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Nfkb1 is a haploinsufficient DNA damage-specific tumor suppressor

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Abstract

NF-κB proteins play a central and subunit-specific role in the response to DNA damage. Previous work identified p50/NF-κB1 as being necessary for cytotoxicity in response to DNA alkylation damage. Given the importance of damage-induced cell death for maintenance of genomic stability, we examined whether *Nfkb1* acts as a tumor suppressor in the setting of alkylation damage. *Hprt* mutation analysis demonstrates that *Nfkb1*$^{-/-}$ cells accumulate more alkylator-induced, but not ionizing radiation (IR)-induced, mutations than similarly treated wildtype cells. Subsequent *in vivo* tumor induction studies reveal that following alkylator treatment, but not IR, *Nfkb1*$^{-/-}$ mice develop more lymphomas than similarly treated *Nfkb1*$^{+/+}$ animals. Heterozygous mice develop lymphomas at an intermediate rate and retain functional p50 in their tumors indicating that *Nfkb1* acts in a haploinsufficient manner. Analysis of human cancers, including therapy-related myeloid neoplasms, demonstrates that *NFKB1* mRNA expression is down regulated compared to control

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CONFLICT OF INTEREST

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samples in multiple hematological malignancies. These data indicate that *Nfkb1* is a haploinsufficient, pathway-specific tumor suppressor that prevents the development of hematologic malignancy in the setting of alkylation damage.

Keywords

NF-κB; tumor suppression; DNA damage; lymphoma

INTRODUCTION

Damage to DNA is a central factor in the acquisition of genomic errors and development of cancer. Chemotherapy represents an exogenous source of DNA damage that is highly genotoxic. Therapy-related myeloid neoplasia (t-MN) is a devastating complication of cytotoxic chemotherapy whereby a secondary malignancy is induced following treatment of an initial malignant or non-malignant disease.[1] The importance of DNA alkylation damage to secondary malignancy formation is underlined by the observation that in the largest study of t-MN reported to date, 78 % of patients received an alkylating agent for their primary tumor.[2]

NF-κB represents a family of proteins comprised of five subunits: p50 (NF-κB1, p105), p52 (NF-κB2, p100), p65 (relA), c-rel, and relB that play a complex role in tumor formation. While the oncogenic effects of this transcription factor are consistent with the propensity of NF-κB to mediate survival signaling,[3, 4] tumor suppressive actions are evident in the finding that various subunits can indirectly mediate the tumor suppressive effects of p53 or ARF.[5–9] Despite indirect tumor suppression, targeted deletion of individual NF-κB proteins has not yet revealed a direct tumor suppressive role for any specific subunit.[10]

We recently reported that p50/NF-κB1 is necessary for cytotoxicity by alkylating agents such as temozolomide (TMZ) or N-methyl-N-nitrosourea (MNU).[11] These agents induce cell death by forming O^6-methylguanine (O^6-MeG) lesions that mispair with deoxythymidine (T) residues and induce cytotoxicity in a mismatch repair-dependent fashion.[12] In cells where damage is tolerated, O^6-MeG:T mismatches lead to mutations that are potentially oncogenic.[13] Given that depletion of p50, or deletion of *Nfkb1*, renders cells tolerant of alkylation damage without affecting damage repair,[11] we hypothesized that this NF-κB subunit acts to maintain genomic stability specifically in the setting of DNA alkylation damage. In this report, we demonstrate that *Nfkb1* protects mice against alkylator-induced, but not ionizing radiation (IR)-induced, lymphoma formation in a haploinsufficient manner.

RESULTS AND DISCUSSION

Alkylating agents are mutagenic and loss of p50/*Nfkb1* leads to increased survival following alkylator treatment.[11] These findings suggest that loss of *Nfkb1* may affect alkylator-induced mutagenesis. To examine mutation formation, the hypoxanthine-guanine phospho-ribosyltransferase (hprt) assay was used in $Nfkb1^{-/-}$ and $Nfkb1^{+/+}$ MEFs. This assay accurately reports alkylator-induced mutations. Primary, low passage MEFs were treated

with alkylating agent and mutation induction assessed. Significantly more 6-TG resistant clones develop following treatment of *Nfkb1*$^{-/-}$ MEFs than *Nfkb1*$^{+/+}$ ($P < 0.03$, Figure 1a), suggesting that *Nfkb1* maintains genomic integrity in the setting of alkylation damage. To examine whether it is specifically p50, the mature protein product of *Nfkb1*, that prevents mutation induction, *Hprt* assay was performed following reexpression of p50. *Nfkb1*$^{-/-}$ MEFs stably expressing p50 acquire less 6-TG resistant clones following alkylator treatment than isogenic cells expressing empty vector (EV) (Figure 1b). Next, to examine DNA strand break induction, alkaline comet assay was performed following treatment. Equal amounts of strand breaks are induced in *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ MEFs within 3 hours of treatment (Figure 1c). This finding suggests that the difference in the mutation frequency following alkylator exposure is not due to quantitative differences in the level of induced DNA damage, a finding supported by previous data showing that loss of *Nfkb1* does not affect damage repair.[11] The above observations, coupled with the finding that loss of *Nfkb1* results in a decrease in alkylation-induced apoptosis,[11] suggest that the difference in mutagenesis is likely due to increased survival of damaged *Nfkb1*$^{-/-}$ compared to *Nfkb1*$^{+/+}$ MEFs.

Increase in mutation formation suggests that *Nfkb1* may be tumor suppressive in the setting of DNA damage. To examine this hypothesis, we attempted to transform MEFs using TMZ or MNU but found that primary cells of both genotypes are resistant to alkylator-induced transformation *in vitro*. Therefore, *in vivo* tumor formation was examined using *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ mice. MNU was used as the inducing carcinogen due to its well-described tumor-forming dose response profile.[14, 15] 6-week old *Nfkb1*$^{-/-}$ and *Nfkb1*$^{+/+}$ mice were injected with either vehicle or MNU and followed for 1 year. At each MNU concentration, significantly more *Nfkb1*$^{-/-}$ than *Nfkb1*$^{+/+}$ mice develop thymic lymphomas ($P < 0.002$ at 30 mg/kg and $P < 0.02$ at 15 mg/kg) (Figure 2a). Next, to examine potential *Nfkb1* gene-dosage effects, *Nfkb1*$^{-/-}$ animals, backcrossed for 12 generations with C57BL/6 mice, were obtained and bred with wildtype C57BL/6 animals. Subsequently, *Nfkb1*$^{-/-}$, *Nfkb1*$^{+/-}$ and *Nfkb1*$^{+/+}$ mice were injected with 50 mg/kg MNU. At this higher MNU concentration, *Nfkb1*$^{-/-}$ mice also form significantly more thymic lymphomas than *Nfkb1*$^{+/+}$ animals ($P < 0.0001$) (Figure 2b and Supplementary Figure 1) while heterozygotes have an intermediate tumor induction rate ($P \leq 0.05$ relative to *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$). FACS analysis demonstrates that the lesions are CD4−/CD8− and CD4+/CD8+ T-cell tumors (data not shown).

The carcinogenic effect of MNU is blocked primarily by O^6-methylguanine-DNA methyltransferase (MGMT), a protein that specifically removes alkyl groups from the O^6 position of guanine.[15] Therefore, MGMT expression level was examined in untreated mice. Equal MGMT protein expression is seen in thymic tissue from *Nfkb1*$^{-/-}$ and *Nfkb1*$^{+/+}$ animals (Figure 2c) suggesting that the difference in tumor formation rate is not due to differences in O^6-MeG repair. In addition, given that *Nfkb1*$^{-/-}$ animals have defects in innate and adaptive immunity,[10, 16] we examined whether tumors from *Nfkb1*$^{-/-}$ animals can be transplanted into mice of both genotypes. *Nfkb1*$^{-/-}$ and *Nfkb1*$^{+/+}$ recipient mice develop disseminated cancer at equal rates following injection of tumor cells from *Nfkb1*$^{-/-}$ animals (Supplementary Figure 2) indicating that *Nfkb1*$^{-/-}$ tumors are equally transplantable.

It was previously noted that loss of *Nfkb1* does not affect the cytotoxic response to IR.[11] We therefore examined tumor formation in response to whole-body IR. Remarkably, at the IR dose used, no significant difference in tumor formation rate is noted between $Nfkb1^{-/-}$ and $Nfkb1^{+/+}$ animals ($P > 0.5$) (Figure 2d). While these data suggest that *Nfkb1* does not protect mice from IR-induced tumor formation, it is possible that at a different dose of IR, or if greater numbers of animals are used, a difference in tumor formation may be noted. Nevertheless, given that the dose of IR used results in approximately 30% tumor formation in both genotypes, a rate comparable to that induced by 30 mg/kg MNU in $Nfkb1^{+/+}$ mice (Figure 2a), it is evident that loss of *Nfkb1* renders animals significantly more sensitive to tumor formation by MNU than IR. Given the findings in response to IR, we also examined mutation induction following IR. In contrast to TMZ, expression of p50 does not affect mutation frequency in response to IR (Figure 2e). Together, these findings indicate that loss of *Nfkb1* leads to an increase in the susceptibility of mice to alkylation-induced tumor formation and moreover, suggest that *Nfkb1* acts as a pathway-specific tumor suppressor.

To examine whether *Nfkb1* functions in a haploinsufficient manner, tumors from mice were harvested. All the tumors from $Nfkb1^{+/-}$ animals retain an *Nfkb1* allele (Figure 3a). As loss of heterozygosity can also occur via post-translational mechanisms, expression of p105 and p50 was examined. In tumors from $Nfkb1^{+/-}$ animals, both p105 and p50 expression are universally retained (Figure 3b). Moreover, immunohistochemical analysis demonstrates that p50 is found in tumor cells from $Nfkb1^{+/-}$ mice and not solely in the stroma (Figure 3c). Finally, to examine the functional status of p50, binding to κB consensus DNA was evaluated using EMSA. Protein from three tumors of each genotype was harvested and examined for subunit binding by supershift analysis. In addition to p50 binding, p52 was examined as this subunit most often cross-compensates when p50 is lost,[1117] and p65 was studied as this is the primary interacting partner of p50. Supershift analysis demonstrates that while tumors from $Nfkb1^{+/+}$ animals contain p50/p65 dimers, in $Nfkb1^{-/-}$ mice although p65 remains, p50 binding is lost and replaced by p52 (Figure 3d). Most significantly, tumors from $Nfkb1^{+/-}$ animals retain p50 that binds DNA. These findings indicate that functional p50, i.e. p50 that does not contain mutations in critical DNA binding regions, is maintained in tumors from heterozygous mice and, when considered with the intermediate tumor formation rate in heterozygote animals (Figure 2b), suggest that *Nfkb1* is a haploinsufficient tumor suppressor.

Tumor suppression by *Nfkb1* raises the question of whether this subunit acts in a similar fashion in man. Although *NFKB1* is rarely mutated in human tumors, the haploinsufficient nature of this factor suggests that reduced expression may be sufficient to facilitate tumor formation. We therefore analyzed *NFKB1* expression data in T-cell lymphomas and noted that 100 % of tumors have at least 2-fold decreased expression relative to $CD8^+$ and $CD4^+$ T-cells from normal human peripheral blood (Figure 4a).[18] Next, a series of other tumors were studied and a highly significant decrease in *NFKB1* expression noted in multiple hematological malignancies ($P < 1\times10^{-7}$), including B-cell ALL (Figure 4b), T-cell ALL (Supplementary Figure 3a),[19] B-cell CLL (Figure 4c and Supplementary Figure 3b),[20, 21] diffuse large B-cell lymphoma (DLBCL) (Supplementary Figure 3c)[22] and AML (Figure 4d).[19] In addition, *P65* expression level was examined. Contrary to *NFKB1*, *P65* expression

is up-regulated in T-cell ALL (2.3-fold), B-cell ALL (1.6-fold) and AML (1.5-fold) (Supplementary Figure 4a), decreased in T-cell lymphoma (Supplementary Figure 4b) and unchanged in DLBCL (Supplementary Figure 4c) suggesting that a general decrease in NF-κB pathway mRNA is not present. The above data demonstrate that in multiple different human hematological malignancies a striking decrease in *NFKB1* mRNA expression is apparent.

Given that *Nfkb1* is tumor suppressive specifically following alkylator treatment and that over 75 % of t-MN patients receive an alkylating agent for their primary tumor,[2] *NFKB1* expression was examined in t-MN. We performed next-generation RNA sequencing on a series of patients with t-MN and *NFKB1* expression level was compared to that in samples from patients with *de novo* AML, which occurs without a history of prior malignancy. t-MN samples have significantly reduced *NFKB1* relative to that found in *de novo* AML (P = 0.026, Figure 4e). While t-MN has a higher frequency of adverse-risk cytogenetics (e.g. −5/del(5q) and −7/del(7q)),[2] *NFKB1* expression in patients with adverse-risk cytogenetics is also decreased compared to *de novo* AML with similar karyotypes (17.68 ± 0.18 sterr, n = 14 vs. 18.17 ± 0.20, n = 5). Although this smaller subset does not achieve statistical significance (P = 0.19), the median values in the subgroups are comparable to those in the complete sample sets. Finally, to examine whether mRNA differences in *de novo* AML and t-MN patients are reflected by differences in protein expression, immunoblotting was performed on the original bone marrow samples from patients with both types of disease. A general trend for less p50 protein is seen in t-MN samples compared to *de novo* AML, an observation confirmed by densitometry following normalization to loading (Figure 4f and Supplementary Figure 4d). Importantly, t-MN samples also have less p105, supporting the observation that *NFKB1* expression is reduced at the mRNA level in these tumors. In addition, it is notable that in myeloid neoplasms decreased *NFKB1* expression is associated with significantly reduced expression of death pathway genes (Supplementary Figure 5), supporting the association of reduced *NFκB1* with increased damage tolerance and cell survival. In sum, the human data are consistent with the animal findings and suggest that chemotherapy exposure and reduced NF-κB1 cooperate to promote malignancy.

The current work uncovers a previously unidentified tumor suppressive role for *Nfkb1*. Specifically, loss of *Nfkb1* increases the susceptibility of animals to alkylator-induced lymphoma formation. Interestingly, tumor formation in response to IR is not increased suggesting that *Nfkb1* mediates its tumor suppressive effects in a pathway-specific manner. Pathway-specific tumor suppression is consistent with the prior finding that the mature product of *Nfkb1*, p50, facilitates cytotoxicity specifically in response to certain types of DNA damage.[11] Moreover, the observation that *Nfkb1*$^{-/-}$ animals do not have increased susceptibility to tumor formation by the carcinogen, diethylnitrosamine,[23] an agent that induces tumors by forming O^4-ethylthymidine adducts, further emphasizes the damage-specific nature of tumor suppression by *Nfkb1*. Despite tumor suppression, it is notable that *Nfkb1*$^{-/-}$ animals are not tumor prone at baseline.[16] While lack of spontaneous tumor formation in animals deficient for a tumor suppressor is not uncommon,[24] the absence of increased tumorigenesis may be related to the fact that *Nfkb1*$^{-/-}$ animals are housed in a pathogen-free environment. The primary phenotype of *Nfkb1*$^{-/-}$ mice is their deficiency in

innate and adaptive immunity.[10, 16] Although alterations in the inflammatory environment likely contribute to tumor formation, the damage-specific nature of tumor suppression, coupled with the increased mutagenesis *in vitro*, argues against tumor suppression by *Nfkb1* being solely an immune-mediated phenomenon.

The data suggest that tumor suppression occurs in a haploinsufficient manner indicating that reduced expression, without complete loss, is sufficient to facilitate oncogenesis. This observation is supported by prior work indicating that even partial decrease in p50/p105 is sufficient to compromise the damage response.[11] From a mechanistic prospective, a decrease in p50 level results in formation of compensatory p52/p65 heterodimers, as noted on EMSA in the tumor tissue (Figure 3d). While p52 can cross compensate for p50 in certain respects,[17] it cannot functionally compensate for p50 in the response to DNA damage.[11] Haploinsufficiency is particularly relevant to man because complete loss of *NFKB1*/p50 in human tumors is rare, likely because of the critical role of NF-κB in normal cellular physiology. The lack of significant *NFKB1* gene deletion or mutation in cancer raises the question of how *NFKB1* expression is reduced in human malignancy. While decreased expression may be a result of epigenetic factors such as promoter methylation, several oncogenes implicated in hematological malignancy, including tal1, lmo1, bcl-6 and myc negatively regulate *Nfkb1* expression.[25–27] On the other hand, low *NFKB1* expression may be a normal variant found in the general population, an observation supported by the description of a functional *NFKB1* promoter polymorphism, −94ins/delATTG, that is associated with reduced *NFKB1* expression.[28]

Therapy-related malignancy is a particularly severe complication of cancer treatment that is becoming more prevalent as patients survive for longer time.[1] In fact, up to one in six cancers is thought to be therapy-related.[29] Many chemotherapeutic agents that induce t-MN have mechanisms of action that involve the NF-κB1/p50 pathway. From a clinical perspective, *NFKB1* expression, like other predisposing factors, may be especially important in patients with non-malignant lesions where the risk/benefit ratio for treatment with a cytotoxic agent can be unclear. In patients that have a higher risk of secondary tumor formation, caution should be taken prior to the use of a cytotoxic agent whose beneficial effects may be slim. In this regard, therapy-related lymphoma was recently reported in several patients with low-grade gliomas that were treated with an alkylator.[30]

The ability to maintain genomic integrity in the face of DNA damage is a feature of ‘caretaker’ genes;[31] however, NF-κB1 does not appear to be directly involved in damage repair. Nevertheless, *Nfkb1* is tumor suppressive in the setting of DNA damage. These observations suggest that NF-κB1 may belong to the growing group of low penetrance cancer susceptibility genes that act in combination to determine the overall cellular response to genomic insults.[32] Future work will examine the mechanism by which this ubiquitous, yet under-examined NF-κB subunit modulates the response to DNA damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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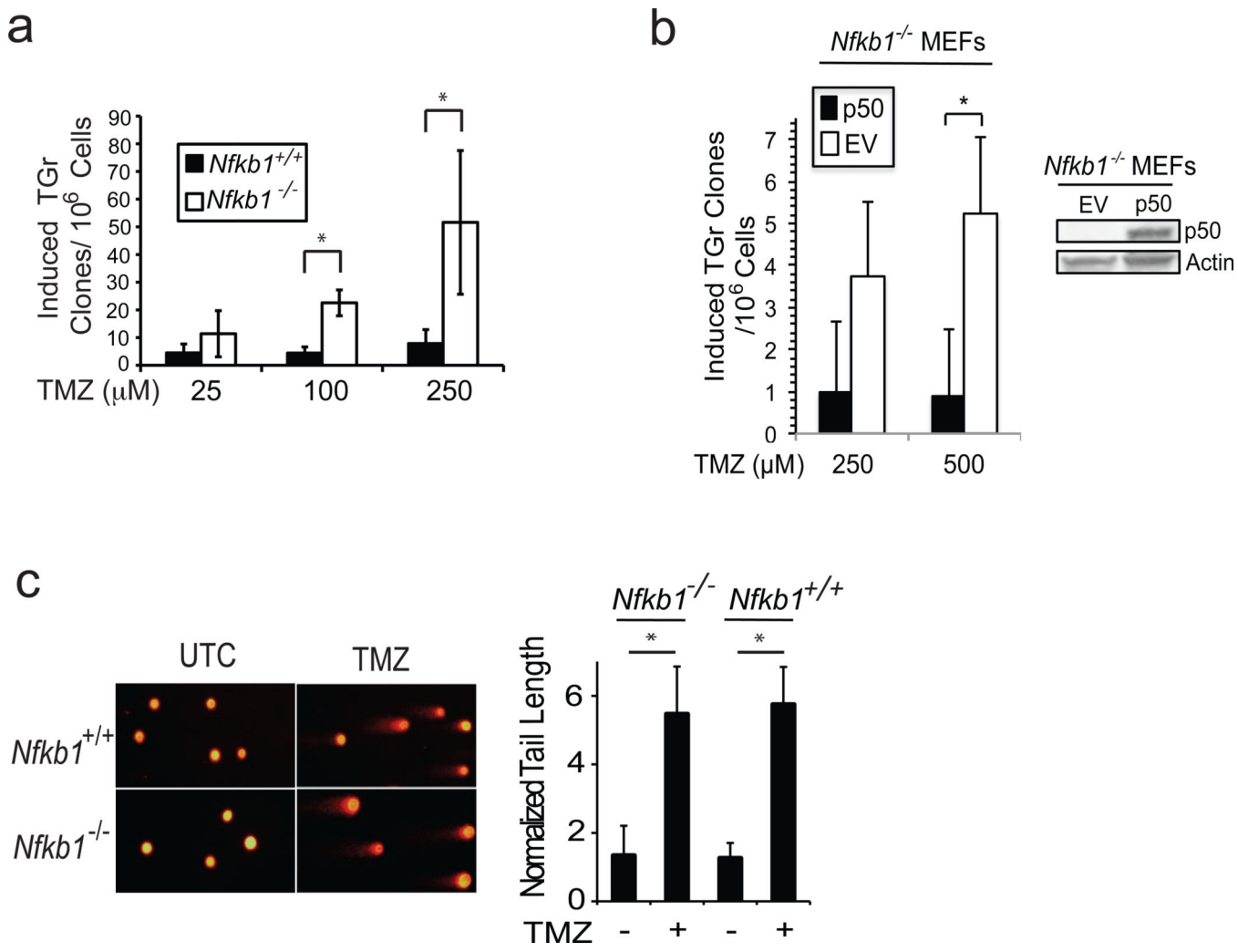

Figure 1.

Nfkb1 prevents alkylator-induced mutation. (**a**) *Hprt* mutation assay in primary *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ MEFs treated with increasing concentrations of TMZ and selected in 5 μg/ml 6-TG. Data show mean number of 6-TG resistant (TGr) clones ± SEM of 4 independent experiments performed at least in duplicate. * $P < 0.05$. (**b**) *Hprt* assay in *Nfkb1*$^{-/-}$ MEFs stably expressing p50 or empty vector (EV) following treatment with TMZ. * $P < 0.05$. (**c**) Alkaline comet assay in *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ MEFs treated with vehicle (UTC) or 100 μM TMZ for 3 hours. Graph demonstrates quantification of average tail length normalized to nucleus diameter ± SD. * $P < 1\times10^{-5}$. N = 50 cells. Experiment was repeated. *Hprt* assay in (**a**) was performed using early passage primary MEFs, harvested from day 14 embryos, treated with TMZ and maintained in exponential growth for 7 days. Cells were subcultured in growth medium alone (plating efficiency) or in the presence of 5 μg/ml 6-TG for selection of mutants. Induced *Hprt* mutations were calculated as the number of 6-TG resistant colonies per 10^6 cells plated after correction for plating efficiency. Importantly, 5 ug/ml 6-TG is 100 % lethal to un-mutated MEFs of both genotypes. For re-expression of p50, immortal *Nfkb1*$^{-/-}$ MEFs were infected with the retroviral vector MigR1-p50 or MigR1-EV. MigR1-p50 was created by liberating p50 from pcDNA3.1-p50[33] by digestion with PmeI

and XhoI, and ligated into the BglII site of pMSCV-MigR1 containing an IRESGFP insert. Retrovirus was produced with Platinum-GP packaging cells following transfection of MigR1-p50 or MigR1-EV using XtremeGENE transfection reagent (Roche). MEFs were then spinoculated with virus/polybrene-containing supernatant and colonies sorted by FACS. Alkaline single cell gel electrophoresis (Comet assay) was performed as described.[34] Briefly, MEFs were treated with TMZ for 3 hours, trypsinized and resuspended in low-melting point agarose. A single cell suspension in the agarose was layered on glass slides and following cell lysis, submerged in alkaline (pH > 13) buffer and electrophoresis performed until slight migration pattern observed in the untreated nuclei. Dried slides were stained with ethidium bromide and visualized by fluorescence microscopy. Tail length was quantified as tail length normalized to nuclear diameter.

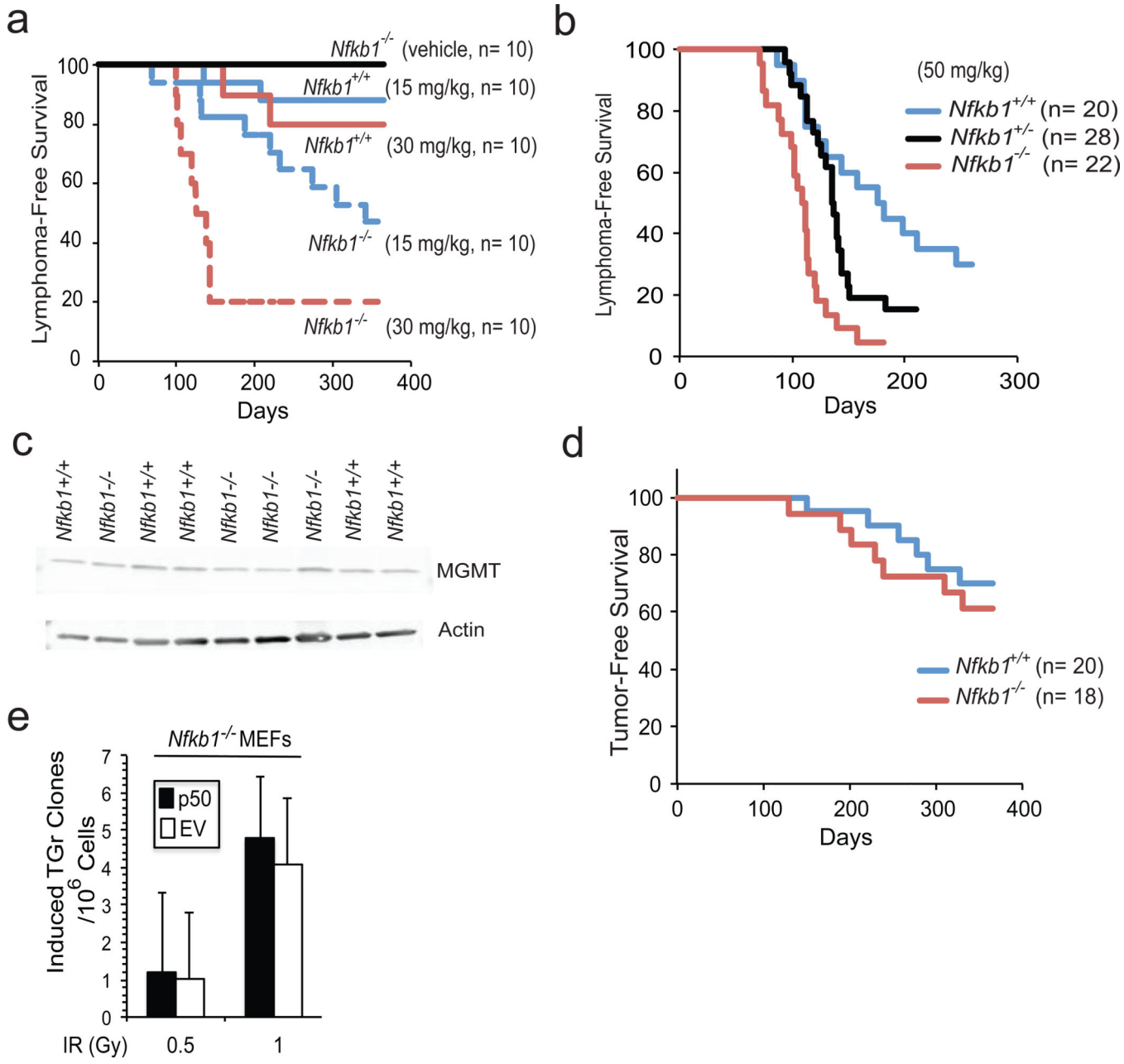

Figure 2.

Nfkb1 prevents alkylator-induced tumor formation. (**a**, **b** and **d**) Kaplan-Meier survival curves in mice following a single i.p. injection of MNU. (**a**) B6/129 *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ animals treated with two concentrations of MNU. Statistical significance: $P < 0.002$ at 30 mg/ kg MNU and $P < 0.02$ at 15 mg/kg. (**b**) C57BL/6 mice treated with 50 mg/kg MNU. Statistical significance: $P < 0.0001$: *Nfkb1*$^{+/+}$ vs. *Nfkb1*$^{-/-}$; $P = 0.05$: *Nfkb1*$^{+/+}$ vs. *Nfkb1*$^{+/-}$; and $P = 0.002$: *Nfkb1*$^{+/-}$ vs. *Nfkb1*$^{-/-}$. (**c**) Western blot with anti-MGMT antibody in thymus extract of untreated C57BL/6 *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ mice. (**d**) C57BL/6 *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ animals treated with whole body IR. Statistical significance: $P > 0.5$. (**e**) *Hprt* assay in *Nfkb1*$^{-/-}$ MEFs stably expressing p50 or empty vector (EV) following

treatment with IR. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Chicago. *Nfkb1*$^{-/-}$ mouse strains used include B6;129P-*Nfkb1*^{tm1Bal}/J and B6.Cg-*Nfkb1*^{tm1Bal}/J that have been backcrossed for 12 generations with C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine, USA). The respective *Nfkb1*$^{+/+}$ controls include: B6;129PF1/J and C57BL/6J. For tumorigenesis in the B6;129P strain, male mice were used and for tumorigenesis in C57BL/6J mice, equal ratios of male to female animals of all genotypes were used. Animals were housed in a pathogen-free, biosafety level II facility. MNU was dissolved in DMSO and diluted in PBS within 10 min of injection. The final preparation was delivered in a total volume of 500 μl per 25 g mouse. Six-week old mice were given a single i.p. injection of MNU or vehicle. For radiation carcinogenesis, equal ratios of male to female animals were treated with four weekly doses of 1 Gy total body irradiation using a Phillips RT250 x-ray generator. Animals were observed at least 2 times per week and sacrificed when they exhibited signs of cachexia, hunched position, respiratory distress or a visible mass. Following euthanasia, autopsy was performed. For evaluation of thymic MGMT expression, 5– 8 week old male C57BL/6 mice were sacrificed and the thymus harvested. Immunoblot was performed on cell lysates with anti-MGMT antibody (sc-8828, Santa Cruz Biotechnology, Dallas, Texas, USA). For histological analysis, tumors were removed and either snap frozen, disaggregated for FACS analysis or immersed in paraformaldyhyde prior to sectioning. Statistical analysis was performed using one-sided Mantel-Cox Log rank analysis.

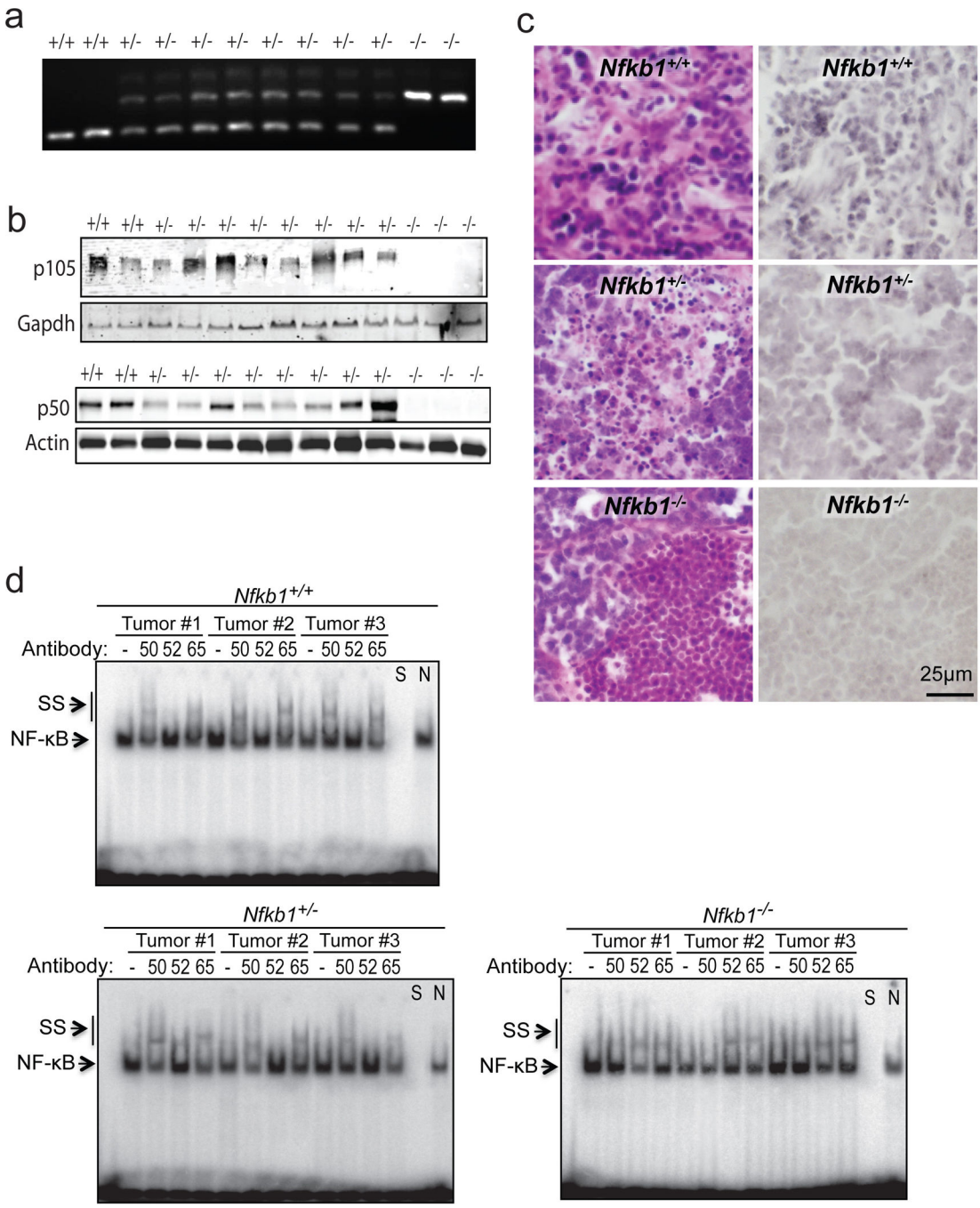

Figure 3.

Thymic tumors from *Nfkb1*$^{+/-}$ animals retain heterozygosity. (**a**) Tumors from *Nfkb1*$^{-/-}$, *Nfkb1*$^{+/-}$ and *Nfkb1*$^{+/+}$ mice were examined by pcr using genotype specific primers. (**b**) Immunoblot of tumor lysate from the indicated mice with anti-p105 (C-terminal) antibody (upper) and anti-p50 antibody (lower). (**c**) Immunohistochemical analysis, using anti-p50 antibody, of tumors harvested from *Nfkb1*$^{+/+}$, *Nfkb1*$^{+/-}$ and *Nfkb1*$^{-/-}$ mice. Hematoxylin and eosin sections from the same tumors are also shown (left). (**d**) EMSA using the Ig-κB probe of tumor lysates from three separate tumors from mice of each genotype. Supershift

(SS) was performed with anti-p50 (50), anti-p52 (52) or anti-p65 (65) antibody where shown. To confirm the band specificity, competition with specific (S) and nonspecific (N) DNA was performed as indicated.

Genotyping was performed using snap frozen tumor samples following thawing and lysis at 55°C for 18 hours. Nucleic acid was extracted and DNA amplified by PCR using primers described for *Nfkb1*$^{-/-}$ animals (Jackson Laboratory). Amplified DNA was run through 2 % agarose gel. For tumor protein, snap frozen samples were thawed, homogenized in 700 ml RIPA lysis buffer and incubated on ice for 5 minutes. Subsequently, samples were centrifuged (14000g × 5 minutes) and the supernatant used for immunobloting and EMSA. Immunoblots were performed with anti-p105 C-terminal (#4717, Cell Signaling Technologies), anti-p50 (sc-8414, Santa Cruz Biotechnology), anti-actin (sc-7210) or anti-gapdh (sc-137179) antibodies. EMSA was performed as previously described[11] using a probe bearing the decameric Ig-κB consensus sequence (5'-GGGACTTTCC-3'). Supershift was performed by pre-incubation with anti-p50 (sc-1190x), anti-p52 (sc-298x) or anti-p65 (sc-8008) antibodies for 30 min on ice.

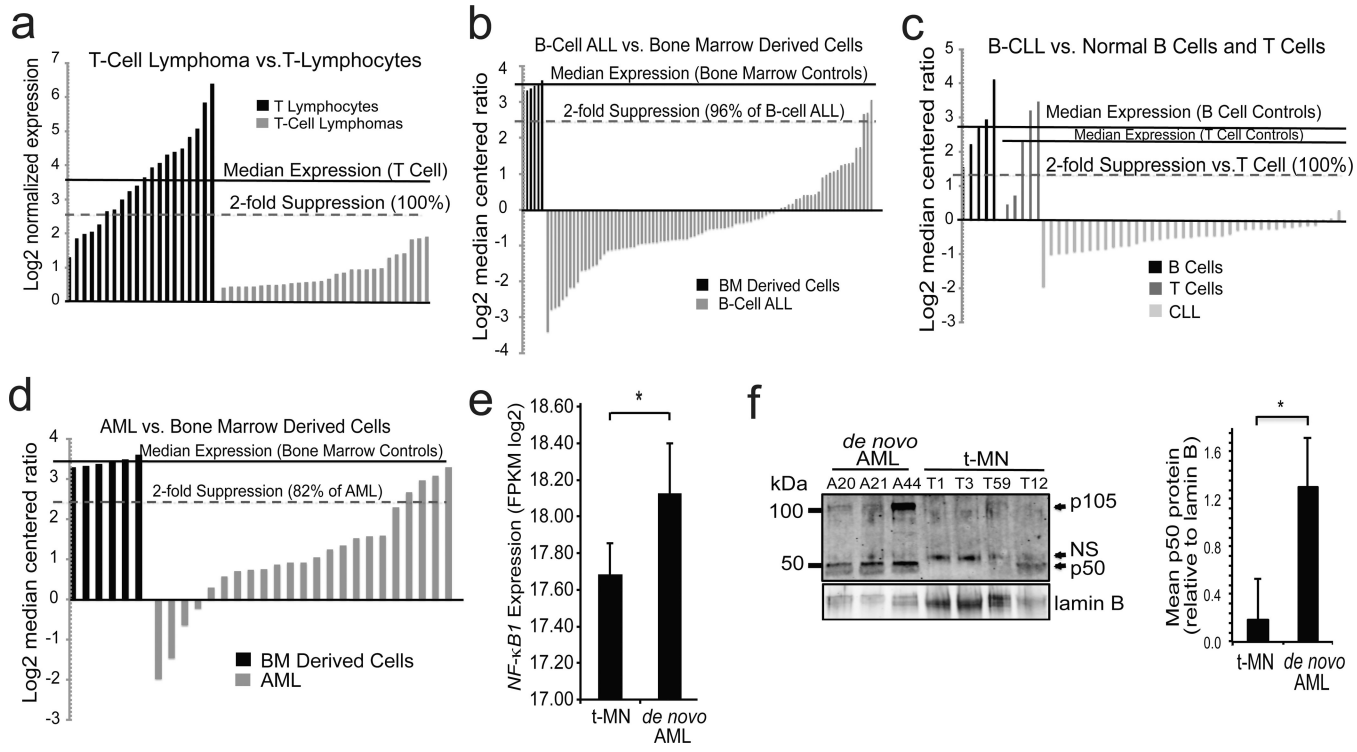

Figure 4.

NFKB1 mRNA is decreased in hematological malignancy. (**a–d**) Waterfall plots of data from human tumor databases. Median expression in the control samples (solid line) and % below 2-fold suppression (dashed line) are indicated for each dataset. (**a**) T-cell lymphoma samples vs. circulating T cells of healthy volunteers. $P = 8\times10^{-13}$. (**b**) Pediatric B-cell ALL bone marrow (BM) samples vs. BM of healthy volunteers. $P = 1.1\times10^{-11}$. (**c**) Adult B-cell CLL peripheral blood samples vs. B cells isolated from healthy adults, or T cells isolated from 1 neonate, 2 fetal and 2 adult samples. $P < 1\times10^{-12}$ (CLL vs. B or T cells). (**d**) Pediatric AML BM vs. BM of healthy volunteers. $P = 2.7\times10^{-7}$. (**e**) *NFKB1* expression level assessed by RNA sequencing in t-MN (N =18) compared to *de novo* AML (N = 10). *NFKB1* expression is output as Fragments per Kilobase per Million mapped reads (FPKM). * $P = 0.026$. RNA sequencing data are extracted from our previous study,[35] plus five previously unpublished t-MN samples. (**f**) Immunoblot (left) with anti-p50 antibody in bone marrow samples from patients with *de novo* AML (n=3) and t-MN (n= 4). Loading was examined with anti-lamin B. Quantification of p50 protein level (right) is shown relative to lamin B. * $P < 0.05$. *NFKB1* expression level in human lymphomas and leukemias was determined from published datasets. Oncomine (Compendia Bioscience, Ann Arbor, MI, USA) was initially used for screening of data and preliminary analysis. Subsequently, identified datasets [GEO accession: GSE7186;[19] GSE2466;[20] and GSE6338[18]] were downloaded from the Gene Expression Omnibus using the Bioconductor package *GEOquery*.[36, 37] Data for DLBCL by Rosenwald *et. al.*[22] was downloaded from the Lymphoma/Leukemia Molecular Profiling Project Gateway (http://llmpp.nih.gov/DLBCL/). Data was quantile normalized and log2 transformed as-needed using the *limma* and *lumi* packages.[38] The median *NFKB1* expression level of control samples was calculated, and the percentage of tumor samples

with 2-fold lower expression determined. Means and standard deviations of *NFKB1* log2 normalized expression or log2 median centered ratios for patient samples and controls in each dataset were calculated using Graphpad Prism (San Diego, CA, USA). Five t-MN samples were processed for next-generation RNA sequencing on the Illumina platform as previously described.[35] Patient characteristics and read depth are provided (Supplemental Table 1). Data analysis and expression level estimates were performed as previously described.[35] Raw sequencing data will be deposited in the NCBI Short Read Archive. Two-tailed Student's *t* tests were used with an alpha of < 0.05. For t-MN, one-sided Wilcoxon rank sum test was used. For immunoblotting, bone marrow samples were thawed, washed in PBS and lysed with RIPA buffer prior to SDS-PAGE and immunoblotting. Anti-lamin B antibody (sc-6216). p50 quantification was performed by densitometry by measuring band intensity with ImageJ freeware (W. S. Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) and the data plotted as ratio over loading after compensating for background intensity taken in a non-immunoreactive region of the blot.