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A non-redundant function of cyclin E1 in hematopoietic stem cells

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Abbreviations: HSC, hematopoietic stem cell; LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent precursors; 5-FU, 5-fluorouracil; LTC-IC, long-term culture initiating cell; BMCs, bone marrow mononucleated cells; CFU, colony-formation init; CRU, competitive repopulation unit; WBCs, white blood cells; CDK, cyclin-dependent kinase; MEF, mouse embryonic fibroblast; KO, knockout; GFP, green fluorescent protein

A precise balance between quiescence and proliferation is crucial for the lifelong function of hematopoietic stem cells (HSCs). Cyclins E1 and E2 regulate exit from quiescence in fibroblasts, but their role in HSCs remains unknown. Here, we report a non-redundant role for cyclin E1 in mouse HSCs. A long-term culture-initiating cell (LTC-IC) assay indicated that the loss of cyclin E1, but not E2, compromised the colony-forming activity of primitive hematopoietic progenitors. *Ccne1*$^{-/-}$ mice showed normal hematopoiesis in vivo under homeostatic conditions but a severe impairment following myeloablative stress induced by 5-fluorouracil (5-FU). Under these conditions, *Ccne1*$^{-/-}$HSCs were less efficient in entering the cell cycle, resulting in decreased hematopoiesis and reduced survival of mutant mice upon weekly 5-FU treatment. The role of cyclin E1 in homeostatic conditions became apparent in aged mice, where HSC quiescence was increased in *Ccne1*$^{-/-}$ animals. On the other hand, loss of cyclin E1 provided HSCs with a competitive advantage in bone marrow serial transplantation assays, suggesting that a partial impairment of cell cycle entry may exert a protective role by preventing premature depletion of the HSC compartment. Our data support a role for cyclin E1 in controlling the exit from quiescence in HSCs. This activity, depending on the physiological context, can either jeopardize or protect the maintenance of hematopoiesis.

Introduction

Hematopoietic stem cells (HSCs) are rare cells endowed with multipotency and self-renewing ability that are capable of generating every specialized cell of the blood system.[1] HSCs exist mainly in a quiescent state in vivo, while their immediate descendants, the early hematopoietic progenitors, constitute a highly proliferative compartment.[2,3] Precise regulation of HSC proliferation and cell fate decisions are necessary for production of mature blood cells throughout adult life, either under homeostasis or in instances that require rapid regenerative responses.[4] Several positive and negative regulators of the cell cycle machinery are critical for various aspects of HSC and/or progenitor proliferation, including the cyclin-dependent kinase inhibitors p21,[5-7] p27,[8,9] p57,[9,10] p16,[11] and p18,[12] as well as the D-type cyclins[13] and their catalytic partners Cdk4 and Cdk6.[14]

Cyclins associate with cyclin-dependent kinases (CDKs), thereby activating their kinase activity, which is key to the regulation of biological processes, such as cell cycle progression and regulation of transcription.[15,16] In mammals, the cyclin E subfamily includes cyclins E1 and E2, which preferentially associate with—and activate—the catalytic subunit Cdk2.[17-20] In physiological conditions, cyclin E/Cdk2 complexes show periodic activation during the cell cycle, starting at the G_1/S transition and persisting for the duration of the S phase.[21] The genes encoding cyclins E1 and E2 (*Ccne1* and *Ccne2*) show similar and widespread tissue distributions,[22] and deletion of either gene alone revealed no obvious phenotype in mice.[23,24] Combined knockout of *Ccne1* and *Ccne2* did not impair embryonic development per se, but caused lethality at mid-gestation owing to a placental defect, following from the failure of giant trophoblastic cells to undergo endo-replication.[23,24] Characterization of double-knockout (dKO) mouse embryo fibroblasts (MEFs) confirmed that cyclins E1 and E2 are not essential for cell proliferation altogether, but unraveled their requirement for cell cycle re-entry upon growth factor stimulation of quiescent cells.[23] Given the prominent role of quiescence and cell cycle re-entry in HSC biology, we decided to analyze the role of E-type cyclins in this particular cellular

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compartment. Our results reveal an unexpected non-redundant requirement for cyclin E1 in HSCs.

Results

Cylin E1 deficiency affects early hematopoietic progenitors in vitro

Since regulation of the cell cycle is germane to the tight balance between differentiation and self-renewal, we decided to assess the function of cyclins E1 and E2 in murine HSCs and hematopoietic progenitors. We first probed primitive hematopoietic cells in a long-term culture initiating cell (LTC-IC) assay. Bone marrow mononucleated cells (BMCs) were grown for 5 weeks on a feeder layer of stromal cells in order to allow proliferation and terminal differentiation of hematopoietic progenitors. The cells were then transferred onto a semisolid medium supplemented with a cocktail of cytokines promoting proliferation and differentiation of primitive hematopoietic cells (in particular HSCs). Colonies formed in these secondary cultures arise from HSCs and hematopoietic progenitors that have withstood the initial 5 weeks due to their low mitotic activity and their high self-renewal potential. Loss of cyclin E1 significantly reduced the number of colonies scored in the LTC-IC assay, while loss of cyclin E2 had no effect (**Fig. 1A**). The few colonies scored in plates seeded with *Ccne1*$^{-/-}$ BMCs were also much smaller, a feature typical of primitive colonies composed of poorly differentiated hematopoietic cells (**Fig. 1B**). This reduction in colony formation observed with *Ccne1*$^{-/-}$ cells correlated with a reduction in areas of hematopoiesis (cobblestone areas)[25] observed at 5 weeks of culture on feeder layers (**Fig. 1C**). In contrast with the result of the LTC-IC assay, the frequency and proliferation of committed progenitors upon direct plating were unaffected by the loss of either cyclin E1 or E2, as measured by a colony-forming unit (CFU) assay (**Fig. 1D**; **Fig. S1**). Consistent with this result, FACS analysis showed that common lymphoid progenitors[26] and myeloid progenitors[27] occurred at similar frequencies in the bone marrow of wild-type and *Ccne1*$^{-/-}$ animals (**Fig. 1E**; **Fig. S2**).

Altogether, our data pointed to a selective requirement for cyclin E1, but not cyclin E2, in cytokine-mediated proliferation of primitive hematopoietic progenitors. We thus focused our attention on possible hematopoietic defects in *Ccne1*$^{-/-}$ mice in vivo.

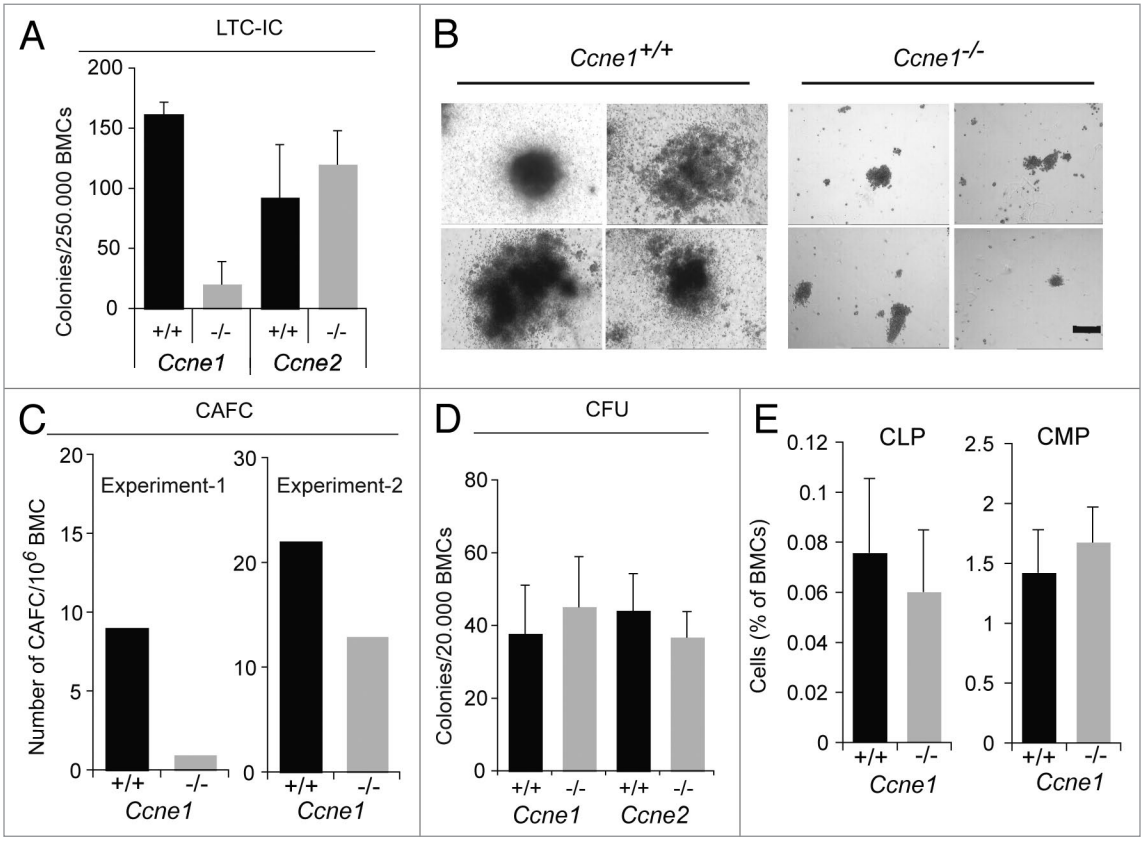

Figure 1. Analysis of progenitors and primitive hematopoietic cells derived from cyclin E mice of mixed background. (**A**) LTC-IC assay performed with 250 000 BMCs isolated from 3 independent mice of the indicated genotypes. The graph reports the average number of colonies scored for the appropriate genotypes, ± stdv. (**B**) Representative pictures of the colonies obtained in an LTC-IC assay using BMCs derived from *Ccne1*$^{+/+}$ or *Ccne1*$^{-/-}$ mice. Scale bar is 400 µm. (**C**) CAFC assay; 2 independent experiments are shown performed on *Ccne1*$^{+/+}$ or *Ccne1*$^{-/-}$ derived bone marrow mononucleated cells (BMCs). Cobblestone areas were assessed after 5 wk of culture on stromal feeder layers. (**D**) CFU assay performed with 20 000 bone marrow mononucleated cells (BMCs) isolated from 3 independent mice of the indicated genotypes. Colonies were counted after 10 d of culture. The graph represents the average number of colonies scored. The error bar indicates the standard deviation (stdv). (**E**) FACS analysis of common lymphoid progenitors (CLP: Lin$^-$, ILR7$^+$, Kit^{low}, and Sca1^{low}) and common myeloid progenitors (CMP: Lin$^-$, ILR7$^-$, FcγRII/III^{Low}, Kit$^+$, and Sca1$^-$) in *Ccne1* mice. Bar graphs are the average frequencies ± stdv.

Normal HSC frequencies in young *Ccne1*$^{-/-}$ mice

The reduction of colonies scored in the LTC-IC assay in *Ccne1*$^{-/-}$ BMCs could be interpreted either as a numerical or a functional deficiency of primitive hematopoietic progenitors and HSCs in *Ccne1*$^{-/-}$ mice. FACS analysis based on surface markers revealed similar frequencies of long-term (LT) or short-term (ST) HSCs, as well as of multipotent-precursors (MPP) in *Ccne1*$^{-/-}$ and wild-type animals (**Fig. 2A**; **Fig. S3A**). Furthermore, assessment of dormant HSCs using the CD150 and CD48 marker[28,29] did not reveal any difference between *Ccne1*$^{-/-}$ and wild-type littermates (**Fig. 2B**; **Fig. S3B**). Next we assessed the cell cycle status of HSCs and progenitor populations by FACS by combining the analysis of DNA content (Hoechst) with intracellular expression of Ki67, a protein expressed in cycling cells, but not in quiescent G_0 cells. Loss of cyclin E1 did not significantly alter the cell cycle distribution profiles of LT-HSCs, ST-HSCs, MPPs or myeloid progenitors (MPs) (**Fig. 2C**). Given the lack of numerical differences in HSCs or progenitor cells isolated from *Ccne1*$^{-/-}$ mice compared with wild-type animals as assessed by FACS, we wondered whether loss of cyclin E1 would affect the function of these cells. For this purpose, we performed a competitive repopulation unit (CRU) assay, which is an in vivo limiting dilution assay that allows the evaluation of functional HSCs.[30] The CRU assay revealed similar frequencies of functional HSCs in wild-type and *Ccne1*$^{-/-}$ mice (1:20047 vs 1:23740, *P* value:0.809, details in **Table S1**). Altogether, we conclude that, in vivo, loss of cyclin E1 affected neither the frequency nor the function of primitive hematopoietic cells at steady state. Thus, the reduction in colonies measured in the LTC-IC assay when culturing WBCs derived from *Ccne1*$^{-/-}$ mice is more likely to reflect an impairment of *Ccne1*$^{-/-}$ HSCs in undergoing cytokine-mediated proliferation: this condition may be distinct from homeostasis, and may rather correspond to a stress response.

Cyclin E1 regulates HSC responses to systemic aplasia

Based on the above data, we hypothesized that, while not essential for homeostasis in vivo, cyclin E1 might be limiting in situations that require prompt cell cycle re-entry of HSCs, such as when the hematopoietic system is facing severe aplasia. To address this question, we assessed the recruitment of quiescent HSCs into the cell cycle following a single administration of the anti-metabolite 5-fluorouracil (5-FU). We first focused our analysis at 24 h post-5-FU injection, when HSCs are starting to be recruited into the cell cycle in response to treatment.[31] At this time point, HSCs were still prospectively identifiable, since they still presented high levels of c-Kit detectable by FACS (**Fig. 3A**). The frequencies of LT/ST-HSCs or MPPs (**Fig. 3A**), as well as total BMC counts (**Fig. 3B**), were not significantly different between *Ccne1*$^{+/+}$ and *Ccne1*$^{-/-}$ mice, indicating that loss of cyclin E1 did not affect the number of hematopoietic cells surviving to 5-FU treatment. Since 5-FU selectively eliminates cycling cells[32,33] this suggests that there is no difference in the number of quiescent HSCs in *Ccne1*$^{+/+}$ and *Ccne1*$^{-/-}$ mice prior to treatment. This is in line with the observation that at steady-state HSC quiescence is not altered by cyclin E1 loss (**Fig. 2C**; **Fig. S3**). However, 24 h post-5-FU treatment LT-HSCs and ST-HSCs isolated from *Ccne1*$^{-/-}$ mice were significantly more quiescent compared with their *Ccne1*$^{+/+}$ counterparts (**Fig. 3C and D**), suggesting that cyclin E1 may control the kinetics of cell cycle re-entry following myeloablation. Of note, the more differentiated MPPs, showed comparable cell cycle distribution regardless the cyclin E1 status (**Fig. 3D**). Similar results were replicated in the inbred C57/Bl6 background: again, no differences were recorded in HSC frequencies (**Fig. S4A**) while a higher fraction of quiescent HSCs was measured in *Ccne1*$^{-/-}$ mice at 24 h post-5-FU treatment (**Fig. S4B**).

At longer time points after 5-FU treatment (i.e., 48 h), HSCs respond to the myeolablation by starting a self-renewing proliferation and, as a consequence of their cytokine-dependent proliferative state,[31] display lower level of c-Kit cell surface expression. Thus at this stage HSCs can be identified by FACS within a population of primitive hematopoietic cells that are defined as Lin$^-$, Sca1$^+$, and c-Kit^{low} (LSK^{low}, as shown in **Fig. 3E**).

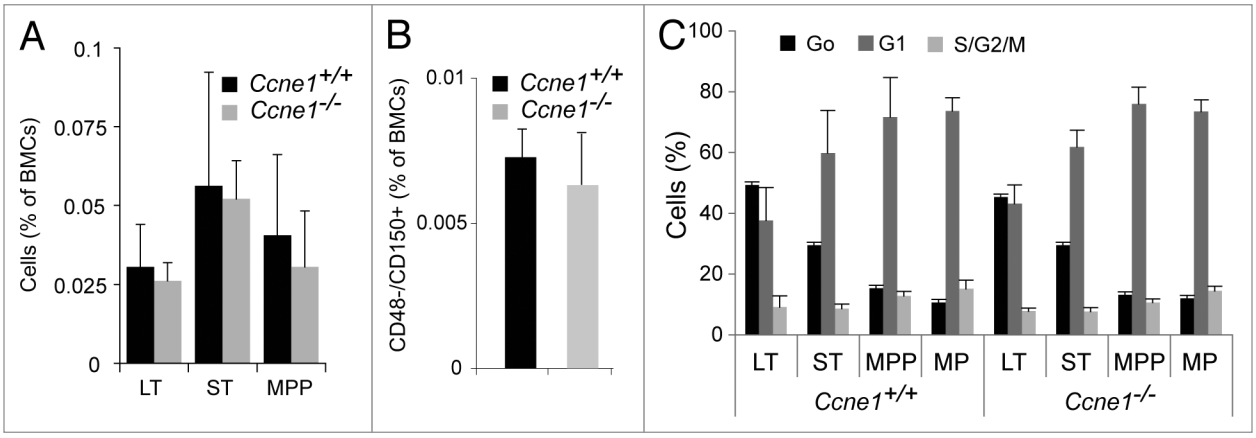

Figure 2. FACS analysis of HSCs derived from *Ccne1*$^{-/-}$ mice. (**A**) FACS analysis of bone marrow HSCs subpopulations belonging to the Lin$^-$, Sca1$^+$, Kit$^+$ (LSK) population by using the Flk2 and CD34 surface markers. MP, myeloid progenitors (Lin$^-$, Sca$^-$ and Kit$^+$); MPP, multipotential precursors (LSK, Flk2$^+$, CD34$^+$); ST, short-term HSCs (LSK, Flk2$^-$, CD34$^+$); LT, long-term HSCs (LSK, Flk2$^-$, CD34$^-$). (**B**) HSC analysis using the SLAM markers CD48 and CD150 to enrich in "dormant" HSCs (LSK, CD34$^-$, CD48$^-$ and CD150$^+$). (**C**) Cell cycle analysis (Ki67/Hoechst) of the different HSC populations and progenitors. Five mice for each genotype were used in the experiments described in panels (**A–C**).

Remarkably, the kinetics of accumulation of these cells depended on cyclin E1, with LSK^{low} cells accumulating at lower levels in *Ccne1*$^{-/-}$ compared with wild-type mice (**Fig. 3E**). Thus, the lower efficiency of *Ccne1*$^{-/-}$ HSCs in being recruited into the cell cycle after 5-FU treatment correlated with a lower production of LSK^{low} cells, which are responsible for the wave of hematopoiesis that follows severe aplasia.[31] Accordingly, 4 d after 5-FU administration, *Ccne1*$^{-/-}$ mice showed lower bone marrow cellularity than *Ccne1*$^{+/+}$ littermates (**Fig. 3F**; **Fig. S4D**), suggesting that the impairment in cell cycle entry of *Ccne1*$^{-/-}$ HSCs results in reduced de novo hematopoiesis. To verify this hypothesis, we evaluated the kinetics of hematopoiesis by monitoring the abundance of white blood cells (WBCs) in peripheral blood for up to 24 d following a single 5-fluorouracil treatment. While

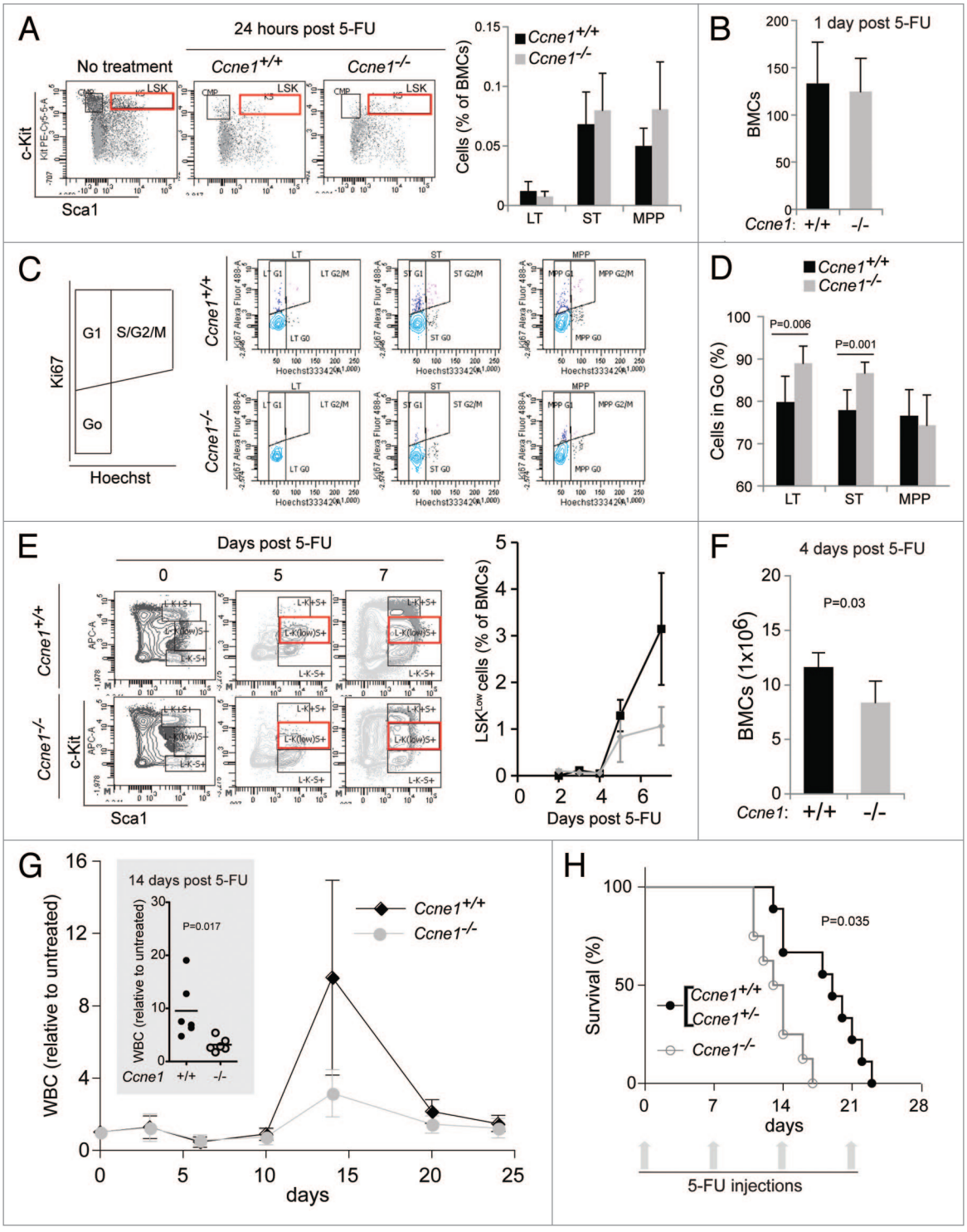

Figure 3. For figure legend, see page 3667.

Ccne1$^{+/+}$ animals showed the characteristic transient increase (rebound) in circulating WBCs, due to HSCs differentiating along the myeloid and lymphoid lineages, this effect was blunted in *Ccne1*$^{-/-}$ mice. Thus, cyclin E1-null HSCs have a reduced ability to enter the cell cycle and give rise to LSK^{low} HSCs following myeloablation, leading to reduced de novo hematopoiesis.

The defective hematopoiesis of *Ccne1*$^{-/-}$ mice following myeloablation would be predicted to lead to an enhanced sensitivity to sustained aplasia. To verify this, we subjected mice to weekly 5-FU treatments and monitored their survival over time. Indeed, *Ccne1*$^{-/-}$ mice showed a significantly reduced overall survival compared with wild-type or heterozygous littermates (**Fig. 3H**; **Fig. S5**). Altogether, our data suggest that the defective cell cycle re-entry of quiescent *Ccne1*$^{-/-}$ HSCs affects their ability to resume efficient hematopoiesis following myeloablating treatment, thus reducing their ability to effectively face sustained myelotoxic stress.

Increased HSC quiescence in aged *Ccne1*$^{-/-}$ mice

Given the lack of a detectable cell cycle phenotype at steady-state in the HSCs from young *Ccne1*$^{-/-}$ animals (8–120-wk-old), and considering that the control of cell cycle dynamics in HSCs is age-dependent,[11,34] we studied a cohort of 23-mo-old mice. FACS analysis showed equal frequencies of LT/ST-HSCs, MPPs or MPs in aged *Ccne1*$^{-/-}$ and wild-type animals (**Fig. 4A**). Yet, we measured a statistically significant increase in the proportion of quiescent LT/ST-HSCs, with an average of 75% of quiescent LT/ST-HSCs in *Ccne1*$^{-/-}$ compared with 60% in *Ccne1*$^{+/+}$ animals (**Fig. 4B and C**). Of note the cell cycle distribution of MPPs and myeloid progenitors (MPs) was not affected by cyclin E1 loss (**Fig. 4B and C**), thus indicating a specific function of cyclin E1 in regulating the cell cycle in the HSC compartment.

Loss of cyclin E1 provides competitive advantage to HSCs

We finally assessed HSC function in competitive bone marrow transplants. *Ccne1*$^{-/-}$ or wild-type "test" BMCs (expressing the Ly5.2 epitope) were transplanted together with equal amounts of competitor congenic BMCs (expressing the Ly5.1 epitope). In line with the results of the CRU assay, mutant and wild-type cells were equally efficient in producing long-term hematopoiesis in primary recipients. After long-term engraftment, BMCs were transplanted from the primary into secondary recipients, and the same procedure was repeated serially into tertiary and quaternary recipients. At each passage, we evaluated long-term hematopoiesis in peripheral blood by monitoring the relative proportions of Ly5.1^{+} and Ly5.2^{+} WBCs. Loss of cyclin E1 endowed HSCs with a competitive advantage upon serial transplantation, since ternary and quaternary recipient of cyclin E1-deficient BMCs displayed stronger long-term hematopoiesis compared with mice that had received wild-type BMCs (**Fig. 5A**). Similar results were obtained when using wild-type or cyclin E1-null mice of a C57/129 mixed background that were also transgenic for GFP (**Fig. 5B**). Altogether, our data suggest that loss of cyclin E1 limits the exhaustion of the HSC compartment when exposed to the highly proliferative response that follows bone marrow transplantation, resulting in increased resilience of HSCs upon serial transplantation.

Discussion

We have used mice lacking cyclin E1 to study the role of this protein in HSCs biology. Our data show that cyclin E1 regulates the kinetics of cell cycle entry of quiescent stem cells following severe pancytopenia caused by myeloablating treatments such as 5-FU injection, thereby affecting their efficiency in generating differentiated progenitors. Indeed, the slower cell cycle entry measured in *Ccne1*$^{-/-}$ HSCs following 5-FU treatment correlated with a lower accumulation of LSK^{low} cells, a population of cells comprising self-renewing HSCs and highly proliferating early progenitors which are generated following aplasia.[31] A reduction of the LSK^{low} cells is likely to impact on downstream hematopoiesis, and, in fact, we consistently measured a lower rebound of circulating WBCs in *Ccne1*$^{-/-}$ mice, thus suggesting that cell cycle entry and proliferation rates of primitive progenitors (i.e., LSK^{low} cells) affect the efficiency of hematopoiesis.

It is important to note that *Ccne1*$^{-/-}$ HSCs are not inherently sensitized to 5-FU treatment since: (1) in homeostatic conditions they are as quiescent as their wild-type counterpart (**Fig. 2C**); and (2) upon myeloablation, the fraction of surviving HSCs is comparable in either genetic background (**Fig. 3A**). Moreover, the relative increase in quiescence observed in *Ccne1*$^{-/-}$ HSCs at 24 h post 5-FU (**Fig. 3B**; **Fig. S4B**) is not expected to provide any protective effect upon repeated 5-FU administration: indeed, despite the lower kinetics of cell cycle exit, by the time the subsequent treatment was administered all the *Ccne1*$^{-/-}$ HSCs had entered the cell cycle and had converted into LSK^{low} (**Fig. 3E**). The impairment of stress-induced hematopoiesis observed in

Figure 3 (See opposite page). Cyclin E1 regulates HSCs following myeloablation. (**A**) Prospective identification of HSCs and progenitors in *Ccne1* mice by FACS analysis of bone marrow mononucleated cells (BMCs) isolated from *Ccne1* mice 24 h following 5-FU administration. On the left, FACS profiles of Lin^{-} cells stained with c-Kit and Sca1 are shown. The red box outlines the gating for the LSK cells used to define LT-HSC, ST-HSC and MPP (as described to in **Fig. 2**). Frequencies of the different LSK cells are represented in the bar graph on the right. The bar graph represents the average values relative to the different LSK population of cells; error bars are the standard deviation, n = 7. (**B**) Assessment of BMCs, at 1 d post-5-FU treatment (n = 7). Values are the average of the total count of cells isolated from 2 tibias and 2 femurs/mouse. (**C**) Cell cycle distribution by FACS analysis of the different HSC/progenitor populations of cells as shown in (**A**), stained for the proliferation marker Ki67 and the DNA dye Hoechst. (**D**) Analysis of quiescence (i.e., percentage of cells in G_0) based on the FACS profiles shown in (**C**). Bar graph is the average percentage of quiescent cells ± stdv, n = 7. (**E**) Analysis of the kinetics of accumulation of the LSK^{low} following a single injection of 5-FU. FACS profiles of Lin^{-} cells stained with c-Kit and Sca1 are shown on the left; the red box highlights the LSK^{low} gate used. Quantitation of LSK^{low} cells is shown on the right as average cells ± stdv, n = 3. (**F**) Assessment of BMCs 4 d post 5-FU treatment (n = 4). Values are the average of the total count of cells isolated from 2 tibias and 2 femurs/mouse. (**G**) A cohort of *Ccne1*$^{+/+}$ or *Ccne1*$^{-/-}$ mice (n = 6) was injected with 5-FU (200 mg/Kg) at day 0. Peripheral white blood cells (WBCs) were counted at defined days; data were normalized to values measured before 5-FU treatment and plotted as average ± stdv. The count of white blood cells at day 14 is also reported in the inset. (**H**) Kaplan–Mayer survival curves of different cohorts of mice of the indicated genotypes subjected to 5-FU injections (150 mg/Kg) every 7 d, as indicated by the arrows. *Ccne1*$^{-/-}$ = 8, *Ccne1*$^{+/+}$ = 5, and *Ccne1*$^{+/-}$ = 4.

Ccne1$^{-/-}$ mice also accounts for their sensitivity to a regimen of continuous 5-FU administration: since this treatment causes the progressive erosion of HSCs, *Ccne1*$^{-/-}$ mice, which show defective stress-induced hematopoiesis, are likely to fall below the survival threshold sooner than wild-type mice.

Contrary to what observed in young animals, where cyclin E1 is dispensable for controlling HSC homeostasis, loss of cyclin E1 resulted in a higher proportion of quiescent HSCs in aged mice compared with aged matched littermates. This effect of cyclin E1 most probably reflects intrinsic changes in the way the cell cycle is controlled in aged HSCs, as exemplified by the accumulation of the CDK inhibitors (CKI) p21 and p18.[34] Likewise, p16 expression has been reported to increase with HSCs aging,[11] although whether this may represent a general feature of aging HSCs is still debated.[35]

Such alterations bear direct consequences on cell cycle dynamics, since p21 directly inhibits cyclin E/CDK2 activity, while inhibition of cyclin D/CDK4,6 complexes by p18 would be expected to render cell cycle progression critically dependent on cyclin E activity, as demonstrated by studies on CDK4,6 double KO and cyclin D1–3 triple KO cells.[13,14] In this setting, the reported downregulation of cyclin E2 expression in aged HSCs[34] may explain why cyclin E1 becomes limiting.

We also notice that our aged mice, regardless of their *Ccne1* status, showed increased HSC quiescence compared with young animals of the same genotype: while this observation is coherent with studies showing a progressive decrease in the proliferative and self-renewal potential of HSCs during aging,[11,34,36-38] it is in contrast with early work reporting increased proliferation of aged HSCs.[39] Methodological differences may account in part for this discrepancy: here, we used Ki67 as a marker for defining quiescent cells in HSCs, as opposed to BrDU incorporation, which was used in earlier studies and is now known to intrinsically alter the cell cycle distribution in HSCs.[29] This notwithstanding, our analysis indicates that loss of cyclin E1 affects quiescence of LT/ST HSCs during aging.

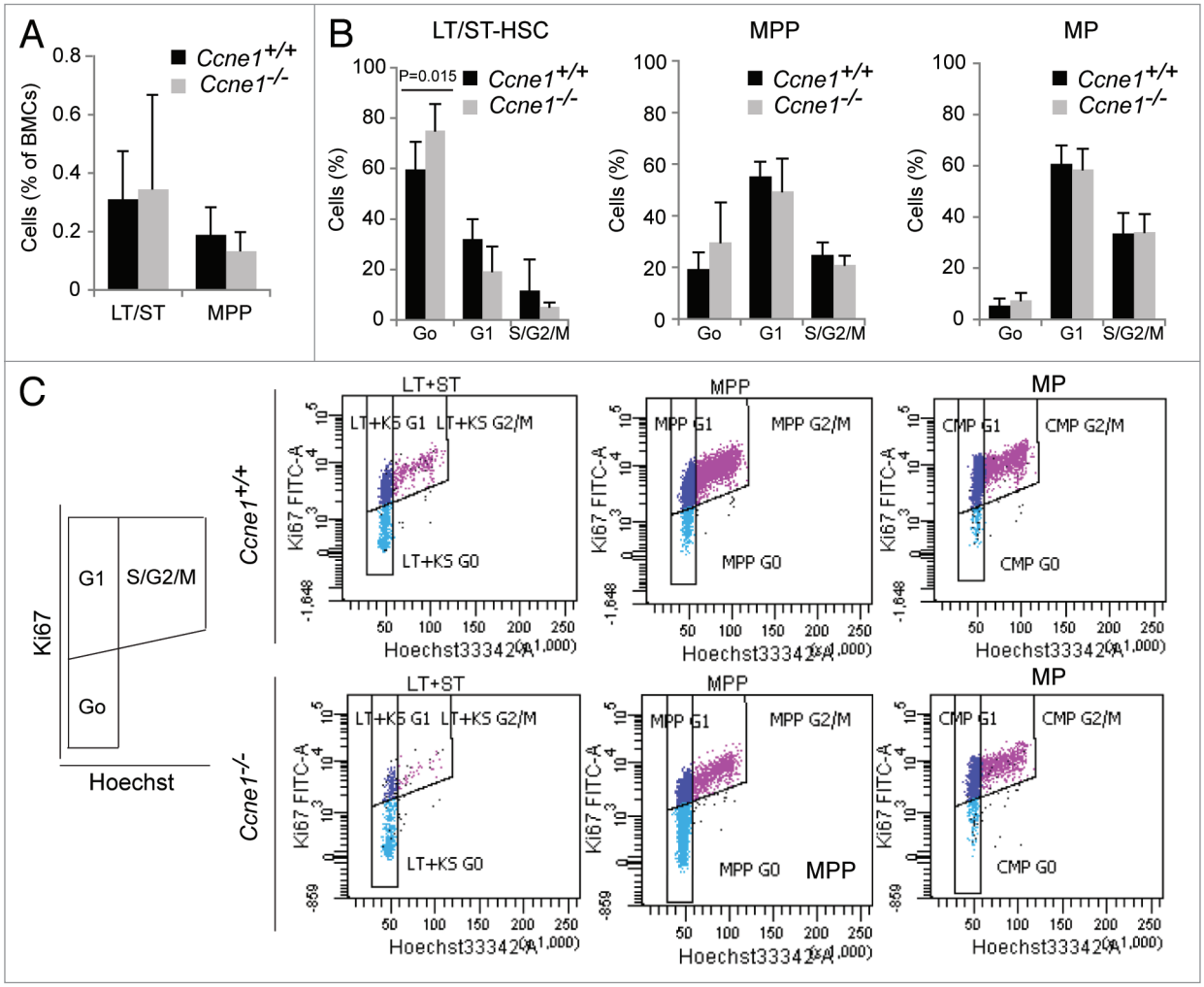

Figure 4. Cyclin E1 regulates quiescence of aged HSCs. (**A**) Prospective identification of HSCs and progenitors in cyclin E1 mice of C57/Bl6 background by FACS analysis. Bone marrow mononucleated cells (BMCs) were isolated from *Ccne1*$^{+/+}$ (n = 10) or *Ccne1*$^{-/-}$ (n = 6) mice aged for 23 mo. Bar graphs report the average value, error bars are the standard deviation. HSCs, LSK (Lin^{-}, Sca1^{+}, Kit^{+}); MPP, multipotential precursors, (LSK, Flk2^{+}); LT/ST, short-term and long-term HSCs (LSK, Flk2^{-}). (**B**) Cell cycle analysis of HSCs and myeloid progenitors (MP), cell cycle distribution was determined by FACS based on the Hoechst/Ki67 staining of the different cell populations. (**C**) FACS profiles of the analysis shown in (**B**)

In previous studies, neither *Ccne1*$^{-/-}$ nor *Ccne2*$^{-/-}$ mice showed detectable phenotypes.[23,24] The lack of redundancy that we report here for cyclin E1 in HSCs may in principle be due to: (1) a peculiar function of cyclin E1 not shared by cyclin E2, or (2) differential expression of the *Ccne1* and *Ccne2* genes in HSCs, such that cyclin E2 is not available to compensate for the absence of cyclin E1. In favor of the latter hypothesis, the *Ccne1* but not the *Ccne2* mRNA is upregulated in HSCs following cyclophosphamide and G-CSF administration,[40] which similarly to 5-FU activates quiescent HSCs and induces cell cycle entry.[41]

At a mechanistic level, we speculate that the defective cell cycle entry of *Ccne1*$^{-/-}$ HSCs may be related to the requirement of cyclins E1 and E2 for the loading of MCM complexes onto replication origins during cell cycle entry but not in the continuous cell cycle, as demonstrated in MEFs,[42] a role for which cyclin E1 alone may be rate-limiting in HSCs.

Although endowed with self-renewal capacity and generally considered long-lasting cells, HSCs are not immortal and are subjected to aging and exhaustion following serial transplantation.[43-45] HSCs will self-renew at each transplant[46] to recreate the HSC pool and at the same time will differentiate to support systemic hematopoiesis.[47] These reiterated cycles of self-renewal and hematopoiesis have an impact on the lifespan of HSCs, and upon serial transplantation, HSCs will reach a point in time at which

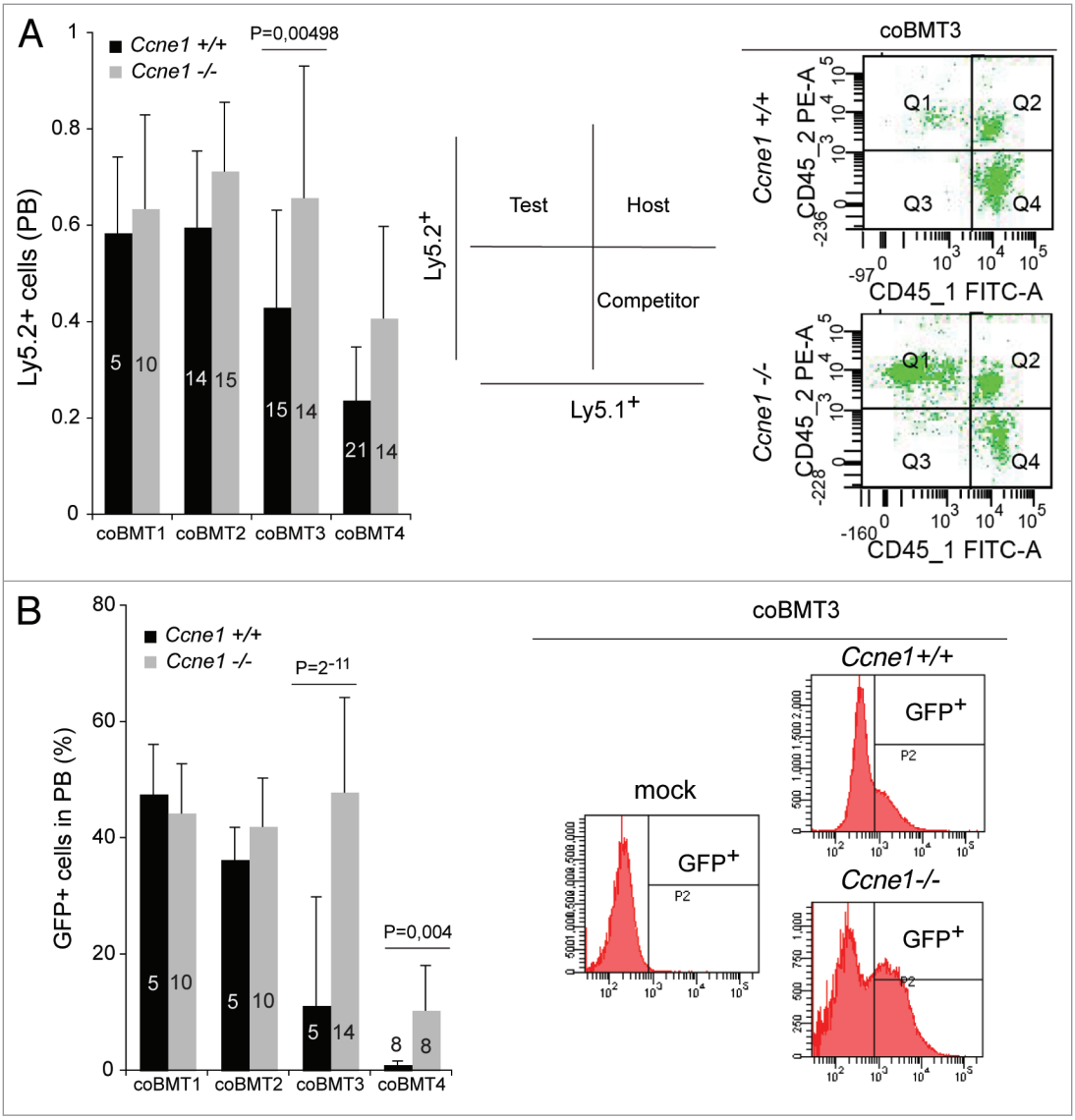

Figure 5. Serial bone marrow transplants of *Ccne1*$^{+/+}$ or *Ccne1*$^{-/-}$ BMCs (**A**) Ly5.2 BMCs from *Ccne1*$^{+/+}$ or *Ccne1*$^{-/-}$ mice were transplanted in a 1:1 ratio with competitor cells derived from Ly5.1 C57/Bl6 mice into Ly5.2/Ly5.1 recipient mice. After long-term reconstitution (5 months), peripheral blood chimerism was assessed by FACS. BMCs derived from primary recipients were then transplanted into secondary recipients. The process was repeated until the 4th bone marrow transplant. Data were plotted as averages of the Ly5.2^{+} peripheral blood nucleated cells normalized to the total cells transplanted (Ly5.1^{+} + Ly5.2^{+} cells). Numbers within graph bars indicate the number of recipients used in each transplant. (**B**) GFP^{+} BMCs from *Ccne1*$^{+/+}$ or *Ccne1*$^{-/-}$ mice were transplanted in a 1:1 ratio with competitor BMCs from C57/Bl6 mice into C57/129 F1 hybrids. The experiment was performed as in (**A**). After long-term reconstitution (5 mo) GFP^{+} cells were determined in peripheral blood by FACS analysis and reported as average percentage ± standard deviation. Numbers within graph bars indicate the number of recipients used in each transplant.

they will be unable to support hematopoiesis. In this context, a defective exit from quiescence of HSCs, while still permissive for efficient hematopoiesis, would be expected to limit the self-renewing proliferation, thereby delaying their exhaustion upon serial transplantation. Thus, paradoxically, a moderate inefficiency in cell cycle entry will exert a protective effect and will endow cells with a competitive advantage, as we indeed observed with *Ccne1*$^{-/-}$ relative to wild-type HSCs. Alternatively, a reduction of cyclin E levels may limit the hyper-mitogenic drive that follows HSCs transplantation, thus preventing replicative stress responses and accelerated aging due to cellular senescence.[48,49] It is noteworthy that the mTOR pathway plays a relevant role in controlling such activities, since mTOR inhibition prevents replicative senescence[50] and reverts some of the phenotypes associated with HSCs aging.[51] In a mirror image to our findings on cyclin E1, a similar mechanism has been proposed to explain the protective roles of the CDK inhibitors p16 and p21 in limiting HSC depletion during serial transplantation.[5,11]

Logically, while protective upon serial bone marrow transfer, the reduced cell cycle entry of *Ccne1*$^{-/-}$ HSCs constitutes a disadvantage for hematopoiesis following acute myeloablation. By analogy, such a dual effect may apply to chemical inhibition of CDK activities in cancer cells, where inhibition of cell cycle entry may turn out to be protective to cancer stem cells.[19]

Materials and Methods

Mice

Ccne1- and *Ccne2*-knockout (KO) mice of C57/129 mixed background[24] were backcrossed into a C57BL/6J background for nine generations. To perform some of the bone marrow transplant experiments described, *Ccne1*-null mice of C57/129 mixed background, were crossed with GFP transgenic mice (C57BL/6TgN[ACTbEGFP]). Unless otherwise stated, all experiments were performed on 8–12-week-old mice of C57/129 background. Mouse colonies were maintained in a certified animal facility in accordance with national guidelines.

FACS analysis

The following antibodies were used: anti-mouse CD117 (clone 2B8; PE, APC, or PE-Cy5.5 conjugated; eBioscience), anti-mouse Ly-6A/E (clone D7; FITC, PE, PE-Cy5.5, or Biotin conjugated; eBioscience), anti-mouse CD34 (clone RAM34; Biotin, FITC, or APC conjugated; eBioscience), anti-mouse CD135 (clone A2F10, PE conjugated; eBioscience), anti-mouse CD127 (Interleukin-7 Receptor, A7R34, PE conjugated; eBioscience), anti-mouse CD48 (clone HM48-1, PE or Biotin conjugated, eBioscience), anti-mouse CD150 (clone TC15–12F12.2, PerCP/Cy5.5 conjugated, Biolegend) anti-mouse CD16/32 (blocks Fc binding, clone 93, PE conjugated; eBioscience), anti-mouse CD45.1 (clone A20, FITC conjugated; eBioscience), anti-mouse CD45.2 (clone104, PE conjugated; eBioscience). For lineage-specific staining the following lineage specific antibodies were used: anti-mouse Cd11b, anti-mouse GR1, anti-mouse Ter-119, anti-mouse CD3ε, anti-mouse CD4, anti-mouse CD8 and anti-mouse B220, (PE-CY7 conjugated, eBioscience). Cell cycle analysis was performed by FACS as previously described.[29]

CFU, CAFC, and LTC-IC assay

Stromal layers were prepared from the wild-type bone marrow cells, cultured in IMDM supplemented with 12.5% horse serum, 12.5% FBS, penicillin, streptomycin, 1 μM hydrocortisone, and 50 μM 2-mercaptoethanol at 37 °C. After 3 wk, when stromal layers are typically confluent, cells were irradiated (15 Gy) and sub-cultured in 6-well plates. Feeder layers were seeded with bone marrow cells (2.5×10^5 per well) and cultured long term. For LTC-IC (long-term culture initiating cell) assay,[52] bone marrow mononucleated cells (BMCs) cultured for 5 wk on stromal layers were seeded in methylcellulose (Methocult SF M3226, Stem Cell Technology) supplemented with 15% FCS, 2 ng ml^{-1} IL3, 2 ng ml^{-1} IL6, 50 ng ml^{-1} SCF, 60 ng ml^{-1} G-CSF, and 20 ng ml^{-1} GM-CSF (Peprotech). Colonies were scored after 10 d. The CAFC (cobblestone area forming cell) assay was performed after 5 wk of culture on feeder layers as previously described.[25] For CFU (colony-forming unit) assay, 20 000 bone marrow nucleated cells were seeded in methycellulose media supplemented with different sets of differentiating cytokines. Myeloid differentiation was assessed as described above. For lymphoid differentiation, methylcellulose cultures were supplemented with 20 ng/mL Flt3 and 10 ng/mL IL-7 (Peprotech), while erythroid differentiation medium was assessed seeding cells in MethoCult® M3334 supplemented with 2 ng ml^{-1} IL3 and 50 ng ml^{-1} SCF. Colonies were typically scored after 10–12 d of culture.

Bone marrow transplants experiments

One million test cells (Ly5.2$^+$) (3 mice for each genotype) and 1×10^6 cells derived from C57BL/6-CD45.1 congenic mice (Ly5.1$^+$) were pooled and injected into the tail vein of lethally irradiated C57BL/6-CD45.1/CD45.2 F1-hybrids. Recipients' PB was analyzed for donor contribution by flow cytometric analysis. After long-term reconstitution (5–6 mo), 2×10^6 bone marrow cells derived from primary recipients were injected into lethally irradiated secondary recipients. This process was reiterated until reaching the fourth round of transplants. To minimize clonal dominance, each serial transplant was repeated twice using as donors 2 different pools of BMCs derived from 3 independent donors.

A similar protocol was used for cyclin E1 mice of mixed background where 10^6 *Ccne1*$^{+/+}$ or *Ccne1*$^{-/-}$ GFP-positive cells were transplanted in competition with 10^6 bone marrow cells derived from C57BL/6 mice. Chimerism was assessed by FACS analysis of GFP-positive cells in peripheral blood. For competitive repopulation unit assay (CRU assay) recipients, C57BL/6 Ly5.1 mice lethally irradiated (9.5 Gy) the day before transplantation were injected with 15×10^3, 5×10^4, or 2×10^5 Ly5.2$^+$ test bone marrow cells, together with 2×10^5 congenic BMCs (Ly5.1$^+$). Peripheral blood chimerism was determined 4–6 mo post-transplant by FACS analysis to assess relative percentages of Ly5.2- and Ly5.1-positive cells in the peripheral blood. A recipient with less than 2% of Ly5.2$^+$ leukocytes was defined as negative. CRU numbers were determined by Poisson statistical analysis (L-Calc software, StemCell Technologies).

5-FU treatments

5-FU (TEVA, 5 g/mL) was administered intraperitoneally at a dose of either 200 mg/Kg, for single injections, or 150 mg/Kg, for weekly treatments.

Statistical analysis

In all figures, data are presented as the mean ± standard deviation. Statistical significance was calculated using a 2-tailed Student *t* test. Results from survival experiments were analyzed with a log-rank non-parametric test and expressed as Kaplan–Meier survival curves.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/26584

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