SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS AND FIGURES

PROTEIN MODELING

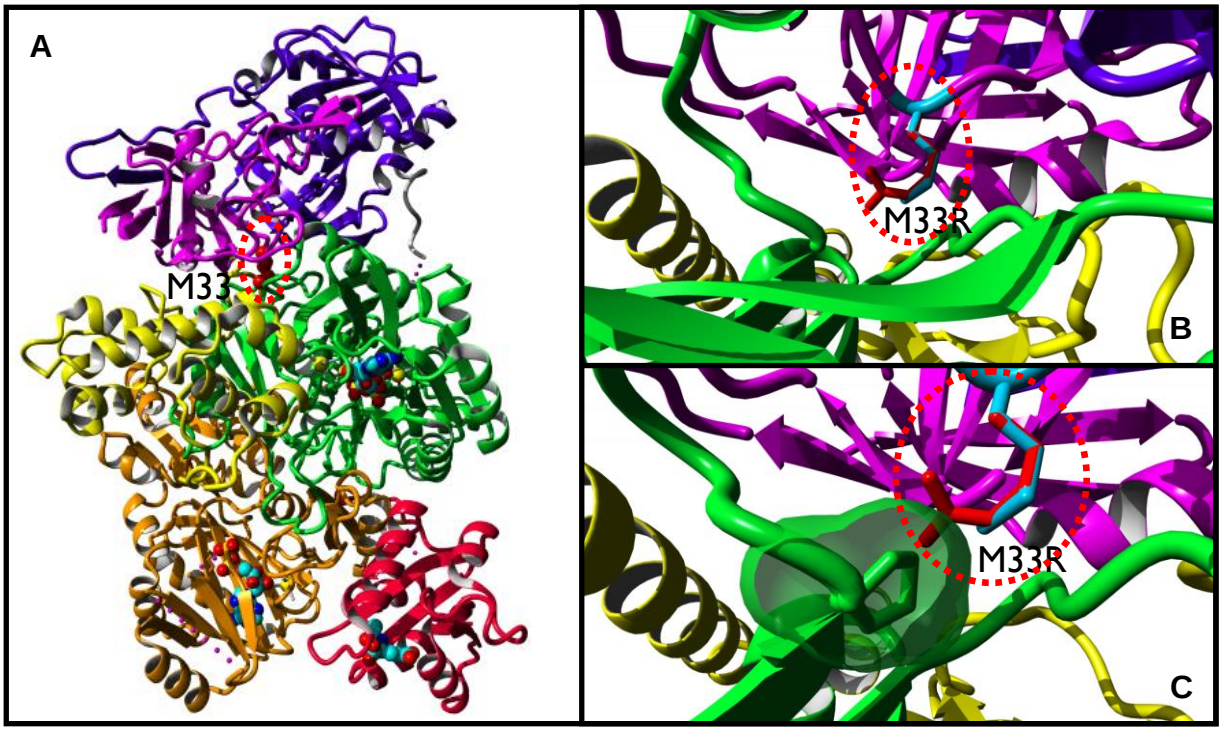
Method

To further evaluate the molecular pathology of the homozygous p.Met33Arg missense mutation in CAD, we modeled the effects of the amino acid substitution using the crystal structure of human CPS1 (Protein Data Bank [PDB] accession number 5DOU, (de Cima *et al.* , 2015)) as a template. The model and template sequence showed 51% sequence identity over 1458 residues. Modeling and subsequent analysis was done using the WHAT IF & YASARA Twinset with standard parameters (Krieger *et al.* , 2002, Vriend, 1990).

Result

The p.Met33Arg mutation affects the glutamine amidotransferase portion of the CPS2 domain of CAD. In the predicted three-dimensional structure of the CPS2 domain, Met33 is located at the interface between subdomains S1, L1 and L2 (Supplementary Figure 1A). A close-up of this position shows a hydrophobic pocket in which the Met33 residue is embedded. Substitution to a positively charged arginine would disrupt the hydrophobic character of this conformation. Additionally, arginine would be sterically unfavorable because of the increased size of its side chain, which is predicted to penetrate the electron-dense surface of an adjacent proline (amino acid 636 of CAD) of the L1 subdomain (Supplementary Figure 1B, C). Taking these effects into account, we hypothesize that the p.Met33Arg mutation destabilizes

subdomain interactions within the CPS2 moiety of CAD, which likely results in inadequate enzymatic function due to altered tertiary protein configuration.



Supplementary Figure 1

Structural impact of the p.Met33Arg change (families I and III) on the Glutamine Amidotransferase/Carbamoyl-phosphate synthase domain (CPS2) of human CAD as predicted by protein modeling.

(A) Overview of predicted CPS2 ligand-bound structure, with individual subdomains shown in different colors (pink: S1 domain, purple: S2 domain, green: L1 domain, yellow: L2 domain, orange: L3 domain and red: L4 domain, as described by de Cima *et al.*). The position of p.Met33 on the interface between S1, L1 and L2 is depicted in red.

(B) Visualization of p.Met33 (blue) change to Arg (red).

(C) Further zoom-in on p.Met33 (blue) to Arg (red) substitution, showing electron density surface of adjacent Pro in green.

NUCLEOTIDE SUGAR ANALYSIS

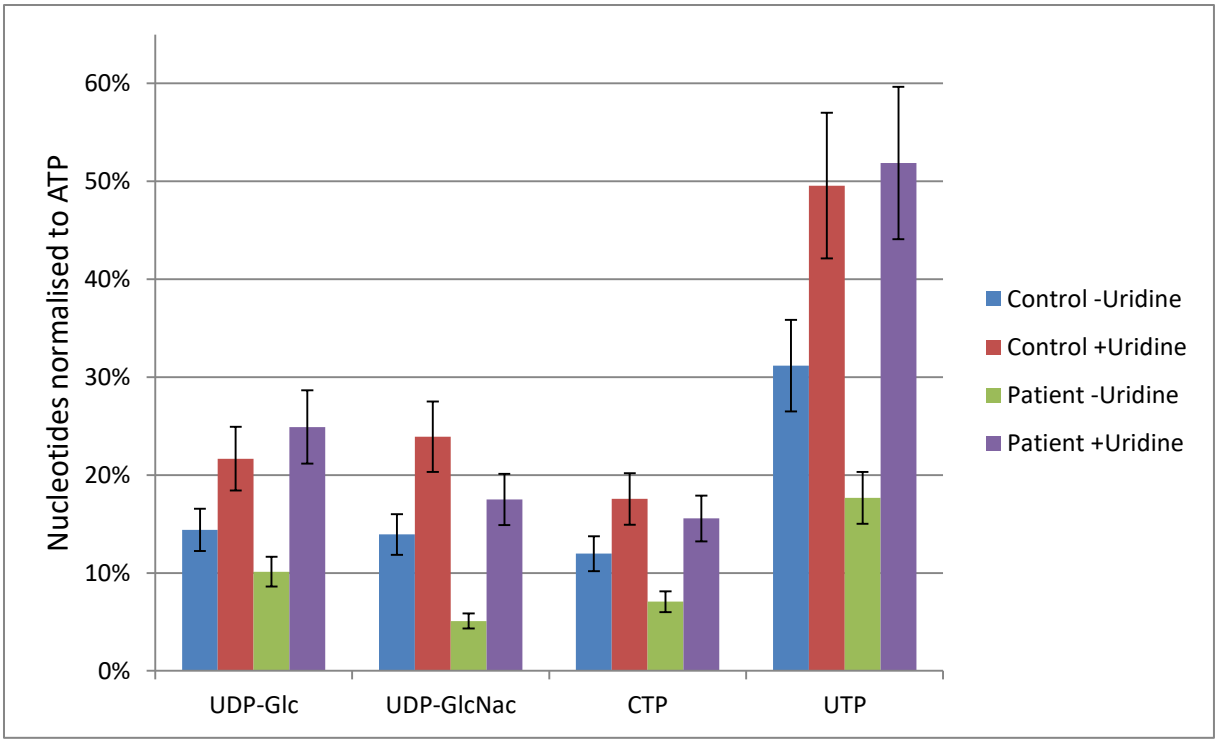
Method

Ion-pairing reversed-phased ultra-performance liquid chromatography analysis of nucleotide sugars from fibroblasts was performed as described previously (Kevelam *et al.* , 2015). In brief, skin fibroblasts of patient F1:II.2 were cultured in AMNIOMAX II medium with and without supplementation with 2 µmol/l (50 µg/ml) uridine. Cells were harvested on the first day of confluence following trypsinization and washed once with phosphate-buffered saline. The cell pellet was resolved in 200 µl 0.4 M ice-cold perchloric acid and incubated in melting ice for 10 minutes. Subsequently, the lysate was centrifuged and the supernatant was neutralized by adding 8 µl 5M K_2CO_3 incubated for 10 minutes in melting ice and centrifuged. The protein pellet was dissolved in 0.2 M NaOH overnight at room temperature and used for protein determination. The supernatant was stored at -80℃ until analysis. Prior to analysis the supernatant was thawed and cleared using a SpinX centrifuge filter.

Nucleotides were quantified using an ion-pairing reversed-phased ultra-performance liquid chromatography method on a LC-18 column and a buffer system consisting of 30 mM KH2PO4 and 10 mM tributylsulfononium in methanol. Detection was done using UV-detection and a single point calibration was used.

Result

Ion-pairing reversed-phased ultra-performance liquid chromatography analysis of nucleotide sugars showed a reduced levels of UDP, UDP-glucose (UDP-glc), UDP-N-acetylglucosamine (UDP-GlcNac), CTP, and UTP in patient’s cells compared to control cells. Uridine supplementation restored all reduced metabolites in patient’s fibroblast to control levels (Supplementary Figure 2).



Supplementary Figure 2

Sugar nucleotide and uridine treatment.

Levels of sugar nucleotides were found decreased in patient's fibroblasts compared to a control cell line but were restored to normal upon uridine supplementation.

Web Resources

The URLs for data presented herein are as follows:

Protein Data Bank (PDB, *http://www.rcsb.org/pdb/home/home.do*)

References

de Cima S, Polo LM, Diez-Fernandez C, Martinez AI, Cervera J, Fita I, et al. Structure of human carbamoyl phosphate synthetase: deciphering the on/off switch of human ureagenesis. Scientific reports. 2015;5:16950.

Krieger E, Koraimann G, Vriend G. Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. Proteins. 2002 May 15;47(3):393-402.

Vriend G. WHAT IF: a molecular modeling and drug design program. Journal of molecular graphics. 1990 Mar;8(1):52-6, 29.

Kevelam SH, Bierau J, Salvarinova R, Agrawal S, Honzik T, Visser D, et al. Recessive ITPA mutations cause an early infantile encephalopathy. Annals of neurology. 2015 Oct;78(4):649-58.

SUPPLEMENTARY TABLES

Supplementary Table 1. Exome sequencing statistics

ID	Pedigree	Sex	libpair	type	Duplicates	Reads	Mapped	Mapped (%)	Seq (Gb)	Avg cov (exome)	Cov 8x	Cov 20x
F1:II.2	ExDiag0134	male	paired-end	SureSelect50Mbv5	8.00	101290786	100640334	99.36	10.230	124.57	99.46	97.83
F1:II.3	ExDiag0134	female	paired-end	SureSelect50Mbv5	11.00	135951288	135756030	99.86	13.730	170.86	99.55	98.67
F2:II.2	ExDiag0671	female	paired-end	SureSelect50Mbv5	9.00	142700042	141625529	99.25	14.340	164.38	99.62	98.91
F3:II.4	ExDiag0227	male	paired-end	SureSelect50Mbv5	11.00	103999500	103869071	99.87	10.500	131.96	99.31	97.33

Supplementary Table 2. Annotation of identified *CAD* mutations.

Chr (hg19)	Gene symbol	Function	pph2	Sift	Cadd	dbSNP 142	1000 genomes AF	Allel count ExAC (het/total)	Depth	%Var	UCSC Transcripts
chr2:27440760-27440760	*CAD*	missense	probably damaging(1)	0	31		0	1 /116914	121	100	uc002rji.3,CAD, c.98T>G, p.Met33Arg; uc010eyw.3,CAD, c.98T>G, p.Met33Arg:
chr2:27448996-27448996	*CAD*	splice site			16.65		0	0	61	44	uc002rji.3,CAD, C>T,near splicesite: uc010eyw.3,CAD, intronic,C>T:
chr2:27462310-27462310	*CAD*	nonsense		1	38		0	1 /120886	118	50	uc002rji.3,CAD, c.5365C>T, p.Arg1789Stop: uc010eyw.3,CAD, c.5176C>T, p.Arg1726*