A Gain-of-Function Mutation in Adenylate Cyclase 3 Protects Mice from Diet-Induced Obesity

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Abstract

In a screen for genes that affect the metabolic response to high-fat diet (HFD), we selected one line of N-ethyl-N-nitrosourea (ENU)-mutagenized mice, Jll, with dominantly inherited resistance to diet-induced obesity (DIO). Mutant animals had dramatically reduced body weight and fat mass, and low basal insulin and glucose levels relative to unaffected controls. Both white adipose tissue (WAT) and brown adipose tissue (BAT) depots were smaller in mutant animals. Mutant animals fed a HFD gained only slightly more weight than animals fed regular chow, and were protected from hepatic lipid accumulation. The phenotype was genetically linked to a 5.7-Mb interval on chromosome 12, and sequencing of the entire interval identified a single coding mutation, predicted to cause a methionine-to-isoleucine substitution at position 279 of the Adcy3 protein (Adcy3M279I, henceforth referred to as Adcy3³). The mutant protein is hyperactive, possibly constitutively so, producing elevated levels of cyclic AMP in a cell-based assay. These mice demonstrate that increased Adcy3 activity robustly protect animals from diet-induced metabolic derangements.

Introduction

Obesity is both a national and worldwide epidemic. The prevalence of obesity (body mass index (BMI) >30) is greater than 30% in the United States, and is even higher in some parts of the world with the highest prevalence in the Pacific Islands [1]. Obesity increases the risk of heart disease, type 2 diabetes, hypertension, stroke, colon cancer, and early mortality [2,3].

Complex genetic and environmental factors affect the risk of developing obesity. Evidence for a genetic contribution to obesity comes from animal models, monogenic obesity in humans, twin and adoption studies, and genome-wide association studies (GWAS). Single-gene mutations that cause obesity in mice or rats have long been known, establishing clearly that genetic mutations can cause obesity, and elucidating the pathways that control energy homeostasis [4]. Central among these is leptin, which is produced by adipose tissue in proportion to total body fat and acts in the hypothalamus to regulate energy intake and expenditure [5]. Cases of monogenic human obesity have been traced to mutations in LEPTIN, genes in the leptin signaling pathway, and other genes not directly implicated in leptin biology [6]. In the case of prohormone convertase 1 (PC1), monogenic obesity was identified in humans before a corresponding, obesity-causing mutation was identified in mice [7,8]. While these studies establish a genetic cause in rare, severe obesity, evidence for a genetic contribution to common obesity comes from adoption and twin studies, which show that 60–80% of variation in BMI is hereditary [9]. GWAS have identified many known and novel genes that contribute to variation in BMI. The genetic variants identified by GWAS so far, however, account for only a small percentage of the heritability in BMI [10].

One of the obesity loci identified by GWAS maps at or near the ADCY3 gene, which encodes a member of the adenylate cyclase (AC) family of proteins [11–14]. Additional genetic evidence supports an association between ADCY3 variation and obesity in both Swedish and Han Chinese populations [15,16]. Animal models have also highlighted the importance of Adcy3 signaling in energy homeostasis, with Adcy3-null mice having more fat mass under basal conditions and being more susceptible to obesity induced by high-fat feeding [17].

Proteins of the AC family catalyze the production of cAMP, a second messenger in many signal transduction pathways. Cyclic AMP is involved in the control of energy homeostasis by many neurotransmitters and (neuro)endocrine peptides including α-melanocyte-stimulating hormone (α-MSH), the Orexins, GLP-1, Ghrelin, and others [18,19]. Signaling from β-adrenergic receptors causes elevated cAMP and subsequent lipolysis in WAT and BAT; mice lacking all three β-adrenergic receptors (β1-, β2-, and β3-AR) have reduced metabolic rate and are slightly obese on chow, and massively obese on HFD [20]. Conversely, β3-adrenergic stimulation by a synthetic ligand promotes energy expenditure and protects against diet-induced obesity in both mice and primates [21]. Cyclic AMP responsive element binding protein (Creb) and Creb-regulated transcription co-activator 1 and...
3 (Crtc1 and 3) mediate transcriptional responses to cAMP. Crtc3 is important in attenuating β-adrenergic signaling in adipose tissue as Crtc3−/− mice have an increased response to catecholamine and are resistant to DIO [22]. Crtc1, on the other hand, is required for proper Leptin response within the hypothalamus and Crtc1−/− mice fail to up-regulate the anorexigenic peptides CARTP and Kiss1 in response to leptin, and are hyperphagic and obese [23].

Upon activation of beta-adrenergic receptors, heterotrimeric G-proteins dissociate and the α subunits signal to effector molecules. Gaα – encoded by the Gnas gene – is the stimulatory subunit that activates AC to promote production of cAMP. Deletion specifically of Gaα leads to mice that are obese and insulin resistant [24]. Knockout of Adcy3 itself also leads to obesity in mice [17]. cAMP activates AMP-dependent protein kinase (PKA), which is composed of two regulatory and two catalytic subunits. cAMP binds the regulatory subunits causing their dissociation from the catalytic subunits, which are then activated. Knockout of RIIb, a regulatory subunit of protein kinase A, leads to increased cAMP signaling and a lean mouse that is resistant to genetic or diet-induced obesity [25,26].

Thus, human genetics and animal models have converged on cAMP signaling in general, and Adcy3 in particular, as central players in energy homeostasis. Here we describe a novel allele of Adcy3, identified through forward genetic screening of mutagenized mice, which protects mice from diet-induced obesity. No difference in food intake was found between mutant mice and their wild-type littermates, but mutant mice expended more energy. The mutant allele was hyperactive relative to the wild-type allele in particular, as central players in energy homeostasis. Here we describe a novel allele of Adcy3, identified through forward genetic screening of mutagenized mice, which protects mice from diet-induced obesity. No difference in food intake was found between mutant mice and their wild-type littermates, but mutant mice expended more energy. The mutant allele was hyperactive relative to the wild-type allele in generating cAMP in response to forskolin.

Materials and Methods

Animals and ENU mutagenesis

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Use and Care Committee of the Scripps Research Institute or Genomics Institute of the Novartis Research Foundation. All mice were housed under a standard 12-hour light cycle with free access to food and water. A subset of mice were given a HFD, which derived 60% of its calories from fat (TD.06414, Teklad), beginning at three to five weeks of age. Body weight and body composition were assessed at two-week intervals from 5 to 17 weeks, or at six- to eight-week intervals from 17 to 54 weeks. Fat and lean mass of live mice was measured by NMR on an EchoMRI 3-in-1. Following completion of body composition analysis at 54 weeks, animals were sacrificed, and fat pads and other tissues were dissected for RNA analysis.

Body composition analysis

Male and female wild-type and heterozygous Jll littermates (N= 2 to 4 in each group), were weaned and placed on HFD at five weeks of age. Body weight and body composition were assessed at two-week intervals from 5 to 17 weeks, or at six- to eight-week intervals from 17 to 54 weeks. Fat and lean mass of live mice was measured by NMR on an EchoMRI 3-in-1. Following completion of body composition analysis at 54 weeks, animals were sacrificed, and fat pads and other tissues were dissected for RNA analysis.

Plasma metabolite and protein determinations

Animals were fasted for six hours prior to orbital bleed. Blood was collected by retro-orbital bleed and stored on ice in EDTA tubes. Plasma was assessed for leptin, insulin, glucose, and cholesterol levels by ELISA (insulin, leptin), or using an Olympus AU400 Autoanalyzer (glucose and cholesterol) as described [28].
Whole-animal metabolic measurements (indirect calorimetry)

Animals used were males, either wild-type (N = 7) or Adcy3\textsuperscript{Jll/+} heterozygous (N = 6), from the sixth generation of outcrossing to 129. Animals were weaned onto regular chow (not HFD) to minimize differences due to obesity. All animals were nine weeks old at the start of the experiment. Animals were housed separately for the experiment, and allowed to acclimate to new cages before the observation period. After an initial three-day acclimation period, observation and measurement lasted seven days, and took place at the TSRI Mouse Behavioral Assessment Core.

Statistical Analysis

All statistical analyses were done by Student’s t-test. In mice, all comparisons were within sex to wild-type mice. In other cases the relevant comparisons are described in the figure legend. Statistically significant differences are noted.

Results

\textbf{Jll} mutation dominantly suppresses diet-induced obesity

The \textit{Jll} phenotype was initially identified by screening families of ENU-mutagenized C57BL/6j mice for changes in body composition or fasting plasma insulin. All animals were placed on HFD at 4 weeks of age and then assessed for total body weight, fat mass, and insulin levels at 12 weeks of age. Family 755 was enriched for mice with low fat mass despite eight weeks of high-fat feeding (Figure 1A, left panel). The same mice also had low fasting insulin levels (Figure 1A, right panel).

Affected mice were either backcrossed to B6, to maintain mutant mice on a pure B6 background with minimal contributions from other ENU-induced mutations, or outcrossed to 129, to map the \textit{Jll} mutation on a hybrid B6.129 background. Since successive litters of outcrossed B6 mice contain roughly 50% affected animals (Figure S1A), the \textit{Jll} phenotype appeared to be dominantly inherited, such that mice heterozygous for the mutation were resistant to HFD-induced obesity.

\textit{Jll} mutant mice have less fat and improved metabolic parameters

The dominant \textit{Jll} mutation was mapped by outcrossing an affected (resistant to DIO) mouse from family 755 to 129. Outcrossing the affected F1 progeny to 129 generated N2 and N3 mice, which allowed genetic mapping of the mutation. Whole-genome scans were performed on B6.129 N2 and N3 mice, and resistance to DIO was compared to inheritance of B6-specific single nucleotide polymorphisms (SNPs). This revealed a strong correlation between inheritance of the low-fat-mass phenotype and inheritance of B6-specific SNPs from an approximately 5.7-Mb interval of chromosome 12, from 3.9 to 9.6 Mb (Figure S1B). This genomic region defined the minimal \textit{Jll} mutant interval. These SNP markers were then used to tag the mutated locus and allow further characterization of the \textit{Jll} phenotype on the B6.129 hybrid background.

Twelve-week-old \textit{Jll} heterozygous animals had significantly lower body weights and fat mass than did their wild-type siblings (p<0.001, Figure 1B), after eight weeks of high-fat feeding, without a difference detectable in lean mass. Genotype-specific differences in body and fat mass were much larger in males than females. Mice homozygous for the \textit{Jll}-containing mutant interval also had significantly lower total body mass and fat mass than wild-type mice (p<0.001, Figure 1B). Homozygous \textit{Jll/Jll} mice did not significantly differ from \textit{Jll/+} mice in body composition, confirming a dominant phenotype (Figure 1B). We therefore compare \textit{Jll/+} heterozygous animals to their wild-type counterparts hereafter.

Both males and females heterozygous for \textit{Jll} have substantially reduced percent body fat (fat mass as a percentage of total body mass, p<0.001, Figure 1C). Other metabolic parameters were also notably improved in B6.129 \textit{Jll} mutant mice: fasting plasma leptin levels were dramatically lower in \textit{Jll} heterozygous males (five-fold) and females (three-fold) relative to wild-type at p<0.001 (Figure 1D); and fasting levels of plasma insulin were significantly lower in \textit{Jll} heterozygous males versus wild-type males (p<0.05), and no difference was detectable in females (Figure S2A). No significant changes in plasma triglycerides, total cholesterol, or HDL cholesterol concentrations were detectable in \textit{Jll} heterozygotes (Figures S2B-D).

\textit{Jll} is a Met-to-Ile mutation in \textit{Adcy3}

Since the inheritance of the B6 alleles at the chromosome 12 locus reproducibly associated with the \textit{Jll} mutant phenotype, we analyzed genomic DNA from a mouse homozygous for B6 markers across this interval, using next-generation sequencing to assess single base changes across the entire 5.7-Mb \textit{Jll} region. Sequencing reads were then compared to GenBank reference genomic sequence to identify potential mutations. Table 1 lists all sequence variants present in at least five total reads, in which departure from reference sequence was present in at least 75% of reads.

Eleven variants meeting this standard were present, of which only one was present in protein-coding sequence, a G-to-A transition at base 827 of mouse \textit{Adcy3} (Table 1). This resulted in a methionine-to-isoleucine change at amino acid 279 (Figure 2A), which mapped to a relatively conserved region among mouse adenylate cyclases (Figure 2B, upper panel). The analogous region has been shown to be important for activity in other ACs. Met279 sits between the sixth transmembrane domain and the first catalytic C1 region, lying 10 amino acids amino-terminal to a known Gx2 -contacting residue [32]. Surprisingly, the particular amino acid at this position is not absolutely conserved among distant Adcy3 orthologs: while proximal amino acids are mostly invariant, and while Met279 is conserved in other vertebrates, both honeybees (\textit{Apis mellifera}) and fruit flies (\textit{Drosophila melanogaster}) have a leucine at this position (Figure 2B, lower panel).

In order to verify that this mutation does, in fact, confer the \textit{Jll} phenotype, body composition was measured in mice from the original (B6 background) family 755, that had been backcrossed to B6 for five generations. When maintained on HFD, mice heterozygous for the \textit{Adcy3\textsuperscript{M279I}} mutation had significantly lower total mass (p<0.001), absolute fat mass (p<0.001), and relative fat mass (p<0.001 for males and p<0.05 for females) than their wild-type littermates (Figure 2C, D). This is true for both males and females, and is indistinguishable from the \textit{Jll} phenotype observed in the mixed B6.129 background. The \textit{Adcy3\textsuperscript{M279I}} mutation is therefore henceforth referred to as \textit{Adcy3\textsuperscript{Jll}}.

\textit{Jll} mice do not expand their adipose tissue in response to HFD

To investigate the dynamic changes in body composition between \textit{Adcy3\textsuperscript{Jll/+}} and \textit{Adcy3\textsuperscript{M279I/+}} mice during growth, we placed mice on HFD for twelve weeks beginning at five weeks of age. Mice normally gain lean mass during growth and when challenged
with HFD, male and female Adcy3^{+/+} also rapidly gained fat mass (Figure 3A). Their Adcy3^{Jll/+} littermates however, did not accrue fat mass despite an almost identical gain in lean mass. Both male and female Adcy3^{Jll/+} mice had significantly lower total body weights and fat mass, compared to wild-type controls, after as little as four weeks on HFD. These differences grew even more pronounced as the 12-week-HFD analysis period progressed, with the fat mass in wild-type animals increasing more than 10-fold, while that in Adcy3^{Jll/+} mice remained virtually unchanged (Figure 3A). The difference in fat mass was also evident in dissected gonadal (gWAT, p<0.001) and inguinal (iWAT, p<0.01) fat pads (Figure S3B).

Cells in interscapular BAT were smaller in mutant than in wild-type mice, presumably due to a lesser accumulation of lipid on HFD (Figure 3B). A similar reduction of cross-sectional adipocyte area was also observed in heterozygous Jll white adipocytes from both the gonadal (p<0.001) and inguinal (p<0.01) white fat depots (Figure 3B and Figure S3C). We did not see evidence for
Whole-body metabolic measurements: Jll mice are more active and expend more energy

To better understand the effects of the Jll mutation on overall metabolism, we analyzed a total of 13 mice (seven wild-type and six Adcy3<sup>Jll</sup> heterozygous animals) for seven days using the Oxymax Comprehensive Live Animal Monitoring System. We chose young, age-matched animals that had been raised on chow, in an attempt to minimize changes in activity resulting from obesity, and better focus on the primary effects of the Jll mutation. The animals chosen did not differ significantly in overall body weight (Figure 4A), nor did we find a difference in food consumption between Adcy3<sup>+/+</sup> and Adcy3<sup>Jll/+</sup> mice (Figure 4B). There was however a small, but statistically significant, difference in activity that was detectable only during the dark phase (p < 0.05, Figure 4C). This may explain the increased energy expenditure (VO<sub>2</sub>) we observed in Adcy3<sup>Jll/+</sup> compared to Adcy3<sup>+/+</sup> mice, which was also detectable only in the dark phase (Figure 4D). This trend is more apparent when measured over a 48-hour period (p < 0.05, Figure 4E).

Adcy3<sup>Jll</sup> encodes a gain-of-function adenylate cyclase

Adenylate cyclase is activated by G<sub>a</sub> proteins, which promote the interaction of the cytoplasmic 1 and 2 domains (C1 and C2, respectively). Forskolin is a small-molecule activator of AC and acts similarly to G<sub>a</sub> proteins. We therefore transfected HuH7 cells with an expression plasmid containing the Jll mutation (M279I) into the promoter of the C1 domain. The Jll mutation increases cAMP production in cells transfected with any of the three expression constructs, but cells expressing Adcy3<sup>Jll</sup> are more responsive to forskolin than cells expressing Adcy3<sup>+/+</sup> or Adcy3<sup>+/+</sup> (Figure 5). Forskolin increases cAMP production in cells transfected with any of the three expression constructs, but cells expressing Adcy3<sup>Jll</sup> are more responsive to forskolin than cells expressing Adcy3<sup>+/+</sup>, demonstrating higher activity of the Jll allele, and consistent with the dominant nature of this allele.

**Discussion**

We have identified a dominant, gain-of-function point mutation in a component of the cAMP signaling pathway that promotes whole-body energy metabolism. Mice carrying one or more Adcy3<sup>Jll</sup> mutant alleles are protected from high fat diet-induced obesity, are more active, and have lower circulating leptin and insulin levels. We did not detect a difference in plasma TG between WT and HET mice on HFD, suggesting that VLDL production by the liver is normal and supporting the idea that the
difference between genotypes is in lipid catabolism. The \textit{Adcy3}\textsuperscript{Jll} mutation, though coding for a conservative amino acid substitution, increases \textit{Adcy3} activity in response to forskolin.

Following ligand engagement of a G protein-coupled receptor, cAMP acts as a second messenger when AC is activated by GS\textsubscript{a}. Cyclic AMP then activates cAMP-dependent protein kinase (PKA), which phosphorylates many substrates that affect energy, glucose, and lipid metabolism. The energy metabolism pathways affected by cAMP include \( \alpha \)-MSH, MCH, and the \( \beta \)-adrenergic pathway, all of which are important intermediates in leptin signaling in the hypothalamus. While \textit{Adcy3} expression is highest in BAT, it is also expressed in several regions of the brain, including hypothalamus. Furthermore, \textit{Jll} mice show no clear expansion of or increased activity – at the level of \textit{Ucp1} or \textit{Dio2} induction in BAT and no “browning” of WAT (Figure 3 and Figure S3). The site of action of \textit{Adcy3}\textsuperscript{Jll} therefore remains unresolved, though BAT and/or hypothalamus are likely candidates.

Uncertainty over the critical affected organ is also apparent for \textit{Prkar2b} knockout mice, which, like \textit{Adcy3}\textsuperscript{Jll} mice, are lean and protected from genetic and diet-induced obesity [25,26]. \textit{Prkar2b} encodes RII\textsubscript{b}, which is a regulatory subunit of PKA. Its knockout leads to an isoform switch from type II to type I PKA, which binds cAMP more avidly and is more readily activated. \textit{Ucp1} is induced in \textit{Prkar2b} knockout mice and metabolic rate and temperature are increased [25]. Double knockout mice, however, lacking both RII\textsubscript{b} and \textit{Ucp1}, retain the lean, resistant-to-DIO phenotype [33]. \textit{Ucp1} therefore is not necessary for the phenotype and BAT may not be involved. Indeed, re-expression of RII\textsubscript{b} in neurons but not adipocytes reversed the resistance-to-DIO phenotype, indicating that the brain is the critical site of cAMP signaling in \textit{Prkar2b} knockout mice [34].
Adcy3-/- mice are obese because mice eat more and are less active than their wild-type littermates [17]. Underlying the reduced activity and increased appetite may be the reduced response to injected leptin, which leads to appetite suppression and weight loss in wild-type mice, but not in Adcy3-/- littermates. In turn, this reduced leptin response may be attributed to reduced AC activity, which is apparent in hypothalami of Adcy3-/- compared to hypothalami of wild-type mice; even more apparent is reduced AC activity in response to forskolin. In the ventral medial hypothalamus, AC is expressed in primary cilia and its absence may be the reason for a lack of response to leptin [17]. Of course, none of this excludes a role for Adcy3 in BAT or other tissues and tissue-specific knockouts will be needed to determine the precise site of action of Adcy3.

In mammals, there are nine Adcy family members, each of which is composed of homologous amino- and carboxy-terminal portions, and each homologous half contains a cluster of six membrane-spanning domains and a large cytoplasmic loop, called C1 or C2 [35]. Enzymatic activity requires association of the C1 and C2 domains, which normally have a low affinity for one another. Both forskolin and GTP-bound Gsα promote the association of C1 and C2; GTP-Gsα also alters the shape of the active site to promote catalysis and this may be why forskolin and Gsα act synergistically [32]. The Jll mutation (M279I) lies in C1, just upstream of the cyclase catalytic domain and this region was not included in in vitro activity assays or in the crystal structures [32]. Given its proximity to Gsα and forskolin binding sites however, one would speculate that it affects binding of one or both of these activators.

Human genetics and gain- and loss-of-function alleles in the mouse have identified Adcy3 as a central, non-redundant player in energy homeostasis. The challenges that now remain include elucidating the precise site of action and mechanism of Adcy3 that make it an important contributor to obesity or resistance to obesity, identifying the exact genetic variation in human populations that contributes to or protects from obesity, and designing pharmacological interventions based on Adcy3 that promote fitness. While isoform-specific activators or inhibitors of Adcy3...
seem unlikely as the active site is highly conserved, a specific inhibitor has recently been identified [36]. It has also recently become apparent that this family of enzymes is subject to regulation by allosteric effectors [37]. One or more of these approaches may allow Adcy3-targeted therapies to slow the obesity epidemic.

Supporting Information

Figure S1 Discovery of Jll. A: Sustained detection of affected mice in outcrossed Jll litters. Outcrossing of Jll (Family 755) to the 129 background results in approximately half the progeny with low fat mass (2.5 g or below), at both the second generation of outcrossing (N2) and the fifth (N5). In contrast, a broad distribution of fat masses is observed in N2 progeny when wild-type (non-ENU-treated) B6 animals are outcrossed to 129 (Control). B: Genome-wide SNP analysis reveals the interval containing Jll on mouse chromosome 12. Inheritance of one copy of B6-specific SNPs between 3912084 Mb and 9569145 Mb on mouse chromosome 12 correlates with the Jll low fat (fat mass < 5 g) and low % fat (below 20%) phenotypes. Living individual animals were phenotyped for fat and lean mass, insulin, and glucose. Genomic tail DNA was extracted and sequenced for an array of SNPs that diverge between the B6 and 129 backgrounds, to detect inheritance from either the ENU-mutagenized (B6) and outcross stock (129) parents. SNPs homozygous for 129 markers are shown in red and marked “C,” SNPs that showed heterozygosity for B6 and 129 markers are shown in orange and marked “H.” “Affected” (aff) and “unaffected” (unaff) phenotypic calls were made based on fat mass, then affected animals were sorted to be on the left of the table, to assist visualizing the genomic interval. The flanking boundaries of the interval are marked with heavy lines.

Figure S2 Assessment of additional metabolic parameters in Jll mice. Plasma was prepared from retro-orbital blood of wild-type (WT) and heterozygous Adcy3Jll/+ (HEt) mice fed a HFD, and assessed for levels of other metabolically relevant markers. A modest decrease in fed glucose levels (A) was detectable. Both sets of animals consumed similar amounts of food during the seven days of the experiment. C. Increased movement in HET animals was visible, only in the dark phase. D. HET animals consumed more oxygen during the dark phase than WT animals. E. The increased level of O2 consumption (VO2) in HET animals, particularly in the dark, is visible in this 48-hour observation period. All data are shown as mean +/- SEM, * p< 0.05 by one-tailed Student’s t-test. doi:10.1371/journal.pone.0110226.g004

Figure 4. Increased oxygen consumption in Jll mice. A. Young, male, chow-fed B6.129 N6 animals were used in this experiment, to minimize activity differences resulting from obesity. No significant difference in body weight between Adcy3+/+ (WT, black) and Adcy3Jll/+ (HET, gray) mice was detectable. B. Both sets of animals consumed similar amounts of food during the seven days of the experiment. C. Increased movement in HET animals was visible, only in the dark phase. D. HET animals consumed more oxygen during the dark phase than WT animals. E. The increased level of O2 consumption (VO2) in HET animals, particularly in the dark, is visible in this 48-hour observation period. All data are shown as mean +/- SEM, * p< 0.05 by one-tailed Student’s t-test. doi:10.1371/journal.pone.0110226.g004
levels of triglycerides (B), total cholesterol (C) or HDL-cholesterol (D). All data are shown as mean +/− SEM. * p<0.05, ** p<0.01, *** p<0.001. (TIF)

Figure 5. Jll expression leads to increased Adcy3 signaling. Treatment of HuH7 cells transiently co-transfected with both a cAMP-responsive luciferase reporter and an empty expression vector (Empty vector), a wild-type mouse Adcy3 cDNA expression vector (Adcy3-WT), or the same vector with a Jll mutation substituted (Adcy3-Jll), revealed increased reporter activity in response to increasing doses of forskolin. For each expression vector used, activity was normalized to the level observed when cells were treated with vehicle (DMSO) alone. All data are shown as mean +/− SEM. ** - p<0.01, *** - p<0.001. doi:10.1371/journal.pone.0110226.g005

Author Contributions
Conceived and designed the experiments: JLP MGW NG. Performed the experiments: RJL RJG NG. Analyzed the data: JLP MGW JBW RJG NG. Contributed reagents/materials/analysis tools: DJL RJG. Wrote the paper: JLP NG.

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References
1. Nguyen DM, El-Serag HB (2010) The epidemiology of obesity. Gastroenterol Clin North Am 39: 1–7.
2. McGee DL, Diverse Populations C (2005) Body mass index and mortality: a meta-analysis based on person-level data from twenty-six observational studies. Ann Epidemiol 15: 87–97.
3. Field AE, Cookley EH, Must A, Spadano JL, Laird N, et al. (2001) Impact of overweight on the risk of developing common chronic diseases during a 10-year period. Arch Intern Med 161: 1581–1586.
4. Leibel RL, Chung WK, Chua SC Jr (1997) The molecular genetics of rodent single gene obesity. J Biol Chem 272: 31937–31940.
5. Friedman JM (2009) Leptin at 14 y of age: an ongoing story. Am J Clin Nutr 89: 973S–978S.
6. O’Rahilly S (2009) Human genetics illuminates the paths to metabolic disease. Nature 462: 307–314.
7. Jackson RS, Creemers JW, Oghazi S, Raflin-Sanson ML, Sanders L, et al. (1997) Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. Nat Genet 16: 303–306.
8. Lloyd BJ, Bohan S, Gekakis N (2006) Obesity, hyperphagia and increased metabolic efficiency in Pe1 mutant mice. Hum Mol Genet 15: 1894–1895.
9. Hanoune J, Defer N (2001) Regulation and role of adenyl cyclase isoforms. Annu Rev Pharmacol Toxicol 41: 145–174.
10. Xiao Q, Grant SF (2013) The genetics of human obesity. Annu N Y Acad Sci 1251: 178–190.
11. Speleios EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, et al. (2010) Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet 42: 937–948.
12. Wen W, Cho YN, Zheng W, Dorapoo R, Kato N, et al. (2012) Meta-analysis identifies common variants associated with body mass index in east Asians. Nat Genet 44: 307–311.
13. Monda KL, Chen GK, Taylor KC, Palmer C, Edwards TL, et al. (2013) A meta-analysis identifies new loci associated with body mass index in individuals of African ancestry. Nat Genet 45: 690–696.
14. Coussinier DL, Bery DJ, Timpson NJ, Ang W, Thiering E, et al. (2013) Genome-wide association and longitudinal analyses reveal genetic loci linking pubertal height growth, pubertal timing and childhood adiposity. Hum Mol Genet 22: 2733–2747.
15. Nordman S, Absalat A, Hilinding A, Langberg EC, Humphreys K, et al. (2008) Genetic variation of the adenyl cyclase 3 (AC3) locus and its influence on type 2 diabetes and obesity susceptibility in Swedish men. Int J Obes (Lond) 32: 407–412.
16. Wang H, Wu M, Zhu W, Shen J, Shi X, et al. (2010) Evaluation of the Association between the AC3 Genetic Polymorphisms and Obesity in a Chinese Han Population. PLoS ONE 5: e13851.
17. Wang Z, Li V, Chen GC, Phan T, Nudelman AS, et al. (2009) Adult type 3 adenyl cyclase-deficient mice are obese. PLoS ONE 4: e6979.
18. Xu TR, Yang Y, Ward R, Gao L, Liu Y (2013) Orexin receptors: Multi-functional therapeutic targets for sleeping disorders, eating disorders, drug addiction, cancers and other physiological disorders. Cell Signal.
19. Wheeler MB, Lu M, Dillon JS, Leng XH, Chen C, et al. (1993) Functional expression of the rat glucagon-like-peptide-1 receptor, evidence for coupling to both adenylyl cyclase and phospholipase-C. Endocrinology 133: 57–62.
20. Bachman ES, Dhallon H, Zhang CY, Cinti S, Bianco AC, et al. (2002) betaAR signaling required for diet-induced thermogenesis and obesity resistance. Science 297: 843–845.
21. Nagase I, Yoshihira T, Kumamoto K, Umemura T, Sakane N, et al. (1996) Expression of uncoupling protein in skeletal muscle and white fat of obese mice treated with thermogenic beta 3-adrenergic agonist. J Clin Invest 97: 2898–2904.
22. Song Y, Altarejos J, Goedzius MO, Issue H, Guo X, et al. (2006) The CRTC3 coactivator Crtc1 is required for energy balance and fertility. Nat Med 12: 1112–1117.
23. Chen M, Gavrilova O, Liu J, Xie T, Deng C, et al. (2005) Alternative Gnas gene products have opposite effects on glucose and lipid metabolism. Proc Natl Acad Sci U S A 102: 7386–7391.
24. Cummings DE, Brandon EP, Plans Jv, Motamed K, Idereda RL, et al. (1996) Genetically lean mice result from targeted disruption of the RIIbeta subunit of protein kinase A. Nature 382: 622–626.
25. Newhall KJ, Cummings DE, Nolan MA, McKeown GS (2005) Deletion of the RIIbeta-subunit of protein kinase A decreases body weight and increases energy expenditure in the obese, leptin-deficient ob/ob mouse. Mol Endocrinol 19: 982–991.
26. Lloyd DJ, Hall FW, Tarantino LM, Gekakis N (2005) Diabetes insipidus in mice with a mutation in aquaporin-2. PLoS Genet 1: e20.
27. Lloyd DJ, Wheeler MC, Gekakis N (2010) A Point Mutation in Sec61alpha1 Leads to Diabetes and Hepatosteatosis in Mice. Diabetes 59: 460–470.
28. Choe M, Scollo UI, Ji W, Liu T, Tikhonova IR, et al. (2009) Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. Proc Natl Acad Sci U S A 106: 19096–19101.
30. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437: 376–380.
31. Conkright MD, Guzman E, Flechner L, Su AI, Hogenesch JB, et al. (2003) Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness. Mol Cell 11: 1101–1108.
32. Tesmer JJ, Sunahara RK, Gilman AG, Sprang SR (1997) Crystal structure of the catalytic domains of adenyl cyclase in a complex with Galpha.GTPgammaS. Science 278: 1907–1916.
33. Nolan MA, Sikorski MA, McKnight GS (2004) The role of uncoupling protein 1 in the metabolism and adiposity of RII beta-protein kinase A-deficient mice. Mol Endocrinol 18: 2302–2311.
34. Zheng R, Yang L, Sikorski MA, Eams LC, Czyzyk TA, et al. (2013) Deficiency of the RIIbeta subunit of PKA affects locomotor activity and energy homeostasis in distinct neuronal populations. Proc Natl Acad Sci U S A 110: E1631–1640.
35. Hurley JH (1999) Structure, mechanism, and regulation of mammalian adenyl cyclase. J Biol Chem 274: 7599–7602.
36. Conley JM, Brand CS, Bogard AS, Pratt EP, Xu R, et al. (2013) Development of a high-throughput screening paradigm for the discovery of small-molecule modulators of adenyl cyclase: identification of an adenyl cyclase 2 inhibitor. J Pharmacol Exp Ther 347: 276–287.
37. Seifert R, Beste KY (2012) Allosteric regulation of nucleotidyl cyclases: an emerging pharmacological target. Sci Signal 5: pe37.