

# Integrative proteomic network analyses support depot-specific roles for leucine rich repeat LGI family member 3 in adipose tissues

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**Abstract.** LGI family member 3 (LGI3) is a member of the LGI protein family. In our previous studies, LGI3 was determined to be expressed in adipose tissues, skin and the brain, where it served as a pleiotropic cytokine. The results indicated that LGI3 levels are increased in adipose tissues of obese individuals in comparison with control individuals and that LGI3 suppressed adipogenesis via its receptor, disintegrin and metalloproteinase domain-containing protein 23. Additionally, it was reported that LGI3 upregulates tumor necrosis factor- $\alpha$  and downregulated adiponectin and hypothesized that LGI3 may act as a proinflammatory adipokine involved in adipose tissue inflammation. In the present study, cytokine arrays were used to analyze cytokine levels in adipose tissues and plasma of LGI3-knockout mice and signaling protein arrays used to analyze the expression and phosphorylation of these proteins in LGI3-treated preadipocytes. The results suggested that expression levels of 129 gene products (24 cytokines and 105 signaling proteins) were altered in response to LGI3 deficiency or LGI3 treatment, respectively. Protein-protein interaction network analysis of LGI3-regulated gene products revealed that 94% of the gene products (21 cytokines and 100 signaling proteins) formed an interaction network cluster. Functional enrichment analysis for the LGI3-regulated gene products, including those from our previous studies, revealed an association with numerous biological processes, including inflammatory responses, cellular differentiation and development and metabolic regulation. Gene co-expression network analysis revealed that these LGI3-regulated gene products were involved in various biological processes in an overlapping and differential manner between subcutaneous and visceral adipose tissues. Notably, inflammatory responses were more strongly associated with the LGI3-regulated gene

co-expression network in visceral adipose tissues than in subcutaneous adipose tissues. Analysis of expression quantitative trait loci identified four single nucleotide variants that affect expression of LGI3 in an adipose depot-specific manner. Taken together, the results suggested that LGI3 may serve depot-specific roles as an adipokine in adipose tissues.

## Introduction

Leucine-rich repeat LGI family member 3 (LGI3; formerly known as leucine-rich glioma inactivated 3) is a secretory protein belonging to the vertebrate LGI family that is abundantly expressed in the brain (1). LGI3 expression in the brain has been suggested to be regulated by activating enhancer-binding protein 2 and neuron-restrictive silencer at the transcriptional level (1). Our research group previously reported that LGI3 regulates neuronal exocytosis and differentiation (2,3). Additionally, LGI3 expression in the epidermal layer of the skin has been identified, where it may act as a cutaneous cytokine (4). Our group previously established that LGI3 is secreted by keratinocytes in response to ultraviolet B irradiation, protecting cells (4). It was also determined that LGI3 promotes the migration, differentiation and inflammatory responses of keratinocytes (5-8) and melanocyte pigmentation (9). LGI3 may also be associated with the cytokine network in cancer (10-12) and its expression is associated with the prognosis of patients with glioma and non-small cell lung cancer (11,13). Notably, the expression and genetic variations of LGI3 may serve potential prognostic roles in various types of cancer (10).

Earlier studies have indicated that LGI3 is expressed in adipose tissues in mice and that its expression is reduced during adipogenesis and increased in the adipose tissues of obese mice (14,15). It has also been demonstrated that LGI3 suppresses adipogenesis via its receptor, disintegrin and metalloproteinase domain-containing protein 23 (ADAM23) (14). Moreover, LGI3 upregulates the expression of proinflammatory genes, including TNF- $\alpha$  in macrophage cells (14), and downregulates adiponectin (15). Notably, LGI3 and TNF- $\alpha$  are mutually upregulated via NF- $\kappa$ B, suggesting their cooperative role in metabolic inflammation in obesity (16). It is hypothesized that LGI3 is a multifunctional cytokine and proinflammatory adipokine that functionally interacts with various cytokines, adipokines, chemokines and signaling proteins (12).

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To gain an insight into the functional network of LGI3 in adipose tissues, integrative analyses were performed based on protein expression and phosphorylation arrays, gene co-expression networks (GCNs), protein-protein interaction networks and expression quantitative trait loci (eQTL). In the present manuscript, evidence was presented to support the hypothesis that LGI3 has differential functions in subcutaneous adipose tissues (SATs) and visceral adipose tissues (VATs).

## Materials and methods

**Animals and cell culture.** All animal protocols were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (Seoul, Korea). All animal studies complied with the ARRIVE guidelines (17). Briefly, all animal welfare considerations were taken including daily monitoring of health and behavior and minimizing suffering and distress by practicing euthanasia as described below. LGI3-knockout mice were generated by MacroGen, Inc. (15). Adipose tissue samples at autopsy and plasma collected before euthanasia were obtained from 6 10-week-old mice (mean body weight, 26 g) bred and maintained in a rodent facility under a 12 h light/dark cycle, at a relative humidity of 55±15% and a constant temperature (23±3°C). The animals were provided with food and water *ad libitum*. A total of 6 male mice (three wild type and three LGI3 homozygous knockout mice) were euthanized by cervical dislocation and death was confirmed by loss of respiration and heartbeat. The duration of the experiment was 10 weeks and no mice were found dead during the period. White adipose tissues (WATs, epididymal fat) and plasma were obtained from mice and 3T3-L1 cells (American Type Culture Collection) were cultured as previously described (14).

**Preparation of recombinant LGI3 and protein array analysis.** Recombinant LGI3 protein was purified as previously described (3). Briefly, LGI3-His<sub>6</sub> protein was expressed in *E. coli* BL21 (DE3) using the pET28a(+) expression vector (Novagen; Sigma-Aldrich; Merck KGaA) and chaperone system (pGro7; Takara Bio., Inc.). The protein was purified using TALON Metal Affinity Resin (Clontech Laboratories, Inc.). 3T3-L1 cells were treated with LGI3 (10 ng/ml) at 37°C for 1 and 24 h for phosphoprotein array analysis and signaling protein analysis, respectively. Phosphoprotein array analysis was performed using the Phospho Explorer Antibody Array (cat. no. PEX100; Full Moon BioSystems, Inc.). Signaling protein analysis was performed using the Signaling Explorer Antibody Array (cat. no. SET100; Full Moon BioSystems, Inc.) and the Explorer Antibody Array (cat. no. ASB600; Full Moon BioSystems, Inc.). All assays were performed in accordance with the manufacturer's instructions. Cell extracts were prepared using the protein extraction buffer (EXB050; Full Moon BioSystems, Inc.) and Antibody Array Assay Kit (cat. no. KAS02; Full Moon BioSystems, Inc.) and analyzed using ExDEGA 1.1.9.0 (eBiogen, Inc.) according to the manufacturer's protocol. Data normalized using  $\log_2(\text{fold change}) \geq 1$  ( $P < 0.05$ ) were used for integrative analyses.

**Preparation of adipose tissues and cytokine array analysis.** Adipose tissue extracts were prepared by homogenizing tissues

in Dulbecco's phosphate-buffered saline (Sigma-Aldrich; Merck KGaA) containing a cocktail of protease inhibitors (Roche Diagnostics). After homogenization, Triton X-100 was added to a final concentration of 1% and the samples were frozen at -70°C, thawed and centrifuged at 10,000 x g for 5 min at 4°C. The supernatants were then used to investigate the cytokine profile by employing the Mouse XL Cytokine Array kit (cat. no. ARY028; R&D Systems, Inc.) according to the manufacturer's protocol. The Mouse XL Cytokine Array kit differs from the Adipokine and Cytokine Array Kits used in our previous study (12). These contain 111 capture antibodies, including 76 cytokine antibodies that were not included in the arrays of the previous study.

**Protein-protein interaction network and functional enrichment analysis.** A protein-protein interaction network was generated using data from the Search Tool for the Retrieval of Interacting Genes/Proteins (version 11.0; <http://string-db.org>) (18) and visualized by Cytoscape 3.8.0 (<https://cytoscape.org>) using an interaction degree-sorted circle layout (19). Network centrality was analyzed by Cytoscape 3.8.0 using the closeness centrality method (20). Functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using the Database for Annotation, Visualization and Integrated Discovery (version 6.8; <https://david.ncifcrf.gov>) (21). The results were sorted based on the P-values and entries with  $P < 0.05$  were presented.

**Gene co-expression network (GCN) analysis.** GCN analysis was performed using the GCNs of human SATs (UUID: 7054a727-5ca3-11e7-8f50-0ac135e8bacf) and VATs (omentum; UUID: c8749eba-5ca3-11e7-8f50-0ac135e8bacf) (22) obtained from the Network Data Exchange (NDEx; version 2.4.5; <http://www.ndexbio.org>) (23) and visualized by Cytoscape 3.8.0 using the prefuse force-directed layout. The gene ontology (GO) categories associated with the GCN were mapped using BiNGO 3.0.4 (<http://apps.cytoscape.org/apps/bingo>) and visualized by Cytoscape 3.8.0 using the edge-weighted spring-embedded layout. The hypergeometric test and Bonferroni correction were used to obtain P-values (24). Comparative analysis of categorized gene groups was presented as a Venn diagram generated using Venny 2.1 (<http://bioinfogp.cnb.csic.es/tools/venny>). The association between genes and transcription factors with respect to transcriptional regulation was assessed using transcription factor affinity prediction (<http://trap.molgen.mpg.de>) tools (25). RNA-seq data were obtained from the Human Protein Atlas (<https://www.proteinatlas.org>) and the GTEx project (<https://www.gtexportal.org>).

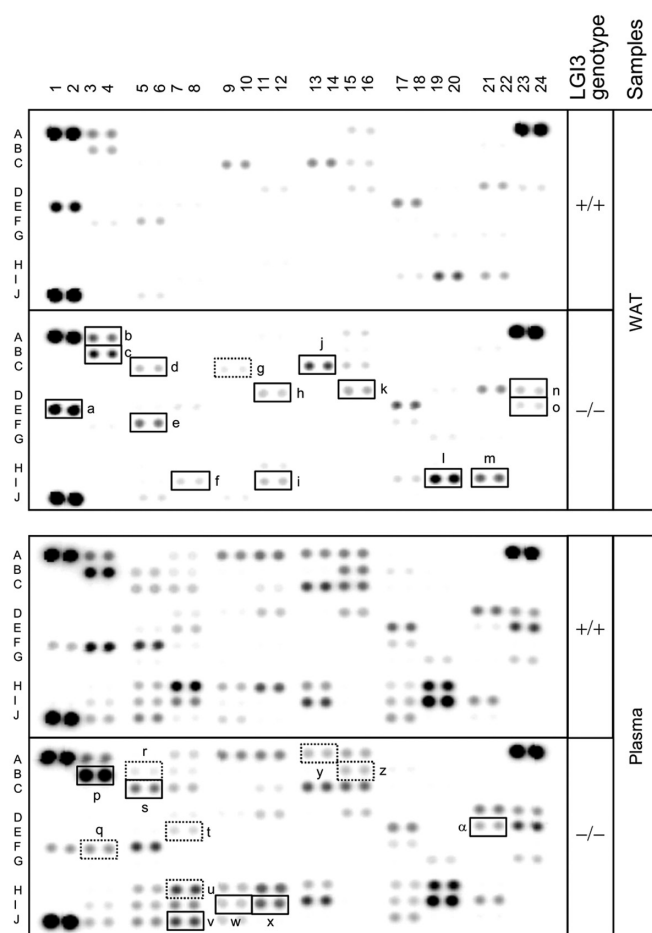
**Analysis of eQTL.** Single-tissue eQTL of adipose tissues were identified by searching the Genotype-Tissue Expression (GTEx) portal (<https://www.gtexportal.org>) (26). RNA sequencing (RNA-seq) data for the gene expression levels in subcutaneous (n=581) and visceral (omentum; n=469) adipose tissues were used for evaluation of the effect of eQTL on LGI3 expression. Single nucleotide variants (SNVs) in eQTL that affect LGI3 expression with  $P < 0.01$  and false discovery rate  $< 0.05$  were considered to be statistically significant.

**Statistical analysis.** Significance was assessed using ANOVA with Bonferroni correction. The results were considered significant at  $P < 0.05$ . Statistical analyses were conducted using SPSS version 26 (IBM Corp.) and all statistical tests were two-sided. The hypergeometric test and Bonferroni correction were used to obtain P-values in BiNGO analysis and nominal P-values were generated by the linear regression model between genotype and expression in eQTL analysis.

## Results

**Effect of LGI3 knockout on cytokine profiles.** The WATs and plasma derived from wild-type and LGI3-knockout mice were employed as samples for analyzing cytokine profiles using protein arrays (Fig. 1). The results indicated that the expression levels of various cytokines were increased or decreased in LGI3-knockout mice in comparison with WT mice (Fig. 1). The cytokines with increased expression included fibroblast growth factor 1 (FGF1), adiponectin (C1Q and collagen domain containing; ADIPOQ), chemokine (C-C motif) ligand 6 (CCL6), retinoic acid receptor responder 2 (RARRES2), insulin-like growth factor binding protein 6 (IGFBP6), periostin (POSTN), cystatin C (CST3), prolactin family 2 subfamily c member 2 (PRL2C2), complement factor D (CFD), dipeptidyl peptidase 4 (DPP4), regenerating islet-derived 3 $\gamma$  (REG3G), resistin (RETN),  $\alpha$ -2-HS glycoprotein (AHSG), insulin-like growth factor binding protein 2 (IGFBP2), serpin family E member 1 (SERPINE1), delta-like noncanonical notch ligand 1 (DLK1) and insulin-like growth factor binding protein 1 (IGFBP1) (Fig. 1 a-f, h-p, s, v-x; Table I). The cytokines with reduced levels of expression included coagulation factor III, tissue factor (F3), insulin-like growth factor binding protein 5 (IGFBP5), C-C motif chemokine ligand 11 (CCL11), growth arrest specific 6 (GAS6), C-X-C motif chemokine ligand 5 (CXCL5), TNF superfamily member 13b (TNFSF13B) and C-C motif chemokine ligand 21 (CCL21) (Table I; Fig. 1 g, q, r, t, u, y, z). CCL6, PRL2C2 and RARRES2 were found to be increased in the WATs as well as in the plasma of LGI3-knockout mice (Fig. 1; CCL6, c, p; PRL2C2, i, x; RARRES2, d, s). Several cytokines (IGFBP5, ADIPOQ, DLK1, IGFBP1 and SERPINE1) had previously been reported to be regulated by LGI3 (Table I) (12).

**Effect of LGI3 on the phosphorylation and expression of signaling proteins.** LGI3 and its receptor ADAM23 have been demonstrated to be expressed predominantly in 3T3-L1 preadipocytes, with their expression declining during differentiation into adipocytes (14). Thus, LGI3 may transduce intracellular signaling in preadipocytes in an autocrine and paracrine manner. To explore the LGI3-stimulated intracellular signaling pathway, phosphoprotein array and signaling protein array analyses were performed using extracts from 3T3-L1 preadipocytes treated with the LGI3 protein (Fig. 2). Expression levels of 105 proteins were found to be increased or decreased by LGI3 in comparison with a control (Fig. 2; Tables I and SI, SII). Additionally, 15 proteins showed upregulation following LGI3 phosphorylation and 18 proteins showed downregulation following LGI3 phosphorylation (Fig. 2; Tables I and SI). KEGG pathway analysis of these gene products revealed that the proteins expressed at increased levels were



**Figure 1.** Effect of LGI3 knockout on cytokine profiles. WATs and plasma from wild-type (+/+) and homozygous LGI3-knockout (-/-) mice were analyzed using cytokine XL arrays. Solid-line box, increased proteins in knockout mice. Numbers and capital letters represent the labels of array coordinates (Mouse XL Cytokine Array ARY028, R&D Systems, Inc., [https://www.rndsystems.com/products/proteome-profiler-mouse-x1-cytokine-array\\_ary028](https://www.rndsystems.com/products/proteome-profiler-mouse-x1-cytokine-array_ary028)). Array coordinate is listed in Table SII. Dotted-line box, decreased proteins in knockout mice. The list of proteins has been described in the Results and in Tables I and SI. a, FGF1; b, ADIPOQ; c and p, CCL6; d and s, RARRES2; e, IGFBP6; f, POSTN; g, F3; h, CST3; i and x, PRL2C2; j, CFD; k, DPP4; l, REG3G; LGI3, leucine-rich repeat LGI family member 3; m, RETN; n, AHSG; o, IGFBP2; q, IGFBP5; r, CCL11; t, GAS6; u, CXCL5; v, SERPINE1; w, DLK1; WATs, White adipose tissues; y, TNFSF13B; z, CCL21;  $\alpha$ , IGFBP1.

associated with the terms ‘neurotrophin signaling pathway’, ‘osteoclast differentiation’, ‘B cell receptor signaling pathway’, ‘PI3K/Akt signaling pathway’, ‘NF- $\kappa$ B signaling pathway’, ‘TNF signaling pathway’, ‘insulin resistance’ and additional terms related to various infectious diseases and cancer-related pathways (Table SIII). The gene products with reduced expression levels were found to be associated with the terms ‘PI3K/Akt signaling pathway’, ‘mTOR signaling pathway’ and various cancer-related pathways (Table SIII). Signaling protein array analysis demonstrated that the expression of 27 proteins was increased, while that of 45 proteins was reduced (Fig. 2; Tables I and SI). Functional enrichment analysis of these gene products revealed that the upregulated gene products were involved in tyrosine kinase signaling pathways, cell proliferation, PI3K signaling pathway, and growth hormone receptor and melanosome functions (Table SIV). The down-regulated gene products were associated with transcriptional

Table I. Summary of LIG3-regulated gene products identified in the present study and in previous studies.

Array	Upregulated gene products (reference no.)	Downregulated gene products (reference no.)
Cytokine XL Array <sup>a</sup>	CCL21, CXCL5, F3, IGFBP5 <sup>d</sup> , TNFSF13B, CCL11, GAS6	ADIPOQ <sup>d</sup> , AHSG, CCL6, CFD, CST3, DLK1 <sup>d</sup> , DPP4, FGF1, IGFBP1 <sup>d</sup> , IGFBP2, IGFBP6, POSTN, PRL2C2, RARRES2, REG3G, RETN, SERPINE1 <sup>d</sup>
Phospho Explorer Antibody Array <sup>b</sup>	BLNK, BRCA1, BTK, CALM1, CREB1, ERBB2, FOXO3, GRK2, IKKBK, IL2RA, JUN, LIMK1, RELA, RPS6KA1, TP73	ALK, CAV1, CBL, CD5, DOK1, ESR1, HSP90AB1, IRS1, KIT, KRT18, MAPK14, MTOR, PDGFRA, PRKCA, PRKCD, RYR2, STMN1, ZAP70
Signaling Explorer Antibody Array CYP39A1,	ADCK2, AFP, CASP1, CD37, CD80, ERBB3, ERN1, F10, F12, GAD1, GH1, GPR151, LYN, MSTN, PTGS1, PTK6, RCBTB1, RPS27, SND1, TUBB3, TYK2, TYRO3	ADCK1, AKT2, AXL, C1S, CAMKV, CD247, CD3E, CFB, COL4A3, CRYAB, CYP2S1, DCC, DDX4, DNAL4, EEF1G, EGF, EPHB1, EPN3, EXOG, F2R, FLI1, FN1, FOXA2, KAT8, KDR, KLK3, LAMC3, MATK, MUC16, NCR1, NEUROG3, NFKBIA, POLR3D, POU3F1, PRPF19, RCHY1, SLU7, SNAI2, TAF4, TBP, TP63, USP13 INSR, MAP2K2
Explorer Antibody Array	CD63, E2F2, MLH1, SEMA4D, SLC3A2	
Previously reported	AKT1 (3), CCL12 (12), CCL2 (16), CD68 (12), CSF3 (12), CTNNA1 (5), CXCL13 (12), CXCL2 (12), CYBA (12), CYBB (12), EMR1 (12), FLG(6), IGF1 (12), IGFBP5 (12), IL6 (12), ITGAX (12), IVL (6), KRT10 (6), LOR (6), MAPK1 (12), MAPK3 (12), MDM2 (4), MITF (9), NCF1 (12), NCF2 (12), NFKB1 (16), NOS2 (14), PIK3CA (3), PRKAA1 (12), PTEN (12), PTGS2 (14), PTK2 (3), TGM1 (6), TIMP1 (12), TNF (14,16)	ADIPOQ (15), BAD (12), C5 (12), CEBPA (14), CRP (12), CSF1 (12), DLK1 (12), EIF4EBP1 (12), ESM1 (12), FABP4 (14), GSK3A (12), GSK3B (5), IGFBP1 (12), LPL (14), PPARG (14), SERPINE1 (12), STX1A <sup>c</sup> (2), TP53 (4)

<sup>a</sup>Upregulated proteins in LIG3-knockout mice were presumed to be downregulated by LIG3, and vice versa for downregulated proteins in LIG3-knockout mice. <sup>b</sup>Classified by the changes in protein activities that are upregulated or downregulated by phosphorylation. <sup>c</sup>Syntaxin 1 (STX1A) was presumed to be functionally downregulated by LIG3 (2). <sup>d</sup>Previously reported LIG3-regulated gene products. Gene product names are the official gene symbols. The official full names of the gene symbols are listed in Table SI.

regulation, PI3K/Akt signaling pathway, T cell receptor signaling pathway, focal adhesion, and various cancer-related pathways (Table SIV).

*Protein-protein interaction network of LIG3-regulated gene products.* A protein-protein interaction network of LIG3-regulated gene products, based on both the findings of the present study (Tables I and SI) and those of our previous study (12), was constructed and visualized based on the interaction degree (Fig. 3). In total, 169 gene products (95% of 177 LIG3-regulated gene products) were associated with the protein-protein interaction network cluster; 81 upregulated gene products and 88 downregulated gene products were involved in the interaction network cluster; and 42 gene products in the cluster were cytokines, adipokines, and chemokines (Fig. 3; inner circular subnetwork). Of the gene products, 94% (21 cytokines and 100 signaling proteins) were identified and included the interaction network cluster, and 6% of the gene products were not associated with the network cluster. Notably, all cytokines, adipokines, and chemokines that were regulated by LIG3, with the exception of REG3G, CCL6 and PRL2C2, were included in the interaction network cluster (Fig. 3, inner

circular subnetwork). Transcriptional regulatory association analysis of this subnetwork indicated that the member genes may be regulated by NF- $\kappa$ B, C/EBP $\alpha$ , Pax-8, TFIIA- $\alpha/\beta/\gamma$  and TBP ( $P < 0.01$ ; Table SV). A total of 1,906 interactions were found in the LIG3-regulated protein-protein interaction network. The gene products with the highest interaction degrees (>50) included IL6, AKT1, TP53, EGF, TNF, MAPK3, FN1, MAPK1, IGF1, PTEN, JUN, CCL2, MTOR, CTNNA1, IRS1, MAPK14 and ERBB2. The cytokines, adipokines and chemokines in the network with the highest interaction degrees (>30) included IL6, EGF, TNF, IGF1, CCL2, CRP, TIMP1, SERPINE1, ADIPOQ, CSF1 and CSF3.

*Functional enrichment analysis of LIG3-regulated genes.*

To elucidate the biological functions associated with the protein-protein interaction network of LIG3-regulated gene products, a functional enrichment analysis was performed using the gene members of the network. GO categories were mapped using statistically overrepresented functional themes in a hierarchical manner (Fig. 4). GO terms with the highest significance were associated with inflammatory responses, hormonal responses, epithelial differentiation and

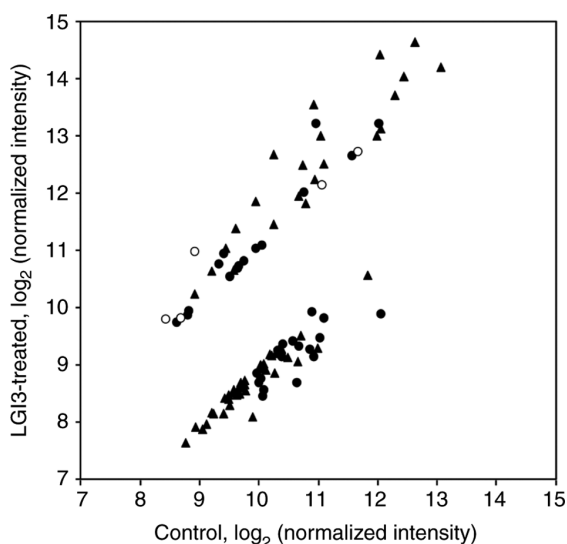


Figure 2. Effect of LGI3 on the phosphorylation and expression of signaling proteins. Scatter plots of  $\log_2$  normalized intensities. Black circle, phosphorylation data from Phospho Explorer Antibody Array (PEX100). Triangle, data from Signaling Explorer Antibody Array (SET100). White circle, data from Explorer Antibody Array (ASB600). Data with  $|\log_2(\text{fold change})| \geq 1$  are indicated.

development, protein phosphorylation and signaling, metabolic and transcriptional regulation, programmed cell death, and protein transport (Fig. 4). Notably, inflammatory response was the most significant and highly represented GO category.

**Gene co-expression network analysis.** All LGI3-regulated gene products were queried against the GCNs of SATs and VATs. GCNs are networks of genes connected by significant co-expression relationships that provide insights into the tissue-specific functions of gene sets, as co-expressed genes are regulated by common transcriptional regulatory programs and are components of the same protein complex or signaling pathway (27). A total of 121 and 114 gene products in the LGI3-regulated gene set were identified in the GCNs of SATs and VATs, respectively (Fig. 5A, group a; Fig. 5B, group c; Table SVI). These LGI3-regulated gene product subsets were associated with 5,904 gene products in the SAT GCN and with 3,743 gene products in the VAT GCN (Fig. 5A, group b; Fig. 5B, group d; Table SVI). Notably, these subnetworks of first-neighboring co-expressed genes (Fig. 5A, group b; Fig. 5B, group d) revealed distinct distributions between the GCNs of SATs and VATs. Comparative analysis was performed with the sum of the sets for LGI3-regulated genes and their first-neighboring co-expressed genes in the GCNs of SATs and VATs (Fig. 6A). The intersection of the sets included 2,669 genes, with 3,356 genes and 1,188 genes occurring only in the GCNs of SATs and VATs, respectively (Fig. 6A). A GO category map of the sum of gene co-expression subnetworks (Fig. 5, groups a-d; Fig. 6A) demonstrated that the gene products were involved in inflammatory and immune system, metabolic processes, apoptosis and vascular development (Fig. 6B). It was also observed that inflammatory response is a predominant GO term in the LGI3-regulated GCNs of VATs and that apoptosis and vascular development are preferentially associated with the LGI3-regulated GCNs of SATs (Fig. 6B; Table SVII). Transcriptional regulatory association analysis of

these subnetworks predicted that distinct and common transcription factors may be involved in the LGI3-regulated GCNs of SATs and VATs (Table SVIII).

Venn diagram analysis of LGI3-regulated genes and their associated GCNs of adipose tissues revealed that six LGI3-upregulated genes and 10 LGI3-downregulated genes belonged to the set of SAT GCN, and that five LGI3-upregulated genes and four LGI3-downregulated genes belonged to the set of VAT GCN (Fig. 7A). Protein-protein interaction network analysis of the 25 LGI3-regulated genes associated with adipose tissue GCNs demonstrated that 21 gene products formed an interaction network cluster (Fig. 7B). The interaction network cluster included 14 SAT-specific and seven VAT-specific LGI3-regulated genes in GCNs (Fig. 7B). Functional enrichment analysis of these genes revealed their involvement in various biological processes in a depot-specific manner (Table II). Notably, hematopoietic cell lineage, immune response and cytokine-cytokine receptor interaction were predominant functions of LGI3-regulated genes in VAT GCN.

In the present study, GCN analysis of the LGI3-regulated gene products demonstrated distinct co-expression network profiles between SATs and VATs. RNA-seq data of adipose tissues from the Human Protein Atlas indicated that LGI3 transcripts were expressed in adipocytes [40-60% of the total transcripts per million (TPM), fibroblasts (20-40%), smooth muscle cells (15%) and other cell types (5%)]. Moreover, RNA-seq data from the GTEx project of human adipose tissues revealed that the expression of LGI3 was higher in SATs [average protein transcripts per million (pTPM)=0.5] than in VATs (average pTPM=0.2). The gene products in the LGI3-regulated gene co-expression subnetworks may be regulated by common transcriptional regulatory programs.

**Expression quantitative trait loci for LGI3 expression in adipose tissues.** To explore the adipose depot-specific regulation of LGI3, single tissue eQTL were analyzed for the genomic loci that affect the expression levels of LGI3 in adipose tissues. eQTL are SNVs in genomic loci that account for variation in expression levels of mRNA (26). It was identified that three SNVs (Fig. 8A-D) in SAT and one in VAT (Fig. 8A and E) significantly altered LGI3 (Table III). These SNVs were cis-eQTL located 0.2-1.1 Mb apart from the LGI3 gene on chromosome 8. Analysis of single tissue RNA-seq data revealed that two SNVs (chr8\_23149508\_G\_A\_b38 and chr8\_23149665\_G\_A\_b38) increased LGI3 expression in SAT (Fig. 8C and D), and two SNVs (chr8\_21801331\_T\_G\_b38 and chr8\_22361155\_C\_T\_b38) decreased LGI3 expression in SAT and VAT (Fig. 8B and E).

## Discussion

The proinflammatory adipokine hypothesis for LGI3 is supported by the increased levels of LGI3 in adipose tissues in conditions of genetic obesity or high-fat-diet-induced obesity (14,15). Furthermore, our previous results revealed that LGI3 downregulated adiponectin and upregulated TNF- $\alpha$  (15,16). These results led to the hypothesis that LGI3 serves a regulatory role in the cytokine network of adipose tissues. It was further determined that LGI3-regulated gene products formed a protein-protein interaction network

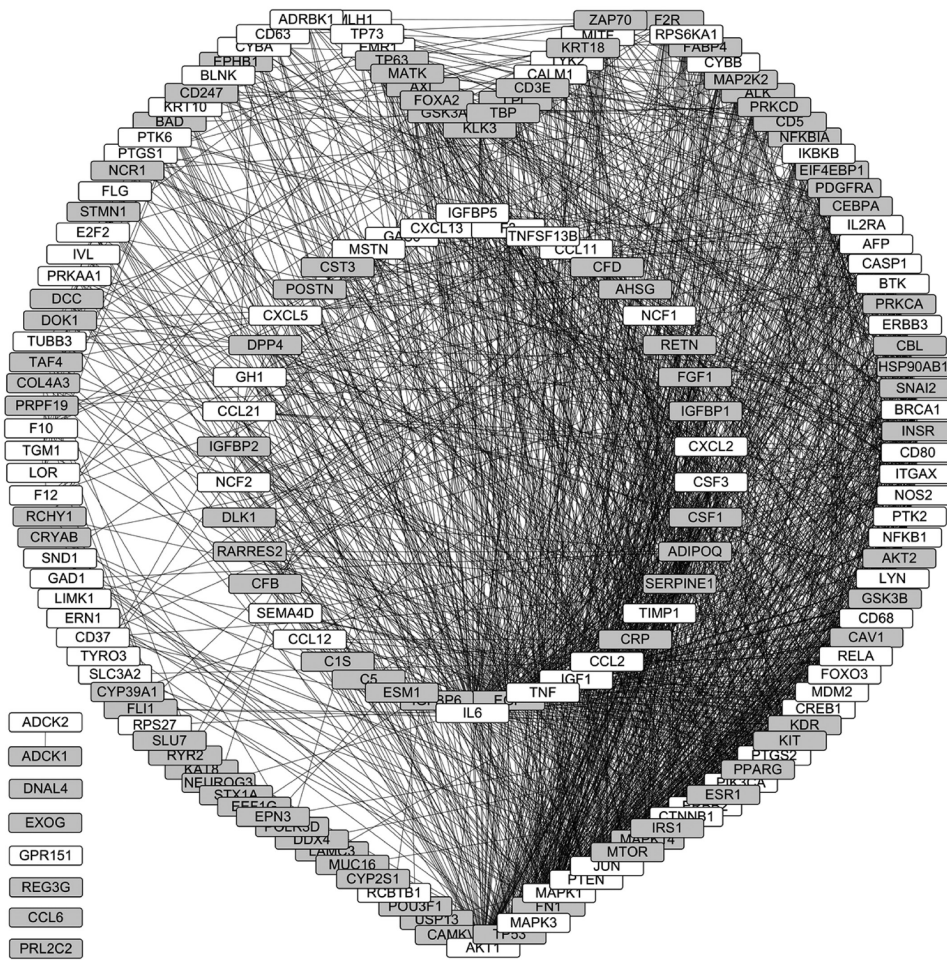


Figure 3. Protein-protein interaction network of leucine-rich repeat LGI3-regulated gene products. The network is represented by nodes (box, gene products) and lines (pairwise protein-protein interactions). The nodes are sorted based on the interaction degree. The white nodes indicate the upregulated gene products, whereas the gray nodes indicate the downregulated gene products. The inner circular network indicates a subnetwork consisting of cytokines, adipokines and chemokines.

cluster (12). The present study revealed an extended LGI3-regulated protein-protein interaction network and depot-specific association of LGI3 with adipose tissues by employing GCN and eQTL analyses in addition to functional enrichment analysis used in the previous study (12). In addition to adipose tissues, LGI3-regulated protein-protein interaction networks have been implicated in the prognosis of patients with glioma (11) and non-small cell lung cancer (13). Thus, LGI3-regulated functional networks may be large with numerous gene product members in multiple tissues.

The altered expression of various cytokines in the adipose tissue and plasma of LGI3-knockout mice suggests that the regulatory interactions of LGI3 with these factors may have both local and systemic effects (12). Moreover, the increased expression of cytokines in LGI3-knockout mice may be due to compensatory upregulation in response to LGI3 deficiency or may represent cytokines that are negatively regulated by LGI3 in wild-type mice (12). The downregulated cytokines in LGI3-knockout mice suggest that LGI3 may upregulate these factors in wild-type mice (12). Moreover, the protein-protein interaction network cluster of LGI3-regulated cytokines supports the critical role of LGI3 in the homeostasis of adipose tissues through the cytokine network. It has been predicted that the LGI3-regulated cytokine network is primarily regulated

by NF- $\kappa$ B, a key transcription factor in immune and inflammatory processes (28). It has also been demonstrated that LGI3 is a target gene and activator of NF- $\kappa$ B (8,16). Multiple genes with altered expressions in obesity have been found to be regulated by NF- $\kappa$ B (29). Thus, the increased expression of LGI3 observed in obesity may perturb the cytokine network largely through NF- $\kappa$ B and lead to metabolic inflammation in obese adipose tissues.

The components of the intracellular signaling pathways induced by LGI3 have been explored in various cell types. In neuronal cells, it has been revealed that LGI3-induced neurite outgrowth is mediated by Akt and focal adhesion kinase (3). p53 and MDM2 have also been determined to be involved in LGI3-promoted survival in ultraviolet B-irradiated keratinocytes (4). Moreover, it has been demonstrated that GSK3 $\beta$  and  $\beta$ -catenin mediated LGI3-promoted keratinocyte migration (5). Multiple signaling proteins (Erk1/2, AMPK, Bad, PTEN, 4E-BP1, Akt and GSK3 $\beta$ ) are regulated in LGI3-treated preadipocytes (12). Notably, the suppressive effect of LGI3 on adipogenesis in 3T3-L1 cells is mediated by its receptor, ADAM23 (14), whereas ADAM22 was determined to be the primary receptor for LGI3 in keratinocyte inflammatory signaling (8). Thus, LGI3 may transduce intracellular signaling through distinct receptors with common or unique

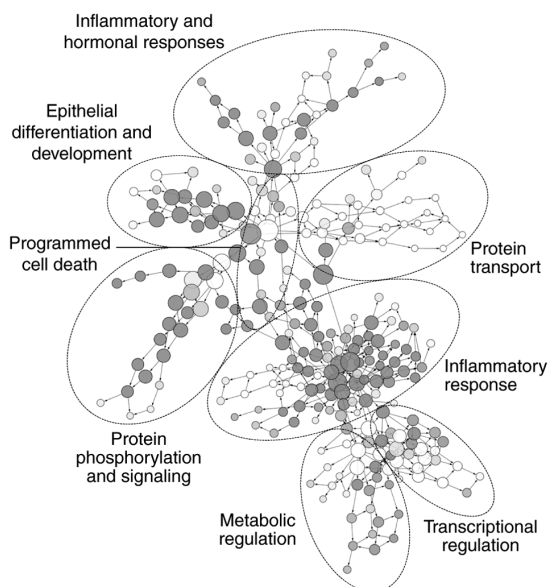


Figure 4. Functional enrichment analysis map of leucine-rich repeat LGI family member 3-regulated gene products. The nodes represent GO terms, and the arrow lines indicate parental relationships in the network hierarchy. The darker nodes represent lower P-values in the BiNGO analysis and indicate statistically more overrepresented GO terms ( $P < 0.01$ ). The dotted circles indicate the groups of GO category names that represent the GO term clusters. GO, Gene Ontology.

signaling components in various target cell types. As 3T3-L1 cells express ADAM23 but not ADAM22, the LGI3-regulated signaling protein network of the present study may represent the components and crosstalk effectors of the LGI3-ADAM23 signaling pathways.

The proximal signaling mechanisms of ADAM23 are still largely unknown. However, ADAM23 associates with  $\alpha\beta 3$  integrin to promote cell adhesion (30), with  $\alpha\beta 3$  integrin regulating macrophage inflammatory responses via PI3K/Akt-dependent NF- $\kappa$ B activation (31). Presumably, short-term upregulation by LGI3-induced protein phosphorylation occurred in the gene products that were predicted to be involved in immune response, inflammatory response and the PI3K/Akt signaling pathway, whereas gene products down-regulated in response to phosphorylation were predominantly associated with cancer-related pathways (11,13,15,16). These results support the hypothesis that LGI3 is a proinflammatory adipokine upregulated in conditions of obesity-associated metabolic inflammation and downregulated in cancer (10,11,13-15). In the current study, long-term upregulation of expression in LGI3-treated 3T3-L1 cells was demonstrated in the gene products predicted to be associated with receptor protein tyrosine kinase/non-membrane spanning protein tyrosine kinase signaling pathway, cell proliferation, PI3K signaling pathway and growth hormone receptor function. Notably, gene products with downregulated expression were predicted to be involved in the positive regulation of transcription, cancer pathways, nuclear chromatin function, and cell migration. It was also observed that the PI3K signaling pathway and cancer-related pathways were regulated by phosphorylation and expression levels. Presumably, the suppressive effect of LGI3 on adipogenesis (14) may be mediated by ADAM23 via NF- $\kappa$ B and PI3K/Akt pathways. NF- $\kappa$ B may be activated cooperatively

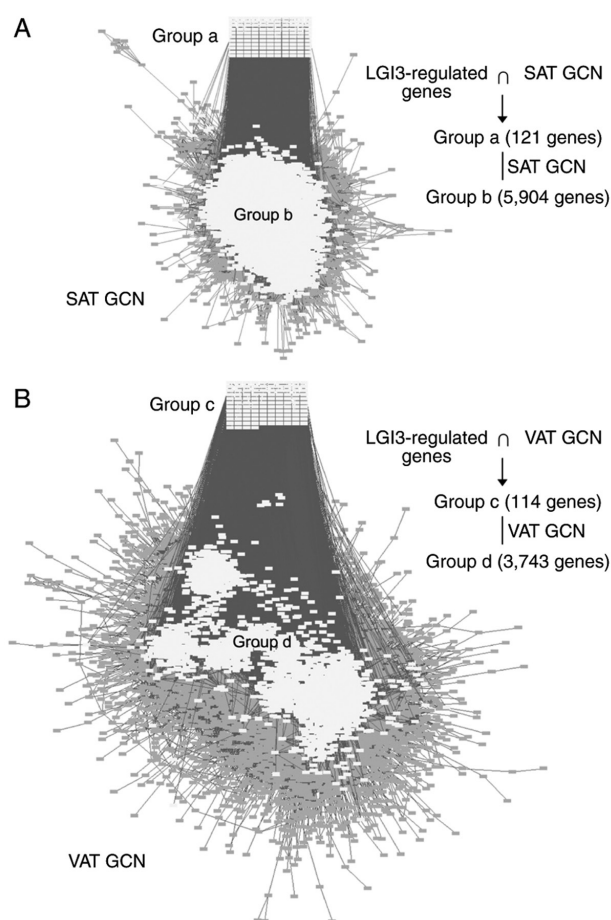


Figure 5. GCN analysis of LGI3-regulated gene products in adipose tissues. (A) LGI3-regulated gene products associated with the co-expression subnetwork of SAT GCN (group a) and a group of their first-neighboring co-expressed genes of SAT GCN (group b). (B) LGI3-regulated gene products associated with the co-expression subnetwork of VAT GCN (group c) and a group of their first-neighboring co-expressed genes of VAT GCN (group d). GCN, gene co-expression network; LGI3, leucine-rich repeat LGI family member 3; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

by LGI3 and TNF- $\alpha$  to antagonize insulin signaling in adipogenesis (14). LGI3 may also regulate the PI3K/Akt pathway, a component of adipogenic signaling pathways, by an unknown mechanism that may counteract adipogenesis.

The majority of LGI3-regulated gene products were involved in a protein-protein interaction network cluster with high interaction degrees. In the present study, the LGI3-regulated interaction network reported in our previous study (12) was extended by  $\sim 4$  fold in node number. Statistical analysis of network centrality to identify critical gene products in the network revealed that the gene products with the highest centrality in rank order were AKT1, IL6, TP53, EGF, TNF, FN1, MAPK3, MAPK1, PTEN, IGF1, JUN, CCL2, MTOR, CTNBN1, MAPK14, ESR1, IRS1, ERBB2, PIK3CA and PPARG (data not shown). These gene products have previously been reported to be involved in obesity-associated metabolic disorders (32-51). Moreover, a subset of these gene products has been previously reported to be regulated by LGI3; these included AKT1, TP53, TNF, MAPK3, MAPK1, PTEN, IGF1, CTNBN1, PIK3CA and PPARG (4-6,12,16).

The functional enrichment map of LGI3-regulated gene products demonstrated that the LGI3-regulated protein-protein

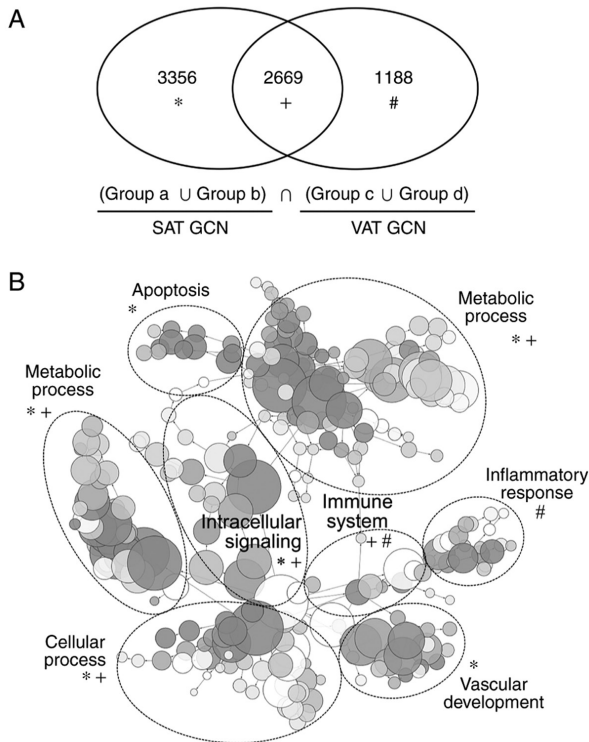


Figure 6. Comparative and functional enrichment analyses of the gene co-expression subnetworks of LGI3-regulated gene products and their first-neighboring co-expressed genes in adipose tissue GCNs. (A) Venn diagram indicating the distribution of genes in LGI3-regulated SAT and VAT GCNs. (B) Functional enrichment analysis map of LGI3-regulated SAT and VAT GCNs. The nodes represent GO terms and the arrow lines indicate parental relationships in the network hierarchy. The darker nodes represent lower P-values ( $P < 0.01$ ). The dotted circles indicate the groups of GO category names that represent the GO term clusters. Differences (\*) and intersections (+) of gene sets (Fig. 6A) in LGI3-regulated GCNs are indicated. GCN, gene co-expression network; LGI3, leucine-rich repeat LGI family member 3; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

interaction network was associated most significantly with inflammatory responses, epithelial differentiation and development, and metabolic regulation. Previous results indicated that LGI3 may serve a proinflammatory role by upregulating COX-2, iNOS, MCP-1, TNF- $\alpha$  and NF- $\kappa$ B (14,16). Notably, LGI3 differentially increases the expression of multiple inflammatory genes in preadipocytes, adipocytes and macrophages (12). These LGI3-regulated inflammatory gene products are known to be involved in obesity-associated metabolic disorders (36,52-61). It was also previously reported that LGI3 regulated the differentiation of keratinocytes (6), neuronal cells (3) and adipocytes (14), as well as the inflammatory response of keratinocytes (8), preadipocytes, adipocytes and macrophages (12). These results support the multitarget and pleiotropic nature of LGI3.

Comparative analysis of the LGI3-regulated gene products in the GCNs of SATs and VATs revealed common and distinct subsets of gene products. A larger number of gene products were involved in the LGI3-regulated GCNs in SATs than in VATs. The larger size of the LGI3-regulated SAT GCN may be due to the relatively higher expression of LGI3 in SATs than in VATs, and the difference in the transcription factor repertoires that control LGI3-regulated GCNs. The functional enrichment map of LGI3-regulated GCNs of SATs

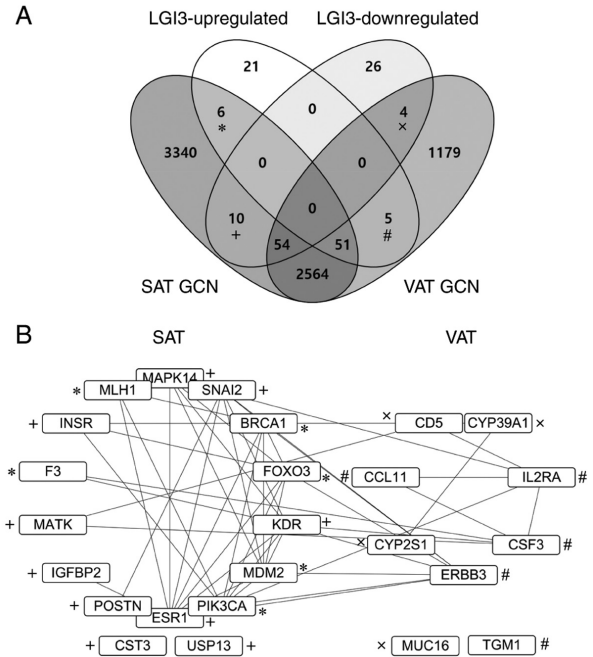


Figure 7. Comparative analysis of the upregulated or downregulated genes by LGI3 and the gene sets of adipose tissue GCNs associated with LGI3-regulated genes. (A) Venn diagram presenting the sets of regulated gene categories. (B) Protein-protein interaction network of LGI3-regulated genes in adipose tissue GCNs. The network is depicted by nodes (gene products) and lines (pairwise protein interactions) sorted based on interaction degrees. The symbols (\*, x, + and #) indicate the gene products in the common sets of the gene categories indicated in Fig. 7A. GCN, gene co-expression network; LGI3, leucine-rich repeat LGI family member 3; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

and VATs revealed that vascular development and inflammatory responses are the predominant function categories in SATs and VATs, respectively. These results imply that LGI3 may serve as a multifunctional cytokine in cellular differentiation and development (3,6,12) in SATs and as a proinflammatory adipokine in obesity-associated metabolic disorders in VATs (14-16). GCN analysis of the metabolic disorder-associated modules of the co-expressed genes in adipose tissues demonstrated enrichment of the immune response and oxidative phosphorylation pathways (62). Moreover, GCN and protein interaction network analyses indicated that NF- $\kappa$ B was implicated in angiogenesis and inflammation in adipose tissue (29). Transcription factor GCN of adipose tissue RNA-seq data also indicated that the obesity-associated network module was enriched for regulation processes in the immune system (63). Protein-protein interaction networks of LGI3-regulated gene products associated with adipose tissue GCNs further supported that these gene products may be differentially regulated in SAT and VAT in a cooperative manner. Hence, LGI3-regulated GCNs may serve multiple and differential roles in the homeostasis and dysregulation of metabolic, cellular and inflammatory processes in SATs and VATs.

Whole-genome analysis of eQTL revealed four SNVs that account for the regulation of LGI3 expression in adipose tissues. All eQTL were located within ~1.1 Mb of the LGI3 gene and may serve as a cis-acting regulatory elements for LGI3 expression. These eQTL were located in intergenic regions



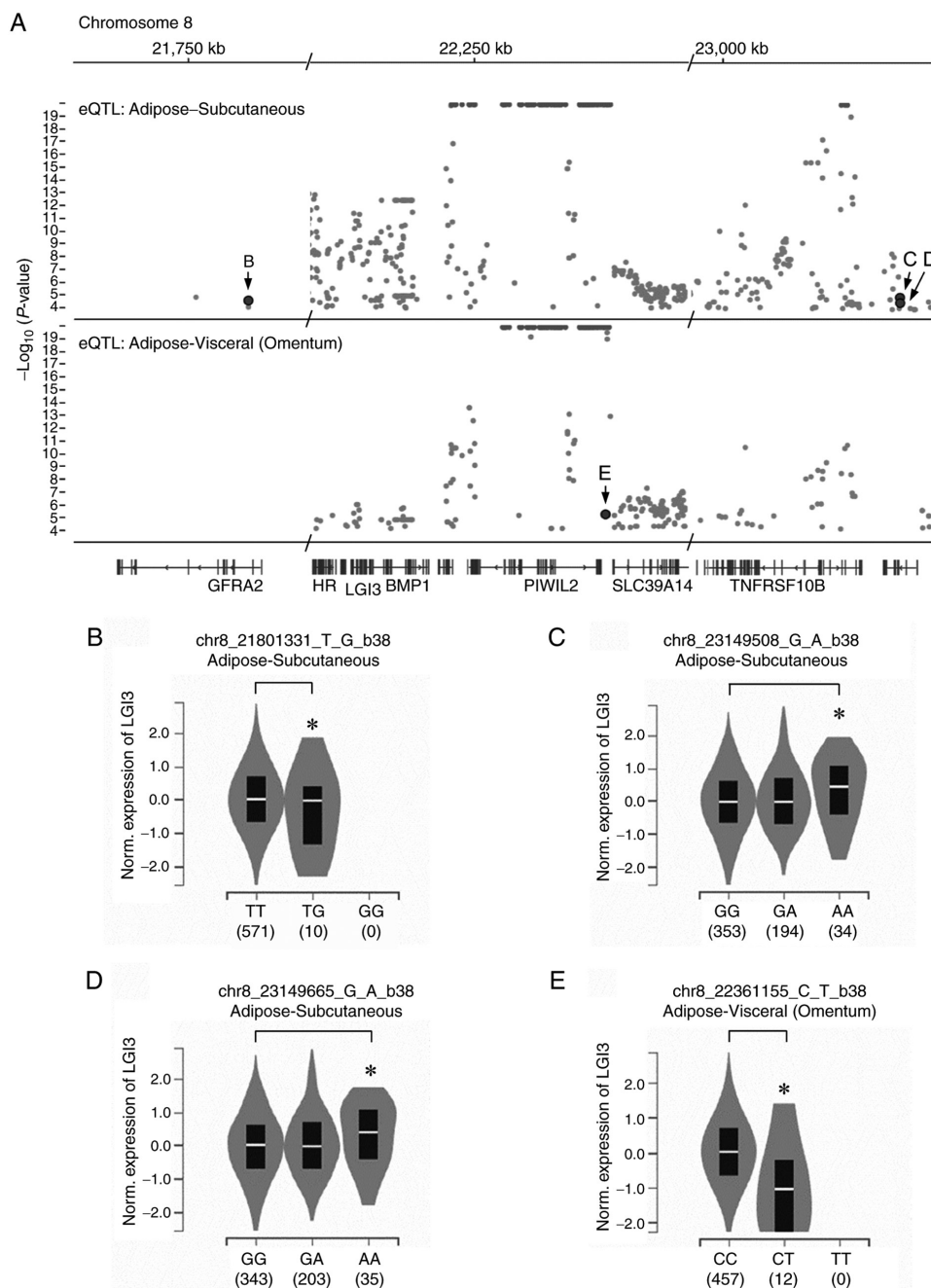


Figure 8. eQTL that affect the expression of LGI3 in adipose tissues. (A) Single-tissue eQTL tracks. Each dot in the scatter plots represents a significant eQTL (FDR <0.05). Large dots (B-E) indicate significant eQTL for LGI3. Slash lines in horizontal axis indicate the regions of chromosome omitted in the diagram to show widespread eQTLs collectively. (B-E) eQTL violin plots for significant eQTL that affect LGI3 expression in a depot-specific manner. The x-axis is the variant alleles and sample numbers and the y-axis is normalized expression values of LGI3. The eQTL for LGI3 are summarized in Table III. Variant IDs of eQTL are as follows: (B) chr8\_21801331\_T\_G\_b38; (C) chr8\_23149508\_G\_A\_b38; (D) chr8\_23149665\_G\_A\_b38; and (E) chr8\_22361155\_C\_T\_b38. \*FDR <0.05 and P<0.01. eQTL, expression quantitative trait loci; LGI3, leucine-rich repeat LGI family member 3; Norm, normal.

(chr8\_21801331\_T\_G\_b38, chr8\_22361155\_C\_T\_b38) and in introns of the TNFRSF10D gene (chr8\_23149508\_G\_A\_b38, chr8\_23149665\_G\_A\_b38). Notably, an intergenic SNV (chr8\_22361155\_C\_T\_b38) decreased the expression of LGI3 in VAT. These results suggested that genetic variations in SNVs near the LGI3 gene are associated with adipose depot-specific regulation of LGI3.

Macrophage polarization in adipose tissues is a hallmark of the proinflammatory switch in obesity (64-66), and the predominance of proinflammatory M1-type macrophages over those of the anti-inflammatory M2-type represents a major

component of metabolic inflammation in VATs (67,68). Our previous studies indicated that LGI3 upregulated M1-polarized macrophage markers (TNF- $\alpha$ , iNOS, CCL-2/MCP-1, IL-6, CD68 and CD80) (12,14,16). The increased expression of LGI3 in adipose tissue macrophages is observed in obesity and may serve a critical regulatory role in M1 macrophage polarization and proinflammatory transition of cytokine networks (12,14,16). It was hypothesized that LGI3 may contribute to innate tumor immunity in the cancer microenvironment by promoting M1 polarization of the tumor-associated macrophages (13). Thus, LGI3-regulated cytokine networks may serve pathological

Table II. Functional enrichment analysis of the LIG3 family member 3-regulated gene products associated with GCNs of SAT and VAT.

A, SAT			
Category	Term	Count	P-value
GO TERM_MF_DIRECT	GO:0005515 protein binding	16	5.53x10 <sup>-5</sup>
KEGG_PATHWAY	hsa04068:FoxO signaling pathway	5	6.04x10 <sup>-5</sup>
GO TERM_BP_DIRECT	GO:0032355 response to estradiol	4	6.68x10 <sup>-5</sup>
KEGG_PATHWAY	hsa04151:PI3K-Akt signaling pathway	6	1.82x10 <sup>-4</sup>
KEGG_PATHWAY	hsa04917:Prolactin signaling pathway	4	2.17x10 <sup>-4</sup>
KEGG_PATHWAY	hsa05205:Proteoglycans in cancer	5	2.86x10 <sup>-4</sup>
GO TERM_BP_DIRECT	GO:0090090 negative regulation of canonical Wnt signaling pathway	4	3.75x10 <sup>-4</sup>
GO TERM_BP_DIRECT	GO:0030335 positive regulation of cell migration	4	5.34x10 <sup>-4</sup>
GO TERM_BP_DIRECT	GO:0038083 peptidyl-tyrosine autophosphorylation	3	5.70x10 <sup>-4</sup>
GO TERM_BP_DIRECT	GO:0008284 positive regulation of cell proliferation	5	6.27x10 <sup>-4</sup>
B, VAT			
Category	Term	Count	P-value
KEGG_PATHWAY	hsa04640:Hematopoietic cell lineage	3	2.29x10 <sup>-3</sup>
GOTERM_BP_DIRECT	GO:0006955 immune response	3	1.59x10 <sup>-2</sup>
KEGG_PATHWAY	hsa04060:Cytokine-cytokine receptor interaction	3	1.70x10 <sup>-2</sup>
GOTERM_CC_DIRECT	GO:0005615 extracellular space	4	1.70x10 <sup>-2</sup>
GOTERM_BP_DIRECT	GO:0030838 positive regulation of actin filament polymerization	2	2.12x10 <sup>-2</sup>
GOTERM_MF_DIRECT	GO:0016705 oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	2	2.67x10 <sup>-2</sup>
GOTERM_BP_DIRECT	GO:0043547 positive regulation of GTPase activity	3	2.77x10 <sup>-2</sup>
GOTERM_BP_DIRECT	GO:0014068 positive regulation of phosphatidylinositol 3-kinase signaling	2	3.06x10 <sup>-2</sup>
GOTERM_CC_DIRECT	GO:0031090 organelle membrane	2	3.76x10 <sup>-2</sup>
GOTERM_CC_DIRECT	GO:0016021 integral component of membrane	6	4.59x10 <sup>-2</sup>

GCN, gene co-expression network; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; MF, molecular function; CC, cellular components.

Table III. Summary of depot-specific expression quantitative trait loci that regulate the expression of LIG3 family member 3.

Variant ID	SNV ID	P-value	NES	MAF	Tissue
chr8_21801331_T_G_b38	rs373132699	2.9x10 <sup>-5</sup>	-0.9	0.0018	SAT
chr8_23149508_G_A_b38	rs13256716	1.7x10 <sup>-5</sup>	0.18	0.2514	SAT
chr8_23149665_G_A_b38	rs13257094	4.7x10 <sup>-5</sup>	0.17	0.2618	SAT
chr8_22361155_C_T_b38	rs56251182	6.4x10 <sup>-6</sup>	-0.79	0.0174	VAT (Omentum)

Nominal P-values were generated by the linear regression model between genotype and expression (<https://gtexportal.org/home/documentationPage>). SNV, single nucleotide variations; NES, normalized effect size; MAF, minor allele frequency; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

and prognostic roles in obesity-associated metabolic diseases through macrophage polarization in VATs. The limitations of the present study were that mouse epididymal adipose tissues and 3T3-L1 cells were used for array analyses and GCN and eQTL databases for integrative analysis. The present results

warrant further studies to validate the predominant proinflammatory role of LIG3 in VAT via M1 macrophage polarization using VAT from obese animals and humans.

In conclusion, the present study provided an integrative insight into LIG3-regulated gene products and the association

of their protein-protein interaction and co-expression networks in adipose tissues with common and differential biological processes in SATs and VATs. It is hypothesized from these data that LGI3 may serve a homeostatic and pathological role in adipose tissues in a depot-specific manner.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are not deposited in public repositories due to pending patent but are available from the corresponding author on reasonable request.

### Authors' contributions

HYY conceived and designed the study, performed the data analysis and wrote the manuscript. HAK performed the experiments and data analysis. KJB contributed to the analysis and interpretation of data. HYY, HAK and KJB confirm the authenticity of all the raw data. All the authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The datasets used and/or analyzed during the current study are partially related with pending patent (HYY, Korean patent application reference no. 10-2018-0060762. 2018.5.28). The authors declare that they have no other competing interests.

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