Supplemental Materials

Materials and methods

Real Time PCR. Total RNA was isolated from cells after different treatments, using the TRIzol (Invitrogen) protocol. Reverse transcription of 3 μg of total RNA was performed in a 20 μl reaction using SuperscriptII reverse transcriptase (Invitrogen) reagent, dNTP, and oligo(dT)15 primer. 1 μl of RT product was used in the real time PCR. A 15 μl reaction was performed using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol, and amplified on the Applied Biosystems 7300 Real-Time PCR system. Threshold cycles (*Ct*) for three replicate reactions were determined by SDS2. The relative transcript abundance was calculated following normalization with the GAPDH amplicon. The data were collected at 80 °C. The following primers were used: *p21* F, 5'-CTGGACTGTTTTCTCTCGGCTC-3', and R, 5'-TGTATATTCAGCATTGTGGGAGGA-3'; *gapdh* F, 5'-GATTCCACCCATGGCAAATTC-3', and R, 5'-AGCATCGCCCCACTTGATT-3'.

Immunofluorescent staining and fluorescent microscopic analysis. MEF $^{p53-/-, MDM2-/-}$ cells were fixed for immunofluorescent staining with monoclonal anti MDMX antibody (8C6) and the Alexa Fluor 546 (red) goat anti-mouse antibody (Molecular Probes, Oregon). DNA was stained by DAPI. Stained cells were analyzed under the Zeiss Axiovert 200M fluorescent microscope (Zeiss, Germany).

Figure legends

Fig. S1. The sub-celluar localization of endogenous MDMX and the effects of MDMX levels on p21 mRNA. (A) The cellular localization of endogenous MDMX in

MEF $^{p53-/-, MDM2-/-}$ cells. MEF $^{p53-/-, MDM2-/-}$ cells were fixed for immunofluorescent staining with monoclonal anti MDMX antibody (8C6) and the Alexa Fluor 546 (red) goat anti-mouse antibody (Molecular Probes, Oregon). DNA was stained by DAPI. One set of cells were pretreated with 0.2% Triton-X-100 for 3 seconds to remove the soluble cytoplasmic proteins. (**B**) Over-expressed MDMX does not alter the mRNA level of p21. H1299 cells were transfected as indicated. Total RNA was prepared from the cells 48 hours post transfection, using the TRIzol (Invitrogen) protocol. Reverse transcription of 3 μg of total RNA was performed in a 20- l reaction using SuperscriptII reverse transcriptase (Invitrogen) reagent, dNTP, and oligo(dT)15 primer. 1μl of RT product was used in the real time PCR. A 15- l reaction was performed using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol, and amplified on the Real-Time PCR system (Applied Biosystems 7300). The results were analyzed as mentioned in materials and methods. (**C**) Knowing-down of MDMX by siRNA marginally increases p21 mRNA. H1299 cells were transfected with scramble siRNA or siMDMX. The cells were harvested 48 hours post transfection. The total RNA preparation, reverse transcription and real-time PCR were performed as in (B). The results were analyzed as mentioned in materials and methods.

Fig. S2. MDMX binds p21 and reduces, instead of promotes, p21 ubiquitylation. (**A**) Endogenous p21 and MDMX bind to each other. H1299 cell lysate (400 μg) was immunoprecipitated with polyclonal anti-p21 antibody, or control IgG, and probed for p21 and MDMX, as shown on the left. * indicates the light chain of IgG. (**B**) Purified MDMX physically associates with p21 *in vitro*. The preparation of GST fusion protein beads and the purification of his-MDMX were previously described. His-MDMX (100 ng)

was incubated with beads conjugating 500 ng GST0 or GST-fused p21 or fragments in lysis buffer. 30 min after incubation at room temperature, the mixtures were washed with lysis buffer once, SNNTE buffer twice and lysis buffer again. The samples, together with 10% his-p21 input, were resolved by SDS-PAGE, followed by WB for p21. **(C) and (D) The increase in GFP-MDMX level reduces the ubiquitylation of p21 in cells.** H1299 cells (C) or MEF $^{p53-/-, MDMX-/-}$ cells (D) were transfected with a combination of plasmid encoding His6X -ubiquitin, human p21, or GFP-MDMX, as indicated. 1X and 2X respectively stand for 1 μg and 2 μg of plasmid encoding GFP-MDMX in the transfection. The cells were treated with 10 μM MG132 for 16 hours prior to harvest. Cell lysates were incubated with Ni-NTA beads and washed intensively for SDS-PAGE and WB analyses using antibodies against p21 or MDMX. The cell lysates (50 μg /sample) were used for straight WB, as indicated. 14-3-3γ was detected for loading control.

Fig. S3. The mapping of S2 interaction domain on MDMX. H1299 cells were transfected with V5-S2 alone or together with myc-tagged wild type or deletion mutants of MDMX. The cells were treated and lysed as in (S3A). The lysates were immunoprecipitated with monoclonal anti myc antibody (9E10), followed by WB analyses, as indicated on the right. * indicates the residue signal from the anti-V5 WB.

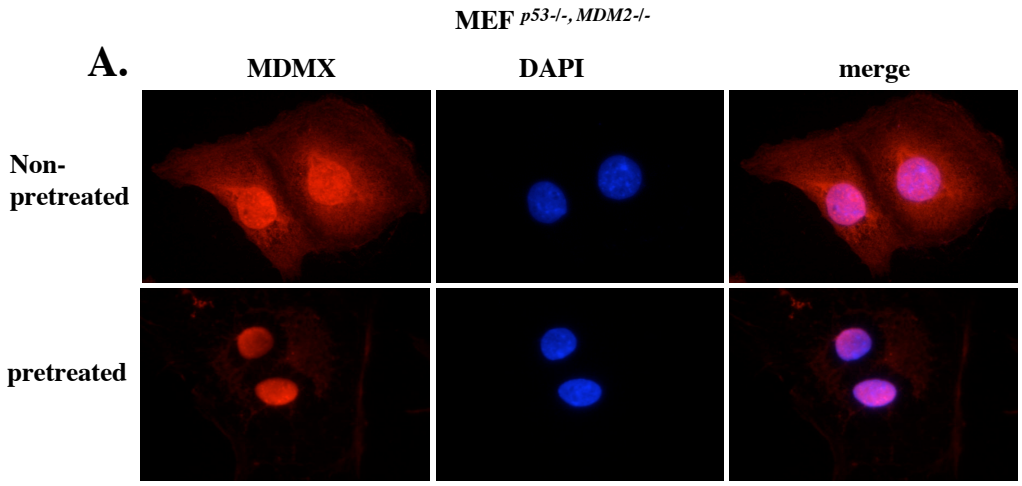
Fig. S4 p21 interplays with multiple subunits of 19S regulatory particles of the 26S proteasome and the lysine residues of p21 are dispensable for its interaction with S2. (**A**) p21 interacts with multiple subunits of 19S particle. H1299 cells were transiently transfected as indicated. 2 μg of each plasmid was used. The transfected cells were treated with 10 μM MG132 for 16 hours and harvested at 48 hours post transfection. The cell

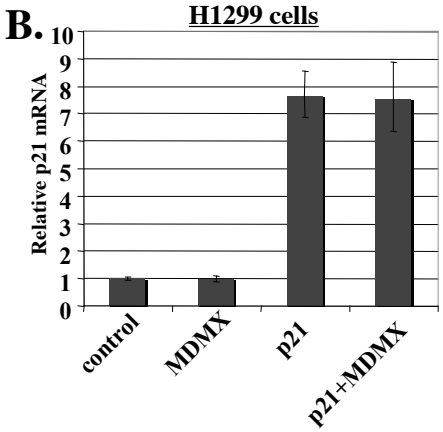
lysates (300 μg/sample) were immunoprecipitated with polyclonal anti-V5 antibody. The cell lysates (50 μg/sample) were also used for WB, as indicated on the right. (**B**) The lysine free mutant of p21 is able to associate with S2 in cells. H1299 cells were transiently transfected as indicated. 2 μg of each plasmid was used. The transfected cells were treated, lysed and immunoprecipitated, as in (A). WB analyses were indicated on the right.

Fig. S5 The molecular-weight distribution profiles of individually over-expressed p21, MDMX, and MDM2 after the size exclusion chromatography. H1299 cells were transfected with vectors as indicated on the left, and treated with 10 μM MG132 for 16 hours. The size exclusion chromatography was carried out as described in Figure 5A. The selected fractions (30 μl/fraction) were revolved by 12% SDS-PAGE followed by WB, as indicated on the right.

Fig. S6. MDM2 and MDMX alter p21 protein levels in G1/ early S phase of the cell cycle. (A) and (B) Depletion of MDMX or MDM2 by siRNA increases p21 protein level. The scramble siRNA, siMDMX or siMDM2 transfected H1299 cells were synchronized by a double-thymidine arrest and harvested at 0, 2, 4, 6, and 8 after the second release. The cell pellets were split to two aliquots: one for the cell cycle by FACS analysis (Fig7 D, E and F) and the other for WB to determine protein levels. Control (c) refers to non-synchronized cells. (C) The protein level of p21 is higher in MDMX or MDM2 null MEF cells. MEF cells with different genetic backgrounds of p53, MDMX or MDM2 were treated with a double-thymidine arrest and harvested at 0, 2, 4, 6, and 8 hours after the second release. 80 μg of protein per cell lysate were used for WB analyses, as shown on the right. Control (c) refers to non-synchronized cells.

Fig. S1


MEF p53-/-, MDM2-/-
A.
MDMX
DAPI
merge
Non-pretreated
pretreated


B.
H1299 cells
Relative p21 mRNA
0
1
2
3
4
5
6
7
8
9
10
control
MDMX
p21
p21+MDMX

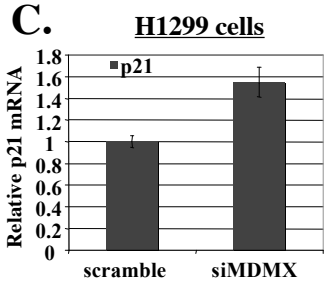

C.
H1299 cells
p21
Relative p21 mRNA
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
scramble
siMDMX

Fig. S2

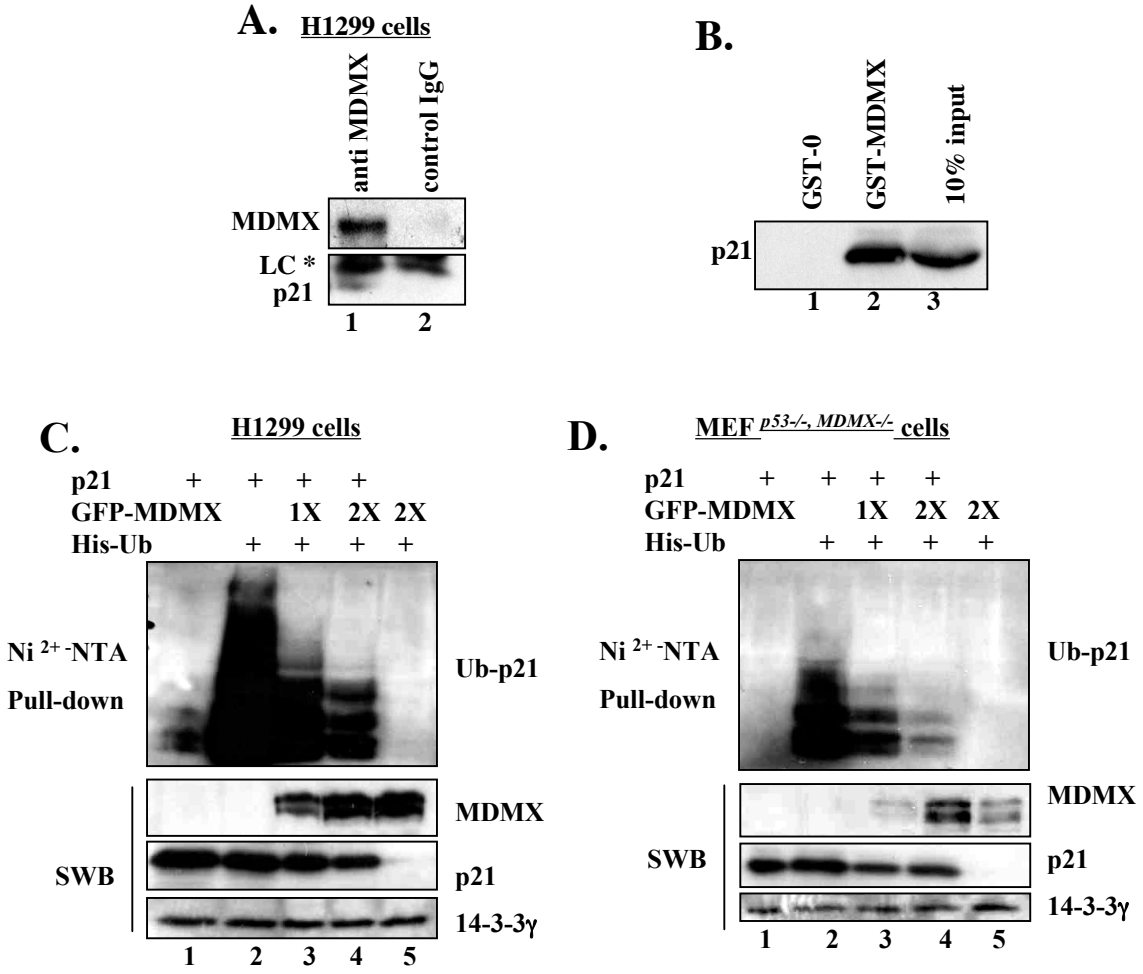

A.
H1299 cells
anti MDMX
control IgG
MDMX
LC *
p21
1
2
B.
GST-0
GST-MDMX
10% input
p21
1
2
3
C.
H1299 cells
p21 + + + +
GFP-MDMX 1X 2X 2X
His-Ub + + + +
Ni 2+ -NTA
Pull-down
Ub-p21
SWB
MDMX
p21
14-3-3γ
1
2
3
4
5
D.
MEF p53-/-, MDMX-/- cells
p21 + + + +
GFP-MDMX 1X 2X 2X
His-Ub + + + +
Ni 2+ -NTA
Pull-down
Ub-p21
SWB
MDMX
p21
14-3-3γ
1
2
3
4
5

Fig. S3

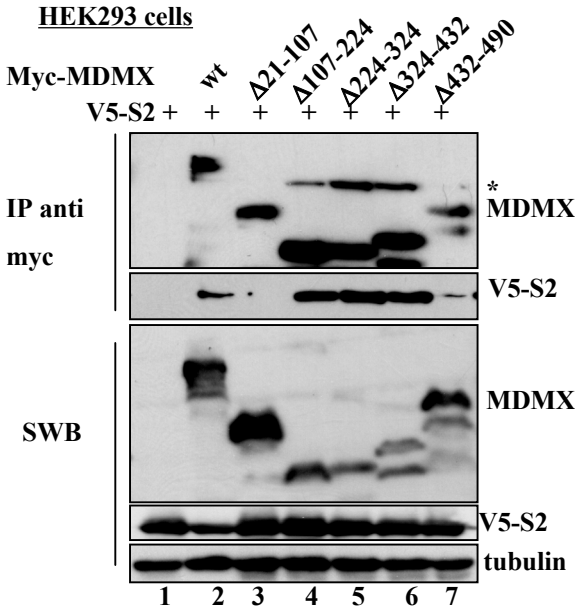
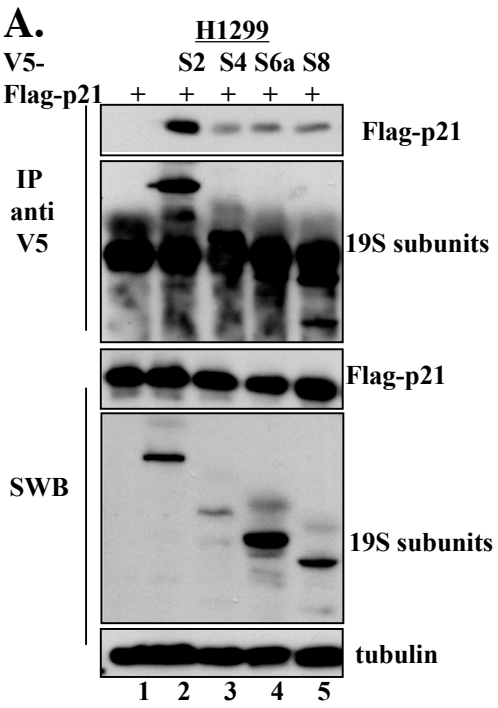

HEK293 cells
Myc-MDMX
wt
Δ21-107
Δ107-224
Δ224-324
Δ324-432
Δ432-490
V5-S2 + + + + + + +
IP anti myc
*
MDMX
V5-S2
SWB
MDMX
V5-S2
tubulin
1 2 3 4 5 6 7

Fig. S4


A.
H1299
V5-
S2 S4 S6a S8
Flag-p21 + + + + +
IP
anti
V5
Flag-p21
19S subunits
SWB
Flag-p21
19S subunits
tubulin
1 2 3 4 5

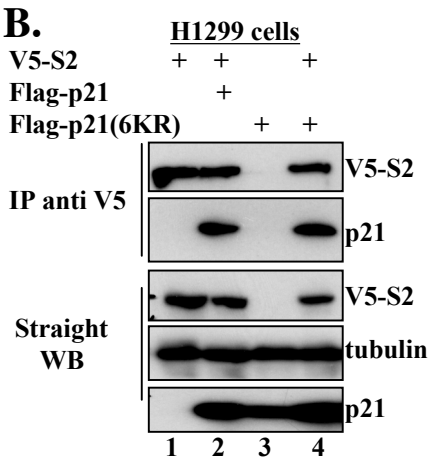

B.
H1299 cells
V5-S2 + + +
Flag-p21 +
Flag-p21(6KR) + +
IP anti V5
V5-S2
p21
Straight
WB
V5-S2
tubulin
p21
1 2 3 4

Fig. S5

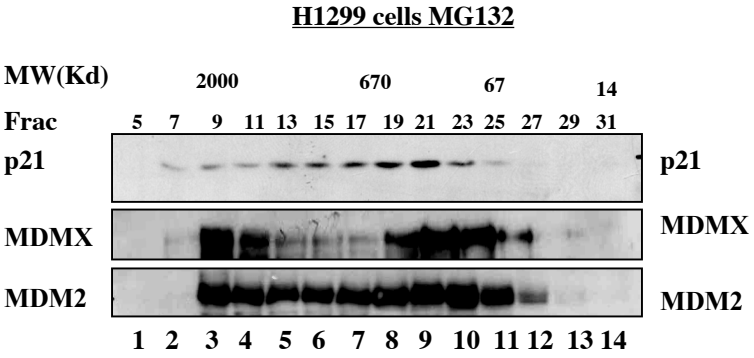
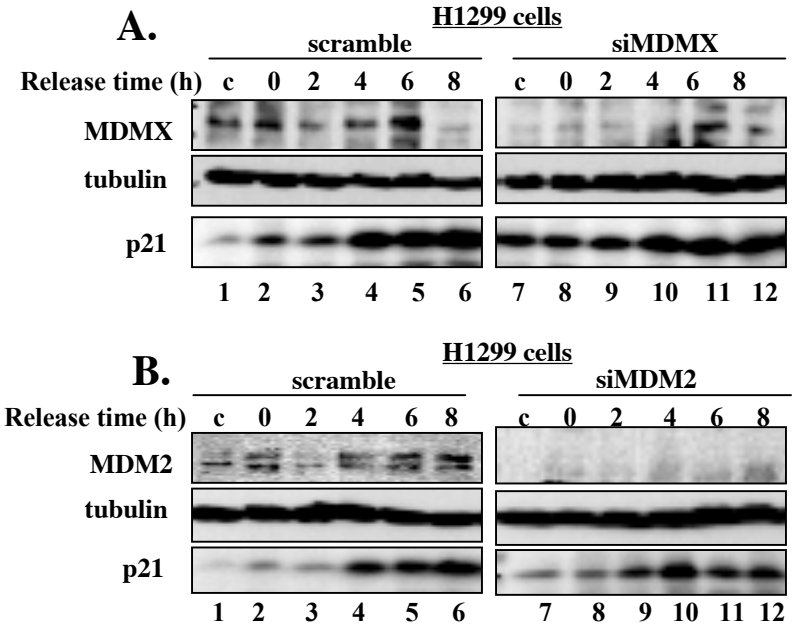
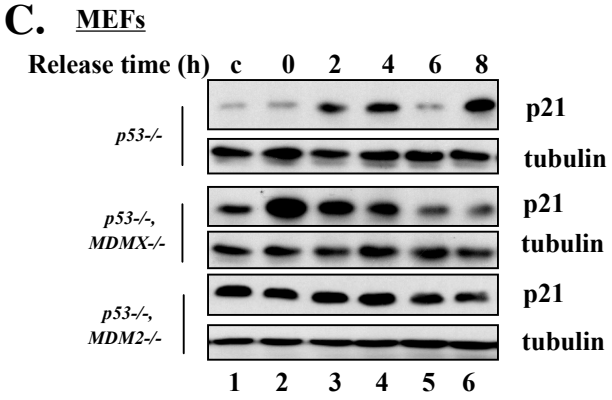

H1299 cells MG132
MW(Kd)
2000
670
67
14
Frac
5 7 9 11 13 15 17 19 21 23 25 27 29 31
p21
MDMX
MDM2
p21
MDMX
MDM2
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. S6


A.
H1299 cells
scramble
siMDMX
Release time (h) c 0 2 4 6 8 c 0 2 4 6 8
MDMX
tubulin
p21
1 2 3 4 5 6 7 8 9 10 11 12
B.
H1299 cells
scramble
siMDM2
Release time (h) c 0 2 4 6 8 c 0 2 4 6 8
MDM2
tubulin
p21
1 2 3 4 5 6 7 8 9 10 11 12


C. MEFs
Release time (h) c 0 2 4 6 8
p53-/-
p21
tubulin
p53-/-, MDMX-/-
p21
tubulin
p53-/-, MDM2-/-
p21
tubulin
1 2 3 4 5 6