Trm28 Haploinsufficiency Triggers Bi-stable Epigenetic Obesity

Highlights

- *Trm28* haploinsufficiency triggers stochastic bi-stable obesity or polyphenism
- Non-classical imprinted gene dysregulation specifies “on” versus “off” obese states
- *Peg3* and *Nnat* perturbation trigger stochastic bi-stable obesity
- Human BMI distributions and transcriptomes suggest Trm28-associated subpopulations

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In Brief

TRM28 insufficiency in both mouse and human leads to polyphenism, wherein lean and obese phenotypes can arise from the identical genotypes through dysregulation of an imprinted gene network.
Trim28 Haploinsufficiency Triggers Bi-stable Epigenetic Obesity

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SUMMARY

More than one-half billion people are obese, and despite progress in genetic research, much of the heritability of obesity remains enigmatic. Here, we identify a Trim28-dependent network capable of triggering obesity in a non-Mendelian, “on/off” manner. Trim28−/−/+D mouse mutant mice exhibit a bi-modal body-weight distribution, with isogenic animals randomly emerging as either normal or obese and few intermediates. We find that the obese-“on” state is characterized by reduced expression of an imprinted gene network including Nnat, Peg3, Cdkn1c, and Plag1 and that independent targeting of these alleles recapitulates the stochastic bi-stable disease phenotype. Adipose tissue transcriptome analyses in children indicate that humans too cluster into distinct sub-populations, stratifying according to Trim28 expression, transcriptome organization, and obesity-associated imprinted gene dysregulation. These data provide evidence of discrete polyphenism in mouse and man and thus carry important implications for complex trait genetics, evolution, and medicine.

INTRODUCTION

Complex traits such as height, shape, and weight emerge from the integration of multiple genetic and epigenetic determinants. They underpin susceptibility to and severity of virtually all disease. Current estimates place obesity incidence at more than 600 million individuals worldwide (WHO, 2015). As a prime risk factor for heart disease, stroke, cancer, type 2 diabetes, and neurodegeneration, obesity poses a major socio-economic challenge. Although studies over the last decades have provided a genetic framework for understanding obesity, the contribution of epigenetic regulation remains poorly understood. Measurements in monozygotic twins and inbred mouse strains indicate that epigenetic control can have substantial effects on body-mass outcomes. Isogenic C57Bl6/J mice, for instance, can vary by as much as 100% in body weight when fed a high-fat diet, even when reared in highly standardized laboratory conditions (Koza et al., 2006). Experiments in multiple model organisms suggest that pre-conceptual and early-life environment contribute to variability by reproducibly shifting offspring phenotype (reviewed in Patti, 2013; Daxinger and Whitelaw, 2012; Rando and Simmons, 2015). Also, epidemiological data suggest that similar regulatory mechanisms determine human phenotypic outcomes. Despite many investigations, we still know little about the mechanisms by which developmental trajectories are canalized and how these states are reproducibly altered. Changes in imprinting
RESULTS

Trim28 Haploinsufficiency Induces a Stochastic Bi-stable Obesity

Whitelaw et al. previously reported exaggerated variability in body mass in Trim28+/D9 haploinsufficient mice (Whitelaw et al., 2010). In an effort to understand the nature of the variable phenotype, we generated large cohorts of Trim28+/D9 animals well-controlled for parental and offspring litter size (7–10 pups), lactation sufficiency, and housing conditions, factors all known to influence growth and metabolism and thus to add noise to phenotypic data. As examples, individually housed mice eat ~25% more than those housed in groups of 4; also, small litter size (calorie excess during lactation) is used as a model for metabolic reprogramming toward obesity.

Rather than simple amplification of random (Gaussian-distributed) variation, we found that highly backcrossed and inbred (F10+N20+ FVB/NJ) Trim28+/D9 individuals clustered into two discrete sub-populations, one heavy (obese-Trim28+/D9) and one normal (lean-Trim28+/D9) (Figure 1A). The resulting body-weight frequency distribution was bi-modal (Figure 1B). Interestingly, this on/off phenotype was observed not only when merging data from multiple litters but also within individual litters, i.e., when parental and developmental environment are maximally controlled. In addition to increased body mass, the obese-Trim28+/D9 sub-population was slightly longer (~1%–2% increase in nose-to-tail length; Figure 1C) relative to both their lean-Trim28+/D9 and wild-type siblings. Trim28 was equally expressed in the lean versus obese sub-groups (Figure S1A). Obese-Trim28+/D9 animals exhibited increased adipose tissue mass in all measured depots including epididymal, inguinal, and brown fat pads (Figures 1D and 1E) and showed no observable difference in brain, kidney, spleen, muscle, or liver weights, indicating that bi-stability was at least partially restricted to adiposity. Documenting many individuals and litters, we observed that the Trim28+/D9-sensitized obesity emerged during a relatively short window in early adulthood (8–12 weeks of age; Figure 1F) and then remained stable. Of potential interest, non-significant trends toward increased body mass and length were also observed in lean-Trim28+/D9 relative to wild-type siblings (Figures 1A–1F).

Notably, inheritance of obesity was non-Mendelian. To date, the bi-stability phenotype has survived >7 years through breeding schemes including maternal and paternal transmission of the Trim28+/D9 allele, as well as extensive backcrossing (> N10), inbreeding (> F20), three different mouse houses (QIMR, Australia; IMBA, Austria; and MPI-IE, Germany; Figure 1G), and embryo transfer. Comparison of interquartile ranges, coefficient of variation, and standard deviation against large, independently obtained non-early-life-controlled cohorts confirmed the heightened variation specifically of the Trim28+/D9 heterozygotes (Figure S1B). We have observed average facility-specific obesity “on” rates ranging from as low as ~5%–20% (MPI-IE, Freiburg) to nearly ~50% (QIMR, Australia) of the heterozygote Trim28+/D9 population, suggesting that external factors can reproducibly influence the on/off decision. Though periods of apparent sex bias have been observed, the phenotype has been observed in both male and female animals and, as mentioned above, passes through both the male and female germlines. Importantly, the non-Mendelian bi-stability has survived dedicated attempts to segregate it; obese-Trim28+/D9 fathers show no evidence of producing more obese offspring relative to their lean-Trim28+/D9 siblings.

Importantly, conditional homozygous deletion of Trim28 in muscle (Mck-Cre), adipose (Adipoq-Cre), liver (Alb-Cre), and...
the satiety regulating POMC- (POMC-Cre), and AgRP-neurons (AgRP-Cre) have revealed no obvious effect on adiposity (Figure 1J). These data indicate that TRIM28 is largely dispensable in fully differentiated adult tissues and support a role, consistent with much literature, in transcriptional programming in development. Thus, Trim28<sup>+/D9</sup> mice exhibit non-Mendelian bi-stable obesity.

Obese-Trim28<sup>+/D9</sup> Animals Are Metabolically “Healthy”
To better understand the adiposity phenotype, we metabolically phenotyped cohorts of Trim28<sup>+/D9</sup> animals. Examining oral glucose tolerance (Figure S1C), serum-free fatty acids, triglycerides (Figures S1D and S1E), as well as fasting glucose and insulin levels (Figures S1F and S1G), we found no evidence of major anomalies in the obese-Trim28<sup>+/D9</sup> animals nor in all Trim28<sup>+/D9</sup> animals grouped as a whole. Key determinants of metabolic health in obesity include efficient adipocyte turnover and function, as well as resistance to chronic metabolic inflammation (Gregor et al., 2013; Teperino et al., 2012; Jais et al., 2014). Consistent with the relatively protected metabolic phenotype, obese-Trim28<sup>+/D9</sup> animals showed no significant changes in adipocyte size (Figures 1H and 1I). Given the measured white adipose tissue mass differences (Figure 1D), the data are consistent with an approximate doubling of adipocyte number in the obese-Trim28<sup>+/D9</sup> animals. Plasma levels of the pro-inflammatory adipokines TNF-α and resistin, of C-reactive protein (Ouchi et al., 2011), and of the plasma-soluble receptor RAGE (Alexiou et al., 2010) were unremarkable (Figure S1H). Further, no evidence was found of secondary metabolic complications such as hepatosteatosis (Figure S1I), though our own previous work examining older cohorts has shown this as a potential endpoint (Whitelaw et al., 2010). mRNA expression profiling of epidydimal

Figure 1. Trim28 Haploinsufficiency Induces Stochastic, Bi-stable Obesity in the Mouse
(A and B) Body mass of wild-type littermates and Trim28<sup>+/D9</sup> mice at 14–18 weeks of age (A) and frequency distribution of body weight normalized to wild-type littermates (B).
(C) Obese-Trim28<sup>+/D9</sup> mice are heavier and longer relative to wild-type and lean-Trim28<sup>+/D9</sup> mice.
(D and E) Increased mass in obese-Trim28<sup>+/D9</sup> results from expansion of fat depots (eWAT, scWAT, and BAT) and not organomegaly.
(F) Trim28<sup>+/D9</sup> mice are lighter at weaning relative to wild-type littermates; body-weight differences between lean- and obese-Trim28<sup>+/D9</sup> mice emerge near adulthood.
(G) The Trim28<sup>+/D9</sup> colony body-mass distributions at three different sites (MPI-IE, Germany; IMBA, Austria; QIMR, Australia) with a variable frequency ranging from 10%-50%.
(H and I) H&E staining of epidymidal adipose (scale bar: 200 μm) shows no sign of adipocyte hypertrophy in Trim28<sup>+/D9</sup> mice.
(J) Tissue-specific knockout of Trim28 in liver (Alb-Cre), muscle (McK-Cre), adipose (Adipoq-Cre), POMC (POMC-Cre), or AgRP (AgRP-Cre) neurons does not impact body mass.
Data are mean ± SEM (*p < 0.05). See also Figure S1.
adipose by RNA sequencing revealed moderate expression changes associated with both positive (elevated Fgf21, Fndc5; decreased Retn; Figure S1J) and negative metabolic outcomes (increased Lep, Tnfa, IL1b, and Hmox1; Figure S1K), indicating that although largely normal, the animals are likely not immune to metabolic complication.

Characterizing energy homeostasis has been difficult. Specifically, anxious and stress-sensitive behavior of the Trim28+/D9 line has restricted successful analysis to a select few individuals despite use of stress-limiting home-cage indirect calorimetry. The data indicate no changes in food intake, expected alterations in VO2 and VCO2, as well as a tendency toward reduced activity (Figures S1L–S1O). Statistical detection of differences in energy expenditure will require a high sampling number, which, given the stochasticity and stress sensitivity of the model, is an ongoing challenge.

Non-classical Imprinted Gene Dysregulation Specifies the Obesity “On” State

To find causal underpinnings for the obesity induction, we intersected RNA-sequencing datasets comparing epididymal adipose tissue gene expression in our obese- and lean-Trim28+/D9 animals with comparable data contrasting high-fat diet (HFD)-induced obese animals with chow-fed controls (GSE38337). Global analysis of the two differential obesity datasets revealed low overall correlation (Pearson R = 0.16; Spearman R = 0.11; Figure 2A), indicating that the Trim28+/D9-induced obese state is distinct from that induced by over-eating.
Because Trim28\textsuperscript{-/C24} was originally identified as a silencing “E(var)” mutation (Whitelaw et al., 2010), we focused on genes downregulated in obese-Trim28\textsuperscript{-/C24} animals but unaltered or increased in HFD-induced obesity. Querying the top 250 such genes against the MSigDB collection of curated gene pathway annotations, we observed enrichment in genesets associated with proliferation/cell cycle/cancer, with adipogenesis/stem cell differentiation, and interestingly with Polycomb and HDAC-associated chromatin regulation (Figures 2B and 2C and Tables S1, S2, and S3).

One of the most enriched signals, top among chromatin-related pathways, was the geneset “Brideau_Imprinted Genes” (Brideau et al., 2010) (Figures 2B and S2C). Imprinted genes comprise ∼150 genomic loci whose expression follows a DNA methylation-associated parental-origin pattern, such that only one of the two alleles, either the maternally or paternally inherited one, is expressed. We built custom pathway annotations comprising all known paternally and maternally expressed imprinted genes (PEGs and MEGs, respectively) and probed for enrichment in our RNA-seq data using gene set enrichment analysis (GSEA). Whereas MEGs as a group exhibited less remarkable regulation, PEGs showed marked pathway downregulation specifically in obese-Trim28\textsuperscript{-/C24} samples (Figures 2D, 2E, and S2D). Heatmap and GSEA enrichment score profiles suggested that the pathway contained two roughly equally sized gene subsets: one, prominently downregulated (“leading edge”; Figures 2D and 2E and Table S4), and a second, apparently non-regulated, subset with random distribution. Al Adhami et al. recently used an unbiased in silico approach to show that imprinted genes cluster into at least three co-regulated groups, suggesting functional compartmentalization beyond paternal and maternal expression (Al Adhami et al., 2015). Querying our data against these non-classical “imprinted gene network” (IGN) annotations at the pathway level, we found almost exclusive regulation of IGN1, a predominantly paternally expressed cluster previously implicated in body size/weight control (Varrault et al., 2006; Gabory et al., 2009; Al Adhami et al., 2015) (Figure 2F). The core enrichment signal of this analysis was driven by downregulation of Plagl1, Dlk1, Cdkn1c, Nnat, Igf2, Peg3, Ppp1r9a, Ndn, and Grb10 in obese-Trim28\textsuperscript{-/C24} animals (Figure 2F and Table S4). The downregulation of Nnat, Plagl1, and Peg3 was confirmed by qPCR and at the protein level for Nnat (Figures S3E and S3F). Comparable regulation was not observed in data from HFD-induced obese adipose tissue (Figure S2G). Thus, an IGN1-centric gene signature characterizes Trim28\textsuperscript{-/C24}-induced obesity.

Mechanistically, imprinting results from discordant maternal versus paternal DNA methylation patterning at germline-defined imprinting control regions (ICRs). We next examined DNA methylation levels at IGN1 ICRs. Included were ICRs of three of our most differentially regulated IGN1 genes, including two moderately (Plagl1, Peg3) and one highly expressed (Nnat) gene as well as ICRs of unaffected imprinted loci within (H19 and Dlk1-Meg3) and outside (Snrpn) the IGN1 network. Changes in H19 and Snrpn have previously been linked to Trim28 dysregulation in vivo (Messerschmidt et al., 2012). Importantly, we found no evidence of altered DNA methylation at any of these loci (Figure 2G). These findings indicated intact imprinting control and were true for assessments of both purified white epididymal adipocytes (Figure 2G) and stromal vascular preparations including adipocyte progenitor cells and pre-adipocytes (Figure S2H). Extension “genome-wide” using reduced representation bisulfite sequencing (RRBS) provided no evidence of significant changes in DNA methylation also beyond ICRs, including at promoters or gene bodies (Figures 2H and 2I). These findings were consistent with a lack of DMR-associated reciprocal gene-expression patterns that would be expected from classical loss of imprinting. Thus, a non-classical imprinted gene signature specifies Trim28\textsuperscript{-/C24}-dependent bi-stable obesity.

**IGN1 PerturbationInducesBi-stableObesity**

To test whether IGN1 dysregulation could cause obesity, we examined the phenotypic outcomes of targeted deletion of relevant IGN1 genes. We used Gateway-based “knockout-first” conditional targeting of exons 2 and 3 in JM8.F6 C57Bl6/N embryonic stem cells (ESCs) according to standard EUCOMM protocols to generate a knockout allele of our most strongly dysregulated IGN1 gene, Nnat (Figure 3A). ESCs were injected into blastocysts and implanted into pseudo-pregnant females, and chimeric offspring were screened for germline transmission. Deletion was confirmed on the RNA and protein levels (Figures 3B and 3C), and the newly derived Nnat knockout line was backcrossed more than ten generations to C57Bl6/J.

Nnat mutant offspring of both maternal and paternal transmission were born at Mendelian ratios. In support of a causal role for Nnat downregulation in Trim28\textsuperscript{-/C24}-induced obesity and bi-stability, paternal deletion mutants (Nnat\textsuperscript{+/P}) exhibited a hyper-variable adiposity phenotype, again clearly emerging in adulthood (Figure 3D). Obese individuals were observed in both male and female offspring, and consistent with Nnat being paternally expressed, obesity was only observed upon paternal inheritance of the deleted allele. No evidence of obesity was detected in either wild-type littermates or cohorts receiving the imprinted deletion allele from their mothers (Nnat\textsuperscript{+/M}; i.e., normal paternal Nnat expression) (Figure 3D). Fitting the body-weight distribution to the sum of two Gaussians (R\textsuperscript{2} = 0.98) indicated an obesity-on rate of ∼26% of the mutant population, with a 40% increase in body weight between the obese- and lean-Nnat\textsuperscript{+/P} animals (Figure 3E). Similar to the Trim28\textsuperscript{-/C24} line, the bi-stable phenotype has been confirmed in more than one facility (MRC Cambridge and MPI-IE Freiburg; Figure 3F) and has survived embryo transfer and backcrossing onto pure C57Bl6/J as well as mixed C57Bl6/J:FVBN/J F1 backgrounds. Obesity in Nnat\textsuperscript{+/P} animals was characterized by increased adipose tissue mass in epididymal adipose pads and on the whole animal level by dual-emission X-ray absorption analysis (Figures 3G and S3A). These findings implicate Nnat in buffering non-Mendelian bi-stable adiposity.

We also found evidence of potential bi-stability in an additional IGN1-targeted mouse model, a conventional knockout of the paternally expressed Peg3 allele (Peg3\textsuperscript{+/P}). Curley et al. reported increased body-fat accumulation in both male and female Peg3\textsuperscript{+/P} mutants (Curley et al., 2005). Re-analysis of those data revealed a population-level fat-mass distribution consistent with two adiposity-discordant sub-populations with an estimated obesity-on rate of ∼20% and a near-doubling in body...
fat between lean and obese mutants (N = 80) (Figure 3H). Interestingly, Peg3\(^{+/−}p\) mice were reported to be uniformly smaller, with concomitant reductions in body size, weight, and skeletal muscle mass, indicating that gene dosage among IGN1 members might be important for phenotypes beyond adiposity (Figure 3I). To the best of our knowledge the Peg3 deletion strain was not maintained by the community. We are now in the process of backcrossing a re-derived line from the original targeting construct.

During development, TRIM28 controls endogenous retroviruses and germline ICRs in the context of TRIM28-ZFP complexes. Hoping for additional mechanistic insight into the obesity decision, we also mapped our RNA-seq reads to a compendium of genomic repeat sequences as TRIM28 has previously been implicated in control of repeat expression (Rowe et al., 2010). Examining ERV families and classes and uniquely mapping reads and chimeric reads between genes and transposable element regions, we found few significant changes. Indeed, the number and degree of repeat expression alterations were lower than in comparable mutants of cellular metabolism (data not shown). Interestingly though, when examining RNA expression of the imprint-regulating TRIM28-ZFP57 complex, and related genes, in adipose of the Trim28 and Nnat mutant lines, we observed consistent dysregulation. Obese-Trim28\(^{+/−}d9\) animals exhibited decreased expression of the guide/recruitment factors Zfp57, Hp1α, and Hp1γ, concomitant with increased expression of Dnmt1, 3a, and 3L relative to their lean siblings (Figure 3J). The same trend was observed in Nnat\(^{+/−}m\) animals, except that instead of Zfp57, Trim28 was reduced in obese individuals (Figure 3K). These data are consistent with a model wherein relative dosage of TRIM28-ZFP57 complex recruitment and silencing functions underpin the switch between lean and obese phenotypes.
Evidence of TRIM28-Associated Phenotypic Bi-potential (Polyphenism) in Humans

We reasoned that if present, evidence of bi-stability and Trim28/IGN1-associated obesity would be most evident in childhood because this represents a window of tight environmental control in humans (i.e., essentially all children in the developed world exhibit largely coincident and enforced circadian, feeding, and activity patterns imposed through parenting, primary care, and education). We examined gene expression by Taqman qPCR in subcutaneous adipose tissue samples from a cohort of pre-pubertal Caucasian children of European ancestry entering the clinic for elective surgery (typically orthopedic); the cohort included 22 lean and 18 obese individuals. The children were disease and medication free and have been described in detail as part of the Leipzig Childhood AT cohort (Landgraf et al., 2015).

When measured against three housekeeping genes (ACTB, HPRT, TBP), we found both a significant reduction in adipose tissue TRIM28 levels in obese children (Figure 4A) and an apparent cluster of very low TRIM28-expressing individuals in the obese group. Individuals in the lower 50th percentile of TRIM28 expression appeared more likely to be obese than high TRIM28 expressors (Figure 4B). Mimicking analysis of the Trim28+, D9 haploinsufficient mouse scenario, we sub-divided all individuals into obese or lean groups of comparably high or low TRIM28 levels (Trim28_High and Trim28_Low, respectively) and used qPCR to measure expression of six of our leading-edge, Trim28+/D9-obesity-associated IGN1 genes (Figures 4C and 4D). IGN1 genes correlated with TRIM28 expression (Figure 4D). Whereas Trim28_High individuals showed essentially identical IGN1 expression levels irrespective of adiposity (Figure 4C, upper...
BMI Bi-modality at the Population Level in Humans

Next, we examined publically available datasets for signs of bi-modal body-weight distributions in the general population. We first examined adipose tissue microarray data from 13 discordant monozygotic (MZ) twin pairs each comprising one obese and one normal co-twin (Pietiläinen et al., 2008). Importantly, we found suggestions of both reduced mean TRIM28 levels (Figure 4G) and reduced IGN1 pathway expression (GSEA; Figure 4H) specifically in obese relative to lean isogenic co-twins. This indicates that Trim28-IGN1 expression correlates with epigenetically rooted human obesity.

We analyzed BMI distributions of ~4,000 Caucasian children, 6–11 years old, surveyed by the National Health and Nutrition Examination Survey (NHANES) between 1999 and 2012 (Center for Disease Control (CDC, 2012). As is well described for the general population, BMI distributions were positively “skewed.” We observed, however, that this positive bias was not gradual but rather contained a distinct inflection point suggestive of a mixed distribution (Figure 5A). We noted during the analysis that bar-chart visualization, as well as large and/or irregular bin-sizings often used in BMI analysis, mask this inflection. Importantly, where a single Gaussian failed to accurately model the data (and in particular the positive skew), we found that a mixed model assuming two independent and potentially overlapping Gaussian sub-populations fit ~99.2% of the data distribution (R² = 0.99; sum-of-two-Gaussians: R² = 0.91; single Gaussians) (Figures 5A and 5B). Inspection on a log-scale revealed that only the most obese children (>~30 BMI; <1% of the population) fell outside confidence intervals (Figures 5A and 5B). Of note, analyses compensating for the complex NHANES sampling strategy provided equivalent results. Most importantly, we observed the same pattern and goodness-of-fit when independently analyzing BMI distributions of males and females, of Mexican American, Hispanic, Caucasian, and African American children (Figure 5C), as well as of adult populations examined inside and outside North America, including a cohort of ~10,000 Han Chinese individuals (Figures 5D and 5E).

Finally, we found evidence of a marked frequency transition between sub-populations over recent decades. We compared BMI distributions from NHANES data gathered between 1963 and 1994 (CDC, 1994) with the more recent 1999–2012 (continuous NHANES) data (CDC, 2012). Both datasets followed a sum-of-two-Gaussians fit with an R² > 0.99. In contrast to the popularized notion that the population as a whole is significantly gaining weight, we observed that the calculated mean BMI of the major (lean) population increased only 0.07 BMI units from 1963–1994 (BMI = 15.81; males 6–11 years of age) to 1999–2012 (BMI = 15.87), i.e., +0.4%. Instead, the profiles indicated an increase in the relative fraction of individuals falling into the second (heavier) sub-population. The heavy sub-population, with a mean BMI 4–5 points above “normal,” more than tripled, from ~12% to ~38% of all individuals. These findings agree with other descriptions of childhood obesity (Körner et al., 2007) and suggest increased incidence of a distinct category of “triggered” individuals that would be consistent with the notion of polyphenism. Given that mean normalizations do not effectively accommodate variable bimodality, these data suggest that normalizations to age- and sex-specific medians, modes, or Winsorized means may more accurately align population-level BMI variation.

DISCUSSION

Phenotypic variation describes the extent and character of variability in a given phenotype in the population and is thought to be a platform for adaptation and evolution. It can be of genetic or epigenetic origin, or both (Simpson et al., 2011). Polyphenism refers specifically to the case where individuals of the same genotype can exhibit multiple discrete phenotypic end-points without intermediates and has been most heavily studied in insects. Classical examples include seasonal morphs in butterflies, caste morphs in eusocial insects (worker, soldier, queen), and the intriguing intergenerational reproductive morphs of aphids. The observation of two distinct phenotypic end-points in isogenic Trim28- Nnat- and possibly Peg3- mutant mice indicates similar phenotypic bi-potential in mice and identifies a genetic network that buffers against emergence of divergent states.

Although unclear whether socially, genetically, or epigenetically underpinned, the relatively inbred wild mole-rat exhibits a somewhat parallel dispersal morph that arises in times of plenty, exhibits increased adipose tissue mass, and shows a behavioral phenotype that ultimately leads it to leave the colony (O’Riain et al., 1996). The uncommon phenotype is thought to provide populations with the cooperative advantages of eusocial behavior while avoiding complete inbreeding. Similarly, increased energy storage induced via the Trim28ADE29-sensitive axis described here might be expected to protect from predation panel), obese Trim28_Low subjects showed clear reductions in CDKN1C, PLAGL1, NNAT, and PEG3 expression when compared to equally Trim28_Low lean subjects (Figure 4C, lower panel). These data recapitulate our observations of enhanced IGN1 downregulation in Trim28−/− haploinsufficient mice and are consistent with a conserved role for a Trim28-IGN1 axis in human adiposity regulation.

To test whether the observations reflected differences beyond IGN1 output, we performed RNA sequencing of the same adipose samples and analyzed transcriptome organization with principal-component analysis (PCA) of the 6,000 most variable, expressed genes. The PCA revealed a non-homogeneous population distribution with two apparent sub-populations (Figure 4E). Plotting individuals according to TRIM28 expression revealed that the clusters did not discriminate obese from lean or male from female subjects (Figures S4A and S4B) but rather Trim28_High from Trim28_Low individuals (Figure 4E). These findings recapitulate PCA analysis of the IGN1 gene qPCR data from the same individuals (insets, Figures 4E, S4A, and S4B) and indicated that on the transcriptome level Trim28_High and_Low individuals are more different than lean versus obese or male versus female individuals. These patterns were equally clear when assessing the data in a Pearson correlation matrix (Figure 4F) and by hierarchical clustering (Figure S4C). Thus, humans appear to stratify into sub-populations defined by adipose TRIM28 expression, with Trim28_Low individuals exhibiting distinct transcriptional complexity, IGN1 dysregulation, and increased obesity incidence.

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and starvation. Although the nature of triggers for our observed obese state is not clear, we have observed extended periods with reduced obese individuals correlating with housing density and reduced temperature. At the site of generation of the Trim28+/D9 line (QIMR, Australia), obese individuals were observed at higher rates; that facility was warmer but also had different microflora, rodent diet, and staff and is a non-SPF facility. Preliminary experiments examining maternal HFD effects in Trim28+/D9 animals suggest possible increased obesity rates.

Polyphenisms can also be genetically influenced. Our first efforts to map the molecular switch at work reveal coordinate suppression of IGN1 members. The mutant data implicate Trim28, Nnat, and Peg3 dosage and function in triggering bi-potential. Human genetic variants near NNAT associate in some populations with NNAT expression and with obesity, also in children (Vrang et al., 2010), and paternal transmission of obesity in F1 background crosses has been associated with Peg3 variation (Morita et al., 2014). Together, these findings indicate multiple genetic entry points for sensitization of the described epigenetic obese state. The bi-modal fit of human population-level BMI distributions would suggest that genome-wide association study (GWAS) efforts performed to date might already contain substantial signals for comparable non-Mendelian obesity. Generating tools to stratify and filter these datasets for epigenetic versus genetically driven phenotype would markedly enhance their power.

Figure 5. BMI of the General Population Is Consistent with a Bi-modal Distribution
(A) BMI distribution of 6- to 11-year-old non-hispanic white males from the continuous NHANES 1999–2012 survey (CDC, 2012). Data are fit to a single Gaussian (gray) and a double Gaussian (blue).
(B) Individual Gaussian components of the double Gaussian from (A).
(C) Near-perfect double-Gaussian fit is observed across children of five major ethnicity classes, as well as in adult cohorts from (D) continuous NHANES 1999–2012 (CDC, 2012) and (E) Han chinese populations.
(D and E) Shown are age-normalized BMI distributions for females aged 25–50.
(F) Comparison of similar fitting of childhood data from continuous NHANES 1999–2012 (CDC, 2012) and prior NHANES/NHES surveys (1963–1994) (CDC, 1994) shows a marked shift in recent decades where the heavy sub-population triples in size (pie charts).

See also Figure S5.
animals has yet to reveal any marked increase in obesity incidence and therefore suggests that Nnat and Trim28 lie in the same genetic pathway. IGNI genes have been implicated in placentaion (Sekita et al., 2008), development, growth, and importantly metabolic control (see Cleaton et al., 2014 for review), and five of the nine IGNI genes show energy homeostasis defects when deleted: Peg3 (Curley et al., 2005), Plag1 (Kamiya et al., 2000), Grb10 (Smith et al., 2007), Dik1 (Moon et al., 2002), and Nnat (this manuscript). TRIM28 loss has previously been linked to dysregulation of imprinted genes during development (Messerschmidt et al., 2012), and interestingly, DNMT3A, the methyltransferase in part responsible for proper imprint deposition, has also been tied to buffering phenotypic variability (Whitelaw et al., 2010). Also noteworthy, combined heterozygous mutation of the insulin and insulin-like growth factor 1 receptors leads to reduced expression of a subset of IGNI-overlapping imprinted genes (Boucher et al., 2014), suggesting insulin and IGF1 signaling as potential regulatory candidates for the phenotypes observed here.

Currently we cannot rule out largely on/off environmental signals (e.g., parental smoking) as direct effectors of bi-modality in the human data presented, nor can we rule out somatic mutations or Trim28-stabilized mutational hotspots and transposon activity as genetic underpinnings for the observations presented here. If substantiated, however, the impact of phenotypic bi-stability for humans is substantial. They include academic, ethical, and therapeutic aspects, and understanding the number, nature, and disease associations of possible states will be paramount. Trim28 mutant mice show increased cancer susceptibility (Herquel et al., 2011) as well as anxiety and behavioral phenotypes (Whitelaw et al., 2010; Jakobsson et al., 2008). Critical next steps for us will include testing whether comparable behavioral, cancer-prone, or alternate epigenetic trajectories exist in the sensitized contexts presented here and ultimately also in humans.

EXPERIMENTAL PROCEDURES

Generation of Trim28 and Nnat Mutant Mice

The generation of Trim28<sup>−/−</sup> has been described elsewhere (Blewitt et al., 2005). For information on the generation of Trim28 tissue-specific knockout mice and of Nnat null mice, see the Supplemental Experimental Procedures. All mouse models are described in the Supplemental Experimental Procedures. Animals were kept on a 12 hr light/dark cycle with free access to food and water and housed in accordance with international guidelines.

Human Study Population

Subcutaneous adipose tissue samples were obtained from 18 obese and 22 lean subjects of the Leipzig Childhood AT cohort (NCT02208141) (Landgraf et al., 2019). Children were 2–15 years old and included if they were in pre-puberty. For further information, see the Supplemental Experimental Procedures.

Glucose Tolerance Test

Oral glucose (1 g/kg) tolerance test was performed on 25-week-old animals as described (Boucher et al., 2014), suggesting insulin and IGF1 signaling as potential regulatory candidates for the phenotypes observed here.

Statistical Analysis

Data are expressed as mean ± SEM unless otherwise specified. Statistical analyses were performed as described in the Supplemental Experimental Procedures. All animal experiments included at least four biological replicates, and all reported p values are two-tailed unless stated otherwise. p < 0.05 was used as a cutoff for statistical significance.

Indirect Calorimetry

To measure basal metabolic rate, 10- to 14-week-old animals were singly housed in a home-cage indirect calorimetry system (TSE Systems). Animals were monitored over a 6 day period and fed an ad libitum chow diet. Data from the first day were discarded to reduce variation introduced by acclimatization. Data from consecutive days were treated as technical replicates, and data were binned in 2 hr intervals. Food consumption was measured directly as accumulated data.

Mouse Laboratory Parameters and Cytokines

Fasted (6 hr fast) blood plasma were obtained, and a panel of hormones, fatty acids, and adipokines were measured as described in the Supplemental Experimental Procedures.

Histology, Immunofluorescence, Adipocyte Size, and Number

We used semi-automated morphometry with H&E-stained paraffin sections of perigonadal WAT pads to perform adipocyte number and size analyses. Images were analyzed using ImageJ. Immunofluorescence was performed as described in the Supplemental Experimental Procedures.

Isolation of Mouse Primary White Pre-adipocytes and Mature Adipocytes

Primary preadipocytes and mature adipocytes were obtained by collagenase digestion from perigonadal white adipose tissue in mice as described (Pospisil et al., 2010).

qRT-PCR

For qRT-PCR of mouse and human samples, analysis of total RNA was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Mouse primers were designed using qPrimerDepot, and human “Best-Collectors" Taqman probes were purchased from Life Technologies. Threshold cycles (Ct-values) of all replicate analyses were normalized to TBP (mouse) or to TBP, ACTB, HPRT (human) housekeeping genes. To compare the effect of various conditions with controls, 2<sup>DD</sup> values were calculated to obtain fold expression levels. For further information, see the Supplemental Experimental Procedures.

RNA Sequencing

Trizol-purified RNA was poly(A)-enriched, and libraries were prepared with a TruSeq Sample Prep v2 kit (Illumina) and sequenced on a HiSeq 2500 (Illumina). Greater than 15 million reads per mouse sample and 10 million reads per human sample were mapped using TopHat v2.0.8b with -G option against Mus musculus genome (mm9, iGenome UCSC) and Homo sapiens genome (hg19, iGenome UCSC), respectively. Gene expression values and significantly differentially expressed genes were calculated using Cuffnorm and Cuffdiff v2.2.1 with geometric normalization and multi-read correction (-u option).

Reduced Representative Bisulfite Sequencing

Total genomic DNA was digested overnight with MspI (NEB), and sequencing libraries were prepared with NEBNext DNA Library kit (NEB). Samples were sequenced on a HiSeq 2500 (Illumina). For further information, see the Supplemental Experimental Procedures.

Bioinformatic Analyses

Gene set enrichment analysis used GSEA 2.0 with default parameters (permutation type: gene_set, Collapse dataset to gene symbols: false).

Statistical Analysis

Data are expressed as mean ± SEM unless otherwise specified. Statistical analyses were performed as described in the Supplemental Experimental Procedures. All animal experiments included at least four biological replicates, and all reported p values are two-tailed unless stated otherwise. p < 0.05 was used as a cutoff for statistical significance.

Other Methods

See Supplemental Experimental Procedures.

ACCESSION NUMBERS

Raw RNA-seq data from Trim28<sup>−/−</sup> adipose tissue are available via the ENA, under project “PRJEB11740“ (http://www.ebi.ac.uk/ena/data/view/PRJEB11740). Processed data are available in Tables S2 and S3.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.12.025.

AUTHOR CONTRIBUTIONS

Conceptualization, K.D. and J.A.P.; Methodology, K.D., J.A.P., A.C.F.-S., E.W., I.Z., and D.R.; Formal Analysis, Investigation, and Visualization, K.D., K.L., S.H., J.L., K.G., M.R., M.O., R.S., M.S., T.T.-H.L., E.C., R.T., I.Z., D.R., Y.O.L.T., B.L., R.L., and A.P.C.; Writing – Original Draft, K.D., J.A.P., and A.L.; Writing – Review & Editing, K.D., J.A.P., A.L., A.S., A.C.F.-S., A.P.C., and S.O.R.; Supervision, Resources, and Funding Acquisition, J.A.P., A.C.F.-S., T.V., J.M.P., T.J., C.-L.C., A.K., S.O.R., G.S.H.Y., and A.P.C.

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