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Abstract:
Maintenance of quiescence and DNA replication dynamics are two paradoxical requirements for the distinct states of dormant and active hematopoietic stem cells (HSCs), which are required to preserve the stem cell reservoir and replenish the blood cell system in response to hematopoietic stress respectively. Here, we show that key self-renewal factors, β-catenin or Hoxa9, largely dispensable for HSC integrity in fact have dual functions in maintaining quiescence and enabling efficient DNA replication fork dynamics to preserve the functionality of hematopoietic stem and progenitor cells (HSPCs). While β-catenin or Hoxa9 single knockout (KO) exhibited mostly normal hematopoiesis, their co-inactivation led to severe hematopoietic defects stemmed from aberrant cell cycle, DNA replication and damage in HSPCs. Mechanistically, β-catenin and Hoxa9 function in a compensatory manner to sustain key transcriptional programs that converge on the pivotal downstream target and epigenetic modifying enzyme, Prmt1, which protects the quiescent state and ensures an adequate supply of DNA replication and repair factors to maintain robust replication fork dynamics. Inactivation of Prmt1 phenocopied both cellular and molecular phenotypes of β-catenin/Hoxa9 combined KO, which at the same time could also be partially rescued by Prmt1 expression. The discovery of the highly resilient β-catenin/Hoxa9/Prmt1 axis in protecting both quiescence and DNA replication dynamics essential for HSCs at different key states provides not only novel mechanistic insights into their intricate regulation but also a potential tractable target for therapeutic intervention.

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Data availability

All RNA-seq, ATAC-seq and CUT&RUN-seq data are available in GEO under accession code GSE235821.

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Abstract

Maintenance of quiescence and DNA replication dynamics are two paradoxical requirements for the distinct states of dormant and active hematopoietic stem cells (HSCs), which are required to preserve the stem cell reservoir and replenish the blood cell system in response to hematopoietic stress respectively. Here, we show that key self-renewal factors, β-catenin or Hoxa9, largely dispensable for HSC integrity in fact have dual functions in maintaining quiescence and enabling efficient DNA replication fork dynamics to preserve the functionality of hematopoietic stem and progenitor cells (HSPCs). While β-catenin or Hoxa9 single knockout (KO) exhibited mostly normal hematopoiesis, their co-inactivation led to severe hematopoietic defects stemmed from aberrant cell cycle, DNA replication and damage in HSPCs. Mechanistically, β-catenin and Hoxa9 function in a compensatory manner to sustain key transcriptional programs that converge on the pivotal downstream target and epigenetic modifying enzyme, Prmt1, which protects the quiescent state and ensures an adequate supply of DNA replication and repair factors to maintain robust replication fork dynamics. Inactivation of Prmt1 phenocopied both cellular and molecular phenotypes of β-catenin/Hoxa9 combined KO, which at the same time could also be partially rescued by Prmt1 expression. The discovery of the highly resilient β-catenin/Hoxa9/Prmt1 axis in protecting both quiescence and DNA replication dynamics essential for HSCs at different key states provides not only novel mechanistic insights into their intricate regulation but also a potential tractable target for therapeutic intervention.

Key points:

- Novel complementary functions between β-catenin and Hoxa9 in regulating Prmt1 to protect stem cell quiescence and DNA replication dynamics

- Prmt1 KO phenocopies and its re-expression partially rescues cellular and molecular defects of β-catenin/Hoxa9 KO

Keywords: quiescence, β-catenin, Hoxa9, Prmt1, DNA replication stress,

Introduction

Life-long regeneration of the blood system relies on a rare population of self-renewing hematopoietic stem cells (HSCs) that are capable of upkeeping faithful genome replication and integrity[1-6]. Maintenance of quiescence/dormancy not only allows cells to persist in nondividing state over extended time to preserve self-renewal potential, but also protects against DNA damage and functional exhaustion[1,2,7]. However, HSCs must also respond efficiently to hematological stress such as infection, blood loss or exposure to cytotoxic agents by re-entering active cell cycle to rapidly generate functional progenitors and progenies of different lineages. In contrast to quiescent HSCs (qHSCs), active HSCs (aHSCs) must robustly replicate their genome to replenish the hematopoietic system without depleting the stem population[8-10]. So far factors have been identified to enable either but not both features (e.g., CDK inhibitors for quiescence, MCM proteins for genome replication)[3,11,12], suggesting the maintenance of each cell status by distinct molecular pathways. While this stepwise regulation of different pathways in governing status switching is in line with linear discrete hematopoietic differentiation model, emerging evidence indicate that HSC differentiation and acquisition of lineage-specific fates is a continuous process characterized by a highly coordinated transcriptional program[4,13,14]. Given the interfluent requirement of quiescent and active HSC states that enable swift adaptation to changing requirements[13,15], it remains to be determined if there are indeed common pathways operating to safeguard both quiescence and efficient DNA replication[4,7,12].

To gain novel insights into the molecular regulation of HSC functions, signaling pathways such as Homeobox, Wnt, Notch and Hedgehog, which are frequently dysregulated in cancer, have been shown to govern self-renewal in hematopoietic stem and progenitor cells (HSPCs)[16]. Ectopic expression of Hoxa9[17-20] or β-catenin[21-26], the key component of canonical Wnt pathway[27], promotes HSC expansion and eventually leukemic transformation[16]. Importantly, overexpression of either factor is highly predictive of poor patient prognosis, whereas their inhibitions suppress leukemia development, leading to intense therapeutic interests in targeting these factors[28,29]. However, inactivation of β-catenin or Hoxa9 has only modest impacts on adult HSCs, suggesting 1) the existence of alternative pathways that support hematopoietic self-renewal in the absence of either factor, or 2) their otherwise distinct physiological functions in normal HSCs that are different from those upon their over-expression. Extensive searches for such alternative pathways over the past decade (e.g., γ-catenin in β-catenin knockout (KO), Hoxb3/4 in Hoxa9 KO) have so far yielded little success, casting doubts about the physiological roles of these key self-renewal molecules in HSC functions[30, 31]. Clearly, resolving this dilemma will have profound implications on stem cell biology and also strategic therapeutic design. How HSCs sustain their functions in the absence of either factor and what the underlying mechanisms are remains enigmatic.

Here we establish, for the first time, how endogenous β-catenin and Hoxa9 function in a novel complementary manner with dual roles in protecting HSCs from exit of quiescence and accumulation of replication stress, where activation of key downstream epigenetic enzyme Prmt1 is responsible, at least in part, for circumventing functional decline of HSCs.

Methods

Mouse models and transplantation studies

β-catenin^{fl/fl} mice[32] were crossed with *Hoxa9*^{−/−} knockout mice[19,33] to generate *Hoxa9*^{−/−} *β-catenin*^{fl/fl} Rosa-CreER mice. Conditional *Prmt1* knockout mouse models were generated using targeted ES cell clones of C57BL/6, as described previously[34]. Further details in supplemental Methods. $1x10^6$ CD45.2[+] donor bone marrow cells were transplanted intravenously into lethally irradiated (13Gy total body Y-irradiation) C57BL/6 congenic strain expressing pan-leukocyte marker CD45.1 (Jackson Lab; JAX:002014) recipient mice along with $2x10^5$ CD45.1[+] helper bone marrow cells. Deletion of floxed alleles were achieved via daily intraperitoneal injection of Tamoxifen (60 μl at 20 mg/ml) for five consecutive days.

Immunophenotype analysis and sorting

Femora and tibiae were isolated from transplanted mice and gently crushed in staining medium (Phosphate buffered saline (PBS) + 2% fetal bovine serum (FBS)) prior to red blood cell lysis. Detailed staining procedure for stem and progenitor populations can be found in supplemental Methods. Stained cells were analyzed on a BD LSRII flow cytometer using the gating strategy illustrated in supplemental Figure 1F and described previously[35].

Ki67 and BrdU immunostaining

For *in vivo* BrdU incorporation analysis, transplanted mice were intraperitoneally injected with 150 μl (10 mg/ml solution) BrdU (BD Biosciences) two weeks post induction of target gene deletion. After 48 h, mice were sacrificed and femora, tibiae and pelvis were isolated and LSKs sorted by FACS. For Ki67 staining, LSK cells were isolated from primary transplanted mice two weeks post targeted gene deletion. Isolated LSKs were fixed, permeabilized and stained as outlined in supplemental Methods.

Immunofluorescent staining

Bone marrow isolated from knockout mouse models were enriched for cKit^{+} cells using CD117 Micro-Beads (Miltenyi Biotec) by positive selection according to manufacturer's instructions. ckit[+] HSPCs were treated with either 4-hydroxytamoxifen (4-OHT, 25 ng/ml, Sigma) or EtOH (vehicle) for 48 h prior to fixation and staining as outlined in supplemental Methods.

DNA fiber assay

CD45.2$^+$ ckit$^+$ HSPCs were isolated from transplanted mice, 5000 cells per sample were incubated at 37 °C for 30 min in StemSpan medium (StemCell Technologies). Subsequently, 19mM 5-Chloro-20 - deoxyuridine (CldU, Sigma) was added to the medium for 30 min. Medium was exchanged and cells incubated with 28mM 5-iodo-20 -deoxyuridine (IdU, Sigma) for 30 min. DNA fibers were then spread on glass slides and stained as described previously[36,37].

RNA-sequencing

Total RNA from LSK cells was isolated using the RNeasy Plus Micro Kit (Qiagen) and Library preparation was performed using Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs, E7760L), according to manufacturer's instructions. Detailed information in supplemental Methods.

ATAC-sequencing

A total of 50,000 LSK cells from WT or KO mouse models were used for ATAC-seq which was performed as described previously with minor modifications[38]. Peaks were detected using function callpeak from the MACS2 tool with '--format BAMPE' option[39]. Profile plots were generated with computeMatrix and plotProfile from deepTools[40].

CUT&RUN-sequencing

CUT&RUN was performed using 0.5 million HSPCs from WT BL6 mice or Ctnnb1-Biotin-3xFLAG knockin mice (JAX:029511) according to Epicypher CUT&RUN Protocol V2.0. Detailed information in supplemental Methods.

Results

Co-inactivation of Hoxa9 and β-catenin induces severe defects in hematopoietic stem populations

To identify potential molecular pathways that sustain stem cell integrity in the absence of Hoxa9 or β-catenin, RNA-sequencing was performed on LSKs (Lin$^-$c-Kit$^+$Sca1$^+$) harboring inactivation of either factor. To our surprise, among the top candidate pathways involved in maintaining HSC functions are canonical Wnt signaling and Hoxa9 pathways upon the reciprocal inactivation of Hoxa9 and β-catenin respectively (supplemental Figure 1A and B). While no prior molecular link has been reported in HSCs between these two pathways, the hypothesis of their novel crosstalk in preserving HSC functionality is consistent with our recent discovery of the co-regulation of posterior *Hoxa* loci and *Ctnnb* by long noncoding RNA, HOTTIP[17,26]. To further investigate this novel crosstalk, we generated a Hoxa9$^{-/-}$ *Ctnnb1*$^{fl/fl}$ Cre ER mouse model by crossing our previously described *Hoxa9* KO mice[33] with *Ctnnb1* floxed mice carrying Rosa26-CreER allele[23] for serial bone marrow transplantation assays (Figure 1A and supplemental Figure 1C-E). In contrast to single Hoxa9 or β-catenin

inactivation where we observed only mild hematopoietic phenotypes with a noticeable reduction of common myeloid progenitor (CMP) as previously reported[33,41], combined inactivation of Hoxa9/β-catenin (dKO) resulted in severe hematopoietic defects at all measured time points (Figure 1B and supplemental Figure 1F-P. Defects originated as early as the LT-HSC stage with a substantial 3.5-fold reduction and a 5-fold reduction in ST-HSCs at 12-week time point compared to wild-type (WT) counterparts. Consistent with a stem cell defect, we also observed significant reductions in downstream myeloid progenitor populations including common myeloid progenitor (CMP) (3.5-fold), granulocyte-monocyte progenitor (GMP) (5-fold) and megakaryocyte and erythrocyte progenitors (MEP) (4-fold). In line with these findings, the absolute number and percentage of total CD45.2$^+$ donor cells were drastically reduced in the dKO bone marrow (17%) compared to single KOs (67%) (Figure 1C and supplemental Figure 1Q and R). At a functional level, *in vitro* clonogenic assays revealed that dKO bone marrow cells generated markedly reduced myeloid colony numbers of which mature CFU-G and CFU-E were the predominant composition (supplemental Figure 1S). LSKs isolated from dKO mice generated significantly fewer colonies and were devoid of multipotential progenitors (CFU-GEMM) in contrast to the diverse colony composition of control and single KO cells (supplemental Figure 1T). The frequency of long-term culture initiating cells (LTC-IC) was also significantly diminished in dKO HSPCs (Figure 1D), consistent with functional decline of HSCs. Finally, the ability of dKO cells to engraft and reconstitute the bone marrow of secondary recipient mice was severely impaired compared to WT and single KO cells (supplemental Figure 1U and V). Together, these data suggest that co-inactivation of β-catenin/Hoxa9 results in significant functional defects in the stem population.

To gain further insight into the mechanisms underlying the stem cell defects in dKO HSCs, RNA-sequencing analysis revealed a significant reduction of stem related gene sets in dKO LSKs compared to either β-catenin KO or Hoxa9KO alone. Indeed, genes down-regulated in HSCs (Jaatinen_Hematopoeietic_Stem_Cell_DN, Boquest_Stem_Cell_DN) were positively enriched in dKO LSKs with additional enrichment in stem cell differentiation pathways (WP_Stem_Cell_Differentiation) (Figure 1E and supplemental Tables 1), suggesting their roles in maintaining stemness. Comparison of differentially expressed genes (DEG ≥1.5 fold, padj ≤0.05) in Hoxa9 KO and β-catenin KO LSKs revealed that a small subset (n=226) is co-ordinately regulated by the single KOs consistent with an independent and compensatory relationship between these two pathways in governing HSC functions (Figure 1F). Strikingly, GO analysis revealed that the DEGs exclusively regulated by dKO LSKs (n=877) were overwhelmingly correlated with cellular processes involved in regulating cell cycle, DNA replication, double-strand break repair and genomic stability (Figure 1F), suggesting their key functions in regulating cell cycle, DNA replication and repair. Further mechanistic insight into the Hoxa9/β-catenin crosstalk was revealed by Cleavage Under Targets and Release Using Nuclease (CUT&RUN) sequencing on endogenous Hoxa9 and

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endogenously FLAG-tagged β-catenin in HSPCs. Genome-wide chromatin mapping of Hoxa9 and β-catenin binding demonstrated a striking correlation in binding site occupancy (supplemental Figure 1W). Consistent with RNA sequencing analysis, the regions of Hoxa9/β-catenin co-occupancy were enriched in pathways regulating cell cycle, DNA repair and replication in addition to stem cell pluripotency pathways with co-occupancy detected at the loci of HSC regulators including Myc, Fos, Jarid2 and Stat3 (supplemental Figure 1X and Y).

Concurrent Hoxa9/β-catenin deletion disrupts HSC quiescence, induces replication stress and DNA damage accumulation

We next sought to functionally investigate the effect of β-catenin/Hoxa9 co-inactivation on quiescent state of HSCs which greatly aids their ability to preserve their genomic integrity and that of all future downstream lineages. LSKs were isolated from single and dKO transplanted mice two weeks post deletion, and stained with the proliferation marker, Ki67, and the DNA content dye, Hoechst 33342, to examine their quiescent vs proliferation status. As a control, WT mice were treated with the thymidylate synthase inhibitor, fluorouracil (5-FU), known to induce replication stress resulting in exit of HSCs from quiescence and entry into cell activation programs[42]. Similarly, dKO LSKs displayed a marked reduction in the frequency of cells within the G_0 subset or Ki67$^-$ fraction and a concomitant increase in Ki67$^+$ fraction compared to WT or single Hoxa9/β-catenin KO (Figure 2A-C, supplemental Figure 2A). To further confirm the increase in the fraction of actively cycling LSKs, *in vivo* bromodeoxyuridine (BrdU) incorporation analysis was performed. Consistent with Ki67 staining, dKO LSKs exhibited a significantly greater frequency of BrdU$^+$ cells compared to WT or single β-catenin/Hoxa9 KO (Figure 2D and supplemental Figure 2B). Together, these findings indicate that while the complementary functions between β-catenin and Hoxa9 are capable of resiliently preserving HSC functionality upon its single knockout, their combined inactivation drive HSCs out from their quiescent state into active cycling.

Effective and faithful DNA replication is fundamental for genomic integrity and cell cycle progression, which is particularly important after HSCs exit from quiescence. In order to functionally investigate if DNA replication dynamics were indeed altered upon inactivation of β-catenin/Hoxa9 as suggested by the RNA-sequencing analyzes, HSPCs isolated from primary transplanted mice were pulse-labeled with nucleotide analogues (CldU, IdU), and DNA fibers were spread to monitor replication fork progression at the single-molecule level (supplemental Figure 2C and D). Combined inactivation of β-catenin and Hoxa9 significantly compromised the speed of replication forks evident by reduced fork track length in dKO cells compared to WT or single KO cells (Figure 2E and F and supplemental Figure 2E). Consistent with their heightened levels of replication stress, dKO HSPCs displayed asymmetrical progression of sister forks from recently fired origins, and an overall higher frequency of stalled forks in the population of events (Figure 2G and H and supplemental Figure 2F-

H). Moreover, dKO HSPCs displayed an accumulation of γH2AX foci indicative of double-strand breaks and fork collapse (Figure 2I and J and supplemental Figure 2I). Together these results are consistent with a novel function of β-catenin/Hoxa9 in protecting HSPCs from replication stress resulting in replication fork stalling, accumulating DNA damage and functional decline of HSCs.

Prmt1 KO phenocopies the severe hematopoietic, quiescent, DNA replication and damage defects associated with β-catenin/Hoxa9 KO

To identify key downstream players that mediate the dKO phenotype, further investigation of gene set enrichment analysis (GSEA) of RNA-sequencing data generated from dKO LSKs revealed a significant negative enrichment in arginine histone methylation (Figure 3A). Protein arginine methyltransferase 1 (PRMT1) is the predominant epigenetic enzyme that catalyzes the asymmetric dimethylation of arginine and its overexpression has been implicated in numerous solid and hematological cancers leading to significant clinical interest in developing PRMT-targeted therapeutics[34,43,44]. Further evaluation of Prmt1 in dKO LSKs revealed a substantial reduction in Prmt1 expression compared to single KO LSKs (Figure 3B and supplemental Figure 3A). CUT&RUN sequencing revealed co-occupancy of Hoxa9 and β-catenin at Prmt1 transcription start site (TSS) (Figure 3C). At the same time, Assay for Transposase Accessible Chromatin (ATAC)-sequencing analysis revealed reduced chromatin accessibility at the *Prmt1* promoter region in dKO LSKs (Figure 3D), consistent with epigenetic downregulation of Prmt1 expression upon co-inactivation of β-catenin/Hoxa9. To further investigate the role of Prmt1 in hematopoietic development, we generated a novel Prmt1 KO model where Prmt1 can be conditionally inactivated in HSCs upon Tamoxifen treatment. Deletion of *Prmt1* alone phenocopied the β-catenin/Hoxa9 dKO, resulting in severe reductions in LT-HSC, ST-HSC and downstream progenitor populations at all analyzed time points (Figure 3E-H and supplemental Figure 3B-F). Both *in vitro* clonogenic assays and LTC-IC assays demonstrated functional decline of HSPCs with impaired colony forming ability and significantly reduced frequency of LTC-IC (supplemental Figure 3G and Figure 3I). Knockout of *Prmt1* resulted in severe stem cell defects as evidenced by the inability of *Prmt1* KO cells to engraft and reconstitute in secondary recipient mice (supplemental Figure 3H).

Importantly, Prmt1 deficient LSKs displayed features of hematopoietic and replication stress consistent with the observed β-catenin/Hoxa9 (dKO) phenotypes. These included a diminishing G_0 (quiescent) fraction (Figure 4A and B) and corresponding increases in Ki67+ (Figure 4C) and BrdU+ (Figure 4D and supplemental Figure 4A) fractions. Perturbations in replication dynamics were evidenced by reduced replication fork speed (Figure 4E and F and supplemental Figure 4B) and stability (Figure 4G and H and supplemental Figure 4C and D), leading to the accumulation of DNA breaks (increased γH2AX foci, Figure 4I and J and supplemental Figure 4E). These findings consistently suggest that Prmt1 may serve as a functional downstream mediator of the β-

catenin/Hoxa9 complementary signaling axis that preserves DNA replication dynamics and long-term self-renewal potential that underpin stem cell functionality.

Prmt1 KO replicates the transcriptomic and epigenetic alterations induced by dKO

Further comparison of the DEGs revealed a striking correlation between β-catenin/Hoxa9 and Prmt1 regulated targets in LSKs, where over 50% of β-catenin/Hoxa9 targets were also identified as Prmt1 targets with approximately 90% of their common targets regulated in the same direction, consistently suggesting Prmt1 as a key transcriptional mediator for β-catenin/Hoxa9 in HSCs (Figure 5A). In addition to the expected negative enrichment in arginine histone methylation, Prmt1 KO replicated the pathways deregulated in dKO LSKs including abnormal cell cycle, DNA replication, DNA repair (GO: pathway) and chromosomal instability (GO: mouse phenotype) (supplemental Figure 5A). GSEA of Prmt1 KO LSKs also revealed enrichment in stem cell differentiation pathways and positive enrichment in genes normally down-regulated in HSCs (Figure 5B), although it can be possible that this reduction in stemness occurs as a consequence of dysfunctional DNA replication and repair rather than direct regulation of stemness related genes.

We then focused our analysis to identify possible transcriptomic alterations that may underpin the observed perturbations to replication dynamics and genomic stability. ATAC- and RNA-sequencing analyzes revealed that both dKO and Prmt1 KO LSKs were characterized by significant deficiencies in both chromatin accessibility and expression of molecules involved in DNA replication and repair compared to WT and single KOs (Figure 5C-F and supplemental Figure 5B-C). Of note, numerous members of the minichromosome maintenance complex (MCM2-7) which binds and licenses origins of replication essential for HSC functionality[11] were among the targets co-regulated by β-catenin/Hoxa9 and Prmt1 including Mcm2, Mcm3, Mcm4, Mcm5 and Mcm7. Additionally, targets such as Fancd2, Brca1 and Pcna play a dual role in canonical HR-mediated repair of stalled forks and replication stress tolerance reflecting the extensive crosstalk between the processes of DNA replication and repair. These findings indicate that Prmt1 functions as a pivotal target for the convergence of the β-catenin and Hoxa9 compensatory pathways to maintain an adequate repertoire of replication and repair factors required for protection against the detrimental effects of replication stress after HSCs exit from quiescence.

Prmt1 rescues both cellular and molecular defects of β-catenin/Hoxa9 KO HSPCs

To further interrogate the integral downstream role of Prmt1 in the β-catenin/Hoxa9 signaling axis, we performed ectopic expression of PRMT1 in dKO HSPCs (supplemental Figure 6A). Functional assays demonstrated that PRMT1 could significantly rescue the colony forming ability of dKO LSK cells (Figure 6A) and markedly restore the frequency of LTC-IC (Figure 6B). In addition, dKO HSPCs transduced with WT but not catalytically dead mutant of PRMT1 (Mut) displayed a significantly reduced shift in BrdU incorporation and maintained a profile highly reflective of WT HSPCs, whereas

expression of WT PRMT1 in WT HSPCs did not result in further reduction of BrdU+ cells (Figure 6C and supplemental Figure 6B). Importantly, ectopic expression of WT PRMT1 also protected against perturbations to replication dynamics seen in dKO HSPCs transduced with empty vector (EV) or PRMT1 Mut. Replication track length in dKO HSPCs was fully restored by expression of WT but not PRMT1 Mut (Figure 6D and E, supplemental Figure 6C). Similarly, sister fork progression and the frequency of stalled forks in dKO HSPCs were rescued to levels observed in control HSPCs when WT, but not PRMT1 Mut, was ectopically expressed (Figure 6F and G, supplemental Figure 6D and E). Intriguingly, similar to the results from BrdU studies above, overexpression of WT PRMT1 in WT HSPCs did not confer a replicative advantage as no increase in fork speed and no alteration in bidirectional fork symmetry was observed, suggesting that PRMT1 functions in a protective manner to safeguard against replication stress rather than conferring a generalized proliferative or replicative advantage (supplemental Figure 6F and G). Correspondingly, PRMT1 protected against the accumulation of YH2AX foci induced by β-catenin/Hoxa9 co-inactivation in HSPCs (Figure 6H and I, supplemental Figure 6H).

At the gene expression level, we also demonstrated that enforced expression of WT PRMT1 could restore all the major transcriptomic alterations in the dKO. In addition to stem related genesets (Figure 6J), PRMT1 also rescued gene expression programs for DNA replication and repair processes including those associated with DNA helicase activity, methylation-dependent protein binding (GO: Molecular Function), DNA replication and repair (GO: Biological Process) (Figure 6K and L, supplemental Figure 6I and J). In contrast, genes upregulated upon expression of PRMT1 Mut were not associated with any of these functions or processes (supplemental Figure 6K). Together, these functional and transcriptomic data indicate that Prmt1 regulates DNA replication dynamics, genomic integrity and gene expression programs of HSPCs, thereby preserving stem cell functionality.

Discussion

The maintenance of quiescence and replication potential are two paradoxical requirements of HSCs that underpin their functionally distinctive dormant and active states, imperative to the lifetime supply of hematopoietic cells. In this study, we depict novel functions of the β-catenin/Hoxa9/Prmt1 axis that robustly preserves HSC functionality by protecting both stem cell quiescence and DNA replication dynamics. We propose that the complementary regulation[17,26] and functions between β-catenin and Hoxa9 can provide a resilient safeguard mechanism to protect HSCs against suppression of either single factor (Figure 7, top panel). Only co-inactivation of β-catenin and Hoxa9 drives HSCs out of their quiescent state into active cycling (Figure 7, bottom panel). Upon their exit from quiescence, accurate genome duplication which requires the orchestrated activation and maintenance of replication forks emanating from thousands of origins of replication during S-phase of the cell cycle then immediately becomes one of the most important challenges faced by aHSCs[6,45].

Perturbations to normal replication fork progression due to exogenous and endogenous stress such as DNA lesions, insufficient nucleotides, chemical compounds or a shortage of replication factors lead to fork stalling[46,47]. Sustained replication stress or the inability to resolve replication obstacles can result in fork collapse, via fork inactivation or endonucleolytic cleavage leading to double-strand breaks[48]. Here we show that the shortage of replication factors such as Mcm2, Mcm3, Mcm4, Mcm5, Mcm7, cdc7, Fancd2 and Brca1 as a result of Prmt1 downregulation in the β-catenin/Hoxa9 dKO directly impedes replication fork speed and stability, ultimately resulting in an accumulation of double-strand breaks and functional decline of the stem cell population (Figure 7, bottom panel). Indeed, old cycling HSCs in mice were shown to have heightened levels of replication stress associated with cell cycle defects, chromosome breaks and altered DNA replication fork dynamics, which have been attributed to decreased expression of key replication factors such as MCM helicase components[3,11]. The spontaneous recombination events and genomic instability induced by perturbations to replication dynamics are associated with many pathological conditions. For example, homozygous inactivation of any member of the Fanconi anaemia replication factors leads to the pediatric syndrome, Fanconi anemia, characterized by progressive bone marrow failure, chromosomal instability and high cancer predisposition[49,50].

Increasing evidence has revealed that protein arginine methylation plays critical roles in regulating DNA damage response including methylation of canonical DDR molecules such as MRE11, USP11, BRCA1 and 53BP1[51,52,44,53,54]. At the same time, Prmt1 mediated H4R3 methylation has been correlated with active transcription[55-57] and our preliminary unpublished CUT&RUN sequencing data also showing potential direct binding of PRMT1 to key DNA replication/repair loci, raises a possibility of transcriptional regulation of DNA replication/repair pathways by PRMT1. Here, we reveal a novel role of Prmt1 as a key, albeit likely not the only, mediator of β-catenin and Hoxa9 functions to preserve stem cell quiescence and DNA replication dynamics in part by maintaining adequate expression of DNA replication and repair factors at a transcriptional level, thereby alleviating DNA damage accumulation and preserving HSC integrity (Figure 7). Given the importance of quiescence and replication dynamics in maintaining stem cell functionality, it is not surprising that HSCs have developed intricate crosstalk and compensatory signaling pathways to protect such critical cellular processes in the events of genetic and/or epigenetic inactivation of either of the key players. Our finding of novel functions of the β-catenin/Hoxa9/Prmt1 axis in safeguarding both stem cell quiescence and DNA replication fork processivity also provide support for a continuum model to enable dynamic switching between qHSC and aHSC states[4,58,59]. Given that the current study was conducted solely using mouse models, further future studies in human HSCs will be required to confirm our findings although previous studies have shown highly conserved hematopoietic functions of Hoxa9 and β-catenin in both mouse and human[20,23,60-62]. While the proto-oncogenic and generally undruggable natures of transcription factors make β-catenin and Hoxa9 challenging targets,

identification of Prmt1 with tractable enzymatic activity as a key mediator in safeguarding HSC functionality provides a novel avenue for therapeutic intervention[63]. Since many of these molecules are also frequently hijacked during malignant transformation and responsible for treatment resistance[19,23,64], a deeper understanding of their roles in maintaining stem cell functions will also aid in the development of anti-leukemia targeted therapies.

Authorship

Contribution: JL performed majority of the experiments and prepared the manuscript draft; ET, TKF, CV, PL, NC carried out some of the experiments; KG, BZ and JWHW conducted bio-informatics analyzes; ML and SH advised on experimental design and data interpretation; CWES designed and oversaw the execution of project and preparation of the manuscript.

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Figure Legends

Figure 1. Co-inactivation of Hoxa9/β-catenin induces severe HSC defects. (A) Schematic representation of experimental procedures. (B) Absolute number of HSPC populations and (C) percentage CD45.2$^+$ donor cells from indicated mice at 12 week time point (at least n=3 mice/group). Floxed allele deletion was achieved by intraperitoneal injection of Tamoxifen for five consecutive days. β-catenin$^{fl/fl}$ mice treated with corn oil (vehicle) served as WT control as indicated. (D) Long-term culture initiating cell (LTC-IC) frequency from HSPCs as indicated. Enrichment in stem related genesets (E) and gene ontology analysis (F) of LSKs isolated from transplanted mice. All data represent mean ±SEM. P values calculated by Student's *t* test. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$, ns p > 0.05.

Figure 2. Disruption of stem cell quiescence and DNA replication dynamics by concurrent Hoxa9/β-catenin deletion. (A) Representative FACS plots of Ki67 and Hoechst 33342 staining of LSKs isolated from indicated mice. (B) Percentage of LSKs in G_0 cell cycle phase. (C) Percentage Ki67$^+$ and (D) BrdU$^+$ LSKs. n=3 mice per group. (E) CldU track length of DNA fibers from HSPCs isolated from indicated mice. (F) Representative images of replication fork lengths. Scale bar, 10μm (G) Ratio of IdU track lengths of bidirectional forks. (H) Percentage of stalled forks (green only) present in single/combined KO cells. Fiber data from one representative experiment is shown, n=3 data is shown in supplemental Material. Immunofluorescent images (I) and foci counts (J) of YH2AX staining on HSPCs isolated from indicated mice. Several fields of cells were scored from two independent experiments. Scale bar, 25μm. WT BL6 mice treated with corn oil (vehicle) are indicated as WT control**.**

Figure 3. Prmt1 KO phenocopies the hematopoietic defects associated with dKO. (A) Negative enrichment in arginine methyltransferase activity in isolated dKO LSKs. (B) Independent qPCR validation of Prmt1 expression in indicated LSKs. Data represents n=2 independent experiments. (C) Co-occupancy of HOXA9 and β-CATENIN at the Prmt1 transcription start site (TSS) detected by CUT&RUN-sequencing in HSPCs. (D) Chromatin accessibility at the Prmt1 locus visualized by ATAC-sequencing of LSKs. (E) Percentage of donor cells in bone marrow of transplanted mice at indicated time points. Absolute number of HSPCs at 4 week (F), 8 week (G) and 12 week (H)

harvests. n=3 mice per group per time point. (I) LTC-IC frequency of HSPCs isolated from transplanted mice. Prmt1$^{fl/fl}$ mice treated with corn oil (vehicle) are indicated as WT control.

Figure 4. Impaired quiescence and DNA replication dynamics induced by Prmt1 KO. (A) Representative FACS plots of Ki67 and Hoechst 33342 staining of LSKs isolated from indicated mice 4 weeks post Tamoxifen treatment. (B) Percentage of LSKs in G_0 cell cycle. Percentage Ki67$^+$ (C) and BrdU$^+$ (D) LSKs from indicated mice. n=3 per group. (E) CldU track length of DNA fibers from HSPCs isolated from indicated mice. (F) Representative images of elongating fork lengths. Scale bar, 10μm. (G) Ratio of IdU (red) track lengths of bidirectional forks. (H) Percentage of stalled forks (green only). Fiber data from one representative experiment is shown, n=3 data is shown in supplemental Material. Representative immunofluorescent images (I) and YH2AX foci counts (J) of ckit$^+$ Prmt1 WT and KO cells. Several fields of cells were scored from two independent experiments. Scale bar, 25μm. WT BL6 mice treated with corn oil (vehicle) are indicated as WT control.

Figure 5. Prmt1 KO replicates the transcriptomic alterations induced by dKO. (A) Overlap of DEGs from Prmt1 KO and dKO LSKs. (B) GSEA of Prmt1 KO LSKs. Heatmap illustrating the log$_2$fold suppression of DNA replication genes (C) and DNA repair genes (D) in single and dKO LSKs compared to WT LSKs. Prmt1$^{fl/fl}$ and β-catenin$^{fl/fl}$ mice treated with corn oil (vehicle) served as WT control for Prmt1 KO and Hoxa9/β-catenin KO, respectively. Asterisks indicate significant change in gene expression (padj ≤0.05). (E) Profile plots of ATAC-seq data (RPKM-normalized) around peak center of DNA replication and repair genes. (F) Chromatin accessibility at Mcm5, Mcm2 and Pold3 loci. WT control represents BL6 mice treated with corn oil (vehicle).

Figure 6. Ectopic expression of PRMT1 phenotypically and transcriptomically rescues dKO HSC defects. (A) Total number and composition of colonies produced by WT, and dKO LSKs transduced with Empty Vector control (EV) or PRMT1. Data from three independent experiments are shown. (B) Long-term culture initiating cell (LTC-IC) frequency from HSPCs as indicated. (C) Percentage BrdU$^+$ HSPCs from indicated samples labeled overnight with BrdU analogue *in vitro*. Data from three independent experiments are shown. (D) CldU track length of fibers from HSPCs isolated from WT or dKO transplanted mice (+EV or PRMT1). (E) Representative images of elongating fork lengths. Scale bar, 10 μm (F) Bidirectional fork symmetry. (G) Frequency of stalled fork present in indicated samples. Fiber data from one representative experiment is shown, n=3 data is shown in supplemental Material. (H) Immunofluorescent images and (I) foci counts of YH2AX staining of indicated HSPCs. Several fields of cells were scored from two independent experiments. Scale bar, 25μm. (J) Enrichment plots of stem-related genesets in RNA-seq data sets as indicated. (K) Fold change in expression of DNA replication transcripts. (L) GO analysis of significantly upregulated genes from HSPCs isolated from dKO transplanted mice and transduced with PRMT1 WT compared to EV (n=433).

Figure 7. Schematic summary of Hoxa9/β-catenin/Prmt1 mediated regulation of hematopoietic stem cell quiescence and DNA replication dynamics. Top panel illustrates the maintenance of HSC functionality in the absence of either Hoxa9 or β-catenin. Bottom panel depicts the perturbance to quiescent HSC (qHSC) and active HSC (aHSC) equilibrium and DNA replication dynamics induced by co-inactivation of Hoxa9 and β-catenin.

Regulation of Hematopoietic Stem Cell (HSC) Quiescence and DNA Replication Dynamics by the Hoxa9/β-Catenin/Prmt1 Axis

HSC with Hoxa9 and/or β-catenin expression
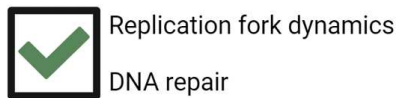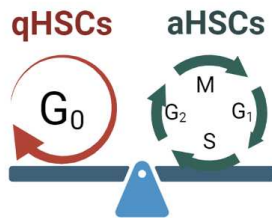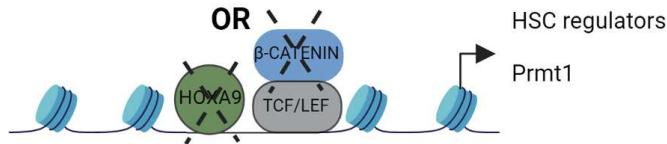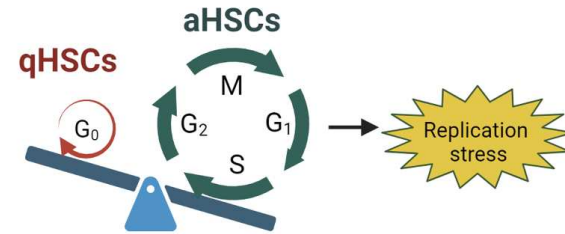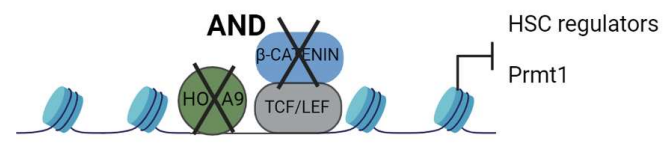
Maintenance of HSC functionality

HSC with Hoxa9 and β-catenin deletion
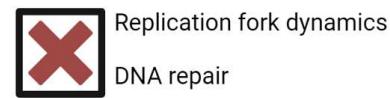
HSC functional decline

Conclusions: Co-inactivation of Hoxa9 and β-catenin perturbs the equilibrium between quiescent HSC (qHSC) and active HSC (aHSC), resulting in DNA damage accumulation and loss of HSC functionality.

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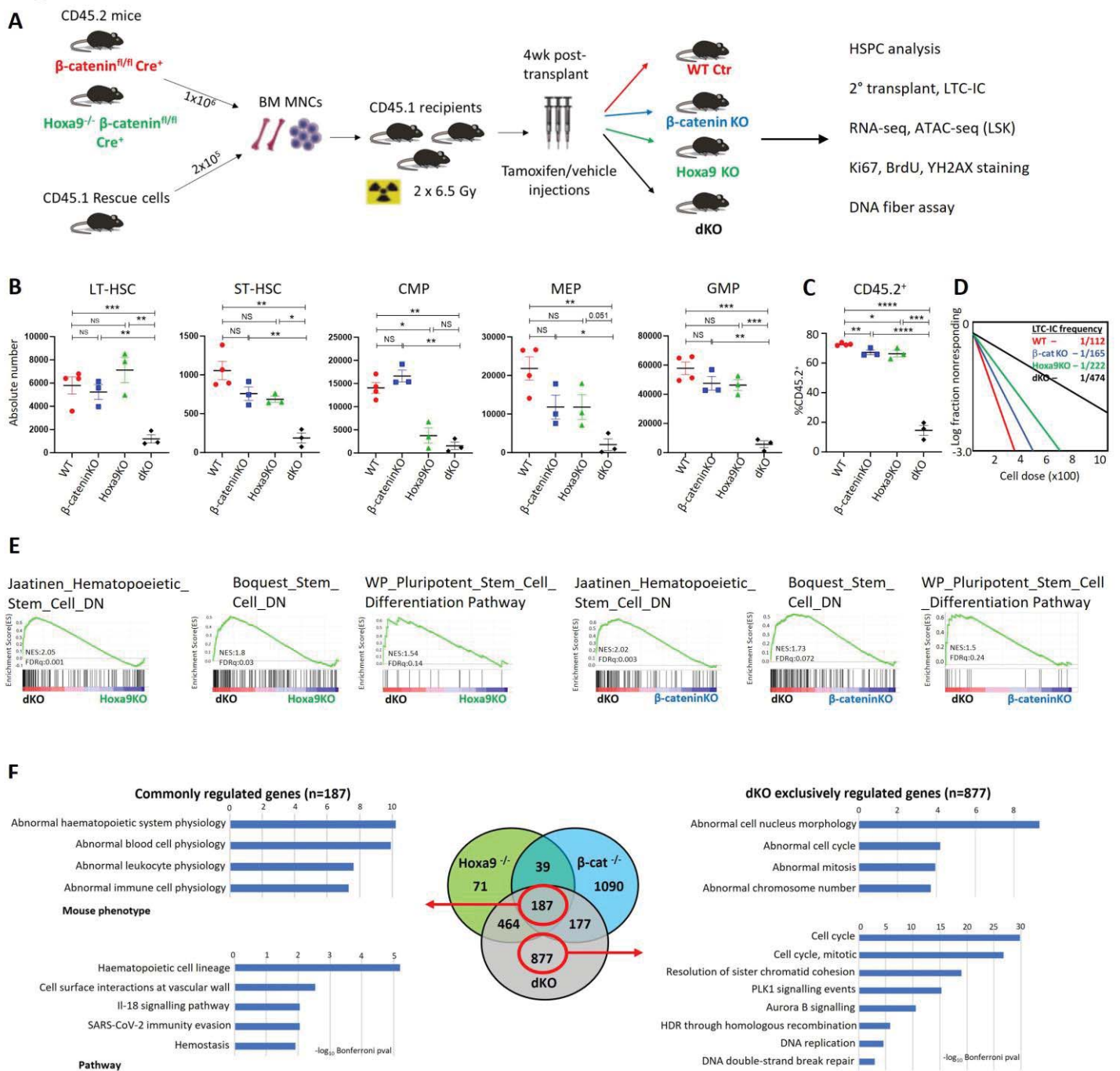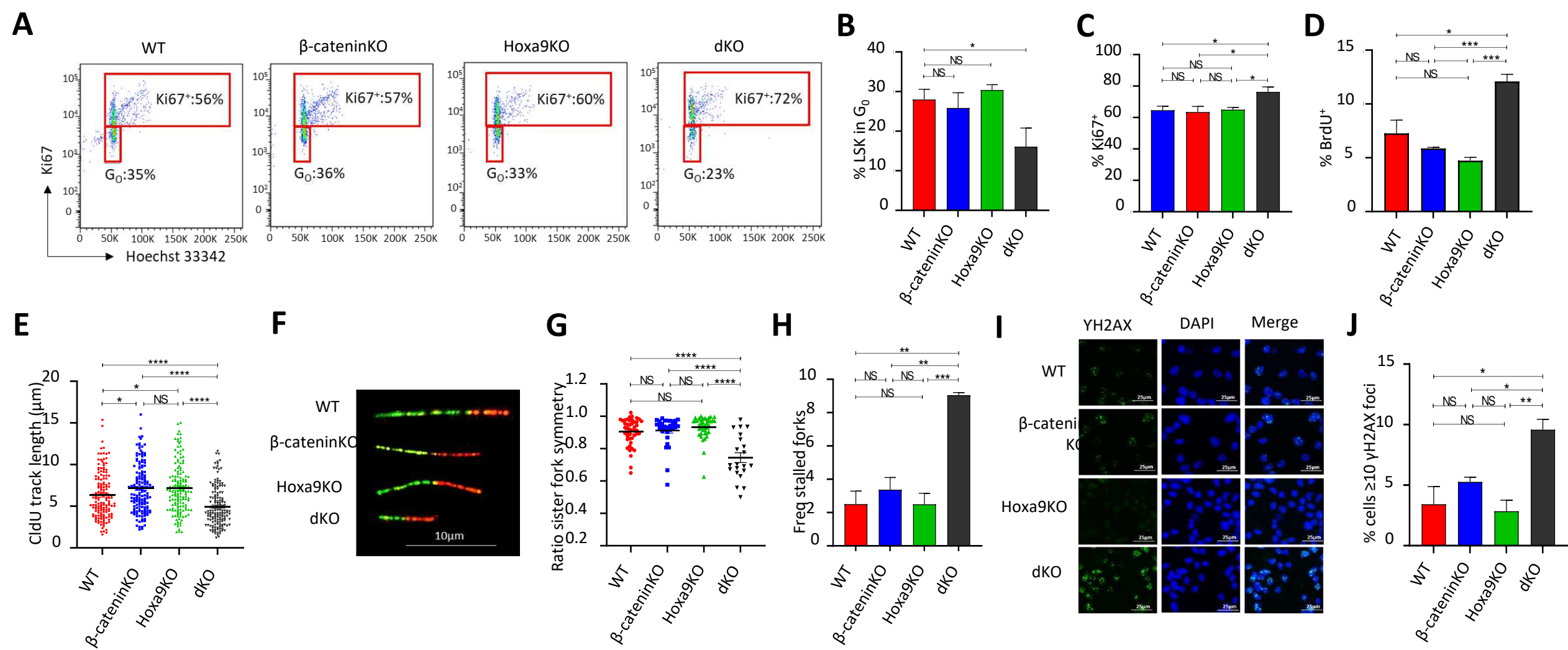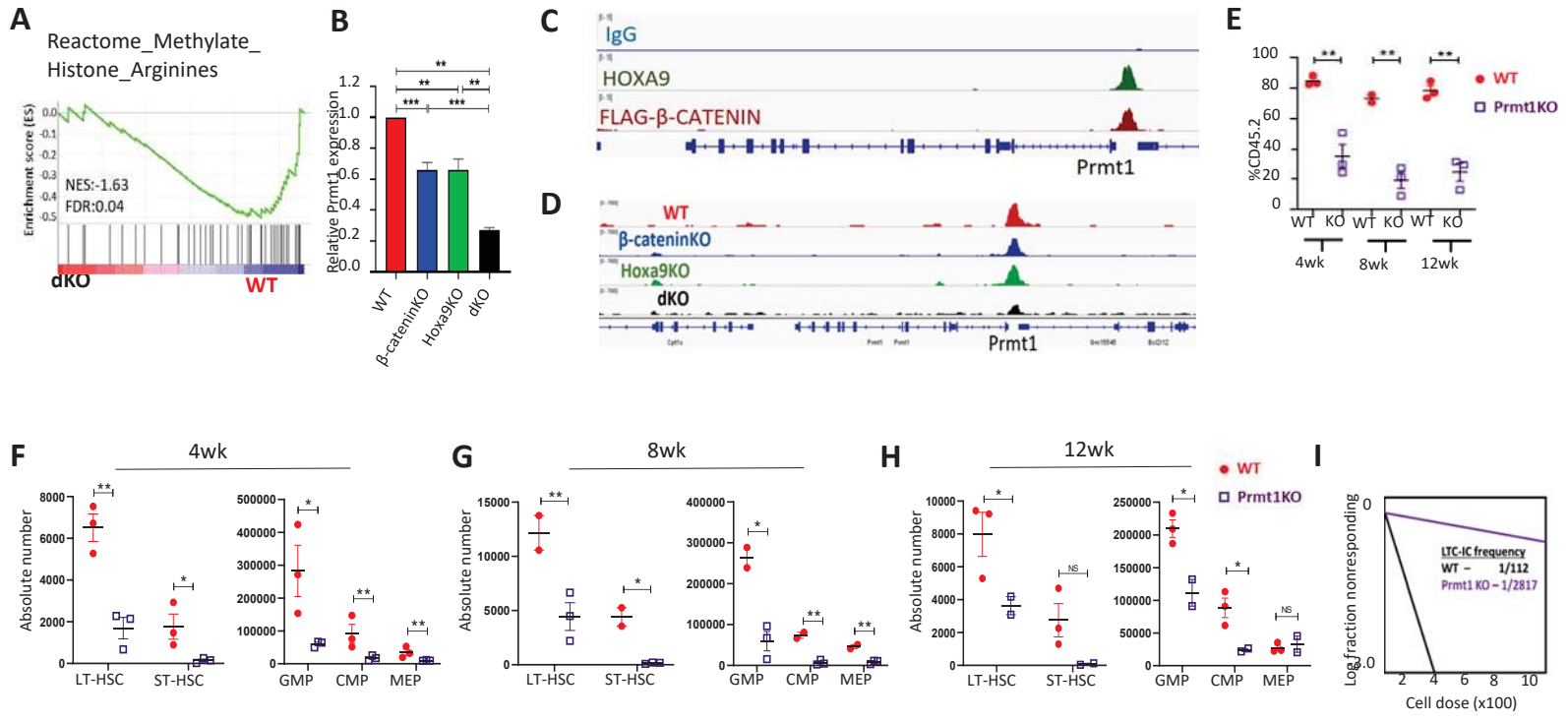**Blood Visual Abstract**

Figure 1

A

CD45.2 mice

β-catenin^fl/fl Cre^+

Hoxa9^-/- β-catenin^fl/fl Cre^+

CD45.1 Rescue cells

1x10^6

2x10^5

BM MNCs

CD45.1 recipients

2 x 6.5 Gy

4wk post-transplant

Tamoxifen/vehicle injections

WT Ctr

β-catenin KO

Hoxa9 KO

dKO

HSPC analysis

2° transplant, LTC-IC

RNA-seq, ATAC-seq (LSK)

Ki67, BrdU, YH2AX staining

DNA fiber assay

B

LT-HSC, ST-HSC, CMP, MEP, GMP plots (Absolute number vs WT, β-cateninKO, Hoxa9KO, dKO)

C CD45.2^+ (%CD45.2^+)

D Log fraction nonresponding vs Cell dose (x100)

LTC-IC frequency
WT – 1/112
β-cat KO – 1/165
Hoxa9KO – 1/222
dKO – 1/474

E

Jaatinen_Hematopoeietic_Stem_Cell_DN — NES:2.05 FDRq:0.001 — dKO / Hoxa9KO

Boquest_Stem_Cell_DN — NES:1.8 FDRq:0.03 — dKO / Hoxa9KO

WP_Pluripotent_Stem_Cell_Differentiation Pathway — NES:1.54 FDRq:0.14 — dKO / Hoxa9KO

Jaatinen_Hematopoeietic_Stem_Cell_DN — NES:2.02 FDRq:0.003 — dKO / β-cateninKO

Boquest_Stem_Cell_DN — NES:1.73 FDRq:0.072 — dKO / β-cateninKO

WP_Pluripotent_Stem_Cell_Differentiation Pathway — NES:1.5 FDRq:0.24 — dKO / β-cateninKO

F

Commonly regulated genes (n=187)

Mouse phenotype:
- Abnormal haematopoietic system physiology
- Abnormal blood cell physiology
- Abnormal leukocyte physiology
- Abnormal immune cell physiology

Pathway:
- Haematopoietic cell lineage
- Cell surface interactions at vascular wall
- Il-18 signalling pathway
- SARS-CoV-2 immunity evasion
- Hemostasis

-log₁₀ Bonferroni pval

Venn diagram: Hoxa9^-/- 71, β-cat^-/- 1090, 39, 187, 464, 177, dKO 877

dKO exclusively regulated genes (n=877)

- Abnormal cell nucleus morphology
- Abnormal cell cycle
- Abnormal mitosis
- Abnormal chromosome number

- Cell cycle
- Cell cycle, mitotic
- Resolution of sister chromatid cohesion
- PLK1 signalling events
- Aurora B signalling
- HDR through homologous recombination
- DNA replication
- DNA double-strand break repair

-log₁₀ Bonferroni pval

Figure 2

Figure 3

Figure 4
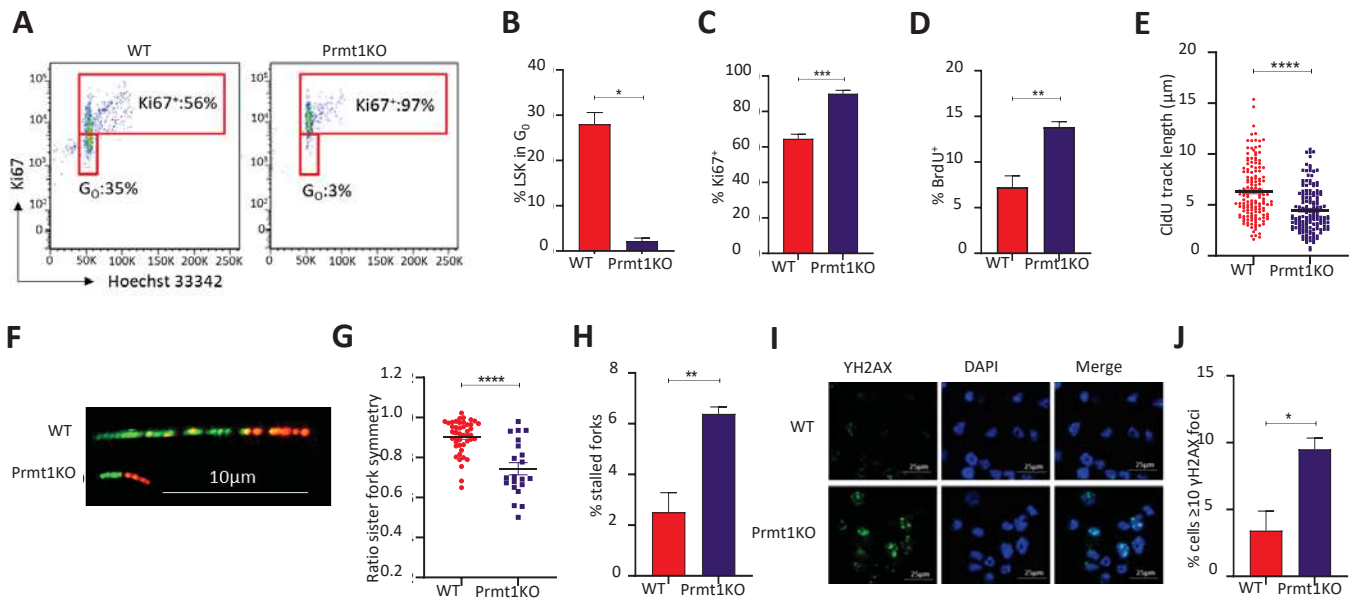
Figure 5

Figure 6

Figure 7