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A Stratified Transcriptomics Analysis of Polygenic Fat and Lean Mouse Adipose Tissues Identifies Novel Candidate Obesity Genes

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Abstract

Background: Obesity and metabolic syndrome results from a complex interaction between genetic and environmental factors. In addition to brain-regulated processes, recent genome wide association studies have indicated that genes highly expressed in adipose tissue affect the distribution and function of fat and thus contribute to obesity. Using a stratified transcriptome gene enrichment approach we attempted to identify adipose tissue-specific obesity genes in the unique polygenic Fat (F) mouse model. The model allowed us to further filter genes for increased likelihood of being causal or secondary for obesity. This successfully identified several genes previously linked to obesity (C1qr1 and Np3r) as positional QTL candidate genes elevated specifically in F line adipose tissue. A number of novel obesity candidate genes were also identified (Thbs1, Ppp1r3d, Tmepai, Tp53inp2, Ttc7b, Tubalga, Fgf13, Fmr) that have inferred roles in fat cell function. Quantitative microarray analysis was then applied to the most phenotypically divergent adipose depot after exaggerating F and L strain differences with chronic high fat feeding which revealed a distinct gene expression profile of line, fat depot and diet-responsive inflammatory, angiogenic and metabolic pathways. Selected candidate genes Npr3 and Thbs1, as well as Gys2, a non-QTL gene that otherwise passed our enrichment criteria were characterised, revealing novel functional effects consistent with a contribution to obesity.

Results: To enrich for adipose tissue obesity genes a ‘snap-shot’ pooled-sample transcriptome comparison of key fat depots and non adipose tissues (muscle, liver, kidney) was performed. Known obesity quantitative trait loci (QTL) information for the model allowed us to further filter any genes that were identified as positional QTL candidate genes and thus narrow down the list of genes that are most likely to be causally related to obesity. This analysis identified several genes that have previously been linked to obesity (C1qr1 and Np3r) as well as several novel obesity candidate genes in the distinct F line adipose depot. Quantitative microarray analysis was then applied to the most divergent adipose depot after exaggerating F and L strain differences with chronic high fat feeding which revealed a distinct gene expression profile of line, fat depot and diet-responsive inflammatory, angiogenic and metabolic pathways. Selected candidate genes Npr3 and Thbs1, as well as Gys2, a non-QTL gene that otherwise passed our enrichment criteria were characterised, revealing novel functional effects consistent with a contribution to obesity.

Conclusions: A focussed candidate gene enrichment strategy in the unique F and L model has identified novel adipose tissue-enriched genes contributing to obesity.

Introduction

Obesity and its co-associated metabolic diseases result from a complex interaction between environmental and genetic factors [1–3]. The polygenic Fat (F) and Lean (L) mouse models were selectively bred for divergent body fat mass [4] and thus model complex polygenic human obesity. Four major obesity QTLs in the F line were initially described in an F2 cross of the out-bred F and L models and thus model complex polygenic human obesity. Four major obesity QTLs in the F line were initially described in an F2 cross of the out-bred F and L models and thus model complex polygenic human obesity. Four major obesity QTLs in the F line were initially described in an F2 cross of the out-bred F and L models and thus model complex polygenic human obesity. The polygenic Fat (F) and Lean (L) mouse models were selectively bred for divergent body fat mass [4] and thus model complex polygenic human obesity. Four major obesity QTLs in the F line were initially described in an F2 cross of the out-bred F and L models and thus model complex polygenic human obesity. The polygenic Fat (F) mouse strain generated by selective breeding over 60 generations for divergent adiposity from a common ancestor is one of the most extensively studied obesity models [5]. Indeed, F mice have a lower caloric intake than L mice [6,7]. Since the divergent adiposity of the model was selected using fat pad mass rather than food intake, and because several studies, including recent meta-genome wide association, link lipid metabolism and intrinsic molecular pathways within the adipose tissue to fat mass regulation [8–12] we reasoned that the F and L adipose tissue would be a rich resource for identifying obesity-susceptibility genes.

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We describe a stratified microarray analysis of gene expression using first a qualitative, comparative 'snap-shot' pooled transcriptomic approach across several adipose tissue depots and non-adipose metabolic tissues to broadly identify adipose tissue-specific gene expression differences between F and L lines. We used previously defined QTL information [3] to enrich for genes with an increased causal likelihood. This was followed by quantitative gene chip validation in subcutaneous fat, where the largest divergence in fat mass in response to chronic high fat (HF) feeding was observed [13]. Having defined adipose-tissue specific pathways and positional candidate obesity genes we performed real time PCR (RT-PCR) validation of key affected pathways. We then functionally linked two of the QTL-associated candidate obesity genes – and one non-QTL associated gene that had an expression profile parallel to that of the candidate genes – to fat cell function.

Results and Discussion

Insights into adipose tissue and depot-specificity of gene expression: Snap-shot results

We applied a 4 step filtering strategy to narrow our search for key adipose-specific obesity-associated genes (Figure 1) as described in Methods and Materials. The number of genes that were differentially expressed between the lines across any of the three fat depots (3WATs) was markedly reduced after the first filter was applied to enrich for genes co-ordinately regulated in 3WATs (Table 1, Figure 1). Notably, in terms of overall adiposity (3 WATs) there were 10 times as many genes elevated in F (102) than L mice (9). This may reflect that causal gene variants raising the upper set-point of body fat mass are not as strongly proscribed, in the absence of predation, in contrast to genes conferring a potentially threatening low fat mass [14]. Despite EPI fat being the sole selection criteria for the first 20 generations of the breeding [4] there were fewer differences in gene expression in the EPI of F mice (300 versus SC 426 and MES 450). This may reflect the induction of secondary inflammatory, metabolic, angiogenic and tissue remodelling programmes that are more pronounced in the F line SC and MES adipose tissues (see below). This is also consistent with the SC being the most divergently HF responsive tissue [13] and with visceral (MES) fat being highly vascularised and immune cell-rich. In contrast, twice as many differentially expressed genes were elevated in the EPI of L mice (104 versus SC 262 and MES 201) which may reflect an EPI depot-selective process that is relatively quiescent in other L fat depots.

The genes found by the relaxed 3WAT criteria were exported to Webgestalt and screened against the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Gene Ontology database to look for enrichment of functional pathways that were over-represented in the F adipose tissue (Table 2). Database for Annotation, Visualization and Integrated Discovery (DAVID) was also used for pathway analysis with similar findings (data not shown).

Table 2 shows the list of genes identified from more stringent criteria (>2-fold difference between the lines in 3WATs) that were additionally positioned within major QTL boundaries [5]. This further reduced the chance of selecting functionally neutral or secondary genes. Three of these genes (C1qr1, Np3r and Thbs1) have been previously linked to obesity or adipose tissue function (Table 3). Several novel adipose-tissue specific mechanisms potentially contributing to fat mass accumulation and/or its associated metabolic consequences are inferred from functions ascribed to the other genes or closely-related functionally characterised genes or disease processes (Table 3).

'Snap-shot' filters for non-adipose specific divergently expressed genes

The base population for the F and L strains was created by crossing two inbred and one out-bred strain [4]. Line-specific
differences in gene expression that are potentially unrelated to adiposity (neutral) may therefore co-segregate with QTLs in an F2 cross or are secondary due to vast differences in final phenotype/pathology between the lines. Differential expression due to sequence divergence between F and L genomes may also result in false-positive expression differences due to altered microarray hybridisation efficiency. To help address this, the ‘snap-shot’ analysis was used to exclude genes that were differentially expressed across all tissues. Thus, gene expression was compared between 3 representative adipose depots versus muscle, liver and kidney using tissues pooled from 3 mice from each line as described in Materials and Methods. Examples of divergently expressed, but non-adipose-specific, genes (Depdc6 [15,16], Gsn, Table 4) were not further pursued in the present study, although we do not discount their potential functional relevance to the phenotype of F or L lines [17].

Modelling a gene-by-diet interaction: further insight from quantitative microarray

To model a gene-by-diet interaction and potentially amplify responses of obesity-susceptibility genes we performed quantitative analysis (n = 4 per group) of the transcriptome in the SC fat depot. SC fat was chosen as it showed the greatest phenotypic divergence (fat mass) in response [13] to the obesigenic stimulus of chronic HF feeding (Figure 2, Table 5). Notably, with the statistical power of the quantitative microarray, far fewer genes were differentially expressed in subcutaneous fat between the lines under control conditions (Table 1) demonstrating the importance of the quantitative approach for reducing false positives. The baseline gene expression differences were clearly amplified by diet (Table 5). As with basal differences between the lines, the F line had a more marked gene expression response to the diet than the L line (Figure 2, Table 5). The top functional pathways affected in F line obesity are listed in Table 2 and reflect an accentuation of changes in angiogenesis, cell migration, UDP-glycosylation, prostaglandin synthesis, triglyceride and glycogen synthesis, collagen formation, ER and stress fibre pathways and membrane peptidases.

In the snapshot data, enriched categories are shown for genes differentially expressed in all three white adipose tissues (3WAT; >2-fold; >100AU in higher expressing strain) between fat and lean mice. In the quantitative array data, enriched categories are shown for genes differentially expressed between Fat mice on a high fat diet and Fat mice on a control diet (>2-fold; p-value <0.05, >100AU in higher condition). GOBP = Gene Ontology Biological Process; KEGG = Kyoto Encyclopedia of Genes and Genomes pathway database. Data analysed in Webgestalt, similar results using DAVID and Metacore (not shown).

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Table 2. Gene set enrichment analyses of ‘snapshot’ and quantitative array data.

| Database           | Category                                | Observed number | Expected Number | P-Value |
|--------------------|-----------------------------------------|-----------------|-----------------|---------|
| **Upregulated 3WAT (368 genes)** | GOBP fat cell differentiation             | 8               | 1               | 0.002   |
|                    | GOBP cell adhesion                       | 22              | 7               | 0.002   |
|                    | GOBP cell death                         | 26              | 11              | 0.007   |
|                    | GOBP cell-matrix adhesion                | 6               | 1               | 0.007   |
|                    | KEGG ECM-receptor interaction            | 10              | 1               | 2e-6    |
|                    | KEGG PPAR signaling pathway              | 6               | 1               | 0.005   |
| **Downregulated 3WAT (113 genes)** | GOBP antigen processing and presentation of peptide antigen | 5               | <1              | 0.0001  |
|                    | GOBP immune response                     | 11              | 2               | 0.0005  |
|                    | KEGG Antigen processing and presentation | 4               | <1              | 0.0008  |
| **Upregulated 3WAT not regulated in liver, muscle, kidney (165 genes)** | GOBP cell adhesion             | 13              | 4               | 0.05    |
|                    | KEGG ECM-receptor interaction            | 7               | <1              | 1e-5    |
| **Downregulated 3WAT not regulated in liver, muscle, kidney (14 genes)** | No significant enrichment           |                 |                 |         |

In the quantitative array data, enriched categories are shown for genes differentially expressed between Fat mice on a high fat diet and Fat mice on a control diet (>2-fold; p-value <0.05, >100AU in higher condition). GOBP = Gene Ontology Biological Process; KEGG = Kyoto Encyclopedia of Genes and Genomes pathway database. Data analysed in Webgestalt, similar results using DAVID and Metacore (not shown).

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Validation of key changes in gene expression within the context adipose tissue dysfunction in obesity

Line differences in key genes involved in the inflammatory (Figure 3), metabolic and anti-oxidant (Figure 4) and hypoxia/angiogenic (Figure 5) pathways were validated by RT-PCR in a larger cohort (n = 8–11). This gave insight into line and diet-specific regulation of key genes contributing to obesity and the...
associated functional changes in the adipose tissue in the most phenotypically divergent fat depot.

Line and diet effects on inflammatory genes

Our analyses revealed a clear line divergence of inflammatory gene expression with distinct and sometimes unexpected HF diet responses. Most of the genes in this pathway were not obesity QTL-associated and hence represent secondary responses (Figure 3 and microarrays). Many inflammatory genes that were more highly expressed in F adipose tissue have been linked with obesity and/or insulin resistance, \(\text{Ccl3/MIP1a}^\text{[18]}\), \(\text{C3ar1}^\text{[19]}\), \(\text{Ccl2/MCP1}^\text{[20]}\), \(\text{spp1/osteopontin}^\text{[21]}\). Others appear to link obesity and inflammation with vascular function and haemostasis \(\text{Ptgds2}\), \(\text{Tbaxs1}\), and \(\text{Pla2g7}^\text{[note Pla2g16 has been linked with obesity ref. 10]}\), \(\text{Tlr13}\). Surprisingly, \(\text{Tlr13}\) (mapping to \(\text{Fob4}\)) expression, but not the \(\text{Tlr2}\) or \(\text{Tlr4}\) genes encoding the canonical proinflammatory bacterial lipotoxin receptors (e.g. lipopolysaccharide) recently shown to mediate adipocyte pro-inflammatory and insulin

Table 3. Genes with increased causal likelihood as indicated by a stringent stratified criteria.

| Gene (Mean fold-expression difference across 3 WATs) | function(s)? (gene functions abridged from iHOP: http://www.ihop-net.org). Some functions are inferred from associated phenotypes and are denoted by (?) | Linked with obesity? (mean expression intensity across 3WAT ‘snap-shot’ arbitrary units) | [Refs] |
|---|---|---|---|
| Fob1 | Thbs1 (3.4) Angiogenesis, platelet and macrophage function, fibrogenesis Yes \(~\sim 402\) | [39–42] |
| C1qr1 (2) Mac/NC cell phagocytosis, lectin binding Yes \(~\sim 300\) | [19] |
| Ppp1r3d (2.5) Regulatory subunit of phosphatase1, glyocogen synthesis. There are \(~\sim 16\) Ppp1rs No \(~\sim 100\) | [61: Ppp1r3a] |
| Tmepai (4) Transmembrane egf/androgen response, cell cycle. Obesity and androgen signalling (?) No \(~\sim 390\) | [62] |
| Tp53inp2 (3) Embryogenesis: neural tissue, p53-inducible No \(~\sim 3060\) | [63] |
| Fob2 | Ttc7b (2.5) Unknown. Related WD40/tetracopeptide involved in lipid storage(?) No \(~\sim 560\) | [64] |
| Tuba1e (2) Cytoskeleton/structural/transport/lipid droplet-associated No \(~\sim 4265\) | [65] |
| Fob3 | Npr3 (7) Natriuretic peptide clearance receptor Yes \(~\sim 560\) | [36–37] |
| Fob4 | Fgf13 (8) Morphogenesis, tissue repair. Other Fgfs have a role in adipose tissue and metabolism? No \(~\sim 425\) | [66–67] |
| Frmr1 (4.5) Cell signalling, RNA binding, neuronal: Related to Prader-Willi –like obesity syndrome in fragile X syndrome? No \(~\sim 1485\) | [68–69] |

A stringent search, based on QTL boundaries \(\text{(Horvat et al., 2000, ref 5)}\) produces a limited set of genes with \(\geq 2\) fold increased expression levels in all 3 WATs of F when the difference in gene expression is set between \(\sim 2\) and \(\sim 2\) in muscle, liver and kidney. Only genes with a higher mean intensity of 100 a.u. in the 3 WATs were selected (more likely to be of functional relevance in adipose tissue).

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Table 4. Exclusion of non-adipose-specific (all-tissue) divergently expressed genes.

| Gene | SC | EPI | MES | LIV | MUSCLE | KIDNEY |
|---|---|---|---|---|---|---|
| Depdc6 F-line gene expression | | | | | | |
| Fat | 1392 | 1506 | 947 | 791 | 888 | 263 |
| Lean | 10 | 11 | 14 | 18 | 12 | 12 |
| fold-change | 138 | 133 | 68 | 44 | 75 | 23 |
| Gsn L-line gene expression | | | | | | |
| Fat | 10 | 6 | 6 | 9 | 9 | 6 |
| Lean | 8455 | 10420 | 4408 | 51 | 444 | 227 |
| fold-change | \(-864\) | \(-1646\) | \(-613\) | \(-6\) | \(-444\) | \(-41\) |

Numbers represent absolute chip expression intensity (AU) values from pooled transcriptome Genechip 2.0 array of subcutaneous (SC) epididymal (EPI) mesenteric (MES) and liver, muscle and kidney. Note, due to the mean expression intensity being \(<100\) AU, \(\text{Depdc6}\) and \(\text{Gsn}\) are effectively switched on in the Fat and Lean line tissues, respectively. Positive fold-change numbers indicate elevated in F line. Negative Ratio numbers indicate elevated in L line.

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resistance effects of free fatty acids [22], was higher in F adipose tissue. Tlr13 is most closely related to Tlr3 that modulates innate immune responses [23] and, unlike TLRs1-9 [24], has not been linked to adipose tissue biology. Of note, Tlr13 mRNA expression is higher in extreme obesity/diabetes mouse strains (Supplementary Figure S1). Notably, some immunomodulatory genes were expressed more highly in L adipose tissue indicating an alternate inflammatory response (Ccl5/RANTES [25]).

Striking diet-sensitive gene expression patterns were also observed. For example, mRNA levels for Gz2 (CD21) and Ccl19 (MIP3β) that link innate and adaptive immunity and dendritic cell migration [26] were markedly suppressed by HF diet in the F line. In contrast, HF diet induced proinflammatory Ptger3 expression in F adipose tissue but suppressed obesity-associated [27] serum proinflammatory Saa3 expression. The divergence in discrete immune responses may help identify pathological versus reparative processes and informs the simplistic view of a general increase in inflammatory gene expression in adipose tissue in obesity.

Overall we saw evidence of a selectively activated proinflammatory adipose environment which is modulated by HF diet in F adipose tissue but also an anti-inflammatory profile in adipose tissue of obesity-resistant L mice with very little response to HF diet.

Line and diet effects on metabolic genes

We found a major divergence in gene expression for lipid and carbohydrate metabolism (Figure 4 and microarray). Genes involved in fat oxidation (Cpt1) and energy dissipation (Ucp2) were...
increased (mean intensity fold-change of up to lactate for intermediary metabolism in the development of obesity. Consistent with a major, though likely secondary role for genes of (non-QTL) but adipose-specific change in adipose tissue intermediary metabolism.

2.6) but not in liver muscle or kidney, exemplifying a secondary feature of adipose tissue in obesity [9]. Lower mRNA levels of the obesity-induced gene. Altered retinol metabolism appears to be a path that showed the most divergent response to high fat (HF) feeding. FC; Fat line control diet, FF; Fat line HF diet, LC; Lean line control diet, LF; Lean line HF diet. The left column shows the total number of differentially expressed genes (Genome wide) and then those that lie within the 95% confidence interval of the major QTLs Fob1-4 [5]. This number is also expressed as a percentage of the Genome wide number (%).

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Table 5. Quantitative gene-chip analysis results showing the numbers of genes differentially expressed >2 fold with an adjusted p = value<0.05, and where the mean expression is >100 a.u. intensity for the higher group.

| number of upregulated genes matching criteria | Genomewide | Fob1 (%) | Fob2 (%) | Fob3 (%) | Fob4 (%) |
|---------------------------------------------|------------|---------|---------|---------|---------|
| FC vs LC                                    | 98         | 3(3)    | 0(0)    | 0(0)    | 5(5)    |
| FF vs LF                                    | 254        | 13(5)   | 4(2)    | 3(1)    | 12(4)   |
| FF vs FC                                    | 95         | 4(4)    | 0(0)    | 2(2)    | 5(5)    |
| LF vs LC                                    | 27         | 0(0)    | 0(0)    | 2(7)    | 3(1)    |

| number of downregulated genes matching criteria | Genomewide | Fob1 (%) | Fob2 (%) | Fob3 (%) | Fob4 (%) |
|------------------------------------------------|------------|---------|---------|---------|---------|
| FC vs LC                                    | 87         | 7(8)    | 2(2)    | 1(1)    | 2(2)    |
| FF vs LF                                    | 225        | 6(3)    | 2(1)    | 5(2)    | 7(3)    |
| FF vs FC                                    | 21         | 0(0)    | 0(0)    | 1(5)    | 1(5)    |
| LF vs LC                                    | 16         | 1(6)    | 0(0)    | 2(13)   | 0(0)    |

Upregulated (A) refers to genes that are higher in the first group in the row compared to (vs) the second group in the row. Downregulated (B) refers to genes that are lower in the first group in the row compared to (vs) the second group in the row. For example upregulated in FC vs LC means higher expression in Fat line Control fed than in (vs) Lean line Control fed. Only diet based (high fat v control diet) and strain based (Fat v Lean) comparisons are shown. All results are from the subcutaneous fat pad that showed the most divergent response to high fat (HF) feeding. FC; Fat line control diet, FF; Fat line HF diet, LC; Lean line control diet, LF; Lean line HF diet. The left column shows the total number of differentially expressed genes (Genome wide) and then those that lie within the 95% confidence interval of the major QTLs Fob1-4 [5]. This number is also expressed as a percentage of the Genome wide number (%).

Pl применяется в крае сенсорной митохондрии (гликолиз в цикл сукцинат) – был выше в F adipose tissue (4). Увеличение Rbp4 может быть включено в oXeO-индуцированную в F adipose tissue и было в дальнейшем связано с HF. Rbp7, который представляет собой новый seO-индуцированный ген. Адаптированный RETINOL metabolism appears to be a feature of adipose tissue in obesity [9]. Lower mRNA levels of the HDL-associated anti-oxidant factor Poni 31 [31] were found in F adipose tissue. HF diet suppressed Poni further in F adipose tissue but induced Poni expression in the L adipose tissue (Figure 4) suggesting an enhanced anti-oxidant response in L adipose associates with their improved metabolic profile.

Consistent with impaired carbohydrate metabolism, pyruvate dehydrogenase kinase 4 (Pdk4) mRNA levels – encoding a protein that inactivates pyruvate dehydrogenase and hence the initial step in mitochondrial (glycolysis to citrate cycle) oxidation – was higher in F adipose tissue (Figure 4). Indeed, Pdk4 was higher across all 3WATs in F adipose tissue (3WAT averaged mean intensity fold-change: 2.6) but not in liver muscle or kidney, exemplifying a secondary (non-QTL) but adipose-specific change in adipose tissue intermediary metabolism. Ldh2 encoding lactate dehydrogenase (pyruvate to lactate for de novo lipogenesis) showed a similar a 3WAT-specific increase (mean intensity fold-change of ~5) in F adipose tissue, consistent with a major, though likely secondary role for genes of intermediary metabolism in the development of obesity.

Line and Diet Effects on angiogenic/hypoxia genes

Key changes were observed in the angiogenesis pathway (Angi1, Angi4 and Angi1; Hif1a, Apg4, Hif2b) in F adipose tissue (Figure 5 and microarray) consistent with adipose tissue hypoxia being an early molecular link between obesity and insulin resistance [32–34]. Distinct matrix metalloproteases associated with the remodeling of adipose tissue [35] were selectively elevated in F (Mmp3, Mmp12) or L (Mmp9) adipose tissue (Figure 5 and microarray). Thus, alternate inflammatory, angiogenic and adipose remodeling mechanisms are active in the two lines that may impact upon the severity of metabolic disease.

Functional validation of key divergently expressed genes

To further validate our stratified obesity gene enrichment approach we investigated the functional impact of 3 genes implicated in F-line obesity on fat cell function. We chose natriuretic peptide receptor C (Npr3), a gene associated with the Fob3b QTL (Figure 6) and thrombospondin-1 (Thbs1; Figure 7) a Fob1 QTL-associated gene as candidates that had passed all the selection criteria. We also investigated one non-QTL associated gene, glycoen synthase 2 (Gys2; Figure 8) as it had passed all but one (within QTL) of our stringent adipose enrichment criteria. Although more likely a secondary gene response, the conspicuously large fold-change (15, versus 6 and 4 for Npr3 and Thbs1, respectively), supporting evidence from functionally related gene expression changes (Pdk4, Ldh2) and the additional possibility that Gys2 may fall within an un-characterised minor QTL, we wished to maintain scientific balance in our approach by including it.

Investigation of Npr3 uncovers of a novel role for the ANP system in rodent lipolysis in vivo

Natriuretic peptide receptors (NPRs) regulate blood pressure and are particularly relevant to exercise-induced lipid mobilisation [36]. The receptor encoded by Npr3 is a signalling deficient isoform, highly expressed in adipose tissue and kidney which is involved in clearance of active atrial natriuretic peptide (ANP).
Npr3 was elevated in all 3 fat depots of F mice in the snap-shot array and in the quantitative array (Figure 6A–B). RT-PCR validation showed that Npr3 expression (Fig. 6C) closely followed the changes in subcutaneous adipose tissue mass in response to HF feeding in F and L mice (Figure 6D) and showed a trend towards being increased in 4 week HF-fed C57BL/6J mice (Figure 6E).

ANP was shown to induce lipolysis in primate, but not rodent, adipocytes through activation of the NPRA (Npr1) [37]. We investigated the effects of the NPR3-selective agonist cyclic ANP (cANP) on lipolysis in vivo by implanting cANP-releasing minipumps into C57BL/6J mice, a standard model of high fat-diet induced obesity. Plasma ANP levels were elevated in cANP-treated mice (saline, fed state: 17±3 ng/ml versus cANP infusion, fed state: 125±21 ng/ml, P<0.001), as expected from its blockade of Npr3-mediated ANP clearance [38]. This did not affect body weight gain with short-term (4 week) HF feeding (data not shown).

To test if the ANP system might be involved in adipocyte lipolysis we challenged the mice with a 24 hour fast during week 3 of the HF experiment. Fasting glucose levels were in the normal range for loss of an overnight feeding bout (saline treated: 6.1±0.4 mmol/L, cANP treated: 5.8±0.2 mmol/L). We found a novel effect of fasting to elevate endogenous ANP levels. This was exaggerated in cANP-infused mice (saline fasted state: 61±11 ng/ml, cANP fasted state: 436±54 ng/ml, P<0.001). In addition, cANP-treated mice showed a significant increase in fasting NEFA levels that was not apparent in the fed state (Figure 6F). Our data suggest that fasting is permissive for a physiological lipolytic effect of ANP in rodents which is exaggerated by blocking ANP clearance with cANP in vivo. High NPR3 levels in adipose tissue of Fat mice may abrogate the lipolytic effect of ANP locally through increased clearance of lipolytic ANP and thus contribute to obesity. The lipolytic effect of ANP may only be manifest in states of low insulin action such as fasting or, perhaps more relevant, in combination with other factors (eg obesity) that contribute to pre-existing insulin resistance [13].

Functional insight into a novel obesigenic role for elevated Thbs1 on adipocytes

Control of vascular growth during adipose tissue expansion is a major regulator of adiposity [39]. We noted that a number of genes in the angiogenic/angiostatic cascade were differentially expressed between the F and L lines (Figure 5 and microarray). Thrombospondin1 (Thbs1) was elevated across the three fat depots in the snap-shot analysis and this was confirmed in the quantitative microarray (Figure 7 A–B). Thbs1 is positioned in the Fob1 QTL [5]. During the course of validation of this array work, others published [40] that thrombospondin-1 was elevated in obesity.
and was an adipocyte-derived cytokine (adipokine). Although realtime PCR analysis did not show any Thbs1 change in L adipose tissue (not validated) the increase of Thbs1 in F mice was validated (Fig. 7C). Thbs1 mRNA was also elevated in adipose tissue from HF-induced- and genetically-obese mice (Figure 7D–E). Critically, the angiostatic peptide fragment [41] of thrombospondin-1 (ABT-510) had distinct concentration-dependent effects, reducing fatty acid uptake into 3T3-L1 adipocytes in vitro at low concentrations (0.1–1 nM) but increasing fatty acid uptake at high physiological (100 nM) concentrations (Figure 7F). Our data suggest Thrombospondin-1 may have a dual effect. In the adipose vasculature it may curtail angiogenesis [41] and potentially prevent fatty acid uptake through its CD36 cell surface receptor [42] at low concentrations. However, at high concentrations thrombospondin-1 may have a direct hypertrophic effect on adipocyte lipid accumulation thus promoting obesity.

Elevated Gys2 is associated with abnormal carbohydrate metabolism and increased glycogen deposition in adipose tissue

Despite not being positioned within any of the 4 major QTLs, Gys2 gene mRNA was markedly higher (~14-fold) selectively in F adipose tissues in the snap-shot experiment and this was confirmed with the quantitative microarray and RT-PCR validation (Figure 8A–C). Glycogen content was also elevated in the F adipose tissue and in Lepob mice, a monogenic model of morbid obesity (Fig. 8D) suggesting ectopic glycogen is a feature of adipose tissue in obesity. Notably, mRNA levels of the regulatory subunit of phosphatase 1 (Ppp1r3d) that targets glycogen synthase is one of the novel Fob1 obesity candidate genes (Table 3). This finding is consistent with a switch in intermediary metabolism from carbohydrate oxidation (eg Pdk4) to inefficient lipid oxidation which leads to a net increase in lipid synthesis (eg Ldh2; lactate, driving glyceroneogenesis) and ectopic carbohydrate storage in adipose tissue. Of note, glycogen complexes with water and thus has a higher mass per mole than lipid. Ectopic glycogen deposition may therefore make an important contribution to fat pad mass per unit stored.

Conclusions

We have gained insight into metabolic, inflammatory and angiogenic remodelling responses characteristic of obesity, some of which overlap with those described by others [43–45] and that are consistent with broad changes found in microarray studies on adipose tissue from obese rodents and humans [46–52]. Crucially, some of our observations are unique to our model. This is very likely due to the polygenic nature and unique selection-based
origin of the divergent lines which reveals obesity (and indeed leanness)-related changes beyond other commonly used models (eg monogenic Lep-driven or HF-fed C57BL/6J), with relevance to human obesity and its associated metabolic disturbances. Devising a stratified systems approach integrating gene expression, tissue-specific, depot-specific and functional data with positional (QTL) information allowed a more rigorous test to identify and validate candidate obesity genes. We have been able to show that a prime obesity candidate gene (Npr3), that fulfilled all the required criteria, turned out to have a complex, context-dependent effect on fat mobilisation in mice in vivo. Npr3 may also link obesity-related hypertension [13] in the model. Our approach has led to identification of a novel direct effect of thrombospondin-1 on fat cell hypertrophy. The exact mechanism whereby Thbs1 promotes obesity will require further study due to its complex modular structure, functions and multiple receptors [53]. Nevertheless the effect of the type 1 repeat of thrombospondin-1 (ABT-510) suggests a functional cross-talk between adipose tissue endothelial cells and fat cells. Given the success of our stratified enrichment strategy in linking candidate genes with fat cell function (Tic7h, Tabal) or obesity-associated insulin resistance (Ppp1r3d, Tip33np2, Fgfl3, Fnr). Our results highlight the benefit of using transcriptomics in addition to F₂ QTL information to identify secondary, adipose-enriched genes (Gys2, Pdk4, Ldh2; intermediary fat and carbohydrate metabolism in fat cells) in parallel with positional candidate genes. These genes likely contribute to obesity (eg ectopic glycogen storage) and its consequences regardless of the mechanistic origin of excessive weight gain. The original QTL mapping study [5] was done using the outbred F and L lines and a low-density genetic marker spacing with the power to detect only major QTL effects. Our current study may therefore have uncovered novel minor QTLs that were here regarded as ‘non-QTL-associated’, and so it was important to document a prime example of at least one gene (Gys2) that otherwise, and rather conspicuously (large fold change across 3WATs, validated in quantitative array), passed our filtering criteria. Potentially, Gys2 or other genes could be confirmed as true expression (e)QTLs in future high resolution F₂ mapping. Of further note, many of the inflammatory changes are non-QTL associated, suggesting that these genes are secondary, though still important, responders to obesity. Interestingly, there were no major expression differences in well-characterised transcriptional pathways regulating adipocyte formation (C/EBPs, PPARs, GATAs, RIP1-40, ERRs, etc.).

Figure 5: RT-PCR validation of angiomodulatory genes differentially expressed in the subcutaneous adipose tissue of Fat and Lean mice. Fat and Lean mice were fed control (FC; black bars, LC; grey bars) or high fat diet (FF; black hatched bars, LF; grey hatched bars) for 18 weeks. RNA was extracted and the gene specified above the graphs was measured by RT-PCR. Target gene expression was corrected to the expression level of the housekeeping gene tata-binding protein (TBP) and is expressed arbitrarily as an adjusted ratio. * = P<0.05 significant effect of diet within a line. † = P<0.05, ††† = P<0.001 significant difference between lines.

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KLFs, WNTs, STATS, E2Fs, Dlk, Dlp, Foxos, PRDM16), or fat accumulation and synthesis (SREBPs). The L mice remarkably lose fat mass with HF feeding and may model beneficial adipose tissue changes with weight loss [13]. After bariatric surgery in obese patients there is a switch in gene expression from prominent stress-related pathways to genes consistent with remodelling of the adipose tissue [54]. L mice show a similar response with alternate-inflammatory, adipose remodelling and lipolytic effects alongside improved metabolic function with HF diet [13]. Thus the gene pathways changing in L mice losing fat mass with HF feeding are not akin to a ‘lipodystrophic’ re-distribution of calories into non-adipose tissues with consequent worsening of metabolic disease. This data may point to important leanness genes that act independently in adipose tissue to help cope with cellular stress and this is an area of important future investigation.

Future work in the adipose tissues of refined congenic lines with discrete QTLs introgressed on the comparator genetic background will help apply additional filters for gene expression and functional characterisation of candidate adiposity genes. This approach has been successful in identifying 6p2 as an important obesity candidate gene, albeit of hypothalamic origin, in a C57BL/6J subcongenic line carrying a 7.4 Mb region of chromosome 2 from SPRET/Ei mice [55]. A similar approach led to the positional cloning and identification of the Prca gene as being causal for a hypothalamic mechanism of leanness involving α-MSH degradation [56]. An optimal test to prove that a candidate gene is causal for the QTL effect is quantitative complementation [57], which requires knockout models in particular genetic backgrounds. These resources are currently unavailable for the F and L lines. Therefore, the approach of developing novel subcongenic lines with ever smaller donor segments combined with bioinformatics, sequence, expression, and functional analyses remains an optimal strategy to find causal sequence variation for obesity candidate genes identified in this study. Recent SNP genome-wide association studies (GWAS) in humans revealed that quantitative variation in obesity is due to the action of numerous QTLs of relatively small effect; each of the obesity loci detected generally account for less than 1% of the phenotypic variance [1–2,12]. In an extremely large scale GWAS study exploring a well characterised quantitative trait, height, the 180 loci uncovered accounted for only 10% of phenotypic variation [58]. Quantitative

**Figure 6. Validation and functional investigation of the candidate obesity gene Npr3.**

A. Snap-shot pooled transcriptome microarray chip intensities (arbitrary units) across subcutaneous (SC), epididymal (EPI), mesenteric (MES) adipose tissues versus liver, muscle and kidney in Fat (F) and Lean (L) mice with the rounded expression ratio in bold. B. Mean chip intensity from quantitative microarray (n = 4) in Fat and Lean mice fed control (FC, LC) or high fat diets (FF, LF) with relevant P value for significant differences. C. RT-PCR validation of changes in Npr3 gene expression along with its relationship to D. Altered fat mass in response to high fat diet in subcutaneous adipose tissue of control diet-fed Fat (FC, black bars), high fat diet-fed Fat (FF, black hatched bars), control diet-fed Lean (LC, grey bars) and high fat diet-fed Lean (LF, grey hatched bars) mice. * = P<0.05 significant effect of diet within a line. E. Expression levels of Npr3 in subcutaneous fat of control diet (horizontal hatched bar) or HF fed (heavily stippled bar) C57BL/6J mice (C57). F. The effects of fasting (fast) on plasma free fatty acid levels (NEFA) in C57BL/6J mice implanted with minipumps releasing saline (S) or 100 ng/day cyclic ANP3-23 (cA). ** = P<0.01 for an effect of cA treatment of fasting NEFA.

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traits like obesity are therefore controlled by many more genes than initially predicted and highly statistically-powered animal studies in defined models such as the one used here are likely to uncover additional obesity loci that would remain undetected in human GWAS.

The unique selection basis of the Fat and Lean strains on fat pad mass divergence highlights some previously unidentified molecular mechanisms contributing to fat mass accumulation and its downstream metabolic sequelae that may be amenable to therapeutic intervention. The genes and pathways identified by our stratified enrichment approach may prove important over and above the genetically determined appetitive, energy expenditure and activity-mediated drivers of fat mass in rodents and humans.

**Methods**

**Experimental Design**

**Experiment 1. ‘Snap-shot’ pooled transcriptome microarray.** Our first experiment was designed to look across a panel of tissues of the F and L mice including 3 white adipose tissue depots, liver, muscle and kidney in Fat (F) and Lean (L) mice with the rounded expression ratio in bold. **A.** Mean chip intensity from quantitative microarray (n = 4) in Fat and Lean mice fed control (FC, LC) or high fat diets (FF, LF) with relevant P value for significant differences. **B.** Thbs1 mRNA levels in control-fed C57BL/6J (C57), horizontal striped bars and genetically obese leptin-deficient Lepob mice (Ob, lightly stippled bars) and **C.** Thbs1 mRNA levels in control-fed C57BL/6J (C57), horizontal striped bars and high fat diet fed C57BL/6J mice (C57 HF, heavily stippled bars). **D.** Thbs1 mRNA levels in control-fed C57BL/6J (C57), horizontal striped bars and genetically obese leptin-deficient Lepob mice (Ob, lightly stippled bars) and **E.** Thbs1 mRNA levels in control-fed C57BL/6J (C57), horizontal striped bars and high fat diet fed C57BL/6J mice (C57 HF, heavily stippled bars). **E.** The effects of the thrombospondin-1 type 1 repeat ABT-510 (0.1–100 nM, grey bars) on 3H-palmitate uptake into fully differentiated 3T3-L1 adipocytes. **P<0.01** versus control (Ctrl, black bar), non-ABT510 treated cells. doi:10.1371/journal.pone.0023944.g007

Figure 7. Validation and functional investigation of the candidate obesity gene Thbs1. A. Snap-shot pooled transcriptome microarray chip intensities (arbitrary units) across subcutaneous (SC), epididymal (EPI), mesenteric (MES) adipose tissues versus liver, muscle and kidney in Fat (F) and Lean (L) mice with the rounded expression ratio in bold. B. Mean chip intensity from quantitative microarray (n = 4) in Fat and Lean mice fed control (FC, LC) or high fat diets (FF, LF) with relevant P value for significant differences. C. RT-PCR validation of changes in Thbs1 gene expression in subcutaneous adipose tissue of control diet-fed Fat (FC, black bars), high fat diet-fed Fat (FF, black hatched bars), control diet-fed Lean (LC, grey bars) and high fat diet-fed Lean (LF, grey hatched bars) mice. **P<0.01** versus FC. D. Thbs1 mRNA levels in control-fed C57BL/6J (C57), horizontal striped bars and genetically obese leptin-deficient Lepob mice (Ob, lightly stippled bars) and E. Thbs1 mRNA levels in control-fed C57BL/6J (C57), horizontal striped bars and high fat diet fed C57BL/6J mice (C57 HF, heavily stippled bars). **P<0.05** versus lean control. E. The effects of the thrombospondin-1 type 1 repeat ABT-510 (0.1–100 nM, grey bars) on 3H-palmitate uptake into fully differentiated 3T3-L1 adipocytes. **P<0.01** versus control (Ctrl, black bar), non-ABT510 treated cells.

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Stratified Identification of Adipose Obesity Genes

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specifically altered in the 3 white fat depots but not in the other metabolic tissues to increase the likelihood of identifying adipose-specific causal obesity genes. Note the original selection criterion of the F and L mice was on divergent fat pad mass and that the obesity is not the result of increased food intake [6,7]. Moreover, this was useful since the mixed genetic background of the base population may have carried ‘bystander’ genes that are differentially expressed between the lines in both adipose and non-adipose tissues, but that are not related to the divergent obesity and metabolic phenotype. 4. Obtain information on possible adipose tissue depot-specific changes in gene expression that are informative as regards the impact of different fat depots on metabolic disease.

Experiment 2. Exon-chip microarray with dietary intervention: Our second experiment was designed to look at the adipose tissue depot which showed the greatest divergence in mass between the lines in response to chronic high fat feeding [13]. We took subcutaneous fat from control diet and high fat fed F and L mice after 18 weeks on the diets (n = 4) which allowed us to:

1. Validate changes in subcutaneous fat from experiment 1 and 2.

Perform quantitative analyses on the changes in gene expression between the lines and identify line-specific effects of the diet.

Bioinformatics analysis of microarray data. 1. Experiment 1 looked at qualitative fold changes in gene expression between the lines in the 3 WAT depots (subcutaneous (SC), epididymal (EPI) and mesenteric (MES), liver (L), muscle (M) and kidney (K). We set the fold-difference threshold for changes of interest to > ±1.5 (where denoted a ‘−’ refers to genes that are up regulated in the Lean line and, positive numbers denotes genes that are up regulated in the F lines, respectively). Initially we did not specify any range limitations for gene expression changes in L, M or K. A second filter set the fold change within L, M or K to be within ≤ ±1.5. This search narrowed our targets to white adipose tissue-specific changes in gene expression, with the caveat that many gene pathways maybe linked between or operate differently/reciprocally from white fat and non-adipose tissues. An example would be the opposing effects of glucocorticoids in adipose versus liver for 11β-HSD1 expression [13]. Further, for candidate genes we excluded all genes whose absolute signal was below the threshold of 100 (genes unlikely to be meaningfully expressed in the adipose) in one or both lines, with the caveat that it is possible some genes may be effectively switched on or off in adipose tissue of one or other line (eg Depdc6 and Gsn, see text).

2. Quantitative microarray analysis of subcutaneous fat in experiment 2 looked at fold changes in gene expression in a single fat depot, but allowed us to further assess the effects of chronic high fat feeding between and within the lines. We set the fold difference threshold to ≤ ±1.5 as before.

QTL-informed analysis. Analysis of both experiment 1 and experiment 2 benefited from the previously identified major QTL information [5], which we introduced as a filter to provide more information on which genes were more likely to represent causal genes (found within the 95% confidence interval of the four major QTL boundaries) and those which were more likely secondary (not found within the 95% confidence interval of the four major QTLs).
Validation. Our validation steps consisted of realtime PCR (RT-PCR) for ‘landmark’ genes on RNA from adipose tissues, functional assessment of biochemical changes (glycogen) in the adipose tissue of the lines and a number of in vitro and in vivo studies to test distinct hypotheses (Natriuretic peptide receptor and thrombospordin1).

F and L mice

Ethics Statement. All animal experiments were performed according to local ethical guidelines of The University of Edinburgh Ethics Committee and those of the (Scientific Procedures) Act (1986) of the UK Government Home Office under the auspices of an approved Home Office Project License (60/3962).

The long-term selection and further development of the F and L lines and details of the genetic basis of the line divergence and the inbreeding period are described elsewhere [4–7,15–16]. Mice derived from the inbred lines were used in this study [4]. Extensive characterisation of our lines in previous studies determined that body weight gain is highly correlated with fat mass accretion in fat mice [4–7]. This was substantiated in several genetic mapping experiments [3,15–16] demonstrating that the fat% trait and body weight trait co-localise to the same QTL regions with significant LOD scores. We followed bodyweight change closely and interpreted the changes in our lines as being primarily due to altered body fat mass. Animals were fed on pelleted Rat and Mouse breeder and grower diet (Special Diets Services, SDS, UK Ltd., Witham, Essex, UK) or with defined low (11% calories as fat with 48% carbohydrates, 31% protein) diets (Research Diets, New Brunswick, New Jersey).

Tissue and plasma measurements

At the end of the experiment, mice were killed within 1 minute of disturbing the home cage by cervical dislocation, to avoid stress-induced changes in metabolic parameters. Blood was collected in EDTA coated tubes (Sarstedt, Numbrecht, Germany). Plasma insulin was measured by ELISA (Crystalchem, Downers Grove, IL, USA), glucose by (Infinity reagent, Sigma, Dorset, UK) and free fatty acids (Wako Diagnostics, Neuss, Germany) levels (FFA) were measured with a colorimetric method. Liver (L), muscle (quadriceps, M), kidney (K), and epididymal (E), subcutaneous (S), and mesenteric (M) adipose tissues were collected, weighed and stored at −80°C.

RNA extraction and analysis

RNA was extracted using 800 µl TRIzol reagent (Invitrogen, Paisley, UK) per 50 mg tissue. Briefly, 200 µl of chloroform was added to the homogenate then centrifuged at 12,000 rpm for 1 min to remove cell debris. The supernatant was then vortexed for 15 s and centrifuged at 12,000 rpm for 15 min at 4°C. The upper (aqueous) layer was removed, mixed with 30 µl of RNAid matrix (Anachem, Luton, UK) and agitated for 5 min before centrifugation at 12,000 rpm for 1 min. The supernatant was removed and washed 3 times with 500 µl of RNA wash (Anachem, Luton, UK), resuspended in 20 µl of RNAid matrix (Anachem, Luton, UK) or with defined low (11% calories as fat with 48% carbohydrates, 31% protein) diets (Research Diets, New Brunswick, New Jersey).

Microarray analysis

For the snapshot experiment, tissue RNAs were prepared using Qiagen RNeasy kits, processed through standard Affymetrix protocols, and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips (n = 4 per group, Affymetrix, Santa Clara). Raw CEL file data were imported into BioConductor for background subtraction and normalization with the Robust Multichip Average (RMA) algorithm. Differential expression was determined using the Bioconductor Limma tool and the Benjamini and Hochberg FDR method. Annotation data for the genes were obtained from NetAffx. Webgestalt (http://bioinfo.vanderbilt.edu/webgestalt, Vanderbilt University, USA), DAVID (http://david.abcc.ncifcrf.gov/, National Institute of Allergy and Infectious Diseases) were used to cross validate clustering and pathways analyses. Full microarray data are MIAME compliant and are available in the ArrayExpress database under the accession number M-EXP-3091. For the quantitative microarray RNA was prepared with Qiagen RNAeasy kits and hybridised to Affymetrix mouse ST 1.0 GeneChips. Data were imported into the Onechannelgui package of Bioconductor, normalised with RMA with background subtraction and analysed with LIMMA and Benjamini and Hochberg FDR multiple testing correction. Data are available in ArrayExpress with accession number E-MEXP-3094. Microarray processing was by the ARK Genomics team at the Roslin Institute.

Real-time PCR

cDNA was synthesised from 2 µg RNA using Reverse Transcription system (Promega, Southampton, UK) with oligo(dT) primer, according to the manufacturer’s instructions. Gene-specific mRNA levels were measured using the LightCycler® 480 Real-Time PCR system (Roche Diagnostic Ltd, West Sussex, UK) with light cycler 480 probe master (Roche Diagnostic Ltd, West Sussex, UK) and TaqMan® Gene expression Assays (Applera, Cheshire, UK). Samples were analysed in triplicate with each PCR reaction containing 4.5 µl cDNA, 5 µl master mix and 0.5 µl primer-probe. Results are expressed as the ratio of gene of interest corrected to the housekeeping gene TBP mRNA levels as an internal control. TaqMan® Gene expression Assays were inventoried and are available from the Applera website (http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr.html)
3T3-L1 cell culture

The 3T3-L1 cell line is a preadipocyte cell line derived from the Swiss 3T3 mouse fibroblast cell line [58]. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Cambrex, Verviers, Belgium) supplemented with 10% new born calf serum (NCS), 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml) (Invitrogen, Paisley, UK) at 37°C in humidified atmosphere with 5% CO2. Confluent 3T3-L1 cells were subjected to differentiation protocol. Maintenance medium was changed at day 0 to differentiation DMEM but with additional 10% FBS to replace NCS and supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 0.25 mM dexamethasone (Dex), 1 μg/ml insulin and 100 nM Rosiglitazone.

Palmitate uptake

Pre-adipocytes (passage 8–11) were seeded onto 12 well plates at a density of 2.5×10^4 cells/well. Adipocytes were then fully differentiated with the above protocol and incubated with charcoal-stripped fetal calf serum overnight. ABT-510 1–100 nM (dissolved in sterile PBS) was incubated in stripped serum with the adipocytes for 6 hours at 37°C. Palmitate uptake was measured by the addition of unlabeled palmitate, dissolved in ethanol, and tracer (final 200 nM) radiolabelled [3H] palmitate obtained by counting aliquots of both the medium and in the cells scraped with 10% SDS in 2 mls of aqueous scintillation fluid (GE healthcare) in a Beckman LS330 scintillation counter.

Cyclic atrial natriuretic peptide (cANP) studies in vivo

13 C57/BL6J male (in house colony) at approximately 4-month of age were used for the study. Mice were weighed and divided into two groups to match initial body weight. Allowing for variations in body weight, mini pumps were prepared using sterile saline or varying amounts of cANP3-23 (Bachem, Switzerland) dissolved in sterilized saline to allow infusion of ~100 ng/day cANP3-23 a dose chosen to cause maximal NP3R occupancy [38]. The pumps were primed in saline and stored in the refrigerator for three days prior to implantation. On the day of surgery, animals were fasted for 4 hours, weighed and blood samples were taken from tail nicks to measure basal ANP, glucose and circulating FFAs. Animals were inserted subcutaneously. After recovery, animals were started on a 58 kcal% fat w/sucrose high fat diet (Research Diets). Body weight and food consumption were measured routinely twice a week. At week 3, a physiological challenge to elevate free fatty acids was performed (24 hour fast) and the change in body weight and food consumption were measured twice a week. At week 3, a physiological challenge to elevate free fatty acids was performed (24 hour fast) and the change in body weight and food consumption were measured routinely twice a week.

Glycogen assays

Tissue glycogen was assessed using a modified acid hydrolysis extraction protocol [60]. Briefly, 500 μl 0.03 M HCl were added to approximately 200 mg of adipose tissue, homogenized and then boiled for 45 min at 80–90°C in a water bath. Sample pH was adjusted to pH 5 by adding a small amount of either 1.1 M HCl. The samples were incubated water bath shaker at 37°C for at least 2 h. Glucose levels were determined at room temperature as described above.

Statistical analyses

Gene expression differences in validation real time was analysed using 2-way ANOVA for line and diet effects followed by post-hoc Holm-Sidak multiple comparison tests using Sigmastat version 3.5 (Systat Software Inc). Effects of treatments on biological parameters such as fatty acids, tissue glycogen or palmitate uptake were assessed using 1-way ANOVA. P-values below 0.05 were accepted as statistically significant.

Supporting Information

Table S1 Selected strains with high fasting plasma glucose levels after chronic HF feeding.

Figure S1 Toll-like receptor 13 (Tlr13) mRNA levels in adipose tissues from obese diabetic mouse strains. The bars show the relative expression of Tlr13 in the adipose tissues from multiple strains (http://biogps.gnf.org). Tlr3 expression patterns are shown from 2 eQTL analyses (gnf1m32524_at and 1457753_at) from the tissue gene expression/activity chart of biogps from ‘Fat’ or ‘Adipose’. High Tlr13 expression is found in mouse strains that are also obese and diabetic (Supplemental Table S1 [70]).

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Author Contributions

Conceived and designed the experiments: NMM CJK DRD. Performed the experiments: NMM YBN ZM EMDR LR. Analyzed the data: NMM. Contributed reagents/materials/analysis tools: LB. Wrote the paper: NMM SH CJK DRD. Reviewed and edited the manuscript: PFWH LB JRS.

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