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Variants in *CPA1* are strongly associated with early onset chronic pancreatitis

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Chronic pancreatitis is an inflammatory disorder of the pancreas. We analyzed *CPA1*, encoding carboxypeptidase A1, in subjects with nonalcoholic chronic pancreatitis (cases) and controls in a German discovery set and three replication sets. Functionally impaired variants were present in 29/944 (3.1%) German cases and 5/3,938 (0.1%) controls (odds ratio (OR) = 24.9, $P = 1.5 \times 10^{-16}$). The association was strongest in subjects aged ≤10 years (9.7%; OR = 84.0, $P = 4.1 \times 10^{-24}$). In the replication sets, defective *CPA1* variants were present in 8/600 (1.3%) cases and 9/2,432 (0.4%) controls from Europe ($P = 0.01$), 5/230 (2.2%) cases and 0/264 controls from India ($P = 0.02$) and 5/247 (2.0%) cases and 0/341 controls from Japan ($P = 0.013$). The mechanism by which *CPA1* variants confer increased pancreatitis risk may involve misfolding-induced endoplasmic reticulum stress rather than elevated trypsin activity, as is seen with other genetic risk factors for this disease.

Chronic pancreatitis is an inflammatory condition that is characterized by abdominal pain and progressive damage to both exocrine and endocrine components of the pancreas, resulting in insufficiency of the organ with maldigestion and diabetes. Although alcohol abuse has long been recognized as a major risk factor for chronic pancreatitis, genetic susceptibility has emerged during the last two decades as a strong determinant of disease risk, particularly in the pediatric population[1].

Genetic studies performed so far have suggested that the development of intrapancreatic trypsin activity has a central role in disease pathogenesis. Thus, gain-of-function mutations in the gene encoding cationic trypsinogen (*PRSS1*, MIM276000) as well as loss-of-function variants in the genes encoding the pancreatic secretory trypsin inhibitor (*SPINK1*, MIM167790) and the trypsinogen-degrading enzyme chymotrypsin C (*CTRC,* MIM601405) increase the risk for chronic pancreatitis[2–8]. Consistent with the proposed pathogenic role of trypsin, a rapidly autodegrading variant of the gene encoding anionic trypsinogen (*PRSS2*, MIM601564) and a common *PRSS1* promoter variant protect against chronic pancreatitis[9,10].

Despite these recent advances, we still find that many individuals with chronic pancreatitis do not carry mutations in any of the known susceptibility genes, suggesting the involvement of other yet unidentified genes. In the present study, we investigated the role of *CPA1* in individuals with chronic pancreatitis. Digestive carboxypeptidases are pancreatic metalloproteases that hydrolyze C-terminal peptide bonds in dietary polypeptide chains[11]. Three different isoforms have been described in human pancreatic juice. A-type carboxypeptidases (CPA1 and CPA2) act on aromatic and aliphatic amino acid residues that are exposed by the action of chymotrypsins and elastases, whereas the B-type carboxypeptidase (CPB1) hydrolyzes C-terminal lysine and arginine residues generated by tryptic cleavages[11]. The gene encoding human CPA1 (MIM114850) maps to 7q32.2, spans approximately 8 kb and contains 10 exons. The inactive preproprotein comprises 419 amino acids, including a

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Table 1 Nonsynonymous *CPA1* variants in German subjects with nonalcoholic chronic pancreatitis and healthy controls

Exon	Nucleotide change	Amino acid change	Cases (%) (n = 944)	Controls (%) (n = 3,938)	P	OR	95% CI	Apparent activity	Secretion level
1	c.5G>A	p.Arg2Gln	0 (0)	2 (0.05)	1.0	–	–	103	92
2	**c.79C>T**	**p.Arg27Ter**	**0 (0)**	**2 (0.05)**	**1.0**	**–**	**–**	**0**	**0**
2	c.101C>T	p.Ala34Val	0 (0)	1 (0.03)	1.0	–	–	98	97
3	c.197G>A	p.Arg66Gln	0 (0)	2 (0.05)	1.0	–	–	60	55
3	c.281A>G	p.Gln94Arg	1 (0.1)	13 (0.3)	0.5	–	–	57	57
3	c.321C>G	p.Phe107Leu	0 (0)	1 (0.03)	1.0	–	–	112	100
3	c.371C>T	p.Thr124Ile	8 (0.9)	45 (1.1)	0.6	–	–	23	27
4	c.410C>G	p.Ala137Gly	1 (0.1)	0 (0)	0.2	–	–	52	56
5	c.497G>A	p.Gly166Asp	5 (0.5)	20 (0.5)	1.0	–	–	73	66
5	**c.542G>A**	**p.Arg181Gln**	**0 (0)**	**1 (0.03)**	**1.0**	**–**	**–**	**1**	**39**
6	c.622G>A	p.Ala208Thr (het)	71 (7.5)	266 (6.8)	0.4	–	–	81	73
6	c.622G>A	p.Ala208Thr (hom)	1 (0.1)	1 (0.03)	0.4	–	–	–	–
6	c.622G>T	p.Ala208Ser	0 (0)	1 (0.03)	1.0	–	–	91	83
6	**c.673G>A**	**p.Gly225Ser**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**4**	**12**
7	**c.710G>A**	**p.Arg237His**	**0 (0)**	**2 (0.05)**	**1.0**	**–**	**–**	**0**	**81**
7	**c.751G>A**	**p.Val251Met**	**2 (0.2)**	**0 (0)**	**0.1**	**–**	**–**	**0**	**0**
7	**c.758C>G**	**p.Pro253Arg**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**0**
7	**c.768C>G**	**p.Asn256Lys**	**7 (0.7)**	**0 (0)**	**9.9×10^{-6}**	**NC**	**NC**	**0**	**0**
7	c.775G>A	p.Ala259Thr	0 (0)	1 (0.03)	1.0	–	–	85	82
8	**c.811T>C**	**p.Cys271Arg**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**1**	**0**
8	**c.829G>A**	**p.Gly277Ser**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**0**
8	**c.839C>A**	**p.Ala280Asp**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**5**
8	**c.847G>A**	**p.Glu283Lys**	**2 (0.2)**	**0 (0)**	**0.1**	**–**	**–**	**0**	**0**
8	**c.982G>A**	**p.Glu328Lys**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**9**	**42**
9	c.1009G>C	p.Val337Leu	0 (0)	1 (0.03)	1.0	–	–	64	61
Intron 9	**c.1073-2A>G**	**p.Tyr358fs[a]**	**3 (0.3)**	**0 (0)**	**0.007**	**NC**	**NC**	**0**	**0**
10	**c.1085G>A**	**p.Gly362Glu**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**6**
10	**c.1126T>C**	**p.Ser376Pro**	**2 (0.2)**	**0 (0)**	**0.1**	**–**	**–**	**0**	**7**
10	**c.1144C>T**	**p.Arg382Trp**	**5 (0.5)**	**0 (0)**	**0.0003**	**NC**	**NC**	**0**	**31**
10	c.1157G>A	p.Arg386His	0 (0)	1 (0.03)	1.0	–	–	92	97
10	c.1193C>T	p.Pro398Leu	0 (0)	1 (0.03)	1.0	–	–	42	64
10	c.1217C>G	p.Ala406Gly	0 (0)	1 (0.03)	1.0	–	–	137	114
10	**c.1247delA**	**p.Asn416fs**	**1 (0.1)**	**0 (0)**	**0.2**	**–**	**–**	**11**	**15**
10	c.1251C>A	p.His417Gln	0 (0)	1 (0.03)	1.0	–	–	62	54
10	c.1253C>T	p.Pro418Leu	0 (0)	1 (0.03)	1.0	–	–	91	99
All variants with apparent activity <20%			**29 (3.1%)**	**5 (0.1)**	**1.5×10^{-16}**	**24.9**	**9.6–64.6**	**–**	**–**

P values were determined by Fisher's exact test. Apparent CPA1 activity and secretion levels are expressed as a percentage of the wild-type values. Apparent activity corresponds to the CPA1 activity measured in the conditioned medium of transfected cells after activation with trypsin and CTRC (Online Methods). Thus, the apparent activity reflects the combined effects of the variants on secretion, catalytic activity and degradation by trypsin and/or CTRC. Secretion level indicates the concentration of proCPA1 in the conditioned medium measured by SDS-PAGE and densitometry (Online Methods). Alterations in bold indicate variants with less than 20% apparent activity.

[a]A splice-site variant that was modeled functionally as intron retention (as described in the **Supplementary Note**). Het, heterozygous; hom, homozygous; NC, not calculated, as the variant was not detected in the controls, rendering the OR infinite.

16-amino-acid secretory signal peptide and a 94-amino-acid propeptide. Activation of the proenzyme (proCPA1) to CPA1 is catalyzed by the sequential action of trypsin and CTRC, which cleave and degrade the propeptide[12]. After trypsinogens, proCPA1 is the second largest component of pancreatic juice, contributing more than 10% of the total protein[13].

We performed direct DNA sequencing of all ten *CPA1* exons in 944 cases and 3,938 control subjects of German origin. Considering variants in the coding regions and flanking splice sites, we identified 31 missense variants, 1 nonsense variant, 1 frame-shift variant and 1 splice-site variant and found that 3 variants were significantly enriched in cases (**Table 1**). Functional analysis demonstrated that 17/34 (50%) variants resulted in a marked (>80%) loss of apparent CPA1 activity, a term we use to describe the combined effects of the variants on secretion, proteolytic stability and catalytic competence (**Table 1**, **Supplementary Fig. 1** and Online Methods). The majority of these variants were located in exons 7, 8 and 10. Notably, 14 out of 17 (82%) functionally impaired variants were present in cases exclusively, including the c.768C>G (p.Asn256Lys) variant, which we detected in seven cases. Thus, *CPA1* variants with less than 20% apparent activity were significantly over-represented in the chronic pancreatitis group (29/944, 3.1%) as compared to in controls (5/3,938, 0.1%) (OR = 24.9, 95% confidence interval (CI) 9.6–64.6, $P = 1.5 \times 10^{-16}$) (**Table 1**). No individual was compound heterozygous or

Table 2 Distribution of functionally impaired *CPA1* variants in different age groups of German subjects with nonalcoholic chronic pancreatitis

Age of cases	Cases (%)	Controls (%)	P	OR	95% CI
All	29/944 (3.1)	5/3,938 (0.1)	1.5×10^{-16}	24.9	9.6–64.6
>20 years	2/358 (0.6)	5/3,938 (0.1)	0.2	–	–
≤20 years	27/586 (4.6)	5/3,938 (0.1)	6.8×10^{-20}	38.0	14.6–99.1
≤10 years	22/228 (9.7)	5/3,938 (0.1)	4.1×10^{-24}	84.0	31.5–224.1

P values were determined by Fisher's exact test. Alterations with less than 20% apparent activity were included.

Table 3 Nonsynonymous *CPA1* variants in the European replication study

Exon	Nucleotide change	Amino acid change	Cases (%) (n = 600)	Controls (%) (n = 2,432)	P	OR	95% CI	Apparent activity	Secretion level
1	c.5G>A	p.Arg2Gln	0 (0)	6 (0.2)	0.6	–	–	103	92
2	**c.79C>T**	**p.Arg27Ter**	**1 (0.2)**	**5 (0.2)**	**0.7**	**–**	**–**	**0**	**0**
2	**c.80G>C**	**p.Arg27Pro**	**0 (0)**	**1 (0.04)**	**1.0**	**–**	**–**	**0**	**0**
Intron 2	**c.148-1G>A**	**p.Leu50_Glu127del**[a]	**0 (0)**	**1 (0.04)**	**1.0**	**–**	**–**	**0**	**0**
3	c.197G>A	p.Arg66Gln	0 (0)	1 (0.04)	1.0	–	–	60	55
3	c.241T>C	p.Ser81Pro	0 (0)	1 (0.04)	1.0	–	–	53	57
3	c.281A>G	p.Gln94Arg	0 (0)	9 (0.4)	1.0	–	–	57	57
3	c.313T>C	p.Phe105Leu	1 (0.2)	0 (0)	0.2	–	–	109	99
3	c.334C>T	p.Arg112Cys	1 (0.2)	0 (0)	0.2	–	–	69	78
3	c.371C>T	p.Thr124Ile	3 (0.5)	14 (0.6)	1.0	–	–	23	27
4	c.389A>C	p.Asp130Ala	0 (0)	1 (0.04)	1.0	–	–	77	68
5	c.497G>A	p.Gly166Asp	1 (0.2)	11 (0.5)	0.5	–	–	73	66
6	c.604C>A	p.Gln202Lys	1 (0.2)	0 (0)	0.2	–	–	114	104
6	c.622G>A	p.Ala208Thr	45 (7.5)[b]	143 (5.9)[b]	0.1	–	–	81	73
6	**c.686C>T**	**p.Thr229Met**	**0 (0)**	**1 (0.04)**	**1.0**	**–**	**–**	**0**	**0**
6	c.695C>T	p.Thr232Met	1 (0.2)	0 (0)	0.2	–	–	87	80
7	**c.751G>A**	**p.Val251Met**	**1 (0.2)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**0**
8	**c.809C>G**	**p.Pro270Arg**	**1 (0.2)**	**0 (0)**	**0.2**	**–**	**–**	**9**	**14**
8	**c.941A>G**	**p.Tyr314Cys**	**1 (0.2)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**23**
8	**c.954_955delCA**	**p.Tyr318Ter**	**2 (0.3)**	**0 (0)**	**0.04**	**NC**	**NC**	**0**	**0**
9	c.1010T>C	p.Val337Ala	0 (0)	1 (0.04)	1.0	–	–	63	90
Intron 9	**c.1072+1G>T**	**p.Asp330fs**[a]	**0 (0)**	**1 (0.04)**	**1.0**	**–**	**–**	**0**	**0**
Intron 9	**c.1073-2A>G**	**p.Tyr358fs**[a]	**1 (0.2)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**0**
10	c.1115G>A	p.Gly372Asp	1[c] (0.2)	0 (0)	0.2	–	–	25	34
10	c.1203G>C	p.Lys401Asn	1 (0.2)	0 (0)	0.2	–	–	115	103
10	**c.1217C>T**	**p.Ala406Val**	**1 (0.2)**	**0 (0)**	**0.2**	**–**	**–**	**0**	**87**
All variants with apparent activity <20%			**8 (1.3)**	**9 (0.4)**	**0.01**	**3.6**	**1.4–9.5**	**–**	**–**

P values were determined by Fisher's exact test. Apparent CPA1 activity and secretion levels were measured as described in **Table 1** and are expressed as a percentage of the wild-type values. Alterations in bold indicate variants with less than 20% apparent activity.
[a]The functional effects of the splice-site variants c.148-1G>A, c.1072+1G>T and c.1073-2A>G were modeled as skipping of exon 3, skipping of exon 9 and retention of intron 9, respectively (as described in the **Supplementary Note**). [b]One individual was homozygous for p.Ala208Thr. [c]This individual was homozygous for this variant. NC, not calculated, as the variant was not detected in controls, rendering the OR infinite.

homozygous for a defective *CPA1* variant. Variants located in noncoding regions and synonymous variants located in coding regions are listed in **Supplementary Table 1**.

We observed that cases bearing a defective *CPA1* variant were younger than those without a *CPA1* alteration. In the German chronic pancreatitis group, the majority of *CPA1* variants with less than 20% apparent activity were present in cases at or below 20 years of age (27/586, 4.6%) (OR = 38.0, 95% CI 14.6–99.1, $P = 6.8 \times 10^{-20}$). This finding was even more significant in a subgroup of cases at or below 10 years of age. In this group, 22/228 (9.7%) carried an impaired *CPA1* variant (OR = 84.0, 95% CI 31.5–224.1, $P = 4.1 \times 10^{-24}$) (cases ≤10 years of age compared to cases ≤20 years of age, $P = 0.007$; cases ≤10 years of age compared to all cases, $P = 7.6 \times 10^{-5}$) (**Table 2**).

We also investigated all *CPA1* exons in 465 German subjects with alcohol-related chronic pancreatitis. Only 2/465 (0.4%) of these individuals were heterozygous for a defective *CPA1* variant: c.954_955delCA (p.Tyr318Ter) in one individual and c.811T>C (p.Cys271Arg) in the other. This indicates that loss-of-function alterations in *CPA1* have a minor role in alcoholic pancreatitis.

To confirm the association of nonalcoholic chronic pancreatitis and *CPA1* in an independent European cohort, we sequenced all *CPA1* exons in 600 cases and 2,432 control subjects originating from France, the Czech Republic and Poland. Again, variants with less than 20% apparent activity were significantly over-represented in chronic pancreatitis cases (8/600, 1.3%) compared to in ethnically matched controls (9/2,432, 0.4%) (OR = 3.6, 95% CI 1.4–9.5, $P = 0.01$) (**Table 3**). One subject with chronic pancreatitis was homozygous for the c.1115G>A (p.Gly372Asp) variant.

To investigate the significance of *CPA1* variants in subjects of non-European descent, we sequenced all ten exons in 230 cases and 264 controls of Indian origin and 247 cases and 341 controls from Japan. Overall, 2.2% (5/230) of the Indian cases but none of the controls

Table 4 Nonsynonymous *CPA1* variants in Indian subjects with nonalcoholic chronic pancreatitis and healthy controls

Exon	Nucleotide change	Amino acid change	Cases (%) (n = 230)	Controls (%) (n = 264)	P	OR	95% CI	Apparent activity	Secretion level
2	c.94G>C	p.Asp32His	1 (0.4)	0 (0)	0.5	–	–	79	75
5	c.506G>A	p.Arg169His	4 (1.7)	0 (0)	0.046	NC	NC	24	23
6	c.622G>A	p.Ala208Thr	6 (2.6)	7 (2.7)	1.0	–	–	81	73
8	**c.922T>C**	**p.Tyr308His**	**5 (2.2)**	**0 (0)**	**0.02**	**NC**	**NC**	**3**	**17**
All variants with apparent activity <20%			**5 (2.2)**	**0 (0)**	**0.02**	**NC**	**NC**	**–**	**–**

P values were determined by Fisher's exact test. Apparent CPA1 activity and secretion levels were measured as described in **Table 1** and are expressed as a percentage of the wild-type values. Alterations in bold indicate variants with less than 20% apparent activity. NC, not calculated, as the variant was not detected in controls, rendering the OR infinite.

Table 5 Nonsynonymous *CPA1* variants in Japanese subjects with nonalcoholic chronic pancreatitis and healthy controls

Exon	Nucleotide change	Amino acid change	Cases (%) (n = 247)	Controls (%) (n = 341)	P	OR	95% CI	Apparent activity	Secretion level
4	c.410C>G	p.Ala137Gly	1 (0.4)	0 (0)	0.42	–	–	52	56
7	**c.713A>T**	**p.Lys238Met**	**1 (0.4)**	**0 (0)**	**0.42**	**–**	**–**	**0**	**3**
7	**c.751G>A**	**p.Val251Met**	**2 (0.8)**	**0 (0)**	**0.18**	**–**	**–**	**0**	**0**
7	**c.764G>T**	**p.Arg255Met**	**1 (0.4)**	**0 (0)**	**0.42**	**–**	**–**	**0**	**86**
9	c.1021G>A	p.Ala341Thr	37 (15.0)	53 (15.5)	1.0	–	–	99	85
10	**c.1079-27_1111dup60**	**p.Thr368_Tyr369ins20**[a]	**1 (0.4)**	**0 (0)**	**0.42**	**–**	**–**	**0**	**49**
All variants with apparent activity <20%			**5 (2.0)**	**0 (0)**	**0.013**	**NC**	**NC**	**–**	**–**

P values were determined by Fisher's exact test. Apparent CPA1 activity and secretion levels were measured as described in **Table 1** and are expressed as a percentage of the wild-type values. Alterations in bold indicate variants with less than 20% apparent activity.
[a]The functional effect of the variant c.1079-27_1111dup60 was modeled as an insertion of 20 amino acids between Thr368 and Tyr369 (as described in the **Supplementary Note**).
NC, not calculated, as the variant was not detected in controls, rendering the OR infinite.

carried a defective *CPA1* variant (P = 0.02) (**Table 4**). In the Japanese sample collection, 2.0% (5/247) of the cases but none of the controls carried an impaired *CPA1* variant (P = 0.013) (**Table 5**). No individual from India or Japan was compound heterozygous or homozygous for a defective *CPA1* variant.

Chronic pancreatitis is a complex multigenic disease, and affected individuals often carry mutations in several disease-associated genes. To elucidate the relationship between *CPA1* alterations and *PRSS1*, *SPINK1*, *CTRC* and *CFTR* variants, we investigated all German subjects with chronic pancreatitis for variants in *PRSS1* (encoding p.Ala16Val, p.Asn29Ile and p.Arg122His), *SPINK1* (encoding p.Asn34Ser and c.194+2T>C), *CTRC* (encoding p.Arg254Trp and p.Lys247_Arg254del) and *CFTR* (encoding p.Phe508del). In total, 50/944 (5.3%) individuals carried a heterozygous *PRSS1* variant, 147/944 (15.6%) were positive for *SPINK1* p.Asn34Ser (121 heterozygotes and 18 homozygotes) and c.194+2T>C (20 heterozygotes, 12 of which were compound heterozygous with p.Asn34Ser), 28/944 (3.0%) were positive for a *CTRC* variant (21 instances of p.Arg254Trp and 7 occurrences of p.Lys247_Arg254del), and 42/944 (4.5%) were positive for *CFTR* p.Phe508del. Together, 273/944 (28.9%) of the cases showed at least one of the above-mentioned genetic alterations, and 24/944 (2.5%) of the cases were *trans* heterozygous. However, only 1/29 (3.6%) cases with a defective *CPA1* variant was *trans* heterozygous; this subject carried the *CPA1* c.1073-2A>G alteration (inherited from the mother) and the *SPINK1* p.Asn34Ser variant (inherited from the father). This suggests a limited interaction of *CPA1* variants with variants in other susceptibility genes and stands in contrast with the high number of *trans* heterozygotes for *SPINK1*, *CTRC* and/or *CFTR* variants, as was described recently[14].

The mechanism by which loss-of-function *CPA1* variants predispose to chronic pancreatitis is not intuitively apparent. We found no detectable effect of CPA1 on trypsinogen activation, trypsin activity or degradation of trypsin and trypsinogen by CTRC (**Supplementary Fig. 2**), indicating that *CPA1* variants do not exert their effect by increasing intrapancreatic trypsin activity. However, the low apparent activity of most of the defective variants was due to markedly reduced secretion (**Tables 1**–**5** and **Supplementary Figs. 1** and **3**), raising the possibility that CPA1 mutants misfold in the endoplasmic reticulum and cause endoplasmic reticulum stress, as has been demonstrated previously for some PRSS1 and CTRC mutants[15,16]. Indeed, expression of the most frequently found p.Asn256Lys variant in AR42J rat acinar cells resulted in endoplasmic reticulum stress, as evidenced by increased splicing of *Xbp1* and elevated mRNA levels of the chaperones *Hspa5* (encoding BiP) and *Calr* (encoding calreticulin) (**Fig. 1**). Considering that CPA1 is one of the most abundant proteins synthesized by the pancreas, misfolding-induced endoplasmic reticulum stress seems to be a plausible mechanism to explain the clinical effects of heterozygous CPA1 variants.

In summary, loss-of-function *CPA1* variants are strongly associated with nonalcoholic chronic pancreatitis, especially early onset disease. Although there was evidence of heterogeneity in the spectrum of variants present in different populations, the identification of functionally impaired *CPA1* variants in both European and non-European sample collections establishes its global role in the pathogenesis of chronic pancreatitis.

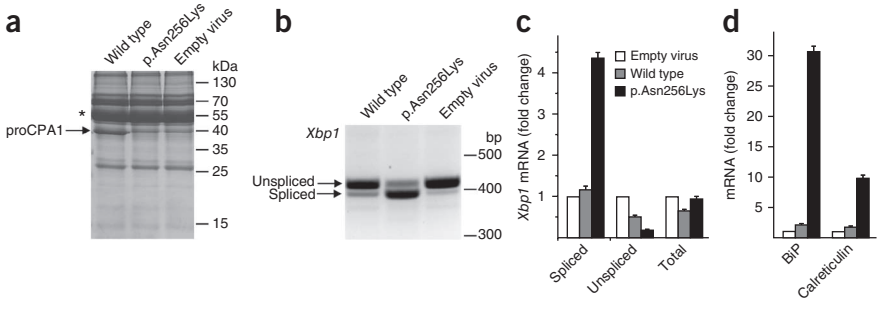

Figure 1 Endoplasmic reticulum stress induced by the p.Asn256Lys CPA1 variant. (**a**) AR42J rat acinar cells were transfected with the indicated wild-type, mutant or empty adenovirus vectors for 24 h using 4 × 10^7 plaque-forming units (pfu) per ml of virus. Conditioned media (200 µl) were precipitated with trichloroacetic acid (10% final concentration) and analyzed by SDS-PAGE and Coomassie blue staining. The p.Asn256Lys mutant had a complete lack of secretion. The faint band at 40 kDa represents an endogenous protein also found in the medium from cells infected with the empty virus. The asterisk indicates the characteristically strong amylase band. See the Online Methods for experimental details. A representative gel of three independent transfections is shown. (**b**) *Xbp1* splicing was assessed by RT-PCR and agarose gel electrophoresis with ethidium bromide staining. A representative gel of three independent experiments is shown. (**c**) Levels of spliced, unspliced and total *Xbp1* mRNA were measured by quantitative real-time PCR and are expressed as fold changes relative to the levels measured in cells transfected with empty adenovirus. (**d**) Quantitative real-time PCR measurements of *Hspa5* (encoding BiP) and *Calr* (encoding calreticulin) mRNA were performed as described in the Online Methods and are expressed as fold changes relative to the levels measured in cells transfected with empty adenovirus. Error bars (**c**,**d**), s.d. (n = 3 independent experiments).

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GenBank: carboxypeptidase A1 (*CPA1*), NT_007933.15 (*Homo sapiens* chromosome 7 genomic contig, GRCh37.p5); NM_001868.2 (human *CPA1* mRNA sequence).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.W. and M.S.-T. conceived, designed and directed the study. G.R.C., J.-M.C., J.R., A.M. and H.W. designed, performed and interpreted genetic analyses with substantial contributions from D.B., F.B., M. Braun, S. Bhaskar, C.D., D.L., E.M., S.P., S.S., A.S.-T., K.K., E.N., Y.K., T.S., J.T. and A. Schneider. S. Beer, M. Bence, R.S., A. Szabó, A. Schnúr and M.S.-T. carried out functional characterization of CPA1 variants. H.W., M.S.-T. and S. Beer wrote the manuscript with substantial contributions from G.R.C., J.-M.C., J.R. and A.M. O.L. provided oligonucleotides. All other coauthors recruited study subjects, collected clinical data and provided genomic DNA samples. All authors approved the final manuscript and contributed critical revisions to its intellectual content.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study population. The medical ethical review committees of all participating study centers approved this study. All study subjects gave informed consent. We enrolled 944 unrelated German individuals with a diagnosis of nonalcoholic chronic pancreatitis and 465 subjects with alcohol-related chronic pancreatitis. In the replication study, we investigated 600 unrelated subjects with nonalcoholic chronic pancreatitis originating from France (n = 456), the Czech Republic (n = 21) and Poland (n = 123). In addition, we investigated unrelated subjects affected with nonalcoholic chronic pancreatitis from India (n = 230) and Japan (n = 247). The diagnosis of chronic pancreatitis was based on two or more of the following findings: presence of a typical history of recurrent pancreatitis, pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreaticography or by magnetic resonance imaging of the pancreas, and/or pathological sonographic findings. Alcoholic chronic pancreatitis was diagnosed in subjects who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than 2 years. Control subjects were recruited from Germany (n = 3,938), France (n = 2,000), the Czech Republic (n = 235), Poland (n = 197), India (n = 264) and Japan (n = 341).

Mutation screening. We designed primers complementary to intronic sequences flanking *CPA1* exons on the basis of the published nucleotide sequence (GenBank NT_007933.15) (**Supplementary Table 2**). After PCR amplification, the entire coding region and the exon-intron boundaries were sequenced. All mutations were confirmed with a second independent PCR reaction. In the German laboratories, we performed PCR using 0.75 U AmpliTaq Gold polymerase (Perkin Elmer), 400 µmol/l deoxynucleoside triphosphates and 0.1 µmol/l primers in a total volume of 25 µl. The cycle conditions were as follows: initial denaturation for 12 min at 95 °C; 48 cycles of 20 s denaturation at 95 °C, 40 s annealing at 64 °C and 90 s primer extension at 72 °C; and a final extension step for 2 min at 72 °C. PCR products were digested with Antarctic phosphatase (New England Biolabs) or shrimp alkaline phosphatase (USB) and exonuclease I (New England Biolabs). Cycle sequencing was performed using BigDye terminator mix (Applied Biosystems) with a 56 °C annealing temperature. The reaction products were purified with ethanol precipitation and loaded onto an ABI 3730 or ABI 3100-Avant fluorescence sequencer (Applied Biosystems).

Functional characterization of CPA1 variants. We investigated the functional consequences of *CPA1* alterations by transient transfection of HEK 293T cells (#Q401, GenHunter) with wild-type and mutant constructs and analyzing the conditioned medium for the amount of proCPA1 protein constitutively secreted using densitometry of stained gels and CPA1 activity after activation with trypsin and CTRC.

Expression plasmids, mutagenesis and adenoviruses. Construction of the pcDNA3.1(-) human *CPA1* expression plasmid has been reported previously[12]. The coding DNA in this plasmid was derived from IMAGE clone #3949850 (GenBank accession BC005279), which contains a c.827A>G (p.His276Arg) alteration. This error was corrected by back mutating Arg276 to histidine. *CPA1* mutants were created by PCR mutagenesis and ligated into the pcDNA3.1(-) expression plasmid. Recombinant adenoviruses carrying wild-type *CPA1* or the p.Asn256Lys mutant were generated by Viraquest. Details regarding the construction of the *CPA1* splice-site and duplication mutant expression plasmids are provided in the **Supplementary Note**.

Cell culture and transfection. HEK 293T cells were cultured in six-well tissue culture plates (1.5×10^6 cells per well) in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 4 mM glutamine and 1% penicillin and streptomycin at 37 °C. Transfections were carried out at 90% confluence using 10 µl Lipofectamine 2000 (Invitrogen) and 4 µg expression plasmids in a final volume of 2 ml DMEM. After overnight incubation, cells were washed, and the transfection medium was replaced with 2 ml OPTI-MEM I Reduced Serum Medium (Invitrogen). The conditioned OPTI-MEM media were harvested after 48 h of incubation. AR42J rat pancreatic acinar cells (American Type Culture Collection #CRL-1492) were maintained in DMEM supplemented with 20% fetal bovine serum, 4 mM glutamine and 1% penicillin and streptomycin at 37 °C. Before transfection, cells were plated in six-well plates (10^6 cells per well) and grown in the presence of a 100 nM concentration of dexamethasone for 48 h to induce differentiation. Infections with adenoviruses were performed using 4×10^7 pfu per ml of the final adenovirus concentrations in a total volume of 1 ml OPTI-MEM in the presence of dexamethasone (100 nM final concentration).

CPA1 activity assay. The enzymatic activity of CPA1 was determined after activation with trypsin and CTRC using the N-[4-methoxyphenylazoformyl]-L-phenylalanine substrate[17] with minor modifications of our previously published conditions[12]. The CPA1 activity measured in the conditioned medium of transfected cells is referred to as the 'apparent activity' and reflects the combined effects of the variants on the secreted amounts of proCPA1, the proteolytic degradation during activation and the catalytic activity of the activated CPA1. To activate proCPA1, an aliquot (20 µl) of the conditioned medium was supplemented with 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05% Tween 20, 100 nM human cationic trypsin and 50 nM human CTRC (final concentrations in a final volume of 40 µl) and incubated at 37 °C for 60 min. CPA1 activity was then measured by adding 50 µl assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 0.05% Tween 20) and 10 µl substrate (final concentration of 60 µM) to the activation mix. The decrease in absorbance was followed at 350 nm for 2 min. Rates of substrate cleavage were calculated from fits to the initial linear portion of the curves and are expressed as a percentage of the wild-type rate, which was set to 100%. The wild-type activity corresponded to 116 ± 34 milliOD min^{-1} (average ± s.d.), which equals a 262 ± 77 nM s^{-1} substrate cleavage rate.

Measurement of proCPA1 secretion. Secreted amounts of proCPA1 protein in the conditioned medium were determined by SDS-PAGE and densitometry. An aliquot (200 µl) of the medium was precipitated with trichloroacetic acid (10% final concentration), the precipitate was recovered by centrifugation, dissolved in 20 µl Laemmli sample buffer containing 100 mM dithiothreitol (DTT) (final concentration) and heat denatured at 95 °C for 5 min. Electrophoretic separation was performed on 15% SDS-PAGE mini gels in standard Tris-glycine buffer, and gels were stained with Brilliant Blue R-250. Quantification of bands was carried out with the GelDocXR+ gel documentation system and Image Lab 3.0 software (Bio-Rad).

Measurement of endoplasmic reticulum stress. To study endoplasmic reticulum stress, we generated recombinant adenoviruses carrying either wild-type *proCPA1* or the p.Asn256Lys mutant, infected AR42J rat pancreatic acinar cells (#CRL-1492, American Type Culture Collection (ATCC)) with these viruses and measured endoplasmic reticulum stress markers as described below.

RT-PCR analysis and real-time PCR. Total RNA was extracted from AR42J cell lysates using an RNeasy mini kit (Qiagen). RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *Xbp1* (encoding X-box binding protein 1) splicing was studied by PCR using a primer set that flanked the spliced region and amplified both the spliced and unspliced forms (**Supplementary Table 3**). PCR was carried out using the *Taq* DNA Polymerase kit (Qiagen) with the following conditions: 10 min initial denaturation at 95 °C followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 52 °C, 30 s extension at 72 °C and a final extension at 72 °C for 5 min. The PCR products were resolved on 2% agarose gels and stained with ethidium bromide. Quantification of mRNA expression was performed by real-time PCR (7500 Real Time PCR System, Applied Biosystems). *Xbp1* expression was measured with SYBR Green (PCR Master Mix, Applied Biosystems) using different primer sets for the spliced, unspliced and total mRNA (**Supplementary Table 3**). Levels of *Hspa5* (encoding the immunoglobulin-binding protein BiP) and *Calr* (encoding calreticulin) mRNA were determined using TaqMan primers (rat *Hspa5*, Rn00565250_m1; rat *Calr*, Rn00574451_m1) with TaqMan Universal PCR Mastermix (Applied Biosystems). Real-time PCR conditions were as follows: 2 min equilibration at 50 °C, 10 min denaturation and enzyme activation at 95 °C followed by 40 two-step cycles of 15 s at 95 °C and 60 s at 60 °C. Gene expression was quantified using the comparative C_T method ($\Delta\Delta C_T$ method). Threshold cycle (C_T) values were determined using the 7500 System Sequence Detection Software 1.3.

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Expression levels of target genes were first normalized to the *Gapdh* internal control gene (ΔC_T) and then to the expression levels measured in cells infected with empty adenovirus ($\Delta\Delta C_T$). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$.

Statistics. The significance of the differences between mutation frequencies in affected individuals and controls was tested by two-tailed Fisher's exact test. Additional ORs were calculated using SAS/STAT software (v 9.1) and GraphPad Prism (v 4.03).

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