Supplementary File:

Whole Exome Sequencing Identifies *ADRA2A* mutation in Atypical Familial Partial Lipodystrophy

Abhimanyu Garg,[1*] Shireesha Sankella,[1] Chao Xing,[2] and Anil K. Agarwal[1]

Supplementary Methods:

Human omental and subcutaneous abdominal adipose tissue total RNA extraction:

Total RNA was extracted using RNeasy Lipid Tissue Midi kit (Qiagen) according to the manufacture's protocol. Approximately 0.5 g adipose tissue was homogenized in 5 ml of QIAzol lysis reagent followed by separation on the nucleic acid binding column. Residual DNA was removed by digesting the content with DNase I on the column. RNA was then eluted with the elution buffer. Total RNA was further concentrated using RNeasy MinElute cleanup kit (Qiagen). RNA concentration was measured using Nanodrop.

ADRA2 isoform expression analysis by Real-time Reverse Transcriptase-PCR (RT-PCR).

An equal amount of total RNA (3 samples for males and 4 samples for females) was pooled and cDNA was generated using TaqMan® reverse transcription reagents kit (Applied Biosystems-Roche, Branchburg, NJ). Real-time PCR was carried out according to the manufacture's protocol in 96-well plates, read and analyzed using the ABI PRISM 7700HT Sequence Detection System (Applied Biosystems, Foster City, CA). Human omental and subcutaneous abdominal adipose tissue was obtained from 4 females and 3 males undergoing bariatric surgery (Supplementary Table 3). Primers used to amplify individual ADRA isoforms are shown in Supplementary Table 4A. The primers were obtained from Realtimeprimers.com (Real Time Primers, LLC, Elkins Park, PA**)**. The primers used in the study were individually amplified by conventional PCR and sequenced to verify the ADRA2 isoform-specific amplification. Q-PCR expression levels were normalized to the expression level of 18S and the fold change was estimated by $\Delta\Delta C_T$ method.

Amplification of wild type (WT) human *ADRA2A*:

The human *ADRA2A* gene is encoded by a single exon which was amplified using touchdown PCR from genomic DNA obtained from human lymphoblasts using primer pairs 1 and 2 located in the 5' and 3' regions of the gene (all primers are listed in Supplementary Table 3). The PCR product (~1.6 kb) was gel purified and ligated into pDrive. The insert was released from the recombinant clones by digesting them with *BamHI* and *HindIII* and ligated into *BamHI* and *HindIII* digested pcDNA3.1 vector. The recombinant plasmid pcDNA3.1-*ADRA2A*_WT was amplified and isolated by an extraction kit Qiagen (Valencia, CA). This *ADRA2A* clone consists of 165 bp of 5' untranslated region which was included in the wild type (WT) construct. The generated recombinant clone was confirmed by sequencing.

Generation of human *ADRA2A* WT and mutant (L68F) constructs with V5 epitope:

Human *ADRA2A* WT construct with a V5 epitope tag at the amino terminus was generated by amplifying the pcDNA3.1-*ADRA2A*_WT using primer pairs 3 and 4 (Supplementary Table 3). The PCR product was gel purified using extraction kit from Qiagen and ligated into pDrive and sequenced for nucleotide error. The insert was released by digesting them with *EcoRI* and *XhoI* and ligated into *EcoRI* and *XhoI* digested pcDNA3.1 vector and sequenced for PCR errors. The expression vector was designated *ADRA2A*_V5_WT (*ADRA2A*_WT_N-terminus_V5).

The generation of mutant *ADRA2A_L68F* with a V5 epitope in the amino terminus was carried out by performing two successive PCR reactions: the 1st set of PCR reactions used primer pairs 3 and 8; and another PCR reaction included primer pairs 4 and 7 (Supplementary Table 3). Both the PCR products were gel purified using extraction kit from Qiagen. The purified PCR products were mixed in equimolar concentration and an overlap PCR (2nd PCR reaction) was carried out using primer pairs 3 and 4. The desired PCR product was gel purified and digested with *EcoRI* and *XhoI* and ligated into similarly digested pcDNA3.1 vector.

Supplementary Tables

Supplementary Table 1. Filtering of the variants seen on whole exome sequencing in FPLD 122

Total variants		50,452
Potentially functional variants	missense, nonsense, splice site, and frameshift indels	11,724
Dominant model	heterozygotes in F122.2 and F122.3, but wild type in F122.9	938
Frequency	absent in 1000 Genome and MAF < 0.001 in ExAC	25
Linkage region	Lod score >0.8	3
Conservation	GERP++ conservation score > 2.0	3

Supplementary Table 2. Potential candidate variants from whole exome sequencing in the linked regions

Chromo-some	Position	Refe-rence	Variant	dbSNP	Function	Gene	Coding Variant	Protein Variant	Frequency in ExAC	GERP	CADD
10	**112837956**	**C**	**T**	**N/A**	**Missense**	***ADRA2A***	**NM_000681.3:c.202C>T**	**p.Leu68Phe**	N/A	**2.89**	**20.8**
11	119549163	G	A	rs377427305	Missense	*PVRL1*	NM_002855.4:c.392C>T	p.Thr131Met	6.589e-05	4.78	19.84
22	19163726	G	C	N/A	Missense	*SLC25A1*	NM_005984.4:c.853C>G	p.Arg285Gly	N/A	5.64	35.00

GERP, Genomic Evolutionary Rate Profiling; CADD, Combined Annotation-Dependent Depletion
Sanger sequencing of these candidate variants in the entire family revealed lack of segregation of *PVRL1* and *SLC25A1* variants among affected and unaffected adults from the family.

Supplementary Table 3A. Primers used in this study.

#	Primer name	**Forward primer sequence**	**Reverse primer sequence**
1	*ADRA2A*_Ex2.2.1.1_S	CCGGAGGAAGAGGAGGAC	
2	*ADRA2A*_Ex2.2.4.1_R		GTTTCCTCGTCTGGGGTG
3	h*ADRA2A*_EcoRI_V5_F	CGGAATTCATGGGTAAGCCTATCC CTAACCCTCTCCTCGGTCTCGATTC TACGATGTTCCGCCAGGAGCAG	
4	h*ADRA2A*_XhoI_R		CCGCTCGAGTCACACGATCCGCTTCCTG
5	h*ADRA2A*_EcoRI_ F	CGGAATTCATGTTCCGCCAGGAGC AG	
6	h*ADRA2A*_XhoI_V5_R		CCGCTCGAGTCACGTAGAATCGAGACCGA GGAGAGGGTTAGGGATAGGCTTACCCACG ATCCGCTTCCTG
7	h*ADRA2A*_L68F_F	CGGCGATGATGACGAACACGTTGC CGAACAC	
8	h*ADRA2A*_L68F_R		GTGTTCGGCAACGTGTTCGTCATCATCGC CG

Supplementary Table 3B. Real time PCR primers used in this study.

Gene	**Accession #**	**Size**	**Location**	**Forward Primer 5'>3'**	**Reverse Primer 5'>3'**
ADRA2A	NM_000681	213	420-632	GAGAGTCGGTAATCGCTTCG	ATCGGTGGCCTGATAACTTG
ADRA2B	NM_000682	245	554-798	GCCTCATCATGATCCTTGTC	CCCAGTATCTTCAGGGGTCT
ADRA2C	NM_000683	242	1338-1579	CCGGTCATCTACACGGTCTT	ATCTCTCTGCCAAGCTCCTG

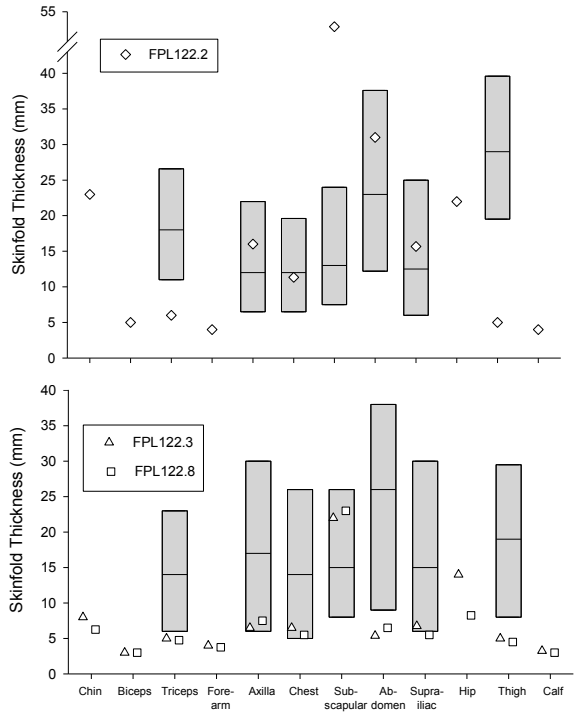
Supplementary Table 4: Demographics of the subjects from which subcutaneous abdominal and omental adipose tissue was obtained.

Age	Gender	Race	Procedure
45	M	W	RYGB
49	M	W	RYGB
68	M	W	Gastroplasty Revision
49	F	AA	RYGB
57	F	W	Gastric banding
30	F	W	RYGB
58	F	W	RYGB

AA, African-American; F, female; M, male; RYGB, roux-en-Y gastric bypass surgery; W, white.

Supplementary Figure 1:

Suppl Fig. 1



Supplementary Figure 1. Body fat distribution in affected subjects. Skinfold thickness at various anatomic sites in an affected female (A) (FPLD122.2, shown in unfilled diamond) and two affected males (B) (FPLD122.3 shown in unfilled triangles and FPLD122.8 shown in unfilled squares) with *ADRA2A* mutation. The bars represent median, 10th and 90th percentile values of skinfold thickness for the normal women aged 18-55 years (1) and normal men aged 18-61 years (2). Affected subjects from FPLD122 pedigree, had considerably reduced peripheral skinfold thickness but normal to increased central skinfold thickness.

References:

1. Jackson, A.S., Pollock, M.L., and Ward, A. 1980. Generalized equations for predicting body density of women. *Medicine & Science in Sports & Exercise* 12:175-181.
2. Jackson, A.S., and Pollock, M.L. 1978. Generalized equations for predicting body density of men. *British Journal of Nutrition* 40:497-504.