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<td>Fløyel, Tina; Brorsson, Caroline; Nielsen, Lotte B.; Miani, Michela; Bang-Berthelsen, Claus Heiner; Friedrichsen, Martin; Overgaard, Anne Julie; Berchtold, Lukas A.; Wiberg, Anna; Poulsen, Pernille; Hansen, Lars; Rosinger, Silke; Boehm, Bernhard O.; Ram, Ramesh; Nguyen, Quang; Mehta, Munish; Morahan, Grant; Concannon, Patrick; Bergholdt, Regine; Nielsen, Jens H.; Reinheckel, Thomas; Herrath, Matthias von; Vaag, Allan; Eizirik, Decio Laks; Mortensen, Henrik B.; Størling, Joachim; Pociot, Flemming</td>
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CTSH regulates β-cell function and disease progression in newly diagnosed type 1 diabetes patients

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Edited by Donald F. Steiner, University of Chicago, Chicago, IL, and approved June 3, 2014 (received for review February 12, 2014)

Over 40 susceptibility loci have been identified for type 1 diabetes (T1D). Little is known about how these variants modify disease risk and progression. Here, we combined in vitro and in vivo experiments with clinical studies to determine how genetic variation of the candidate gene cathepsin H (CTSH) affects disease mechanisms and progression in T1D. The T allele of rs3825932 was associated with lower CTSH expression in human lymphoblastoid cell lines and pancreatic tissue. Proinflammatory cytokines decreased the expression of CTSH in human islets and primary rat β-cells, and overexpression of CTSH protected insulin-secreting cells against cytokine-induced apoptosis. Mechanistic studies indicated that CTSH exerts its antiapoptotic effects through decreased JNK and p38 signaling and reduced expression of the proapoptotic factors Bim, Dps, and c-Myc. CTSH overexpression also up-regulated Ins2 expression and increased insulin secretion. Additionally, islets from Ctsht−/− mice contained less insulin than islets from WT mice. Importantly, the TT genotype was associated with higher daily insulin dose and faster disease progression in newly diagnosed T1D patients, indicating agreement between the experimental and clinical data. In line with these observations, healthy human subjects carrying the T allele have lower β-cell function, which was evaluated by glucose tolerance testing. The data provide strong evidence that CTSH is an important regulator of β-cell function during progression of T1D and reinforce the concept that candidate genes for T1D may affect disease progression by modulating survival and function of pancreatic β-cells, the target cells of the autoimmune assault.

The T1D susceptibility locus on chromosome 15q25.1 contains four known protein-coding genes: ADAMTS7, cathepsin H (CTSH), MORF4L1, and RASGRF1 (3, 7). The most significantly associated SNP, rs3825932, is located in intron 1 of the CTSH gene (7) and has been reported to correlate with the CTSH transcript level (http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/). CTSH is a ubiquitously expressed lysosomal cysteine protease. The family of cathepsins is involved in overall protein turnover and specific cellular processes, such as apoptosis, antigen presentation, and prohormone processing (8, 9). Experiments with nonobese diabetic mice deficient in cathepsin B, L, or S showed that these proteases are important for the onset of autoimmune diabetes (10, 11). Additionally, several cathepsins have been implicated in proinflammatory processes (12) or in insulin-secreting β-cells (13). CTSH has been implicated in apoptosis (14–16), cancer development (17), and processing of neurotransmitters (18).

Significance

In type 1 diabetes (T1D), the insulin-producing pancreatic β-cells are destroyed by the immune system. Both genetic and environmental factors contribute to T1D risk. Candidate genes for T1D identified by genome-wide association studies have been proposed to act at both the immune system and the β-cell levels. This study shows that the risk variant rs3825932 in the candidate gene cathepsin H (CTSH) predicts β-cell function in both model systems and human T1D. Collectively, our data indicate that higher CTSH expression in β-cells may protect against immune-mediated damage and preserve β-cell function, thereby representing a possible therapeutic target. Our study reinforces the concept that candidate genes for T1D may affect disease progression by modulating survival and function of the β-cells.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1402571111/-/DCSupplemental.
Against this background, we investigated how genetic variations affect *CTSH* expression, disease mechanisms, and disease progression in children with newly diagnosed T1D. By combining in vitro and in vivo experimental studies and functional observations in healthy and T1D individuals, we show that the T1D candidate gene *CTSH* is an important regulator of β-cell function during progression of T1D.

### Results

**rs3825932 Genotypes Correlate with *CTSH* mRNA and Protein Expression.** Previous expression quantitative trait locus (eQTL) studies have reported effects of rs3825932 on the *CTSH* transcript level (19, 20). These datasets show a clear signal surrounding the linkage disequilibrium (LD) block at the transcriptional start site of *CTSH*, where rs3825932 is among the SNPs with the most significant eQTL signals in *CTSH* (Fig. S1). There was no eQTL effect on the expression of the three additional genes at the 15q25.1 locus.

Real-time quantitative PCR (qPCR) analysis of the HapMap B-lymphoblastoid cell lines (BLCLs) confirmed that the T allele of rs3825932 correlated with decreased *CTSH* mRNA in an allele dose-dependent manner (*P = 1.3 × 10^{-7}, ANOVA*) (Fig. 1A). Of all analyzed SNPs in the LD block, rs3825932 was most significantly associated with *CTSH* expression (Table S1) and captured the eQTL effect of the top SNPs, which was shown by forward stepwise regression. When tag SNPs for the entire region were included, rs3825932 explained most of the eQTL effects observed.

Additionally, rs3825932 also affected the protein expression of *CTSH* in the HapMap BLCLs. The TT genotype was associated with a lower CTSH protein level compared with the CC and CT genotypes (*P = 0.014, ANOVA*) (Fig. 1B and C). Similar to the HapMap BLCLs, the T allele of rs3825932 was correlated with a decreased *CTSH* mRNA level in BLCLs from patients with T1D (*P = 0.006*). Analysis of pancreatic tissue from five donors with T1D supported that the effect is present in primary disease-relevant tissue, because the *CTSH* mRNA level was one-third in the three TT genotype carriers compared with the two CC/CT carriers (Fig. S2).

**Cytokines Suppress *CTSH* Expression in Human Islets and Rat β-cells.** Proinflammatory cytokines, particularly IL-1β, IFN-γ, and TNF-α, contribute to β-cell dysfunction and apoptosis in T1D (1, 21). We, therefore, investigated if cytokines affect the expression of *CTSH* in pancreatic islets. Exposure of human islets to IL-1β, IFN-γ, and TNF-α decreased the expression of *CTSH* compared with untreated islets (Fig. 2A). The cytokine-mediated suppression of *CTSH* expression was confirmed specifically in β-cells, which was shown by a decrease in *Ctsh* mRNA in purified primary rat β-cells after exposure to IL-1β and IFN-γ (Fig. 2B).

To confirm that *CTSH* is expressed at the protein level in human β-cells, we performed immunohistochemical staining of pancreatic sections from non-diabetic donors. *CTSH* was expressed in both exocrine tissue and pancreatic islets (Figs. S3 and S4), including β-cells, where it co-stained with insulin (Fig. S3). Overexpression of *CTSH* Decreases Cytokine-Induced Apoptosis. To investigate whether *CTSH* is involved in cytokine-induced β-cells apoptosis, insulin-secreting INS-1 cells with stable overexpression of *CTSH* were generated, and two *CTSH*-overexpressing clones [plasmid encoding *CTSH* #1 (pCTSH #1) and pCTSH #2] were selected (Fig. 3A). Overexpression of *CTSH* decreased cytokine-induced apoptosis by ~50% compared with cells transfected with an empty vector (Fig. 3B). Basal apoptosis rate was not significantly affected. These findings were supported by data showing that overexpression of *CTSH* decreased cytokine-induced caspase-3/7 activity (Fig. 3C).

**Cytokines Regulate Cytokine Signaling.** NF-κB, signal transducer and activator of transcription 1, and the MAPKs JNK, p38, and ERK are critical mediators of cytokine signal transduction in β-cells (21, 22). We, therefore, examined if *CTSH* affects cytokine signaling through these components. Cytokine-induced degradation of IκB, phosphorylation of signal transducer and activator of transcription 1, and phosphorylation of ERK1/2 were unaffected by *CTSH* overexpression (Fig. S5). In contrast, phosphorylation of JNK1/2 and p38 was diminished in cells overexpressing *CTSH* compared with control cells (Fig. 4A and B). In line with these data, phosphorylation of c-Jun and JunD, transcription factors downstream of JNK, was decreased in CTSH-overexpressing cells after exposure to cytokines (Fig. 4C and D). A downstream target of NF-κB and MAPKs in β-cells is inducible nitric oxide synthase (iNOS) (21, 22). Overexpression of *CTSH* caused an early transient reduction in cytokine-induced iNOS mRNA expression, which translated into reduced iNOS protein (Fig. 4E and F).

Finally, we examined if overexpression of *CTSH* affects the expression of the proapoptotic factors Bim, Bak, Bid, death protein 5 (DPP5), p53 up-regulated modulator of apoptosis (Puma), and c-Myc. Although overexpression of *CTSH* abolished cytokine-induced expression of *Bcl2* (Bim), c-Myc, and DPP5 (Hrk) mRNA (Fig. 4 G–I), it failed to significantly affect Bak, Bid, and Puma (Blc3) mRNA expression (Fig. S5).

**Cytokines Regulate Insulin Transcription.** We also examined if *CTSH* affects insulin secretion. Overexpression of *CTSH* increased the

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**Fig. 1.** rs3825932 affects *CTSH* mRNA and protein levels. (A) *CTSH* mRNA was examined in HapMap BLCLs by qPCR and normalized to the geometric mean of *GAPDH*, *ACTB*, and *UBC*. Data are relative expression with median, SD, and 5–95 percentiles (*n = 25* (CC), *n = 24* (CT), and *n = 7* (TT)). (B) *CTSH* protein was examined in HapMap BLCLs by immunoblotting and normalized to β-actin. Data are relative expression (fold) with median, SD, and 5–95 percentiles (*n = 6* (CC), *n = 5* (CT), and *n = 6* (TT)). (C) A representative immunoblot with *n = 2* for each genotype. *P < 0.05; **P < 0.01; ***P < 0.001.

**Fig. 2.** Cytokines suppress *CTSH* expression. (A) Human islets were untreated or exposed to IL-1β (1 ng/mL), IFN-γ (20 ng/mL), and TNF-α (8 ng/mL) (MIX) for 24 or 48 h. *CTSH* was examined by microarray. Data are means ± SEMs of four individuals. (B) Purified primary rat β-cells were untreated or exposed to IL-1β (50 U/mL) and IFN-γ (500 U/mL) (MIX) for 24 h. Ctsh was examined by qPCR and normalized to *Gapdh*. Data are means ± SEMs (*n = 6*). CTRL, control. **P < 0.01; ***P < 0.001.
INS2 and WT mice (Fig. 6). 

To further validate our findings, mirror experiments were performed in primary islets from Cts/h−/− and WT mice. Medium-accumulated insulin was lower in isolated islets from Cts/h−/− mice compared with WT littermates (Fig. 5D). These data are in line with a trend for a higher fasting blood glucose level in Cts/h−/− mice (P = 0.2) (Fig. 5E). Additionally, the insulin intensity was significantly lower in islets from Cts/h−/− mice compared with islets from WT mice (Fig. 6 and Table S2), which was evaluated by immunohistochemical staining of pancreatic sections. The difference was not caused by a deficit of Ins1 in Cts/h−/− mice, which was shown by an identical ratio of Ins1 and Ins2 mRNA in pancreas from Cts/h−/− and WT mice. In contrast, there was no difference in the glucagon intensity between Cts/h−/− and WT mice (Fig. 6).

**rs3825932 Affects Disease Progression in Children with T1D.** To gain insight into possible in vivo effects caused by the rs3825932 variant, we examined if rs3825932 affects residual β-cell function and glycemic control in children with newly diagnosed T1D. These children display a varying degree of decline in residual β-cell function, which could partly be determined by genetic risk factors. Of interest, carriers of the TT genotype required a higher daily insulin dose 9 and 12 mo after disease onset compared with the CC and CT carriers (9 mo: estimate: 0.18 U/kg, P = 0.009; 12 mo: estimate: 0.22 U/kg, P = 0.009) (Fig. 7A). There was no difference in glycemic control (HbA1c) between the groups. The insulin dose-adjusted HbA1c (IDAA1c) levels were, however, higher 9 and 12 mo after disease onset in carriers of the TT genotype compared with the CC and CT carriers (9 mo: estimate: 0.8, P = 0.042; 12 mo: estimate: 1.1, P = 0.026) (Fig. 7B). Of note, there was a tendency toward fewer TT carriers entering remission (as identified by an IDAA1c ≤ 9) (23), which is a state of temporary improvement in disease progression caused by improved β-cell function. Indeed, by 12 mo after disease onset, none of the TT carriers were in remission compared with 15% and 21% of the CC and CT carriers, respectively (Fig. 7C) (P = 0.068, recessive model).

**rs3825932 Affects β-Cell Function in Healthy Subjects.** We next investigated if rs3825932 affects β-cell function in a group of young, clinically well-characterized healthy twins. Insulin secretion was assessed by oral (OGTT) (Table 1) and i.v. (IVGTT) (Table S3), whereas insulin sensitivity was evaluated by a euglycemic–hyperinsulinemic clamp (Table S3). There was a significant effect of rs3825932 on the insulinogetic index, a measure of β-cell function. The effect size was 0.79 (P = 0.03), corresponding to a 21% decrease in β-cell function for each T allele (Table 1). However, rs3825932 did not affect insulin sensitivity (Table S3), suggesting that the observed effect is caused by differences in β-cell function.

**Discussion**

We have shown that CTS/H is a T1D candidate gene that regulates β-cell function and disease progression in newly diagnosed patients. We confirmed that the previously reported eQTL signals for SNPs located in the T1D susceptibility locus on chromosome 15q25.1 affect the expression of CTS/H. Specifically, we observed that the T allele and the TT genotype of rs3825932 significantly correlate with a lower CTS/H expression at the mRNA and protein levels, respectively; rs3825932 has no effect on the expression of the other candidate genes in the region, supporting CTS/H as the causal gene of this locus. Although rs3825932 is superior in explaining the eQTL signal in CTS/H expression, it cannot be excluded that other SNPs or structural variants in tight LD with this variant also carry causal effects.

Exposure of isolated human islets and purified primary β-cells to proinflammatory cytokines decreased CTS/H mRNA expression. This observation is in concordance with recently published RNA sequencing data used to identify transcripts under cytokine regulation in human islets (4). Importantly, we confirmed that CTS/H is expressed at the protein level in human β-cells in pancreatic sections, which is consistent with a previous study that showed that CTS/H is localized to the secretory granules of α- and β-cells (24).

The overexpression experiments revealed CTS/H as a negative regulator of cytokine-induced apoptosis in insulin-secreting cells. Interestingly, deletion of Cts/h in a mouse model of pancreatic islet cell cancer led to a reduction in tumor incidence and tumor growth because of increased apoptosis (17). Taken together, these results support an antiapoptotic effect of CTS/H. Subsequent mechanistic experiments indicated that CTS/H exerts its antiapoptotic effects through inhibition of specific cytokine-induced signals in β-cells. Thus, overexpression of CTS/H in INS1 cells diminished signaling through the JNK and p38 pathways, key modulators of cytokine-induced β-cell apoptosis (21). We also observed a clear inhibitory effect of CTS/H overexpression on cytokine-induced transcription of the proapoptotic factors Bim, DP5, and c-Myc. The expressions of Bim, c-Myc, and DP5 are induced by cytokines in β-cells, and Bim and DP5 are regulated, at least in part, through JNK and p38 pathways, key modulators of cytokine-induced β-cell apoptosis (21). We also observed a clear inhibitory effect of CTS/H overexpression on cytokine-induced transcription of the proapoptotic factors Bim, DP5, and c-Myc. The expressions of Bim, c-Myc, and DP5 are induced by cytokines in β-cells, and Bim and DP5 are regulated, at least in part, through JNK and p38 pathways, key modulators of cytokine-induced β-cell apoptosis (21).

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genotype differs from the CC and CT genotypes. The lack of association between CTSH genotypes and C-peptide or pro-insulin levels might relate to the fact that these patients already suffer from severe β-cell loss, rendering it difficult to detect subtle differences in the β-cell mass and function. Nevertheless, the results indicate that TT carriers have a faster disease progression, leading to a more prominent β-cell dysfunction, which is consistent with our observation that fewer TT carriers enter remission.

In line with these observations, healthy human subjects carrying the T allele had lower β-cell function. We found no effect of rs3825932 on the first-phase insulin secretion, which was assessed through the IVGTT, but in the OGTT, rs3825932 significantly affected the insulinogenic index, showing that the β-cell function was decreased by 21% for each T allele. The data suggest that rs3825932 primarily affects insulin secretion in response to oral as opposed to i.v. glucose ingestion. It is conceivable that the differences between the two tests are explained by rs3825932 primarily affecting the second-phase insulin secretion response. Importantly, a recent study identified a type 2 diabetes candidate gene that only affects second-phase insulin secretion (29).

Several T1D candidate genes have been implicated in β-cell function and survival (28, 30–32), but to our knowledge, this study is the first demonstration that a genome-wide association study-identified genetic variation affects β-cell function in both model systems and human T1D. Thus, this study strengthens CTSH as a causal risk gene in T1D and reinforces the concept that candidate genes for T1D may affect disease progression by modulating survival and function of the pancreatic β-cells (33).

We suggest that SNPs, which cause a low expression of CTSH, sensitize β-cells to immune-mediated β-cell dysfunction and death, leading to a more rapid and severe loss of functional β-cell mass. This functional knowledge of CTSH may be used for development of new diagnostic and preventive strategies to preserve β-cell function in T1D.

Fig. 4. CTSH overexpression decreases cytokine signaling. INS-1 pcDNA and pCTSH cells were untreated or exposed to IL-1β (150 pg/mL) and IFN-γ (5 ng/mL) (MIX). (A–D and F) Immunoblotting was performed after stimulation for 0 h, 30 min, or 6 h. Data are means ± SEMs (n = 3–4). (E and G–I) qPCR was performed after stimulation for 2, 4, or 8 h; data are normalized to Actb and presented as fold induction. Data are means ± SEMs (n = 4). CTRL, control. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 5. CTSH regulates insulin transcription and secretion. (A) Medium insulin accumulation over 24 h from INS-1 pcDNA and pCTSH cells. Data are insulin (micrograms per liter) divided by DNA content with median, SD, and 5–95 percentiles (n = 7). (B and C) INS-1 pcDNA and pCTSH cells were untreated or exposed to IL-1β (150 pg/mL) and IFN-γ (5 ng/mL) (MIX) for 24 h. Ins1 and Ins2 were examined by qPCR and normalized to Hprt1. Data are mean ± SEM (n = 4). (D) Medium insulin accumulation over 24 h from islets of WT and Ctsh−/− (KO) mice. Data are insulin (micrograms per liter) divided by DNA content with median, SD, and 5–95 percentiles (n = 4). (E) Blood glucose (milligrams per deciliter) was measured in WT and Ctsh−/− (KO) mice after overnight fasting with ad libitum water access. Median, SD, and 5–95 percentiles (n = 7 in each group). CTRL, control. **P < 0.01; ***P < 0.001.
All human studies were approved by the regional ethical board, Freiburg or the Danish Animal Experiment Inspectorate as well as the local ethical committees and conducted according to the principles of the Declaration of Helsinki. Animal studies were approved by the local ethical committees as described in detail above.

Statistical Analyses. Statistical analysis was performed using a two-tailed t test unless otherwise stated. P < 0.05 was considered statistically significant. Genotype-specific levels of CTSH were analyzed using two-way ANOVA. The difference in intensity of the insulin staining between islets from WT and Cts/h−/− mice was evaluated using 2\(\chi\)2 test with 5 degrees of freedom (groups 6 and 7 were merged because of few measurements). The distributions are shown in Table S2. All statistical tests on the two study populations were performed in SAS (version 9.1; SAS Institute). In the Hvidore Study Group, stimulated C peptide (logarithmic), proinsulin (logarithmic), HbA\(\text{1c}\) (logarithmic), and IDAA1c (logarithmic) were analyzed as dependent variables in separate multiple regression repeated measurement models with unstructured variance, with sex, age, HLA risk groups, and CTSH genotype as explanatory variables. The assumption of constant effect of genotype was checked by first allowing for interaction between genotype and disease duration. In the twin study population, the proc mixed procedure was used to adjust for twin pair and zygosity status as well as other contributing variables, including age, sex, VQ\text{max}, and total fat percentage. All response variables were log-transformed to avoid skewness of the residuals, which also resulted in per-allele effects expressing percentage-wise and not absolute changes of the response variable.
Table 1. Oral glucose tolerance test measures in healthy subjects

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<td>51 (22/29)</td>
<td>17 (17/0)</td>
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<td>-</td>
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<td>79 (29)</td>
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<td>62.1 (23.1)</td>
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Data are mean (SD). P values were calculated using the mathematical model Ln(response variable) = age + sex + V0max = total fat percentage + rs3825932 genotype.

1. Insulinogenic index was calculated as (InsAUC − InsGlu)/Glu.
2. The total insulin area under the curve (AUC) was calculated for the initial 30-min period.
3. Homeostasis model assessment (HOMA) of β-cell function was calculated as InsM, 0.144 × (2 × Glu) − 3.5.

ACKNOWLEDGMENTS. We thank Novo Nordisk for support to The Hvidore Study Group on Childhood Diabetes, Nils Billestrup for RNA from human islets, Katja Serena and Anne Mette Trauelsen for assistance with mice, Anders Mellerup for sample preparation, and Klaus Stenggaard Frederiksen for help with the microarrays on human islets. The technical assistance of Bodil Bosmann Jørgensen, Oda Troest, Jacqueline Tyljberg, and Fie Hillersø is acknowledged. T.F., C.B., and F.P. were funded by National Institute of Health Grant 1 DP3 DK085678, Juvenile Diabetes Research Foundation Grant 33-2008-391, the A. P. Møller Foundation, and the Poul and Erna Sehested Hansen Foundation. R.R., Q.N., M. Mehta, and G.M. were funded by National Health and Medical Research Council of Australia Grant S16700 and the Diabetes Research Foundation of Western Australia. M. Miani and D.L.E. were funded by the Communauté Française de Belgique Actions de Recherche Concer tes, Fonds National de la Recherche Scientifique Belgium, and the European Union (NAIMIT and BetaBat in the FP7 of the European Community). T.R. was funded by the Excellence Initiative of the German Federal and State Governments (EXC 294).