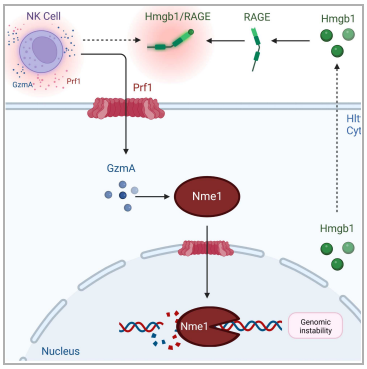

Pancreatic β cell-specific deletion of Helicase-like transcription factor (Hltf) activates the Hmgb1-Rage axis and granzyme A-mediated killing of pancreatic β cells resulting in neonatal lethality V.1

Gurvinder Kaur[1], Rebecca A. Helmer[2,3], Dalia Martinez-Marin[2,4], Souad R. Sennoune[5], Rachel L. Washburn[2], Raul Martinez-Zaguilan[5], Jannette M. Dufour[2], Beverly S. Chilton[2]

[1]Department of Medical Education, Texas Tech University Health Sciences Center, Lubbock, TX, USA;
[2]Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX;
[3]Garrison Independent School District, Garrison, Texas, USA, USA;
[4]Department of Immunology and Molecular Microbiology, Texas Tech University-Health Sciences Center, Lubbock, TX, USA;
[5]Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX, USA

Beverly S Chilton

VERSION 1

APR 24, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.kxygx9yqdg8j/v1

External link: http://Hltf, pancreatic beta cell-specific, diabetes

Protocol Citation: Gurvinder Kaur, Rebecca A. Helmer, Dalia Martinez-Marin, Souad R. Sennoune, Rachel L. Washburn, Raul Martinez-Zaguilan, Jannette M. Dufour, Beverly S. Chilton 2023. Pancreatic β cell-specific deletion of Helicase-like transcription factor (Hltf) activates the Hmgb1-Rage axis and granzyme A-mediated killing of pancreatic β cells resulting in neonatal lethality. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygx9yqdg8j/v1

License: This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Protocol status: Working
We use this protocol and it's working

Created: Dec 04, 2022

Last Modified: Apr 24, 2023

PROTOCOL integer ID: 73532

Keywords: Hltf, pancreatic beta cells, diabetes

ABSTRACT

Insulin-producing pancreatic β cells maintain glucose homeostasis and their loss of function causes diabetes. Epigenetic mechanisms that do not alter the DNA sequence are integral to β cell function. Promoter hypermethylation of the helicase like-transcription factor (HLTF) gene—a component of the cellular DNA damage response that contributes to genome stability—has been implicated in age-associated changes in β cell function. To study helicase-like transcription factor (HLTF) we generated global and β cell-specific (β) *Hltf* knockout (KO) immune competent (IC) and immune deficient (ID) *Rag2-/IL2-*mice. IC global and β *Hltf* KO mice were neonatal lethal whereas ID global and β *Hltf* KO newborn mice had normal survival. This focused our investigation on the effects of Rag2 interruption with common gamma chain interruption on β cell function/survival. Three-way transcriptomic (RNAseq) analyses of whole pancreata from IC and ID newborn β *Hltf* KO and wild type (*Hltf* +/+) controls *combined with spatially resolved transcriptomic analysis of formalin fixed paraffin embedded tissue, immunohistochemistry and* laser scanning confocal microscopy showed DNA damage caused by β *Hltf* KO in IC mice upregulated the Hmgb1-Rage axis and a gene signature for innate immune cells. Perforin-delivered granzyme A activation of the DNase, Nme1, damaged nuclear single-stranded DNA (γH2AX immunostaining). This caspase-independent method of cell death was supported by transcriptional downregulation of the *Serpinc1* gene that encodes a serine protease inhibitor of GzmA. Increased transcriptional availability of the complement receptors *C3ar1* and *C5ar1* likely invited crosstalk with Hmgb1 to amplify inflammation. This study explores the complex dialog between β cells and immune cells during development, and has implications for the initiation of type I diabetes in utero, when a gene deletion that compromises genome stability invokes a localized inflammatory response.

MATERIALS

Spatial transcriptomics library slides from 10X Genomics

Reagents and Kits

1 Infrared warming pads were from Kent Scientific (Torrington, CT). OneTouch Ultra Mini and OneTouch Ultra Mini Blue test strips for the measurement of blood sugar were from LifeScan (Malpitas, CA). MiniCollect® red top capillary blood collection system (Z serum Clot Activator 450470, Greiner Bio-One) in combination with the MiniCollect® capillary tubes (450431) were from Summus Henry Schein (Melville, NY). Genomic DNA from tail biopsies was isolated with the DNeasy® Blood & Tissue Kit (69506) purchased from Qiagen (Valencia, CA). RNeasy® FFPE Kit (73504) and Deparaffinization Solution (19093) were also purchased from Qiagen. Invitrogen RNAlater stabilization solution (7020), and SequalPrep™ Long PCR Kit with dNTPs (A10498) were from ThermoFisher Scientific (Grand Island, NY). Midland Certified Reagent Company (Midland, TX) synthesized the PCR primers. MetaPhor™ Agarose (50180) was from LONZA (Rockland, ME). Promega (Madison, WI) was the source of the Lambda DNA/EcoRI + HindIII agarose gel markers

(G173A). ALPCO was the source of mouse ultrasensitive insulin ELISA kits (80-INSMSU-E01). Vectastain® ABC-HRP Kit, Peroxidase (Guinea Pig IgG; PK-4007), Vectastain® ABC-HRP Kit, Peroxidase (Standard; PK-4000), and Hematoxylin QS (H-3404) were purchased from Vector Laboratories (Burlingame, CA). Diaminobenzidine (HK542-XAKE) was from BioGenex (Fermont, CA). Invitrogen by Thermo Fishcer Scientific was the source of ProLong™ Gold antifade reagent with DAPI (P36935). The DeadEnd™ Fluorometric TUNEL assay (Apoptosis Detection System, G3250) was from Promega Corporation (Madison, WI). 10X Genomics (Pleasanton, CA) was the source of all Visium reagent kits for whole transcriptome profiling of intact formalin fixed paraffin embedded (FFPE) tissue sections including test slide (PN-1000347), slide kit (PN-1000188), reagent kit (PN-1000361), mouse transcriptome probe kit (PN-1000365), accessory kit (PN-1000194) and dual index kit TS Set A (PN-1000251). KAPA SYBR FAST qPCR master mix (KK4600) was purchased from Roche Diagnostic Corporation (Indianapolis, IN). SPRIselect (B23317) was purchased from Beckman Coulter Life Sciences (Indianapolis, IN). IgG-free, protease-free bovine serum albumin (BSA, 001-000-162) was purchased from Jackson ImmunoResearch (West Grove, PA), ProLong Gold antifade reagent with DAPI (P36935) was from Invitrogen by Thermo Fisher Scientific. Primary and secondary antibodies used with FFPE tissue sections in immunohistochemistry (IHC-P) and immunofluorescence (IF) are listed in Table 1.

1.1 Table 1. Source, application and concentration of antibodies

Primary Antibodies	Secondary Antibodies
Dako/Agilent Technologies (Santa Clara, CA) R.T.U. (ready-to-use) polyclonal guinea pig anti-swine insulin #IR002, IHC-P/IF 1:1000	IHC-P: Vector Laboratories RTU Biotinylated Goat Anti-rabbit IgG (H+L) (BP-9100-50) Alexa Fluor 594 goat anti-guinea pig (A-11076) from ThermoFisher Scientific (1:200) Alexafluor 488-Phalloidin (1:500) in PBS-BSA 1%
Abcam (Cambridge, MA) rabbit monoclonal anti-γH2AX (phospho S139) ab81299 IHC-P 1:50 Bioss Antibodies Inc. (Woburn, MA) Rabbit polyclonal anti-Granzyme A (BS-2578R) from ThermoFisher IHC-P 1:200 Proteintech Rabbit polyclonal anti-NME1 (11086-2-AP) from Life Technologies Corp. IHC-P 1:20	IHC-P: Vector Laboratories RTU Biotinylated Goat Anti-rabbit IgG (H+L) (BP-9100-50)

Hltf-deleted mouse models and controls

2 IC global *Hltf* KO mice were developed in collaboration with genOway (Lyon, France) as previously described [15]. Mice with either the *Hltf*-deletion or floxed *Hltf*-gene were bred to the recombinase activating gene 2 (*Rag2*)/common gamma (*IL2rg*) double knockout mice [16], thereby generating ID *Hltf* KO or *Hltf-fl/fl* mice. Mice with a floxed Hltf-gene that were either IC or ID were bred to Rip-Cre^{TG} mice (The Jackson Laboratory, Stock No. 003573) thereby deleting Hltf selectively in β cells (β *Hltf* KO)

All mice are in sentinel-monitored, rodent housing in the Laboratory Animal Resource Center (LARC) at Texas Tech University Health Sciences Center (TTUHSC). Additionally, mice on the *Rag2*)/*IL2rg* double knockout background are in bioBubble™-husbandry conditions in the LARC. Pathogen free mice were able to access food and water *ad libitum*. All studies were in accord with the NIH Guidelines for the Care and Use of Laboratory Animals, as reviewed and approved by the Animal Care and Use Committee at TTUHSC [NIH Assurance of Compliance A3056-01; USDA Certification 74-R-0050, Customer 1481]. TTUHSC's IACUC specifically approved this study. Pain and suffering were always minimal. Term pregnant females received an IP injection of a Ketamine/Xylazine cocktail at 100 microliters per 20 g body weight. The cocktail contained 87.5 mg/kg Ketamine and 12.5 mg/kg Xylazine. Following surgical removal of unborn pups and their placentae, previously pregnant females were euthanized by drug overdose followed by cervical dislocation.

Genotyping

3 PCR screening reaction to authenticated the ID *Hltf* KO genotype is as previously described [15-17] except we used SequalPrep reagents. PCR screening reactions were used to detect amplicons unique to the *Hltf floxed* allele (329-bp wildtype, 329/424-bp heterozygous, 424-bp floxed), and the *rIPCre* transgene (550-bp). Each 50 µl PCR reaction consisted of genomic DNA (60 ng), primer pairs (15 pmol each, Table 2), SequalPrep Long Reaction Buffer with nucleotides (5 µl of 10X), SequalPrep Long Enhancer B (2.5 µl), DMSO (1 µl), SequalPrep Long Polymerase (1 µl = 5 U). Reaction conditions for the *Hltf* floxed allele were as follows: 120 sec at 94ºC, followed by 35 cycles of 94ºC for 30 sec, 65ºC for 30 sec, and 68ºC for 120 sec, and a final extension for 480 sec at 68ºC.Reaction conditions for the rIPCre transgene were as follows: 360 sec at 94ºC followed by 40 cycles of 94ºC for 60 sec, 60ºC for 30 sec, and 72ºC for 30 sec, and a final extension for 420 sec at 72ºC. At the conclusion of each reaction, samples were cooled rapidly to 4ºC, and amplicons were resolved/visualized by MetaPhor™ agarose (2%) gel electrophoresis with ethidium bromide (0.05 µg/ml).

3.1 Table 2. PCR primers for genotyping and gender authentication

Primers	
Hltf-floxed forward	5'-ACC TCA ATT GAC ATC TTA ATC GGT CG-3'

Hltf-floxed reverse	5'-CTG CCA AGA TAC TCC AAA TCT GTT CAC TAC-3'
rIPCre forward	5'-CTC TGG CCA TCT GCT GAT CC-3'
Cre 102 reverse	5'-CGC CGC ATA ACC AGT GAA AC-3'
Myog forward	5'-TTA CGT CCA TCG TGG ACA GC-3'
Myog reverse	5'-TGG GCT GGG TGT TAG TCT TA-3'
Sry forward	5'-TCA TGA GAC TGC CAA CCA CAG-3'
Sry reverse	5'-CAT GAC CAC CAC CAC CAC CA-3'

Serum collection from newborn mice

4 Postprandial newborn mice are unable to thermoregulate, and were placed on infrared warming pads (37ºC) to avoid the negative effects of hypothermia on blood glucose prior to decapitation with surgical scissors. Blood glucose in trunk blood was measured immediately in all members of each litter with the exception of pups that were already dead. Low (≤15 mg/dL) blood sugar in global and β *Hltf*KO mice affected anywhere from one pup in the litter to the entire litter. We used the MiniCollect® capillary blood collection system to collect trunk blood. Serum was removed from clotted blood after centrifugation. Serum samples (5-30 µl) were stored frozen (-20ºC) until use in an ultrasensitive insulin test. Tails from pups were used for genotyping and gender authentication.

Mouse Ultrasensitive Insulin ELISA

5 The Mouse Ultrasensitive Insulin ELISA (Alpco) quantified the concentration of insulin protein from mouse I and mouse II proinsulin genes according to the manufacturer's instructions. There was no cross reactivity with mouse C-peptide 1 or 2, or mouse IGF 1 or 2. Because 25 µl of serum was required for hypoinsulinemic samples, it was necessary to pool serum samples in a gender specific manner as shown in Table 3.

5.1 **Table 3. Number (N) of samples in blood sugar and serum insulin calculations.**

Genotype	N animals = N blood glucose values	N = Insulin from pooled serum
IC *Hltf +/+* (control)	231	69
IC Global *Hltf* KO	209	26

IC β *Hltf* KO	182	22
IC *Hltf fl/fl* (control)	193	43
IC *rIPCre Hltf +/+ (control)*	191	43
ID *Hltf+/+* (control)	87	27
ID *Global Hltf* KO	183	27
ID β *Hltf* KO	303	109

Analysis of pancreatic tissue

6 Abdominal segments of IC Hltf +/+ (control) and global *Hltf*KO E18.5 mice (N=8/group) were formalin fixed overnight at 4ºC, paraffin embedded, and serially sectioned (4 µm). Tissue sections were either stained with Hematoxylin and Eosin (H&E), or immunostained for insulin. Diaminobenzidine was the chromogen.

Abdominal segments of newborn IC pups were infused with formalin-based fixative (n=12 each for global *Hltf*-KO with low (≤15 mg/dL) blood sugar, *Hltf* +/+ control, and β *Hltf* KO with low (≤15 mg/dL) blood sugar. In companion experiments, abdominal segments of ID new born pups of the same three genotypes were infused with formalin-based fixative. All tissues were fixed overnight at 4ºC, paraffin embedded, and serially sectioned (4 µm).

For tissue insulin quantification, tissue sections were subjected to heat-induced epitope retrieval (HIER) with citrate buffer pH 6.0, then immunolabeled (Table 1). Slides were then incubated with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1 µg/mL) to detect cell nuclei. Images at 20x magnification were merged, and quantified with Image J software.

Immunocytochemistry (Table 1) for laser scanning confocal microscopy was performed with serial sections from the above described groups of newborn pups with HIER, aldehyde quench (50 mM NH_4Cl in PBS), and ProLong Gold DAPI.

TUNEL Assay

7 Apoptosis was determined using the DeadEnd^{TM}Fluorometric TUNEL assay with slides from the samples used for quantification of insulin expression (above) according to the manufacturer's instructions. Negative controls included sections incubated without the TdT enzyme, and were devoid of a positive reaction. For quantification, the area of TUNEL positive cells was determined using particle analysis (internal function of Image J) in pixels^2 for each image. Contrast enhancement expanded the dynamic range of images, and color threshold was set at a constant value, which only selected the positive staining areas. To control for tissue size, the total area of TUNEL positive cells was normalized to the total tissue area.

Statistical analysis

8 All values are expressed as the mean ± standard error of the mean (SEM) of *n*independent experiments. With the exception of RNA-seq and spatial transcriptomics data analyses, all data analyses were conducted with GraphPad Prism version 9.1.1 software. For multiple comparisons, we performed a one-way analysis of variance (ANOVA) with an appropriate *post hoc* test as described for each experiment, $p<0.05$ was significant.

Pancreatic transcriptome (RNAseq)

9 Because pancreata are ribonuclease-rich [18], trunks of decapitated newborn mice were perfused *in situ*with RNAlater by insertion of a 20g-1-inch needle attached to a 5 ml syringe into the abdomen [19] via the crural (posterior) attachment of the diaphragm [20]. RNA stabilization occurred concomitant with the initial stretching of the pancreas. Pancreata were stored in RNA later at -70C until total RNA was isolated. RNA integrity and purity were assessed (Agilent Bioanalyzer) for 10 samples, i.e. 3 from β *Hltf*KO IC mice with low (≤15 mg/dL) blood sugar, 3 from *Hltf+/+* controls, and 4 from β *Hltf*KO IDmice. cDNA was generated from Ribo-Zero Plus rRNA-depleted samples and subjected to Illumina library preparation. Libraries were sequenced utilizing Illumina sequencing technology. Paired-end 100 nucleotide reads were aligned to reference mouse genome C57BL/6J (GRCm38/mm10) and analyzed using the platform provided by DNAnexus, Inc. (Mountain View, CA) to generate three-way transcriptomic (RNAseq) analyses of whole pancreata from β *Hltf*KO IC and ID newborn mice, and wild type (*Hltf* +/+) controls. The analysis included alternative splicing analysis in control (*Hltf +/+*) pancreata. The power in detecting alternative splicing was dramatically increased by paired-end sequencing relative to single-end sequencing. FPKM (fragments per kilobase of transcript per million mapped reads) were mapped against mm10 with Tophat (V1.3.3) to obtain .bam mapping files that were input into Cufflinks for transcript assembly. Cuffdiff (V 1.3.0), part of the Cufflinks package, used the alignment reads for rigorous statistical comparison of the three genotypes. The depth of sequencing (Table 3) was a minimum of 20 million sequencing reads per sample [90% Power, 5% significance level: 91+/- 4% of all annotated genes are sequenced at a frequency of 0.1 times/10^3bases X 3 x 10^9 bases/sequencing read x 3 samples = 9 x10^4reads/gene]. All RNA-seq data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE137060. (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE137060). Data were imported into iPathwayGuide (Advaita Corporation) a next-generation pathway analysis tool. Standard enrichment parameters (log2 fold change, log2 FC = 0.6, p<0.05) were used.

9.1 **Table 4. Sample quality control and RNA-seq outcome for *Hltf*-deleted, i.e. knockout (KO) and control (*Hltf* +/+) samples**.

Spatial Transcriptomics

10 **Work flow –** five basic steps were necessary to implement spatial transcriptomics technology. Step 1, placement of FFPE tissue (abdominal segments) on capture areas of a Visium gene expression (GEX) slide. Step 2, H&E staining followed by brightfield microscopic imaging with ZEISS Axioscan 7 high-performance slide scanner (White Plains, NY). Step 3, permeabilization of tissue and construction of barcoded libraries with a final sample index PCR all according to the manufacturer's instructions. Step 4, NGS short-read sequencing (Illumina NovaSeq) of barcoded libraries by Genewiz (Azenta US, Inc, South Plainfield, NJ). Step 5, data analysis of tissue images and sequencing files in FASTQ format with Space Ranger run on Ubuntu 22.04 LTS –Thelio Mira-b3 by System76, Inc. (Denver, CO). The space ranger aggr pipeline was used to aggregate data from replicate samples and from samples from the different biological conditions (IC, ID). Loupe browser visualization software was accessed in a desktop application via Windows (Dell Optiplex 990).

FFPE sections – abdominal tissue sections (5 µm) from IC and IDβ *Hltf* KO newborn mice were processed with the RNeasy FFPE kit for DV200 analysis. Replicate sections from IC and IDβ *Hltf*KO newborn mice were placed within fiducial frames of capture areas A,B and C,D respectively, on Visium GEX slide V11D13-089-A1. 10X Genomics best practices guide helped to maintain tissue adhesion and RNA integrity before and after sectioning.

GEX slide – 4 capture areas (6.5 x 6.5 mm each) inside fiducial frames that measures 8 x 8 mm. Each capture area contains 5,000 gene expression spots (55 µm in diameter) spaced with a distance of 100 µm between the centers of each spot and captures gene expression data for 1-10 cells. Visium for FFPE uses RNA-templated ligation (RTL) probes targeting the whole transcriptome. The assay does not capture transcripts directly, but captures probes via a capture sequence, e.g. poly-A for Visium for FFPE probes. Each gene expression spot has primers with a unique spatial barcode Probes are designed against the entire mouse genome, each with primers that include Illumina TruSeq Read 1 (partial read 1 sequencing primer), 16 nt spatial barcode (all primers in a specific spot share the same spatial barcode), 12 nt unique molecular identifier (UMI), and 30 nt poly(dT) sequence (captures ligation product). Spatially barcoded, ligated products were released from the slide, and harvested for qPCR with KAPA SYBR Fast qPCR master mix. The threshold for determining the Cq value for each sample was set along the exponential phase of the amplification plot at ~25% of the peak fluorescence value with QuantStudio 12 K Flex real-time PCR system (ThermoFisher Scientific). Sample index sets were selected to distinguish each of the 4 samples in a multiplexed sequencing run. Samples were amplified using Ilumina-compatible indexing primers, cleaned up with SPRIselect reagent, and bi-directionally sequenced.

Mouse Probe Set – Visium Mouse Transcriptome Probe Set v1.0 contains 20,551 gene ids targeted by 20,873 probes. Gene ids (1,086, 5.3%) targeted by 1,110 probes were excluded by default due to predicted off-target activity to a different gene. As a result, 19,465 gene_ids (targeted by 19,763 probes) were present in the final filtered output. During data analysis, read 2 sequences were mapped against the reference mouse genome C57BL/6J (GRCm38/mm10) and read 1 sequences were used for UMI filtering to obtain spatial information.

Sequencing – Illumina NovaSeq at GenWiz (Azenta Life Sciences, South Plainfield, NJ). Unique dual indexing — unique identifiers on both ends of the sample — allows for an increase in the number of samples sequenced per run and reduce per-sample cost compared to other indexing strategies. Sequencing depth was a minimum of 50k read pairs per spot covered with tissue. This was calculated by estimating the percent of capture area covered by the tissue section based upon the H&E brightfield image. Actual values are provided in Table 5.

Bioinformatics analysis utilizes the Visium Spatial Gene Expression Software Suite that includes Space Ranger and Loupe Browser. Space Ranger has five pipelines relevant to spatial gene expression experiments. Three are available for FFPE data analysis.

Spaceranger mkfastq demultiplexed the Illumina sequencer's base call files (BCLs) for each flow cell directory into FASTQ files. Spaceranger count combined a brightfield microscope slide image and FASTQ files from spaceranger mkfastq and performed alignment, tissue detection, fiducial detection, barcode/UMI counting., and prepared a full resolution slide image for visualization in Loupe Browser.

The pipeline used the Visium spatial barcodes to generate feature-spot matrices, determine clusters, and perform gene expression analyses. The pipeline uses a probe aligner algorithm for FFPE tissues. Outputs were delivered in BAM, MEX, CSV, HDF5, TIFF, PNG, JPEG and HTML formats. Spaceranger aggr used the output of multiple runs of spaceranger count from related samples and aggregated their input, normalizing those runs to the same sequencing depth, and then recomputed the feature-barcode matrices and the analysis on the combined data. The aggr pipeline combined data from multiple samples into an experiment-wide feature-barcode matrix and analysis. Loupe Browser was used to interrogate significant genes, characterize and refine gene clusters, and perform differential expression analyses.

10.1 **Table 5. Statistics for spatial transcriptomics outcome for Visium_FFPE_Mouse_Pancreas in ID β*Hltf*-KO (Sample ID, A and B) and IC β *Hltf*-KO (Sample ID, C and D).**