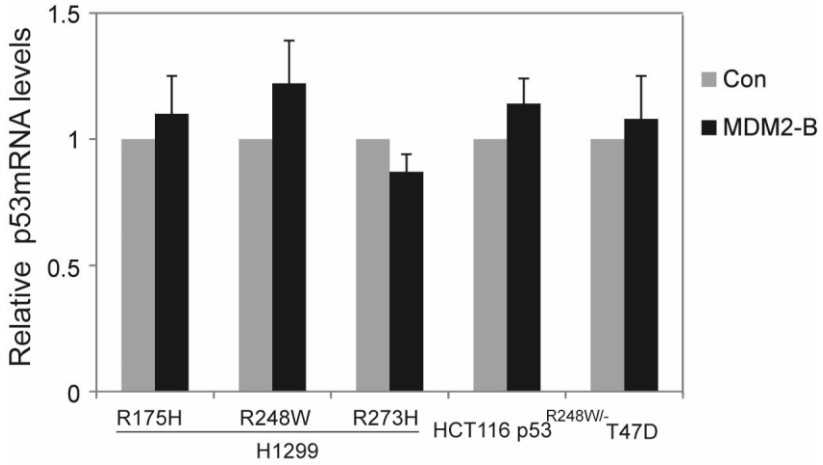
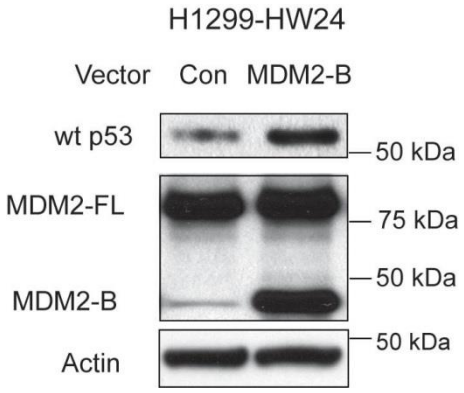
Supplementary Information

Spliced MDM2 isoforms promote mutant p53 accumulation and gain-of-function in tumorigenesis

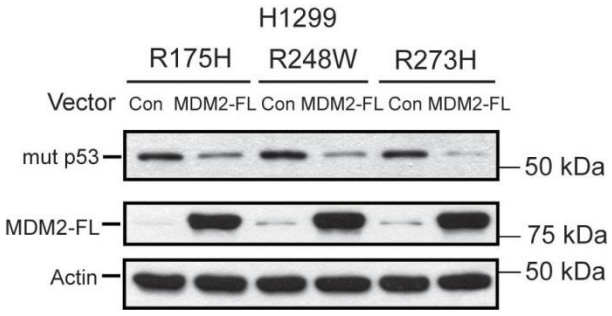
Tongsen Zheng, Jiabei Wang, Yuhan Zhao, Cen Zhang, Meihua Lin, Xiaowen Wang, Haiyang Yu, Lianxin Liu, Zhaohui Feng, Wenwei Hu



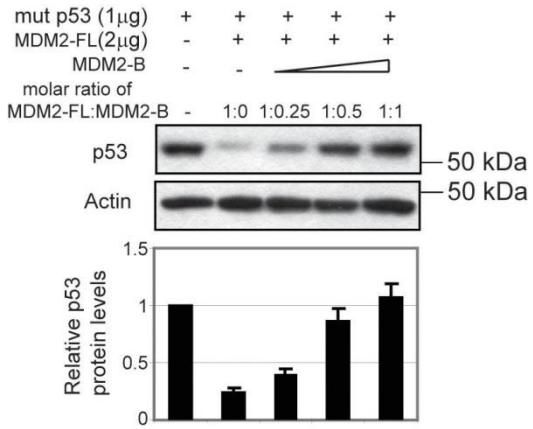
Supplementary Figure S1. MDM2-B does not change the mRNA levels of mutp53 in cells. Ectopic expression of MDM2-B-Flag by transfection of MDM2-B-Flag expression vectors (MDM2-B) did not have an obvious effect on the mRNA levels of mutp53 in H1299 cells with stable ectopic expression of mutp53 (R175H, R248W and R273H), HCT116 $p53^{R248w/-}$, and T47D cells as determined by Taqman real-time PCR. Cells were transfected with control vector (Con) or MDM2-B-Flag expression vector 24 h before assays. The mRNA levels of mutp53 were normalized with actin. Data are presented as mean ± SD (n=3).



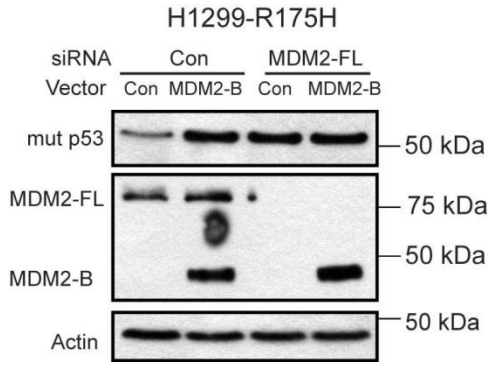
Supplementary Figure S2. MDM2-B promotes wtp53 protein accumulation in H1299-HW24 cells. H1299-HW24 cells expresses wtp53 under a tetracycline-regulated promoter. Wtp53 was expressed when cells were cultured in the presence of 5 ng/ml tetracycline. H1299-HW24 cells cultured in the presence of 5 ng/ml tetracycline were transfected with control (Con) or MDM2-B-flag (MDM2-B) expression vectors, and the protein levels of p53, MDM2-FL and MDM2-B were determined by western-blot assays.



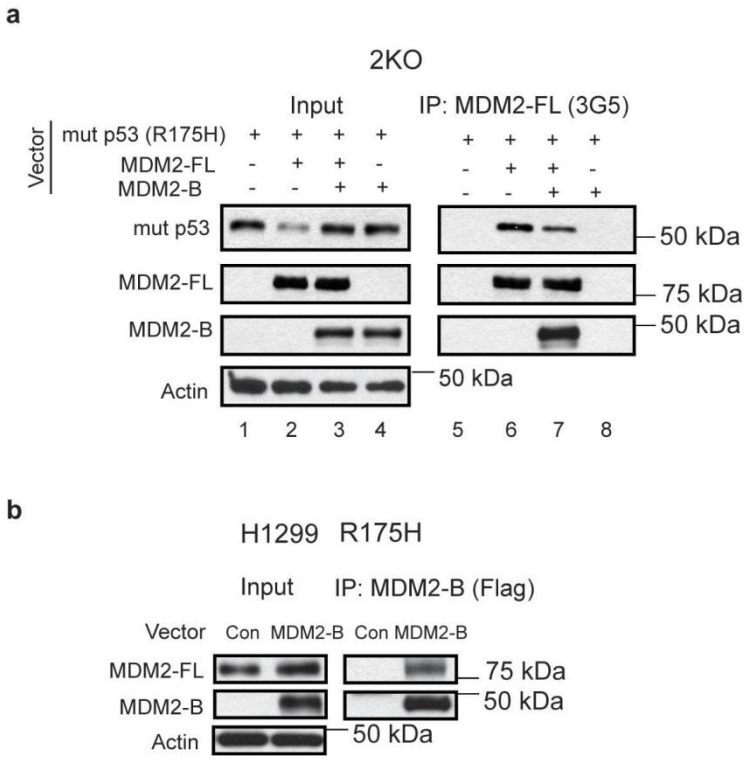
Supplementary Figure S3. MDM2-FL negatively regulates mutp53 protein in H1299 cells. Ectopic expression of MDM2-FL by transfection of a MDM2-FL expression vector down-regulated mutp53 protein levels in H1299 cells with stable ectopic expression of mutp53 (R175H, R248W and R273H). Cells were transfected with control vectors (Con) or MDM2-FL expression vectors at 24 h before assays.



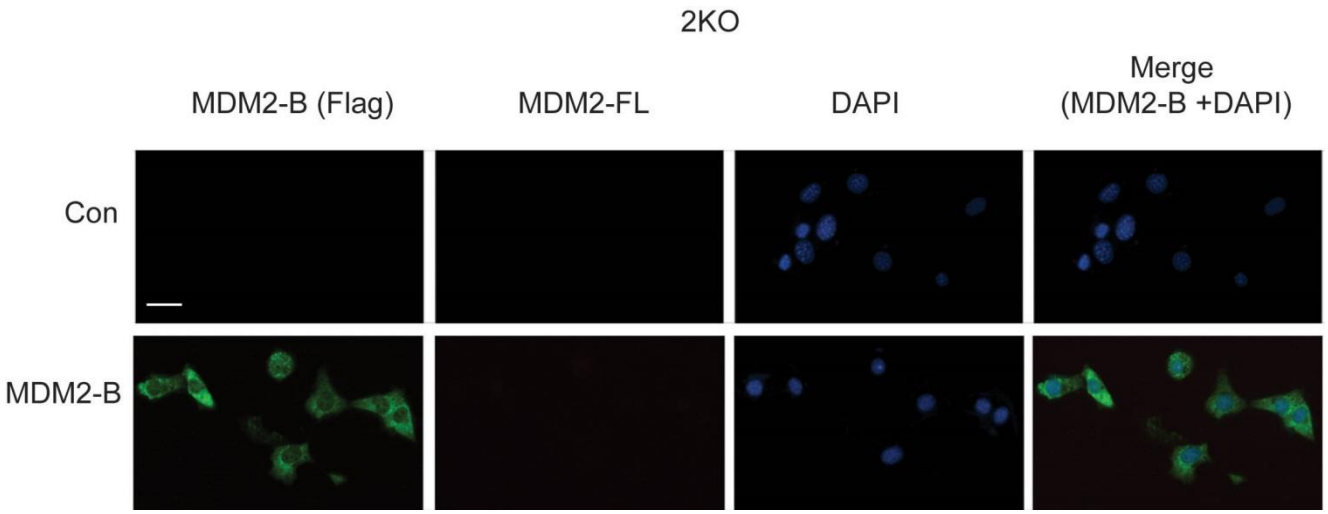
Supplementary Figure S4. MDM2-B inhibits MDM2-FL-mediated degradation of mutp53 protein in 2KO cells. Indicated combination of expression vectors of MDM2-B-Flag (MDM2-B), MDM2-FL and mutp53 (R248W) were co-transfected into 2KO cells. The inhibitory effect of MDM2-B on MDM2-FL-mediated degradation of mutp53 was observed when the molar ratio of MDM2-FL *vs.* MDM2-B-Flag is 1:0.25, and reached 90% when the ration of MDM2-FL *vs.* MDM2-B-Flag is 1:0.5. Data are presented as mean ± SD (n=3).



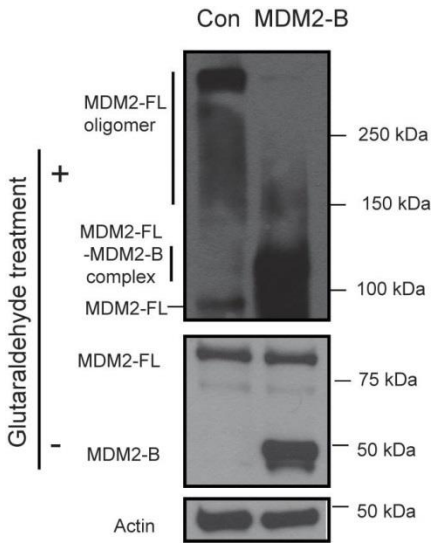
Supplementary Figure S5. MDM2-B increases mutp53 protein levels through the inhibition of MDM2-FL function in H1299-R175H cells. Ectopic expression of MDM2-B-Flag (MDM2-B) increased mutp53 protein levels in H1299 cells with stable ectopic expression of mutp53 (R175H) (H1299-R175H), but not in H1299-R175H cells with knockdown of endogenous MDM2-FL by siRNA. Con: Control. Two different siRNA oligos against MDM2-FL were used, and similar results were obtained.



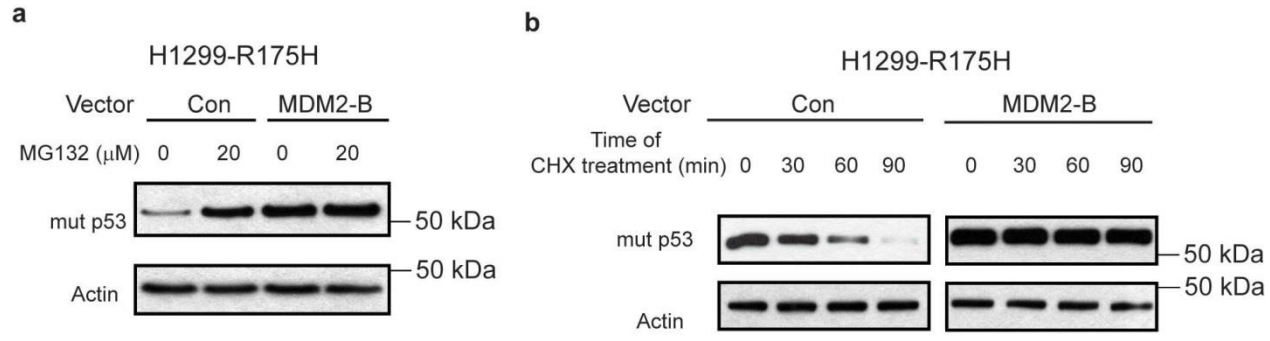
Supplementary Figure S6. MDM2-B interacts with MDM2-FL and blocks the binding of MDM2-FL to mutp53 (R175H). a. 2KO cells were transfected with expression vectors of mutp53 (R175H), MDM2-FL and/or MDM2-B-Flag (MDM2-B). Cell lysates were immunoprecipitated with 3G5, a MDM2 antibody recognizing MDM2-FL but not MDM2-B, and analyzed by Western-blot assays for indicated proteins. **b.** H1299-R175H cells were transfected with control (Con) or MDM2-B-Flag (MDM2-B) expression vectors. Cell lysates were immunoprecipitated with an anti-Flag antibody, and analyzed by western-blot assays for indicated proteins.



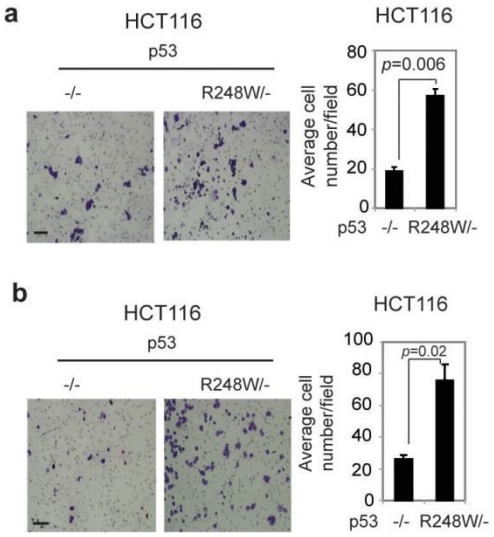
Supplementary Figure S7. Subcellular localization of MDM2-B in 2KO cells. 2KO cells were transfected with control (Con) or MDM2-B-Flag (MDM2-B) expression vectors. MDM2-B was predominantly localized in cytoplasm in 2KO cells as determined by IF staining. Antibodies used for IF: 3G5 for MDM2-FL; Flag for MDM2-B-Flag. Nuclei were stained with DAPI. Scale bar: 20 μm.



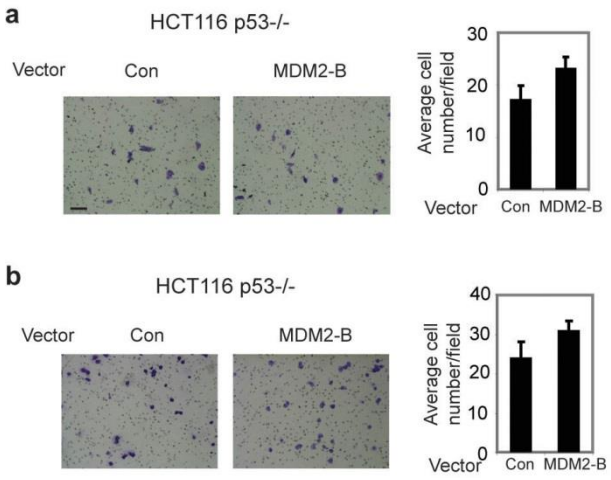
Supplementary Figure S8. MDM2-B reduces the oligomerization of MDM2-FL in HCT116 p53$^{R248w/-}$ cells. Top panel: HCT116 p53$^{R248w/-}$ cells with and without ectopic expression of MDM2-B were analyzed for oligomerization status of MDM2-FL by glutaraldehyde (0.02%) cross-linking and Western-blot assays using 3G5 antibody which specifically detects MDM2-FL but not MDM2-B. Middle and lower panel: MDM2-FL, MDM2-B and actin protein levels were determined by Western-blot assays using antibodies against MDM2 (2A10 for MDM2-FL and MDM2-B) and actin, respectively, in cells without glutaraldehyde cross-linking treatment.



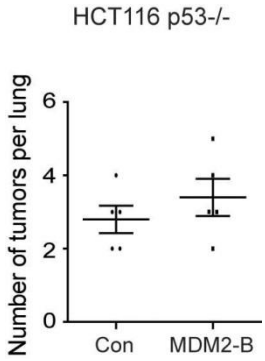
Supplementary Figure S9. MDM2-B decreases the degradation of mutp53 (R175H) and increases the half-life of mutp53 (R175H) in H1299-R175H cells. a. Blocking proteasomal degradation by MG132 increased mutp53 levels in H1299-R175H but not in H1299-R175H with ectopic expression of MDM2-B-Flag (MDM2-B). Con: Control. **b.** MDM2-B increased the half-life of mutp53 protein (R175H). H1299-R175 cells with and without ectopic expression of MDM2-B-Flag were treated with cycloheximide (CHX, 10 μM), and the mutp53 protein levels were determined at indicated time points by Western-blot assays.



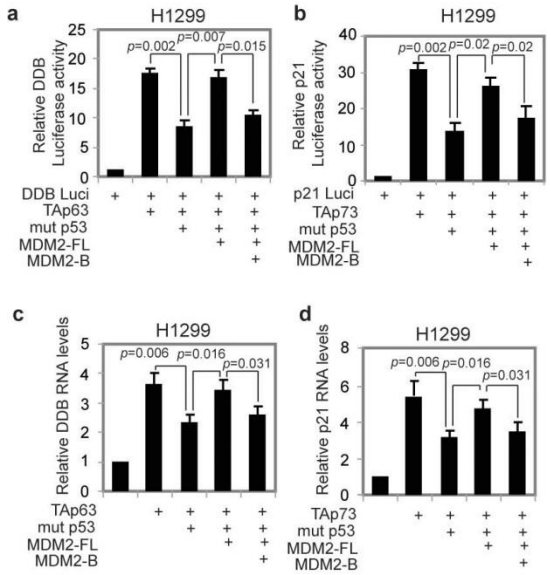
Supplementary Figure S10. Mutp53 promotes invasion and migration in HCT116 cells. The impact of mutp53 upon invasion (**a**) and migration (**b**) abilities was determined in HCT116 cells by trans-well assays. HCT116 p53-/- and HCT116 $p53^{R248w/-}$cells were seeded into the upper chambers with matrigel coating (for invasion) or without matrigel (for migration) and cultured at 37°C for 24 h. Cells on the lower surface of the upper chamber were counted. **a.** Mutp53 promoted invasion in HCT116 cells. HCT116 $p53^{R248w/-}$ cells displayed higher invasion ability compared with HCT116 p53-/- cells. **b.** Mutp53 promoted migration in HCT116 cells. HCT116 $p53^{R248w/-}$ cells displayed higher migration ability than HCT116 p53-/- cells. Data are presented as mean ± SD (n=3). p value was obtained by using student *t*-test. Scale bar in **a & b**: 50 µm



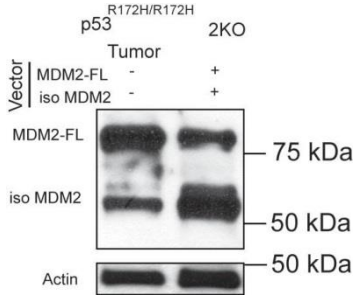
Supplementary Figure S11. The promoting effect of MDM2-B on invasion and migration is largely abolished in HCT116 p53-/- cells. The impacts of MDM2-B upon the abilities of invasion (**a**) and migration (**b**) were determined in HCT116 p53-/- cells by trans-well assays. HCT116 p53-/- cells with or without ectopic expression of MDM2-B-Flag (MDM2-B) were seeded into the upper chambers with matrigel coating (for invasion) or without matrigel (for migration) and cultured at 37°C for 24 h. Cells on the lower surface of the upper chamber were counted. Ectopic expression of MDM2-B-Flag did not significantly promote invasion (**a**) and migration (**b**) of HCT116 p53-/- cells. Con: Control. Data are presented as mean ± SD (n=3). Scale bar: 50 μm.



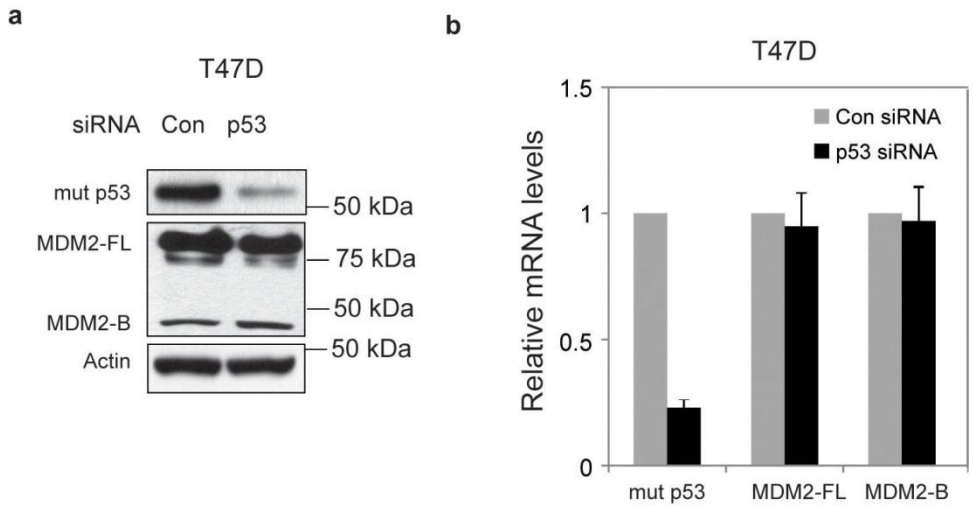
Supplementary Figure S12. The promoting effect of MDM2-B on metastasis is largely abolished in HCT116 p53-/- *in vivo*. HCT116 p53-/- cells with or without stable ectopic expression of MDM2-B-Flag (MDM2-B) were injected into nude mice *via* tail vein and the number of lung metastatic tumors was determined after 6 weeks. Con: Control. n=6/group. Data are presented as mean ± SD.



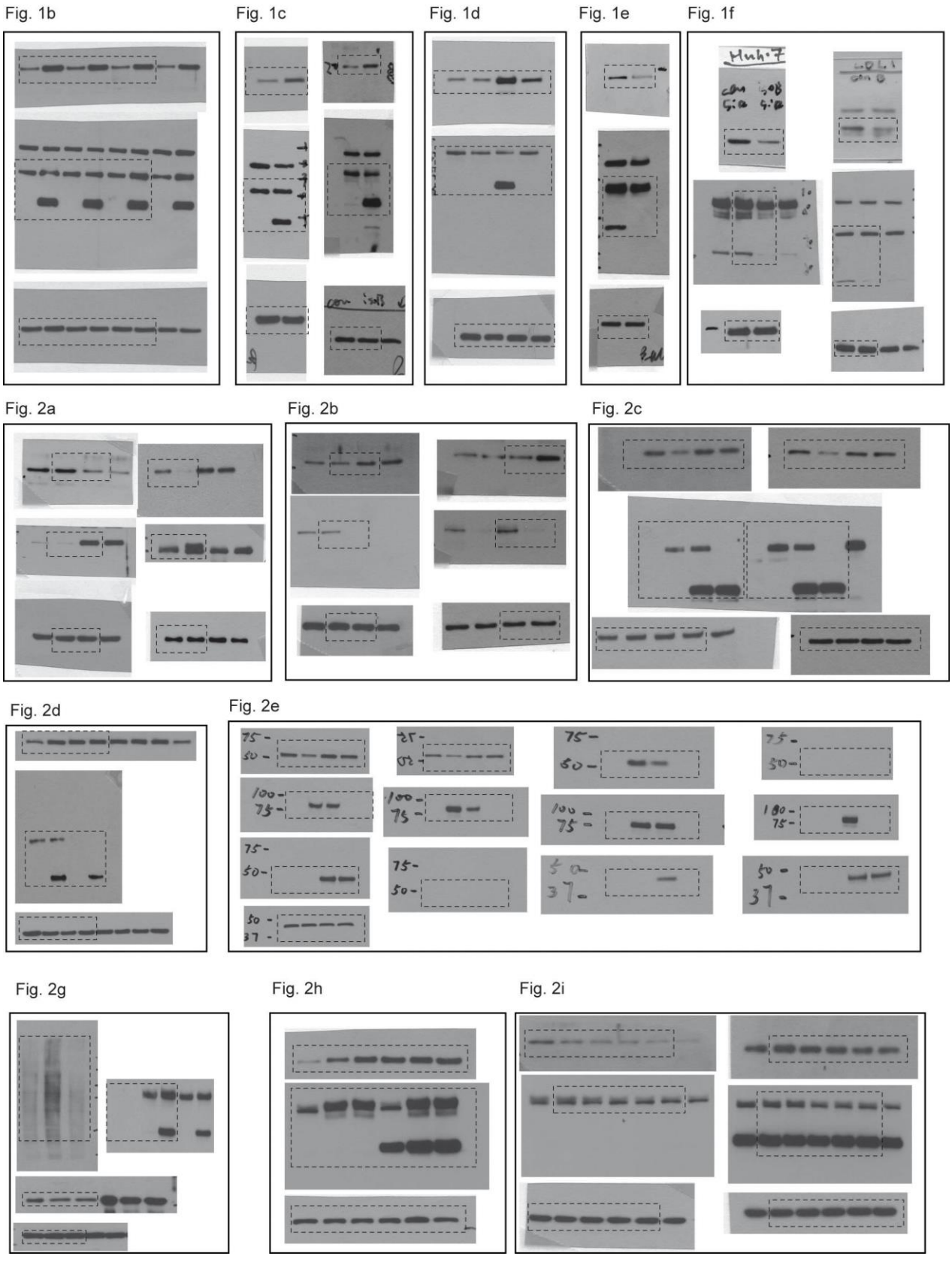
Supplementary Figure S13. MDM2-B increases the inhibitory effect of mutp53 on the transcriptional activities of TAp63 and TAp73 through inhibition of MDM2-FL function. a&b. MDM2-B increased the inhibitory effect of mutp53 on the transcriptional activities of TAp63 (**a**) and TAp73 (**b**) through inhibition of MDM2-FL function as determined by the luciferase activity assays. H1299 cells were transiently co-transfected with the indicated combination of expression vectors of TAp63 (**a**) or TAp73(**b**), mutp53 (R248W), MDM2-FL, and MDM2-B-Flag (MDM2-B) along with a luciferase (Luci) reporter vector containing p53-responsive DNA binding element of the DDB2 promoter which can be transactivated by p63 (**a**) or the p21 promoter which can be transactivated by p73 (**b**). pRL-SV40 vectors were co-transfected as an internal standard. The luciferase activity was normalized to the activity of Renilla luciferase expressed by pRL-SV40. Data are presented as mean ± SD (n=3). **c&d.** MDM2-B increased the inhibitory effect of mutp53 on the transcriptional activities of TAp63 (**c**) and TAp73 (**d**) through inhibition of MDM2-FL function as determined by Taqman real-time PCR. H1299 cells were transiently co-transfected with the indicated combination of expression vectors of TAp63 (**c**) or TAp73(**d**), mutp53 (R248W), MDM2-FL, and MDM2-B-Flag. The mRNA levels of endogenous DDB2 (**c**) and p21 (**d**) were determined by Taqman real-time PCR and normalized with actin. Data are presented as mean ± SD (n=3). p value was obtained by student *t*-test.



Supplementary Figure S14. Ectopic expression of the mouse MDM2-FL and MDM2 isoform in 2KO cells. 2KO cells were transiently transfected with expression vectors of MDM2-FL and the MDM2 isoform over-expressed in tumors from $p53^{R172H/R172H}$ mice. Ectopic expression of the mouse MDM2 isoform (right lane) was confirmed at the protein levels by Western-blot assays. The mouse MDM2 isoform expressed a protein similar to the protein overexpressed in tumors from $p53^{R172H/R172H}$ mice (left lane) at the protein size.



Supplementary Figure S15. Mutp53 does not regulate MDM2-B expression levels in T47D cells. Knockdown of endogenous mutp53 in T47D cells by siRNA did not have an obvious effect on the expression levels of MDM2-B mRNA and protein as determined by Taqman real-time PCR and Western-blot assays, respectively. Two different siRNA oligos were used and similar results were obtained. Data are presented as mean ± SD (n=3).



Supplementary Figure S16. Full scans of Western-blot data shown in Figures 1&2. Rectangles delimit cropped areas used in the indicated panels in Figures 1 & 2.

Fig. 3c

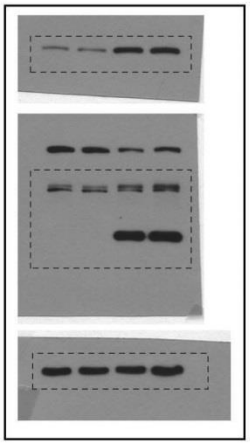


Fig. 4a

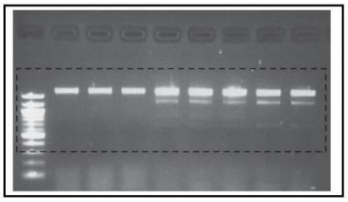


Fig. 4e

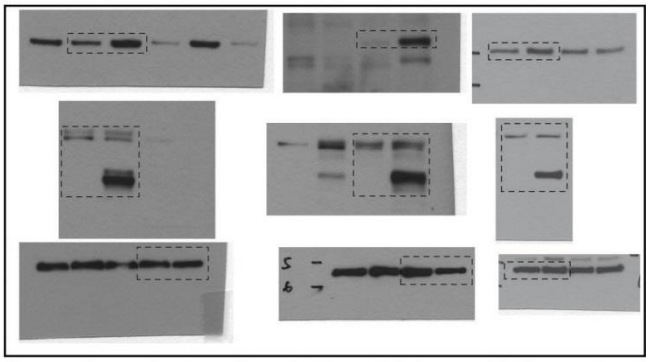


Fig. 4c

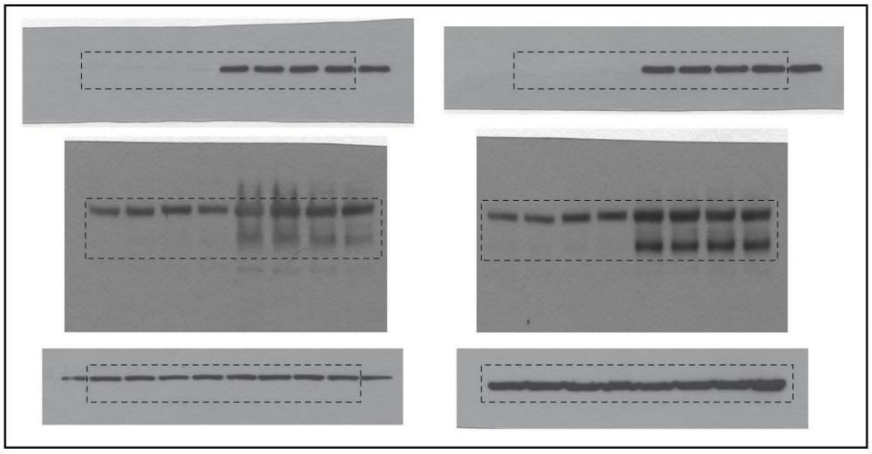


Fig. 4f

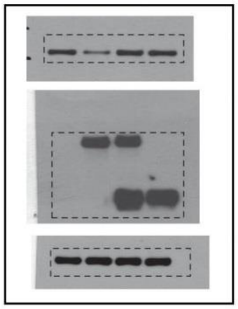
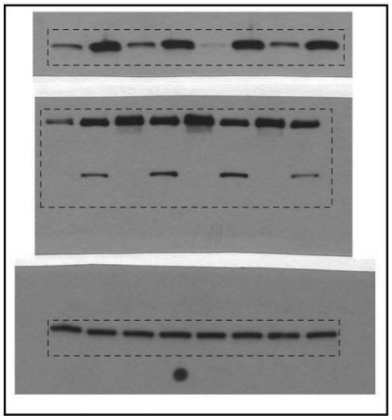
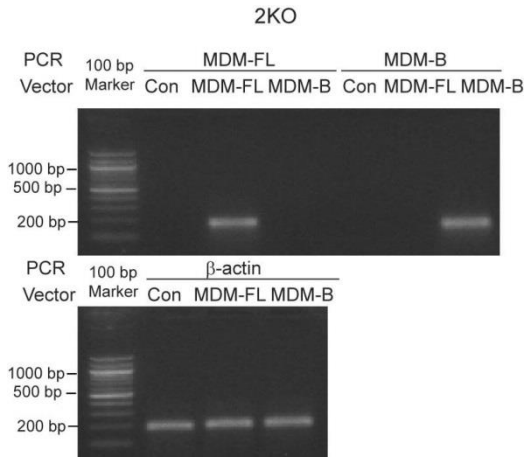


Fig. 5b



Supplementary Figure S17. Full gel image of PCR and full scans of Western-blot data shown in Figures 3-5. Rectangles delimit cropped areas used in the indicated panels in Figures 3-5.



Supplementary Figure S18. The specificity of MDM2-B and MDM2-FL primers were confirmed in 2KO cells by agarose gel electrophoresis. 2KO cells were transiently transfected with expression vectors of control (Con), MDM2-FL or MDM2-B-Flag (MDM2-B). The mRNA expression levels of MDM2-B, MDM2-FL and β-actin were determined by Taqman real-time PCR analysis. The PCR products were analyzed by agarose gel electrophoresis.

Supplementary Table S1. The sequences of the primers used for p53 mutation analysis

Exon	Primer
2-3	Forward: 5’ TCTCATGCTGGATCCCCACT 3’ Reverse: 5’ AGTCAGAGGACCAGGTCCTC 3’
4	Forward:5’CCTGGTCCTCTGACTGCTCTTTTCACCCA 3’ Reverse: 5’ GGCCAGGCATTGAAGTCTCAT 3’
5	Forward: 5’ CAACTCTGTCTCCTTCCT 3’ Reverse: TGTCGTCTCTCCAGCCCC 3’
6	Forward: 5’ CAGGCCTCTGATTCCTCACT 3’ Reverse: CAGTTGCAAACCAGACCTCA 3’
7	Forward: 5’ CCTCATCTTGGGCCTGTGTT 3’ Reverse: 5’ ATGGTGCAGGGTGGCAAGTG 3’
8	Forward: 5’ CCTTACTGCCTCTTGCTTCT 3’ Reverse: 5’ ATAACTGCACCCTTGGTCTC 3’
9	Forward: 5’ GGAGACCAAGGGTGCAGTTATGCCTCAG 3’ Reverse: 5’ CCCAATTGCAGGTAAAACAG 3’
10	Forward: 5’ CAATTGTAACTTGAACCATC 3’ Reverse: 5’ GGATGAGAATGGAATCCTAT 3’
11	Forward: 5’ AGACCCTCTCACTCATGTGA 3’ Reverse: 5’ TGACGCACACCTATTGCAAG 3’