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Recommended Citation
Carr, Michael I.; Roderick, Justine E.; Zhang, Hong; Woda, Bruce A.; Kelliher, Michelle A.; and Jones, Stephen N., "Phosphorylation of the Mdm2 oncoprotein by the c-Abl tyrosine kinase regulates p53 tumor suppression and the radiosensitivity of mice" (2016). UMass Metabolic Network Publications. 7.
http://escholarship.umassmed.edu/metnet_pubs/7

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Keywords
DNA damage, Mdm2, c-Abl, p53, tumorigenesis

Comments
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Phosphorylation of the Mdm2 oncoprotein by the c-Abl tyrosine kinase regulates p53 tumor suppression and the radiosensitivity of mice

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Edited by Carol Prives, Columbia University, New York, NY, and approved November 15, 2016 (received for review July 21, 2016)

The p53 tumor suppressor acts as a guardian of the genome by preventing the propagation of DNA damage-induced breaks and mutations to subsequent generations of cells. We have previously shown that phosphorylation of the Mdm2 oncprotein at Ser394 by the ATM kinase is required for robust p53 stabilization and activation in cells treated with ionizing radiation, and that loss of Mdm2 Ser394 phosphorylation leads to spontaneous tumorigenesis and radioresistance in Mdm2S394A mice. Previous in vitro data indicate that the c-Abl kinase phosphorylates Mdm2 at the neighboring residue (Tyr393) in response to DNA damage to regulate p53-dependent apoptosis. In this present study, we have generated an Mdm2 mutant mouse (Mdm2Y393F) to determine whether c-Abl phosphorylation of Mdm2 regulates the p53-mediated DNA damage response or p53 tumor suppression in vivo. The Mdm2Y393F mice develop accelerated spontaneous and oncogene-induced tumors, yet display no defects in p53 stabilization and activity following acute genotoxic stress. Although apoptosis is unaltered in these mice, they recover more rapidly from radiation-induced bone marrow ablation and are more resistant to whole-body radiation-induced lethality. These data reveal an in vivo role for c-Abl phosphorylation of Mdm2 in regulation of p53 tumor suppression and bone marrow failure. However, c-Abl phosphorylation of Mdm2 Tyr393 appears to play a lesser role in governing Mdm2-p53 signaling than ATM phosphorylation of Mdm2 Ser394. Furthermore, the effects of these phosphorylation events on p53 regulation are not additive, as Mdm2S393F/S394A mice and Mdm2Y393F mice display similar phenotypes.

Significance

The p53 transcription factor is stabilized in response to cellular stress and regulates the expression of genes involved in numerous biological activities, thereby suppressing tumorigenesis. DNA damage and other stress signals upregulate p53, in part, by freeing p53 from negative regulation imposed by the Mdm2 and MdmX (Mdm4) oncoproteins. MDM proteins are subject to posttranslational modification, and accumulating evidence indicates that phosphorylation of Mdm2 by different stress-activated kinases such as ATM or c-Abl alters Mdm2-p53 signaling and profoundly affects p53 function. A better understanding of the in vivo effects of Mdm2 phosphorylation may facilitate the development of novel therapeutics capable of stimulating p53 antitumor activity or alleviating p53-dependent toxicities in nonmalignant tissues.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1611798114/-/DCSupplemental.
coexpression of c-Abl overcomes MDM2-mediated ubiquitination and nuclear export of p53 (20). c-Abl phosphorylates MDM2 Tyr394 as well as Tyr276 and Tyr405 (12, 13), and c-Abl phosphorylation of MDM2 Tyr394 impairs the ability of MDM2 to inhibit p53 stabilization and transactivation and p53-mediated apoptosis (12). More recently, it was proposed that c-Abl phosphorylation of MDM2 increases MDM2–MDMX binding and promotes MDM2-directed MDMX ubiquitination, and that this ultimately destabilizes the MDM2–MDMX complex, promoting p53 stabilization (21).

As we have shown that ATM phosphorylation of Mdm2 Ser394 profoundly impacts the p53 response to DNA damage in mice, we sought to determine whether c-Abl phosphorylation of Mdm2 Tyr393 similarly regulates p53 functions in vivo. To this end, we generated a knockin mouse model in which Mdm2 Tyr393 is substituted with phenylalanine (Mdm2Y393F), as well as a mouse in which both the c-Abl target residue Mdm2 Tyr393 and the adjacent ATM target residue Mdm2 Ser394 are mutated (Mdm2Y393F/S394A), enabling the study of whether phosphorylation of these residues has additive or redundant effects.

**Results**

**Mdm2Y393F** Mice Are Viable and Display Increased Spontaneous and Oncogene-Induced Tumorigenesis. To investigate the role of Mdm2 Tyr393 phosphorylation under physiological conditions, we generated a mouse model in which this tyrosine residue is substituted with a phenylalanine residue (Y393F). Site-directed mutagenesis was performed to introduce an A-to-T missense mutation in the 393 codon, and a synonymous G-to-C mutation in the 397 codon of Mdm2 exon 12 (Fig. 1A). A gene-replacement vector was constructed to replace the endogenous Mdm2 exon 12 sequences with the mutated exon 12 (Fig. S1A). Gene targeting was performed in PC3 (1295V) embryonic stem (ES) cells (22), and homologous recombination was confirmed in G418-resistant clones by Southern blotting (Fig. S1 A–C). Blastocyst injection of targeted ES clones produced several high-degree male chimeras that passed the Mdm2 Y393F allele through their germ line. Southern blotting further confirmed proper targeting in F1 and F2 generation mice, along with protamine-Cre-directed deletion of the floxed neomycin cassette (Fig. S1 D and E). The presence of the additional synonymous G-to-C mutation within the 397 codon of the targeted allele introduced a novel BssHII restriction digest site that allowed for identity confirmation by PCR-digest strategy (Fig. L4 and Fig. S1F). Mdm2 transcripts from spleens of Mdm2Y393F mice were sequenced and confirmed as containing only the targeted mutations (Fig. L4). Heterozygous intercrosses yielded homozygous Mdm2Y393F mice at Mendelian ratios, indicating that Mdm2Y393F is not compromised in its function during development (Fig. SIG).

Additionally, no differences were observed in average litter size, body weights at 6 wk of age, or male-to-female sex distribution.

We have previously shown that mice deficient for phosphorylation of the neighboring residue (Mdm2 Ser394) by ATM are prone to spontaneous tumorigenesis (11). Therefore, we sought to examine whether c-Abl phosphorylation of Mdm2 Tyr393 impaired tumor suppression. Cohorts of WT and Mdm2Y393F mice were established and monitored for tumor formation. During the 24-mo tumor assay, 8 of 21 (38%) Mdm2Y393F mice developed spontaneous tumors (Fig. 1B), whereas only 1 of 28 (4%) WT mice presented with a tumor at 20 mo of age. Mdm2Y393F tumors arose between 14.5 and 24 mo of age, a similar latency as seen in Mdm2Y393A mice (11).

The majority of tumors arose in lymphatic tissues, and 5 of the 6 tumors (83%) were identified as B-cell lymphomas, with one of the B-cell lymphomas also containing atypical T cells. Other tumor types seen in the cohort included a myeloid sarcoma, a hepatocellular carcinoma, and a papillary tumor of lacrimal origin (Fig. 1 C–E).

As Mdm2Y393F mice were more prone to develop spontaneous tumors of lymphoid origin, we further examined the effects of Mdm2-Y393 phosphorylation on tumor suppression using the Eμ-Myc mouse model (23). Cohorts of Eμ-Myc and Eμ-Myc;
Mdm2<sup>Y393F</sup> mice were exposed to 5 Gy IR and spleens were analyzed for p53 and Mdm2 levels by immunoblotting (Fig. 2A). In both genotypes, c-Abl cleavage was observed in response to IR, similar to previous observations in cells undergoing stress-induced apoptosis (25, 26). c-Abl phosphorylation at Tyr245 confirmed kinase activation. No qualitative differences were detected in basal or IR-induced p53 protein levels. Similarly, no obvious differences were observed in p53 activation as indicated by levels of p53-Ser18 phosphorylation. No differences were observed in the levels of Mdm2 protein, which increased at 2-4 h following IR, before decreasing as p53 activity diminished, similar to what has previously been reported in this tissue (17). The lack of a difference in p53 stabilization was confirmed quantitatively in biological triplicates at 0, 2, and 4 h (Fig. S2A). Similar results were observed in thymi from these same animals, with no qualitative differences detected in the levels of total p53, phosphorylated p53 (S18), or Mdm2, for slightly higher basal Mdm2 levels in Mdm2<sup>Y393F</sup> mice (Fig. S2B). Consistent with equal levels of basal and IR-induced p53 protein stabilization and phosphorylation, real-time quantitative PCR (RT-qPCR) performed on cDNA from untreated and irradiated spleens of WT and Mdm2<sup>Y393F</sup> mice detected no differences in the basal or DNA damage-induced expression levels of a selection of p53-target genes involved in growth arrest, apoptosis, DNA repair, or metabolic regulation (Fig. 2B). Similar results were observed in thymi (Fig. S2C).

Hematopoietic organs are highly radiosensitive, and p53 activation in these tissues triggers widespread apoptosis following IR (28). In keeping with the absence of observable differences in p53 stabilization and activation in Mdm2<sup>Y393F</sup> spleens and thymi, we detected no differences in splenic or thymic apoptosis in untreated and irradiated Mdm2<sup>Y393F</sup> mice detected no differences in the basal or DNA damage-induced expression levels of a selection of p53-target genes involved in growth arrest, apoptosis, DNA repair, or metabolic regulation (Fig. 2B). Similar results were observed in thymi (Fig. S2C).

We also examined whether Mdm2 Tyr393 phosphorylation affected p53-dependent growth arrest. MEFs from WT, Mdm2<sup>Y393F</sup>, and p53<sup>−/−</sup> mice were untreated or exposed to either 5 Gy IR or 150 nM doxorubicin for 18 h. Flow cytometric cell-cycle analysis revealed no differences in growth arrest in Mdm2<sup>Y393F</sup> MEFs following either irradiation or treatment with doxorubicin (Fig. S2G). Furthermore, WT and Mdm2<sup>Y393F</sup> MEFs exhibited similar growth rates in standard cell proliferation assays (Fig. S2F).

**Mdm2<sup>Y393F</sup> Mice Are Radioreistant and Display Enhanced Bone Marrow Repopulating Abilities Following IR Exposure.** The proposed effects of c-Abl phosphorylation of Mdm2 on p53 are similar to those of ATM phosphorylation of Mdm2, namely p53 stabilization and activation. Conversely, our findings that DNA damage-induced p53 stabilization and activation in spleen and thymus, and p53-dependent apoptosis and growth arrest in Mdm2<sup>Y393F</sup> tissues and cells, are unaltered contrasts with what we have reported with Mdm2<sup>Y394A</sup> mice. Thus, c-Abl’s phosphorylation of Mdm2-Y393 may either have limited effect on the p53-dependent DDR or may be adequately compensated for by ATM phosphorylation of Mdm2 Ser394. However, when we challenged cohorts of WT and Mdm2<sup>Y393F</sup> mice to a series of IR doses spanning the threshold-lethal range, we observed a significant resistance to whole-body IR-induced lethality in Mdm2<sup>Y393F</sup> mice (Fig. 3A). Following treatment with 8 Gy IR, 74% of Mdm2<sup>Y393F</sup> mice survived to 4 wk compared with 22% survival of WT mice. Similarly, 29% of Mdm2<sup>Y393F</sup> mice survived to 4 wk after exposure to 9 Gy IR compared with 7% of WT mice. All mice survived 7 Gy IR for both genotypes, whereas no mice of either genotype survived past 2 wk following 10 Gy IR. The majority of mice succumbed between 1 and 3 wk post-IR. Lethality in mice treated with this range of IR doses is routinely attributed to p53-dependent bone marrow failure, referred to as “hematopoietic syndrome” (29). As we have described a similar resistance to whole-body IR in Mdm2<sup>Y394A</sup> mice (11), we next compared the effects of IR on bone marrow from WT, Mdm2<sup>Y393F</sup>, Mdm2<sup>S394A</sup>, and p53<sup>−/−</sup> mice. Expression of p53-target genes Puma and p21 are unaltered in Mdm2<sup>Y393F</sup> bone marrow, in agreement with what was observed in irradiated spleen and thymus (Fig. S3A). Conversely, expression levels of those same target genes are significantly reduced in Mdm2<sup>Y394A</sup> bone marrow, in keeping with the previously described reduced IR-induced p53 responses in these animals (11). However, similar initial reductions in bone marrow cellularity were observed in both mutants, as well as in WT mice and in p53<sup>−/−</sup> mice following treatment with 5 Gy IR (Fig. 3B). Thus, the initial reduction in gross cellularity appears, to some extent, to be independent of p53. Flow cytometry analysis of untreated bone marrow from WT, Mdm2<sup>Y393F</sup>, Mdm2<sup>S394A</sup>, and p53<sup>−/−</sup> mice revealed no differences in the populations of lineage-defined, mature hematopoietic cells, save for statistically fewer Cd11b<sup>+</sup> cells in p53<sup>−/−</sup> mice (Fig. S3B). In agreement with the observed reduction in bone marrow cellularity in all genotypes, IR treatment induced a cumulative decline in populations of mature cells in bone marrow from each genotype (Fig. S3C).
Only p53−/− mice displayed significantly more B220+ cells. Again, no differences were observed in the absence of treatment, in the more primitive Lin− Sca1+ cKit+ (L− S− K+) progenitor populations of WT, Mdm2Y393F/S394A, or p53−/− mice (Fig. 3D). However, whereas L− S− K+ cell numbers decreased 70–80% in WT, Mdm2Y393F, and Mdm2S394A bone marrow, bone marrow from p53−/− mice retained significantly more L− S− K+ cells following IR. This finding suggests that increased survival of hematopoietic progenitors underlies the resistance to IR-induced bone marrow failure in mice with compromised p53. Indeed, when Lin− Sca1+ cKit+ (L− S− K−) hematopoietic stem and progenitor cells (HSPCs) were quantified in these same animals, we observed no decrease following irradiation in p53−/− mice (Fig. 3C). Whereas greater numbers of HSPCs were observed in untreated p53−/− mice, no differences were observed in HSC number in untreated WT, Mdm2Y393F, and Mdm2S394A mice. However, there were significantly more HSPCs in Mdm2S394A bone marrow following IR, and HSPC levels also appeared slightly (although not statistically) elevated in irradiated Mdm2Y393F/S394A bone marrow. We examined whether the resistance to acute whole-body IR-associated lethality observed in Mdm2Y393F and Mdm2S394A mice was due to an increased capacity to repopulate irradiated marrow by performing H&E stains on bone marrow from mice either untreated or 6 and 9 d following 8 Gy IR (Fig. 3D). At 6 d post-IR, whereas mice of all three genotypes displayed evidence of a significant decrease in cellularity, both p53−/− and Mdm2Y393F/S394A bone marrow, and to a lesser extent Mdm2S394A bone marrow, contained multiple colonies of hematopoietic cells that were not apparent in WT bone marrow, as well as visibly more erythrocytes. By 9 d post-IR, coinciding with the period of observed morbidity in threshold-lethally irradiated animals, an even larger discrepancy in cellularity was observable between WT and mutant bone marrows. Few hematopoietic colonies were visible in WT marrow, whereas colonies present in p53−/− and Mdm2Y393F/S394A mice had expanded significantly and largely replenished the medullary cavity. Whereas there was greater visible repopulation in Mdm2Y393F bone marrow compared with WT, this repopulation appeared intermediate to that observed in Mdm2S394A bone marrow, trending with the observed improved HSPC survival following IR.

**Fig. 3.** Mdm2Y393F mice are radioresistant and display improved bone marrow repopulation following IR exposure. (A) Kaplan–Meier survival curves of WT (n = 7–18) and Mdm2Y393F (n = 6–19) mice exposed to 7, 8, 9, and 10 Gy whole-body IR. WT and Mdm2Y393F mice were compared by log-rank test: 7 Gy (n.s.), 8 Gy (P = 0.003), 9 Gy (P = 0.030), and 10 Gy (n.s.). (B) Quantification of numbers of nucleated cells in bone marrow from both hindlimbs of WT, Mdm2Y393F, Mdm2S394A, and p53−/− mice either untreated or 8 h after exposure to 5 Gy IR. (n = 3–6, ± SEM). *P < 0.05, **P < 0.01 (Student’s t-tests). (C) Quantification of LSK HSPCs in bone marrow of WT, Mdm2Y393F, Mdm2S394A, and p53−/− mice treated as described in B. *P < 0.05, **P < 0.01 (Student’s t-tests). (D) H&E stained bone marrow from WT, Mdm2Y393F, Mdm2S394A, and p53−/− mice exposed to 8 Gy IR. Yellow arrows indicate nascent hematopoietic cell colonies. (Scale bars, 100 μm.)
staining followed by flow cytometry of Mdm2<sup>Y393F</sup>/S394A thymi relative to those seen in Mdm2<sup>S394A</sup> thymi (Fig. S5 C and D).

Finally, we examined whether the radioresistant phenotypes observed in Mdm2<sup>Y393F</sup> and Mdm2<sup>S394A</sup> mice were exacerbated in Mdm2<sup>Y393F/S394A</sup> mice. Mdm2<sup>S394A</sup> and Mdm2<sup>Y393F/S394A</sup> mice were exposed to 9 Gy whole-body IR (as used for Mdm2<sup>Y393F</sup> mice) and monitored for signs of morbidity (Fig. 4F). No significant difference in survival was observed between wild-type and Mdm2<sup>Y393F/S394A</sup> and Mdm2<sup>Y393F/S394A</sup> mice at this dose, with 82% of Mdm2<sup>S394A</sup> mice and 69% of Mdm2<sup>Y393F/S394A</sup> mice surviving at 4 wk, respectively. However, both genotypes are significantly more radioresistant than Mdm2<sup>Y393F</sup> mice, which are themselves significantly more radioresistant than WT mice at this dose. Thus, radioresistance resulting from the loss of either Mdm2 Tyr393 phosphorylation by c-Abl or Mdm2 Ser394 phosphorylation by ATM is not increased by mutation of both phospho-target residues. Accordingly, IR-induced expression levels of p53-target genes in irradiated bone marrow of Mdm2<sup>S394A</sup> and Mdm2<sup>Y393F/S394A</sup> mice are similarly reduced (Fig. S6A). The equivalent deficiency in the p53 response in Mdm2<sup>S394A</sup> and Mdm2<sup>Y393F/S394A</sup> bone marrow follows with comparatively increased numbers of LSK HSPCs surviving in Mdm2<sup>S394A</sup> and Mdm2<sup>Y393F/S394A</sup> bone marrow following IR (Fig. 4G). Furthermore, bone marrow cells from Mdm2<sup>Y393F/S394A</sup> mice exhibit similar hematopoietic repopulating abilities in vivo (Fig. S6B). As was observed with Mdm2<sup>S394A</sup> and Mdm2<sup>Y393F</sup> mice, no differences were seen in the number of lineage-defined or LSK hematopoietic cells (Fig. S6 C and D).

Discussion

These findings provide further evidence of the significance of phosphorylation of Mdm2 by DNA damage-activated kinases in regulating p53-dependent organismal responses. Mdm2<sup>Y393F</sup> mice are viable and display no developmental defects, yet they are significantly more prone to developing spontaneous tumors over their lifespan (Fig. 1B). The spontaneous tumors arising in Mdm2<sup>Y393F</sup> mice are primarily hematopoietic in nature and led us to examine whether Mdm2 Tyr393 phosphorylation impacted oncogene-induced tumorigenesis using the Eμ-Myc allele. Indeed, Eμ-Myc/Mdm2<sup>Y393F</sup> mice developed tumors at a significantly accelerated rate (Fig. 1F).

The increased spontaneous tumorigenesis rates and accelerated B-cell lymphomagenesis are a likely result of reduced p53 activity in Mdm2<sup>Y393F</sup> and Eμ-Myc/Mdm2<sup>Y393F</sup> mice, as p53-dependent apoptosis and senescence have been shown to inhibit spontaneous tumorigenesis and B-cell tumors induced by aberrant Myc activity (24, 30–32). However, we observed no defects in p53-dependent apoptosis in spleens or thymi of Mdm2<sup>Y393F</sup> mice following IR, or p53-dependent growth arrest in MEFs treated with IR or doxorubicin (Fig. 2 and S2). It is conceivable that Mdm2 phosphorylation by c-Abl can regulate p53 tumor suppressing effects other than apoptosis or growth arrest, and/or that subtle differences in these p53 functions in Mdm2<sup>Y393F</sup> mice are not detectable by examining the effects of acute damage on p53 tumor suppression. Furthermore, it is possible that additional c-Abl target residues on Mdm2 can compensate for the loss of Mdm2 Tyr393 phosphorylation (12, 13).}

Seemingly incongruously, Mdm2<sup>Y393F</sup> mice are resistant to threshold-lethal doses of radiation (Fig. 3A). This radioresistance parallels our previous observations with Mdm2<sup>S394A</sup> mice, which display profound defects in p53-dependent apoptosis and tissues. HSPC or bone marrow cells from both mutants display improved repopulating functions following IR exposure, albeit to a greater extent in Mdm2<sup>S394A</sup> mice (Fig. 3D). In keeping with this difference, HSPCs in Mdm2<sup>S394A</sup> bone marrow display a significant survival advantage following IR, whereas Mdm2<sup>Y393F</sup> HSPCs display only a marginal increase in survival after DNA damage. However, the slight increase in the survival of Mdm2<sup>Y393F</sup> HSPCs manifests increased radioresistance in Mdm2<sup>Y393F</sup> mice relative to WT mice.
The generation of  \( \text{Mdm}^2Y393F/S394A \) mice allowed us to examine whether the common effects of Mdm2 Tyr393 phosphorylation by c-Abl and Mdm2 Ser394 phosphorylation by ATM are additive or redundant. We observed no additive or synergistic effects of the loss of both phosphorylation events on the incidence of spontaneous tumorigenesis, with  \( \text{Mdm}^2Y393F/S394A \) mice developing spontaneous tumors at a frequency and latency that nearly overlap with what we have reported with  \( \text{Mdm}^2Y393F \) mice (Fig. 4B). Similarly, we observed no additive effects on radiosensitivity, with  \( \text{Mdm}^2Y393F/S394A \) and  \( \text{Mdm}^2S394A \) mice displaying comparable survival, HSPC numbers, and bone marrow reconstitution following whole-body IR (Fig. 4 F and G and Fig. S6). Hence, there is an apparent redundancy of the shared phenotypes between  \( \text{Mdm}^2Y393F \) and  \( \text{Mdm}^2S394A \) mice, with tumorigenesis and radioresistance in  \( \text{Mdm}^2Y393F/S394A \) mice never exceeding that observed in  \( \text{Mdm}^2S394A \) mice. This may reflect the proposed interdependence of c-Abl and ATM for their respective activities (16, 33, 34). However, ATM phosphorylation of Mdm2 Ser394 clearly has a predominant effect on Mdm2-p53 signaling and p53 functions, relative to the effects induced by c-Abl phosphorylation of Mdm2 Tyr393.

**Methods**

**Mice and Animal Studies.** All animals described in this study were on a C57BL/6 background. Mice and cells were irradiated with a cesium-137 source (Gammacell 40). The generation of  \( \text{Mdm}^2Y3946 \) mice has previously been described (11).  \( \text{E}_{\text{nu}}-\text{Myc} \) mice were a gift from Christine Eischen, Vanderbilt University, Nashville, TN. A detailed description of the generation and genotyping of  \( \text{Mdm}^2Y393F \) and  \( \text{Mdm}^2S394A \) mice is provided in SI Methods. All animals used in this study were maintained and assayed in accordance with federal guidelines and those established by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (UMMS).

**Immunoblotting.** Tissues and cells were lysed in Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors. A detailed description of the methods used, including antibodies and clones, is provided in SI Methods.

**Gene Expression Analysis and Sequencing.** Total RNA was isolated from tissues by RNAeasy Mini Kit (Qiagen) and cDNA synthesized by the SuperScript III First Strand Synthesis System (Invitrogen). qPCR was performed using SYBR Select Master Mix (Applied Biosystems) in conjunction with a 7300 Real-Time PCR System (Applied Biosystems). A detailed description of the methods used for qPCR and sequencing is provided in SI Methods.

**Histopathology.** Tissue samples were fixed in 10% (vol/vol) formalin for 24 h. The UMMMS Morphology Core Laboratory performed embedding, sectioning, and staining. TUNEL staining was performed using the In Situ Cell Death Detection Kit, POD (Roche) according to manufacturer’s instructions. Immunohistochemistry was performed with antibodies specific for B220 (S50286; BD Pharmingen) and CD3 (A0452; Dako). Naphthol chloroacetate esterase staining was performed to detect cells with myeloid differentiation. Stained tissue was analyzed using an Olympus CX41 microscope fitted with a PixeLINK camera and software.

**Bone Marrow Analysis.** Total bone marrow from both hind limbs was harvested, RBCs were lysed, and single-cell suspensions were stained with cell-surface antibodies for Gr-1, CD11B, CD3, and B220. All samples were run on a BD LSRII flow cytometer (BD Biosciences) and analyzed using Flowjo software (Tree Star). A complete list of antibodies including clone numbers is given in Table S1.

**Statistical Analysis.** Statistical analyses were performed using GraphPad Prism software, version 6.0d. Kaplan–Meier survival curves were analyzed by log-rank test. A P value of <0.05 was considered statistically significant for Student t tests.

**ACKNOWLEDGMENTS.** This research was supported by NIH Grants R01-CA077735 (to S.N.J.) and R01-CA096899 (to M.A.K.). J.E.R. was supported by Postdoctoral Fellowship 125087-PF-13-247-01-LIB from the American Cancer Society.