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ARTICLE

Glycerol kinase deficiency alters expression of genes involved in lipid metabolism, carbohydrate metabolism, and insulin signaling

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Glycerol kinase (GK) is at the interface of fat and carbohydrate metabolism and has been implicated in insulin resistance and type 2 diabetes mellitus. To define GK's role in insulin resistance, we examined gene expression in brown adipose tissue in a glycerol kinase knockout (KO) mouse model using microarray analysis. Global gene expression profiles of KO mice were distinct from wild type with 668 differentially expressed genes. These include genes involved in lipid metabolism, carbohydrate metabolism, insulin signaling, and insulin resistance. Real-time polymerase chain reaction analysis confirmed the differential expression of selected genes involved in lipid and carbohydrate metabolism. PathwayAssist analysis confirmed direct and indirect connections between glycerol kinase and genes in lipid metabolism, carbohydrate metabolism, insulin signaling, and insulin resistance. Network component analysis (NCA) showed that the transcription factors (TFs) PPAR-γ, SREBP-1, SREBP-2, STAT3, STAT5, SP1, CEBPα, CREB, GR and PPAR-α have altered activity in the KO mice. NCA also revealed the individual contribution of these TFs on the expression of genes altered in the microarray data. This study elucidates the complex network of glycerol kinase and further confirms a possible role for glycerol kinase deficiency, a simple Mendelian disorder, in insulin resistance, and type 2 diabetes mellitus, a common complex genetic disorder.
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Glycerol kinase (GK) catalyzes the phosphorylation of glycerol to glycerol 3-phosphate (G3P) which is important in the formation of triacylglycerol (TAG) and fat storage.[1] GK is at the interface of fat and carbohydrate metabolism. GK deficiency (GKD) is an X-linked inborn error of metabolism that is characterized biochemically by hyperglycerolemia and glyceroluria and is due to mutations within or deletions of the *GK* gene on Xp21.[1] Isolated GKD can be symptomatic or asymptomatic and we have previously shown that there is no genotype–phenotype correlation in isolated GKD.[2,3] We hypothesize that this lack of genotype–phenotype correlation makes even

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simple Mendelian disorders complex traits and that this complexity is due to the role of modifier genes, metabolic flux through related pathways, systems dynamics, thresholds of protein functions, networks that the protein functions within as well as the moonlighting (alternative) functions of the enzyme.[2–5]

There is an emerging role for GK in type 2 diabetes mellitus (T2DM) as individuals with a GK missense mutation, N288D, have the asymptomatic form of isolated GKD, increased risk for obesity, insulin resistance and T2DM.[6] In addition, hepatocyte nuclear factor 4 alpha (HNF4α) is important for GK expression[7] and mutations in HNF4α are associated with maturity onset diabetes of youth (MODY).[8] HNF4α is an orphan nuclear receptor involved in regulating gluconeogenesis in the liver,[9] insulin secretion, and directly activates the insulin gene.[10,11] Recently many studies have focused on the role of HNF4α variants and polymorphisms on T2DM.[12–17] Thiazolidinediones (TZDs) are drugs used to treat T2DM[18] and have been shown to induce GK expression in adipocytes, which reduces free fatty acid (FA) secretion and increases insulin sensitivity.[19,20] Insulin resistance and T2DM results from a network of interactions between many genes and environmental factors.[21] Identification of these interactions will allow a better understanding of the molecular mechanisms leading to insulin resistance and T2DM.

GK is not normally expressed in white adipocytes but it is induced by TZD's in this tissue.[22] However, GK is expressed in brown adipose tissue (BAT) which is metabolically active.[23] BAT expresses LPL (lipoprotein lipase) which releases FA from lipoproteins and increases FA uptake. TZDs also affect BAT[24,25] and decreased BAT activity is associated with obesity, insulin resistance, and hyperlipidemia.[26] Maintenance of adequate stores of TAG, through esterification of G3P is essential for BAT functioning.[27] In addition, insulin deficiency induces BAT glyceroneogenesis to produce more G3P which is important to preserve the normal metabolic activity of BAT. Therefore, we hypothesize that GK has a role in BAT energy homeostasis and insulin resistance.

To relate gene expression data to protein function, we used network component analysis (NCA) which reduces dimensionality of high-dimensional microarray data to a lower dimension.[28,29] This allows identification of hidden dynamics and patterns such as transcription factor activities, (TFAs) which may not be found by microarray analysis.

In this study, we investigated the role of the murine ortholog of *GK*, *Gyk*, in metabolism (fat and carbohydrate), and insulin sensitivity using microarray analysis of BAT from wild-type (WT) and *Gyk* knockout (KO) mice. We determined that *Gyk* deletion causes alterations in expression of genes involved in carbohydrate and lipid metabolism as well as insulin signaling. NCA determined *Gyk*'s role in adipocyte-specific transcription. Our work confirms a role for GK in metabolism and insulin resistance and helps to understand the complexity of this single gene disorder.

Materials and methods

Animal care

Gyk-deficient mice (courtesy of W J Craigen, Baylor College of Medicine) were generated using 129/SvJ embryonic stem cells, bred to a C57B1/6J mouse, and then backcrossed onto C57B1/6J to make a congenic strain on C57B1/6J.[30] WT controls are male littermates of the KO mice and were born to carrier mothers. The mice were on a normal 3.5% fat diet (Harlan Tekland) and experiments were per a UCLA Chancellor's Animal Research Committee approved protocol.

RNA isolation

Day three of life WT and *Gyk* KO mice in the fed state were killed and interscapular BAT was extracted, homogenized in Trizol reagent (GibcoBRL Life Technologies, Rockville, MD, USA), frozen in liquid nitrogen, and stored at -80°C. Three to five tissues were pooled, then RNA was isolated (Trizol reagent, Gibco BRL Life Technologies), purified (RNeasy minielute, Qiagen, Valencia, CA, USA), and DNase treated (Turbo DNA-free, Ambion Inc., Austin, TX, USA).

cDNA synthesis and hybridization

cDNA synthesis was performed on pooled RNA and hybridized to Affymetrix Mouse Genome 430 2.0. Gene chips (three WT and four KO) as described.[31]

Microarray analysis

Microarray data was analyzed using DNA-Chip (dChip) analyzer software.[32] Two unsupervised learning methods (multidimensional scaling and hierarchical clustering with an Euclidean distance measure) were used on genes with a coefficient of variance between 0.3 and 10, and a percent present call of 20% to define the 'most varying probesets' (3776 probesets). Differentially expressed genes were filtered out using the criteria: fold change >1.5 between baseline (WT) and experimental (KO), absolute difference in the expression level between WT and KO >100, Student's *t*-test *P*-value <0.05, and percent present call of $\geqslant 20\%$.

Gene annotation

Genes that met the above criteria were used to identify enriched biological themes by Expression Analysis Systemic Explorer (EASE)[33] using the categorical overrepresentation function and the one-tailed Fisher exact probability for overrepresentation.

Real-time polymerase chain reaction

RNA was extracted as described above. KO and WT samples included some of the samples from microarray analysis as well as single animal samples. Gene expression assays (Applied Biosystems Inc. (ABI), Foster City, CA, USA) were used for all genes. Predeveloped Taqman assay reagents for *18 s rRNA* (ABI) were the endogenous controls. cDNA synthesis was performed using Superscript III (Invitrogen Corp., Carlsbad, CA, USA) per the manufacturer's instructions with random primers. The reaction was carried out using Taqman master mix (ABI) and Real-time polymerase chain reaction (RT-PCR) products detected using ABI prism 7700/7500 Sequence detection system. Fold differences for each of the genes were calculated using the $2^{-\Delta\Delta C}$T method.[34]

PathwayAssist analysis

Altered genes were analyzed using PathwayAssist (version 3.0,. Stratagene, La Jolla, CA, USA) by searching for connections of the genes listed in Tables 1 and 2 by looking for common regulators, and finding the shortest paths between nodes.

NCA

PUBMED was used to construct the connectivity matrices between transcription factors (TFs) important in adipocytes and genes differentially expressed in the microarray analysis. NCA was performed with criteria of 1.2-fold change, absolute difference in the expression level between the WT and KO larger than 100, Student's *t*-test *P*-value <0.05, and percent present call $\geqslant 20\%$. Ten TF were used to construct the final connectivity matrix. The data matrices were decomposed and the control strengths (CS), and transcription factor activity (TFA) matrices and contribution plots were obtained using the NCA toolbox (http://www.seas.ucla.edu/~liaoj/downloads/htm).

Results

Three-day-old mice were chosen for this study because this is the first day that the mice have statistically significant clinical symptoms including hypoglycemia, lower pH, lower bicarbonate, and lower base excess, which mimics the human disease.[35] It was felt that on day of life two the important changes from glycerol kinase deficiency would not be present without the clinical changes seen in mice (and humans). We believe that the majority of the changes seen on day 3 will be owing to the effects of GKD, however one cannot rule out the possibility that some will be due to the perimortum state of the mice as they die on DOL4. Measurements of the brown fat pads in subsequent WT and KO mice have shown no statistical difference in weight (in g) (data not shown).

Unsupervised hierarchical clustering and multidimensional scaling using the 3776 most varying probesets showed that the gene expression in BAT in a *Gyk* KO mouse model grouped together according to KO and WT status (Figures 1a and b). In both the hierarchical clustering tree (Figure 1a) and the multidimensional scaling (Figure 1b) the bfko2 sample is linked to the WT samples however, it is still distinct from them. This unsupervised learning analysis demonstrates that Gyk KO mice have a distinct global gene expression profile compared to WT. Differential gene expression analysis (gene filtering)

Table 1 EASE analysis grouping of biological processes and molecular functions using the filtered 888 probesets

Gene category	*List hits*	*List total*	*Population hits*	*Population total*	*Probability*
GO biological process					
cell proliferation	58	441	758	10415	7E-06
cell cycle	41	441	531	10415	0.0001
regulation of cellular process	37	441	481	10415	0.0003
regulation of cell cycle	25	441	281	10415	0.0004
regulation of cell proliferation	16	441	147	10415	0.0005
deoxyribonucleotide metabolism	4	441	10	10415	0.0005
G1/S transition of mitotic cell cycle	5	441	18	10415	0.0007
mitotic cell cycle	14	441	125	10415	0.0008
actin cytoskeleton organization and biogenesis	8	441	66	10415	0.0065
glutamine family amino-acid biosynthesis	3	441	11	10415	0.0096
carbohydrate transport	6	441	44	10415	0.0100
negative regulation of cell proliferation	7	441	59	10415	0.0119
lipid metabolism	27	441	402	10415	0.0119
GO molecular function					
DNA-dependent ATPase activity	5	463	14	10918	0.0002
sugar porter activity	6	463	36	10918	0.0037
sugar transporter activity	6	463	36	10918	0.0037
beta-adrenergic receptor activity	2	463	3	10918	0.0052
carbohydrate transporter activity	6	463	42	10918	0.0081

Table 2A List of genes involved in lipid metabolism altered in the KO male vs the WT male mice

Lipid metabolism			
Gene	*Description*	*Accession ID*	*Fold change*[a]
Downregulated in Gyk KO mice			
Gyk	Glycerol kinase	BF683028	10 (3.73, 1.0E+08)
Elovl1	Elongation of very long chain fatty acids like 1	BB041150	2.2 (1.5, 3.1)
Thea	Thioesterase, adipose associated	AW060409	2.1 (1.6, 3.1)
Lip1	Lysosomal acid lipase 1	AI596237	2.0 (1.5, 3.1)
Pla2g7	Phospholipase A2, group VII	AK005158	1.9 (1.5, 2.4)
Srebf2	Sterol regulatory element binding factor 2	BM123132	1.9 (1.6, 2.2)
Lep	Leptin	U18812	1.8 (1.4, 2.8)
2310032D16Rik	RIKEN cDNA 2310032D16 gene	AV291259	1.7 (1.4, 2.0)
Plaa	Phospholipase A2, activating protein	BM198417	1.6 (1.3, 2.2)
Ltc4s	Leukotriene C4 synthase	NM_008521	1.6 (1.3, 2.1)
Cryl1	Crystallin, lamda 1	C85932	1.6 (1.4,1.9)
Prkag2	Protein kinase, AMP-activated, gamma 2	BB756794	1.6 (1.2, 2.2)
Fasn	Fatty acid synthase	AF127033	1.6 (1.2, 2.1)
Dgat2	Diacylglycerol O-acyltransferase 2	AK002443	1.5 (1.2, 2.2)
Abcd2	ATP-binding cassette, subfamily D (ALD), member 2	BB197269	1.5 (1.2, 1.9)
2310015N07Rik	RIKEN cDNA 2310015N07 gene	AK009370	1.5 (1.2, 2.0)
Upregulated in Gyk KO mice			
Lpin1	Lipin 1	NM_015763	1.5 (1.3, 1.8)
Vldlr	Very low-density lipoprotein receptor	BE647363	1.5 (1.3, 1.9)
Lpin1	Lipin 1	AK014526	1.5 (1.2, 1.8)
C20orf155	RIKEN cDNA 0610009I22 gene	AK016165	1.5 (1.3, 1.8)
Fdft1	Farnesyl diphosphate farnesyl transferase 1	BB028312	1.6 (1.3, 2.1)
Stard5	StAR-related lipid transfer (START) domain containing 5	BI076697	1.6 (1.3, 1.9)
Hpgd	Hydroxyprostaglandin dehydrogenase 15	AV026552	1.7 (1.4, 2.1)
Pitpnm1	phosphatidylinositol membrane-associated	BB206460	1.7 (1.5, 2.0)
Plcd3	Phospholipase C, delta 3	AK011892	1.7 (1.5, 2.0)
Adipor1	Adiponectin receptor 1	BC014875	1.7 (1.4, 2.2)
Sult1a1	Sulfotransferase family 1A, member 1	AK002700	1.8 (1.4, 2.4)
Sgpl1	Sphingosine phosphate lyase 1	NM_009163	1.9 (1.4, 2.6)
Zap128	Cytosolic acyl-CoA thioesterase 1	NM_012006	1.9 (1.4, 2.7)
Pld2	Phospholipase D2	NM_008876	2.3 (1.4, 4.2)

[a]The numbers in parentheses represent the lower and upper limits of the fold change.
Student's *t*-test *P*-value <0.05.

Table 2B List of genes involved in carbohydrate metabolism altered in the KO male vs the WT male mice

Carbohydrate metabolism			
Gene	*Description*	*Accession ID*	*Fold change*[a]
Downregulated in Gyk KO mice			
Gyk	Glycerol kinase	BF683028	10 (3.73, 1.0E+08)
Gys2	Glycogen synthase 2	BC021322	2.8 (2.0, 4.4)
1110032E23Rik	RIKEN cDNA 1110032E23 gene	AK008987	3.5 (2.0, 9.4)
1110032E23Rik	RIKEN cDNA 1110032E23 gene	NM_133187	2.3 (1.6, 3.5)
Slc35a2	Solute carrier family 35, member 2	BB375649	1.8 (1.4, 2.5)
Naglu	Alpha-N-acetylglucosaminidase	NM_013792	1.7 (1.3, 2.5)
Ppp1r3b	Protein phosphatase 1, regulatory subunit 3B	BG071940	1.6 (1.4, 1.9)
Gale	Galactose-4-epimerase, UDP	BC027438	1.6 (1.3, 1.9)
Flj10986	RIKEN cDNA 2310009E04 gene	AK009249	1.6 (1.3, 2.0)
Pdhb	Pyruvate dehydrogenase beta	AK011810	1.5 (1.3, 1.8)
Upregulated in Gyk KO mice			
Rpe	Ribulose-5-phosphate-3-epimerase	AV023018	1.5 (1.4, 1.7)
Neu1	Neuraminidase 1	AI649303	1.6 (1.4, 1.9)
Sdh1	Sorbitol dehydrogenase 1	BI143942	1.7 (1.5, 2.1)
Lyz	Lysozyme	AV058500	1.9 (1.4, 2.7)

[a]The numbers in parentheses represent the lower and upper limits of the fold change.
Student's *t*-test *P*-value <0.05.

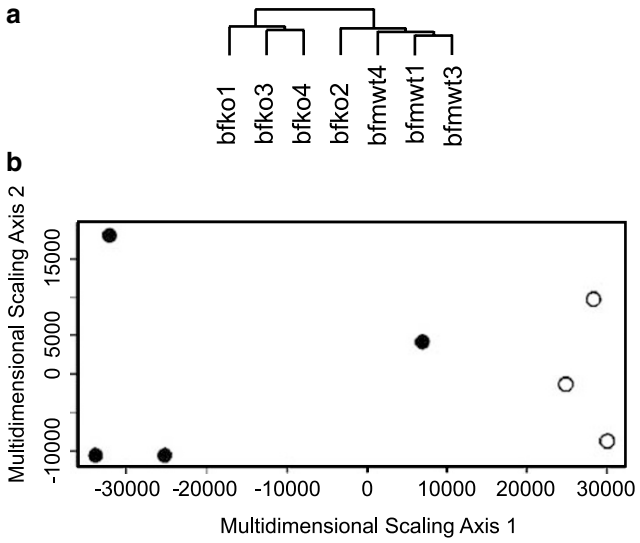

Figure 1 Unsupervised learning analysis of KO and WT mice samples using the 3777 most varying genes. (**a**) Average linkage hierarchical clustering tree of KO samples and WT samples. (**b**) Multidimensional scaling plot of the same samples. Black circles are KO, and open circles are WT. bfko represents brown fat knockout and bfmwt represent brown fat male wild type.

revealed 888 probesets (668 genes) significantly differentially expressed between KO and WT mice with a median false discovery rate of 5%. Of the 668 genes, 388 genes were downregulated and 280 were upregulated.

To uncover enriched biological themes among the differentially expressed genes we used the gene ontology analysis EASE software[33] (Table 1). The analysis identified the most significant enrichment of differentially expressed genes to be in cell proliferation ($P<0.001$). Other suggestive (P-value $\leqslant 0.02$) biological and molecular themes included cell cycle, regulation of cellular process, regulation of cell cycle, regulation of cell proliferation, carbohydrate transport, lipid metabolism, sugar porter activity, and sugar transporter activity. EASE analysis of genes involved in lipid and carbohydrate metabolism revealed 40 genes (excluding *Gyk*) differentially expressed between the KO and WT mice (Tables 2A and B). Of these, 28 were in lipid metabolism and 12 in carbohydrate metabolism. Fifteen genes involved in lipid metabolism were downregulated in the KO mouse including lysosomal acid lipase 1 (*Lip1*), fatty acid synthase (*Fasn*), and leptin (*Lep*), (Table 2A), and 13 genes involved in lipid metabolism were upregulated, which included Lipin 1 (*Lpin1*), very low density lipoprotein receptor (*Vldlr*), and adiponectin receptor 1

Table 3 Genes involved in insulin signaling and insulin resistance altered in the KO male vs the WT male mice

Gene name	*Description*	*Accession ID*	*Fold change*[a]
Downregulated in Gyk KO mice			
Igf1	Insulin-like growth factor 1	BG092677	2.2 (1.5, 3.9)
Igf1	Insulin-like growth factor 1	AF440694	2.1 (1.7, 2.9)
Igf1	Insulin-like growth factor 1	BG075165	2.0 (1.4, 3.9)
Eif4e	Eukaryotic translation initiation factor 4E	BB406487	1.9 (1.6, 2.3)
Srebf2	Sterol regulatory element binding factor 2	BM123132	1.9 (1.6, 2.2)
Lep	Leptin	U18812	1.8 (1.4, 2.8)
Igf1	Insulin-like growth factor 1	NM_010512	1.8 (1.4, 2.3)
Jun	Jun oncogene	NM_010591	1.7 (1.4, 2.1)
Mapk6	Mitogen-activated protein kinase 6	BC024684	1.7 (1.4, 1.9)
Fasn	Fatty acid synthase	AF127033	1.6 (1.2, 2.1)
Upregulated in Gyk KO mice			
Ptpn11	Protein tyrosine phosphatase, non-receptor type 11	BC003980	1.5 (1.3, 1.7)
Map3k4	Mitogen activated protein kinase kinase kinase 4	AV079128	1.5 (1.2, 2.0)
Prkcm	Protein kinase C, mu	AV297026	1.6 (1.4, 1.8)
Slc2a1	Solute carrier family 2, member 1	BM209618	1.6 (1.3, 2.0)
Acacb	Acetyl-coenzyme A carboxylase beta	BC022940	1.8 (1.4, 2.4)
Adrb1	Adrenergic receptor, beta 1	AK018378	1.9 (1.5, 1.9)
Adrb3	Adrenergic receptor, beta 3	BB224790	1.9 (1.5, 2.3)
Cited2	Cbp/p300-interacting transactivator 2	Y15163	2.0 (1.4, 2.6)
Cited2	Cbp/p300-interacting transactivator 2	NM_010828	2.0 (1.6, 2.5)
Adrb3	Adrenergic receptor, beta 3	NM_013462	2.2 (1.6, 3.4)
Glcci1	Glucocorticoid-induced transcript 1	AA152997	2.3 (1.8, 3.0)
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	BB831146	2.8 (2.0, 3.6)
Map3k6	Mitogen-activated protein kinase kinase kinase 6	NM_016693	2.8 (2.0, 3.7)
Igfbp3	Insulin-like growth factor binding protein 3	AI649005	3.4 (2.2, 5.2)
Igfbp3	Insulin-like growth factor binding protein 3	AV175389	4.3 (2.8, 7.1)

Student's t test P-value <0.05.
[a]The numbers in parentheses represent the lower and upper limits of the fold change.

(*Adipor1*) (Table 2A). Twelve genes involved in carbohydrate metabolism were differentially expressed (excluding *Gyk*); eight of which were downregulated including glycogen synthase 2 (*Gys2*), alpha-N-acetylglucosaminidase (*Naglu*), and pyruvate dehydrogenase beta (Table 2B). Four genes were upregulated including sorbitol dehydrogenase 1 (Table 2B).

To assess GK's role in insulin resistance and T2DM, we examined the differential gene expression of genes involved in insulin sensitivity including genes encoding insulin-receptor-associated proteins, components of and downstream effectors of the phosphatidylinositol 3-kinase (PI3K) and MAP kinase pathways, primary target genes for insulin resistance, effectors of insulin signaling, and target genes for peroxisome proliferator-activated receptor gamma (PPAR-γ). We found 25 probesets (19 genes) that were differentially expressed between KO and WT mice (Table 3) that relate to insulin signaling or insulin resistance. Ten probesets (7 genes) were downregulated including insulin-like growth factor 1 (*Igf1*), eukaryotic translation initiation factor 4E (*Eif4e*), sterol regulatory element binding factor 2 (*Srebf2*), leptin (*Lep*), jun oncogene (*Jun*), mitogen-activated protein kinase 6, and *Fasn*. Fifteen probesets (12 genes) were upregulated in the Gyk KO mouse including adrenergic receptor beta 1 and 3 (*Adrb1* and *Adrb3*), CCAAAT/enhancer binding protein delta (*Cebpd*), and insulin-like growth factor binding protein 3 (*Igfbp3*).

Supervised hierarchical clustering based on the 28 and 12 differentially expressed genes (29 and 13 probesets) belonging to lipid and carbohydrate metabolism, respectively showed a clustering distinction between WT and KO mice (Figure 2a). The 19 genes (25 probesets) differentially expressed involved in insulin signaling and insulin resistance also showed distinct clustering of the WT and KO mice (Figure 2b).

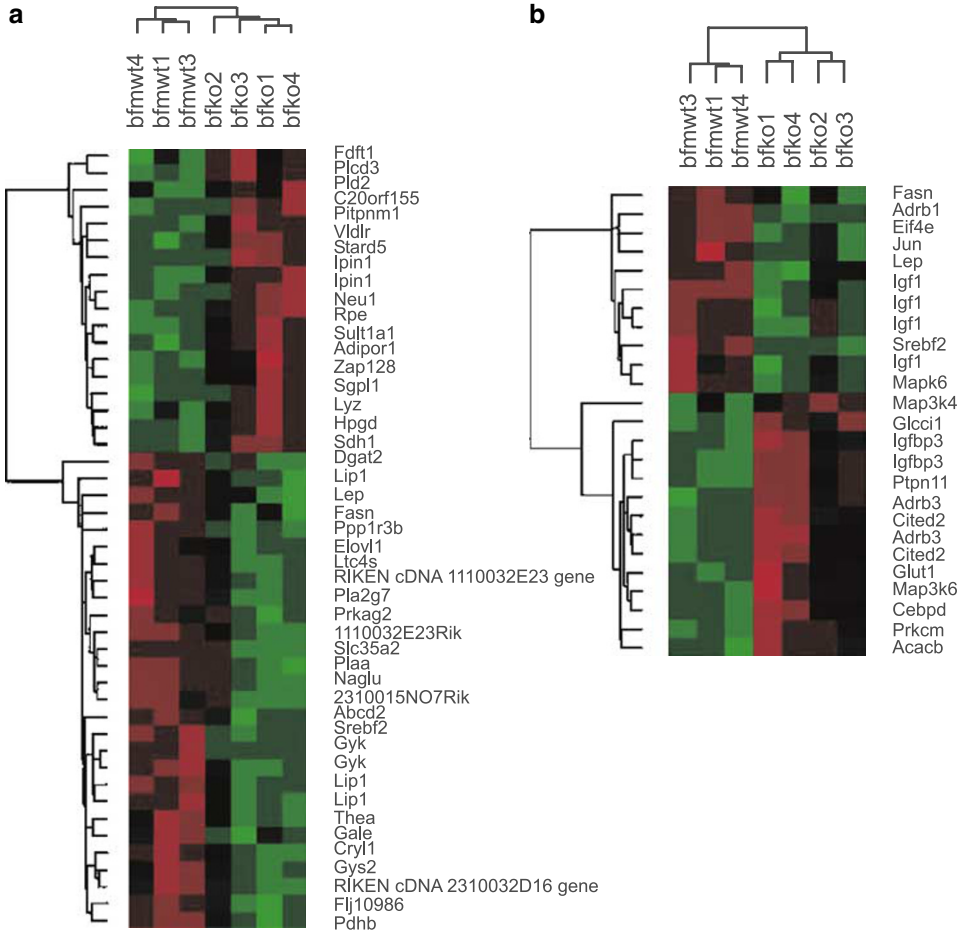

Figure 2 Supervised hierarchical clustering of KO and WT samples; (**a**) dChip clustering tree of top 46 most significant differentially expressed probesets involved in lipid and carbohydrate metabolism. (**b**) dChip clustering tree of top 25 most significant differentially expressed probesets involved in insulin signaling and insulin resistance. The top genes for both clusters were filtered out using fold change larger than 1.5, absolute difference in the expression level larger than 100, Student's *t*-test *P*-value smaller than 0.05, and percent present call of at least 20%. bfko represents brown fat knockout and bfmwt represent brown fat male wild type.

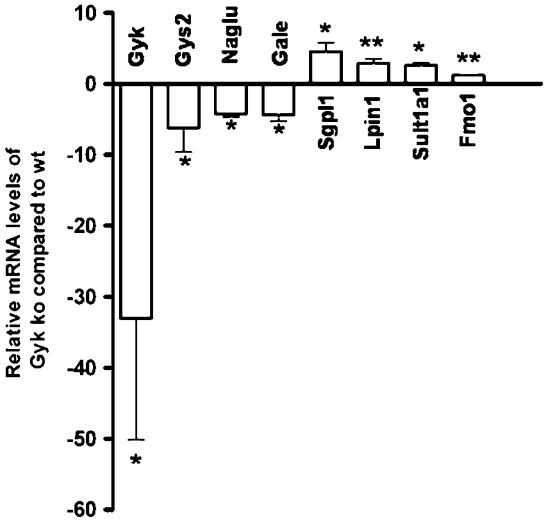

Figure 3 RT- PCR data (mean ± SEM) expressed as relative mRNA levels of Gyk KO compared to WT mice. Glycerol kinase (*Gyk*) ($n=4$), *Gys2* ($n=8$), *Naglu*($n=4$), UDP *Gale* ($n=4$), *Sgpl1* ($n=4$), *Lpin1* ($n=4$), *Sult1a1* ($n=8$), and *Fmo1* ($n=4$). *$P<0.01$, ** $P<0.05$.

RT-PCR was performed on 30 genes found to be differentially expressed by microarray analysis. RT-PCR confirmed 20 of these 30 genes including the down-regulation of carbohydrate and lipid metabolism genes including *Gyk, Gys2, Naglu*, and galactose-4-epimerase (*Gale*) (Figure 3 and Table 4). Sphingosine phosphate (*Sgpl1*), *Lpin1,* sulfotransferase family 1A, phenol-preferring, member 1 (*Sult1a1*), and flavin containing monooxygenase 1 (*Fmo1*) were upregulated in the KO mice by both RT-PCR and microarray expression data (Figure 3 and Table 4).

PathwayAssist analysis was used to represent the networks affected by *Gyk* deletion (Figure 4). Genes involved in lipid and carbohydrate metabolism, insulin signaling, and insulin resistance that were found to be altered in the microarray were used to create the pathway. For example, *Gyk* deletions lead to disruption of genes such as leptin that is implicated in insulin resistance. Central regulators in the pathway include PPAR-γ, PPAR-α, jun oncogene (JUN), tumor necrosis factor (TNF), glucose, and glucocorticoid. JUN is the most highly connected node, which likely reflects its global role as a transcription factor and it's involvement in a wide variety of biological processes.[36–39]

We elucidated transcription factor activities of TFs important in adipose tissue in the absence of Gyk using NCA of our microarray data as described in methods. Sixty-seven genes and 10 TFs resulted from the analysis. PPAR-γ, *trans-acting* transcription factor 1 (SP1), CCAAT/enhancer binding protein alpha, and glucocorticoid receptor (GR) activities were increased in the KO compared to the WT (Figure 5) whereas SREBP-2, SREBP-1, signal transducer and activator of transcription 3 (STAT3), STAT5, cAMP responsive element binding protein 1 (CREB), and PPAR-α activities were decreased in the KO (Figure 5). The connectivity, CS, expression matrices, and references used to deduce TFA and control strengths (CS) are provided as supplemental material. In order to rule out false discovery, we performed 100 permutations by shuffling the gene expression data and tested for significance. All activities of the 10 TFs showed perturbations when compared to the 100 permutated (shuffled gene expression) data (*P*-value <0.05) (data not shown). Contribution plots for phosphogluconate dehydrogenase (*Pgd*), angiopoietin-like 4 (*Angptl4*), fatty acid binding protein 4, adipocyte (*Fabp4*), *Cebpd, Fasn*, solute carrier family 2 (facilitated glucose transporter) member 3 (*Slc2a3*), insulin-like growth factor 1 receptor (*Igf1r*), and *Igf1* are depicted in Figure 6. The graph shows the contribution to the total gene expression of the specific gene by each TF (hatched and white bars each represent a TF) and the actual gene expression from the microarray data is represented by black bars.

Table 4 Real-time PCR and microarray expression data

Gene	*RT-PCR*	*Microarray*
Sult1a1	2.6	1.81
Gys2	−2.38	−2.84
Lpin1	2.3	1.53
Sgpl1	4.5	1.88
Gyk	−17.24	−9.975
Naglu	−4.2	−1.72
Fmo1	1.2	2.05
Gale	−3.8	−1.55

P-value = 0.027, Spearman correlation = 0.786, Spearman rank order correlation test.

Discussion

We report gene expression profiles in the *Gyk* KO mouse to provide insights into the role of GK in brown fat, insulin resistance, and T2DM. The *Gyk* KO mouse model mimics human GKD in that the mice have hyperglycerolemia, metabolic acidosis, and hypoglycemia.[30,35] However, the *Gyk* KO mouse has a more severe phenotype as the mice have growth retardation and die in the neonatal period.[30]

Of the biological themes identified using EASE analysis, cell proliferation was the most highly enriched (*P*-value <0.0001). Two related biological themes, regulation of cell proliferation (*P*-value <0.001) and negative regulation of cell proliferation (*P*-value = 0.01), were also significantly enriched in the KO mouse. This may relate to the emerging role of GK in apoptosis[40] and energy metabolism at the outer mitochondrial membrane.[41] GK binds to the voltage dependent anion channel (VDAC or porin) of the outer mitochondrial membrane,[41,42] a component of the

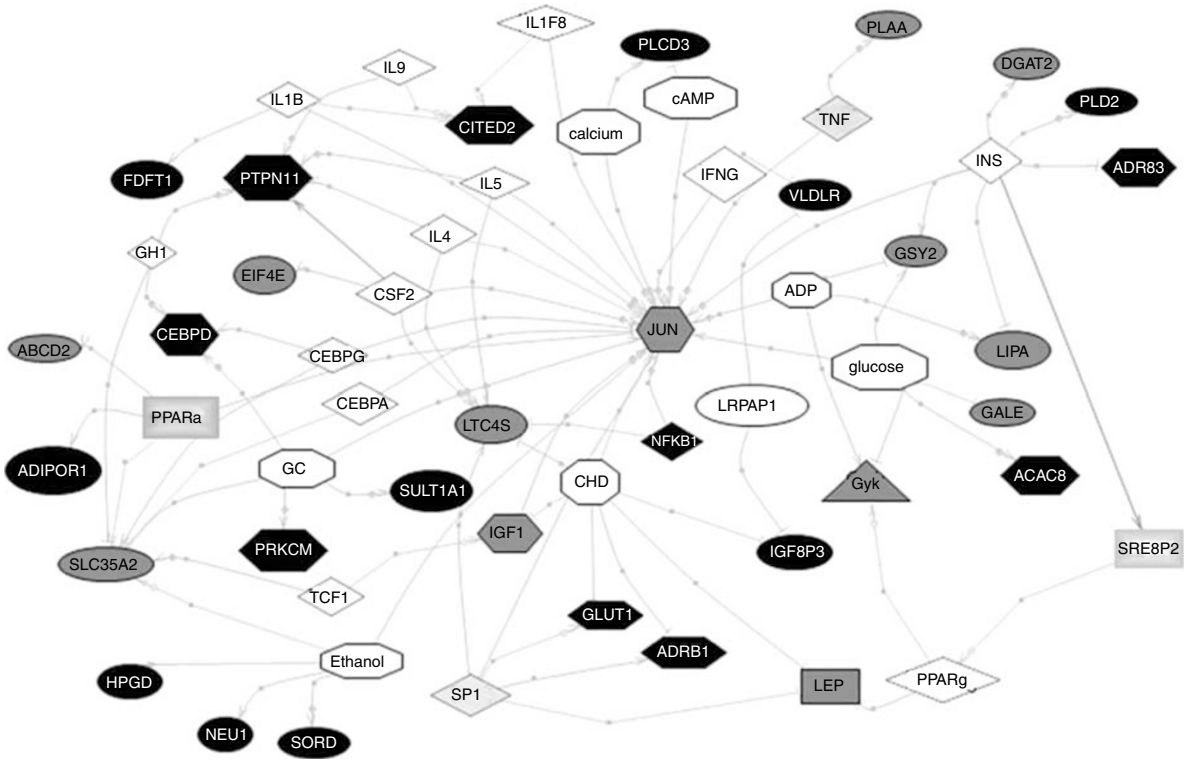

Figure 4 PathwayAssist analysis involving differentially expressed genes from carbohydrate and lipid metabolism groups and from the insulin signaling and insulin resistance groups. Black nodes represent proteins coded by genes upregulated in the microarray analysis, gray nodes represent proteins coded by genes that were downregulated, and white nodes represents no change in the micorarray. Ovals represent genes involved in lipid and carbohydrate metabolism, hexagons represent genes involved in insulin and insulin resistance, rectangles represent genes that belong to both groups, diamonds represent common regulators of the differentially expressed genes, and octagonal nodes represent small molecules. Gyk is denoted by a triangle. ABCD2, ATP-binding cassette subfamily D member 2; ACACB, acetyl-Coenzyme A carboxylase beta; ADIPOR1, ADRB1, adrenergic receptor beta 1; ADRB3, adrenergic receptor beta 3; CEBPA, CCAAT/enhancer binding protein, alpha; CEBPD, CCAAT/enhancer binding protein delta; CEBPG, CCAAT/enhancer binding protein gamma; CITED2, Cbp/p300-interacting transactivator 2; CSF2, colony-stimulating factor 2; PRKCM, DGAT2, diacylglycerol *O*-acyltransferase 2; EIF4E, FDFT1, farnesyl diphosphate farnesyl transferase 1; GALE, galactose-4-epimerase, UDP; GH1, growth hormone 1; Glut1, glucose transporter 1; GC, glucocorticoid; GYS2, glycogen synthase 2; HPGD, hydroxyprostaglandin dehydrogenase 15; IFNG, interferon gamma; IGF1, insulin-like growth factor 1; IGFBP3, insulin-like growth factor binding protein 3; IL1B, interleukin 1 beta; IL1F8, interleukin 1 family, member 8; IL4, interleukin 4; IL5, interleukin 5; IL9, interleukin 9; INS, insulin; JUN, jun Oncogene; Lep, leptin; LIPA, lipaseA; LRPAP1, lipoprotein receptor-associated protein; LTC4S, leukotriene C4 synthase; NEU1, sialidase 1; NF κB1, nuclear factor kappa-B, subunit 1; PLAA, phospholipase A2-activating protein; PLCD3, phospholipase C, delta 3; PLD2, phospholipase d2; PPARA, peroxisome proliferator-activated receptor alpha; PPARG, peroxisome proliferator-activated receptor gamma; PRKCM, protein kinase C, mu; PTPN11, protein tyrosine phosphatase non-receptor type 11; SLC35A2, solute carrier family 35, member 2; SORD, sorbital dehydrogenase; SP1, sp1 transcription factor; SREBF2; SULT1a1, sulfotransferase family 1A; TCF1, transcription factor 1; TNF, VLDLR.

mitochondrial permeability transition pore complex (PTPC) involved in cytochrome c release and apoptosis.[41,43]

A large number of enriched biological themes were identified in the *Gyk* KO mouse (Table 1). This attests to the complex biological network, within which GK is functioning and may be due in part to its role in phosphorylating glycerol. Of particular interest is carbohydrate and lipid metabolism, which we focused on for further analysis.

Within the category of carbohydrate metabolism, the levels of *Gys2* were downregulated in the KO mice. Glycogen synthase (GYS) is expressed in the liver, catalyzes the incorporation of a glycosyl residue into glycogen, and is regulated by insulin, glucose, and glucose-6- phosphate.[44,45] In the absence of GK, the cell may become more dependent upon glucose metabolism to drive cellular processes. Therefore, glucose will not be converted into glycogen and a reduction of glycogen synthase would be expected.

In the lipid metabolism group, *Lpin1*, involved in fat adipocyte differentiation, is upregulated in the Gyk KO, whereas *Lip1*, involved FA release, is downregulated. LIP1 catalyzes hydrolysis of cholesteryl esters and triglycerides, and is suppressed by insulin.[46] These findings suggest that GK deletion possibly stimulates adipocyte differentiation and decreases fat hydrolysis. The stimulation of adipocyte differentiation is intriguing given the role of PPAR-γ in adipocyte differentiation and stimulation of *Gyk* expression.[19,20] The levels of PPAR-γ mRNA were not changed in

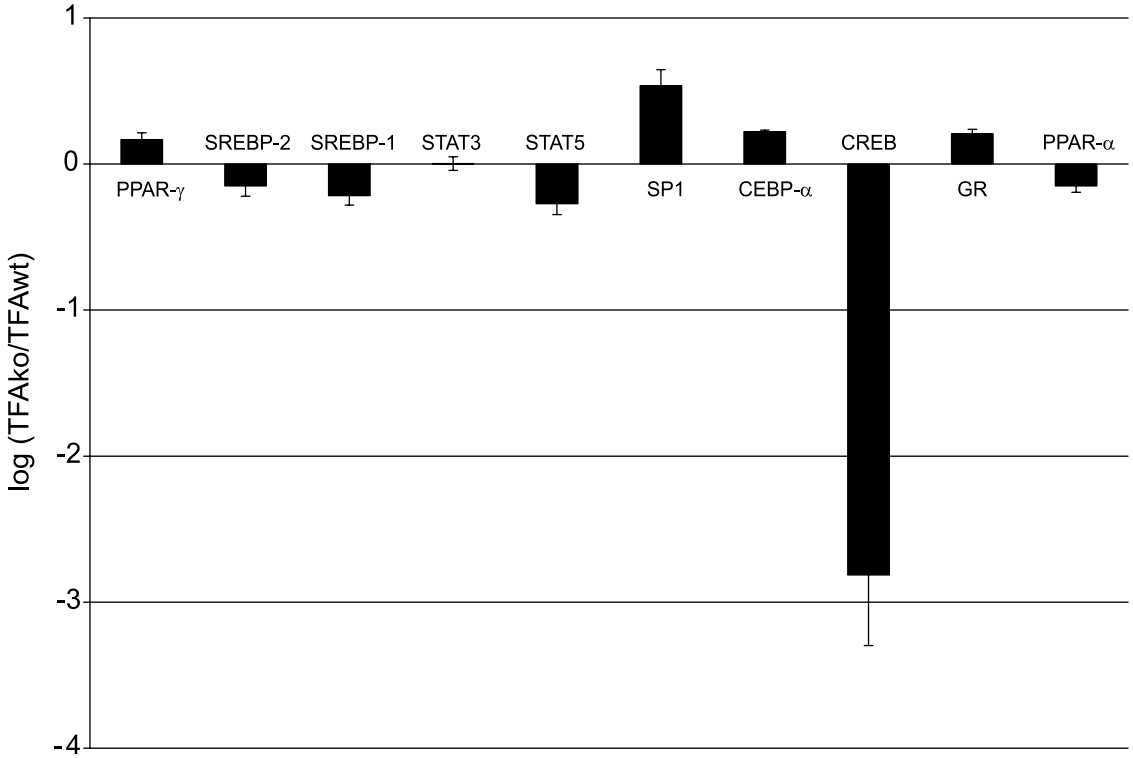

Figure 5 TFA of selected TFs important in adipose tissue. The transcriptional network containing 10 TF and 67 genes resulting from microarray analysis of 1.2-fold change. Peroxisome proliferator-activated receptor gamma (PPAR-γ), sterol regulatory element binding factor 2 (SREBP-2), sterol regulatory element binding factor 1 (SREBP-1), signal transducer and activator of transcription 3 (STAT3), signal transducer and activator of transcription 5 (STAT5), *trans*-acting transcription factor 1 (SP1), CCAAT/enhancer binding protein (C/EBP), alpha (CEBP-α), cAMP responsive element binding protein 1 (CREB), glucocorticoid receptor (GR), and peroxisome proliferator-activated receptor alpha (PPAR-α).

the *Gyk* KO mice compared to the WT, although PPAR-γ is activated as determined by NCA thus demonstrating the importance of NCA. These studies suggest that GK has a role in adipocyte differentiation and may relate to its role in T2DM.

Differentially expressed genes involved in insulin signaling, insulin resistance, and T2DM identify a link between GK and T2DM. Phospholipase d2 (*Pld2)* and protein kinase C (*Pkc)* mRNA levels were upregulated in *Gyk* KO mice 2.3- and 1.5- fold respectively. PLD2 releases diacylglycerol (DAG) during the breakdown of phospholipids. DAG is involved in many different signal transduction pathways including the activation of PKC,[47] which ultimately inhibits insulin action in muscle.[48–50] The increase of PLD2 and PKC in the absence of GK suggests that the presence of GK is important for maintaining normal insulin action, which is consistent with insulin resistance in individuals with GKD.[6]

The pathways affected by GK deletion were examined (Figure 4). Pathway Assist analysis showed that JUN was the node with the highest connectivity. This is possibly due to the increase of leptin in the KO mice because leptin increases JUN transcriptional activity.[51] JUN's central role is likely due to its extensive role as a transcription factor in numerous mechanisms.[36–39] SP1 was implicated to regulate five of the genes that were altered by *Gyk* deletions: *Lep, Adrb1*, solute carrier family 2 (facilitated glucose transporter) (*Slc2a1*), member 1, Jun, and leukotriene C4 synthase. This confirms SP1's role as a key mediator of 'cross-talk' between signaling pathways such as insulin sensitivity, and gene transcription.[52] Although SP1 regulates genes that are affected by GK deletions, the direct role of GK on SP1 remains unknown. Insulin was also implicated in the regulation of five of the genes differentially expressed in the microarray: *Adrb3*, *Pld2*, *Dgat2*, *Gys2*, *and Lip1*. Other key regulators in the pathway include PPAR-γ, PPAR-α, glucose, glucocorticoid, and TNF. PathwayAssist analysis provides a better understanding of the complexity of the network that GK functions in and suggests that GK is a critical component in multiple pathways.

NCA determined the TFA of TFs important in BAT when GK is deleted including PPAR-γ, SREBP-1, SREBP-2, STAT3, STAT5, SP1, CEBPα, CREB, GR, and PPAR-α. The gene expression level of *Srebf2,* which codes for SREBP-2 was significantly decreased in the KO mice *vs* the WT mice (1.86-fold). All other TFs gene expression levels were not significantly altered. This variance of gene expression level

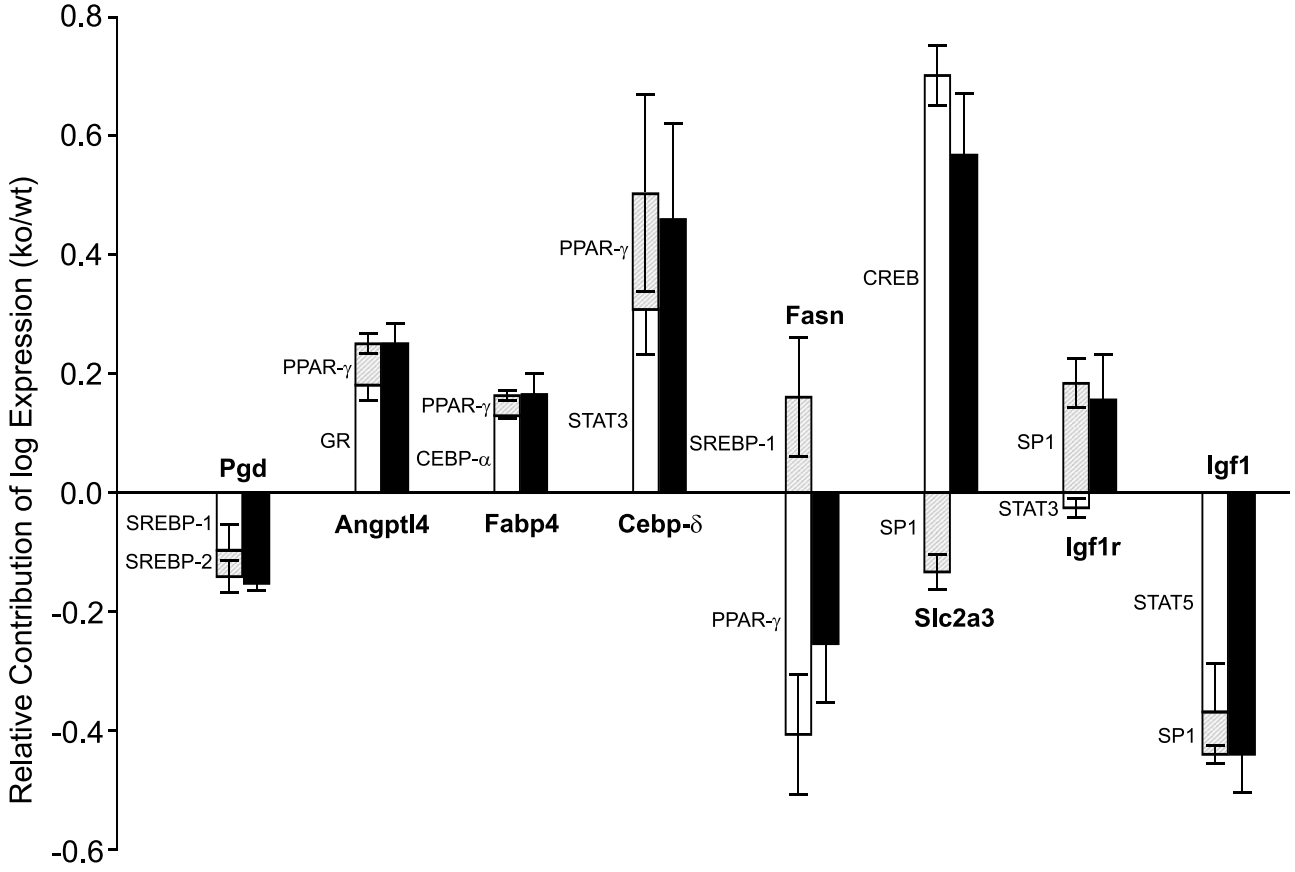

Figure 6 Scaling-independent contribution plots of a subset of genes. TF gene expression contribution for phosphogluconate dehydrogenase (*Pgd*), angiopoietin-like 4 (*Angptl4*), fatty acid binding protein 4, adipocyte (*Fabp4*), CCAAT/enhancer binding protein (C/EBP), delta (*Cebp*), *Fasn*, solute carrier family 2 (facilitated glucose transporter), member 3 (*Slc2a3*), insulin-like growth factor I receptor (*Igf1r*), and *Igf1*. Each gene is represented by two bars, white/hatched, and black. The white and hatched bar represents two TFs as indicated contributing to the gene expression of that gene; the sum of the white and hatched areas represents the fitted gene expression computed by the NCA software. The black bar represents the actual gene expression data from the microarray analysis.

and TFA shows the importance of NCA to determine TF protein function from gene expression data.

A total of 12 PPAR-γ target genes were altered in our microarray data including *Angptl4* and *Adipor1,* which are both upregulated. *Angptl4* has been shown to be upregulated during fasting.[53] GKD is similar to fasting in that glycerol and FA are released. *Fasn*, uncoupling protein 3 (*Ucp3*), and *Dgat2* are downregulated in the KO mice. *Fasn* is involved in FA synthesis and is probably decreased in the KO as a feedback response by the cells to compensate for the absence of GK, which blocks the esterification of FA. *Dgat2* is responsible for the synthesis of triglycerides and needs G3P.[54] We would predict TFs such as PPAR-γ involved in adipocyte differentiation to be altered in the KO mice as the cells attempt to compensate for the lack of triglyceride synthesis by activating adipocyte differentiation.

Aquaporin 7 (*Aqp7*), a PPAR-γ target gene, functions to transport glycerol into the blood stream when needed.[55] *Aqp7* is decreased in the KO mice, which is intriguing, as previous studies have shown that *Aqp7* disruption leads to increased GK levels in adipocytes.[56] The studies show that *Aqp7* deficiency leads to obesity by increasing GK levels. The decrease in *Aqp7* in the *Gyk* KO mice is most likely in response to the excess glycerol in the bloodstream (hyperglycerolemia). Therefore, glycerol does not need to be transported into the bloodstream and *Aqp7* is not expressed.

We assessed the relative contributions of various TFs on specific gene expression. The expression of *Slc2a3*, a glucose transporter gene is increased in the Gyk KO mice. *Slc2a3* gene expression is controlled by SP1 (inhibitor) and CREB (activator). The actual levels of *Slc2a3* expression are higher in the KO because the activation of *Slc2a3* by CREB is greater than the inhibition by SP1 (Figure 6). *Fabp4* gene expression is increased in the *Gyk* KO mouse owing to the activation by both PPAR-γ and CEBP-α (Figure 6). *Fasn* encodes Fasn, which catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA into long-chain

saturated FA. PPAR-γ antagonizes SREBP-1 to contribute to an overall decrease of *Fasn* gene expression (Figure 6). For all of the TFA, the fitted expression represented by the contributions of the TFs (hatched and white bars) correlated with the actual expression from the microarray data (represented by the black bars). This study confirms and expands our previous work indicating that NCA can be used in mammalian systems[31] and allows better definition of the transcriptional network within which GK functions.

This study confirmed that GK has an integral part in the overall metabolic network including insulin signaling and begins to elucidate the relationship between GKD, insulin resistance, and T2DM. Specifically, our study showed that GK deletion causes alterations in gene expression levels of genes involved in lipid and carbohydrate metabolism, as well as other metabolic and transcriptional networks including genes involved in insulin resistance. Further investigations into the mechanisms of these alterations may provide greater insight into the links between glycerol and glucose metabolism, and the role of GK in insulin resistance and T2DM. Such investigations will help us understand the complexity of GKD and serve as a model for other complex genetic diseases.

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