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Impaired DNA damage responses and inflammatory signaling underpin hematopoietic stem cell defects in *Gata2* haploinsufficiency

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SUMMARY

Clinical *GATA2* haploinsufficiency results in immunodeficiency that evolves to leukemia. How *GATA2* haploinsufficiency disrupts the functionality of hematopoietic stem/progenitor cells (HSCs/HSPCs) to facilitate pre-leukemia development is poorly defined. Using a hematopoietic-specific conditional mouse model of *Gata2* haploinsufficiency, we identified pervasive defects in HSPC differentiation in young adult *Gata2* haploinsufficient mice and perturbed HSC self-renewal following transplantation. These alterations aligned with deregulated global DNA damage responses and inflammatory cell signaling from *Gata2* haploinsufficient HSCs. We also discovered genetic interplay between *Gata2* and *Asxl1*, a secondary mutation leading to leukemia in *GATA2* deficiency syndromes. HSCs from young adult compound *Gata2/Asxl1* haploinsufficient mice were hyperproliferative, functionally compromised after transplantation, and displayed a broad pre-leukemia transcriptomic program. Thus, *Gata2* haploinsufficiency triggers HSC genomic instability. Our data further suggest that secondary mutations like *ASXL1* exploit this impaired HSC genomic integrity to nurture a pre-leukemic state in *GATA2* haploinsufficiency syndromes.

INTRODUCTION

Hematopoietic cells with wide-ranging purpose including immune defense, blood clotting and oxygen supply to tissues originate from scarce, self-renewing hematopoietic stem cells (HSCs) housed in the bone marrow (BM) (Rieger and Schroeder, 2012). Cell-intrinsic genetic and epigenetic mechanisms combine with cell-extrinsic signaling relayed from the BM niche to regulate HSC cell cycle and apoptotic fates that assure maintenance of genomic stability in HSCs for life (Rieger and Schroeder, 2012). However, mechanisms critical for maintaining HSC integrity—governed principally by nuclear transcription factors (TFs)—can become corrupted through the acquisition and functional propagation of genetic and epigenetic mutations in HSCs and their descendants, hematopoietic stem/progenitor cells (HSPCs), increasing the probability of immunodeficiency, BM failure syndromes, hematologic malignancies, and other non-hematopoietic disorders, such as cardiovascular disease (Abdelfattah et al., 2021; Rieger and Schroeder, 2012). Aging is a major determinant for the emergence of these conditions, including leukemia. Yet leukemia can arise *de novo* in childhood or at a relatively young age through inherited genetic predisposition, exemplified by hereditary myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML) caused by germline mutations in *RUNX1*, *CEBPA*, or *GATA2* TFs (Khoury et al., 2022).

As a member of the GATA (guanine/adenine/thymine/adenine) family of zinc-finger TFs that regulate gene expression by binding to the DNA sequence WGATAR (W:A/T, R:A/G) (Tsai et al., 1989), *Gata2* is most prominently expressed in the HSC compartment, and, reflecting this expression pattern, extensive experimental modeling using loss- and gain-of-function approaches has identified *Gata2* as an indispensable regulator of HSC development from hemogenic endothelium and of adult HSC maintenance in the BM (de Pater et al., 2013; Menendez-Gonzalez et al., 2019b; Rodrigues et al., 2005). Though *Gata2* expression variably diminishes during differentiation to select hematopoietic lineages, critical roles for *Gata2* in lineage specification have been defined in granulocyte-macrophage progenitor generation and terminal differentiation of megakaryocytes, basophils, and mast

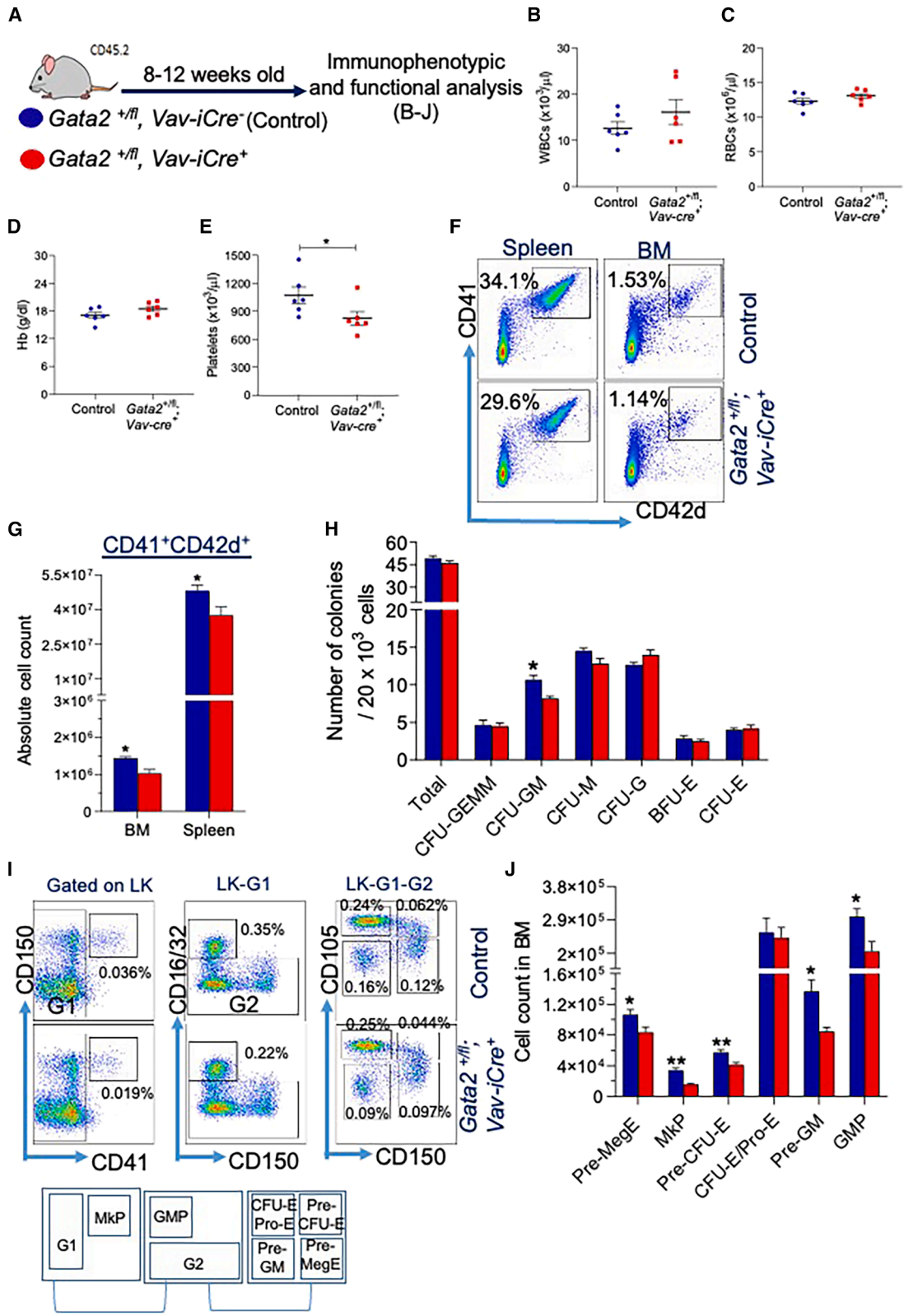
cells (Huang et al., 2009; Li et al., 2015; Rodrigues et al., 2008). These data highlight that *Gata2* acts as a crucial regulator of HSC generation during embryonic development, adult HSC maintenance, and differentiation to select hematopoietic lineages.

Mirroring the similar functional outcomes of down-regulating and up-regulating GATA2 expression level in HSPCs in loss and gain-of-function studies, respectively (Rodrigues et al., 2005, 2008; Tipping et al., 2009), deregulation of *GATA2* expression in the clinical setting has been shown to have both tumor suppressor and oncogenic roles in AML. Overexpression of *GATA2* confers poor prognosis in approximately 25% of all AML cases, implying an oncogenic role for GATA2 in AML (Vicente et al., 2012). Studies where *Gata2* was genetically deleted in mouse models of AML lead to enhanced apoptosis specifically in the therapy-resistant leukemic stem cell (LSC) compartment and reactivation of myeloid differentiation, suggesting that the *Gata2* transcriptional network may be an exploitable therapeutic vulnerability in AML (Menendez-Gonzalez et al., 2019b). As proof of concept supporting this notion, pharmacologic inhibition of GATA2 in combination with AML chemotherapy has been shown to enhance apoptosis of AML cells (Menendez-Gonzalez et al., 2019a, 2022).

In striking contrast, a tumor suppressor role for *GATA2* has also been established in MDS/AML. Hereditary *GATA2* haploinsufficiency mutations are observed throughout the *GATA2* locus, yet approximately 70% of mutations arise in the DNA-binding zinc-finger domains (Collin et al., 2015; Wlodarski et al., 2017), disrupting GATA2 chromatin binding activity and the expression of normal GATA2 target genes in hematopoiesis (Hahn et al., 2011). Germline *GATA2* haploinsufficiency mutations are largely categorized into four groups: (1) truncated mutations (i.e., nonsense, in-frame deletion and in-frame insertion mutations), which represent approximately 60% of *GATA2* haploinsufficiency cases; (2) missense mutations reported in approximately 30% patients; (3) non-coding mutations in intron-4 (+9.5kb *cis*-element), which represent about 10% of cases; and (4) sporadic cases of whole-locus deletions, N/C terminals, and untranslated region regions (Collin et al., 2015; Johnson et al., 2012; Wlodarski et al., 2017). These *GATA2* mutations, which largely result in loss of function, initially lead to primary immunodeficiency disorders such as monocytopenia and mycobacterial infection (MonoMAC); dendritic cell, monocyte, B/natural killer (NK) lymphoid (DCML) syndromes, which are characterized by a severe reduction in peripheral monocytes, $CD4^+$ lymphocytes, B cells, NK cells, and dendritic cells; and Emberger syndrome, an autosomal dominant disorder characterized by primary lymphedema of the lower limbs, cutaneous warts, and deafness (Dickinson et al., 2011; Hsu et al., 2011; Ostergaard et al., 2011; Rodrigues et al., 2012). The development of Emberger syndrome in the context of *GATA2* haploinsufficiency reflects the role of *Gata2* in lymphatic vessel development during embryogenesis (Ostergaard et al., 2011). *GATA2* haploinsufficiency-driven MonoMAC, DCML, and Emberger syndromes ultimately evolve to MDS/AML on acquisition of additional co-operating mutations, such as *ASXL1*, with variable latency and presentation (Ostergaard et al., 2011; West et al., 2014).

There are compelling imperatives to understand the mechanisms underscoring *GATA2* haploinsufficiency in HSCs/HPCs. First, widespread myeloid and lymphoid differentiation defects are observed in the setting of *GATA2* haploinsufficiency-mediated MonoMAC/DCML immunodeficiency disorder, and pan-cytopenia is similarly observed in Emberger syndrome (Dickinson et al., 2011; Hsu et al., 2011), suggesting a functional defect originating in the HSC/HSPC compartment; such a postulate is supported by extensive modeling of haploinsufficiency in genetic mouse models, zebrafish models, and short hairpin RNA (shRNA)-mediated knockdown in cord blood HSPCs (Ling et al., 2004; Menendez-Gonzalez et al., 2019b; Rodrigues et al., 2005, 2008, 2012). Furthermore, while most cases of MonoMAC as a result of *GATA2* deficiency evolve to myeloid neoplasms, some patients advance to B-cell acute lymphoblastic leukemia (Koegel et al., 2016; Nováková et al., 2016). Second, variable patient disease presentation and latency, even for similar types of *GATA2* haploinsufficiency mutations, may be ascribed to the differential genetic/epigenetic impact of similar *GATA2* mutations occurring within the heterogeneous HSC/HSPC compartment (Collin et al., 2015; Wlodarski et al., 2017). Alternatively, variations in patient progression could be due to the influence of secondary mutations that are adopted in HSCs/HSPCs from *GATA2* haploinsufficiency patients. Finally, HSC/HSPC-derived pre-LSCs and LSCs in MDS/AML provide a reservoir of leukemic cells that are not cleared by current AML treatment regimens, forming the cellular basis for therapy resistance, relapse, and fatality in AML (West et al., 2014). How the pre-LSC/LSC transcriptional program mediated by *GATA2* haploinsufficiency shapes initiation, propagation, or maintenance of leukemia in these patients remains unclear. Understanding the functional and transcriptional networks operating in *GATA2* haploinsufficient HSCs and the impact of secondary co-operating mutations in HSCs/HSPCs in *GATA2* deficiency is therefore required to conceive and identify targeted, non-toxic curative treatments rather than the prevailing, imperfect standard of care for these patients—allogeneic BM transplantation (West et al., 2014).

Germline and conditional genetic mouse models of *Gata2* haploinsufficiency have recapitulated the hereditary nature and select biological functions underlying clinical *GATA2* mutation-driven syndromes that, in the vast majority of



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patients, correlates with *GATA2* haploinsufficiency and reduced *GATA2* expression (Abdelfattah et al., 2021; de Pater et al., 2013; Ling et al., 2004; Rodrigues et al., 2005, 2008). By breeding *Gata2* "floxed" allele mice (*Gata2*$^{fl/fl}$) with constitutively active, hematopoietic-specific *Vav-Cre* mice (*Vav-iCre*$^{+}$), we previously generated *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice, which harbored a genetic deletion in a single *Gata2* allele from the onset of HSC emergence *in utero* at embryonic day 11 (Abdelfattah et al., 2021). Through analysis of young adult *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice, in this report, we expose the hitherto poorly understood mechanistic impact of *Gata2* haploinsufficiency in HSCs/HPCs.

RESULTS

Generation and validation of *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ haploinsufficient mice

To explore the function of *Gata2* haploinsufficiency in the adult hematopoietic system, *Gata2*$^{+/+}$*; Vav-iCre*$^{+}$ with *Gata2*$^{fl/fl}$*; Vav-iCre*$^{-}$ mice were crossed to produce *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ (heterozygote) and *Gata2*$^{+/fl}$*; Vav-iCre*$^{-}$ (control) littermates (Figure 1A), which led to efficient genetic deletion of a single *Gata2* allele where *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice were born in the expected Mendelian ratio and appeared phenotypically normal when compared with their control littermates (Abdelfattah et al., 2021).

Gata2 haploinsufficiency causes defective early erythroid commitment and impairs platelet numbers mapping to an early defect in megakaryocyte progenitor generation

We initially evaluated the hematopoietic potential of *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ and control (*Gata2*$^{+/fl}$*; Vav-Cre*$^{-}$) mice by complete blood count analysis. While white blood cells (WBCs), red blood cells (RBCs), and hemoglobin levels were equivalent between both genotypes, the number of platelets was significantly decreased in *Gata2*$^{+/fl}$*;Vav-Cre*$^{+}$ mice when compared to control mice (Figures 1B–1E), indicating that *Gata2* haploinsufficiency disrupts megakaryopoiesis in agreement with data implicating *Gata2* as a key regulator of megakaryopoiesis (Huang et al., 2009). To uncover the cellular basis for the defect in platelet generation in *Gata2*$^{+/fl}$*; Vav-Cre*$^{+}$ mice, we evaluated the frequency of mature CD41^{+}CD42d^{+} megakaryocyte cells in BM and spleen, which were significantly reduced in BM and spleen in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice (Figures 1F and 1G). Thus, *Gata2* haploinsufficiency perturbs megakaryocytic maturation.

To explore whether other lineage-specific hematopoietic cells in the peripheral blood (PB), BM, spleen, and thymus were affected in *Gata2*$^{+/fl}$*; Vav-Cre*$^{+}$ mice, flow cytometric analysis was performed for myeloid, erythroid, and T and B cell markers. We first assessed cellularity in hematopoietic organs in *Gata2*$^{+/fl}$*; Vav-Cre*$^{+}$ mice and found that BM, spleen, and thymic cellularity were equivalent between genotypes (Figure S1A). Furthermore, frequencies of fully differentiated myeloid, lymphoid, and erythroid cells in the PB, BM, spleen, and thymus were similar in both *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ and control mice (Figures S1B–S1E). Functional analysis of BM myelo-erythroid lineage output in colony-forming cell (CFC) assays was comparable between genotypes, except for CFC-granulocyte/macrophage (GM) frequency, which was significantly reduced in *Gata2* haploinsufficient mice as previously demonstrated (Rodrigues et al., 2008) (Figure 1H).

We next assessed the impact of *Gata2* haploinsufficiency on committed myeloid and lymphoid progenitors in BM. Committed myeloid-restricted progenitor immunophenotyping has been defined by Pronk et al. (2007). In this paradigm of hematopoietic cell differentiation, common myeloid progenitors (CMPs) give rise to either primitive granulocyte/macrophage progenitors (Pre-GMs, LK_CD150^{-}_CD41^{-}_CD16/32^{-}_CD105^{-}) that differentiate into granulocyte/macrophage progenitors (GMPs) (LK_CD150^{-}_CD41^{-}_CD16/32^{+}) or primitive megakaryocyte/erythroid progenitors (Pre-MegEs, LK_CD150^{+}_CD41^{-}_CD16/32^{-}_CD105^{-}). Pre-MegE cells segregate into either primitive erythroid-forming colonies (Pre-CFU-Es, LK_CD150^{+}_CD41^{-}_CD16/32^{-}_CD105^{+}) that further differentiate into erythroid-forming colonies and pro-erythroblasts (CFU-Es and Pro-Es, LK_CD150^{-}_CD41^{-}_CD16/32^{-}_CD105^{+}) or megakaryocyte progenitors (MkPs, LK_CD150^{+}_CD41^{+}). Our analysis revealed that Pre-GMs, GMPs, Pre-MegEs, MkPs, and Pre-CFU-Es were significantly reduced in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice (Figures 1I and 1J). These data suggest that *Gata2* haploinsufficiency causes defects in early erythroid

Figure 1. *Gata2* haploinsufficiency impairs megakaryopoiesis and myeloid progenitor development

(A) Experimental design: *Gata2*$^{+/fl}$*; Vav-iCre*$^{-}$ (control) and *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ littermates analyzed at 8–12 weeks

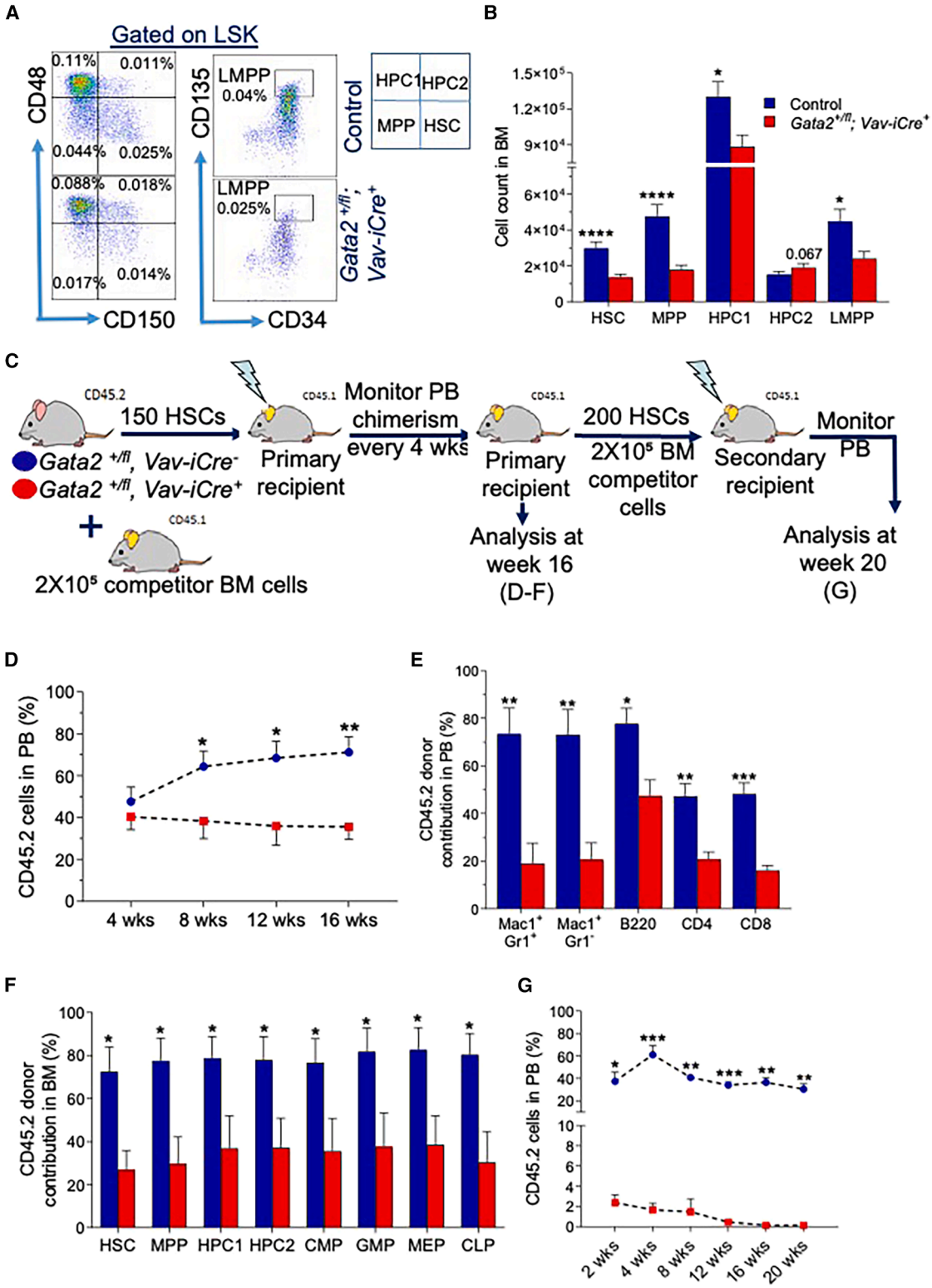
(B–E) Peripheral blood analysis: WBCs (B), RBCs (C), hemoglobin (D), and platelets (E); n = 6/genotype.

(F and G) Flow cytometry of mature megakaryocytes (CD41^{+}CD42d^{+}) in BM and spleen: frequency (F) and absolute counts (G); n = 4/genotype.

(H) CFU assay from BM cells; colonies counted at day 12; n = 6/genotype.

(I and J) Immunophenotyping and quantification of BM progenitors: myeloid (Pre-GMs, GMPs), erythroid (Pre-MegEs, Pre-CFU-Es, CFU-Es/Pro-Es), and megakaryocyte (Pre-MegEs, MkPs); n = 6/genotype.

Data: mean ± SEM. Statistical test: Mann-Whitney U; *p < 0.05, **p < 0.01.



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commitment. In contrast, common lymphoid progenitors (CLPs), defined as Lin$^-$_Sca-1^{low}_c-Kit^{low}_CD127$^+$ (Kondo et al., 1997), were unchanged between the two genotypes (Figures S1F and S1G). Early myeloid progenitor commitment in the BM, including MkP generation, was therefore compromised in the setting of *Gata2* haploinsufficiency. Together, these data suggest that reduced platelets observed in *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ mice arise from an early Pre-MegE and MkP defect and a subsequent block in differentiation toward maturing CD41$^+$CD42d$^+$ megakaryocytes.

Reduced immunophenotypic HSPC abundance in *Gata2* haploinsufficient mice

To assess if the alteration in lineage-specific hematopoietic progenitors was caused by a disruption in the frequencies of BM HSPCs in *Gata2* haploinsufficient mice, we analyzed the HSPC compartment by flow cytometry. LSK (Lin$^-$_Sca-1$^+$_c-Kit$^+$) and SLAM markers (CD150 and CD48) were used to dissect HSPC compartments as follows: HSC, LSK_CD150$^+$_CD48$^-$; hematopoietic progenitor cell 1 (HPC1), LSK_CD150$^-$_CD48$^+$; hematopoietic progenitor cell 2 (HPC2), LSK_CD150$^+$_CD48$^+$; and multi-potent progenitor (MPP), LSK_CD150$^-$_CD48$^-$ (Kiel et al., 2005; Oguro et al., 2013) (Figure 2A). HPC1 differentiates into lymphoid/myeloid restricted progenitors and are incapable of advancing to megakaryocytes/erythrocytes, whereas HPC2 differentiate to all restricted hematopoietic progenitors (Oguro et al., 2013). In parallel, HSCs give rise to lympho-myeloid progenitors (LMPPs), defined immunophenotypically as LSK_CD135^{hi}_CD34$^+$, that possess dual lymphoid/myeloid potential and overlap functionally with HPC1 (Adolfsson et al., 2005). Frequencies of HSCs, MPPs, LMPPs, and HPC1 were significantly reduced in BM from *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ compared to control mice, whereas the proportion of HPC2 was marginally increased in *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ as compared with control littermates, suggestive of a differentiation block at the transition from LMPP/HPC1 to HPC2 (Figure 2B).

Gata2 haploinsufficient HSCs exhibit a functional defect in multi-lineage repopulation ability

Having shown that *Gata2* haploinsufficiency reduces immunophenotypic HSPC abundance in BM, we assessed HSPC function *in vivo* using competitive transplantation experiments (Duran-Struuck and Dysko, 2009). Donor CD45.2 HSCs were prospectively isolated by fluorescence-activated cell sorting (FACS) from either *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ or control mice and were mixed with competitor BM cells from CD45.1 mice and injected intravenously into CD45.1-irradiated recipient mice (Figure 2C). Overall, donor cell contribution from the *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ genotype to the PB was significantly decreased compared to the control group from week 8 post transplant onward (Figure 2D) and reflected multi-lineage engraftment defects in the PB, BM, spleen, and thymus of recipients of *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ HSCs at week 16 post transplantation (Figures 2D, 2E, and S1H–S1J). To assess if *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ HSC repopulation defects stem from reduced engraftment defects of immunophenotypically defined HSPCs and committed progenitors, we immunophenotyped HSPC donor contribution and found a significant reduction spanning all HSPC populations and myeloid/lymphoid-committed progenitors from *Gata2* haploinsufficient HSCs donor cells (Figure 2F). Thus, *Gata2* haploinsufficient HSCs lack competence for multi-lineage differentiation *in vivo* post transplantation.

Gata2 haploinsufficiency abolishes HSC self-renewal potential *in vivo*

Given that *Gata2* haploinsufficiency impairs HSC-mediated multi-lineage differentiation post transplantation with a reduction in HSC engraftment (Figure 2F), we next assessed the impact of *Gata2* haploinsufficiency on HSC self-renewal, as judged by serial transplantation experiments (Duran-Struuck and Dysko, 2009). At week 16 of primary transplantation, FACS-purified HSCs from *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ or control (CD45.2 cells) primary recipients were mixed with fresh competitor BM cells (CD45.1 cells) and subsequently re-transplanted to lethally irradiated secondary recipient mice (CD45.1 background) (Figure 2C). Donor chimerism in PB of secondary recipients receiving *Gata2*$^{+/fl}$*; Vav-iCre*$^+$ HSCs was severely reduced from week 2 post transplant when compared to the control group and, remarkably, was nearly undetectable by week 20 post transplantation in both PB and BM (Figures 2G and S1K). These data indicate that *Gata2* haploinsufficiency abolishes HSC self-renewal *in vivo*.

Figure 2. *Gata2* haploinsufficiency disrupts HSPC function

(A and B) FACS and quantification of BM HSPCs: HSCs, MPPs, HPC1, HPC2, LMPPs; n = 9–10 (HSCs–HPC2), n = 8 (LMPPs).

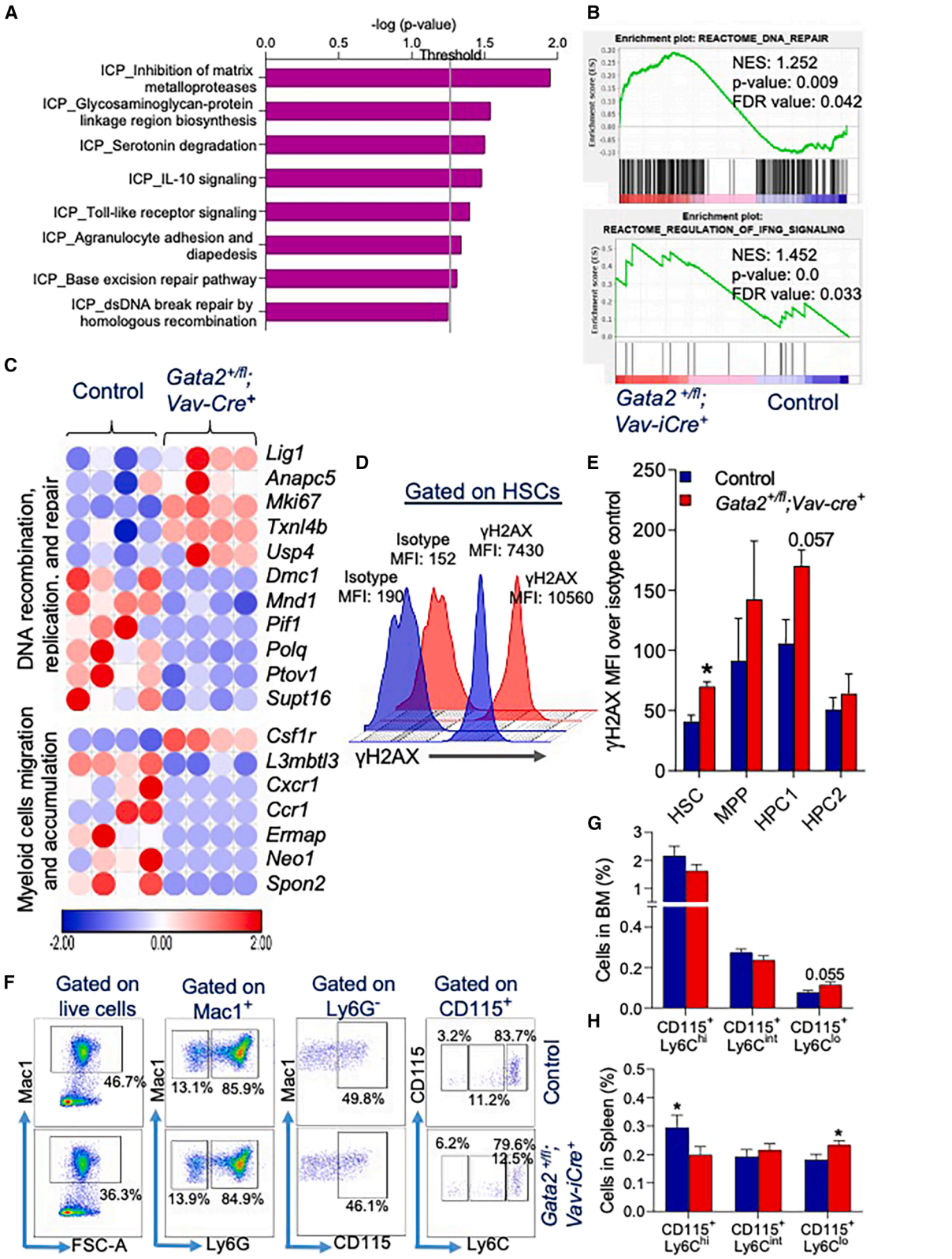
(C) Competitive transplantation: 150 CD45.2$^+$ HSCs + 2 × 10^5 CD45.1$^+$ support cells into lethally irradiated CD45.1$^+$ recipients; n = 8/group. Secondary transplant: 200 CD45.2$^+$ HSCs + 2 × 10^5 competitors; n = 7/group.

(D and E) Donor chimerism in PB (D) and lineage-specific contribution in PB (E).

(F) Donor-derived HSPCs and progenitors in BM at 16 weeks post transplant.

(G) PB chimerism post secondary transplant (2–20 weeks).

Data: mean ± SEM. Statistical tests: unpaired t test for HSCs, MPPs, HPC1, and HPC2 and Mann-Whitney U test for LMPPs (A–B), Mann-Whitney U (others); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.



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Gata2 haploinsufficient HSCs display disrupted B cell development, global DNA damage responses, and inflammatory cell signaling

To investigate the molecular underpinnings of *Gata2* haploinsufficiency-induced defects in HSC function, we conducted genome-wide transcriptomic analysis by RNA sequencing (RNA-seq) of highly purified HSCs (LSK_CD150^{+}CD48^{-}) from *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ and control mice. 117 statistically significant differentially expressed genes (DEGs) were identified in *Gata2* haploinsufficient HSCs relative to control HSCs, of which 74 genes were down-regulated and 43 genes were up-regulated (Figure S2A).

Ingenuity Pathway Analysis (IPA) of differentially regulated genes from *Gata2* haploinsufficient HSCs indicated molecular function affected by *Gata2* in normal hematopoiesis (e.g., cell death and apoptosis/survival, cellular growth and proliferation, and hematological system development and function) (Figures S2B and S2C). Notably, cell-to-cell signaling and interaction, as evidenced by deregulated cytoskeletal organization, and cell adhesion genes (Figures S2B and S2C), both of which are required for maintaining HSC integrity (Almotiri et al., 2021; Schreck et al., 2017), were also differentially deregulated in *Gata2* haploinsufficient HSCs. Pathways directly related to *GATA2* clinical haploinsufficiency syndromes were observed in *Gata2* haploinsufficient HSCs, including B cell development/humoral responses (Koegel et al., 2016; Nováková et al., 2016). Confirming the impact of this transcriptional signature on the B cell developmental program in *Gata2* haploinsufficient mice, by flow cytometry we found a significant decrease in Pro-B cells (IgM^{-}CD43^{+}CD19^{+}BP1^{-}) and an expansion in early pre-B cells (IgM^{-}CD43^{+}CD19^{+}BP1^{+}) in BM (Figures S2D and S2E) and a specific decrease in Pre-Pro B cells (IgM^{-}CD43^{+}CD19^{-}BP1^{-}) in the spleen of *Gata2* haploinsufficient mice (data not shown).

By analysis of Ingenuity Canonical Pathway (ICP), gene set enrichment analysis (GSEA), and Molecular Signatures Database (hallmark gene sets and C2-curated gene sets), we found that *Gata2* haploinsufficient HSCs displayed altered DNA repair pathways, specifically double-stranded (ds) DNA-break repair by homologous recombination and base-excision repair, and pro-inflammatory signaling (e.g., Toll-like receptor signaling, interferon-gamma (IFN-γ) signaling, interleukin-10 signaling, and granulocyte adhesion/diapedesis) as well as extracellular matrix components pathways implicated in maintenance of HSC integrity (de Bruin et al., 2013; Schuettpelz and Link, 2013) (Figures 3A–3C and S2F).

To validate altered transcriptional programming in DNA damage response pathways at the protein level in *Gata2* haploinsufficient HSPCs, we used flow cytometry-based analysis of γH2AX, a member of the histone H2A family that becomes phosphorylated at Ser139 following dsDNA break damage and accumulates at locations of DNA damage to attract DNA damage response (DDR) proteins (Mah et al., 2010). γH2AX expression, as gauged by median fluorescence intensity (MFI), was selectively increased in BM HSCs in *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ mice (Figures 3D and 3E), demonstrating that *Gata2* haploinsufficiency induces dsDNA break damage in HSCs. Next, the molecular mechanisms operating in response to dsDNA break damage in *Gata2* haploinsufficient HSCs were analyzed. Transcriptomic analysis of *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs revealed reduced expression of the critical *ATR-Chk1* and *ATM-Chk2* axes of the DDR (Smith et al., 2010) (Figure S2F and data not shown). By phospho-specific flow cytometry, we detected a significant reduction in the activity of ATR, Chk1, ATM, and Chk2 in *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs and 53BP, another crucial mediator of dsDNA break damage repair (Gupta et al., 2014; Smith et al., 2010) (Figure S2G). DNA ligase IV activity, which is integral for repair of dsDNA breaks in HSCs (Nijnik et al., 2007), remained intact in *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs. Therefore, *Gata2* haploinsufficiency disrupts the activity of select DDR proteins in HSCs.

As IPA identified deregulated GATA2-mediated IFN-γ target genes and cell adhesion molecules in HSCs (Figures 3B and S2C), both of which are involved in the recruitment of macrophages that modulate inflammation (Figures 3B and 3C), we sought to assess the impact of altered inflammatory signaling in *Gata2* haploinsufficient HSCs on the generation of downstream inflammatory cell subsets in BM and spleen. By flow cytometry, we evaluated functionally distinct Mac1^{+} Ly6G^{-} CD115^{+} macrophage populations by Ly6C expression that discriminate between

Figure 3. *Gata2* haploinsufficiency in HSCs deregulates transcriptional programs linked to DNA repair and inflammation

(A) IPA pathway analysis of differentially expressed genes (DEGs) from *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs relative to control. Data presented: $-\log_{10}(p)$. n = 4/genotype.

(B) GSEA plots of enriched up-regulated pathways in *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs.

(C) Heatmaps of DEGs (Z score scale); red = up-regulated, blue = down-regulated.

(D and E) γH2AX expression in BM LSK cells: histogram (D) and MFI ratio (E); n = 4/genotype.

(F–H) Flow cytometry of BM and spleen macrophage subsets: phagocytic/pro-inflammatory (Ly6C^{high}), pro-inflammatory (Ly6C^{int}), and anti-inflammatory (Ly6C^{low}); n = 5–8/genotype.

Data (D–H): mean ± SEM. Statistical test: Mann-Whitney U; *p < 0.05.

phagocytic and pro-inflammatory macrophages (Mac1$^+$ Ly6G$^-$ CD115$^+$ Ly6C^{high}), pro-inflammatory macrophages (Mac1$^+$ Ly6G$^-$ CD115$^+$ Ly6C^{intermediate}), and anti-inflammatory macrophages (Mac1$^+$ Ly6G$^-$ CD115$^+$ Ly6C^{low}) (Yang et al., 2014). While pro-inflammatory macrophage populations were unchanged in *Gata2* haploinsufficient mice, anti-inflammatory macrophages were marginally increased within the BM of *Gata2* haploinsufficient mice (Figures 3F and 3G). Phagocytic and pro-inflammatory macrophages were significantly decreased in the spleen of *Gata2* haploinsufficient mice while splenic anti-inflammatory macrophages were significantly increased (Figure 3H). In agreement with attenuated inflammatory responses to infectious stimuli in *Gata2* haploinsufficient mice (Takai et al., 2021), these data suggest that altered inflammatory signaling in *Gata2* haploinsufficient HSCs reduces the generation of inflammatory cell populations in BM and spleen.

Ex vivo knockdown of *Asxl1* in Gata2 haploinsufficient BM cells reduces multi-potent hematopoietic progenitor generation

Next, we sought to explore potential genetic pathways contributing to impaired DNA damage and inflammatory responses in *Gata2* haploinsufficient mice by assessing the role of *Asxl1*, an epigenetic modifier involved in the regulation of Polycomb genes in normal hematopoiesis and hematological neoplasms (Abdel-Wahab et al., 2013; Wang et al., 2014). *ASXL1* deregulation has been implicated in DNA damage accrual in HSCs, and somatic *ASXL1* mutations are a frequent abnormality observed in clonal hematopoiesis of indeterminate potential that drives deregulated inflammation and the propensity to develop AML during aging (Abdel-Wahab et al., 2013; Wang et al., 2014). We also elected to study *Asxl1* as acquired *ASXL1* mutations have been detected in approximately 30% of MDS/AML cases with germline *GATA2* haploinsufficiency mutations (West et al., 2014).

To mimic *GATA2* haploinsufficiency with acquired *ASXL1* somatic mutations and explore the interaction between GATA2 and ASXL1 pathways in HSPCs, we used shRNA-mediated knockdown of *Asxl1* in *Gata2*$^{+/fl}$; *Vav-iCre*$^+$ or control (wild type [WT]) BM HPSCs (LSK cells) to functionally analyze the impact of *Gata2/Asxl1* deficiency on hematopoietic progenitor function (Figure 4A). Using a lentiviral vector linking a mCherry reporter to *Asxl1* shRNAs (*Asxl1*-sh1 and *Asxl1*-sh2) or empty vector (EV) control, we initially validated knockdown of *Asxl1* in WT LSK cells transduced with each vector (Figure 4A). Equivalent transduction efficiency, nearing 60%, was observed in WT LSK cells transduced with EV and *Asxl1* shRNA groups (Figure 4B). In two independent experiments, *Asxl1*-sh1 and *Asxl1*-sh2 displayed an approximately 2-fold and 4-fold reduction in *Asxl1*-mRNA, respectively, compared to EV-transduced WT LSK cells (Figure 4C). After transduction of LSK cells harvested from WT or *Gata2*$^{+/fl}$; *Vav-iCre*$^+$ mice with EV or *Asxl1-sh1 and Asxl1-sh2*, mCherry$^+$ cells were isolated by FACS and plated in a CFC assay, where total CFC number was significantly decreased in all experimental groups when compared to EV-transduced WT LSK cells (Figure 4D). As expected, total CFC number was reduced in WT LSK cells transduced with *Asxl1-sh1 and Asxl1-sh2* and, to a lesser extent, in single haploinsufficient *Gata2* transduced with EV (Figure 4D). Notably, there was a significant reduction in total CFCs in the *Gata2*$^{+/fl}$; *Vav-iCre*$^+$-*Asxl1*-sh1/sh2 group compared to single haploinsufficient *Gata2* transduced with EV or WT LSK cells transduced with *Asxl1* shRNA (Figure 4D). This reduction in CFCs from *Gata2*$^{+/fl}$; *Vav-iCre*$^+$-*Asxl1*-sh1/sh2 LSK cells reflected a substantial reduction across multipotential progenitors (CFU-GEMM) and bi-potent myeloid progenitors (CFC-GMs) compared to single haploinsufficient *Gata2* transduced with EV or WT LSK cells transduced with *Asxl1* shRNA (Figure 4E). All lineage-specific progenitors (CFU-GMs, CFU-M, CFU-G, and CFU-E) were reduced in *Gata2*$^{+/fl}$; *Vav-iCre*$^+$-*Asxl1*-sh1/sh2 LSK cells compared to single haploinsufficient *Gata2* transduced with EV (Figure 4E). Thus, knockdown of *Asxl1* in *Gata2* haploinsufficient LSK cells functionally reduces the differentiation capacity of multipotent progenitors *in vitro*. These data further suggest that knockdown of *Asxl1* potentiates a defective differentiation program already established in *Gata2* haploinsufficient mice (Figures 1 and S1) and observed following transplantation of *Gata2* haploinsufficient HSCs (Figures 2 and S1).

Immunophenotypic HSPC and progenitor defects in *Gata2* and *Asxl1* double haploinsufficient mice mirror those found in *Gata2* haploinsufficient mice

That knockdown of *Asxl1* in *Gata2* haploinsufficient LSK cells results in a reduction of multipotent progenitors beyond that observed in single haploinsufficient *Gata2* or *Asxl1* LKS cells suggests genetic interaction between GATA2→ASXL1 pathways in HSPCs (Lawson et al., 2021; Menendez-Gonzalez et al., 2019b). To formally test this *in vivo*, we assessed whether combined *Gata2* and *Asxl1* haploinsufficiency causes exacerbated loss of HSPCs when compared to either *Gata2* or *Asxl1* haploinsufficiency. To generate compound *Gata2/Asxl1* haploinsufficient mice, *Gata2*$^{+/fl}$, *Asxl1*$^{+/fl}$, *Vav-iCre*$^-$ males were bred with *Gata2*$^{+/+}$; *Asxl1*$^{+/+}$; *Vav-iCre*$^+$ females to generate *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^+$ (double *Gata2/Asxl1* heterozygote mice), *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^-$ (control mice), *Gata2*$^{+/fl}$; *Vav-iCre*$^+$ (single *Gata2* heterozygote), and *Asxl1*$^{+/fl}$; *Vav-iCre*$^+$ (single *Asxl1* heterozygote) (Figure 5A). All littermates were born healthy, in the predicted Mendelian proportions. Genotyping PCR of ear notch biopsies

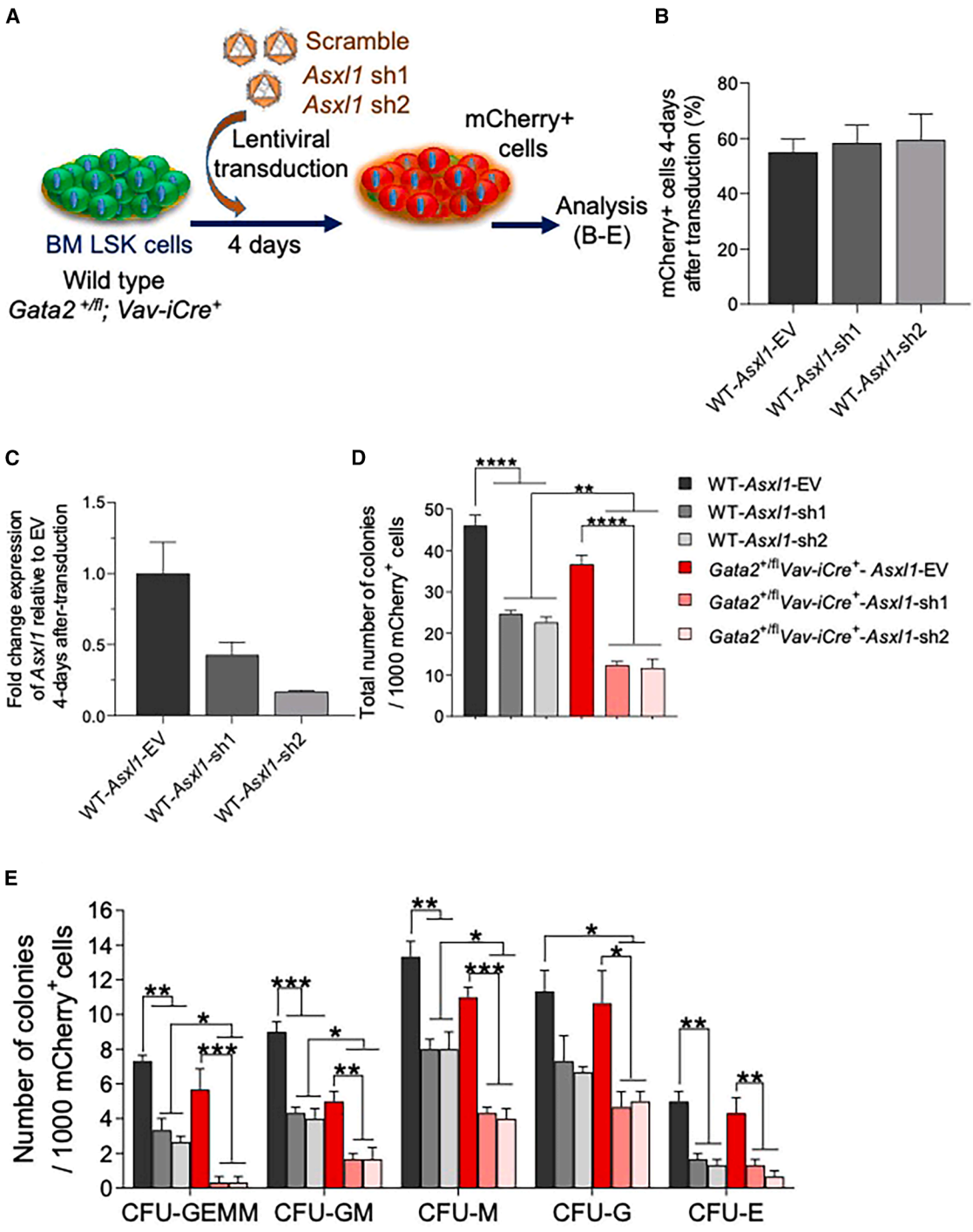


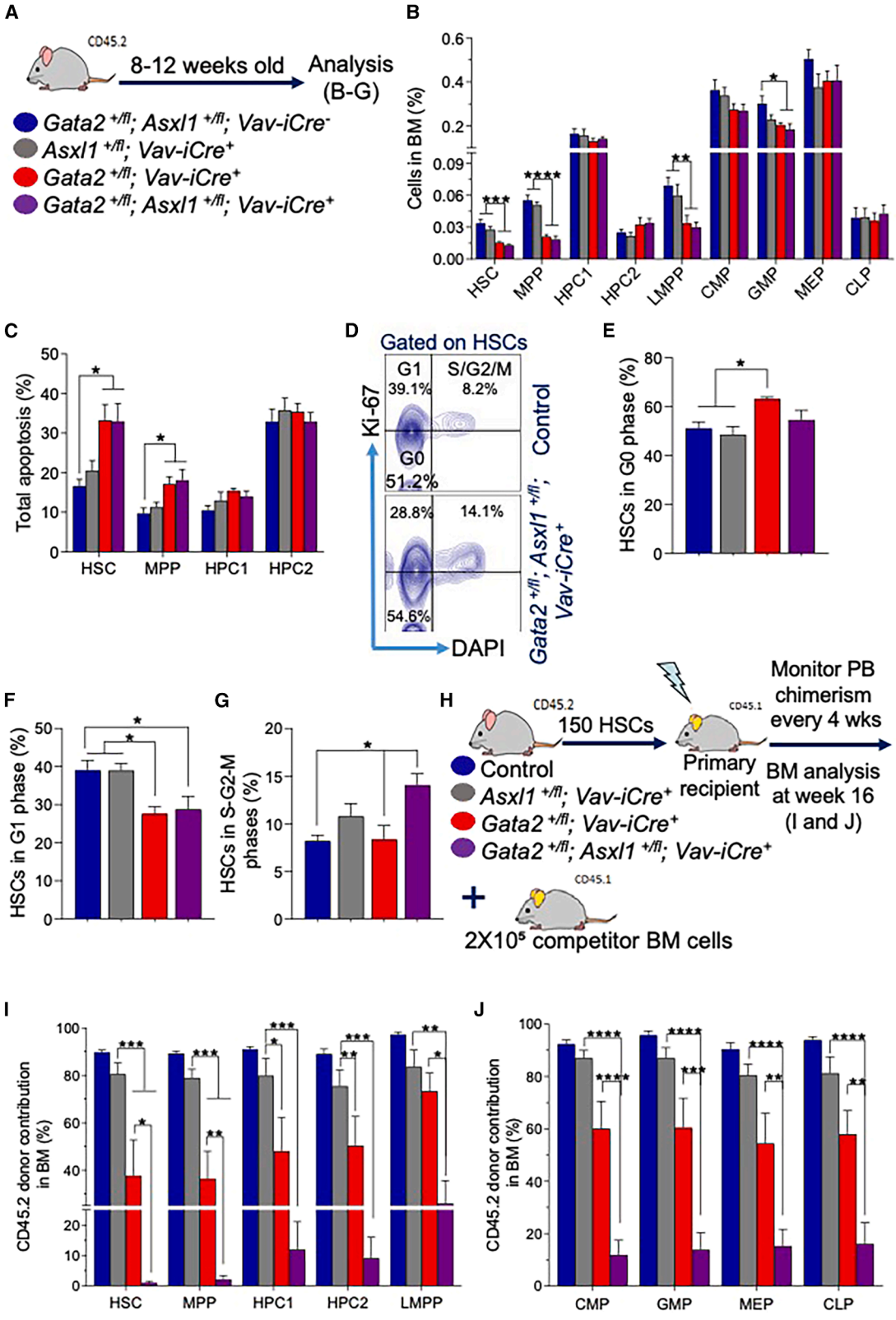
Figure 4. *Asxl1* knockdown in *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ HSPCs impairs myeloid differentiation *in vitro*

(A) Experimental design: LSK cells transduced with *Asxl1-EV*, -sh1, or -sh2; mCherry^{+} cells collected by FACS for qPCR, and CFC analysis.

(B and C) Transduction efficiency (B) and *Asxl1* mRNA knockdown (C); n = 2–3 independent experiments.

(D and E) Colony counts and myeloid differentiation in methylcellulose; n = 3/genotype.

Data: mean ± SEM. Statistical test: one-way ANOVA with Tukey's post hoc; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



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and BM cells confirmed complete excision of one allele of both *Gata2* and *Asxl1* genes (Figure S3A), and mRNA analysis confirmed reduced *Asxl1* expression in total BM cells from *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ and a decrease in *Asxl1* and *Gata2* mRNAs in HSPC-enriched c-kit^{+} cells from *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ mice (Figures S3B and S3C).

Fully differentiated hematopoietic cells from PB, BM, and spleen and cellularity of BM and spleen from young adult (8–12 weeks old) *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ mice mirrored hematopoietic potential in their single heterozygote counterparts, indicating that haploinsufficiency of both *Gata2* and *Asxl1* does not disrupt steady-state terminal differentiation of hematopoietic cells in PB, BM, and spleen nor the cellularity of BM and spleen (Figures S3D–S3H). Yet, the frequencies of HSCs, MPPs, LMPPs, and GMPs in the BM of double haploinsufficient mice were reduced compared to *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ and control littermates and, notably, mirrored the HSPC/progenitor defects found in *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ mice (Figure 5B). No differences were observed in CMP, megakaryocyte-erythroid progenitor (MEP), and CLP frequency comparing experimental genotypes to *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{-}$ controls (Figure 5B). Thus, *Gata2*/*Asxl1* double haploinsufficiency *in vivo* impacts immunophenotypic HSPCs/progenitors in a manner closely resembling that observed in *Gata2* haploinsufficient mice.

Increased HSC proliferation potential in *Gata2* and *Asxl1* double haploinsufficient mice

While double *Gata2* and *Asxl1* haploinsufficiency replicated the HSPC immunophenotype found in *Gata2* haploinsufficient mice, it was unclear whether cellular mechanisms regulating HSPC genomic integrity, namely apoptosis and cell cycle status, were similar in both genotypes. Annexin V analysis revealed a significant increase in total apoptosis from HSCs and MPPs in *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ mice when compared to *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ or control littermates, which, importantly, correlated with enhanced HSPC apoptosis observed in *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ mice (Figure 5C). Determination of HSC cell cycle status revealed an unchanged proportion of quiescent HSCs and an enhancement in S/G2/M phase HSCs from *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ mice compared to *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ mice or control littermates (Figures 5D, 5E, and 5G). The proportion of G1 HSCs was similar in *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ and *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ mice and reduced compared to *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ or control littermates (Figures 5D and 5F). Thus, *Gata2* and *Asxl1* haploinsufficiency together cause an increase in HSC proliferation, contrasting sharply with an increase in HSC quiescence observed from *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$ mice (Figures 5D and 5E). These data further suggest that combined *Gata2* and *Asxl1* haploinsufficiency compromises both unique and complementary mechanisms that regulate genomic integrity in the HSC pool.

Functional exhaustion of HSCs following competitive transplantation of HSCs from *Gata2* and *Asxl1* double haploinsufficient mice

Having shown that *Gata2/Asxl1* double haploinsufficient HSCs were more proliferative, we evaluated how this would impact *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ HSC function under conditions of proliferative stress using competitive transplantation experiments. We transplanted HSCs from control, *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$, *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$, or *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ CD45.2 mice alongside 2 × 10^{5} CD45.1 competitor BM cells into irradiated CD45.1 recipient mice, monitoring PB engraftment every 4 weeks and chimerism in BM and spleen of recipients at week 16 following transplantation (Figure 5H).

While equivalent PB engraftment was noted initially for all genotypes at week 4 post transplant, from week 8 onward, recipients of *Gata2/Asxl1* double haploinsufficient HSCs experienced a progressive decrease in PB chimerism that was statistically significant compared to recipients of control or *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs (Figure S4A). In comparison to their control or *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ counterparts, recipients of *Gata2/Asxl1* double haploinsufficient HSCs were significantly reduced in PB myeloid (Mac-1^{+}Gr-1^{+} and Mac-1^{+}Gr1^{-}) engraftment capacity at week 16 (Figure S4B). As expected, recipients of HSCs from

Figure 5. *Gata2/Asxl1* double haploinsufficient mice demonstrated increased HSC cycling, decreased HSC survival, and exhausted HSC pool size after HSC transplantation

(A) Experimental design: *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{-}$ (control), *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$, *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$, and *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ mice assessed at 8–12 weeks

(B) Frequency of BM HSPCs (HSCs, MPPs, HPC1, HPC2, and LMPPs) and committed progenitors (CMPs, GMPs, MEPs, and CLPs); *n* = 5–6/genotype.

(C) Apoptosis in BM HSPCs using Annexin V and DAPI; *n* = 5–6/genotype.

(D–G) Cell cycle analysis of BM HSPCs: representative plots (D), G0 (E), G1 (F), and S/G2/M (G) phases; *n* = 4/genotype. G0: Ki-67^{-}DAPI^{-}; G1: Ki-67^{+}DAPI^{-}; S/G2/M: Ki-67^{+}DAPI^{+}.

(H) Competitive HSC transplantation: 150 CD45.2^{+} HSCs + 2 × 10^{5} CD45.1^{+} BM cells into lethally irradiated CD45.1^{+} recipients; 4 donors/group.

(I and J) CD45.2 donor contribution at 16 weeks in BM HSPCs (I) and committed progenitors (J); *n* = 6–7/group.

Data: mean ± SEM. Statistical analysis: one-way ANOVA with Tukey's test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Gata2$^{+/fl}$*; Vav-iCre*$^{+}$ displayed a significant reduction in PB engraftment from week 8 onward when compared to mice receiving control HSCs (Figures 2D and S4A); at week 16, this led to diminishment of all lympho-myeloid engraftment in PB (Figure S4B). In contrast, PB chimerism in recipients of *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs was more moderately reduced over time compared to recipients of control HSCs (Figure S4A). At 16 weeks post transplant, this was reflected by a significant reduction only in T cell engraftment to the PB of mice transplanted with HSCs from the *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ genotype (Figure S4B). Overall, *Gata2/Asxl1* double haploinsufficient HSCs were severely compromised in their PB engraftment capacity after transplantation. Notably, recipients of *Gata2/Asxl1* double haploinsufficient HSCs exhibited lower PB engraftment than recipients of either *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ or *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs starting from week 8, though this reduction did not reach statistical significance compared to recipients receiving *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs (Figure S4A).

To evaluate the provenance of defective PB engraftment in transplant recipients of *Gata2/Asxl1* double haploinsufficient HSCs, we assessed donor HSPCs and committed hematopoietic population contribution to the BM of recipients at week 16. Remarkably, HSCs and MPPs were barely detectable in *Gata2*$^{+/fl}$*; Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ genotype recipients compared to control, *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$, or *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ recipients (Figure 5I). Other HSPC (HPC1, HPC2, and LMPPs) and committed progenitor (CMPs, GMPs, MEPs, and CLPs) donor cell engraftment were markedly attenuated in *Gata2*$^{+/fl}$*; Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ recipients compared to control or *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ genotype recipients (Figures 5I and 5J). A statistically significant reduction in LMPP, CMP, GMP, MEP, and CLP donor cell engraftment was also noted in *Gata2*$^{+/fl}$*; Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ recipients in comparison to *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ recipients (Figures 5I and 5J). As predicted, the frequency of HSPCs and hematopoietic progenitor donor cells in recipients of HSCs from *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice was significantly lower than their control counterparts, whereas recipients of *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs showed comparable HSPC and committed progenitor repopulation potential to recipients of control HSCs (Figures 5I and 5J). Thus, engraftment of HSPC compartments to the BM of recipients from *Gata2*$^{+/fl}$*; Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ was markedly reduced when compared to their single haploinsufficient counterparts. Overall, double haploinsufficiency of *Gata2/Asxl1* leads to HSC/MPP exhaustion after transplantation.

RNA-seq analysis of *Gata2/Asxl1* double haploinsufficient HSCs identifies common *Gata2* and *Asxl1* target genes/pathways and a unique transcriptomic signature

Gata2/Asxl1 haploinsufficiency augments HSC proliferation and causes HSPC functional defects beyond that observed in their single haploinsufficient counterparts, suggesting cooperative genetic interaction between *Gata2* and *Asxl1* pathways. To explore the transcriptional programs underpinning genetic interaction between *Gata2* and *Asxl1* pathways, we conducted RNA-seq analysis of purified BM HSCs from control, *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$, *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$, and *Gata2*$^{+/fl}$*; Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ mice.

1,251 down-regulated genes and 1,355 up-regulated genes were found in double *Gata2/Asxl1* haploinsufficient HSCs, tallying up to 2,606 DEGs in *Gata2/Asxl1* double haploinsufficient HSCs when compared to control HSCs (Figure S5A). 2,083 down-regulated genes and 2,135 up-regulated genes, a total of 4,218 DEGs, were identified in HSCs from *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ mice in comparison to control HSCs (Figure S5B). In agreement with the notion that *ASXL1* mutations act as a pre-leukemic initiator (Abdel-Wahab et al., 2013; West et al., 2014; Zhang et al., 2018), several affected pathways that are involved in HSC integrity were detected in *Asxl1* haploinsufficient HSCs, including DNA damage repair, cellular survival, and AML signaling (Figure S5D). Approximately 50% (2,139) of DEGs were shared between *Gata2/Asxl1* double haploinsufficient HSCs and *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs. Of 117 DEGs identified from *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs (Figure S5C), approximately 39% (46 DEGs) were shared with *Gata2/Asxl1* double haploinsufficient HSCs (Figure S5C). 39 DEGs were common to *Gata2* and *Asxl1* single haploinsufficient HSCs and *Gata2/Asxl1* double haploinsufficient HSCs (Figure S5C). Together, these data demonstrate a core common transcriptional signature specifically related to GATA2 and ASXL1 target genes in *Gata2/Asxl1* double haploinsufficient HSCs. Yet, 16% (421) of all DEGs were unique to *Gata2/Asxl1* double haploinsufficient HSCs, indicating that combined *Gata2 and Asxl1* haploinsufficiency can in parallel generate a transcriptomic signature in HSCs distinct from that observed in either *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs or *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs (Figure S5C).

Gata2/Asxl1 double haploinsufficient HSCs display transcriptomic deregulation related to multi-lineage differentiation, membrane transport proteins, genomic integrity, and inflammatory cell signaling

Using ICP analysis, we initially surveyed the entire transcriptomic signature of 2,606 DEGs identified in *Gata2/Asxl1* double haploinsufficient HSCs, which consisted of DEGs that overlapped with *Gata2* and *Asxl1* single haploinsufficient HSCs. Consistent with hematopoietic differentiation defects observed in pre-leukemia (Abdel-Wahab et al., 2013; West et al., 2014; Zhang et al., 2018), transcriptional programs specifying multi-lineage differentiation capacity were highly deregulated in *Gata2/Asxl1* double haploinsufficient HSCs (Figure 6A). Myriad solute carrier (SLC) membrane transport proteins were both up- and down-regulated

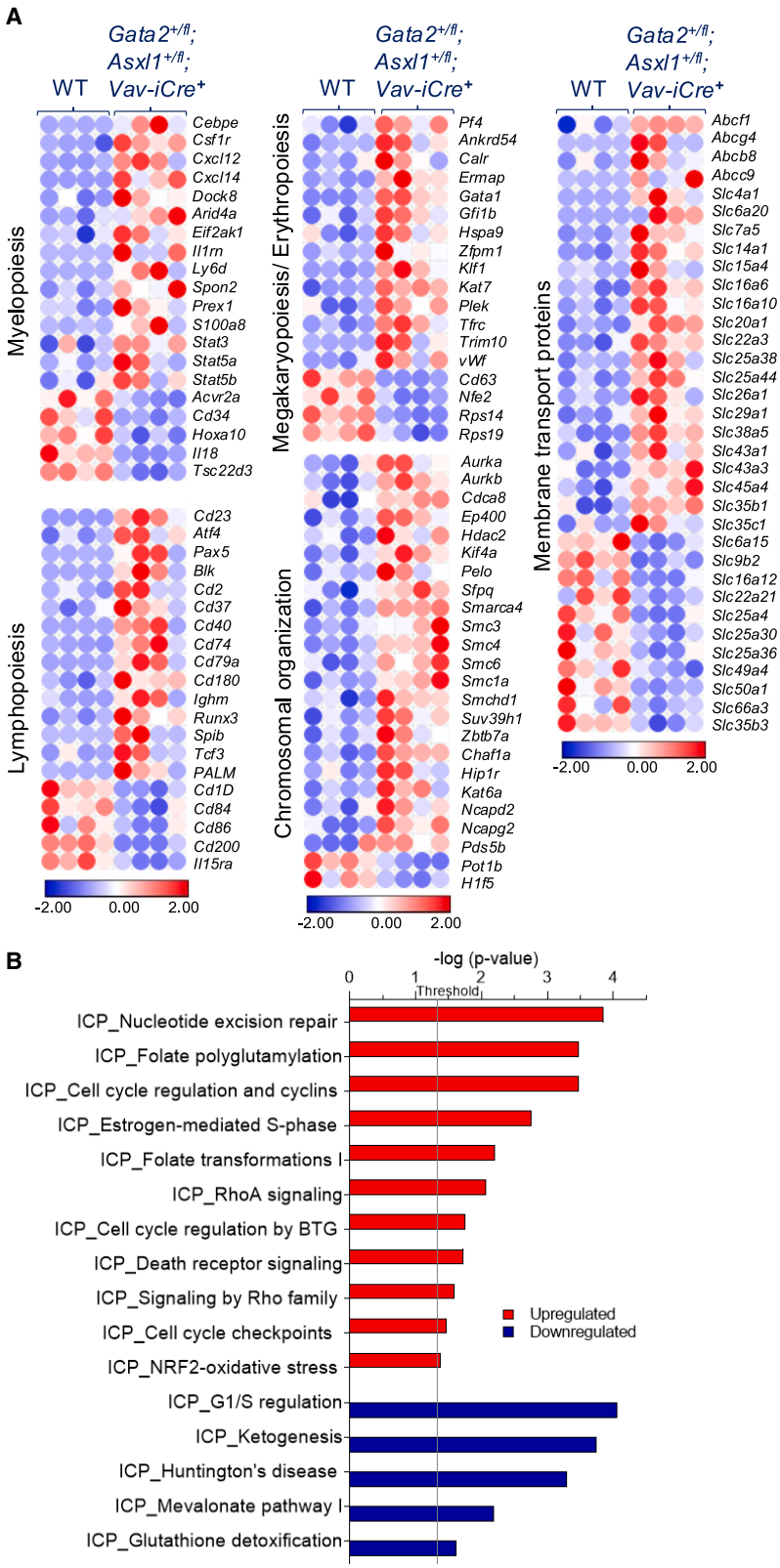


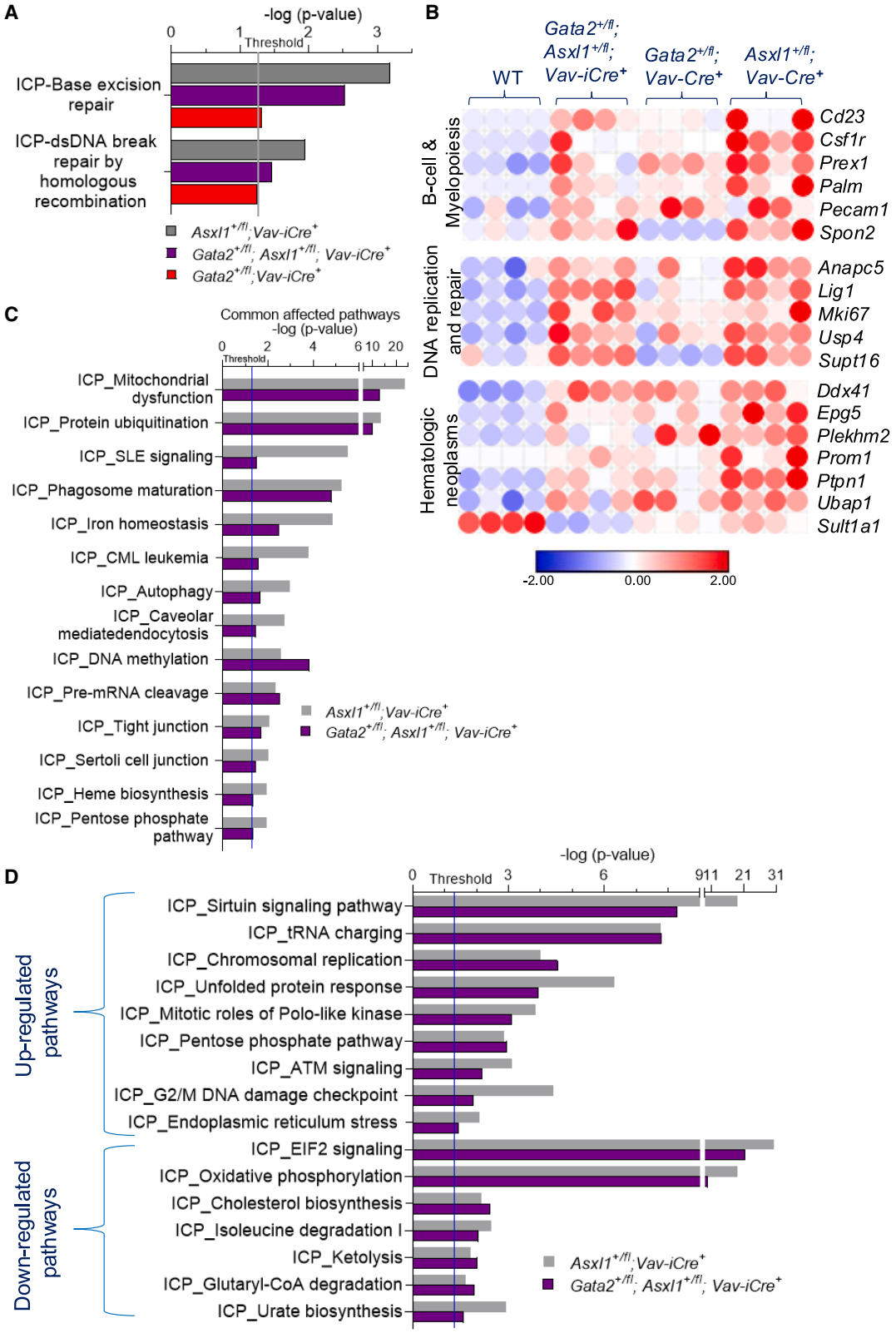
Figure 6. *Gata2/Asxl1* double haploinsufficient HSCs display transcriptomic deregulation related to multi-lineage differentiation, membrane transport proteins, genomic integrity, and inflammatory cell signaling

RNA-seq of purified HSCs (LSKCD150^{+}CD48^{-}) from *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{-}$, *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$, *Gata2*$^{+/fl}$; *Vav-iCre*$^{+}$, and *Gata2*$^{+/fl}$; *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$; n = 4/genotype.

(A) Heatmaps of DEGs (p < 0.05, false discovery rate < 0.05) using DESeq2 and Morpheus; red = high, blue = low expression.

(B) Canonical pathway analysis of dysregulated genes using IPA.

Data as $-\log_{10}(p)$; gray line = p = 0.05. ICP: Ingenuity Canonical Pathway. Statistical analysis: Fisher's exact test.



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in *Gata2/Asxl1* double haploinsufficient HSCs, which likely reflects the paradoxical function of various SLCs to import nutrient substrates across cellular membranes in both normal and cancer settings (Chigaev, 2015) (Figure 6A). In *Gata2/Asxl1* double haploinsufficient HSCs, a smaller selection of membrane ABC proteins, which, in contrast, act as export transporters to protect cells from undesirable metabolites and toxins, were up-regulated (Chigaev, 2015; Raaijmakers, 2007) (Figure 6A). In a similar vein to the transcriptional program in *Gata2* haploinsufficient HSCs (Figures 3 and S2), DNA damage and repair pathways (e.g., chromosomal organization genes, nucleotide excision repair, cell cycle checkpoints, and G1/S regulation) and inflammatory pathways (e.g., death receptor signaling) were predominantly deregulated in *Gata2/Asxl1* double haploinsufficient HSCs (Figure 6B). Reflecting heightened cell cycling potential and impairment of DNA damage responses in *Gata2/Asxl1* double haploinsufficient HSCs, a proliferative transcriptomic signature was observed with up-regulation in pathways correlating with cell cycle regulation/cyclins and S-phase (Figure 6B). Therefore, *Gata2/Asxl1* double haploinsufficiency in HSCs causes transcriptional defects in DNA damage pathways and inflammation, which likely reflect genetic crosstalk between *Gata2* and *Asxl1*-mediated biological pathways.

Common GATA2 and ASXL1 target genes enforce an oncogenic and proliferation-biased transcriptional signature to *Gata2/Asxl1* double haploinsufficient HSCs

Next, we asked which shared *Gata2* and *Asxl1* biological pathways are involved in the 39 DEG core transcriptional signatures found in *Gata2/Asxl1* double haploinsufficient HSCs, *Gata2* haploinsufficient HSCs, and *Asxl1* haploinsufficient HSCs. ICP analysis uncovered that common biological pathways affected in each genotype were associated with DNA damage repair, hematopoietic differentiation to B cell/myeloid lineages, and deregulation of genes critical to development of hematopoietic neoplasms (Figures 7A and 7B). These data demonstrate that *Gata2/Asxl1* double haploinsufficient HSCs deregulate *Gata2* and *Asxl1* biological pathways essential for maintaining genomic and functional integrity of HSCs.

ICP analysis revealed that certain biological pathways (e.g., DNA repair) were enhanced in *Gata2/Asxl1* double haploinsufficient HSCs beyond that found in single haploinsufficiency HSCs (Figure 7A). By complementary bioinformatic analysis using GSEA, we explored whether *Gata2* or *Asxl1* target genes/pathways were up-regulated by *Gata2/Asxl1* double haploinsufficient HSCs. Target genes of MYC, a proto-oncogene involved in maintenance of HSC integrity and AML development (Ohanian et al., 2019), were highly enriched in double *Gata2/Asxl1* haploinsufficient HSCs compared to either *Gata2* or *Asxl1* haploinsufficient HSCs (Figures S6A and S6B). Consistent with increased HSC proliferation in *Gata2/Asxl1* double haploinsufficient mice (Figure 5), G2M checkpoints, E2F target genes, and mitotic spindle pathways, all of which are also required to maintain genome instability (Ren et al., 2002), were up-regulated compared to *Gata2* haploinsufficient HSCs (Figure S6B). Furthermore, spliceosome and adipogenesis pathways, which are deregulated in the context of MDS/AML (Pellagatti et al., 2018; Sabbah et al., 2023), were enriched in *Gata2/Asxl1* double haploinsufficient HSCs compared with *Gata2* haploinsufficient HSCs (Figure S6B).

As approximately 50% of DEGs were shared between *Gata2/Asxl1* double haploinsufficient HSCs and *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs, we also explored the commonality in biological pathways between these two genotypes. By ICP analysis, common affected pathways in *Gata2/Asxl1* double haploinsufficient HSCs and *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs broadly encompassed metabolism (mitochondrial dysfunction), protein ubiquitination, immune cell homeostasis (phagosome maturation), cellular stress responses (autophagy), DNA methylation, and RNA-processing pathways (Figure 7C). By segregating these common affected pathways into specific up-regulated and down-regulated pathways in ICP analysis, we found that G2M DNA damage checkpoints and ATM signaling were among some key pathways that were up-regulated in *Gata2/Asxl1* double haploinsufficient HSCs and *Asxl1*$^{+/fl}$; *Vav-iCre*$^{+}$ HSCs (Figure 7D). Notably, the common feature of affected, up-regulated and down-regulated pathways in these two genotypes was their role in MDS/AML pathogenesis (Brendolan and Russo, 2022; Ren et al., 2002; Soltani et al., 2021). Overall, this analysis suggests that common GATA2 and ASXL1 target genes/pathways in *Gata2/Asxl1* double haploinsufficient HSCs co-operate to fortify an oncogenic and proliferation-biased transcriptomic program.

Figure 7. Shared GATA2 and ASXL1 target genes embed oncogenic and proliferative transcriptomic programs in double haploinsufficient HSCs

(A) Pathway analysis of shared dysregulated pathways across *Gata2* and *Asxl1* haploinsufficient HSCs and double haploinsufficient HSCs ($-\log_{10}p$; threshold = 0.05; Fisher's exact test).

(B) Heatmaps of shared DEGs across genotypes (*Z* score scale; red = up-regulated, blue = down-regulated).

(C and D) IPA of shared dysregulated genes in *Asxl1* haploinsufficient HSCs and double haploinsufficient HSCs vs. controls, showing altered, up and down-regulated biological processes ($-\log_{10}p$; threshold = 0.05; Fisher's exact test). ICP, Ingenuity Canonical Pathway.

Gata2/Asxl1 double haploinsufficient HSCs formulate a unique impaired DNA damage, inflammatory response, and pre-leukemic transcriptional network

Having established common GATA2 and ASXL target genes/pathways in *Gata2/Asxl1* double haploinsufficient HSCs, we conducted separate pathway analysis on the unique 421 DEGs identified in *Gata2/Asxl1* double haploinsufficient HSCs. Analysis of these DEGs in ICP and GSEA databases unveiled biologic pathways recurrently linked to the HSC transcriptional signature of all genotypes, namely DNA damage responses/proliferation (e.g., GADD45 signaling, MYC target genes, apoptosis, and mitotic spindle) and inflammatory responses (e.g., neutrophil degranulation, IFN-γ response, and TNF-α signaling via nuclear factor κB) (Figure S7A). By utilizing both *Gata2/Asxl1*-dependent and -independent mechanisms, impaired DNA damage and inflammatory responses therefore appear to be critical to the formulation of the *Gata2/Asxl1* double haploinsufficient HSC transcriptional network. Finally, we also found evidence for *Gata2/Asxl1* double haploinsufficient HSC-specific DEGs contributing to a robust gene signature related to the pathogenesis of MDS. This signature consisted of deregulated *Prmt5*, *Hmgcs1*, *Lamp2*, *Gdf15*, and *Mlf1* expression (Hu et al., 2020) as well as aberrant Wnt signaling (Liu et al., 2022) and mRNA spliceosome pathways (pre-mRNA splicing) (Pellagatti et al., 2018) (Figures S7A and S7B). These data indicate that *Gata2/Asxl1* double haploinsufficient HSCs express a unique pre-leukemic transcriptional signature that may be germane to the progression of clinical *GATA2* haploinsufficiency syndromes.

DISCUSSION

Predating the discovery of familial *GATA2* haploinsufficiency syndromes, where patients develop primary immunodeficiency syndromes that transform to MDS/AML (Dickinson et al., 2011; Hahn et al., 2011; Hsu et al., 2011), mouse models of germline *Gata2* haploinsufficiency demonstrated underlying HSC/HSPC functional defects (Ling et al., 2004; Rodrigues et al., 2005). Using sophisticated genetic mouse models, work since then has demonstrated that *Gata2 cis*-regulatory elements, which are mutated in *GATA2*-deficient patients, are essential for HSC genesis in the embryo and necessary for HSPC regeneration in response to genotoxic insults (Johnson et al., 2012). Yet, our understanding of the effects of *GATA2* haploinsufficiency in HSCs/HSPCs and the impact of secondary mutations acquired by *GATA2*-deficient HSCs/HPCs during pre-leukemic evolution remains ill-defined. Using conditional mouse genetics to model *Gata2* haploinsufficiency alone or combined with deficiency in a known secondary mutation, *Asxl1* (West et al., 2014), this report documents *Gata2* haploinsufficiency as an initiator of HSC genome instability and that genetic interplay between *Gata2* and *Asxl1* compromises HSC genome integrity further to enforce the development of a robust pre-leukemic transcriptional program.

GATA2 haploinsufficient patients display both myeloid and lymphoid lineage-specific defects, and *Gata2* haploinsufficient mouse models have been partially successful in modeling defects linked to myeloid lineage commitment (de Pater et al., 2013; Ling et al., 2004; Rodrigues et al., 2008). Here, in addition to the expected reduction in HSCs and progenitors related to the myeloid lineage in *Gata2* haploinsufficient mice (Rodrigues et al., 2005, 2008), we identified selective B cell development blocks in BM and spleen, preceded by attenuation in lymphoid primed progenitor (LMPP/HPC1) abundance and deregulation of the B cell transcriptional program that begins in HSCs. While we did not observe defects in mature B cells in *Gata2* haploinsufficient mice, these data provides the basis for understanding the differentiation stage-specific dependency for *GATA2* in B cell maturation in *GATA2* haploinsufficient patients, where the incipient stages of B cell differentiation from HSCs/multi-potent progenitors are known to be disrupted (Koegel et al., 2016; Nováková et al., 2016). We also identified megakaryocytic lineage-specific alterations in *Vav-Cre*-mediated *Gata2* haploinsufficiency, which has a provenance within MPPs. MPP2, a Meg/E-biased MPP compartment (Pietras et al., 2015), was expanded whereas downstream failure of megakaryocyte/erythroid-committed progenitors to mature into megakaryocytes likely caused the reduction in platelets observed in *Gata2* haploinsufficient mice. Despite defects in early erythroid commitment, evidenced by Pre-Meg/E and Pre-CFU-E reduction in *Gata2* haploinsufficient mice, later erythropoiesis, as measured by CFU-E/Pro-E, was broadly unimpacted by *Gata2* haploinsufficiency, likely due to compensation from other indispensable erythroid factors including the GATA family member, *Gata1* (Suzuki et al., 2013). Overall, these data support studies implicating a pivotal role for *Gata2* in megakaryocyte differentiation (Huang et al., 2009). Since HSCs can directly generate platelets independently of MPPs (Pietras et al., 2015), we cannot exclude the possibility of a parallel reduction in platelets caused exclusively by HSC reduction observed in *Vav-Cre*-mediated *Gata2* haploinsufficiency. Given that megakaryocytes regulate HSC quiescence by secreting important factors like CXCL4, thrombopoietin, and transforming growth factor β1 (Bruns et al., 2014), enhanced HSC quiescence observed in *Gata2* haploinsufficient mice appears to be independent of megakaryocytes, as the observed reduction in megakaryocytes would instead be predicted to drive HSC proliferation (Huang et al., 2009). Notably, our model

of *Gata2* haploinsufficiency reflects crucial features of deregulated megakaryocyte biology and early erythroid commitment observed in *GATA2* deficiency patients including skewed HSC differentiation toward megakaryocyte/erythrocyte progenitors in MDS-driven familial *GATA2* haploinsufficiency (Wu et al., 2020), frequently observed BM erythroid/megakaryocytic features of dysplasia in *GATA2* haploinsufficiency patients, and peripheral thrombocytopenia, seen in about 20% of patients harboring *GATA2* mutations that have evolved to MDS/AML (Huang et al., 2009).

Functional analysis of *Gata2* haploinsufficient HSCs revealed defects in self-renewal and multi-lineage differentiation capability that were underpinned by impaired DDR. In *Gata2* haploinsufficient HSCs, we showed reduced activity of global DDR proteins ATR, Chk1 (ATR-Chk1 axis) and ATM, Chk2 (ATM-Chk2 axis), which collaboratively regulate both error-prone non-homologous end-joining (NHEJ) DNA repair and high-fidelity DDR mediated by homologous recombination (HR) (Smith et al., 2010; Sørensen et al., 2005). 53BP, another crucial regulator of NHEJ and HR (Gupta et al., 2014; Smith et al., 2010), also demonstrated attenuated activity in *Gata2* haploinsufficient HSCs. Given that DNA damage in quiescent HSCs is largely rectified by NHEJ that can enhance mutagenesis (Mohrin et al., 2010; Zhou et al., 2015), it is possible that enhanced HSC quiescence observed in *Gata2* haploinsufficient mice (Abdelfattah et al., 2021; Rodrigues et al., 2005) makes HSCs more susceptible to acquisition of secondary mutations that drive malignant transformation (Mohrin et al., 2010). Since the G0/G1 checkpoint is the major restriction checkpoint before entering mitosis (Pietras et al., 2011), in the setting of *Gata2* haploinsufficiency NHEJ could potentially expand the pool of quiescent HSCs with genomic instability, and, if those HSCs are not completely eliminated by apoptosis, on re-entry into cycle they can export genomic instability to both proliferating and differentiating HSPC pools. Together with deregulation of HR, which in contrast operates exclusively in proliferating cells (Zhou et al., 2015), our data suggest that genome instability builds across both quiescent and proliferating HSC compartments in *Gata2* haploinsufficiency. Future investigations will be required to identify the specific NHEJ and HR mechanisms deregulated in *Gata2* haploinsufficient HSCs and how this contributes to differentiation-specific impacts in HSPCs and their downstream progeny.

HSCs from *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice demonstrated deregulated inflammatory signaling, typified by up-regulation of IFN-γ signaling that may explain the observed HSC self-renewal defect (de Bruin et al., 2013; Schuettpelz and Link, 2013). It is also of interest to contemplate whether enhanced inflammatory signaling in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSC is driven by the deregulated DDR or vice versa. In the former scenario, induced quiescence and apoptosis in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs in response to DNA damage stimuli are known to elicit inflammatory cytokine secretion (de Bruin et al., 2013; Schuettpelz and Link, 2013). Alternatively, the chronic inflammatory tone evident in HSCs may initiate DNA damage genomic instability via the production of reactive oxygen species (de Bruin et al., 2013; Schuettpelz and Link, 2013). In whatever order deregulated DNA damage and inflammatory signaling pathways happens in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs, a perpetual feedback loop and crosstalk may be maintained between these pathways, thereby promoting mutagenesis (de Bruin et al., 2013; Schuettpelz and Link, 2013). Further studies to elucidate the mechanistic relationship between genome stability and inflammation in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice will be of interest.

Surprisingly, the enhanced inflammatory tone in HSCs shifts to the production of anti-inflammatory cells in the BM/spleen and down-regulation of pro-inflammatory cells in the spleen of *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ mice. Notably, however, altered production of macrophage inflammatory subsets is likely consistent with monocytopenia in *GATA2* haploinsufficiency patients and impaired inflammatory responses to pathogenic insults observed in *Gata2* haploinsufficient mice and patients alike (Dickinson et al., 2011; Hsu et al., 2011; Takai et al., 2021). Why this occurs is unclear, but there are several possible explanations. Anti-inflammatory cell generation or down-regulation of pro-inflammatory cells during HSC differentiation may be a compensatory mechanism to correct for enhanced inflammatory signaling from *Gata2* haploinsufficient HSCs. Second, the anti-inflammatory cell feedback may operate to preserve the growth and expansion of some abnormal HSC clones providing an advantage for leukemic transformation, whereas overall growth of *Gata2* haploinsufficient HSCs via self-renewal is curtailed (Avagyan et al., 2021). Finally, heightened inflammatory signaling in *Gata2* haploinsufficient HSCs may simply cause disordered differentiation to inflammatory cell lineages in the BM and spleen (de Bruin et al., 2013; Schuettpelz and Link, 2013).

We conducted analysis of *Gata2/Asxl1* double haploinsufficient mice and shRNAi *Asxl1* knockdown experiments in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs in order to understand how secondary mutations capitalize on genomic instability and deregulate inflammatory signaling in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs to facilitate the evolution of *GATA2* haploinsufficiency to MDS/AML. *Gata2/Asxl1* double haploinsufficient mice largely phenocopied the HSPC characteristics of *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSPCs and transcriptomic signatures that overlapped considerably with their single heterozygote counterparts, including DNA damage response and inflammatory signaling. This suggests that *Gata2* and *Asxl1*

cooperate to reenforce genomic instability via DNA damage responses and inflammatory signaling. However, in contrast to their single heterozygote counterparts, *Gata2/Asxl1* double haploinsufficient mice HSCs were more proliferative and engrafted poorly in transplantation experiments. Hyper-proliferation of *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs was consistent with progression through cell cycle, supported by transcriptomic profiling indicating increased deregulation of key later stage cell cycle regulators (e.g., G1/S, G2M checkpoints, DNA replication initiators, and mitosis-related pathways) linked to an increased oncogenic potential. For example, MYC target genes were enhanced in *Gata2/Asxl1* double haploinsufficient HSCs and are implicated in cell cycle regulation, yet persistent expression of MYC target genes also positively correlates with tumorigenesis (Mannava et al., 2008). Thus, our data point to HSC proliferation enabling the development of a pre-leukemia transcriptomic signature in young *Gata2/Asxl1* double haploinsufficient mice, an idea that is further supported by the unique MDS transcriptional signature observed in young *Gata2/Asxl1* double haploinsufficient mice and functional assays showing reduced differentiation to committed progenitors in CFCs from shRNA *Asxl1* knockdown experiments in *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$ HSCs and following transplantation of HSCs from *Gata2/Asxl1* double haploinsufficient mice. Future work will be focused on evaluating hematopoietic potential and disease progression in aged *Gata2/Asxl1* double haploinsufficient mice.

GATA2 haploinsufficient patients with somatic *ASXL1* mutations have a rapid onset of MDS/AML and poor prognosis (Abdel-Wahab et al., 2013; West et al., 2014). Our study offers insight into the mechanisms that may operate to facilitate progression of clinical *GATA2* haploinsufficiency syndromes from immunodeficiency to hematologic malignancy and provide a framework to understand the basis for poorer prognosis in these patients. A case in point is our finding that ABC export proteins were up-regulated in *Gata2/Asxl1* double haploinsufficient HSCs, providing a plausible basis for chemoresistance in these patients (Chigaev, 2015; Raaijmakers, 2007) and an opportunity for pharmacologic intervention to eradicate pre-LSCs/LSCs (Chigaev, 2015; Raaijmakers, 2007). Thus, the wealth of transcriptomic data presented in our analysis of young *Gata2/Asxl1* double haploinsufficient HSCs should also inform early therapeutic interventions to overcome the challenge of poor prognosis in these patients.

METHODS

Animals

Generation of *Gata2*$^{fl/fl}$, *Asxl1*$^{fl/fl}$, and *Vav-iCre* mice has been previously described (Abdel-Wahab et al., 2013; Charles et al., 2006; Stadtfeld and Graf, 2005). PB was collected by the lateral tail venesection using EDTA microvette capillary collection tubes (Sarstedt) (see supplemental information).

Preparation of BM, spleen, and thymus cells

Femurs and tibias were dissected, cleaned, and crushed in PBS with 2% FBS (Gibco). Cell suspensions were filtered through 70 μm strainers (Miltenyi Biotec). Spleen and thymus were minced in PBS/2% FBS using a syringe plunger and filtered similarly. Cells from BM, spleen, and thymus were counted using a BD Accuri flow cytometer (BD Biosciences).

Flow cytometry, magnetic c-kit^{+} cell enrichment, and cell sorting

Methods are provided in supplemental information.

In vitro colony-forming cell assay

Methods are provided in supplemental information.

Lentiviral production and transduction

Methods are provided in supplemental information.

Genomic PCR and reverse-transcription qPCR

Methods are provided in supplemental information.

Transplantation experiments

CD45.1 mice were lethally irradiated (2 × 450 cGy, 4 h apart) and used as recipients. CD45.2 BM cells were enriched for c-kit^{+}, stained for HSPC markers, and FACS sorted for HSCs (Lin^{-}c-kit^{+}Sca-1^{+}CD48^{-}CD150^{+}). For primary transplants, 150 CD45.2^{+} HSCs from various genotypes were mixed with 2 × 10^{5} CD45.1^{+} BM cells and injected via tail vein. For secondary transplants, 200 CD45.2^{+} HSCs from week-16 primary recipients were combined with 2 × 10^{5} CD45.1^{+} support cells and injected into new irradiated hosts. Donor chimerism was monitored over time; BM, spleen, and thymus were analyzed at week 16.

RNA-seq and analysis

Methods are provided in supplemental information.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Neil P. Rodrigues (rodriguesN@cardiff.ac.uk).

Material availability

All reagents generated in this study are available from the lead contact without restriction.

Data and code availability

Data are available from the lead contact. The accession numbers for *Gata2*$^{+/fl}$*; Vav-iCre*$^{+}$, *Asxl1*$^{+/fl}$*; Vav-iCre*$^{+}$, and *Gata2*$^{+/fl}$*, Asxl1*$^{+/fl}$*, Vav-iCre*$^{+}$ HSC RNA-seq reported in this paper are as follows: GSE133248, GSE283398, and GSE283399.

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AUTHOR CONTRIBUTIONS

A. Abdelfattah conducted experiments, data analysis (including RNA-seq), figure prep, and contributed to manuscript writing. A.H., L.-a.T., J.B.M.-G., and A. Almotiri performed experiments, analyzed data, and reviewed the manuscript. H. Alqahtani, H.L., S.T., M.S., R.A., H. Alzahrani, A. Alshahrani, and A.C. performed experiments and analysis. A.G. and P.G. conducted initial RNA-seq analysis. A.T., A.S.B., and K.R.K. contributed significantly to design, analysis, and reviewing the manuscript. N.P.R. conceived and led the project, designed the experiments, analyzed the data, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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