

Mdm2 Is Required for Survival and Growth of p53-Deficient Cancer Cells

Kyle P. Feeley¹, Clare M. Adams², Ramkrishna Mitra², and Christine M. Eischen^{1,2}



Abstract

p53 deletion prevents the embryonic lethality of normal tissues lacking Mdm2, suggesting that cells can survive without Mdm2 if p53 is also absent. Here we report evidence challenging this view, with implications for therapeutically targeting Mdm2. Deletion of *Mdm2* in T-cell lymphomas or sarcomas lacking p53 induced apoptosis and G₂ cell-cycle arrest, prolonging survival of mice with these tumors. *p53*^{-/-} fibroblasts showed similar results, indicating that the effects of Mdm2 loss extend to premalignant cells. *Mdm2* deletion in *p53*^{-/-} cells upregulated p53 transcriptional target genes that induce apoptosis and cell-cycle arrest. *Mdm2* deletion also increased levels of p73, a p53 family

member. RNAi-mediated attenuation of p73 rescued the transcriptional and biological effects of Mdm2 loss, indicating that p73 mediates the consequences of *Mdm2* deletion. In addition, *Mdm2* deletion differed from blocking Mdm2 interaction with p53 family members, as Nutlin-3 induced G₁ arrest but did not activate apoptosis in *p53*^{-/-} sarcoma cells. Our results indicate that, in contrast to current dogma, Mdm2 expression is required for cell survival even in the absence of p53. Moreover, our results suggest that p73 compensates for loss of p53 and that targeting Mdm2 in p53-deficient cancers has therapeutic potential. *Cancer Res*; 77(14); 3823–33. ©2017 AACR.

Introduction

Mdm2 negatively regulates the p53 tumor suppressor by inhibiting its transcriptional activity and targeting it for degradation via ubiquitination (1). During development in mice, global *Mdm2* deletion results in embryonic lethality, which is rescued with accompanying *p53* deletion (2, 3), genetically establishing Mdm2 regulation of p53. Deletion of *Mdm2* in specific tissues (e.g., cardiomyocytes, central nervous system, and hepatocytes) induces apoptosis that is rescued with *p53* deletion (4–6). Induced *Mdm2* deletion in adult mice resulted in abnormalities in multiple tissues (e.g., spleen, liver, and kidney), which were not evident when performed on a *p53*-null background (7). Fibroblasts from *Mdm2/p53*-double null mice were viable and grew at similar rates as fibroblasts from *p53*-null only mice (8, 9). Furthermore, the rate of tumor development was analogous between *Mdm2*^{-/-}*p53*^{-/-} and *p53*^{-/-} mice (8). These data led to the conclusion that the primary function of Mdm2 was to regulate p53 and that the deleterious effects of *Mdm2* loss are p53 dependent.

MDM2 is frequently overexpressed in human malignancies (10), making MDM2 an attractive therapeutic target. Recently,

drugs such as Nutlin-3 have been developed that interfere with Mdm2:p53 binding, thereby activating p53 and killing cancer cells (11). However, *p53* is mutated or deleted in half of human cancers, making compounds that disrupt Mdm2:p53 binding not viable for these malignancies (12). In addition, resistance to these compounds develops through p53 inactivation (13–15). p73, a p53 family member, is rarely mutated in human cancers (16). Both p53 and p73 activation upregulate transcriptional targets that induce cell-cycle arrest and/or apoptosis (17). Mdm2 can bind and regulate p73 (18–21); yet, the circumstances under which this takes place remains incompletely resolved. Insight into their interaction may be exploited therapeutically in tumors with inactivated p53 (16). For example, high concentrations of Nutlin-3 induced apoptosis of *p53*-null HCT116 colon carcinoma cells partially through activation of p73 (22). Therefore, identifying alternative approaches to activate p73-induced apoptosis in cells lacking functional p53 may be beneficial.

Because *Mdm2* loss in the context of p53 inactivation could hold therapeutic promise and has not been thoroughly examined outside of development, we utilized a conditional *Mdm2* deletion mouse model to determine the effect of *Mdm2* loss on *p53*^{-/-} cells. Unexpectedly, both T-cell lymphoma and sarcoma cells lacking *p53* underwent apoptosis when *Mdm2* was deleted, resulting in significantly diminished cancer cell growth, reduced tumor burden, and extended survival. *p53*^{-/-} adult mouse fibroblasts were similarly affected by *Mdm2* deletion. Mechanistically, we determined p73 mediated the effects of *Mdm2* deletion. Thus, Mdm2 is critical for cell survival independent of p53. Therefore, targeting Mdm2 directly may offer therapeutic potential for cancers that have deleted *p53* by activating p73.

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Materials and Methods

Mice, cells, and tumor development

C57Bl/6 *Mdm2*^{fl/fl} and *p53*^{-/-} mice (2) provided by Dr. Guillermina Lozano (MD Anderson Cancer Center, Houston, TX) were

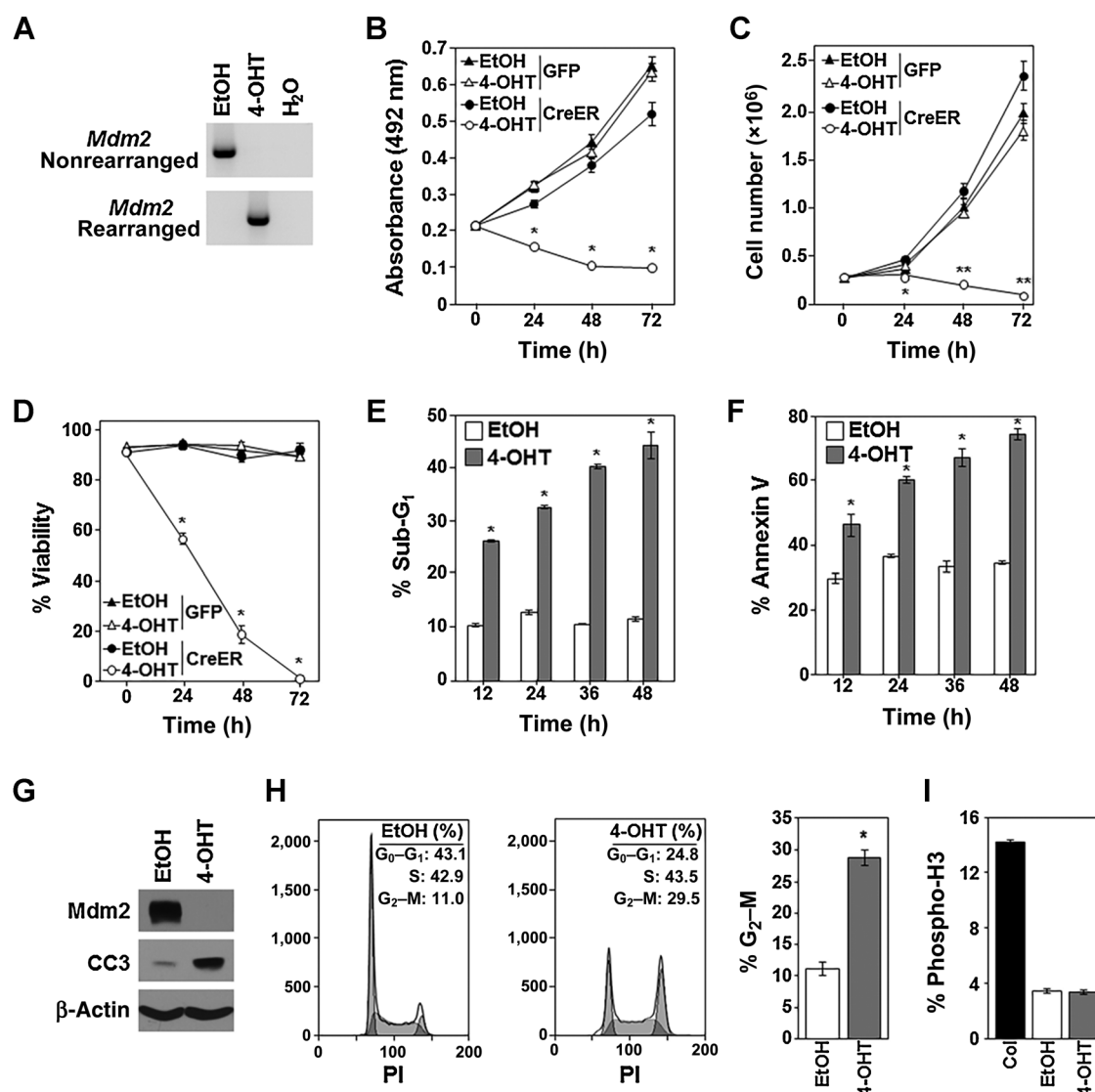


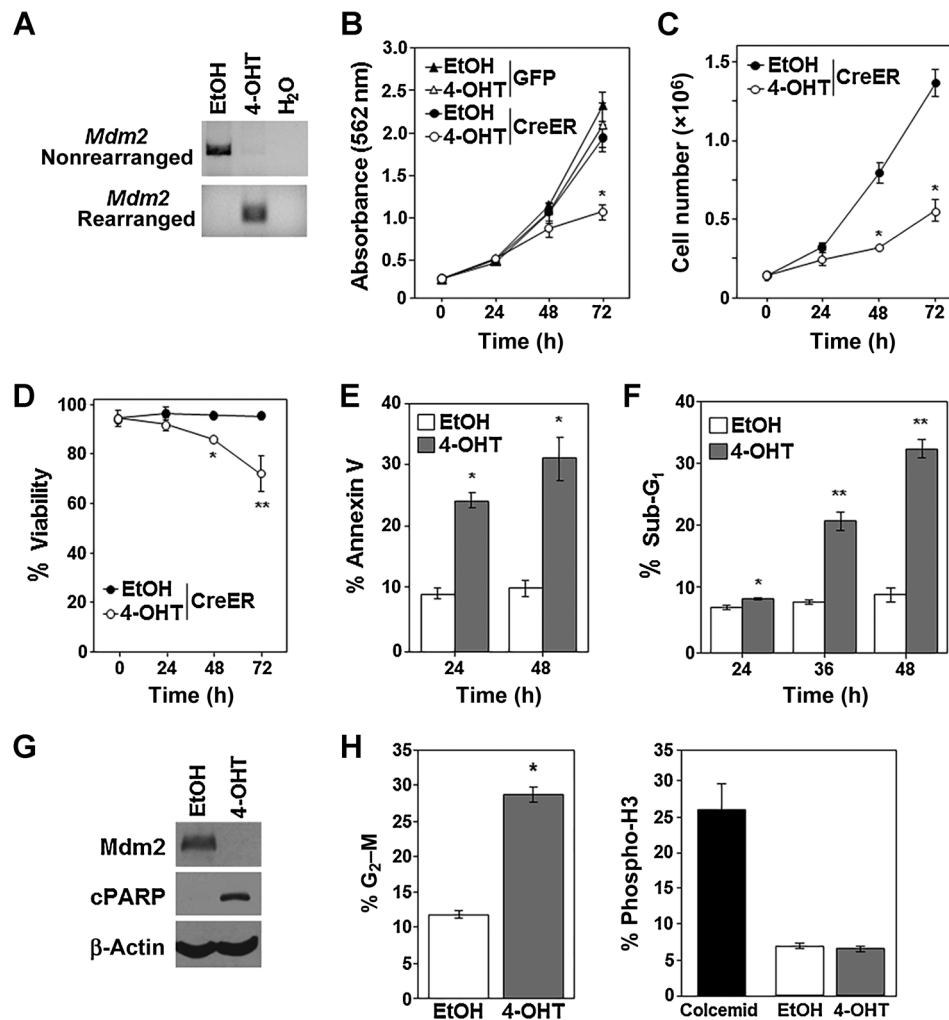
Figure 1. Deletion of *Mdm2* inhibits growth and survival of *p53*-null lymphomas. Vehicle control (EtOH) or 4-OHT was added to *Mdm2*^{fl/fl}*p53*^{-/-} lymphomas expressing CreER^{T2} and GFP or GFP alone. **A-F**, *Mdm2* gene rearrangement PCR (**A**), proliferation (MTS assay, quadruplicate; **B**), cell number (**C**), viability (**D**), sub-G₁ DNA (**E**), and Annexin-V (**F**) measured (**C-F**, triplicates). **G**, Western blotting 16 hours after EtOH or 4-OHT addition. CC3, cleaved caspase-3. **H** and **I**, Cell cycle (representative histograms; values in inset, left; G₂-M mean values, right; **H**) and phospho-histone H3 (**I**) 12 hours after EtOH or 4-OHT (triplicate). Col, colcemid. *, *P* < 0.0002 (**B**); *, *P* = 0.011; **, *P* < 0.0002 (**C**); *, *P* < 0.0002 (**D**); *, *P* < 0.00009 (**E**); *, *P* < 0.006 (**F**); *, *P* = 0.00102 (**H**).

mated and offspring intercrossed to generate *Mdm2*^{fl/fl}*p53*^{-/-} mice. Ear punches to derive fibroblasts or tumors (T-cell lymphoma and sarcoma) that developed in *Mdm2*^{fl/fl}*p53*^{-/-} mice were harvested and placed in short-term culture (see Supplementary Information). The cultured cells were confirmed mycoplasma negative (Mycosensor Mycoplasma Detection PCR Assay Kit; Agilent Technologies). Female nude mice (6–7 weeks old; Envigo) were injected subcutaneously with 1×10^6 *Mdm2*^{fl/fl} *p53*^{-/-} T-cell lymphoma or sarcoma cells expressing CreER^{T2} (23). Tamoxifen (2 mg) or corn oil (vehicle) was injected (intraperitoneal) once daily for 3 days after lymphomas became palpable and once daily for 4 days beginning the day of sarcoma cell injection. Tumor volumes were calculated from caliper measurements. Mice were sacrificed at humane endpoints and tumors harvested for analy-

ses. In a second cohort of mice, when tumors were palpable, tamoxifen or corn oil was injected into mice with size-matched tumors and the tumors harvested 48 hours (lymphoma) or 72 hours (sarcoma) later. All studies complied with state and federal guidelines and were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Proliferation

T-cell lymphoma, sarcoma, and fibroblast cells expressing CreER^{T2} and GFP or GFP alone (retrovirus) were plated and 1 μ mol/L 4-hydroxytamoxifen (4-OHT) or EtOH vehicle or Nutlin-3 (10, 20, or 30 μ mol/L; Sigma) or DMSO vehicle added. Cell number and viability were determined by Trypan Blue Dye exclusion (triplicate). Proliferation determined by MTS (492 nm;

**Figure 2.**

Mdm2 loss significantly impedes growth and survival of *p53*-null sarcomas. Vehicle control (EtOH) or 4-OHT was added to *Mdm2*^{fl/fl}*p53*^{-/-} sarcoma cells expressing CreER^{T2} and GFP or GFP alone. **A–F**, *Mdm2* gene rearrangement PCR (**A**), proliferation (MTT assay, quadruplicate; **B**), cell number (**C**), viability (**D**), Annexin-V (**E**), and sub-G₁ DNA (**F**) measured (**C–F**, triplicates). **G** and **H**, Following 36 hours of EtOH or 4-OHT addition, Western blotting (**G**; cPARP, cleaved-PARP) was performed, and cells in G₂-M (left) and phospho-histone H3 (**H**, triplicate) determined. *, $P < 0.002$ (**B** and **C**); *, $P < 0.005$; **, $P < 0.02$ (**D**); *, $P < 0.0003$ (**E**); *, $P < 0.02$; **, $P < 0.0067$ (**F**); *, $P = 0.00086$ (**H**).

Promega) or MTT (562 nm; Sigma) assays (quadruplicate), as we previously reported (24, 25).

Cell cycle and apoptosis

Cell-cycle and fragmented (sub-G₁) DNA was analyzed by flow cytometry following propidium iodide staining as we described (24). The percentage of viable cells in each phase of the cell cycle was determined using the Dean–Jett–Fox model on FlowJo (TreeStar). Phosphorylated histone H3 (phospho-S10, Abcam) was detected according to manufacturer's protocol; colcemid (4 hours, 0.05 μg/mL; Gibco) treated cells were a positive control for cells in M phase. Annexin-V/7-AAD (BD Pharmingen) staining was performed as described previously (24). All assays (triplicate) were conducted following addition of 1 μmol/L 4-OHT, 30 μmol/L Nutlin-3, or vehicle control (EtOH or DMSO, respectively).

Western blot analysis

Whole-cell protein lysates were Western blotted as described previously (26), except for Fig. 6H and I that were performed as previously described (27). Antibodies used include: Mdm2 (2A10; Calbiochem), cleaved caspase-3 (D175) and cleaved PARP

(Asp214) from Cell Signaling Technology, p73 (EP436Y; Abcam), and β-actin (Sigma).

PCR, qRT-PCR, and RNA sequencing

PCR was performed on genomic DNA with primers specific for unrearranged and rearranged *Mdm2* as published (28). PCR genotyping was also used to confirm the T-cell lymphoma, sarcoma, and fibroblasts evaluated were *p53*-null (Supplementary Fig. S1). Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. qRT-PCR (triplicate) for mRNA analysis was performed as we described (primers in Supplementary Information; ref. 29). mRNA levels were normalized to β-actin levels and then made relative to vehicle control and presented as $2^{-\Delta\Delta C_t}$. Following RNA isolation, samples were subjected to RNA sequencing using the Illumina NextSeq500 platform; GEO accession number is GSE98705.

Bioinformatic analysis

RNA-sequencing data were analyzed by Kallisto v0.43.0 (30). Murine transcript definitions (Ensembl release 85) were used for transcriptome quantification. Tximport (31) was used to summarize transcript-level estimates for gene-level analysis. Differential gene expression analysis was performed using the R package

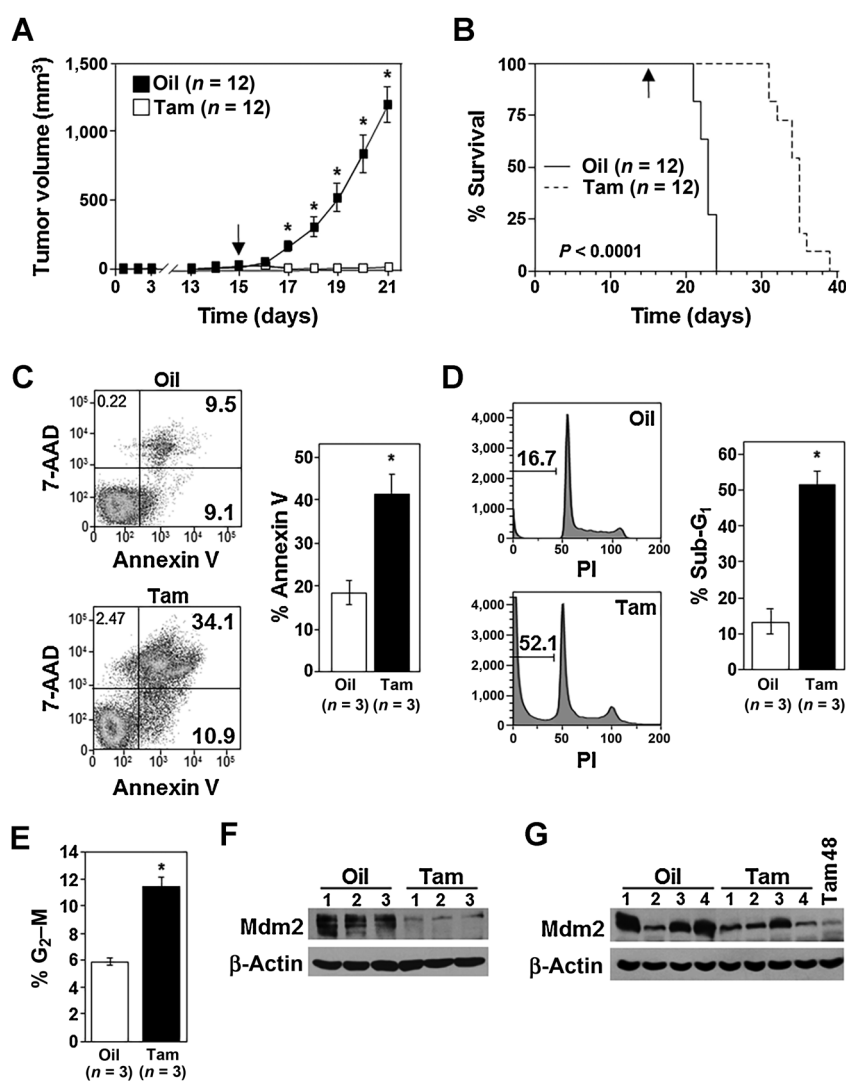


Figure 3.

Loss of *Mdm2* inhibits lymphoma growth *in vivo*. **A**, Tumor volumes in mice (number indicated) injected (subcutaneously) with CreER^{T2} expressing *Mdm2*^{fl/fl}*p53*-null lymphoma cells and administered tamoxifen (Tam) or vehicle control (corn oil, Oil) starting day 15 were measured (*, $P < 0.0006$). **B**, Kaplan–Meier survival curves of the mice in **A** ($P < 0.0001$). Arrow, the day tamoxifen or oil administration began. **C–E**, Annexin-V (**C**), sub-G₁ DNA (**D**), and cells in G₂-M (**E**) measured in extracted lymphoma cells 48 hours after tamoxifen or vehicle control administration to mice. For **C** and **D**, representative data, left: mean values, right: $P = 0.006$ (**C**); $P = 0.0008$ (**D**); $P = 0.013$ (**E**). **F** and **G**, Western blots of lymphomas harvested after 48 hours (**F**) or at humane endpoints (**G**) following tamoxifen (Tam) or vehicle control (Oil) administration. For **G**, a lymphoma harvested 48 hours after tamoxifen administration (Tam 48) included for comparison.

edgeR (32) as indicated by Tximport (31). Details are in Supplementary Information.

shRNA knockdown

Lentiviral vectors for two p73 shRNA and their respective control nontargeting shRNA were provided by Jennifer Pietsenpol (Vanderbilt University, Nashville, TN). Infected sarcoma cells were selected with puromycin (2.5 μg/mL) for 3 days prior to CreER^{T2} activation.

Statistical analysis

Means ± SEM are plotted. Log-rank tests used for Kaplan–Meier survival analyses. All other statistical analyses used the Student *t* test, except for RNA-sequencing analysis (see Supplementary Information).

Results

Mdm2 is required for p53-null T-cell lymphoma survival

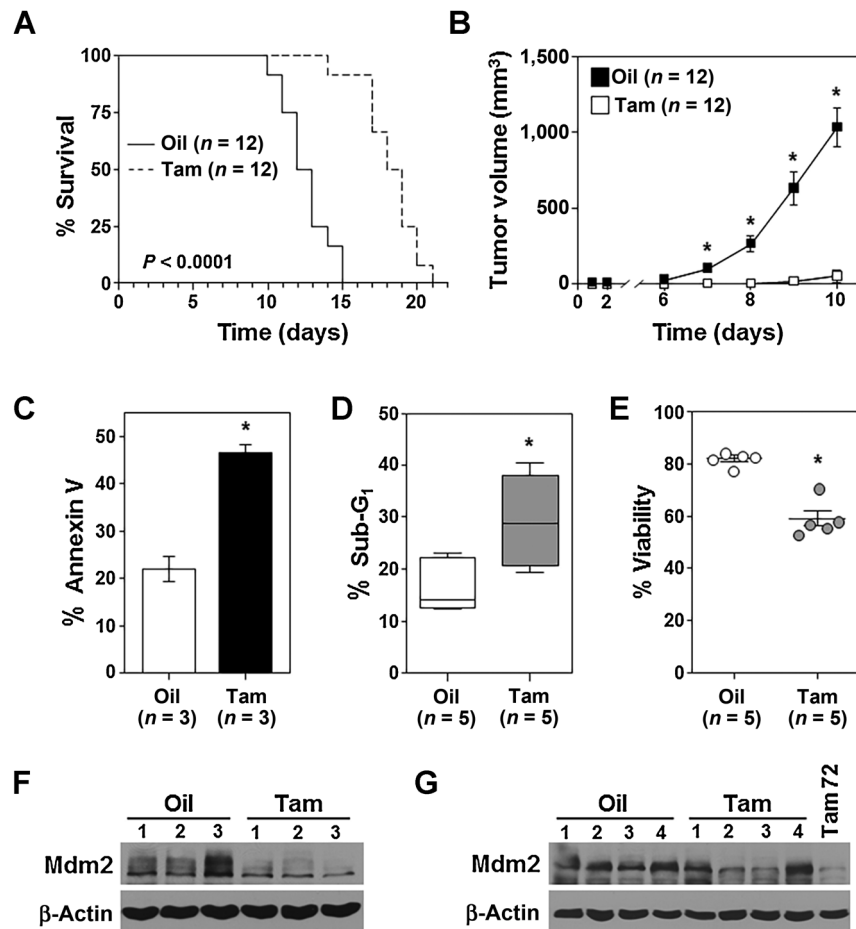
Previous studies showed cell death from loss of Mdm2 during development is rescued by p53 deletion (2, 3), indicating cells can

survive without Mdm2 if p53 is also absent. We questioned whether there would be a consequence to deleting *Mdm2* in mature, fully developed cells that lacked p53. In addition, because many human cancers delete *p53* (12), we sought to determine whether Mdm2 loss in p53-null malignant cells would affect their growth and/or survival. We generated p53-null mice homozygous for the conditional *Mdm2* knockout alleles (28). Because p53^{-/-} mice develop T-cell lymphoma (33), we isolated and cultured a T-cell lymphoma that arose in an *Mdm2*^{fl/fl}*p53*^{-/-} mouse. CreER^{T2}, a 4-OHT inducible form of Cre (23), was expressed (retrovirally) in the lymphoma cells. Rearrangement of the *Mdm2* loci occurred within 24 hours after 4-OHT addition (Fig. 1A).

To determine whether Mdm2 loss affects lymphoma cell growth, proliferation was assessed by MTS assay. Surprisingly, p53-null lymphoma cells with CreER^{T2}-mediated *Mdm2* deletion showed reduced proliferation compared with vehicle control treated lymphoma cells (Fig. 1B). Lymphoma cell numbers and viability significantly declined following CreER^{T2} activation with half the cells dead by 24 hours and <5% alive by 72 hours, whereas numbers of vehicle control treated lymphoma cells increased (Fig. 1C and D). Within 12 hours of CreER^{T2} activation,

Figure 4.

Mdm2 loss inhibits sarcoma growth *in vivo*. **A**, Kaplan-Meier survival curves of mice (number indicated) injected (subcutaneously) with CreER^{T2} expressing *Mdm2*^{fl/fl}*p53*^{-/-} sarcoma cells and administered tamoxifen (Tam) or vehicle (corn oil, Oil) control starting the day of cell injection ($P < 0.0001$). **B**, Tumor volumes in mice in **A** measured at intervals (*, $P < 0.00003$). **C-E**, 72 hours following tamoxifen or vehicle control administration to mice, Annexin-V (**C**), sub-G₁ DNA (**D**), and viability (**E**) were measured in extracted sarcoma cells. For **D**, whiskers represent the minimum and maximum, the line the median, and the box the 25th to 75th percentiles. *, $P = 0.001$ (**C**); *, $P = 0.012$ (**D**); *, $P < 0.0001$ (**E**). **F** and **G**, Western blots of sarcoma harvested after 72 hours (**F**) or at humane endpoints (**G**) after tamoxifen (Tam) or vehicle control (Oil) administration. For **G**, a sarcoma harvested 72 hours after tamoxifen administration (Tam 72) included for comparison.



the percentage of cells with fragmented (sub-G₁) DNA and that were Annexin-V positive was significantly higher in those that deleted *Mdm2*, and these differences increased over 48 hours (Fig. 1E and F). *Mdm2*-deleted lymphoma cells also showed the appearance of cleaved caspase 3 (Fig. 1G). Previously, we reported activation of CreER^{T2} in lymphoma cells lacking p53 had no effect (24), indicating the apoptosis in the CreER^{T2} activated *Mdm2*^{fl/fl}*p53*^{-/-} lymphoma cells was specific to *Mdm2* loss. Interestingly, there were significantly more cells in the G₂-M phase of the cell cycle 12 hours following CreER^{T2} activation in the *Mdm2*^{fl/fl}*p53*^{-/-} lymphoma cells, indicating cell-cycle arrest may be occurring (Fig. 1H). To differentiate G₂ from M phase, phosphorylated histone H3, a marker of M phase, was measured after *Mdm2* deletion. No difference in the percentage of cells in M phase between control and CreER^{T2}-activated lymphomas was detected (Fig. 1I), indicating the cells were likely arresting in G₂ and not M phase. Our data indicate *Mdm2* deletion in *p53*^{-/-} T-cell lymphoma inhibits growth and survival by initiating a G₂ cell-cycle arrest and apoptosis.

p53-null sarcoma cells require *Mdm2* for growth and survival

To test the reproducibility of our results and determine whether the apoptosis and cell-cycle arrest observed following *Mdm2* deletion was specific to *p53*^{-/-} T-cell lymphoma, we evaluated a different *p53*-null cancer. Because *p53*^{-/-} mice also develop sarcomas, we isolated and cultured a spindle-cell sarcoma from an

Mdm2^{fl/fl}*p53*^{-/-} mouse. Following retroviral expression of CreER^{T2} in the sarcoma cells, *Mdm2* was deleted within 24 hours of 4-OHT addition (Fig. 2A). There was significantly reduced proliferation by 72 hours (Fig. 2B). Consistent with this, the *Mdm2*-deleted sarcoma cells showed reduced cell number (Fig. 2C) and viability (Fig. 2D) compared with controls. Within 24 hours following CreER^{T2} activation, there were significantly elevated levels of Annexin-V positive sarcoma cells (Fig. 2E) and within 36 hours, increased sub-G₁ DNA content (Fig. 2F). Cleaved PARP was also evident within 36 hours of 4-OHT addition (Fig. 2G). Thus, apoptosis of the sarcoma cells was occurring upon *Mdm2* deletion.

Similar to the lymphoma cells, there were more sarcoma cells in the G₂-M phase of the cell cycle 36 hours after CreER^{T2} activation compared to cells with vehicle control (Fig. 2H and Supplementary Fig. S2). Analysis of phosphorylated histone H3 showed no difference in the percentage of cells in M phase between control and CreER^{T2}-activated sarcomas, indicating the increase of cells in G₂-M is likely due to a G₂ cell-cycle arrest. Therefore, when *Mdm2* was deleted in either *p53*^{-/-} sarcoma or lymphoma cells, growth and survival are greatly diminished by a combination of apoptosis and G₂ cell-cycle arrest.

Mdm2 deletion inhibits *p53*^{-/-} T-cell lymphoma growth *in vivo*

To test whether *Mdm2* deletion would impact *p53*-null tumor growth *in vivo*, we injected CreER^{T2} expressing *Mdm2*^{fl/fl}*p53*^{-/-} T-

cell lymphoma cells subcutaneously into nude mice. Following detection of palpable tumors at day 15 (Fig. 3A), tamoxifen or corn oil vehicle control was administered to all mice once daily for 3 days. Lymphomas in the vehicle control group continued to increase in volume, whereas the lymphomas in the mice administered tamoxifen regressed and were still undetectable at day 21 when the first vehicle control treated mouse was sacrificed (Fig. 3A and B). This reduced lymphoma tumor burden in mice receiving tamoxifen resulted in a significant increase in survival compared with mice receiving vehicle control (Fig. 3B).

In a separate cohort of mice, T-cell lymphomas were excised and evaluated 48 hours after the first injection of tamoxifen to activate CreER^{T2} or vehicle control. In mice that received tamoxifen, there were significantly more lymphoma cells that were Annexin-V positive (Fig. 3C), that had increased sub-G₁ DNA (Fig. 3D), and that were in G₂-M of the cell cycle (Fig. 3E and Supplementary Fig. S3). Only trace amounts of Mdm2 protein were detectable in the lymphomas harvested from mice 48 hours after receiving tamoxifen compared with controls (Fig. 3F). However, the lymphomas that ultimately grew in mice receiving tamoxifen in Fig. 3B showed Mdm2 protein was present (Fig. 3G), indicating the tumors formed from cells that had not deleted *Mdm2*. Therefore, deletion of *Mdm2* in *p53*^{-/-} lymphoma *in vivo* causes an increase in cells in G₂-M and apoptosis, dramatically diminishing their growth and prolonging survival of the mice.

Mdm2 deletion in *p53*^{-/-} sarcomas inhibits growth *in vivo*

We next evaluated *Mdm2* deletion in *Mdm2*^{fl/fl}*p53*^{-/-} sarcoma cells *in vivo*. CreER^{T2} expressing *Mdm2*^{fl/fl}*p53*^{-/-} sarcoma cells were injected subcutaneously into nude mice and tamoxifen or corn oil vehicle control was administered. Survival was significantly extended in mice administered tamoxifen to induce *Mdm2* deletion (Fig. 4A), as vehicle-treated sarcoma tumors grew significantly larger more quickly than those that had deleted *Mdm2* (Fig. 4B).

In a separate cohort of mice, 72 hours following the first administration of tamoxifen or vehicle control, sarcomas were excised and evaluated. In mice that received tamoxifen to delete *Mdm2*, there were significantly more sarcoma cells that were Annexin-V positive (Fig. 4C and Supplementary Fig. S4), contained sub-G₁ DNA (Fig. 4D), and had decreased viability (Fig. 4E). There was little *Mdm2* protein in the tumors 72 hours after tamoxifen (Fig. 4F). However, the sarcoma tumors that developed in the mice receiving tamoxifen in Fig. 4A retained *Mdm2* (Fig. 4G). Our data show that similar to *p53*-null lymphoma, loss of *Mdm2* in *p53*^{-/-} sarcoma cells induces apoptosis and significantly inhibits tumor cell growth *in vitro* and *in vivo*.

Mature *p53*-null immortalized fibroblasts also require *Mdm2*

Our data with hematopoietic and nonhematopoietic cancer cells indicate *Mdm2* expression is required for malignant cell survival even when *p53* is absent. To determine whether the effects of *Mdm2* loss extended to noncancerous cells, we evaluated deletion of *Mdm2* in *p53*^{-/-} adult mouse fibroblasts. Activation of CreER^{T2} deleted *Mdm2* in *Mdm2*^{fl/fl}*p53*^{-/-} fibroblasts, resulting in reduced *Mdm2* protein (Fig. 5A). *Mdm2* loss significantly decreased proliferation (Fig. 5B), cell number (Fig. 5C), and viability (Fig. 5D) within 48 hours. Following 24 hours of CreER^{T2} activation, Annexin-V positivity and sub-G₁ DNA content were significantly elevated and this increased over 72 hours (Fig. 5E and F, respectively). Interestingly, as with the

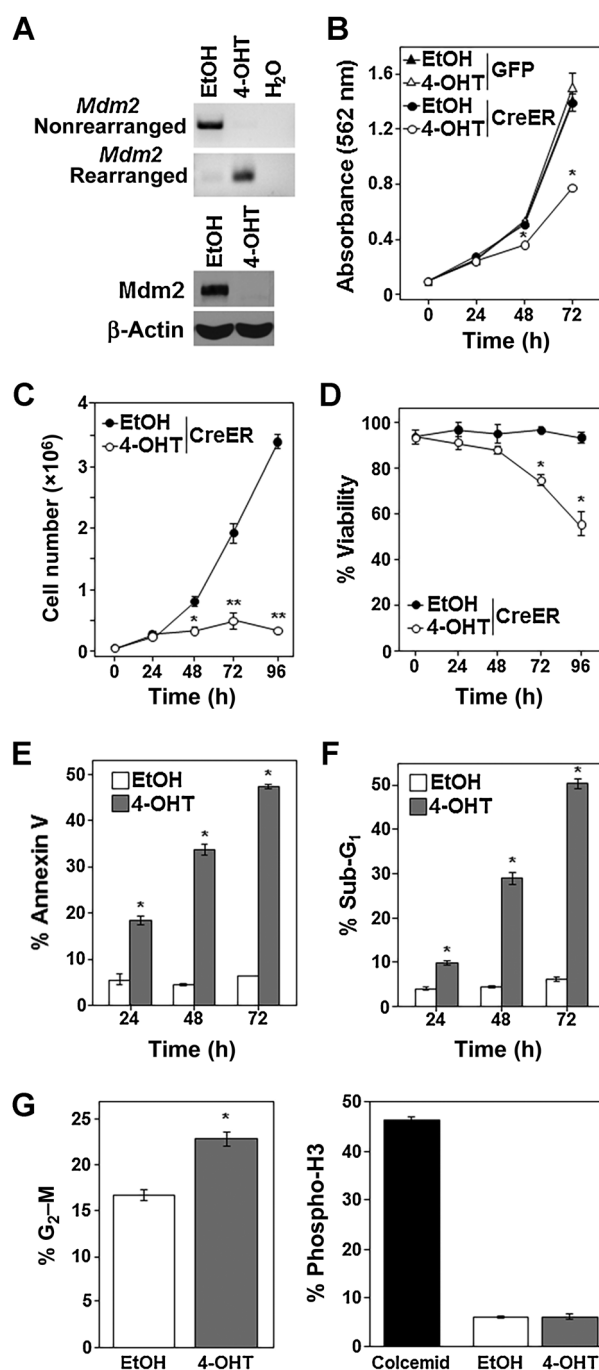


Figure 5. *Mdm2* deletion in nontransformed *p53*-null fibroblasts inhibits growth and survival. Vehicle control (EtOH) or 4-OHT was added to *Mdm2*^{fl/fl}*p53*^{-/-} fibroblasts expressing CreER^{T2} and GFP or GFP alone. **A**, *Mdm2* gene rearrangement PCR (top) and Western blotting (bottom) were performed. **B-F**, Proliferation (MTT assay, quadruplicate; **B**), cell number (**C**), viability (**D**), Annexin-V (**E**), and sub-G₁ DNA (**F**) were measured. **G**, Cells in G₂-M and phospho-histone H3 evaluated 24 hours following 4-OHT or EtOH administration. **C-G**, triplicates. *, *P* < 0.009 (**B**); *, *P* < 0.003, **, *P* < 0.0009 (**C**); *, *P* < 0.006 (**D**); *, *P* < 0.0004 (**E**); *, *P* < 0.00009 (**F**); *, *P* = 0.0042 (**G**).

cancer cells, the immortalized *p53*-null fibroblasts had increased cell numbers in G₂ after *Mdm2* deletion (Fig. 5G). Therefore, loss of Mdm2 results in reduced cell survival characterized by cell-cycle arrest and apoptosis in both malignant and immortalized cells lacking *p53*.

***Mdm2* deletion activates p53/p73 transcriptional target genes, inducing apoptosis and cell-cycle arrest**

Because it was unexpected for loss of Mdm2 to kill *p53*-null cells, to gain insight into the mechanism responsible for the apoptosis induced by Mdm2 loss, we performed RNA-sequencing analysis following CreER^{T2} activation to delete *Mdm2* or vehicle control to leave *Mdm2* intact in all three *p53*-null cell types. Gene ontology analysis showed 52 of the 312 significantly upregulated genes (>two-fold) following *Mdm2* deletion were linked to apoptotic processes ($P = 2.63 \times 10^{-15}$). In addition, gene set enrichment analysis showed genes linked to apoptosis (Fig. 6A and Supplementary Fig. S5), and pathway analysis of the genes significantly upregulated indicated genes involved in apoptosis ($P = 1.19 \times 10^{-7}$). Unexpectedly, pathway analysis also showed genes significantly upregulated in the *Mdm2*-deleted *p53*-null cells were in the p53 pathway ($P = 2.62 \times 10^{-8}$). Gene set enrichment analysis revealed p53-regulated gene sets in all three cell types after *Mdm2* deletion (Fig. 6B and Supplementary Fig. S5). Although the cells were *p53*-null, p53 target genes showed differential expression in cells that deleted *Mdm2* (Fig. 6C). Many of the upregulated genes are linked to apoptosis and cell-cycle arrest (Fig. 6D). In independent experiments, qRT-PCR was used to verify the RNA-sequencing results and evaluate a subset of the upregulated p53 target genes that mediate apoptosis and cell-cycle arrest that spanned the range of expression observed (gray bars in Fig. 6D). Following CreER^{T2} activation to delete *Mdm2* in the lymphoma cells, the proapoptotic Bcl-2 family members, *Bax* and *Puma*, were significantly elevated, whereas *Noxa*, also a pro-apoptotic Bcl-2 family member remained undetectable (Fig. 6E). Levels of the cell-cycle inhibitor, *p21* also significantly increased (Fig. 6E). *Mdm2* deletion in *p53*^{-/-} sarcoma significantly elevated levels of *Bax*, *Noxa*, *Puma*, and *p21* (Fig. 6F), and similar results were obtained from *Mdm2* loss in fibroblasts (Fig. 6G). Our data show *Mdm2* deletion in *p53*^{-/-} lymphoma, sarcoma, and precancerous fibroblasts decreased growth and survival, consistent with the upregulation of proapoptotic and cell-cycle arrest genes known to be transcriptionally regulated by p53.

p73, a p53 family member, can bind Mdm2 and is capable of mediating apoptosis and cell-cycle arrest by transactivating p53 target genes (18–21). Because we observed upregulation of genes transcriptionally regulated by p53 following *Mdm2* deletion in cells lacking p53, we investigated whether p73 was involved in transcriptionally activating these genes. We first evaluated whether p73 levels were altered upon *Mdm2* deletion. Loss of Mdm2 increased p73 protein (Fig. 6H). Next, to test whether p73 was mediating the negative effects of *Mdm2* deletion, we used two different shRNA to knockdown p73 (Fig. 6I). Following CreER^{T2} activation to delete *Mdm2* in the sarcoma cells (Fig. 6I), decreased cell growth was only observed in cells expressing the nontargeting control shRNA (Fig. 6I). In contrast, cells with p73 knockdown grew at the same rate as vehicle control cells (Fig. 6I). In addition, after CreER^{T2} activation, cleaved PARP was absent in the sarcoma cells with p73 knockdown, but present in the nontargeting shRNA controls (Fig. 6I). Evaluation of *Bax*, *Noxa*, *Puma*, and *p21* mRNA levels

showed that following p73 knockdown, *Mdm2* deletion did not increase these mRNA, whereas these mRNA cells with nontargeting shRNA were upregulated (Fig. 6K). Our data indicate the apoptosis and cell-cycle arrest induced by loss of *Mdm2* is transcriptionally mediated by p73 in cells lacking p53.

***Mdm2* deletion is not the same as blocking Mdm2 interaction with p53 family members**

High concentrations (e.g., 30 μmol/L) of Nutlin-3 inhibit the growth and survival of *p53*^{-/-} colon cancer cells, in part by activating p73 (22). We evaluated Nutlin-3 treatment of *Mdm2*^{fl/fl}*p53*^{-/-} sarcoma cells to determine whether the effects of inhibiting Mdm2 interaction with p53 family members pharmacologically were analogous to deleting *Mdm2*. Although neither 10 μmol/L nor 20 μmol/L of Nutlin-3 affected sarcoma proliferation over 72 hours (Fig. 7A), 30 μmol/L of Nutlin-3 significantly inhibited proliferation by 48 hours (Fig. 7B). The decrease in proliferation was not due to apoptosis, as neither cleaved caspase 3 nor cleaved PARP were detected (Fig. 7C), and there was no change in sub-G₁ DNA content after 30 μmol/L Nutlin-3 treatment (Fig. 7D). Mdm2 protein levels were increased in *p53*-null sarcoma cells treated with Nutlin-3 (Fig. 7C), as we and others previously reported in other cell types (11, 34, 35). Notably, sarcoma cells treated with 30 μmol/L Nutlin-3 showed no change in cell number in G₂-M over 48 hours relative to control; however, there was a significant increase of cells in G₁ of the cell cycle (Fig. 7D). Therefore, the increase of cells in G₁ and not apoptosis accounted for the reduction in proliferation caused by Nutlin-3.

It was previously reported that p53 family target genes, *Noxa*, *Puma*, and *p21*, were upregulated upon high-dose Nutlin-3 exposure in *p53*-null colon cancer cells (22). We assessed these mRNA and *Bax* following 30 μmol/L Nutlin-3 treatment in the sarcoma cells. Consistent with the increase of cells in G₁ of the cell cycle and no induction of apoptosis, *p21* expression was elevated 3.3-fold relative to control, but there were no significant changes in the proapoptotic Bcl-2 family genes *Bax*, *Noxa*, or *Puma* mRNA (Fig. 7E). Transcriptional targets of p53/p73 involved in G₂ cell-cycle arrest, including *14-3-3-σ* and *Gadd45a* (36), also remained unchanged following addition of 30 μmol/L Nutlin-3 (Fig. 7E). In contrast, *14-3-3-σ*, *Gadd45a* and *p21* were significantly increased following deletion of *Mdm2* (Fig. 7F). Our results indicate *Mdm2* deletion in *p53*^{-/-} cells inhibits proliferation and induces apoptosis, which is distinct from blocking Mdm2 from binding p53 family members with Nutlin-3, leading to increased levels of Mdm2 and G₁ cell-cycle arrest. Thus, loss of *Mdm2* and elevated levels of Mdm2 unable to interact with p53 family members produce different intracellular responses, with loss of *Mdm2* being cytotoxic in a *p53*-null background.

Discussion

Prior to this study, it was thought that Mdm2 was unnecessary for survival of cells lacking p53. However, our unanticipated results that deletion of *Mdm2* in *p53*-null cells induces apoptosis is a significant, unexpected finding with important clinical ramifications. While inhibiting Mdm2 interaction with p53 family members in p53 wild-type containing tumors is being clinically pursued (37), evidence indicates that resistance develops through p53 inactivation (13–15). In addition, many human cancers

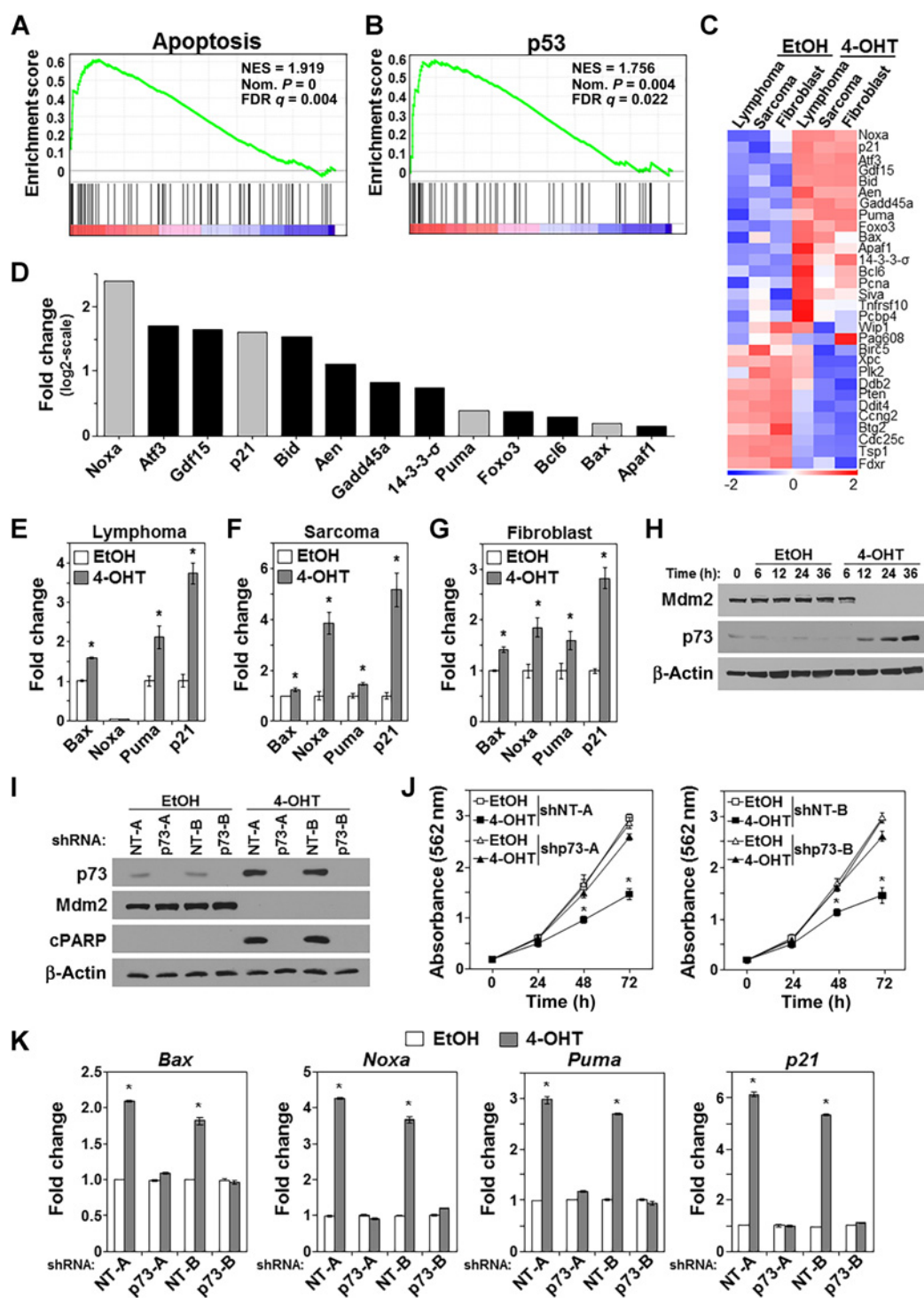


Figure 6.

Mdm2 loss leads to activation of p73. *Mdm2*^{fl/fl}*p53*^{-/-} cells expressing CreER^{T2} received 4-OHT or vehicle control (EtOH). **A-D**, RNA-sequencing was performed after 6 hours (lymphoma) or 48 hours (sarcoma and fibroblasts) of treatment. Gene set enrichment analysis of pooled samples (**A** and **B**). NES, normalized enrichment score; Nom., nominal. P value, false discovery rate (FDR). **C** and **D**, Heatmap of individual samples (**C**) and fold change of significantly increased p53/p73 target genes in 4-OHT-treated pooled samples relative to vehicle-treated pooled samples (**D**). Genes verified with qRT-PCR are in gray. **E-G**, qRT-PCR (triplicate) assessed specific mRNA levels. **E**, *Bax* *, $P = 0.00008$; *Puma* *, $P = 0.045$; *p21* *, $P = 0.0009$; **F**, *Bax* *, $P = 0.035$; *Noxa* *, $P = 0.001$; *Puma* *, $P = 0.003$; *p21* *, $P = 0.0005$; **G**, *Bax* *, $P = 0.0007$; *Noxa* *, $P = 0.009$; *Puma* *, $P = 0.03$; *p21* *, $P = 0.0001$. **H**, Western blotting CreER^{T2} expressing *Mdm2*^{fl/fl}*p53*^{-/-} sarcoma cells after 4-OHT or EtOH. **I-K**, CreER^{T2} expressing *Mdm2*^{fl/fl}*p53*^{-/-} sarcoma cells expressing p73 shRNA (shp73-A or shp73-B) or nontargeting controls (shNT-A or shNT-B). Western blotting following 48 hours of 4-OHT or EtOH (**I**; cPARP, cleaved PARP). Proliferation (**J**; MTT assay, quadruplicate); *, $P < 0.002$. qRT-PCR (triplicate) for mRNA following 48 hours of 4-OHT or EtOH (**K**). *Bax* *, $P < 0.002$; *Noxa* *, $P < 0.0009$; *Puma* *, $P < 0.0007$; *p21* *, $P < 0.0002$.

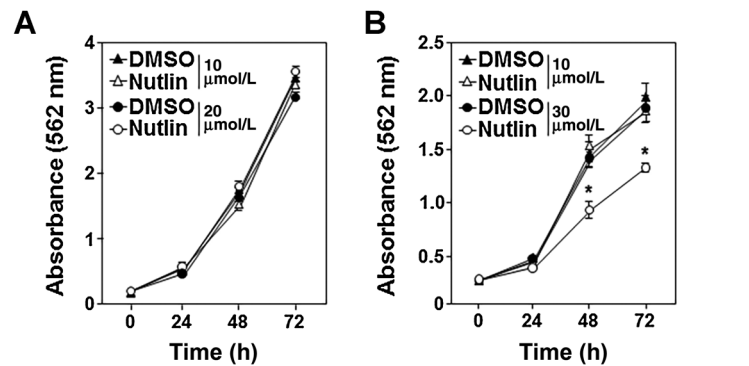
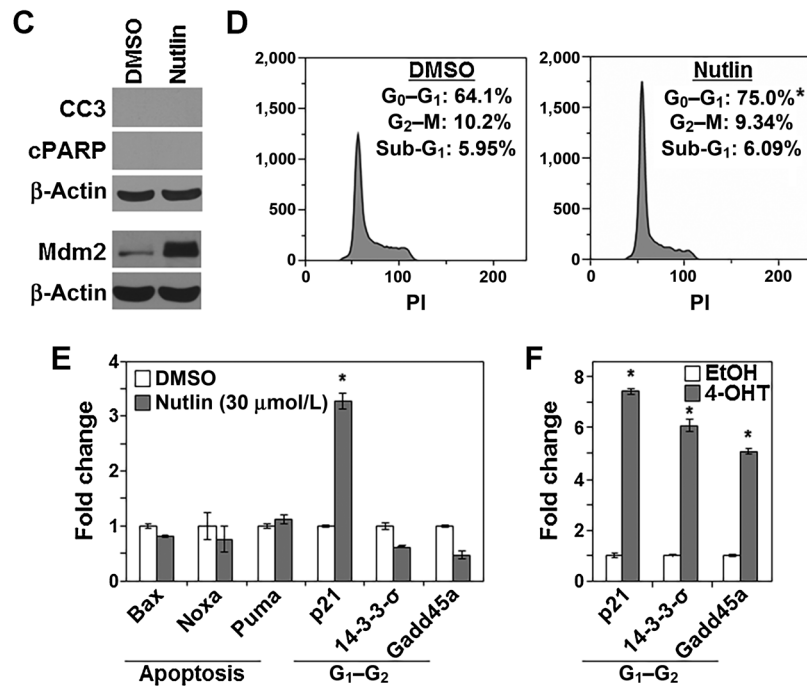


Figure 7.

Inhibition of Mdm2 is not the same as *Mdm2* deletion. **A–E**, *Mdm2^{fl/fl}p53^{-/-}* sarcoma cells treated with the indicated concentrations or 30 $\mu\text{mol/L}$ of Nutlin-3 or DMSO control. **A** and **B**, Proliferation (MTT assays, quadruplicate) measured; *, $P < 0.003$. **C**, Western blots performed following 48 hours of treatment (same lysates run into two different gels). CC3, cleaved caspase-3; cPARP, cleaved PARP. **D**, Cell cycle and sub- G_1 DNA were measured (triplicate) at 48 hours. Representative histograms with mean values inset; G_0 – G_1 , *, $P < 0.0009$; G_2 – M , $P = 0.401$. **E**, qRT-PCR (triplicate) was performed. **F**, CreER^{T2} expressing *Mdm2^{fl/fl}p53^{-/-}* sarcomas received 4-OHT or EtOH control and qRT-PCR (triplicate) was performed. *, $P < 0.0003$ (**E**); *, $P < 0.0006$ (**F**).



present with *p53* deletions (12). Therefore, better treatment options are needed for patients with *p53*-null cancers. We describe a mechanism through which Mdm2 loss in hematopoietic and nonhematopoietic malignancies lacking *p53* results in activation of *p73* and upregulation of its target genes that induce apoptosis and G_2 cell-cycle arrest, causing *p53*-null cancer cells to die. *In vivo*, when *Mdm2* was deleted, *p53*-null lymphoma and sarcoma had dramatically reduced tumor growth, extending survival of the mice. Given this surprising requirement of Mdm2 for *p53*-null cancer cell survival, our results demonstrate that targeting Mdm2, leading to its reduced expression in cancer cells, is likely to be therapeutic in cancers that have deleted *p53*. Furthermore, analogous results were obtained in immortalized *p53*-null cells, suggesting sensitivity for pre-cancerous cells to *Mdm2* loss as well.

Numerous studies show that when wild-type *p53* is present, loss of Mdm2 induces a *p53*-dependent death (2, 3), leading to the belief that Mdm2 is unnecessary if *p53* is inactivated. Therefore, our data showing *Mdm2* deletion in *p53*-null cells led to apoptosis was not predicted. This was not just evident in primary

cell culture, as our *in vivo* experiments showed that although Mdm2 protein was lost initially after receiving tamoxifen to activate CreER^{T2} and delete *Mdm2*, Mdm2 was detectable at levels similar to vehicle-treated controls in the tumors that ultimately emerged. Therefore, the tumors developed from a population of cells that retained Mdm2, indicating Mdm2 expression, is required for the growth and survival of *p53*-null lymphoma and sarcoma *in vivo*. Consistent with this is that *MDM2* is not deleted in any human cancers lacking *p53* (38). We similarly demonstrated that mature fibroblasts lacking *p53* are also dependent on Mdm2 for survival. These results are in contrast to an *in vivo* study showing induced deletion of *Mdm2* in multiple tissues of *p53^{-/-}* mice did not cause tissue defects or lethality of the mice (7). Our data suggest a possible explanation for the discrepancy between these studies. Both the lymphoma and sarcoma cells that survived CreER^{T2} activation *in vivo* had retained Mdm2. Therefore, we postulate in the Zhang and colleagues study that any cells that died from loss of *Mdm2* in the *p53*-null mice were replaced in a timely manner with cells that contained Mdm2, so that no overt tissue damage occurred and mice survived. Another possibility is

that proliferating immortalized or malignant cells may be more reliant on Mdm2 than less proliferative, nontransformed cells *in vivo* and therefore, be more sensitive to Mdm2 loss. Future studies are warranted to further investigate the cell types and conditions that confer sensitivity to Mdm2 loss in the absence of p53.

The p53 family member p73 shares significant homology to p53 and many of the same target genes (16, 17). p73 is rarely inactivated in human cancers, in contrast with the high frequency of p53 mutation or deletion (16). Our data show that Mdm2 loss in p53-null cancer cells upregulates p53/p73 transcriptional targets that mediate cell-cycle arrest (*p21*, *Gadd45a*, and *14-3-3-σ*) and apoptosis (*Bax*, *Puma*, and *Noxa*), both of which occurred. p21 is known to exert its influence at the G₁ phase of the cell cycle, but can also mediate progression through G₂ (39–42). Gadd45a and 14-3-3-σ are established mediators of the p53-induced G₂ checkpoint (36). The proapoptotic Bcl-2 family members, Bax, Puma, and Noxa, are effective at inducing apoptosis (43). Here, we show that Mdm2 loss induces apoptosis and G₂ cell-cycle arrest in p53-null cancer cells and that loss of p73 was sufficient to rescue these effects, indicating that p73 mediates the negative consequences of Mdm2 deletion when p53 is absent. Mdm2 can regulate p73 stability and transcription (18–21), but the conditions in which this occurs remained incompletely resolved. Most importantly, what was not known or predicted that our data revealed was that cells lacking p53 would die when Mdm2 was deleted and that this was mediated by p73. Our data indicate p73 functionally replaced p53 in p53-null cancer cells and mature fibroblasts and consequently, p73 was stabilized and activated upon Mdm2 deletion. Our results also imply that during embryogenesis, p73 does not replace p53, because *Mdm2*^{-/-}*p53*^{-/-} embryos survive; however, additional investigations will be needed to test Mdm2 and p73 interaction during embryogenesis.

Since the discovery of Nutlin-3, a compound that blocks Mdm2:p53 interactions, there has been increased effort examining the therapeutic utility of activating the p53 pathway in tumor cells by blocking Mdm2 binding (37). In addition, compounds that bind the p53-binding pocket of Mdm2 can cause apoptosis and/or cell-cycle arrest in p53-null cancer cells through other mechanisms, such as activation of p73 and inhibition of DNA break repair (22, 34). Nutlin-3 can activate p73 in malignant cells lacking p53, but this required high concentrations of Nutlin-3 (22). Consistent with this report, we observed the standard 10 μmol/L of Nutlin-3 and 20 μmol/L had no effect, but 30 μmol/L induced a G₁ cell-cycle arrest of p53-null sarcoma cells. In contrast to blocking p53 family

members from binding Mdm2, Mdm2 deletion induced a G₂ arrest and apoptosis in p53-null cancer cells, indicating different mechanisms or different target genes involved. We detected differences in the target genes induced by Nutlin-3 and Mdm2 deletion that explain these different outcomes. In addition, we previously reported a side effect of Nutlin-3 binding to Mdm2 is Mdm2 stabilization, leading to increased Mdm2 levels (34), which we also observed in this study. Elevated Mdm2 levels can inhibit DNA break repair (34); thus, stabilization of Mdm2 with compounds, such as Nutlin-3, can have consequences other than p53/p73 activation that can induce different cellular responses. Our data indicate that inhibiting Mdm2 from binding p53 family members and Mdm2 deletion have distinctly different effects, but that both are deleterious for the cell. Collectively, our results demonstrate that p53-null cancer cells require Mdm2 for growth and survival, and consequently, this could be exploited therapeutically.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K.P. Feeley, C.M. Adams, C.M. Eischen

Development of methodology: C.M. Eischen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.P. Feeley, C.M. Adams

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.P. Feeley, C.M. Adams, R. Mitra, C.M. Eischen

Writing, review, and/or revision of the manuscript: K.P. Feeley, C.M. Adams, C.M. Eischen

Study supervision: C.M. Eischen

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