

Measurement of the nuclear concentration of α -ketoglutarate during adipocyte differentiation by using a fluorescence resonance energy transfer-based biosensor with nuclear localization signals

Tomohiro Suzuki^{1)*}, Mayuko Hayashi^{1)*}, Tetsuro Komatsu¹⁾, Akiko Tanioka¹⁾, Masahiro Nagasawa¹⁾, Kyoko Tanimura-Inagaki^{1),2)}, Mohammad Sharifur Rahman¹⁾, Shinnosuke Masuda¹⁾, Kosuke Yusa³⁾, Juro Sakai^{4),5)}, Hiroshi Shibata¹⁾ and Takeshi Inagaki¹⁾

¹⁾ Laboratory of Epigenetics and Metabolism, Institute for Molecular and Cellular Regulation, Gunma University, Gunma 371-8512, Japan

²⁾ Division of Diabetes, Endocrinology, and Metabolism, Department of Medicine, Nippon Medical School, Tokyo 113-8602, Japan

³⁾ Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

⁴⁾ Division of Metabolic Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan

⁵⁾ Division of Molecular Physiology and Metabolism, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

Abstract. α -Ketoglutarate (α -KG) also known as 2-oxoglutarate (2-OG) is an intermediate metabolite in the tricarboxylic acid (TCA) cycle and is also produced by the deamination of glutamate. It is an indispensable cofactor for a series of 2-oxoglutarate-dependent oxygenases including epigenetic modifiers such as ten-eleven translocation DNA demethylases (TETs) and JmjC domain-containing histone demethylases (JMJDs). Since these epigenetic enzymes target genomic DNA and histone in the nucleus, the nuclear concentration of α -KG would affect the levels of transcription by modulating the activity of the epigenetic enzymes. Thus, it is of great interest to measure the nuclear concentration of α -KG to elucidate the regulatory mechanism of these enzymes. Here, we report a novel fluorescence resonance energy transfer (FRET)-based biosensor with multiple nuclear localization signals (NLSs) to measure the nuclear concentration of α -KG. The probe contains the α -KG-binding GAF domain of NifA protein from *Azotobacter vinelandii* fused with EYFP and ECFP. Treatment of 3T3-L1 preadipocytes expressing this probe with either dimethyl-2-oxoglutarate (dimethyl-2-OG), a cell-permeable 2-OG derivative, or citrate elicited time- and dose-dependent changes in the FRET ratio, proving that this probe functions as an α -KG sensor. Measurement of the nuclear α -KG levels in the 3T3-L1 cells stably expressing the probe during adipocyte differentiation revealed that the nuclear concentration of α -KG increased in the early stage of differentiation and remained high thereafter. Thus, this nuclear-localized α -KG probe is a powerful tool for real-time monitoring of α -KG concentrations with subcellular resolution in living cells and is useful for elucidating the regulatory mechanisms of epigenetic enzymes.

Key words: α -Ketoglutarate, Subcellular concentration, Fluorescence resonance energy transfer, Nuclear probe, Adipocyte differentiation

α -KETOGLUTARATE (α -KG), which is also known as 2-oxoglutarate (2-OG), has recently gained interest as a mediator of cellular functions by affecting the activity of 2-oxoglutarate-dependent oxygenases (2OGDs) [1].

Submitted Apr. 15, 2021; Accepted Jun. 17, 2021 as EJ21-0255
Released online in J-STAGE as advance publication Jul. 15, 2021
Correspondence to: Takeshi Inagaki, M.D., Ph.D., Laboratory of Epigenetics and Metabolism, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-machi, Maebashi, Gunma 371-8512, Japan.

E-mail: inagaki@gunma-u.ac.jp

*These authors contributed equally to this work.

The 2OGD family includes members which regulate a variety of biological processes, including regulation of transcription and protein biosynthesis, nucleic acid repair, hypoxia sensing, collagen synthesis, and metabolism of lipid and growth factors. Most 2OGDs require both α -KG and oxygen as co-substrates, use ferrous iron as a cofactor, and produce CO₂ and succinate as by-products. Since the activity of 2OGDs depends on the availability of these co-substrates and the cofactor, 2OGDs sense cellular metabolism and redox status by detecting the concentration of α -KG, oxygen, and iron [2]. For example, prolyl hydroxylases (PHDs) reduce

their activity to hydroxylate the hypoxia-inducible factors (HIFs) by sensing hypoxia, thus avoiding the association of HIFs with von Hippel-Lindau (VHL) tumor suppressor pVHL, a substrate recognition component of the E3 ubiquitin ligase complex, thereby increasing stability of HIF proteins [2]. Another important example is that epigenetic modifying 2OGDs such as JmjC domain-containing histone lysine demethylases (JMJDs), the ten-eleven translocation family of DNA demethylases (TETs), and RNA demethylases including the fat mass and obesity-associated gene product (FTO, also known as ALKBH9), sense the intracellular concentration of α -KG, a central metabolic intermediate in the tricarboxylic acid (TCA) cycle, and regulate both transcription and translation [3, 4]. Since the methylation levels of histone and DNA affect the transcription of genes in vicinity regions, and the methylation of mRNA, such as the N⁶-methyladenosine (m⁶A) modification, affects its splicing and stability, the α -KG level may be sensed and it may influence the transcriptional and translational regulation by epigenetic enzymes.

α -KG is generated in the TCA cycle from citrate by isocitrate dehydrogenases (IDHs) according to the chemical equilibrium in the mitochondria matrix. It is also produced by the deamination of glutamate in amino acid metabolism. Studies in cancer cells and lineage-specific progenitor cells harboring IDH mutations have revealed that α -KG levels regulate cell differentiation [5, 6]. IDH mutants in various cancers inhibit the activity of epigenetic modifying 2OGDs by converting α -KG to 2-hydroxyglutarate (2HG), thereby promoting tumor progression. Mutated IDHs also prevent terminal differentiation of hematopoietic cells [7] and 3T3-L1 preadipocytes [6]. It is also reported that α -KG maintains the pluripotency of embryonic stem cells by modulating the activity of JMJDs and TETs [8]. These findings indicate that α -KG plays a pivotal role in the regulation of cellular functions through epigenetic control.

Considering that epigenetic modifications of histone and genomic DNA occur in the nucleus, it is inevitable to measure the concentration of α -KG in the nucleus because there may be a discrepancy in α -KG concentration between the organelles that produce α -KG and those that consume it. The regulation of cellular function by 2OGDs is at least partially dependent on their specific subcellular localization and differences in K_m values over a range of several hundred folds. As examples of organelle-specific functions of 2OGDs, procollagen C-4 prolyl hydroxylase (CP4H) catalyzes the *trans*-C-4 hydroxylation of procollagen in the endoplasmic reticulum (ER) [2, 9], and aspartyl/asparaginyl beta-hydroxylase (ASPH) catalyzes the post-translational hydroxylation of epidermal growth factor (EGF) in the

ER [2, 10]. Therefore, it would be of great importance to measure the organelle-specific concentrations of α -KG, which is sensed by 2OGDs at a specific subcellular location to mediate cellular function. However, with the conventional approach using cell extracts, only the total cellular amount of α -KG can be obtained, making it difficult to determine the α -KG concentration at the subcellular (organelle) level. Here, we report a novel fluorescence resonance energy transfer (FRET)-based biosensor [11, 12] for α -KG that enables the determination of the nuclear concentrations of α -KG. By using this FRET probe, we measured the changes in the nuclear concentration of α -KG during adipocyte differentiation.

Materials and Methods

Cell culture

3T3-L1 preadipocytes (kindly provided by Howard Green, Harvard Medical School) [13] were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (4.5 g/L D-glucose) (FUJIFILM Wako, Osaka, Japan) supplemented with 10% calf serum at 37°C in 5% CO₂. The cells were plated onto a 35 mm glass-bottom dish (MatTek, MA, USA) to observe fluorescence. For adipocyte differentiation, 2 days after confluence (day 0), 3T3-L1 preadipocytes were treated with DMEM-high glucose supplemented with 10% fetal bovine serum (DMEM-FBS) containing 1.67 μ M insulin (Sigma-Aldrich, MO, USA), 1 μ M dexamethasone (Sigma-Aldrich), and 115 μ g/mL isobutylmethylxanthine (IBMX) (Sigma-Aldrich) (DMI mixture) for 48 hours followed by 1.67 μ M insulin treatment for 48 hours. Subsequently, the cells were cultured in DMEM-FBS with medium replacement every 2 days. Deferoxamine treatment was performed by adding to the medium at the concentration of 100 μ M for 2 days from day 0 of differentiation. The expression levels of the genes of interest during the differentiation of 3T3-L1 cells were analyzed using the microarray dataset previously reported in [14].

Plasmid construction

The GAF-AAA⁺ sequence coding 1–400 amino acid (a.a.) of NifA (*A. vinelandii*, UniProt: P09570-1) [15, 16] with codon-optimization was synthesized commercially (GenScript, NJ, USA). An expression plasmid for the GAF-AAA⁺ fused with an enhanced cyan fluorescent protein (ECFP) and an enhanced yellow fluorescent protein (EYFP) (pcDNA3-EYFP-GAF-AAA⁺-ECFP plasmid) was prepared by replacing Epacl of pcDNA3-EYFP-Epacl-ECFP [17] with GAF-AAA⁺ using restriction-enzyme-based technique at the EcoRI and XbaI sites. To construct a plasmid containing multiple nuclear localization signals (NLSs), a DNA sequence of

three copies of NLS from SV40 (3xSV40 NLS) was synthesized commercially (Bioneer, Daejeon, Korea) and amplified by PCR. The PCR product and a HindIII-digested pcDNA3-EYFP-GAF-AAA+-ECFP fragment were assembled by Gibson assembly in the buffer containing 4 mU/ μ L T5 exonuclease (NEB, MA, USA), 4 U/ μ L Taq DNA ligase (NEB), 0.25 U/ μ L Phusion polymerase (NEB), 0.1 mM each dNTP, 0.1 M Tris-HCl (pH 7.5), 5 mM MgCl₂, 10 mM dithiothreitol, 6.3 mM polyethylene glycol 8,000, and 1 mM β -nicotinamide adenine dinucleotide. The obtained plasmid was named pcDNA3-3xSV40 NLS-EYFP-GAF-AAA+-ECFP plasmid. For producing a *piggyBac* plasmid, the sequence of 3xSV40 NLS-EYFP-GAF-AAA+-ECFP was amplified by PCR and subcloned by Gibson assembly into a CMV promoter-driven *piggyBac* plasmid (pPBpuro-MCS; kindly provided by Dr. Michiyuki Matsuda, Kyoto University) linearized by BamHI and XhoI digestion (pPB-3xSV40 NLS-EYFP-GAF-AAA+-ECFP). Subsequently, to exclude the original XhoI site on the backbone plasmid and introduce a unique XhoI site between the ECFP sequence and the stop codon, a partial AAA+ and ECFP sequence was amplified by PCR using a primer containing an additional XhoI site, and the PCR product was assembled by Gibson assembly with a digested fragment of pPB-3xSV40 NLS-EYFP-GAF-AAA+-ECFP plasmid at the EcoNI and AflIII sites. Additionally, a RexNLS sequence was amplified by PCR using the pHRdSV40-scFv-GCN4-sfGFP-VP64-GB1-NLS plasmid (Addgene #60904) as a template and was inserted into the newly introduced XhoI site by Gibson assembly. To produce a plasmid containing an additional SV40 NLS (pPB-3xSV40 NLS-EYFP-GAF-AAA+-ECFP-RexNLS-SV40 NLS), a double-stranded sequence for SV40 NLS was prepared by primer annealing and elongation with Phusion polymerase and was inserted at the end of RexNLS with the XhoI site by Gibson assembly. For plasmid preparation, all plasmids were transformed into DH5 α , amplified, and purified using either the LaboPass Plasmid Mini (Hokkaido System Science, Hokkaido, Japan) or the PureLink HiPure Plasmid Midiprep Kit (Invitrogen, CA, USA).

Transient transfection by electroporation

The GenePulser Xcell (BioRad, CA, USA) was used for transfection of the pcDNA3-based expression vectors. In detail, 4×10^6 3T3-L1 preadipocytes were pelleted by centrifugation at 800 rpm for 3 minutes at 4°C and resuspended in 750 μ L GenePulser Electroporation Buffer (BioRad). The cell suspension was mixed with 20 μ g of either pcDNA3-EYFP-GAF-AAA+-ECFP or pcDNA3-3xSV40 NLS-EYFP-GAF-AAA+-ECFP, and electroporation was performed in a 0.4 cm-gap

cuvette with the setting at voltage: 0.2 kV, time constant: 28 milliseconds, and gap: 0.4 cm. The cells were spread on a 35 mm glass-bottom dish and cultured for 24 hours before observation.

Establishment of stable cell line

For the establishment of 3T3-L1 preadipocytes stably expressing 3xSV40 NLS-EYFP-GAF-AAA+-ECFP-Rex NLS-SV40 NLS, the plasmids were transfected using 4D-Nucleofector (Lonza, Basel, Swiss). In detail, 5×10^4 3T3-L1 preadipocytes were pelleted by centrifugation at $100 \times g$ for 3 minutes at 4°C and resuspended in a buffer containing 16.4 μ L of SE Cell Line Solution (Lonza) and 3.6 μ L of Supplement 1 (Lonza) from the Amaxa SE Cell line 4D-Nucleofector X Kit (Lonza). The cell suspension was mixed with 700 ng of pCMV-mPBase [18] and 1,750 ng of pPB-3xSV40 NLS-EYFP-GAF-AAA+-ECFP-Rex NLS-SV40 NLS, transferred to a 16-well Nucleocuvette Strip (Lonza), and then transfected using the program CM137 with 4D-Nucleofector. The cells were spread on the cell culture plate and screened by puromycin at 4 μ g/mL for 3 days to obtain the stable cell line.

Cell imaging

Culture medium was replaced with Hank's balanced salt solution containing 20 mM HEPES/NaOH, pH 7.4 (HBSS) before imaging. For localization analysis of the EYFP-GAF-AAA+-ECFP, cells were imaged with a 3 CCD-based fluorescent microscope system (Hamamatsu Photonics, Hamamatsu, Japan) equipped with a 438/24 nm excitation filter, a 458 nm dichroic mirror, and emission filters (483/32 nm for ECFP and 542/27 nm for EYFP). To study the time-lapse changes in FRET, 3T3-L1 cells were treated with the indicated concentrations of dimethyl-2-oxoglutarate (dimethyl-2-OG) (Sigma-Aldrich, #349631) or citrate (FUJIFILM WAKO, Osaka, Japan, #035-03495) diluted in HBSS with pH adjustment to 7.4. For dual-emission ratio imaging of ECFP and EYFP, the cells were observed at room temperature on a fluorescence microscope BZ-X710 (KEYENCE, Osaka, Japan) equipped with a 436/20 nm excitation filter, a 455 nm dichroic mirror, and two emission filters (480/40 nm for ECFP, 535/30 nm for EYFP). The image data was then analyzed with the OLYMPUS FLUOVIEW software (ver.4.2b). Regions of interest (ROIs) were set on each cell with the same-sized background (BG) regions corresponding to each ROI in the intercellular area in the vicinity of each cell, and the relative fluorescence intensity was calculated after subtracting the corresponding background value from the fluorescence value of each cell (ROI-BG). Image processing (e.g. pseudo-color assignment) was carried out using ImageJ/Fiji

(<https://imagej.nih.gov/ij/>). For generating YFP/CFP ratio images, the pixel which presented the lowest signal in each image/channel was subtracted as a background from the signals of other pixels in the image, and the signal values in each pixel in YFP was divided by that of CFP using the ImageJ plugin Ratio Plus with the setting of clipping values at 7,500 (YFP) for the experiment of dimethyl-2-OG treatment and 10,000 (YFP) for the experiment of adipocyte differentiation.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from 3T3-L1 cells at each time point during differentiation using TRIpure reagent (Meridian Bioscience). Obtained RNA was treated with DNase I (Nippon Gene) and subsequently reverse-transcribed into cDNA using SuperScript II (Invitrogen) and oligo dT₁₅ primer. RT-qPCR was performed using QuantStudio 6 Flex (Applied Biosystems) and each reaction contained cDNA, PowerUp SYBR Green Master Mix (Applied Biosystems), and a specific primer set as below in a total volume of 10 μ L. The primers used were as follows: *Cyclophilin B* (*Ppib*) forward: 5'-GGAGATG GCACAGGAGGAA-3' and *Cyclophilin B* reverse: 5'-G CCGTAGTGTCTTCAGCTT-3'; *Aco2* forward: 5'-GGC TGCCAGTATGACCAAGTG-3' and *Aco2* reverse: 5'-G GCCCATTGATATGTGGCTTT-3'; *Idh3a* forward: 5'-A ACGGTGTTGCCATCTTTGAA-3' and *Idh3a* reverse: 5'-CATGTCCTTGCTGCAATGT-3'; *Idh3b* forward: 5'-CAAGAAAGGGCGGAGCAA-3' and *Idh3b* reverse: 5'-GCCATCCCCTAGTTTCATGATG-3'; *Idh3g* forward: 5'-CAACACAACAATGCAGCTGGTA-3' and *Idh3g* reverse: 5'-ATTAGGCATCACCATGACATCAA-3'; *Gpt* forward: 5'-CGCGTCTTTCCACTCAGTCTCT-3' and *Gpt* reverse: 5'-TCCACATAGCCACCACGAAAC-3'; *Pparg* forward: 5'-CACAATGCCATCAGGTTTGG-3' and *Pparg* reverse: 5'-GCTGGTCGATATCACTGGAGA TC-3'. All reactions were performed in triplicate. A standard curve for each primer set was generated in each assay and the expression level of the gene of interest was normalized to that of *Cyclophilin B*.

Statistics

The data for the time- and dose-dependent FRET changes in response to either dimethyl-2-OG or citrate was analyzed using two-way repeated measures analysis of variance (two-way RM ANOVA) by GraphPad Prism software (ver.9.1.0) (GraphPad Software, CA, USA) with Dunnett's *post hoc* analysis. The temporal changes in the FRET ratio and the expression of mRNA during adipocyte differentiation were analyzed using one-way ANOVA followed by Tukey's test. The two-tailed Student's *t*-test was performed for changes in response to deferroxamine treatment to determine the statistical significance.

Results

Establishment of a FRET probe harboring nuclear localization signals for measurement of α -KG

Several attempts have been made to establish intracellular biosensors for α -KG by focusing on its important roles in balancing the nutritional status of nitrogen and carbon, such as nitrogen assimilation reactions. Candidate proteins that may act as α -KG sensors include the P_{II} protein family which is widely distributed in bacteria and plants [19], the nitrogenase transcriptional regulator protein NifA [16, 20], the NtcA transcriptional regulator belonging to the Crp-Fnr family in Cyanobacteria, the archaeal transcriptional repressor NprR, the carbohydrate phosphotransferase system in bacteria species, the *Bacillus subtilis* transcriptional regulator GltC, and the KguS/KguR in uropathogenic *E. coli* (reviewed in [1]). Among these, we employed NifA protein for establishing an intra-nuclear FRET biosensor by adding multiple NLSs to the previously reported sensor which contains the α -KG binding domain of the NifA protein from *Azotobacter vinelandii* and is highly selective for α -KG and did not exhibit an apparent response to the related metabolites such as citrate, isocitrate, glutamate, and glutamine [16, 21]. NifA is a transcriptional activator of nitrogen fixation (*nif*) genes in response to the levels of fixed nitrogen by the regulation of P_{II} proteins [1, 22]. Its N-terminal GAF domain regulates the catalytic activity by binding directly to α -KG with a dissociation constant of ~ 60 μ M [20]. Firstly, the DNA sequence coding GAF-AAA+ domains (400 a.a.) of NifA protein from *Azotobacter vinelandii* was tagged with EYFP on the 5' end and ECFP on the 3' end (YFP-GAF-AAA+-CFP: NifA-FRET-NLS(-)) to prepare an expression plasmid as previously reported. Next, three copies of NLS from SV40 were added on the 5' end of the coding sequence of EYFP to generate an NLS-containing NifA-FRET probe (NifA-FRET-NLS(+)) (Fig. 1A). The obtained plasmids were transiently transfected by electroporation to 3T3-L1 preadipocytes to confirm the subcellular distribution of the probes by detecting CFP fluorescent signal. After a recovery period from the electroporation, the incubation medium was changed to HBSS and either the NifA-FRET-NLS(-) probe or the NifA-FRET-NLS(+) probe were excited with the wavelength of 438/24 nm and detected using a 483/32 nm emission filter. The acquired image of CFP fluorescence confirmed that the NifA-FRET-NLS(-) probe was predominantly distributed throughout the cytosol, while the NifA-FRET-NLS(+) probe was accumulated in the nucleus (Fig. 1B, left panels). Additionally, the YFP image obtained with a 542/27 nm emission filter with the same excitation wavelength (438/24 nm) indicated an efficient FRET between CFP

and YFP in both probes at their subcellular localization in 3T3-L1 preadipocytes (Fig. 1B, right panels). Next, the response of the NifA-FRET-NLS(+) probe to α -KG was examined by treating with dimethyl-2-OG which is a cell-permeable compound to be converted to α -KG in the cell [19, 23]. The nuclear FRET signal of the NifA-FRET-NLS(+) probe was monitored in the 3T3-L1 preadipocytes by acquiring a series of FRET images every 30 seconds after the addition of 40 mM dimethyl-2-OG. ROIs were set on three individual cells and the fluorescence values of CFP and YFP in each ROI were recorded. To subtract the background levels, ROIs with the same size were placed in the extracellular area in the vicinity of each cell (Fig. 1C, left). The corresponding background values were subtracted from the YFP and CFP fluorescence values acquired in each ROI and then the ratio of YFP/CFP was calculated as a FRET ratio. The YFP/CFP ratio (FRET ratio) from all three ROIs set on the NifA-FRET-NLS(+) expressing 3T3-L1 preadipocytes showed a $\sim 17\%$ reduction at one minute after the addition of 40 mM dimethyl-2-OG. Thus, the NifA-FRET-NLS(+) probe detected the changes of the nuclear α -KG concentration in a living cell. By using this FRET-based probe, we subsequently examined the changes in the nuclear α -KG concentrations during adipocyte differentiation. For this aim, we established a 3T3-L1 cell line which stably expresses the NifA-FRET-NLS(+) probe. We employed the *piggyBac* transposon system [18] to deliver and mobilize a long sequence (6.8 kb) encoding the NifA-FRET-NLS(+) probe into the genome of 3T3-L1 cells instead of using a retrovirus-mediated gene transfer system which incidentally causes recombination between *CFP* and *YFP* genes during the reverse-transcription process due to the high sequence similarity between them [24]. The 3T3-L1 adipocytes transduced with the NifA-FRET-NLS(+) probe and a puromycin resistant sequence were screened by treatment with 4 μ g/mL puromycin for 3 days. Next, the selected cells were differentiated into mature adipocytes with the induction cocktail consisting of insulin, dexamethasone, and IBMX. When monitoring the distribution of the NifA-FRET-NLS(+) probe, the fluorescent signal of the probe gradually leaked to the cytosol during the adipocyte differentiation. More precisely, the leak was observed from 1 to 2 days after induction and by day 8 of differentiation, most cells presented a fluorescent signal throughout the cytosol and the nucleus (data not shown). This unfavorable observation suggested a possibility of activated nuclear export with differentiation of 3T3-L1 cells. However, the treatment of cells on day 4 or day 8 of differentiation with 20 ng/mL of leptomycin B which is a specific inhibitor of nuclear export machinery [25, 26] for either 30 minutes or 2 hours did not improve the

leak of the probe. To overcome this problem, a different approach was adopted. Several additional NLSs was tagged, such as an arginine-rich NLS of Rex and an SV40 NLS on the 3' end of the probe to increase the number of NLSs (Fig. 1A, bottom). The new probe constructed (NifA-FRET-NLS(++)) predominantly localized to the nucleus throughout the time course of adipocyte differentiation (Fig. 1A and shown later).

Characterization of nuclear FRET probe for monitoring the concentrations of α -KG

By using the newly established 3T3-L1 preadipocyte line which stably expresses NifA-FRET-NLS(++), the response of the FRET biosensor was titrated with serial concentrations of dimethyl-2-OG ranging from 0 to 40 mM. The FRET ratio (YFP/CFP) was dropped in response to the treatment with dimethyl-2-OG by $\sim 8\%$ at 20 mM and $\sim 17\%$ at 40 mM in a couple of minutes. These responses were comparable to the reduction levels of the formerly reported non-organelle-specific P_{II} FRET probe which showed a decrease of FRET ratio by $\sim 5\%$ in human embryonic kidney (HEK293T) cells and by $\sim 10\%$ in U87-MG glioblastoma cancer cells in response to 20 mM dimethyl-2-OG [19] (Fig. 2A and B).

Similarly, the addition of citrate, which is converted to α -KG in the TCA cycle, reduced the FRET ratio although the dose-dependency of the FRET ratio was different from that of dimethyl-2-OG. The reduction of FRET ratio within one minute after the addition of citrate was $\sim 7.5\%$ at 20 mM and $\sim 10\%$ at 40 mM (Fig. 2C). When compared to the dose-dependent reduction of the FRET ratio in response to dimethyl-2-OG, which was $\sim 8\%$ at 20 mM, almost the half level of $\sim 17\%$ at 40 mM, the reduction of the FRET ratio at 40 mM citrate ($\sim 10\%$) was lower than expected from the result at 20 mM ($\sim 7.5\%$) (Fig. 2B and C). Additionally, the recovery rate of FRET ratio from the initial reduction ($\sim 10\%$ at 1 minute) with 40 mM citrate was slower compared to those with lower concentrations of citrate as well as with 40 mM dimethyl-2-OG. These differences in the responses to dimethyl-2-OG and citrate may be partly attributed to the limited and slow conversion rate from citrate to α -KG in 3T3-L1 cells when cells are treated with an excessive amount of citrate [27].

Nuclear α -KG concentration is elevated during 3T3-L1 adipocyte differentiation

Using the established 3T3-L1 cells stably expressing the NifA-FRET-NLS(++), the nuclear α -KG concentrations were monitored during adipocyte differentiation. The FRET ratio in the nuclear region decreased after the induction of adipocyte differentiation and reached a plateau level by 2–4 days after the induction

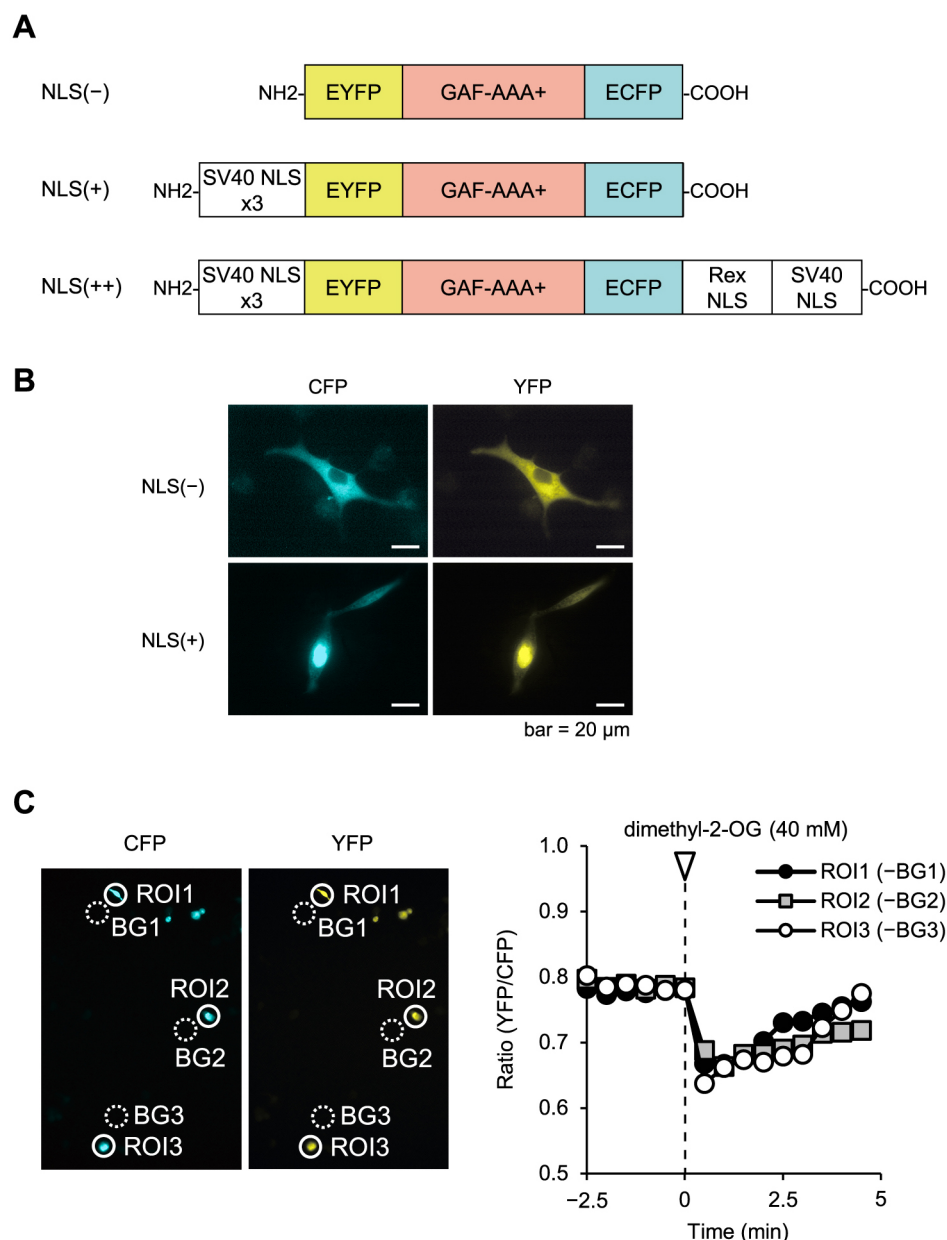


Fig. 1 Establishment of nuclear α -KG sensor

Schematic representations of the α -KG FRET probes with or without nuclear localization signals (NLS). The α -KG FRET probe without NLS (NifA-FRET-NLS(-)) is composed of a partial amino acid (a.a.) sequence encoding the GAF and the AAA+ domains with an extra sequence from NifA protein of *Azotobacter vinelandii* (GAF-AAA+) which is sandwiched between EYFP and ECFP as the donor and acceptor fluorescent proteins, respectively. Three copies of NLS from SV40 (SV40 NLS x3) were fused to the N-terminus of the NifA-FRET-NLS(-) probe (NifA-FRET-NLS(+)), which is additionally fused to the tandem sequence of an NLS of Rex and an SV40 NLS to its C-terminus (NifA-FRET-NLS(++)). (B) Subcellular localization analysis for both the NifA-FRET-NLS(-) and the NifA-FRET-NLS(+) probes. The fluorescence of CFP and YFP were detected by a 483/32 nm emission filter and a 542/27 nm emission filter, respectively, in response to the excitation using a 438/24 nm filter. (C) The response of the NifA-FRET-NLS(+) probe to the dimethyl-2-OG was determined by replacing the cell culture medium with HBSS and then adding dimethyl-2-OG. For the analysis of the acquired image, a region of interest (ROI) was set on each cell with the background (BG) region corresponding to each ROI in its vicinity area (ROI-BG) (left panel). The fluorescent values of CFP and YFP in each ROI were subtracted by the corresponding background and subsequently calculated YFP/CFP as a FRET ratio. The changes in the FRET ratio of three individual cells were measured every 30 seconds before and after the treatment of dimethyl-2-OG at 40 mM (right).

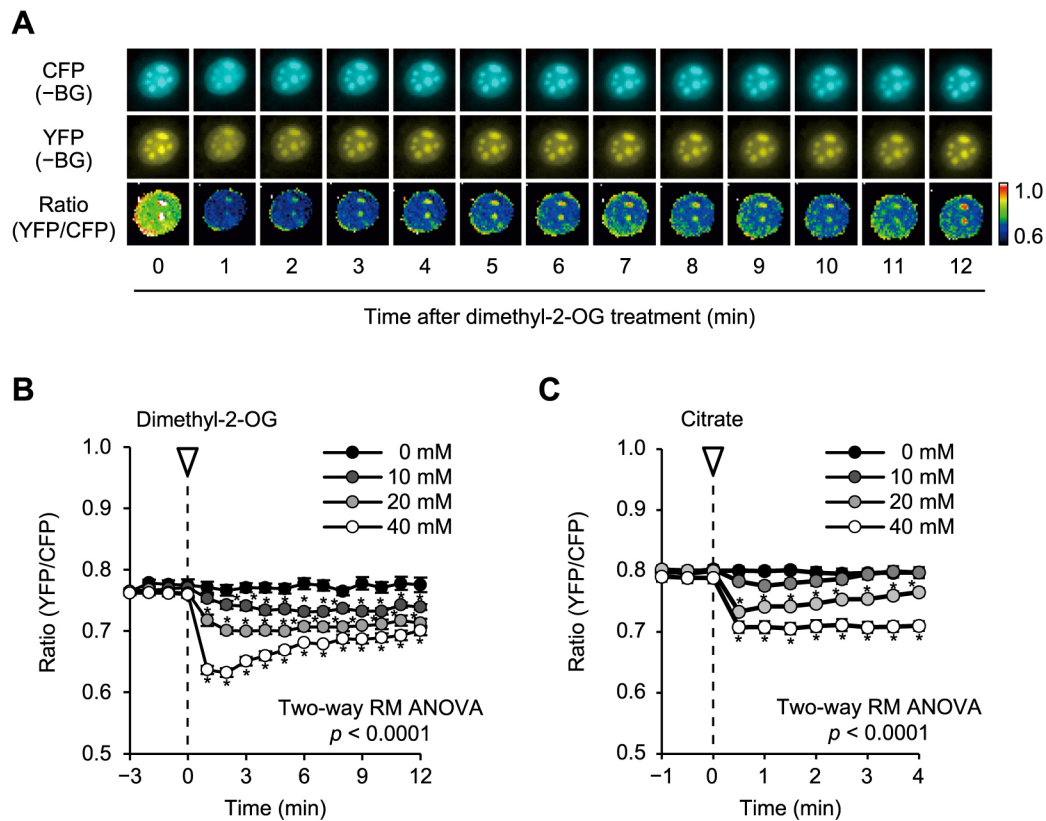


Fig. 2 Stably expressed nuclear α -KG sensor shows time- and dose-dependent FRET changes

(A–C) The 3T3-L1 cell line stably expressing NifA-FRET-NLS(++) probe was established and conducted to the analysis of the time-course of FRET change in the individual cells after the stimulation with 40 mM dimethyl-2-OG (A) or with serial concentrations of dimethyl-2-OG (B) or citrate (C). (A) The representative captured images were analyzed to visualize the FRET ratio (YFP/CFP) using the prepared individual images of CFP and YFP subtracted by their corresponding background levels (–BG). Heatmap represents the level of FRET ratio. (B–C) The time-dependent changes in the FRET ratio were measured after treatment with dimethyl-2-OG at 0 mM ($n = 10$ ROIs), 10 mM ($n = 14$ ROIs), 20 mM ($n = 14$ ROIs), or 40 mM ($n = 13$ ROIs) (B) or citrate at 0 mM ($n = 13$ ROIs), 10 mM ($n = 13$ ROIs), 20 mM ($n = 11$ ROIs), or 40 mM ($n = 10$ ROIs) (C) (mean $\pm$ s.e.m.). Statistical analysis was performed using two-way RM ANOVA ($p < 0.0001$) with Dunnett's *post hoc* analysis compared to 0 mM at each time point ($*p < 0.05$). The experiments were performed five times for dimethyl-2-OG and three times for citrate and similar results were obtained.

(Fig. 3A and B, left panel). Since the FRET ratio is inversely correlated with the concentration of α -KG, the data indicates a rapid increase of the nuclear α -KG concentrations in the early stage of differentiation as shown by the reciprocal plot of FRET ratio (Fig. 3B, right panel). This increasing trend during adipocyte differentiation is consistent with the changes in the total cellular amount of α -KG determined by the gas chromatography-mass spectrometry method in a recent report [28]. While the regulation mechanisms of the nuclear α -KG concentration during adipocyte differentiation are to be elucidated, its elevation was associated with increased expression of genes encoding the TCA cycle enzymes such as *Aco2*, *Idh3a*, *Idh3b*, and *Idh3c* and alanine aminotransferase 1 (*Gpt*) (Fig. 3C and D). Additionally, the α -KG concentration on day 2 was significantly decreased by treatment with deferoxamine, which chelates iron, an indispensable

metal cofactor for mitochondrial aconitase (*Aco2*) (Fig. 3E). Thus, increased α -KG production may contribute to an increased concentration of α -KG in the nucleus.

Discussion

In recent years, α -KG has been attracting more attention as a regulator of epigenetic enzymes including JMJDs, TETs, and RNA demethylases [2–4]. Since genomic DNA and histone locate in the nucleus, it would be of great interest to measure the nuclear concentration of α -KG in order to elucidate the regulatory mechanism of these enzymes. In the present study, we established a FRET-based nuclear localizing biosensor for α -KG, which enabled the measurement of the α -KG concentration in the nucleus in living cells.

This nuclear localizing α -KG probe was generated by

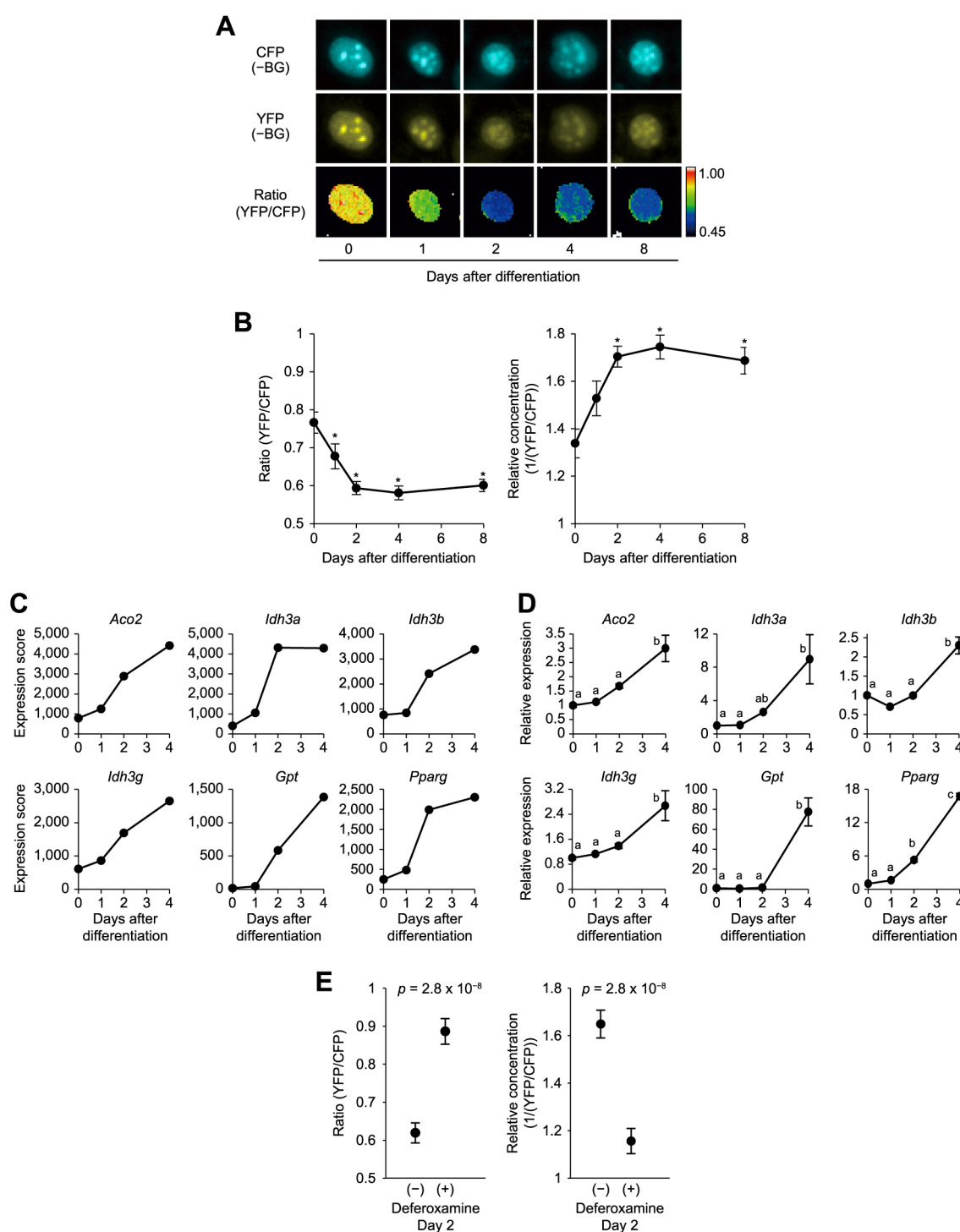


Fig. 3 Nuclear concentration of α -KG increases during adipocyte differentiation

(A–B) The changes in α -KG concentration during the 3T3-L1 differentiation were monitored by measuring the FRET ratio of the NifA-FRET-NLS(++) probe. (A) The representative captured images of CFP (top panel) and YFP (middle panel) after subtracting their representative background (–BG) and the visualized images of FRET ratio (bottom panel) are shown. (B) The temporal changes in the FRET ratio during adipocyte differentiation were measured (left panel) and the relative concentration of α -KG is presented as reciprocal of FRET ratio value (right panel) (mean $\pm$ s.e.m., $n = 15$ ROIs). One-way ANOVA followed by Tukey's test was performed ($*p < 0.05$ compared to day 0). (C–D) The expression levels of the indicated genes during the differentiation of 3T3-L1 cells (0, 1, 2, and 4 days after induction of differentiation) were analyzed using the previously reported microarray dataset ($n = 1$) (C) and further confirmed by RT-qPCR ($n = 3$) (D). (D) One-way ANOVA followed by Tukey's test was performed and the presence of different lowercase letters indicates statistical significance ($p < 0.05$). (E) The FRET ratio in the 3T3-L1 cells on day 2 of differentiation with or without treatment of deferoxamine (100 μ M) was measured (left panel) and the relative concentration of α -KG is presented as reciprocal of FRET ratio value (right panel) (mean $\pm$ s.e.m., $n = 20$ ROIs). The two-tailed Student's *t*-test was performed.

adding multiple NLSs to the FRET-based α -KG sensor originally reported by Zhang *et al.* [16], in which the α -KG-binding GAF-AAA+ domain of NifA protein from *Azotobacter vinelandii* was inserted between EYFP and ECFP. When expressed in 3T3-L1 preadipocytes, the FRET ratio of this nuclear α -KG probe decreased in a dose-dependent manner with dimethyl-2-OG or citrate treatment. Considering that the reported dissociation constant for α -KG of NifA protein is $\sim 60 \mu\text{M}$ [20], the sensitivity of our probe to exogenous dimethyl-2-OG is lower (above mM range). There are several possible reasons for the lower sensitivity of this probe to dimethyl-2-OG. First, the intracellular level of α -KG may be lower than the extracellular dimethyl-2-OG concentration due to inefficient conversion. Second, the binding constant of α -KG to GAF domain may be altered when expressed in mammalian cells. Finally, the design of the probe may not be optimal for the detection of sub-mM levels of α -KG. Nevertheless, the α -KG probe enabled detection of an increase of α -KG concentrations in the nucleus at the early stage (first two days after induction) of adipocyte differentiation in 3T3-L1 cells, indicating that the probe is useful for measuring α -KG levels in living cells with subcellular resolution. Also, these results are consistent with the changes in the total cellular amount of α -KG during adipocyte differentiation previously measured by mass spectrometry [28]. This system is applicable for measuring α -KG concentration in other organelles by adding organelle-specific targeting signals. For instance, the addition of a mitochondrial localization signal to the FRET probe will elucidate the concentrations of α -KG in the mitochondria, which will be a useful tool to study the

subcellular balance of mitochondrial production, consumption, and transport of α -KG. Thus, the measurement of α -KG concentrations at the organelle-specific level will provide a deeper insight into the relationship between cellular metabolism and epigenetic regulation.

Acknowledgments

We thank Dr. Michiyuki Matsuda for providing the plasmids for the *piggyBac* system, advice, and technical supports, Dr. Atsushi Miyawaki for helpful suggestions, the IMCR Joint Usage/Research Support Center (IMCR-JURSC) for the research equipment, and all the members of the Inagaki laboratory for helpful discussions. This work was supported by JSPS KAKENHI Grant Numbers JP17H03631, JP18H04796, JP19K22633, JP 20H03729, the shared research equipment JPMXS0420600121, and the Advanced Bioimaging Support (ABiS) JP16H06280,

Author Contributions

T.I. designed the study and T.I. and H.S. wrote the paper. T.S., M.H., A.T., M.N., K.T-I., and M.S.R. carried out experiments. M.H., T.K., and S.M. carried out analyses. K.Y. provided materials and technical supports and contributed to discussions. J.S. conducted transcriptome analysis

Disclosure

The authors have no competing interests to declare.

References

1. Huergo LF, Dixon R (2015) The emergence of 2-oxoglutarate as a master regulator metabolite. *Microbiol Mol Biol Rev* 79: 419–435.
2. Islam MS, Leissing TM, Chowdhury R, Hopkinson RJ, Schofield CJ (2018) 2-oxoglutarate-dependent oxygenases. *Annu Rev Biochem* 87: 585–620.
3. Inagaki T, Sakai J, Kajimura S (2016) Transcriptional and epigenetic control of brown and beige adipose cell fate and function. *Nat Rev Mol Cell Biol* 17: 480–495.
4. Herr CQ, Hausinger RP (2018) Amazing diversity in biochemical roles of Fe (II)/2-oxoglutarate oxygenases. *Trends Biochem Sci* 43: 517–532.
5. Xiao M, Yang H, Xu W, Ma S, Lin H, *et al.* (2012) Inhibition of α -KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. *Genes Dev* 26: 1326–1338.
6. Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, *et al.* (2012) IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* 483: 474–478.
7. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, *et al.* (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* 18: 553–567.
8. Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB (2015) Intracellular α -ketoglutarate maintains the pluripotency of embryonic stem cells. *Nature* 518: 413–416.
9. Shoulders MD, Raines RT (2009) Collagen structure and stability. *Annu Rev Biochem* 78: 929–958.
10. Gronke RS, VanDusen WJ, Garsky VM, Jacobs JW, Sardana MK, *et al.* (1989) Aspartyl beta-hydroxylase: *in vitro* hydroxylation of a synthetic peptide based on the structure of the first growth factor-like domain of human factor IX. *Proc Natl Acad Sci U S A* 86: 3609–3613.

11. Förster T (2012) Energy migration and fluorescence. *J Biomed Opt* 17: 011002.
12. Xia Z, Liu Y (2001) Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. *Biophys J* 81: 2395–2402.
13. Djian P, Phillips M, Green H (1985) The activation of specific gene transcription in the adipose conversion of 3T3 cells. *J Cell Physiol* 124: 554–556.
14. Inagaki T, Iwasaki S, Matsumura Y, Kawamura T, Tanaka T, *et al.* (2015) The FBXL10/KDM2B scaffolding protein associates with novel polycomb repressive complex-1 to regulate adipogenesis. *J Biol Chem* 290: 4163–4177.
15. Dixon MP, Pau RN, Howlett GJ, Dunstan DE, Sawyer WH, *et al.* (2002) The central domain of Escherichia coli TyrR is responsible for hexamerization associated with tyrosine-mediated repression of gene expression. *J Biol Chem* 277: 23186–23192.
16. Zhang C, Wei ZH, Ye BC (2013) Quantitative monitoring of 2-oxoglutarate in Escherichia coli cells by a fluorescence resonance energy transfer-based biosensor. *Appl Microbiol Biotechnol* 97: 8307–8316.
17. Nikolaev VO, Bünemann M, Hein L, Hannawacker A, Lohse MJ (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. *J Biol Chem* 279: 37215–37218.
18. Yusa K, Zhou L, Li MA, Bradley A, Craig NL (2011) A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci U S A* 108: 1531–1536.
19. Lüddecke J, Francois L, Spät P, Watzer B, Chilczuk T, *et al.* (2017) P II protein-derived FRET sensors for quantification and live-cell imaging of 2-oxoglutarate. *Sci Rep* 7: 1437.
20. Little R, Dixon R (2003) The amino-terminal GAF domain of *Azotobacter vinelandii* NifA binds 2-oxoglutarate to resist inhibition by NifL under nitrogen-limiting conditions. *J Biol Chem* 278: 28711–28718.
21. Zhang C, Ye BC (2014) A single fluorescent protein-based sensor for *in vivo* 2-oxoglutarate detection in cell. *Biosens Bioelectron* 54: 15–19.
22. Rudnick P, Kunz C, Gunatilaka MK, Hines ER, Kennedy C (2002) Role of GlnK in NifL-mediated regulation of NifA activity in *Azotobacter vinelandii*. *J Bacteriol* 184: 812–820.
23. Pan M, Reid MA, Lowman XH, Kulkarni RP, Tran TQ, *et al.* (2016) Regional glutamine deficiency in tumours promotes dedifferentiation through inhibition of histone demethylation. *Nat Cell Biol* 18: 1090–1101.
24. Komatsubara AT, Matsuda M, Aoki K (2015) Quantitative analysis of recombination between YFP and CFP genes of FRET biosensors introduced by lentiviral or retroviral gene transfer. *Sci Rep* 5: 13283.
25. Wolff B, Sanglier JJ, Wang Y (1997) Leptomycin B is an inhibitor of nuclear export: inhibition of nucleocytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem Biol* 4: 139–147.
26. Ando R, Mizuno H, Miyawaki A (2004) Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science* 306: 1370–1373.
27. Anderson NM, Mucka P, Kern JG, Feng H (2018) The emerging role and targetability of the TCA cycle in cancer metabolism. *Protein Cell* 9: 216–237.
28. Okabe K, Nawaz A, Nishida Y, Yaku K, Usui I, *et al.* (2020) NAD⁺ metabolism regulates preadipocyte differentiation by enhancing α -ketoglutarate-mediated histone H3K9 demethylation at the PPAR γ promoter. *Front Cell Dev Biol* 8: 586179.