SUPPORTING INFORMATION

DARPins as pan-reactivators of temperature-sensitive p53 cancer mutants

Philipp Münick[a,1], Dimitrios-Ilias Balourdas[b,c,1], Julianne S. Funk[d], Büşra Yüksel[a,e], Danai Mavridi[b,c], Justin Heftel[a,e], Birgit Dreier[f], Jonas V. Schaefer[f], Birgit Schäfer[a], Stefan Knapp[b,c], Tümay Telatar[g], Baki Akgül[g], Andreas Plückthun[f], Thorsten Stiewe[d,h,i], Andreas C. Joerger[b,c,*] and Volker Dötsch[a,*]

[a]*Institute of Biophysical Chemistry and Center for Biomolecular Magnetic Resonance, Goethe University, 60438 Frankfurt, Germany*

[b]*Institute of Pharmaceutical Chemistry, Goethe University, 60438 Frankfurt, Germany*

[c]*Structural Genomics Consortium (SGC), Goethe University, 60438 Frankfurt, Germany*

[d]*Institute of Molecular Oncology, Universities of Giessen and Marburg Lung Center (UGMLC), Member of the German Center for Lung Research (DZL), Philipps-University, 35043 Marburg, Germany*

[e]*IMPRS on Cellular Biophysics, 60438 Frankfurt, Germany*

[f]*Department of Biochemistry, University of Zurich, 8057 Zurich, Switzerland*

[g]*Institute of Virology, Medical Faculty and University Hospital Cologne, University of Cologne, 50935 Cologne, Germany*

[h]*Genomics Core Facility, Philipps-University, 35043 Marburg, Germany*

[i]*Institute for Lung Health (ILH), Justus Liebig University, 35392 Giessen, Germany*

[1]Contributed equally to this work

*To whom correspondence may be addressed. Email: Joerger@pharmchem.uni-frankfurt.de or vdoetsch@em.uni-frankfurt.de

SUPPLEMENTAL MATERIALS AND METHODS

Protein expression and purification

DARPins and p53 DBD variants were expressed and purified following published protocols (1).

For the DARPins, the respective pET15b expression plasmid was transformed into *E. coli* BL21(DE3) Rosetta cells, and cells were grown in 2xYT medium until an optical density of 0.8 was reached. Then, protein expression was induced with 0.6 mM IPTG for 16 h at 16 °C. Cells were harvested, resuspended in buffer (50 mM Tris, pH 8.0, 400 mM NaCl, 30 mM imidazole) and lysed by sonication. After centrifugation, the protein was purified from the supernatant using an immobilized metal affinity chromatography (IMAC) column (HiTrap IMAC Sepharose FF, Cytiva), and the polyhistidine tag was removed by TEV protease cleavage. The cleaved DARPin was further purified by reverse IMAC and final size-exclusion chromatography (SEC) with SEC buffer I (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM TCEP) using a Superdex 75 10/300 column (Cytiva).

For p53 DBD variants, the pET15b derived expression plasmids were transformed and expressed in *E. coli* OverExpress C41(DE3) (Lucigen) cells. Cells were grown in 2xYT medium at 37 °C until an optical density of 0.8 was reached. The expression medium was supplemented with 100 µM $ZnCl_2$ upon induction with 0.5 mM IPTG for 16 h at 18 °C. The cells were harvested, resuspended in buffer A (50 mM HEPES (pH 7.5), 300 mM NaCl, 10 mM imidazole, 0.5 mM TCEP), and protease inhibitor cocktail (250 mM AEBSF, 25 mM leupeptin, 25 mM bestatin, 0.75 mM aprotinin, 12.5 mM E-64 and 2.5 mM pepstatin A) was added, followed by lysis via sonication. Polyethyleneimine was added, and the debris was separated by centrifugation. The supernatant was loaded onto a preequilibrated Ni-IMAC column, the column was washed with buffer A and the polyhistidine-tagged protein was eluted with buffer B (50 mM HEPES (pH 7.5), 300 mM NaCl, 300 mM Imidazole, 0.5 mM TCEP). TEV protease was added to cleave the tag overnight. The protein solution was then diluted 1:8 with HAC buffer A (25 mM HEPES pH 7.5, 0.5 mM TCEP) and loaded on a HiTrap heparin HP column (Cytiva). Bound protein was eluted by applying an increasing gradient of HAC buffer B (25 mM HEPES pH 7.5, 1 M NaCl, 0.5 mM TCEP) using an ÄKTA purifier system. This was followed by a final SEC step on a HighLoad

16/600 Superdex 75 pg column using SEC buffer II (25 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP). For the highly unstable E285K mutant, the buffer was adjusted to buffer SEC III (25 mM HEPES, pH 7.5, 300 mM NaCl, 1 mM TCEP, and 5% v/v glycerol). Proteins were concentrated (AmiconUltra, MWCO: 10K) to 2.7-6.5 mg/mL based on their solubility, flash-frozen in liquid nitrogen and stored at -80 °C. Purity and molecular size of all purified proteins were analyzed by SDS-PAGE and LC-ESI-TOF-mass spectrometry.

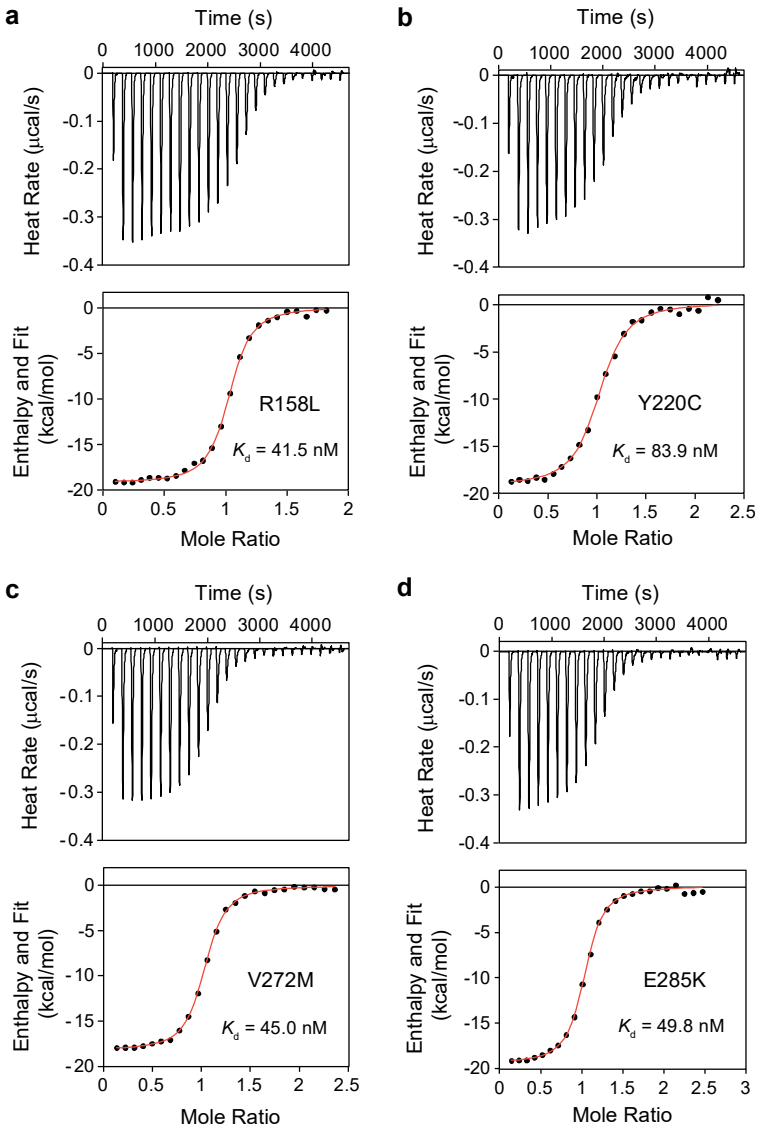

Figure S1. Representative ITC data and derived binding constants, K_ds, of DARPin C10-H82R with different p53 DBD mutants, determined at 20 °C. **a**, R158L mutant. **b**, Y220C mutant. **c**, V272M mutant, **d**, E285K mutant.

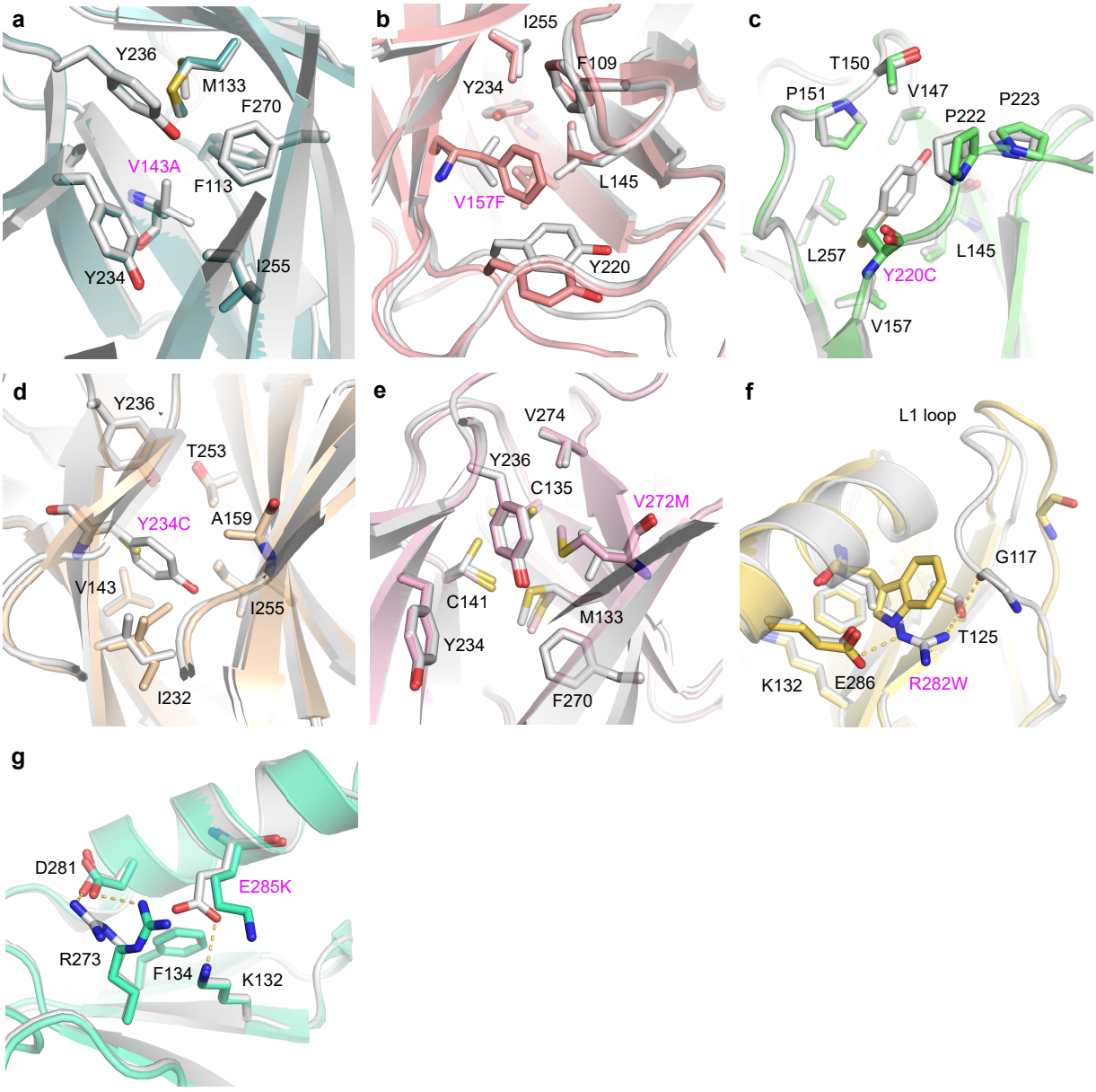

Figure S2. Structural effects of p53 cancer mutations. Structures of mutant p53-DARPin C10 complexes were superimposed onto the DNA-binding domain of wild-type p53 (PDB entry 2XWR; (2)), shown in gray. Selected residues around each mutation site are shown as stick models, and specific polar interactions are highlighted with yellow dashed lines. **a**, Large-to-small substitution V143A creating an internal cavity. **b**, Small-to-large V157F mutation leading to steric clashes in the hydrophobic core of the β-sandwich and structural rearrangements. **c**, Creation of an extended surface crevice in the Y220C mutant. **d**, Y234C mutation creating an energetically unfavorable cavity in the hydrophobic core of the β-sandwich. **e**, V272M mutation perturbing the hydrophobic core close to the DNA-binding surface. **f**, R282W mutant with perturbed L1 region and loss of polar interactions. **g**, E285K mutation resulting in the loss of the stabilizing salt bridge between Glu285 and Lys132.

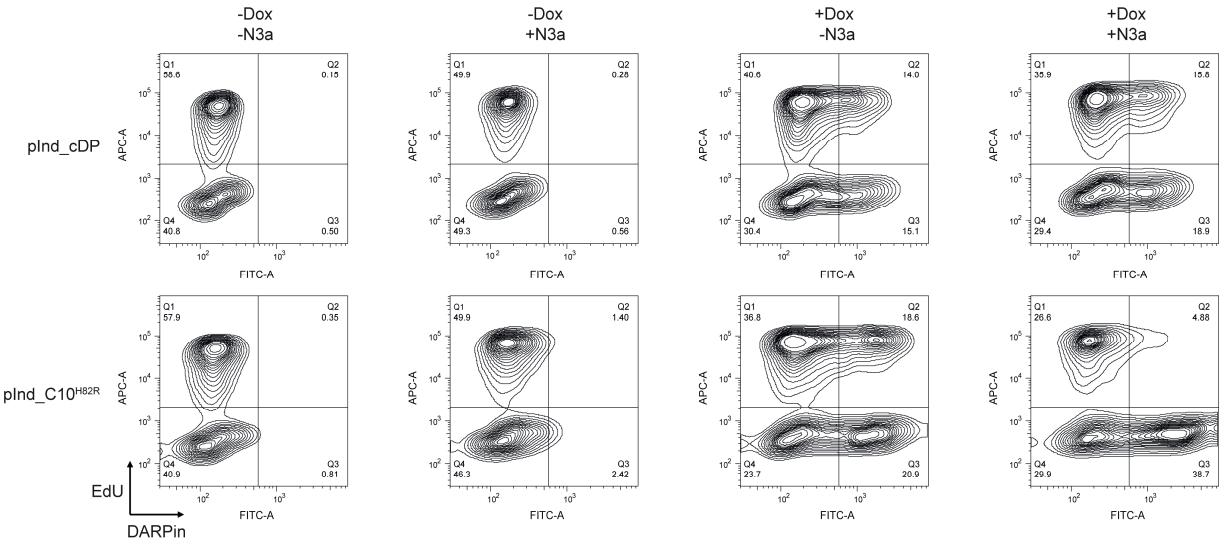

Figure S3. DARPin-induced reactivation of mutant p53 in CRISPR-engineered cell lines. Contour plots of HCT116 *TP53*$^{\Delta/T256A}$ pInd_cDP or pInd_C10^{H82R} cells after Dox and N3a treatment, analyzed by parallel DARPin and EdU staining. Dox, doxycycline; N3a, Nutlin-3a.

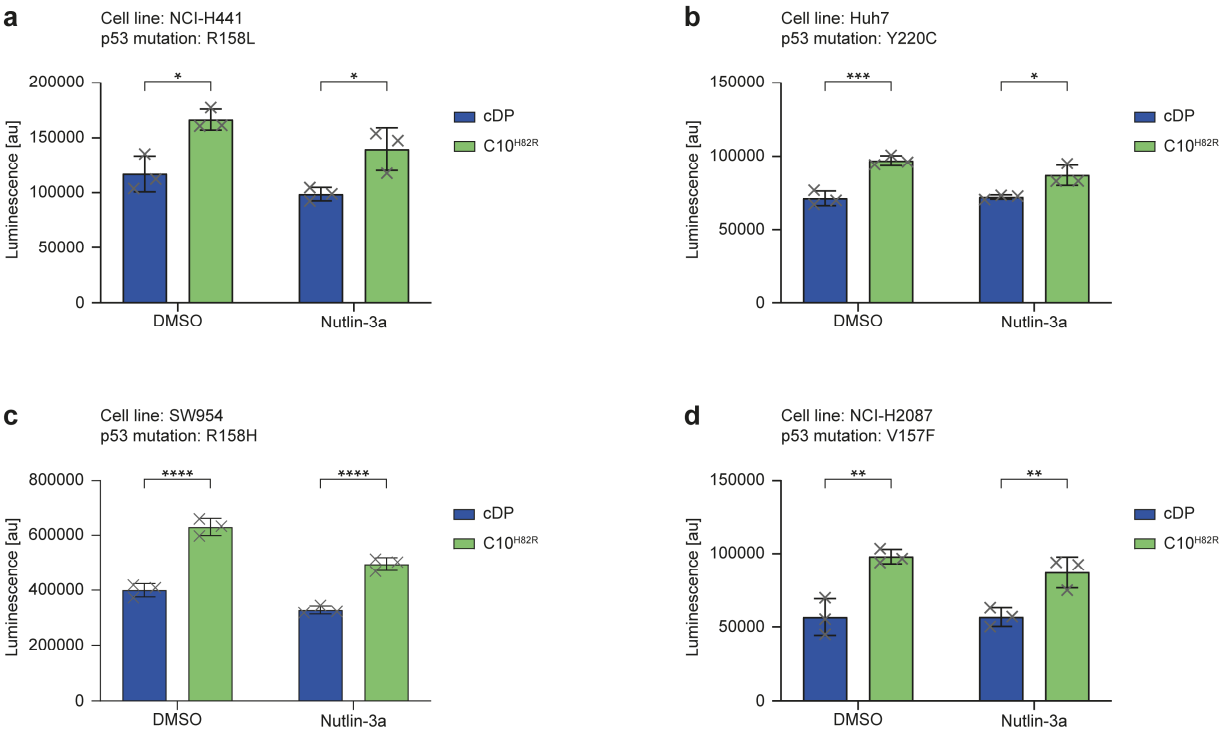

Figure S4. Caspase Glo3/7 Assay to detect induction of apoptosis in patient-derived cancer cell lines expressing either the control DARPin or C10-H82R, with and without Nutlin-3a co-treatment. **a**, NCI-H441 (p53 mutant R158L). **b**, Huh7 (p53 mutant Y220C). **c**, SW954 (p53 mutant R158H). **d**, NCI-H2087 (p53 mutant V157F). Bar diagrams represent the mean values and the error bars the corresponding s.d. of three biological replicates. An ordinary one-way ANOVA was performed to assess the statistical significance (n.s., $P > 0.05$; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

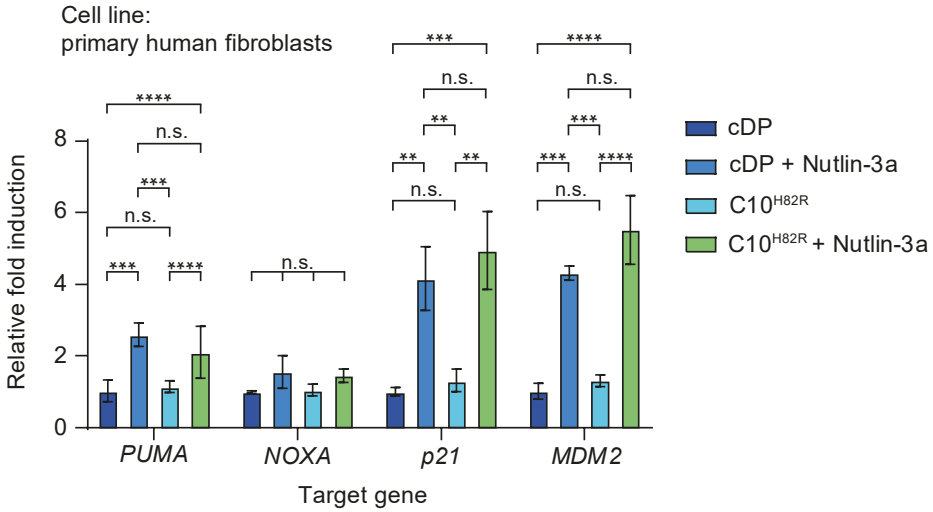

Figure S5. Effect of DARPins on p53 activity in primary human fibroblast cells. Reverse transcription qPCR (RT–qPCR) analysis of the expression of the *PUMA*, *NOXA*, *p21*, and *MDM2* gene in primary human fibroblast cells expressing either control DARPin (cDP) or DARPin C10-H82R, with and without MDM2 inhibitor Nutlin-3a. The total RNA was isolated from the cells and reverse transcribed into complementary DNA before analysis by qPCR. Gene expression was referenced to the housekeeping gene HPRT-1. An ordinary one-way ANOVA was performed to assess the statistical significance (n.s., $P > 0.05$; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

Table S1. Crystallization conditions of p53 DBD mutant - DARPin complexes

Complex	Protein concentration (mg/mL)	Reservoir buffer	Drop volume ratio (protein:reservoir solution)
p53(V143A)-C10	0.75	20% PEG smear broad, 0.1 M HEPES pH 7.5, 0.2 M ammonium nitrate	1:1
p53(V157F)-C10	0.60	20% PEG3350, 10% ethylene glycol, 0.2 M sodium/potassium phosphate	2:1
p53(Y220C)-C10	2.3	20% PEG3350, 10% ethylene glycol, 0.1 M bis-tris-propane pH 8.5, 0.2 M sodium formate	2:1
p53(Y234C)-C10	1.5	15% PEG3350, 0.1 M magnesium formate	2:1
p53(V272M)-C10	0.9	2.4 M sodium malonate	2:1
p53(R282W)-C10	1.8	25% PEG3350, 0.1 M HEPES pH 7.5, 0.2 M lithium sulfate	1:1
p53(E285K)-C10(H82R)	0.54	25% PEG3350, 0.2 M lithium sulfate monohydrate, 0.1 M Tris pH 8.5	2:1

All crystal plates were incubated at 20 °C, and grown crystals were mounted after 2-5 days. For cryo protection of the crystals, the mother liquor was supplemented with 23% (v/v) ethylene glycol.

Table S2. X-ray data collection and refinement statistics of mutant p53 DBD-DARPin complexes

Mutant-DARPin complex	V143A-C10	V157F-C10	Y220C-C10	Y234C-C10	V272M-C10	R282W-C10	E285K-C10^{H82R}
Data Collection							
Space Group	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
a, b, c (Å)	37.8, 93.7, 53.1	38.2, 92.9, 54.7	37.8, 93.8, 53.3	37.7, 93.8, 53.2	38.0, 93.9, 53.6	37.6, 93.8, 53.3	37.7, 93.9, 53.3
α, β, γ (°)	90.0, 110.3, 90.0	90.0, 108.2, 90.0	90.0, 110.2, 90.0	90.0, 110.2, 90.0	90.0, 109.8, 90.0	90.0, 109.9, 90.0	90.0, 110.2, 90.0
Complexes/AU	1	1	1	1	1	1	1
Resolution (Å)[a]	46.86-1.71 (1.74-1.71)	46.45-1.93 (1.98-1.93)	46.92-1.48 (1.51-1.48)	46.89-1.47 (1.50-1.47)	46.94-2.20 (2.27-2.20)	46.92-1.66 (1.69-1.66)	28.25-1.42 (1.45-1.42)
Unique reflections[a]	36,732 (1,909)	26,770 (1,785)	57,810 (2,747)	58,708 (2,899)	17,985 (1,558)	40,703 (2,011)	65,164 (3,204)
Completeness (%)[a]	98.2 (98.1)	99.9 (100)	99.7 (96.0)	99.5 (99.9)	99.7 (99.8)	99.4 (98.7)	99.9 (98.0)
Multiplicity[a]	5.2 (5.4)	5.1 (5.2)	5.0 (3.9)	5.0 (5.2)	5.1 (5.2)	5.0 (5.3)	6.9 (6.3)
R_{merge}[a]	0.090 (0.875)	0.074 (0.863)	0.049 (0.531)	0.051 (0.799)	0.135 (0.901)	0.069 (0.628)	0.053 (0.648)
R_{pim}[a]	0.044 (0.409)	0.036 (0.419)	0.023 (0.303)	0.025 (0.398)	0.066 (0.440)	0.033 (0.295)	0.022 (0.278)
CC(1/2)[a]	0.998 (0.762)	0.998 (0.745)	0.999 (0.839)	0.999 (0.657)	0.995 (0.706)	0.998 (0.858)	0.999 (0.789)
Mean $I/\sigma(I)$[a]	10.4 (2.0)	12.2 (2.0)	15.7 (2.3)	15.5 (2.1)	8.6 (2.0)	11.1 (2.0)	18.2 (2.6)
Refinement							
R_{work}, (%)[b]	17.9	19.1	14.8	15.6	17.5	15.1	14.6
R_{free}, (%)[b]	21.8	22.3	18.2	19.2	22.2	20.1	17.7
No. of atoms							
Protein	2471	2405	2517	2485	2429	2497	2515
Water/zinc/other ligands	267/1/28	137/1/4	317/1/36	342/1/32	148/1/8	265/1/24	333/1/0
RMSD bonds (Å)	0.006	0.007	0.005	0.005	0.007	0.05	0.005
RMSD angles (°)	0.82	0.83	0.74	0.77	0.83	0.75	0.75
Mean B (Å^2)	28.3	38.1	27.2	25.7	33.6	34.2	22.9
Ramachandran statistics							
Allowed/disallowed (%)	99.7/0.0	97.8/0.0g	100.0/0.0	99.7/0.0	98.4/0.0	98.8/0.0	100.0/0.0
PDB entry	9SPM	9SPN	9SPO	9SPP	9SPQ	9SPR	9SPS

[a]Values in parentheses are for the highest-resolution shell. [b]R_{work} and $R_{free} = \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}|$, where R_{free} was calculated with 5 % of the reflections chosen at random and not used in the refinement. [c]Number includes

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1. Munick P*, et al.* (2025) DARPin-induced reactivation of p53 in HPV-positive cells. *Nat Struct Mol Biol*. 32: 790-801.
2. Natan E*, et al.* (2011) Interaction of the p53 DNA-binding domain with its n-terminal extension modulates the stability of the p53 tetramer. *J Mol Biol* 409:358-368.