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NKAP must associate with HDAC3 to regulate hematopoietic stem cell maintenance and survival[1]

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Abstract

NKAP is a multifunctional nuclear protein that associates with the histone deacetylase HDAC3. While both NKAP and HDAC3 are critical for hematopoietic stem cell (HSC) maintenance and survival, it was not known whether these two proteins work together. To assess the importance of their association *in vivo*, serial truncation and alanine scanning was performed on NKAP to identify the minimal binding site for HDAC3. Mutation of either Y352 or F347 to alanine abrogated the association of NKAP with HDAC3, but did not alter NKAP localization or expression. Using a linked conditional deletion/re-expression system *in vivo*, we demonstrated that re-expression of the Y352A NKAP mutant failed to restore HSC maintenance and survival in mice when endogenous NKAP expression was eliminated using Mx1-cre and poly-IC, while re-expression of WT NKAP maintained the HSC pool. However, Y352A NKAP did restore proliferation in murine embryonic fibroblasts (MEFs) when endogenous NKAP expression was eliminated using ER-cre and tamoxifen. Therefore, Y352 in NKAP is critical for association with HDAC3 and for HSC maintenance and survival, but is not important for proliferation of MEFs, demonstrating that NKAP functions in different complexes in different cell types.

Introduction

Hematopoiesis is initiated by hematopoietic stem cells (HSCs), which must tightly balance differentiation with proliferation and self-renewal (1). Defects in this balance can lead to loss of HSCs and subsequent hematopoietic failure. Previous work demonstrated that NKAP is critical for the maintenance and survival of HSCs and for hematopoiesis in adult mice (2). HSCs were rapidly depleted from Mx1-cre NKAP conditional knockout (cKO) mice after poly-IC induction. NKAP-deleted HSCs displayed increased apoptosis, decreased proliferation, and upregulation of several mediators of cell cycle arrest at early time points, prior to their disappearance. After poly-IC induction, Mx1-cre NKAP cKO mice exhibited severe thrombocytopenia and anemia, as well as reductions in multiple hematopoietic lineages, leading to euthanasia within two weeks. Bone marrow chimera experiments demonstrated that the lethality was due to cell intrinsic hematopoietic effects, and did not

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stem from NKAP deficiency in other tissues. Hence, NKAP is a critical mediator of HSC maintenance and survival. In addition to this role in HSCs, NKAP has been found to be required for the normal development and maturation of conventional T cells, iNKT cells, and regulatory T cells (3-7).

NKAP functions as a transcriptional repressor, and associates both with CIR, a component of the Notch corepressor complex, and with Histone Deacetylase 3 (HDAC3), an important regulator of chromatin structure (4). NKAP also has been implicated in RNA processing (8), and has been found to promote proper chromosome alignment during mitosis (9). Taken together, these *in vitro* studies suggest that NKAP is a multifunctional protein that may act *in vivo* through multiple molecular mechanisms and pathways. Of particular interest was the association with HDAC3, as similar phenotypes are observed when either HDAC3 or NKAP are conditionally deleted in mice. Both NKAP and HDAC3 are required to generate iNKT cells (5). Deficiencies in either NKAP (3) or HDAC3 (10) at the double positive stage resulted in similar impairments in post-positive selection T cell maturation. HDAC3 deficient HSCs also displayed reduced proliferation and a block in DNA replication, and were unable to reconstitute hematopoiesis after transplantation into irradiated recipients (11). Thus, the function of NKAP in HSCs and other hematopoietic cell types may depend on its interaction with HDAC3.

To determine whether NKAP and HDAC3 work together to regulate HSC maintenance and survival *in vivo*, the sites of HDAC3 interaction with NKAP were mapped using serial truncation analysis and alanine scanning mutagenesis. Alanine substitutions at either Y352 or F347 in NKAP abrogated its interaction with HDAC3 without altering expression, nuclear localization, or association with CIR. We generated a novel linked conditional deletion/re-expression system, in which deletion of the genomic allele of NKAP was coupled to induction of a transgene encoding either wild type (WT) NKAP or the Y352A mutant NKAP which is defective in HDAC3 binding. Re-expression of WT NKAP, but not the Y352A mutant, reversed all effects of conditional deletion of NKAP in Mx1-cre NKAP cKO mice, including depletion of HSCs from the bone marrow. Thus, Y352 of NKAP, which mediates the interaction with HDAC3, is critical for the function of NKAP in HSCs. In contrast, the NKAP Y352A was able to functionally substitute for WT NKAP to promote proliferation in mouse embryonic fibroblasts (MEFs). Hence, NKAP participates in both HDAC3 dependent and independent processes, dependent on the cellular context.

Materials and Methods

Plasmids, immunoprecipitation, and microscopy.

YFP-tagged NKAP, Myc-tagged CIR, and Myc-tagged HDAC3 plasmids were described previously (4). Site-directed mutagenesis was performed on the YFP-NKAP plasmid to either change particular codons to alanine or to create a C-terminal truncation by introducing a stop codon. Sequencing was performed to confirm the presence of desired mutations without other alterations. See figures for nomenclature describing the various mutants. 293T transfection, immunoprecipitation, and western blotting were performed as described previously (4). For microscopy, 293T cells were seeded to 6 well dishes, transfected, fixed in 10% formalin, and overlaid with PBS before analysis. MEFs were seeded to glass slides,

fixed in 10% formalin, and permeabilized in 0.1% Triton before mounting in Slowfade reagent with DAPI (Invitrogen). Cells were analyzed using a Leica DMI3000B microscope with FITC/GFP (for YFP) and DAPI filter cubes and a Q-Imaging Q1-Click camera. Q Capture Pro 6 software was used to apply a HiGauss deconvolution filter to each image (to sharpen nuclear punctae) and to convert from TIFF to JPEG format. Canvas software was used to prepare the figure.

Knock-in mouse generation and other mice.

YFP-NKAP [WT] and YFP-NKAP [Y352A] knock-in mice were generated in partnership with the Mayo Clinic Transgenic and Knockout Mouse Core Facility. These genes were inserted specifically into the ColA1 locus by FLP recombinase (12) using a system that would allow Tetracycline Response Element (TRE) regulated expression (13). Briefly, YFP-NKAP [WT] or [Y352A] was subcloned into the EcoRI site of the pBS31 tetO vector which was cotransfected with pCAGGS-FLPe into KH2 murine ES cells. Hygromycin resistant clones with normal karyotype and YFP-NKAP expression after treatment with doxycycline were selected for blastocyst injection. Progeny of resulting chimeric mice were screened for germline transmission of the YFP-NKAP transgene by PCR, as well as loss of the Rosa26 rtTA (reverse tetracycline transactivator) present in KH2 ES cells. Mice were then interbred with those with a lox-neo-lox tetracycline transactivator (LNL-tTA) expression cassette (14) to allow cre-mediated but dox-independent induction of the YFP-NKAP transgene. These mice were then interbred to introduce a floxed NKAP allele (4) and an Mx1-cre gene. Mx1-cre, LNL-tTA, and estrogen receptor cre (ER-cre) mice were all obtained from Jackson Labs. The “WT” control mice used in various experiments were littermates that either lacked a cre transgene, or had neither an NKAP floxed allele nor LNL-tTA, and therefore could not delete nor re-express NKAP after poly-IC induction. All mouse work was performed with approval from the Mayo Clinic Institutional Animal Care and Use Committee.

Cre induction experiments in mice.

Mice expressing Mx1-cre were treated with poly-IC as previously described (2). To examine YFP-NKAP induction prior to the onset of hematopoietic failure, poly-IC injections were performed at days 0 and 2 and mice were examined at day 3. For a cohort of mice examined at day 6 after poly-IC initiation, injections were performed at days 0, 2, and 4. For endpoint experiments, injections were performed at days 0, 2, 4, and 6 and mice were examined daily for up to 28 days. Lethality was recorded if mice were either found dead or required euthanasia due to severe morbidity. Surviving mice were euthanized at day 28. Bone marrow was harvested from euthanized mice and a complete blood count (CBC) of peripheral blood was performed as described previously (2).

Flow cytometry.

Single cell suspensions from bone marrow were prepared and erythrocytes were lysed in ACK lysis buffer. Live single cells were analyzed by flow cytometry to quantify the Lin $^{-}$Sca-1^{+}c-Kit^{+} (LSK) population. Fixable viability dye was from Tonbo. The lineage cocktail was comprised of APC-conjugated antibodies against CD8α (clone 53-6.7, Tonbo), TCRβ (clone H57-597, Tonbo), TCRγδ (clone Gl3, Biolegend), CD3ε (clone 145-2C11, Tonbo), B220 (clone RA3-682, Tonbo), CD19 (clone 1D3, Tonbo), CD11c (clone N418, Tonbo),

Gr-1 (clone RB6-8C5, Tonbo), NK1.1 (clone PK136, Tonbo) and Ter119 (clone Ter119, Tonbo). Sca-1 (clone D7) and c-Kit (clone 2B8) were purchased from Biolegend or eBioscience. Analysis was performed on an Attune NxT cytometer (Life Technologies) and was analyzed using FlowJo (TreeStar).

Fibroblast experiments.

MEFs from ER-cre NKAP cKO pups were established after timed mating and immortalized by transduction with retrovirus encoding SV40T antigen (15). YFP, YFP-NKAP[WT], and YFP-NKAP[Y352A] genes were subcloned into the MIGR1 retroviral vector (16) using restriction sites chosen to eliminate the original GFP reporter within MIGR1. The resulting plasmids were transfected into 293T cells along with helper plasmid and virus containing supernatants were harvested. Next, the MEFs were transduced and individual sublines were cloned by limiting dilution. Clones expressing WT and mutant NKAP at similar levels were chosen for the experiments presented. Cells were cultured in DMEM with 10% FCS and endogenous NKAP was deleted using 0.1 μM 4-hydroxytamoxifen. For analysis of fibroblast proliferation, cells were detached, counted using a Countess device (Invitrogen), and lysed directly in SDS sample buffer. Western blot analysis utilized antibodies to Ser10 phosphorylated Histone H3 (Cell Signaling #53348) and actin (Sigma A3854).

NKAP deletion analysis.

Genomic DNA was purified from bone marrow with a Qiamp Mini blood kit (Qiagen). Quantitative PCR was performed using SYBR Green master mix (Applied Biosystems) and a Step One Plus instrument (Applied Biosystems). Primers were used to detect a region of the NKAP gene that is removed upon cre recombination (and absent from the YFP-NKAP cDNA in knock-in mice) as previously described (4). Primers that recognize a region of the HES1 promoter were also used to normalize for total genomic DNA content (17). Differences in genomic NKAP abundance between samples were then calculated by the ΔΔCT method (18). NKAP primers: 5’-GAACCACAGGACCAACATAGAGG, 5’-CAACAGAGGCTAGAAAGACATCAG. HES1 primers: 5’-GCTCCAGATCCTGTGTGATCC, 5’-CTGTGGGAAAGAAAGTTTGGGAAG.

Results

To understand the physiological importance of the interaction between NKAP and HDAC3 in regulating HSC maintenance and survival, serial truncation analysis and alanine scanning was performed to identify the site(s) of NKAP association with HDAC3. Previously, we demonstrated that the C-terminal 142 amino acids of NKAP interact with HDAC3 but not CIR (4). Progressive YFP-NKAP C-terminal truncation mutants were cotransfected into 293T cells with Myc-tagged HDAC3 and immunoprecipitated with an antibody to YFP. As shown in Figure 1A, HDAC3 co-immunoprecipitated with WT YFP-tagged NKAP (YFP-NKAP [WT]), but not with YFP. HDAC3 also associated with NKAP mutants truncated after amino acid 394 or 364, but not with mutants truncated after amino acid 341 or 264. Thus, the segment of NKAP between amino acids 341 and 364 is critical for association with HDAC3. Next, alanine substitutions were made in blocks of between 2 and 5 amino acids within this segment (shown schematically in Figure 1B). Replacement of either the

SFE sequence beginning at position 346 or the GYVM sequence beginning at position 351 eliminated the interaction between NKAP and HDAC3 (Figure 1C). However, none of the other alanine substitutions within this region exhibited any reduction in HDAC3 binding. Single alanine substitutions were made in the SFE and GYVM regions (shown schematically in Figure 1D) and examined for association with HDAC3. Replacement of either F347 or Y352 with alanine eliminated the association between NKAP and HDAC3, while HDAC3 binding to the other mutations were unaffected (Figure 1E).

To rule out the possibility that mutation of either F347 or Y352 of NKAP perturbed its association with HDAC3 as an indirect result of a more general defect, the expression, subcellular localization, and CIR association of these NKAP mutants was examined. The F347A and Y352A mutations did not cause any apparent alteration in the expression of NKAP in 293T cells, and both displayed a subcellular localization pattern indistinguishable from WT (Figure 2A). In addition, both F347A and Y352A NKAP mutants retained the ability to associate with CIR (Figure 2B). These results show that the two closely located aromatic residues, F347 and Y352, are critical for the physical association of NKAP with HDAC3, but that mutating these residues to alanine does not interfere with NKAP expression or with its ability to localize to the nucleus in a punctate pattern or associate with CIR.

Having identified amino acid substitutions capable of abrogating the interaction between NKAP and HDAC3, we examined whether the association of NKAP with HDAC3 was required for HSC maintenance and survival. A system was created to assess the ability of an NKAP mutant with this defect to functionally substitute for native NKAP *in vivo* using a cre-mediated deletion/re-expression system (shown schematically in Figure 3A). YFP-NKAP [WT] or YFP-NKAP [Y352A] transgenes under the control of a tet-responsive element (TRE) were knocked into the ColA1 locus (12, 13) in ES cells using FLP-mediated recombination. After germline transmission, these lines were interbred with floxed NKAP (NKAP fl) mice, Mx1-cre transgenic mice, and LNL-tTA (lox-Neo-Lox tet transactivator) mice to produce Mx1-cre NKAP cKO LNL-tTA YFP-NKAP [WT] or Mx1-cre NKAP cKO LNL-tTA YFP-NKAP [Y352A] mice. For simplicity, these mice will be referred to as either “cKO+YFP-NKAP [WT]” or “cKO+YFP-NKAP [Y352A].” In these mice, poly-IC treatment induces cre expression, resulting in the simultaneous deletion of the endogenous floxed NKAP allele (2) as well as removal of the Neo sequence within the LNL-tTA cassette, allowing for tTA expression (14). Once expressed, the tTA protein (which is active in the absence of tetracycline) induces expression of the TRE regulated YFP-NKAP transgene. Neither of these novel mouse strains displayed any gross phenotype or significant differences in peripheral blood hematopoietic populations compared to WT mice prior to poly-IC treatment (Figure 3B). In addition, there was no YFP expression in either line prior to induction with poly-IC (data not shown). To confirm expression of the YFP-NKAP transgenes, mice were treated with poly-IC and YFP-NKAP expression was analyzed in bone marrow LSK cells ($Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$) after three days. As shown in Figure 3C, cre induction efficiently induced expression of YFP-NKAP [WT] or YFP-NKAP [Y352A] in nearly all LSK cells. YFP-NKAP [Y352A] was expressed at slightly higher levels than YFP-NKAP [WT], although there was considerably overlap between the two peaks by flow

cytometry. Therefore, *in vivo*, YFP-NKAP [Y352A] is not expressed at lower levels as compared to YFP-NKAP [WT] in the functional experiments below.

To determine if its association with HDAC3 is required for NKAP to support HSC maintenance and survival, cKO+YFP-NKAP [WT] or cKO+YFP-NKAP [Y352A] mice were treated with poly-IC along with WT and Mx1-cre NKAP cKO mice. After six days, the LSK pool of hematopoietic progenitors, which contains both long-term and short-term HSCs, was analyzed. This time point is just prior to the onset of lethality previously observed in Mx1-cre NKAP cKO mice (2). At day 6, deletion of endogenous NKAP resulted in a near complete loss of the LSK pool in Mx1-cre NKAP cKO mice (Figure 4A), leading to a severe reduction in both frequency and absolute number of LSKs (Figure 4B). In cKO +YFP-NKAP [WT] mice, in which endogenous NKAP is deleted and a YFP-NKAP [WT] transgene is re-expressed, there was no difference in either frequency or absolute number of LSKs as compared to WT mice (Figures 4A and 4B). Thus, the YFP-NKAP [WT] transgene is able to functionally substitute for endogenous NKAP upon conditional deletion/re-expression. In contrast, the LSK pool was severely reduced in the cKO+YFP-NKAP [Y352A] mice, similar to that observed in Mx1-cre NKAP cKO mice. Therefore, in LSKs, NKAP [Y352A], which cannot bind to HDAC3, cannot functionally substitute for endogenous NKAP for HSC maintenance and survival. Day 6 peripheral blood counts were also analyzed (Supplemental Figure 1). Paralleling the changes observed in LSKs, the number of platelets was strongly and significantly reduced in both Mx1-cre NKAP cKO and cKO+YFP-NKAP [Y352A] mice as compared to WT mice. However, platelet numbers were unaffected in cKO+YFP-NKAP [WT] mice (Figure 4C). Thus, YFP-NKAP [Y352A], which cannot bind HDAC3, cannot functionally substitute for endogenous NKAP in regulating the maintenance and survival of hematopoietic progenitors *in vivo*.

Mx1-cre NKAP cKO mice undergo hematopoietic failure after poly-IC induction, succumbing approximately 8-14 days after poly-IC induction (2). Two separate cohorts of WT, Mx1-cre NKAP cKO, cKO+YFP-NKAP [WT] and cKO+YFP-NKAP [Y352A] mice were treated with poly-IC and monitored daily for up to 28 days (Figure 5). Poly-IC treatment did not cause lethality in WT mice and all survived to day 28 (data not shown). Consistent with previous results (2), poly-IC treatment in Mx1-cre NKAP cKO mice resulted in rapid lethality (Figure 5A). All mice in the poly-IC treated cKO+YFP-NKAP [WT] group survived to day 28 and deletion of the endogenous NKAP gene in the bone marrow was confirmed by QPCR (Figure 5B). Thus, the rapid lethality induced after poly-IC treatment in Mx1-cre NKAP cKO mice is reversed by expression of the YFP-NKAP [WT] transgene *in vivo*. In contrast, all mice in the cKO+YFP-NKAP [Y352A] cohort succumbed within two weeks after the initial poly-IC treatment, in a time frame indistinguishable from the Mx1-cre NKAP cKO mice. The frequency of LSK cells in the bone marrow was analyzed in moribund cKO+YFP-NKAP [Y352A] mice at the time of euthanasia as well as in WT and cKO+YFP-NKAP [WT] mice that were euthanized at day 28 (Figure 5C). The LSK frequency was higher in cKO+YFP-NKAP [WT] mice compared to WT mice, though there was not a statistically significant difference in absolute numbers of LSK cells when these mice were compared. In contrast, both the LSK frequency and absolutely number were significantly lower in cKO+YFP-NKAP [Y352A] mice compared to cKO+YFP-NKAP [WT] mice (Figure 5C). While the mechanism for the increase in frequency in the LSK pool

caused by induction of YFP-NKAP [WT] is unknown, YFP-NKAP [Y352A] clearly exhibits a loss of function in comparison. Together, the lethality and reduced LSK pool observed in poly-IC treated cKO+YFP-NKAP [Y352A] mice clearly demonstrate a loss of biological function in an NKAP mutant incapable of association with HDAC3.

Previously, we demonstrated that poly-IC treated Mx1-cre NKAP cKO mice displayed severe reductions in peripheral blood platelets and red blood cells (RBCs), leading to thrombocytopenia and anemia which likely contribute to morbidity and mortality (2). Peripheral blood neutrophils and monocytes were also significantly decreased in these mice. Thus, peripheral blood counts were examined in the mice from the endpoint experiment described in detail above. Peripheral blood was taken from Mx1-cre NKAP cKO and cKO +YFP-NKAP [Y352A] mice at the time of euthanasia while peripheral blood from WT and cKO+YFP-NKAP [WT] mice was taken at day 28 (Figure 5D). Thus, peripheral blood was not isolated and examined at the same time point for each group. As previously described, there were significant decreases in every parameter examined in peripheral blood from Mx1-cre NKAP cKO mice as compared to WT mice. This was entirely reversed when WT NKAP was re-expressed in cKO+YFP-NKAP [WT] mice, with no statistically significant differences in counts between WT and cKO+YFP-NKAP [WT] mice with the exception of monocytes which were higher in cKO+YFP-NKAP [WT] mice. In contrast, there was variability in the effect of YFP-NKAP [Y352A] re-expression on peripheral blood populations. Platelet, white blood cell (WBC), and lymphocyte populations were significantly reduced in cKO+YFP-NKAP [Y352A] mice in comparison to WT or cKO +YFP-NKAP [WT] mice, albeit not to the same extent as observed in Mx1-cre NKAP cKO mice. In contrast, there were no significant differences in neutrophil, monocyte or RBC numbers from cKO+YFP-NKAP [Y352A] mice when compared to WT or cKO+YFP-NKAP [WT] mice. Thus, although YFP-NKAP [Y352A] is defective in supporting the survival and maintenance of hematopoietic progenitors within the LSK pool, it may be able to substitute for endogenous NKAP in the generation and regulation of other lineages such as the erythroid lineage, indicating that the requirement for NKAP to associate with HDAC3 may be dependent on cell type and developmental stage.

To test this idea, we examined whether there was a requirement for NKAP association with HDAC3 in MEFs. An immortalized MEF cell line was generated from ER-cre NKAP cKO mice. Stable cell lines were then generated to constitutively express YFP, YFP-NKAP [WT] or YFP-NKAP [Y352A] by retroviral transduction. Cell lines with similar expression of YFP-NKAP [WT] or YFP-NKAP [Y352A] were used in subsequent experiments (Figure 6A). As was observed in 293T cells (Figure 2a), WT and Y352A NKAP also exhibited indistinguishable patterns of punctate nuclear localization in these cell lines. Treatment of YFP expressing ER-cre NKAP cKO MEFs with tamoxifen led to a ten-fold decrease in cell numbers relative to untreated controls after seven days in culture (Figure 6B). NKAP deletion by tamoxifen greatly decreased phosphorylation of Histone H3 at Ser10 (pH3S10), which is present in mitotic cells, as compared to untreated controls (Figure 6C). Therefore, in the YFP expressing Mx1-cre NKAP cKO MEFs, tamoxifen treatment caused a pronounced reduction in the frequency of mitotic cells, indicating that NKAP deficiency promotes cell cycle withdrawal in MEFs. However, both cell numbers and pH3S10 levels were restored in the tamoxifen-treated ER-cre NKAP cKO MEFs which were stably

transduced with either YFP-NKAP [WT] or YFP-NKAP [Y352A]. Thus, the Y352A mutant can functionally substitute for endogenous NKAP in MEFs, showing that its function in this context does not require an association with HDAC3. The results presented here show that the association of NKAP with HDAC3 is required for maintenance and survival of LSKs, but is not required in MEFs.

Discussion

To understand the contribution of its association with HDAC3 to the function of NKAP in the regulation of HSC maintenance and survival, we systematically performed truncation analysis and alanine scanning to identify the minimal region of NKAP required for this association. Substitution of alanine at either F347 or Y352 was found to be sufficient to abrogate the interaction with HDAC3. Neither mutation altered NKAP expression or localization in 293T cells, or abrogated the association of NKAP with CIR. To understand the requirement for this interaction in regulating HSC maintenance and survival, novel lines of knock-in mice were generated in which expression of cre recombinase induces both deletion of endogenous NKAP and re-expression of either YFP-NKAP [WT] or YFP-NKAP [Y352A]. Previously, we demonstrated that the LSK pool of hematopoietic progenitors is lost when Mx1-cre NKAP cKO mice were treated with poly-IC (2). After 6 days of poly-IC treatment, there were almost no cells in the LSK pool from Mx1-cre NKAP cKO mice, but this loss was abrogated when YFP-NKAP [WT] was re-expressed. Therefore, YFP-NKAP [WT] could functionally substitute for endogenous NKAP for HSC maintenance and survival. However, re-expression of YFP-NKAP [Y352A] could not functionally substitute for endogenous NKAP and the reduction in the LSK pool was similar between poly-IC treated Mx1-cre NKAP cKO and cKO+YFP-NKAP [Y352A] mice. The loss of the LSK pool was not due to decreased expression of the YFP-NKAP [Y352A] transgene relative to the YFP-NKAP [WT] transgene. Therefore, Y352 of NKAP, which mediates its association with HDAC3, is critical for the function of NKAP in HSC maintenance and survival. Endpoint experiments were also performed, as poly-IC treated Mx1-cre NKAP cKO mice became moribund within two weeks due to hematopoietic failure and severe decreases in peripheral blood counts (2). Similar to the observations made at day 6, YFP-NKAP [WT] but not YFP-NKAP [Y352A] was able to functionally substitute for endogenous NKAP in all parameters examined. At 28 days after poly-IC treatment, cKO+YFP-NKAP [WT] mice were healthy, without any decrease in the number of LSK cells in the bone marrow or any decreases in peripheral blood populations determined by CBC. However, cKO+YFP-NKAP [Y352A] mice became moribund with a similar time course as Mx1-cre NKAP cKO mice after poly-IC treatment. In contrast, while the Y352A mutant could not substitute for endogenous NKAP in HSC maintenance and survival, it did functionally substitute for endogenous NKAP for proliferation of MEFs. Hence, the effect of the single Y352A amino acid substitution is not due to a general disruption of NKAP function, such as causing a general structural change. Instead, it demonstrates that an association with HDAC3 is critical for NKAP function in some cell types but not others. It is also important to note that Y352 and F347 in NKAP, which are critical for its association with HDAC3, as well as several surrounding residues, are perfectly conserved in NKAP orthologues across all species which have NKAP including mammals, Drosophila, Dictyostelium, and Arabidopsis (19). Hence,

determining the biological consequence of disrupting this region of NKAP serves to critically evaluate the significance of this evolutionary conservation.

A surprising finding was that there was not a perfect phenocopy between poly-IC treated Mx1-cre NKAP cKO mice and cKO+YFP-NKAP [Y352A] mice with respect to peripheral blood populations. Consistent with our previously published results, there were significant decreases in each peripheral blood cell type from Mx1-cre NKAP cKO mice as compared to WT mice at endpoint, and in all cell types except RBC at day 6. As six days is insufficient for HSCs to terminally differentiate into different hematopoietic lineages, this implies that the decreased numbers of peripheral blood cells cannot simply be due to the loss of LSKs at day six in Mx1-cre NKAP cKO mice. Rather, this indicates that NKAP may have additional roles in regulating the differentiation or survival of many different hematopoietic lineages. Previously, we demonstrated that NKAP is required in thymic development at the DN3 to DP transition (4). However, the requirement for NKAP in erythroid, platelet or myeloid cell development is not yet known. Interestingly, at endpoint, statistically significant reductions were only observed in peripheral blood platelet and lymphocyte counts in cKO+YFP-NKAP [Y352A] mice while peripheral blood counts for monocytes, neutrophils, and red blood cells were not significantly different from these levels in poly-IC treated WT mice. One potential explanation may be that although NKAP is required for differentiation of many hematopoietic lineages, functions mediated by Y352 may not be required for the differentiation of erythroid or myeloid lineages. This will be examined through the generation of lineage specific NKAP cKO mice, along with re-expression of YFP-NKAP [WT] or YFP-NKAP [Y352A], which is currently being pursued.

It is also striking that cKO+YFP-NKAP [Y352A] mice become moribund within a similar time course as Mx1-cre NKAP cKO mice after poly-IC treatment, although there was no anemia as red blood cell numbers did not decrease and the thrombocytopenia was not as dramatic (average of 22 K/μl at endpoint in Mx1-cre NKAP cKO mice and 314 K/μl at endpoint in cKO+YFP-NKAP [Y352A] mice). However, the poly-IC used in this system to delete NKAP may exacerbate effects of thrombocytopenia by altering platelet function. Diminished platelet function was observed in poly-IC treated mice resulting from induction of type I interferons (20). In addition, type I interferons can induce thrombotic microangiopathy due to alterations in the vasculature (21). Therefore, the lethality in Mx1-cre NKAP cKO mice might be due to synergy between decreased platelet numbers due to loss of NKAP and decreased platelet function due to induction of type I interferons by poly-IC. However, we cannot exclude that NKAP may have a cell-intrinsic role in platelet function as well, which is also under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CBC	complete blood count
cKO	conditional knockout
ER-cre	estrogen receptor cre
HDAC3	Histone Deacetylase 3
HSC	hematopoietic stem cell
LNL-tTA	lox-neo-lox tetracycline transactivator
LSK	$Lin^{-}Sca1^{+}cKit^{+}$
MEF	murine embryonic fibroblast
RBC	red blood cell
TRE	tetracycline response element
WBC	white blood cell
WT	wild type

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Key Points

1. NKAP Y352 is required for association with HDAC3.
2. The interaction between NKAP and HDAC3 is critical for its function in HSCs.
3. The interaction between NKAP and HDAC3 is unimportant in some cell types.

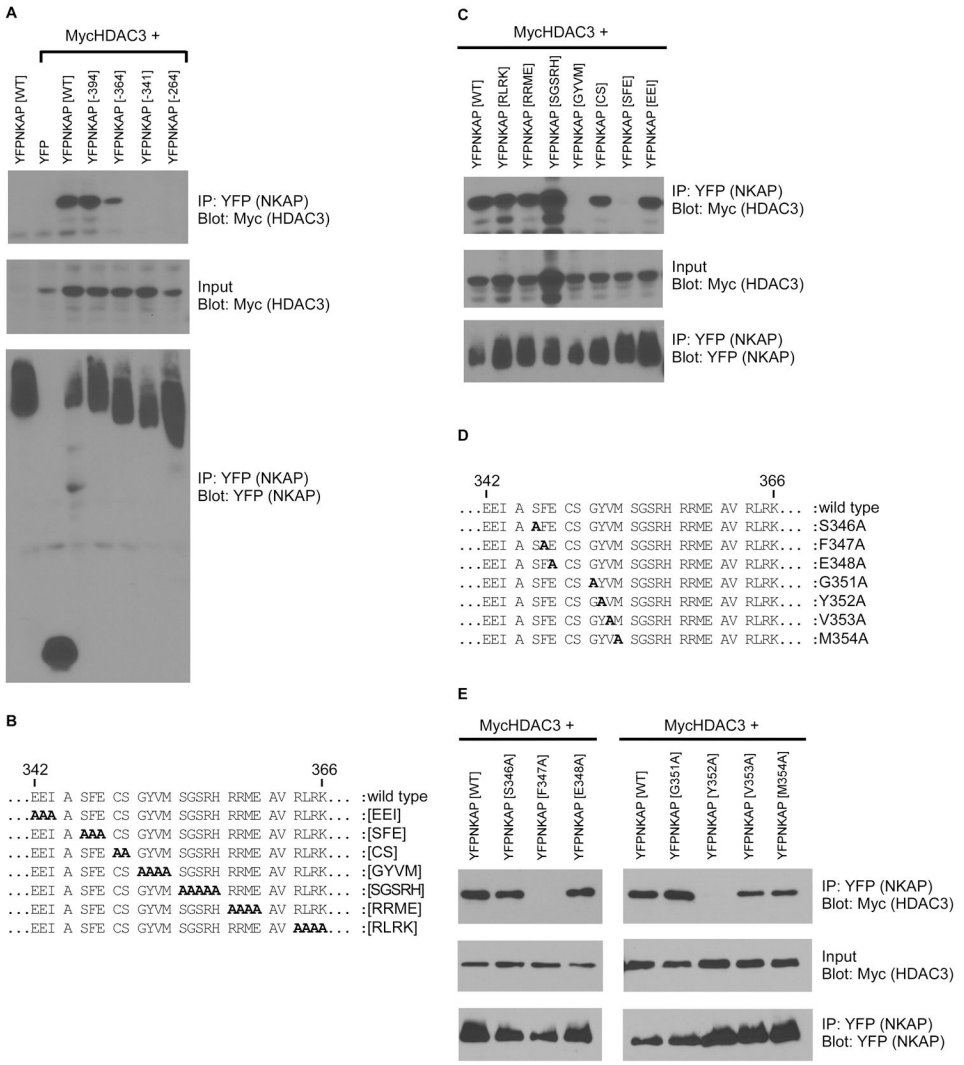


Figure 1. Identification of single amino acid substitutions in NKAP that abrogate its association with HDAC3.

(A) 293T cells were transfected with expression constructs for YFP, WT YFP-tagged NKAP (YFP-NKAP [WT]), or truncation mutants containing NKAP sequence extending from the N-terminus to the residue indicated in the bracket. Cells were cotransfected with Myc-tagged HDAC3 where indicated. Lysates were immunoprecipitated with antibodies to YFP. Input (whole cell lysates) and immunoprecipitated (IP) proteins were examined for YFP (NKAP) or Myc (HDAC3). **(B)** A schematic of blocks of NKAP alanine substitution mutants is presented with WT sequence in the top row. In subsequent rows, blocks of residues that were simultaneously mutated are indicated. **(C)** Immunoprecipitation experiments performed as in (A) with NKAP block mutants in which all residues in the sequence indicated in the bracket were replaced with alanine. Data shown is representative of two independent experiments. **(D)** A schematic of individual NKAP alanine substitution mutants is presented with WT sequence in the top row. **(E)** Immunoprecipitation experiments performed as in (A) with NKAP point mutants in which the single residue listed

in the bracket was replaced by alanine. Data shown is representative of at least three independent experiments.

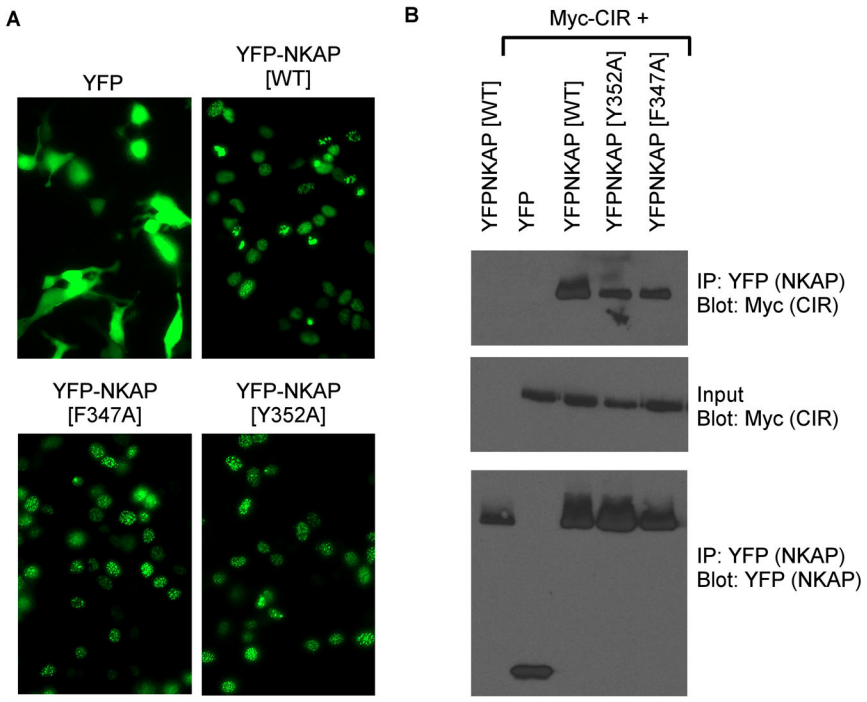
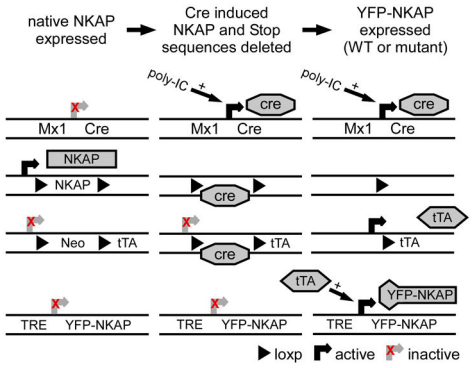


Figure 2. NKAP mutants defective in HDAC3 association do not exhibit defects in localization or in CIR association.

Cells were prepared for microscopy as described in methods. Representative fields are shown. **(A)** Images were acquired with a 20X lens. 293T cells were transfected with either YFP, YFP-NKAP [WT], or one of the two NKAP point mutants that failed to associate with HDAC3 (Y352A or F347A). YFP localized throughout the cell, while YFP-NKAP localized to nuclei in a highly variable punctate pattern. Expression and localization of the two mutants was indistinguishable from WT. Data shown is representative of at least three independent experiments. **(B)** 293T cells were transfected with expression constructs for YFP or YFP tagged NKAP (WT or indicated mutants) and cotransfected with Myc-tagged HDAC3 where indicated. Cells were lysed and immunoprecipitation performed as in Figure 1A. CIR expression was also examined in input (whole cell extract) to demonstrate equivalent expression. Data shown is representative of three independent experiments.

A



B

genotype:	WT	cKO	cKO+WT	cKO+Y352A
poly-IC:	-	-	-	-
Platelets (K/μl)	846 ± 154	874 ± 157	842 ± 147	847 ± 204
WBC (K/μl)	8.60 ± 1.61	8.80 ± 2.98	9.89 ± 2.58	8.31 ± 1.08
Lymphocytes (K/μl)	6.13 ± 1.22	6.41 ± 2.32	7.05 ± 2.24	5.87 ± 0.88
Neutrophils (K/μl)	2.26 ± 0.57	2.23 ± 0.88	2.53 ± 0.55	2.23 ± 0.60
Monocytes (K/μl)	0.19 ± 0.07	0.15 ± 0.09	0.25 ± 0.10	0.18 ± 0.06
RBC (M/μl)	9.41 ± 0.25	9.92 ± 1.59	11.16 ± 3.01	9.47 ± 0.27
Hemoglobin (g/dL)	12.04 ± 0.30	12.53 ± 1.51	13.96 ± 3.19	12.56 ± 0.54
	n=11	n=14	n=8	n=9

C

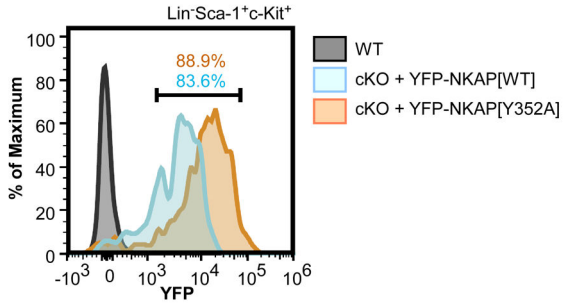


Figure 3. Establishment of a conditional deletion/re-expression system to replace endogenous NKAP with a mutant defective in HDAC3 binding

(A) Schematic of conditional deletion/re-expression system. **(B)** Peripheral blood was isolated from mice of the indicated genotypes and CBCs determined (prior to poly-IC treatment). The total number of mice in each group is indicated in the bottom row. For each parameter indicated to the left, the average and standard deviation is shown. One way analysis of variance was performed (GraphPad Prism software) and no significant differences between the groups were observed for any parameter ($p>0.05$). **(C)** Mice were treated with poly-IC on days 0 and 2 and analyzed on day 3. Bone marrow was harvested and LSK cells examined for YFP expression. YFP expression in cells in the LSK gate was measured to monitor induction of YFP-NKAP [WT] or YFP-NKAP [Y352A] protein. Histograms for YFP expression in cells from poly-IC treated mice of indicated genotypes

are overlaid. Representative data from three cKO+YFP-NKAP [WT] and four cKO+YFP-NKAP [Y352A] mice analyzed in two independent experiments is shown.

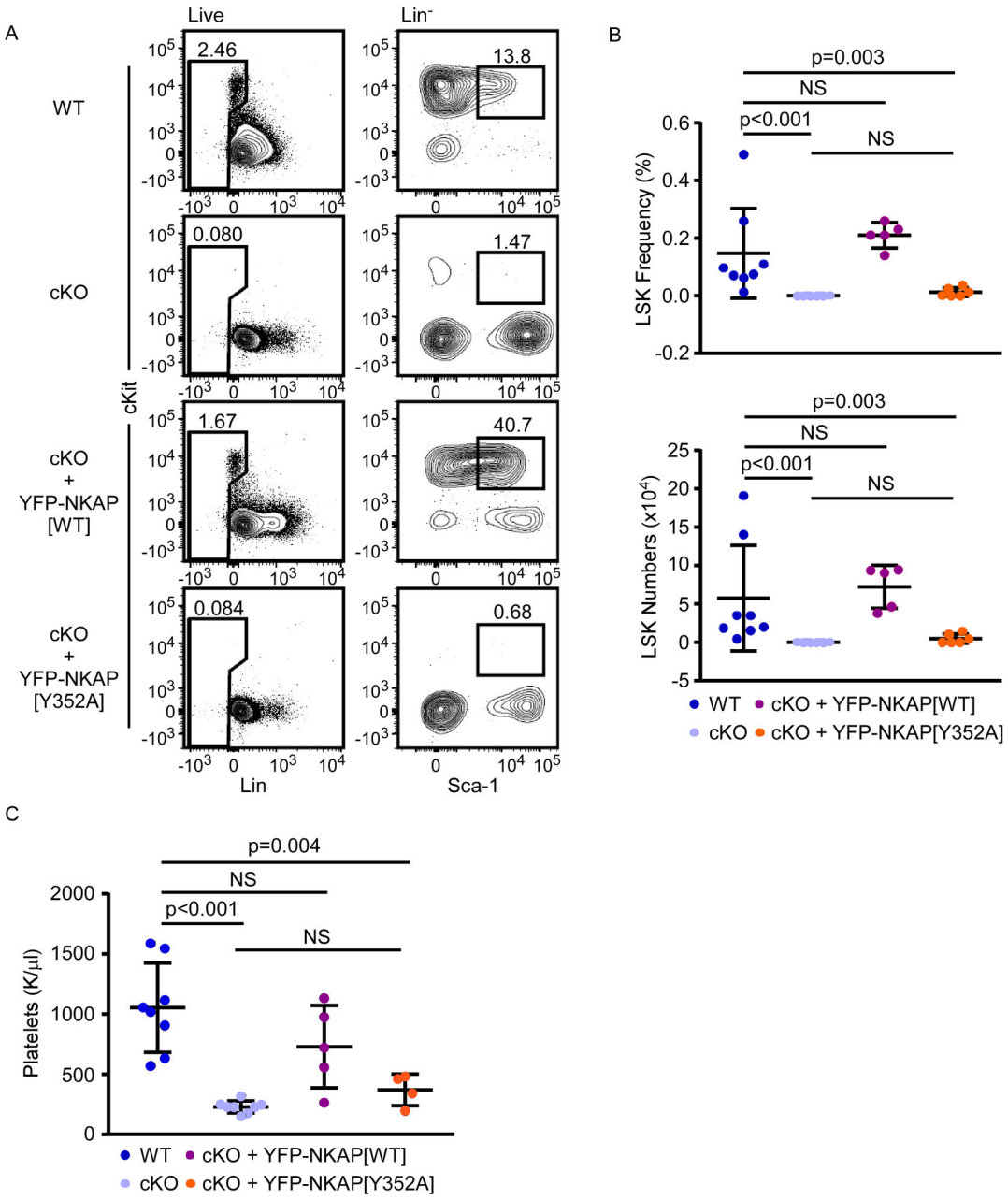
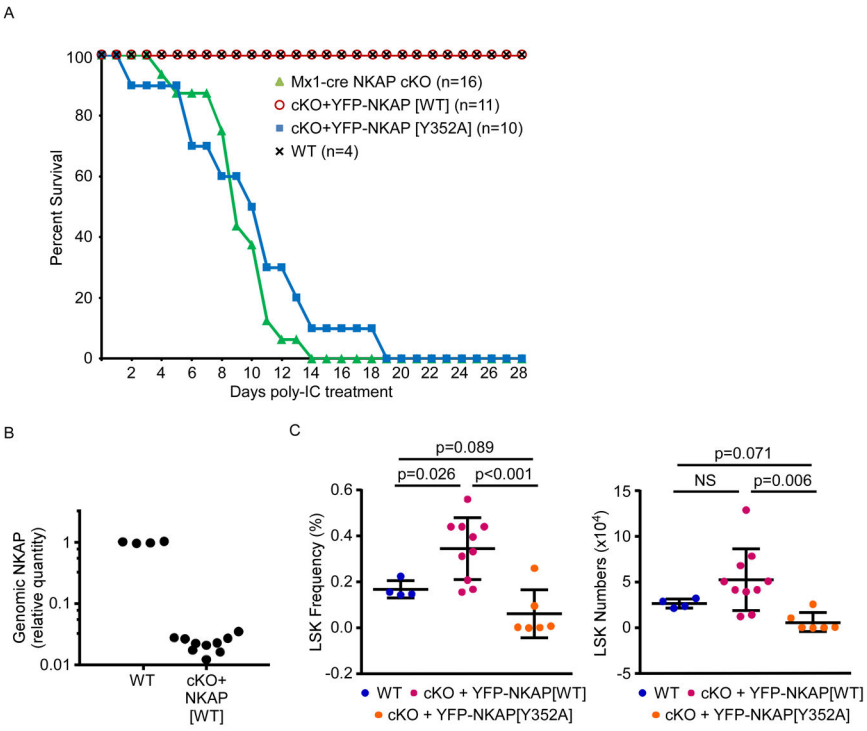


Figure 4. Severe reduction in the LSK pool of hematopoietic progenitors and in platelet counts in mice in which the Y352A NKAP mutant is expressed.

(A) Mice of the indicated genotypes were treated with poly-IC on days 0, 2, and 4 and examined on day 6. Bone marrow was harvested and cells stained with markers to identify the LSK population as described in methods. Viable, lineage negative cells are indicated by the gate drawn within the left set of boxes and c-Kit/Sca-1 high cells within this population are indicated in the gate drawn within the right set. Representative plots are shown. **(B)** The frequency and number of LSK cells, analyzed as in (A) were calculated. Data shown is from a grand total of 8 WT, 8 Mx1-cre NKAP cKO, 5 cKO+YFP-NKAP [WT], and 6 cKO+YFP-NKAP [Y352A] mice from 3 independent experiments. Scatter plots with averages and

standard deviations for each group are shown. Significance of the comparisons indicated by the bars was determined by Mann-Whitney rank sum test (GraphPad Prism software). Please note that the Y axis scale includes negative numbers to allow identification of symbols close to zero that would otherwise be against the axis. (C) Platelet counts from peripheral blood was measured in 8 WT, 8 Mx1-cre NKAP cKO, 5 cKO+YFP-NKAP [WT], and 4 cKO +YFP-NKAP [Y352A] mice treated with poly-IC as described above. Scatter plots with averages and standard deviations for each group are shown. Significance of the comparisons indicated by bars was determined by Mann-Whitney rank sum test (GraphPad Prism software).



D

genotype:	WT	cKO	cKO+WT	cKO+Y352A
poly-IC:	+	+	+	+
Platelets (K/μl)	820 ± 123	22 ± 12 p<0.001	911 ± 331 NS	314 ± 224 p=0.036
WBC (K/μl)	6.40 ± 2.63	1.00 ± 0.48 p=0.026	9.93 ± 3.43 NS	3.31 ± 1.57 p=0.046
Lymphocytes (K/μl)	4.59 ± 2.14	0.95 ± 0.47 p=0.007	6.25 ± 2.17 NS	1.67 ± 0.72 p=0.013
Neutrophils (K/μl)	1.64 ± 0.51	0.02 ± 0.02 p<0.001	3.19 ± 1.36 NS	1.24 ± 0.98 NS
Monocytes (K/μl)	0.14 ± 0.04	0.02 ± 0.01 p<0.001	0.39 ± 0.15 p=0.013	0.27 ± 0.24 NS
RBC (M/μl)	9.42 ± 0.43	2.68 ± 0.59 p<0.001	9.51 ± 0.60 NS	8.97 ± 1.95 NS
Hemoglobin (g/dL)	12.78 ± 0.78	2.94 ± 0.73 p<0.001	11.62 ± 0.93 NS	11.35 ± 2.60 NS
	n=4	n=5	n=6	n=6

Figure 5. Expression of an NKAP mutant defective in HDAC3 binding does not prevent rapid lethality upon deletion of native NKAP.

(A) Mice were treated with poly-IC at days 0, 2, 4, and 6 and monitored daily for up to 28 days. A grand total of 4 WT mice, 16 Mx1-cre NKAP cKO mice, 11 cKO+YFP-NKAP [WT] mice, and 10 cKO+YFP-NKAP [Y352A] mice from two independent experiments were examined. For each day of the time course, the percentage of live mice in each group is indicated. (B) Endogenous NKAP gene deletion in 4 male WT and 10 cKO+YFP-NKAP [WT] mice on day 28 after initiation of poly-IC induction was determined as described in methods. The relative quantity of the NKAP gene in each mouse was normalized to the average relative quantity in the WT mice examined in the same cohort. The data, presented on a log scale, shows that the native NKAP gene was deleted in at least 95% of the total bone marrow cells isolated from each of the cKO+YFP-NKAP [WT] mice. (C) The

frequency and number of LSK cells was determined as in Figure 4 from a total of 4 WT, 10 cKO+YFP-NKAP [WT], and 6 cKO+YFP-NKAP [Y352A] mice analyzed in two independent experiments. Bone marrow cells were examined from either moribund mice at the time of euthanasia or surviving mice euthanized at day 28. Scatter plots with averages and standard deviations for each group are shown. Significance of the comparisons indicated by bars was determined by unpaired Student's t test (GraphPad Prism software). Please note that the Y axis scale includes negative numbers to allow identification of symbols close to zero that would otherwise be against the axis. (D) CBCs were performed on blood from 4 poly-IC treated WT mice, 5 Mx1-cre NKAP cKO, 6 cKO+YFP-NKAP [WT] and 6 cKO +YFP-NKAP [Y352A] mice. Peripheral blood was obtained either from moribund mice at the time of euthanasia or surviving mice euthanized at day 28. Each value shows the average with standard deviation. Significance listed underneath the values was determined by comparison to WT by unpaired Student's t test (GraphPad Prism software).

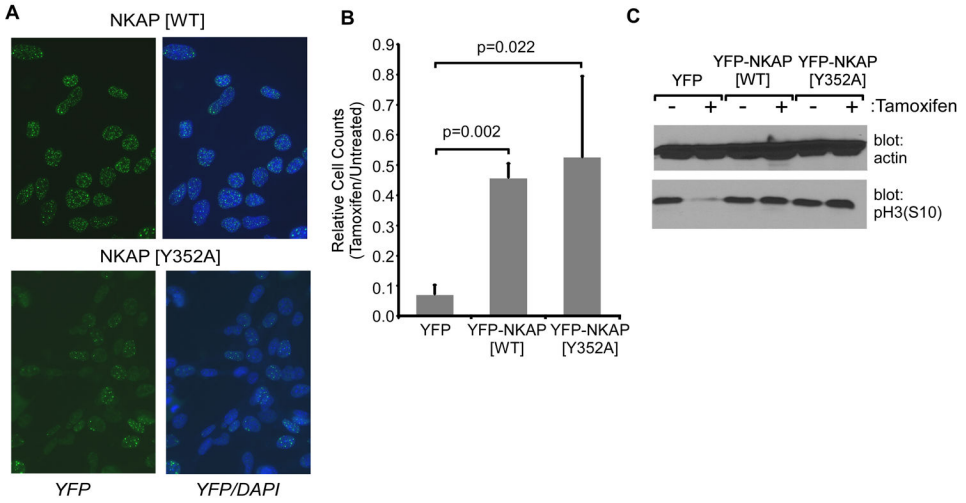


Figure 6. Y352A mutant NKAP supports proliferation in MEFs.

(A) An ER-cre NKAP cKO MEF cell line was transduced with retrovirus encoding either YFP-NKAP [WT] or YFP-NKAP [Y352A] and individual clones isolated. Clones expressing WT and Y352A NKAP at similar levels are shown. Cells were fixed and permeabilized, and DAPI applied to visualize nuclei. The fields shown display YFP fluorescence only (left) or YFP overlaid with DAPI fluorescence in the same cells (right). Images were acquired with a 40X lens and are representative of three independent experiments. **(B)** Equal numbers of ER-cre NKAP cKO MEF cell lines which stably express YFP, YFP-NKAP [WT], or YFP-NKAP [Y352A] were plated in the presence or absence of tamoxifen to induce deletion of endogenous NKAP. Cells were harvested and counted. After 7 days, the average number of cells in the treated relative to untreated cultures, determined from three separate experiments, is shown along with standard deviations. Statistical significance was determined by unpaired Student's t test (GraphPad Prism software). **(C)** Lysates from cells treated in (B) were examined by western blot for phospho-histone H3 serine 10 (pH3-S10) and actin as a loading control. The membrane was cut after transfer to allowing simultaneous examination of both proteins. Data shown is representative of three independent experiments.