Supplementary Online Material for

# Chymotrypsin C (CTRC) alterations that diminish activity or secretion are associated with chronic pancreatitis

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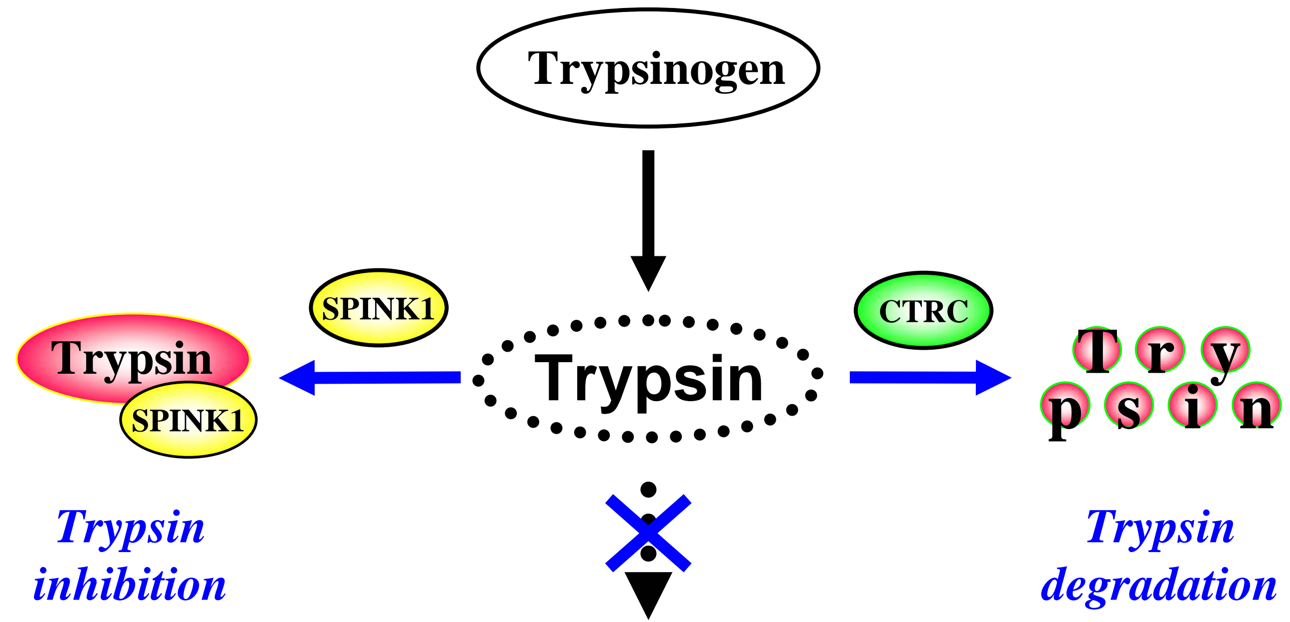
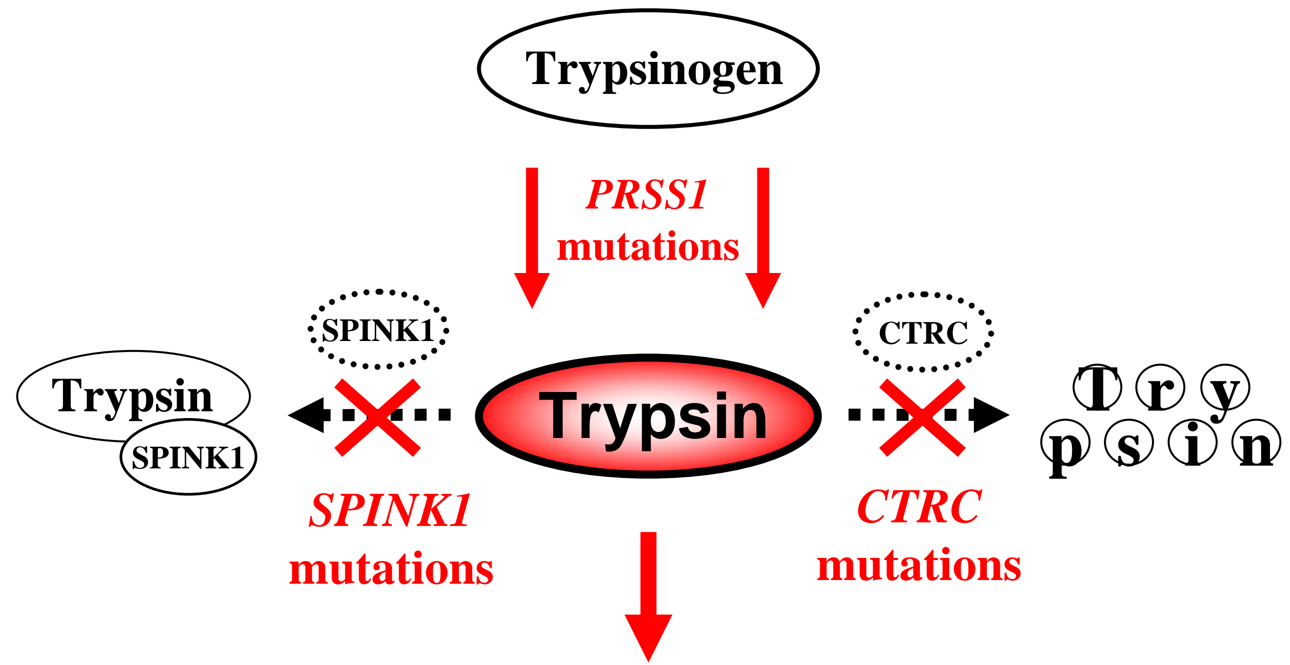
## A. Normal Pancreas




**Autodigestion/Pancreatitis**

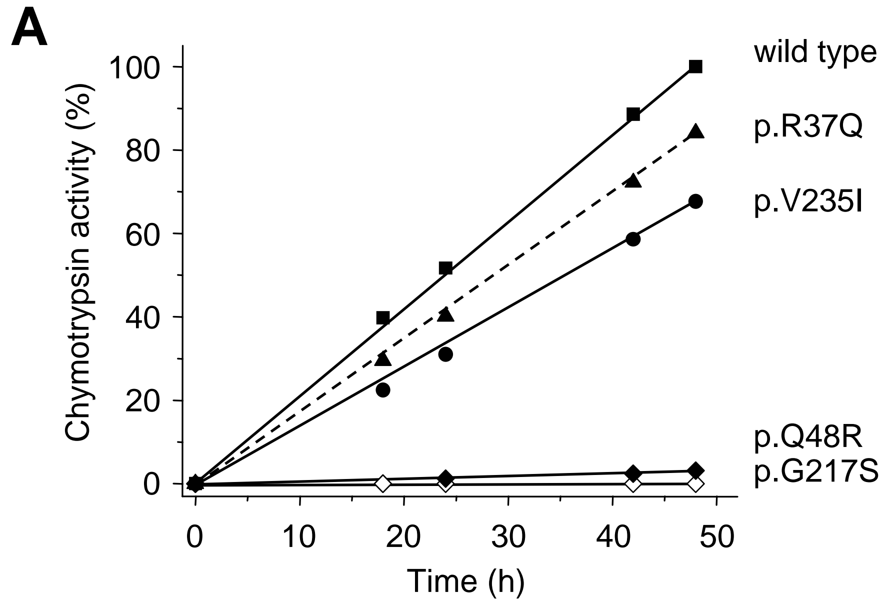
## B. Pancreatitis

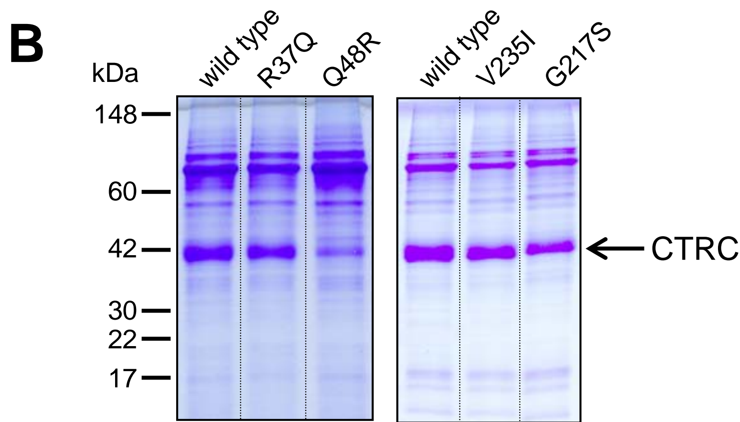


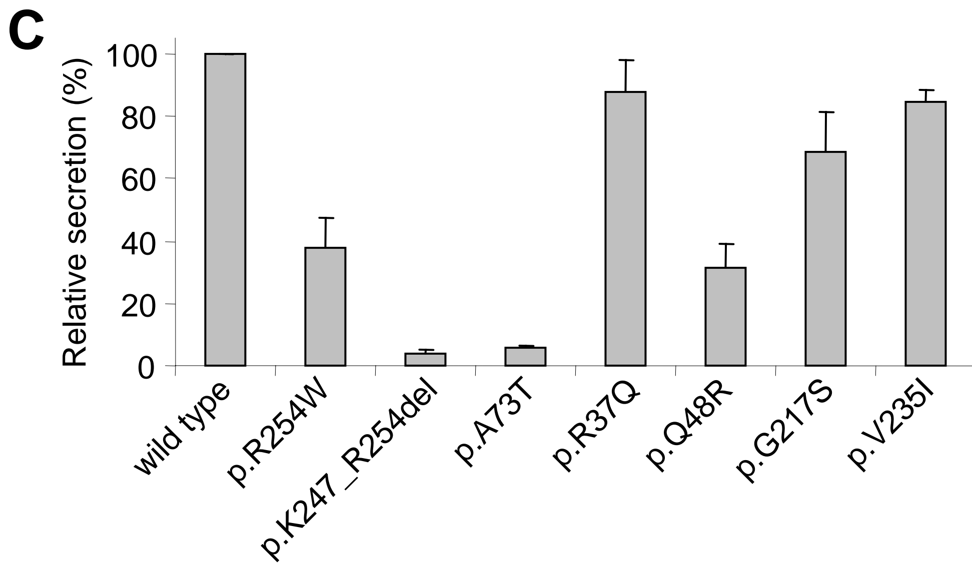

**Autodigestion/Pancreatitis**

Supplementary Figure 1

**Supplementary Figure 1.** The trypsin dependent pathological model of chronic pancreatitis. Sustained, intrapancreatic conversion of trypsinogen to trypsin plays a central role in the development of chronic pancreatitis. Pancreatic defense mechanisms against unwanted trypsin activity include trypsin inhibition by SPINK1 and trypsin degradation by CTRC. *PRSS1* missense variants stimulate activation of trypsinogen to trypsin or block degradation of active trypsin, whereas *SPINK1* alterations reduce inhibitor levels and thus compromise trypsin inhibition. *CTRC* variations abolish activity or decrease secretion of CTRC and thereby impair trypsin degradation.




Supplementary Figure 2

**Supplementary Figure 2.** Effect of different *CTRC* variants on chymotrypsinogen C secretion. **A.** HEK 293T cells were transfected with the indicated constructs, and CTRC activity in the conditioned media was determined as described in Fig 1. **B.** Aliquots (0.15 mL) of conditioned media of transfected HEK 293T cells were precipitated with 10% trichloroacetic acid (final concentration) and analyzed by SDS-PAGE and Coomassie Blue staining. **C.** Secretion of chymotrypsinogen C variants relative to wild type. The amount of secreted CTRC protein was determined by densitometry of dried gels stained with Coomassie Blue. Gels were scanned at 300 dpi resolution and CTRC bands were quantitated using the Volume Analysis tool of the Bio-Rad Quantity One software (ver. 4.4.0). The average of at least 3 experiments is shown; the standard deviation was within 15 %.

**Supplementary Table 1**. Secretion and activity of CTRC mutants. All values are percentages, given relative to wild type (100 %). N.D., not determined. p.DEL, mutant p.K247_R254del.

| | [a]Activity secreted from HEK 293T cells | [b]CTRC protein secreted from HEK 293T cells | [c]Specific activity of secreted protein | [d]CTRC protein secreted from AR42J cells |
|---|---|---|---|---|
| wild type | 100 | 100 | 100 | 100 |
| p.R254W | 50 | 38 | 84 | 49 |
| p.DEL | 0 | 4 | 0 | 0 |
| p.A73T | 1.5 | 6 | 18 | 0 |
| p.R37Q | 85 | 88 | 87 | N.D. |
| p.Q48R | 3.5 | 31 | 13 | N.D. |
| p.G217S | 0 | 68 | 7 | N.D. |
| p.V235I | 69 | 85 | 83 | N.D. |

a. Chymotrypsin activity secreted from transfected HEK 293T cells is given as the slopes of linear fits to the time-courses of secretion shown in Fig 1A and Supplementary Fig 2A. The slope of wild type was 300 nM p-nitroaniline / min / min.

b. The amount of secreted CTRC protein was determined by densitometry of dried gels stained with Coomassie Blue. Gels were scanned at 300 dpi resolution and CTRC bands were quantitated using the Volume Analysis tool of the Bio-Rad Quantity One software (version 4.4.0). The average of at least 3 experiments is shown; the standard deviation was within 15 %. The data are also shown in graph format in Supplementary Fig 2C, and representative gels are shown in Fig 1B and Supplementary Fig 2B.

c. Specific activity of the secreted protein was determined as follows. First, chymotrypsin activity of conditioned media was measured using the Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate at 22 $^{o}$C. Mutants with no measurable activity or low activity were concentrated 10-fold using a Vivaspin 20 concentrator (5000 MWCO) and re-assayed. Second, the concentration of wild-type and mutant CTRC protein was measured by immunoblots, using purified CTRC as standards. Finally, specific activity was calculated by dividing the chymotrypsin activity with the protein concentration. The specific activity of wild-type CTRC was 148 mM p-nitroaniline / min / mg CTRC protein.

d. The amount of GluGlu-tagged CTRC protein secreted by AR42J cells was determined by densitometry of the blot shown in Fig 1B.

**Supplementary Table 2.** Kinetic parameters of wild-type CTRC and mutants p.R254W and p.V235I on peptide substrates in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, at 22 °C.

Suc-Ala-Ala-Pro-Phe-p-nitroanilide

| | **wild type** | **R254W** | **V235I** |
|---|---|---|---|
| $K_M$ | 10.3 ± 0.4 μM | 11.0 ± 1.1 μM | 13.8 ± 1.4 μM |
| $k_{cat}$ | 4.7 ± 0.1 $s^{-1}$ | 4.1 ± 0.1 $s^{-1}$ | 4.9 ± 0.2 $s^{-1}$ |
| $k_{cat}/K_M$ | $4.6*10^5$ $M^{-1}$ $s^{-1}$ | $3.7*10^5$ $M^{-1}$ $s^{-1}$ | $3.6*10^5$ $M^{-1}$ $s^{-1}$ |

Glt-Ala-Ala-Pro-Leu-p-nitroanilide

| | **wild type** | **R254W** | **V235I** |
|---|---|---|---|
| $K_M$ | 2.3 ± 0.2 μM | 3.9 ± 0.5 μM | 3.4 ± 0.3 μM |
| $k_{cat}$ | 2.3 ± 0.1 $s^{-1}$ | 2.2 ± 0.1 $s^{-1}$ | 2.3 ± 0.1 $s^{-1}$ |
| $k_{cat}/K_M$ | $1.0*10^6$ $M^{-1}$ $s^{-1}$ | $5.6*10^5$ $M^{-1}$ $s^{-1}$ | $6.8*10^5$ $M^{-1}$ $s^{-1}$ |

**Supplementary Methods**

**Mutation screening**. We designed primers complementary to intronic sequences flanking exons 1-8 of *CTRC* based on the published nucleotide sequence (GenBank # NT_004873). The annealing temperatures used in the PCR reactions are also indicated.

**Oligonucleotides used for PCR amplification and sequencing of *CTRC* (Berlin)**

| **PCR primers** | | |
|---|---|---|
| promoter region and exon 1 | forward: 5´-CTCATCTCACCTCAGAGCAG-3´ | 64°C |
| | reverse: 5´-TAATCTGGGAAAACGCCACC-3´ | |
| exons 2-3 | forward: 5´-ACAGGTGTACCGGGCACTCG-3´ | 64°C |
| | reverse: 5´-TCACTGCTGGTTCCTGGCAC-3´ | |
| exons 4-5 | forward: 5´-CAAGGGCGGTGGAAGCTTGG-3´ | 64°C |
| | reverse: 5´-ATACCACAGATGTCATCCCC-3´ | |
| exon 6 | forward: 5´-TGACCCTCTGGGACCTTGGC-3´ | 64°C |
| | reverse: 5´-TGTGGGTTGGCTTCTCAGCC-3´ | |
| exons 7-8 | forward: 5´-GCTAGTCTGACACCCCAGGG-3´ | 64°C |
| | reverse: 5´-GCAGCTTGTGAGATGGAGCG-3´ | |
| **Sequencing primers** | | |
| promoter region | forward: 5´-GGCTGAGACAGGAGAATCGC-3´ | 56°C |
| exon 1 | forward: 5´-GTAACCACCCAAGGTCAGGG-3´ | 56°C |
| exons 2-3 | forward: 5´-GCCCAGCCCCAACTCTGTGC-3´ | 56°C |
| exon 4 | forward: 5´-GCTACACAGCCAGGAGCAGC-3´ | 56°C |
| exon 5 | forward: 5´-TCCACTCTCACCTCCCTCTG-3´ | 56°C |
| exon 6 | forward: 5´-ATCTGCTCCCTGAAGGCCTG-3´ | 56°C |
| exon 7 | forward: 5´-CCCTCACCATGGGCAGGCTG-3´ | 56°C |
| exon 7 | reverse: 5´-GTGAATGAGTGAGGGGATGG-3´ | 56°C |
| exon 8 | forward: 5´-CTGAGGGTATGGCCCAGCAG-3´ | 56°C |

**Oligonucleotides used for PCR amplification and sequencing of *CTRC* (Leipzig)**

| **PCR primers** | | |
|---|---|---|
| promoter region and exon 1 | forward: 5´-CTCATCTCACCTCAGAGCAG-3´ | 62°C |
| | reverse: 5´-CTGGTTGGTAGCATCTGAAC-3´ | |
| exons 2-3 | forward: 5´-CTTTCCCCGTGGGCTACCA-3´ | 62°C |
| | reverse: 5´-GAAGCTGCTCAGAAAAAGCAGAGTA-3´ | |
| exons 4-5 | forward: 5´-GGAACTGAGGCACAAGGCTAC-3´ | 62°C |
| | reverse: 5´-TAGAGTGTCTGTCACATGGT-3´ | |
| exon 6 | forward: 5´-CAGCCTGAGCAACAGAGTGA-3´ | 62°C |
| | reverse: 5´-CAGTGAAGCCTCTTCTCTGT-3´ | |
| exon 7 | forward: 5´-GCCTCCCAGAATAAGGCCAAG-3´ | 62°C |
| | reverse: 5´-GCTACTGGAAGCACTCAACCA-3´ | |
| exon 8 | forward: 5´-GGACAAGGCTGGCATGTGA-3´ | 60°C |
| | reverse: 5´-GGCTCACTAAACACTTCTCA-3´ | |
| **Sequencing primers** | | |
| promoter region and exon 1 | reverse: 5´-CTGGTTGGTAGCATCTGAAC-3´ | 62°C |
| exons 2-3 | forward: 5´-GTGGGCTACCAGCCCTATTCA-3´ | 62°C |
| exon 4 | reverse: 5´-GCCTTCCTTGTGGACTTTCCT-3´ | 64°C |
| exon 5 | forward: 5´-TCCACTCTCACCTCCCTCTG-3´ | 62°C |
| exon 6 | forward: 5´-TGACCCTCTGGGACCTTGGC-3´ | 62°C |
| exon 7 | forward: 5´-GCTAGTCTGACACCCCAGGG-3´ | 62°C |
| exon 8 | forward: 5´-AACTGGCTGAGTGGGGTCTC-3´ | 62°C |

After PCR amplification, the entire coding region and the exon-intron transitions were sequenced. In all patients analyzed in Berlin, both strands of exon 7 were sequenced. All mutations were confirmed with a second independent PCR reaction. PCR reactions were performed using slightly different conditions at the two centers. In Leipzig, 0.75 U AmpliTaq Gold polymerase (Applied Biosystems), 450 µmol/L deoxynucleoside triphosphates, and 0.3 µmol/L of each primer were used in a total volume of 25 µL. In Berlin, 0.5 U AmpliTaq Gold polymerase, 400 µmol/L deoxynucleoside triphosphates and 0.1 µmol/L primers were used. Cycle conditions were as follows: initial denaturation for 6 min at 95°C; 40 cycles of 20 s denaturation at 95°C, 40 s annealing (see temperatures in Table above) and 90 s primer extension at 72°C; and a final extension step for 6 min at 72°C. In Berlin, 12 min initial denaturation and 2 min final extension was used. PCR products were digested with shrimp alkaline phosphatase (USB) and exonuclease I (USB). Cycle sequencing was performed using BigDye terminator mix (Applied Biosystems). The reaction products were purified with ethanol precipitation or on a Sephadex G-50 column (Amersham) and loaded onto an ABI 3100-Avant or an ABI 3730 fluorescence sequencer (Applied Biosystems).