A turn-on split-luciferase sensor for the direct detection of poly(ADP-ribose) as a marker for DNA repair and cell death

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Supplementary Information

Experimental Methods

Cell Culture. HeLa cells were maintained at 37 °C and 5% CO_2 in 90% DMEM/F12 1:1 media (Lonza) supplemented with 10% FBS (Lonza), penicillin-streptomycin (Mediatech), and ampotericin B (JR Scientific). MCF7 cells were cultured in 90% RPMI-1640 (HyClone) media supplemented with 10% FBS, penicillin-streptomycin, and ampotericin B.

Cloning and Protein Translation. All cloning enzymes were purchased from New England Biolabs. The firefly luciferase (Fluc) halves utilized in all biosensors were split as previously described.[1] APLF (residues 376-441) was generated by Klenow extension of overlapping primers, followed by ligation into vectors (Invitrogen) containing CLuciferase (residues 398-550) or NLuciferase (residues 2-416), generating pEF6-V5/His TOPO-CLuciferase-APLF (Figure S1) and pcDNA3.1-V5/His TOPO-APLF-NLuciferase (Figure S2). All sequences were confirmed using dideoxynucleotide sequencing. Genes encoding the split-proteins CLuciferase-APLF and APLF-NLuciferase were PCR amplified, and the corresponding products served as templates for in vitro transcription using a T7 Ribomax RNA production kit (Promega) according to the manufacturer's suggestions. Generally, 3 µg of amplified DNA template was incubated at 37 °C for 3 h in the presence of 1x T7 transcription buffer, 7.5 mM rNTPs, and T7 enzyme mix. The transcribed RNA was purified over illustra ProbeQuant G-50 Micro Columns (GE Healthcare) and analyzed by agarose gel electrophoresis. To generate the split-proteins, mRNA encoding CLuciferase-APLF and APLF-NLuciferase was translated in the Flexi Rabbit Reticulocyte Lysate System (Promega). Initial reactions were performed using ^{35}S-methionine to confirm protein expression. Two 20 µL reactions were performed at 30 °C for 1.5 h and consisted of the following components: 66% lysate, 20 µM each amino acid except methionine, 70 mM KCl, 10 µM $ZnCl_2$, 0.8 U/µL RNasin, 1 µCi ^{35}S-

methionine (PerkinElmer) and 2 pmol CLuciferase-APLF or 2 pmol of APLF-NLuciferase mRNA. The translations were analyzed by SDS-PAGE, followed by exposure to a Storage Phosphor Screen (Molecular Dynamics) that was scanned using a Typhoon 9410 Variable Mode Imager (GE Healthcare).

In Vitro Detection of Poly(ADP-Ribose). mRNA encoding CLuciferase-APLF and APLF-NLuciferase was translated in the Flexi Rabbit Reticulocyte Lysate System (Promega). A typical 20 µL reaction was performed at 30 °C for 1.5 h and consisted of the following components: 66% lysate, 20 µM each amino acid, 70 mM KCl, 10 µM $ZnCl_2$, 0.8 U/µL RNasin, 0.4 pmol CLuciferase-APLF, and 0.7 pmol of APLF-NLuciferase mRNA. Following translation, 4.5 µL (50 pg) poly(ADP-ribose) (PAR) (Trevigen) or H_2O was added to 18 µL of the translation and binding was allowed to occur for 45 min at 4 °C. To determine an optimum concentration of $ZnCl_2$ for use in PAR detection, translations were prepared as above with 10, 25, 50, 100, or 200 µM $ZnCl_2$. The limit of PAR detection was determined by adding decreasing amounts of PAR (50, 25, 12.5, or 6.25 pg) following translation with 50 µM $ZnCl_2$. In all cases activity was monitored as a luminescent signal produced upon addition of Steady-Glo Luciferase Assay System (Promega), where 20 µL of each translation was added to 80 µL of Steady-Glo reagent. Luminescent readings were acquired 1 min after mixing using a Turner TD-20e Luminometer with a 10 second integration time.

PAR Detection is Dependent on APLF. Duplicate 25 µL translations were carried out in the Flexi Rabbit Reticulocyte Lysate System according to the manufacturer's protocol using 1.3 pmols of APLF-NLuciferase and CLuciferase-APLF, or 2 pmols of PBSII-NLuciferase and CLuciferase-Zif268 mRNA (Zif268 and PBSII are Cys2-His2 zinc fingers that bind dsDNA),[2] 10 µM $ZnCl_2$, and 0.8 U/µL RNasin and allowed to incubate for 90 min at 30 °C. Following translation, 23.75 µL was added to 1.25 µL of 1 µM poly(ADP-ribose) (BioMol International), 1 µM Zif268-0-PBSII dsDNA, or water for 30 min at RT. Samples were assayed by the addition of 80 µL of Steady-Glo Luciferase Assay System to 20 µL of translated lysate. Light emission was monitored 1 min after Steady-Glo addition using a Turner TD-20e luminometer with a 10 second integration time (Figure S3).

Monitoring Poly(ADP-Ribose) Glycohydrolase. To observe poly(ADP-ribose) glycohydrolase (PARG) activity in vitro, 14 ng PAR (BioMol International) was incubated in 1× PARG assay buffer (50 mM KH_2PO_4/K_2HPO_4, 50 mM KCl, pH 7.2) with 0.5 mU PARG (BioMol International) for 5, 30, 60, or 120 min at 30 °C, followed by heat inactivation of PARG at 95 °C for 5 min. Translations were prepared in 20 µL reactions containing 66% lysate, 20 µM each amino acid, 70 mM KCl, 10 µM $ZnCl_2$, 0.8 U/µL RNasin, 0.6 pmol CLuciferase-APLF, and 0.6 pmol of APLF-NLuciferase mRNA and incubated at 30 °C for 1.5 h. Following translation, 4.5 µL of PARG-treated PAR was added to 18 µL of the translation and incubated at room temperature for 30 min. Activity was monitored as a luminescent signal produced upon addition of Steady-Glo Luciferase Assay System (Promega), where 20 µL of each translation was added to 80 µL of Steady-Glo reagent. Luminescent readings were acquired 1 min after mixing using a Turner TD-20e Luminometer with a 10 second integration time.

Detection of Poly(ADP-Ribose)-Doped Cell Lysates. HeLa cells were harvested from a T-75 flask by trypsinization, and live cells were counted by tryptan blue exclusion. Duplicate aliquots of 1.6×10^5 cells were lysed using 100 µL M-PER Mammalian Protein Extraction Reagent (Pierce) according to the manufacturer's instructions. Poly(ADP-ribose) or an equivalent volume of water was added to cell lysate to achieve 11.1 pg/µL PAR. mRNA encoding CLuciferase-APLF and APLF-NLuciferase was translated in the Flexi Rabbit Reticulocyte Lysate System (Promega). A typical 20 µL reaction was performed at 30 °C for 1.5 h and consisted of the following components: 66% lysate, 20 µM each amino acid, 70 mM KCl, 50 µM $ZnCl_2$, 0.8 U/µL RNasin, 0.4 pmol CLuciferase-APLF, and 0.7 pmol of APLF-NLuciferase mRNA. Following translation, 4.5 µL of PAR-doped lysate was added to 18 µL of the translation and binding was allowed to occur for 45 min at 4 °C. Activity was monitored as a luminescent signal produced upon addition of Steady-Glo Luciferase Assay System (Promega), where 20 µL of each translation was added to 80 µL of Steady-Glo reagent. Luminescent readings were acquired 1 min after mixing using a Turner TD-20e Luminometer with a 10 second integration time. Results are presented as the average of duplicate 50 pg PAR-doped cell aliquots.

Detection of Poly(ADP-Ribose) Induction in Cells. To induce PAR formation in culture, HeLa cells were plated in complete medium at 8×10^4 cells per well in a 24-well plate 24 h prior to treatment. Cells were exposed to 100 μM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (TCI America) or 1% DMSO for 3 min. For recovery experiments, cells were returned to 37 °C and 5% CO_2 for various time points (0, 5, 10 min) before harvest. Each well was washed with PBS, and cells were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce) according to the manufacturer's instructions. To determine the relative amount of cells in each lysate, the relative total protein concentration was determined using the Micro BCA™ Protein Assay Kit (Peirce) according to the manufacturer's instructions. PAR content in the lysate was determined by adding 4.5 μL cell lysate to 18 μL of in vitro translated CLuciferase-APLF and APLF-NLuciferase. Activity was monitored as a luminescent signal produced upon addition of 80 μL Steady-Glo Luciferase Assay System to 20 μL of each translation. Luminescent readings were acquired 1 min after mixing using a Turner TD-20e Luminometer with a 10 second integration time. Data is presented as a luminescent reading relative to protein concentration as determined by the BCA assay. Results are presented as the average of at least two independent experiments.

Detection of PAR in HeLa Cells Treated with H_2O_2. To demonstrate PAR detection in cells challenged with a second DNA damaging agent, we selected H_2O_2. To induce PAR formation in culture, HeLa cells were plated in complete medium at 8×10^4 cells per well in a 24-well plate 24 h prior to treatment. Media was removed and replaced with PBS. Cells were exposed to 100 μM H_2O_2 (J.T. Baker) for 5 min at 37 °C and 5% CO_2. Each well was washed with PBS, and cells were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce) according to the manufacturer's instructions. To determine the relative amount of cells in each lysate, the relative total protein concentration was determined using the Micro BCA™ Protein Assay Kit (Peirce) according to the manufacturer's instructions. PAR content in the lysate was determined by adding 4.5 μL cell lysate to 18 μL of in vitro translated CLuciferase-APLF and APLF-NLuciferase. Data is presented as a luminescent reading relative to protein concentration as determined by the BCA assay. A 2-fold signal was observed for H_2O_2-treated

cells, providing a result comparable to that observed when using MNNG treatment to induce PAR formation (Figure S5).

Figures and Legends

Fig. S1 CLuciferase-APLF sequence. CLuciferase is shown in red, and APLF is shown in green. The linker is black.

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ATGTCCGGTTATGTAAACAATCCGGAAGCGACCAACGCCTTGATTGACAAGGATGGATGGCTACATTCT
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GGAGACATAGCTTACTGGGACGAAGACGAACACTTCTTCATCGTTGACCGCCTGAAGTCTCTGATTAAG
 G  D  I  A  Y  W  D  E  D  E  H  F  F  I  V  D  R  L  K  S  L  I  K
TACAAAGGCTATCAGGTGGCTCCCGCTGAATTGGAATCCATCTTGCTCCAACACCCCAACATCTTCGAC
 Y  K  G  Y  Q  V  A  P  A  E  L  E  S  I  L  L  Q  H  P  N  I  F  D
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 A  G  V  A  G  L  P  D  D  D  A  G  E  L  P  A  A  V  V  V  L  E  H
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 R  E  I  L  I  K  A  K  K  G  G  K  I  A  V  G  G  G  S  S  G  G  G
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 E  T  D  D  R  P  E  C  P  Y  G  P  S  C  Y  R  K  N  P  Q  H  K  I
GAGTACAGACACAACTGA
 E  Y  R  H  N  -
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Fig. S2 APLF-NLuciferase sequence. APLF is shown in green and NLuciferase is shown in red. The linker is black.

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 H  P  G  D  S  D  Y  G  G  V  Q  I  V  G  Q  D  E  T  D  D  R  P  E
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 C  P  Y  G  P  S  C  Y  R  K  N  P  Q  H  K  I  E  Y  R  H  N  Q  I
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 S  Y  A  S  R  G  G  G  S  S  G  G  G  E  D  A  K  N  I  K  K  G  P
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 L  V  P  G  T  I  A  F  T  D  A  H  I  E  V  D  I  T  Y  A  E  Y  F
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 E  M  S  V  R  L  A  E  A  M  K  R  Y  G  L  N  T  N  H  R  I  V  V
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 C  S  E  N  S  L  Q  F  F  M  P  V  L  G  A  L  F  I  G  V  A  V  A
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 P  A  N  D  I  Y  N  E  R  E  L  L  N  S  M  G  I  S  Q  P  T  V  V
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 F  V  S  K  K  G  L  Q  K  I  L  N  V  Q  K  K  L  P  I  I  Q  K  I
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 F  S  H  A  R  D  P  I  F  G  N  Q  I  I  P  D  T  A  I  L  S  V  V
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 P  F  H  H  G  F  G  M  F  T  T  L  G  Y  L  I  C  G  F  R  V  V  L
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 M  Y  R  F  E  E  E  L  F  L  R  S  L  Q  D  Y  K  I  Q  S  A  L  L
GTGCCAACCCTATTCTCCTTCTTCGCCAAAAGCACTCTGATTGACAAATACGATTTATCTAATTTACAC
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 E  I  A  S  G  G  A  P  L  S  K  E  V  G  E  A  V  A  K  R  F  H  L
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 P  G  I  R  Q  G  Y  G  L  T  E  T  T  S  A  I  L  I  T  P  E  G  D
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 D  K  P  G  A  V  G  K  V  V  P  F  F  E  A  K  V  V  D  L  D  T  G
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 K  T  L  G  V  N  Q  R  G  E  L  C  V  R  G  P  M  I  M  S  G  Y  V
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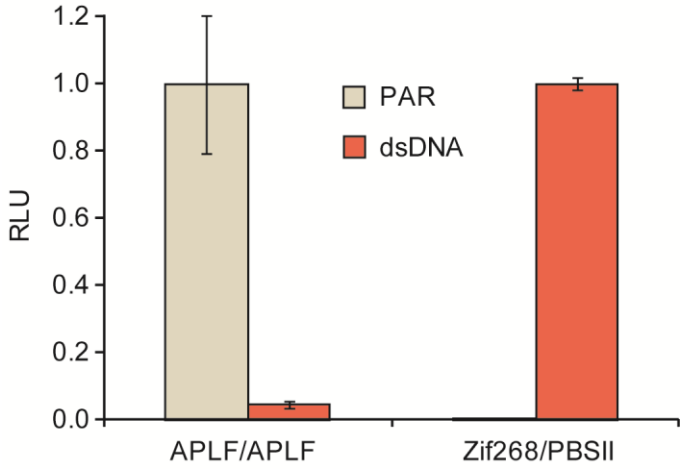

Fig. S3 Specific detection of PAR by APLF. Translations of CLuciferase-APLF + APLF-NLuciferase were incubated with 10 nM PAR or 10 nM of a DNA oligonucleotide. Translations of CLuciferase-PBSII + Zif268-NLuciferase were also incubated with 10 nM PAR or 10 nM of a dsDNA target that contains Zif268 and PBSII binding sites. APLF domains selectively recognize PAR, leading to luciferase reassembly. The Cys2-His2 zinc fingers, Zif268 and PBSII, selectively recognize the dsDNA target, leading to luciferase reassembly.

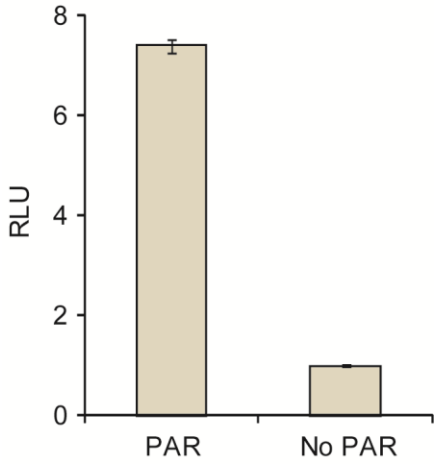

Fig. S4 Split-luciferase detection of PAR-doped cell lysates. CLuciferase-APLF and APLF-NLuciferase were reassembled in the presence of HeLa cell lysate spiked with 50 pg PAR or an equivalent volume of water, followed by luminescence readings.

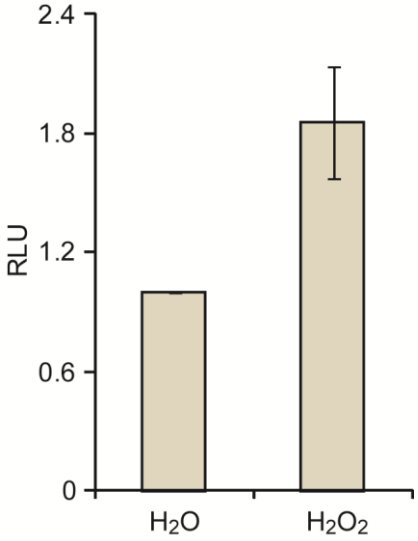

Fig. S5 Detection of PAR in H_2O_2-treated HeLa cells. Translations of CLuciferase-APLF and APLF-NLuciferase were incubated with lysates from HeLa cells treated with H_2O or 100 μM H_2O_2.

References

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2. C. I. Stains, J. R. Porter, A. T. Ooi, D. J. Segal and I. Ghosh, *J. Am. Chem. Soc.*, 2005, **127**, 10782-10783.