学位論文

Regulation of growth hormone biosynthesis by Cdk5 regulatory subunit associated protein 1-like 1 (CDKAL1) in pituitary adenomas
（下垂体腺腫におけるCDKAL1による成長ホルモン生合成の制御）

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ORIGINAL

Regulation of growth hormone biosynthesis by Cdk5 regulatory subunit associated protein 1-like 1 (CDKAL1) in pituitary adenomas

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Abstract. CDK5 regulatory subunit associated protein 1-like 1 (CDKAL1) is a tRNA-modifying enzyme that catalyzes 2-methylthiolation (ms^2) and has been implicated in the development of type 2 diabetes (T2D). CDKAL1-mediated ms^2 is important for efficient protein translation and regulates insulin biosynthesis in pancreatic cells. Interestingly, an association between T2D and release of growth hormone (GH) has been reported in humans. However, it is unknown whether CDKAL1 is important for hormone production in the pituitary gland. The present study investigated the role of CDKAL1 in GH-producing pituitary adenomas (GHPAs). CDKAL1 activity was suppressed in GHPAs, as evidenced by a decrease in ms^2, compared with non-functioning pituitary adenomas (NFPAs), which do not produce specific hormones. Downregulation of *Cdkal1* using small interfering and short hairpin RNAs increased the biosynthesis and secretion of GH in rat GH3 cells. Depletion of *Cdkal1* increased the cytosolic calcium level *via* downregulation of DnaJ heat shock protein family (Hsp40) member C10 (Dnajc10), which is an endoplasmic reticulum protein related to calcium homeostasis. This stimulated transcription of *GH via* upregulation of Pit-1. Moreover, CDKAL1 activity was highly sensitive to proteostatic stress and was upregulated by suppression of this stress. Taken together, these results suggest that dysregulation of CDKAL1 is involved in the pathogenesis of GHPAs, and that modulation of the proteostatic stress response might control CDKAL1 activity and facilitate treatment of GHPAs.

Key words: tRNA, Hypophyseal tumor, Pituitary tumor, Growth hormone, Protein synthesis

CDK5 REGULATORY SUBUNIT-ASSOCIATED PROTEIN 1-LIKE 1 (*CDKAL1*) is a risk gene for the development of type 2 diabetes (T2D) [1-4]. Genetic variations in the intronic region of *CDKAL1* are associated with decreased insulin secretion [5]. For instance, rs7756992 is one of the most reproducible T2D-associated single-nucleotide polymorphisms (SNPs) [1]. Insulin secretion is 22% lower in homozygous carriers of the risk alleles (GG) of rs7756992 than in noncarriers [1]. The odds ratio for development of T2D in homozygous carriers of rs7756992 is 1.5~1.55 in European and Hong Kong populations.

The molecular function of CDKAL1 and its relevance to T2D have been elucidated using *Cdkal1*-knockout mice [6, 7]. CDKAL1 is a tRNA-modifying enzyme that specifically recognizes $tRNA^{Lys(UUU)}$ as a substrate and converts N^6-threonylcarbamoyladenosine (t^6) into 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A) at position A37 adjacent to the anticodon [8]. The modified site can directly interact with the first nucleotide of Lys codons (AAA or AAG). This interaction stabilizes codon-anticodon binding and contributes to accurate and efficient decoding of Lys codons [6]. In *Cdkal1*-knockout mice, insulin biosynthesis is impaired at Lys

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codons, which causes aberrant insulin processing and ultimately leads to decreased insulin secretion and glucose intolerance [6]. Similar to the mouse model, CDKAL1 activity is decreased in individuals carrying the risk alleles of rs7756992 [9]. Furthermore, low CDKAL1 activity is associated with decreased insulin secretion in humans [9].

Various hormones, such as growth hormone (GH), modulate the action of insulin [10]. GH is a multifunctional hormone that is secreted from the anterior lobe of the pituitary gland and acts on various tissues to stimulate metabolism and cell growth [10]. GH directly induces insulin release by pancreatic β-cells [11]. On the other hand, GH also elicits diabetogenic effects by inhibiting glucose uptake and increasing the blood glucose level [12, 13]. Neoplasms arising from GH-producing pituitary adenomas (GHPAs) are characterized by excessive GH secretion, leading to the development of acromegaly [14, 15]. Interestingly, abnormal secretion of GH in GHPAs is occasionally accompanied by T2D [16, 17]. For example, a previous study has shown that approximately 55% of patients with acromegaly have T2D or impaired glucose tolerance [18]. Moreover, 0.6% of hospitalized T2D patients were reported to have pituitary adenomas and mild acromegalic features [18].

Although *CDKAL1* is ubiquitously expressed, the function of CDKAL1 in endocrine cells is unclear. Given the fundamental role of tRNA modification in the regulation of protein synthesis, we investigated whether CDKAL1 is involved in secretion of GH. Here, we show that downregulation of CDKAL1 increases secretion of GH.

Materials and Methods

Patients

Tissues samples of GHPAs, non-functioning pituitary adenomas (NFPAs), and adrenocorticotropic hormone-producing pituitary adenomas (ACTH-PAs) were collected during neurosurgeries at Kumamoto University Hospital in 1999–2014 and 2012–2014, 2007–2014, respectively. Informed consent was obtained from each patient before surgery. All procedures were approved by the Institutional Review Board (IRB) of Kumamoto University (Approval number: 231).

Cell culture

Rat GH3 cells (JCRB9047) were purchased from the JCRB Cell Bank. Cells were cultured in Ham's F10 medium (Thermo Fisher Scientific, Waltham, MA) containing 15% horse serum (Thermo Fisher Scientific) and 2.5% fetal bovine serum (Corning, Corning, NY), and were maintained at 37°C in 5% CO_2 and 95% air.

Knockdown of Cdkal1

To knockdown *Cdkal1* using small interfering RNA (siRNA), cells were transfected with *Cdkal1*-targeting Silencer Select pre-designed siRNA (Cdkal1 siRNA; s167039; Ambion, Thermo Fisher Scientific) using RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. To knockdown *Cdkal1* using short hairpin RNA (shRNA), an oligonucleotide targeting *Cdkal1* (5'-GCTTGCTGCCTATGGCTATAA-3') was cloned between the AgeI and EcoRI sites of the pLKO.1-puro plasmid (Addgene, Watertown, MA) according to the manufacturer's instructions. pLKO.1-puro containing scrambled shRNA (shScramble; Sigma-Aldrich Japan, Tokyo, Japan) was used as a negative control. Lentiviral particles were generated by co-transfecting 293FT cells with the packaging vectors psPAX2 and pMD2.G using TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI).

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio, Kusatsu, Shiga, Japan). Quantitative real-time PCR was performed using TB Green Premix Ex Taq II (Takara Bio) according to the manufacturer's instructions. Primers used for the relative quantification are shown in Supplementary Table 1. The expression levels of human *CDKAL1* and rat *Cdkal1* were normalized to housekeeping genes 18S rRNA. The expression levels of *XBP1* and *ATF4* were normalized to the geometric mean of the levels of *GAPDH*, *GUSB*, or *HPRT1*. Data were analyzed by a ddCt method as described elsewhere.

Analysis of 2-methylthiolation (ms^2)

The level of ms^2 at position A37 of $tRNA^{Lys(UUU)}$ was measured by a quantitative PCR-based method as described previously [19]. Briefly, total RNA was isolated from tumors or cultured cells using TRIzol. Two $tRNA^{Lys(UUU)}$-specific oligonucleotide primers were used in the reverse transcription reaction: reverse primer r1, which was complementary to the region including A37, and reverse primer r2, which was complementary to the downstream region of A37. Total RNA was mixed with 20 pmol of primer r1 or primer r2, and reverse transcription was initiated by adding 0.5 units of recombinant reverse transcriptase (Transcriptor First Strand cDNA Synthesis Kit; Roche Applied Science, Germany). An aliquot of the synthesized cDNA was mixed with a $tRNA^{Lys(UUU)}$-specific forward primer and reverse primer r1, and then subjected to quantitative PCR using the SYBR premix Ex Taq Kit (Takara). For an individual

sample, the threshold cycle number was obtained by quantitative PCR using a cDNA template that was synthesized using reverse primer r1 or primer r2 (referred to as CTr1 or CTr2). The difference between CTr1 and CTr2 was calculated by subtracting CTr1 from CTr2 and was proportional to the level of ms^2 [19]. The following primers were used to detect ms^2 in $tRNA^{Lys(UUU)}$: forward primer, GTCGGTAGAGCATCAGACTT; reverse primer r1, CCTGGACCCTCAGATTAAAA; reverse primer r2, GAACAGGGACTTGAACCCTG.

Western blotting

Cells were collected and lysed in RIPA buffer (1 M Tris-HCl, pH 8.0; 0.5% NP-40; 150 mM NaCl; and protease inhibitors) and sonicated for 10 s. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific). Samples were electrophoresed in 12% polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were incubated with anti-GH (AF1067; Bio-techne, Minneapolis, MN), anti-CDKAL1 (sc-135456; Santa Cruz Biotechnology, Dallas, TX), anti-DnaJ heat shock protein family (Hsp40) member C10 (Dnajc10; 13101-1-AP; Proteintech, Rosemont, IL), anti-Sarco/Endoplasmic Reticulum Ca-ATPase 2 (SERCA2; ab3625; Abcam, Cambridge, MA), and anti-Pit-1 (sc-25258; Santa Cruz Biotechnology) antibodies diluted in phosphate-buffered saline (PBS) overnight at 4°C. Signals were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunofluorescence microscopy

Coverslips were coated with 10% Matrigel matrix (Corning) prepared in PBS (Thermo Fisher Scientific) at 37°C for 1 h. GH3 cells treated with siRNA or shRNA were seeded onto the coverslips, fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical, Osaka, Japan), stained with phalloidin (Thermo Fisher Scientific) to label actin, and observed under a confocal microscope (FV3000; Olympus, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA) of rat GH

Cell culture medium was collected and diluted 1:100 with sterilized distilled water. The level of GH in the medium was analyzed using a Rat Growth Hormone ELISA Kit (BioVender, Karasek, Brno, Czech Republic) according to the manufacturer's instructions.

Intracellular calcium imaging

The calcium level in GH3 cells was monitored using a Calcium Kit-Fluo 4 (Dojindo, Mashiki, Kumamoto, Japan). Cells were washed thrice with PBS, loaded with 2.5 μM Fluo-4 for 30 min, and then washed again with PBS. Fluo-4 fluorescence was observed using a FV3000 confocal microscope.

Electron microscopy

Tumor samples were collected during surgery, immediately fixed with 2% paraformaldehyde and 2% glutaraldehyde as described previously [6], and imaged by transmission electron microscopy.

Statistical analysis

All data were analyzed using GraphPad Prism 6 software. Data are expressed as the mean ± standard error of the mean. Two groups were compared using the Student's *t* test. Multiple groups were compared using a one-way analysis of variance, and then two groups were compared using the Student's *t* test. A two-tailed *p*-value less than 0.05 was considered significant.

Results

CDKAL1-mediated ms^2 is decreased in GHPAs

We investigated CDKAL1 activity by measuring the level of ms^2 in total RNA isolated from growth hormone-producing pituitary adenomas (GHPAs) and non-functioning pituitary adenomas (NFPAs) using a quantitative PCR-based method (Fig. 1A). The modification index represents the level of ms^2 in cytosolic $tRNA^{Lys(UUU)}$. The level of ms^2 was significantly lower in GHPAs than in NFPAs (Fig. 1A). By contrast, expression of *CDKAL1*, which encodes the enzyme that mediates this modification, did not significantly differ between GHPAs and NFPAs (Fig. 1B). In addition, we analyzed the levels of ms^2 and *CDKAL1* mRNA in ACTH-PAs. Similar to GHPAs, the level of ms^2 was significantly lower in ACTH-PAs than in NFPAs (Supplementary Fig. 1). The level of *CDKAL1* mRNA did not differ between ACTH-PAs and NFPAs (Supplementary Fig. 1).

Next, we examined whether the rs7756992 SNP of *CDKAL1* is associated with this difference in the level of ms^2 between GHPAs and NFPAs. rs7756992 is one of the most reproducible T2D-related SNPs [1]. The risk alleles (AG and GG) of rs7756992 are associated with the development of T2D as well as a decrease in ms^2 [9]. The number of patients carrying these risk alleles (AG and GG) did not significantly differ between those with GHPAs and those with NFPAs (Fig. 1C). Nevertheless, the level of ms^2 was significantly lower in GHPAs than in NFPAs for each genotype (Fig. 1D). We did not analyze the genotype effect in ACTH-PAs because the sample size was small (rs7756992: AA = 2, AG = 2, GG = 4). Taken together, these results suggest that a decrease

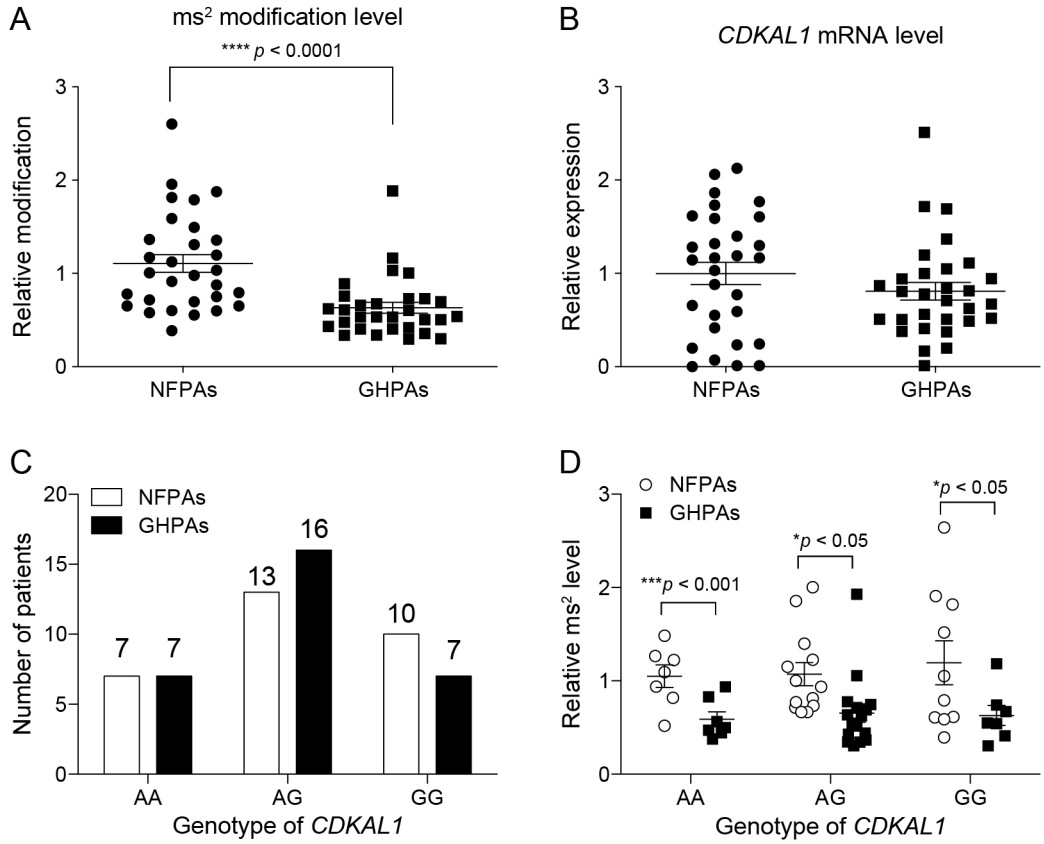

Fig. 1 The level of CDKAL1-mediated ms^2 is decreased in GHPAs

(A) The level of CDKAL1-mediated ms^2 in NFPAs and GHPAs extracted from patients was measured using a quantitative PCR-based method. Note that the level of ms^2 is significantly decreased in GHPAs. $n = 30$ per group. ****$p < 0.0001$. (B) The mRNA level of *CDKAL1* was compared between NFPAs and GHPAs. $n = 30$ per group. (C) The numbers of patients carrying risk and non-risk alleles of *CDKAL1* (rs7756992) are shown. Note that the G allele is the T2D-risk allele. (D) The level of ms^2 in NFPAs and GHPAs of patients with various genotypes are shown. *$p < 0.05$, ***$p < 0.001$.

in the ms^2 level is involved in the pathogenesis of GHPAs; however, this decrease is unlikely due to the risk genotype of *CDKAL1* or a reduction in mRNA expression of *CDKAL1*.

Knockdown of Cdkal1 increases GH biosynthesis

To elucidate the effect of CDKAL1 downregulation in GHPAs, we knocked down *Cdkal1* in rat GH3 pituitary tumor cells by transfecting Cdkal1-targeting siRNA (Cdkal1 siRNA). Transfection of Cdkal1 siRNA significantly decreased mRNA expression of *Cdkal1* (Fig. 2A) and the level of ms^2 (Fig. 2B) in these cells. Western blotting demonstrated that the protein level of GH was markedly higher in cells transfected with Cdkal1 siRNA than in cells transfected with control siRNA (Fig. 2C). In addition, cell culture medium was subjected to an ELISA to examine the effect of Cdkal1 on the secretion of GH. The level of GH was significantly higher in the culture medium of cells transfected with Cdkal1 siRNA than in the culture medium of cells transfected with control siRNA (Fig. 2D).

Next, we generated lentivirus carrying *Cdkal1*-targeting shRNA (shCdkal1). mRNA expression of *Cdkal1* and the level of ms^2 were significantly lower in cells infected with the lentivirus carrying shCdkal1 than in cells infected with the control lentivirus carrying shScramble (Supplementary Fig. 2A and B). Accordingly, shRNA-mediated knockdown of *Cdkal1* decreased and increased the protein levels of CDKAL1 and GH in GH3 cells, respectively (Fig. 3A). Consistently, the level of GH was significantly higher in the culture medium of cells infected with the lentivirus carrying shCdkal1 than in the culture medium of cells infected with the lentivirus carrying shScramble (Fig. 3B). Furthermore, immunocytochemistry demonstrated that the fluorescence intensity of GH-containing granules was higher in cells infected with the lentivirus carrying shCdkal1 than in cells infected with the lentivirus carrying shScramble (Fig. 3C). Thus, knockdown of *Cdkal1* increases GH biosynthesis in GH3 cells.

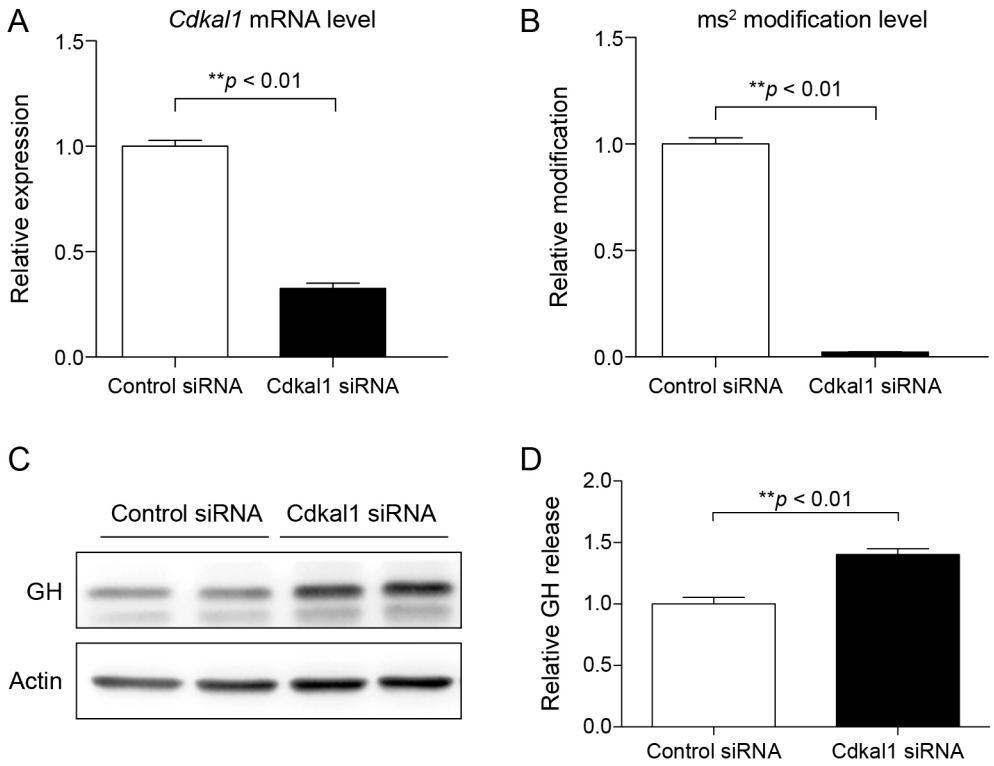

Fig. 2 siRNA-mediated knockdown of *Cdkal1* increases GH biosynthesis in GH3 cells

(A) Relative mRNA expression of *Cdkal1* in GH3 cells transfected with control or Cdkal1 siRNA was measured by quantitative PCR. $n = 6$ per group. $^{**}p < 0.01$. (B) The level of ms^2 was measured in GH3 cells transfected with control or Cdkal1 siRNA. Note that knockdown of *Cdkal1* significantly decreased the level of ms^2. $n = 6$ per group. $^{**}p < 0.01$. (C) The protein level of GH in GH3 cells transfected with control or Cdkal1 siRNA was measured by Western blotting. Note that knockdown of *Cdkal1* increased the protein level of GH. (D) The level of GH in the culture medium of GH3 cells transfected with control or Cdkal1 siRNA was measured using an ELISA, and normalized against the cell number. $n = 6$ per group. $^{**}p < 0.01$.

Knockdown of Cdkal1 alters calcium signaling

Next, we investigated the molecular pathway by which downregulation of *Cdkal1* increases GH biosynthesis. A complex mechanism involving calcium signaling regulates the biosynthesis of GH [20]. Thus, we used the Fluo-4 calcium indicator to determine the cytosolic calcium level in GH3 cells in which *Cdkal1* had been downregulated using siRNA (Fig. 4A and B) or shRNA (Fig. 4C and D). The basal cytosolic calcium level was significantly higher in cells transfected with Cdkal1 siRNA than in cells transfected with control siRNA. Under normal conditions, the majority of cytosolic calcium is transported into the endoplasmic reticulum (ER) by SERCA proteins, and consequently the cytosolic calcium level remains low [21]. SERCA2, a member of the SERCA family, is ubiquitously expressed in organs and tissues of mammals. Dnajc10 (also known as Erdj5) physically interacts with SERCA2, and regulates the calcium pump function of SERCA2 in a redox-dependent manner [22]. Western blotting demonstrated that the protein level of Dnajc10, but not SERCA2, was markedly decreased in cells infected with the lentivirus carrying shCdkal1 (Fig. 4E and F). Calcium not only triggers release of GH but also enhances its transcription *via* Pit-1, which is the major transcription factor for pituitary hormones [23]. Consistently, knockdown of *Cdkal1* increased the protein level of Pit-1 (Fig. 4F), resulting in upregulation of *GH* mRNA (Fig. 4G). These results suggest that upregulation of GH in *Cdkal1*-knockdown GH3 cells is partly due to altered calcium signaling.

Proteostatic stress regulates the function of CDKAL1

We finally investigated the molecular mechanism underlying the differential regulation of CDKAL1 activity in GHPAs and NFPAs. In general, synthesis of large amounts of secretory proteins inevitably leads to production of aberrant proteins, which causes proteostatic stress in secretory cells [24]. In contrast with NFPAs, the majority of protein synthesis in GHPAs is dedicated to biosynthesis of GH [15]. Electron microscopy demonstrated that the ER was markedly dilated in the pituitary tumor cells from biopsies of GHPAs, which is indicative of proteostatic stress (Fig. 5A). By contrast, the ER had a normal morphology or was moderately dilated in pitui-

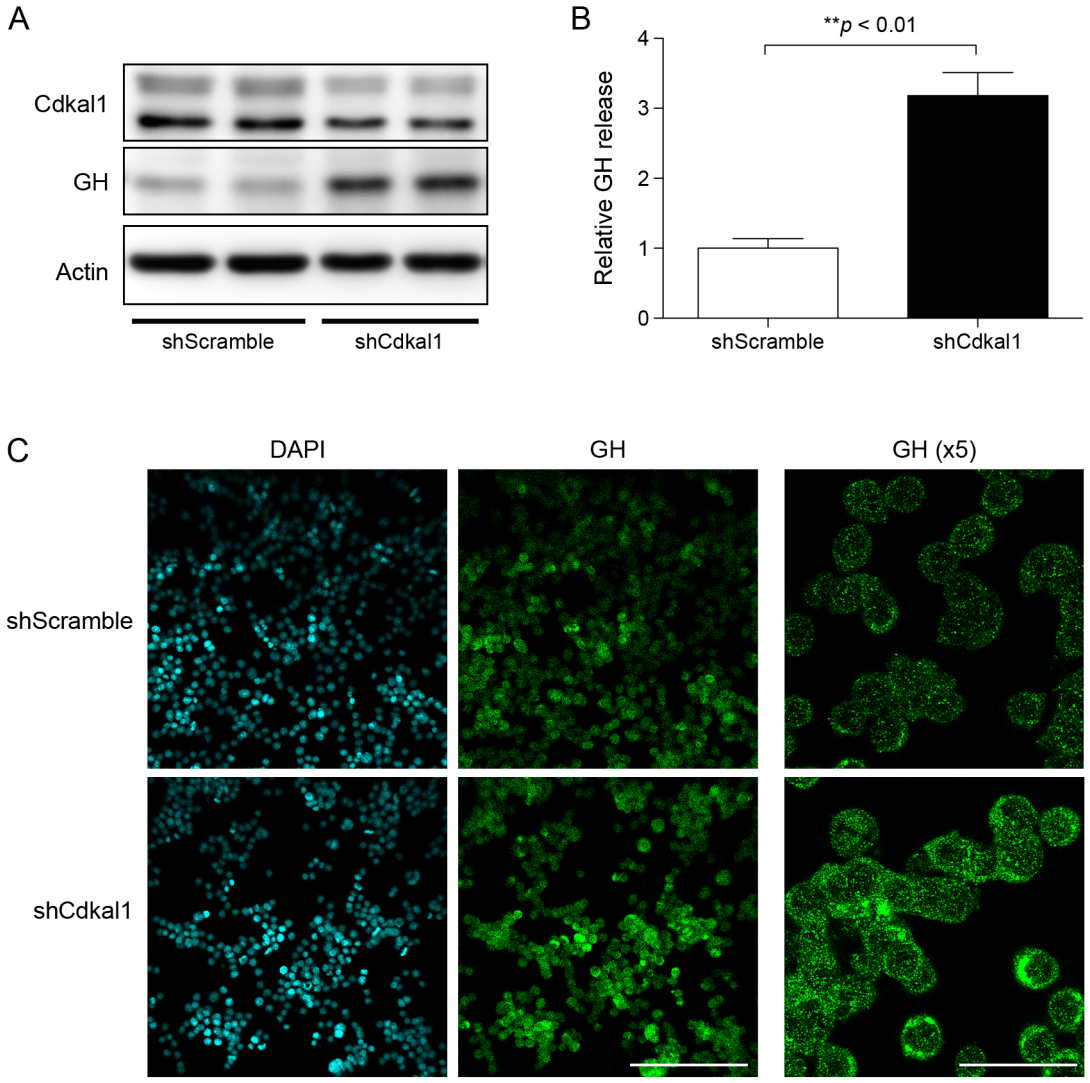

Fig. 3 shRNA-mediated knockdown of *Cdkal1* increases GH biosynthesis in GH3 cells

(A) The protein levels of Cdkal1 and GH in GH3 cells infected with lentivirus carrying shCdkal1 or shScramble were examined by Western blotting. (B) The level of GH in the culture medium of GH3 cells infected with a lentivirus carrying shCdkal1 or shScramble was measured using an ELISA, and normalized against the cell number. $n = 6$. $^{**}p < 0.01$. (C) Immunofluorescence staining of GH was performed in GH3 cells infected with lentivirus carrying shCdkal1 or shScramble. Fluorescence corresponding to GH staining was strong and punctate in *Cdkal1*-knockdown cells. Magnified images are shown on the right. Bars = 200 μm (middle panel) and 40 μm (right panel).

tary tumor cells from biopsies of NFPAs (Fig. 5A). Consistently, the expression levels of genes related to the proteostatic stress response, such as *XBP1* and *ATF4*, were significantly higher in GHPAs than in NFPAs (Fig. 5B). Furthermore, the expression level of *XBP1* was negatively associated with the level of ms^2 (Fig. 5C). These results suggest that proteostatic stress is closely correlated with the decrease in CDKAL1 activity. Finally, to examine whether proteostatic stress is capable to affect CDKAL1 activity, we treated GH3 cells with tunicamycin (TM) and thapsigargin (TG) to induce proteostatic stress. Treatment with TM and TG significantly decreased the level of ms^2 (Fig. 5D). Conversely, treatment with Sal003, which prevents proteostatic stress by inhibiting dephosphorylation of eukaryotic initiation factor 2 subunit α, significantly increased the level of ms^2 (Fig. 5D). TM, TG, and Sal003 did not affect the expression level of *Cdkal1* in GH3 cells (Supplementary Fig. 3); therefore, these reagents likely altered the ms^2 level *via* a post-transcriptional mechanism.

Discussion

The present study reveals the molecular function of the tRNA-modifying enzyme CDKAL1 in the pathogenesis of GHPAs. CDKAL1 activity was suppressed in

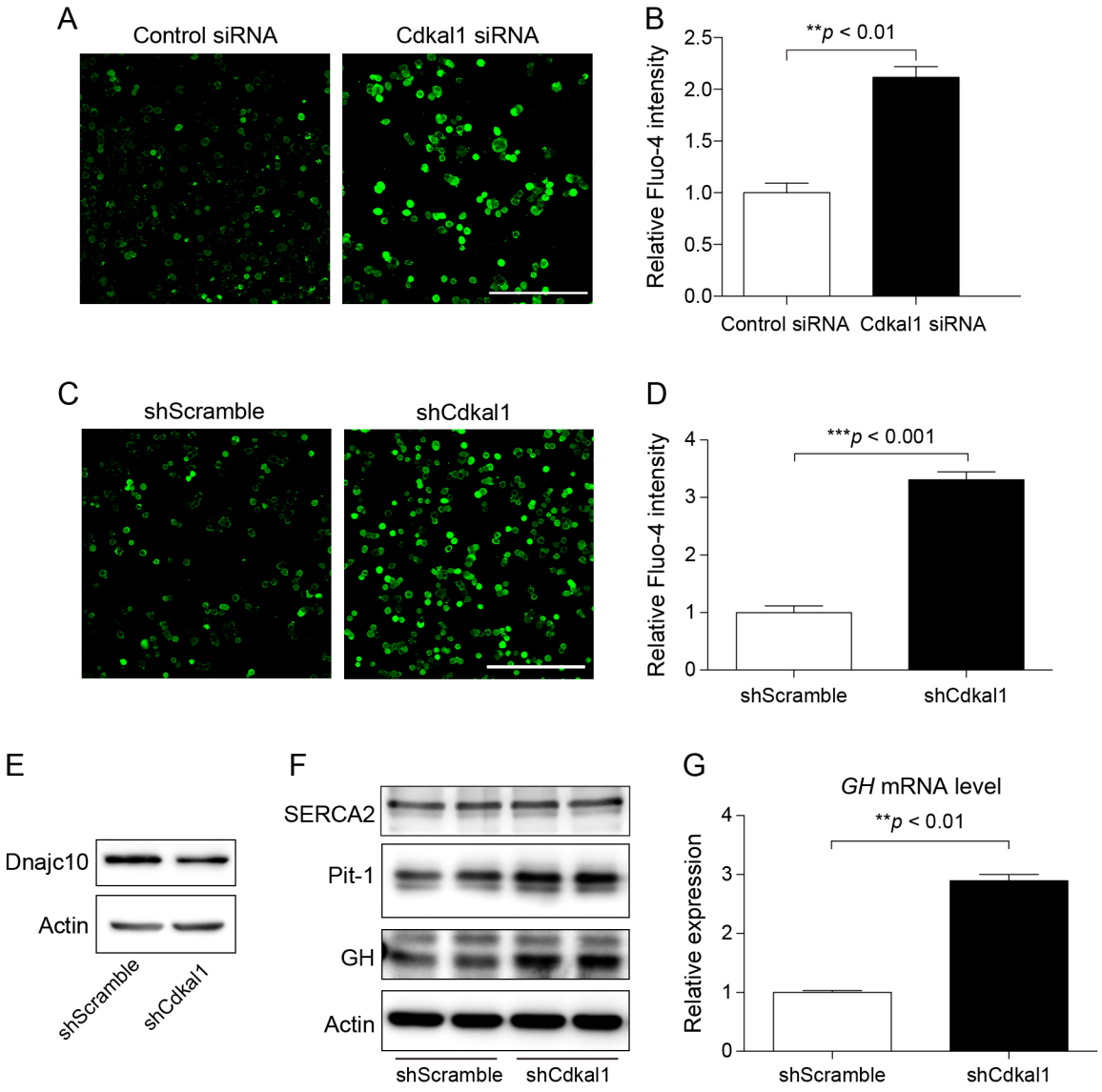

Fig. 4 Knockdown of *Cdkal1* alters calcium homeostasis in GH3 cells

(A and B) GH3 cells were transfected with control or Cdkal1 siRNA. Intracellular calcium was visualized using Fluo-4 (A). Bar = 200 μm. The fluorescence intensity of Fluo-4 was quantified (B). $n = 4$, $^{**}p < 0.01$. (C and D) GH3 cells were infected with lentivirus carrying shScramble or shCdkal1. Intracellular calcium was visualized using Fluo-4 (C). Bar = 200 μm. The fluorescence intensity of Fluo-4 was quantified (D). $n = 6$, $^{***}p < 0.001$. (E–F) Protein levels of Dnajc10, SERCA2, Pit-1, and GH in GH3 cells infected with lentivirus carrying shScramble or shCdkal1 were examined by Western blotting. (G) mRNA expression of *GH* was significantly increased in GH3 cells infected with lentivirus carrying shCdkal1. $n = 6$, $^{**}p < 0.01$.

GHPAs due to proteostatic stress. Knockdown of *Cdkal1* increased the biosynthesis and secretion of GH in GH3 cells. Downregulation of *Cdkal1* suppressed expression of Dnajc10, which increased the cytosolic calcium level, leading to upregulation of *GH* expression through Pit-1.

An important finding of this study is that downregulation of CDKAL1 in pituitary adenomas was associated with increased biosynthesis of GH. However, upregulation of GH secretion upon knockdown of *Cdkal1* is surprising at the molecular level because loss of CDKAL1 perturbs ms^2 of $tRNA^{Lys(UUU)}$, leading to impaired insulin biosynthesis in mouse and human pancreatic β-cells [6, 9]. Proinsulin contains two Lys residues, one of which is required for cleavage of this protein to generate mature insulin. Deficiency of CDKAL1 causes mistranslation of Lys, which impairs proinsulin processing and thereby decreases the mature insulin content. On the other hand, GH is cleaved between Ala26 and Phe27 to generate the mature form [25]. Thus, CDKAL1 dysfunction likely has less impact on production of GH due to the differential significance of Lys translation in hormone processing.

The present study showed that Cdkal1 is involved in

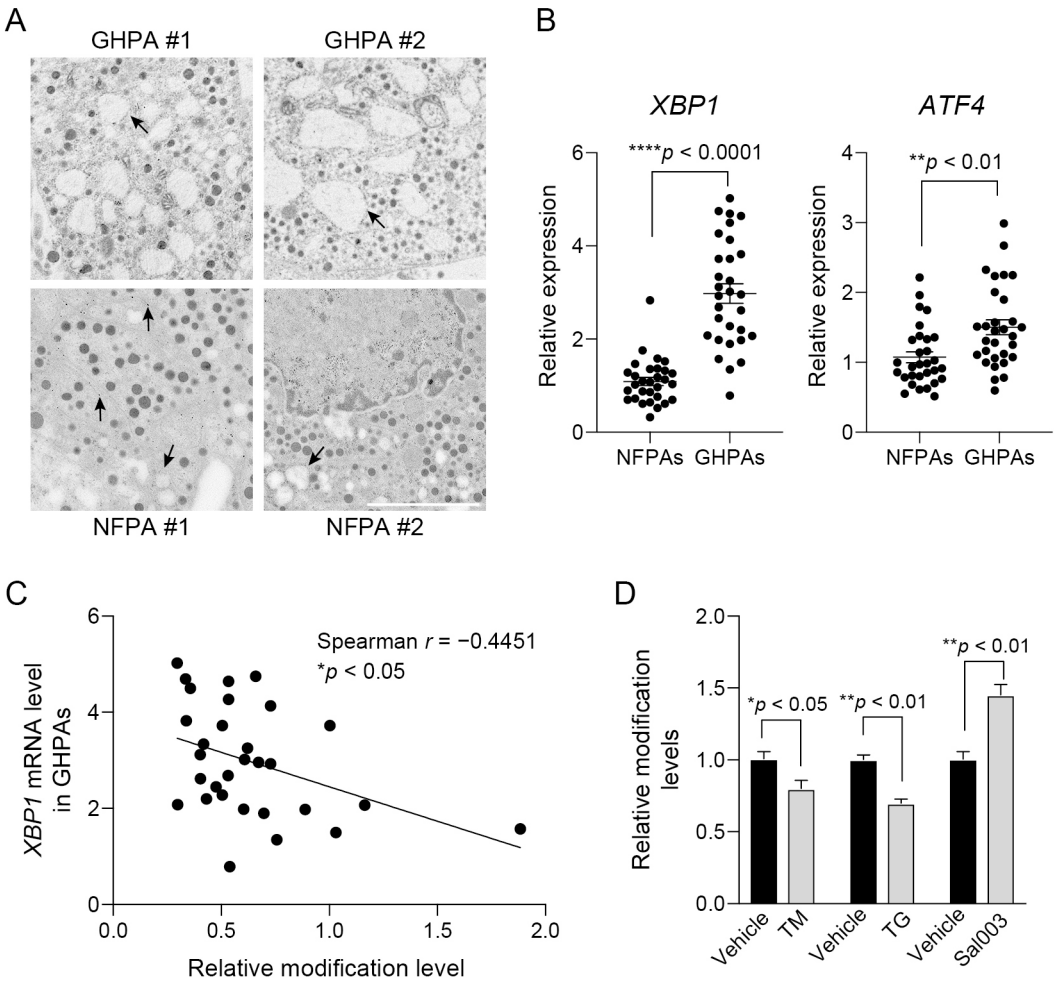

Fig. 5 Proteostatic stress regulates CDKAL1 activity

(A) Electron micrographs of GHPAs and NFPAs are shown. White arrows indicate dilated ER structures. Note that GHPA#1 and GHPA#2 were derived from two patients, while NFPA#1 and NFPA#2 were derived from one patient. Black arrows indicate normal ER structures. Bar = 2 μm. (B) Expression levels of *XBP1* and *ATF4* in NFPAs and GHPAs were examined by quantitative PCR. $n = 30$ per group. $^{**}p < 0.01$, $^{****}p < 0.0001$. (C) The expression level of *XBP1* was negatively associated with the level of ms^2 in GHPAs. (D) The level of ms^2 was measured in GH3 cells treated with 10 μM TM, 10 μM TG, or 2 μg/mL Sal003. $n = 6$ per group, $^{*}p < 0.05$, $^{**}p < 0.01$.

the regulation of intracellular calcium homeostasis through Dnajc10, which functions in GH biosynthesis. Dnajc10 is a disulfide reductase located in the ER and is the master regulator of calcium homeostasis in a redox-dependent manner [22, 26]. Specifically, Dnajc10 directly reduces the disulfide bond of SERCA2, leading to influx of calcium from the cytosol into the ER. Dysfunction of Dnajc10 or SERCA2 alters calcium homeostasis [22, 27]. Consistently, knockdown of *Cdkal1* markedly reduced the protein level of Dnajc10, which was associated with an increased level of cytosolic calcium. Notably, Dnajc10 is highly expressed in secretory cells, including those in the hypothalamus, brain stem, and adrenal gland of mice [26, 28]. According to a human RNA database, the level of Dnajc10 transcripts is high in the pituitary gland and low in the pancreas. These results suggest that dysregulation of Dnajc10 upon downregulation of CDKAL1 is involved in the pathogenesis of GHPAs. A further study is required to investigate whether Dnajc10 is controlled at transcriptional or post-translational level by CDKAL1.

Ca^{2+} is a major second messenger that not only controls exocytosis of hormones but also regulates gene expression. In the pituitary gland, Ca^{2+} is absolutely required for release of GH [20]. Moreover, an increase in Ca^{2+} is associated with upregulation of *GH* expression in GH3 cells [29]. Various transcription factors, including Pit-1 and CREB, regulate transcription of *GH*. Importantly, Ca^{2+} is required for Pit-1-mediated transcription in GH3 cells [30]. Consistently, knockdown of *Cdkal1* increased the level of Pit-1 in GH3 cells, which was associated with increased mRNA expression of *GH*.

This study revealed that the proteostatic stress status regulates CDKAL1 activity in pituitary cells. In general, tRNA modifications are considered to be static [31]. However, CDKAL1-mediated ms^2 is rapidly reactive to proteostatic stress. The level of ms^2 is decreased by proteostatic stress and increased by inhibition of this stress. Proteostatic stress is usually triggered by protein misfolding in the ER, which induces various cellular responses at the translational and transcriptional levels [32]. Notably, proteostatic stress is closely coupled with the oxidative stress response because reactive oxygen species are usually generated as byproducts during protein folding [32]. Interesting, iron-sulfur clusters and reactive sulfur species, which are highly sensitive to oxidative stress, are required for CDKAL1 activity [33]. Indeed, oxidative stress effectively suppresses CDKAL1 function, which might explain the reduction in CDKAL1 activity in GHPAs. A further study is required to elucidate the potential role of oxidative stress in the regulation of CDKAL1 activity during the pathogenesis of GHPAs. CDKAL1 activity was increased in GH3 cells treated with Sal003, which inhibits proteostatic stress. Interestingly, Sal003 effectively induces cell death in various cancer cells such as leukemia and glioblastoma cells, suggesting that proteostatic stress is a therapeutic target in GHPAs [34, 35].

GHPAs can be microscopically categorized into two types: densely and sparsely granulated [36]. Densely granulated pituitary adenomas are strongly immunopositive for GH and contain large secretory granules. On the other hand, sparsely granulated pituitary adenomas are weakly immunopositive for GH and only contain a few small granules. Given the high demand for biosynthesis of GH, densely granulated pituitary adenomas are likely exposed to a high level of proteostatic stress, which might affect CDKAL1 activity. A further study is required to investigate whether CDKAL1 activity and the mRNA expression level of *CDKAL1* are differentially regulated in densely and sparsely granulated pituitary adenomas.

Excess GH affects insulin sensitivity and can alter the function of pancreatic β-cells [16, 17]. Chronic GH excess induces insulin resistance by promoting gluconeogenesis in the liver and suppressing glucose uptake in muscles. Importantly, prolonged hyperglycemia can exaggerate oxidative stress in pancreatic β-cells, leading to impairment of insulin secretion [37]. Given the susceptibility of CDKAL1 activity to oxidative stress, it is conceivable that chronic GH excess indirectly decreases CDKAL1 activity in pancreatic β-cells, which ultimately impairs biosynthesis and secretion of insulin.

In conclusion, we demonstrated that downregulation of CDKAL1 is involved in the pathogenesis of GHPAs. Proteostatic stress suppressed CDKAL1 activity, which led to dysregulation of calcium homeostasis *via* Dnajc10 downregulation and, consequently, upregulation of GH production.

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Disclosure

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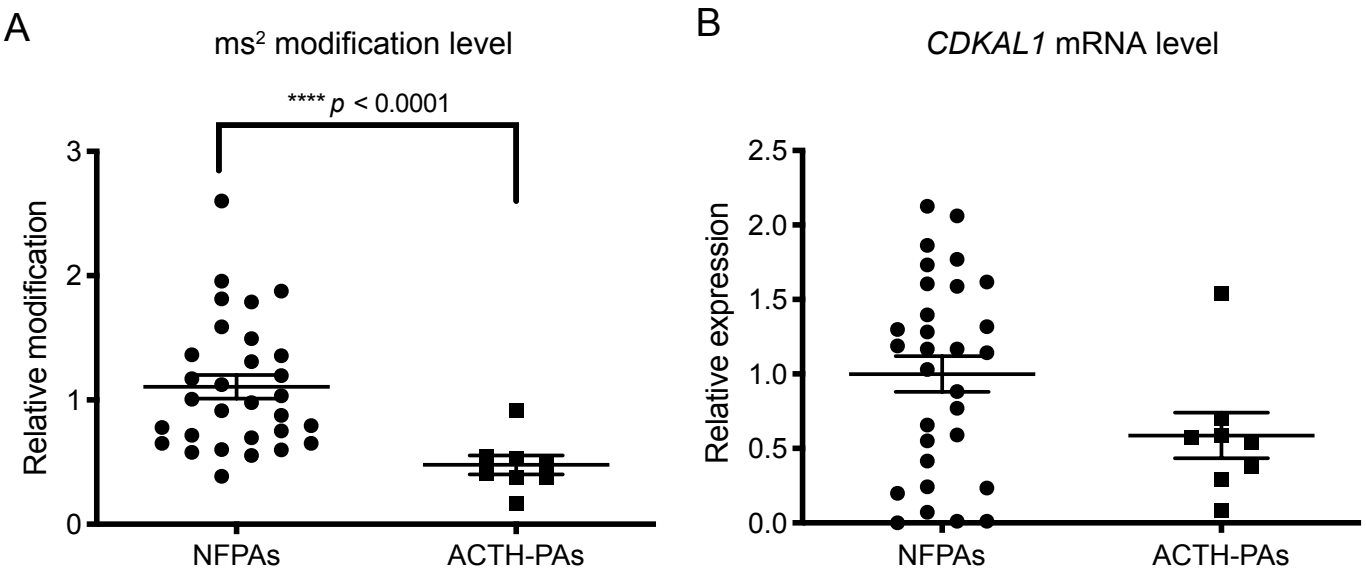
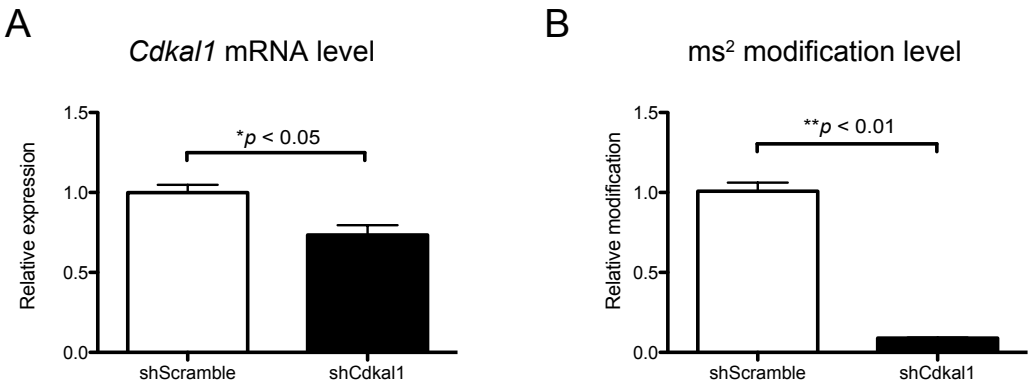
Supplementary Table 1 Sequences of primers used for quantitative PCR

Primer Name	Primer Sequence
Human_ATF4_Forward	GGTTCTCCAGCGACAAGG
Human_ATF4_Reverse	TCTCCAACATCCAATCTGTCC
Human_XBP1_Forward	CCTGGTTGCTGAAGAGGAGG
Human_XBP1_Reverse	CCATGGGGAGATGTTCTGGAG
Human_CDKAL1_Forward	ATGTTGTCCCGAAGGTACGAA
Human_CDKAL1_Reverse	GCTGTCACTTGGTGGACTGTT
Human_GAPDH_Forward	CTGGGCTACACTGAGCACC
Human_GAPDH_Reverse	AAGTGGTCGTTGAGGGCAATG
Human_GUSB_Forward	GTCTGCGGCATTTTGTCGG
Human_GUSB_Reverse	CACACGATGGCATAGGAATGG
Human_HPRT1_Forward	CCTGGCGTCGTGATTAGTGAT
Human_HPRT1_Reverse	AGACGTTCAGTCCTGTCCATAA
Human/Rat_18S rRNA_Forward	GTAACCCGTTGAACCCCATT
Human/Rat_18S rRNA_Reverse	CCATCCAATCGGTAGTAGCG
Rat_Cdkal1_Forward	GATGGAGAATACATGGCTGGA
Rat_Cdkal1_Reverse	CTGTTCAGGAGCCACAGATCT
Rat_GH_Forward	CAAAGAGTTCGAGCGTGCCTA
Rat_GH_Reverse	TGGGATGGTCTCTGAGAAGCA

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3