Supplemental Information Inventory (CELL-D-12-00930R2)

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Developmental delay and congenital malformations in litters from *Mtrr*$^{+/+}$ intercrosses at E10.5 suggest an intergenerational effect, related to Figure 2.

The genotypes of the maternal grandparents were determined retrospectively. Data represented as the average number of conceptuses/litter (±s.e.) followed by the percentage of total conceptuses (brackets) unless otherwise indicated. Second pedigree from the left: the genotype of the maternal grandfather was either *Mtrr*$^{+/+}$ or *Mtrr*$^{+/gt}$. Statistical analyses: independent comparison of the average number of conceptuses/litter from each pedigree compared to C57Bl/6 (black asterisk) or from each pedigree compared to conceptuses with an *Mtrr*$^{+/+}$ maternal grandmother and mother (red asterisk) using Mann-Whitney test. [a]P=0.05; *P<0.05; **P<0.01; ***P<0.001. [b]Phenotype frequencies of conceptuses from *Mtrr*$^{+/+}$ maternal grandmothers and mothers were statistically different from those with *Mtrr*$^{+/gt}$ maternal grandparents (Kruskal-Wallis test). No maternal effect was observed (Kruskal-Wallis test). Pedigree key: circle, female; square, male; blue outline, C57Bl/6 mice; black outline, *Mtrr* mouse line; white fill, *Mtrr*$^{+/+}$; half black/half white, *Mtrr*$^{+/gt}$; black fill, *Mtrr*$^{gt/gt}$.

Figure S2. Frequencies of developmental delay and congenital abnormalities in conceptuses at E10.5 derived from *Mtrr*$^{+/gt}$ maternal grandmothers or maternal grandfathers, related to Figure 3.

Data represented as the average number of conceptuses/litter (±s.e.) that displayed the phenotype followed by the percentage of total conceptuses in brackets. C57Bl/6, *Mtrr*$^{+/gt}$ paternal and *Mtrr*$^{gt/gt}$ pedigrees were controls. Some conceptuses with severe abnormalities displayed >1 phenotype and were scored more than once (e.g., embryo with neural tube and heart defects was

counted in each category). However, these conceptuses were represented once in the overall total of severely abnormal conceptuses. Statistical analyses (Mann-Whitney test): Independent comparison of the average number of conceptuses/litter from each pedigree compared to C57Bl/6 (black asterisk) or from *Mtrr*$^{+/gt}$ maternal grandmothers and *Mtrr*$^{+/+}$ mothers to those from *Mtrr*$^{+/gt}$ maternal grandmothers and *Mtrr*$^{+/gt}$ mothers (red asterisk). *P<0.05, **P<0.01, ***P<0.005. E, embryonic day; s, somite pairs; CA, chorioallantoic attachment; NTD, neural tube defects including neural tubes that did not close by the appropriate developmental stage; twins/triplets indicates more than one conceptus per implantation site; [a,b,c]Number of [a]maternal grandmothers, [b]maternal grandfathers or [c]fathers assessed. Pedigree key: circle, female; square, male; blue outline, C57Bl/6 mice; black outline, *Mtrr* mouse line; white fill, *Mtrr*$^{+/+}$; half black/half white, *Mtrr*$^{+/gt}$; black fill, *Mtrr*$^{gt/gt}$.

Figure S3. The degree of epigenetic instability increases with severity of phenotype in the *Mtrr* model in placentas at E10.5, related to Figure 4.

(A) Graphs showing the proportion of total DMRs (N=20), somatic DMRs (N=8) and germline DMRs (N=12) with a particular range of abnormally methylated CpG sites. Whole placentas (E10.5) of grandprogeny derived from the indicated *Mtrr* pedigree were analyzed and compared to C57Bl/6. Each DMR was categorized based on the percentage of its CpG sites with abnormal methylation. CpG site methylation that was statistically different from C57Bl/6 (P<0.05) was considered abnormal. (**B**) Average DNA methylation (±s.e.) at specific CpG sites within the *Igf2* DMR1, *Igf2* DMR2 and *H19* DMR (CTCF2) observed in whole phenotypically normal (n), growth-restricted (gr), growth-enhanced (ge) and severely affected (a) *Mtrr*$^{+/+}$ embryos at E10.5 derived from the specified pedigree (left panel). Relative levels of *Igf2* and *H19* mRNA expression (mean±s.e.) in embryos at E10.5 derived from the specified phenotype and pedigree

(N=3-6 embryos) compared to C57Bl/6 levels (normalized to 1) (right panel). Legend: Each colored box denotes the pedigree from which the conceptuses analyzed were derived as well as their phenotype. Statistical analysis: Methylation percentages at each CpG site were independently compared to C57Bl/6 using a Mann-Whitney test; RNA levels between each phenotype/pedigree and C57Bl/6 were compared using independent t-tests. *P<0.05, **P<0.01, ***P<0.005. See also Table S3.

Figure S4. *Mtrr*^{gt} mutation does not cause a direct paramutation effect, related to Figure 5.

Relative expression of wildtype *Mtrr* mRNA in (**A**, **C**) placentas (N=3-8) and (**B**, **D**) embryos (N=4-9) at E10.5 derived from the specified *Mtrr* genetic crosses compared to C57Bl/6 controls (N=7-10 placentas, N=4-8 embryos; normalized to 1). The genotypes of the conceptuses (*Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$ or *Mtrr*$^{gt/gt}$) are noted along with their phenotypes (phenotypically normal [n], growth-restricted [gr], or severely affected [a]). RNA levels of each group were compared to C57Bl/6 using independent t-tests. *P<0.05, **P<0.01, ***P<0.005.

Figure S5. Frequencies of congenital abnormalities in wildtype grandprogeny derived from an *Mtrr*$^{+/gt}$ maternal grandparent after embryo transfer into recipient females, related to Figure 6.

Data is represented as the average number of conceptuses/litter (±s.e.) that displayed the designated phenotype at E10.5 followed by the percentage of total conceptuses in brackets. Some conceptuses with severe abnormalities displayed >1 phenotype and were scored more than once (e.g., embryo with neural tube and heart defects was counted in each category). However, these conceptuses were represented once in the overall total of severely abnormal conceptuses. Independent comparison of the average number of conceptuses/litter from each pedigree compared to C57Bl/6 using a Mann-Whitney test. [a]P=0.05, *P<0.05, **P<0.01. E, embryonic

day; s, somite pairs; CA, chorioallantoic attachment; NTD, neural tube defects including neural tubes that did not close by the appropriate developmental stage; twins/triplets indicates more than one conceptus per implantation site. Pedigree key: circle, female; square, male; blue outline, C57Bl/6 mice; black outline, *Mtrr* mouse line; red outline, B6D2F1 mice; white fill, $Mtrr^{+/+}$; half black/half white, $Mtrr^{+/gt}$.

Figure S6. Severe abnormalities persist in wildtype conceptuses from generations III, IV and V derived from an $Mtrr^{+/gt}$ maternal ancestor, related to Figure 7.

Data is represented as the average number of wildtype conceptuses/litter (±s.e.) from generations III, IV and V derived from an $Mtrr^{+/gt}$ maternal grandmother (MGM) or an $Mtrr^{+/gt}$ maternal grandfather (MGF) that displayed the phenotype followed by the percentage of total conceptuses in brackets. Dissections occurred at E10.5. Some conceptuses with severe abnormalities displayed >1 phenotype and were scored more than once but were represented only once in the overall total. Statistical analyses (Mann-Whitney test): independent comparison of the average number of conceptuses/litter with a phenotype compared to C57Bl/6. *P<0.05, **P<0.01, ***P<0.005. E, embryonic day; s, somite pairs; CA, chorioallantoic attachment; NTD, neural tube defects including neural tubes that did not close by the appropriate developmental stage; twins/triplets indicates more than one conceptus per implantation site.

Table S1. Even *Mtrr*$^{+/+}$ conceptuses from *Mtrr*$^{+/gt}$ intercrosses had congenital malformations at E10.5, related to Figure 2.

			C57Bl/6 x C57Bl/6 †	*Mtrr*$^{+/gt}$ x *Mtrr*$^{+/gt}$ †	Phenotypic frequencies of each genotype arising from *Mtrr*$^{+/gt}$ intercrosses‡			
Embryonic genotype			***Mtrr*$^{+/+}$** N=93	**All** N=155	***Mtrr*$^{+/+}$** N=36	***Mtrr*$^{+/gt}$** N=77	***Mtrr*$^{gt/gt}$** N=37	**N.A.** N=5
Normal stage			**8.9±0.4** (95.7%)	**5.1±0.7**** (55.5%)	**19/36** (52.8%)	**44/77** (57.1%)	**23/37** (62.2%)	**-**
Developmental delay		≥E9.5-E10.0 (21-29 s)	**0.0**	**1.7±0.6*** (18.8%)	**9/36** (25.0%)	**13/77** (16.9%)	**7/37** (24.2%)	**-**
		≥E8.5-E9.5 (8-20 s)	**0.0**	**0.1±0.1** (0.6%)	**0/36**	**1/77** (1.3%)	**0/37**	**-**
		<E8.5 (<8 s)	**0.0**	**0.1±0.1** (0.6%)	**1/36** (2.8%)	**0/77**	**0/37**	**-**
		Total	**0.0**	**1.8±0.6*** (20.0%)	**10/36** (27.8%)	**14/77** (18.2%)	**7/37** (24.2%)	**-**
Severe abnormalities	**Overall total**		**0.0**	**1.2±0.3***** (12.9%)	**6/36** (16.7%)	**8/77** (10.4%)	**5/37** (13.5%)	**1/5** (20.0%)
	>1 phenotype§		**0.0**	**0.3±0.1** (3.2%)	**2/36** (5.6%)	**3/77** (3.9%)	**0/37**	**-**
	Placenta	Off-centered CA	**0.0**	**0.5±0.2** (5.2%)	**2/36** (5.6%)	**3/77** (3.9%)	**2/37** (5.4%)	**1/5** (20.0%)
		No CA	**0.0**	**0.1±0.1** (0.6%)	**1/36** (2.8%)	**0/77**	**0/37**	**-**
		Total	**0.0**	**0.5±0.2** (5.8%)	**3/36** (5.6%)	**3/77** (3.9%)	**2/37** (5.4%)	**1/5** (20.0%)
	Heart	Pericardial edema	**0.0**	**0.1±0.1** (1.3%)	**2/36** (5.6%)	**0/77**	**0/37**	**-**
		Reversed looping	**0.0**	**0.1±0.1** (0.6%)	**1/36** (2.8%)	**0/77**	**0/37**	**-**
		Enlarged heart	**0.0**	**0.3±0.1** (3.2%)	**1/36** (2.8%)	**2/77** (2.6%)	**2/37** (5.4%)	**-**
		Total	**0.0**	**0.5±0.2*** (5.2%)	**4/36** (11.1%)	**2/77** (2.6%)	**2/37** (5.4%)	**-**
	Neural tube	Cranial NTD	**0.0**	**0.2±0.1** (2.9%)	**1/36** (2.8%)	**2/77** (2.6%)	**1/37** (2.7%)	**-**
		Spinal NTD	**0.0**	**0.0**	**0/0**	**0/0**	**0/0**	**-**
		Total	**0.0**	**0.2±0.1** (2.9%)	**1/36** (2.8%)	**2/77** (2.6%)	**1/37** (2.7%)	**-**
	Hemorr-hages	Placenta	**0.0**	**0.3±0.1** (3.2%)	**2/36** (5.6%)	**3/77** (3.9%)	**0/37**	-
		Embryo	**0.0**	**0.1±0.1** (0.6%)	**0/36**	**1/77** (1.3%)	**0/37**	-
		Total	**0.0**	**0.4±0.1*** (3.9%)	**2/36** (5.6%)	**4/77** (5.2%)	**0/37**	**-**
Resorptions			**0.4±0.2** (4.3%)	**1.1±0.3** (11.6%)	**1/36** (2.8%)	**11/77** (14.3%)	**2/37** (5.4%)	**4/5** (80.0%)

†Average number of conceptuses/litter (±s.e.m.) followed by the percentage of total conceptuses in brackets.

‡The number conceptuses that displayed the phenotype out of the total number of conceptuses in that genotypic category. No significant difference between the embryonic genotypes (Fisher's exact test).
§Some severely affected conceptuses displayed more than one phenotype and were scored more than once but were represented only once in the overall total for severely affected conceptuses.
Statistical analysis: independent comparison of the average number of conceptuses/litter from *Mtrr*$^{+/gt}$ intercrosses compared to C57Bl/6 using Mann-Whitney test (*P<0.05; **P<0.01; ***P<0.001)
N.A., genotype not available; E, embryonic day; s, somite pairs; CA, chorioallantoic attachment; NTD, neural tube defect including only neural tubes that did not close by the appropriate developmental stage.

Table S2. The frequency of growth phenotypes in wildtype grandprogeny (i.e., generation III) did not correlate with litter size at E10.5, related to Figure 3.

Generation I (Female x Male)	**C57Bl/6** x **C57Bl/6**	***Mtrr*$^{+/gt}$** x **C57Bl/6**	***Mtrr*$^{+/gt}$** x **C57Bl/6**		**C57Bl/6** x ***Mtrr*$^{+/gt}$**	
Generation II (Female x Male)	**C57Bl/6** x **C57Bl/6**	**C57Bl/6** x ***Mtrr*$^{+/gt}$**	***Mtrr*$^{+/+}$** x **C57Bl/6**	***Mtrr*$^{+/gt}$** x **C57Bl/6**	***Mtrr*$^{+/+}$** x **C57Bl/6**	***Mtrr*$^{+/gt}$** x **C57Bl/6**
Generation III Frequency of growth restriction vs. litter size[a,b]						
R^2	0.074	**0.647**	0.343	0.027	0.004	0.063
P value	0.477	**0.009**	0.098	0.573	0.879	0.485
Generation III Frequency of growth enhance-ment vs. litter size[a,b]						
R^2	0.000	0.069	0.013	0.000	0.000	0.001
P value	1.000	0.495	0.768	1.000	1.000	0.947

[a]Embryos deemed growth-restricted or -enhanced had a somite pair number within the normal range for E10.5 (30-40 somite pairs) and a crown-rump length that was more than two standard deviations from the mean of control C57Bl/6 embryo head-rump lengths.
[b]Correlation coefficients (R^2) were generated using a linear regression model. R^2>0.5 indicated that there was good correlation between litter size and the growth defect. *P* value <0.05 indicated that the slope of the line was significantly non-zero.

Table S3. Placentas but not embryos derived from an *Mtrr*$^{+/gt}$ maternal grandparent displayed epigenetic instability at specific imprinted loci associated with changes in gene expression, related to Figures 4 and 5.

Please refer to Excel file (Padmanabhan Table S3).

Table S4. Comparison of the components of each normal breeding diet used, related to Figure 1.

Dietary component[†]	Pico-Vac Lab Rodent Diet (5061)[‡,*]	Rat/Mouse Breeding Diet 3 (RM3 P)[§,**]
Calories provided		
Protein (%)	24.65	27.28
Fat (%)	13.21	11.48
Carbohydrates (%)	62.14	61.24
Gross energy (kcal/g)	4.07	3.61
Metabolizable energy (kcal/g)	3.07	3.29
Nitrogen-free extract (%)	52.9	50.4
Moisture (%)	10.0	10.0
Amino Acids		
Arginine (%)	1.22	1.42
Cystine (%)	0.28	0.35
Glycine (%)	0.96	1.85
Histidine (%)	0.50	0.55
Isoleucine (%)	0.97	0.98
Leucine (%)	1.56	1.87
Lysine (%)	1.16	1.34
Methionine (%)	0.70	0.37
Phenylalanine (%)	0.90	1.23
Tyrosine (%)	0.59	0.87
Threonine (%)	0.77	0.88
Tryptophan (%)	0.26	0.27
Valine (%)	1.00	1.15
Serine (%)	1.03	1.01
Aspartic Acid (%)	2.19	1.40
Glutamic Acid (%)	4.34	4.39
Alanine (%)	1.15	0.27
Proline (%)	1.47	1.56
Taurine (%)	0.02	<0.01
Fat		
Cholesterol (mg/kg)	141	Not indicated
Linoleic Acid (%)	2.19	1.26
Linolenic Acid (%)	0.26	0.17
Arachidonic Acid (%)	<0.01	0.12
Omega-3 Fatty Acids (%)	0.33	Not indicated
Total Saturated Fatty Acids (%)	0.93	0.70
Total Monosaturated Fatty Acids (%)	0.99	1.11
Carbohydrates, Fiber and Non-starch polysaccharides		
Total Dietary Fiber (%)	Not indicated	16.15
Fiber (Crude) (%)	4.7	4.42
Neutral Detergent Fiber (%)	16.4	15.3
Acid Detergent Fiber (%)	6.0	Not indicated
Pectin (%)	Not indicated	1.53
Starch (%)	33.9	33.9
Sugar (%)	4.94	4.37

Minerals		
Ash (%)	6.1	8.05
Macro Minerals		
Calcium (%)	0.81	1.24
Total Phosphorus (%)	0.63	0.83
Phytate Phosphorus (%)	0.30	0.26
Available Phosphorus (%)	0.33	0.56
Sodium (%)	0.30	0.24
Chlorine (%)	0.51	0.36
Potassium (%)	1.07	0.81
Magnesium (%)	0.22	0.29
Sulfur (%)	0.34	Not indicated
Micro Minerals		
Fluorine (mg/kg)	10	8.67
Iron (mg/kg)	220	163.44
Zinc (mg/kg)	87	48.67
Manganese (mg/kg)	85	102.71
Copper (mg/kg)	13	20.53
Cobalt (mg/kg)	0.71	0.60
Iodine (mg/kg)	0.97	0.87
Chromium (mg/kg)	0.81	Not indicated
Selenium (mg/kg)	0.30	0.39
Vitamins		
Choline (mg/kg)	2000	1422.4
Folic acid (mg/kg)	3.0	2.99
Carotene (mg/kg)	1.5	1.7
Vitamin K (as Menadione) (mg/kg)	3.3	4.14
Thiamine (mg/kg)	17	28.4
Riboflavin (mg/kg)	8.0	10.28
Niacin (mg/kg)	90	85.7
Pantothenic acid (mg/kg)	17	40.8
Pyridoxine (mg/kg)	9.6	18.87
Biotin (mg/kg)	0.30	0.32
Vitamin B_{12} (µg/kg)	51	19.23
Vitamin A (IU/g)	15	22.2
Retinol (mg/kg)	Not indicated	6.67
Vitamin D_3 (IU/g)	2.2	2.9
Vitamin E (IU/kg)	99	111
Vitamin C (mg/g)	0.0	1.33
Cholecalciferol (mg/kg)	Not indicated	0.07
α-Tocopherol (mg/kg)	Not indicated	100.9
Inositol (g/kg)	Not indicated	1.84

†Nutrients expressed as a percent of ration except where otherwise indicated. Moisture content is 10%.
‡Further information: http://www.labdiet.com/rodent_diet.html
§Further information: www.sdsdiets.com/products_and_data_sheets/rodent/
*Experiments completed in University of Calgary included Figures 2, 6, S1, S5, Table S1.
**Experiments completed in University of Cambridge included Figures 1, 3-5, 7 S2-S4, S6, Tables S2, S3.

Table S5. A list of the qRT-PCR and bisulfite pyrosequencing primers used in this study, related to Figure 5.

Please refer to excel file (Padmanabhan Table S5).

EXTENDED EXPERIMENTAL PROCEDURES

Diet

For the analysis completed at the University of Calgary (Figures 2, 5, S1 and S5, Table S1), mice were fed normal breeding Pico-Vac® Lab Rodent Diet (5061, PMI Nutrition International). For the analysis completed at the University of Cambridge (Figures 1, 3, 4, 6, S2, S3, S4 and S6, Tables S2 and S3), mice were fed Rodent No. 3 breeding chow (Special Diet Services). The dietary components are comparable (Table S4). Data generated from mice in Canada were never pooled with data from mice in the UK.

SUPPLEMENTAL REFERENCES

Charalambous, M., Ferron, S.R., da Rocha, S.T., Murray, A.J., Rowland, T., Ito, M., Schuster-Gossler, K., Hernandez, A., and Ferguson-Smith, A.C. (2012). Imprinted gene dosage is critical for the transition to independent life. Cell Metab *15*, 209-221.

Fauque, P., Ripoche, M.A., Tost, J., Journot, L., Gabory, A., Busato, F., Le Digarcher, A., Mondon, F., Gut, I., Jouannet, P., *et al.* (2010). Modulation of imprinted gene network in placenta results in normal development of in vitro manipulated mouse embryos. Hum Mol Genet *19*, 1779-1790.

Green, K., Lewis, A., Dawson, C., Dean, W., Reinhart, B., Chaillet, J.R., and Reik, W. (2007). A developmental window of opportunity for imprinted gene silencing mediated by DNA methylation and the Kcnq1ot1 noncoding RNA. Mamm Genome *18*, 32-42.

Kamei, Y., Suganami, T., Kohda, T., Ishino, F., Yasuda, K., Miura, S., Ezaki, O., and Ogawa, Y. (2007). Peg1/Mest in obese adipose tissue is expressed from the paternal allele in an isoform-specific manner. FEBS Lett *581*, 91-96.

Lui, J.C., Finkielstain, G.P., Barnes, K.M., and Baron, J. (2008). An imprinted gene network that controls mammalian somatic growth is down-regulated during postnatal growth deceleration in multiple organs. Am J Physiol Regul Integr Comp Physiol *295*, R189-196.

Messerschmidt, D.M., de Vries, W., Ito, M., Solter, D., Ferguson-Smith, A., and Knowles, B.B. (2012). Trim28 is required for epigenetic stability during mouse oocyte to embryo transition. Science *335*, 1499-1502.

Radford, E.J., Isganaitis, E., Jimenez-Chillaron, J., Schroeder, J., Molla, M., Andrews, S., Didier, N., Charalambous, M., McEwen, K., Marazzi, G., *et al.* (2012). An unbiased assessment of the role of imprinted genes in an intergenerational model of developmental programming. PLoS Genet *8*, e1002605.

Sun, B., Ito, M., Mendjan, S., Ito, Y., Brons, I.G., Murrell, A., Vallier, L., Ferguson-Smith, A.C., and Pedersen, R.A. (2012). Status of genomic imprinting in epigenetically distinct pluripotent stem cells. Stem Cells *30*, 161-168.

Tunster, S.J., Tycko, B., and John, R.M. (2010). The imprinted Phlda2 gene regulates extraembryonic energy stores. Mol Cell Biol *30*, 295-306.

Varrault, A., Gueydan, C., Delalbre, A., Bellmann, A., Houssami, S., Aknin, C., Severac, D., Chotard, L., Kahli, M., Le Digarcher, A., *et al.* (2006). Zac1 regulates an imprinted gene network critically involved in the control of embryonic growth. Dev Cell *11*, 711-722.

Wultsch, T., Grimberg, G., Schmitt, A., Painsipp, E., Wetzstein, H., Breitenkamp, A.F., Grundemann, D., Schomig, E., Lesch, K.P., Gerlach, M., *et al.* (2009). Decreased anxiety in mice lacking the organic cation transporter 3. J Neural Transm *116*, 689-697.

Yamasaki, Y., Kayashima, T., Soejima, H., Kinoshita, A., Yoshiura, K., Matsumoto, N., Ohta, T., Urano, T., Masuzaki, H., Ishimaru, T., *et al.* (2005). Neuron-specific relaxation of Igf2r imprinting is associated with neuron-specific histone modifications and lack of its antisense transcript Air. Hum Mol Genet *14*, 2511-2520.

Zaitoun, I., Downs, K.M., Rosa, G.J., and Khatib, H. (2010). Upregulation of imprinted genes in mice: an insight into the intensity of gene expression and the evolution of genomic imprinting. Epigenetics *5*, 149-158.

Zhu, J., and Mackem, S. (2011). Analysis of mutants with altered shh activity and posterior digit loss supports a biphasic model for shh function as a morphogen and mitogen. Dev Dyn *240*, 1303-1310.