A Series of BRAF- and NRAS-Driven Murine Melanoma Cell Lines with Inducible Gene Modulation Capabilities

Ilah Bok¹,², Ariana Angarita¹, Stephen M. Douglass³, Ashani T. Weeraratna³,⁴ and Florian A. Karreth¹

Murine cancer cell lines are powerful research tools to complement studies in genetically engineered mouse models. We have established 21 melanoma cell lines from embryonic stem cell-genetically engineered mouse models driven by alleles that model the most frequent genetic alterations in human melanoma. In addition, these cell lines harbor regulatory alleles for the genomic integration of transgenes and the regulation of expression of such transgenes. In this study, we report a comprehensive characterization of these cell lines. Specifically, we validated melanocytic origin, driver allele recombination and expression, and activation of the oncogenic MAPK and protein kinase B pathways. We further tested tumor formation in syngeneic immunocompetent recipients as well as the functionality of the integrated Tet-ON system and recombination-mediated cassette exchange homing cassette. Finally, by deleting the transcription factor MAFG with an inducible CRISPR/Cas9 approach, we show the utility of the regulatory alleles for candidate gene modulation. These cell lines will be a valuable resource for studying melanoma biology and therapy.

INTRODUCTION

Genetically engineered mouse models (GEMMs) have significantly advanced our understanding of the pathobiology of melanoma; however, the use of GEMMs is expensive and cumbersome. The generation of GEMM-derived melanoma cell lines (Hooijkaas et al., 2012; Jenkins et al., 2014; Koya et al., 2012; Meeth et al., 2016; Pérez-Guijarro et al., 2020; Wang et al., 2017), especially those that can be allografted into syngeneic immunocompetent recipients, has alleviated some of the shortcomings of GEMMs. Indeed, the Yale University Mouse Melanoma series (Meeth et al., 2016) as well as the UV-irradiated derivative YUMMERT1.7 (Wang et al., 2017) are widely used tools in basic and preclinical melanoma research. Most cell lines were derived from melanoma GEMMs where BrafV600E is expressed from its endogenous locus. Only a few Nras-mutant cell lines have been established, all of which were derived from models where a transgenic Nras oncogene is under the control of the tyrosinase promoter (Tyr::NrasQ61R) (Dorard et al., 2017; Lindsay et al., 2011; Petit et al., 2019; Swoboda et al., 2021). Murine cell lines derived from melanomas driven by the endogenous LSL-NrasQ61R allele (Burd et al., 2014) have not been reported. Moreover, the field would benefit from murine melanoma cell lines harboring alleles that enable the efficient modulation of genes of interest.

We recently established a speedy mouse modeling platform that relies on efficient targeting of multiallelic embryonic stem cells (ESCs) to produce chimeras as experimental animals (Bok et al., 2020). These newly derived ESCs contain driver alleles that model the most common genetic alterations in human melanoma (BrafV600E, NrasQ61R, PtenΔD, Cdkn2aΔD). Moreover, the ESCs contain alleles enabling the integration of transgenes through recombination-mediated cassette exchange (RMCE) and the regulation of such transgenes through the Tet-ON system. We previously derived 17 cell lines from melanomas that arose in chimeras generated from untargeted ESCs (Bok et al., 2020). In this study, we report the establishment of another 4 murine melanoma cell lines and the systematic characterization of key features for the 21 cell lines. Furthermore, by inducible CRISPR/Cas9-mediated depletion of MAFG, a transcription factor we recently identified as a potential vulnerability of melanoma (Vera et al., 2021), we show the utility of the integrated regulatory elements for the characterization of genes of interest.

RESULTS AND DISCUSSION

We previously reported the derivation of 17 melanoma cell lines (Table 1) from ESC-derived GEMMs chimeras (Bok et al., 2020). These lines harbor allele combinations that model the
most frequent genetic alterations in human melanoma (Braf\(^{V600E}\), Nras\(^{Q61R}\), Pten\(^{ΔΔ}\), Cdkn2a\(^{ΔΔ}\), the Tyr-CreERT2 and CAGs-LSL-rtTA3 regulatory alleles, and the collagen homing cassette (CHC) for RMCE (Bok et al., 2020). We established four additional mouse melanoma cell lines (Table 1) for a total of 11 Braf\(^{V600E}\); Pten\(^{ΔΔ}\), Cdkn2a\(^{ΔΔ}\), Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CAGs-LSL-rtTA3, CHC) melanomas (Figure 2c), and we therefore did not examine their expression in the cell lines. We further performed genotyping PCR reaction (Figure 1c). However, these two cell lines express mutant Nras\(^{Q61R}\), as confirmed by Sanger sequencing of exon 2 (Figure 1d). The M175M1 and M175M2 cell lines were derived from two different tumors from the same mouse. We validated the lack of the aberrant recombination in the ESC line used to generate this NPP chimera (data not shown), indicating that the recombination occurred in the mouse. It seems unlikely, albeit possible, that the aberrant recombination occurred in parallel in two melanocytes that then gave rise to two independent tumors. Rather, the aberrant recombination may have occurred during development in a common melanocyte precursor, or one tumor is a skin metastasis of the other. Either way, because the LSL was partially removed, and mutant Nras\(^{Q61R}\) is expressed, the M175M1 and M175M2 cell lines are equivalent to the other NPP cell lines that underwent the canonical LSL excision.

The requirement for BPP and NPP cell lines to undergo an in vivo passage indicates that additional genetic changes

Table 1. Overview of the 21 Established Murine Melanoma Cell Lines

| Genotype | Cell Lines | Grows in BL/6 | rTA3 Expression | CHC Targeting | Source |
|----------|------------|---------------|-----------------|---------------|--------|
| BPP      | M10M1      | Yes           | Yes             | No            | Bok et al., 2020 |
| BPP      | M10M2      | Yes           | No              | No            | Bok et al., 2020 |
| BPP      | M10M3      | Yes           | Yes             | No            | Bok et al., 2020 |
| BPP      | M10M4      | No            | Yes             | No            | Bok et al., 2020 |
| BPP      | M10M5      | Yes           | No              | No            | Bok et al., 2020 |
| BPP      | M10M6      | Yes           | Yes             | No            | Bok et al., 2020 |
| BPP      | M10M7      | Yes           | Yes             | No            | Bok et al., 2020 |
| BPP      | M27M1      | Yes           | No              | Yes           | Bok et al., 2020 |
| BPP      | M33M1      | Yes           | Yes             | No            | Bok et al., 2020 |
| BPP      | M33M2      | No            | No              | No            | Bok et al., 2020 |
| BCC      | M161M1     | Yes           | No              | Yes           | Bok et al., 2020 |
| BCC      | M161M2     | No            | Yes             | No            | Bok et al., 2020 |
| BCC      | M167M1     | Yes           | No              | Yes           | Bok et al., 2020 |
| NPP      | M171M1     | Yes           | No              | No            | Bok et al., 2020 |
| NPP      | M173M1     | Yes           | No              | No            | Bok et al., 2020 |
| NPP      | M173M2     | Yes           | No              | Yes           | Bok et al., 2020 |
| NPP      | M175M1     | No            | Yes             | Yes           | Bok et al., 2020 |
| NPP      | M175M2     | Yes           | Yes             | No            | Bok et al., 2020 |
| NCC      | M133M1     | No            | No              | No            | This paper |
| NCC      | M133M2     | Yes           | Yes             | No            | This paper |

Abbreviations: BCC, Braf\(^{V600E}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Braf\(^{V600E}\); Cdkn2a\(^{ΔΔ}\); CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette; NCC, Nras\(^{Q61R}\); Cdkn2a\(^{ΔΔ}\); Nras\(^{Q61R}\); Pten\(^{ΔΔ}\); Tyr-CreERT2; CHC, collagen-homing cassette.
need to occur to enable the establishment of cell lines, most likely alterations in the \textit{Cdkn2a} and or \textit{p53} genes. We therefore assessed the expression levels of p16Ink4a, p19Arf, and p53. Interestingly, except for one cell line, p16Ink4a, p19Arf, and p53 expression was undetectable in BPP and NPP cell lines (Figure 1e). As expected, BCC and NCC cell lines do not express p16Ink4a, p19Arf, or p53 (Figure 1e). We also analyzed the expression of PTEN and found that it was retained in one BPP cell line, whereas PTEN was undetectable in all the other BPP and NPP cell lines (Figure 1e). All BCC cell lines retained PTEN expression as did one NCC cell line, whereas the second NCC cell line surprisingly lost PTEN.
expression (Figure 1e). We next tested the activation of oncogenic pathways in the melanoma cell lines and found that, as expected, BPP and NPP cell lines displayed increased protein kinase B activation compared with BCC and NCC lines (Figure 1e). Extracellular signal–regulated kinase activation was increased compared with that in mouse embryonic fibroblasts, varied between cell lines, and was not associated with a particular genotype (Figure 1e). Thus, our cell lines are of melanocytic origin, have recombined the relevant alleles, and exhibit activation of the appropriate oncogenic pathways. Moreover, BPP and NPP cell lines inactivated p16Ink4a, p19Arf, and p53 expression.

We next explored the utility of the regulatory alleles, Tyr-CreERT2, CAGS-LSL-rtTA3, and CHC. Cre expression was lost in all the 21 cell lines (Bok et al., 2020 and Figure 3a), and given that tyrosinase is undetectable in ESC-derived GEMM melanomas (Figure 2c), this indicates that Tyr-CreERT2 expression is attenuated as well. To test whether the rtTA3 transactivator is active, we transduced all cell lines with a doxycycline (Dox)-inducible lentiviral TRE-GFP construct. Adding Dox to the culture media and analyzing GFP expression revealed rtTA3 activity in eight cell lines (Table 1). Lack of rtTA3 activity in the remaining cell lines is likely due to failed recombination of the CAGS–LSL-rtTA3 allele. Accordingly, rtTA3 activity was readily induced by delivery of adenoviral Cre recombinase to a TRE-GFP–transduced BPP cell line as measured by GFP positivity (Figure 3b). Furthermore, using a constitutively active EF1α-GFP construct, we found that seven lines are amenable to CHC targeting by RMCE (Table 1). We surmise that optimizing the transfection and selection conditions will enable successful CHC targeting in the remaining lines. Thus, although Tyr-CreERT2 is inactivated in all the cell lines, rtTA3 is active in eight cell lines and can likely be activated in the remaining lines. We found CHC targeting by RMCE to be possible in several lines and further optimization could improve the targeting success rate.

The ESCs used to generate chimeras and thus the melanomas from which we derived the cell lines are on an almost pure C57BL/6 background. To determine the suitability of the murine cell lines for syngeneic allografts, we subcutaneously transplanted 1 × 10⁶ cells of each line into C57BL/6 recipient mice and found that 16 cell lines formed tumors (Table 1). Three of the five cell lines (M10M4, M36M3, and M175M1) did not form tumors in C57BL/6 recipient mice despite forming tumors in Nu/Nu mice when we established them. This suggests that antitumor immunity prevents the growth of these cell lines in C57BL/6 mice. The remaining two cell lines, M161M2 and M133M1, have not been transplanted into Nu/Nu or C57BL/6 recipients, and their ability to form tumors in immunocompromised hosts is currently unknown. To further analyze potential antitumor immunity, we injected 0.25 × 10⁶ cells of a BCC line (M167M1) into C57BL/6 recipients. This resulted in initial tumor growth, followed by transient tumor regression (Figure 4a), suggesting some antitumor immunity. Interestingly, antitumor immunity was
boosted when M167M1 tumor-bearing C57BL/6 recipients were treated with anti–PD-1 checkpoint inhibitor (Figure 4b). Another BCC cell line, M161M1, did not respond to anti–PD-1 treatment (Figure 4c), indicating that sensitivity to checkpoint inhibition is not a universal feature of our murine melanoma cell lines.

We next assessed how the regulatory alleles, specifically the rtTA3 transactivator, can be employed for inducible gene modulation in the murine melanoma cell lines. To this end, we delivered Dox-inducible lentiviral TRE-Cas9 and tested the effect of continuous Cas9 expression on tumor growth. Although Dox treatment had no effect on parental M167M1 cells (Figure 4a and b), Dox moderately slowed tumor growth of cells stably transduced with Dox-inducible TRE-Cas9. This occurred in immunocompromised NSG mice and syngeneic C57BL/6 mice (Figure 4c and d), indicating that in this context, Cas9 does not elicit an immune response but rather cell-intrinsically affects growth. A similar effect was observed for M10M3 cells when transplanted into NSG mice (Figure 4e and f). Thus, transient expression of Cas9 may be desirable. We confirmed that Dox withdrawal quickly turned off Cas9 expression in four cell lines (Figure 4g). To test the efficiency

Figure 4. Melanoma growth in C57BL/6 mice and response to anti–PD-1 checkpoint inhibitor. (a) Different numbers of M167M1 (BCC) cells were injected into C57BL/6 mice. Whereas injecting 1 x 10^6 cells led to continuous tumor growth, injecting only 0.25 x 10^6 cells resulted in initial growth, followed by transient regression on day 14 and recovery after approximately 1 week. (b, c) M167M1 or M161M1 (BCC) cells were injected into C57BL/6 mice and treated with anti–PD-1 or IgG. Anti–PD-1 slowed tumor growth of the M167M1 model but had no effect on M161M1 tumor growth. BCC, Braf^V600E; Cdkn2a^D/D; NPP, Nras^Q61R; Pten^D/D.

Figure 3. Loss of Cre expression and rtTA3 activation by exogenous Cre. (a) Cre expression was tested by western blot and found to be absent in the 21 cell lines. Nevi isolated from hyperpigmented mouse skin of a BPP chimera was used as a positive control. (b) Parental M10M7 cells showed no rtTA3 activity likely owing to failed excision of the LSL cassette in the CAGS–LSL–rtTA3 allele. M10M7 cells were infected with lentivirus carrying TRE-GFP, followed by AdCre recombinase. On Dox treatment, cells infected with AdCre expressed GFP, indicating successful LSL removal and rtTA3 expression. Bar = 2 mm. AdCre, adenoviral Cre; BCC, Braf^V600E; Cdkn2a^D/D; BPP, Braf^V600E; Pten^D/D; Dox, doxycycline; NCC, Nras^Q61R; Cdkn2a^D/D; NPP, Nras^Q61R; Pten^D/D.
of inducible TRE-Cas9, we delivered a CRISPR/Cas9 reporter consisting of a GFP cDNA and a GFP-targeting sgRNA (Figure 6a). On Dox treatment, GFP expression was reduced (Figure 6b), thus validating Cas9 activity. To demonstrate the utility of Dox-inducible TRE-Cas9, we delivered a sgRNA targeting Mafg to two murine melanoma lines, a transcription factor we recently found to be important for the proliferation of melanoma cells (Vera et al., 2021) (Figure 6a). Dox treatment reduced Mafg expression in TRE-Cas9 melanoma lines harboring a Mafg-targeting sgRNA (Figure 6c). Interestingly,
MAFG depletion impaired colony formation and anchorage-independent growth in BPP cells (M10M3) but had only modest effects in BCC cells (M167M1) (Figure 6d–g). This may suggest the possibility of the genotype-specific significance of Mafg, which warrants further investigation. Overall, the regulatory alleles enable inducible gene depletion by CRISPR/Cas9 and could prove useful for reversible expression of cDNA or short hairpin RNA constructs.

In summary, we generated 21 mouse melanoma cell lines harboring clinically relevant genetic drivers. Most of these cell lines form tumors when allografted into syngeneic recipients. In addition, these lines contain CHC and rtTA3 alleles for the genomic insertion and regulation of transgenic constructs. The utility of these cell lines may be further assessed and improved, for instance, by optimizing CHC targeting, by generating UV-irradiated derivatives, by isolating single-cell clones with high Cas9 activity, or by testing the sensitivity of additional lines to checkpoint inhibition. This cell line panel includes the first seven cell lines derived from melanomas driven by the LSL-NrasQ61R allele (Burd et al., 2014), whereas previously reported Nras-mutant cell lines were derived from Tyr::NrasQ61K melanomas (Dorard et al., 2017; Lindsay et al., 2011; Petit et al., 2019; Swoboda et al., 2021). Interestingly, melanomas in LSL-BrafV600E and LSL-NrasQ61R mice are almost uniformly amelanotic. This could be explained by the downregulation of Mitf (Figure 2a and b) in these tumors, which also results in the downregulation of transgenes under the control of the tyrosinase promoter such as Tyr-CreERT2 (Bok et al., 2020 and Figure 3a). Thus, our murine melanoma cell lines may...
represent human melanomas having low MITF expression. It is possible that in melanomas driven by the \( \text{Tyr-::Nras}^{Q61K} \) transgene, where mutant \( \text{Nras} \) is under the control of the tyrosinase promoter, the MITF-regulated gene program remains active to maintain the expression of mutant \( \text{Nras} \). However, whether \( \text{Tyr-::Nras}^{Q61K} \) tumors and cell lines model MITF-high human melanomas and whether these two \( \text{Nras} \) alleles model different subtypes of human melanoma remain to be investigated. With the establishment of the LSL-\( \text{Nras}^{Q61R} \) cell lines, comparative studies are now possible. Taken together, this panel of murine melanoma cell lines will further expand the resources available for investigating melanoma biology and therapies, and it will be made available to the melanoma research community.

**MATERIALS AND METHODS**

**Mouse melanoma cell line generation**

Melanoma cells were isolated from ESC-derived GEMMs and cell lines established as previously described (Bok et al., 2020). Briefly, tumor tissues were collected and washed in 70% ethanol for 10 seconds and rinsed in PBS. Diced tumor tissues were then digested in 1 mg/ml collagenase/disappease (catalog number 10269638001; Sigma-Aldrich, St. Louis, MO) for 20 minutes in a humidified incubator at 37 °C. Then tissues were then washed with PBS and further digested in 0.25% Trypsin (catalog number VWR1054-0100; VWR, Radnor, PA) for 30 minutes in the incubator. Cells were spun down and plated in RPMI containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells isolated from Braf\(^{V600E}\), Pten\(^{-}\) and \( \text{Nras}^{Q61R} \), Pten\(^{-}\) tumors were expanded in vitro for several days, followed by subcutaneous injection into athymic nude mice (J:NU Stock number 007850; The Jackson Laboratory, Bar Harbor, ME) before the onset of in vitro growth arrest. Cell lines derived from allografted tumors were established similar to spontaneous ESC-derived GEMM melanomas.

**Cell culture**

Mouse melanoma cell lines and SW1 cell line (a gift from Eric Lau) were cultured in RPMI containing 5% FBS and 1% penicillin/streptomycin. Cells were cultured in RPMI containing 5% FBS and 1% penicillin/streptomycin supplemented with 200 nM 12-O-Tetradecanoylphorbol-13-acetate (catalog number P8139; Sigma-Aldrich), 200 pM Cholera Toxin (catalog number C8052; Sigma-Aldrich), 0.25% Trypsin (catalog number VWRL0154-0100; VWR, Radnor, PA) for 30 minutes in the incubator. Cells were spun down and plated in RPMI containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells isolated from Braf\(^{V600E}\), Pten\(^{-}\) and \( \text{Nras}^{Q61R} \), Pten\(^{-}\) tumors were expanded in vitro for several days, followed by subcutaneous injection into athymic nude mice (J:NU Stock number 007850; The Jackson Laboratory, Bar Harbor, ME) before the onset of in vitro growth arrest. Cell lines derived from allografted tumors were established similar to spontaneous ESC-derived GEMM melanomas.

**Genomic DNA isolation and LSL-recombination genotyping**

PCR Mouse melanoma cells were harvested by scraping. Cells were lysed in lysis buffer (5% 2 M Tris, pH 7.5, 2% 5 M sodium chloride, 1% 0.5M EDTA, 2% of 10% SDS, and 90% double distilled water) containing proteinase K overnight at 56 °C. DNA was precipitated by adding 100% ethanol, and the pellet was washed with 70% ethanol. The DNA pellet was then air dried and resuspended in DNase/RNase-free water. The PCR primers used for LSL-recombination genotyping of LSL-\( \text{Bra}^{V600E} \) were as follows: 5'-CAAA-CACCCTGAGTCTAGGG-3' (common forward) for LSL-\( \text{Bra}^{V600E} \) and 5'-CCTTGGATCCAGCAATTGATG-3' (reverse) for LSL and 5'-GATT-CACATTGGGACCTGAAC-3' (reverse) for wild-type. The primers for LSL-\( \text{Nra}^{Q61R} \) were reported previously (Burd et al., 2014).

**RNA isolation and Nras exon amplification**

Mouse melanoma cells were lysed with QIAzol lysis reagent (catalog number 79306; Qiagen, Hilden, Germany), and RNA was isolated as per the manufacturer’s instructions. cDNA synthesis was performed using 500 ng RNA and the PrimeScript RT master mix (catalog number RR036A; Takara Bio, Kusatsu, Japan). PCR was performed using the Go-Taq Green master mix (catalog number M7123; Promega, Madison, WI) with the following primers to amplify \( \text{Nras} \) exons 1–3: 5'-GACTGAGTAACACTGTGG-3' (forward) for \( \text{Nras}_6 \) and 5'-GCCGCTCTTGGTCACGTC-3' (reverse) for \( \text{Nras}_6 \). The PCR products were separated by gel electrophoresis and purified using the E.Z.N.A. Gel extraction kit (catalog number 101318-972; Omega Bio-Tek, Norcross, GA). Sanger sequencing was performed by Eton Bioscience (San Diego, CA).

**Allograft experiments**

All animal experiments were conducted in accordance with an Institutional Animal Care and Use Committee protocol (R-I500005420) approved by the University of South Florida (Tampa, FL). Mouse melanoma cells were injected into C57BL/6j (stock number 00664; The Jackson Laboratory) or NSG gamma (stock number 005557; The Jackson Laboratory) bred in house. Tumors were measured using calipers, and volume was calculated using the formula \( \text{volume} = \text{width}^2 \times \text{length} / 2 \). Mice were fed chow containing 200 mg/kg Dox purchased from Envigo (Indianapolis, IN).

**Anti–PD-1 and IgG treatment**

C57BL/6j mice were purchased from the Charles River National Cancer Institute facility. Mice were aged 10 weeks at the time of tumor implantation. Melanoma cell lines were intradermally injected onto the backs of mice. Mice were given a total of five injections of 200 μg/ml IgG2AK (#400566; BioLegend, San Diego, CA) or anti–PD-1 (#114115, clone RMP1-14; BioLegend) commencing on the day of tumor implantation. The generation of pLenti-TRE-GFP-Blast was previously described (Bok et al., 2020). Briefly, cells were cotransfected with 2 μg pCAGGS-FLPe plasmid and 4 μg COL1A1-EF1-GFP targeting vector at a 1:2 ratio using FuGENE HD Transfection Reagent (catalog number E2311; Promega) according to the manufacturer’s instructions, followed by selection in 50 μg/ml hygromycin. GFP-positive cells were assessed after 3 weeks.

**Plasmids and virus transduction**

High-fidelity, codon-optimized Cas9 was cloned from pLenti-HF1RA-PGK-Puro (Zafra et al., 2018), a gift from Lukas Dow (plasmid number 110860; Addgene, Watertown, MA), by replacing GFP in pLenti-TRE-GFP-Blas. The U6-sgRNA cassette was validated by western blot of mouse embryonic fibroblasts infected with sgRNA_lentiCRISPRv2 (Sanjana et al., 2014), a gift from Feng Zhang (plasmid number 52961, Addgene; provided by Tyler Jacks). sgRNA cutting efficiency of Cas9 was validated by western blot of mouse embryonic fibroblasts infected with sgRNA_lentiCRISPRv2. The U6-sgRNA cassette
was then cloned by InFusion cloning (U6 5′-CAACCCCGAGGG-
GACCGAGGCGCTATT-3′ [forward] and U6 5′-CGGGCTCTG
TCGGGTTGCTAGCAGGTTCAAAAAAGGACCCG-3′ [reverse]) in the
KHi restriction site of pLenti-GFP-Hygro. The U6-sgGFP-gRNA
scaffold cassette from pXPR_011 (Doench et al., 2014), a gift from
John Doench and David Root (plasmid number 59702, Addgene;
provided by Gina DeNicola), was cloned into pLenti-GFP-Hygro
using the same approach. Lentivirus production and transduction
were performed as previously described (Bok et al., 2020). M10M7
cells were infected with Ad5CMVCre adenovirus purchased from
the University of Iowa Viral Vector Core (https://vector-core.medicine.
uiowa.edu/).

Immunoblotting
Immunoblotting was performed as previously described (Bok et al.,
2020). Antibodies against FLAG (1:1,000, catalog number 14793S;
Cell Signaling Technology, Danvers, MA), GFP (1:2,000, catalog
number 2956S; Cell Signaling Technology), MAFG (1:1,000, catalog
number ab154318; Abcam, Cambridge, United Kingdom),
p16INK4a (1:2,000, catalog number ab211542; Abcam), p19ARF
(1:1,000, catalog number ab80; Abcam), p53 (1:1,000, catalog
number 3036-100; BioVision, Milpitas, CA), phosphorylated AKT
S473 (1:2,000, catalog number 9188S; Cell Signaling Technology),
phosphorylated AKT T308 (1:500, catalog number 13038T; Cell
Signaling Technology), AKT (1:5,000, catalog number 4691T; Cell
Signaling Technology), phosphorylated ERK T202/Y204 (1:1,000,
catalog number 9101S; Cell Signaling Technology), ERK (1:1,000,
catalog number 4695S; Cell Signaling Technology), S100B (1:1,000,
catalog number ab52942; Abcam), MART1 (1:1,000, catalog
number SAB4500949-100UG; Sigma-Aldrich), Cre (1:1,000, catalog
number ab190177; Abcam), MITF (1:1,000, catalog number
12590S; Cell Signaling Technology), and β-actin (1:10,000, catalog
number AM4302; Thermo Fisher Scientific, Waltham, MA) were
used.

Colony formation assay
Cells were treated with 0.5 μg/ml Dox (catalog number D9891-1G;
Sigma-Aldrich) for 3 days, followed by culture in Dox-free media for
2–3 days to inactivate Cas9 expression. A total of 500 cells were
seeded in six-well plates in triplicates and incubated for 8 days. Cells
were fixed in cold 4% paraformaldehyde (catalog number 101176-
014; VWR) and stained with 0.5% crystal violet (catalog number
97061-850; VWR). Percent area of crystal violet staining was
quantified using ImageJ (National Institutes of Health, Bethesda,
MD).

Soft agar assay
For the bottom layer, 0.8% SeaPlaque agarose (catalog number
50101, Lonza, Basel, Switzerland) in RPMI containing 10% FBS and
1% penicillin/streptomycin was plated onto six-well plates and so-
lidified at room temperature. For the upper layer, 0.4% agarose in
RPMI containing 10% FBS and 1% penicillin/streptomycin mixed
with 3,000–5,000 cells were plated and overlaid with 1 ml RPMI
with 5% FBS and 1% penicillin/streptomycin once solidified. After
14 days, plates were stained with 0.001% crystal violet overnight at
4 °C. The number of colonies was quantified using ImageJ.

Statistical analysis
Statistical analysis was performed using GraphPad Prism software
(GraphPad Software, San Diego, CA) and Microsoft Excel (Microsoft,
Redmond, WA). Data were analyzed with unpaired two-tailed t-test,
and P < 0.05 was considered statistically significant.

Data availability statement
No datasets were generated or analyzed during this study.

ORCIDs
Ilah Bok: http://orcid.org/0000-0001-8958-5161
Ariana Angarita: http://orcid.org/0000-0001-6795-4640
Stephen M. Douglass: http://orcid.org/0000-0003-1562-2235
Ashani T. Weeraratana: http://orcid.org/0000-0003-0448-6952
Florian A. Karreth: http://orcid.org/0000-0002-2350-9809

AUTHOR CONTRIBUTIONS
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CONFLICT OF INTEREST
ATW is on the Board of Directors for ReGAIN therapeutics. The remaining
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