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Beyond “to divide or not to divide”: kinetics matter in Haematopoietic Stem Cells

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Highlights

- Distinct quiescent states exist in the hematopoietic stem cell (HSC) pool to maintain life-long function.
- The CDK4/CDK6/Cyclin D complex is key to maintain the balance between HSC quiescence and division.
- The kinetics of quiescence exit and early G_1 influence HSC differentiative output *ex vivo*.
- Modulating the quiescence to division kinetics has key implications for HSC expansion and gene therapy.

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Abstract

Lifelong blood production is ensured by a population of rare and largely quiescent long-lived Haematopoietic Stem Cells (HSCs). The advent of single cell technologies has recently highlighted underlying molecular and functional heterogeneity within the HSC pool. Despite heterogenous HSC behaviours, quiescence remains as the most uncontroversial unifying property of HSCs. Nonetheless, a multifaceted and complex continuum of states has recently been identified within what was previously described as just "quiescent". Here we review such evidence and discuss how it challenges pre-conceived ideas on the contribution of cell cycle kinetics to HSC function. Specifically, we detail how both the frequency and kinetics of HSC division, largely determined by a network of molecular regulators linked to early G_1, influence long-term HSC function *in vivo*. In addition, we present data which show that that lengthening the duration of G_1 by inhibiting CDK6 decreases lymphoid differentiation of a subset of lymphoid-primed human HSCs, thus linking cell cycle kinetics to cell fate decisions in HSCs. Finally, we reflect on how these new insights can be helpful to fully harness HSC potential in clinical applications that require *ex vivo* culture.

Introduction:

A trillion blood cells are produced daily to maintain blood function at steady state. This immense regenerative output is achieved by the concerted action of rare and infrequently dividing hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs). Decades of work, primarily from mouse models, have built complex roadmaps of the first steps of hematopoiesis by dissecting the cascade of cell divisions occurring from the most potent HSCs to multi- and in turn unilineage progenitors. Recently, single cell RNA-seq and clonal tracking have complemented these by defining differentiation trajectories at single cell resolution [1]. All evidence to date agrees that unilineage progenitor cell types typically divide continuously, re-entering G_1 after mitosis. In contrast, HSCs and MPPs do not; they divide occasionally, spending the majority of their cellular life outside of the cell cycle, in a reversible state termed quiescence or G_0. As HSCs and MPPs largely differ at single cell resolution in their self-renewal capacity and lineage preferences [1], this places quiescence as the only fundamental common property of all HSC/MPPs.

Quiescence can be conceptualized as a poised, restrained and actively maintained molecular state [2]. Classical hallmarks of quiescence are shared by many adult stem cell types and include low protein synthesis and reliance on glycolytic metabolism. Consistently, *in vivo*, the maintenance of HSCs in quiescence relies on a specific metabolic context and organelle biology. Initially thought to have low mitochondrial mass, quiescent HSCs are now known to contain relatively high numbers of inactive mitochondria [3] with an abundance of large inactive lysosomes [4]. Long-term maintenance of HSC potency is reliant on restrained glycolytic metabolism [4,5], and a balanced metabolic state maintained by autophagic recycling [6,7]. Protein synthesis rates are low in quiescent HSCs [8,9], yet there are high thresholds of protein quality control ensured by high basal expression of Endoplasmic Reticulum Associated Degradation (ERAD) [10,11] and Integrated Stress Response pathways [12,13]. Similarly, cellular responses to DNA damage are distinct in HSCs compared to progenitors [14,15]. A consequence of the longevity of HSCs is the accumulation of genotoxic, proteotoxic and oxidative stress damage which, in absence of cell division, cannot be distributed to progeny. Quiescent HSCs are thus wired to maintain the highest standards of genomic, proteostatic and organelle integrity resulting in preferential culling of damaged HSCs to mitigate the risk of leukemic transformation and reduced HSC pool fitness.

Maintenance of HSC quiescence, exit from quiescence, division and subsequent return to quiescence must be tightly controlled. This balance has emerged to be intimately linked to HSC function. In the past decade, single cell resolution and novel tools have shown that the overall picture is complex and cannot be oversimplified to: “HSCs are either more quiescent or more proliferative” in the particular instance studied. Here we focus on a few important findings that have emerged from these studies. We provide definitions and a framework in which to interpret the complex relationship between quiescence, cell cycle kinetics and HSC

fate choices; a key step towards new translational opportunities for HSC expansion, transplantation and gene therapy.

Quiescence is a collection of actively maintained cellular states

In hierarchically organised stem cell systems such as blood, muscle [16] and the subventricular zone of the brain [17], there is now ample evidence that quiescence is not a uniform state and that varying depths of quiescence allow cells to differently and appropriately contribute to tissue maintenance and regeneration. Across all these tissues, the most immature stem cells reside in a deeper quiescent state than their progeny. Depth of quiescence is primarily measured via the time a cell takes to exit this state upon receiving a mitogenic stimulus (quiescence exit: from G_0 to end of early G_1), often approximated to the time of first division *ex vivo*. Quiescence is an integral state existing in a wide range of organisms; there is much to be learnt from seminal work in bacteria, yeast and cell lines that applies to adult stem cells, including HSCs.

First, protein levels of the CDK4/CDK6 complex are a faithful reporter of quiescence depth. CDK4/CDK6 activation is the key trigger for cells to enter G_1 whether they have just divided (from M to G_1) or have been quiescent for a given period of time (from G_0 to G_1). CDK4/CDK6 activity sustains retinoblastoma protein (Rb) hyperphosphorylation in early G_1, which is necessary for a cell to pass the restriction point (R point) and commit to division [18]. In human and mouse HSCs, CDK6 levels govern the degree to which a cell is primed to respond to a mitogenic stimulus. Quiescent long-term HSCs (LT-HSCs, capable of forming blood over serial transplantation) possess undetectable levels of CDK6 protein, whereas quiescent short-term HSCs (ST-HSCs, with transient regenerative potential upon transplantation) express higher CDK6 protein levels to allow faster cell cycle entry following mitogenic stimulation [19,20] (**Figure 1**) . Recent work has shown that reaching the R point is best described as a probabilistic process dependent on levels of CDK4/CDK6 activity in single cells [21]. This implies that for a specific level of mitogenic stimulus, cells with high levels of CDK4/CDK6 such as ST-HSCs have a higher probability of commitment to division than cells with low levels (like LT-HSCs). It is thus tempting to speculate that LT-HSCs may also require a stronger/longer mitogenic stimulus in order to divide. LT-HSCs may also be more likely to return to quiescence, as inhibition of CDK4/CDK6 is required for cells to return to G_0 after mitosis [22]. In any case, maintenance of distinct depths of quiescence in LT-HSCs and ST-HSCs and the associated division kinetics are necessary to control HSC pool size and effective tissue regeneration [20].

Second, distinct stressors can induce metabolically different states of quiescence in yeast and bacteria [23,24]. It is therefore unsurprising that alternative states of quiescence are also rendered accessible by injury in mammals. Neural stem cells exist in graded states of activation with characteristic metabolic profiles and acquire a primed-state of quiescence

after ischemia [17]. Similarly, quiescent muscle stem cells sensing an injury in a distant muscle can reversibly switch into a cell-cycle primed "G_{alert}" phase, marked by mTORC1 activation, where cells are quiescent but possess enhanced tissue regeneration properties [16]. Mouse HSCs can access an mTORC1-high "G_{alert}" state following injury, priming them to divide faster upon successive challenges for example with interferon-gamma [16]. The "G_{alert}" phase is distinct from the primed quiescent state of ST-HSCs, that actually shows minimal levels of mTORC1 activity [20]. The molecular mechanisms as well as the range of stimuli that push HSCs into "G_{alert}" remain to be studied. Nevertheless, these data demonstrate that specific quiescent states may provide at least transient cellular memory for stem cells to respond more efficiently to subsequent injury signals. Given recent evidence of long-term epigenetic memory of mouse HSCs to inflammatory signals [25,26], it will be interesting to study if and how alternative quiescent states play a role in such responses.

Finally, a molecular checkpoint that separates the end of G_0 from the beginning of G_1 has not been identified to date in any organism. Such a checkpoint may very well not exist and the transition from quiescence to early G_1 may instead occur over a continuum of molecular states, with increasing activation of G_1 genes and gradual establishment of a distinct metabolic activity to that of quiescence. Consistently, in the mouse, there is a continuum of transcriptional activation from dormant HSCs to quiescent cell-cycle primed HSCs to cell-cycle active HSCs [19]. Whether this activation continuum corresponds to that of the lineage-bias/restriction observed in the HSC/MPP compartment at single cell level [27,28] remains to be explored.

All HSC/MPPs are quiescent but very few are truly dormant

All HSC/MPPs predominantly reside in G_0 and divide much more infrequently than progenitors (approximately once every 30 days compared to once every 1-2 days for the latter). Dormant HSCs are defined as the subset of HSCs which reside in the deepest state of quiescence, divide the most infrequently (**Figure 1**) and have long been exclusively defined functionally, using inducible label retention assays in mice. These studies have allowed careful analysis of the heterogeneity in division frequencies observed in HSCs and MPPs *in vivo* under homeostatic and stress conditions. Dormancy correlates with the longest duration of quiescent exit and the highest degree of long term repopulation capacity [19,20,29–33]. In fact, the correlation is so strong that both long quiescence exit duration and extremely infrequent divisions can be used as surrogate markers for the long-term repopulation capacity of an HSC. The dormant HSC subset in mouse cycles less than once every 120 days [33], has superior serial repopulation capacity to all other HSC/MPP subsets and is only recruited into cycling upon transplantation or severe stress [19,33]. Only recently, reporter mice allowing prospective identification of dormant HSCs have been developed. These respectively trace

dormant HSCs based on either Gprc5c expression [19], p27 activity [34] or retinoic acid signalling [35].

Self-renewal capacity has been suggested to decline with each successive division, with dormant HSCs becoming less likely to return to quiescence [29,31]. The number of symmetric divisions sufficient to exhaust self-renewal capacity is still under debate [29,36]. Extrinsic stresses such as those induced by 5-FU [33], LPS [32] or pI-pC [19] to mimic bacterial and viral infections, force dormant HSCs to re-enter the cell cycle and become activated. Importantly, injury activated HSCs are later able to return to dormancy and restored proportions of dormant HSCs can be detected within days of the initial stress signal [19,33]. However, a recent study has shown that HSCs permanently accumulate dysfunctional mitochondria upon return to quiescence [37], providing mechanistic insights into how HSC divisional histories contribute to functional decline.

Altogether, label retention studies indicate that the contribution of dormant HSCs to daily or steady state hematopoiesis is minimal, complementing insights obtained from clonal tracking experiments. This reinforces the concept that diversity of quiescent states within the HSC/MPP compartment is key for the resilience of blood production, with distinct dynamics underlying both a rapid stress response and protection from untoward HSC/MPP exhaustion. However, if and how the balance of quiescent states is altered with ageing or disease remains poorly characterised. Interestingly, in the ageing brain, changes in the niche enforce increased neuronal stem cell quiescence [38]. Future studies will have to consider the further complexity that arises in humans with age, due to niche-driven effects, life histories of infection/inflammation [39,40] as well as age-related clonal hematopoiesis [19].

The importance of the early G_1 regulatory network for HSC function

Many studies have investigated whether manipulating the levels of cell cycle regulators can influence HSC self-renewal and/or differentiation (reviewed in [41–43]). It is no surprise that complex control of the molecular network promoting Rb hyperphosphorylation, and hence commitment to division, is absolutely fundamental to regulate how often and how fast HSCs exit from quiescence (as discussed above), in addition to the kinetics of successive divisions. This network includes, in addition to Rb, CDK4/CDK6, their Cyclin Ds partners, as well as their specific CDK inhibitors (p16, p18, p27, p57; **Figure 2**).

Given the importance of maintaining the life-long capacity for HSC division, it is expected that there is a high degree of redundancy between members of the CDK4/CDK6-Rb network, with phenotypes observed only when several homologues are genetically deleted in mice [44–46]. Constitutive deletion of *Cdk6* has minor effects on hematopoiesis, likely due to compensatory effects from *Cdk4*. However, Cdk6 is strictly necessary for HSC exit from quiescence and

activation under stress conditions. Cdk6 action during this transition cannot be compensated by Cdk4, as demonstrated with constitutive [47] and conditional *Cdk6* knock-out models [48].

In addition, when HSC function fails either in the context of an experimental model, or in patients, teasing apart whether cell cycle changes are drivers of such loss or merely consequences of other signalling events is an ongoing challenge in the field. Indeed, many cell cycle regulators possess functions beyond pushing cells towards division [49,50]. CDK6 can directly phosphorylate EGR1 [47], a transcription factor involved in HSC function, but also TSC2, thereby promoting mTORC1 activity and linking cell cycle with cell growth [51]. CDK6 can directly block RUNX1 transcriptional activity [52] or contact the chromatin modulating NFkB-p65 [53], p16, VEGF-A [54], and p53 antagonists activity [55], broadly affecting HSC function in a cell-cycle independent manner.

The whole picture is further complicated by the fact that the cell cycle machinery is activated or repressed by a multitude of external stimuli. A very substantial body of work has collectively identified more than 100 genes and dozens of environmental components [56] that contribute to regulating the delicate balance between HSC quiescence and division. A common interpretation of all these studies is that excessive division *in vivo* almost inevitably leads to loss of HSC self-renewal. We would argue that this is an oversimplification. This can certainly be driven by exhaustion through excessive division coupled to differentiation (loss of self-renewal, "locked out" of quiescence). Nonetheless, failure to divide and produce differentiated progeny ("locked in" quiescence) eventually leads to the same failure in blood production. Most experimental assays require division to read out HSC function, so caution must be taken when interpreting HSC phenotypes with regards to the effects of a particular perturbation on the quiescence to division balance.

Is there a causal relationship between G_1 length and HSC fate decisions?

A possible causal relationship between cell cycle and fate decisions has long been pursued in stem cell biology. The position in the cell cycle may control how cells respond to external stimuli [57] and the duration of a particular cell cycle phase [58] may influence cell fate. The duration of G_1 causally influences cell fate decisions in embryonic stem cells, specifically which differentiation route cells take [59–61]. Lengthening of G_1 is also associated with commitment in neural stem cells [62,63]. Whether similar mechanisms are at play in HSCs is still under debate. In human LT-HSCs, overexpression of CDK6 alone leads to shorter early G_1 and confers a competitive advantage over serial transplantation without causing any overt changes in differentiation ability [20]. Overexpression of both CDK4 and Cyclin D1 shortened early G_1 further and increased myeloid differentiation upon xenotransplantation *in vivo*, but this was not supported *in vitro* [64]. Given the pleiotropic effects of CDKs and Cyclins, it is difficult to conclude from these genetic experiments if the slight bias in lineage differentiation is caused by different cell cycle lengths or by other factors.

We reasoned that we could gain insights into the causal relationship between G_1 length and human HSC differentiation capacity by lengthening their time of first division. We chose to use Palbociclib, a dual and highly specific CDK4/CDK6 inhibitor (hereafter referred to as CDK6i), which inhibits the kinase-dependent effects of these proteins without affecting their kinase-independent functions. Our group recently identified a subpopulation of long-term repopulating cells within the phenotypic human LT-HSC ($CD49f^+$ $CD90^+$) compartment [65], that produce lymphoid and myeloid (My) cells but no erythroid (Ery) or megakaryocytic (Meg) cells *in vitro* and upon xenotransplantation [27]. This population, that we termed $CLEC9A^{lo}$ LT-HSCs (purified as $CD34^{hi}CD38^-CD45RA^-CD90^+CD49f^+CLEC9A^{lo}$) was also characterised by shorter quiescence exit and higher CDK6 levels than LT-HSCs or $CLEC9A^{hi}$ LT-HSCs ($CD34^{lo}CD38^-CD45RA^-CD90^+CD49f^+CLEC9A^{hi}$) [27]. We thus sorted single $CLEC9A^{lo}$ LT-HSCs and cultured them in conditions that sustain simultaneous differentiation of My, Ery, megakaryocytic (Meg) and lymphoid (NK) cells [27], in the presence or absence of 200nM CDK6i for the first 3 days (Supplementary Methods). Single $CLEC9A^{hi}$ LT-HSCs were also cultured in parallel as a control. Time to first division was recorded and the differentiation output of each cell was determined 3 weeks later by flow cytometry. First, CDK6i lengthened the duration of $CLEC9A^{lo}$ LT-HSCs' first division to that of $CLEC9A^{hi}$ LT-HSCs (**Figure 3A)**, without altering clonogenic efficiency **(Figure 3B)**. Interestingly, upon CDK6i, the proportion of $CLEC9A^{lo}$ LT-HSCs' derived colonies containing NK cells decreased significantly with CDK6i ($p=0.027$); **Figure 3C**), principally due to a decrease in colonies containing both My and NK cells **(Figure 3D)**. However, colony output of CDK6i $CLEC9A^{lo}$ LT-HSCs was still vastly different from that of $CLEC9A^{hi}$ LT-HSCs **(Figure 3D)**. These results suggest that $CLEC9A^{lo}$ LT-HSCs' inability to produce Ery and Meg cells is not caused by their shorter time of quiescence exit. Rather we propose that restriction to the myelo-lymphoid lineages is irreversibly determined epigenetically and transcriptionally, in line with $CLEC9A^{lo}$ LT-HSCs possessing transcriptional features intermediate between $CLEC9A^{hi}$ LT-HSCs and lymphoid-primed multipotent progenitors [27]. However, when G_1 was lengthened, $CLEC9A^{lo}$ LT-HSCs commitment to the myeloid and lymphoid lineages was significantly imbalanced *in vitro*. More broadly this suggests that changes in cell cycle duration may also influence lineage differentiation in hematopoietic stem and progenitor cells and warrant further investigation.

Understanding quiescence exit and division ex vivo is essential to improve HSC clinical approaches

Recent years have seen important milestones towards clinical applications of HSCs that require *ex vivo* culture of HSCs, such as HSC gene therapy and protocols for HSC expansion [66,67]. One critical challenge remains: prolonged culture *ex vivo* leads to a net decline in HSC long-term repopulation capacity [68–70], as a result of divisions shifted to producing daughter cells destined for differentiation but also reduced homing. The causes of such decline are

multiple and highly dependent on the culture medium composition and culture duration [71,72]. Interestingly though, whereas *in vivo* self-renewing HSCs return to quiescence after division, *ex vivo* HSCs fail to do so. We would argue that understanding how quiescence exit and division differ ex vivo from in vivo will be essential to successfully recreate conditions maintaining divisions where one (self-renewal) or both (expansion) daughter cells retain HSC identity. This will be equally beneficial in the context of gene therapy, where significant improvement to gene transfer [73] and editing rates [74] has been achieved by manipulating cell-cycle related parameters.

Evidence has accumulated both *in vivo* [75–78] and *ex vivo* [79–81] that self-renewal and differentiation can under certain circumstances be uncoupled from division. This implies that there is no theoretical impossibility for HSC maintenance or expansion *ex vivo*, as long as survival can be guaranteed. Strategies mimicking quiescence-inducing factors normally found in the *in vivo* niche have shown success in minimising the decline in long-term repopulation ability of cultured HSCs [82–84], most likely by favouring self-renewing asymmetric divisions. Recent strategies with small molecule inhibitors [85,86], aimed at removing differentiation factors [71,82,87], limiting accumulation of damaging agents [88] and/or retaining quality control mechanisms associated with quiescence *in vivo* [89] have managed to obtain fully functional HSCs in numbers similar or higher than those at the start of the cultures. The most impressive expansion to date has been reported to be around 550-fold over 2 months of mouse HSC culture with polyvinyl alcohol (PVA) [87]. Of note, no such expansion has been obtained with human cells so far, and that all protocols above generate mixed cultures where functional HSCs remain a minority. Nonetheless these descriptive studies represent major steps forward. All measure HSC expansion by assessing HSC function through transplantation rather than mere phenotypic characterisation, which is absolutely essential for this field to progress. Altogether, these studies indicate that the entire underlying molecular network, stress response regulation and macromolecular organelle biology associated with quiescence in vivo should be preserved when aiming to maintain or expand self-renewal *ex vivo*.

There is much to be learnt from how each of these protocols mechanistically act on HSC identity and how they affect quiescence exit and division. For instance, we do not know if successful expansion strategies are effectively allowing at least some of the HSCs to return to quiescence *ex vivo* and potentially maintain a hierarchy of cells characterised by accelerated division kinetics and progressively decreasing self-renewal as *in vivo*. There is no doubt that better understanding quiescence exit and return to quiescence (or G_1) at single cell resolution both *in vivo* as well as in clinically relevant *in vitro* settings will have a major impact not only on our understanding of HSC biology but also on HSC transplantation success rates.

Conclusions

Quiescence unites but also divides HSCs. Subtle differences in metabolism, quality control mechanisms and organelle biology during quiescence lead to seemingly small (several hours) yet important differences in division kinetics and distinct division frequency over very long timeframes (several months). Together these two parameters control how likely a cell is to divide and contribute to blood formation both at steady state and under stress. Given the extent to which divisional histories contribute to HSC function [29,31,36,37], and the fact that cellular memories of injury may affect subsequent divisions, we propose that diversity in quiescent states is perhaps the most relevant aspect of HSC heterogeneity. It is already established that the reversible shift from quiescence to division plays a key role in leukemia, particularly influencing drug resistance and relapse [90]. HSC quiescent states, quiescence exit kinetics and potentially the lineage-biases associated to the latter are likely to shift over time, with indications that such shifts may be detrimental in aging and for progression from clonal hemopoiesis to overt malignancy. This is a field that we bet will not become dormant in the near future.

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Data statement: all raw data is available upon request to the authors

FIGURE LEGENDS

Figure 1: HSC quiescence is a continuum of molecular states associated with specific functional features. The cell cycle has four distinct phases indicated by the inner circle, Mitosis (M), Gap 1 (G_1), the DNA synthesis phase (S) and Gap 2 (G_2). Quiescence (G_0) is a collection of stable states (schematized by colour shading) where Retinoblastoma protein (Rb) is unphosphorylated and levels of CDK proteins are low. Dormant HSCs divide very infrequently, reside in a particularly deep state of quiescence and are largely resistant to activation. Dormant, long term HSCs (LT-HSCs) and short term HSCs (ST-HSCs) reside in sequentially shallower states of quiescence, each becoming more primed for cell cycle re-entry. Quiescence exit (indicated by arrow, arrow thickness indicating strength of activation signal required for cell cycle re-entry) in HSCs is predominantly mediated by levels of CDK6 which form a complex with Cyclin D to allow cell cycle re-entry. During early G_1, Rb is progressively re-phosphorylated until hyperphosphorylation at the "restriction point" (denoted as R) whereby cell lose dependency on extracellular mitogens for cell cycle progression and commit to complete the cell cycle. Text indicates molecular and functional characteristics associated with different depths of quiescence. ST-HSC: Short term HSCs; LT-HSC: Long term HSC; ERAD: Endoplasmic Reticulum Associated Degradation; Rb: Retinoblastoma protein; R: restriction point.

Figure 2: Quiescence exit and cell cycle re-entry in HSCs is regulated by CDK6/CCND mediated Rb phosphorylation. After dephosphorylation in M, G_0 and early G_1 unphosphorylated Rb is physically associated to E2F factors blocking the transactivation domain and inhibiting cell cycle progression. Upon cell cycle re-entry from quiescence, Rb is sequentially phosphorylated by kinase complexes of Cyclin D (CCND) with CDK6 and later Cyclin E (CCNE) with CDK2. Hyperphosphorylation of Rb causes a conformational change permitting E2F release and transcription of CDK2, Cyclin E (CCNE), Cyclin A (CCNA), MYC and other genes involved in cell cycle progression and nucleotide biosynthesis. At this "restriction point" (denoted as R) cells lose dependency on extracellular mitogenic signals and commit to entering G_1/S transition and to complete the cell cycle. Gradients of colour indicate the regions of the cell cycle indistinguishable by current functional assays whereas cells can be allocated to respective portions of the cell cycle by current methods in fill coloured regions. Edge and fill colours for each protein respectively indicate the cell cycle progression/frequency of division and long-term repopulation phenotypes observed when the corresponding genes were knocked-out as reported in for: p16 [91], p18 [92], p27[93], p57 [94], p27/p57 [44], CDK6 [47], CycD triple KO [45], Rb/p107/p130 [46]. White proteins: not determined. KO: knock-out; Cyc: cyclin; Rb: Retinoblastoma protein; R: restriction point.

Figure 3: Lengthening the time to first division decreases lymphoid differentiation of Ly-ST-HSCs. Single cells from the indicated populations were cultured for 3 days in presence or absence of CDK6i (Palbociclib, 200 nM), then without CDK6i for 3 weeks. **(A-B):** Mean time to first division (EC50 of non-linear fit of cumulative first division kinetics) **(A)**, and clonogenic efficiency of single cells from the indicated populations **(B)**. n= 2 experiments with independent CB samples, 144 total cells plated for CD49f+ Subset1, 336 for CD49f+ Subset2, 192 for CD49f+ Subset2 CDK6i. **(C-D):** Percentage of colonies containing differentiated cells of the indicated lineages **(C)** and of the indicated type generated by single cells of the indicated populations **(D)**. n =2 experiments with independent CB samples, n = 128 colonies from CD49f+ Subset1, n = 105 colonies from CD49f+ Subset2, n=161 colonies from CD49f+ Subset2 CDK6i). Statistical significance shown was calculated by Fisher test using the number of colonies obtained from both experiments. (NK: expmt 1 p =0.048, expmt 2 p=0.024; My: expmt 1 p=0.039, expmt 2 p=0.056; MyNK: expmt 1 p<0.001, expmt 2 p=0.003; NK only: expmt 1 =0.016, expmt 2 p=0.418). Mean ± SEM is shown

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Figure 1

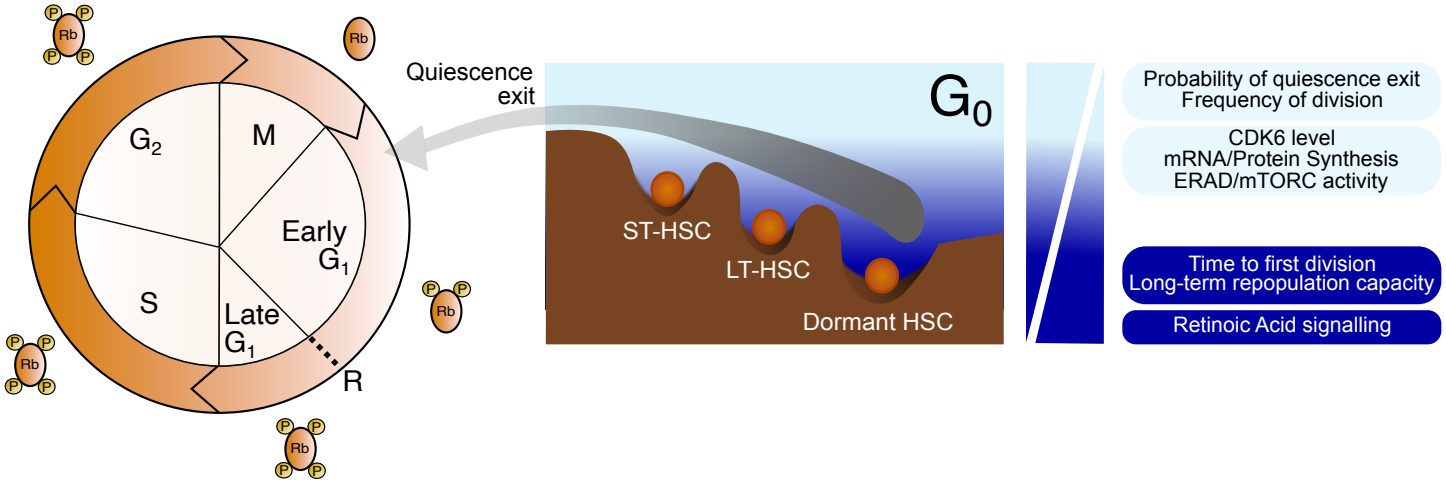
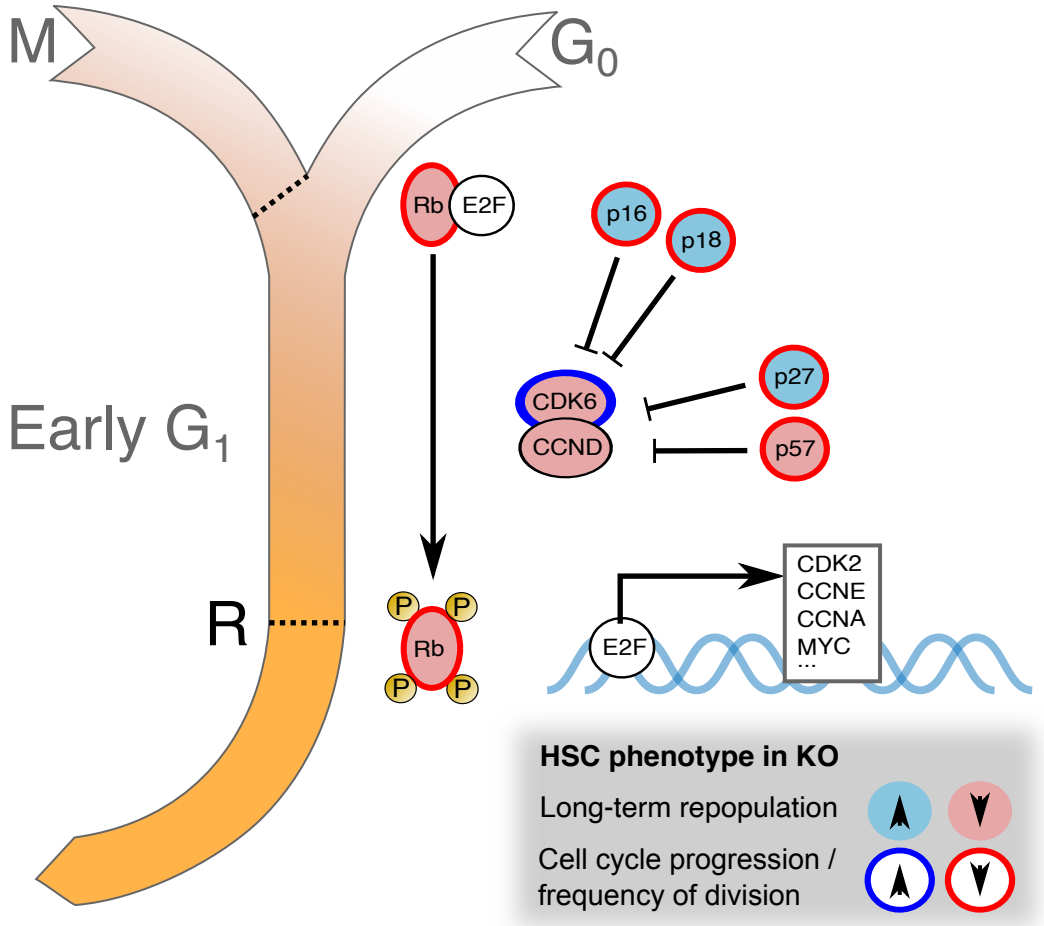

Rb
Rb
Quiescence exit
G2
M
Early G1
S
Late G1
R
G0
ST-HSC
LT-HSC
Dormant HSC
Probability of quiescence exit
Frequency of division
CDK6 level
mRNA/Protein Synthesis
ERAD/mTORC activity
Time to first division
Long-term repopulation capacity
Retinoic Acid signalling

Figure 2

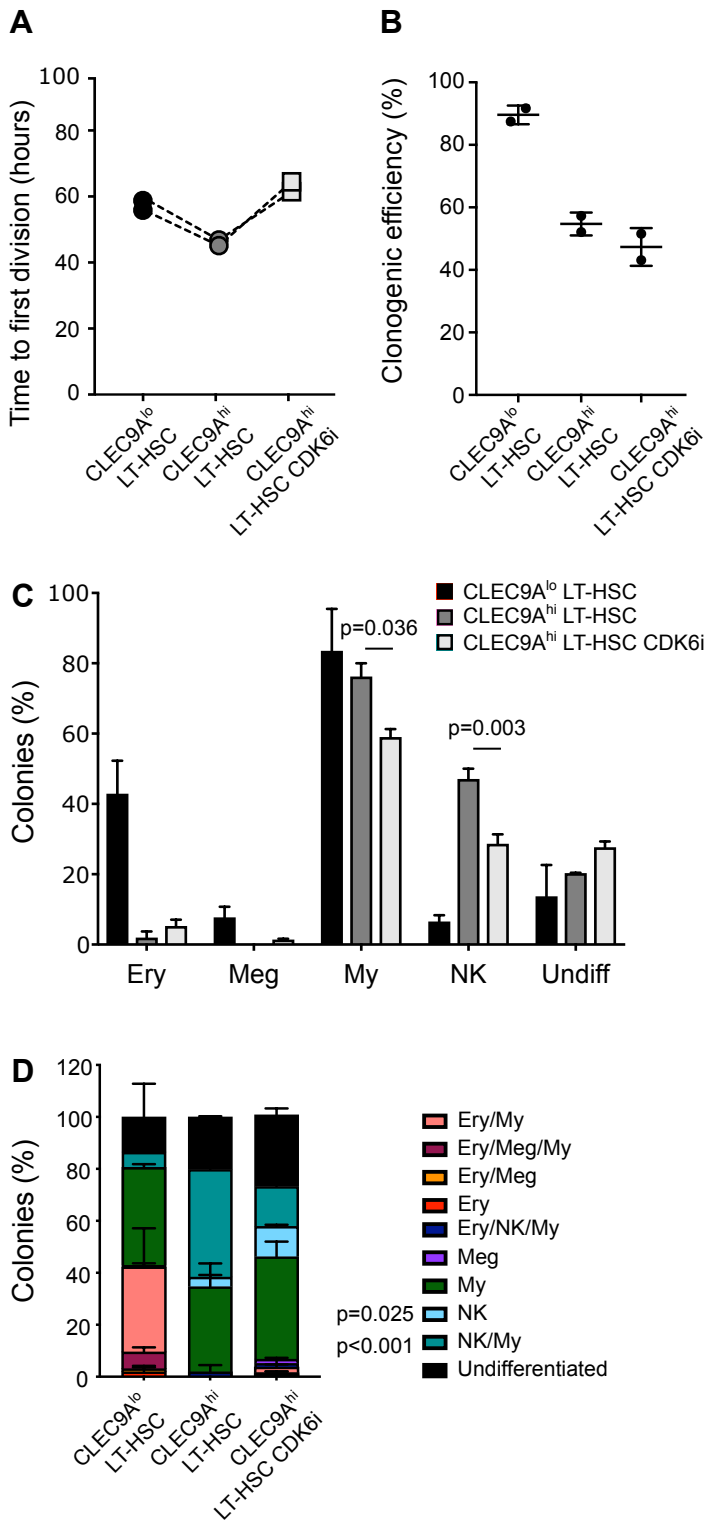


HSC phenotype in KO

Long-term repopulation

Cell cycle progression / frequency of division

Figure 3


A
Time to first division (hours)
100
80
60
40
20
0
CLEC9A^lo LT-HSC
CLEC9A^hi LT-HSC
CLEC9A^hi LT-HSC CDK6i
B
Clonogenic efficiency (%)
100
80
60
40
20
0
CLEC9A^lo LT-HSC
CLEC9A^hi LT-HSC
CLEC9A^hi LT-HSC CDK6i
C
Colonies (%)
100
80
60
40
20
0
CLEC9A^lo LT-HSC
CLEC9A^hi LT-HSC
CLEC9A^hi LT-HSC CDK6i
p=0.036
p=0.003
Ery
Meg
My
NK
Undiff
D
Colonies (%)
120
100
80
60
40
20
0
p=0.025
p<0.001
Ery/My
Ery/Meg/My
Ery/Meg
Ery
Ery/NK/My
Meg
My
NK
NK/My
Undifferentiated
CLEC9A^lo LT-HSC
CLEC9A^hi LT-HSC
CLEC9A^hi LT-HSC CDK6i

Artwork for cover

Active HSC

Depth of Quiescence

Dormant HSC