

A discovery-based proteomics approach identifies protein disulphide isomerase (PDIA1) as a biomarker of β cell stress in type 1 diabetes



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Summary

Background Stress responses within the β cell have been linked with both increased β cell death and accelerated immune activation in type 1 diabetes (T1D). At present, information on the timing and scope of these responses as well as disease-related changes in islet β cell protein expression during T1D development is lacking.

Methods Data independent acquisition-mass spectrometry was performed on islets collected longitudinally from NOD mice and NOD-SCID mice rendered diabetic through T cell adoptive transfer.

Findings In islets collected from female NOD mice at 10, 12, and 14 weeks of age, we found a time-restricted upregulation of proteins involved in stress mitigation and maintenance of β cell function, followed by loss of expression of protective proteins that heralded diabetes onset. EIF2 signalling and the unfolded protein response, mTOR signalling, mitochondrial function, and oxidative phosphorylation were commonly modulated pathways in both NOD mice and NOD-SCID mice rendered acutely diabetic by T cell adoptive transfer. Protein disulphide isomerase A1 (PDIA1) was upregulated in NOD islets and pancreatic sections from human organ donors with autoantibody positivity or T1D. Moreover, PDIA1 plasma levels were increased in pre-diabetic NOD mice and in the serum of children with recent-onset T1D compared to non-diabetic controls.

Interpretation We identified a core set of modulated pathways across distinct mouse models of T1D and identified PDIA1 as a potential human biomarker of β cell stress in T1D.

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Research in context**Evidence before this study**

Type 1 diabetes (T1D) develops over a time frame of many years and as a result of a complex and bidirectional interaction between the pancreatic β cell and the immune system. Findings from ex vivo models of T1D and pancreatic sections from human organ donors with diabetes have linked changes in β cell mass and function with activation of a variety of stress pathways, many of which are thought to accelerate β cell death and increase β cell immunogenicity. However, the majority of existing studies have examined tissues at a single time-point, resulting in a limited view of disease pathogenesis that lacks resolution on the timing and scope of β cell responses during disease evolution. In addition, few findings have been translated between mouse and human tissues and into potential biomarker assays.

Added value of this study

To characterize changes in the islet proteome during T1D development, islets from NOD mice and NOD-SCID mice rendered acutely diabetic through T cell adoptive transfer were isolated and analysed using data independent acquisition-mass spectrometry (DIA-MS). In islets collected longitudinally from the chronic progressive NOD mouse model, there was a time-restricted upregulation of proteins involved in the maintenance of β cell function and stress mitigation, followed by loss of expression of several protective proteins preceding diabetes development. At diabetes onset, proteomics analysis identified a common set

of modulated pathways in both NOD and NOD-SCID islets. Pathways implicated across the two models included EIF2 signalling and the unfolded protein response, mTOR signalling, mitochondrial dysfunction, and oxidative phosphorylation. To translate our findings into humans, we focused on protein disulphide isomerase A1 (PDIA1), which is a highly abundant ER localized and secreted thiol oxidoreductase that plays a role in insulin secretion, proinsulin processing, and protection against β cell ER stress. In immunofluorescence experiments, we verified a biphasic pattern of expression of PDIA1 during T1D progression in NOD islets, and we found increased PDIA1 expression in human islets treated ex vivo with cytokines and in islets from pancreas tissue collected from human organ donors with autoantibody positivity and with T1D. Moreover, plasma levels of PDIA1 were elevated in pre-diabetic NOD mice and in the serum of children with recent-onset T1D compared to age and sex-matched non-diabetic controls.

Implications of all the available evidence

Our study highlights the value of applying unbiased proteomics approaches in preclinical models to identify key β cell pathways involved in the temporal evolution of T1D. Utilizing this strategy, we identified a common set of modulated pathways across several distinct mouse models of T1D and identified PDIA1 as a potential T1D associated biomarker in humans.

Introduction

Type 1 diabetes (T1D) results from immune-mediated destruction of the insulin-producing β cells and manifests clinically after there is a threshold reduction in β cell mass and function. Data from clinical cohorts of autoantibody positive individuals suggest there are several predictable metabolic checkpoints during T1D progression. In early stage disease and following the development of autoantibodies, there is measurable loss of early C-peptide responses and decreased C-peptide secretion during oral glucose tolerance testing (OGTT) that can be detected up to six years prior to clinical diagnosis.^{1,2} This is followed by a second phase of relatively stable OGTT C-peptide measurements.^{1,2} Metabolic deterioration accelerates one year to six months prior to the clinical diagnosis of T1D and is characterized by a marked decline in β cell glucose sensitivity coupled with decreased insulin sensitivity and rising blood glucose levels.¹⁻³ In parallel, histologic studies performed on pancreatic sections from organ donors with autoantibody positivity and with T1D demonstrate variable reductions in β cell mass before and at diabetes

onset.⁴⁻⁶ Findings from ex vivo disease models and pancreatic sections from human organ donors with diabetes have linked changes in β cell mass and function with activation of a variety of cell intrinsic stress pathways, such as endoplasmic reticulum (ER) and mitochondrial dysfunction, HLA Class I overexpression, and changes in alternative splicing patterns, all of which have been implicated in β cell death and increased β cell immunogenicity.⁷⁻¹⁴

At present, a major limiting factor in the treatment or prevention of T1D is a lack of knowledge regarding the timing and nature of β cell dysfunction prior to the onset of clinical disease.¹⁵ Addressing this knowledge gap is important, as recent clinical studies suggest this accelerated disease state during the peri-diagnostic period likely represents a key window for effective therapeutic intervention.¹⁵ Longitudinal imaging of the β cell compartment and sampling of the pancreas in living individuals is not clinically feasible. Studies performed using isolated islets or pancreatic sections from organ donors with diabetes provide critical information about disease pathogenesis; however, they enable only a single

snapshot view of disease pathogenesis. Improved temporal resolution of the molecular programs modulated within the β cell during T1D development could inform therapeutic and biomarker strategies in humans.^{16–18} Therefore, there is a critical need to interrogate T1D progression in alternative model systems that lend themselves to longitudinal studies.

The non-obese diabetic (NOD) mouse has been widely used to study T1D pathogenesis for over three decades.^{19–21} Islets from female NOD mice show evidence of immune cell infiltration as early as four weeks of age,²² and a majority of female NOD mice develop diabetes. In contrast, a majority of male NOD mice remain diabetes-free.^{19,23} Consistent with patterns observed in humans, β cell function and mass decline in female NOD mice during the pre-diabetes phase. In cross-sectional analyses, subsets of overlapping stress pathways have been identified in β cells from NOD mice and in human islets from organ donors with diabetes.^{20,24,25} Therefore, longitudinal analysis of pancreatic islets of female NOD mice during the progression to T1D may allow for identification of stress pathways that are activated prior to β cell destruction, thus enabling the identification of clinical biomarkers and the development of potential therapeutics.

To gain insight into the time course of molecular changes in the β cell during T1D progression, we used a data independent acquisition-mass spectrometry (DIA-MS) based approach to monitor longitudinal changes in the islet proteome during early and late disease progression in NOD mice. To illustrate the utility of this approach in prioritizing β cell proteins as T1D biomarkers, we focused on protein disulphide isomerase A1 (PDIA1) as an example of a secreted protein that was differentially expressed in NOD islets during diabetes progression. We demonstrated increased islet expression of PDIA1 in NOD mouse islets during the evolution of T1D and in pancreatic sections from human organ donors with autoantibody positivity or with T1D. In addition, we developed a high sensitivity electrochemiluminescence assay to measure circulating PDIA1 levels. Using this assay, we demonstrated increased plasma PDIA1 levels in pre-diabetic NOD mice and in the serum of children with recent-onset T1D compared to age- and sex-matched paediatric controls.

Methods

Animals and experimental procedures

Female NOD/ShiLTJ (NOD), NOD-BDC2.5, and NOD-SCID mice were purchased from Jackson Laboratory. Female outbred CD1 mice were purchased from Charles River Laboratories. Only female NOD mice were utilized for this study, as spontaneous T1D development occurs almost exclusively in female mice (~80–85% diabetes incidence in our vivarium), making

it very difficult to study diabetes pathogenesis in male NOD mice (~10% diabetes incidence).^{26,27} Mouse studies were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.²⁸ Mice were allowed to acclimate for at least two weeks upon arrival prior to the initiation of experiments. Blood glucose was monitored weekly in all the mouse models and diabetes was defined as a blood glucose >250 mg/dL for two consecutive measurements. Blood glucose and body weight were recorded on the day of islet isolation for each age group of mice used for downstream analysis (Supplementary Figure S1). Pancreatic islets were isolated or the pancreas was harvested at the indicated time points using methods described previously.^{29,30}

Mouse islets were handpicked, washed twice with PBS, and stored as pellets at -80°C until use. Blood for plasma analysis was obtained at the time of euthanasia via cardiac puncture, transferred to a Becton Dickinson Vacutainer K2EDTA tube (Cat# 365974), and centrifuged at 5000 rpm for 10 min at 4°C . The separated plasma samples were aliquoted into 1.5 mL cryotubes and stored at -80°C until use.

Single-cell splenocyte suspensions were prepared for adoptive transfer experiments from 12-week-old male NOD-BDC2.5 mice, as previously described.³¹ CD4⁺ T cells were purified by negative selection (Cat# 558131, BD Biosciences), activated in 6-well plates (5×10^6 cells/well), coated with anti-CD3 and anti-CD28 antibody (1 mg/mL each), and expanded for 72 h in T-75 flasks (5×10^6 cells/flask) in complete RPMI 1640 medium (1% penicillin/streptomycin and 10% FBS) containing 100 U/mL IL-2. Cells were subsequently collected, washed twice with Hanks' balanced salt solution (HBSS), and diluted to 5×10^6 cells/mL in HBSS.³² Recipient 8-week-old immunodeficient male NOD-SCID mice received 1×10^6 T cells via intraperitoneal injection, and blood glucose was measured daily for 21 days. Age-matched NOD-SCID mice that received HBSS alone were used as controls. The onset of diabetes was defined as two consecutive blood glucose readings of ≥ 250 mg/dL.

Immunofluorescence staining and quantification

Immunofluorescence (IF) was performed to investigate key findings from the MS analysis. Briefly, formalin-fixed paraffin-embedded (FFPE) pancreatic tissues from an independent cohort of pre-diabetic age-matched NOD mice, obtained at 7, 9, 11, 13 weeks of age and mice that developed diabetes, were sectioned at a thickness of $5\ \mu\text{m}$ and deparaffinized. The sections were hydrated twice with fresh Xylene for 5 min and a series of decreasing ethanol concentrations (100–70%). Antigen retrieval was performed using citrate buffer and stained using antibodies against PDIA1 (Cell Signaling, Cat# 3501S, RRID:AB_2156433), PRDX3 (Abcam, Cat#

ab73349, RRID:AB_1860862), 14-3-3 β /YWHAB (Sigma, Cat# HPA011212, RRID:AB_1844334), insulin (Dako, Cat# IR002, RRID:AB_2800361), glucagon (Abcam, Cat# ab10988, RRID:AB_297642), CHOP (ThermoFisher Scientific, Cat# MA1-250, RRID:AB_2292611), and BIP (Cell Signaling Technology, Cat# 3177S, RRID:AB_2119845). Similarly, human pancreatic tissue sections from non-diabetic cadaveric organ donors, organ donors with autoantibody positivity but no diabetes, and organ donors with T1D (Supplemental Table S5) were received from the Network of Pancreatic Organ Donors (nPOD) Biorepository and stained for PDIA1, insulin, and glucagon using the above-mentioned primary antibodies. Secondary antibodies included Alexa 488-labeled goat anti-guinea pig, Alexa 568-labeled donkey anti-rabbit, and Alexa 647-labeled donkey anti-mouse antibodies. Nuclei were stained with DAPI. Images were acquired using an LSM800 confocal microscope (Zeiss, Germany) and quantified using Image-J software (NIH) as described previously.³⁰ From each mouse (4–7 mice/group), 3–7 islets were randomly selected for imaging, and for human pancreatic sections, 5–10 islets from every donor were randomly selected for imaging. Normalized total islet cell fluorescence intensity was calculated independently by two individuals working in a blinded fashion.

Collection of human serum samples

Fasting serum was obtained from children with recent-onset T1D and age- and sex-matched non-diabetic healthy controls (Supplemental Table S1). Written informed consent or parental consent and child assent were obtained from all participants.³³ Children with T1D had been newly diagnosed within 48 h of blood collection and were hospitalized at the Riley Hospital for Children. Control paediatric subjects were from outpatient clinic, did not take any chronic prescription medications, and were free of any chronic or acute illness within two weeks preceding sampling.

Measurement of serum and plasma PDIA1

Thirty μ L of two-fold diluted mouse plasma samples or thirty μ L of four-fold diluted human serum samples were assayed. A four-fold serially diluted rPDIA1 protein with a starting concentration of 2500 ng/mL was used to generate a standard curve. Since the human and mouse PDIA1 protein share 93.725% sequence homology, human rPDIA1 protein was used as the standard to measure human and mouse samples. To quantitate circulating levels of PDIA1 in human serum and mouse plasma samples, standard one spot MSD plates were incubated with 5 μ g/mL of capture antibody (Sigma, Cat# HPA018884, RRID:AB_1854896) overnight at 4 °C, and the same procedures described above under “Assay development” were followed. Following sample

incubation, plates were washed as described above and incubated with mouse PDIA1 detection antibody (Thermo Fisher Scientific, Cat# MA3-019, RRID:AB_2163120) for 1 h in an orbital shaker at RT. The plates were then washed and incubated with an MSD mouse Sulfo-Tag for 1 h at RT in a shaker. Finally, the plates were read using 150 μ L of read-buffer in a Quick Plex SQ 120 plate reader (MSD), and the data were analysed as described above.

Details of mass spectrometry sample processing and analysis, human islet culture and immunoblot, and assay development to measure plasma and serum PDIA1 is provided in the Supplemental Materials and Methods and Supplemental Table S3.

Ethics

Mice were maintained at the Indiana University School of Medicine Laboratory Animal Resource Center under protocols that were approved by the Indiana University Institutional Animal Care and Use Committee (protocol number: 20104 MD/R/HZ/E/AR). Fasting serum was obtained from children with recent-onset T1D and age- and sex-matched non-diabetic healthy controls under protocols approved by the Indiana University Institutional Review Board (protocol number: 1411938757). Written informed consent or parental consent and child assent were obtained from all participants.³³

Statistics

For sample size determination, we used <https://clincalc.com/stats/samplesize.aspx> software. At study outset, we collected islet samples from a total of 79 mice for DIA-MS analysis. Using the stringent quality control criteria described in the Supplemental Methods, a total of 18 samples were excluded following mass spectrometry analysis. Despite these exclusions, the sample size for each group was at or above 3 mice/group, which provided 80% power to detect differences between groups assuming a type I error α of 0.05, based on the established disease incidence of 80–85% in our NOD colony. Protein abundance changes in islet proteomics in different sample groups were analysed using principal component analysis (PCA). Differences in protein abundance between two groups was determined using Student's *t*-tests; differences between two or more groups were tested using one-way ANOVA. A $P \leq 0.05$ was considered significant. Differentially expressed proteins were visualized using heatmaps (<https://software.broadinstitute.org/morpheus/>) with unsupervised hierarchical clustering analysis. The median normalized up- and down-regulated pathways were filtered using 1.5 fold change on log₂ transformed data and functional enrichment of protein sets with differential expression was performed using Ingenuity Pathway Analysis. Fishers exact test was used to examine the significance of enrichment using

Bonferroni-corrected $P < 0.05$. For analysis of PDIA1 in human cohorts, we used the mean \pm S.D. of PDIA1 fluorescent intensities ($n = 4$ mice/group) to calculate sample size requirements. Based on these calculations, a sample size of 10 per group provided 80% power with a type I error α of 0.05. Data other than mass spectrometry were analysed using GraphPad Prism version 9. Data are presented as mean \pm S.E.M or mean \pm S.D.

Role of funders

The funding sources for this project played no role in the study design, data collection, analysis, interpretation, writing, or editing of the manuscript.

Results

Analysis of temporal changes in the NOD proteome during disease progression

To characterize temporal changes in islet protein expression during diabetes progression, pancreatic islets were isolated from age-matched CD1 and NOD mice at 10, 12, and 14 weeks of age and at the time of diabetes onset (mean \pm S.D. age of diabetes development was 17 ± 3.3 weeks) and analysed using DIA proteomics (Fig. 1). [Supplementary Figure S1](#) shows the blood glucose and body weight data for mice on the day of islet isolation. An average of 1160 proteins and 897 overlapping proteins were quantified in NOD and CD1 mouse islets ([Supplementary Figure S3](#)). Since CD1 mice are not diabetes-prone and exhibit tightly regulated blood glucose levels, we used sex- and age-matched CD1 mice to normalize protein abundance in NOD islets. To identify differentially expressed proteins at each time point, results were analysed using median normalization, a filtering criterion of a 1.5-fold change in protein abundance, and $P \leq 0.05$. As shown in [Supplementary Figure S4 \(a and b\)](#) biological replicates across each experimental group clustered together, indicating the reproducibility of our proteomics approach. Panels c–e in [Supplementary Figure S4](#) illustrate patterns of differentially expressed proteins and hierarchical clustering between the experimental groups. The complete list of differentially expressed proteins between experimental groups is included in [Supplemental Table S3](#). We identified 411 up-regulated and 502 down-regulated proteins in islets from 10 week old NOD mice; 364/524 at week 12 and 530/220 at week 14. A total of 344 up-regulated and 584 down-regulated proteins were identified in islets from NOD mice at the time of diabetes development. Similar results (434/275) were observed in islets from NOD-SCID-BDC2.5 mice rendered acutely diabetic. Finally, a total of 428 up- and 474 down-regulated proteins were identified in islets from the diabetes-resistant NOD mice.

In principal component analysis (PCA), NOD mice at different pre-diabetic ages (10, 12, and 14 weeks)

clustered primarily as one group, whereas diabetic mice were distinctly clustered (Fig. 2a). The top 30 differentially expressed (DE) proteins from individual mice are shown in Fig. 2b. Unsupervised hierarchical clustering analysis of these DE proteins revealed a time-dependent loss of insulin and islet amyloid polypeptide (IAPP) expression (Fig. 2c). In addition, we observed a time-restricted upregulation of several proteins implicated in stress mediation and the maintenance of normal β cell function during the prediabetic phase (Fig. 2c). Proteins exhibiting this pattern of expression included actin-related protein 2/3 complex 2 (ARPC2), which regulates actin cytoskeleton-mediated transport of secretory vesicles,^{34–36} collagen 1A1 (COL1A1), and collagen 1A2 (COL1A2), which are extracellular matrix proteins,^{37,38} and the metallothioneins MT1 and MT2, which suppress immune responses.^{39,40} A similar pattern was observed for peroxiredoxin 3 (PRDX3), a protein that regulates mitochondrial function, and 14-3-3 β , which plays a role in metabolic processes, including mTOR signalling, amino acid metabolism, and mitochondrial function.^{41,42} Protein disulphide isomerase A1 (PDIA1) was upregulated similarly during weeks 12–14. Notably, PDIA1 is a thiol reductase that plays a critical role in proinsulin folding and the regulation of ER function.⁴³ Overall, this pattern of upregulation was observed through the 14-week timepoint followed by declining expression of several of proteins with potentially protective roles that heralded diabetes development (Fig. 2c).

Fig. 3a shows the top 10 upregulated pathways, while Fig. 3b shows the top 10 downregulated pathways in longitudinal analysis of islets from NOD mice using Ingenuity Pathway Analysis. During diabetes progression, pathways related to Cdc42, integrin signalling, actin, epithelial adherens, and mTOR signalling were upregulated (Fig. 3a). EIF2 signalling, which is involved in global mRNA translation initiation and is a target during the unfolded protein response and ER stress,^{44,45} was markedly upregulated at weeks 12 and 14 and in diabetic mice (Fig. 3a). Changes in mitochondrial function were represented in both up- and down-regulated pathways, while significantly downregulated pathways encompassed several metabolic pathways, including the TCA cycle, oxidative phosphorylation, fatty acid oxidation, and glutathione redox reactions. Sirtuin signalling and phagosome maturation were also downregulated (Fig. 3b and [Supplementary Figures S5 and S6](#)).

Comparison of spontaneous and induced models of T1D

To identify commonalities and differences in the islet proteome between the chronic, spontaneous NOD model and an aggressive, acute model of immune-mediated β cell destruction, we compared proteomics

Schematic workflow

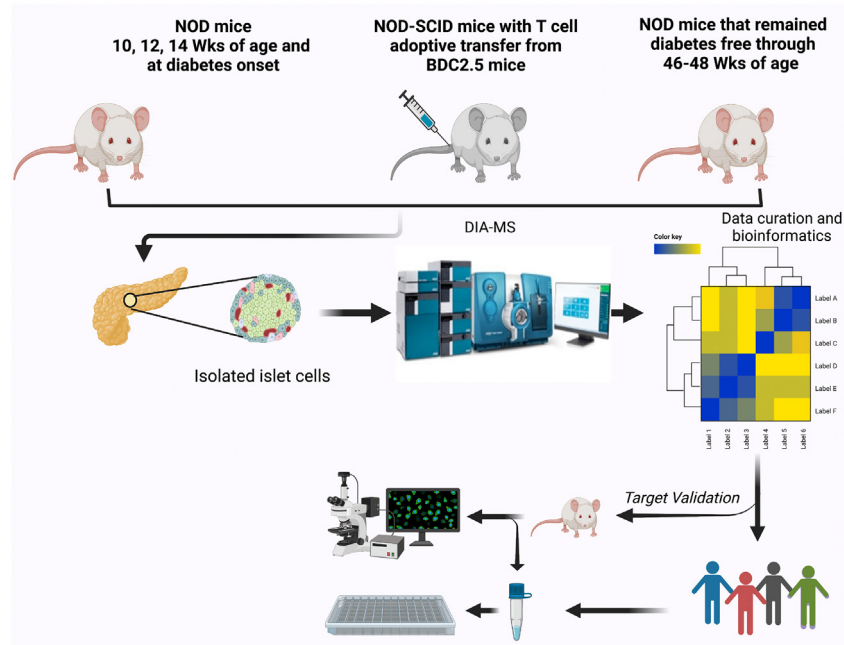


Fig. 1: Schematic representation of study design and experimental workflow (created using BioRender.com).

results from islets isolated from diabetic NOD mice and islets isolated at the time of diabetes development from NOD-SCID mice that had undergone adoptive transfer of CD4⁺ T-cells from NOD.BDC2.5 mice. Mice in the latter group develop significant hyperglycaemia around 7 days following adoptive transfer (Supplementary Figure S1). Despite a significantly different time-course of diabetes development compared to the chronic NOD model, ~65% of identified proteins were common to both models (Fig. 4a). In addition, a comparison of functional canonical pathways suggested that similar pathways were activated in both models. Key overlapping pathways included modulation of EIF2 signalling and the unfolded protein response, mitochondrial dysfunction, oxidative phosphorylation, and mTOR signalling (Fig. 4b). These results suggest that irrespective of the type of inflammation (acute or chronic), similar patterns of β cell stress are activated, underscoring the importance of this core set of pathways in T1D pathogenesis.

Proteome comparison of NOD mice that developed diabetes and that remained diabetes-free

We reasoned that comparing diabetic NOD mice and NOD mice that remained diabetes-free through extended follow-up might highlight protective pathways within the β cell during immune activation. Proteomic analysis was performed on islets from 46 to 48 week old

NOD mice who remained diabetes-free, and results were compared to islets collected from NOD mice at the time of diabetes development. To account for differences that may be driven by age, data from each NOD group was normalized to their respective CD1 age-matched controls. Compared to NOD mice that developed diabetes, NOD mice that remained diabetes free had markedly fewer proteins that were differently expressed relative to their age-matched CD1 controls (Supplementary Figure S4a). Principal component analysis indicated a clear separation between the diabetes-resistant group and diabetic NOD mice (Fig. 5a); data from individual biological replicates shown in Fig. 5b. Next, unsupervised hierarchical clustering analysis was performed using the Euclidian distance and average linkage method (Fig. 5c). Data from this analysis revealed upregulation of several unique proteins previously linked with the mitigation of β cell stress. Among the top proteins upregulated in diabetes resistant mice and down-regulated in diabetic mice were IAPP and antioxidant-1 (ATOX1), a copper chaperone shown to be protective against hydrogen peroxide and superoxide mediated-oxidative stress.⁴⁶ Other key proteins showing this pattern of expression were proteasome subunit beta 10 (PSB10), which is involved in the maintenance of protein homeostasis,⁴⁷ coactosin like protein (COTL1), an F-actin-binding protein that plays a role in cellular growth,⁴⁸ and S100A4, which functions as an intracellular cytosolic calcium sensor.^{49,50}

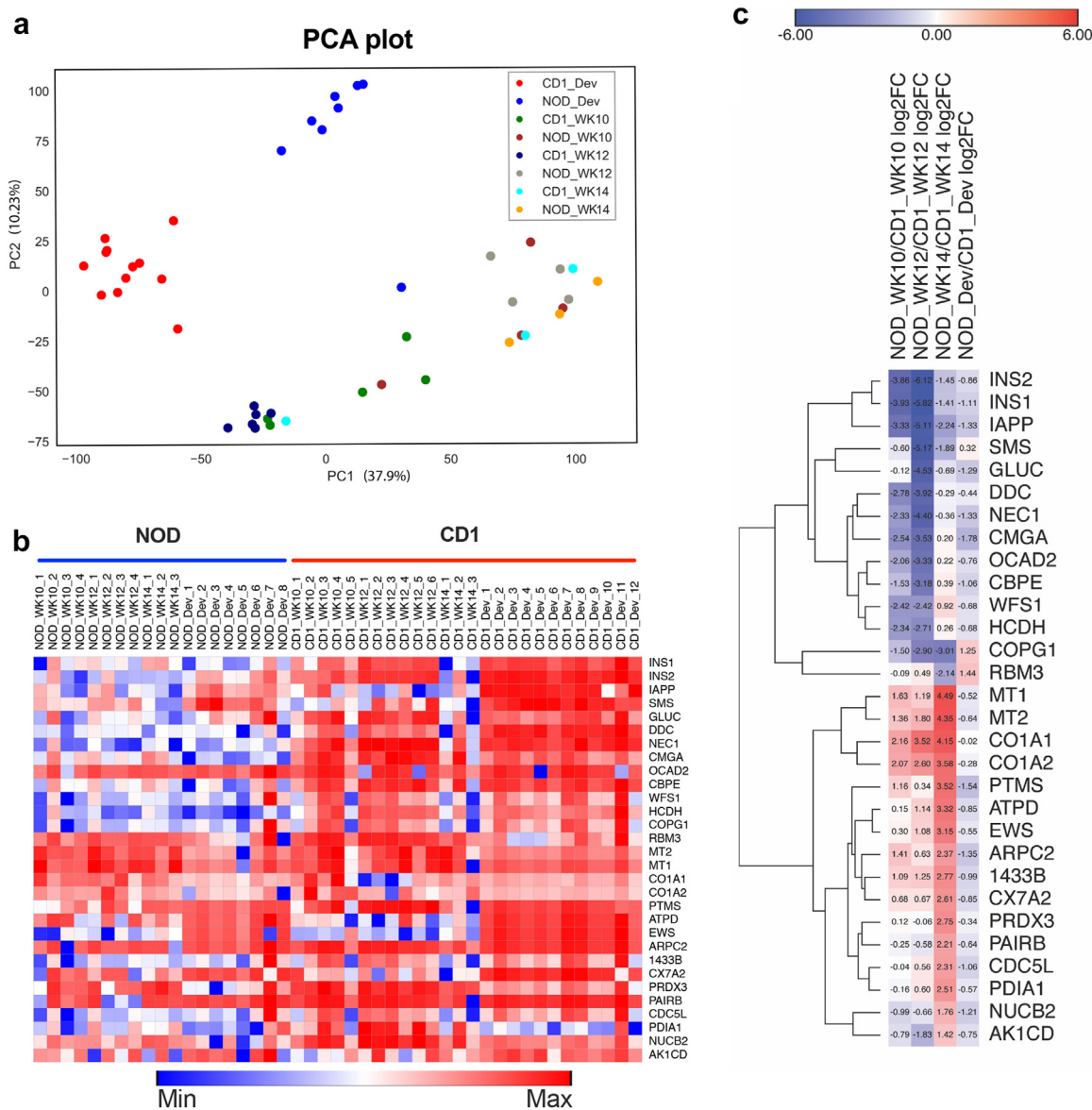


Fig. 2: Proteomic analysis of pancreatic islets in NOD and CD1 mice over time. (a) Principal component analysis (PCA) of all quantified islet proteins from NOD and CD1 mice at 10, 12, and 14 weeks of age and during diabetes onset. (b) Heatmap showing expression patterns of the top 30 differentially expressed proteins from each individual biological replicate at 10, 12, and 14 weeks of age and at diabetes onset. (c) Unsupervised hierarchical clustering analysis of the top 30 differentially expressed proteins in NOD mice compared to age- and sex-matched CD1 mice from 10, 12, and 14 weeks of age, and during diabetes onset ($n = 3-12$ animals/per group).

In Fig. 5d, pathway enrichment analysis shows the top ten significantly upregulated pathways in diabetes-resistant NOD mice compared to NOD mice that developed diabetes. In diabetes-resistant NOD mice, there was notable modulation of pathways involved in maintaining cellular homeostasis, tissue repair, tissue clearance (i.e., phagocytosis in macrophages and monocytes), and aryl hydrocarbon receptor signalling, which is linked with mitigation of insulinitis in NOD

mice.⁵¹ Consistent with this, we observed down-regulation of pathways related to mitochondrial dysfunction, phagosome maturation, oxidative phosphorylation, and the unfolded protein response in islets of diabetes-resistant NOD mice (Fig. 5e). Interestingly, actin cytoskeleton signalling, and epithelial adherens junction signalling were identified among both the up- and down-regulated pathways, with distinct proteins implicated within the up- and down-regulated categories.

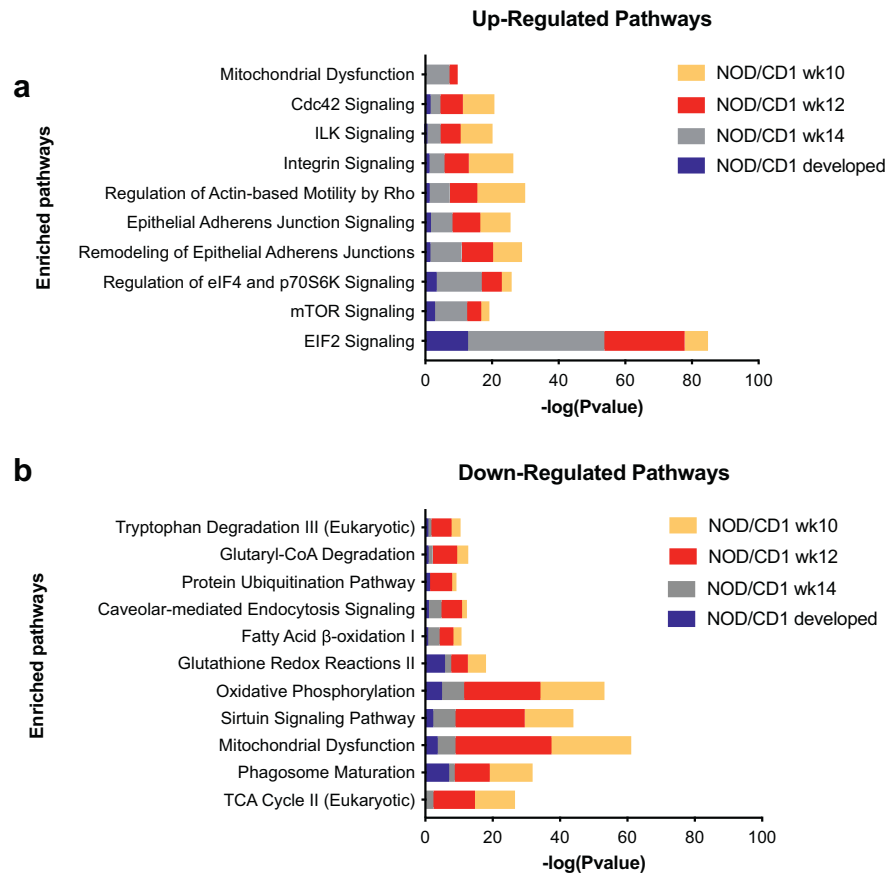


Fig. 3: Ingenuity pathway analysis of islet proteome. The top 10 upregulated (a) and downregulated (b) canonical pathways modulated during diabetes progression in islets collected from NOD mice compared to age-matched CD1 mice.

Analysis of identified protein targets by immunofluorescence and immunoblot

To verify key findings from the proteomic analysis, immunofluorescence staining of pancreatic tissue sections was performed using a separate cohort of NOD mice aged 9–13 weeks and at diabetes onset. Three protein targets, PDIA1, 14-3-3 β , and PRDX3, were selected for validation experiments based on top hits from the analysis shown in Fig. 2b and their known roles in maintaining β cell function.^{42,43,52,53} Similar to the proteomics data, the staining intensity of islet PDIA1 (Fig. 6a) and 14-3-3 β (Fig. 6b) increased from 9 to 13 weeks in NOD mice, followed by a significant loss of target protein expression at T1D onset. Changes in the staining intensity of PRDX3 did not change significantly during the prediabetic timepoints; however, PRDX3 expression was significantly decreased at T1D onset in NOD mice (Fig. 6c).

To test the relevance our findings in human T1D, pancreatic tissue sections were obtained from non-diabetic organ donors, organ donors with autoantibody positivity (AAb+), and organ donors with established

T1D. Immunofluorescence analysis of PDIA1, insulin, and glucagon was performed and revealed a significant increase in PDIA1 expression in pancreatic islets of individuals with AAb+ and with T1D compared to non-diabetic control donors (Fig. 7a and b).

PDIA1 is an ER resident protein with an established role in proinsulin maturation.⁴³ Therefore, to understand whether there was an association between ER stress and PDIA1 under conditions of β cell stress, we took an *in vitro* approach by treating human islets with or without pro-inflammatory cytokines (IL-1 β + IFN γ) or high glucose (22.5 mM) for 1 h and 24 h. Under both chronic stress conditions (i.e. 24 h treatment), we observed a parallel upregulation of PDIA1 and IRE1 α (Fig. 7c and d). BIP expression was increased but not to a significant extent. In line with this data, immunofluorescence analysis of NOD pancreatic tissue sections showed a significant upregulation of CHOP at week 11 (Supplementary Figure S7a and b). However, there was no difference in BIP expression between different age groups of NOD mice.

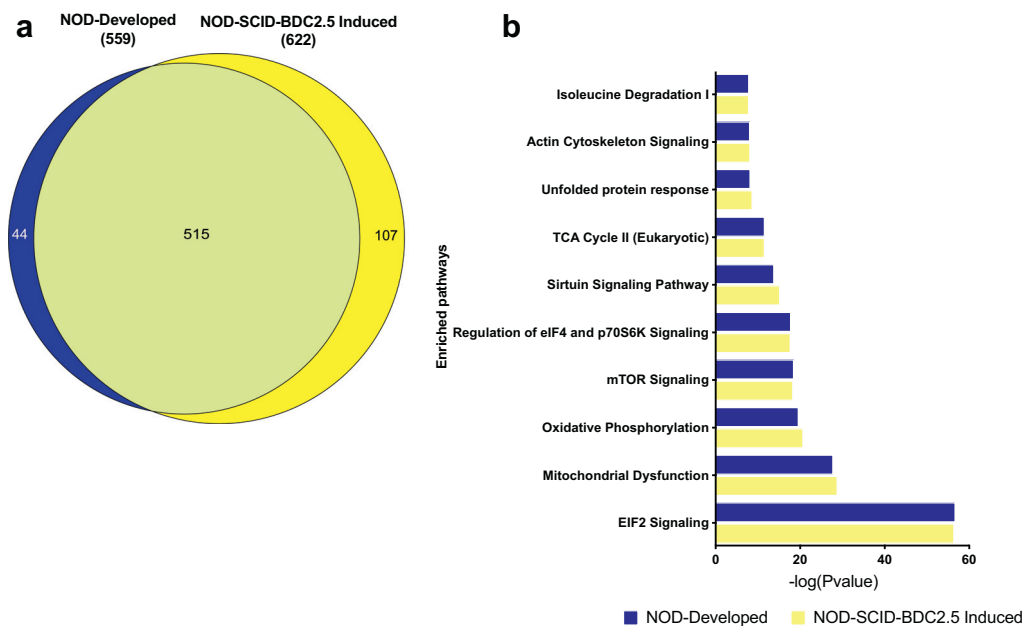


Fig. 4: Comparison of the islet proteome between acute and chronic models of T1D. (a) Venn diagram showing protein overlap between the spontaneous and inducible models of T1D. (b) Pathway enrichment analysis identified common pathways that are significantly activated during the pathogenesis of T1D ($n = 4\text{--}8$ mice/per group).

Analysis of circulating PDIA1 as a T1D associated biomarker

In addition to its intracellular role as a thiol reductase,^{43,54,55} PDIA1 is a secreted protein.⁴³ To determine whether the islet-specific upregulation of PDIA1 identified in the proteomics and immunofluorescence analyses was linked with changes in circulating PDIA1 levels, we developed a high-sensitivity electrochemiluminescence assay using Meso Scale Discovery technology. PDIA1 was measured using serially diluted (1:4) recombinant PDIA1, and this analysis showed that PDIA1 could be detected in the range of 0.152 ng/mL to 2500 ng/mL (Fig. 8a). Using plasma collected from the same mice used in the longitudinal proteomics analysis shown in Fig. 2, we found that the plasma levels of PDIA1 were significantly increased in pre-diabetic NOD mice compared to CD1 mice at 10 and 14 weeks of age (Fig. 8b–d). However, PDIA1 levels were not different between NOD mice at the time of diabetes onset and age-matched CD1 mice (Fig. 8e). In addition, PDIA1 levels were below the detectable range in plasma from diabetic NOD-SCID mice that had undergone T-cell adoptive transfer.

Next, we applied this assay to serum samples collected from children within 48 h of the clinical onset of T1D ($n = 14$; average age (mean \pm SD) = 11.57 \pm 4.05 yrs; 8 male; 6 female) and in serum collected from non-diabetic paediatric controls ($n = 10$; average age = 12.1 \pm 4.20; 6 male; 4 female) (Supplemental Table S1). Serum levels of PDIA1 were significantly

higher in paediatric subjects with recent-onset T1D compared to controls, suggesting PDIA1 may have utility as a clinical, human T1D biomarker (Fig. 8f).

Discussion

In this study, we identified temporal changes in islet β cell protein expression during the evolution of T1D using three distinct mouse models of T1D and high-throughput DIA proteomics. Additionally, we illustrated the utility of an unbiased approach to prioritize β cell proteins as T1D biomarkers in humans, identifying PDIA1 as one example of a secreted protein that was differentially expressed in NOD islets during diabetes progression and in human islets from organ donors with autoantibody positivity and diabetes. Finally, using a high-sensitivity electrochemiluminescence assay to measure circulating PDIA1 levels, we demonstrated increased plasma PDIA1 levels in pre-diabetic NOD mice and in the serum of children with recent-onset T1D compared to age- and sex-matched paediatric controls.

Proteomics analysis of the three mouse models revealed several notable themes. In the dataset obtained from the longitudinal NOD cohort, we observed an early but time-restricted increase in the expression of several proteins linked with secretory function, proinsulin folding, and stress mitigation, including proteins involved in ER and oxidative stress signalling. Interestingly, we observed week-14 as a potential inflection

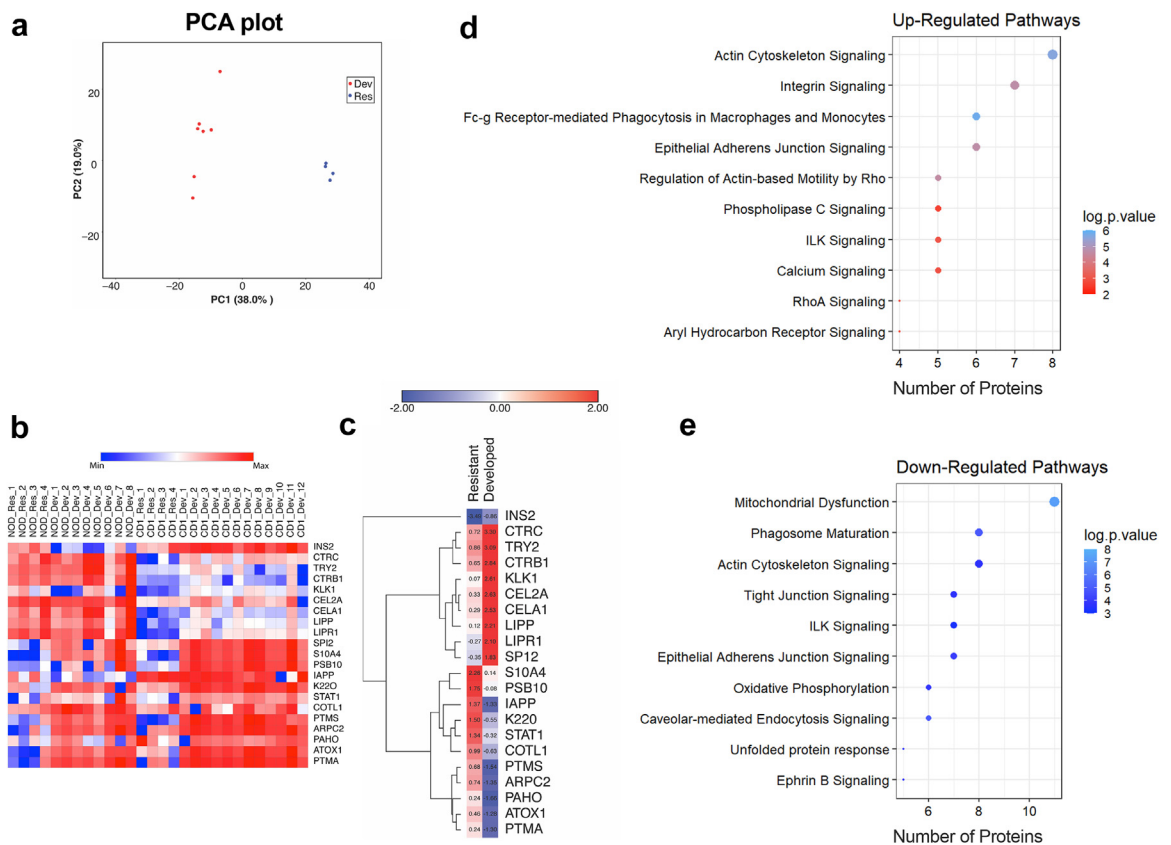


Fig. 5: Proteomics analysis of diabetes-resistant NOD mice and NOD mice that developed diabetes. (a) Principal component analysis (PCA) of the islet proteome in NOD mice that remained diabetes-free through 46–48 weeks of age (Res) and NOD mice at the time of diabetes development (Dev). (b) Heatmap showing top differentially expressed islet proteins in diabetes-resistant mice and mice at the time of diabetes onset and (c) median normalized hierarchical clustering of differentially expressed proteins. Shown are up-regulated (d) and down-regulated (e) pathways (y-axis) and the corresponding number of proteins (x-axis) differentially expressed in islets from diabetes-resistant mice ($n = 4$ –8 mice/per group).

point, where loss of expression of these protective proteins heralded T1D onset. Consistent with this observation, canonical pathway analysis of differentially expressed proteins from weeks 10, 12, and 14 and diabetes onset identified upregulation of pathways associated with defective insulin synthesis and several β cell stress pathways, including mitochondrial dysfunction, ER stress, and UPR activation. We observed a down-regulation of signalling pathways that were crucial for the mitigation of ongoing cellular stress, including glutathione redox signalling, which is known to counteract the effects of reactive oxygen species,^{56,57} and phagosome maturation, which is involved in the clearing of cellular debris.^{58,59}

This biphasic pattern is reminiscent of metabolic data from natural history cohorts of autoantibody-positive individuals who progress to T1D, where there are compensatory changes in the architecture of insulin secretion that largely maintain glycemia until

~12 months prior to disease onset, followed by loss of insulin secretion and rapid worsening of glycaemic control 12–6 months prior to diabetes diagnosis.² Our findings are also consistent with cross-sectional studies that have analysed gene and protein expression patterns in pancreatic sections from human donors with diabetes^{60–63} and in previous studies in mouse models of diabetes, where a prominent role for ER and mitochondrial dysfunction has been identified.^{8,12,64,65} We found these pathways are activated early in the disease process, and there is continued overlap between several of these key activated stress pathways in the NOD mouse model and in the acute, inducible model of T1D at the time of diabetes onset. The similarities between the proteomic analysis of these two models highlight the importance of this core set of pathways in T1D pathogenesis.

To verify findings from the proteomics analysis, we performed immunofluorescence experiments, focusing

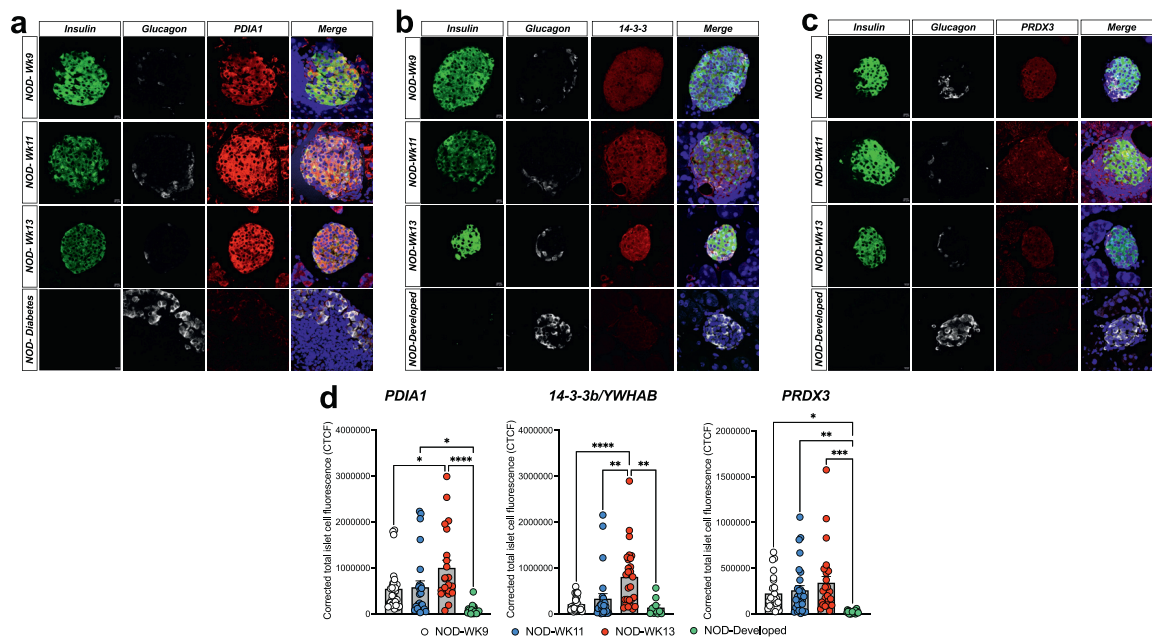


Fig. 6: Immunofluorescence-based verification of protein targets in NOD mice. (a–c) Pancreas sections from 9, 11, and 13 week old NOD mice and NOD mice that developed diabetes were immunostained for PDIA1 (a), 14-3-3 β (b), and PRDX3 (c) (red), and co-stained with insulin (green) and glucagon (white). Scale bar = 10 μ m. The bar graphs show the quantitation of fluorescence intensity for each protein target calculated using the corrected total islet cell fluorescence ($n = 4$ –7 mice/age-group; 3–7 islets/mice were used for quantification; all the values were presented as mean \pm SEM; (one-way ANOVA), * $P < 0.05$, ** $P < 0.001$).

on three targets identified in the longitudinal NOD cohort, 14-3-3 β , PRDX3, and PDIA1. Members of the 14-3-3 protein family have been implicated in various metabolic signalling pathways and have been linked with protection against apoptosis in pancreatic β cells.⁴² PRDX3 prevents mitochondrial dysfunction, and its overexpression has been shown to be protective against oxidative stress induced by insulin resistance and hyperglycaemia.^{52,66} PDIA1 is a highly abundant ER localized thiol oxidoreductase that has been implicated in glucose-stimulated insulin secretion, proinsulin processing, and protection against ER stress.⁴³ In agreement with previous reports, we observed a positive correlation between the expression of ER stress markers and PDIA1 in human islets treated with proinflammatory cytokines or high glucose. Moreover, we observed increased PDIA1 levels in pancreatic sections from human organ donors with autoantibody positivity and with diabetes.

Of note, PDIA1 is a secreted protein. In other cell types, PDIA1 release is increased in the setting of injury and stress.^{67,68} Extracellular PDIA1 has been linked with the regulation of thrombus formation during vascular inflammation,^{69,70} but a complete understanding of the extracellular role of this protein is lacking. Interestingly, anti-PDIA1 antibodies have been identified in individuals with recent-onset T1D,⁷¹ suggesting that β cell-

derived PDIA1 serves as a T1D autoantigen. Therefore, we hypothesized that increased β cell expression of PDIA1 may be reflected in the circulation and that measurement of PDIA1 may have utility as a T1D biomarker. To test this possibility, we developed a high sensitivity electrochemiluminescence assay to measure serum and plasma levels of PDIA1. Using this assay, we documented an increase in plasma PDIA1 in pre-diabetic NOD mice and in serum of children with recent-onset T1D.

Our study has several limitations that should be acknowledged. First, there are significant differences between mouse models and human T1D,^{72–74} underscoring the need to cross-validate findings from mice in human samples. We verified key findings by using human islets and pancreatic sections from human organ donors. Given the progressive nature of islet destruction in our mouse models, we were constrained by the amount of starting material in our proteomics analysis, which was reflected in the fact that 18 islet samples failed to reach our stringent QC standards. This limitation, combined with a lack of a defined proteomic library for murine islet cells, limited the total number of proteins we confidently identified. However, we overcame this constraint by using a library-free DIA-Umpire search and a strategy involving downstream validation of our candidate marker. Finally, a limitation of all

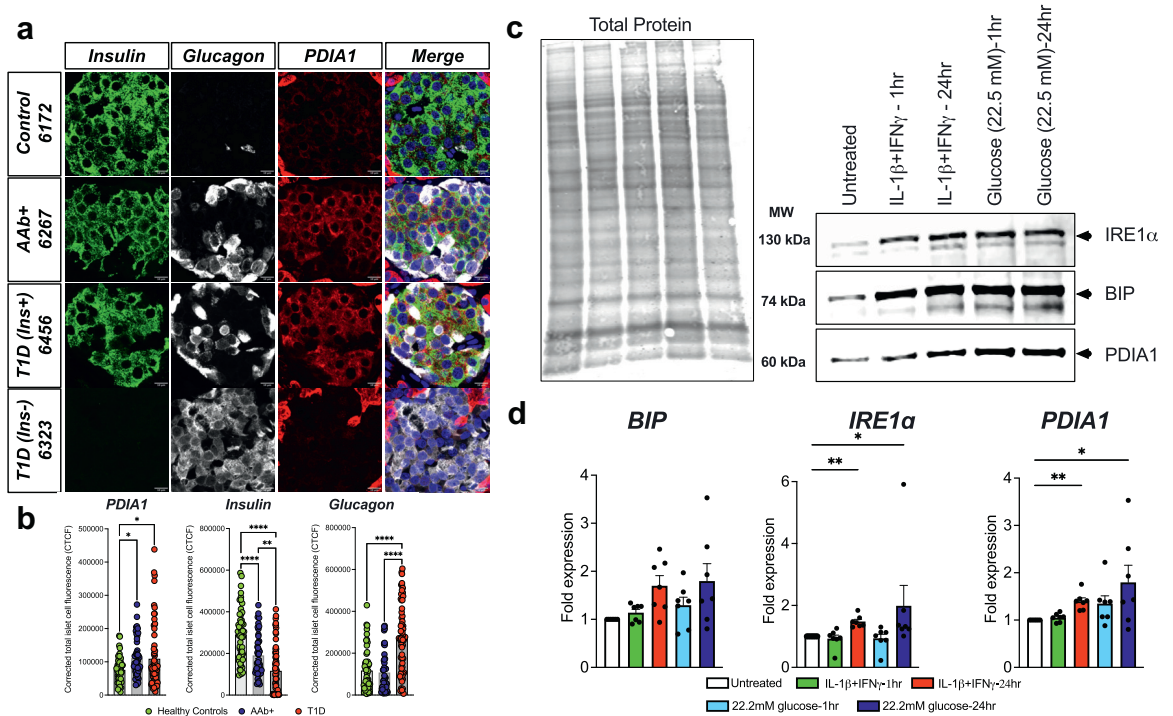


Fig. 7: Islet PDIA1 expression is increased in human pancreatic tissue sections from organ donors with autoantibody positivity and with T1D. (a) Human pancreatic tissue sections from non-diabetic human organ donors, organ donors who were autoantibody positive (AAb+) but without a diagnosis of T1D, and organ donors with established T1D were immunostained for PDIA1 (red) and co-stained for insulin (green), glucagon (white), and DAPI (blue). Scale bar = 10 μm. (b) The bar graphs show the quantitation of fluorescence intensity for each protein target calculated using the corrected total islet cell fluorescence (n = 5–7 donors/per group; 5–10 islets/donor). (c) Western blot analysis of human islets (n = 7 donors) treated with or without pro-inflammatory cytokines (IL-1β + IFNγ) or high glucose (22.5 mM) for 1 h or 24 h, (d) Protein expression was normalized to Revert™700 total protein staining and is presented as fold expression compared to untreated controls, all the values are presented as mean ± SEM (one-way-ANOVA); *P < 0.05, **P < 0.001.

proteomics studies employing intact islets is that the data represent a heterogeneous group of endocrine and immune cells. Therefore, findings may not only reflect changes in the β cell proteome.

Our study documented increased serum levels of PDIA1 in a small cohort of paediatric subjects with recent-onset T1D. While biomarkers with the ability to non-invasively monitor β cell stress are lacking in T1D, it is important to note that ours is a small cross-sectional study and validation in larger cohorts should be performed. It will be essential to test PDIA1 levels in samples collected from clinical cohorts followed longitudinally during T1D progression. Such an analysis will provide necessary insight into whether PDIA1 can predict T1D risk. Our analysis of human pancreatic sections indicated that PDIA1 levels are increased in the pancreatic islets in at-risk individuals who are autoantibody positive. Whether PDIA1 is purely a marker of β cell stress or may reflect β cell mass changes is not clear from our data and should be tested in follow-up studies. It is noteworthy that PDIA1 levels were higher in

children with new onset T1D, whereas plasma elevations in PDIA1 were most different at the pre-diabetic timepoints in NOD mice. This data is consistent with more recent findings showing that there is substantial β cell mass remaining in humans at T1D onset,^{75,76} whereas in both of the mouse models studied here, β cells are nearly completely destroyed by the time of diabetes onset.^{26,77}

Notwithstanding these limitations, our study highlights the value of unbiased proteomics approaches for identifying β cell pathways involved in the temporal evolution of T1D. Utilizing this strategy, we identified a common set of modulated and disease-related pathways across several distinct mouse models of T1D. Finally, we identified increased PDIA1 expression as a marker of early β cell stress in T1D, and our data indicates that measurement of serum or plasma PDIA1 may serve as a clinically useful biomarker that merits additional follow-up testing in populations at risk of developing T1D.

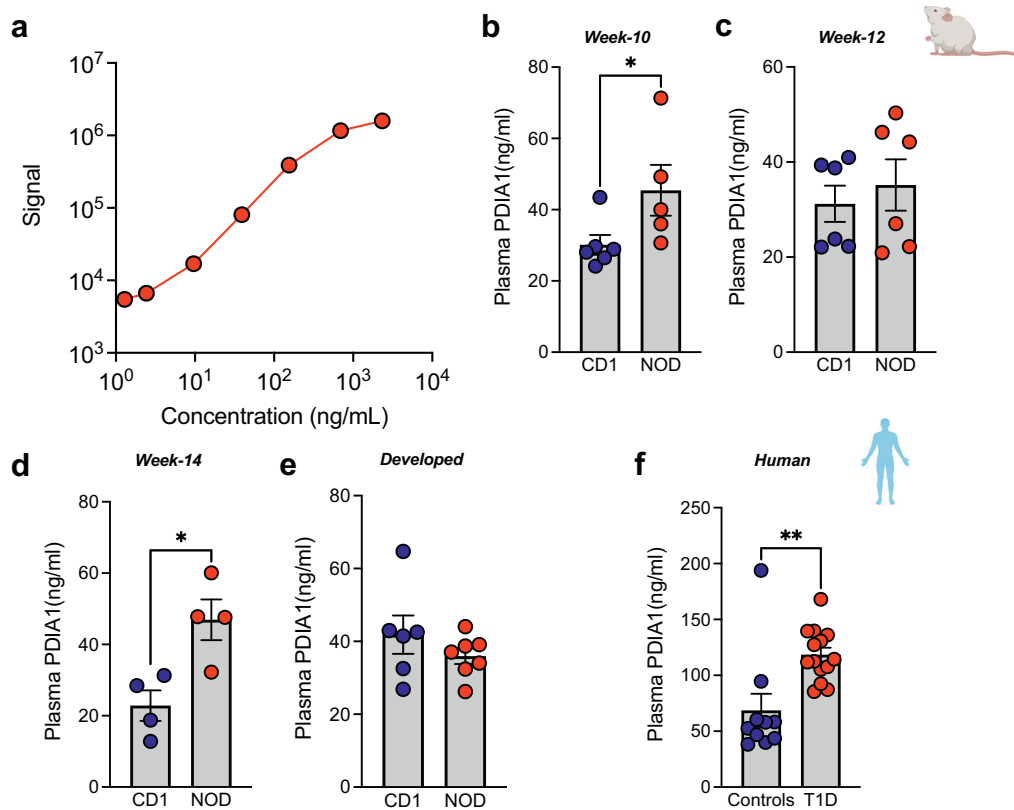


Fig. 8: Circulating PDIA1 levels are increased in pre-diabetic NOD mice and in children with recent-onset T1D. (a) Standard curve generated using a serial dilution of recombinant PDIA1 protein showing higher and lower detection limits of assay sensitivity (b–e) Measurement of circulating PDIA1 levels in plasma samples of sex- and age-matched CD1 and NOD mice at different ages. $n = 4\text{--}7$ mice/group, * $P < 0.05$, ** $P < 0.001$. (f) Plasma concentration of PDIA1 in non-diabetic control subjects ($n = 10$, average age 12 ± 4.2 yrs) and paediatric subjects with recent-onset T1D ($n = 14$, average age 11.57 ± 4.05 yrs), all the values are presented mean \pm SEM (t-test), ** $P = 0.001$.

Contributors

FS and CEM conceived and designed the study. FS and RNB performed experiments. KR and JV prepared and ran LC-MS/MS samples. DS, XL, HW, and PK performed computational analyses. MRM, RGM, M-LY, and MJM interpreted results. FS and CEM interpreted the data and wrote the manuscript. All authors provided critical revisions and edits to the manuscript. All authors read and approved of the final manuscript. CEM is the guarantor of this work. Both FS and CEM have verified the underlying data of this manuscript.

Data sharing statement

Data presented as part of this manuscript are available from the corresponding author upon request. The proteomics data has been deposited to the PRIDE proteomics database (accession ID: PXD035504).

Declaration of interests

CEM and FS have filed a provisional patent application for the use of PDIA1 as a diabetes biomarker. CEM has served on advisory boards related to T1D research clinical trial initiatives: Prevention Bio, Dompe, Isla Technologies, and MaiCell Technologies. CEM serves as President of the Immunology of Diabetes Society (IDS), Co-Executive Director of the Network for Pancreatic Organ Donors with Diabetes (nPOD), Scientific Advisor in TrialNet, and PI of the NIH Integrated Islet

Distribution Program (IIDP). These activities have not dealt directly with topics covered in this manuscript. All other authors declare no competing interests.

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research resources are listed at <http://www.jdrfnpod.org/for-partners/npod-partners>. The authors acknowledge the support of the Islet and Physiology Core and the Translation Core of the Indiana Diabetes Research Center (P30DK097512). We thank Dr. Emily Anderson-Baucum and Dr. Jan Kajstura for their assistance in editing the text of this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2022.104379>.

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