	*2/57 (4%)*	*4/51 (8%)*	*10/74 (14%)*	*18/50 (36%)*
	Hypoplasia or agenesis		-	1 (1%)	19 (29%)	-	-	10 (15%)	-	3 (6%)	5 (7%)	17 (34%)
	Dysmorphic (duplex, hydronephrosis)		1 (1%)	4 (4%)	2 (3%)	20 (14%)	1 (2%)	1 (2%)	2 (4%)	1 (2%)	9 (12%)	1 (2%)

Tail‡	*Total*	-	*1/92 (1%)*	*27/65 (42%)*	-	-	*9/65 (14%)*	-	*2/51 (4%)*	-	*16/50 (32%)*
	Caudal agenesis	-	1 (1%)	27 (42%)	-	-	9 (14%)	-	2 (4%)	-	16 (32%)
Limbs	*Total*	-	-	*7/65 (11%)*	*1/142 (1%)*	-	*4/65 (6%)*	-	*1/51 (2%)*	*1/74 (1%)*	*13/50 (26%)*
	Talipes	-	-	5 (8%)	1 (1%)	-	3 (5%)	-	1 (2%)	1 (1%)	2 (4%)
	Short limbs	-	-	2 (3%)	-	-	1 (2%)	-	-	-	11 (22%)
Digits§	*Total*	-	*6/92 (7%)*	*36/65 (55%)*	*5/142 (4%)*	-	*10/65 (15%)*	-	*4/51 (8%)*	*6/74 (8%)*	*9/50 (18%)*
	Polydactyly	-	6 (7%)	33 (51%)	-	-	8 (12%)	-	4 (8%)	1 (1%)	-
	Oligodactyly	-	-	2 (3%)	5 (4%)	-	-	-	-	4 (5%)	9 (18%)
	Syndactyly	-	-	2 (3%)	1 (1%)	-	2 (3%)	-	-	1 (1%)	-
	Triphalangeal thumb	-	-	-	-	-	-	-	-	1 (1%)	-
Palate	*Total*	-	-	*15/65 (23%)*	-	-	*5/65 (8%)*	-	*2/51 (4%)*	*4/74 (5%)*	*19/50 (38%)*
	Cleft palate	-	-	15 (23%)	-	-	5 (8%)	-	2 (4%)	4 (5%)	19 (38%)
Eyes	*Total*	-	*11/92 (12%)*	*30/65 (46%)*	*6/142 (4%)*	-	*8/65 (12%)*	-	*5/51 (10%)*	*10/74 (14%)*	*12/50 (24%)*
	Microphthalmia	-	10 (11%)	30 (46%)	6 (4%)	-	8 (12%)	-	5 (10%)	6 (8%)	12 (24%)
	Coloboma	-	3 (3%)	-	-	-	-	-	-	4 (5%)	-
Abdominal wall	*Total*	-	*1/92 (1%)*	*9/65 (14%)*	*4/142 (3%)*	-	*5/65 (8%)*	-	*1/51 (2%)*	*2/74 (3%)*	*12/50 (24%)*
	Omphalocele or Gastroschisis	-	1 (1%)	9 (14%)	4 (3%)	-	5 (8%)	-	1 (2%)	2 (3%)	12 (24%)
Skull	*Total*	-	*2/92 (2%)*	*1/65 (2%)*	*1/142 (1%)*	-	-	-	-	*2/74 (3%)*	*1/50 (2%)*
	Exencephaly	-	2 (2%)	1 (2%)	1 (1%)	-	-	-	-	2 (3%)	1 (2%)

For representative images of some commonly occurring malformations, see *SI Appendix* Fig. S1. M, maternal *Haao* genotype; P, paternal *Haao* genotype; -, malformation was not observed in the respective treatment group. All percentages are percent of live embryos. For each "Location", the Total refers to the total number of affected embryos, irrespective of the cumulative number of malformations in the given location.

*Isolated abnormalities in the vertebrae C1 and/or C2 were not counted, because C1 and C2 were occasionally removed during dissection. Ossification point abnormalities (e.g. flattened shape, less compaction, dumbbell shape, smaller than usual, two separate ossification points) were not counted, as they can result from delayed ossification and do not necessarily lead to a

skeletal defect (4). Vertebral abnormalities observed and counted included vertebral fusions, butterfly vertebrae, and hemivertebrae.

[†]We determined that kidneys of wildtype E18.5 embryos developed under a normal unrestricted diet are consistently around 3 mm long measured from tip to tip (average 2.98 mm; range 2.75 – 3.375 mm; n = 90) and classified kidneys ≤ 1.5 mm in length as malformed (hypoplastic).

[‡]The skeletal staining procedure did not reliably allow visualization of caudal vertebra defects. Therefore, tails were assessed at the whole embryo level. Short and curly tails that did not extend past the toes when stretched out were classified as caudal agenesis.

[§]Polysyndactyly is counted as polydactyly and syndactyly.

Table S2. Summary of the embryo counts and survival at E11.5 with the tested treatments

	***Haao* Genotype**		**Treatment**	**Embryos**	
	M	**P**		**Alive**	**Dead**
a	+/+	+/+	Standard	105 (92%)	9 (8%)
b	+/+	+/+	NTF+TW600	33 (75%)	11 (25%)
c	+/+	+/+	NTF+TW500	23 (53%)	20 (47%)
e	+/+	+/+	NTF+TW600+HYP	39 (67%)	19 (33%)
f	+/−	+/−	Standard	42 (93%)	3 (7%)
g	+/−	+/−	NTF+TW600	81 (74%)	28 (26%)
h	+/−	+/−	NTF+TW600+NW15	46 (81%)	11 (19%)
k	−/−	+/−	NF+NW6	39 (75%)	13 (25%)
	P (a, b, c): Diet effect			<0.0001	
	P (a, f): Genotype effect with sufficient NAD precursors			ns	
	P (b, g): Genotype effect under NAD precursor restriction			ns	
	P (b, e): Hypoxia effect under NAD precursor restriction			ns	
	P (g, h): Effect of nicotinic acid supplementation			ns	

P values were calculated by two-sided Fisher's exact test when comparing two groups or Chi-square test when comparing multiple groups.
M, maternal *Haao* genotype; P, paternal *Haao* genotype; ns = not significant.

Table S3. Maternal liver NAD levels of pregnant mice under different treatments

Treatment	*Haao* genotype	NAD (nmol/g tissue ± SD)	n	*P*
Standard	+/+	561 ± 65	6	
NTF+TW600	+/+	481 ± 68	4	0.3885
NTF+TW500	+/+	413 ± 81	5	0.0088
NTF+TW600+HYP	+/+	497 ± 86	7	0.4725
Standard	+/−	579 ± 70	4	0.9994
NTF+TW600	+/−	493 ± 77	9	0.3521
NTF+TW600+NW15	+/−	489 ± 39	6	0.3919
NF+NW6	−/−	415 ± 19	5	0.0098
Standard (non-preg.)	+/+	673 ± 86	8	0.0345

Livers of pregnant mice were collected at E11.5. Non-pregnant female mice on Standard diet were of similar age as the pregnant females.
P values were calculated by ANOVA followed by Dunnett's multiple comparisons test, comparing all treatment groups to the pregnant wildtype mice on Standard diet (first row).

Table S4. Embryo NAD levels at E9.5 with different maternal treatments during pregnancy

Treatment	Maternal genotype	Paternal genotype	NAD (nmol/mg protein ± SD)	n	*P*
Standard	+/+	+/+	6.01 ± 1.30	18	
NTF+TW600	+/+	+/+	4.66 ± 1.46	17	0.0030
NTF+TW600+HYP	+/+	+/+	4.29 ± 0.84	23	<0.0001

P values were calculated by ANOVA followed by Dunnett's multiple comparisons test, comparing all treatment groups to the pregnant wildtype on Standard diet control group (first row).

Table S5. Overview of the approximate NAD precursor levels mice receive from the diets used in this study and their human equivalents

Treatment	Mouse NAD precursors (mg/kg)*	Human equivalent dose (mg/kg)†	Mouse NAD precursor supply per day (µg/day)	Human equivalent NAD precursor supply per day (mg/day)‡
Standard	12.42	1.01	298.0	60.56
NTF+TW600	2.81	0.23	67.5	13.71
NTF+TW500	2.38	0.19	57.1	11.61
NTF+TW400	1.95	0.16	46.8	9.51
NTF+TW600+NW15	6.69	0.54	160.5	32.61
NF+NW6	6.65	0.54	159.7	32.45

*NAD precursors (=niacin equivalents) are based on an average consumption of 3.9 g food and 6.2 mL water per day and on the approximation that 60 mg of dietary tryptophan is equivalent to 1 mg of NA for the conversion to NAD (5, 6)

†Human equivalent doses were calculated using the approximation that the mouse dose is 12.3-times the human dose (7).

‡The recommended daily intake (RDI) of NAD precursors for humans is 18 mg/day (8, 9), indicating that NTF+TW400, NTF+TW500, and NTF+TW600 would potentially also represent a nutritional deficiency in humans, when converted to the human equivalents.

Supplementary References

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