

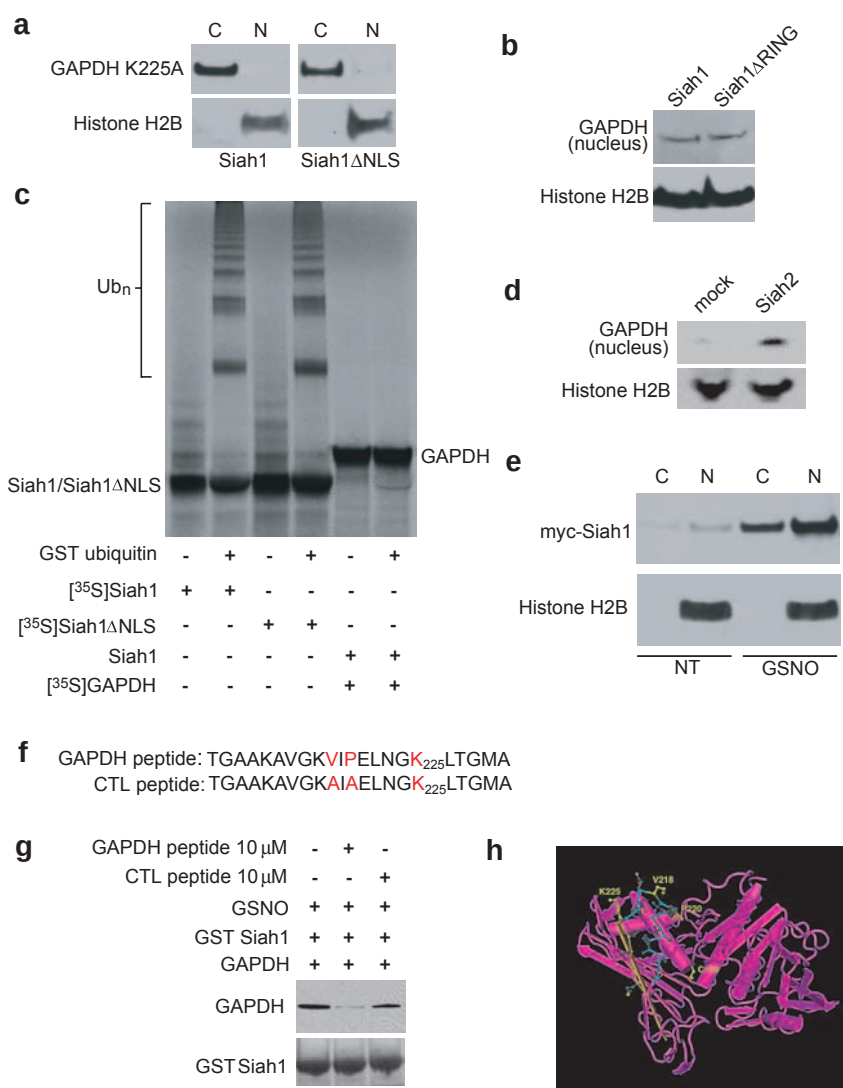


Figure S1 a, Subcellular distribution of GAPDH K225A mutant was examined in cells transfected with Siah1 or Siah1ΔNLS. C: supernatant after 100,000 X g centrifugation, reflecting cytosol. N: purified nuclei obtained by sucrose gradient centrifugation. The result was replicated two times. **b**, Siah1ΔRING mediates GAPDH's translocation to the nucleus as well as wild-type Siah1. HEK293 cells were transfected with either Siah1 or Siah1ΔRING. Forty eight hours after transfection, cells were harvested and crude nuclear fraction was purified. **c**, *In vitro* ubiquitination assay of Siah1. *In vitro* transcribed and translated [³⁵S]Siah1, [³⁵S]Siah1ΔNLS, or [³⁵S]GAPDH was used in the assay. Ub_n indicates the migration position of polyubiquitin conjugates. The result was replicated three times. **d**, Siah2 enhances GAPDH's translocation to the nucleus. HEK293 cells were mock transfected or transfected with myc-Siah2 (human). Cells were harvested and the nuclear fraction obtained. The result was replicated two times. **e**, Effect of an NO donor

on Siah1 protein levels. myc-Siah1 was transfected into HEK293 cells. Transfected cells were either mock treated or 750 μM GSNO treated for 24 h. Augmentation of cytosolic Siah1 presumably reflects Siah1's capacity to shuttle between the cytosol and nucleus. The result was replicated two times. **f**, Amino acid sequence of synthetic GAPDH peptide, which contains Siah binding motif, and control (CTL) peptide, which contains replaced amino acids in Siah binding motif². **g**, GAPDH peptide inhibits the binding of GAPDH and Siah1. GAPDH was pretreated with 200 μM GSNO. GST-Siah1 (amino acid 109-282 of rat Siah open reading frame: cf. Fig. 1a) was added, and binding was assessed by GST-agarose pulldown followed by Western blotting. The result was replicated three times. **h**, Crystal structure of the rabbit GAPDH^{3,4}. The Siah binding domain and C150 are depicted by a ball and stick model.

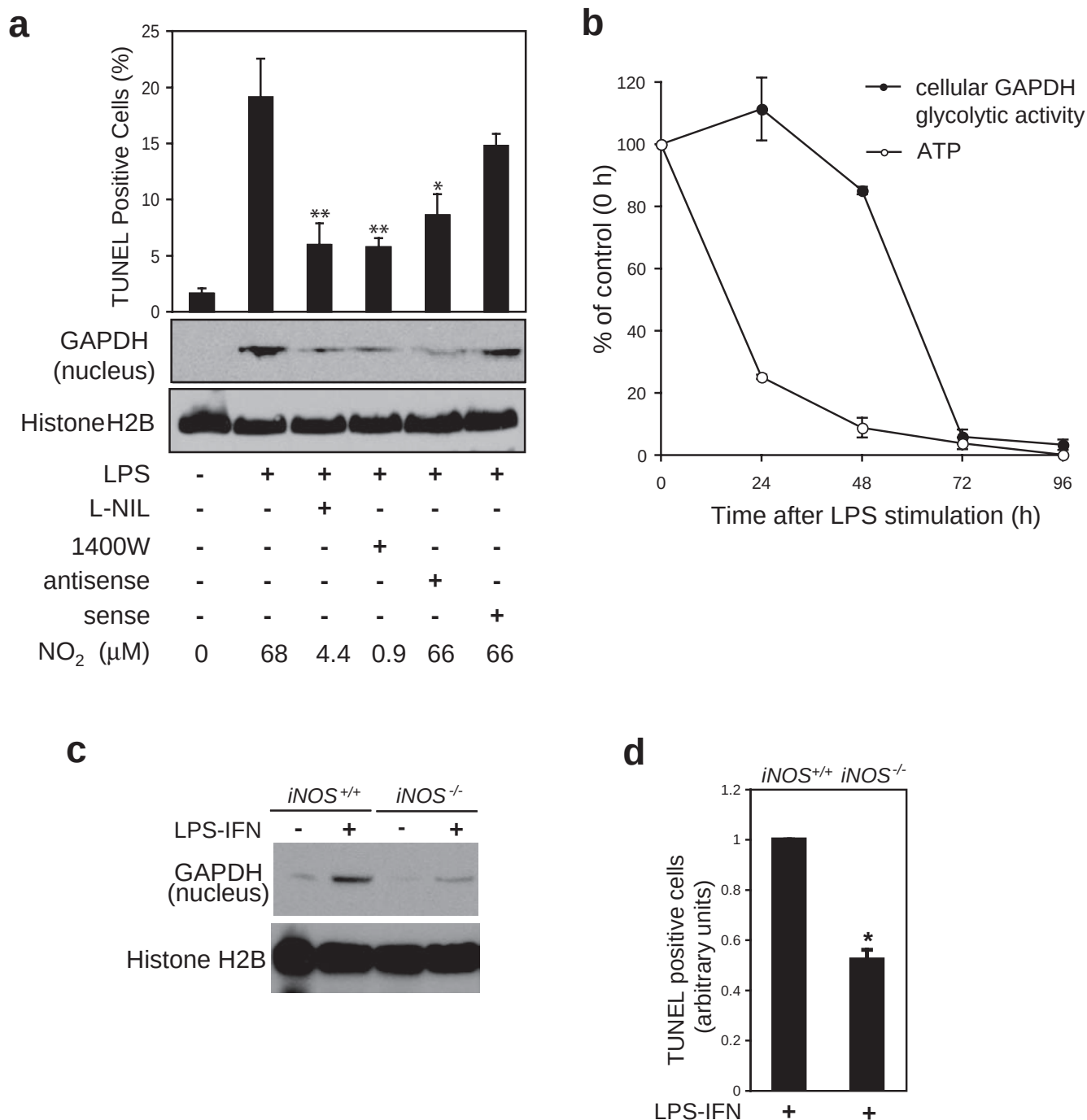


Figure S2 a, Depleting nuclear GAPDH protects macrophages from cell death caused by NO. RAW264.7 cells were activated with LPS for 72 h. iNOS inhibitors L-N^G-(1-iminoethyl)lysine (L-NIL) and 1400W were added together with LPS. Antisense (5 μM) or sense (5 μM) oligonucleotides to GAPDH were added 1 h before stimulation with LPS. Cell death was assessed by TUNEL assay (n = 4–5 ± SEM, *p < 0.05 versus LPS; **p < 0.01 versus LPS; p = 0.3 for LPS + sense versus LPS only). Nuclear fractions were analyzed by Western blotting. Densitometry analysis reveals that 90 (10% (n = 2) of GAPDH translocates to the nuclear fraction 72 h after LPS treatment, consistent with decreased cellular GAPDH glycolytic activity as in **b**. NO₂ concentration was measured by the Griess reagent (n = 4). **b**, Cellular GAPDH glycolytic activity and ATP in RAW264.7 cells after LPS stimulation.

RAW264.7 cells were treated with LPS for the indicated period. GAPDH glycolytic assay: n = 3 ± SEM. Cellular ATP: n = 2 ± SEM. **c**, Decreased translocation of GAPDH to the nucleus in peritoneal macrophages from iNOS knockout mice. Peritoneal macrophages were treated with LPS and IFN-γ for 24 h. The nuclear fractions were analyzed by Western blotting. The remaining nuclear translocation of GAPDH in iNOS KO mice is presumably due to reactive oxygen species produced from NADPH oxidase, which is also activated by LPS-IFN-γ treatment. The experiment was replicated two times. **d**, Endogenous NO is involved in cell death of peritoneal macrophages. Peritoneal macrophages were treated as in **c**. Cell death was assessed by TUNEL assay (n = 3 ± SEM, *p < 0.0005).

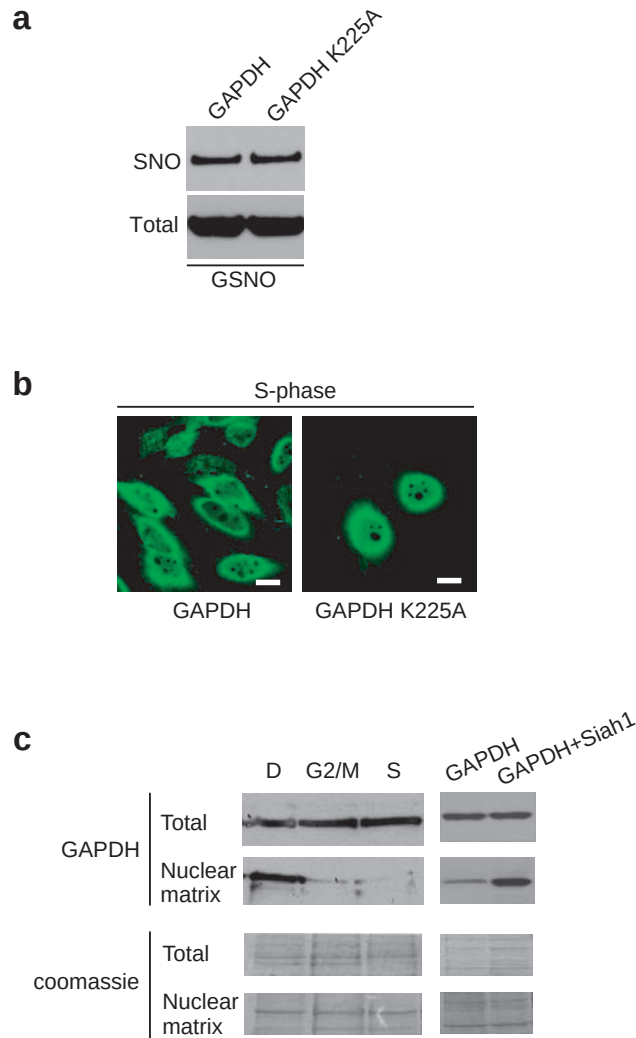


Figure S3 a, S-nitrosylation of GAPDH K225 mutant. HEK293 cells were transfected with either HA-GAPDH or HA-GAPDH K225A mutant. Cell lysates were treated with 50 μ M GSNO for 1 h at 37 $^{\circ}$ C and subjected to the biotin switch assay. SNO: S-nitrosylated target proteins. The result was replicated two times. **b**, GAPDH K225 mutant translocates to the nucleus during S-phase. HeLa cells, transfected with HA-GAPDH K225A, were synchronized in S-phase and stained with anti-HA antibody. Cells were examined under confocal microscope. As a control, endogenous GAPDH during S-phase was stained in parallel. The scale bar indicates 20 μ m.

Representative pictures from two experiments are presented. **c**, GAPDH in the nuclear matrix: Substantial levels of endogenous GAPDH were observed in the nuclear matrix during apoptotic cell death (16 h after 100 μ M etoposide treatment) in HeLa cells (**D**: Death). Nuclear GAPDH in HeLa cells synchronized in the S-phase is soluble and does not occur in the nuclear matrix (**G₂/M**, **S**). GAPDH overexpressed with Siah1 in HEK293 cells is concentrated in the nuclear matrix (**GAPDH**, **GAPDH + Siah1**). The result was replicated two times.

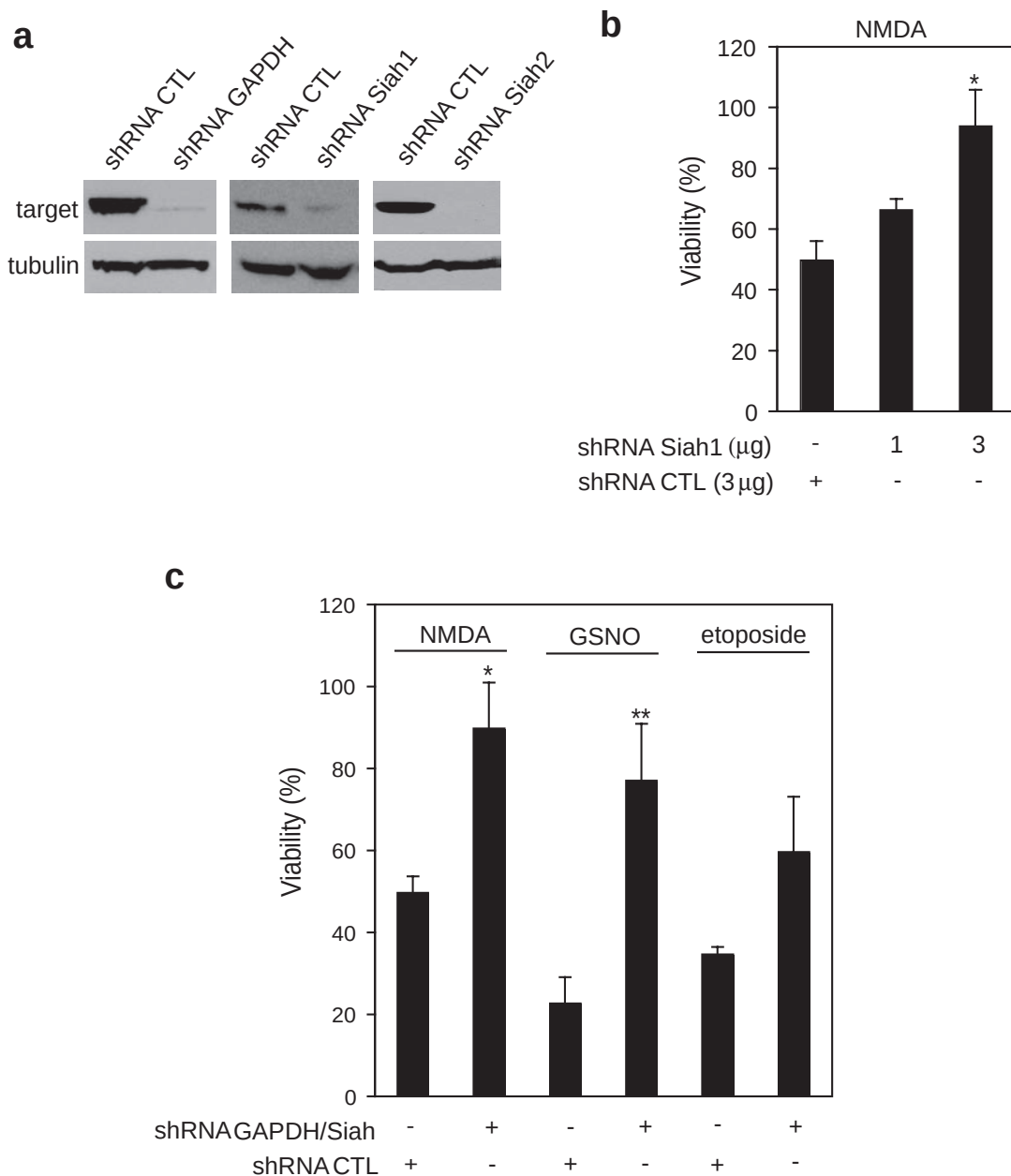


Figure S4 a, Target protein depletion by shRNA examined by Western blotting. Expression constructs to a target molecule (GAPDH, Siah1, Siah2) with myc-tag were transfected into HEK293 cells together with a plasmid construct expressing shRNA to each target or control shRNA (shRNA CTL). Two days after transfection, total cell extracts were analyzed on SDS-PAGE followed by Western blotting with an anti-myc antibody. The experiment was replicated three times. **b**, Dose dependent protection of shRNA-Siah1 against NMDA neuronal toxicity. Neurons were treated with NMDA as in Fig. 7c, except transfection of either 3 μg shRNA CTL (n = 5 ± SEM), 1

μg shRNA Siah1 (n = 2 ± SEM), or 3 μg shRNA Siah1 (n = 4 ± SEM, *p < 0.01). Viability was assessed by GFP staining. **c**, Comparison of shRNA to GAPDH and Siah in preventing cell death elicited by NMDA, GSNO or etoposide. Primary cerebellar granule neurons were transfected with plasmid expressing shRNA to CTL or combination of GAPDH, Siah1 and Siah2 (shRNA Siah: shRNA Siah1 + shRNA Siah2). Transfected cells were treated either with NMDA (n = 5 ± SEM, *p < 0.01), same as in Fig. 7c, treated with 200 μM GSNO for 48 h (n = 3 ± SEM, *p < 0.05), or 100 μM etoposide for 24 h (n = 2 ± SEM). Viability was assessed by GFP staining.

SUPPLEMENTARY METHODS

Reagent. GAPDH peptide [TAT domain (YGRKKRRQRRR)-TGAAKAVG KAVGKVIPELNGKLTGMAK-5FAM] and a control peptide [TAT domain (YGRKKRRQRRR)-TGAAKAVGKAVGKAIAELNGKLTGMAK-5FAM] were produced by GenScript Corporation. Antisense and sense phosphorothioate oligonucleotides to mouse GAPDH were produced in the DNA synthesis facility at Johns Hopkins University. The oligonucleotide sequences for mouse GAPDH were 5'-GACCTTCACCATTTGTCTA-3' for antisense, and 5'-TAGACAAGATGGTGAAGGTC-3' for sense. L-NIL was purchased from Alexis.

TUNEL assay. Cell death of RAW264.7 cells was assayed 72 h after LPS (1 µg/ml) exposure. Antisense (5 µM) or sense (5 µM) oligonucleotides to GAPDH were added to the media 1 h before the LPS exposure. L-NIL (100 µM) or 1400W (100 µM) was added together with LPS. Total and dead cells were determined by counting nuclei stained with 100 ng/ml DAPI and TUNEL, respectively. Cells were fixed in 4% paraformaldehyde and then stained using the *In Situ* Cell Death Detection Kit (Roche) following protocols provided by the manufacturer. The cells were examined under a fluorescence microscope (Zeiss). Cell death was determined as the ratio of dead to total cells.

Cell synchronization. The well-established protocol using thymidine was used as described¹. In brief, HeLa cells were treated with 2 mM thymidine for 16 h to initiate an S-phase block. Cells were released from the initial block by washing off thymidine and incubating in medium containing 24 mM deoxycytidine for 9 h, allowing progression through the cell cycle. A second block with thymidine was for 16 h to synchronize cells at the G1/S boundary.

In vitro ubiquitination assay. Siah1, Siah1ΔNLS, and GAPDH were labeled with [³⁵S]methionine by *in vitro* translation by using TnT kits (Promega).

Two µl of *in vitro* translated sample were mixed with 100 ng E1 (BostonBiochem), 200 ng UbcH5b (BostonBiochem), and 10 µg GST-Ubiquitin (BostonBiochem) in a reaction mixture (50 mM Tris pH 7.4, 2 mM ATP-Mg²⁺, 2 mM DTT, 5 mM MgCl₂). The sample was incubated for 60 min at 30 °C, resolved by SDS-PAGE and analyzed by autoradiograph. For examining ubiquitination of GAPDH by Siah1, Siah1 was *in vitro* translated with cold methionine and 4 µl of the sample were used for ubiquitination assay.

Enzymatic assay for GAPDH. Cells were harvested and lysed in HEN buffer (250 mM HEPES pH 7.7, 1 mM EDTA and 0.1 mM neocuproine). Crude lysates were cleared of insoluble debris by centrifugation at 1,000 X g. The assay mixture contained 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium arsenite, 2 mM NAD⁺, 500 µM DTT and 10 µg of cell lysates. After incubating in a spectrophotometer at 37 °C for 5 min to achieve temperature equilibrium, the reaction was initiated with the addition of glyceraldehyde 3-phosphate to 2 mM. A₃₄₀ from 0 to 10 min was recorded.

Measurement of ATP. ATP concentration was measured by chemiluminescence using Bioluminescent Somatic Cell Assay Kit (Sigma) following the manufacturer's protocol.

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4. Cowan-Jacob, S. W., Kaufmann, M., Anselmo, A. N., Stark, W. & Grutter, M. G. Structure of rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase. *Acta Crystallogr D Biol Crystallogr* **59**, 2218–27 (2003).