

## Supplementary Methods

### Homozygous frame shift variant in *ATP7B* exon 1 leads to bypass of nonsense-mediated mRNA decay and to a protein capable of copper export

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### Vector constructs

#### ATP7B expression vectors

ATP7B expression vectors were assembled using two fragments: (i) a gBlocks fragment (IDT, Leuven, Belgium) containing exon 1-2 and the 5' part of exon 3 of the *ATP7B* gene as well as the 5'UTR and a truncated form of intron 2 and (ii) a PCR product containing the 3' part of exon 3 and exon 4-21 of the *ATP7B* gene.

The gBlocks fragment was introduced into pcDNA3 (Invitrogen/Life Technologies, Carlsbad, USA) using *KpnI* and *XhoI* restriction sites (pcDNA3\_gBlocks).

Sequence of the gBlocks fragment used for cloning into pcDNA3 vector:

CGCGGGTACCTTCCCAGACCCCTGTTTGCTTTAGAGCCGAGCCGCGCCGATGCCCTCACACTCTGCGCCTC  
CTCTCCCAGGACTTTAACACCCCGCTCTCCTCCACCGACCAGGTGACCTTTTGCTCTGAGCCAGATCAGAG  
AAGAATTCGGTGTCCGTGCGGGACGATGCTGAGCAGGAGAGAATGATCACAGCCAGAGAAGGGGCCAGTCGGA  
AAATCTTATCTAAGCTTTCTTTGCCTACCCGTGCCTGGGAACCAGCAATGAAGAAGAGTTTGTCTTTGACAATG  
TTGGCTATGAAGGTGGTCTGGATGGCCTGGGCCCTTCTTCTCAGGTGGCCACCAGCACAGTCAGGATCTTGGGCA  
TGACTTGCCAGTCATGTGTGAAGTCCATTGAGGACAGGATTTCCAATTTGAAAGGCATCATCAGCATGAAGGTTT  
CCCTGGAACAAGGCAGTGCCACTGTGAAATATGTCCATCGGTTGTGTGCCTGCAACAGGTTTGCCATCAAATTG  
GGGACATGGGCTTCGAGGCCAGCATTGCAGAAGGAAAGGCAGCCTCCTGGCCCTCAAGGTCTTGCTGCCCAGG  
AGGCTGTGGTCAAGCTCCGGGTGGAGGGCATGACCTGCCAGTCCTGTGTGCTCAGCTCCATTGAAGGCAAGGTCCGGA  
AACTGCAAGGAGTAGTGAGAGTCAAAGTCTCACTCAGCAACCAAGAGGCCGTCATCACTTATCAGCCTTATCTCA  
TTCAGCCCGAAGACCTCAGGGACCATGTAAATGACATGGGATTTGAAGCTGCCATCAAGAGCAAAGTGGCTCCCT  
TAAGCCTGGGACCAATTGATATTGAGCGGTTACAAAGCACTAACCACAAAGAGACCTTTATCTTCTGCTAACCAGA  
ATTTTAATAATTCTGAGACCTTGGGGCACCAAGGAAGCCATGTGGTCAACCTCCAAGTGAAGATAGATGGAATGC  
ATTGTAAGTCTTGCCTTGAATATTGAAGAAAATATTGGCCAGCTCCTAGGGGTTCAAAGTATTCAAGTGTCTT  
TGGAGAACAAACTGCCCAAGTAAAGTATGACCCTTCTTGTTACCAGCCAGTGGCTCTGCAGAGGGCTATCGAGG  
CACTTCCACCTGGGAATTTTAAAGTTTCTTCTCCTGATGGAGCCGAAGGGAGTGGGACAGATCACAGGTCTTCCA  
GTTCTCATTCCCCTGGCTCCCCACCGAGAAACCAGGTCCAGGGCACATGCAGTACCACTCTGATTGCCATTGCCG  
GTCAGACCTGTGCATCCTGTGTCCATTCCATTGAAGGCATGATCTCCCAACTGGAAGGGGTGCAGCAAATATCGG  
TGCTTTGGCCGAAGGACTGCAACAGTTCTTTATAATCCCTCTGTAATTAGCCAGAAAGTACAGCTGCTA  
TAGAAGACATGGGATTTGAGGCTTCAGTTCGTTTCTGgtacgtagtgtgttgaggcatgtcctgagccttgt/cctcacc  
aagagccctgaaacctctgttctgaaaaacatatatttggctcttctaatttgaatattttctgacattttatcctagAAA  
GCTGTTCTACTAACCCTCTTGGAAACCACAGTGCTGGGAATTCCATGGTGCAAACTACAGATGGTACACCTACAT  
CTGTGCAGGAAGTGGCTCCCCACACTGGGAGGCTCCCTGCAACCATGCCCGGACATCTTGGCAAAGTCCCCAC  
AATCAACCAGAGCAGTGGCACCGCAGAAGTGCTTCTTACAGATCAAAGGCATGACCTGTGCATCCTGTGTGTCTA  
ACATAGAAAGGAATCTGCAGAAAGAAGCTGGTGTCTCTCCGTGTTGGTTGCCTTGATGGCAGGAAAGGCAGAGA  
TCAAGTATGACCCAGAGGTATCCAGCCCCCTCGAGATAGCTCAGTTCATCCAGGACC...

5'UTR

Exon 1

Exon 2

Intron 2

Exon 3

**ATG**: Start codon

**CA**: deleted in index patient (c.19\_20delCA)

**TAA**: Premature termination codon generated in c.19\_20delCA p.(Gln7Aspfs\*14)

**T**: mutated to „A“ in control c.915T>A p.(Cys305\*)

**...cttgt/cctcacc...**: between t/c a large part of the original intron 2 was deleted

**GGTACC**: *KpnI* site

**CTCGAG**: *XhoI* site

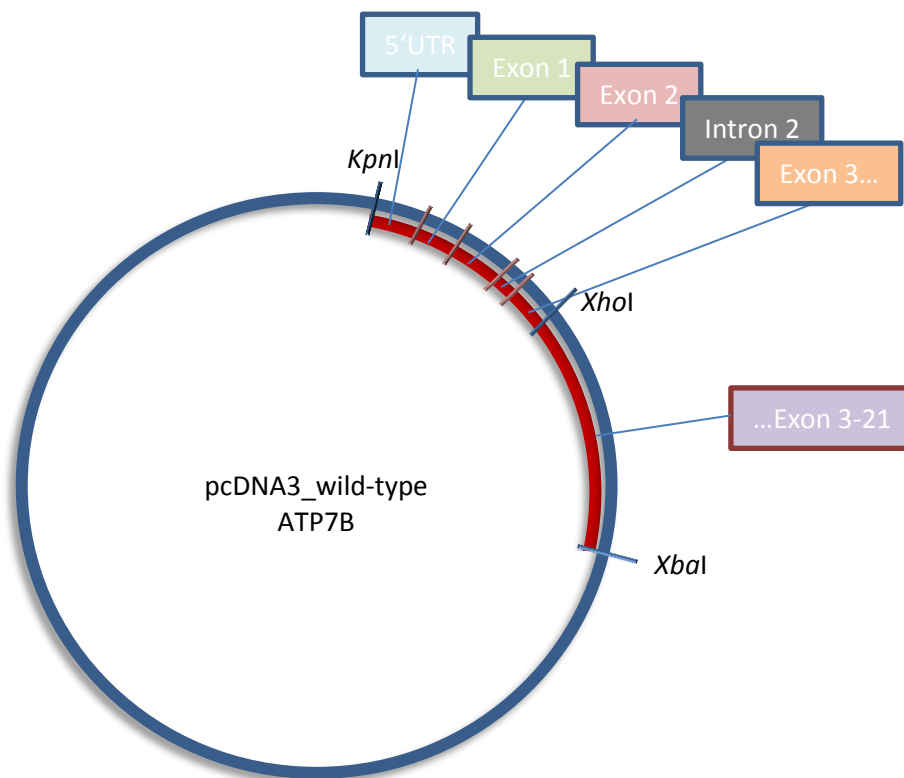
The 3' part of exon 3 and exon 4-21 of the *ATP7B* gene were amplified from pLB1080 (a gift from Ann Hubbard, Addgene plasmid # 37826) <sup>1</sup> using Phusion High Fidelity DNA-Polymerase (NEB, Frankfurt am Main, Germany). The following primers were used:

Fwd: CAGCCC**CTCGAG**ATAGCT;

Rev: AAGGAAT**CTAGAT**CAGATGTACTGCTCCTCATC

(underlined sequences show *XhoI* and *XbaI* restriction sites, respectively).

The PCR fragment was introduced to pcDNA3\_gBlocks using *XbaI* and *XhoI* restriction sites.



### Site-directed mutagenesis

Variants c.19\_20del p.(Gln7Aspfs\*14) and c.915T>A p.(Cys305\*) were introduced using Quick change site directed mutagenesis kit (Agilent) with the following primers:

#### For c.19\_20del

Forward: 5' CTGAGCAGGAGAGAGATCACAGCCAGAG 3'

Reverse: 5' CTCTGGCTGTGATCTCTCTCCTGCTCAG 3'

#### For c.915T>A

Forward: 5' GTATGACCCTTCTTGAACCAGCCCAGTGGC 3'

Reverse: 5' GCCACTGGGCTGGTTCAAGAAGGGTCATAC 3'

### Reporter vector

A sequence containing 4 tandem repeats of metal responsive element d of the mouse metallothionein I<sup>2</sup> promoter and a TATA box was synthesized by genscript (Piscataway, NJ, USA) and introduced into pGL3 basic vector (Promega) using *KpnI* and *NheI* restriction sites. The principle of the method was already described by van den Berghe et al<sup>3,4</sup>.

Sequence of the gBlocks fragment used for cloning into pGL3 basic vector:

TTAGTCTAGggtaccCTGCACTCCGCCCGAGCTCTGCACTCCGCCCGAGCTCTGCACTCCGCCCGAGC  
TCTGCACTCCGCCCGTCTGAGTCTAGAGGGTATATAATGGATCccgggctcgagatctgcgatctaagt  
aagcttggcattccgggtactggttggttaaagccacgctagcAGTCTAGAT

ggtacc: *KpnI* site

gctagc: *NheI* site

TATAA: TATA box

TGCACTC: metal responsive element d from mouse metallothionein I

All sequences of introduced fragments and variants were verified by Sanger sequencing.

### **Cell culture**

HEK293T cell line was kindly provided by Johann Meyer (Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany) and cultured in DMEM containing 10% FCS, 1% sodium pyruvate, and 100 U/mL penicillin/streptomycin at 37°C and 5% CO<sub>2</sub> in a humidified incubator.

### **Extraction of total RNA**

To isolate RNA, cells were lysed in TRIzol (Invitrogen/Life Technologies) or RLT buffer from RNeasy Mini Kit (Qiagen, Hilden, Germany). Snap-frozen liver tissue (stored at -80°C) was crushed in liquid nitrogen using a pestle and mortar before lysis and lysates were applied to QIAshredder Homogenizer columns (Qiagen). (Homogenized) lysates were further processed with the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA concentration and purity was measured

using nanophotometer P3000 (Implen, München, Germany). RNA integrity was routinely determined using the Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

### qRT-PCR

1,000 ng total RNA were reverse transcribed by M-Mul reverse transcriptase using random hexamer primers (Revert Aid First Strand cDNA synthesis kit, Thermo Fisher Scientific, Braunschweig, Germany). Real-Time PCR was performed in duplicates or triplicates with a LightCycler 1.0 (Roche, Basel, Switzerland). Primers and HybProbes were synthesized by TIB MOLBIOL (Berlin, Germany). The Master Mix for the PCR was prepared using the LightCycler FastStart DNA Master<sup>Plus</sup> HybProbe kit (Roche). To determine the expression values, a calibrator normalized relative quantification with efficiency correction was performed using *TBP* (TATA box-binding protein) as the reference gene. Patient samples were normalized to cDNA of Universal Human Reference RNA (Agilent, Santa Clara, USA). Cell line samples were normalized to pcDNA3\_empty vector.

### Primers and probes for qPCR

Gene	Name	Sequence	Function
<i>ATP7B</i>	ATP7B S	GACAGACCACGAGATGAAAGGAC	forward primer
	ATP7B A	AATAGCTCTGGCTGTCTTCCG	reverse primer
	ATP7B FL	TGTGCTCTGTGGGATGATCGCAA-FL	donor probe
	ATP7B LC	LC640-CGCAGACGCTGTCAAGCAGGAG-PH	acceptor probe
<i>TBP</i>	TBP F	TTCGGAGAGTTCTGGGATTGTA	forward primer
	TBP R	TGGACTGTTCTTCACTCTTGGC	reverse primer
	TBP FL	CCAAGCGGTTTGCTGCGGTAATC-FL	donor probe
	TBP LC	LC640-TGAGGATAAGAGAGCCACGAACCACG-PH	acceptor probe

FL: Fluorescein, PH: Phosphat, LC: LightCycler Red 640

### Northern blot

Northern blot probe was generated from RNA using SuperScriptII reverse transcriptase (Invitrogen/Life Technologies, Carlsbad, USA) and primer ATP7B RT-PCR rev. CDNA was amplified using primers ATP7B RT-PCR fwd, ATP7B RT-PCR rev and AmpliTaq Gold™ DNA Polymerase (Thermo fisher Scientific, Braunschweig, Germany).

### Primers to generate Northern blot probe

Gene	Name	Sequence	Function
<i>ATP7B</i>	ATP7B RT-PCR fwd	GTGGCTATTGACGGTGTGCT	forward primer
	ATP7B RT-PCR rev	GTGGTGAGTGGAGGCAAGTC	reverse primer

## Western Blot

Harvested cells were lysed in RIPA buffer. Protein of equal amounts of cells were electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel, and transferred onto a nitrocellulose membrane (GE Healthcare, Freiburg, Germany) by tank blotting. After Ponceau staining, the membrane was blocked for 1 h in non-fat milk. After incubation with primary antibody over night at 4 °C and secondary antibody at room temperature for 1 h the membrane was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).  $\beta$ -Actin served as loading control.

Antibody	Manufacturer
Anti-ATP7B Antibody, rabbit, polyclonal PA2262	Boster, Pleasonton, USA
Anti- $\beta$ -Actin Antibody mouse, monoclonal, #3700	Cell Signaling Technolog, Leiden, Netherlands
Goat anti-rabbit IgG-HRP, sc-2004	Santa Cruz Biotechnology, Heidelberg, Germany
Goat anti-mouse Ig-HRP, 554002	Becton Dickinson, Heidelberg, Germany

## Supplementary Methods - References

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3. van den Berghe PV, Folmer DE, Malingre HE *et al*: Human copper transporter 2 is localized in late endosomes and lysosomes and facilitates cellular copper uptake. *Biochem J* 2007; **407**: 49-59.
4. van den Berghe PV, Stapelbroek JM, Krieger E *et al*: Reduced expression of ATP7B affected by Wilson disease-causing mutations is rescued by pharmacological folding chaperones 4-phenylbutyrate and curcumin. *Hepatology* 2009; **50**: 1783-1795.