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Hyper-Variability in Circulating Insulin, High Fat Feeding Outcomes, and Effects of Reducing *Ins2* Dosage in Male *Ins1*-Null Mice in a Specific Pathogen-Free Facility

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Abstract

Insulin is an essential hormone with key roles in energy homeostasis and body composition. Mice and rats, unlike other mammals, have two insulin genes: the rodent-specific *Ins1* gene and the ancestral *Ins2* gene. The relationships between insulin gene dosage and obesity has previously been explored in male and female $Ins2^{-/-}$ mice with full or reduced *Ins1* dosage, as well as in female $Ins1^{-/-}$ mice with full or partial *Ins2* dosage. We report herein unexpected hyper-variability in *Ins1*-null male mice, with respect to their circulating insulin levels and to the physiological effects of modulating *Ins2* gene dosage. Two large cohorts of $Ins1^{-/-}$:$Ins2^{+/-}$ mice and their $Ins1^{-/-}$:$Ins2^{+/+}$ littermates were fed chow diet or high fat diet (HFD) from weaning, and housed in specific pathogen-free conditions. Cohort A and cohort B were studied one year apart. Contrary to female mice from the same litters, inactivating one *Ins2* allele on the complete *Ins1*-null background did not consistently cause a reduction of circulating insulin in male mice, on either diet. In cohort A, all HFD-fed males showed an equivalent degree of insulin hypersecretion and weight gain, regardless of *Ins2* dosage. In cohort B the effects of HFD appeared generally diminished, and cohort B $Ins1^{-/-}$:$Ins2^{+/-}$ males showed decreased insulin levels and body mass compared to $Ins1^{-/-}$:$Ins2^{+/+}$ littermates, on both diets. Although experimental conditions were consistent between cohorts, we found that HFD-fed $Ins1^{-/-}$:$Ins2^{+/-}$ mice with lower insulin levels had increased corticosterone. Collectively, these observations highlight the phenotypic characteristics that change in association with differences in circulating insulin and *Ins2* gene dosage, particularly in male mice.

Introduction

Variations in circulating insulin levels have far-reaching metabolic consequences. In addition to the expected fluctuations in insulin secretion that are associated with blood glucose, circulating insulin levels are affected by a number of hormones and circulating factors, including

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amino acids, fatty acids, estrogen, melatonin, leptin, growth hormone, glucose-dependent insulinotropic polypeptide, and glucagon-like peptide-1 [1]. In mice, the mean 5-h fasted insulin of non-obese, 12 week-old males ranges from 0.5 to 1.2 ng/mL across four commonly used strains [2]. In humans, fasting insulin levels can range from 0.04 to 3.43 ng/mL in a nondiabetic adult population [3,4], and evidence suggests that less than half of the variance in fasting insulin can be can be accounted for by genetic variability [5,6].

Mice and rats have two non-allelic insulin genes, with a rodent-specific *Ins1* gene that likely arose from the transposition of a reverse-transcribed, partially processed mRNA of the ancestral *Ins2* [7]. *Ins1* and *Ins2* genes reside on different chromosomes in mice [8]. While *Ins1* lacks one of the two introns found in *Ins2*, the murine *Ins* genes share high homology up to 500 base pairs preceding the transcription initiation site [9]. However, *Ins1* and *Ins2* have distinct promoter elements, tissue- and temporal-specific expression patterns, and imprinting status [10–14]. In addition, differential translation or processing rates of the two murine preproinsulins have been reported [15,16]. It is therefore possible that levels of the fully processed murine insulin 1 and insulin 2 peptides are divergently susceptible to modulation under various conditions, which could underlie the evolutionary retention of both genes [17]. When one insulin gene is inactivated, elevated transcript and protein level of the non-deleted insulin gene can at least partially compensate for the loss [18], although the exact nature of this reciprocal relationship remains understudied.

We performed a series of investigations to examine how murine *Ins1* and *Ins2* gene dosage impacts the onset of high fat diet-induced hyperinsulinemia and the development of obesity. Previous work in our laboratory showed that reducing *Ins1* gene dosage (on an *Ins2*-null background) resulted in continuous suppression of fasting hyperinsulinemia in male mice, thereby preventing diet-induced obesity [14]. Interestingly, circulating insulin levels were not similarly modulated in the female littermates from this study [14], suggesting the possibility of sex-specific differences in the relationship between insulin gene dosage and circulating insulin levels. In the converse genetic manipulation, reduced *Ins2* dosage (on an *Ins1*-null background) led to high-fat fed female $Ins1^{-/-}$:$Ins2^{+/-}$ mice having lower insulin secretion than their $Ins1^{-/-}$:$Ins2^{+/+}$ controls at a young age, which again corresponded with attenuated obesity [19]. The phenotype of the female $Ins1^{-/-}$:$Ins2^{+/-}$ mice was highly consistent between the two large cohorts of animals studied under specific pathogen-free (SPF) conditions, and was congruent to preliminary evaluations in a conventional facility.

We report herein on circulating insulin levels and the metabolic phenotype of the male $Ins1^{-/-}$:$Ins2^{+/-}$ and $Ins1^{-/-}$:$Ins2^{+/+}$ littermates of the female mice that were the subject of our recent investigation [19]. Contrary to our expectations, inactivating one *Ins2* allele did not consistently cause a reduction of circulating insulin in *Ins1*-null male mice, which precluded us from properly testing the hypothesis that reduced *Ins2* dosage and lower insulin levels would lead to protection from obesity in males. We found that there were discrepancies between cohorts with respect to the effects of high fat feeding on glucose homeostasis, insulin sensitivity, and the weight gain experienced by $Ins1^{-/-}$:$Ins2^{+/-}$ versus $Ins1^{-/-}$:$Ins2^{+/+}$ male mice. Moreover, circulating insulin levels were hyper-variable across cohorts in *Ins1*-null male mice, pointing to sex-specific compensation of insulin homeostasis in these animals. Alongside our previous published work [14,19], this report illustrates that the physiological outcomes of *Ins2* haploinsufficiency in male *Ins1*-null mice are dramatically variable relative to female littermates, and relative to mice with reduced *Ins1* gene dosage on an *Ins2*-null background. This phenotypic hyper-variability is evident despite the controlled environment of a SPF facility.

Materials and Methods

Experimental Animals, Body Composition and Metabolic Cage Experiments

All animal procedures were approved by the University of British Columbia Animal Care Committee (animal care certificates A11-0390 and A14-0197), following Canadian Council for Animal Care guidelines. Mice with *Ins1*-null and *Ins2*-null alleles were generated previously [20] and were roughly equal parts C57BL/6 and 129 background. Data presented here are from the male littermates of previously described $Ins1^{-/-}$:$Ins2^{+/+}$ and $Ins1^{-/-}$:$Ins2^{+/-}$ females [19], and were therefore collected in the same time-frames and conditions. All animals were predominately handled by the same female researcher. The mice tracked across time were in two major cohorts, born a year apart (cohort A, born October 2011 –December 2011, and cohort B, born October 2012 –February 2013; Fig 1A). The dams and sires of cohort B experimental mice were themselves born of parents from cohort A stock, thus minimizing the chance of significant genetic drift, and the same room was used for breeding across both cohorts. Two discrete groups of animals (25 week-old mice, born April 2014, and 70 week-old mice, born April 2012) were used for some terminal experiments, such as islet-level analyses. Shortly after weaning, all experimental mice were assigned a moderate-energy chow diet (CD, 25% fat content; LabDiet 5LJ5; PMI Nutrition International, St. Louis, MO, USA) or a high-energy high fat diet (HFD, 58% fat content; Research Diets D12330; Research Diets, New Brunswick, NJ, USA) provided *ad libitum*, except during fasting periods (Fig 1A). Diet assignments were distributed within each litter, based on approximately matching starting body weights between diet groups. Mice were housed under SPF conditions at 21°C, on a 12:12 h light:dark cycle, in the same room for both cohorts. Cages were always enriched with crinkle paper, nesting material, and plastic huts. Unlike their female littermates [19], the vast majority of male mice from both cohorts were individually housed, due to fighting between young cage-mates.

After weaning, all mice were weighed weekly, although a subset of pups from cohort B was also weighed prior to weaning. *In vivo* body composition was periodically assessed in cohort B mice using dual-energy X-ray absorptiometry (DEXA; Lunar PIXImus densitometer; GE Medical Systems LUNAR, Madison, WI, USA). A subset of 17 week-old HFD-fed mice from cohort B was evaluated in PhenoMaster metabolic cages (TSE Systems, Chesterfield, MO, USA) as described [21] after individual housing for at least a week, and acclimation to the metabolic cage environment for at least four days. Values were averaged from 48–84 h of continual data acquisition.

Glucose Homeostasis and Plasma Analytes

Mice were fasted for 4 h in fresh, clean cages during the light period to ensure a postprandial state for all blood sampling. Fasting and glucose-stimulated (2 g/kg) insulin secretion was assessed, as well as blood glucose response to intraperitoneal delivery of glucose (2 g/kg) or an insulin analog (0.75 U/kg of Humalog; Eli Lilly, Indianapolis, IN, USA). OneTouch Ultra2 glucose meters (LifeScan Canada Ltd, Burnaby, BC, Canada) were used for blood glucose assessments, and plasma insulin was measured with a mouse insulin ELISA (Alpco Diagnostics, Salem, NH, USA), according the manufacturers' instructions. Area over the curve or area under the curve were calculated to evaluate statistical differences in these glucose-stimulated or insulin-stimulated tests. Corticosterone was measured in plasma collected in the early afternoons across multiple weeks for each cohort, and assessed using a mouse/rat corticosterone ELISA (Alpco Diagnostics) according the manufacturers' instructions. In plasma from 40 week-old cohort B mice, we used colorimetric kits to measure total cholesterol (Cholesterol E

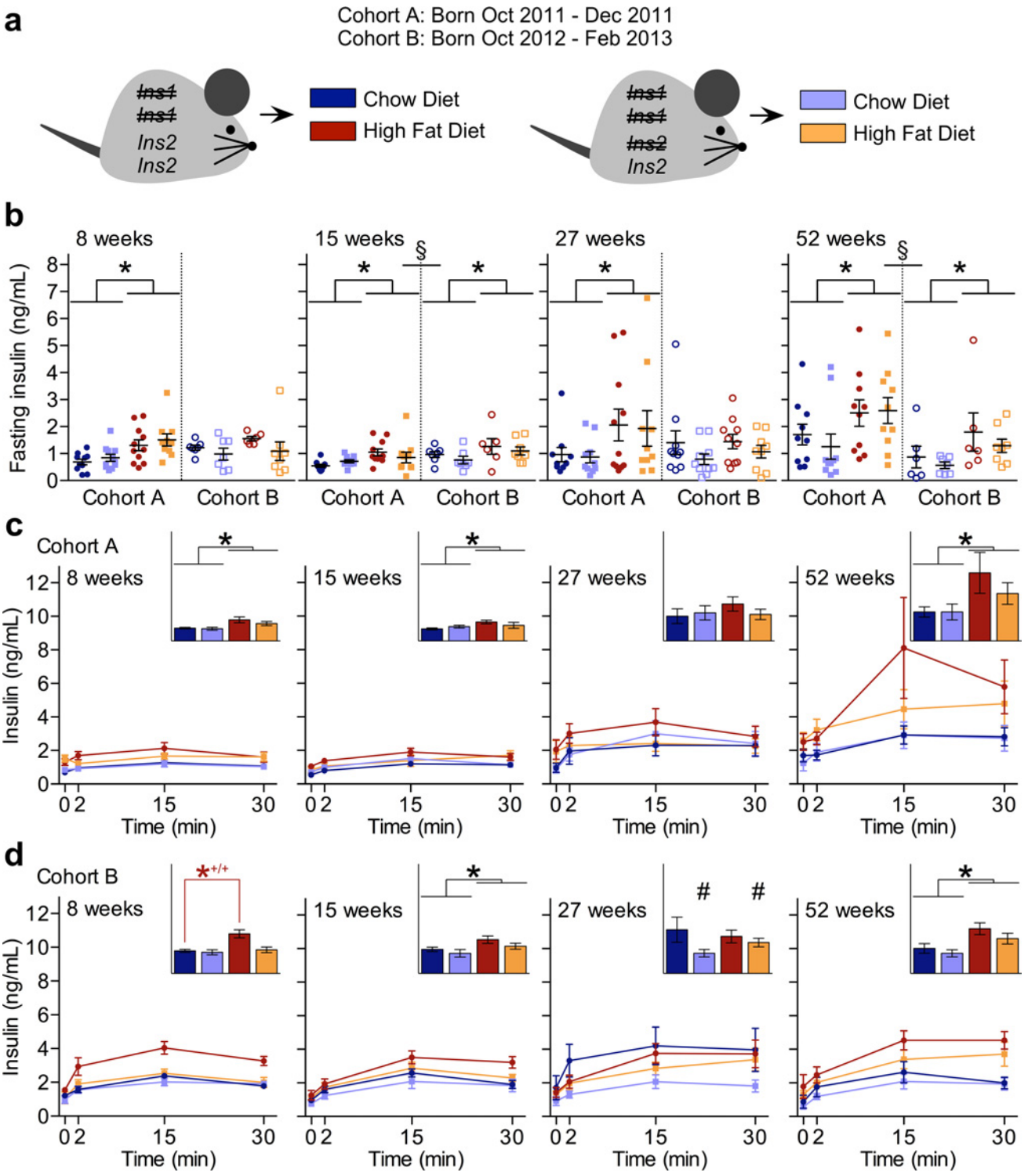

Fig 1. Hyper-variability in Insulin Secretion Across Two Cohorts. (**a**) Experimental design showing two cohorts (A and B) of *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male littermates fed chow diet (CD) or high fat diet (HFD). (**b**) Periodic measurements of 4-h fasted insulin levels is shown for both cohorts, with scatter points to indicate individual values (cohort A: closed points, n = 10–11; cohort B: open points, n = 6–10). In addition, periodic measurements of glucose-stimulated insulin secretion in (**c**) cohort A (n = 10–11) and (**d**) cohort B (n = 6–8) is shown, with area under the curve (y-axis units of ng/mL•min) in panel insets. Data are means ± SEM. Dark blue and red represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, *$^{+/+}$ for CD- vs HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice, # for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$, § for cohort A vs cohort B.

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kit; Wako Chemicals, Richmond, VA, USA), triglycerides (Serum Triglyceride kit; Sigma-Aldrich, St Louis, MO, USA), and non-esterified fatty acid levels (NEFA-HR[2] kit; Wako Chemicals), in addition to a mouse magnetic bead panel assay utilizing Luminex technology (Millipore, St. Charles, MO, USA) to evaluate leptin, resistin, interleukin 6, glucose-dependent insulinotropic polypeptide, peptide YY, and glucagon levels, in accordance with manufacturers' instructions.

Islet Analyses

Islet *Ins2* mRNA, insulin content, and function were assessed in mice that were discrete from the other experimental groups, as the A and B cohorts were assessed longitudinally. 25 week-old mice were humanely euthanized via CO_2 and cervical dislocation after 4 h of fasting in the light period, and HFD-fed 70 week-old mice were similarly euthanized after a minimum of 1 h of fasting. After pancreatic tissue was removed, wet masses of the inguinal, epigonadal, and mesenteric white adipose tissue depots, liver, interscapular brown adipose tissue, and the triceps surae mixed muscle group (comprised of the gastrocnemius, plantaris, and solaris muscles) were measured from 25 week-old mice. Islets were isolated with collagenase and filtration, using a previously described protocol [22] with minor modifications. Islets were handpicked using brightfield microscopy, and cultured at 37°C and 5% CO_2 for at least 16 h prior to any analyses, in RPMI-1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 11 mM glucose, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% (volume/volume) fetal bovine serum. Islet perifusion experiments were carried out as previously described [23], using groups of 150 size-matched islets and evaluating stimulatory conditions of 15 mM glucose or 30 mM KCl. For analyzing secretory responses, phase I was defined as the initial 10 minutes of stimulation, including the immediate peak in insulin release (transient rise and then decline), and phase II was defined as the subsequent 20 min, consisting of the prolonged period of insulin secretion until the stimulation was removed. Islet insulin content were averaged from 10 size-matched islets per mouse, incubated at -20°C in 0.02 M HCl in 70% ethanol, and sonicated a minimum of 30 s before dilution for measurement with a mouse insulin ELISA kit (Alpco Diagnostics).

Quantitative reverse transcription PCR was used for the relative quantification of *Ins2* in islets, normalized against the reference gene ß-actin. Total RNA was extracted from islets using the Qiagen RNeasy Mini kit (Qiagen, Mississauga, ON, Canada), according to manufacturers' instructions. RNA was quantified at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and cDNA was generated using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Transcript levels were measured with Taqman assays (*Ins2*: forward primer 5'-GAAGTGGAGGACCCACAAGTG-3', reverse primer 5'-GATCTACAATGCCACGCTTCTG-3'; Integrated DNA Technologies, Toronto, ON, Canada; ß-actin: assay catalog number 4352341E, Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate on a StepOnePlus real-time PCR system (Applied Biosystems), using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and a fast-mode thermal program consisting of a 20-s activation at 95°C, then 40 cycles of 95°C melting for 1 s and 60°C annealing for 20 s. Values were normalized using the $2^{-\Delta Ct}$ method.

Statistical Analyses

Statistical analyses were performed with SPSS 15.0 software, and a critical α-level of $p \leq 0.05$. For most analyses, we used two-way analysis of variance (ANOVA) models within each cohort to assess factors of genotype and diet, and a significant interaction led to one-way ANOVAs

comparing HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice, CD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice, HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice, and CD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice, with Bonferroni corrections. Three-way ANOVAs were used for incorporating the additional factor of cohort or parental effect, and 2-tailed independent *t*-tests were used to assess differences if there were only two groups to be compared. Analysis of covariance was employed to test energy expenditure with covariates of lean and fat mass. In all cases, we used Levene's test to evaluate the assumption of homogeneity of variance, and where the assumption was violated, logarithmic transformations were applied, generally stabilizing data variance. Graphpad Prism 6.0 software was used to generate and assess the linear regressions.

Results

Variability in Insulin Secretion Between Cohorts of Male Mice

We have recently studied the effects of reducing *Ins2* gene dosage in *Ins1*-null female mice, and found consistent outcomes for insulin secretion and body composition across cohorts [19]. While characterizing male siblings from the same cohorts, we noticed that a number of measured parameters showed dramatic inconsistencies between cohorts A and B, precluding us from pooling the data from these two cohorts. For instance, we observed that in cohort A males, fasting insulin was significantly higher in HFD-fed mice than CD-fed mice at all measured time points across a year (Fig 1B), and glucose-stimulated insulin secretion was higher at 8, 15, and 52 weeks of age (Fig 1C). Unexpectedly, there were no significant differences on either diet in circulating insulin levels (either fasting or glucose-stimulated) between *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice, at any point up to one year in cohort A (Fig 1B and 1C). In contrast, cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice tended to have lower fasting insulin levels than their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermates (Fig 1B), and at 27 weeks of age they had significantly reduced glucose-stimulated insulin secretion (Fig 1D). In addition, *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice were the only cohort B males showing more glucose-stimulated insulin secretion on HFD than CD at the early 8-week time point (Fig 1D). However, the HFD-induced elevation of basal and glucose-stimulated insulin secretion was, in general, quite modest for most males in cohort B (Fig 1B and 1D). Notably, by 52 weeks all groups of cohort B male mice clearly had lower average insulin levels than cohort A males (Fig 1B).

To evaluate potential mechanisms underlying cross-cohort changes in circulating insulin levels in male mice, we assessed insulin mRNA, protein, and islet function in two distinct groups of mice. Due to the longitudinal nature of the experiments, we could not use islets from cohort A or B mice. However, a separate group of 25 week-old male mice resembled cohort B males in that they tended to show a genotype effect for *in vivo* fasting insulin ($p = 0.056$), as well as similar raw insulin values to those of cohort B mice (Figs 1B and 2A). Islets from these 25 week-old *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice had an expected reduction in *Ins2* mRNA compared to *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ islets (Fig 2B). Interestingly, the significant reduction in *Ins2* mRNA did not correspond to significant genotype differences in islet insulin protein content (Fig 2C), suggesting the involvement of post-transcriptional compensation. Consistent with the lack of a difference between genotypes for islet insulin content, dynamic secretion by islets from 25 week-old HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice was not reduced compared to *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ islets, and in fact 25 week-old *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ islets appeared to have the capacity for a marginally increased second-phase response to KCl stimulation (Fig 2D).

We also evaluated 70 week-old islets from a group of HFD-fed mice (body masses: *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ 40.5 ± 2.4, *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ 38.6 ± 2.4; $p = 0.60$) that had shown no obvious genotype differences in fasting insulin levels at 52 weeks of age (Fig 2E), similar to cohort A males. This allowed us to confirm that the genetic manipulation also led to reduced *Ins2* mRNA in older HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice (Fig 2F), with a comparable capacity for compensation at the

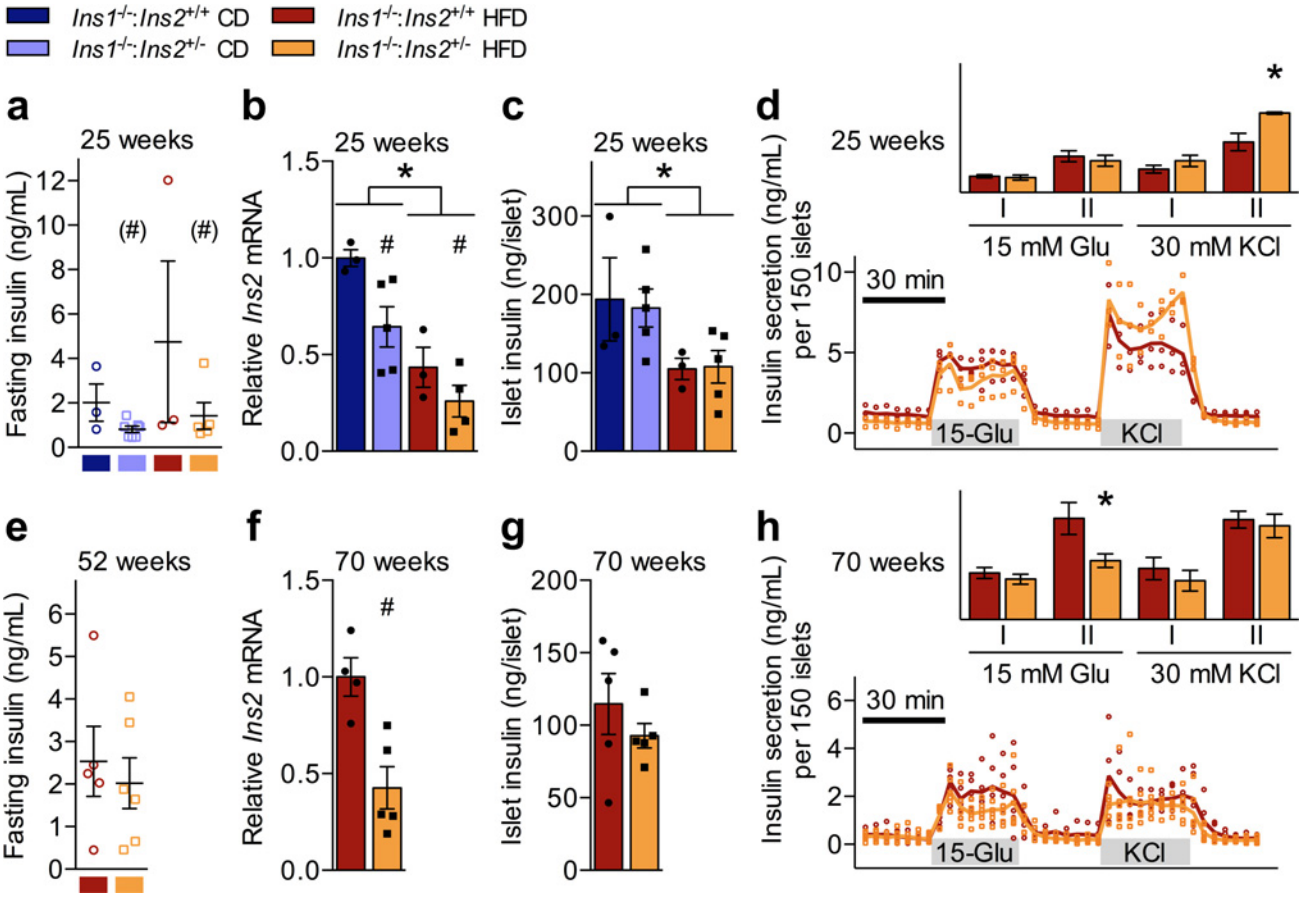

Fig 2. Characterization of *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ Islets. Separate groups of male mice (discrete from cohorts A and B) were used for islet analyses. (**a**) *In vivo* 4-h fasted insulin is shown from 25 week-old mice prior to collection of islets to assess (**b**) *Ins2* mRNA, corrected against ß-actin and normalized to CD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice, (**c**) islet insulin content, and (**d**) insulin secretion by 150 islets perifused with 3 mM glucose (basal), 15 mM glucose (Glu), and 30 mM KCl, with area under the curve (y-axis units of ng/mL•min) depicted for phases I/II of glucose and KCl stimulation, minus basal secretion. (**e**) *in vivo* 4-h fasted insulin is shown from 52 week-old mice prior to collection of islets at 70 weeks to assess (**f**) *Ins2* mRNA, corrected against ß-actin and normalized to HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice, (**g**) islet insulin content, and (**h**) insulin secretion by 150 islets perifused with 3 mM glucose (basal), 15 mM glucose (Glu), and 30 mM KCl, with area under the curve (y-axis units of ng/mL•min) depicted for phases I/II of glucose and KCl stimulation, minus basal secretion. n = 3–7. Data are means ± SEM, with scatter points to indicate individual values. Dark blue and red represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, # for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$. (#) indicates $p = 0.056$ for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$.

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level of islet insulin protein content (Fig 2G) as was evident at 25 weeks (Fig 2C). The only detected differences in dynamic *in vitro* islet secretion were minimal, showing that at 70 weeks, *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ islets did not secrete quite as much insulin in the second phase of glucose stimulation as *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ islets (Fig 2H). Collectively, these data show that although the experimental genetic manipulation did successfully reduce *Ins2* expression in the male mice, there was evidence of compensation with respect to insulin protein content and islet secretory capacity. The capability for insulin production and/or secretory compensation may have accounted for the lack of consistent differences in circulating insulin levels between *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice.

Variability in Glucose Homeostasis Between Cohorts of Male Mice

We also observed heterogeneity between cohorts in a longitudinal analysis of glucose homeostasis, as might be expected from the cross-cohort variability in insulin levels. In conjunction

with the sustained HFD-induced elevation of circulating insulin in cohort A mice, from 14 weeks onwards HFD-fed males from cohort A showed significant whole-body insulin resistance compared to their CD-fed littermates (Fig 3A). HFD-fed mice in cohort A also had a modest degree of glucose intolerance compared to mice on CD, at 8 and 50 weeks (Fig 3B). In contrast, there were no statistically significant differences in insulin sensitivity or glucose tolerance observed between CD- and HFD-fed groups of cohort B mice, consistent with a limited response to HFD-feeding (Fig 3C and 3D).

Reduced *Ins2* dosage and the slight differences in circulating insulin levels observed between *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ cohort B mice did not appear to cause robust negative repercussions for glucose homeostasis (Fig 3C and 3D). However, in cohort A, *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice were slightly but significantly more glucose intolerant than their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermate controls at each measured time point across a year. Closer examination of the responses to intraperitoneal glucose stimulation shows a trend for a delayed or sustained peak in blood glucose in cohort A *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice (Fig 3B). This suggests that although cumulative insulin levels appeared nearly matched between genotypes in cohort A, at least across the first 30 minutes post-stimulation (Fig 1B and 1C), subtle differences in insulin secretory patterns could have affected glycemic control (*e.g.* Fig 2H; although *in vitro* insulin secretion was assessed in a discrete group of mice, they appeared phenotypically similar to cohort A males). Indeed, in addition to the long-term elevation of fasting glucose evident in HFD-fed mice compared to CD-fed mice in cohort A, there were periods where cohort A *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males had higher fasting glucose levels than their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermates (Fig 4A). On the other hand, all groups of cohort B mice had largely comparable fasting glucose levels throughout the year (Fig 4B).

Heterogeneity Between Cohorts in Body Mass of *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ Male Mice

Perhaps one of the most striking differences noted between cohorts A and B was the differential impact of reducing *Ins2* gene dosage on body mass, particularly in HFD-fed mice. The loss of one *Ins2* allele did not notably affect either circulating insulin levels (Fig 1B and 1C) or HFD-induced growth (Fig 4C) in cohort A males, since all HFD-fed mice showed equivalent weight gain compared to CD-fed littermates, particularly from 20 weeks onward (Fig 4C). Although young *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice were smaller than their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermates for a limited period in cohort A, this did not persist (Fig 4C). HFD-fed mice were heavier than CD-fed mice for a similar duration in cohort B as in cohort A, but the significantly smaller mass of cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice compared to their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermates continued throughout most of the year (Fig 4D). Notably, a difference in body mass between *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice of cohort B was detectable in male pups as young as 2 days of age (Fig 4E), and it is possible that there could have also been similar size differences in neonatal pups of cohort A (not measured) that might have contributed to the genotype effect on body mass observed in young cohort A mice (Fig 4C).

We attempted to evaluate different factors that could have contributed to phenotypic differences between cohorts A and B. We did not detect obvious means by which parental imprinting of the *Ins2* allele might have accounted for the observed variability, as both breeding pair options (*i.e.* either the dam or the sire donating the disrupted *Ins2* allele) contributed to cohort A and B experimental animals. There were no statistically significant differences between offspring of dams versus sires with the disrupted *Ins2* allele when the factor of "parental effect" was incorporated into analyses of body mass or fasting insulin levels (with both cohorts pooled); any patterns suggestive of a possible parental effect in one cohort were either not

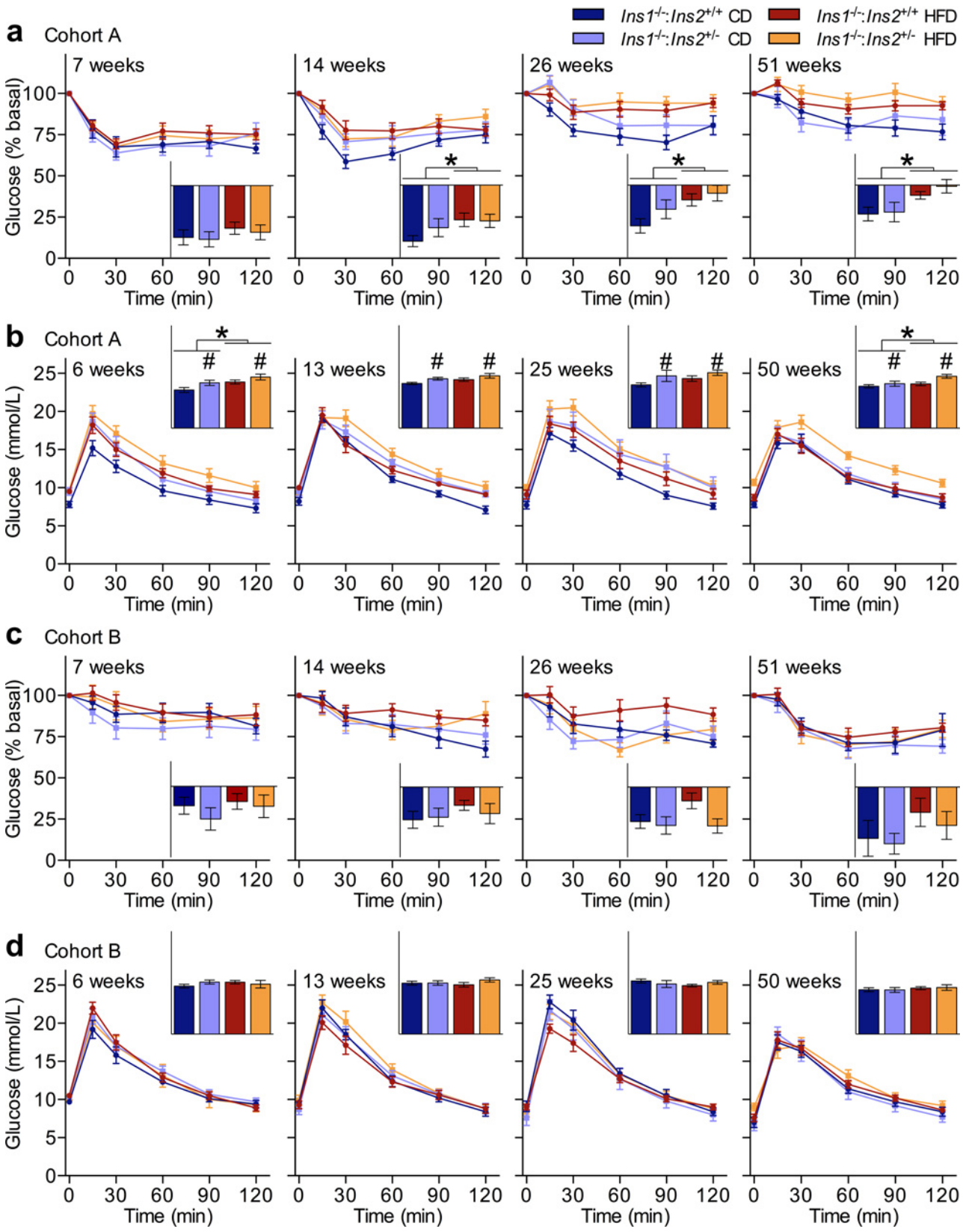
a
Cohort A
Ins1-/-:Ins2+/+ CD
Ins1-/-:Ins2+/- CD
Ins1-/-:Ins2+/+ HFD
Ins1-/-:Ins2+/- HFD
7 weeks
14 weeks
26 weeks
51 weeks
Glucose (% basal)
Time (min)
b
Cohort A
6 weeks
13 weeks
25 weeks
50 weeks
Glucose (mmol/L)
c
Cohort B
d
Cohort B

Fig 3. Variability in Glucose Homeostasis Across Two Cohorts. Periodic measurements of blood glucose response to intraperitoneal delivery of (**a**) an insulin analog (n = 10–19) in cohort A mice, and (**b**) glucose (n = 11–19) in cohort A mice, as well as (**c**) an insulin analog (n = 7–11) in cohort B mice, and (**d**) glucose (n = 7–11) in cohort B mice. Area under or over the curve (y-axis units of **a**,**c** percent•min, **b**,**d** mmol/L•min) is shown in panel insets. Data are means ± SEM. Dark blue and red represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, # for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$.

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present or showed opposite trends in the other cohort. There was also no apparent distinction in the mean or distribution of litter sizes between cohorts A and B (Fig 4F).

As an indicator of environmental stressors, we also measured 4 h-fasted corticosterone levels at 27 weeks. Interestingly, there tended to be a greater number of mice with elevated corticosterone levels in cohort B, and modest trends suggested that there were higher average corticosterone levels in HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males from cohort B, compared to cohort A (Fig 4G). Closer examination of HFD-fed animals showed that across both cohorts, fasting insulin levels in HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice were inversely correlated to corticosterone levels ($r^2 = 0.38$, $p < 0.01$), and this relationship was not evident in HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice ($r^2 = 0.00$, $p = 0.95$; Fig 4H). Interpretation of these data is limited, as it is based on using a single measurement of corticosterone for each individual animal as a marker of 'stress' during an extended time period. However, it appears that those HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice that reached the highest fasting insulin levels at 27 weeks (predominately individuals from cohort A) tended to have lower corticosterone levels, whereas increased stress may have dampened the capacity of HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice to compensate for reduced *Ins2* dosage at the level of basal insulin secretion. If mice in cohort B did experience more stressful conditions than cohort A animals (Fig 4G), this could be one of potentially many contributing factors underlying phenotypic differences in circulating insulin levels between cohorts A and B.

Heterogeneity in our data precluded pooling cohorts A and B to generate averaged results. However, although experimental genetic and dietary manipulations did not have consistent effects in both cohorts, we did observe a comparable range of fasting insulin values and body masses for both cohorts (Figs 1B, 4C and 4D). To indirectly evaluate whether differences in fasting insulin might be underlying body weight alterations in this model, we examined the relationship between these two variables across all 1 year-old mice. There was a positive correlation between body mass and fasting insulin levels at this age ($r^2 = 0.55$, $p<0.0001$; Fig 4I). Therefore, while we did not observe consistent effects of reducing *Ins2* gene dosage on circulating insulin and obesity in male mice, in general, these data support the concept that reduced insulin levels are associated with attenuated body weight and obesity.

Attenuated Obesity and Fat-free Mass in Cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ Males

We further characterized cohort B mice, as they showed a sustained divergence in body mass between *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ groups (Fig 4D). First, we used metabolic cages to examine the *in vivo* energy balance of HFD-fed mice at 17 weeks, an age when both cohorts showed similar trends for body masses. Although HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice exhibited a slight elevation in activity levels during the early hours of the dark period (Fig 5A), it did not appear that there were genotype differences in whole-body energy expenditure (Fig 5B), respiratory exchange ratio (Fig 5C), or food intake (Fig 5D) to account for the disparities in weight gain between HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ and *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ males.

We assessed body composition longitudinally with DEXA. Consistent with the evidence suggesting that lower *Ins2* dosage led to generally reduced growth in male neonatal pups (Fig 4E), reductions in both adiposity and fat-free mass contributed to the smaller size of cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice. *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ animals had significantly higher fat mass than *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males on both diets, at all measured time points (Fig 6A). A similar pattern was also

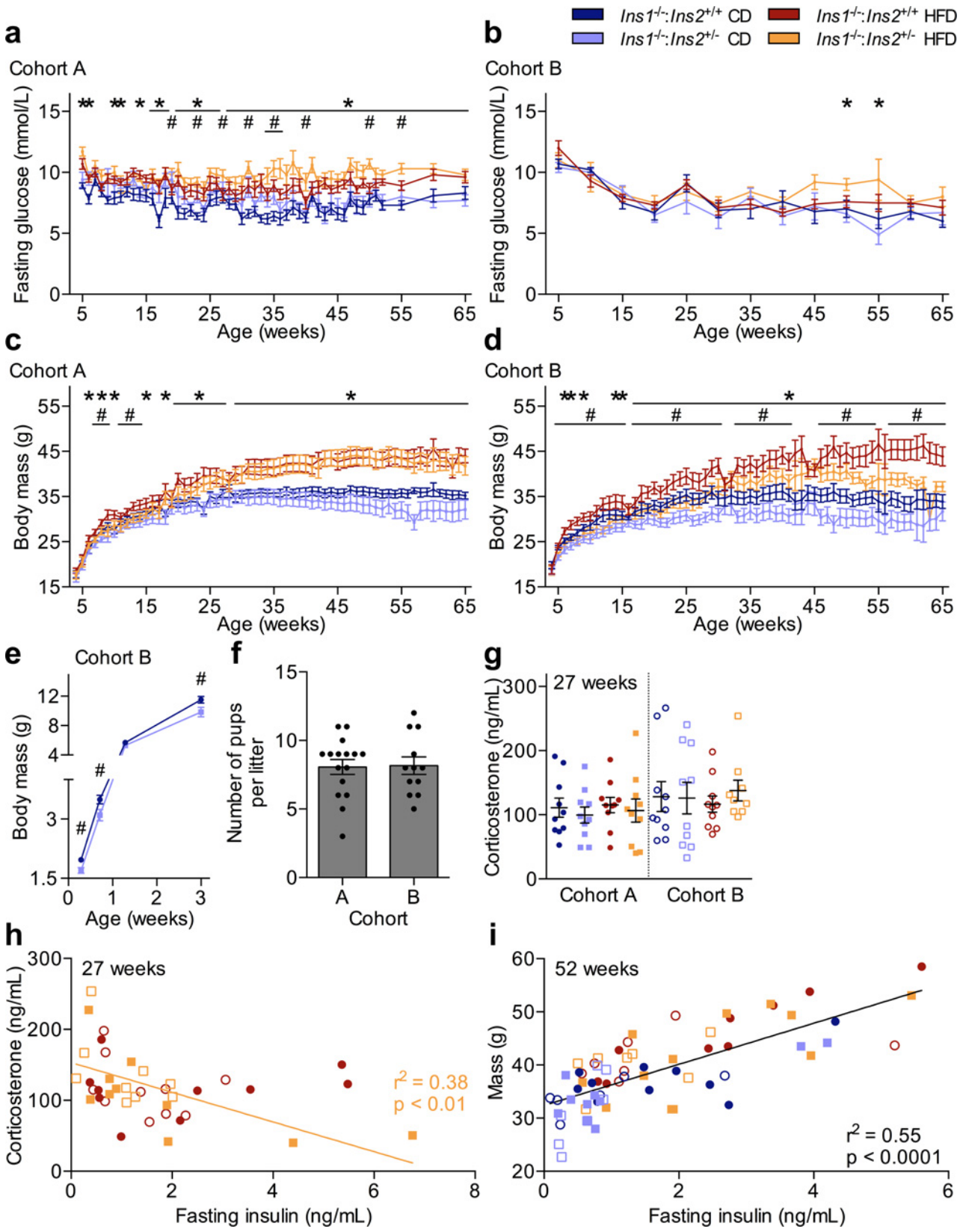
a
Cohort A
Fasting glucose (mmol/L)
Age (weeks)
b
Cohort B
Fasting glucose (mmol/L)
Age (weeks)
Ins1-/-:Ins2+/+ CD
Ins1-/-:Ins2+/- CD
Ins1-/-:Ins2+/+ HFD
Ins1-/-:Ins2+/- HFD
c
Cohort A
Body mass (g)
Age (weeks)
d
Cohort B
Body mass (g)
Age (weeks)
e
Cohort B
Body mass (g)
Age (weeks)
f
Number of pups per litter
Cohort
A
B
g
27 weeks
Corticosterone (ng/mL)
Cohort A
Cohort B
h
27 weeks
Corticosterone (ng/mL)
r2 = 0.38
p < 0.01
Fasting insulin (ng/mL)
i
52 weeks
Mass (g)
r2 = 0.55
p < 0.0001
Fasting insulin (ng/mL)

Fig 4. Cross-cohort Variation in Fasting Glucose, Body Mass, and Corticosterone. Periodic measurements of 4-h fasted blood glucose in (**a**) cohort A (n = 12–18, most time points) and (**b**) cohort B (n = 8–10, most time points) is shown, in addition to weekly body mass in (**c**) cohort A (n = 12–18, most time points) and (**d**) cohort B (n = 8–10, most time points). (**e**) Body mass was also tracked in a subset of pre-weaned male pups from cohort B (n = 12–22). (**f**) Litter sizes were compared between cohorts, with scatter points indicating the number of pups per individual litter (n = 12–16). (**g**) 4-h fasted corticosterone levels, measured in plasma collected during early afternoon from 27 week-old mice, were assessed between cohorts. Data are means ± SEM. (**h**) The relationship between corticosterone and insulin levels across cohorts in 27 week-old HFD-fed males (cohort A: closed points, n = 10; cohort B: open points, n = 9-10), with $r^2 = 0.38$ and $p < 0.01$ for *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice. (**i**) The relationship between body mass and 4-h fasted insulin levels at one year of age, $r^2 = 0.55$ and $p < 0.0001$ (cohort A: closed points, n = 9–12; cohort B: open points, n = 6–7). Dark blue and red represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, # for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$.

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evident for fat-free masses, particularly at the older ages (Fig 6B). However, since reductions in fat mass were proportionally greater than reductions in fat-free mass for *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ versus *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ males up to 60 weeks of age (Fig 6A and 6B), it is clear that an attenuation in adiposity contributed to the reduced body mass of cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice, compared to their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ controls. In the group of 25 week-old males with similar insulin levels (Fig 2A) and body masses (CD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ 36.8 ± 1.4, CD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ 32.6 ± 0.6, HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ 41.8 ± 1.8, HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ 37.2 ± 1.6; $p \leq 0.05$ for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ and for CD vs HFD) as cohort B male mice, both subcutaneous and visceral white adipose tissue depots were smaller in *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ versus *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ mice, in addition to the reduced sizes of these depots in CD-mice compared to HFD-fed mice (Fig 6C). Furthermore, the *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice had smaller interscapular brown adipose tissue depots than their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermates, and a slight reduction in the size of a mixed triceps surae muscle group (Fig 6C).

Despite the attenuated adiposity and fat-free mass of cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males compared to their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermates, we did not observe notable genotype differences in the fasting levels of circulating lipids and metabolic factors at 40 weeks of age. All HFD-fed mice had higher cholesterol and non-esterified fatty acids than CD-fed animals, without significant differences in triglycerides levels (Fig 6D–6F). In spite of differences in adipose tissue mass, leptin was similarly elevated in all cohort B HFD-fed mice compared to CD-fed mice (Fig 6G), as was resistin (Fig 6H). Based on levels of interleukin 6, there did not appear to be significant

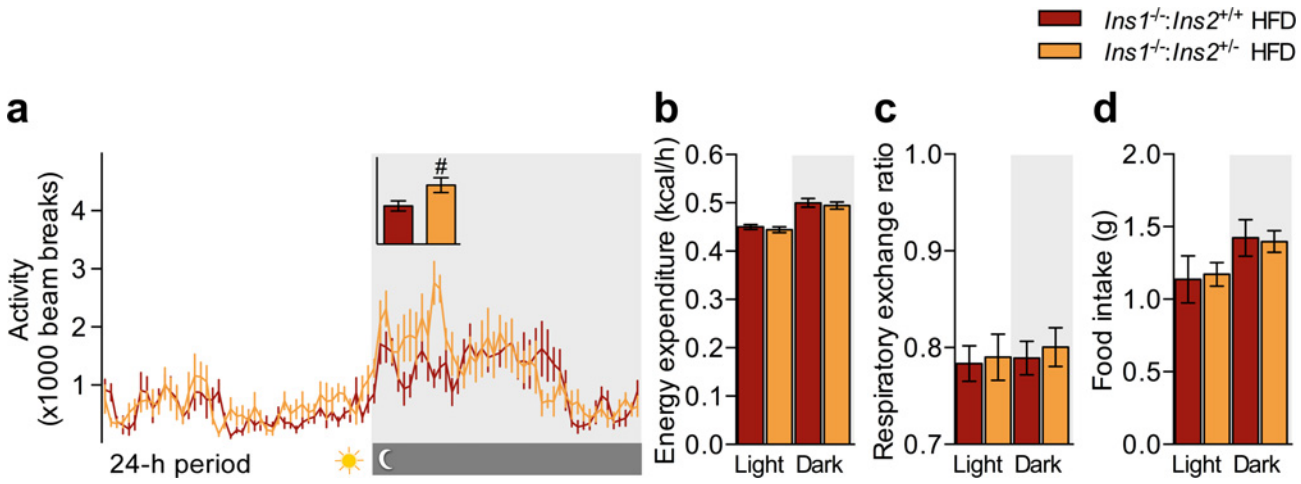

Fig 5. *In Vivo* Energy Homeostasis of HFD-fed Cohort B Males. In HFD-fed 17 week-old mice (n = 6–7), (**a**) 24-h activity, with inset showing mean beam breaks across the first 4 h of the dark period (y-axis units of beam breaks), as well as (**b**) energy expenditure, (**c**) respiratory exchange ratio, and (**d**) food intake were averaged across 48–84 h, with grey indicating dark cycles. Energy expenditure is shown as estimated marginal means ± SEM, adjusted for covariates of lean and fat mass; other data are simple means ± SEM. Dark red represents HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ male mice; orange represents HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice. $p \leq 0.05$ denoted by # for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$.

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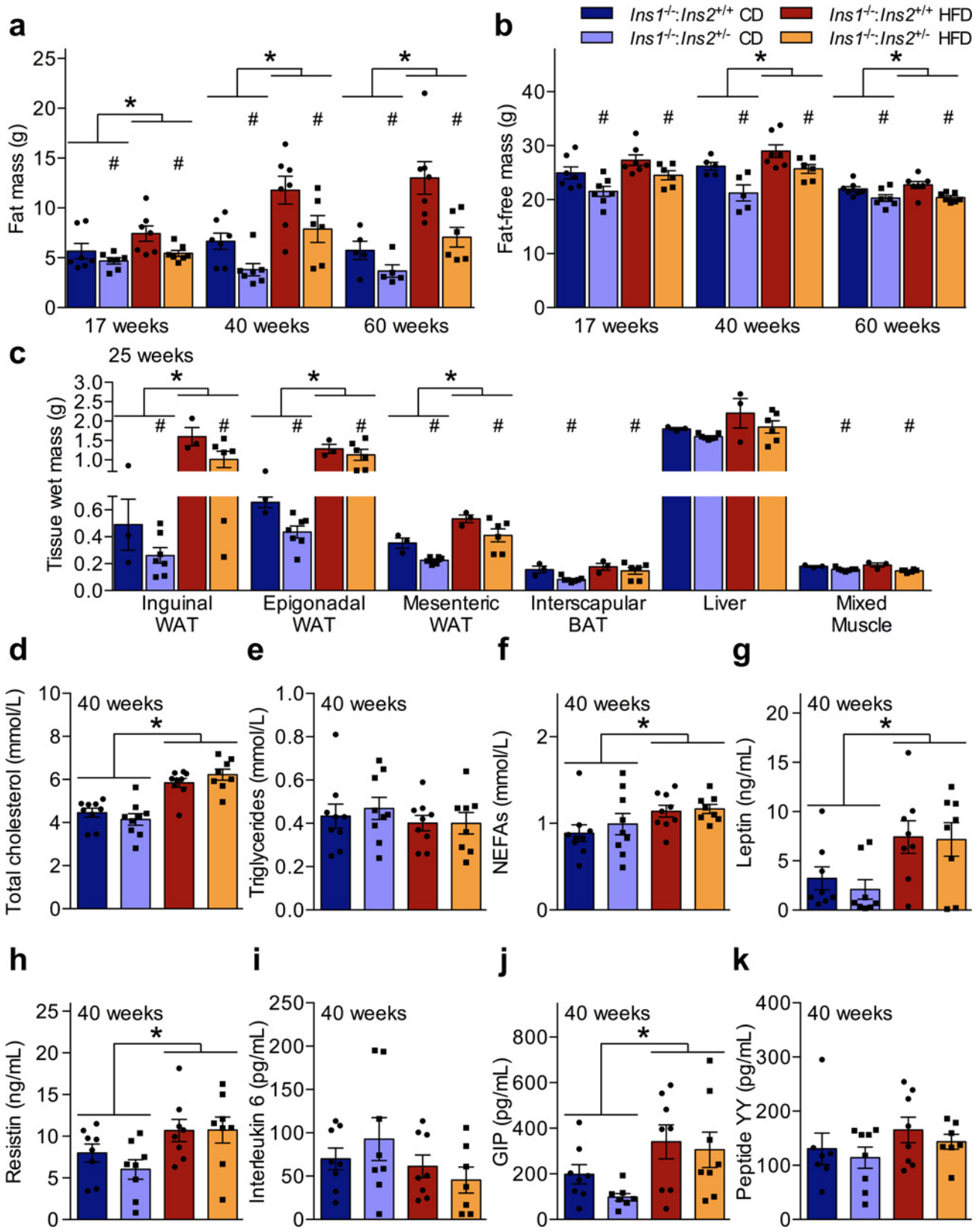
a
b
Ins1-/-:Ins2+/+ CD
Ins1-/-:Ins2+/- CD
Ins1-/-:Ins2+/+ HFD
Ins1-/-:Ins2+/- HFD
Fat mass (g)
Fat-free mass (g)
17 weeks
40 weeks
60 weeks
c
25 weeks
Tissue wet mass (g)
Inguinal WAT
Epigonadal WAT
Mesenteric WAT
Interscapular BAT
Liver
Mixed Muscle
d
e
f
g
Total cholesterol (mmol/L)
Triglycerides (mmol/L)
NEFAs (mmol/L)
Leptin (ng/mL)
40 weeks
h
i
j
k
Resistin (ng/mL)
Interleukin 6 (pg/mL)
GIP (pg/mL)
Peptide YY (pg/mL)

Fig 6. Attenuated Adiposity and Fat-free Mass in Cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ Males. Periodic DEXA-measured (**a**) fat mass and (**b**) fat-free mass is shown in cohort B male mice (n = 5–7). This corresponded to (**c**) wet masses of inguinal, epigonadal, and mesenteric WAT depots, the interscapular BAT depot, whole liver, and the triceps surae hindlimb mixed muscle group, with all tissues from a group of 25 week-old male mice (n = 3–7) that was separate yet similar in phenotype to cohort B mice. In addition, 4-h fasted (**d**) cholesterol, (**e**) triglycerides, (**f**) non-esterified fatty acids (NEFAs), (**g**) leptin, (**h**) resistin, (**i**) interleukin 6, (**j**) glucose-dependent insulinotropic polypeptide (GIP), and (**k**) peptide YY, is shown in 40 week-old mice from cohort B (n = 7–9). Data are means ± SEM, with scatter points to indicate individual values. Dark blue and red represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, # for *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ vs *Ins1*$^{-/-}$:*Ins2*$^{+/-}$.

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differences in inflammatory state between groups (Fig 6I). Glucose-dependent insulinotropic polypeptide levels were similarly elevated in all HFD-fed mice, compared to CD-fed animals (Fig 6J), but no significant differences were detected in concentrations of peptide YY (Fig 6K). These data suggest that while a combination of attenuated adiposity and reduced fat-free mass contributed to the smaller body weights of cohort B *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males compared to their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermate controls, cohort B HFD-fed *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males nonetheless displayed many of the expected characteristics of high fat feeding.

Discussion

The initial aim of our work was to test the hypothesis that reducing *Ins2* gene dosage on an *Ins1*-null background would prevent HFD-induced hyperinsulinemia, and thereby protect against obesity in male mice. Contrary to our expectations, inactivating one *Ins2* allele did not consistently cause a reduction of circulating insulin in *Ins1*-null male mice–not even the transient suppression of insulin hypersecretion that was consistently evident in their female *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ littermates at a young age [19]. We report that under some conditions, *Ins1*-null males with reduced *Ins2* mRNA were capable of producing nearly equivalent circulating insulin levels as *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ males, albeit possibly with subtle differences in secretory patterns that could have contributed to modest glucose intolerance. This clearly distinguishes these *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males from the *Ins2*-null male mice with reduced dosage of the *Ins1* gene, as *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ male mice experienced a sustained suppression of hyperinsulinemia [14]. Our findings show that *Ins1*-null male mice exhibit phenotypic hyper-variability across cohorts with respect to insulin levels, glucose homeostasis, and effects of *Ins2* gene dosage.

In the current study, all HFD-fed mice in the first experimental cohort, cohort A, showed notable insulin hypersecretion and weight gain, without significant effects of reduced *Ins2* dosage. In contrast, cohort B tended towards a less pronounced degree of HFD-induced insulin hypersecretion and peripheral insulin resistance. In addition, in cohort B there seemed to be a sustained reduction in insulin levels and body mass in *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ mice compared to their *Ins1*$^{-/-}$:*Ins2*$^{+/+}$ littermate controls, without detected changes in food intake or energy expenditure. These two cohorts from the same colony were studied approximately one year apart, under consistent experimental conditions in a controlled SPF facility. Despite this, by one year of age the average differences in fasting insulin levels between the two cohorts were considerably more pronounced than the difference between having one or two functional *Ins2* alleles (in either cohort).

It is important to note that pronounced phenotypic variability between cohorts of *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ male mice, particularly with respect to body mass, was also evident within another animal facility [24]. We cannot explain the widely diverse phenotypic responses to reduced *Ins2* gene dosage in male mice. However, it is clear that cross-cohort phenotypic variability in *Ins1*$^{-/-}$:*Ins2*$^{+/-}$ males has been observed in two distinct facilities to a degree that was not observed in their female littermates [19], nor in *Ins2*-null male or female mice with full or partial *Ins1* expression [14], despite the fact that these similar mouse models were studied by our group in the same time frames and under similar conditions [14,19]. Therefore, in *Ins1*-null

male mice, the phenotypic outcomes of *Ins2* gene modulation appear to be susceptible to a wide range in variability.

Phenotypic variability, in general, is poorly understood, but likely affects many long-term animal studies. There is evidence that the *in utero* and neonatal environments (*e.g.* [25–28]), gut microbiome composition (*e.g.* [29–31]), and exposure to different stressors, including temperature (*e.g.* [32–34]), noise (*e.g.* [35,36]), social hierarchy (*e.g.* [37,38]), and even the sex of the researchers working with animal subjects [39], can have far-reaching effects on many physiological parameters. Additional considerations include animal background strain or substrain, and genetic drift within a colony (*e.g.* [2,40–43]). These variables can confound experimental results through such means as altering the endocrine milieu, or changing gene expression levels, directly or via the epigenome. However, it should be noted that we attempted to control for many of these potentially confounding variables at a reasonable level in our investigation.

There are numerous other factors that may have played a part in the observed phenotypic heterogeneities in our investigation. For instance, the murine *Ins2* gene has been shown to be subject to developmental stage-dependent and tissue-specific genomic imprinting [11,44,45]. We considered the possibility that the disrupted *Ins2* allele may have had variable effects depending on whether it was inherited from the maternal or the paternal side, particularly as our mice lacked the potential for compensatory *Ins1* expression. Although we did not observe consistent parental effects on the experimental animals, genomic imprinting is a complex system that has not yet been fully elucidated (see [45–47]), and a potential role cannot be fully ruled out.

As the experimental cohorts were separated temporally, another potential explanation for cross-cohort variability is subtle environmental changes across the years (although the cohorts were roughly matched for seasons, since they were approximately one year apart). There were no differences between cohorts with respect to the average number of siblings sharing their *in utero* and neonatal environments, nor in numerous controlled parameters. However, in long-term experiments it is not always possible to avoid such environmental perturbations as minor earthquakes, construction periods around a facility, and so forth. At 27 weeks, an age when it was becoming clear that the two cohorts were diverging in their patterns of insulin secretion and weight gain, a single blood sample per mouse provided limited indication that levels of the stress hormone corticosterone might have been slightly higher in cohort B males, compared to mice from cohort A. Effects may vary depending on duration or type of stressor, but there is evidence that chronic stress [48,49] or glucocorticoid exposure itself [50–53] can lead to reduced insulin secretion in rodents. Interestingly, the glucocorticoid receptor has been shown to bind to a negative regulatory element of the human *INS* gene [54]. In our results, there was a negative correlation between basal insulin levels and corticosterone across both cohorts of HFD-fed $Ins1^{-/-}$:$Ins2^{+/-}$ males. We suggest that in the current study, reduced exposure to stress, signified by decreased plasma corticosterone, may have partially accounted for some HFD-fed $Ins1^{-/-}$:$Ins2^{+/-}$ male mice having the ability to produce nearly equivalent amounts of fasting insulin as their $Ins1^{-/-}$:$Ins2^{+/+}$ littermates. However, multiple other contributing factors likely influenced these outcomes.

The hypothetically environmental-dependent or stress-dependent ability to compensate for reduced *Ins2* dosage at the level of insulin translation and/or secretion was only observed in male $Ins1^{-/-}$:$Ins2^{+/-}$ mice. Although the female littermates experienced largely consistent conditions under the same experimental time frame, they did not exhibit similar patterns in insulin levels. These findings thereby point to the possibility of sex-specific regulation of murine insulin 2 production or secretion. Both testosterone and estrogen have the capacity to stimulate insulin production and secretion [55–57], so if the gonadal steroids have differential effects on

Ins2 and or insulin 2 peptide, it might have contributed to the observed male-specific variability in insulin levels for $Ins1^{-/-}$:$Ins2^{+/-}$ mice. However, there are numerous other sex-specific physiological differences that could have also played a role.

Overall, results from this investigation should serve to highlight the range of insulin's physiological effects, and the phenotypic characteristics that can change in association with variability in insulin levels. We were unable to properly test the hypothesis that prevention of HFD-induced hyperinsulinemia would protect against obesity using this male mouse model, due to non-uniform effects of reducing *Ins2* gene dosage on circulating insulin in *Ins1*-null males. However, we did observe a positive correlation between body mass and fasting insulin, which supports the concept that HFD-fed mice with lower endogenously produced circulating insulin also tend to have reduced obesity [14,19]. In addition, in cohort B experimental mice we show that reduced *Ins2* expression and subtle reductions in insulin levels can be associated with a long-term attenuation of whole body growth, including both fat-free mass and adipose tissue. While it might be expected that differences in insulin levels would primarily affect circulating glucose and other metabolites, we found that anabolic repercussions could be detected in the absence of significant alterations to glucose homeostasis.

Our study shows that circulating insulin levels varied widely in male mice lacking the potentially stabilizing effects of *Ins1* expression. Indeed, measured insulin levels in humans and wild-type mice have been shown to be subject to considerable variation [2–4], and our results suggest that inadvertent stress exposure may be one factor underlying variability in insulin levels. In addition, we show that it is important to look beyond insulin's well-characterized effects on glucose homeostasis when evaluating the physiological effects of divergent insulin levels.

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Author Contributions

Conceived and designed the experiments: NMT AEM JDJ. Performed the experiments: NMT. Analyzed the data: NMT. Contributed reagents/materials/analysis tools: JDJ. Wrote the paper: NMT AEM JDJ.

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