Supporting Information

Sen Santara et al. 10.1073/pnas.1304145110

SI Methods

Protein Expression and Purification. For expression of recombinant full-length heme containing adenylate cyclase from *Leishmania major* (HemAC-Lm) and the catalytic domain (Δ360HemAC-Lm), the corresponding plasmids were transformed separately into competent *Escherichia coli* BL21(DE3). Overnight-grown culture was inoculated in 500 mL of modified terrific broth, incubated at 37 °C for 5 h, and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside followed by addition of 0.4 mM δ-aminolevulinic acid. *E. coli* cells were grown at 22 °C and 180 rpm for 20 h, pelleted, and resuspended in 50 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM EDTA, 10% glycerol, 150 mM NaCl, protease inhibitor mixture (Roche), 0.5 mM PMSF, and 1.0 mg/mL lysozyme. The resuspended solution was kept for 1 h on ice at 4 °C, and then the cells were broken by sonication. The lysate was centrifuged at 14,000 × *g* for 1 h. The supernatant or the crude extract was loaded onto a Ni^{2+}-nitrilotriacetate column. After loading the crude extract, the column was washed with 10 column volumes of washing buffer [50 mM Tris·HCl buffer (pH 7.5), 150mM NaCl, 10% glycerol, and 1 mM PMSF]. The enzyme was eluted with the washing buffer supplemented with 150 mM imidazole and then dialyzed three times against 50 mM Tris·HCl buffer (pH 7.5), 150 mM NaCl, and 10% glycerol. Then the dialyzed fraction was concentrated and loaded into Superdex 200 Columns of ÄKTAprime plus system (GE Healthcare). Purification of Δ360HemAC-Lm catalytic domain was carried out in the same way as above except δ-aminolevulinic acid was excluded during induction and no gel filtration was performed after elution from the Ni^{2+}-nitrilotriacetate column. Molecular weight and purity of the proteins were confirmed by 12% SDS PAGE.

Enzyme Assay. Enzymatic reactions were initiated by addition of 100–150 μg of enzyme to the reaction mixture (final volume of 175 μL) containing 20 mM Tris·HCl buffer (pH 7.5), 1 mM l-methyl-3-isobutyl- xanthine, 5 mM ATP, 1 mM $MgC1_2$, 10 mM $MnC1_2$, and 10 mM NaCl. Assay incubations were routinely conducted for 15 min at 37 °C, terminated by placing into boiling water bath for 1 min and filtered by Amicon ultra 3.0 kDa (millipore). Sixty microliters of the filtrate was injected in an HPLC chromatographic system (Waters 1525 binary HPLC pump), connected to a Symmetry 300 5-μm C-18 (250 × 4.6 mm) column with a C-18 guard column (Waters). The HPLC run was conducted at isocratic mode with 50 mM phosphate buffer (pH 4.3) containing 0.2% triethylamine-methanol (90:10) at a flow rate of 1 mL/min. Peaks were monitored with a Waters 2489 UV/Visible Detector at 260 nm. The amount of cAMP produced during the enzymatic reaction was determined by external standardization, being the linearity between peak areas and the masses of cAMP injected in the range 0.1–500 nano moles ($r = 0.999$).

Preparation of Deoxy Protein. Deoxy ferrous form of HemAC-Lm was prepared by incubating the protein with the strong reducing agent sodium dithionite inside the anaerobic chamber (atmosbag, Aldrich) followed by immediate removal of the excess dithionite by gel filtration on a Sephadex-G25 column (2 mL) equilibrated with N_2-equilibrated anaerobic buffer containing 50 mM Tris·HCl buffer (pH 7.4), 50 mM NaCl, and 10% (vol/vol) glycerol. The ferrous deoxygenated sample was collected into an anaerobic rubber-sealed cuvette. Then the UV-visible spectrum was taken for verification of full conversion from ferrous oxygenated form to ferrous deoxygenated form and for measuring protein concentration. For preparation of anaerobic Sephadex-G25 column within the anaerobic chamber, the column was washed twice with 100 μl sodium dithionite solutions (1 mg/mL) followed by extensive washing with N_2-equilibrated anaerobic buffer before sample loading.

Subcellular Fractionation and Western Blotting. Subcellular fractionation was performed to separate mitochondria, nuclei, cytosol, and microsomal fractions by the mitochondria isolation kit (Qiagen). The purity of each fraction was checked by Western blot analysis with organelle-specific marker antibodies. The primary antibodies used were as follows: mouse anti–(*L. major*)-HemAC antibody (1:100), rabbit anti-(*L. major*) ascorbate peroxidase (1:50), rabbit anti–(*Leishmania donovani*)-adenosine kinase (1:50), and rabbit anti-(*Trypanosoma brucei*) Bip antibody (1:1,000). The HRP-conjugated secondary antibodies used were anti-rabbit (1:10,000), and anti-mouse (1:6,000).

Generation of Stable Knockout Strain for HemAC-Lm Alleles. To generate the constructs pXG-NEO HemAC-Lm and pXG-HYG HemAC-Lm, 1.007 kb from the 5′ region and 1 kb from the 3′ region of the HemAC-Lm gene were inserted into the modified pXG-NEO and pXG-HYG vectors (1), respectively. Primers 7/8 (Table S1) were used for amplifying the 5′ flank, and primers 9/10 (Table S1) were used for amplifying the 3′ flank of the gene. The HindIII/SalI-digested 5′ flanking region and the SmaI/BamHI-digested 3′F flanking region were cloned on either side of ORFs encoding neomycin and hygromycin resistance genes of pXG-NEO and pXG-HYG vectors, respectively. Both constructs were then digested with HindIII and BamHI to get linear fragments of gene deletion constructs HemAC-Lm::NEO and HemAC-Lm::HYG. HemAC-Lm::NEO construct was transfected in log-phase promastigotes to generate heterozygous knockouts (HKOs). HKO strain with HemAC-Lm::NEO construct was maintained at 200 μg/mL neomycin. To generate double-knockout parasites, HemAC-Lm::HYG construct was transfected into the HKO parasites. HKO was confirmed by PCR using primers 11/12 as external to the gene to detect proper insertion of cassettes into the transformant genomic DNA. The double-knockout strain was unable to grow in growth medium containing 60 μg/mL neomycin and 100 μg/mL hygromycin drug.

Inducible Gene Expression Strain (LmT7.TR) Construction. A 2.7-kbp fragment encoding T7 RNA polymerase containing N-terminal nuclear localization signal (NLS) was amplified by PCR using primers 13/14 from pLew13 plasmid (Addgene plasmid 24007). The resulting PCR product was digested with BglII and NotI and cloned into *Leishmania* expression vector pLEXSY-hyg2 (Jena Bioscience) with the same enzymes to generate pLEXSY-hyg2-T7polNLS construct. *L. major* promastigotes were transfected with SwaI linearized pLEXSY-hyg2-T7polNLS plasmid. Linearized pLEXSY-hyg2-T7polNL plasmid contains T7 RNA polymerase and its 5′ssu and 3′ssu regions. The 5′ssu and the 3′ ssu regions are responsible for homologous recombination into 18S rRNA locus of host chromosome. Hygromycin-resistant transformants were maintained at 200 μg/mL hygromycin. Finally, hygromycin-resistant transformants were further transfected with AatII-digested pLew114hyg5′vector for the generating LmT7.TR cells. The pLew114hyg5′vector contains tetracycline repressor sequence, and it has the capability for homologous recombination into hygromycine locus of hygromycine cassette. LmT7.TR clones were maintained in 200 μg/mL neomycin. The incorporation of T7RNA polymerase gene and tetracycline repressor

sequence in LmT7.TR clones were confirmed by PCR using primers 15/16 and primers 17/18, respectively.

Construction of Inducible Knockdown Cell Line for HemAC-Lm. An antisense construct of HemAC-Lm (pLewHemAC-Lm) was generated by cloning a segment from the 5′ terminus using primers 19/20 and cloned in reverse orientation into the HindIII- and BamHI-digested pLEW82v4 vector which contains T7 promoter and a tetracycline operator sequence. The pLewHemAC-Lm was transfected in the LmT7.TR promastigotes by electroporation to generate tetracycline inducible knockdown for HemAC-Lm gene (IKO) cell line. The IKO cell line was selected by using 25 μg/mL phleomycin and 200 μg/mL neomycin.

Detection of Intracellular cAMP in *L. major* Cells. Intracellular concentration of cAMP in control (CT), IKO, HKO, and overexpressing (OE) cells, incubated either at normoxic or hypoxic condition, were determined by cAMP complete ELISA kit (Enzo Life Sciences). Briefly, 2×10^7 cells were centrifuged and washed twice with Tris buffer saline and homogenized in 400 μL of 0.1 M HCl. Homogenized samples were centrifuged at 10,000 rpm to collect the supernatant. The 200-μL test samples were mixed with 10 μL acetylating reagent mix and vortexed immediately to acetylate cAMP. Then 50 μL of neutralizing reagent was added to a goat anti-rabbit IgG–coated 96-well plate followed by the sequential addition of 100 μL of the acetylated test samples or standards, 50 μL of cAMP conjugate, and 50 μL of cAMP Antibody. The plate was then incubated for 2 h with mild shaking. The plate was then washed five times with wash buffer and was developed by incubating with p-nitrophenyl phosphate solution for 1 h followed by addition of 50 μL stop solution to each well. Reading was taken at 405 nm. All incubations were performed at room temperature.

PKA Activity Assay. PKA activity was assayed in *L. major* lysates as described by Malki-Feldman et al. (2) by phosphorylation of the specific fluorescent PKA substrate kemptide using the PepTag Non-Radio-active PKA Assay (Promega) kit. Briefly, log-phase cells were harvested and washed twice at $1,200 \times g$ (4 °C) for 5 min with washing buffer [20 mM Tris·HCl (pH 7.5), 100 mM NaCl]. The cell pellets were suspended in wash buffer supplemented with 1:25 diluted protease inhibitors (complete-protease inhibitor tablets; Roche). Using a 26-gauge needle and freeze–thawing three times, 5.0×10^6 cells per μL were disrupted. Extracts were precleared by centrifuging at $17,000 \times g$ for 15 min at 4 °C. The aliquots were assayed in 25 μL reaction volume for 30 min at 30 °C. Phosphorylated and nonphosphorylated PepTag peptides were separated by electrophoresis (100 V, 20–30 min) in 0.8% agarose gels in 50 mM Tris·HCl buffer, pH 8. UV-illuminated gels were photographed, and the digital images were analyzed by densitometry using ImageJ software. Kemptide phosphorylation by control cell lysate in the absence of inhibitors or activators was arbitrarily set, and the PKA activity of other cell types was calculated relative to this value and presented as the mean ± SD. Experiments were repeated at least twice.

Metacyclic Purification Assay. To study the metacyclogenesis in promastigotes, log-phase promastigote cultures of each type of cells were washed with PBS, resuspended to 10^8/mL cell density, and incubated with 50 μg/mL of peanut agglutinin (PNA). After 30 min of incubation at room temperature, the suspension was centrifuged at $40 \times g$ for 5 min. The nonagglutinated promastigotes (PNA^-) were collected from supernatant by centrifugation for 5 min at $1,200 \times g$ and washed twice with PBS. Cells were resuspended in PBS and analyzed for light scatter by flow cytometry. Dot plots of forward-angle light scatter vs. side-angle light scatter of the whole sample were taken.

Cell Death Assessment by PI and Annexin V Staining. Phosphatidylserine exposure was assessed by ApoAlert annexin V apoptosis kit (Clontech). After the incubation in the hypoxic condition, cells were harvested at 4 °C by centrifugation for 5 min at $1,200 \times g$ and washed twice with cold 1× binding buffer. The cells were resuspended in 200 μL of 1× binding buffer, following the addition of 5 μL of FITC-conjugated annexin V (20 μg/mL) and 10 μL of propidium iodide (50 μg/mL). Cells were incubated at room temperature for 5–15 min in the dark and analyzed by flow cytometry.

For microscopy, FITC-conjugated annexin V and PI-labeled equivalent cells were adhered to poly-L-lysine-coated slide and visualized with an OlympusIX81 microscope. For each sample, ~20 fields were observed.

Cell Cycle Analysis. Flow cytometric analysis of cell cycle was performed as follows: about 1×10^7 cells were centrifuged and transferred into fresh M199 medium containing 200 μg/mL hydroxyurea and incubated at 25 °C for 12 h for synchronization. The cells were washed and transferred into fresh M199 media containing 10% FCS without hydroxyurea. Aliquotes were taken at 2 h interval and harvested by centrifugation. Cells were then washed twice with PBS and suspended in 200 μL PBS. For fixation, 2 mL of ice cold 70% ethanol was added dropwise to cell suspension with gentle vortexing and left overnight at 4 °C. Before analysis, the cells were treated with 20 mg/mL RNase for 1 h at 37 °C. Subsequently, DNA was stained using propidium iodide (200 μg/mL). The samples were analyzed on a FACS Calibur (Becton Dickinson), and the proportions of G1, S, and G2-M populations were determined using ModFit software. Around 20,000 events were collected for each sample.

Quantitative Real-Time PCR. Oxidative stress response of CT, HKO, IKO, and OE was analyzed by relative quantification mRNA levels of five important antioxidant genes of *Leishmania*: non-selenium glutathione peroxidase (LmjF.26.0810), iron superoxide dismutaseA (LmjF08.0290), peroxidoxin tryparedoxin peroxidase (LmjF23.0040), superoxide dismutase (LmjF30.2770), and tryparedoxin peroxidase (LmjF.15.1120). Total RNA was extracted from CT, HKO, IKO, and OE *Leishmania* cells using an RNAqueous-4PCR kit (Ambion) according to the manufacturer's protocol. RNA quantification was measured by comparing absorbance at 260/280-nm wavelengths. Then the cDNA synthesis from 2 μg of RNA of each cell type was done by using a High Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR was then done with the cDNA on StepOne instrument (Applied Biosystems) using SYBR Green dye (Applied Biosystems) as the reporter dye according to the manufacturer's protocol. The mRNA quantification was done by comparative C_T method using 18 S rRNA as the reference gene.

1. Pal S, Dolai S, Yadav RK, Adak S (2010) Ascorbate peroxidase from Leishmania major controls the virulence of infective stage of promastigotes by regulating oxidative stress. *PLoS ONE* 5(6):e11271.

2. Malki-Feldman L, Jaffe CL (2009) Leishmania major: Effect of protein kinase A and phosphodiesterase activity on infectivity and proliferation of promastigotes. *Exp Parasitol* 123(1):39–44.

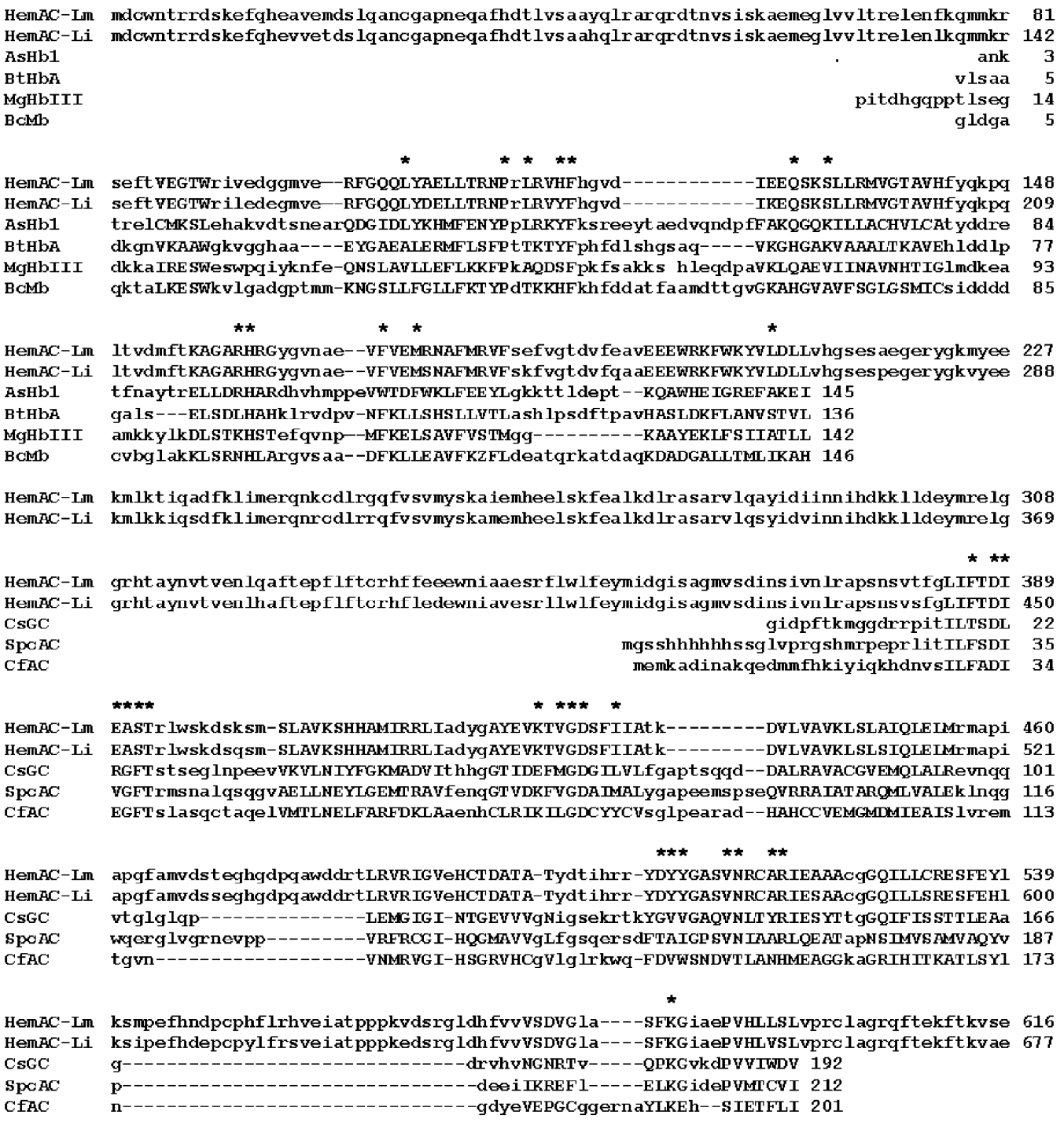

Fig. S1. Sequence analysis of HemAC-Lm. Amino acid sequence of HemAC-Lm was aligned with heme containing adenylate cyclase from *Leishmania infantum* (HemAC-Li), *Ascaris suum* Hemoglobin Domain I (AsHb1), *Bos taurus* deoxyhemoglobin A (BtHbA), *Myxine glutinosa* hemoglobin III (MgHbIII), *Busycotypus canaliculatus* myoglobin (BcMb), *Cyanobacterium synechocystis* sp. PCC guanylyl cyclase (CsGC), *Spirulina platensis* soluble adenylate cyclase Cyac (SpcAC), and *Canis lupus familiaris* adenylate cyclase (CfAC). Lowercase amino acids are unaligned. Dashes indicate variations in sequence length among aligned proteins. Uppercase amino acids are aligned and used to generate a position-specific scoring matrix. Asterisk indicates heme-binding site and nucleotidyl-binding site residue.

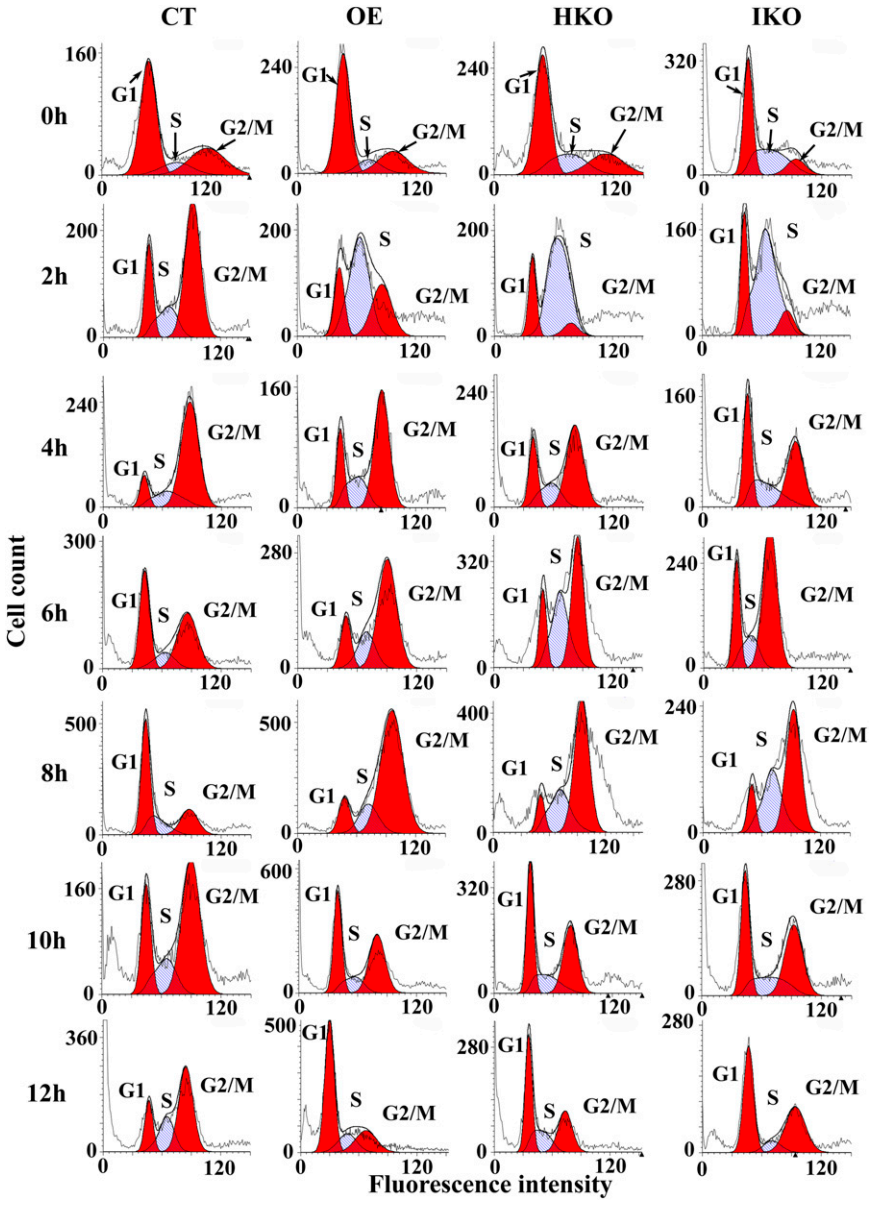

Fig. S2. DNA content distribution of CT, OE, HKO, and IKO cells after removing the hydroxyurea block. The log-phase cells ($<10^7$/mL) were synchronized at the G1–S border with 200 μg hydroxyurea for 12 h. DNA content was measured after staining with propidium iodide, and the cell cycle phases were analyzed by flow cytometry at different time intervals up to 12 h. The fraction of cells in G1/G0 and G2/M phases of the cell cycle (red) and the fraction of cells in S phase of the cell cycle (blue hatched areas) were calculated by Mod-fit software.

Table S1. Oligonucleotides used in this paper

Primer	Primer sequence*	Purpose
Primer 1	5′-AAAAGGATCCGACTGTTGGAACACGCGA-3′	Cloning of HemAC-Lm
Primer 2	5′-AAAACTCGAGTTATTCCGATACCTTCGT-3′	
Primer 3	5′-AAAAGGATCCTCGGCCGGTATGGTGAGC-3′	Cloning of Δ360HemAC-Lm
Primer 4	5′-AAAAAAGCTTTTATTCCGATACCTTCGT-3′	
Primer 5	5′-AAAACCCGGGCGCATGGACTGTTGGAACACGCGA-3′	Cloning of OE construct
Primer 6	5′-AAAAGGATCCTTATTCCGATACCTTCGT-3′	
Primer 7	5′-AAAAAAGCTTTTCGCCACGATCTCG-3′	5′ flanking region
Primer 8	5′-AAAAGTCGACAGTGGACGCGCAGAC-3′	
Primer 9	5′-AAAACCCGGGCACTCAGTGCGCTAC-3′	3′ flanking region
Primer 10	5′-AAAAGGATCCCCTCGATACAATATGTG-3′	
Primer 11	5′-TTCGCCACGATCTCG-3′	Testing primers of HKO
Primer 12	5′-CCTCGATACAATATGTG-3′	
Primer 13	5′-AAAAAGATCTGTCGCCACCATGAACACGATTAACATCGC-3′	Cloning of pLEXSY-hyg2-T7polNLS
Primer 14	5′-AAAAGCGGCCGCGGAGTCGTATTGATTTG-3′	
Primer 15	5′-GAACACGATTAACATCGCTAAG-3′	Testing primers for T7 gene
Primer 16	5′-GCCTGATTCGGCTGAGTGA-3′	
Primer 17	5′-TAGAGCTGCTTAATGAGGTCG-3′	Testing primers for TetR gene
Primer 18	5′-CACAGCGCTGAGTGCATATAATG-3′	
Primer 19	5′-AAAAGGATCCGCGTCTGCGCGTCCACTTTCAC-3′	Cloning of antisense
Primer 20	5′-AAAAAAGCTTCCGTGCACGAGCAGATCTAG-3′	

*Restriction sites in primer sequences are underlined.