# Electronic Supplementary Information

**Selection of MDM2-binding peptides in *Escherichia coli* using an engineered split intein and aminoglycoside phosphotransferase**

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## Experimental Section

### Materials and methods

All chemicals and solvents were of reagent or HPLC grade. The plasmids, pET22b(+), pET28a(+) and pCDF-1b and BL21(DE3)pLysS were purchased from Novagen. BL21-Gold(DE3)pLysS was purchased from Agilent Technologies. Oligo DNAs were purchased from Japan Bio Services and Greiner Japan. KOD FX DNA polymerase was purchased from TOYOBO. All restriction enzymes were purchased from New England BioLabs. Certified PCR Agarose and Certified Megabase agarose were purchased by Bio-Rad Laboratories. Annexin V-FITC Apoptosis Detection Kit was purchased from Nacalai Tesque. Anti-p53 and anti-β-actin monoclonal antibodies were purchased from Medical & Biological Laboratories. Amino acid derivatives and reagents for peptide synthesis were purchased from Watanabe Chemical and AAPPTEC. Other chemicals and reagents were purchased from Tokyo Chemical Industry, Nacalai Tesque, Sigma Aldrich, Wako Chemical, TOYOBO and QIAGEN. Electrospray ionisation mass spectrometry was measured on a Shimadzu LCMS-2020 single quadrupole liquid chromatography mass spectrometer. HPLC was performed on Cosmosil C18 AR-II (10 × 250 mm) and Daisopak SP-120 C4-Bio (4.6 × 150 mm and 10 × 250 mm, Daiso Chemical) packed columns by employing Hitachi L-7100. PCR was performed on a MJ mini thermal cycler (Bio-Rad Laboratories). Electrophoresis was performed using PowerPac Basic Power Supply (Bio-Rad Laboratories). DNA sequence was analysed using BigDye Terminator 3.1 cycle sequencing kit. UV-visible absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer using a quartz cell with 1.0 cm or 0.5 cm pathlength. Histidine-tagged proteins were purified on a Ni-NTA agarose resin (QIAGEN). Size-exclusion chromatography was performed on a HiLoad 16/600 Superdex 200

pg (GE Healthcare) by employing a Pharmacia LKB LCC-501 plus FPLC system. Bio-layer interferometry (BLI) was performed using a BLItz system (Fortebio). Isothermal titration calorimetry (ITC) was performed using a Microcal VP-ITC microcalorimeter.

**Construction of the plasmid encoding APHN-IntNwt and IntCwt-APHC**

The gene encoding APHN was prepared by PCR from pET28a(+), which contains a kanamycin resistance (KanR) gene, using primers 5′-GGGGTGC-CATGGCCCATATTCAACGGGAAAC-3′ and 5′-GGGCAGCTTAAGCAATGA-AACTGCAATTTATTCATATCAGG-3′. The PCR product was restricted by NcoI and AflII. The gene encoding *Npu* DnaE wild type intein (1-102) was prepared by PCR from a pSKDuet16 vector[1] as a template using primers 5′-GAAGGATCCTGCTTAAGCTATGAAACGG-AAATATTGAC-3′ and 5′-GGAGGCGAGCTCTTAATTCGGCAAATTATCAA-CCCGCATCAAATCC-3′. The PCR product was restricted with AflII and SacI. These restricted DNA products were ligated into pETDuet-1 vector via the NcoI/SacI restriction site. The product plasmid encoding APHN-IntNwt was confirmed by sequencing.

The gene encoding *Npu* DnaE wild type intein (103-137) and APHC was prepared by PCR from a pSKDuet16 vector as a template using primers 5′-GGAGCGCATATGATCAAAATAGCCACACGTAAATATTTAGGC-3′ and 5′-GGAGGCCTCGAGTTAGAAAAACTCATCGAGCATGCAATTAGAAGCTAT GAAGCCATTTTTG-3′. The PCR product was restricted with NdeI and XhoI. The restricted DNA product was ligated into the NdeI/XhoI site of the plasmid encoding APHN-IntNwt. The product plasmid encoding APHN-IntNwt and IntCwt-APHC was confirmed by DNA sequencing.

**Active APH generation by PTS between APHN-IntNwt and IntCwt-APHC**

BL21(DE3)pLysS cells were transformed by the plasmid encoding APHN-IntNwt and IntCwt-APHC, APHN-IntNwt alone or APH full. The *E. coli* cells were grown in LB medium supplemented with 100 μg $mL^{-1}$ ampicillin and 34 μg $mL^{-1}$ chloramphenicol. When $OD_{600}$ reached 0.5–0.6, expression of the protein was induced at 37°C for 30 min or 18°C for 4 h by addition of a final concentration of 0.2 mM IPTG. The cells were spread on LB agar medium containing IPTG (0.2 mM) and kanamycin (0, 10, 20, 40, 80, 160, 320 and 640 μg $mL^{-1}$). The plates were incubated at 37°C for 1 d or 18°C for 3 d.

**Construction of the plasmid encoding tgn-MDM2 and PMI-tgc**

The gene encoding MDM2(18-125) was prepared by PCR from a human placenta cDNA library using 5′-CAGATTCCAGCTTCGGAACAAGAG-3′ and 5′-GTTCTCACTCACAGATGTACCTGAG-3′. The PCR product was further amplified by PCR using 5′-GGGAGGCATATGCAGATTCCAGCTTCGGAAC-AAGAG-3′ and 5′-GGGAGGCTCGAGGTTCTCACTCACAGATGTACCTGAG-3′. The PCR product was restricted with NdeI and XhoI. The product was ligated into the NdeI/XhoI site of pET22b(+). The product plasmid encoding MDM2(18-125) was confirmed by DNA sequencing and restricted with XbaI and NdeI. The gene encoding the engineered 304-2 DnaE intein (1-131) was prepared by the restriction of a reported plasmid encoding 304-2 DnaE intein (1-131) using AflII and NdeI.[2] The plasmid encoding APHN-IntNwt, described above, was restricted with XbaI and AflII. The DNAs encoding 304-2(1-131) and APHN were ligated into XbaI/NdeI site of the plasmid encoding MDM2(18-125). The ligation product was amplified by PCR using primers 5′-GGGGTGCCATGGCCCA-TATTCAACGGGAAAC-3′ and 5′-GGGGCTTGTCGACTTTGTTAGCAGCCGG-ATCTCAGTGG-3′. The PCR product was restricted with NcoI and SalI and

ligated into NcoI/SalI site of pETDuet-1. The product plasmid, termed pET-tgnMDM2, was analysed by sequencing.

The DNA encoding PMI-tgc (MTSFAEYWNLLSP-GGSGGSGGS-GFIASN-CMLDEFF) was prepared by PCR using four oligo DNAs (5′-CCAGCTTTGCCGAATATTGGAACTTACTTTCGCCGGGCGGAAGCGGTGG CTCTGGAGGGAGCGGG-3′, 5′-GAAAAATTCATCCAGCATGCAGTTTGAT-GCAATAAACCC-GCTCCCTCCAGAGCCACCGC-3′, 5′-GGCAGGCATATG-ACCAGCTTTGCC-GAATATTGGAAC-3′ and 5′-CTCGAGTTAGAAAAATT-CATCCAGCATG-3′). The product DNA was restricted with NdeI and XhoI and ligated into the NdeI/XhoI site of pETDuet-1. The plasmid was confirmed by DNA sequencing. The plasmid was restricted with NcoI and SalI and inserted by ligation with the restriction product of pET-tgnMDM2 (NcoI/SalI). The plasmid, termed pET-tgnMDM2-PMItgc, was analysed by DNA sequencing.

**Construction of the plasmids encoding PMI analogue sequences**

The DNAs encoding alanine analogue sequences of PMI were prepared by PCR from pET-tgnMDM2-PMItgc using primers as follows.

S2A_F 5′-CATATGACCGCCTTTGCCAATATTGG-3′

S2A_R 5′-CAATATTCGGCAAAGGCGGTCATATGTATATC-3′

F3A_F 5′-GACCAGCGCTGCCGAATATTGGAACTTAC-3′

F3A_R 5′-CCAATATTCGGCAGCGCTGGTCATATGTATATC-3′

W7A_F 5′-CTTTGCCGAATATGCGAACTTACTTTC-3′

W7A_R 5′-GAAAGTAAGTTCGCATATTCGGCAAAGC-3′

L9A_F 5′-GAATATTGGAACGCACTTTCGCCGGGCGG-3′

L9A_R 5′-CGGCGAAAGTGCGTTCCAATATTCGG-3′

L10A_F 5′-GAATATTGGAACTTAGCTTCGCCGGGCGG-3′

L10A_R 5′-CCCGGCGAAGCTAAGTTCCAATATTC-3′

pET-1F 5′-GATGCGTCCGGCGTAGAGG-3′

T7 Terminator 5′-GCTAGTTATTGCTCAGCGGT-3′

The DNAs were restricted with NotI and AvrII and ligated into the NotI/AvrII site of pET-tgnMDM2. The product DNAs were analysed by DNA sequencing. A plasmid encoding a PTS-inactive mutant, termed PMI-tgc(Ala) (MTSFAEYWNLLSP-GGSGGSGGS-GFIASN-AMLDEFF), in which Ala substitutes for Cys+1 residue of DnaE intein, was prepared by PCR using 5′-GGACGGTCTCGAGTTAGAAAAATTCATCCAGCATGGCGTTTGATGCAATAAACCCGC-3′.

***E. coli* survival assay by PTS reaction of tgn-MDM2 with peptide-tgc**

BL21(DE3)pLysS was transformed with the plasmids encoding tgn-MDM2 and peptide-tgc sequences or PMI-tgc(Ala). The *E. coli* cells were grown in LB medium supplemented with 100 μg $mL^{-1}$ ampicillin and 34 μg $mL^{-1}$ chloramphenicol. When $OD_{600}$ reached 0.5–0.6, expression of the protein and peptide was induced at 18°C for 4 h by addition of a final concentration of 0.2 mM IPTG. The cells were spread on LB agar medium containing IPTG (0.2 mM) and kanamycin [0, 10, 20, 40, 80, 160, 320 and 640 μg $mL^{-1}$ for peptide-tgc; 0 and 10 μg $mL^{-1}$ for PMI-tgc(Ala)]. The plates were incubated at 18°C for 3 d.

**Western blotting analysis of the PTS reaction of tgn-MDM2 with peptide-tgc**

The DNAs encoding a DYKDDDDK (FLAG tag) sequence at the C-terminus of peptide-tgc (peptide-tgc-FLAG) were prepared by PCR from the plasmids encoding tgn-MDM2 and peptide-tgc-sequences and PMI-tgc(Ala) using 5′-CGGCGTCCTCGAGTTATTTGTCGTCATCGTCTTTGTAGTCTCCGAAAAAT

TCATCCAGCATGCAGTTTGATG-3′. BL21(DE3)pLysS was transformed the plasmids encoding tgn-MDM2 and peptide-tgc-FLAG or PMI-tgc(Ala)-FLAG. The *E. coli* cells were grown in LB medium supplemented with 100 μg $mL^{-1}$ ampicillin and 34 μg $mL^{-1}$ chloramphenicol. When $OD_{600}$ reached 0.5–0.6, expression of the protein and peptide was induced at 18°C by addition of a final concentration of 0.2 mM IPTG. After 20 h, the cells were harvested by centrifugation and resuspended with a sample buffer [62.5 mM Tris-HCl (pH 6.8), 1% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 10 mM DTT]. The lysates were separated on 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to the polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 0.3% (w/v) skim milk in PBS-T [10 mM phosphate (pH 7.4), 150 mM NaCl, 0.1% (v/v) Tween 20] at room temperature for 1.5 h. Anti-DYKDDDDK antibody conjugated with horseradish peroxidase (HRP) (1/2500) in PBS-T containing 0.3% (w/v) skim milk was added to the membrane and incubated at room temperature for 1 h. After washing with PBS-T three times, the proteins were visualised with a chemiluminescence detection kit (Supersignal West Femoto Maximum sensitivity substrate kit, Pierce).

**Construction of the plasmid encoding a 12-residue peptide library bearing tgc**

To construct the DNA encoding the peptide library bearing tgc, DNA encoding tgc (GFIASN-CMLDEFF) and having a dummy sequence was constructed by Klenow fragment using 5′-GGAGGCCTCTAGAGGCCATCGAGGCCTACTT-TAAGCTCTGGAGGCCAGGGAGGCCAGGGGAGCGGGTTTATTGC-3′ and 5′-CCGTCCCTCGAGTTAGAAAAATTCATCCAGCATGCAGTTTGATGCAA-TAAACCCGCTCCCCTGGCCTCCCTGG-3′. The product dsDNA was treated with XbaI and XhoI and ligated into XbaI/XhoI site of pET22b(+). C2925 cells were transformed with the plasmid and cultured in LB medium (100 mL) containing

ampicillin (100 μg $mL^{-1}$) at 37°C for overnight. The cells were harvested by centrifugation. The plasmid was extracted and purified form the cells using NucleoBond PC100 (Takara Bio). The purified plasmid was restricted with SfiI at 50°C for 20 h. After the reaction, dephosphorylation at 5′ site of the restricted plasmid was performed using calf intestinal phosphatase.

The DNAs encoding a 12-residue peptide library were prepared by Klenow fragment using 5′-GGCGAGGCCATCGAGGCCTACTTTAAGAAGG-AGATATACATATGNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNN KNNKGGCGGAAGCGGTGGCTCTGG-3′ and 5′-CAGCTTGGCCTCCCTGGC-CTCCAGAGCCACCGCTT CCGCC-3′ (N = A, G, C, or T; K = G or T). The dsDNAs were restricted with SfiI at 50°C for 20 h and purified by 2% (w/v) agarose gel electrophoresis. The restricted DNAs (1.9 μg) were ligated with the restricted and dephosphorylated plasmid (5.2 μg) using T4 DNA ligase (1,200 units) in 1 x T4 DNA ligase ligation buffer (total volume = 85 μL) at 16°C for 16 h. The enzyme was inactivated at 65°C for 10 min. The product plasmids were purified by phenol/chloroform extraction and ethanol precipitation. The purified plasmids were dissolved in sterile water (72 μL).

**PTS-based screening of peptides that bind to MDM2**

The plasmid pET-tgnMDM2 was restricted with NcoI and SalI. The product DNA encoding tgn-MDM2 was ligated into the NcoI/SalI site of pCDF-1b vector. The product plasmid, termed pCDF-tgnMDM2 was confirmed by DNA sequencing.

BL21-Gold(DE3)pLysS was transformed with pCDF-tgnMDM2. The *E. coli* cells were grown in 300 mL LB medium supplemented with 50 μg $mL^{-1}$ spectinomycin and 34 μg $mL^{-1}$ chloramphenicol. When $OD_{600}$ reached 0.55–0.60, the cells were harvested by centrifugation (3,000 g, 4°C, 15 min) and resuspended with 10% glycerol in water (260 mL). After centrifugation (4,000 g, 4°C, 15 min),

the cells were resuspended with 10% glycerol in water (128 mL). After centrifugation (3,000 rpm, 4°C, 15 min), the cells were resuspended with 10% glycerol in water (8 mL). After centrifugation (4,000 rpm, 4°C, 15 min), the cells were resuspended with 10% glycerol in water (1.5 mL). The suspension was dispensed and stored at -80°C.

The prepared electrocompetent cells were transformed by electroporation with the plasmids encoding the 12-residue peptide library and tgc. The plasmid solution (2 μL) was mixed with the competent cells (60 μL), and the mixture was cooled on ice for 1 min. Electroporation was performed on a Bio-Rad MicroPulser using 0.1 cm cuvette (1.8 kV, 1 pulse). The cells were immediately rescued with 1 mL of SOC medium and then incubated at 37°C for 1 h. The cells were cultured on LB-agar medium supplemented with 100 μg $mL^{-1}$ ampicillin, 50 μg $mL^{-1}$ spectinomycin and 34 μg $mL^{-1}$ chloramphenicol at 37°C for overnight. To determine the library size, 10 μL aliquots of the dilution samples (1/10, 1/100, 1/1000) were also cultured on LB-agar medium supplemented with 100 μg $mL^{-1}$ ampicillin, 50 μg $mL^{-1}$ spectinomycin and 34 μg $mL^{-1}$ chloramphenicol at 37°C for overnight. After counting the number of colonies, the library size was calculated as $1.12 \times 10^6$. The cells were harvested by scraping and diluted in LB medium supplemented with 100 μg $mL^{-1}$ ampicillin, 50 μg $mL^{-1}$ spectinomycin and 34 μg $mL^{-1}$ chloramphenicol at 37°C. When $OD_{600}$ reached 0.5–0.6, expression of the protein and peptide was induced at 18°C for 4 h by addition of a final concentration of 0.2 mM IPTG. The cells were cultured on LB-agar medium supplemented with 0.2 mM IPTG and several concentrations of kanamycin (160, 320 and 640 μg $mL^{-1}$). The plates were incubated at 18°C for 5 d. The number of colonies were shown in Table S1. Plasmids from the colonies in the LB-agar medium containing 640 μg $mL^{-1}$ were analysed by sequencing.

**Peptide synthesis**

Peptides were synthesised by 9-fluorenylmethoxycarbonyl (Fmoc) solid phase method on a Rink amide MBHA resin (AAPPTEC) using *O*-(7-azabezotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HATU) as a coupling reagent. Side-chain protections for amino acids were as follows: Arg(Pbf), Asp(OtBu), His(Trt), Gln(Trt), Ser(tBu), Thr(tBu), Trp(Boc) and Tyr(tBu). Fmoc group was removed with 20% piperidine in NMP at room temperature (1 min × 1, 15 min × 1). The coupling condition of Fmoc amino acids was as follows: Fmoc-AA-OH (3 eq), HATU (3 eq) and diisopropyl ethylamine (DIEA; 6 eq) in 1-methyl-2-pyrrolidone (NMP) at room temperature for 40 min. The reaction completion was confirmed by Kaiser test. Acetylation was performed using acetic anhydride (10 eq) and DIEA (2 eq) at room temperature for 40 min. The peptidyl resin was washed with NMP and dichloromethane and dried *in vacuo*. The peptidyl resin was treated with 2.5% (v/v) ethanedithiol, 2.5% (v/v) water and 1.0% triisopropylsilane (TIS) in TFA at room temperature for 2 h. The resin was removed by filtration, and the filtrate was added into $Et_2O$ to precipitate the peptide. The solid was washed with $Et_2O$ and dried *in vacuo.* The crude peptides were purified with RP-HPLC on a Cosmosil 5C18 AR-II semi-preparative column (10 × 250 mm) using a linear gradient of acetonitrile/0.1% TFA at a flow rate of 3.0 mL $min^{-1}$ under 40°C. The purified peptides were lyophilised. The peptides were identified by ESI-MS; aeea-BP1, *m*/*z* calcd for $C_{97}H_{138}N_{20}O_{25}S_2$ $[M+2H]^{2+}$ 1025.20, found 1024.81. aeea-BP3, *m*/*z* calcd for $C_{97}H_{140}N_{27}O_{22}S$ $[M+3H]^{3+}$ 690.47, found 690.46. aeea-PMI, *m*/*z* calcd for $C_{80}H_{117}N_{17}O_{25}$ $[M+H]^{+}$ 1717.89, found 1717.69. Ac-BP1, *m*/*z* calcd for $C_{87}H_{118}N_{18}O_{20}S_2$ $[M+H]^{+}$ 1801.11, found 1800.43. Ac-BP3, *m*/*z* calcd for $C_{87}H_{121}N_{25}O_{17}S$ $[M+2H]^{2+}$ 911.56, found 911.23. Ac-PMI, *m*/*z* calcd for $C_{70}H_{97}N_{15}O_{20}$ $[M+H]^{+}$ 1468.71 (exact mass), found 1468.37. HPLC

chromatogram and ESI-MS spectrum of each peptide was shown in Fig. S6 and S7.

The peptides were dissolved in dimethylsulphoxide (DMSO) to prepare stock solutions. The concentrations of the peptides were determined by amino acid analysis using the phenyl isothiocyanate (PITC) method on a Wakopak WS-PTC column (4.0 × 200 mm).

**Expression of MDM2**

The DNA encoding MDM2(18-125) was prepared by PCR from pCDF-tgnMDM2 as a template using 5′-GGGAGGCTATGCAGATTCCAGCTTCGGAAC-AAGAG-3′ and 5′-GGACGGCTCGAGTCAGTTCTCACTCACAGATGTACCT-G-3′. The product was restricted with NdeI and XhoI and ligated into NdeI/XhoI site of pColdII (Takara Bio). The plasmid was analysed by DNA sequencing. BL21-pG·Tf2 was transformed with the plasmid. The cells were grown in 900 mL LB medium supplemented with 100 μg $mL^{-1}$ ampicillin and 20 μg $mL^{-1}$ chloramphenicol at 37°C. When $OD_{600}$ reached 0.15, expression of chaperon proteins was induced by addition of a final concentration of 20 ng $mL^{-1}$ tetracycline at 37°C. When $OD_{600}$ reached 0.45, the culture was cooled at 4°C for 30 min. The protein expression was induced by addition of a final concentration of 0.4 mM IPTG at 15°C for 24 h. The cells were harvested by centrifugation (7,500 rpm, 5 min, 4°C) and resuspended in 36 mL of bind buffer [50 mM phosphate (pH 8.0), 500 mM NaCl, 5 mM imidazole]. The cells were disrupted by sonication, and the cell debris was removed by centrifugation (10,000 g, 4°C, 1 h). The supernatant was applied on a column with 0.75 mL of Ni-NTA agarose resin (Qiagen) equilibrated with bind buffer. The agarose resin was washed with bind buffer and wash buffer [50 mM phosphate (pH 8.0), 500 mM NaCl, 30 mM imidazole]. MDM2 was eluted with elute buffer [50 mM phosphate (pH 8.0), 500

mM NaCl, 250 mM imidazole]. MDM2 was further purified by a HiLoad 16/600 Superdex 75 pg (GE Healthcare) equilibrated with assay buffer [10 mM phosphate (pH 7.4), 150 mM NaCl, 0.1 mM tris(carboxyethyl)phosphine (TCEP)]. Peak fractions containing MDM2 were pooled and the concentration of MDM2 was determined by absorbance at 280 nm with an extinction coefficient of 10,430 $M^{-1}$ $cm^{-1}$.

**BLI measurements**

Amine Reactive 2nd Generation (AR2G) biosensors were hydrated in water for 10 min and activated by 5-min immersion of 10 mM *N*-hydroxysulfosuccinimide and 20 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in water. Subsequently, the biosensors were incubated with Aeea-modified peptides (5–20 μM) in 10 mM acetate buffer (pH 4.0) for 10 min, and unreacted active ester was quenched using 1 M aminoethanol (pH 8.5). The biosensors were equilibrated with assay buffer containing 0.025% (v/v) tween 20, and associated with 4 μL of several concentrations of MDM2 in the same buffer at room temperature for 2 min. Subsequently, the biosensors were incubated with the buffer for 3 min. The biosensors were regenerated by 6 M guanidine hydrochloride and 0.2 M acetic acid for 30 s and equilibrated with the buffer. BLI signals were analysed using BLItz Pro 1.2 software (Fortebio).

**ITC measurements**

2 μM each peptide in assay buffer containing 1% (v/v) DMSO was load in the cell with 23 μM MDM2 in the same buffer in the titration syringe. The titration experiments were performed at 25°C with an initial 3 μL injection with duration 7.2 s, followed by 22 12 μL injections with a duration of 28.7 s. The spacing between each injection was 300 s, and the stirring speed was 260 rpm. Reference

titrations were carried out by injecting 23 μM MDM2 in assay buffer containing 1% (v/v) DMSO into assay buffer containing 1% (v/v) DMSO in the absence of the peptide in the cell, and heat of dilution was subtracted from the corresponding peptide-MDM2 titration data. Data was analysed by Origin 7 software by fitting to a single-site binding model. Errors correspond to the standard deviation of the nonlinear least-squares fit of the data points of the titration curve.

**Competitive binding assay**

A fluorescently labeled p53 peptide (TAMRA-Ahx-ETFSDLWRLLPEN-$NH_2$; Ahx = 6-aminohexanoyl) was synthesised by Fmoc solid phase method described above on a Rink amide PEG resin XV. After deprotection of Fmoc group at the N-terminus, 5-carboxytetramethylrhodamine (2 eq), HATU (2 q), and DIEA (4 eq) in NMP were added and the mixture was stirred at room temperature for 2 h. The peptidyl resin was treated with TFA and purified by RP-HPLC described above. TAMRA-p53, *m*/*z* calcd for $C_{105}H_{143}N_{22}O_{27}$ $[M+H]^{2+}$ 1073.20, found 1072.89 (Fig. S8).

To determine the $K_d$ value of TAMRA-p53 peptide with MDM2, 50 nM TAMRA-p53 in assay buffer was mixed with several concentrations of MDM2 using a black 96-well half area plate (Corning). The fluorescence polarization was recorded on a multi-detection microplate reader (Synergy 2, BioTek) with the 530 nm excitation and 590 nm emission filters. The $K_d$ value was calculated using the following equation and KaleidaGraph (Synergy), in which a 1:1 stoichiometry was assumed.

$$FP = FP_f + (FP_b - FP_f)\frac{[T]_0 + [M]_0 + K_d - \sqrt{([T]_0 + [M]_0 + K_d)^2 - 4[T]_0[M]_0}}{2[T]_0}$$

Where *FP* is the measured polarization value, $FP_b$ is the polarization of the bound TAMRA-p53, $FP_f$ is the polarization of the free TAMRA-p53, $[T]_0$ is the total concentration of TAMRA-p53 and $[M]_0$ is the total concentration of MDM2.

To determine the $K_d$ values by the competitive binding assays, several concentrations of the peptides (BP1, BP3 and PMI) were mixed with 50 nM TAMRA-p53 and 2 μM MDM2 in assay buffer. The fluorescence polarization was measured, and the obtained data were analysed according to a literature[3] using KaleidaGraph. Each data point represents the average of an experimental condition performed in triplicate.

**Cell viability assay**

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay was performed to determine the cell viability in the presence and absence of the peptides. SJSA-1 or SW480 cells were maintained in RPMI-1640 or Leibovitz's L-15 medium, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a humidified atmosphere of 5% $CO_2$. SJSA-1 cells were seeded at 5,000 cells per well in 96-well tissue culture plates and cultured in RPMI-1640 supplemented with 10% FBS and 1% P/S for 24 h. SW480 cells were seeded at 10,000 cells per well in 96-well tissue culture plates and cultured in Leibovitz's L-15 supplemented with 10% FBS and 1% P/S for 24 h. Cells were then treated with BP1, BP3, PMI, Nutlin-3 or DMSO (1% DMSO) in the culture medium for 3 d. The solutions were changed into the medium supplemented with 10% FBS, 1% P/S and MTT (0.5 mg/mL), and the cells were further incubated at 37°C for 4 h. The precipitated formazan was dissolved by 0.04 M HCl in 2-propanol. Absorbance at 570 nm was then measured. Cell viability was analysed at the ratio of the absorbance at 570 nm of the cells treated with the compounds to the cells with the DMSO vehicle.

**Annexin V binding assay**

SJSA-1 cells were seeded at 25,000 cells per well in 24-well tissue culture plates and cultured in RPMI-1640 supplemented with 10% FBS and 1% P/S for 24 h. SW480 cells were seeded at 50,000 cells per well in 24-well tissue culture plates and cultured in Leibovitz's L-15 supplemented with 10% FBS and 1% P/S for 24 h. Cells were then treated with 50 μM BP3, 10 μM Nutlin-3 or DMSO (1% DMSO) in the culture medium for 2 d. The cells were detached by treatment with a trypsin/EDTA solution at 37°C for 5 min. The cells were collected with the culture medium in microtubes, and then centrifuged and washed with PBS twice. Cells were suspended in the Annexin V binding buffer. Annexin V-FITC and propidium iodide (PI) solutions were added to the cells. The mixtures were incubated at room temperature for 15 min, and then transferred into 24-well tissue culture plates. The annexin V-positive cells and PI positive cells were counted on a fluorescence microscope (FLoid Cell Imaging Station, ThermoFisher Scientific). The experiments were conducted in triplicate.

**Western blotting**

SJSA-1 cells were seeded at 25,000 cells per well in 24-well tissue culture plates and cultured in RPMI-1640 supplemented with 10% FBS and 1% P/S for 24 h. Cells were then treated with 50 μM BP3, 10 μM Nutlin-3 or DMSO (1% DMSO) in the culture medium for 24 h. The culture media were removed, and the cells were washed with PBS twice. Cell lysis buffer [50 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) deoxycholic acid and 0.1% (w/v) SDS] containing 1 x protease inhibitor cocktail (Roche) was added and incubated at 4°C for 30 min. The cell lysates were collected in microtubes, and the insoluble materials were removed by centrifugation to obtain the supernatants. Total

protein concentrations were estimated by BCA assay. The cell lysates containing 1 μg of whole-cell protein were separated on 12% SDS-PAGE, and transferred to the PVDF membrane. The membranes were blocked with 0.3% (w/v) skim milk in PBS-T at room temperature for 1.5 h. Anti-p53 (1/4000) or anti-β-actin (1/4000) antibody in PBS-T containing 0.3% skim milk was added to the membrane and incubated at room temperature for 1 h. After washing with PBS-T three times, anti-mouse IgG conjugated with HRP in PBS-T containing 0.3% skim milk was added and incubated at room temperature for 1 h. After washing with PBS-T three times, the proteins were visualised with a chemiluminescence detection kit (Supersignal West Femoto Maximum sensitivity substrate kit, Pierce).

**Cell penetration of peptides**

SJSA-1 cells were seeded at 25,000 cells per well in 24-well tissue culture plates and cultured in RPMI-1640 supplemented with 10% FBS and 1% P/S for 24 h. Cells were then treated with 50 μM BP1, BP3 and PMI in the culture medium for 30 min. The cells were detached by treatment with a trypsin/EDTA solution at 37°C for 5 min. The cells were collected with the culture medium in microtubes, and then centrifuged and washed with PBS three times. The cells were mixed with 0.1% (v/v) TFA (100 μL) and disrupted by sonication. After centrifugation, the lysates were analysed by RP-HPLC using the tryptophan fluorescence with a fluorescence detector (Shimadzu, RF20A). An analytical column (Daisopak SP-120 C4-Bio, 4.6 × 150 mm) was used. The excitation and emission wavelength are 295 nm and 350 nm, respectively.

## References


1. S. Ellilä, J. M. Jurvansuu and H. Iwaï, *FEBS Lett.*, 2011, **585**, 3471–3477.
2. M. Kawase, M. Fujioka and T. Takahashi, *ChemBioChem*, 2021, **22**, 577–584.
3. M. H. A. Michael, J. Y. Wang and G. Wagner, *Biochemistry*, 2004, **43**, 16056–16066.

**Table S1**. The number of colonies by screening

| kanamycin conc. (μg $mL^{-1}$) | number of colonies |
|---|---|
| 160 | > 100 |
| 320 | 80 |
| 640 | 25 |

**Table S2**. Kinetic parameters of peptides with MDM2

| peptide | $k_{on}$ (× $10^5$ $M^{-1}$ $s^{-1}$) | $k_{off}$ (× $10^3$ $s^{-1}$) | $K_d$ (nM)[a] |
|---|---|---|---|
| BP1 | 4.05 ± 0.06 | 2.39 ± 0.04 | 5.90 ± 0.14 |
| BP3 | 3.63 ± 0.08 | 1.72 ± 0.08 | 4.74 ± 0.23 |
| PMI | 10.59 ± 0.32 | 11.67 ± 0.26 | 11.0 ± 0.40 |

[a]$K_d$ was calculated by the ratio of $k_{off}/k_{on}$.

**Table S3**. Thermodynamic parameters of peptides with MDM2

| peptide | ΔH (kcal $mol^{-1}$) | TΔS (kcal $mol^{-1}$) | $K_d$ (nM)[a] |
|---|---|---|---|
| BP1 | -18.4 ± 0.15 | -6.80 | 3.24 ± 0.63 |
| BP3 | -18.7 ± 0.19 | -7.99 | 15.1 ± 2.05 |
| PMI | -17.7 ± 0.17 | -6.77 | 9.17 ± 1.59 |

[a]$K_d$ was calculated by the ΔG value.

APHN-IntNwt

MAHIQRETSCSRPRLNSNMDADLYGYKWARDNVGQSGATIYRLYGKPDAPELFLKHGKG
SVANDVTDEMVRLNWLTEFMPLPTIKHFIRTPDDAWLLTTAIPGKTAFQVLEEYPDSGE
NIVDALAVFLRRLHSIPVCNCPFNSDRVFRLAQAQSRMNNGLVDASDFDDERNGWPVEQ
VWKEMHKLLPFSPDSVVTHGDFSLDNLIFDEGKLIGCIDVGRVGIADRYQDLAILWNCL
GEFSPSLQKRLFQKYGIDNPDMNKLQFHCLSYETEILTVEYGLLPIGKIVEKRIECTVY
SVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGSLIRATKDHKFMTVDGQMLPIDEIFER
ELDLMRVDNLPN

IntCwt-APHC

MIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASNCMLDEFF

**Fig. S1** The amino acid sequences of APHN-IntNwt (upper) and IntCwt-APHC (lower). The sequences corresponding to APH and *Npu* DnaE wt were shown in green and blue, respectively.

MAHIQRETSCSRPRLNSNMDADLYGYKWARDNVGQSGATIYRLYGKPDAPELFLKHGKG
SVANDVTDEMVRLNWLTEFMPLPTIKHFIRTPDDAWLLTTAIPGKTAFQVLEEYPDSGE
NIVDALAVFLRRLHSIPVCNCPFNSDRVFRLAQAQSRMNNGLVDASDFDDERNGWPVEQ
VWEEMHKLLPFSPDSVVTHGDFSLDNLIFDEGKLIGCIDVGRVGIADRYQDLAILWNCL
GEFSPSLQKRLFQKYGIDNPDMNKLQFHCLSYDTEILTVEYGLLPIGKIVEERIECTVY
SVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGSLIRATRDHKFMTVDGQMLPIDEIFER
ELDLMRVDNLPSIKIATRKYLGKQKVYDIGVERDHNFALKNGGTSGGSGGSGGSGGSGG
SGGSGGSGGSGGSGGSGGSGGSHMQIPASEQETLVRPKPLLLKLLKSVGAQKDTYTMKE
VLFYLGQYIMTKRLYDEKQQHIVYCSNDLLGDLFGVPSFSVKEHRKIYTMIYRNLVVVN
QQESSDSGTSVSENLEHHHHHH

**Fig. S2** The amino acid sequence of tgn-MDM2. The sequences corresponding to APHN(1–164), 304-2 DnaE(1–131) and MDM2(18–125) were shown in green, purple and red, respectively.

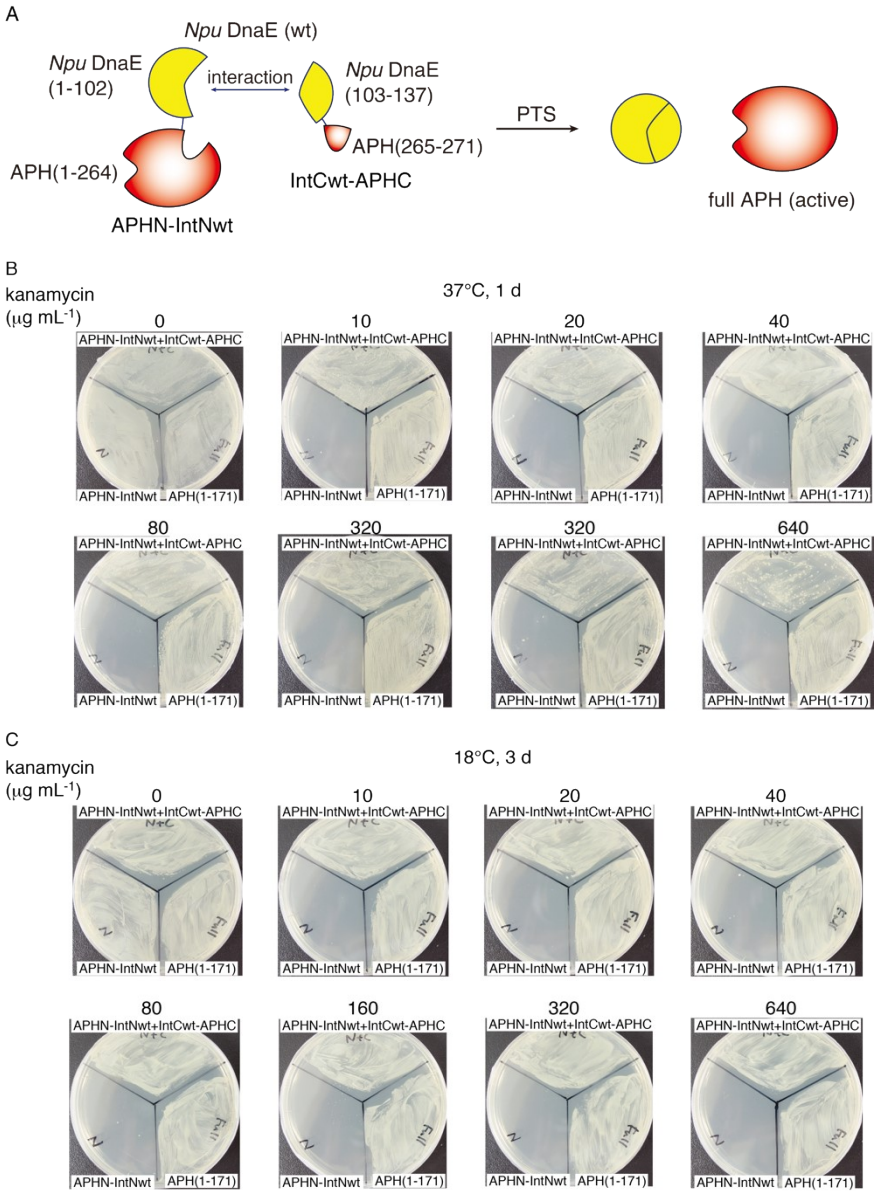



**Fig. S3** PTS reaction and active APH generation using *E. coli* cells expressing APHN-IntNwt and IntCwt-APHC. (A) Schematic representation of the PTS reaction of APHN-IntNwt with IntCwt-APHC. (B, C) The reaction was examined by *E. coli* cell growth in LB-agar medium containg 0, 10, 20, 40, 80, 160, 320 and 640 μg $mL^{-1}$ kanamycin at (B) 37°C for 1 d and (C) 18°C for 3 d. *E. coli* cells expressing full length APH(1–271) and APHN-IntNwt alone were used as positive and negative controls, respectively.

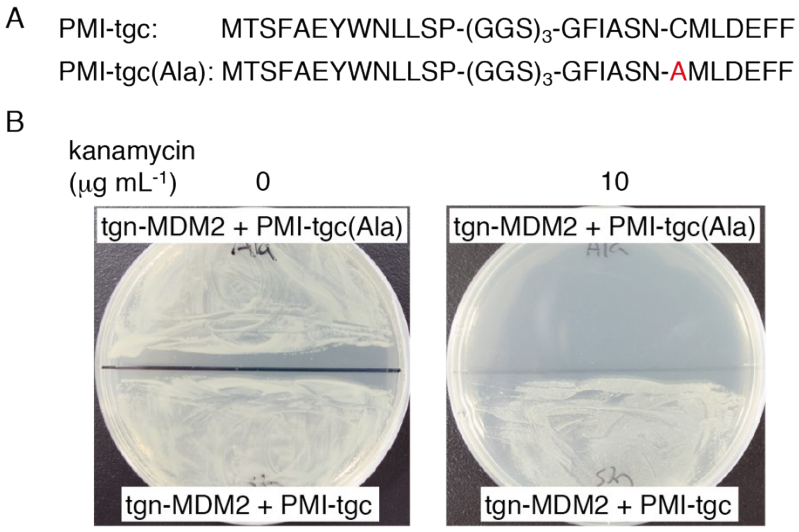
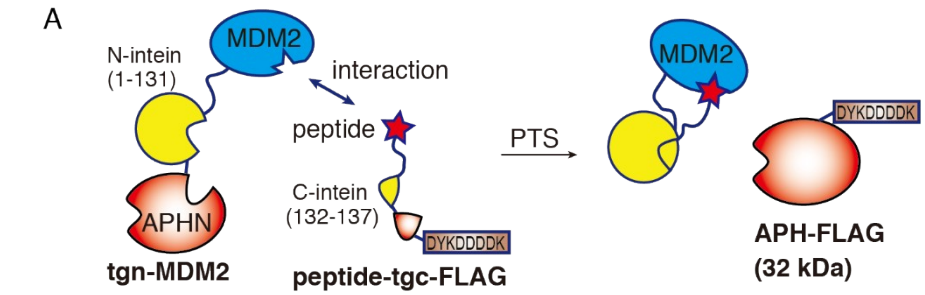



**Fig. S4** (A) Amino acid sequences of PMI-tgc and a PTS-inactive mutant PMI-tgc(Ala). (B) Examination of PTS reaction and active APH generation using *E. coli* cells expressing tgn-MDM2 and PMI-tgc(Ala) and PMI-tgc. *E. coli* cells were grown in LB-agar medium containg 0 and 10 μg $mL^{-1}$ kanamycin at 18°C for 3 d.




B

peptide-tgc-FLAG

PMI: MTSFAEYWNLLSP-$(GGS)_3$-GFIASN-CMLDEFF-G-DYKDDDDK

S2A: MTAFAEYWNLLSP-$(GGS)_3$-GFIASN-CMLDEFF-G-DYKDDDDK

F3A: MTSAAEYWNLLSP-$(GGS)_3$-GFIASN-CMLDEFF-G-DYKDDDDK

W7A: MTSFAEYANLLSP-$(GGS)_3$-GFIASN-CMLDEFF-G-DYKDDDDK

L9A: MTSFAEYWNALSP-$(GGS)_3$-GFIASN-CMLDEFF-G-DYKDDDDK

L10A: MTSFAEYWNLASP-$(GGS)_3$-GFIASN-CMLDEFF-G-DYKDDDDK

peptide-tgc(Ala)-FLAG

PMI(Ala): MTSFAEYWNLLSP-$(GGS)_3$-GFIASN-AMLDEFF-G-DYKDDDDK

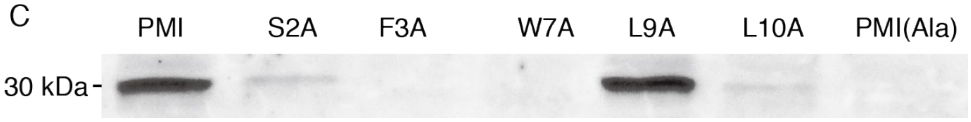



**Fig. S5** (A) Schematic illustrations of the PTS reaction between tgn-MDM2 and peptide-tgc-FLAG. A product protein, termed APH-FLAG can be detected by western blotting using an anti-DYKDDDDK antibody. (B) Amino acid sequences of peptide-tgc-FLAG and peptide-tgc(Ala)-FLAG. (C) Western blotting analysis of the PTS reaction between tgn-MDM2 and peptide-tgc-FLAG or peptide-tgc(Ala)-FLAG.

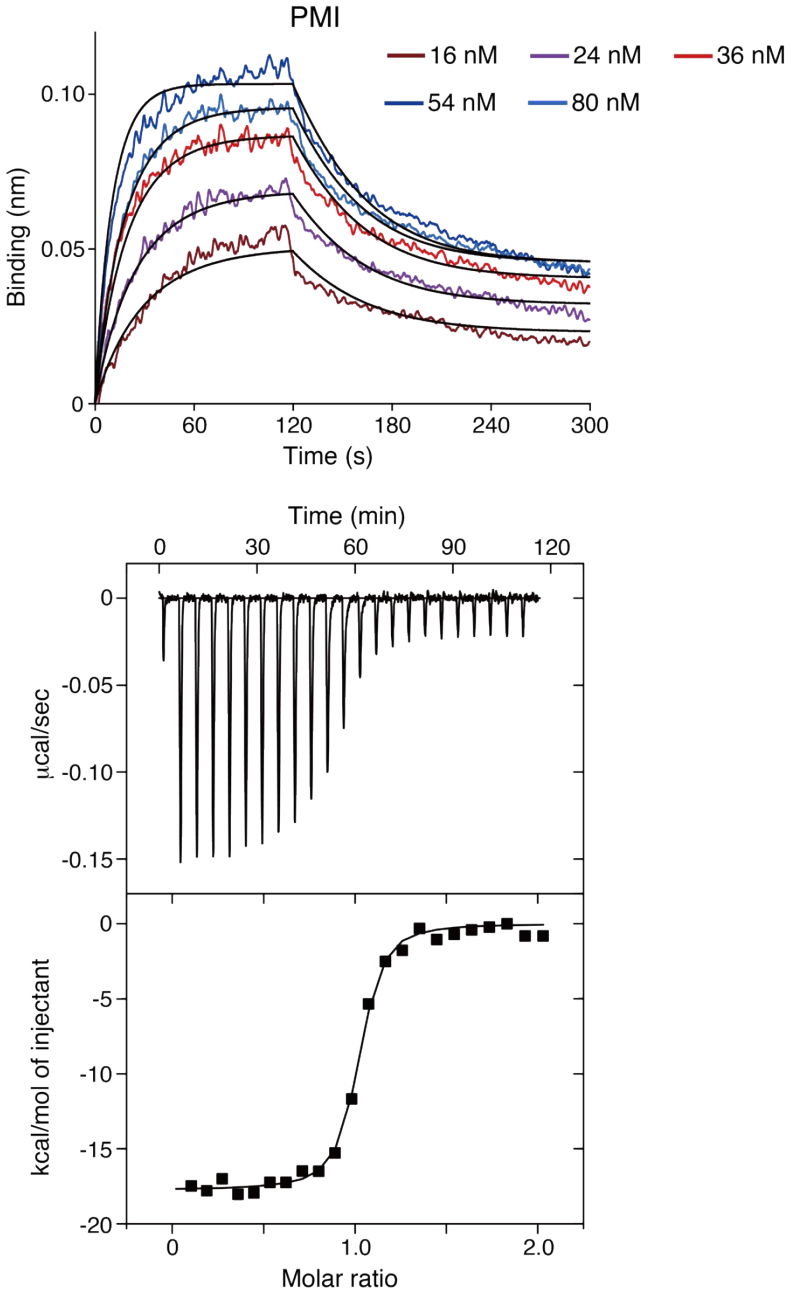



**Fig. S6** (A) BLI and (B) ITC measurements of PMI with MDM2.

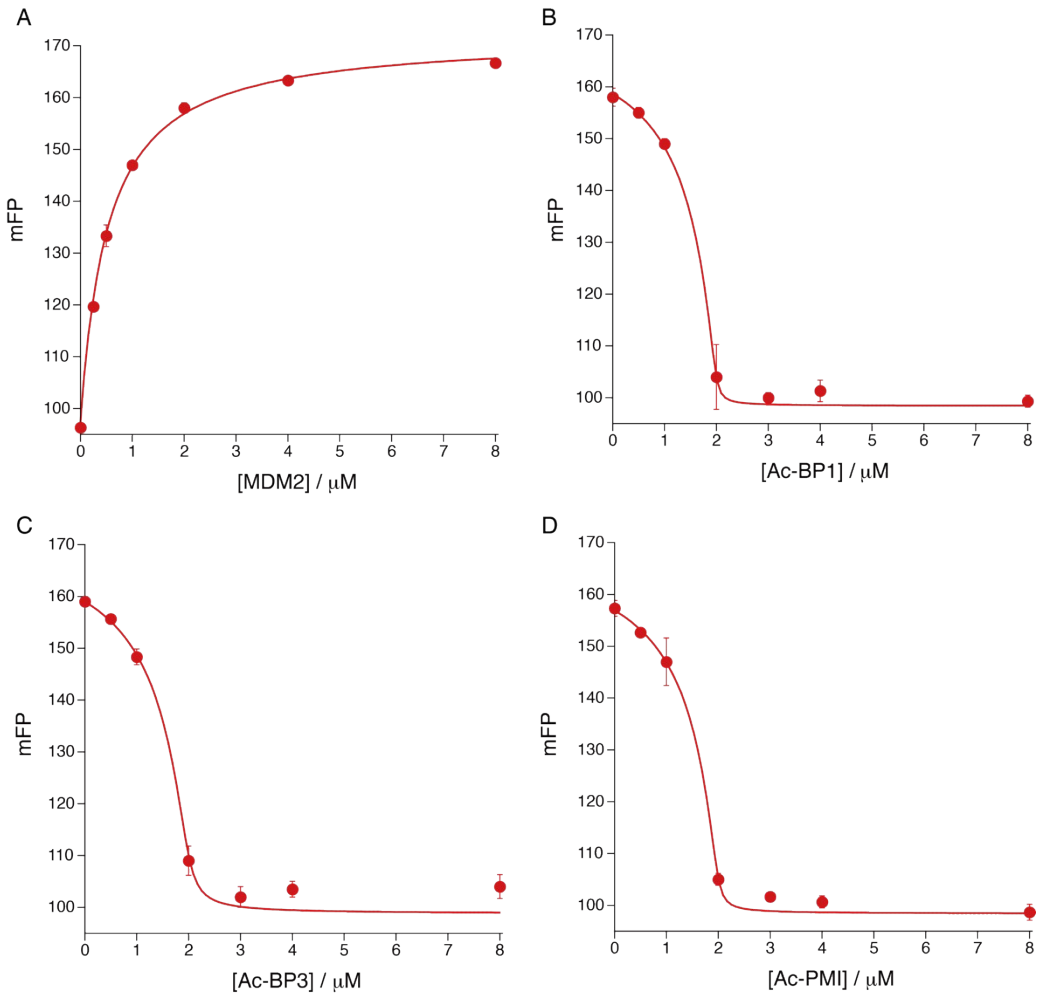



**Fig. S7** (A) Fluorescence polarization of TAMRA-p53 (50 nM) of increasing concentration of MDM2. (B–D) Fluorescence polarization of TAMRA-p53 (50 nM) with MDM2 (2.0 μM) of increasing concentrations of (B) Ac-BP1, (C) Ac-BP3 and (D) Ac-PMI.

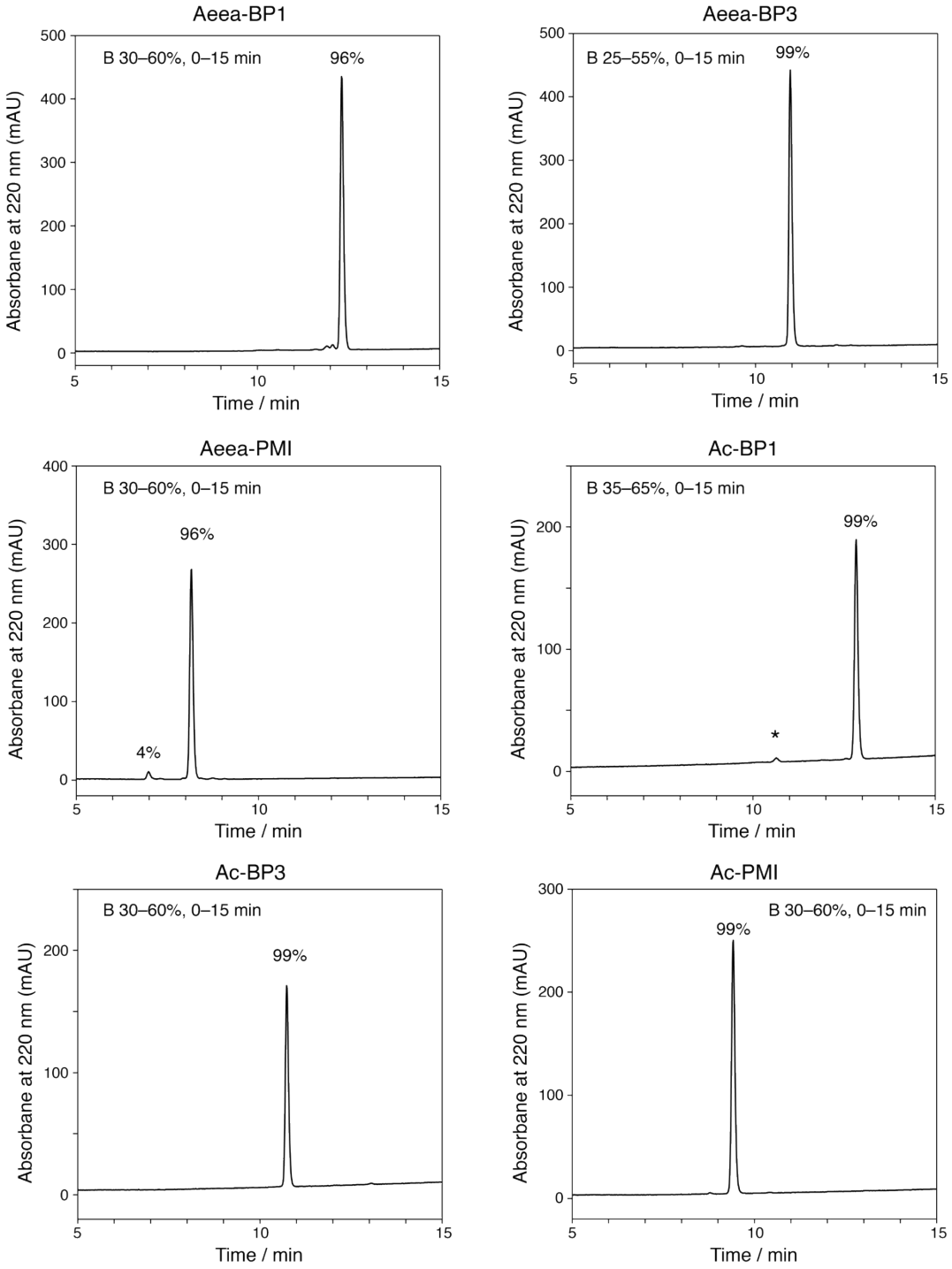



**Fig S8** HPLC chromatograms of purified peptides, Aeea-BP1, Aeea-BP3, Aeea-PMI, Ac-BP1, Ac-BP3 and Ac-PMI. Purity of each peptide was shown in the chromatograms. Asterisk indicates the peak corresponding to the impurity from the column.

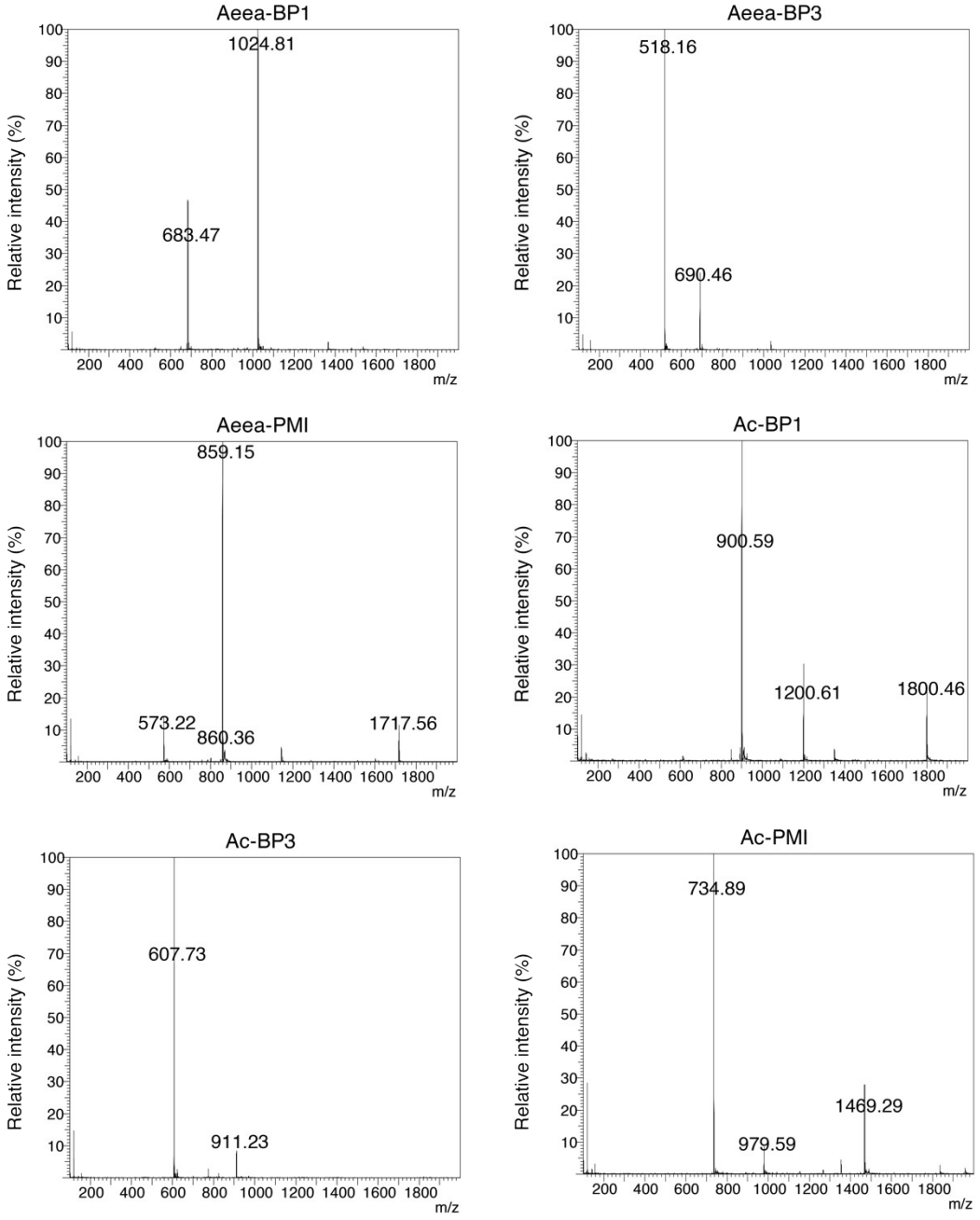



**Fig S9** ESI-MS of purified peptides, Aeea-BP1, Aeea-BP3, Aeea-PMI, Ac-BP1, Ac-BP3 and Ac-PMI.

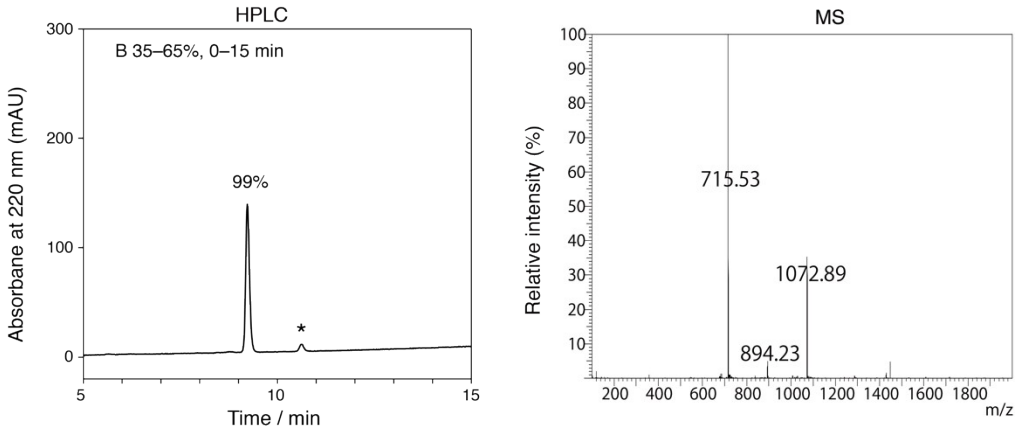



**Fig S10** HPLC chromatogram (left) and ESI-MS (right) of TAMRA-p53.

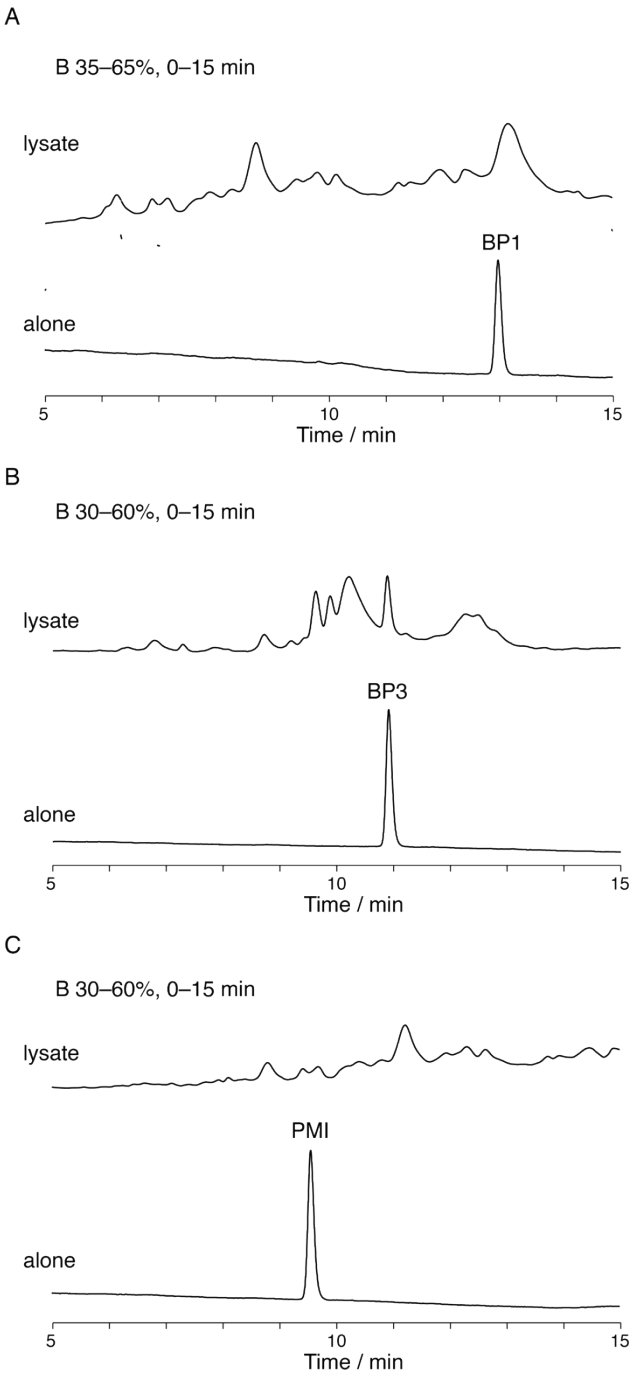



**Fig S11** HPLC chromatograms of the cell lysates when BP1, BP3 and PMI were incubated with SJSA-1 cells. λex = 295 nm and λem = 350 nm.