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Supplemental Data

Bi-allelic *TARS* Mutations Are Associated with Brittle Hair Phenotype

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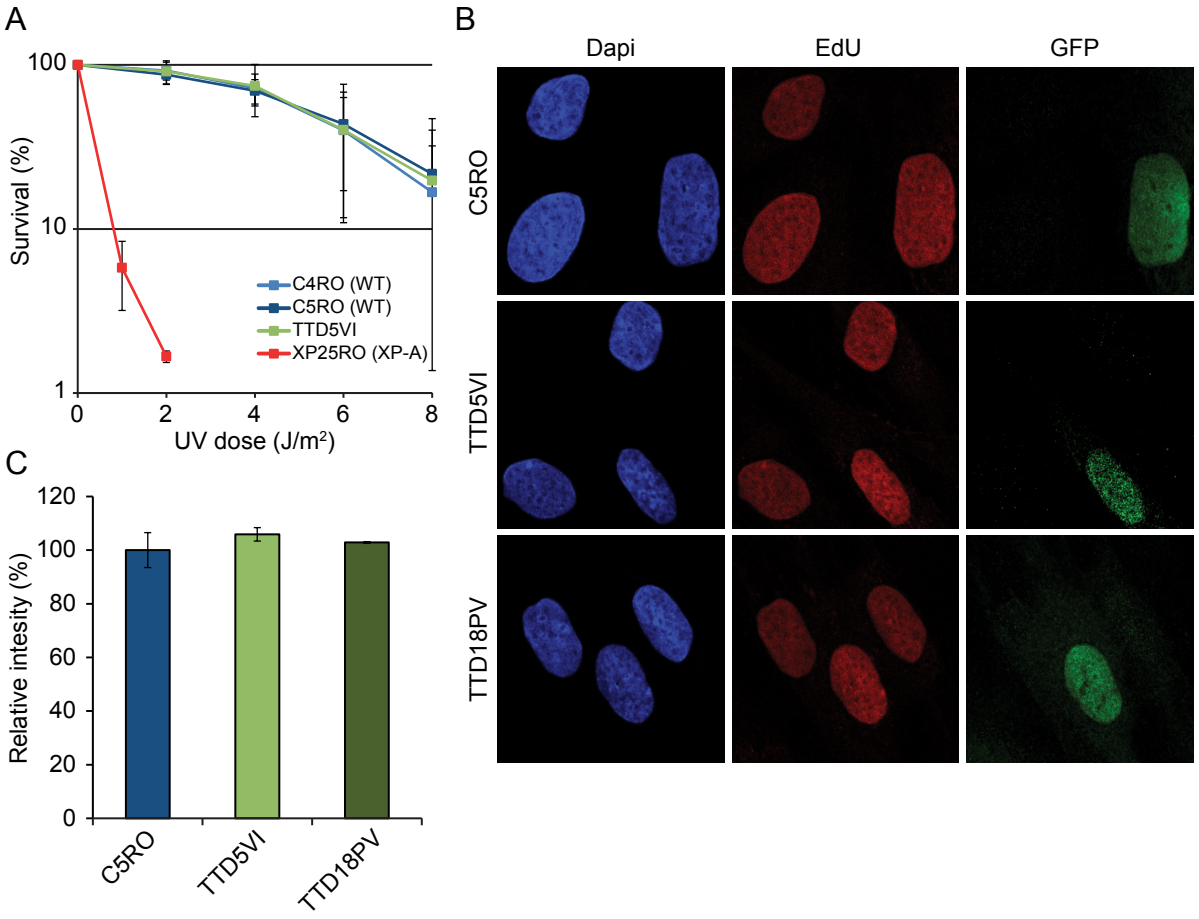

Figure S1. Characterization of DNA Repair capacity. (A) Clonogenic UV-survival assay to measure UV-sensitivity. One day after seeding of respectively TTD5VI, NER-defective XP-A (XP25RO) and NER-proficient fibroblasts (C4RO and C5RO), the cells were irradiated with the indicated (X-axis) doses of UV and cultures incubated for two weeks to grow colonies. Survival was plotted as the percentage of colonies obtained after treatment compared to the mean number of colonies from the mock-treated cells (set at 100%). The error bars indicate SEM of three independent experiments. (B) Global NER activities measured as UV-induced unscheduled DNA synthesis (UDS), using EdU incorporation after UV-irradiation, visualized by fluorescence-conjugated azide (Click-iT assay). Shown are representative pictures from the UDS experiment performed on primary fibroblasts of TTD5VI or TTD18PV and compared to NER-proficient (wild-type) control primary fibroblasts C5RO. To directly compare UDS levels with known NER-proficient cells, the wild-type fibroblasts that stably express GFP were mixed with the test cells. UDS-derived fluorescence is shown in red (middle panel) and nuclear staining in blue (DAPI)(left panel), NER-proficient cells can be distinguished by the GFP signal (green, right panel). (C) Quantification of the UDS experiments. Mean intensities of at least 50 nuclei are expressed as percentages of those in normal cells assayed in parallel. The error bars indicate SEM of three independent experiments.

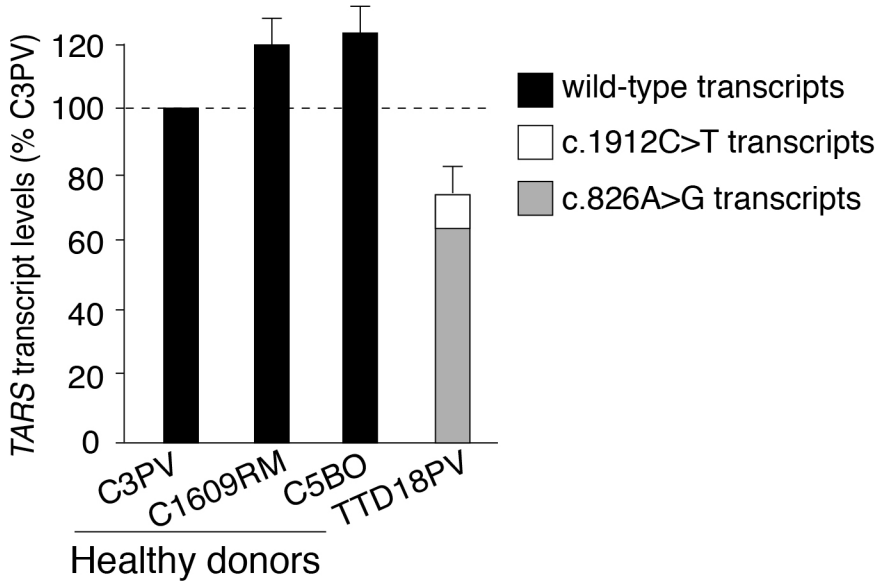

Figure S2. Analysis of mutant *TARS* transcript levels. Cellular levels of *TARS* mRNA assessed by qRT-PCR. Total TARS transcript levels were first normalized to the levels of *GAPDH* mRNA and then expressed as percentages of the corresponding value in the normal C3PV cells. The relative percentage of the mutant *TARS* transcripts in TTD18PV cells (c.1912C>T and c.826A>G) was assessed by allele-specific qRT-PCR. The reported values are the means of two independent experiments, each done in triplicate. The error bars indicate SEM.

Table S1: Hair analysis subject TTD18PV.

Aminoacid	**Normal Value**	**TTD18PV**
Aspartic acid	4,9	8,5
Threonine	6,9	3,0
Serine	10,8	10,1
Glutamic acid	11,4	16,8
Proline	9,0	8,6
Glycine	5,5	6,6
1/2 Cysteine	20,8	8,5
Valine	4,9	6,1
Methionine	0,3	0,2
Isoleucine	2,5	3,6
Leucine	6,0	9,1
Tyrosine	1,9	1,6
Phenylalanine	1,6	2,6
Histidine	0,9	1,1
Lysine	2,7	4,6
Arginine	5,0	6,5

Aminoacid content in the hair of the subject TTD18PV compared to a normal hair sample. Hair aminoacid content was determined using chromatography and values obtained for individual aminoacids were expressed as percentage.

Table S2. cDNA and genomic primers used in this study.

Assay[a]	Gene	Position[b]	Primer	Sequence 5'-3'
cDNA PCR	*TARS*	c.-9_-28	TARS_F1	GGTTCTCTCATCGCTTCGTC
		c.664_687	TARS_R1	CATTGCCAGTAAAGTTTCTTTCTT
		c.371_390	TARS_F2	ATGTTGTGTGGGACCTGGAC
		c.1167_1186	TARS_R2	TGTTCTCGCTGTAGTGCTGC
		c.970_989	TARS_F3	AGGAAAATTGGCAGGGACCA
		c.1771_1790	TARS_R3	ACTGATCCCAAGATGGCTCG
		c.1488_1507	TARS_F4	AAACCTTTCTACTCGCCCGG
		c.+11_+30	TARS_R4	TTCCATGGAGCCAATCTGGG
Genomic DNA PCR	*TARS*	16417_16436	TARS_int8F2	GGGGCATTTTTGAGGATAGA
		16764_16784	TARS_int9R2	TCATGCAGCTGAATTTCAAAC
		25989_26008	TARS_int18F2	AGAGGAGCTGGCCTTTTTGA
		26452_26473	TARS_int19R2	CCCAGTATGTATGCATGAGCCT
qRT-PCR	*TARS*	c.-73_-53	TARSv1_F1	CCTCTTGGCTCCTCTCCTCT
		c.320_339	TARSv1_R1	CAGGCCTTGACTAATTCCAC
		c.1763_1782	TARS_F1763	TTGTTCATCGAGCCATCTTG
		c.1912_1931	TARS_R1912G	GCATCGTGGAATTGTTG**G**C<u>G</u>
			TARS_R1912A	GCATCGTGGAATTGTTG**G**C<u>A</u>
	GAPDH	c.20_39	GAPDH_F2	GAGTCAACGGATTTGGTCGT
		c.204_185	GAPDH_R2	GACAAGCTTCCCGTTCTCAG

[a] PCR amplified cDNA and genomic DNA fragments were sequenced using the corresponding F and R PCR primers.

[b] Positions of cDNA PCR primers and qRT-PCR primers refer to the GenBank reference mRNA sequence NM_152295.4 (*TARS* variant 1, CDS 312-2483). For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence. For primers TARS_R1912G and TARS_R1912A underlined nucleotides indicate wild-type and mutant nucleotides, respectively. In order to prevent the amplification of the non-matching primer, an additional nucleotide mismatch located three bases from the 3' termini was incorporated (indicated in bold).

Positions of genomic DNA primers refer to the *TARS* GenBank reference genomic sequence NC_000005.10 nt 33440696-33468091.

Supplemental Material and Methods

Whole genome sequencing (WGS)

Nanoball based massive parallel sequencing (software version 2.5.0.44) was done as described previously [1]. Image data analyses including base calling, DNB mapping, and sequence assembly. Reads were mapped to the National Center for Biotechnology Information (NCBI) reference genome, build 37. Variants were annotated using NCBI build 37 and dbSNP build 137. Data were provided as lists of sequence variants (SNPs and short indels) relative to the reference genome. Analysis of the massive parallel sequencing data was performed using Complete Genomics analysis tools (cga tools version 1.8.0 build 1; http://www.completegenomics.com/sequence-data/cgatools/) and TIBCO/Spotfire version 7.0.1 (http://spotfire.tibco.com/).

Whole exome sequencing (WES)

Exonic DNA was captured using the Agilent SureSelect Human All Exon target enrichment kit (Agilent Technologies, Santa Clara, CA) and sequencing was performed with paired-end 100bp reads on one-quarter of an Illumina Analyzer IIx (Illumina, San Diego, CA). About 50 M reads/individual were generated resulting in approximately 30-40x coverage of the targeted exome. The Genome Analysis Toolkit (GATK v2.5) was used to perform variant discovery and genotyping. SNPs and Indels were called according to the GATK's Best Practices. Variants were filtered assuming a recessive inheritance model. Given the rare incidence of TTD (estimated to be 1.2 per million livebirths in Western Europe [2]) only variants with a frequency ≤ 0.001 were considered. Variants prioritization was based on computationally predicted pathogenicity scores in SIFT and PolyPhen2.

Sanger validation sequencing

In TTD18PV the *TARS* mutation was confirmed in the relevant genomic DNA region based on WES findings. 24 additional TTD cases were screened for mutations in *TARS* by directed sequencing of either the cDNA or genomic DNA using PCR and sequencing primers (Table S2).

Quantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Transcript levels of *TARS* and *GAPDH* (for normalization) were measured on a LightCycler 480 (Roche, Mannheim, Germany). Primer sequences are in Table S2.

Cell culture

Primary fibroblasts: TTD18PV (NPS-TTD), TTD5VI (NPS-TTD), C3PB, C1609RM, C5BO, C4RO and C5RO, were cultured in Ham's F10 medium (BE02-014F, Lonza) supplemented with 10% fetal bovine serum (S1810, Biowest) and 1% penicillin-streptomycin (P0781, Sigma-Aldrich) at 37°C, 20% O2 and 5% CO2.

Antibodies

Primary antibodies used: α-TARS (ab50795; Abcam), α-Tubulin (T5168, Sigma-Aldrich) and α-GFP (11814460001, Roche Diagnostics). Secondary antibodies used: CF680 anti-mouse (SAB4600199, Sigma-Aldrich), IRDye 800CW Donkey anti-mouse (926-32212,LI-COR) and Alexa Fluor 488 goat anti-mouse (A11001, Invitrogen).`

Colony forming ability/survival

Cells were plated in 10 cm dishes (1500 cells/dish), in triplicate. After 24 h, cells were irradiated with different doses of UV-C irradiation (0-8 J/m^2) and incubated for approximately 2 weeks. Colonies were fixed and stained with 0.1% Brilliant Blue R (Sigma) and counted (Gelcount, Oxford Optronix Ltd.). The survival was plotted as the percentage of colonies obtained after treatment compared to the mean number of colonies from the mock-treated cells (set at 100%).

Unscheduled DNA synthesis (UDS) assay

For UDS, GFP (Green Fluorescent protein)-expressing control fibroblasts were mixed with fibroblasts from subject TTD5VI, subject TTD18PV, a healthy individual (C5RO) or a DNA repair deficient subject (XP-A), and seeded onto 24 mm cover slips. Two days later adherent cells were washed with PBS and UV-C irradiated with 16 J/m^2. Thereafter, fibroblasts were incubated for 3 h in medium containing 0.1 µM 5-ethynyl-2'-deoxyuridine (EdU, A10044, Invitrogen). After EdU incorporation, fibroblasts were fixed in 2% formaldehyde/PBS, washed with 3% BSA/PBS, permeabilized for 20 min in 0.1% Triton/PBS and washed once with PBS. Samples were incubated for 30 min with fluorescent dye coupling buffer containing 10 mM CuSO4 and Atto 594 azide (AD 594-105, ATTO-TEC), washed with 0.1% Triton X-100/PBS, rinsed for 15 min in PBS+ (PBS containing 0.15% glycine and 1% BSA). For visualizing GFP-expressing cells, samples were incubated for 2 h with monoclonal GFP antibody diluted in PBS/Triton X-100 in a moist chamber, washed with PBS+ and incubated for 1 h with secondary antibodies diluted in PBS/Triton X-100 at room temperature in moist chamber. After washing in PBS/Triton X-100 samples were embedded in Vectashield mounting medium with DAPI (H-1200, Vector Laboratories). UDS levels were expressed as

the average fluorescence intensity in the nucleus of subject fibroblasts versus GFP-expressing control fibroblasts, which was set at 100%. The mean fluorescence is determined with a confocal microscope (Zeiss LSM 700) from at least 50 cells and three independent experiments. Images were processed using ImageJ.

Immuno-blot analysis

Whole cell extracts were isolated as follows: cells were washed with PBS and directly lysed in SDS-PAGE Laemmli sample buffer, separated on a Mini-PROTEAN TGX Gel (#456-1084, BIO-RAD), blotted onto Immobilon-FL membrane (IPFL00010, Merck Millipore Ltd.), stained with specific primary and secondary antibodies and analyzed using an Odyssey imager (LI-COR). Immunoblots were quantified using ImageStudio Lite (ver. 5.2, LI-COR Biosciences).

Yeast Complementation Assays

Complementation assays were performed by plasmid shuffling experiments. Briefly, yeast cells (haploid *S. cerevisiae* BY4743 ths1- strain harbouring a pRS426 maintenance vector expressing the wild-type yeast *THS1* and *URA3*) were transformed with *LEU2* pRS425 vectors expressing either wild-type or mutated human *TARS* cDNA and then the yeast strain was grown in selective medium lacking leucine and uracil (-LU) to select for the presence of both vectors. Next, the cells were spotted on plates containing 0.1% 5-Floroorotic acid (5-FOA) or -LU medium and incubated at 30°C for 96 hours. 5-FOA is toxic to yeast cells expressing URA3 and thus select for cells that have spontaneously lost the maintenance vector. Only those cells containing a functional gene expressed from the LEU2 plasmid are

able to complement the cell lethality caused by the deletion of endogenous gene and survive on 5-FOA plates. Survival was determined by visual inspection of growth.

Aminoacylation reaction

Assays were performed at 37°C in reaction buffer (50 mM Tris buffer pH 7.5, 12 mM MgCl , 25 mM KCl, 1 mg/mL bovine serum albumin, 0.5 mM spermine, 1 mM ATP, 0.2 mM yeast total tRNA, 1 mM dithiotreitol, 0.3 mM [$^{13}C_4$,^{15}N] threonine and [D_4] lysine. The aminoacylation reaction was terminated by adding trichloroacetic acid (TCA) and subsequently washed. Ammonia was added in order to release [$^{13}C_4$,^{15}N] threonine and [D_4] lysine from the charged tRNAs. [D_2] glycine and [$^{13}C_6$] arginine were added as internal standards. Labeled amino acids were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Supplemental References

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