Jarid2 regulates hematopoietic stem cell function by acting with Polycomb Repressive Complex 2

Running title: Jarid2 regulates HSCs with PRC2

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Depletion of Jarid2 in mouse and human HSCs enhances their activity
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Abstract
Polycomb Repressive Complex 2 (PRC2) plays a key role in hematopoietic stem and progenitor cell (HSPC) function. Analyses of mouse mutants harboring deletions of core components have implicated PRC2 in fine-tuning multiple pathways that instruct HSPC behavior, yet how PRC2 is targeted to specific genomic loci within HSPCs remains unknown. Here we use shRNA-mediated knockdown to survey the function of PRC2 accessory factors that were defined in ES cells, by testing the competitive reconstitution capacity of transduced murine HSPCs. We find that similar to the phenotype observed upon depletion of core subunit Suz12, depleting Jarid2 enhances the competitive transplantation capacity of both fetal and adult mouse HSPCs. Furthermore, we demonstrate that depletion of JARID2 enhances the in vitro expansion and in vivo reconstitution capacity of human HSPCs. Gene expression profiling revealed common Suz12 and Jarid2 target genes that are enriched for the H3K27me3 mark established by PRC2. These data implicate Jarid2 as an important component of PRC2 that has a central role in coordinating HSPC function.
**Introduction**

Polycomb repressive complexes are major epigenetic regulators that control multiple aspects of stem cell fate\(^1\). PRC2 consists of three core polycomb group proteins: Eed, Suz12 and the histone methyltransferase Ezh2 or Ezh1, which catalyze the di- and trimethylation of histone H3 lysine 27\(^2-4\), the latter which is enriched at transcriptionally silent loci\(^5\). The generic histone chaperone proteins Rbbp4 and Rbbp7 are also often considered core PRC2 components\(^2,3\).

The majority of studies regarding the molecular mechanism of PRC2 targeting have been performed in ES cells, where PRC2 represses a number of key developmental regulators to safeguard pluripotency\(^6,7\). Although core PRC2 components lack DNA binding ability, in ES cells several accessory factors, including Jarid2 and the mammalian orthologs of the *Drosophila* Polycomb-like (Pcl) protein, Phf1, Mtf2 and Phf19, are important both for PRC2 recruitment to target genes and modulating its histone methyltransferase activity.

Jarid2 is a catalytically inactive jumonji family histone demethylase that is essential for PRC2 recruitment in ES cells\(^8-12\). Jarid2 has ARID DNA binding and zinc finger domains that demonstrate low affinity binding to DNA with a preference for CpG-rich regions, although this alone cannot explain the specificity of its genomic distribution\(^9,10\). Jarid2 additionally exhibits nucleosome and lncRNA binding capabilities that promote PRC2 assembly, association with chromatin and stimulation of methyltransferase activity\(^13-15\).

The Pcl proteins are also enriched at some PRC2 targets in ES cells, but predominantly form distinct complexes to PRC2-Jarid2\(^16-21\). Pcl proteins bind the active H3K36me3 mark via their Tudor domain thereby recruiting PRC2 to transcriptionally active chromatin\(^18-21\). While these ES cell studies have formed the basis for the paradigms of PRC2 accessory factor function, the extent to which they hold true in other cell types, particularly other rare adult stem cell populations, is unknown.

Hematopoietic stem cells (HSCs) are a well-characterized, clinically relevant stem cell population. HSCs generate the full array of mature blood cell types in a tightly regulated
process that balances self-renewal and differentiation, however alterations to PRC2 disrupt this delicate balance. Although somewhat controversial, Ezh2 appears to be important in highly proliferative fetal HSCs, yet dispensable in their adult counterparts. By contrast, Ezh1 is critical for adult HSCs; Ezh1 knockout results in bone marrow failure, due to Cdkn2a-induced senescence and reduced homing capacity. Eed knockout leads to adult HSC exhaustion through the disruption of self-renewal, differentiation and apoptosis. Therefore HSCs represent a relevant and interesting population in which to study PRC2 accessory factor function.

While complete loss of PRC2 core components compromises HSPC function and viability, seemingly contradictorily, heterozygous deletion or depletion by shRNA-mediated knockdown leads to enhanced progenitor proliferation and contribution in competitive transplantation assays. Therefore, we have surveyed the function of known PRC2 accessory factors in HSPCs, using shRNA-mediated knockdown and competitive reconstitution assays to determine which factors behave similarly to Suz12 knockdown and demonstrate enhanced contribution to all hematopoietic lineages.

We report that similar to Suz12 knockdown, Jarid2/JARID2 knockdown leads to enhanced capacity for transplantation in fetal and adult HSPCs in mouse, and in human cells cultured in vitro or transplanted into recipient mice. Gene expression profiling of knockdown HSPCs revealed that Jarid2 represses a subset of Suz12 targets in this population, suggesting that Jarid2 plays a dose sensitive role as a PRC2 accessory factor in HSPCs.

**Methods**

**Murine knockdown and transplantation assays**

Animal studies were approved by the Walter and Eliza Hall Institute Animal Ethics Committee (AEC 2011.027). Gene-specific shRNA sequences (Supplemental Table 1) were designed using the DSIR website and subcloned into the LMS-GFP or LMP-BFP vectors, adapted from with selectable markers EGFP or EBFP/puromycin, respectively. Retrovirus production, E14.5 fetal liver
(FL) cell transduction and transplantation were performed as described\textsuperscript{26,27}. Lethally irradiated (11Gy) secondary transplant recipients were injected with 1-3 million BM cells isolated from FL primary recipients, 2 or 3 recipients per donor.

Adult BM was harvested from the iliac, femur and tibia of CD45.1\textsuperscript{+} C57BL/6 donor mice. Cells were stained with rat monoclonal antibodies against lineage markers (Ter119, B220, CD19, Mac1, Gr1, CD2, CD3, CD8) then incubated with BioMag goat anti-rat IgG beads (Qiagen) for magnetic depletion. Lineage-depleted cells were stained with fluorochrome-conjugated anti-rat IgG, c-Kit and Sca-1 and sorted on the FACSARia or BD InFlux (BD Biosciences). HSPCs were incubated for 16h in StemSpan SFEM (StemCell Technologies) with cytokines (50ng/ml SCF, 10ng/ml each of TPO, IL6 and Flt3), placed on viral-coated dishes and incubated for 24h before intravenous injection into lethally irradiated CD45.2\textsuperscript{+} recipients with buffer whole BM cells.

**Human CD34\textsuperscript{+} cell in vitro expansion and transplantation**

Xenograft transplantation experiments were performed at Lund University, approved by the Lund/Malmö Ethical Committee (M39-13). Approval for the use of the human cell samples from healthy donors was given by the Ethical Committee at Lund University Hospital (2010/696). Human CD34\textsuperscript{+} cord blood cell isolation, transduction, in vitro expansion and transplantation were performed as described\textsuperscript{30}. Human FL cells were obtained from Novogenix Laboratories LLC, CA, USA. Human BM cells were obtained from healthy donors aged 20-25 after informed consent. Bone marrow aspirations were performed from the posterior superior iliac spine at the Lund University Hospital. Bone marrow derived CD34\textsuperscript{+} were acquired as for cord blood. Human CD34\textsuperscript{+} were cultured in StemSpan SFEM with 100ng/ml each of recombinant human TPO, SCF and Flt3L (Peprotech). Cells (200,000 per well) were transduced overnight in 96-well viral-loaded plates. Cells were re-plated in fresh media and maintained with biweekly half media changes and wells split as required. For transplantation, CD34\textsuperscript{+} cells were further enriched for HSCs by flow sorting for CD34\textsuperscript{+}38\textsuperscript{low}90\textsuperscript{+}45RA\textsuperscript{−} cells. After transduction and following a 36h culture as above, transduced GFP\textsuperscript{+} cells were sorted and intravenously injected (2000 cells/mouse) into the tail vein of 8-12 week old sublethally irradiated (300
cGy) NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>im1Wjl</sup>/SzJ (NSG) recipients. For secondary transplantations, a half femur equivalent of BM from primary recipients was injected into new mice. Contribution of human cells was measured by flow cytometry for human CD45 (huCD45) in the peripheral blood at 8 weeks and in the BM at 16-18 weeks post-transplant.

**RNA-sequencing**

Donor<sup>+</sup> Lineage<sup>-</sup> c-Kit<sup>+</sup> Sca-1<sup>-</sup> GFP<sup>+</sup>/BFP<sup>+</sup> HSPCs were isolated from BM of recipient mice with 30,000 to 100,000 HSPCs pooled from 6 to 10 recipients per sample. We generated 5 shRNA-Nons, 4 shRNA-Suz12, 4 shRNA-Jarid2.1 and 3 shRNA-Jarid2.2 transduced HSPC samples. Sequencing libraries were prepared using the TruSeq mRNA Sample Prep Kit (Illumina) and 100bp single-end read sequencing was performed on a HiSeq2000 or HiSeq2500 (Illumina).

The reads were aligned to mm10 using TopHat version 2.0.8 with the --b2-very-sensitive preset, then summarised over exons using featureCounts from the Rsubread package. Differential analysis was performed using Voom<sup>31</sup> together with Limma<sup>32</sup> on genes with a count per million (cpm) larger than 1 in at least 2 samples. The data is deposited in GEO (GSE60808).

Further methods and reagents are detailed in the Supplemental Material.

**Results:**

A functional survey of PRC2 accessory factors identifies Jarid2 as a critical regulator of murine hematopoiesis

First, we confirmed expression of all candidate PRC2 accessory factors (Rbbp4, Rbbp7, Phf1, Mtf2, Phf19, Jarid2) in FL-derived Lineage<sup>-</sup> Sca1<sup>+</sup> c-Kit<sup>+</sup> HSPCs (Fig. 1A). The rarity of long-term HSCs precludes the use of biochemical techniques to identify PRC2 binding partners, so we tested the function of these factors in an in vivo assay of stem cell function. FL cells were transduced with retrovirus containing validated shRNAs (Supplemental Fig. 3A) to deplete the expression of the candidate PRC2 accessory factors.
within these donor cells and their progeny. We analyzed the repopulating capacity of transduced cells in competition with non-transduced cells in reconstitution assays and compared this to mice receiving cells transduced with a negative control (non-silencing, Nons). At 10 weeks post-transplant, the contribution of transduced (GFP⁺ or BFP⁺) cells to the B and T lymphocyte and myeloid lineages in peripheral blood was analyzed, reasoning that an effect in multiple lineages was likely indicative of an effect in the HSPC population.

To account for differences in transduction efficiency, the percentage of transduced cells within each lineage at 10 weeks post-transplant (output) was compared to the percentage of transduced FL cells at the time of transplant (input). The output to input ratio for each test shRNA was compared to the output to input ratio for the negative control recipients generated from the same original donor FL cells, allowing for comparison between different cohorts. This is expressed as the ‘relative contribution’, where ‘1’ means no difference to negative controls (Fig. 1B).

Suz12 knockdown led to enhanced contribution of transduced cells to both lymphoid and myeloid lineages (Fig. 1C), as expected\textsuperscript{26,27}. Of the six factors assessed, only Jarid2 knockdown induced a similar phenotype (Fig. 1C), suggesting Jarid2 may act with PRC2 in hematopoiesis. We assessed the contribution of Rbbp4, Rbbp7, Phf1, Mtf2 or Phf19 depleted donor cells to hematopoietic cell populations in the spleen, thymus and BM at 16 weeks post-transplant (data not shown) including the HSPCs (Supplemental Fig. 4). No consistent change to relative contribution to HSPCs by both validated shRNAs for any of these five accessory factors was found, thus we focused on Jarid2. The efficacy of all hairpins was confirmed by RT-qPCR on splenic lymphocytes (Supplemental Fig. 3A) and Jarid2 and Suz12 knockdown confirmed by Western blot on transduced thymocytes, isolated at 16 weeks post-transplant (Supplemental Fig. 3B).

Depleting Jarid2-interacting H3K9 methyltransferases does not enhance repopulation capacity of FL donor cells
In addition to PRC2, Jarid2 interacts with several H3K9 methyltransferases: Setdb1, Ehmt1 and Ehmt2\(^{33,34}\). To assess whether Jarid2 may function with these proteins in hematopoiesis, validated knockdown constructs against each (Supplemental Fig. 3C) were used in competitive transplantation assays (Fig. 1B). In contrast to Suz12 or Jarid2 knockdown, H3K9 methyltransferase knockdown cells displayed a reduced relative contribution to all cell lineages analysed in the peripheral blood at 10 weeks post-transplant (Fig. 1D). These data suggest Jarid2 is not predominantly functioning in concert with H3K9 methyltransferases, and indicate previously uncharacterized roles for these enzymes in hematopoiesis.

Jarid2 knockdown enhances FL-derived donor cell contribution in competitive transplants

We analyzed the relative contribution of Suz12 or Jarid2 knockdown FL cells to hematopoietic cell populations within the thymus, spleen and BM at 6 and 16 weeks post-transplant. We observed increased relative contribution of both Suz12 and Jarid2 knockdown cells to the HSPC compartment from 6 weeks post-transplant (Fig. 2A). Despite this increase, total peripheral blood counts were normal at 6 and 10 weeks post-transplant, there was no significant difference in the total cell number of the thymus, spleen or BM between recipients of different constructs at 16 weeks post-transplant, nor did any recipients develop leukemia or lymphoma during these experiments (data not shown).

At 16 weeks post-transplant, a time point that evaluates the contribution of long-term repopulating HSCs\(^{35}\), the relative contribution of Suz12 or Jarid2 knockdown cells was enhanced for all cell types examined, including the HSPC compartment (Fig. 2B, Supplemental Fig. 1 & Supplemental Fig. 5A). While the phenotype was similar between both Suz12 and Jarid2 knockdown FL cell recipients, Jarid2 knockdown resulted in a marked increase in relative contribution to the T cell lineage (Fig. 2B), evident from the early double negative stage of thymocyte development and maintained in mature splenic T cells (Supplemental Fig. 1).
**Suz12 or Jarid2 knockdown augments contribution of FL-derived donor cells to the long-term HSC pool**

We used the SLAM cell surface markers CD48 and CD150 to delineate the long-term HSCs (LT-HSC), short term HSCs (ST-HSC) and multipotent progenitors (MPP) within the HSPC population (Fig. 2C)\(^3^6\). Within all these sub-populations both Suz12 and Jarid2 knockdown led to an elevated relative contribution, most markedly with Suz12 knockdown (Fig. 2D & Supplemental Fig 6).

To confirm that transduced HSCs were capable of maintaining hematopoiesis through serial transplantation, whole BM from recipients of transduced FL cells was injected into lethally irradiated secondary recipients (Fig. 2E). Relative contribution was calculated using the percentage of transduced cells in the HSPC compartment of the primary donor as ‘input’.

Jarid2-depleted donor cells contributed to secondary recipients, but no longer demonstrated an augmented repopulating capacity in competition with non-transduced cells, except within the T cell lineage (Fig. 2E & Supplemental Fig. 5B). As expected\(^2^6\), the relative contribution of Suz12 knockdown cells to recipient hematopoiesis increased with serial competitive transplantation, particularly in the myeloid and progenitor compartments (Fig. 2E). As the proportion of Suz12-depleted cells within the secondary transplant recipients increased, peripheral blood platelet count also increased (Figure 2F). This is the phenotype by which a Suz12 ENU-induced point mutation was first identified on a sensitized genetic background\(^2^6\). Target gene knockdown was maintained in secondary recipients at 16 weeks post-transplant (Supplemental Fig. 3D).

These data suggest that while Jarid2 has a role in controlling HSPC behavior, as a PRC2 accessory factor the extent of its role may be more specialized compared to that of core PRC2 component Suz12. Furthermore, it appears that Jarid2 has an additional function within the T cell lineage; interesting in light of the JARID2, SUZ12, EED and EZH2 mutations found in acute T lymphoblastic leukemia\(^3^7-3^9\).
Adult HSPC function is sensitive to Jarid2 knockdown

Fetal HSCs are known to functionally and transcriptionally differ from adult HSCs\(^{40}\). Previous studies imply they may also have a distinct dependency on PRC2: fetal HSCs on PRC2-Ezh2 and adult on PRC2-Ezh1\(^{22,24}\). Jarid2 is associated with PRC2-Ezh2, rather than PRC2-Ezh1 in ES cells\(^{11}\), and is only required for PRC2-Ezh2 nucleosome binding\(^{13}\). Jarid2 has, however, been shown to stimulate the methyltransferase activity of both complexes\(^{13}\). Based on these reports plus our secondary transplant data where Suz12 and Jarid2 knockdown donor cells behaved differently, we examined whether Jarid2 knockdown affects adult HSPCs.

We confirmed Jarid2 expression in fetal and adult LT-HSCs, ST-HSCs and MPPs (Fig. 3A). Jarid2 expression was comparable between fetal and adult LT-HSCs. Transduced adult BM HSPCs were used to reconstitute lethally irradiated recipients and the relative contribution of Suz12 or Jarid2 knockdown cells to hematopoiesis analyzed (Fig. 3B). Both Suz12 and Jarid2 knockdown resulted in enhanced contribution to all cell types examined at early time-points in the peripheral blood (Fig. 3C) and at 16 weeks post-transplant in the thymus, spleen and BM (Fig. 3D). Again, the most pronounced effect of Jarid2 knockdown was within the T cell lineage (Fig. 3D). Target gene knockdown was confirmed in transduced BM cells isolated at 16 weeks post-transplant (Supplemental Fig. 3E). These data suggest that, like Suz12, Jarid2 plays a pivotal role in restraining both fetal and adult HSPC repopulation capacity. This may be due to an increased proliferative burden induced by in vitro culture and/or reconstitution that leads to an increased dependence of PRC2-Ezh2 and thus Jarid2 in adult HSPCs.

**JARID2 knockdown enhances human HSPC expansion in vitro and in vivo**

We next tested whether JARID2 has a role in human HSCs. JARID2 expression was detected in HSPC populations isolated from human cord blood, which contains a mixture of fetal and adult cell types (Fig. 4A & Supplemental Fig. 2A). Two validated shRNA constructs (Fig. 4B) were used to transduce cord-blood derived HSPC-enriched CD34\(^+\) cells that normally demonstrate limited survival in vitro\(^{41}\). We observed a dose-dependent outgrowth of JARID2 knockdown GFP\(^+\) CD34\(^+\) cells relative to untransduced GFP\(^-\) cells,
which was not observed in the negative control (shRNA-Scramble, Fig. 4C & Supplemental Fig. 2B). A similar phenotype was observed with transduced FL, cord blood and adult BM CD34+ cells after 14 and 21 days in culture (Fig. 4D).

To test the effect of JARID2 knockdown in vivo, transduced cord blood-derived cells enriched for human HSC markers (CD34+CD38lowCD90+CD45RA-) were transplanted into sub-lethally irradiated NSG recipient mice. Primary recipients receiving shRNA-JARID2 transduced cells showed an increased percentage of human CD45+ cells in the peripheral blood and BM compared to the negative control (Fig. 4E & Supplemental Fig. 2C), with a normal distribution of lymphoid and myeloid cells (Supplemental Fig. 2D). Upon secondary transplantation, shRNA-JARID2 transduced human CD45+ cells were still detectable in the peripheral blood and BM of secondary recipients at 18 weeks post-transplant, whereas negative control human CD45+ cells could not be detected in the BM at this time-point (Fig. 4F). These data suggest that JARID2 functions in both fetal and adult human HSPCs. The continued presence of detectable JARID2 knockdown human cells in the BM of secondary transplant recipients likely indicates that JARID2 affects long-term HSCs.

**Jarid2 and Suz12 share common H3K27me3-enriched target genes in HSPCs**

We next investigated the molecular effects of Jarid2 knockdown in murine HSPCs. The gene expression profiles of Suz12 and Jarid2 knockdown HSPCs isolated from primary recipients of transduced FL cells at 16 weeks post-transplant were compared to the equivalent negative controls. Suz12 and Jarid2 transcripts were reduced to half that of control samples (Fig. 5A & B); however depletion of Jarid2 or Suz12 did not alter transcript levels of PRC2 core components (Fig. 5A), other accessory factors (Fig. 5B) or other jumonji domain proteins (data not shown).

In ES cells, Suz12 and Jarid2 share common targets8,10,12. In HSPCs, 529 genes were differentially expressed between Suz12 knockdown and negative controls (FDR<0.05, Supplemental Table 4), 78% of which were up-regulated (Supplemental Fig. 7A & B). Jarid2 knockdown (shRNA-Jarid2.1 and shRNA-Jarid2.2 grouped) had a more muted
effect, with 76 genes attaining genome-wide significance (FDR<0.05, Supplemental Table 5, Supplemental Fig. 7C & D). While 14 genes were significantly up-regulated in both comparisons, a simple overlap test is biased due to each knockdown being compared with the same control samples and because highly expressed genes have increased power to detect differential expression\textsuperscript{42}. Therefore to study the correlation between the effect of the knockdowns in an unbiased manner, we firstly split the control samples between the two comparisons. Secondly we selected genes with evidence of differential expression in both comparison (p<0.01, Fisher’s method of combining p-values) and finally we compared the log\textsubscript{2} fold-change between the Jarid2 and Suz12 comparisons. Genes up-regulated in the Suz12 knockdown were significantly up-regulated in the Jarid2 knockdown compared with genes that were down-regulated in the Suz12 knockdown (p<10\textsuperscript{-5}, Mann Whitney test, Fig. 5C). This suggests there are common targets of Jarid2 and Suz12.

We contemplated the biological significance of the differentially expressed genes. Both \textit{Lin28b} and \textit{Hmga2}, genes important for the high self-renewal of fetal compared with adult HSCs\textsuperscript{43}, were significantly up-regulated in Suz12 knockdown HSPCs (Supplemental Table 4). GSEA demonstrated that both Jarid2 and Suz12 knockdown produce a significant up-regulation of genes expressed in fetal HSCs\textsuperscript{44} (Fig. 5D), suggesting Jarid2-PRC2 is responsible for repressing the fetal HSC expression signature. We broadened our analysis to a further 15 hematopoietic gene sets and found a significant enrichment for genes up-regulated in HSCs compared with other hematopoietic cells in both Suz12 and Jarid2 knockdown HSPCs (Supplemental Table 6). Furthermore, an LT-HSC specific gene signature\textsuperscript{45} was significantly and strongly up-regulated in Suz12 knockdown HSPCs, and modestly up-regulated upon Jarid2 knockdown (FDR q=0.061, Fig. 5E). These effects had strong signals (Supplemental Table 6) and were driven by alteration in expression of different groups of genes (data not shown), shown by leading edge analysis.

We examined whether genes up-regulated in Suz12 or Jarid2 knockdown HSPCs were H3K27me3 marked using published chromatin immunoprecipitation followed by next
generation sequencing (ChIP-seq) data generated from wild-type adult BM HSPCs and our own ChIP-seq data from E14.5 FL HSPCs (Fig. 6A & B). Genes repressed by Suz12 and Jarid2 (positive log2-fold change, p<0.01) showed significant H3K27me3 enrichment in adult (Fig. 6A & B) and fetal HSPCs (Fig. 6A). These genes are likely direct PRC2 targets that are sensitive to reduced Suz12 and/or Jarid2 expression.

To investigate whether Jarid2 and Suz12 directly interact in hematopoetic progenitors, we performed co-immunoprecipitation assays in an ES cell-derived hematopoietic progenitor cell line, HPC7, that can partially sustain hematopoiesis when transplanted into lethally irradiated recipient mice. Immunoprecipitation of Suz12 pulled down Jarid2 (Supplemental Fig. 8A), an interaction that was validated by the reverse immunoprecipitation (Supplemental Fig. 8B). This confirms Jarid2 binds PRC2 in a relevant hematopoietic progenitor population. Furthermore, we found Suz12 knockdown depleted Jarid2 in transduced thymocytes isolated 16 weeks post-transplant (Supplemental Fig. 3B). Since Suz12 is required for complex stability, this suggests Jarid2 is also part of PRC2 in thymocytes.

Discussion
Here, we investigated the role of PRC2 accessory factors in HSPCs using sensitive, dose-dependent competitive reconstitution assays. Of the factors examined, only knockdown of Jarid2 altered the reconstitution capacity of transduced cells. In both FL and BM HSPC primary transplant recipients, depleting Jarid2 mirrored the phenotype observed upon knockdown of PRC2 core component Suz12; both knockdowns increased contribution to the HSC compartment, compared with the negative control. Furthermore, JARID2 knockdown in human cord blood, FL and adult BM CD34+ cells enhanced their capacity to be cultured in vitro, and augmented the contribution of HSC-enriched cord blood cells to transplant recipient mice. These data indicate Jarid2/JARID2 is a newly characterized regulator of HSPC function in mice and humans.

The function of Jarid2 in HSC activity has previously been examined in an RNAi screen targeting Jumonji domain-containing methyltransferases. Although two unvalidated
Jarid2 targeting hairpins scored in their assay, which is similar to ours, Jarid2 was not selected for further study. Mice carrying Jarid2 (Jmj) gene trap alleles have also been used to investigate hematopoiesis; however transplantation assays were qualitative so does not contradict our observation of enhanced contribution by Jarid2 knockdown cells\textsuperscript{50}.

While Jarid2 was the only PRC2 accessory factor to influence hematopoiesis in our assay, this does not exclude a role for the other accessory factors. Other factors may require depletion below a threshold level for a functional outcome. Factors such as histone chaperone proteins Rbbp4 and Rbbp7 or the Pcl proteins may be redundant in HPSCs. Indeed, compensation by another accessory factor might also account for the differences between Jarid2 and Suz12 knockdown in HSCs upon secondary transplantation. While we did not observe increased mRNA expression of other accessory factors in Jarid2 knockdown HSPCs, there may be increased protein stability upon long-term Jarid2 knockdown, or other as yet unidentified compensatory factors playing a role.

In HSPCs, Jarid2 appears to function as an integral component of PRC2. In addition to the similar phenotypic outcomes of Suz12 or Jarid2 knockdown in primary transplants, we observed a direct interaction between Suz12 and Jarid2 in a hematopoietic progenitor cell line and a significant overlap in genes regulated by Suz12 and Jarid2 in HSPCs, that are also H3K27me3-enriched. Both Jarid2 and Suz12 knockdown resulted in significant up-regulation of a fetal HSC expression signature\textsuperscript{44}; a set of genes where some account for the high self-renewal of fetal compared with adult HSCs\textsuperscript{43}. Moreover, Suz12 knockdown resulted in significant alterations in the expression of genes involved in LT-HSCs\textsuperscript{45}, while Jarid2 depletion led to modest changes in these genes. These findings raise the intriguing possibility that while Suz12 and Jarid2 depletion do not alter the proportion of LT-HSCs within the HSPC compartment, as determined by cell surface markers, they may increase the self-renewal capacity of LT-HSCs. This may occur in part via de-repression of the fetal HSC program, without overly compromising differentiation, consistent with what occurs in B cell progenitors depleted of Suz12\textsuperscript{51}.
While there was overlap in Jarid2 and Suz12 regulated genes, there were also Suz12 regulated genes not affected by Jarid2 knockdown. This suggests that Jarid2 may be present in only a proportion of PRC2 complexes within HSCs and therefore not affect all aspects of PRC2 function observed with Suz12 knockdown. Alternatively, a reduced level of an accessory factor may have less impact on complex stability or absolute activity than depletion of a core component. Certainly, the contribution of Suz12 knockdown cells to the HSPC compartment, particularly the LT-HSC population, was greater than that observed for Jarid2 knockdown, despite a significant enhancement compared to the negative control. The more muted effect of Jarid2 depletion on HSPC behavior and gene expression in primary recipients may account for the differing phenotypic outcome between Suz12 and Jarid2 knockdown in secondary recipients; a small enrichment of Jarid2 depleted cells may be too small to be observed above the high variance inherent in competitive transplantation. Alternatively, Jarid2 knockdown may even result in mild exhaustion of the HSPCs in secondary recipients, again with a small effect size making it indistinguishably different from controls.

Our study has identified Jarid2/JARID2 as a regulator of HSC function, and highlights JARID2 as a potential therapeutic target for HSC manipulation. Our data suggest that Jarid2 performs this role via interaction with PRC2, providing insight into the mechanistic basis of PRC2 targeting in HSPCs and broadening our understanding of the circumstances in which Jarid2 behaves as a PRC2 component. Interestingly, depletion of PRC2 has different cellular and gene expression outcomes compared to deletion of PRC2 core component Eed\textsuperscript{22}. It is worth considering these differing phenotypes in the context of inhibitors that target EZH2\textsuperscript{52}, where activity is likely to be reduced but not eliminated. Additionally, accessory factors and non-enzymatic components of these complexes are emerging as promising therapeutic targets\textsuperscript{53,54} so these differing phenotypes may be informative regarding how such drugs, when administered systemically, may affect HSC behavior. In the future it will be interesting to determine whether deletion of Jarid2 in HSCs has a similar effect to depletion.
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Author Contributions
SAK, RG, CF, KC, WSA, MD, IJM, AO, JL and MEB designed the experiments. SAK, RG, AK, OG, SL, JL, KB, LJG, DLM and MEB performed the experiments. CF and AO performed the bioinformatic analysis. SAK and MEB wrote the manuscript with input from RG, CF and JL.

All authors contributed in discussions and declare no conflict of interest.
References:


Figure legends

Figure 1: Analysing the effect of PRC2 accessory factor depletion in competitive transplantation assays. (A) RT-qPCR analysis of PRC2 accessory factor expression in HSPCs (Lin‐ Sca1+ c-kit+) isolated from E14.5 FLs, embryonic stem cells (ESC) and E14.5 murine embryonic fibroblasts (MEF) from C57BL/6 mice. Results are normalised to housekeeping genes Hprt and Hmbs, expressed relative to a HSPC sample. Data are shown as mean ± SD, n = 3. (B) Design of FL competitive transplantation assays. (C) The relative contribution of shRNA-transduced cells to the B, T and myeloid cell lineages in the peripheral blood at 10 weeks post-transplant for validated hairpins targeting PRC2 accessory factors compared to a non-silencing (Nons) control. Relative contribution is defined as the output to input ratio for each test hairpin normalised to the output to input ratio for Nons controls from the same cohort. (D) Relative contribution of FL cells transduced with shRNAs against Jarid2-interacting H3K9 methyltransferases at 10 weeks post-transplant. Data from recipients transduced with hairpins targeting the same gene have been combined. Graphs shows mean + SD, recipient mouse numbers per hairpin are shown, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 calculated using a two-way ANOVA with multiple comparisons in GraphPad Prism5 on logit transformed data (see supplementary information).

Figure 2: Jarid2-depleted cells show enhanced contribution to hematopoietic progenitors and mature cell lineages. (A) Relative contribution (mean + SD) of FL cells transduced with shRNA-Nons (white), shRNA-Suz12 (hatched), shRNA-Jarid2.1 (dark grey) or shRNA-Jarid2.2 (light grey) to HSPCs at 6 weeks post-transplant. 13-16 recipients per shRNA from 3 independent cohorts. (B) Relative contribution of transduced cells to the bone marrow (HSPCs: Lin‐ cKit+ Sca1+, myeloid progenitors: Lin‐ cKit+ Sca1‐, mature myeloid cells: Mac1+ Gr1+), spleen (B220+/CD19+ B cells) and thymus (CD4+ SP thymocytes) at 16 weeks post-transplant. Recipients are from 7 independent cohorts. (C) Representative FACS plots showing gating strategy to identify

(D) Relative contribution (mean + SD) of transduced cells to HSPC sub-populations at 16 weeks post-transplant. Recipients are from 3 independent cohorts. (E) Relative contribution (mean + SD) of transduced cells to the bone marrow at 16 weeks post-transplant. Secondary recipients are from 3 independent cohorts. (F) Peripheral blood platelet counts for secondary recipients at 6 (black) and 10 (grey) weeks post-transplant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 calculated using a two-way ANOVA with multiple comparisons in GraphPad Prism5 on logit transformed data.

**Figure 3: Adult HSPC function is sensitive to Jarid2-depletion.** (A) RT-qPCR analysis of Jarid2 expression in LT-HSC, ST-HSC and MPP purified from E14.5 FL or 8 week old adult BM from C57BL/6 mice. Results are normalised to housekeeping gene Hprt, expressed relative to a calibrator FL LT-HSC sample. Data represents mean ± SD, n = 3. (B) Experimental design for adult HSPC retroviral infection and transplantation into lethally irradiated recipients. (C) Peripheral blood analysis showing the relative contribution of transduced cells to the T, B and myeloid cell lineages (as in Fig. 2), at 6 and 10 weeks post-transplant. (D) Relative contribution (mean + SD) of transduced adult BM HSPCs to major cell types within the bone marrow, spleen and thymus (as in Fig. 2), at 16 weeks post-transplant. Recipients are from 2 independent cohorts. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 calculated using a two-way ANOVA with multiple comparisons in GraphPad Prism5 on logit transformed data.

**Figure 4: JARID2-depletion enhances human HSC expansion in vitro and in vivo.** (A) RT-qPCR analysis of JARID2 expression in human hematopoietic progenitor cells. Results are normalised to housekeeping gene HPRT1. Data represent mean ± SD, n = 3. (B) JARID2 knockdown confirmed by RT-qPCR and Western blot after 3 days of in vitro culture. Results for qPCR are normalised to housekeeping gene HPRT1. Data represent mean ± SD, n = 5-9. (C) Dose-dependent enrichment of CD34^+ cells after three weeks in culture within the JARID2-depleted GFP^+ population compared to GFP^+ and shRNA-Scramble controls. Data represent mean ± SD, n = 3. (D) Relative enrichment of CD34^+
cells from fetal liver, cord blood and adult BM after 14 and 21 days in culture. Data represent mean + SD, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 calculated using a two-way ANOVA with multiple comparisons in GraphPad Prism5. (E, F) Graphs showing the percentage of human cell chimerism (huCD45⁺) in the peripheral blood and BM of mice transplanted with shRNA-JARID2.1, shRNA-JARID2.2 or shRNA-Scramble transduced GFP⁺ cells in (E) primary and (F) secondary recipients. Primary animals used for secondary transplant have square data points.

**Figure 5: Suz12 and Jarid2 regulate common genes in HSPCs, enriched for genes important in fetal and long-term HSCs.** (A, B) Expression of PRC2 core components (A) and accessory factors (B) as measured by RNA-seq performed on transduced HSPCs sorted from FL primary recipients at 16 weeks post-transplant. There were 5 shRNA-Nons, 4 shRNA-Suz12, 4 shRNA-Jarid2.1 and 3 shRNA-Jarid2.2 transduced HSPC samples used for the analysis. Expression is shown in RPKM (reads per kilobase per million reads). (C) Box plots showing that genes up-regulated in Suz12 knockdown HSPCs compared with Nons are significantly up-regulated in Jarid2 knockdown (shRNA-Jarid2.1 and shRNA-Jarid2.2 samples combined) HSPCs (LFC is log₂ fold-change, Mann Whitney test). Genes differentially expressed in Suz12 and Jarid2 knockdown HSPCs (p<0.01, Fisher’s method of combining p-values) were split into those with positive LFC (Suz12 up) or negative LFC (Suz12 down) in Suz12 knockdown compared with half of the control samples. The LFC for Jarid2 knockdown compared with the other half of the controls was plotted for the same genes (y-axis), and a significantly higher mean LFC in the Jarid2 knockdown HSPCs was observed for genes up-regulated in Suz12 knockdown HSPCs (p=9.9 x 10⁻⁶), suggesting Jarid2 and Suz12 share common targets. (D-E) GSEA performed on Suz12-depleted (top panel) and Jarid2-depleted (bottom panel) HSPCs demonstrates significant enrichment of a fetal HSC signature (D, p<0.0001 Suz12 shRNA, p=0.02 Jarid2 shRNA) and LT-HSC genes (E, FDR<0.0001 Suz12 shRNA, FDR=0.061 Jarid2 shRNA). NES is normalised enrichment score.
Figure 6. Suz12 and Jarid2 share common H3K27me3 marked target genes in HSPCs. (A) Plots comparing gene expression profiles (shown as LFC) of Suz12-depleted (x-axis, n=4 samples) versus Jarid2-depleted (y-axis, n=7 samples) HSPCs compared to separate Nons controls (n=2-3), using RNA-seq data shown in Figure 5. Each dot represents a gene, with the size of the dot corresponding to significance of differential expression and the color corresponding to the rank of the H3K27me3 enrichment (adult HSPCs at left, fetal HSPCs at right). The enrichment of the gene ranked halfway through the list (median H3K27me3 enrichment) is colored grey and the genes that are H3K27me3 enriched or depleted genes are colored increasingly red or cyan respectively. Larger dots, indicating more significant differences in expression, are predominantly found for genes that are red (most H3K27me3 enriched), and these are generally up-regulated in both Jarid2 and Suz12 knockdown compared with negative controls (upper right quadrant). (B) Box plots showing that genes that are up-regulated when Suz12 and Jarid2 are depleted are enriched for H3K27me3 (log2 H3K27me3/input), using adult HSPC H3K27me3 ChIP-seq data. Genes were divided into those that were up-regulated in Suz12 (at left) or Jarid2 (at right) knockdown HSPCs compared with controls (positive LFC, p<0.01), or those that were not differentially expressed by Suz12 or Jarid2 knockdown (non DE, p>0.01). These genes were scored for their H3K27me3 enrichment in adult HSPCs (y-axis). There was a significantly higher H3K27me3 enrichment for genes that are up-regulated compared to genes not differentially expressed, following both Suz12 knockdown (p=1.5 x 10^{-67}) and Jarid2 knockdown (p=5.2 x 10^{-17}).
Figure 1

A

Harvest CD45.1 E14.5 FL
Infect with shRNA-GFP or -BFP retrovirus

Ter119 depletion
Culture overnight

Transplant cultured
FL cells into
lethally irradiated
CD45.2 recipient

Determine % infected FL cells at
time of transplant (input)

Measure % transduced
cells at 10 wks
post-transplant (output)

Results displayed as:
Relative contribution =
Test Output / Test Input
Nons Output / Nons Input

cf.

GFP or BFP

Gated on donor cells:

Gated on T cells:

Gated on B cells:

B220/CD19
FSC
CD45.1

CD4/CD8

GFP or BFP

Input

Output

Peripheral blood at 10 weeks post-transplant

Cell type

Relative expression

HSPC ESC MEF

Rbbp4

Rbbp7

Phf1

Mtf2

Phf19

Jarid2

HSPC ESC MEF

Cell type

Relative expression

Cell type

Relative expression

Cell type

Relative expression

C

D

Relative contribution

Relative contribution

shRNA

shRNA

n = 83 30 17 16 12 12 7 8 12 11 8 8 31 26

n = 7 8 7 7

B cells

T cells

Myeloid cells
Figure 2

A Primary recipients, 6 weeks post-transplant

B Primary recipients, 16 weeks post-transplant

C Gated on lineage^neg donor cells:

D Secondary recipients, 16 weeks post-transplant

E Bone marrow

F Platelets

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**A**  
Jarid2 expression

<table>
<thead>
<tr>
<th>Relative expression</th>
<th>LT-HSC</th>
<th>ST-HSC</th>
<th>MPP</th>
<th>LT-HSC</th>
<th>ST-HSC</th>
<th>MPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal liver</td>
<td></td>
<td></td>
<td></td>
<td>Bone marrow</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**  
Harvest CD45.1 adult BM
Sort HSPCs
Culture overnight
Lineage depletion
Infect with shRNA-GFP retrovirus
Culture overnight
Determine % infected cells at time of transplant (input)
Transplant cultured cells into lethally irradiated CD45.2 recipients
*Inject cell culture progeny of 10,000 HSPCs with 10⁵ CD45.2 buffer BM cells
*Measure contribution of transduced GFP+ cells to mature cell populations at various time points post-transplant (output)

**C**  
Adult BM HSPC transplant peripheral blood analysis

6 weeks post-transplant  
10 weeks post-transplant

**D**  
Adult BM HSPC transplant at 16 weeks post-transplant
**A**

**PRC2 core components**

![Graph showing RPKM values for Suz12, Eed, Ezh1, Ezh2, Rbbp4, and Rbbp7 genes.](image)

**B**

**PRC2 accessory factors**

![Graph showing RPKM values for Phf1, Mtf2, Phf19, and Jarid2 genes.](image)

**C**

![Box plot showing Jarid2 - Nons (LFC) with Suz12 down and up.](image)

**D**

**Fetal HSC gene set**

- Suz12 knockdown vs control
  - Suz12 kd > Nons
  - Suz12 kd < Nons
  - p < 0.0001
  - NES = 2.57

**E**

**LT-HSC gene set**

- Jarid2 knockdown vs control
  - Jarid2 kd > Nons
  - Jarid2 kd < Nons
  - p = 0.024
  - NES = 1.6

- Suz12 knockdown vs control
  - Suz12 kd > Nons
  - Suz12 kd < Nons
  - p = 9.9e-06
  - NES = 2.57

- Jarid2 knockdown vs control
  - Jarid2 kd > Nons
  - Jarid2 kd < Nons
  - p = 0.024
  - NES = 1.6

- Suz12 knockdown vs control
  - Suz12 kd > Nons
  - Suz12 kd < Nons
  - p = 9.9e-06
  - NES = 2.57

- Jarid2 knockdown vs control
  - Jarid2 kd > Nons
  - Jarid2 kd < Nons
  - p = 0.024
  - NES = 1.6

Figure 5
Figure 6

A

**H3K27me3 adult HSPCs**

- Red dots: most enriched
- Black dots: median enriched
- Turquoise dots: least enriched

**H3K27me3 fetal HSPCs**

- Red dots: p-value < 1e-6
- Black dots: p-value = 1e-4
- Turquoise dots: p-value > 0.01

B

**Suz12 targets**

- p-value = 1.5e-67

**Jarid2 targets**

- p-value = 5.2e-17
Jarid2 regulates hematopoietic stem cell function by acting with polycomb repressive complex 2