HHS Public Access

Author manuscript

*Leukemia*. Author manuscript; available in PMC 2023 February 01.

Published in final edited form as:

*Leukemia*. 2022 August ; 36(8): 2032–2041. doi:10.1038/s41375-022-01632-y.

# Combinatorial genetics reveals the *Dock1-Rac2* axis as a potential target for the treatment of *NPM1;Cohesin* mutated AML

**Alison E. Meyer**[1], **Cary Stelloh**[1], **Kirthi Pulakanti**[1], **Robert Burns**[1], **Joseph B. Fisher**[2], **Katelyn E. Heimbruch**[1,3], **Sergey Tarima**[4], **Quinlan Furumo**[5], **John Brennan**[6], **Yongwei Zheng**[7], **Aaron D. Viny**[8], **George S. Vassiliou**[9], **Sridhar Rao**[1,3,10]

[1]Blood Research Institute, Versiti, Milwaukee, WI USA

[2]Current address: Department of Natural Sciences, Concordia University Wisconsin, Mequon, WI USA

[3]Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, WI USA

[4]Department of Biostatistics, Medical College of Wisconsin, Milwaukee, WI USA

[5]Current address: Department of Biology, Boston College, Chestnut Hill, MA USA

[6]Current address: Department of Pathology and Lab Medicine, University of North Carolina School of Medicine, Chapel Hill, NC USA

[7]Current address: Guangzhou Bio-gene Technology Co., Ltd. Guangzhou, China

[8]Department of Medicine, Division of Hematology and Oncology and Department of Genetics and Development, Columbia University Irving Medical Center, New York, NY USA

[9]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, United Kingdom

[10]Department of Pediatrics, Division of Hematology, Oncology, and Bone Marrow Transplantation, Medical College of Wisconsin, Milwaukee, WI USA

## Abstract

Acute myeloid leukemia (AML) is driven by mutations that occur in numerous combinations. A better understanding of how mutations interact with one another to cause disease is critical to developing targeted therapies. Approximately 50% of patients that harbor a common mutation in *NPM1* (*NPM1cA*) also have a mutation in the cohesin complex. As cohesin and Npm1 are known to regulate gene expression, we sought to determine how cohesin mutation alters the transcriptome in the context of *NPM1cA*. We utilized inducible $Npm1^{cAflox/+}$ and core

*Corresponding author: Sridhar Rao, sridhar.rao@versiti.org, Phone: (414) 937-3841.

AUTHOR CONTRIBUTIONS

Alison E. Meyer designed research studies, conducted experiments, acquired and analyzed data, and wrote the manuscript. Cary Stelloh conducted experiments and acquired data. Kirthi Pulakanti, Robert Burns, Quinlan Furumo, and John Brennen analyzed data. Sergey Tarima provided statistical advice and analyzed data. Joseph B. Fisher designed research studies, conducted experiments, acquired data, and analyzed data. Katelyn E. Heimbruch conducted experiments and acquired data. Yongwei Zheng conducted experiments. Aaron D. Viny and George S. Vassiliou created and provided mouse models for the experiments and provided comments and edits to the manuscript. Sridhar Rao designed research studies and aided in writing the manuscript.

Competing interests statement

The authors have nothing to disclose.

cohesin subunit *Smc3*$^{flox/+}$ mice to examine AML development. While *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ mice developed AML with a similar latency and penetrance as *Npm1*$^{cA/+}$ mice, RNA-seq suggests that the *Npm1*$^{cA/+}$; *Smc3*$^{\Delta/+}$ mutational combination uniquely alters the transcriptome. We found that the Rac1/2 nucleotide exchange factor *Dock1* was specifically upregulated in *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ HSPCs. Knockdown of *Dock1* resulted in decreased growth and adhesion and increased apoptosis only in *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ AML. Higher Rac activity was also observed in *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ vs. *Npm1*$^{cA/+}$ AMLs. Importantly, the Dock1/Rac pathway is targetable in *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ AMLs. Our results suggest that Dock1/Rac represents a potential target for the treatment of patients harboring *NPM1cA* and cohesin mutations and supports the use of combinatorial genetics to identify novel precision oncology targets.

## INTRODUCTION

Acute myeloid leukemia (AML) is a high-risk disease with an overall poor prognosis, even with aggressive chemotherapy treatment and allogeneic bone marrow transplantation (1). The low survival rates of AML are due, in part, to the genetic complexity of the disease. While 76 recurrently mutated genes are found in the majority of patients, most patients harbor 5–10 different mutations, making the spectrum of mutational combinations very large (2,3). Understanding how different mutational combinations work synegistically to promote AML is critical to the development of precision oncology approaches. Indeed, the careful study of different mutational combinations may identify targetable pathways, even if the genetic mutations themselves are not druggable. Four commonly mutated genes in AML encode members of the cohesin complex, which is comprised of STAG2, SMC3, SMC1A, and RAD21. Only one of the four cohesin subunits is somatically mutated in a patient, resulting in haploinsufficiency (2–4). The overall rate of mutation of each subunit in AML is STAG2 (3.2%), RAD21 (2.3%), SMC3 (1.9%), and SMC1A (1.7%) (2–4). Cohesin mutations result in haploinsufficiency of the entire complex, suggesting that a loss of complex activity contributes to AML development or progression (2–5). Decreased expression of cohesin genes in patients lacking cohesin mutations has also been observed, with reduced expression of one subunit often resulting in reduced expression of other subunits (5). Collectively, this demonstrates that loss of cohesin, either through loss-of-function mutations or decreased expression, is critical in AML pathogenesis.

The cohesin complex canonically functions during mitosis to promote proper sister chromatid segregation (6). Cohesin also cooperates with CTCF to organize the genome through chromatin looping into topologically associated domains (TADs), thereby separating the genome into distinct regions (1). Cohesin can also facilitate long-range interactions between enhancers and promoters within TADs, thereby regulating the expression of genes involved in HSPC maintenance and self-renewal such as *HOXA7/9* (7). Cohesin mutations alone are insufficient to cause AML, although they do result in increased HSPC self-renewal and impact gene expression (8–12). Because AMLs that harbor cohesin mutations are rarely aneuploid and cohesin haploinsufficency does not appear to induce mitotic defects, cohesin mutations likely promote AML by altering gene expression (2–5, 8–13).

Cohesin mutations are strongly associated with mutations in *NPM1*, the protein product of which is involved in several cellular processes including ribosome biogenesis, histone chaperoning, centrosome duplication, and the DNA damage response (2–5, 13). The most common *NPM1* mutation is an insertion which results in cytoplasmic accumulation (*NPM1cA*) (2, 3, 14–16). Similar to cohesin-mutated HSPCs, $Npm1^{cA/+}$ HSPCs exhibit increased self-renewal and elevated expression of *HOXA9* (17–20). Mice carrying the $Npm1^{cA/+}$ allele develop AML with a prolonged latency (~18 months) and incomplete penetrance (31%) (17). The addition of a second driver such as $NRas^{G12D}$ or $Flt3^{ITD}$, decreases latency and increases penetrance (21). Due to the co-occurrence of *Npm1cA* and cohesin mutations in AML patients, we examined how *Npm1cA* and cohesin haploinsufficiency cooperate to alter the transcriptome and AML development. Here, we combined two genetic models, an $Npm1^{cA/+}$ mouse model (17) and an $Smc3^{\Delta/+}$ mouse model (9) in an effort to identify a precision oncology target for the treatment of $Npm1cA;Cohesin^{mut}$ AML.

## MATERIALS AND METHODS

Detailed methods are provided in the supplemental material.

## RESULTS

### Cohesin haploinsufficiency enhances Npm1$^{cA/+}$ HSPC self-renewal

$Npm1^{cA/+}$ and cohesin$^{\Delta/+}$ HSPCs individually show increased self-renewal (8–12, 17, 19, 22), suggesting that their combination may enhance this phenotype. We compared the self-renewal of HSPCs isolated from wild type (WT), $Npm1^{cA/+}$ (N), $Smc3^{\Delta/+}$ (S), and $Npm1^{cA/+};Smc3^{\Delta/+}$ (NS) mice four weeks post knock-in (*Npm1cA* allele) or gene excision ($Smc3^{fl}$ allele) by PIpC treatment (Supplemental Figure 1). Consistent with previous reports, S and N cells showed enhanced self-renewal compared to WT cells (Figure 1A). At tertiary and quaternary passages, NS cells showed enhanced self-renewal over each single mutation (Figure 1A). We next tested whether the two mutations cooperated *in vivo*, following mice for 18 months post PIpC treatment. Excess death was not observed in either WT or S mice (Figure 1B)(9). By contrast, 25/40 (63%) of N and 44/58 (76%) of NS mice died of AML (Figure 1B,C and Supplemental Table 1), although a detailed necropsy was not able to be performed on all animals. While we observed a trend toward decreased latency (61 weeks for NS vs. 66 for N) and increased penetrance (76% for NS vs. for 63% N), statistical significance was not reached (p=0.14 for latency and 0.18 for penetrance). 3/3 NS AMLs were transplantable with significantly reduced latency (average of 11 weeks, Figure 1D). This was not statistically different from that reported for $Npm1^{cA/+}$ mice (average of 13 weeks) (17). Immunophenotyping revealed that AMLs from N or NS mice had a similar profile (17), indicating they were both AML with maturation based upon CD4-,Cd8a-,B220-,c-Kit+/Gr1+/Cd11b+ (Supplemental Figure 2A and Supplemental Table 2). In addition, NS mice with AML had enlarged livers and spleens in comparison with WT animals, which were positive for myeloperoxidase, similar to that reported for N leukemic mice (17)(Supplemental Figure 2B,C). Our data indicate that $Npm1^{cA}$ and

*Smc3*$^{\Delta/+}$ synergize to promote abnormal self-renewal *in vitro* without altering the latency or penetrance of AML *in vivo*.

## Smc3 haploinsufficiency influences the spectrum of acquired mutations in Npm1$^{cA/+}$ AML

Given that the addition of *NRas*$^{G12D}$ or *Flt3*$^{ITD}$ affected the mutations seen in *Npm1*$^{cA}$ animals (17, 21), we wondered if the AMLs that developed in N vs. NS animals had distinct mutational spectrums. To address this, we performed whole exome sequencing on 7 primary and 4 secondary NS leukemias and reanalyzed previously published data for 12 N leukemias (21)(Supplemental Tables 3 and 4). While the number of mutations was not dissimilar (Figure 2A), the spectrum was distinct (Figure 2B, C), with many of the genes mutated being annotated in AML patients (Supplemental Table 5). Importantly, 3/4 secondary leukemias (all from the same primary AML source) gained a Flt3 missense mutation, which occurred in the activation loop at Ser844, consistent with activated signaling contributing to mutational synergy. To determine if the number of acquired mutations in our NS animals correlated with time to disease, we examined the latency of the 7 primary NS AML samples on which exome sequencing was performed. 4/7 AMLs had a latency under 200 days, with 3/7 having a latency over 200 days. No correlation was observed between the two groups regarding latency or the number of acquired mutations (Supplemental Table 6). We also performed gene ontology (GO) analysis on the genes mutated in the short and long latency groups. While our gene lists are small, we did find 4 genes associated with cell differentiation and 3 genes associated with cell adhesion in the shorter latency group (Supplemental Table 6), suggesting that disruption of these pathways may contribute to shortened latency. We conclude that the addition of *Smc3*$^{\Delta/+}$ to *Npm1*$^{cA/+}$ results in a different mutational profile than that observed for *Npm1*$^{cA/+}$ alone.

## Smc3 haploinsufficiency alters the transcriptional profile of Npm1$^{cA/+}$ HSPCs

Because cohesin regulates gene expression, we hypothesized that *Smc3* haploinsufficiency might alter the transcriptome in the presence of *Npm1*$^{cA/+}$. However, given the known influence of Npm1cA on nuclear vs. cytoplasmic localization, we first confirmed that the NS combination did not alter the exclusive nuclear localization of the Smc3 protein (Supplemental Figure 3A) or affect Smc3 protein levels (Supplemental Figure 3B,C). Additionally, our RNA sequencing data showed the expected reduction in read density at exon 4 in S and NS HSPCs only, which is the region targeted by Cre-excision (Supplemental Figure 3D). This indicates that *Npm1*$^{cA}$ does not alter *Smc3* mRNA expression, protein levels, or nuclear localization.

To understand how the combination of *Smc3*$^{\Delta/+}$ and *Npm1*$^{cA}$ affects gene expression, we performed RNA-seq on HSPCs isolated from each genotype (WT, N, S, and NS) four weeks post Cre induction (Supplemental Tables 7, 8, 9). We chose this time point to determine the influence of the two alleles on the transcriptome prior to leukemia development or the acquisition of additional somatic mutations. Differential expression (DE) analysis comparing S vs. WT HSPCs identified 50 genes (fold cutoff > 2, adj p val < 0.05), with the majority (42) being upregulated (Figure 3A, left and Supplemental Tables 7 and 10). Surprisingly, these same genes were largely downregulated in N vs. WT HSPCs (Figure 3A, middle). When comparing NS vs. WT HSPCs, the general pattern of upregulation was maintained

(Figure 3A, right), suggesting that *Smc3* haploinsufficiency dominantly affects the pattern of gene transcription. Comparing N vs. WT HSPCs, we found 75 genes were differentially expressed in the N genotype, the majority of which (42) were downregulated (Figure 3B, left and Supplemental Tables 7 and 10). Most of the 75 genes showed a relative increase in expression in S HSPCs (Figure 3B, middle). Generally, the addition of $Smc3^{\Delta/+}$ to $Npm1^{cA/+}$ dampened or reversed the changes in gene expression observed with $Npm1^{cA/+}$ alone (Figure 3B, right). Notable exceptions were genes in the *Hoxa* cluster, which remained highly upregulated in the NS HSPCs (Figure 3B). This is consistent with previous reports linking cohesin haploinsufficiency and *Npm1cA* with *Hoxa* cluster expression and self-renewal (Figure 1A) (8–10, 17, 20, 21, 23). We conclude that the addition of $Smc3^{\Delta/+}$ to $Npm1^{cA/+}$ alters the transcriptional landscape to favor the upregulation of a subset of genes which are normally repressed by $Npm1^{cA/+}$. Surprisingly, although both $Npm1^{cA/+}$ and $Smc3^{\Delta/+}$ increase *Hoxa* expression, we found little overlap in the differentially expressed genes between any of the genotypes as compared to WT (Figure 3C). The total number of differentially expressed genes was increased in NS vs. either N or S HSPCs (Supplemental Table 7), the majority of which were unique to the NS genotype. Thus, the combination of $Smc3^{\Delta/+}$ and $Npm1^{cA/+}$ results in transcriptome changes which are not simply additive between the two mutations.

We next directly compared NS vs. S and NS vs. N by DE analysis. While only 11 genes were differentially expressed in the NS vs. S genotype, 576 genes were differentially expressed in NS vs. N HSPCs, with the majority being upregulated. Consistent with our results in Figure 3A and B, the overall pattern of gene upregulation in NS vs. N was maintained in S vs. N (Supplemental Figure 4A), highlighting the contribution of $Smc3^{\Delta/+}$ to gene upregulation. Gene ontology analysis of the NS vs. N DE genes showed an enrichment in processes such as actin-based cell projections, cortical cytoskeleton, filopodium, and actin filament bundles, suggesting that changes in the actin cytoskeleton occur specifically in NS HSPCs (Figure 3D). Gene Set Enrichment Analysis (GSEA) yielded a similar result (Supplemental Figure 5A, B, and C), and was consistent with some of the somatic mutations we identified by exome sequencing (Supplemental Table 6). We also performed GSEA on NS vs. S, the results of which are shown in Supplemental Figure 4B–F. We found fewer genesets of an overall lower significance enriched in NS vs. S compared to NS vs. N. We conclude that the combination of $Smc3^{\Delta/+}$ and $Npm1^{cA/+}$ in HSPCs results in the altered expression of a unique set of genes involved in actin cytoskeletal regulation that is not observed with either single mutation.

## Dock1 regulates $Npm1^{cA/+}$;$Smc3^{\Delta/+}$ AML biology

Given our exome and transcriptome results, we examined the DE genes in NS vs. N HSPCs for actin regulators linked to AML. We found that *Dock1* and *Elmo1* were upregulated in the NS HSPCs (Supplemental Table 7). Dock1 partners with Elmo1 to act as a bipartite guanine nucleotide exchange factor (GEF) for Rac1/2 (24, 25). Rac1/2 signaling is an essential regulator of the actin cytoskeleton, with roles in proliferation, apoptosis, differentiation, adhesion and homing/migration of HSPCs (26). *DOCK1, ELMO1*, and *RAC1/2* are elevated in AML (27–30), and high expression of *DOCK1* is associated with decreased survival and *NPM1* mutations (30, 31). Given this, we evaluated DOCK1/RAC as a potential targetable

pathway in *NPM1cA;Cohesin*$^{mut}$ AML. We focused on Dock1 rather than Elmo1 as Dock1 contains the domains responsible for GEF activity (24, 25). First, we verified by qPCR that *Dock1* was upregulated in multiple AML samples from NS, but not N, mutant mice (Supplemental Figure 6A). By contrast, we observed decreased expression of related family members *Dock2* or *Dock5* in NS vs. N AMLs (Supplemental Figure 6B–C). While we observed upregulation of *Dock4* in our NS HSPCs by RNA sequencing, no difference in expression of *Dock4* was observed in our leukemic cells, and expression of *Dock4* is quite low (Supplemental Figure 6D and Supplemental Table 8), consistent with limited expression in AML (30). Thus, *Dock1* expression is specifically increased in NS vs. N HSPCs and AML blasts.

As Dock1 regulates apoptosis in normal and cancerous cells (32–37), we examined the effect of *Dock1* depletion by RNAi on the growth and apoptosis of leukemic cells isolated from NS vs. N mice. We used two independent lentiviral shRNA constructs toward *Dock1* which resulted in ≈ 47 and 37% mRNA reduction (Supplemental Figure 6E). Compared to empty vector (EV), both shRNAs significantly reduced the growth of NS leukemic cells (Figure 4A). Similar effects were observed with two other shRNAs, minimizing the likelihood of off-target effects (Supplemental Figure 6F). By contrast, *Dock1* knockdown resulted in increased growth in N cells (Figure 4A). *Dock1* knockdown led to a significant increase in early apoptosis (Figure 4B, Supplemental Figure 7A,B), with minimal changes in cell cycle (Supplemental Figure 7C). These results suggest that *Dock1* may be a viable and specific target for the treatment of *NPM1cA;Cohesin*$^{mut}$ AMLs.

We next sought to confirm these results using human OCI-AML3 cells which harbor an *NPM1*$^{cA}$ mutation but are cohesin wild type. We first used CRISPR/Cas9 to target *SMC3* and successfully isolated a clone that exhibited a 50% loss of SMC3 protein compared to a non-targeting control (NTC) line (Supplemental Figure 8A). Next, we targeted *DOCK1* by CRISPR, resulting in an approximate 50% loss of *DOCK1* mRNA (Supplemental Figure 8B). We were unable to locate an antibody to *DOCK1* which could be used to measure endogenous protein levels. Having established these lines, we compared the growth of cells with or without *SMC3* and/or *DOCK1* gene editing. We found that the OCI-AML3 NTC line did not differ in growth compared to the OCI-AML *SMC3* edited line (Supplemental Figure 8C), consistent with our mouse model survival and *in vitro* data. By contrast, *DOCK1* targeted cells exhibited reduced growth only in combination with *SMC3*$^{+/-}$ (Figure 4C). We also observed increased apoptosis only upon *DOCK1* editing of the *SMC3*$^{+/-}$ line (Figure 4D, Supplemental Figure 8D,E). These results confirm that *DOCK1* knockdown is effective at reducing cell growth through enhanced apoptosis in human and mouse *NPM1cA;SMC3* haploinsufficient AMLs. We conclude that *Dock1* expression is increased by cohesin haploinsufficiency in the *Npm1*$^{cA/+}$ background and that loss of *DOCK1* inhibits the growth of leukemias harboring both *NPM1cA* and *SMC3* mutations.

## Dock1 functions through Rac2 to regulate apoptosis in Npm1$^{cA/+}$;Smc3$^{\Delta/+}$ AML cells

Increased Rac activity results in enhanced proliferation and survival in many blood cancers (38, 39). Consistent with the known role of Dock1 as a Rac GEF, we observed higher Rac activity in NS vs. N leukemias (Figure 5A). Furthermore, *Dock1* knockdown reduced

Rac activity (Figure 5B) without affecting Rac1/2 protein levels (Supplemental Figure 9). Collectively, these data indicate that Dock1 is a direct modulator of Rac activity in NS leukemic cells. Mice and humans predominantly express Rac1 and 2 in their hematopoietic system (26), as is seen in our RNA-seq data (Supplemental Figure 10A). While both Rac1 and Rac2 regulate HSPC growth, Rac1 predominantly influences cell cycling while Rac2 regulates apoptosis (26). To determine whether Rac1 or Rac2 controlled apoptosis in NS leukemic cells, we tested if conditional expression of dominant negative (DN) Rac1 or Rac2 affected NS cell growth or apoptosis. A more significant reduction in growth was observed with DN Rac2 vs. Rac1 in NS cells (Figure 5C). The effect of DN Rac1 is likely due to effects on cell cycling (Supplemental Figure 10B). However, only DN Rac2 resulted in increased early apoptosis, consistent with the effects of *Dock1* knockdown (Figure 5D, Supplemental Figure 10C,D). Rac2 also plays a prominent role in HSPC adhesion to fibronectin (26). We observed that *Dock1* knockdown resulted in decreased adhesion (Figure 5E). Collectively, our results suggest that Dock1 primarily regulates Rac2 to control apoptosis and cell adhesion in NS leukemic cells and that this pathway represents a viable target for the treatment of *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ AML.

## The Dock1/Rac2 pathway is targetable in Npm1$^{cA/+}$;Smc3$^{\Delta/+}$ AML

To determine if Dock1 is pharmacologically targetable in NS leukemias we utilized a commercially available Dock inhibitor, CPYPP, which inhibits Dock1, 2, and 5 (40). We treated N and NS AML cells with a range of CPYPP doses. To test for specificity, we examined the effect of CPYPP on a murine MLL-AF9 cell line, which lacks N and S mutations but does express high levels of *Hoxa9*. We found that CPYPP dose-dependently reduced the growth of NS leukemic cells and was more effective at lower doses in NS vs. N cells (Figure 6A). By contrast, CPYPP increased the growth of MLL-AF9 cells at the highest dose (Figure 6A). Consistent with our *Dock1* knockdown results, CPYPP resulted in increased early apoptosis in NS leukemic cells (Figure 6B, Supplemental Figure 11A,B). We also tested the pan-Rac inhibitor EHT 1864, which blocks Rac1/2 activity by inhibiting guanine nucleotide binding (41). Similar to CPYPP, EHT 1864 had a dose-dependent effect on the growth of NS leukemic cells (Figure 6C) and enhanced apoptosis, although stronger effects on late (not early) apoptosis were observed (Figure 6D, Supplemental Figure 11C,D). Although the development of isoform-specific inhibitors would aid in specificity, our data suggest that the Dock1/Rac2 pathway is pharmacologically targetable.

We next sought to determine if *Dock1* knockdown would prolong the latency of NS AML using an *in vivo* transplant model. NS AML blasts were freshly isolated and infected with lentivirus expressing EV, *shDock1#1*, or *shDock1#2*. Following selection, cells were transplanted alongside competitor cells into lethally irradiated recipients. Compared to the EV control animals, animals receiving *shDock1* AML cells exhibited significantly prolonged latency, with 4/6 animals bearing no evidence of disease 100 days post-transplant (Figure 6E). In comparison, all animals receiving EV control blasts succumbed to disease by 59 days, confirming that targeting *Dock1* is effective against *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ AML. Collectively, these data suggest that the Dock1-Rac2 pathway is targetable in *Npm1cA;Cohesin*$^{mut}$ leukemias, and that the development of isoform-specific Dock1 or

Rac2 small molecule inhibitors may have enhanced specificity in AML patients harboring these two genetic alterations.

## DISCUSSION

Due to advances in genomic sequencing, the genetic mutations that occur in AML have been carefully detailed (2, 3). Current precision oncology approaches focus on a single lesion, which is effective for drivers like activated *FLT3*. However, AML is a complex disease, and multiple mutations exist in a variety of combinations. Combinatorial genetic models are thus necessary to determine how different mutations interact to promote AML and to uncover potential therapeutic targets. Our results show that *Npm1cA* and cohesin haploinsufficiency combine to uniquely alter the transcriptome and the genetic evolution of AML without altering disease penetrance or latency. Importantly, key molecular differences exist between N and NS leukemias. Here, we have identified the DOCK1-RAC2 axis as a potential specific therapeutic target in *NPM1*$^{cA}$*;Cohesin*$^{mut}$ AML.

We initially hypothesized that cohesin haploinsufficiency and *Npm1*$^{cA/+}$ would cooperate to drive higher levels of *Hoxa* expression. While we do see increased *Hoxa* cluster expression in HSPCs harboring both mutations, expression is not higher in NS vs. N only HSPCs (Supplemental Table 8). This indicates that factors beyond elevated *Hoxa* expression drive the synergistic increase in *in vitro* self-renewal that we observed in NS HSPCs (Figure 1A). Additionally, neither *Hoxa* expression nor enhanced self-renewal was sufficient to alter AML latency or penetrance. These data suggest that factors other than *Hoxa* cluster expression and self-renewal influence *NPM1cA;Cohesin*$^{mut}$ AML evolution and biology. Consistent with this, we observed little overlap between genes differentially expressed in S and N HSPCs, with most of the differentially expressed genes being unique to the NS genotype (Figure 3C). Furthermore, we observed a unique mutational profile in NS AML samples (Figure 2C, Supplemental Table 4). Importantly, our data reveal that *Smc3*$^{\Delta/+}$ alters the transcriptional landscape differentially in WT versus *Npm1*$^{cA/+}$ HSPCs, suggesting that cohesin loss may have unique transcriptional effects depending upon the “context” of the co-occurring driver mutation. It will thus be important to determine how cohesin loss impacts the transcriptome in the presence of other driver mutations, such as AML1-ETO where cohesin mutations are common (42).

How might *Npm1cA* and *Smc3* haploinsufficiency result in the deregulation of a unique set of genes? Interestingly, both NPM1 and cohesin interact with the PRC2 complex, a key epigenetic regulator (1, 43). Knockdown of *NPM1* and cohesin have each been shown to decrease levels of the PRC2 repressive mark, H3K27me3 (1, 43). While PRC2 loss has been linked to *HOXA* gene upregulation (1), given the effects of *Npm1*$^{cA/+}$*;Smc3*$^{\Delta/+}$ on gene expression observed in this study, it would be particularly interesting to determine if cohesin haploinsufficiency and *Npm1cA* cooperatively alter H3K27me3 levels or PRC2 recruitment to a unique set of genes. Given the known antagonism between the repressive PRC2 and activating DOT1L, which deposits H3K79me2, it is not surprising that both *NPM1*$^{cA}$ and cohesin mutations have been identified as potential targets of DOT1L inhibitors (7, 23). Thus, cells harboring both NPM1cA and cohesin mutations may be exquisitely sensitive to DOT1L inhibition.

Although many genes were uniquely deregulated in NS HSPCs, we focused our analysis on the Dock1/Rac pathway as increased expression of these factors have been independently associated with AML in several patient cohorts (27–31) and because deregulation of actin-associated pathways was common in our exome and RNA sequencing analyses. While we do not know the exact mechanism by which *Dock1* expression becomes elevated in NS HSPCs and AMLs, inhibition of *Dock1* through shRNA or CPYPP treatment results in decreased growth and increased apoptosis of NS AMLs compared to N only AMLs (Figures 4 and 6). Although the effects of CPYPP were consistent with our targeted shRNA-based approach, CPYPP also inhibits Dock1 related family members Dock2 and Dock5 (40, 44, 45). Thus, the use of a pan-inhibitor may have unintended consequences on healthy cells. Indeed, the lack of specificity of CPYPP is highlighted by its moderate effects on the growth of N only leukemic cells (Figure 6A), while shRNA-mediated knockdown of *Dock1* increased the growth of this same leukemia (Figure 4A). We did attempt to treat NS leukemic mice with CPYPP *in vivo* but observed high toxicity related to the solubility of CPYPP in DMSO. It should also be noted CPYPP is metabolized very quickly (40), making it a poor candidate for *in vivo* use. Careful medicinal chemistry approaches will be needed to modify CPYPP to make it potentially useful in preclinical testing.

It is important to note that the pan-Rac inhibitor used here, EHT 1864, had effects on the proliferation of both N and NS cells (Figure 6C and data not shown). Thus, while NS leukemic cells do have a higher level of Rac activity vs. N cells, our isoform-targeted experiments show that genotype specificity occurs at the level of Dock1. While we suggest that inhibition at the Dock1 level may be the most beneficial for targeted treatment of *NPM1cA*;*Cohesin*$^{mut}$ AML, Rac inhibition may also be useful in the treatment of all *NPM1* mutant AML. However, as EHT 1864 is a pan-Rac inhibitor, and Rac1 and 2 have multiple, often opposing effects on hematopoietic cells *in vivo* (26), the development of a Rac2-specific inhibitor may be critical to minimize potential toxicities. Further, the pharmacokinetic properties of EHT 1864 have not been clearly defined in a preclinical animal model. As a next step in preclinical testing, the delivery route and dosing schedule, as well as any genotype-specific effects of EHT 1864 should be rigorously tested in an AML model.

In conclusion, the addition of cohesin haploinsufficiency to the *Npm1*$^{cA/+}$ background has profound effects on gene expression that influence HSPC and AML cell biologies. Although no difference in latency was observable between *Npm1*$^{cA/+}$ and *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$ animals, our studies show clear value in the use of combinatorial genetics to uncover novel, specific targets that may result in tailored therapies. Our data also argue that common co-occurring AML mutations should be studied in detail, as the effect of each mutation may not simply be additive and, in fact, may alter the fundamental biology of the disease. These results underline the usefulness of combinatorial genetics not just for the identification of novel therapeutic targets, but also for a deeper understanding of how different mutations may influence disease presentation or progression in a particular patient.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

The authors would like to acknowledge Scott Armstrong and Michael Kühn for providing the OCI-AML3 Cas9 cell line and Benedetta Bonacci for aid with flow cytometry experiments. This work was funded by: NCI R01 CA204231 and the Midwest Athletes against Childhood Cancer to S. Rao. AEM is supported by a generous gift from Ms. Nan Gardetto.

### Funding

This work was funded by: NCI R01 CA204231 and the Midwest Athletes against Childhood Cancer to S. Rao. AEM is supported by a generous gift from Ms. Nan Gardetto.

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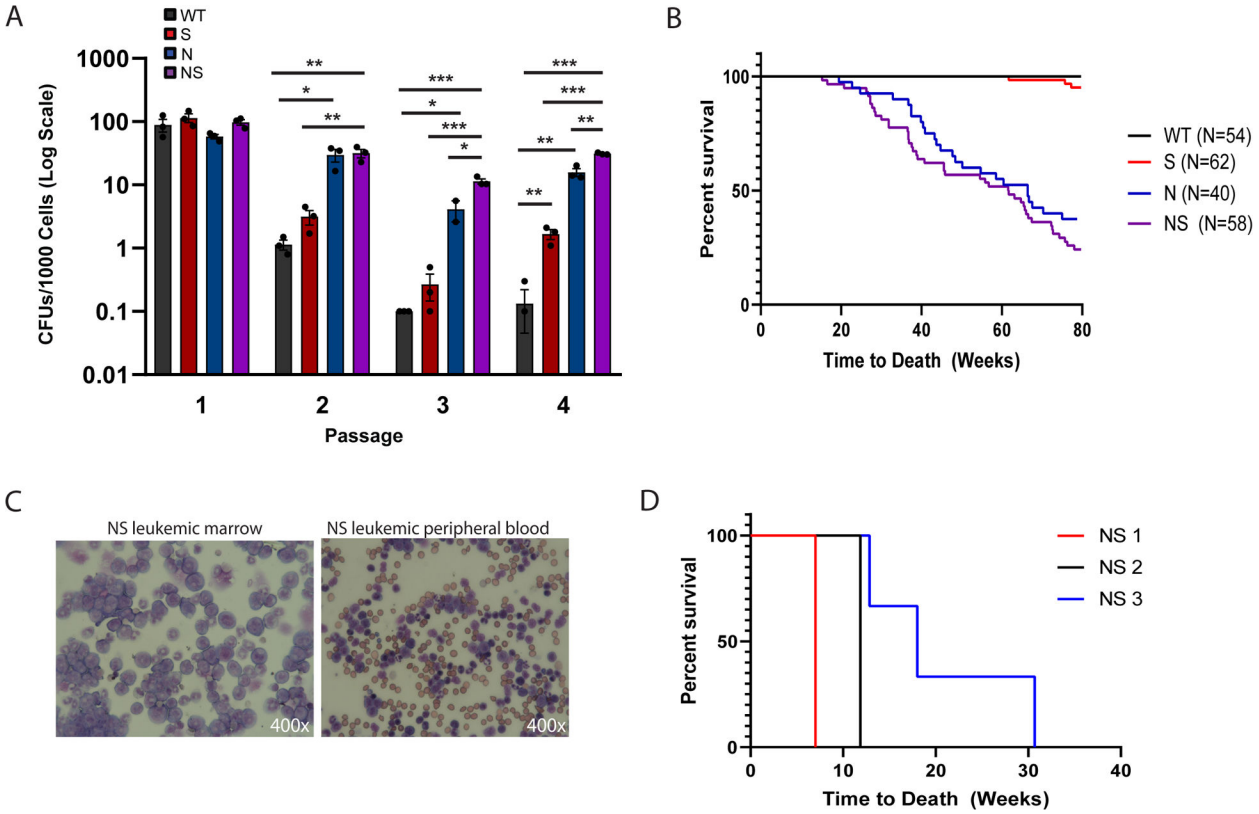


**Figure 1. Cohesin haploinsufficiency enhances Npm1$^{cA/+}$ HSPC self-renewal.**

**A.** HSPCs were isolated from 3 mice of each genotype (WT: wildtype, S: *Smc3*$^{\Delta/+}$, N: *Npm1*$^{cA/+}$, NS: *Npm1*$^{cA/+}$;*Smc3*$^{\Delta/+}$). 2,000 cells were plated in triplicate in methylcellulose. Colonies were counted weekly and passaged. Results are graphed as the number of CFUs (colony forming units)/1000 cells plated. Data represent the mean ± SEM. Statistical significance was determined using Student's t-test (two-tailed, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **B.** Survival curve showing decreased survival of N and NS animals compared to WT and S animals. **C.** Example of leukemic bone marrow (left) and peripheral blood (right) from an NS animal. Images are 400x. **D.** Survival curve showing decreased latency with secondary leukemic transplants. 500,000 AML blasts were isolated from 3 moribund NS animals and transplanted into sublethally irradiated recipients (3 recipients per AML source).

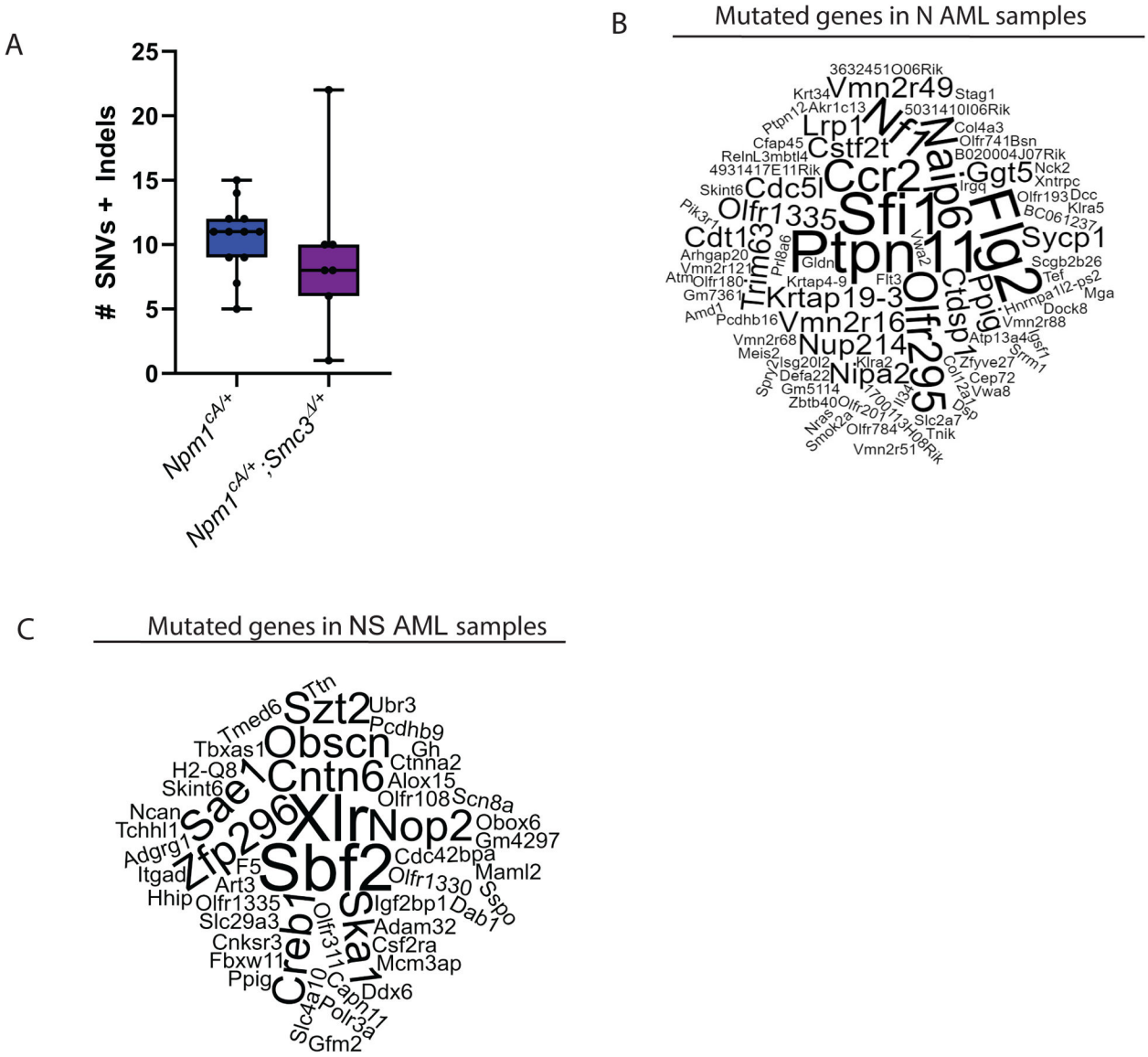


**Figure 2. Smc3 haploinsufficiency influences the spectrum of acquired mutations in Npm1$^{cA/+}$ AML.**

**A.** The average number of SNVs+indels observed by exome sequencing. Results for NS primary AMLs were compared with previously published results for N (21). N: 10.6 ± 0.8, NS: 8.6 ± 1.6 (mean ± SEM). Statistical significance was determined using the Mann-Whitney test, two-tailed. Total number of AML samples sequenced: N: 12, NS: 7. **B** and **C.** Word clouds representing the mutations found in N (B) and NS primary AMLs (C). Text size corresponds to the number of times a mutation in the gene was detected.

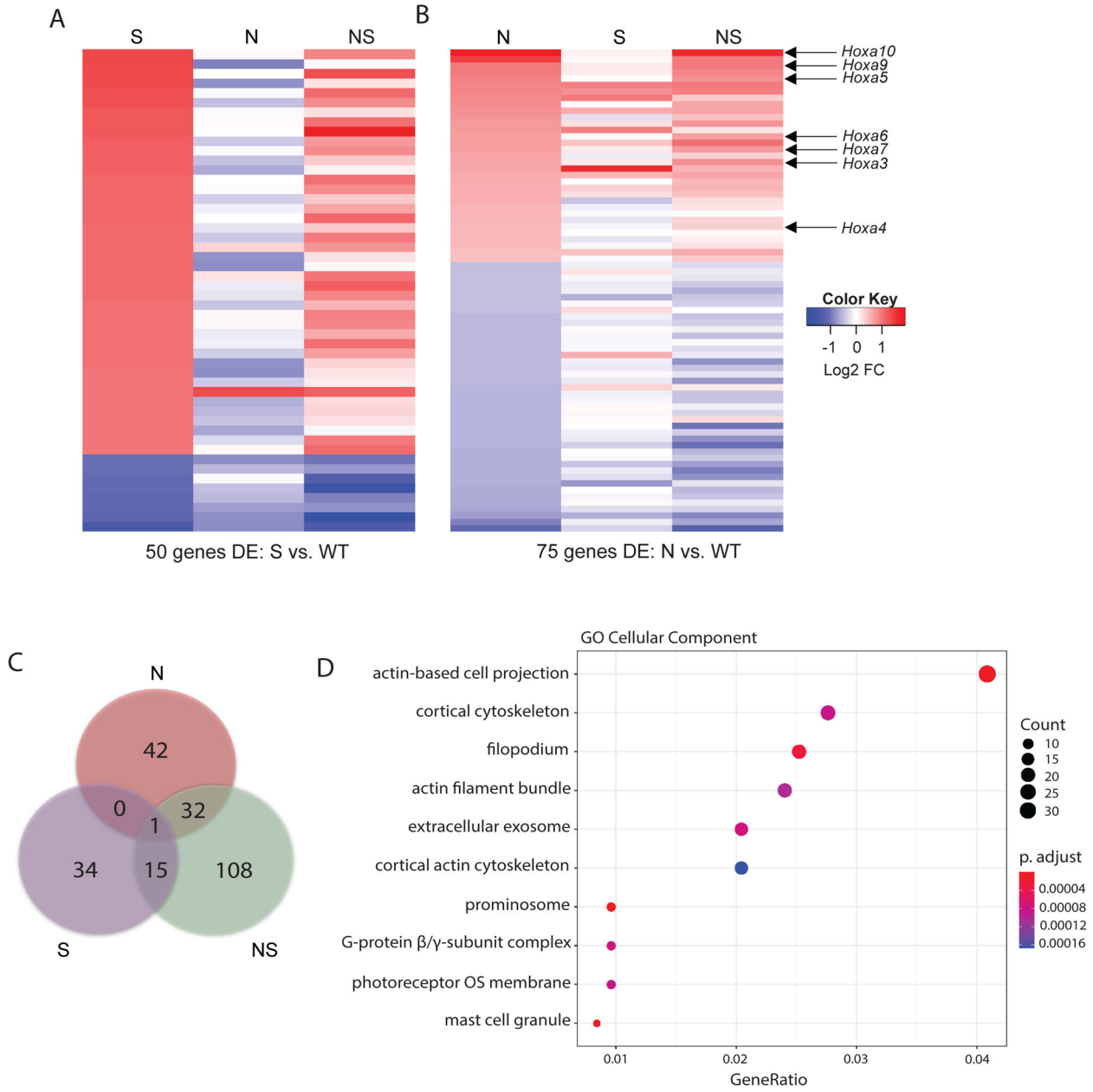


**Figure 3. Smc3 haploinsufficiency alters the transcriptional profile of Npm1$^{cA/+}$ HSPCs.**

**A.** Heatmap showing the 50 significantly differentially expressed genes (2-fold change, p-val adj<0.05) in HSPCs from S animals as compared to WT (left-most column). The middle and right columns show the expression of these same genes in N and NS HSPCs, respectively. **B.** Heatmap showing the 75 significantly differentially expressed genes in HSPCs from N animals as compared to WT (left-most column). The middle and right columns show the expression of these same genes in S and NS HSPCs, respectively. **C.** Venn diagram showing the overlap of differentially expressed genes in each genotype as compared to WT. **D.** GO analysis was performed on the differentially expressed genes in NS vs. N HSPCs, with a focus on cell component. OS=outer segment. Data from 3 animals of each genotype were analyzed for all parts of this figure.

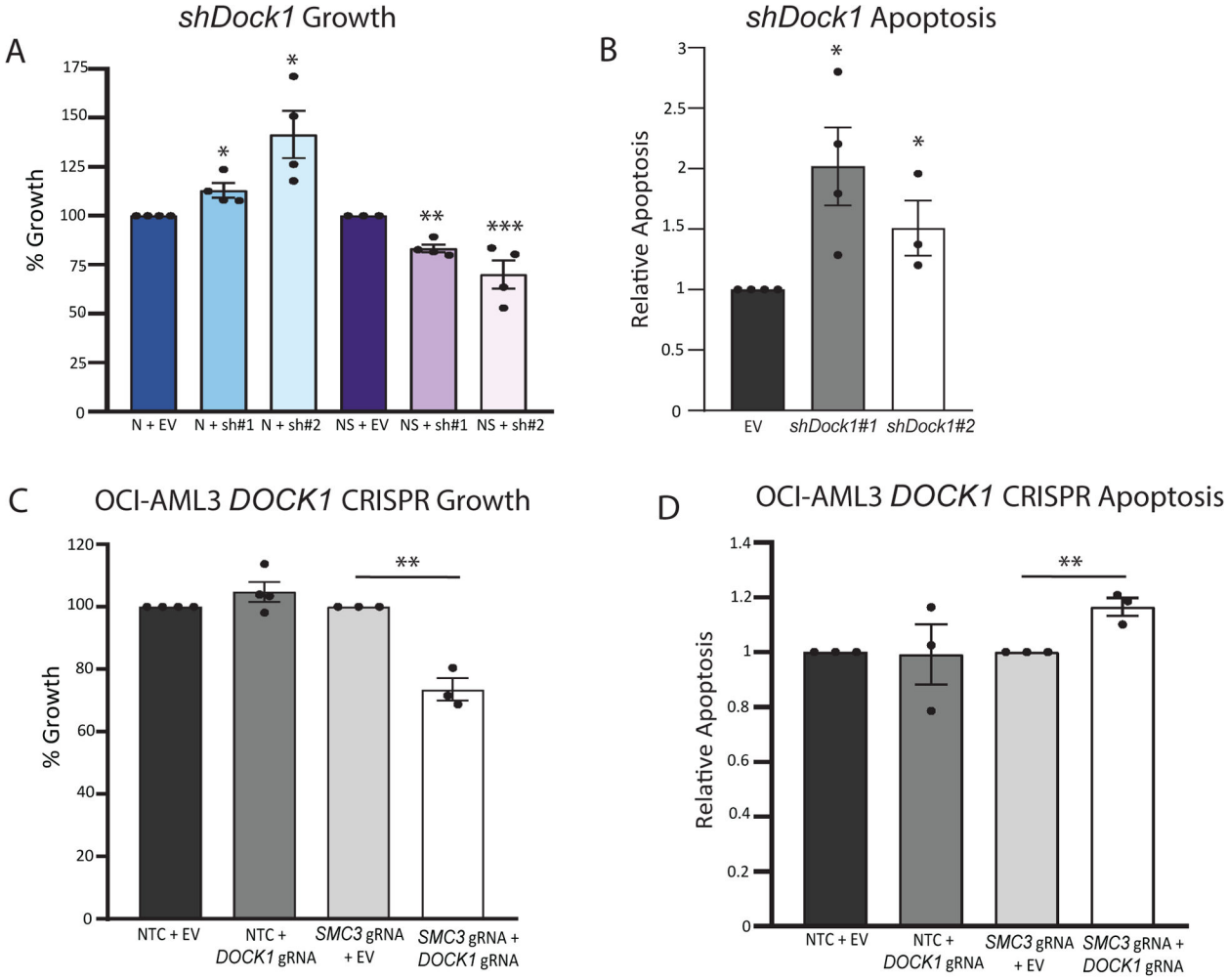


**Figure 4. Dock1 regulates Npm1$^{cA/+}$;Smc3$^{\Delta/+}$ AML biology.**

**A.** N or NS leukemic cells were infected with EV, *shDock1* construct #1 or #2. Following selection, 100,000 N or NS cells were plated and allowed to expand for 72 hours. Live cells were counted and the % growth relative to EV was graphed for each condition. **B.** NS leukemic cells from A were stained with Annexin V and PI and analyzed by flow cytometry. Cells positive for Annexin V only are shown and were considered early apoptotic. Apoptosis relative to EV was graphed. **C.** OCI-AML3 cells were modified using CRISPR-Cas9. As in A, the growth of cells with non-targeting control and EV was compared to that of cells edited for *DOCK1* only (NTC+*DOCK1* gRNA), *SMC3* only (*SMC3* gRNA+EV), and for both (*SMC3* gRNA+*DOCK1* gRNA). **D.** OCI-AML3 cells from C were analyzed for apoptosis by Annexin V staining. Note we were unable to use PI due to the cells being dual positive for GFP and RFP. Apoptosis relative to EV was graphed. A minimum of 3 replicates were performed for all assays in this figure. Data represent the mean ± SEM. Statistical significance was determined using Student's t-test (two-tailed, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

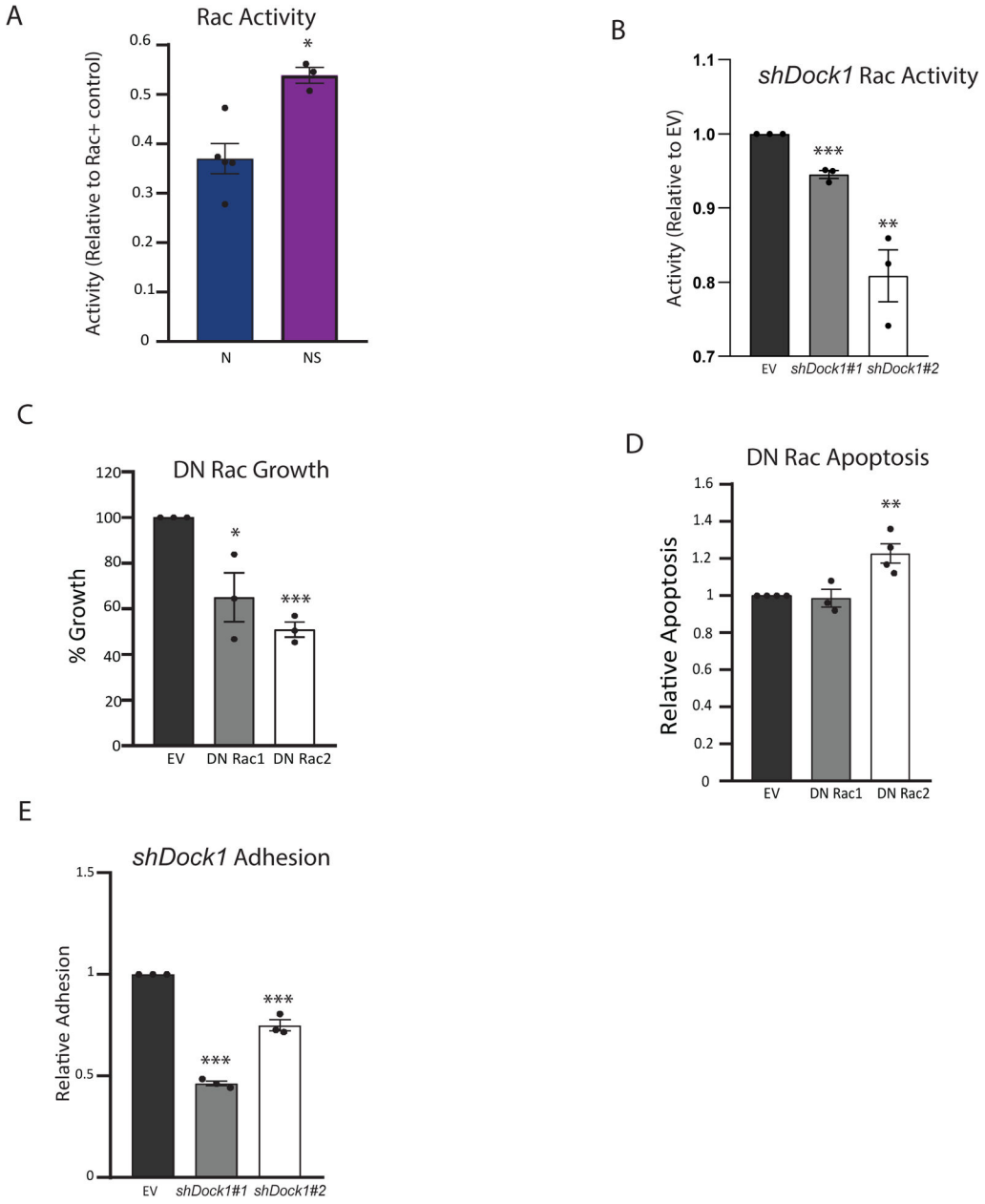


**Figure 5. Dock1 functions through Rac2 to regulate apoptosis in Npm1$^{cA/+}$;Smc3$^{\Delta/+}$ AML cells.**

**A.** Rac activity in N and NS leukemic cells were compared to the positive control included in the assay kit (see methods for details). **B.** Rac activity was examined as in A for NS cells expressing EV, *shDock1 #1*, and *shDock1#2*. **C.** NS leukemic cells were infected with cumate-inducible dominant negative (DN) Rac1, Rac2, or EV lentiviral constructs. Following selection, 100,000 cells were plated, and DN Rac1/2 were induced with 50 μg/mL cumate for 72 hours. 96 hours after plating, live cells were counted and results were graphed compared to the EV control. **D.** Apoptosis was measured 96 hours after plating with Annexin V/PI staining by flow cytometry for EV, DN Rac1, and DN Rac2 cells. Results were graphed relative to the EV control. Cells positive for Annexin V only are shown and were considered early apoptotic. **E.** NS leukemic cells were infected with EV, *shDock1*

construct #1 or #2. Following selection, 50,000 cells were plated on fibronectin-coated dishes and allowed to adhere for 18 hours. Adherent cells were trypsinized and counted by flow cytometry. Adhesion relative to EV was graphed. A minimum of 3 replicates were performed for all assays in this figure. Data represent the mean ± SEM. Statistical significance was determined using Student's t-test (two-tailed, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

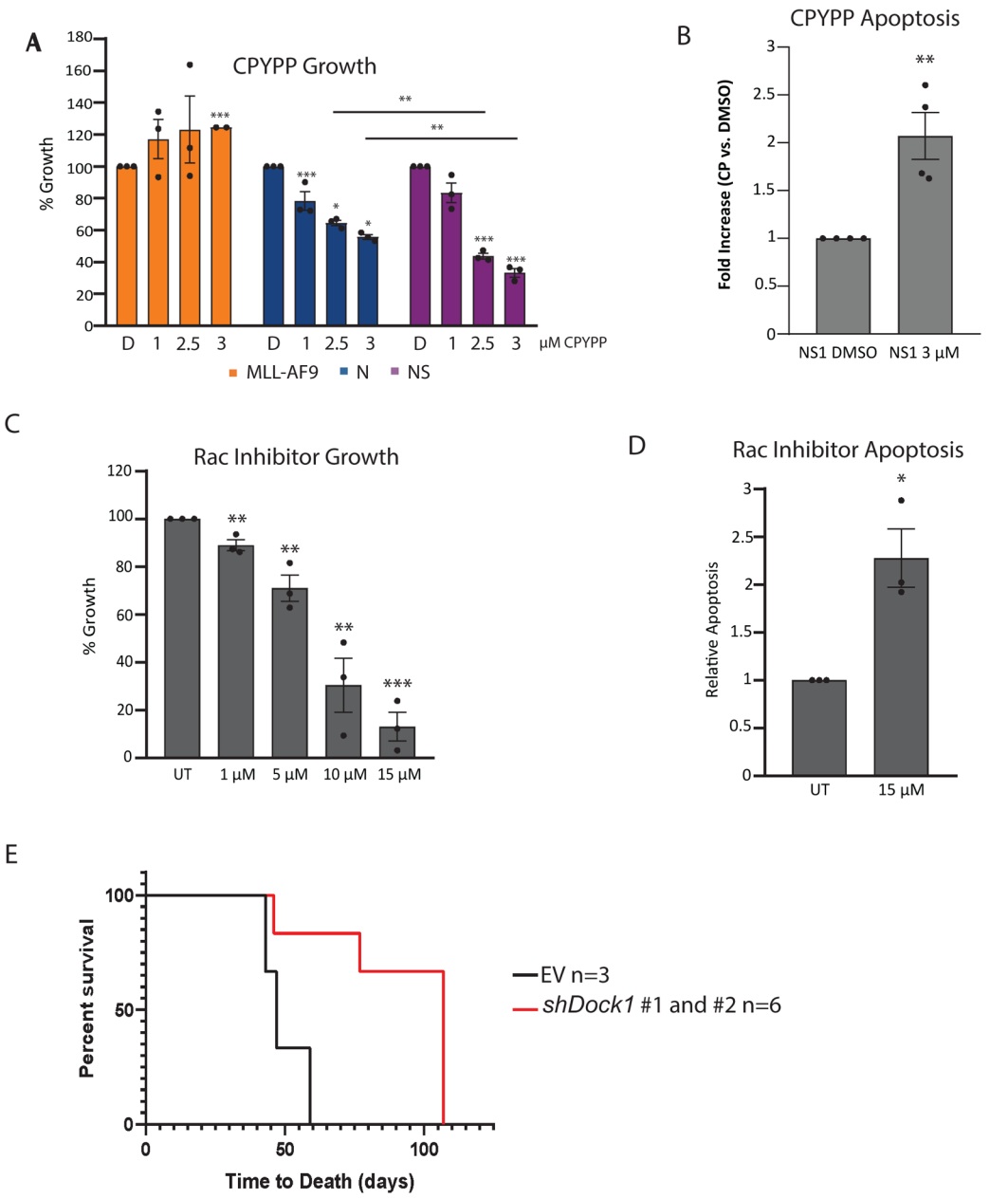


**Figure 6. The Dock1/Rac2 pathway is targetable in Npm1$^{cA/+}$;Smc3$^{\Delta/+}$ AML.**

**A.** 100,000 MLL-AF9, N and NS leukemic cells were plated and treated with vehicle (DMSO, D) or the indicated concentrations of CPYPP. After 72 hours, live cells were counted, and the % growth relative to the DMSO control was graphed. **B.** Apoptosis was measured with Annexin V/PI staining for the NS cells after 72 hours of treatment with 3 μM CPYPP. Cells positive for Annexin V only are shown and were considered early apoptotic. **C.** Growth of NS leukemic cells was carried out as in A, with the indicated concentrations of the Rac inhibitor EHT 1864. UT=untreated. **D.** Apoptosis was measured as in B after 72 hours of treatment with 15 μM EHT 1864. Cells positive for both Annexin V and PI are shown and were considered late apoptotic. **E.** Survival curve showing prolonged survival upon knockdown of *Dock1* in NS leukemia. A minimum of 3 replicates were performed for

all assays in this figure. Data represent the mean ± SEM. For A-D, statistical significance was determined using Student's t-test (two-tailed, unpaired). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.