Genes and Pathways for Plasma Lipid Traits via Multi-tissue Multi-omics Systems Analysis

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Abstract

Genome-wide association studies (GWAS) have implicated ~380 genetic loci for plasma lipid regulation. However, these loci only explain 17-27% of the trait variance and a comprehensive understanding of the molecular mechanisms has not been achieved. In this study, we utilized an integrative genomics approach leveraging diverse genomic data from human populations to investigate whether genetic variants associated with various plasma lipid traits, namely total cholesterol (TC), high and low density lipoprotein cholesterol (HDL and LDL), and triglycerides (TG), from GWAS were concentrated on specific parts of tissue-specific gene regulatory networks. In addition to the expected lipid metabolism pathways, gene subnetworks involved in 'interferon signaling', 'autoimmune/immune activation', 'visual transduction', and 'protein catabolism' were significantly associated with all lipid traits. Additionally, we detected trait-specific subnetworks, including cadherin-associated subnetworks for LDL, glutathione metabolism for HDL, valine, leucine and isoleucine biosynthesis for TC, and insulin signaling and complement pathways for TG. Finally, utilizing gene-gene relations revealed by tissue-specific gene regulatory networks, we detected both known (e.g. *APOH*, *APOA4*, and *ABCA1*) and novel (e.g. *F2* in adipose tissue) key regulator genes in these lipid-associated subnetworks. Knockdown of *F2* gene (Coagulation Factor II, Thrombin) in 3T3-L1 adipocytes reduced gene expression of *Abcb11*, *Apoa5*, *Apof*, *Gc*, *Fabp*, *Hrg*, *Proc*, and *Cd36*, several of which are important in lipoprotein transport and fatty acid uptake, providing evidence for a link between adipose thrombin and plasma lipid regulation. Our results shed light on the complex mechanisms underlying lipid metabolism and highlight potential novel targets for lipid regulation and lipid-associated diseases.

Keywords: Lipid metabolism; Integrative genomics; GWAS; Pathway and network analysis; Coagulation Factor II

Introduction

Lipid metabolism is vital for organisms as it provides energy as well as essential materials such as membrane components and signaling molecules for basic cellular functions. Lipid dysregulation is closely related to many complex human diseases, such as atherosclerotic cardiovascular disease (1), Alzheimer's disease (2, 3), type 2 diabetes (T2D) (4), and cancers (5). The notion of targeting lipid metabolism to treat human diseases has been reinforced by the fact that many disease-associated genes and drug targets (e.g., *HMGCR* as the target of statins and *PPARA* as the target of fibrates) are involved in lipid metabolic pathways (6-8).

Accumulating evidence supports that plasma lipids are complex phenotypes influenced by both environmental and genetic factors (9, 10). Heritability estimates for main plasma lipids are high (e.g. ~70% for low density lipoprotein cholesterol [LDL] and ~55% for high density lipoprotein cholesterol [HDL]) (11), indicating that DNA sequence variation plays an important role in explaining the inter-individual variability in plasma lipid levels. Indeed, genome-wide association studies (GWAS) have pinpointed a total of 386 genetic loci, captured in the form of single nucleotide polymorphisms (SNPs) associated with lipid phenotypes (12-16). For example, the most recent GWAS on lipid levels identified 118 loci that had not previously been associated with lipid levels in humans, revealing a daunting genetic complexity of blood lipid traits (16).

However, there are several critical issues that cannot be easily addressed by traditional GWAS analysis. First, even very large GWAS may lack statistical power to identify SNPs with small effect sizes and as a result the most significant loci only explain a limited proportion of the genetic heritability, for example, 17.2 – 27.1% for lipid traits (17). Second, the functional consequences of the genetic variants and the causal genes underlying the significant genetic loci are often unclear and await elucidation. To facilitate functional characterization of the genetic variants, genetics of gene expression studies (18, 19) and the ENCODE efforts (20) have documented tissue- or cell-specific expression quantitative trait loci

63 (eQTLs) and functional elements of the human genome. These studies provide the much-needed bridge
64 between genetic polymorphisms and their potential molecular targets. Third, the molecular mechanisms
65 that transmit the genetic perturbations to complex traits or diseases, that is, the cascades of molecular
66 events through which numerous genetic loci exert their effects on a given phenotype, remain elusive.
67 Biological pathways that capture functionally related genes involved in molecular signaling cascades and
68 metabolic reactions, and gene regulatory networks formed by regulators and their downstream genes can
69 elucidate the functional organization of an organism and provide mechanistic insights (21). Indeed,
70 various pathway- and network-based approaches to analyzing GWAS datasets have been developed (18,
71 22-24) and demonstrated to be powerful to capture both the missing heritability and the molecular
72 mechanisms of many human diseases or quantitative phenotypes (18, 23, 25, 26). For these reasons,
73 integrating genetic signals of blood lipids with multi-tissue multi-omics datasets that carry important
74 functional information may provide a better understanding of the molecular mechanisms responsible for
75 lipid regulation as well as the associated human diseases.

76 In this study, we apply an integrative genomics framework to identify important regulatory genes,
77 biological pathways, and gene subnetworks in relevant tissues that contribute to the regulation of four
78 critical blood lipid traits, namely TC, HDL, LDL, and TG. We combine the GWAS results from the
79 Global Lipids Genetics Consortium (GLGC) with functional genomics data from a number of tissue-
80 specific eQTLs and the ENCODE project, and gene-gene relationship information from biological
81 pathways and data-driven gene network studies. The integrative framework is comprised of four main
82 parts (**Figure 1**): 1) Marker Set Enrichment Analysis (MSEA) where GWAS, functional genome, and
83 pathways or co-regulated genes are integrated to identify lipid-related functional units of genes, 2)
84 merging and trimming of identified lipid gene sets, 3) key driver analysis (KDA) to pinpoint important
85 regulatory genes by further integrating gene regulatory networks, and 4) validation of key regulators
86 using genetic perturbation experiments and *in silico* evidence. This integrated systems biology approach

87 enables us to derive a comprehensive view of the complex and novel mechanisms underlying plasma lipid
88 metabolism.
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Materials and Methods

GWAS of lipid traits

The experimental design, genotyping, and association analyses of HDL, LDL, TC, and TG were described previously (12). The dataset used in this study is comprised of > 100,000 individuals of European descent (sample size 100,184 for TC, 95,454 for LDLC, 99,900 for HDLC and 96,598 for TG), ascertained in the United States, Europe, or Australia. More than 906,600 SNPs were genotyped using Affymetrix Genome-Wide Human SNP Array 6.0. Imputation was further carried out to obtain information for up to 2.6 million SNPs using the HapMap CEU (Utah residents with ancestry from northern and western Europe) panel. SNPs with minor allele frequency (MAF) < 1% were removed. Finally, a total of ~ 2.6 million SNPs tested for association with each of the four lipid traits were used in our study.

Genetic association study of lipid traits using MetaboChip

The experimental design, genotyping, and association analyses of the lipid MetaboChip study were described previously (27). The study examined subjects of European ancestry, including 93,982 individuals from 37 studies genotyped with the MetaboChip array, comprised of 196,710 SNPs representing candidate loci for cardiometabolic diseases. There was limited overlap between the individuals involved in GWAS and those in MetaboChip.

Knowledge-based biological pathways

We included canonical pathways from the Reactome (version 45), Biocarta, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (28, 29). In addition to the curated pathways, we constructed four positive control pathways based on known lipid-associated loci ($p < 5.0 \times 10^{-8}$) and candidate genes from the GWAS Catalog (30). These gene sets were based on 4, 11, 13, and 13 studies for TC, TG, LDL,

112 and HDL, respectively (full lists of genes in each positive control sets are in **Supplemental Table S6**)
113 and serve as positive controls to validate our computational method.

114 **Data-driven modules of co-expressed genes**

115 Beside the canonical pathways, we used co-expression modules that were derived from a collection of
116 genomics studies (**Supplement Table S7**) of liver, adipose tissue, aortic endothelial cells (HAEC), brain,
117 blood, kidney, and muscle (31-40). A total of 2706 co-expression modules were used in this study.
118 Although liver and adipose tissue are likely the most important tissues for lipid regulation, we included
119 the other tissue networks to confirm whether known tissue types for lipids could be objectively detected
120 and whether any additional tissue types are also important for lipids.

121 **Mapping SNPs to genes**

122 Three different mapping methods were used in this study to link SNPs to their potential target genes.

123 *Chromosomal distance-based mapping*

124 First, we used a standard distance-based approach where a SNP was mapped to a gene if within 50 kb of
125 the respective gene region. The use of ± 50 kb to define gene boundaries is commonly used in GWAS.

126 *eQTL-based mapping*

127 The expression levels of genes can be seen also as quantitative traits in GWAS. Hence, it is possible to
128 determine eQTLs and the expression SNPs (eSNPs) within the eQTLs that provide a functionally
129 motivated mapping from SNPs to genes. Moreover, the eSNPs within the eQTL are specific to the tissue
130 where the gene expression was measured and can therefore provide mechanistic clues regarding the tissue
131 of action when intersected with lipid-associated SNPs. Results from eQTL studies in human adipose
132 tissue, liver, brain, blood, and HAEC were used in this study (31, 33, 34, 41-49). We included both *cis*-
133 eSNPs (within 1 Mb distance from gene region) and *trans*-eSNPs (beyond 1 Mb from gene region), at a
134 false discovery rate < 10%.

135 *ENCODE-based mapping*

136 In addition to eQTLs and distance-based SNP-gene mapping approaches, we integrated functional data
137 sets from the Regulome database (20), which annotates SNPs in regulatory elements in the *Homo sapiens*
138 genome based on the results from the ENCODE studies (50).

139 *Nine unique combinations of SNP-gene mapping*

140 Using the above three mapping approaches, we derived nine unique sets of SNP-gene mapping. These are:
141 eSNP adipose, eSNP liver, eSNP blood, eSNP brain, eSNP HAEC, eSNP all (i.e., combining all the
142 tissue-specific eSNPs above), Distance (chromosomal distance-based mapping), Regulome (ENCODE-
143 based mapping), and Combined (combining all the above methods).

144 **Removal of SNPs in linkage disequilibrium**

145 We observed a high degree of linkage disequilibrium (LD) in the eQTL, Regulome, and distance-based
146 SNPs, and this LD structure may cause artifacts and biases in the downstream analysis. For this reason,
147 we devised an algorithm to remove SNPs in LD while preferentially keeping those with a strong
148 statistical association with lipid traits. Technical details are available in Supplementary Methods. We
149 chose a LD cutoff ($R^2 < 0.5$) to remove redundant SNPs in high LD.

150 **Marker Set Enrichment Analysis (MSEA)**

151 We applied a modified MSEA method (24, 51) to find pathways/co-expressed modules associated with
152 lipid traits (**Supplemental Methods**). False discovery rates (FDR) were estimated with the method by
153 Benjamini and Hochberg (52). Pathways or co-expression modules with FDR < 10% were considered
154 statistically significant. MSEA were applied to both the GLGC GWAS dataset and the MetaboChip
155 dataset. The combined FDR from these two datasets was expected to be < 1% (10% * 10% = 1%).

156 **Comparison between MSEA and other computational method**

157 To ensure that the pathway results from MSEA are reproducible, we used the improved gene-set-
158 enrichment analysis approach (iGSEA) (53). In the iGSEA analysis, we generated gene sets using the
159 same canonical pathways and co-expression modules in MSEA. The SNPs were mapped to genes using
160 the default settings of iGSEA. For each given gene set, significance proportion-based enrichment score
161 was calculated to estimate the enrichment of genotype–phenotype association. Then, iGSEA performed
162 label permutations to calculate nominal P-values to assess the significance of the pathway-based
163 enrichment score and FDR to correct multiple testing, with FDR < 0.25 (default setting) regarded as
164 significant pathways. Considering that MSEA and iGSEA were independent, the combined FDR from
165 these two methods of analysis was expected to be < 5% (10% x 25% = 2.5%).

166 **Construction of independent supersets and confirmation of lipid association**

167 Because the pathways or co-expression modules were collected from multiple sources, there were
168 overlapping or nested structures among the gene sets. To make the results more meaningful, we
169 constructed relatively independent supersets that captured the core genes from groups of redundant
170 pathways and co-expression modules (**Supplemental Methods**). After merging, we annotated each
171 superset based on function enrichment analysis of the known pathways from the Gene Ontology and
172 KEGG databases (P < 0.05 in Fisher's exact test after Bonferroni correction). The supersets were given a
173 second round of MSEA to confirm their significant associated with lipids using P < 0.05 after Bonferroni
174 correction as the cutoff.

175 **Key driver analysis (KDA)**

176 We adopted a previously developed KDA algorithm (54-56) of gene-gene interaction networks to the
177 lipid-associated supersets in order to identify the key regulatory genes (**Figure 1**). In the study, we
178 included Bayesian regulatory networks from diverse tissues, including adipose tissue, liver, blood, brain,
179 kidney and muscle (31-39). A key driver was defined as a gene that is directionally connected to a large
180 number of genes from a lipid superset, compared to the expected number for a randomly selected gene

181 within the Bayesian network (details in **Supplemental Methods**). The MSEA, merging, and KDA were
182 performed using R.

183 **Enrichment analysis of lipid-associated subnetworks in human complex diseases**

184 We collected disease susceptibility genes from GWAS Catalog with GWAS P<10E-5 for four human
185 complex diseases, including cardiovascular diseases ('myocardial infarction', 'myocardial infarction
186 (early onset)', 'coronary artery calcification', and 'coronary heart disease'), Alzheimer's disease, type 2
187 diabetes, and cancer ('colon cancer', 'breast cancer', 'pancreas cancer', 'prostate cancer', and 'chronic
188 lymphocytic leukemia'). Fisher's exact test was used to explore the enrichment of genes in the lipid-
189 associated subnetworks in the disease gene sets. Bonferroni-corrected $p < 0.05$ was considered significant.

190 **3T3-L1 cell culture and transient transfection with *F2 siRNA***

191 The mouse preadipocytes 3T3-L1 cells were obtained from ATCC and maintained and differentiated to
192 adipocytes according to the manufacturer's instruction. For knockdown experiments, 3 predesigned
193 siRNAs targeting *F2* gene (GenePharma, Paramount, CA) were tested and the most effective one was
194 selected for the experiment; 3T3-L1 adipocytes were transfected with 50 nM of *F2 siRNA* using
195 lipofectamin2000 on day 7 of the differentiation. As control, 50 nM of scrambled *siRNA* (GenePharma,
196 Paramount, CA) was transfected. Followed by 72 hrs of siRNA treatment, adipocytes were processed for
197 the experiments. To decide the changes in lipid accumulation, adipocytes were stained by Oil Red O stain
198 solution according to the protocol. After obtaining the pictures, Oil Red O was eluted in isopropyl alcohol
199 and OD values were measured at 490 nm.

200 **RNA extraction and Real-time PCR**

201 Total RNA was extracted from the adipocytes (Zymo Research, Irvine, CA), and quantitative PCR was
202 performed using the primers shown in **Supplemental Table S8**. Relative quantification was calculated

203 using the 2 ^ (-ΔΔ CT) method (57). Statistical significance was determined by Two-tailed unpaired
204 Student's t test. In all graph, *P < 0.05 and **P < 0.01. Data are mean ± s.e.m of n = 3-4.

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222 **Results**

223 **Identification of pathways and gene co-expression modules associated with lipid traits**

224 To asses biological pathway enrichment for the four lipid traits with GLGC GWAS, we curated a
225 total of 4532 gene sets including 2705 tissue-specific co-expression modules (i.e., highly co-regulated
226 genes based on tissue gene expression data) and 1827 canonical pathways from Reactome, Biocarta and
227 the Kyoto Encyclopedia of Genes and Genomes (KEGG). These gene sets were constructed as data- and
228 knowledge-driven functional units of genes. Four predefined positive control gene sets for HDL, LDL,
229 TC, and TG were also created based on candidate genes curated from the GWAS catalog (58). To map
230 potential functional SNPs to genes in each gene set, tissue-specific eQTLs, ENCODE functional
231 genomics information, and chromosomal distance-based mapping were used (details in Methods). Tissue-
232 specific eQTL sets were obtained from the GTEx database from studies on human adipose tissue, liver,
233 brain, blood, and human aortic endothelial cells (HAEC), and a total of nine SNP-gene mapping methods
234 were created. The liver and adipose tissues have established roles in lipid regulation, whereas the other
235 tissues are included for comparison.

236 Integrating the datasets mentioned above using MSEA, we identified 65, 86, 90, and 92 gene sets
237 whose functional genetic polymorphisms showed significant association with HDL, LDL, TC, and TG,
238 respectively, in GLGC GWAS (FDR < 10%; **Supplemental Table S1**). The predefined positive controls
239 for the four lipid traits were among the top signals for their corresponding traits (**Table 1**), indicating that
240 our MSEA method is sensitive in detecting true lipid trait-associated processes. Compared with other
241 tissues, more pathways were captured when using liver and adipose eSNPs to map GWAS SNPs to genes
242 (**Supplemental Table S1**). For example, 56 out of the 86 LDL-associated pathways were found when
243 liver and adipose eSNPs were used in our analysis. These results confirmed the general notion that liver
244 and adipose tissue play critical roles in regulating plasma lipids, leading us to focus the bulk of our
245 analysis on these two tissues, with the remaining tissues serving as supplement.

246 Among the significant gene sets, 39 were shared across the four lipid traits. These gene sets
247 represented the expected lipid metabolic pathways as well as those that are less known to be associated
248 with lipids, such as 'antigen processing and presentation', 'cell adhesion molecules (CAMs)', 'visual
249 phototransduction', and 'IL-5 signaling pathway' (summary in **Table 1**; details in **Supplemental Table**
250 **S1**). We broadly classified the common gene sets detected into 'positive controls', 'lipid metabolism',
251 'interferon signaling', 'autoimmune/immune activation', 'visual transduction', and 'protein catabolism'
252 (**Table 1**).

253 Beside the common gene sets described above, we also detected 18, 5, 6, and 17 trait-specific
254 pathways/modules for HDL, LDL, TC, and TG, respectively (**Table 2**; **Supplement Table S1**),
255 suggesting trait-specific regulatory mechanisms. Among the 18 pathways for HDL were 'cation-coupled
256 chloride transporters', 'glycerolipid metabolism' and 'negative regulators of RIG-I/MDA5 signaling'
257 across analyses using different tissue eSNP mapping methods, 'alcohol metabolism' from brain-based
258 analysis, 'packaging of telomere ends' in adipose tissue, 'glutathione metabolism' in liver, and
259 'cobalamin metabolism' and 'taurine and hypotaurine metabolism' in both adipose and liver-based
260 analyses. LDL-specific pathways included the 'platelet sensitization by LDL' pathway and a liver co-
261 expression module related to cadherin. TC-specific pathways included 'valine, leucine and isoleucine
262 biosynthesis' across tissues and 'wound healing' in the brain-based analysis. When looking at the TG-
263 specific pathways, gene sets associated with 'cellular junctions' were consistent across tissues whereas
264 'insulin signaling' and complement pathways were exclusively seen in adipose tissue-based analysis.

265 **Replication of lipid-associated pathways using additional dataset and method**

266 To replicate our results from the analysis of GLGC GWAS datasets, we utilized an additional lipid
267 genetic association dataset based on a MetaboChip lipid association study (15) which involved
268 individuals independent of those included in GLGC. The gene sets detected using this independent dataset
269 highly overlapped with those from the GLGC dataset (**Table 1**; **Supplemental Figure 1**; Overlap p

270 values < 10^{-20} by Fisher's exact test). We also utilized a different pathway analysis method iGSEA (53)
271 and again many of the gene sets were found to be reproducible (**Table 1**; **Supplemental Figure 1**;
272 overlapping p values < 10^{-20}).

273 **Construction of non-overlapping gene supersets for lipid traits**

274 As the knowledge-based pathways and data-driven co-expression modules used in our analysis can
275 converge on similar functional gene units, some of the lipid-associated gene sets have redundancies. We
276 therefore merged overlapping pathways to derive independent, non-overlapping gene sets associated lipid
277 traits. For the 39 shared pathways/co-expression modules across the four lipid traits described earlier, we
278 merged and functionally categorized them into five independent supersets (**Table 1; Table 3**). For the
279 significant gene sets for each lipid trait, we merged them into 17, 16, 18, and 14 supersets for HDL, LDL,
280 TC, and TG, respectively (**Table 3**), and confirmed that the merged supersets still showed significant
281 association with the corresponding lipid traits in a second round of MSEA ($p < 0.05$ after Bonferroni
282 correction for the number of supersets tested; **Table 3**).

283 **Identification of central regulatory genes in the lipid-associated supersets**

284 Subsequently, we performed a key driver analysis (KDA; **Figure 1**) to identify potential regulatory genes
285 or key drivers (KDs) that may regulate genes associated with each lipid trait using Bayesian networks
286 constructed from genetic and gene expression datasets of multiple tissues (detailed in Methods; full KD
287 list in **Supplemental Table S3**). The top adipose and liver KDs for the shared supersets of all four lipid
288 traits and the representative Bayesian subnetworks are shown in **Figure 2**.

289 In adipose tissue (**Figure 2A**), the top KDs for the 'lipid metabolism' subnetwork include well-
290 known lipoproteins and ATP-binding cassette (ABC) family members that are responsible for lipid
291 transport, such as *APOF*, *APOA5* and *ABCB11*. We also found several KDs that are less known to be
292 associated with lipid metabolism, particularly *F2* (coagulation Factor II or thrombin). For the
293 'autoimmune/immune activation' subnetwork, *CD86*, *HCK*, and *HLA-DMB* were identified as KDs.

294 *PSMB9* was a KD for the 'protein catabolism' subnetwork, whereas *NUP210* is central for the 'interferon
295 signaling' subnetwork. Moreover, the *SYK* gene is a shared KD between 'lipid metabolism' and
296 'autoimmune/immune activation'.

297 In the liver (**Figure 2B**), the top liver KDs for the 'lipid metabolism' subnetwork are enzymes
298 involved in lipid and cholesterol biosynthesis and metabolism, such as *FADS1* (fatty acid desaturase 1),
299 *FDFT1* (farnesyl-diphosphate farnesyltransferase 1), *HMGCS1* (3-hydroxy-3-methylglutaryl-CoA
300 synthase 1), and *DHCR7* (7-dehydrocholesterol reductase). We also identified more KDs for the
301 'interferon signaling' subnetwork in the liver compared to the adipose tissue, with *MX1*, *MX2*, *ISG15*,
302 *IFI44*, and *EPSTI1* being central to the subnetwork. Similar to the adipose network, *PSMB9* and *HLA-*
303 *DMB* were also identified as KDs for 'protein catabolism' and 'autoimmune/immune activation'
304 subnetworks in liver, respectively. We did not detect key driver genes for the 'visual transduction'
305 subnetwork in either tissue, possibly as a result that the networks of liver and adipose tissues did not
306 capture gene-gene interactions important for this subnetwork.

307 In addition to the KDs for the subnetworks shared across lipid traits as discussed above, we
308 identified tissue-specific KDs for individual lipid traits (**Supplemental Table S3**). In adipose, *PANK1*
309 and H2B histone family members were specific for the HDL subnetworks (**Figure 3A**); *HIPK2* and *FAU*
310 were top KDs for the LDL subnetworks (**Figure 3B**); genes associated with blood coagulation such as
311 *KNG1* and *FGL1* were KDs for the TC and TG subnetworks (**Figure 3C-3D**). Interestingly, genes related
312 to insulin resistance, *PPARG* and *FASN*, were KDs for both HDL and TG subnetworks. Similarly, trait-
313 specific KDs and subnetworks were also detected in the liver; 37 KDs were identified for the TG
314 subnetwork including *ALDH3B1* and *ORM2*, whereas *AHSG*, *FETUB*, *ITIH1*, *HP*, and *SERPINC1* were
315 KDs found in the LDL subnetwork. We note that most of the KDs are themselves not necessarily GWAS
316 hits but are surrounded by significant GWAS genes. For example, gene *F2* is centered by many GWAS
317 hits in the adipose subnetwork (*APOA4*, *APOC3*, *APOA5*, *LIPC*, etc.; **Figure 2; Supplemental Figure**
318 **S2**). The observation of GWAS hits being peripheral nodes in the network is consistent with previous

319 findings from our group and others (59-64), and again supports that important regulators may not
320 necessarily harbor common variations due to evolutionary constraints.

321 **Experimental validation of *F2* KD subnetworks using 3T3-L1 adipocytes**

322 Taking into account that the *F2* gene is surrounded by various significant GWAS hits within its
323 subnetwork, we aimed to validate the role of the *F2* gene subnetwork in lipid regulation through a siRNA-
324 mediated knockdown experiment in 3T3-L1 adipocytes. We found that *F2* gene expression was low in
325 preadipocytes, but gradually increased according to adipogenesis. In fully differentiated adipocytes at day
326 10, *F2* gene expression level was higher than preadipocytes by 12-fold (**Supplemental Figure S3)**.
327 Subsequently, we tested the effect of *F2* gene siRNA knockdown on ten neighbors of the *F2* gene in the
328 adipose network (selected from **Figure 2A**). With 60% knockdown efficiency of *F2* siRNA, seven *F2*
329 network neighbors (*Abcb11, Apoa5, Apof, Fabp, Hrg, Gc* and *Proc*) showed significant changes in
330 expression levels (**Figure 4A**), and several of these genes are involved in lipoprotein transport. In contrast,
331 none of the four negative controls (random genes not in F2 network neighborhood) showed significant
332 changes in their expression levels. These results support our computational predictions on the structures
333 of *F2* gene subnetworks. As *F2* expression increases during adipogenesis, we performed Oil red O
334 staining of the *F2* siRNA treated adipocytes. A significant decrease ($p < 0.01$) in lipid accumulation in
335 adipocytes was observed compared with scrambled siRNA control (**Figure 4B**). Next, we measured
336 expression levels of genes related with adipogenesis (*Pparr*, *Cepba*, *Srepb1*, *Fas*), lipolysis (*Lipe*), fatty
337 acid transport (*Cd36*, *Fabp4*), and other adipokines following *F2* siRNA treatment. We found no change
338 in the expression of these tested genes, with the exception of *Cd36*, which encodes fatty acid translocase
339 facilitating fatty acid uptake. *Cd36* expression was decreased by 15 % ($p < 0.05$) compared with control
340 (**Figure 4C**). The decreases in *Cd36* and lipoprotein transport genes after *F2* knockdown suggest that
341 cholesterol and fatty acid transport/uptake by adipocytes is compromised, which could contribute to
342 alterations in circulating lipid levels.

343 **The association between the lipid subnetworks and human diseases**

344 Epidemiological studies consistently show that plasma lipids are closely associated with human
345 complex diseases. For example, high TC and LDL levels are associated with increased risk of
346 cardiovascular disease (CVD). Here, we examined the association between the lipid subnetworks
347 identified in our study and four human complex diseases, namely, Alzheimer's disease, CVD, T2D, and
348 cancer (Materials and Methods). We found that the gene supersets identified for each lipid traits were
349 significantly enriched for GWAS candidate genes reported by GWAS catalog for the four diseases at
350 Bonferroni-corrected $p < 0.05$ (**Figure 5; Supplemental Table S4**). The superset 'lipid metabolism',
351 which was shared across lipid traits, was associated with Alzheimer's disease and CVD. When trait-
352 specific subnetworks were considered, those associated with TC, LDL, and TG had more supersets
353 associated with CVD compared to those associated with HDL, a finding consistent with recent reports (15,
354 65, 66). In addition, supersets of each lipid trait, except HDL, were also found to be significantly
355 associated with cancer, whereas supersets associated with HDL, LDL, and TG but not TC, were linked to
356 T2D.

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365 **Discussion**

366 To gain comprehensive insights into the molecular mechanisms of lipid traits that are important for
367 numerous common complex diseases, we leveraged the large volume of genomic datasets and performed
368 a data-driven multi-omics study combining genetic association signals from large lipid GWAS, tissue-
369 specific eQTLs, ENCODE functional data, known biological pathways, and gene regulatory networks.
370 We identified diverse sets of biological processes, guided by their tissue-specific gene-gene interactions,
371 to be associated with individual lipid traits or shared across lipid traits. Many of the lipid associated gene
372 sets were significantly linked to multiple complex diseases including CVD, T2D, cancer, and Alzheimer's
373 disease. More importantly, we elucidated tissue-specific gene-gene interactions among the gene sets and
374 identified both well characterized and novel KDs for these lipid-associated processes. Our results offer
375 new insight into the molecular regulation of lipid metabolism, which would not have been possible
376 without the systematic integration of diverse genetic and genomic datasets.

377 We identified shared pathways associated with all four lipid traits, including 'lipid metabolism'
378 and 'autoimmune/immune activation', which have been consistently linked to lipid phenotypes, as well as
379 additional pathways such as 'interferon signaling', 'protein catabolism', and 'visual transduction'.
380 Interferon factors have previously been linked to lipid storage attenuation and differentiation in human
381 adipocytes (67). Protein catabolism has only recently been identified to be important in regulating lipid
382 metabolism through the PSMD9 protein, which had no previously known function but was shown to
383 cause significant alterations in lipid abundance in both a gain of function and loss of function study in
384 mice (68). The 'visual transduction' superset contains retinol-binding proteins, which are carrier proteins
385 involved retinol transport, and play key roles in gene expression regulation and developmental processes
386 (69). 'visual transduction' also shares lipoprotein genes with 'lipid metabolism', suggesting that retinol-
387 related signal transduction is intimately linked to lipoprotein transport and hence plasma lipid levels.

388 Furthermore, our results indicate that the trait-specific supersets are tissue-specific. For example,
389 most TG-specific pathways were found to be significant when adipose eSNPs were used, and complement
390 and insulin signaling pathways in the adipose tissue were specific for TG. This is in line with adipose
391 tissue functioning as the major storage site for TG and the regulatory role of immune system and insulin
392 signaling in adipocyte functions and fat storage (70). We also found five HDL-specific pathways, most of
393 which are associated with glucose, lipid, and amino acid metabolism, and were signals derived from liver
394 eSNPs. As HDL acts as the major vehicle for transporting cholesterol to the liver for excretion and
395 catabolism, the critical role of liver as well as the connections between major metabolic pathways in HDL
396 regulation is recapitulated by our analysis. Interestingly, the TC-specific pathways can be only found
397 when brain eSNPs are used. While the brain accounts for 2% of body weight, it contains 23% of TC in the
398 body (71) and deregulated cholesterol trafficking appears to be involved in the pathogenesis of
399 neurodegenerative diseases, such as Parkinson's and Alzheimer's disease (72). These tissue- and trait-
400 specific pathways or processes support the unique features of each lipid species and point to tissue-
401 specific targeting strategies to modulate levels of individual lipid traits and the associated diseases.

402 In addition to detecting trait- and tissue-specific causal pathways for the lipid traits, our study
403 attempted to delineate the interactions between lipid genes and pathways through gene network analysis.
404 Indeed, the tissue-specific gene networks revealed in our study highlight the regulatory connections
405 between lipid genes and pathways, and thus put individual genes in a broader context. The identification
406 of KDs in a network is essential for uncovering key regulatory components and for identifying drug
407 targets and biomarkers for complex diseases (24, 73). Here we adopted data-driven Bayesian gene
408 regulatory networks that combine various genomic data (54) to detect the central genes in plasma lipid
409 regulation. The power of this data-driven objective approach has been demonstrated recently (24, 55, 62,
410 63, 74, 75) and is again supported in this study by the fact that many KDs detected are known regulators
411 for lipids or have served as effective drug targets based on the DrugBank database (76). For instance, for
412 the shared 'lipid metabolism' subnetwork, four top KDs (*ACAT2*, *ACSS2*, *DHCR7*, and *FADS1*), are

413 targeted by at least one FDA approved anti-cholesteremic drugs. Another KD, *HMGCS1*, is a rate-
414 limiting enzyme of cholesterol synthesis, and is considered a promising drug target in lipid-associated
415 metabolic disorders (77). These lines of evidence lead us to speculate the other less-studied KDs are also
416 important for lipid regulation.

417 Among the top network KDs predicted, several including *F2, KLKB1 and ANXA4* are involved in
418 blood coagulation. A previous study revealed polymorphisms in the anticoagulation genes seemed to
419 modify the efficacy of statins in reducing risk of cardiovascular events (78), which in itself is not
420 surprising. However, the intimate relationship between a coagulation gene *F2* and lipid regulation
421 predicted by our analysis is intriguing (**Figure 4)**. We found that the partner genes in the adipose *F2*
422 subnetwork tend to be differentially expressed after *F2* knockdown in adipocytes, with several of the
423 altered genes (*APOA5*, *APOF*, *ABCB11*, *FABP1,* and *CD36*) closely associated with cholesterol and fatty
424 acid transport and uptake, and *F2* knockdown affects lipid storage in adipocytes. These results indicate a
425 largely untapped role of adipose *F2* gene in lipid transport and storage, and provide a novel target in the
426 *F2* gene.

427 In addition to shared KDs such as *F2* for different lipids, it may be also of value to focus on the
428 trait-specific KDs as numerous studies have revealed these lipid phenotypes play different roles in many
429 human diseases. For example, LDL and TC are important risk factors for CVD (79) and TG has been
430 linked to T2D (80), while the role of HDL in CVD has been controversial (81). We detected 37 genes as
431 TG-specific KDs in liver regulatory subnetworks. Among these, *CP (*ceruloplasmin) and *ALDH3B1*
432 (aldehyde dehydrogenase 3 family, member B1) were clinically confirmed to be associated with T2D (82,
433 83) while most of the other genes such as *DHODH* and *ANXA4* were less known to be associated with TG
434 and thus may serve as novel targets. In adipose tissue, genes important for insulin resistance and diabetes
435 such as *PPARG* and *FASN* were found to be KDs for TG, further supporting the connection between TG
436 and diabetes. Additionally, *FASN* has been implicated as a KD in numerous studies for non-alcoholic

437 fatty liver disease (64, 75, 84), again highlighting the importance of this gene in common metabolic
438 disorders.

439 We acknowledge some potential limitations to our study. First, the GWAS datasets utilized are
440 not the most recently conducted and therefore provides the possibility of not capturing the full array of
441 unknown biology. However, despite this our results are consistent with the biology found more recently
442 including overlapping signals in pathways for chylomicron-mediated lipid transport and lipoprotein
443 metabolism (85) as well as more novel findings such as visual transduction pathways. In addition, one of
444 our key drivers *KLKB1*, which was not found to be a GWAS hit in the dataset we utilized, has since been
445 found to pass the genome wide significance threshold in more recent larger GWAS and is a hit on
446 apolipoprotein A-IV concentrations, which is a major component of HDL and chylomicron particles
447 important in reverse cholesterol transport (86). This further exemplifies the robustness of our integrative
448 network approach to find key genes important to disease pathogenesis even when smaller GWAS were
449 utilized.

450 In summary, we used an integrative genomics framework to leverage a multitude of genetic and
451 genomic datasets from human studies to unravel the underlying regulatory processes involved in lipid
452 phenotypes. We not only detected shared processes and gene regulatory networks among different lipid
453 traits, but also provide comprehensive insight into trait-specific pathways and networks. The results
454 suggest there are both shared and distinct mechanisms underlying very closely related lipid phenotypes.
455 The tissue-specific networks and KDs identified in our study shed light on molecular mechanisms
456 involved in lipid homeostasis. If validated in additional population genetic and mechanistic studies, these
457 molecular processes and genes can be used as novel targets for the treatment of lipid-associated disorders
458 such as CVD, T2D, Alzheimer's disease and cancers.

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461 **Data Availability**

462 All genomic data utilized in the analysis were previously published and were downloaded from public
463 data repositories. All experimental data were presented in the current manuscript. Mergeomics code is
464 available at R Bioconductor DOI: 10.18129/B9.bioc.Mergeomics.

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Author contributions

XY designed and directed the study. YZ, MB, ZS, ISA and HL conducted the analyses. VPM contributed analytical methods and tools. YZ, MB, ZS, ISA and XY wrote the manuscript. ISA conducted the validation experiments. All authors edited and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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708 **Tables**

709 **Table 1. Common pathways shared by the four lipid traits in SNP set enrichment analysis.**

Categories	Descriptions	Traits*				MetaboChip	iGSEA
		HDL	LDL	TC	TG		
Positive Controls	Positive control gene set for TG	1,2,3,5,6,7,8,9	2,3,5,6,7,8,9	2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
	Positive control gene set for LDL	5,6,7,8,9	1,2,3,4,5,6,7,8,9	1,2,3,4,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
	Positive control gene set for TC	3,5,6,7,8,9	1,2,3,4,5,6,7,8,9	1,2,3,4,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
	Positive control gene set for HDL	1,2,3,4,5,6,7,8,9	2,6,7,8,9	2,5,6,7,8,9	1,2,5,6,7,8,9	YES	YES
Lipid metabolism	Lipoprotein metabolism	1,2,5,6,7,8,9	5,6,7,8,9	5,6,7,8,9	5,6,7,8,9	YES	YES
	Chylomicron-mediated lipid transport	5,6,7,8,9	7,8,9	5,6,7,8,9	5,6,7,8,9	YES	YES
	LDL-mediated lipid transport	6,7,9	6,7,9	6,7,9	6,7,9	NO	YES
	HDL-mediated lipid transport	1,2,5,6,7,8,9	5,7,8,9	5,7,8,9	5,7,8,9	YES	YES
Protein catabolism	ER-Phagosome pathway	1,5,8,9	1,3,5,6,8,9	1,2,3,5,6,8,9	1,3,5,6,8,9	YES	YES
	Antigen processing and presentation	5,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
Interferon Signaling	Interferon Signaling	7,9	1,3,5,6,8,9	1,2,3,5,6,8,9	1,3,5,8	YES	YES
Autoimmune /Immune activation	Type I diabetes mellitus	1,5	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
	Scavenging by Class B Receptors	6,7,8,9	7,9	7,9	7,9	NO	YES
	Asthma	6	1,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
	IL 5 Signaling Pathway	5	1,5,6,8,9	1,5,6,8,9	5,6,8	NO	NO
	Th1/Th2 Differentiation	3	1,3,5,6,8	1,3,5,6,8,9	1,3,5,6,8	NO	YES
	Natural killer cell mediated cytotoxicity	5	1,3,5	1,3,5,6,9	1,3,5	YES	YES
	HLA genes	1,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
	Cell adhesion molecules (CAMs)	5	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,3,5,6,8,9	YES	NO
	Autoimmune thyroid disease	1,3,5,6,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	YES	YES
Visual transduction	Diseases associated with visual transduction	7	7,8,9	7,8,9	7,9	YES	YES
	Visual phototransduction	7	7,8,9	7,8,9	7,9	YES	YES

710 Note: *: The trait columns represent in which methods the MSEA of the pathways is significant with FDR

711 < 10%. Number 1 to 9 represent: adipose eSNP (1), blood eSNP (2), brain eSNP (3), human aortic

712 endothelial cells (HAEC) eSNP (4), liver eSNP (5), all eSNP (6), Distance (7), Regulome (8), and

713 Combined (9), respectively. The Metabochip and iGSEA columns tell whether the gene set can also be

714 detected as statistically significant in the analysis.

715 **Table 2. Trait-specific pathways identified in the SNP set enrichment analysis for four lipid traits.**

Traits	Modules	Descriptions	Methods*
HDL	rctm0846	Packaging of telomere ends	1
	Haec:M1+	(Cholesterol biosynthesis)	9
	M12882	Taurine and hypotaurine metabolism	1,5
	rctm0060	Activation of Genes by ATF4	9
	rctm0216	Cation-coupled Chloride cotransporters	7,8,9
	rctm0697	Metabolism of water-soluble vitamins and cofactors	5
	Cerebellum:M1+	(Alcohol metabolism)	3
	Cerebellum:M2+		3
	rctm0507	Glutathione synthesis and recycling	5
	Liver:M1+	(Transition metal ion homeostasis)	2,9
	rctm0937	RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways	7,8,9
	rctm0772	Negative regulators of RIG-I/MDA5 signaling	7,8,9
	rctm0255	Cobalamin (Cbl, vitamin B12) transport and metabolism	1,5
	M15902	Glycerolipid metabolism	6,7,9
	rctm1178	Striated muscle contraction	9
	rctm0696	Metabolism of vitamins and cofactors	5
LDL	Haec:M2+	(Positive regulation of cellular metabolism)	3
	Liver:M2+	(Cadherin)	6
	Cerebellum:M3+	(Immunity and defense)	8
	M6831	The citric acid cycle	6
	rctm0876	Platelet sensitization by LDL	7,9
TC	M17946	Valine, leucine and isoleucine biosynthesis	1,6,9
	PC:M1+	(Chaperone)	3
	Cerebellum:M4+	(Response to wounding)	9
	Adipose:M1+		8
	Omental:M1+		3
	rctm1111	Signal transduction by L1	3
TG	rctm1276	Tight junction interactions	1,6,8,9
	rctm0589	Initial triggering of complement	1
	rctm0235	Cholesterol biosynthesis	2
	M18155	Insulin signaling pathway	1
	Blood:M1+	(Carbohydrate metabolism)	1,6
	rctm0225	Cell-cell junction organization	1,6,8
	Blood:M3+	(Transferase activity, transferring glycosyl groups)	1
	M7146	Classical complement pathway	1
	rctm0059	Activation of Gene Expression by SREBP (SREBF)	2
	M917	Complement pathway	1
	M5872	Steroid biosynthesis	2
	Omental:M2+	(hemopoietic or lymphoid organ development)	8
	M2164	Leukocyte transendothelial migration	1

716 Note: *: The method column represents in which methods the MSEA of the pathways is significant with

717 FDR < 10%. Number 1 to 9 represent: adipose eSNP (1), blood eSNP (2), brain eSNP (3), human aortic

718 endothelial cells (HAEC) eSNP (4), liver eSNP (5), all eSNP (6), Distance (7), Regulome (8), and
719 Combined (9), respectively. [+]: Co-expression modules. The statistically overrepresented Gene Ontologies
720 satisfying $p < 0.01$ in Fisher's exact test after Benjamini-Hochberg correction within the modules are
721 listed in the parentheses. PC: prefrontal cortex. #: The column tells whether the trait-specific pathways
722 can also be detected as trait-specific ones in either Metabochip and/or iGSEA.

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738 **Table 3. Supersets shared by four lipid traits and key driver genes.**

Supersets	No. Genes	Methods[a]				Top Adipose KDs	Top Liver KDs
		HDL	LDL	TC	TG		
Lipid metabolism	793	1,2,3,5	1,2,3,5	1,2,3,5	1,2,3,5	APOH, ABCB11, F2, ALB, APOA5, APOC4, DMGDH, SERPINC1, APOF, HADHB, ETFDH, KLKB1	HMGS1, FDFT1, FADS1, DHCR7, ACAT2, ACSS2
Protein catabolism	253	1,3,4,5,6,7,8,9	1,3,5,6	1,3,5,6,9	1,3,5,6,8	PSMB9	PSMB9
Interferon Signaling	171	1,3,5,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,7,8,9	1,2,3,5,6,8,9	NUP210	MX1, ISG15, MX2, IFI44, EPSTI1
Autoimmune/ Immune activation	152	1,3,4,5,6,7,8,9	1,2,3,4,5,6,7,8,9	1,2,3,4,5,6,7,8,9	1,2,3,4,5,6,7,8,9	HLA-DMB, HCK, SYK, CD86	HLA-DMB, CCL5, HLA-DQA1
Visual transduction	86	7,9	7,8,9	7,8,9	7,8,9	-	-

739 **Note:** [a] The method column represents in which methods the MSEA of the pathways is significant with

740 Bonferroni-adjusted P<0.05. Number 1 to 9 represent: adipose eSNP, blood eSNP, brain eSNP, haec

741 eSNP, liver eSNP, all eSNP, Distance, Regulome, and Combined, respectively.

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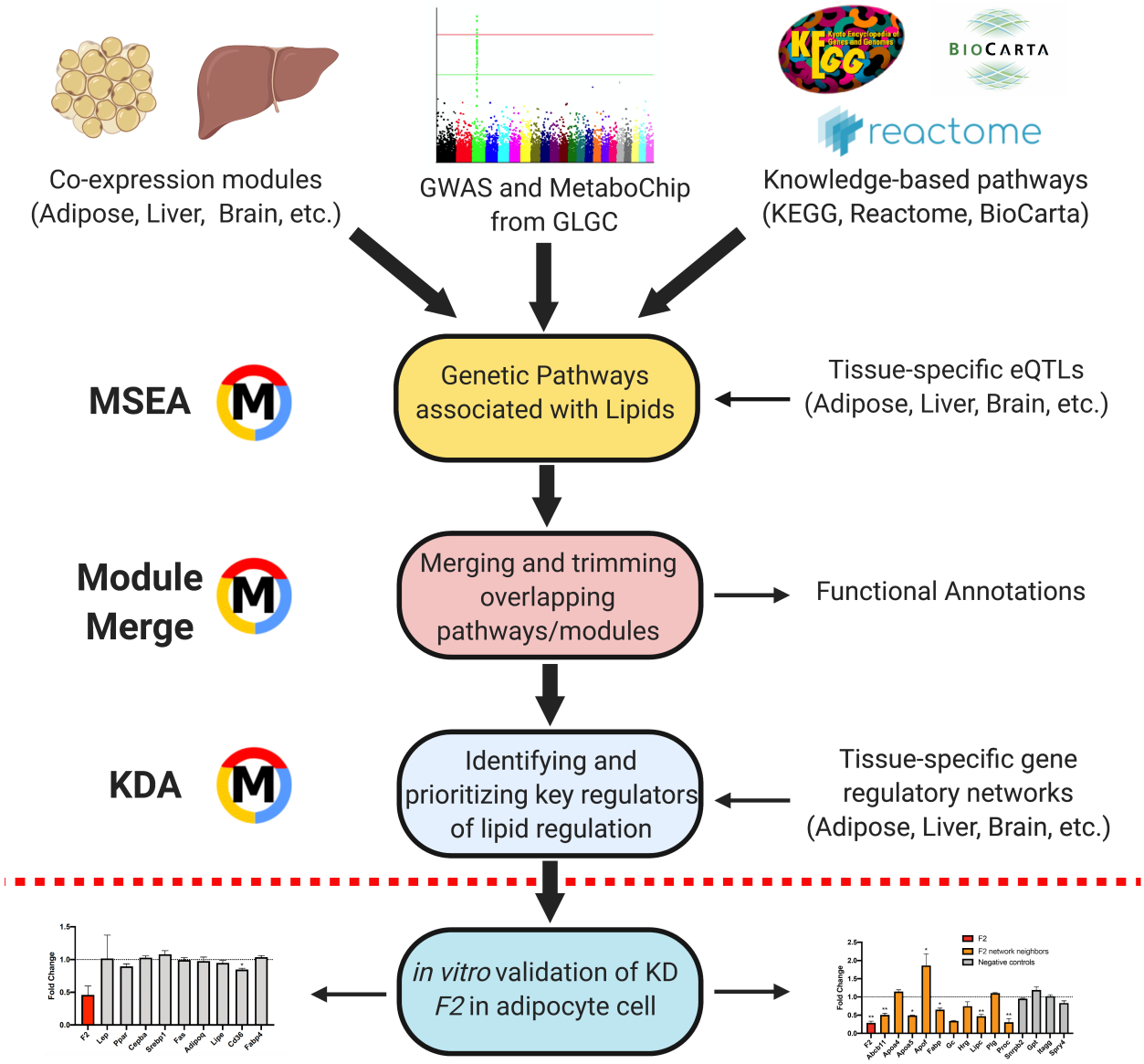
746 **Figures**



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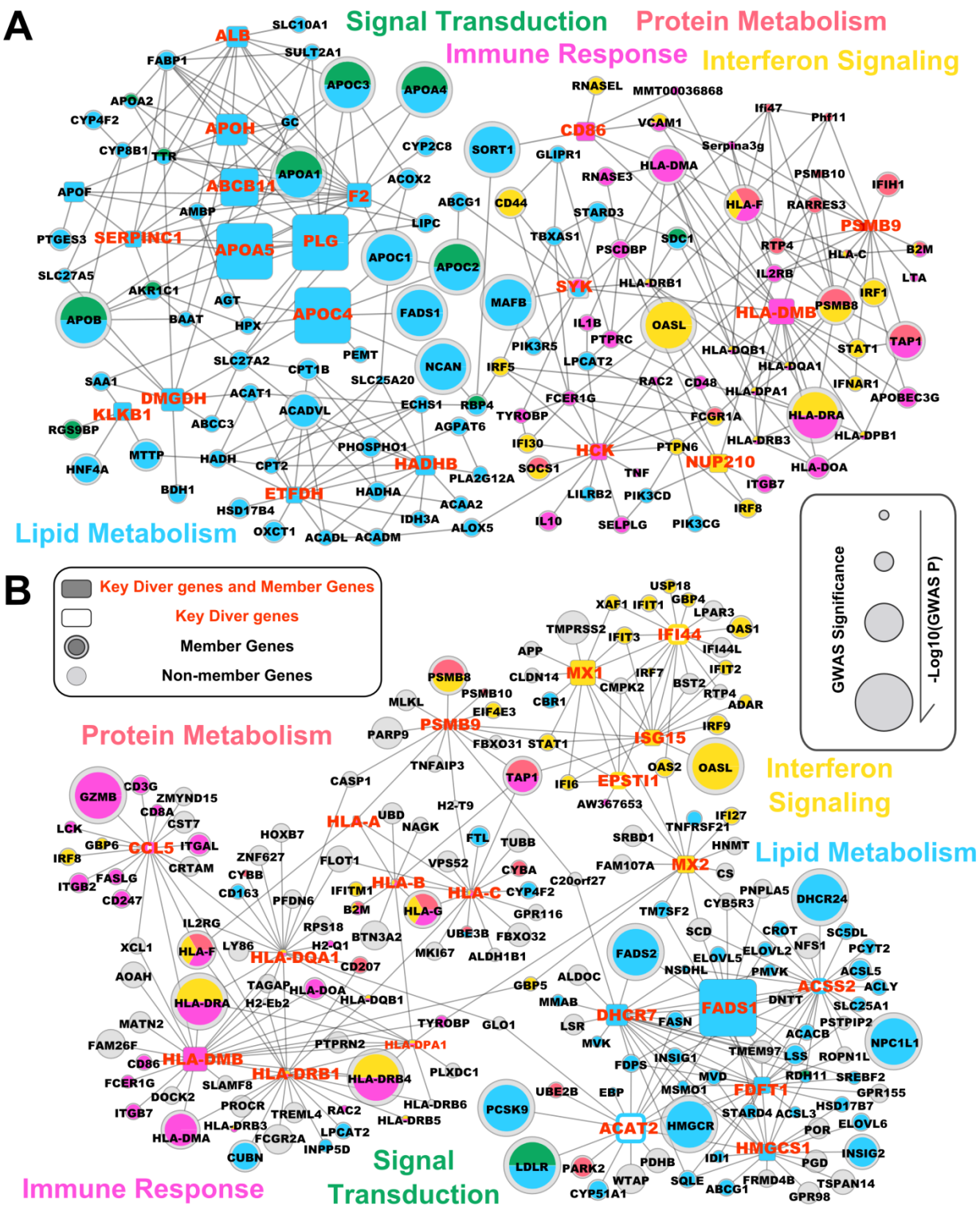
748 **Figure 1. Overall design of the study.** The statistical framework can be divided into four main parts,
749 including Marker Set Enrichment Analysis (MSEA), merging and trimming of gene sets, Key Driver
750 Analysis (KDA), and validation of the key regulators using *in vitro* testing.



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752 **Figure 2. Common KDs and their neighboring genes in the shared lipid- associated subnetworks.** A)

753 Adipose KDs and subnetworks. B) Liver KDs and subnetworks. The subnetworks shared by HDL, LDL,

754 TC, and TG are depicted by different colors according to the difference in their functional categories.
755 Nodes are the KDs and their adjacent regulatory partner genes, with KDs depicted as larger nodes. Only
756 network edges that were present in at least two independent network studies were included. The node size
757 corresponds to the GWAS significance.

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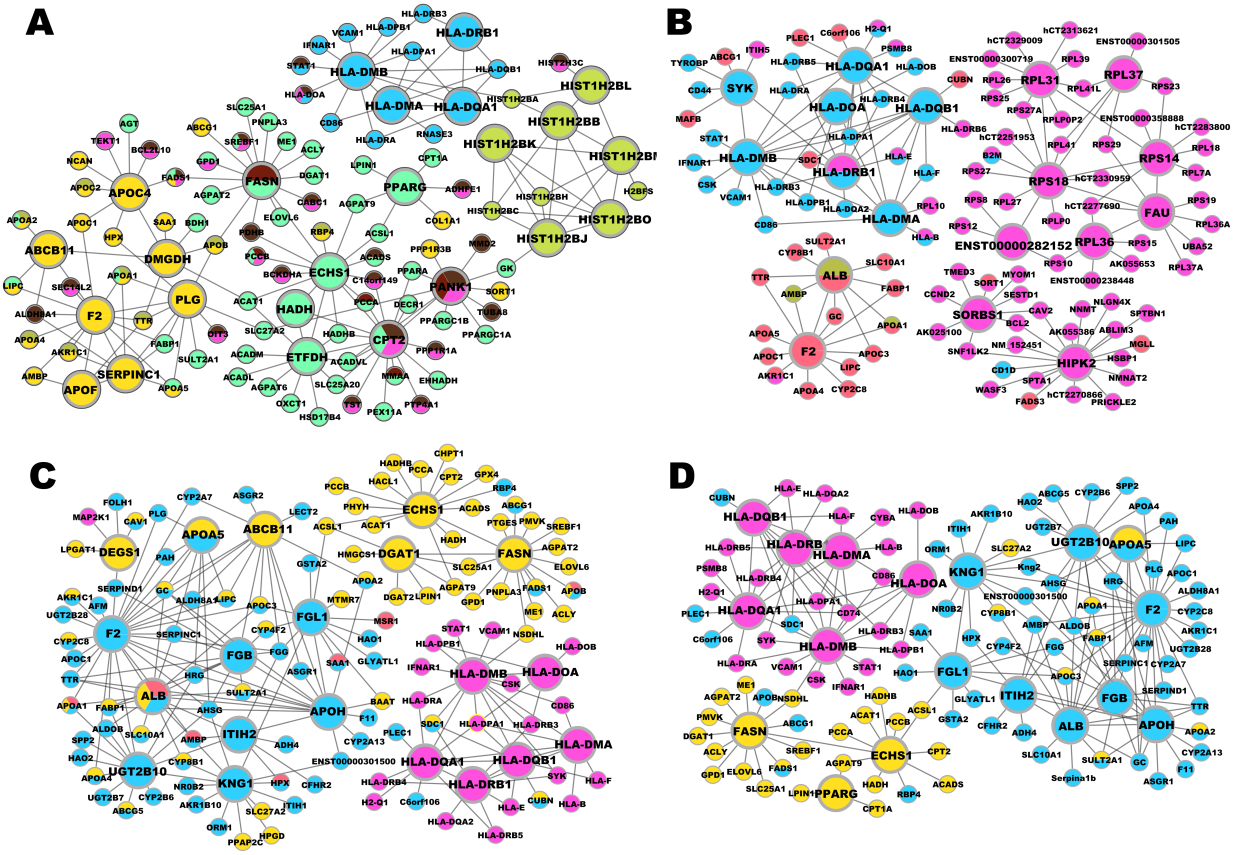
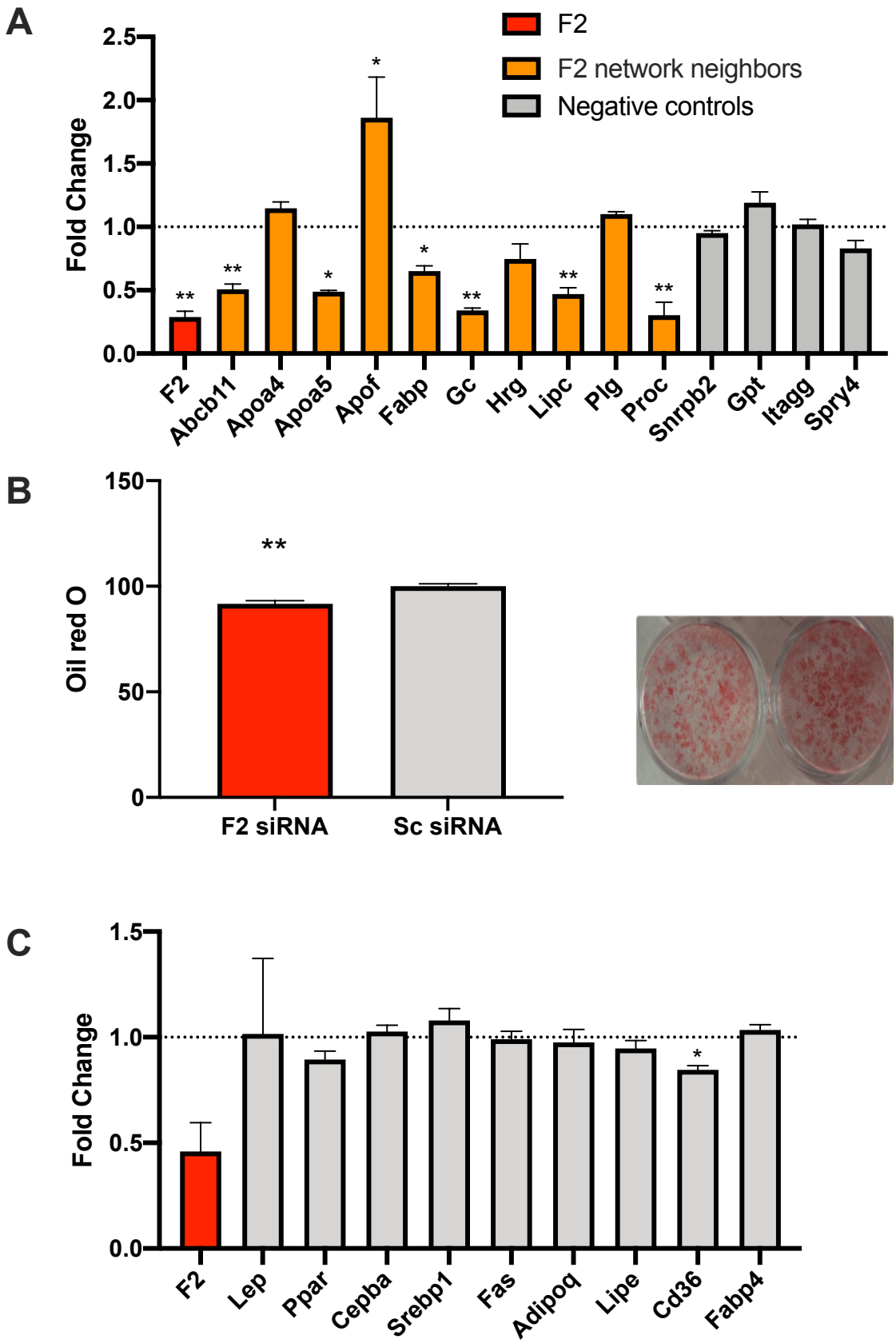


Figure 3. Adipose KDs and subnetworks for each lipid trait. Panel (A)-(D) represent HDL, LDL, TC, and TG subnetworks. Nodes are the KDs and their adjacent regulatory partner genes, with KDs depicted as larger nodes. The yellow color signifies networks associated with interferon signaling, blue with lipid metabolism, pink with immune response, green with protein metabolism, red with lipoprotein metabolism and brown with fatty acid oxidation.


A
F2
F2 network neighbors
Negative controls
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
F2
Abcb11
Apoa4
Apoa5
Apof
Fabp
Gc
Hrg
Lipc
Plg
Proc
Snrpb2
Gpt
Itagg
Spry4
B
Oil red O
150
100
50
0
F2 siRNA
Sc siRNA
C
Fold Change
1.5
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0.5
0.0
F2
Lep
Ppar
Cepba
Srebp1
Fas
Adipoq
Lipe
Cd36
Fabp4

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772 **Figure 4. Validation of *F2* subnetwork using in vitro siRNA knockdown (A) and possible role of *F2***
773 **in adipocytes (B, C).** At day 7 of differentiation, 3T3-L1 adipocytes were transfected with F2 siRNA for
774 the knockdown experiments. A) Fold change of expression level for F2 adipose subnetwork genes and
775 negative control genes. Ten F2 neighbors were randomly selected from the first and second level
776 neighboring genes of F2 in adipose network. Four negative controls were randomly selected from the
777 genes not directly connected to F2 in adipose network. B) Visualization and quantification (calculated by
778 OD value/total RNA and converted to percentage) of lipid accumulation by Oil Red O staining. C) The
779 fold changes of adipogenesis-related genes were determined by RT-qPCR, normalized to beta-actin. The
780 fold changes were expressed relative to scrambled siRNA control. Results represent mean ± s.e.m.
781 Statistical significance was determined by Student's t-test (*$p < 0.05$ and **$p < 0.01$).

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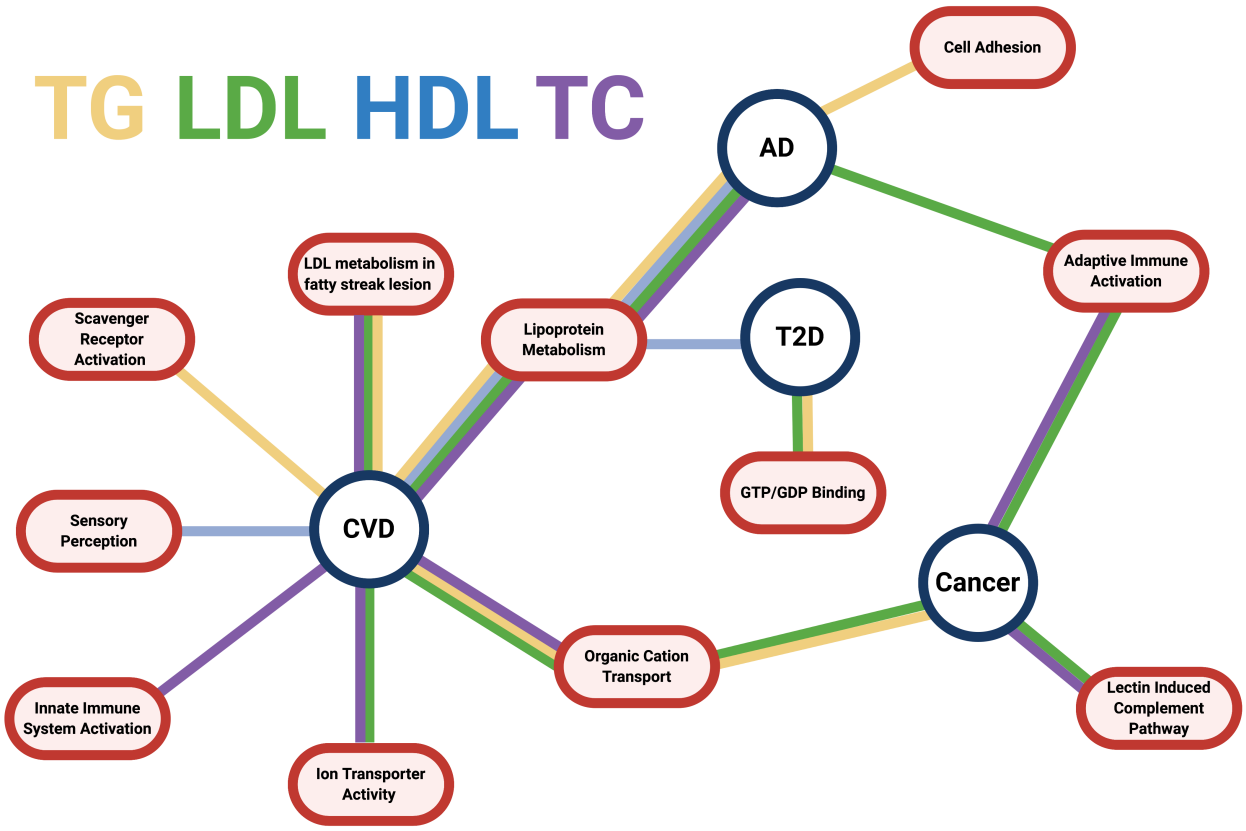

Figure 5. The associations between lipid-associated supersets and human complex diseases. The edges represent the associations between supersets for the specific lipid classes matched by color and diseases (p value < 0.05; Fisher exact test with Bonferroni correction). AD: Alzheimer's disease; CVD: cardiovascular diseases; T2D: type 2 diabetes.