Supplementary Figure Legends and Tables for

Intermittent leucine deprivation produces long-lasting improvement in insulin sensitivity by increasing hepatic *Gcn2* expression

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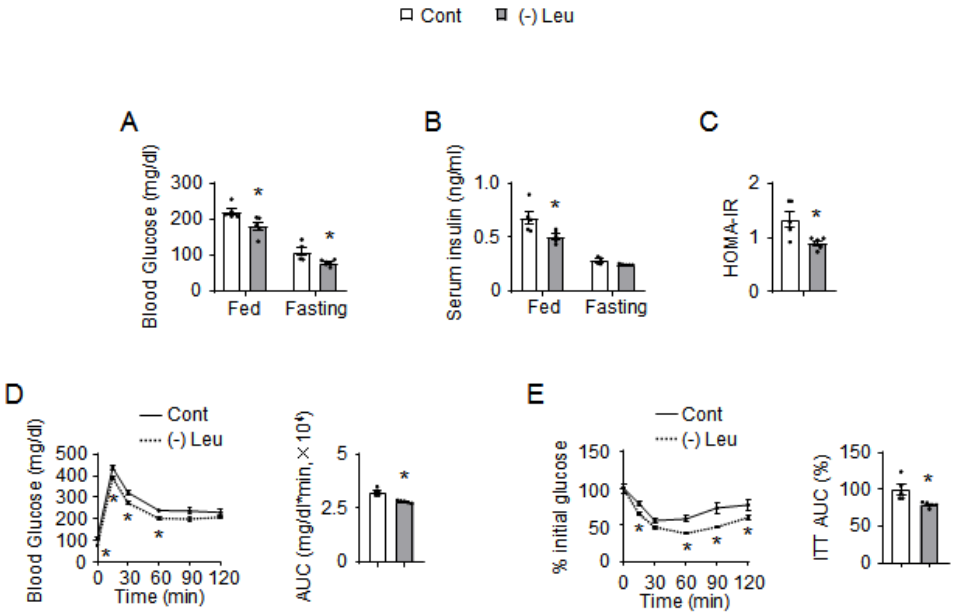
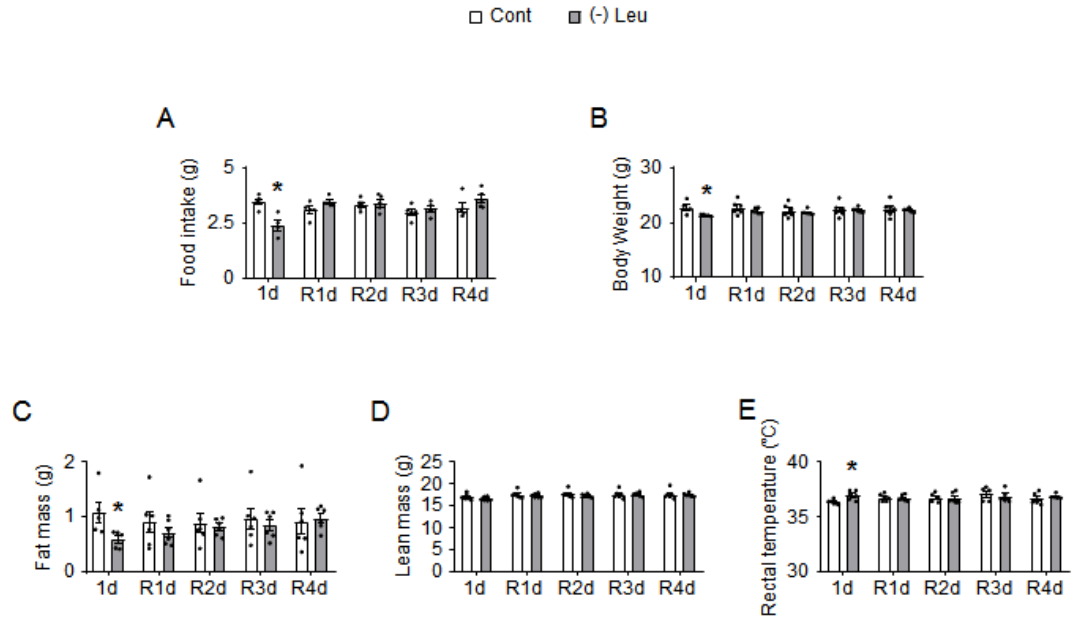
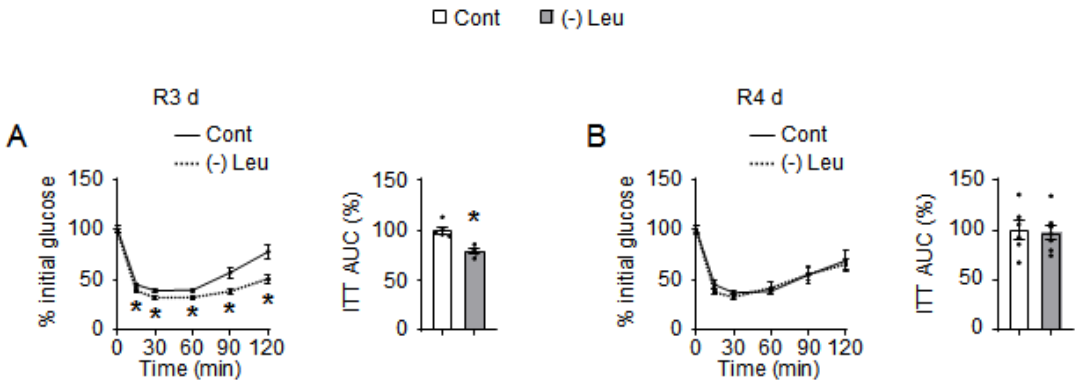
Running title: ILD persistently increases insulin sensitivity



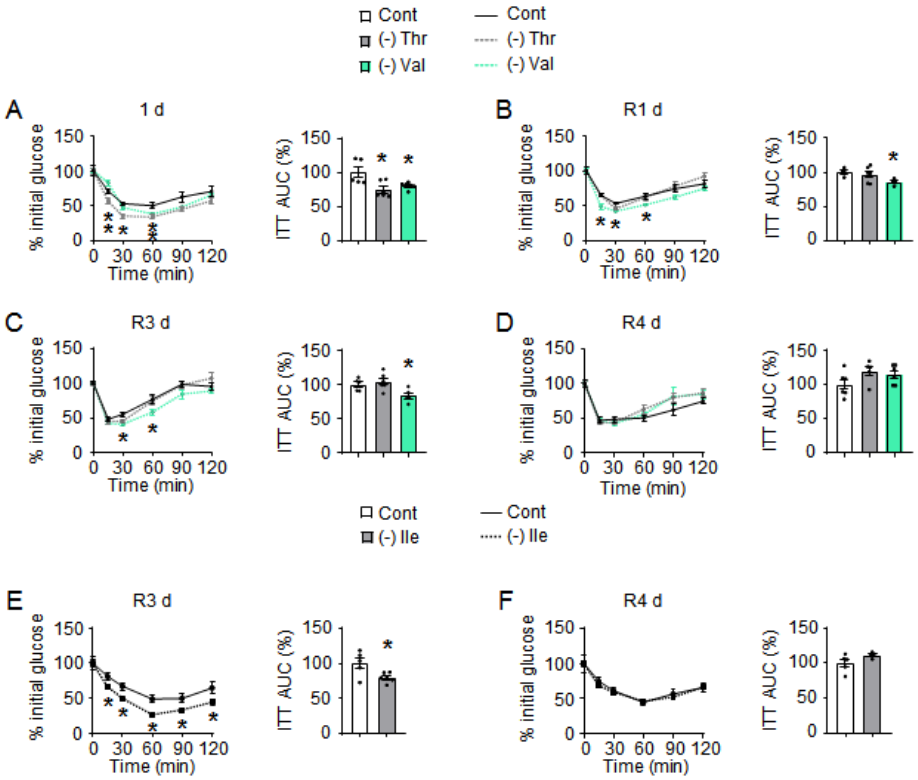
Supplementary Figure 1: One day of leucine deprivation improves whole-body insulin sensitivity. (A) Fed and fasting blood glucose levels. (B) Fed and fasting serum insulin levels. (C) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (D) Glucose tolerance tests (GTTs); AUC: area under the curve. (E) Insulin tolerance tests (ITTs). Studies were conducted using 8-week-old male wild-type mice fed a control diet for 1 days (Cont) or fed a leucine-deficient diet for 1 day [(-) Leu]. Data are expressed as the mean ± SEM (n = 4–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of (-) Leu group versus Cont group.



Supplementary Figure 2: The effects of 1-day leucine deprivation on lipid and energy metabolism. (A) Daily food intake. (B) Body weight. (C) Fat mass. (D) Lean mass. (E) Rectal temperature. Studies were conducted using 8-week-old male wild-type mice fed a control diet (Cont) or fed a leucine-deficient diet [(-) Leu] for 1 day and resuming a control diet for days as indicated (R1/2/3/4 d: 1, 2, 3 or 4 days). Data are expressed as the mean ± SEM (n = 4–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of (-) Leu group versus Cont group.

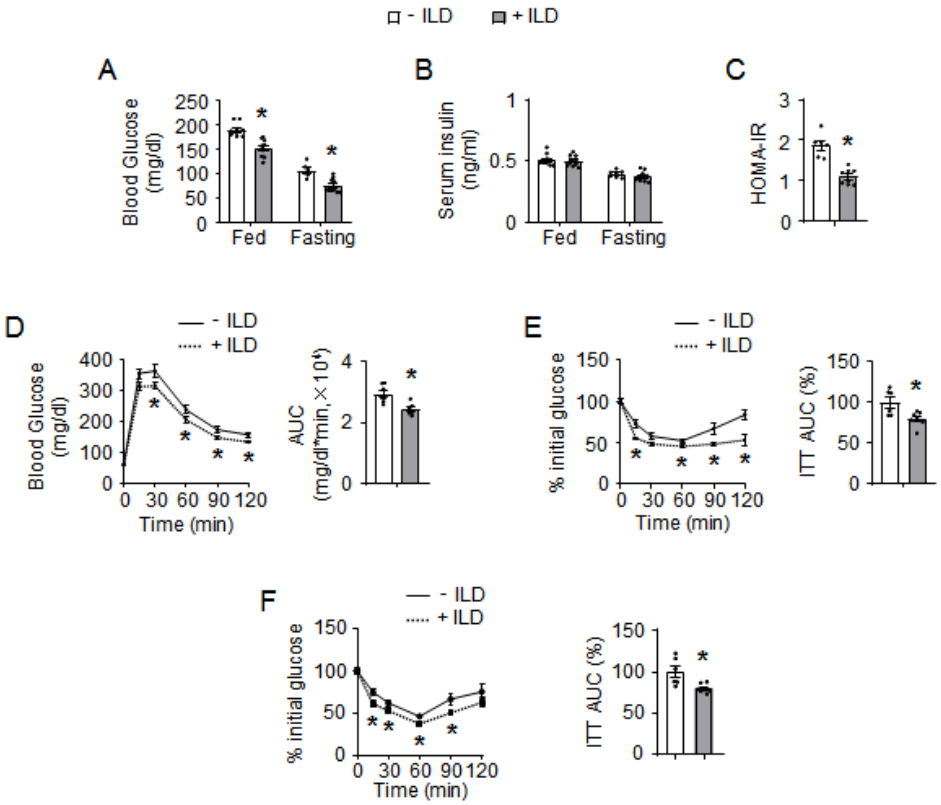


Supplementary Figure 3: Seven days of leucine deprivation improves whole-body insulin sensitivity for 3 days. (A–B) Insulin tolerance tests (ITTs); AUC: area under the curve. Studies were conducted using 8-week-old male wild-type mice fed a control diet (Cont) or fed a leucine-deficient diet [(-) Leu] for 7 days and resuming a control diet for days as indicated (R3/4 d: 3 or 4 days). Data are expressed as the mean ± SEM (n = 4–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of (-) Leu group versus Cont group.



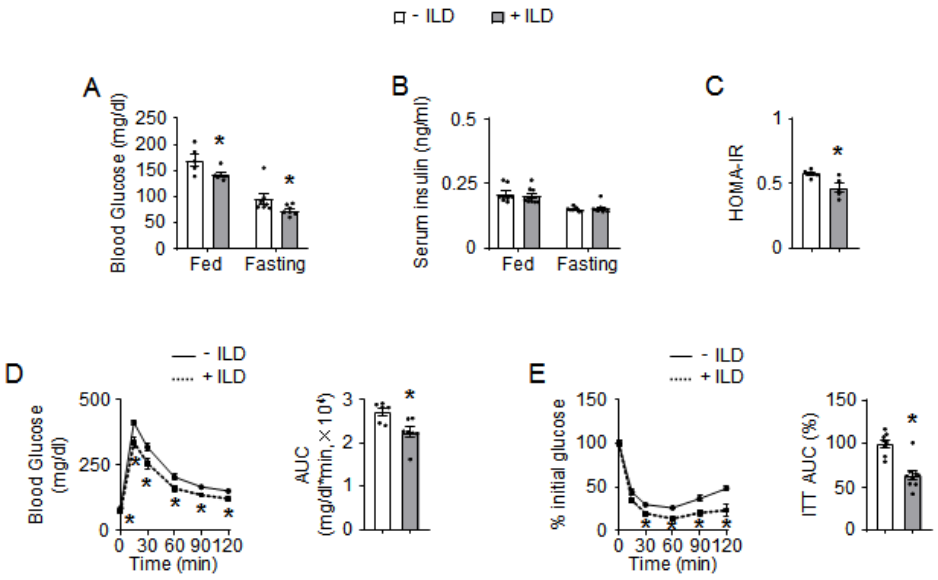
Supplementary Figure 4: Effects of 1 day of single BCAA deprivation on insulin sensitivity. (A–D) Insulin tolerance tests (ITTs) for threonine and valine deprivation; AUC: area under the curve. (E–F) Insulin tolerance tests (ITTs) for isoleucine deprivation Studies were conducted using 8-week-old male wild-type mice fed a

control diet (Cont), or fed a threonine deficient or a valine deficient diet or a isoleucine deficient diet [(-) Thr or (-) Val or (-) Ile] for 1 day and resuming a control diet for days as indicated (R1/3/4 d: 1, 3 or 4 days). Data are expressed as the mean ± SEM (n = 4–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of any group versus Cont group on the indicated day.



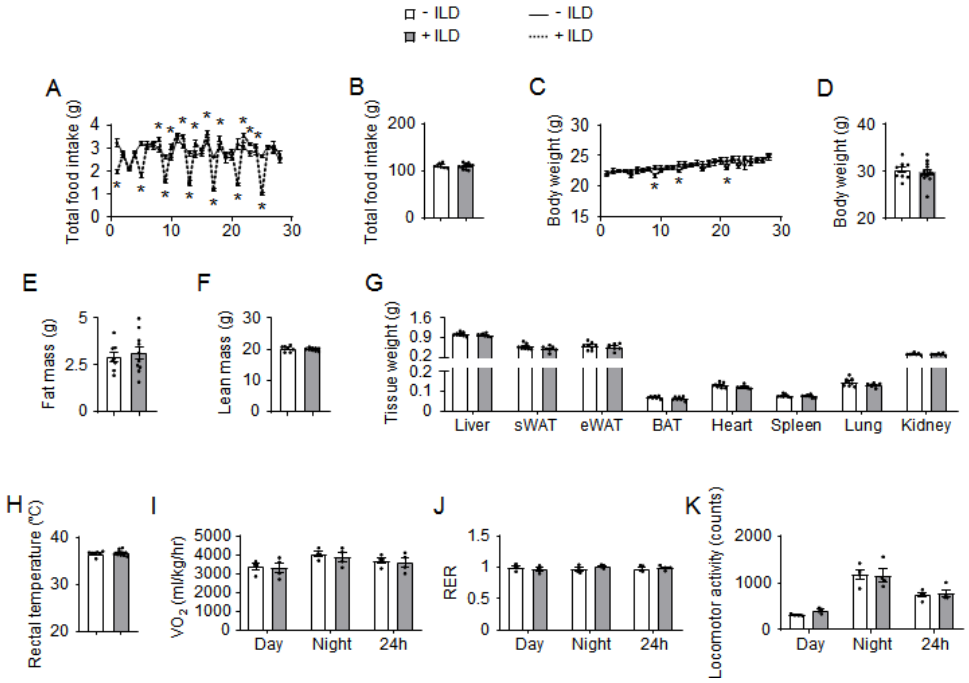
Supplementary Figure 5: ILD improves whole-body insulin sensitivity. (A) Fed and fasting blood glucose levels. (B) Fed and fasting serum insulin levels. (C) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (D) Glucose tolerance tests (GTTs); AUC: area under the curve. (E) Insulin tolerance tests (ITTs). (F) Insulin tolerance tests (ITTs). Studies for A–E were conducted using 8-week-old

male wild-type mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 7 days. Studies for F were conducted using 8-week-old male mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 84 days. Data are expressed as the mean ± SEM (n = 6–10 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.

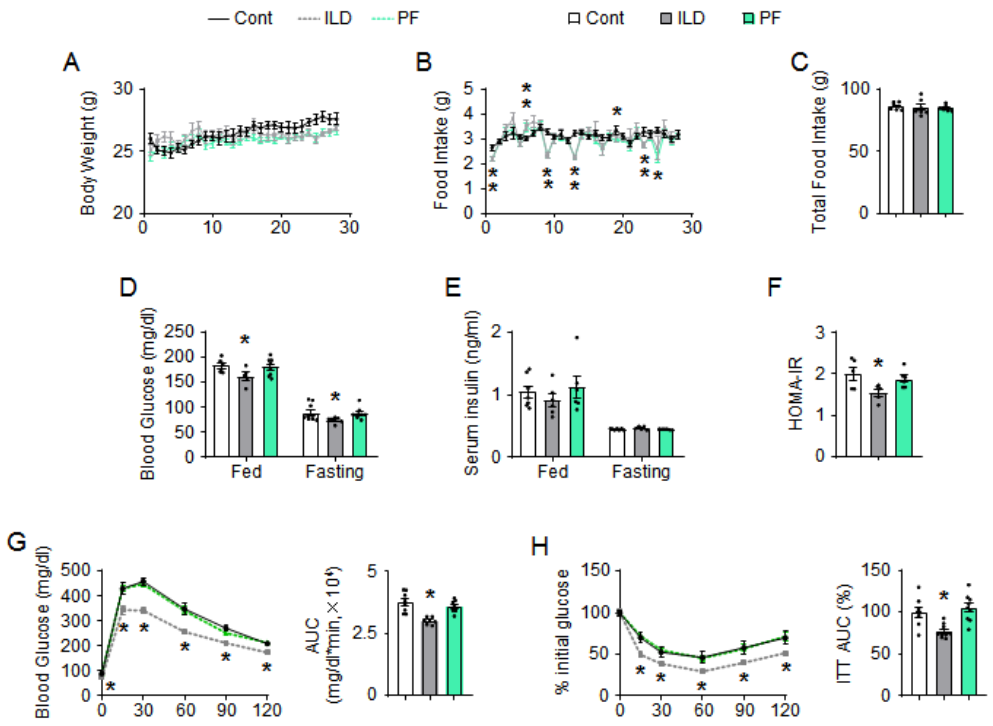


Supplementary Figure 6: **Intermittent leucine deprivation (ILD) improves long-lasting (on day 21) insulin sensitivity in female mice**. (A) Fed and fasting blood glucose levels. (B) Fed and fasting serum insulin levels. (C) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (D) Glucose tolerance tests (GTTs); AUC: area under the curve. (E) Insulin tolerance tests (ITTs). Studies were conducted using 8-week-old female wild-type mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 21 days. Data are expressed as the mean ± SEM (n = 5–8 per group, as indicated), with individual data points. *P < 0.05

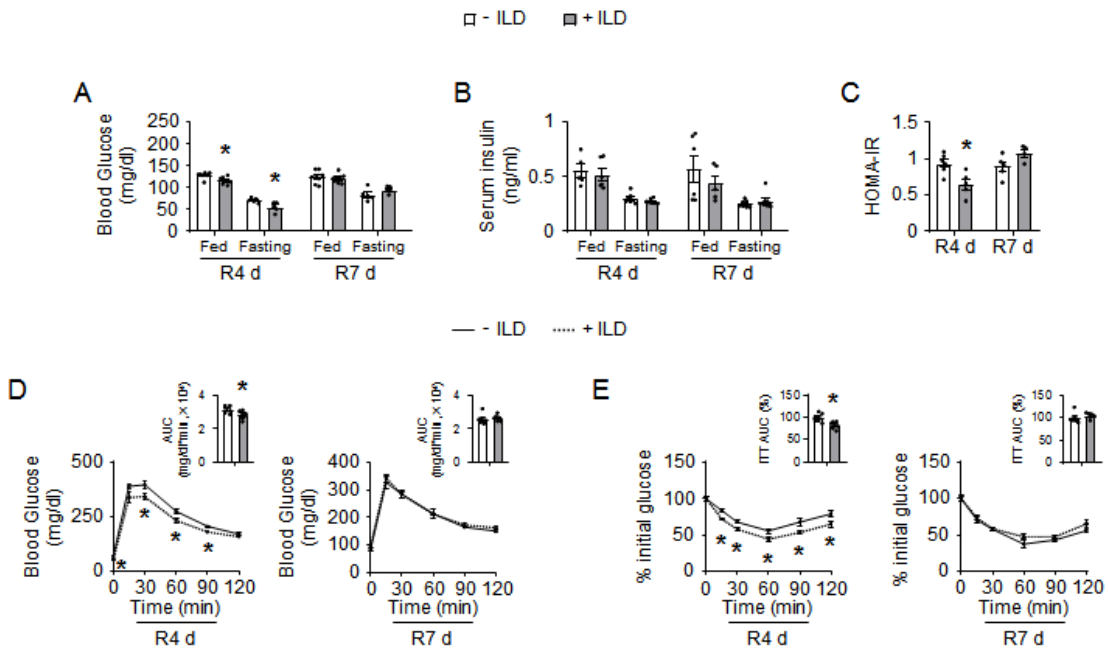
for the effect of + ILD group versus - ILD group.



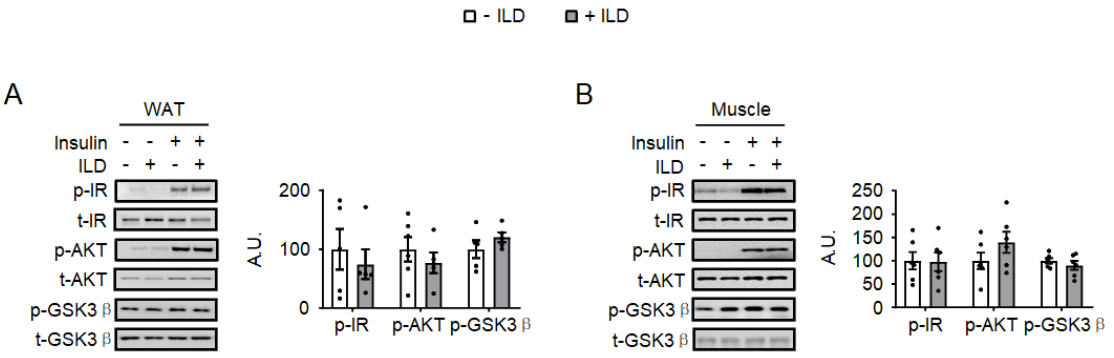
Supplementary Figure 7: Metabolic parameters of mice treated with ILD (on day 49). (A) Daily food intake. (B) Total food intake. (C) Daily body weight. (D) Body weight on day 49. (E) Fat mass. (F) Lean mass. (G) Tissue weight. (H) Rectal temperature. (I) 24-h oxygen consumption normalized by lean mass measured by the comprehensive lab animal monitoring system (CLAMS). (J) Respiratory exchange ratio (RER, V_{CO2}/V_{O2}) measured by CLAMS. (K) Locomotor activity measured by CLAMS. Studies were conducted using 8-week-old male mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 49 days. Data are expressed as the mean ± SEM (n = 4–10 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.



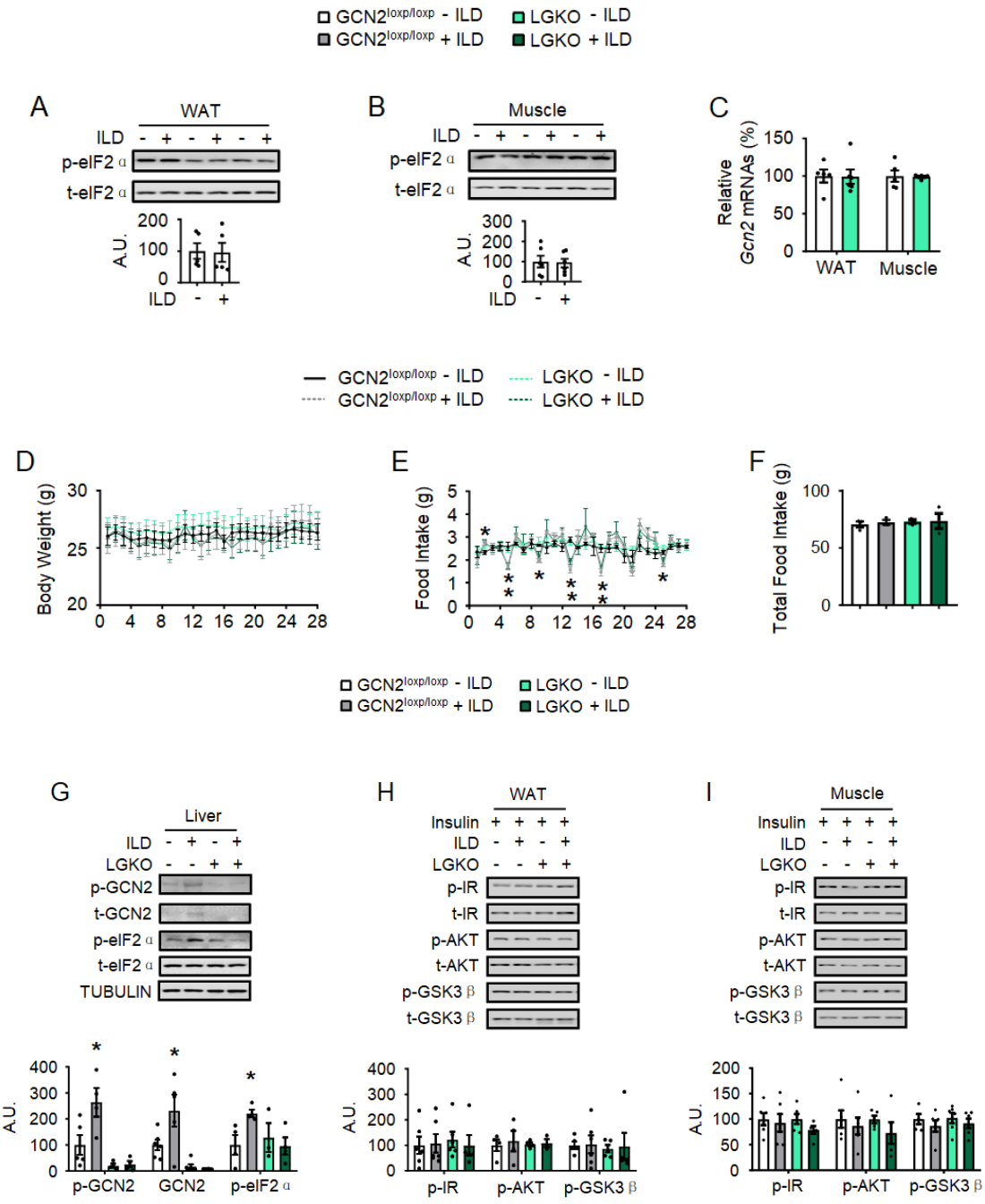
Supplementary Figure 8: Pair-feeding has no beneficial effect on improved glucose metabolism as intermittent leucine deprivation (ILD). (A) Daily body weight. (B) Daily food intake. (C) Total food intake. (D) Fed and fasting blood glucose levels. (E) Fed and fasting serum insulin levels. (F) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (G) Glucose tolerance tests (GTTs); AUC: area under the curve. (H) Insulin tolerance tests (ITTs). Studies were conducted using 8-week-old male wild-type mice receiving a control diet (Cont) or ILD diet (ILD) or pair-fed diet (PF). Data are expressed as the mean ± SEM (n = 5–9 per group, as indicated), with individual data points. *P < 0.05 for the effect of ILD group versus control group.



Supplementary Figure 9: 4 cycles of ILD has no long-lasting effect on improved whole-body insulin sensitivity. (A) Fed and fasting blood glucose levels. (B) Fed and fasting serum insulin levels. (C) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (D) Glucose tolerance tests (GTTs); AUC: area under the curve. (E) Insulin tolerance tests (ITTs). Studies were conducted using 8-week-old male wild-type mice receiving a control diet (- ILD) or four cycles of ILD diet (+ ILD) and resuming a control diet for days as indicated (R4/7 d: 4 or 7 days). Data are expressed as the mean ± SEM (n = 4–10 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.

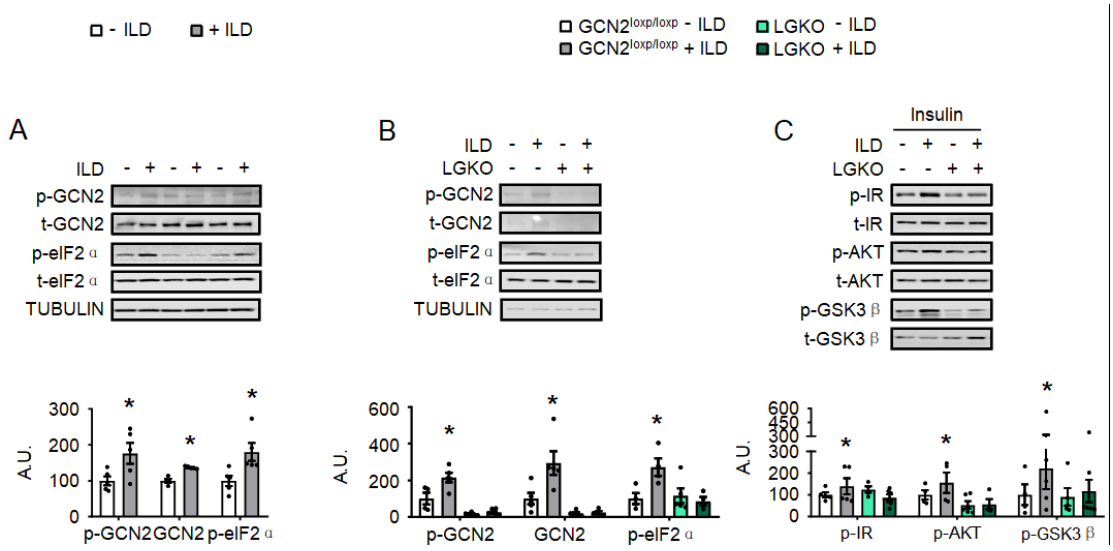


Supplementary Figure 10: ILD has no effect on insulin sensitivity of WAT and muscle. (A-B) P-IR, t-IR, p-AKT, t-AKT, p-GSK3β and t-GSK3β proteins in the WAT (A) and muscle (B) shown by western blotting (left) and quantified by densitometric analysis (right); A.U.: arbitrary units. Studies were conducted by using 8-week-old male wild-type mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 49 days. The insulin signaling pathway in the WAT and muscle was examined before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3−5 min. Data are expressed as the mean ± SEM (n = 5–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.



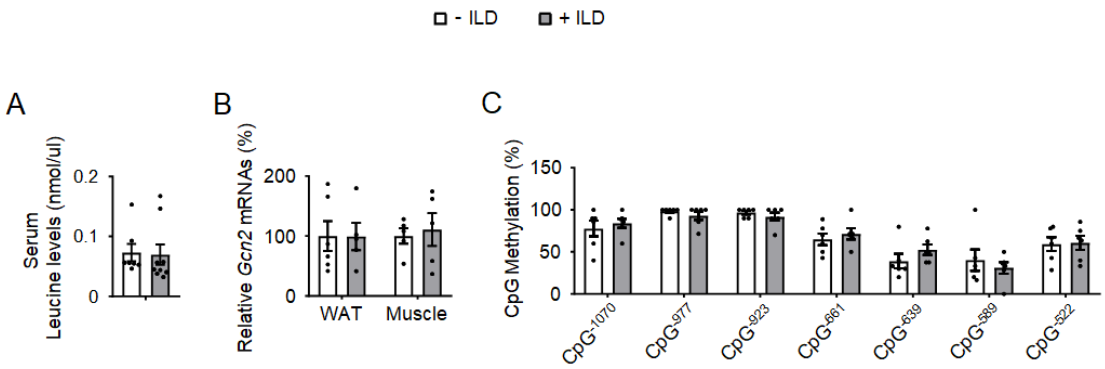
Supplementary Figure 11: Improved glucose metabolism of ILD depends on hepatic GCN2. (A–B) P-eIF2α and t-eIF2α proteins in the WAT (A) and muscle (B) shown by western blotting (top) and quantified by densitometric analysis (bottom); A.U.: arbitrary units. (C) Gene expression of *Gcn2* in the WAT and muscle by RT-PCR. (D) Daily body weight. (E) Daily food intake. (F) Total food intake. (G)

P-GCN2, t-GCN2, p-eIF2α, t-eIF2α and TUBULIN proteins in the liver shown by western blotting (top) and quantified by densitometric analysis (bottom). P-GCN2 and t-GCN2 were normalized to TUBULIN, p-eIF2α was normalized to t-eIF2α. (H–I) P-IR, t-IR, p-AKT, t-AKT, p-GSK3β and t-GSK3β proteins in the WAT (H) and muscle (I). Studies for A-B were conducted by using 8-week-old male wild-type mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 49 days; studies for C–I were conducted using 8-week-old male $GCN2^{loxp/loxp}$ mice ($GCN2^{loxp/loxp}$) or hepatic GCN2 deletion mice (LGKO) receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 49 days. Data are expressed as the mean ± SEM (n =3–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.



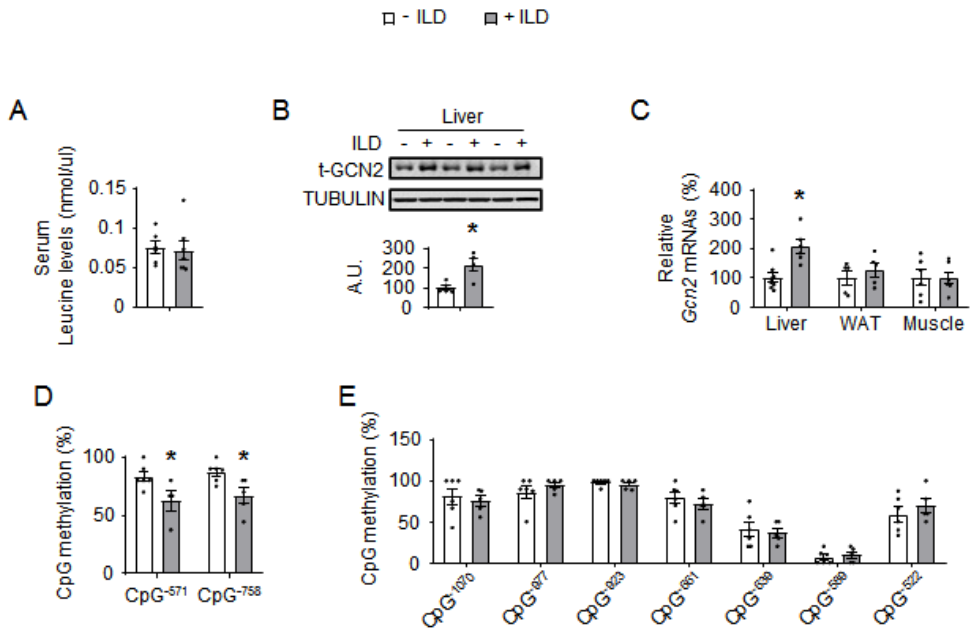
Supplementary Figure 12: Improved insulin sensitivity of ILD depends on hepatic GCN2 in vitro. (A–B) P-GCN2, t-GCN2, p-eIF2α, t-eIF2α and TUBULIN proteins in primary hepatocytes of male wild-type mice (A) and primary hepatocytes

of GCN2$^{loxp/loxp}$ and LGKO mice (B) shown by western blotting (top) and quantified by densitometric analysis (bottom); A.U.: arbitrary units. P-GCN2 and t-GCN2 were normalized to TUBULIN, p-eIF2α was normalized to t-eIF2α. (C) P-IR, t-IR, p-AKT, t-AKT, p-GSK3β and t-GSK3β proteins in primary hepatocytes shown by western blotting (top) and quantified by densitometric analysis (bottom). Studies were conducted in primary hepatocytes of male wild-type mice or GCN2$^{loxp/loxp}$ and LGKO mice treated with a control medium (- ILD) or seven cycles of 10 min leucine deprivation medium and 30 min control medium (+ ILD) followed by a control medium for 8 h and then by stimulation with 100 nM insulin for 10 min. Data are expressed as the mean ± SEM (n =3–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.

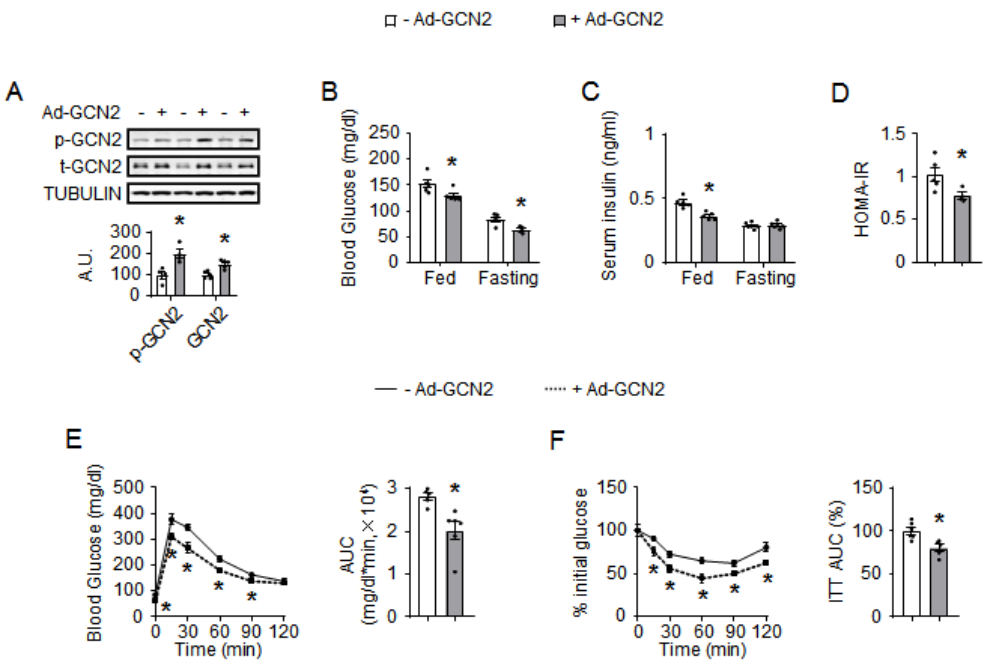


Supplementary Figure 13: ILD has no effect on *Gcn2* expression in WAT and muscle (on day 49). (A) Serum leucine levels. (B) Gene expression of *Gcn2* in the WAT and muscle by RT-PCR. (C) Methylation status of other CpG sites in the hepatic GCN2. Studies were conducted by using 8-week-old male wild-type mice

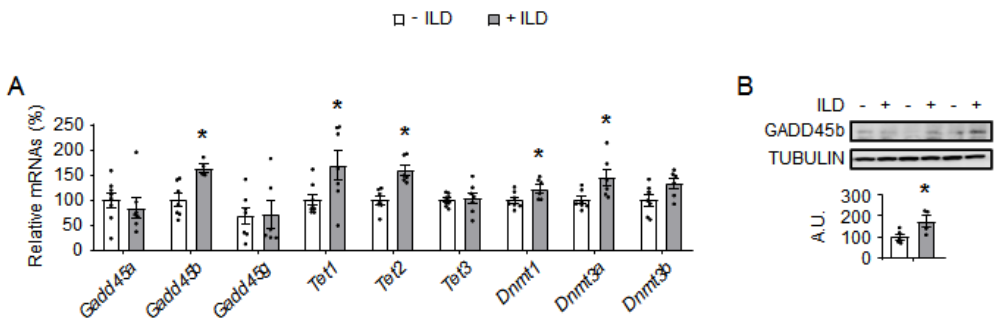
receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 49 days. Data are expressed as the mean ± SEM (n = 5–9 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.



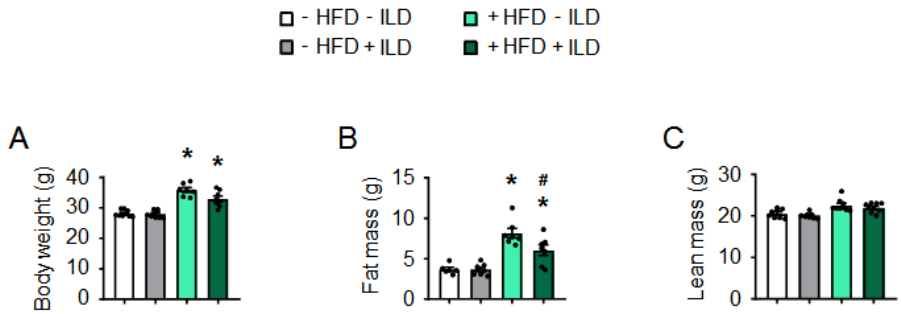
Supplementary Figure 14: ILD upregulates GCN2 expression via reducing its DNA methylation (on day 7). (A) Serum leucine levels. (B) T-GCN2 and TUBULIN proteins in liver shown by western blotting (top) and quantified by densitometric analysis (bottom); A.U.: arbitrary units. (C) Gene expression of *Gcn2* in the liver, WAT and muscle by RT-PCR. (D) Levels of CpG^{-571} and CpG^{-758} methylation discovered by BSP sequencing in the liver. (E) Methylation status of other CpG sites. Studies were conducted using 8-week-old male wild-type mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 7 days. Data are expressed as the mean ± SEM (n = 4–8 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.



Supplementary Figure 15: Over-expression of GCN2 in liver improves insulin sensitivity. (A) P-GCN2 and t-GCN2 proteins in liver shown by western blotting (top) and quantified by densitometric analysis (bottom); A.U.: arbitrary units. (B) Fed and fasting blood glucose levels. (C) Fed and fasting serum insulin levels. (D) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (E) Glucose tolerance tests (GTTs); AUC: area under the curve. (F) Insulin tolerance tests (ITTs). Studies were conducted using 8-week-old male wild-type mice receiving adenovirus expressing GFP (-Ad-GCN2) or GCN2 (+Ad-GCN2) by caudal vein. Data are expressed as the mean ± SEM (n =4–6 per group, as indicated), with individual data points. *P < 0.05 for the effect of + Ad-GCN2 group versus - Ad-GCN2 group.

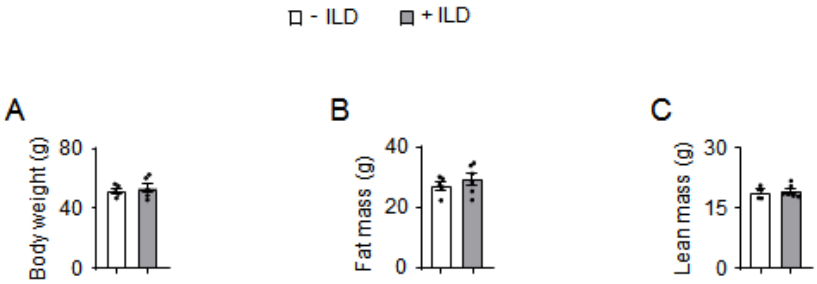


Supplementary Figure 16: Expression of demethylases and methylases in liver of ILD mice (on day 7). (A) Gene expression of *Gadd45a, Gadd45b, Gadd45g, Tet1, Tet2, Tet3, Dnmt1*、*Dnmt3a*、*Dnmt3b* in the liver by RT-PCR. (B) GADD45b, TUBULIN proteins in the liver shown by western blotting (top) and quantified by densitometric analysis (bottom); A.U.: arbitrary units. Studies were conducted using 8-week-old male wild type mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 7 days. Data are expressed as the mean ± SEM (n = 4–8 per group, as indicated), with individual data points. *P < 0.05 for the effect of + ILD group versus - ILD group.



Supplementary Figure 17: The effect of ILD on body composition in HFD mice. (A) Body weight. (B) Fat mass. (C) Lean mass. Studies were conducted using

4-week-old male wild-type mice receiving an 8-week control diet or high-fat diets (HFD) in advance. The mice treated with control diet were then treated with either a control diet (- HFD - ILD) or ILD diet (- HFD + ILD) followed by a control diet for 49 days. The mice treated with the HFD (+ HFD - ILD) were then treated with either a HFD or a HFD without leucine (- leu HFD) in a manner as ILD (+ HFD + ILD) followed by a HFD diet for 49 days. Data are expressed as the mean ± SEM (n = 6–9 per group, as indicated), with individual data points. *P < 0.05 for the effect of any group versus - HFD - ILD group. #P < 0.05 for the effect of any group versus + HFD - ILD group.



Supplementary Figure 18: ILD has no effect on body composition in *db/db* mice. (A) Body weight. (B) Fat mass. (C) Lean mass. Studies were conducted using 8-week-old male *db/db* mice receiving a control diet (- ILD) or ILD diet (+ ILD) followed by a control diet for 49 days. Data are expressed as the mean ± SEM (n = 5–6 per group, as indicated), with individual data points.

Supplementary Table 1. Diet composition used in current study

Ingredient (gm）	control diet	(-) leu diet	(-) thr diet	(-) val diet	HFD	(-) leu HFD
L-Arginine	10	10	10	10	10	10
L-Histidine-HCl-H2O	6	6	6	6	6	6
L-Isoleucine	8	8	8	8	8	8
L-Leucine	12	0	12	12	12	0
L-Lysine-HCl	14	14	14	14	14	14
L-Methionine	6	6	6	6	6	6
L-Phenylalanine	8	8	8	8	8	8
L-Threonine	8	8	0	8	8	8
L-Tryptophan	2	2	2	2	2	2
L-Valine	8	8	8	0	8	8
L-Alanine	10	10	10	10	10	10
L-Asparagine-H2O	5	5	5	5	5	5
L-Aspartate	10	10	10	10	10	10
L-Cystine	4	4	4	4	4	4
L-Glutamic Acid	30	30	30	30	30	30
L-Glutamine	5	5	5	5	5	5
Glycine	10	10	10	10	10	10
L-Proline	5	5	5	5	5	5
L-Serine	5	5	5	5	5	5
L-Tyrosine	4	4	4	4	4	4
Total L-Amino Acids	170	158	158	158	170	158
Corn Starch	550.5	562.5	558.5	558.5	102.3	114.3
Maltodextrin 10	125	125	125	125	125	125
Cellulose	50	50	50	50	50	50
Corn Oil	50	50	50	50	18	18
Hygrogenated Coconut Oil	0	0	0	0	229	229
Mineral Mix S10001	35	35	35	35	35	35
Sodium Bicarbonate	7.5	7.5	7.5	7.5	7.5	7.5
Vitamin Mix V10001	10	10	10	10	10	10
Choline Bitrartrate	2	2	2	2	2	2
Red Dye, FD&C #40	0	0.025	0.025	0.025	0.05	0.025
Blue Dye, FD&C #1	0.05	0	0	0	0	0.025
Yellow Dye, FD&C #5	0	0.025	0.025	0.025	0	0
Total	1000.05	1000.05	1000.05	1000.05	753.85	753.85

Supplementary Table 2. Primers used for gene amplification.

Gene	Direction	Primer sequence 5'→3'
GCN2	F	CCTGCACCATGAGAACATTG
	R	CTGCCCAGTTCTTCAGTGT
Gadd45a	F	GCTACTGGAGAACGACAAGAG
	R	CCATTGTGATGAATGTGGGTTC
Gadd45b	F	GCTGGCCATAGACGAAGAAG
	R	GCCTGATACCCTGACGATGT
Gadd45c	F	GTCTACGAGTCAGCCAAAGTC
	R	AAAGCCTGGATCAGCGTAAAAT
Tet1	F	GAGCCTGTTCCTCGATGTGG
	R	CAAACCCACCTGAGGCTGTT
Tet2	F	AACCTGGCTACTGTCATTGCTCCA
	R	ATGTTCTGCTGGTCTCTGTGGGAA
Tet3	F	TCCGGATTGAGAAGGTCATC
	R	CCAGGCCAGGATCAAGATAA
Dnmt1	F	AAAGTGTGATCCCGAAGATCAAC
	R	TGGTACTTCAGGTTAGGGTCGTCTA
Dnmt3a	F	TGCTACATGTGCGGGCATAA
	R	GGAGTCGAGAAGGCCAGTCTT
Dnmt3b	F	CCCAAGTTGTACCCAGCAATTC
	R	TGCAATTCCATCAAACAGAGACA