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A patient with homozygous nonsense variants in two Leigh syndrome disease genes: distinguishing a dual diagnosis from a hypomorphic protein-truncating variant

Nicole J. Lake[1,2,13], **Luke E. Formosa**[3], **David A. Stroud**[4], **Michael T. Ryan**[3], **Sarah E. Calvo**[5,6,7], **Vamsi K. Mootha**[5,6,7], **Bharti Morar**[8,9], **Peter G Procopis**[10,11], **John Christodoulou**[1,2,11,12], **Alison G. Compton**[1,2], **David R. Thorburn**[1,2,12]

[1]Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, VIC 3052, Australia

[2]Department of Paediatrics, University of Melbourne, Melbourne, VIC 3052, Australia

[3]Department of Biochemistry and Molecular Biology, Monash Biomedicine Discovery Institute, Monash University, Clayton Campus, Melbourne, VIC, 3800, Australia

[4]Department of Biochemistry and Molecular Biology and The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria 3010, Australia

[5]Howard Hughes Medical Institute, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA

[6]Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA

[7]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

[8]Centre for Clinical Research in Neuropsychiatry, School of Psychiatry and Clinical Neurosciences, University of Western Australia, Perth, Australia

[9]Harry Perkins Institute of Medical Research and Centre for Medical Research, University of Western Australia, Nedlands, Western Australia 6009, Australia

[10]Department of Neurology, Children's Hospital at Westmead, Sydney, NSW 2145, Australia

[11]Discipline of Child and Adolescent Health, Sydney Medical School, University of Sydney, Sydney, NSW 2006, Australia

[12]Victorian Clinical Genetic Services, Royal Children's Hospital, Melbourne, VIC 3052, Australia

[13]Now at Department of Genetics, Yale School of Medicine, New Haven, CT 06519

Abstract

Leigh syndrome is a mitochondrial disease caused by pathogenic variants in over 85 genes. Whole exome sequencing of a patient with Leigh-like syndrome identified homozygous protein-

CORRESPONDING AUTHOR Prof. David Thorburn, Murdoch Children's Research Institute, Royal Children's Hospital, 50 Flemington Rd, Parkville, Melbourne, VIC 3052, Australia, david.thorburn@mcri.edu.au.

DISCLOSURE

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truncating variants in two genes associated with Leigh syndrome; a reported pathogenic variant in *PDHX* (NP_003468.2:p.(Arg446*)), and an uncharacterized variant in complex I (CI) assembly factor *TIMMDC1* (NP_057673.2:p.(Arg225*)). The *TIMMDC1* variant was predicted to truncate 61 amino acids at the C-terminus and functional studies demonstrated a hypomorphic impact of the variant on CI assembly. However, the mutant protein could still rescue CI assembly in *TIMMDC1* knockout cells and the patient's clinical phenotype was not clearly distinct from that of other patients with the same *PDHX* defect. Our data suggest that the hypomorphic effect of the *TIMMDC1* protein-truncating variant does not constitute a dual diagnosis in this individual. We recommend cautious assessment of variants in the C-terminus of TIMMDC1 and emphasize the need to consider the caveats detailed within the American College of Medical Genetics and Genomics (ACMG) criteria when assessing variants.

Keywords

TIMMDC1; Leigh syndrome; complex I; protein truncation; ACMG guidelines

The American College of Medical Genetics and Genomics (ACMG) guidelines are a widely recognized framework for variant interpretation (Richards et al., 2015). A predicted null variant can be classified as pathogenic or likely pathogenic by the ACMG criteria in the absence of functional data if it is (a) in a gene where loss-of-function (LoF) is an established mechanism of disease (PVS1), (b) present at extremely low frequency in controls (PM2), and (c) predicted to be pathogenic by multiple *in silico* analyses (PP3). However, caution is advised for the application of criterion PVS1 to nonsense variants predicted to escape nonsense-mediated decay (NMD) according to the '50-bp rule' (Nagy & Maquat, 1998). This caveat is not listed in the table detailing the criteria, or within the ClinGen Pathogenicity Calculator used to apply the ACMG guidelines (Patel et al., 2017), but rather relies on the assessor being familiar with the caveats for each criterion.

Leigh syndrome (MIM# 256000) is the most common pediatric presentation of mitochondrial disease (Lake, Compton, Rahman, & Thorburn, 2016). It can be caused by mutations in over 85 genes encoding proteins required for the activity of either oxidative phosphorylation (OXPHOS) or pyruvate dehydrogenase (PDH) complexes (Lake et al., 2016; J. Rahman, Noronha, Thiele, & Rahman, 2017). Here we report a patient diagnosed with Leigh-like syndrome, characterized by persistent lactic acidosis, seizures, spastic quadriplegia, and neuroradiological abnormalities (Supp. Table S1). Whole exome sequencing identified homozygous nonsense variants in two genes, *PDHX* and *TIMMDC1*, where recessive LoF mutations are a known cause of Leigh syndrome (Ivanov et al., 2014; Kremer et al., 2017). These were a reported pathogenic variant in PDH subunit *PDHX* (GRCh37 chr11:35016549C>T; NM_003477.2:c.1336C>T; NP_003468.2: p.(Arg446*)) (Ivanov et al., 2014) and an uncharacterized variant in OXPHOS complex I (CI) assembly factor *TIMMDC1* (GRCh37 chr3:119236128C>T; NM_016589.3:c.673C>T; NP_057673.2:p.(Arg225*)). The *TIMMDC1* p.(Arg225*) mutation was a strong candidate disease-causing variant that could potentially be classified as pathogenic or likely pathogenic by the ACMG guidelines (PVS1, PM2 and PP3). Whole exome sequencing of cohorts has shown that up to 7% of patients may have dual or blended phenotypes caused by mutations

in two or more genes (Balci et al., 2017). Cognizant therefore of the possibility of our patient having two monogenic disorders, we performed functional studies to determine if the *TIMMDC1* p.(Arg225*) variant had a damaging effect on protein function.

The p.(Arg446*) variant in *PDHX* is a founder mutation reported to cause PDH deficiency and Leigh syndrome in the European Roma population (Ivanov et al., 2014). The variant was confirmed in the patient by Sanger sequencing (Supp. Figure S1a). Parental samples were unavailable. A dipstick enzyme assay revealed that PDH activity in patient fibroblasts was reduced by 95% relative to the mean control measurement (Figure 1a), confirming PDH deficiency. Screening of the *TIMMDC1* c.673C>T variant in 16 reported individuals homozygous for the *PDHX* p.(Arg446*) variant and of Roma ancestry (Ivanov et al., 2014) revealed none had the *TIMMDC1* variant.

The *TIMMDC1* c.673C>T, p.(Arg225*) nonsense variant causes C-terminal truncation of 61 amino acids. The variant was confirmed in the patient by Sanger sequencing (Supp. Figure S1b) and is reported in the Genome Aggregation Database (gnomAD) (2 of 246,016 alleles examined, no homozygotes) and dbSNP (rs149481081). The variant has been submitted to ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) under accession number SCV000778864. Although the truncated protein encoded by the *TIMMDC1* p.(Arg225*) variant would retain its conserved transmembrane domains, most of the C-terminal mitochondrial matrix domain is lost, including many conserved residues (Figure 1b and Supp. Figure S2). The *TIMMDC1* p.(Arg225*) variant was predicted as ‘Disease Causing’ by MutationTaster (probability value 0.999) (Schwarz, Cooper, Schuelke, & Seelow, 2014), in the top 0.1% most deleterious substitutions by the Combined Annotation Dependent Depletion (CADD) tool (PHRED score 35) (Kircher et al., 2014), and as a recessive disease-causing variant by the Annotation of Loss-of-Function Transcripts (ALoFT) method (Recessive score 0.72) (Balasubramanian et al., 2017). Since the *TIMMDC1* c.673C>T variant lies in the penultimate exon and <50 bps from the last exon junction, the mutant transcript is predicted to escape NMD (Nagy & Maquat, 1998). Analysis of PCR products synthesized from control and patient fibroblast cDNA confirmed the stability of the *TIMMDC1* c.673C>T transcript (Supp. Figure S3). Immunoblotting of fibroblast lysates using an antibody recognizing a C-terminal region of TIMMDC1 showed no detectable protein, while an antibody recognizing an N-terminal region of TIMMDC1 revealed that the truncated p.(Arg225*) protein is stable (Figure 1c).

Since TIMMDC1 is a CI assembly factor (Sanchez-Caballero, Guerrero-Castillo, & Nijtmans, 2016), we sought to determine whether the C-terminal truncation caused defective CI assembly. The steady-state level of assembled CI in fibroblasts from the patient was mildly reduced relative to control, as demonstrated by BN-PAGE (Figure 2a). A dipstick enzyme assay revealed partial (35%) reduction of CI activity (relative to complex IV activity) in patient fibroblasts, relative to the average of three controls (Figure 1a). Spectrophotometric analysis of OXPHOS enzymes in a pathology-certified diagnostic laboratory found that CI activity in patient fibroblasts was 33% lower than the control mean relative to citrate synthase (31% lower than control mean relative to complex IV), however this value was within the control reference range determined from thirty-five controls (Supp. Table S2). Immunoblotting of several CI subunits and assembly factors demonstrated that

most were present in the patient fibroblasts at levels comparable to control, although subunits NDUFS5 and MT-ND1 were present at approximately half the level of control (Figure 2b and Supp. Figure S4). Finally, complementation studies were performed using *TIMMDC1* knockout HEK293T cells to determine if the truncated TIMMDC1 protein could facilitate CI assembly. Remarkably, overexpression of the TIMMDC1 p.(Arg225*) mutant or wild-type protein in *TIMMDC1* knockout cells showed comparable restoration of CI assembly (Figure 2c).

To determine if the clinical presentation of our patient was consistent with that observed for the homozygous *PDHX* p.(Arg446*) mutation, we compared the clinical features against the 23 published cases (Supp. Table S1). This revealed that the patient's age of onset, survival and clinical features were consistent with the reported patients, and thus not suggestive of a blended phenotype resulting from an additive effect of two monogenic disorders. The consistency of clinical features, in combination with our functional data, mean that we found no reason to conclude that the *TIMMDC1* p.(Arg225*) variant is contributing significantly to the patient's phenotype. Closer examination of *TIMMDC1* variants reported in gnomAD revealed that 50% of all nonsense and frameshift variants lay downstream of the p.(Arg225*) variant in the last 20% of the coding region (Supp. Table S3 and Figure 1b), implying that the C-terminus is more tolerant of these variant types at a population level.

Pathogenic variants in *TIMMDC1* have only recently been reported, where a homozygous intronic splicing variant resulting in LoF (c.596+2146A>G; p.(Gly199_Thr200ins5*)) was identified in three unrelated individuals with CI deficiency and early-onset encephalopathy, including one patient diagnosed with Leigh syndrome (Kremer et al., 2017). Here we report the characterization of a nonsense variant in *TIMMDC1* (c.673C>T; p.(Arg225*)). In the absence of functional data, there were multiple lines of evidence supporting the prediction of pathogenicity of the *TIMMDC1* p.(Arg225*) variant, which could have enabled a classification of pathogenic or likely pathogenic by the ACMG criteria (PVS1, PM2 and PP3) if supported by functional studies. Our functional analyses of the *TIMMDC1* variant demonstrate the need for caution in selecting appropriate functional tests for validation of protein truncating variants. For example, immunoblotting with two different antibodies gave markedly different results; if it had not been recognised that one was raised to a C-terminal epitope, the results in Figure 1C could have been interpreted as providing support for loss of function, substantiating application of the PVS1 criterion.

Overall, our functional studies suggest the *TIMMDC1* p.(Arg255*) variant is a hypomorphic allele. Immunoblotting data showed that C-terminal truncation of TIMMDC1 may slow the incorporation of subunits NDUFS5 and MT-ND1 into CI, consistent with the proposed role of TIMMDC1 in CI assembly (Guarani et al., 2014; Sanchez-Caballero et al., 2016); however, this appeared to have little overall impact on CI assembly. Levels of assembled CI were only mildly reduced in patient fibroblasts relative to control, in contrast to the near complete loss of assembled CI seen in fibroblasts from the reported patients with pathogenic *TIMMDC1* variants (Kremer et al., 2017). Furthermore, CI activity in patient fibroblasts measured by diagnostic spectrophotometric enzyme assays was within the control reference range.

Since only fibroblasts were available from the patient, we cannot exclude the possibility that the *TIMMDC1* mutation has a tissue-specific impact on CI. However, the identification of the homozygous *PDHX* p.(Arg446*) variant and PDH deficiency in the patient appears to adequately explain all aspects of the clinical presentation, given that the age of onset, disease course and neuroradiological abnormalities are consistent with those observed in reported patients. The truncated TIMMDC1 protein retains all four conserved transmembrane domains, which could explain the hypomorphic nature of the p.(Arg225*) variant. The relative dispensability of TIMMDC1's C-terminus may be further supported by the observation that the C-terminus of TIMMDC1 is enriched with protein-truncating variants at a population level. Although nonsense mutations are often referred to as LoF or null variants, this study supports the term 'protein-truncating' variant being a more accurate description (Rivas et al., 2015).

In finalizing our classification of the *TIMMDC1* p.(Arg225*) variant, we noted that the homozygous *PDHX* mutation provides an alternate molecular basis for disease given the clinical phenotype was indistinguishable from other patients with the homozygous *PDHX* p.(Arg446*) variant, satisfying BP5 criterion. Patient fibroblasts showed relatively minor impact on CI enzyme activity and CI assembly compared to findings in fibroblasts from patients with pathogenic *TIMMDC1* variants. Together with the rescue results of CI assembly in *TIMMDC1* knockout cells by overexpression of the mutant p.(Arg225*) protein, it could be argued that these data warrant a strong benign support (BS3) criterion for functional studies in the ACMG guidelines. This would result in an overall classification of likely benign. However, on balance we took the more conservative view that the functional data provide only moderate rather than strong support for benign impact, in acknowledgement that there is evidence of a hypomorphic impact, that an overexpression system may not wholly reflect endogenous expression, and that a tissue-specific effect cannot be excluded. The presence of both benign and pathogenic evidence (BS3 downgraded to moderate weight, BP5, PM2, and PP3) support a classification of the *TIMMDC1* p.(Arg225*) variant as being of uncertain significance.

Discussion between molecular diagnostic laboratories on the ACMG criteria led to several suggestions for modification, including that criteria PVS1 should not be applied to variants that are predicted to escape NMD (Amendola et al., 2016). Our study emphasizes that cautious assessment of nonsense variants predicted to escape NMD is warranted, and highlights the importance of functionally characterizing a novel variant prior to concluding pathogenicity. It also demonstrates that different functional studies can provide different levels of evidence for and against pathogenicity. It has been suggested that most rare missense variants are deleterious in humans (Kryukov, Pennacchio, & Sunyaev, 2007), however, most do not appear to be causing severe, penetrant monogenic disease. Hence it is not surprising that for hypomorphic variants, whether missense, protein-truncating or affecting splicing, *in vitro* functional data will not necessarily provide clear distinction between a pathogenic or benign clinical impact. We therefore recommend caution when assessing putative pathogenic variants in the C-terminus of TIMMDC1. Given that up to 7% of patients undergoing exome sequencing are thought to have two monogenic disorders (Balci et al., 2017), careful assessment of variant pathogenicity where multiple putative genetic diagnoses are identified is warranted, as misclassification can impact patient

management, genetic counseling, prenatal diagnosis, and the diagnosis of subsequent unrelated patients.

Finally, it is well known that mitochondrial disorders often show variability in age of onset, survival and the range of symptoms. This occurs even in affected siblings and patients with homozygous founder mutations, as seen in patients with homozygous mutations in *PDHX*, *PET100, POLG* and *SURF1* (Ivanov et al., 2014; Lim et al., 2014; Neeve et al., 2012; Wedatilake et al., 2013). Some of this variability can be attributed to environmental factors such as intercurrent infections or exposure to drugs such as valproate or aminoglycosides (Parikh et al., 2017; Wedatilake et al., 2013). Genetic and epigenetic modifiers will also contribute to clinical variability of mitochondrial disorders but it is difficult to identify modifier genes in rare diseases (Neeve et al., 2012). It is possible that the presence of hypomorphic variants such as the *TIMMDC1* p.(Arg225*) variant could have a negative or positive modifier effect that contributes to clinical variability in patients with mitochondrial disease. However, the absence of the *TIMMDC1* p.(Arg225*) variant in 16 of 16 other reported *PDHX* patients and the comparable clinical features in our patient compared to them suggest that this variant is unlikely to contribute substantially to clinical variability of *PDHX* disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Amendola LM, Jarvik GP, Leo MC, McLaughlin HM, Akkari Y, Amaral MD, … Rehm HL (2016). Performance of ACMG-AMP Variant-Interpretation Guidelines among Nine Laboratories in the Clinical Sequencing Exploratory Research Consortium. American Journal of Human Genetics, 99(1), 247. doi:10.1016/j.ajhg.2016.06.001 [PubMed: 27392081]

Balasubramanian S, Fu Y, Pawashe M, McGillivray P, Jin M, Liu J, … Gerstein M (2017). Using ALoFT to determine the impact of putative loss-of-function variants in protein-coding genes. Nature Communications, 8(1), 382. doi:10.1038/s41467-017-00443-5

Balci TB, Hartley T, Xi Y, Dyment DA, Beaulieu CL, Bernier FP, … Boycott KM (2017). Debunking Occam's razor: Diagnosing multiple genetic diseases in families by whole-exome sequencing. Clinical Genetics, 92(3), 281–289. doi:10.1111/cge.12987 [PubMed: 28170084]

Guarani V, Paulo J, Zhai B, Huttlin EL, Gygi SP, & Harper JW (2014). TIMMDC1/C3orf1 functions as a membrane-embedded mitochondrial complex I assembly factor through association with the MCIA complex. Molecular and Cellular Biology, 34(5), 847–861. doi:10.1128/MCB.01551-13 [PubMed: 24344204]

Ivanov IS, Azmanov DN, Ivanova MB, Chamova T, Pacheva IH, Panova MV, … Kalaydjieva L (2014). Founder p.Arg 446* mutation in the PDHX gene explains over half of cases with congenital lactic acidosis in Roma children. Molecular Genetics and Metabolism, 113(1–2), 76–83. doi:10.1016/j.ymgme.2014.07.017 [PubMed: 25087164]

Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, & Shendure J (2014). A general framework for estimating the relative pathogenicity of human genetic variants. Nature Genetics, 46(3), 310–315. doi:10.1038/ng.2892 [PubMed: 24487276]

Kremer LS, Bader DM, Mertes C, Kopajtich R, Pichler G, Iuso A, … Prokisch H (2017). Genetic diagnosis of Mendelian disorders via RNA sequencing. Nature Communications, 8, 15824. doi:10.1038/ncomms15824

Kryukov GV, Pennacchio LA, & Sunyaev SR (2007). Most rare missense alleles are deleterious in humans: implications for complex disease and association studies. American Journal of Human Genetics, 80(4), 727–739. doi:10.1086/513473 [PubMed: 17357078]

Lake NJ, Compton AG, Rahman S, & Thorburn DR (2016). Leigh syndrome: One disorder, more than 75 monogenic causes. Annals of Neurology, 79(2), 190–203. doi:10.1002/ana.24551 [PubMed: 26506407]

Lim SC, Smith KR, Stroud DA, Compton AG, Tucker EJ, Dasvarma A, … Thorburn DR (2014). A founder mutation in PET100 causes isolated complex IV deficiency in Lebanese individuals with Leigh syndrome. American Journal of Human Genetics, 94(2), 209–222. doi:10.1016/j.ajhg.2013.12.015 [PubMed: 24462369]

Nagy E, & Maquat LE (1998). A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends in Biochemical Sciences, 23(6), 198–199. [PubMed: 9644970]

Neeve VC, Samuels DC, Bindoff LA, van den Bosch B, Van Goethem G, Smeets H, … Horvath R (2012). What is influencing the phenotype of the common homozygous polymerase-gamma mutation p.Ala467Thr? Brain, 135(Pt 12), 3614–3626. doi:10.1093/brain/aws298 [PubMed: 23250882]

Parikh S, Goldstein A, Karaa A, Koenig MK, Anselm I, Brunel-Guitton C, … Chinnery PF (2017). Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. Genetics in Medicine, 19(12). doi:10.1038/gim.2017.107

Patel RY, Shah N, Jackson AR, Ghosh R, Pawliczek P, Paithankar S, … ClinGen R (2017). ClinGen Pathogenicity Calculator: a configurable system for assessing pathogenicity of genetic variants. Genome Med, 9(1), 3. doi:10.1186/s13073-016-0391-z [PubMed: 28081714]

Rahman J, Noronha A, Thiele I, & Rahman S (2017). Leigh map: A novel computational diagnostic resource for mitochondrial disease. Annals of Neurology, 81(1), 9–16. doi:10.1002/ana.24835 [PubMed: 27977873]

Rahman S, Blok RB, Dahl HH, Danks DM, Kirby DM, Chow CW, … Thorburn DR (1996). Leigh syndrome: clinical features and biochemical and DNA abnormalities. Annals of Neurology, 39(3), 343–351. doi:10.1002/ana.410390311 [PubMed: 8602753]

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, … Committee, A. L. Q. A. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine, 17(5), 405–424. doi:10.1038/gim.2015.30 [PubMed: 25741868]

Rivas MA, Pirinen M, Conrad DF, Lek M, Tsang EK, Karczewski KJ, … MacArthur DG (2015). Human genomics. Effect of predicted protein-truncating genetic variants on the human transcriptome. Science, 348(6235), 666–669. doi:10.1126/science.1261877 [PubMed: 25954003]

Sanchez-Caballero L, Guerrero-Castillo S, & Nijtmans L (2016). Unraveling the complexity of mitochondrial complex I assembly: A dynamic process. Biochimica et Biophysica Acta, 1857(7), 980–990. doi:10.1016/j.bbabio.2016.03.031 [PubMed: 27040506]

Schwarz JM, Cooper DN, Schuelke M, & Seelow D (2014). MutationTaster2: mutation prediction for the deep-sequencing age. Nature Methods, 11(4), 361–362. doi:10.1038/nmeth.2890 [PubMed: 24681721]

Wedatilake Y, Brown RM, McFarland R, Yaplito-Lee J, Morris AA, Champion M, … Rahman S (2013). SURF1 deficiency: a multi-centre natural history study. Orphanet J Rare Dis, 8, 96. doi: 10.1186/1750-1172-8-96 [PubMed: 23829769]

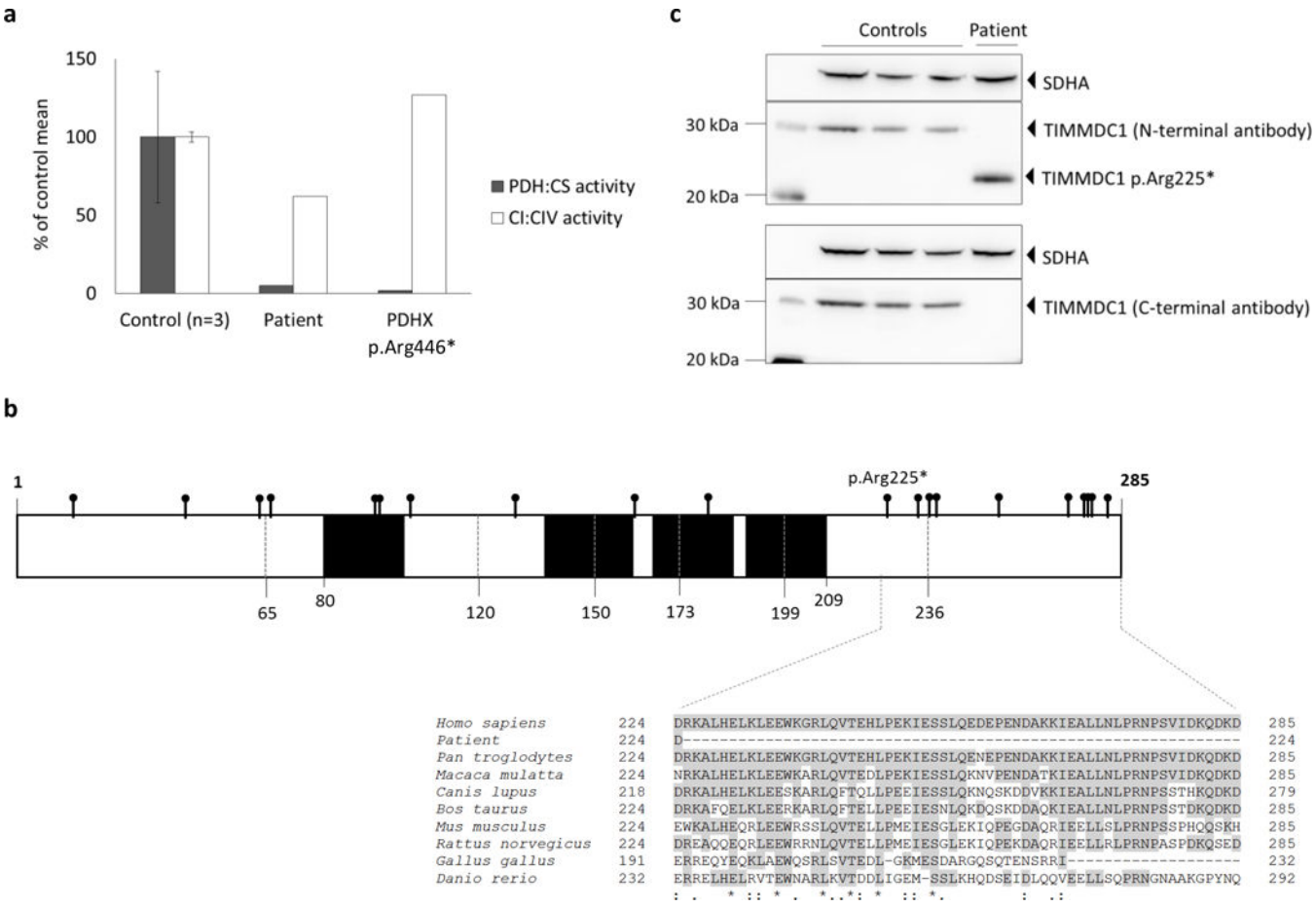

Figure 1. Identification of pyruvate dehydrogenase deficiency and truncated TIMMDC1 protein in patient fibroblasts.

a. Pyruvate dehydrogenase (PDH), complex I (CI) and complex IV (CIV) activities were measured in fibroblasts from controls, the patient and a previously reported patient with PDH deficiency (patient 26 in the reference) (S. Rahman et al., 1996) who is also homozygous *PDHX* p.(Arg446*). PDH activity was normalized to citrate synthase (CS), and CI activity was normalized to CIV activity. Results are presented as percent of mean control activity. The control data represent the mean of three control cell lines (each measured in triplicate) ± SEM, while the patient data are represented as the mean of triplicate measurements.

b. Schematic diagram depicting the TIMMDC1 protein. The black regions represent the four transmembrane domains, and the grey dashes represent the exon junctions. The corresponding amino acid number of each exon junction is described. The p.(Arg225*) variant identified in the patient is denoted. Other exonic nonsense or frameshift variants leading to premature protein truncation of TIMMDC1 reported in the Genome Aggregation Database are shown (circle symbols); these variants are provided in Supp. Table S3. The protein sequence alignment of human TIMMDC1 with its homologs in 8 other vertebrate species illustrates the conservation of the C-terminus missing from the p.(Arg225*) truncated protein in the patient. Asterisk (*), colon (:), and period (.) indicate amino acids that are identical, strongly similar, and weakly similar, respectively.

c. SDS-PAGE western blot of TIMMDC1 in fibroblasts using an antibody targeting the N-terminus (amino acids 1–100) identified the truncated p.(Arg225*) protein in the patient, while no signal was detected in the patient when an antibody targeting the C-terminus of TIMMDC1 (amino acids 212–284) was used. Complex II subunit SDHA represents the loading control. The first lane represents the protein ladder. Expected size of wild-type and TIMMDC1 p.(Arg225*) protein is 32 kDa and 25 kDa, respectively.

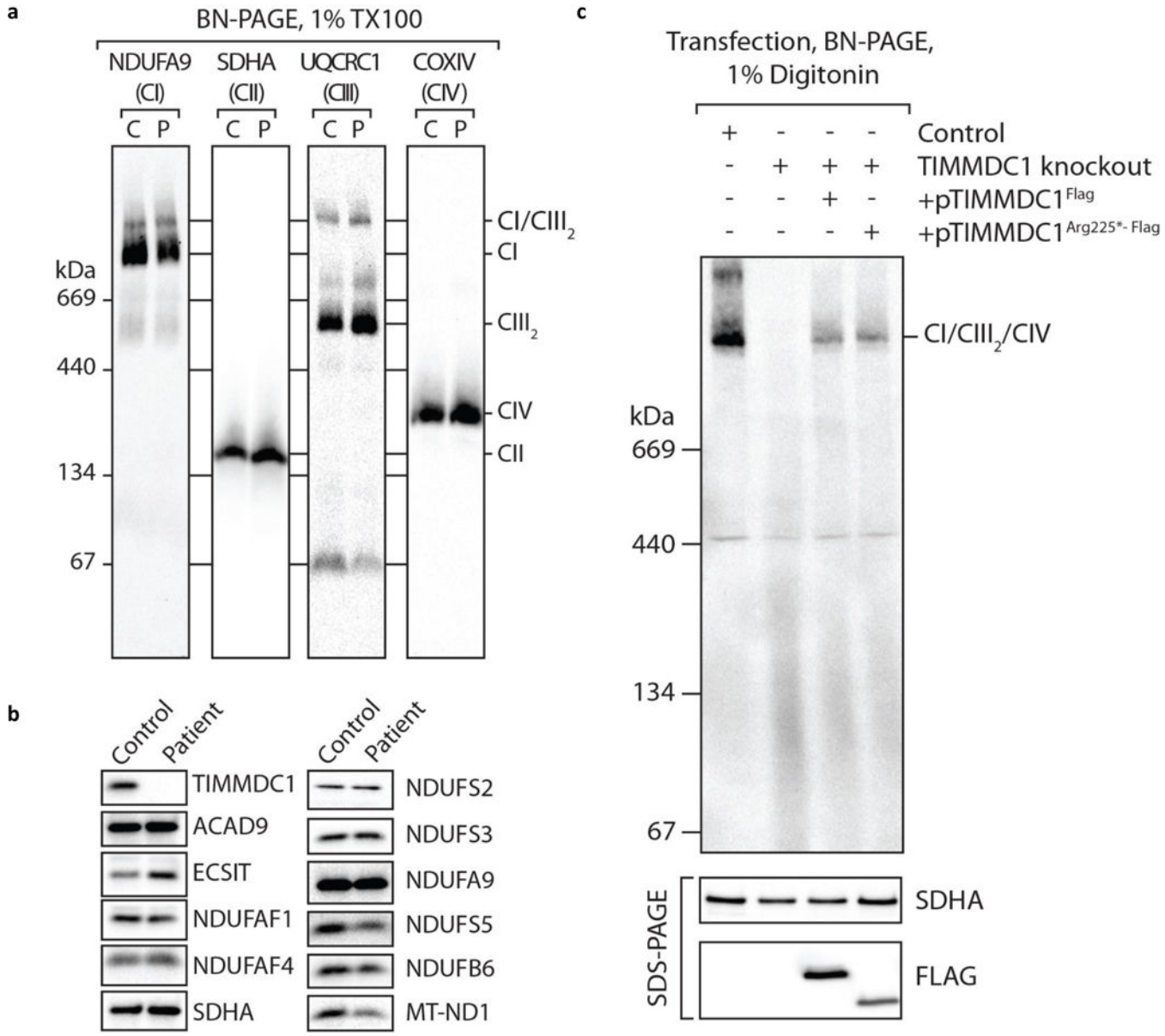

Figure 2. Characterizing the effect of the *TIMMDC1* p.(Arg225*) variant on complex I assembly.

a. BN-PAGE western blot of fibroblast protein showed a mild reduction of complex I (detected using antibody against NDUFA9) in the patient (P) relative to control (C). Levels of complex III (UQCRC1) and complex IV (COXIV) in the patient were comparable to control. Complex II (SDHA) is indicative of loading.

b. SDS-PAGE western blot of protein from fibroblasts showing protein levels of complex I subunits (MT-ND1, NDUFS2, NDUFS3, NDUFS5, NDUFA9 and NDUFB6) and complex I assembly factors (ACAD9, ECSIT, NDUFAF1 and NDUFAF4) in the patient and a control; subunits NDUFS5 and MT-ND1 were most reduced in the patient relative to control. An antibody targeting the C-terminus of TIMMDC1 (amino acids 212–284) was used. Complex II subunit SDHA was used as a loading control.

c. BN-PAGE western blot of control and *TIMMDC1* knockout HEK293T cells expressing wild-type or TIMMDC1 p.(Arg225*) protein with C-terminal FLAG-tag. Overexpression of the wild-type and TIMMDC1 p.(Arg225*) mutant protein restored complex I assembly equally in *TIMMDC1* knockout cells. An antibody against NDUFA9 was used to detect complex I. SDS-PAGE western blot using an anti-FLAG antibody was used to confirm expression of the FLAG-tagged TIMMDC1 proteins. Expected size of the FLAG-tagged wild-type and TIMMDC1 p.(Arg225*) protein is 33.2 kDa and 26.1 kDa, respectively. Complex II subunit SDHA is indicative of loading.