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Chelsea D. Mullins  
*Washington University School of Medicine in St. Louis*

Mack Y. Su  
*Washington University School of Medicine in St. Louis*

Vishwanathan Hucthagowder  
*Washington University School of Medicine in St. Louis*

Liang Chu  
*Washington University School of Medicine in St. Louis*

Lan Lu  
*Washington University School of Medicine in St. Louis*

See next page for additional authors

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Germinal Center B-Cells Resist Transformation by Kras Independently of Tumor Suppressor Arf

Chelsea D. Mullins¹, Mack Y. Su¹, Vishwanathan Huchtagowder², Liang Chu¹, Lan Lu¹, Shashikant Kulkarni²,³, Deborah Novack², Ravi Vij¹, Michael H. Tomasson¹*

1 Department of Internal Medicine, Washington University School of Medicine, Saint Louis, Missouri, United States of America, 2 Department of Pathology & Immunology, Washington University School of Medicine, Saint Louis, Missouri, United States of America, 3 Department of Genetics, Washington University School of Medicine, Saint Louis, Missouri, United States of America

Abstract

Activating mutations in Ras (N- and K-) are the most common point mutations found in patients with multiple myeloma (MM) and are associated with poor clinical outcome. We sought to directly examine the role of Ras activation in MM pathogenesis and used two different tissue-specific Cre recombinase mouse lines (Cj1-Cre and AID-Cre), to generate mice with mutant Kras (KrasG12D) activated specifically in germinal center B-cells. We also generated mice with activation of the KrasG12D allele in a tumor-prone Arf-null genetic background. Surprisingly, we observed no significant disruption in B-cell homeostasis in any of these models by serum immunoglobulin ELISA, SPEP, flow cytometry and histological examination. We observed development of non-overlapping tumor types due to off-target Cre expression, but despite successful recombination in germinal center and later B-cell populations, we observed no B-cell phenotype. Together, these data demonstrate that Ras activation is not sufficient to transform primary germinal center B-cells, even in an Arf-null context, and that the temporal order of mutation acquisition may be critical for myeloma development. Specific pathways, yet to be identified, are required before Kras can contribute to the development of MM.

Introduction

Multiple myeloma (MM) is an incurable malignancy of antibody-secreting plasma B-cells, whose etiology remains poorly understood. Mutations in Ras genes, encoding key proteins regulating cell growth, differentiation and survival, occur commonly in MM with a prevalence of 20–39% [1–3]. Indeed, using a targeted sequencing approach to screen highly expressed tyrosine kinase and cytokine signaling genes in primary human patient myeloma, we previously identified mutations at codon 12 and 61 in N- and KRAS as being the only recurrent variation in our sample set [4]. Recent genome sequencing efforts also found Ras mutations to be the most common single nucleotide variant (SNV) in MM [4], suggesting that Ras activation is an important event in MM pathogenesis. The somatic SNVs found most frequently in MM are gain-of-function mutations in Ras oncogenes (Kras and Nras), causing constitutive activation of the Ras protein [5].

Despite the genomic evidence for Ras pathogenesis, the functional role of Ras activation in MM has not previously been tested. This issue is not trivial as the induction of neoplasia by Ras activation is highly dependent on cellular context [6]. Understanding the effects of Ras activation in mature B-cells will allow us to better define the downstream pathways critical for development of MM. Moreover, pharmaceutical approaches to target cancers with mutant Ras are underway [7–10], and a pre-clinical model faithfully replicating Ras-driven myeloma would be critical in evaluating the therapeutic potential of these agents in myeloma.

Post-germinal center (GC) B-cells are strongly implicated as the cell of origin in MM by demonstration of stable immunoglobulin (Ig) switch clonotypes over the course of disease [11,12]. To test if expression of oncogenic Ras in GC B-cells was sufficient to induce myeloma, we utilized transgenic mice harboring a constitutively active Kras (G12D mutation) knocked-in to the endogenous Kras locus and flanked by a Lox-Stop-Lox cassette [13]. The Kras mouse model has been successfully used in several labs in developing cancer models [14,15] [13,16]. These mice were crossed with two different mature B cell-specific Cre recombinase (Cre) mouse strains (Cj1-Cre and AID-Cre) to definitively test the effects of Ras activation in post-GC B-cells, including downstream memory B and plasma cells [17,18]. As Ras activation can induce cellular senescence [19] and often requires cooperating mutations to induce transformation, so we also generated a strain of triple transgenic mice by crossing KrasG12D mice with mice null for the P19ARF tumor-suppressor gene (Arf−/−) [20]. Arf (P14ARF in humans) is a potent tumor suppressor gene that cooperates with Ras activation in cellular transformation and carcinogenesis [21,22]. In patients with myeloma, the P14/P16 locus is methylated in 42% [23], although the biological significance of this epigenetic modification is contested [24].
Surprisingly, in these settings we found B-cell development to be only subtly perturbed, even in the setting of AID deficiency. Conversely, mice frequently developed tumors harboring Cre-recombined Kras alleles in non-B-cell tissues due to small amounts of off-target Cre expression. These data demonstrate that post-GC B-cells are resistant to transformation by mutations that are strongly oncogenic in other cellular contexts and that Ras activation must likely cooperate with tissue-specific mutations or epigenetic events to induce myeloma.

Results

Cry1-Cre Kras<sup>G12D</sup> Mice Develop Thymic Lymphomas and Lung Adenomas but not Myeloma

To examine the effect of Kras in plasma cells, we generated double transgenic mice. In Kras<sup>G12D</sup> mice, the G12D mutation is knocked-in to the endogenous Kras locus, upstream of the Lox-Stop-Lox cassette (Figure 1A,B). Kras<sup>G12D</sup> mice were crossed with mice expressing Cre recombinase (Cre) under control of the Ig heavy chain locus (Cry1-Cre) reported to express Cre selectively in a subset of germinal center B-cells (Figure 1C).

We first confirmed that wild type Kras is strongly expressed in murine B-lineage cells; naive splenic B-cells, germinal center B-cells, memory B-cells and plasma cells from C57BL/6 mice (Figure 2A) [25]. As expected, Cre-mediated excision of the Kras allele stop cassette was robust and specific to B-lineage cells undergoing class-switch recombination in vitro (Figure 2B and Figure S1). We also confirmed Cre-recombination in vivo in mature B-cell populations isolated from Cry1-Cre Kras<sup>G12D</sup> mice by fluorescence associated cell sorting (FACS). Splenic germinal center B-cells (B220<sup>+</sup>/IgM<sup>-</sup>/GL7<sup>+</sup>) and class switched memory/plasma cells (IgG<sup>+</sup>) demonstrated clear, albeit low-level recombination, as did bone marrow plasma cells (B220<sup>+</sup>/CD138<sup>+</sup>), Figure 2C).

We aged Cry1-Cre Kras<sup>G12D</sup> mice, both naive and immunized with chicken gamma globulin to expand plasma cells, to monitor the development of disease. After 100 days, 58% (n = 12) of naive mice developed weight loss, ruffled fur and shortness of breath and were found on necropsy to have thoracic cavity tumors. Unexpectedly, these tumors were T-lymphoblastic in phenotype (CD4<sup>+</</sup>CD8<sup>+</sup>) by flow cytometry (Figure S2A). Additionally, 42% (n = 12) of naive Cry1-Cre Kras<sup>G12D</sup> mice and 66% (n = 7) CGG-immunized Cry1-Cre Kras<sup>G12D</sup> mice were found to have lung nodules at autopsy (300 day endpoint). Sections of lung from immunized Cry1-Cre Kras<sup>G12D</sup> mice show well-demarcated nodules composed mostly of sheets of bronchial epithelial cells and some “signet ring” cells with bland nuclear features and absence of mitotic figures consistent with adenomas or low-grade adenocarcinomas (Figure S2B-E). Tissue from lung tumors in two independent Cry1-Cre Kras<sup>G12D</sup> mice shows partial recombination of the Kras allele (Figure S2F). The immunized and unimmunized negative control Cry1-Cre mice showed no evidence of disease (Figure 3A). Tissue from T-cell lymphomas found in two separate unimmunized Cry1-Cre Kras<sup>G12D</sup> mice showed complete Kras allele recombination, suggestive of loss of the wild-type allele, whereas spleen showed a partial recombination pattern consistent with infiltration of the spleen with these same cells (Figure 3B). Despite extensive analysis, no B-lineage oncogenic transformation was observed in any Cry1-Cre Kras<sup>G12D</sup> mice. B-cell subsets in spleen and bone marrow and serum immunoglobulin levels were all normal (data not shown). Taken together, these data suggest that Kras<sup>G12D</sup> allele activation in germinal center B-cells failed to perturb B-cell homeostasis in Cry1-Cre Kras<sup>G12D</sup> mice.

AID-Cre-YFP Kras<sup>G12D</sup> Mice Develop Focal Epidermal Papillomas

Noting the low level of in vivo recombination in Cry1-Cre Kras<sup>G12D</sup> mice (Figure 2C), and the lack of appreciable B- or plasma cell phenotype, we generated a second strain of mice using an independent tissue specific Cre allele. We crossed the Kras<sup>G12D</sup> mice with mice expressing Cre recombinase under the control of the activation-induced cytosine deaminase (AID) gene (Figure 1D). AID is expressed with exquisite specificity in B-cells undergoing the germinal center reaction where it mediates class switch recombination and somatic hypermutation. To facilitate our analysis, this strain of mice also included the Rosa26-EYFP reporter allele, which allowed us to effectively track B-cells where recombination had occurred (AID-Cre-EYFP Kras<sup>G12D</sup>). Upon cre-mediated recombination, YFP marks cells where Kras<sup>G12D</sup> is also expressed. In an attempt to stimulate malignant B-cell transformation in AID-Cre-EYFP Kras<sup>G12D</sup> mice, vitamin D deficient chow and/or sub-lethal radiation was given to cohorts of mice after immunization.

Robust Kras<sup>G12D</sup> allele recombination was induced in AID-Cre-EYFP Kras<sup>G12D</sup> splenic B-cells undergoing plasmacytic differentiation and class switch recombination ex vivo (Figure 4A). In contrast to the weak levels of in vivo recombination observed in Cry1-Cre Kras<sup>G12D</sup> mice, germinal center splenocyte populations and post germinal center cells isolated from AID-Cre-EYFP Kras<sup>G12D</sup> mice showed robust Cre-mediated recombination at both the Kras<sup>G12D</sup> locus (Figure 4B) and the YFP reporter in the spleen and to lesser extent in the bone marrow (Figure 4C).

At 3 weeks of age, 100% (n = 20) AID-Cre-EYFP Kras<sup>G12D</sup> mice lacked fur on the ventral neck and developed small growths, compared to control mice (Figure 5A,B). Radiation and Vitamin D deficient chow (RV) treatments increased the number and size of growths on AID-Cre-EYFP Kras<sup>G12D</sup> mice as early as 17 weeks, compared to AID-Cre-EYFP Kras<sup>G12D</sup> given neither (Figure 5C,D). By 26 weeks of age, all AID-Cre-EYFP Kras<sup>G12D</sup> mice receiving both irradiation and vitamin D deficient chow (100%, n = 5) were hunched with ruffled fur and had infected lesions over the cutaneous growths with a median

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**Figure 1. Schematic of alleles used in generating transgenic mice.**

A) Floxed Kras allele with exons 0, 1, and 2, under the endogenous Kras locus. Asterisk represents G12D mutation in exon 1. B) Excision of the stop cassette of the Kras allele by Cre recombinase allows the G12D mutation to activate. C) The Cre-coding sequence is knocked in downstream of the last coding exon of the Kras locus. Expression of Cre recombinase is induced by transcription of the Ig γ1 constant region. D) After the floxed neomycin gene is deleted by Cre-mediated, the YFP is expressed alongside AID-expressing B cells. doi:10.1371/journal.pone.0067941.g001
AID-Cre-YFP Kras G12D mice with no treatment (besides immunization) at 26 weeks had an increase in the number of growths similar in appearance to that at 17 weeks. At 17 weeks, KrasG12D mice given both irradiation and vitamin D deficient chow appeared healthy without growths, similar to the 26 week timepoint (data not shown). All AID-Cre-YFP KrasG12D mice regardless of irradiation or vitamin deficient chow subsequently died or were sacrificed due to persistent skin infections associated with fungating skin lesions (Figure 6A). The cutaneous lesions were identified by histological examination to be benign papillomas (data not shown). Papillomas from 3 separate AID-Cre-YFP Kras G12D mice showed strong Cre-mediated recombination by PCR (Figure 6B). A small increase in total serum gamma region protein level achieved statistical significance in AID-Cre-YFP Kras G12D mice fed vitamin deficient chow (Figure S3A, middle panel), however the increase was not maintained over time, and mice treated with radiation, or no treatment at all had no significant changes in total serum gamma protein levels at any time point (Figure S3A). Serum ELISA showed small changes among the antibody subtypes in AID-Cre-YFP Kras G12D mice, but no evidence of plasma cell transformation or any B-cell malignancy was found (Figure S3B and data not shown).

Figure 2. Kras expression in B-cell subsets and tissue-specific recombination in Ccr1-Cre KrasG12D mice. A) Expression of Ras genes by microarray in primary mature B-cell subsets; naïve splenic B-cells, germinal center B-cells, memory B-cells, and plasma cells. B) Successful Lox-Stop-Lox excision from Kras locus in B cells of Ccr1-Cre KrasG12D mice following class switch recombination. PCR of Kras G12D allele in B-cells of Ccr1-Cre KrasG12D mice stimulated to undergo class switch recombination ex vivo. Naïve splenic B-cells were stimulated to undergo class switch recombination with lipopolysaccharide (LPS) alone or LPS plus interleukin-4 (IL-4). Successful recombination was observed upon switch to IgG1 induced by LPS plus IL-4. C) Fluorescence activated cell sorting (FACS) isolation of mature B-cell subsets directly from Ccr1-Cre KrasG12D mice. In the first panel, bone marrow mononuclear cells; second and third panels, splenic mononuclear cells, both panels gated for B220+. Red arrows indicate lanes with detectable recombination. Recombination was low but detectable in bone marrow plasma cells (CD138+/B220low, lane 1); germinal center B-cells (B220+/GL7+/IgMlow, lane 5) and IgG1 class switched splenic B-cells (B220+/IgM+/IgG1+, lane 9).

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AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) Mice Develop Fatal Epidermal Papillomas and Derivative Carcinomas

We reasoned that a lack of a detectable B-cell phenotype in C\(^{c1}\)-Cre Kras\(^{G12D}\) and AID-Cre-YFP Kras\(^{G12D}\) mice was most likely due to a requirement for a cooperating “second hit” to induce cellular transformation. Therefore, to test the effects of a second mutation known to cooperate with Kras\(^{G12D}\), we crossed AID-Cre-YFP Kras\(^{G12D}\) mice into a tumor-prone Arf-H12/2 background (Figure 1). All AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) mice developed rapidly progressive papillomas and by 12 wks, 66% of AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) mice (n = 3) developed cutaneous sarcomas (Figure 7A), while AID-Cre-YFP Arf\(^{-/-}\) control mice remained disease-free (Figure 7B). Histopathological sections of spleen from control mice show typical red pulp, white pulp and germinal center structures (Figure 7D); whereas AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) spleen showed defacement of splenic architecture with loss of distinction between red and white pulp and a paucity of germinal centers (Figure 7C). Sections of sarcomas from AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) showed characteristic undifferentiated spindle cells (Figure 7E), consistent with tumors previously described in Arf-deficient mice (10). The only abnormalities attributable to B-cells that we identified were small but significant increases in polyclonal antibody responses over time. The gamma protein fraction by SPEP was higher in AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) at 12 weeks compared to AID-Cre-YFP Arf\(^{-/-}\) controls (Figure S4C), but none of the mice developed multiple myeloma or monoclonal gammopathy. AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) and AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{+/+}\) mice also showed significant changes in total serum gamma region protein levels between baseline and 12 weeks (Figure S4A). Serum ELISA of antibody subtypes from AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\), AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{+/+}\), and control AID-Cre-YFP Arf\(^{-/-}\) also showed small but significant changes between baseline and 12 weeks in IgM and IgG isosubtypes (Figure S4B), perhaps related to infected, fungating papillomas in these mice. Flow cytometric immunophenotyping of bone marrow and splenic mononuclear cells failed to detect the abnormal growth in any B-cell populations in AID-Cre-YFP Kras\(^{G12D}\) Arf\(^{-/-}\) mice.

**Discussion**

Kras is the oncogene most frequently mutated in MM, yet its role in the pathogenesis of the disease has yet to be elucidated. Here, we used a mouse model of activated Kras to directly test the effect of activated Kras in post-germinal center B-cells using two different Cre recombinases reported to be specific to germinal center B-cells. These mice developed T-cell lymphomas, lung.
adenomas, and sarcomas but no plasma cell tumors despite evidence of activated Kras in vivo B-lineage cells. The Kras allele was recombined in T-cell lymphomas and lung tumors, suggesting these tumors developed as a consequence of off-target Cre expression. In fact, T-cell lymphomas and lung adenomas have been described in KrasG12D mice with Cre expressed via adenovirus and Mx-1 respectively [14,15,26].

For malignant transformation in many contexts, activated Ras requires cooperation with additional mutations [27] and we tried several strategies to accelerate disease in AID-Cre-YFP KrasG12D mice. Cohorts of AID-Cre-YFP KrasG12D mice were subjected to vitamin D deficient chow or sub-lethal radiation or both in an attempt to generate additional mutations and increase the proliferation of pre-malignant B-cells. The combination of vitamin D deficiency and radiation significantly accelerated and worsened the development of skin tumors in AID-Cre-YFP KrasG12D mice, but we observed no B-cell phenotype in any of these mice, despite extensive analysis.

Lastly, we engineered mice with a specific cooperating mutation, germinal center expression of KrasG12D in an Arf-null background. The Ink4a gene locus encoding both Ink4a and Arf is frequently silenced by hypermethylation in MM [28–30] and mutated in some cases of MM (51) and COSMIC database). Germline mutations in Ink4a affect predisposition to plasmacytomas in mice [32] and to MM in people [33].

We observed significant acceleration of skin tumors and progression to invasive carcinomas, demonstrating the successful cooperation between the Kras and Arf pathways, but again, these mice failed to demonstrate a significant B-cell phenotype. The development of non-overlapping off-target tumors demonstrates that KrasG12D can mediate oncogenicity, but germinal center B-cells seem to possess an inherent resistance to its oncogenic effects.

Figure 4. Efficient tissue specific recombination of Kras in class switched B cells of AID-Cre-YFP KrasG12D mice. A) PCR of KrasG12D allele in B-cells of AID-Cre-YFP KrasG12D mice stimulated to undergo class switch recombination ex vivo. Splenic B-cells were stimulated to undergo class switch recombination with lipopolysaccharide (LPS) alone or LPS plus interleukin-4 (IL-4). In contrast to C57-Cre KrasG12D mice in Figure 2B, recombination was seen following stimulation with LPS-IL-4 or with LPS alone. B) FACS-purification of mature B-cell subsets from AID-Cre-YFP KrasG12D mice and detection of recombination by PCR. High-levels of Cre-mediated recombination in B220+/CD138+ bone marrow plasma cells (lane 1), B220+/IgM−/GL7+ splenic germinal center B-cells (lane 5) and B220+/IgM−/IgG1+ class switched memory B-cell populations (lane 9) in AID-Cre-YFP KrasG12D mice. C) Detection of Cre-activated YFP reporter in cells isolated from spleen and bone marrow of AID-Cre-YFP KrasG12D mice given radiation and vitamin D deficient chow. Recombined, YFP-positive cells are plentiful in spleen (6.4%) but rare in the bone marrow (0.20%). Experiment was repeated with three mice and a representative example is shown.

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We conclude that activation of Kras alone or in the context of Arf pathway inactivation is insufficient to disrupt B-cell homeostasis. These negative data demonstrate that GC B-cells are refractory to mutations which are sufficient to transform other murine tissues, and suggest that distinct tumor suppressor pathways may be active in post-GC B-cells.

The temporal order of acquisition of mutations is likely to be important in the development of some cancers. Observational

Figure 5. Gross appearance of cutaneous papillomas in AID-Cre-YFP Kras<sup>G12D</sup> mice is enhanced by tumor-promoting treatments. A) By 3 weeks of age, AID-Cre-YFP Kras<sup>G12D</sup> mice uniformly have hair loss and a single papilloma localized to the ventral neck; B) control Kras<sup>G12D</sup> mouse shows normal hair pattern and no papilloma; C) By 17 weeks, AID-Cre-YFP Kras<sup>G12D</sup> mice given radiation and vitamin D deficient chow (RV) had numerous fungating papillomas and more hair loss at the same site on the ventral neck; D) AID-Cre-YFP Kras<sup>G12D</sup> mice without tumor-promoting treatments also had progressive papillomas but much fewer and with less hair loss associated; E) AID-Cre-YFP Kras<sup>G12D</sup>+RV mice aged to 26 weeks showed confluent fungating and ulcerated masses at the ventral neck with spread to paws; F) age-matched control Kras<sup>G12D</sup>+RV mouse shows no similar signs.

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studies have suggested that Ras activation is a “late event” in myeloma pathogenesis [34]. Ras mutations are significantly less common in patients with monoclonal gammopathy of uncertain significance (MGUS), and are not found in the memory B-cell population of patients with MM, [34] and our data directly supports the model that the temporal order of these events is important to the development of myeloma disease. The lack of a significant B-cell phenotype in our mice is reminiscent of the intrinsic resistance to the effects of KrasG12D displayed by intestinal cells. Intestinal homeostasis is unperturbed in mice by expression of KrasG12D alone [33], but carcinogenesis occurs with concurrent inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene [36]. Mutations in APC do not occur with significant frequency in MM, and it remains unclear what specific mutations cooperate with Ras in myeloma development. In ongoing work, it will be important to determine the pathways that cooperate with Ras activation to transform germinal center B-cells.

**Methods**

**Mouse Strains**

KrasG12D mice [13] (on C57BL/6 background) were crossed to C57 mouse knock-in mice [17] (on C57BL/6 background) or AID-Cre-YFP transgenic mice [18] (on 129/SvJ × C57BL/6 backgrounds) to obtain double transgenic mice. Triple transgenic mice were created by crossing KrasG12D mice to AID-Cre-YFP and Arf-/- mice [20] (on 129/SvJ × C57BL/6 backgrounds) (Figure 1). All mice were routinely observed up to 1 year after birth in a specific pathogen-free facility.

**Ethics Statement**

This study was performed in strict accordance with animal use protocols approved by the Washington University Institutional Animal Care and Use Committee (IACUC, protocol number 20120152). Mice were euthanized if they met any early removal

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**Figure 6.** Cutaneous papillomas in AID-Cre-YFP KrasG12D mice and acceleration of lethality by tumor-promoting treatments. A) Kaplan-Meier survival curves of AID-Cre-YFP KrasG12D mice and control KrasG12D mice. Cohorts of AID-Cre-YFP KrasG12D and KrasG12D mice were subjected to vitamin D deficient chow continuously from 8.5 weeks of age or a single dose of sub-lethal gamma irradiation or vitamin D deficiency. Mice were sacrificed when morbidity developed, defined by weight loss, unkempt coat, hunched posture, and lethargy. Each AID-Cre-YFP KrasG12D group had (n = 5) and developed papillomas, leading to infection, whereas every KrasG12D (n = 5) survived to day 352 endpoint. No B-cell phenotype was observed in any cohort. B) Cre-mediated recombination of Kras locus in DNA from papillomas was detected by PCR in three separate papilloma samples from AID-Cre-YFP KrasG12D mice. WT, wild-type control. doi:10.1371/journal.pone.0067941.g006
criteria (weight loss, lethargy, hunched posture, and/or ruffled coat) to limit suffering, in accordance with NIH-approved institutional animal care guidelines.

Kras Expression

Raw dataset files of Kras, Hras, and Nras expression in murine B cells was accessible through www.ncbi.nlm.nih.gov/geo (accession GSE4142).

Stimulation of Germinal Centers

1 mg of (4-hydroxy-3-nitrophenyl) acetyl conjugated chicken gamma globulin (NP-CGG; Biosearch Technologies, Novato, California) was mixed with Freud's Adjuvant, Complete (Sigma-Aldrich, St. Louis, Missouri) for primary immunization or Freud's Adjuvant, Incomplete (Sigma-Aldrich) for boosting immunization in 100 ul to inject intraperitoneally at specific time points (Figure S5). Where indicated, AID-Cre-YFP KrasG12D mice were given a Vitamin D deficient diet (Harlan, Madison, Wisconsin) beginning at 8.5 weeks of age and/or 4 Gy of sub-lethal ionizing radiation at 12 weeks of age.

Molecular Genotyping of Mouse Strains

For genotyping by PCR, genomic DNA was extracted from tail tissue using Extract-N-Amp Tissue Kit (Sigma-Aldrich). All primer sequences used for genotyping are available upon request. To detect Cre-mediated somatic recombination in bone marrow, spleen, and tumors, genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland), then amplified using PCR to yield expected 622-bp WT and 650-bp loxP, signifying recombination of the KrasG12D allele. Mature B cell populations sorted from spleen and bone marrow DNA was extracted using prepGEM Tissue Kit (ZyGEM, New Zealand).

Ex vivo Class Switch Recombination Assay

Splenocytes from 8–12 week old mice were purified by immunomagnetic depletion of CD43 positive cells (Miltenyi, Auburn, California). CD43 negative splenic naive B cells were cultured with 15 ng/ml of IL-4 (R&D systems, Minneapolis, Minnesota) and 20 ug/ml of LPS or LPS alone (Sigma-Aldrich) in B cell medium (RPMI-1640 with L-Glutamine (Cellgro, Manassas, Virginia), 1% HEPES, 1% penicillin/streptomycin/amphotericin B, 10% FBS (HyClone, South Logan, Utah)) at 8x10^5 cells/well of a 6-well plate. Cells were removed from culture at days 0, 3, 5, and 7 for flow cytometric analysis and DNA extraction (Qiagen). On day 5 and 7, cells were split and given 15 ng/ml of IL-4 and 20 ug/ml of LPS or LPS alone.

Histopathology

Mouse tissues were fixed in 10% neutral buffered formalin for at least 48 hours, dehydrated in an alcohol gradient, cleared in
xylene, and infiltrated and embedded in paraffin. Sections were stained for hematoxylin/eosin (H&E).

Flow Cytometric Analysis

Single cell suspensions of bone marrow and spleen briefly underwent red blood cell lysis. 1x10^6 cells were pre-incubated for 3 minutes on ice with Fc block (CD16/CD32; BD Pharmingen, Franklin Lakes, New Jersey), stained for 25 min on ice with specific antibodies and washed twice in PBS/0.5 M EDTA/0.5 g BSA. The following antibodies were used from BD Pharmingen, unless noted otherwise: FITC-B220 (RA3-6B2), PE- IgM (H/41; eBioscience), APC-IgG1 (X56), AlexaFluor-647 GL7 (eBioscience), PE-CD138 (281-2), PECy7-B220 (RA3-6B2), PE-CD4 (GK1.5), and FITC-CD8. Flow cytometric analysis was performed using FACSscan (Becton Dickinson, Franklin Lakes, New Jersey), modified with additional lasers (Cytek Development). Flowjo software (Tree Star, Ashland, Oregon) was used to analyze a minimum of 10,000 events acquired during collection.

MoFlo

Two 8-week-old C57-1-Cre KrasG^{12D} or Kras AID-Cre mice were stimulated with 100 ug CGG intraperitoneally and sacrificed 14 days later. Preparation of spleen and bone marrow was previously described. Flow cytometric analysis was performed with the MoFlo single-cell sorter (Becton-Coulter, Brea, California).

ELISA and SPEP

Total IgA, IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 levels in serum were measured by enzyme-linked immunosorbent assay (ELISA, Bethyl Laboratories, Inc, Montgomery, TX). Serum protein electrophoresis (SPEP) quantified albumin and globulin proteins from serum using the spife3000 (Helena Laboratories, Beaumont, Texas). Gels were scanned and analyzed using Quicksan2000 software (Helena Laboratories).

Supporting Information

Figure S1 Flow cytometry of C57-1-Cre KrasG^{12D} mouse splenocytes undergoing class switch recombination ex vivo. C57-1-Cre KrasG^{12D} mouse splenocytes negatively selected for CD43 and plated in media supplemented with LPS+IL-4. Flow cytometry shows increase of B220+ IgM+ IgG1+ splenocytes at day 3 with LPS+IL-4, compared to day 0. (TIF)

Figure S2 Analysis of T-cell lymphomas and lung tumors arising in C57-1-Cre KrasG^{12D} mice. A) Flow cytometry of single cell suspension of naive C57-1-Cre KrasG^{12D} mouse that developed fatal thymus tumor. Lymphoma cells appear to be homogeneous and composed of CD4+ and double positive CD4/CD8 populations present in both tumor and spleen. Similar results were obtained in 2 additional naive C57-1-Cre KrasG^{12D} mice that developed thymus tumors. B-E) Hematoxalin & eosin stains of lung sections from immunized C57-1-Cre KrasG^{12D} (B,C) and control naive C57-1-Cre mice (D,E) showing incidentally discovered lung tumors. Original magnification, x4 and x40. Scale bar: 500 um and 50 um. F) PCR of two lung nodule samples from 2 different C57-1-Cre KrasG^{12D} mice show recombination of KrasG^{12D} locus. (TIF)

Figure S3 Subtle changes in immunoglobulin isotype responses in AID-Cre-YFP Kras^{G12D} mice detected by enzyme linked immunosorbent assay (ELISA). A) Total serum gamma region protein levels from AID-Cre-YFP Kras^{G12D} and control Kras^{G12D} mice calculated from total serum protein multiplied by the percentage of protein in the gamma region of serum protein electrophoresis (SPEP) divided by 100. Results are shown from untreated AID-Cre-YFP Kras^{G12D} vs Kras^{G12D} mouse cohorts (immunization protocol only; left panel), AID-Cre-YFP Kras^{G12D} vs Kras^{G12D} cohorts fed vitamin D deficient chow (middle panel) and AID-Cre-YFP Kras^{G12D} vs Kras^{G12D} cohorts given radiation (right panel). B) Serum ELISA of indicated immunoglobulin isotypes of untreated Kras^{G12D} and AID-Cre-YFP Kras^{G12D} mice. All changes were small in magnitude, but statistically significant differences were noted at baseline in IgM, IgA and IgG3 isotypes, at 9 month IgG2b timepoint and total IgG at endpoint. Serum samples were taken at baseline, prior to immunization with NP-CGG; PPI, post-primary immunization; PBI, post-boosting immunization; 9 mo, 9 month time point; Endpt, endpoint prior to sacrifice. Student’s T-test, *, p<0.05, **, p<0.01, *** p<0.001 (TIF)

Figure S4 AID-Cre-YFP Kras^{G12D} Arf^{+/−} shows minimal changes in ELISA and serum protein electrophoresis (SPEP). A) Total gamma region protein levels from serum of AID-Cre-YFP Kras^{G12D} Arf^{+/−} (DKA, n = 3), AID-Cre-YFP Kras^{G12D} Arf^{−/−} (DKA, n = 2), and control AID-Cre-YFP Arf^{−/−} (DA, n = 1) at baseline and 12 weeks, with no immunization. B) Serum ELISA of IgM and IgG isotypes of AID-Cre-YFP Kras^{G12D} Arf^{+/−} (DKA, n = 3), AID-Cre-YFP Kras^{G12D} Arf^{−/−} (DKA, n = 2), and control AID-Cre-YFP Arf^{−/−} (DA, n = 1), with statistical significance of IgM isotype of DKA and IgG isotype of DKA^{+/−}. Student’s T-test, *, p<0.05, **, p<0.01, *** p<0.001 C) SPEP gel and representative graph showing a low gamma protein of control AID-Cre-YFP Arf^{−/−} at 12 weeks, compared to AID-Cre-YFP Kras^{G12D} Arf^{−/−}. (TIF)

Figure S5 Protocol of immunization used in this study. Mice were injected intraperitoneally with NP-CGG in Freund’s complete adjuvant for primary immunization, followed 4 weeks later by boosting immunization with NP-CGG in Freund’s incomplete adjuvant (arrows). Serum was sampled (arrow heads) at baseline prior to PI, four weeks after primary immunization (post-primary immunization; PPI), four weeks after boosting immunization (post-boosting immunization; PBI), at nine months and prior to sacrifice. (TIF)

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Author Contributions

Conceived and designed the experiments: CDM MYS LL. Performed the experiments: CDM MYS LL. Analyzed the data: CDM DN LL. Wrote the paper: CDM MHT.
References


