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The cohesin subunit *Rad21* is a negative regulator of hematopoietic self-renewal through epigenetic repression of *HoxA7* and *HoxA9*

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Abstract

Acute myelogenous leukemia (AML) is a high-risk hematopoietic malignancy caused by a variety of mutations, including genes encoding the cohesin complex. Recent studies have demonstrated that reduction in cohesin complex levels leads to enhanced self-renewal in hematopoietic stem and progenitors (HSPCs). We sought to delineate the molecular mechanisms by which cohesin mutations promote enhanced HSPC self-renewal since this represents a critical initial step during leukemic transformation. We verified that RNAi against the cohesin subunit *Rad21* causes enhanced self-renewal of HSPCs *in vitro* through derepression of Polycomb Repressive Complex 2 (PRC2) target genes, including *Hoxa7* and *Hoxa9*. Importantly, knockdown of either *Hoxa7* or *Hoxa9* suppressed self-renewal, implying both are critical downstream effectors of reduced cohesin levels. We further demonstrate that the cohesin and PRC2 complexes interact and are

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bound in close proximity to *Hoxa7* and *Hoxa9. Rad21* depletion resulted in decreased levels of H3K27me3 at the *Hoxa7 and Hoxa9* promoters, consistent with Rad21 being critical to proper gene silencing by recruiting the PRC2 complex. Our data demonstrates that the cohesin complex regulates PRC2 targeting to silence *Hoxa7 and Hoxa9* and negatively regulate self-renewal. Our studies identify a novel epigenetic mechanism underlying leukemogenesis in AML patients with cohesin mutations.

Introduction

Acute Myelogenous Leukemia (AML) is an aggressive myeloid malignancy[1,2]. Even when patients are treated with highly intense chemotherapy regimens, including consolidation with allogeneic bone marrow transplantation (BMT), long-term survival rates remain poor. The cause of this poor prognosis for patients with AML is multifactorial. One common finding hindering the development of novel therapeutics is high genetic variability among patients[3]. A subset of patients have recurrent chromosomal translocations which generate driver oncogenic fusion proteins such as *RUNX1-RUNX1T1* or *MYH11-CBFB*[3]; however, the majority of patients do not have a clear structural abnormality driving their leukemia. Many karyotypically normal AML patients have mutations in genes including *FLT3, TET2, NPM1, CEBPA,* or *DNMT3A*. A study in which 200 patients with *de novo* AML were sequenced[3] revealed that patients have on average 13 distinct somatic mutations within the coding regions of >250 well-annotated genes, indicating AML arises from a diverse combination of mutations. A subset of the genes uncovered were not previously described in AML patients including subunits of the cohesin complex (*RAD21*, *STAG2*, *SMC1A*, *SMC3*)[3–7]. Cohesin mutations tend to occur early within disease pathogenesis[3,6] implying that these mutations are critical events during leukemia development. Subsequent studies have confirmed that cohesin mutations are relatively common in AML, and frequently co-occur with known driver mutations including *NPM1* and *FLT3*-ITD[3,4,6–8].

Complete loss of cohesin function causes an embryonic lethal phenotype in knock-out mice, implying that some level of cohesin function is absolutely required for normal development[9–12]. Interestingly, somatic cohesin mutations in AML are uniformly heterozygous and not associated with dramatic changes in chromosomal number, implying that cohesin mutations promote AML through a function independent of their role during mitosis[3,6,7,13,14]. Cohesin plays a critical role in controlling gene expression[13,15,16]. The complex facilitates DNA looping that permits interactions between distal genomic segments and gene promoters facilitating transcriptional regulation in *cis*[17–19]. The cohesin complex interacts with CCCTC-binding factor (CTCF) to organize the genome and recruit *cis* regulatory elements which promote gene silencing[17,20–22]. Given the importance of transcriptional regulators in the pathogenesis of AML[3], it has been hypothesized that cohesin mutations promote AML through altered gene expression.

Recent studies demonstrate that reduced cohesin expression confers enhanced self-renewal on hematopoietic stem and progenitor cells (HSPCs) both *in vitro* and *in vivo*[23–26]. These studies demonstrated that reducing expression of single cohesin genes induces genome-wide changes in chromatin accessibility, and when combined with Flt3-internal tandem

duplications (ITD) mutations it resulted in potentiated Stat5 signaling and 100% penetrant leukemia with an average latency of 6 months[24]. Other studies observed altered numbers of higher order hematopoietic stem and progenitor cells (LT- and ST-HSCs) *in vivo,* after prolonged cohesin-depletion[23–25], whereas more differentiated progenitor populations (CMP, GMP, MEP) remained unchanged[23,25]. Prolonged cohesin-depletion causes increased HSPC self-renewal, a potential mechanism to leukemic transformation[23,26]. Thus, while multiple groups have demonstrated cohesin mutations promote enhanced HSPC self-renewal and altered gene expression, the precise molecular mechanism connecting cohesin depletion to these changes remains unclear.

Materials and Methods

A detailed Materials and Methods section can be found in the supplemental material.

Results

Rad21 depletion augments self-renewal and proliferation of murine HSPCs *in* vitro

Rad21 is a critical component of the cohesin complex and to define its role in normal hematopoiesis *in vitro*, we transduced murine HSPCs with puromycin-resistance encoding lentiviruses containing two independent shRNAs targeted to non-overlapping regions of *Rad21*. RT-qPCR and immunoblotting confirmed *Rad21* mRNA (Figure S1A and 1A) and protein (Figure 1B, C) was efficiently depleted in shRNA treated samples 24 hours after viral transduction and after primary and secondary passages in methylcellulose.

To assess whether *Rad21* depletion altered self-renewal *in vitro*, we performed serial replating assays[23–26]. Through serial replating, *Rad21*-depleted cells maintained the ability to generate CFUs, and showed enhanced proliferation (Figure 1D, E). During the fourth replating *Rad21*-depleted colonies were larger and denser than control colonies (Figure 1F). Based on recent findings that chronic cohesin depletion in HSPCs causes elevated erythroid potential[25], we monitored granulocyte/marcophage (CFU-GM), granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM), and erythroid blast forming unit (CFU-BFU-E) colonies at the end of primary methylcellulose passage. We observed no statistically significant differences between the *Rad21*-depleted HSPCs compared to controls (Figure 1G). From these data we conclude that *Rad21* depletion confers enhanced self-renewal to murine HSPCs *in vitro* consistent with published studies[23–26].

One possible explanation for the enhanced self-renewal is that *Rad21* depletion selectively depletes lower order progenitors, thereby enriching for short term- or long term-hematopoietic stem cells (LT-HSCs, ST-HSCs). Thus, we monitored percentages of c-kit-negative/lineage-positive and c-kit-positive/lineage-negative cells, and observed no significant differences between the *Rad21*-depleted and empty vector cells (Figure S2A and S2B). Importantly, this assay is not sensitive enough to distinguish between subtypes of higher order HSPCs (LT-HSCs, ST-HSCs, and MPPs). These data are consistent with previous studies which observed minimal changes in differentiation of cohesin-deficient HSPCs at 1 week during *ex vivo* culture[26].

Rad21 depletion does not induce aneuploidy

Given that patients with germline heterozygous cohesin mutations are not predisposed to aneuploidy[11,27], we hypothesized that *Rad21* depletion of 50% would not induce aneuploidy[28]. We identified cells in G_0/G_1 (2N DNA content), S-phase (2N-4N), Mitosis (4N), and aneuploid cells (<2N or >4N) by flow cytometry. In accordance with published studies[7,23–26], we observed no significant increases in aneuploid cells following *Rad21* depletion, but did observe enhanced self-renewal evidenced by decreased cells in G_0/G_1 with increased cells in S and M-phase following *Rad21* depletion (Figure S3).

Rad21 depletion causes increased expression of the self-renewal factor *Hoxa9*

We hypothesized that *Rad21* depletion increases self-renewal through alterations in gene expression. Next, we isolated mRNA from HSPCs at the end of the primary passage and conducted targeted transcriptome analyses by microarray. Overall, approximately 1,000 probes (384 probes UP and 660 DOWN) demonstrated a minimum 2-fold change following *Rad21* depletion, consistent with reduced *Rad21* levels causing broad changes in gene expression (Table S5). Among genes with a statistically significant change following *Rad21* depletion (Figure 2A and Table S5), we noted an approximately 10-fold increase in the expression of *Hoxa9*, a homeodomain-containing transcription factor that is over-expressed in about half of human AMLs. *HoxA9* expression is associated with a poor prognosis[29–31] and is known to confer enhanced self-renewal on HSPCs *in vivo* and *in vitro*[32,33]. We reasoned that if *Rad21*/cohesin negatively regulates *Hoxa9*, *its* expression would be elevated rapidly and chronically following *Rad21* depletion. We noted a statistically significant increase in *Hoxa9* expression within 24 hours of *Rad21* depletion (Figure 2B), which was maintained through secondary replatings (Figure 2C). Next we reanalyzed microarray data sets in which a 4-hydroxytamoxifen (4-OHT)-inducible *Hoxa9* expression vector was used[34] (GSE21299) to identify genes activated *(Hoxa9*-Up*)* or repressed (*Hoxa9*-Down) by *Hoxa9* overexpression. We analyzed *Hoxa9*-altered genes using Gene Set Enrichment Analysis (GSEA) to determine if they demonstrated a statistically significant, concordant difference with the gene lists generated from *Rad21*-depleted HSPCs[35]. We observed a significant concordance with *Rad21* depletion (Figure 3A), specifically that *Hoxa9*-activated genes were elevated and *Hoxa9*-repressed genes were repressed following *Rad21* depletion. To confirm that a small subset of genes was not driving this phenomenon, we selected the top 100 genes increased or decreased by *Hoxa9* overexpression and monitored changes in the same probes after *Rad21* depletion. Additionally, we compared our microarray data to a recently published RNA-seq data set in which cohesin subunits (Rad21 and Smc3) were depleted and found that in all samples, *Hoxa9* target genes showed a similar pattern of expression[25] (Figure 3B). More broadly we found substantial overlap between our microarray data and previously published RNA-seq data[25] (Figure S4).

Given the increased self-renewal phenotype we wondered whether *Meis1*, which cooperates with Hoxa9 to induce AMLs, was also overexpressed by Rad21 depletion[36–38]. RT-qPCR analyses did not reveal any differences in *Meis1* expression (Figure S5). Collectively, these data show that *Rad21* depletion induces aberrant expression of *Hoxa9* and its downstream targets, but has no effect on *Meis1* expression, which is consistent with the observation that cohesin depletion alone is insufficient to drive leukemic transformation.

Additional HoxA family members exhibit increased expression in *Rad21*-depleted HSPCs

Because the entire *Hoxa* locus is silenced by PRC2 during hematopoiesis[21,39], we speculated that additional *Hoxa* factors would exhibit elevated expression. Thus, we reanalyzed published RNA-seq data from cohesin-depleted HSPCs[25], and found elevated expression of *Hoxa7* and *Hoxa9*, with smaller changes in other *HoxA* genes (Figure S6A). We confirmed these data by RT-qPCR and included *Hoxb4* as a negative control since it was unchanged following cohesin depletion[25] (Figure S6B). Given the known role of the cohesin complex in modulating expression of genes within chromatin domains[40,41], these data imply that *Rad21* is required to repress the chromatin domain containing *Hoxa7* and *Hoxa9*.

AML patients with cohesin mutations have elevated levels of *Hoxa7* and *Hoxa9*

Given changes in *Hoxa7* and *Hoxa9* expression following cohesin depletion, we hypothesized that AML patients harboring cohesin mutations would express higher levels of both *HOXA7* and *HOXA9* compared to AML patients lacking cohesin mutations. Next, we reanalyzed transcriptome datasets from the Cancer Genome Atlas (TCGA), of which 47% were karyotypically normal[3,4]. Among patients with (25) or without (172) cohesin mutations (*SMC1A, SMC3, STAG2,* or *RAD21)* we observed a statistically significant increase in both *HOXA7* and *HOXA9* expression in cohesin-mutated AML (Figure 3C). Consistent with a multiple hit model of AML, cohesin mutations co-occur most frequently with mutations in *NPM1* (p-value $6.32\times10^{-33,4}$).

Depletion of *Hoxa7* or *Hoxa9* diminshes HSPC self-renewal

Given the elevated expression of *Hoxa7* and *Hoxa9*, we wondered whether depletion of either of these two factors could rescue the enhanced self-renewal phenotype elicited by *Rad21* depletion. Thus, we transduced HSPCs with empty vector alone, *Rad21*-shRNAs alone, or *Rad21-shRNAs* combined with *Hoxa9*- or *Hoxa7*-shRNAs. At the end of primary methylcellulose plating we observed fewer colonies present in the *Rad21*/*Hoxa7 or Rad21*/*Hoxa9* co-depleted cultures (Figure S7A, Figure S8A). This reduction in colony number was associated with overall reduced cell numbers, and the phenotype was maintained in secondary platings (Figure S7B, C, D, Figure S8B, C, D). These data are consistent with previously published reports highlighting a role of *Hoxa7* and *Hoxa9* in regulating HSPC self-renewal[37,42–44]. Importantly, depletion of *Hoxa7* or *Hoxa9* alone (Figure S9A–B) diminishes HSPC self-renewal (Figure S9C–G). Thus, both *Hoxa7* and *Hoxa9* are required for HSPC self-renewal, and their effects following *Rad21* depletion are not a form of epistasis.

Rad21 cooperates with PRC2 to repress target gene expression

Previous studies have shown that *Hoxa7* and *Hoxa9* expression are repressed by the PRC2 complex[39,45], and selectively induced in myeloid- and erythroid-committed progenitor cells by removal of its associated histone mark H3K27me3[46,47]. To identify pathways altered by *Rad21* depletion in an unbiased fashion, we performed GSEA and screened 3,395 different pathways (gene sets). Each curated gene set was generated by perturbing a biological system with different chemical, RNAi, or genetic means. We narrowed the list (Table S6) by focusing on gene sets derived from hematopoietic tissues and noted an upregulation of 38

genes repressed by Ezh2 (Figure 4A), the catalytic subunit of the PRC2 complex[48]. We confirmed this derepression by a differential expression analysis (Figure 4B). Collectively, these data imply that *Rad21* depletion causes derepression of PRC2 target genes.

The derepression of a large number of its target genes suggests either reduced PRC2 levels or impaired activity. To investigate the former we performed immunoblotting for two members of the PRC2 complex and observed no significant difference in the levels of Ezh2 or Suz12 (Figure 4C–E). Next, we assessed if global levels of histone H3K27me3 were altered by *Rad21* depletion. Following *Rad21* depletion we observed an approximately 40%–50% reduction in histone H3K27me3 levels (Figure 4C&F). From these data we conclude that *Rad21* depletion reduces levels of the repressive mark H3K27me3 without disrupting PRC2 expression.

Cohesin and PRC2 complexes interact in murine myeloid cells

Given our results, we hypothesized that cohesin and PRC2 interact to mediate epigenetic silencing. First, we reanalyzed a published proteomics study that characterized binding partners of the PRC2 complex[49] and noted the core PRC2 subunit Eed interacted with Rad21 in two murine cell types (Figure S10). Next, we performed co-immunoprecipitation experiments using lysates from immortalized murine myeloblast cells (32D cells). We observed interactions between members of the cohesin and PRC2 complexes, and could identify these interactions by immunoprecipitating either complex (Figure 5A). Members of the cohesin and PRC2 complexes are shown in Figure 5B for reference. Whether the interactions observed represent direct or indirect association between the two complexes will require additional studies.

Rad21 mediates PRC2 recruitment to the *Hoxa9* locus and deposition of H3K27me3

Given that Rad21 and PRC2 interact in myeloid cells and that *Hoxa* genes are silenced by PRC2[30,45], we hypothesized that cohesin would be bound near the *Hoxa7* and *Hoxa9* locus. Using data from both the ENCODE projects, we identified Rad21/RAD21 occupancy between the *Hoxa7*/*HOXA7* and *Hoxa9*/*HOXA9* genes at a Ctcf/CTCF binding site. The entire *Hoxa*/*HOXA* locus exhibits high levels of enrichment of the repressive histone mark H3K27me3 consistent with PRC2-mediated silencing. These patterns were observed in both mouse (Figure S11) and human (Figure S12) hematopoietic and non-hematopoietic cell lines. The proximity of a cohesin:CTCF site was intriguing; in other contexts CTCF mediates gene silencing, and in the *Hoxa* cluster facilitates PRC2 targeting[17,21,22]. Next, we hypothesized that *Rad21* depletion induces *HoxA7&9* expression through loss of H3K27me3. ChIP analyses revealed a significant reduction in H3K27me3 at the promoters of both *Hoxa7* and *Hoxa9* following *Rad21* depletion (Figure 6A). In summary, these data demonstrate that Rad21 facilitates PRC2-mediated silencing of *Hoxa7* and *Hoxa9* (Figure 6B).

Given the colocalization of cohesin, PRC2, and CTCF at the *Hoxa* locus (Figure S11 and S12) we wondered whether depletion of *Ctcf* in HSPCs would phenocopy the enhanced self-renewal observed following *Rad21* depletion. *Ctcf* depletion had minimal effect on *Hoxa7* (Figure S13B) or *Hoxa9* (Figure S13C) expression. The lack of elevated *Hoxa7* and *Hoxa9*

expression, was accompanied by a reduced number of CFUs and cells numbers (Figures S13D, E). These data suggest that although CTCF likely plays a role in regulating PRC2 and cohesin occupancy at the *Hoxa* locus its functions are so diverse that its depletion is not tolerated by HSPCs.

Discussion

AML is a complex myeloid malignancy hallmarked by genetic heterogeneity, and as with most cancers, multiple cooperating mutations are involved in oncogenic transformation. This implies that different genetic mutations disrupt similar pathways inducing a shared phenotype such as enhanced self-renewal. In this study, we focused on cohesin mutations, which are frequently observed in AML. Recent studies have shown that reduced cohesin promotes hematopoietic self-renewal, and when combined with FLT3-ITD causes 100% penetrant AML[23–26]. In addition, these studies observed changes in chromatin accessibility following cohesin depletion. In one study, genes displaying increased chromatin accessibility around their TSS demonstrated increased occupancy by transcription factors critical to self-renewal such as Gata2, Erg, and Runx1[23]. Our study lends further mechanistic, in that the PRC2 histone mark H3K27me3 classically promotes recruitment of the PRC1 complex which mediates polynucleosome compaction[50,51]. Thus, cohesin loss may ultimately impart enhanced self-renewal by preventing proper chromatin compaction around genes that need to be silenced for proper differentiation.

One open question is how the cohesin complex is targeted to the *Hoxa* locus to recruit the PRC2 complex to mediate epigenetic silencing. One possible explanation is that cohesin regulates gene expression by modulating genomic architecture. Key insights into the role of cohesin in regulating gene expression were uncovered with the discovery of a direct interaction between cohesin and the DNA binding protein CTCF[17]. CTCF plays a central role in regulating gene expression at multiple levels, including acting as an insulator to block enhancer:gene interactions or to organize the genome into topologically associated domains (TADs[52–55]). The interaction between CTCF and cohesin was initially thought to be critical in regulating genomic architecture, specifically establishment of TADs. Interestingly, seminal studies from the Merkenschlager lab demonstrated that homozygous deletion of *Rad21* in non-cycling thymocytes does not alter TAD architecture, but rather disrupts long-range interactions within TAD compartments (sub-TADs[41]). The authors found that TAD architecture overall remained intact, but that cohesin loss resulted in compression of the dynamic range of gene expression through loss of sub-TAD interactions. This is interesting because other studies have shown that the *Hoxa* cluster is regulated in a tissue-specific manner by CTCF and *cis*-regulatory elements located within sub-TADs surrounding the *Hoxa* locus which ultimately results in PRC2-mediated gene silencing[21,56–58] Our data supports a model whereby cohesin is essential for proper PRC2 recruitment and inhibition of *Hoxa7* and *Hoxa9* expression, which may be the result of a sub-TAD interaction dependent upon cohesin. Unlike the results of Seitan *et al*[41], we did not see broad increases across all *Hox* clusters, as would be predicted if all sub-TAD interactions were disrupted. The discrepancy is likely secondary to differences in the level of cohesin loss, specifically our use of an approximately 50% reduction in *Rad21* levels versus complete deletion of *Rad21*. Alternatively, it may be secondary to our use of cycling bone marrow cells, as opposed to

non-cycling thymocytes. Importantly, this raises the intriguing hypothesis that some cohesin-dependent chromatin interactions require a higher level of cohesin than others.

Our data demonstrates an interaction between the cohesin and PRC2 complexes, disruption of which increases expression of the self-renewal factor *Hoxa9*. Determining whether the interactions between the cohesin and PRC2 complexes are direct or indirect will require additional studies. Modulators of this pathway could provide possible therapeutic targets for patients with cohesin-mutated AML. *Hoxa9* expression has been shown to be, in part, activated by di- or tri-methylation of histone H3 on lysine 79 (H3K79me2/me3) catalyzed by Dot1l, which is blocked by PRC2 activity[59–61]. Dot1l inhibitors are already in clinical trials for MLL rearranged AML; therefore, these could provide a possible therapeutic approach that could reverse the aberrant expression of *Hoxa* genes in cohesin-mutated AML. Whether or not these histone marks are altered upon *Rad21*/cohesin depletion remains unknown, but provide testable hypotheses to identify therapeutic targets for patients with cohesin-mutated AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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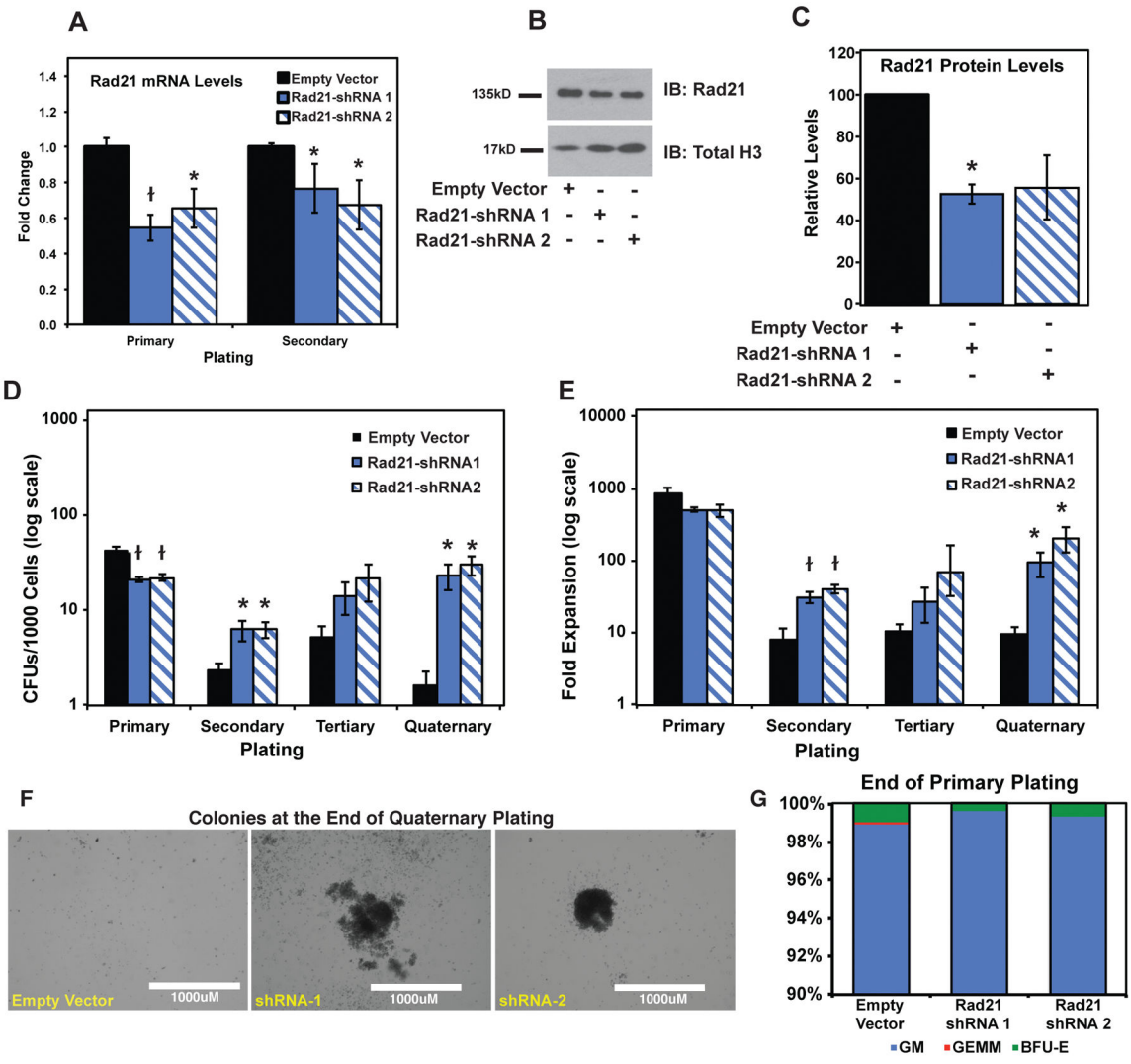

Figure 1. *Rad21* depletion enhances self-renewal of primary HSPCs

(A) qRT-PCR analysis of control and *Rad21*-depleted lineage negative HSPCs at the end of primary and secondary methylcellulose platings. (B) Immunoblotting of *Rad21*-depleted and control HSPCs using antibodies against Rad21 and Total Histone H3. (C) Densitometry of the blot shown in panel B. (D+E) Control and *Rad21*-depleted HSPCs were serially passaged in methylcellulose and after each passage colony number (D: CFUs/1000 cells) and total cell numbers (E: fold expansion) were determined. (F) Representative images of colonies from empty vector and *Rad21*-depleted HSPCs at the end of quaternary plating. (G) Colony counts (GM, BFU-E, GEMM) of empty vector and Rad21-depleted HSPCs at the end of primary methylcellulose culture.

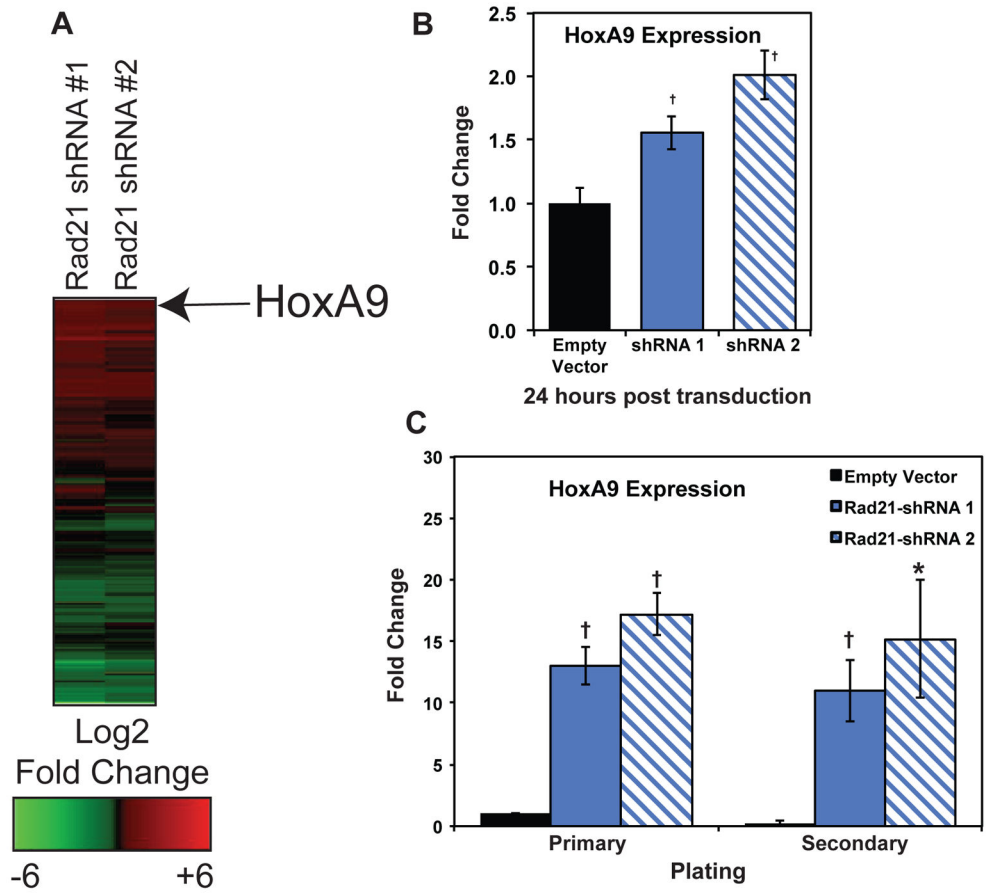

Figure 2. ***Rad21*** **depletion increases** ***Hoxa9*** **expression**

(A) Microarray heat map showing the gene expression changes following *Rad21* depletion. (B+C) qRT-PCR analysis of empty vector and *Rad21*-depleted HSPCs after (B) 24 hours, or (C) after culture in methylcellulose.

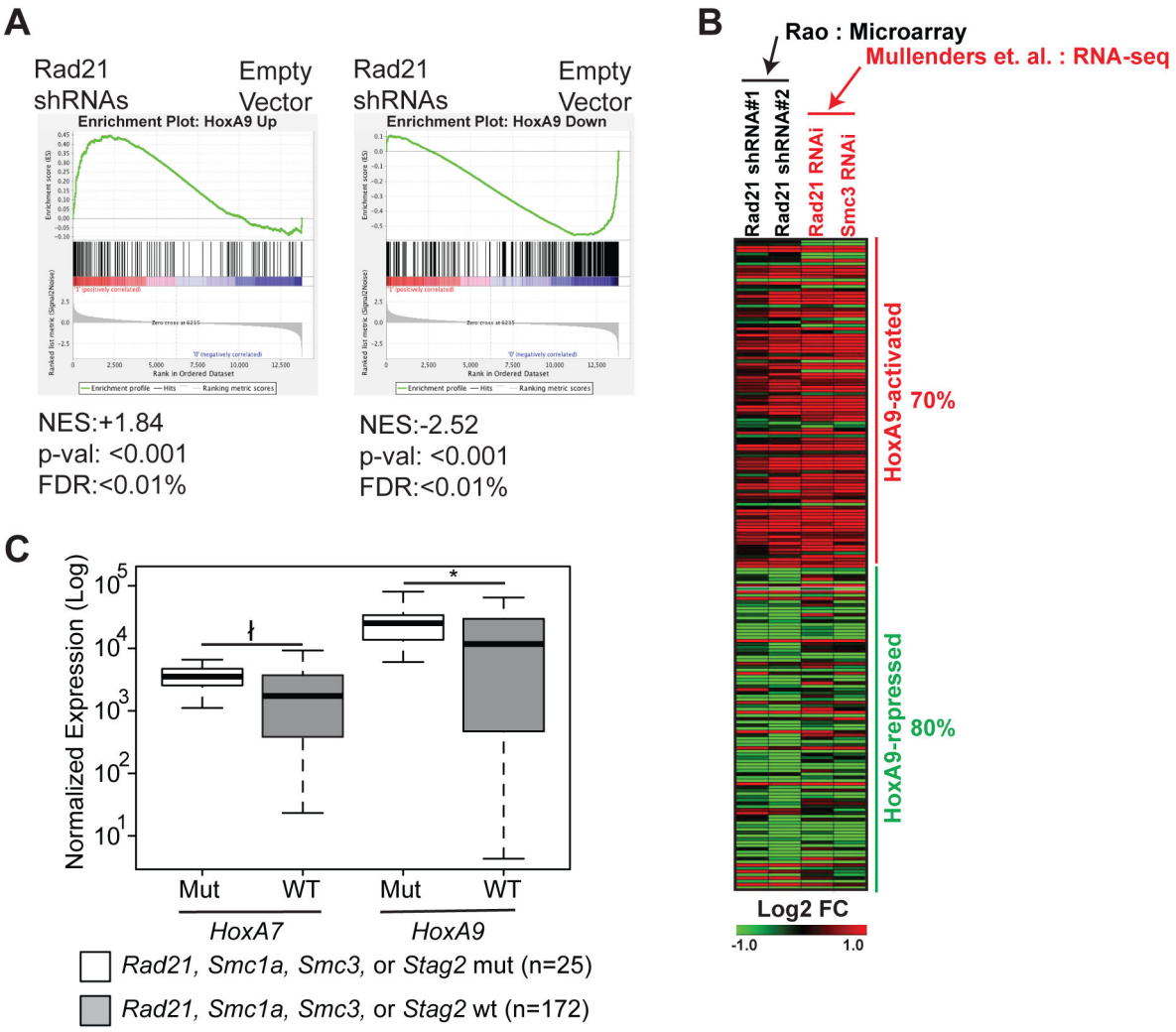

Figure 3. *Rad21* depletion alters expression of Hoxa9 target genes

(A) GSEA was performed comparing control and *Rad21*-depleted HSPCs against a gene set compiled from[34] consisting of genes that exhibit increased (*Hoxa9* Up) or decreased (*Hoxa9* Down) expression following forced *Hoxa9* expression. (B) Heat map showing fold changes of *Rad21*-shRNA1 and shRNA2 over empty vector using the list of genes in Panel A. RNA-sequencing data from[25] was included for comparison, in which either *Rad21* or *Smc3* were depleted. The percent concordance between the microarray and RNA-seq datasets, is shown on the right. (C) *Hoxa7* and *Hoxa9* probes were analyzed from cohesin-mutant and cohesin-WT AML patients. () indicates # of patients.

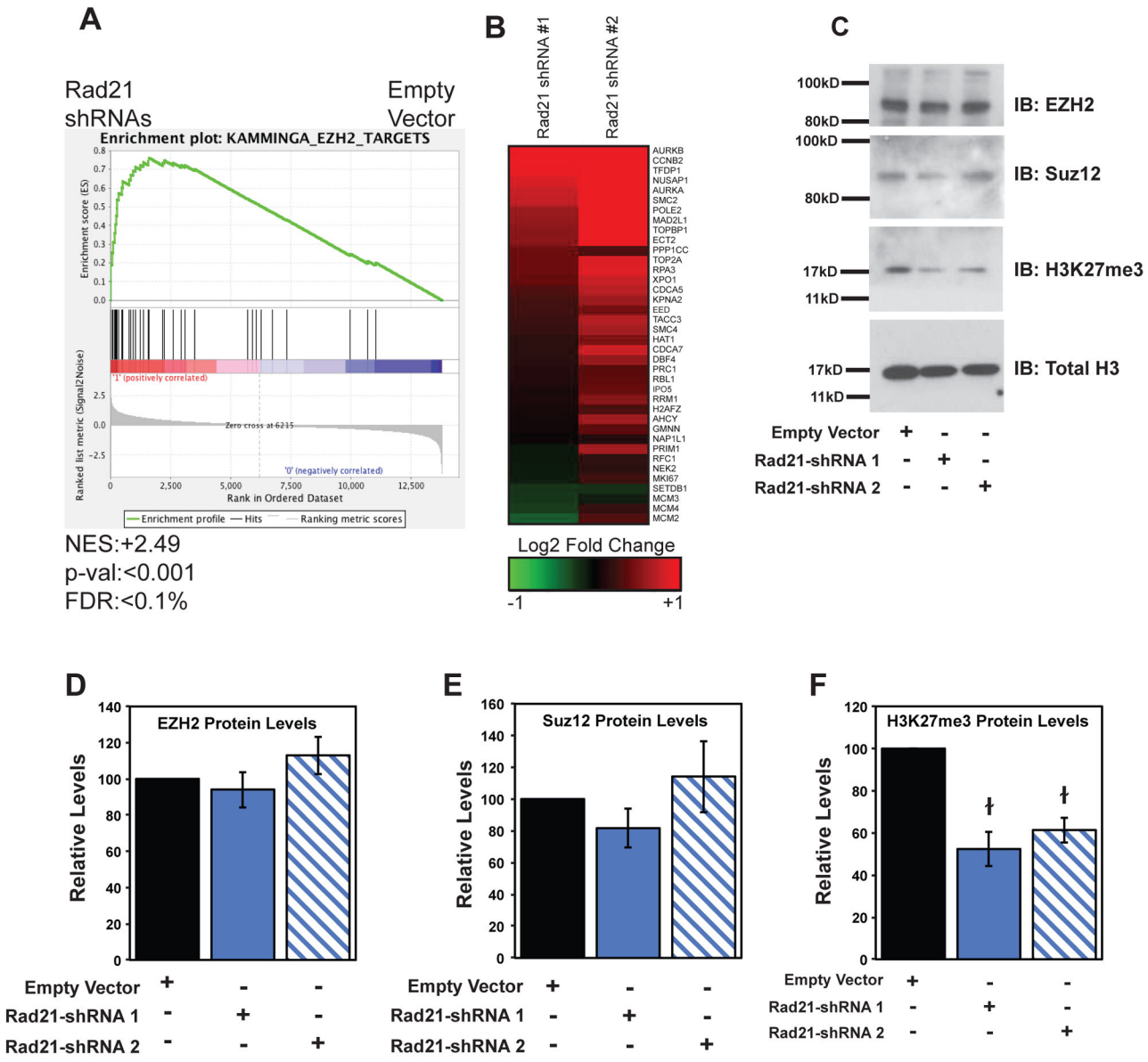

Figure 4. *Rad21* depletion causes de-repression of PRC2 target genes

(A) GSEA showing the correlation between *Rad21*-depleted HSPCs and genes repressed by the PRC2 subunit Ezh2. (B) Heat map showing increased expression of Ezh2-repressed genes. (C) Immunoblot analysis of lysates from empty vector and *Rad21*-depleted cells at the end of primary methylcellulose plating. (D–F) Densitometry of blots shown in panel C showing quantitation of 3 independent biological replicates for Ezh2 (D), Suz12 (E), and H3K27me3 (F).

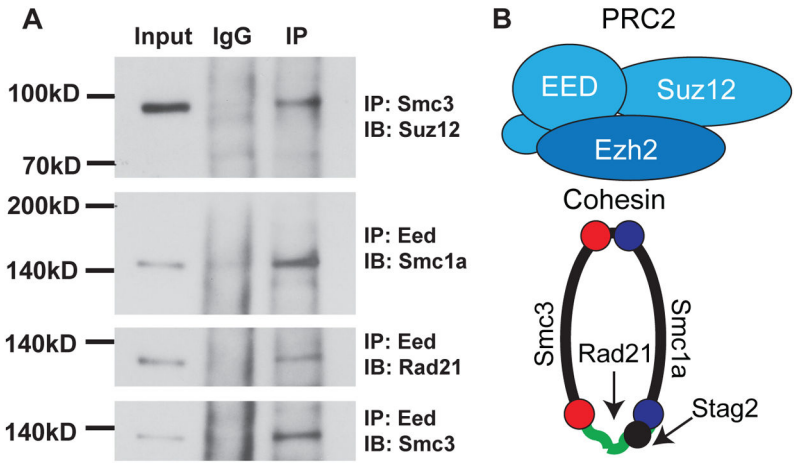

Figure 5. Cohesin and PRC2 interact in murine myeloid cells

(A) Endogenous immunoprecipitation from mouse myeloid progenitor cells. Precipitated proteins were analyzed by western blotting using antibodies against various cohesin and PRC2 complex members. (B) Schematic of the cohesin and PRC2 complexes.

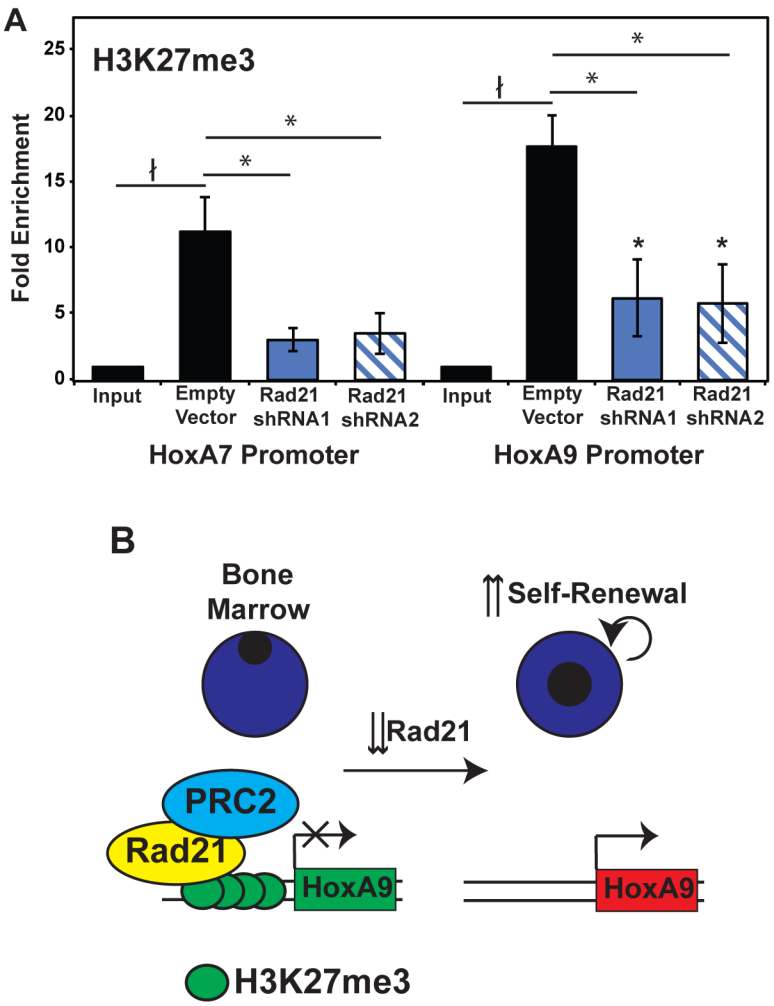

Figure 6. *Rad21* depletion decreases histone H3K27me3 deposition at the *Hoxa9* locus

(A) ChIP was performed on control and *Rad21*-shRNA1 and shRNA2 HSPCs at the end of primary methylcellulose culture. Chromatin was precipitated using an antibody against histone H3K27me3 and purified DNA was analyzed by qPCR using primers against the *Hoxa7* and *Hoxa9* promoters. Each data point represents 6 and 3 independent biological replicates for *Hoxa7* and *Hoxa9* respectively. (B) Model for how Rad21 regulates expression of *Hoxa7* and *Hoxa9*.