MiR-20b, miR-296 and Let-7f expression in human adipose tissue is related to obesity and type 2 diabetes

Adriana-Mariel Gentile*[1], Said Lhamyani*[1], Leticia Coín[2], Mercedes Clemente-Postigo[2], Wilfredo Oliva Olivera[2], Silvana-Yanina Romero-Zerbo[3], Sara García-Serrano[3], Eva García-Escobar[3], Francisco-Javier Bermúdez-Silva[3], Mora Murri[2], Francisco Tinahones[2] and Rajaa El Bekay[4].

[1]Unidad de Gestión Clínica de Endocrinología y Nutrición, Instituto de Investigación Biomédica de Málaga (IBIMA), Hospital Regional Universitario. Universidad de Málaga. Campus Teatinos s/n - 29010 - Málaga, España

[2]Departament of Clinical Endocrinology and Nutrition, Institute of Biomedical Research of Málaga (IBIMA), Clinical Hospital of Málaga (Virgen de la Victoria). University of Málaga (UMA).CIBERThe Spanish Biomedical Research Centre in Physiopathology of Obesity and Nutrition. Institute of Health Carlos III, Spain.

[3]Unidad de Gestión Clínica de Endocrinología y Nutrición, Instituto de Investigación Biomédica de Málaga (IBIMA), Hospital Regional Universitario, Málaga, Spain; CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Malaga, Spain.

[4]Unidad de Gestión Clínica de Endocrinología y Nutrición, Instituto de Investigación Biomédica de Málaga (IBIMA), Hospital Regional Universitario, Málaga, Spain. CIBERThe Spanish Biomedical Research Centre in Physiopathology of Obesity and Nutrition. Institute of Health Carlos III, Spain

Abbreviated title: M*icroRNAs obesity and type 2 diabetes*

Key terms: MicroRNAs, human adipose tissue, obesity, type 2 diabetes,

Word count: 3779

Numbers of figures and tables: 6

***Authors collaborated equally**

Corresponding Author*:*

Rajaa El Bekay elbekay@gmail.com

Francisco J Tinahones fjtinahones@hotmail.com

Abstract

BACKGROUND: Obesity is very often accompanied by other diseases such as type 2 diabetes (T2D) or insulin resistance (IR). Appropriate adipose tissue (AT) functioning is essential to avoid these metabolic disorders. MiRNAs have emerged as key regulators for multiple signaling pathways in AT and others tissues and could be involved in AT expandability regulation.

The aim of the study was to analyze the relationship between obesity, T2D, miR-20b, miR-296, Let-7f and their corresponding target genes.

METHODS: The expression levels of miR-20b, miR-296, Let-7f and their corresponding target genes were assessed by real-time quantitative PCR in visceral (VAT) and subcutaneous (SAT) AT from obese and lean subjects classified according to their glycemic status, normoglycemic (NG) or T2-D.

RESULT: MiR-20b, miR-296 and Let-7f expression levels in AT were statistically different between NG and type 2 diabetic (T2-D) subjects. An interaction between obesity and AT depot was observed, finding that the expression levels of miR-20b, miR-296 and Let-7f in VAT from T2-D and NG obese subjects were higher than in SAT. On the other hand, the expression levels of miR-20b, miR-296 and Let-7f in VAT were higher in NG obese subjects (NG-OB) than in T2-D obese subjects.

CONCLUSION: Taken together our results suggest the potential relationship between miR-20b, miR-296, Let-7f, obesity and T2D, and highlight that these microRNAs could be acting on VAT and SAT functionality through the regulation of their target genes *CDKN1A, CX3CL1, HIF1A, PPP2R1B, STAT3* and *VEGFA*, which are key regulators of angiogenesis and Wnt signaling pathways.

INTRODUCTION

The prevalence of obesity has increased dramatically in the last decades and is now considered as a major health problem. In fact, the current epidemic of obesity has been suggested as the main cause of decreased life expectancy by next ages[1]. Obesity is very often accompanied by other diseases such as type 2 diabetes (T2D), insulin resistance (IR) and cardiovascular complications[2].

The mechanisms underlying IR and T2D development are not fully understood. Thus, there is currently an intense research aiming to understand these mechanisms and several hypothesis propose AT as a key determinant for metabolic dysregulation[3,4]. Several lines of evidence indicate that the physiopathology of these diseases involves mechanisms other than fat accumulation. AT is recognized to be highly active metabolic and endocrine organ, and its normal function is altered in obesity. Thus, accumulating evidence suggests that AT dysfunction represent primary defects in obesity and may link obesity to IR and T2D[5].

Inappropriate angiogenesis may underlie AT dysfunction, which in turn increases T2D risk[6]. Vascular endothelial growth factor (VEGF) is one of the most relevant factors known to play a pivotal role in angiogenesis regulation. Also, Wnt signaling pathway has been described to contribute to angiogenic process regulation in AT[7,8], and to be involved in the regulation of gluconeogenesis and glycolysis[9].

MicroRNAs (miRNAs) are small non-coding RNAs involved in RNA silencing[10]. Moreover, miRNAs are described to be involved in the development and function of AT and thus to be potentials therapeutic molecules in mitigating obesity and its associated[11]. The major function of miRNAs in AT is to stimulate or inhibit the differentiation of adipocytes, and to regulate specific metabolic and endocrine functions[12]. Various

miRNAs have been associated with physiopathological disorders related to obesity such as oxidative stress, impaired adipogenesis, insulin signaling, apoptosis, angiogenesis, and inflammation[12,13]. Specifically, miR- 20b, miR-296 and Let-7f have been described to be involved in the regulation of WNT signaling pathway and angiogenesis through the regulation of multiple target genes belonging to these pathways[14].

In the present study we set out to analyze the expression levels of miR-20b, miR-296 and Let-7f and of their corresponding target genes in VAT and SAT, and to study the interaction of these microRNAs, their corresponding target genes with obesity and T2D

MATERIAL AND METHODS

Patients and adipose tissue collection

The study included obese patients (BMI>30kg/m^2) undergoing bariatric surgery at the Virgen de la Victoria University Hospital (Malaga, Spain) and normo weight subjects (BMI= <30kg/m^2) who underwent laparoscopic surgery for hiatus hernia or cholelithiasis. Participants were classified into four groups according to their BMI and glycemic status in normoglycemic and normoweight subjects (NG-NW), type 2 diabetic normoweight subjects (T2-D-NW), normoglycemic obese subjects (NG-OB) and type 2 diabetic obese subjects (T2-D-OB).

World Health Organization (WHO) criteria for T2D diagnosis ((a) random blood glucose ≥ 200 mg/dl; (b) glycosylated hemoglobin (HbA1c) ≥ 6.5%; (c) blood glucose after oral glucose overload 75g ≥ 200 mg/dl; (d) fasting glucose after at least 8 hours fasting ≥ 110 mg/dl) were used to assign participants to the NG or the T2-D groups.

Patients were excluded if they had insulin treatment for T2D, cardiovascular disease in the 6 months prior to the inclusion in the study, evidence of acute or chronic inflammatory disease, infectious disease or if patient refused to participate in the study.

This study was approved by the ethic and research committee of Virgen de la Victoria University Hospital (Malaga, Spain), and all participants gave their written informed consent.

Anthropometric and biochemical characteristics of the study groups are shown in Table1. Both SAT and VAT were obtained at the beginning of the surgical procedure, washed in physiological saline, immediately frozen in liquid nitrogen, and stored at -80ºC until analysis.

Laboratory measurements

After an overnight fast and before surgery, blood samples were obtained from the antecubital vein and placed in vacutainer tubes (BD vacutainer™). The serum was separated by centrifugation for 10 min at 4000 rpm and immediately frozen at -80ºC until analysis. Serum glucose, cholesterol, triglycerides and HDL-cholesterol were measured in a Dimension autoanalyzer (Dade Behring Inc., Illinois, USA) by enzymatic methods (Randox Laboratories Ltd., County Antrim, UK and Wako Bioproducts). LDL-cholesterol was calculated with the Friedewald equation. Insulin was quantified by radioimmunoassay (BioSource International, California, USA) and insulin resistance calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) [15]. Leptin and adiponectin were analyzed by ELISA kits (Mediagnost, Reutlingen, Germany).

miRNA isolation and real-time quantitative PCR (qPCR). The miRNA were extracted from AT fragments using *mir*Vana™ miRNA Isolation Kit (Ambion), according to the manufacturer's guidelines. miRNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer V3.7.1 cDNA was obtained using TaqMan®

MicroRNA Reverse Transcription Kit (Applied Biosystems, California, USA) and specific primers and probes for each miRNA were used (TaqMan® MicroRNA Assay, Applied Biosystems). A constant amount of 5ng of miRNA was used to perform reverse transcription carried out in compliance the proportion as follow: 5 µl RNA: 7 µl RT-MasterMix: 3 µl RT-primers. End volume 15µl. 2720 Thermal Cycler (Applied Biosystems). The cycle program consisted of 30 minutes at 16ºC, 30 minutes at 42ºC and 5 minutes at 85ºC. miRNAs expression levels was assessed by real-time qPCR using Applied Biosystems 7500 Fast Real-Time PCR System. Relative quantification of the expression levels was performed by the comparative threshold cycle (Ct) method according to the manufacturer's guidelines, and each sample were quantified by duplicate. Two small noncoding RNA genes (miR-16 and RNU6B) were assessed using Bestkeeper software to determine their usability as reference genes (http://www.gene-quantification.de/bestkeeper.html). The BK value found for miR-16 was the best and was used as the endogenous reference control for all miRNAs assessed in this study. Commercially available and prevalidated TaqMan primer/probe sets used were as follows: hsa-miR-16 (000391), RNU6B (4373381), hsa-miR-20b (001014), hsa-miR-296 (000527), hsa-let-7f (000382).

Prediction of putative miRNA target genes

The miRTarBase 4.0 web site (http://mirtarbase.mbc.nctu.edu.tw/) and the miRWalk 2.0 (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html) databases were used for prediction of validated and not validated target genes of the three miRNAs analyzed. The PANTHER Database (Protein Analysis through Evolutionary Relationship) Classification System (http://www.pantherdb.org/) and DAVID

(https://david.ncifcrf.gov/) were applied to annotate the biological process of the predicted targets.

In order to reduce the false positive target prediction as a high degree of confidence of miRNA target genes for subsequent analysis, we choose miRNA target genes as follows:

a) For each miRNA the target genes that were validated in two prediction algorithms.

b) The target genes which were validated in one database and that are common to at least two studied miRNA.

c) The not validated target genes which are common to three miRNA.

mRNA isolation and real-time qPCR

Total mRNA was isolated using the RNA Stat 60 Reagent (Ams Biotechnology, Abingdom, UK). RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer V3.7.1 (Thermo Scientific, MA, USA).

A constant amount of 1µg of total RNA was reverse transcribed using Transcriptor reverse Transcriptase kit (Roche Diagnostic, Barcelona, Spain) in a 2720 Thermal Cycler (Applied Biosystems). Primers used for qPCR reactions are detailed in Table S1. Primers for qPCR were designed based on NCBI database sequences and examined for specificity with BLAST software from NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers were pre-mixed with Brillant III Ultra-Fast SYBRGreen QPCR MasterMix (Agilent Technologies, INC. Santa Clara, CA 95051, USA. Ref. 600882) and aliquots of 18 µl were applied to Mx3000P®/Mx3005PTM 96-Well Tube Plates (Agilent Technologies,INC., Santa Clara, CA 95051, USA. Cat. 410088). Aliquots of 2 µl cDNA (5ng cDNA/well) was added in duplicate. Thermal cycling conditions were as follows: 3 min at 95ºC and 40

cycles of 15 s at 95ºC and 20s at 60ºC on a Mx3005P QPCR Systems (Agilent Technologies, INC.). Negative controls (without template) were included in each assay. ΔCt values for each amplified product were determined using a threshold value of 5000. The threshold cycle (Ct) for Elongation factor 1 alpha (EF1α) was subtracted from the Ct of the target gene to adjust variations in the cDNA synthesis. Bestkeeper software was used to determine EF1α usability as reference gene (http://www.gene-quantification.de/bestkeeper.html).

Interaction between microRNAs and differentially expressed target genes in human AT

The interaction among miR-20b, miR-296, Let-7f, biologic processes and the differentially expressed target genes were visualized with Cytoscape v.3.2.1 software (http://www.cytoscape.org/).

Statistical analysis

The results are expressed as means ± SEM. Data were analyzed by one way analysis of variance (ANOVA). We tested normality of the distribution of continuous variables using the Shapiro-Wilk statistics. Pos hoc statistical analysis was performed by using Bonferroni test. Moreover, data were analyzed by multivariate and univariate general linear models. The group of diabetes (NG and T2-D), obesity (NW and OB), subject type (NG-NW, NG-OB, T2-D-OB) and the group of tissue (VAT and SAT) were introduced as independent variables. The expressions of the studied microRNAs and target genes were introduced as dependent variables. Differences were considered statistically significant at $p<0.05$. Statistical analyses were carried out with the statistical software package SPSS (version 22.0; SPSS Inc, Chicago, IL).

RESULTS

MiRNA expression levels in AT.

A general linear multivariate model, including simultaneously the expression of all the miRNAs studied, showed the existence of statistically significant differences when all variables are considered as a whole, and are expressed by the intercept of the model (Wilks' λ=0.330, F=9.486, p=0.001). In addition, an interaction between obesity and tissue was observed (Wilks' λ=0.531, F=4.120, p=0.027), which indicates that the differences between NW and OB were not the same in VAT and SAT (miR-20b, p=0.020; miR-296, p=0.022; Let-7f, p=0.009) (Fig. 1A). In addition, differences in the expression levels of each miRNA separately were analyzed through the univariate general linear model. Fig. 1A shows that, Let-7f expression levels were significantly higher in OB compared to NW subjects in VAT (p=0.011). However, no significant differences in Let-7f expression levels were observed between these two groups in SAT. Thus, the kind of tissue significantly affected the expression of Let-7f consisting of a completely different behavior among NW and OB subjects in VAT and SAT (p=0.012).

Prediction of putative target genes of miRNA-20b, miRNA-296, Let-7f and their Biological functions

Bioinformatic analysis highlighted 7038 putative target genes for miR-20b, miR-296 and Let-7f (Table 2). The criteria followed for target genes election are described in materials and methods and the new list of predicted target genes included 98 genes (Table S2). These target genes were introduced in the PANTHER and DAVID Databases to highlight the biological process in which they are involved (Fig. 1B).

Analysis of target gene expression levels in human SAT

The expression levels in SAT of 15 target genes (*ECE1, ERBB2, KDR, NAT10, NOS3, RET, SSSCA1, TNFRSF1B, ZNF512, CDKN1A, CX3CL1, HIF1A, PPP2R1B, STAT3, VEGFA*) were analyzed. An analysis with ANOVA showed statistically significant differences in mRNA expression levels of *CDKN1A* (p=0.007), *NAT10* (p=0.036), and SSSCA1 (p=0.039) between NW and OB subjects. On the other hand, *CDKN1A* (p=0.009), *ECE1* (p=0.028), NOS3 (p=0.041), *PPP2R1B* (p=0.040) and *KDR* (p=0.038) mRNA levels were significantly different between NG and T2-D-OB. Specifically, *CDKN1A* gene expression showed statistically significant differences between NW vs. T2-D-OB and *ECE1* gene expression showed statistically significant differences between NW vs NG-OB and between NG-OB and T2-D-OB (Fig. 2A). The relationship between the six target genes and the three miRNAs are shown in Fig.2B. A multivariate general linear model was used to analyze the expression of all target genes as a whole, considering at the same time the effect of obesity and diabetes. Thus, an interception among type of subjects, obesity and diabetes, was observed when six genes are expressed as a whole (PP*2R1B, NOS3, CX3CL1, NAT10, RET, VEGFA*) (Wilks' λ=0.025, F=26.37, p=0.004) (Fig. 2C), indicating that differences in gene expression between OB and NW subjects are not the same when the glycemic status is taken into consideration. On the other hand, univariate general linear models served to analyze the differences in the expression of each of the target gene separately. The analysis between couples (NW vs. OB subjects) showed a statistically significant difference in obesity; specifically, obesity significantly decreased the expression of NAT10 (p=0.037, F=5.955). The analysis between couples (NW vs. T2-D-OB) showed a statistically significant difference in diabetes; specifically, diabetes significantly increased the

252 expression of PPP2R1B (p=0.045, F=5.393) and decreased NOS3 expression (p=0.046,
253 F=5.341).
254 Fig. 2D summarizes the relationship between the six target genes, the three miRNAs
255 and their corresponding biological processes. The scheme shows that the relationship
256 between 296 and the NOS3 and PPP2R1B genes could be involved in the signaling
257 pathways of the FGF, Wnt and interleukins.
258
259 ***Analysis of target gene expression levels in human VAT***
260 The expression levels in VAT of 17 target genes (*CDKN1A, CX3CL1, HIF1A,*
261 *PPP2R1B, STAT3, VEGFA, TNF, MYC, MPP3, DENNDC1, GLUL, GPI, IL1B,*
262 *LRRC33, RNASEN, WDR20, ZNF274*) were analyzed. An analysis with ANOVA
263 showed statistically significant differences in mRNA expression levels of *GLUL*
264 (p=0.026), GPI (p=0.033) and *VEGFA* (p=0.037) between NW and OB subjects. On the
265 other hand, *GPI* (p=0.028), *ZNF274* (p=0.019) and *VEGFA* (p=0.002) mRNA levels
266 were significantly different between NG and T2-D-OB. Specifically, *CDKN1A, MPP3,*
267 *GLUL, IL1B, STAT3, WDR20* and *ZNF274* showed statistically differences between
268 NW and NG-OB; *GLUL, GPI* and *VEGFA* between NW and T2-D-OB and *CDKN1A,*
269 *PPP2R1B, CX3CL1, VEGFA, WDR20* and *ZNF274* showed statistically differences
270 between NG-OB and T2-D-OB (Fig. 3A). The relationship between these eleven target
271 genes and the three microRNAs are shown in Fig. 3B.
272 A multivariate general linear model was used to analyze the expression of all target
273 genes as a whole, considering at the same time the effect of obesity and diabetes. Thus,
274 an interception among type subjects, obesity and diabetes groups was observed when
275 seven genes are expressed as a whole (*TNF, MYC, IL1B, WDR20, CX3CL1, HIF1A,*
276 *GPI*) (Wilks' λ=0.022 , F=18.620 , p=0.018), indicating that differences in gene

277 expression between OB and NW subjects are not the same when the glycemic status is
278 taken into consideration (Fig. 3C).
279 On the other hand, univariate general linear model served to analyze the differences in
280 the expression of each target gene separately. The inter-subject effect led to statistically
281 significant differences in *IL1B* (p=0.018, F=6.532), *GPI* (p=0.038, F=4.819), *CX3CL1*
282 (p=0.014, F=7.031) and *WDR20* (p=0.006, F= 9.771). The analysis between couples is
283 showed in Table S3. Moreover, the obesity effect (NW vs. OB subjects) led to
284 statistically significant differences in *GPI* (p=0.026, F=7.044) and *WDR20* (p=0.035,
285 F=6.159) expression levels. The T2D effect (NW vs. T2-D subject) also led to
286 statistically significant differences in *GPI* (p=0.024, F=7.09) and *CX3CL1* (p=0.022,
287 F=7.588) expression levels.
288 Fig. 3D summarizes the relationship between the seven target genes, three microRNAs
289 and their corresponding biological processes. The scheme shows that the relationship
290 between miR-20b and Let-7f and the MYC gene could be involved in the signaling
291 pathways of the Wnt and interleukins.
292
293 ***Analysis of target gene expression levels in human SAT and VAT***
294 We have found that *CDKN1A, CX3CL1, HIF1A, PPP2R1B, STAT3, VEGFA* are
295 differentially expressed in VAT and SAT. A multivariate general linear model was used
296 to analyze the expression of these six genes as whole, considering at the same time the
297 effect of tissue type, obesity and T2D. An effect of tissue was observed (Wilks'
298 λ=0.194, F=8.973, p=0.001), indicating that differences in gene expression between OB
299 and NW subjects, from VAT and SAT, are not the same when the glycemic status is
300 taken into consideration (Fig. 4A). Specifically, the effect of tissue on the interaction of
301 each target showed the following results: *CX3CL1* (p=0.006, F=9.595), *CDKN1A*

(p=0.000, F=20.850), *HIF1A* (p=0.028, F=5.714), *PPP2R1B* (p=0.007, F=9.168) *STAT3* (p=0.000, F= 28.115), *VEGFA* (p =0.000, F=30.384). Specifically, an inter-subject effect was observed *CX3CL1* (p=0.003, F=5.681), *CDKN1A* (p = 0.000, F= 13.140), *HIF1A* (p=0.079, F=2.391), *PPP2R1B* (p=0.008, F=4.79) *STAT3* (p=0.000, F=9.550) and *VEGFA* (p=0.000, F=11.972). The analysis between couples is showed in Table S4. On the other hand, univariate general linear models served to analyze differences in the expression of each target gene separately. The analysis between couples, NW vs. OB subjects showed a statistically significant difference in obesity (Wilks' λ=0.153, F=12.001, p=0.000) (Fig. 4A). In addition, the analysis between couples, also showed a statistically significant difference in T2D (Wilks' λ= 0.438, F=12.001, p = 0.057) (Fig. 4A). Fig. 4B summarizes the relationship between the six target genes, three miRNAs and their corresponding biological processes. The scheme shows that the relationship between miR-20b and miR-296 and the PPP2R1B, VEGFA and HIF1A genes could be involved in the signaling pathways of the Wnt and angiogenesis.

Analysis of target gene expression levels related with miRNAs, obesity and T2D

Concordantly to the proposed roles of these genes our study describes that *CDKN1A, CX3CL1, MPP3, STAT3, VEGFA* and *ZNF274* in VAT and *EC*E1 in SAT displayed high expression levels in obesity, pointing to the potential connection between obesity, miR-20b and these gene targets. On the other hand, *CDKN1A, CX3CL1, VEGFA* and *ZNF274* in VAT, and *CDKN1A* and *ECE1* in SAT displayed lower expression levels in T2-D subjects, pointing to the potential connection between obesity-associated T2D, miR-20b and expression levels of these target genes.

In accordance with all these data, we observed that the genes *CX3CL1, IL1B, MPP3, VEGFA, WDR20* and *ZNF274* in VAT and *ECE1* in SAT displayed higher expression

levels in obese subjects, pointing to the potential connection between obesity, miR-296b and these target genes. On the other hand, *CX3CL1, GPI, PPP2R1B, VEGFA, WDR20* and *ZNF274* in VAT and *ECE1, KDR* and *NOS3* in SAT showed lower expression levels in T2-D obese subjects, pointing to the potential relationship between T2D, miR-296 and these target genes.

Moreover, *GLUL* in VAT and *SSSCA1* and *NAT10* in SAT had low expression levels in both metabolically healthy and T2-D obese subjects, suggesting the potential connection between obesity, T2D, miR-296 and these genes. Moreover, our results showed that *CDKN1A, MPP3, STAT3* y *ZNF274* displayed high expression levels in the VAT of obese subjects, pointing to the potential relationship between obesity, Let-7f and these target genes. *GPI* and *ZNF274* displayed low expression levels in AT from T2-D obese subjects, pointing to the potential connection between T2D, Let-7f and these two gene target. Moreover, *GLUL* displayed low expression levels in AT from both metabolically and T2-D obese subjects, suggesting the possible relationship between obesity, T2D, Let-7f and *GLUL*.

DISCUSSION

The present study shows, for first time, an influence of obesity and T2D on the expression of miR-20b, miR-296 and Let-7f in human VAT and SAT. Concordantly, target genes of these miRNAs were also differentially regulated in obesity and T2D depending on the AT depot. These target genes are related to different biological processes relevant for AT physiology which reinforce the putative role of these miRNAs in the regulation of AT function and the maintenance of metabolic homeostasis.

351 It has been shown that miRNA expression in AT is related to fat depots, to the
352 parameters of AT morphology, to obesity and to glucose metabolism[16,17]. Some
353 miRNAs has also been involved in the regulation of preadipocyte proliferation and
354 differentiation in obese subjects compared to leans [18,19]. In accordance with our results,
355 it has been described that miR-296 is involved in the regulation of Ovine AT
356 functionality and that diet could affect its expression level in both SAT and VAT[20].
357 To our knowledge, till the date there are no studies that describe expression levels of
358 miR-20b, miR-296 and Let-7f in human VAT and SAT in relation to obesity and T2D.
359 Our results suggest the potential relationship between miR-20b, miR-296 and Let-7f
360 and obesity. In accordance with our suggestion, Shi and co-authors have highlighted the
361 potential connection between Let-7f and adipogenesis in relation to obesity and
362 associated metabolic alterations[21]. Moreover, other study has shown that placenta and
363 plasma miR-296 levels could be related to gestational obesity[22]
364 Other study has described that miR-20b expression levels decreased in SAT with weight
365 loss induced by hypocaloric diet and exercise[23]. Our results also suggest the existence of
366 a potential relationship between miR-20b, miR-296 and Let-7f and T2D. In accordance
367 with this suggestion, it has been described that circulating Let-7f overexpression is
368 related to insulin secretion and T2D in rodent[24]. Other study carried out in carotid artery
369 plaques has described the protective effect of Let-7 family regulating the inflammatory
370 response associated to T2D[25]. Moreover, miR-20b, miR-296 and Let-7f have been
371 described to be biomarkers for obesity or T2D in rodents. Concretely, Let-7f has been
372 related to obesity and weight loss in rodents, showing that circulating Let-7f decreased
373 in high fat diet fed rodents and increased in obese rodents that reduced their
374 bodyweight[26]. Other studies have identified miR-20b as a biomarker of T2D, because it
375 showed lower expression levels in the plasma of diabetic subjects compared to healthy

individuals[27]. Circulating miR-20b has also been described to be highly expressed in IR subjects in a study carried out by Flowers and co-authors[28], whilst other study has described the contrary trend showing that circulating levels of this miRNA were lower in T2D subjects compared to healthy individuals[29].

Importantly, miR- 20b, miR-296 and Let-7f have been described to be involved in the regulation of WNT signaling pathway and angiogenesis through the regulation of multiple target genes belonging to these pathways[30,31,32,33,34]. Specifically, it has been described that *PPP2R1B, MYC* and *TNF* are key regulators of WNT signaling pathway and *HIF1A, VEGFA* and *STAT3* are the key regulators of angiogenesis and VEGF signaling pathway.

CDKN1A and *MYC* have been described to be involved in adipocyte proliferation and differentiation in relation to obesity[35,36]; *CX3CL1, ECE1, STAT3* and *TNF* are related to obesity and T2D-associated inflammation[37,38,39,40,11,41]. *HIF1A, KDR* and *VEGFA* are related to IR and obesity-associated angiogenesis[41,42,43] and *ZNF274* has been described to be involved in the epigenetic regulation of non-alcoholic hepatic steatosis associated with obesity and IR[44]. *MPP3* has been described to be associated to cell adhesion and extracellular matrix restructuration of the epithelial cells and in osteosarcoma cells[45]. NAT10 has been showed to be involved in apoptosis pathways[46].

GLUL, *IL1B* and *TNF* have been described to be associated to inflammation and insulin signaling pathway in relation to obesity, IR and T2D[47,48,40,41] ; *GPI* and *NOS3* are related to AT vascularity in obesity and T2D[49]; *PPP2R1B* is associated to cellular connection[50], to apoptosis[51] and to Wnt pathway[52]. It has also been described that *RET* is involved in the regulation of cellular proliferation[53,54], and that *WDR20* is likely associated with apoptosis in renal cell carcinoma[55]. Our study showed that the expression levels of these genes depend on the presence of obesity and diabetes and

thus, support the hypothesis that there is a connection between miR-20b, miR-296 and Let-7f, obesity and T2D through the regulation of these target genes.

In conclusion, our results suggest the potential relationship between miR-20b, miR-296, Let-7. These microRNAs could be acting together on VAT and SAT functionality through the regulation of their target genes *CDKN1A, CX3CL1, HIF1A, PPP2R1B, STAT3* and *VEGFA* which are key regulators of angiogenesis and Wnt signaling pathways.

Acknowledgments

The authors wish to thank all the participants for their collaboration. CIBER Fisiopatología de la Obesidad y Nutrición (Pathophysiology of Obesity and Nutrition, CIBEROBN) and CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM) are part of the Instituto de Salud del Carlos III (Institute of Health Carlos III, ISCIII) Project.

Funding sources

This work was co-funded by the European Union through the European Regional Development Fund (FEDER) and supported by grants from the Ministry of Economy and Competitiveness, Institute of Health Carlos III (PI15/01114, PI13/02628; PI12/02355) and the Ministry of Economy and Knowledge (PI-CTS-08181/2011; CTS-7895/2011). R.E is under a contract of a 'Nicolas Monarde' programme from the 'Servicio Andaluz de Salud

R.E.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest: The authors have nothing to disclose.

References

1. Ahima RS. Digging deeper into obesity. *J. Clin. Invest.* **121**, 2076–9 (2011).
2. Hajer GR, van Haeften TW & Visseren FLJ. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur. Heart J.* **29**, 2959–71 (2008).
3. Gustafson B, Hedjazifa, S, Gogg S, Hammarstedt A & Smith U. Insulin resistance and impaired adipogenesis. *Trends Endocrinol. Metab*. **26**, 193–200 (2015).
4. Jabbarzadeh E, Starnes T, Khan YM, Jiang T, Wirtel AJ, Deng M, *et al.* Induction of angiogenesis in tissue-engineered scaffolds designed for bone repair : A combined gene therapy – cell transplantation approach *SCIENCES.* (2008).
5. Blüher M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. *Best Pract Res Clin Endocrinol Metab*. **27**:163-77 (2013).
6. Corvera S & Gealekman O. Biochimica et Biophysica Acta Adipose tissue angiogenesis : Impact on obesity and type-2 diabetes ☆. BBA - *Mol. Basis Dis*. (2013). doi:10.1016/j.bbadis.2013.06.003
7. Parmalee NL & Kitajewski J. Wnt signaling in angiogenesis. *Curr. Drug Targets* **9**, 558–64 (2008).
8. Reis M & Liebner S. Wnt signaling in the vasculature. *Exp. Cell Res*. **319**, 1317–1323 (2013).
9. Kozińsk, K & Dobrzyń A. [Wnt signaling pathway--its role in regulation of cell metabolism]. *Postepy Hig. Med. Dosw*. (Online) **67**, 1098–108 (2013).
10. Ha M & Kim VN. Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* **15**, 509–524 (2014).

11. Alexander R, Lodish H & Sun L. MicroRNAs in adipogenesis and as theraeutic targets for obesity. *Expert Opin Ther Targets.* **15**:623-36 (2011).

12. Arner P & Kulyté A. MicroRNA regulatory networks in human adipose tissue and obesity. *Nat. Rev. Endocrinol.* **11**, 276–288 (2015).

13. Ge Q1, Brichard S2, Yi X3 & Li Q. MicroRNAs as a new mechanism regulating adipose tissue inflammation in obesity and as a novel therapeutic strategy in the metabolic syndrome. *J Immunol Res.* 987285. doi: 10.1155/2014/987285 (2014).

14 Huang T, Alvarez AA, Pangeni RP, Horbinski CM, Lu S, Kim SH *et al.* A regulatory circuit of miR-125b/miR-20b and Wnt signalling controls glioblastoma phenotypes through FZD6-modulated pathways. *Nat. Commun.* **7**, (2016).

15 Clemente-Postigo M, Roca-Rodriguez M del M, Camargo A, Ocaña-Wilhelmi L, Cardona F & Tinahones FJ. Lipopolysaccharide and lipopolysaccharide-binding protein levels and their relationship to early metabolic improvement after bariatric surgery. *Surg. Obes. Relat. Dis*. **11**, 933–939 (2015).

16. Klöting N, Berthold S, Kovacs P, Schön MR, Fasshauer M, Ruschke K *et al.* MicroRNA expression in human omental and subcutaneous adipose tissue. *PLoS One* **4**, e4699 (2009).

17 Viesti A Collares R, Salgado W, Pretti da Cunha Tirapelli D & dos Santos JS. The expression of LEP, LEPR, IGF1 and IL10 in obesity and the relationship with microRNAs. *PLoS One* **9**, e93512 (2014).

18. Chen L, Dai YM, Ji CB, Yang L, Shi CM, Xu GF *et al.* MiR-146b is a regulator of human visceral preadipocyte proliferation and differentiation and its expression is altered in human obesity. *Mol. Cell. Endocrinol.* 393, 65–74 (2014).

19. Peng Y, Yu S, Li H, Xiang H, Peng J & Jiang S. MicroRNAs: Emerging roles in

adipogenesis and obesity. *Cell. Signal.* **26**, 1888–1896 (2014).

20. Meale SJ, Romao JM, He ML, Chaves AV, McAllister TA & Guan LL. Effect of diet on microRNA expression in ovine subcutaneous and visceral adipose tissues. *J. Anim. Sci*. **92**, 3328–37 (2014).

21. Shi C, Huang F, Gu X, Zhang M, Wen J, Wang X *et al.* Adipogenic miRNA and meta-signature miRNAs involved in human adipocyte differentiation and obesity. *Oncotarget* **5**, 1–16 (2016).

22. Carreras-Badosa G, Bonmatí A, Ortega FJ, Mercader JM, Guindo-Martínez M, Torrents D *et al*. Dysregulation of Placental miRNA in Maternal Obesity Is Associated With Pre- and Postnatal Growth. *J. Clin. Endocrinol. Metab.* **102**, 2584–2594 (2017).

23. Kristensen MM, Davidsen PK, Vigelsø A, Hansen CN, Jensen LJ, Jessen N *et al.* miRNAs in human subcutaneous adipose tissue: Effects of weight loss induced by hypocaloric diet and exercise. *Obesity* **25**, 572–580 (2017).

24. Frost RJ & Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 21075–80 (2011).

25. Brennan E, Wang B, McClelland A, Mohan M, Marai M, Beuscart O *et al.* Protective Effect of Let-7 miRNA Family in Regulating Inflammation in Diabetes-Associated Atherosclerosis. *Diabetes* db161405 (2017). doi:10.2337/db16-1405

26. Hsieh CH, Rau CS, Wu SC, Yang JC, Wu YC, Lu TH *et al.* Weight-reduction through a low-fat diet causes differential expression of circulating microRNAs in obese C57BL/6 mice. (2011). doi:10.1186/s12864-015-1896-3

27. Villard A, Marchand L, Thivolet C& Rome S. Diagnostic Value of Cell-free

Circulating Micrornas for Obesity and Type 2 Diabetes: A Meta-analysis. *J. Mol. Biomark. Diagn.* (2015).

28. Flowers E, Aouizerat BE, Abbasi F, Lamendola C, Grove KM, Fukuoka Y *et al.* Circulating microRNA-320a and microRNA-486 predict thiazolidinedione response: Moving towards precision health for diabetes prevention. *Metabolism.* **64**, 1051–9 (2015).

29. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M *et al.* Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ. Res.* **107**, 810–7 (2010).

30. Wang W, Feng L, Zhang H, Hachy S, Satohisa S, Laurent LC *et al.* Preeclampsia up-regulates angiogenesis-associated microRNA (i.e., miR-17, -20a, and -20b) that target ephrin-B2 and EPHB4 in human placenta. *J. Clin. Endocrinol. Metab.* **97**, E1051–9 (2012).

31. Zhang YH, Yang Y, Zhang C, Sun YF, Zhu W, Ma CL *et al.* [Prediction of microRNA-296-5p target genes and its application in lung development]. *Zhongguo Dang Dai Er Ke Za Zhi* **18**, 1302–1307 (2016).

32. Feng J, Huang T, Huang Q, Chen H, Li Y, He W *et al.* Pro□angiogenic microRNA□296 upregulate vascular endothelial growth factor and downregulates Notch1 following cerebral ischemic injury. *Mol. Med. Rep*. **12**, 8141–7 (2015).

33. Egea V, Zahler S, Rieth N, Neth P, Popp T, Kehe K, *et al*. Tissue inhibitor of metalloproteinase-1 (TIMP-1) regulates mesenchymal stem cells through let-7f microRNA and Wnt/β-catenin signaling. doi:10.1073/pnas.1115083109

34. Dhahri W, Dussault S, Haddad P, Turgeon J, Tremblay S, Rolland K *et al*. Reduced expression of let-7f activates TGF-β/ALK5 pathway and leads to

impaired ischaemia-induced neovascularization after cigarette smoke exposure. *J. Cell. Mol. Med.* (2017). doi:10.1111/jcmm.13144

35. Boqué N, Campión J, Milagro FI, Moreno-Aliaga M.-J & Martinez JA. Some cyclin-dependent kinase inhibitors-related genes are regulated by vitamin C in a model of diet-induced obesity. *Biol. Pharm. Bull.* **32**, 1462–8 (2009).

36. Deisenroth C, Black MB, Pendse S, Pluta L, Witherspoon SM, McMullen PD *et al.* MYC is an early response regulator of human adipogenesis in adipose stem cells. *PLoS One* **9**, e114133 (2014).

37. Shah R, O'Neill SM, Hinkle C, Caughey J, Stephan S, Lynch E *et al.* Metabolic Effects of CX3CR1 Deficiency in Diet-Induced Obese Mice. *PLoS One* **10**, e0138317 (2015).

38. Jensen DD, Halls ML, Murphy JE, Canals M, Cattaruzza F, Poole DP *et al.* Endothelin-converting enzyme 1 and β-arrestins exert spatiotemporal control of substance P-induced inflammatory signals. *J. Biol. Chem.* **289,** 20283–94 (2014).

39. Priceman SJ, Kujawski M, Shen S, Cherryholmes GA, Lee H, Zhang C *et al.* Regulation of adipose tissue T cell subsets by Stat3 is crucial for diet-induced obesity and insulin resistance. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 13079–84 (2013).

40. Barbarroja N1, Lopez-Pedrera C, Garrido-Sanchez L, Mayas MD, Oliva-Olivera W, Bernal-Lopez MR *et al.* Progression from high insulin resistance to type 2 diabetes does not entail additional visceral adipose tissue inflammation. *PLoS One* **7**, e48155 (2012).

41. Song MG, Lee HJ, Jin BY, Gutierrez-Aguilar R, Shin KH, Choi SH *et al*. Depot-specific differences in angiogenic capacity of adipose tissue in differential susceptibility to diet-induced obesity. *Mol. Metab*. **5**, 1113–1120 (2016).

552 42. García-Fuentes E, Santiago-Fernández C, Gutiérrez-Repiso C, Mayas
553 MD, Oliva-Olivera W, Coín-Aragüez L *et al.* Hypoxia is associated with a lower
554 expression of genes involved in lipogenesis in visceral adipose tissue. *J. Transl.*
555 *Med.* **13**, 373 (2015).
556 43. Tinahones FJ, Coín-Aragüez L, Mayas MD, Garcia-Fuentes E, Hurtado-Del-
557 Pozo C, Vendrell J *et al.* Obesity-associated insulin resistance is correlated to
558 adipose tissue vascular endothelial growth factors and metalloproteinase levels.
559 *BMC Physiol.* **12**, 4 (2012).
560 44. Ahrens M, Ammerpoh O, von Schönfels W, Kolarova J, Bens S, Itzel T *et al.*
561 DNA Methylation Analysis in Nonalcoholic Fatty Liver Disease Suggests
562 Distinct Disease-Specific and Remodeling Signatures after Bariatric Surgery.
563 *Cell Metab.* **18**, 296–302 (2013).
564 45. Murakami S, Sakurai-Yageta M, Maruyama T & Murakami Y. Correction:
565 Trans-Homophilic Interaction of CADM1 Activates PI3K by Forming a Complex
566 with MAGuK-Family Proteins MPP3 and Dlg. *PLoS One* **9**, e110062 (2014).
567 46. Liu X, Tan Y, Zhang C, Zhang Y, Zhang L, Ren P *et al.* NAT10 regulates p53
568 activation through acetylating p53 at K120 and ubiquitinating Mdm2. *EMBO*
569 *Rep.* **17**, 349–66 (2016).
570 47. Wang Y, Fan S, Lu J, Zhang Z, Wu D, Wu Z *et al.* GLUL Promotes Cell
571 Proliferation in Breast Cancer. *J. Cell. Biochem.* **118**, 2018–2025 (2017).
572 48. Nandipati KC, Subramanian S & Agrawal DK. Protein kinases: mechanisms and
573 downstream targets in inflammation-mediated obesity and insulin resistance.
574 *Mol. Cell. Biochem.* **426**, 27–45 (2017).
575 49. Georgescu A, Popov D, Constantin A, Nemecz M, Alexandru N, Cochior D *et al.*
576 Dysfunction of human subcutaneous fat arterioles in obesity alone or obesity

associated with Type 2 diabetes. *Clin. Sci.* **120**, 463–472 (2011).

50. Liao ZJ, Ma WL, Liang S, Meng W, Shang T & Zheng WL. [Transcriptional regulation of genes involved in liver-selective cell communication]. *Nan Fang Yi Ke Da Xue Xue Bao* **28**, 1582–5 (2008).

51. Feng J, Li A, Deng J, Yang Y, Dang L, Ye Y *et al.* miR-21 attenuates lipopolysaccharide-induced lipid accumulation and inflammatory response: potential role in cerebrovascular disease. *Lipids Health Dis.* **13**, 27 (2014).

52. Suraweera N, Robinson J, Volikos E, Guenther T, Talbot I, Tomlinson I *et al*. Mutations within Wnt pathway genes in sporadic colorectal cancers and cell lines. *Int. J. Cancer* **119**, 1837–1842 (2006).

53. Alao, JP, Michlikova S, Dinér P, Grøtli M & Sunnerhagen P. Selective inhibition of RET mediated cell proliferation in vitro by the kinase inhibitor SPP86. *BMC Cancer* **14**, 853 (2014).

54. Franz H, Greschik H, Willmann D, Ozretić L, Jilg CA, Wardelmann E *et al*. The histone code reader SPIN1 controls RET signaling in liposarcoma. *Oncotarget* **6**, 4773–4789 (2015).

55. Takahashi M, Tsukamoto Y, Kai T, Tokunaga A, Nakada C, Hijiya N *et al.* Downregulation of WDR20 due to loss of 14q is involved in the malignant transformation of clear cell renal cell carcinoma. *Cancer Sci.* **107,** 417–23 (2016).

FIGURE LEGENDS

Figure 1. A. Analysis of miRNA gene expression in human VAT and SAT: miRNAs were extracted from VAT and SAT (n= 12). The gene expression was measured by qPCR and relative quantification of the expression levels was carried out with the comparative threshold cycle (Ct) method. miR-16 was used as endogenous control. Data are expressed as means ± SEM and were submitted to multivariate and univariate general linear models introducing groups of tissue (VAT vs. SAT), diabetes (NG vs. type 2 Diabetic subjects) and Obesity (NW vs. OB) as independent variables. # $p< 0.05$ for the difference in obesity group NW(NG and Diabetic) vs. OB (NG and Diabetic). ‡ $p< 0.05$ for the difference in tissue group (VAT vs. SAT). § $p< 0.05$ for the interaction between Obesity and tissue. **C**. Bioinformatic analysis: a total of 98 putative target genes for miR-20b, miR-296 and Let-7f were introduced in PANTHER and DAVID databases to annotate the biological process in which they are involved.

Figure 2. A. Differentially expressed target genes in human SAT. mRNA was extracted from SAT (n=12). The *CDKN1A, SSSCA1, NAT10, ECE1, NOS3* and *KDR* expression levels was measured by qPCR and relative quantification of the expression levels was carried out with the comparative threshold cycle (Ct) method. EF1α was used as endogenous control. Samples were quantified in duplicate and data are expressed as means ± SEM. * $p< 0.05$ and ** $p<0.01$ according to ANOVA test and Bonferroni's pos hoc test; # $p< 0.05$ for the difference in obesity group NW vs. OB and + $p< 0.05$ for the difference in diabetes group, NG vs. D-OB. **B.** Interaction network between miRNAs and differentially expressed target genes in human SAT was created using Cytoscape software. This network is made of gray nodes for miR-20b, miR-296 and Let-7f, and white nodes for each regulated target gene. Edges shown interaction between miRNAs

and target genes that were differentially expressed in SAT in relation to obesity and type 2 diabetes. **C.** A multivariate general linear model including simultaneously the expression of six target genes was performed as described in material and methods. ∞ $p < 0.05$ interception of model. **D**. Interaction network between miRNAs and target genes were created using Cytoscape software. This network is made of gray nodes for miR-20b, miR-296 and Let-7f and white nodes for each regulated target gene. Edges shown interaction between miRNA and target genes that were differentially expressed in human SAT and their biological function (Forward Slash: Inflammation; Equal Dash: Angiogenesis; Sinewave: Wnt via; Dots: FGF signaling; Solid: Interleukin Signaling; Parallel Lines: Ribosome Biogenesis; Dash Dots: MAPK cascade).

Figure 3. A. Differentially expressed target genes in human VAT. mRNA was extracted from VAT (n=12). The *CDKN1A, SSSCA1, MPP3, GLUL, PPP2R1B, GPI, CX3CL1, VEGFA, IL1B, STAT3, WDR20* and *ZNF274* expression levels was measured by qPCR and relative quantification of the expression levels was carried out with the comparative threshold cycle (Ct) method. EF1α was used as endogenous control. Samples were quantified in duplicate and data are expressed as means ± SEM. * $p< 0.05$ and ** $p<0.01$ according to ANOVA test and Bonferroni's pos hoc test; # $p< 0.05$ for the difference in obesity groups NW vs. OB and + $p< 0.05$ for the difference in diabetes groups, NG vs. D-OB. **B.** Interaction network between miRNAs and differentially expressed target genes in human SAT was created using Cytoscape software. This network is made of gray nodes for miR-20b, miR-296 and Let-7f, and white nodes for each regulated target gene. Edges shown interaction between miRNAs and target genes that were differentially expressed in SAT in relation to obesity and type 2 diabetes. **C.** A multivariate general linear model including simultaneously the expression of seven

target genes was performed as described in material and methods. ∞ $p<0.05$ interception of model. **D**. Interaction network between miRNAs and target genes were created using Cytoscape software. This network is made of gray nodes for miR-20b, miR-296 and Let-7f and white nodes for each regulated target gene. Edges shown interaction between miRNA and target genes that were differentially expressed in human SAT and their biological function (Forward Slash: Inflammation; Equal Dash: Angiogenesis; Sinewave: Wnt via; Solid: Interleukin Signaling; Separate Arrow: Apoptosis; Contiguous Arrow: Glycolisis; Zigzag: Phosphoprotein).

Figure 4. A. Differentially expressed target genes in human SAT and VAT. *CDKN1A, CX3CL1, HIF1A, PPP2R1B, STAT3* and *VEGFA* are expressed in both tissues (SAT and VAT) as shown in Fig. 2 and 3. A multivariate general linear model, including simultaneously the expression of these six target genes in SAT and VAT, was performed as described in material and methods. # $p<0.05$ for the difference in obesity groups (NW vs. OB). ‡$p< 0.05$ for the difference in tissue groups (VAT vs. SAT). †$p<0.05$ for the difference in diabetes (NG vs. D-OB). **B.** Interaction network between miRNAs and target genes were created using Cytoscape software. This network is made of gray nodes for miR-20b, miR-296 and Let-7f. White nodes for each regulated target gene. Edges shown interaction between miRNA and target genes that were differentially expressed in SAT and VAT and their biological function.

Table 1. Anthropometric and biochemical characteristics of the study groups.

	NG-NW (n=7)	D-NW (n=3)	NG-OB (n=7)	D-OB (n=7)
BMI (kg/m^2)	22.00 ± 0.46 [a, b]	25.00 ± 0.32 [a, c]	33.97 ± 2.70 [b]	41.33 ± 3.36 [c]
Age (years)	42.29 ± 4.52 [a]	64.00 ± 2.00 [a]	45.83 ± 5.89	52.00 ± 5.69
Waist (cm)	80.14 ± 2.84 [b]	87.67 ± 1.20	105.83 ± 5.09 [b]	86.43 ± 23.77
Hip (cm)	94.14 ± 3.12 [b]	100.33 ± 1.76	114.67 ± 6.71 [b]	85.86 ± 22.53
Glucose (mg/dL)	90.57 ± 3.12 [a]	175.33 ± 19.92 [a]	92.00 ± 6.24 [d]	121.29 ± 13.00 [d]
HOMA-IR	1.44 ± 0.28	2.60 ± 0.525	2.18 ± 0.45	2.40 ± 0.61
Cholesterol (mg/dL)	189.43 ± 13.71	228.67 ± 31.17	197.67 ± 14.22	210.14 ± 16.35
Triglycerides (mg/dL)	73.71 ± 9.68 [a]	163.67 ± 21.88 [a]	106.00 ± 14.06	104.29 ± 8.29
HDL-c (mg/dL)	62.71 ± 4.00 [b]	51.00 ± 3.46	47.33 ± 3.84 [b, d]	58.00 ± 3.82 [d]
LDL-c (mg/dL)	116.89 ± 10.92	121.00 ± 21.22	134.5 ± 10.60	125.43 ± 12.92

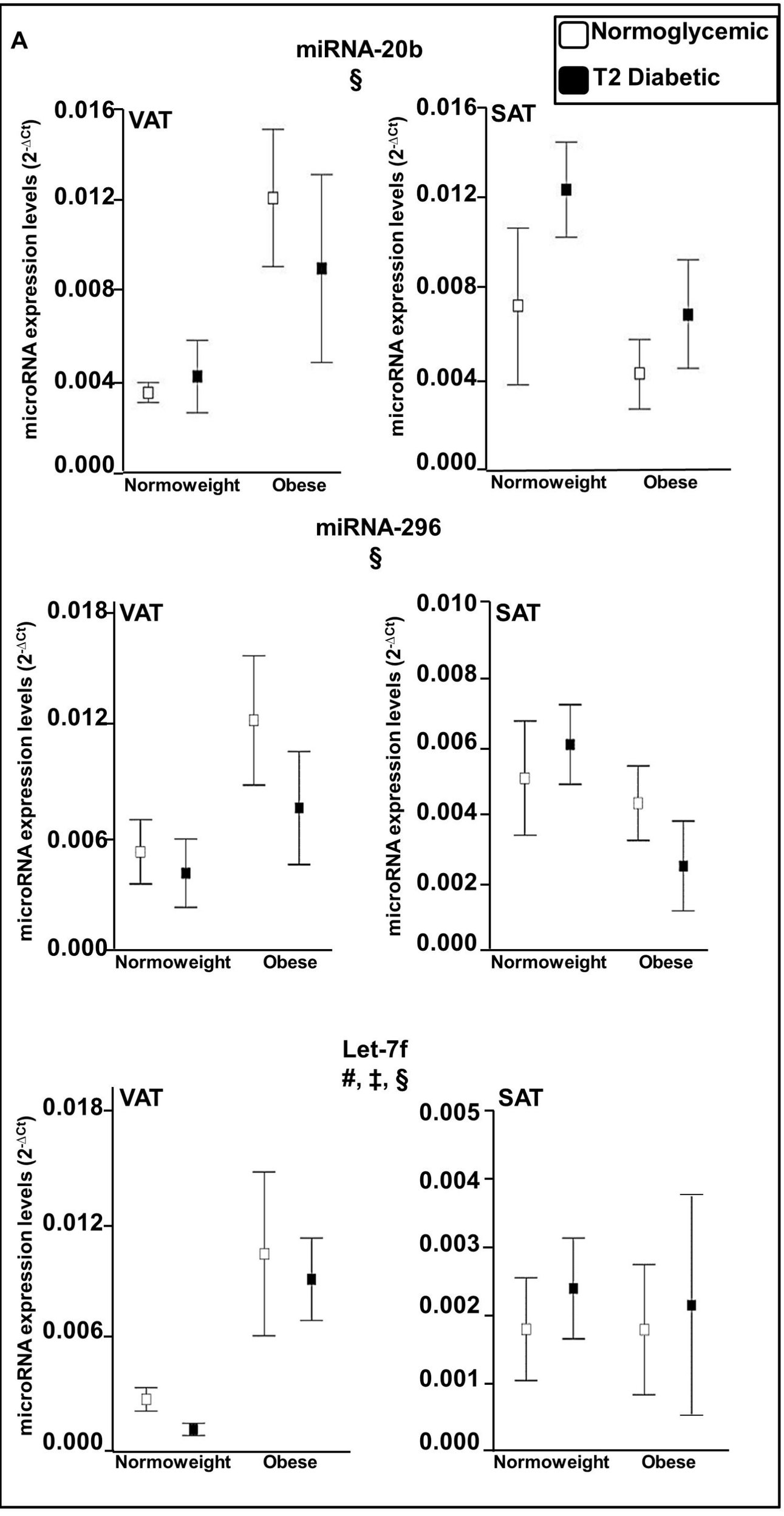
Donors (n = 24) were selected according to BMI and glycemic state. Data are expressed as the mean ± SEM. Comparison among groups was performed by Mann-Whitney U test. Different superscript letters represent statistically significant differences between groups ($p<0.05$). BMI: body mass index, HOMA index: homeostasis model assessment index; NG-NW: normoglycemic normoweight subjects; D-NW: diabetic normoweight subjects; NG-OB: normoglycemic-Obese subjects; D-OB: diabetic obese subjects.

Table 2. Bioinformatic analysis highlighted 7038 putative target genes for miR-20b, miR-296 and Let-7f.

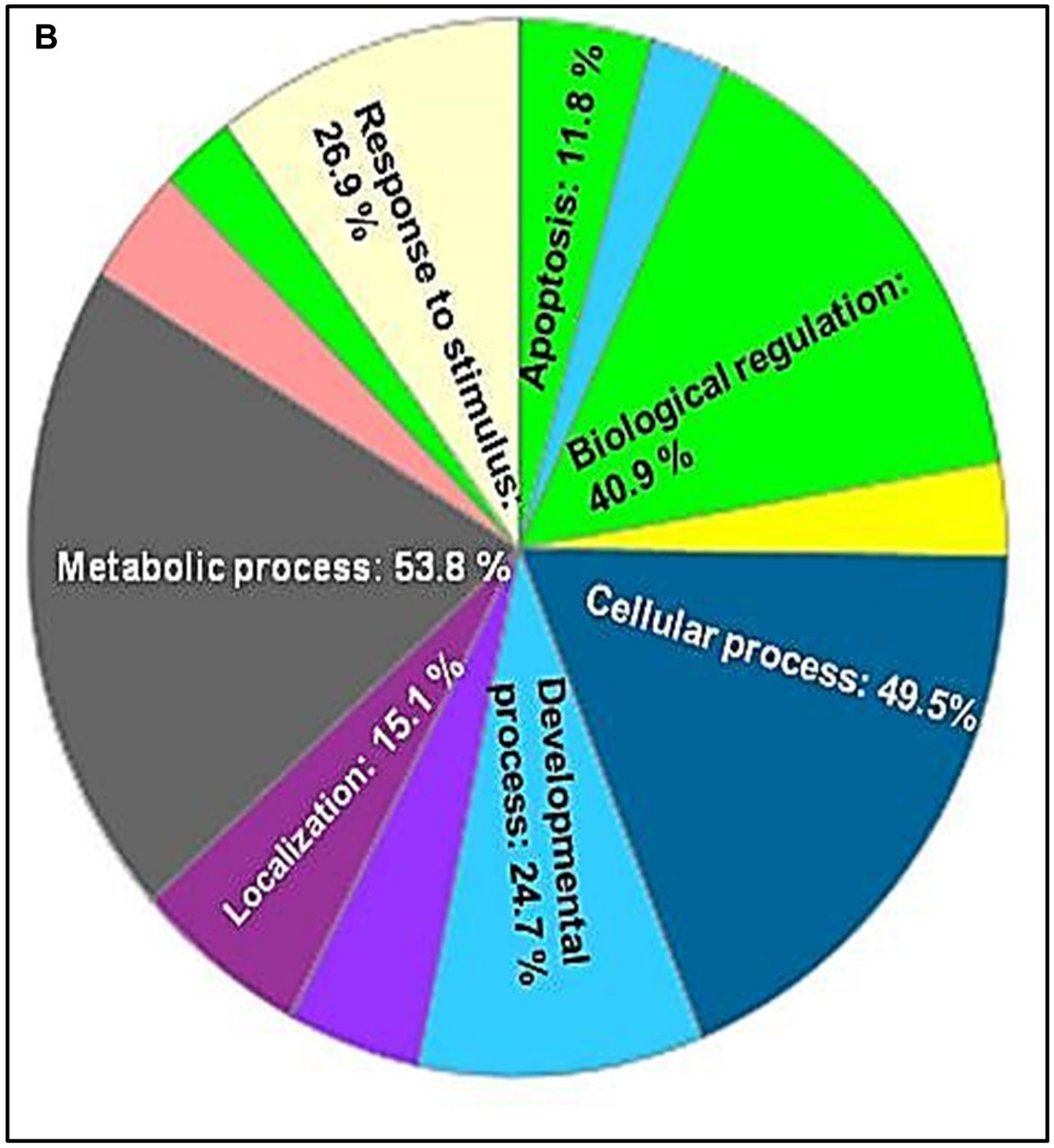
miRNA	Validated		No Validated	Total
	miRTarBase	miRWalk	miRWalk	
20b	17	25	2163	2205
Let-7f	88	28	1244	1360
296	191	92	3190	3473
Total	296	145	6597	7038
Selected		73	25	98
Total analyzed genes		Total differencial expression		
SAT	15	12		
VAT	17	14		
SAT/VAT	32	6		

MiRTarBase 4.0 and miRWalk 2.0 databases were used for prediction of target genes for miR-20b, miR-296 and Let-7f. A selection of them highlighted 98 putative candidates and the expression level of 32 target genes were analyzed in VAT and SAT by qPCR analysis.

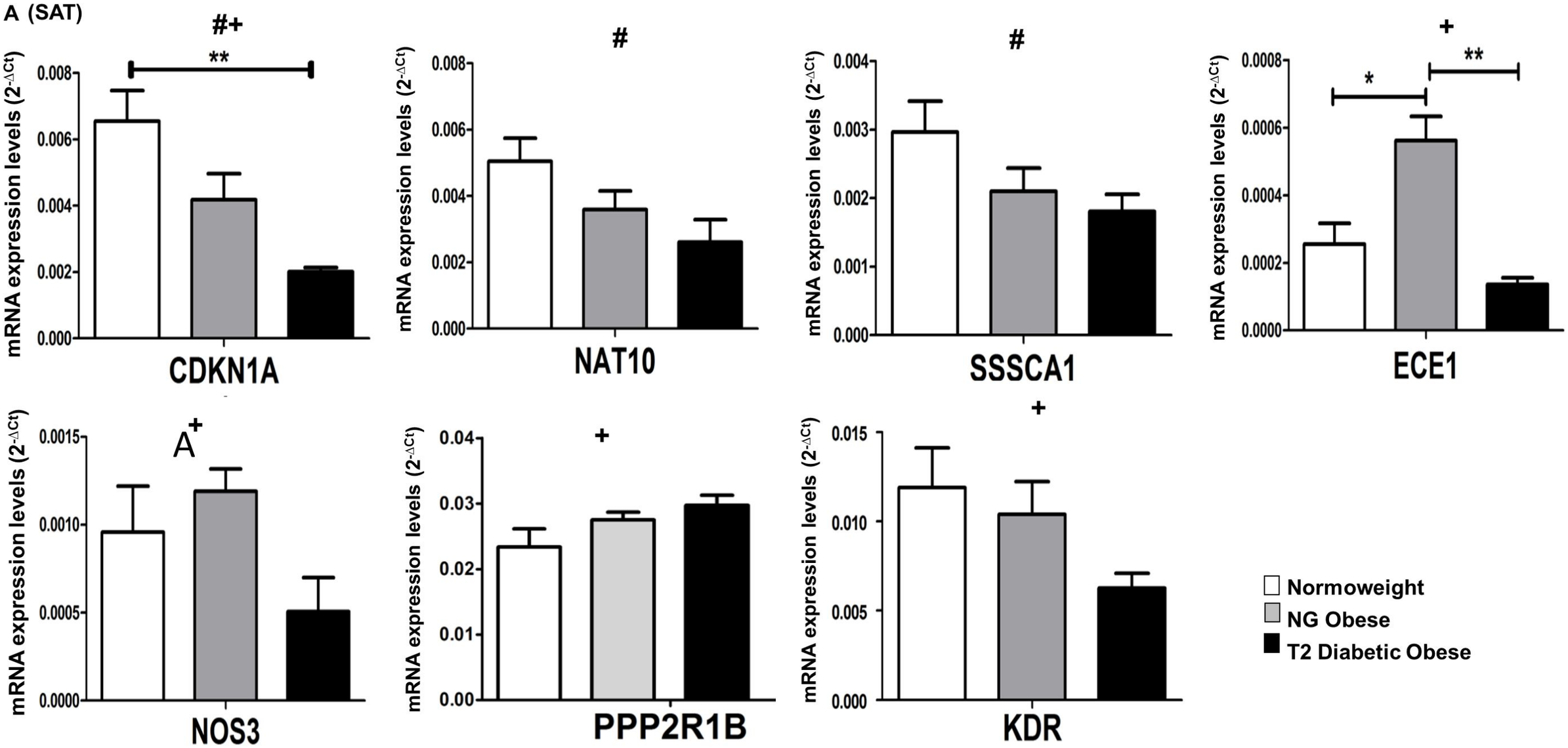
A


Normoglycemic
T2 Diabetic
miRNA-20b
§
VAT
SAT
microRNA expression levels (2-ΔCt)
0.016
0.012
0.008
0.004
0.000
Normoweight
Obese
miRNA-296
§
0.018
0.010
0.006
0.002
Let-7f
#, ‡, §
0.005
0.003
0.001

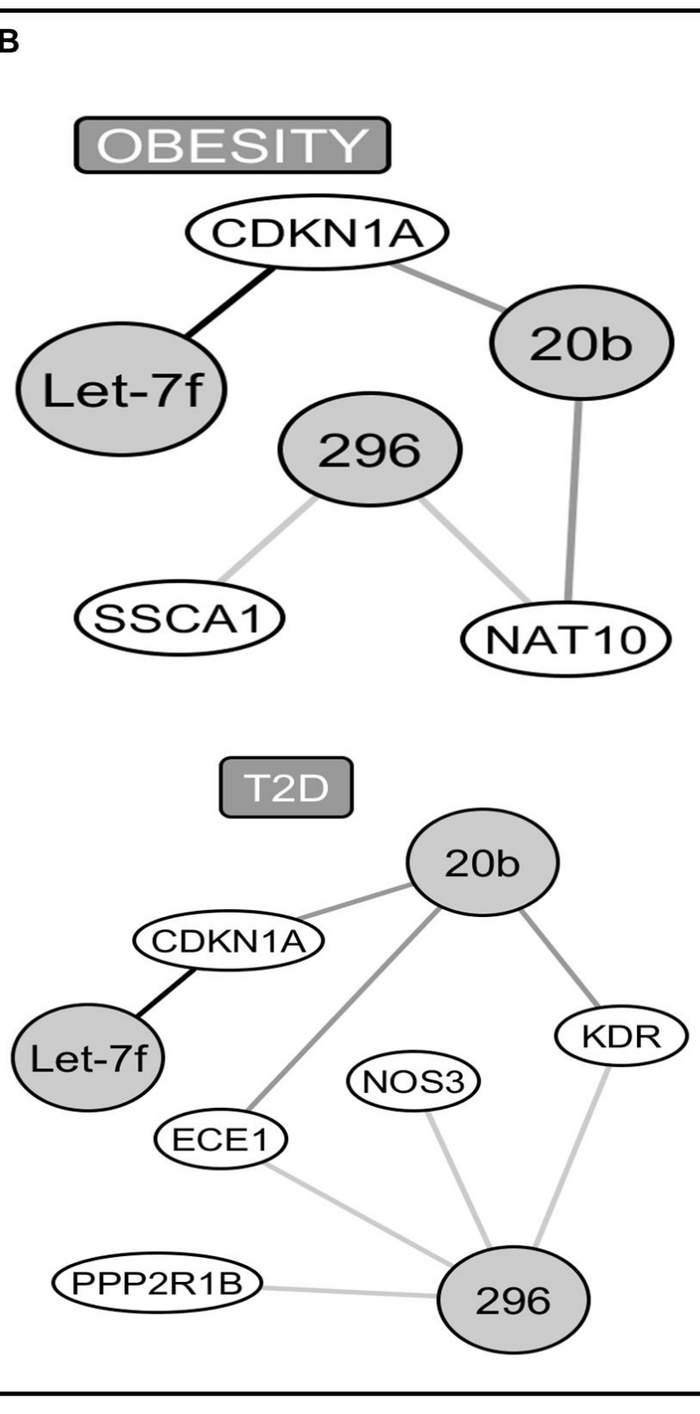
B


Response to stimulus: 26.9 %
Apoptosis: 11.8 %
Biological regulation: 40.9 %
Metabolic process: 53.8 %
Cellular process: 49.5%
Localization: 15.1 %
Developmental process: 24.7 %

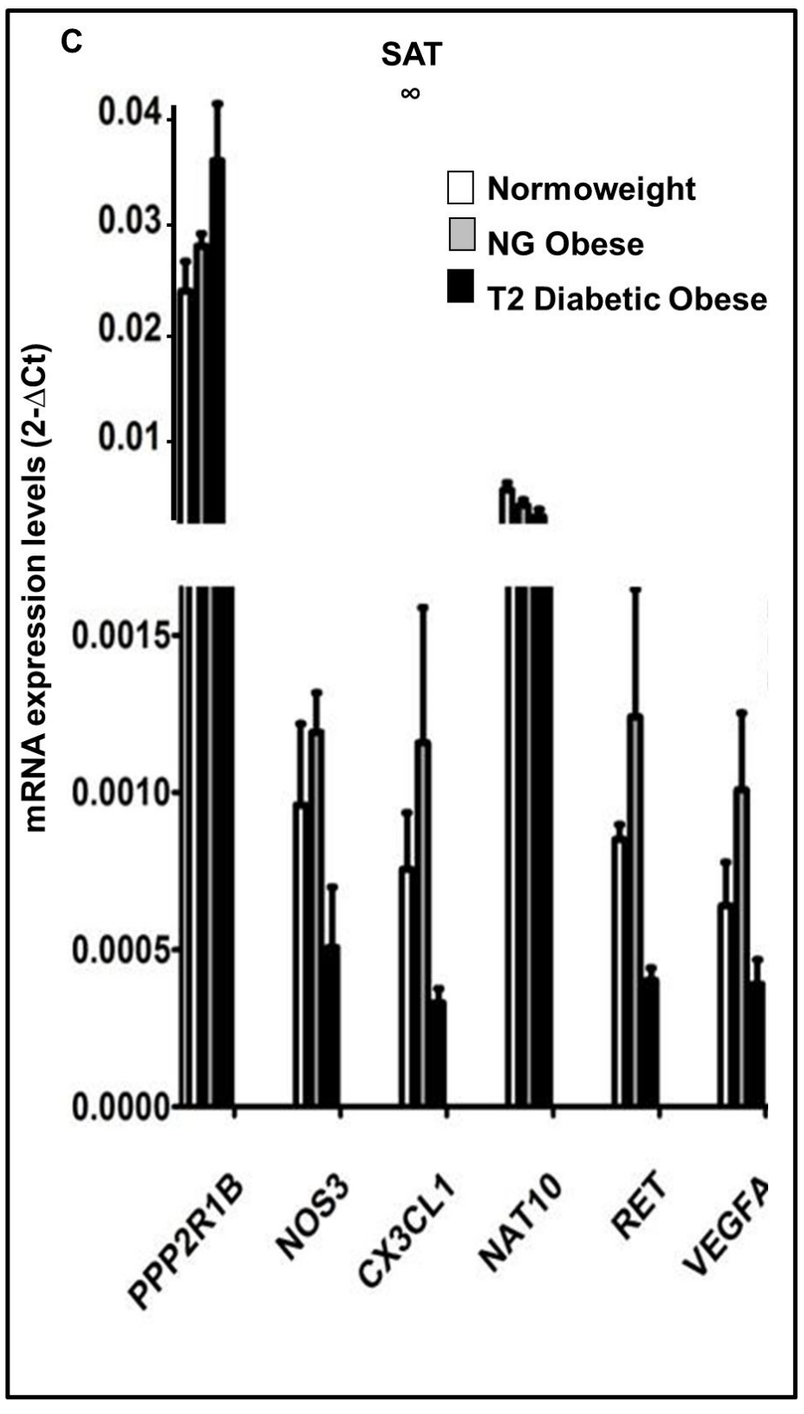
A (SAT)


CDKN1A
NAT10
SSSCA1
ECE1
NOS3
PPP2R1B
KDR
mRNA expression levels (2-ΔCt)
Normoweight
NG Obese
T2 Diabetic Obese

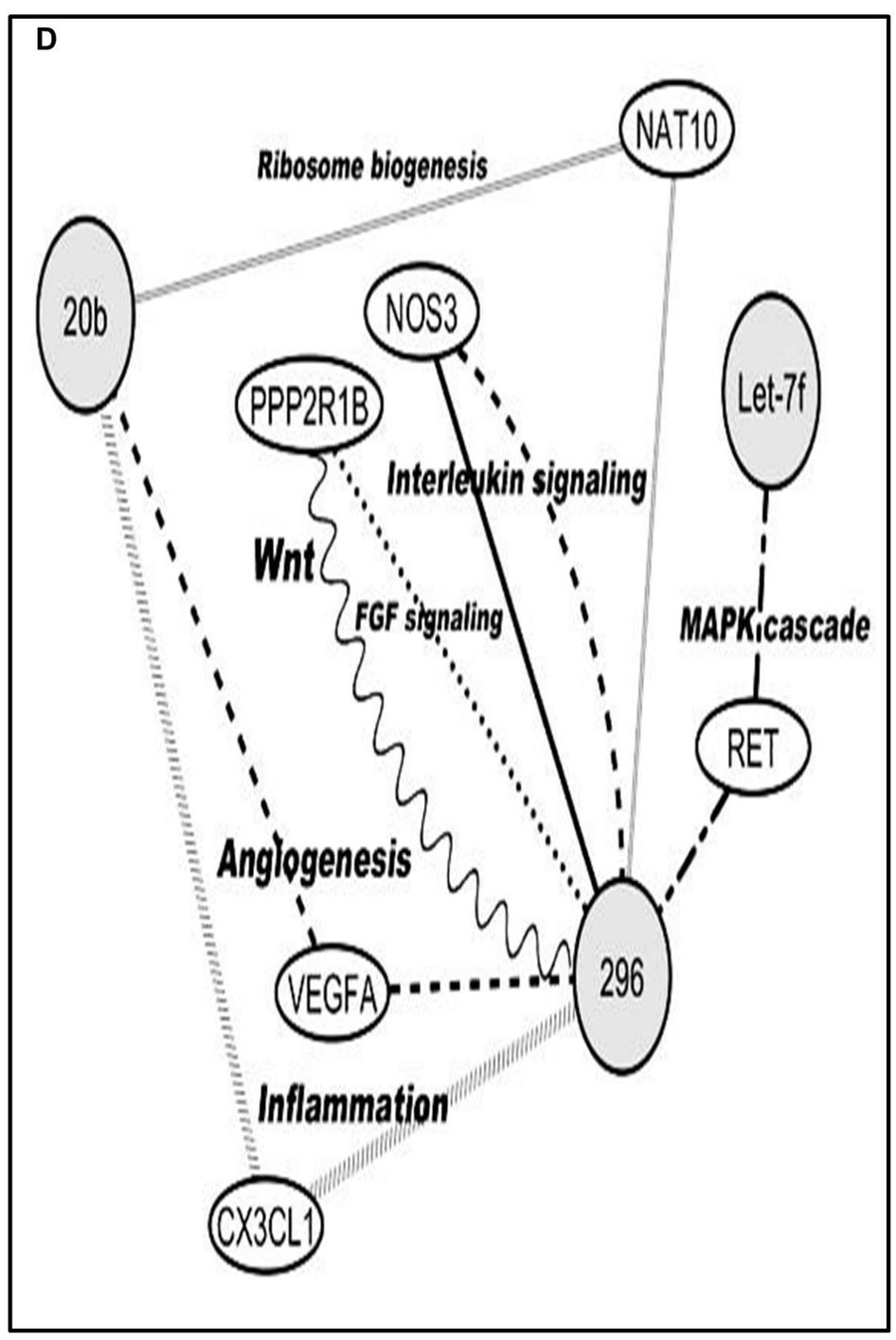
B


OBESITY
CDKN1A
20b
Let-7f
296
SSCA1
NAT10
T2D
20b
CDKN1A
Let-7f
KDR
NOS3
ECE1
PPP2R1B
296

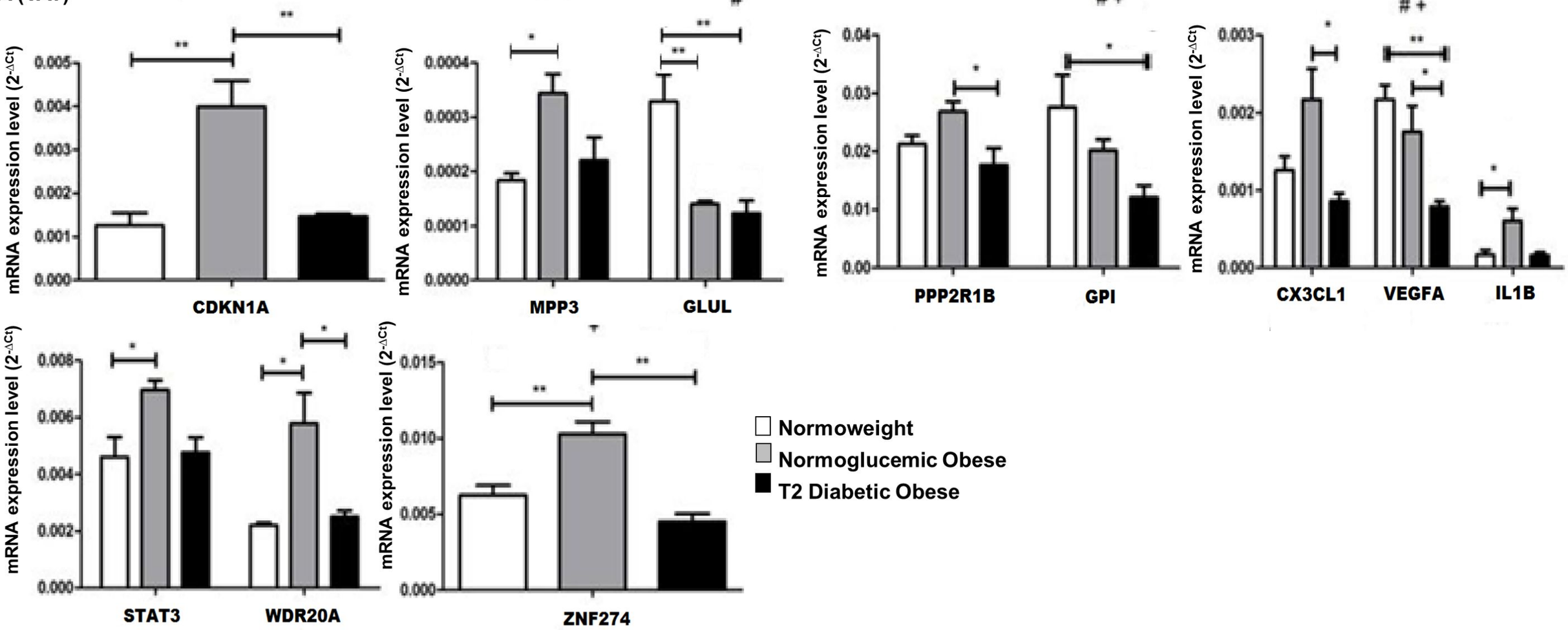
C


SAT
Normoweight
NG Obese
T2 Diabetic Obese
mRNA expression levels (2-ΔCt)
PPP2R1B
NOS3
CX3CL1
NAT10
RET
VEGFA

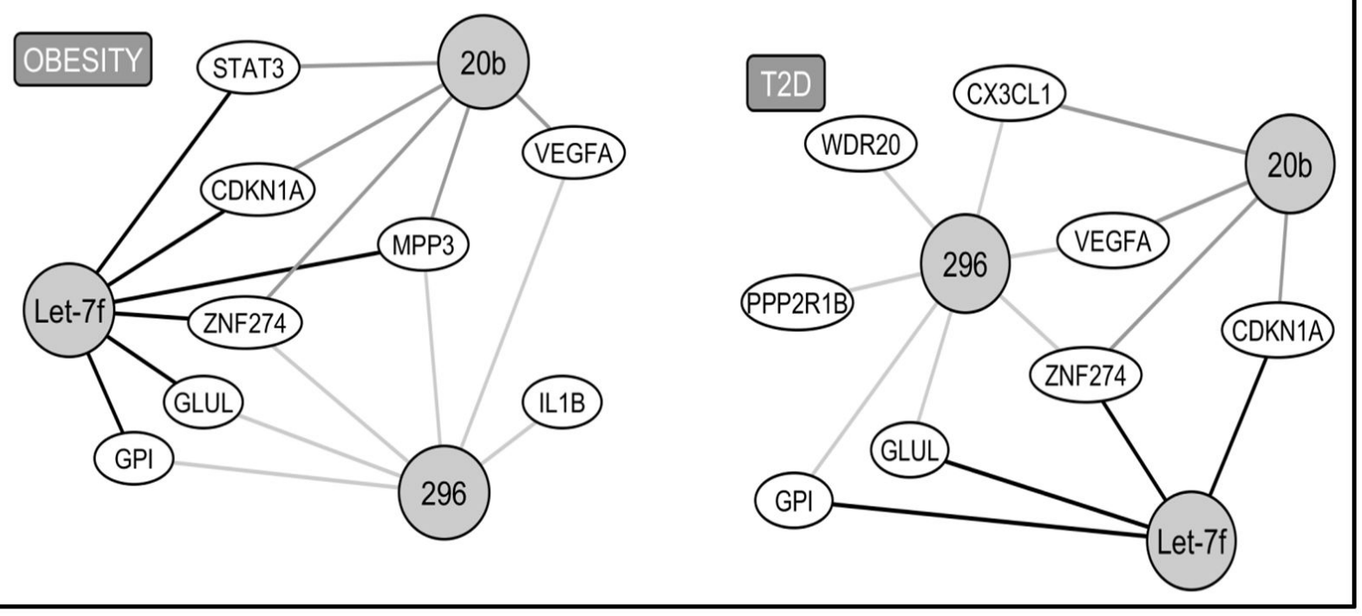
D


NAT10
Ribosome biogenesis
20b
NOS3
PPP2R1B
Let-7f
Interleukin signaling
Wnt
FGF signaling
MAPK cascade
RET
Angiogenesis
VEGFA
296
Inflammation
CX3CL1

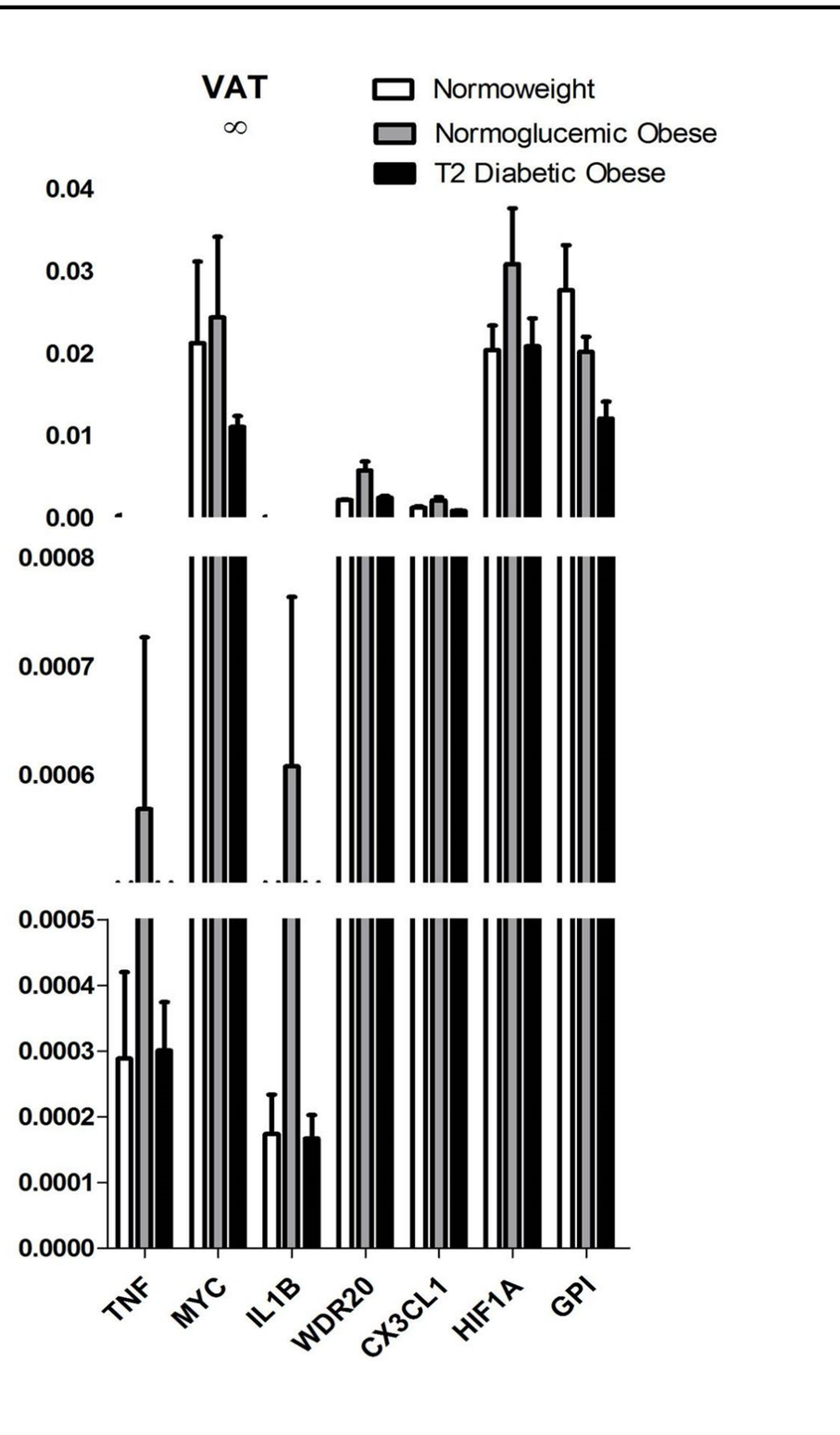
A (VAT)


mRNA expression level (2-ΔCt)
CDKN1A
MPP3
GLUL
PPP2R1B
GPI
CX3CL1
VEGFA
IL1B
STAT3
WDR20A
ZNF274
Normoweight
Normoglucemic Obese
T2 Diabetic Obese

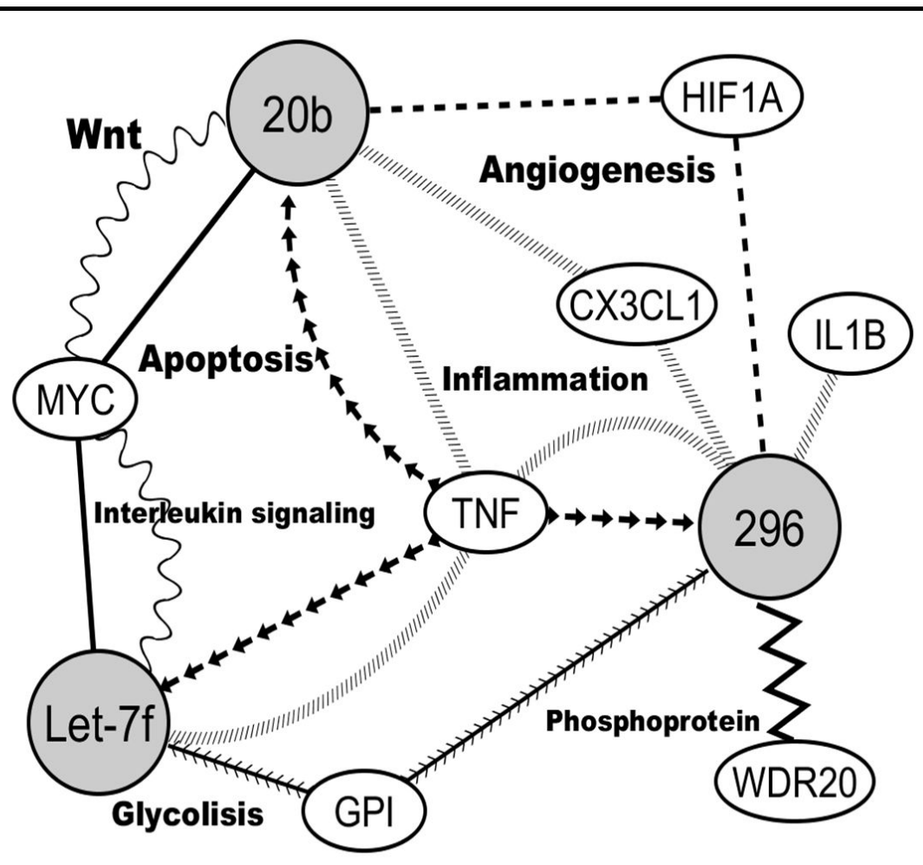
B


OBESITY
STAT3
20b
VEGFA
CDKN1A
MPP3
Let-7f
ZNF274
GLUL
IL1B
GPI
296
T2D
CX3CL1
WDR20
20b
296
VEGFA
PPP2R1B
CDKN1A
ZNF274
GLUL
GPI
Let-7f

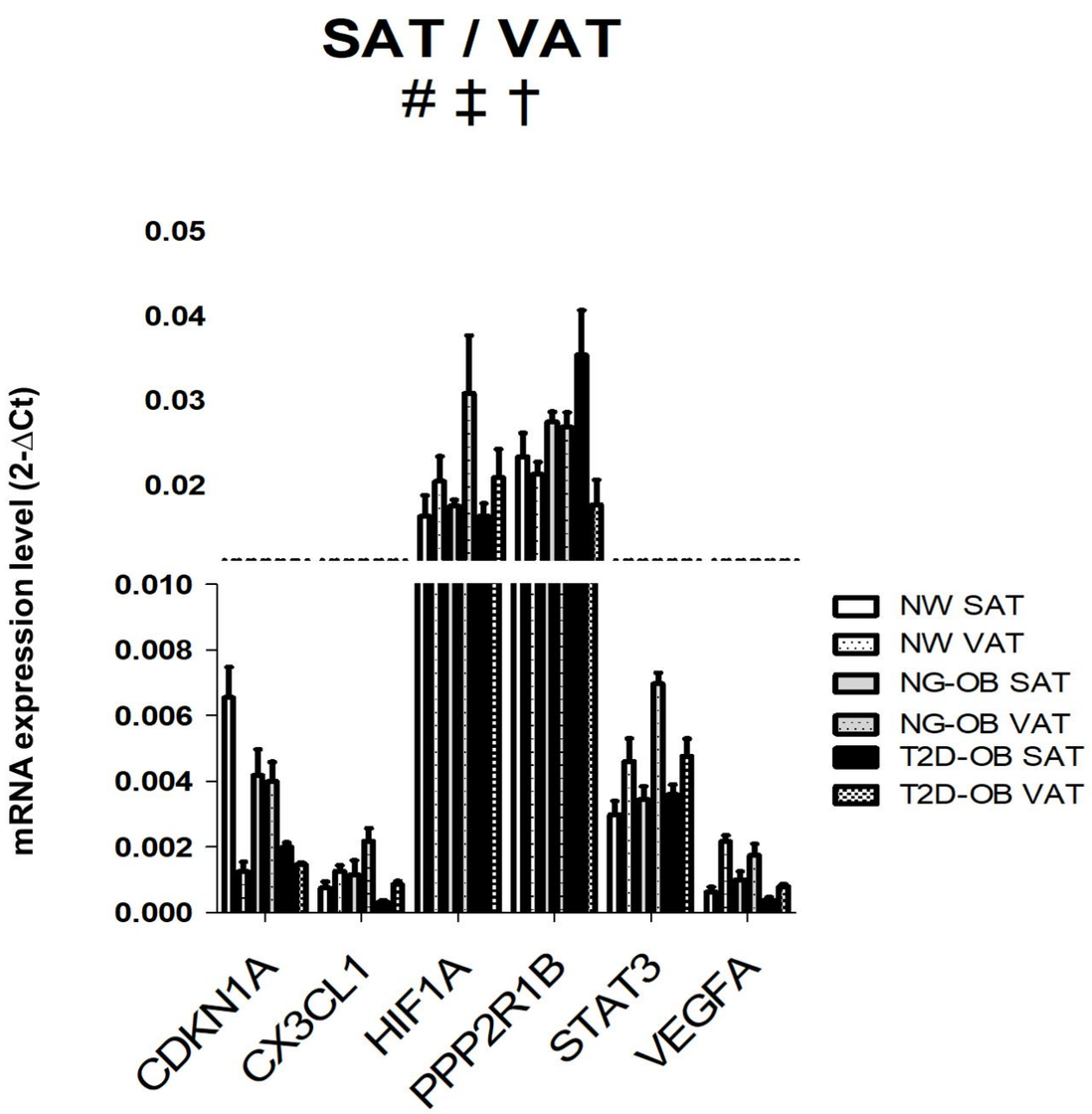
C


VAT
∞
Normoweight
Normoglucemic Obese
T2 Diabetic Obese
TNF
MYC
IL1B
WDR20
CX3CL1
HIF1A
GPI

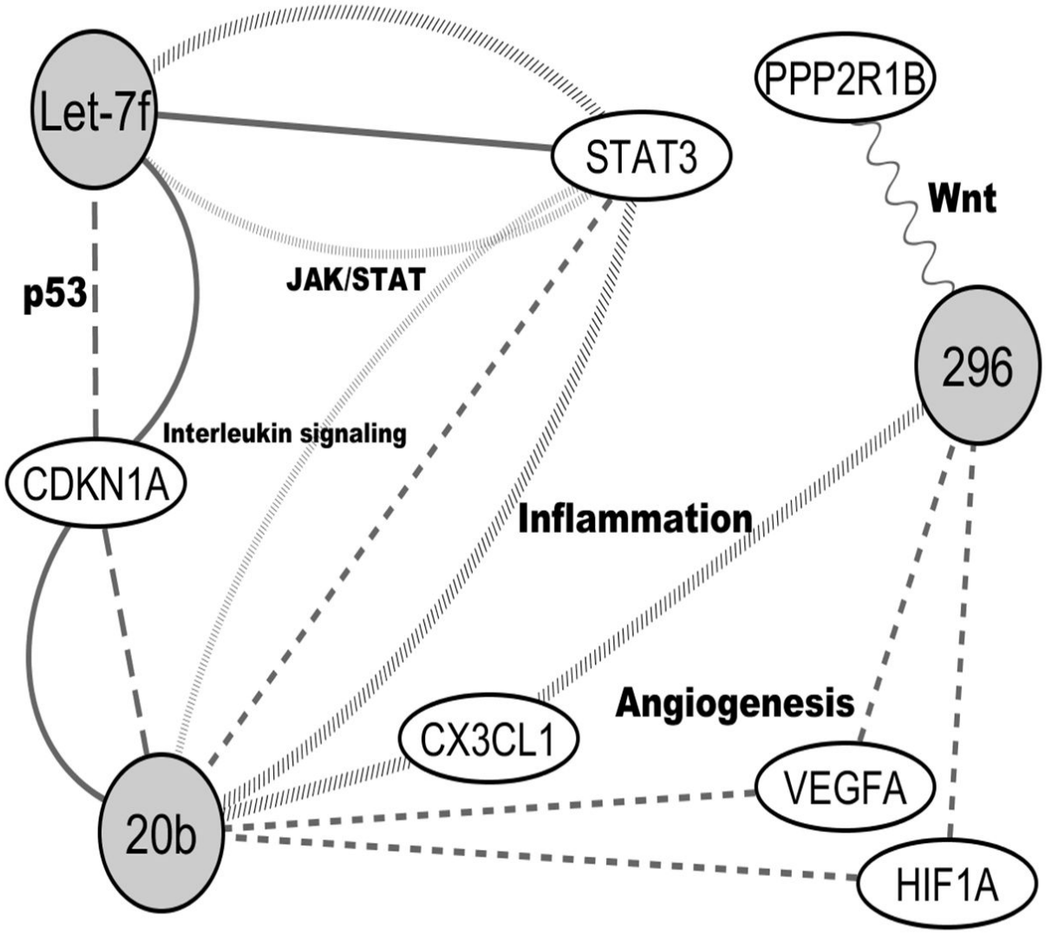
D


20b
HIF1A
Wnt
Angiogenesis
CX3CL1
IL1B
Apoptosis
Inflammation
MYC
Interleukin signaling
TNF
296
Let-7f
Phosphoprotein
WDR20
Glycolisis
GPI

A


SAT / VAT
‡ †
mRNA expression level (2-ΔCt)
0.05
0.04
0.03
0.02
0.010
0.008
0.006
0.004
0.002
0.000
NW SAT
NW VAT
NG-OB SAT
NG-OB VAT
T2D-OB SAT
T2D-OB VAT
CDKN1A
CX3CL1
HIF1A
PPP2R1B
STAT3
VEGFA

B


Let-7f
STAT3
PPP2R1B
Wnt
p53
JAK/STAT
296
Interleukin signaling
CDKN1A
Inflammation
Angiogenesis
CX3CL1
VEGFA
20b
HIF1A