Supporting Information (SI) for

Dynamic imaging of MYC and CDKN1A mRNA as an indicator of cell G1-phase arrest

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Table of contents

Experimental Section

Materials and Reagents. All molecular beacons (MBs)—MYC MBs, CDKN1A MBs, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) MBs—and their complementary oligonucleotides and primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). GAPDH MBs were used as control molecular beacons. Details of sequence information were seen in Supplementary Table 1. The G-rich oligodeoxynucleotides (ODNs) were HPLC purified. All the oligonucleotides were dissolved in TE buffer (10mM Tris，1mM EDTA，pH 8.0) as stock solution and stored at -20 °C. Biodegradable PEIs (bPEIs) was a friendly gift from Wisegen Biotechnology (Nanjing, China). Rabbit anti-human β-actin antibody and antibodies of HIF-1α and MYC are purchased from Abcam Inc.(Cambridge, MA). RIPA lysis buffer (including complete protease inhibitor cocktail) are from Applygen Technologies Inc. (Beijing, China). The source of Porphyrin compounds TMPyP4 is Sigma-Aldrich. Human umbilical Vein Endothelial Cells (HUVECs), human cancer cell lines MCF7, U251 and Caski were purchased from Cell Resource Cencer (Beijing, China).

Cell culture and treatment. MCF7, Caski cells and HUVECs were cultured in DMEM with 10% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin in 5% CO_2 at 37 °C. U251 cells were cultured in RPMI-1640 with 10% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin in 5% CO_2 at 37 °C. TMPyP4 dissolved in DMSO was diluted into 25 μM and 100μM working solution with complete medium. The MCF7 cells were treated by 0, 25, and 100 μM of TMPyP4 for 0, 12, 24, 36 and 48 h. The U251 cells were treated by 100 μM $CoCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) for 24 h before adding 100 μM of TMPyP4 for 36 h. The Caski cells and HUVECs were treated by 100 μM of TMPyP4 for 36 h.

Preparation and delivery of bPEIs/MBs indicators. Cells that were seeded into a 24-well plate were cultured for 48 h before the experiment. The molecular beacons (MBs, included MB1 and MB2) were dissolved and diluted with phosphate buffer saline (PBS) buffer. Effective upload of MBs onto the bPEIs was carried out by mixing them in 100 μl of PBS and standing for 15 minutes before diluted to 300 μl with culture medium. The final concentration of MBs was at 300 nM, and 3 μl of bPEIs were added to 1 μg MBs to

form the complex. Cells were washed twice with 1ml of PBS buffer containing 5 mM $MgCl_2$ and then incubated with the 300 μl of diluted bPEIs/MBs for 2 h at 37 °C in 5% CO_2 before diluted with fresh medium for continue incubation. For the following imaging assay, the cells were washed twice with PBS buffer and finally incubated in DMEM or RPMI-1640 medium with 10% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin.

Fluorescence measurements and DNA retarding assay. To test the specificity of molecular beacons (MBs) for the target mRNAs by in vitro fluorescence measurements: MYC MBs were diluted to a concentration of 200 nM in PBS containing 5 mM $MgCl_2$ and treated with desired amount of complementary strand. The maximum excitation wavelength is at 488 nm and the scanning range of emission wavelength is from 500 to 600 nm. The loading number of MBs was determined by DNA retarding assay: The MBs without biodegradable polyethylenimines (bPEIs) were used as control. The MBs were mixed with bPEIs at the ratio of 1:1, 1:3 and 1:5 were separated on MultiNA MCE 202 microchip electrophoresis (Shimadu, Japan) in condition as described in previous work.[1] Briefly, the DNA-500 separation buffer mixed with SYBR gold dye was firstly filled into the chip separation channel. The samples were then introduced for 50 s. A blue light emitting diode (LED) at 470 nm was equipped at the detection window, and florescence at 525 nm was collected as response from the samples. The chip channels were washed three times with ultrapure water between any two analyses.

Qualitative imaging of MBs hybridization in cells and for corresponding cell cycle arrest. MCF7 cells were cultured for 24 h prior to experiments. All microscopy measurements were performed on Zeiss780 inverted fluorescence microscope. The MCF7 cells were delivered with bPEIs/MB1 and bPEIs/MB2 indicators usingthe same above protocol. The imaging of cell cycle arrest imaging was first displayed in fixed MCF7 cells： The cells were treated with or without 100 μM of TMPyP4 for 36 h. After fixed with 4% triformol, the cells were incubated with 50 nM MB1 and 50 nM MB2 for 30 min at 37°C. Before observation with fluorescence microscope, all sample cells were washed with PBS buffer.

Imaging of cell cycle arrest. Dynamic imaging of cell population results were achieved by high content screening (HCS) on ArrayScan VTI 700. Cells were cultured on

24-well plates for 24 h prior to experiments. These cells were firstly stained with Hochest 33342 (Beyotime, Shanghai, China) for 30 min and then with indicators. For the imaging of cell cycle arrest in live cells, the selected cells were treated with or without 100 μM of TMPyP4 for a period of time before delivered with bPEIs/MBs indicators. Continuously imaging was achieved over 2 h at 15 min intervals or over 12 h at 1 h intervals. For time-lapse imaging on individual cells, the cells were grown on a 35 mm glass-bottom dish in phenol red-free Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Cells were labeled with Hochest and then delivered with MB1/MB2 indicators and continuously observed by a high-resolution imaging system (DeltaVision, Elite) equipped with a SSI lamp, a LED, two CCD camera (sCMOS and EMCCD), differential interference contrast (DIC) optical components, and interference filters. Continuously imaging was achieved over 24 h at 30 min intervals. Image acquisition and analysis were performed by SoftWoRx Suite software.

Western blot analysis. TMPyP4 treated cells and control cells were lysed with cell lysis buffer, and complete protease inhibitor cocktail (Roche Applied Science, No. 04693116001), followed by incubation at 4 °C for 1 h. The lysates were ultra-sonicated and centrifuged at 12,000 g for 10 min. Protein concentrations were determined by BCA methods. Fifty micrograms of protein was separated on 10% polyacrylamide-SDS gel and electro-blotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia, Piscataway, NJ). After blocking with TBS/5% skim milk, Millipore (Temecula, CA, USA). Blots were then incubated with primary antibody and β-actin (Sigma) overnight in a cold room. Blots were washed with Tris-buffered saline Tween-20 three times and subjected to secondary anti-rabbit IgG by incubating for 1 hour at room temperature before the final detection. Blots were washed with Tris-buffered saline Tween-20 and detected with the chemiluminescent kit (Amersham Biosciences).

Flow cytometry. For cell cycle analysis by prodium Iodide (PI) dye, cells were collected and washed twice by cold PBS, the cells were then fixed by 70% ethanol at 4 °C overnight. PI dye solution (contain 50 μg/ml of PI, 100 /ml of RNase A and 0.2% of Triton X-100) were added to fixed cells, incubating for 30 minutes at 4 °C before detection on flow cytometry. The results were analyzed by ModFit. For DNA contents analysis, Hoechst 33342 solution was added to the mRNA indicator-containing MCF7

cells. After incubation for 30 min at 37 °C, cells were harvested and analyzed using BD LSRFortessa. MB1 and MB2 were excited by a 488 nm and a 561 nm laser line. Hoechst 33342 was excited by a 325 nm laser line. Fluorescence signals were collected at 530 nm for MB1, at 575 nm for MB2, and at 400 nm for Hoechst33342. The data were analyzed using FlowJo software.

Cytotoxicity analysis. The cytotoxicity assays were based on the CCK8 test. Assays were performed in sterile 96-well plates. Cells in the logarithmic growth phase were seeded on the plate at 1×104 cells/well and cultured by complete RPMI 1640. Cells were incubated for 24 h at 37 °C, in 5% CO_2. After 24 h cells were treated with an increasing concentration of MYC indicators (bPEIs/MBs) for 2 h before diluted with fresh medium for continue incubation. At the indicated time, the cells were washed twice with PBS buffer, and 10 μl of CCK solution was added to each well and incubated for 4 h at 37 °C, in 5% CO_2. Absorbance at 450 nm was measured.

Real-time qPCR analysis. Total RNA was isolated from cells using TRIZOL Reagent (Invitrogen) and stored at −80 °C. Real-time RT-PCR analysis of HIF-1α, VEGF, MYC and CDKN1A mRNA levels was performed using SuperReal PreMix Plus (SYBR Green) from Tiangen Biotech Co., Ltd. (Beijing, China) according to the manufacturer's instructions. The thermocycling conditions were as follows: 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, and 60 °C for 32 s. The relative HIF-1α, MYC, VEGF, and CDKN1A mRNA levels were normalized to GAPDH. The experiment was repeated in triplicate.

Supplementary Figures and Legends

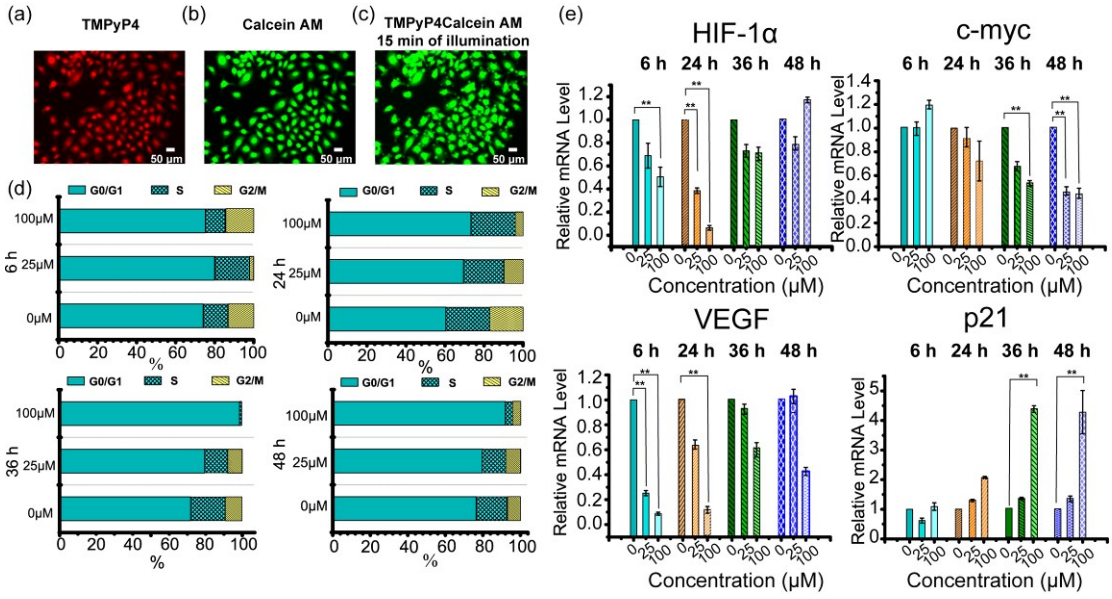

Fig. S1 Establishment model of G1-phase arrest on MCF7 cells by TMPyP4. Fluorescence images of (a) TMPyP4 in MCF7 cells, (b) Calcein AM in MCF7 cells, and TMPyP4 and Calcein AM in MCF7 cells treated by illumination treatment for 15 min. (d) Cell cycle analysis for MCF7 cells treated by TMPyP4 at the concentration of 0, 25 μM and 100 μM at 6, 24, 36 and 48 h. (e) Real-time PCR analysis for MCF7 cells treated by TMPyP4. The symbol ** means statistically significant difference (n=3, $P<0.01$). One-tailed unpaired Student's t-test was used to compare data sets.

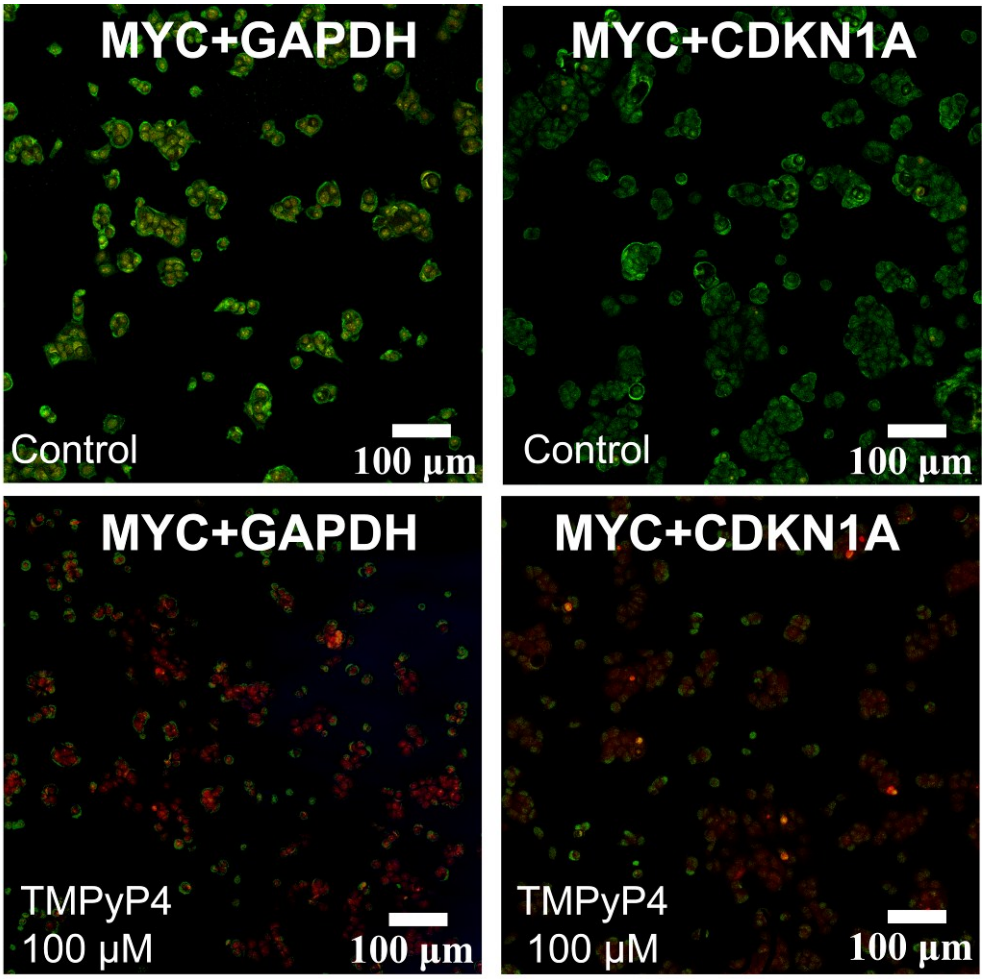

Fig. S2 Merging images of MYC with GAPDH and MYC with CDKN1A in MCF7 cells treated with or without 100 µM of TMPyP4. MYC mRNAs are imaged with green probes, while GAPDH and CDKN1A mRNAs are imaged with red probes.

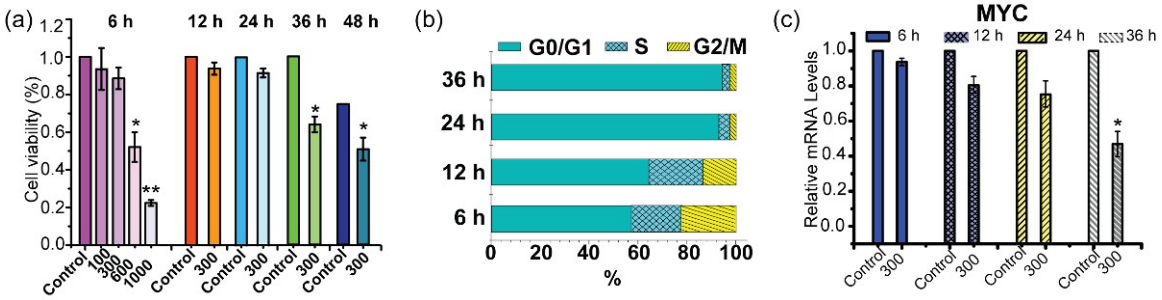

Fig. S3 *MYC* molecular beacons (MBs) affect MCF7 cell viability in terms of concentration and incubation time. (a) Cell viability analysis of MCF7 cells incubated with 100, 300, 600, 1000 nM of MYC MBs for 6 h; with 300 nM of MYC MBs for 12 h; with 300 nM of *MYC* MBs for 24 h; with 300 nM of *MYC* MBs for 36 h; with 300 nM of *MYC* MBs for 48 h. MCF7 cells incubated without *MYC* MBs for 6 h, 12 h, 24 h, 36 h and 48 h were observed as control. *MYC* MBs have dose-dependent and time-dependent inhibition effect, and 300 nM is the concentration threshold that would not cause inhibition effect. (b) Cell cycle analysis for MCF7 incubated with 300 nM of *MYC* MBs for 6 h, 12 h, 24 h and 36 h. (c) Real-time qPCR analysis for *MYC* mRNA level in MCF7 cells incubated without *MYC* MBs (control) and with 300 nM of *MYC* MBs for 6 h, 12 h, 24 h and 36 h. All data presented are the mean of three separate experiments. *P<0.05, **P<0.01.

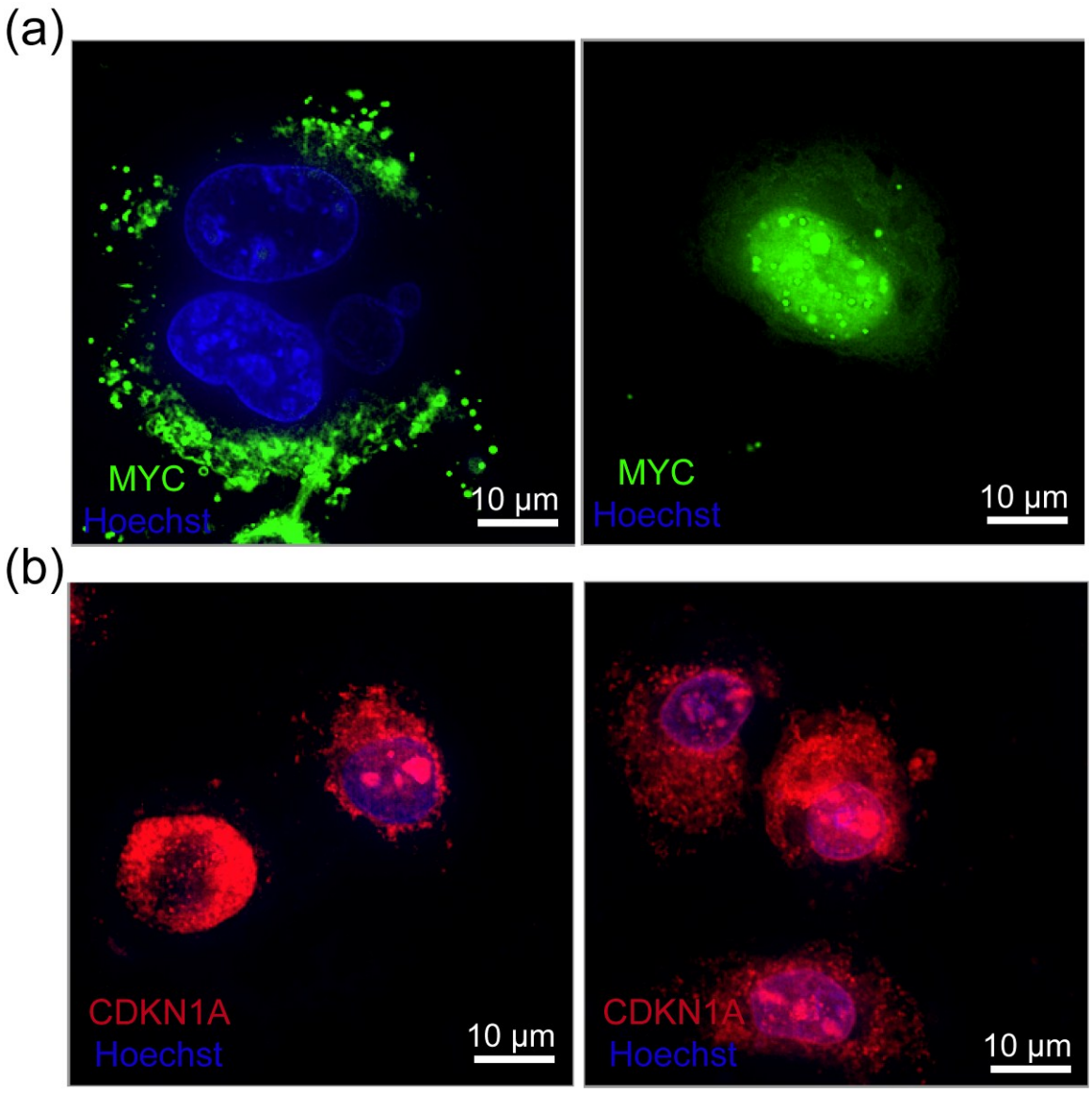

Fig. S4 Distribution patterns of MYC and CDKN1A mRNAs imaged by their specific probes MB1 (green) and MB2 (red). Nucleic were labelled by Hoechst and imaged in blue. (a) Distribution patterns of MYC mRNAs. (b) Distribution patterns of CDKN1A mRNAs.

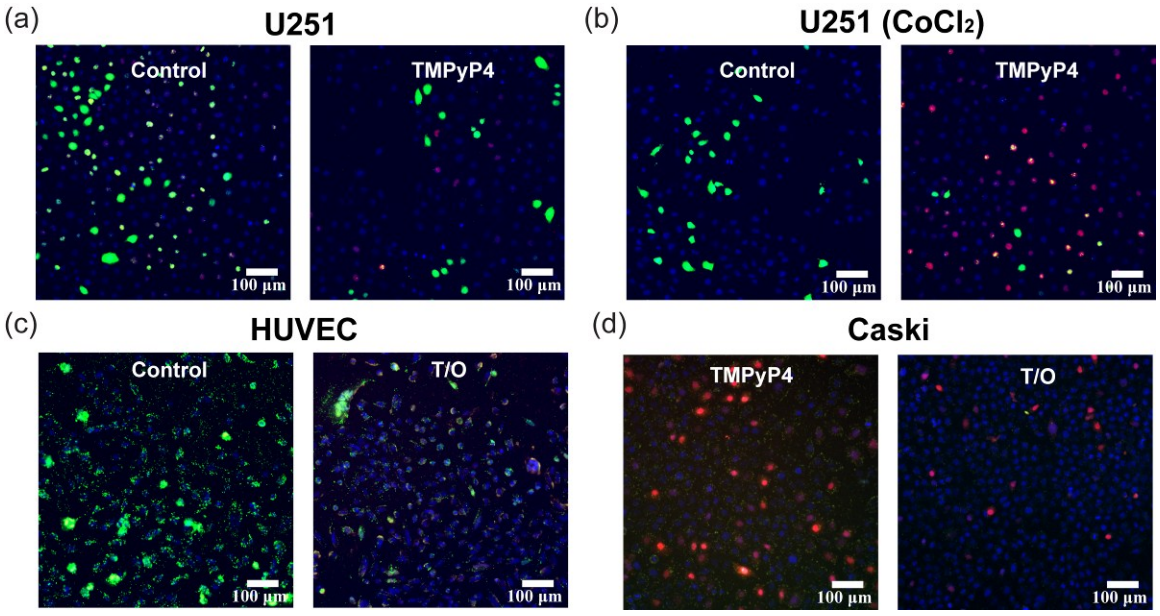

Fig. S5 Observation of different cycle arrest in different cells. The merged fluorescent images of Hoechst (blue), MYC molecular beacons (green) and CDKN1A molecular beacons (red) for (a) U251 cells upon no drug treatment (control) and TMPyP4 treatment; (b) U251 cells treated by CoCl2 (control) and followed by TMPyP4; (c) HUVEC upon no drug treatment (control) and TMPyP4/G-rich oligodeoxynucleotide (T/O) treatment; and (d) Caski cells treated with TMPyP4 and T/O.

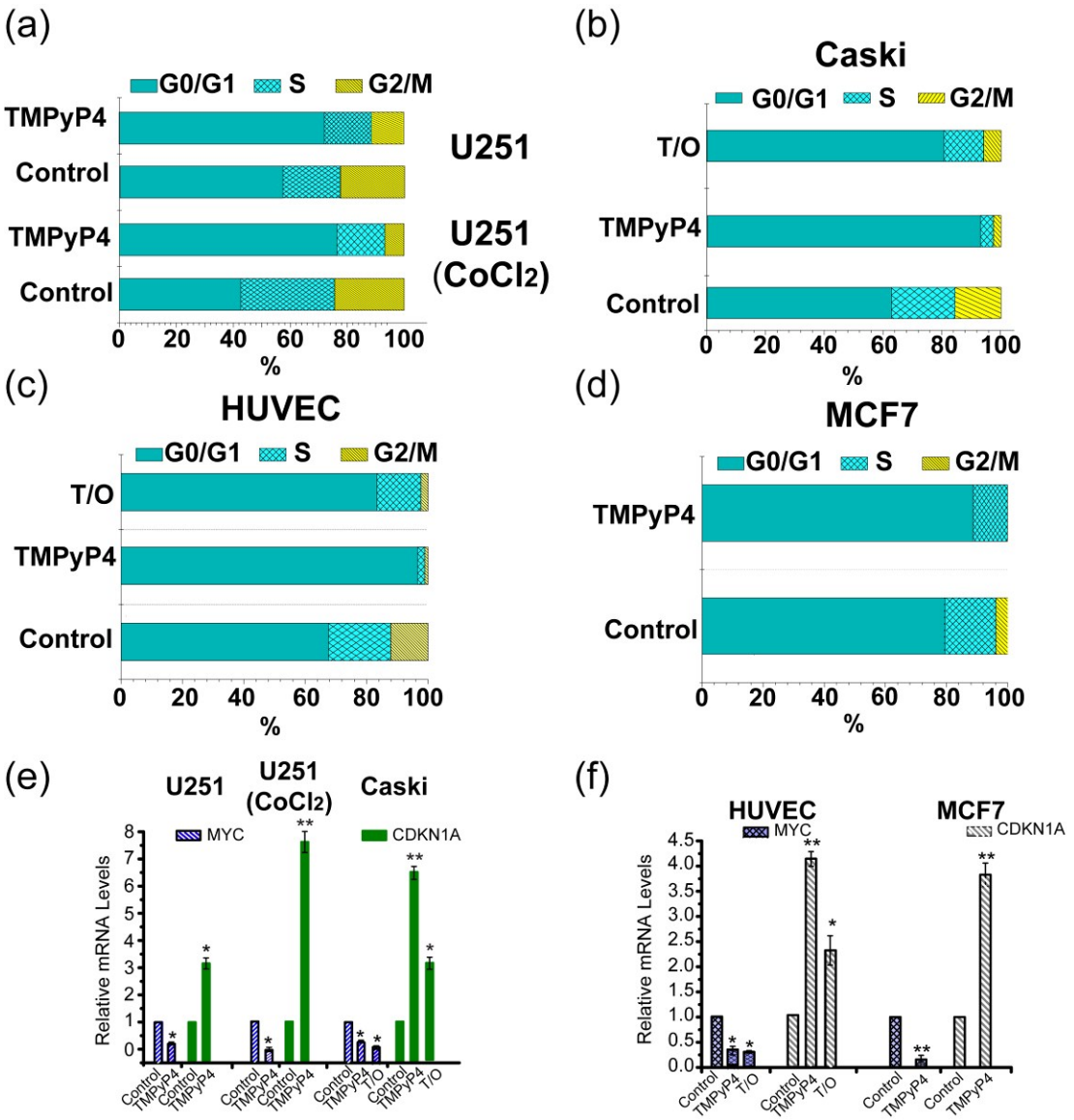

Fig. S6 Cell cycle analysis by flow cytometry and real-time qPCR analysis. (a) Results of cell cycle analysis for U251 cells without treatment of drug and upon treatment of TMPyP4, and U251 cells (treated by $CoCl_2$) without treatment of drug and upon treatment of TMPyP4. (b) Results of cell cycle analysis for Caski cells without treatment of drug, upon treatment of TMPyP4, and upon treatment of TMPyP4/ODN (T/O). (c) Results of cell cycle analysis for HUVEC without treatment of drug, upon treatment of TMPyP4, and upon treatment of TMPyP4/ODN (T/O). (d) Results of cell cycle analysis for MCF7 cells without treatment of drug and upon treatment of TMPyP4. qPCR analysis of MYC and mRNAs level for U251 cells, U251 cells (treated by $CoCl_2$), Caski cells, (e) HUVEC, and MCF7 cells. All data presented are the mean of three separate experiments. *$P<0.05$, **$P<0.01$.

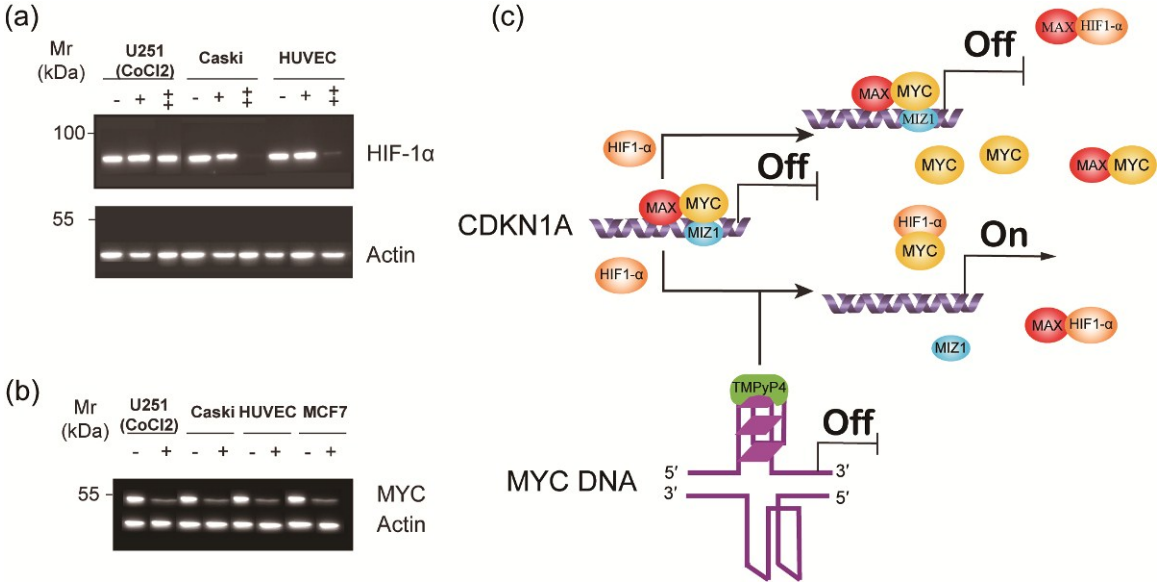

Fig. S7 A model involving functionally counteraction between *MYC* and *HIF-1α*, *MYC* mRNA degradation by *APE1*, and activation of *p53* by *APE1*. (a) Western blotting analysis of *HIF-1α* protein and (b) *MYC* protein. HU represents HUVEC cells. The mark – represents no treatment, + represents TMPyP4 treatment, and double + represents T/O treatment. (c) Schematic representation of up-regulation of CDKN1A by TMPyP4.

Table

Table S1. Detailed sequence information for all oligonucleotide probes.[2-6]

Name	Sequence(5'to3')
HIF-1α primers	Upstream TCT GGG TTG AAA CTC AAG CAA CTG Downstream CAA CCG GTT TAA GGA CAC ATT CTG
MYC primers	Upstream TCG GAA CTA TCC TGC TG Downstream GTG TGT TCG CCT CTT GAC ATT
CDKN1A primers	Upstream TTA GCA GCG GAA CAA GGA GT Downstream AGC CGA GAG AAA ACA GTC CA
VEGF primers	Forward TGC TTC TGA GTT GCC CAG GA Reverse TGG TTT CAA TGG TGT GAG GAC ATA G
GAPDH primers	Upstream CAT GAG AAG TAT GAC AAC AGC CT Downstream AGT CCT TCC ACG ATA CCA AAG T
MYC molecular beacon	6-FAM- ***CC***GAGCACGTTGAGGGGCATCTC***GG*** -BHQ1 [a] Complementary strand GATGCCCCTCAACGTFC
CDKN1A molecular beacon	Cy3- ***CG***CTAGGACACATGGGGAGCCGAAG***CG*** -BHQ2 [a] Complementary strand TCGGCTCCCCATGTGTCCT
GAPDH molecular beacon	Cy3-**CG**AGTCCTTCCACGATACCCACT***CG***-BHQ2 [a]

[a]Underline denotes base pairs in the stem.

ATCG: phosphorothioated backbone. Because it will reduce the binding affinity of MBs by replacing all of the phosphate group with sulfur, we just deal with two bases at 5' end

and 3’ end by phosphothioate (PS) modification.

Movie Description

Movie 1: Imaging of cytoplasmic *MYC* signal
Description: The merged fluorescent images of cell nuclei (Hoechst blue), *MYC* molecular beacons (green) and *CDKN1A* molecular beacons (red) in live MCF7 cells. In MCF7 cells that highly express *MYC*, MB1 shows increased cytoplasmic signal while MB2 nearly shows no signal.
Movie 2: Time-lapse merged imaging of fluorescence and DIC in MCF7 cells without TMPyP4 treatment
Description: Intense green fluorescence was continuously observed in the whole cells. The lasting-observing time is 24 h.
Movie 3: Nuclear *CDKN1A* signal
Description: The dynamic fluorescent imaging of *CDKN1A* signal in live MCF7 cells that up-regulated *CDKN1A*. The blue indicate cell nuclei (Hoechst), the green are signals of MYC molecular beacons (MB1), and the red indicate the *CDKN1A* signals (MB2).
Movie 4: Time-lapse imaging of G1-phase arrest
Description: A significant green-to-red conversion was observed during 24 h in TMPyP4 treated MCF7 cells, indicating that the G1-phase arrest happened.
Movie 5: Time-lapse imaging of relief from G1-phase arrest
Description: A red-to-green conversion was observed during 24 h in MCF7 cells relieved from TMPyP4 effect.

Supplementary References

1. L. Yi, X. Xu, X. Lin, H. Li, Y. Ma and J.-M. Lin, *The Analyst*, 2014, **139**, 3330-3335.
2. H. Luo, G. O. Rankin, N. Juliano, B.-H. Jiang and Y. C. Chen, *Food Chem.*, 2012, **130**, 321-328.
3. G. Li, L. He, E. Zhang, J. Shi, Q. Zhang, A. D. Le, K. Zhou and X. Tang, *Cancer Lett.*, 2011, **311**, 160-170.
4. T. Chen, C. S. Wu, E. Jimenez, Z. Zhu, J. G. Dajac, M. You, D. Han, X. Zhang and W. Tan, *Angewandte Chemie-International Edition*, 2013, **52**, 2012-2016.
5. A. K. Chen, M. A. Behlke and A. Tsourkas, *Nucleic Acids Res.*, 2007, **35**.
6. X. H. Peng, Z. H. Cao, J. T. Xia, G. W. Carlson, M. M. Lewis, W. C. Wood and L. Yang, *Cancer Res.*, 2005, **65**, 1909-1917.