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Transcriptional memory of cells of origin overrides β-catenin requirement of MLL cancer stem cells

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Abstract

While β-catenin has been demonstrated as an essential molecule and therapeutic target for various cancer stem cells (CSCs) including those driven by MLL fusions, here we show that transcriptional memory from cells of origin predicts AML patient survival and allows β-catenin-independent transformation in MLL-CSCs derived from hematopoietic stem cell (HSC)-enriched LSK population but not myeloid–granulocyte progenitors. Mechanistically, β-catenin regulates expression of downstream targets of a key transcriptional memory gene, *Hoxa9* that is highly enriched in LSK-derived MLL-CSCs and helps sustain leukemic self-renewal. Suppression of *Hoxa9* sensitizes LSK-derived MLL-CSCs to β-catenin inhibition resulting in abolishment of CSC transcriptional program and transformation ability. In addition, further molecular and functional analyses identified Prmt1 as a key common downstream mediator for β-catenin/*Hoxa9* functions in LSK-derived MLL-CSCs. Together, these findings not only uncover an unexpectedly important role of cells of origin transcriptional memory in regulating CSC self-renewal, but also reveal a novel molecular network mediated by β-catenin/Hoxa9/Prmt1 in governing leukemic self-renewal.

Keywords cells of origin; Hoxa9; MLL leukemia; Prmt1; Wnt/β-catenin
Subject Categories Cancer; Stem Cells; Transcription
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Introduction

Self-renewal is a critical feature of stem cells, but is diminished upon differentiation into their progenitors. During the differentiation process, gene expression programs responsible for self-renewal are down-regulated and frequently replaced by lineage-specific transcriptional programs. Increasing evidence suggests that genes involved in promoting normal stem cell self-renewal are commonly hijacked in cancer stem cells (CSCs), which are believed to sustain the disease and be responsible for relapse of various cancers including acute myeloid leukemia (AML; Zeisig *et al*, 2012; Fung *et al*, 2013). Among them, one of the most striking molecules is β-catenin, which is required for leukemic stem cells (LSCs) driven by MLL-fusion proteins or their downstream targets, Meis1/Hoxa9 (Wang *et al*, 2010; Yeung *et al*, 2010). Suppression of β-catenin reversed MLL-LSC to pre-LSC stage (Yeung *et al*, 2010), and its complete inactivation prevented development of leukemia driven by MLL fusions or Meis1/Hoxa9 (Wang *et al*, 2010; Yeung *et al*, 2010). While β-catenin is critical for embryonic and a number of somatic stem cells including fetal hematopoietic stem cells (HSCs; Zhao *et al*, 2007; Malhotra & Kincade, 2009), it is largely dispensable for adult HSCs, which can function normally when β-catenin alone or even together with γ-catenin is deleted (Cobas *et al*, 2004; Jeannet *et al*, 2008; Koch *et al*, 2008), highlighting the therapeutic potentials of targeting β-catenin for eradication of LSCs.

In spite of the fundamental difference between stem cells and their progenitors in self-renewal ability, we and others have shown that phenotypically and genetically indistinguishable cancers, including *MLL*-rearranged leukemia, can arise from not only stem cells, but also their immediate downstream short-lived progenitors with distinctive transcriptional programs (Cozzio *et al*, 2003; So *et al*, 2003; Huntly *et al*, 2004; Krivtsov *et al*, 2006; Visvader, 2011; Blanpain, 2013). Consistently, gene expression signatures associated with stem cells and progenitors correlate with different clinical outcomes in AML (Eppert *et al*, 2011; Krivtsov *et al*, 2013). MLL leukemia derived from mouse HSC-enriched $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) populations can be more aggressive and less responsive to standard chemotherapy than those derived from granulocyte–myeloid

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progenitors (GMPs; Krivtsov *et al*, 2013). In line with this, a more recent study also reveals that HSC-derived leukemia drives an invasive EMT-related gene expression program, which may partly account for the aggressive nature of the disease (Stavropoulou *et al*, 2016). In spite of these recent evidences indicating the importance of cancer cells of origin in disease pathogenesis, we still do not know whether and how they may govern the utilization of molecular pathways for self-renewal, which is a defining feature of CSC and has been a major focus for development of effective cancer therapeutics in the past decade.

Given the important function of β-catenin in CSC biology, we carried out detailed functional biology and molecular studies examining β-catenin requirement in MLL-CSCs originated from different cells of origin. Here, we report that transcriptional memory from cells of origin that robustly predicts AML patient survival can govern and help to override the β-catenin requirement in MLL-CSCs. Mechanistically, we identify a novel transcriptional network mediated by *Hoxa9/Prmt1* in sustaining leukemic self-renewal in the absence of β-catenin in HSC-derived MLL-CSCs. These findings reveal previously unrecognized functions and molecular networks from cancer cells of origin that allow override of β-catenin-dependent leukemic self-renewal, adding a new dimension to the ongoing research efforts in developing effective therapeutics for eradication of CSCs.

Results

LSK- but not GMP-derived MLL-CSCs can override β-catenin requirements for leukemic self-renewal

To determine the functional requirement of β-catenin in MLL-CSCs derived from different cells of origin, we employed the previously described retroviral transduction/transformation assays (RTTA; Yeung & So, 2009; Zeisig & So, 2009) using HSC-enriched $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ population (LSK), granulocyte/macrophage progenitors (GMPs), and control c-Kit+ cells (mixed population consisting of mostly progenitors) from *Ctnnb1*$^{fl/fl}$ CreER (Brault *et al*, 2001) conditional knockout mice (Fig 1A, Appendix Fig S1A and B). Consistent with previous findings (Yeung *et al*, 2010), β-catenin was not required for MLL-ENL *in vitro* transformation of $c\text{-}Kit^{+}$ cells (Appendix Fig S1C–E), but essential for *in vivo* development of CSCs (Appendix Fig S1F). Similarly, MLL-ENL could transform LSK and GMPs independently of β-catenin *in vitro* and formed compact colonies with early myeloid phenotypes (Fig 1B–D, Appendix Fig S1G and H). However, while β-catenin deletion in GMP-MLL-ENL abolished its leukemogenic potentials *in vivo* (Fig 1E), β-catenin deletion had little impact on LSK-MLL-ENL, which could still induce leukemia with indistinguishable phenotypes and largely similar latencies as compared with the wild-type controls (Fig 1F–H). More importantly, LSK-MLL-ENL β-catenin-deficient cells could competently induce AML upon secondary transplant (Fig 1F–H, Appendix Fig S1I and J), which readout the self-renewal property of CSCs and indicate the largely uncompromised CSC property in the absence of β-catenin in LSK-derived but not GMP-derived MLL-CSCs. The results could also be reproduced using a different MLL-ENL construct carrying the minimal transformation domain (Slany *et al*, 1998) and MLL-AF9 (Smith *et al*, 2011), and were not due to different expression levels of the MLL fusions in these populations (Appendix Fig S1K–M).

To gain further insights into the role of β-catenin in disease development, we followed the *in vivo* kinetics of the MLL-transformed cells derived from different cellular origins with or without β-catenin. The results showed a similar percentage of engraftment across all samples of different cellular origins and genotypes at 16 and 96 h post-transplant (Fig 1I), suggesting that β-catenin deletion did not significantly affect homing and early *in vivo* proliferation abilities. In contrast to LSK-derived MLL-CSCs that continued to expand and induced leukemia in the absence of β-catenin, the expansion of GMP-MLL-ENL *Ctnnb1*$^{del/del}$ cells slowed down at 15 days and were gradually lost *in vivo* over a 4-month period (Fig 1I), consistent with an impaired self-renewal.

β-Catenin is also not required for leukemia maintenance by LSK-derived MLL-CSCs

To explore the function of β-catenin in the maintenance of leukemia derived from different origin-specific CSCs, full-blown leukemic cells harvested from primary leukemic mice carrying *Ctnnb1*-floxed alleles were then treated with either EtOH or tamoxifen prior to transplantation into secondary recipients (Fig 1A). As expected, both EtOH-treated LSK- and GMP-derived MLL leukemic cells could competently induce leukemia. Inactivation of β-catenin in GMP-MLL-ENL totally abolished their leukemogenic potential (Fig 1J), while β-catenin-deficient LSK-MLL-ENL still efficiently induced leukemia and even with a slightly shorter latency in secondary recipients (Fig 1K). In contrast to the absolute requirement of β-catenin for development of various LSCs (Zhao *et al*, 2007; Wang *et al*, 2010; Yeung *et al*, 2010), the finding of largely dispensable function of β-catenin in LSK-derived MLL-CSCs reveals an unexpected and previously unrecognized role of cells of origin in governing leukemic self-renewal for both cancer initiation and maintenance.

Genomic variations do not account for contrasting β-catenin dependence in MLL-CSCs from different cells of origin

To assess whether the observed cell type-specific differences could be explained by random genetic changes associated with particular cell types, nucleotide variations were called from all actively transcribed genes using RNA-Seq. Among 23,766,084 high-quality base pairs (depth ≥ 10 and quality ≥ 30), the vast majority of sites (23,747,763) were invariant in all our primary cell lines, and identical to the reference genome GRCm38, while a very small proportion, 4,663 SNPs (single nucleotide polymorphisms), were invariant and different from GRCm38. In-depth variance analysis revealed that the difference between the LSK-MLL-ENL and GMP-MLL-ENL cells was not larger than between normal cells (Fig 2A, Dataset EV1A–C), and there were no fixed differences between the samples that could have caused the observed phenotypic differences. To further profile the non-coding genomes, whole-genome sequence analysis on LSK-MLL-ENL and GMP-MLL-ENL cells covering 918,583,518 high-quality base pairs revealed 917,764,811 invariant sites, and a very small number of variants in the samples; 39,846 variants were different between the two biological replicates (mice), and only 17,225 were found different between the two cell types (Fig 2B, Dataset

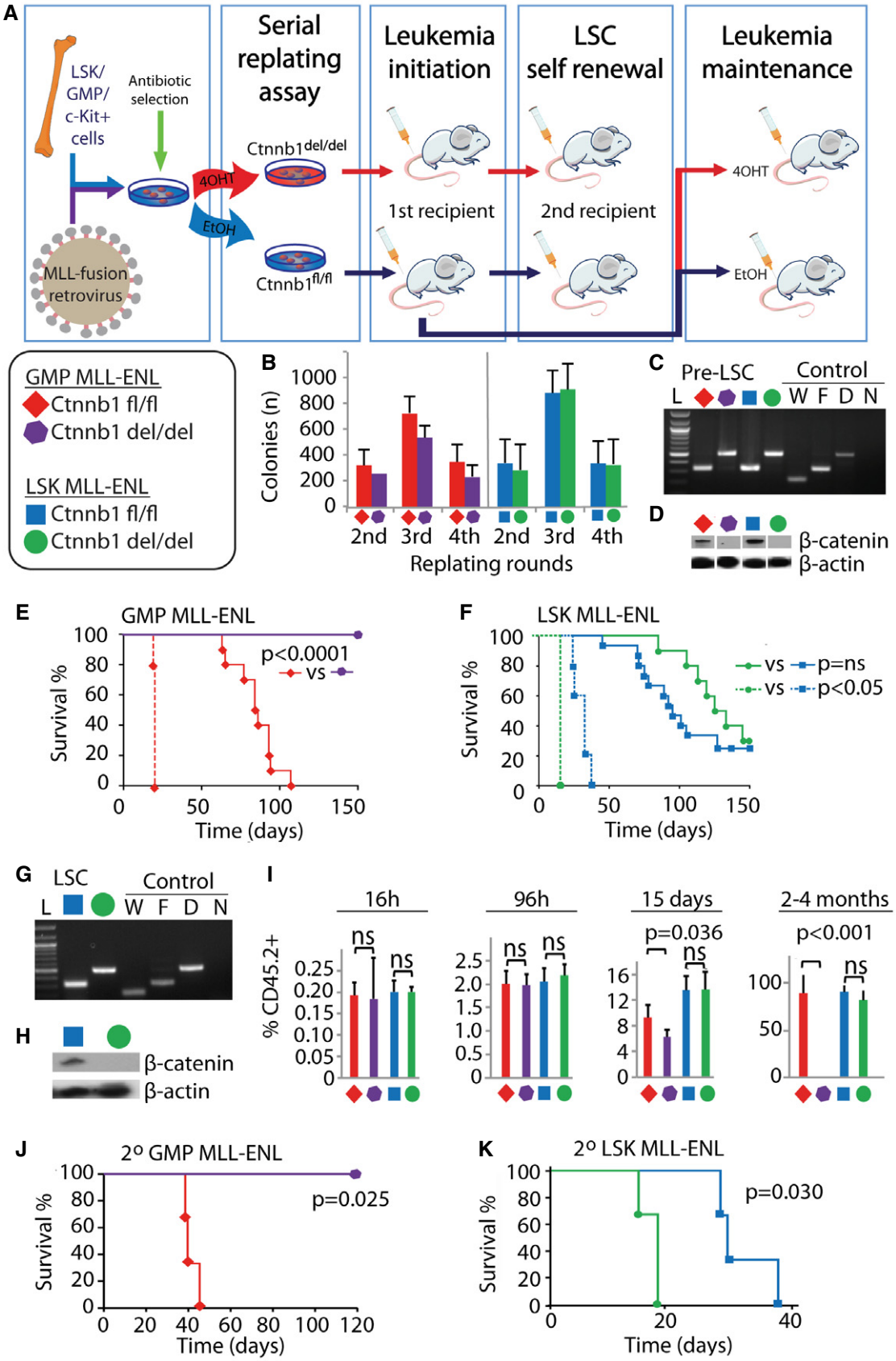

Figure 1.

Figure 1. MLL-ENL leukemic stem cells derived from LSK or GMP populations have contrasting functional requirements of β-catenin for their initiation and maintenance of disease.

A Schematic overview of the experimental procedures. Keys and color codes in the legend box indicate the cells of origin and the β-catenin status of MLL-ENL-transduced cells in the following experiments (B–K).

B Colony numbers in serial replating assay of the different MLL-ENL-transduced cells. Data are represented as mean ± SD ($n = 4$).

C PCR validation of *Ctnnb1* deletion on genomic DNA isolated from the indicated MLL-ENL-transduced GMP and LSK cells. L, 100-bp ladder; W, wild-type control; F, *Ctnnb1*-floxed allele; D, *Ctnnb1*-deleted allele; N, negative control.

D Cell lysates from indicated MLL-ENL-transduced GMP and LSK cells after the fourth round of plating were blotted with anti-β-catenin (top) and anti-actin (bottom) antibodies.

E, F Kaplan–Meier survival curves of indicated MLL-ENL-transduced cells transplanted into primary recipient (solid lines) and secondary recipient mice (dotted lines). $N = 10$ mice per group were used in primary transplants (solid lines) in (E). $N = 15$ mice were used for $Ctnnb1^{fl/fl}$ (blue solid line), and $n = 10$ mice were used for $Ctnnb1^{del/del}$ (green solid line) in (F). For all secondary transplants (dotted lines), $n = 5$ mice were used per group.

G PCR validation of *Ctnnb1* deletion on genomic DNA isolated from leukemic cells. L, 100-bp ladder; W, wild-type control; F, *Ctnnb1*-floxed allele; D, *Ctnnb1*-deleted allele; N, negative control.

H Cell lysates from indicated leukemic mice were blotted with anti-β-catenin (top) and anti-actin (bottom) antibodies.

I Percentage of $CD45.2^+$ donor cells in the bone marrow of recipient mice at the indicated time points post-transplantation of the indicated GMP-MLL-ENL- and LSK-MLL-ENL-transduced cells. Data are represented as mean ± SD. $N = 4$, two-tailed *t*-test was performed.

J, K Kaplan–Meier survival curves of secondary transplanted GMP-MLL-ENL (J) and LSK-MLL-ENL (K) primary leukemia bone marrow cells treated with DMSO as floxed controls or tamoxifen for β-catenin deletion prior to secondary transplantation ($n = 3$ per group).

Data information: See also Appendix Fig S1.
Source data are available online for this figure.

EV1C). Interestingly, the distribution of SNPs differing between cell types or mice was comparable for both comparisons in non-coding and coding regions (Appendix Fig S2A). Consistently, SNPs in coding regions occurred in similar proportions in exon, intron, and UTR regions in both comparisons (Appendix Fig S2B). Moreover, the number of derived SNPs in GMP-MLL-ENL (9,309) was higher than those in LSK-MLL-ENL cells (7,916) that exhibited β-catenin-independent phenotypes (Dataset EV1C). Additionally, we examined and compared the copy number variances (CNVs) between the LSK-MLL-ENL and GMP-MLL-ENL genomes (Fig 2C and D). As a result, we observed very little CNVs in both genomes. There is only a very small genomic region of about 1 kb showing CNV between same cell types (i.e., LSK-MLL-ENL vs. GMP-MLL-ENL), whereas multiple chromosomal regions of about 50 kb exhibiting CNV were detected between samples (i.e., mouse Exp60 vs. mouse Exp69 in Dataset EV1D). Importantly, there is also no known coding gene in the 1-kb CNV region shared between cell types (Fig 2D), consistently indicating insignificant genomic difference between LSK-MLL-ENL vs. GMP-MLL-ENL cells, which could account for their contrasting β-catenin dependence. Together, these data reveal relatively few genomic variation in LSK-MLL-ENL compared with GMP-MLL-ENL and the controls, suggesting that non-genomic influence from the cells of origin can be a key factor in governing the self-renewal property of genetically and phenotypically indistinguishable cancers.

Transcriptional memory from cells of origin governs self-renewal pathways and predicts AML patient survival

As self-renewal in normal stem cells is maintained by specific transcriptional programs, we hypothesized that the transcriptional memories from LSK and GMPs would be partially preserved even after transformation, resulting in transcriptional and functional differences observed in the respective CSCs (Zeisig *et al*, 2012). Thus, RNA-seq analyses of normal LSK, GMPs, and their MLL-ENL-transformed counterparts were carried out. There were, as expected, large transcriptional differences between normal LSK and GMPs with 4,768 significantly differentially expressed genes, including *Hox* genes, *Meis1* and *Evi1* (Fig 2E, Appendix Fig S2C, Dataset EV2A and B), while overall gene expression differences between cells of different origin decreased after MLL-ENL transformation (Fig 2E, Appendix Fig S2D). Nevertheless, a significantly larger than expected by chance number of genes remained differentially expressed between LSK and GMP even after transformation (Fig 2F, Appendix Fig S2C, Dataset EV2C), indicating the presence of "transcriptional memory" retained from the cells of origin. Toppgene functional annotation revealed genes associated with AML are consistently present in both signatures (Appendix Fig S2F–I, Dataset EV2D).

To further investigate the relevance of this cells of origin transcriptional memory gene signature in human leukemia, we employed it to stratify 1,290 human AML patients from multiple independent centers (Valk *et al*, 2004, Raponi *et al*, 2007, 2008; Metzeler *et al*, 2008; Wouters *et al*, 2009; Cancer Genome Atlas Research N, 2013; Dataset EV2E). AML patients with LSK-transcriptional memory signature had much worse prognosis with a median survival 14.5 months as compared to patients with GMP-transcriptional memory signature with median survival 22.7 months (Fig 2G), even though the two groups had similar WBC count (means = 40.3, 45.3, *t*-test $P = 0.30$), age distributions (means = 48.0, 50,0, *t*-test $P = 0.07$), and cytogenetic risk (cytogenetic risk (1/2/3) = 71/199/85, 82/165/66, chi-square test $P = 0.16$). When compared with the previously identified human HSC signature (Eppert *et al*, 2011) and MLL leukemic-GMP (LGMP) signatures from different cells of origin (Krivtsov *et al*, 2013), the current transcriptional memory signature represents a stronger predictor to stratify patients into different prognostic subgroups based on both resultant median survivals and *P*-values (Appendix Fig S2J). Moreover, multivariate analyses consistently resulted in significant Cox proportional hazards ratios > 1; *z*-score < 0.1 with both human HSC signature and transcriptional memory signature (Dataset EV2F). Together, these data indicate functional and pathological relevance of the newfound cells of origin transcriptional memory in governing human cancer biology beyond known cytogenetic/genetic risk factors.

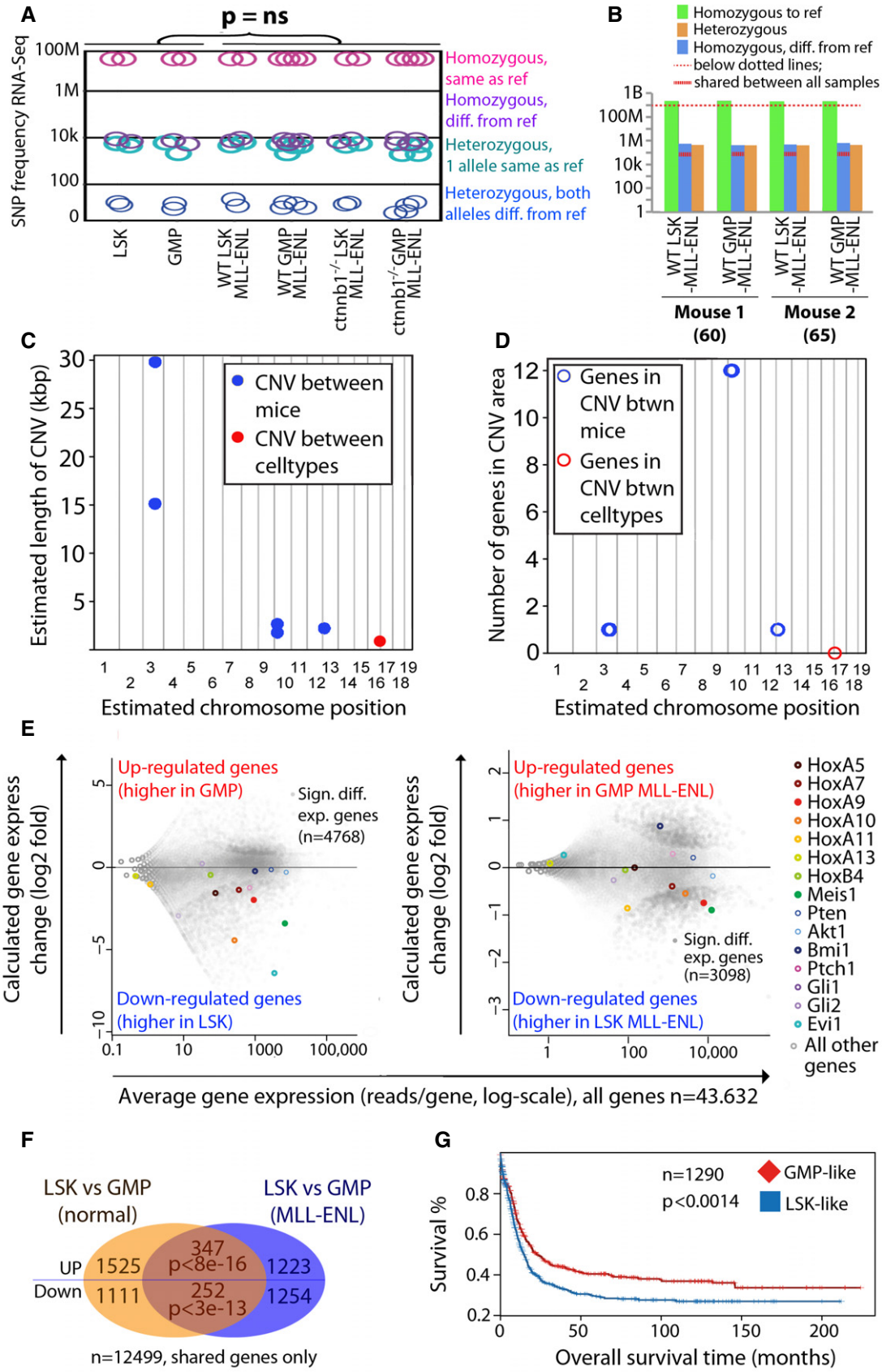

Figure 2.

Figure 2. Cells of origin transcriptional memory predicts survival in AML patients.

A, B Number of identified genomic variants in indicated MLL-ENL transformed cells using RNA-Seq (A) and genomic sequencing (B). A two-tailed *t*-test was performed in (A). LSK, wild-type LSK cells; GMP, wild-type GMP cells.

C, D Manhattan plots indicating estimated length of CNVs (C) or number of genes in CNV areas (D) on the *y*-axis in the respective chromosomal positions shown in the *x*-axis.

E MA-plots showing the log2-fold gene expression changes in the normal (left panel) and MLL-ENL transformed (right panel) cells as indicated.

F Transcriptional memory signature; the overlap of differentially expressed genes in GMP-LSK in normal vs. MLL-ENL transformed cells is significantly enriched using a hypergeometric test.

G Survival differences between patients clustered using transcriptional memory signatures with a log-rank test.

Data information: See also Appendix Fig S2.

Hoxa9 as a key transcriptional memory gene phenocopies β-catenin function in development of origin-specific MLL leukemia

Given the largely dispensable function of β-catenin in LSK-derived MLL-CSCs, we hypothesize that some self-renewal programs from normal stem cells may persist after transformation, and can sustain self-renewal in the absence of β-catenin. In the transcriptional memory signature, there were a small number of self-renewal genes such as *Hoxa9*, *Hoxa10,* and *Meis1* (Fig 2E and F), which are known downstream targets of MLL fusions (Milne *et al*, 2002; Zeisig *et al*, 2004; Huang *et al*, 2012), indicating that their degrees of activation are in part also determined by the cellular origins. Strikingly, RNA-sequencing analysis on MLL-ENL transformed cells upon β-catenin inactivation revealed a specific up-regulation of targets genes suppressed by Hoxa9/Meis1, suggesting a critical function of β-catenin in regulation Hox/Meis1 axis for leukemic self-renewal (Fig 3A, Dataset EV3A–C). Moreover, various stem cell-related gene sets were positively enriched in β-catenin-deleted LSK-MLL-ENL cells as compared with β-catenin-deleted GMP-MLL-ENL (Fig 3B, Appendix Fig S3A, Dataset EV3C). β-catenin-deleted LSK-MLL-ENL not only expressed higher levels of *Hoxa9* (Fig 3C and D) but also showed a negative enrichment for genes repressed by *Hoxa9* (Fig 3E, Dataset EV3C). Together, the data consistently suggest a potential *Hoxa9* complementation function in replacing β-catenin in LSK-derived MLL-CSCs.

Similar to β-catenin, activation of *Hoxa9* enhances self-renewal, while its deletion does not have significant impact on HSCs (So *et al*, 2004; Lawrence *et al*, 2005; Smith *et al*, 2011), consistent with the existence of multiple complementary self-renewal pathways in HSCs. We hypothesize whether there is indeed a functional complementation between Hoxa9 and β-catenin, *Hoxa9* requirement for MLL transformation may also be influenced by cells of origin. To address this issue, we used purified hematopoietic populations from *Hoxa9*-knockout mice for RTTA. While MLL-ENL could competently transform wild-type LSK and GMPs *in vitro*, only LSK but not GMPs could be transformed by MLL-ENL in the absence of Hoxa9 (Fig 3F, Appendix Fig S3B). More importantly, LSK-MLL-ENL $Hoxa9^{-/-}$ similar to wild-type LSK-MLL-ENL could induce serially transplantable leukemia in recipient mice (Fig 3G, Appendix Fig S3C), strongly suggesting that Hoxa9 requirement, similar to β-catenin, is also largely determined by cancer cells of origin. These results not only assert the critical function of the newfound cells of origin transcription memory in governing the biology of the resultant disease, but also suggest that LSK-MLL-CSCs may be able to utilize the *Hoxa9*-mediated self-renewal pathways as a molecular mechanism to overcome targeted disruption of β-catenin function.

Suppression of Hoxa9 abolishes β-catenin-independent transformation in LSK-derived MLL-CSCs

To gain further molecular insights into the functional interplay between β-catenin and Hoxa9 in mediating self-renewal in origin-specific CSCs, we generated a novel compound $Hoxa9^{-/-}Ctnnb1^{fl/fl}$ Rosa-CreER Rosa-YFP mouse by crossing $Hoxa9^{-/-}$ mice (Smith *et al*, 2011) with $Ctnnb1^{fl/fl}$ Rosa-CreER Rosa-YFP mice for RTTA and RNA-sequencing analysis. While MLL-ENL-transduced LSK and GMPs isolated from compound $Hoxa9^{-/-}Ctnnb1^{fl/fl}$ Rosa-CreER Rosa-YFP mice produced a similar number of first round colonies (Appendix Fig S4A), a further β-catenin inactivation significantly compromised their transformation ability resulting in reduced number and size of colonies with early myeloid phenotypes (Fig 4A and B, Appendix Fig S4B–D).

LSK-MLL-ENL $Hoxa9^{-/-}Ctnnb1^{-/-}$ displayed a higher percentage of apoptosis (Fig 4C), and an increase in G2/M arrest at the expense of S-phase (Fig 4D), which might help explain their reduced numbers and colony sizes (Fig 4A). Upon transplantation, both LSK-MLL-ENL $Hoxa9^{-/-}Ctnnb1^{fl/fl}$ and $Hoxa9^{-/-}Ctnnb1^{del/del}$ engrafted in comparable levels into the bone marrow and were able to proliferate and transiently expand (Fig 4E). However, inactivation of β-catenin in LSK-MLL-ENL $Hoxa9^{-/-}$ led to a gradual loss of their self-renewal property and failed to induce leukemia (Fig 4E and F). Further *in vivo* limiting dilution analysis revealed similar frequency of CSCs found in wild-type, $Hoxa9^{-/-}$, or $Ctnnb1^{-/-}$ LSK-MLL-ENL, ranging from 1/3,381 to 1/8,625 (Fig 4G and Appendix Fig S4E–G), indicating a rather limited impact of single inactivation of Hoxa9 or β-catenin on LSK-derived MLL-CSCs. In contrast, deletion of both proteins in LSK-MLL-ENL resulted in a drastic reduction of CSC frequency (estimated to be below 1/1,669,041; Fig 4G and Appendix Fig S4H), consistent with a critical functional crosstalk between Hoxa9 and β-catenin, in which a high level of *Hoxa9* expression allows β-catenin-independent transformation in LSK-derived MLL-CSCs.

β-catenin and Hoxa9 co-regulate Prmt1 in LSK-MLL-ENL cells

To gain further insights into the molecular pathways underlying β-catenin- and Hoxa9-mediated leukemic self-renewal, we sought to define common gene sets that were deregulated upon the loss of leukemic self-renewal (i.e., β-catenin inactivation in $Hoxa9^{-/-}$ LSK-MLL-ENL, Hoxa9 inactivation in $Ctnnb1^{-/-}$ LSK-MLL-ENL cells). As a result, 38 gene sets were commonly up-regulated (Fig 5A) and 33 were commonly down-regulated (Fig 5B, Dataset EV4A and B) in compound $Hoxa9^{-/-}Ctnnb1^{-/-}$ LSK-MLL-ENL cells. Consistent with the loss of leukemic self-renewal, LSC_maintenance

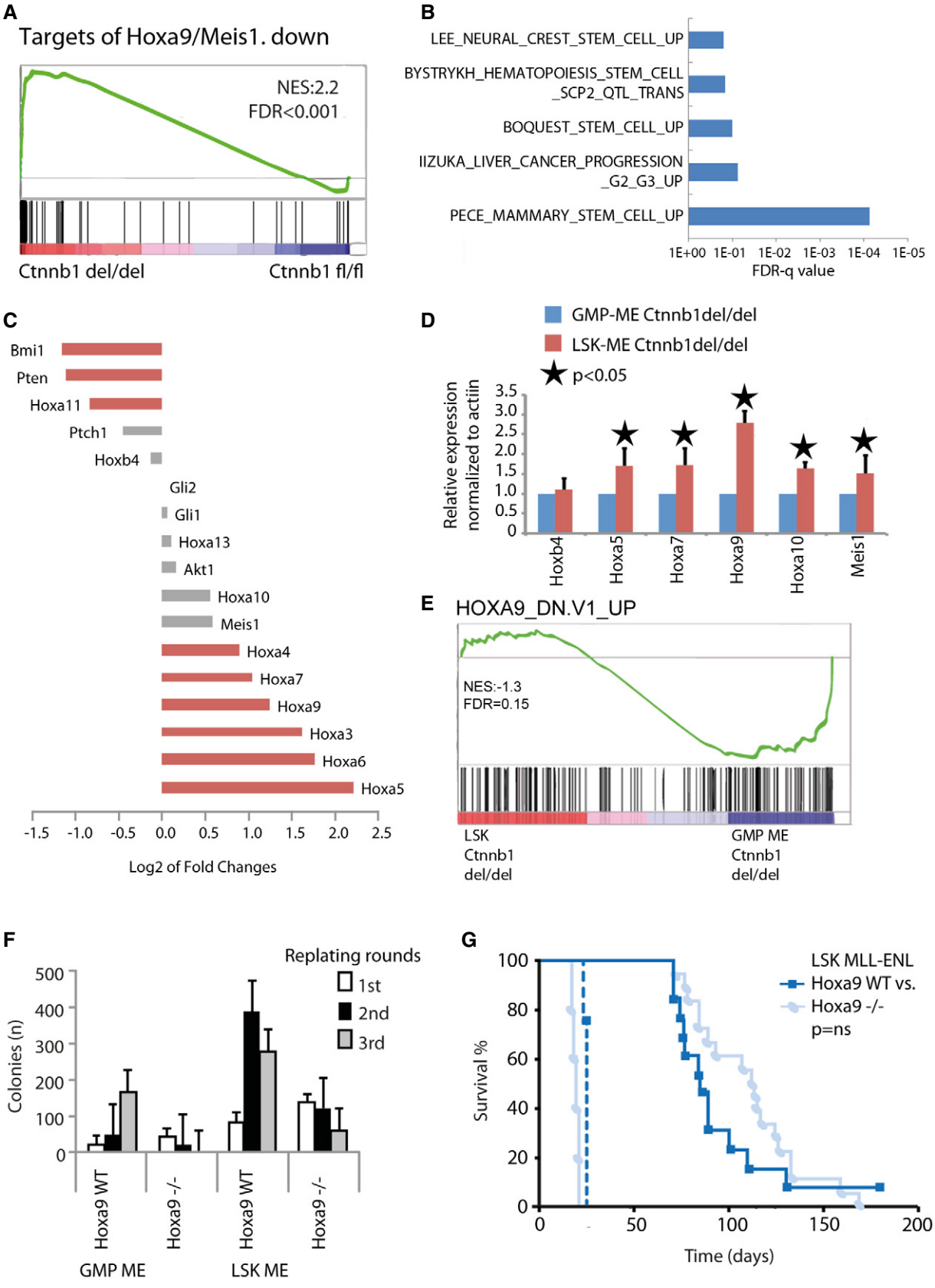

Figure 3.

Figure 3. Key transcriptional memory gene *Hoxa9* may help to overcome β-catenin-dependent transformation in LSK-derived MLL-CSCs.

A Gene set enrichment analysis (GSEA) shows "Targets of Hoxa9/Meis1, down" (Hess *et al*, 2006) for the indicated comparison.

B Log10-fold FDR *q*-values of the indicated gene sets positively enriched in β-catenin-deleted LSK-MLL-ENL compared to β-catenin-deleted GMP-MLL-ENL.

C–E RNA-seq log2-fold change of key self-renewal genes (C), RT–qPCR validation of *Hox/Meis1* expression represented as mean ± SD of three independent experiments. Two-tailed *t*-test was performed (D), and GSEA showing "Hoxa9_dn.v1_up" (Faber *et al*, 2009) for the β-catenin-deleted LSK-MLL-ENL to β-catenin-deleted GMP-MLL-ENL comparison (E).

F Colony numbers in serial replating assay of indicated MLL-ENL-transduced cells. Data are represented as mean ± SD ($n = 3$).

G Kaplan–Meier survival curve of mice transplanted with *Hoxa9*$^{-/-}$ ($n = 18$) or WT LSK-MLL-ENL ($n = 13$) transformed cells (solid lines) and secondary recipient mice ($n = 5$ for *Hoxa9*$^{-/-}$ and $n = 4$ for wt, dotted lines) as indicated. Comparisons between *Hoxa9* WT and *Hoxa9*$^{-/-}$ were not significantly different (ns).

Data information: See also Appendix Fig S3.

signatures (Somervaille *et al*, 2009) were inversely enriched. Moreover, the Hoxa9/Meis1 targets (Hess *et al*, 2006) were also inversely enriched, supporting the hypothesis that β-catenin and Hoxa9 may co-regulate common sets of genes critical for self-renewal.

In order to specifically identify β-catenin and Hoxa9 co-regulated targets in LSK-MLL-ENL, we performed global quantitative expression analyses using RNA-Seq and revealed a small fraction of genes ~1% ($n = 525$) showing a synergistic pattern in the double knockout compared to single knockouts alone (Fig 5C, Appendix Fig S5, Dataset EV4C). Different protein classes are present in the 321 synergistically up- and 204 synergistically down-regulated gene lists. While hydrolase and cysteine protease inhibitors were enriched in the up-regulated list, methyltransferases among others were enriched in the down-regulated list (Fig 5D). These methyltransferases were arginine-specific Prmt1, Prmt5, and Prmt7. Consistently, H4-R3-specific histone methyltransferase activity was among the enriched GO:Molecular functions (Fig 5E, Dataset EV4D), suggesting that arginine methylation may be co-regulated by Hoxa9 and Ctnnb1. Indeed, when the targets of Hoxa9/Meis1 gene sets were overlapped with the synergistically up- and down-regulated genes, 19 out of 111 (17%) genes from the Hoxa9/Meis1 targets (Fig 5F, Dataset EV4E), were synergistically regulated by β-catenin and Hoxa9 in LSK-MLL-ENL (χ^2, $P = 1.4e\text{-}22$). Moreover, among them is again Prmt1, which had also been independently confirmed by RT–qPCR (Fig 5F). Strikingly, Prmt1 is a key epigenetic modifying enzyme known to be recruited by various fusion proteins involved in AML pathogenesis (Cheung *et al*, 2007, 2016; Shia *et al*, 2012), leading us to examine its role in mediating β-catenin/Hoxa9 functions in LSK-MLL-ENL cells.

Prmt1 regulates similar and overlapping transcriptional programs mediated by β-catenin in *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells

To investigate the transcriptional functions and potential molecular crosstalk between Prmt1 and β-catenin, global transcriptional analyses by RNA sequencing were performed in *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells in the presence or absence of shRNA-mediated Prmt1 knockdown using previously validated shRNAs (Cheung *et al*, 2007). As a result, we identified 1,416 differentially expressed genes, including 686 differentially up- and 730 differentially down-regulated genes from two biological replicates upon Prmt1 inactivation (Fig 6A, Dataset EV5A). Similar transcriptomic analyses were then performed between *Hoxa9*$^{-/-}$ LSK-MLL-ENL and *Hoxa9*$^{-/-}$ *Ctnnb1*$^{-/-}$ LSK-MLL-ENL, where we identified 342 differentially up-regulated genes and 134 differentially down-regulated genes from two biological replicates (Fig 6A). To assess whether Prmt1 and β-catenin may regulate common transcriptional targets, we compared the differentially expressed genes from both analyses. As a result, we revealed similar and highly significant overlapping gene expression signatures associated with the loss of Prmt1 vs. β-catenin in Hoxa9$^{-/-}$ LSK-MLL-ENL cells (Fig 6A, Dataset EV5A). While 24 genes were commonly down-regulated by β-catenin and Prmt1 ($P < 2.4E\text{-}7$), 68 genes showed the opposite pattern ($P < 2.7E\text{-}22$) upon their individual inactivation (Fig 6A). Functional annotation analysis revealed increased myeloid differentiation and apoptosis but reduced histone binding, chromatin silencing, and negative regulation of gene expression as dominant GO:Molecular functions and GO:Biological processes upon Prmt1 knockdown and β-catenin knockout (Fig 6B). Strikingly, GSEA revealed that about 75% of the gene sets/pathways affected by Prmt1 knockdown were also regulated by β-catenin knockout in *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells (Fig 6C, Dataset EV5B) including those involved in leukemic/normal stem cell functions and stemness (Fig 6D), consistent with the hypothesis that Prmt1 mediates β-catenin functions in *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells. Together, these results strongly suggest a molecular and functional overlap between Prmt1 and β-catenin in regulating critical transcriptional programs in LSK-MLL-ENL cells.

Suppression of Prmt1 abolishes Hoxa9-independent transformation in LSK-derived MLL-CSCs

To finally evaluate the biological function of Prmt1 as a critical mediator for β-catenin/Hoxa9 functions, we first assessed its requirement in mediating *Hoxa9*$^{-/-}$ LSK-MLL-ENL transformation. Prmt1 expression was independently down-regulated in *Hoxa9*$^{-/-}$ LSK-MLL-ENL by two different shRNAs, which also resulted in reduction of H4R3 asymmetric dimethylation mark (H4R3me2as) specifically conferred by Prmt1 (Appendix Fig S6A and B). Inhibition of Prmt1 expression compromised *in vitro* MLL-ENL transformation of *Hoxa9*$^{-/-}$ LSK cells (Fig 7A, Appendix Fig S6C) and mimic *in vivo* inactivation of β-catenin in LSK-MLL-ENL. Similar to *Hoxa9*$^{-/-}$*Ctnnb1*$^{-/-}$ LSK-MLL-ENL cells (Fig 4G), *Hoxa9*$^{-/-}$Prmt1 KD LSK-MLL-ENL cells were able to engraft and proliferate short term, but gradually lost their self-renewal ability (Fig 7B). Crucially, Prmt1 knockdown suppressed oncogenic potential of *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells, which could otherwise induce leukemia within a month (Fig 7C).

To further determine whether Prmt1 as a key mediator for β-catenin/Hoxa9 can also replace the function of Hoxa9, Prmt1 expression was suppressed in *Ctnnb1*$^{-/-}$ LSK-MLL-ENL (Appendix Fig S6A and B). As a result, Prmt1 inhibition mimic Hoxa9 inactivation leading to suppression of colony formation

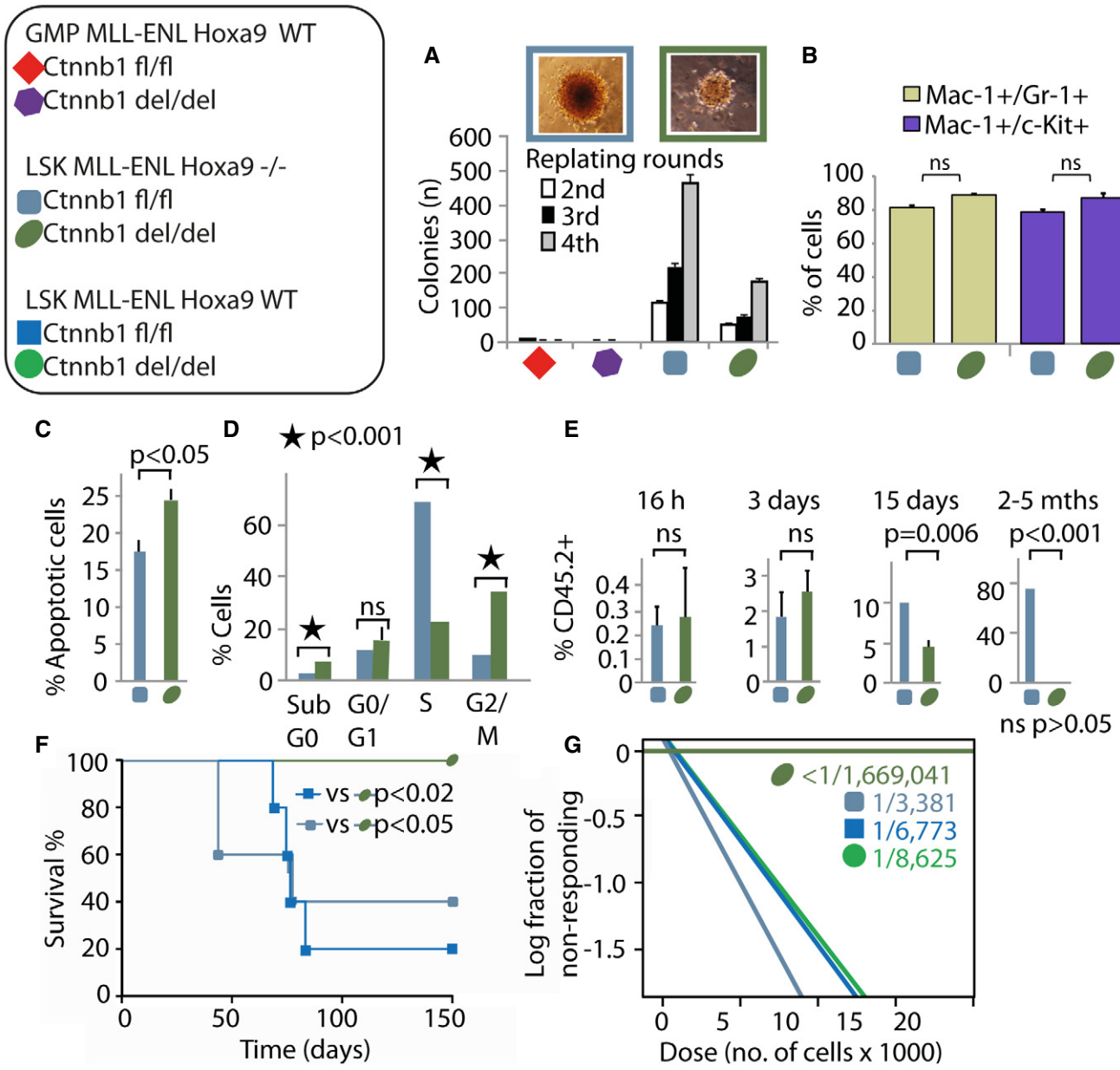

Figure 4. Deletion of both β-catenin and Hoxa9 abrogates leukemia development in LSK-MLL-ENL cells.

Keys and color code in the left top corner indicate the origin and the β-catenin and Hoxa9 status of MLL-ENL-transduced cells.

A Colony numbers in serial replating assay of MLL-ENL-transduced cells. Images of typical 4th round colonies shown above. Data are represented as mean ± SD ($n = 3$).

B Summary of immunophenotypic analysis (Appendix Fig S4D) of LSK-MLL-ENL *Hoxa9*$^{-/-}$*Ctnnb1*$^{fl/fl}$ with or without 72 h of tamoxifen treatment. Data are represented as mean ± SD. $N = 3$; two-tailed *t*-test was performed.

C–E Percentage of apoptotic cells (AnnexinV assay) (C), cells in the indicated cell cycle phases (BrdU assay) (D) and CD45.2$^+$ donor cells in the bone marrow of recipient mice at the indicated time points post-transplantation (E) of LSK-MLL-ENL-transduced cells. Data are represented as mean ± SD. $N = 4$, two-tailed *t*-test was performed.

F Kaplan–Meier survival curve of mice transplanted with LSK-MLL-ENL carrying different *Hoxa9* and *Ctnnb1* genotypes. $N = 5$ per genotype was used.

G Summary of the LSC frequencies obtained from the *in vivo* limiting dilution experiments (Appendix Fig S4E–H) using the LSK-MLL-ENL leukemic cells with different genotypes.

Data information: See also Appendix Fig S4.
Source data are available online for this figure.

ability of *Ctnnb1*$^{-/-}$ LSK-MLL-ENL cells (Fig 7D), abolished their self-renewal potentials (Fig 7E) and oncogenic ability *in vivo* (Fig 7F). Together with the comprehensive global gene expression network analyses, these results consistently indicate Prmt1 as a key player and novel downstream target mediating β-catenin/Hoxa9 functions in LSK-derived MLL-CSCs.

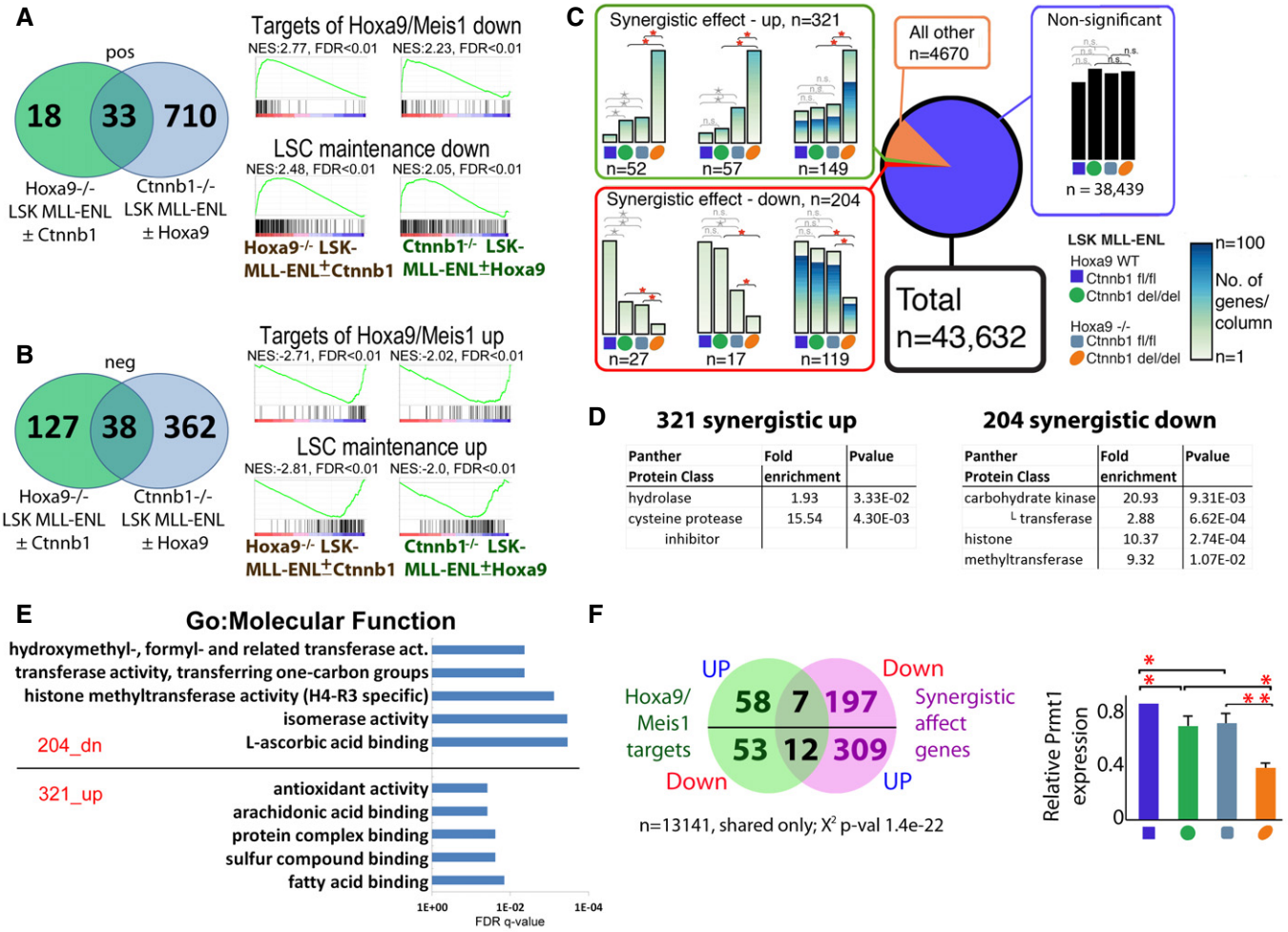

Figure 5. β-catenin and Hoxa9 co-regulate Prmt1 in LSK-MLL-ENL cells.

A, B Venn diagram showing the overlap of 33 commonly enriched up-regulated (A) and 38 commonly enriched down-regulated (B) highly significant (FDR < 0.05) gene sets between indicated comparisons (i.e., β-catenin inactivation in *Hoxa9*$^{-/-}$ LSK-MLL-ENL, Hoxa9 inactivation in *Ctnnb1*$^{-/-}$ LSK-MLL-ENL cells), including "Hoxa9/Meis1_DN" (Hess *et al*, 2006), "LSC_mainentence_down" (Somervaille *et al*, 2009), "Hoxa9/Meis1_UP" (Hess *et al*, 2006), and "LSC_maintenance_up" (Somervaille *et al*, 2009) as indicated.

C A small set of genes is synergistically regulated by β-catenin and Hoxa9 (the effect of knockout of both genes is smaller or larger than could have been predicted from their single knockouts). Example sets of genes synergistic up-regulated (top), down-regulated (bottom) and non-significantly regulated (bottom right) are shown (all sets are in Appendix Fig S5). Keys and color code in the top corner indicate the origin and the β-catenin and Hoxa9 status of MLL-ENL-transduced cells. Five differential expression comparisons were performed using DESeq2 with negative binomial GLM fitting and Wald statistics; comparisons are indicated with braces. All asterisks mark significant ($P_{adjusted}$ < 0.05) differential expression between the indicated conditions. Colored asterisks highlight differential expression which defines them as synergistically regulated genes. The height of the column represents the sum of normalized counts of all genes following the selected pattern of gene expression for a particular condition. The gradient fill of the columns indicates the number of genes represented in that column.

D Pantherdb protein classes (http://pantherdb.org/) are shown for the 321_synergistic_up and 204_synergistic_down genes as indicated. *P*-value was obtained after Bonferroni correction.

E The FDR *q*-values of the top 5 Toppgene GO:Molecular function (https://toppgene.cchmc.org/enrichment.jsp) are shown for the synergistic up and down-regulated genes as indicated.

F Statistically significant directionality in the overlap of the synergistic genes with the Hox/Meis1_DN gene set (Dataset EV5B) including Prmt1, which has also been independently validated by RT–qPCR (right panel: $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test).

Data information: See also Appendix Fig S5.

Discussion

Self-renewal as a defining feature of normal and cancer stem cells is tightly regulated by complex transcriptional networks. Most of the current targeted therapies and their intended clinical utility are developed without considering the cancer cells of origin, which can have distinctive self-renewal and transcriptional properties. This traditional view has been challenged by the identification of phenotypically indistinguishable leukemia from different cells of origin (Cozzio *et al*, 2003; So *et al*, 2003; Huntly *et al*, 2004), which exhibit different responses to standard chemotherapy treatment (Krivtsov *et al*, 2013; George *et al*, 2016; Stavropoulou *et al*, 2016).

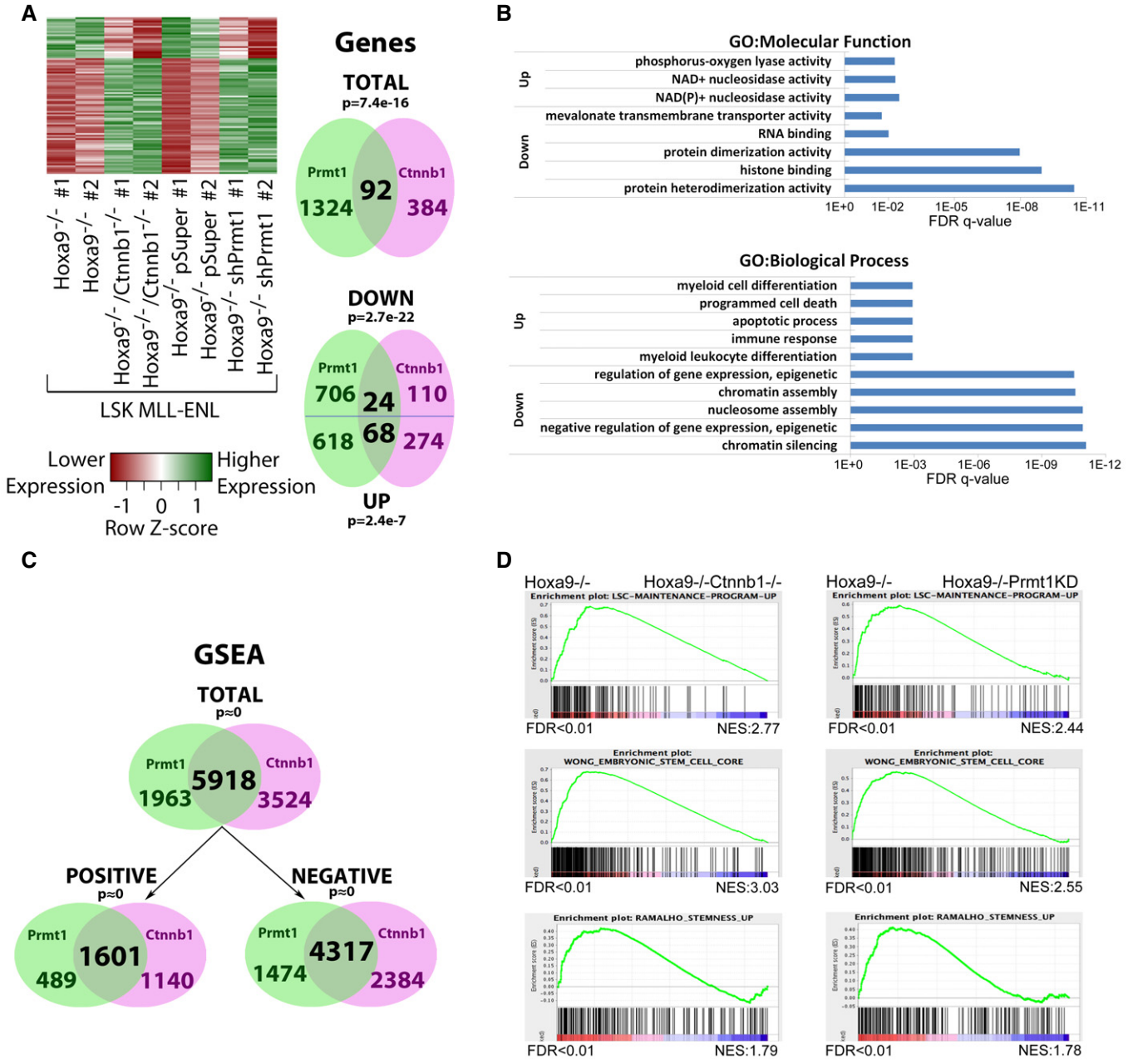

Figure 6. Prmt1 regulates similar and overlapping transcriptional programs mediated by β-catenin in LSK-MLL-ENL cells.

A Heatmap analysis and Venn diagram showing the commonly regulated genes after the loss of function of Prmt1 and β-catenin in *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells.

B The FDR *q*-values of the top GO:Molecular function (top panel) and GO:Biological process (bottom panel) (https://toppgene.cchmc.org/enrichment.jsp) for the indicated up- and down-regulated genes is shown.

C Venn diagrams showing the total, positively and negatively regulated genesets identified by GSEA upon the loss of function of Prmt1 and β-catenin in *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells.

D Examples of stem cell and stemness signatures which were commonly higher expressed in the single knockout *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells compared to Prmt1 down-regulated or β-catenin inactivated *Hoxa9*$^{-/-}$ LSK-MLL-ENL cells.

However, until now, very little is known about impact of cells of origin on cancer self-renewal and the molecular pathways underpinning this defining feature of CSCs. By performing comprehensive genomic and transcriptomic analyses on origin-specific CSCs in combination with various *in vitro* and *in vivo* functional genomic assays, here we provide the experimental evidence for the presence of cells of origin transcriptional memory governing molecular pathways available for CSC self-renewal, urging that both genetic mutations and transcriptional memory inherited from cells of origin determine the resultant CSC biology and heterogeneous responses

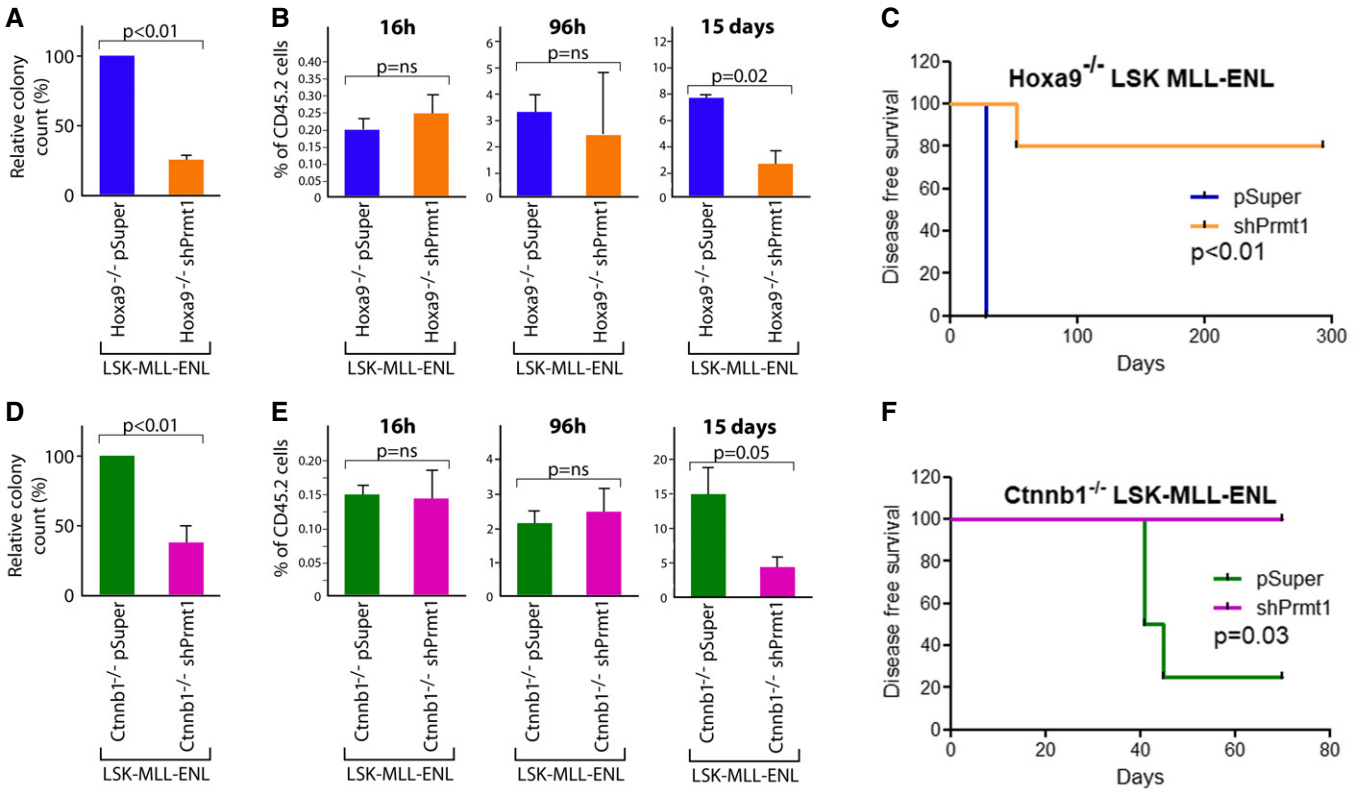

Figure 7. Suppression of Prmt1 abolishes β-catenin- or Hoxa9-independent transformation in HSC-derived MLL-CSCs.

A The relative number of colonies from *Hoxa9*$^{-/-}$ LSK-MLL-ENL leukemic cells with empty vector or shPRMT1 ($n = 3$, *t*-test).

B The % of CD45.2^{+} donor cells in the bone marrow of recipient mice at the indicated time points post-transplantation of *Hoxa9*$^{-/-}$ LSK-MLL-ENL leukemic cells with empty vector or shPrmt1 ($n = 4$, *t*-test).

C Kaplan–Meier survival curve of secondary recipient mice transplanted with *Hoxa9*$^{-/-}$ LSK-MLL-ENL leukemic cells with vector control or shPrmt1 ($n = 5$/cohort, log-rank test).

D The relative number of colonies from *Ctnnb1*$^{-/-}$ LSK-MLL-ENL leukemic cells with empty vector or shPRMT1 ($n = 3$, *t*-test).

E The % of CD45.2^{+} donor cells in the bone marrow of recipient mice at the indicated time points post-transplantation of *Ctnnb1*$^{-/-}$ LSK-MLL-ENL leukemic cells with empty vector or shPrmt1 ($n = 4$, *t*-test).

F Kaplan–Meier survival curve of secondary recipient mice transplanted with *Ctnnb1*$^{-/-}$ LSK-MLL-ENL leukemic cells with vector control or shPrmt1 ($n = 4$/cohort, log-rank test).

Data information: Data are represented as mean ± SD. See also Appendix Fig S6.

to treatment. Key components of the canonical Wnt/β-catenin signaling pathway are recurrently deregulated in various human cancers, and a number of inhibitor are in early-phase clinical trials (Anastas & Moon, 2013). This is particularly relevant to leukemia as normal HSCs remain largely intact upon a complete inactivation of β-catenin and targeting β-catenin represents a promising venue for eradication of LSCs (Fung *et al*, 2013). However, our results reveal an added dimension of cancer heterogeneity conferred by cells of origin transcriptional memory and predict that pharmacological targeting of β-catenin is unlikely to be effective in MLL leukemia originated from HSCs. We would like to point out that all the published literatures including our studies related to cancer cells of origin were performed using mouse cells, which could have different features from the human counterparts. Given the recent evidences from mouse models indicate the importance of the cells of origin in governing the resultant cancer biology, future relevant studies in human cell systems are paramount to give necessary insights and to improve our understanding of the cancer cell biology and designing effective therapeutics in the human diseases.

In contrast to its essential function in embryonic and other somatic stem cells, β-catenin is dispensable for adult HSCs, suggesting the presence of residual canonical Wnt signaling for normal HSC function (Malhotra & Kincade, 2009) or an alternative molecule/pathway compensated for β-catenin in adult hematopoiesis. Interestingly, most of the known molecules/pathways involved in canonical Wnt signaling predominately identified in epithelial cells or ES cells are not significantly affected upon β-catenin deletion in MLL leukemia regardless of their cells of origin (Datasets EV2A and EV3C). In contrast, we identified a number of novel β-catenin targets including those downstream of Meis1/Hoxa9 that are critical for HSC self-renewal, consistently indicating co-regulation of common self-renewal pathways by β-catenin and Hoxa9 in hematopoietic cells. Hoxa9 recently proposed as a key component of human LSC signature in AML (Jung *et al*, 2015) can mediate Bmi-1-independent leukemic self-renewal (Smith *et al*, 2011) and

resistance to PARPi treatment in AML (Esposito *et al*, 2015). In line with this finding, we and others have also reported the ability of β-catenin or Hoxa9 in promoting HSC self-renewal (Argiropoulos & Humphries, 2007; Malhotra & Kincade, 2009; Zeisig *et al*, 2012), but deletion of either one of them yields only mild hematopoietic phenotypes (Cobas *et al*, 2004; So *et al*, 2004; Lawrence *et al*, 2005; Jeannet *et al*, 2008; Koch *et al*, 2008; Smith *et al*, 2011), suggesting their overlapping function in HSC self-renewal. This is further supported by identification of Prmt1 as a key common downstream target that mediates their transcriptional and self-renewal functions. However, the lack of good-quality ChIP-grade antibodies against Hoxa9 and β-catenin makes it unfeasible to reliably determine whether they might directly bind to Prmt1 regulatory regions. While the lack of known consensus binding sites of Hox or β-catenin/Tcf by in silico analysis (data not shown) suggests an intermediate instead of direct regulation, future investigation is critical to gain further insights into any potential direct functional interactions and regulations. Nevertheless, our comprehensive transcriptomic analyses in combined with functional genomic studies have revealed the novel molecular networks mediated by β-catenin/Hoxa9/Prmt1 in regulating leukemic self-renewal in LSK-derived MLL-CSCs, and exemplify the intricate diversity in molecular pathways utilized by cancer cells to evade therapies, and underscores that simultaneous targeting of multiple self-renewal pathways may be required for successful elimination of certain CSCs.

Intriguingly, activation of canonical Wnt/β-catenin in MLL-CSCs has also recently been identified as a major mechanism for development of resistance to pharmacological inhibition of BET (Fong *et al*, 2015; Rathert *et al*, 2015), a targeted therapy at its early clinical phase for MLL leukemia, further highlighting the importance of adequately targeting β-catenin in the context of cells of origin for future targeted cancer therapies. Given the challenges involved in developing clinically effective inhibitors to β-catenin (Fung *et al*, 2013), the identification of Prmt1 as a nexus for mediating leukemic self-renewal in LSK-MLL-ENL transformed cells not only provides novel mechanistic insights into the downstream targets and molecular networks regulated by β-catenin, but also suggests an alternative avenue for targeting β-catenin in MLL-CSCs.

Materials and Methods

Animals and transplantation studies

All experimental procedures were approved by King's College London ethics committees and conform to the UK Home Office regulations. For all *in vivo* experiments, mice were distributed into their respective groups randomly. Investigators were not blinded to the sample identity. Mice were considered leukemic when an engraftment of donor cell (> 30%) was detected in the bone marrow. *Ctnnb1*$^{fl/fl}$ mice (Brault *et al*, 2001) were crossed with Rosa26-CreER Rosa26-YFP mice to generate *Ctnnb1*$^{fl/fl}$ Rosa-CreER RosaYFP mice. These mice were crossed with Hoxa9$^{-/-}$ knockout mice (So *et al*, 2004; Smith *et al*, 2011) to generate *Hoxa9*$^{-/-}$ *Ctnnb1*$^{fl/fl}$ Rosa-CreER RosaYFP mice. Compound homozygous animals were used for experiments. C57BL/6 or SJL mice were given 11 Gy total body γ-irradiation and injected via tail vein with test cells mixed with C57BL/6 or SJL bone marrow nuclear cells. Mice were culled when sign of sickness appeared. Survival curves were produced using GraphPad Prism software, and survival differences tested with the log-rank test. For leukemia development experiments, up to 500,000 test cells mixed with 200,000 C57BL/6 or SJL bone marrow nuclear cells were transplanted. For primary transplants n = 5–18 mice/cohort and for secondary transplants n = 3–10 mice/cohort were used. For *in vivo* limiting dilution assays, varying numbers of cells from indicted populations were transplanted into n = 5 sublethally irradiated syngeneic mice/cohort and monitored for disease development. ELDA was used for statistical analysis (Hu & Smyth, 2009). For *in vivo* homing experiments, 5 million test cells were transplanted into $n \geq 4$ (when studying genetic ablation) and n = 2 (when studying shPRMT1 knockdown) sublethally irradiated syngeneic mice/cohort/time point. Homing differences were statistically tested using a two-tailed *t*-test in Excel.

Hematopoietic stem and progenitor purification

Mouse femur and tibias were prepared and c-Kit$^+$ (CD117) cells isolated (Zeisig & So, 2009) using MACS (Miltenyi Biotech Technology, Germany). LSK and GMP populations were isolated as previously described (Yeung & So, 2009). Briefly, LSK (Lin$^-$, Sca-1$^+$, c-Kit$^+$) and GMP (Lin$^-$, Sca-1$^-$, c-Kit$^+$, CD34$^+$, CD16/32^{lo}) were isolated from lineage-negative cells after lineage (Gr-1, Mac-1, B220, Ter119, CD3e, CD4, CD8) (Biolegend/eBiosciences) depletion using a BD FACS ARIA cell sorter. Post-sort purity of > 97% was routinely achieved. For functional analysis, sorted LSK and GMP were plated in Methocult M3434 (Stem Cell Technologies, Canada), and after 7–10 days of incubation, different colony types were scored.

Retroviral transduction and transformation assays

Retroviral transduction and transformation assays (RTTA) were performed as previously described with some modifications (Zeisig & So, 2009). The MSCV-MLL-ENL construct has been described previously. The two independent shRNAs against murine Prmt1 have been reported previously (Cheung *et al*, 2007). Briefly, isolated c-Kit$^+$, LSK, or GMP cells were cultured overnight in RPMI + 10% FBS supplemented with 20 ng/ml SCF, 10 ng/ml IL3, and 10 ng/ml IL6 prior to viral transduction with virus particles carrying MLL-ENL by centrifugation at 800 × *g* at 32°C for 2 h. Cells were plated in M3234 Methylcellulose medium supplemented with 20 ng/ml SCF, 10 ng/ml of each IL3, IL6, and GM-CSF and appropriate antibiotic selection on the following day. Colonies were scored after 7 days and replated every 7 days. To induce the deletion of Ctnnb1, 20 nM tamoxifen (Sigma, USA) was added to the methylcellulose medium in the second round of plating and YFP positive were sorted after the second round of plating using a BD FACS ARIA and plated into the 3rd round. After the 4th round of plating, cells were cultured in R20/20 to establish cell lines as previously described (Yeung *et al*, 2010). Differences in colony numbers were statistically tested using a two-tailed *t*-test in Excel.

Phenotypic analysis

Immunophenotypic analysis was performed by FACS using fluorochrome-conjugated monoclonal antibodies to murine c-Kit (2B8

clone), Mac-1 (M1/70), and Gr-1 (RB6-8C5) (eBiosciences). Staining was generally performed on ice for 15 min and washed once before analysis using a BD LSR II system. Differences in surface marker expression were statistically tested using a two-tailed *t*-test in Excel.

Western blot

Cell lysates from primary transformed cells or sorted leukemic cells were isolated and subjected to Western blot as described (Yeung *et al*, 2010).

Histone extraction and detection

Histone proteins were prepared by acid extraction (Abcam protocol, http://www.abcam.com/protocols/histone-extraction-protocol-for-western-blot). Briefly, cells were lysed for 10 minutes on ice in TEB buffer (PBS/0.5% Triton X-100/protease inhibitors) at a cell density of 10^7 per ml. The nuclei were harvested by centrifugation and washed in half the volume of TEB. Pellets were resuspended in 0.2 N HCl at a density of 4×10^7 nuclei per ml, and histones were extracted overnight at 4C. Samples were centrifuged to pellet debris, and the supernatant containing the histones was transferred into a new Eppendorf tube. 10 μl of histones was mixed with 40 μl PBS and 50 μl 2× SDS loading buffer and incubated at 95°C for 8 min. 25 μl of the denatured histones were subjected to Western blot using a 10% Next gel (AMRESCO LLC). Antibodies against H4R3me2a (Active Motif) and Histone H3 (Abcam) were used.

Genotyping PCR

Genomic DNA was isolated, and β-catenin genotyping PCR was performed as described previously (Brault *et al*, 2001). Primer sequences for Hoxa9 are available on request. Differences in gene expression were statistically tested using a two-tailed *t*-test in Microsoft Excel.

qRT–PCR

qRT–PCR was performed on StepOne qPCR machine (Applied Biosystems) using TaqMan or SYBR green chemistries. See Appendix Table S1 for the primer sequences used throughout the paper for validation.

Apoptosis and cell cycle analysis

MLL-ENL-transduced Hoxa9$^{-/-}$*Ctnnb1*$^{fl/fl}$ cells were treated for 72 h with or without tamoxifen and stained with AnnexinV for apoptosis. Cell cycle was analyzed using the BRDU Flow kit (BD Pharmingen) according to manufacturer's instructions. Cells were analyzed using a BD LSR II system (BD, USA), and differences were statistically tested using a two-tailed *t*-test in Excel.

RNA sequencing

300 ng to 1 μg of mirVANA (Ambion) isolated total RNA was used for RNA-Seq library preparation using TruSeq Stranded Total RNA kit (Illumina) and sequenced on HIseq2000 platform (Illumina) as per manufacturers recommendations. All samples are listed in Dataset EV1A.

Whole-genome sequencing

Genomic DNA was isolated with the QIAamp DNA Micro kit (Qiagen) according to the manufacturer's instructions, and 30 ng of genomic DNA was used as input for each library preparation. Whole-genome sequencing libraries were generated using the tagmentation method as previously described (Wang *et al*, 2013), but without bisulfite treatment and with minor modifications. Briefly, genomic DNA was subjected to tagmentation with a hyperactive Tn5 transposase (Epizyme), which fragmented the DNA and appended sequencing adaptors in a single step. After PCR amplification of libraries, DNA fragments of 200–800 bp were double-side selected using SPRI Ampure XP beads, with left–right ratios of 1.5–0.55. Purified libraries were subjected to 125-bp paired-end sequencing on an Illumina HiSeq2500 machine.

Bioinformatic analyses

Mapping and read counts

The FASTQ files were de-tagged, and the quality of the FASTQ files inspected using FastQC v.0.11.2 (Andrews, 2015). Remaining adapters were trimmed using TRIMGalore! v.0.3.7 (Kreuger, 2015). Whole-genome sequencing reads were mapped to the Ensembl mouse genome GRCm38 (Aken *et al*, 2016) using Bowtie2 v.2.2.5 (Langmead & Salzberg, 2012). RNA-Seq reads were mapped to the Ensembl mouse genome using TopHat2 v2.0.13 (Kim *et al*, 2013), and reads were filtered for quality and counted using samtools v.0.1.18 and bedtools v2.23.0-10-g447cb97 (Li *et al*, 2009; Quinlan & Hall, 2010; Dataset EV1B).

SNP calling

SNP calling (for both RNA-Seq and WGS) was performed using samtools v.0.1.18 mpileup and bcftools v.1.2 (using htslib v.1.2.1) (Li, 2011), SNPs were leniently filtered only for quality ≥ Q30, and custom scripts used to produce summary stats (Dataset EV1C). To summarize the regions in which SNPs fell, variant call files and Ensembl GTF annotation (Mus_musculus.GRCm38.82.gtf) were converted to bed format using custom scripts and compared using bedtools intersect v2.23.0. Bedtools complement v2.23.0 (Quinlan & Hall, 2010) was used to extract intronic and intergenic coordinates.

Gene differential expression

Differential expression was determined either using DESeq2 v.1.10.1 (Love *et al*, 2014), negative binomial GLM fitting and Wald statistics; or with limma v.3.26.9 (Ritchie *et al*, 2015) using voom to normalize read counts, and eBayes to determine differential expression (as detailed in Expanded View Datasets). Functional enrichment analysis was conducted using the GSEA software, using various Molecular Signature Databases (c2 set version 5.0, version 6.0 and all_gene_sets) (MSigDB) (Subramanian *et al*, 2005) appended with a custom-made gene set for LSC stem cell maintenance (Somervaille *et al*, 2009; Dataset EV3B), comparing log2-fold changes in gene expression as the ranking metric. For the GSEA analysis, human-mouse gene orthologues were identified

using MGI list of orthologous genes (Blake *et al*, 2014), and Ensembl bioMart used to transfer MGI IDs to Ensembl gene IDs (Kinsella *et al*, 2011). In order to find genes which showed a synergistic effect of simultaneous knockout of Cbnnt1 and Hoxa9, differential expression was tested with DESeq2 Negative Binomial GLM fitting and Wald statistics. The data were subjected to five DE analyses, which served to categorize the genes into 78 different classes. These comparisons are as follows: 1. LSK WT to Ctnnb1 KO, 2. LSK WT to HoxA9 KO, 3. Additive effect LSK WT to Ctnnb1 KO and Hoxa9 KO, 4. LSK Ctnnb1 KO to Ctnnb1:HoxA9 interaction, 5. LSK HoxA9 KO to Ctnnb1: HoxA9 interaction. For overlapping significantly differentially expressed genes, heatmaps were plotted using the function heatmap.2 from the R-package gplots v3.0.1 (Warnes *et al*, 2016) with *Z*-score scaling of rows and/or columns.

Survival analysis

Statistical analysis and data visualization were performed using R (R Core Team, 2014). Survival and Cox proportional hazards ratio analysis were conducted using R-packages survival v.2.39.5 (Therneau, 2015) and survcomp v.1.20.0 (Schroder *et al*, 2011) on all patients which had complete survival and expression data from the following datasets: GSE1159 ($n = 293$), GSE12417; GPL570 ($n = 79$), GPL96 ($n = 163$), GSE14468 + GSE6891 ($n = 602$), GSE5122 ($n = 58$), GSE8970 ($n = 34$) available from the Gene Expression Omnibus database ncbi.nlm.nih.gov/geo/ and The Cancer Genome Atlas AML dataset ($n = 183$; Cancer Genome Atlas Research N, 2013). Summary data for patient cohorts are presented in Dataset EV2D. Samples were normalized using the GENENORM algorithm from the R-package inSilicoMerging v.1.15.0 (Taminau *et al*, 2012) with prior DESeq2 Vst transformation of RNA-Seq samples used in this study to derive the cell of origin-specific signature (Love *et al*, 2014). All probe intensities were translated into human genes and averaged prior to merging. MLL patients in the datasets were identified from associated karyotyping and PCR diagnosis.

Data availability

All WGS has been submitted to the ENA study PRJEB14461. RNA-Seq is available on ArrayExpress E-MTAB-3647.

Statistical analysis

All the experimental results were analyzed using two-tailed Student's *t*-test, chi-square test, hypergeometric test, as indicated in the figure legends and Expanded View Datasets. Groups that were statistically compared shared a similar variance, as shown in the figures. $P < 0.05$ was considered as statistically significant. For GSEA analysis, FDR *q*-values < 0.25 were considered as statistically significant (Subramanian *et al*, 2005). The log-rank test was used to compare survival curves.

Expanded View for this article is available online.

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Author contributions

TS, BBZ, TKF, MM, C-TT, PNIL, PV, JB, and AW performed experiments, BBZ contributed to writing the manuscript, MZ, QCH, CL, and BL contributed the bioinformatics analyses and editing the manuscript, and CWES contributed to overall research design, direction, and writing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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