Supplementary Information

Mdm2 requires Sprouty4 to regulate Focal Adhesion Formation and Metastasis independent of p53

Rafaela Muniz de Queiroz, Gizem Efe, Asja Guzman, Naoko Hashimoto, Yusuke Kawashima, Tomoaki Tanaka, Anil K Rustgi and Carol Prives

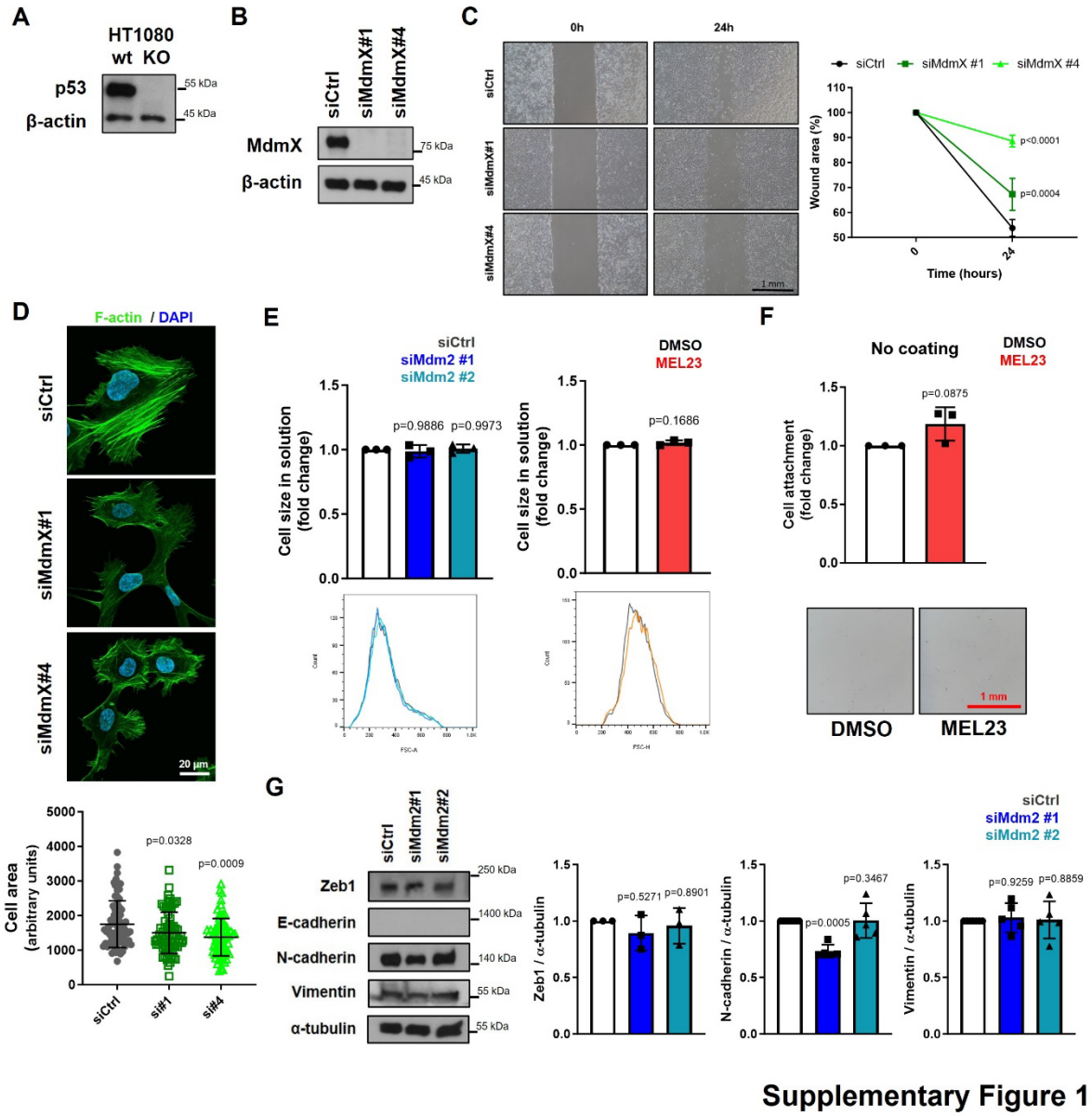
List of content:

Supplementary Table 1: Expression of EMT markers in HT1080 p53KO cells in response to Mdm2 silencing using siRNAs or treatment with MEL23 for 24 h analyzed by mass spectrometry. Green rows represent mesenchymal markers while orange rows represent epithelial markers. The experiment was performed in 3 independent experimental replicates. FC: fold change; Uniprot Ac.: Uniprot accession number; FDR: false discovery rate.

		MEL23		siMdm2#1		siMdm2#2	
UniProt Ac.	Gene symbol	Average FC	FDR value	Average FC	FDR value	Average FC	FDR value
O95863	SNAI1	1.228	0.242	0.963	0.806	0.878	0.256
P08670	VIM	0.994	0.472	1.048	0.975	1.036	0.998
P02751	FN1	0.531	0.088	1.148	0.923	1.204	0.925
Q99958	FOXC2	0.727	0.314	1.867	0.032	1.431	0.865
P08253	MMP2	0.611	0.348	1.057	0.993	1.084	0.993
P14780	MMP9	2.304	0.166	0.973	0.963	1.321	0.942
P37275	ZEB1	1.211	0.109	1.119	0.905	1.219	0.645
O60315	ZEB2	1.062	0.259	1.113	0.906	1.112	0.967
P05783	KRT18	0.898	0.948	1.092	0.932	1.049	0.994
P13647	KRT5	0.733	0.692	0.816	0.909	5.174	0.775
P05787	KRT8	0.816	0.296	1.020	0.450	1.033	0.949
O95832	CLDN1	0.839	0.766	0.914	0.829	0.932	0.988
P15924	DSP	1.079	0.426	0.745	0.686	2.068	0.907
Q07157	TJP1	0.897	0.777	1.028	0.881	1.075	0.903

Supplementary Table 2: Integrin expression in HT1080 p53KO cells in response to Mdm2 silencing using siRNAs or treatment with MEL23 for 24 h analyzed by mass spectrometry. Experiment was performed in 3 independent experimental replicates. FC: fold change; Uniprot Ac.: Uniprot accession number; FDR: false discovery rate.

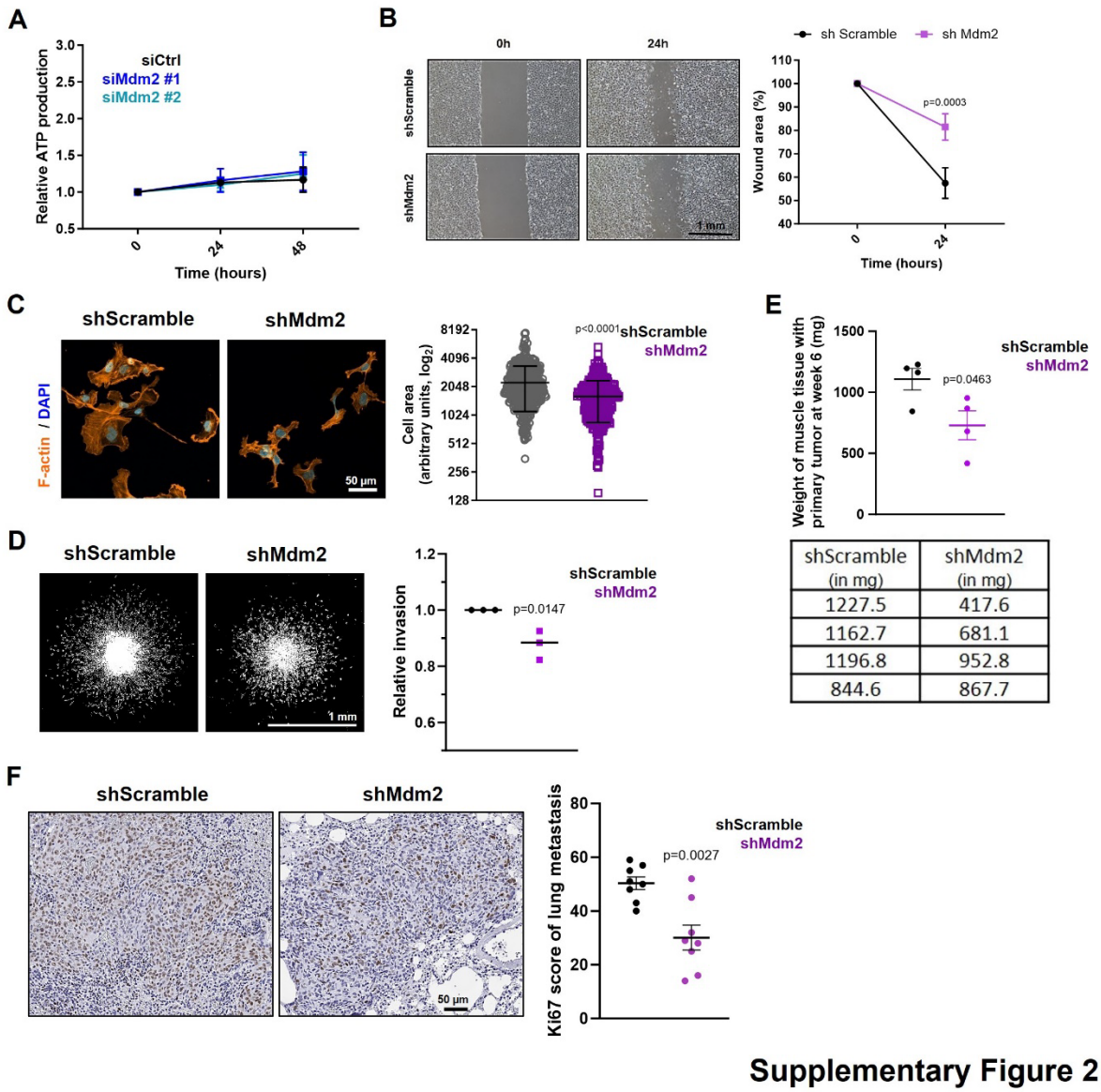
		MEL23		siMdm2#1		siMdm2#2	
UniProt Ac.	Gene symbol	Average FC	FDR value	Average FC	FDR value	Average FC	FDR value
P56199	ITGA1	0.817	0.626	1.284	0.520	1.330	0.626
O75578	ITGA10	0.520	0.476	0.971	0.787	1.349	0.476
P17301	ITGA2	0.953	0.995	1.017	0.955	1.087	0.995
P26006	ITGA3	0.773	0.126	1.035	0.907	0.837	0.126
P08648	ITGA5	1.066	0.208	1.020	0.909	1.110	0.884
P23229	ITGA6	0.748	0.913	0.898	0.473	1.032	0.998
P06756	ITGAV	0.920	0.969	1.074	0.966	1.028	0.998
P05556	ITGB1	0.884	0.998	1.062	0.991	1.022	0.998
O14713	ITGB1BP1	1.083	0.788	0.773	0.441	0.793	0.788
P05107	ITGB2	0.855	0.942	0.939	0.848	1.245	0.942
P05106	ITGB3	0.953	0.977	0.966	0.896	0.912	0.978
P18084	ITGB5	0.773	0.938	1.009	0.860	1.083	0.938
P26012	ITGB8	0.686	0.991	1.236	0.937	1.234	0.991



Supplementary Figure 1

Supplementary Fig. 1 Functional aspects of ablation of Mdm2 and MdmX in HT1080 p53KO cells. (A) p53 protein levels in HT1080 parental (p53 wild-type, wt) and HT1080 p53KO cells. β-actin was used as loading control. **(B-D)** HT1080 p53KO cells were silenced for MdmX using siRNAs. (B) Protein levels of MdmX after transfection. β-actin was used as loading control. (C) Cell migration assay. Quantification and representative images of wound scratch migration assay, n=3 groups. Scale bar equivalent to 1 mm. (D) Representative images of morphology of cells attached to collagen coated coverslips and quantification of cell area after silencing of MdmX, n=3 groups. Scale bar equivalent to 50 µm. **(E)** Representative histogram and quantification of HT1080 p53KO cell size in solution in response to

Mdm2 knockdown or MEL23 treatment (7 μM) for 24 h, n=3 samples. **(F)** Attachment assay in MEL23 treated cells. Quantification and representative images of control for no-coating plates, n=3 samples. Scale bar equivalent to 1 mm. **(G)** Protein levels of EMT markers in HT1080 p53KO cells treated with control (siCrtl) or Mdm2 siRNAs. α-tubulin was used as loading control, n=3 samples for Zeb1 and n=5 samples for N-cadherin and vimentin. Graphs shown represent mean ±SD of independent experimental replicates**.** More details about statistical tests used can be found in Source Data file.

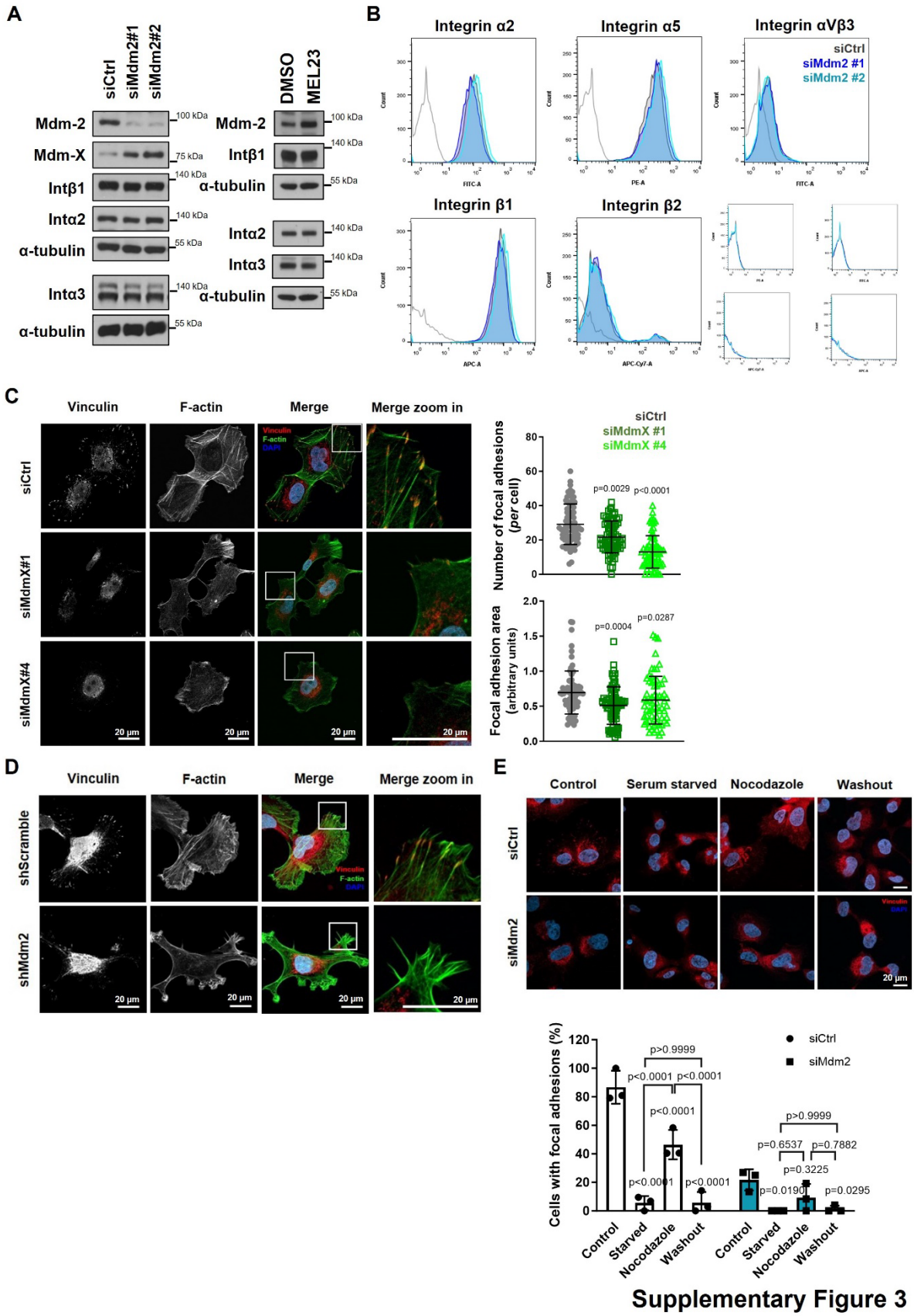


shScramble (in mg)	shMdm2 (in mg)
1227.5	417.6
1162.7	681.1
1196.8	952.8
844.6	867.7

Supplementary Figure 2

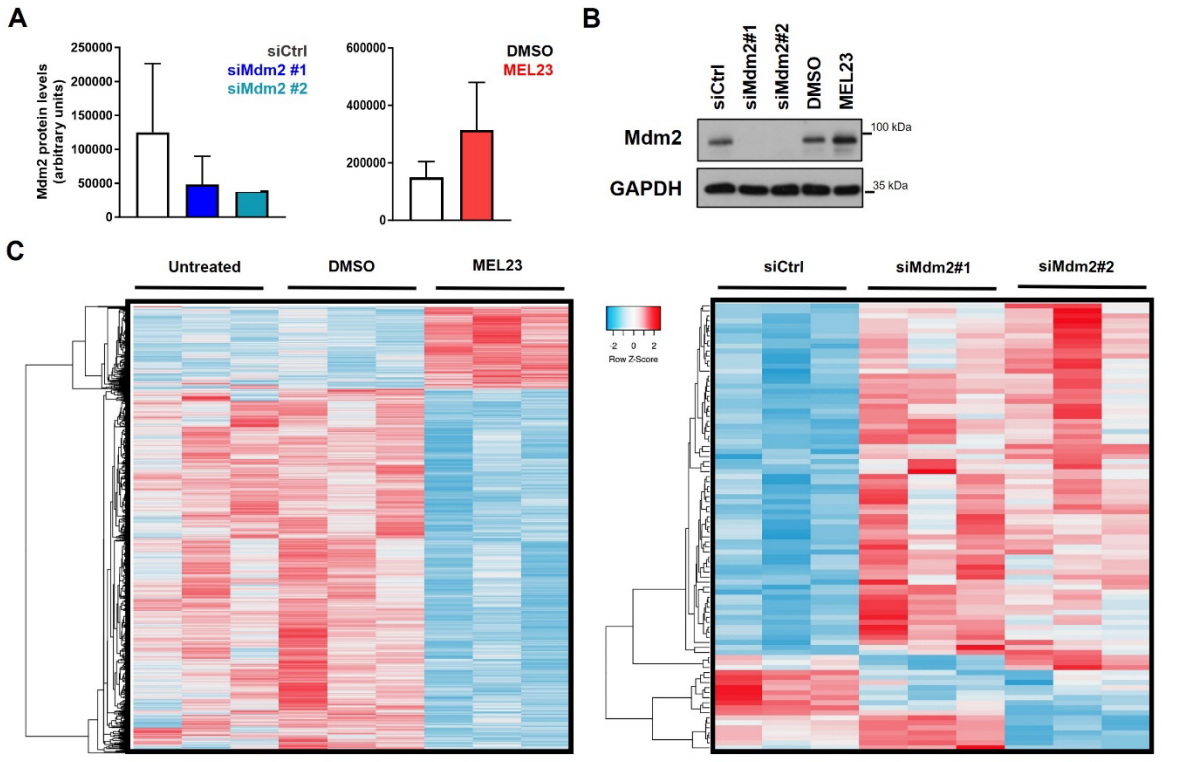
Supplementary Fig. 2 Mdm2 depletion in growth and metastasis-related processes. (A) Effects of transient Mdm2 silencing on growth of tumor spheroids of HT1080 p53KO cells measured by CellTiter Glo 3D, n=3 samples. **(B-F)** Functional aspects of HT1080 p53KO cells stably expressing shScramble or a pool of shRNAs against Mdm2. (B) Cell migration assay. Quantification and representative images of wound scratch migration assay, n=3 groups. Scale bar equivalent to 1 mm. **(C)** Representative images (left) and quantification (right) of cell area of HT1080 p53KO stable cell lines, n=3 groups. Scale bar is equivalent to 50 µm. **(D)** Invasion assay in collagen I matrix using stable cell lines. Representative images (left) and quantification (right), n=3 groups. Scale bar equivalent to 1 mm. **(E)** Quantification of tumor weight at week 6 in the orthotopic mouse model, n=4 mice/group. **(F)** Representative images

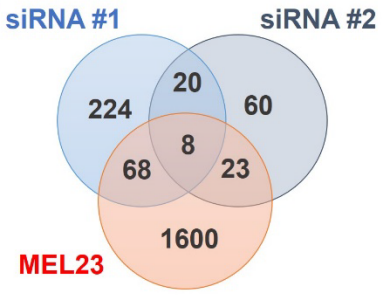
and score of Ki67 staining in lung metastasis 8 weeks after metastasis assay using tail vein injection, n=7 fields. Scale bar is equivalent to 50 μm. Graphs shown represent mean ±SD of independent experimental replicates**.** More details about statistical tests used can be found in Source Data file.



Supplementary Figure 3

Supplementary Fig. 3 Changes in integrin expression and FA formation in response to Mdm2 loss. (A) Protein levels of key expressed integrins by immunoblot in HT1080 p53KO cells in response to Mdm2 knockdown by siRNA (left) or MEL23 treatment (7 μM) for 24 h (right). α-tubulin was used as loading control. **(B)** Expression of different integrins in the cell surface in response to Mdm2 knockdown. Representative histograms of the presence of 5 different integrin or integrin complexes in the plasma membrane of HT1080 p53KO cells, n=3 samples. Light grey line represents unstained cells transfected with siCtrl, while dark gray and blue curves represent the conditions tested after incubation with the fluorescent antibodies. Smaller histograms on the bottom right are the curves of unstained samples for all 3 conditions for each fluorophore utilized. **(C-D)** Immunofluorescence showing FA foci by vinculin staining (red), cell surface was outlined by phalloidin staining (green), and nuclei by DAPI staining of DNA (blue). (C) Representative images and quantification of FA parameters of HT1080 p53KO cells silenced for MdmX using siRNAs, n=3 groups. (D) Representative images of HT1080 p53KO cells stably expressing shRNAs against Mdm2, n=3 groups. Scale bar equivalent to 20 μm. **(E)** FA disassembly assay. Immunofluorescence shows FA foci by vinculin staining (red), and nuclei by DAPI staining of DNA (blue). Representative images and quantification of number of FAs of HT1080 p53KO cells silenced for Mdm2 using siRNA, n=3 groups. Graphs shown represent mean ±SD of independent experimental replicates**.** More details about statistical tests used can be found in Source Data file.





E

		MEL23		siRNA#1		siRNA#2	
UniProt Ac.	Gene symbol	Average FC	FDR value	Average FC	FDR value	Average FC	FDR value
O14578	CIT	0.788	0.119	1.606	0.031	1.311	0.070
Q14687	GSE1	0.743	0.186	2.002	0.025	1.977	0.070
Q9NUD5	ZCCHC3	0.700	0.162	1.917	0.040	1.950	0.066
Q14562	DHX8	0.791	0.094	1.487	0.025	1.227	0.125
O15392	BIRC5	1.143	0.160	0.640	0.044	0.660	0.067
Q9Y4C1	KDM3A	1.050	0.145	1.546	0.035	1.361	0.081
Q9C004	SPRY4	1.166	0.076	1.571	0.149	1.733	0.168
Q5VV17	OTUD1	1.525	0.142	2.003	0.106	2.981	0.061

F

GO Cellular Components

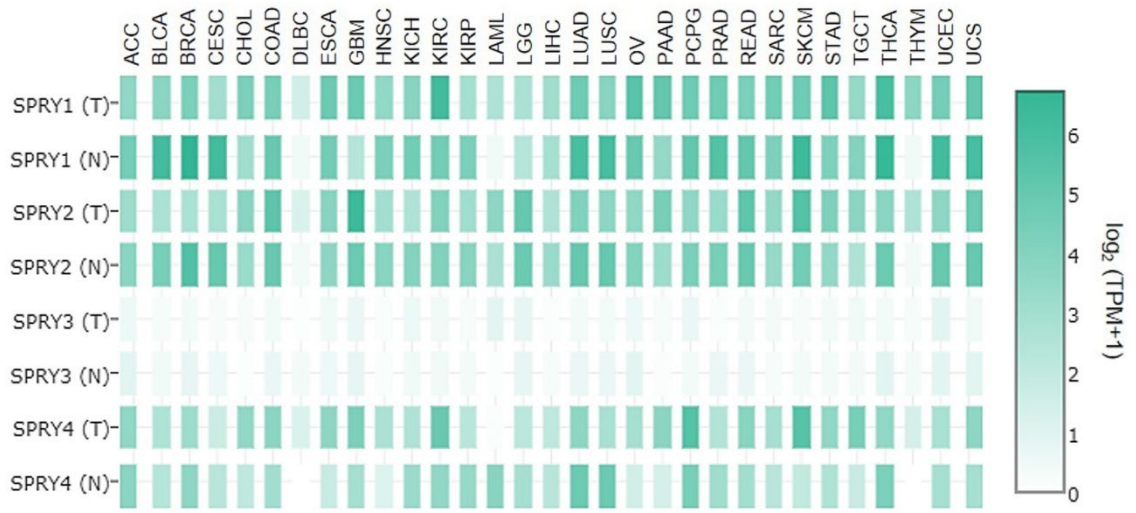
Index	Name	p-value	Adjusted p-value	Odds ratio	Combined score
1	Intracellular membrane-bounded organelle (GO:0043231)	$76x10^{-7}$	0.0005	2.31	27.25
2	Nucleus (GO:0005634)	$85x10^{-7}$	0.0004	2.31	27.43
3	Cytoplasmic side of plasma membrane (GO:0009898)	0.0009	0.033	7.28	51.26
4	Heterochromatin (GO:0000792)	0.003	0.088	11.15	64.42
5	Endoplasmic Reticulum tubular network (GO:0071782)	0.010	0.237	14.14	64.59
6	Intermediate filament cytoskeleton (GO:0045111)	0.012	0.2378	6.57	28.76
7	Membrane raft (GO:0045121)	0.018	0.2703	4.16	16.6
8	Focal adhesion (GO:0005925)	0.028	0.2703	2.72	9.69
9	Postsynaptic recycling endosome (GO:0098837)	0.029	0.2703	42.11	148.52
10	Box C/D RNP complex (GO:0031428)	0.029	0.2703	42.11	148.52

KEGG 2021 Human

Index	Name	p-value	Adjusted p-value	Odds ratio	Combined score
1	Apoptosis	0.0002	0.021	7.71	65.39
2	Hippo signaling pathway	0.0004	0.021	6.67	51.64
3	TNF signaling pathway	0.0005	0.021	8.11	60.78
4	Transcriptional misregulation in cancer	0.001	0.03	5.62	38.71
5	ECM-receptor interaction	0.0019	0.043	8.2	51.40
6	Small cell lung cancer	0.0022	0.043	7.82	47.79
7	Notch pathway	0.0052	0.087	9.16	48.08
8	Focal adhesion	0.007	0.103	4.4	21.81
9	p53 signaling pathway	0.0094	0.123	7.32	34.13
10	Pathways in cancer	0.0141	0.165	2.67	11.37

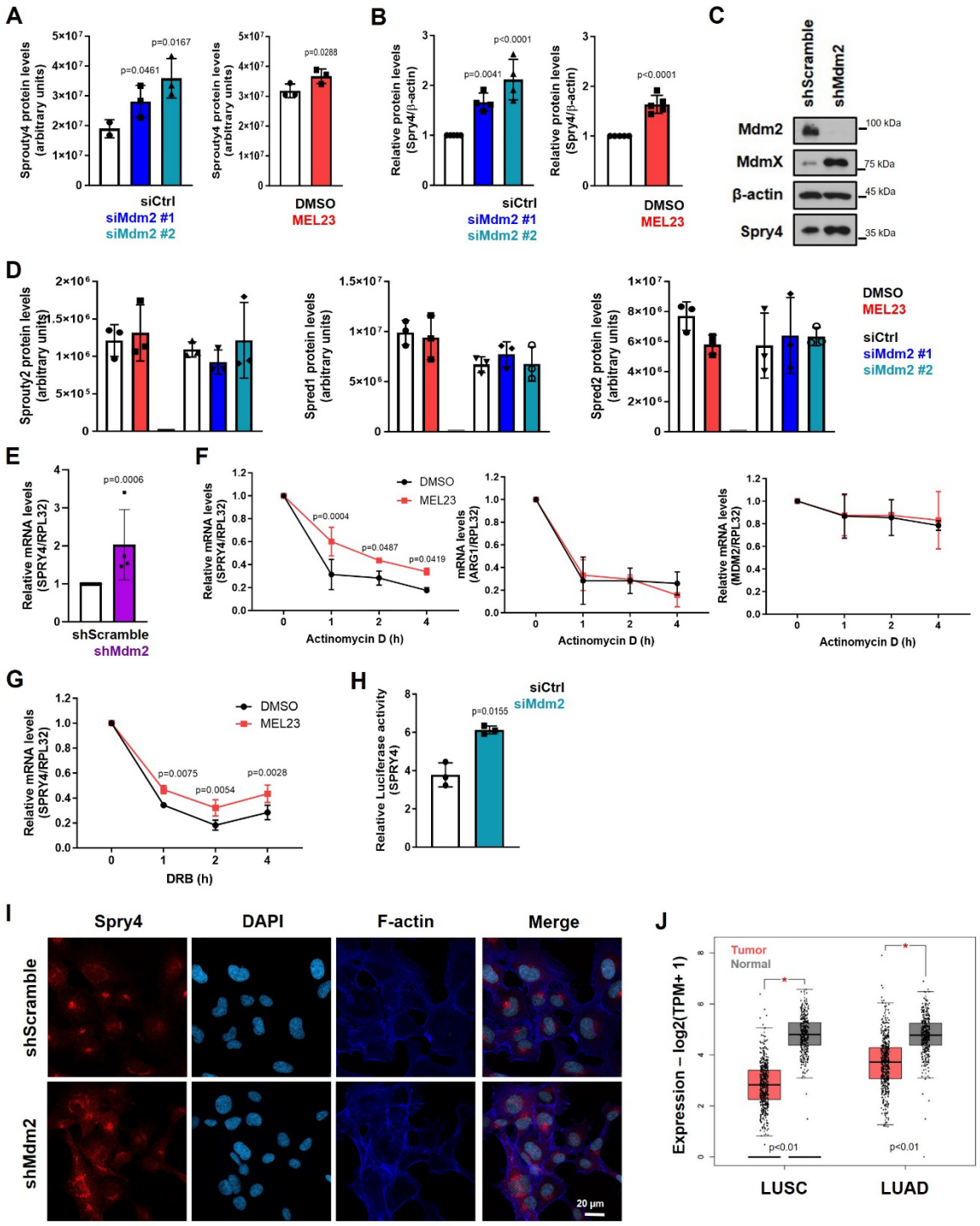
Supplementary Figure 4

Supplementary Fig. 4 Proteomic analysis of HT1080 p53KO cells in response to Mdm2 inhibition using siRNAs and pharmacological inhibitor, MEL23. (A-B) Validation of conditions by quantification of Mdm2 expression in proteomics samples through (A) mass spectrometry (n=3 samples) and (B) immunoblot. GAPDH was used as loading control for the immunoblot. Graphs shown in A represent mean ±SD of independent experimental replicates**. (C)** Heatmaps of significantly differentially expressed proteins in each experimental replicate indicated at top. **(D)** Venn diagram of proteins significantly differentially expressed after transfection with siRNA#1, siRNA#2 or after treatment with 7 μM MEL23. Significance cutoff FDR<0.2. **(E)** Table containing statistical analysis of the 8 proteins commonly significantly regulated by siRNA#1, siRNA#2 and MEL23. Proteins found to be upregulated in the siRNA group but downregulated after treatment with the pharmacological inhibitor are shown in orange background, proteins found to be downregulated in the siRNA group but upregulated after treatment with MEL23 are shown in green background, proteins found to be upregulated in all three conditions are shown in blue background. No proteins were found significantly downregulated by all 3 conditions. **(F)** Pathway analysis of genes commonly modulated by at least 2 out of the 3 conditions. The proteomics analysis was performed in three independent independent experimental replicates. UniProt Ac. UniProt accession number, FC fold change, FDR false discovery rate.



Supplementary Figure 5

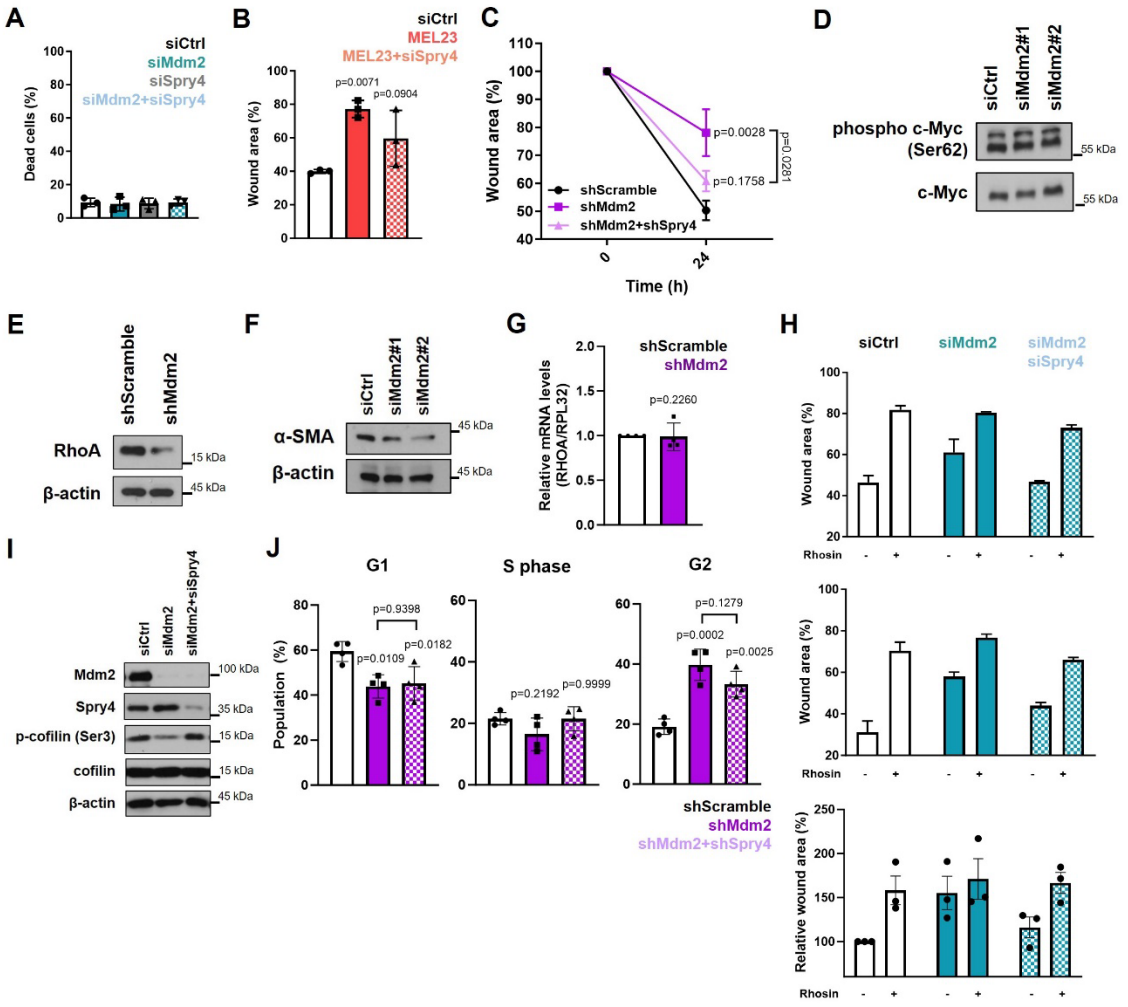
Supplementary Fig. 5 Different members of the Sprouty family and their expression in patient samples. Expression of Sprouty family members in patient samples from TCGA and GTEx databases. Rows designated (T, tumor) regard cancer patients, rows designated (N, normal) regard patients with no tumors. ACC: adrenocortical carcinoma; BLCA: bladder urothelial carcinoma; BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: cholangio carcinoma; COAD: colon adenocarcinoma; DLBC: lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: esophageal carcinoma; GBM: glioblastoma multiforme; HNSC: head and neck squamous cell carcinoma; KICH: kidney chromophobe; KIRC: kidney renal clear cell carcinoma; KIRP: kidney renal papillary cell carcinoma; LAML: acute myeloid leukemia; LGG: brain lower grade glioma; LIHC: liver hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; OV: ovarian serous cystadenocarcinoma; PAAD: pancreatic adenocarcinoma; PCPG: pheochromocytoma and paraganglioma; PRAD: prostate adenocarcinoma; READ: rectum adenocarcinoma; SARC: sarcoma; SKCM: skin cutaneous melanoma; STAD: stomach adenocarcinoma; TGCT: testicular germ cell tumors; THCA: thyroid carcinoma; THYM: thymoma; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma.



Supplementary Figure 6

Supplementary Fig. 6 Sprouty-family modulation in response to Mdm2 knockdown in HT1080 p53KO cells and patients' samples. (A-B) Quantification of protein levels of Spry4 measured by (A) mass spectrometry (n=3 samples) and (B) densitometry of immunoblots (n=4 samples with siRNAs; n=5 samples with MEL23 treatment), in HT1080 p53KO cells silenced for Mdm2 (left) or treated with MEL23

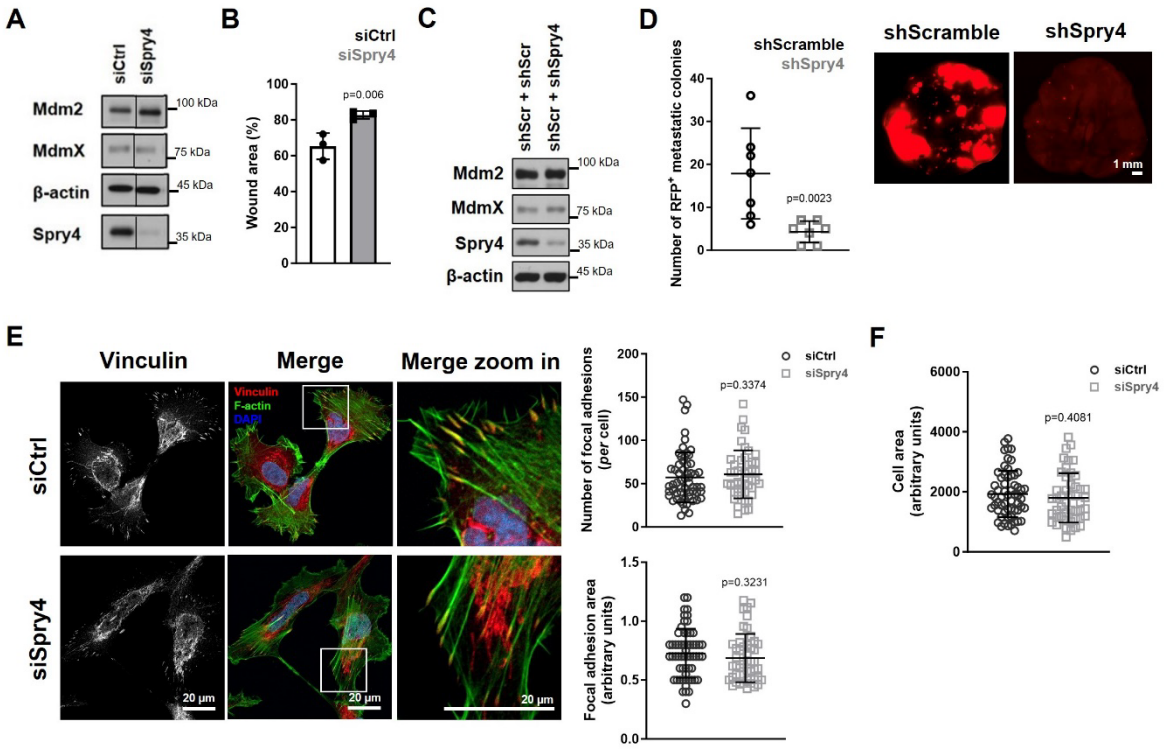
(right). **(C)** Expression of Spry4 in stable Mdm2 knockdown HT1080 p53KO cells. β-actin was used as loading control. **(D)** Quantification of the three Sprouty family members expressed in HT1080 cells in response to treatment with MEL23 or Mdm2 KD measured by mass spectrometry, n=3 samples. **(E)** mRNA levels of SPRY4 in Mdm2 knockdown HT1080 p53KO stable cell line, n=4 samples. **(F)** Half-life of Spry4 mRNA in cells treated with 7 μM MEL23 or vehicle in response to actD treatment (10 μg/ml), n=3 samples. ARG1 was used as control for short-lived mRNA, and MDM2, as control for more stable mRNAs. **(G)** Half-life of Spry4 mRNA in cells treated with 7 μM MEL23 or vehicle in response to DRB treatment (100 μM), n=3 samples. **(H)** *Spry4* luciferase promoter assay in cells silenced for Mdm2 using siRNA, n=3 samples. **(I)** Localization of Spry4 in stable control and Mdm2 KD cells. Immunofluorescence showing Spry4 staining (red), cell surface was outlined by phalloidin staining of F-actin (dark blue), and nuclei (cyan) detected by DAPI staining of DNA, n=3 groups. Scale bar equivalent to 20 μm. **(J)** Expression of Spry4 in lung cancer. Analysis of samples from the TCGA and GTEx database. LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma. Graphs shown represent mean ±SD of independent experimental replicates. More details about statistical tests used can be found in Source Data file.



Supplementary Figure 7

Supplementary Fig. 7 Experiments with Mdm2 and/or Spry4 silencing or with MEL23 treatment to test cell parameters related to Figure 6 and 7. (A) Cell viability of HT1080 p53KO cells in response to Mdm2 knockdown as measured by Trypan Blue, n=3 samples. **(B)** Quantification of migration of HT1080 p53KO cells silenced or not for Spry4 in the presence of absence of Mdm2 inhibitor MEL23, n=3 samples. **(C)** Quantification of migration of H1299 cells in response to knockdown of Mdm2 alone or co-knockdown of Mdm2 and Spry4 using siRNAs in HT1080 p53KO cells, n=3 samples. **(D)** Levels of c-Myc phosphorylation at serine 62 and total c-Myc in response to knockdown of Mdm2 using siRNAs. **(E)** RhoA protein levels in shMdm2 stable HT1080 p53KO cells. **(F)** Protein levels of alpha-SMA in HT1080 p53KO cells silenced against Mdm2. **(G)** RhoA mRNA levels in shMdm2 stable HT1080 p53KO cells, n=5 samples. **(H)** Cell migration assay of HT1080 p53KO cells in response to knockdown

of Mdm2 alone or co-knockdown of Mdm2 and Spry4 in the presence or absence of the RhoA inhibitor Rhosin (50 μM) for 24 h. Top and middle graphs: Quantification of the other two independent experimental replicates of the experiment shown in Fig.7, panel G. N=3 technical replicates in each graph. Bottom graph: Quantification of all 3 independent experimental replicates normalized by the control condition (cells treated with DMSO and siCtrl). The graph shown represents mean ±SEM of independent experimental replicates. Statistical analysis was performed using the Kruskal-Wallis test with p=0.048, n=3 samples. **(I)** Levels of cofilin phosphorylation at serine 3 in response to knockdown of Mdm2 alone or co-knockdown of Mdm2 and Spry4 in H1299 cells. **(J)** Cell cycle analysis of HT1080 p53KO cells stably expressing shRNAs against Mdm2 alone or co knockdown of Mdm2 and Spry4, n=4 samples. Graphs shown represent mean ±SD of independent experimental replicates**.** More details about statistical tests used can be found in Source Data file.



Supplementary Figure 8

Supplementary Fig. 8 Experiments performed in HT1080 p53KO cells using Spry4 siRNAs or shRNAs alone. (A-F) HT1080 p53KO cells were silenced for Spry4 transiently or stable using a pool of si- or shRNAs, respectively. (A) Protein levels of Spry4 as well as Mdm2 and MdmX after transient transfection with a pool of siRNAs against Spry4. α-tubulin was used as loading control, n=4. (B) Quantification of wound scratch migration assay of Spry4-depleted cells using siRNAs, n=3. (C) Protein levels of Spry4, Mdm2 and MdmX in HT1080 p53KO cells stably expressing shRNAs against Spry4, n=3. β-actin was used as loading control. (D) Analysis of metastatic burden *in vivo* using tail-vein model of stable Spry4 KD HT1080 p53KO cells. Representative images (right) and quantification (left) of metastatic foci in the lungs after 8 weeks of injection, n=7 mice. (E) Immunofluorescence showing FA foci by vinculin staining (red), cell surface was outlined by phalloidin staining of F-actin (green), nuclei are detected by DAPI staining (blue) of cells transiently transfected with a pool of siRNAs against Spry4 or siCtrl. Representative images (left) and quantification (right) of FA parameters, n=3. Scale bar equivalent to 20 µm. (F) Quantification of cell area of HT1080 p53KO cells transiently transfected with a pool of siRNAs against Spry4 shown in panel E, n=3. Graphs shown represent mean ±SD of at least 3 independent

experimental replicates**.** More details about statistical tests used can be found in Source Data file.