Human Genetic Variation at rs10071329 Correlates With Adiposity-Related Traits, Modulates *PPARGC1B* Expression, and Alters Brown Adipocyte Function

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Human genetic variation in *PPARGC1B* has been associated with adiposity, but the genetic variants that affect *PPARGC1B* expression have not been experimentally determined. Here, guided by previous observational data, we used clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) to scarlessly edit the alleles of the candidate causal genetic variant rs10071329 in a human brown adipocyte cell line. Switching the rs10071329 genotype from A/A to G/G enhanced *PPARGC1B* expression throughout the adipogenic differentiation, identifying rs10071329 as a *cis*-expression quantitative trait loci (eQTL). The higher *PPARGC1B* expression in G/G cells coincided with greater accumulation of triglycerides and higher expression of mitochondria-encoded genes, but without significant effects on adipogenic marker expression. Furthermore, G/G cells had improved basal- and norepinephrine-stimulated mitochondrial respiration, possibly relating to enhanced mitochondrial gene expression. The G/G cells also exhibited increased norepinephrine-stimulated glycerol release, indicating improved lipolysis. Altogether, our results showed that rs10071329 is a *cis*-eQTL, with the G/G genotype conferring enhanced *PPARGC1B* expression, with consequent improved mitochondrial function and response to norepinephrine in brown adipocytes. This genetic variant, and as yet undetermined eQTLs, at *PPARGC1B* could prove useful in genotype-based precision medicine for obesity treatment.

ARTICLE HIGHLIGHTS

- Elucidating the effects of common genetic variation on metabolism could guide future genotype-based precision medicine.
- We highlighted the function of common genetic variation in *PPARGC1B*, a gene important for metabolism.
- We experimentally identified a causal expression quantitative trait loci, rs10071329, common in sub-Saharan populations.
- Clustered regularly interspaced short palindromic repeats-mediated allele substitution of rs10071329 in human brown fat cells altered *PPARGC1B* expression, oxidative phosphorylation protein content, mitochondrial respiration, and norepinephrine-stimulated lipolysis.
- rs10071329 associated with adiposity in a cohort lookup.

Obesity and its comorbidities are predicted to progressively increase (1,2), prompting the development of precision medicine approaches that leverage person-specific genetic variation effects on weight loss. While multiple studies have correlated genetic variation with adiposity,

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experimental evidence of causality between the two variables is frequently absent.

Genes regulating energy metabolism, including *PPARGC1A* and *PPARGC1B*, are downregulated in people with diabetes, and their genetic variants are associated with insulin resistance (3,4). *PPARGC1B* genetic variants are also associated with obesity (Ala203Pro) (5) and with altered subcutaneous adiposity after lifestyle intervention aimed at weight loss (rs10071329) (6). The rs10071329 variant is biallelic, downstream of *PPARGC1B*, with a minor allele frequency of ∼1% globally, but higher in African populations, reaching 7–8% in Esan in Nigeria and Yoruba populations (7). Nevertheless, whether rs10071329 is merely correlated or also causative of altered *PPARGC1B* expression is unknown, thus explaining the role of this polymorphism on energy metabolism and adiposity.

Here, we hypothesized that rs10071329 is a causal expression quantitative trait loci (eQTL) and tested this hypothesis using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-mediated allele editing of rs10071329 in a human brown preadipocyte (hBA) cell line. The allele editing effects on *PPARGC1B* expression, mitochondrial function, and norepinephrine response were subsequently examined.

RESEARCH DESIGN AND METHODS

For detailed protocols and reagent lists see the Supplementary Research Design and Methods.

Cell Culture

Immortalized hBAs were from Professor Yu-Hua Tseng, (Joslin Diabetes Center, Harvard Medical School, Boston, MA) (8). For adipogenic differentiation, a previously described protocol (9) was used.

CRISPR/Cas9 Allele Editing of rs10071329 in hBAs

CRISPR/Cas9 guide RNAs and donor templates for allele editing were designed for use with Cas9 D10A nickase (IDT, Leuven, Belgium) to decrease CRISPR-mediated off-targeting risk. All primers, single-guide RNA, and donor templates were ordered from IDT. A detailed editing protocol is provided in the Supplementary Research Design and Methods.

RNA Extraction and RT-Quantitative PCR

RNA was extracted with QIAzol buffer and RNeasy Mini Kit (Qiagen). cDNA was synthesized using SuperScript IV VILO Master Mix (Thermo Fisher) and used with TaqMan quantitative (q)PCR assays (Thermo Fisher).

Immunoblots

Polyvinylidene fluoride membranes with transferred proteins were blocked with 5% BSA in Tris-buffered saline (TBS), incubated with 1 μg/mL antibodies in 5% BSA in TBST (TBS plus 0.1% v/v Tween-20), washed in TBST, incubated with horseradish peroxidase-conjugated 0.2 μg/mL secondary antibodies in 5% BSA in TBST, washed in TBST, and developed using Clarity Western ECL Substrate (Bio-Rad).

Electrophoretic Mobility Shift Assays

Nuclear proteins were extracted from differentiated hBAs and used with LightShift electrophoretic mobility shift assays (EMSA) optimization and control kit (Thermo Fisher). Samples were run on 6% Tris-borate-EDTA polyacrylamide gels, transferred to nylon membranes, and developed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher).

Oil Red O or Boron-Dipyrromethene Staining

Differentiated hBAs in 24-well plates were washed with PBS, fixed with 4% formalin, and stained with Oil Red O and hematoxylin or with boron-dipyrromethene.

Lipolysis Assay and Total Triglyceride Content

Differentiated cells were washed with PBS and incubated with DMEM with 2% free fatty acid-free BSA, with or without norepinephrine. Lipolysis (glycerol release) was measured using the Glycerol-Glo Assay kit (Promega). Triglyceride content was quantified using the Triglyceride-Glo Assay kit (Promega).

Bioenergetic Profiling of Differentiated hBAs

Seahorse Extracellular Flux Analyzer (Seahorse Bioscience) determined the mitochondrial oxygen consumption rate (OCR) in differentiated hBAs, in which norepinephrine was used to activate UCP1, oligomycin to inhibit ATP synthase, and rotenone/antimycin A to inhibit electron transport chain.

Mitochondrial Content Quantification

Relative mtDNA content was quantified using qPCR, as previously described (10). Briefly, total DNA was extracted with QIAamp DNA Mini Kit (Qiagen) and used as a qPCR template with SYBR Green master mix (Thermo Fisher) and primers targeting mitochondrial *MT-CYB*, *MT-ND1*, and *MT-TQ*, and nuclear genes *APP* and *B2M* (for normalization). Relative mtDNA content was calculated as Δcycle threshold (Ct; Ct of nuclear target − Ct of mitochondrial target).

Epidemiology Lookups

The FOetal Exposure and Epidemiological Transitions: the role of Anaemia in early Life for Non-Communicable Diseases in later life (FOETALforNCD) study is described elsewhere (11). Briefly, genome-wide genotyping was performed on samples from 132 Tanzanian women using the Illumina Infinium OmniExpressExome-8 v1.4 using Illumina iScan. Quality control was performed with a previously described pipeline (12). rs10071329 was assessed for association with log-transformed BMI and midupper arm circumference using linear regression adjusting for age after log transforming the outcome variables. The Tanzanian

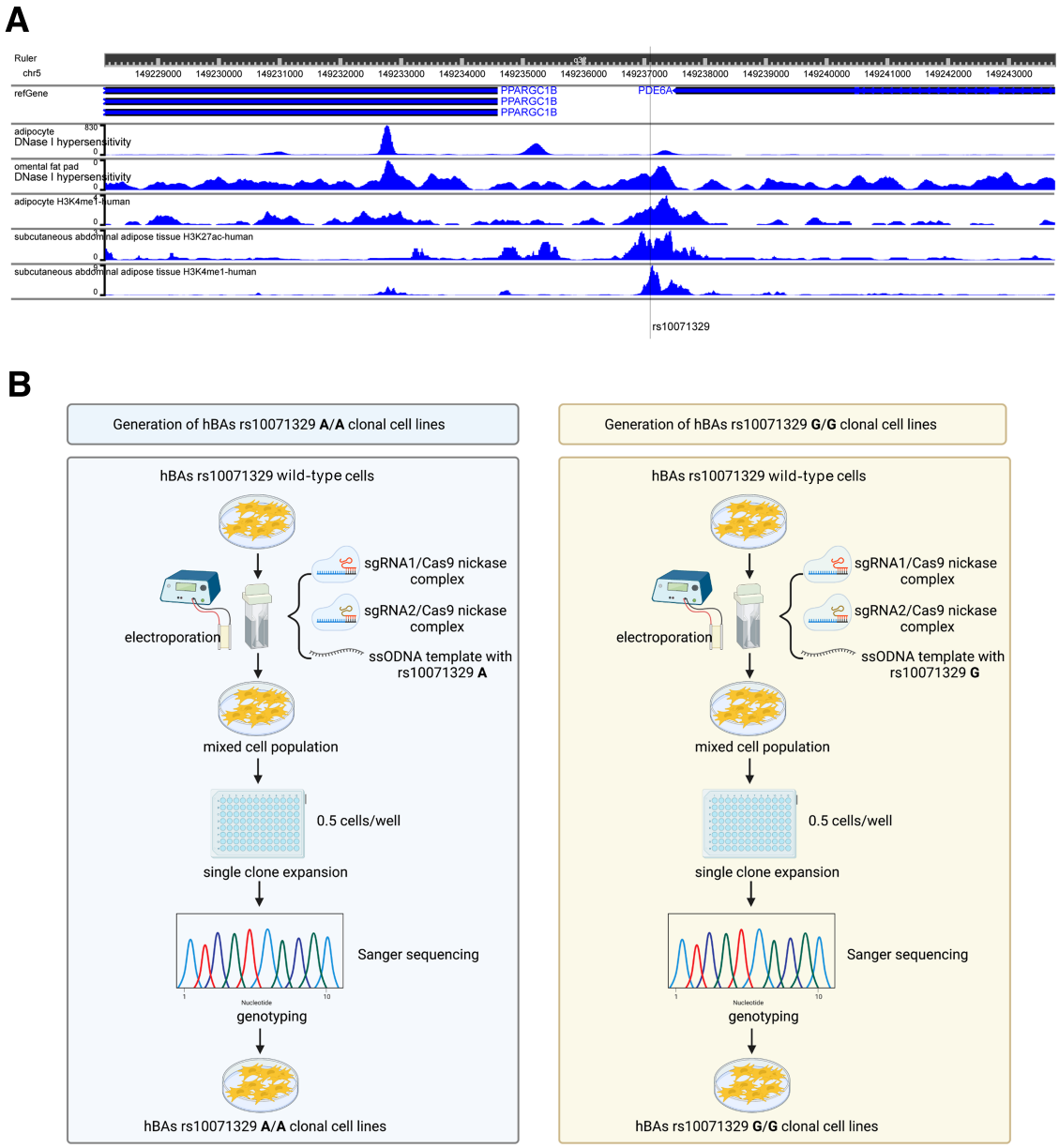

Figure 1—*A*: Chromatin features around the rs10071329 locus in human adipocytes and adipose tissue. The screenshot was taken from the Washington University at St. Louis (WashU) Epigenome Browser, using hg19 genome tracks, showing features commonly associated with enhancer elements, including DNase I hypersensitivity, H3K27Ac, and H3K4me1 peaks. The rs10071329 location is marked by the vertical black line across all tracks. The SNP is flanked by the genes *PPARGC1B* and *PDE6A* (in refGene track). *B*: Workflow for allele editing of rs10071329 in hBA cells. Wild-type (A/A) cells were electroporated with ribonucleoprotein complexes of CRISPR/Cas9 nickase and two different single-guide (sg)RNAs, together with a single-stranded DNA donor template based on the rs10071329 locus sequence, carrying either the A (mock) or the G allele. The cell pool, containing both correctly and incorrectly edited cells, was seeded at 0.5 cells/well density in 96-well plates, to obtain single-cell clonal populations. Each resulting population was Sanger sequenced to find correctly allele-edited cells (A/A and G/G) without indels. *C*: Representative Sanger sequencing traces for rs10071329 A/A or G/G single-cell clonal populations. ssODNA, single-stranded oligo DNA.

Medical Research Coordinating committees approved the study, and all participants gave written informed consent.

Statistics

The number of replicates, SD, and statistical significance are reported in the figure legends. Hypothesis testing was performed using a two-tailed Student *t* test. The hBAs cell line was obtained from one female subject, and sex was not considered a factor in the statistical analyses undertaken on data from hBAs.

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding

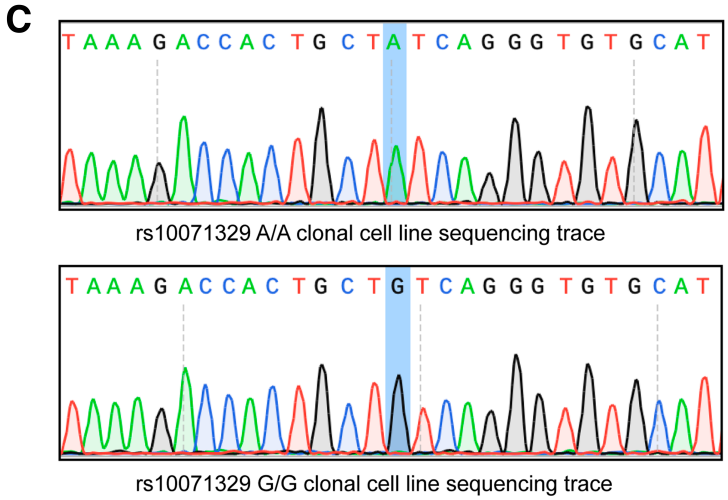

Figure 1—Continued

author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

Chromatin Feature Lookups for rs10071329

The rs10071329 single nucleotide polymorphism (SNP) has no common variants in linkage disequilibrium $r^2 >$ 0.8, resides 10 kilobase downstream of *PPARGC1B*, and overlaps with enhancer marks (DNase I hypersensitivity, H3K27Ac, and H3K4me1) in human adipocytes or subcutaneous fat (Fig. 1*A*). We therefore hypothesized this SNP to be a causal eQTL.

Establishment of rs10071329 Allele-Edited Cells

To test our hypothesis, we performed CRISPR/Cas9-mediated rs10071329 allele editing in the brown preadipocyte cell line hBAs (8), generating homozygous G/G or mock A/A cells through single-cell cloning (Fig. 1*B* and *C*). We chose hBAs cells because *PPARGC1B* plays a key role in mitochondria-rich brown adipocytes (13,14). We obtained 3 A/A and 4 G/G clones of 95 and 90 screened single-cell clones, respectively. Prior to functional studies, the clones were validated for their adipogenic differentiation capacity and lipolytic response to norepinephrine.

Establishment of the rs10071329 Locus As Causal *cis*-eQTL in hBAs

After subjecting A/A and G/G cells to adipogenic differentiation, we observed that *PPARGC1B* mRNA levels were more than fourfold higher in G/G cells (Fig. 2*A*) at differentiation days 7, 12, and 28. They also had approximately twofold higher PGC1B protein content (Fig. 2*B*). Notably, the expression of the rs10071329-vicinal or predicted three-dimensional (3D) loop-linked genes *PDE6A*, *SLC26A2*, *TIGD6*, and *HMGXB3* was either not detectable or unaffected (data not shown). (The 3D loop-linked genes were predicted using the 3DSNP tool [15].)

EMSA on rs10071329-Derived DNA Probes

Since our data showed rs10071329 to be a causal eQTL, we used EMSA to test whether DNA probes based on the rs10071329 locus sequence captured nuclear proteins extracted from hBAs. The G allele-carrying probes—more than the A allele-carrying probes—captured at least one nuclear protein. The observed DNA-protein interaction could be specifically inhibited by nonlabeled probes, more so by the G allele-carrying probes (Fig. 2*C*). While we could not experimentally identify the interacting protein, bioinformatics analysis identified the G allele as important for binding of several proteins, including CTCF, MEIS2, MYOG, and ATF2 (Fig. 2*D*).

Adipogenic Differentiation and Adipogenic Marker Expression in rs10071329 Allele-Edited hBAs

To assess any effects of rs10071329 allele editing on adipogenic differentiation, we examined the differentiated hBAs for triglyceride content and expression of adipocyte-related genes. G/G cells accumulated more fat droplets than the A/A cells (Fig. 3*A* and Supplementary Data 1) and had higher triglyceride content (Fig. 3*B*). The expression of thermogenesis and adipocyte function-specific markers was generally comparable between the genotypes, except for increased *PNPLA2*, *SCD*, *PPARG*, and *ADIPOQ* transcripts in G/G cells (Fig. 3*C*).

Mitochondrial Gene Expression and Mitochondrial Content in rs10071329 Allele-Edited hBAs

Next, we examined the effect of rs10071329 on the expression of nuclear and mitochondrial encoded genes. Differentiated G/G cells had higher *MT-ND3* and *MT-CO3* expression, but not *TFAM*, *TOMM20*, and *CS* expression (Fig. 3*D*), and had unchanged mtDNA content (data not shown). The electron transport chain (OXPHOS) content of selected proteins (ATP5A, UQCRC2, SDHB, and COX II) was generally higher in G/G cells (Fig. 3*E*).

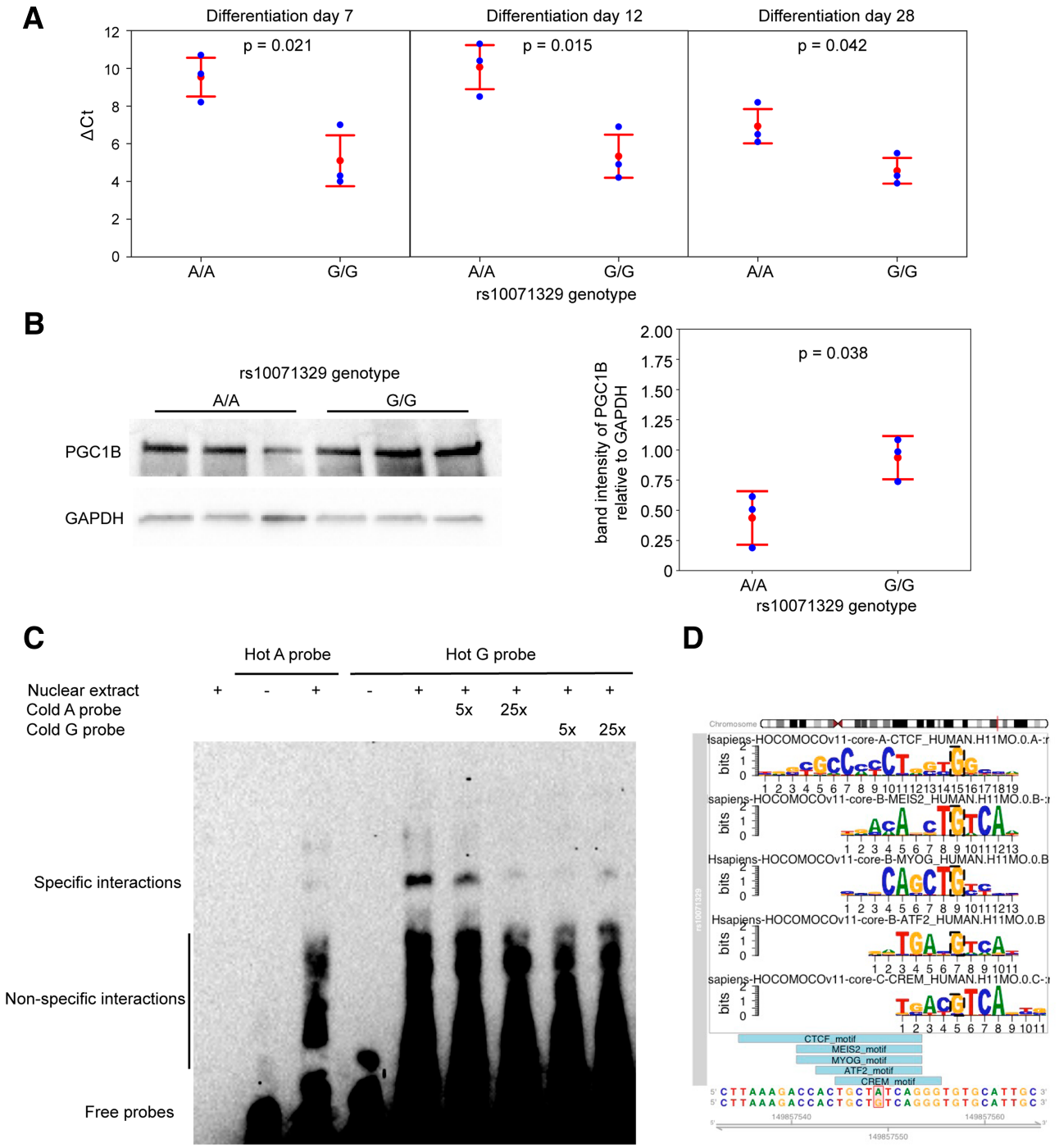

Figure 2—rs10071329 alleles modulate *PPARGC1B* expression in hBA cells. *A*: rs10071329-edited A/A and G/G hBA cells were differentiated to brown adipocytes, and expression of *PPARGC1B* as ΔCt was measured by RT-qPCR at differentiation days 7, 12, and 28. *B*: The cells were as in *A* from differentiation day 28, lysed in SDS, and immunoblotted for PGC1B and GAPDH. The graph on the right shows quantified band intensity of PGC1B normalized to GAPDH. For *A* and *B*, values for each clonal population are plotted as blue dots, mean values as red dots, and SD as red error bars ($n = 3$ clonal populations per genotype). *P* values are from the Student *t* test; similar data were observed in two independent differentiation experiments. *C*: EMSA on biotinylated rs10071329 locus-based 30 base pair probes carrying the A or G allele. The probes were incubated without (lanes 2 and 4) or with nuclear extracts from differentiated hBA cells (lanes 3 and 5). Cold, nonbiotinylated probes (carrying the A or G allele) were added at 5- and 25-fold molar excess to biotinylated G probe samples (lanes 6–9) to assess the specificity of the DNA-protein interaction. Similar results were obtained in three independent experiments. *D.* DNA-interacting proteins identified using motifbreakR and the HOmo sapiens COmprehensive MOdel COllection (HOCOMOCO) database. Several proteins were identified as binding to the rs10071329 locus with the G allele, but not with the A allele, at the *P* value cutoff <0.001.

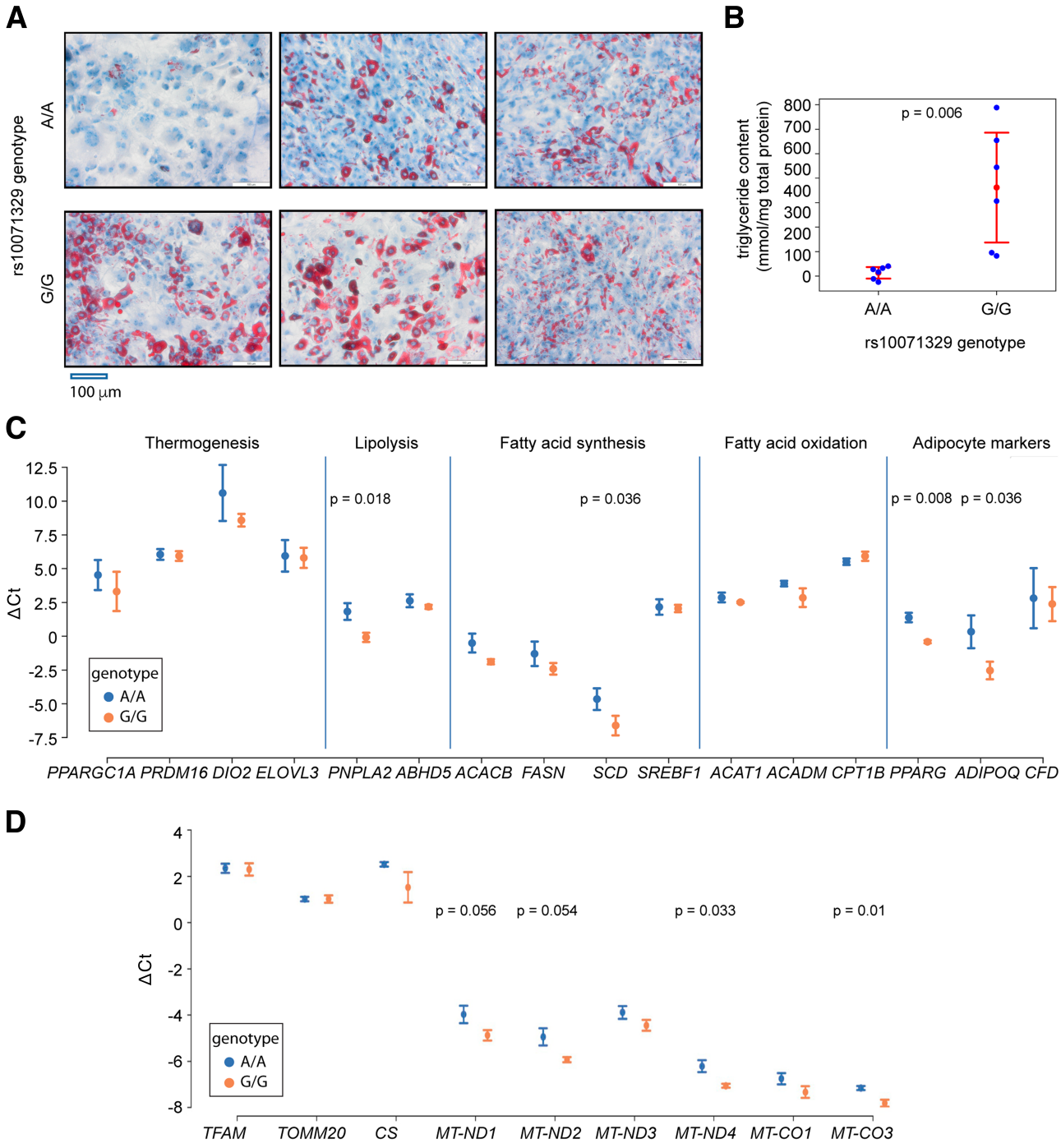

Figure 3—rs10071329 alleles influence lipid accumulation and mitochondrial gene expression in hBA cells. *A*: rs10071329-edited A/A and G/G hBA cells were differentiated to brown adipocytes and stained with Oil Red O to assess lipid accumulation. Representative images are shown, and similar data were obtained in three independent experiments. *B*: Triglyceride content measured in cells differentiated as in *A* was measured by lipase-released glycerol quantification, normalized to total protein content (n = 3 clonal populations per genotype; data are from two independent experiments). *P* values are from the Student *t* test. *C*: Cells differentiated as in *A* were assessed by RT-qPCR for expression of *x*-axis–denoted genes involved in different brown adipocyte-relevant functions (denoted above the graph) (n = 3 clonal populations per genotype). *P* values are from the Student *t* test, only $P < 0.05$ are shown in the graph; similar data were observed in two independent differentiation experiments. *D*: rs10071329-edited A/A and G/G hBA cells were differentiated to brown adipocytes, and expression of the *x*-axis–denoted nuclear- and mitochondria-encoded (prefix *MT*) genes was measured using RT-qPCR. *E*: The cells were as in *D*, but here DNA was extracted, and the content of three different *x*-axis–denoted mitochondrial genes was assessed using qPCR. *F*: The cells were as in *D*, lysed in SDS, and the samples were immunoblotted for OXPHOS proteins. The graph below the blot shows quantified band intensity of each OXPHOS protein normalized to GAPDH. In all graphs, dots are mean values, and error bars are SDs (n = 3 clonal populations per genotype). *P* values are from the Student *t* test, *P* values <0.05 or with trending significance are printed in the graphs; similar data were observed in two independent differentiation experiments.

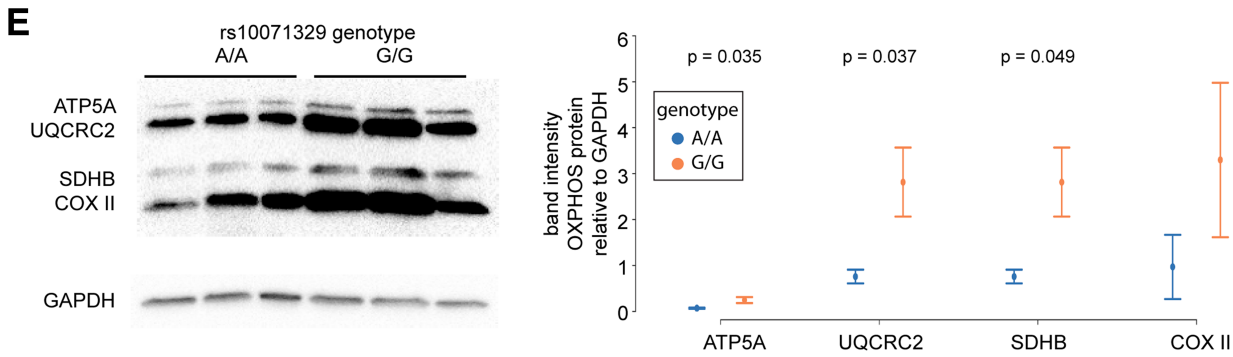

Figure 3—Continued

Mitochondrial Respiration at Basal and Norepinephrine-Stimulated Conditions in rs10071329 Allele-Edited hBAs

hBAs mitochondria respond to norepinephrine or to cold exposure by shifting to adaptive thermogenesis mediated by UCP1 (16). Since the rs10071329 G/G cells appeared to have higher mitochondrial gene expression, we assessed their respiration at basal and norepinephrine-stimulated conditions (Fig. 4*A*): mitochondrial respiration (OCR) changes upon the sequential addition of compounds used for thermogenesis activation (norepinephrine) (17), ATP synthase inhibition (oligomycin), and electron transport chain inhibition (rotenone/antimycin A), respectively. G/G cells had a higher basal mitochondrial respiration (Fig. 4*B*), improved norepinephrine response (thermogenesis) (Fig. 4*C*), and higher norepinephrine-stimulated–to–basal OCR (Fig. 4*D*). Lastly, the proton leak was more pronounced in G/G cells (Fig. 4*E*), whereas ATP production was comparable between the genotypes (Fig. 4*F*).

Norepinephrine-Stimulated Lipolysis in rs10071329 Allele-Edited hBAs

We also examined the rs10071329 effect on lipolytic activity before and after addition of norepinephrine, which is

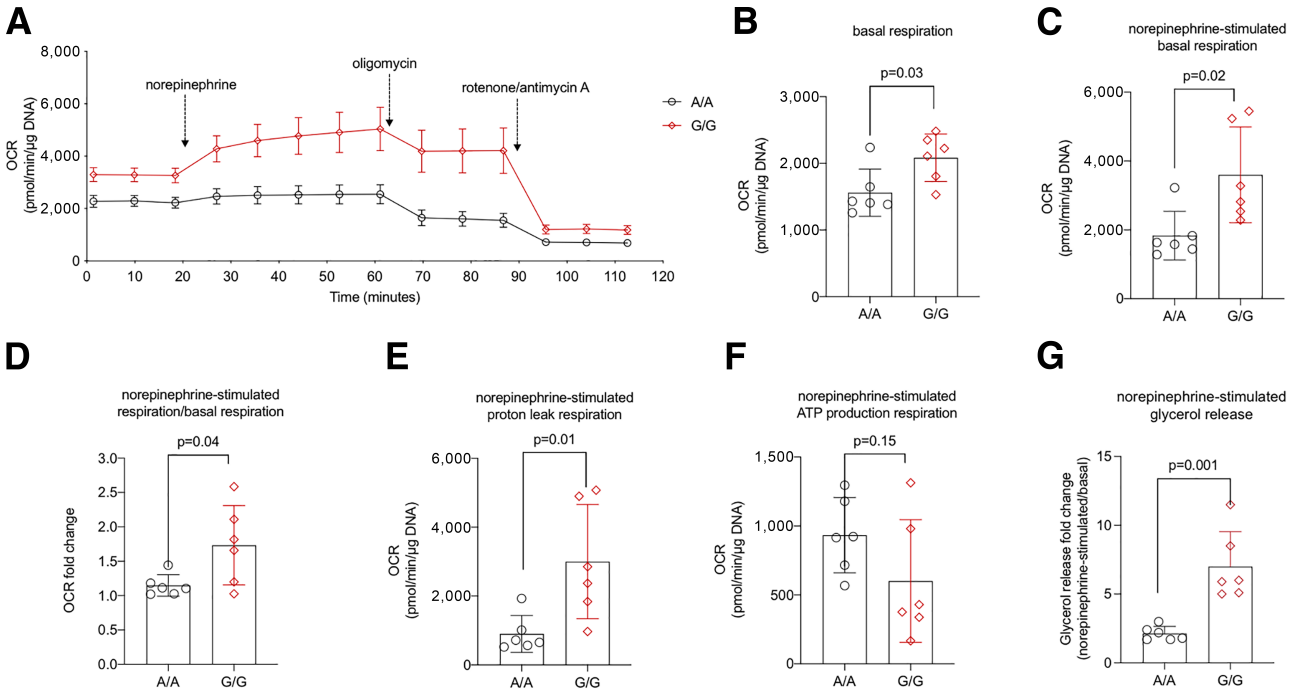

Figure 4—rs10071329 alleles modulate norepinephrine response on mitochondrial function and lipolysis. *A*: rs10071329-edited A/A and G/G hBA cells were differentiated to brown adipocytes and examined for mitochondrial function (OCR) using the Seahorse bioanalyzer at basal condition and with sequential exposure to norepinephrine, oligomycin, and rotenone/antimycin A. *B–F*: Data extracted from *A* on basal OCR (*B*), norepinephrine-stimulated OCR (*C*), fold change of norepinephrine-to-basal OCR (*D*), norepinephrine-stimulated proton leak (*E*), and norepinephrine-stimulated ATP production (*F*). *G*: Norepinephrine-stimulated-over-basal glycerol release (lipolysis) in rs10071329 A/A and G/G differentiated hBAs cells (n = 3 clonal populations per genotype were used in two independent experiments). The error bars show the SD, and P values were calculated by the Student t test and are shown in the graph.

normally released during cold exposure and exercise. We measured the fold-change of norepinephrine-stimulated–to–basal lipolysis: G/G cells released more glycerol than A/A cells (Fig. 4*G*).

DISCUSSION

The SNP rs10071329 has been associated with change in subcutaneous adiposity in response to lifestyle intervention (aimed at ∼7% weight loss and ∼150 min physical activity/week) (6). Here, we used CRISPR/Cas9-mediated allele editing to demonstrate that rs10071329 is a causal SNP, probably part of an enhancer element, binding an experimentally yet unidentified transcription factor (Figs. 1*A* and 2*C*). The minor G allele increases *PPARGC1B* expression (Fig. 2*A* and *B*), leading to higher content of mitochondrial electron transport chain proteins and improved norepinephrine response (Figs. 3 and 4). This might explain the association between rs10071329 and adiposity, with the G allele mimicking the outcomes of overexpressing *PPARGC1B*, previously shown to increase mitochondrial respiration (18) or conferring resistance to obesity in mice (19).

We show that G allele-altered *PPARGC1B* expression is correlated with improved mitochondrial gene expression, mitochondrial function, and response to norepinephrine. This agrees with the observations of *Ppargc1b* deficiency causing lower expression of mitochondrial genes and a reduced response to norepinephrine (20–22). Furthermore, total mitochondrial respiration and uncoupled, norepinephrine-induced respiration were improved in rs10071329 G/G cells, which is in line with the previously reported improved mitochondrial respiration after *PPARGC1B* overexpression (18,23).

The higher *PPARGC1B* content in rs10071329 G/G cells did not markedly affect the expression of brown adipogenic differentiation markers (Fig. 3*C*), a finding consistent with an earlier report on *Ppargc1b* knockdown in mouse brown preadipocytes (24). Interestingly, the higher *PPARGC1B* expression appeared to enhance lipid droplet accumulation (Fig. 3*A* and *B*); this could be attributed to increased ATP (Figs. 3 and 4), consequent to improved mitochondrial function, where ATP drives lipogenesis. Indeed, it appeared that the improved norepinephrine response and mitochondrial function related to *PPARGC1B* underpinning the expression of mitochondrial oxidative phosphorylation genes.

Owing to the resource-demanding nature of allele editing experiments, we focused on just one cell type (hBAs), but similar allele-dependent effects in other cell types are conceivable (e.g., in mitochondria-rich muscle). Another limitation was the difficulty in obtaining heterozygous cells, precluding the assessment of allele additive versus dominant effects. This limitation is common to CRISPR-mediated allele editing, where correct editing of one chromosome is often accompanied by CRISPR-induced random indels on the second chromosome. Lastly, single-cell cloning introduces some random variation to adipogenic differentiation capacity, and thus also to triglyceride content, as well as expression of some pan-adipocyte markers. Since *PPARGC1B* itself is an energy metabolism-related gene, its expression also contributes to these readouts. rs10071329 being a causal genetic variant, affecting *PPARGC1B* expression, appears to be a robust finding in itself and is observable at different differentiation days; importantly, rs10071329 does not affect expression of the neighboring genes.

To complement our experimental findings, we also performed an epidemiological lookup for rs10071329 in a small cohort (n = 132) of Tanzanian women, where previous association between this SNP and adiposity (6) appears to be replicated (Supplementary Data 2).

In summary, we have experimentally demonstrated that rs10071329 is a *cis*-eQTL with effects on mitochondrial function, suggesting that manipulating *PPARGC1B* expression could be useful in treating type 2 diabetes and obesity. Conceivably, one could also leverage the gain-of-function effect of genetic variants in *PPARGC1B* carried by specific individuals: previous observational data, and now this experimental validation, suggest that rs10071329 G allele carriers may experience a more beneficial response to lifestyle interventions focused on weight loss or prevention of weight regain.

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Author Contributions. M.H. researched data and wrote the manuscript. R.B.P. researched data and reviewed the manuscript. D.E.C. researched data. L.H. collected the data and edited the manuscript. D.T.R.M. collected the data and reviewed the manuscript. H.M. contributed to discussion and edited the manuscript. P.W.F. researched data and wrote the manuscript. S.K. researched data and wrote the manuscript. S.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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