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Mutations in *TRNT1* cause congenital sideroblastic anemia with immunodeficiency, fevers, and developmental delay (SIFD)

Pranesh K. Chakraborty,[1] Klaus Schmitz-Abe,[2] Erin K. Kennedy,[3] Hapsatou Mamady,[1] Turaya Naas,[1] Danielle Durie,[1] Dean R. Campagna,[4] Ashley Lau,[4] Anoop K. Sendamarai,[4] Daniel H. Wiseman,[5] Alison May,[6] Stephen Jolles,[7] Philip Connor,[8] Colin Powell,[9] Matthew M. Heeney,[10] Patricia-Jane Giardina,[11] Robert J. Klaassen,[12] Caroline Kannengiesser,[13] Isabelle Thuret,[14] Alexis A. Thompson,[15] Laura Marques,[16] Stephen Hughes,[17] Denise K. Bonney,[5] Sylvia S. Bottomley,[18] Robert F. Wynn,[5] Ronald M. Laxer,[19] Caterina P. Minniti,[20] John Moppett,[21] Victoria Bordon,[22] Michael Geraghty,[23] Paul B. M. Joyce,[24] Kyriacos Markianos,[2] Adam D. Rudner,[3] Martin Holcik,[25] and Mark D. Fleming[4]

[1]Newborn Screening Ontario, Department of Pediatrics, Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, ON, Canada; [2]Division of Genetics and Genomics, Department of Medicine, Boston Children's Hospital, Boston, MA; [3]Ottawa Institute of Systems Biology and BMI, University of Ottawa, Ottawa, ON, Canada; [4]Department of Pathology, Boston Children's Hospital, Boston, MA; [5]Department of Haematology, Royal Manchester Children's Hospital, Manchester, United Kingdom; [6]Department of Haematology, Cardiff University School of Medicine, Cardiff, United Kingdom; [7]Immunodeficiency Centre for Wales, University Hospital of Wales, Cardiff, United Kingdom; [8]Department of Paediatrics, Children's Hospital for Wales, Cardiff, United Kingdom; [9]Department of Child Health, Institute of Molecular and Experimental Medicine, School of Medicine, Cardiff University, Children's Hospital for Wales, Cardiff, United Kingdom; [10]Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, MA; [11]Division of Hematology-Oncology, Children's Cancer and Blood Foundation Laboratories, Weill Cornell Medical College, New York, NY; [12]Department of Pediatrics, Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, ON, Canada; [13]INSERM UMR773, Université Paris Diderot, Assistance Publique des Hospitaux de Paris, Département de Génétique, Hôpital Xavier Bichat, Paris, France; [14]Department of Pediatric Hematology, Hôpital de la Timone, Marseille, France; [15]Department of Pediatrics, Northwestern University Feinberg School of Medicine, Ann & Robert H. Lurie Children's Hospital, Chicago, IL; [16]Paediatric Department, Infectious Diseases and Immunodeficiencies Unit, Centro Hospitalar do Porto, Porto, Portugal; [17]Department of Immunology, Royal Manchester Children's Hospital, Manchester, United Kingdom; [18]Department of Medicine, Hematology-Oncology Section, University of Oklahoma College of Medicine, Oklahoma City, OK; [19]Department of Paediatrics and Medicine, The Hospital for Sick Children, University of Toronto, ON, Canada; [20]Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD; [21]Bristol Children's Hospital, Bristol, United Kingdom; [22]Department of Pediatric Hemato-Oncology and Stem Cells Transplant, Universitair Ziekenhuis Ghent, Belgium; [23]Department of Pediatrics, Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, ON, Canada; [24]Department of Chemistry and Biochemistry and Center for Structural and Functional Genomics, Concordia University, Montreal, QC, Canada; and [25]Molecular Biomedicine Program, Children's Hospital of Eastern Ontario Research Institute, Department of Pediatrics, University of Ottawa, Ottawa, ON, Canada

Key Points

- SIFD is a syndromic form of congenital sideroblastic anemia associated with immunodeficiency, periodic fevers, and developmental delay.
- SIFD is due to partial loss-of-function mutations in the CCA-adding enzyme TRNT1.

Mutations in genes encoding proteins that are involved in mitochondrial heme synthesis, iron-sulfur cluster biogenesis, and mitochondrial protein synthesis have previously been implicated in the pathogenesis of the congenital sideroblastic anemias (CSAs). We recently described a syndromic form of CSA associated with B-cell immunodeficiency, periodic fevers, and developmental delay (SIFD). Here we demonstrate that SIFD is caused by biallelic mutations in *TRNT1*, the gene encoding the CCA-adding enzyme essential for maturation of both nuclear and mitochondrial transfer RNAs. Using budding yeast lacking the *TRNT1* homolog, *CCA1*, we confirm that the patient-associated *TRNT1* mutations result in partial loss of function of TRNT1 and lead to metabolic defects in both the mitochondria and cytosol, which can account for the phenotypic pleiotropy. (*Blood*. 2014;124(18):2867-2871)

Introduction

The congenital sideroblastic anemias (CSAs) are a heterogeneous group of syndromic and nonsyndromic inherited diseases characterized by pathologic iron deposition in the mitochondria of red blood cell precursors in the bone marrow. All identified causative CSA genes encode structural RNAs or proteins that are involved in 1 of 3 mitochondrial pathways: heme synthesis, mitochondrial

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There is an Inside *Blood* Commentary on this article in this issue.

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Table 1. SIFD patients and *TRNT1* mutations

Patient	Consanguinity	Ethnicity	Allele 1				Allele 2			
1A	Yes	South Asian (Pakistani)	c.569G>T	p.R190I	Exon 5	Missense	c.569G>T	p.R190I	Exon 5	Missense
1B	Yes	South Asian (Pakistani)	c.569G>T	p.R190I	Exon 5	Missense	c.569G>T	p.R190I	Exon 5	Missense
2	No	Caucasian	c.668T>C	p.I223T	Exon 6	Missense	c.1057-7C>G	NA	ivs7	Splicing
3	No	Caucasian	c.668T>C	p.I223T	Exon 6	Missense	c.1057-7C>G	NA	ivs7	Splicing
4	No	Caucasian	c.668T>C	p.I223T	Exon 6	Missense	No mutation/deletion detected.			
5	No	Caucasian	c.218_219ins22	NA	Exon 3	Frameshift	c.668T>C	p.I223T	Exon 6	Missense
6	Yes	Caucasian (Hispanic)	c.668T>C	p.I223T	Exon 6	Missense	c.668T>C	p.I223T	Exon 6	Missense
7	No	Caucasian	c.497T>C	p.L166S	Exon 5	Missense	c.461C>T	p.T154I	Exon 4	Missense
8	Yes	South Asian (Pakistani)	c.569G>T	p.R190I	Exon 5	Missense	c.569G>T	p.R190I	Exon 5	Missense
9	No	Caucasian	c.668T>C	p.I223T	Exon 6	Missense	c.1057-7C>G	NA	ivs7	Splicing
10	No	Caucasian	c.977T>C	p.I326T	Exon 7	Missense	c.472A>G	p.M158V	Exon 4	Missense
11	No	Caucasian	c.608+1 G>T	NA	ivs5	Splicing	c.461C>T	p.T154I	Exon 4	Missense
12A	No	Caucasian	c.1246A>G	p.K416E	Exon 8	Missense	c. del1054_1056+10	NA	Exon 7	Splicing
12B	No	Caucasian	c.1246A>G	p.K416E	Exon 8	Missense	c. del1054_1056+10	NA	Exon 7	Splicing
13	No	Afro-Caribbean	c.668T>C	p.I223T	Exon 6	Missense	c.1142insATGT	p.W381fs	Exon 8	Frameshift
14	No	Caucasian	c.668T>C	p.I223T	Exon 6	Missense	c.1252_1253insA	S418fs	Exon 8	Frameshift

Patient 2 had a similarly affected sibling; however, DNA was not available for study. Complementary DNA numbering is based on NM_182916.2. Protein numbering is based on NP_886552.2.

A and B, sibling pairs; NA, not applicable.

iron-sulfur cluster biogenesis, and mitochondrial protein synthesis.[1] We recently described a syndromic form of CSA associated with B-cell immunodeficiency, periodic fevers and developmental delay (SIFD). Variably severe sensorineural hearing loss, cardiomyopathy, and central nervous system abnormalities also occurred in some patients.[2] SIFD pedigrees indicated an autosomal recessive mode of inheritance. Here, we extend the cohort of patients described with SIFD, and, using whole exome sequencing and a novel method of identity by descent mapping, identify the causative gene as *TRNT1,* a template-independent RNA polymerase required for the maturation of cytosolic and mitochondrial transfer RNAs (tRNAs).

Materials and methods

Ethics approval

The work was completed with the approval of the institutional review boards at Boston Children's Hospital and the Children's Hospital of Eastern Ontario.

Genomic analyses

We automated the discovery process using a custom-built, rule-based "Variant Explorer" pipeline using copy number variation, family linkage as well as population level homozygosity to aid interpretation of the results (K.S.-A. and K.M., unpublished). For the analysis, we analyzed 180 (113 affected) samples from multiplex families or singletons with CSA.

siRNA knockdowns

Small interfering RNA (siRNA) transfections of fibroblasts were performed using lipofectamine RNAiMAX according to the protocol provided by the manufacturer (Invitrogen). The cells were transfected TRNT1 siRNA (Hs_TRNT1, Qiagen SI00751464 [#4], SI04142691 [#6], SI04235056 [#7], SI04301857 [#8]), or a nonsilencing control siRNA (Dharmacon, 5′-UUC UCCGAACGUGUCACGUdTdT-3). Cells were collected for analysis at 24, 48, or 72 hours posttransfection.

Immunoblotting

See supplemental Methods on the *Blood* Web site.

Kinetic measurement of cytotoxicity and Caspase-3/7 activation

Normal human skin fibroblast cells were transfected with increasing concentrations of siRNA for 24 hours in a 96-well plate. Cells were then incubated with either 100 nM YOYO-1 dye (Life Technologies) or 1 μM of Cell Player reagent (Essen BioScience) and the was incubation monitored for 48 hours using the INCUCYTE ZOOM Live-Cell Imaging System as described by the manufacturer (Essen BioScience, MI). The fraction of YOYO-1 and Caspase 3/7–positive cells was measured after treatment with 0.0625% Triton X-100X for the YOYO-1 assay or 1 μM Vybrant Green DNA (Life Technologies, Invitrogen) for the Caspase 3/7 assay.

Overexpression and purification of native and variant TRNT1 proteins

HEK293T cells were transfected with either wild-type TRNT1-FLAG plasmid (pTrueORF, Origene, USA) or mutated plasmid DNA in antibiotic-free Opti-MEM with lipofectamine 2000 (Invitrogen). After 48 hours, lysates were immunoprecipitated with Flag-beads (Sigma-Aldrich) as previously described.[3] Before assay, protein was quantified using BCA protein assay (Pierce), checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

CCA-adding enzyme assays

In vitro transcription of *Bacilla subtilis* $tRNA^{Asp}$ lacking 3 nucleotides (CCA) at the 3′-terminus was performed using the plasmid G73 (a gift of Dr. Alan M. Weiner, Department of Biochemistry, University of Washington, Seattle, WA)[4] and CCA-adding enzyme activity assayed using either in-gel or glass fiber assays measuring the incorporation of [α^{32}P]-adenosine triphosphate as described in detail in the supplemental Methods.

Yeast strain construction and plate-based assays

See the supplemental Methods and supplemental tables.

Statistical analyses

All results are expressed as mean ± standard error of the mean with a minimum of 3 biological replicates, unless otherwise noted. The Student *t* test was used to determine statistical significance (Graph Pad Prism 5).

Results and discussion

We performed genome-wide Affymetrix 6.0 SNP analysis on 6 SIFD probands (Table 1; patients 2, 3, 6, 7, 8, and 10) and 10 of their

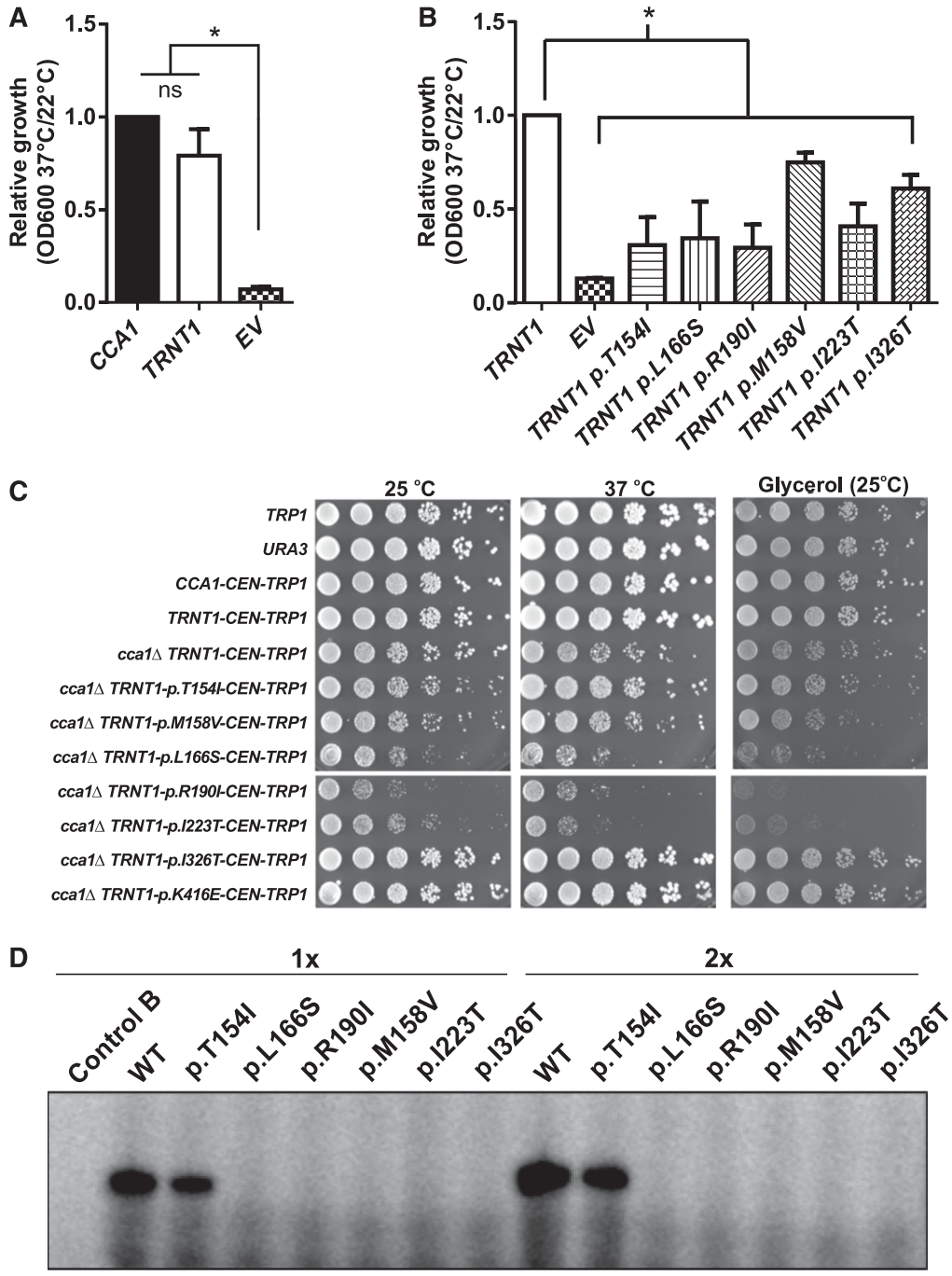

Figure 1. Mutations in *TRNT1* in SIFD patients are loss-of-function alleles. (A) Yeast *cca1-1* cells were transformed with centromeric plasmids encoding yeast *CCA1*, human *TRNT1*, or an empty vector control (EV) and grown at permissive (22°C) or nonpermissive (37°C) temperatures overnight in selective media. The optical density of the sample measured at a wavelength of 600 nm (OD_{600}) value for the empty vector was subtracted from each value and the ratio of OD_{600} at 37°C/22°C was calculated and normalized so that the relative growth rate of cells harboring a *CCA1* plasmid is 1 (mean ± standard error of the mean, with minimum n = 5). The ability of human TRNT1 to rescue growth at the nonpermissive temperature was not statistically different from *CCA1* as determined by Student paired *t* test. (B) Patient TRNT1 mutants partially rescue growth impairment of *cca1-1* cells. *cca1-1* cells were transformed with EV, wild-type *TRNT1*, or *TRNT1* mutants (p.T154I, p.L166S, p.R190I, p.M158V, p.I223T, and p.I326T) and grown as in (a). The OD_{600} value for the EV control was subtracted from each value and the ratio of OD_{600} at 37°C/22°C was calculated and normalized so that the relative growth rate of cells harboring a TRNT1 plasmid is 1 (mean ± standard error of the mean, with minimum n = 3; Student paired *t* test $*P \leq .05$). (C) Complemented *cca1*Δ strains that contain only the *TRNT-CEN-TRP1* plasmid were isolated from SC-trp + 5-FOA plates (supplemental Figure 15B). Ten-fold serial dilutions of 10^6 cells of the indicated genotypes were spotted on YEP + 2% dextrose or 3% glycerol plates at the indicated temperatures. Wild-type and most *TRNT1* mutants have growth defects and temperature sensitivity, with the exception of *TRNT1-p.K416E* and *TRNT-p.I326T*, which are indistinguishable from *CCA1* cells. (D) Two different concentrations of purified TRNT1-FLAG protein with patient-specific mutations were incubated with [α^{32}P]-adenosine triphosphate and $tRNA^{Asp}$ lacking the terminal CCA sequence at 37°C for 5 minutes as described in the supplemental Methods and the reactions were terminated by addition of loading dye and resolved on a denaturing 5% polyacrylamide/8M urea gel, dried, and exposed to a phosphoimager screen. Other than the T154I variant, all of the mutants have negligible activity in this assay.

parents. We mapped the disease locus by analyzing genotypes for overlapping regions of identity by descent. We identified a region on chromosome 3p26.1 in which both consanguineous probands (patients 6 and 8) were homozygous for distinct haplotypes (supplemental Figure 1). Subsequent linkage analysis in another consanguineous Pakistani kindred (patients 1A and 1B, parents, and 2

unaffected siblings) was also consistent with linkage to 3p26.1, resulting in a cumulative logarithm of odds score of 3.38. Affected members of this pedigree shared a partially overlapping, identical homozygous haplotype with patient 8, also Pakistani. On this basis, we were able to narrow the disease interval to a 1.04-Mb, 5-cM region containing 4 genes, including *TRNT1*. Given its role in mitochondrial tRNA metabolism, *TRNT1* was considered the primary candidate gene. Independently, whole exome sequencing was performed on patient 7. Rare variants in mitochondrial proteins annotated in the MitoCarta[5] database were examined, and revealed biallelic, missense variants in *TRNT1*. By Sanger sequencing amplified exons, we found biallelic *TRNT1* mutations in all but 1 of the other SIFD patients previously described (Table 1) as well as 5 additional affected individuals from 4 families (Table 1, patients 11, 12A, 12B, 13, and 14). In patient 4, we identified only 1 uncommon variant. We did not identify a small deletion or copy number variation in this patient using a custom Nimblegen 720k array spanning the *TRNT1* locus. In aggregate, we identified 3 frameshift alleles, 3 splicing variants, and 7 unique missense TRNT1 alleles: p.T154I, p.M158V, p.L166S, p.R190I, p.I223T, p.I326T, and p.K416E. Except for p.K416E, each of the missense variants occurs in a highly conserved residue and received a PolyPhen2 score >0.95, indicating a likely damaging mutation.[6] Nonetheless, K416 is highly conserved in mammals, suggesting its potential pathogenicity. Five of the missense mutations (p.T154I, p.M158V, p.L166S, p.R190I, and p.I223T) cluster in the active site, and 2 (p.I326T and p.K416E) are in the less well-conserved C-terminal region (supplemental Figure 2).[7] Only 1 missense variant, p.I223T, occurs in the National Heart, Lung, and Blood Institute Exome Sequencing Variant Project (http://evs.gs.washington.edu/EVS/), and then only at an allele frequency of 0.0077%. We did not identify any uncommon variants in *TRNT1* in 58 other unrelated probands with nonsyndromic and phenotypically distinct syndromic CSA.[8]

TRNT1 encodes the human CCA-adding enzyme, an RNA polymerase required for the posttranscriptional, template-independent addition of 2 cytosines and 1 adenosine to the 3′ end of all tRNA molecules, which is necessary for tRNA aminoacylation.[9] CCA-adding enzymes are also implicated in tRNA quality control and the stress response.[10-13] *TRNT1* encodes the only human CCA-adding activity and is responsible for the maturation of both cytosolic and mitochondrial tRNAs.[11]

siRNA knockdown of TRNT1 in wild-type human fibroblasts caused cytotoxicity and apoptosis (supplemental Figure 3), suggesting that eliminating TRNT1 function altogether is lethal, whereas mutations only impairing function could be disease-associated. To determine the functional significance of the disease-associated variants, we examined the effect of the variants in patient-derived skin fibroblasts and in yeast. Only full-length *TRNT1* transcript was detected from control human skin fibroblasts (supplemental Figure 4a). The c.608+1 G>T and del1054_1056+10 variants resulted in aberrant splicing (supplemental Figure 4a-c). The c.1057-7C>G mutation found in 3 individuals (patients 2, 3, and 9) is strongly predicted by the Human Splicing Finder[14] to result in a new splice acceptor site that, if employed, inserts 6 nucleotides in the complementary DNA upstream of exon 8, encoding the insertion of sequential threonine and TAG stop codons (p.D352_S353insTX) and premature termination of the protein (supplemental Figure 4d). Unfortunately, no primary material was available from any of these patients to validate this prediction.

We examined the ability of TRNT1 mutants to complement budding yeast cells harboring a temperature-sensitive mutation in *CCA1* (*cca1-1,* originally named ts352),[7,15] and in a *CCA1* deletion strain. Although expression of the wild-type human TRNT1 in the *cca1-1* strain fully restored growth at a nonpermissive temperature (Figure 1A), expression of the mutant TRNT1 alleles provided only partial rescue (Figure 1B). Furthermore, as demonstrated by tetrad dissections, only the wild-type yeast protein and the human p.I326T mutant were able to rescue the lethal deletion of *CCA1* through meiosis in haploid yeast (supplemental Figure 5a). Consequently, we rescued the lethal deletion of *CCA1* with wild-type and mutant TRNT1 plasmids using a plasmid shuffle assay (Figure 1C; supplemental Figure 5b,c). We found that *TRNT1* with any of the 5 missense mutations that cluster near the active site of the enzyme all complemented growth to varying degrees as compared with wild-type human TRNT1. The p.R190I and p.I223T variants grew particularly poorly on nonfermentable glycerol medium, indicating a defect in mitochondrial function. Two mutants, p.I326T and p.K416E, not near the active site, complemented the *cca1*Δ strain as efficiently as yeast CCA1 itself, suggesting that they may be milder alleles than the other missense mutations.

Although *TRNT1* mRNA levels were comparable between wild type and mutants, protein levels were variable in wild-type and *cca1-1* yeast (supplemental Figure 6a,b). This was equally true of TRNT1 expression in patient fibroblast cells (supplemental Figure 6c-e), suggesting that mutations in *TRNT1* may also affect TRNT1 proteostasis. In support of this hypothesis, we also found that rescued *cca1*Δ strains selected for high expression of *TRNT1* (supplemental Figure 1a,b).

All but 1 of the missense mutations impaired the ability of TRNT1 to catalyze the formation of the CCA trinucleotide, in vitro (Figure 1D; supplemental Figure 8a-c). Most mutant proteins had no detectable activity, whereas the activity of the p.T154I and p.K416E alleles were ~60% and equal to the wild-type, respectively. Because of the limited numbers of patients, we were unable to perform a systematic genotype-phenotype correlation; however, we note that the siblings with the p.K416E allele have a mild phenotype, with predominantly neurological abnormalities, periodic fevers, and infections, but minimal anemia and longer survival. Similarly, patient 7, carrying the p.T154I variant, is neither transfusion- nor intravenous immunoglobulin–dependent. Patient 10, carrying the p.I326T allele, also has a variant phenotype having few neurological issues and not requiring intravenous immunoglobulins. Altogether, these genetic and functional assays indicate that all SIFD patients have *TRNT1* mutations and an individual patient may present with a range of clinical severity of the constituent phenotypes depending upon the degree of CCA-adding enzyme loss of function.

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Authorship

Contribution: P.K.C., K.M., A.D.R., M.H., and M.D.F. designed and supervised the study; R.F.W., D.H.W., A.M., S.J., P.C., C.P., M.M.H., P.J.G., R.J.K., C.K., I.T., A.A.T., L.M., S.H., D.K.B., S.S.B., R.M.L., C.P.T., J.M., C.M., V.B., M.T.G., P.K.C., and M.D.F. characterized SIFD syndrome and collected clinical data and patient samples; K.S.-A. was supervised by K.M. and developed the informatics pipeline and identified the causative genetic defect by way of linkage studies; D.R.C. and A.L. performed mutation analysis in CSA patients; P.K.C. and M.T.G. identified the causative genetic defect post exomic sequencing; E.K.K., supervised by A.D.R., M.H., and P.K.C., designed and performed the yeast CCA knockout and rescue experiments; T.N., supervised by M.H. and P.K.C., designed and performed the siRNA knockdown experiments; H.M., supervised by A.D.R., M.H., and P.K.C., designed and performed the adenylation assays; D.D., supervised by A.D.R., M.H., and P.K.C., performed the temperature-sensitive yeast rescue experiments with advice from A.D.R. and P.B.M.J.; A.K.S. created the figure depicting the locations of the missense mutations on the TRNT1 X-ray crystal structure; E.K.K., H.M., T.N., and D.D. prepared the draft methods; and P.K.C., A.D.R., M.H., and M.D.F. prepared the final manuscript.

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Correspondence: Pranesh K. Chakraborty, Newborn Screening Ontario, Department of Pediatrics, Children's Hospital of Eastern Ontario, 401 Smyth Rd K1H 8L1, Ottawa, ON, Canada; e-mail: pchakraborty@cheo.on.ca; and Mark D. Fleming, Department of Pathology, Boston Children's Hospital, 300 Longwood Ave, Bader 124.1, Boston, MA 02115; e-mail: mark.fleming@childrens.harvard.edu.

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