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Supplementary Materials for

Protein Kinase G Controls Brown Fat Cell Differentiation and Mitochondrial Biogenesis

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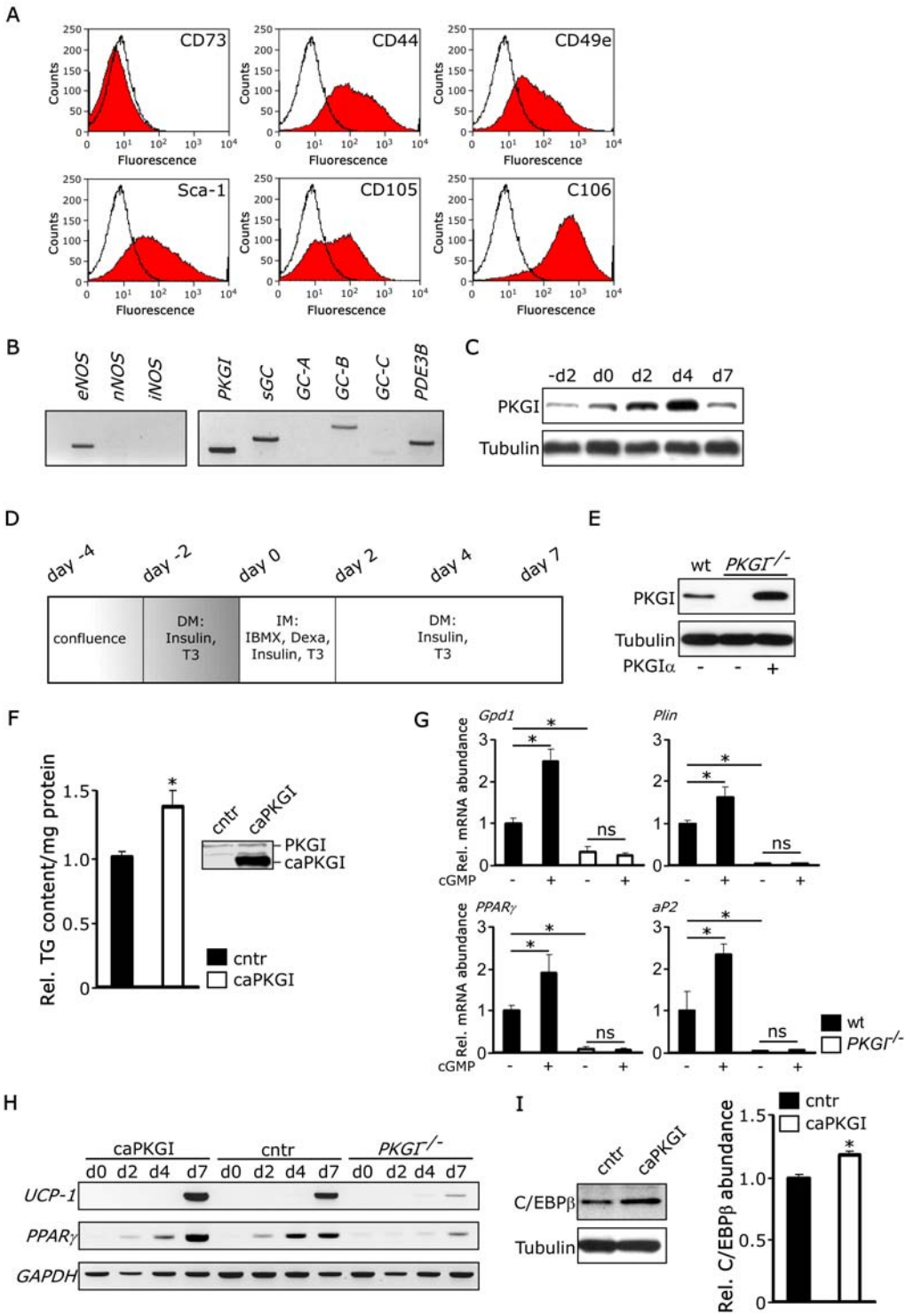

Fig. S1. Characterization of preadipocytes and mRNA and protein abundance of adipogenic factors. **(A)** Flow cytometry analysis of immortalized preadipocytes; note the presence of mesenchymal stem cell markers (Sca-1, CD44, CD49e, CD105, and CD106). **(B)** Abundance of

NO and cGMP signaling molecules in brown preadipocytes (day -2) as assessed by RT-PCR. *NOS*, *NO-synthase*; *eNOS*, *endothelial NOS*; *iNOS*, *inducible NOS*; *nNOS*, *neuronal NOS*; *GC*, *guanylyl cyclase*; *sGC*, *soluble GC*. **(C)** Time course of changes in PKGI protein abundance during brown fat cell differentiation. **(D)** Stages of brown adipocyte differentiation (day -4 to day 7) and the media used (DM, differentiation medium; IM, induction medium; IBMX, isobutylmethylxanthine; Dexa, dexamethason; T3, triiodothyronine). **(E)** Lentiviral reexpression of PKGIα in *PKGI*$^{-/-}$ cells. After differentiation, wild-type and *PKGI*$^{-/-}$ cells and *PKGI*$^{-/-}$ cells reexpressing PKGIα were lysed and subjected to Western blotting with PKGI and tubulin antibodies. **(F)** TG content (left) and Western blot (right) of differentiated wild-type brown adipocytes infected with either control (cntr) or caPKGI lentivirus as indicated (n=4 independent experiments). **(G)** Analysis of fat cell markers *Gpd1*, *Plin*, *aP2*, and *PPARγ* by RQ-PCR (n=5 independent experiments). **(H)** Time course of changes in *UCP-1* and *PPARγ* mRNA abundance (RT-PCR) during differentiation of wild-type cells transduced with cntr or caPKGI lentivirus and *PKGI*$^{-/-}$ cells. **(I)** Protein abundance of C/EBPβ post infection with either cntr or caPKGI lentivirus (day 0, left). Densitometric analysis of C/EBPβ normalized to tubulin (n=3 independent experiments, right). Data are represented as means ± SEM; *, $P < 0.05$; ns, not significant.

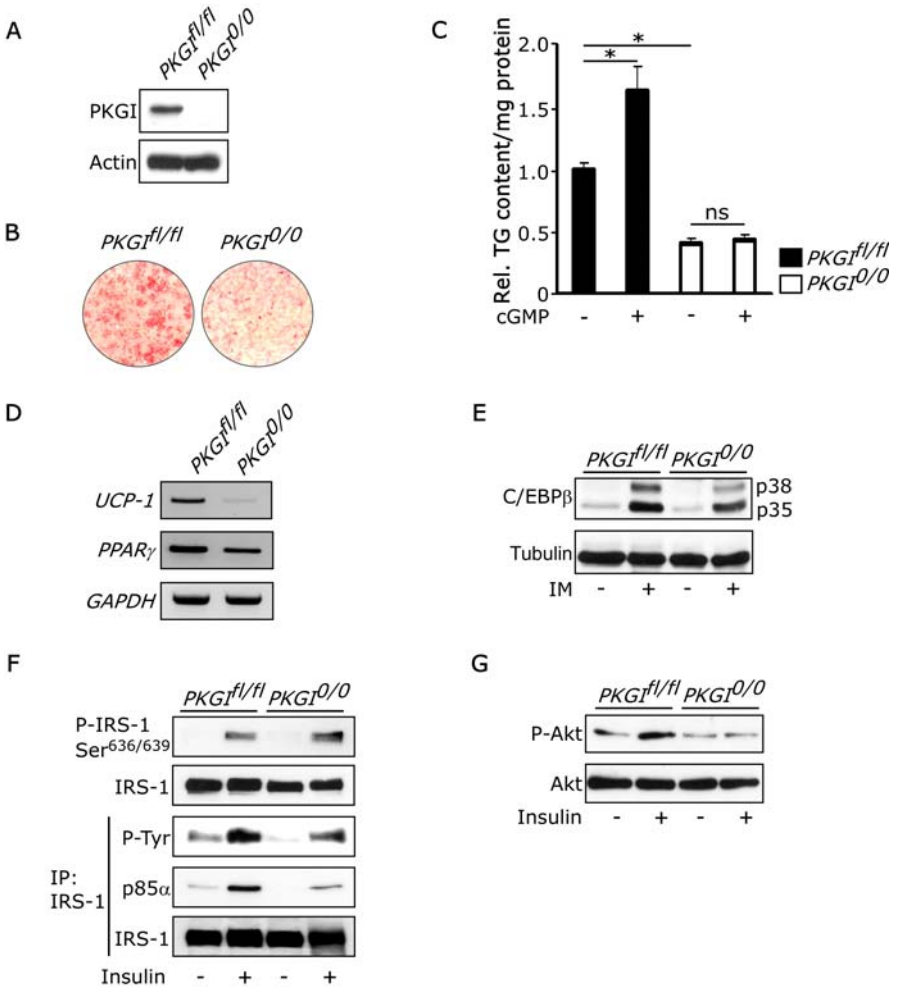

Fig. S2. Analysis of *PKGI floxed* cells. **(A)** Western blot for PKGI in *PKGI*$^{fl/fl}$ and *PKGI*$^{0/0}$ preadipocytes 72 hours post infection with a self-deleting Cre lentivirus. **(B)** Oil Red O staining of differentiated *PKGI*$^{fl/fl}$ and *PKGI*$^{0/0}$ brown adipocytes. **(C)** TG content of differentiated PKGI$^{fl/fl}$ and *PKGI*$^{0/0}$ brown adipocytes treated with cGMP as indicated (n=4 independent experiments). **(D)** Abundance of *UCP-1* and *PPARγ* in differentiated *PKGI*$^{fl/fl}$ and *PKGI*$^{0/0}$ brown adipocytes as assessed by RT-PCR. **(E)** Induction of C/EBPβ protein at day 0 of differentiation in *PKGI*$^{fl/fl}$ and *PKGI*$^{0/0}$ preadipocytes (6 hours IM). **(F)** IRS-1 (Ser$^{636/639}$) phosphorylation (P-IRS-1) in cell lysates of serum starved *PKGI*$^{fl/fl}$ and *PKGI*$^{0/0}$ brown preadipocytes (day 0) treated with 100 nM insulin as indicated (upper panels). Cell lysates of serum starved *PKGI*$^{fl/fl}$ and *PKGI*$^{0/0}$

brown preadipocytes treated with 100 nM insulin as indicated were subjected to immunoprecipitation with an IRS-1 antibody. Phospho-tyrosine (P-Tyr), p85α, and IRS-1 were visualized by immunoblotting with specific antibodies (lower panels). **(G)** Insulin-induced Akt (Ser^{473}) phosphorylation (P-Akt) after incubation with 10 nM insulin in cell lysates of serum starved $PKGI^{fl/fl}$ and $PKGI^{0/0}$ brown preadipocytes (day 0). Data are represented as means ± SEM; *, $P < 0.05$; ns, not significant.

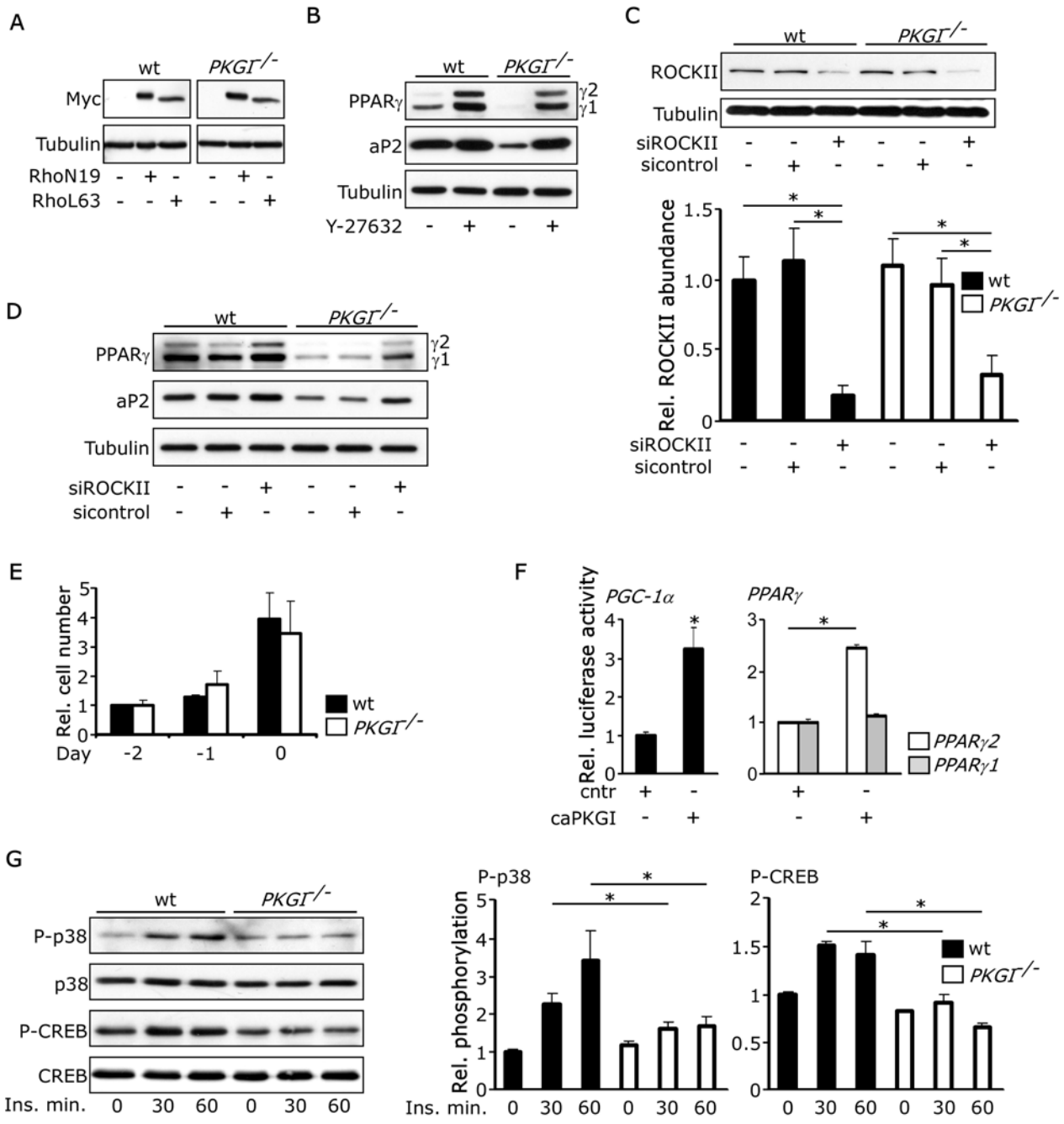

Fig. S3. Analysis of RhoA–ROCK and insulin signaling. **(A)** Expression of Myc-tagged RhoA mutants RhoN19 and RhoL63 72 hours post infection as assessed by Western blot analysis with a Myc antibody. **(B)** Western blot of PPARγ and aP2 in differentiated wild-type and *PKGI*$^{-/-}$ brown adipocytes treated with 20 µM Y-27632 during differentiation as indicated (one representative

Western blot of three independent experiments is shown). **(C)** Western blot showing the abundance of ROCKII in differentiated wild-type and *PKGI*$^{-/-}$ brown adipocytes transduced with lentiviral vectors encoding ROCKII or control siRNAs as indicated (upper panels). Densitometric analysis of ROCKII normalized to tubulin (n=4 independent experiments, lower graph). **(D)** Western blot of PPARγ and aP2 in differentiated wild-type and *PKGI*$^{-/-}$ brown adipocytes transduced with siRNAs against ROCKII or control siRNA (one representative western blot of three independent experiments is shown). **(E)** Relative cell numbers of wild-type and *PKGI*$^{-/-}$ preadipocytes during early stages of differentiation (day -2 to day 0). Proliferation was assessed by counting cells at the indicated times (n=3 independent experiments per time point). **(F)** Luciferase activity of *PGC-1α* and *PPARγ1* and *2* promoter fragments in HIB1B preadipocytes transfected with either caPKGI or cntr (n = 6 independent experiments). **(G)** Time course of insulin-induced phosphorylation of p38 MAPK (Thr^{180}/Tyr^{182}) and CREB (Ser^{133}) in serum-starved wild-type and *PKGI*$^{-/-}$ brown adipocytes (day 7, left). Densitometric analysis of P-p38 normalized to total p38 and P-CREB normalized to total CREB (n=3 independent experiments, right). Data are represented as means ± SEM; *, $P < 0.05$.

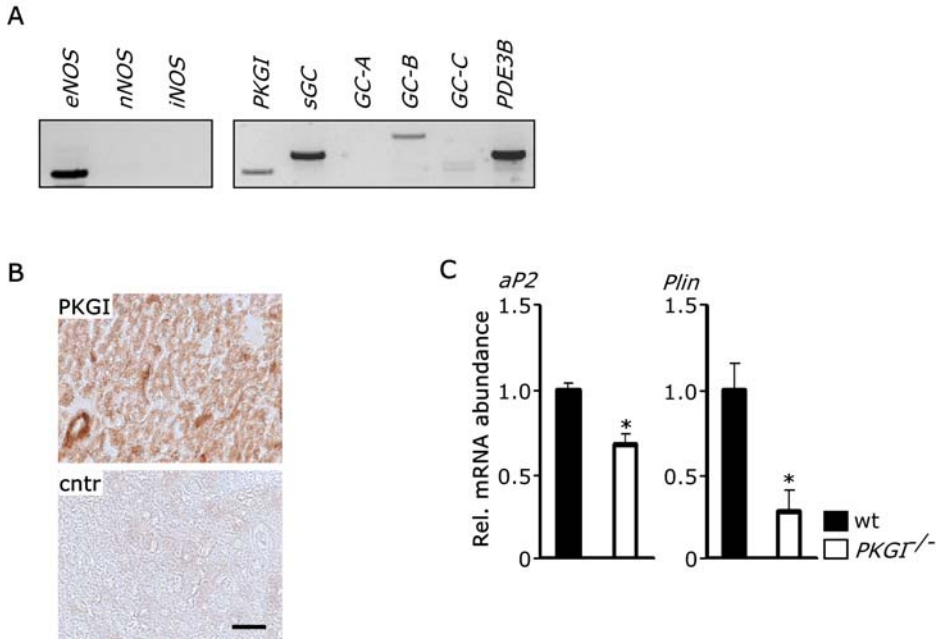

Fig. S4. In vivo analysis of *PKGI*$^{-/-}$ mice. **(A)** Abundance of NO and cGMP signaling molecules in BAT of newborn wild-type mice as assessed by RT-PCR. **(B)** Immunohistochemical analysis of PKGI abundance (anti-PKGI staining, brown; cntr, secondary antibody alone) in BAT sections of newborn wild-type mice; scale bar, 20µm. **(C)** Analysis of fat cell markers *aP2* and *Plin* by RQ-PCR in BAT of newborn wild-type and *PKGI*$^{-/-}$ mice (n=5 mice per group). Data are represented as means ± SEM; *, $P < 0.05$ as compared to wt.

Table S1. RT-PCR primer sequences.

gene	primer sequence
UCP-1 forward	5’ - GTG AAC CCG ACA ACT TCC GAA GTG - 3´
UCP-1 reverse	5’ - CAT GAG GTC ATA TGT CAC CAG CTC - 3´
PPARγ forward	5’ - ATG CCA TTCTGG CCC ACC AAC TTC - 3´
PPARγ reverse	5’ - CAT AAA TAA GCT TCA ATC GGA TGG - 3´
PKGI forward	5’ - ACT GTA TGT ACC CCG TGG AAT - 3´
PKGI reverse	5’ - TTG GTG AGT CTT CTC GAG TAA - 3´
GC-A forward	5’ - ATC GGG GTG AAG GAT GAG TAC G - 3´
GC-A reverse	5’ - AGT ACT CAG GAT TAT CGG GTT C - 3´
GC-B forward	5’ - CAT GGC AGG ACA ATC GAA CC - 3´
GC-B reverse	5’ - TGC CTG CAC CCT TGT GAT AG - 3´
GC-C forward	5’ - TCC AGG TGG CCT ACG AAG AC - 3´
GC-C reverse	5’ - GAT TCT CCG AAT GGT GTC AC - 3´
sGC forward	5’ - TCA CTC TGG CTA ACA AAT TTG AAT C - 3´
sGC reverse	5’ - ACA ATG TGC TGG ATT TTG AGT GCA G - 3´
PDE3B forward	5’ - CAG GAA GGA TTC TCA GTC AG - 3´
PDE3B reverse	5’ - GTA CTC TGG GCG AGA AAG AT - 3´
nNOS forward	5’ - CAA ACG CAA AGT GGG AGG TC - 3´
nNOS reverse	5’ - TTG CCG TCG AGG TCT CTG TC- 3´
iNOS forward	5’ - GTT CTC AGC CCA ACA ATA CA - 3´
iNOS reverse	5’ - AGG CAG TGC ATA CCA CTT CA - 3´
eNOS forward	5’ - CTG GCA AGA CAG ACT ACA CGA - 3´
eNOS reverse	5’ - CGC AAT GTG AGT CCG AAA ATG T - 3´
GAPDH forward	5’ - CGG CAA ATT CAA CGG CAC AGT CA - 3´
GAPDH reverse	5’ - GGT TTC TCC AGG CGG CAC GTC A - 3´

Table S2. RQ-PCR primer sequences.

gene	primer sequence
UCP-1 forward	5’ - GGT GAA CCC GAC AAC TTC CGA AGT G - 3´
UCP-1 reverse	5’ - GGG TCG TCC CTT TCC AAA GTG TTG A - 3´
PPARγ forward	5’ - TCC GTA GAA GCC GTG CAA GAG ATC A - 3´
PPARγ reverse	5’ - CAG CAG GTT GTC TTG GAT GTC CTC G - 3´
PGC-1α forward	5’ - GCA CAC ACC GCA ATT CTC CCT TGT A - 3´
PGC-1α reverse	5’ - ACG CTG TCC CAT GAG GTA TTG ACC A - 3´
HSL forward	5’ - GAG GCT CAG ACG AGA GGG AGA A - 3´
HSL reverse	5’ - CTA CGG GAA GGA CAG GAC AGC AAG G - 3´
Plin forward	5’ - CTC TGG GAA GCA TCG AGA AGG TGG T - 3´
Plin reverse	5’ - CCT TCA GGG CAT CGG ATA GGG ACA T - 3´
Gpd1 forward	5’ - TGT TAA ATA CCT GCC AGG GCA CAA GC - 3´
Gpd1 reverse	5’ - AGT TGG GTG TCT GCA TCA GGT CCT TC - 3´
Cidea forward	5’ - ATT TAA GAG ACG CGG CTT TGG GAC A - 3´
Cidea reverse	5’ - TTT GGT TGC TTG CAG ACT GGG ACA T - 3´
aP2 forward	5’ - TGA AAG AAG TGG GAG TGG GCT TTG C - 3´
aP2 reverse	5’ - CAC CAC CAG CTT GTC ACC ATC TCG T - 3´
β3-AR forward	5’ - ATC TTC TCT CTG TGC TGG CTG CCC T- 3´
β3-AR reverse	5’ - CAT CGG TTC TGG AGC GTT GGA GAG T- 3´
HPRT forward	5’ - ACA TTG TGG CCC TCT GTG TGC TCA - 3´
HPRT reverse	5’ - CTG GCA ACA TCA ACA GGA CTC CTC GT - 3´
mt-Co1 forward	5’ - CCC AAT CTC TAC CAG CAT C - 3´
mt-Co1 reverse	5’ - GGC TCA TAG TAT AGC TGG AG - 3´
mt-Nd1 forward	5’ - AAT CGC CAT AGC CTT CCT AAC AT - 3´
mt-Nd1 reverse	5’ - GGC GTC TGC AAA TGG TTG TAA - 3´
mt-Cytb forward	5’ - TTC TGA GGT GCC ACA GTT ATT - 3´

mt-Cytb reverse	5’ - GAA GGA AAG GTA TTA GGG CTA AA - 3´
H19 forward	5’ - GTA CCC ACC TGT CGT CC - 3´
H19 reverse	5’ - GTC CAC GAG ACC AAT GAC TG - 3´