Molecular synergy underlies the co-occurrence patterns and phenotype of NPM1-mutant acute myeloid leukemia.

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*Npm1c* and *Nras-G12D* co-mutation in mice leads to AML with a longer latency and a more mature phenotype than the *Npm1c/Flt3-ITD* combination. Mutant *Flt3* or *Nras* allele amplification is the dominant mode of progression in *Npm1c/Flt3-ITD* and *Npm1c/Nras-G12D* murine AML.
Abstract

*NPM1* mutations define the commonest subgroup of acute myeloid leukemia (AML) and frequently co-occur with *FLT3* internal tandem duplications (ITD) or, less commonly, *NRAS* or *KRAS* mutations. Co-occurrence of mutant *NPM1* with *FLT3-ITD* carries a significantly worse prognosis than *NPM1-RAS* combinations. To understand the molecular basis of these observations we compare the effects of the two combinations on hematopoiesis and leukemogenesis in knock-in mice. Early effects of these mutations on hematopoiesis show that compound *Npm1cA/++;NragsG12D/+* or *Npm1cA;Flt3ITD* share a number of features: *Hox* gene over-expression, enhanced self-renewal, expansion of hematopoietic progenitors and myeloid differentiation bias. However, *Npm1cA;Flt3ITD* mutants, displayed significantly higher peripheral leucocyte counts, early depletion of common lymphoid progenitors and a monocytic bias compared to the granulocytic bias in *Npm1cA/++;NragsG12D/+* mutants. Underlying this was a striking molecular synergy manifested as a dramatically altered gene expression profile in *Npm1cA;Flt3ITD*, but not *Npm1cA/++;NragsG12D/+*, progenitors compared to wild type. Both double-mutant models developed high penetrance AML although latency was significantly longer with *Npm1cA/++;NragsG12D/+*. During AML evolution, both models acquired additional copies of the mutant *Flt3* or *Nras* alleles, but only *Npm1cA/++;NragsG12D/+* mice showed acquisition of other human AML mutations, including *IDH1 R132Q*. We also find, using primary Cas9-expressing AMLs, that *HoxA* genes and selected interactors or downstream targets are required for survival of both types of double-mutant AML. Our results show that molecular complementarity underlies the higher frequency and significantly worse prognosis associated with *NPM1c/FLT3-ITD* versus *NPM1/NRAS-G12D*-mutant AML and functionally confirm the role of HOXA genes in NPM1-driven AML.

[247 words]
Introduction

Advances in genomics have defined the somatic mutational landscape of acute myeloid leukemia (AML), leading to a detailed characterisation of their prognostic significance and patterns of mutual co-occurrence or exclusivity.\(^1\) Mutations in \textit{NPM1}, the gene for Nucleophosmin, characterise the most common subgroup of AML representing 25-30\% of all cases, result in cytoplasmic dislocation of the protein (\textit{NPM1c}) and are mutually exclusive of leukemogenic fusion genes.\(^1\) As is often the case for fusion genes, progression to AML after the acquisition of mutant \textit{NPM1} is contingent upon the gain of additional somatic mutations such as those that activate STAT and/or RAS signalling.\(^3\)\(^4\). For reasons that are not clear, this transforming step favours acquisition of internal tandem duplications in \textit{FLT3} (\textit{FLT3-ITD}) over other somatic mutations with similar effects such as those involving \textit{NRAS} or \textit{KRAS}.\(^1\)\(^4\) Furthermore, the \textit{NPM1c/FLT3-ITD} combination is associated with a significantly worse prognosis compared to combinations of \textit{NPM1c} with mutant \textit{NRAS}, \textit{KRAS} or other mutations.\(^2\)

Whilst the adverse prognostic impact of \textit{NPM1/FLT3-ITD} vs \textit{NPM1/RAS} co-mutation influences clinical decisions in AML, its molecular basis and that of the frequent co-occurrence of \textit{NPM1c} and \textit{FLT3-ITD} in AML are unknown. Here, in order to investigate these phenomena, we compare the interaction of \textit{Npm1c} with \textit{Flt3-ITD} to its interaction with \textit{Nras\textsuperscript{G12D}} in knock-in mice. Individually, knock-in models of \textit{NPM1c}, \textit{FLT3-ITD} and \textit{NRAS-G12D} display enhanced myelopoiesis and progression to myeloproliferative disorders or AML in a significant proportion of animals.\(^5\)\(^7\) Also, we and others have previously shown that \textit{Npm1c} and \textit{Flt3-ITD} synergise to drive rapid-onset AML\(^8\)\(^9\), but the interaction between \textit{Npm1c} and mutant \textit{Nras\textsuperscript{G12D}} has not, to our knowledge, been previously investigated in knock-in mice\(^10\). Our findings reveal that the combination of \textit{Npm1c} and \textit{Flt3-ITD} has an early profound effect on gene expression and hematopoiesis, whilst \textit{Npm1c} and \textit{Nras-G12D} display only modest molecular synergy and subtler cellular changes. Also, whilst both types of co-mutation drove AML in the majority of mice, the leukemias in \textit{Npm1c;Flt3-ITD} mice were more aggressive and undifferentiated than those which developed in \textit{Npm1c;Nras-G12D} animals. At the genomic level, there was frequent amplification in both models of the mutant \textit{Flt3-ITD} or \textit{Nras-G12D} allele, however additional somatic mutations in AML driver genes (e.g. \textit{Idh1} and \textit{Ptpn11}) were seen only in \textit{Npm1c;Nras-G12D} AMLs. Our findings propose that the molecular synergy between \textit{Npm1c} and \textit{Flt3-ITD} underpin the co-occurrence patterns, phenotype and prognosis of NPM1-mutant AML.
Materials and methods

Animal husbandry

*Mx1-Cre*⁺; *Npm1*⁺/⁻*Fox-cA/+* were crossed with *Nras*⁻⁻⁻⁻*SL-G12D* or *Flt3ITD* mice, to generate triple transgenic animals (*Mx1-Cre; Npm1*⁺/⁻*Fox-cA/+; Nras*⁻⁻⁻⁻*SL-G12D/+ and Mx1-Cre; Npm1*⁺/⁻*Fox-cA/+; Flt3ITD/+*). To activate conditional alleles (*Npm1cA* and *NrasG12D*) in approximately 12-14 week old *Mx1-Cre; Npm1*⁺/⁻*Fox-cA/+; Nras*⁻⁻⁻⁻*SL-G12D/+ mice, *Mx1-Cre* was induced by administration of pIpC. As described previously, *Mx-1Cre; Npm1Flox-cA/+; Flt3ITD/+* mutants do not require pIpC induction of *Mx1-Cre* and recombination of the *Npm1Flox-cA* allele.⁸ For pre-leukemic analyses *Npm1cA/+; NrasG12D/+* were sacrificed 4-5 weeks post pIpC and *Npm1cA/+; Flt3ITD/+* were sacrificed at 5 weeks of age. Genotyping for mutant alleles was performed as previously described.⁵-⁷

Hematological measurements

Blood counts were performed on a VetABC analyzer (Horiba ABX).

Histopathology

Formalin fixed, paraffin embedded (FFPE) sections were stained with hematoxylin and eosin. Samples from leukemic mice were also stained with anti-CD3, anti-B220 and anti-myeloperoxidase. All material was examined by two experienced histopathologists (P.W. and M.A.) blinded to mouse genotypes.

Colony-forming assays and serial re-plating

Nucleated cells (3 x 10⁴) from bone marrow (BM) aspirates of mutant and wild-type mice were suspended in cytokine-containing methylcellulose-based media (M3434, Stem Cell Technologies) and plated in duplicate wells of 6-well plates. Colony-forming units (CFUs) were counted 7 days later. For serial re-plating, 3 x 10⁴ cells were re-seeded and colonies counted after 7 days.

Flow cytometry and cell sorting

Single cell suspensions of BM cells or splenocytes were incubated in 0.85% NH₄Cl for 5 minutes to lyse erythrocytes. Cells were then suspended in Hank’s Balanced Salt Solution (HBSS) supplemented with 2% FCS and 10µM HEPES. Progenitor populations were defined and stained as described in supplementary methods. Gated cellularity was calculated by multiplying the percentage of gated cells by the total number of nucleated cells from BM samples after erythrocyte depletion.
Viral transduction of BM progenitors and AML cell culture.

Lineage depleted BM aspirates, isolated from wildtype and Flt3|ITD/+ mice, were transduced with MSCV-Hoxa9-GFP and/or MSCV-Nkx2-3-CFP retroviruses and expanded for 7 days in liquid culture (X-Vivo, Lonza, supplemented with 10ng/ml IL-3, 10ng/ml IL-6 and 50ng/ml SCF, Peprotech). CFP, GFP or double positive cells were FACS sorted and 2.5 x10⁴ cells re-plated in semi-solid media as previously described. BM-derived AML cells from Roas26-EF1-Cas9 mice were cultured in vitro in the presence of cytokines. Disruption of individual candidate genes was performed by transduction with lentivirus expressing gene-specific guide RNA (gRNA) and blue fluorescent protein (BFP). The impact of gene disruption on AML cell growth was determined using competitive co-culture of transduced (BFP+) vs non-transduced (BFP-) cells as described previously11 (Figure 6A, Supplemental methods).

Microarray and comparative genomic hybridization analysis

Mouse gene expression profiles (GEPs) were generated using the Illumina MouseWG-6 v2 Expression BeadChip platform (Illumina). DNA copy number variation in leukemic samples was assessed with Mouse Genome Comparative Genomic Hybridization 244K Microarray (acGH, Agilent Technologies). Full details of analysis are provided in supplemental methods. For mouse gene expression profiling, n=4-10 (Lin-) or n=3-5 (MPP).

AML exome sequencing and mutation calling

Whole exome sequencing (WES) of AML BM and control C57BL/6N or 129Sv tail DNA was performed using the Agilent SureSelect Mouse Exon Kit (Agilent Technologies) and paired-end sequencing on a HiSeq2000 sequencer (Illumina). Validation of mutations was performed using MiSeq sequencing (Illumina) of amplicon libraries as previously described (See Supplemental Methods Figure S1 and Supplemental Tables 6 and 7 for primer sequences).12,13 Full details of analysis are provided in supplemental methods.

Datasets

Microarray data were deposited at Array Express (accession number E-MTAB-5356), and RNA sequencing (accession numbers ERS1732539 to ERS1732546, ERS812461 and ERS812462) as well as exome and Miseq sequencing (accession numbers PRJEB18526 and ERP020464) at EBI ENA.
Results

Mutant Npm1 co-operates with Nras-G12D and Flt3-ITD to increase self-renewal of hematopoietic progenitors and expand myelopoiesis

To understand the impact of the studied mutations, we analyzed hematopoietic cell compartments of Npm1<sup>CA/+</sup>;Nras<sup>G12D/+</sup>, Npm1<sup>CA/+</sup>;Flt3<sup>ITD/+</sup>, Nras<sup>G12D/+</sup>, Flt3<sup>ITD/+</sup> and wild type (WT) mice 4-6 weeks after activation of conditional mutations (Figure 1). Compared to Flt3<sup>ITD/+</sup> single mutants, Npm1<sup>CA/+</sup>;Flt3<sup>ITD/+</sup> mice displayed higher white cell counts (WCC) (56±13.4 vs 6.5±0.5 x 10<sup>6</sup> g/L, p<0.001) and spleen weights (0.63g vs 0.16g, p<0.001), but not BM cellularity (Figure 1B). By contrast, both Nras<sup>G12D/+</sup> and Npm1<sup>CA/+</sup>;Nras<sup>G12D/+</sup> mutants exhibited subtler increases in spleen weight (WT: 0.12g, Nras<sup>G12D/+</sup>: 0.18g, Npm1<sup>CA/+</sup>;Nras<sup>G12D/+</sup>: 0.19g, p<0.01 and p<0.001 respectively), but increased BM cellularity (WT: 28.1±1.9 x10<sup>6</sup>, Nras<sup>G12D/+</sup>: 43.7±2.6 x10<sup>6</sup> and Npm1<sup>CA/+</sup>;Nras<sup>G12D/+</sup>: 41.3±3.2 x10<sup>6</sup>, p<0.01 for either comparison vs WT) (Figure 1B).

Expanded myelopoiesis and myeloproliferation were previously documented in single Nras<sup>G12D/+</sup> and Flt3<sup>ITD/+</sup> mutant mice.5,6 Mutant Npm1 augmented these phenotypes with increases in total Mac-1<sup>+</sup> splenocytes (from 27% to 50% for Nras<sup>G12D/+</sup>; and 57% to 73% for Flt3<sup>ITD/+</sup>). Notably, these cells were predominantly granulocytic (Mac-1<sup>+</sup>/Gr-1<sup>+</sup>) in Npm1<sup>CA/+</sup>;Nras<sup>G12D/+</sup> and predominantly monocytic (Mac-1<sup>+</sup>/Gr-1<sup>-</sup>) in Npm1<sup>CA/+</sup>;Flt3<sup>ITD/+</sup> mice (Supplemental Figure S1A).

Nras<sup>G12D/+</sup> mice have been shown to have increased hematopoietic stem (HSC) and progenitor cell numbers, due to increased proliferation and self-renewal of the HSC and multipotent progenitor (MPP) compartments.14,15 Our results confirm these data demonstrating significant increases in total myeloid progenitors i.e. granulocyte-macrophage (GMP) and common-myeloid progenitors (CMP). Total numbers of Sca-1/Kit positive early progenitors (LSK) and MPPs are also increased in both Npm1<sup>CA/+</sup>;Nras<sup>G12D/+</sup> and Nras<sup>G12D/+</sup> BM cells (Figure 1C and Supplemental Figure S2A). However, Nras<sup>G12D/+</sup> progenitor cell composition was largely unaltered by the addition of mutant NPM1.

Concordant with previous studies, hematopoiesis in Flt3<sup>ITD/+</sup> mice was characterised by increased numbers of total myeloid progenitors (LK p<0.05 and GMPs p<0.01) and early progenitor populations (LSK, MPP and LMPP, p<0.01, p<0.01 and p<0.05 respectively) (Figure 1C and Supplemental Figure S2A).16,17 Of note, there were detectable decreases in the size of the common lymphoid progenitor (CLP) population in Flt3<sup>ITD/+</sup> and Npm1<sup>CA/+</sup>;Flt3<sup>ITD/+</sup> mice (Figure 1C) (in part due to the reduction in IL-7Ra-positive cells) (Figure S2B). Npm1<sup>CA/+</sup>;Flt3<sup>ITD/+</sup> mice also exhibited robust increases in numbers of LK, LSK, MPP and LMPP populations, above what was observed with Flt3<sup>ITD/+</sup>, when compared to WT. In direct comparison with Flt3<sup>ITD/+</sup> mutants, numbers of CMP and MEP progenitors in Npm1<sup>CA/+</sup>; Flt3<sup>ITD/+</sup>
mice were reduced (from $55 \times 10^3$ to $16 \times 10^3$, $p<0.05$ and from $61 \times 10^3$ to $17 \times 10^3$, $p<0.05$), yet GMPs, proposed as direct descendants of CMPs\(^{18}\), are significantly increased. This demonstrates that Flt\(^{3+/+}\) mutant myelopoiesis is dramatically altered by the addition of Npm\(^{1+/+}\). In direct comparison with Npm\(^{1+/+}\);Nras\(^{G12D/+}\) and Flt\(^{3+/+}\) mice showed increased LMPP and GMP populations with reduced numbers of lymphoid progenitors (CLP) (Figure 1E).

In order to assess the effects on the earliest detectable hematopoietic stem cell compartment (HSC) we opted to perform E-SLAM staining (CD45\(^+\)/EPCR\(^+\)/CD48\(^-\)/CD150\(^+\)).\(^{19}\) Importantly, this does not rely on cell surface expression of FLT3, and reveals the percentage of E-SLAM detectable HSCs is decreased in Npm\(^{1+/+}\);Nras\(^{G12D/+}\) mice and further so in Npm\(^{1+/+}\);Flt\(^{3+/+}\) mutants (Figure 1D). Finally, using serial re-plating of BM cells in semi-solid media we show that Npm\(^{1+/+}\) co-mutation markedly increased self-renewal of Flt\(^{3+/+}\) (as shown previously\(^8\)) and of Nras\(^{G12D/+}\) cells (Figure 1F).

**An Npm\(^{1+/+}\) transcriptional signature persists in double mutant hematopoietic progenitors**

To examine their combined effects on transcription we performed comparative global gene expression profiling of lineage negative (Lin\(^-\)) BM cells using microarrays. Npm\(^{1+/+}\);Nras\(^{G12D/+}\) and Npm\(^{1+/+}\);Flt\(^{3+/+}\) cells displayed a dramatically altered GEP compared to single Nras\(^{G12D/+}\) or Flt\(^{3+/+}\) mutants (Figure 2A and Supplemental Figure S3B). Previously, we showed that mouse Npm\(^{1+/+}\) Lin\(^-\) cells overexpressed several homeobox (Hox) genes (in particular overexpression of Hoxa5, Hoxa7, Hxa9 and two other homeobox genes, Hopx and Nkx2-3).\(^7\) Here, we show that this signature, absent from Nras\(^{G12D/+}\) or Flt\(^{3+/+}\) singular mutant mice, persists in compound Npm\(^{1+/+}\);Nras\(^{G12D/+}\) and Npm\(^{1+/+}\);Flt\(^{3+/+}\) Lin\(^-\) progenitors. (Figure 2A, Supplemental Figure S3A-C). Gene Set Enrichment Analysis (GSEA) of Npm\(^{1+/+}\) single and compound mutant cell GEPs, showed significant enrichment for genes up-regulated in NPM1-mutant and MLL-fusion gene positive human leukemias (Figure 2A).

**Overexpression of the homeobox gene NKX2.3 in human NPM1-mutant AML**

Using the human TCGA AML dataset, we compared GEPs of NPM1 mutant (NPM1c\(^{+}\)) to NPM1 wildtype (NPM1\(^{-}\)) AML.\(^1\) In agreement with previously published analyses, both HOXA and HOXB genes were significantly overexpressed in NPM1c\(^{+}\) AML (Figure 2B).\(^{20}\) We also noted NKX2-3 was also overexpressed in keeping with our findings in Npm\(^{1+/+}\) mice (Figure 2A). Recently, NKX2-3 overexpression was shown to be the most effective discriminant of MLL-MLLT4 (MLL-AF6)-driven AML from AMLs driven by other MLL-fusion genes.\(^{21}\) Whilst overexpression of Hox genes such as Hoxa9 has been shown to impart increased self-renewal and proliferation of hematopoietic progenitors, the effects of Nkx2-3 overexpression are unknown.\(^{22}\) To study this we performed retroviral gene transfer of fluorescently tagged Nkx2-3-CFP and Hoxo9-GFP into wildtype and Flt\(^{3+/+}\) Lin\(^-\) cells. Cells were
subsequently sorted and plated in semi-solid methylcellulose for colony formation assays (Figure 2Ci).
We find that overexpression of \textit{Nkx2-3} increases clonogenic potential, albeit to a lesser extent compared to \textit{Hoxa9} overexpression, in both wildtype and \textit{Flt3}^{ITD/+} progenitors. Notably, this is not augmented in combined transfected cells. (Figure 2Cii).

**\textit{Hoxa} gene expression is unaltered in mutant NPM1 early multipotent progenitors**

In order to mitigate the impact of the studied driver mutations on cell surface phenotypes, we performed transcriptome analysis on a homogeneous population of early progenitors, purified LSK-MPPs, (Figure 2D). \textit{Hox} gene expression was not significantly altered in this population in any of the \textit{Npm1}^{ca/+} models when compared to wildtype or single \textit{Nras}^{G12D/+} and \textit{Flt3}^{ITD/+} mutants (Figure 2E and Figure S3C). These results are in agreement with observations that \textit{Hox} gene expression in human NPM1c AML blasts is comparable to that seen in WT human HSCs and myeloid progenitors.\textsuperscript{20} As we do not observe statistically significant expansion in total (Lin-) progenitors in single \textit{Npm1}^{ca/+} mice (figure 1C), these data propose that, unlike HSCs, the observed pattern of \textit{Hox} overexpression in these progenitors is a molecular consequence of NPM1c rather than a change in cellular composition. This concurs with our published observations that the \textit{Hox} signature is detectable even in CD19-positive B-cells\textsuperscript{7}.

MPPs from single \textit{Nras}^{G12D/+} or \textit{Flt3}^{ITD/+} and the respective \textit{Npm1}^{ca/+} compound mutant MPPs also had distinct transcriptional changes. Compared to WT, both \textit{Nras}^{G12D/+} and \textit{Npm1}^{ca/+};\textit{Nras}^{G12D/+} MPPs displayed small numbers of differentially expressed genes yet only ~20% of these were shared (Figure 2Di). GSEA did not uncover significant overlap with any pre-established expression signatures (data not shown). In contrast, the “addition” of \textit{Npm1}^{ca/+} to \textit{Flt3}^{ITD/+} in MPPs led to differential expression of a large number of additional genes, whilst also retaining most of the transcriptional changes attributable to \textit{Flt3}^{ITD/+} (Figure 2Dii, Table S2) demonstrating the powerful synergy between \textit{Npm1}^{ca/+} and \textit{Flt3}^{ITD/+}. Pathway analysis of genes differentially expressed in \textit{Npm1}^{ca/+};\textit{Flt3}^{ITD/+} MPPs revealed enrichment of genes in the JAK-STAT pathway (Supplemental Figure 3E, Supplemental Tables S4), including the negative regulators \textit{Cish} and \textit{Socs2} (Figure 2F). A number genes, encoding proteins involved in MAPK signaling were also deregulated, as were genes involved in chromatin regulation/organisation and hematopoietic/myeloid differentiation (Figure 2F, Supplemental Figure 3D). Many of the genes in our \textit{Npm1}^{ca/+};\textit{Flt3}^{ITD/+} dataset were also found deregulated in a recently published Tet2\textsuperscript{-/-};\textit{Flt3}^{ITD/+} mouse model of AML (, Supplemental Figure 3F and Supplemental Table 6,), which serves to verify our mouse dataset technically, but also reveals a distinguishing expression signature of FLT3-ITD which includes \textit{Socs2}, \textit{Id1}, \textit{Csfr3r} and \textit{Bcl11a}.\textsuperscript{17} In contrast a lack of correlation
between deregulated gene sets of \( Npm1^{CA/} ; Flt3^{ITD/+} \) and \( Npm1^{CA/+} ; Nras^{G12D/+} \) MPPs (Supplemental Figure S3D) emphasises the molecular distinction between these compound mutants.

**\( Npm1^{CA/+} \) and \( Nras^{G12D} \) collaborate to promote high penetrance AML**

To understand the leukemogenic potential of combined \( Npm1^{CA/+} \) and \( Nras^{G12D} \) mutations, we aged combined and single mutant cohorts. Compound \( Npm1^{CA/+} ; Nras^{G12D/+} \) and \( Npm1^{CA/+} ; Flt3^{ITD/+} \) mice had significantly reduced survival (median 138 and 52.5 days respectively) when compared to wildtype (618 days), \( Npm1^{CA/+} \) (427 days), \( Nras^{G12D/+} \) (315 days) and \( Flt3^{ITD/+} \) (also 315 days) (Figure 3A, Supplemental Figure S4A). No difference in the survival of \( Nras^{G12D/+} \) and \( Flt3^{ITD/+} \) mutant mice was observed (p=0.85, see Supplemental Figure S4A for all comparisons). At time of sacrifice, blood counts and tissues were collected and subjected to histopathological analysis. Aged \( Npm1^{CA/+} ; Nras^{G12D/+} \) and \( Npm1^{CA/+} ; Flt3^{ITD/+} \) mice exhibited characteristic AML pathological findings at a much higher frequency than single mutant mice. These included significantly higher WCC, reduced platelet numbers and substantial organ infiltration with leukemic cells (Supplemental Figure S4B-D). Histological analysis verified the increased AML incidence from 41% (\( Flt3^{ITD/+} \)) to 100% in \( Npm1^{CA/+} ; Flt3^{ITD/+} \) samples and from 13% (\( Nras^{G12D/+} \)) to 85% in \( Npm1^{CA/+} ; Nras^{G12D/+} \) samples (45% AML with maturation, AML+ and 40% AML without maturation, AML− as defined by the Bethesda classification23 (Figure 3B).

**Additional somatic mutations are required for progression to AML in \( Npm1^{CA/+} ; Nras^{G12D/+} \) mice.**

\( Npm1^{CA/+} ; Flt3^{ITD/+} \) mice succumb to AML significantly more rapidly, compared to \( Npm1^{CA/+} \) and \( Npm1^{CA/+} ; Nras^{G12D/+} \) mice. We hypothesised that the slower onset of AML in the latter two genotypes may be due to the requirement for additional cooperating mutations. To test this, we performed aCGH and WES of AMLs from \( Npm1^{CA/+} \), \( Npm1^{CA/+} ; Flt3^{ITD/+} \) and \( Npm1^{CA/+} ; Nras^{G12D/+} \) mice. We first confirmed the frequent development of loss-of-heterozygosity (LOH) at the \( Flt3 \) locus in \( Npm1^{CA/+} ; Flt3^{ITD/+} \) AMLs 8,24 and verified this by quantifying \( Flt3^{ITD} \) variant allele fractions (VAFs) using PCR-MiSeq (Figure 4Ai). aCGH showed that LOH was copy-neutral and due to uniparental disomy of \( Flt3^{ITD} \) (Supplementary Figure 4Aii). Interestingly, aCGH of \( Npm1^{CA/+} ; Nras^{G12D/+} \) samples revealed amplification of chr3 in 5/10 samples tested (Figure 4Bi). This was exclusive to \( Npm1^{CA/+} ; Nras^{G12D/+} \) AMLs and mapped to a minimally amplified region (chr3: 102743581-103470550) containing \( Nras \) (Supplementary Table S10). We confirmed these \( Nras^{G12D} \) copy gains using PCR-MiSeq and also found copy neutral LOH for \( Nras^{G12D} \) in 3/10 AMLs. In addition, we found copy neutral LOH in 3 of 4 \( Npm1^{CA/+} ; Nras^{G12D/+} \) AMLs not studied by aCGH. In summary, increased \( Nras^{G12D} \) dosage was detected in 11/14 \( Npm1^{CA/+} ; Nras^{G12D/+} \) AMLs (Figure 4Bii), and this correlated with levels of RAS pathway activation as measured by pERK1/2 staining (Figure 4C).
WES revealed that the average number of single nucleotide variants (SNVs) and small insertions/deletions (indels) per AML sample correlated positively to survival (Figure 5A). Npm1CA/AMLs spontaneously acquired mutations in genes involved in RAS signaling (Nras-p.Q61H, Cbl-p.S374F, Ptpn11-p.S502L, Nf1-p.W1260* and Nf1-R683*) confirming this genetic interaction. Likewise, we detected a spontaneous tyrosine kinase domain mutation in Flt3, (Flt3-p.D842G) confirming the importance of FLT3 mutations in progression of NPM1-mutant AML (Figure 5B-C, Supplemental Table 9). Interestingly, a single Npm1CA+/NrasG12D/AML harbored an Idh1-p.R132Q mutation and mirroring the R132H/R132C mutations commonly seen in human AML1 whilst IDH1-R132Q itself was reported in human chondrosarcoma.25 aCGH also revealed complete or partial gain of a minimally amplified region on chr7 in 7/8 Npm1CA/ and 4/9 Npm1CA+/NrasG12D/AMLs containing genes implicated in leukemogenesis including Nup98, Wee1 and Eed, (Supplemental Figure SSC).7,26-28 Single copy loss of a region containing the epigenetic modifiers Wt1, Asxl1, Dnmt3a (1/8 Npm1CA/) and a focal deletion of Ezh2 (1/9 Npm1CA+/; NrasG12D/) were also detected (Figure 5C and Supplemental Figure SSC).

MLL, Hox genes and their partners are required for the survival of Npm1CA-driven AML cells.

To assess their contribution to AML maintenance in Npm1CA+/NrasG12D/ and Npm1CA;Flt3ITD mice, we employed CRISPR-Cas9 to disrupt selected deregulated genes identified by our pre-leukemic GEP studies. For this, we bred with Rosa26-EF1-Cas9 animals11 to generate Rosa26Cas9+/Npm1CA/;NrasG12D/; and Rosa26Cas9+/Npm1CA +/-Flt3ITD/ mice. Competitive co-culture of gRNA transduced and non-transduced BM cells from these mice revealed that Hoxa10 and to a lesser degree Hoxa9, but not Hoxa7 are required for Npm1CA+/NrasG12D/ and Npm1CA;Flt3ITD/ AML maintenance (Figure 6B). In contrast, all three Hox genes were required for growth of AMLs generated by retroviral MLL-AF9 transformation of Flt3ITD/ BM cells (Supplementary Figure S7C).11,29,30 Notably, although Nkx2-3 overexpression enhanced colony-forming ability of wild type and Flt3ITD/ BM (Figure 2C), disruption of endogenous Nkx2-3 did not significantly affect proliferation of Npm1CA+/NrasG12D/ or Npm1CA;Flt3ITD/ AMLs in vitro. Other genes whose disruption reduced proliferation of Npm1CA-driven AMLs included Mll (Kmt2a) gene, recently shown to be a therapeutic target in this AML type31, Hoxa9/10 partners or co-factors including Meis1, Pbx1 and Pbx3, the HOXA9 targets Bcl2 and Lmo2.32-34 A number of genes with altered expression in mutant pre-leukemic MPP cells, were not required for survival of AML cells in vitro (Figure 6C). However, we cannot exclude a potential role for these in leukemia initiation.

We also wanted to investigate potential differences in JAK/STAT vs RAS signaling in our AMLs in a similar way. FLT3-ITD leads to constitutive activation of JAK/STAT signaling, driving growth and transformation of hematopoietic cells.35-37 In keeping with this, our transcriptome analysis revealed that genes involved in JAK/STAT signaling (Stat5a, Cish, Socs2) were differentially expressed in Npm1CA;Flt3ITD but not in Npm1CA;NrasG12D Lin- progenitors. Nevertheless, CRISPR-targeting of Jak2 and
Stat5a/b genes inhibited the growth of both Npm1cA;Flt3ITD/+ and Npm1cA/+;NrasG12D/+ AML cells (Supplemental Figure S8B). We confirmed by RNA-seq that this was due to activation of a JAK/STAT programme in Npm1cA/+;NrasG12D/+ AML cells (Figure S9). In this light we conclude that the cytokines required for culturing primary AML cells in vitro (IL-3, IL-6 and SCF), precludes the assessment of signaling genes in AML growth and proliferation.

Discussion

Whilst the mutational drivers of AML and their patterns of co-occurrence are well understood, the molecular basis for the frequency and prognostic impact of these patterns remain unknown. Of particular clinical relevance are the co-occurrence patterns of mutant NPM1 mutations, which characterize the most common AML subtype. Co-mutation of NPM1 with FLT3-ITD is both significantly more frequent and carries a worse prognosis than co-mutation with RAS genes. To understand the basis of this observation we investigated the interactions of these mutations in bespoke experimental models (Figure 1A). Analysis of the short-term impact of these mutations on hematopoiesis confirmed that single Npm1cA/+ mutant mice have normal BM cellularity, WCC and splenic weight. As described before, single Flt3ITD/+ and NrasG12D/+ had moderate but significant increases in splenic size, whilst NrasG12D/+ had raised WCC and BM cellularity. Introduction of Npm1cA/+ into the Nras G12D/+ background did not alter these parameters significantly, yet the Npm1cA/+;Flt3ITD/+ co-mutation led to a dramatic rise in WCC and splenic size (Figure 1B). At the cellular level, the Npm1cA/+;NrasG12D/+ combination did not change progenitor and stem cell numbers when compared to NrasG12D/+ alone. In contrast, when compared to Flt3ITD/+ mutants, Npm1cA/+;Flt3ITD/+ mice displayed reductions in CMP and MEP, and increases in LSK progenitors. Furthermore, Npm1cA/+;Flt3ITD/+ mice showed a profound reduction in phenotypic HSCs (Figure 1C–E).

The differential impact of Npm1cA/+ on Flt3ITD/+ versus NrasG12D/+ was reflected in marked differences in GEPs between double mutant mice. The Npm1cA/+;NrasG12D/+ model displayed only minimal differences to single NrasG12D/+, whilst Npm1cA/+;Flt3ITD/+ lin- progenitors had profoundly different GEPs to Flt3ITD/+.

From these and complimentary analyses of human NPM1c AML we identify NKKX2-3 as a marker of this type of AML. Expression of NKKX2-3 distinguishes MLL-AF6 and MLL-ENL from other forms of MLL-mutant leukemia, highlighting the mechanistic links between NPM1c- and MLL-fusion genes. Here, we show that whilst potent overexpression of Nkx2.3 by lentivirus may have an impact on self-renewal, genetic disruption of the endogenous Nkx2.3 did not inhibit AML cell growth (Figure 6).

We went on to age double mutant mice and report that, like Npm1cA/+;Flt3ITD/+ animals, Npm1cA/+;NrasG12D/+ mice also develop highly penetrant AML, albeit with a much longer latency and a
more mature phenotype overall. Interestingly single mutant Flt3\textsuperscript{ITD/+} and Nras\textsuperscript{G12D/+} mice had similar survival (Figure 3A), indicating that the interaction with Npm1\textsuperscript{cA} was central to this difference. To understand the genetic events involved in leukemic progression, we performed exome sequencing and copy number analysis of Npm1\textsuperscript{cA/+};Flt3\textsuperscript{ITD/+} and Npm1\textsuperscript{cA/+};Nras\textsuperscript{G12D/+} AMLs. Interestingly, the commonest somatic event during AML progression was an increase in Nras\textsuperscript{G12D/+} or Flt3\textsuperscript{ITD/+} mutant allele burden, through copy-neutral LOH or copy number gain. In human AML, copy-neutral LOH is common for FLT3-ITD, but less so for mutant NRAS; for example in a recent study we identified only one such LOH event amongst 13 RAS mutant human AMLs. Nevertheless, in keeping with our findings, studies using the Nras\textsuperscript{G12D/+} model, in combination with retroviral insertional mutagenesis, resulted in high penentrance AML with frequent LOH for Nras-G12D when combined with overexpression of oncogenes such as Evi1. The different incidence of LOH for mutant RAS between murine and human AML may operate through the fact that, compared to the acquisition of other oncogenic mutations (e.g. Idh1-R132Q in our study), LOH for Nras-G12D may be more expedient in mice given the large numbers of Npm1\textsuperscript{cA/+}/Nras\textsuperscript{G12D/+} pre-leukemic HSCs. Other possible reasons may relate to the differences in human-mouse synteny and the fact that mice are inbred potentially making recombination events more likely. Notwithstanding mouse-human differences in LOH frequencies, our data provide strong evidence that increased mutant Flt3 and Ras gene dosage are important for leukemic transformation/progression.

Finally, in order to investigate their role in Npm1c AML, we use CRISPR-Cas9 to disrupt selected genes in Cas9-expressing primary mouse leukemia cells. Using this approach we confirmed the requirement for the HoxA9/10 functional gene network in Npm1c AML maintenance. Interestingly, although it is widely appreciated that overexpression of Hoxa9 stimulates leukemic transformation,\textsuperscript{22,29,33} in our model disruption of Hoxa10 has a more detrimental impact on survival, mirroring our recent genome wide essentiality screen in the NPM1c-harboring OCI-AML3 cell line.\textsuperscript{11}

Our study describes the first faithful mouse model of the interaction of Npm1c with Nras-G12D, the preferred form of oncogenic NRAS in human AML.\textsuperscript{2} Both NPM1c models share a number of salient characteristics, which are imparted by mutant Npm1, such as homeobox gene overexpression and increased self-renewal of hemopoietic progenitors. However, we demonstrate that the co-occurrence of Npm1c/Flt3-ITD is significantly more leukemogenic and leads to strikingly different molecular and cellular consequences compared to Npm1c/Nras-G12D, providing a mechanistic explanation for the higher frequency and worse prognosis of NPM1c/FLT3-ITD AML. Furthermore, through the generation of Cas9-expressing AML models, we also present a versatile approach for the study of genetic interactions in primary mouse leukemias using CRISPR. Whilst our non-Cas9-expressing Npm1c/Flt3-
ITD model was helpful in recent studies of new anti-AML therapies, these Cas9-expressing models can be utilized to study both genetic and pharmacological interactions in parallel, and also to perform targeted mechanistic studies.

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Authorship

Contribution: O.M.D., J.L.C., A.M., C.S.G., C.L., P.G. and G.S.V. performed mouse experiments. O.M.D. and G.S.V. analyzed results; P.W. and M.A. performed histopathological analysis of mouse samples; O.M.D., N.C., R.M.A. and MS. V. performed transcriptome analysis; I.V. performed analysis of next generation sequencing; O.M.D. S.P. and K.T. performed CRISPR-CAS9 experiments; O.M.D. and G.S.V. designed the study. O.M.D. and G.S.V. wrote the paper with the help of R.R., P.W., M.A. and A.B.

Conflict of interest disclosure: GSV is a consultant for and holds stock in Kymab Ltd, and receives an educational grant from Celgene. All other authors declare no competing financial interests.

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**Figure Legends**

**Figure 1.** Mutant Npm1 co-operates with Nras-G12D and Flt3-ITD to enhance myeloid differentiation and enhance progenitor self-renewal.

(A) Schema for Mx-1 Cre, Npm1<sup>flox-cA</sup>, Nras<sup>G12D</sup> and Flt3<sup>ITD</sup> inter-crosses. (B) Nras<sup>G12D/cA</sup> mice show a subtle and Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/ITD</sup> mice a marked increase in white cell count (WCC), compared to wildtype. Splenic sizes were significantly increased in all mutant genotypes except Npm1<sup>cA/cA</sup>, with Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/ITD</sup> showing the most striking phenotype. Bone marrow cellularity was increased only in the presence of the Nras<sup>G12D/cA</sup> allele. (C) FACS analysis at 4-5 weeks after mutation induction. Gating strategies depicted are from wildtype mice. Significant differences in the stem and progenitor cell compartments of Nras<sup>G12D/cA</sup> and Flt3<sup>ITD/cA</sup>, but not Npm1<sup>cA/cA</sup> single mutant mice, as previously reported. In double mutant mice, the Npm1<sup>cA/cA</sup>; Nras<sup>G12D/cA</sup> combination was not significantly different to Nras<sup>G12D/cA</sup>, in contrast to Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/ITD</sup> which was markedly different to both Flt3<sup>ITD/ITD</sup> and Npm1<sup>cA/cA</sup> single mutants. (D) Using a cell surface phenotype independent of FLT3 staining, we found that CD45+/EPCR+/CD150+/CD48- HSCs were reduced slightly in Npm1<sup>cA/cA</sup>; Nras<sup>G12D/cA</sup> and markedly in Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/cA</sup> mice. (E) Summary of hematopoietic effects of Npm1<sup>cA/cA</sup>; Nras<sup>G12D/cA</sup> and Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/cA</sup> double mutations in mice. (F) Single Npm1<sup>cA/cA</sup> and double Npm1<sup>cA/cA</sup>; Nras<sup>G12D/cA</sup> or Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/cA</sup> mutant hematopoietic progenitors show increased self-renewal potential in whole bone marrow serial replating assays (n=4-8). Mean ±SEM are plotted. Significant values are reported for one-way analysis of variance (ANOVA, Bonferroni adjusted); * P<0.05 vs wildtype, ** P<0.01 vs wildtype, *** P<0.001 vs wildtype, (Δ) P<0.05 vs Flt3<sup>ITD/cA</sup>, (ΔΔ) P<0.01 vs Flt3<sup>ITD/cA</sup>, (ΔΔΔ) P<0.001 vs Flt3<sup>ITD/cA</sup>, (♣) P<0.05 vs Nras<sup>G12D/cA</sup>, (♣♣) P<0.01 vs Nras<sup>G12D/cA</sup>, (♣♣♣) P<0.001 vs Nras<sup>G12D/cA</sup>, (†) P<0.05 Npm1<sup>cA/cA</sup>; Nras<sup>G12D/cA</sup> vs Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/cA</sup>, (♣♣♣) P<0.01 Npm1<sup>cA/cA</sup>; Nras<sup>G12D/cA</sup> vs Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/cA</sup>, (♣♣♣♣) P<0.001 Npm1<sup>cA/cA</sup>; Nras<sup>G12D/cA</sup> vs Npm1<sup>cA/cA</sup>; Flt3<sup>ITD/cA</sup>.

**Figure 2.** Impact of Npm1<sup>cA/cA</sup> on the transcriptome of Nras<sup>G12D/cA</sup> and Flt3<sup>ITD/cA</sup> mutant hematopoietic progenitors.

(A) Overlap of differentially expressed mRNAs reveals that Npm1<sup>cA/cA</sup> has a dramatic impact on Lin-progenitor GEPs when combined with Flt3<sup>ITD/cA</sup>, but only a modest impact when combined with Nras<sup>G12D/cA</sup>. Nonetheless, the characteristic hallmarks of Npm1<sup>cA/cA</sup> are retained in both double mutant progenitors, namely overexpression of Hoxa genes and of the homeobox genes Hopx and Nkx2-3 (also seen in single Npm1<sup>cA/cA</sup> progenitors). Gene Set Enrichment Analysis reveals enrichment of differentially expressed genes from these models in human AMLs harboring mutant NPM1 or MLL gene fusions (B) Comparison of human NPM1-mutant (NPM1<sup>c</sup>) versus NPM1-wildtype (NPM1<sup>WT</sup>) normal karyotype AML (NK-AML) also shows marked overexpression of HOXA and HOXB genes, as well as of NKKX2.3 raising the possibility that the latter may mediate some of the effect of NPM1<sup>c</sup>. (C) Effects of Nkx2-3 and Hoxa9 over-expression on mouse hematopoietic progenitors. (i) Lin<sup>-</sup> bone marrow progenitors
from wildtype and Flt3ITD/+ mice were transduced with MSCV-Nkx2.3-CFP and/or MSCV-Hoxa9-GFP constructs, maintained in liquid culture for 7 days, FACS sorted for CFP and GFP single and for double transfected cells and plated in semi-solid media. (ii) Colony assays of 2,500 transduced cells show that both MSCV-Hoxa9 and MSCV-Nkx2-3 conferred an increase in self-renewal of both wildtype and Flt3ITD/+ cells. However, double MSCV-Hoxa9/MSCV-Nkx2-3 transfected cells showed no further changes in self-renewal when compared to MSCV-Hoxa9 alone. Mean ± SEM (n=3); *p<0.05; **p<0.01; ***p<0.001; students t-test). (D) Sorting strategy for LSK/CD34+/Flt3+/CD48+ progenitor cells and overlap of differentially expressed genes (Illumina MouseWG-6 v2 Expression BeadChip) for (i) NrasG12D/+ vs Npm1ca/++;NrasG12D/+ and (ii) Flt3ITD/+ vs Npm1ca/++;Flt3ITD/+ MPPs datasets. (E) Heat map of normalised Hox gene expression in purified (i) MPP and (ii) Lin+ populations reveal that Npm1ca/+ mutants (single or double) have similar patterns of Hox gene expression to wildtype (normalised average expression values are used to generate heat map values). (F) Differentially expressed genes in Npm1ca/++;Flt3ITD/+ MPPs vs wildtype controls.

Figure 3. Npm1ca and NrasG12D co-operate to drive high penetrance AML.

(A) Kaplan Meier survival curves of wildtype (n=23), Npm1ca/+ (n=34), NrasG12D/+ (n=40), Flt3ITD/+ (n=39), Npm1ca/++;NrasG12D/+ (n=46) and Npm1ca/++;Flt3ITD/+ (n=40). Double mutant (Npm1ca/++;NrasG12D/+ and Npm1ca/++;Flt3ITD/) mice had a significantly shortened survival when compared to single mutants, whilst Npm1ca/++;Flt3ITD had significantly shorter survival than Npm1ca/++;NrasG12D/+ mice. (B) Results of independent histopathological analysis of aged moribund mice. Incidence of AML in compound Npm1ca/++;NrasG12D/+ and Npm1ca/++;Flt3ITD/+ mice is increased compared to Npm1ca/+, NrasG12D/+ and Flt3ITD/+ mice. Examples of complete effacement of splenic tissue and infiltration of myeloid blast cells in liver tissue from Npm1ca/++; NrasG12D/+ and Npm1ca/++; Flt3ITD/+ AMLs are presented. Reduced MPO staining in diseased tissues is observed in samples categorized as AML without maturation (AML-) compared to those categorized as AML with maturation (AML+). H&E, Haematoxylin and eosin; MPO, myeloperoxidase.

Figure 4. Leukemic progression in double mutant mice involves increased NrasG12D or Flt3ITD allele dosage

(A) Increase in Flt3ITD allele burden in AMLs from Npm1ca/++; Flt3ITD mice through loss of heterozygosity for the locus. (i) Flt3ITD amplicon sequencing (MiSeq) of leukemic bone marrow or spleen DNA (FN2-FN7). Tail DNA amplified from 2-week-old Flt3+/+, Flt3ITD/+, Flt3ITD/ITD mice was used as control. (ii) Normalised Log2 ratio plots show copy neutrality of chr5 and the Flt3 locus in 7/7 Npm1ca/++; Flt3ITD murine AMLs (FN-AMLs) tested. (B) (i) Summary of aCGH showing copy number gain at the Nras locus in AMLs RN6-10. (ii) Allele fractions for Nraswt vs NrasG12D show that copy number gains in RN6-10 involved NrasG12D, and that an additional 3 cases (RN3-5) show copy-neutral loss-of-heterozygosity. In addition, two more RN AMLs show gains in mutant NRAS when measuring Nraswt vs NrasG12D allele fractions (aCGH was not performed on these). Results of two Npm1ca/+ samples are also shown for comparison purposes (N6, N7). (C) Increased gene dosage of NrasG12D correlates with increased levels.
of phosphorylated RAS effectors pERK1/2. FN2,3,4,6,7= Npm1C;Flt3ITD AML, RN1-14= Npm1C/++;NrasG12D/+ AML.

Figure 5. Somatic mutations in Npm1C, Npm1C/++; NrasG12D/+ and Npm1C; Flt3ITD AMLs. (A) Exome sequencing identifies an increased number of somatic nucleotide variants (SNVs) and small indels in Npm1C/+, compared to Npm1C/++; NrasG12D/+ (RN-AML) and Npm1C; Flt3ITD (FN-AML) AML samples. Npm1C/+ 6.8±0.9, Npm1C/++; NrasG12D/+ 3.3±0.5 and Npm1C/++; Flt3ITD 2.6±0.7 (mean±SEM) (** p<0.01 vs Npm1C/+ one way ANOVA, Bonferroni adjusted). Total AMLs sequenced; Npm1C/+ (n=12), Npm1C/++; NrasG12D/+ (n=14) and Npm1C/++; Flt3ITD (n=7). (B) Summary of SNVs/Indels detected in AMLs from each genotype as indicated. Those in blue are genes mutated in the TCGA AML dataset. Those in red are exact or synonymous mutations detected in the TCGA AML dataset. (C) Co-occurrence of SNVs and CNVs. Depicted are SNVs and focal copy number variations (CNVs) which have been formally detected in the TCGA AML dataset or detected as common insertion sites (CIS) in our previously published Npm1C/+ Sleeping Beauty Transposon screen. Mutant allele copy gains, chromosome gains and losses depicted. For copy number variation, colour coded boxes are based on log2 ratios (aCGH) and are not representative of CNV size. For a complete overview of all CNV and SNV co-occurrence see Supplemental Figure S6.

Figure 6. MLL, Hox genes and their partners are required for the survival of Npm1C-driven AML cells. (A) Schematic depicting the derivation and liquid culture of Rosa26-EF1-Cas9 expressing AML cell lines. CRISPR-EF1-Cas9 based assessment of individual genes aberrantly expressed in Npm1C/++; NrasG12D/+ and Npm1C; Flt3ITD mice. CAS9 activity of these mouse AML cell lines was validated as described previously (Supplementary Figure S7A). Individual Rosa26-EF1-Cas9 expressing cell lines were derived from two mice of each genotype. In vitro competitive assays were performed over a 23 day period using AML cell lines transduced with lentivirus expressing gRNAs for the indicated gene, and the BFP-positive fraction compared with the non-transduced population. Results were normalized to day 3 for each gRNA. Results from AML cell lines transduced with guide RNAs targeting Hoxa-related (B) and non-Hoxa related (C) genes. gRNA sequences were selected from a previously published library and are detailed in Supplementary Table S15. Guides against the pan essential Npm1 gene are used as a control.
References


