Cdkal1, a type 2 diabetes susceptibility gene, regulates mitochondrial function in adipose tissue

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Colin J. Palmer [1], Raphael J. Bruckner [2], Joao A. Paulo [2], Lawrence Kazak [2,3], Jonathan Z. Long [2,3], Amir I. Mina [1], Zhaoming Deng [1], Katherine B. LeClair [1], Jessica A. Hall [1], Shangyu Hong [1], Peter-James H. Zushin [1], Kyle L. Smith [4], Steven P. Gygi [2], Susan Hagen [4], David E. Cohen [5], Alexander S. Banks [1,*]

ABSTRACT

Objectives: Understanding how loci identified by genome wide association studies (GWAS) contribute to pathogenesis requires new mechanistic insights. Variants within *CDKAL1* are strongly linked to an increased risk of developing type 2 diabetes and obesity. Investigations in mouse models have focused on the function of *Cdkal1* as a tRNA^{Lys} modifier and downstream effects of *Cdkal1* loss on pro-insulin translational fidelity in pancreatic β−cells. However, *Cdkal1* is broadly expressed in other metabolically relevant tissues, including adipose tissue. In addition, the *Cdkal1* homolog *Cdk5rap1* regulates mitochondrial protein translation and mitochondrial function in skeletal muscle. We tested whether adipocyte-specific *Cdkal1* deletion alters systemic glucose homeostasis or adipose mitochondrial function independently of its effects on pro-insulin translation and insulin secretion.
Methods: We measured mRNA levels of type 2 diabetes GWAS genes, including *Cdkal1*, in adipose tissue from lean and obese mice. We then established a mouse model with adipocyte-specific *Cdkal1* deletion. We examined the effects of adipose *Cdkal1* deletion using indirect calorimetry on mice during a cold temperature challenge, as well as by measuring cellular and mitochondrial respiration *in vitro*. We also examined brown adipose tissue (BAT) mitochondrial morphology by electron microscopy. Utilizing co-immunoprecipitation followed by mass spectrometry, we performed interaction mapping to identify new CDKAL1 binding partners. Furthermore, we tested whether *Cdkal1* loss in adipose tissue affects total protein levels or accurate Lys incorporation by tRNA^{Lys} using quantitative mass spectrometry.
Results: We found that *Cdkal1* mRNA levels are reduced in adipose tissue of obese mice. Using adipose-specific *Cdkal1* KO mice (A-KO), we demonstrated that mitochondrial function is impaired in primary differentiated brown adipocytes and in isolated mitochondria from A-KO brown adipose tissue. A-KO mice displayed decreased energy expenditure during 4 °C cold challenge. Furthermore, mitochondrial morphology was highly abnormal in A-KO BAT. Surprisingly, we found that lysine codon representation was unchanged in *Cdkal1* A-KO adipose tissue. We identified novel protein interactors of CDKAL1, including SLC25A4/ANT1, an inner mitochondrial membrane ADP/ATP translocator. ANT proteins can account for the UCP1-independent basal proton leak in BAT mitochondria. *Cdkal1* A-KO mice had increased ANT1 protein levels in their white adipose tissue.
Conclusions: Cdkal1 is necessary for normal mitochondrial morphology and function in adipose tissue. These results suggest that the type 2 diabetes susceptibility gene *CDKAL1* has novel functions in regulating mitochondrial activity.

1. INTRODUCTION

Translating results from genome wide association studies (GWAS) into understanding of disease pathogenesis is often hindered by three critical bottlenecks: identifying the genes affected by noncoding variants, ascertaining the tissues affected, and characterizing the molecular function of poorly characterized genes. The *CDKAL1* locus, implicated in type 2 diabetes, is a perfect example of these issues. Polymorphic variants within the *CDKAL1* locus are strongly associated with increased risk of developing type 2 diabetes by GWAS and dozens of replication studies in diverse populations [1—7]. However, the mechanism linking non-coding variants within *CDKAL1* with diabetes

[1]Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital, Boston, MA 02115, USA [2]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA [3]Dana-Farber Cancer Institute, Boston, MA 02115, USA [4]Department of Surgery, Beth Israel Deaconess Medical Center, Boston, MA 02115, USA [5]Division of Gastroenterology & Hepatology, Weill Cornell Medical College, New York, NY 10065, USA

*Corresponding author. Brigham and Women's Hospital, 77 Avenue Louis Pasteur, HIM 643, Boston, MA 02115, USA. E-mail: abanks@bwh.harvard.edu (A.S. Banks).

susceptibility remains unclear. Because the disease-associated single nucleotide polymorphisms (SNP) fall within an intronic region of the 700 kb *CDKAL1* locus, in a region sparsely populated by genes, most studies have assumed these SNPs cause changes in *CDKAL1* gene expression. In non-diabetic subjects, *CDKAL1* disease-associated SNPs correlate with impaired insulin secretion, suggesting that β-cells in pancreatic islets may be disproportionately affected [8,9]. Impaired insulin secretion was similarly observed in *CDKAL1*$^{-/-}$ human ESC differentiated into β cells [10]. Consequently, prior studies on Cdkal1 have focused on *Cdkal1* loss in pancreatic islets and its effects on insulin secretion, although defects in liver, muscle, or adipose tissue, among other tissues, may also impinge on β-cell function [11,12].

Phenotypes in *Cdkal1* deficient mice are attributed to Cdkal1 function in regulating a specific modification of the cytoplasmic tRNA$^{Lys(UUU)}$. Chemical modifications of tRNAs are an ancient, evolutionarily conserved mechanism to maintain the accuracy of codon recognition [13]. CDKAL1, along with its homolog CDK5RAP1, shares protein domain architecture and 18–23% amino acid identity with the bacterial methyl-thiol transferase (MTT) proteins MiaB, MtaB, and RimO. MTT enzymes utilize two [4Fe-4S] cluster cofactors, bound to an N-terminal MTT domain and a central radical S-adenosyl methionine (SAM) domain, to add a methylthiol moiety (-SCH$_3$) to a non-activated carbon on their substrates [14]. Their C-terminal TRAM domains confer substrate specificity. In *Escherichia coli*, RimO is also capable of transferring a methyl-thiol group onto ribosomal protein S12, a post-translational modification not found in eukaryotes [15]. Prokaryotic MtaB and MiaB complete the hyper-modification of tRNA adjacent to the anticodon, a reaction thought to enhance base pairing between RNA codons and associated tRNAs [14,16]. MtaB performs the final modification of threonylcarbamoyl-modified adenosine (t^{6}A$_{37}$) to 2-methylthio-N^6-threonylcarbamoyl adenosine (ms^{2}t^{6}A$_{37}$), affecting anticodon pairing with ANN codons [17]. MTT proteins are extensively implicated in affecting protein translation at the ribosome [14]. Cdkal1 in mammals is annotated as a t^{6}A$_{37}$ MTT, and decreased levels of the associated ms^{2}t^{6}A$_{37}$ modification are found in *Cdkal1* knockout mice [18]. Dysfunctional translation of critical Lys residues in pro-insulin and ER stress are reported to cause the impaired insulin secretion from β-cells observed in mice lacking *Cdkal1* [18].

Mice with whole-body *Cdkal1* deletion have been previously described and were observed to be developmentally and phenotypically normal on a standard diet, a finding which is seemingly at odds with *Cdkal1* as a generalized guardian of Lys translational fidelity [19]. These mice exhibit mild impairment of glucose tolerance when challenged with 20 weeks on a high fat diet (HFD) [19,20]. Mice with pancreatic β-cell specific *Cdkal1* knockout have also been previously characterized. Those mice displayed a more pronounced phenotype, with strongly impaired glucose tolerance on both normal chow and HFD, a finding attributed to defects in pro-insulin translation [18].

Here we investigate the biological role of Cdkal1 in adipose tissue *in vivo* using a mouse model with adipocyte-specific knockout (A-KO) of *Cdkal1*. Mice lacking adipose *Cdkal1* exhibit features of impaired brown adipose tissue (BAT) mitochondrial function including decreased energy expenditure when challenged with cold temperature. Primary brown adipocytes differentiated *ex vivo* from digested *Cdkal1* knockout BAT had decreased rates of respiration. In addition, electron micrographs of *Cdkal1* A-KO BAT revealed highly disrupted mitochondrial morphology, and respiration experiments in isolated A-KO BAT mitochondria confirmed functional defects. Interestingly, we did not observe differences in Lys codon utilization by mass spectrometry in adipose tissue, as would be predicted based on its function as tRNA

modifier, nor were differences in glucose homeostasis apparent in *Cdkal1* A-KO mice. To identify potential novel functions for CDKAL1 independent of tRNA^{Lys} modification, we performed unbiased protein interaction mapping to find new CDKAL1 binding partners. Through these studies, we identified the interaction between CDKAL1 and ANT1/Slc25a4, the mitochondrial adenine nucleotide translocator protein, which may provide a mechanistic link between CDKAL1 and mitochondrial dysfunction. Taken together, these findings in adipose tissue suggest that the type 2 diabetes GWAS candidate gene *Cdkal1* has a functional role in regulating mitochondrial function *in vivo*.

2. MATERIALS & METHODS

2.1. Animals
Mice with a conditional allele of *Cdkal1* (*Cdkal1*^{flox}) were generated by crossing the "null-first" mice Cdkal1$^{tm2a(EUCOMM)Wtsi}$ (Emma European Mouse Mutant Archive) to mice expressing enhanced FLP1 recombinase (Jackson Labs, 005703) [21]. *Cdkal1*^{flox} mice were bred with adipocyte-specific Adipoq-Cre$^{1Evdr/J}$ [22] (Jackson Labs, 010803) to generate mice with adipocyte-specific deletion of *Cdkal1* (A-KO) (Supplementary Figure 2A). Routine genotyping was performed with the following three primers, 1608 (sense):5′-CTTCTTGTGACTCCTTGGTTA-3′, 1609 (antisense): 5′-CAACGGGTTCTTCTGT-TAGTCC-3′, 1610 (antisense):5′-GCTGTCCAGCCATGTATTCTC-3′. The wild-type 1608–1610 amplicon is 670 bp. The 1608–1609 amplicon from the floxed allele is 750 bp. Unless stated otherwise, 8–14 week old male mice were examined against littermates while maintained on a standard chow diet (PicoLab Rodent Diet 20, #5053). Control mice are *Cdkal1*$^{flox/flox}$ genotype, while A-KO mice are Adipoq-Cre$^+$::*Cdkal1*$^{flox/flox}$. For chow vs high-fat diet studies in wild type mice, as well as Cdkal1 mRNA tissue distribution, C57BL/6J mice were purchased from Jackson Laboratories (Jackson Labs, 000664). High fat diet with 60% of calories from fat was obtained from Research Diets (Research Diets, #D12492, irradiated). All animal studies were approved by the Brigham and Women's Hospital IACUC.

2.2. Indirect calorimetry
Control and A-KO male mice were implanted with temperature probes 5–7 days before the beginning of indirect calorimetry experiments. Mice were maintained on a standard chow diet and housed at thermoneutrality (30 °C) for 48–72 h prior to experiment start. The temperature transition from 30 °C to 4 °C was performed over a period of 3 h. Oxygen consumption, CO$_2$ emission, food consumption, movement, and energy expenditure were measured using a 24 cage CLAMS apparatus (Columbus Instruments) available to the Brigham and Women's Hospital Metabolic Phenotyping Core. Mice were implanted with intraperitoneal telemetry temperature probes one week prior to the start of measurement. Statistical analysis and plotting was performed in the R programming language with CalR, a custom package for analysis of indirect calorimetry using analysis of covariance with a graphical user interface.

2.3. Glucose tolerance tests and insulin tolerance tests
Diet-induced obesity (DIO) in *Cdkal1* A-KO mice, along with littermate controls, was generated by feeding mice a high fat, high sugar diet (HFD) which delivers 60% of its caloric content as fat (Research Diets, D12492). Mice were established on HFD feeding after weaning, at 3–5 weeks of age. Body mass was monitored weekly. Body composition of DIO mice was measured by EchoMRI at 10–11 weeks of HFD feeding. Intraperitoneal glucose tolerance test (IP-GTT) on HFD-fed mice was conducted after 11–13 weeks on HFD feeding. Mice were given one