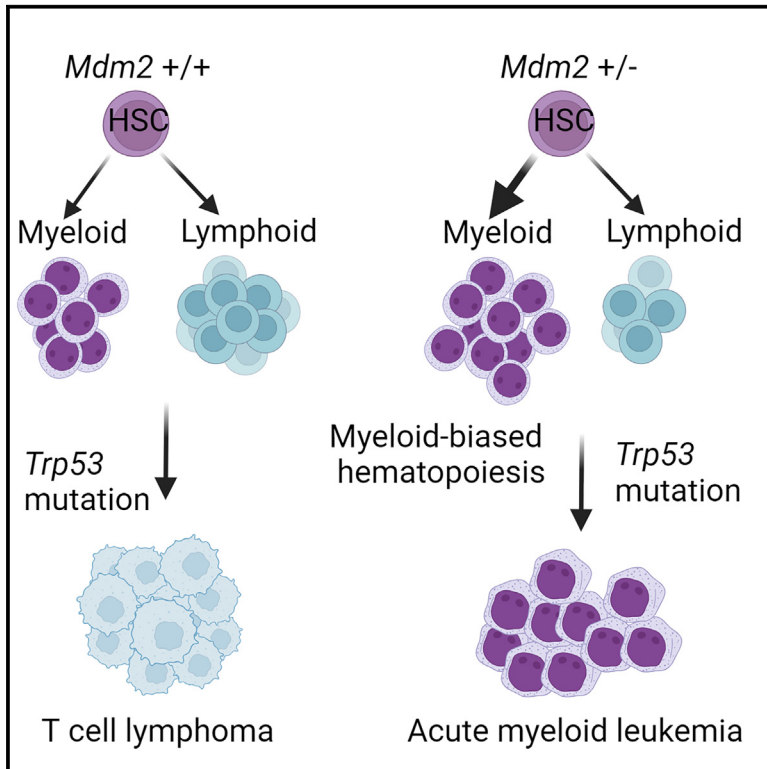


# Age-specific induction of mutant p53 drives clonal hematopoiesis and acute myeloid leukemia in adult mice

## Graphical abstract



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## In brief

Pourebrahim et al. develop two mouse models for p53-mutant acute myeloid leukemia, revealing how Mdm2 interaction and the timing of mutations in blood stem cells influence disease onset and type. This work offers new insights into AML pathogenesis and potential treatment strategies.

## Highlights

- Allele-specific loss of *MDM2* in *TP53*-mutant acute myeloid leukemia (AML)
- *Mdm2* haploinsufficiency collaborates with mutant *Trp53* in AML
- p53-independent mechanism of Mdm2 in controlling the mevalonate pathway
- Targeting the mevalonate pathway enhances the efficacy of MDM2 inhibitors in AML



## Article

# Age-specific induction of mutant p53 drives clonal hematopoiesis and acute myeloid leukemia in adult mice

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## SUMMARY

The investigation of the mechanisms behind p53 mutations in acute myeloid leukemia (AML) has been limited by the lack of suitable mouse models, which historically have resulted in lymphoma rather than leukemia. This study introduces two new AML mouse models. One model induces mutant p53 and *Mdm2* haploinsufficiency in early development, showing the role of *Mdm2* in myeloid-biased hematopoiesis and AML predisposition, independent of p53. The second model mimics clonal hematopoiesis by inducing mutant p53 in adult hematopoietic stem cells, demonstrating that the timing of p53 mutation determines AML vs. lymphoma development. In this context, age-related changes in hematopoietic stem cells (HSCs) collaborate with mutant p53 to predispose toward myeloid transformation rather than lymphoma development. Our study unveils new insights into the cooperative impact of HSC age, *Trp53* mutations, and *Mdm2* haploinsufficiency on clonal hematopoiesis and the development of myeloid malignancies.

## INTRODUCTION

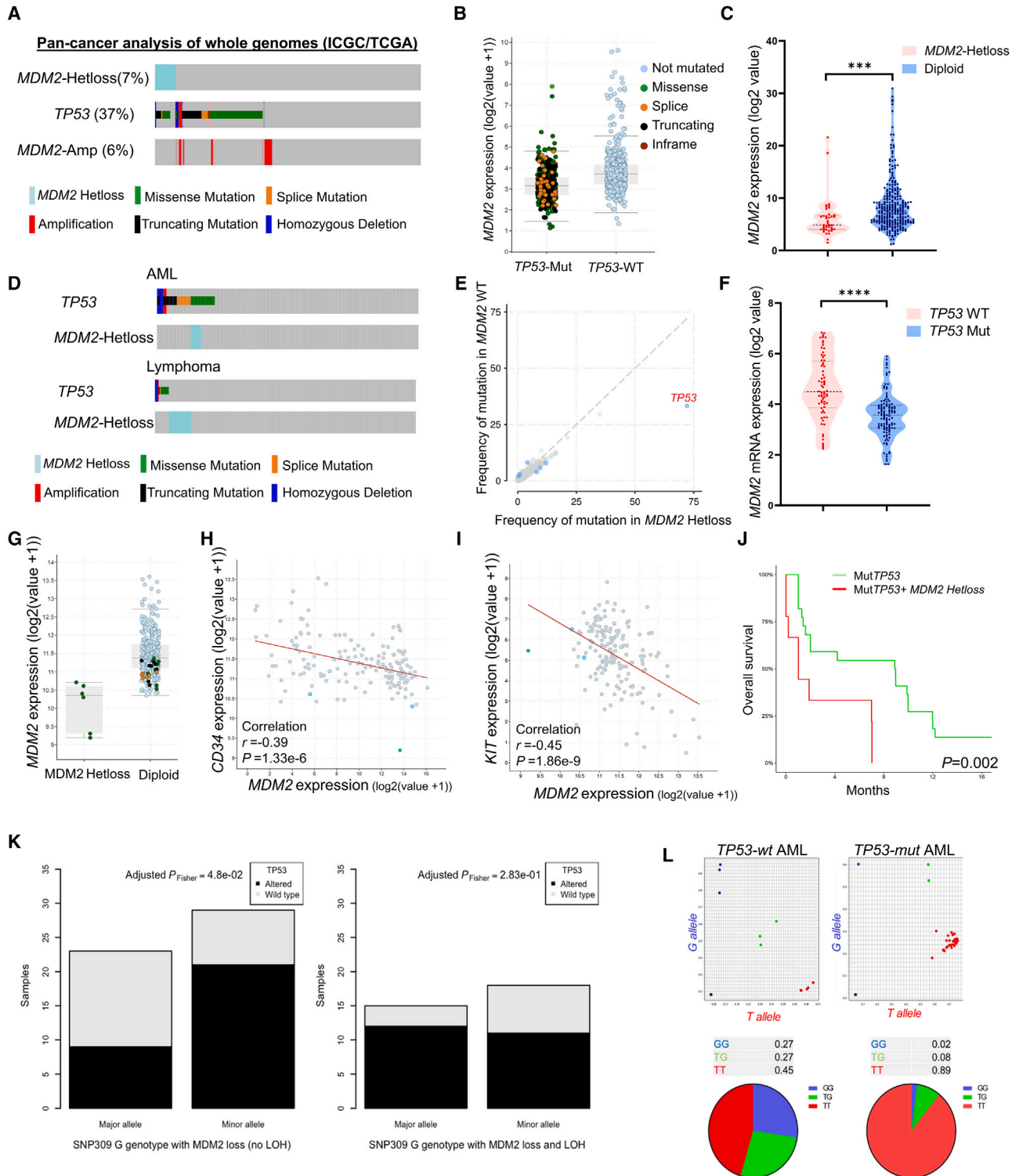
*TP53* abnormalities are observed in 5%–10% of *de novo* myelodysplastic syndrome (MDS) and AML patients, but the frequency increases up to 40% in older patients or those with therapy-related myeloid malignancies.<sup>1</sup> The mechanisms by which *TP53* mutations lead to the development of AML are not well understood. Previous data from studies with *Trp53* and *Mdm2* knockout mice suggested that once p53 is inactivated, *Mdm2* becomes redundant in cells.<sup>2,3</sup> However, recent findings have challenged this established belief, revealing that *Mdm2* plays a crucial role in the survival and proliferation of p53-mutated and deleted human cancer cells, such as triple-negative breast cancer.<sup>4</sup>

The regulation of p53 protein by *Mdm2* plays a crucial role in maintaining cellular integrity following DNA damage.<sup>5,6</sup> *Mdm2* is an E3 ubiquitin ligase that targets p53 for degradation in the 26S proteasome, providing a mechanism for fine-tuning p53 levels after DNA damage.<sup>7–9</sup> Li-Fraumeni syndrome (LFS), caused by germline mutations in the *TP53* gene, is associated with increased susceptibility to developing lymphoid malig-

nancies at a young age, and myeloid malignancies in the setting of therapy-related disease and old age.<sup>10</sup> Similarly, mice with germline *Trp53* mutations also predominantly develop lymphomas rather than leukemias,<sup>11</sup> further emphasizing the role of germinal p53 mutations in lymphoma predisposition. Yet, the role of p53 and *Mdm2* in hematopoietic differentiation and their link to the development of specific hematological malignancies have yet to be fully elucidated.

In this study, we explored the impact of *Mdm2* haploinsufficiency on the development of lymphoma vs. AML in the presence of *Trp53* R172H mutation. Our analysis revealed concurrent heterozygous loss of *MDM2* in *TP53*-mutated AML, along with a significant enrichment of *Mdm2*<sup>SNP309</sup> in *TP53*-mutated AML. We show that *Mdm2* haploinsufficiency leads to myeloid-biased hematopoiesis, which cooperates with somatic *Trp53*-mutations in the development of AML. Furthermore, we uncovered a novel function of *Mdm2* in regulation of the mevalonate pathway and demonstrate that its inhibition promotes myeloid differentiation, independently of its role in p53 regulation. Through a series of complementary genetic models, we show that the age of mice is a critical factor in determining the transformation to myeloid





**Figure 1. Analysis of TP53 mutations, MDM2 alterations, and MDM2 expression patterns in cancer**

(A) Frequency of TP53 mutations and MDM2 copy number alterations across various cancer types using whole-genome analysis of ICGC/TCGA Pan-Cancer data.

(B) Expression of MDM2 in TP53-wild-type (WT) and TP53-mutant (Mut) cases in pan-cancer analysis of whole genomes.

(C) Relative expression of MDM2 mRNA based on MDM2 allele status pan-cancer analysis of whole genomes, \*\*\* $p < 0.001$ .

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vs. lymphoid malignancies in the context of p53 mutations. Together, our findings present a valuable mouse model for dissecting the role of mutant p53 in AML and offer important insights into the impact of *Mdm2* haploinsufficiency on myeloid-biased hematopoiesis, potentially guiding the development of therapeutic strategies for *TP53*-mutant AML.

## RESULTS

### Frequency and association of *MDM2* alterations and *TP53* mutations and allele-specific characteristics of *MDM2*<sup>SNP309</sup> in AML

To assess the significance of *MDM2* alterations and the potential implications of *MDM2* haploinsufficiency in AML, we conducted an analysis using data from the Pan-Cancer Analysis of Whole Genomics study.<sup>12</sup> Our objective was to determine the frequency of *MDM2*-Hetloss specifically in *TP53* mutant tumors. Among the 2,583 patients examined, we observed *MDM2*-Hetloss in approximately 7% of all cancers. However, *TP53*-mutant cases exhibited *MDM2* Hetloss in more than 17% of instances, indicating a strong association (log<sub>2</sub> odds ratio = 2.04,  $p < 0.001$ ) (Figure 1A). It is worth noting that no homozygous deletions of *MDM2* were detected in any cancer types, suggesting the essential nature of *MDM2*, even in *TP53*-mutant tumors, which has been previously reported.<sup>13</sup> We also assessed the transcript levels of *MDM2* with respect to *TP53* mutation status and found that *MDM2* expression levels are significantly lower in *TP53*-mutant tumors (t test,  $p = 0.0001$ ) (Figure 1B). In the *TP53*-mutant group, *MDM2* expression was significantly lower in *MDM2*-Hetloss cases compared with *MDM2*-diploid cases (t test,  $p = 0.001$ ) (Figure 1C). Additionally, we searched for available AML datasets on cBioPortal (400 cases) and found that *MDM2*-Hetloss was concomitant with *TP53* missense mutations (log<sub>2</sub> odds ratio >3,  $p < 0.001$ ) (Figure 1D). This association was not present in lymphomas, where we found *MDM2*-Hetloss and *TP53* mutations to be mutually exclusive, suggesting that the observed *MDM2* copy number changes may have a specific link to the myeloid lineage (Figure 1D). Among AML patients with *MDM2*-Hetloss, *TP53* mutations were the most frequently observed mutation (Figure 1E). To validate these genomic findings, we expanded our analysis to a *TP53*-mutant AML dataset consisting of 187 AML cell lines documented in the Cancer Cell Line Encyclopedia.<sup>14</sup> The AML cell lines with *TP53* mutations exhibited significantly lower *MDM2* expression compared with *TP53* wild-type cell lines ( $p = 0.0001$ ) (Figure 1F).

To investigate if the reduced expression of *MDM2* in *TP53*-mutant patients is due to *MDM2*-Hetloss or impaired transcrip-

tional activity of *TP53* resulting from mutations, we compared *MDM2* expression in subjects with *MDM2*-Hetloss with those with a diploid *MDM2* status. Significantly lower *MDM2* expression was observed in *TP53*-mutant/*MDM2*-Hetloss cases compared with *TP53*-mutant/*MDM2*-diploid cases ( $p = 0.001$ ) (Figure 1G). This indicates that in *TP53*-mutant patients, *MDM2* levels are predominantly influenced by *MDM2* copy number. Further analysis was conducted to determine whether *MDM2*-Hetloss is linked to a specific phenotype in *TP53*-mutant AML. As depicted in Figures 1H and 1I, an inverse relationship was found between *MDM2* levels and the expression of *CD34* and *KIT*, suggesting a possible role in the differentiation of leukemia cells. Moreover, a significant correlation was found between *MDM2*-Hetloss and lower survival rates ( $p = 0.002$ ) (Figure 1J), suggesting the potential prognostic significance of *MDM2*-Hetloss in *TP53*-mutant AML.

Next, we aimed to investigate whether *MDM2* loss of heterozygosity (LOH) exhibited allele-specific characteristics or varied between alleles of *MDM2* single nucleotide polymorphisms (SNPs), with particular focus on *MDM2*<sup>SNP309</sup> (rs2279744; T>G). *MDM2*<sup>SNP309</sup> is located in the intronic promoter region of *MDM2* (Figure S1A) and has been associated with increased *MDM2* expression and earlier cancer onset in patients with LFS and sporadic cancers.<sup>15</sup> We analyzed the *MDM2*<sup>SNP309</sup> genotype within the pan-cancer analysis of whole genomes (PCAWG) cohort. Due to the limited availability of *TP53*-mutant AML cases (only eight) in a curated list of 2,658 PCAWG cases, we leveraged whole-genome sequencing data to achieve sufficient statistical power. Our analysis revealed similar patterns across the T/T, T/G, and G/G genotypes, indicating no significant association between the *MDM2*<sup>SNP309</sup> genotype and *TP53* inactivation, alteration, or wild-type status ( $P_{\text{ch}^2} = 0.71$ ; Figure S1B). We also examined the relationship between *MDM2* copy number status and *TP53* mutational status within the T/T and G/G genotypes. In a wild-type *TP53* context, we observed a higher frequency of *MDM2* losses in the G/G genotype (13.22%) compared with the T/T genotype (7.66%) (adjusted  $P_{\text{Fisher}}$  test =  $3.99\text{e-}2$ ; Figure S1C). Subsequently, we assessed *MDM2* allelic imbalances in T/G germline cases and found that the G allele was retained on the major allele in 64% of wild-type *TP53* cases, whereas only 30% of altered-*TP53* cases exhibited this pattern in losses without LOH (Figure 1K). Of note, no significant differences in T/G were observed in losses with LOH.

Additionally, we investigated the *MDM2*<sup>SNP309</sup> T/G genotype in bone marrow samples from AML patients, distinguishing between those with wild-type ( $n = 10$ ) and mutant *TP53* ( $n = 46$ ).

(D) Frequency of *TP53* mutations and *MDM2* copy number alterations in patients with AML and lymphoma, sourced from cBioPortal for Cancer Genomics.

(E) Frequency of mutations in different genes categorized by *MDM2* WT and *MDM2* LOH, retrieved from cBioPortal.

(F) Relative expression of *MDM2* based on *TP53* status using data from the Cancer Cell Line Encyclopedia, \*\*\*\* $p < 0.0001$ .

(G) *MDM2* expression in AML samples, highlighting cases with mutant *TP53*: green for missense, brown for splice, and black for truncation mutations.

(H and I) Scatter diagram graphs the correlation of *CD34* and *KIT* versus *MDM2* at the mRNA levels in patients with AML (TCGA).

(J) Overall survival of p53 mutant AML patients with or without *MDM2* LOH.

(K) Bar plot displaying the distribution of the G allele on the major or minor allele in germline T/G cases and in the context of somatic *MDM2* loss, depending on *TP53* mutational status (altered or wild type) and the presence or absence of the T allele (no LOH; left) or its absence (LOH; right). This demonstrates a significant enrichment for the G allele on the minor allele in altered *TP53* cases without LOH (adjusted  $P_{\text{Fisher}} = 4.8\text{e-}2$ ).

(L) Allele-specific expression analysis of GG (blue), TG (green), and TT (red) in indicated patient samples.



Our findings revealed that the T/T genotype was present in 45% of wild-type *TP53* cases, while 89% of mutant *TP53* cases exhibited the T/T genotype, indicating selective loss of the G allele in *TP53*-mutant AML (Figure 1L). These results suggest an association between allele-specific loss of the G allele and *TP53* mutations in various cancer types and AML patients. Together, these data reveal a strong association between *MDM2*-Het/loss and *TP53* mutations in AML, indicating functional implications. Additionally, allele-specific loss of the G allele suggests a link between *MDM2* expression levels and *TP53* mutations across various cancer types and AML.

### **Mdm2 haploinsufficiency cooperates with mutant p53 to promote the development of AML**

Building on the observed correlation between *MDM2* expression levels and *TP53* mutations in AML, we developed specific genetic models to further elucidate the potential interplay between *Mdm2* levels and *Trp53* mutations, thereby enhancing our understanding of AML pathogenesis. As a brief introduction, the gain-of-function (GOF) activity of mutant p53 has been largely studied through comparison of mice harboring a conditional mutant *Trp53* allele known as *LSL-Trp53<sup>R172H</sup>*.<sup>16</sup> Hematopoietic-specific induction of *LSL-Trp53<sup>R172H</sup>* results in thymic lymphoma in most animals.<sup>17</sup> However, the LSL cassette in *LSL-Trp53<sup>R172H</sup>* mice leads to *Trp53* heterozygosity in the whole mouse, allowing for a heterozygous *Trp53* microenvironment that facilitates tumorigenesis.<sup>18</sup> To tackle this challenge, we utilized an inducible mutant *Trp53<sup>R172H</sup>* allele, which we refer to as “wmR172H” (where “wm” denotes the transition from wild type to mutant). This allele features an R172H mutation, analogous to the R175H mutation in humans, positioned downstream of a wild-type *Trp53* cDNA sequence, which is in turn flanked by *loxP* sites (Figure 2A). This design ensures the maintenance of wild-type *Trp53* expression until recombination occurs.<sup>19,20</sup> To evaluate the impact of *Trp53* LOH and *Mdm2* haploinsufficiency in our model, we utilized the *Trp53<sup>fl</sup>*<sup>21</sup> and *Mdm2<sup>fl22</sup>* respectively. To monitor recombined cells in mice, we utilized an *mTmG* allele, a fluorescent reporter with a tdTomato (mT) cassette. Upon *Cre*-mediated recombination, this cassette is replaced by a green fluorescent protein (mG) cassette (Figure 2A).

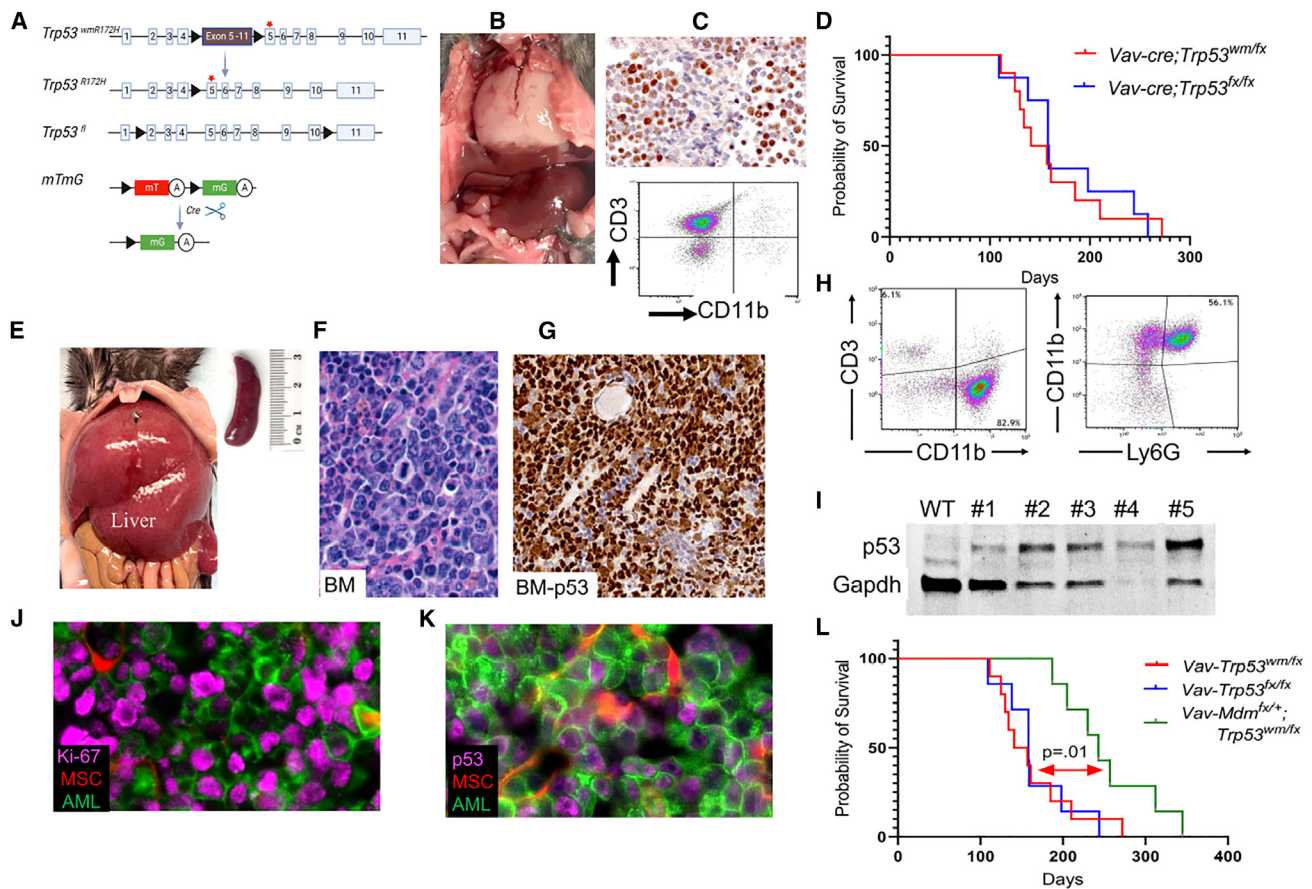
We initially assessed the impact of *Trp53* mutation/loss in early hematopoietic stem cells (HSCs) using the *Vav-Cre* system. Two mouse cohorts were generated: one with a conditional *Trp53* mutant allele exhibiting *Trp53* LOH (*Vav-Cre;Trp53<sup>wmR172 H/fl</sup>*), and another with homozygous *Trp53* floxed alleles (*Vav-Cre;Trp53<sup>fl/fl</sup>*), allowing us to compare the effects of p53 mutation vs. complete loss of p53 in HSCs. Some mice also carried the *mTmG* allele for tracking. To validate our model, we analyzed E10.5 *Vav-Cre-mTmG* embryos and confirmed successful recombination of the *mTmG* allele in the aortogonadal mesenchyme and fetal liver by fluorescence microscopy (Figure S2). Mice with *Trp53* mutations and *Trp53* deletion introduced early in life developed lymphoma with complete penetrance, suggesting that the mutation or deletion of *Trp53* at an early age is more likely to result in the development of lymphoma (Figure 2B). Immunohistochemical analysis of lymphoma tissue showed nuclear p53 staining and flow cytometry confirmed CD3 expression and absence of CD11b in lymphoma cells (Figure 2C). The

Kaplan-Meier survival analysis revealed comparable survival of *Vav-Cre;Trp53<sup>wmR172 H/fl</sup>* mice to that of *Vav-Cre;Trp53<sup>fl/fl</sup>* mice, indicating that there was no GOF of mutant p53 in this model (Figure 2D). Both models had full penetrance of thymic lymphoma, suggesting that the loss of p53 function alone is sufficient to drive lymphoma development in these mice. This observation is consistent with previous reports comparing *Trp53<sup>null</sup>* with *Trp53<sup>R172H</sup>* mice in which *Trp53* was deleted or mutated in the whole body.<sup>23–25</sup> The absence of AML in both cohorts indicates that additional genetic aberrations may be necessary in this context for the development of AML.

Next, we aimed to explore the phenotype of *Mdm2* haploinsufficiency and *Trp53* mutations in early HSCs. We crossed mice carrying the *Mdm2<sup>fl</sup>* alleles,<sup>22</sup> with progeny from *Trp53<sup>wmR172 H/fl</sup>* to generate *Vav-Cre;Mdm2<sup>+/fl</sup>; Trp53<sup>wmR172 H/fl</sup>* mice (referred to as *Vav-Mdm<sup>fl/+</sup>;p53<sup>mut/fl</sup>*), some of which also carried the mTmG allele for tracing. *Vav-Mdm<sup>fl/+</sup>;p53<sup>mut/fl</sup>* mice developed AML with full penetrance, characterized by myelomonocytic leukemia cells infiltrating both bone marrow (BM) and liver (Figure 2E; Table S1). Three out of 12 mice succumbed to lymphoma. However, analysis of DNA isolated from tumor tissue revealed a lack of recombination of the *Mdm2* allele in the tumor. Histopathological analysis of BM, spleen, and liver samples revealed myeloid-derived leukemias (Figure 2F). Some mice showed multiclonality with the presence of more than one type of morphologic nonlymphoid hematopoietic neoplasm. Immunostaining analysis showed strong nuclear expression of mutant p53 in the BM (Figure 2G). Flow cytometry analysis revealed a CD3<sup>+</sup>, CD11b<sup>+</sup>, Ly6G<sup>+</sup> phenotype, consistent with our histopathology findings (Figure 2H). Western blot analysis of a handful of spleen samples revealed high levels of mutant p53 (Figure 2I). Myelomonocytic AML features were observed in BM samples from *Vav-Mdm<sup>fl/+</sup>; p53<sup>mut/fl</sup>* mice, with GFP<sup>+</sup> leukemic cells invading periosteum and muscles (Figure S3A). Immunostaining displayed a highly proliferative disease, with p53 accumulation in most malignant BM cells (Figures 2J and 2K). We observed a few clusters of CD3<sup>+</sup> cells in the BM indicating suppressed lymphoid lineage (Figure S3B). Clonal expansion of p53-mutant AML cells was heterogeneously distributed throughout the BM (Figures S3C and S3D). *Mdm2* haploinsufficiency significantly extended survival of p53-mutant mice compared with *Mdm2*-diploid p53-mutant mice ( $p = 0.01$ ) (Figure 2L). Notably, mice with the deletion of both *Mdm2* alleles (*Vav-Mdm<sup>fl/fl</sup>;p53<sup>mut/fl</sup>*) developed lymphoma, a phenotype comparable to that of *Vav-cre;p53<sup>mut/fl</sup>* mice, suggesting that the complete loss of *Mdm2* does not directly contribute to the development of AML. Together, these findings confirm that *Mdm2* haploinsufficiency and *Trp53* mutations synergistically induce aggressive AML with full penetrance.

### **Trp53 somatic mutation in adult HSCs leads to pre-leukemic clonal expansion**

To explore the clonal evolution of p53 mutant subclones and their progression into myeloid malignancies, we created an inducible mouse model using the *Mx1-cre* driver and polyinosinic:polycytidylic acid (pIpC) induction. The inducible conditional mouse model replicates clonal hematopoiesis by inducing somatic mutations in a fraction of adult HSCs at about 4 months of age, which is equivalent to young adult humans, and track



**Figure 2. Overview of alleles and pathological findings in a novel p53 mutant AML mouse model**

(A) Schematic representation of the genetic modifications in the mouse model. The activation of Cre recombinase under the *Vav1* promoter leads to deletion of wild-type *Trp53* cDNA flanked by *loxP* sites (black triangles) resulting in a *Trp53*-mut allele. The diagram also shows the recombination of the *mTmG* reporter allele, facilitating GFP expression in hematopoietic cells, with “mT” for membranous tomato, “mG” for membranous GFP, and “A” indicating a polyadenylation sequence. The red star marks the *Trp53* R172H mutation on exon 5.

(B) Image of a white thoracic lymphoma mass in a *Vav-Cre;Trp53<sup>fl/wmR172H</sup>* mouse.

(C) Immunohistochemistry for p53, showing nuclear accumulation in lymphoma cells. Flow cytometry analysis identifying a distinct CD3<sup>+</sup> T lymphocyte population, with absence of CD11b<sup>+</sup> myeloid cells.

(D) Kaplan-Meier survival curve with a *p* value of 0.2 from the log rank (Mantel-Cox) test, showing the overall survival rate of the mice.

(E) Representative image of AML infiltration in liver and spleen of moribund *Vav-Cre;Mdm2<sup>+/fl</sup>;Trp53<sup>fl/wmR172H</sup>* mice.

(F) Hematoxylin and eosin (H&E)-stained bone marrow (BM) section from the AML mouse depicted in (J), showing characteristic features of AML.

(G) Immunostaining for p53 protein, visualized as brown staining, demonstrates the presence of p53 in leukemia cells within bone marrow (BM).

(H) Flow cytometry analysis of CD3, CD11b, and Ly6G markers in the bone marrow of AML mice.

(I) Western blot analysis of p53 protein expression in BM samples comparing p53 wild-type control and five AML samples.

(J) Immunofluorescence analysis of Ki-67 in BM. MSCs, mesenchymal stromal cells.

(K) Mutant p53 accumulation in GFP-positive AML cells in BM sections.

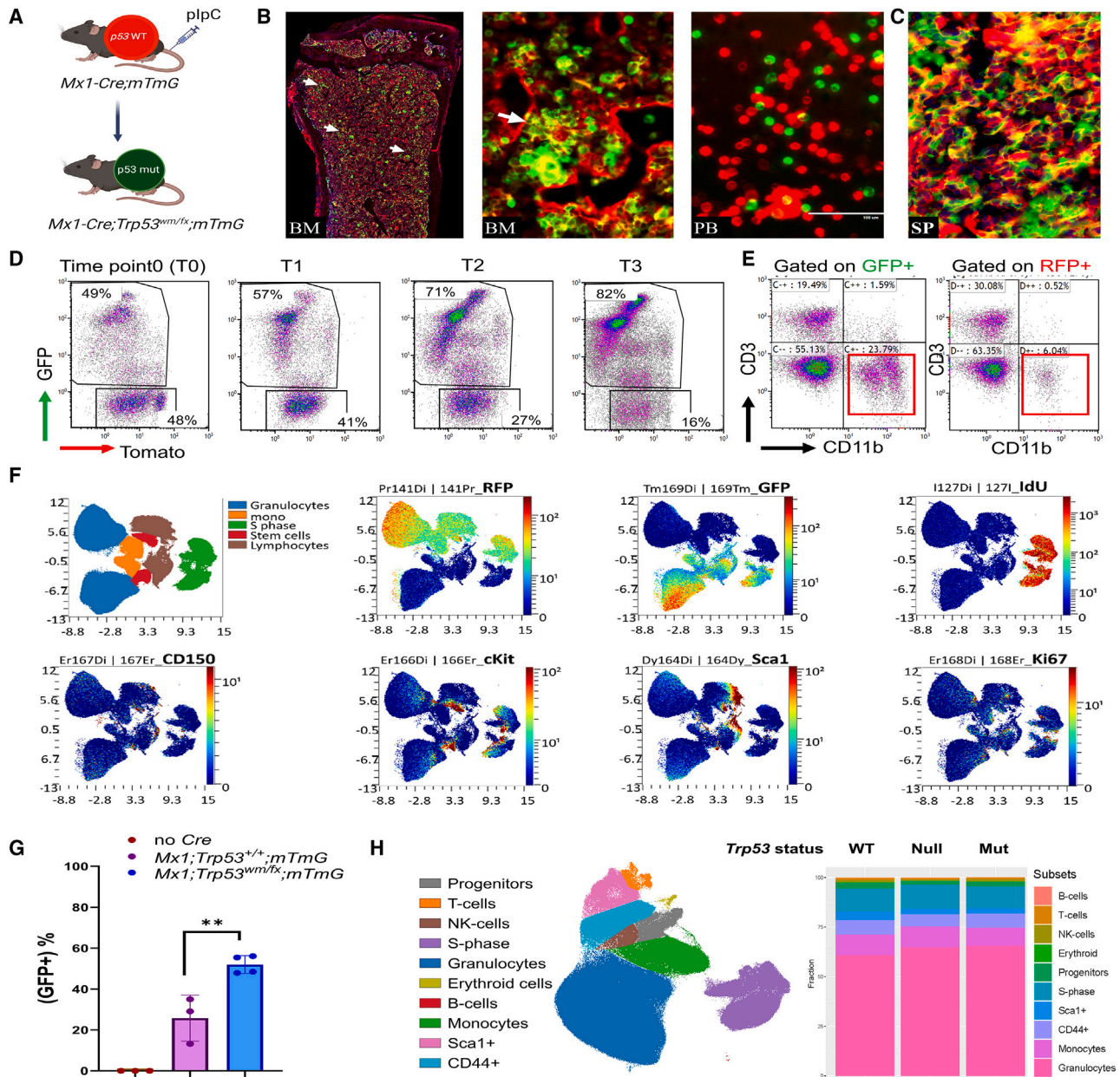
(L) Kaplan-Meier analysis of overall survival of the indicated mice. *p* = 0.01, log rank (Mantel-Cox) test.

the recombined cells using the *mTmG* allele (Figure 3A). Initially, we confirmed that *Mx1-cre* effectively targets HSCs in the BM of *Mx1-cre;mTmG* mice. Analysis of BM cells from these mice revealed that the GFP<sup>+</sup> population was present within the CD150<sup>+</sup>/Lin<sup>-</sup>/Sca1<sup>+</sup>/c-Kit<sup>+</sup> (LSK) cell population, indicating the successful targeting of the hematopoietic stem cell compartment by the *Mx1-cre* driver (Figure S4A). This finding is consistent with previous reports demonstrating the effectiveness of the *Mx1-cre* system *in vivo*.<sup>26</sup> Fluorescence microscopy analysis of the BM displayed clear evidence of efficient recombination of the *mTmG* allele, as indicated by the presence of

clonal clusters of GFP<sup>+</sup> cells (Figure 3B). The GFP<sup>+</sup> cells were detectable throughout the BM and formed distinct colonies. The presence of GFP<sup>+</sup> cells was also detected in the peripheral blood (PB), and spleen (Figure 3C).

Next, we investigated the clonal evolution of p53 mutant clonal hematopoiesis by generating cohorts of *Mx1-Cre;mTmG* and *Mx1-Cre;Trp53<sup>wmR172 H/fl</sup>;mTmG* mice, and analyzing PB samples on a monthly basis, starting at 4 months of age. The population of p53-mutant (GFP<sup>+</sup>) cells increased over time (Figure 3D). Flow cytometry analysis of PB after 3 months revealed an increased myeloid population defined as CD11b<sup>+</sup> (Figure 3E).





**Figure 3. Clonal evolution and generation of a p53 mutant clonal hematopoiesis mouse model**

(A) The schematic outlines the creation of the *Trp53<sup>R172H</sup>* clonal hematopoiesis model in adult HSCs. Following plpC injection, *Cre* recombinase expressed under the *Mx1-Cre* promoter induces recombination in hematopoietic cells, leading to a fluorescence switch in the *mTmG* allele from red (tdTomato) to green (GFP), simultaneously converting wild-type *p53* to its mutant form.

(B and C) (B) Direct fluorescence image of the bone marrow of *Mx1-Cre;Trp53<sup>wmR172 H/H</sup>;mTmG* mouse showing the distribution of GFP+ cells in the bone marrow (BM), peripheral blood (PB), and (C) spleen (SP). High magnification displaying a colony of GFP+ cells marked by white arrow.

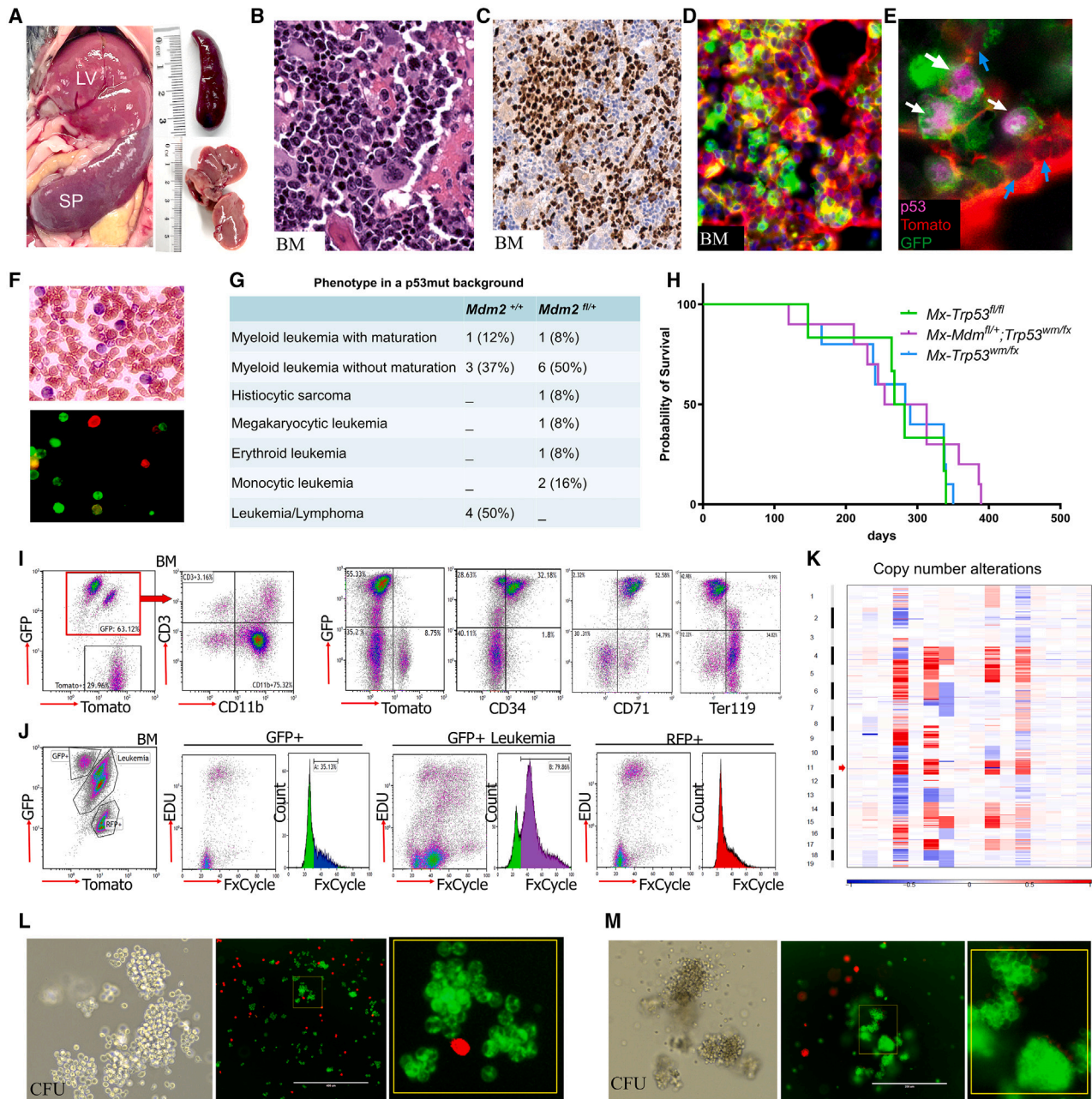
(D) Flow cytometry plots showing the expression of GFP and Tomato in the peripheral blood of *Mx1-Cre;Trp53<sup>wmR172 H/H</sup>;mTmG* mice at different time points.

(E) Flow cytometry plots of CD3 and CD11b expression gated on GFP+ or RFP+ cells.

(F) tSNE plots representing a CyTOF analysis of the indicated marker distribution in BM.

(G) Graph showing the percentage of GFP+ cells in the peripheral blood of indicated mice over 3 months after plpC injection. The data are presented as mean ± standard deviation.

(H) UMAP plot showing the cell subsets detected in pooled BM samples isolated from *Mx1-Cre;mTmG* (*p53* wild-type), *Mx1-Cre;Trp53<sup>fl/fl</sup>;mTmG* (*p53* null), and *Mx1-Cre;Trp53<sup>wmR172 H/H</sup>;mTmG* (*p53* mutant) mice subjected to UMAP dimension reduction. Each lineage is marked by colors indicating cell type. The stacked bar plots summarize the subset frequencies in indicated genotypes.



**Figure 4. Characterization of AML development in *Mx1-Cre;Trp53<sup>wmR172 H/Fl</sup>;mTmG* mice**

(A) Infiltration of AML cells into the liver (LV) and spleen (SP), indicative of AML development, is shown.  
 (B) Hematoxylin and eosin (H&E)-stained sections of bone marrow (BM) showing extensive leukemia cell infiltration.  
 (C) p53 protein immunostaining in leukemia cells in BM.  
 (D) Direct fluorescence image of BM depicting colonies of GFP+ cells. The stromal cells and normal hematopoietic cells are labeled with tomato fluorescence.  
 (E) Fluorescent imaging of p53 immunostaining in BM shows GFP+ hematopoietic cells expressing mutant p53 protein (magenta) among Tomato+ stromal cells.  
 (F) Peripheral blood smear and fluorescence imaging of GFP+ blast cells.  
 (G) Table representing the phenotype of different leukemia subtypes in p53 mutant AML mice with indicated *Mdm2* genotypes.  
 (H) Kaplan-Meier analysis of overall survival of the indicated mice.  
 (I) Flow cytometry analysis of BM for indicated markers showing the expansion of myeloid and erythroid markers within the GFP+ population.  
 (J) DNA content analysis in selected BM cell populations showing higher DNA content in p53 mutant AML cells (GFP+) compared with normal BM cells (RFP+).  
 (K) Heat map of copy number variation in bone marrow cells derived from *Mx1-Cre;Trp53<sup>wmR172H/Fl</sup>;mTmG* mice with AML. Each row represents a separate bone marrow sample, while each column represents specific genomic regions. The color scale indicates the relative copy number, with shades ranging from blue (deletion) to red (amplification).

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To identify specific lineages that were expanded in p53 mutant clonal hematopoiesis, we isolated BM samples from *Mx1-Cre;mTmG* (p53 wild-type), *Mx1-Cre;Trp53<sup>wmR172 H/fl</sup>;mTmG* (p53 mutant) and *Mx1-Cre;Trp53<sup>fl/fl</sup>;mTmG* (p53 null) mice and performed CyTOF analysis using a panel of 31 antibodies (Table S2). Antibodies specific to RFP and GFP were employed to distinguish the p53-mutant (GFP+) and p53 wild-type (RFP+) populations (Figure 3F). The p53 wild-type group showed relatively stable population levels over time, whereas the p53 mutant population displayed a significant expansion with increasing duration following polyinosinic:polycytidylic acid (plpC) injection (Figure 3G). The CyTOF analysis revealed a slight increase in the myeloid lineage in p53 mutant and null mice compared with p53 wild-type (Figures 3H and S4B). We did not observe a significant expansion of a specific stem/progenitor cell population in p53 mutant GFP+ cells. These findings indicate that loss of p53 function may contribute to alterations in hematopoietic lineage development, without causing a dramatic shift in the stem cell population. The overall increase in the myeloid population may indicate a transformation occurring in the myeloid progenitors. Together, these data highlight the significant impact of *Trp53* mutation on the clonal expansion and evolution of HSCs into myeloid lineage within an adult mouse model.

### Clonal expansion of p53 mutant cells and development of myeloid malignancies in adult HSCs

We expanded the cohorts of *Mx1-Cre;Trp53<sup>wmR172H/+</sup>;mTmG* mice (referred to as *Mx-p53<sup>H/+</sup>*), *Mx1-Cre;Trp53<sup>wmR172 H/fl</sup>;mTmG* mice (referred to as *Mx-p53<sup>mut/fl</sup>*), and *Mx1-Cre;Mdm2<sup>fl/+</sup>;Trp53<sup>wmR172 H/fl</sup>;mTmG* mice (referred to as *Mx-Mdm-p53<sup>mut/fl</sup>*) and injected them with plpC to induce the *Mx1-Cre*. Then, we closely monitored them to observe the development of leukemia in these mice cohorts. After a follow-up period of over a year, the *Mx-p53<sup>mut/fl</sup>* and *Mx-Mdm-p53<sup>mut/fl</sup>* mice developed various myeloid malignancies (Figure 4A). In contrast, the *Mx-p53<sup>H/+</sup>* mice did not develop similar transformations during the observation period. These findings suggest that the loss of the wild-type *Trp53* allele was a crucial factor in the transformation process. *Mx-p53<sup>mut/fl</sup>* mice displayed a significant expansion of myeloid cells in the BM, categorized as myeloid leukemia with or without maturation, aligning with the Bethesda proposals for classifying nonlymphoid hematopoietic neoplasms in mice<sup>27</sup> (Figures 4B and S5A). Immunostaining of the BM confirmed the accumulation of the mutant p53 in leukemic clones (Figure 4C), which were scattered throughout the bone marrow (Figure S5B), while normal stromal and hematopoietic cells (Tomato-positive) did not show p53 positivity (Figure 4E). GFP+ leukemia blast cells were observed in the PB, indicating the progression and systemic spread of the disease (Figures 4D and 4F). The *Mx-p53<sup>mut/fl</sup>* mice exhibited a significant incidence of mixed leukemia/lymphoma phenotype (50%). In contrast, all *Mx-Mdm-p53<sup>mut/fl</sup>* mice presented with myeloid malignancies, showing no expansion of the lymphoid lineage (Figure 4G). The *Mx-Mdm-p53<sup>fl/fl</sup>* mice ( $n = 3$ ) developed leukemia with the same

penetrance as *Mx-Mdm-p53<sup>mut/fl</sup>*, suggesting that the role of *Mdm2* haploinsufficiency in this context was p53-independent.

To investigate the gain of function of mutant p53 in our AML model, we compared the survival rates of *Mx-p53<sup>mut/fl</sup>* mice with those of *Mx-p53<sup>fl/fl</sup>* mice. The findings suggest that the mutant p53 does not exhibit a pronounced GOF effect beyond the loss of wild-type p53 tumor-suppressing functions (Figure 4H). The survival rates of *Mx-Mdm-p53<sup>mut/fl</sup>* mice were also similar to those of *Mx-p53<sup>mut/fl</sup>* mice, suggesting that in this AML model, *Mdm2* haploinsufficiency did not alter survival (Figure 4H). Further characterization of each BM sample by flow cytometry showed that the myeloid leukemia cells were mainly CD11b<sup>+</sup>Ly6G<sup>+</sup>CD3<sup>-</sup>, while the erythroid leukemia cells were CD34<sup>+</sup>CD71<sup>+</sup> (Figure 4I). We observed that p53 mutant cells (GFP+) were highly enriched in CD71 population whereas the population of GFP<sup>neg</sup>RFP<sup>neg</sup> cells was predominantly Ter119 positive, indicating the expansion of mature erythroblasts (Figure 4I). To evaluate aneuploidy, which is a common feature of p53 mutant AML, we initially performed DNA content analysis. The population of GFP+ AML cells had higher DNA content compared to Tomato-positive p53 wild-type cells (Figure 4J). Further, whole exome sequencing analysis conducted on 16 AML samples with p53 mutations, and five control samples identified significant copy number alterations across various chromosomes (Figure 4K). Interestingly, we observed a recurrent microdeletion at 11q, and a deletion at 6q in 12 out of 16 (75%) AML samples, resembling the 7q deletion commonly found in human *TP53* mutant AML. The Integrative Genomics Viewer analysis of the sequencing data verified the presence of the *Trp53* R172H mutation in all 16 samples (Figure S5C).

Colony-forming analysis of BM cells from the *Mx-p53<sup>mut/fl</sup>* mice revealed granulocyte-macrophage (GM) colonies of GFP+ cells (Figure 4L), while erythroid leukemia cells showed BFU colonies (Figure 4M). In subsequent secondary transplant studies conducted in the syngeneic background or NSG mice, no leukemia developed even after a period of 6 months, despite the presence of detectable GFP and RFP cells in the recipients, suggesting that the p53 mutant leukemia cells may possess a more mature phenotype.

In conclusion, these data demonstrate the role of p53 mutations in the development of myeloid malignancies in adult HSCs, and that *Mdm2* haploinsufficiency in adult HSCs leads to the development of myeloid, but not lymphoid malignancies.

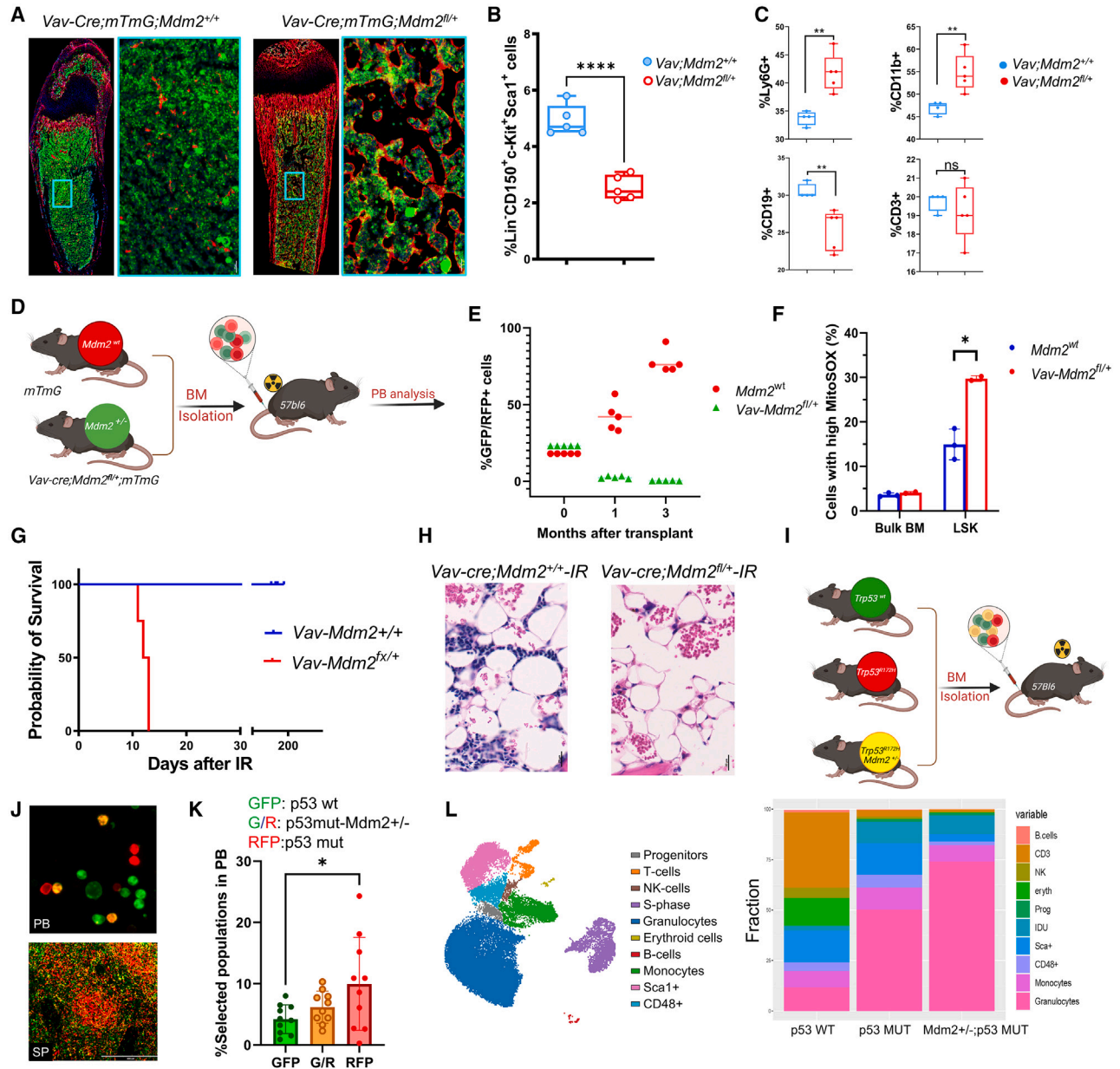
### *Mdm2* haploinsufficiency leads to myeloid-biased hematopoiesis

Both *Mdm2* and p53 play crucial roles in the regulation of HSC differentiation and hematopoiesis.<sup>28</sup> To explore the role of *Mdm2* in hematopoietic differentiation and myeloid-biased hematopoiesis, we focused on studying the hematopoietic phenotype of *Mdm2* haploinsufficient hematopoietic cells using *Vav-Cre;Mdm2<sup>fl/+</sup>;mTmG* which will hereafter be referred to as *Vav-Mdm<sup>fl/+</sup>* mice. Evaluation of BM cells of *Vav-Mdm<sup>fl/+</sup>* mice at 10 days post-birth showed a reduction in cellularity compared

(L) Colony forming unit (CFU) assay shows granulocyte macrophage (GM) colonies under brightfield and fluorescence, highlighting the clonogenic ability of GFP+ cells, with Tomato+ cells showing no colony formation.

(M) Erythroid colony formation in AEL mice is shown through brightfield and direct fluorescence images, showing expansion of GFP+ colonies.





**Figure 5. Myeloid-biased hematopoiesis resulting from *Mdm2* haploinsufficiency**

(A) Direct fluorescence images of longitudinal sections of femurs derived from the indicated mice on postnatal day 10. GFP marks hematopoietic cells. A high-magnification view of the boxed region in the left panel is shown in the right panel.

(B) Quantification of the expression of the LSK population expression in the indicated mice. Each data point represents a measurement from an individual mouse; bars indicate means, and whiskers indicate SDs. \*\*\*\* $p < 0.0001$ ,  $n = 5$ .

(C) Expression of CD19, CD11b, Ly6G, and CD3 presented as percentages of total white blood cells (means  $\pm$  SDs). \*\* $p < 0.01$ .

(D) Schematic depiction illustrating the experimental setup wherein BM cells from indicated donor mice were transplanted into recipient mice.

(E) Dot plot showing the percent of chimerism in indicated time points ( $n = 5$ ).

(F) Quantification of ROS levels (means  $\pm$  SDs) in indicated populations of BM isolated from indicated mice. \* $p < 0.01$ ,  $n = 3$ .

(G) Kaplan-Meier analysis of the overall survival of indicated mice after irradiation.

(H) Representative histologic images of hematoxylin and eosin (H&E)-stained bone marrow sections from the indicated mice, taken 1 week after irradiation (IR).

(I) Schematic view of the transplant strategy. Mice were transplanted with a mixture of bone marrow cells-GFP (Trp53wt), Tomato (p53 mut), and GFP+Tomato (p53 mut, *Mdm2* het) in equal proportions.

(J) Fluorescence images of peripheral blood (PB) and spleen show clone distribution.

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to the control mice (Figure 5A). Furthermore, BM cells from *Vav-Mdm<sup>fl/+</sup>* mice showed a significant reduction in LSK cells (Figure 5B), and a significant reduction in PB counts, coupled with an increase in the myeloid lineage and a decrease in the erythroid lineage (Figures S6A and S6B). Flow cytometry analysis of BM-derived cells indicated a significant increase in CD11b<sup>+</sup> and Ly6G<sup>+</sup> cell populations in *Vav-Mdm<sup>fl/+</sup>* mice, suggesting myeloid-biased hematopoiesis (Figure 5C).

To investigate the effects of *Mdm2* haploinsufficiency on hematopoietic stem/progenitor cells, we conducted a comprehensive analysis using both *in vitro* (colony formation and serial replating assays) and *in vivo* (competitive repopulation) functional assays. LSK cells were isolated from *Vav;Mdm<sup>fl/+</sup>* and control mice to evaluate their colony-forming capabilities. Notably, the *Vav;Mdm<sup>fl/+</sup>* mice demonstrated small colonies and increase in colony-forming unit-granulocyte-macrophage (CFU-GM) progenitors, and a significant reduction in colony formation in serial replating assays (Figures S6C–S6E). These findings suggest that *Mdm2* haploinsufficiency specifically impacts the formation of GM progenitors, potentially leading to alterations in myeloid lineage development.

Long-term repopulation assay in irradiated recipient mice over 3 months was performed to evaluate the HSC self-renewal and differentiation potential. Equal numbers of BM cells from *Vav;Mdm<sup>fl/+</sup>* (GFP+) and control *mTmG* mice (Tomato+) were transplanted into lethally irradiated recipients and monitored to assess engraftment and reconstitution ability (Figure 5D). A significant decrease in engraftment and multilineage reconstitution ability was observed in *Vav;Mdm<sup>fl/+</sup>* compared with control mice (Figure 5E). Flow cytometry analysis of BM cells derived from *Vav-Mdm2<sup>fl/+</sup>* mice further revealed higher levels of reactive oxygen species (ROS) in the LSK population (Figure 5F), suggesting that *Mdm2* haploinsufficiency may contribute to increased oxidative stress and impaired HSC function. In addition, *Vav;Mdm<sup>fl/+</sup>* mice displayed heightened radiosensitivity and lethal hematopoietic failure following irradiation (Figure 5G). Histological examination of the BM in *Vav;Mdm<sup>fl/+</sup>* mice after irradiation revealed a broad loss of hematopoietic lineage, indicating the essential role of *Mdm2* levels in maintaining hematopoietic integrity after DNA damage (Figure 5H). These data indicate that *Mdm2* haploinsufficiency in *Vav-Mdm2<sup>fl/+</sup>* mice leads to impaired HSC function and maintenance in response to DNA damage stress.

Next, we examined if the observed phenotype in *Vav;Mdm<sup>fl/+</sup>* mice was p53 dependent. Deletion of p53 in *Vav;Mdm<sup>fl/+</sup>* mice rescued the LSK population and improved colony formation, showing that p53 loss can counteract some defects in *Mdm2<sup>+/-</sup>* HSCs (Figures S6F and S6G). To evaluate the phenotype *in vivo*, we conducted a competitive transplant experiment using BM cells from *Vav;mTmG* (p53WT-green), *Vav;Rosa<sup>Lsl-TdTomato</sup>;p53<sup>mut/fl</sup>* (p53-mutant, red) and *Vav;Rosa<sup>Lsl-TdTomato</sup>;mTmG;Mdm-p53<sup>mut/fl</sup>* (p53-mutant-*Mdm2<sup>+/-</sup>*,

yellow) cells (Figure 5I). Six weeks post-transplant, these transplanted populations were detectable in the PB and spleen (Figure 5J). Notably, the population of p53 mutant cells was significantly higher than the other groups (Figure 5K). To further investigate the impact of *Mdm2* haploinsufficiency on the hematopoietic hierarchies, we performed multiplexed CyTOF analysis of the BM in indicated mice. We employed unsupervised analysis to identify distinct lineages in the BM and assessed the impact of each genetic modification on cell composition. Notably, our analysis revealed the preferential expansion of granulocytic lineages in p53-mutant-*Mdm2<sup>+/-</sup>* BM cells (Figure 5L). This expansion occurred at the expense of lymphoid lineage and other lineages, indicating a significant skewing of BM differentiation trajectories toward myeloid differentiation. This result further supports our findings that the *Mdm2* haploinsufficiency promotes differentiation toward the myeloid lineage, and that this effect is p53-independent.

Our collective findings suggest that *Mdm2* haploinsufficiency leads to impaired HSC function, including myeloid-biased differentiation, engraftment defect, and increased oxidative stress. Moreover, these effects on differentiation appear to be independent of p53 function.

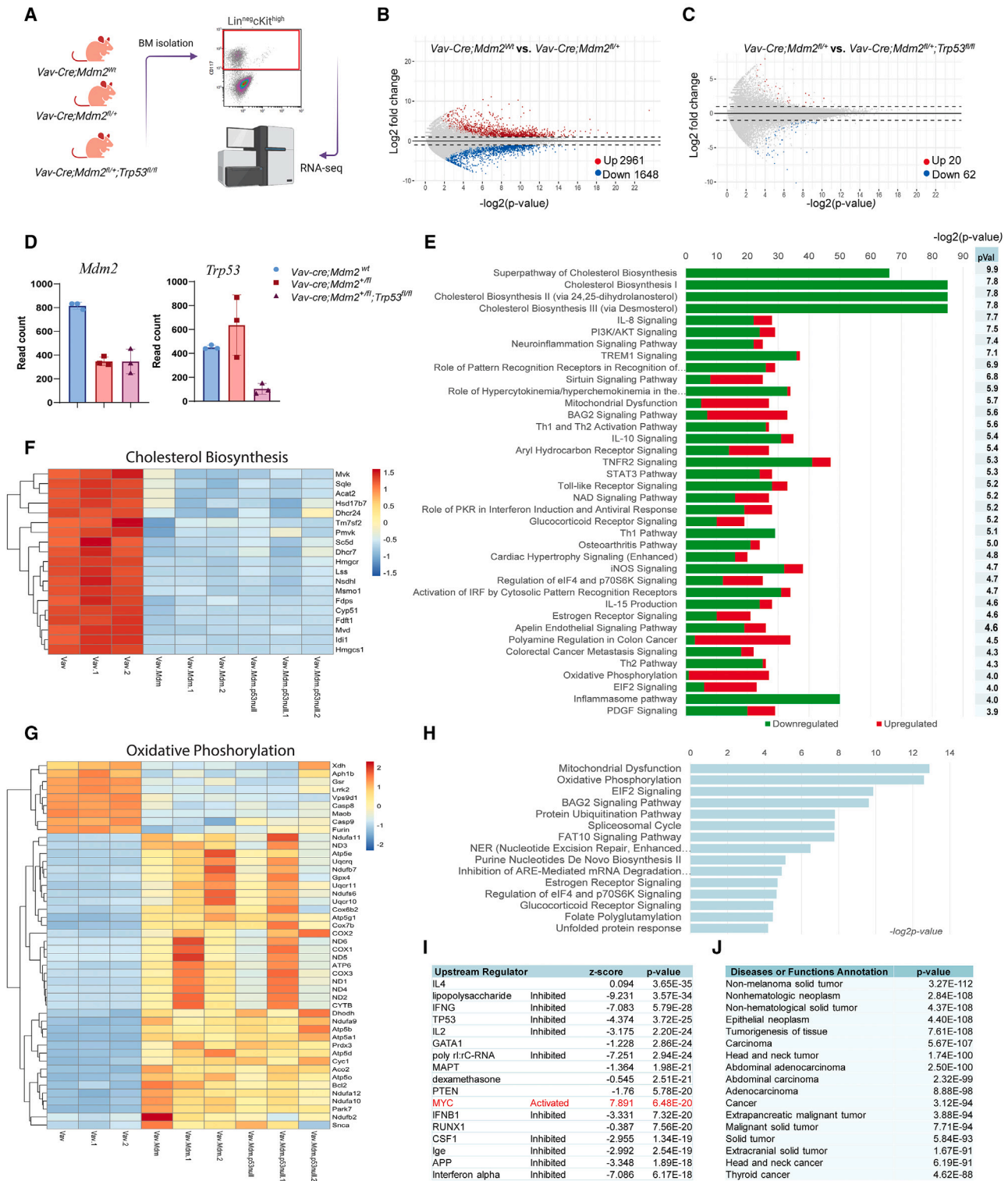
### Mechanisms of *Mdm2* haploinsufficiency in myeloid-biased hematopoiesis

To investigate how *Mdm2* haploinsufficiency results in myeloid-biased hematopoiesis, we conducted RNA sequencing (RNA-seq) on Lin-ckit<sup>+</sup> (LK) cells isolated from the BM of adult *Vav;Mdm<sup>Wt</sup>*, *Vav;Mdm<sup>fl/+</sup>*, and *Vav;Mdm<sup>fl/+</sup>;p53<sup>fl/fl</sup>* mice (Figure 6A). We observed 2,961 upregulated and 1,648 downregulated genes ( $\pm 1.5$ -fold, false discovery rate [FDR] = 0.05) in *Vav;Mdm<sup>fl/+</sup>* compared with *Vav;Mdm<sup>Wt</sup>* mice (Figure 6B). Surprisingly, the gene expression signature of LK cells isolated from *Vav;Mdm<sup>fl/+</sup>;p53<sup>fl/fl</sup>* mice was very similar to that of *Vav;Mdm<sup>fl/+</sup>* mice (Figure 6C), indicating that the gene signature associated with *Mdm2* haploinsufficiency was independent of p53. Deletion of *Trp53* in *Vav;Mdm<sup>fl/+</sup>* mice only led to upregulation of 20 genes and downregulation of 62 genes (Figure 6C). The transcript level of *Mdm2* was reduced by 50% in *Vav;Mdm2<sup>fl/+</sup>* and *Vav;Mdm<sup>fl/+</sup>;p53<sup>fl/fl</sup>* mice, implying that *Mdm2* copy numbers, and not p53 levels, determine its expression levels (Figure 6D).

Ingenuity pathway analysis (IPA) of differential gene expression revealed significant downregulation of cholesterol biosynthesis and the mevalonate pathway ( $-\log_2 p = 20$ ) in *Vav;Mdm<sup>fl/+</sup>* mice compared with *Vav;Mdm<sup>Wt</sup>* mice (Figure 6E). In addition, TREM1 signaling, Th1 and Th2 activation, and IL8 signaling, were significantly enriched in the downregulated gene set (Figure 6E). Strikingly, 85% of genes involved in cholesterol biosynthesis, including seven encoding enzymes within the mevalonate pathway (*Hmgcr*, *Mvk*, *Mvd*, *Fdps*, *Sqle*, *Lss*, *Dhcr7*), were downregulated in *Vav;Mdm<sup>fl/+</sup>* mice (Figure 6F). Importantly, deletion of *Trp53* in

(K) The bar plot shows cell population frequencies in peripheral blood 4 weeks after transplant, identified by flow cytometry. Each data point represents a measurement from an individual mouse; bars indicate means. \* $p < 0.01$ .

(L) UMAP visualization depicting distinct cell subsets identified in pooled BM samples cells-GFP (Trp53wt), Tomato (p53 mut), and GFP+Tomato (p53 mut, *Mdm2* het). Different cell lineages are color-coded for easy identification. The stacked bar plots provide a summary of subset frequencies across the specified genotypes.



**Figure 6. Mdm2 haploinsufficiency represses the mevalonate pathway independently of p53**

(A) Schematic representation of experimental design. The total RNA extracted from Lin<sup>-</sup> cKit<sup>high</sup> cells sorted from the indicated mice were analyzed by RNA sequencing.

(B and C) Mean-average plots of Log2 mean expression vs. Log2 fold change in mice with the indicated genotypes. Red dots indicate genes that were upregulated (Log2FC > 1 and p < 0.05). Blue dots indicate genes that were downregulated (Log2FC < -1 and p < 0.05).

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*Vav*; *Mdm2*<sup>fl/+</sup> mice (*Vav*; *Mdm2*<sup>fl/+</sup>; *p53*<sup>fl/fl</sup>) did not change this signature, indicating that the mevalonate pathway downregulation was p53 independent. Furthermore, the gene signature associated with oxidative phosphorylation (OxPhos) was significantly upregulated in *Vav*; *Mdm2*<sup>fl/+</sup> mice independent of p53 (Figures 6G and 6H), suggesting a potential compensatory mitochondrial stress response in HSCs with *Mdm2* haploinsufficiency.

The key regulatory factors influencing the observed gene expression in *Vav*; *Mdm2*<sup>fl/+</sup> mice (lipopolysaccharide, IFNG, TP53, and IL2) were all downregulated (Figure 6I). Notably, the expression of *Myc* and its associated signature was upregulated in *Vav*; *Mdm2*<sup>fl/+</sup> mice (Figure 6I). IPA also showed a strong correlation between the *Mdm2* signature with the gene signature observed in non-hematological malignancies (Figure 6J), suggesting that the *Mdm2* haploinsufficiency-associated gene signature is highly enriched in cancers. In summary, these data demonstrate that *Mdm2* haploinsufficiency leads to significant changes in gene expression in HSCs, particularly in the downregulation of cholesterol biosynthesis and the upregulation of oxidative phosphorylation. These changes appear to be independent of p53, as deletion of *Trp53* did not alter the gene signature associated with *Mdm2* haploinsufficiency.

### Disruption of the mevalonate pathway by *Mdm2* haploinsufficiency, and synergistic induction of apoptosis through combined inhibition of mevalonate pathway and *Mdm2*

The mevalonate pathway, regulated by *Srebp*, is responsible for synthesizing cholesterol and isoprenoids. Statin drugs inhibit the mevalonate pathway, affecting CoQ production and blocking squalene monooxygenase (*Sqle*)-mediated cholesterol synthesis (Figure 7A). To elucidate the effects of *Mdm2* haploinsufficiency on the mevalonate pathway and its impact on hematopoietic differentiation, we compared the levels of p21, known to regulate HSC differentiation,<sup>29</sup> p53, and *Sqle* in *Vav-Mdm2*<sup>fl/+</sup> mice and control mice. The *Vav-Mdm2*<sup>fl/+</sup> mice exhibited a notable reduction in *Sqle* protein (Figure 7B). In *Mdm2* haploinsufficient mice, p53 levels were comparable to those in wild-type mice, yet p21 levels were elevated compared with the control mice (Figures 7B and 7C). Analysis of BM cells derived from irradiated mice revealed an upregulation of p21 and downregulation of *Srebp2* precursor levels in *Vav-Mdm2*<sup>fl/+</sup> mice (Figure 7D). The observed decrease in *Srebp2* expression further supports the idea that *Mdm2* haploinsufficiency may downregulate the mevalonate pathway via *Srebp2* levels.

To assess the RNA-seq findings that suggested *Eif2* signaling activation in *Mdm2* haploinsufficient HSC/progeni-

tor cells (Figure 6H), we conducted western blot analysis on spleen cells from *Vav-Mdm2*<sup>fl/+</sup> and control mice. This analysis revealed higher levels of *p-Eif2a* in the *Vav-Mdm2*<sup>fl/+</sup> mice, suggesting that *Mdm2* haploinsufficiency may activate the integrated stress response (ISR) pathway (Figures S7A and S7B). To evaluate if inhibition of the mevalonate pathway may play a role, K562 cells were treated with the ROR $\gamma$  inhibitor XY018, which is known to decrease cholesterol biosynthesis rate without affecting host cholesterol homeostasis.<sup>30</sup> This treatment led to significant decreases in *Hmgcs* and *Sqle* levels and an increase in *p-Eif2a* levels, indicating that cholesterol metabolism inhibition might trigger ISR activation (Figure 7E). However, ISR activation through Thapsigargin resulted in increased *Hmgcs* and *Sqle* levels, pointing to a complex regulatory interaction between these pathways (Figure S7C).

Next, we investigated the impact of statin-mediated inhibition of cholesterol biosynthesis on myeloid differentiation. LSK cells isolated from wild-type mice were treated with atorvastatin in colony-forming cultures, and showed a significant increase in myeloid differentiation indicated by CD11b<sup>+</sup> cells (t test, *p* = 0.0001) (Figure 7F), suggesting the involvement of the mevalonate pathway in myeloid differentiation. Furthermore, treating p53-null HL60 cells with atorvastatin promoted myeloid differentiation (Figure 7G), accompanied by a dose-dependent inhibition of cell proliferation (Figure 7H). These data further support the contribution of the mevalonate pathway in myeloid differentiation.

To determine whether atorvastatin could synergize with the MDM2 inhibitor (MDM2i) nutlin3a (N3a) to promote cell apoptosis in AML, we treated three AML cell lines with varying concentrations of atorvastatin as previously described,<sup>31</sup> nutlin3a, or a combination of both. We also examined the effects of atorvastatin and nutlin3a in the presence and absence of CoQ. All three cell lines were relatively sensitive to both nutlin3a and atorvastatin, but the combination was synergistic in all three cell lines (Figures 7I–7K and S7D–S7F). Notably, CoQ treatment reduced apoptotic cell death, especially in the atorvastatin/nutlin3a combination group, suggesting that inhibiting the mevalonate pathway may enhance MDM2i-mediated oxidative stress by depleting CoQ. Additionally, we measured lipid peroxidation and found that the atorvastatin/nutlin3a combination increased lipid peroxidation (Figure 7L). Together, these results reveal a role for the mevalonate pathway in myeloid differentiation and suggest that a combination of statins and MDM2 inhibitors is potentially effective in the treatment of AML.

(D) Bar graphs representing the average read counts of genes in the indicated mice (mean  $\pm$  SD).

(E) Ingenuity pathway analysis (IPA) of differentially expressed genes (DEGs) representing the percentage of significant DEGs out of the total genes in each pathway that were downregulated (green) or upregulated (red) in *Mdm2*-haploinsufficient hematopoietic progenitors, ranked by *p* values (*p*Val).

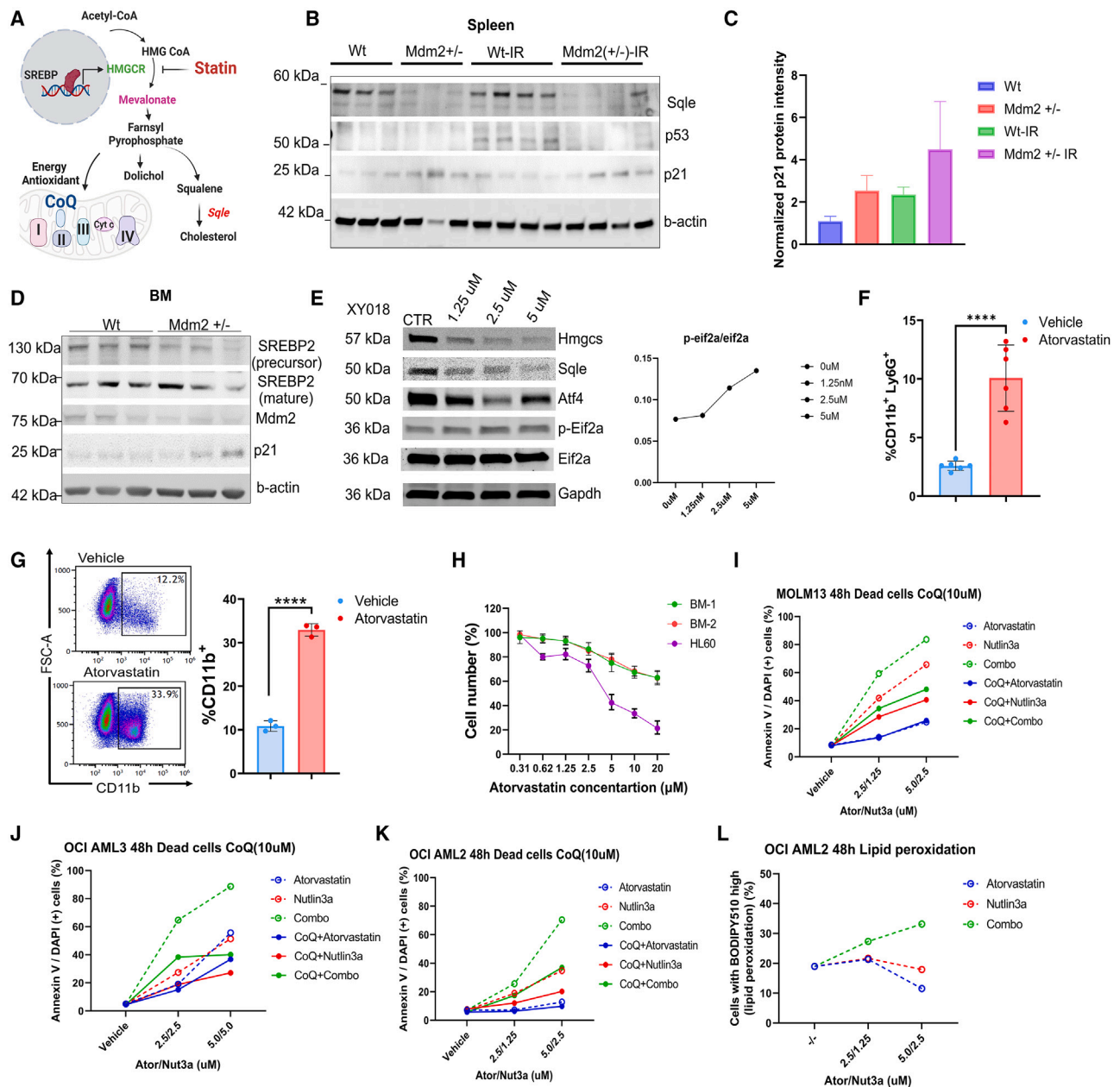
(F) Heatmap representing changes in gene expression of the cholesterol biosynthesis pathway in *Vav-Cre;Mdm2*<sup>fl/+</sup> mice compared with *Vav-Cre;Mdm2*<sup>+/+</sup> mice and *Vav-Cre;Mdm2*<sup>fl/+</sup>; *Trp53*<sup>fl/fl</sup>.

(G) Heatmap representing changes in gene expression of the oxidative phosphorylation (OxPhos) pathway.

(H) IPA analysis of upregulated genes in *Mdm2*-haploinsufficient mice.

(I) IPA upstream regulator analysis of DEGs in *Vav-Cre;Mdm2*<sup>+/fl</sup> vs. *Vav-Cre;Mdm2*<sup>+/+</sup> mice showing inhibition of indicated upstream regulators and activation of *Myc* (red).

(J) IPA disease or functions annotation of DEGs between *Vav-Cre;Mdm2*<sup>+/fl</sup> vs. *Vav-Cre;Mdm2*<sup>+/+</sup> mice.



**Figure 7. Inhibition of mevalonate metabolism induces myeloid differentiation and synergizes with MDM2i to induce apoptosis in AML cells**  
 (A) Schematic representation of the mevalonate pathway. The mevalonate pathway, regulated by SREBP, is responsible for synthesizing cholesterol and isoprenoids. Statin drugs inhibit SREBP activation, affecting CoQ production and blocking Sqle-mediated cholesterol synthesis.  
 (B) Western blot analysis of Sqle, p53, p21, and b-actin levels in spleen cells derived from *Vav-Cre;Mdm2<sup>+/-</sup>* and *Vav-Cre;Mdm2<sup>+/-</sup>/IR*, before and after irradiation (6 Gy). The figure displays bands corresponding to the molecular weights of the respective proteins.  
 (C) Quantification of p21 protein levels in (B), normalized to beta-actin. Bar graphs represent the mean protein expression level relative to the control, with error bars indicating the standard error of the mean (SEM) based on two separate blots.  
 (D) Western blot analysis of SREBP, Mdm2, p21, and  $\beta$ -actin protein levels in bone marrow cells derived from *Vav-Cre;Mdm2<sup>+/-</sup>* and *Vav-Cre;Mdm2<sup>+/-</sup>/IR*, after irradiation (6 Gy). The bands at the corresponding molecular weights are displayed.  
 (E) Western blot analysis of K562 cells treated with indicated concentrations of XY018. The figure displays bands corresponding to the molecular weights of the respective proteins. The normalized levels of phospho-eIF2 $\alpha$  are shown on the right.  
 (F) Quantification of myeloid cells (CD11b<sup>+</sup>) in CFU culture treated with atorvastatin, displaying means  $\pm$  SDs. Each data point represents a measurement from an individual mouse. \*\*\*\**p* < 0.0001.  
 (G) Representative flow cytometry plot and quantification of the frequency of CD11b<sup>+</sup> in HL60 cells after 48 h treatment with atorvastatin. \*\*\*\**p* < 0.0001.

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## DISCUSSION

AML remains a challenging disease with poor outcomes, particularly in cases involving *TP53* mutations, yet the precise molecular mechanisms underlying the disease remain elusive, primarily due to the lack of suitable mouse models. The *Trp53*<sup>R172H</sup> mutation in mice and its human equivalent, the R175H allele, contribute to abnormal self-renewal and enhanced cell proliferation in AML.<sup>17</sup> Our study, utilizing an inducible *Trp53*<sup>R172H</sup> mouse model and *Mdm2* heterozygous deletion models, reveals new insights into the cooperative effects of *Trp53* mutations and *Mdm2* haploinsufficiency on clonal hematopoiesis and myeloid lineage development. While *Mdm2* has previously been associated with cell-cycle progression independently of p53,<sup>32</sup> our findings uncover an additional p53-independent role for *Mdm2* in regulating the mevalonate pathway and cellular stress response, which influence myeloid differentiation in hematopoietic cells.

*Mdm2* plays a critical role in regulating cell fate decisions within the hematopoietic system by modulating p53 levels, thereby balancing self-renewal and differentiation in HSCs.<sup>33,34</sup> Our investigation into the hematopoietic defects observed in conditional *Mdm2* haploinsufficient mice revealed that, contrary to our expectation, p53 levels did not increase in BM cells of these mice, even after irradiation-induced DNA damage, suggesting that the phenotype of *Mdm2* haploinsufficiency in BM cells is not solely attributed to increased p53 activity. However, the upregulation of p21, a p53 target gene, suggests a potential p53-independent mechanism, consistent with previous reports of MDM2 inhibition leading to p53-independent increases in p21 levels.<sup>35</sup> Furthermore, *Mdm2* haploinsufficiency still promoted granulocytic differentiation even in the absence of p53, aligning with previous reports of its p53-independent role in differentiation,<sup>36</sup> such as the induction of granulocytic differentiation in p53 null HL-60 cells by nulin-3a.<sup>37</sup> In the context of hematologic malignancies, the presence of *MDM2*(SNP309) was found as a biomarker associated with worse clinical outcomes in AML patients.<sup>38</sup>

Recent studies underscore the significance of HSC age in determining susceptibility to transformation and the type of malignancy that arises.<sup>39,40</sup> Aging HSCs are associated with myeloid-biased hematopoiesis, linked to myeloid malignancies like AML.<sup>41,42</sup> The observed activation of ISR pathway in *Mdm2* haploinsufficient BM cells suggest an increased state of cellular stress, potentially accelerating the aging process in HSCs. Furthermore, *Mdm2* haploinsufficiency compromises the self-renewal and differentiation potential of HSCs, ultimately leading to a skewing toward myeloid lineage. Therefore, further investigations into how ISR activation influences HSC aging in the context of *Mdm2* haploinsufficiency are essential. These studies provide invaluable insights into the mechanisms underlying age-related hematopoietic disorders.

The genetic mouse model of *Mdm2* haploinsufficiency offers insights into the p53-independent role of *Mdm2* in the maintenance

and differentiation of HSCs. Pharmacological inhibition of MDM2, unleashing the pro-apoptotic effects of p53, holds promise as a treatment approach for malignancies with intact *TP53*.<sup>43,44</sup> However, concerns arise regarding the possible selection of *TP53*-mutant subclones with GOF activities.<sup>45,46</sup> Previous research, including our own, has indicated that MDM2 inhibition may inadvertently promote the expansion of *TP53*-mutant clones.<sup>47–49</sup> Our findings, demonstrating no significant difference between p53 mutant and p53 null groups, suggest that mutant p53 in our model lacks pronounced GOF effects. These results align with recent studies in the field,<sup>50,51</sup> further supporting the notion that the mutant p53 does not exhibit enhanced oncogenic properties beyond loss of wild-type p53 function.

*Mdm2* haploinsufficiency was previously shown to alter the tumor spectrum in p53-null mice, showing lower incidence of lymphoma.<sup>52,53</sup> Importantly, our data clarify the role of *Mdm2* haploinsufficiency in leukemogenesis, demonstrating that *Mdm2* haploinsufficiency can promote granulocytic differentiation of p53 null BM cells partly through inhibition of the mevalonate pathway. While our findings highlight the significance of *Mdm2* haploinsufficiency in myeloid-biased hematopoiesis, its specific role in the transition from clonal hematopoiesis to AML warrants further investigation. We propose that dysregulation of *Mdm2* and p53 pathways may contribute to clonal expansion and genomic instability, facilitating the progression from pre-leukemic states to overt AML. However, elucidating the precise mechanisms underlying this transition requires additional research.

## Limitations of the study

Our study sheds light on the interaction between *Mdm2* haploinsufficiency and mutant p53 in AML development. However, given the heterogeneity of AML in humans, our findings may primarily apply to specific AML subsets, especially those with p53 mutations and *MDM2* LOH, hence our results might not extend to all AML patient scenarios. Moreover, while we provide valuable insights into the roles of *Mdm2* and mutant p53 in the context of animal models, especially regarding myeloid-biased hematopoiesis, we recognize that our study does not fully elucidate the mechanisms of leukemogenesis. Alternative mechanisms and the direct translation of these findings to human disease requires further research.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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(H) Percentage of live cells in indicated cells treated with different concentration of atorvastatin. Error bars indicate the standard error of the mean (SEM), representing the variability within the experimental replicates.

(I–K) Percentage of dead cells defined as Annexin V/DAPI(+) cells in indicated cell lines treated with indicated compounds and concentrations.

(L) Percentage of cells with high peroxidation index treated with indicated compounds.

- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
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  - DNA isolation and SNP genotyping
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xcrm.2024.101558>.

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### AUTHOR CONTRIBUTIONS

Conceptualization: R.P. and M.A. Methodology: R.P. and M.G. Software: B.L., M.M., T.L., and P.V.L. Investigation: R.P., R.H.M., H.A., L.O., S.K., R.Z., J.D.K., N.B., and M.G. Resources: M.A. and R.P. Writing – original draft: R.P. Writing – review & editing: M.A. Supervision: P.V.L. and M.A. Project administration: R.P. Funding acquisition: R.P. and M.A.

### DECLARATION OF INTERESTS

The authors declare no potential conflicts of interest.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
4% paraformaldehyde	Invitrogen	Cat# FB002
Optimal Cutting Temperature (OCT)	Tissue-Tek	Cat# 4583
5-Iodo-2'-deoxyuridine (IdU)	Acros Organics	Cat# AC122350000
MitoSOX Red	Molecular Probes	Cat# M36008
C11 BODIPY 581/591	Molecular Probes	Cat# D-3861
Methylcellulose-based medium (MethoCult)	StemCell Technologies	Cat# M3534 or M3434
RBC lysis buffer	BD Biosciences	Cat# 555899
Protease/Phosphatase Inhibitor Cocktail	Cell Signaling	Cat# 5872
Xylene	Fisher Scientific	Cat# X5-1
Ethanol, Absolute	Fisher Scientific	Cat# E7023
Hydrogen Peroxide, 3% Solution	Sigma-Aldrich	Cat# H1009
Hematoxylin	Sigma-Aldrich	Cat# MHS16
<b>Antibodies</b>		
Rabbit monoclonal anti-Ki67	Abcam	Cat# ab15580
Rabbit polyclonal anti-p53	Leica	Cat# CM5P-L
Rat monoclonal anti-CD3	Biolegend	Cat# 100202
Chicken anti-Goat IgG Alexa Fluor 647	Invitrogen	Cat# A-21469
Donkey anti-Rabbit IgG Alexa Fluor 647	Invitrogen	Cat# A-31573
DAPI	Life Technologies	Cat# D3571
VECTASHIELD Mounting Medium	Fisher	Cat# H-1000
CD117 (c-Kit) PE/Cy7	BioLegend	Cat# 105814
Ly-6A/E (Sca-1) Alexa Fluor 700	BD Biosciences	Cat# 565981-82
Lineage Cocktail Pacific Blue	BioLegend	Cat# 133306
CD150 APC (SLAM)	BioLegend	Cat# 115910
CD19 FITC	BD Biosciences	Cat# 557398
CD11 b PE	BD Biosciences	Cat# 557397
Ly6G APC Cy7	BD Biosciences	Cat# 560600
CD3 PE	BD Biosciences	Cat# 555275
SREBP2 antibody	Invitrogen	Cat# PA1-338
MDM2 antibody	Sigma-Aldrich	Cat# M4308
Sqle antibody	Cell Signaling	Cat# 40659
p21 antibody	Cell Signaling	Cat# 556430
eIF2a antibody	Cell Signaling	Cat# 2103
Phospho-eIF2a (Ser51) antibody	Cell Signaling	Cat# 53085
ATF-4 antibody	Cell Signaling	Cat# 11815
b-actin antibody	Cell Signaling	Cat# A5316
Alpha-tubulin antibody	Cell Signaling	Cat# 2125
Histone H3 antibody	Cell Signaling	Cat# 3638
<b>Software and algorithms</b>		
PhenoChart	PerkinElmer	Available from PerkinElmer
Vectra Polaris	PerkinElmer	PerkinElmer
<b>Experimental models: Organisms/strains</b>		
Mdm2-floxed mice	Jackson Laboratory	Mdm2tm2.1Glo/J

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
mTmG mice	Jackson Laboratory	B6.129(Cg)-Gt(ROSA)26Sortm4 (ACTB-tdTomato,-EGFP)Luo/J
Ai14 reporter	Jackson Laboratory	RosaLsl-TdTomato
Trp53-floxed mice	Jackson Laboratory	B6.129P2-Trp53tm1Brn/J
Vav-Cre mice	Jackson Laboratory	B6.Cg-Commd10Tg(Vav1-icre)A2Kio/J
Mx1-cre mice	Jackson Laboratory	B6.Cg-Tg1Cgn/J
Trp53 <sup>wmR172H</sup> mice	Previously described	Pourebahim et al. <sup>19</sup> and Zhang et al. <sup>20</sup>
<b>Deposited data</b>		
Differential gene expression data of murine LK cells in <i>Mdm2</i> haploinsufficient mice	This paper	Dryad: <a href="https://doi.org/10.5061/dryad.s4mw6m9dr">https://doi.org/10.5061/dryad.s4mw6m9dr</a>

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michael Andreeff ([mandreeff@mdanderson.org](mailto:mandreeff@mdanderson.org)).

**Materials availability**

All unique/stable reagents generated in this study are available from the [lead contact](#) with a completed Materials Transfer Agreement.

**Data and code availability**

- All primary microscopy and western blot data generated in this study are available from the [lead contact](#) upon request.
- This paper does not report original code.
- Any additional information required to reanalyze or reproduce data reported in this paper is available from the [lead contact](#) upon request.

**EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

**Animal studies**

The following mouse models were utilized in this study: *Mdm2*-floxed mice (*Mdm2*<sup>tm2.1Glo/J</sup>), mTmG mice (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J), Ai14 reporter (*Rosa*<sup>lsl-TdTomato</sup>), *Trp53*-floxed mice (B6.129P2-*Trp53*<sup>tm1Brn/J</sup>), *Vav-Cre* mice (B6.Cg-*Commd10*<sup>Tg(Vav1-icre)A2Kio/J</sup>), and *Mx1-cre* (B6.Cg-Tg1Cgn/J), which were obtained from the Jackson Laboratory. *Trp53*<sup>wmR172H</sup> mice were previously described.<sup>19,20</sup> Male and female mice aged between 2 and 6 months were used for the transplantation experiments, ensuring age-matching within each experimental group. Activation of the *Mx1-Cre* recombinase was achieved by intraperitoneal injections of plpC. Genotyping was conducted by PCR analysis using tail DNA samples. All animals were housed in compliance with the approved protocols of the Institutional Animal Care and Use Committee (IACUC) at MD Anderson.

**METHOD DETAILS**

**Fluorescence microscopy**

Isolated tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (Invitrogen, FB002) overnight. Bones were washed in PBS and decalcified in 14% EDTA solution for 10 days at 4°C. Bone samples were subsequently washed in PBS and immersed in 30% sucrose PBS solution overnight. Bones and soft tissues were transferred and submerged in optimal cutting temperature (OCT) compound (Tissue-Tek, 4583). The embedded tissue was cut into 5-µm sections using a cryostat. The sections were then stored as frozen slides at -80°C or further processed. For further processing, tissue slides were washed with PBS and then incubated in 3% BSA for 1 h at room temperature. After three subsequent 5-min washes in PBS, primary antibody incubation against anti-Ki67 antibody (Abcam, ab15580), p53 (CM5, Leica P53-CM5P-L), and CD3 (BioLegend, 100202) was performed at antibody-specific dilutions in a light-protected hydration chamber at 4°C. Secondary antibody staining against the primary antibody host species (Chicken anti-Goat IgG Alexa Fluor 647, Invitrogen, A-21469; and Donkey anti-Rabbit IgG Alexa Fluor 647, Invitrogen, A-31573) was done at dilutions of 1:500 for 1 h at room temperature. Tissue slides were washed thrice in PBS for 5 min, stained with DAPI (Life Technologies, D3571) at 1:750 for 5 min and rinsed with PBS. Slides were mounted with VECTASHIELD Mounting Medium (Fisher, H-1000) and sealed with nail polish.

### CyTOF analysis

Bone marrow samples were collected using aseptic techniques by flushing the long bones with cold PBS. After removing red blood cells (RBC lysis), the bone marrow suspension was transferred to sterile tubes. To eliminate contaminants, the cell pellet was washed multiple times with PBS. A panel of 31 antibodies, targeting specific cellular markers of interest in the bone marrow, was designed and selected (Table S2). Antibody cocktails were prepared by combining the individual antibodies in appropriate dilution buffers. The bone marrow cells obtained from the pellet were resuspended in staining buffer, and the antibody cocktails were added to the cell suspension. The mixture was then incubated in the dark at room temperature to allow the antibodies to bind to the target markers. Next, the stained cell suspension was loaded onto the CyTOF instrument. To mark cells in the S-phase of the cell cycle, the cells were labeled with 5-Iodo-2'-deoxyuridine (IdU) (Acros Organics) at a final concentration of 10  $\mu$ M IdU for 30 min at 37°C. Following intracellular staining, the cells were washed twice and resuspended in an intercalator solution (1.6% paraformaldehyde in PBS with 125 nM iridium nucleic acid intercalator). The suspension was then incubated at 4°C overnight and acquired at a rate of 300 events per second on the Helios instrument, using CyTOF Software version 6.7.1016 (Standard Bio Tools, San Francisco, CA). Subsequently, data processing of samples was performed using FlowJo version 10.8.1.

### Imaging and spectral unmixing

Frozen tissue sections and immunofluorescence-stained slides were imaged using a Vectra Multispectral Imaging System version 2 (Akoya Biosciences) at a magnification of 40 $\times$ . The images were then visualized using Phenochart slide viewer software (Akoya Biosciences). Spectral unmixing was performed using inForm Analysis software (Akoya Biosciences), as previously described. Briefly, the software identifies the spectral signature of each fluorescent probe in the sample and separates overlapping signals into distinct channels based on their unique spectral properties. This process allows for accurate identification and quantification of individual markers in complex samples.

### Flow cytometry and ROS analysis

Bone marrow (BM) single-cell suspensions were obtained by intrafemoral flushing and crushing of bone, followed by filtration through 40- $\mu$ m strainers (Fisher, 08-771-1). Red blood cells (RBCs) were lysed in 1x RBC lysis buffer (BD Biosciences, 555899) for 15 min with slight agitation at room temperature. After two washes in PBS, cells were stained with the following antibodies: CD117 (c-Kit) PE/Cy7 (BioLegend, 105814), Ly-6A/E (Sca-1) Alexa Fluor 700 (BD Bioscience, 565981-82), Lineage Cocktail Pacific Blue (BioLegend, 133306), CD150 APC (SLAM) (BioLegend, 115910), CD19 FITC (BD Biosciences, 557398), CD11 b PE (BD Biosciences, 557397), Ly6G APC Cy7 (BD Biosciences, 560600), and CD3 PE (BD Biosciences, 555275).

To assess oxidative stress upon irradiation, cells were stained with MitoSOX Red (Molecular Probes, M36008) or C11 BODIPY 581/591 (Molecular Probes, D-3861), together with Ghost Dye Violet 540 (Tonbo Biosciences, 13-0879) and the aforementioned surface marker antibodies. Mitochondrial oxidative stress was determined by the percentage of cells exhibiting a high MitoSOX signal, while lipid peroxidation was assessed by the fluorescence ratio of oxidized signal (510 nm) to reduced signal (590 nm) on flow cytometry. The gating strategy is described in the Figure S8. Data were collected and analyzed using the Beckman Coulter Gallios Flow Cytometer and Kaluza Analysis Software.

### Colony assays

Isolated total bone marrow and/or progenitor cells from mice were plated at a concentration of 50,000 cells in 2 mL of methylcellulose methylcellulose-based medium (MethoCult M3534 or M3434; StemCell Technologies, Canada), containing IL-3, IL-6, mSCF, EPO. After 5–7 days, colonies were scored microscopically, harvested, and replated up to 5 times. For liquid culture assays, we washed 30,000 cells in PBS and resuspended them in either RPMI 1640 with FBS, Pen/Strep, and various growth factors, or Xvivo Medium supplemented with Flt3-ligand, mSCF, TPO.

### Immunoblot analysis

Mouse BM cells were collected, and RBCs were lysed in 1x RBC lysis buffer. After *ex vivo* irradiation of 400 cGy, cells were lysed at a density of  $1 \times 10^6/50 \mu$ L in protein lysis buffer (0.25 M Tris-HCl, 2% SDS, 4%  $\beta$ -mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling) and incubated at 95°C for 5 min for denaturing. Immunoblot analysis was performed as previously reported with some modifications. Briefly, an equal amount of protein lysate was resolved using 4–12% Bir-Tris Mini Protein Gels (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were divided, probed for target proteins, and imaged (Odyssey imaging system, LI-COR). Primary antibodies used were as follows: SREBP2 (Invitrogen, PA1-338), MDM2 (Sigma-Aldrich, M4308), Sqle (Cell Signaling #40659), p21 (BD #556430), eIF2 $\alpha$  (cell signaling #2103), Phospho-eIF2 $\alpha$  (Ser51) (cell signaling #53085), ATF-4 (cell signaling #11815),  $\beta$ -actin (Sigma-Aldrich A5316), p53 (Leica, NCL-L-p53-CM5p), alpha-tubulin (Cell Signaling #2125), and histone H3 (Cell Signaling #3638). IRDye 680LT Donkey anti-Mouse IgG and 800CW Donkey anti-Rabbit IgG (LI-COR Biosciences) were used as secondary antibodies.

### Evaluation of cytotoxicity and synergism in AML cell lines

AML cell lines (MOLM13, OCIAML2 and OCIAML3) were seeded at a density of 15,000 cells per well in 96 well plate containing complete RPMI-1640 medium supplemented with 10% fetal calf serum. The cells were treated for 48 h with Atorvastatin at doses ranging

from 0 to 10  $\mu$ M and Nutlin (N3a) at doses ranging from 0 to 5  $\mu$ M, resulting in a 6  $\times$  6 matrix of treatment. Each experiment was conducted independently three times. Viable cell numbers were assessed by quantifying ATP using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The results were normalized to the values obtained for DMSO-treated cells and expressed as a percentage of control. Dose-response curves were analyzed using a curve-fitting routine based on nonlinear regression to calculate the EC50 value. Additionally, synergistic effects of Atorvastatin with Nutlin were determined based on the mean values obtained from three independent experiments. Synergy evaluation was performed by collecting, processing, and visualizing the data using the BLISS Index method with COMBENEFIT software.

### Immunohistochemical analysis

Immunohistochemical analysis was performed to investigate the expression of p53 protein in formalin-fixed paraffin-embedded tissue sections. The VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, Pk-6101) was used for the immunohistochemical staining. Formalin-fixed paraffin-embedded tissue sections were deparaffinized by immersion in two changes of xylene, followed by two changes of 100% ethanol. Subsequently, the sections were passed through 95%, 75%, and 50% ethanol for 5 min per change. To block endogenous peroxidase activity, the tissue sections were immersed in a 3% H<sub>2</sub>O<sub>2</sub> solution in methanol for 10 min. The antigen retrieval process was performed by immersing the slides in Coplin jars containing antigen retrieval solution. The slides were then placed in an IHC-Tek Epitope Retrieval Steamer (IHC World, IW-1102) and steamed for 10 min. Afterward, the slides were allowed to cool down for 20 min at room temperature. To prevent non-specific binding, the slides were blocked for 60 min with 10% serum in 0.1% TBST (Tris-buffered saline with Tween 20). The primary antibody against p53 (CM5, Leica P53-CM5P-L) was diluted in 2% FBS (fetal bovine serum) in 0.1% TBST and incubated overnight at 4°C in a light-protected hydration chamber. Following the primary antibody incubation, the slides were washed twice for 5 min each in Tris-buffered saline (TBS). The slides were incubated with the biotinylated secondary antibody solution for 30 min at room temperature. Slides were washed twice for 5 min each in TBS. Slides were incubated with ABC solution for 30 min and washed twice for 5 min each in phosphate-buffered saline (PBS). The slides were incubated in the 3,3'-Diaminobenzidine (DAB) solution until the desired signal intensity was achieved. Excess DAB solution was washed off with deionized water, and the slides were counterstained with hematoxylin for 80 s. Slides were immersed in lithium carbonate solution for 60 s, followed by immersion in deionized water for 10 min. The slides were sequentially immersed in 95% ethanol and 100% ethanol for 90 s per change (two changes for each). Slides were immersed three times for 5 min each in xylene. Finally, the slides were mounted with Richard-Allan Scientific Mounting Medium (Thermo Scientific, 4112) for preservation and analysis. The immunohistochemically stained slides were then examined under a microscope to assess the expression pattern and intensity of p53 protein in the tissue samples.

### Cell blood counts

Mouse peripheral blood (50  $\mu$ L) was collected into BD Microtainer vials (Fisher, 02-669-33) via retro-orbital bleeding. Blood samples were analyzed on the Horiba ABX SAS PENTRA 60 C+ cell blood counter to identify hematopoietic irregularities.

### RNA sequencing

RNA was extracted utilizing the Direct-Zol RNA Microprep kit (Zymo Research, R2060). Barcoded Illumina-compatible stranded total RNA libraries were prepared using the TruSeq Stranded Total RNA kit (Illumina) as previously described.<sup>19</sup> Library pools were quantified by qPCR and sequenced on the HiSeq 4000 sequencer using the 75-bp paired-end format. The raw RNA-seq readouts were subsequently mapped to the mouse mm10 assembly reference genome using TopHat2 and analyzed with DESeq2 (Bioconductor package).

### Whole exome sequencing

Dual-indexed libraries compatible with Illumina were created using 15ng–50ng of genomic DNA, which had been sheared by a Biorupter Ultrasonicator (Diagenode) and treated with RNase. This DNA was processed using the Twist Library Preparation Kit and the Twist Universal Adapter System, following the manufacturer's instructions. The libraries, indexed and prepared for capture, underwent 6 cycles of PCR amplification with Twist UDI primers. Their fragment size distribution was then evaluated using the 4200 TapeStation High Sensitivity D1000 ScreenTape (Agilent Technologies), and their concentration was determined with the Qubit dsDNA HS Assay Kit (ThermoFisher). After normalizing the concentrations, equal amounts of the uniquely dual-indexed libraries were pooled together, with 7 libraries in each pool. For exon targeting, the Twist Mouse Core Exome kit was employed. Post-capture, the enriched exon library pools were further amplified with 6 PCR cycles. Size distribution was again assessed with the 4200 TapeStation High Sensitivity D1000 ScreenTape (Agilent Technologies), and concentration measurements were taken using the Qubit dsDNA HS Assay Kit. Quantification of the exon-enriched library pools was performed by qPCR utilizing the KAPA Library Quantification Kit (KAPABiosystems). Finally, these libraries were sequenced on a NovaSeq6000 SP-200 flow cell in a 100 nt PE format.

### Copy number analysis

Copy number detection was conducted using the latest version of Control-FREEC.<sup>54</sup> This process involves using aligned reads (bam files) as input, from which Control-FREEC creates copy number and B-allele frequency profiles, incorporating tumor purity estimates and corrections. These profiles undergo normalization, segmentation, and analysis to assign copy number events to specific

genomic regions. With the inclusion of a matched normal sample, Control-FREEC is able to distinguish between somatic and germline events. A threshold representing 50% of a chromosome arm's length serves as the criterion for differentiating between broad and focal events. To mitigate false positives from hyper-segmentation, segments underwent filtering with an amplitude threshold set at a copy-difference of 0.35 on a log scale. The frequency of broad copy number changes was quantified, followed by an analysis of focal copy number changes using the GISTIC methodology, which identifies significant peaks of amplifications and deletions. The identified copy number events were classified into two categories based on their observed frequency: focal events, which are significantly smaller than a chromosome arm, and broad events, encompassing a chromosome arm or an entire chromosome.

### **MDM2<sup>SNP309</sup> exploration**

We used alleleCount (<https://github.com/cancerit/alleleCount>) on PCAWG tumor/germline BAM files at the *MDM2*<sup>SNP309</sup> position (chr12:69,202,580 in hg19 coordinates) to compute variant allele frequency (VAF) (minimum count  $\geq 5$ ). In germlines, genotypes were directly assessed using VAF (T/T: VAF = 0; T/G:  $0.35 \leq \text{VAF} \leq 0.65$ ; G/G: VAF = 1). Copy number profiles and whole-genome doubling (WGD) information were retrieved from consensus copy number data; [https://dcc.icgc.org/releases/PCAWG/consensus\\_cnv](https://dcc.icgc.org/releases/PCAWG/consensus_cnv)). In tumors, the retained allele (either T or G) was assessed in cases with *MDM2* allelic imbalance where VAF was compared to copy number states of major and minor alleles, corrected for tumor purity. *MDM2* copy-number statuses (normal, gain, loss, and copy-neutral) were defined by considering baseline ploidy (adjusted for WGD). *TP53* mutation status was obtained from the PCAWG Drivers and Functional Interpretation Group. *TP53* single nucleotide variations and insertion/deletions were functionally annotated (e.g., missense, nonsense, frameshift) using ANNOVAR.<sup>55</sup>

### **DNA isolation and SNP genotyping**

Genomic DNA was extracted from the BM samples isolated from AML patients at diagnosis using QIAamp DNA Blood Midi kit (Qiagen). Genotyping of SNP rs2279744 (*Mdm2*<sup>SNP309</sup>) in intron 1 of the *MDM2* gene was performed with 50 ng of genomic DNA using TaqMan SNP Genotyping Assay, (human-SNP ID:rs2279744) and TaqMan Genotyping Mastermix (ThermoFisher). PCR reactions were carried out on a Quantstudio 6 Flex instrument and analyzed using TaqMan Genotyper Software (Applied Biosystems Life Technologies).

### **QUANTIFICATION AND STATISTICAL ANALYSIS**

Means and standard deviations were calculated using GraphPad Prism 6 software. The Student's t test was used for comparative analysis between two groups. Analysis of variance was used to compare multiple groups.  $p < 0.05$  was set as statistically significant. A  $p$  value of less than 0.05 was considered statistically significant. In figure legends, symbols \*, \*\*, \*\*\*, and \*\*\*\* denote  $p$  values of less than 0.05, 0.01, 0.001, and 0.0001, respectively, unless stated otherwise. All experimental procedures were replicated three times, and error bars in the figures denote mean  $\pm$  standard deviation (SD) values, unless mentioned otherwise.