Vitamin D₃ Produced by Skin Exposure to UVR Inhibits Murine Basal Cell Carcinoma Carcinogenesis

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The effect of UVR on human basal cell carcinoma (BCC) epidemiology is complex—the incidence rises until approximately 30,000 hours of lifetime sunlight exposure and then plateaus. We hypothesize that UVR has opposing effects on BCC carcinogenesis—stimulatory via mutagenesis and inhibitory via production of hedgehog-inhibiting vitamin D₃ (D₃). We find that UVR exposure of ionizing radiation-treated Ptch1¹⁺⁻ mice accelerates BCC carcinogenesis in male mice, in which UVR does not produce D₃. By contrast, in female mice, in which UVR does produce D₃, UVR fails to accelerate BCC carcinogenesis, thus mirroring the plateauing in humans. However, if D₃ production is attenuated in female mice by deletion of keratinocyte lathosterol 5-desaturase, then UVR accelerates ionizing radiation-induced BCC carcinogenesis. Congruently, chronic topical application of D₃ inhibits ionizing radiation-induced BCC tumorigenesis. These findings confirm that UVR-induced production of D₃ in keratinocytes significantly restrains murine BCC tumorigenesis and demonstrate the counterintuitive conclusion that UVR has anti-BCC carcinogenic effects that can explain, at least in part, the complex relationship between exposure to UVR and BCC incidence.

INTRODUCTION

The epidemiologic relationship between UVR exposure and basal cell carcinoma (BCC) incidence is complex—the incidence of BCCs rises until approximately 30,000 hours of exposure and then plateaus (Kricker et al., 1995; Rosso et al., 1996a). By contrast, the incidence of cutaneous squamous cell carcinomas (SCCs) is proportional to lifetime hours of sunlight, and the incidence rises particularly rapidly as the cumulative lifetime sun exposure approaches 100,000 hours (Rosso et al., 1996b). Thus, in America, the SCC to BCC ratio is considerably higher in the South than in the North. Furthermore, protection against sun damage reduces the incidence of BCCs less than it reduces that of SCCs (Pandey et al., 2005; van der Pols et al., 2006). Nonetheless, both BCCs and SCCs of the skin have extremely high rates of mutations, and in both the majority of these are of the UVR signature type (Jayaraman et al., 2014). The pivotal molecular abnormality underlying all BCCs is aberrant upregulation of hedgehog (HH) signaling (Epstein, 2008). The Ptch1¹⁺⁻ mouse is a highly useful animal model for the study of environmentally induced murine BCC carcinogenesis (Aszterbaum et al., 1999). Unperturbed, these mice develop few BCCs. If exposed to environmental insult, they uniformly develop many skin tumors. With a single dose of ionizing radiation, all visible tumors are BCCs or BCC-like; with chronic UVR, BCCs do form but many of the visible tumors are SCCs or spindle cell tumors (fibrosarcomas). Conditional deletion of Tp53 in keratinocytes in Ptch1¹⁺⁻ K14-CreERT² p53⁰⁰⁰⁰ (PF) mice (Wang et al., 2011) accelerates BCC carcinogenesis so that multiple visible BCCs (vBCCs) appear as early as age 5 months. This model retains clinical relevance, because (i) BCCs arise on chronically sun damaged skin in which many cells carry mutant Tp53 (Jonason et al., 1996) and (ii) human BCCs frequently carry mutations of Tp53, along with those of PTC1 and SMOOTHENED (Ling et al., 2001).

In 1941, Apperly suggested that the previously known latitudinal gradient of cancer mortality is due to differing exposure to ambient sunlight (Apperly, 1941), and in 1980 the Garland brothers suggested that the mechanism underlying this association is the anticancer effect of vitamin D₃ (D₃) (Garland and Garland, 1980). Humans have two major sources of D₃: dietary and “endogenous”; the latter is produced in the skin by UVR conversion of 7-dehydrocholesterol (7-DHC) to pre-D₃. Skin-produced D₃ can then be transferred to the circulation bound to the D₃-binding protein and can be converted to 25-hydroxy-D₃ (25(OH)D₃) in the liver and then to 1,25(OH)₂D₃ in the kidney. The circulating levels of the relatively longer-lasting 25(OH)D₃ are the most commonly used indicator of body D₃ status. Essentially all past studies of the putative anticancer efficacy of D₃ have focused on the transcriptional effects of 1,25(OH)₂D₃ bound to the D₃

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Abbreviations: 7-DHC, 7-dehydrocholesterol; 25(OH)D₃, 25-hydroxy-vitamin D₃; BCC, basal cell carcinoma; CHORI, Children’s Hospital Oakland Research Institute; D₃, vitamin D₃; ER, estrogen receptor; HH, hedgehog; µBCC, microscopic basal cell carcinoma; PF, Ptch1¹⁺⁻ K14-CreERT² p53⁰⁰⁰⁰ mice; PF Sc5d, Ptch1¹⁺⁻ K14-CreERT² p53⁰⁰⁰⁰Sc5d⁰⁰⁰⁰ mice; Sc5d, lathosterol 5-desaturase; SSC, squamous cell carcinoma; vBCC, visible basal cell carcinoma

Received 17 September 2016; revised 16 May 2017; accepted 21 May 2017; accepted manuscript published online 31 July 2017; corrected proof published online 26 October 2017

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receptor. By contrast, in 2006, Bijlsma et al. identified unhydroxylated D₃ as an inhibitor of HH signaling (Bijlsma et al., 2006). Furthermore, Dormoy et al. (2012) found that the ability of D₃ to inhibit HH signaling is limited to unhydroxylated D₃; 25(OH)D₃, 1,25(OH)₂D₃, and 7-DHC are far less effective HH inhibitors. We reported that topical D₃ (26 µg/mouse/d) can inhibit HH signaling acutely when applied directly to murine BCCs (Tang et al., 2011). UVR-induced or topically applied D₃ exposes peripheral tissues, in particular the skin, to considerably higher levels of unhydroxylated D₃ than does oral D₃ ingestion because of the efficient hydroxylation of dietary D₃ during transit through the liver after absorption in the small intestine. We report herein studies that address our hypothesis that UVR-produced skin D₃ significantly inhibits BCC carcinogenesis and thereby contributes to the complex relationship between sun exposure and BCC epidemiology.

RESULTS
See Supplementary Table S1 online for details of mouse cohorts.

Topical but not oral vitamin D₃ delays BCC carcinogenesis
To study the effect of D₃ on BCC carcinogenesis, we treated D-depleted PF mice of mixed genetic background topically with 0.38 µg/mouse/D₃, an amount that in short-term pilot studies in D-depleted mice produced essentially the same circulating blood level of D₃ (13.4 ng/ml) as was produced by studies in D-depleted mice produced essentially the same with 0.38 g/mouse/d. To investigate further the effect of D₃ on cell proliferation, we tested its effect in vitro on cells grown from PF mouse BCCs. D₃ or the small molecule HH signaling pathway inhibitor XL-139 reduced cell proliferation significantly (P < 0.05) as monitored by the water-soluble tetrazolium-1 assay (Figure 2c). This reduction in cell proliferation was associated with downregulation of Gli1 expression by D₃ (Figure 2d), an association that is consistent with the hypothesis that D₃ inhibits BCC proliferation at least in part by suppressing HH signaling. However, the data suggest that D₃ inhibits proliferation as much as does XL-139 despite achieving somewhat less HH inhibition. Thus, D₃ may inhibit proliferation by mechanism(s) in addition to its HH inhibiting activity.

The effects of UVR on BCC carcinogenesis correlate inversely with D₃ production in male versus female PF mice
We treated PF mice fed a normal D₃ diet (1,500 IU/kg chow) with 4 Gy ionizing radiation at age 8 weeks to induce BCC carcinogenesis (Wang et al., 2011) and then with 350 mJ/cm² of UVR three times per week from age 10 weeks until death (Supplementary Table S1). As reported in mice of other genotypes (Gorman et al., 2012; Thomas-Ahner et al., 2007; Xue et al., 2015), in our study acute UVR treatment markedly increased circulating D₃ levels (assessed 6 hours after the third of three every other day treatments) in female (44 ± 11 vs. 4 ± 2 ng/ml, P = 0.000003) but not in male (4.4 ± 1.5 vs. 2.7 ± 1.4 ng/ml, P = 0.08) PF mice (Figure 3a), a difference that has been reported to correlate with lower concentrations of 7-DHC in the skin of male than that of female mice (Xue et al., 2015). Therefore, we compared the effects of UVR on BCC carcinogenesis in male versus female mice to correlate UVR’s production of D₃ with its anti-BCC carcinogenesis efficacy. We found that UVR treatment of PF male mice significantly (P = 0.036) increased µBCC numbers (Figure 3b), shortened the time until mice developed detectable vBCCs (P = 0.047), shortened overall survival (P = 0.004), and increased in later life the numbers of vBCCs per mouse (P ≤ 0.05) (Figure 3c–e). These data are consistent with the hypothesis that the procarcinogenic effects of UVR are unopposed in male mice unable to produce D₃ in the skin. By contrast, UVR treatment of PF female mice affected none of these four outcomes (Figure 3b, 3f–h), data that are consistent with the hypothesis that the procarcinogenic effects of UVR are blunted in female mice by the anticarcinogenic effects of UVR-produced D₃.

Sc5d deletion in the skin of PF mice sensitizes female mice to UVR stimulation of BCC carcinogenesis
Because gender differences in UVR-induced BCC carcinogenesis could be due to mechanisms unrelated to differential D₃ production, we tested the effects of UVR on female PF mice unable to synthesize D₃ in keratinocytes. Sterol C5-
desaturase (Sc5d) (EC: 1.14.19.20) is the enzyme that converts lathosterol to 7-DHC, the substrate that UVR converts to D₃ (Holick et al., 1980; Jones et al., 1998; Vantieghem et al., 2006). Generalized deletion of Sc5d is lethal in both mice and humans (Brunetti-Pierri et al., 2002; Krakowiak et al., 2003), presumably due to the lack of synthesis of cholesterol, to which 7-DHC is converted by 7-DHC reductase. Therefore, we generated mice carrying, to our knowledge, a specifically in keratinocytes (Makarova et al., 2017) and crossed them with PF mice from our colony to enable deletion of Sc5d substrates in the hair, and lack immunohistochemically detectable Sc5d protein in keratinocytes (Makarova et al., 2017).

On the basis of our hypothesis of the opposing effects of UVR on BCC carcinogenesis—stimulatory via mutagenesis, but inhibitory via UVR-produced D₃—we predicted that UVR treatment of female PF Sc5d-deleted mice (to which ionizing radiation had been given to induce tumorigenesis) would significantly increase BCC carcinogenesis, as occurs in PF male mice unable to produce D₃ in the skin. We compared BCC carcinogenesis in three cohorts of PF Sc5d/KO female mice ingesting a normal D₃/normal mineral diet (TD2018) (Supplementary Table S1), all treated with tamoxifen at age 4 weeks to activate Cre, thereby deleting keratinocyte Sc5d and Tp53. Indeed, we found the number of μBCCs/mm at age 4 months, P = 0.0375, log-rank test) of tumor-free survival in mice treated with (3.8 μg/d) topical D₃ compared with the topical vehicle-treated group or the dietary D₃ group. (e) Topical D₃ (3.8 μg/d) reduced the number of vBCCs at age 5–7 months compared with control vehicle-treated mice, whereas dietary D₃ reduced vBCC numbers less robustly, if at all. (f) Representative images showing that topical D₃, in particular at the higher dose, dramatically reduced the number and size of vBCCs in skin biopsies taken at age 4 or 5 months. Hematoxylin and eosin. Scale bar = 50 μm. (g) In D-deficient PF mice topical D₃ (0.38 and 3.8 μg/d 5 times per week applied for 6–7 months) increased the circulating D₃ and 25(OH)D₃ blood levels in D-depleted mice compared with control vehicle-treated mice. D₃ at 3.8 μg/d raised blood 25(OH)D₃ to levels similar to those in mice fed normal D₃/normal minerals diet (TD2018) (D₃ dietary group). Data are shown as mean ± SD. 25(OH)D₃, 25-hydroxy-vitamin D₃; BCC, basal cell carcinoma; PF, Ptch1/KO mice; μBCC, microscopic basal cell carcinoma; vBCC, visible basal cell carcinoma; Sc5d, lathosterol 5-desaturase; SD, standard deviation.

Figure 1. Topical vitamin D₃ treatment in a dose-dependent manner delays tumorigenesis in PF mice. (a) Sites of BCC analysis. The distance from the site of application of D₃ to the site of the biopsy was approximately 3 cm (left). The distance from the site of application to the vBCCs varied from 0 to 10 cm, depending on their location (right). (b) Topical application of 0.38 μg/d D₃ significantly reduced the numbers of μBCCs/mm present in the skin of D-depleted PF mice at age 5 months compared with D-depleted PF mice receiving topical acetone or to normal-D-fed mice. Mean ± SD. (c) Topical application of high dose of 3.8 μg/d D₃ significantly reduced the numbers of μBCCs/mm present in the skin of D-depleted PF mice at age 4 months compared with D-depleted PF mice receiving topical acetone or to normal-D-fed mice. Mean ± SD. (d) Kaplan-Meier analysis of vBCC-free survival showed a significant extension (P = 0.0375, log-rank test) of tumor-free survival in mice treated with (3.8 μg/d) topical D₃ compared with the topical vehicle-treated group or the dietary D₃ group. (e) Topical D₃ (3.8 μg/d) reduced the number of vBCCs at age 5–7 months compared with control vehicle-treated mice, whereas dietary D₃ reduced vBCC numbers less robustly, if at all. (f) Representative images showing that topical D₃, in particular at the higher dose, dramatically reduced the number and size of vBCCs in skin biopsies taken at age 4 or 5 months. Hematoxylin and eosin. Scale bar = 50 μm. (g) In D-deficient PF mice topical D₃ (0.38 and 3.8 μg/d 5 times per week applied for 6–7 months) increased the circulating D₃ and 25(OH)D₃ blood levels in D-depleted mice compared with control vehicle-treated mice. D₃ at 3.8 μg/d raised blood 25(OH)D₃ to levels similar to those in mice fed normal D₃/normal minerals diet (TD2018) (D₃ dietary group). Data are shown as mean ± SD. 25(OH)D₃, 25-hydroxy-vitamin D₃; BCC, basal cell carcinoma; PF, Ptch1/KO mice; μBCC, microscopic basal cell carcinoma; vBCC, visible basal cell carcinoma; Sc5d, lathosterol 5-desaturase; SD, standard deviation.

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BCC progression in UVR-treated female mice, reducing overall survival (mean 25 ± 2 vs. 27 ± 2 weeks, \( P = 0.004 \), Gehan-Wilcoxon pairwise comparison) (Figure 4c) and increasing vBCC tumor volume (2.4 ± 0.5 vs. 0.9 ± 0.3 cm\(^3\) at 26 weeks of age, \( P = 0.022 \)) (Figure 4d).

**DISCUSSION**

We have used the BCC-prone Ptch1\(^{+/−}\) mouse model to test our hypothesis that UVR-produced HH-inhibiting D\(_3\) opposes the procarcinogenic effects of UVR. Our central findings are (i) that topical but not oral D\(_3\) inhibits BCC carcinogenesis, mirroring the differing circulating levels of unhydroxylated D\(_3\) according to the route of administration and (ii) that UVR has little effect on BCC carcinogenesis in (female) mice that can make D\(_3\) on UVR exposure but strongly stimulates BCC carcinogenesis in (male or keratinocyte-specific Sc5d-deficient female) mice unable to make D\(_3\). Hence, our findings indicate that UVR’s induction of skin production of D\(_3\) significantly restrains BCC tumorigenesis, leading to the counterintuitive conclusion that UVR, via production of HH-inhibiting D\(_3\), has anti-BCC carcinogenic effects that explain at least in part the complex relationship between exposure to UVR and human BCC incidence (Figure 4e). Of note, it is well known that in older humans the risk of BCCs increases markedly and the ability to increase circulating D\(_3\) by UVR exposure decreases. Thus, our data suggest a mechanism for this increased risk. Our finding of the lack of the effect of oral D\(_3\) indicates that oral D\(_3\) supplements are unlikely to prevent BCC carcinogenesis.

**MATERIALS AND METHODS**

**Transgenic mice**

We used PF mice (genotype: Ptch1\(^{+/−}\) K14-CreER\(^2\) p53\(^{fl/fl}\) (Tang et al., 2011; Wang et al., 2011) and PF Sc5d\(^{fl/fl}\) mice (genotype: Ptch1\(^{+/−}\) K14-CreER\(^2\) p53\(^{fl/fl}\) Sc5d\(^{fl/fl}\) (PF Sc5D-deleted mice) with wild-type Sc5d or conditional Sc5d deletion in keratinocytes, produced by crossing PF mice with Sc5D\(^{fl/fl}\) mice (Makarova et al., 2017). Tamoxifen-inducible keratinocyte-specific Cre recombinase-expressing mice with a mutated ligand-binding domain for the human estrogen receptor (ER), K14-CreER\(^2\), were originally from Pierre Chambon (University of Strasbourg) (Indra et al., 2000). Genotyping primer sequences and PCR conditions were described previously (Indra et al., 2000; Jonkers et al., 2001; Makarova et al., 2017). On treatment with tamoxifen (Sigma Aldrich, St Louis, MO) at age 4 or 7.5 weeks (see Supplementary Table S1) (100 mg/mouse by intraperitoneal injection once daily for three consecutive days) (Wang et al., 2011), the CreER\(^2\) construct deleted the floxed target region. We repeated a single tamoxifen intraperitoneal injection once daily for three consecutive days (Wang et al., 2011), the CreER\(^2\) construct deleted the floxed target region. We repeated a single tamoxifen intraperitoneal injection monthly for all PF Sc5D mice. We induced BCC formation with a single dose of 4 Gy 160 kV of ionizing radiation (RadSource RS2000 irradiator, Brentwood, TX) at age 8 weeks (Wang et al., 2011).

Mice were housed under standard conditions (fluorescent lighting 12 hours per day, room temperature 23–25 °C, and relative humidity 45–55%). Mice were routinely maintained on a standard normal D\(_3\)/normal mineral diet (D\(_3\) 1500 IU/kg, Ca 1%, phosphate 0.7%; TD2018: Harlan, Madison, WI). We enrolled 6- to 10-week-old mice in all studies unless otherwise specified in the text. For dietary studies, mothers and enrollees were weaned onto and maintained on a D\(_3\) depleted/high minerals diet (D\(_3\) 0 IU/kg, Ca 2%, phosphate 1.25%; TD87095: Harlan). Animal care and use were in
compliance with protocols approved by the Institutional Animal Care and Use Committee of Children's Hospital Oakland Research Institute (CHORI).

Treatment groups and procedures

**Topical D3 treatment.** For pre-enrollment consistency across the differing groups, PF mothers and enrollees before weaning were maintained on D-depleted/high minerals diet (TD2018; Harland Teklad, Madison, WI). At age 6 weeks, D-depleted mice were randomized into four groups, \( n = 17 \) mice per group (see Supplementary Table S1). Mice in groups 2 and 3 received cholecalciferol (unhydroxylated D3, Sigma-Aldrich, St Louis, MO), 0.38 or 3.8 mg/d to a small shaved area of the dorsal neck (Figure 1a), a site less amenable to self-licking, 5 days/wk in 20 µl of acetone, age 6 weeks to 7 months. Control mice received topical acetone 5 days/wk for the same duration. We confirmed that mice do not lick the application site by monitoring the persistence of a water-soluble ink at this site and its disappearance at a lickable site. All enrolled mice were housed singly and were dorsally shaved each week.

**UVR light source and treatment.** A Daavlin Spectra UVB Light Box with FS-40 fluorescent tubes (Daavlin, Bryan, OH) was used as a light source as previously described (Makarova et al., 2017).

To compare the effect of acute UVR on circulating D3 in male versus female PF mice, we used 10-week-old mice ingesting a standard normal D3/normal mineral diet (TD2018; Harland Teklad). Mice were treated acutely with 350 mJ/cm² UVR (280–315 nm) on each of three alternate mornings, and blood was obtained 6 hours after the third treatment.

To study the effect of UVR on BCC carcinogenesis, we used 10-week-old PF Sc5d mice maintained on normal D3/normal mineral diet (TD2018). Mice were treated chronically with 350 mJ/cm² UVR three times a week. The breeding pairs were heterozygous for the floxed Sc5d allele, and offspring from the same breeders were randomized into three treatment groups (Supplementary Table S1).
Skin sampling and β-galactosidase staining
At age 4 or 5 months, mouse dorsal skin biopsies were taken distant from the skin to which topical agents were applied (Figure 1a). β-Galactosidase staining and quantification of μBCCs of skin surface length in five histologic sections prepared from a 1-cm-long skin biopsy were performed as described previously (Wang et al., 2011).

Immunostaining
We used standard immunostaining procedures (Wang et al., 2011) with primary anti-Ki67 rabbit monoclonal (1:200, Thermo Fisher Scientific, Santa Clara, CA) and secondary biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) antibodies.

Serum D3 and 25(OH)D3 analysis
Blood was obtained by terminal cardiac puncture at the time of necropsy, and serum was collected from blood samples by centrifugation in amber tubes or tubes protected from light with foil covers. D3 and 25(OH)D3 levels were measured commercially (Heartland Assays LLC & Metabolic Technologies, Lenexa, KS). 25(OH)D3 was measured by radioimmunoassay; D3 was measured by liquid chromatography tandem-mass spectrometry using an Agilent 1290/6460 series triple quadrupole system and a deuterated internal standard.

Assessment of in vitro effects of D3 on murine BCC cells
We assessed the effects of D3 on murine BCC cells grown from PF mice (Wang et al., unpublished data). Cells were cultured in 154CM supplementary media (Thermo Fisher Scientific) supplemented with transferrin, adenine, hydrocortisone, fibroblast growth factor (Sigma-Aldrich, Carlsbad, CA), 0.05 mM calcium SD-208 (TOCRIS, Minneapolis, MN) and Y-27632 (TOCRIS), and penicillin/streptomycin (UCSF Cell Culture Facility, San Francisco, CA).

Proliferation.
Cells were seeded at 1.5 x 10⁴ cells per well in a 96-well plate and incubated overnight to obtain approximately 70% confluency. On day 1, drugs—vitamin D3 (5 and 10 μM) and XL-139 (0.1 and 0.5 μM)—and vehicle were added. On day 3 (48 hours later),
cell viability was measured using the cell proliferation reagent water-soluble tetrazolium-1 (Sigma-Aldrich). A volume of 10 μl water-soluble tetrazolium-1 (10%) was added to 100 μl culture medium per well and incubated for 3 hours at room temperature. The plate was shaken thoroughly for 10 s and measured at 450 nm using the SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA).

**Gli1 quantitation.** On day 0, cells were seeded at 0.7 × 10^6 cells per well onto a six-well plate and incubated overnight to obtain approximately 70% confluency. On day 1, drugs—vitamin D3 (5 and 10 μM) and XL-139 (0.1 and 0.5 μM)—and vehicle were added. On day 3 (48 hours later), cells were harvested into Trizol (Thermo Fisher Scientific), RNA was extracted, and Gli1 expression assessed by qPCR using the predesigned Taqman expression assay (Thermo Fisher Scientific).

**Statistical analysis**
Statistical analysis was carried out using the GraphPad Prism 6 software (La Jolla, CA). For all measures, the significance of the difference in means between groups was evaluated by Student’s t-test. For survival analysis, time to first tumor and overall survival Kaplan-Meier curve were used to represent the data and log-rank test to compare the survival distributions. A nonparametric Mann-Whitney U-test was performed to analyze tumor multiplicity and to determine the statistical significance of the difference between the groups. All values are presented as mean ± standard deviation; P ≤ 0.05 were considered to be statistically significant. All studies were designed to yield at least n = 15 for each experimental group, which allows for a detection of a 10% change in the difference of the age at which the first tumor is visible with >80% power at the P ≤ 0.05 significance level. Data were collected from multiple litters; mice from the same litter were randomized into different treatment groups. Each data point represents biological replicates. All statistics were done in consultation with the CHORI statistician, G. Gildengorin.

**CONFLICT OF INTEREST**
EHE is a co-founder, board member, stock holder, and officer of PellePharm. All other authors state no conflict of interest.

**ACKNOWLEDGMENTS**
We thank those who contributed mice containing mutant alleles as listed in the Materials and Methods. We also thank Y. Khaimskiy for mouse colony management, G. Gildengorin for discussions on statistical analysis, M. Fry, F. Frascari, and other members of our lab for invaluable assistance, and UC Berkeley student assistants for help with animal monitoring. This work was supported by NIH R01 CA142870 (EHE), The American Institute for Cancer Research 10A103 (EHE), and the joint UCB-CHORI T32 training grant in Tumor Biology (AM).

**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.05.037.

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