Restricted cell cycle is essential for clonal evolution and therapeutic resistance of pre-leukemic stem cells

Cedric S. Tremblay, Jesslyn Saw, Sung Kai Chiu, Nicholas C. Wong, Kirill Tsyganov, Sarah Ghotb, Alison N. Graham, Feng Yan, Andrew A. Guirguis, Stefan E. Sonderegger, Nicole Lee, Paul Kalitsis, John Reynolds, Stephen B. Ting, David R. Powell, Stephen M. Jane, & David J. Curtis

Pre-leukemic stem cells (pre-LSCs) give rise to leukemic stem cells through acquisition of additional gene mutations and are an important source of relapse following chemotherapy. We postulated that cell-cycle kinetics of pre-LSCs may be an important determinant of clonal evolution and therapeutic resistance. Using a doxycycline-inducible H2B-GFP transgene in a mouse model of T-cell acute lymphoblastic leukemia to study cell cycle in vivo, we show that self-renewal, clonal evolution and therapeutic resistance are limited to a rare population of pre-LSCs with restricted cell cycle. We show that proliferative pre-LSCs are unable to return to a cell cycle-restricted state. Cell cycle-restricted pre-LSCs have activation of p53 and its downstream cell-cycle inhibitor p21. Furthermore, absence of p21 leads to proliferation of pre-LSCs, with clonal extinction through loss of asymmetric cell division and terminal differentiation. Thus, inducing proliferation of pre-LSCs represents a promising strategy to increase cure rates for acute leukemia.
The leukemia stem cell (LSC) concept posits the presence of a cell population with stem cell-like properties enabling their ability to generate the full heterogeneity of the tumor and fuel tumor growth during disease progression. These LSCs are intrinsically resistant to therapies via potential mechanisms that include quiescence, low reactive oxygen stress, enhanced DNA repair and expression of adenosine triphosphate-binding cassette transporters. Over recent years, genome-wide studies of matched primary and relapsed leukemia samples strongly support this model wherein the clone responsible for relapse arises from either a pre-existing LSC or an antecedent LSC clone referred to as a pre-leukemic stem cell (pre-LSC)\(^1\)–\(^3\). These pre-LSCs contain the founding genetic mutation but not the full complement of mutations found at diagnosis. Although pre-LSCs retain the ability to differentiate into functional mature blood cells, they also have long-lived self-renewal capacity\(^4\) and their presence in patient remission samples following intensive chemotherapy portends a high risk of relapse\(^5\). In addition to acute leukemia, cells akin to pre-LSCs underpin myelodysplastic syndromes and perhaps even clinical hematopoesis of the elderly, which can evolve into acute leukemia over many months to years\(^6,7\).

Quiescence may be an important mechanism of therapeutic resistance for LSCs, particularly for therapies that rely upon cell proliferation for their activity. Clinically, this concept is exemplified in chronic myeloid leukemia where, even in the era of tyrosine kinase inhibitor therapy, the absence of cure is thought to reside with the inability to eradicate the quiescent clones of LSCs\(^8\)–\(^10\). Perhaps the most convincing in vivo evidence comes from Ebinger et al.\(^11\), who identified a rare subpopulation of dormant and treatment-resistant cells in patient-derived xenografts. They also showed that these chemoresistant cells share the same gene expression profile with primary leukemia cells isolated from patients at minimal residual disease. Moreover, Saito et al.\(^12\) experimentally showed that quiescent leukemic cells residing in the bone marrow niche were protected from chemotherapy. They subsequently showed that overcoming quiescence with cytokine stimulation could sensitize these leukemogenic cells to chemotherapy. However, these and other experimental in vivo studies of LSC quiescence have almost exclusively used label-retaining cell fixation assays with DNA analogs such as bromodeoxyuridine which preclude subsequent functional studies\(^13\).

This major hurdle for the study of quiescence in hematopoietic stem and progenitor cells has recently been overcome by the generation of transgenic mice expressing a doxycycline-regulated fluorescent protein (GFP) retention showed the cell-cycle kinetics of pre-LSCs. Heterozygous TetOP-H2B-GFP\(^{\text{TetO}}\);\(^{\text{Lmo2Tg}}\) mice were treated with doxycycline for 6 weeks to induce expression of H2B-GFP in dividing cells. We then examined GFP expression in thymocytes following withdrawal of doxycycline for 1, 2, 4 and 8 weeks (Fig. 1a), focusing on the DN3 T-cell fraction, which contains all pre-LSC activity\(^19\)\(^,\)\(^22\). At the end of the labeling period, almost all DN3 cells in both control and Lmo2\(^{Tg}\) mice expressed the H2B-GFP division marker, which comprised high and intermediate populations (Fig. 1b). Interestingly, a small proportion of DN3 cells in H2B-GFP;\(^{\text{Lmo2Tg}}\) mice remained GFP negative despite a 6-week labeling period, which suggests the presence of cells that had not divided. Consistent with this highly proliferative stage of T-cell development, withdrawal of doxycycline led to a rapid loss of GFP in DN3 thymocytes from control H2B-GFP mice such that there were no GFP\(^{\text{hi}}\) cells beyond 2 weeks. However, in H2B-GFP;\(^{\text{Lmo2Tg}}\) mice, a small fraction of DN3 cells retained GFP expression for up to 8 weeks (Fig. 1b). In absolute numbers, this rare GFP\(^{\text{hi}}\) population of cells at 8 weeks equated to 3000 cells per whole thymus (Supplementary Fig. 1a). Consistent with the exclusive presence of pre-LSCs within the DN3 thymocyte population, no difference was observed in the proportion of GFP-retaining cells within other T-cell subsets in H2B-GFP;\(^{\text{Lmo2Tg}}\) mice compared with control mice (Supplementary Fig. 1b).

Assuming that the mean cell fluorescence halved with each cell division, we estimated a mean cycling time of 18.2 ± 2.8 h for control DN3 cells and 50.1 ± 13.8 h for Lmo2\(^{Tg}\) DN3. Given there was no obvious long-term plateau of GFP loss (Fig. 1c), we designated GFP\(^{\text{hi}}\) cells as cell-cycle restricted rather than quiescent or dormant. Staining with 4,6-diamidino-2-phenylindole (DAPI) and Ki67, an independent assay for analyzing snapshots of cell cycle, confirmed that most GFP\(^{\text{hi}}\) cells present beyond 2-week chase were in the G0 phase (Fig. 1d), whereas less than 40% of GFP\(^{\text{lo}}\) cells were in the G0 phase when assayed 2 weeks after withdrawal of doxycycline (Fig. 1e). Therefore, all subsequent analyses were performed using DN3 thymocytes from mice 2 weeks after withdrawal of doxycycline and cells that did not divide during the 6-week labeling period were excluded.

Cell-cycle restriction maintains leukemic pre-LSCs. Serial transplantation is the gold-standard assay that defines LSCs\(^23\). Fluorescence-activated cell sorting (FACS)-isolated GFP\(^{\text{hi}}\) and GFP\(^{\text{lo}}\) DN3 cells from 2-month-old H2B-GFP;\(^{\text{Lmo2Tg}}\) mice were injected into sublethally irradiated CD45.1\(^{+}\) recipient mice to examine the importance of cell-cycle kinetics on repopulating activity (Fig. 2a). In primary recipients, GFP\(^{\text{hi}}\) DN3 cells were able to expand 100-fold compared with 10–20-fold for GFP\(^{\text{lo}}\) DN3 cells (Fig. 2b). This decreased capacity to generate DN3 cells...
may be explained in part by the enhanced differentiation of GFPlo DN3 cells into CD4+CD8+ double-positive (DP) thymocytes (Fig. 2c), which lack self-renewal activity. We performed serial transplant to assess long-term self-renewal capacity (Fig. 2a), the quintessential property of all stem cells. A period of 4 weeks between transplants was chosen to allow competition with normal HSCs, which take up to 3 weeks to repopulate the thymus. GFPhi DN3 cells retained an ability to expand for at least four rounds of transplantation (Fig. 2d). In contrast, GFPlo DN3 cells progressively lost the ability to regenerate DN3 cells such that by the fourth passage, there was exhaustion of their expansion potential. Thus, restricted cell cycle was a critical property of self-renewing pre-LSCs.

Given that self-renewal enables pre-LSCs to accumulate additional genetic events necessary for progression to leukemia, we postulated that only the GFPhi DN3 cells would be capable of generating T-ALL. Consistent with this idea, there was increased monoclonality in GFP hi DN3 cells as measured by Tcrβ rearrangement (Supplementary Fig. 2a). Furthermore, a proportion of secondary, tertiary and quaternary recipients of
GFPhi DN3 cells developed T-ALL, whereas no cases of leukemia were observed in mice transplanted with proliferative GFPlo DN3 cells over the 22-week serial transplant period (Fig. 2d and Supplementary Fig. 2b). Given that leukemias only arise in recipients injected with GFPhi cells, our results demonstrate that restricted cell cycle is important for clonal evolution and leukemogenic potential of pre-LSCs.

HSCs can re-enter a dormant state following hematopoietic stress, including chemotherapy\textsuperscript{25,26}. To determine if proliferative pre-LSCs were able to return to a cell cycle-restricted state, we administered doxycycline for 6 weeks to mice transplanted with GFPlo cells. Unlike normal HSCs, proliferative pre-LSCs were unable to generate GFPhi cells (Fig. 2e and S2c). In contrast, GFPhi cells generated a cell cycle-restricted progeny, confirming that they can be maintained even in the setting of proliferative stress. To determine whether cell cycle-restricted pre-LSCs are maintained in disease progression, we determined the numbers of GFPhi DN3 cells in 6-month-old \textit{Lmo2} Tg mice. In these older mice, the GFPhi DN3 cell population had expanded 3-fold, and 10-fold in mice with overt T-ALL (Supplementary Fig. 2d). Thus, cell cycle-restricted pre-LSCs expand with disease progression.

A recent study by Ebinger et al\textsuperscript{11} linked cell-cycle restriction with treatment resistance in human ALL. To determine whether cell-cycle restriction protects pre-LSCs against chemotherapeutic...
agents typically used for human T-ALL, we looked for enrichment of GFP^{phi} DN3 cells following treatment of H2B-GFP;Lmo2^{Tg} mice with a combination of vincristine, dexamethasone and L-asparaginase (VXL)\textsuperscript{27}. Consistent with the cell cycle-dependent effect of chemotherapy, the proportion of cells surviving 24 h after combination therapy was fourfold higher in the GFP^{phi} fraction compared with the GFP^{plo} fraction: 9% of GFP^{phi} cells compared with 2% of GFP^{plo} cells (Fig. 2f). A similar increased resistance of GFP^{phi} cells was observed in response to sub-lethal irradiation (Supplementary Fig. 2e). Given that the H2B-GFP labeling model reflects the history of the cell cycle, the enrichment for GFP^{phi} cells observed cannot be due to therapy-induced senescence and must reflect cells that have not actively divided in the preceding 2 weeks of chase. Thus, cell-cycle restriction of pre-LSCs enhances resistance to therapeutic agents used for treatment of human T-ALL.

Expression profile of cell cycle-restricted pre-LSCs. To investigate the molecular signature of cell cycle-restricted pre-LSCs, we performed gene expression profiling of GFP^{phi} and GFP^{plo} DN3 cells from three independent cohorts of H2B-GFP;Lmo2^{Tg} mice. Overall, there were 853 genes differentially expressed more than twofold using a false discovery rate (FDR) of 0.01: 255 genes increased and 598 genes reduced in the GFP^{phi} cells (Supplementary Fig. 3a and Supplementary Data 1). Gene ontology analysis of upregulated genes showed enrichment for p53 activation (Cdkn1a, Sess2, Ddit4, Pmaip1, Zmat3 and Pldhla3), antigen processing and presentation (H2-DMb2, CD74, H2-Aa, H2-Eb1, H2-Q1, H2-T3 and H2-Q6) and T-cell differentiation (Supplementary Data 1). Gene set enrichment analysis (GSEA) confirmed downregulation of cell-cycle regulators, as well as the activation of p53 apoptosis and the antigen processing pathway (Fig. 3a and Supplementary Data 1) and also identified increased expression of multiple proteasomal activator subunits (Supplementary Fig. 3b and Supplementary Data 1). GSEA also identified reduced expression of multiple DNA polymerase and mini-chromosome maintenance (MCM) genes required for DNA replication (Fig. 3b and Supplementary Data 1). Consistent with the reported error-prone DNA repair in quiescent HSCs\textsuperscript{28}, expression of genes required for high-fidelity homologous recombination and mismatch repair were reduced in GFP^{phi} DN3 cells (Fig. 3c and Supplementary Data 1). To determine if the gene signature of cell cycle-restricted pre-LSCs was similar to dormant HSCs, we utilized expression data generated from H2B-GFP transgenic mice\textsuperscript{29} (Supplementary Data 1). GSEA showed that genes downregulated in dormant HSCs were also significantly reduced in cell cycle-restricted pre-LSCs (Fig. 3d and Supplementary Data 1). In contrast, the genes increased in dormant HSCs were not similar to those increased in cell cycle-restricted pre-LSCs (Supplementary Fig. 3c).

Cell cycle-restricted pre-LSCs acquire Notch1 mutations. One of the most striking changes identified in the GSEA was reduced expression in GFP^{phi} DN3 cells of genes clustering on different regions of human chromosomes (Supplementary Fig. 4a) that assembled on chromosomes 2 and 15 in mice (Fig. 4a) as compared to GFP^{plo} thymocytes. Given that the analysis was performed using the average expression in all samples tested, this striking observation suggested that whole chromosomes were either lost in GFP^{phi} or gained in GFP^{plo} thymocytes. Targeted probe analysis for chromosomes 15 and 2 revealed a high frequency of trisomy 15 and 2 in GFP^{plo} DN3 thymocytes (Fig. 4b). Genomic PCR for the IL7r and Myc loci on chromosome 15 (Supplementary Methods) confirmed increased copy number in GFP^{plo} cells (Supplementary Fig. 4b). Given the RNA-sequencing (RNA-seq) analysis was performed on pooled samples, we also performed whole-exome sequencing (WES) on GFP^{phi} and GFP^{plo} DN3 thymocytes isolated from 5 individual doxycycline-pulsed 2-month-old H2B-GFP;Lmo2^{Tg} mice. In accordance with gene expression data, WES analysis revealed gains of chromosomes 2 and 15 as well as other numerical chromosomal alterations in the GFP^{plo} population from all mice analyzed (Fig. 4c). Thus, cycling is associated with aneuploidy in pre-LSCs.

It is postulated that the quiescent state of long-term repopulating HSCs increases the risk of acquired mutations due to the use of error-prone non-homologous end joining-mediated DNA repair\textsuperscript{28}. In contrast, cycling HSCs or progenitors can utilize high-fidelity homologous recombination for DNA repair. To define the relationship between cell cycle and mutations in...
Fig. 4 Genomic alterations associated with cell cycle in pre-leukemic stem cells (pre-LSCs). a Box plot of the relative expression profiling of genes in GFPlo and GFPhi DN3 thymocytes from H2B-GFP;Lmo2Tg mice 2 weeks after doxycycline pulse, in accordance with their chromosomal location. Median ± whiskers with maximum 1.5 interquartile range (IQR) and outliers are indicated for each chromosome. Log fold change (LogFC) as compared to median expression among all the samples analyzed. b Targeted probe analysis for chromosomes 15 and 2 in GFPlo DN3 thymocytes from H2B-GFP;Lmo2Tg mice 2 weeks after doxycycline pulse. Frequency of diploid (top panel) and aneuploid (bottom panel) cells is indicated. c Mouse chromosomal ideogram showing the chromosomal gain and losses identified in GFPlo subpopulations of DN3 thymocytes by whole-exome sequencing (WES) on 5 individual doxycycline-pulsed 2-month-old H2B-GFP;Lmo2Tg mice. Top red bars represent chromosomal gains and losses are identified by bottom blue bars.

d Diagram showing the position of mutations found in the Notch1 gene sequence in purified GFPlo DN3 thymocytes from 2-month old H2B-GFP;Lmo2Tg mice (n = 5). The amino-acid numbers are shown, with their position in the corresponding exons from the Notch1 locus. GFP green fluorescent protein, L Lin/NOTCH repeats, T transmembrane domain, RAM RAM domain, ANK ankyrin repeat domain, TAD transactivation domain, PEST PEST domain; and the arrow indicated the site of cleavage releasing the Notch1 intracellular domain following activation

pre-LSCs, we used the RNA-seq data to identify variants differentially expressed in GFPlo and GFPhi DN3 cells. Overall, we detected 57 genes with variants predicted to be deleterious due to frameshift, splice or premature stop (Supplementary Data 2). While the majority (n = 43) were found in both GFPlo and GFPhi cell populations, frameshift mutations upstream of the PEST coding region in Notch1 were present only in the cell cycle-restricted GFPlo cells (Supplementary Data 2). Targeted sequencing of the Notch1 locus in GFPlo DN3 thymocytes isolated from 5 individual mice confirmed the presence of activating mutations of Notch1 in cell cycle-restricted pre-LSCs (Fig. 4d). In addition, a stop in Tcrg-V3 indicating Tcrg gene rearrangement was found exclusively in GFPlo cells. The expression of truncated transcripts of Tcrg-V3 represents an early marker of clonal selection in cell cycle-restricted pre-LSCs. Thus, distinct mutations occur in pre-LSCs according to their cell-cycle dynamics with recurrent activating mutations of Notch1 limited to cell cycle-restricted pre-LSCs.

p21 is required for stem cell-like properties of pre-LSCs. We examined expression of the known inhibitors of cell cycle in HSCs to define the mechanism of cell-cycle restriction in pre-LSCs. Consistent with activation of p53, we found a threefold increase in the expression of Cdkn1a (p21) in the DN3 GFPlo subpopulation. In contrast, expressions of Cdkn1b (p27), Cdkn1c (p57) and the Ink4a family members were not altered in GFPlo cell (Fig. 5a and Supplementary Fig. 5a).

To assess the importance of p21 in cell cycle-restricted pre-LSCs, we generated H2B-GFP;Lmo2Tg mice on a p21-deficient (p21−/−) background. Significantly, loss of p21 led to almost complete absence of GFPlo cells by 2 weeks post doxycycline pulse (Fig. 5b and Supplementary Fig. 5b). This loss of cell-cycle restriction was observed in total DN3, where the proportion of cells in G0 was restored to wild-type levels (Supplementary Fig. 5c). Genomic quantification of the Il7r and Myc loci revealed that p21 deficiency increased copy number of chromosome 15 in p21-deficient Lmo2Tg cells lacking p21 (Supplementary Fig. 5d), suggesting that p21 was important for cell-cycle restriction and genomic stability in pre-LSCs. Given the absence of GFPlo cells, we performed serial transplant experiments of total DN3 thymocytes from 2-month-old mice to determine the functional consequences of loss of cell-cycle restriction (Fig. 5b). Interestingly, Lmo2Tg DN3 cells lacking p21 were able to generate sevenfold more DN3 progeny than Lmo2Tg DN3 cells expressing p21 (Supplementary Fig. 5e). In addition, absence of p21 promoted differentiation to DP cells (Fig. 5d). Despite the enhanced repopulation in primary transplants, serial transplant
revealed a progressive loss of DN3 repopulating activity such that there was complete loss by the fourth transplant (Fig. 5e). In contrast, the expansion of Lmo2 Tg DN3 remained relatively constant over serial transplants.

To determine if this pre-LSC exhaustion was only observable in the setting of proliferative stress related to transplantation, analogous to previous reports with p21-deficient HSCs30, we compared the numbers of DN3 cells in 6- and 12-month-old Lmo2 Tg and Lmo2 Tg; p21−/− mice. As previously reported31, there was a progressive increase in the numbers and proportion of DN3 cells in aged Lmo2 Tg mice (Fig. 5f and Supplementary Fig. 5f). In contrast, absence of p21 prevented DN3 expansion with aging. Accordingly, there was marked loss of repopulating activity in 6-month-old Lmo2 Tg; p21−/− thymus compared with age-matched Lmo2 Tg mice (Fig. 5g). Consistent with loss of pre-LSCs, there was reduced monoclonality as assessed by Tcrβ rearrangement (Supplementary Fig. 5g), and most importantly marked reduction of T-ALL penetrance in mice lacking p21 (Fig. 5h).

### Legend for Figures

- **a**: Relative expression of indicated genes.
- **b**: Flow cytometry analysis of GFP expression.
- **c**: Serial transplantation scheme.
- **d**: Proportion of CD45.2+ cells.
- **e**: DN3 fold expansion over time.
- **f**: DN3 absolute numbers over time.
- **g**: 6 Months comparison of DN3 fold expansion.
- **h**: Leukemia-free survival.
- **i**: VXL induction experiment.
- **j**: Survival and DN3 fold expansion in VXL experiment.
showed that Notch1 mutations are acquired during disease progression. To assess the importance of cell cycle in the acquisition of Notch1 mutations, we used the RNA-seq data to identify variants differentially expressed in p21-deficient Lmo2−/− DN3 cells, and found that Notch1 mutations were only present in Lmo2+/− thymocytes (Supplementary Data 2). Targeted sequencing of the Notch1 locus in DN3 thymocytes isolated from 6-month-old mice revealed that the presence of Notch1 mutations was decreased by twofold in pre-LSCs lacking p21 (Supplementary Fig. 5h). In aggregate, these studies show that p21 was required for clonal evolution and leukemia progression of pre-LSCs.

To assess the role of p21 in therapeutic resistance, we measured repopulating activity in the thymus 24 h after multi-agent chemotherapy. At this time point, the proportion of surviving DN3 thymocytes was fourfold lower in Lmo2+/− mice lacking p21 (Fig. 5i). Transplant of these chemoresistant thymocytes showed that repopulating activity was maintained in Lmo2+/− thymocytes but markedly reduced in p21-deficient Lmo2−/− thymocytes (Fig. 5j). The presence of p21 was also important for the resistance of Lmo2+/− DN3 thymocytes to γ-irradiation (Supplementary Fig. 5i). Thus, p21 mediates resistance of pre-LSCs to chemotherapy and radiation.

Given the lack of cell cycle-restricted DN3 cells in Lmo2+/−p21−/− mice, we isolated total DN3 cells for gene expression profiling to gain insight into the role of p21 in pre-LSCs. We restricted studies to 6-week-old wild-type, p21−/−, Lmo2+/− and Lmo2+/−p21−/− mice, an age prior to loss of repopulating activity in p21-deficient Lmo2−/− mice (Fig. 5g). Principal component analysis confirmed clustering according to genotypetype (Fig. 5a). Comparison of wild-type DN3 cells with p21−/− DN3 thymocytes revealed minimal changes with only 3 genes differentially expressed more than twofold (Supplementary Data 3). To determine how p21 abrogates Lmo2-induced leukemogenesis, we compared Lmo2+/− DN3 cells with Lmo2+/−p21−/− DN3 thymocytes. Importantly, there was no difference in the expression of Lmo2 or its downstream targets responsible for self-renewal,32,33 such as Ly11, Hhex and c-Kit (Fig. 6b). Overall, there were 463 differentially expressed genes: 153 increased and 310 decreased more than twofold in Lmo2+/−p21−/− DN3 cells (Fig. 6c and Supplementary Data 3). Gene ontology pathway analysis showed that the reduced genes were enriched for general metabolic pathways of transcription and translation as well as signaling (nuclear factor-κB, mitogen-activated protein kinase, G1/S transition and apoptosis (Supplementary Data 3). These changes were confirmed using GSEA, which revealed a striking reduction in genes involved in DNA replication, splicing and the proteasome (Fig. 6d).

Importantly, these changes were not seen with p21-deficient DN3 cells compared with wild-type DN3 thymocytes (Supplementary Fig. 6a). Thus, p21-mediated cell-cycle restriction was required for widespread metabolic processes in the context of oncogene-transformed cells.

To understand the cellular fate of Lmo2+/− DN3 cells in the absence of p21 (apoptosis or differentiation), we co-cultured sorted DN3 thymocytes on OP9-DL1 stroma cells, which support in vitro division and differentiation of T-cell progenitors.34 Using this approach, we confirmed that absence of p21 promoted the differentiation of Lmo2+/− DN3 thymocytes into DP cells (Fig. 6e). Pre-LSCs develop just prior to the β-selection checkpoint during which T-cell fate is tightly regulated by asymmetric cell division (ACD),35 a homoeostatic cell division process also crucial for self-renewal of HSCs.36,37 ACD can be observed by the polarized segregation of the “differentiation fate determinant” Numb in dividing cells, which generate one identical immature/stem and one differentiated daughter cell.35,38,39 Given that p21 has been associated with a switch from asymmetric to symmetric division in co-cultured stem cells,40 we examined the frequency of ACD in sorted DN3 thymocytes using the Numb distribution in dividing cells (Fig. 6f). Consistent with the stem cell-like phenotype of pre-LSCs, the frequency of ACD was significantly increased in Lmo2+/− DN3 cells compared with wild-type and p21−/− DN3 cells (Fig. 6f and Supplementary Fig. 6b). The increased ACD observed in Lmo2+/− expressing DN3 cells was significantly reduced in the absence of p21, restoring the preponderance of symmetric division observed in wild-type thymocytes. Altogether, these results show that p21-deficiency promotes differentiation of pre-LSCs at the expense of ACD, which correlates with the importance of p21 for the maintenance of self-renewing pre-LSCs during leukemia development.

Discussion

Pre-LSCs are an important cell population during leukemic development, and provide a pool of cells that give rise to relapse.3,41 The clinical relevance of pre-LSCs has recently been highlighted by sensitive molecular assays, where patients with detectable pre-LSCs during complete remission have an increased risk of relapse.3,42 However, the properties of pre-LSCs that allow them to escape high-dose chemotherapy remain unknown. In this paper, we show that clonal evolution and therapeutic resistance can be defined by their cell-cycle characteristics. Specifically, we identify the presence of a rare subpopulation of cell cycle-restricted pre-LSCs that have enhanced therapeutic resistance and most importantly represent the population of cells that acquire
oncogenic Notch1 mutations necessary for clonal evolution to T-ALL. By genetic deletion of the cell-cycle inhibitor p21, we show that overcoming cell-cycle restriction abrogates this therapeutic resistance and significantly reduces clonal evolution of pre-LSCs. Thus, we show that cell-cycle restriction is a fundamental property of pre-LSCs that explains their long-term competitive advantage and potential for causing relapse following high-dose chemotherapy.

We have previously shown that Lmo2 induces aberrant self-renewal of immature T-cell progenitors without preventing T-cell differentiation19, and as such display features typical of pre-LSCs13. We now extend these findings to show that long-term self-renewal necessary for clonal evolution is limited to a rare subpopulation of cell cycle-restricted pre-LSCs. The impaired repopulating activity of GFP10 DN3 thymocytes might be explained by increased cycling leading to impaired homing. However, this is highly unlikely as DN3 cells lacking p21 had increased repopulating activity in primary transplants (Fig. 5c) despite increased cycling. Consistent with their stem cell-like properties, these cells are also more resistant to irradiation and combination chemotherapy. We did not directly examine the leukemic potential of the GFP hi cells enriched by chemotherapy.

Thus, strategies that promote cell cycle prior to chemotherapy may be able to eradicate pre-LSCs without detrimental effects on normal HSCs.

The role of p21 in leukemogenesis is controversial with both tumor suppressive and promoting activity. For example, knockdown of p21 in MLL-AF10-induced leukemia accelerated disease46. In contrast, PML/RAR (promyelocytic leukemia/retinoic acid receptor)-transformed HSCs required p21 for long-term recombination activity of GFP lo DN3 thymocytes might be explained by increased cycling leading to impaired homing. However, this is highly unlikely as DN3 cells lacking p21 had increased repopulating activity in primary transplants (Fig. 5c) despite increased cycling. Consistent with their stem cell-like properties, these cells are also more resistant to irradiation and combination chemotherapy. We did not directly examine the leukemic potential of the GFP hi cells enriched by chemotherapy.

Thus, strategies that promote cell cycle prior to chemotherapy may be able to eradicate pre-LSCs without detrimental effects on normal HSCs.

The role of p21 in leukemogenesis is controversial with both tumor suppressive and promoting activity. For example, knockdown of p21 in MLL-AF10-induced leukemia accelerated disease46. In contrast, PML/RAR (promyelocytic leukemia/retinoic acid receptor)-transformed HSCs required p21 for long-term
The observation that Lmo2DN3 cells lacking p21 have enhanced primary repopulating capacity but loss in subsequent transplants (Fig. 5f) provides one possible explanation for this controversy. The properties identified for cell cycle-restricted pre-LSCs may have many parallels with normal HSCs under conditions of stress or aging. First, p21 is important for HSCs in the setting of stress.29 Second, activation of the p53 axis promotes cell cycle arrest and DNA repair in irradiated HSCs in the setting of stress.30 Third, error-prone DNA repair occurs in stress or aging HSCs due to reduced expression of genes required for high-fidelity homologous recombination and components of the MCM helicase. Thus, we propose that cell cycle-restricted pre-LSCs arising from a committed progenitor behave like normal HSCs following DNA damage.

Mutation analysis of pre-LSCs identified an intriguing relationship between cell cycle and types of genomic mutations. Cell cycle of pre-LSCs was associated with a high frequency of aneuploidy. Almost half of all cells undergoing cell division had trisomy 15 and/or 2 (Fig. 4a). Although it is difficult to know which comes first (aneuploidy or cell cycle), the higher rate of aneuploidy in pre-LSCs unable to arrest (Lmo2−/−p21−/−) suggests that cell cycle induces aneuploidy. Furthermore, studies of cell lines with trisomy generated from transgenic mice suggest that aneuploidy from normal HSCs with regard to their ability to return to a cell cycle-restricted state following stress-induced proliferation. We also demonstrate that pre-LSCs fundamentally diverge from normal HSCs with regard to their ability to return to a cell cycle-restricted state following stress-induced proliferation. We propose the H2B-GFP system will be a powerful in vivo model to determine if similar properties apply to other models of pre-LSCs and identify and test strategies to overcome quiescence of relapse-inducing cells.

### Methods

**Mouse experiments.** All experiments were approved and complied with the ethical regulations mandated by the AMREP Animal Ethics Committee. The

| Gene        | Name of the primer | Sequence               |
|-------------|--------------------|------------------------|
| Cdkn1a (p21) | mCdkn1a_Fw         | taccgtctttcccaagct    |
|             | mCdkn1a_Rv         | acctgtcacccacagatcg   |
|             | mCdkn1b_F          | gttagcgagcaagttcaca   |
|             | mCdkn1b_R          | tctgttctgcccctttt    |
|             | mCdkn1c_F          | caggagagataaagacgca   |
|             | mCdkn1c_R          | gcttggcaagagttcgt    |
| mTcrb       | S16 Fw             | ACGGACGATTGTGCTGGTGG   |
|             | S16 Fv             | GCTACGAGGGCTTTAGAGTG   |
|             | Tcr Vb5 Fw         | CCCAGCAAGATTCTCAGTCCACAG |
|             | Tcr Vb8 Fw         | GCATGAGGGTGAGCTGACACTTA |
|             | Tcr Jb2 Fv         | TGAGCTGTCCTCCTACTATGATT |
| Genomic PCR of loci on Chr. 15 | mTcrb | Sequence               |
| mIl7r       | mIl7r Fw           | ACTGTGGTGGTGCTCTTAG    |
|             | mIl7r Rv           | AAATTGGCAGAACCAACCAG        |
| mMyc        | mMyc Fw            | ACACCGAGGGAAAAGCACAAAG |
|             | mMyc Fv            | TCGTCTGCTGAAAGGACAG      |
| Notch1 sequencing | mMyc | Sequence               |
| mNotch1     | Exon26_4853Fw      | CCGCTATGAGCCCAAGTA     |
|             | Exon26_5250Rv      | ACAGGATGTCCATGGGT      |
|             | Exon27_5253Fw      | CTCATGGTCTACCTG       |
|             | Exon27_5401Rv      | CCTTCAGGGCTCTAACT      |
|             | Exon34_6423Fw      | TCCCTGGTCTGGCGC       |
|             | Exon34_6548Fw      | TGACCCAGCATACCTG      |
|             | Exon34_7265Fw      | ATAGCATGATGCGCCACTA   |
|             | Exon34_8157Rv      | ACCCCATGGTCTACTCATCG   |
|             | Exon34_7284Rv      | CCTCACCTGAGCAGAANAA   |
|             | Exon34_7265Rv      | TTAGGCCCTAACCTATGAT   |
|             | Exon34_7265Rv      | CACCGAGGGAGGGAGTGTTA  |
|             | Exon34_7675Rv      | TGA ATC CTG TT CAT ATT TTA CAG ACA CAC AGG G   |
current study was performed using the previously described TetO-P-H2B-GFPRI, kindly given by H. Hock from the Harvard Stem Cell Institute14,15, p21-deficient (p21/−) C57BL/6J or CD2D-Lmo2/− mouse models. All mouse lines were backcrossed onto a C57BL/6J background for 10 generations and maintained in pathogen-free conditions according to institutional animal care guidelines.

FACS analysis. Flow cytometry analysis and cell sorting were done as previously described16,17 on single-cell suspensions of T-cell progenitors. Thymocytes were stained in BD FACS buffer. Live thymocytes of 0.7×106 sorted cells from 3 to 5 different 6-week-old mice were fixed and permeabilized using the permeable nucleic acid dye 7-aminoactinomycin D (BD Australia) following the permeabilization protocol. Cells were washed, incubated with 1 ng/mL DAPI for 15 min, washed twice and resuspended in BD Perm/Wash buffer and 1% w/v bovine serum albumin (BSA, Thermo Fisher Scientific, Sydney, NSW, Australia). Briefly, library construction was performed by random fragmentation of the DNA or cDNA sample, followed by 5 and 3 adapter ligation. Library quality control used DNA fragmentation, which combined fragmentation and ligation reactions into a single step, for increasing the efficiency of the library preparation process. Adapter-ligated fragments were then amplified by PCR and purified on gel. The whole exome was captured through target enrichment of DNA using the Agilent SureSelectXT Library Prep Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Sequencing was done with HiSeq 4000 instruments in high-output mode with TrueSeq 3000 4000 SBS v3 chemistry. All runs were 101-nt paired-end reads, and data were analyzed with the HSC v3.2 software. Raw data were generated by the Illumina HiSeq 4000, which utilized HiSeq Control Software v3.3 for system control and base calling through the Real Time Analysis v2.7.3 software. The base calls binary was converted into FASTQ utilizing the Illumina bcl2fastq v2.17.1.14 protocol. Exome sequencing was performed on amplified DNA samples from sorted GFPΔ and GFPΔ6 DN3 cells from individual 2-month-old H2B-GFP-Lmo2/− mice by Macrogen Inc. (Macrogen Oceania, Sydney, NSW, Australia).

Chemosensitivity assays. For chemosensitivity assays, 6-week-old mice were intraperitoneally injected with induction therapeutic regimen (VXL) consisting of 0.15 mg/kg vincristine (Vincristine Sulfate, Pfizer), 5 mg/kg dexamethasone (DBL, Dexamethasone, Hospira) and 1000 U/kg L-asparaginase (Leunase, Sanofi-Aventis)27. Thymocytes were harvested, counted and characterized by flow cytometry 24 h after treatment with the VXL regimen.

In vitro differentiation assays. For co-culture assays, 100,000 sorted DN3 cells were washed, and seeded onto OP9-DL1 stromal cells, as previously described2,2. Briefly, thymocytes were co-cultured on stromal cells in reconstituted alpha-minimum essential medium (12561, Gibco, Life Technologies, Scoresby, VIC, Australia) supplemented with 10% heat-inactivated fetal bovine serum and 5 μM p21 (Gibco), 10 mM HEPES (15630-060, Gibco), 1 mM sodium pyruvate (11360-070, Gibco), 55 μM β-mercaptoethanol (21985-023, Gibco), 2 mM Glutamax (15750-060, Gibco), penicillin/streptomycin (15140-122, Gibco), 5 ng/mL FLT-3 Ligand (308-FK-025, R&D Systems, Minneapolis, MN, USA) and 5 ng/mL IL-7 (217-17, Peprotech, Rock Hill, NJ, USA). For differentiation assays, cells were harvested, counted and characterized by flow cytometry after 5 days of co-culture.

Cell division patterning assays. For cell division patterning assays, 7×104 OP9-DL1 stromal cells were first plated onto glass-bottom 8-well culture chamber slides (Thermo Fisher Scientific, Life Technologies, Scoresby, VIC, Australia) and left to adhere overnight, as previously described23. Then, 1–4×105 sorted DN3 thymocytes were added with fresh media and co-cultured for 24–48 h. DN3-stromal cell conjugates were washed and fixed as previously described34. Cells were blocked using phosphate-buffered saline (PBS) 1× + 1% w/v bovine serum albumin (BSA), for 20 min at room temperature, and incubated for 30 min at room temperature in PBS 1× + 0.25% v/v Triton X-100 for permeabilization. Cells were stained in PBS 1× + 1% w/v bovine serum albumin (BSA), for 20 min at room temperature, and incubated for 30 min at room temperature in PBS 1× + 0.25% v/v Triton X-100 for permeabilization. Cells were stained in PBS 1× + 0.25% v/v BSA with anti-Syndecan-1 (1:100; ab1447, Abcam, Sapphire Bioscience Pty. Ltd., Redfern, NSW, Australia) and anti-Tubulin (1:100; sc-32293, Santa Cruz Biotechnology, Dallas, TX, USA). Cells were washed twice using Perm/Wash buffer (BD Australia, North Ryde, NSW, Australia) and incubated in permeabilization buffer with donkey Alexa Fluor 488-conjugated anti-mouse (1:300; A-21202, Molecular Probes, Life Science) and donkey Alexa Fluor 546-conjugated anti-astro (1:300; A-11056, Molecular Probes, Life Science) secondary antibodies for 1 h on ice. Cells were washed, incubated with 1 ng/mL DAPI for 15 min, washed twice and mounted with Mowiol mounting medium (81381, Sigma-Aldrich Pty Ltd, Castle Hill, NSW). At least 20 different fields of view were collected for blinded quantification, for each time point. All images were acquired using a Nikon A1r Plus S1 inverted confocal microscope (Nikon Australia, Rhodes, NSW) using a Plan Apo 60x oil objective.

Data availability

The datasets generated during the current study are available in the Gene Expression Omnibus repository at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110132. All relevant data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 22 March 2018 Accepted: 2 August 2018
Published online: 30 August 2018

References

1. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med. 3, 730–737 (1997).
2. Shlush, L. I. et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature 506, 328–333 (2014).
3. Mullighan, C. G. et al. Genome analysis of the clonal origins of relapsed acute lymphoblastic leukemia. Science 322, 1377–1380 (2008).
4. Sykes, S. M., Kokkaliaris, K. D., Milson, M. D., Levine, R. L. & Majeti, R. Clonal evolution of preleukemic hematopoietic stem cells in acute myeloid leukemia. Exp. Hematol. 43, 989–992 (2015).
5. Kloc, J. M. & Zon, L. I. Association between mutual clearance after induction therapy and outcomes in acute myeloid leukemia. JAMA Oncol. 3, 811–822 (2015).
6. Morsen, M. et al. Mutational hierarchies in myelodysplastic syndromes dynamically adapt and evolve upon therapy response and failure. Blood 128, 1246–1259 (2016).
7. Steensma, D. P. et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood 126, 9–16 (2015).
8. Holtz, M., Forman, S. J. & Bhatia, R. Growth factor stimulation reduces residual quiescent chronic myelogenous leukemia progenitors remaining after imatinib treatment. Cancer Res. 67, 1113–1120 (2007).
9. Holtz, M. S., Forman, S. J. & Bhatia, R. Nonproliferating CML CD34+ progenitors are resistant to apoptosis induced by a wide range of proapoptotic stimuli. Leukemia 19, 1034–1041 (2005).
10. Barnes, D. J. & Melo, J. V. Primitive, quiescent and divided: the role of DNA repair and mutagenesis. Nature 441, 1064–1070 (2006).
11. Kamminga, L. M. et al. Impaired hematopoietic stem cell functioning after serial transplantation and during normal aging. Stem Cells 23, 82–92 (2005).
12. Saito, Y. et al. Maintenance of the hematopoietic stem cell pool in bone marrow niches by EVI1-regulated GPR56. Leukemia 27, 1637–1649 (2013).
13. Tunbar, T. et al. Defining the epithelial stem cell niche in skin. Science 303, 359–363 (2004).
14. Li, Q. et al. Oncogenic Nras has bimodal effects on stem cells that sustainably increase competitiveness. Nature 504, 143–147 (2013).
15. Boehm, T., Foroni, L., Kaneko, Y., Perutz, M. E. & Rabbitts, T. H. The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. Proc. Natl Acad. Sci. USA 88, 4367–4371 (1991).
16. Rahman, S. et al. Activation of the LMO2 oncogene through a somatically acquired neomorph promoter in T-cell acute lymphoblastic leukemia. Blood 120, 3221–3226 (2017).
17. McCormack, M. P. et al. The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. Science 327, 879–883 (2010).
18. Tremblay, M. et al. Modeling T-cell acute lymphoblastic leukemia induced by the SCL and LMO1 oncogenes. Genes Dev. 24, 1093–1105 (2010).
19. Tremblay, C. S. et al. Loss-of-function mutations of Dynamin 2 promote T-ALL by enhancing p73 signaling. Leukemia 30, 1993–2001 (2016).
20. Gerby, B. et al. SCL, LMO1 and Notch1 reprogram thymocytes into self-renewing cells. PLoS Genet. 10, e1004768 (2014).
21. Clarke, M. F. et al. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res. 66, 9339–9346 (2006).
22. Aplin, R. D. et al. An scl gene product lacking the transactivation domain induces bony abnormalities and cooperates with LMO1 to generate T-cell malignancies in transgenic mice. EMBO J. 16, 2408–2419 (1997).
23. Wilson, A. et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135, 1118–1129 (2008).
24. Bernstein, J. M., Kim, H. S., MacArthur, B., Sieburg, H. & Moore, K. Hematopoietic stem cells count and remember self-renewal divisions. Cell 167, 1296–1309 (2016).
25. Szymanska, B. et al. Pharmacokinetic modeling of an induction regimen for in vivo combined testing of novel drugs against pediatric acute lymphoblastic leukemia xenografts. PLoS ONE 7, e3894 (2012).
26. Muhlin, M. et al. Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. Cell Stem Cell 7, 174–185 (2010).
27. Qiu, J., Papatsenko, D., Niu, X., Schaniel, C. & Moore, K. Divisional history and hematopoietic stem cell function during homeostasis. Stem Cell Rep. 2, 473–490 (2014).
28. Cheng, T. et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. EMBO J. 20, 3876–3886 (2001).
29. Larson, B. C. et al. T cell tumours of disparate phenotype in mice transgenic for Rbhtn-2. Oncogene 9, 3675–3681 (1994).
30. McCormack, M. P. et al. Requirement for Ly11 in a model of Lmo2-driven early T-cell precursor ALL. Blood 122, 2093–2103 (2013).
31. Sibilia, A. J. et al. Ihex regulates Kit to promote radioresistance of self-renewing thymocytes in Lmo2-transgenic mice. Leukemia 29, 927–938 (2015).
Fellowship (1047630 (to S.B.T.)) from the Australian National Health and Medical Research Council (NHMRC), and a Senior Medical Research Fellowship from the Sylvia and Charles Viertel Foundation (to D.J.C.).

Author contributions
C.S.T. and D.J.C. designed research, analyzed data and wrote the manuscript; C.S.T., J.S., S.K.C., S.G., A.N.G., A.A.G., S.E.S. and N.L. performed research and analyzed data; P.K., S.B.T. and D.R.P. designed research and analyzed data; N.C.W., K.T., F.Y. and J.R. analyzed data; and S.M.J. provided reagents, designed research and reviewed the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-06021-7.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/ © The Author(s) 2018

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018