NIAM-Deficient Mice Are Predisposed to the Development of Proliferative Lesions including B-Cell Lymphomas

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Abstract

Nuclear Interactor of ARF and Mdm2 (NIAM, gene designation Tbrg1) is a largely unstudied inhibitor of cell proliferation that helps maintain chromosomal stability. It is a novel activator of the ARF-Mdm2-Tip60-p53 tumor suppressor pathway as well as other undefined pathways important for genome maintenance. To examine its predicted role as a tumor suppressor, we generated NIAM mutant (NIAMm/m) mice homozygous for a β-galactosidase expressing gene-trap cassette in the endogenous gene. The mutant mice expressed significantly lower levels of NIAM protein in tissues compared to wild-type animals. Fifty percent of aged NIAM deficient mice (14 to 21 months) developed proliferative lesions, including a uterine hemangioma, pulmonary papillary adenoma, and a Harderian gland adenoma. No age-matched wild-type or NIAM+/+ heterozygous animals developed lesions. In the spleen, NIAMm/m mice had prominent white pulp features indicating early B-cell lymphoma. This correlated with selective expansion of marginal zone B cells in the spleens of younger, tumor-free NIAM-deficient mice. Unexpectedly, basal p53 expression and activity was largely unaffected by NIAM loss in isolated splenic B cells. In sum, NIAM down-regulation in vivo results in a significant predisposition to developing benign tumors or early stage cancers. These mice represent an outstanding platform for dissecting NIAM’s role in tumorigenesis and various anti-cancer pathways, including p53 signaling.

Introduction

The p53 tumor suppressor forms the core of an extensive signaling network that protects cells against genomic instability and neoplastic transformation in response to genotoxic insults [1–3]. Once activated, p53 transactivates or represses a wide array of genes that cause cell cycle arrest, promote DNA repair, restrict metabolism or kill irreparably damaged cells, among other anti-cancer activities [4]. Loss of p53 function occurs in the vast majority of human cancers, if not all, due to TP53 gene mutation or alteration of its many regulators and targets [2,5]. Mouse models that lack p53 or express naturally occurring p53 mutants are highly tumor prone and develop the broad range of malignancies found in humans with impaired p53 signaling [6–8]. Understanding how p53 activity is controlled, and the importance of its regulators in tumor biology, has been a top priority in cancer research for more than two decades [1–3,9].
The interplay of NIAM with p53 and its established partners (ARF, Mdm2 and Tip60), all of which are frequently disrupted in human cancers [3,14,17,21,22], suggests NIAM may have tumor suppressive activity. That idea is supported by in silico evidence from microarray databases suggesting significant down-regulation of NIAM mRNA levels in many advanced human cancers [23,24]. Interestingly, NIAM can act independently of ARF-Mdm2-p53 signaling. It can inhibit proliferation in cells lacking ARF, Mdm2 and/or p53, and its depletion in ARF/Mdm2/p53-null mouse embryo fibroblasts results in chromosomal instability [10]. These findings imply that NIAM plays an important role in other anticancer pathways outside of the ARF-Mdm2-p53 tumor suppressor pathway.

Here, we describe the generation and initial characterization of the first NIAM mutant mouse model. These animals have hypomorphic NIAM alleles that result in greatly impaired expression of NIAM protein in tissues, similar to what may occur in human malignancies in which its mRNA expression is down-regulated. Spontaneous tumor formation was assessed and NIAM down-regulation found to increase tumor susceptibility in aged animals. B-cell lymphoma was among the tumors identified and this correlated with a marked expansion of splenic marginal zone B cells in young, tumor-free NIAM-deficient mice. Interestingly, p53 inactivation in B cells promotes splenic marginal zone B cell expansion and B-cell lymphoma [25,26], implicating impaired p53 function in the NIAM knockout phenotype. At least under non-stressed conditions, however, splenic B cells from young NIAM-deficient mice showed no significant effect on basal p53 activity. We suggest that the NIAM mutant mice described in this study represent a unique model of B-cell lymphoma that should help resolve NIAM’s biological role in p53 signaling and other cancer pathways.

## Results

### Decreased NIAM mRNA expression in human tumors

It is well established that ARF-Mdm2-Tip60-p53 signaling plays a dominant role in carcinogenesis [1–3,17,21,27]. Since NIAM has important roles in regulating this pathway, we probed various online databases for the most recent information on NIAM alterations in human cancers. A previous analysis of the ONCOMINE microarray database in 2007 suggested that NIAM mRNA expression is down-regulated in multiple advanced human cancers [10]. The addition of large amounts of microarray results to ONCOMINE since that time only strengthens that conclusion, once again suggesting significant reduction of NIAM mRNA levels in many cancers including lung, breast, brain, prostate, and B-cell lymphoma (Table 1). Recent RNA sequencing data from The Cancer Genome Atlas (TCGA) project is now available for certain cancers and verifies that there is a marked decrease in NIAM (gene name Tbrg1) mRNA levels in human lung, liver, bladder, and breast cancers as compared to paired, normal tissues (Fig. 1). These data demonstrate that NIAM expression is reduced in many different types of human malignancies, consistent with the prediction that it plays an important role in inhibiting tumorigenesis.

### Generation of NIAM-deficient mice

A NIAM gene targeting construct was inserted between exon 1 (which contains the ATG start site) and exon 2 in C57BL/6N embryonic stem cells by the Knockout Mouse Project (KOMP) (Fig. 2A). The cassette contains a poly (A) adenylation site, a neomycin resistance gene (Neo), and β-galactosidase trap for tracking normal NIAM expression patterns in tissues. It also contains FRT sites for removal of neomycin and β-galactosidase cassettes by flipase, which would restore normal gene function of NIAM. Two intronic LoxP sites enable conditional deletion of exon 2 using Cre-expressing mice should the cassette not lead to sufficient loss of the NIAM gene. This construct is predicted to interfere with splicing or, at minimum, generate a severely

### Table 1. Reduced NIAM mRNA levels in cancer tissues.

| Tumor Type       | No. of Studies* | References |
|------------------|-----------------|------------|
| Brain            | 9               | [63–67]    |
| B-cell Lymphoma  | 7               | [33–37]    |
| Lung             | 2               | [68]       |
| Breast           | 10              | [69–71]    |
| Prostate         | 2               | [72]       |

*Microarray analyses identified through ONCOMINE showing a statistically significant decrease in NIAM mRNA in tumors. (p < 0.05).

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Figure 1. NIAM mRNA expression is downregulated in multiple human cancers. NIAM mRNA levels are significantly reduced in primary tumor (T) samples relative to normal (N) tissue, according to RNA-seq data obtained from the TCGA project database. The number of samples analyzed for each tissue was as follows: lung (N = 58, T = 488), liver (N = 50, T = 147), bladder (N = 19, T = 211), and breast (N = 108, T = 992). Error bars represent a 95% CI as calculated using the standard error, and an unpaired Welch’s T-test was used to calculate statistical significance (*, p < 0.0001).

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truncated chimeric protein containing only 48 N-terminal residues of NIAM.

We obtained chimeric mice expressing the mutant (m) NIAM allele from KOMP and generated NIAM heterozygous (+/+m) mice by crossing them to C57BL/6N breeders. After obtaining germline transmission, we interbred heterozygous mice to obtain homozygous offspring (m/m) (Fig. 2B). Heterozygous mouse matings (n = 91) produced litter sizes between 1 to 9 pups (avg = 5.58). The genotype (+/++, +/m, m/m) was determined for 173 mice and the expected Mendelian ratios of 1:2:1 were not met (p = 0.0035, using the χ²-test) (Fig. 2C). The number of heterozygous NIAM+/m and homozygous NIAMm/m mice was higher than expected, possibly suggesting that NIAM loss may provide a selective advantage during embryogenesis. The NIAMm/m mice also successfully mated and produced offspring demonstrating no crucial effects of NIAM on fertility and viability.

To determine if the gene-trap cassette effectively interfered with NIAM expression, mouse tissues from each genotype were obtained and assessed for levels of NIAM protein. NIAM expression, mouse tissues from each genotype were obtained and assessed for levels of NIAM protein. NIAM protein expression remained in all mouse tissues under non-stressed conditions. Indeed, basal expression of NIAM would be predicted to reduce their levels; however, we did not expect to detect significant changes in their expression since p53 signaling is generally kept off in normal tissues. Changes in the spleens of older NIAMm/m mice were often due to reactive hyperplasia. This, along with perivascular lymphoid aggregates in various visceral organs, was consistent with systemic inflammation in mice [29]. In addition, one NIAMm/m mouse had severe diffuse eosinophilic crystalline pneumonia (ECP) whereas only one control had minor localized ECP disease (Fig. 5D). ECP may be seen in certain strains of mice including these with immunodeficiency and Th2 prone inflammatory environments [30,31]. Altogether, these data suggest that NIAM mutant mice have a pronounced inflammatory response.

Inflammation and early B-cell lymphoma develop in NIAMm/m mice

A prominent finding within the cohort was the pathological changes in the spleens of older NIAM mutant mice, which were significantly increased in size compared to wild-type and heterozygous mouse spleens (p < 0.05) (Fig. 5A). Hematoxylin and Eosin (H&E) staining showed that NIAM deficiency led to a significant expansion of the splenic white pulp compared to control age matched cohorts (Fig. 5B, C). These white pulp changes in NIAMm/m mice were often due to reactive hyperplasia. This, along with perivascular lymphoid aggregates in various visceral organs, was consistent with systemic inflammation in mice [29]. In addition, one NIAMm/m mouse had severe diffuse eosinophilic crystalline pneumonia (ECP) whereas only one control had minor localized ECP disease (Fig. 5D). ECP may be seen in certain strains of mice including those with immunodeficiency and Th2 prone inflammatory environments [30,31]. Altogether, these data suggest that NIAM mutant mice have a pronounced inflammatory response.

Figure 2. Generating and verifying NIAM gene disruption in mice. A. The targeted NIAM gene locus for the conditional-ready mouse knock-out model. Cre-recombinase LoxP sites and Flippase targeted FRT sites are shown, as are locations for the PCR primers to detect the endogenous NIAM allele (a+b) or the mutant allele (a+c). Ex, exon. B. PCR amplification of the mutant (mut) allele results in a 380 bp product whereas the 548 bp product reflects the wild-type (WT) NIAM allele. DNA standards are shown in lane 4. C. Number of mice with each genotype (+/++, +/m, m/m) from heterozygous mouse crossings. Chi-square analysis shows a statistically significant difference from the Mendelian distribution of 1:2:1. doi:10.1371/journal.pone.0112126.g002
proinflammatory phenotype. Additionally, two of the twelve (17%) NIAMm/m mice developed early B-cell lymphoma. Their spleens had multifocal loss of the white pulp architecture (e.g. small lymphocytes, germinal centers and tingible body macrophages) with replacement by highly mitotic centrocytic and centroblastic cells (Fig. 5E) [32]. When considered with the development of various benign neoplasms in other NIAMm/m animals, these results indicate that NIAM expression is required to prevent spontaneous tumorigenesis.

Marginal zone B cells are increased in NIAMm/m mice

Since aged NIAMm/m mice have enlarged spleens and some develop B-cell lymphoma, we evaluated B cell development in younger, tumor-free mice (6 months of age, 3 of each NIAM genotype) to identify potential pre-malignant changes that could give rise to B-cell tumors. Analysis of B cell development in the bone marrow of NIAMm/m mice revealed no alterations compared to wild-type control animals (Fig. 5I). Specifically, there were no significant differences in the frequency of pro/pre (B220+IgM-, p = 0.4783), immature (B220+IgM+, p = 0.7693) or mature (B220-IgM+, p = 0.8048) B cells. Analysis of B cells from the spleen likewise showed no difference in the frequency of transitional (CD21hi/CD23-) or follicular (CD21+/CD23+) B cells (Fig. 6A, B; p values of 0.5068 and 0.9325, respectively). In contrast, splenic marginal zone B cells (CD21hi/CD23-) were significantly increased in frequency (p = 0.0281) and number (p = 0.0144) in NIAMm/m mice compared to wild-type controls (Fig. 6). These results uncover a specific sensitivity of splenic marginal zone B cells to NIAM loss, which may suggest that NIAM normally restricts their proliferation.

Selective expansion of splenic marginal zone B cells also occurs in mice with conditional inactivation of p53 in B cells and it is associated with their development of B-cell lymphomas [25,26]. Since NIAM is a positive regulator of p53 expression and transcriptional activation [12], and some NIAM mice developed B-cell lymphoma, we wondered if the B cell phenotype in NIAMm/m mice could be associated with reduced p53 signaling. Therefore, we examined p53 status in LPS-stimulated splenic B cells isolated from five wild-type NIAM+/+ and five mutant NIAMm/m mice. Western analyses showed complete NIAM loss in NIAMm/m cells compared to wild-type B cells (Fig. 7). We expected NIAM loss would reduce p53 levels and activity, but surprisingly it had no effect on basal expression of p53 or its targets, Mdm2 and p21, in the B cells of most NIAMm/m mice (4 of 5) relative to NIAM-positive B cells (Fig. 7, set A). These results were seen in B cells isolated from both 8 week and 6 month old mice, suggesting the marginal zone B cell expansion in 6 month old NIAM mutant mice is likely independent of p53. Notably, one NIAM mutant mouse had decreased B cell expression of p53 although this did not correlate with reduced expression of Mdm2 or p21, at least under these stress-free conditions (Fig. 7, set B). These analyses show that NIAM is not required for basal p53 activity in splenic B cells but may, depending on the context, predispose to p53 down-regulation. Additional studies examining p53 stimulation and checkpoint activation following DNA damage or other genotoxic insults in NIAM-deficient B cells are warranted.

Discussion

This study is the first investigation of NIAM function in vivo using newly generated NIAM-deficient mice. A tumor suppressor role for NIAM was anticipated given its ability to activate p53 and other currently undefined anti-cancer pathways involved in growth inhibition and genome maintenance [10,12]. Consistent with that prediction, reduced NIAM expression in mice yielded a tumor phenotype characterized by the development of both benign and cancerous lesions, including early B-cell lymphoma. No neoplasms developed in similarly aged heterozygote or wild-type controls, strongly suggesting that tumor formation was due to

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**Table 2. Neoplastic lesions in NIAM-deficient mice.**

| Genotype   | Avg. Age at Necropsy (weeks) | # of mice with Neoplasms* | Types of neoplasms |
|------------|-----------------------------|---------------------------|--------------------|
| NIAMm/m    | 66                          | 6/12                      | Early B-cell lymphoma (2) Uterine hemangioma (1) Preputial gland cyst (1) Harderian gland adenoma (1) Lung adenoma (1) |
| NIAM+/+    | 68.2                        | 0/8                       | -                  |
| NIAMm/m    | 68.5                        | 0/8                       | -                  |

* p < 0.0025 by Fisher’s Exact Test between control (NIAM+/+ and NIAMm/m) mice and NIAMm/m mice.

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**Figure 3. NIAM protein expression in tissues of NIAM mutant mice.** NIAM protein levels were assessed in mouse tissues from two mice of each genotype (+/+, +/m, m/m). Equivalent amounts of protein lysates from spleen, bladder, lung, brain, and pancreas were analyzed by immunoblotting with antibodies to NIAM and γ-tubulin. The relative expression of NIAM in each sample, as compared to the first wild-type (+/+) sample in each tissue set, is denoted below the lanes. Relative NIAM levels were determined by Image J analysis of band intensities followed by normalization of those values to quantified intensities of the loading control (γ-tubulin or the non-specific band in brain lysates) in each sample. doi:10.1371/journal.pone.0112126.g003
NIAM loss. It is noteworthy that systemic inflammation was seen in multiple mice as that often precedes tumorigenesis. Although mice from each genotype (+/+, +/+m, m/m) in our cohort showed signs of heightened inflammation, only the NIAMm/m animals develop lesions. Thus, it is possible that reduced NIAM expression may accelerate or exacerbate the tumor promoting effects of a chronic inflammatory response.

The development of B-cell lymphoma in NIAMm/m mice is consistent with several observations. First, microarray analyses suggest that NIAM mRNA levels are significantly reduced in human B-cell lymphomas [33–37]. Second, NIAM’s functional partners, ARF, Mdm2, and p53 each play a critical role in that disease. ARF and p53 are commonly inactivated whereas Mdm2 is overexpressed in a majority of B-cell lymphomas in patients [38–40], and genetically engineered mice that mimic those alterations effectively model the disease [41–43]. Finally, NIAM is a growth inhibitor whose activation of p53 in cultured cells results in induction of p21 (CDKN1A/Waf1/Cip1) [10,12], a key transcriptional target of p53 [44,45]. Others previously showed that a significant fraction (14%) of tumors that develop in p21 knockout mice are B-cell lymphomas [46], which is similar in incidence (17%) to the B-cell lymphomas arising in NIAMm/m mice.

Our data show that NIAM contributes to tumor suppression but to a lesser extent than p53 or ARF. Half of NIAMm/m mice developed benign and early cancerous lesions at an average age of 16.5 months (66 weeks). By comparison, mice lacking p53 in all tissues develop a range of malignancies (mainly T-cell lymphomas and sarcomas) with full penetrance at approximately 5 months of age [6,7]. Less pronounced in vivo effects are observed for disruption of p53 regulators and targets relative to loss of p53 itself [2]. For example, animals lacking ARF, a major positive regulator of p53, develop tumors at a slower rate (~10 months average age) than p53-null mice [47,48]. Among p53 targets, loss of PUMA causes no increased susceptibility to tumorigenesis [49] while deletion of p21 yields a moderate tumor phenotype [46]. Specifically, p21-null animals develop tumors at an average age of 16 months and with reduced incidence (27% in females, 55% in males) compared to mice lacking p53. Thus, a milder tumor phenotype for NIAMm/m mice is not surprising since it represents just one of many p53 regulators [1,3].

We currently know very little about the normal physiological or pathological signals that control NIAM expression and where its function is required. The expression of β-galactosidase in these mutant mice will provide an excellent surrogate for tracking normal patterns of NIAM expression during development, in particular tissue and cell types, and in response to different stimuli. The interplay between NIAM, p53, and ARF, as well as NIAM’s regulation of p21 expression, may be instructive. For instance, loss of each factor (p53, ARF, or p21) is associated with impaired stem cell maintenance and renewal for a variety of cellular lineages, including hematopoietic and neuronal cells, suggesting a possible contribution by NIAM to those processes and cell types [50–52]. Similarly, one of the most significant cellular stresses that stimulate p53 activity is DNA damage [1]. We recently found that treatment of MCF10A mammary epithelial cells causes NIAM protein upregulation [Fig. S2], implying a role for NIAM in the DNA damage response. The importance of NIAM to p53 activation and maintenance of DNA integrity in different tissues in response to DNA damage or other diverse stimuli may now be evaluated in an in vivo context.

A link between NIAM and p53 in B cell development and lymphoma was strongly suggested by findings that mice lacking those genes each display splenic marginal zone B cell expansion and B-cell lymphoma ([25,26] and this study). Yet NIAM loss had minimal effects on basal p53 expression and activity in B cells from young animals, with just one of five NIAMm/m mice displaying lower p53 levels. That unexpected result suggests the splenic marginal zone B cell enrichment in young NIAMm/m mice is independent of p53. However, it remains to be determined if p53 function in response to DNA damage or other cellular stresses is diminished in NIAMm/m cells and contributes to tumor development in older animals. Indeed, it is conceivable that lower basal p53 expression in splenic B cells observed in some NIAMm/m mice impairs stress-induced p53 signaling and checkpoint activation, ultimately fostering B-cell lymphomagenesis in aging mice. Future studies will assess that possibility.

Crosses between NIAM mutant mice with other genetic models of cancer will be instrumental in determining NIAM’s contribution to p53 signaling as well as other cancer pathways. In that regard, in vivo studies should help resolve the biological relevance of NIAM’s association with the ARF tumor suppressor [10–12]. Initial studies showed NIAM is capable of mobilizing ARF into the nucleoplasm [10], where ARF is known to more effectively bind

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**Figure 4. NIAMm/m mice are predisposed to proliferative lesions.** Histopathologic analyses of multiple mouse tissues. A. A hemangioma (asterisk, right panel) found in the uterus of a NIAMm/m mouse compared to normal uterine tissue (left panel). (H&E, 100x) B. Pulmonary papillary adenoma in a homozygous NIAMm/m mouse (arrow, right panel). A depiction of normal lung tissue from a control mouse is shown (left panel). (H&E, 20x) C. Harderian gland adenoma of a NIAMm/m mouse (asterisks, right panel). A representative image of a normal Harderian gland (asterisk) from a control mouse is depicted (left panel). (H&E, 40x) D. A representative image of a normal liver from a control mouse (left panel) versus a focus of cellular alteration (arrows) in a NIAMm/m mouse (right panel). (H&E, 40x) Controls represent tissues from similarly aged wild-type (panels B and C) and NIAMm/m heterozygous (panels A and D) mice, which were found to be indistinguishable in our analyses.

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Mdm2 and activate p53 [15,16], yet NIAM and ARF can nonetheless act independent of each other to transiently activate p53 [10,12]. It is possible that NIAM’s inhibition of Mdm2 and association with Tip60 may help sustain ARF-p53 signaling and promote senescence in cells following DNA damage or oncogene activation. If so, this would represent an important anti-cancer function since p53-mediated cellular senescence (induced by ARF or other mechanisms) is thought to play a pivotal role in tumor suppression [53–56].

Earlier work on NIAM and much of this Discussion has revolved around the idea that NIAM would prevent tumorigenesis, at least in part, through its effects on p53. However, it is likely that NIAM has significant anti-cancer functions in vivo that are independent of p53. One reason is that NIAM can inhibit proliferation and promote chromosomal stability independent of ARF, Mdm2, and p53, indicating it acts in other important pathways relevant to maintaining genomic stability [10]. Moreover, in this study basal p53 expression in splenic B cells was unchanged by NIAM loss in most animals. One factor besides p53 that could contribute to the tumor phenotype in NIAM−/− mice is transforming growth factor beta 1 (TGF-β1). NIAM was originally identified as a TGF-β1 responsive gene [13] and we previously found it is induced in TGF-β1-arrested cells [10]. TGF-β1 is a potent inhibitor of proliferation in many cell types (epithelial, endothelial, hematopoietic, etc), but alterations of its signaling components in neoplastic cells ultimately causes it to drive proliferation and cancer progression [57,58]. If NIAM is an effector of TGF-β1 signaling, its down-regulation in NIAM+/− mice may diminish the anti-proliferative activities of the pathway and consequently enhance its tumor-promoting effects.

Another factor of interest is the nuclear transcription factor, NF-κB. NF-κB was originally identified in B cells, contributes to marginal zone B cell formation, and is often constitutively activated in numerous inflammatory conditions and human cancers, including B cell lymphomas [59,60]. NF-κB is activated by a multitude of stimuli and cross-talks with many essential molecules (e.g., p53 and STAT3) and complex signaling pathways (including TGF-β) that control inflammation, cell survival and cell proliferation, among other key biological processes. The enrichment of marginal zone B cells as well as progressive expansion of splenic white pulp and development of B-cell lymphomas in NIAM mutant mice supports the prediction that NF-κB signaling may be hyper-activated when NIAM is down-regulated.

Overall, our data show that NIAM deficiency is associated with a proinflammatory phenotype and facilitates spontaneous tumor-ogenesis. Based on the literature, we speculate that the marginal

Figure 5. Loss of NIAM promotes reactive lymphoid hyperplasia and B-cell lymphoma in the spleen. A. Weights of spleens from +/+ and +/+ m mice (CON, n = 15) compared to NIAM+/− mice (m/m, n = 12). Note: one WT (+/+ mouse with an incidental case of severe chronic pyometra was excluded from the analysis. Statistical significance was shown by a Mann-Whitney test (*, p<0.05). B. The percentage of splenic white pulp per total splenic area was calculated for each genotype. NIAM+/− mice (n = 8) are statistically different from both wild-type (+/+, n = 5) and heterozygous (+/m, n = 6) mice as calculated by an unpaired two-tailed T-test. (*, p<0.05) Note: one WT (+/+) and one mutant (m/m) mouse were excluded from the analysis because extensive extramedullary hematopoiesis prevented accurate visualization of the white pulp. C. Representative images of spleens from control wild-type (+/+) and homozygous m/m mice (H&E, 40x). Splenic white pulp (asterisks) was increased in NIAM+/− mice. Note: these samples show a range of variation in the extent of red pulp extramedullary hematopoiesis that was seen for both groups. D. Eosinophilic crystalline pneumonia in the lungs of control NIAM+/− heterozygous and NIAM+/− homozygous mice. Loss of NIAM resulted in more severe disease with extensive pulmonary lymphoid inflammation (arrows) and large extracellular crystals (asterisks) in the affected NIAM+/− mouse. (H&E, 400x). A spleen from a heterozygous mouse with chronic ulcerative dermatitis and reactive hyperplasia of white pulp characterized by a germinal center (GC) and tingible body macrophages full of cellular debris (arrows) on a background of small lymphocytes (SL) (left panel). In two homozygous m/m mice, typical white pulp architecture was multifocally depleted and effaced by centroblasts and centrocytes with multiple mitotic figures (inset and arrows) (right panel). Note that NIAM+/− heterozygous and NIAM−/− wild-type mice appeared identical in our analyses and were therefore used interchangeably as controls.

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Neoplasias in NIAM-Deficient Mice
zone B cell expansion in NIAM mutant mice is intimately related to their development of systemic inflammation [61] and B cell lymphomagenesis [25,26]. We also predict that NIAM, as a regulator of ARF-Mdm2-Tip60-p53 signaling and other undefined pathways affecting maintenance of chromosomal stability, normally cooperates with multiple anti-cancer pathways to suppress tumor development. The NIAMm/m mice described here are an outstanding model to explore those concepts.

Materials and Methods

Animal Husbandry and Ethics Statement

Mice were housed in the University of Iowa Animal Care barrier facility. Mice were kept in rooms with a 12 hour light-dark cycle and free access to water and food. All mouse experiments were conducted according to protocols approved by the University of Iowa Institutional Animal Care and Use Committee (protocol #1204079). All efforts were made to minimize suffering. Mice were euthanized using carbon dioxide inhalation.

Figure 6. Marginal zone B cells are increased in NIAMm/m mice. Flow cytometric analyses of splenic B cell development. Splenic B cells were isolated from young, 6 month old tumor-free wild-type (+/+ ) and NIAM mutant (m/m) mice (three of each genotype). A. Representative flow cytometry plots of NIAM wild-type versus m/m IgM positive splenocytes. Transitional (CD21(lo)/CD23-), follicular (CD21(+) /CD23+) and marginal zone (CD21(hi)/CD23-) B cells are identified. B. Average frequency of follicular (top left), transitional (top right), and marginal zone (bottom left) B cells from mice of the indicated genotypes. Average marginal zone B cell numbers are also shown (bottom right). All p values were calculated using unpaired, two-tailed Student’s T tests. doi:10.1371/journal.pone.0112126.g006

Figure 7. NIAM is not required for basal p53 activity in splenic B cells. Splenic B cells from young, tumor-free wild-type NIAM(+/+) or NIAMm/m homozygous mutant mice were isolated and expression of endogenous NIAM, p53, Mdm2 and p21 proteins was assessed by western blotting. Relative levels of p53, Mdm2 and p21 (normalized to GAPDH loading and compared to the untreated, wild-type control sample) were determined following quantification of bands using Image J. Representative results from 4 of 5 pairs of mice (both 8 week and 6 months of age) are shown in Set A, while Set B shows data for one pair of 6 month old mice. Set A and B samples were analyzed on separate gels, and lanes within each set were spliced together from the same autoradiogram for image clarity. doi:10.1371/journal.pone.0112126.g007

Generation of NIAM Mutant Mice

The Knockout Mouse Project (KOMP) generated male chimeras by injecting embryonic stem cells containing a NIAM(TBRG1) allele with a β-galactosidase cassette and LoxP sites around Exon2 into C57BL/6 blastocysts (www.komp.org, Project ID: CSD141510). Chimeric mice were then bred with C57BL/6/N females to obtain germline transmission of the mutant TBRG1 allele. To confirm mouse genotypes, the REDExtract-N-Amp Tissue PCR Kit (Sigma Aldrich, XNAT-100RXN) was used to isolate DNA from mouse tails and to perform PCR. PCR genotyping primers were:

Primer a: 5′-GGTCAAAGCTGTAAGCATAGAGAGTC-3′
Primer b: 5′-CTTGAGGCTCCTTTCTGGTG-3′
Primer c: 5′-CCAAGCTGCTGGCAGAACAT-3′

PCR reactions consisted of 2 μL DNA added to 5 μl RedExtract PCR Master Mix (Sigma Aldrich), 0.8 μl of each primer and 0.6 μL PCR grade water to a total reaction volume of 10 μL. PCR was performed as follows: 95°C for 5 min, 35 cycles of 95°C for 1 min, annealing of primers at 54°C for 1 min, and extension at 72°C for 1.5 min on a PCR Sprint thermal cycler (Thermo Scientific Hybaid).

Database Analysis

The Cancer Genome Atlas Pan-Cancer analysis project’s individual RNAseq datasets were downloaded from the UCSC Cancer Genome Browser (https://genome-cancer.ucsc.edu/). The UCSC Cancer Browser Team utilized level 3 TCGA RNAseq datasets (available at https://tcga-data.nci.nih.gov/tcga/), log2(x+1) transformed, and renormalized to the expression of all available TCGA cancer cohorts. PanCan normalized, log2(x+1) expression for available paired normal and primary tumor samples were compared for each TCGA cancer cohort utilizing an unpaired Welch’s T-test.

Protein Analyses in Mouse Tissues

Mouse tissues were isolated, flash frozen in liquid nitrogen, and crushed into a powder by mortar and pestle. Tissues were lysed
with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Sodium deoxycholate) supplemented with 1 mM NaF, protease inhibitor cocktail (Sigma, P6304), phosphatase inhibitor cocktail (Sigma, P0044), and 30 μM phenylmethylsulfonyl fluoride for 1 hour on ice. After brief sonication (2.5×5 s pulses), protein lysates were centrifuged at 14,000 rpm for 15 min at 4°C. The concentration of protein for each tissue was assessed by BCA assay (Pierce, Rockford, IL). Equivalent amounts of total cellular protein was loaded and separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and analyzed by immunoblotting. Proteins were detected on membranes by ECL (Amersham Biosciences) with antibodies against NIAM (rabbit polyclonal antibody at 1.5 μg/ml [10] and mouse monoclonal antibody [clone 11E12] at 1:5 dilution [62]) and γ-tubulin (Sigma, T6557, mouse monoclonal antibody, 1:10,000).

Primary B Cell Analyses

Bone marrow and spleens were harvested from wild-type and NIAM mutant mice (three 6 month old littermates males per genotype) and mixed between frosted glass slides to liberate cells. ACK lysis (Lonza, Radnor, PA) was used to lyse red blood cells. For flow cytometric analyses of B cells, one million lymphocytes were washed and resuspended in staining buffer that consisted of balanced salt solution, 5% bovine calf serum and 0.1% sodium azide. Non-specific binding of antibody was blocked using 10% rat serum (Jackson Immunoresearch, West Grove, PA) and 10 μg 2.4G2 (BioXCell, West Lebanon, NH). Cells were then incubated on wet ice in the dark with antibodies to B220-PE-Cy7 (6B2, eBioscience, San Diego, CA), CD23-PE (B3B4, eBioscience), CD21-APC (7G6, BD Biosciences, San Jose, CA), and IgM-FITC (RMM-1, Biologend, San Diego, CA). Samples were run on a BD FACSCANTO II instrument (Becton Dickinson, San Jose, CA) and data were analyzed using FlowJo (Tree Star, Ashland, OR). For analyses of p53, B cells were isolated from spleens of 6 month old and NIAM mutant mice (three 6 month old littermates males per genotype) and mixed between frosted glass slides to liberate cells. ACK lysis (Lonza, Radnor, PA) was used to lyse red blood cells. For flow cytometric analyses of B cells, one million lymphocytes were washed and resuspended in staining buffer that consisted of balanced salt solution, 5% bovine calf serum and 0.1% sodium azide. Non-specific binding of antibody was blocked using 10% rat serum (Jackson Immunoresearch, West Grove, PA) and 10 μg 2.4G2 (BioXCell, West Lebanon, NH). Cells were then incubated on wet ice in the dark with antibodies to B220-PE-Cy7 (6B2, eBioscience, San Diego, CA), CD23-PE (B3B4, eBioscience), CD21-APC (7G6, BD Biosciences, San Jose, CA), and IgM-FITC (RMM-1, Biologend, San Diego, CA). Samples were run on a BD FACSCANTO II instrument (Becton Dickinson, San Jose, CA) and data were analyzed using FlowJo (Tree Star, Ashland, OR).

Mouse Necropsy and Histopathological Analyses

Individual organs were harvested from mice euthanized by CO2 asphyxiation. A total of 28 mice (8 NIAM+/+, 8 NIAM/m+ and 12 NIAM/m−) between the ages of 14 to 21 months were subjected to pathological examination. Organs assessed by macroscopic and histopathological analyses included pancreas, liver, spleen, gastrointestinal tract, reproductive tract for males and females, kidney, urinary bladder, lung, heart, head sections (e.g., eyes, nasal cavity, etc), and sagittal section of the brain (cerebellum, cerebrum and brain stem). Organ weights (e.g., spleen and liver) were collected and then tissues were formalin fixed and paraffin embedded. Tissue sections (~4 μm) were made from paraffin blocks and stained with hematoxylin and eosin (H&E). Mouse tissues were analyzed and reviewed for abnormal findings by a pathologist. Quantification of the white pulp of individual mouse spleens stained with H&E was measured by Image J software. The splenic white pulp was evaluated as a percentage of the total splenic area in tissue sections.

Supporting Information

Figure S1 Early B cell development is not altered in NIAM-deficient mice. Flow cytometric analysis of early B cell development. A. Representative flow cytometry plots of bone marrow lymphocytes from NIAM wild-type (+/+) or mutant (m/m) mice. Early (B220+/IgM−), immature (B220+/IgM+) and mature (B220(high)/IgM+) B cells are identified. B. Average frequency of immature (left), mature (middle) and early (right) B cells in NIAM +/- versus m/m mice. No significant differences were observed. For these studies, bone marrow was isolated and analyzed from six mice total (the same analyzed in Figure 6), three of each genotype.

Figure S2 Sustained DNA damage induces NIAM expression. Western analyses show that endogenous NIAM protein is induced by DNA damage caused by exposure to doxorubicin (Dxn, 66 ng/mL) for the indicated times (hrs) in human MCF10A mammary epithelial cells. GAPDH levels serve as control for equivalent loading.

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