The American Journal of Human Genetics

Supplemental Data

Loss-of-Function Mutations in *APPL1* in Familial Diabetes Mellitus

Sabrina Prudente, Prapaporn Jungtrakoon, Antonella Marucci, Ornella Ludovico, Patinut Buranasupkajorn, Tommaso Mazza, Timothy Hastings, Teresa Milano, Eleonora Morini, Luana Mercuri, Diego Bailetti, Christine Mendonca, Federica Alberico, Giorgio Basile, Marta Romani, Elide Miccinilli, Antonio Pizzuti, Massimo Carella, Fabrizio Barbetti, Stefano Pascarella, Piero Marchetti, Vincenzo Trischitta, Rosa Di Paola, and Alessandro Doria

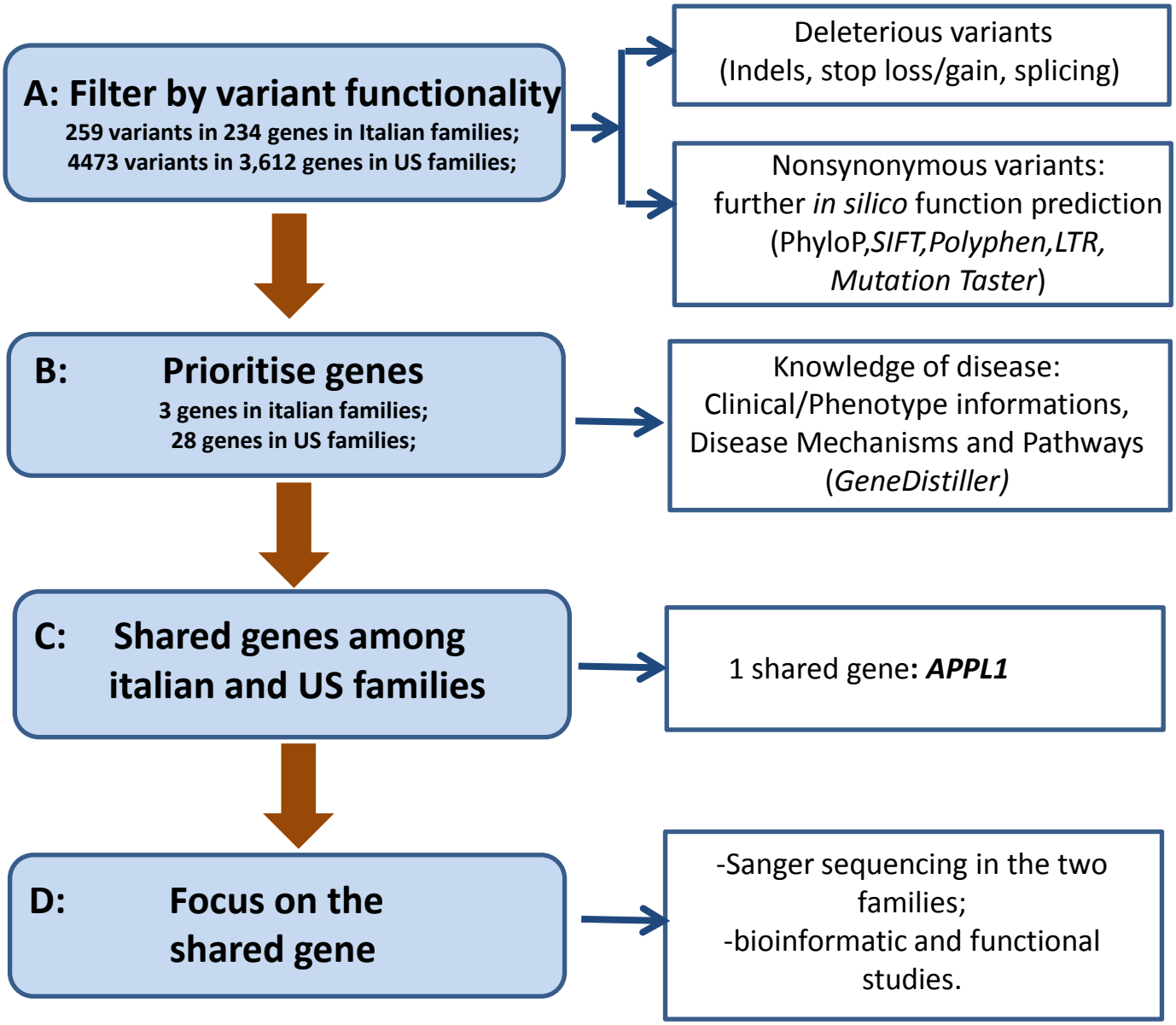

Figure S1. Strategy to filter and prioritize candidate variants in the Italian and US families.
Candidate variants were stratified through a mixed filtering/prioritization strategy taking into account the predicted impact of each variant and the functional relevance of individual genes with regard to the disease of interest (i.e. diabetes mellitus). In Step A, variants were filtered by functionality and only changes in residues conserved among species (as evaluated by Phylop) or predicted to be deleterious by at least of one of four different prediction algorithms (i.e., SIFT, PolyPhen2, LRT and MutationTaster) or "inapplicable" by any predictors were retained. Variants identified in Step A were prioritized in Step B on the basis of the functional relevance of the genes in which they occurred using "GeneDistiller" (Seelow D et al., PLoS ONE 2008; 3:e3874). Genes were ranked based on combinations of terms based on knowledge of disease (including clinical and phenotype information, disease mechanisms and pathways) including non-insulin dependent diabetes mellitus (NIDDM) maturity onset diabetes of the young (MODY), permanent neonatal diabetes mellitus (PNDM), insulin, glucose, pancreas and islets as keywords in the OMIM entries, and using similarity of expression patterns, protein-protein interactions, and specific tissues expression (i.e. liver, pancreatic islets, adipocytes and skeletal muscle) as major weights. By using these filtering criteria, 4 variants in 3 genes and 35 variants in 28 genes were prioritized in the Italian and the US sets, respectively. We focused the present study on the only gene (*APPL1)* that was shared by the two prioritization lists.

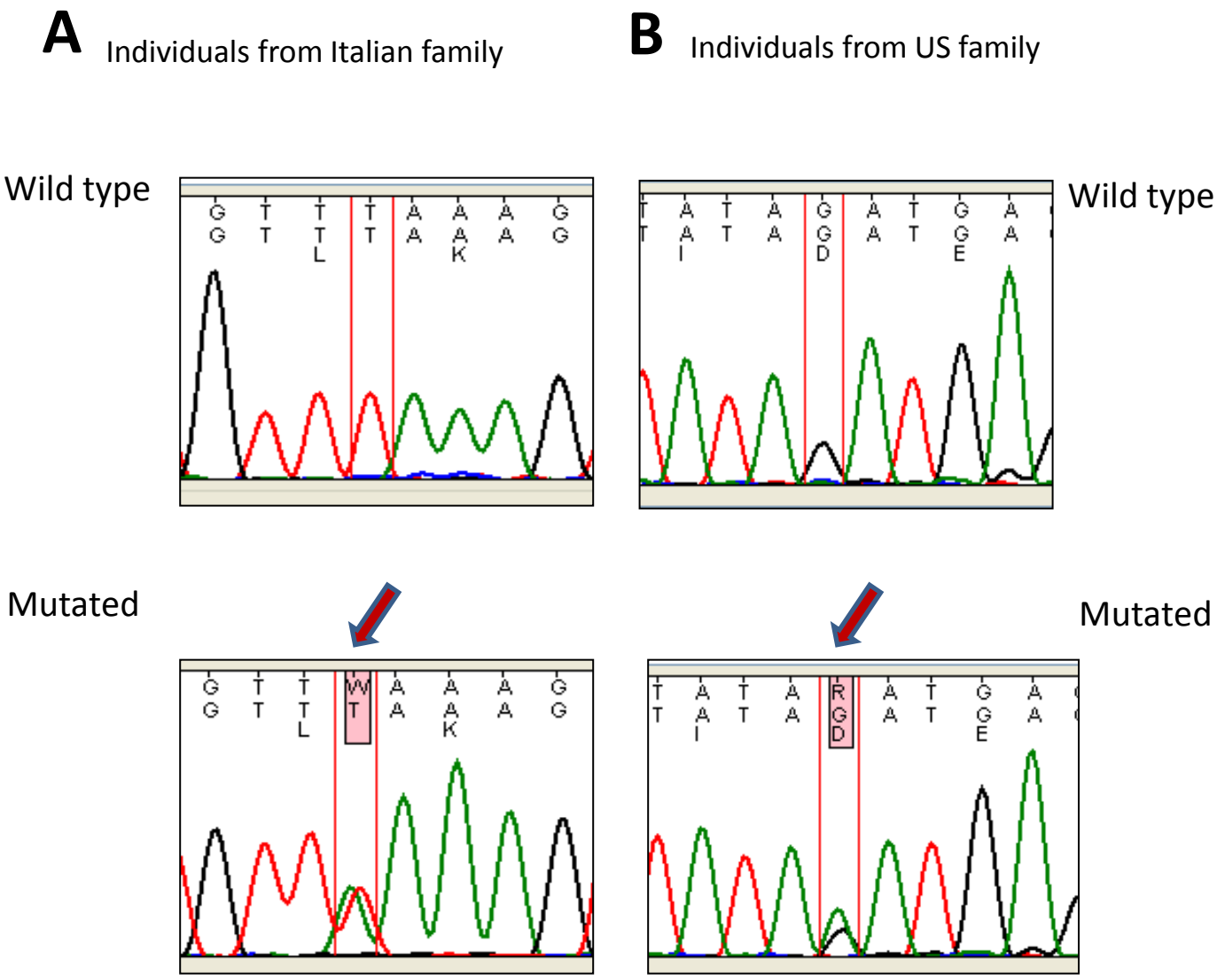

Figure S2. Electropherograms of the *APPL1* mutations identified in two families.

The panel A shows the heterozygous non-sense mutation c.1655T>A: p.Leu552* (marked with an arrow) in exon 17 found in the italian family.
The panel B shows the heterozygous missense mutation c.280G>A: p.Asp94Asn (marked with an arrow) in exon 4 found in the family from US.

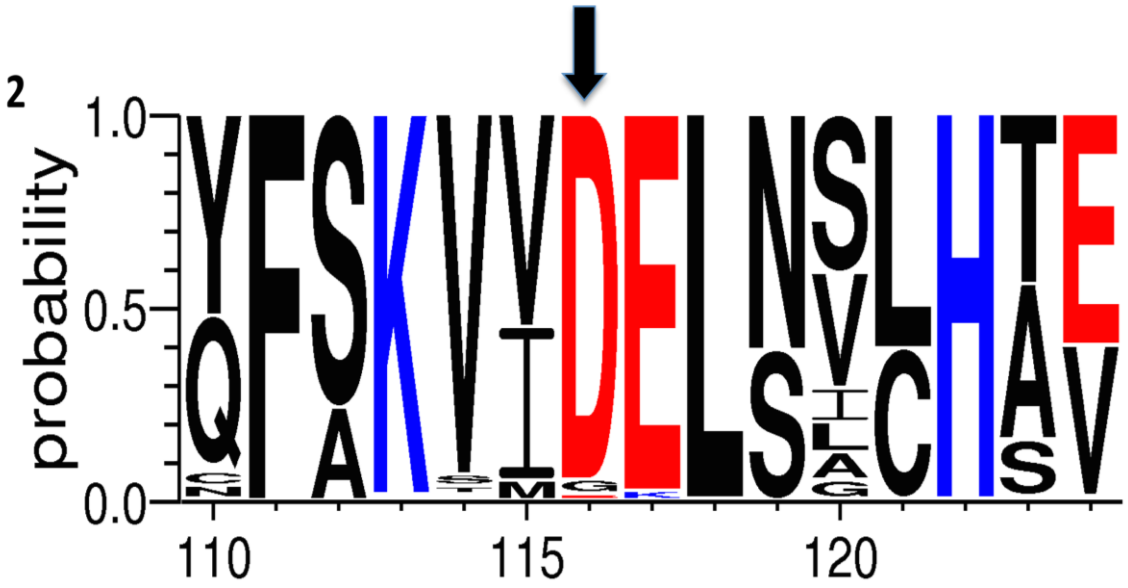

Figure S3. Aminoacid conservation in the APPL1 region encompassing Asp94

Multiple-sequence alignment of the APPL1 BAR domain from 244 homologous sequences was performed with the Clustal Omega sequence-alignment tool. Logo was calculated by means of the WebLogo service. The height of each amino acid one-letter code is proportional to the observed frequency of the residue at that position.
Numbering refers to the alignment.
Asp94 (indicated by the black arrow) appears to be highly conserved.

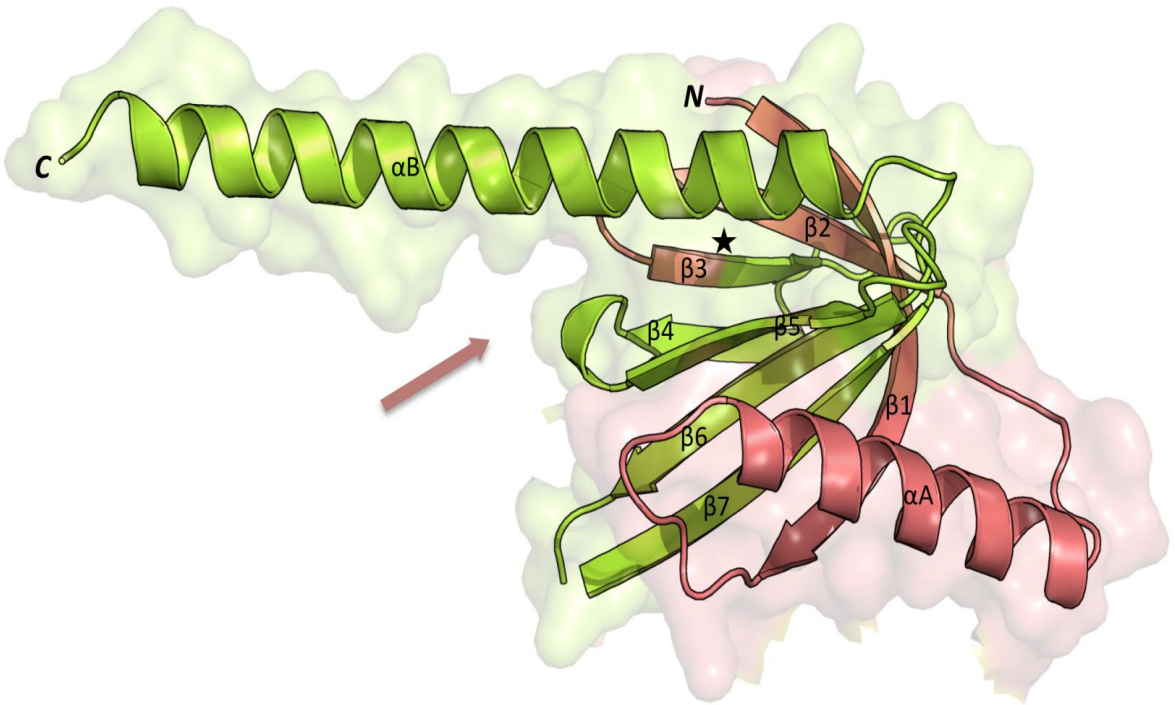

Figure S4. Structure of the PTB domain of human APPL1.

Phosphotyrosine binding domain (PTB) of human APPL1 (Protein Data Bank ID: 2ELA) is represented. The star denotes the position of Leu552 in the third β-sheet of PTB domain: the premature stop codon results in a deletion of the protein structure shown in green, while the region before the mutation is coloured in magenta. The arrow indicates the peptide-binding site (between β5 sheet and C-terminal helix) in most of the proteins containing PTB domain.

Table S1. Summary of whole-exome sequencing (WES) performance in US and Italian families

	Variants (N)	
	Italian families (n=8)	US families (n=52)
Individuals subjected to WES	24	104
All variants	250,174	453,415
Passing primary QC filters	158,695	365,984
Heterozygous	92,043	214,118
Novel,infrequent or rare (MAF <0.01)	14,726	133,742
Shared by two affected members	3,109	30,107
Nonsense, frameshift, missense, splicing	644	7,972

Table S2. Candidate disease genes obtained after prioritization in both the Italian and US families

Italian Families			US Families		
Gene	**Gene ID**	**Gene Distiller Overall Score**	**Gene**	**Gene ID**	**Gene Distiller Overall Score**
APPL1	26060	5898	*GCG*	2641	68431
CPT1A	1374	5548	*ADIPOQ*	9370	31117
TRIB3	57761	271	*MAPK3*	5595	26747
			SREBF1	6720	26410
			GLP1R	2740	24728
			PTPN1	5770	20433
			G6PC2	57818	20359
			PTPN11	5781	19198
			AKT2	208	16489
			FOXA1	3169	12469
			STXBP4	252983	11239
			PRKAA2	5663	8656
			TSC2	7241	7312
			ITPR3	3710	7277
			SORBS1	10580	6141
			APPL1	26060	5848
			MET	4233	4107
			RAPGEF4	11069	3845
			KL	9365	3362
			ANKRD26	22852	3063
			PRKAR1B	5575	2460
			PRKAG3	53632	2320
			MTOR	2474	236
			ADRA2	150	98
			WFS1	7466	89
			CACNAE1	777	41
			RFX6	222546	22
			ASPSCR1	79058	21

Genes prioritization has been carried out by «GeneDistiller» http://www.genedistiller.org

Table S3. Average clinical features of examined members from Italian and US families according to their genetic and glycemic status.

Family	Status	Gender (M/F)	Age	Age at diagnosis	BMI	FPG
Italian	Non affected subjects (non carriers)	1/7	38.5±8.9	-	26.2±4.4	85.6±8.9
	Non affected subjects (carriers)	5/5	32.8±7.6	-	28.2±4.6	81.1±8.8
	Affected subjects (carriers)	4/6	47.9±10.9	37.6±10.1	28.3±2.5	-
US	Non affected subjects (non carriers)	3/3	42.5±20.2	-	28.8±7.6	84.7±11.8
	Non affected subjects (carriers)	1	48.0	-	28.0	72.0
	Affected subjects* (carriers)	3/0	54.0±13.2	37.33±9.2	27.9±2.6	-

BMI: body mass index; FPG: fasting plasma glucose;
*this group comprises neither the patient with type 1 diabetes nor the one with type 2 diabetes who does not carry mutation.

Table S4. Primers and PCR conditions used for *APPL1* gene (NC_000003.12 57227737..57273471) resequencing

Primer Name	Primer sequence	Length	Ta	Product Size
APPL1-Ex1-F	ACGGTCCCCTGCGATTTAG	19	59	590
APPL1-Ex1-R	CCCAGCCTCCACAACTCC	18		
APPL1-Ex2-F	TTGGAAAATACCTCTGCTTTG	21	53	531
APPL1-Ex2-R	ATGCTTCAATCGTCTATCACTTT	23		
APPL1-Ex3-F	GGCAATAATCAGTCATCAACG	21	53	645
APPL1-Ex3-R	GCTCCAGTTCCTCCAATAACAC	22		
APPL1-Ex4-F	GCTGTGACTTGCTGAACATTAC	22	53	632
APPL1-Ex4-R	TCTTCTCTCCCTGCCTACATC	21		
APPL1-Ex5-F	TGTTCCTTGTGCTTTTAGTGTCA	23	53	474
APPL1-Ex5-R	TCCTATAAATGTTTGGTAACTGAGC	25		
APPL1-Ex6-F	CAGAGTAGCTTTTTCACCCTGA	22	63	579
APPL1-Ex6-R	GGGCAGCAGGATCACTACA	19		
APPL1-Ex7-F	TTCAAGCCACAATCATAGCATAC	23	53	702
APPL1-Ex7-R	TCAAAATACAAAGCAACAAAAGG	23		
APPL1-Ex8-F	TTCTAAAAGATCAGAAAACAGGAACA	26	53	496
APPL1-Ex8-R	TGCAAGGAAAACCATTCTTC	20		
APPL1-Ex9-F	TCCTTAATGGCTCCCTGTGCAGT	23	53	495
APPL1-Ex9-R	TCCCATCAAACTCCCACAATTTCA	24		
APPL1-Ex10-F	CCCATGCCGCTCTTCCTCTC	20	68	524
APPL1-Ex10-R	ATGCCCAGCCCTGGTTTCAC	20		
APPL1-Ex11-F	ATTTCCAGCCTTGTTTTGG	19	53	671
APPL1-Ex11-R	AGGATTTTCAGTTCCAGTTCC	21		
APPL1-Ex12-F	TGAGGATCAGCACCTGTTCCCTT	23	53	572
APPL1-Ex12-R	CAGCCTGCCAGACAGCAACA	20		
APPL1-Ex13-F	TGCAGTTGAGTATTGAACATTTGCCT	20	64	534
APPL1-Ex13-R	TCATGGCACATTAAAGCTACTCATTCA	20		
APPL1-Ex14-15-F	CAACACTTGCAGGGAATTTT	20	53	811
APPL1-Ex14-15-R	GAAATGCAGACAGGGGATTA	20		
APPL1-Ex16-F	AGGATGGGGCATTTTAGAGC	20	63	560
APPL1-Ex16-R	CACACAAACACACAACAACTGG	22		
APPL1-Ex17-18-F	GAAAACCTAAAAGGAGGCAGTG	22	53	554
APPL1-Ex17-18-R	CAGCCAAAGACCAATCATAGC	21		
APPL1-Ex19-F	TTCAACAACCTTTTTCTGCTTG	22	53	558
APPL1-Ex19-R	TTGGATGATGGACACCCTAAA	21		
APPL1-Ex20-F	AGGTGAGAGTCAGCAGTGGAA	21	64	479
APPL1-Ex20-R	CAAGTCATTCTCGTGCCTCA	20		
APPL1-Ex21-F	GATGCTTGATTTTCCAAGAATG	22	53	500
APPL1-Ex21-R	TCTGTTTATGAAGTGGCTGAAC	22		
APPL1-Ex22-F	TGGTGTTGTATTGTTTGGGTGT	22	53	583
APPL1-Ex22-R	GAGCAAATGTAATTCCTCAGCA	22		

Table S5. Software prediction algorithms used to assess the Asp94Asn mutation effect on APPL1 protein

Software prediction algorithm	Score	Effect predicted
Sequence based		
SIFT	0.37(cutoff = 0.05)	Neutral
Polyphen-2	0.61(scale 0 to 1)	Possibly damaging
Panther	0.66 (scale 0 to 1)	Deleterious
Structure based		
CUPSAT	$\Delta\Delta G$=- 0.22 kcal/mol	Overall stability = Destabilising Torsion angles = Unfavourable
I-Mutant 2.0	$\Delta\Delta G$=- 1.30 kcal/mol	Decrease stability
MUPRO	Confidence score = -0.96 (scale -1 to 1, -1 = max. decrease stability)	Decrease stability

Table S6. APPL1 mRNA expression levels in transfected HepG2 cells.

	APPL1 expression levels ($2^{-\Delta\Delta CT}$ ± sd)
HepG2 EV	1
HepG2 APPL1	1048 ± 173
HepG2 APPL1_X552	1003 ± 108
HepG2 APPL1_ASN94	1135 ± 144

HepG2 EV: HepG2 cells transiently transfected with control empty vector.
HepG2 APPL1: HepG2 cells transiently transfected with pCMV6-Entry APPL1 myc tagged cDNA.
HepG2 APPL1_X552: HepG2 cells transiently transfected with APPL1 cDNA carrying X552 mutation.
HepG2 APPL1_ASN94: HepG2 cells transiently transfected with APPL1 cDNA carrying ASN94 mutation.
RNA was isolated by using RNeasy Mini kit (Qiagen S.r.l., Milan, Italy), cDNA generated by reverse transcription with iScript Reverse Transcription (Biorad, Hercules, CA) according to the manufacturer's instructions and used as template in the subsequent analyses. Prime Time Std qPCR Assays (IDT, Iowa USA) were used to quantify relative gene expression levels of APPL1, GAPDH, B actin and 18S on ABI-PRISM 7900 (Applera Life Technologies, Carlsbad, CA). Expression levels of APPL1accross different experimental conditions were calculated by using the comparative ΔCT method. Briefly, the amount of APPL1 was normalized to the geometric mean of GAPDH, B actin and 18S expression and related to APPL1 expression in HepG2 EV control cells ($2^{-\Delta\Delta CT}$). Data are presented as $2^{-\Delta\Delta CT}$ ± sd.