Supplemental Methods

Study Participants

The study design was approved by the ethics committees of the participating centres and all subjects gave written informed consent. Clinical characteristics of the study sample are listed in **Table 1**.

Stage 1 study samples

Sample 1, Dutch CVR (cardiovascular risk). A total of 393 unrelated subjects at increased risk for CVD, i.e. with the following risk factors: age 40-70 years and either hypertension (HT), or body mass index (BMI) >25 kg/m^2 from the Cohort study of Diabetes and Atherosclerosis Maastricht (CoDAM) were genotyped. Details of the CoDAM study design are described in detail elsewhere.[1] To eliminate possible interactions with type 2 diabetes (T2DM)[2,3], individuals with T2DM based on an oral glucose tolerance test were excluded from this study sample. Lipid lowering drug treatments were withheld for 2 weeks before fasting blood sample were taken.

Sample 2, Dutch FCHL. A total of 195 unrelated probands and spouses from families with an established diagnosis of FCHL were genotyped. The FCHL families were recruited through the Lipid Clinic of the Utrecht University Hospital (until 1997) and Maastricht University Hospital (1997-2004) as described in detail previously.[4] Lipid phenotypes were measured as described previously[4]. Lipid lowering drug treatments were withheld for 2 weeks before fasting blood sample were taken. Participants were instructed to abstain from alcohol for 72 h and from smoking on the morning of their visit to the lipid clinic.

Stage 2 study samples

Sample 3, Finnish FCHL. A total of 61 Finnish FCHL families with 715 individuals genotyped were recruited in the Helsinki and Turku University Central Hospital, as described in detail previously.[5] Lipid phenotypes were measured as described previously.[5] Lipid lowering drug treatments were withheld for 4 weeks before fasting blood sample were taken.

Finnish population-based cohort, The METSIM (METabolic Syndrome In Men) cohort consists of 5,112 male subjects, age 50-70 years, randomly selected from the population of Kuopio in Eastern Finland (population 95,000). Recruitment, data collection and phenotypic determinations were performed in the University of Kuopio as described previously.[6] Each participant underwent an evaluation of their disease history, drug treatment, cardiovascular risk factors and an extensive panel

of lipid, glucose and other metabolic traits. Subjects reimbursed for lipid lowering therapies (n=599) were excluded from the analyses.

Sample 4, Finnish CVR. A total of 1,371 subjects with CVR (cardiovascular risk) were selected from the METSIM cohort using the same ascertainment criteria used for the Dutch CVR (Sample 1), i.e. either BMI >25 kg/m^2 or reimbursed for medication for the treatment of HT. To eliminate possible interactions with T2DM, individuals with family history of T2DM and/or T2DM based on an oral glucose tolerance test were excluded from this study sample.

SNP selection and genotyping

To select SNPs in the ATF6-gene region (193±3kb) for stage 1 genotyping we utilized the HapMap CEU population with northern and western Europe ancestry. We used the Haploview software[7] to select 13 tag SNPs with minor allele frequency (MAF) > 0.1 and r^2 threshold of 0.85 (rs4657101, rs7553368, rs4579731, rs2134697, rs1503815, rs2340721, rs11581364, rs7554023, rs10918215, rs7514053, rs10918243, rs13401and rs3795649). In addition, 3 coding SNPs were also selected (rs1058405, rs2070150 and rs1135983). The stage 1 genotyping of 16 SNPs was performed using the TaqMan 7900HT (Applied Biosystems) in 588 subjects of Samples 1 and 2. We obtained > 97% success rate for all SNPs. The SNPs rs10918215 and rs7514053 were in complete LD (r^2=1) with rs13401 and the SNP rs2070150, was in complete LD with rs1135983. Therefore we excluded these SNPs, resulting in a total of 13 SNPs analyzed. In stage 2 genotyping the rs1058405 (SNP3) was genotyped in 61 Finnish FCHL families (Sample 3) using the pyrosequencing technique on the PSQ HS96A platform and in the METSIM cohort using the TaqMan assay. The genotype call rate was 98% in Sample 3, and 99% in the METSIM cohort. We replicated 10% of the stage 1 genotypes for SNP3 using different DNA dilutions from the same individual and two different genotyping techniques, i.e. TaqMan and pyrosequencing. Only one of the genotypes did not replicate. All SNPs were in Hardy-Weinberg equilibrium in all subjects of Sample 1 and 4, as well as in the Dutch and Finnish FCHL spouses.

Statistical Analyses

We utilized a two-stage study design, in which all tag-SNPs were first analyzed in stage1. The variant that provided the strongest association signal in the stage 1 analyses was further analyzed in a combined analysis of both stages. This design was originally introduced by Skol et al. to reduce the cost of genotyping while maintaining the overall power of the study.[8] Association analysis of the stage

1 samples with continuous traits (TC, LDL-C and apoB) was performed using PLINK v1.01 software.[9] We tested the genotypic model using multivariate linear regression while including age and sex as covariates. The genotypic test is a two degrees of freedom (d.f) test of of an additive ($\hat{\beta}_{add}$) and a dominance-deviation ($\hat{\beta}_{dev}$) effect. The $\hat{\beta}_{dev}$ coefficient is called dominance-deviation as it reflects a deviation from an additive effect. A recessive character is suggested when the sign of $\hat{\beta}_{dev}$ is opposite of $\hat{\beta}_{add}$, and a dominant character is suggested when the signs of both coefficients are in the same direction. A full recessive or dominant model is observed when the magnitude of the effects is equal $|\hat{\beta}dev| = |\hat{\beta}add|$. To avoid violating the assumptions of the test statistics, we estimated empirical p-values in the combined analysis of Sample 1 and 2 by combining the $\chi 2$ statistics from 20,000 random permutations of each sample weighted by the proportion of individuals examined in each sample.[6] Accordingly, the empirical p-value of each SNP was calculated as (R+1)/(20,000+1) where R is the number of times the permuted combined statistic was greater than the observed combined statistic. Association analysis of the Finnish FCHL families (Sample 3) with residual traits adjusted for age and sex was performed using the genotypic model of the family-based association test (FBAT) software.[10] The genotypic model of FBAT compares all genotypes simultaneously to their null expectation in one test with 2 d.f and the test statistic follows a $\chi 2$ distribution. The PedCheck program[11] was used to detect Mendelian errors in the families. All statistical analyses of the METSIM cohort were performed using the SPSS 15.0 software. We assessed the effect of SNP3 on continuous traits using multivariate linear regression for the genotypic model. Multiplicative interaction with CVR and T2DM and/or family history of T2DM was tested by including in the model the genotypes coded as 0, 1, 2 copy of the Val variant (g); the risk factor (CVR or T2DM) coded as 1 or 0 (r); and their interaction term (gr). The combined analysis of stage 1 and 2 and the meta-analysis were performed using Fisher's method for combining P-value with the proportions of the sample sizes as weights.[12] This statistic follows a chi-square distribution with 2k degrees of freedom, where k is the number of independent tests. The weighted Fisher's $\chi 2$ method [$C_wF = -2w_i\ln(p_i)$] was calculated in R.2.8.0 as follow: w=k*size/sum(size) # where size is the sample size; cwf =-2*sum(w*log(p)); df=2*k ;p.cwf=1 - pchisq(chi, df). We performed a meta-analysis of stage 1, Sample 3 and the entire METSIM cohort as well as while excluding the subjects with T2DM or family history of T2DM from the METSIM cohort.

The proportion of variance explained (R^2) was calculated using univariate general linear model in SPSS 15.0. In the FCHL families (Sample 3), the R^2 values were calculated with adjustment for family membership as a factor.[13]

To test for associations with untyped SNPs within the ATF6 region, we used the Bayesian IMputation-Based Association Mapping (BIMBAM) software version 0.99.[14] We utilized the phased chromosomes of 60 HapMap CEU founders to impute genotypes of 249 SNPs with MAF>5% and genotype call rate>95% in the 254kb region on chromosome 1. We only used the subjects of stage 1 with complete genotype data (n=498) and residual trait (Z-scores) adjusted for age and sex were prepared separately in each sample. Ten SNPs failed the first run of imputations (average of 1,000 imputations), indicated by the standard-error (SE) of the Bayes factor (BF) estimate. The BFs were computed using the prior D2 from Servin and Stephens[14], averaging over σa = 0.05; 0.1; 0.2; 0.4 and $\sigma d = \sigma a/4$ where σa denotes the prior for the additive effect and σd denotes the prior for the dominance-deviation effect. We also used the BFs to compute P-values using 10,000 permutations. The posterior-mean-genotypes were used to calculate the average posterior probability of each SNP, calculated as the percentage of individuals with >80% posterior probability for calling a genotype 0, 1 or 2. Only P-values for those 200 SNPs with at least 80% average posterior probability are presented in **Figure 1**

Cell studies and functional experiments

Isolation and culture of pre-adipocytes

Adipose tissue was collected as described previously.[15] Following the fat biopsy and collagenase treatment, the cell suspension was filtered through 500 µm nylon mesh and spun at 220×g for 1 min to separate pre-adipocytes from mature adipocytes. Pre-adipocytes were suspended in 5 ml DMEM medium containing 10% fetal calf serum (FCS) and 1% glutamine / streptomycine / peniciline (GSP) and cultured during 4 passages under standardized conditions. ATF6 protein levels were measured by western blot in lysates of cultured primary pre-adipocytes, derived from 11 subjects. Clinical characteristics of these subjects at the time of the fat biopsy were as follows: 6 women/5 men, age 50±12 years, BMI 26.8±2.2 kg/m^2, TC 5.7±1.5 mmol/L, LDL-C 4.1±1.7 mmol/L, apoB 1.12±0.33 g/L, TG 2.1±1.8 mmol/L.

HeLa cell culture and transfection

HeLa cells were maintained in DMEM containing 10% fetal calf serum. HeLa cells were resuspended at $2.5*10^6$ cells per 400µl of cold Dulbecco's phosphate-buffered saline and electroporated in a 0.4-cm gap electroporation cuvette at 250V and 950 microfarads using a GenePulser II Electroporator (Bio-Rad). Cells were then plated at a density of $0.5*10^6$ per 24-mm well for luciferase and galactosidase assays, or $1.5*10^6$ per 35-mm well for Western blot experiments.

Plasmids

Cytomegalovirus-Galactosidase: Cytomegalovirus-galactosidase, which is driven by the cytomegalovirus promoter and codes for galactosidase, was used to normalize for transfection efficiency.

Flag-ATF6-(1→373[67]-Met/Val/Leu): These Flag-ATF6 constructs are driven by the cytomegalovirus promoter, and encode amino acid 1-373 of the ATF6 protein that confer strong constitutive transcriptional activity, mimicking all aspects of cleaved endogenous ATF6. All three constructs contain a 3xFLAG-epitope and only differ by their amino acid at position 67 (Met, Val or Leu). Construction of Flag-ATF6-(1→373[67]-Leucine) has been described previously[16]. This parent vector was used as a template to create Flag-ATF6-(1-373[67]-Methionine) and Flag-ATF6-(1→373[67]-Valine) by site-directed mutagenesis (QuickChange kit, Stratagene, Inc.) using the following primers:

[67]-Methionine-sense GATTTGGATTTG**A**TGCCTTGGGAGTCAGAC;

[67]-Methionine-antisense GTCTGACTCCCAAGGCA**T**CAAATCCAAATC;

[67]-Valine-sense GATTTGGATTTG**G**TGCCTTGGGAGTCAGAC;

[67]-Valine-antisense GTCTGACTCCCAAGGCA**C**CAAATCCAAATC.

Nucleotides in **bold** were responsible for the desired amino acid substitutions.

Grp78 promotor(-284→+221)-Luciferase: This construct encodes the GRP78 promoter from -284 to +221 driving luciferase. It was created by PCR using HeLa genomic DNA as a template and a sense primer that begins at base -284 and includes a Kpn1 restriction site and an antisense primer at +221 and includes an Xho1 restriction site. The resulting PCR product was digested with Kpn1 and Xho1, creating the GRP78-promoter (-284 to +221), which was ligated into the vector pGL2 (Promega).

The pGL2 promoter (catalog number E1631; Promega) contains an SV40 promoter upstream of a luciferase reporter gene.

Reporter assays

β-galactosidase: 48 hours after transfection, cells were lysed in 500 µl of ice-cold lysis buffer (25 mM Gly-Gly, pH 7.8, 15 mM MgSO4, 4 mM EDTA, 0.25% Triton X-100, and 1 mM dithiothreitol). Following centrifugation of the cell lysate, 100 µl of supernatant was combined with 500 µl of galactosidase buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1mM MgSO4, 1 mg/ml chlorophenol red-D-galactopyranoside, and 50 mM 2-mercaptoethanol) and after 1h of incubation absorbance was measured at 570 nm.

Luciferase: Following cell lysis and centrifugation as described above, 100µl cell lysate was combined with 100 µl of luciferase buffer (the above described lysis buffer containing 45 mM MgSO4, 0.3 mM D-luciferin, and 3 mM ATP). An Optocompt II luminometer (MGM Instruments, Inc) was used to measure light emission of each sample for 10 seconds. Relative luciferase activities were determined by dividing luciferase values by β-galactosidase values.

Western Blotting: Cell cultures were extracted in lysis buffer composed of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml aprotinin. After clearing by centrifugation, the protein concentration of the lysate was determined. Protein dilutions were prepared with appropriate amount of 2x Laemmli buffer and equal amounts of protein from each sample and then fractionated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were then probed with ATF6-alpha antiserum (Santa Cruz Biotechnology, California), beta-actin antiserum (Biolegend, San Diego), FLAG antiserum (F-3165, Sigma), KDEL antiserum (SPA-827,Stessgen Biotechnologies Inc, San Diego, CA), or GAPDH (RDI-TRK 5G4-65C; Research Diagnostics Inc, Flanders, NJ).

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Supplemental Figure legends

Figure S1

ATF6 protein from cultured pre-adipocytes as measured by western blot using ATF6-specific antiserum antibody. Western blots were quantified by densitometry analysis and ATF6 protein levels were normalized to beta-actin (A.U. indicates arbitrary units). Positive correlation with plasma TC (A) LDL-C (B) and apoB (C) levels is shown. The Pearson correlations with TC, LDL-C and apoB were 0.64, 0.72, and 0.76; respectively with corresponding P-values p=0.032, 0.018, and 0.006.

Figure S2

A. Schematic representation of full-length and cleaved (activated) ATF6.

B. Detailed representation of the VN8-like region (amino acid 61-68) in ATF6. The Met[67]Val amino-acid substitution (grey box), is associated with cholesterol levels.

Table S1. Clinical characteristics of the study samples

Study sample	N (%female)	Age	TC (mmol/L)	LDL-C (mmol/L)	ApoB (g/L)	BMI (kg/m^2)	Glucose (mmol/L)	SBP (mmHg)	DBP (mmHg)	Met[67]Val (MAF)
Stage I study samples										
Sample 1 Dutch CVR	393 (30%)	59±7	5.23±0.91	3.34±0.85	1.12±0.24	28±4.04	5.38±0.50	140±20	84±10	0.30
Sample 2 Dutch FCHL	195 (54%)	51±11	6.06±1.37	4.01±1.22	1.14±0.33	26±3.50	5.11±0.78	133±19	85±12	0.27
Stage II study samples										
Sample 3 Finnish FCHL	715 (51%)	41±17	6.14±1.45	3.89±1.19	1.12±0.38	26±4.78	5.11±1.50	130±17	81±12	0.24
Sample 4 Finnish CVR	1,371 (0%)	59±6	5.49±0.96	3.52±0.85	1.10±0.28	29±3.30	5.72±0.50	142±16	90±9	0.31
Population-based cohort										
METSIM	4,532 (0%)	59±6	5.39±0.96	3.42±0.85	1.06±0.27	27±3.75	5.94±1.17	140±17	88±10	0.31

Trait values represent the mean ± SEM. BMI, indicates body mass index; SBP, systolic blood pressure and DBP, diastolic blood pressure.

Table S2. Association results of ATF6 polymorphisms with TC, LDL-C and apoB levels in stage 1 analyses

SNP	Genotype	N		MAF		TC (mmol/L)		Combined	LDL-C (mmol/L)		Combined	ApoB (g/L)		Combined
		I	II	I	II	Sample I	Sample II	P-value	Sample I	Sample II	P-value	SampleI	Sample II	P-value
SNP 1	C/C	27	12			5.13±0.18	5.98±0.39		3.37±0.16	4.17±0.36		1.14±0.05	1.15±0.10	
rs4657101	A/C	146	57	0.26	0.23	5.31±0.08	6.29±0.18	0.894	3.39±0.08	4.09±0.16	0.834	1.13±0.02	1.18±0.04	0.846
	A/A	205	106			5.21±0.05	5.93±0.13		3.34±0.05	3.94±0.13		1.12±0.02	1.11±0.03	
SNP 2	C/C	32	19			5.05±0.16	6.19±0.31		3.26±0.16	4.04±0.28		1.09±0.04	1.16±0.07	
rs7553368	T/C	165	72	0.31	0.30	5.28±0.08	5.98±0.16	0.559	3.37±0.08	4.01±0.16	0.861	1.13±0.02	1.13±0.04	0.821
	T/T	178	87			5.23±0.08	6.06±0.16		3.34±0.05	3.96±0.13		1.11±0.02	1.13±0.04	
SNP 3	G/G	33	20			5.54±0.16	6.71±0.28		3.73±0.16	4.43±0.26		1.24±0.04	1.31±0.07	
rs1058405	A/G	168	58	0.30	0.27	5.21±0.08	5.91±0.18	**0.009**	3.29±0.05	3.94±0.16	**0.008**	1.10±0.02	1.11±0.04	**0.002**
	A/A	183	103			5.21±0.08	5.96±0.13		3.34±0.05	3.91±0.1		1.12±0.02	1.11±0.03	
SNP 4	T/T	4	1			4.92±0.44	6.55		3.16±0.41	4.77		1.08±0.12	1.29	
rs1135983	C/T	53	23	0.08	0.07	5.21±0.13	6.22±0.28	0.653	3.29±0.10	3.89±0.26	0.697	1.09±0.03	1.12±0.07	0.775
	C/C	324	155			5.26±0.05	6.01±0.1		3.39±0.05	3.99±0.1		1.13±0.01	1.13±0.03	
SNP 5	G/G	8	2			5.26±0.31	5.98±0.96		3.47±0.28	4.09±0.85		1.07±0.08	1.27±0.23	
rs4579731	A/G	86	39	0.14	0.12	5.26±0.10	5.91±0.21	0.997	3.32±0.08	3.78±0.18	0.900	1.12±0.03	1.10±0.05	0.663
	A/A	280	135			5.26±0.05	6.06±0.1		3.37±0.05	4.01±0.1		1.13±0.01	1.14±0.03	
SNP 6	C/C	24	8			5.02±0.18	5.96±0.49		3.19±0.18	3.94±0.44		1.04±0.05	1.11±0.13	
rs2134697	T/C	139	75	0.25	0.25	5.23±0.08	5.8±0.16	0.270	3.32±0.08	3.81±0.16	0.348	1.11±0.02	1.10±0.04	0.133
	T/T	216	100			5.28±0.05	6.27±0.13		3.39±0.05	4.17±0.13		1.14±0.02	1.17±0.03	
SNP 7	T/T	6	3			4.51±0.36	6.22±0.78		2.72±0.34	3.86±0.7		0.96±0.10	1.20±0.23	
rs1503815	C/T	70	40	0.11	0.13	5.26±0.10	5.72±0.21	0.071	3.37±0.10	3.89±0.18	0.114	1.12±0.03	1.09±0.05	0.157
	C/C	302	139			5.26±0.05	6.14±0.1		3.37±0.05	4.01±0.1		1.13±0.01	1.15±0.03	
SNP 8	A/A	44	19			5.00±0.13	5.8±0.31		3.11±0.13	3.89±0.26		1.04±0.04	1.07±0.08	
rs2340721	C/A	171	86	0.34	0.34	5.23±0.08	5.93±0.16	0.063	3.37±0.05	3.91±0.13	0.045	1.12±0.02	1.12±0.04	0.008
	C/C	167	80			5.28±0.08	6.24±0.16		3.42±0.05	4.14±0.13		1.14±0.02	1.18±0.04	
SNP 9	T/T	53	32			5.21±0.13	6.06±0.23		3.39±0.10	3.96±0.21		1.13±0.03	1.14±0.06	
rs11581364	G/T	171	75	0.37	0.39	5.26±0.08	5.98±0.16	0.977	3.34±0.05	3.94±0.13	0.750	1.12±0.02	1.10±0.04	0.727
	G/G	155	70			5.21±0.08	6.14±0.16		3.32±0.08	4.09±0.16		1.11±0.02	1.18±0.04	
SNP 10	G/G	10	7			4.95±0.28	6.19±0.52		3.16±0.26	4.56±0.44		1.11±0.07	1.19±0.12	
rs7554023	A/G	120	47	0.19	0.17	5.34±0.08	6.11±0.21	0.606	3.47±0.08	4.01±0.18	0.417	1.15±0.02	1.16±0.05	0.879
	A/A	246	123			5.21±0.05	5.98±0.13		3.29±0.05	3.94±0.1		1.11±0.01	1.12±0.03	
SNP 11	C/C	42	24			5.41±0.13	6.58±0.26		3.57±0.13	4.33±0.23		1.20±0.04	1.25±0.07	

rs10918243	T/C	183	75	0.35	0.35	5.26±0.05	5.93±0.16	0.033	3.37±0.05	3.89±0.13	0.054	1.12±0.02	1.11±0.04	0.008
	T/T	153	80			5.15±0.08	6.01±0.16		3.26±0.08	4.01±0.13		1.10±0.02	1.13±0.04	
SNP 12	G/G	21	14			5.10±0.18	5.75±0.36		3.19±0.18	3.89±0.34		1.07±0.05	1.06±0.09	
rs13401	A/G	144	65	0.25	0.25	5.18±0.08	6.01±0.18	0.390	3.26±0.08	3.91±0.16	0.390	1.10±0.02	1.12±0.04	0.202
	A/A	210	110			5.28±0.05	6.14±0.13		3.42±0.05	4.09±0.13		1.14±0.02	1.15±0.03	
SNP 13	T/T	57	33			5.18±0.13	6.11±0.23		3.39±0.10	4.07±0.21		1.13±0.03	1.17±0.06	
rs3795649	C/T	186	81	0.40	0.40	5.23±0.08	5.93±0.16	0.856	3.32±0.05	4.01±0.13	0.955	1.12±0.02	1.12±0.04	0.960
	C/C	134	65			5.26±0.08	6.14±0.16		3.37±0.08	3.99±0.16		1.12±0.02	1.15±0.04	

Trait values represent the marginal mean evaluated at the average age and sex ±SEM
The P-values represent the results of the combined analysis of stage 1 and 2, as described in Supplementary methods.

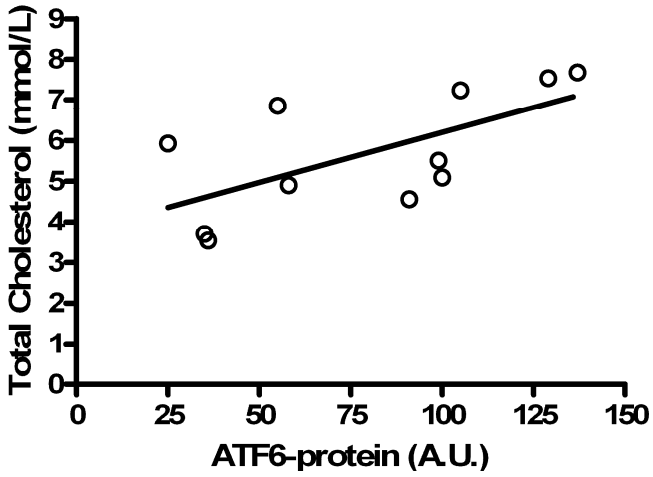
Table S3. Descriptive statistics of glucose traits for the METSIM cohort by family history of T2DM

Trait	FHDM* n=2,099	Non-FHDM n=2,432	P value†
Fasting plasma glucose (mmol/l)	6.08±0.03	5.8±0.02	1.52E-17
Glucose 120min (mmol/l)	6.71±0.06	6.34±0.05	2.45E-06
Fasting serum insulin (mU/l)	10.25±0.34	8.58±0.14	8.17E-07
Insulin 120min(mU/l)	55.9±1.28	52.6±1.15	1.22E-03
Glucose AUC (mmol/l * min)	932±4.94	903±3.82	2.04E-06
Insulin resistance index (HOMA)	3.03±0.12	2.34±0.05	3.82E-10
Insulin sensitivity index (QUICKI)	0.63±0.003	0.65±0.003	6.69E-10

Trait values represent the mean ± SEM. AUC, indicates area under the glucose curve attained during an oral glucose tolerance test; HOMA, Homeostatic model assessment and QUICKI, quantitative insulin sensitivity check index.
*FHDM, indicates subjects with family history of diabetes.
†The P value represents the results obtained by independent t-tests with log transformed values.

Figure S1

A



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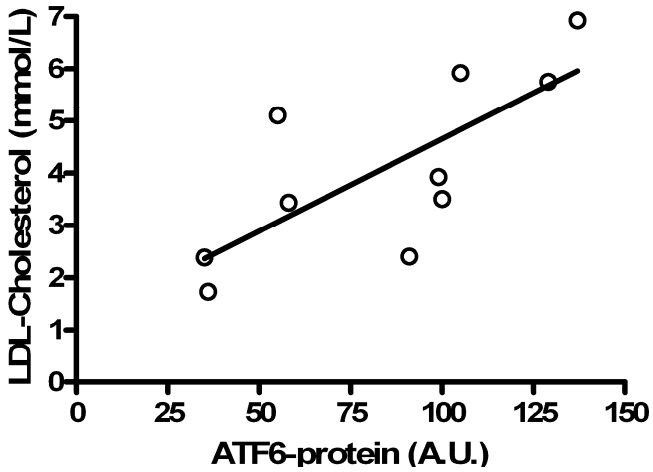


C

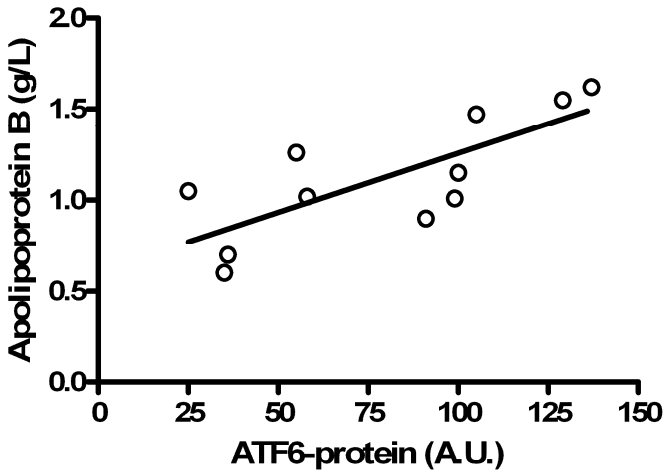
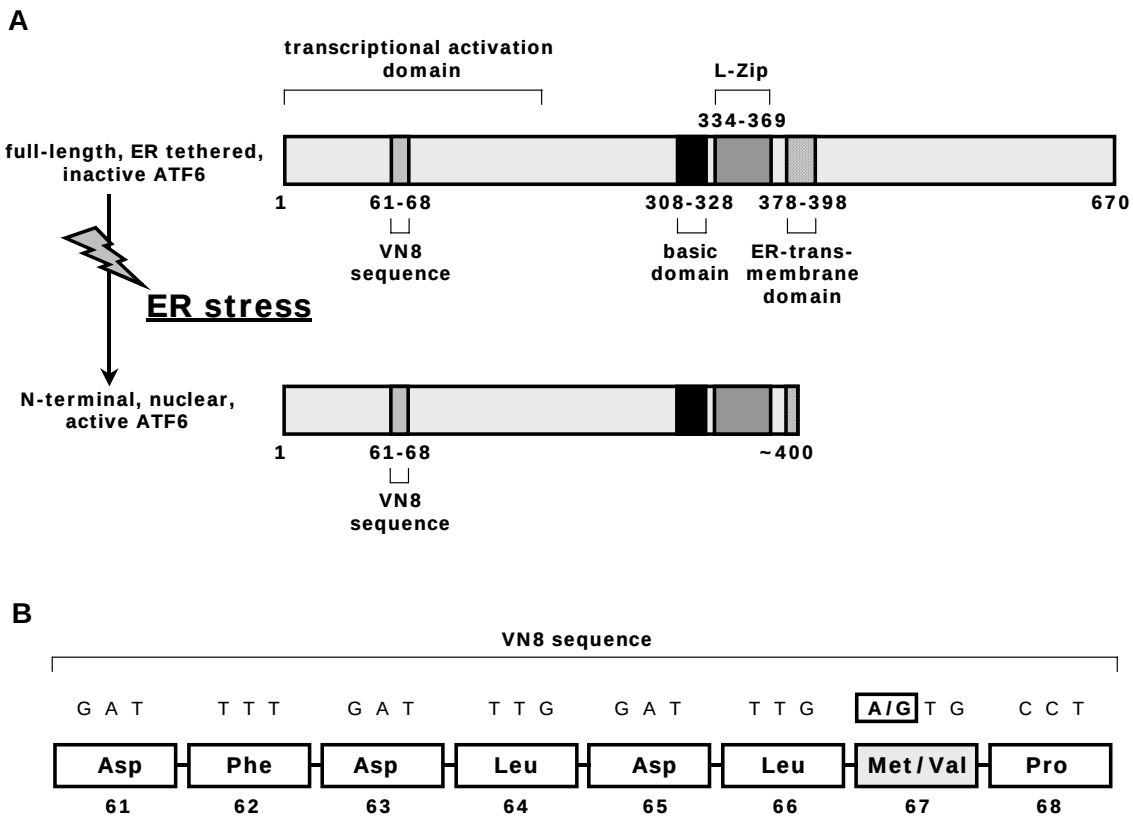


Figure S2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ER stress
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VN8 sequence
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Asp
Leu
Asp
Leu
Met/Val
Pro
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62
63
64
65
66
67
68