Supporting Information

Batuello et al. 10.1073/pnas.1416656112

SI Methods

Cell Culture. Mammalian cells H1299 (p53 null), 239T, MDA231, T47D (mutant p53), and MCF7 and U87 (wild-type p53) were cultured as described (1). Transfection in H1299 cells was performed using the calcium phosphate method and in MCF7 cells using Lipofectamine Plus reagent (Invitrogen). Equal amounts of DNA were transfected into cells and incubated for the indicated times before analysis. PP1 (Calbiochem) and MLN4924 (LifeSensors) were used at concentrations given. Site-directed mutagenesis was performed by PCR, and constructs were sequenced in their entirety. For the luciferase assays, H1299 and MCF7 cells were transfected with PG13-Luc, Mdm2-P2-Luc, Maspin-Luc, or Mutant Maspin-Luc (MT1) along with Myc-LacZ for the determination of β-galactosidase activity, using the calcium phosphate method. Reporter activity was normalized to β-galactosidase activity. Experiments were done in triplicate, and SD was calculated from the mean.

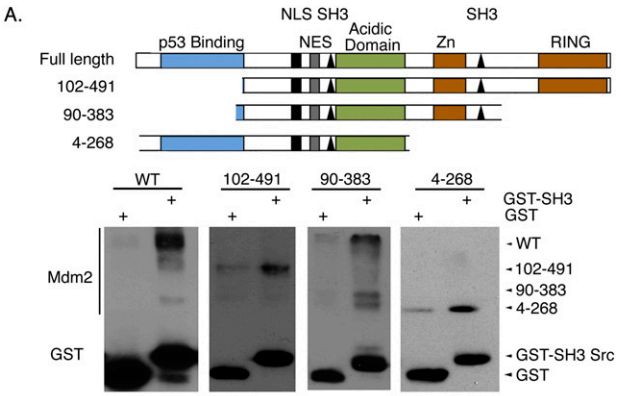
Protein Analysis. Debris was collected by centrifugation, and equal amounts of supernatant protein were determined by BioRad assay. Protein was either used for immunoprecipitation or fractionated by SDS/PAGE and transferred to PVDF membrane (Amersham Biosciences). Antibodies used for immunoblotting were Mdm2 (SMP14, 2A10, and 4B11) c-Src (B_{12}; Santa Cruz), p53 (DO-1), tubulin (TU-02), GAPDH (8C2), and β-actin (C4; Santa Cruz Biotechnology); phosphotyrosine (4G10; Upstate); phospho-c-Src Tyr^{416} (Cell Signaling); Myc (9E10); and Ubc12 (GeneTex) and HA (12CA5). Immunoprecipitation from lysates was performed with SMP14 for Mdm2, HA (12CA5), p53 (DO1), Ubc12, or c-Src (B_{12}) antibody overnight at 4 °C in 700 μL PBS or lysis buffer. Precipitates were washed three times in PBS or lysis buffer and prepared for SDS/PAGE and immunoblot analysis.

For analysis by mass spectrometry, T47D or MDA 231 cells were grown on six 150-mm plates to ∼80% confluency and harvested. Mdm2 was purified from cell lysate by immunoprecipitation and resolved on an 8% SDS page gel. The band was stained by Coomassie brilliant blue and then excised, destained, and digested with GluC. The polypeptides were injected into a C18 column and eluted with a linear gradient of acetonitrile. The effluent was electro-sprayed into the LTQ mass spectrometer. Analysis was performed using neutral loss scan function and SEQUEST algorithm.

In Vitro Reactions. All recombinant proteins were produced and purified from BL21DE3 cells. Proteins were bound to nickel beads in buffer A (25 mM Hepes, 0.2% Triton X-100, 5 mM DTT, 1 M KCL) and then washed extensively in buffer B (buffer A + 10 mM imidazole). Proteins were eluted in buffer C (buffer A + 300 mM imidazole) and dialyzed into buffer D [50 mM Hepes at pH 7.5, 100 mM NaCl, 10% (vol/vol) glycerol, 1 mM DTT]. Recombinant GST-tagged proteins were purified over a 2-mL glutathione column, as previously described (1). In vitro Src and Abl kinase reactions were as described (2), using 0.5 μg Src (Calbiochem) or Abl (Invitrogen). For in vitro ubiquitination assays, 500 ng p53 was incubated with 50 ng E1 (Boston Biochem), 200 ng Ubch5a (Boston Biochem), and 1 μg ubiquitin (Boston Biochem) in the presence of 500 ng phosphorylated or unphosphorylated Mdm2. Reactions were performed for 2 h at 37 °C.

His-Ubiquitin and His-Nedd8 Pull-Downs. H1299 cells were transfected with His-Ubiquitin or His-Nedd8 and other plasmids, as indicated, using calcium phosphate method. Forty-eight hours after transfection, cells were lysed in 1 mL of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris–HCl at pH 8.0 plus 5 mM imidazole and 10 mM β-mercaptoethanol. After sonication, the lysates were mixed with 30 μL Ni^{2+}-NTA-agarose beads (Qiagen), prewashed with lysis buffer and incubated for 2 h at room temperature. The beads were washed and eluted as previously described (3).

1. Lehman JA, et al. (2011) Induction of apoptotic genes by a p73-phosphatase and tensin homolog (p73-PTEN) protein complex in response to genotoxic stress. *J Biol Chem* 286 (42):36631–36640.
2. Waning DL, Lehman JA, Batuello CN, Mayo LD (2011) c-Abl phosphorylation of Mdm2 facilitates Mdm2-Mdmx complex formation. *J Biol Chem* 286(1):216–222.
3. Rodriguez MS, et al. (1999) SUMO-1 modification activates the transcriptional response of p53. *EMBO J* 18(22):6455–6461.



B. T47D

Seq	b^{-2}	Seq	y^{-2}	y^{-3}
P		E		
E		N		699.897
I		C		
S		S		
L		T		596.181
A		C	837.226	
D		K		
phos-Y		W		
W		phos-Y		
K	633.283	D		
C		A		
T		L		
S		S		
C		I		
N	1024.342	E		
E		P		

MDA 231

Seq	b^{+}	b^{+2}
phos-Y		
W	430.116	
K		
C		
T		
S		
C		
N		671.159
E		

Fig. S1. (*A*) Schematic of Mdm2 truncation mutants is shown of Mdm2 (*Top*). GST pull-down assays of GST or GST-SH3 Src were used to map which domain of Mdm2 interacted with Src. An immunoblot was probed with antibodies to Mdm2 and GST (*Bottom*). (*B*) Mdm2 was immunopurified from T47D and MDA 231 cells for mass spectrometry analysis. *y* and *b* ion fragmentation data and the respective mass is shown.

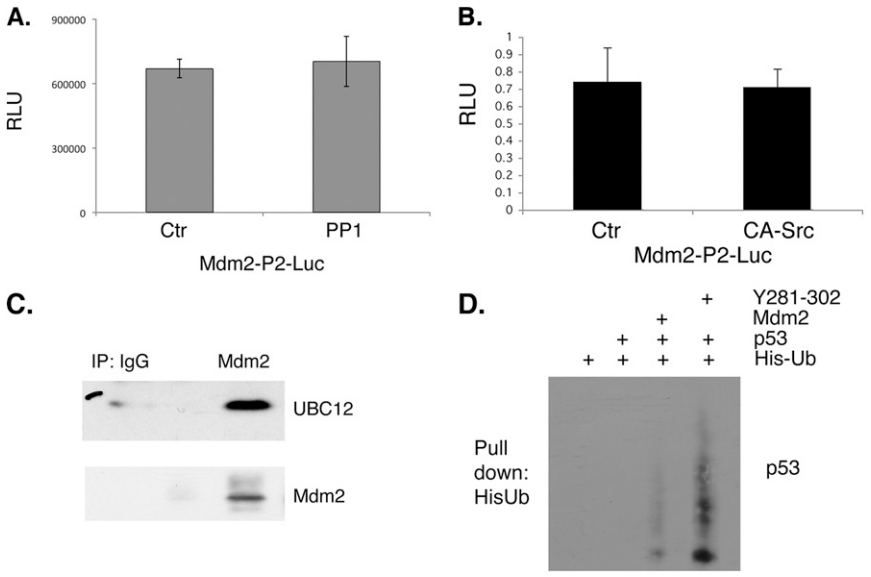


Fig. S2. (*A*) Luciferase activity of the *mdm2*-P2-Luc with Myc-LacZ in MCF7 cells after treatment with 10 μM PP1 or DMSO for 16 h. (*B*) Luciferase assay of the *mdm2*-P2-Luc with Myc-LacZ in H1299 cells alone or expressing CA-Src. (*C*) Immunoblot of Ubc12 (*Top*) or Mdm2 (*Bottom*) of immunoprecipitated, endogenous Mdm2 from MCF7 cells. (*D*) Immunoblot of p53 from a His-pull down assay conducted with lysates isolated from H1299 cells expressing His-Ub, p53, Mdm2, and/or Mdm2-Y281-302.

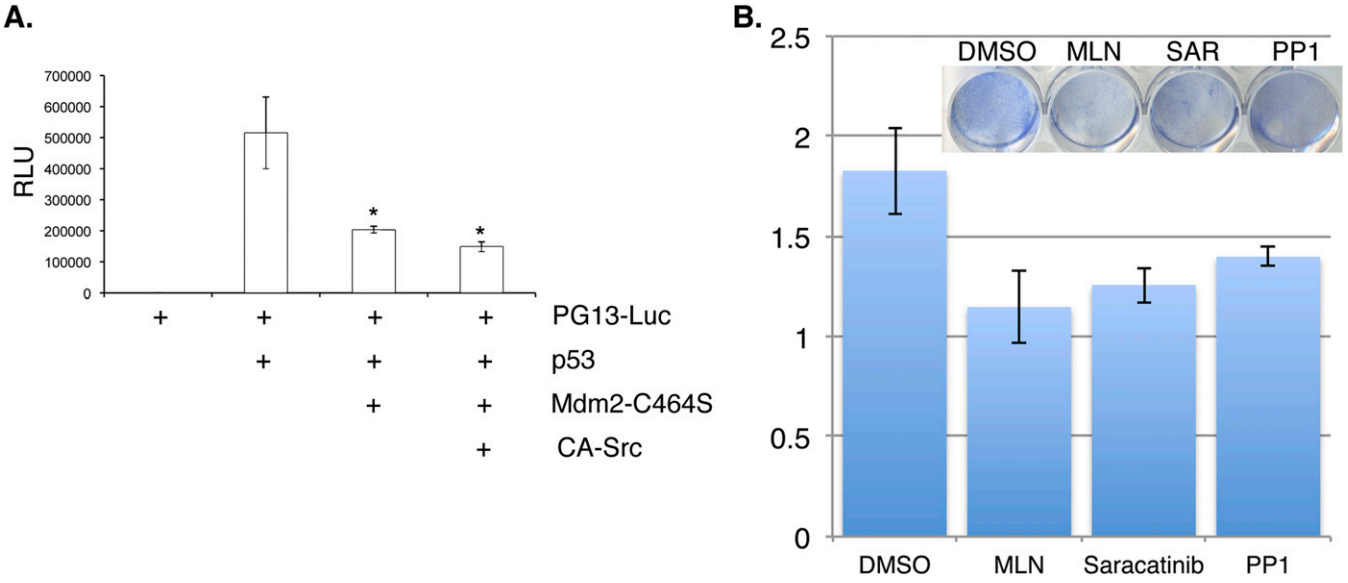


Fig. S3. (*A*) Luciferase assay using PG-13-Luc with Myc-LacZ in H1299 cells transfected with p53, Mdm2-C464S, and CA-Src. $^{*,\#}P < 0.05$. *Y* axis measurements are relative luciferase units (RLU). The RLU was calculated from the ratio of luciferase/β-gal activity. Error bars represent SD of three separate experiments. (*B*) Viability assay of MCF7 cells that had been treated with 0.6 μM MLN, 20 μM saracatanib, 20 μM PP1 or DMSO for 48 h. Cells were eluted (70% ethanol, 5% HCl), and absorbance at 650 nM was graphed. SE was calculated from three replicates.

Table S1. SH3 binding domain array results for tyrosine kinases from Panomics

Symbol	Name of the tyrosine kinase on the SH3 domain array or other SH3 domain protein	Bound by Mdm2
Abl	Ableson tyrosine kinase	*
Abl2	Ableson-related protein (Arg)	*
BLK	Beta lymphocyte-specific protein tyrosine kinase	
BTK	Bruton tyrosine kinase	
Hck	Hematopoietic cell kinase	*
Itk	Interleukin-2-inducible T-cell kinase	
LCK	Human T lymphocyte-specific protein tyrosine kinase (p56)	
cSrc	Cellular Rous sarcoma viral oncogene homolog	*
SLK	Proto-oncogene tyrosine protein kinase FYN	
TXK	Tyrosine-protein kinase	
PEXD	Peroxisomal membrane protein pex13	
Y124	PAK -interacting exchange factor beta	