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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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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 wild-type 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 in the afternoon (2 - 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 µL 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.

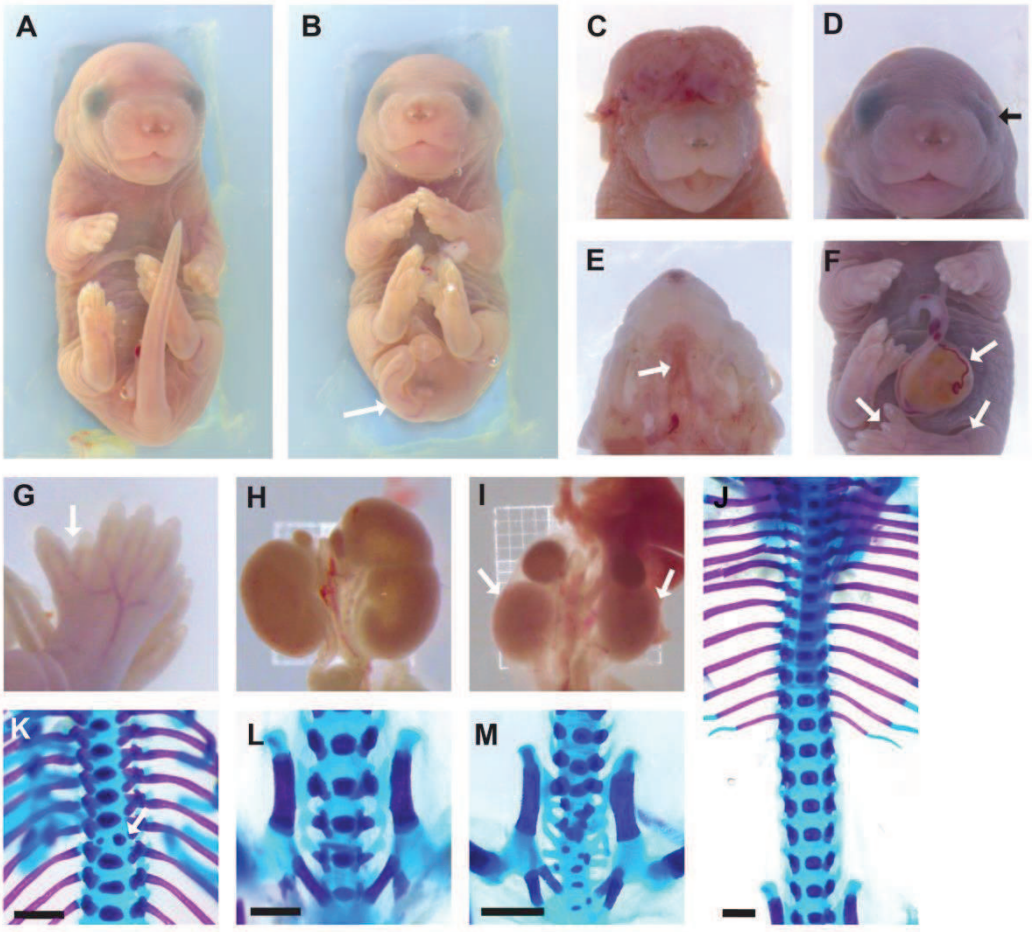

Fig. S1. Representative images of some of the observed embryo phenotypes at E18.5. **A:** Normal E18.5 embryo. **B:** Embryo with caudal agenesis (arrow). **C:** Exencephaly (skull defect). **D:** One-sided microphthalmia (arrow). **E:** Cleft palate (arrow). **F:** Omphalocele (abdominal wall defect), polydactyly, and hind limb talipes (arrows). **G:** Polydactyly (arrow). **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.

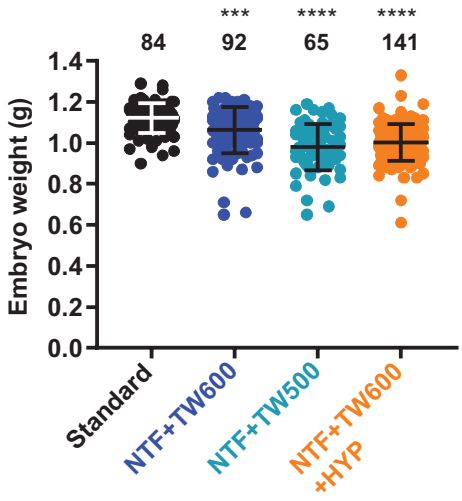

Fig. S2. E18.5 embryos from litters of C57BL/6J wild-type mice that were fed NAD precursor restricted diets throughout pregnancy were significantly lighter compared to pregnant mice fed the Standard diet. Dissected embryos were weighed prior to assessment for congenital malformations. Bars indicate mean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

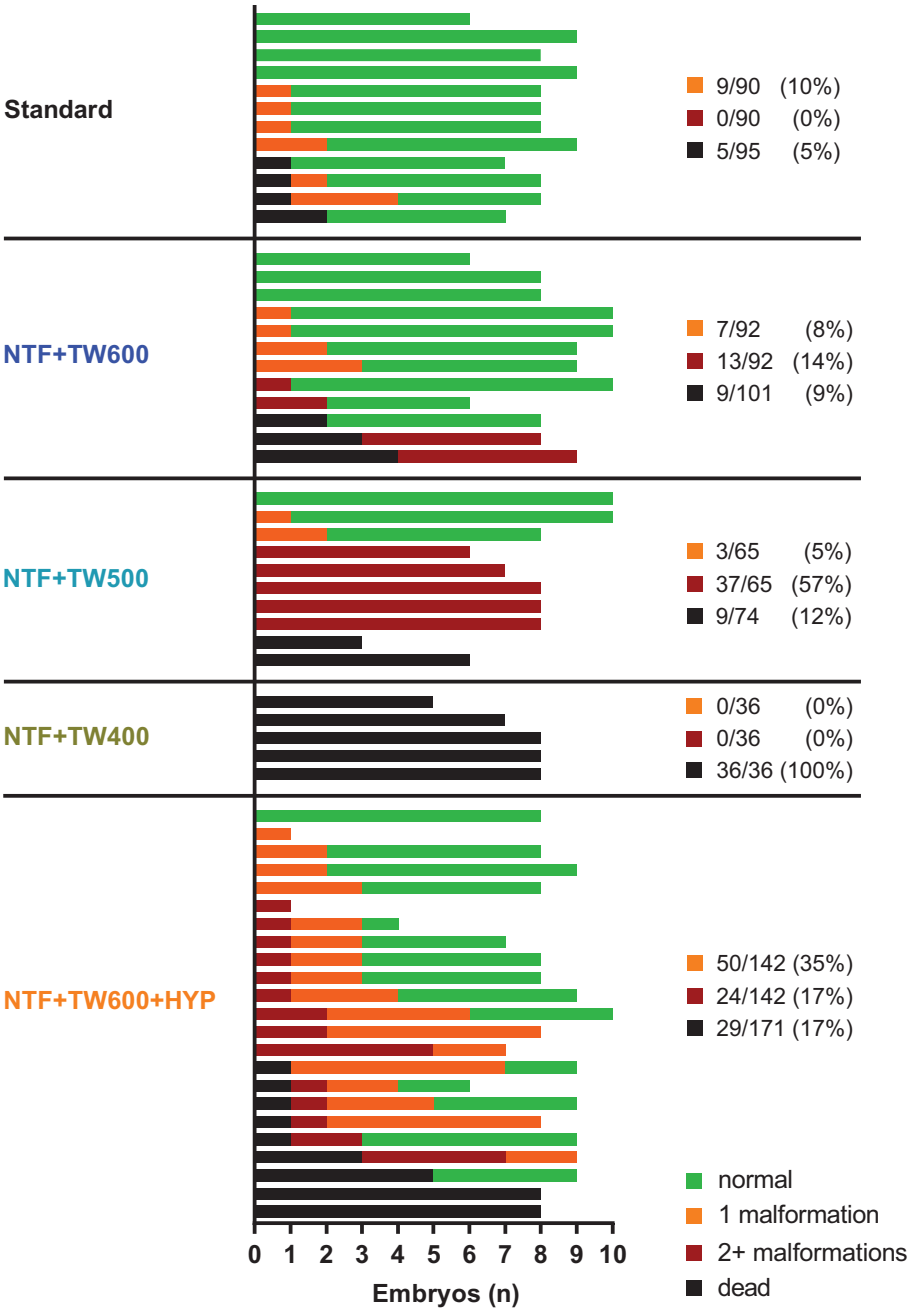

Fig. S3. Phenotypes of C57BL/6J wild-type mouse embryos at E18.5, within the maternal diet treatment groups, as indicated on the left (see Table 1 for a detailed description of diets). Each horizontal bar represents a litter and length of the bars indicates the total number of embryos per litter. All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

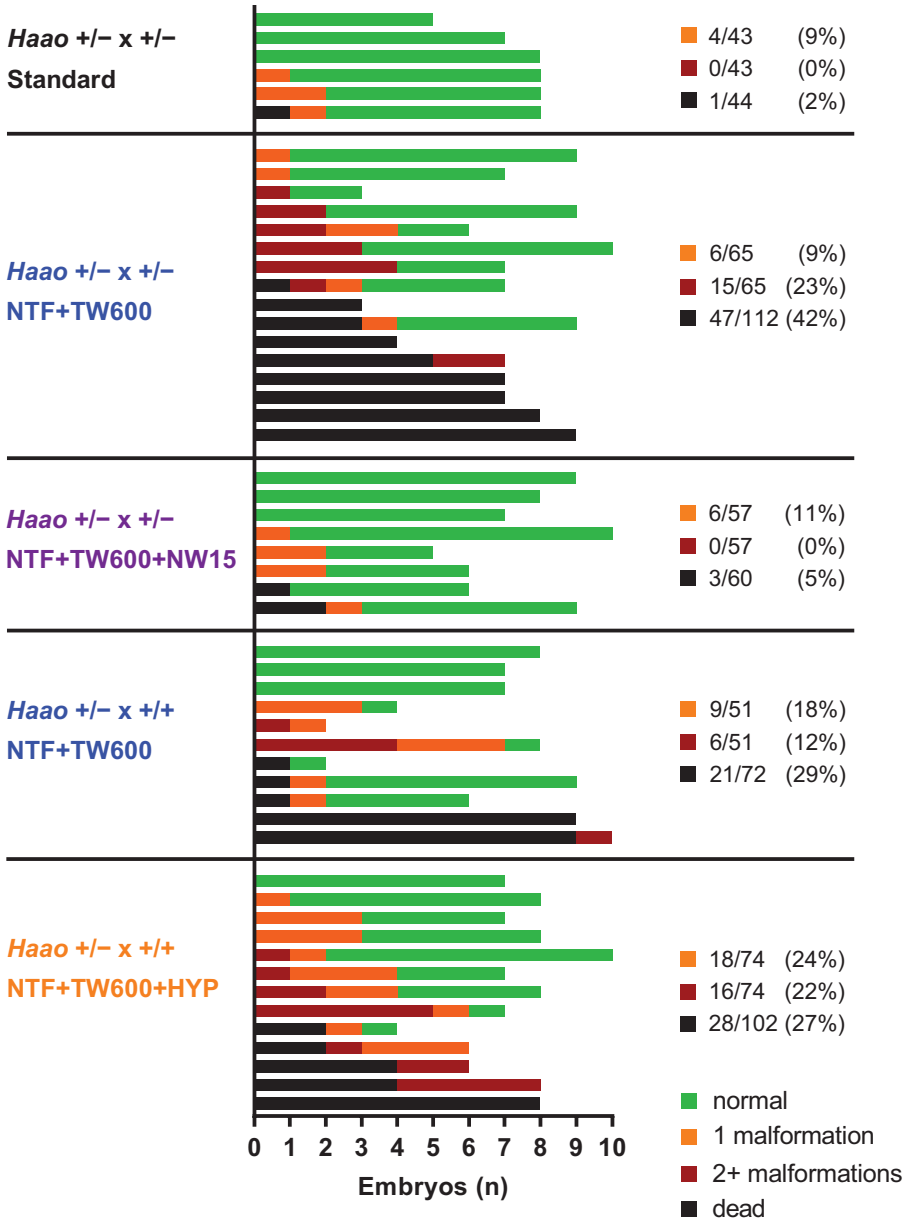

Fig. S4. Phenotypes of embryos at E18.5 within the maternal diet treatment groups that involve mothers with a *Haao*$^{+/-}$ loss-of-function variant. Treatment conditions and the mating scheme (maternal x paternal) are indicated on the left. Each horizontal bar represents a litter and length of the 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.

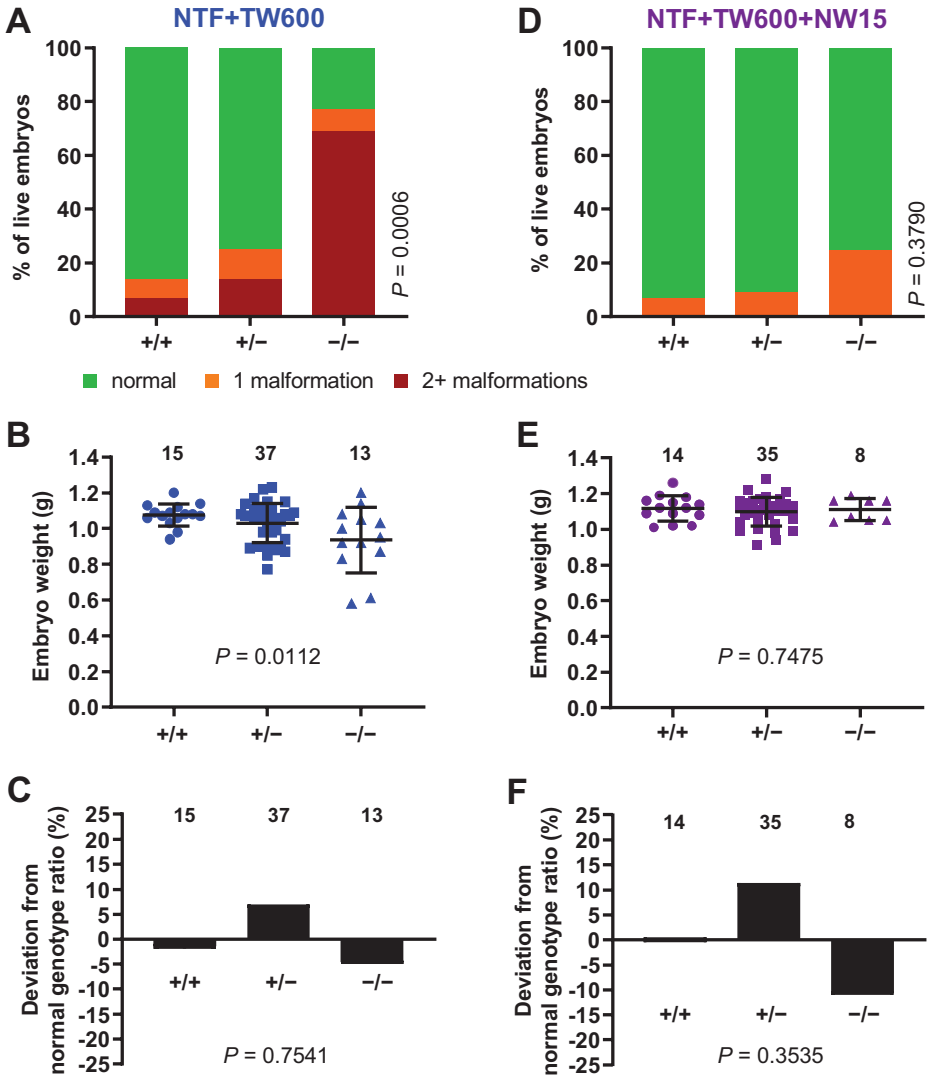

Fig. S5. Embryo genotype influences phenotype of offspring of mice with a *Haao*$^{+/-}$ loss-of-function variant and maternal NAD precursor intake restriction throughout pregnancy. **A:** Summarised live embryo outcomes within the NTF+TW600 treatment group, sorted by *Haao* genotype, as indicated on 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.

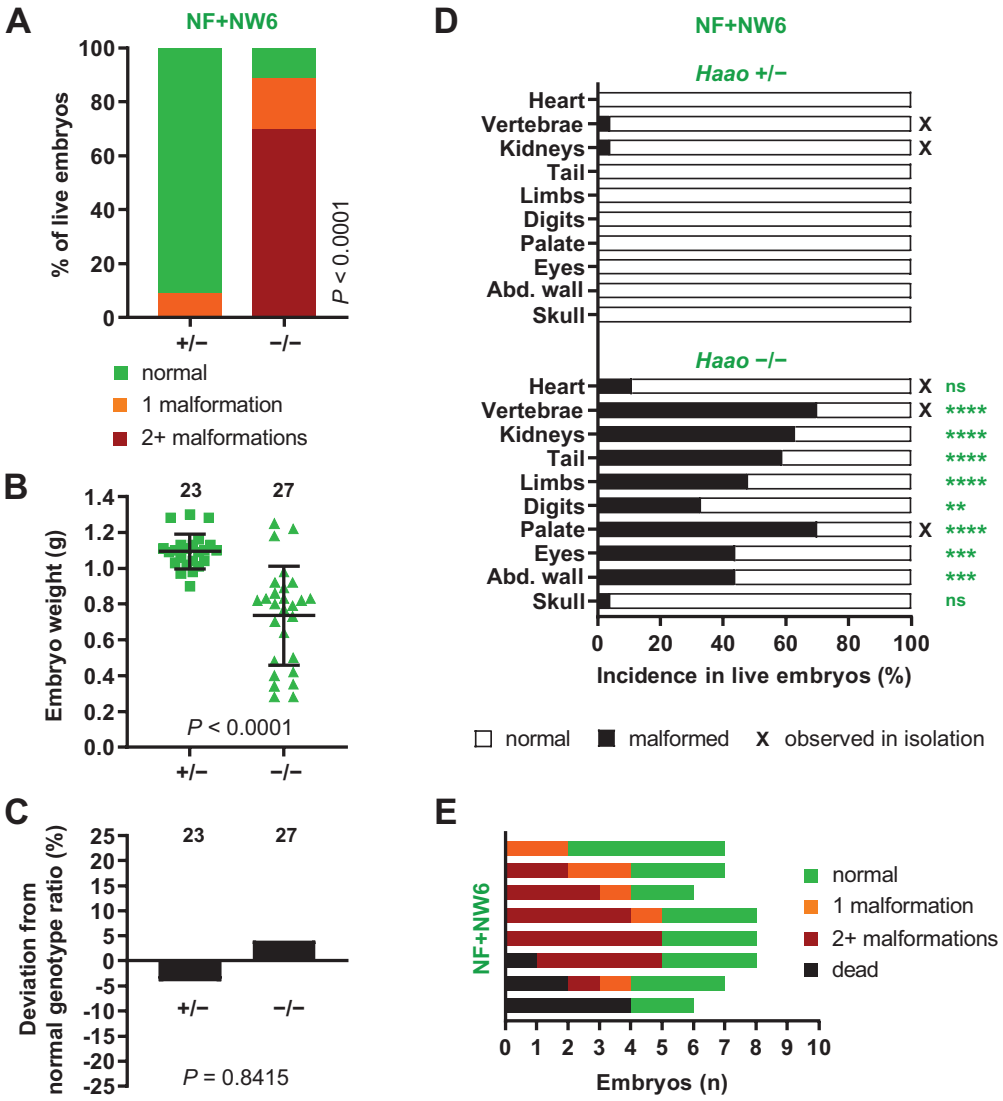

Fig. S6. Embryo genotype strongly influences phenotype of offspring of mice with maternal *Haao*$^{-/-}$ and paternal *Haao*$^{+/-}$ loss-of-function variant, and maternal NAD precursor intake restriction throughout pregnancy with NF+NW6 diet. **A:** Summarised live embryo outcomes of the NF+NW6 group 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). **P < 0.01, ***P < 0.001, ****P < 0.0001. **E:** Embryo outcomes in individual litters. Each horizontal bar represents a litter and the length of bars represents the number of embryos per litter.

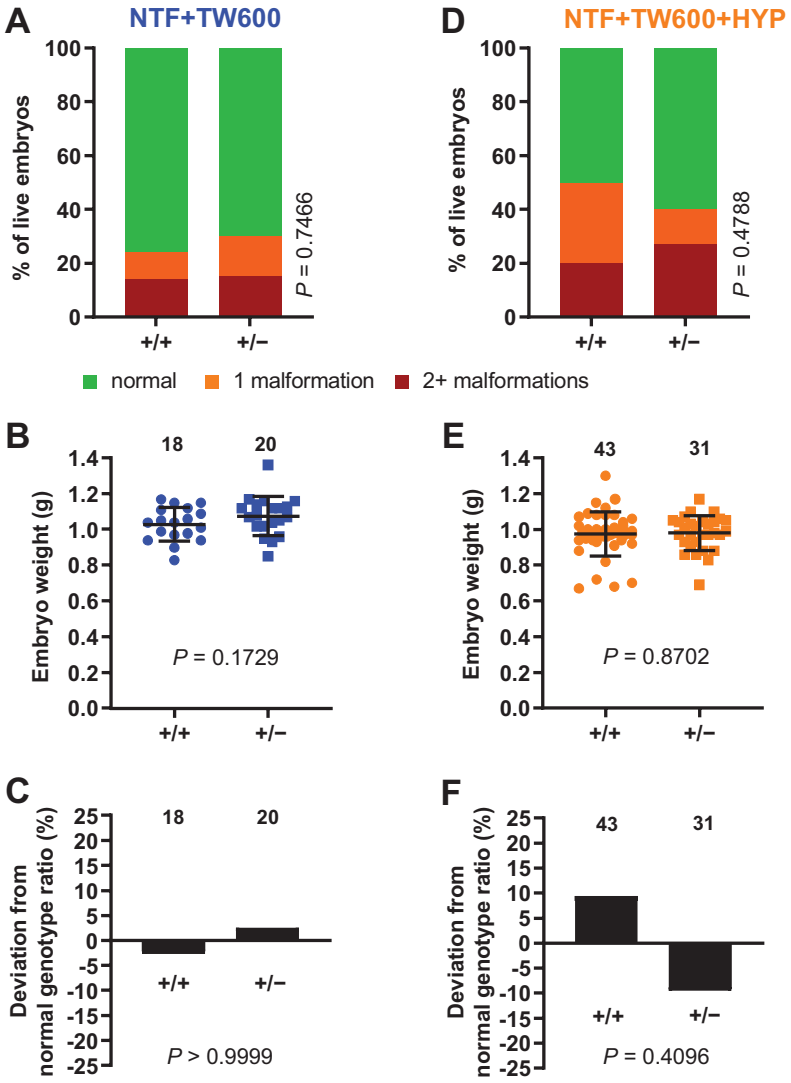

Fig. S7. The embryo genotype has no significant influence on embryo phenotype at E18.5 in embryos from matings that involved a maternal *Haao*$^{+/-}$ loss-of-function variant and maternal NAD precursor restriction throughout pregnancy, with exposure to hypoxia. **A:** Summarised live embryo outcomes 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.

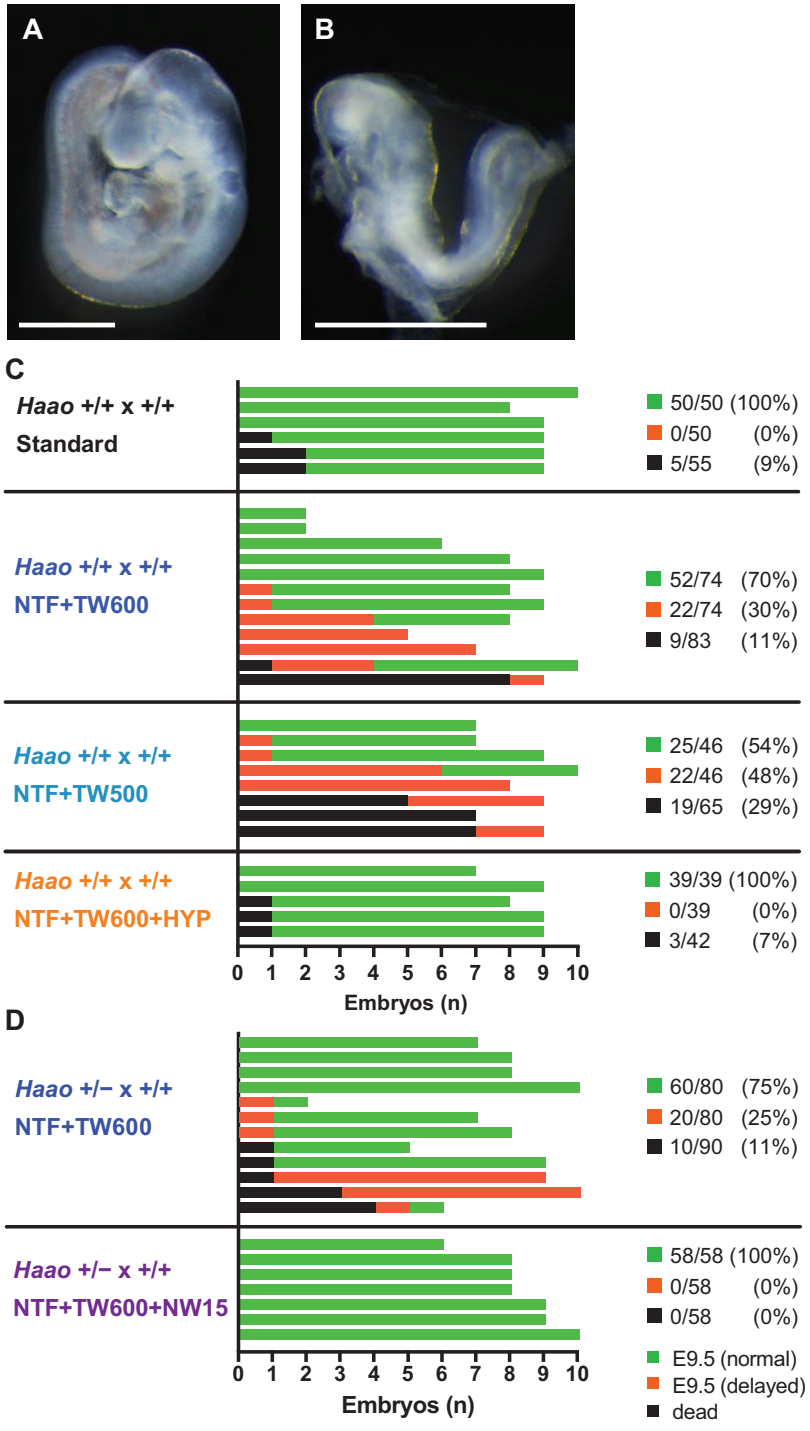

Fig. S8. E9.5 embryos frequently exhibited developmental delay under maternal dietary NAD precursor restriction throughout pregnancy. **A:** normal embryo at E9.5. **B:** Embryo at E9.5 that morphologically resembles an E8.5 embryo. Scale bars 1 mm. **C, D:** Summarised embryo outcomes at E9.5 for each of the mating schemes (maternal x paternal) 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.

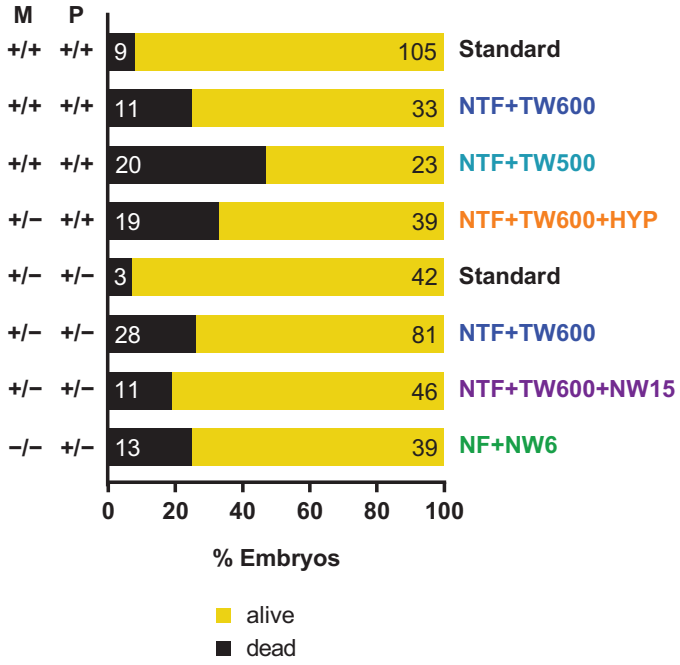

Fig. S9. Summarized embryo outcomes at E11.5 show that dietary NAD precursor restriction during pregnancy is associated with higher rates of embryo death, similar to the observations at E18.5. The maternal dietary treatments throughout pregnancy are indicated on the right. M, maternal *Haao* genotype;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

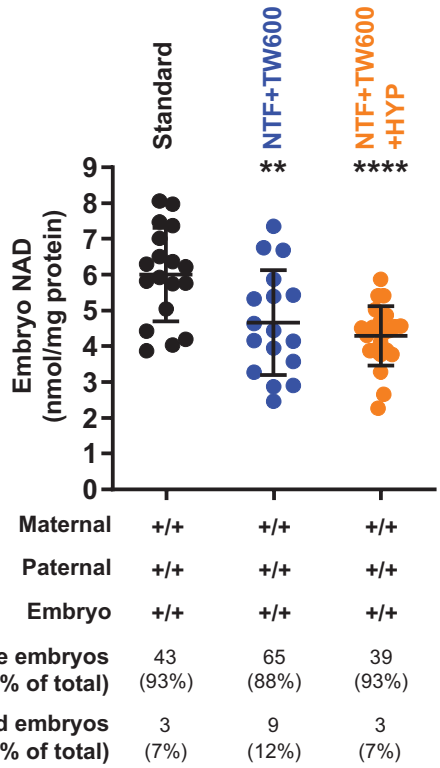

Fig. S10. Whole embryo total NAD levels (NAD^+ and NADH) and embryo outcomes at E9.5. Dots represent NAD levels and bars indicate the mean ± SD. The parental and embryo *Haao* genotypes are indicated at the bottom (all mice were wild-types). The total numbers and percentages of live and dead embryos 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. $^{**}P < 0.01$, $^{****}P < 0.0001$. For a summary of NAD level values, see *SI Appendix* Table S4.

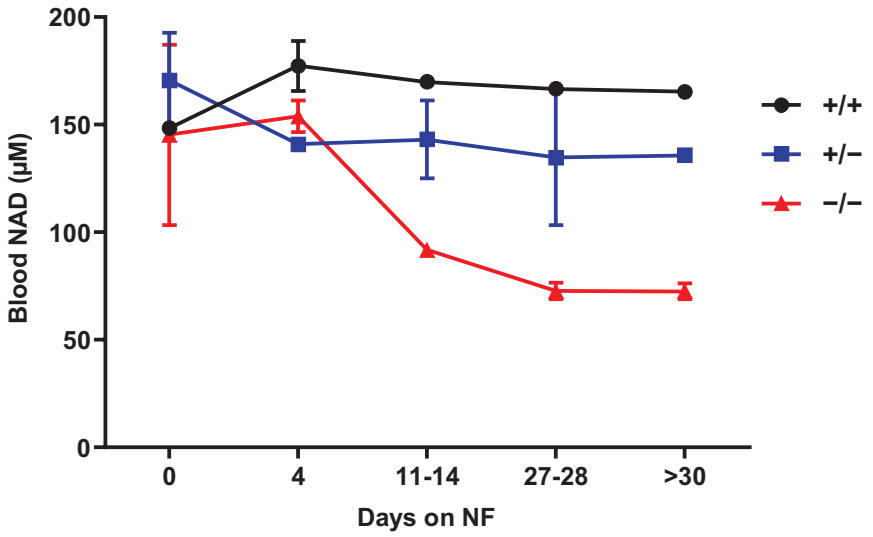

Fig. S11. Feeding mice an NAD precursor restricted diet results in depletion of whole blood NAD levels in *Haao*$^{-/-}$ mice over time but does not affect blood NAD levels in *Haao*$^{+/+}$ and *Haao*$^{+/-}$ mice. Non-pregnant mice were maintained on NF (containing 1.4 mg/kg NA and 1800 mg/kg tryptophan, 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 enough 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. 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 χ^2 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/d)	Human equivalent NAD precursor supply per day (mg/d)‡
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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