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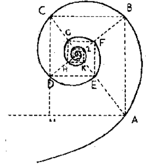

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Role of tRNA modifying enzymes in pancreatic beta cell demise

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"… to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Sir Isaac Newton

SOMMARIO

I transfer RNA (tRNA) sono piccole molecole di circa 70-80 nucleotidi, che hanno un ruolo cruciale nella sintesi proteica. Le molecole di tRNA sono altamente modificate a livello post-trascrizionale; la metilazione di residui nucleotidici è una delle modificazioni più comuni. Diversi enzimi sono responsabili della modificazione di tRNA e la loro funzione è essenziale per il mantenimento delle funzioni cellulari in quanto può regolare la stabilità, l'aminoacilazione e la rigidità della struttura del tRNA. I tRNA, quando de-aminoacilati o degradati in frammenti, sono importanti molecole di segnalazione all'interno della cellula e possono attivare diversi pathways di risposta a condizioni di stress. Per questo motivo non è sorprendente che mutazioni in diversi enzimi che modificano il tRNA siano state associate a patologie di diversa natura nell'uomo. Diversi polimorfismi del gene CDKAL1, codificante la proteina Cdk5 regulatory associated protein 1, sono stati associati a diabete di tipo 2 (T2D) nell'uomo. CDKAL1 codifica per una metil-tio transferasi che modifica il residuo 37 in tRNA che riconoscono il codone UUU specifico per l'aminoacido Lisina. E' stato dimostrato che l'assenza di CDKAL1 e, di conseguenza, della modificazione catalizzata dall'enzima induce una diminuzione dell'efficienza di incorporazione di residui di lisina nella pro-insulina, importanti per la corretta maturazione, a livello delle cellule beta del pancreas. Questo provoca la diminuzione della maturazione di pro-insulina in insulina e peptide-C, con difetti nella secrezione di insulina glucosio-dipendente. Inoltre l'assenza di CDKAL1 porta all'aumento di markers di stress del reticolo endoplasmico. L'attivazione dei processi di stress del reticolo endoplasmico induce il blocco della sintesi proteica e l'attivazione cronica innesca pathways pro-apoptotici. Questi eventi sono alla base dello sviluppo del diabete di tipo 2.

Il presente lavoro ha lo scopo di studiare il ruolo di enzimi che modificano il tRNA a livello delle beta cellule pancreatiche e di investigare le conseguenze di mutazioni in questi enzimi sulla funzione e la sopravvivenza cellulare.

L'Iperinsulinismo Congenito è una malattia rara, caratterizzata da un'inappropriata secrezione di insulina che causa ipoglicemia. Mutazioni in nove geni sono state precedentemente individuate come causative della malattia, nonostante la causa genetica sia ancora ignota nel 50% dei pazienti. Uno studio di Whole Exome Sequencing in pazienti di Iperinsulinismo Congenito, attuato precedentemente nel nostro laboratorio, ha portato all'individuazione di una lista di geni candidati. L'utilizzo di strumenti bioinformatici mi ha permesso di identificare CDKAL1 come uno dei candidati più promettenti. La mutazione individuata porta alla sostituzione S561F con probabili conseguenze sul dominio transmembrana della proteina, che ne assicura la localizzazione nel reticolo endoplasmatico. Allo scopo di studiare le conseguenze della variante di CDKAL1 sulle beta cellule, ho utilizzato tecniche di biologia molecolare per indurre l'overespressione del gene wild type e mutato nella linea cellulare INS-1E, modello di beta cellule ampiamente utilizzato derivata da insulinoma di ratto. La localizzazione di CDKAL1 è stata valutata con l'utilizzo di microscopia ad immunofluorescenza; è stato quindi dimostrato che la variante S561F di CDKAL1 altera la localizzazione della proteina, che, nonostante sia ancora inserita nella membrana del reticolo endoplasmico, tende ad accumularsi in strutture vescicolari in alcune zone della membrana. Per saggiare le conseguenze della mutazione sulla funzione delle beta cellule, ho valutato il contenuto e il

rilascio di insulina. L'overespressione di CDKAL1 porta ad un aumento del contenuto di insulina. Questo effetto non è stato invece osservato in cellule che overesprimono la variante S561F, che induce in contrasto un aumento nella secrezione basale (non stimolata da glucosio) di insulina. Questi dati preliminari suggeriscono che la variante S561F di CDKAL1 possa avere un ruolo nello sviluppo delle disfunzioni della beta cellula che provocano un'alterata secrezione insulinica.

La metiltransferasi TRM10 è un enzima che modifica tRNA in lievito. Una mutazione nel gene omologo umano TRMT10A che porta all'inserimento di un codone di stop e alla conseguente assenza della proteina è stata identificata in pazienti affetti da microcefalia e diabete con insorgenza giovanile. E' stato dimostrato che TRMT10A è una metiltransferasi anche in cellule umane e che modifica residui di guanina. L'assenza della proteina a livello di cellule pancreatiche provoca un aumento dell'apoptosi in condizioni basali e sensibilizza le cellule ad apoptosi indotta da stress del reticolo endoplasmico. Il mio lavoro ha come scopo l'identificazione delle conseguenze dell'assenza di TRMT10A sulle modificazioni e la stabilità di molecole di tRNA. Le molecole di tRNA specifiche per glutammina e metionina sono risultate essere modificate da TRMT10A in cellule umane e lo sviluppo di una tecnica di northern blot ha fornito dati preliminari riguardo alle conseguenze dell'assenza dell'enzima sull'aminoacilazione e la stabilità del tRNA. Lo studio dei meccanismi che portano alla morte cellulare, attivati dall'assenza di TRMT10A, è stato inoltre approfondito nel presente lavoro. A questo proposito è stata utilizzata le linee cellulari INS-1E e EndoC- βH1, linea cellulare umana, in cui è stato indotto il silenziamento del gene TRMT10A. I risultati ottenuti dimostrano che l'assenza di TRMT10A induce un aumento dell'apoptosi mediante l'attivazione del pathway intrinseco dell'apoptosi tramite l'attivazione della proteina pro-apoptotica Bim e l'aumento dell'attivazione di caspasi a valle.

Il lavoro svolto durante il mio dottorato apporta un avanzamento nella comprensione dell'importante ruolo di enzimi che modificano il tRNA nel mantenimento della funzione e sopravvivenza delle beta cellule pancreatiche.

ABSTRACT

Transfer RNAs (tRNAs) are small molecules of 70-80 nucleotides with a crucial role in protein synthesis. tRNAs once transcribed are highly modified and the methylation is the most common modification. Several enzymes are responsible of tRNA modification and their function is necessary to regulate the stability, the aminoacylation and the rigidity of the structure of tRNAs. De-aminoacylated or degraded tRNAs can act as important signal molecules in the cells, activating different pathways of stress response. For this reason is not surprising that mutations in genes codifying for tRNA modifying enzymes have been associated to many human diseases. Polymorphisms in the gene CDKAL1, codifying the Cdk5 regulatory associated protein 1, have been linked to the development of type 2 diabetes (T2D) in human. CDKAL1 is a methyl-thio transferase that modifies the residue in position 37 of tRNAs, which recognize the codon UUU for lysine. The absence of CDKAL1, and consequently of the modification catalyzed by the enzyme, was shown to induces a decrease of incorporation of lysine residues in proinsulin at the level of pancreatic beta cells. Lysine residues are crucial for the correct maturation of proinsulin. It was shown that the absence of CDKLA1 mediated modifications leads to defects in the processing of proinsulin to produce insulin and c-peptide and to impaired glucose-stimulated insulin secretion. Furthermore in CDKAL1 knock out beta cells it's observed an increase of markers of endoplasmic reticulum (ER) stress. The chronic activation of ER stress processes decreases the general protein synthesis and the chronic activations triggers pro-apoptotic pathways. These events have been linked to the development of T2D.

The aim of the present work is to study the role of tRNA modifying enzymes in pancreatic beta cells and to investigate the consequences of mutations in these genes on cell function and survival.

A Whole Exome Sequencing study performed previously from my group produced a list of candidate genes for Congenital Hyperinsulinism (CHI). CHI is a rare disease, characterized by inappropriate insulin secretion leading to hypoglycemia. Mutations in nine genes are already known to be causative of the disease, but in 50% of patients the genetic cause is unknown. Using bioinformatics tool I identified CDKAL1 as one of the most promising candidate genes. The mutation identified leads to the substitution of a Serine with a Phenylalanine in position 561, with probable consequences on the transmembrane domain that ensures the correct localization of the protein in the membrane of the ER. In order to study the consequences of S561F CDKAL1 variant in beta cells, I used molecular biology techniques inducing the overexpression of wild type and mutated gene in INS-1E cell line, derived from rat insulinoma. The localization of CDKAL1 was analyzed by immunofluorescence microscopy: the S561F variant affect the localization of the protein that, although still inserted in the ER, tends to accumulate in vesicular structures in some regions of the ER membrane. I also studied the impact of S561F CDKAL1 overexpression on the beta cell function, by measuring the content and the release of insulin in basal growing conditions. I observed an increase of insulin content induced by the overexpression of the wild type protein while the insulin release was not changed. On the other hand, the S561F variant doesn't affect the insulin content that doesn't change compared to not-transfected

cells, but induces an increase in insulin release. These preliminary results suggest that the S561F CDKAL1 variant could have a role in the development of beta cell dysfunction leading to an inappropriate insulin secretion.

The second part of my project regards the methyl-transferase TRMT10A. A mutation in this gene - the insertion of a stop codon and consequent absence of mature protein - was identified in patients affected by microcephaly and young onset diabetes. It was demonstrated that TRMT10A modifies guanine residues, but its role in tRNA modification in human is still not demonstrated. The absence of the protein leads to an increase of cell death in basal conditions and sensitizes cells to ER stress-induced apoptosis. My work aimed at the characterization of the consequences of TRMT10A deficiency on tRNA modification and stability. I used lymphoblast cells derived from controls subjects and patients to investigate this tRNAs molecules specific for glutamine and methionine were identified to be modified by the enzyme, and the development of a northern blot technique allowed me to obtain preliminary on the aminoacylation and stability of these molecules in TRMT10A deficiency conditions. Furthermore I investigated the mechanisms that lead to beta cell death, triggered by the absence of the protein. With this purpose I induced the silencing of the gene in two different cell lines: INS-1E and EndoC-βH1 (human beta cell line). Results obtained demonstrated that TRMT10A deficiency triggers the activation of the intrinsic pathway of apoptosis through the modulation of Bim expression, a proapoptotic protein of the BH3-only family. The results obtained highlighted the importance of TRMT10A for the survival of the beta cells. Furthermore the activation of the intrinsic pathway of apoptosis is one of the events observed in the development of type 2 diabetes. These findings can give additional proves that the monogenic forms of diabetes can be used as model for the study of mechanisms involved in type 2 diabetes.

Even if further investigations on the complex processes involved are needed, the present work provides important evidences of the role of tRNA modifying enzyme in beta cell homeostasis. Moreover recent reports about the role of tRNAs in signalling pathways support the hypothesis that these molecules can be important mediators of stress response in beta cells, and the tRNA modifying enzymes may act as activators or inhibitors of these responses.

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1 INTRODUCTION

1.1 Diabetes mellitus

Diabetes is characterized by insulin deficiency and consequent hyperglycemia due to progressive failure of pancreatic beta cells. The worldwide incidence of diabetes reached epidemic proportions, with nearly 300 million of people affected (http://www.idf.org). There are two major forms of diabetes; the most diffused form is type 2 (80-85% of patients), while type 1 represents the 10-15% of cases. Several genetic polymorphisms are associated to diabetes, but also specific environmental factors, such as viral infections for T1D and obesity for T2D, participate to the development of the disease. The failure of pancreatic beta cells is a consequence of different mechanisms in the two forms of diabetes. T1D is an autoimmune disease characterized from mononuclear cells infiltration in pancreatic islets and instauration of chronic inflammation (insulitis). Beta cells are progressively destroyed with the loss of most of 70-80% of beta cell mass at the time of diagnosis; this process is triggered by direct contact with immune system cells and through signals released from these cells such as proinflammatory cytokines: interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ[1]. T2D results from alteration of glucose stimulated insulin secretion that is caused from dysfunction of beta cells. Loss of β-cell mass can also play a role in the development of T2D, since post-mortem studies have shown a 25-50% mass reduction[2] and increased apoptosis[3]. High glucose and saturated free fatty acid derived from diet are risk factors for T2D and can activate intracellular pathways that lead to apoptosis[4].

In both forms of diabetes beta cell death is a consequence ER stress and/or activation of the intrinsic pathway of apoptosis. While the extrinsic pathway of apoptosis begins outside the cells through the activation of

death receptors by pro-apoptotic signals, the intrinsic pathway involves non-receptor–mediated intracellular signals, inducing activities in the mitochondria that initiate apoptosis. The Bcl-2 protein family plays a central role in this process. Bcl-2 proteins are classified in three categories: the pro-survival (Bcl-2, Bcl-XL, Mcl-1, Bcl-W and A1), the pro-apoptotic (Bax, Bak and Bok) and the Bcl-2 homology 3 (BH3)-only proteins subfamily. The activation of apoptosis pathway is a matter of balance and interactions between these proteins. Bax and Bak are the real effectors of cell death and their activation is a crucial event. Bax exists as inactive monomer in the cytosol while Bak is always inserted in the mitochondrial outer membrane (MOM) and kept inactive by the interaction with VDAC2. BH3 activators induce the oligomerization of Bax and the insertion in the MOM, where it disrupts the interaction between Bak and VDAC2, allowing Bak oligomerization. These events lead to the formation of pores in the MOM and the release of pro-apoptosis factors such as cytochrome *c* and SMAC/DIABLO[5]. Citochrome c in the cytoplasm binds to APAF1 and triggers the formation of the apoptosome that activates pro-caspases, proteases that cleave important proteins through the cell[6] (Figure 1).
The pathways that induce apoptosis are cell and context specific. In T1D cytokines released from immune system cells bind to beta cells receptors inducing intracellular responses. IL-1β activates the transcription factor NF-kB that modulates the expression of several genes, including cytokines, chemokines and iNOS (nitric oxide synthase)[4]. IFN-γ binding with cell surface receptors induces JAK1 and JAK2 kinases that phosphorylate and activate STAT1 transcription factor. The activation of IFN-γ dependent pathways have a synergic effect on IL-1β induced cellular responses, triggering different mechanisms that can lead to beta cell death. One of the mechanisms involves the member of the MAPK family c-Jun NH_2-terminal

kinase (JNK) that phosphorylates c-Jun inducing the upregulation of DP5 sensitizer BH3 protein.

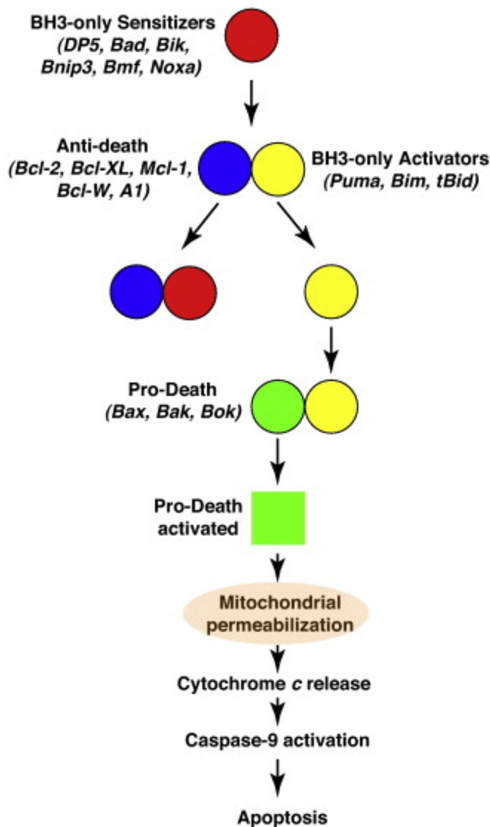


Figure 1: schematic representation of Bcl-2 proteins interactions. There are two groups of BH3-only subfamily members; DP5, Bad, Bik, Bnip3, Bmf and Noxa are named sensitizers and act by binding the pro-survival Bcl2 proteins promoting the release of BH3-only activatprs Puma Bim and tBid. The activators bind the pro-apoptitic effectors Bax, Bak and Bok triggering the mitochondrial pathway of apoptosis[68]. Image adapted from *Gurzov E.N. and Eizirik, D.*, 2011.

Another mechanism involved in cytokines induced apoptosis is the ER stress response that is triggered by NO production via PERK activation. ER stress response leads to apoptosis through the decrease of Mcl-1, pro-survival Bcl-2 protein,[7] and increase of expression of DP5 and PUMA, BH3-only activator[8]. ER stress is a process by which cells respond to the disruption of ER homeostasis. Different factors can induce an accumulation of unfolded proteins in the ER, such as mutations that affect proteins folding or lack of chaperon proteins activity, leading to the activation of the unfolded protein response (UPR). The role of the UPR is to contrast ER

stress by attenuating the general protein translation and the consequent overload of proteins in the ER and triggering the degradation of misfolded proteins[9]. In parallel, the UPR induces the expression of genes, involved in the restoration of ER homeostasis, such as chaperons. If the UPR fails in attenuate ER stress the apoptosis pathway is induced. Three proteins inserted in ER membrane are important mediators of the UPR: inositol requiring ER-to-nucleus signal kinase (IRE) 1, activating transcription factor (ATF) 6 and double-stranded RNA-activated kinase (PKR)- like ER kinase (PERK). These proteins are associated to the chaperon protein Bip and maintained inactive. In presence of unfolded protein Bip dissociates to participate in the protein folding, inducing the activation of the trans-membrane proteins[10]. IRE1α mediates the splicing of the Xbp-1 transcription factor (bZip protein). Spliced XBP-1 enters the nucleus and modulates the transcription of unfolded protein response (UPR) target genes, including ER chaperones; moreover IRE1α induces the phosphorylation of JNK and consequently the activation and upregulation of DP5 via c-Jun triggering the mitochondrial apoptosis pathway[11]. ATF6 once activated by ER stress can translocate to the Golgi, where is cleaved, and then to the nucleus. ATF6 is a transcription factor that binds ER stress response elements (ERSE) in genes codifying for chaperon proteins[12]. ATF6 and IRE1α pathways are strictly connected: ATF6 increase the mRNA expression of XBP-1 increasing the substrate of IRE1α[13]. PERK is protein kinase inserted in the ER membrane that directly phosphorylates the eukaryotic initiation factor 2 alpha (eIF2α) leading to the inhibition of total protein synthesis (Figure 2). When eIF2α is phosphorylated the transcriptional factor ATF4 gene is specifically transcribed and induces the expression of CHOP that seems to contribute to the activation of mitochondrial pathway of apoptosis, even if the mechanism is still not clarified. Other kinases can phosphorylate eIF2α in response to different

kind of stress: PKR is a sensor of double strand RNA, GCN2 is activated by de-aminoacylated tRNAs in aminoacid deprivation condition and by UV light, HRI mediates the response to heme deficiency and oxidative stress[14] (Figure 3).

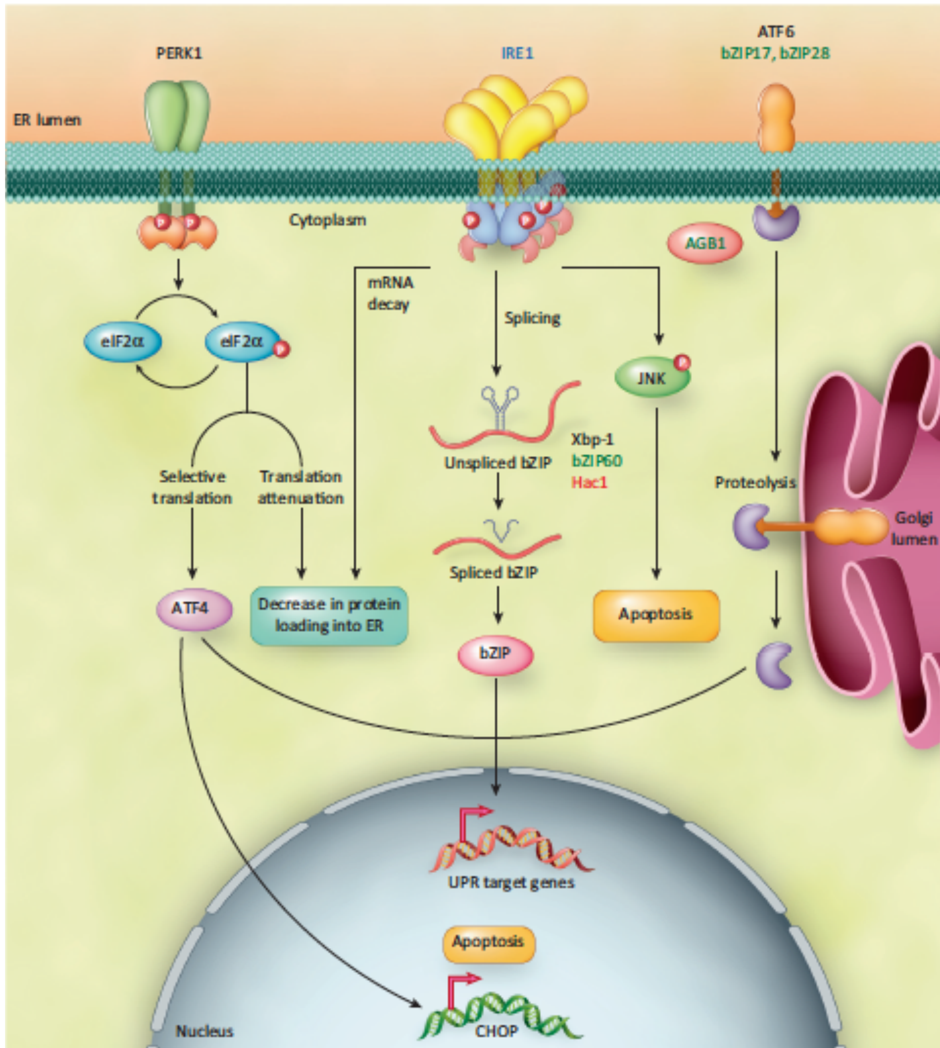


Figure 2: Unfolded protein response. The image from *Chen, Y. and Brandizzi, F.* 2013 shows the three arms of the UPR signaling that involves to activation of PERK, IRE1 and ATF6 pathways[11].

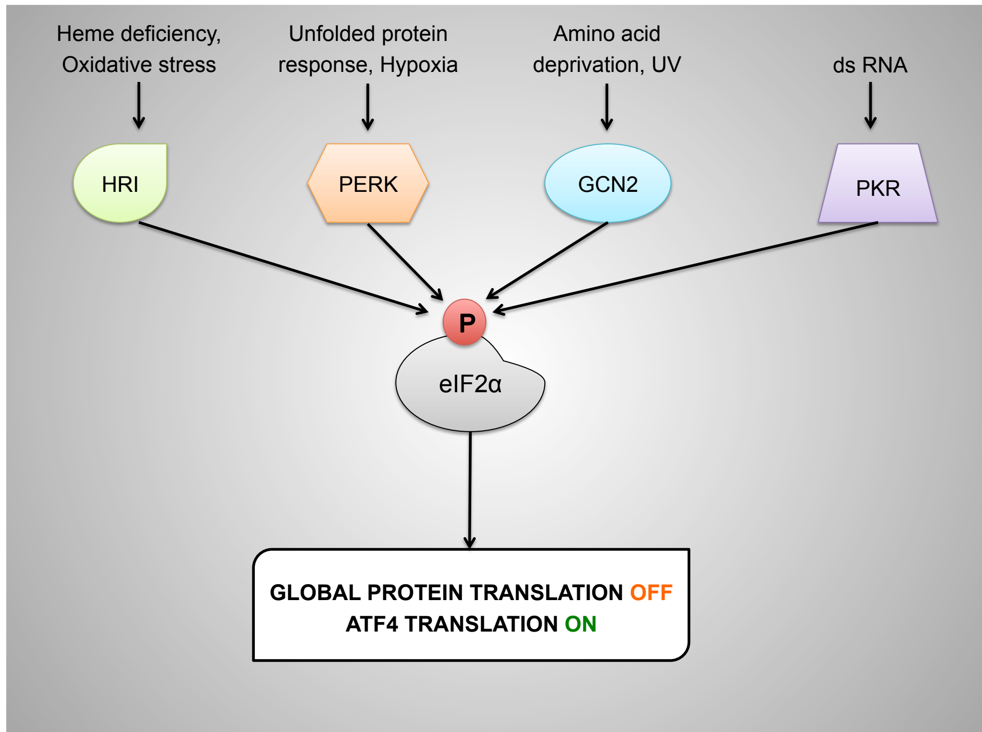


Figure 3: Representation of eIF2 kinases activated by different cellular stress stimuli. The heme regulated inhibitor (HRI), the RNA-dependent kinase (PKR), the PKR-like ER kinase (PERK) and the general control non-derepressible 2 (GCN2) can phosphorylate the Eukaryotic Initiation Factor 2 at the level of the subunit α. the phosphorylation of eIF2α induces the inhibition of general translation and the preferential translation of transcription factors (e.g. ATF4) that initiate a pattern of gene expression that allows the cell to respond to the stress.

In T2D the apoptosis is induced mainly from free fatty acid (FFA) and high glucose; ER stress response seems to be play a major role in the activation of apoptosis pathways. In the case of lipotoxicity, ER stress can be triggered by over-stimulation of FFA esterification in the ER that leads to delay in the export of proteins and high glucose can sensitize the cells to

ER stress by increasing the secretory demand[4]. However several and complex events can lead to ER stress and activation of UPR in beta cells, the mechanisms remain to be elucidated. The described events that lead to development of T2D are triggered by stress conditions, but polymorphisms in several genes contribute to the predisposition to the disease modulating the response to environmental stimuli. Monogenic forms represent a third category of diabetes and comprise single gene disorders with different phenotypes unrelated to auto-immunity. Genes causing monogenic forms of diabetes are involved in developmental and/or functional processes of the pancreatic beta cell physiology. Different genes causative of monogenic forms of diabetes have been associated to T2D. The identification of gene variants and mechanisms leading to monogenic forms of diabetes can serve as model for identifying new targets of beta cell demise in T2D.

1.2 Congenital hyperinsulinism

An altered insulin secretion characterizes the rare genetic disease Congenital Hyperinsulinism (CHI) that causes persistent hypoketotic hypoglycemia (i.e. plasma glucose levels <50 mg/dl and low levels of ketones) in newborns[15]. CHI occurs in 1:30000-1:50000 live births, but the incidence can be higher in population where consanguineous marriages are common[16]. Hypoglycemia can cause brain damage; children affected by CHI can develop neuromotor delay, mental retardation, and epilepsy as a consequence of repeated episodes of hypoglycaemia that may also lead to death. A rapid diagnosis and appropriate management are essential to prevent brain damages[17] with interventions aimed at maintaining blood glucose within normal range, initially through glucose administration and glucagon infusion, and once the diagnosis is set, with specific treatments.

CHI cases differ for histological characteristics: diffuse forms involve the whole pancreas, while focal forms affect a limited pancreatic region (or

more than one in very rare multifocal CHI)[18]. Focal forms can be cured with the surgical excision of the lesion identified and localized with specialized imaging (18F-DOPA-PET), while diffuse cases may need subtotal or total pancreatectomy to control hypoglycemia, with dramatic consequences such as diabetes and malabsorption due to deficit of pancreatic enzymes. Recently, an “atypical” form of focal hyperinsulinism characterized by morphologic mosaicism of pancreatic islets[19] has been also described. The genes involved in the development of the disease and the type of mutations lead to different clinical onset (ranging from mild to severe) of CHI, inheritance and histologic. The majority of CHI cases are caused by mutations of ABCC8 and KCNJ11 genes placed in the short arm of chromosome 11 and encoding the two subunits Sur1 and Kir6.2 of the pancreatic beta cell ATP-sensitive potassium channel (KATP). The KATP channel participates in the regulation of glucose-dependent insulin secretion from the beta cells. In resting conditions the channel is open and when plasma glucose raises, glucose metabolism within the beta cell induces the closure of KATP channel and the consequent membrane depolarization[20]. These events lead to the opening of voltage gated Ca2+ channels, and Ca2+ influx triggers the exocytosis of insulin-containing granules. Genetic defects in these genes affect beta cell membrane depolarization that leads to glucose-independent insulin secretion. Mutations that completely abolish channel function can be heterozygous mutations with dominant-negative effect. Recessively inherited mutations in ABCC8 and KCNJ11 lead to diffuse and severe forms of CHI, usually not responsive to diazoxide and often requiring subtotal or total pancreatectomy[21]. In contrast, patients with dominant-negative heterozygous mutations usually respond to medical treatment, and may present a later onset of hypoglycemia. Paternally inherited mutations in

ABCC8 or KCNJ11 cause the focal forms when a concomitant somatic loss of maternal 11p15 allele occurs within limited regions of the pancreas[22].

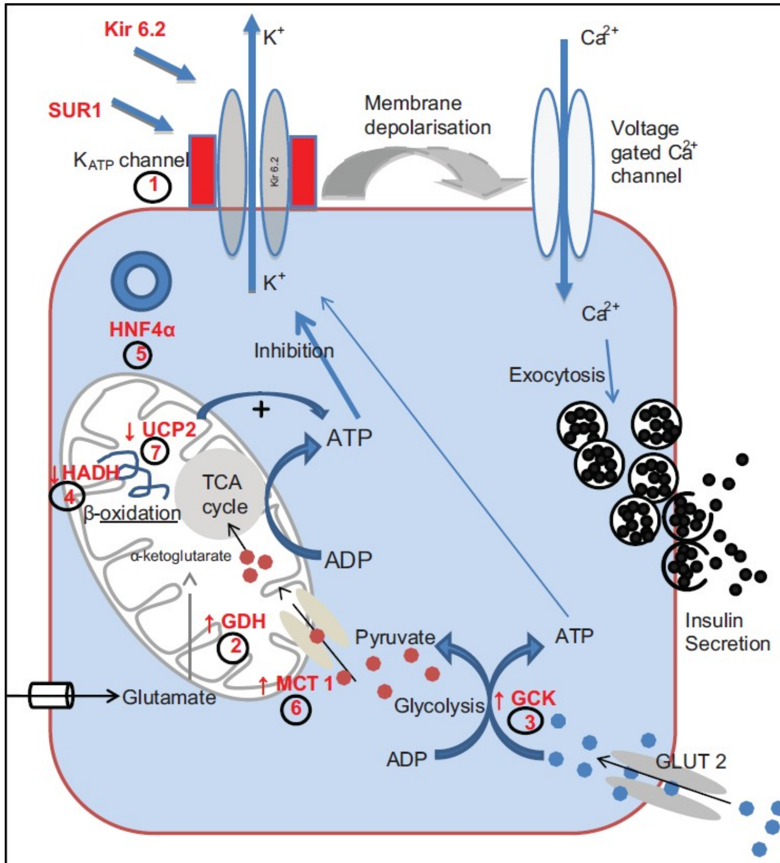


Figure 4: Insulin secretion mechanism in beta cells; Insulin release from beta cells is a two phases mechanism: raising levels of circulating blood glucose trigger the first phase that is more rapid and immediate compare to the second phase, which consists in a slow response involving the formation of new vesicles. In the first phase glucose enters the cells through the GLUT2 transporter and is metabolized by glycolysis and Krebs cycle with the production of ATP. The ATP sensitive potassium channel is inhibited by the increase of ATP and its closure leads to membrane depolarisation. The influx of calcium from voltage gated channels and calcium release from the ER is triggered and this stimulates the release of secretory vesicles previously synthetized. In the figure are highlighted (in red) the proteins codified by genes known to be causative of Congenital hyperinsulinism. Image adapted from *Seniappan, S. et al* 2013.

In addition to ABCC8 and KCNJ11 variants, studies identified other genes as causative of the disease: autosomal recessive mutations of HADH gene and dominant mutations of GCK, GLUD1, SLC16A1, HNF4A, UCP2, HNF1A[23]. All nine genes codify proteins (transcription factors, metabolic enzymes and transporters) necessary for the regulation of the glucose-dependent insulin secretion in pancreatic beta cells - linking the mitochondrial glucose metabolism to insulin secretion events (Figure 4).

The mainstay of medical therapy is diazoxide (DZX), used as a first line drug; it binds and activates intact KATP channels, thereby reducing insulin secretion. The side effects of this treatment include fluid retention and hypertrichosis. However, diazoxide can be ineffective in diffuse CHI due to inactivating mutations in ABCC8 and KCNJ11 and in most patients with focal CHI. Octreotide is a long-acting analogue of somatostatin (SMS) with the ability to inhibit the secretion of several hormones including insulin and is utilised when diazoxide fails to control hypoglycemia[24]. Additional medical options include the Ca2+ channel blocker nifedipine, and more recently, the GLP-1 receptor antagonist, exendin, that showed the ability to acutely elevate fasting blood glucose in individuals with CHI caused by ABCC8 mutations[25].

CHI represents the opposite phenotype of monogenic diabetes, which is characterized by hyperglycemia of early onset (neonatal period/childhood/young adults) due to genetic defects of pancreatic beta cell. The two main clinical forms of monogenic diabetes are neonatal diabetes (NDM) - diabetes onset within 6 months of birth- and maturity onset diabetes of the young (MODY). Interestingly, mutations in genes previously identified as causative of MODY or NDM such as GCK[26,27], HNF4A, and HNF1A[,28,29] can cause CHI[30,31] while genes that were first implicated in CHI, like ABCC8, KCNJ11, have been later found to be involved in NDM and MODY[32,33]. Therefore, the identification of new CHI

genes might simultaneously provide clues about new NDM/MODY genes[18]. In addition, numerous studies have demonstrated that some polymorphisms in genes causing CHI or monogenic diabetes are also associated to type 2 diabetes (T2D), showing that a "gradient" of variants links all these conditions, i.e., CHI, NDM/MODY and T2D[34]. However, it has to be noticed that, differently from T2D, NDM and MODY do not have insulin resistance as pathophysiological trait. Genetic diagnosis of a patient with CHI, along with imaging results, is crucial for the correct clinical management (i.e. drug therapy vs surgery, and type, mode and duration of drug therapy), but no mutations in known genes are found in a high percentage (up to 50% in diazoxide responsive and about 10% of diazoxide unresponsive) of patients clinically diagnosed as CHI.

1.3 Transfer RNA

Transfer RNAs (tRNAs) are the most abundant RNA molecules in the cell (4-10%) and play a key role in protein synthesis process, mediating the translation of messenger RNA (mRNA) codons into specific polypeptides[35]. The function of tRNA in translation is well known since decades but only during the last years it has been shown that these molecules don't act just as mediators but have also many functions in signaling and regulation of cellular pathways[36]. Moreover mutations in genes codifying tRNAs or tRNA-modifying enzymes have been described in different human diseases[37]. The biogenesis of tRNAs is a complex process that involves many steps and checkpoints. The RNA polymerase III is responsible of the transcription of tRNA genes into precursors molecules that need to be processed, spliced, modified and transported to the cytoplasm to participate in translation.

1.3.1 tRNA structure and function in translation

Mature tRNAs are 70 – 80 nucleotides molecules and their functionality depends on the correct three-dimensional structures. In solution, all tRNA molecules fold into a cloverleaf secondary structure (Figure 5 A), characterized by four stems – double helices stabilized by Watson-Crick base pair interactions; three of the four stems have loops and one contains the free 3' and 5' ends of the chain. The central loop contains three nucleotides of the anticodon domain that forms base pairs with the three complementary nucleotides forming a codon in mRNA. Specific aminoacyl-tRNA synthetases recognize the surface structure of each tRNA for a specific amino acid and covalently attach the proper amino acid to the acceptor stem. The folded tRNA molecule acquires an L shape with the anticodon loop and acceptor stem localized at the ends of the two arms (Figure 5 B). tRNA molecules have two basic functions: to bind a particular amino acid and to recognize specific codons in mRNA adding the aminoacid to a growing peptide chain. The correct folding of the molecule is necessary for the interaction with aminoacyl tRNA synthetases, with the ribosome subunits and the mRNA codons. During protein synthesis initiation the Eukaryotic translation initiation factor 2 (eIF2) bound to a GTP molecule and $tRNA^{iMet}$ (ternary complex) associates with a small (40S) ribosomal subunit complexed with two other factors, eIF3 and eIF1A, which stabilize binding of the ternary complex. Phosphorylation of eIF2 is a mechanism that cells use to regulate protein synthesis; this complex is unable to bind $tRNA^{iMet}$, inhibiting protein synthesis. During the elongation phase each incoming aminoacyl-tRNA moves through three ribosomal sites: the A (aminoacyl), P (peptidyl), and E (exit) sites.

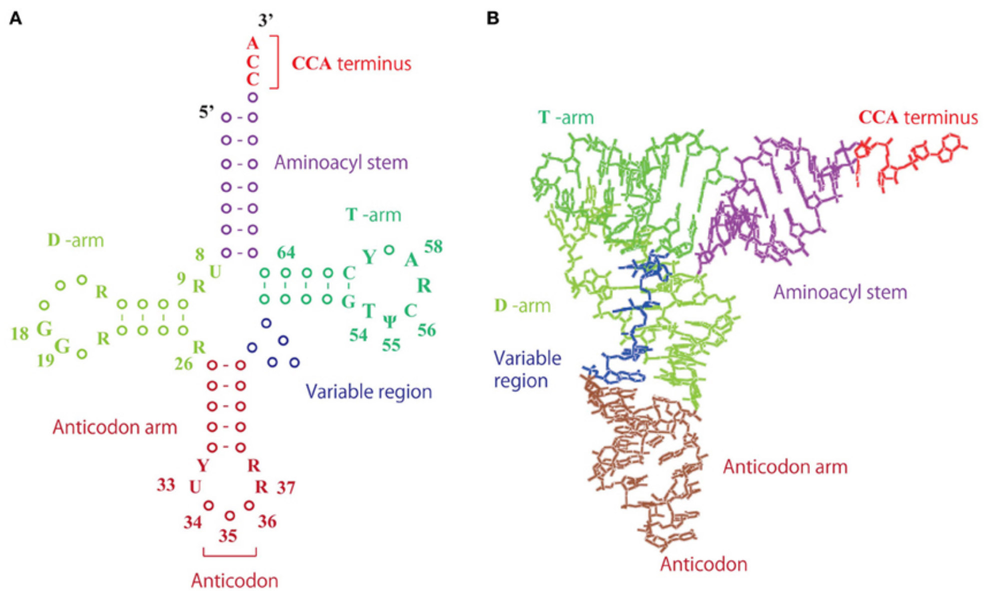


Figure 5: The figure shows the secondary structure **(A)** of tRNA molecules and its folding in the typical L-shaped tertiary structure **(B)**. Image adapted from *Hori, H*. 2014.

1.3.2 tRNA genes transcription

tRNA genes are repeated in the genome and are organized in clusters localized in the nucleolus; this sub-localization suggests a common regulation of transcription[38]. In the human genome there are 513 tRNAs genes encoding for 49 isoacceptors (tRNAs with different anticodons bearing the same amino acid); 22 tRNA genes are also contained in the mitochondrial genome. The presence of a large number of tRNA genes is due to the fact that isoacceptors are encoded by family of genes, and can exist as different isodonors (tRNAs with the same anticodons but with different sequences in the other domains). This variability is not only the consequence of the evolution of genome but seems to be the base for modulation of tRNAs expression in different cell type or state, affecting the efficiency of the RNA polymerase III binding[39]. The first step of tRNA genes

transcription consists in the binding of the transcription factor TFIIIC to the DNA elements A-box and B-box and the recruitment of the RNA polymerase III (Pol III) through the complex TFIIIB. Maf1 negatively regulates pol III by a direct interaction and binding the TFIIIB transcription factor. Maf1 is phosphorylated in positive growth conditions by PKA and TOR dependent kinase Sch9 and dephosphorylated in response to different environmental stresses[40]. The phosphorylation prevents the binding to Pol III and allows the active transcription of tRNAs genes.

1.3.3 tRNA processing and modification

Several events lead to the maturation of pre-tRNAs to functional molecules. Once tRNA is transcribed is processed by the cleavage of the 5' leader sequence catalyzed by RNAseP enzyme. The 3' trailer sequence is cleaved by endonucleases (Ribonuclease Z) and modified with the addition of the CCA end by ATP(CTP)-tRNA-specific nucleotidyl-transferase. tRNAs genes are characterized by introns between the nucletides 37 and 38 that are removed by tRNA splicing endonucleases and ligases[38].

The most complex characteristic of tRNA maturation process is the large number of modifications that can be found in almost 12% of nucleotide residues. In figure 6 are illustrated all the known tRNAs modifications, but different tRNAs have different set of modifications, that can also vary in a tissue specific way or basing on the cell growth state. tRNA modifications can have very different chemical nature and are catalysed by a diversity of enzymes, however the most common is the methylation of nucleotide residues. Modifications at the level of the anticodon domain or in close residues (residues 34 and 37) can affect the accuracy and efficiency of translation, modulating the codon recognition and translational frameshift (codon-anticodon wobbling)[41]. Modifications in the body of the molecule are very important for the folding and the stability; as an example modifications

found at the core of the folded RNA, are thought to predominantly affect the structure rigidity or flexibility. Increased rigidity is a consequence of pseudouridine residues.

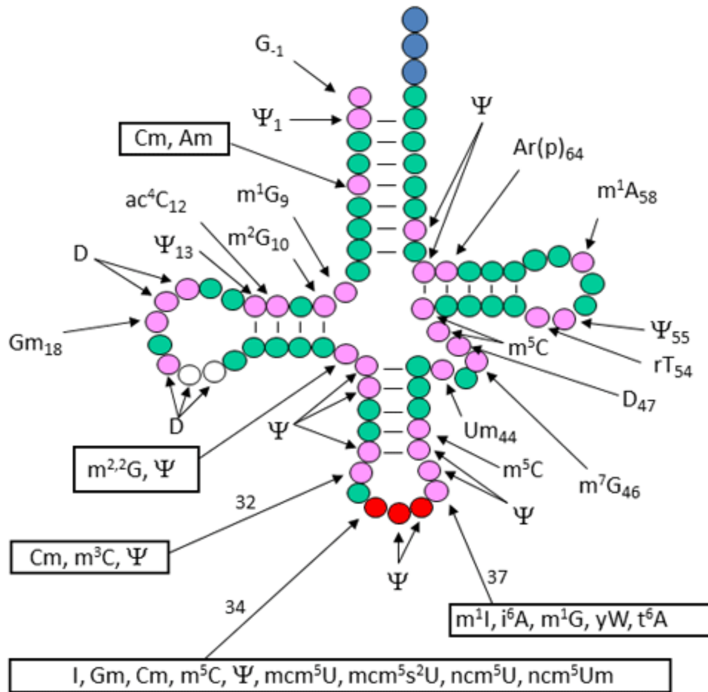


Figure 6: Summary of all known modifications at the level of nucleotide residues in tRNA molecules. Image adapted from *Phizicky, E. M. and Hopper, A. K.* 2010.

tRNA biogenesis is controlled at different levels. A nuclear surveillance system leads to the degradation of pre-tRNAs that show defects in the processing, while hypomodified molecules are eliminated in the cytosol. Only correctly modified tRNAs are charged with the specific cognate aminoacid and can participate to the protein translation process.

tRNA charging process takes place in the cytoplasm by the action of aminoacyl-tRNA synthetases. The synthetase binds ATP and the

corresponding amino acid to form an aminoacyl-adenylate. The complex then binds the appropriate tRNA molecule, and the amino acid is transferred to either the 2'- or the 3'-OH of the last tRNA nucleotide (A76) at the 3'-end. aminoacyl-tRNA synthetases are characterized by a catalytic domain and an anticodon binding domain that recognise the correct tRNA molecule. Some enzymes contain additional RNA binding domains and can edit incorrectly charged tRNA by cleavege[42]. Mitochondrial encoded tRNAs are aminoacylated within the organelle, even if some nuclear encoded tRNAs are imported from the cytosol.

1.3.4 tRNAs as signaling molecules

tRNA molecules are involved in different mechanisms used by the cells in response to environmental stresses.
Under nutrient deprivation conditions cytoplasmic tRNAs are reimported in the nucleus with the consequent repression of general protein translation. Non-charged tRNA molecules still in the cytoplasm bind and activate GCN2 protein kinase that phosphorylates eIF2α triggering the cellular stress response pathway, with the inhibition of general protein synthesis and activation of translation of the mRNA encoding the activating transcription factor 4 (ATF4)[43]. Recent studies have described newly identified pathways activated under specific cellular growth conditions that lead to the cleavage of tRNA molecules at the level of the anticodon loop. Different stress conditions (oxidative stress, heat shock, UV irradiation) trigger the cleavage of tRNAs with the production of small molecules (30-40 nucleotides) called tiRNAs[44]. Angiogenin is the enzyme responsible of this process in human cells; it recognizes preferentially tRNAs with CA sequence in the anticodon. The cleavage at the level of the anticodon generates two tRNA halves and it has been shown that only the 5’ tiRNAs, and not the 3’ tiRNAs, are able to inhibit protein translation. This process is eiF2a-phosphorilation

independent and leads to the formation of stress granules[45]. The function of tiRNA in the inhibition of protein translation is similar to the function of microRNA and small interfering RNA (siRNA) by displacing eukaryotic translation initiator factor 4E (eiF4E) and eiF4G[46]. Intriguingly under stress conditions tiRNAs can interact with specific siRNAs promoting the expression of stress response genes[47]. It's interesting to notice that tRNA halves can be found in serum circulating as part of nucleoprotein complexes, suggesting the importance of tRNAs as signaling molecules and a possible use as biomarkers since the circulating pool changes with age[48]. In addition of tRNA halves, smaller fragment of 13-30 nucleotides have been identified in different species. These fragments can derive from a cleavage of D-loop and T-stem by the angiogenin. It has been shown that small tRNA fragments can repress translation with a microRNA-like activity[36].

1.4 tRNA modification and human diseases

tRNA molecules play a central role in the regulation of translations under different cell growth conditions and can act as signaling factors for the activation of cellular pathways. It was also shown that some modifications modulate the translation of only some mRNAs that are enriched in specific codons; this could represent the way by which the cells modulate the synthesis of proteins involved in the same pathway[49]. Nucleotide modifications are necessary for the correct biogenesis, structure and function of tRNAs suggesting that the absence of tRNA modifications could be deleterious for cells function and survival. Several reports during the last years demonstrated the involvement of mutations of tRNA genes and tRNA modifying enzymes in human diseases[37]. Table 1 from Torres et al, 2014 represents a summary of all human disorders linked with mutations of tRNA modifying enzymes that have been associated with different disease

categories: neurological, cardiac, respiratory, cancer, metabolic and mitochondrial linked. Even if the process of tRNA modification occurs in every cell type and stage, the mutation of some tRNA modifying enzymes affect only subtypes of cells and organs. This is a consequence of transcriptional modulation of tRNA modifying genes that show increased expression in certain tissues or development stages.

Disease category	Disease	Affected tRNA modificatio	Gene involved	Refs
Neurological	Intellectual disability	2'O-methylribose	FTSJ1	Freude, K. et al 2004; Takano, K. et al 2008; Gong, P. et al 2008
		m22G	TRM1	Najmabadi, H. et al 2011
		m5C	NSUN2	Abbasi-Moheb, L. et al 2012; Khan, M.A. et al 2012
		m7G	WDR4	Michaud, J. Et al 2000
		A-to-I editing	ADAT3	Alazami, A.M. et al 2013
	Familial dysautonomia	mcm5s2U	IKBKAP	Slaugenhauupt, S.A. and Gusella, J.F. 2002 Chen, C. et al 2009
	Amyotrophic lateral sclerosis	mcm5s2U	ELP3	Simpson,C.L. et al 2009
	Rolandic epilepsy	mcm5s2U	ELP4	Strug, L.J. Et al 2009
	Dubowitz-like syndrome	m5C	NSUN2	Martinez, F.J. Et al 2012
Cardiac	Noonan-like syndromed	m5C	NSUN2	Fahiminiya, S. et al 2013
Respiratory	Bronchial asthma	mcm5s2U	IKBKAP	Takeoka, S. et al 2001
Cancer	Skin, breast, and colorectal	m5C	NSUN2	Frye, M. and Watt, F.M. 2006 Vachon, C.M. et al 2007
	Breast	wybutosine	TRMT12	Rodriguez, V. et al 2007
		m5U	TRMT2A	Barlett, J.M. et al 2010
	Colorectal	m1G	HRG9MTD2	Berg, M. et al 2010
	Urothelial	mcm5U	HABH8 (HALKBH8)	Shimada, K. Et al 2009
	Breast, bladder, colorectal, cervix, testicular	mcm5U	HTRM9L	Bergley, U.et al 2013
	Epigenetic cancer treatment	m5C	DNMT2	Schaefer, M. et al 2009
Metabolic	Type 2 diabetes	ms2t6A	CDKAL1	Saxena, R. et al 2007 Wei, F.Y. And Tomizawa, K. 2011
Mitochondrial-linked	MELAS	τm5U	mt tRNALeu(UAA)	Yasukawa, T. et al 2000 Kirino, Y. Et al 2005 Kirino, Y. Et al 2006
	MERRF	τm5s2U	mt tRNALys(UUU)	Yasukawa, T. et al 2000
	Infantile liver failure	s2U	MTU1 (TRMU)	Zeharia, A. et al 2009
	Deafness associated with mutations in mitochondrial 12S ribosomal RNA	s2U	MTU1 (TRMU)	Guan, M.X. Et al2006

Table 1: summary of human diseases linked to tRNA modifying enzymes. Table adapted from *Torres, A.G. et al* 2014.

1.5 tRNA modifying enzymes in beta cell dysfunction and demise

1.5.1 CDKAL1

One of the tRNA modifying enzymes most characterized at the level of pancreatic beta cells is the Cdk5 regulatory associated protein 1 (CDKAL1). Whole genome sequencing studies have identified several SNPs in CDKAL1 associated with impaired insulin secretion and type 2 diabetes (T2D)[50,51,52]. CDKAL1 is a member of methylthio-transferase (MTTase) family characterized by the S-adenosyl-methionine (SAM) domain[53] and catalyzes the modification of N6-threonyl carbamoyl adenosine (t6A) in 2-methylthio-N6-threonyl carbamoyl adenosine (ms2t6A) at position 37 of $tRNA^{Lys}$(UUU) in mammals[54]. Wei at al. elegantly clarified the role of CDKAL1 using in vitro and in vivo techniques. They found that the protein localizes in the ER (Figure 7 A) and demonstrated that the 2-methyl-thio modification is necessary to prevent the misreading and frame shifting of $tRNA^{Lys}$(UUU)'s cognate codons during protein translation. The role of CDKAL1 in the development of type 2 diabetes was studied in beta cell specific Cdka1-deficient mice. Beta cell knockout mice show decreased level of C-peptide, accumulation of proinsulin in C-peptide negative granules and impaired glucose stimulated insulin secretion. The transcription of insulin gene produces preproinsulin that is transported to the endoplasmic reticulum through the interaction of signal-recognition molecules with the 24 aa signal peptide. In the ER the signal peptide is cleaved and proinsulin is folded and transferred to the Golgi where is charge in secretory granules. In the granules proinsulin is processed through the cleavage and excision of the C-peptide and the binding of the B and A chains[55]. One specific lysine residue is located in the cleavage site between the C-peptide and the A-chain and is important for the processing of proinsulin. Misreading of lysine residues due to the absence of CDKAL1

can lead to inappropriate proinsulin processing. It was shown that in CDKAL1 knockout islets the incorporation of lysine residues in proinsulin in significantly decreased (Figure 7 B)[54]. Furthermore the absence of CDKAL1 induces the expression of ER stress related genes, probably because of accumulation of misfolded proteins in the ER[56], and this can lead to increased high fat diet susceptibility as was observed in islets-specific knockout mice[54].

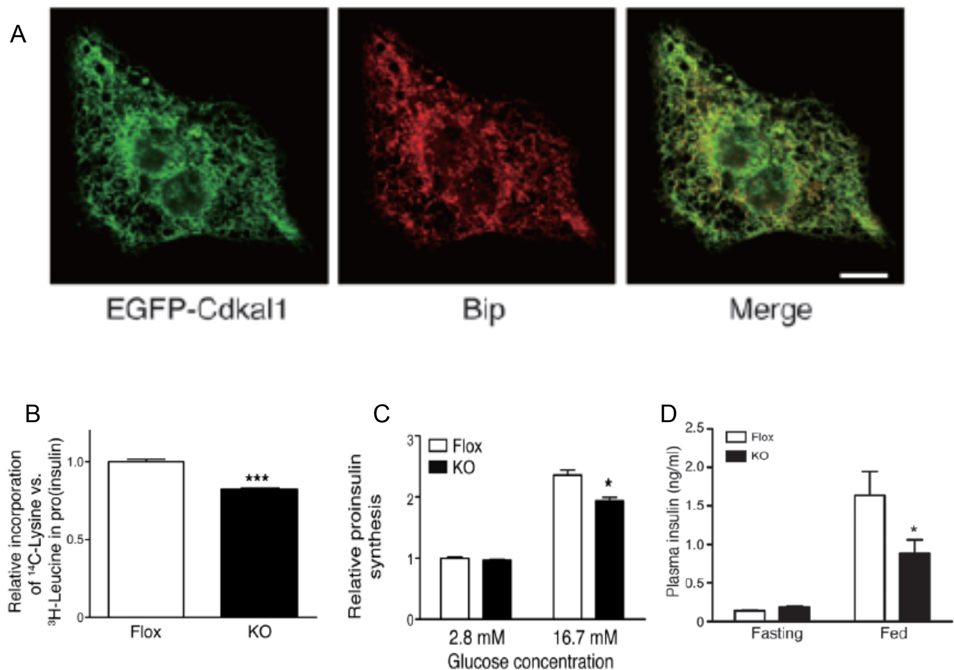


Figure 7: Key results published in *Wei et al*. 2011. **A)**: the colocalization of CDKAL1 with Bip (ER marker) was identified by overexpression of the protein fused to GFP in HEK293 cells. Results show a significant decrease of relative incorporation of 14C-lysine to 3H-lysine in immunoprecipitated pro-insulin **(B)**, a decrease of pro-insulin synthesis with high glucose concentration **(C)** in islets of beta cell CDKAL1 knockout mice compared to Flox controls mice and decreased plasma insulin in knockout mice **(D)**.

1.5.2 TRMT10A

tRNA Methyltransferase 10 Homolog A (TRMT10A) is a protein containing a (guanine-9)methyltransferase domain and is an homolog of the yeast protein TRM10 that modifies tRNAs substrates at the level of G9 residues. Igoillo-Esteve et al in 2013 identified a homozygous non sense mutation in *TRMT10A* gene that leads to young onset diabetes and primary microcephaly in siblings from a consanguineous family. The nucleotide variant G379A in *TRMT10A* gene leads to the insertion of a stop codon at position 127 of the encoded polypeptide (Arg127Stop) and consequently to the absence of the protein in lymphoblasts from patients and reduced mRNA level; this suggests a mechanism of nonsense-mediated mRNA decay. TRMT10A is ubiquitously expressed in rat tissues but is more abundant in pancreas and brain at both mRNA and protein level (Figure 8 A B). The protein localizes predominantly in the nucleolus of beta and non beta cells of rat and human pancreatic islets (Figure 8 C-D)[57]. In vitro studies showed that TRMT10A knockdown induces apoptosis in rat beta cells and dispersed human islets in basal condition and sensitizes the cells to free fatty acids and ER stress mediated apoptosis, while doesn't affect glucose stimulated insulin secretion. Another recent report described the identification of the homozygous TRMT10A mutation Gly206Arg associated with abnormalities in glucose homeostasis, short stature and microcephaly[58]. The mutation was found in three siblings of a consanguineous family. The altered glucose homeostasis was initially manifesting with ketotic and non-ketotic hypoglycaemia with transition to diabetes in adolescence, maybe because of accelerated beta cell apoptosis. The Gly206Arg mutated protein is still synthesized but shows defects in the methylation of tRNA substrates in vitro (<0.1% compared to *wild type* enzyme) even if the binding to tRNA molecules is not altered. Moreover Zung et al reported a case of homozygous deletion of TRMT10A

gene in a patient with diabetes mellitus, delayed puberty and intellectual disability[59]. All these findings suggest that TRMT10A plays a crucial role in beta cell function and survival, but the mechanism by which it regulates cellular responses is still unclear. Furthermore the role of TRMT10A as tRNA modifying enzyme in human *in vivo* remains to be described.

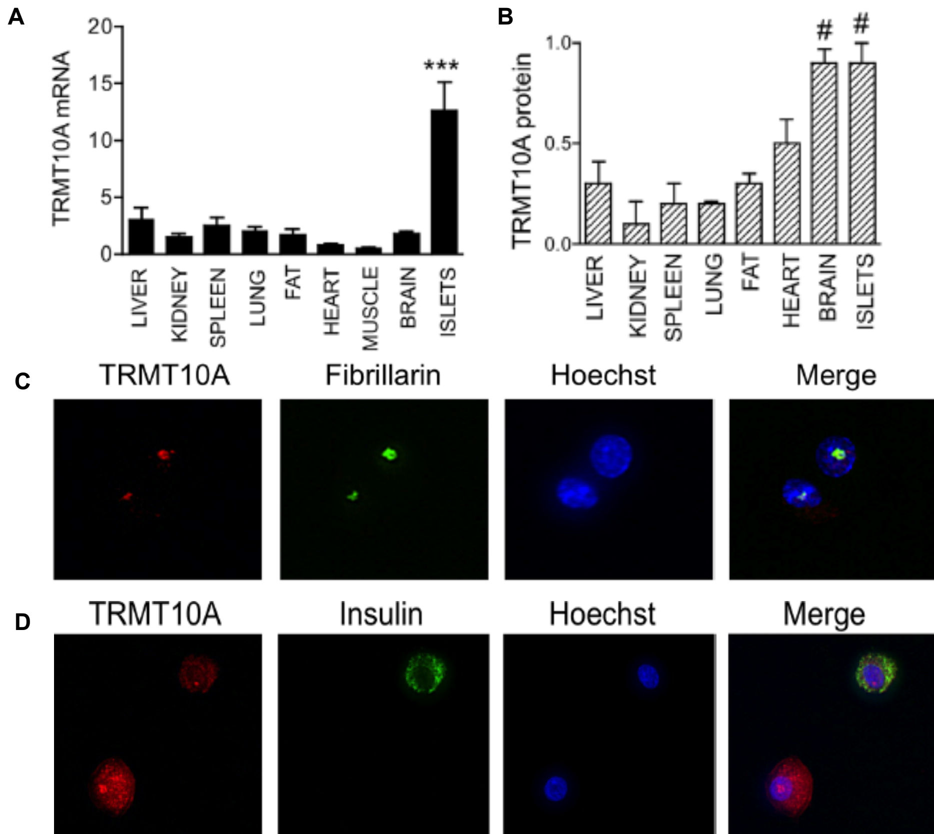


Figure 8: Results published by *Igoillo-Esteve et al* (2013) relative to the expression of TRMT10A in rat tissues **(A-B)**: the expression is enriched in pancreatic islets (mRNA and protein level) and brain (protein level). Immunofluorescence microscopy was used to evaluate the expression of TRMT10A GFP-fused protein in beta and non beta cells in rat **(C)** and human islets **(D)**.

2 AIM

tRNA modifying enzymes have been shown to be crucial to ensure the stability and the correct function of tRNAs. Furthermore several evidences have recently highlighted the role of tRNAs in cell signaling, linking these molecules with the activation of stress response pathways. Basing on these observations it became clear the importance of studying tRNA modifying enzymes role in pancreatic beta cells that are particularly sensitive to environmental stress.

The study of monogenic diseases associated with beta cells dysfunction and demise is a good model for the investigation of essential cellular mechanisms. With this purpose I managed a list of candidate genes of congenital hyperinsulinism derived from a whole exome sequencing study. The objectives of this part of my project are:

- **Identifying new candidate genes for Congenital hyperinsulinism using bioinformatics tools;**
- **Studying the consequences of the variant S561F of CDKAL1, a tRNA methyl-thio transferase, on the protein in a model of beta cell;**
- **Evaluating the S561F CDKAL1 impact on beta cell function**.

The second part of my project regards the characterization of the tRNA modifying enzyme TRMT10A in beta cells. TRMT10A deficiency has been linked to the development of microcephaly and young onset diabetes. My work in this context aimed to investigate

- **The role of TRMT10A in tRNA modification, stability and charging in human cells,**
- **The consequences of TRMT10A deficiency on modulation of apoptosis pathways in beta cells;**
- **The effect of TRMT10A deficiency on ER stress-induce apoptosis in beta cells**;

3 MATERIALS AND METHODS

3.1 Plasmids

Expression plasmid for Human CDKAL1 with HA (hemagglutinin) tag was kindly provided by Dr. Michele Solimena (Universitatsklinikum Carl Gustav Carus an der Technischen Universitat Dresden). The single nucleotide variant C-1682-T found in CHI patients was introduced in the human ORF with QuickChangeII XL site-directed kit (Agilent Technologies®) using the mutagenesis primers:

CT antisense 5'-CAAGCCCACG**A**ACATCCTCAGCGCACAGTC-3'
CT sense 5'-GACTGTGCGCTGAGGATGT**T**CGTGGGCTTG-3'

3.2 Cell culture

Rat INS-1E cells (kindly provided by Prof. Wolheim, Geneva) were cultured in RPMI-1640 GlutaMAX-I medium (Invitrogen) supplemented with 5% FBS as previously described[60]. The EndoC-βH1 human beta cell line was cultured in low glucose DMEM (Invitrogen) in plates coated with Matrigel 100 μg/ml and fibronectin 2 μg/ml as described[61]. Human lymphoblasts from control individuals, two patients and one heterozygous carrier of the mutation were cultured in RPMI 1640 medium supplemented with 20% FBS, 100 mU/ml penicillin and 100 mU/ml streptomycin.

Establishment of CDKAL1 overexpressing INS-1E clones: INS-1E cells were transfected with lipofectamine 2000 (Invitrogen) in dishes of 6 cm diameter. About 16 hours post transfection the medium was supplemented with 0.5 g/L of neomycin. Medium containing neomycin was changed every day for 7 days. Cells were then trypsinized and diluted 3 cells/20μl (150 cells/ml). 20 μl were distributed to each well of the 96 well plates. Cells were cultured in the wells for about 2 weeks until colonies were visible.

RNA interfence: Cells were transfected using Lipofectamine RNAiMAX (Invitrogen). The siRNA-lipid complex was formed in Opti-mem medium (Invitrogen) with an incubation of 20 min at room temperature. The final concentration of the siRNA in the transfection medium was 30nM. Transfection conditions are summarized in table. The transfection was induced overnight and all the experiments were performed after 48 hours from the transfection. Cells were transfected using different siRNAs: control siRNA (Qiagen), three different siRNAs targeting rat TRMT10A (siTRMT10A #B, #C and #D), two siRNAs targeting human TRMT10A (siTRMT10A #1 and #3), siRNA specific for rat Bim and siRNA for human Bim (siBim). The control siRNA is characterized by a random sequence that doesn't interfere with the expression of any gene. The sequences of siRNAs used are reported in table 3.

Molecule transfected	Lipofectamine transfection agent	Plate	Transfection agent volume per well (µl)	Final volume (µl)
siRNA	RNAiMAX	24 wells	1.0	500
siRNA	RNAiMAX	96 wells	0.2	100

Table 2: Transfection conditions used in INS-1E and EndoC-βH1 cells.

Species	Gene name	siRNA name	Sequence
Rat	Bim	siBim	5'-GAGUUCAAUGAGACUUACACGAGGAU CCUCGUGUAAGUCUCAUUGAACUC-3'
Rat	TRMT10A	siTRMT10A #B	5'-CCUAUGUGAUUGGAGGGUUAGUGGAU CCACUAACCCUCCAAUCACAUAGG-3'
		siTRMT10A #C	5'-CACGUUUAAGCAAGCUCCAGUUAUAUA ACUGGAUGCUUGCUUAAACGUG-3'
		siTRMT10A #D	5'-UGCAGAGGCCCAGCCAAUAUCUAAA-3'
Human	Bim	siBim	5'-CACGAAUGGUUAUCUUACGACUGUU-3'
Human	TRMT10A	siTRMT10A #1	5'-CAGAGCACUAUAGUGAACUCAUAAAUUUA UGAGUUCACUAUAGUGCUCUG-3'
		siTRMT10A #3	5'-CCAUCACAAGGGACUCACAUAAUAAAAUUU AUAUGUGAGUCCCUUGUGAUGG-3'

Table 3: sequences of siRNAs used to silence the expression of different genes in rat INS-1E or human EndoC-βH1 cells.

Cells treatment: Palmitate (sodium salt, Sigma) was dissolved in 90% ethanol and diluted 1:100 to a final concentration of 0.5 mM. The chemical ER stressor thapsigargin, a SERCA pump blocker, was used at the concentration 1 μM. Combination of cytokines was used at the following concentrations: recombinant human IL-1β (R&D Systems, Abingdon, UK) 50 U/ml, human IFN-γ (Peprotech, London, UK) 1000 U/ml. For all treatments the control condition contained the same dilution of vehicle.

3.3 Cell viability

The evaluation of cell death was performed staining the cells with DNA dyes Hoechst 342 (20 mg/ml) and propidium iodide (10 mg/ml) (HO/PI). Apoptotic cells, due to a change in membrane permeability, show an increased up-take of the dye Hoechst 342 compared to live cells. Propidium iodide is added to discriminate late apoptotic or necrotic cells, which have lost membrane integrity that leads the entrance of the dye. Live or early apoptotic cells have intact membranes that cannot be crossed by the dye. After 15 min incubation at 37°C with the staining solution the cell death was evaluated using inverted fluorescence microscopy. Cell death was determined in at least 600 cells in each experimental condition by at least two observers.

3.4 Western Blot

Cells were washed once with cold PBS, lysed and collected in Laemmli buffer (60 mmol/l Tris pH 6.8, 10% Glycerol, 1% SDS, 0.001% blue Bromophenol and 5% β-mercaptoethanol). Cell lysates were resolved in SDS-PAGE gels and transferred to nitrocellulose membranes. Immunoblotting was performed using specific primary antibodies for the protein of interest (Table 4). After incubation with secondary antibodies horseradish peroxidase (HRP) conjugated, the membranes were detected adding the chemiluminescent substrate Luminol with the ChemiDoc system

(Biorad). Antibodies used are reported in table. The quantification of detected bands was performed with ImageJ software.

Antibody	Specificity	Ref Number	Company	Band Size (KDa)
Bim	Rat, Human	2819	Cell Signaling	23 (EL), 15 (L), 12 (S)
Cleaved Caspase 9	Rat	9507	Cell Signaling	38
Cleaved Caspase 9	Human	7237	Cell Signaling	37
Phospho-Bad (Ser136)	Rat, Human	4366	Cell Signaling	23
Bad	Rat, Human	9239	Cell Signaling	23

Table 4: List of antibodies used for western blot experiments. WB membranes were incubated with antibodies diluted in TBS-Tween 5% BSA.

3.5 RNA extraction

PolyA$^+$-RNA was isolated from INS-1E and EndoC-βH1 cells using oligo-dT 25-coated polystyrene Dynabeads (DYNAL Oslo, Norway). cDNA was prepared with the GeneAmp RNA PCR Kit (Perkin-Elmer, Norwalk Conn,USA). The cDNA was used to perform Real Time PCR. For northern blot experiments, total RNA from lymphoblast cells was extracted in acid condition to preserve the aminoacylation of tRNA molecules. The cells were pelleted at 4°C, resuspended in 0.3 ml of 0.3 M sodium acetate (pH 4.5) and 10 mM EDTA, and lysed in volume of phenol equilibrated with the same buffer. Cells were vortexed three times for periods of 30, 60, and 60 seconds with 60 seconds intervals between each step. Samples were then centrifuged for 10-15 min and the aqueous phase was transferred to new tubes containing 0.3 ml of phenol, vortexed for 60 seconds, and centrifuged for 10 min. The aqueous layer was transferred to new tubes and the RNA

was precipitated with 2.5 volumes of ethanol, and incubated on ice for 1-2 hours. Total nucleic acids were recovered by centrifugation for 15 min. The pellet was dissolved in 60 µl of 0.3 M sodium acetate (pH 4.5). Nucleic acids were precipitated again with 2.5 volumes of ethanol, incubated on ice for 2-3 hours, and recovered by centrifugation for 15 min. The pellet was dissolved in 20 µl of 10 mM sodium acetate (pH 4.5) and 1 mM EDTA. An aliquot was used to measure absorbance at 260 nm after dilution 1:1 in 100mM Tris-HCl pH 7.0. Another aliquot was used for the alkali treatment in order to induce the complete de-aminoacylation of tRNA. These samples were used as control to detect the position of de-aminoacylated tRNAs in the electrophoretic gels. 50 µg of total RNA were diluted in water to 40 µl and treated with Add 5 µl of 10 mM EDTA (pH 8.0) and 5 µl of 1 M Tris-HCl (pH 8.9) at 37°C for 60 min. After the incubation sodium acetate 3 M (pH 5.0) was added to a final concentration of 0.3M, RNA was precipitated in 2.5 volumes of ice-cold 95%, incubated on ice for 10 min and recovered by centrifugation at 13.000xg for 10 min at 4°C. Pellet was washed once with 70% ethanol and dissolved in 10-20 µl of 10 mM sodium acetate (pH 4.5) and 1 mM EDTA. For primer extension assay total RNA from lymphoblasts was extracted using a variation of the RNAesay kit (Qiagen) protocol. The pellet derived from centrifugation of lymphoblast cells was passed through QiAShered column that allows the fragmentation of long nucleic acid into smaller fragments. Columns were centrifuged at 12000 rpm for 2 min and the homogenates were incubated at room temperature for 5 min. Samples were mixed with 140 µl of chloroform, incubated 23 min at room temperature and the centrifuged. The aqueous phase was transferred to a new tube and RNA was precipitated adding 70% ethanol. Samples were passed through RNeasy Mini spin column that binds long RNAs (longer than 200bp), while the flow-through contains the short RNAs; column was washed and the long RNAs were eluted with RNAse-free water. Short

RNAs contained in the flow-through were precipitated adding 100% ethanol and then passed through RNeasy MinElute column that binds shorter molecules of RNA. Column was washed and then short RNAs were eluted with RNAse-free water and used for the purification of tRNAs. RNA samples were quantified by nanodrop after dilution 1:1 in 100 mM Tris-HCl pH 7.0.

3.6 tRNA purification

tRNA purification protocol was set up adapting the method already described in literature[62]. Column of matrix for the purification were prepared placing Nucleobond XTRA resin (MACHEREY-NAGEL) in Pasteur pipettes equipped with a filter. The matrix was equilibrated with equilibration buffer (50 mM Tris-H_3PO_4 pH 6.3, 15% ethanol, 300 mM KCl). Columns were washed once with equilibration buffer and tRNAs were eluted with elution buffer (100 mM Tris-H_3PO_4 pH 6.3, 15% Ethanol, 650 mM KCl). 20 fractions were collected and the absorbance at 260 nm was monitored. The fractions corresponding to the peak of the absorbance were pooled and the tRNAs were precipitated incubating the samples with 0,7 volumes of isopropanol for 1 hour at 4°C. After centrifugation at 20000 rcf for 30 min at 4°C the pellet was washed once in 70% ethanol and then dried and resuspended in RNA-free water.

3.7 Quantitative real time PCR

Real-time PCR was performed using FastStart SYBR Green on the LightCycler (Roche Diagnostics) or Rotor-Gene SyBR Green on a Rotor-Gene Q cycler (Qiagen). Suitable primers were used to perform a conventional PCR for the preparation of the standards. Using the standard curve approach, gene expression was calculated as copies/ml and the values were normalized with the expression of the reference genes GAPDH and/or b-actin, which resulted to be not modified by the

experimental conditions. The sequences of primers used for Real Time PCR are provided in Table 5.

Species	Gene name	Primer forward	Primer Reverse
Rat	Bad	5'-CCAATAACAGTCATCATGGAG-3'	5'-GTCCTCGAAAAGGGCTAAG-3'
Rat	Bim	5'-AGAGATACGGATCGCACAGG-3'	5'-GTCTTCCGCCTCTCGGTAAT-3'
Rat	Bim S	5'-CAGAATCGCAAGCTTCCATA-3'	5'-GTCTTCCGCCTCTCGGTAAT-3'
Rat	DP5	5'-GCCGTGGTGTTACTTGGACT-3'	5'-GATTGTGCCAGAGCTTCACA-3'
Rat	Puma	5'-AGTGCGCCTTCACTTTGG-3'	5'-CAGGAGGCTAGTGGTCAGGT-3'
Rat	TRMT10A	5'-ATGACTTGATGGTGTTAAAG-3'	5'-ATCCATGTTCTTCTTCAGTTG-3'
Rat	GAPDH	5'-AGTTCAACGGCACAGTCAAG-3'	5'-TACTCAGCACCAGCATCACC-3'
Human	Bim	5'-TTCTTGCAGCCACCCTGC-3'	5'-CTTGCGTTTCTCAGTCCGA-3'
Human	Bim S	5'-GAGCCACAAGCTTCCATGAG-3'	5'-TAACCATTCGTGGGTGGTCT-3'
Human	TRMT10A	5'-ATGTTGTTCATAGCACCCTTC-3'	5'-ATGTTCTTTTTCAGCTGGCCT-3'
Human	β-Actin	5'-CTGTACGCCAACACAGTGCT-3'	5'-GCTCAGGAGGAGCAATGATC-3'

Table 5: Sequences of primers used for quantitative Real Time PCR.

3.8 Radio labeling of primers and RNA probes

Oligonucleotide primers used for primer extension assay and RNA probes used for northern blot experiments were 5'-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. The labeling reaction was performed mixing 20 pmol oligonucleotide, 0,15 mCi [γ-32P]ATP, 2 µl 10X reaction buffer, 10U T4 polynucleotide kinase, DEPC-treated water up to 20 µl. Samples were incubated at 37°C and after 1 hour the reaction was stopped by adding EDTA to 1 mM, pH 8.0 and heating at 95°C for 2 minutes. RNA primers and probes sequences are reported in table 6 and table 7.

tRNA molecule	Primer
tRNA-Histidine	5’-TGCGGCCACAACGCAGAGTA-3’
tRNA-Methionine	5’-TTCTGGGTTATGGGCCCAGC-3’
tRNA-Glutamine	5’-GCTGGATTCAAAGTCCAGA-3’
tRNA-Glycine	5’-CCTCCCGCGTGGCAGGCGAG-3’
tRNA-Asparagine	5’-TTTCGGTTAACAGCCGAACG-3’

Table 6: RNA oligonucleotides used for primer extension assay targeting different tRNAs.

tRNA molecule	Probe sequence
tRNA-Histidine	5’-CGAACCGAGGUUGCUGCGGCCACAACGCAGA GUACUAACCACUAUACGAUCACGGC-3’
tRNA-Methionine	5’-CGAUCCAUCGACCUCUGGGUUAUGGGCCCAG CACGCUUCCGCUGCGCCACUCUGC-3’
tRNA-Glutamine	5’- CUCGGAUCGCUGGAUUCAAAGUCCAGAGUGC UAACCAUUACAC -3’
5s-rRNA	5’-GACCCUGCUUAGCUUCCGAGAUCAGACGAG-3’

Table 7: RNA oligonucleotide probes used for northern blot experiments.

3.9 Primer extension assay

Primer extension assay previously described[63] was used to study the role of TRMT10A in methylation of G9 (m^1G_9) residues of different tRNA species. Purified tRNAs are retrotranscribed using ^{32}P-radiolabeled primers targeting specific tRNAs. The presence of m^1G_9 modification stops the reverse transcription due to the N-1 methyl group that prevents the formation of base pair necessary to continue the extension and thus generating shorter amplicons. The annealing of specific primers was performed mixing 50 mM Tris-HCl pH 8.3, 30 mM NaCl, 10 mM DTT, 1

pmol primer and 0.5 µg of purified tRNA. The mix was heated at 95°C for 3 minutes and then slowly cooled to 37°C. Annealed tRNA/primers were used for reverse transcription reaction. 2 µl of annealing reaction were added to 5 µl reaction mix containing 0.7 µl $MgCl_2$, 0.7 µl dNTPs, 0.7 µl 10X buffer, 0.35 µl MuLV reverse transcriptase, 0.35 µl RNase inhibitor and 2.2 µl water. Samples were incubated 5 minutes at room temperature and then at 37°C for 1 hour. The reaction was stopped adding 7 µl of loading buffer containing 90% formamide, 18.6% EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue and 10% glycerol. The amplicons are separated in 15% polyacrylamide gel containing 4 M urea. Gel was pre-run in 1X TBE buffer (0.09 M Tris base, 0.09 M boric acid and 2 mM EDTA) at 200 V for 30 minutes and before loading the samples the wells were cleaned from excess of urea. After loading the samples, gel was run at 120 V for 30 minutes and then at 150 V for 1 hour. Gel was fixed in 1X TBE 10% methanol and 10% ethanol. Signal was detected by autoradiography.

3.10 Northern Blot

Total RNA samples extracted in acid condition were resolved on acid-urea gels. Samples were diluted 1:1 in acid loading buffer containing 0.1 M sodium acetate (pH 5.0), 8 M urea, 0.05% bromophenol blue, and 0.05% xylene cyanol and loaded into 6.5% polyacrylamide gel (19:1 acrylamide: bisacrylamide) containing 8 M urea in 0.1 M sodium acetate buffer (pH 5.0). Electrophoresis was performed at 80 V (-12 V/cm) was performed in a cold room until the bromophenol blue dye reached the bottom of the gel. The portion of the gel between the xylene cyanol and bromophenol blue dyes, contains the tRNAs of interest. Samples were blotted onto a positively charged nylon membrane (ThermoFisher) using at 20 V overnight with Tris Borate-EDTA buffer (TBE) buffer 0,5X as transfer buffer. Membrane was crosslinked with UV light for 10 minutes. Membranes were pre-hybridized

with North2South hybridization buffer (ThermoFisher) for 30 min and then hybridized overnight with radiolabeled RNA probes at 65°C shaking. The signal of radiolabeled probes was detected by autoradiography.

3.11 Autoradiography

Gels from primer extension assay and membranes from northern blot were placed in intensifying screens TranScreen HE (Kodak) that convert most of the penetrating radiation energy to photons providing maximum sensitivity. The radioactive signals were detected using Amersham Hyperfilm MP (GE Healthcare Limited, UK), placed in a cassette and incubated at -80°C. Before the detection the cassette was defrost. Autoradiography was processed manually using Carestream developer and fixator solutions (Kodak).

3.12 Bioinformatics tools

ExPASy portal (http://expasy.org) was used to access different databases to obtain information about function and domains of proteins of interest. Modomics database (http://modomics.genesilico.pl) was used to investigate the nucleotide sequences of different tRNAs and the specific nucleotide modifications.

The list of genes derived from whole exome sequencing of congenital hyperinsulinism was analyzed using the candidate gene prioritization tool ToppGene (https://toppgene.cchmc.org/prioritization.jsp). ToppGene produces two outputs: the first is the functional profile of a training set of genes, enriched with different annotation categories (training set output)[64]. The training set is a reference list of genes, such as known causative genes of a disease. The second output represents the level of similarity between the test set genes and the training set. The tool produces a similarity score for each functional category and an average of scores, obtained by meta-analysis; genes are ranked for statistical significance of

scores: p values are calculated using a Bonferroni statistical test. Genes used as training set and as test set are reported in table 8.

TRAINING SET LIST	TEST SET LIST
ABCC8, KCNJ11, GLUD1, GCK, HADH, SLC16A1, HNF4A, HNF1A, UCP2, ABCC8	KCNH6, GNAS, ACACB, NOTCH2, SLC37A3, CSMD1,, RYR3, TRPV3, TRPC5, CAMK2D, PIK3R3, CDKAL1, SCN8A, KCNJ10, PDE4C, NOS2, SLC24A6, SULF1, TLL1, CACNA1A, PC

Table 8: Gene lists used for candidate gene prioritization. The training set is the list of known causative genes of congenital hyperisnulinism, the test set is the list of genes derived from whole exome sequencing of congenital hyperinsulinism patients not mutated in known causative genes.

3.13 Statistics

Data are presented as means ± SEM. Non-normally distributed variables were log-transformed before statistical testing. Comparisons between groups were made by ANOVA followed by two-sided Student's paired t test with Bonferroni correction for multiple comparisons. A p-value <0.05 was considered statistically significant.

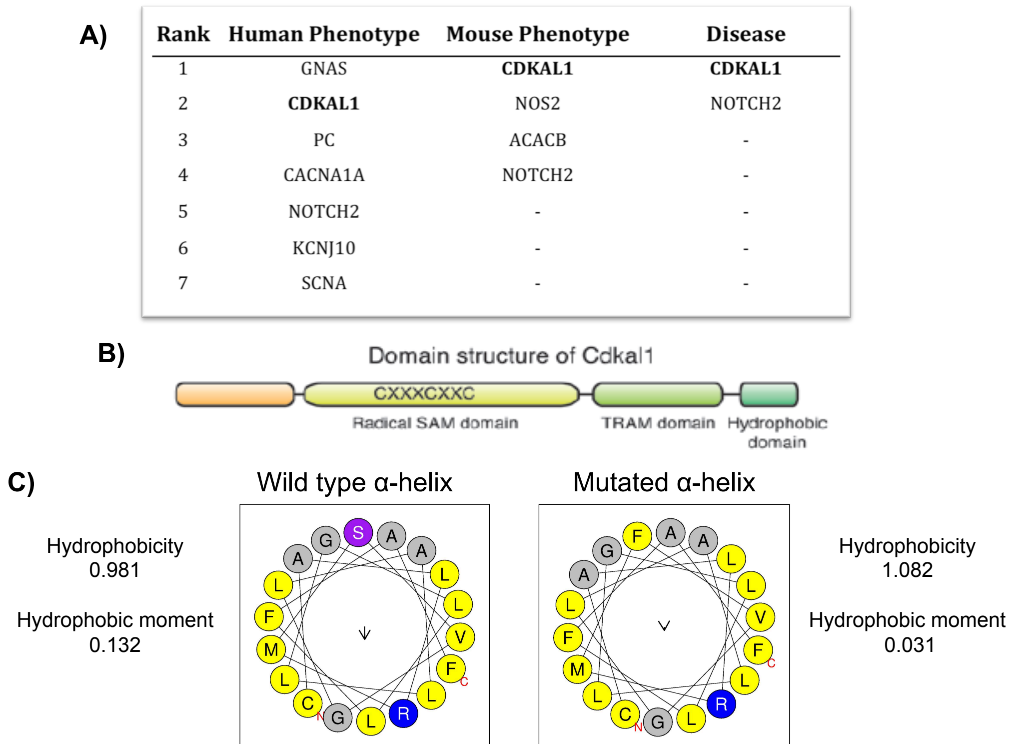
4 RESULTS

4.1 S561F CDKAL1 variant

4.1.1 Identification and in silico study of the variant

Prioritization tools can be useful for searching unknown genes causing or associated with a disease, basing on the hypothesis that similar phenotypes are caused by similar genes. I used the list of congenital hyperinsulinism causative genes as training set and the list of genes derived from the previous WES study as test set. I considered the output relative to three functional categories: disease, human and mouse phenotype. The output of prioritization analysis shows that some genes significantly correlate with phenotypes associated to CHI causative genes, among these *CDKAL1* ranked in the top positions of the three functional categories (Figure 9 A). The nucleotide variant identified by WES is the c1682t that results in the amino acid change S561F. CDKAL1 protein sequence consists in 579 amino acid residues; the Methyl-thiotransferase catalytic domain (MTTAse) is located at the N-terminus (64-172aa); the core of the protein is a radical SAM domain, containing a cysteine motif CxxxCxxC that binds a [4Fe-4S] cluster, used to cleave S-adenosylmethionine (SAM) to generate methionine and a 5'-deoxyadenosyl radical. The TRAM domain predicted to bind tRNA molecules localises between the 431 and the 493 residues. CDKAL1 is a tail-anchored protein inserted in the membrane of the ER through the C-terminus hydrophobic domain[56]. The S561F variant localises in the hydrophobic transmembrane domain comprised between the positions 556-578. I used the HeliQuest (http://heliquest.ipmc.cnrs.fr) tool from ExPASy portal to analyse the interaction of the α-helix residues in the *wild type* and mutated sequence. The substitution of a serine with a phenylalanine, more hydrophobic residues, leads to an increase of hydrophobicity of the transmembrane

domain and decrease of the hydrophobic moment that quantifies the amphiphilicity of a helix (Figure 9 C). These changes could affect the stability of the protein in the membrane and consequently the turnover and the localization.



A)

Rank	Human Phenotype	Mouse Phenotype	Disease
1	GNAS	**CDKAL1**	**CDKAL1**
2	**CDKAL1**	NOS2	NOTCH2
3	PC	ACACB	-
4	CACNA1A	NOTCH2	-
5	NOTCH2	-	-
6	KCNJ10	-	-
7	SCNA	-	-

Figure 9: A) Top position of the rank derived from candidate gene prioritization tool of ToppGene portal; **B)** Prediction of conserved CDKAL1 domains; **C)** prediction of α-helix organization and characteristics in *wild type* and S561F CDKAL1.

4.1.2 Overexpression of CDKAL1 in INS1E cells

In order to perform functional studies in beta cells I obtained stably transfected INS1-E clones expressing the human ORF of CDKAL1 fused to a HA-tag. I selected different clones expressing the *wild type* and the S561F CDKAL1. Since the S561F mutation affects the C-terminal trans-membrane domain the localization of CDKAL1 was monitored by indirect immunofluorescence using a commercial anti-HA antibody and confocal analysis. As reported, *wild type* CDKAL1 localized in a reticular compartment, diffused in the cell cytoplasm. The genetic variant S561F similarly was confined in a reticular compartment, although its localization was enriched in enlarged vesicular structures (Figure 10).

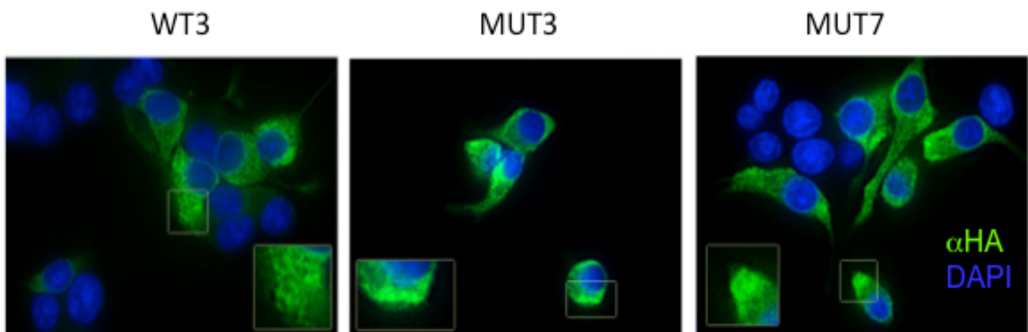


Figure 10: Immunofluorescence staining of wild type (WT3) and mutant CDKAL1 (MUT3 and MUT7) in stably transfected INS-1E cells. Blue: DAPI labelled nuclei; green: CDKAL1 with HA tag is visualized with Alexa Fluor® 488 dye staining.

To investigate if the S561F CDKAL1 variant could affect beta cells function, I evaluated the insulin content and release in INS1E clones. As it was expected the overexpression of *wild type* CDKAL1 leads to an increase of insulin content; the overexpression of the variant S561F abolishes the increase of insulin content. On the other hand, the release of insulin in basal glucose condition is significantly increased in the mutated clones (Figure 11)

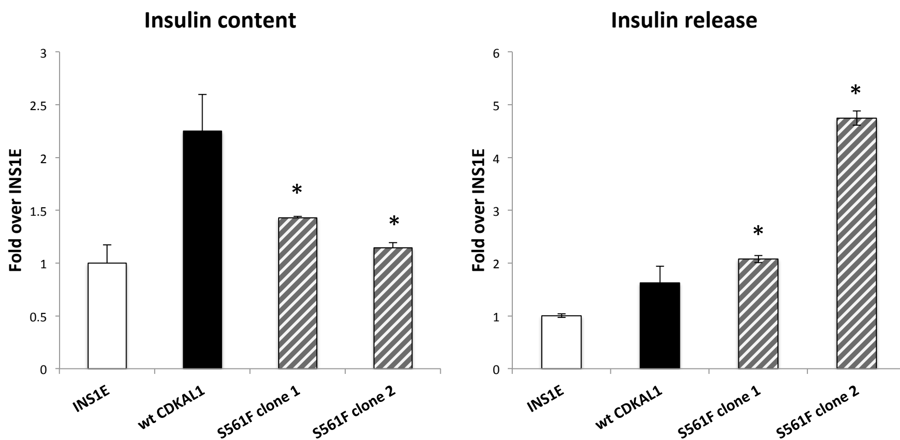


Figure 11: Quantification of insulin content (left) and release (right) in basal conditions (11mM glucose) in INS-1E non-transfected and overexpressing wilt type (wt) and mutated (S561F clone 1 and 2) CDKAL1. Data expressed as means ± SEM * $p<0.05$

4.2 TRMT10A deficiency

4.2.1 *TRMT10A modifies tRNA^{GLN} and tRNA^{iMeth} in human cells*

The G379A variant of TRMT10A leads to the complete absence of the protein in patients affected by microcephaly and young onset diabetes. Different techniques were used to determine the function of TRMT10A in human cells and to understand the consequences of the deficiency of the protein. It was shown previously that TRMT10A is involved in the methylation of guanine residues in human cells (data non published): total RNA was extracted from lymphoblast cells of patients and control subjects and used for a mass spectrometry analysis of nucleotide modifications. The methylation of guanine residues in total RNA resulted to be decreased in lymphoblasts derived from two patients compared to the controls. This observation suggests that TRMT10A can be responsible of the methylation of guanine residues of tRNAs *in vivo* in human. To further investigate the role of TRMT10A, tRNA molecules characterized by a guanine residue in position 9 were identified using Modomics database (http://modomics.genesilico.pl). The tRNAs specific for glycine (Gly), glutamine (Gln), initiator methionine (iMeth) and asparagine (Asp) have a G9 residue and are potential substrates of TRMT10A. The primer extension assay was used to identify tRNA molecules modified by TRMT10A *in vivo*. tRNA faction was purified from total RNA samples derived from lymphoblasts of one patient (PA 1), one heterozygous carrier (HZ) and control subjects (CT1, CT2 and CT3). The sequence of the different tRNAs was amplified using specific ^{32}P-radiolabeled primers; the products of amplification were resolved in urea-polyacrilamide gel and detected by autoradiography; the presence of the methylation in position 9 causes the block of the amplification that will produce amplicons of 35 base pairs. The absence of the modification allows the complete amplification of the tRNA

sequence producing bigger bands (38bp). $tRNA^{Gln}$ and $tRNA^{iMeth}$ resulted to be methylated at the level of G9 residue in lymphoblasts derived from control subjects but not in lymphoblasts from the patient (Figure 12). The G9 residue of $tRNA^{Asp}$ and $tRNA^{Gly}$ is methylated in all the samples suggesting that the methylation of these tRNAs is not mediated by TRMT10A. $tRNA^{His}$ (specific for histidine) was used as negative control since it doesn't present a guanine in position 9. These results demonstrated that TRMT10A deficiency leads to the absence of methylation of G9 residues in $tRNA^{Gln}$ and $tRNA^{iMeth}$ and that these tRNAs are specific substrates of the enzyme *in vivo.* It's known from literature that tRNA modifications can affect the maturation and the stability of the molecules. Also the amino-acylation can be modulated by post-transcriptional modifications. Based on that I set up a northern blot technique in order to detect and quantify aminoacylated and deaminoacylated tRNAs. Radiolabeled probes were used to detect specifically $tRNA^{Gln}$ and $tRNA^{iMeth}$ that were shown to be TRMT10A substrates, and $tRNA^{His}$ as negative control. Total RNA from lymphoblasts was isolated under acid conditions to preserve aminoacylation. Aminoacylated tRNAs are heavier and run slower in the electrophoretic gel, and can be distinguished from deaminoacylated molecules that run faster. To confirm that extracted tRNAs were aminoaylated, part of the sample was treated with alkali pH that induces complete deaminoacylation. These alkali treatment samples were used as controls in electrophoretic gels. Preliminary results showed in Figure 13 suggest that in basal codition the absence of G9 methylation in $tRNA^{Gln}$ and $tRNA^{iMeth}$ may decrease the abundance of these molecules without affecting the aminoacylation. Nevertheless these results need to be confirmed analyzing a second patient and more controls.

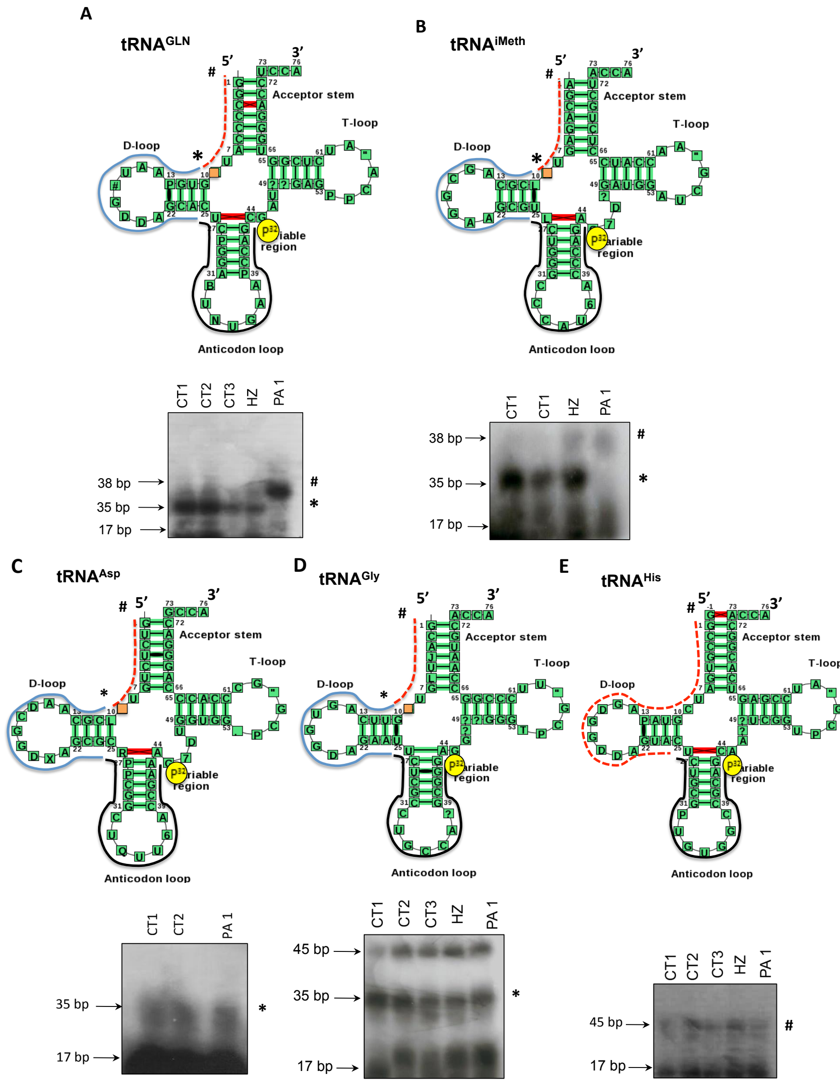


Figure 12: Identification of TRMT10A tRNA targets by primer extension assays. tRNAs purified from lymphoblasts from two controls (CT1-2), one heterozygous carrier (HZ) and one TRMT10A-deficient patient (PA 1) were reverse transcribed using radiolabelled primers. G9 methylation (m1G9) stops the reverse transcription generating short amplicons (*). In the absence of m1G9 the reverse transcription continues up to the 5' end of the tRNA (red dotted lines) generating longer amplification products (#). TRMT10A deficiency only modified the amplification pattern of tRNA^{GLN} **(A)** and tRNA^{iMeth} **(B)** but not of tRNA^{Asp} **(C)** and tRNA^{Gly} **(D)**. As expected, no difference in the amplification pattern of the C9-containing tRNA^{His} **(E)** was observed. In the figure are shown cloverleaf structures of tRNAs and the autoradiography of the amplification products. Black lines represent the oligonucleotides used as primers, blue lines represent the reverse transcribed fragment from the 3' end of the primer to the G9.

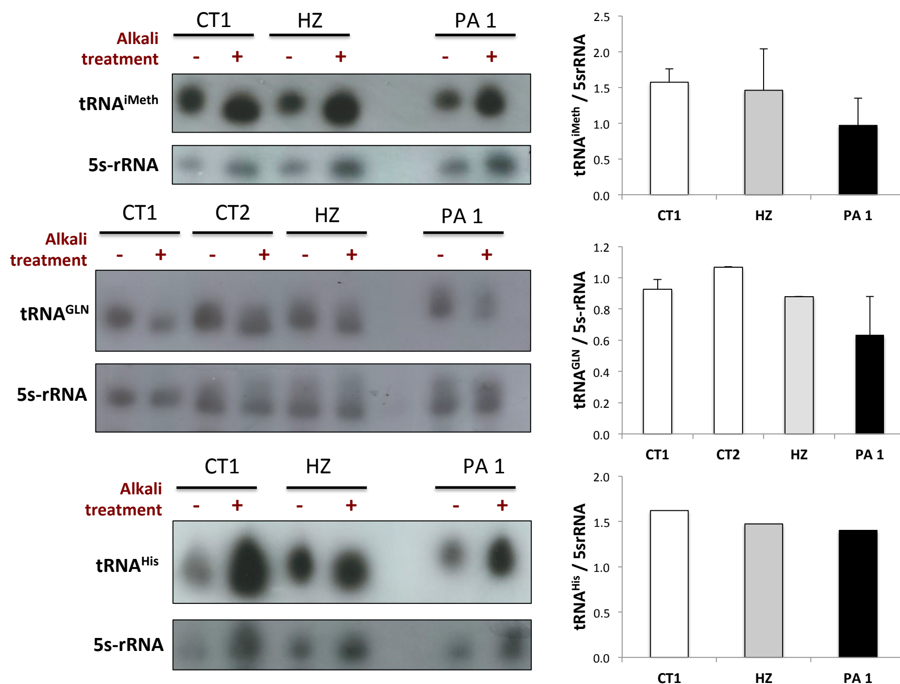


Figure 13: RNA isolated from lymphoblasts from control subjects (CT1, CT2), one heterozygous carrier (HZ) and one patient (PA 1) was analyzed by northern blot. Aliquots of the same samples were treated with alkali pH to induce complete deaminoacylation. $tRNA^{iMeth}$, $tRNA^{GLN}$ and $tRNA^{His}$ were detected by hybridization of northern blot membranes with specific radiolabeled probes. Ribosomal RNA 5s-rRNA was detected and used as loading control. Autoradiography of representative blots is shown on the left. Autoradiography films were scanned and bands were quantified by optical density analysis: results from quantification are plotted in histograms on the right. Data are expressed as mean ± SEM (n=1-5).

4.2.2 TRMT10A deficiency induces apoptosis in beta cells through the activation of the intrinsic pathway of apoptosis

In order to obtain a model of TRMT10A deficiency in beta cells, I induced the knockdown of the gene through RNA interference in two beta cell lines: INS-1E, derived from rat insulinoma, and EndoC-βH1, a human beta cell line. It was already shown that TRMT10A silencing induces apoptosis in INS-1E cells[57]; To study the molecular mechanism of TRMT10A deficiency-

induced apoptosis I have silenced TRMT10A in INS-1E cells using three different siRNAs (siTRMT10A #B, #C and #D) and I confirmed the induction of apoptosis previously shown (Figure 14 A-B). Interestingly, TRMT10A knockdown in EndoC-βH1 cells using two different siRNAs (siTRMT10A-#1, siTRMT10A-#3) also induced apoptosis (Figure 14 C-D). To evaluate which pathway of apoptosis was activated by TRTM10A deficiency I examined the acttivation of Caspase 9, a hallmark of the intrinsic pathway of apoptosis. TRMT10A knockdown increased Caspase 9 cleavage (measured by western blot) in INS1E and EndoC-βH1 (Figure 15) suggesting that the intrinsic pathway of apoptosis is activated.

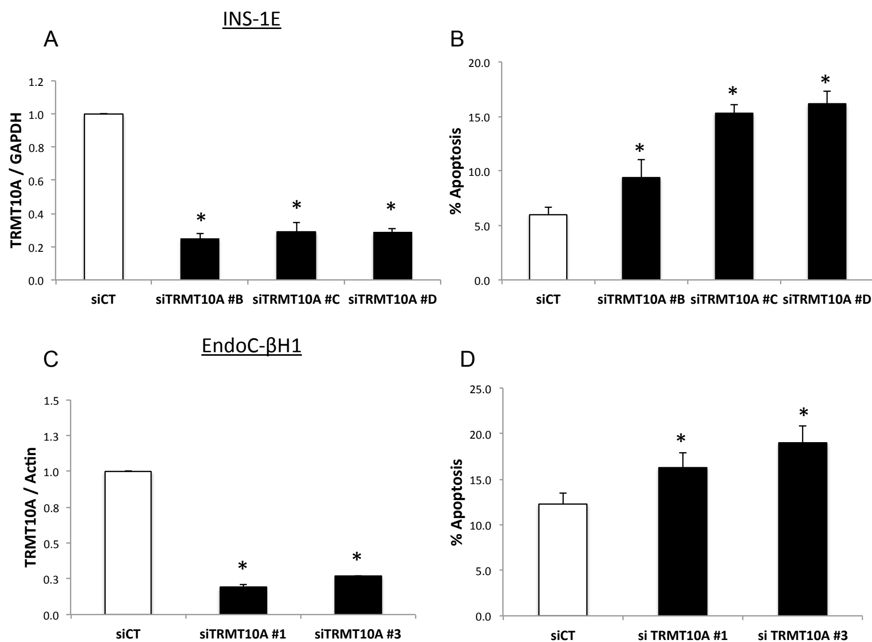


Figure 14: A) TRMT10A mRNA expression normalized to the reference gene GAPDH **B)** percentage of apoptosis in INS-1E transfected with control siRNA (siCT) and siRNAs targeting rat TRMT10A (siTRMT10A #B, #C and #D) **C)** TRMT10A mRNA expression in EndoC-βH1 normalized to reference gene β-actin **D)** percentage of apoptosis in INS-1E transfected with control siRNA (siCT) and siRNAs targeting humanTRMT10A (siTRMT10A #1 and #3). Data are expressed as mean ± SEM (n=9–11). siCT vs siTRMT10A * $p<0.05$

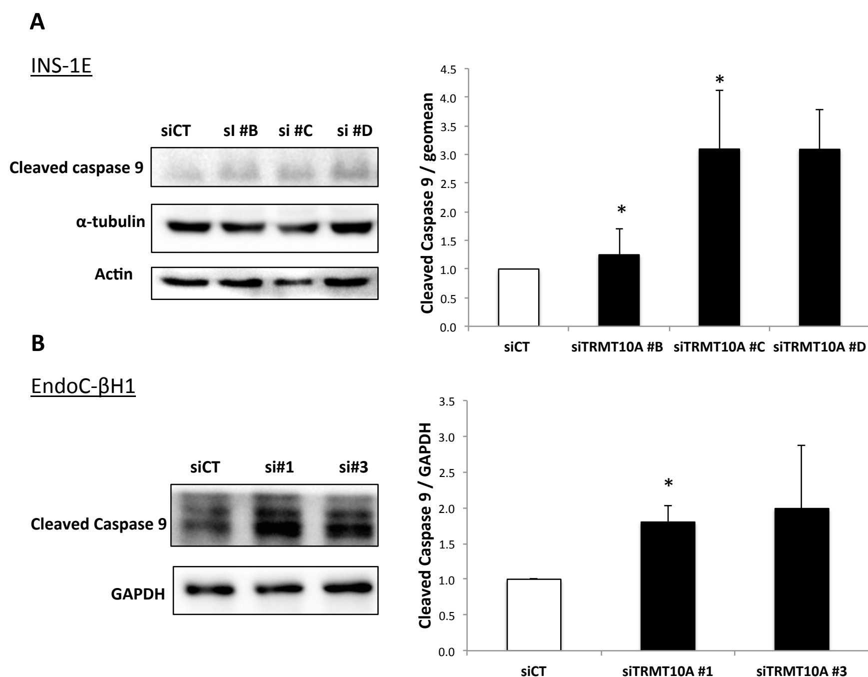


Figure 15: A) Quantification of cleaved Caspase 9 by western blot in INS-1E transfected with control siRNA and siRNAs targeting rat TRMT10A (siTRMT10A #B, #C and #D). **B)** Quantification of cleaved Caspase 9 in EndoC-βH1 transfected with control siRNA and siRNAs targeting human TRMT10A (siTRMT10A #1 and #3). Left panels: representative blots, right panels: optical density analysis. Quantification of cleaved Caspase 9 was corrected by the geometric mean of the expression of the reference proteins α-tubulin and β-actin (INS-1E) or by the expression of the reference protein GAPDH (EndoC-βH1) Data expressed as mean ± SEM (n=4–7). siCT vs siTRMT10A * $p<0.05$

4.2.3 The BH3-only activator Bim is the mediator of TRMT10A deficiency-induced apoptosis

I have previously shown that the intrinsic pathway of apoptosis is activated with TRMT10A deficiency. I wanted next to identify which are the mediators of the process. To pursue this objective I evaluated the expression of the pro-apoptotic BH3-only family members Bim, Bad, DP5 and PUMA at mRNA level in INS-1E cells after TRMT10A silencing. TRMT10A

knockdown induces a significant mRNA increase of Bim and Bad but not of DP5 or PUMA (Figure 16 A-B-C-D) in INS-1E cells. Alternative splicing produces three splice variants of Bim: BimEL, BimL and BimS. The isoform BimS was shown to have the most pro-apoptotic potency[65]. Interestingly, this specific Bim isoform resulted to be induced by TRMT10A knockdown in INS-1E cells (Figure 16 E). The mRNA expression of pro-survival proteins Bcl-2 and Bcl-xL was not modulated by TRMT10A silencing (*data not shown obtained in our lab*).

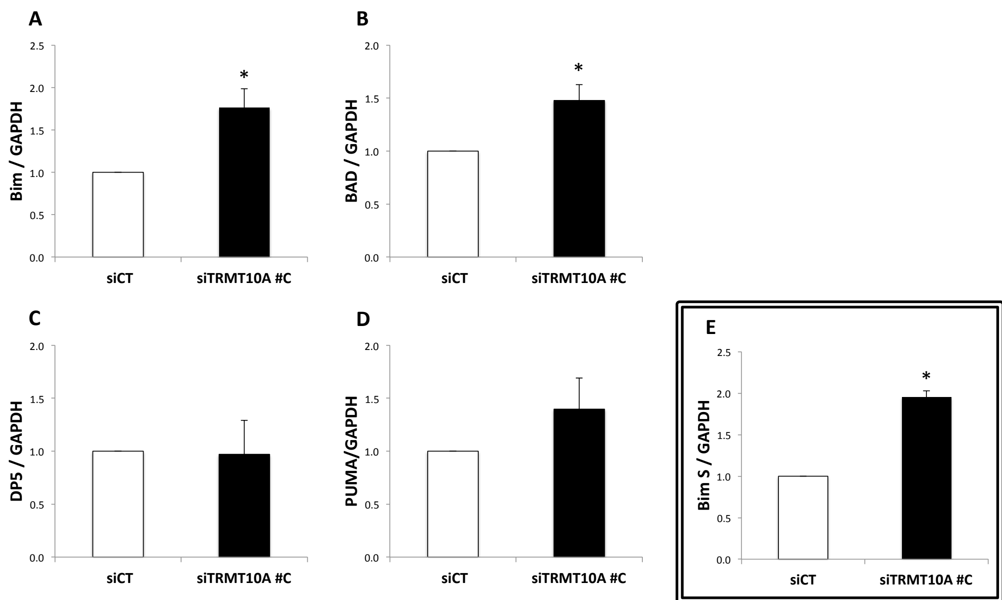


Figure 16: mRNA expression measured by real-time PCR of total Bim **(A)**, Bad **(B)**, DP5 **(C)** and PUMA **(D)** BH3-only family members and Bim S isoform **(E)** in INS-1E cells transfected with control siRNA (siCT) or siRNAs targeting TRMT10A (siTRMT10A #C). Data are normalized to the expression of reference gene GAPDH, and are expressed as mean ± SEM (n=4). siCT vs siTRMT10A * $p<0.05$

The results derived from mRNA expression of BH3-only members suggested that the activator Bim and the sensitizer Bad could mediate the apoptosis induced by TRMT10A deficiency. To investigate this hypothesis I

checked the activation of Bim and Bad at protein level. Transcriptional modulation and alternative splicing regulate the pro-apoptotic function of Bim. I observed a significant increase of the most pro-apoptotic isoform BimS in TRMT10A knockdown-INS-1E cells, while the isoforms Bim L and Bim EL were not modulated (Figure 17 A). The pro-apoptotic action of Bad is controlled by phosphorylation; dephosphorylated Bad can bind and inactivate the pro-survival proteins Bcl-2 and Bcl-xL inducing Bax/Bak-triggered apoptosis. Bad phosphorylation at Ser136 by Akt and at Ser112 by PKA plays a critical role in blocking the dimerization of Bad and Bcl-xL, promoting cell survival. I didn't detect any significant change in Bad phosphorylation at Ser136 site after TRMT10A silencing (Figure 17 B) and in previous experiment from my group the Ser112 phosphorylation was not modulated by TRMT10A knockdown (*data not shown*). This suggests that Bim and not Bad activation is the key event mediating TRMT10A silencing-induced apoptosis. Interestingly, results showed that Bim mRNA expression was significantly induced by TRMT10A knockdown also in human beta cells (Figure 18 A). The isoform BimS showed a trend of increase at mRNA level (Figure 18 B), while was significantly induced at protein level (detected with western blot) after TRMT10A silencing (Figure 18 C).

Since TRMT10A deficiency induces apoptosis via Bim activation, the absence of Bim is expected to be protective against TRMT10A silencing-induced apoptosis. I verified this hypothesis inducing a double knockdown of TRMT10A and Bim in INS-1E cells (Figure 19). Cells were transfected with control siRNA or TRMT10A-specific siRNAs (siTRMT10A #C-#D) alone or in combination with siRNA specific for Bim (siBim). Bim silencing significantly protected TRMT10A deficient rat (Figure 19 A) beta cells from apoptosis.

INS-1E

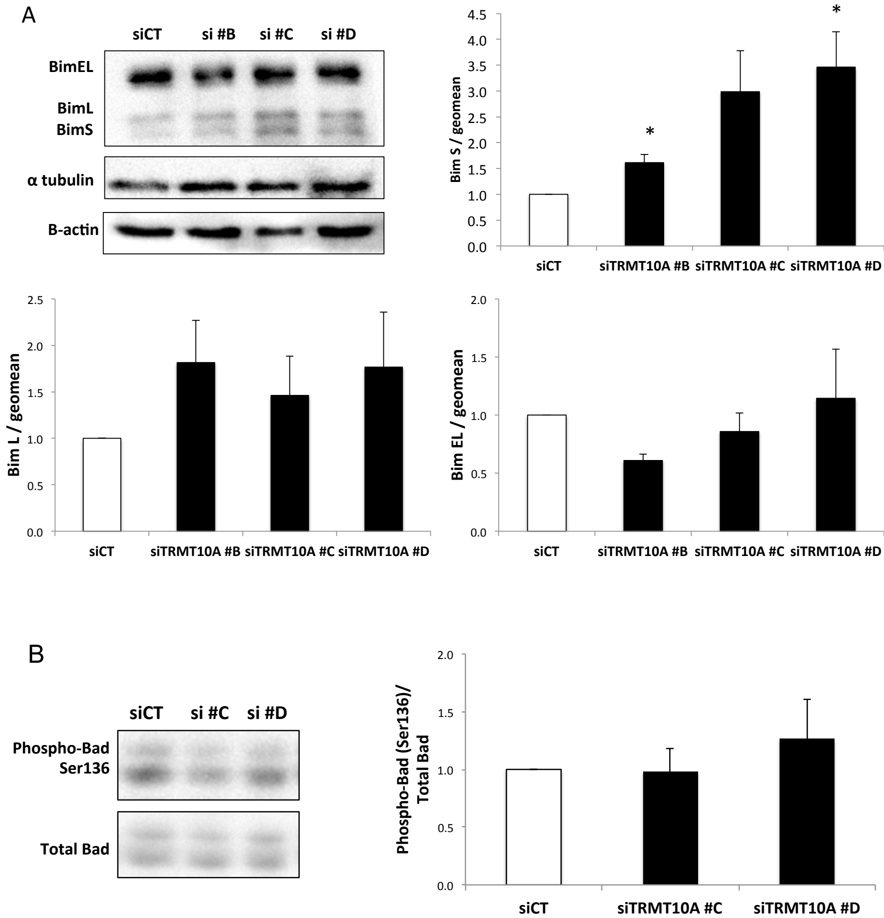


Figure 17: Expression of Bim S, L and EL isoforms **(A)** and Bad phosphorylation at Ser136 **(B),** examined by western blot, in INS-1E cells transfected with control siRNA (siCT) or siRNA targeting TRMT10A (siTRMT10A #B, #C and #D). Representative blots are shown, bands were quantify by optical densitometry analysis. Data are normalized to the geometric mean of expression of reference proteins α-tubulin and β-actin (Bim S, L and EL) or to total Bad expression (Phospho-Bad), Data are expressed as mean ± SEM (n=4–7). siCT vs siTRMT10A * $p<0.05$

EndoC-βH1

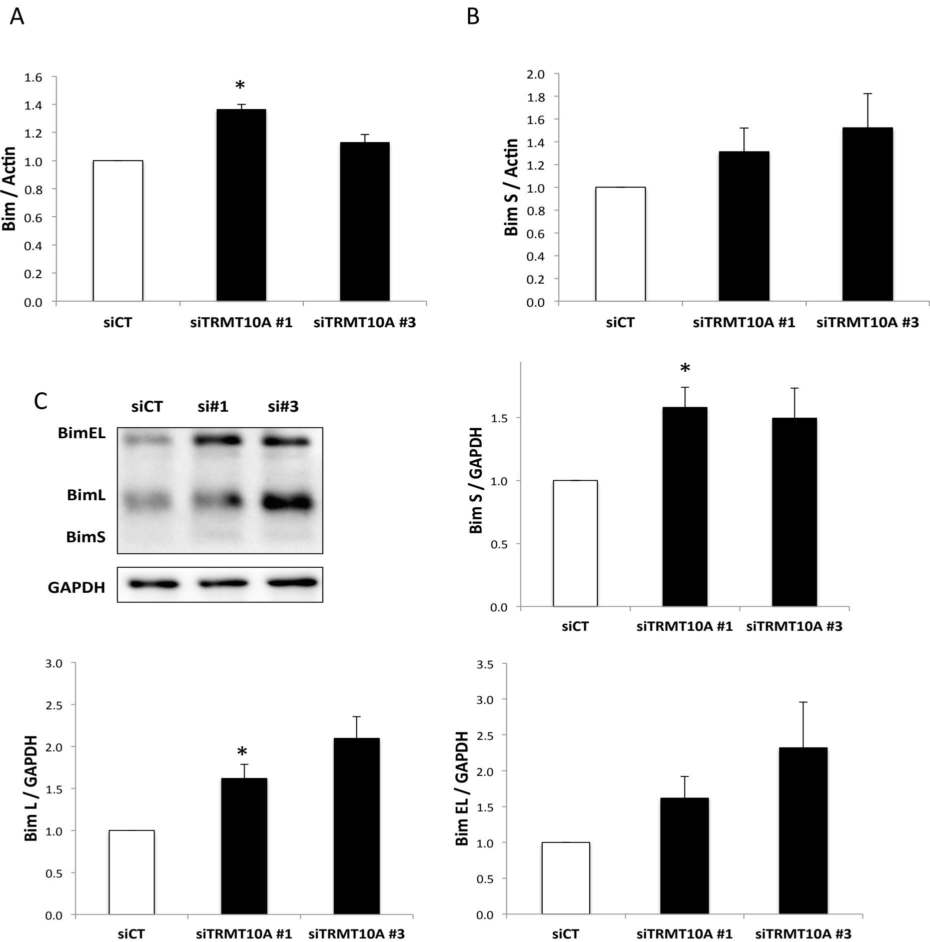


Figure 18: mRNA expression of total Bim **(A)** and BimS isoform **(B)** examined by real-time PCR in EndoC-βH1 cells transfected with control siRNA (siCT) and siRNAs targeting TRMT10A (siTRMT10A #1 and #3). mRNA data were normalized to β-Actin reference gene expression. The expression of Bim isoforms (Bim S, L and EL) was analysed by western blot in EndoC-βH1 cells **(C)** transfected with control siRNA and siRNAs targeting TRMT10A (siTRMT10A #1 and #3). Representative blot is shown; bands were quantified by optical densitometry analysis and normalized to the GAPDH reference protein expression. Data expressed as mean ± SEM (n=3-5). siCT vs siTRMT10A * $p<0.05$

The efficiency of the knockdown was verified at mRNA level by real-time PCR: the expression of both TRMT10A and Bim was decreased by 60 to 70% after transfection with the specific siRNAs compared to the control condition (Figure 19 B-C). I have shown that Bim is induced after TRMT10A silencing also in human beta cells. To further confirm the role of Bim as mediator of TRMT10A deficiency-induced apoptosis in human beta cells, EndoC-βH1 cells were transfected with siRNAs targeting TRMT10A (siTRMT10A #1 and #3) in combination with siRNA specific for Bim (siBim). A significant protection against TRMT10A knockdown-induced apoptosis was observed in cells transfected with siTRMT10A #3, that is the siRNA that induces more apoptosis in basal conditions (Figure 20 A). The analysis of mRNA expression by real-time showed a very good silcencing of TRMT10A (from 60 to 80%), while just a 40% of decrease was observe for Bim mRNA (Figure 20 B-C). A positive control experiment to confirm the effectiveness of Bim knockdown in EndoC-βH1 cells was performed in parallel. It was previously shown that Bim silencing protects EndoC-βH1 cells from cytokines induced apoptosis[66]. For the positive control cells were transfected with siRNA of control (siCT) and siRNA targeting Bim (siBim) and treated with cytokines (h-IFNγ and IL-1β) for 24 hours (Figure 20 D-E). Bim silcencing protects cells from cytokines-induced apoptosis as it was expected, indicating that human Bim siRNA was effectively working despite the low knockdown efficiency observed by real-time PCR.

In conclusion it was confirmed that Bim is the mediator of TRMT10A deficiency-induced apoptosis.

INS-1E

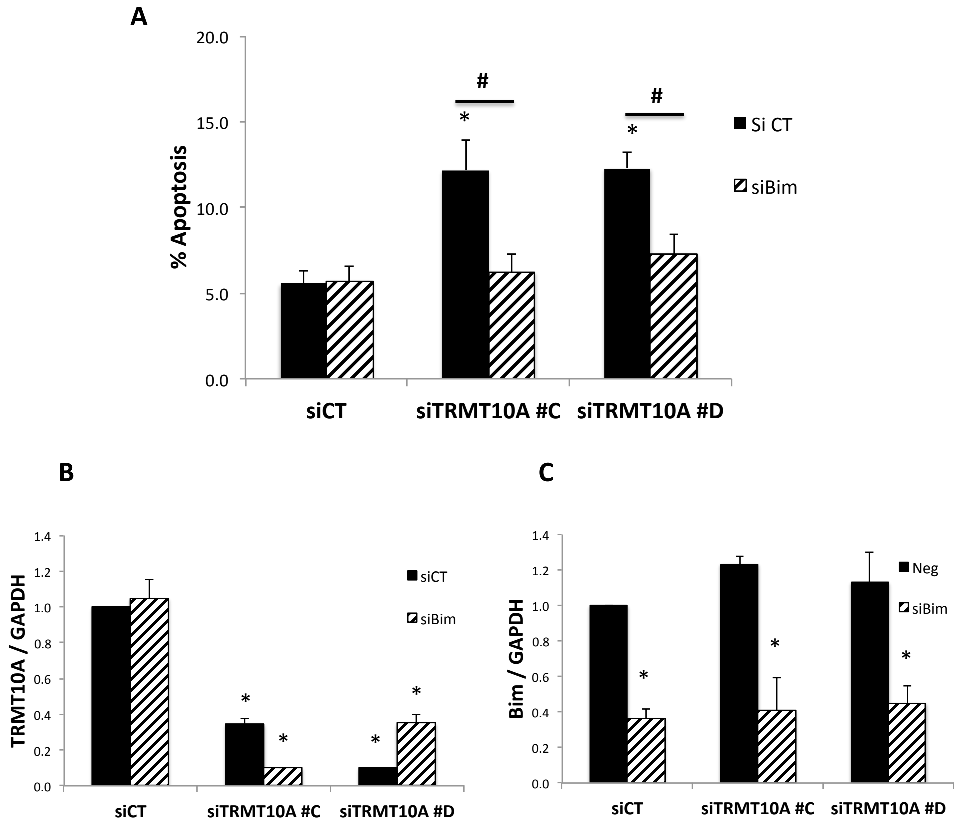


Figure 19: INS-1E cells were transfected with control siRNA (siCT) or siRNAs targeting TRMT10A (siTRMT10A #C and #D) alone or in combination with siRNA targeting Bim (siBim). After 48 hours from transfection, apoptosis was evaluated with HO/PI staining **(A)**. TRMT10A **(B)** and Bim **(C)** mRNA expression was examined by real-time PCR. Data of real-time are normalized to the expression of reference gene GAPDH. Data are expressed as mean ± SEM (n=3–7). *, # p<0,05

EndoC-βH1

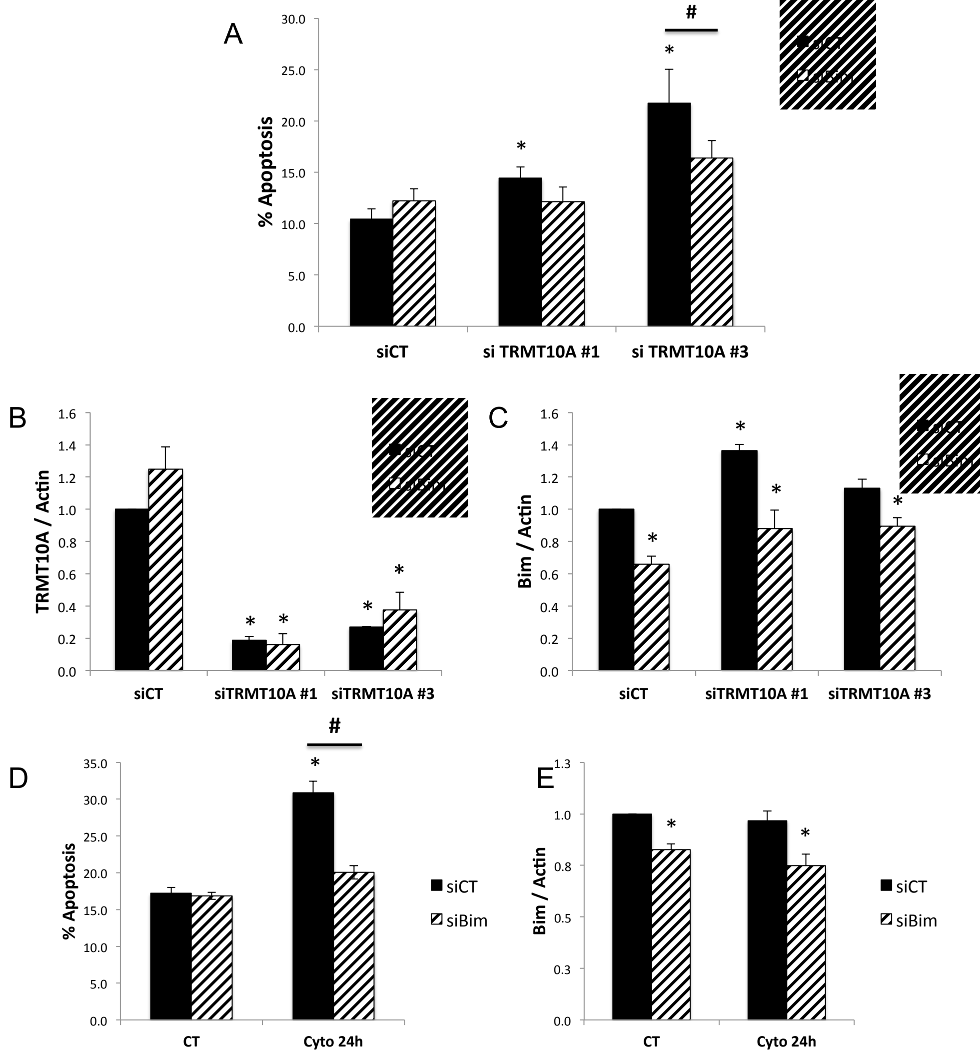


Figure 20: EndoC-βH1 cells were transfected with control siRNA (siCT) or siRNAs targeting TRMT10A (siTRMT10A #1 and #3) alone or in combination with siRNA targeting Bim (siBim). After 48 hours from transfection, apoptosis was evaluated with HO/PI staining **(A)**. TRMT10A **(B)** and Bim **(C)** mRNA expression was examined by real-time PCR. **(D)** EndoC-βH1 cells after 48 hours from transfection with control siRNA or siRNA targeting Bim (siBim) were treated with cytokines (cyto) for 24 hours. Apoptosis was evaluated. **(D)** and mRNA expression of Bim **(E)** was examined. Data of real-time are normalized to the expression of reference gene GAPDH. Data expressed as mean ± SEM (n=3–7). Data are expressed as mean ± SEM (n=3–7). *, # $p<0,05$

4.2.4 TRMT10A deficiency sensitizes beta cells to free fatty acid and ER-stress induced apoptosis

It was previously shown that TRMT10A mRNA expression is induced by treatment with palmitate and the chemical ER stressor cyclopiazonic acid (CPA) in INS-1E cells[57]. CPA is an inhibitor of the sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) and causes the depletion of calcium storage leading to ER stress. Furthermore the TRMT10A knockdown sensitizes INS-1E cells to palmitate, oleate and ER stress induced apoptosis (Figure 21)[57]. To investigate if the BH3-only protein Bim is also the mediator of this process, I evaluated whether Bim silencing protected TRMT10A deficient beta cells from palmitate treatment. Bim knockdown protected INS-1E cells from palmitate-induced apoptosis in control condition and after TRMT10A silencing (Figure 22). In EndoC-βH1 cells, TRMT10A mRNA expression was induced by 24 hours treatment with Thapsigargin (Figure 23 A). Thapsigargin is an inhibitor of the sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) that induces ER stress with the same mechanism of CPA. TRMT10A silencing sensitizes human beta cells to thapsigargin-induced apoptosis (Figure 23 B). Interestingly, silencing Bim had a protective effect against the sensitization to ER stressors induced by TRMT10A deficiency in human beta cells (Figure 24).

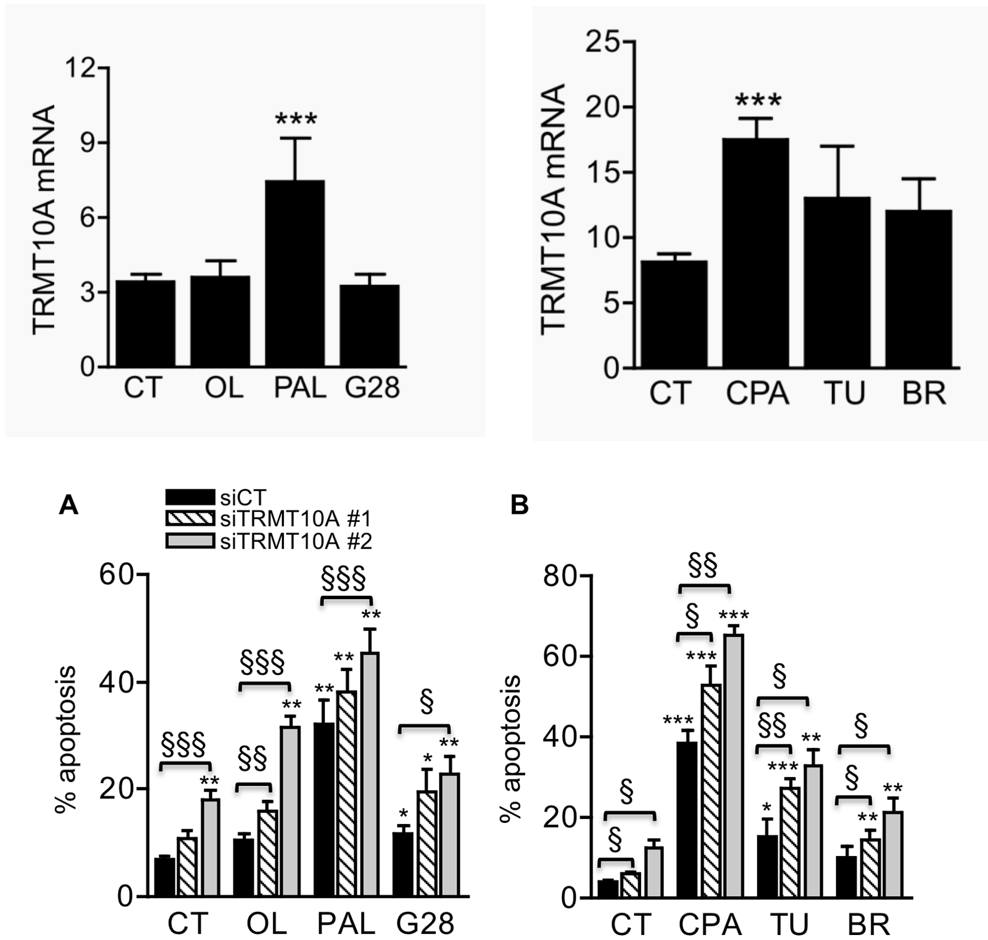


Figure 21: Figure adapted from *Igoillo-Esteve, M*. et al 2013. TRMT10A mRNA expression and percentage of apoptosis in INS-1E cells exposed or not (CT) to oleate (OL), palmitate (PAL), 28 mM glucose (G28), and chemical ER stressors cyclopiazonic acid (CPA), tunicamycin (TU) or brefeldin (BR). mRNA expression data are normalized to the geometric mean of GAPDH, tubulin and OAZ1 mRNA expression. Data expressed as mean ± SEM (n=4–11). *,§ $p<0.05$

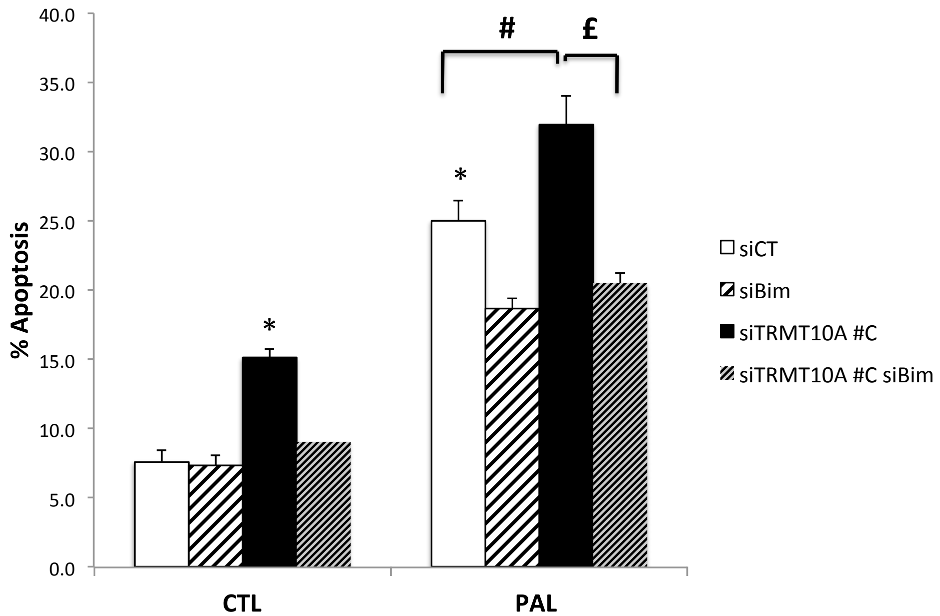


Figure 22: Percentage of apoptosis in INS-1E cells after transfection with control siRNA (siCT), siRNA targeting TRMT10A (siTRMT10A #C) alone or in combination with siRNA targeting Bim (siBim) in cells treated for 16 hours with palmitate (PAL). Data are expressed as mean ± SEM (n= 4-8). * p<0,05 vs siCT(CT), # p<0.05 vs siCT(PAL), £ p<0,05 vs siTRMT10A(PAL)

EndoC-βH1

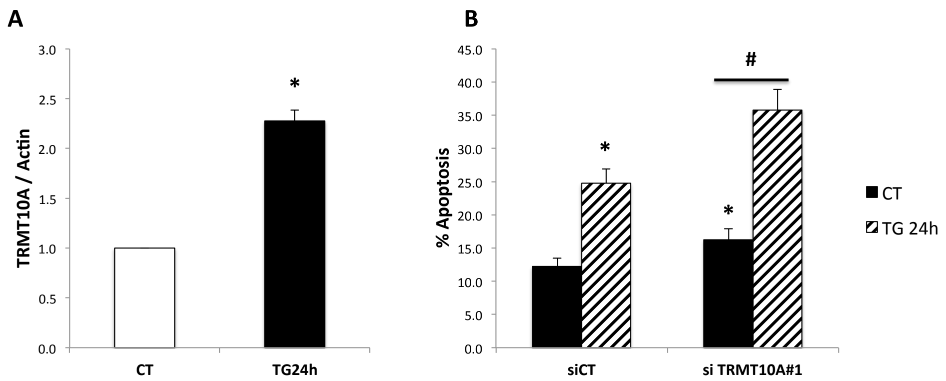


Figure 23: A) TRMT10A mRNA expression in control condition and after 24 hours thapsigargin treatment in EndoC-βH1 cells. Data are normalized to the expression of reference gene β-actin; **B)** Percentage of apoptosis in EndoC-βH1 cells transfected with control siRNA (siCT) or targeting TRMT10A (siTRMT10A #1) in control condition and after 24 hours thapsigargin treatment. Data are expressed as mean ± SEM (n=5-9). *,# p<0,05

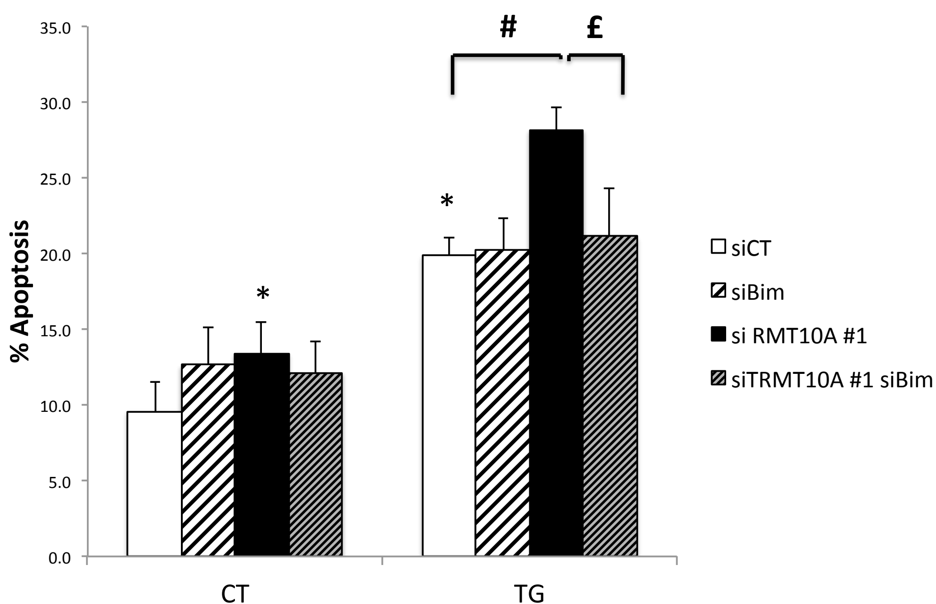


Figure 24: Percentage of apoptosis in EndoC-βH1 cells after transfection with control siRNA (siCT), siRNA targeting TRMT10A (siTRMT10A #1) alone or in combination with siRNA targeting Bim (siBim) in cells treated for 24 hours with palmitate (TG). Data are expressed as mean ± SEM (n= 4-8). * p<0,05 vs siCT(CT), # p<0.05 vs siCT(TG), £ p<0,05 vs siTRMT10A(TG)

5 DISCUSSION

The present work describes the study of tRNA modifying enzymes in pancreatic beta cells. tRNA molecules have a crucial role in protein translation and several reports have recently shown that tRNAs can act as signal molecules in response to environmental stresses. The maturation, the stability and the function of tRNAs are highly regulated by post-transcriptional modifications of nucleotide residues and methylation is the most common modification. Mutations in genes encoding tRNA modifying enzymes have been associated to different diseases. Even if the modification of tRNA residues is a ubiquitous phenomenon, mutations in tRNA modifying enzymes can affect specific tissues or cell types, depending on differential expression levels or on specific feature and functions of cells. The main objectives that have been driving my PhD research project are:

- The identification of a new candidate gene for congenital hyperinsulinism through the characterization of the S561F variant of the methyl-thio transferase CDKAL1;
- The study of the role of the methyl transferase TRMT10A in human cells and the investigation of consequences of TRMT10A deficiency on pancreatic beta cells survival.

The Cdk5 regulatory associated protein 1 (CDKAL1) was previously shown to have a central role in regulation of beta cell function. The enzyme catalyzes the methyl-thiolation of N6-threonylcarbamoyladenosine (t(6)A), leading to the formation of 2-methylthio-N6-threonylcarbamoyladenosine (ms(2)t(6)A) at position 37 in tRNAs. tRNAs specific for lysine are modified by CDKAL1 and this modification is required to ensure fidelity in the incorporation of lysine residues in nascent polypeptides during protein translation. Polymorphisms in CDKAL1 gene are associated with the

development of type 2 diabetes; functional studies using an islets-specific knock out mouse model determined the key role of CDKAL1 in beta cell function. The enzyme resulted to be important for the correct maturation of pro-insulin, regulating the fidelity of lysine residues incorporation in cleavage sites of the protein. CDKAL1 knockout mice show impaired insulin secretion and develop diabetes. The S561F variant of CDKAL1 was identified in a whole exome sequencing (WES) study of congenital hyperinsulinism patients. Congenital hyperinsulinism is a rare disease, caused by mutation in genes involved in different steps of glucose stimulated insulin secretion process. The genetic causes have been defined in only the 50% of patients and WES studies are useful to identify novel causative genes. I managed the gene list derived from WES using a candidate gene prioritization tool, identifying CDKAL1 as one of the most promising genes, significantly enriched in different functional categories. The possible consequences of the variant were investigated using bioinformatics tools and databases. The non-conservative aminoacid change affects the C-terminus of the protein and the residue 561 is part of the hydrophobic domain that forms the trans-membrane α-helix inserted in the endoplasmic reticulum membrane. Using the HeliQuest tool I performed an *in silico* prediction of the organisation and the characteristics of the *wild type* and mutated α-helix, finding that the substitution of a serine residue with a phenylalanine leads to an increase of hydrophobicity and decrease in the hydrophobic moment. The hydrophobic moment is important to predict the amphiphilicity of the α-helix that defines the spatial distribution and the orientation of the hydrophobic and hydrophilic residues of the domain. The conclusions derived from bioinformatics studies are that the mutation identified in CDKAL1 could participate in the development of inappropriate insulin secretion phenotype because of the functional role of CDKAL1 in beta cells; furthermore the S561F variant modifies key features

of the trans-membrane domain, suggesting possible consequences on the localization and retention of the protein in the ER-membrane. The beta cell line INS-1E was used as model for *in vitro* studies of CDKAL1 variant. Cells were stably transfected with expression vectors encoding the *wild type* or the S561F variant of CDKAL1 fused with a hemoagglutinin (HA) tag. Results derived from immunofluorescence microscopy showed that the S561F CDKAL1 variant still localizes in the ER but presents an abnormal accumulation in spots of the ER membrane. It's still unclear if the abnormal localization pattern could have a role on the modulation of protein stability and turnover. To address this question ongoing experiments aim to investigate the stability of the protein in the ER membrane. INS-1E cells will be transfected transiently and collected at different time points. The expression of the protein will be evaluated in total protein lysates and/or in isolated ER fractions. INS-1E clones were also used to investigate whether CDKAL1 variant could affect beta cell function. Interestingly, insulin content is increased by the over-expression of *wild type* but not of mutated CDKAL1. On the other hand the insulin secretion results to be increased by the overexpression of mutated CDKAL1 in basal conditions. Further analysis are necessary to confirm if the mutation can leads to beta cell dysfunction and which is the mechanism involved. To pursue this objective experiments will be performed to evaluate the insulin secretion in a range of glucose concentrations in *wild type* and mutated INS-1E clones. The incorporation of lysine residues in pro-insulin has been shown to be necessary for the correct maturation of the protein, furthermore CDKAL1 was found to be important for the expression of precursors of secretory granules proteins, pro-CGA and pro-ICA512[56]. The modulation of granules proteins expression and maturation can be the mechanism involved in CDKAL1-dependent modulation of insulin secretion. Further studies will investigate the direct consequence of S561F CDKAL1 variant on granules

proteins. Lysine incorporation in immunoprecipitated pro-insulin and granule proteins will be evaluated in cells overexpressing the *wild type* and the mutated form of CDKAL1.

Preliminary results presented in this work suggest that the S561F variant of CDKAL1 could have a role in the development of beta cell dysfunction, but further confirmations are needed to associate this variant with the development of congenital hyperinsulinism.

A mutation in the gene encoding the methyl-transferase TRMT10A was identified in patients affected from a new syndrome of microcephaly and young onset diabetes. Three patients derived from a consanguineous family showed to have the same homozygous non-sense mutation Arg127stop in TRMT10A gene that leads to the complete absence of mature protein and mRNA non-sense mediated decay[57]. In addition a homozygous missense mutation Gly206Arg in TRMT10A was identified in siblings affected by abnormalities in glucose homeostasis and microcephaly[58]. Another recent report described one patient affected by a syndrome of intellectual disability, delayed puberty and diabetes characterized by the deletion of TRMT10A gene[59]. All these evidences support the hypothesis that TRMT10A function is necessary for beta cell homeostasis. However the function of TRMT10A in human cells is still not clarified. The yeast homolog of the protein is a methyl-transferase that modifies tRNAs molecules at the level of G9 residues. Lymphoblast cells derived from control subjects, one heterozygous carrier and one patient with Arg127stop mutation of TRMT10A were used to investigate the function of the protein in human. Lymphoblasts are a good experimental model since they are easy to obtain and maintain in culture and they have the same genetic background of the donors. Mass spectrometry analysis of samples derived from lymphoblasts showed that there was a decrease of tRNA methylation in TRMT10A deficient patient. However the technique

doesn't allow us to identify which tRNA molecules are missing the modification. tRNA purified from lymphoblasts samples was used for the primer extension assays. Between the tRNA molecules that present a guanine residue in position 9, only the tRNAs specific for glutamine ($tRNA^{GLN}$) and for the initiator methionine ($tRNA^{iMeth}$) resulted to not methylated in the patient. This result is the first evidence that human TRMT10A catalyzes the methylation of specific tRNAs *in vivo* in guanine 9 of tRNAs. It's known that nucleotide modifications can affect the function, the stability and the aminoacylation of tRNAs. A northern blot technique was set up in order to detect specific tRNA molecules through the hybridization with radiolabeled probes. The extraction of total RNA from lymphoblasts in acid conditions allows preserving the aminoacylation of the molecules. Preliminary northern blot results showed a slight decrease of the amount of $tRNA^{iMeth}$ and $tRNA^{GLN}$, substrates of TRTM10A, in lymphoblast from one patient. This observation needs to be confirmed analyzing a second patient and more controls. Suitable positive controls are also necessary to verify if the technique allows the identification of partially deminoacylated tRNAs. Previous studies in yeast reported that the absence of non-essential modifications of tRNAs affect the maintenance of tRNA aminoacylation after heat-induced stress that triggers the unfolded protein response[67]. Furthermore TRMT10A deficiency induces a pathologic phenotype that involves mainly pancreatic beta cell dysfunction and neurological abnormalities, suggesting tissue and cell specific consequences of the absence of the enzyme. For this reason it will be necessary to repeat the experiment with samples derived from TMRT10A knock out beta cells in control conditions and treated with different stressors, such as free fatty acid or chemical ER stressors. In addition, improving the northern blot technique may allow the detection of small fragments of tRNAs (tiRNAs). The amount of tiRNAs in cells is modulated

by environmental stress and this mechanism can regulate cell survival[44]. Furthermore tiRNAs can act as microRNA regulating gene expression[46].

Previous *in vitro* studies have demonstrated that TRMT10A deficiency doesn't affect insulin secretion from beta cells, but induces apoptosis and sensitizes cells free fatty acid and chemical ER stressors[57]. The hypothesis derived from these observations is that TRMT10A deficiency can lead to the development of diabetes by affecting the survival of beta cells. To understand which mechanism is involved in TRMT10A deficiency I used TRMT10A knockdown-beta cells as experimental model. TRMT10A silencing-induced apoptosis was confirmed in INS-1E cells using three different siRNAs. Importantly all the results obtained in INS-1E cells were confirmed in the human beta cell model EndoC-βH1 cell line. It was previously shown that activation of the intrinsic pathway of apoptosis leads to beta cells loos during the development of diabetes[68]. My results show that TRMT10A deficiency leads to the increase of Caspase 9 cleavage, a marker of the intrinsic pathway of apoptosis, in rat and human beta cells. The pro-apoptotic BH3-only family members are mediators of the intrinsic pathway of apoptosis activating Bax and Bak effectors that form pores in the mitochondrial outer membrane with consequent cytochrome *c* release and caspases activation. Different pathways, in response to apoptotic stimuli, can regulate the function of BH3-only proteins. I evaluated the expression of the BH3-only members Bim, Bad, DP5 and Puma at mRNA level in INS-1E cells, finding that the expression of the sensitizer Bim and the activator Bad was induced by TRMT10A silencing. However only Bim resulted to be modulated at protein level, with an increase of expression, especially of the most pro-apoptotic isoform BimS, produced by alternative splicing. These findings were confirmed in EndoC-βH1 cells. Furthermore Bim silencing protected cells from TRMT10A knockdown-induced apoptosis. It was previously shown that TRMT10A expression is induced in

INS-1E in response to palmitate and chemical ER stressors and that TRMT10A silencing sensitizes cells to palmitate-induced apoptosis. Palmitate is one of the most common long chain free fatty acid that represent the main environmental factor leading to beta cell apoptosis in type 2 diabetes through ER stress. Bim knockdown resulted to be protective also against TRMT10A knockdown-induced sensitization of INS-1E cells to palmitate. Since EndoC-βH1 cells are not sensitive to free fatty acid (*not published observation from our group*), these cells were treated with the SERCA pump inhibitor thapsigargin to induce ER stress response. My results showed that the expression of TRMT10A was induced by thapsigargin treatment in human beta cells. TRMT10A silencing sensitizes in EndoC-βH1 cells to apoptosis after thapsigargin treatment and the role of Bim in the process was confirmed.

My results demonstrated that TRMT10A deficiency affects beta cell homeostasis mainly through the activation of the intrinsic pathway of apoptosis. It's still unclear which is the mechanism that leads to the activation of Bim. Bim function is modulated though the regulation of gene expression and splicing. Three are the major isoforms and all of them induce apoptosis. However the shortest isoform (Bim S) is the most potent since it can directly bind the pro-apoptotic effector Bax and is not sequestered to the cytoskeleton as the bigger isoforms Bim EL and Bim L[69]. Bim gene transcription is regulated from different transcription factors: FoxO3a, E2F1, STAT1, c-Myc, NF-Y, Smad1/3, Runx1-3, c-Jun and RelA induce Bim while YY1, HoxB8, SPi-1/PU.1, PINCH-1 and Pokemon are inhibitors of Bim expression. Several proteins can also regulate the splicing. In beta cells the splicing factor SRp55 leads to increased expression of BimS and apoptosis. Moreover several microRNAs have been shown to modulate Bim expression. Another major regulatory mechanism of Bim-dependent apoptosis is driven by phosphorylation.

Phosphorylation may either increase its activity (e.g., by JNK), or promote its degradation (e.g., by ERK/MAPK) resulting in pro-survival effect. Due to the complexity of this process further studies are necessary to understand which mechanism regulates Bim function and apoptosis as a consequence of TRMT10A deficiency. Interestingly recent observations showed that tiRNAs can interact directely with cytochrome *c* modulating the intrinsic pathway of apoptosis as a consequence of environmental stress[70]. Hypomethylation also may lead to tRNAs fragmentation triggering cellular stress responses[71]. Based on that future studies will investigate the role of TRMT10A-mediated modification in tRNA cleavage.

During the last years several reports highlighted the crucial role of tRNA modifications in modulation of cellular homeostasis and response to environmental factors. The results achieved and described in the present work supply new evidences of the role of tRNA modifying enzymes in beta cell function and survival even if further studies are necessary to fully understand the molecular mechanisms that are involved. In summary the study of monogenic diseases represents a good model for the identification of novel pathways involved in beta cell demise.

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Research products relative to this project:

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