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Epigenetic changes related to glucose metabolism in type 1 diabetes after BCG vaccinations: A vital role for *KDM2B*

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ABSTRACT

Background: A recent epigenome-wide association study of genes associated with type 2 diabetics (T2D), used integrative cross-omics analysis to identify 22 abnormally methylated CpG sites associated with insulin and glucose metabolism. Here, in this epigenetic analysis we preliminarily determine whether the same CpG sites identified in T2D also apply to type 1 diabetes (T1D). We then determine whether BCG vaccination could correct the abnormal methylation patterns, considering that the two diseases share metabolic derangements.

Methods: T1D (n = 13) and control (n = 8) subjects were studied at baseline and then T1D subjects studied yearly for 3 years after receiving BCG vaccinations in a clinical trial. In this biomarker analysis, methylation patterns were evaluated on CD4+ T-lymphocytes from baseline and yearly blood samples using the human Illumina Methylation EPIC Bead Chip. Methylation analysis combined with mRNA analysis using RNAseq.

Results: Broad but not complete overlap was observed between T1D and T2D in CpG sites with abnormal methylation. And in the three-year observation period after BCG vaccinations, the majority of the abnormal methylation sites were corrected in vivo. Genes of particular interest were related to oxidative phosphorylation (*CPT1A, LETM1, ABCG1*), to the histone lysine demethylase gene (*KDM2B*), and mTOR signaling through the *DDIT4* gene. The highlighted CpG sites for both *KDM2B* and *DDIT4* genes were hypomethylated at baseline compared to controls; BCG vaccination corrected the defect by hypermethylation.

Conclusions: Glycolysis is regulated by methylation of genes. This study unexpectedly identified both *KDM2B* and *DDIT4* as genes controlling BCG-driven re-methylation of histones, and the activation of the mTOR pathway for facilitated glucose transport respectively. The BCG effect at the gene level was confirmed by reciprocal mRNA changes. The *DDIT4* gene with known inhibitory role of mTOR was re-methylated after BCG, a step likely to allow improved glucose transport. BCGs driven methylation of *KDM2B*'s site should halt augmented histone activity, a step known to allow cytokine activation and increased glycolysis.

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1. Introduction

First used in 1921, the Bacillus Calmette-Guerin (BCG) vaccine is primarily used prophylactically for tuberculosis. It is a live attenuated strain of *Mycobacterium bovis* and is one of the safest vaccines in production, with over 4 billion people vaccinated globally and 100 million newborn children vaccinated each year [1,2]. Since the 1970s, the vaccine was proven to be efficacious for intravesical treatment of bladder cancer [3–5], and more recently, has shown surprising off-target human benefits, including protection against diverse infections, protection from allergies, and significant protection against autoimmune diseases such as multiple sclerosis and type 1 diabetes (T1D) [6–23].

Abbreviations: BCG, Bacillus Calmette-Guérin; EWAS, Epigenome-wide association study; *DDIT4*, DNA-damage-inducible transcript 4 protein; *KDM2B*, Lysine demethylase 2B; T1D, Type 1 Diabetes; T2D, Type 2 Diabetes; CpG, Regions of DNA where a cytosine nucleotide is immediately followed by a guanine nucleotide on the same strand in a 5′ to 3′ direction; OXPHOS, Oxidative Phosphorylation; FAO, Fatty Acid Oxidation.

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The mechanisms behind these off-target or heterologous immune benefits appear to be diverse and may partly be derived from the ability of this microorganism to reset host immune and metabolic functions, even at the DNA level. This concept of modulated methylation of host DNA in monocytes by microorganisms originated in plants but also applies to humans in the concept referred to as "trained immunity" a refinement of innate immunity [24–28]. The epigenetic DNA changes in human host DNA have likely evolved to result in long term commensalism by select and often ancient organisms including their close relative tuberculosis i.e. *Mycobacterium tuberculosis*. BCG vaccination or tuberculosis infection, also result in changes to adaptive immunity and this can be measured by the upregulation of host Regulatory T Cells (Treg) genes to prevent host immune detection [9,12]. Additionally, infected host myeloid cells are modified into greater utilization of aerobic glycolysis for preferential sugar metabolism through epigenetic changes. A step believed by some to relate to BCG replication and therapeutic effect on blood sugars in T1D [29,30]. In human clinical trials with adult vaccinations of BCG with underlying T1D, this gradually reestablishes commensalism (over a number of years) and results in predictable epigenetic changes in DNA obtained from various subpopulations of lymphocytes [12,30]. This concept goes hand-in-hand with the hygiene hypothesis. It is only in the modern era that the sustained exposures to these ancient microbes disappeared, such as in the soil or water [31]. Recent human data shows a reset of T cells and monocytes after BCG vaccinations, thus an influence of BCG on innate and adaptive immunity. Furthermore, BCG vaccinations may exert an affect at the level of the hematopoietic progenitor stem cell compartment [32], suggesting a mechanism for the long term and durable effects from BCG.

It is now well-established that human disease can be driven by altered methylation patterns, often triggered by environmental exposures affecting gene expression. Although T1D and type 2 diabetes (T2D) are different diseases, both are modulated by the environment affecting disease incidence and both have glucose and insulin dysregulation and a strong role for epigenetics in the control of disease expression [33–35]. These environment exposures can be in the context of changed methylation patterns. For T2D, the environmental influences are often contributing to worsening disease, such as smoking and obesity [36]. In T1D, the environmental influences are often described as preventative, the basis for the Hygiene Hypothesis and protection after certain beneficial microbiologic exposures [34,35]. Abundant T2D data from cross-omics and pure DNA analysis approaches have identified altered methylation sites of glucose and insulin homeostasis by these massive EWAS studies and this has allowed strong confirmation of the identified methylation errors with very large sample numbers [33]. In T1D reproducibility of epigenetic differences between control and T1D DNA sample have been less confirmed but a study has identified a CpG site in DDIT4 as a potential candidate by comparing DNA samples from discordant identical twins [37].

A recent publication identified a list of 22 differentially methylated CpGs that were associated with early phases of glucose intolerance in an Epigenome-Wide Association Study (EWAS) of 4808 non-diabetic individuals [38]. These CpGs were associated with either fasting glucose, fasting insulin, or both, and 13 of the 22 had been found in previous EWAS. Out of the novel associations, CpGs in LETM1, RBM20, IRS2, MAN2A2, and the 1q25.3 region were associated with fasting insulin, and CpGs in FCRL6, SLAMF1, APOBEC3H and the 15q26.1 region were associated with fasting glucose [38]. Of those previous associations with Type 2 Diabetic (T2D) traits, CpGs in SLC7A11, CPT1A and SREBF1 had been associated with both fasting glucose and insulin, and the CpGs in DHCR24, CPT1A, RNF145, ASAM, KDM2B, MYO5C, TMEM49, two in ABCG1, and the 4p15.33 region were associated with fasting insulin only.

Here we study methylation alterations in gene regions associated with T1D DNA samples compared to control samples. We target the above mentioned CpG associated regions and genes that are differentially methylated and impact fasting insulin, fasting glucose, and glucose metabolism. Next, we study those same insulin, glucose associated CpG regions and genes after multi-dose BCG vaccine therapy in a 3-year clinical trial with adults with long-standing T1D. We also study the downstream effects on mRNA expression. These studies especially ask whether BCG therapy can correct abnormal methylation patterns associated with insulin secretion, fasting glucose and glucose metabolism. In the course of answering these questions, we uncovered an upstream gene that controls BCG-driven histone methylation and demethylation. This likely results in increased levels of histone 3 lysine 4 trimethylation (H3K4me3). We also confirm an undermethylated CpG site in T1D that is related to overlapping mTOR signaling defects in both T1D and T2D and is re-methylated by BCG in vivo therapy.

2. Materials and methods

2.1. Research subjects and human consent

All human studies had full institutional approvals through Massachusetts General Hospital and Partners Health Care, and composed of open and closed label subjects consented through various protocols (Study# 2007P001347, 2012P002243 and 2013P002633). The BCG interventional studies were also formally approved by the FDA (IND#2007P001347 and IND#2013P16434). All blood donors, both T1D and non-diabetic control (NDC) subjects consented through Study #2001P001379. Informed consent was obtained from all subjects and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. This study included 13 T1D subjects, where the timing of BCG vaccinations and serial blood sampling times for visits were baseline (pre-BCG vaccine), year 01, year 02 and year 03. All patients were given two initial intradermal BCG vaccinations, the first at baseline and a second vaccine four weeks later. Non-diabetic control subjects (n = 8) were also studied and compare against T1D patients. The T1D have a mean age of 46 years, average age of disease onset of 25 years, and an average of 20 years of disease duration. All individuals were derived from a random sample of subjects and are unrelated to each other.

2.2. Isolation of lymphocytes from whole blood

Lymphocytes of CD4 subset were isolated from blood that was collected in purple tops (K2-EDTA anticoagulant) using magnetic Easysep Direct Human CD4+ T Cell Isolation Kits from Stem Cell Technologies (Vancouver, BC, Canada), following the instructions of the manufacturer. Briefly, 1200 μL of Isolation Cocktail and 1200 μL of Rapid Spheres were mixed with 24 mL of whole blood in a 50 mL centrifuge tube and incubated for 5 min at room temperature. Twenty-four milliliters of Ca- and Mg-free PBS was then added, and the tube placed into an "Easy 50" magnet (Stem Cell Technologies). This immobilized the unwanted cells at the side of the tube. After 10 min, the CD4 enriched cell suspension was transferred into a new tube and the magnetic separation process repeated for 5 min with fresh Rapid Spheres. The resulting highly CD4 enriched cell suspension was transferred into a new tube and purified for a third time using the magnet. The resulting final CD4 T cell preparations had purities of >95%.

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2.3. Methylation analysis

Genomic DNA (gDNA) from lymphocytes of the CD4 T cell subset was isolated in the laboratory, and methylation patterns were analyzed as described by the Partners Personalized Medicine Translational Genomics Core using the Human Infinium Methylation EPIC Bead Chip Kit (Illumina, San Diego, CA). This chip targets over 850,000 known and potential human DNA methylation sites. DNA methylation levels for particular CpGs (Illumina CpG Database Identifier), were expressed as beta values (β = intensity of the methylated allele (M) / (intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100)). A beta value of 0 defined the CpG to be fully demethylated, and a value of 1 being fully methylated. The data for the relevant genes and the beta values for the CpGs were extracted using fgrep through the Unix command line. Data processing, heatmap creation, and statistical analyses was carried out on Microsoft Excel Version 16.38 (20061401) for Mac, Graph pad Prism Version 8.4.3 (471), the R program and the G* Power 3.1 software.

2.4. mRNA isolations and analysis

Total RNA was extracted from isolated CD4 T cells using the RNeasy Plus Mini Kit of Qiagen (Qiagen Sciences, MD, Cat #74104) and the RNA quality determined using an Agilent 2100 Bioanalyzer. RNAseq was performed at the Center for Cancer Computational Biology (CCCB) of the Dana-Farber Cancer Institute (DFCI) as well as at the BioMicro Center (BMC) of the Massachusetts Institute of Technology. mRNA was isolated from total RNA using NEBNext® Poly(A) mRNA Magnetic Isolation Modules. For library preparation, the CCCB used NEBNext® Ultra™ Directional RNA Library Prep Kits for Illumina®, whereas the BMC used NEBNext® Ultra II™ Kits instead. Sequencing at the CCCB was performed on the Illumina NextSeq 500, whereas the BMC used a NovaSeq 6000 with S4 flowcell as well as a NextSeq 500. Reads were aligned to the GRCh38.95 reference genome using STAR Aligner, primary aligned reads were filtered using Samtools, and subsequent quality control software included FastQC, RSeQC, and MultiQC. The reads were then normalized using DESeq2, and the data analyzed in Microsoft Excel Version 16.38 (20061401) for Mac, Graph pad Prism Version 8.4.3 (471) and the R program.

2.5. Statistical analyses

Normality testing for the CpGs was performed in Prism using Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests. The beta-values of individual CpGs from 13 T1Ds were averaged, a repeated measure one-way ANOVA, followed with a Holm-Šídák's multiple comparisons test was conducted to compare baseline values to subsequent years (year 1, 2 or 3) for parametric CpGs. Sphericity was not assumed, and the Geisser-Greenhouse correction was applied. For CpGs that were observed as non-parametric in at least three of the four normality tests for a given year, a Friedman's test with a Dunn's test for multiple comparison was instead performed when comparing baseline values to subsequent years (years 1, 2 or 3). When comparing T1D baseline to control methylation levels, a Mann-Whitney's *u* test was conducted. Whole gene summary figures for LETM1 and FCRL6 were created by averaging the mean beta values for CpGs across the genes for each year, baseline or control. For the mRNA data, a two tailed Student's *t*-test was conducted to compare baseline values to subsequent years (year 1, 2 or 3).

3. Results

3.1. Baseline evaluation of T1D CpG sites to control DNA methylation sites; relationship to fasting glucose and insulin metabolism

Some DNA methylation alterations in T2D lymphocytes, both hyper and hypo-methylation are associated with dysfunctional fasting insulin, fasting glucose and glucose homeostasis [38]. We first studied T1D DNA compared to control DNA for similar methylation alterations at these strategic CpG sites similarly using DNA samples derived from lymphocytes. Of the 22 T2D identified CpG or genes associated with altered glucose or insulin control, 20 CpGs were represented on our Illumina EPIC Bead Chips, and so were studied in T1D DNA samples compared to control samples. The methylation levels of 13 of the 20 T2D high-risk CpGs were altered in the same direction for T1D as for T2D, either with hypermethylation or with hypomethylation compared to control DNA (Fig. 1, circled values). In three cases these cgs were statistically significant (ABCG cg06500161, CPT1A cg00574958 and CPT1A cg17058475). The CpG within the SLAMF1 gene showed no difference between T1D and control, and was excluded from Fig. 1. The remaining six CpGs were in the opposite direction, of which the CpG for KDM2B cg13708645 was significant (Fig. 1). Of the four significant CpGs (p-value cut off of < 0.05) three met a false discovery rate q-value cut off of < 0.1 (CPT1A cg00574958 (0.002), CPT1A cg17058475 (0.077) and KDM2B cg13708645 (0.086). The results suggest that for 13 out of 20 studied CpG sites in T1D a possible similar directional methylation error was occurring at strategic DNA methylation sites as previously found in the T2D cross-omics study and associated with glucose and insulin control. Of the CpG sites that were not in the same direction in T1Ds, 5 out of 6 of them were associated with high fasting insulin levels, with SLC7A11 cg06690548 also being associated with high fasting glucose. This is an expected outcome due to T1Ds inability to produce insulin.

Table 1 provides a summary of the CpGs identified in T2D and their relevant gene functions (data pulled from GeneCard, [39]), including their association with fasting glucose or fasting insulin. Of particular interest is the observation that three CpGs within two genes (CPT1A and LETM1) were associated with mitochondrial processes. This is of interest since in T1D there is overactive OXPHOS in the mitochondrial organelles of lymphoid cells; cells that are deprived of balanced energy metabolism due to deficient aerobic glycolysis, a state of greater glucose utilization [29,30]. Typically, T2D studies analyze whole blood, this was also the case for the cross-omics paper. In comparison, we specifically used CD4 lymphocytes, which may have resulted in missed trends.

3.2. Clinical impact on altered CpG methylation sites in BCG vaccinated T1D subjects

The BCG vaccine, derived from *Mycobacterium bovis*, is an example of an environmental influence that changes the DNA methylation patterns. T1D with established and long-term disease received at least 2 BCG vaccinations and then were followed for 3 years at yearly intervals. DNA methylation patterns were tracked over the 3-year time course using the Infinium Methylation EPIC Bead Chip Kit. This Illumina methodology identified 20 of 22 CpG DNA sites associated with insulin and glucose homeostasis. The data shows CpG gene regions responding to BCG vaccines in T1D that over the observation period either hypermethylated (Fig. 2 top) or demethylated. (Fig. 2 bottom). Overall, 13 of 20 CpGs showed that BCG treatment could indeed induce significant methylation changes in T1D CD4 lymphocytes at gene sites of interest for insulin and glucose regulation (Fig. 2). All statistics were baseline value

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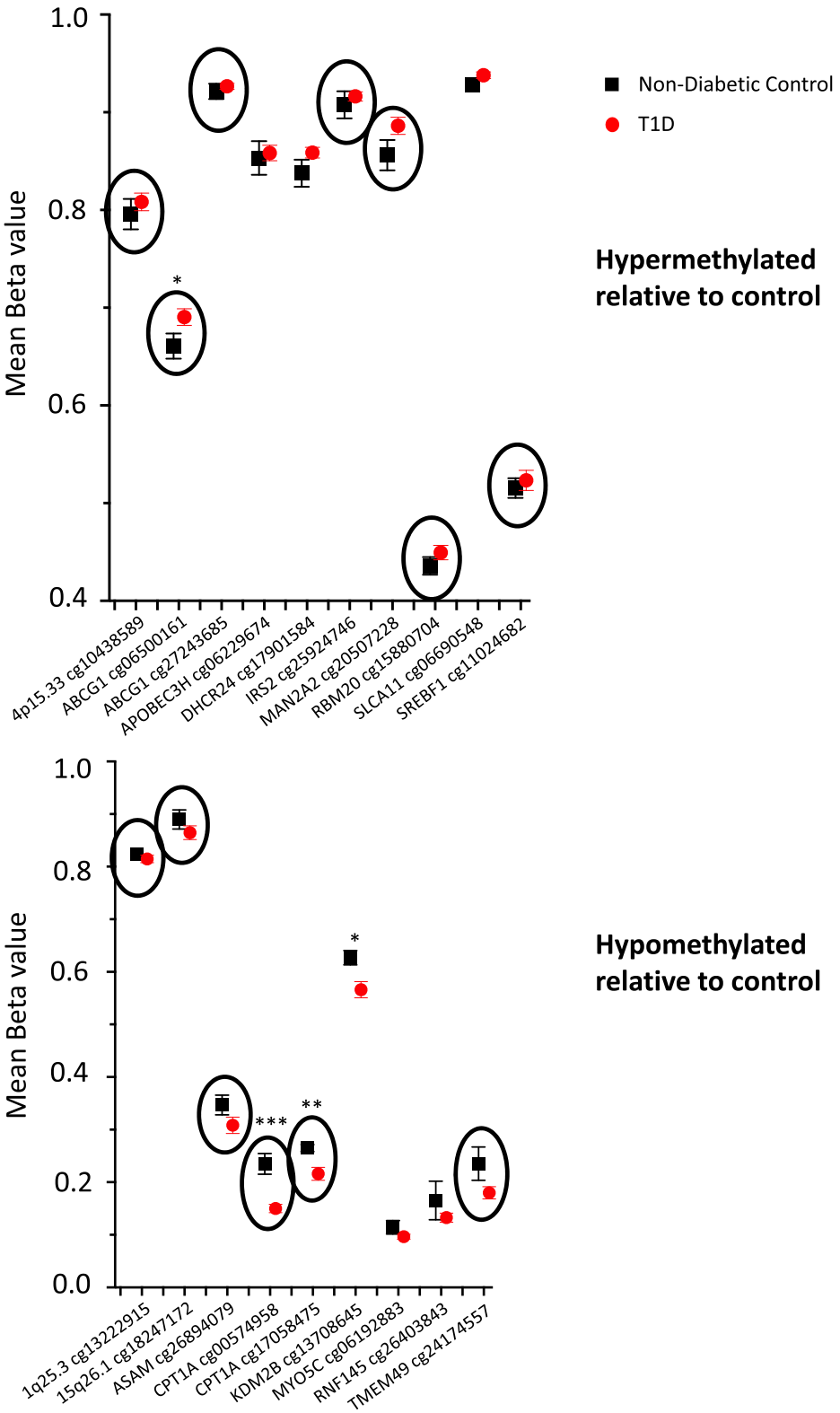

Fig. 1. T1D Baseline methylation changes in genes associated with T2D, either hyper (top) or hypomethylated (bottom), as compared to non-diabetic controls. Altered DNA methylation patterns at gene sites of glucose and insulin homeostasis were studied in the context of hypermethylation (top) or hypomethylation (bottom) in T1D compared to non-diabetic controls. If the T1D differential shift in methylation of select gene regions in T1D compared to controls was in the same abnormal direction as T2D, the paired samples were circled i.e. circled sets are those that were in the same direction as for abnormal T2D traits. Red circles are T1D; black squares are controls. Of the CpG sites listed, the two in CPT1A, the KDM2B CpG and the ABCG1 CpG showed significant differences in T1D compared to controls; the rest of circled data showed directional trends. n = 13 for T1D and n = 8 for controls, a Mann-Whitney's u-test was used to determine significance. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. P-values were 0.0368 (ABCG1 cg0650016), <0.0001(CPT1A cg00574958), 0.0077 (CPT1A cg17058475) and 0.0129 (KDM2B cg13708645). Testing for the false discovery rate yielded Q values with a cut-off as 0.1 for CPT1A cg00574958 (0.002), CPT1A cg17058475 (0.077) and KDM2B cg13708645 (0.086). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 1
CpG sites studied, and the functions for the genes.

Gene/ Region	CpG	Associated with Fasting Glucose (FG) / Fasting Insulin (FI):	Function
4p15.33	cg10438589	FI	
ABCG1	cg06500161	FI	ATP-binding cassette, sub-family G (WHITE), member 1.Encodes a protein belonging to the superfamily of ATP-binding cassette (ABC) transporters which transport different molecules across extra- and intra-cellular membranes. ABCG1 is involved in cholesterol and phospholipids transport and is assumed to regulate cellular lipid homeostasis.
ABCG1	cg27243685	FI	Above
APOBEC3H	cg06229674	FG	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3H. A cytidine deaminase with anti-retroviral activity by generating hypermutations in viral genomes, inhibiting replication and retrotransposon mobility. Is enriched in immune effector processes and innate immune response
DHCR24	cg17901584	FI	24-Dehydrocholesterol Reductase. Contains a Flavin Adenine Dinucleotide (FAD)-dependent oxidoreductase which catalyzes the reduction of the delta-24 double bond of sterol intermediates during cholesterol biosynthesis.
IRS2	cg25924746	FI	Insulin Receptor Substrate 2. Cytoplasmic signaling molecule mediating the effects of insulin, insulin-like growth factor 1 and other cytokines by acting as a molecular adaptor between diverse receptor tyrosine kinases and downstream effectors. This molecule is phosphorylated by the insulin receptor tyrosine kinase upon stimulation
MAN2A2	cg20507228	FI	Mannosidase Alpha Class 2A Member 2. (Glycosylase enzyme). Catalyzes the first committed step in the biosynthesis of complex N-glycans. Regulates conversion of high mannose to complex N-glycans at the final hydrolytic step. Also related to pathways involving metabolism of proteins and Intra-Golgi traffic.
RBM20	cg15880704	FI	RNA Binding Motif Protein 20. Protein that binds mRNA and regulates splicing. Including those of a subset of genes involved in cardiac development. Regulates the splicing of TNN (Titin).
SLC7A11	cg06690548	FI, FG	Solute Carrier Family 7 Member 11. Protein coding gene for a member of a heteromeric, sodium-independent, anionic amino acid transport system that is highly specific for cysteine and glutamate. Glutamate is one pathway for sugar regulation.
SREBF1	cg11024682	FI, FG	Sterol regulatory element binding transcription factor 1. Encodes a transcription factor binding to the sterol regulatory element-1, which is a decamer flanking the low-density lipoprotein receptor gene and some genes involved in sterol biosynthesis.
1q25.3	cg13222915	FI	
15q26.1	cg18247172	FG	
ASAM	cg26894079	FI	(ASAM) CLMP: Coxsackie- And Adenovirus Receptor-Like Membrane Protein. This gene encodes a type I transmembrane protein that is localized to junctional complexes between endothelial and epithelial cells and may have a role in cell–cell adhesion. Expression of this gene in white adipose tissue is implicated in adipocyte maturation and development of obesity.
CPT1A	cg00574958	FI, FG	Carnitine palmitoyltransferase 1a. CPT1A is an isoform of carnitine palmitoyltransferase (CPT) I. By the sequential action of CPT and carnitine palmitoyltransferase II, together with a carnitine-acylcarnitine translocase, the mitochondrial oxidation of long-chain fatty acids is initiated. Deficiency of CPT I leads to a reduced rate of fatty acid beta-oxidation. Rate limiting for mitochondrial fatty acid oxidation.
CPT1A	cg17058475	FI	Above
KDM2B	cg13708645	FI	Lysine Demethylase 2B. Involved in phosphorylation-dependent ubiquitination. Histone demethylase that preferentially demethylates trimethylated H3 'Lys-4′ and dimethylated H3 'Lys-36′. Preferentially binds the transcribed region of ribosomal RNA and represses the transcription of ribosomal RNA genes which inhibits cell growth and proliferation.
MYO5C	cg06192883	FI	Myosin VC. May be involved in transferrin trafficking. Likely to power actin-based membrane trafficking in many physiologically crucial tissues.
RNF145	cg26403843	FI	Ring Finger Protein 145. E3 ubiquitin ligase. In response to bacterial infection, negatively regulates the phagocyte oxidative burst by controlling the turnover of the NADPH oxidase complex subunits. Involved in the maintenance of cholesterol homeostasis. In response to high sterol concentrations ubiquitinates HMGCR, a rate-limiting enzyme in cholesterol biosynthesis, and targets it for degradation. In addition, triggers ubiquitination of SCAP, likely inhibiting its transport to the Golgi apparatus and the subsequent processing/maturation of SREBPF2, ultimately downregulating cholesterol biosynthesis.
TMEM49	cg24174557	FI	(TMEM49) VMP1: Vacuole Membrane Protein 1. Transmembrane protein playing a key regulatory role in autophagy. Plays a role in the initial stages of the autophagic process through its interaction with BECN1.
SLAMF1	cg18881723	FG	Signaling Lymphocytic Activation Molecule 1. Self-ligand receptor involved in modulating the activation and differentiation of a variety of immune cells, and hence involved in the regulation and interconnection of both innate and adaptive immune responses.
FCRL6	cg00936728	FG	Fc Receptor Like 6. A Protein Coding gene. Acts as an MHC class II receptor. When stimulated on its own, does not play a role in cytokine production or the release of cytotoxic granules by NK cells and T cells.
LETM1	cg13729116	FI	Leucine Zipper And EF-Hand Containing Transmembrane Protein 1. This gene encodes a protein that is localized to the inner mitochondrial membrane. The protein functions to maintain the mitochondrial tubular shapes and is required for normal mitochondrial morphology and cellular viability. Crucial for the maintenance of mitochondrial tubular networks and for the assembly of the supercomplexes of the respiratory chain.

of a T1D compared to year 01, year 02 and year 03 of monitoring. Of these 13 CpGs, 8 were in the same altered starting direction for T2D traits; 4p15.33, both ABCG1 CpGs, IRS2, MAN2A2, 15q26.1, and both CPT1A CpGs. Thus, BCG treatment in vivo was directional correct at reversing the abnormal patterning. These CpGs, except the one for 15q26.1, all showed significant changes in methylation status by year 3. In 15q26.1 this change was evident by year 1 instead. This data is additionally reflected in the heatmaps as all

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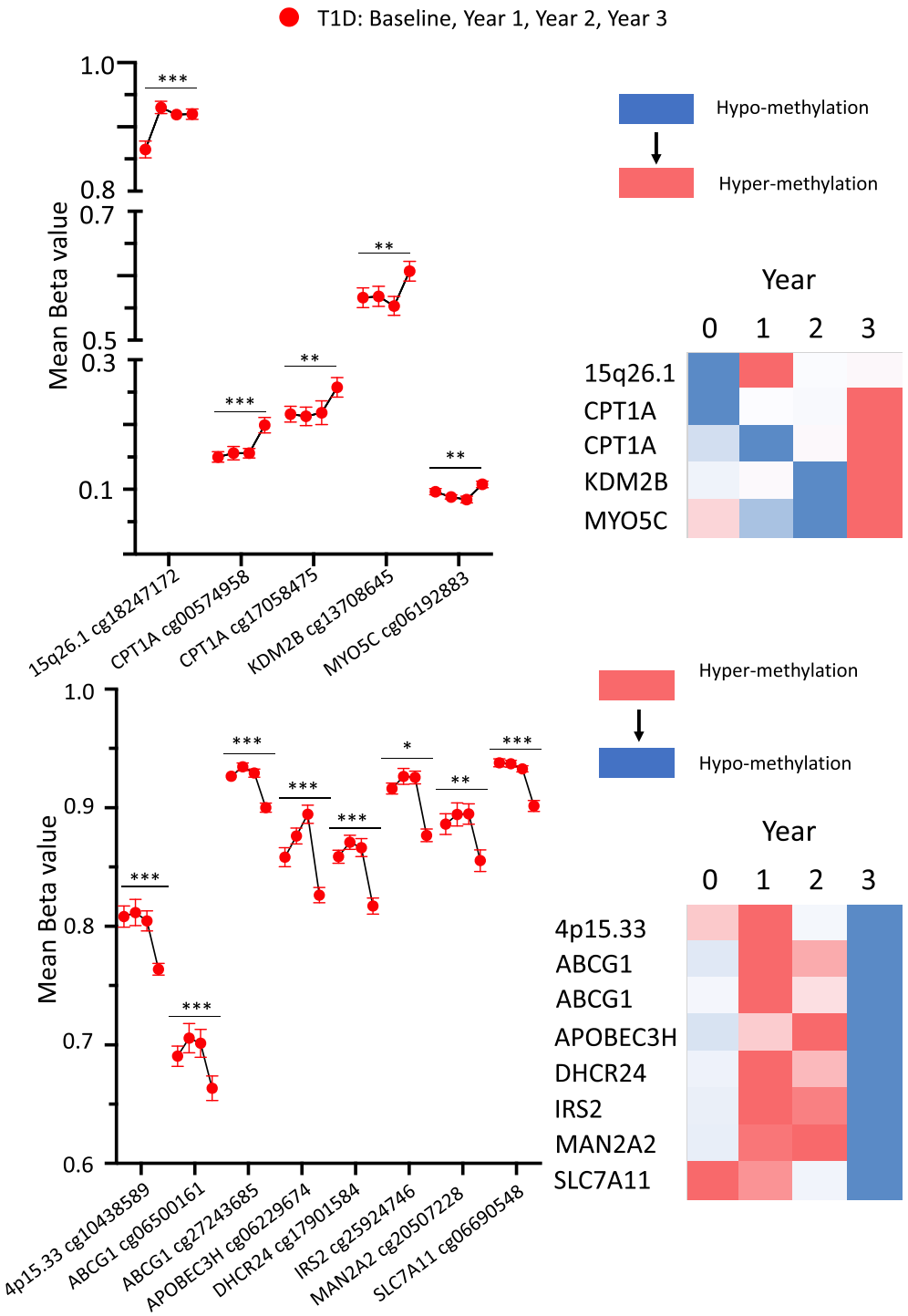

Fig. 2. BCG vaccinations of T1D subjects induce shifts in methylation in restored direction towards normal over a 3-year monitoring time period. Significant yearly and longitudinally followed changes in methylation status of select CpG regions related to glucose and insulin regulation. Homeostasis occurred either in year 01 (region 15q26.1 cg18247172) or by year 3 with the desired hypermethylation (top) of sites or hypomethylation (bottom) of select CpG sites (Red: T1D samples from Baseline – Year 3). Heat maps (right) showed the same methylation data over Year 01–03 for each of the studied CpG sites but for simplicity represented as the gene name for the CpG sites. n = 13 for T1D; Significance was calculated using a repeated measure one-way ANOVA test followed with a Holm-Šídák's multiple comparisons test comparing baseline to each sequential year. For IRS2 cg25924746 a Friedman's test followed with Dunn's test for multiple comparisons was used instead. P-values * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$ are shown for the RM-ANOVA's, and error bars represent standard error of the mean. Significant adjusted P-values for Holm-Šídák's testing are: 15q26.1 cg18247172 (Y1: 0.0004, Y2: 0.0007, Y3: 0.0010), CPT1A cg00574958 (Y3: 0.0002), CPT1A cg17058475 (Y3: 0.0046), KDM2B cg13708645 (Y3: 0.0161), 4p15.33 cg10438589 (Y3: 0.0002), ABCG1 cg06500161 (Y3: 0.0021), ABCG1 cg27243685 (Y3: <0.0001), APOBEC3H cg06229674 (Y1: 0.0226, Y2: 0.0044, Y3: 0.0045), DHCR24 cg17901584 (Y3: <0.0001), IRS2 cg25924746 (Y3: 0.0294), MAN2A2 cg20507228 (Y3: 0.0343), SLC7A11 cg06690548 (Y3: <0.0001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the CpGs, except the one for 15q26.1, show the boldest colors by year 3. As from the past data, this suggested that the beneficial effects on glucose regulation as now showed, by following HbA1c levels, is a slow gradual process that occurs over multiple years [9,12]. Our data shows a similar slow gradual time course occurred at methylation sites as well as a few CpG sites were observed to

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methylate in an unfavorable direction (in relation to that for controls) for the first 2 years before correcting in year 3; an effect that was significant for the CpG in APOBEC3H in years 1 and 2. The CpG for MYO5C decreased significantly in beta value by year 2 in an unfavorable direction, however by year 3 the significant difference compared to baseline was diminished and the methylation level was closer to that of the control. The full effects of BCG on blood sugar control can take up to 3 years to be induced [12]. For the CpGs that corrected, heatmaps of the all the available CpGs from the genes showed that the most dramatic changes in beta values occurred in year 3.

LETM1 is an identified gene from the T2D EWAS but the specific CpG for this gene identified in T2D studies associated with altered insulin was not represented in our Infinium Methylation EPIC Bead Chip for this study. Therefore, we studied the entire methylation patterning after BCG treatment in vivo in T1D for all CpG sites in LETM1 (Fig. 3). The average beta value across all the CpG sites for this gene showed increased methylation compared to controls; but this difference statistically decreased for the pooled sites over the three years of monitoring (Fig. 3A). The BCG treatment in vivo appeared to overall work on the gene's methylation sites with no single site driving the changes. Most LETM1 CpG sites gradually de-methylated towards normal and showed significant net demethylation across available CpGs by year 3 for LETM1.

In contrast, 7 of the T1D CpG sites with abnormal methylation did not show a favorable change after BCG treatment (Supplemental Fig. 1, 2). The CpGs in region 1q25.3 and RBM20 shifted in an unfavorable direction away from control levels, and no change occurred for the CpGs in TMEM49 and SREBF1. The CpGs in ASAM and RNF145 only got back to baseline levels by year 3, and further longitudinal data would be required to confirm if their methylation levels reach the same as controls. Additionally, the CpG in SLAMF1 did not show a difference between baseline and control levels; a significant decrease in methylation was observed by year 1, how-

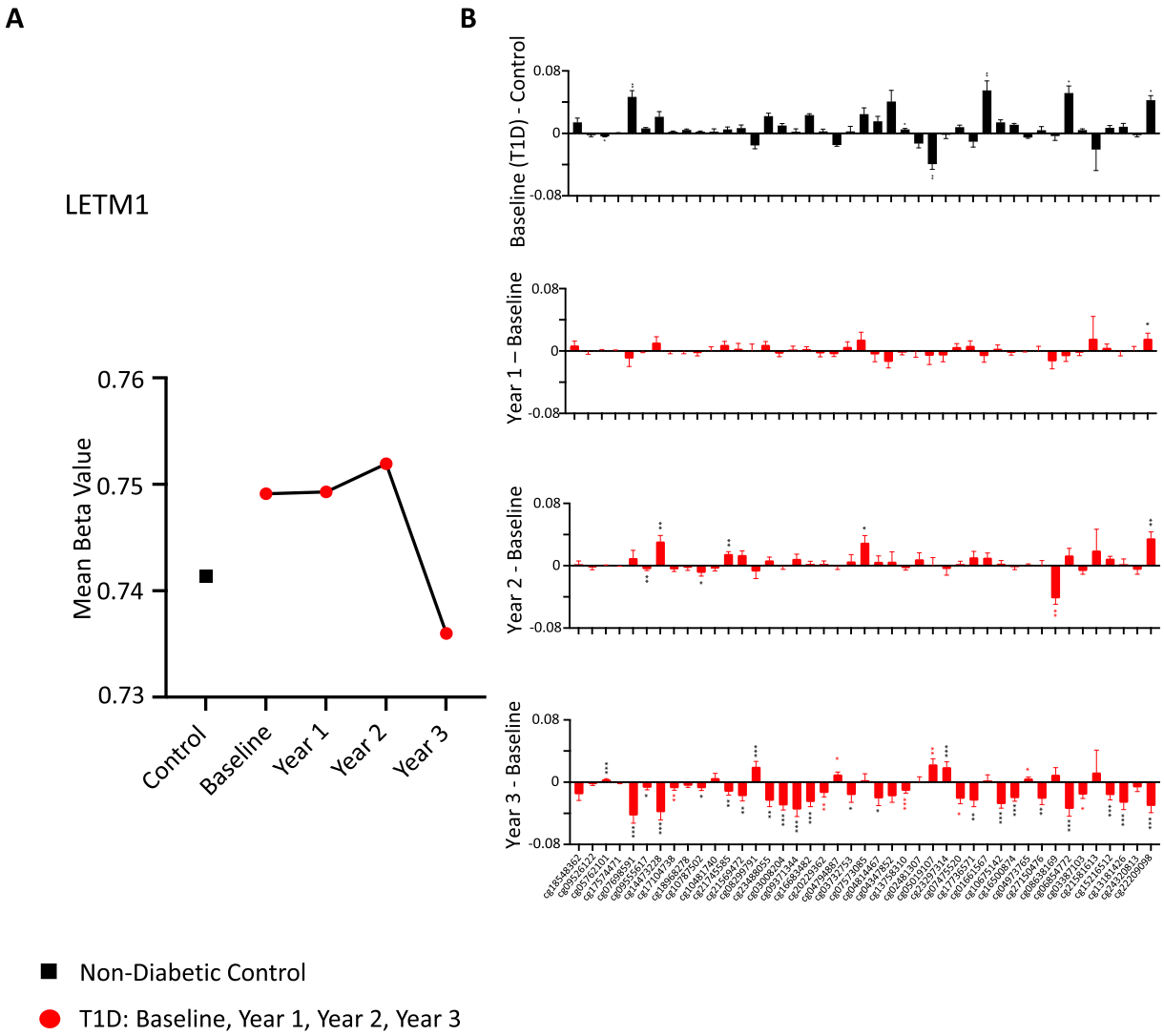

Fig. 3. BCG induces demethylation in the LETM1 gene, an entire gene associated with fasting insulin, towards a favorable direction in T1D. A. Net demethylation in year 3 for the LETM1 gene occurs with BCG vaccinations in T1D (red) towards restored levels like controls (black). These results are mirrored in the individual CpG breakdown, when comparing the difference from each year to baseline (**B**), as significant demethylation is evident for multiple CpGs by year 3. N = 43 for net CpG changes studies. n = 13 for T1Ds; n = 8 for Controls. For Fig. 3B significance for each individual CpG was calculated using a Mann-Whitney's u-test when comparing baseline to controls, and a repeated measure one-way ANOVA test followed with a Holm-Šídák's multiple comparisons test comparing baseline to each sequential year. For non-parametric CpGs a Friedman's test with Dunn's test multiple comparisons was used instead when comparing baseline to each sequential year (Red asterisks). P-values * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$ are shown for the Holm-Šídák's/Dunn's multiple comparison testing in Fig. 3B, and error bars represent standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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ever, the levels did not fully shift back up towards baseline/controls. Similarly, a heatmap of these CpGs (Supplemental Fig. 1, 2) do not show any particular trends.

3.3. The methylation patterns resulting from BCG treatment of T1D correlate with mRNA expression of the genes

We performed a more detailed characterization of genes of particular interest. CPT1A has two CpG regions associated with T2D risk and is associated with glucose regulation pathways associated with OXPHOS, a topic of interest in T1D. In T1D both of these sites are hypermethylated at baseline compared to control samples (Fig. 4). Importantly, both sites acquired methylation over the 3-year observation period after BCG vaccinations in very statistically significant ways (Fig. 4A). To confirm that this gene site and methylation changes might be functionally important, mRNA was prepared from the lymphocytes of treated T1D and analyzed by RNAseq. In a corresponding manner, mRNA levels diminished as methylation increased suggesting the CPT1A CpG sites were associated tightly with the regulation in mRNA production. As mentioned earlier CPT1A has a function related to mitochondrial oxidation and this metabolic process is overzealous in T1D. We should point out here that the benefit of BCG on turning off overzealous OXPHOS could involve this gene, a step that then promoted aerobic glycolysis and accelerated lymphoid ambient sugar usage.

The KDM2B gene, also known as lysine demethylase 2B, was also studied in detail due to its known role in histone methylation and known role in glucose regulation in type 2 diabetes (Fig. 4B). This gene is a mechanism for BCG to modify DNA histones and impact gene expression. T1Ds at baseline were hypomethylated at the cg13708645 site compared to controls samples and BCG triggered a gradual 3-year correction by hypermethylation. This shift was reflected in the corresponding CD4 mRNA samples that showed decreased KDM2B expression. After BCG vaccinations, it is already known that in monocytes, an increase in H3K4me3 histone modifications occurs and is associated with the promoters of TNF, IL6 and TLR4. Thus our in vivo BCG methylation studies confirm this intricate control of gene expression.

The KDM family of lysine demethylase genes has many gene members, so we also explored the other mRNA changes in the other KDM family members, not just the KDM2B gene associated with glucose metabolism. Nine different KDM-related genes showed decreasing mRNA expression over the 3-year monitoring course (Fig. 5). It should be noted that none of these other KDM related genes have a single CpG site that has been associated with T2D. For the KDM genes with suppressed mRNA from BCG we do not know the epigenetic sites for control. Not all members of the KDM family of genes showed decreased mRNA; non-responding mRNA levels were also identified (Supplemental Fig. 3).

A summary of KDM family members, their mRNA response after BCG, and their known associations with select lysines on histones 3 and 4 is shown in Table 2. Many experiments in trained immunity, typically on monocytes with various bacterial and yeast ligand showed that H3K4s are modulated by BCG responsive cytokine or glycolytic genes. This analysis showed a similar but not identical overlap to KDM and known H3K4 histones in T1D.

To further buttress the functional link between T1D CpG site methylation changes due to BCG and mRNA changes, three additional CpG sites associated with insulin and glucose control were analyzed (Fig. 6). For MAN2A2 at baseline T1D lymphocytes were hypermethylated (similar to T2D) but demethylated by year 3. This was associated with a corresponding upward trend in mRNA also isolated from lymphocytes. A heat map of all CpG sites in MAN2A2 showed BCG by year 03 was in general hypomethylating the gene as well as the specific CpG site under study. MYO5C corrects with hypermethylation of the select CpG site but the gene overall showed dominant hypomethylation although the mRNA corresponded to the CpG site methylation patterns in response to BCG. The CpG site for SLAMF1 does not appear to be abnormal in T1D, and after BCG treatment, a significant decrease in methylation is evident for year 1 and year 2, reflecting the changes in the mRNA profiles for the gene. Contrastingly, only 54% of the CpG sites in the SLAMF1 gene were observed to have the strongest changes by year 3.

Taken together the results indicate that BCG treatment in T1D induces significant changes of the methylation status of the majority of CpGs associated with T2D, although this is a gradual years-long process after vaccine administration.

3.4. Baseline under-methylation of the DDIT4 gene is confirmed; BCG vaccine treatment re-methylates this mTOR inhibitory gene

Epigenome wide association studies are difficult and demonstrate why large integrative cross omics of many T2D patients are needed to show reproducibility [38]. Primary DNA methylation variability in T1D, has been tackled most successfully using epigenome wide association studies in monozygotic twin pairs discordant for T1D and isolated CD4 T cells [37]. A single observation that reaches statistical significance is cg01674036 in CD4 T cells. This is a controlling region for the *DDIT4* gene, an inhibitor of mTOR. Although many prior CpG sites were not found especially in discordant identical T1D, we were able to show and confirm at baseline similar methylation of this gene (Fig. 7). With BCG vaccine therapy and observations for 3 years, BCG re-methylated this inhibitory gene. Based on the known signaling pathways of mTOR this would be predicted to increase cellular glucose utilization in CD4 T cells [30].

4. Discussion

This study is a secondary analysis of a clinical trial evaluating the benefits of BCG for the treatment of T1D. Patients with long-term T1D were dosed with at least 2 BCG vaccinations and then followed yearly for 3 years to look for the systemic methylation changes in lymphocytes at CpG sites related to glucose and insulin homeostasis taken from a cross-omics study of T2D. Five CpG sites demonstrated over the 3-year time period re-methylation to control levels. Eight CpG sites demonstrated de-methylation to levels close to control samples (Supplemental Table 1). We unexpectedly identified KDMB2 as a key upstream regulator for BCG's effect on histone activity such as H3K4me3, a finding that is discussed further below.

The protective effect of the BCG vaccine from unrelated infections and in autoimmunity are now well documented from clinical trial experiences [6–23]. Infections as well as hyperglycemia itself can change the patterns of epigenetics through histone modifications. Hyperglycemia is associated with increased H3K4 and reduced H3H9 histone methylation patterns in the NFkB gene sets [40]. Bacterial ligands such as Lipopolysaccharide and β-glucan (bacterial cell wall component) can also change the methylation patterns on histone genes [41,42]. It is appreciated that each extracellular ligand, whether Lipopolysaccharide or β-glucan, utilizes different functional programs and uses different cellular pathways to achieve epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity [43,44]. BCG itself also alters histone methylation patterns, thus increasing expression of cytokine genes and glycolytic pathways [41,42]. Each ligand or microorganism is expected to have shared as well as unique sets of histone modifications specific to select gene regulation.

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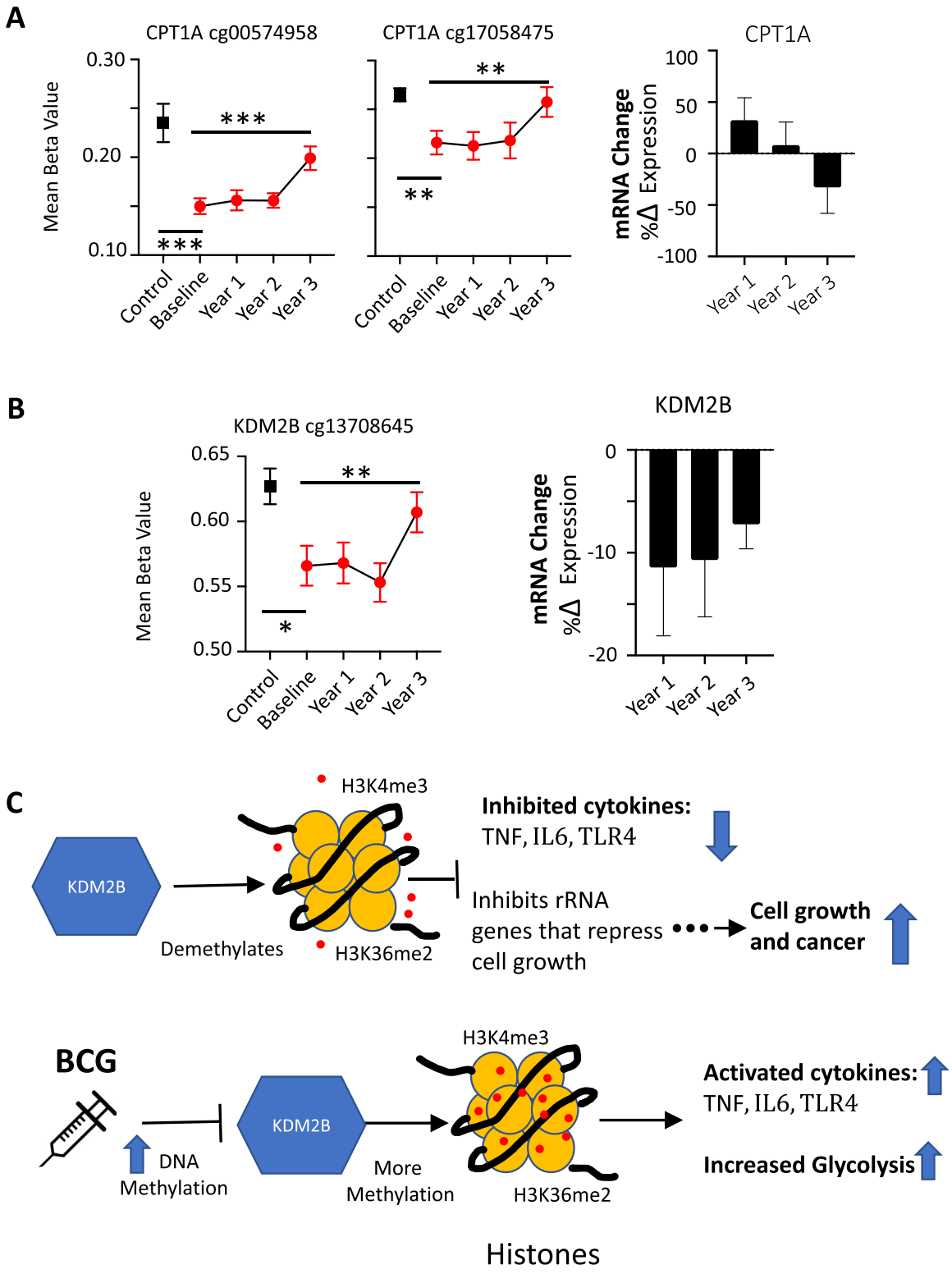

Fig. 4. Impact of BCG vaccines in T1D on the methylation and mRNA patterns for the CpG CPT1A sites and CpG KDM2B site; yearly patterns after in vivo BCG treatment. In T1Ds at baseline, both CPT1A (**A**) and KDM2B (**B**) CpG regions are hypomethylated compared to controls. The KDM2B gene is known as Lysine Demethylase 2B. With BCG treatment and monitoring for 3 years after BCG vaccinations of T1D, the CpG sites of these genes become hypermethylated to adopt patterns that more closely align with control DNA patterns. Hypermethylation for both the CPT1A gene sites and the KDM2B sites are associated with decreasing levels of mRNA over a 3-year period in the T1D (percent change in expression compared to T1D baseline). The mRNA expression profiles nominally match their methylation longitudinal changes after BCG vaccinations, suggesting functional significance. (**C**). Increased DNA methylation from BCG treatment at the KDM2B region may silence the gene. This likely results in increased levels of methylation at Histone 3 Lysine 4 (H3K4me3) and Histone 3 Lysine 36me2 (H3K36me3) (red dots), allowing downstream activation of cytokines associated with BCG therapy e.g TNF, IL6, TLR4 and genes involved in glycolysis. For the methylation n = 13 for T1Ds; n = 8 for controls regarding the methylation data. Significance presented is from a repeated measure one-way ANOVA test followed with a Holm-Šídák's multiple comparisons test comparing baseline and sequential years, and a Mann-Whitney's *u* test when comparing T1D baseline to control. mRNA expression percent change was calculated comparing baseline to each sequential year; A paired Student's *t*-test was performed between T1Ds baseline and sequential years, N = 10 for T1Ds. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$, and error bars represent standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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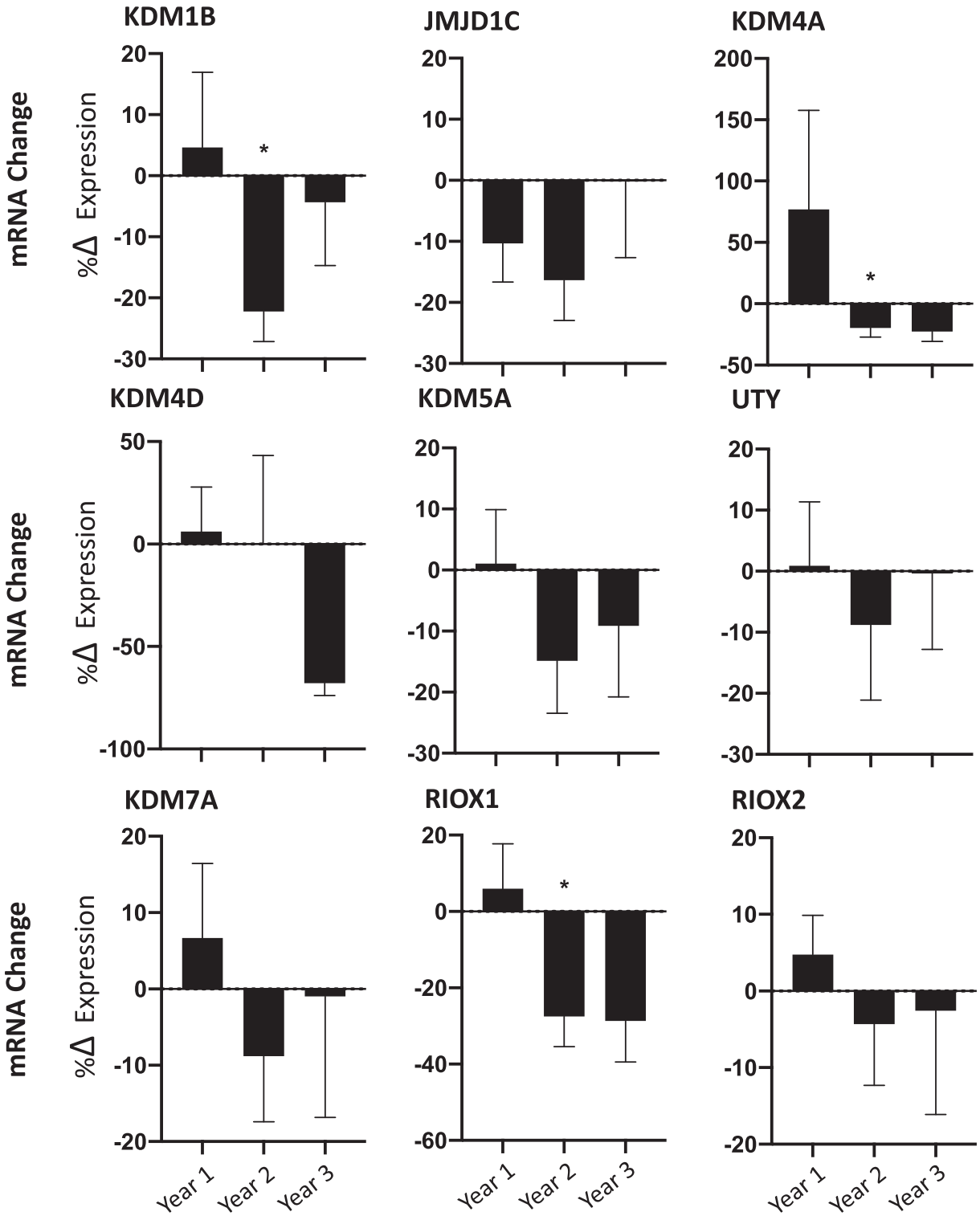

Fig. 5. Impact of BCG vaccinations in vivo on the mRNA patterns of KDM-related gene members in T1D after BCG vaccinations. Similar to KDM2B, KDM gene family members were studied for mRNA expression over the 3-year BCG clinical trial. This figure shows the related members that similarly showed less mRNA expression in lymphocytes over the 3-year monitoring time period. mRNA expression percent change was calculated comparing baseline to each sequential year; A paired student's *t*-test was performed between baseline and sequential years, n = 10 for T1Ds. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$, and error bars represent standard error of the mean.

Here we extend these findings to human studies, to the adaptive immune response within T cells and add to the cascade of controlling proteins to this histone control of critical BCG activation. At baseline compared to control subjects, T1D lymphocytes have reduced methylation of the KDM2B gene, monitored in this study on the cg13708645 methylation site previously identified and related to glucose control in T2D. It is known that methylation of select genes are associated with glycolysis; over-methylation of these genes is an underlying defect in diabetes [12,29,30].

With repeat BCG vaccinations in T1D, the critical glucose regulating CpG site of the KDM2B gene becomes re-methylated. This is confirmed in parallel lymphocyte samples where BCG functionally

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Table 2
Members of the KDM family, RIOX1 was associated to KDM2B through an Ingenuity Pathway Analysis, of which RIO2 was a strong paralog. Information retrieved from genecards.com.

Gene	Expression trend:	Histone Lysine Demethylase Function
KDM1A		Demethylates H3K4me1/me2 and H3K9.
KDM1B	Decreases	Demethylates H3K4me1/me2
KDM2A		Demethylates H3K36me2, weakly H3K36me1
KDM2B	Decreases	Demethylates H3K4me3, H3K36me2
KDM3A		Demethylates H3K9me1/me2
KDM3B		Demethylates H3K9
JMJD1C	Decreases	Demethylates H3K9
KDM4A	Decreases	Demethylates H3K9me3, H3K36me3
KDM4B		Demethylates H3K9me3
KDM4C		Demethylates H3K9me3, H3K36me3
KDM4D	Decreases	Demethylates H3K9me2/me3
KDM4E		Demethylates H3K9me2/me3
KDM5A	Decreases	Demethylates H3K4me2/me3
KDM5B		Demethylates H3K4me1/me2/me3
KDM5C		Demethylates H3K4me2/me3
KDM5D		Demethylates H3K4me2/me3
KDM6A		Demethylates H3K27me2/me3
KDM6B		Demethylates H3K27me2/me3
UTY	Decreases	Demethylates H3K27me3
KDM7A	Decreases	Demethylates H3K9me2, H3K27me2, H4K20me1
PHF8		Demethylates H3K9me1/me2, H3K27me2, H4K20me1
KDM8		Can induce demethylation at H3K36me2/me3
RIOX1	Decreases	Demethylates H3K4me1/me3, H3K36me2
RIOX2	Decreases	Demethylates H3K9me3

Methylation at H3K4, H3K36 and H3K79 is often association with transcriptional activation; methylation at H3K9, H3K27 and H4K20 is usually associated with transcriptional silencing. H3K27 demethylation appears to be concomitant with H3K4 methylation.

decreases mRNA levels in KDM2B. KDM2B could be the upstream gene for BCG training to the already well-documented histone changes for various ligand experiments studying trained immunity, especially for cytokine and glucose regulating genes. Human monocytes in culture exposed to either C. albicans (yeast) or β-glucan show genome-wide changes in epigenetic marks, including histones H3K4me1 and H3K4me3 [45]. For β-glucan exposures to monocytes in culture, the cell surface binding involves dectin 1 (β-glucan receptor), AKT, mTOR and HIFl and cellular metabolism switches from oxidative phosphorylation to glycolysis with epigenetic reprogramming by inhibiting KDM5 histone demethylases [45]. BCG in vitro and in vivo does not bind to the β-glucan receptor but these pathways overlap downstream to similarly change glycolysis [29,30] and now a different central KDM histone lysine demethylase is identified. For cultured human monocytes, H3K4 type histones have been shown to have increased methylation that in return increases cytokines and glycolysis after BCG exposures in culture [27].

In this study we identify the known glucose regulating gene KDM2B as defective in T1D and responsive to BCG, but we also identify close family members are also function by changing mRNA levels over the 3-years of monitoring, including KDM1B, JMJD1C, KDM4A, KDM4D, KDM5A, UTY, KDM7A, RIOX1 and RIOX2. From prior studies we know which KDM family members are associated with histone lysine demethylase targets (Table 2). There is excellent pairing of these histone methylation sites with past data on the patterning of these histones on cytokine genes and glycolysis genes. The current study suggests the central role of KDM2B and some closely related family members in the correction of blood sugars.

The role of KDM family, and KDMB2 specifically, in the BCG mechanism of action is buttressed by another clinical example, BCG for cancer. BCG is an approved drug for bladder cancer at early stages of disease. However, some patients fail BCG therapy and thereby progress to invasive bladder cancer. In this BCG resistant bladder cancer, constitutive activation of KDM2 family members can occur with mutations resulting in overexpression of the KDM2B proteins. This then transforms the bladder tumors to invasive carcinomas [46]. KDM2B likely is the upstream regulatory for the BCG effect on histone modifications for subsequent gene control, especially since it targets the identified BCG histone modulator, H3K4me3. KDM2B dysregulation in T1D may be a primary step in the therapeutic action of BCG in diabetes for the epigenetic changes in cytokines but more importantly the clinically relevant changes in glycolysis thus restoring lymphoid sugar utilization. Some other KDM family members also showed decreasing mRNA levels after BCG therapy in T1D (Fig. 5) although the fit with known BCG driven histones was not as tight (Fig. 5, Table 2). Also, for these other KDM family members, still with decreased levels with BCG therapy, the exact CpG site driving the downregulation of mRNA by epigenetics is not known and genome wide studies have not been associated with glycolysis control.

Traditionally BCG or other microorganisms have been thought to interact with only the innate immune system of human hosts and this has focused studies on monocyte changes in vivo and in vitro, the term trained immunity. The data in this study uses CD4 lymphocyte DNA and the referenced data set was using cells from whole blood. Lymphocytes are part of the adaptive immune system, not the innate immune system. This present data therefore speaks to the broader role of BCG vaccines in both innate and adaptive immunity. To further add to the heterologous benefits of BCG, our phase I trial showed that BCG reduced glycated hemoglobin (HbA1c) levels after 3 years in advanced T1D, increased the numbers of beneficial suppressive T-regs through methylation changes in the promoters of over six central genes for optimal Treg function [12]. In the same publication, BCG treatment was shown to induce DNA demethylation to signature Treg genes, which correlated to their increased expression [12]. The reduction in HbA1c levels from BCG treatment can be explained also by the upregulation of the transcription factor *Myc*, a signature regulator of aerobic glycolysis, glutaminolysis, polyamine synthesis and the mTOR/Hif-α pathways. This resulted in a shift from overactive OXPHOS (a state of low glucose utilization) to aerobic glycolysis (a state of high glucose utilization) [30]. These shifts in glucose utilization were observed both in T cells (adaptive immunity) and in monocytes (innate immunity). BCG therefore in vivo appears to include both the innate and adaptive immune systems through altered methylation patterns.

The CpGs identified in the cross-omics paper were involved in a range of functions, including, metabolism, immune responses, sterol/lipid regulation, sugar regulation and as transporters (Table 1). Of these 20+ CpGs, 13 visually displayed the same differential methylation in T1D as for T2D traits in our study, indicating a strong similarity in the progression of the diseases, at least as it relates to methylation defects associated with insulin and glucose control. The 13 CpGs included: 4p15.33, both ABCG1 CpGs, IRS2, MAN2A2, RBM20, SERBF1, 1q25.3, 15q26.1, ASAM, both CpGs in CPT1A, and TMEM49. In particular, the CpGs for CPT1A, a very well-studied gene related to methylation defects in T2D, were statistically significant for this difference in this study. The CpG in KDM2B was hypomethylated abnormally as compared to controls at baseline, and also corrected with BCG therapy in a statistically significant manner.

Complementary to our work in T1D, the cross-omics analysis of DNA methylation sites related to fasting glucose and insulin identified 3 CpGs genes related to defects in OXPHOS, two of which were localized to the mitochondria. The sites CPT1A cg00574958 and ABCG1 cg06500161 were found to be expression quantitative trait methylations (eQTMs), meaning that there is a correlation

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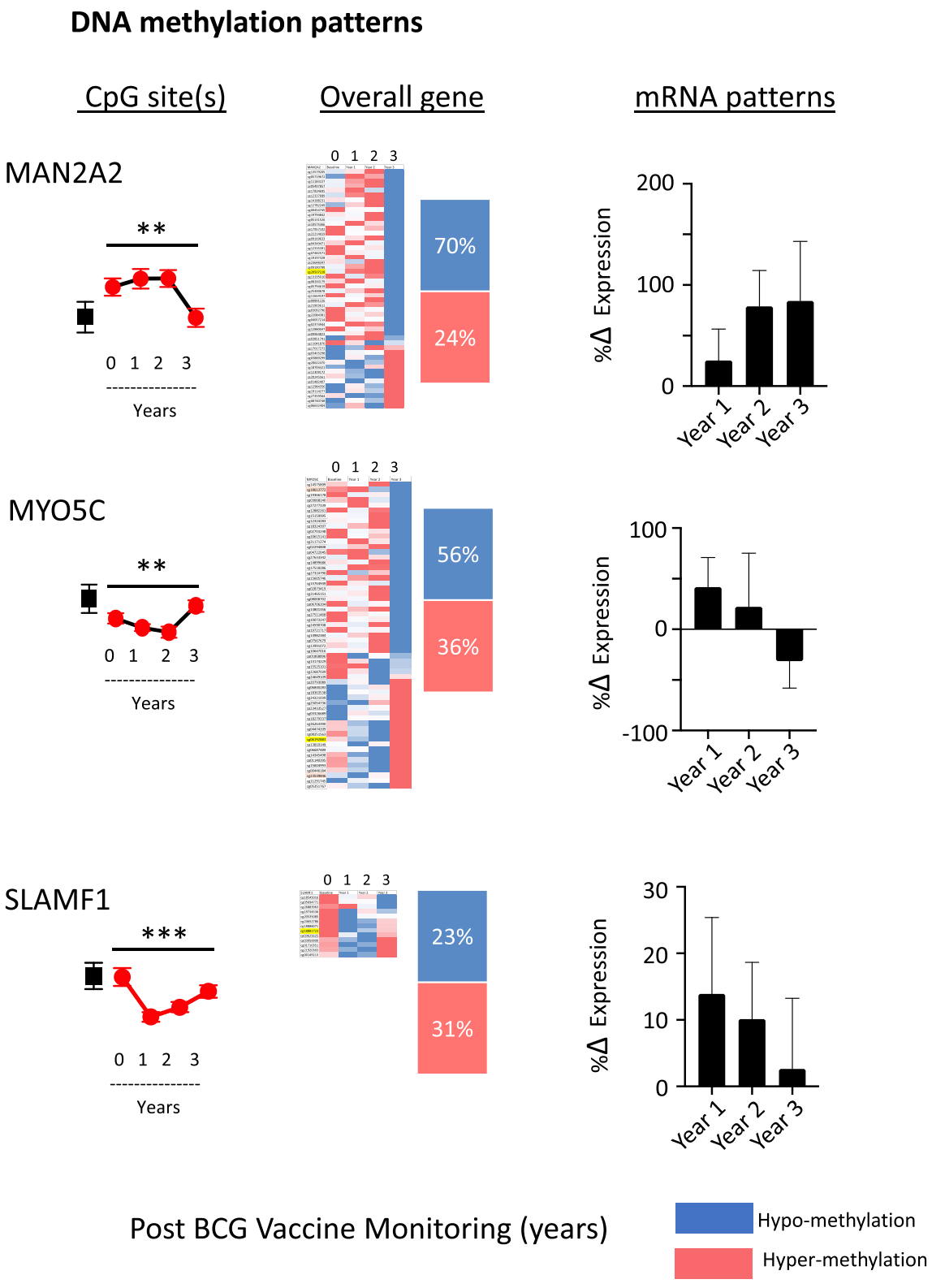

Fig. 6. Impact of BCG vaccinations in vivo on the methylation and mRNA patterns compared to untreated controls for MAN2A2, MYO5C and SLAMF1 CpG sites; yearly patterns after in vivo BCG treatment. For the MAN2A2 CpG site, the MYO5C CpG site, and the SLAMF1 CpG site, BCG treatment of T1D over the 3-year studied time course nearly restores the methylation patters to control levels. MAN2A2 is demethylated, MYO5C is methylated and no difference is seen for SLAMF1. A study of the overall methylation pattern of all CpG sites for the three genes only shows a mild correlation for MAN2A2 but not for MYO5C. For SLAMF1, only just over 50% of the CpG sites show the biggest changes by year 3 (the studied CpGs are highlighted in yellow); for some genes a single CpG site can confer dominant function. In a reciprocal manner the measurement of mRNA in peripheral T cells over the same time course shows the impact of the changing methylation patterns for these genes is reflected by a similar reciprocal change in mRNA expression. For the methylation analysis: n = 13 for T1Ds; n = 8 for controls. Significance shown was calculated using a Mann-Whitney's *u* test when comparing baseline to controls, a repeated measure one-way ANOVA test for MAN2A2 cg20507228 and MYO5C cg06192883, and a Friedman's test for SLAMF1 cg18881723 comparing baseline to each sequential year. mRNA expression percent change was calculated comparing baseline to each sequential year; A paired Student's *t*-test was performed between baseline and sequential years, n = 10 for T1Ds. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$, and error bars represent standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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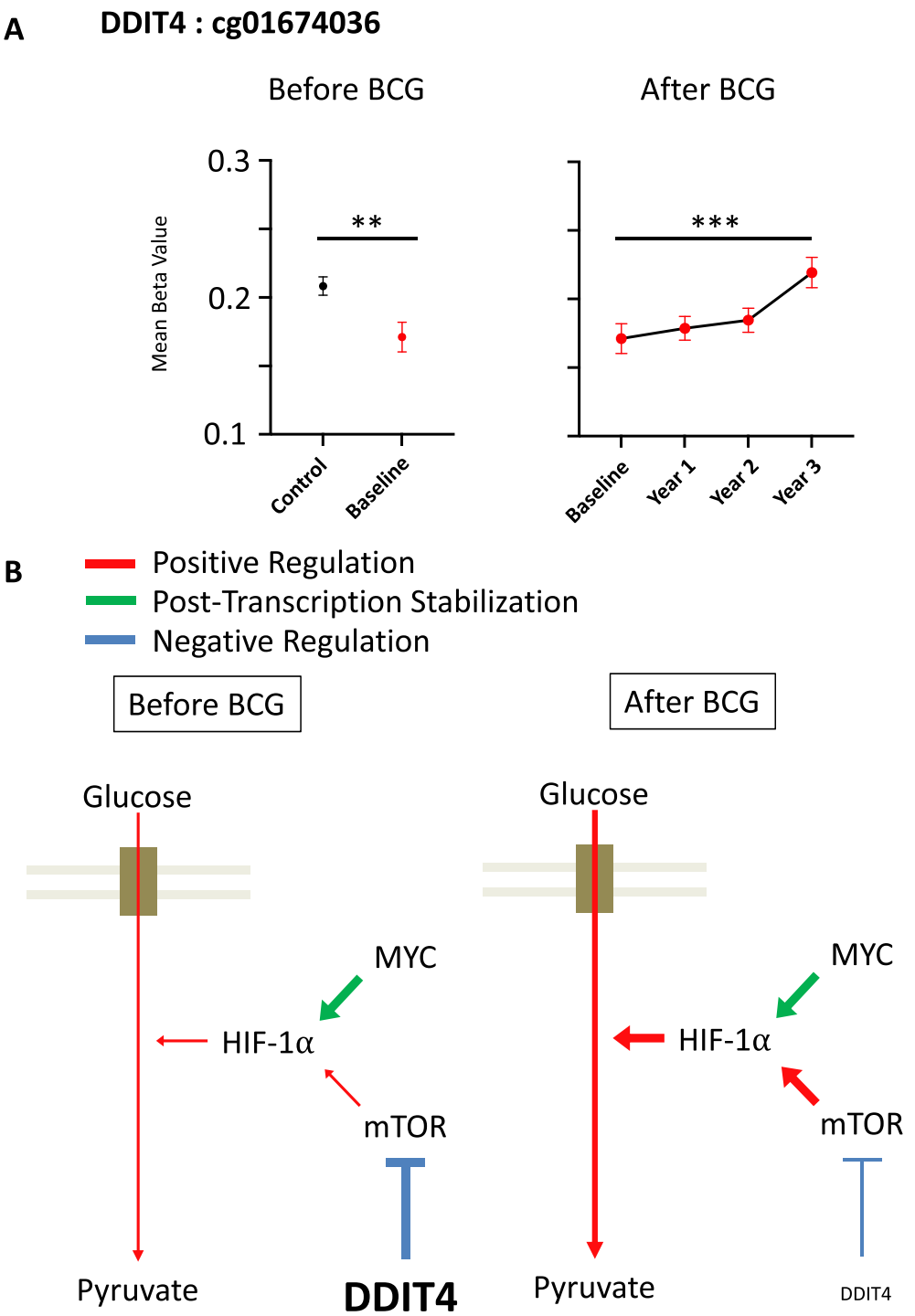

Fig. 7. Baseline undermethylation of a critical CpG site of DDIT4 Gene; Impact of BCG vaccinations in vivo on re-methylation of the gene. (A) At baseline the cg site of the DDIT4 gene (cg01674036), a site associated with T1D progression, is undermethylated. After repeat BCG vaccinations in vivo, the CpG site of the DDIT4 gene is gradually re-methylated, almost to control levels. (**B**) The DDIT4 gene is a known inhibitor of mTOR. mTOR is an activator of HIF-1α and thus facilitates glucose transport. The observed under-methylation of the CCIT4 gene would be predicted to hamper glucose transport. After BCG therapy in vivo, the CD4 lymphoid cells have restored glucose transport from the re-methylation of DDIT4. Significance was calculated using a repeated measure one-way ANOVA test followed with a Holm-Šídák's multiple comparisons test comparing T1D baseline to sequential years, and a Mann-Whitney's u-test when comparing T1D baseline to control. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$, and error bars represent standard error of the mean. ANOVA P-value = <0.0001, Holm-Šídák's P-value comparing T1D Baseline to Year 3 = <0.0001.

between their methylation status and the gene's expression level. CPT1A is a rate-limiting enzyme for fatty acid oxidation, the protein converts acyl-coenzyme A into acyl-carnitines, allowing for migration across membranes into the mitochondria. The beta values for both CPT1A CpGs at baseline were lower than controls implying an increased level of expression for the gene, and thus potentially increasing the rate of fatty acid oxidation. There is also an increase in CPT1A expression (and other fatty acid oxidation (FAO) genes) in the liver of T1D mice models [47], a possible cause for the increased ketogenesis in T1D patients; this increase could be to compensate for the lack of glucose uptake in T1D patients. Three years after BCG treatment, the methylation levels for both the CPT1A CpGs corrected, suggesting a lowered reliance on fatty acid oxidation for energy. Likewise, the relevant CpGs in ABCG1

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were in the same direction for T1D and T2D traits and also corrected by year 3. ABCG1 is a cholesterol and phospholipid transporter in macrophages and prevents cell death by transporting cytotoxic 7-beta-hydroxycholesterol, but its role may also extend to other cell types. Moreover, streptozotocin-induced diabetic mice have shown reduced levels of ABCG1 [48], supporting the data that the CpG may be an eQTM. Conjointly with CPT1A, the CpGs in both genes also showed a correction by year 3, suggesting that these epigenetic changes do impact the biology and may contribute towards the reversal of diabetic traits.

LETM1 displayed significant net demethylation towards controls by year 3. The gene encodes a proton/calcium antiporter protein also located in the inner mitochondrial membrane and is crucial for maintaining the tubular mitochondrial structures. If the methylation difference translated to the gene's expression, T1D at baseline would have reduced levels of this protein available, which suggests dysregulated OXPHOS. A publication from Lin et al 2018 [47] provided data that FAO can promote reprogramming of somatic cells to induced pluripotent stem cells by enhancing OXPHOS (and inhibiting protein kinase C); it was found that in the early stages CPT1B (an isoform of CPT1A) was significantly upregulated. The methylation data regarding CPT1A, ABCG1 and LETM1 could contribute towards why T1Ds overuse OXPHOS compared to controls.

Although this paper was centered on the massive integrative cross-omics analysis of DNA methylation sites, now applied to T1D, we also investigated the single CpG site statistically identified in T1D twin samples with strong methylation differences related to disease expression [37]. In only T1D twins that progress to diseases, the cg01674036 of the *DDIT4* gene is under-methylated. This gene is an inhibitory protein of mTOR, a protein that facilitates glucose transport. We confirm at baseline the gene is undermethylated in our data set. (Fig. 7A). With BCG therapy the gene becomes more methylated at this T1D linked CpG disease risk site (Fig. 7A). The role of the mTOR pathway both in BCG biology and also the use of this pathway to facilitate enhanced glucose uptake has been established [30] (Fig. 7B). This confirms the mTOR derangements are present at baseline in T1D and BCG appears through modulations of methylation impact well known pathway important for cellular glucose transport.

There are limitations of this study. Epigenetic studies comparing T1D to controls populations are difficult to do and often require massive numbers of individuals in the replication. For our data for this comparison (Fig. 1), the power was low for the majority of the CpGs except CPT1A cg00574958 (0.9815793), CPT1A cg17058475 (0.866527) and KDM2B cg13708645 (0.7715659): (ABCG1cg06500161 was 0.4618398). Power calculations for the data in Fig. 2, comparing baseline to each subsequent year, attained acceptable values of over 0.77 for all CpGs bar MYO5C cg06192883, where the highest power was 0.53 comparing baseline to year 2. For DITT4 cg01674036 the power was 0.8281764 comparing T1D baseline to controls, and 0.9999994 comparing T1D baseline to T1D year 3 (Fig. 7). In the past, methylation studies of T1D DNA compared to control populations have been conducted. Probably some of the best controlled studies in T1D and epigenetics are the classic studies comparing discordant twin pair methylation patterns [37,49,50]. We present this early data in this paper (T1D versus controls) as an introduction of epigenetic changes that might directly match similar alterations found in fasting glucose and insulin derangements [38] (Fig. 1). Using this limited data set, 65% overlap was observed; it is not known if our patient sample numbers were larger if this overlap would be higher. The strength of this investigation is the multi-year longitudinal study of T1D compared to self with BCG treatment which affords a more robust power analysis (Figs. 2-7). These comparisons to self eliminate the careful and painstaking matching of control and patient DNA data sets. Additional consideration of this study involves the time frame of observations and comparison of past in vitro data to this in vivo study. Commonly histone modifications of monocytes come from in vitro BCG exposures. This study looks at the histone modifications from humans treated with BCG vaccinations and the common lymphocyte under study are T cells analyzed over years of time post systemic exposures.

In conclusion, many of the CpGs identified in the Cross-omics paper had similar defects in T1D and following BCG treatment (Supplemental Table 3), 13 of the CpGs showed corrections in methylation levels back towards those of controls. The results from this study beg the question whether BCG treatment would be beneficial in T2D. What amount of information would suffice to further pursue studies of BCG treatment in T2D? In a mouse model, BCG reduces plasma High Density Lipoproteins and cholesterol levels and also delays atherosclerotic lesions formed in hyperlipidemic mice, showing the dramatic ability of BCG to change the predominantly mitochondrial driven fatty acid synthesis pathways [51]. Also, BCG ameliorates metabolic syndrome in nonalcoholic fatty liver disease in obese, diabetic ob/ob mice [52]. Finally, it is not yet known in T2D whether like T1D, the starting set point of the immune system similarly shares the marked defects of overabundant OXPHOS and deficient aerobic glycolysis [12,29,30].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.04.011.

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