Stepwise multi-polyubiquitantion of p53 by the E6AP-E6 ubiquitin ligase complex

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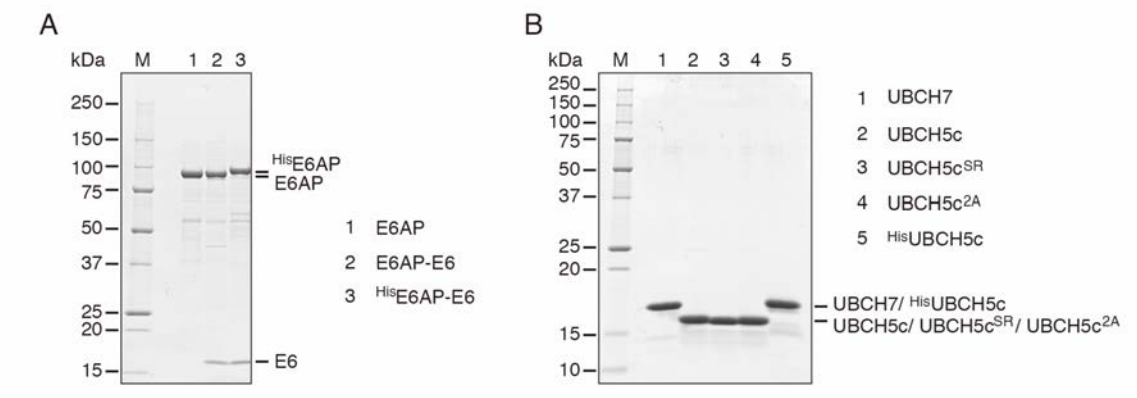
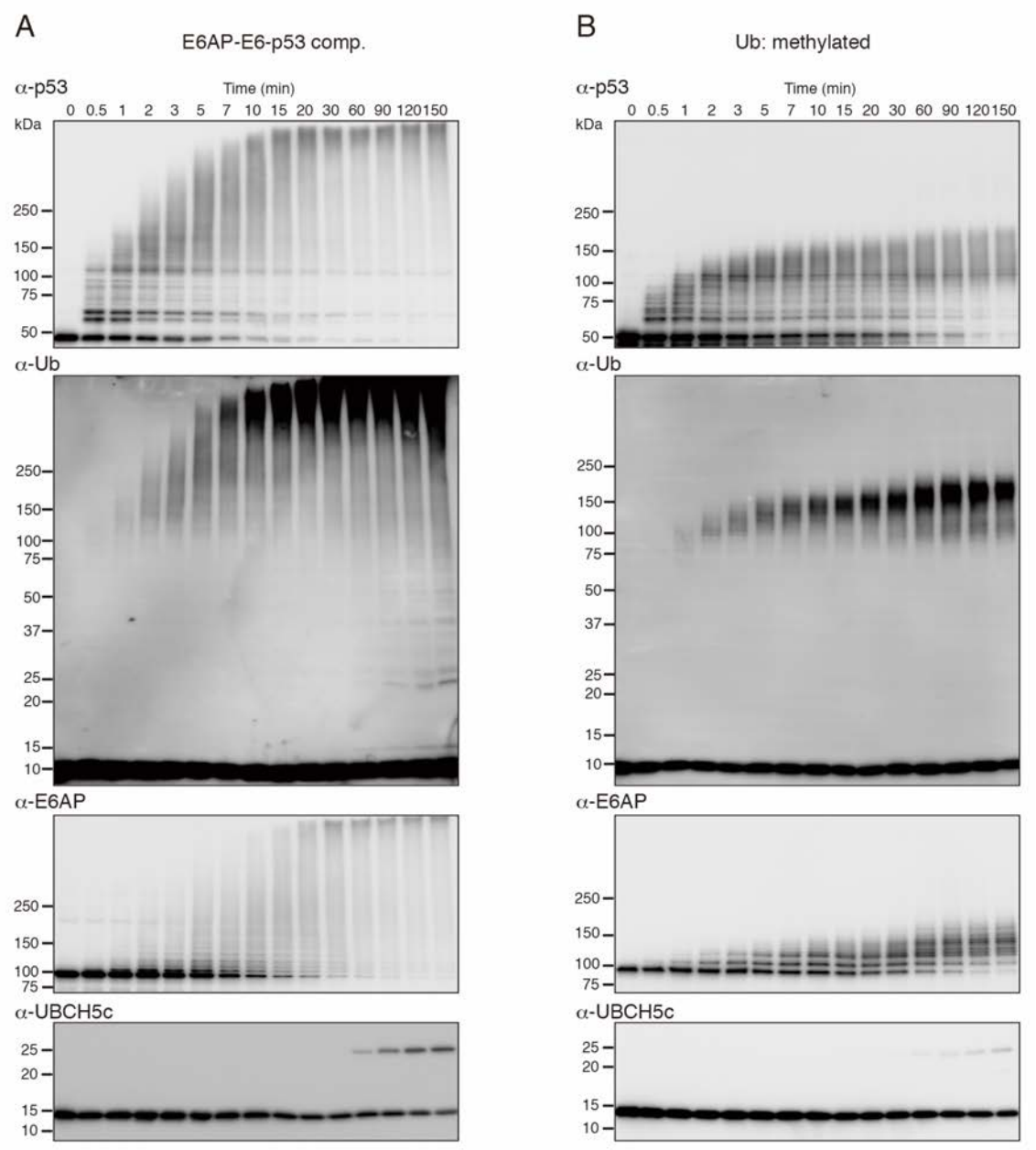
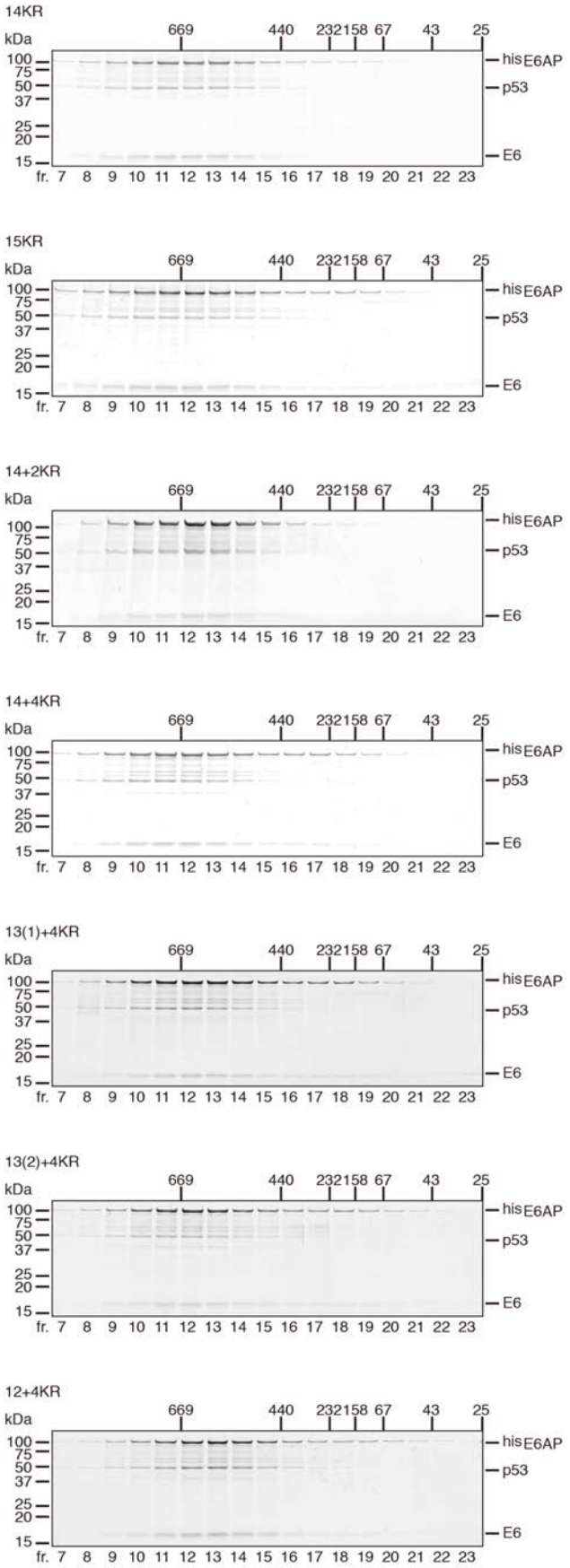
Supporting information includes the following:



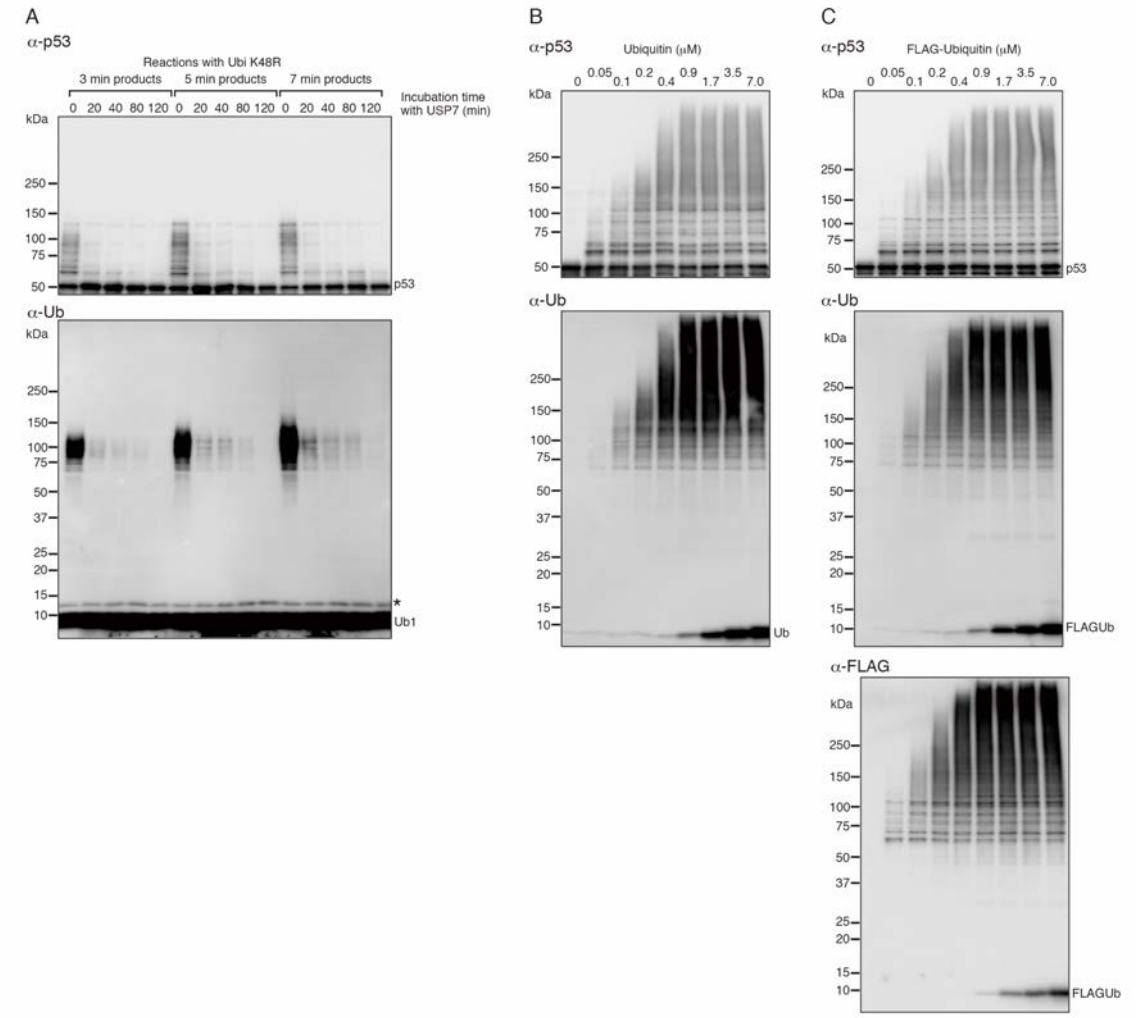
Supporting Figure S1. Recombinant proteins used in this study. SDS-PAGE analysis of the purified proteins. The indicated E6AP and E6AP-E6 complexes (**A**), and E2 proteins (**B**) (500 ng each) were analysed by SDS-PAGE with Coomassie Brilliant Blue (CBB) staining.



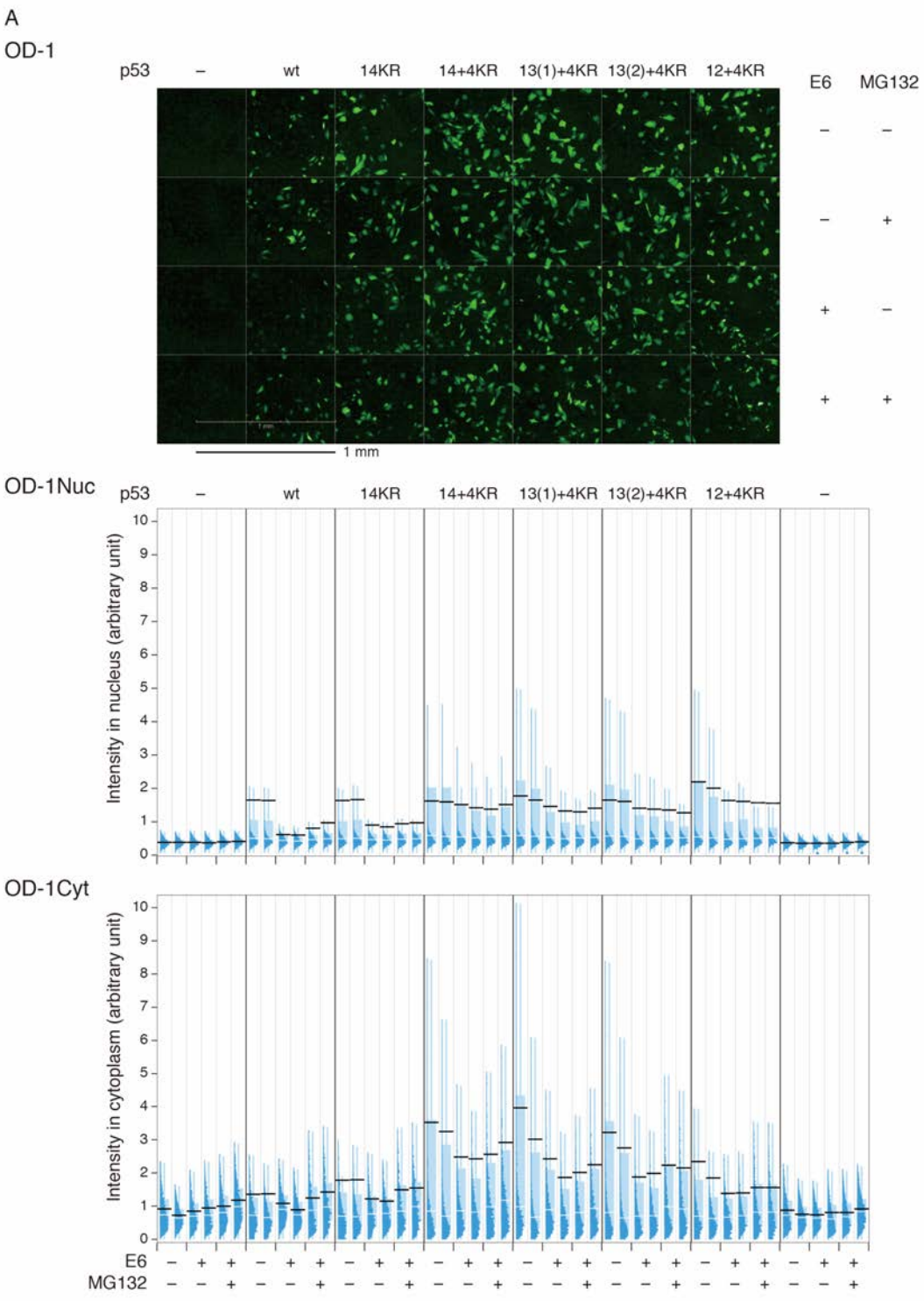
Supporting Figure S2. Time courses of the ubiquitination reactions. **(A)** Reaction with the ^{his}E6AP-E6-p53 ternary complex. The reaction in a volume of 250 µl under standard conditions was started by adding fraction 12 from the gel filtration column (Figure 2B, upper panel) instead of E6AP-E6 and ^{his}p53, and 15 µl aliquots were withdrawn at the indicated times. (**B**) The reaction was performed as in Figure 4A except for the use of methylated Ub instead of wild-type Ub. The reaction products were visualized by western blotting with the indicated antibodies.

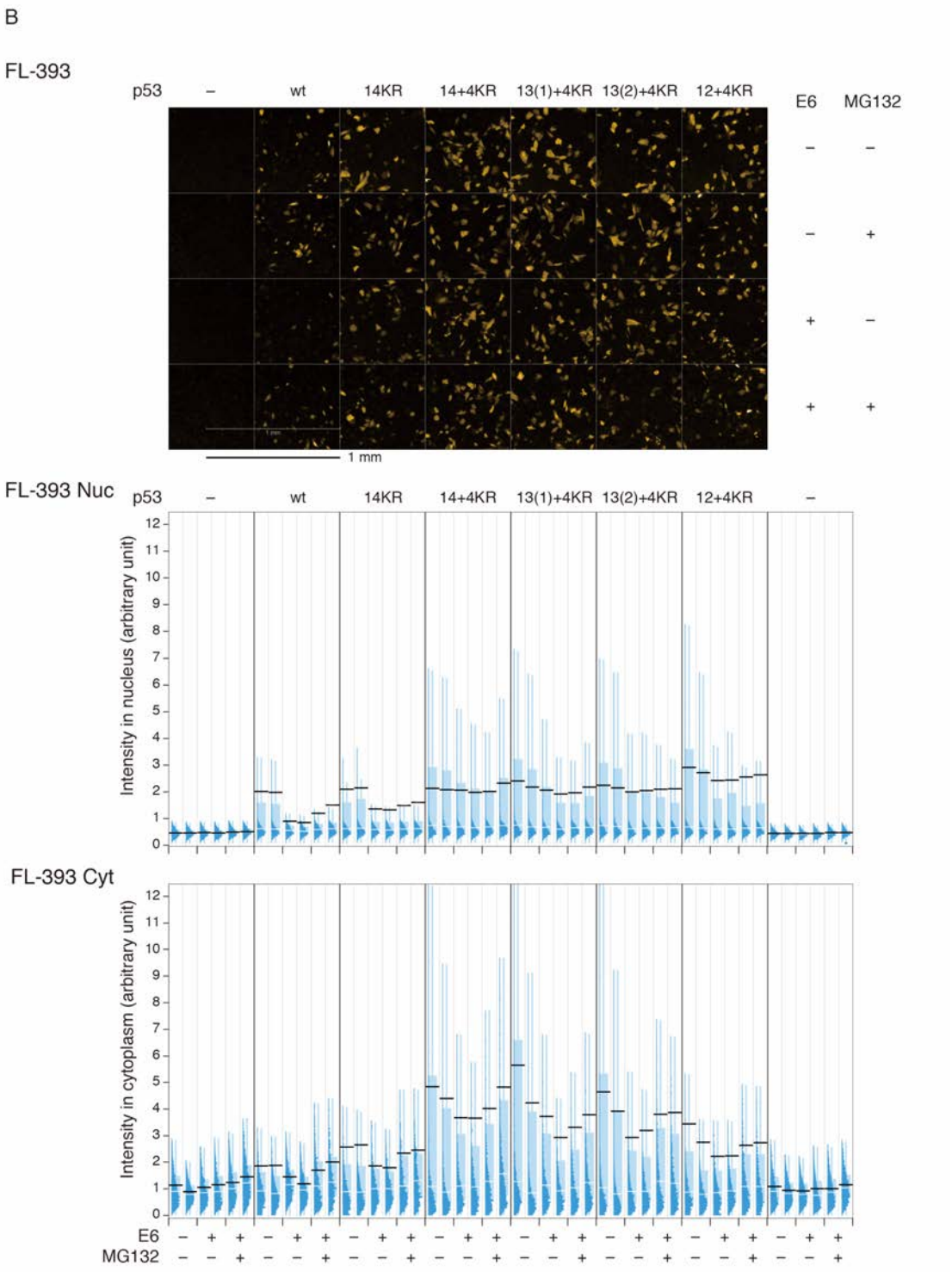


Supporting Figure S3. Gel filtration chromatography of ternary complexes consisting of ^{his}E6AP-E6 and the indicated mutants of p53. Each fraction was analysed by SDS-PAGE and Coomassie Brilliant Blue staining.



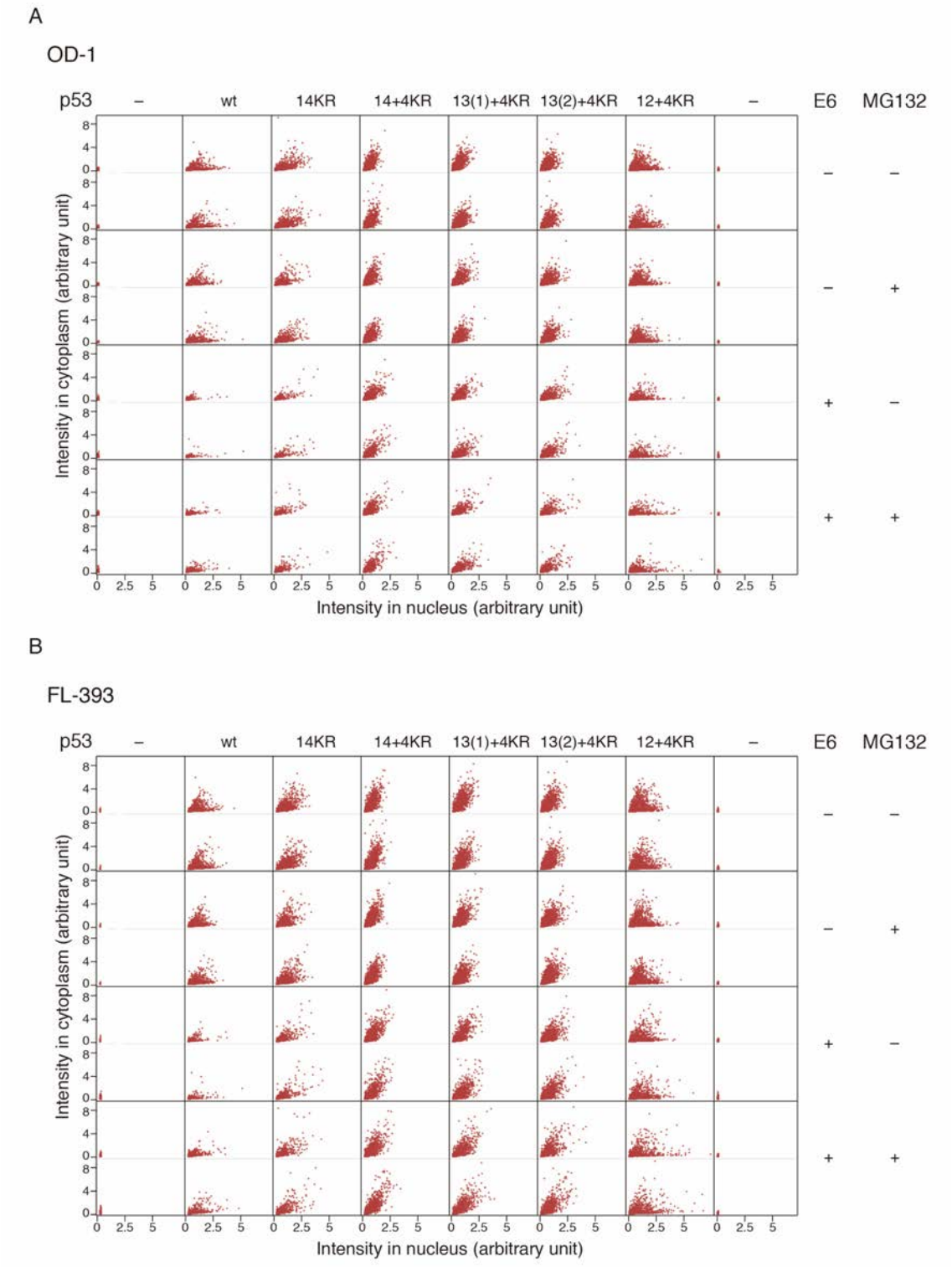
Supporting Figure S4. Analysis of Ub chains. (**A**) The ubiquitination products obtained with Ub^{K48R} for 3, 5 and 7 min were digested with ^{his}USP7 for the indicated times. The reaction products were visualised by western blotting with the indicated antibodies. Note that because of cross-reacting material in the commercial UbK48R preparation (indicated by the asterisk) (see also legend of Figure 4D), we were unable to determine whether di-ubiquitin, which might be K11-linked di-ubiquitin, was released by USP7 digestion. (**B–C**) Titration of Ub. Reactions were performed under standard conditions with Ub (**B**) or ^{FLAG}Ub (**C**). The reaction products were visualised by western blotting with the indicated antibodies.


A
OD-1
p53
–
wt
14KR
14+4KR
13(1)+4KR
13(2)+4KR
12+4KR
E6
MG132
1 mm
OD-1Nuc
Intensity in nucleus (arbitrary unit)
OD-1Cyt
Intensity in cytoplasm (arbitrary unit)



Supporting Figure S5. Automated fluorescence microscope analysis of expression levels of ^{FLAG}p53 and its KR mutants. (**A, B**) Saos-2 cells were transfected with the indicated ^{FLAG}p53 expression plasmids together with an E6 expressing plasmid (+) or the empty vector (-), and were then treated with (+) or without (-) MG132 for 6 h. The cells were subjected to automated fluorescence microscope analysis after immunofluorescence staining with an anti-p53 antibody

OD-1 (Alexa Fluor 488 labelled 2nd antibody, green) (**A**) and FL-393 (Alexa Fluor 555 labelled 2nd antibody, yellow) (**B**). Representative fields acquired from the microscope analysis are shown. The fluorescence intensities in the nucleus (*Nuc*) and cytoplasm (*Cyt*) in each cell were individually measured and data obtained from 2000- to 4000-cell images under each condition are shown as box plots superimposed with histograms showing the cell distributions. Note that outliers are not shown. Black bars indicated average intensities. The results obtained from two independent experiments are shown in the graphs.



Supporting Figure S6. Subcellular distribution of ^{FLAG}p53 and its KR mutants. (**A, B**) Fluorescence intensities in the cytoplasm of each cell was plotted versus that in the nucleus as scatter plots. The data shown in Supporting Figure S5A (**A**) and S5B (**B**) were used. The area of each nucleus/cytoplasm/cell was determined from the signal of Hoechst 33342 staining.

Supporting Table S1. Parameters used in the Ub-AQUA/PRM analysis

Abbreviation	Peptide sequence	Precursor m/z (charge state)	Time window (min)
TITLE	TITLEVEPSDTIENVK	894.467 (+2)	43–63
	TITLEVEPSDTIENV[HeavyV]K	897.474 (+2)	
K6(GG)	M[Oxid]QIFVK[di-GlyGly]TLTGK	465.927 (+3)	21–41
	M[Oxid]QIFVK[di-GlyGly]TL[HeavyL]TGK	468.266 (+3)	
K11(GG)	TLTGK[di-GlyGly]TITLEVEPSDTIENVK	801.427 (+3)	43–63
	TLTGK[di-GlyGly]TITLEVEPSDTIENV[HeavyV]K	803.431 (+3)	
K27(GG)	TITLEVEPSDTIENVK[di-GlyGly]AK	701.0.9 (+3)	36–63
	TITLEVEPSDTIENVK[HeavyV]K[di-GlyGly]AK	703.044 (+3)	
K29(GG)	AK[di-GlyGly]IQDK	408.732 (+2)	5–21
	AK[di-GlyGly]I[HeavyI]QDK	412.241 (+2)	
K33(GG)	IQDK[di-GlyGly]EGIPPDQQR	546.613 (+3), 819.416 (+2)	8–31
	IQDK[di-GlyGly]EGIPP[HeavyP]DQQR	548.618 (+3), 822.423 (+2)	
K48(GG)	LIFAGK[di-GlyGly]QLEDGR	487.600 (+3)	23–43
	LIFAGK[di-GlyGly]QL[HeavyL]EDGR	489.939 (+3)	
K63(GG)	TLSDYNIQK[di-GlyGly]ESTLHLVLR	748.738 (+3)	44–64
	TLSDYNIQK[di-GlyGly]ESTLHLVL[HeavyL]R	751.077 (+3)	
M1(GG)	GGM[Oxid]QIFVK	448.239 (+2)	11–31
	GGM[Oxid]QIFV[HeavyV]K	451.246 (+2)	

Isotopically labelled amino acids (Heavy: $^{13}C^{15}N$), ubiquitination sites (di-GlyGly) and Met oxidation (Oxid) in the peptide sequences are indicated by brackets. Multiple fragment ions were used for quantification using PinPoint software.