

# UCLA

## UCLA Previously Published Works

### Title

Myeloid Malignancies with Chromosome 5q Deletions Acquire a Dependency on an Intrachromosomal NF- $\kappa$ B Gene Network

### Permalink

<https://escholarship.org/uc/item/73n1j8w4>

### Journal

Cell Reports, 8(5)

### ISSN

2639-1856

### Authors

Fang, Jing  
Barker, Brenden  
Bolanos, Lyndsey  
[et al.](#)

### Publication Date

2014-09-01

### DOI

10.1016/j.celrep.2014.07.062

Peer reviewed

Published in final edited form as:

*Cell Rep.* 2014 September 11; 8(5): 1328–1338. doi:10.1016/j.celrep.2014.07.062.

## Myeloid malignancies with chromosome 5q deletions acquire a dependency on an intrachromosomal NF- $\kappa$ B gene network

Jing Fang<sup>1</sup>, Brenden Barker<sup>1</sup>, Lyndsey Bolanos<sup>1</sup>, Xiaona Liu<sup>1</sup>, Andres Jerez<sup>2</sup>, Hideki Makishima<sup>2</sup>, Susanne Christie<sup>1</sup>, Xiaoting Chen<sup>3,4</sup>, Dinesh S Rao<sup>5</sup>, H. Leighton Grimes<sup>1,6</sup>, Kakajan Komurov<sup>1</sup>, Matthew T. Weirauch<sup>3,7</sup>, Jose A. Cancelas<sup>1,8</sup>, Jaroslaw P. Maciejewski<sup>2</sup>, and Daniel T. Starczynowski<sup>1,9</sup>

<sup>1</sup>Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA 45229

<sup>2</sup>Department of Translational Hematology and Oncology Research, Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH, USA 44195

<sup>3</sup>Center for Autoimmune Genomics and Etiology (CAGE), Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA 45229

<sup>4</sup>School of Electronic and Computing Systems, University of Cincinnati, Cincinnati, OH, USA 45229

<sup>5</sup>Department of Pathology and Laboratory Medicine, UCLA, Los Angeles, CA, USA 90095

<sup>6</sup>Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA 45229

<sup>7</sup>Divisions of Biomedical Informatics and Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA 45229

<sup>8</sup>Hoxworth Blood Center, University of Cincinnati College of Medicine, Cincinnati, OH USA 45219

<sup>9</sup>Department of Cancer Biology, University of Cincinnati College of Medicine, Cincinnati, OH, USA 45267

### Summary

Chromosome 5q deletions (del(5q)) are common in high-risk (HR) myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML); however, the gene regulatory networks that sustain these aggressive diseases are unknown. Reduced miR-146a expression in del(5q) HR-MDS/AML and miR-146a<sup>-/-</sup> hematopoietic stem/progenitor cells (HSPC) results in TRAF6/NF- $\kappa$ B activation. Increased survival and proliferation of HSPC from miR-146a<sup>low</sup> HR-MDS/AML is sustained by a

© Published by Elsevier Inc. All rights reserved.

Correspondence: Daniel Starczynowski, Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA 45229, 513-803-5317, Daniel.Starczynowski@cchmc.org.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors declare no competing financial interests.

neighboring haploid gene, *SQSTM1* (*p62*), expressed from the intact 5q allele. Overexpression of *p62* from the intact allele occurs through NF- $\kappa$ B-dependent feedforward signaling mediated by miR-146a deficiency. *p62* is necessary for TRAF6-mediated NF- $\kappa$ B signaling, as disrupting the *p62*-TRAF6 signaling complex results in cell cycle arrest and apoptosis of MDS/AML cells. Thus, del(5q) HR-MDS/AML employs an intrachromosomal gene network involving loss of miR-146a and haploid overexpression of *p62* via NF- $\kappa$ B to sustain TRAF6/NF- $\kappa$ B signaling for cell survival and proliferation. Interfering with the *p62*-TRAF6 signaling complex represents a therapeutic option in miR-146a-deficient and aggressive del(5q) MDS/AML.

## Introduction

Deletions involving the long arm of chromosome 5, del(5q), are the most common cytogenetic abnormalities in MDS and secondary AML (Ebert, 2009). 5q- syndrome, a clinical entity defined by an isolated del(5q) with a blast count of <5% confers a favorable prognosis and responsiveness to lenalidomide (Greenberg et al., 2012; List et al., 2006). However, a large proportion of del(5q) patients have bone marrow (BM) blasts exceeding 5%, complex cytogenetics, extremely poor prognosis, and are refractory to available treatments (Giagounidis et al., 2006). Minimally-deleted regions (MDR) have been mapped and extensively studied (Ebert, 2009). The distal MDR (5q33.3) on chr 5q is associated with a favorable outcome, while the proximal MDR (5q31.1) is associated with worse prognosis and leukemic transformation. More recently, high-resolution genomic analyses on an extensive patient cohort has added new insight into the clinical and genomic correlates of del(5q) myeloid malignancies. It was shown that the extent of the deleted region on 5q determines clinical characteristics (Jerez et al., 2012); that is, chr 5q deletions extending beyond q34 portend a worse overall survival and are associated with high-risk (HR) disease. One potential candidate on chr 5q is miR-146a, a miRNA implicated in del(5q) MDS/AML pathogenesis, which resides on band q34. From previous work, a subset of miR-146a<sup>-/-</sup> mice develop MDS, a myeloid proliferative disease, and/or myeloid tumors resembling AML, in part by derepression of Tumor necrosis factor receptor associated factor 6 (TRAF6) and persistent NF- $\kappa$ B activation (Boldin et al., 2011; Starczynowski et al., 2010; Zhao et al., 2011; Zhao et al., 2013). The mechanism accounting for the variable phenotype and disease latency of miR-146a<sup>-/-</sup> mice is still unresolved, particularly as it relates to disease initiation versus disease maintenance. In addition, a small molecule inhibitor of IRAK1 suppresses TRAF6 and NF- $\kappa$ B activation, and induces apoptosis of primary MDS-propagating cells, but is less effective as a single agent in primary AML (Rhyasen et al., 2013). Therefore, understanding the contribution of miR-146a deficiency to the maintenance of HR MDS/AML and uncovering novel therapeutic opportunities is warranted.

## Results

Loss of miR-146a expression either by miRNA decoy or by genetic deletion in mouse hematopoietic stem/progenitor cells (HSPC) results in features of 5q- syndrome, fatal myeloid malignancies, and altered hematopoietic proportions (Supplemental Figure 1A-F) (Boldin et al., 2011; Zhao et al., 2011; Zhao et al., 2013); however, the effects of miR-146a loss that explain the clinical findings and sustained malignant cell growth in aggressive/HR

del(5q) MDS/AML are not known. To investigate a connection between miR-146a loss and the cellular phenotype of del(5q) MDS/AML cells, gene expression patterns and Gene Ontology (GO) categories using ToppGene(Chen et al., 2009) were compared between HSPC from miR-146a<sup>low</sup> del(5q) MDS/AML patients (Supplemental Table 1) and miR-146a<sup>-/-</sup> mice. The expression of miR-146a in this subset of del(5q) MDS/AML patients, which is ~80% lower as compared to age-matched controls, is best approximated by miR-146a<sup>-/-</sup> mice for functional comparisons as miR-146a expression in miR-146<sup>+/-</sup> (Het) BM cells is similar to WT mice (Supplemental Figure 1A,G). The overlapping pathways were nearly identical between the two groups, which included overexpression of cell cycle, innate immune response, and survival pathway genes (Figure 1A). In support of the gene expression patterns, HSC from miR-146a<sup>-/-</sup> mice have increased PyroninY (Figure 1B,C) and reduced AnnexinV staining (Figure 1D, P = 0.09), suggesting that miR-146a-deficient HSC are less quiescent and have increased survival. Consistent with increased HSPC proliferation and survival, miR-146<sup>-/-</sup> mice exhibit myeloid expansion (increased CD11b<sup>+</sup> cells as compared to WT HSPC; Supplemental Figure 1D,E) and impaired competitive engraftment (reduced CD45.2% chimerism in the PB; Supplemental Figure 1F). As reported previously(Boldin et al., 2011; Starczynowski et al., 2010; Taganov et al., 2006), loss of miR-146a in leukemic cells results in derepression of TRAF6, a mediator of NF-κB activation (Supplemental Figure 1H-K), implicating this molecular complex in the aggressive nature of the del(5q) MDS/AML phenotype.

Inhibiting the TRAF6/NF-κB axis may represent a therapeutic opportunity for aggressive forms of del(5q) MDS/AML with low miR-146a expression, unfortunately the success of direct inhibitors of NF-κB for human cancers have yet to be proven. Chromosome deletions that target tumor suppressor genes also involve multiple neighboring genes, such as with del(5q), and loss of certain neighboring genes may expose cancer-specific vulnerabilities and potential therapeutic targets(Muller et al., 2012; Nijhawan et al., 2012). To overcome the limitations of NF-κB inhibitors and identify novel mechanisms of disease dependency, we examined the expression of all genes residing within chr 5q from del(5q) and control CD34<sup>+</sup> cells (Supplemental Figure 2A). Of 1528 chr 5q genes, 374 genes were significantly lower (<0.5-fold) and 27 were higher (e.g., overexpressed/restored from the intact allele >1.5-fold) in del(5q) cells (Figure 2A, Supplemental Table 2). We also examined the 27 overexpressed genes in non-del(5q) MDS (Supplemental Figure 2B). Interestingly, a subset of these genes were also overexpressed in non-del(5q) MDS, but not to the same extent as in del(5q) MDS (Supplemental Figure 2C). In addition, we utilized GeneConnector functionality in NetWalker to build molecular networks (Supplemental Figure 2A)(Komurov et al., 2012). A single major “NF-κB” molecular node and a minor node related to “coagulation response” formed corresponding to the overexpressed chr 5q genes (Figure 2B). To determine whether the “NF-κB node” is important for the proliferative phenotype in aggressive del(5q) MDS/AML, human leukemic and normal CD34<sup>+</sup> cells were treated with an NF-κB inhibitor (BAY 11-7085) and then analyzed for cell cycle kinetics and viability. Following treatment with BAY 11-7085 or by knockdown of *TRAF6*, all MDS/AML cells exhibited a G2/M arrest and apoptosis (Supplemental Figure 2D-H). In addition, miR-146a<sup>-/-</sup> HSPC were more sensitive to NF-κB pathway inhibition by BAY 11-7085 than WT HSPC, as evident by enhanced G2/M arrest (Supplemental Figure 2G) and apoptosis

(Supplemental Figure 2I). These findings indicate that human and mouse miR-146a<sup>low</sup> HSPC are sensitive to genetic and pharmacologic inhibition of the TRAF6/NF-κB pathway, suggesting an acquired dependency on TRAF6.

Given the paradoxical overexpression of genes from the intact chr 5 allele, we hypothesized that the 27 overexpressed genes might be coregulated by a common transcription factor (TF). We compiled a dataset containing 1867 DNA binding motifs representing the binding preferences of 623 human TFs to search for enriched TF binding sites in the promoter regions of the 5q overexpressed genes (Zambelli et al., 2009). Of all TF motifs, NF-κB binding sites are the most strongly enriched in the entire overexpressed/compensated gene set (Figure 2C; P = 0.0001) and even further enriched in the “NF-κB node” gene set (Figure 2D; P = 0.0007) (Supplementary Table 3). To further explore the broader regulation of NF-κB-target gene expression, we examined two canonical NF-κB gene targets in normal CD34<sup>+</sup>, del(5q) MDS, and non-del(5q) MDS. Despite the enrichment of NF-κB binding sites in a subset of genes residing on chr 5q, not all NF-κB-regulated genes are overexpressed in del(5q) MDS (Supplemental Figure 2C). To determine which genes within the “NF-κB node” are necessary for del(5q) leukemic cell function, we knocked down the expression of each gene and examined leukemic progenitor function (Figure 2E). Based on previous reports, knockdown of *TRAF6* or *IRAK1* significantly impaired HL60 colony formation (Figure 2E). Of the genes within the “NF-κB node” that reside on chr 5q, only knockdown of sequestosome 1 (*SQSTM1/p62*) resulted in reduced colony formation. Knockdown of the remaining “NF-κB node” genes did not result in impaired leukemic progenitor function, suggesting these genes are not sufficient to sustain the NF-κB-mediated survival signal. Among the overexpressed genes residing on chr 5q and within the NF-κB node, *p62* (5q35, P = 0.0054; Adjusted P = 0.023) emerged as an obvious candidate (Supplemental Table 2), as it is required for leukemic progenitor function and is a cofactor for NF-κB activation through recruitment of TRAF6 (Duran et al., 2008; Linares et al., 2011; Moscat and Diaz-Meco, 2009; Wooten et al., 2005).

miR-146a regulates several genes within the innate immune pathway; however, an analysis of the 3' untranslated region of *p62* did not reveal miR-146a binding sites, suggesting an alternative mechanism of *p62* overexpression in miR-146a<sup>low</sup> cells. Examination of the *p62* promoter region using ENCODE functional genomics data (Rosenbloom et al., 2013) revealed the presence of two NF-κB (p50) ChIP-seq binding peaks (κB site motif: NGGGACTTTCN) (Chen et al., 1998) in hematopoietic cells (Figure 3A). Both NF-κB binding motifs directly overlap DNase I hypersensitive, H3K4 mono- and tri-methylation, and H3K27 acetylation regions in hematopoietic/leukemic cells, reaffirming the prediction analysis (Figure 2) and confirming that NF-κB also binds these regions in leukemic cells. Therefore, these analyses suggest that NF-κB transcription factors regulate a subset of overexpressed genes on chr 5q, one of which (*p62*) is a signaling mediator of the canonical NF-κB pathway. Analysis of an independent cohort of patients revealed that *p62* expression inversely correlated with miR-146a expression in HR del(5q) MDS/AML (P = 0.013; Figure 3B). Consistent with RNA expression in primary MDS/AML cells, *p62* protein is expressed at normal or even at elevated levels despite deletion of one *p62* locus in certain MDS/AML samples and cell lines (Figure 3C; Supplemental Figure 3). To further examine the

molecular relationship between miR-146a, NF- $\kappa$ B, and p62, additional knockdown of miR-146a resulted in elevated p62 expression following IL-1 $\beta$ -mediated (TRAF6-dependent) NF- $\kappa$ B activation, but not with TNF $\alpha$  stimulation (TRAF6-independent) (Figure 3D). Similarly, miR-146a<sup>-/-</sup> marrow cells exhibit elevated p62 mRNA and protein expression following IL-1 $\beta$ -induced NF- $\kappa$ B activation (Figure 3E,F). We sought to investigate whether compensation of p62 in this subset of del(5q) MDS/AML modified clinical outcome. Del(5q) MDS/AML patients were divided into miR-146a<sup>low</sup>/p62<sup>high</sup> and miR-146a<sup>high</sup>/p62<sup>low</sup> cohorts. Based on this stratification, del(5q) MDS/AML with miR-146a<sup>low</sup>/p62<sup>high</sup> had a significantly worse overall survival (Figure 3G, P = 0.0064), further implicating p62 as an essential cofactor in aggressive forms of del(5q) MDS/AML.

We next determined the function of p62 in miR-146a<sup>low</sup>-expressing HSPC. Overexpression of p62 in miR-146a<sup>-/-</sup> HSPC further increased cell proliferation as the proportion of cells in S phase increased and the proportion in G2/M decreased, whereas overexpression of p62 did not affect WT HSPC (Figure 3H). Conversely, knockdown of p62 in miR-146a<sup>-/-</sup> HSPC (Figure 3I) reversed the proliferative phenotype by arresting cells in G2/M (Figure 3J,K). However, knockdown of p62 in WT HSPC resulted in minor changes in cell cycle progression (Figure 3J,K). Since transplantation of miR-146a<sup>-/-</sup> BM results in diminished competitive myeloid reconstitution in recipient mice (Supplemental Figure 1F), we evaluated whether knockdown of p62 can restore these cellular consequences. As expected, knockdown of p62 in WT HSPC did not alter proportions of CD11b<sup>+</sup> or CD3<sup>+</sup> cells, while transplantation of miR-146a<sup>-/-</sup> HSPC transduced with control shRNA (shCTL) resulted in diminished myeloid reconstitution in recipient mice (Figure 3L). In contrast, knockdown of p62 in miR-146a<sup>-/-</sup> HSPC restored normal proportions of CD11b<sup>+</sup> cells (Figure 3L; P = 0.04). Therefore, p62 is essential in miR-146a<sup>low</sup>-expressing pre-malignant mouse HSPC by regulating cell cycle progression and myeloid cell development.

p62 is essential in certain solid tumors (Duran et al., 2008; Ling et al., 2012); therefore, we investigated whether p62 serves a similarly essential role in human myeloid malignancies. Utilizing lentiviral vectors encoding shRNAs targeting p62 (Supplemental Figure 4A), knockdown of p62 significantly reduced TRAF6 activation by ~75%, as measured by K63-linked autoubiquitination, in two human del(5q) MDS/AML cell lines (Starczynowski et al., 2011) (Figure 4A). Knockdown of p62 also resulted in impaired NF- $\kappa$ B/p65 nuclear localization, however only following loss of miR-146a (Figure 4B). To examine the effects of p62 depletion in vivo, the MDS/AML cell lines were transduced with shp62 or control (shCTL) and then xenografted into NSG mice. By 6–7 weeks, control leukemic cells expanded ~8-fold in the peripheral blood, while leukemic cells with p62 knockdown did not expand and remained below 10% chimerism (Figure 4C). Mice with HL60 or MDSL xenografts expressing shCTL succumbed to leukemic cell burden by 7 and 10 weeks, respectively (Figure 4C,D; Supplemental Figure 4B). In contrast, p62 knockdown prevented expansion of engrafted leukemic cells, and significantly delayed mortality in mice (Figure 4C,D). To investigate the cellular effects of p62 depletion in vitro, miR-146a<sup>low</sup> MDS/AML cell lines and primary del(5q) AML samples were examined for progenitor function, survival, and proliferation. All MDS/AML cell lines with knockdown of p62 exhibited >80% reduction of leukemic progenitor colonies in methylcellulose (P < 0.05 for all)

(Supplemental Figure 4C,D). In addition, p62 regulates leukemic cell proliferation as knockdown of *p62* resulted in delayed growth in vitro, fewer cells in S-phase, and accumulation of G0/G1 and G2/M cells (Supplemental Figure 4E). MDS/AML cell lines with knockdown of p62 correlated with a significant increase in apoptosis (increased AnnexinV<sup>+</sup> cells; Figure 4E). Loss of p62 did not coincide with a significant increase in apoptosis or changes in cell cycle of control CD34<sup>+</sup> cells (Figure 4E–G), suggesting that malignant HSPC are more sensitive to p62 inhibition. For primary del(5q) MDS/AML cells, knockdown of *p62* also resulted in increased apoptosis (increased AnnexinV<sup>+</sup> cells; Figure 4F; Supplemental Figure 4F), and suppression of cell cycle (Figure 4G). Despite the differential sensitivity of normal and leukemic cells to *p62* knockdown, treatment with BAY 11-7085 induced apoptosis in both cell types (Figure 4F), revealing that broadly inhibiting NF-κB results in cytotoxicity of normal cells, and as such, the therapeutic limitations of NF-κB inhibitors. Given the role of p62 in regulating autophagy in certain cell types, we examined autophagy in leukemic cells. Notably, knockdown or overexpression of p62 did not change the autophagy status in leukemic cells (Supplemental Figure 4G).

The p62 signaling domains have been extensively mapped (Moscat and Diaz-Meco, 2009) (Figure 5A). For NF-κB signaling, p62 recruits and activates TRAF6, via its TRAF6-binding (TB) domain (aa 228–242), which then results in signaling to IKKα/β and NF-κB (Figure 5A) (Seibenhener et al., 2004; Wooten et al., 2005). To determine whether TRAF6 recruitment is essential for p62 function in MDS/AML cells, WT p62 or a mutant p62 lacking the TB domain (p62<sup>TB</sup>) were co-expressed with shp62. Cell viability was restored in shp62-expressing cells when WT p62 was expressed, but not p62<sup>TB</sup>, suggesting that interaction with TRAF6 is essential for the effects of p62 in leukemic cells (Figure 5B,C). We hypothesized that interfering with the p62-TRAF6 signaling complex in leukemic cells would be sufficient to inhibit TRAF6-mediated activation of NF-κB and induce cell death and cell cycle arrest. The TB domain, which consists of 15 aa, was fused to a hemagglutinin epitope tag (HA), and then cloned into a retroviral expression vector for sustained expression in leukemic cells. A stop codon (TGA) was inserted following the last amino acid (Lys-242). As a negative control, two critical residues (Phe-232 and Glu-234) were changed to alanine (Figure 5A). Expression of WT (TB<sup>WT</sup>) and control (i.e., inactive mutant: TB<sup>MUT</sup>) TB peptide was confirmed by dot blot immunoassay in transduced HL60 and transfected HEK293 cells (Figure 5D). To verify that the WT TB peptide inhibits p62-mediated activation of TRAF6 (as measured by K63-linked autoubiquitination), FLAG-TRAF6 and p62 were co-transfected with TB<sup>WT</sup> or TB<sup>MUT</sup> into HEK293 cells followed by immunoprecipitation of TRAF6. TRAF6 autoubiquitination (IP: FLAG; IB: Ub) was increased following co-transfection of p62, but was then significantly reduced in cells expressing TB<sup>WT</sup> (Figure 5E). TB<sup>WT</sup> also suppressed downstream NF-κB signaling as evident by ~25% reduced κB-site reporter gene activation (Figure 5F). To examine the molecular and cellular effects of the TB<sup>WT</sup> peptide in disease-relevant cells, we transduced HL60 cells with TB<sup>WT</sup>- and TB<sup>MUT</sup>-expressing vectors (Figure 5D). Examination of NF-κB activation showed less phosphorylated IKKα/β in TB<sup>WT</sup>-expressing HL60 cells (Figure 5G). Consistent with suppression of NF-κB/IKK activation, immunoprecipitation of TRAF6 from TB<sup>WT</sup>-expressing HL60 cells revealed a 50% reduction in ubiquitinated TRAF6, as compared to control (TB<sup>MUT</sup>) (Figure 5H). Inhibition of TRAF6/NF-κB with TB<sup>WT</sup> in

HL60 cells coincided with impaired G2/M exit (Figure 5I; 26.8% versus 15.2%), apoptosis (Figure 5J; 16.2% versus 3.7%), and impaired leukemic progenitor function (Figure 5K; 144 vs 307 colonies;  $P = 0.001$ ), as compared to normal CD34<sup>+</sup> cells. These findings are consistent with the cellular effects observed after depleting p62 (Figure 4E–G; Supplemental Figure 4C–F). Importantly, TB<sup>WT</sup> expression also resulted in apoptosis of three independent del(5q) AML patient-derived cells, but had no noticeable effect on normal CD34<sup>+</sup> cells (Figure 5J–L). Thus, the TB<sup>WT</sup> motif exerts anti-leukemic effects by interfering with TRAF6 and p62 signaling and resulting in cell cycle arrest, impaired leukemic progenitor function, and increased apoptosis.

## Discussion

Several haploinsufficient genes have been identified and verified in the pathogenesis of del(5q) MDS/AML (Ebert, 2009). Despite the progress, our knowledge on genes and gene networks that are necessary for sustaining the del(5q) MDS/AML-propagating cells is insufficiently described. In this study, we show that deletion of miR-146a in del(5q) MDS/AML cells is associated with sustaining the disease phenotype by increasing cell survival and proliferation of the propagating cells through the TRAF6/p62/NF- $\kappa$ B complex. TRAF6 signaling is implicated in del(5q) myeloid malignancies (Starczynowski et al., 2010; Zhao et al., 2013) and an important target in low risk-MDS (Rhyasen et al., 2013), however this study uncovers the mechanism by which del(5q) cells have acquired and sustained their dependency on the NF- $\kappa$ B network in HR-MDS patients (with elevated blasts) and AML. As part of our effort to uncover del(5q) MDS/AML-specific vulnerabilities and potential therapeutic targets, we identified a subset of 5q genes in which their expression is restored from the intact 5q allele and form an NF- $\kappa$ B signaling node in del(5q) MDS/AML. Furthermore, a detailed promoter analysis confirmed enrichment of NF- $\kappa$ B binding motifs within the promoters of the overexpressed “NF- $\kappa$ B node” genes, suggesting that an NF- $\kappa$ B feed-forward loop exists and may be necessary to sustain the NF- $\kappa$ B signaling pathway following deletion of miR-146a in MDS/AML. *p62*, a key gene within the NF- $\kappa$ B signaling node and an important signaling mediator of TRAF6, is expressed from the intact allele, forming an intrachromosomal gene network with miR-146a to regulate TRAF6 and NF- $\kappa$ B activation (Figure 5M). Importantly, depletion of *p62* has minimal effects on survival, proliferation, or function of normal HSPC. Although our focus was on p62, we predict that the remaining genes within the “NF- $\kappa$ B node” are also important for sustaining NF- $\kappa$ B activation (Figure 2). NF- $\kappa$ B is one of the major pathways regulated by activation of TRAF6; however additional critical pathways regulated by TRAF6 in low-risk (LR) MDS and HR MDS/AML may be different, particularly as they relate to disease initiation versus maintenance. Further investigation into deciphering these molecular pathways during disease evolution and within distinct MDS/AML subtypes is warranted.

Mouse genetic studies have shown that p62 regulates several cellular functions, including bone remodeling, metabolism, and cancer (Linares et al., 2013; Moscat and Diaz-Meco, 2009; Seibenhener et al., 2013). p62 protein contains 5 signaling and/or interaction modules, including PB1, ZZ-type zinc finger domain (ZZ), TRAF6-binding (TB) domain, LC3-interacting region (LIR), and ubiquitin-associated domain (UBA) (Figure 5A) (Moscat and Diaz-Meco, 2009). Through the TB domain, p62 facilitates K63-linked polyubiquitination of



TRAF6 and consequently initiates NF- $\kappa$ B signaling(Sanz et al., 2000). Although the TB domain of p62 has been shown to directly bind TRAF6, overexpression of the TB peptide does not appear to be sufficient to disrupt this interaction in leukemic cells despite efficiently inhibiting p62/TRAF6/NF- $\kappa$ B signaling activity. In our findings, the TB peptide inhibits TRAF6 activation (as measured by TRAF6 autoubiquitination; Figure 5E,H) and signaling to NF- $\kappa$ B (Figure 5F,G). Collectively, these observations suggest that the TB peptide inhibits p62-mediated activation of TRAF6, however the mechanism by which TB peptide inhibits TRAF6/NF- $\kappa$ B does not exclusively depend on disrupting the TRAF6-p62 interaction. Nevertheless, we show that the TB peptide exerts anti-leukemic effects by interfering with TRAF6 and p62 signaling and resulting in cell cycle arrest, impaired leukemic progenitor function, and increased apoptosis. An essential role of p62 in cancer-associated TRAF6/NF- $\kappa$ B has been also described in solid tumors(Beg et al., 1995; Duran et al., 2008). In one of these studies, p62 is required for RAS to activate IKK through polyubiquitination of TRAF6 in lung adenocarcinomas. In normal and RAS-transformed cells, p62 controls timely transit of cells through mitosis and tumor cell proliferation(Linares et al., 2011). These previous findings are consistent with our observation that p62 is important for increased cell proliferation and G2/M transition in miR-146a<sup>low</sup>-expressing leukemic cells. Selectively inhibiting TRAF6 has not been feasible, as it does not possess druggable protein interfaces; therefore, interfering with the p62-TRAF6 interaction may be a novel therapeutic option, particularly in miR-146a<sup>low</sup>-expressing leukemia. Similar approaches using a deliverable peptide to prevent protein interactions have been proposed and successfully implemented in human cancer(Thundimadathil, 2012).

Since del(5q) MDS/AML is associated with a variety of other cytogenetic changes, for this study we focused on differentially expressed genes residing within chr 5. A focused analysis examining just chr 5q genes allowed for a simpler interpretation while providing a proof-of-concept that intra- and/or inter-chromosomal gene networks may expose therapeutic vulnerabilities. Two recent studies have proposed that passenger deletions, which in certain cancers involve functionally redundant gene families or necessary house-keeping genes, create therapeutic vulnerabilities (Muller et al., 2012; Nijhawan et al., 2012). This concept is exemplified by the restored expression of p62 from the intact chr 5q allele in order to sustain NF- $\kappa$ B signaling by recruiting TRAF6 in miR-146a-deficient HSPC (Figure 5M). Del(5q) MDS/AML patients with low miR-146a expression nearly always have normal/elevated p62 highlighting the pathologic connection between miR-146a and p62. In addition, this study reinforces synthetic dependencies based on intrachromosomal gene networks associated with a specific genomic alteration. Although our findings revealed an important role of p62 in sustaining NF- $\kappa$ B-mediated proliferation and survival pathways in HR del(5q) MDS/AML, based on p62 knockdown experiments, we also anticipate that p62 will similarly sustain NF- $\kappa$ B signaling in non-del(5q) AML cells with low miR-146a expression. Not all NF- $\kappa$ B-target genes are overexpressed in del(5q) MDS as one might have expected following deletion of miR-146a (Supplemental Figure 2C). This observation suggests that there is selective regulation of certain NF- $\kappa$ B-target genes, such as the ones described as part of the intra-chromosomal network within chr 5 (Figure 2A,B). Lastly, we also postulate that non-chr 5 genes may equally contribute to the del(5q) network, and that intra- and

interchromosomal gene networks may expose therapeutic vulnerabilities in MDS/AML with other cytogenetic alterations (i.e., monosomy 7).

## Material and Methods

See supplemental files for additional material and methods.

### Mice and primary murine bone marrow cell culture

Animals were bred and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility of Cincinnati Children's Hospital Medical Center. Whole body miR-146a<sup>-/-</sup> mice were a kind gift from Dr. Dinesh Rao (UCLA). All experiments were carried out under approved animal protocols by the Cincinnati Children's Hospital Institutional Animal Committee. BM cells were obtained by crushing the femur, tibia, and pelvic bone into RPMI1640 containing 2% bovine serum, 1% penicillin-streptomycin, and 1mM EDTA, and then filtered through a 40- $\mu$ m cell strainer. The cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum, 1% PS, recombinant mouse stem cell factor (rmSCF, 02931, StemCell Technologies), mouse interleukin 3 (rmIL-3, 02903, StemCell Technologies) and human interleukin 6 (rhIL-6, 02606, StemCell Technologies) at 10ng/mL.

### Patient samples

Informed consent was obtained according to protocols approved by the review boards of Cleveland Clinics. Diagnoses were reviewed at Cleveland Clinics and adapted, when required, to WHO 2008 criteria. For qRT-PCR analysis, bone marrow aspirates were collected from 41 patients and 15 age-matched healthy controls (Supplemental Table 1). For functional studies, MDS/AML peripheral MNCs (Supplemental Table 1) were cultured in StemSpan Serum-Free Expansion Media (SFEM, Stemcell Technologies) supplemented with 100ng/mL of recombinant human stem cell factor (rhSCF, 02830, StemCell Technologies), human Flt3 ligand (rhFL, 02840, StemCell Technologies), human thrombopoietin (rhTPO, 02522, PeproTech) for 48hours before transduction. Cells were cultured in rhSCF, rhFlt3L, rhTPO, rhIL-3, and rhIL-6 at 10ng/mL.

### Transcription factor bioinformatic analysis

To identify transcription factors (TFs) that might be regulating the compensated genes on chr 5q, we searched for enriched TF binding motifs in their promoter regions. For each gene, we defined the promoter region as the 1000 bases upstream of the start of the longest gene transcript, as annotated in the RefSeq database (Pruitt et al., 2009). We compiled a total of 1867 TF binding motif models (in the form of position weight matrices) from the following sources (Transfac (Matys et al., 2006); JASPAR (Portales-Casamar et al., 2010); FactorBook (Wang et al., 2013); UniProbe (Robasky and Bulyk, 2011); hmCHIP (Chen et al., 2011); Jolma (Jolma et al., 2013)), which in total cover 623 distinct human TFs. We then used the Pscan software package (Zambelli et al., 2009) to rank all TF motifs based on their enrichment in the chr 5q gene set, as compared to random expectation based on background distributions obtained by scanning all RefSeq promoters.

## Statistical Analysis

Results are depicted as the mean  $\pm$  standard error of the mean. Statistical analyses were performed using Student's *t*-test. For microarray data, Benjamin Hochberg correction was applied based on the total number of gene expression probes analyzed for the region. Probes with an adjusted P value  $\leq 0.05$  were considered significant (Supplemental Table 2). GraphPad Prism (v5, GraphPad) was used for statistical analysis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by Cincinnati Children's Hospital Research Foundation, American Society of Hematology (ASH), National Institute of Health (RO1HL111103), Gabrielle's Angel Foundation, and Department of Defense grants to D.T.S. The umbilical cord blood samples were received through the Normal Donor Repository in the Translational Core Laboratory at Cincinnati Children's Research Foundation, which is supported through the NIDDK Center's of Excellence in Experimental Hematology (P30DK090971). We thank the Mt. Auburn Ob-Gyn associates and delivery nursing staff at Christ Hospital, Cincinnati for collecting cord blood (CD34<sup>+</sup>) samples from normal deliveries. We thank Jeff Bailey and Victoria Summey for assistance with transplantations and xenotransplantations (Comprehensive Mouse and Cancer Core). MDSL cells were kindly provided by Dr. Kaoru Tohyama. We thank Dr. Ruhikanta Meetei for technical recommendations.

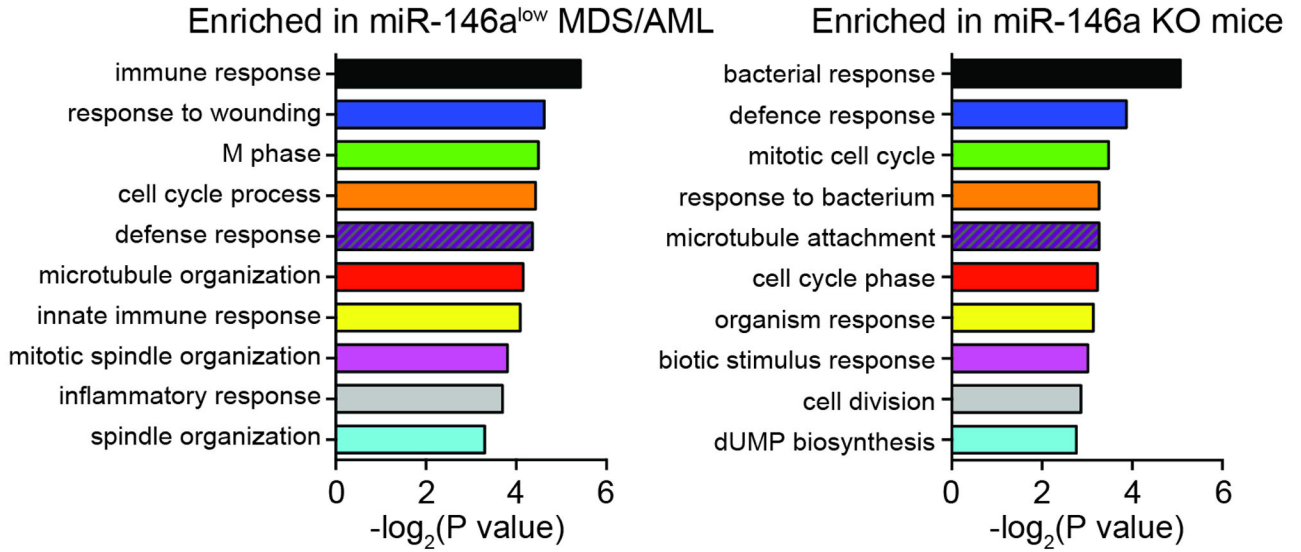
## References

- Beg AA, Sha WC, Bronson RT, Baltimore D. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev.* 1995; 9:2736–2746. [PubMed: 7590249]
- Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, Garcia-Flores Y, Luong M, Devrekanli A, Xu J, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med.* 2011; 208:1189–1201. [PubMed: 21555486]
- Chen FE, Huang DB, Chen YQ, Ghosh G. Crystal structure of p50/p65 heterodimer of transcription factor NF-kappaB bound to DNA. *Nature.* 1998; 391:410–413. [PubMed: 9450761]
- Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res.* 2009; 37:W305–W311. [PubMed: 19465376]
- Chen L, Wu G, Ji H. hmChIP: a database and web server for exploring publicly available human and mouse ChIP-seq and ChIP-chip data. *Bioinformatics.* 2011; 27:1447–1448. [PubMed: 21450710]
- Duran A, Linares JF, Galvez AS, Wikenheiser K, Flores JM, Diaz-Meco MT, Moscat J. The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis. *Cancer Cell.* 2008; 13:343–354. [PubMed: 18394557]
- Ebert BL. Deletion 5q in myelodysplastic syndrome: a paradigm for the study of hemizygous deletions in cancer. *Leukemia.* 2009; 23:1252–1256. [PubMed: 19322210]
- Giagounidis AA, Germing U, Aul C. Biological and prognostic significance of chromosome 5q deletions in myeloid malignancies. *Clin Cancer Res.* 2006; 12:5–10. [PubMed: 16397017]
- Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Sole F, Bennett JM, Bowen D, Fenaux P, Dreyfus F, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood.* 2012; 120:2454–2465. [PubMed: 22740453]
- Jerez A, Gondek LP, Jankowska AM, Makishima H, Przychodzen B, Tiu RV, O'Keefe CL, Mohamedali AM, Batista D, Sekeres MA, et al. Topography, clinical, and genomic correlates of 5q myeloid malignancies revisited. *J Clin Oncol.* 2012; 30:1343–1349. [PubMed: 22370328]
- Jolma A, Yan J, Whittington T, Toivonen J, Nitta KR, Rastas P, Morgunova E, Enge M, Taipale M, Wei G, et al. DNA-binding specificities of human transcription factors. *Cell.* 2013; 152:327–339. [PubMed: 23332764]

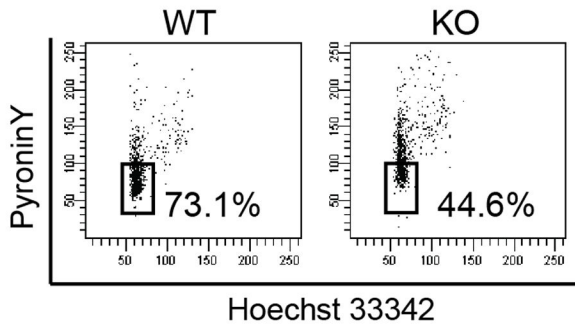
- Komurov K, Dursun S, Erdin S, Ram PT. NetWalker: a contextual network analysis tool for functional genomics. *BMC Genomics*. 2012; 13:282. [PubMed: 22732065]
- Linares JF, Amanchy R, Greis K, Diaz-Meco MT, Moscat J. Phosphorylation of p62 by cdk1 controls the timely transit of cells through mitosis and tumor cell proliferation. *Mol Cell Biol*. 2011; 31:105–117. [PubMed: 20974803]
- Linares JF, Duran A, Yajima T, Pasparakis M, Moscat J, Diaz-Meco MT. K63 Polyubiquitination and Activation of mTOR by the p62-TRAF6 Complex in Nutrient-Activated Cells. *Mol Cell*. 2013; 51:283–296. [PubMed: 23911927]
- Ling J, Kang Y, Zhao R, Xia Q, Lee DF, Chang Z, Li J, Peng B, Fleming JB, Wang H, et al. KrasG12D-induced IKK2/beta/NF-kappaB activation by IL-1alpha and p62 feedforward loops is required for development of pancreatic ductal adenocarcinoma. *Cancer Cell*. 2012; 21:105–120. [PubMed: 22264792]
- List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, Powell B, Greenberg P, Thomas D, Stone R, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *N Engl J Med*. 2006; 355:1456–1465. [PubMed: 17021321]
- Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, Barre-Dirrie A, Reuter I, Chekmenev D, Krull M, Hornischer K, et al. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res*. 2006; 34:D108–D110. [PubMed: 16381825]
- Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. *Cell*. 2009; 137:1001–1004. [PubMed: 19524504]
- Muller FL, Colla S, Aquilanti E, Manzo VE, Genovese G, Lee J, Eisenson D, Narurkar R, Deng P, Nezi L, et al. Passenger deletions generate therapeutic vulnerabilities in cancer. *Nature*. 2012; 488:337–342. [PubMed: 22895339]
- Nijhawan D, Zack TI, Ren Y, Strickland MR, Lamothe R, Schumacher SE, Tsherniak A, Besche HC, Rosenbluh J, Shehata S, et al. Cancer vulnerabilities unveiled by genomic loss. *Cell*. 2012; 150:842–854. [PubMed: 22901813]
- Portales-Casamar E, Thongjuea S, Kwon AT, Arenillas D, Zhao X, Valen E, Yusuf D, Lenhard B, Wasserman WW, Sandelin A. JASPAR 2010: the greatly expanded open-access database of transcription factor binding profiles. *Nucleic Acids Res*. 2010; 38:D105–D110. [PubMed: 19906716]
- Pruitt KD, Tatusova T, Klimke W, Maglott DR. NCBI Reference Sequences: current status, policy and new initiatives. *Nucleic Acids Res*. 2009; 37:D32–D36. [PubMed: 18927115]
- Rhyasen GW, Bolanos L, Fang J, Jerez A, Wunderlich M, Rigolino C, Mathews L, Ferrer M, Southall N, Guha R, et al. Targeting IRAK1 as a Therapeutic Approach for Myelodysplastic Syndrome. *Cancer Cell*. 2013; 24:90–104. [PubMed: 23845443]
- Robasky K, Bulyk ML. UniPROBE, update 2011: expanded content and search tools in the online database of protein-binding microarray data on protein-DNA interactions. *Nucleic Acids Res*. 2011; 39:D124–D128. [PubMed: 21037262]
- Rosenbloom KR, Sloan CA, Malladi VS, Dreszer TR, Learned K, Kirkup VM, Wong MC, Maddren M, Fang R, Heitner SG, et al. ENCODE data in the UCSC Genome Browser: year 5 update. *Nucleic Acids Res*. 2013; 41:D56–D63. [PubMed: 23193274]
- Sanz L, Diaz-Meco MT, Nakano H, Moscat J. The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. *EMBO J*. 2000; 19:1576–1586. [PubMed: 10747026]
- Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, Wooten MW. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. *Mol Cell Biol*. 2004; 24:8055–8068. [PubMed: 15340068]
- Seibenhener ML, Du Y, Diaz-Meco MT, Moscat J, Wooten MC, Wooten MW. A role for sequestosome 1/p62 in mitochondrial dynamics, import and genome integrity. *Biochim Biophys Acta*. 2013; 1833:452–459. [PubMed: 23147249]
- Starczynowski DT, Kuchenbauer F, Argiropoulos B, Sung S, Morin R, Muranyi A, Hirst M, Hogge D, Marra M, Wells RA, et al. Identification of miR-145 and miR-146a as mediators of the 5q-syndrome phenotype. *Nat Med*. 2010; 16:49–58. [PubMed: 19898489]

- Starczynowski DT, Morin R, McPherson A, Lam J, Chari R, Wegrzyn J, Kuchenbauer F, Hirst M, Tohyama K, Humphries RK, et al. Genome-wide identification of human microRNAs located in leukemia-associated genomic alterations. *Blood*. 2011; 117:595–607. [PubMed: 20962326]
- Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A*. 2006; 103:12481–12486. [PubMed: 16885212]
- Thundimadathil J. Cancer treatment using peptides: current therapies and future prospects. *J Amino Acids*. 2012; 2012:967347. [PubMed: 23316341]
- Wang J, Zhuang J, Iyer S, Lin XY, Greven MC, Kim BH, Moore J, Pierce BG, Dong X, Virgil D, et al. [Factorbook.org](http://Factorbook.org): a Wiki-based database for transcription factor-binding data generated by the ENCODE consortium. *Nucleic Acids Res*. 2013; 41:D171–D176. [PubMed: 23203885]
- Wooten MW, Geetha T, Seibenhener ML, Babu JR, Diaz-Meco MT, Moscat J. The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. *J Biol Chem*. 2005; 280:35625–35629. [PubMed: 16079148]
- Zambelli F, Pesole G, Pavesi G. Pscan: finding over-represented transcription factor binding site motifs in sequences from co-regulated or co-expressed genes. *Nucleic Acids Res*. 2009; 37:W247–W252. [PubMed: 19487240]
- Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D. NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. *Proc Natl Acad Sci U S A*. 2011; 108:9184–9189. [PubMed: 21576471]
- Zhao JL, Rao DS, O'Connell RM, Garcia-Flores Y, Baltimore D. MicroRNA-146a acts as a guardian of the quality and longevity of hematopoietic stem cells in mice. *Elife*. 2013; 2:e00537. [PubMed: 23705069]

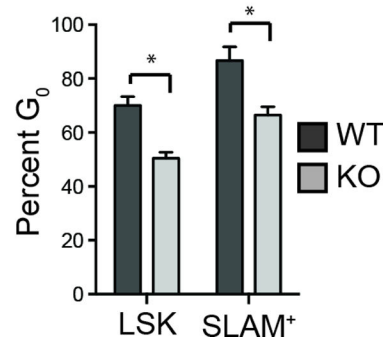
A



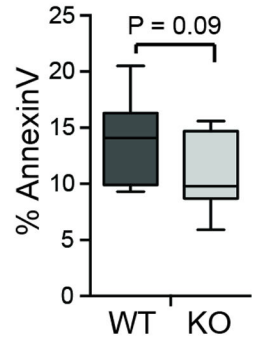
B



C

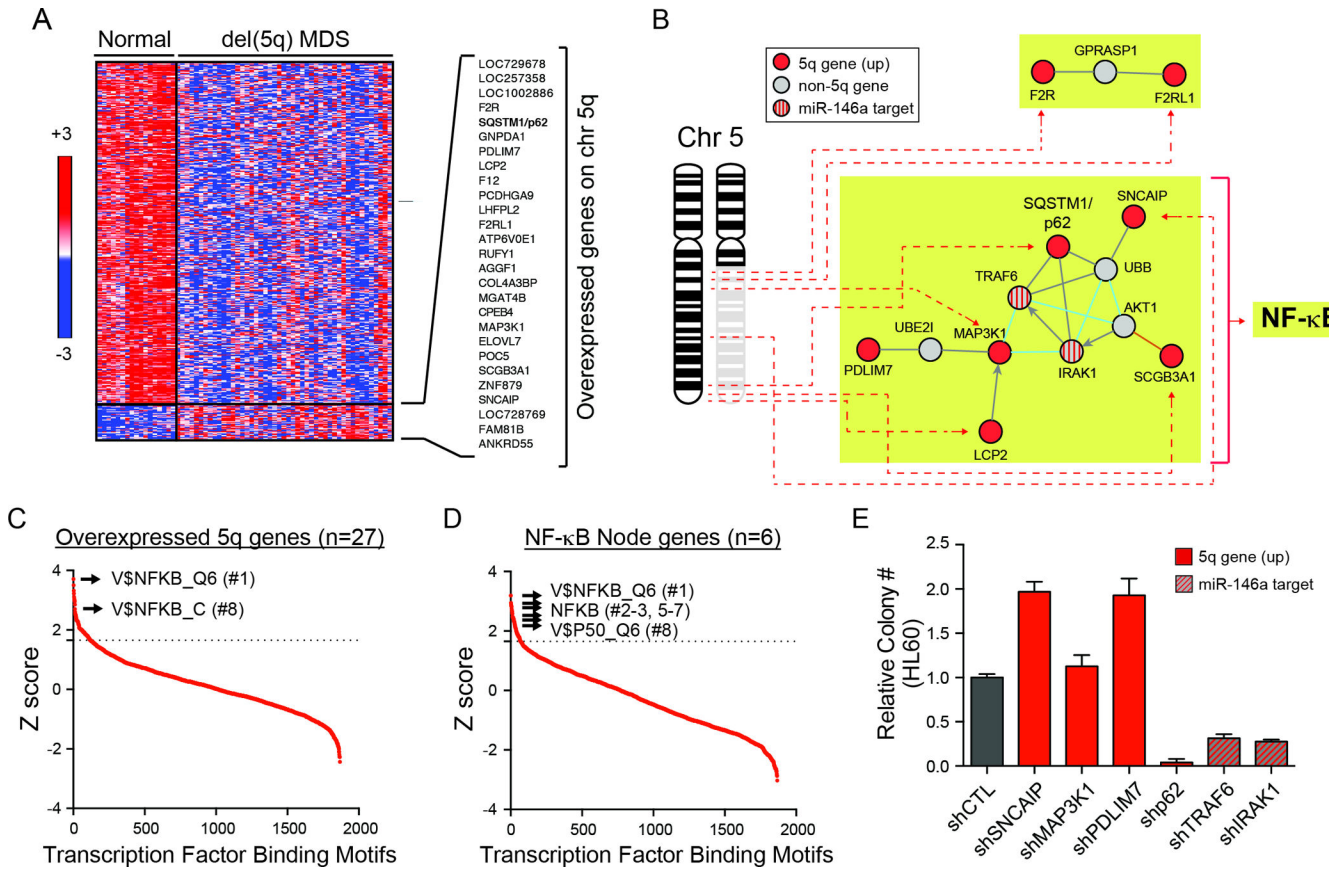


D



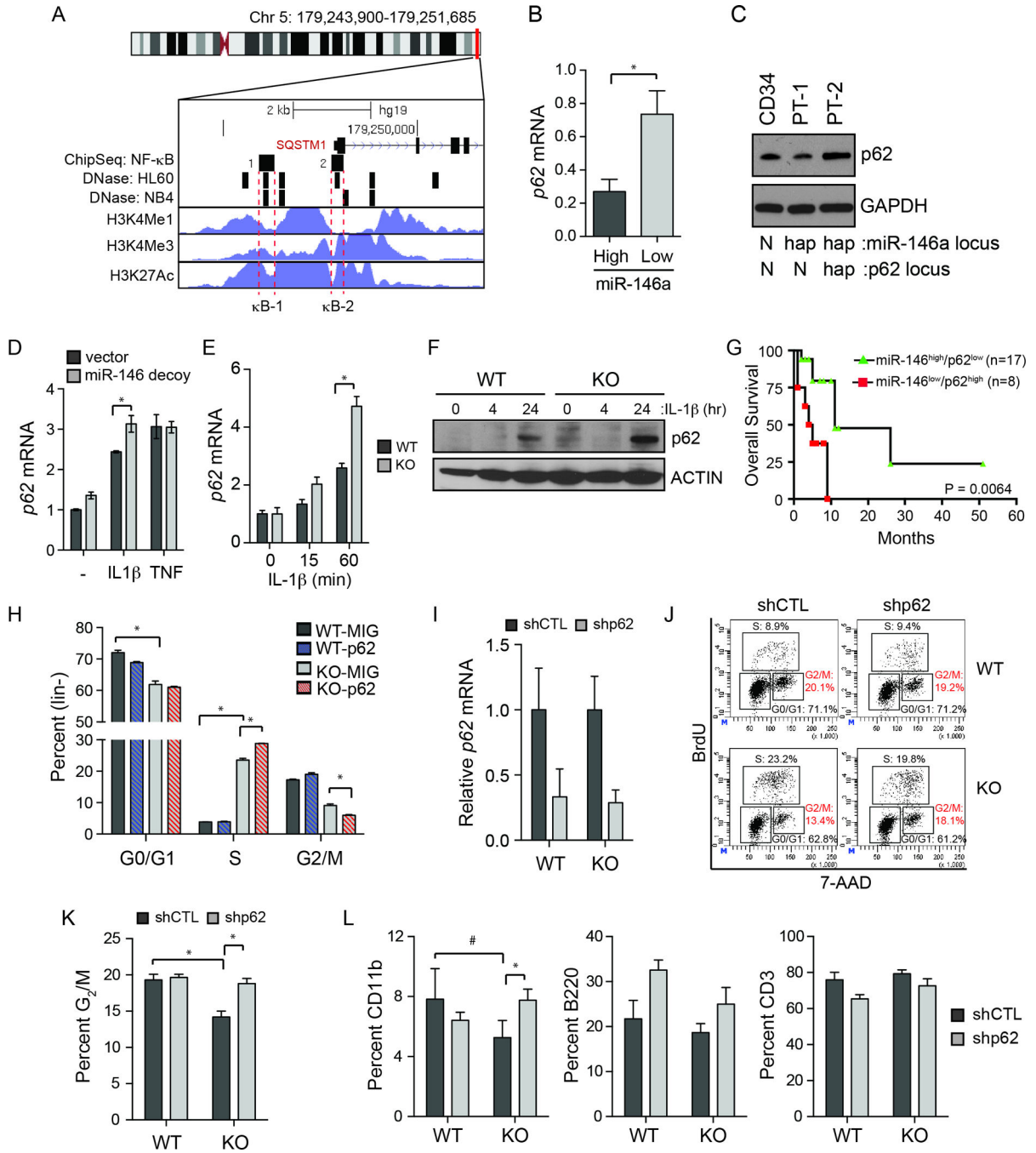
**Figure 1. miR-146a deletion mediates HSPC hyperproliferation**

(A) Overexpressed genes from low miR-146a-expressing del(5q) MDS/AML patients and miR-146a<sup>-/-</sup> (KO) marrow cells were compared using ToppGene. (B–C) Cell cycle analysis was performed on LSK and SLAM<sup>+</sup> cells from WT (n = 4) and KO (n = 6) mice by Pyronin Y/Hoechst 33342 staining. Shown is a representative plot for LSK (B) and summary for LSK and SLAM<sup>+</sup> cells (C). \*, P < 0.05. (D) AnnexinV staining was performed on LK cells from WT (n=4) and KO (n = 6) mice. See also Supplemental Figure 1 and Table 1.



**Figure 2. Del(5q) MDS/AML are associated with an intrachromosomal NF-κB feed-forward gene network**

(A) Gene expression profiling of differentially expressed chr 5q genes (q11-q35) in del(5q) MDS (n = 47) as compared to normal control CD34<sup>+</sup> cells (n = 17). (B) GeneConnector functionality in NetWalker was used to build a molecular network of genes that are overexpressed in del(5q) MDS (see Supplemental Table 2 for all genes used in the analysis). Red nodes indicate genes overexpressed in del(5q) MDS. Grey nodes indicate molecularly-connected genes. Grey nodes with red stripes indicate validated miR-146a gene targets. Dotted lines indicate the chromosome position of each gene. (C–D) Enrichment scores for DNA binding motifs in the promoter regions of overexpressed 5q genes (C) and NF-κB node genes (D). Each point represents the enrichment Z-score for a single transcription factor (TF) binding motif. Enriched motifs for NF-κB are indicated with arrows, along with their overall rank amongst the 1867 total human TF motifs analyzed. Horizontal dashed line indicates the (uncorrected) significance threshold corresponding to a P value cutoff of 0.01 (see Table 3). (E) Knockdown of *SNCAIP*, *MAP3K1*, *PDLIM7*, *p62*, *TRAF6*, and *IRAK1* in HL60 cells was achieved by shRNAs. Solid red histograms represent genes within the “NF-κB node” that reside on chr 5q. Hatched red histograms represent genes within the “NF-κB node” but do not reside on chr 5q (see Figure 2B). Transduced HL60 cells were evaluated for colony formation in methylcellulose following knockdown of each indicated gene. Colonies were scored and normalized to a control shRNA. See also Supplemental Figure 2 and Tables 2–3.

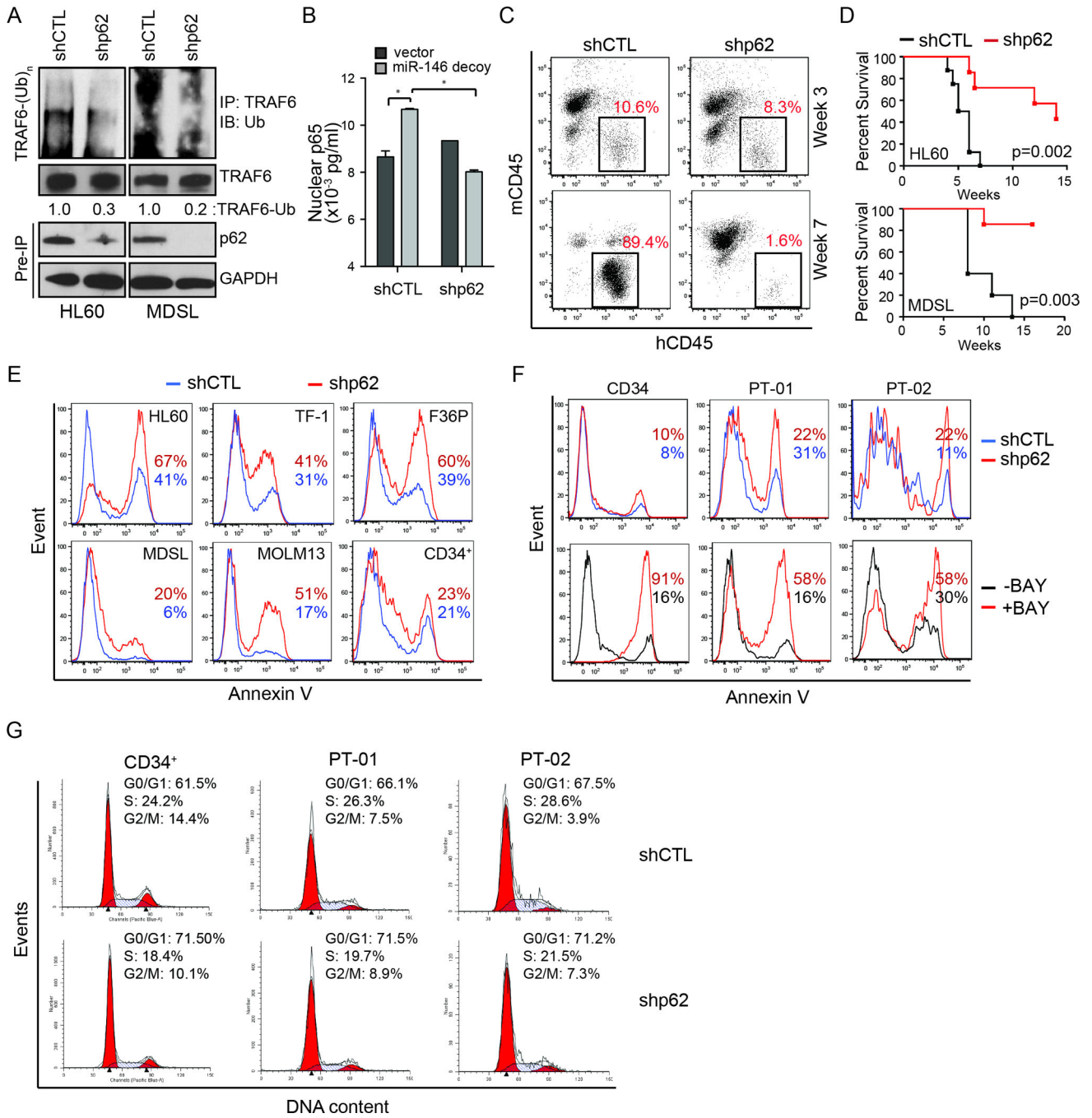


**Figure 3. p62 is overexpressed through NF- $\kappa$ B activation and is an essential cofactor in miR-146a<sup>-/-</sup> HSPC**

(A) Schematic of the *p62* (*SQSTM1*) promoter region. UCSC Genome Browser image displays ENCODE consortium data indicating (top to bottom): location of *SQSTM1* gene (exons depicted as filled boxes, introns as lines with arrows); location of two NF- $\kappa$ B binding regions, based on ChIP-seq data in B cell lines; location of DNaseI hypersensitive regions (indicative of open chromatin) in HL60 and NB4 cells; various histone marks (all indicative of regulatory regions) in K562 cells. (B) *p62* expression in AML marrow cells with high



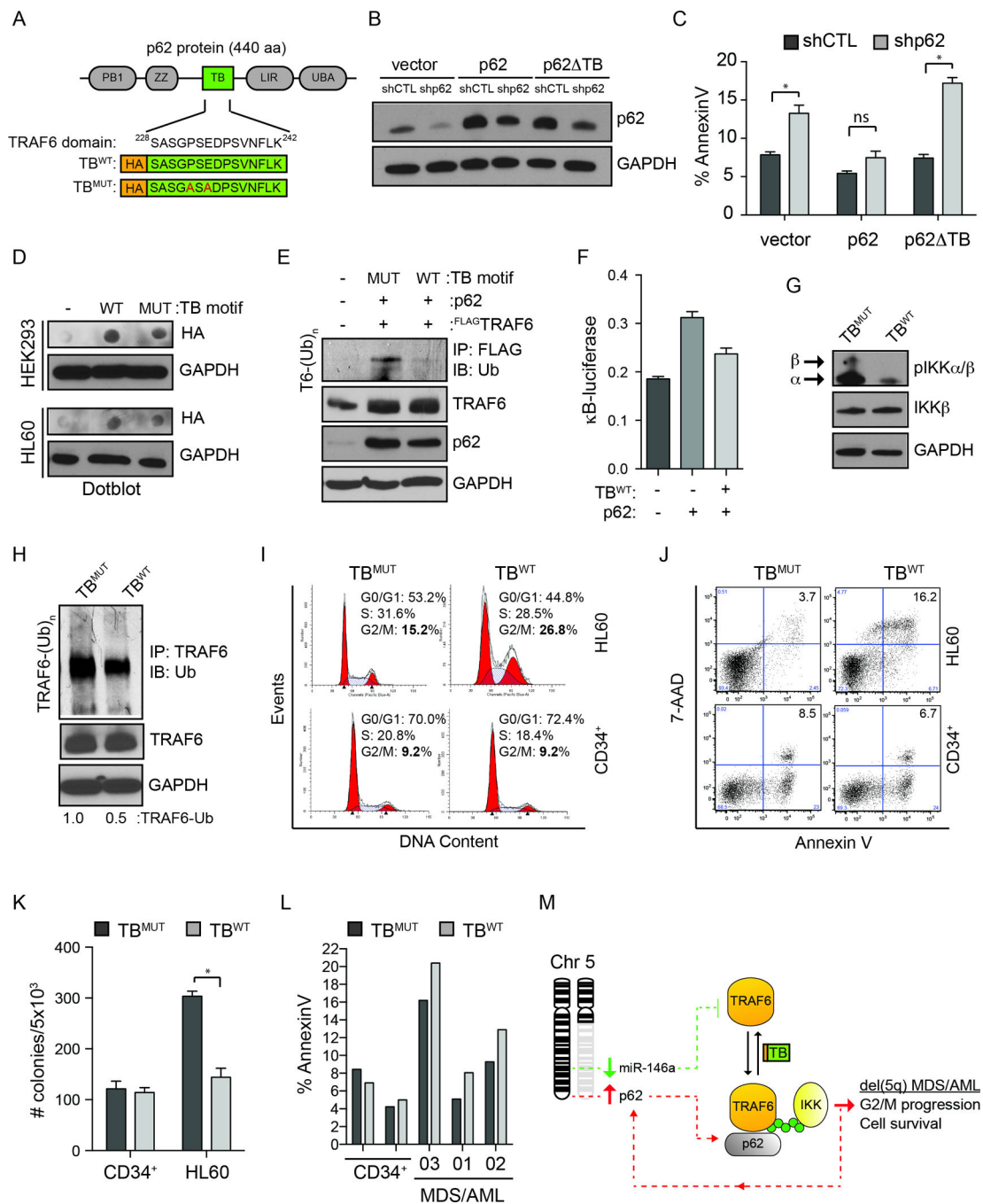
(top 50<sup>th</sup> percentile) or low (bottom 50<sup>th</sup> percentile) miR-146a. **(C)** Immunoblot analysis of p62 in del(5q) AML/MDS patient BM cells and normal CD34<sup>+</sup> cells. The miR-146a and p62 locus status is indicated below (N, normal diploid; hap, haploid). **(D)** qRT-PCR expression of *p62* in TF1 leukemic cells transduced with vector or miR-146 decoy and then treated with IL-1 $\beta$  or TNF $\alpha$ . **(E)** qRT-PCR expression of *p62* in HSPC from WT and miR-146<sup>-/-</sup> mice after treatment with IL-1 $\beta$  at indicated time points. **(F)** p62 immunoblot analysis on marrow cells from WT and miR-146<sup>-/-</sup> mice after treated with IL-1 $\beta$  at indicated time points. **(G)** Del(5q) MDS/AML patients were stratified into miR-146a<sup>high</sup>/p62<sup>low</sup> and miR-146a<sup>low</sup>/p62<sup>high</sup> RNA expression. Overall survival for del(5q) MDS/AML patients based on high miR-146a/low p62 (green, n = 17) and low miR-146a/high p62 (red, n = 8). **(H)** Cell cycle analysis of WT and miR-146a<sup>-/-</sup> BM transduced with vector (MSCV-IRES-GFP; MIG) or p62. **(I)** Validation of *p62* knockdown in WT and miR-146a<sup>-/-</sup> BM cells by qRT-PCR. **(J–K)** Cell cycle analysis of WT and miR-146a<sup>-/-</sup> BM transduced with shCTL or shp62 (J) and summary of replicate experiments (K). **(L)** WT and miR-146a<sup>-/-</sup> HSPC (CD45.2) transduced with shCTL or shp62 were mixed with 1 $\times$ 10<sup>5</sup> WT (CD45.1) marrow cells and then transplanted into lethally-irradiated recipients (n = 8/group). Myeloid and lymphoid proportions in the blood were analyzed 4 weeks post transplantation. \*, P < 0.05; #, P < 0.1. See also Supplemental Figure 3.



**Figure 4. p62 is necessary for MDS/AML cell survival and cell proliferation**

(A) p62 knockdown was confirmed by immunoblotting lysates isolated from cells expressing a control or shp62 lentiviral vector. TRAF6 ubiquitination was measured following transduction with shCTL or shp62. (B) TF1 cells cotransduced with the indicated vectors were examined for p65 DNA binding. (C) HL60 cells transduced with shCTL or shp62 were engrafted into NSG mice (n=5-8/group) and monitored for chimerism at the indicated time points. (D) Survival curves for mice receiving shCTL- or shp62-transduced HL60 (top) or MDSL (bottom) cells. (E) AnnexinV staining of the indicated cells was

determined following transduction with shCTL or shp62. **(F)** AnnexinV staining of control CD34<sup>+</sup> and two del(5q) AML patient samples (PT-01 and PT-02) was determined following transduction with shCTL or shp62. As a positive control, treatment with an NF- $\kappa$ B inhibitor (BAY, BAY 11-7085) resulted in non-specific cell death. **(G)** Cell cycle analysis of CD34<sup>+</sup> and two patient samples (PT-01 and PT-02) transduced with shCTL or shp62 was determined by Hoechst 33342 incorporation. \*,  $P < 0.05$ . See also Supplemental Figure 4.



**Figure 5. p62 sustains NF-κB activation and leukemic cell function via its TRAF6-binding domain**

(A) Schematic of the p62 protein and its domains: PB1, ZZ-type zinc finger domain (ZZ), a TRAF6-binding (TB) domain, and LC3-interacting region (LIR), and a ubiquitin-associated domain (UBA). The amino acid sequence of the TB domain and the schematic of a WT and mutant TB constructs are shown below. (B–C) HL60 expressing MIG, MIG-p62, or MIG-p62 TB retroviral vectors were transduced with shCTL or shp62. p62 expression (B) and cell survival (C) was analyzed 4 days post transduction. (D) Expression of empty vector

(MSCV-pGK-GFP), TB<sup>Mut</sup>, or TB<sup>WT</sup> by transient transfection in HEK293 or by transduction in HL60 was confirmed by dot blot immunoassay. (E) HEK293 cells transfected with the indicated constructs were analyzed by co-immunoprecipitation and immunoblotting. (F) NF- $\kappa$ B activation was measured in HEK293 cells transfected with a  $\kappa$ B site-luciferase and the indicated vectors (n=3/group). Values represent  $\kappa$ B-site firefly over *Renilla* luciferase. (G) IKK $\alpha$ / $\beta$  phosphorylation was measured in HL60 cells transduced with TB<sup>Mut</sup> or TB<sup>WT</sup> by immunoblotting. (H) TRAF6 ubiquitination (TRAF6 IP; Ub IB) was measured in HL60 transduced with TB<sup>Mut</sup> and TB<sup>WT</sup>. (I) Cell cycle analysis of HL60 and CD34<sup>+</sup> cells transduced with TB<sup>Mut</sup> or TB<sup>WT</sup> was determined by Hoechst 33342 incorporation. (J) AnnexinV staining of HL60 and control CD34<sup>+</sup> cells was determined following transduction with TB<sup>Mut</sup> or TB<sup>WT</sup>. (K) HL60 and CD34<sup>+</sup> cells were transduced with TB<sup>Mut</sup> or TB<sup>WT</sup> and then plated in methylcellulose for progenitor colony formation (n=3/group). Colonies were scored 10–14 days after plating. (L) AnnexinV staining of control CD34<sup>+</sup> cells and MDS/AML patient samples (PT-01, PT-02, and PT-03) was determined following transduction with TB<sup>Mut</sup> or TB<sup>WT</sup>. (M) Model of an intrachromosomal network involving miR-146a/p62/TRAF6/NF- $\kappa$ B in HR del(5q) MDS/AML. miR-146a deletion results in derepression of TRAF6 protein. TRAF6 overexpression results in its autoubiquitination (green circles), which serves to recruit and then activate the NF- $\kappa$ B kinase complex (IKK $\alpha$ / $\beta$ ). NF- $\kappa$ B transcription factors induce p62/SQSTM1 expression from the remaining allele within del(5q). p62 is an important cofactor to sustain NF- $\kappa$ B activation through its TRAF6-binding domain (TB). The p62/TRAF6 signaling complex and subsequent NF- $\kappa$ B activation can be inhibited by expressing the TB motif. \*, P < 0.05.