Supplemental Materials

Molecular Biology of the Cell

Gotoh et al.

Supplemental Figures

FIGURE S1: A. The hp53/hPer2 complex is present in the nuclear compartment. HCT116 cells were transfected with pCS2+FLAG-hPer2 and total, cytosolic, and nuclear fractions (T, C, and N, respectively) were immunoprecipitated using α-p53 antibody. Complex components were identified by immunoblotting using specific antibodies (*right panels*). Input samples are shown on *left panels*. Asterisk indicates a non-specific signal. B. Input controls of hPer2 and hp53 expressing proteins. HCT116 cells were transfected with pCS2+*myc*-hp53 and either pCS2+FLAG-hPer2 (+) or empty vector (-) and maintained in complete media for 20 h before adding, or not (control; -MG132), MG132 (50 μM) and ubiquitin aldehyde (5 nM) as described in Fig. 4.D legend. Cells were maintained four additional hours before harvesting. Lysates equivalent to $2.56x10^4$ cells were used to prepare the cytosolic (C) and total (T) fractions whereas $1.28x10^4$ cells were used for nuclei (N) preparation. Protein expression was evaluated in total, cytosolic, and nuclear fractions and input amounts detected by immunoblotting using α-Per2, -p53, -lamin A/C, and -tubulin antibodies. C. Input controls of hPer2 and Mdm2 expressing proteins. HCT116 cell lysates ($3.5x10^4$ cells for T/C; $15.7x10^4$ cells for N) from pCS2+3xFLAG-Mdm2 and pCS2+*myc*-hPer2 cotransfected cells treated, or not (control), with MG132 and ubiquitin aldehyde as described in Fig. 4.E legend, were analyzed for protein expression by immunoblotting using α–p53 (*middle panel*), -FLAG, and -*myc* (*upper two panels*). In all experiments, lamin A/C and tubulin were used as loading controls for nuclear and cytoplasmic fractions, respectively (lower two panels).

FIGURE S2: *In vitro* transcribed and translated FLAG-hp53(ch)hPer2, FLAG-hp53(ch)GST, *myc*-Mdm2, and *myc*-hPer2 proteins were used for ubiquitination experiments. When indicated, *myc*-hPer2 and FLAG-hp53(ch)GST were pre-incubated, thus, the complex was formed before adding *myc*-Mdm2. For the FLAG-hp53(ch)hPer2 chimera, the translated protein and *myc*-Mdm2 were incubated together before the ubiquitination reaction took place. Ubiquitination was carried out as described in the "Materials and Methods" section. FLAG-tagged proteins were immunoprecipitated with α-FLAG/protein A-beads and blotted using α-ubiquitin antibody. Membranes were then stripped and re-probed with α-p53 and –*myc* antibodies to detect complex bound proteins. Asterisk indicates IgG heavy chain. The figure shows immunoblot data from a single experiment that was repeated three times with similar results. Quantification of the sample's ubiquitinated signal was performed using ImageJ Software v1.45 (*bar graph*). Statistical comparisons were evaluated by *t*-test. ##: $p<0.005$.

FIGURE S3: Profile plots of signal intensity across H1299 cells transfected with *myc*-tagged forms of hp53, hp53(ch)GST, hp53(ch)hPer2(356-574/683-872), NLS-hp53(ch)GST, and NLS-hp53(ch)hPer2(356-574/683-872) (Figure 3, *panels i-v*), or the untagged form of hp53(ch)hPer2 (Figure 3, *panel vi*). Recombinant proteins (in red) and DNA levels (in blue) were scored along the white lines shown in each of the image panels located on the left. Fluorescence was visualized using a Nikon Eclipse TE2000-E microscope equipped with a Cascade II E2V CCD97 camera (Photometrics). Images were processed using NIS-Elements AR 3.0 Nikon software and quantified using ImageJ software v1.45.

FIGURE S4: A. *In vitro* association of Cry1 to hPer2/hp53 complex. *In vitro* transcribed and translated FLAG-hp53, FLAG-hPer2, *myc*-hp53, *myc*-hPer2, and *myc*-hCry1 proteins were pre-incubated as follows: FLAG-hp53 with either *myc*-hPer2 (ratio 1:2; lane 1) or *myc*-hCry1 (ratio 1:1; lane 2) and FLAG-hPer2 with either *myc*-hp53 or *myc*-hCry1 (ratio 2:1; lanes 3-4). Complexes were allowed to form by incubating the proteins at room temperature for 20 min. Immunoprecipitations were performed using α-FLAG-conjugated beads followed by washing in NP40 lysis buffer. Associated proteins were detected by immunoblotting using α-FLAG (*upper panel*) or α-*myc* (*lower panel*) antibodies. In lanes 5-6,

complex of *myc*-hp53 with FLAG-hPer2 (ratio 1:2) or *myc*-hCry1 with FLAG-hPer2 (ratio 1:2) were formed by incubation at room temperature before adding *myc*-hCry1 or *myc*-hp53, respectively. Complexes were immunoprecipitated and analyzed as described above. FLAG- and *myc*-inputs are indicated in *top* and *right* panels. Asterisk indicates IgG heavy chain. B. Binding of hPer2 to hp53 prevents hp21 from being expressed. H1299 cells were transfected with pCS2+FLAG-hp53, -hp53(ch)GST, -hp53(ch)hPer2, or -hp53(ch)hPer2(356-574/683-872) and harvested 24 h later. Cell lysates (~40 μg) were resolved by SDS-PAGE and recombinant (*upper left* and *right panels*) and endogenous proteins (*middle* and *lower left* and *right panels*) detected by immunoblotting using α-p21, -p53, -FLAG, -tubulin antibodies.

FIGURE S5: H1299 cells (~ $8x10^5$) were transfected with empty vector, pCS2+FLAG-hp53, pCS2+FLAG-hp53(ch)GST, or hp53(ch)hPer2 and maintained for 24 h before irradiation (5 Gy). Samples were harvested every 12h for 3 days after treatment (t=0 before treatment) and analyzed for viability using a MTT viability assay (Abnova) following manufacturer's instructions. Absorbance was measured at OD_{570nm} in a SPECTRA MAX 190 plate reader (Molecular Devices).

FIGURE S6: Expression levels of recombinant proteins in samples from experiments shown in Figures 5B (panel A) and S7E (panel B). In all cases, cell lysates (40 μg) were resolved by SDS-PAGE and recombinant proteins were detected by immunoblotting using an α-FLAG antibody (*upper panel*). Tubulin was used as the loading control (*lower panel*). EV: empty vector.

FIGURE S7: H1299 cells were transfected with either pCS2+FLAG-hp53, pCS2+FLAG-hp53(ch)GST, hp53(ch)hPer2, or empty vector (EV) and treated (+γ-IR), or not (-γ-IR), with different doses of radiation (0.5, 2.5, or 5 Gy). Cell lysates (50 μg) were collected 2 h after irradiation and proteins resolved by SDS-PAGE and blotted using an α-Chk1-Ser^{345} antibody for phosphorylation in Ser^{345} of endogenous Chk1 as described in the "Materials and Methods" section (*top panels*). Tubulin was used as the loading control (*lower panel*). Protein levels were quantified using ImageLab version 5.1 (Bio-Rad, *middle bar graph*) and values are represented as the mean ± SEM from three independent experiments. Real-time qRT-PCR data were normalized to the levels of expression in untreated empty vector. Data are presented as the mean ± SEM from three independent experiments performed in triplicate (*lower bar graph*). Statistical comparisons were done by *t-test*. NS: indicates not significant; #: indicates $p \leq 0.02$; ##: indicates $p \leq 0.05$.

FIGURE S8: H1299 cells were transfected with: *i*) empty vector (EV, A), *ii*) pCS2+FLAG-hp53 or pCS2+FLAG-hp53(ch)GST (B and C, respectively), or *iii*) pCS2+FLAG-hp53 or pCS2+FLAG-hp53(ch)hPer2(356-574/683-872) (D). Cells were treated (+γ-IR, A, C, D) or not (-γ-IR, A, B, D) with radiation and harvested as indicated in Fig. 6.A legend. Total RNA was purified using TRIzol and cDNA synthesized as described in the "Materials and Methods" section. Real-time qRT-PCR data were normalized to the levels of expression in untreated empty vector (A) or FLAG-hp53 transfected cells (B-D). Data are presented as the mean ± SEM from three independent experiments performed in triplicate. Statistical comparisons were done by either *two-tailed unpaired t-test* (A-C) or ANOVA using *Bonferroni* or *Games-Howell post-hoc* analyses when needed [D; SPSS; IBM Statistics]. NS: indicate not significant; #: indicates $p \leq 0.05$; ##: indicates $p \leq 0.01$; ###: indicates $p \leq 0.001$. E. H1299 cells were co-transfected with the reporter $hp21^{WAF1/CIP1}$-luc construct cloned in pGL2 and pCS2+FLAG-hp53, pCS2+FLAG-hp53(ch)hPer2(356-574/683-872) or empty vector (~200 ng) plus pCMV-β-gal (~200 ng) as internal control. Extracts from cells treated (+γ-IR), or not (-γ-IR), with radiation were assayed for luciferase and β-galactosidase activities. The experiment was replicated thrice; error bars indicate SEM and data evaluated by ANOVA using *Bonferroni post-hoc* test [SPSS; IBM Statistics]. ##: indicates $p<0.01$; ###: indicates $p<0.001$. F. H1299 cells were transfected with either pCS2+FLAG-

hp53(ch)hPer2(356-574/683-872) or empty vector (EV) and treated (+γ-IR), or not (-γ-IR), with radiation as indicated in Fig. 6.A and in the "Materials and Methods" section. Aliquots of lysates taken at different times (20 μg) were resolved by SDS-PAGE and blotted using specific antibodies [α-Chk1 and α-p21 for endogenous Chk1 kinase and $hp21^{WAF1/CIP1}$, respectively; α-FLAG for FLAG-hp53(ch)hPer2(356-574/683-872); α-Chk1-Ser^{345} for phosphorylation in Ser^{345} of endogenous Chk1, and α-p53-Ser^{15} for phosphorylation in Ser^{15} in FLAG-hp53(ch)hPer2(356-574/683-872)]. Tubulin was used as loading control (*lower panel*). Asterisk indicates nonspecific signal.

FIGURE S9: A. HCT116 cells were transfected with either FLAG-hPer2 or siRNAhPer2 and collected at 24 and 48 h post-transfection, respectively. Empty vector (EV) and mock samples were controls. qRT-PCR data are presented as the mean ± SEM from three independent experiments performed in triplicate. Statistical comparisons were done by *two-tailed unpaired t-test* and analyses performed using SPSS (IBM Statistics). NS: indicates not significant; ##: indicates $p \leq 0.01$. B. Lysates (250 μg) from HCT116 cells transfected with either FLAG-hPer2 or empty vector were incubated with α-Per2 antibody and protein A-beads in NP40 lysis buffer for immunodepletion Unbound fraction (supernatants) were analyzed by immunoblotting using specific antibodies. C. H1299 cells were transfected with pCS2+FLAG-hPer2, pCS2+FLAG-hp53, empty vector (EV), or a combination of plasmids. Cells were harvested 24 h after transfection and aliquots were analyzed by immunoblotting and quantified using ImageLab version 5.1 (Bio-Rad). Data are presented as the mean ± SEM from three independent experiments performed in triplicate.

FIGURE S10: A. HCT116 lysates ($15x10^5$ cells) from non-irradiated (-) or γ-IR (+, 10Gy) cells were used to prepare the cytosolic (C) and total (T) fractions whereas $45x10^5$ cells were used for nuclei (N) preparation. Endogenous proteins were identified in total, cytosolic, and nuclear fractions and input amounts detected by immunoblotting using α-Per2, -p53, -lamin A/C, and -tubulin antibodies (*upper panels*). Total, cytosolic, and nuclear extracts were incubated with α-Per2 antibody (0.7 μg) and protein A-beads in NP40 lysis buffer for 3h at 4ºC. Washed samples were analyzed by immunoblotting using specific antibodies. The figure shows immunoblot data from a single experiment that was repeated twice times with similar results. Increased levels of hp53 were expected in the nuclear fraction as result of stabilization and in response to γ-IR [for review see Kruse, (2009)]. B. HEK293 lysates (200 μg) from pCS2+3xFLAG-Mdm2, pCS2+3xFLAG-hp53, and pCS2+*myc*-hPer2 cotransfected cells treated, or not (0 h), with 20 Gy of γ-IR were immunoprecipitated using α-FLAG and protein A-beads. Immunoblotting was performed using specific antibodies to detect *myc*-hPer2 association and 3xFLAG-expressed proteins (*upper* and *lower panels*, respectively). Twenty μg of whole lysates were tested for protein expression levels and are shown on the left.

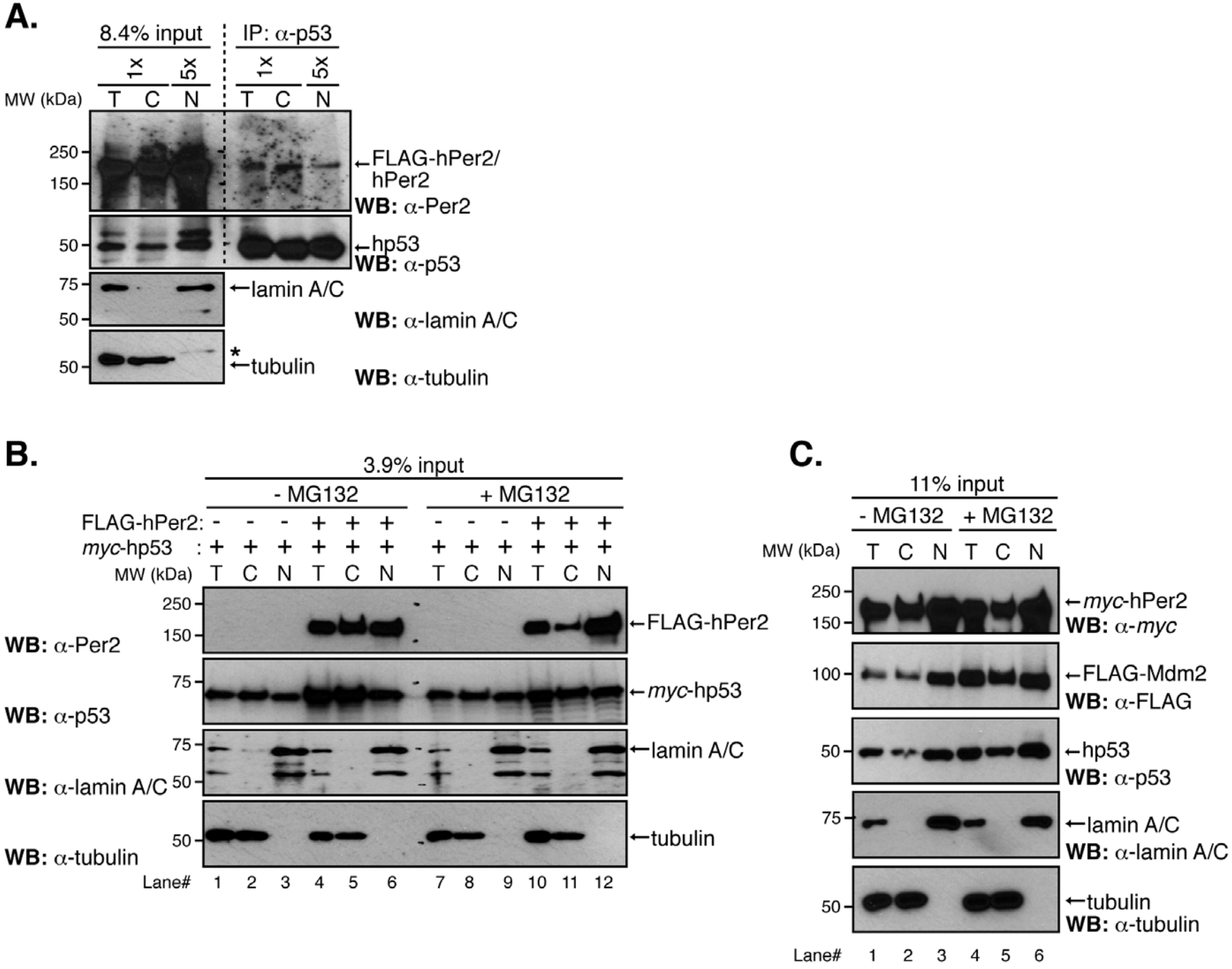
Supplemental Material

Table

Table S1 ***Primer sequences for qRT-PCR reactions.*** Primers used throughout are summarized. Sequences were retrieved from their corresponding GeneBank accession number and primers designed using Beacon Design™ (Premierbiosoft) software.

Table S1. ***Primer sequences for qRT-PCR reactions.***

Gene	Encoding Protein		Primer Sequence 5'-3'	GeneBank Accession Number
ACTB	β-actin	Forward	TCAGAAGGATTCCTATGTGGGCGA	NM_001101.3
		Reverse	TTTCTCCATGTCGTCCCAGTTGGT	
BAX	Bax	Forward	GTTGTCGCCCTTTTCTACTTTGCC	NM_004324.3
		Reverse	TGTCCAGCCCATGATGGTTCTGAT	
CLOCK	Clock	Forward	AGTTCAGCAACCATCTCAGGCTCA	NM_004898.3
		Reverse	TTGCTGGTGATGTGACTGAGGGAA	
CRY1	Cry1	Forward	ATCATTGGTGTGGACTAC	NM_021117.3
		Reverse	TCTGCTTCATTCGTTCA	
GADD45α	Gadd45α	Forward	TGCTGGTGACGAACCCACATTCAT	NM_001924.3
		Reverse	CACCCACTGATCCATGTAGCGACTTT	
GAPDH	GAPDH	Forward	CTCTGGTAAAGTGGATATTGT	NM_002046.4
		Reverse	GGTGGAATCATATTGGAACA	
MYC	c-myc	Forward	AGGAGACATGGTGAACCAGAGTTT	NM_002467.4
		Reverse	AGAAGCCGCTCCACATACAGTCCT	
PER2	Per2	Forward	TGAGAAGAAAGCTGTCCCTGCCAT	NM_022817.2
		Reverse	GACGTTTGCTGGGAACTCGCATTT	
CDKN1α	$p21^{WAF1/CIP1}$	Forward	TCCAGCGACCTTCCTCATCCAC	NM_000389.4
		Reverse	TCCATAGCCTCTACTGCCACCATC	
TP53	p53	Forward	GCGTGTGGAGTATTTGGATGA	NM_000546.5
		Reverse	AGTGTGATGATGGTGAGGATGG	
NR1D1	Rev-erbα	Forward	AGCATGACCAAGTCACCCTGCTTA	NM_021724.3
		Reverse	TGCGGCTTAGGAACATCACTGTCT	
TBP	Tbp	Forward	CACGAACCACGGCACTGATT	NM_003194.4
		Reverse	TTTTCTTGCTGCCAGTCTGGAC	
SFN	14-3-3 σ	Forward	GCAAGACCGAGATTGAGG	NM_006142.3
		Reverse	TGTCACAGGGGAACTTTATTG	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8.4% input
IP: α-p53
1x
5x
MW (kDa)
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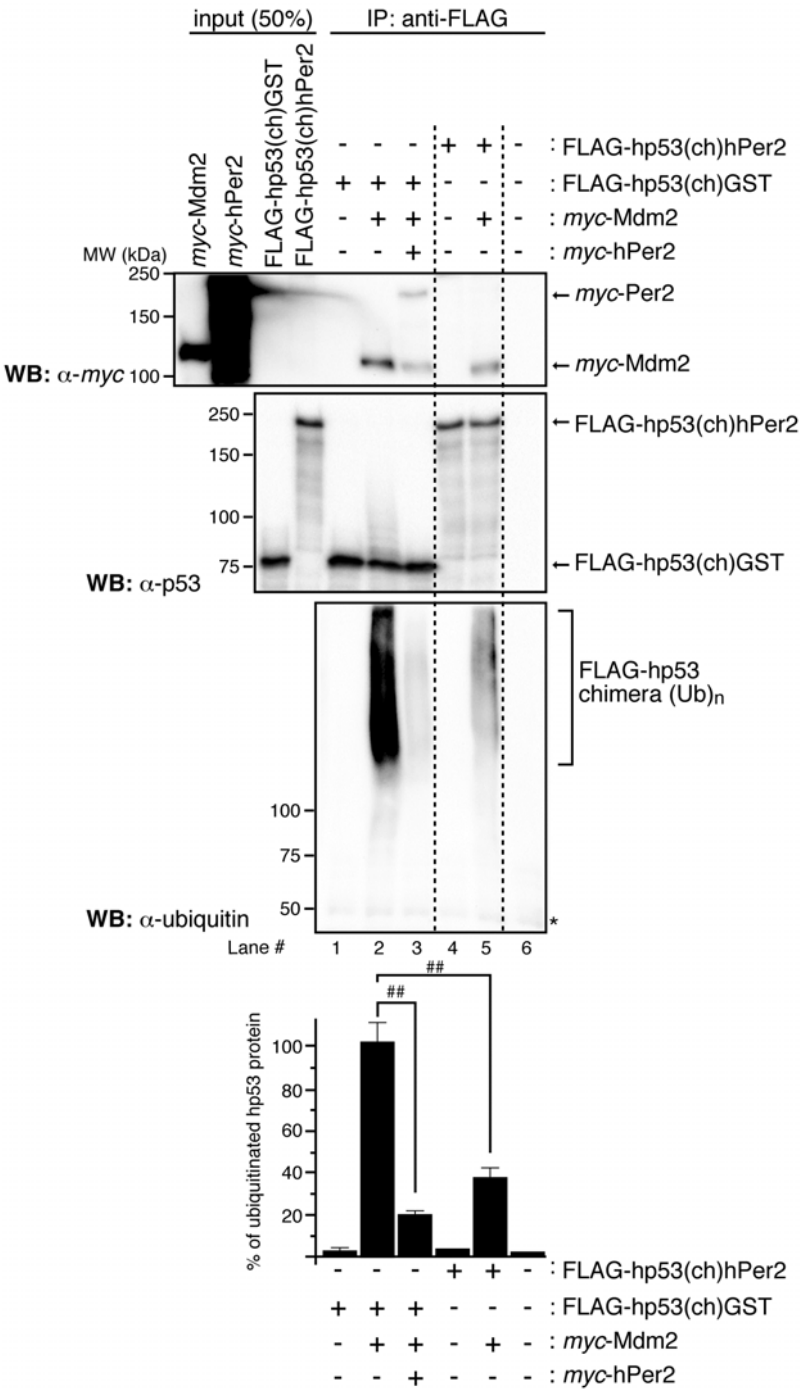
250
150
←FLAG-hPer2/
hPer2
WB: α-Per2
50
←hp53
WB: α-p53
75
←lamin A/C
50
WB: α-lamin A/C
50
*
←tubulin
WB: α-tubulin
B.
3.9% input
- MG132
+ MG132
FLAG-hPer2: - - - + + + - - - + + +
myc-hp53 : + + + + + + + + + + + +
MW (kDa)
T C N T C N T C N T C N
250
150
WB: α-Per2
←FLAG-hPer2
75
←myc-hp53
WB: α-p53
75
50
←lamin A/C
WB: α-lamin A/C
50
←tubulin
WB: α-tubulin
Lane# 1 2 3 4 5 6 7 8 9 10 11 12
C.
11% input
- MG132
+ MG132
MW (kDa)
T C N T C N
250
150
←myc-hPer2
WB: α-myc
100
←FLAG-Mdm2
WB: α-FLAG
50
←hp53
WB: α-p53
75
←lamin A/C
WB: α-lamin A/C
50
←tubulin
WB: α-tubulin
Lane# 1 2 3 4 5 6


input (50%)
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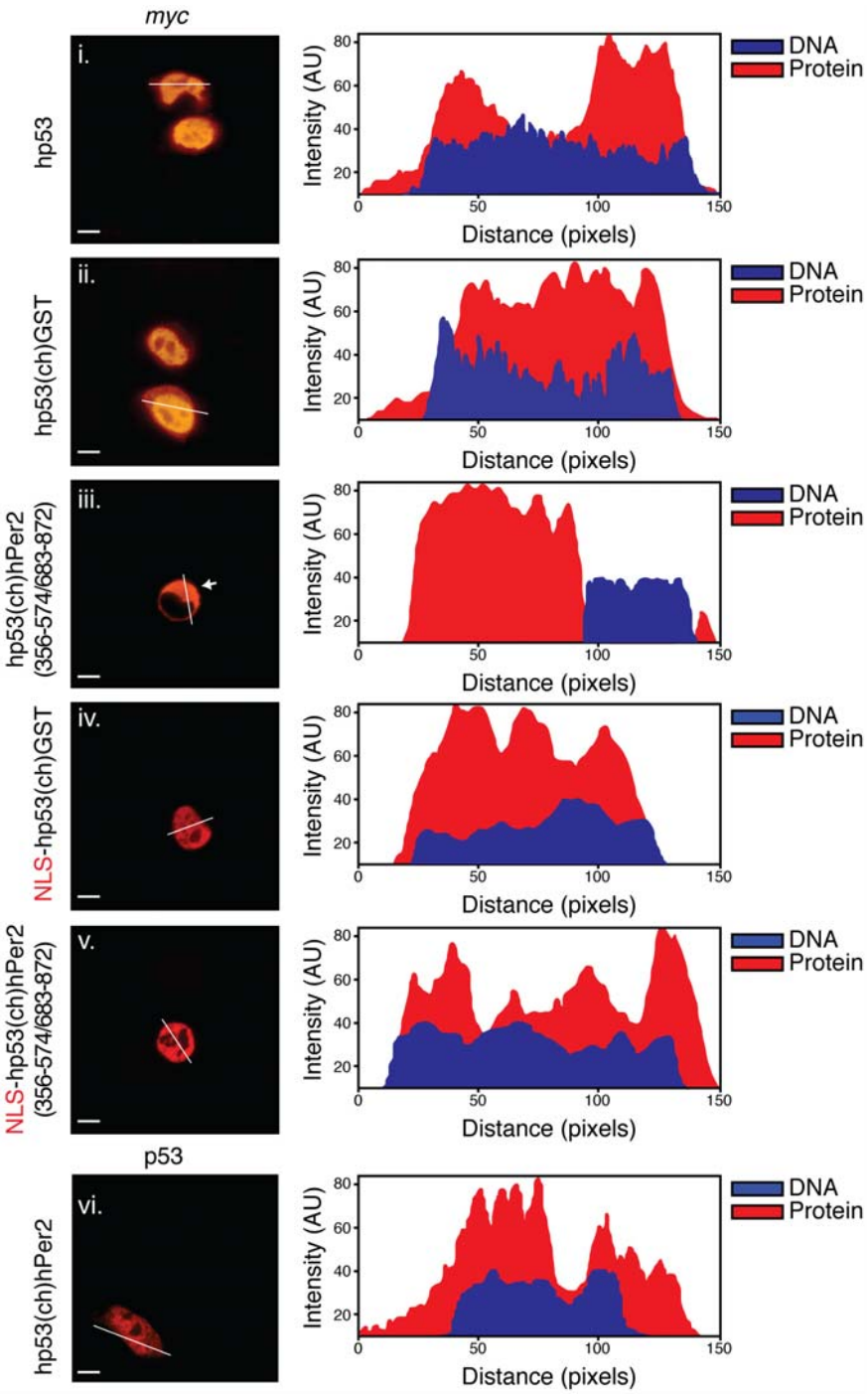
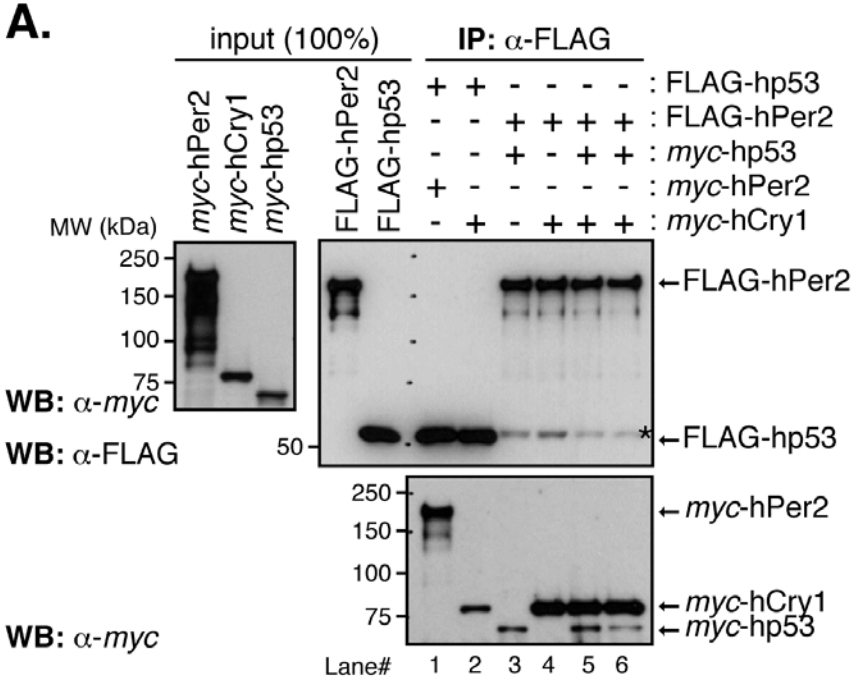
myc-Mdm2
myc-hPer2
FLAG-hp53(ch)GST
FLAG-hp53(ch)hPer2
: FLAG-hp53(ch)hPer2
: FLAG-hp53(ch)GST
: myc-Mdm2
: myc-hPer2
MW (kDa)
WB: α-myc
← myc-Per2
← myc-Mdm2
WB: α-p53
← FLAG-hp53(ch)hPer2
← FLAG-hp53(ch)GST
WB: α-ubiquitin
FLAG-hp53 chimera (Ub)n
Lane # 1 2 3 4 5 6
% of ubiquitinated hp53 protein
##
##


myc
i.
hp53
ii.
hp53(ch)GST
iii.
hp53(ch)hPer2
(356-574/683-872)
iv.
NLS-hp53(ch)GST
v.
NLS-hp53(ch)hPer2
(356-574/683-872)
p53
vi.
hp53(ch)hPer2
Intensity (AU)
Distance (pixels)
DNA
Protein
0
20
40
60
80
50
100
150

A.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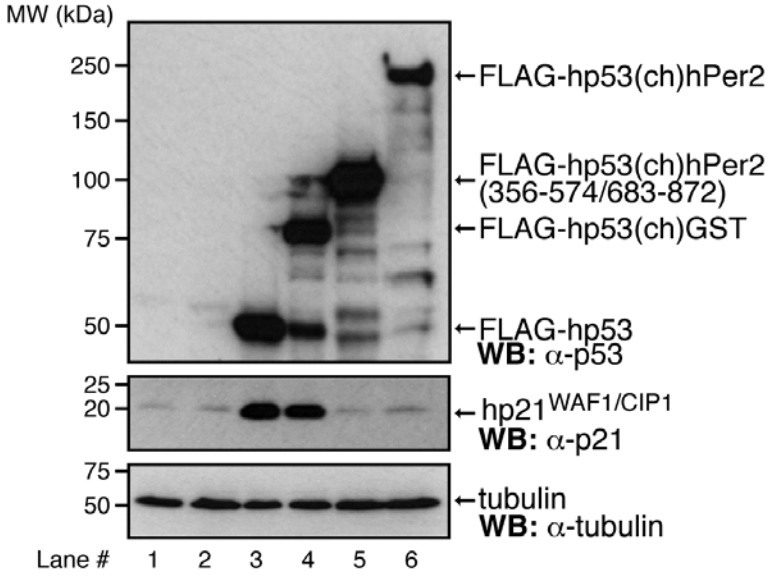
myc-hPer2
myc-hCry1
myc-hp53
FLAG-hPer2
FLAG-hp53
+ + - - - - : FLAG-hp53
- - + + + + : FLAG-hPer2
- - + - + + : myc-hp53
+ - - - - - : myc-hPer2
- + - + + + : myc-hCry1
MW (kDa)
250
150
100
75
50
WB: α-myc
WB: α-FLAG
←FLAG-hPer2
←FLAG-hp53
*
←myc-hPer2
←myc-hCry1
←myc-hp53
WB: α-myc
Lane# 1 2 3 4 5 6

B.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150
100
75
50
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←FLAG-hp53(ch)hPer2 (356-574/683-872)
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←FLAG-hp53
WB: α-p53
25
20
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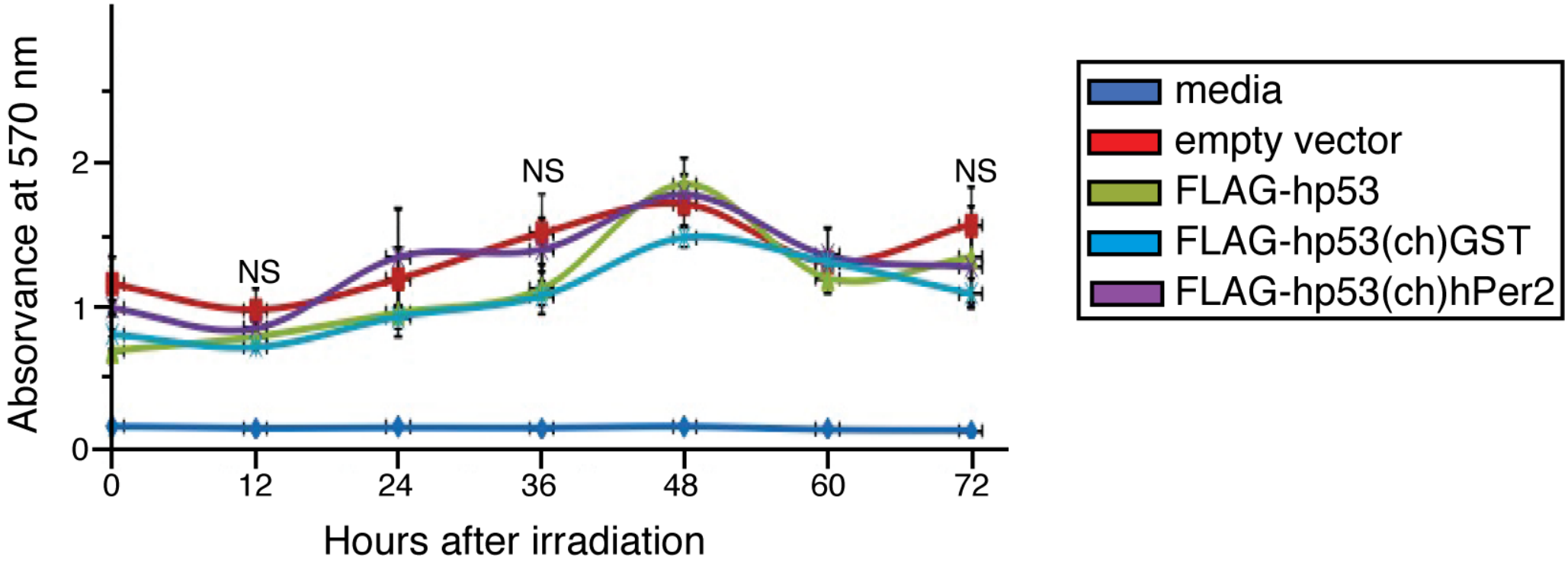
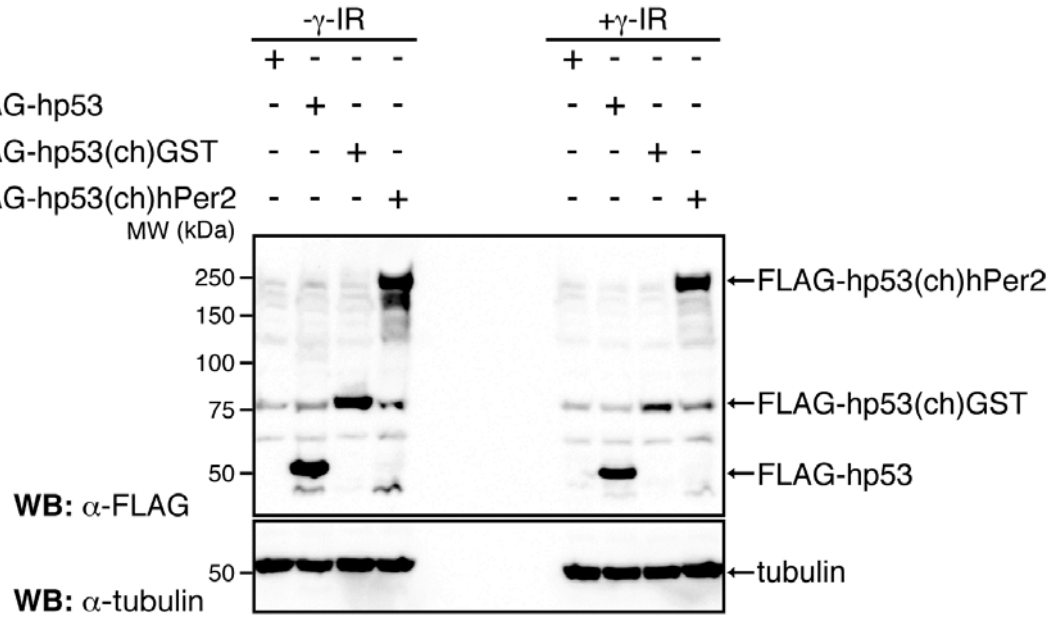
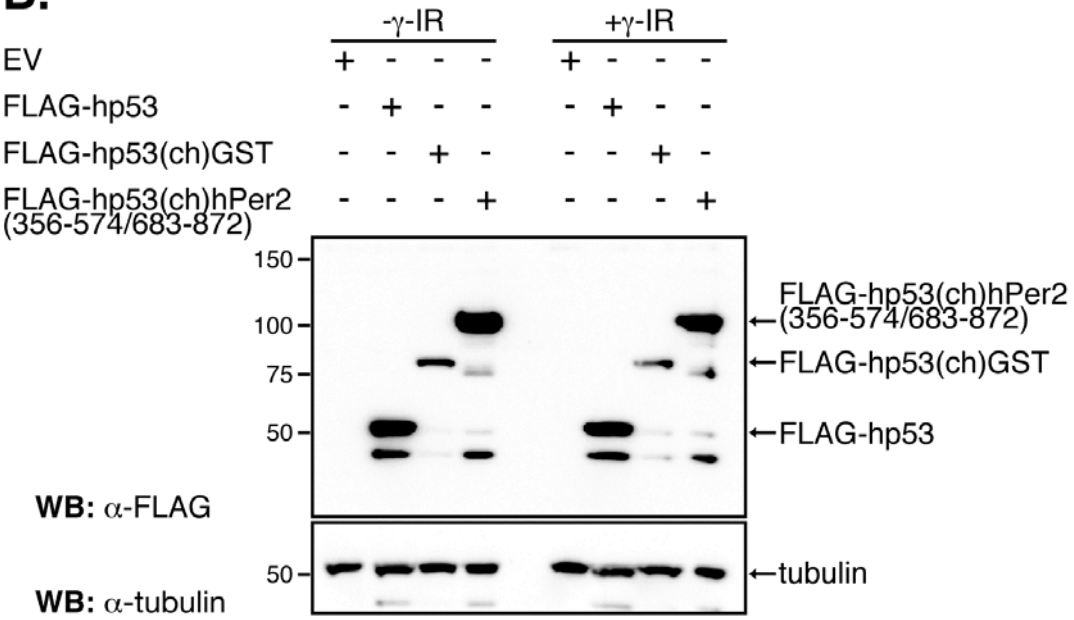
WB: α-p21
75
50
←tubulin
WB: α-tubulin
Lane # 1 2 3 4 5 6


Absorvance at 570 nm
2
1
0
NS
NS
NS
0
12
24
36
48
60
72
Hours after irradiation
media
empty vector
FLAG-hp53
FLAG-hp53(ch)GST
FLAG-hp53(ch)hPer2

A.


-γ-IR
+γ-IR
EV
FLAG-hp53
FLAG-hp53(ch)GST
FLAG-hp53(ch)hPer2
MW (kDa)
250
150
100
75
50
FLAG-hp53(ch)hPer2
FLAG-hp53(ch)GST
FLAG-hp53
WB: α-FLAG
50
tubulin
WB: α-tubulin

B.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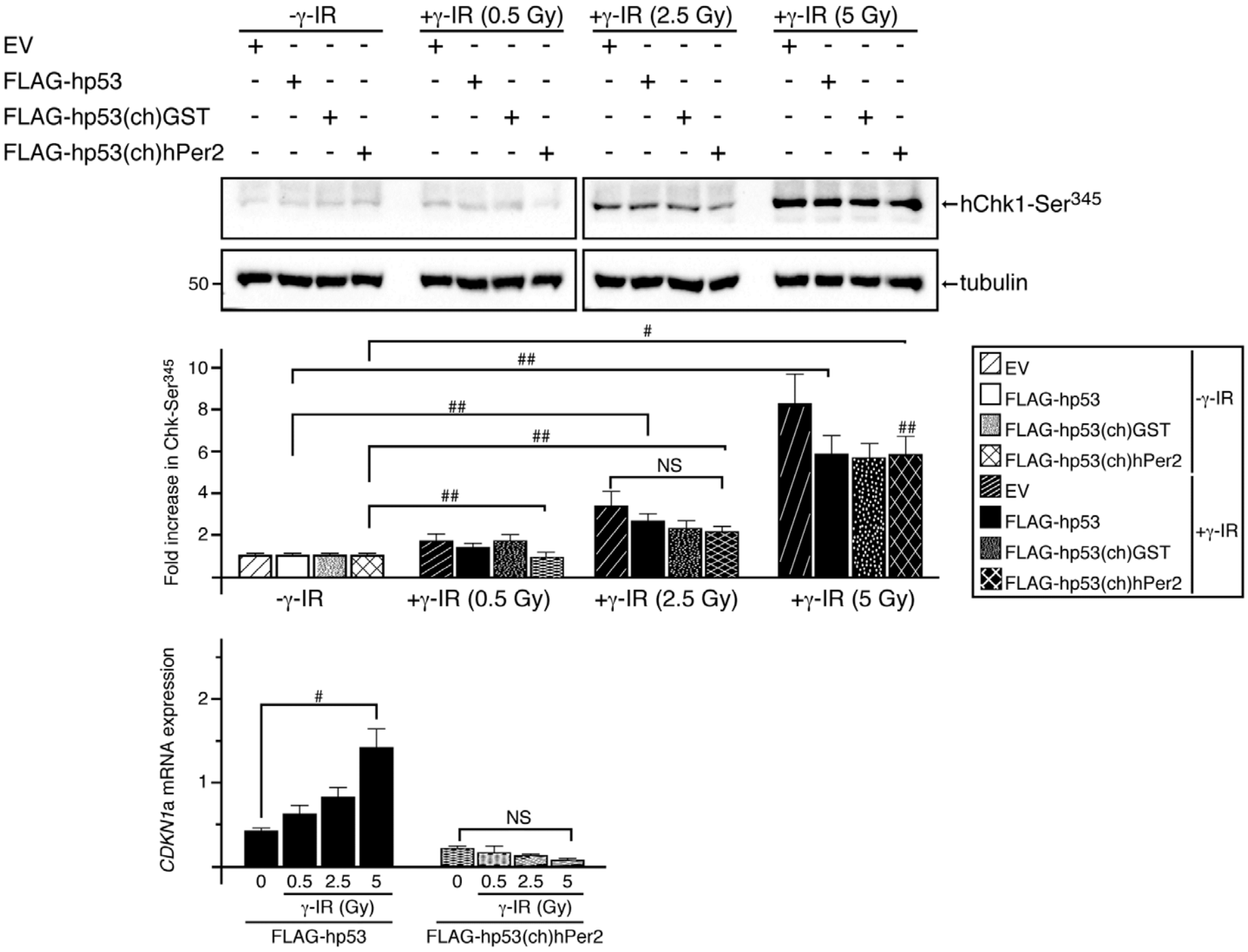
+γ-IR
EV
FLAG-hp53
FLAG-hp53(ch)GST
FLAG-hp53(ch)hPer2
(356-574/683-872)
150
100
75
50
FLAG-hp53(ch)hPer2
(356-574/683-872)
FLAG-hp53(ch)GST
FLAG-hp53
WB: α-FLAG
50
tubulin
WB: α-tubulin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+γ-IR (2.5 Gy)
+γ-IR (5 Gy)
EV
FLAG-hp53
FLAG-hp53(ch)GST
FLAG-hp53(ch)hPer2
←hChk1-Ser345
50
←tubulin
Fold increase in Chk-Ser345
#
##
NS
-γ-IR
+γ-IR (0.5 Gy)
+γ-IR (2.5 Gy)
+γ-IR (5 Gy)
EV
FLAG-hp53
FLAG-hp53(ch)GST
FLAG-hp53(ch)hPer2
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+γ-IR
CDKN1a mRNA expression
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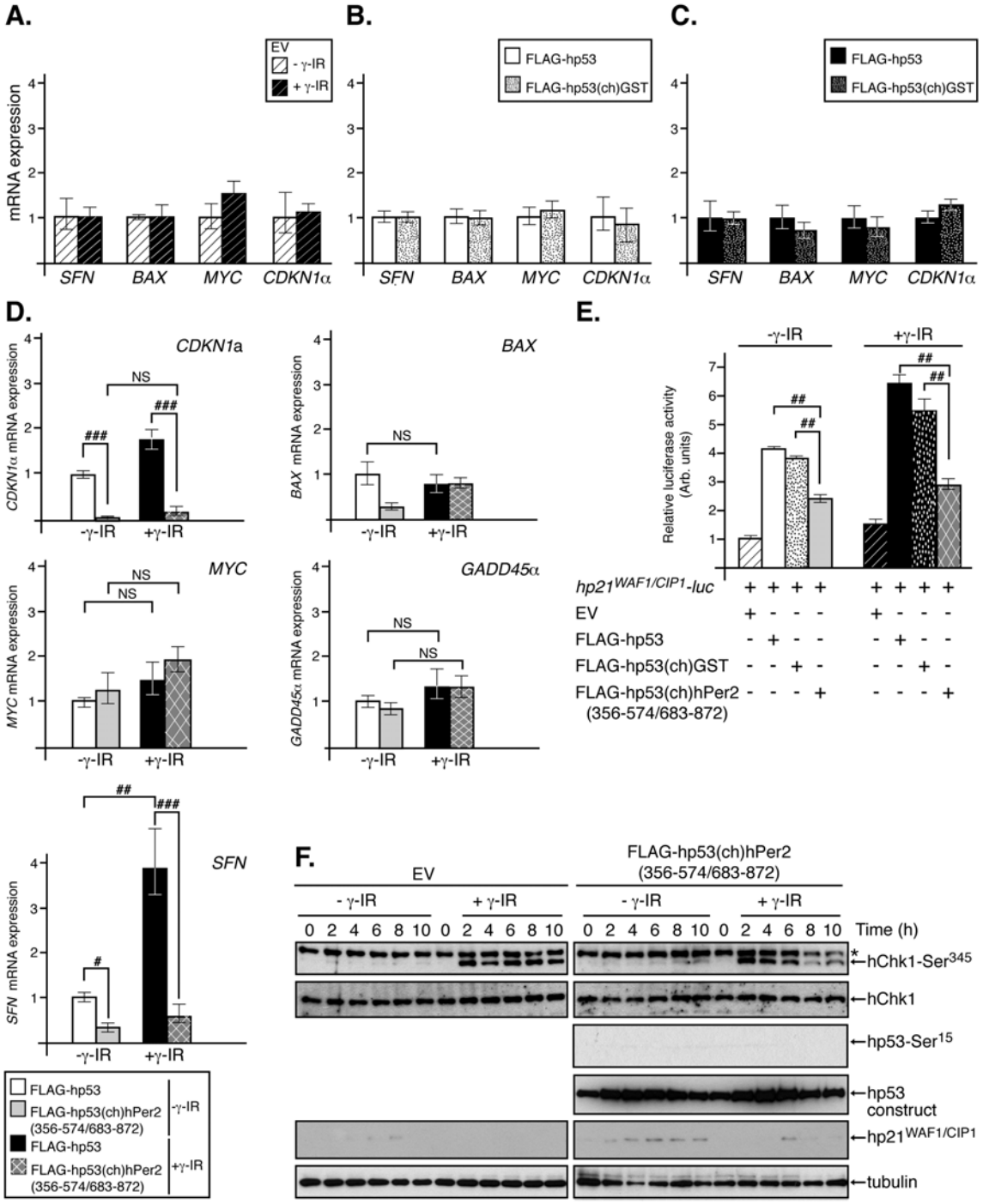
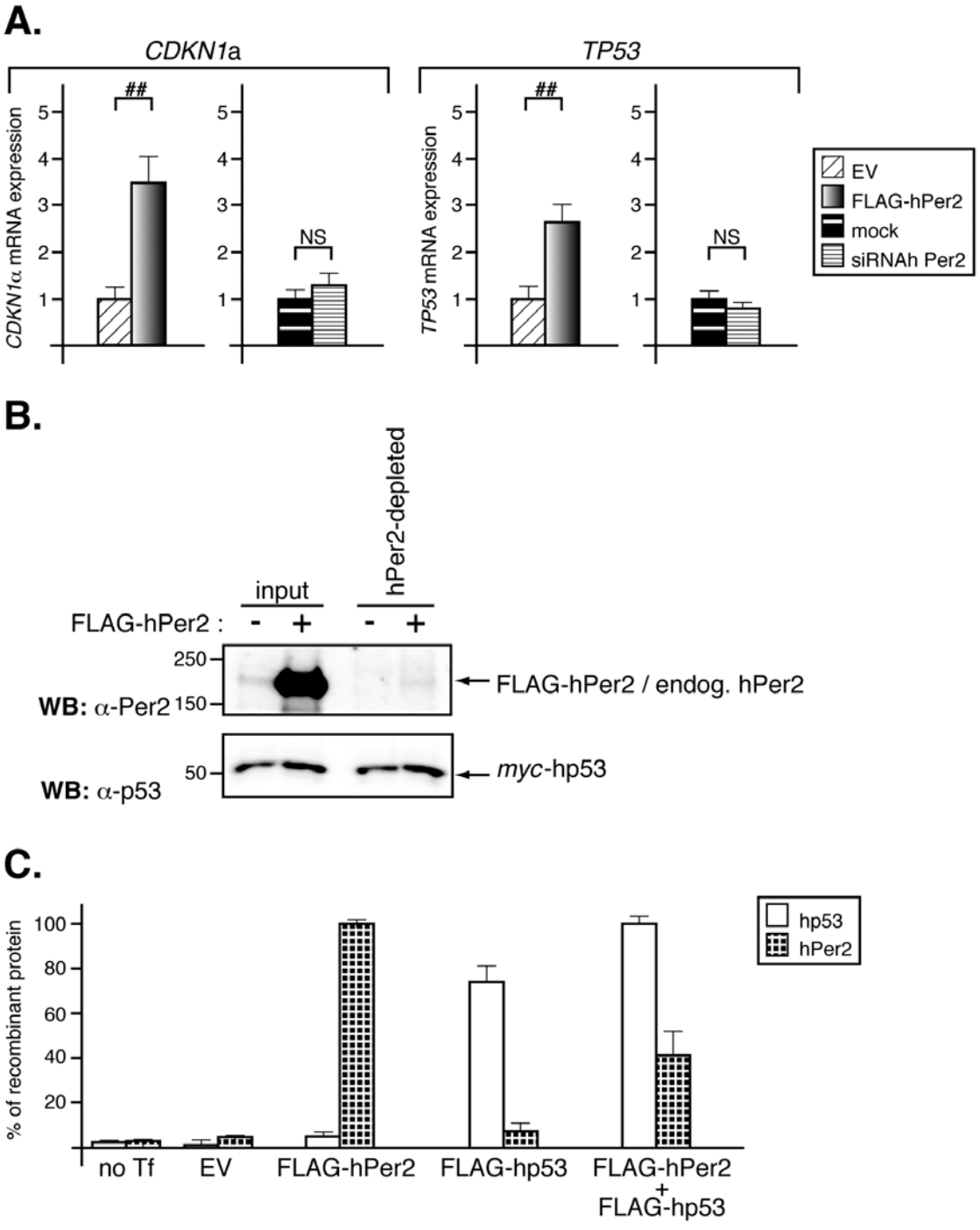
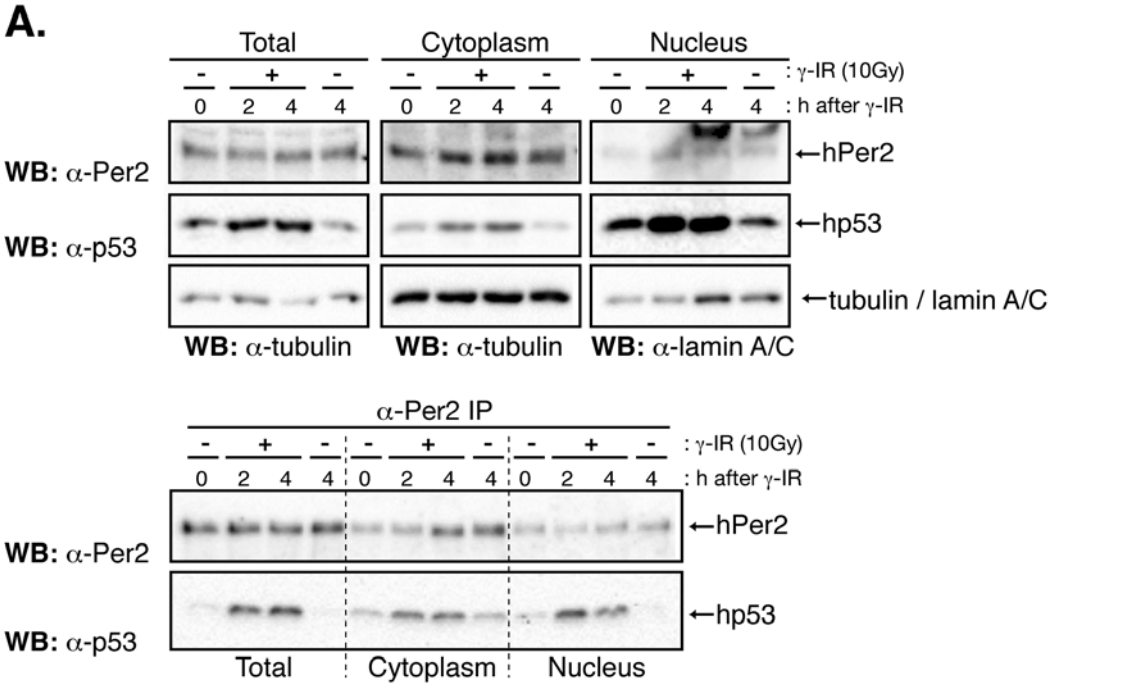
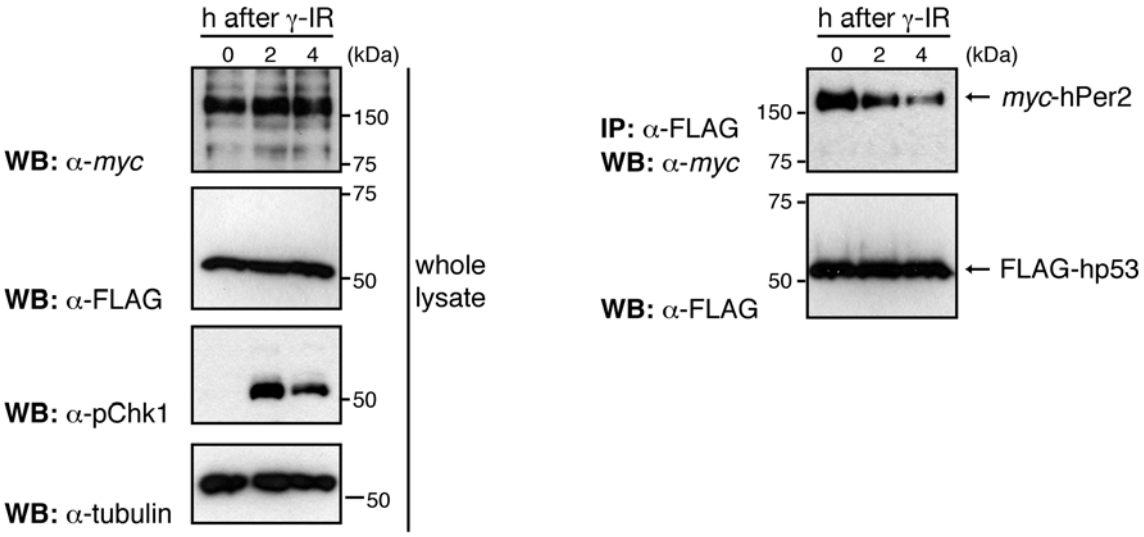
γ-IR (Gy)
FLAG-hp53
FLAG-hp53(ch)hPer2


A.
B.
C.
mRNA expression
EV
- γ-IR
+ γ-IR
FLAG-hp53
FLAG-hp53(ch)GST
SFN
BAX
MYC
CDKN1α
D.
CDKN1a
BAX
MYC
GADD45α
SFN
NS
###
##
#
-γ-IR
+γ-IR
FLAG-hp53
FLAG-hp53(ch)hPer2 (356-574/683-872)
E.
Relative luciferase activity (Arb. units)
hp21WAF1/CIP1-luc
EV
FLAG-hp53
FLAG-hp53(ch)GST
FLAG-hp53(ch)hPer2 (356-574/683-872)
F.
FLAG-hp53(ch)hPer2 (356-574/683-872)
Time (h)
hChk1-Ser345
hChk1
hp53-Ser15
hp53 construct
hp21WAF1/CIP1
tubulin


A.
CDKN1a
TP53
##
NS
CDKN1α mRNA expression
TP53 mRNA expression
EV
FLAG-hPer2
mock
siRNAh Per2
B.
input
hPer2-depleted
FLAG-hPer2 : - + - +
250
150
WB: α-Per2
FLAG-hPer2 / endog. hPer2
50
WB: α-p53
myc-hp53
C.
% of recombinant protein
hp53
hPer2
no Tf
EV
FLAG-hPer2
FLAG-hp53
FLAG-hPer2 + FLAG-hp53


A.
Total
Cytoplasm
Nucleus
: γ-IR (10Gy)
: h after γ-IR
WB: α-Per2
WB: α-p53
←hPer2
←hp53
←tubulin / lamin A/C
WB: α-tubulin
WB: α-tubulin
WB: α-lamin A/C
α-Per2 IP
Total
Cytoplasm
Nucleus


B.
h after γ-IR
(kDa)
WB: α-myc
WB: α-FLAG
WB: α-pChk1
WB: α-tubulin
whole lysate
IP: α-FLAG
WB: α-myc
← myc-hPer2
WB: α-FLAG
← FLAG-hp53