SUPPLEMENTARY INFORMATION

RYBP stabilizes p53 by modulating MDM2

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SUPPLEMENTARY MATERIALS AND METHODS

Plasmids and reagents. The preparation of pGBKT7-MDM2, T7-MDM2, GST-MDM2, HA-p53, His-Ub, HA-Ub, *p21*^{Waf1} promoter luciferase reporter, the Renilla luciferase vector and the deletions of CMV-MDM2 were described previously (Chen *et al*, 2007). Myc-MDM2 C464A was produced by proof-reading PCR using Flag-MDM2 C464A plasmid as a template, The Flag-MDM2 C464A plasmid was provided by Dr. C.G. Maki (Harvard). GFP-RYBP and deletions of GFP-RYBP were produced by proof-reading PCR using the pGADT7-RYBP plasmid as a template. The RYBP siRNA pool and control siRNA pool were from Dharmacon. Cycloheximide (CHX), MG132, doxorubicin, etoposide, glutathione-agarose beads, protease inhibitor and SYBR® Green JumpStart™ Taq ReadyMix™ were all purchased from Sigma. Lipofectin® was from Invitrogen. Ni-NTA agarose resin and protein G beads were from Qiagen and GE Healthcare, respectively.

Cells and antibodies

U2OS and HCT116 cells with or without p53 were kindly provided by Dr. X. Chen (UC Davis) and Dr. B. Vogelstein (Johns Hopkins), respectively. COS7, MCF7, A549 and PC3 cells were purchased from the ATCC. MCF7 cells with p53 knockdown were described previously (Zhang

and Zhang, 2008). Antibodies were purchased from Santa Cruz (MDM2 H221, p53 FL393, p53 DO-1 and Ub), Novagen (T7), Covance (HA.11), Calbiochem (MDM2 2A10 and Myc 9E10), Sigma (GFP, β-actin, mouse and rabbit IgG), and eBioscience (RYBP).

Immunoprecipitation and immunoblotting. Cells were transfected with plasmids as indicated in each figure legend using Lipofectin® according to the manufacturer's protocol (Invitrogen). Cells were seeded at a density of ~1×10^5/well of 6-well plates, ~3×10^5/60-mm dish or 5×10^5/100-mm dish, and were harvested at indicated times post-transfection and lysed in NP-40 lysis buffer consisting of 50 mM Tris/HCl (pH 8.0), 1.0 % Nonidet P-40, 1 mM EDTA, 137 mM NaCl, and protease inhibitor mixture from Sigma. Equal amounts of clear cell lysates were used for immunoblot analysis as described previously (Zhang and Zhang, 2008). Immunoprecipitation was conducted using the antibodies indicated in the figure legends. Beads were washed with lysis buffer three times, and bound proteins were detected by immunoblotting using antibodies as reported previously (Zhang and Zhang, 2008).

Ni-NTA ubiquitination assay. Briefly, A549 cells were transfected with combinations of the following plasmids as indicated in the figure legends: His_6-ubiquitin (4 μg), HA-p53 (2 μg), T7-MDM2 (8 μg), Myc-RYBP (8 μg), GFP-RYBP (4, or 8 μg), or GFP-RYBP 74-143 (4, or 8 μg) in 100-mm dishes using Lipofectin® (Invitrogen). At 24 h after transfection, cells were lysed in buffer A (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol). The cell lysates were incubated with 50 μl of Ni-NTA beads (Qiagen) for 4 h at room temperature, then beads were washed with buffer A, buffer B (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol), and buffer C (8 M

urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl (pH 6.8), 10 mM β-mercaptoethanol). The bound proteins were eluted by 5 × SDS sample buffer supplemented with 250 mM imidazole at 100°C for 5 min, resolved on SDS-PAGE, and detected using the appropriate antibodies.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded sections were first incubated in an oven at 67°C for 8 h. The sections were then deparaffinized, rehydrated and washed with PBS (pH 7.4) for 3 × 5 min. The sections were mounted in 0.01 M citrate buffer (pH 6.0) and boiled in an autoclave for 3 min, then allowed to cool to room temperature. Following washes with PBS, the primary rabbit anti-human RYBP antibody was applied at a 1:750 dilution, and slides were incubated at 37°C for 1.5 h; then washed and incubated in envision secondary antibody at 37°C for 40 min. After washing, the sections were incubated in DAB chromogen for 5 min. The sections were counterstained with hematoxylin, dehydrated and mounted.

Quantitative RT-PCR (qRT-PCR) analysis. Total RNA was isolated from cells using the Trizol reagent (Invitrogen). Reverse transcription of 2.0 μg of total RNA was performed in a total volume of 20 μl using SuperScript TM II Reverse Transcriptase (Invitrogen). 0.3 μl of cDNA was PCR-amplified in 15-μl reactions containing primers at 500 nM in SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma). The primers for *CDKN1A* (p21), *BAX* (Bax) and *GAPDH* were as followings: *p21* forward, 5'-TGT GGA CCT GTC ACT GTC TT-3', reverse, 5'-GGA TTA GGG CTT CCT CTT G-3'; *Bax* forward, 5'-TTC ATC CAG GAT CGA GCA G-3', reverse, 5'-TCC TCT GCA GCT CCA TGT TA-3'; *GAPDH* forward, 5'-GGA GTC CAC TGG CGT CTT CAC-3', reverse, 5'-GAG GCA TTG CTG ATG ATC TTG AGG-3'. PCR was performed for 40 cycles consisting of 94 °C for 15 sec, 58°C for 15 sec and 60 °C for 60 sec

using an iQ5 machine (Bio-Rad, USA). All samples were analyzed in triplicate and adjusted with Rox and normalized to *GAPDH* mRNA levels. The results are expressed as -fold change compared with the normal control.

SUPPLEMENTARY FIGURE LEGENDS

Fig S1 RYBP binds to MDM2. (A) A549 cells (5×10^5 cells/well) were transfected with 10 μg of T7-MDM2, Myc-RYBP or both for 24 h. The lysates were immunoprecipitated with T7 (left panel) or Myc (middle panel) antibody. Then the purified proteins were detected with T7 and Myc antibodies, or the lysates were directly immunoblotted with T7 and Myc antibodies (right panel). (B and D) A series of truncated GFP-RYBP proteins was generated (B). A plasmid expressing T7-MDM2 was co-transfected into COS7 cells with plasmids expressing GFP or the deletions of GFP-RYBP. Sequential immunoprecipitation with GFP antibody, and immunoblotting assays with T7 antibody were performed, and the capacities of the deletion proteins for binding MDM2 are shown in (D). (C and E) A series of truncated Myc-MDM2 plasmids was generated previously (C) (Chen *et al*, 2007). A plasmid expressing GFP-RYBP was co-transfected into COS7 cells with plasmids expressing Myc or the deletions of Myc-MDM2. Sequential immunoprecipitation with Myc antibody, and immunoblotting assays with GFP antibody were performed, and the capacities of the deletions of MDM2 for binding to RYBP are shown in (E)

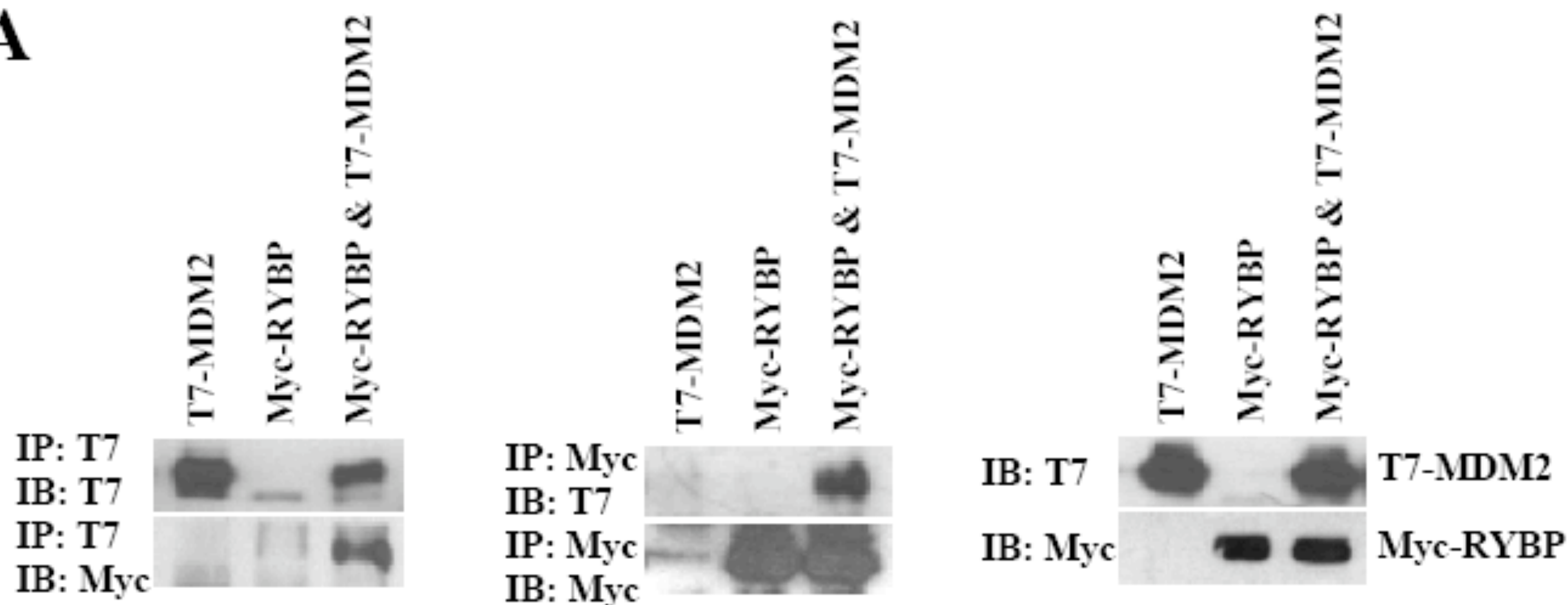
Fig S2 RYBP stabilizes p53. (A and B) A549 cells were transfected with a CMV vector or CMV-RYBP (4 μg) for 24 h followed by exposure to CHX (10 μg/ml) for indicated times. The

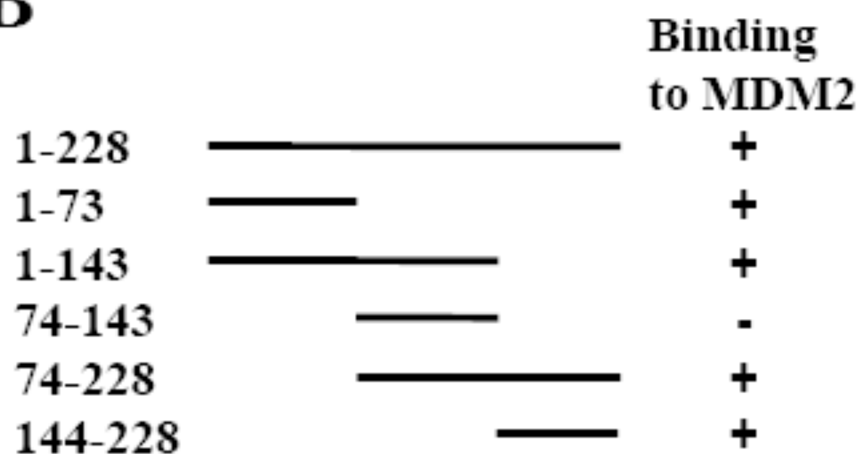
cell lysates were harvested and examined for target protein expression (A). The intensity of the MDM2 bands was analyzed by densitometry (Bio-Rad Model GS-670 Imaging Densitometer; Bio-Rad). The relative density at each time point is expressed as a percentage of the density at time 0 after normalization to the corresponding β-actin level (B). (C) U2OS cells were exposed to either control or RYBP siRNA pools (100 nM) for 72 h, followed by exposure to CHX (10 μg/ml) for the specified times. Cell lysates were harvested and resolved by SDS-PAGE, and target proteins were detected by immunoblotting.

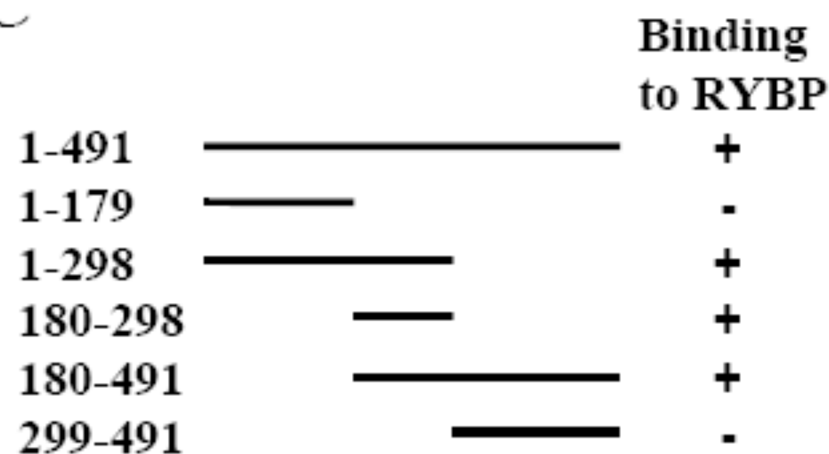
Fig S3 RYBP inhibits MDM2-mediated p53 ubiquitination. (A) COS7 cells were co-transfected with T7-MDM2, HA-p53 and Myc-RYBP as indicated. 24 h later, lysates were either separated by SDS-PAGE followed by immunoblotting, or immunoprecipitated by Myc, T7 or HA antibody, followed by immunoblotting with Myc, T7, or HA antibody. (B) COS7 cells were transfected with MDM2 or a mutant of MDM2 lacking the RYBP binding domain (10 μg), together with HA-p53 and GFP-RYBP (10 μg) for 24 h. The lysates were subjected to immunoprecipitation with GFP antibody, and corresponding proteins were analyzed by immunoblotting. (C) U2OS cells were transfected with plasmids expressing a GFP or GFP-RYBP vector (10 μg) for 24 h, or transfected with either the control or RYBP siRNA pools (100 nM) for 72 h. Cells were then exposed to MG132 (20 μM) for an additional 4 h before harvest. The cell lysates were immunoprecipitated with p53 FL393, and the MDM2 and p53 protein levels were determined using MDM2 2A10 or p53 DO-1, or directly immunoblotted for corresponding protein expression. (D) COS-7 cells were transfected with 4 or 10 μg of GFP-RYBP, together with T7-MDM2 and HA-p53 (10 μg) for 24 h, and the lysates were immunoprecipitated with a GFP antibody. The bound proteins were detected by T7, HA and GFP antibodies. (E) A549 cells were

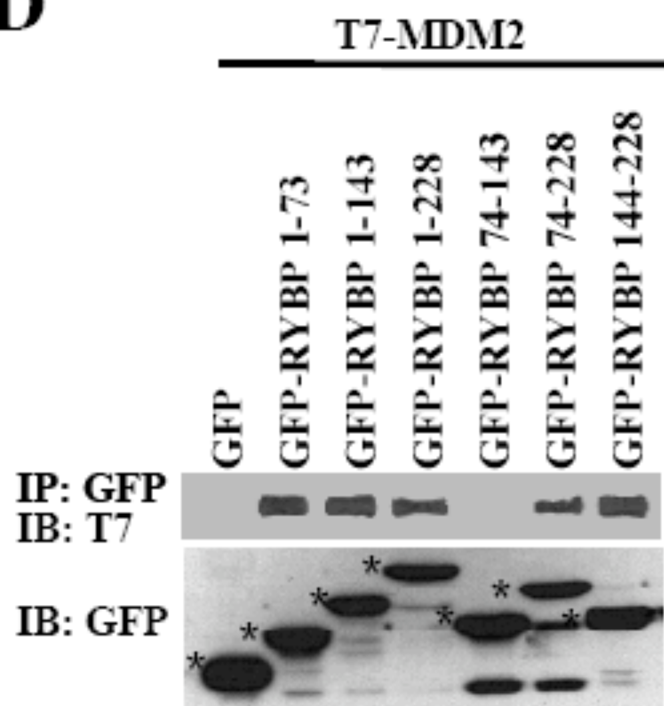
transfected with HA-Ub, HA-p53, T7-MDM2 and Myc-RYBP as indicated in the figure. 24 h after transfection, the cell lysates were cleared and directly detected by immonoblotting (left and right panels), or were first immunoprecipitated with p53 DO-1, followed by immunoblotting with Ub antibody or target protein antibodies (middle panel). (F) U2OS cells were transfected with the control or RYBP siRNA pool (100 nM) for 48 h. Then the cells were transfected with His-Ub for an additional 24 h, and p53 ubiquitination was analyzed by the Ni-NTA method.

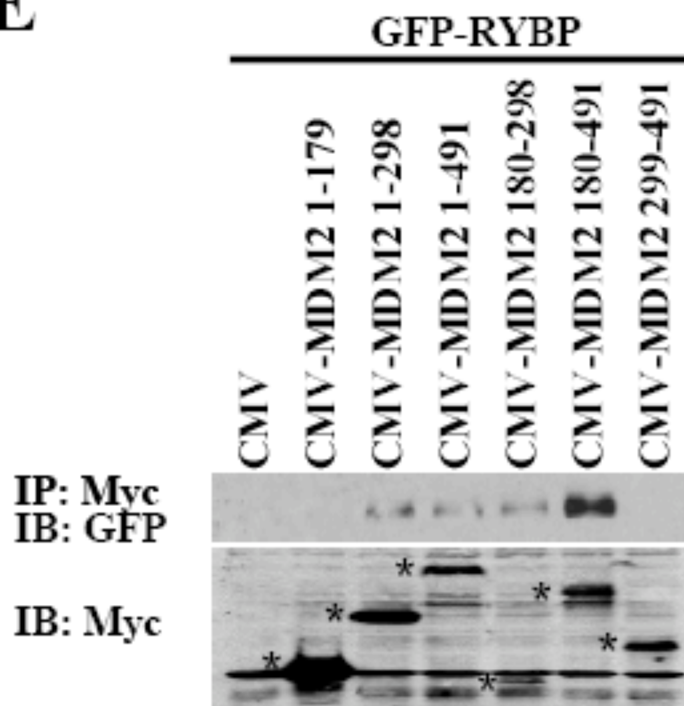
Fig S4 RYPB regulates p53 activity. (A and B) U2OS cells (A), or a pair of MCF7 cells ($p53^{+/+}$ and p53 KD) (B) were transfected with plasmids expressing increasing levels of Myc-RYBP, a *p21*^{Waf1} promoter luciferase reporter, and the Renilla luciferase vector. The luciferase activities were measured 24 h later. The results are presented as means ± SD of duplicate samples. * $p<0.05$ (upper panels of A and B). The expression levels of MDM2 and p53 in the cell lysates under the same transfection conditions are shown (lower panels of A and B). (C and D) U2OS cells were exposed to either doxorubicin (Dox) (C) or etoposide (D) at different doses for 24 h, or with the same dose for different times as indicated in the figures, then the expression of target proteins was evaluated.

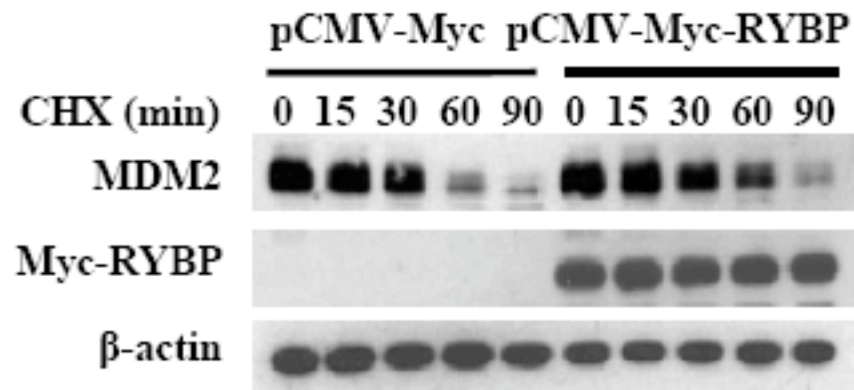

A
T7-MDM2
Myc-RYBP
Myc-RYBP & T7-MDM2
IP: T7
IB: T7
IP: T7
IB: Myc
IP: Myc
IB: T7
IP: Myc
IB: Myc
IB: T7
IB: Myc
T7-MDM2
Myc-RYBP

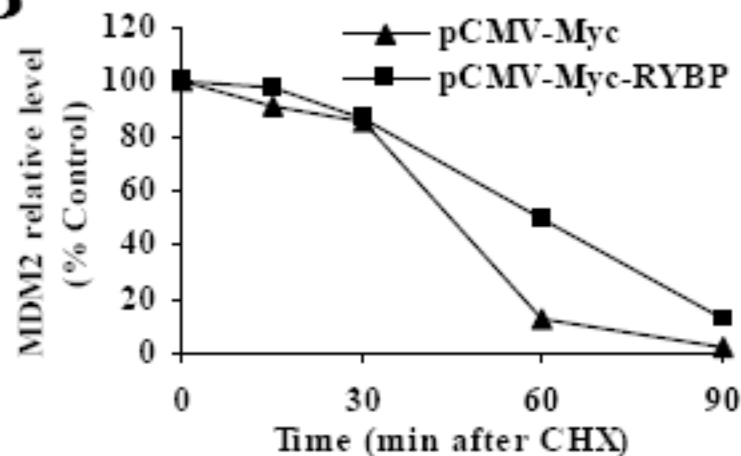

B
Binding to MDM2
1-228 +
1-73 +
1-143 +
74-143 -
74-228 +
144-228 +

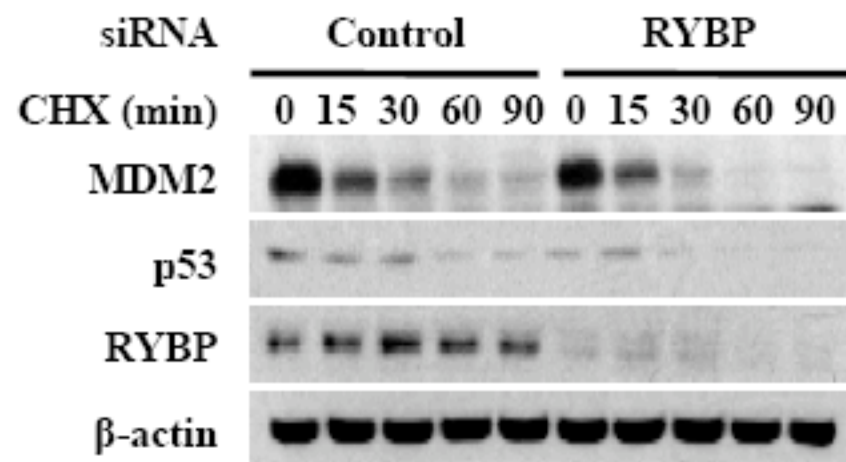

C
Binding to RYBP
1-491 +
1-179 -
1-298 +
180-298 +
180-491 +
299-491 -

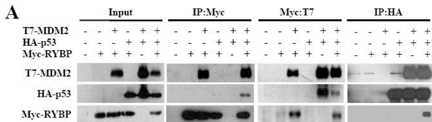

D
T7-MDM2
GFP
GFP-RYBP 1-73
GFP-RYBP 1-143
GFP-RYBP 1-228
GFP-RYBP 74-143
GFP-RYBP 74-228
GFP-RYBP 144-228
IP: GFP
IB: T7
IB: GFP

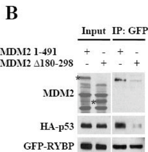

E
GFP-RYBP
CMV
CMV-MDM2 1-179
CMV-MDM2 1-298
CMV-MDM2 1-491
CMV-MDM2 180-298
CMV-MDM2 180-491
CMV-MDM2 299-491
IP: Myc
IB: GFP
IB: Myc

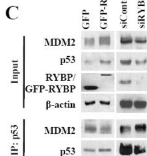

A
pCMV-Myc
pCMV-Myc-RYBP
CHX (min)
0 15 30 60 90 0 15 30 60 90
MDM2
Myc-RYBP
β-actin

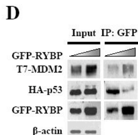

B
pCMV-Myc
pCMV-Myc-RYBP
MDM2 relative level
(% Control)
120
100
80
60
40
20
0
0
30
60
90
Time (min after CHX)

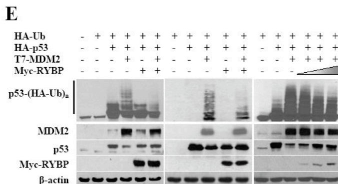

C
siRNA
Control
RYBP
CHX (min)
0 15 30 60 90 0 15 30 60 90
MDM2
p53
RYBP
β-actin

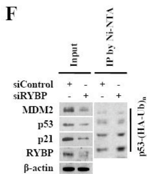

A
Input
IP:Myc
Myc:T7
IP:HA
T7-MDM2
HA-p53
Myc-RYBP
T7-MDM2
HA-p53
Myc-RYBP


B
Input
IP: GFP
MDM2 1-491
MDM2 Δ180-298
MDM2
HA-p53
GFP-RYBP


C
GFP
GFP-RYBP
siControl pool
siRYBP pool
Input
MDM2
p53
RYBP/
GFP-RYBP
β-actin
IP: p53
MDM2
p53


D
Input
IP: GFP
GFP-RYBP
T7-MDM2
HA-p53
GFP-RYBP
β-actin


E
HA-Ub
HA-p53
T7-MDM2
Myc-RYBP
p53-(HA-Ub)n
MDM2
p53
Myc-RYBP
β-actin


F
Input
IP by Ni-NTA
siControl
siRYBP
MDM2
p53
p21
RYBP
β-actin
p53-(HA-Ub)n

A

U2OS

RLU(% Control)

300
200
100
0

RYBP 0µg
RYBP 0.5µg
RYBP 1.0µg

*

RYBP (µg) 0 0.5 1.0
MDM2
p53
Myc-RYBP
β-actin

B

MCF7 p53 KD
MCF7 p53 $^{+/+}$

RLU (% Control)

300
200
100
0

RYBP 0µg
RYBP 0.5µg
RYBP 1.5µg

*

p53 wt
p53 KD
RYBP (µg) 0 0.5 1.0 0 0.5 1.0
MDM2
p53
Myc-RYBP
β-actin

C

Dox (nM)
0 10 50 100
100 nM Dox (h)
0 1 4 8 16 24
MDM2
p53
RYBP
β -actin

D

Etoposide (µM)
0 0.01 0.1 1.0 10
1.0 µM Etoposide (h)
0 1 4 8 16 24
MDM2
p53
RYBP
p21
β-actin