Supplemental Materials

Molecular Biology of the Cell

Gotoh et al.

Supplemental Material

Materials and Methods

Cell culture and transfections. The *c*hinese *h*amster *o*vary-*K1* (CHO-K1), *h*uman *e*mbryonic *k*idney *293* (HEK293), *h*uman *c*olorec*t*al carcinoma-*116* (HCT116), and *h*uman non-small cell lung carcinoma-*1299* (H1299) cell lines were purchased from the *A*merican *T*ype *C*ulture *C*ollection (ATCC) and maintained according to manufacturer's recommendations. Briefly, CHO-K1, HEK-293, HCT116, and H1299 were propagated in ATCC-formulated F-12K medium, *D*ulbecco's *m*odified *E*agle's *m*edia (DMEM), HyClone McCoy's 5a medium (Thermo Scientific), or ATCC-formulated RPMI-1640 medium supplemented with 10% *f*etal *b*ovine *s*erum (FBS) and 50 IU/ml penicillin and 50 μg/ml streptomycin, respectively. For transfection experiments, cells were seeded in 6- or 12-well plates until they reached 50-80% confluence. Transfections were optimized using either Lipofectamine (Life Technologies) for HEK293 and CHO-K1 cells or Lipofectamine LTX (Life Technologies) for HCT116 and H1299 following manufacturer's instructions. Otherwise, transfections in all cell lines were in HyClone HyQ-RS reduced serum medium (Thermo Scientific) for either 3h (for HEK293), 4 h (for H1299 and HCT116), or 5 h (for CHO-K1) at 37°C/5% CO_2. Proteins were then allowed to express at 37°C/5% CO_2 in the appropriate media containing 10%FBS without antibiotics after which they were either collected or further synchronized. Circadian synchronizations were performed by incubating cells in serum-free media for 12 h or by serum-shock treatment (Balsalobre *et al.*, 1998), respectively. For siRNA experiments, HCT116 cells ($2x10^5$ cells) were seeded in 12-well plates and transfections optimized using DharmaFECT2 (Thermo Scientific) following manufacturer's instructions. *H*uman *Per*iod *2* (hPer2) target sequences were as follows: *i*) 5'-CAACAAGGTGCTGAGAGTCAGCTTT-3' and *ii*) 5'-ACTCTGGTTATGAAGCCCCTAGAAT-3' (Life Technologies). Otherwise, all siRNA experiments were performed using siRNA Per2 (*i*). Except for CHO-K1 cells, extracts for protein analysis were prepared in NP-40 lysis buffer containing 10 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 80 mM β-glycerophosphate, 1mM Na_3VO_4, 10 mM NaF, and protease inhibitors (10 μM leupeptin, 1 μM aprotinin A, and 0.4 μM pepstatin). To prepare CHO-K1extracts, cells were lysed in 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, and protease inhibitors as above.

Plasmid constructs. The pBT/pTRG plasmid system (Stratagene) was used for the identification of protein interactors of hPer2. The pBT-hPer2 construct was generated by cloning a PCR-amplified full-length fragment of hPer2 into the *NotI*/*BglII* sites of the vector's polylinker. The pBT-LGF2 (encodes the dimerization domain of the Gal4 transcriptional activator protein) and pTRG-$Gal11^P$ (encodes a domain of the mutant form of the Gal11 protein) were control plasmids provided by the manufacturer. A pTRG cDNA BacterioMatch II premade library from human liver was purchased from Stratagene.

The hPer2, hp53, Mdm2, and mouse β-TrCP full-length cDNAs were cloned downstream from a tag encoding sequence into pCS2+FLAG-, 3xFLAG-, and *(myc)*$_6$-tag vectors (FLAG-hPer2, FLAG-hp53, *myc*-hPer2, *myc*-hp53, 3xFLAG-Mdm2, *myc*-Mdm2, *myc*-β-TrCP) modified for ligation-independent cloning (LIC, Novagen). A non-tagged version of the hPer2 clone was generated by LIC cloning in pCS2+ (named hPer2-tf). Various hPer2 and hp53 cDNA fragments were cloned into the *SalI*/*NotI* sites of pGEX-4T-3. Fragments of hPer2 comprising residues 1-172, 173-355, 356-574, 575-682, 683-872, 873-1,120, and 1,121-1,255 are referred in the text as: GST-hPer2(1-172), GST-hPer2(173-355), GST-hPer2(356-574), GST-hPer2(575-682), GST-hPer2(683-872), GST-hPer2(873-1120), GST-hPer2(1121-1255), respectively. Fragments of hp53 comprising residues 1-200, 1-296, 1-325, 1-364, 100-310, 200-393, and 300-393 are referred in the text as: GST-hp53(1-200), GST-hp53(1-296), GST-hp53(1-325), GST-hp53Δ30, GST-hp53(100-310), GST-hp53(200-393), GST-hp53(300-393).

The *hp21*$^{WAF1/CIP1}$-Luc reporter plasmid was a kind gift from Dr. Liao and is described in (Zhao *et al.*, 2003).

Supplemental Figures

FIGURE S1: A. Direct interaction between hPer2 and hp53 proteins within the complex. Recombinant GST-hp53-bound beads (~5 μg) were incubated in the presence of radiolabeled [^{35}S]-*myc*-hPer2 and increasing amounts of untagged hp53 (0-20 μg). Recombinant GST-bound beads were used as a negative control. Bound proteins were detected by autoradiography (*upper panel*) and Coomassie staining (*lower panel*). Bands were quantified using an AlphaImager and normalized to the input amount (- hp53 addition). The figure shows data from a single experiment that was repeated three times with similar results. The arrows on the right denote radiolabeled protein (*upper panel*) and recombinant proteins (*lower panel*). Molecular mass markers (in kDa) are indicated on the left. B. Mapping of hp53 binding regions in hPer2. Schematic representation of hPer2 (1,255 residues) architecture including the PAS domain (residues 186 to 473) and the PAS-A (residues 186 to 235) and –B (residues 322 to 374) subdomains. Recombinant GST- tagged fragments of hPer2 (residues 1-172, 173-355, 356-574, 575-682, 683-872, 873-1,120, and 1,121-1,255) are represented below. Fragments were purified by affinity chromatography and samples were analyzed for hp53 binding by pull-down assays as described in the "Materials and Methods" section. C. Mapping of hPer2 binding regions in hp53. Schematic representation of hp53 (393 residues) architecture including the transactivation (TAD; residues 1-42), proline-rich (PRD; residues 61-92), DNA-binding (residues 101-300), and tetramerization domain (TD; residues 326-356). Recombinant GST-tagged fragments of hp53 (residues 1-200, 1-296, 1-325, Δ30, 100-310, 200-393, and 300-393) are represented below. Fragments were purified and bound beads were analyzed for hPer2 binding by pull-down assays as described in "Materials and Methods". Results are summarized as follows: (+), strong interaction, (+/-), weak interaction, (-) no interaction detected (*right box*). Abbreviations: NES, nuclear export signal; NLS, nuclear localization signal; CLD, cytoplasmic localization domain.

FIGURE S2: A. hPer2, hp53, and Mdm2 co-localize in HCT116 cells. Endogenous hp53, hPer2, and recombinant Mdm2 protein localizations were monitored in HCT116 cells using specific primary antibodies. Cells were fixed with PFA/PBS and protein localization visualized using α-p53 and -Per2 primary antibodies and Alexa488 or Cy3-coupled secondary antibodies. Cell nuclei were visualized with Syto60 (Life Technologies). Arrows indicate the position of the nuclei. Scale bars correspond to 10 μm. B. The hPer2 and Mdm2 proteins associate even in the absence of hp53. H1299 cells were transfected with pCS2+*myc*-Mdm2, pCS2+*myc*-βTrCP, pCS2+FLAG-hp53, pCS2+FLAG-hPer2, or a combination of plasmids (ratio 1:2 for either hPer2:Mdm2 or hPer2:β-TrCP; ratio 1:2 for hp53:Mdm2) using Lipofectamine LTX. Cells were harvested 24h after transfection and extracts immunoprecipitated using α-FLAG antibody and protein A-beads. Anti-FLAG and *-myc* antibodies were used for immunoblotting. Asterisk indicates non-specific signal. C. The order of each component binding does not alter the formation of the hPer2/hp53/Mdm2 complex. Two *in vitro* transcribed and translated recombinant proteins (indicated on the top right panel as +) were incubated at room temperature to allow the heterodimer to form. The third component was incorporated to the reaction (labeled as a + symbol within a circle) and maintained at room temperature for additional 15 min. Samples were immunoprecipitated by using α-FLAG and protein A-beads and resolved by SDS-PAGE and immunoblotting (*upper* and *lower panels*). Input proteins are shown on the *left panel*. Asterisk indicates IgG heavy chain. D. Expression controls of recombinant proteins. HEK293 cells were transfected with pCS2+*myc*-Mdm2, pCS2+FLAG-hp53, pCS2+*myc*-hPer2, or a combination of plasmids as described in Fig. 3.D. Twelve hours after transfection, cells were treated with MG132 (10 μM) and harvested. Cell lysates (20 μg for α-myc and –tubulin; 80 μg for α-FLAG) were resolved by SDS-PAGE and immunoblotting using α-*myc* (upper panel), α-FLAG (middle panel), or α-tubulin (lower panel) antibodies. Tubulin was used as loading control. Asterisk indicates a non-specific signal.

FIGURE S3: A. hPer2 overexpression influences hp53 stability. HCT116 cells were transfected with either pCS2+FLAG-hPer2 or empty vector (EV) using Lipofectamine LTX and maintained in serum-free media for 4 h before media exchange. A sample equivalent to t=0 was collected 20 h later. Complete media containing cycloheximide (CHX, 100 μg/ml) was added and cells were harvested at different times (t=0.5 to 4 h). Extracts were resolved by SDS-PAGE and immunoblotting using α-FLAG, -p53, and -tubulin (loading control) antibodies (*upper panels*). Protein levels (hp53) from EV and FLAG-hPer2-transfected samples were quantified using ImageJ Software v1.45 and values normalized to tubulin levels. Bar graph indicates the percentage of hp53 protein remaining plotted as a function of time (*lower panel*). Gray box indicates the window of time while FLAG-hPer2 was readily detected in transfected samples. The figure shows data from a single experiment that was repeated three times with similar results. In all cases, EV and FLAG-hPer2 are represented by solid and dashed lines, respectively, and hp53 protein is symbolized by ●.

FIGURE S4: A. Ubiquitin levels from immunoprecipitated hp53 complexes shown in Figure 2C were quantified using ImageJ Software v1.45 and values were normalized to the signal from lane 1 (neither Mdm2 nor Per2 addition). Bar graphs indicate fold increase in hp53-ubiquitination (*bar graph*). B. Ubiquitin levels are represented as described in (A) but were obtained from samples shown in Figure 2D.

FIGURE S5: A. Isogenic clones of HCT116 cells (wt and p53-/-) were harvested and samples subjected to qRT-PCR as described in "Materials and Methods." Data are presented as mean ± SEM from three independent experiments performed in triplicate. B. Three isolated clones of each wt (p53+/+) and p53 -/- HCT116 cells (named #1 to #3) were harvested and lysates (~60 μg) resolved by SDS-PAGE and analyzed for expression of endogenous proteins by immunobloting using specific antibodies (α-Per2, *upper panel*; α-tubulin, *middle panel*; α-p53, *bottom panel*). Bands corresponding to hPer2 protein were quantified using an AlphaImager and normalized to tubulin levels for wt and p53 -/- HCT116 cells (*bar graph*). The figure summarizes the data from all three clones shown above. NS: indicates not significant.

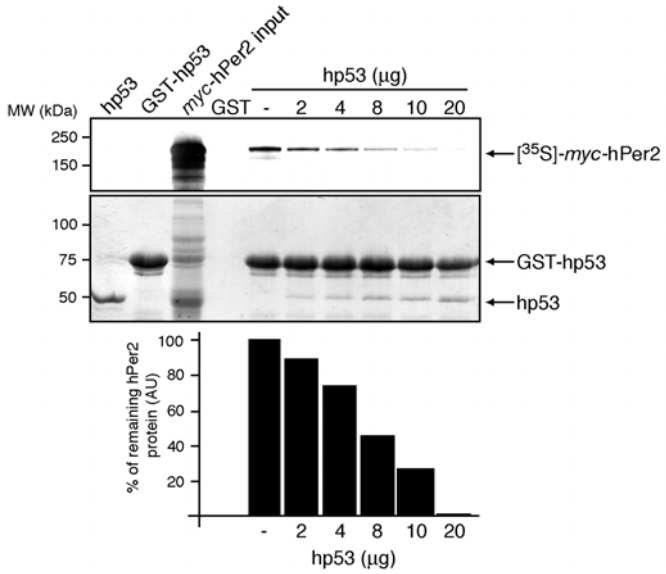
Table

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.

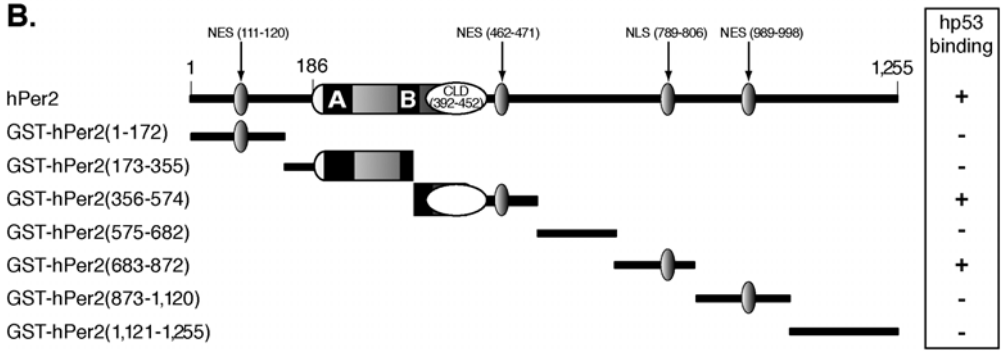
TABLE S1: Primer sequences for qRT-PCR reactions.

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	

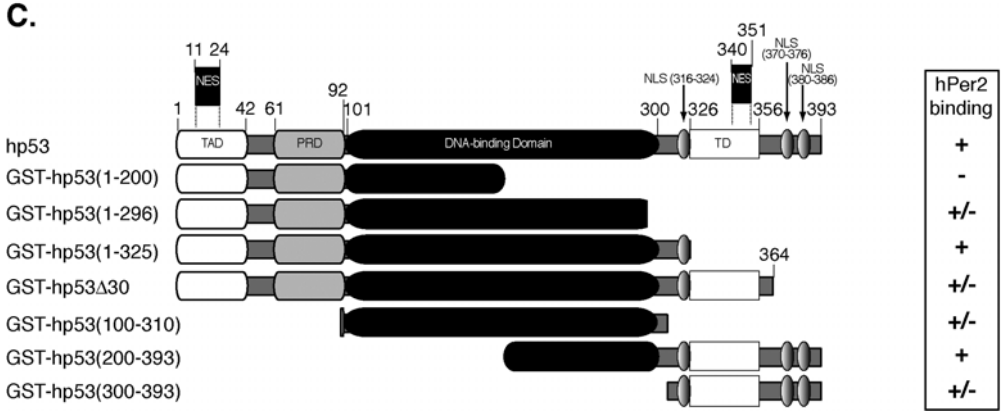
A.


hp53
GST-hp53
myc-hPer2 input
hp53 (μg)
MW (kDa)
GST - 2 4 8 10 20
250
150
100
75
50
←[35S]-myc-hPer2
←GST-hp53
←hp53
% of remaining hPer2 protein (AU)
100
80
60
40
20
- 2 4 8 10 20
hp53 (μg)

B.


NES (111-120)
NES (462-471)
NLS (789-806)
NES (989-998)
1
186
1,255
A
B
CLD (392-452)
hPer2
GST-hPer2(1-172)
GST-hPer2(173-355)
GST-hPer2(356-574)
GST-hPer2(575-682)
GST-hPer2(683-872)
GST-hPer2(873-1,120)
GST-hPer2(1,121-1,255)
hp53 binding
+
-
-
+
-
+
-
-

C.


11 24
NES
92
1
42 61
101
300
NLS (316-324)
326
340
351
NES
NLS (370-376)
NLS (380-386)
356
393
TAD
PRD
DNA-binding Domain
TD
hp53
GST-hp53(1-200)
GST-hp53(1-296)
GST-hp53(1-325)
364
GST-hp53Δ30
GST-hp53(100-310)
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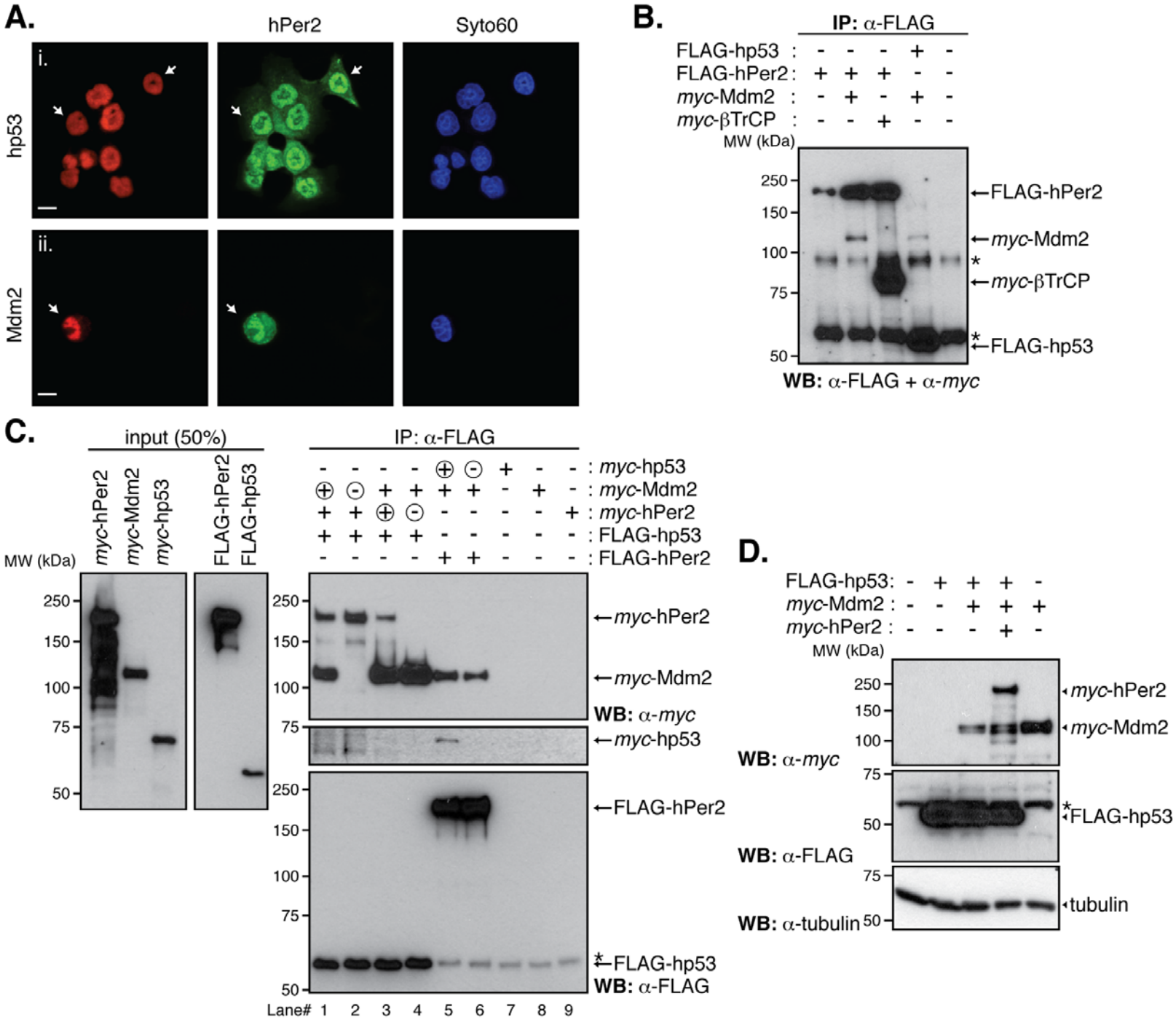
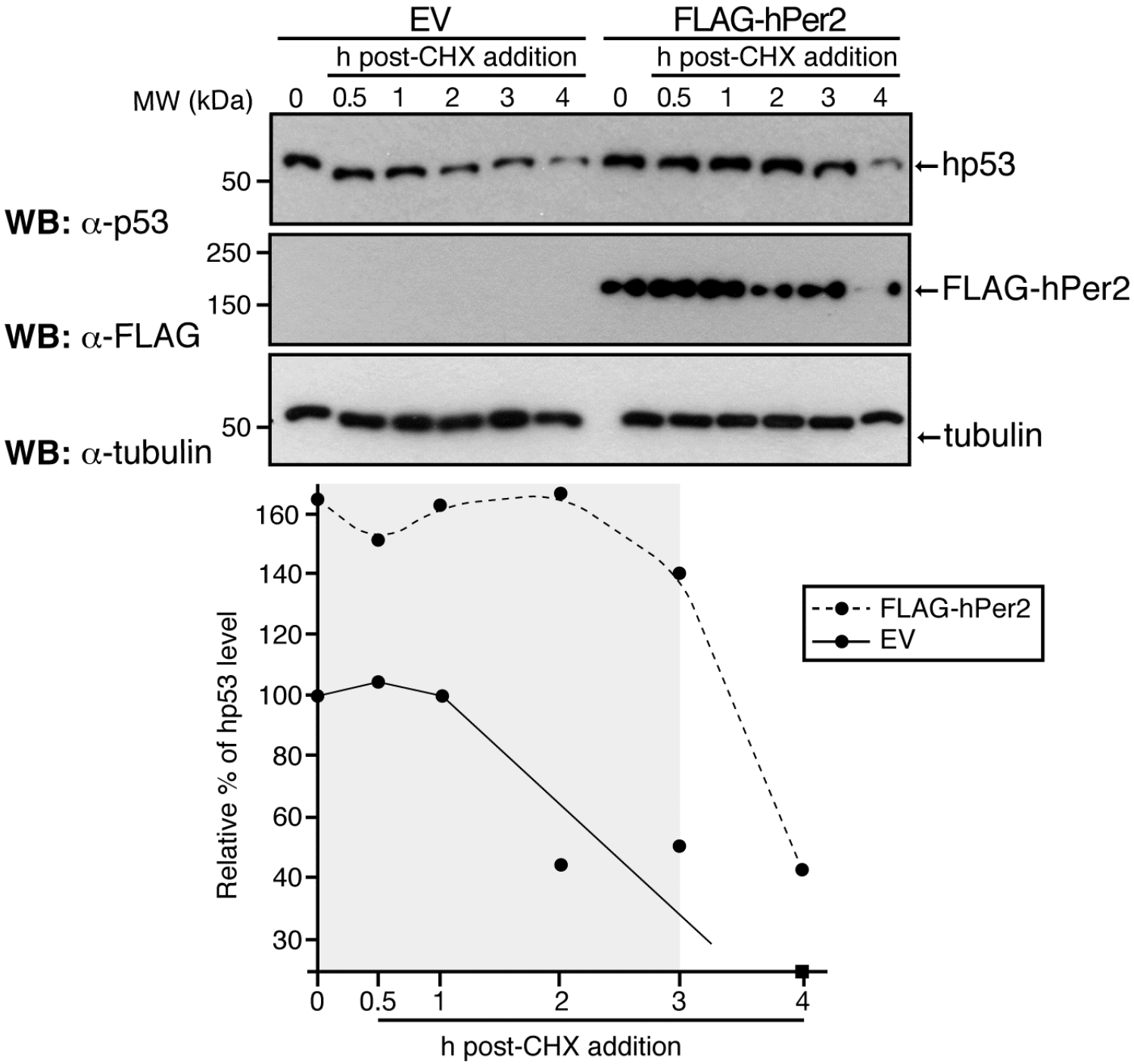
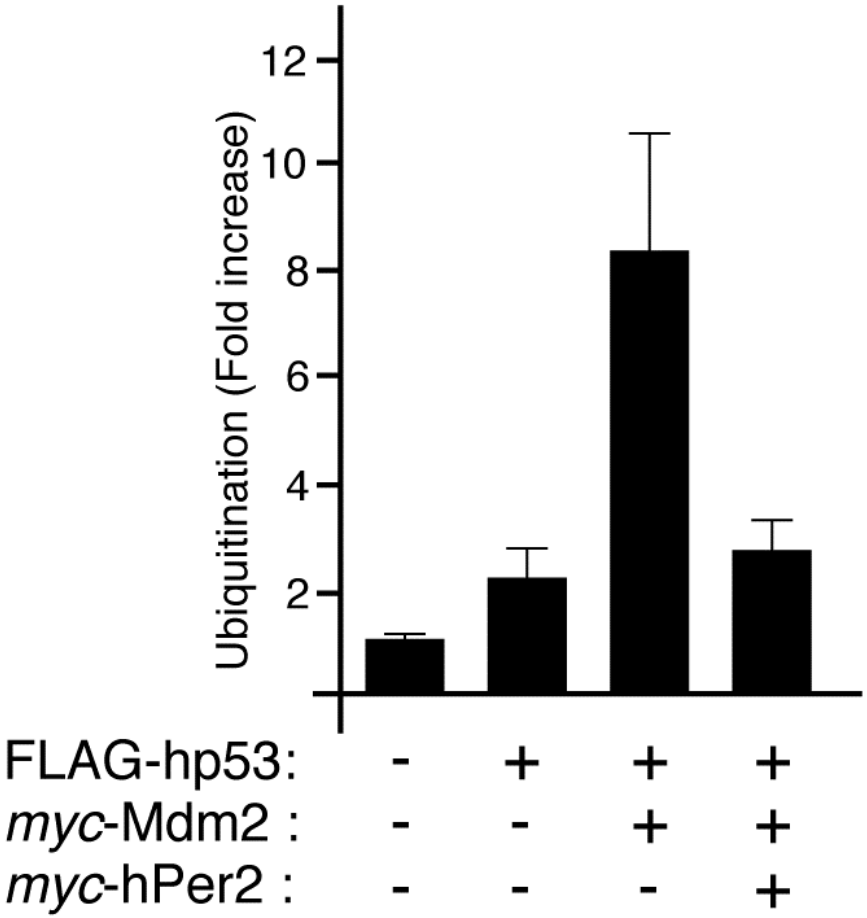
GST-hp53(300-393)
hPer2 binding
+
-
+/-
+
+/-
+/-
+
+/-

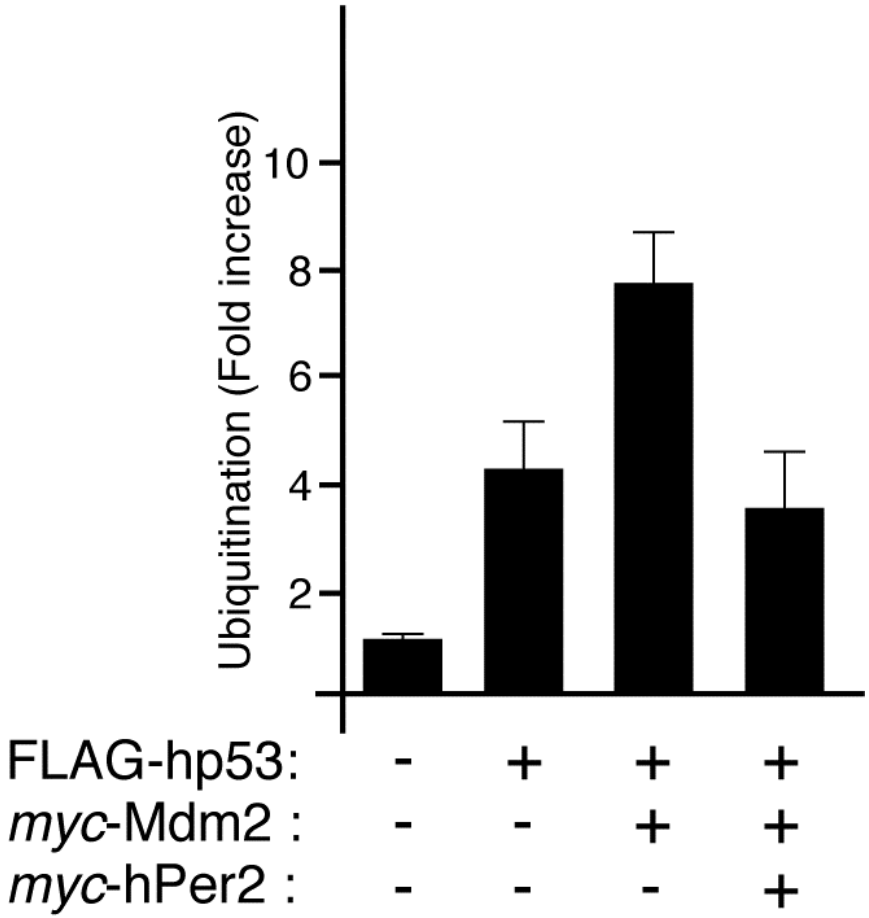

A.
hPer2
Syto60
i.
hp53
ii.
Mdm2
B.
IP: α-FLAG
FLAG-hp53 : - - - + -
FLAG-hPer2 : + + + - -
myc-Mdm2 : - + - + -
myc-βTrCP : - - + - -
MW (kDa)
250
150
100
75
50
←FLAG-hPer2
←myc-Mdm2
*
←myc-βTrCP
*←FLAG-hp53
WB: α-FLAG + α-myc
C.
input (50%)
IP: α-FLAG
myc-hPer2
myc-Mdm2
myc-hp53
FLAG-hPer2
FLAG-hp53
: myc-hp53
: myc-Mdm2
: myc-hPer2
: FLAG-hp53
: FLAG-hPer2
MW (kDa)
←myc-hPer2
←myc-Mdm2
WB: α-myc
←myc-hp53
←FLAG-hPer2
*←FLAG-hp53
WB: α-FLAG
Lane# 1 2 3 4 5 6 7 8 9
D.
FLAG-hp53: - + + + -
myc-Mdm2 : - - + + +
myc-hPer2 : - - - + -
MW (kDa)
myc-hPer2
myc-Mdm2
WB: α-myc
*FLAG-hp53
WB: α-FLAG
tubulin
WB: α-tubulin


EV
FLAG-hPer2
h post-CHX addition
h post-CHX addition
MW (kDa)
0 0.5 1 2 3 4 0 0.5 1 2 3 4
50
WB: α-p53
←hp53
250
150
WB: α-FLAG
←FLAG-hPer2
50
WB: α-tubulin
←tubulin
160
140
120
100
80
60
40
30
Relative % of hp53 level
FLAG-hPer2
EV
0 0.5 1 2 3 4
h post-CHX addition

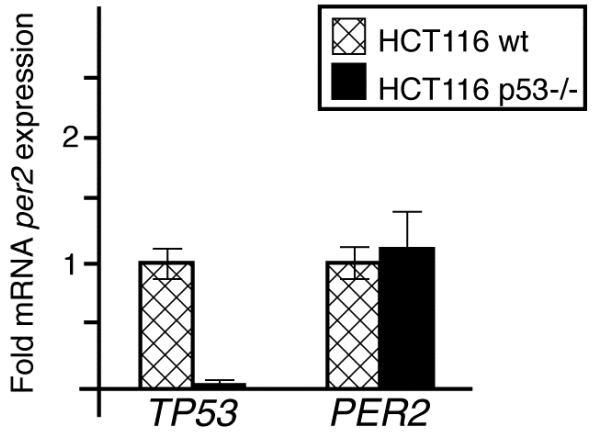
A.


Ubiquitination (Fold increase)
12
10
8
6
4
2
FLAG-hp53: - + + +
myc-Mdm2 : - - + +
myc-hPer2 : - - - +

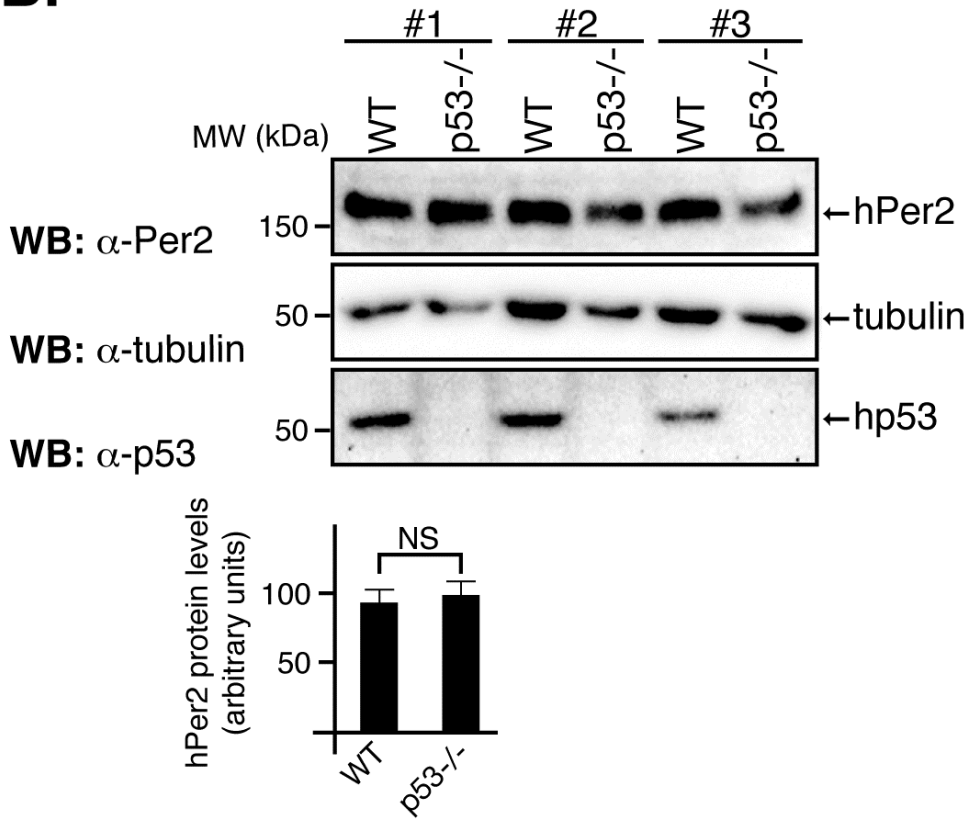
B.


Ubiquitination (Fold increase)
10
8
6
4
2
FLAG-hp53: - + + +
myc-Mdm2 : - - + +
myc-hPer2 : - - - +

A.


HCT116 wt
HCT116 p53-/-
Fold mRNA per2 expression
2
1
TP53
PER2

B.


#1
#2
#3
WT
p53-/-
WT
p53-/-
WT
p53-/-
MW (kDa)
WB: α-Per2
150
←hPer2
WB: α-tubulin
50
←tubulin
WB: α-p53
50
←hp53
hPer2 protein levels (arbitrary units)
NS
100
50
WT
p53-/-