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Cks1 is a critical regulator of hematopoietic stem cell cycling, quiescence and homeostasis, operating upstream of CDK inhibitors

Viktoriya Tomiatti

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1. apl. Prof. Dr. Ulrich Keller
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Für meine Eltern Pavlina und Georgi

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Zusammenfassung

Der Prozess der Blutbildung, Hämatopoese beinhaltet eine hieratische Abfolge hematopoetischer Stammzellen (HSC), Progenitorzellen (HPC) und adulter Blutzellen. Zellzyklusregulation spielt eine Schlüsselrolle während des stationären Ruhezustandes von HSC, der Induktion der Blutproduktion und der Differenzierung. Sie ist ein wichtiger Mechanismus, der unkontrollierter Zellproliferation und Tumorentstehung entgegenwirkt. Cyclin/Cyclin dependent kinase (Cyclin/CDK) Komplexe und die CDK Inhibitoren (CKI) sind wichtige Elemente, die die Übergänge der Zellzyklusphasen kontrollieren. Regulation der CKI Level geschieht vornehmlich auf post-translationaler Ebene, durch Kontrolle der Proteinstabilität. Dieser Mechanismus wurde am gründlichsten für den CKI p27 untersucht, dessen Abbau durch den E3 Ubiquitinligase Komplex SCF^{Skp2} veranlasst wird. Die effiziente Ubiquitinylierung von phosphoryliertem p27 durch den SCF^{Skp2} benötigt zusätzlich das Protein Cks1, welches ebenfalls für die SCF^{Skp2}-vermittelte post translationale Regulation der CKI p21, p57 und des Mitglieds der Rb-Familie, p130, benötigt wird.

Aufgrund der hohen Bedeutung der CKI für die Hämatopoese und des SCF^{Skp2}-vermittelten Abbaus von CKI für den Zellzyklusablauf, wurde im Rahmen dieses Projektes die hämatopoetische Rolle des limitierenden Faktors des SCF^{Skp2}-Komplexes, Cks1 untersucht. Um p27-abhängige Effekte des Cks1-Verlustes zu bestimmen, wurden zusätzlich Experimente mit Doppel-Knockouts für Cks1 und p27 durchgeführt.

Die Ergebnisse zeigen, dass die Expression von Cks1 in den frühen, „long-term“ HSC (LT-HSC) erhöht war. Der Verlust von Cks1 in HSC/HPC resultierte in erhöhten Proteinleveln von p21, p27, p57 und p130. Diese Akkumulation war in den frühesten HSC Populationen, den CD150+ LSK am stärksten ausgeprägt. Während *Cks1*-/- Mäuse eine normale adulte Hämatopoese aufwiesen, wurde im Knochenmark ein Abfall der Vorläufer-Populationen und eine Akkumulation der LT-HSC beobachtet. Dies deutete auf einen verzögerten Austritt der Cks1-defizienten HSC aus dem Ruhezustand hin. Diese Annahme wurde durch 5-FU induzierte Stresshämatopoese Experimente und Transplantationsversuche bewiesen. Der Verlust von Cks1 führte zu einer verzögerten Stress-Antwort nach 5-FU Behandlung und zu einer erhöhten LT-HSC Dichte nach Transplantation.

Beide Beobachtungen wurden von einem Abfall der BrdU-Inkorporation in den *Cks1*-/- LT-HSC begleitet, was auf einen verlangsamten Zellzyklus hindeutet. *In vitro*, führte der Verlust von Cks1 zu einer Sensibilisierung der HPC gegenüber Apoptose und einem verringerten Koloniewachstum. Andererseits konnten die *Cks1*-/- LSK nach Replatierungen länger bestehen und mehr Kolonien in den späteren Passagen bilden, was im Einklang mit den Transplantationsversuche war. Dies bestätigt die Annahme, dass Cks1 den Ruhezustand der HSC kontrolliert.

Da *Cks1*-/-*p27*-/- Mäuse keinen Wildtyp ähnlichen Phänotyp aufwiesen oder *p27*-/- Zellen keinen reziproken Phänotyp gegenüber *Cks1*-/- Zellen zeigten, war keiner der oben erwähnten Effekte des Cks1-Verlustes ausschliesslich auf p27 Akkumulation zurückzuführen. Durch Cks1 bedingte Regulation des p27 Proteinlevels wurde die Verteilung der adulten hämatopoetischen Populationen nach 5-FU induziertem Stress gesteuert. Eine 5-FU vermittelte Ablation der reifen, sich teilenden Zellen und die dadurch bedingte Ausdifferenzierung der HSC, resultierte in einem Anstieg der *Cks1*-/- B-Lymphocyten und einem Abfall der *Cks1*-/- Granulocyten. Der entgegengesetzte Effekt wurde in *p27*-/- Mäusen beobachtet, während die Doppel-Knockouts einen Wildtyp-ähnlichen Phänotyp aufwiesen.

Im letzten Teil des Projektes wurde die Rolle von Cks1 in der hämatopoetischen Stammzellerkrankung CML untersucht. Es konnte gezeigt werden, dass Cks1 Expression durch die Aktivität der konstitutiv aktiven Kinase BCR-ABL hochreguliert wird, während Behandlung mit dem ABL Inhibitor Imatinib zu einer Unterdrückung der Cks1-Level führte. Durch *In vitro* Analysen wurde gezeigt, dass Cks1 die BCR-ABL induzierte klonogene Aktivität beeinflusst.

Die Daten in dieser Studie zeigen, dass Cks1 ein kritischer Regulator der Homöostase in HSC/HPC ist. Diese Regulation geschieht wahrscheinlich durch die Kontrolle der CKI-Proteinlevel als Teil des SCF^{Skp2} Komplexes. Cks1 ist möglicherweise ein wichtiges Zwischenglied der Oncogen-induzierten Zellzyklusregulation in hämatologischen Erkrankungen und somit ein therapeutischer Ansatzpunkt.

Summary

Hematopoiesis, the process of blood production, involves a hierarchy of hematopoietic stem cells (HSC), progenitor cells (HPC) and mature blood cells. Regulation of the cell cycle plays a key role during steady state and challenged blood production and also serves as a mechanism towards uncontrolled cell proliferation that might lead to blood malignancies. Cyclin/Cyclin-dependent kinase (Cyclin/CDK) complexes and their inhibitors (CKI) are the major directing elements in cell cycle transition.

The regulation of the CKI occurs mainly at the post-translational level through control of protein stability. This mechanism has been best elucidated for the CKI p27, the degradation of which is controlled by the E3 ubiquitin ligase complex SCF^{Skp2}. For efficient ubiquitylation of phosphorylated p27, SCF^{Skp2} requires the presence of a small protein, the Cyclin-dependent kinase subunit 1 (Cks1). In addition to the regulation of p27 protein levels, SCF^{Skp2} has further been associated with the post-translational regulation of other CKI including p21, p57 and the Rb family member p130.

Considering the importance of SCF^{Skp2}-mediated degradation of CKI for the proper cell cycle progression of HSC/HPC, the hematopoietic role of Cks1, the rate-limiting component of the SCF^{Skp2}, was studied during this project. To reveal which effects of Cks1 loss in hematopoiesis were p27 dependent, additional experiments with double knockouts for Cks1 and p27 were performed.

Cks1 expression was increased in the early, long-term HSC (LT-HSC) and loss of Cks1 resulted in up-regulated p21, p27, p57 and p130 levels in HSC/HPC. This accumulation was most pronounced in one of the earliest HSC subsets, the CD150+ LSK. While *Cks1*-/- mice exhibited normal adult hematopoiesis, a significant decline in the progenitor populations and an accumulation in the LT-HSC fractions were observed, indicating, that *Cks1*-/- HSC could be decelerated in the exit of the quiescence mode. This assumption was proven through 5-FU induced stress hematopoiesis experiments and transplantation assays. Loss of Cks1 led to a decelerated hematopoietic stress response after 5-FU treatment and increased LT-HSC frequency upon transplantation. Both observations were accompanied by decreased BrdU integration in *Cks1*-/- HSC indicating slower cycling of these cells. *In vitro*, loss of Cks1 led to sensitization towards apoptosis

in HPC and decreased colony forming capability. On the other hand, consistent with the transplantation analysis, after serial replates *Cks1*-/- LSK persisted longer and formed more colonies in the later replates, confirming the suggestion, that Cks1 regulates HSC dormancy.

None of the observed effects in the *Cks1*-/- cells seemed to be dependent on sole p27 accumulation, since either experiments with double knockouts did not resemble the wild type phenotype or *p27* knockout did not oppose the *Cks1*-/- phenotype. Only the lineage distribution upon hematopoietic stress seemed to be controlled through Cks1 in a p27 dependent manner. Ablating the adult hematopoiesis and inducing HSC differentiation with 5-FU resulted in an increase of *Cks1*-/- B-lymphocytes and a decrease in *Cks1*-/- granulocytes. The opposite effect was observed in *p27*-/- mice, whereas double knockouts displayed a phenotype similar to wild type.

In the last part of this study, the role of Cks1 in the HSC disease chronic myeloid leukemia (CML) was investigated. Cks1 was shown to be up-regulated by the constitutively active tyrosine kinase BCR-ABL in CML and suppressed upon treatment with the ABL inhibitor imatinib. *In vitro* analysis showed that Cks1 acted as a mediator of BCR-ABL induced clonogenic activity.

Taken together, the data in this study establish Cks1 as a crucial regulator of HSC/HPC homeostasis, acting upstream of CKI. Cks1 is proposed to be a therapeutic target as a central intermediate for oncogene-induced cell cycle regulation in hematopietic malignancies.

1. Introduction

1.1. Cell cycle

The cell cycle is an essential program, which cells necessarily undergo to reproduce and maintain a living organism. It involves a series of events in order to duplicate the cell DNA and to deliver copies of two identical daughter cells. The cell phases are strongly controlled by certain proteins and their regulation is of major significance for the proper function of cell division and proliferation. Failures in control of the cell cycle are crucial for the cell fate determination transformation and cancer [1].

1.1.1. Phases and checkpoints of the cell cycle

Each cell is born at the end of the mitosis phase, a process which describes the cell division. The chromosomal DNA is replicated in the S phase (synthetic phase). The other two phases are gaps (G1 and G2) between mitosis and the S phase. The G1 phase, of various duration where the cell exerts its functions, is the interval between mitosis and S phase and comparatively short G2 phase is between the DNA replication and mitosis [2].

The transition between the different cell phases is controlled at specific checkpoints. These are biochemical pathways which modulate the progression through the cell cycle in response to external and internal signals. The restriction point in the G1 phase is a response to size and physiological state of the cell and the influence of the extracellular matrix. The DNA damage checkpoint monitors the integrity of the DNA. The metaphase checkpoint (also spindle assembly checkpoint) controls the attachment of the chromosomes to the mitotic spindle [3, 4].

A special compartment of the cell cycle is the G0 phase. A lot of cells with specific functions stay at the G0 phase and could be either dormant or actively engaged in protein synthesis and secretion. In response to certain stimuli G0 cells can enter the cell cycle [2]. The G1 phase is the longest and most variable cell phase, at which the cell is growing, preparing for divisions and undergoing the restriction checkpoint and the G1 DNA damage checkpoints [3, 4]. Both

control points are defect in many cancer types, leading to deregulated division of the cells independent from external signals and existing DNA damage [1, 5].
Successful entry into the S phase is followed by replication of DNA, a complicated process, which is crucial for the cell's survival. The replication of the large chromosomes of higher eukaryotes is initiated at many different sites along the chromosomal DNA, called origins of replication. As replication is initiated at each origin, components of the prereplication complex (formed in the G1 phase) are deactivated, preventing any region of the chromosome from replicating twice [2].
The following G2 phase is the transition into mitosis and comprises the G2 DNA damage checkpoint, an essential control of the replicated DNA. Defects at this checkpoint can lead to cancer and if the control is working properly, any kind of damage of the DNA leads to G2 delay [3, 4].
Mitosis is defined as the actual splitting of the cell into two daughter cells and consists of prophase, prometaphase, metaphase, anaphase, telophophase and cytokinesis. Mitosis involves a dramatic reorganization of the nucleus and the cytoplasm, controlled by a number of enzyme complexes, the regulation of which is essential for the correct completion of the division of the cell and the cell cycle [6].

1.1.2. Cell cycle control: Cyclin/CDK comlexes

Precise control during all cell cycle phases and checkpoints is highly important for maintaining the physiological functions of a living organism. The main controlling proteins in the progression through the cell cycle are Cyclin/Cyclin-dependent kinase complexes and their inhibitors cyclin dependent kinse inhibitors [2].
Cyclin-dependent kinases (CDK) are enzymes acting on the transition point of the cell cycle and always need to form a complex with a regulatory subunit, a cyclin, in order to be active. The interaction of cyclin/CDK complexes serves to phosphorylate intracellular proteins, which are involved in the regulation of different cell cycle events. Assembly and disassembly of phase specific cyclin/Cdk-complexes regulates the progression through the cell cycle [7].
Tree kinds of D-type cyclins (D1, D2 and D3) form a complex and regulate the early G1 phase with one of the two CDK subunits CDK4 or CDK6. Activated

CDK4 or CDK6 phosphorylate transcriptional repressors from the retinoblastoma (Rb) family (pRb, p103 and p107) releasing their inhibitory effect on E2F transcription factors [7-10]. Active E2F transcription factors trigger the expression of cell cycle genes like cyclin E and A. The E-type cyclins (E1 and E2) interact with CDK2 in the late G1 phase and are necessary for further Rb phosphorylation and activation of E2F regulated genes and for passing through the restriction point, initiating S Phase entry [7-9]. A or B-type cyclins in complex with CDK1 are required for transit through G2-M. A-type cyclins bind to CDK2 at the end of the S phase and to CDK1 at the beginning of G2, whereas B-type cyclins activate CDK1 during the end of the G2 phase and the transition to mitosis [9, 11, 12] (Fig. 1).

1.1.3. Cell cycle control: CDK inhibitors

Correct functioning of the cell cycle machinery requires precise up- or down regulation of CDK activity. One of the strategies for negative regulation involves the binding of small inhibitory subunits of the CKI (cyclin-dependent kinase inhibitor) families (Fig. 1). CKI belong to either the INK4- or the CIP/KIP family [2].

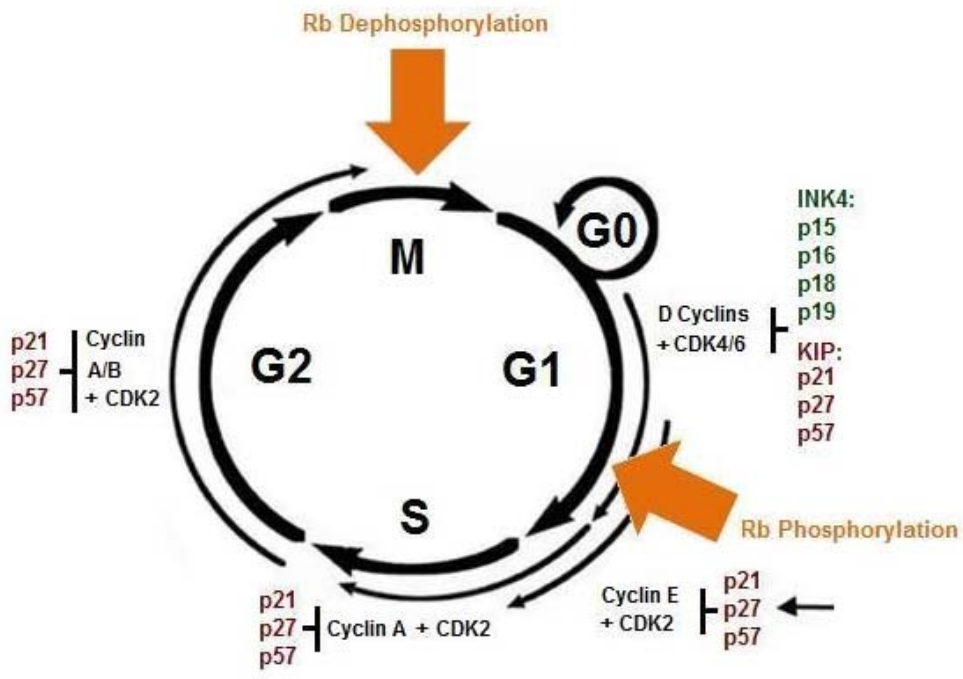


Figure 1: Regulators of the cell cycle

(Modified from Donovan and Slingerland, 2000 [13]).

INK4 Family

The INK4 family consists of $p16^{Ink4a}$ (p16), $p15^{Ink4Bb}$ (p15), $p18^{Ink4c}$ (p18) and $p19^{Ink4d}$ (p19), which bind CDK4 and CDK6 and inhibit their activation in complexes with D-type cyclins [8] and $p19^{ARF}$, which is not a CKI and positively regulates the transcription factor p53 [14].

CIP/KIP Family

The CIP/KIP family members $p21^{Cip}$ (p21), $p27^{Kip1}$ (p27) and $p57^{Kip2}$ (p57) can deactivate all of the CDK involved in cell cycle progression [8]. They display different affinity to the cyclin-CDK complexes, play different roles in cell cycle regulation and bind, in contrast to INK4 family members, to the whole cyclin/CDK complex [15-17]. The CKI from the CIP/KIP family inhibit mainly G1 cyclin/CDK complexes and cyclinB/CDK1 complexes [8, 18]. The CKI p21 acts also as inhibitor of DNA expression by repressing the proliferating cell nuclear antigen (PCNA) [19, 20]. CKI themselves are regulated upon external or internal signals. For example, the p21 promotor comprises a p53 binding site, so that p53 can activate the expression of the p21 gene causing a DNA damage induced cell cycle arrest in G1 or G2 [21, 22], whereas expression of p27 is mostly increased in the absence of external mitogen signals and at quiescence phases [23, 24]. The CKI p57 is specifically expressed in certain tissues during the embryonic development and in the adult organism [16, 25] and is the only CKI required in embryogenesis [26, 27].

Phenotypes of mice lacking the different CKI from the CIP/KIP family underline their role and importance in the regulation of cell reproduction. Knockouts for p27 exhibit multiple organ hyperplasia and increased body size [28]. Cells from *p21*-/- mice are disturbed at the G1 checkpoint and not capable to undergo a DNA-damage-induced cell cycle arrest [29]. And *p57*-/- embryos display hyperplasia in different organs and altered differentiation and proliferation [27].

1.2. Hematopoiesis

The mammalian blood system consists of more than 10 different mature cell types, including erythrocytes, thrombocytes, T- and B lymphocytes, natural killer cells, granulocytes, monocytes and dendritic cells. Different kinds of blood cells are required for different functions in the organism. The red blood cells

(erythrocytes) are responsible for oxygen transport, the white blood cells (leukocytes) are important for the immune response and the platelets (thrombocytes) are involved in blood clotting. The strictly regulated process of blood cells formation is called hematopoiesis. All mature blood cell types arise from a small population of multipotent hematopoietic stem cells (HSC) which reside in the bone marrow, persist for a life-time and form the beginning of a hierarchical organization [30].

1.2.1. Hematopoietic stem cells

When blood cells are lost or turned over, the dormant HSC are initiated to divide. Toward internal and external signals, the multipotent HSC can differentiate and develop to hematopoietic progenitor cells (HPC), which on their side, differentiate further, become lineage restricted and give rise to all mature blood cells [31, 32]. Since HSC replenish the blood system throughout lifetime, they need to self-renew in order to maintain their stem cell character. Self-renewal is together with quiescence the most important characteristic of HSC and is experimentally defined as the capacity for long term reconstitution of all blood lineages upon transplantation into a recipient [33].

Developmental origin of HSC

Before reaching their quiescent state in the adult bone marrow, HSC pass through an active cell cycling and proliferation phase during embryogenesis in order to generate the blood system [32, 34]. Primitive hematopoiesis in the mice embryo involve rapid production of red blood cells for oxygen transport and little HSC activity and takes place in the yolk sac at embryonic day E7,5 [35, 36]. The so called “definitive” hematopoiesis, the generation of all blood lineages, is a longsome process and occurs in different regions of the embryo. Definitive HSC could be isolated from the aorta-gonad-mesonephros (AGM) region and the placenta by E8,5 and from the yolk sac by E10 [37-39]. Later, HSC expand in the fetal liver till the end of the embryonic life [36]. By E17,5 and through the first two weeks of postnatal life, HSC leave the liver and colonize the bones [32, 40].

The hematopoietic hierarchy

During years of research, using cell surface marker phenotype, scientists identified and isolated distinct subpopulations of blood cells. Specific assays delivered information about function, developmental stage and self-renewal, respectively differentiation ability of the different blood cell types. The analysis suggested a hierarchical organization during hematopoietic development with progressive restriction of self-renewal capacity at each further hierarchical step (Fig. 2 and [41]).

Long-term HSC (LT-HSC) are the most primitive compartment in the hierarchy (Fig. 2). LT-HSC can undergo asymmetric divisions and either self-renew into identical copies of themselves and maintain their stem cell qualities, or differentiate into short term HSC (ST-HSC) [31, 42]. ST-HSC have a shorter life span and lower self-renewal capacity and can differentiate into multipotent progenitor cells (MPP). MPP possess very low to no self-renewal potential and are capable of differentiation and commitment into either lymphoid or myeloid lineage restricted progenitors: common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLP and CMP give rise to all of the mature blood cell types [31, 42]. T, B lymphocytes, natural killer cells and the antigen presenting dendritic cells develop from CLP. The CMP commit either to GMP (granulocyte monocyte committed progenitors) or to MEP (megakaryocyte erythrocyte progenitors). The GMP develop to granulocytes, monocytes and dendritic cells and the MEP give rise to erythrocytes and platelets [31, 42].

Surface marker in HSC/HPC

The different blood cell types can be identified on the basis of phenotypic surface markers using flow cytometry. All HSC are negative for lineage markers (Lin-) and express the stem cell antigen 1 (Sca1 or Ly6A/E) and the receptor for stem cell factor (c-Kit or CD117) on their surface (Fig. 1). Therefore HSC are also called LSK (Lin-Sca1+cKit+) [31, 43, 44]. During the last decade, further surface markers were established and the definition of LT- and ST-HSC has been defined more and more precisely.

LT-HSC are enriched in the CD34-/low and receptor tyrosine kinase, (Flk2) negative LSK fraction, while ST-HSC can be found in the CD34+ Flk2+ LSK subset [44, 45]. Although low expression of Thy1.1 is a marker in all LSK cells

[42], the loss of Thy1.1 surface protein together with gain of Flk2 is shown to mark the loss of self-renewal potential in HSC [45]. LT-HSC can be distinguished from ST-HSC also through expression of the SLAM-family markers CD150 and CD48, whereas the early LT-HSC are negative for CD48 or CD34 and positive for CD150 [46-48].

The further differentiation state in the hierarchy, the MPP, is also defined as Lin- and c-Kit+, but the cells express either low or no Sca1 on their surface (Sca1 $Sca1^{lo}$/-) and interleukin 7 receptor α (IL7Rα) is a typical marker used to differentiate the myeoloid and lymphoid progenitor lineages, as CLP are shown to be Thy1.1- and IL7Rα+ [49]. The IL7Rα- fraction of MPP includes the three kinds of myeloid progenitors which on their side are being distinguished by the expression of CD34 and Fcγ Receptor (CD16/32). CMP are CD34+ CD16/23-, GMP are CD34+ $CD16/32^{hi}$ and MEP are CD34- $CD16/32^{lo}$ [42, 50].

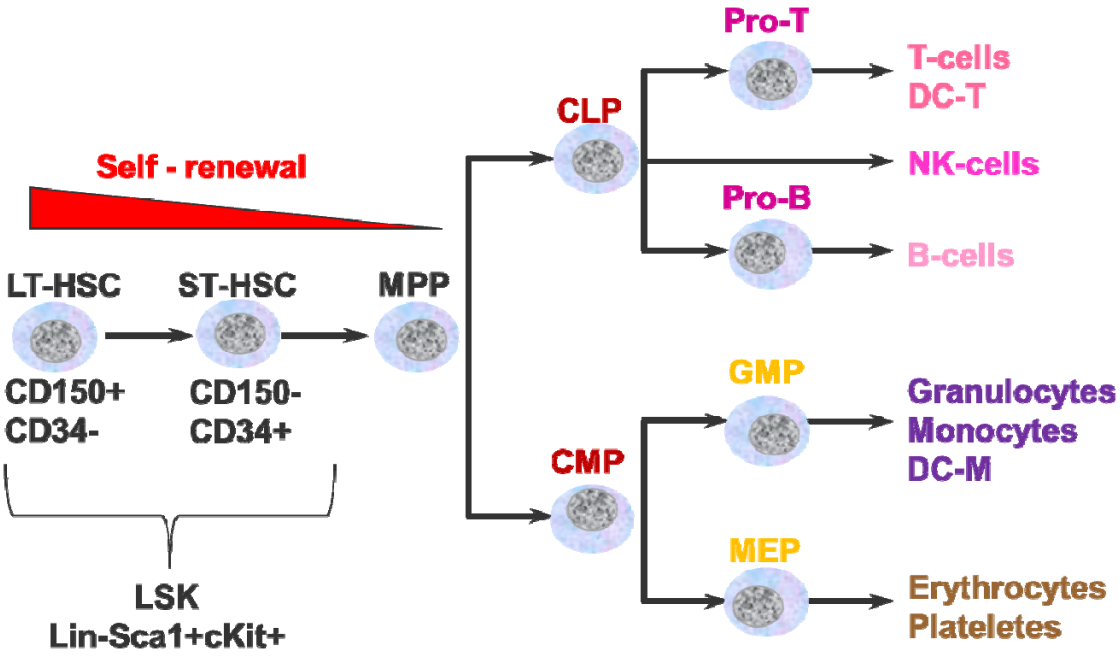


Figure 2: Hematopoietical tree in mice
(Modified from Shizuru et. Al., 2005 [42]).

1.2.2. Regulatory signals in HSC

The production of close to 10^{12} mature blood cells per day from the HSC/HPC pool requires a tightly regulated adjustment to intrinsic and environmental stimuli [30, 51, 52]. Regulation of the cell cycle plays a key role at each step of

hematopoiesis and also serves as a checkpoint towards uncontrolled cell proliferation that might lead to blood malignancies [32].

HSC reside in a specific microenvironment in the bone marrow, the so called niche, which is highly important for the regulation of HSC behavior through extrinsic signals. The hematopoietic stem cell niche is a complex microenvironment, consisting of different cell types and extracellular elements [53, 54]. Molecular cross-talk between HSC and the cells in the niche environment is carried out through a direct cell contact, growth factors and cytokines or components of the extracellular matrix and is of major importance for the developmental fate of the HSC [53, 55].

Growth factors and cytokines

A vast number of growth factors and cytokines promoting hematopoiesis is produced in the HSC niche environment. For instance, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interleukin 6 (IL6), FMS related tyrosine kinase 3 ligand (Flt3L) and stem cell factor (SCF) are expressed in the bone marrow endothelial cells [56, 57]. SCF, which binds and activate the c-Kit receptor, plays an important role in maintaining the long term repopulating activity of HSC [58, 59]. In the bone marrow, SCF is also being produced in osteoblastic cells and nestin-expressing mesenchymal stromal cells (MSC) [57]. Osteoblastic cells express other important factors for maintenance of the long term repopulating activity and quiescence in HSC such as thrombopoietin (TPO) and angiopoietin 1 (Ang-1), whereas Ang-1 is also expressed in nestin-expressing MSC [57]. Some inflammatory cytokines are also playing role in regulating HSC, like the chemokine C-X-C motive chemokine 12 (CXCL12), which is expressed in CXCL12-abundant reticular cells (so called CAR cells), nestin expressing MSC, osteoblastic cells and endothelial cells. CXCL12 regulates the pool size, the homing and the retention of HSC [57, 60, 61].

TGF-β pathway

Transforming growth factor β (TGF-β) is a protein with a lot of functions, including cell differentiation, embryonic development and regulation of the

immune system, tumor development and angiogenesis. TGF-β plays a key role in regulating HSC [62, 63]. TGF-β exists in three isoforms, TGF-β1, TGF-β2 and TGF-β3, which bind to two different receptors, TβRI and TβRII and activate intracellular proteins, so called Smads. Smads are organized in two branches Smad2/3 and Smad 1/5/8, which transduce the TGF-β signal downstream to Smad4, which translocates to the nucleus and activates gene transcription [62]. A number of studies prove that TGF-β signaling can maintain HSC quiescence during steady state hematopoiesis [63-68].

Pten/Akt pathway

Protein kinase B (Akt) signaling, the so called phosphatidylinositol-3-OH kinase (PIK3)-Akt pathway is activated by growth factors and regulates proliferation, survival, growth, metabolic functions and resistance to stress. The phosphatase and tensin homolog (Pten) negatively regulates the PIK3-Akt pathway by inhibiting the activation of Akt. [62]. Pten and Akt have been shown to be important regulators of hematopoiesis and HSC. Pten influences the LT-HSC pool and loss of Pten leads to myeloproliferative disorder [69-71], while both members of the Akt family, Akt1 and Akt2, are essential for maintaining the HSC functions [72]. Furthermore, a downstream target in the Pten/Akt pathway, Forkhead box O (FoxO) transcription factor, is also a significant regulator of the HSC pool, self-renewal and reconstitution ability [73, 74].

Wnt pathway

The Wnt protein family include secreted ligand molecules, which can bind to surface receptors and subsequently induce canonical and noncanonical branches of the Wnt- dependent pathway [62]. Wnt sinaling is highly important in emryogenesis and tumorigenesis [75]. A vast number of research in the field of Wnt signaling and hematopoiesis demonstrate the significance of the Wnt pathway in regulating the HSC. For instance, the canonical Wnt signaling is shown to be important for regulation of proliferation and maintenance of the HSC through the niche [76]. Besides, loss of β-catenin, a protein downstream in the canonical pathway, leads to decreased long-term repopulation efficiency of HSC [77]. Furthermore, Sugimura et. al. demonstrate, that the noncanonical Wnt-signalling maintains HSC in the niche [78].

1.2.3. Regulation of the cell cycle in HSC

An essential characteristic of the HSC is the balance between dormancy and active cycling. Depending on cell extrinsic and cell intrinsic factors, HSC can remain quiescent, self-renew, differentiate, migrate or die. In a steady state condition adult HSC are most of the time quiescent [79]. HSC are constantly required to replenish the blood system since mature blood cells have a short life span [42]. Also, in case of injuries and other hematopoietic stress conditions, quiescent HSC are induced upon external and internal signals to enter the cell cycle [42, 80]. The intrinsic mechanisms regulating the cell cycle in HSC include Cyclin/CDK complexes, Rb proteins and CKI and are together with the extrinsic factors subject of intensive research (Fig. 1).

Studies of knockout mice for the protein of interest deliver knowledge about the role of the different cell cycle regulators in the complex hematopoietic machinery. Mice, that lack cell cycle regulators, display modest to severe phenotypes which affect different levels of the HSC/HPC hierarchy [11, 12, 81].

Cyclin/CDK complexes

Since activating the switch from dormancy to cell division involves entering the cell cycle in G1 phase, the G0-G1 checkpoint is the most important part of HSC regulation. Progression to G1 is controlled by CDK4 and CDK6 kinases and the cyclin D family members cyclin D1, cyclin D2 and cyclin D3 [82]. Knockout mice for all three cyclin D family members die during embryogenesis because of heart defects and hematopoietic failure [83]. The absolute numbers and the frequency of cycling HSC and HPC cells are reduced in *cyclin D1/2/3*-/- fetal liver (FL), where at embryonic days 13-14 the massive production of various fetal HSC cells occurs [83, 84]. Furthermore, *cyclin D1/2/3*-/- FL cells could not reconstitute irradiated recipient mice [83] while one of the most important HSC characteristics is the ability to long-term reconstitute the hematopoietic system in irradiated host [85]. Double knockouts for CDK4/CDK6 display similar defects in the FL hematopoiesis as the cyclin D knockouts [86]. In contrast, CDK2 and CDK6 loss together or separately seem not to play a role in adult HSC regulation [86, 87].

Rb family

The Rb family members (pRb, p103 and p107) play an essential role in progression through G1 phase. Only upon phosphorylation by cyclin D-CDK4/6 complexes and releasing the inhibitory effect of Rb family members on E2F transcription factors, target genes that are necessary for the progression through G1 can be expressed [8]. Interestingly, deleting only one of the family members did not result in severe defects [82, 88-90]. Though a triple knockout mouse for all three family members led to death of the animals at week 4-12 after gene inactivation caused by myeloid expansion. The triple pRb, p107 and p103 knockout HSC were increased in absolute cell number, exhibited severe defects in self-renewal and highly impaired reconstitution potential [91].

CKI from the INK4 family

Studies with knockout mice for different members of the INK4 family, demonstrate, that p16, p18 and p19 are differentially regulated in HSC to maintain balance between quiescence and proliferation [32, 82]. Deletion of both p16 and p19 has no severe effect on HSC activity [82], whereas HSC lacking p18 display elevated engraftment capability in serial transplantations [92]. Nevertheless, a regulation of the HSC by p16 was demonstrated in older mice, where loss of p16 led to increase in the HSC pool [92, 93].

CKI from the CIP/KIP family

Since CKI from the CIP/KIP family also regulate the entry into G1, their effects on HSC regulation has also been an attractive target for investigation. Reports from mutant mouse strains have demonstrated that although all members are involved in the regulation of quiescence of hematopoietic cells under steady state conditions [94], the main function of the CIP/KIP family members appears to be the regulation of cell cycle during periods of hematopoietic stress [95, 96]. For instance, p21 was associated with regulation of HSC at the entry into the cell cycle [97], though a more recent study demonstrates a role of p21 in conditions of stress and DNA damage [98]. Another CIP/KIP family member, p27, has been shown to be important for regulation of cell cycle activity of more committed HPC and lack of p27 alone did not affect HSC number or cycling [99]. In contrast, p57 seem to play a key role in regulating HSC and p57

expression is shown to be highest in this early subpopulation of hematopoietic cells [100, 101]. Conditional deletion of p57 in hematopoietic cells resulted in decreased adult HPC pool and loss of HSC quiescence demonstrating, that p57 is a critical mediator of HSC quiescence [100]. Also, the *p57*-/- phenotype could be compensated with overexpression of p27, but not p21 [100, 101]. Interestingly, p21 and p57 seem to be direct targets for growth repressive signals, such as TGF-β, demonstrating a role of the hematopoietic niche in CKI regulation [102]. In particular, the regulation of the HSC quiescence through p57 and p27 seems to take place upstream of the Rb family [101]. Taken together, these studies implicate overlapping and unique roles of different CIP/KIP family members in regulation of HSC.

The vast number of studies on cell cycle regulators such as CKI and Rb family members reveal in general, that loss of cell cycle inhibitors lead to increased cycling and decreased quiescence in HSC and therefore to loss or decrease of self-renewal potential.

1.2.4. Chronic myeloid leukemia, a HSC disease

Cancer of the blood or bone marrow is characterized by an abnormal proliferation and increase of white blood cells. Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder, which originates from a HSC and is caused by the so called Philadelphia chromosome [103-105]. The Philadelphia chromosome is generated by a reciprocal translocation between the Abelson (*ABL*) gene on chromosome 9 and the brake point cluster region (*BCR*) on chromosome 22, creating *BCR-ABL*, a hybrid oncogene coding for the BCR-ABL protein, a constitutively active tyrosine kinase [106-108]. Deregulated signaling through the BCR-ABL kinase leads to uncontrolled increase in proliferation and survival of leukemic cells. Chronic phase of CML is characterized by an increase of predominantly myeloid precursors, which differentiate to abnormal numbers of granulocytes [109]. Untreated chronic phase leads to the accelerate phase, where irregular amount of progenitor/precursor cells are generated, followed by a terminal phase, called blast crisis [109]. The blast crisis is defined by expansion of myeloid or lymphoid

differentiation-arrested blast cells, presence of immature cells in the blood and lower response to treatment [109, 110].

Currently there are several tyrosine kinase inhibitors applied in the treatment against CML [111]. Imatinib (also STI571 or Gleevec), for instance, is a first generation tyrosine kinase inhibitor, which acts by binding to the catalytic side of BCR-ABL and prevents initiation of the signaling pathway [112]. Imatinib is used successfully to treat patients in the chronic phase of CML since 2001 but may have to be applied through a lifetime, since it does not always cure CML, but only inhibits the proliferation of the leukemic cells [113]. Also, in some cases resistance against imatinib is developed [111]. Quiescent leukemic stem cells are very often insensitive against the drug and capable to sustain CML by providing a pool of leukemic cells [114-116]. Second class tyrosine kinase inhibitors are more specific against progenitors, but also do not always cure the disease and resistances are still possible [111]. Thus, since uncontrolled HSC/HPC regenerative activity is a hallmark of CML and often the cause for resistance against the common drugs, it is important to investigate the driving forces in these cells in order to target CML stem cells for providing cure against the disease.

1.3. SCF^{Skp2}-Cks1

CDK-regulatory subunit 1 (CKS1), a small 9 kDa protein, is a member of the Suc1 (suppressor of *Cdc2* mutation)/Cks family and was discovered in budding yeast [117] as a homolog of the Suc1 gene, which was found in fission yeast [118]. Suc1 and Cks1 are shown to exhibit high affinity against CDK1 [119, 120] and to regulate the cell cycle and especially mitosis [118, 121]. Mutant Cks1 deprived of its function results in severe G1/S and G2/M transition defects as well as in mitotic failure in yeast and *Xenopus* extracts [121, 122].

The Cks family in mammalian cells consists of two members, Cks1 and Cks2, [123]. Concomitant loss of both proteins is lethal for mouse embryos at very early stage [124]. Isolation and analysis of human Cks proteins revealed, that Cks1 and Cks2 are highly conserved in eukaryotes and possess affinity against CDK1, CDK2 and CDK3 [123]. More precisely, Cks1 seems to affect the activity of cyclin-CDK1 towards specific targets [125] and enhances phosphorylation of

some CDK1 substrates [120, 125]. Another significant role of Cks proteins in mitosis involves checkpoint independent anaphase-promoting complex/cyclosome (APC/C) control through targeting cyclin A/CDC20 complexes [126]. Additionally, it was demonstrated that Cks1 is important for targeting cyclin B to the proteasome for degradation in budding yeast [127].

Nevertheless, Cks1 exhibit CDK independent functions in mammalian cells as a part of a complex, which is involved in degradation of CKI [128]

1.3.1. Ubiquitination and the SCF^{Skp2} complex

Protein turnover through synthesis and degradation is of major significance for accomplishing the majority of cellular processes. Degradation of most of the intracellular proteins in eukaryotes is processed through the ubiquitin (Ub) proteasome pathway [129]. In order to be recognized and degraded by the 26S proteasome, proteins need to be labeled with covalently bound Ub molecules, a process called ubiquitination [129]. Ubiquitination involves three major enzymes E1 activating enzyme, E2 conjugating enzyme and E3 ubiquitin ligases [130, 131]. E3 ligases are responsible for transferring activated Ub molecules from the E2 conjugating enzyme yielding an Ub chain onto proteins targeted for degradation [130, 131]. Most of the E3 ligases contain so called RING finger domains and are either monomeric enzymes or many subunits containing complexes [129]. Cullin-RING ligases built the largest group of E3 enzymes [132] and a subgroup of cullin-RING ligases, the Skp1-Cul1-Fbox (SCF) complexes are the best understood ones [129]. The F-Box protein in the complex is a variable component which contains a substrate-binding motive and function as substrate recognition subunit [130, 133]. In many cases, phosphorylation of the substrate is needed in order to be recognized by the F-Box subunit [130]. For instance, in the SCF^{Skp2} complex, T187 phosphorylated p27 is targeted by the F-Box protein Skp2 (Fig. 3 and [134]). Importantly, an additional protein in the complex was found to be essential for the recognition and the following ubiquitination of phosphorylated p27, namely Cks1 [128, 135].

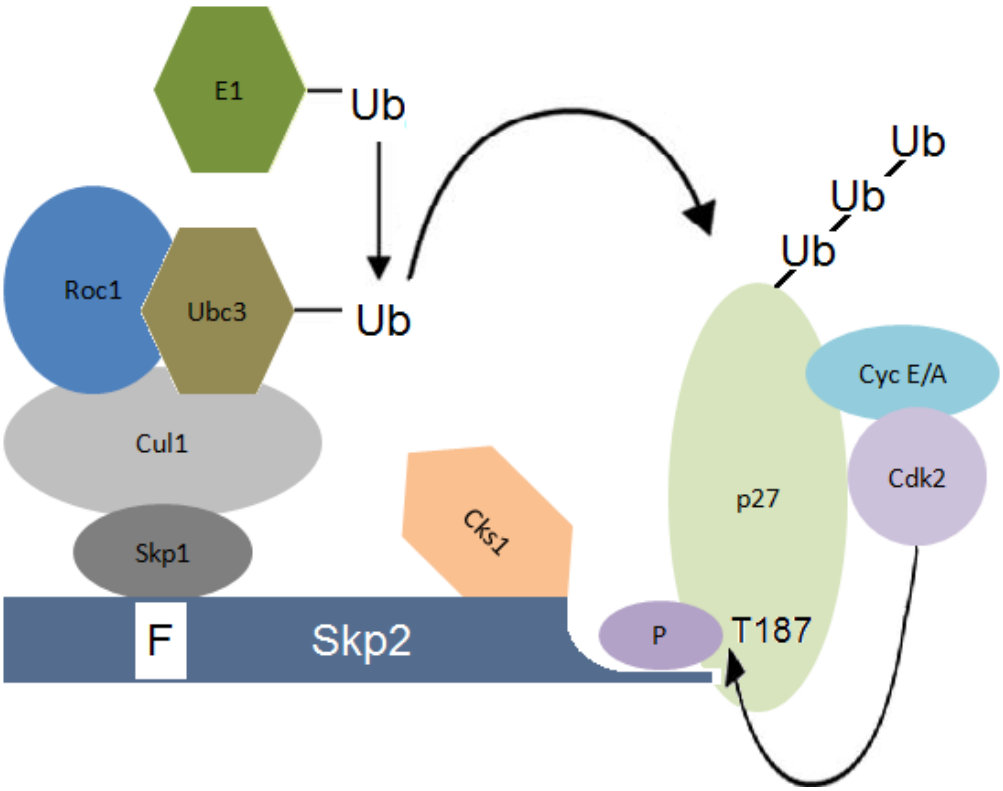


Figure 3: SCF^{Skp2} – induced p27 degradation
(Modified from Bartek and Lukas 2001b [136]).

1.3.2. SCF^{Skp2}-Cks1 and its role in cell cycle control

Since regulation of CKI occurs mainly at post-translational level through control of protein stability, ubiquitination and subsequent degradation of these cell cycle regulators is of major importance in controlling the cycling process.

As part of the SCF^{Skp2} complex, Cks1 interacts with Skp2, inducing allosteric changes in the F-box protein and increasing its affinity to cycline A/E CDK2 kinase phosphorylated p27 [137, 138]. Thus, Cks1 enables the ubiquitination of p27 through the SCF^{Skp2} ubiquitin ligase complex [136]. Therefore, loss of Cks1 in mice results in reduced body size and decreased proliferation associated with an accumulation of p27 [128].

In addition to the regulation of p27 protein levels, SCF^{Skp2} has further been associated with the post-translational regulation of other CKI including p21 [139, 140], p57 [141], and the Rb family member p130 [142]. The SCF^{Skp2} subunit Cks1 is not only a crucial component of SCF^{Skp2}- mediated ubiquitination but is also involved in regulation of CDK1 transcription [143]. Furthermore, loss of Cks1 results in reduced CDK2 kinase activity and defect S Phase entry [144].

1.3.3. Regulation of Cks1 expression

The SCF^{Skp2} ubiquitin ligase complex is involved in the cell cycle through targeting cell cycle regulators for degradation, hence transcriptional regulation of Skp2 and Cks1 is part of the cell cycle control. The forkhead box m1 (*Foxm1*) gene, which functions are important for the G1/S transition and especially for mitotic progression, regulates amongst others transcription of Skp2 and Cks1 [145]. Additionally, Cks1 is regulated through the cycle-dependent element and cell cycle gene homology region, the so called CDE/CHR tandem element in the Cks1 promoter [146]. Promoters containing CDE/CHR element control cell cycle genes and their expression depending on the cell cycle status and are typically activated through several CCAAT elements [146]. Trimeric nuclear factor-Y (NF-Y) binds to CCAAT boxes and acts as main activator, hence NF-Y could be observed as the main activator of Cks1 [146]. On the other hand, p53 downregulates the expression of *Cks1,* whereas its repression is not influenced by NF-Y [146]. Furthermore, Cks1 regulation occurs through TGF-β signaling [147]. As pointed out before (chapter 1.2.2), the TGF-β pathway plays an important role in regulating the cell cycle and is important for maintaining quiescence in HSC. Through downregulation of Cks1 and Skp2 TGF-β signaling also inhibits the SCF^{Skp2} ubiquitin ligase activity [147]. The oncogenic transcriptional factor Myc is inducing Cks1, Skp2 and Cul1 expression and thus initiates p27 degradation, cell proliferation and lymphomagenesis [148].
Regulation of Cks1 also occurs on protein level through the ubiquitin-proteasome pathway and in a cell cycle dependent manner [149]. Protein levels of the substrate recognition subunits of the SCF^{Skp2} ubiquitin ligase, Cks1 and Skp2 are also shown to decrease in late M phase and G1 and increase again in S phase [150]. Degradation of these proteins via the APC/C(Cdh1) ubiquitin ligase at the specific time point of the cell cycle prohibit degradation of SCF^{Skp2} targets, such as CKI, and premature entering in the next cell cycle phase [151].

1.3.4. Cks1 in cancer

Cancer is caused by genetic defects and following failure to control cell proliferation [2]. Since Cks1 is an essential regulative element in the complicated organization of cell cycle control, its expression is disturbed in vast

number of cancer types. In general, Cks1 is overexpressed and correlates with poor prognosis in cancer [152].

For instance, Cks1 is overexpressed in gastric carcinoma [153], non-small cell lung carcinomas [154], colorectal carcinoma [155], ovarian cancer [156] as well as in breast tumors [157]. Gene amplification and overexpression of Cks1 in breast tumors is associated with decrease in p27 levels, lymph node metastasis and poor prognosis [157, 158]. Furthermore, the resistance of esophageal squamous cell carcinoma against radiotherapy is increased in patients with high Cks1 protein levels [159].

Searching for a mechanism of action of Cks1 in cancer revealed that up-regulated Cks1 as well as Cks2 levels induce uncontrolled cell division probably partly through disturbing the S Phase checkpoint by binding to CDK2 and hindering inhibitory phosphorylation [160]. Also, it was shown recently that inhibitors of the SCF^{Skp2} complex block estrogen induced growth and p27 degradation [161], providing a possible treatment in cancers characterized by SCF^{Skp2} induced p27 degradation.

Moreover, Cks1 controls p27 levels in premalignant Eμ-*Myc* B cells, and loss of Cks1 reduces proliferation and delays lymphoma development and dissemination of the disease [148]. Despite the fact that Cks1 is required for tumor cell proliferation, Cks1 on its own is not sufficient to induce hematopoietic malignances [162].

1.4. Aim of this thesis

Cyclin/CDK complexes and their inhibitory CKI are important factors for the proper cell cycle progression in HSC/HPC. Considering the importance of SCF^{Skp2}-mediated degradation of CKI family members in regulation of the cell cycle in HSC/HPC, this project investigates the role of Cks1, the rate limiting component of the SCF^{Skp2} complex in early hematopoiesis.

The aim of this project was to analyze the effects of Cks1 loss on the quality and quantity of HSC/HPC by using *in vitro* and *in vivo* methods. In addition, using double knockouts for Cks1 and p27, it was evaluated, which of the observed effects after Cks1 loss were p27 dependent. Furthermore, the regulation of the human *CKS1B* gene in the myeloproliferative disorder CML

was studied and the role of Cks1 for BCR-ABL mediated clonogenic activity was examined.

2. Materials

2.1. Mice

All animal experiments were performed in accordance with the regional animal ethics committee approvals.
Cks1-/- mice [128] and control WT littermates were bred on a (129S x C57BL/6J) F1 (129B6, Ly5.2) background.
Cks1-/- mice (C57Bl6-129 mixed background) were bred to $p27^{Kip1}$-/- mice (C57BL/6J) [28] for >6 generations to generate *Cks1-/- p27*^{Kip1}*-/-* (DKO) and control animals on a mixed C57BL/6-129 background. Since loss of p27 leads to ovulatory effect and sterility [28], only p27+/- female mice were used in the breeding.
In transplantation experiments 129S2xC57BL/6.SJL/J (129Ly5.1) mice were used as recipients.

2.2. Cell lines

EL08-1D2 [163]	stromal cell line derived from murine embryonic liver (PD Dr. Robert Oostendorp, Klinikim rechts der Isar)
NX (Phoenix) Eco 293T [164]	cell line with vectors for retroviral packaging and envelope protein infectious for murine cells (Dr. Garry P. Nolan Ph D, Standford, USA)
BaF3-p210 [165, 166]	murine bone marrow-derived pro-B-cell line expressing WT p210 Bcr-Abl (Prof. Dr. Nikolas von Bubnoff, Albert-Ludwigs-Universität, Freiburg)
BaF3-*ts* p210 [165, 167]	murine bone marrow-derived pro-B-cell line expressing termo sensitive mutant of p210

Bcr-Abl, which is active at 23°C (Prof. Dr. Nikolas von Bubnoff, Albert-Ludwigs-Universität, Freiburg)

2.3. Bacteria

One Shot TOP10 Chemically Competent *E. coli* (Life Technologies)

2.4. Vectors

MSCV-IRES-Bcr-Abl-p210-GFP was kindly provided from Prof. Dr. Nikolas von Bubnoff, Albert-Ludwigs-Universität, Freiburg

2.5. Antibodies

Table1: Antibodies used for surface staining in flow cytometry

Antigen	Clone	Colour	Company
B220	RA3-6B2	PE-Cy7	eBioscience
CD4	GK1.5	PE-Cy5	eBioscience
CD8a	53-67	PE-Cy5	eBioscience
CD16/32	93	PE	eBioscience
CD34	RAM34	FITC	eBioscience
		APC	eBioscience
CD45.1	A20	PE	eBioscience
		eFluor®780	eBioscience
CD45.2	104	FITC	eBioscience
cKit	2B8	PE	eBioscience
		APC	eBioscience
		APC-Cy7	eBioscience
Sca1	D7	PE-Cy7	eBioscience
CD11B	M1/70	APC-eFluor®780	eBioscience
Gr1	RB6-8C5	PE	eBioscience
		eFluor®450	eBioscience
CD150 (SLAM)	TC15-12F12.2	APC	BioLegend
lineage (CD3ε, B220, Ter119, Gr-1, Cd11b)		biotinylated	eBioscience

Table 2: Antibodies used for intracellular staining in flow cytometry

Antigen	Clone	Colour	Company
p21	C-19	AlexaFluor®488	Santa Cruz
p27	F-8	AlexaFluor®488	Santa Cruz
p57	H-91		Santa Cruz
p130	C-20		Santa Cruz

Table 3: Antibodies used for western blotting

Antigen	Clone	species	Company
p21	C-19	rabbit	Santa Cruz
p27	F-8	mouse	Santa Cruz
p57	H-91	rabbit	Santa Cruz
p130	C-20	rabbit	Santa Cruz
Cks1		rabbit	Life Technologies
Abl	8E9	mouse	BD Bioscience

Secondary Antibodies:

Straptavidine, eFluor®450 conjugated, eBioscience

Anti-rabbit IgG, AlexaFluor®488 conjugated, Life Technologies

Anti-rabbit IgG, Santa Cruz

Anti-rabbit IgG, AlexaFluor®488 conjugated, Santa Cruz

Anti-mouse IgG, AlexaFluor®488 conjugated, Santa Cruz

Anti-rabbit IgG, HRP conjugated, GE Heatlthcare UK limited

Anti-mouse IgG, HRP conjugated, GE Heatlthcare UK limited

2.6. Primer

Primer used for genotyping and (B)Real Time PCR:

Name	Sequence 5'→3'
Cks1-1	CGGAGGATGTAAAACAATGAAA
Cks1-2	GGGAAATCGCCACAACAAAG
Cks1-Neo	CTGTGGTTTCCAAATGTGTCA
mgK3	TGGAACCCTGTGCCATCTCTAT
mcK5	GAGCAGACGCCCAAGAAGC
p27-Neo	CCTTCTATGGCCTTCTTGACG

Primer used for Real Time PCR:

Name	Sequence 5'→3'
CKS1B (human) forward	GCTGAACGCCAAGATTCCTCCATT
CKS1B (human) reverse	TTCGGACAAATACGACGACGAGGA
UBIQUITIN (human) forward	ACCTGACCAGCAGCGTCTGATATT
UBIQUITIN (human) reverse	TCGCAGTTGTATTTCTGGGCAAGC
Cks1 (murine) forward	CGTTCAGCAGAGTCAGGGATGG
Cks1 (murine) reverse	AGTGAGGACAGCTGTGGACCG
Rpl13a (murine) forward	CCCTCCACCCTATGACAAGA
Rpl13a (murine) reverse	TTCTCCTCCAGAGTGGCTGT

2.7. Kits

Lineage cell depletion Kit, mouse	Miltenyi Biotec
CD117 MicroBeads, mouse	Miltenyi Biotec
FITC BrdU Flow Kit BD	Pharmingen
Qiagen® HiSpeed® Plasmid Maxi Kit	Qiagen
Bio-Rad Protein Assay	Bio-Rad
RNeasy Mini Kit	Qiagen
Dynabeads mRNA DIRECT	Life Technologies
Omniscript RT	Qiagen
QuantiTect RT	Qiagen
Qiaschredder	Qiagen
CYBR® Green PCR Master Mix	Life Technologies
Power SYBR® Green PCR Master Mix-	Applied Biosystems

2.8. Chemicals

β-Glycerophosphate	Sigma-Aldrich
2-Mercaptoethanol 50 mM	Life Technologies
Acryalamid/Bisacrylamid	Sigma-Aldrich
Agarose NEEO ultra quality Roti®Garose	Carl Roth

Ammoniumchloride (NH_4Cl)	Sigma-Aldrich
Ammoniumpersulfate (APS)	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Aphidicollin	Sigma-Aldrich
Bovine Serumalbumin (BSA)	Sigma-Aldrich
Bromphenol blue	Sigma-Aldrich
complete Mini (Protease Inhibitor Cocktail)	Roche
D-desthiobiotin solution (10x Buffer E)	IBA
Dimethylsulfoxid (DMSO)	Riedel-de Haën
Dithiothreitol (DTT)	Sigma-Aldrich
dNTP Mix (10mM each)	Fermentas
Dulbecco's phosphate buffered saline (DPBS)	Life Technologies
Ethanol	Riedel-de Haën
Ethidiumbromide	Carl Roth
Ethylene diamine tetra acetic acid (EDTA)	Carl Roth
Fetal bovine serum (FCS)	PAA Laboratories GmbH
Formaline (4 %)	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycin	Sigma-Aldrich
HEPES	Sigma-Aldrich
Isopropanol	Fluka
L-Glutamin 200 mM	Life Technologies
Lipofectamine® 2000	Life Technologies
Methanol	Sigma-Aldrich
Oligo(dT)12-18 Primer	Life Technologies
Opti-Mem®	Life Technologies
Penicillin/Streptomycin (P/S)	Life Technologies
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich
Platimum SYBR Green qPCR SuperMix-UDG	Life Technologies
Polybrene	Sigma-Aldrich
Propidium iodide (PI)	Sigma-Aldrich
RestoreTM Western Blot Stripping Buffer	Thermo Scientific
Ribonuclease A	Sigma-Aldrich
RNase OUT	Life Technologies

Sodium azide (NaN_3)	Sigma-Aldrich
Sodium chloride (NaCl)	Carl Roth
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium fluoride (NaF)	Sigma-Aldrich
Sodium orthovanadate (Na3VO4)	Sigma-Aldrich
SuperSignal West (Pico/Dura/Femto)	Pierce
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Tris(hydroxymethyl)-aminomethan	Carl Roth
Trypan blue 0,4 %	Life Technologies
Tween 20	Carl Roth

2.9. Equipment

CyAn ADP Lx 9 color analyzer	Beckman Coulter
MoFlo legacy 14 color cell sorter	Beckman Coulter
Animal Blood Counter (Vet abc™)	ScilVet
Cell incubator (Heraeus Hera cell 240)	Heraeus
Real time PCR machine (StepOnePlus™)	Applied Biosystems
PTC 200 thermal cycler	BioRad
4°C Fridge (Liebherr Premium)	Liebherr Hashaltsgeräte GmbH
-20°C Freezer (Liebherr Premium)	Liebherr Hausgeräte GmbH
-80°C Freezer(V.I.P.TM -86°C ULT, MDF-U52V)	SANYO Electric Biomedical Co.,
SDS Gelelectrophoresis chamber (Multigel Long)	Biometra GmbH
Glass plates (Multigel Long)	Biometra GmbH
Glassware Labware	SCHOTT AG
Incubator for bacteria (Infors HAT Minitron)	Infors GmbH
Microfuge (MiniSpin)	Eppendorf AG
Pipetboy Integra	Biosciences AG
Dissection instruments	Fine Science Tools GmbH
Laminar flow (HeraSafe HSP 18)	Heraeus
Radiographic film developer (Hyperprocessor)	Amersham Life Science
SONOPLUS Homogenisator (SONOPLUS HD 2070)	Bandelin electronic

Spectrophotometer (SmartSpecTM PLUS)	Bio-Rad
Nitrogen tank	Taylor-Wharton Germany GmbH
Thermomixer comfort	Eppendorf AG
Microfuge (Heraeus Biofuge fresco)	Heraeus
Microfuge (Heraeus Megafuge 3.0 RS)	Heraeus
Microfuge (Heraeus Multifuge 3s)	Heraeus
Counting cell chamber (Neubauer improved)	LO-Laboroptik

2.10. Software programs

FlowJo	TreeStar Inc
Graph Pad Prism	GraphPad Software Inc
Microsoft Excel	Microsoft Inc.
Microsoft PowerPoint	Microsoft Inc.
Microsoft Word	Microsoft Inc.
Adobe Acrobat	Adobe Systems Software Ireland Ltd.
RStudio	RStudio Inc.

3. Methods

3.1. Genotyping

The polymerase chain reaction (PCR) was used to determine the genotype of the bred mice (wild type (WT), Cks1 knockout (*Cks1*-/-), p27 knockout (*p27*-/-) and double knockouts for both Cks1 and p27 (DKO)). Small pieces of the ear (from ear clipping) were used as samples. 50 µl lysis buffer ((100 mM Tris/HCl (Carl Roth), pH 8.3, 500 mM KCl (Sigma-Aldrich), 0.1 mg/ml gelatine (Carl Roth), 1% NP40 (Carl Roth), 1% Tween 20 (Carl Roth)) and 200 µg/ml Proteinase K (Life Technologies) was added to the samples. In order to decompose the tissue, the samples were incubated at 50°C with constant shaking overnight. The next day, the Proteinase K was deactivated by the incubation of the samples at 95°C for 5 min, centrifuged at 15 000 g for 10 min and the supernatant, containing the DNA, was used for a PCR analysis.

Cks1 genotyping Master Mix:

1 µl DNA
0,6 µl Primer Cks1-2 [100 pmol/µl]
0,3 µl Primer Cks1-1 [100 pmol/µl]
0,3 µl Primer Cks1-Neo [100 pmol/µl]
2,5 µl10x Buffer
5 µl5x Q-Solution
0,7 µl dNTP [10 mM each]
1 µl $MgCl_2$
0,3 µl Taq
13,1 µl H_2O

Cks1 genotyping PCR program:

94°C 2 min
30 sec. 94°C
30 sec. 55°C } 30x
1 min. 72°C
10 min. 72 °C
4°C forever

For the mutant allele a fragment of approximately 200 bp should be amplified and for WT allele a fragment of approximately 150 bp should be amplified (Fig. 4A).

p27 genotyping Master Mix:

1 µl DNA
0,3 µl Primer 1 [100 pmol/µl]
0,3 µl Primer 2 [100 pmol/µl]
2 µl10x Buffer
4 µl5x Q Solution
0,7 µl dNTPs Mix (10 mM each)
0,3 µl Taq polymerase
11,4 µl H_2O
Primer Mix for WT allele: primer 1: mgK3, primer 2: mcK5.
Primer Mix for mutant allele: primer 1: mgK3, primer 2. p27-Neo

p27 genotyping PCR program:

preheating 95 °C
30 sec. 96°C
30 sec .57°C } 4x
2 min 65°C
30 sec. 93°C
30 sec. 57°C } 36x
2 min. 65°C
2 min. 65°C
4°C forever

The reaction for WT allele should amplify a fragment of 1 kB.
The reaction for mutant allele should amplify a fragment of 0,5 kB (Fig. 4B).

5 µl of loading buffer (Quiagen) was added to the samples before loading on a 1% agarose gel made with NaB buffer (0,01 M di-sodium tetraborate (Sigma-Aldrich)) and ethidium bromide (0.5 µg/ml, Carl Roth). The gel was run with 300 V and analyzed with a BioRad Gel-Doc XR Imaging System.

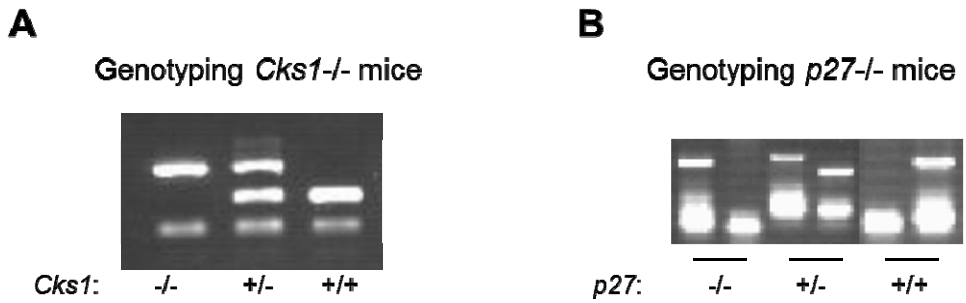


Figure 4: Genotyping of (A) *Cks1-/-* and (B) *p27-/-* mice.

3.2. Preparation of murine tissues

In this project, the organs to be analyzed were bone marrow, spleen, lymph nodes and blood.

For the bone marrow analysis, the 4 long bones (femurs and tibia) of both hind legs were extracted and flushed with HF2 buffer (HBSS (Life Technologies), 10 mM HEPES (Life Technologies), 2 % FCS (PAA), 100 U Penicillin, 100 µg streptomycin (Life Technologies)). The cell suspension was homogenized with a blunt needle and filtered through a 30 µm filcon (BD Biosciences).

For an analysis of spleen or lymph nodes, the organs were squished, and after homogenizing filtered through 30 µm filcons.

Homogenized bone marrow or spleen samples were centrifuged at 300 g for 5 min; the pellets were resuspended in 2 ml of ACK lysing buffer (Life Technologies) and incubated on ice for 5 min in order to lyse the erythrocytes. Samples were then centrifuged at 300 g for 5 min, resuspended in HF2 buffer and filtered again through a 30 µm filcon in order to remove chunk that developed after the erythroid lysation.

Blood was obtained from mice by punctuating the facial vein and collected in EDTA-coated vials. Blood samples were mixed with 10 x volume of ACK lysing buffer and incubated on ice for 15 min in order to lyse the erythrocytes. Samples were then centrifuged at 300 g for 5 min and resuspended in HF2 buffer and filtered through a filcon.

Fetal liver (FL) cells from WT and *Cks1-/-* 14,5 days old embryos were used in a colony forming assay. FL were squished, and after homogenizing filtered through 30 µm filcons. The cells were than directly plated in methylcellulose medium. The heads were used to genotype the embryos.

3.3. Transplantation assays

A transplantation assay is a fundamental method for studying hematopoietic differentiation and specifically HSC self-renewal potential when performed as serial transplantations [12]. The recipients are being lethally irradiated, which leads to ablation of most of the hematopoietic tissue and allows the transplant to engraft. Whole bone marrow samples or single sorted hematopoietic population are transplanted into the recipients in order to replenish the hematopoietic system. Serial transplantations, using donor cells from the primary recipients provide information about the self-renewal capacity of the HSC from the primary donor, since these cells sustain and repopulate the new donor only if they are able to self-renew in the previous recipient. Serial transplantations can be performed until the HSC from the primary donor are exhausted and no longer capable to repopulate an irradiated host [12]. In order to distinguish donor and recipient cells throughout the transplantation assays, mice with congenic expression of different isotypes of the hematopoietic CD45 marker were used.

In all transplantations *129S2xLy5.1* recipients were lethally irradiated with 9Gy (Gulmay irradiation unit). Donor cells were injected into the tail vain of the recipients. Serial whole bone marrow transplantations were performed using $2x10^5$ donor BM cells for primary transplants and $1x10^6$ for secondary transplants. In both cases $4x10^5$ helper spleen cells from Ly5.1 mice were co-transplanted. 300 CD150+ FACS-sorted LSK cells were transplanted with $5x10^5$ helper spleen cells and $1x10^5$ BM cells delivered from mice with the same background as the recipients. For secondary transplantations 300 donor-derived CD150+ LSK cells from the primary recipients were used with the same number of helper and competitor cells used in the primary transplantation.

3.4. 5-FU and BrdU injections

5-FU (Sigma-Aldrich) was diluted in HF2 Buffer and 150µg/g were injected intraperitoneally (i.p.). The mice were sacrificed 6 days after injection.

For proliferation analysis BrdU (BD Bioscience, 50µg/g body weight) was injected i.p. 12 hours before sacrificing mice.

3.5. Colony forming assay

In the colony forming assay, hematopoietic cells in low density are plated in a semi-solid medium (methylcellulose), substituted with a particular cocktail of cytokines, which allows the building of a colony from a single cell. The colony forming assay tests the progenitor number and identity as well as the clonogenetic capacity of hematopoietic cells and has been used successfully for decades [12, 168].

In this study, colony formation analyses with freshly isolated FL, BM or lineage depleted cells were performed with MethoCult M3434 (Stem Cell Technologies) methylcellulose medium. Cells were plated into methylcellulose in 250 µl IMDM (Gibco life technologies) supplemented with 20% FCS and cultured at 37°C with 5% CO_2 for 12 days.

In replating experiments sorted cells were initially cultured for 12 days, and were replated each 7 days for the following analysis. The replating steps were performed by homogenizing the methylcellulose samples with blunt needles.

In case of BCR-ABL-GFP-infected BM, cells were sorted for GFP-positivity and the indicated surface markers, and plated into methyl cellulose without growth factors (MethoCult M3434, Stem cell Technologies), thus allowing proliferation of only BCR-ABL transformed cells.

3.6. Co-cultures of bone marrow cells with a stromal cell line

The stromal cell line EL08-1D2, which is known to maintain hematopoietic stem and progenitor cells *in vitro* [163] was used for culturing of BM cells. Lineage-depleted bone marrow cells (Lin-) were co-cultured with 30 Gy irradiated confluent stromal cells. Irradiation of the stromal cells was performed in order to prevent overgrowing of the cells. The lineage fraction was negatively selected from flushed bone marrow (Lineage depletion kit, Miltenyi Biotec) using a mixture of primary biotinylated antibodies (CD5, B220, CD11b, Gr-1 and Ter119) and subsequently Streptavidin-coupled magnetic MicroBeads separation. The labeled cells were run through columns in a magnetic field. Cells coated with MicroBeads were restrained in the column and the lineage negative, unlabeled cells were collected. 5000 Lin- cells were plated on the confluent and irradiated stromal cells in a 3 cm dish. The cells were cultured in

long-term culture medium (M5300 (StemCell Technologies) with added 200 µM Glutamax (Life Technologies),100 U Penicillin (Sigma Aldrich) and 100 µg streptomycin (Life Technologies)).

3.7. Culture of bone marrow cells

For culturing *in vitro*, freshly isolated BM cells were depleted from lineage-commited cells, using lineage cell depletion kit (Miltenyi Biotec), resuspended in MyeloCult™ 5300 (Stem cell Technologies), supplemented with SCF (100 ng/ml), TPO (20 ng/ml) and Flt3L (50 ng/ml) (all from R&D Systems) and cultivated at 37°C with 5% CO_2.

The cells were than used in apoptosis assays or BrdU was added to assess the proliferation.

3.8. Cell culture

All cell lines (except activation of BaF3-*ts* p210 at 32°C) were cultured at 37°C with 5% CO_2.

The stromal cell line EL08-1D2 was cultured on 0.1 % gelatine-coated dishes in stroma medium (AlphaMEM (Life Technologies), 15 % FCS (PAA), 5 % HS (BioWhittaker), 100 U Penicillin,100 µg streptomycin (Life Technologies), 8 µM folic acid (Sigma-Aldrich), 80 µM inositol (Sigma-Aldrich), 10 µM β-mercaptoethanol (Life Technologies)) with 20 % conditioned medium from the previous passage.

NX (Phoenix) Eco 293T cells were cultured in DMEM (Life Technologies) with 10 % FCS (PAA).

BaF3-p210 and BaF3-*ts* p210 were cultured in RPMI supplemented with 10% FCS (PAA). At 37°C, the BaF3-*ts* p210 cell line was additionally cultivated with IL3. To activate expression of BCR-ABL in the BaF3-*ts* p210 line, cells were cultivated at 32°C without IL3.

3.9. Determination of cell number and cell vitality

The number of vital cells was identified using a hemocytometer and 0,5% Trypan blue solution. The cell suspension was resuspended in the Trypan blue solution in 1:1 dilution and a small drop of the mixture was applied in the

hemocytometer. Dead cells absorb Trypan blue and are stained blue. Vital cells possess intact cell membranes, and exclude the dye therefore they appear in a bright white color under the microscope. The unstained cells in the four square were counted and the number of vital cells was determined by multiplication of the number per square with the dilution factor 2 and the factor 10^4.

3.10. Cell cycle analysis

DNA synthesis and cell proliferation can be measured through integration of the thymidine-analog bromodeoxyuridine (BrdU) into the DNA of cells during S Phase progression. In order to perform full cell cycle analysis, the DNA binding dye, 7-Aminoacctinomycin (7-AAD) can be added after the BrdU staining. 7-AAD labels all DNA, in this way, the different cell phases can be determined and quantitated according to the fluorescence intensity.

For *in vivo* analysis, BrdU was injected in the mice as described in chapter 3.4. For *in vitro* labeling, cells were incubated for 1 hour at a final concentration of 10 µM BrdU. Prior BrdU detection, fresh isolated or cultured bone marrow cells were stained for surface markers and subsequently fixed and permeabilized. To assess the incorporation of BrdU in the DNA, a fluorochrome-conjugated (FITC or APC) antibody against BrdU was used. The BrdU staining was performed following the manufacturer's instructions (BrdU Flow Kit, BD Biosciences).

3.11. Apoptosis assay

Apoptosis in early stages at a cellular membrane level is characterized through phosphatidyl serine molecules (PS) flipping [169]. In healthy cells, PS are located in the inner layer of the cell membrane. At the early stage of apoptosis PS flip on the outer layer of the cell membrane and serve as a signal for macrophages to eliminate the apoptotic cell at later stage [169]. Annexin V-FITC (BioLegend) is a protein dye with strong affinity to PS in the presence of Ca^{2+}. Once attached to PS of apoptotic cells, the cells stay positive with Annexin V-FITC and can be detected with FACS.

In order to induce apoptosis, cultivated BM cells were washed from the supplemented factors and incubated for 24 hours. The cells were then stained as described in 3.13. After the last washing step, however, cells were

resuspended in 500 µl annexin buffer (10 mM HEPES pH 7.4 (Life Technologies), 140 mM NaCl (Carl Roth), 2.5 mM CaCl2 (Sigma-Aldrich)) with added Annexin V-FITC (BioLegend) and PI. The cells were incubated at 4°C for 15 min before measurement.

3.12. Retroviral transduction

In this project BM cells were transduced with p210-BCR-ABL to be used in colony forming assays. The retroviral packaging cell line Phoenix-Eco-293T (Phoenix-E) was transfected with the MSCV-p210-GFP vector. The virus was collected and used for retroviral transduction of 5-FU-mobilized BM cells.

Transfection

Transfection is the process of introducing exogenous DNA into eukaryotic cells. Phoenix-E cells were transfected using the cationic liposome substance, Lipofectamine^{TM} 2000 (Life technologies). In this method, the genetic material is introduced into the cells through liposomes. The used cationic lipids build a compact structure with the negatively charged DNA molecules. Because of their cationic charge on the outside and their lipophilic structure, these complexes interact with the negatively charged, hydrophobic cell membrane and enter the cells trough phagocytosis. For this purpose, at the day before the transfection 2,2x10^6 Phoenix-E cells were plated into a 6 cm dish. 10 µg DNA was used to transfect one dish of Phoenix-E cells. DNA and Lipofectamine 2000 master mix was prepared in a serum reduced medium Opti-MEM® (Life technologies) according to the manufacturer's instructions. Six hours after the transfection procedure, the medium of the Phoenix-E cells was changed with fresh culture medium.

Collecting of the retrovirus

Transfection of the Phoenix-E cell line provides the cells with the information needed to express the structural genes needed to form an infectious virus particle. The introduced DNA is transcribed to complementary RNA, packed into the virus particles and released into the medium. To generate the retrovirus the supernatant from the transfected cells was collected, filtered through 0,45 µm cell filters to exclude eventually detached Phoenix-E cells and the medium was

changed with fresh culture medium. This process was repeated 3 times at every twelfth hour.

Retroviral transduction

Retroviral transduction is a process, in which target cells are infected with DNA constructs by using retroviruses. Characteristic for retroviruses is the enzyme reverse transcriptase, which transcribes the virus RNA into DNA. The DNA can then be integrated into the genome of dividing cells. Primary BM material was prepared for the infection by injecting the mice with 5-FU (described in chaper 3.4.) 4 days before collecting the BM. 5-FU kills actively cycling cells, thus ablating the progenitor pool and all dividing hematopoietic cells but sparing the pool of quiescent, non-dividing HSC [170, 171]. The HSC were activated to divide and replenish the hematopoietic system. 4 days after 5-FU injection, the BM is rich in progenitor dividing cells, which are then cultivated with cytokines to further stimulate the cell division. For retroviral transduction, about $5x10^5$ BM cells were resuspended in 500 µl culture medium and 2 ml retrovirus with added Polybrene (4µg/ml). Polybrene is a cationic polymer and serves to improve the transduction efficiency. The cells were centrifuged for 90 min at 2400 rpm at 32°C and then incubated for 12 hours at 37°C. This process was repeated four times. The transfection efficiency was determined by FACS.

3.13. Cell sorting and surface staining for flow cytometry

For staining of surface markers (antibodies listed in chapter 2.5, table 1) the cells were resuspended in 100 µl staining buffer (0.5 % BSA (Sigma-Aldrich) in PBS (Life Technologies)) with primary antibodies. For the staining of mature populations, $1x10^6$ cells were used, for HSC/HPC stains and BrdU analysis 5-$10x10^6$ cells were stained. The cells were incubated with the antibodies for 15 min at 4°C and after that washed with staining buffer. If necessary, a secondary antibody was added (also diluted in staining buffer, 0.5 µl antibody per 1x106 cells). The cells were incubated again for 15 min at 4°C and then washed with staining buffer. Finally, the stained cells were resuspended in staining buffer with 1 µg/ml PI (Life Technologies) and analyzed on CyAn ADP Lx P8 (Coulter-Cytomation). Cell sorting was performed using the MoFlo legacy 14 colour cell sorter (Beckman Coulter).

3.14. Intracellular staining for flow cytometry

For intracellular staining, freshly isolated BM cells were first depleted from lineage cells, using lineage cell depletion kit (Miltenyi Biotec), than stained for surface markers (as explained in 3. 13). Additionally cells were fixed and permeabilized using the FIX & PERM cell permeabilization reagents (Life Technologies) according to the manufacture's instrucions and subsequently stained with antibodies for intracellular markers (chapter 2.5, table 2).

3.15. Plasmid amplification

Bacterial cells, treated chemically or physically to obtain permeable cell walls are called competent bacteria. These cells are able to include exogenous circular DNA better than conventional bacteria. Competent bacteria are stored at -80°C and thawed slowly on ice prior use. In this project, One Shot TOP10 Chemically Competent *E. coli* (Life Technologies) were used.

In order to amplify DNA-vectors, competent bacteria were transfected with the requested plasmid, incubated over-night and the plasmid DNA was extracted using Maxi-preparation.

Transformation is the process of uptake, incorporation and expression of exogenous genetic material into bacterial cells. For this purpose, the plasmid DNA is mixed with the bacterial suspension and incubated on ice for 30 min. Next, the bacteria are exposed to a heat shock for 30 sec at 42°C. The abrupt heating enables entering of the plasmid DNA into the cells. Immediately after the heat shock the bacteria are put on ice for 2 min. Than the cells are plated on ampicillin culture plates (1,5% Bactoagar in LB-Medium, autoclaved; ampicillin 50 mg/ml) and incubated for 12 hours at 37°C. The antibiotic in the medium serves for selective growth of only those bacteria which were successfully transfected with the DNA plasmid, containing an ampicillin resistance gene. After twelve hours, one colony is picked and seeded into a fluid culture (1% Bacto-Trypton; 1% NaCl; 0,5% yeast extract, autoclaved; ampicillin 50 mg/ml, pH 7,0) Culturing of the cells overnight (37°C, shaking with 250 upm) amplifies the bacteria, hence the transfected plasmid DNA.

The target plasmid DNA was isolated using the Qiagen® HiSpeed® Plasmid Maxi Kit (Qiagen) according to the manufacturer's instruction

3.16. RNA isolation

RNA isolation was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. In brief, pelleted cells were resuspended in RLT buffer containing guanidine-thiocyanate, the cells are thereby lysed and RNases are deactivated. Subsequently, the samples are homogenized by running through QIAshredder spin columns. The homogenized samples are mixed with ethanol to optimize binding conditions and loaded on RNeasy Mini spin columns where the RNA binds to the silica-based membrane. Contaminants were washed away by the use of the wash buffers RW1 and RPE and finally, the total RNA was eluted under low-salt conditions.

For gene expression analysis sorted LSK and progenitor cells, RNA was isolated with the Dynabeads mRNA DIRECT Micro Kit (Life Technologies), following the protocol given in the kit. In brief, pelleted cells were resuspended for cell lysis in Lysis/Binding buffer and connected with the Dynabeads which bind to the poly-adenine tails of the mRNAs. The Dynabeads are magnetic, therefore the samples were placed in a magnetic field where the Dynabeads with the bound mRNA congregated at one side of the tube, which allowed the removal of the supernatant with its contaminants. After two washing steps, the Dynabeads with the bound mRNAs were resuspended in 10 mM Tris/HCl pH 7.5 and directly used for reverse transcription.

3.17. Reverse Transcriptation

RNA from BaF3 cells was transcribed to cDNA by the use of the Omniscript RT Kit (Qiagen).

For gene expression analysis of sorted LSK and progenitor cells, RNA was transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

3.18. Real Time PCR

Real Time PCR (RT PCR) is a method used to determine the expression levels of genes. It measures how many cycles are needed before the amount of amplified DNA reaches certain value.

For cDNA samples of sorted cells, RT PCR was performed using the StepOnePlus Real Time System (Applied Biosystems) and the Power SYBR Green PCR Master Mix (Applied Biosystems). The CYBR Green PCR Master Mix (Life Technologies) was used for samples of CML patients and cell culture. When mature cells were analyzed, *Ubiquitin* was chosen for use as housekeeping gene for normalization. In the case of early hematopoietic cells, *Rpl13a* was used as housekeeping gene

10 µl Power Sybr Green PCR Master Mix
1 µl Primer (forward and reverse, each 10 µM)
1 µl template
8 µl H2O
20 µl total volume

Program:
50°C 2 min
95°C 5 min
95°C 15 s
58°C 20 s
72°C 30 s 40 cycles

3.19. Western blotting

A gold standard to analyze protein levels in cell lysates is the SDS (sodium dodecyl sulfate) Polyacrylamide gel electrophoresis (SDS-PAGE). SDS is an anionic detergent, which is applied to denaturize proteins SDS in order to linearize and negatively charge the protein chains. The denaturized, negatively charged proteins are loaded on a polyacrylamide gel with an electric field applied, causing their transition to the positive electrode. This leads to the separation of the proteins according to their size. Small proteins are transferred to the lower part of the gel, whereas bigger proteins remain at the top of the gel. The level of acrylamide in the gel determines the size of the micro pores. The proteins are then transferred to polyvinylidene difluoride (PVDF) membranes (western blot) where they can be detected using primary antibodies against the

certain protein to be analyzed and secondary peroxidase conjugated antibodies, which bind to the primary antibody. The detection occurs through a reaction, product of which is a luminescence in proportion with the amount of protein.

Cell lysis

Prior lysing, freshly isolated BM cells were depleted of lineage-positive cells and enriched for c-Kit using magnetic beads separation (Miltenyi Biotec). Cell lysis for immunoblotting was performed with lysis buffer containing 50mM HEPES (pH 7.5), 150mM NaCl, 1mM EDTA, 2.5mM EGTA, 0.1% Tween-20 (all from Sigma-Aldrich, Germany) and protease inhibitors (Roche, Germany) followed by sonification (SONOPLUS HD 2070 (Bandelin), 10 pulses). The lysates were centrifugated at maximum speed for 10 min. The supernatant was used to determine the protein concentration with the help of the Protein assay Kit from BioRad, according to the manufacturer's instructions. The proper amount (15-60 µg protein) of the supernatant was mixed with 5x loading buffer (250 mM Tris/HCl (Carl Roth), pH 6.8, 50 % Glycerol (Sigma-Aldrich), 10 % SDS (Sigma-Aldrich), 0.5 M DTT (Sigma-Aldrich), 0,125 % Bromophenolblue (Sigma-Aldrich)), heated for 5 min at 95°C for denaturation and used for SDS-PAGE.

SDS-PAGE

For polyacrylamide gels the gel electrophoresis system from BioRad was used. The Precision Plus Protein Dual Color Standard (BioRad) was used as a molecular weight marker. The samples were loaded onto the gel and the gel was run with 120 V.

Western blot

Afterwards, the proteins were blotted on PVDF membranes (0.45 µm, Millipore) The membrane was blocked with 5 % milk powder (Carl Roth) and 0.1 % Tween 20 in PBS for 1 hour and then transferred into 50 ml falcons containing the primary antibody diluted in blocking buffer and incubated overnight. The next day the membranes were washed twice with PBS with 0.1 % Tween 20 and then secondary antibody was added diluted in blocking buffer. The membranes were incubated for 1 hour at room temperature and then washed twice with PBS with 0.1 % Tween 20 and finally twice with PBS. Detection was

done with Super Signal West Dura or Femto solutions from Pierce.

3.20. Statistics

Statistical analyses were performed using the statistical functions of Excel or GraphPad Prism (GraphPad Software, La Jolla, CA). The bars shown represent the standard error of the mean (SEM). All statistical analyses were *t*-tests. Only *P* values <0.05 were considered statistically significant.

4. Results

4.1. Effects of Cks1 loss on CKI levels and expression of *Cks1* in HSC/HPC

Cks1 has been recognized as a rate-limiting component of the SCF^{Skp2} E3 ubiquitin ligase complex which regulates proteosomal degradation of cell cycle inhibitory CIP/KIP family members [128, 134, 135]. Thus, Cks1 is a significant component of the cell cycle regulatory machinery. Since cell cycle control is from a major importance in hematopoietic regulation, it was hypothesized that Cks1 plays an important role in hematopoiesis. In the beginning of this project the level of SCF^{Skp2} regulated proteins in the hematopoietic subsets of *Cks1*-knockout mice was established. Also, the *Cks1* transcript level in bone marrow (BM) cells of WT mice was analyzed.

4.1.1. Cks1 loss correlates with accumulation of CKI in early hematopoietic subsets

To assess whether Cks1 deficiency leads to alterations in the expression of cell cycle inhibitors in early BM populations, *Cks1-/-* and WT control BM enriched for Lin- cKit+ cells and control Lin+ cells was analyzed for CKI protein levels using western blot (Fig. 5A, B). Indeed, there was a significant accumulation of all three CIP/KIP family members, p57, p27, p21 and also of another cell cycle regulator and known SCF^{Skp2} target, p130, in *Cks1*-/- hematopoietic cells as compared to their WT counterparts (Fig. 5B). Interestingly, this accumulation was more pronounced in the Lin- c-Kit+ subset, and Cks1 was stronger expressed just in the Lin- c-Kit+ fraction, suggesting a more prominent role for Cks1 in the control of CKI protein levels in earlier, c-Kit expressing HSC/HPC.

To track CKI protein changes in distinct HSC/HPC subsets, intracellular staining was used. Lineage-depleted BM cells from WT and *Cks1*-/- mice were stained with antibodies against p57, p27, p21 and p130. Accumulation of p21 and p27 was observed in all analyzed *Cks1*-deficient subsets and, consistent with the western blot analysis, was more pronounced in the early [46] CD150+ LSK (Fig. 5C, D). Interestingly, p57 and p130 protein levels were significantly increased only in the HSC including the CD150- and CD150+ LSK subsets (Fig. 5C, D).

These data suggest a prominent function for Cks1 in the control of CKI protein levels and HSC homeostasis

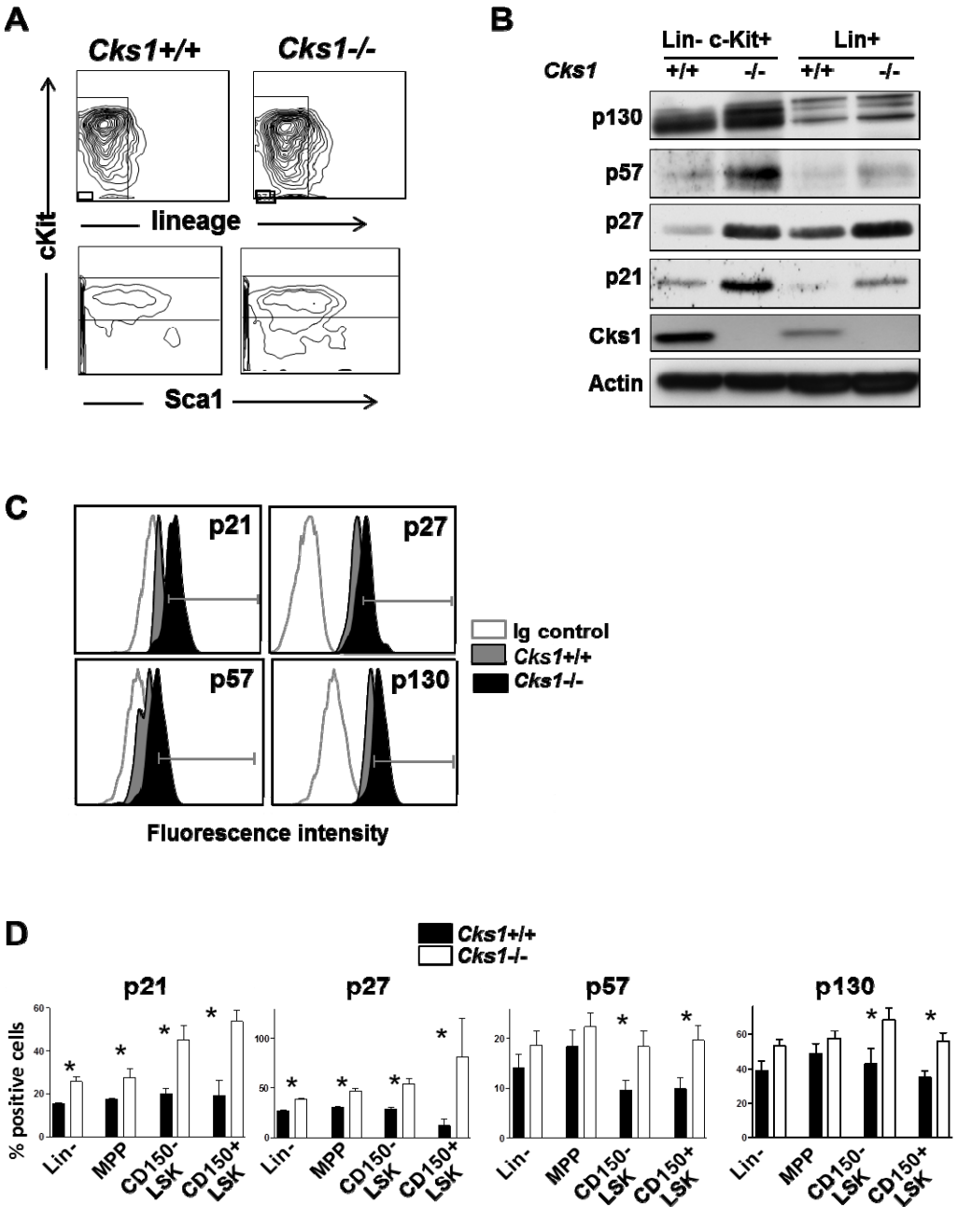


Figure 5: Loss of Cks1 results in accumulation of SCF^{Skp2} substrates.

(**A**) Efficiency of the isolation, analyzed by FACS. BM cells from 5 WT and 5 *Cks1-/-* mice were depleted of lineage-committed cells and positively enriched for c-Kit expression using immunomagnetic beads (Lin- c-Kit+). (**B**) Western blot analysis of the indicated proteins. (**C**) Quantification of intracellular staining of p21, p27, p57 and p130 in the CD150+ LSK population using flow cytometry. (**D**) Summarized results of intracellular staining of different subpopulations for the indicated SCF^{Skp2} targets (*Cks1+/+* n=6, *Cks1-/-* n=5, 3 independent experiments).

4.1.2. High expression of Cks1 in early hematopoietic subsets

Since CKI regulated through the SCF^{Skp2} E3 ubiquitin ligase complex were strongly accumulated in *Cks1*-/- LSK cells, the level of Cks1 in these populations needed to be tested in WT mice. For this purpose, flow cytometric cell sorting on WT BM cells was performed (Fig. 6A) and *Cks1* transcript levels in HSC/HPC subsets were evaluated using real time PCR (Fig. 6B). In contrast to the intracellular staining, where the LT-HSC population was indicated by expression of CD150+ [46], the earliest subset in this case was defined as CD34- LSK [44]. In accordance with the pronounced accumulation of SCF^{Skp2} regulated proteins in absence of Cks1 in early hematopoietic stages (Fig.5 C and D), *Cks1* mRNA was expressed highest in the more immature CD34- LSK cells in WT mice (Fig. 6 B), confirming the suggestion that Cks1 might be an important regulator in early hematopoiesis.

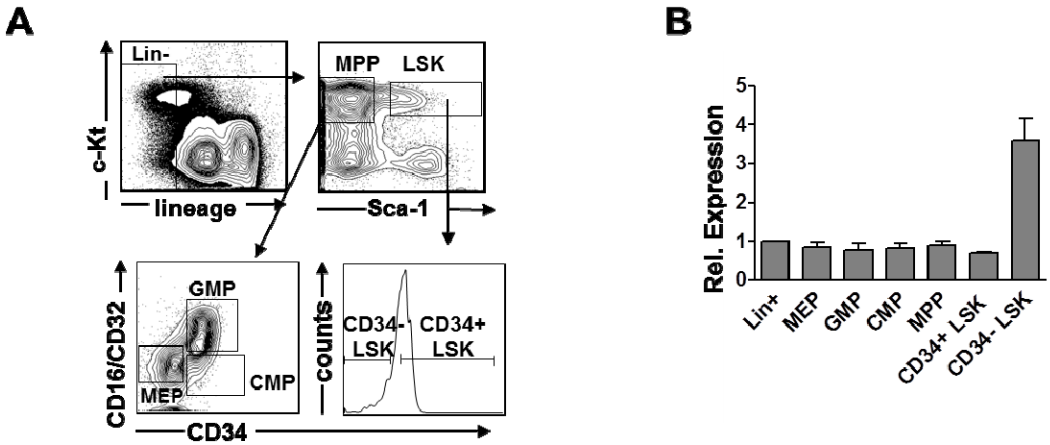


Figure 6: High *Cks1* transcript levels in the CD34- LSK subset of WT mice.

(**A**) Representative FACS-Sort diagram for (**B**) real time PCR analysis of *Cks1* mRNA expression in the indicated WT FACS-sorted subgroups. Shown are the results from n=2 independent experiments. For each experiments the pooled bone marrow of n=6 mice was used.

4.2. Role of Cks1 in steady state and stress hematopoiesis

Taken together, the initial experiments revealed that Cks1 expression is highest in early LSK populations and loss of Cks1 results in a pronounced accumulation of CKI (p21, p27 p57) and p130 in these cells compared to MPP and more mature hematopoietic populations. Based on these results, it was hypothesized, that Cks1 is involved in the regulation of early hematopoiesis.

The next part of this study focused on experiments comparing WT and Cks1-deficient mice in order to study the role of Cks1 in hematopoiesis.

4.2.1. Normal steady state adult hematopoiesis in Cks1-deficient mice

Mice lacking Cks1 are viable and in normal health condition [128]. Though, they are abnormally small (10-20% smaller than their littermates of the same gender) and *Cks1*-/- -derived mouse embryonic fibroblasts proliferate poorly and accumulate p27 [128]. Hematopoiesis in Cks1-deficient mice has not been studied in detail.

In the beginning of this part of the study blood and bone marrow of the knockout mice was analyzed and compared to WT. For the blood analysis blood from WT and knockout mice was measured with blood counter and analyzed by FACS after staining with the cell surface markers as follows: CD45 for leukocytes, combination of CD4 and CD8a for T cells, B220 for B cells and Gr1 and CD11B to distinguish granulocytes and monocytes. No significant differences were observed either in the blood cell counts (Fig. 7A), nor in the different mature blood cell populations (Fig. 7B, C). Hematoxylin-Eosin stainings (Collaboration with PD Dr. Leticia Quintanilla-Fend) showed no abnormalities in the bone marrow structure of the knockout mice (Fig. 7D).

Interestingly, the absolute cell numbers in the hematopoietic tissues BM an fetal liver (FL) of the *Cks1*-/- mice was strongly reduced (Fig. 7E). It is known that *Cks1*-/- mice are smaller in size [144, 172] and respectively have smaller organs. Though the reduction of the absolute cell number was enhanced (36-38%) compared to the reduction of body size (10-20%), hinting towards a pronounced role of Cks1 in hematopoiesis.

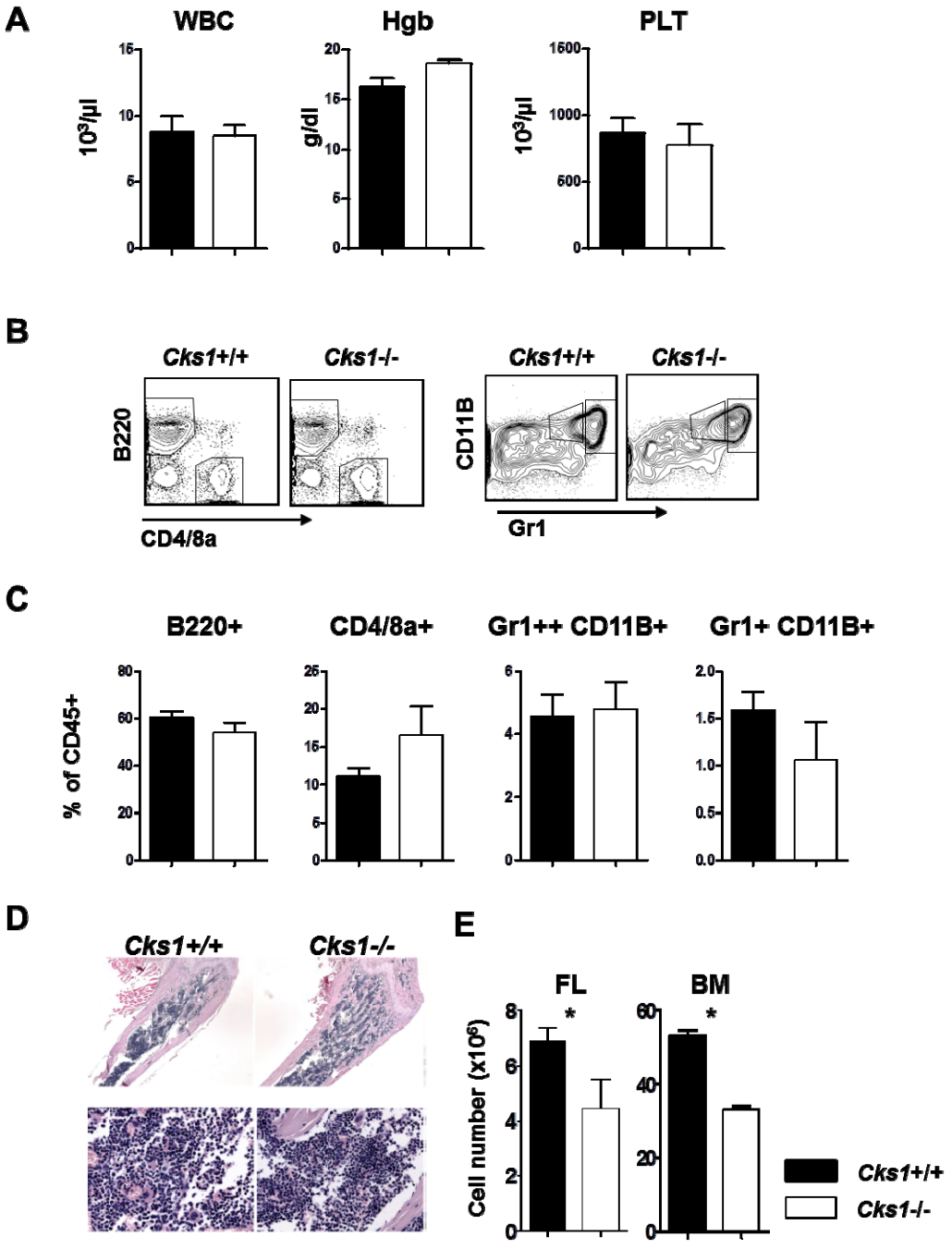


Figure 7: Normal steady state adult hematopoiesis in *Cks1*-deficient mice.

(**A**) Blood counts (WBC: white blood cells; Hgb: hemoglobin; PLT: platelets) in *WT* and *Cks1*-/- mice, n=6. The differences are not statistically significant. (**B**) Representative flow cytometry analysis of the indicated surface markers for adult hematopoietic cells, gated on CD45+ cells. (**C**) Percentage of B cells (B220+), T cells (CD4/8a+), granulocytes (Gr1++ CD11B+) and monocytes (Gr1+ CD11B+) in the periferal blood of wild type and knock out animals at steady state (*Cks1*+/+ n=5; *Cks1*-/- n=6). The differences are not statistically significant. (**D**) Hematoxylin-Eosin staining of bone marrow section from mice of the indicated genotype (Collaboration with PD Dr. Leticia Quintanilla-Fend). Representative sections are shown. (**E**) Absolute cell counts of bone marrow (n=30) and fetal liver (*Cks1*+/+ n=10; *Cks1*-/- n=8).

4.2.2. Decreased absolute HPC numbers and increased relative HSC numbers in *Cks1-/-* mice

The absolute cell number was drastically decreased in FL as well as in the BM of *Cks1-/-* animals (Fig.7E), suggesting altered hematopoiesis in absence of Cks1. To gain further insight into the early hematopoietic subsets of *Cks1-/-* mice, FACS analysis with WT and *Cks1-/-* BM cells was performed. Figure 8 demonstrates the gating strategy which was used based on earlier studies [31, 43, 44, 46]. A biotinylated cocktail for the lineage marker Terr119 (erythroid cells), CD11B (myeloid cells), Gr1 (myeloid cells), CD3 (lymphoid T-cells), B220 (lymphoid B cells) stained secondary with straptavidine-conjugated antibody was used for the lineage gating (Fig. 8A). c-Kit and Sca1 were used to differentiate the lineage negative cells into MPP and LSK (Fig. 8A). The MPP were then distinguished into GMP, CMP and MEP by the expression of CD16/32 and CD34 (Fig. 8B) and LSK were further gated either into CD34- or CD34-CD150+ LSK (Fig. 8C).

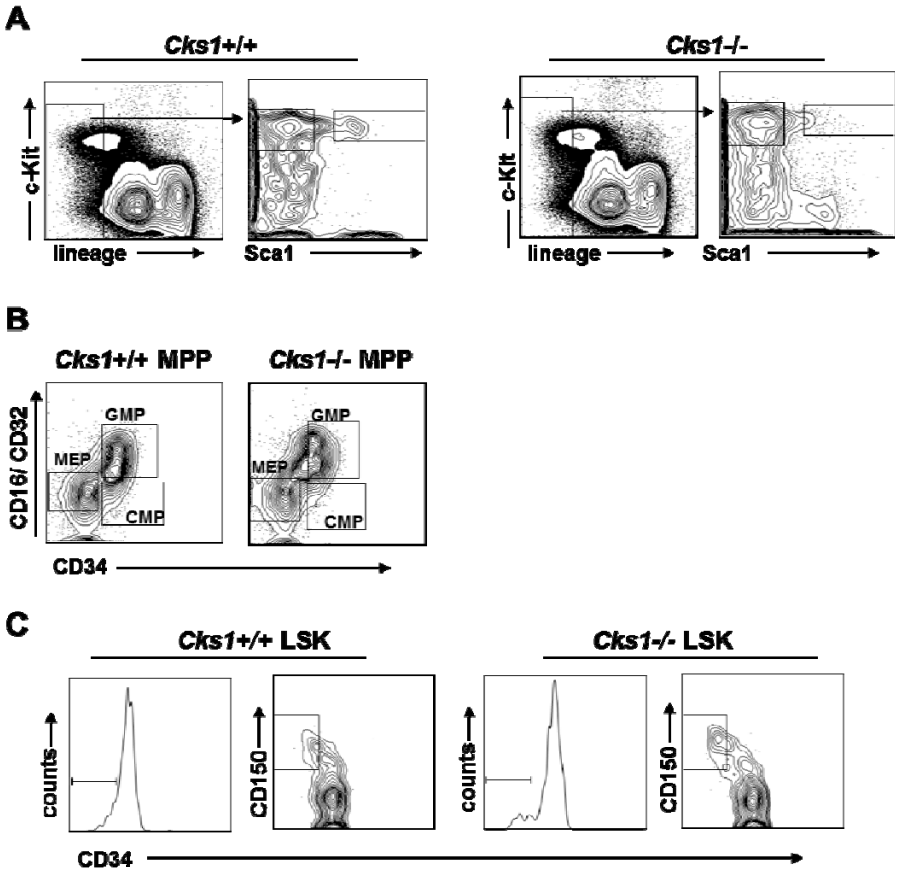


Figure 8: BM analysis in WT and *Cks1-/-*mice. Representative FACS Analysis.
(**A**) Lineage, MPP, and LSK gating. (**B**) CMP, GMP and MEP gating. (**C**) CD34- LSK and CD34-CD150+ LSK gating.

Mice LSK cells expressing high levels of the SLAM family receptor CD150 are known to possess high self-renewal activity and one of the most immature HSC populations is defined as CD34-CD150+ LSK [46, 48, 173]. Flow cytometry analysis of the WT control and *Cks1*-/- mice demonstrated that the $CD150^{high}$ LSK cells are part of the CD34- LSK fraction (Fig. 8C). Based on that finding, CD150+ LSK in this study are referred as LT-HSC.

In order to determine how the different subsets are represented in a steady state mice lacking Cks1, the absolute numbers and the percentages of HSC/HPC from *Cks1*-/- mice was compared to those of WT animals. Consistent with the decreased total cell numbers in *Cks1*-/- BM (Fig. 7E), the absolute MPP, CMP and GMP numbers were significantly decreased (Fig. 9A), whereas the frequencies of these subpopulations were unaffected in *Cks1*-/- BM (Fig. 9A). No significant changes were observed in the LSK subsets of Cks1-deficient mice compared to WT (Fig. 9B). However, analysis of the very early hematopoietic stem cell subsets of CD34- LSK and CD34-CD150+ LSK revealed a significant increase in their frequency in the BM of Cks1-deficient mice (Fig. 9B). This observation led to the assumption that the early stage of hematopoiesis is disturbed at steady state in the absence of Cks1.

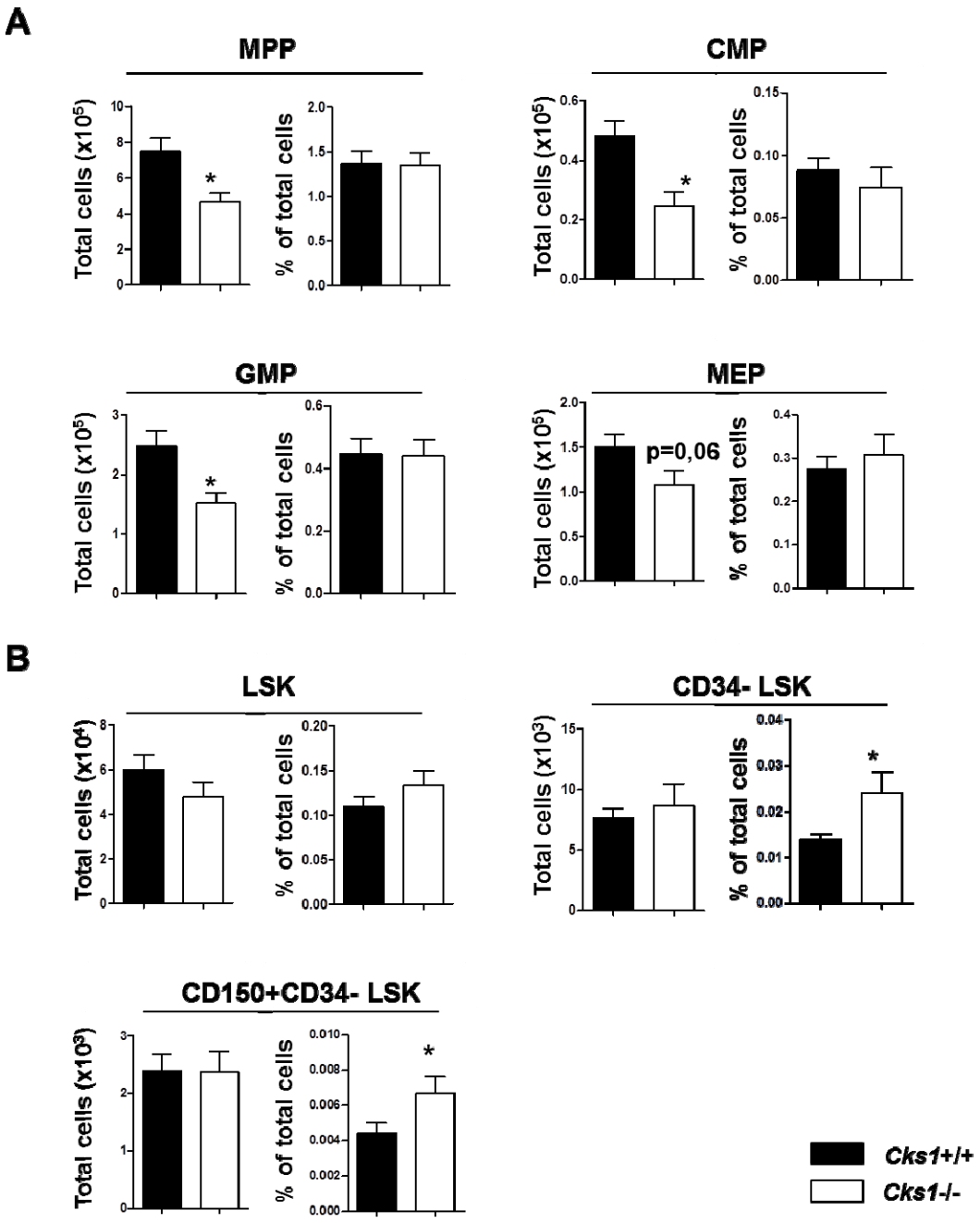


Figure 9: Decreased absolute HPC numbers and increased relative HSC numbers in *Cks1-/-* mice.

(**A**) Total cell number and percentage of total cell number of progenitor populations (n=20 for each genotype, 6 independent experiments). (**B**) Total cell number and percentage of total cell number of LSK; CD34-LSK (n=20 for each genotype, 6 independent experiments) and CD34-CD150+ LSK (n=15 for each genotype, 5 independent experiments).

4.2.3. Loss of Cks1 results in decreased hematopoietic colony formation

A well-established method to test the clonogenetic capacity of hematopoietic cells is the methylcellulose assay [168].To investigate whether the quality of colony forming hematopoietic cells was affected by the loss of Cks1, methyl cellulose assays with freshly isolated FL, BM and Lin- BM cells were performed and the number of colonies for the respective genotype was determined. A significantly reduced ability of *Cks1-/-* BM and FL cells to form colonies was observed (Fig. 10), indicating reduced clonogenetic capacity in the absence of Cks1. Since an equal number of cells were used for both genotypes, and there were no significant differences in the percentage of colony forming progenitors (Fig. 9A) and even an increase in the percentage of stem cells (Fig. 9B), it was assumed that HPC/HSC lacking Cks1 are impaired in their differentiation capacity, proliferation or survival.

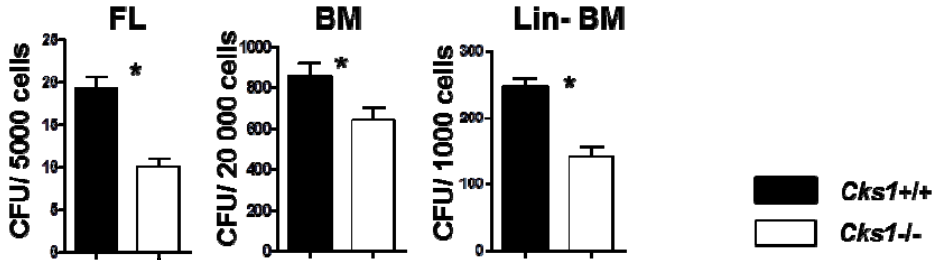


Figure 10: Loss of Cks1 results in decreased colony formation.
Colony count from methylcellulose assays with fetal liver cells (*Cks1*+/+ n=10; *Cks1*-/- n=9), bone marrow (*Cks1*+/+ n=12; *Cks1*-/- n=11) and lineage-depleted cells (*Cks1*+/+ n=20; *Cks1*-/- n=18).

4.2.4. Decreased colony formation and HPC number after cultivation of Lin- *Cks1-/-* cells on stromal cells

The stromal cell line EL08-1D2 is known to maintain hematopoietic stem and progenitor cells *in vitro* [163]. Co-cultures of *Cks1*-/- and WT cells were established to further observe the behavior of the colony forming cells. Therefor the EL08-1D2 cell line was used to support 5000 lineage-depleted WT or *Cks1*-/- BM cells. Freshly isolated cells were incubated with irradiated (30 Gy, in order to restrain overgrow) stromal cells for 14 days (Fig. 11A). Afterwards, the bone marrow cells were harvested, half well was seeded in methyl cellulose medium, and the rest was used for FACS analysis. Consistent with the results at steady

state (Fig. 10), significantly fewer colonies were formed from Lin- *Cks1*-/- cells after co-culture than from the WT cells (Fig. 11B). Furthermore, FACS analysis revealed that there was a correspondingly significant decrease in the remaining *Cks1*-/- MPP cells after the 14 days of co-culture (Fig. 11C). Interestingly, similar to the steady state condition (Fig. 9B), there was no drop in the LSK subset, but a slight increase compared to the WT LSK (Fig. 11C), proving that Cks1-deficient hematopoietic cells tend to accumulate in the early hematopoietic subsets.

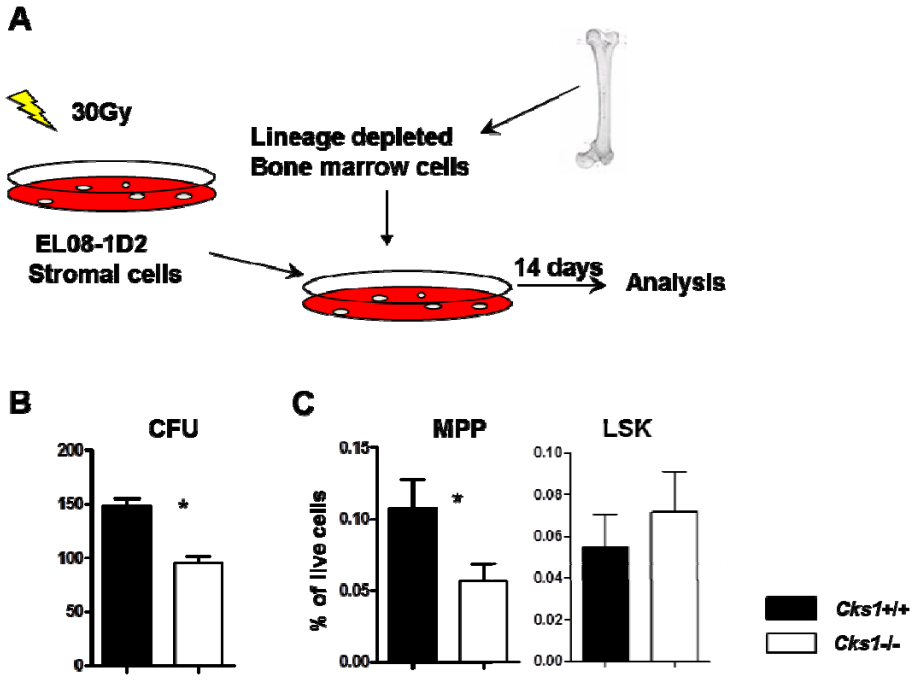


Figure 11: Decreased colony formation and progenitor cell number after cultivation of Lin- *Cks1*-/- cells on stromal cells.

(**A**) Scheme of the experiment: 5000 Lin- BM cells from WT and *Cks1*-/- mice were cultivated for 14 days on irradiated stromal cells. (**B**) CFU Assay after cultivation on stromal cells. (**C**) FACS analysis representing the remaining LSK and MPP cells after 2 weeks of cultivation on stromal cells (*Cks1*+/+ n=6; *Cks1*-/- n=6; 3 independent experiments).

4.2.5. *Cks1*-/- hematopoietic cells proliferate slower in culture

Since *Cks1*-/- cells formed fewer colonies at steady state condition and after co-cultivating them with stromal cells, and the *Cks1*-/- MPP pool was decreased *in vivo*, it was hypothesized, that the cells lacking Cks1 are impaired in their proliferation or in their survival. To test this, *ex vivo* experiments were

performed (Fig. 12A, 13A). First, the cell cycle in cultivated bone marrow cells was analyzed. Bone marrow cells from WT and *Cks1*-/- mice were isolated and depleted of lineage-committed cells using micro beads. The lineage negative cells were then cultivated in the presence of cytokines, which have been shown to maintain early hematopoietic cells in culture [174-176]. To analyze cell cycle progression an assay with the synthetic nucleoside BrdU was applied. FACS analysis of the cells after 5 days of cultivation showed a significantly reduced S phase fraction in *Cks1*-/- Lin- cells as compared to WT Lin- cells (Fig. 12B, C), indicating that Cks1 is important for entry into S phase. A similar effect can be observed in WT vs. *Cks1*-/- MEFs [177].

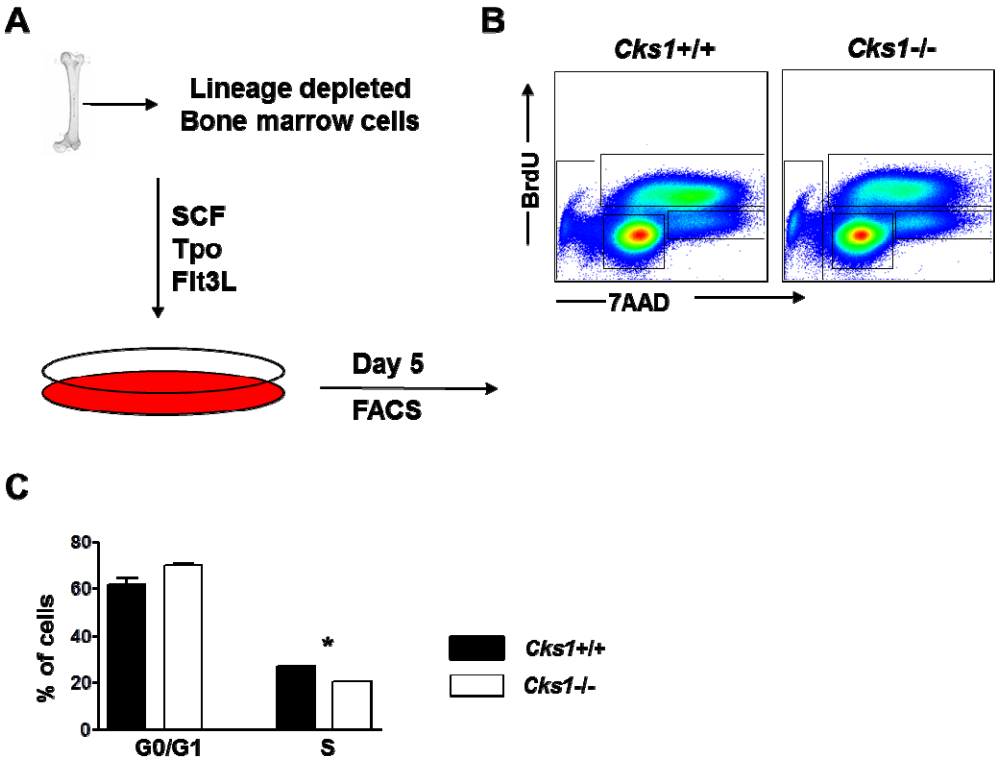


Figure 12: *Cks1*-/- cells proliferate slower in culture.

(**A**) Experimental procedure: Lin- BM WT and *Cks1*-/- cells were cultivated for 5 days with supplemented growth factors. (**B**) Representative dot blots from cell cycle analysis with BrdU. (**C**) Frequency of Lin- *Cks1*+/+ and *Cks1*-/- cells in the different cell phases (n=2 for each genotype).

4.2.6. Cks1 regulates survival of progenitor cells *in vitro*

The lower percentage of cells in the S phase (Fig. 12C) implied that the decrease in MPP numbers *in vivo* (Fig. 9A) and *in vitro* (Fig. 11C) and the decline in *Cks1*-/- colonies of BM or FL cells (Fig. 10) or after co-culture (Fig. 11B) are due to decelerated proliferation in the cells lacking Cks1. Consistent with reduced S Phase progression, significantly lower cell numbers were recovered after 5 days of cytokine stimulated culture of lineage depleted *Cks1*-/- deficient cells compared to WT cells (Fig. 13A, B). In particular, the number of *Cks1*-/- MPP and LSK was significantly reduced (Fig. 13B). An alternative or accessory explanation for the observed differences in HSC/HPC generated in culture is that *Cks1-/-* cells could be more susceptible to the induction of apoptosis. To find out whether this was the case, an apoptosis analysis was performed (Fig. 13A). To induce apoptosis the stimulated *Cks1-/-* or WT cell cultures were deprived of cytokines for 24 hours and subsequently stained with Annexin V and PI for FACS analysis. The cytokine depletion induced a significantly increased apoptotic rate in the *Cks1*-/- Lin- and MPP fraction but not in the Cks1-deficient LSK (Fig. 13C).

Taken together, loss of Cks1 resulted in accumulation of LT-HSC at steady state, reduced proliferation of BM cells and sensitization of MPP towards apoptosis upon growth factor withdrawal. This finding suggests that Cks1 function is required for optimal growth and survival of hematopoietic cells.

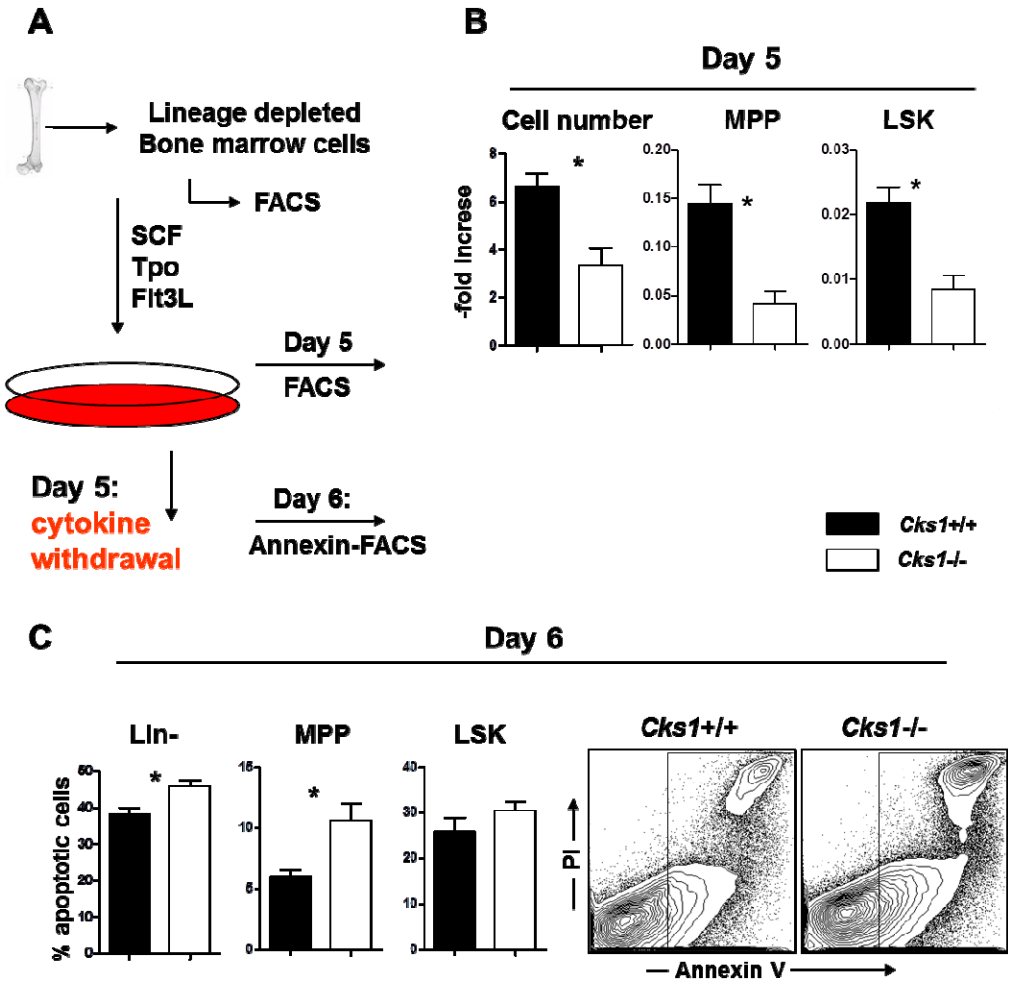


Figure 13: Cks1 regulates survival of progenitor cells *in vitro.*

(**A**) Experimental procedure: Lin- BM WT and *Cks1-/-* cells were cultivated for 5 days with supplemented growth factors and subsequently remaining cells were counted and analyzed by FACS for LSK and MPP populations. In the second part of the experiment, cells were washed from the growth factors and analyzed for apoptosis 12 hours later. (**B**) Cell number and remaining LSK and MPP cells after 5 days cultivation with growth factors (n=7 for each genotype; 3 independent experiments). (**C**) On the left: percentage of apoptotic cells after growth factor withdrawal (n=8 for each genotype, three independent experiments); on the right: representative FACS dot blots (Lin- fraction) of the Annexin V/PI apoptosis analysis.

4.2.7. Increased B-Lymphocytes- and decreased granulocytes frequency after cytotoxic stress in *Cks1*-knockout mice

Cell culture is an *in vitro* model for cellular stress and the results in this study so far demonstrate that Cks1 affects the response to hematopoietic stress *in vitro*. To further analyze whether Cks1 is also involved in the response towards hematopoietic stress *in vivo*, *Cks1-/-* and WT control mice were injected with the cytotoxic agent 5-Fluorouracil (5-FU). 5-FU kills actively cycling cells, thus ablating the progenitor pool and all dividing hematopoietic cells but sparing the pool of quiescent, non-dividing HSC [171, 178]. Such chemo-ablative stress has been shown to efficiently activate the proliferation of the dormant HSC in order to replenish the blood system [7, 179, 180]. Using cell counting and flow cytometry the regeneration of the mature blood cells and of the HSC/HPC pool was determined on day 6 after 5-FU injection. To facilitate the detection of dividing cells, the mice were injected with BrdU 12 hours before sacrifice (Fig. 14A). No differences were observed in the regeneration of the white blood cells (WBC) and the lymphoid and myeloid blood populations in WT and Cks1-deficient mice 6 days after 5-FU injection (Fig. 14B, C). Though, detailed FACS analysis revealed significantly decreased granulocytes and a significantly increased B cell fraction (Fig. 14D, E) in the blood of *Cks1*-/- mice, indicating a disturbed distribution of the different mature populations.

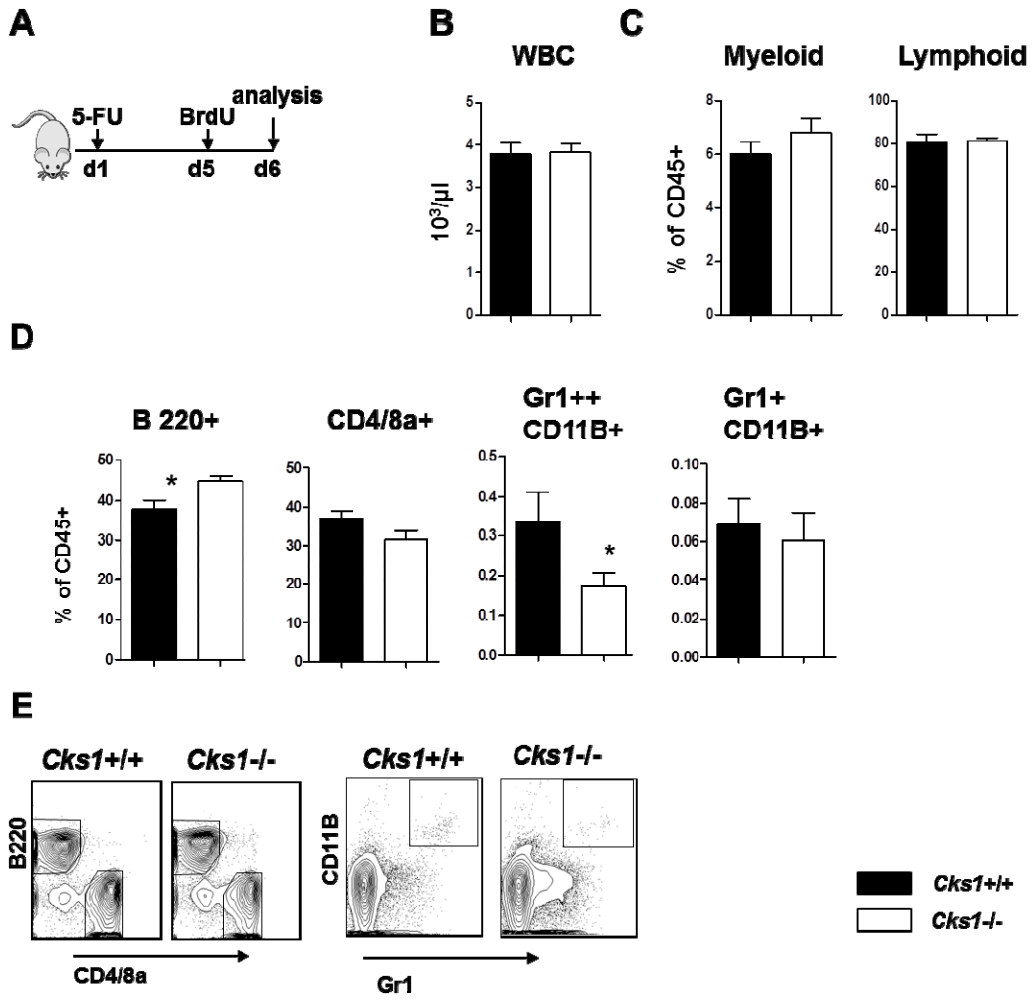


Figure 14: Increased B cells- and decreased granulocytes frequency after cytotoxic stress in *Cks1-/-* mice.

(**A**) Schematic representation of the experiment: WT and *Cks1-/-* mice were treated i.p. with 5-FU (150 µg/g body weight); after 6 days BrdU was injected and 12 hours later the peripheral blood, MPP and LSK subsets were analyzed by FACS. (**B**) WBC counts 6 days after 5-FU injection. (**C**) Fractions of recovered myeloid (B220-CD4/8a-) and lymphoid (Gr1-CD11B-) cells. (**D**) Detailed FACS analysis of the B cells (B220+), T cells (CD4+ CD8a+), granulocytes (Gr1++CD11B+) and monocytes (Gr1+CD11B+) 6 days after 5-FU treatment. (**E**) Representative FACS dot blots from the blood analysis in (D) (*Cks1*+/+ n=14 *Cks1-/-* n=15, 3 independent experiments).

4.2.8. Cks1 controls stress hematopoiesis in LSK cells: impaired regeneration after cytotoxic stress

Consistent with the steady state results (Fig. 8), the CFU potential was significantly reduced in the BM of 5-FU-treated *Cks1*-/- mice as compared to the WT controls (Fig. 15A). Also, the absolute BM and specifically MPP and LSK cell numbers were reduced in 5-FU treated *Cks1*-/- animals and the reduction was significant in the LSK population (Fig. 15B). To further estimate the recovery of HSC/HPC after cytotoxic stress, the ratio of absolute numbers of BM cells, MPP, and LSK (determined using flow cytometry) on day 6 to the absolute number of a matched control group of the respective genotype that was left untreated was calculated. The ratio of BM cells in the *Cks1*-/- mice was comparable to that in the control mice and the ratio of *Cks1*-/- MPP was decreased but not significantly (Fig. 15C). However, there was a striking decay in the ratio of the LSK (Fig. 15C). These results confirm that lack of Cks1 predominantly affects the immature hematopoietic compartment. Most importantly, there was a significant decay in the incorporation of BrdU in *Cks1*-/- LSK (Fig. 15D) strongly suggesting that the reduced numbers of LSK cells was the result of reduced cell cycling. Collectively, these results point to Cks1 being a crucial regulator of the regenerative response of LSK and colony-forming cells after cytotoxic stress.

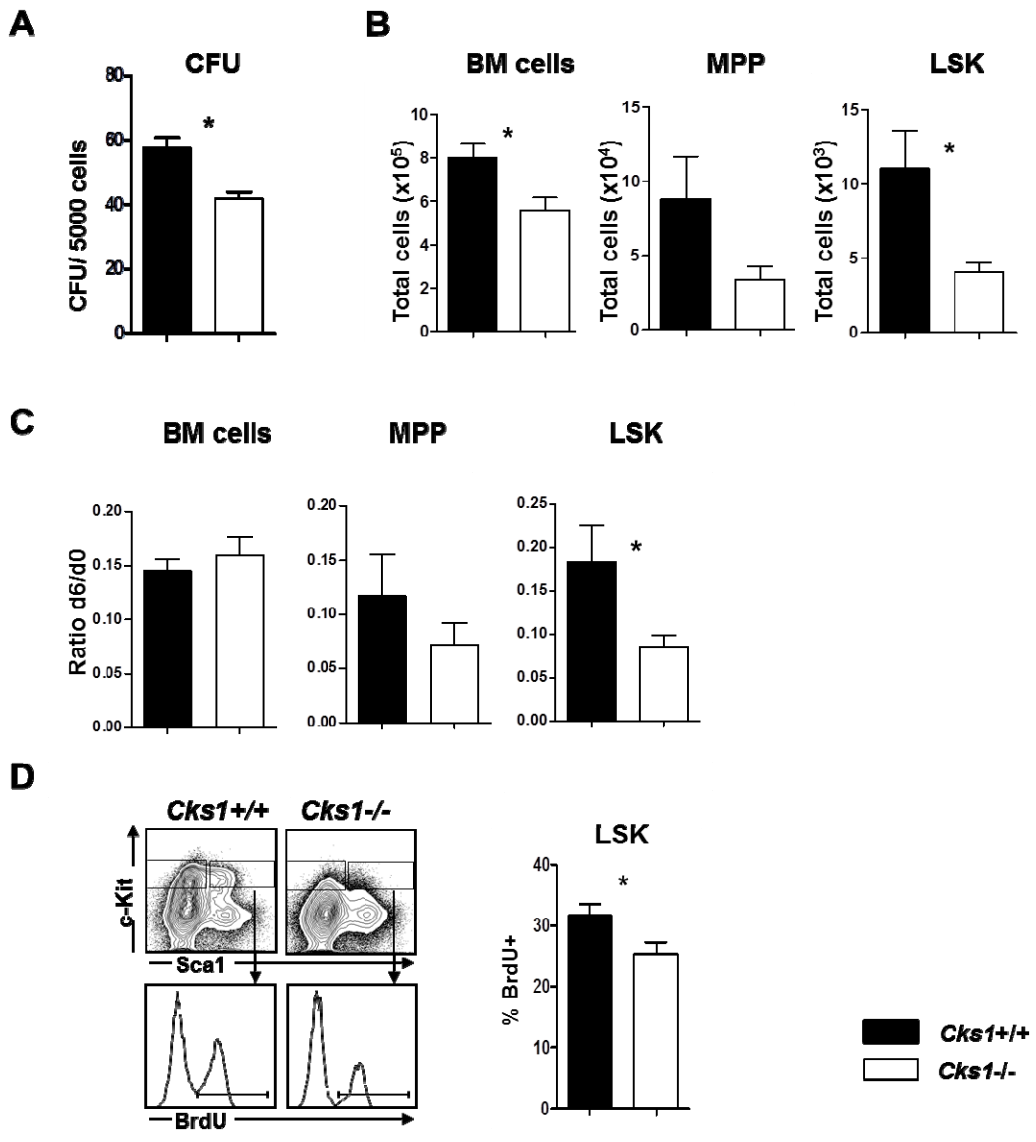


Figure 15: Cks1 controls stress hematopoiesis in LSK cells: impaired LSK regeneration after cytotoxic stress.

(**A**) Colony counts after methyl cellulose assays with 5-FU treated WT and *Cks1*-/- bone marrow cells 6 days after 5-FU injection (n=8 for each genotype). (**B**) Total BM, MPP and LSK cell number 6 days after BrdU treatment (*Cks1*+/+ n=14 *Cks1*-/- n=15, 3 independent experiments). (**C**) Ratio (day 6/day 0) of absolute cell numbers. (**D**) On the left: representative dot blots from the analysis of LSK cells after 5-FU and subsequent BrdU treatment; on the right: BrdU incorporation in the LSK of wild type and *Cks1*-/- mice (*Cks1*+/+ n=14 *Cks1*-/- n=15, 3 independent experiments).

4.2.9. Bone marrow transplantations: Cks1-/- bone marrow cells are able to reconstitute recipient mice

5-FU treatment experiments address the initial stages of hematopoietic regeneration after challenge. The most articulate parameter for enduring hematopoietic recovery is, however, long-term engraftment of HSC. The gold standard for analyzing engraftment and self-renewal capability of HSC is the transplantation of BM cells into lethally irradiated hosts [31, 33]. The irradiation of the recipients leads to cell death of most of the hematopoietic cells, therefore the transplanted BM cells are initiated to divide and replenish the hematopoietic system of the host. Serial transplantations of BM cells deliver information about the self-renewal potential of HSC since only the early (LT-HSC) are capable of long-term engraftment [33].

To study engraftment and self-renewal capacity of Cks1-deficient hematopoietic cells, serial BM transplantations were performed. In order to track donor, recipient and helper hematopoietic cells the CD45 congenic system was used: WT and *Cks1*-/- donor cells express the CD45.2 surface marker. Helper spleen cells were derived from CD45.1 mice and recipients express both CD45.1 and CD45.2. Bone marrow cells ($2x10^5$ per recipient mouse) from WT and Cks1-deficient mice were transplanted together with helper spleen cells ($4x10^5$ per recipient mouse) in lethally irradiated (9Gy) recipients (Fig. 16A). The analysis of the primary recipients did not show significant changes in the engraftment of *Cks1*-/- donor cells compared to WT donor cells in blood, bone marrow, spleen and lymph nodes (LN) (Fig. 16B, C). The absolute numbers of donor-derived Lin-, MPP, LSK and CD150+ LSK cells were comparable in both groups except for few individual mice (Fig. 16D,E). These analysis demonstrate that *Cks1*-deficient BM cells can reconstitute the hematopoiesis in irradiated mice.

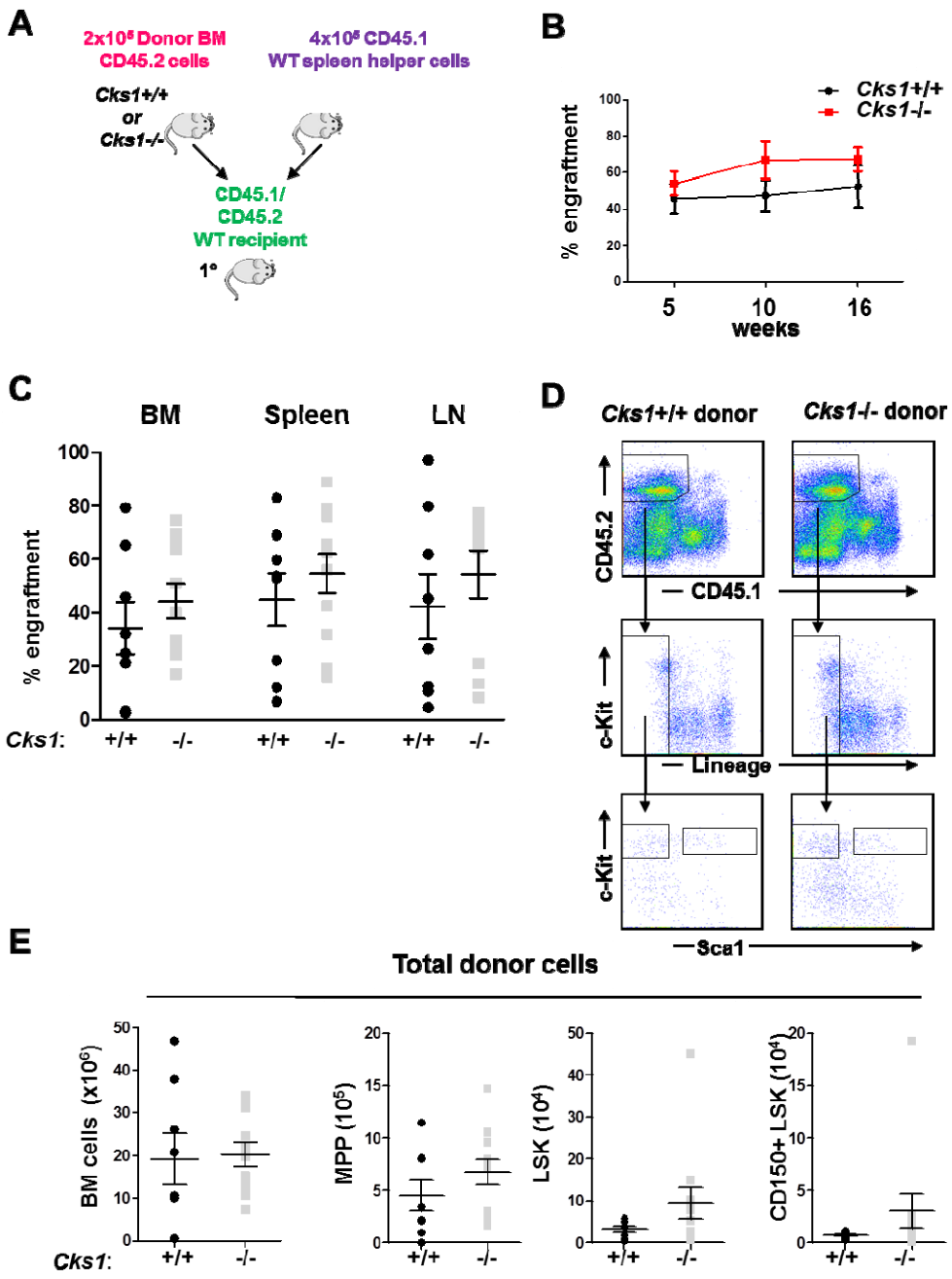


Figure 16: Primary BM Transplantations. *Cks1-/-* BM cells are able to reconstitute recipient mice.

(**A**) Scheme of the transplantation model: $2x10^5$ donor BM cells were transplanted in lethally irradiated recipients together with $4x10^5$ spleen helper cells. (**B**) Blood engraftment at week 5, 10 and 16 after transplantation of $2x10^5$ WT or *Cks1*-/- BM cells. (**C**) Engraftment in different organs: BM, spleen and LN respectively. (**D**) Representative dot blots from the analysis of donor CD45.2+ LSK and MPP cells in the bone marrow of recipient mice 16 weeks after transplantation. (**E**) Absolute numbers of donor BM, MPP, LSK and CD150+ LSK on week 16 after transplantation (recipients of *Cks1*+/+ cells: n=8, recipients of *Cks1*-/- cells: n=11; 2 independent experiments).

4.2.10. Secondary bone marrow transplantations: increased engraftment of *Cks1-/-* cells

To test whether absence of Cks1 affects the self-renewal capability of HSC, secondary transplantations were performed (Fig. 17A). For this purpose $1x10^6$ BM cells from the primary recipients of WT or *Cks1*-/- BM cells (week 16 after transplantation) together with $4x10^5$ WT helper spleen cells were transplanted in lethally irradiated recipients. Surprisingly, five weeks after the secondary transplantation the percentage of *Cks1*-/- donor cells in the blood of the recipients was increased and at week 10 and 22 the donor cells engraftment of the *Cks1-/-* secondary recipients was significantly higher as compared to the WT secondary recipients (Fig.17B). Flow cytometry analysis of the recipient mice at week 22 post transplantation also revealed a significantly higher percentage of donor *Cks1*-/- cells in the bone marrow (Fig. 17C). There were no significant differences in the spleen engraftment (Fig. 17C). Most importantly, the absolute cell numbers of *Cks1*-/- donor BM cells, as well as the single populations: MPP, LSK and CD150+ LSK, was significantly increased compared to WT recipient controls (Fig. 17D, E). These results suggest that Cks1 is a crucial regulator in the LT-HSC.

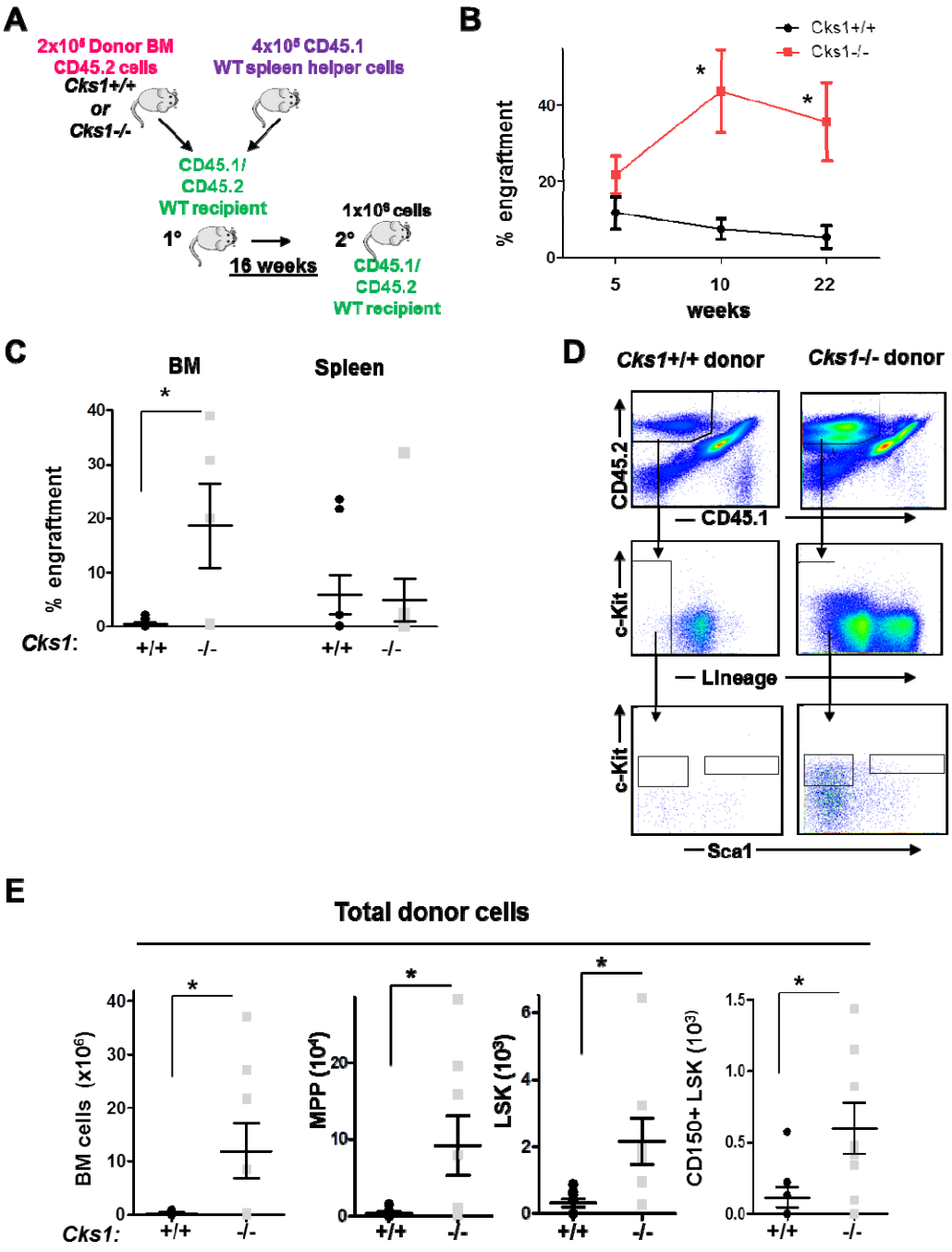


Figure 17: Secondary BM Transplantations. Increased engraftment of *Cks1-/-* cells

(**A**) Scheme of the transplantation model: 1x10^6 primary recipient delivered BM cells were transplanted in lethally irradiated secondary recipients together with 4x10^5 helper spleen cells. (**B**) Blood engraftment at week 5, 10 and 22 after secondary transplantation of 1x10^6 BM cells from primary recipients of WT or *Cks1*-/- BM cells. (**C**) Engraftment in BM and spleen of the secondary recipients. (**D**) Representative dot blots from the analysis of CD45.2+ donor LSK and MPP cells in the bone marrow of the secondary recipients 22 weeks after transplantation. (**E**) Absolute number of donor BM, MPP, LSK and CD150+ LSK at week 22 after secondary transplantation (recipients of *Cks1*+/+ cells: n=8, recipients of *Cks1*-/- cells: n=8; 2 independent experiments).

4.2.11. Loss of Cks1 results in increased LSK frequency and increased replating capability after cultivating *in vitro*

The secondary BM transplantation experiments suggest that lack of Cks1 result in the prolonged ability to maintain the HSC. To further test this possibility, an *in vitro* cell culture experiment was performed, in which FACS-sorted HSC (CD150+ and CD150- LSK) from WT and *Cks1-/-* mice were activated to proliferate using the cytokines SCF, TPO and Flt3L and the frequency of the remaining LSK was analyzed 48 hours after cultivation. Flow cytometric analysis revealed that culture of *Cks1*-/- CD150+ and CD150- LSK cells contained a larger proportion of cells maintaining the HSC phenotype as compared to WT cells (Fig 18A). Next, an *in vitro* surrogate assay for self-renewal again using FACS-sorted CD150+ and CD150- LSK from WT and *Cks1*-/- was performed. The LSK were plated into growth factor-supplemented methyl cellulose. After initial colony formation, cells were recovered from the methyl cellulose and plated at equal numbers for several passages (Fig 18B). As expected, WT HSC lost the ability to form colonies after 3 passages (Fig 18C and [181]). In contrast, *Cks1*-/- HSC cells, which formed fewer colonies after the first plating, continued to form detectable numbers of colonies until up to the sixth round of replating as opposed to WT HSC (Fig 18C).

Taken together, the *in vitro* and *in vivo* experiments show that Cks1 expression is a decisive factor for the maintenance of HSC dormancy.

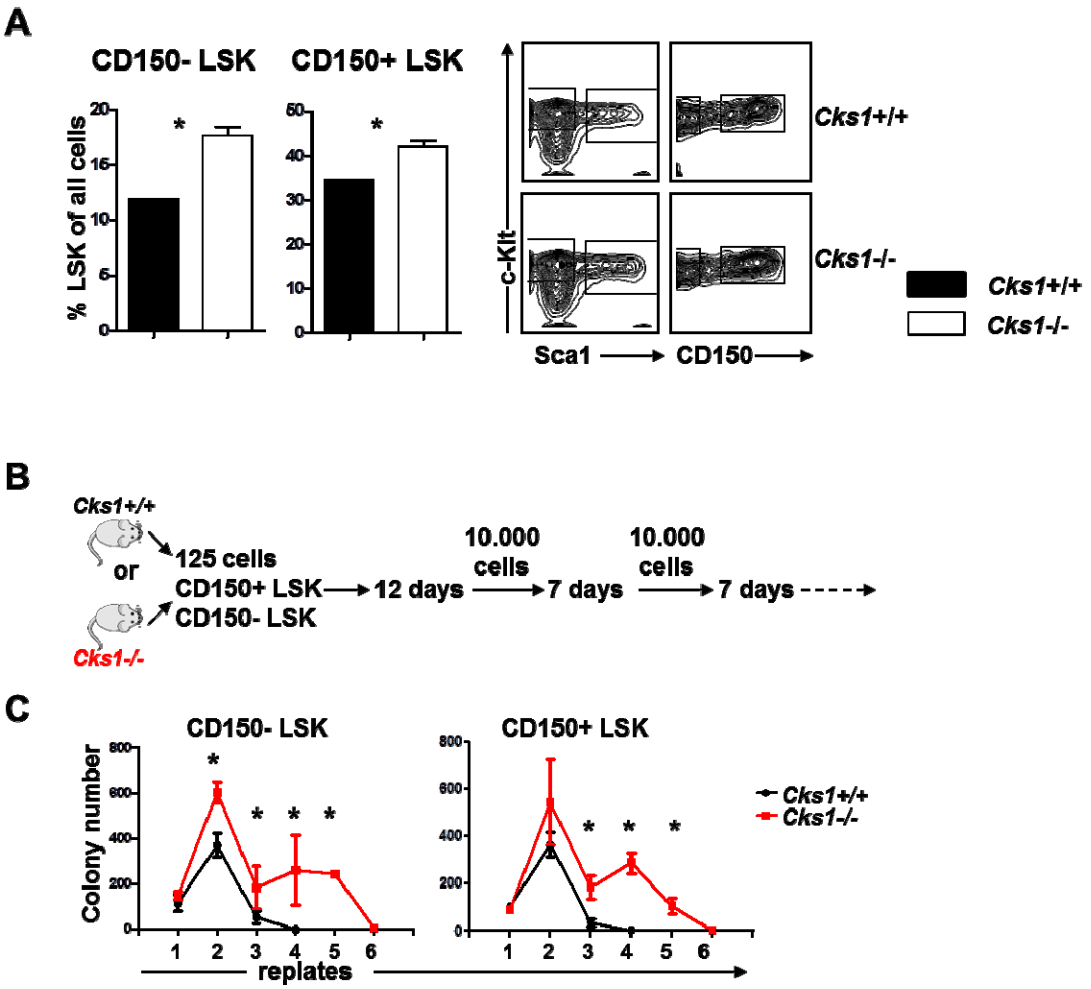


Figure 18: Loss of Cks1 in LSK results in increased replating capability and increased LSK frequency after cultivating in vitro.

(**A**) On the left: frequency of remaining CD150+ and CD150- LSK 48 hours after cultivation in medium with supplemented SCF, TPO and Flt3L analyzed by FACS (2 independent experiments); on the right: representative FACS analysis of the experiment. (**B**) Scheme of methylcellulose replating experiment: sorted CD150+ und CD150- WT and *Cks1*-/- LSK were cultivated in methylcellulose for 12 days. Colonies were counted, the cells were homogenized and equal numbers of cells were replated. This procedure was repeated until no colonies were detected. (**C**) Colony numbers in the replating experiment (2 independent experiments).

4.2.12. Transplantations of CD150+ LSK cells: loss of Cks1 leads to accumulation of LT-HSC

The serial BM transplantations (Fig. 17) suggest that *Cks1*-/- donor HSC possess an enhanced ability to self-renew. Though, since the percentage of CD34- LSK as well as the percentage of CD150+CD34- LSK of absolute cell number at steady state was significantly increased in the *Cks1*-/- mice (Fig. 9B), the *Cks1*-/- transplant contained more CD150+ LSK. Calculated 22,17 *Cks1*-/- CD150+ LSK vs. 16,82 WT CD150+ LSK were transplanted per recipient mice in the primary transplantation. Also, the frequency of these early subpopulations, which are responsible for the secondary engraftment, was already slightly increased (not significant) at week 16 after primary transplantation (Fig. 16E). To avoid aberration and transplantation of unequal HSC numbers and to test the quality of LT-HSC lacking Cks1 *in vivo*, serial transplantations with sorted CD150+ LSK were performed. The bone marrow from 2 WT or 2 *Cks1*-/- was pooled, depleted for lineage-committed cells and stained with cell surface antibodies. CD150+ LSK cells were sorted and 300 CD150+ LSK were transplanted together with $1x10^5$ recipient-derived BM and $5x10^5$ recipient-derived helper spleen cells into lethally irradiated CD45.1xCD45.2 recipients (Fig. 19A, B). The CD150+ LSK cells from both genotypes were able to engraft and repopulate the blood system of the recipient mice. Strikingly, the engraftment in the recipients of *Cks1*-/- delivered CD150+ LSK was significantly decreased in blood, BM, spleen and LN (Fig. 19C, D). This finding is consistent with the slower cycling of cells lacking Cks1 (Fig. 12C and 15D). Different organs of the recipient mice were also analyzed for the donor delivered mature populations. Interestingly, a similar effect as after chemo-ablative stress (Fig. 14D) was observed. The fraction of the *Cks1*-/- CD150+ LSK derived B cells was significantly higher in the blood, bone marrow and the lymph node. On the other hand, the fraction of T cells was significantly decreased in the spleen and lymph nodes (Fig. 19E).

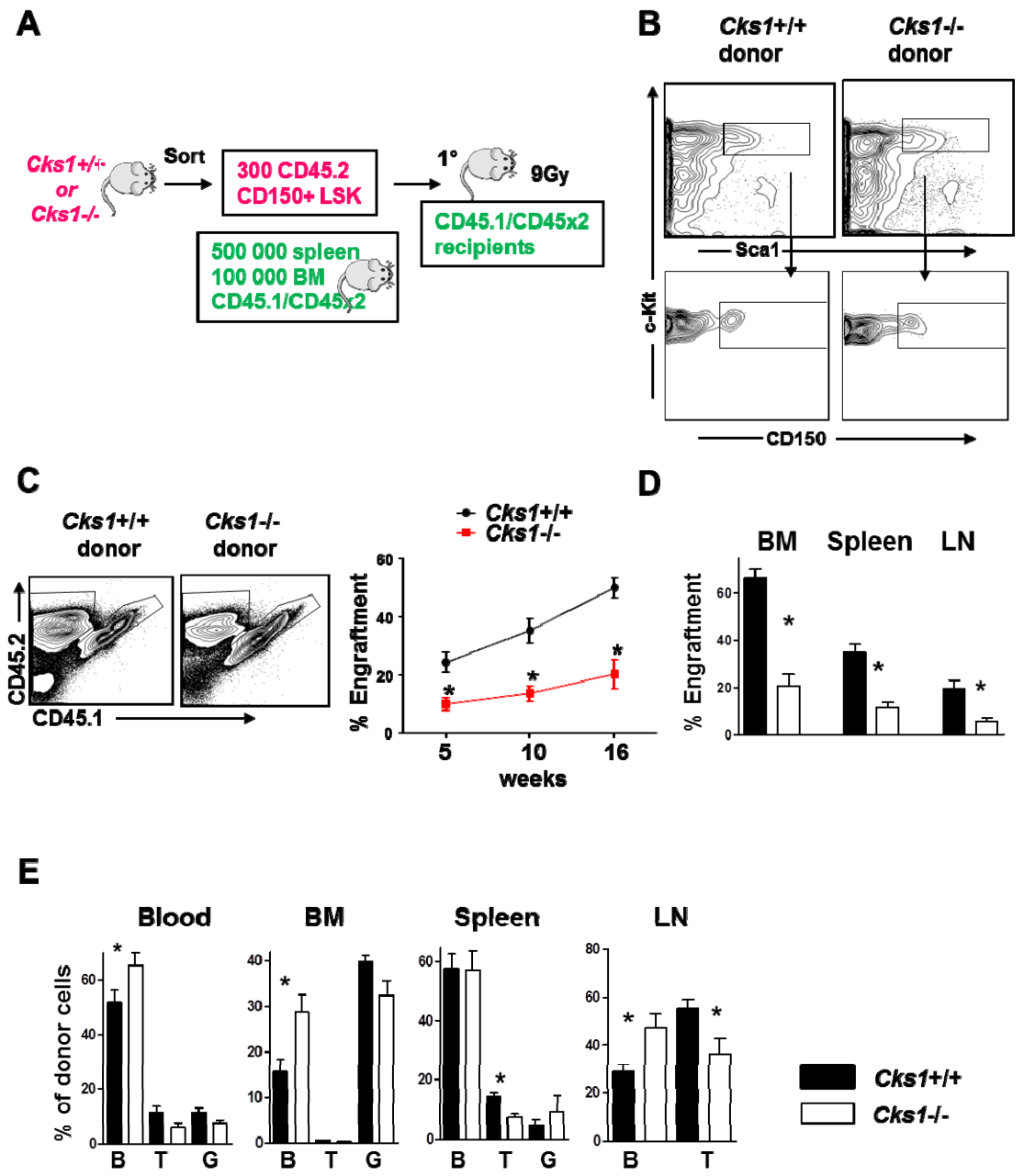


Figure 19: Transplantations of CD150+ LSK cells. Decreased engraftment of Cks1-deficient cells and increase in the *Cks1-/-* donor-delivered B cell population.

(**A**) Scheme of the transplantation experiment: 300 sorted CD150+ LSK from WT and *Cks1*-/- mice were transplanted in lethally irradiated recipients together with $5x10^5$ helper WT spleen cells and $1x10^5$ helper WT BM cells. (**B**) Sorting strategy for the transplantation of 300 CD150+ LSK, gated on Lin- cells. (**C**) On the left: representative dot blots from the blood FACS analysis of recipient mice. On the right: Engraftment (% CD45.2+ cells) in the peripheral blood of the recipients 4, 10 and 16 weeks after transplantation. (**D**) Engraftment in BM, spleen and LN 16 weeks after transplantation. (**E**) Frequency of donor-derived B cells (B), T cells (T) and granulocytes (G) in the indicated tissues (recipients of *Cks1*+/+ cells: n=8, recipients of *Cks1*-/- cells: n=11; 2 independent experiments).

Further flow cytometric analysis of the donor-derived early hematopoietic subsets in the sacrificed recipients at week 16 after transplantation revealed a significant decrease in the MPP population within the lineage negative subset in the recipients of *Cks1*-/- HSC (Fig. 20A, B). At the same time, the fraction of recovered *Cks1*-/- donor-derived CD150+ LSK was increased (Fig. 20A, B). Furthermore, the frequency of the *Cks1*-/- donor-derived CD150+ cells within the donor LSK population was increased (Fig. 20C). The decrease in engraftment capacity of the LT-HSC lacking Cks1 and the accumulation of these cells 16 weeks after transplantation confirmed the suggestion, that Cks1 deficiency alters the function of the early hematopoietic cells probably through maintaining quiescence and thus prohibiting cell cycle entry and proliferation.

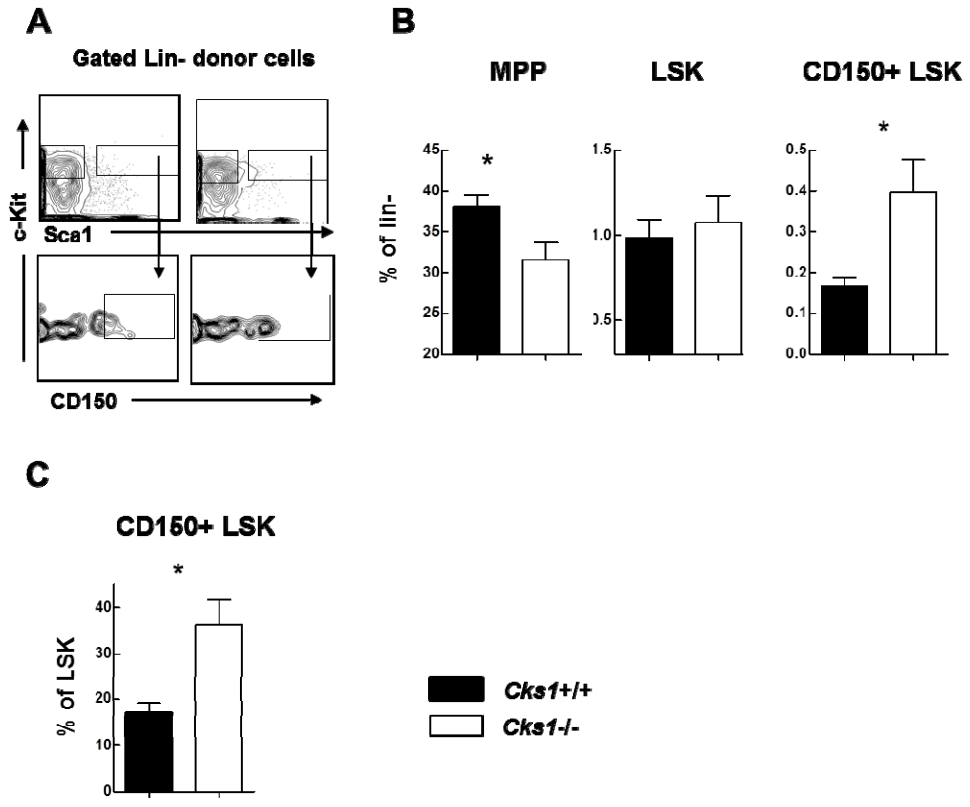


Figure 20: Transplantations of CD150+ LSK cells. Loss of Cks1 leads to decreased engraftment and accumulation of CD150+ LSK.

(**A**) Representative FACS analysis 16 week after transplantation of WT respectively *Cks1*-/- CD45.2+ CD150+ LSK in CD45.1xCD45.2 recipients, gated on Lin- donor cells. (**B**) Relative number of MPP, LSK and CD150+ LSK within the donor Lin- population 16 weeks after transplantation of 300 CD150+ LSK. (**C**) Relative number of CD150+ LSK within the donor LSK population (recipients of *Cks1*+/+ cells: n=8, recipients of *Cks1*-/- cells: n=11; 2 independent experiments).

4.2.13. Secondary transplantations of CD150+ LSK cells: *Cks1-/-* HSC proliferate slower

HSC self-renewal is experimentally defined as the capacity for long-term reconstitution of all blood lineages upon transplantation into recipient [33]. To look further into the self-renewing potential and persistence of WT vs. *Cks1*-/- CD150+ LSK, serial transplantations were performed. 300 donor-derived CD150+ LSK from the primary recipients were transplanted together with $1x10^5$ recipient-derived BM and $5x10^5$ recipient-derived helper spleen cells into lethally irradiated CD45.1xCD45.2 secondary recipients (Fig. 21A, B). Similar effects as after the primary transplantations were observed. The *Cks1*-/- cells engraftment was decreased compared to the control WT cells but the difference was not significant (Fig. 21C, D). Again, more B cells and partly less T cells arose from donor delivered *Cks1*-/- CD150+ LSK (Fig. 21E). Interestingly, in contrast to the primary CD150+ LSK transplantation, also the myeloid fraction was affected and *Cks1*-/- donor-delivered granulocyte population were decreased in the blood and BM of the secondary recipients (Fig. 21E). The analysis of the secondary recipients at week 16 post transplantation also revealed similar to the primary transplantations results for the donor delivered early hematopoietic populations. The frequency of the *Cks1*-/- donor-delivered MPP in the Lin- subset was decreased and the one of LSK was increased (Fig. 22A, B). To further look in the proliferation of the donor cells the secondary recipients were intraperitoneally injected with BrdU 12 hours before sacrifice. As expected, the BrdU incorporation in *Cks1*-/- donor-derived LSK populations was decreased (Fig. 22A, C), proving the assumption that these cells divide slower, thus possess reduced engraftment capacity.

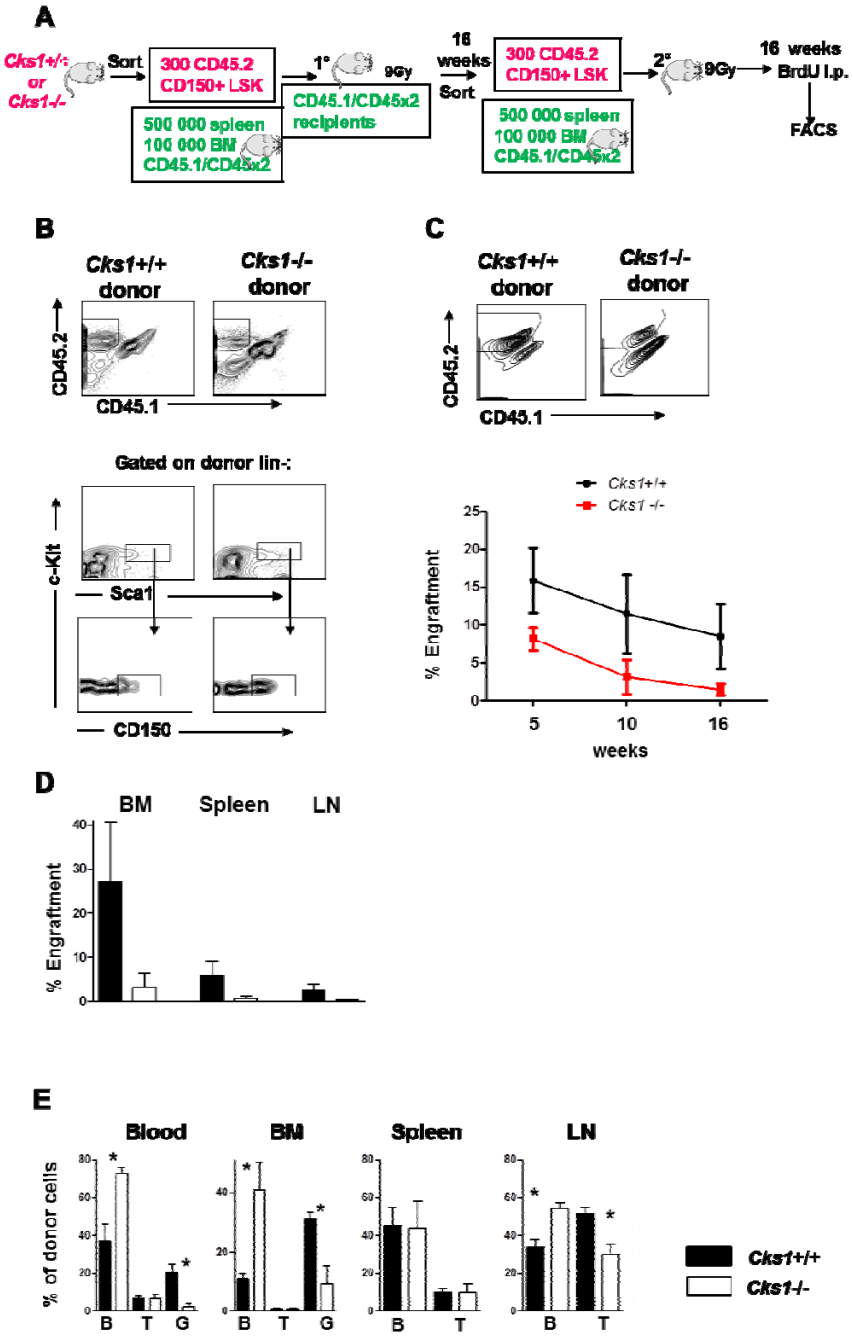


Figure 21: Secondary transplantations of CD150+ LSK cells: Cks1 controls lineage differentiation.

(**A**) Scheme of the experiment: 300 donor-derived CD150+ LSK were FACS-sorted from primary recipients of WT and *Cks1*-/- CD150+ LSK cells 16 weeks after transplantation and were secondary transplanted in lethally irradiated WT recipients together with $5x10^5$ WT helper spleen cells and $1x10^5$ WT helper BM cells. 12 hours before the analysis of the recipient mice, BrdU was injected i.p. (**B**) Sorting strategy for the secondary transplantation of 300 donor-derived CD150+ LSK, gated on Lin- cells. (**C**) Engraftment (% CD45.2+ cells) in the peripheral blood of the recipients 4, 10 and 16 weeks after transplantation. (**D**) Engraftment in BM, spleen and LN. (**E**) Donor-derived B cell (B), T cell (T) and granulocyte (G) populations (recipients of *Cks1*+/+ cells: n=8, recipients of *Cks1*-/- cells: n=9; 2 independent experiments).

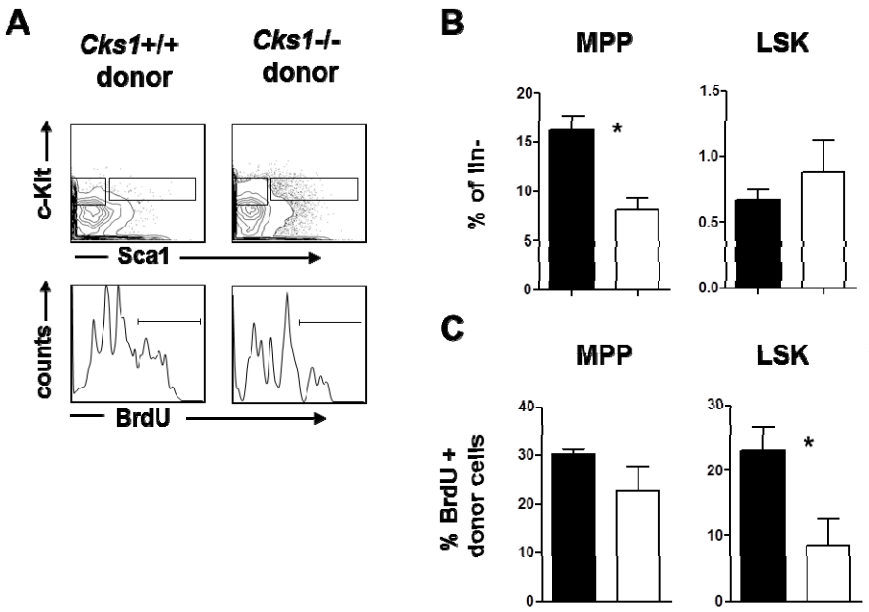


Figure 22: Secondary transplantations of CD150+ LSK cells: *Cks1-/-* HSC proliferate slower.

(**A**) Representative FACS analysis 16 weeks after secondary transplantation of 300 donor-derived CD45.2+ CD150+ LSK in CD45.1xCD45.2 recipients, gated on Lin- donor cells. Lower panel: BrdU incorporation in the donor-delivered LSK fractions. (**B**) Relative number of MPP and LSK within the donor Lin- population 16 weeks after secondary transplantation of 300 CD150+ LSK. (**C**) Percentage of BrdU+ MPP and LSK cells after the secondary transplantation (recipients of *Cks1*+/+ cells: n=8, recipients of *Cks1*-/- cells: n=9; 2 independent experiments).

4.3. p27 dependent and independent effects of Cks1 loss in the hematopoiesis: analysis of double knockouts

In this work, it was proven that the SCF^{Skp2} regulated CKI (p21, p27, p57) and the transcription repressor from the retinoblastoma family p130 accumulate most pronounced in the early hematopoietic cells (Fig. 5). The highest accumulation was observed for p27 with a 6,9 fold upregulation in *Cks1*-/- CD150+ LSK cells compared to WT CD150+ LSK (Fig. 5C, D). Based on this finding, the fact that Cks1 is strongly associated with p27 control [135, 182] and the role of p27 in hematopoiesis [101, 183, 184], the next part of this study focused on the question, if the observed effects of Cks1 as being a regulator of HSC/HPC homeostasis, correlate with the regulation of p27.

For this purpose, double knockouts (DKO) were generated by crossing *Cks1*-/- with *p27*-/- mice [177].

4.3.1. Steady state hematopoiesis in *p27-/-* and *Cks1/p27* DKO

Consistent with the role of p27 as a negative regulator of the cell cycle, mice lacking the *p27* gene are bigger than their WT littermates [28] as opposed to Cks1-deficient mice, which are abnormally small and accumulate p27 in various organs ([128] and this work). The generated DKO displayed normal body size and weight, indicating the direct connection between p27 degradation under Cks1 control.

Initially, the total cell number of bone marrow cells (2 femur and 2 tibia per mice) in the *p27*-/- and DKO mice was defined (Fig. 23A). The number correlated with the size of the animals. The *Cks1*-/- BM cell number was significantly decreased and *p27*-/- showed a significant increase in the BM cell number, while Cks1 loss in the DKO saved the observed effects and the total cell number in these mice was comparable with their WT littermates.

It was also necessary to determine how HSC/HPC populations in adult *p27*-/- and DKO mice behave at steady state conditions compared to WT and *Cks1*-/- populations. For this purpose, FACS analyses were performed with bone marrow cells from the different phenotypes. A comparable increase in the absolute number of MPP, LSK and LSK subpopulations was observed in *p27*-/- and DKO compared to WT and *Cks1*-/- (Fig. 23A, B), meaning that the loss of p27 has stronger impact and is the major event influencing the absolute number of HSC/HPC populations in DKO mice at steady state. Though, calculation of the proportion of one of the earliest HSC subsets (CD34- LSK and CD150+CD34- LSK) revealed that DKO HSC behave similar as *Cks1*-/- HSC and are present at higher rate in the BM. This result signifies a p27 independent effect in the distribution of early HSC.

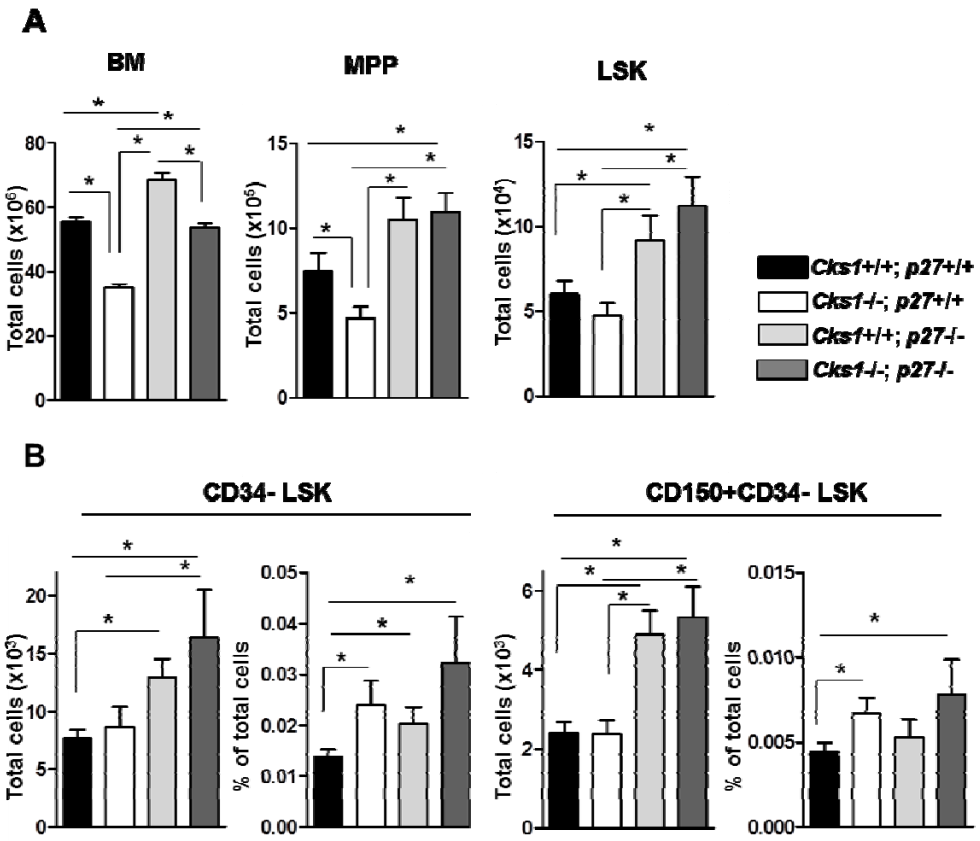


Figure 23: Bone marrow analysis in WT, *Cks1-/-*, *p27-/-* and DKO mice

(**A**) Total cell number of BM, MPP and LSK in different genotypes (WT n=20, *Cks1*-/- n=20, *p27*-/- n=16, DKO n=10, analyzed in 6 independent experiments). (**B**) Total cell number and percentage of total cell number of CD34-LSK (WT n=20, *Cks1*-/- n=20, *p27*-/- n=16, DKO n=10, analyzed in 6 independent experiments) and CD34-CD150+ LSK (WT n=15, *Cks1*-/- n=15, *p27*-/- n=11, DKO n=8; analyzed in 5 independent experiments).

4.3.2. Concomitant loss of Cks1 and p27 in BM cells results in better survival *in vitro* compared to *Cks1-/-* or *p27-/-* cells

In the first part of this study, a role of Cks1 in regulating apoptosis was established (Fig. 13). Similar experiments as described earlier (Fig. 13A) were performed with *p27*-/- and DKO mice. Analyzing the increase of total cultured cells, MPP and LSK after cultivating revealed that cells from DKO mice behave in culture similar as their *p27*-/- counterparts (Fig. 24A). Interestingly, after growth factors withdrawal and apoptosis induction, DKO MPP and LSK showed phenotype similar to WT cells and a better resistance to apoptosis than *Cks1*-/- or *p27*-/- cells (Fig. 24B). The fact that loss of p27 did not result in a reciprocal to *Cks1*-/- effect (Fig. 24B), leads to the assumption that other mechanisms

could be involved, when both Cks1 and p27 are deleted.

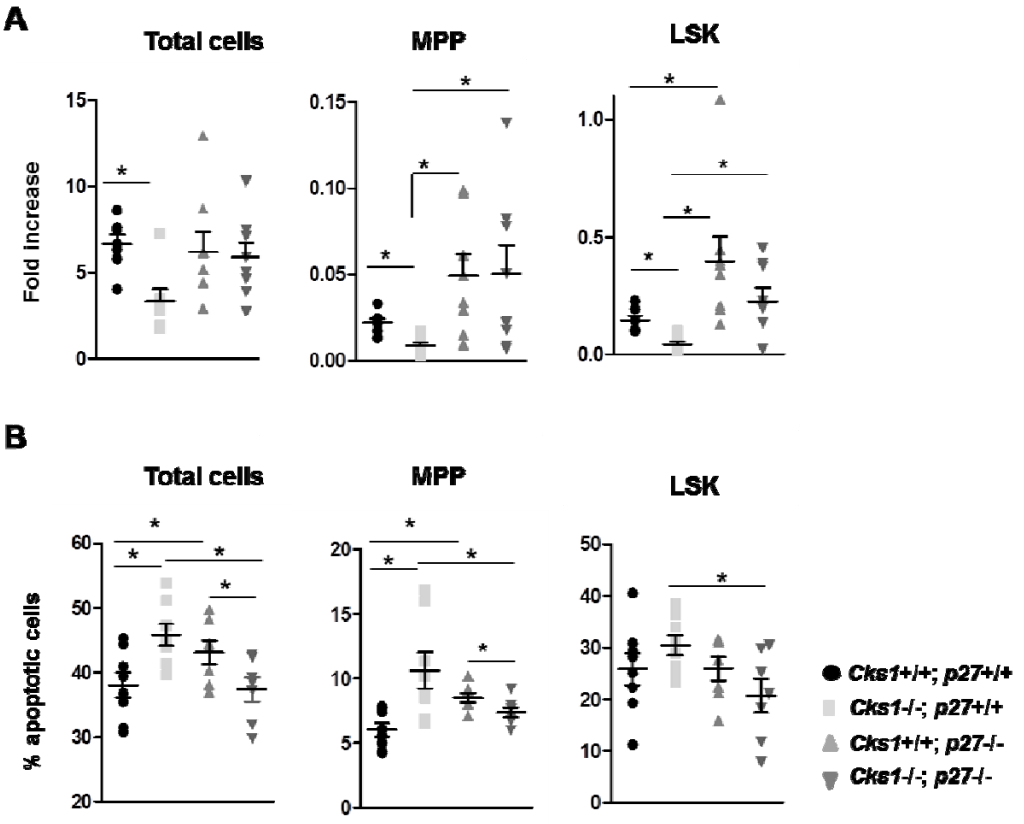


Figure 24: Concomitant loss of Cks1 and p27 in BM cells results in better survival *in vitro* compared to *Cks1-/-* or *p27-/-* cells.

(**A**) Growth of total cells, MPP and LSK (fold increase) after 5 days of incubation of lineage-depleted cells with supplemented growth factors. (**B**) Apoptosis analysis after growth factor withdrawal (WT n=8, *Cks1*-/- n=8, *p27*-/- n=7, DKO n=7; 3 independent experiments).

4.3.3. Cks1 controls the regeneration of mature lineages after cytotoxic stress through p27 regulation

Cytotoxic stress *in vivo* led to decelerated HSC response in *Cks1*-/- mice due to reduced cell cycle. To determine if the observed effects after 5-FU treatment of *Cks1*-/- mice were due to p27 accumulation, the same experiments as described in Fig. 14A were performed with *p27*-/- and DKO mice. First, the regeneration of the mature cell types was determined 6 days after chemo-ablation with 5-FU. The peripheral blood of *p27*-/- mice contained more T cells and less B cells compared to WT cells and reciprocal to *Cks1*-/- mice, which displayed an increase in the frequency of B cells and a decrease in the frequency of T cells (Fig. 25A). The same observation was made in the myeloid

fraction, where the *Cks1*-/- mice showed a decrease and *p27*-/- an increase in the granulocytes (Fig. 25A). The observed effects in the myeloid and lymphoid fractions were rescued by simultaneous deletion of Cks1 and p27 in DKO mice (Fig. 25A), pointing toward a p27 dependent effects in the distribution of mature hematopoietic subsets under stress conditions in *Cks1*-/- mice.

The regeneration of BM, MPP and LSK after the chemo-ablation was analyzed by calculating the ratio of total cell numbers from 5-FU treated and untreated mice (Fig. 25B). There were no significant differences in the ratios of BM cells in all analyzed genotypes. Surprisingly, the p27-deficient MPP displayed very low regeneration ability, an effect, which was rescued upon Cks1 loss in DKO MPP (Fig. 25B). As expected, *Cks1*-/- LSK regenerated slower, whereas DKO LSK displayed a phenotype similar to WT and exhibited the highest regeneration capacity of all analyzed genotypes (Fig 25B). It appears that the deletion of both, p27 and Cks1 lead to the normalization in stress hematopoiesis, even though deletion of p27 alone did not result in a reciprocal effect to the one seen in *Cks1*-/- LSK.

The same effect was observed after BrdU/7AAD cell cycle analysis of 5-FU treated mice (Fig. 25C). Consistent with the poor regeneration of LSK in *Cks1*-/- mice, the percentage of S Phase cells in this subset was decreased, whereas DKO LSK were proliferating more rapidly, comparable to WT LSK (Fig. 25C).

In conclusion, the analysis of the peripheral blood after cytotoxic stress demonstrates that Cks1 controls the distribution of mature hematopoietic populations after cytotoxic stress probably through regulating the p27 levels. Though, the effects observed in the HSC are most likely independent from a p27 accumulation, since loss of p27 did not result in a reciprocal phenotype to *Cks1*-/-.

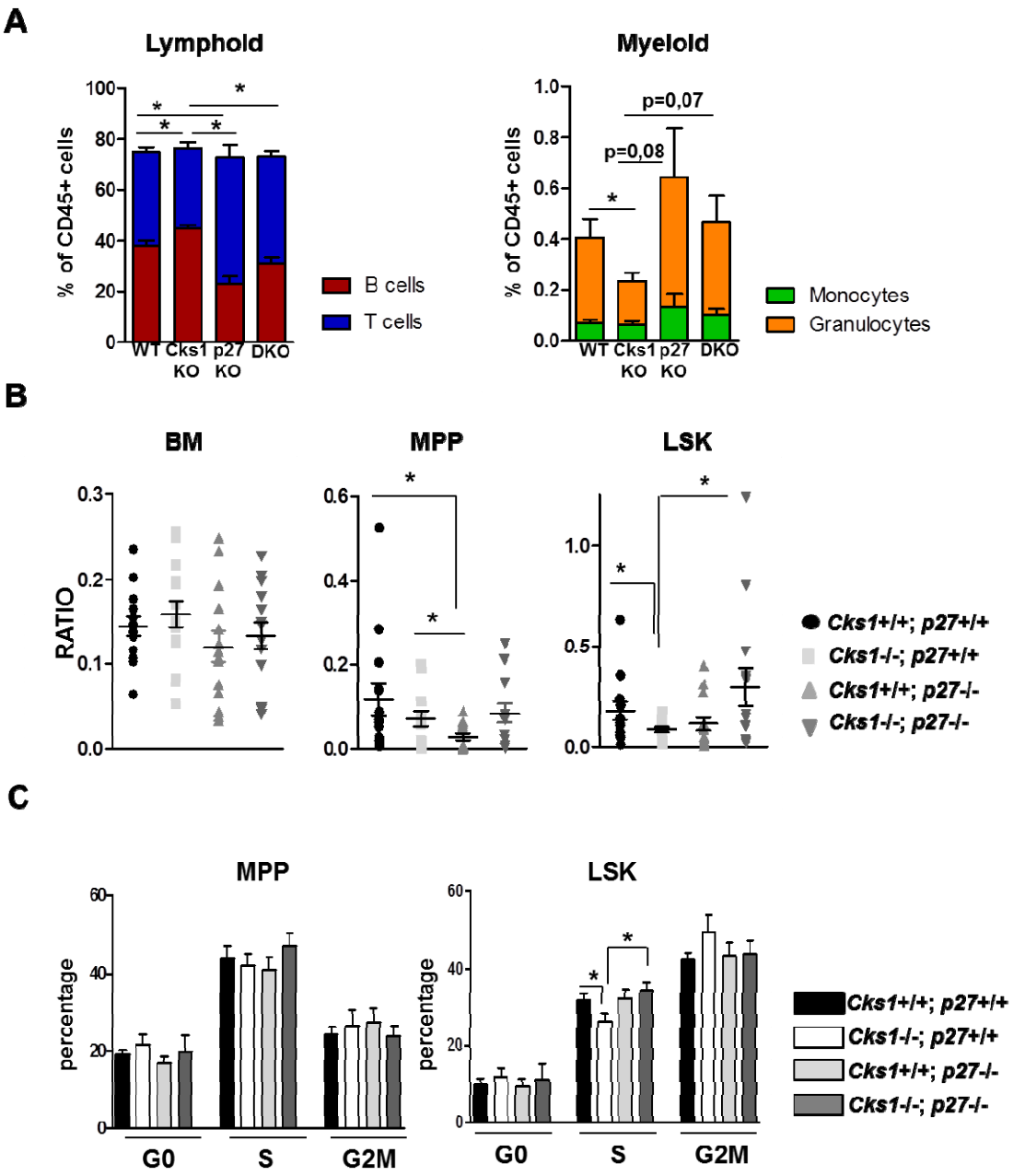


Figure 25: Cks1 controls the regeneration of mature lineages after cytotoxic stress through p27 regulation.

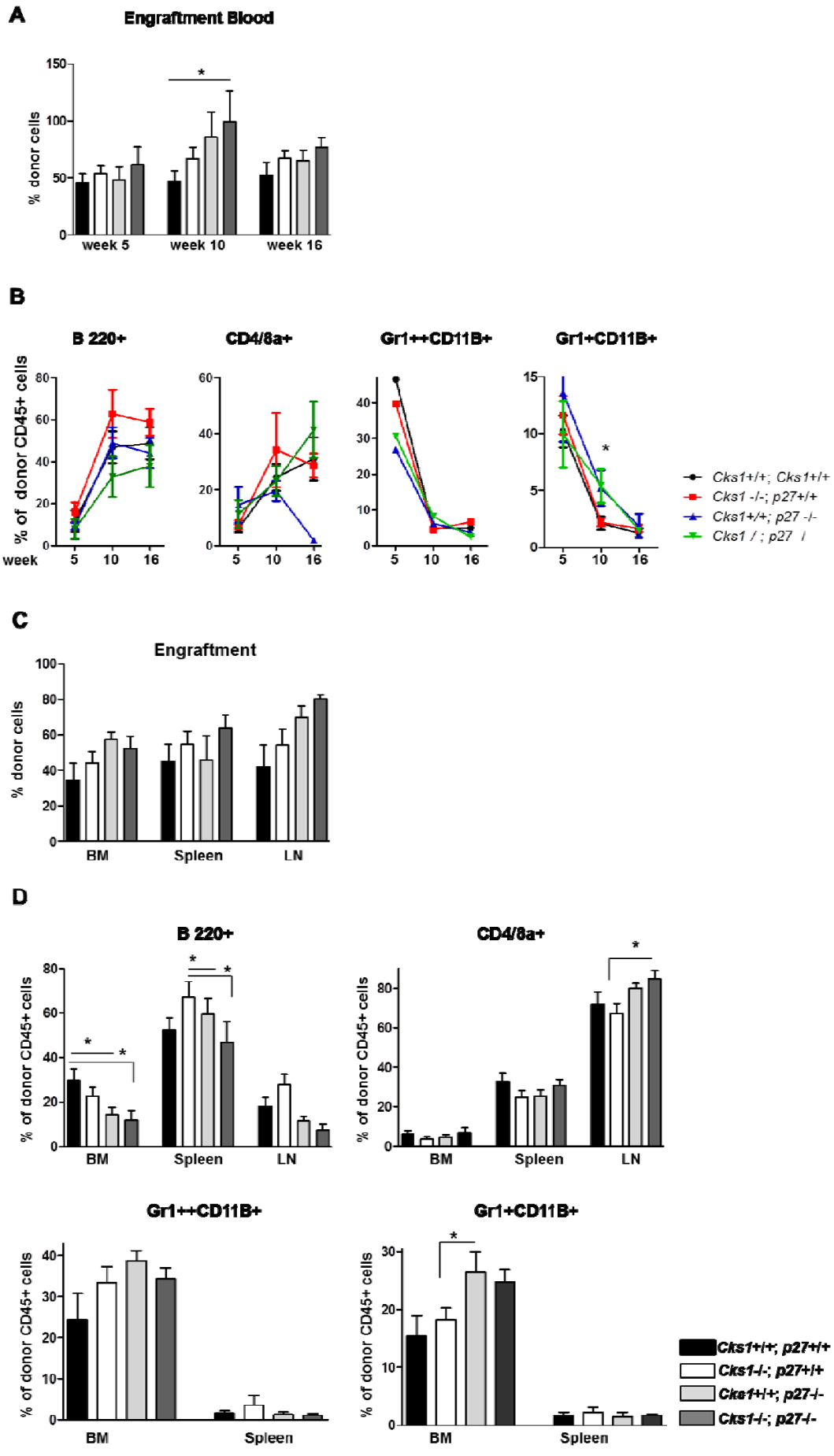
Mice of the indicated genotypes were treated i.p. with 5-FU (150 µg/g body weight). After 5 days, BrdU was injected i.p. and 12 hours later the mice were sacrificed. Peripheral blood, MPP and LSK subsets were analyzed by FACS. Additionally BM was stained with BrdU-antibody and cell cycle analysis of MPP and LSK cells was performed. (**A**) Detailed analysis of the B cells (B220+), T cells (CD4/8a+), granulocytes (Gr1++CD11B+) and monocytes (Gr1+CD11B) 6 days after 5-FU treatment. (**B**) Ratio (day 6 after 5-FU treatment/day0) of absolute cell numbers. (**C**) Cell cycle analysis of MPP and LSK from the indicated genotype (WT n=14, *Cks1*-/- n=15, *p27*-/- n=15, DKO n=14, 3 independent experiments).

4.3.4. Serial transplantations with *p27-/-* and *Cks1/p27* DKO BM

Performing serial transplantations assays with BM and sorted CD150+ LSK, a role of Cks1 in regulating HSC quiescence and self-renewal was established (Chapters 4.2.9/10. and 4.2.12/13.). Lack of Cks1 resulted in increased engraftment and an increase of total CD150+ LSK cell number after secondary transplantation of BM. In order to examine if the observed effect was due to the p27 accumulation in the *Cks1-/-* cells, serial BM transplantations were performed with additionally *p27-/-* and DKO as donors (Fig. 17A, scheme of the experiment). Detailed analysis was performed after primary and secondary transplantations of BM. Cells from all analyzed genotypes were able to engraft and restore hematopoiesis in the irradiated recipient animals. Similar to the earlier results in the first part of the study (Fig. 16B, C), no significant differences between the different genotypes were observed in the engraftment of the blood (Fig. 26A), the fractions of donor delivered B-, T cells, granulocytes and monocytes (Fig. 26B) and the engraftment in the BM, spleen and LN (Fig. 26C). The percentage of donor delivered B cells was decreased in the BM and spleen of recipients of DKO BM and increased in the spleen of mice which received *Cks1-/-* BM (Fig. 26D). The recipients of *Cks1-/-* BM also showed a decrease in the donor-delivered T cells in the LN compared to recipients of DKO BM (Fig. 26D).

Figure 26 (on page 87): Primary BM Transplantations. Analysis of the mature populations.

Lethally irradiated WT recipients were injected with $2x10^5$ BM cells from donor mice of the indicated genotypes and $4x10^5$ WT helper spleen cells. (**A**) Blood engraftment at week 5, 10 and 16 after transplantation. (**B**) Detailed analysis of donor-derived B cells (B220+), T cells (CD4/8a+), granulocytes (Gr1++CD11B+) and monocytes (Gr1+CD11B+) in the blood of recipients at week 5, 10 and 16 after transplantation. (**C**) Engraftment in BM, spleen and LN 16 weeks after transplantation. (**D**) Detailed analysis of the mature hematopoietic populations in BM, spleen and LN at week 16 after transplantation (n=8 recipients of WT cells, n=11 recipients of *Cks1-/-* cells, n=5 recipients of *p27-/-*cells, n=4 recipients of DKO cells; 2 independent experiments).


A
Engraftment Blood
% donor cells
week 5
week 10
week 16
B
B 220+
CD4/8a+
Gr1++CD11B+
Gr1+CD11B+
% of donor CD45+ cells
week
Cks1+/+; Cks1+/+
Cks1 -/-; p27+/+
Cks1+/+; p27 -/-
Cks1 / ; p27 /
C
Engraftment
% donor cells
BM
Spleen
LN
D
B 220+
CD4/8a+
Gr1++CD11B+
Gr1+CD11B+
% of donor CD45+ cells
BM
Spleen
LN
Cks1+/+; p27+/+
Cks1-/-; p27+/+
Cks1+/+; p27-/-
Cks1-/-; p27-/-

Analysis of the early subpopulations 16 weeks after the primary BM transplantations revealed that the absolute number of *Cks1*-/- and DKO donor delivered LSK and CD150+ LSK was increased, the increase being significant in the DKO donor subset (Fig 27). This observation correlates with the steady state analysis of the DKO mice, which showed a marked increase of the percentage of CD34- and CD34-CD150+ LSK of BM cells, indicating that the primary transplants contained higher numbers of HSC stem cells.

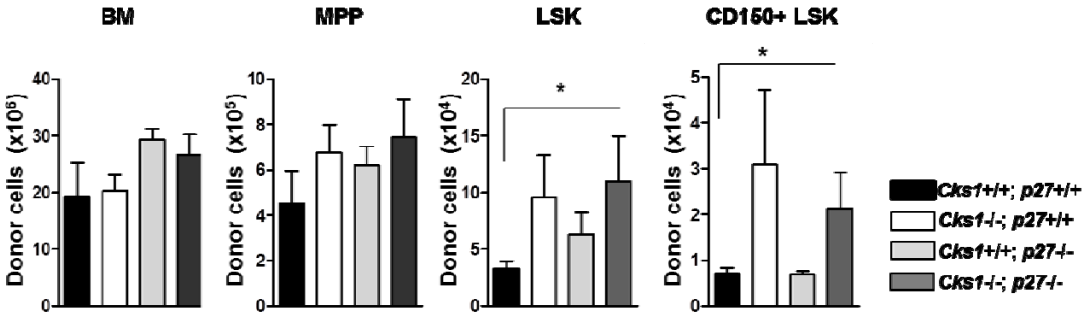


Figure 27: Primary BM Transplantations. Analysis of HSC/HPC.

Primary transplantations were performed as described in figure legend 26.

Absolute number of donor bone marrow cells, MPP, LSK and CD150+ LSK on week 16 after BM transplantation of the indicated genotype (n=8 recipients of WT cells, n=11 recipients of *Cks1*-/- cells, n=5 recipients of *p27*-/- cells, n=4 recipients of DKO cells; 2 independent experiments)

Additionally, secondary transplantations were performed with BM of mice that received WT, *Cks1*-/-, *p27*-/- and DKO BM in a primary transplantation. As already observed, the engraftment of the *Cks1*-/- cells was increased in the blood of the reconstituted mice at week 22 post transplantation (Fig. 28A). The population of donor delivered B and T cells behaved similar in *p27*-/- and DKO recipients in contrast to recipients of WT and *Cks1*-/- cells (Fig. 28B). The percentage of *p27*-/- and DKO donor B cells was decreased, while the fraction of WT and *Cks1*-/- donor B cells was increased. Similarly, an increase in the *p27*-/- and DKO donor T cells correlated with a decrease of this fraction in recipients of WT and *Cks1*-/- (Fig 28B). These results point out that p27 is a regulator of lymphoid differentiation.

Subsequently, engraftment analysis in the BM and spleen of the secondary recipients revealed that the observed increase of engraftment capability of *Cks1*-/- in the BM occurred in a p27 independent manner (Fig. 29A). Consistent

with the blood analysis (Fig. 28B), the percentage of *Cks1*-/- delivered granulocytes was increased in the BM of secondary recipients (Fig. 29B). The percentage of donor-derived B cells was elevated in the BM of secondary recipients of *Cks1*-/- cells (Fig. 29B). Furthermore, the fraction of donor-derived WT and *Cks1*-/- T cells in BM and spleen was decreased, whereas this population was elevated in secondary recipients of *p27*-/- and DKO BM (Fig. 29B).These results confirmed the observations made in the peripheral blood of the secondary recipients and strongly suggest p27 as a regulator of lymphoid progenitors and their differentiation.

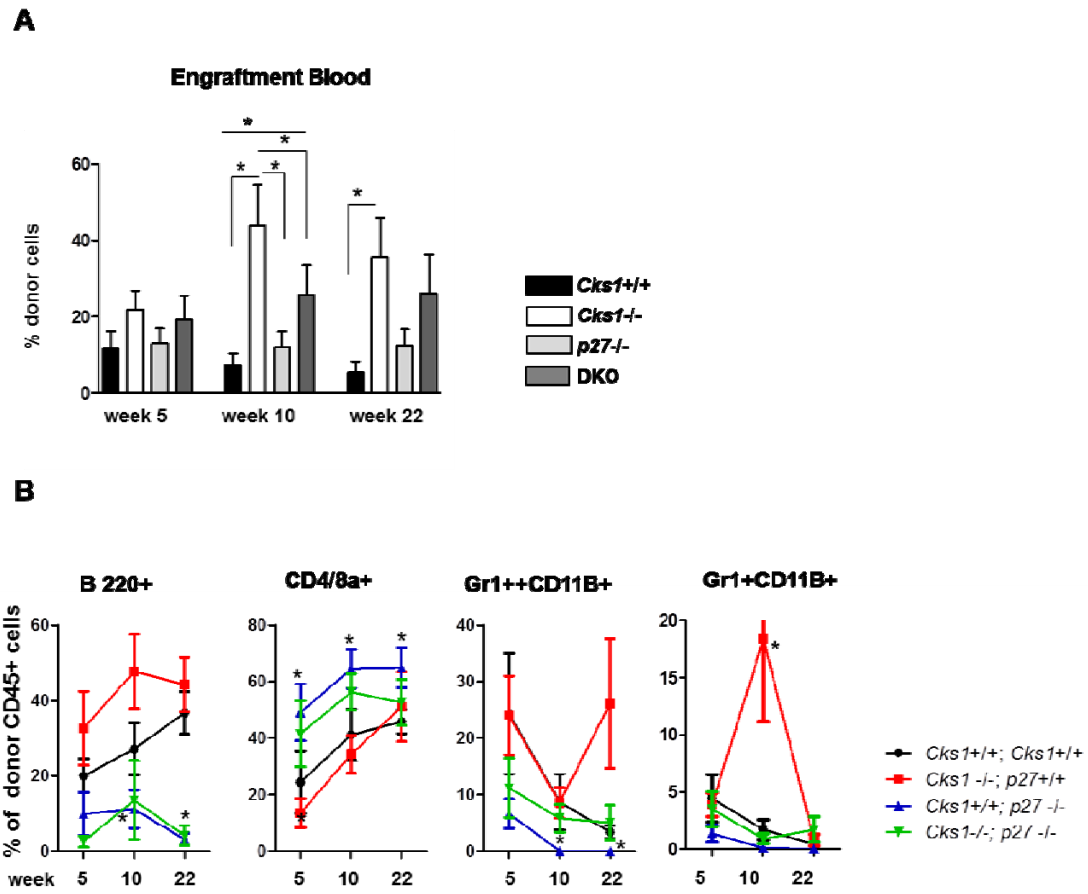


Figure 28: Blood analysis of secondary BM Transplantations. p27 controls lymphoid lineage differentiation.

Lethally irradiated WT recipients on mixed background were injected with $1x10^6$ BM cells delivered from primary recipients of WT, *Cks1*-/-, *p27*-/- and DKO BM cells together with $4x10^5$ WT delivered helper spleen cells. (**A**) Blood engraftment at week 5, 10 and 22 after transplantation. (**B**) Detailed analysis of the blood at week 5, 10 and 22 after transplantation of B cells (B220+), T cells (CD4/8a+), granulocytes (Gr1++CD11B+) and monocytes (Gr1+CD11B+) (n=8 secondary recipients of WT cells, n=8 secondary recipients of *Cks1*-/- cells, n=7 secondary recipients of *p27*-/- cells, n=8 secondary recipients of DKO cells; 2 independent experiments).

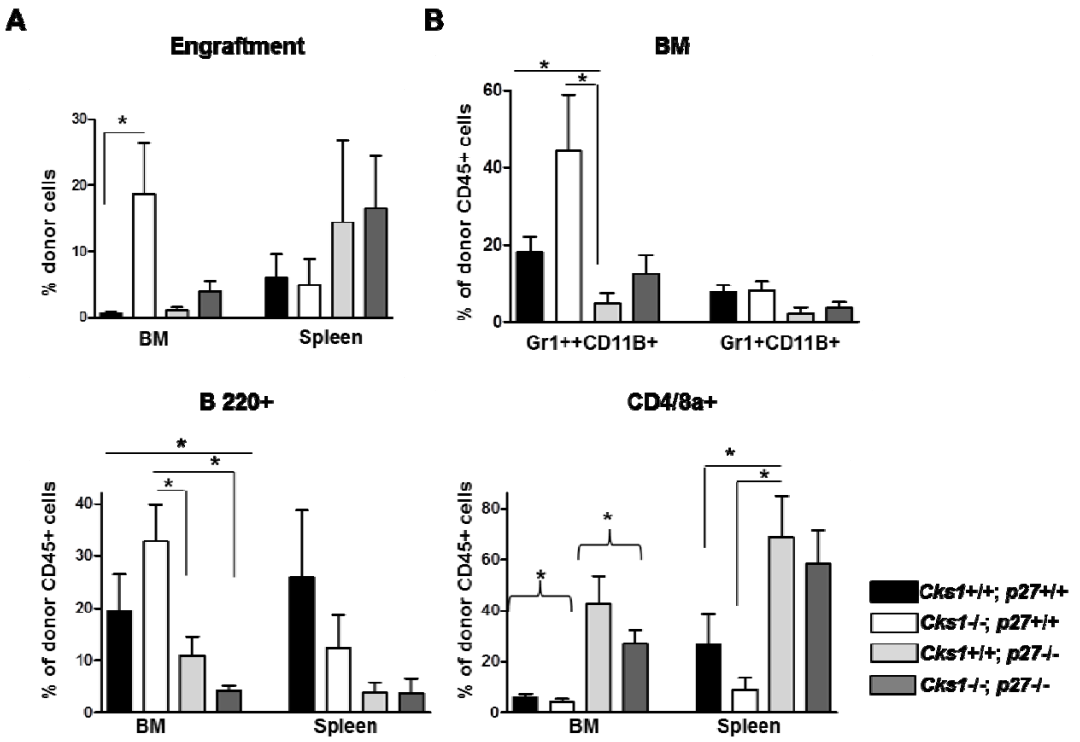


Figure 29: BM and spleen analysis of secondary BM Transplantations. p27 controls lymphoid lineage differentiation

Secondary transplantations were performed as described in figure legend 28. (**A**) Engraftment in BM and spleen 22 weeks after transplantation. (**B**) Detailed analysis of the mature populations in BM and spleen at week 22 after transplantation. (n=8 secondary recipients of WT cells, n=8 secondary recipients of *Cks1*-/- cells, n=7 secondary recipients of *p27*-/- cells, n=8 secondary recipients of DKO cells; 2 independent experiments).

Finally, the donor delivered absolute numbers of BM cells, HPC and HSC populations in the BM of secondary recipients was calculated (Fig. 30). As already proven in the first part of the study, *Cks1*-/- HPC/HSC were detected in significant high levels after the secondary BM transplantation. Remarkably, the observed tendency in the DKO delivered HSC, which were elevated after the primary transplantation (Fig. 27) did not persist and they displayed a *p27*-/- similar phenotype, indicating that these cells exhausted faster than cells lacking Cks1 only.

Taken together, most of the experiments with *p27*-/- and DKO mice indicate that the observed effects in Cks1-deficient mice are not due to sole p27 accumulation and only the lineage distribution upon hematopoietic stress seem to be controlled through Cks1 in a p27 dependent manner.

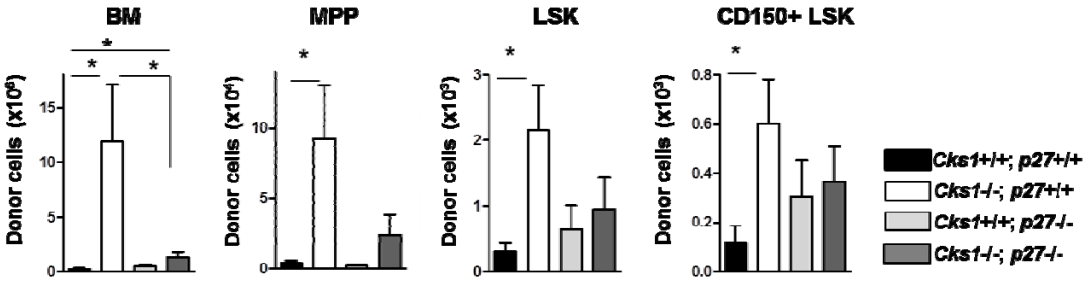


Figure 30: Secondary Transplantations of whole bone marrow. Analysis of HSC/HPC

Secondary transplantations were performed as described in figure legend 28.

Absolute numbers of donor delivered BM, MPP, LSK and CD150+ LSK cells at week 22 after secondary transplantation (n=8 secondary recipients of WT cells, n=8 secondary recipients of *Cks1*-/- cells, n=7 secondary recipients of *p27*-/- cells, n=8 secondary recipients of DKO cells; 2 independent experiments)

4.4. The role of Cks1 in a HSC disease

In the course of this project an important role of Cks1 in HSC homeostasis was established. Considering that uncontrolled HSC/HPC regenerative activity is a hallmark of the prototypic HSC disorder chronic myeloid leukemia [185, 186], it was tempting to analyze the role of Cks1 in CML regulation. For this purpose, assays with a BCR-ABL expressing cell line, human samples and methylcellulose with mouse BCR-ABL expressing HSC/HPC cells were performed.

4.4.1. Cks1 transcript levels are regulated by BCR-ABL acitivity

First, the expression of *Cks1* (*CKS1B* in humans) in CML patients was assessed. *CKS1B* transcription from imatinib treated and untreated patients analyzed by real-time PCR revealed that *CKS1B* transcript levels were significantly elevated in untreated CML (Fig. 31A). Also, publicly available gene expression data with the accession numbers GSE24739 [187] and GSE12211 [188] was downloaded from the gene expression omnibus website and analyzed using the software program R-studio. In this case, CKS1B expression in chronic and accelerated phase (Fig. 31B) as well as in treated and untreated CML (Fig. 31C) was examined in association with the CKI p21 (*CDKN1A*), p27 (*CDKN1B*), p57 (*CDKN1C*) and p130 (*RBL2*). The CKI and p130 gene expression levels correlated negatively with CKS1B expression (Fig. 31B, C).

Correlating with the results in Fig. 31A, *CKS1B* expression was significantly increased in chronic and accelerated phase CML versus normal control cells (Fig. 31B). Furthermore, the transcription levels of *CKS1B* were significantly suppressed upon imatinib treatment (Fig. 31C). This result was confirmed *in vitro* using BaF3-p210 cells, which ectopically overexpress BCR-ABL. Immunoblot analysis with BaF3-p210 cell pellets demonstrate that Cks1 protein levels were reduced after treatment with imatinib (Fig. 31D). On the other hand, activation of BCR-ABL activity in the thermo-sensitive cell line BaF3-*ts* p210 (cultivating at 32°C) led to an increase in *Cks1* transcript levels (Fig. 31E). Thus, Cks1 expression is associated with BCR-ABL activity in the highly proliferative stem cell disorder CML.

4.4.2. BCR-ABL expression in *Cks1-/-* BM cells results in decreased colony forming capability

Since expression of Cks1 is being regulated by BCR-ABL activity in CML, it was important to further evaluate the role of Cks1 in BCR-ABL mediated clonogenetic activity. For this purpose, 5-FU mobilized WT and *Cks1*-/- bone marrow was infected with BCR-ABL-GFP retrovirus. Methyl cellulose assays on medium without any supplemented factors were preformed either with sorted GFP+ cells, GFP+ MPP or GFP+ LSK (Fig. 32A). The medium is eligible only for transformed cells, since normal cells need growth factor in *in vitro* culture in order to survive and proliferate. In all cases, BCR-ABL expressing cells lacking Cks1 displayed significantly reduced ability to form hematopoietic colonies (Fig. 32B). This result points out that Cks1 is a mediator of BCR-ABL induced cytokine-independent clonogenetic activity, indicating a possible role of Cks1 in CML regulation.

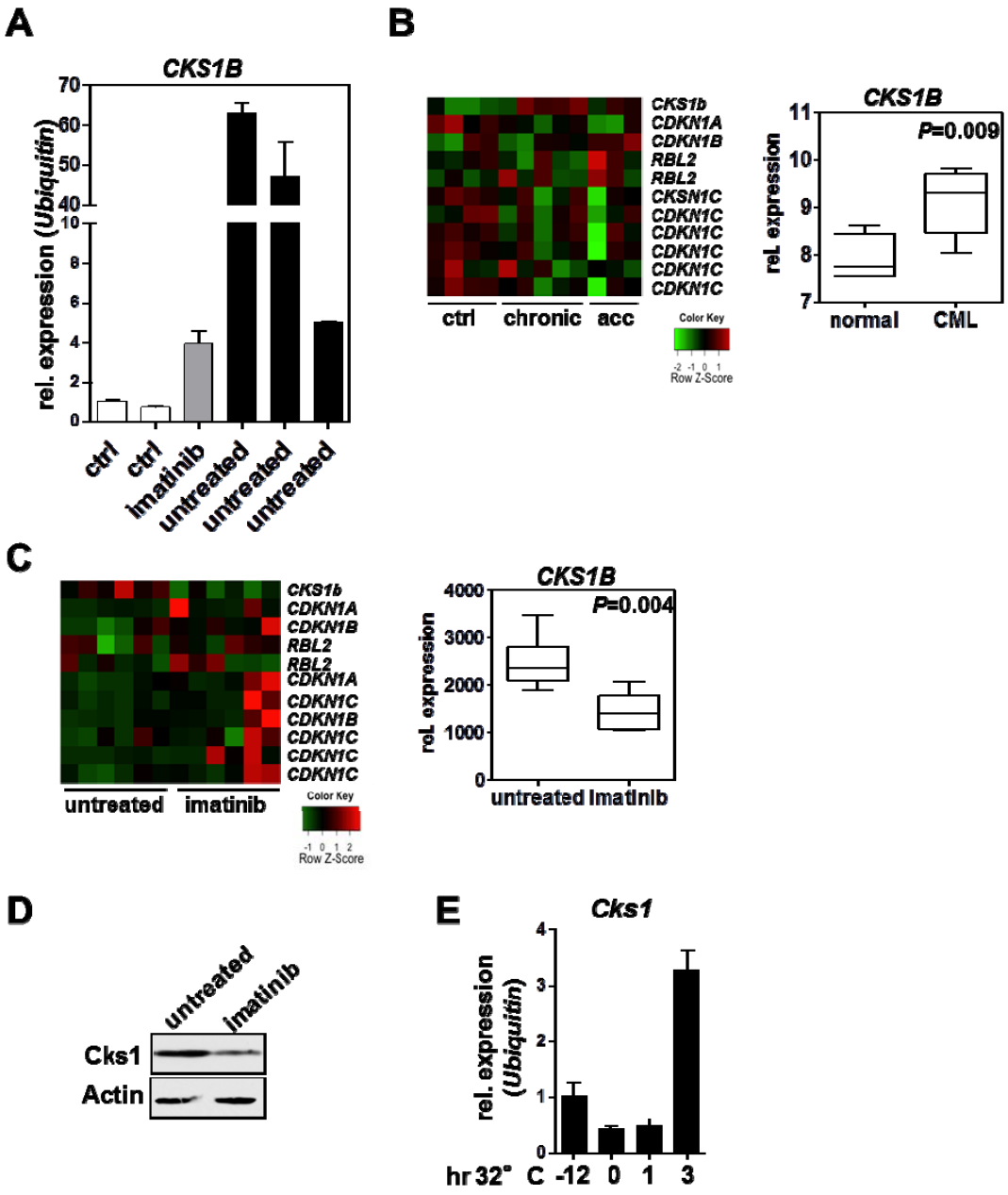


Figure 31: Cks1 is up-regulated in CML.

(**A**) mRNA was isolated from peripheral blood mononuclear cells from healthy donors (ctrl), one imatinib treated patient in complete hematological remission (imatinib), or untreated CML patients (untreated), and analyzed for *CKS1B* transcript levels by real-time PCR. Shown is the relative *CKS1B* expression. (**B**) Expression of the indicated genes in control (ctrl), chronic and accelerated (acc) phase CML samples (source: www.oncomine.org). Left panel: heatmap; right panel: box plots. (**C**) Expression of the indicated genes in HSC/HPC cells of untreated or imatinib treated patients (source: www.oncomine.org). Left panel: heatmap; right panel: box plots. (**D**) Immunoblot analysis of BaF3-p210 cells treated with imatinib. (**E**) BaF3-*ts* p210 (active at 32°C) were cultured at 37°C (-12 to 0 hr) and transferred to 32°C. Shown is the relative expression of *Cks1* assessed by real-time PCR.

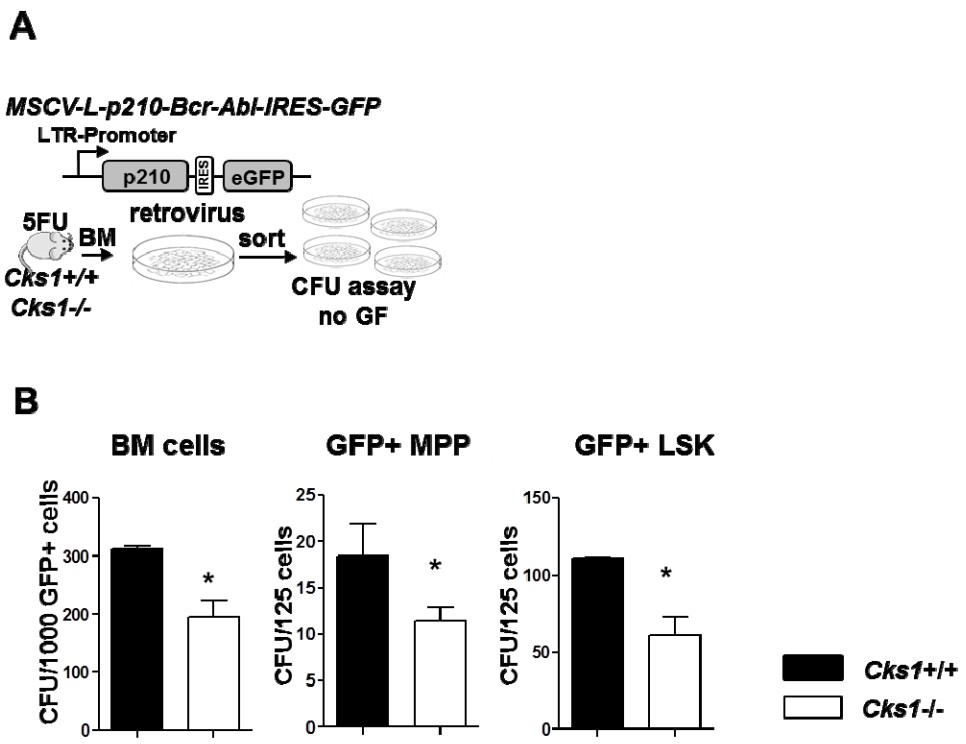


Figure 32: Bcr-Abl expression in *Cks1-/-* BM cells results in decreased colony forming capability.

(**A**) Scheme of the experiment: First, Phoenix-Eco 293T cell line was transfected with BCR-ABL-GFP expressing vector and used for virus production, which was used to infect BM cells from 5-FU treated WT and *Cks1*-/- mice. Subsequently, methyl cellulose assays in absence of cytokines with sorted BCR-ABL expressing GFP+ cells, GFP+ MPP or GFP+ LSK were performed. (**B**) Numbers of formed colonies from BCR-ABL expressing BM, MPP and LSK from WT or *Cks1*-/- mice in cytokine deficient methyl cellulose medium (n=4 for each genotype; 2 independent experiments).

5. Discussion

In this study, genetic *in vitro* and *in vivo* evidence is provided, that the rate-limiting component of the SCF^{Skp2} ubiquitin ligase, Cks1, is a critical mediator of size and activity of the HSC/HPC pool at steady state and in stress hematopoiesis. CKI regulated by SCF^{Skp2} were shown to accumulate in the early hematopoietic subsets in mice lacking Cks1. One of the downstream SCF^{Skp2} targets, p27, was studied more closely in order to determine if the observed effects were due to disturbed p27 regulation. The experiments revealed that lineage differentiation was indeed controlled by p27. However, the role of Cks1 in HSC self-renewal and quiescence seemed to be p27 independent. Nevertheless, evidence was provided that Cks1 is being regulated by BCR-ABL, pointing Cks1 out as a possible regulator in the HSC disorder CML.

5.1. Cks1 controls the HSC/HPC pool at steady state *in vivo* and protects HPC from apoptosis *in vitro*

The majority of HSC at steady state conditions is in G0 phase and only a small number is cycling and responsible for stem cell maintenance and mature cells production [31, 189]. It is known, that disruption of HSC quiescence leads to defects in self-renewal and may result in HSC exhaustion [12, 32]. On the other hand, if HSC are arrested in the G0 phase and enter the cell cycle more infrequently, accumulation in the early subsets and deficiency in the more mature HPC may appear. Such a scenario can be the explanation of the phenotype in *Cks1*-/- mice where lack of Cks1 resulted in reduction of HPC numbers, but an increase in the percentages of early HSC at steady state *in vivo* (Chapter 4.2.2.). The decline in the absolute BM and FL cell numbers (Chapter 4.2.1.) as well as in all HPC subpopulations (Chapter 4.2.2.) could be also related to the reduced body size of the *Cks1*-knockout animals [128, 177]. However, the reduction in the hematopoietic subsets was more enhanced compared to the body size decrease, pointing out the importance of Cks1 in the regulation of the hematopoietic subsets. Since the number of HSC/HPC is also controlled by their capacity to survive during homeostasis or under conditions of stress [52], in addition to the slower exit from the HSC stage, deregulated

apoptosis in the early *Cks1*-/- progenitors might also be playing a role in conserving LT-HSC. And indeed, *in vitro* investigation revealed higher susceptibility of *Cks1*-/- HPC to apoptosis compared to LT-HSC (Chapter 4.2.6.). An opposite effect was observed after cytokine withdrawal in *p27*-/- BM cell cultures [190], pointing p27 out as a possible negative regulator of survival in hematopoietic cells. This stands in contrast to the analysis of *p27*-/- hematopoietic cells in this study. Even the contrary effect was observed, after cytokine withdrawal in Lin- cell cultures, *p27*-/- MPP were more susceptible to apoptosis than WT MPP (Chapter 4.3.2.). This contradiction may be due to different methodology used in both experiments. While Dijkers et. al. used Sca+ BM cells, excluding just the MPP fraction, in this project Lin- cells, including the Lin- c-Kit+ Sca1- MPP fraction were cultured prior apoptosis analysis. Interestingly, ablation of both, Cks1 and p27 resulted in WT similar phenotype (Chapter 4.3.2.), suggesting that Cks1 regulates apoptosis in a p27 dependent manner. Although a discrepancy occurs in this observation since *p27*-/- cells did not show increased apoptotic resistance. Further analysis could provide a possible explanation of the observed effect. For better understanding of the role of Cks1 and p27 in apoptosis in hematopoietic cells, *in vivo* analysis, for instance using fluorochrom-labeled inhibitors of caspases [191] should be performed.

The CDK inhibitor p27 is a critical regulator of cell growth and proliferation and increased body size is a significant feature of the *p27-/-* phenotype that highlights the key role of p27 in maintenance of the G0 state, tissue homeostasis and prevention of malignant growth [28, 192-194]. Concomitant loss of Cks1 in *p27*-/- animals rescues the size defect back to wild type levels indicating a p27 dependent function of Cks1. But in the case of HPC pool at steady state, DKO mice exhibit a phenotype similar to *p27*-/- and possess increased total HPC numbers narrowing the role of Cks1 in regulating p27 protein turnover in those cells (Chapter 4.3.1). Also, when monitored in the presence of cytokines, MPP and LSK cells were better sustained in both, *p27*-/- and DKO cultures (Chapter 4.3.2). These findings support other studies, which define p27 as a regulator of the HPC alone [184] and of the HSC, together with p57 [101]. However, the increase in the percentage of early LSK observed in *Cks1*-/- mice was even more prominent in DKO mice (Chapter 4.3.1), clearly

indicating a p27 independent role of Cks1 at least in the regulation of the HSC pool.

5.2. Downstream targets of the SCF^{Skp2}-Cks1 complex are strongly involved in self-renewal and quiescence balance in HSC

The cell cycle is perhaps the most tightly regulated part of HSC regulation. The balance between self-renewal and differentiation is critical for the maintenance of the HSC throughout life [31, 52] and deviations from that balance can contribute to hematopoietic insufficiencies and the development of hematopoietic malignancies [195]. Phosphorylation through the activity of CDK and targeting for protein degradation through ubiquitin ligases such as SCF complexes are the most prominent factors involved in the regulation of HSC cycling [8, 196]. The present study demonstrates that several substrates of SCF^{Skp2}-Cks1 (the CKI p21, p27, p57 and the Rb family member p130) are strongly regulated by the ubiquitin ligase in the HSC/HPC subsets (Chapter 4.1.1). These CKI [94, 99-101] and p130 [91] have been identified as important regulators of HSC/HPC homeostasis by means of gene knock-out studies [32, 62]. CKI loss results in increased HSC cycling, an effect opposite to that observed in the *Cks1*-/- (Chapters 4.2.8 and 4.2.13) or *Skp2*-/- genotype [197, 198]. For instance, Cheng et al. demonstrated, that HSC in p21-deficient mice are increased in numbers at steady state due to accelerated proliferation [97]. However, more recent studies show p21 to have rather limited function in HSC regulation at steady state [47, 98], diminishing the possibility, that p21 alone is responsible for the observed effects in *Cks1*-/- mice. Thus, it is most likely that other SCF^{Skp2} targets are involved in the regulation of HSC/HPC homeostasis. Supporting this hypothesis, studies with double knockout of p57 and p27 [101] and triple knockout of pRb, p107and p130 [91] show, that these cell cycle inhibitors cooperate to maintain HSC quiescence. No hematopoietic phenotype was observed in p130 deficient mice [88], but conditional deletion of all tree Rb family members results in severe defects in self-renewal [91]. Besides, Rb family members collectively seem to maintain balance between lymphoid and myeloid cell fates in the hematopoietic system [91]. Analysis of p27-deficient mice suggest rather limited functions of p27 in HSC and more pronounced role in regulation of HPC [184]. On the other hand, p57 is shown to be critical for the

maintaining of HSC quiescence [100]. Interestingly, the defect in *p57*-/- HSC quiescence is enhanced by concomitant deletion of either p21 or p27 and only p27 overexpression compensates for the loss of p57 [100, 101]. The central finding in this project that p21, p27 and p57 accumulate most strongly in the *Cks1*-/- CD150+ LSK (Chapter 4.1.2.) and Cks1 deficiency causes an opposite effect (Chapters 4.2.10.-4.2.13.) than p27/p57 deletion [101] is in accord with the idea that Cks1 regulation is a determining factor in the early hematopoiesis upstream of CDK inhibitors. These findings clearly indicate that the control of CKI by SCF^{Skp2} is of paramount importance for maintenance of HSC self-renewal and quiescence. Complex studies with additional knockout animals and respectively double or triple knockouts, can provide definitive evidence for the link between Cks1 and CDK inhibitors. Another option to test if the observed effects in *Cks1*-/- HSC are due to p27 and p57 accumulation is by following the downstream targets of these CKI in the quiescent machinery. Zou et. al demonstrated, that concomitant loss of p27 and p57 results in nuclear import of an Hsc70/cyclin D complex and Rb phosphorylation [101], hence Cks1 deficiency might show cytoplasmic retention of Hsc70/cyclinD and reduced Rb phosphorylation in HSC.

Additionally, the fact that p21, p27 and p57 can compensate for each other could explain the results in this study with *p27*-/- and double knockouts for Cks1 and p27 (Chapter 4.3). Although some effects (body size, lymphoid differentiation after 5-FU treatment) seem entirely p27 dependent, the majority of the tests confirmed that in absence of p27, Cks1 loss affects most likely other downstream targets.

In Addition to the control via ubiquitin ligase mediated proteosomal degradation of the CIP/KIP family of CDK inhibitors and p130, SCF^{Skp2} controls other proteins that may also have important roles in HSC/HPC, such as IkB [199, 200], β-catenin [200] and E2F1 [201]. IkB is an inhibitory protein in the canonical NF-kB pathway, degradation of which is necessary to induce NF-kB activity [202]. Recently, NF-kB was shown as a positive regulator of HSC quiescence [203] through noncanonical signaling, which does not involve IkB [204]. This finding indicates that the control of the SCF^{Skp2} in the NF-kB pathway probably does not apply in HSC, which on the other hand correlates with the observed effects in this study, since lack of Cks1 resulted in increased HSC

quiescence (Chapters 4.2.10.-4.2.13.) and a hypothetical NF-kB inhibition would lead to the opposite effect. β-catenin, another SCF^{Skp2} target [200] is shown to promote HSC self-renewal in a contact dependent manner through the Wnt signaling pathway [205]. However, stabilization of β-catenin in the HSC resulted in HSC exhaustion [205], excluding SCF^{Skp2} as a possible regulator of this pathway in HSC, since the opposite effect was observed in this study (Chapters 4.2.10.-4.2.13.).

Cks1 also functions in regulating CDK expression and activity at least partly independent of SCF^{Skp2} [143, 206], thus additional studies in this direction might reveal a complex role of Cks1 in regulating HSC homeostasis.

Figure 33 represents the hypothetical involvement of the SCF^{Skp2} targets, which were shown to accumulate in *Cks1*-/- HSC/HPC (Chapter 4.1.1.) during early hematopoiesis.

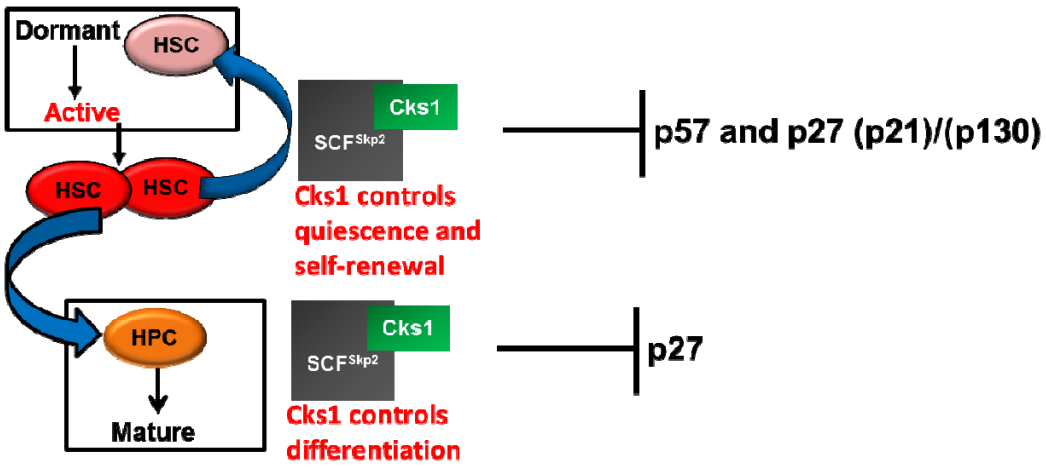


Figure 33: Hypothetical model representing the role of SCF^{Skp2}-Cks1 in early hematopoiesis and the possible CKI involvement.

5.3. Cks1 mediates the balance between lymphoid and myeloid lineage differentiation through regulation of p27

In addition to engraftment and self-renewal studies using transplantation assays, 5-FU analysis provides information about the regenerative capacity of HSC and progenitor differentiation after ablation of mature hematopoietic subsets [171, 178-180]. A remarkable observation was made after 5-FU treatment of Cks1 respectively p27-deficient mice and mice lacking both, Cks1 and p27 (Chapter 4.3.3). While loss of Cks1 resulted in an increase of B-

lymphocytes and decrease in T-lymphocytes and granulocyte percentage, in *p27*-/- BM the opposite effect was observed. The simultaneous loss of Cks1 and p27 resulted in a phenotype similar to WT, indicating that Cks1 regulation of the differentiation after chemo-ablative stress occurs probably through the downstream target p27. A study showing increased T-lymphocyte proliferation in *p27*-/- mice supports this finding [28]. Also, after transplantation analysis, *Cks1*-/- B-lymphocytes were increased and *Cks1*-/- -delivered T-lymphocyte were decreased (Chapter 4.3.4.). However in this case, the opposed effect after the transplantation of *p27*-/- cells was sustained in the DKO group, indicating a stronger effect of the p27 loss in the DKO cells after serial transplantations. Interestingly, serial BM transplantations of *p27*-/- /*p57*-/- induced the same phenotype in the adult hematopoietic populations as 5-FU treatment of *p27*-/- mice, T cell and myeloid populations were increased and B cell population were decreased [101].This finding and the fact that the opposite effect in mice lacking Cks1 was observed after CD150+ LSK transplantations (4.2.12. and 4.2.13.), clearly indicates the Cks1 involvement in differentiation after hematopoietic stress as a regulator of p27 (Figure 33).

The HSC population itself might be heterogeneous and can be subdivided into myeloid-biased and lymphoid-biased stem cells with different properties [207]. Myeloid-biased stem cells, which preferentially differentiate to myeloid cells display improved engraftment and self-renewal capacities compared to lymphoid-biased stem cells [208]. The transplantations with sorted HSC in this project (4.2.12. and 4.2.13.) reveal *Cks1*-/- HSC as mainly lymphoid-biased. They were more quiescent, displayed decreased engraftment and delivered more B-lymphocytes. It is therefore possible that Cks1 affects the distribution of myeloid- and lymphoid-biased HSC, an observation that provides an interesting field for further research.

5.4. E3 ubiquitin ligases are critical factors in the control of HSC fate

The observations made in Cks1-deficient mice show overlap with those reported for the *Skp2-/-* mice [197, 198]. These studies revealed that *Skp2*-deficient mice possess decreased marrow cellularity and increased frequencies of LT-HSC, which seem to have an increased ability to self-renew in vivo. Both Skp2 and Cks1 function in the same SCF complex [136]. The similarity in the

functional modulations of hematopoiesis in *Cks1-/-* animals compared to Skp2 deficiency demonstrates that the function of Skp2 in hematopoiesis is uniquely dependent on Cks1 and that the SCF^{Skp2} ubiquitin ligase is critically important in the control of HSC fate. The present study additionally reveals that the accumulation of SCF^{Skp2}-Cks1 targets is most prominent in the earliest phenotypic hematopoietic population (CD150+ LSK) (Chapter 4.1.1.). These results suggest that the E3 ligase is specifically active in the earliest HSC. Since deficiency of either Skp2 or Cks1 reduces cell cycle entry and maintains a larger population of self-renewing cells, rapid up-regulation of SCF^{Skp2}-Cks1 might be at the center for the recruitment of quiescent cells into active cell cycle which is required to initiate a regenerative response.

Deletion of E3 ubiquitin ligases such as Asb2 [209], c-Cbl [210], Itch [211] and VHL [212] results in similar phenotype as in *Cks1-/-* or *Skp2-/-* mice, where HSC engraftment or self-renewal is increased. This is remarkable, because these ligases target different molecules in HSC than SCF^{Skp2}-Cks1. For instance, c-Cbl acts as negative regulator of HSC self-renewal probably through the TPO/cMpl signaling pathway, which is known to be crucial for the maintenance of HSC [210, 213] and Itch seems to play a similar role to c-Cbl, but via the Notch signalling pathway [211]. VHL is the E3 ligase involved in hypoxia-inducible factor-1alpha (HIF-1a) protein degradation [212] and HIF-1a is needed in HSC to maintain their quiescence [212].

On the other hand, deficiency of other E3 ubiquitin ligases such as Fancl [214] and Fbxw7 [215] result in contrary effects to Skp2 and Cks1 deficiency, as their deletion causes stem cell exhaustion. For instance, Fancl ubiquitinates β-catenin, however the ubiquitin chain, which Fancl adds to β-catenin, possesses an atypical expansion with nonproteolytic functions, increasing β-catenin activity and inducing expansion of multilineage progenitors [214]. A recent study shows the PI3K/Akt pathway, which is involved in self-renewal and survival of HSC, to induce Fancl levels [216] supporting the idea that this E3 ligase plays a contrary role than the SCF^{Skp2} complex. One of the Fbxw7 ligase targets is c-Myc, which overexpression leads to HSC exhausting due to accelerated differentiation [217]. And loss of Fbxw7 in BM resulted in c-Myc accumulation [215] explaining the similarity to the c-Myc overexpression phenotype in Fbxw7 knockouts.

Taken together, these studies demonstrate that E3 ligases play a central role in the control of HSC behaviour and are strongly involved in the fine-tuning of the complex HSC homeostasis.

5.5. Potential regulators upstream of Cks1 in HSC

SCF ubiquitin ligase complexes use a family of F-box proteins as substrate adaptors to mediate the degradation of a large number of regulatory proteins involved in diverse processes. The coordinate expression of such substrate adaptors is therefore of utmost importance [196, 218]. In the case of SCF^{Skp2}, Cks1 adds an additional level of substrate specificity [172, 218], implying that the regulation of Cks1 transcript and protein levels should be tight.

The transcription factor c-Myc, a key protein for acquisition and maintenance of stem cell properties [217, 219] has also been identified as a positive transcriptional regulator of Cks1 [148]. Conditional loss of c-Myc in the BM resulted in accumulation of HSC, which were diminished in their ability to differentiate [217]. Since lack of Cks1 also resulted in elevated HSC quiescence (Chapters 4.2.10.-4.2.13.), it is possible, that c-Myc is one of the positive regulators of Cks1 activity in HSC.

While Myc regulation of Cks1 is indirect [148], another transcription factor, NF-Y, binds directly to the Cks1 promoter to regulate its expression during the cell cycle [146]. Deficiency for Cks1 resulted in higher sensitivity of HPC against apoptosis (Chapter 4.2.6.), slower cycling (Chapters 4.2.5., 4.2.8. and 4.2.13.) and increased persistence of CD150+ HSC (Chapters 4.2.10-4.2.13). NF-Ya deletion is shown to create an accumulation of HSC in G2/M, prompts apoptosis and causes hematopoietic failure [220], a phenotype that could involve reduced levels of Cks1.

One known regulator of HSC quiescence is TGF-β [66]. TGF-β signaling limits the activation of Akt, induced by SCF or TPO stimulation and promotes HSC survival but not proliferation, thus maintaining quiescence [66]. Besides, TGF-β induces p57 expression and prevents cyclin D1 sequestration [66, 102], supporting the finding, that p57 is required for the quiescence in HSC [100]. Interestingly, TGF-β stimulation leads to transcriptional down-regulation of Cks1 [147, 221]. If this mechanism applies to HSC, then Cks1 loss would mimic

constitutive TGF-β stimulation, thus explaining the increased quiescence in *Cks1-/-* HSC (Chapters 4.2.10.-4.2.13.).

Another recently identified regulator of the SCF^{Skp2} complex is FoxO3a [222], a forkhead transcription factor that acts downstream of the PTEN/PI3K/Akt pathway [223, 224]. FoxO3a negatively regulates SCF^{Skp2}, inhibiting the E3 ligase activity and promoting p27 stability [222]. Since FoxO3a is known to be essential for maintenance of HSC quiescence and HSC pool [73] and lack of FoxO3a results in decreased expression of the promoters of quiescence p57 and p27 [73, 101], the transcription factor is also a possible SCF^{Skp2}-Cks1 regulator in early haematopoiesis.

The potential regulators of Cks1 in early hematopoiesis represent an interesting subject of further investigation. A hypothetical model of the upstream regulators of *Cks1* in early hematopoiesis, based on published studies is represented in Fig. 34.

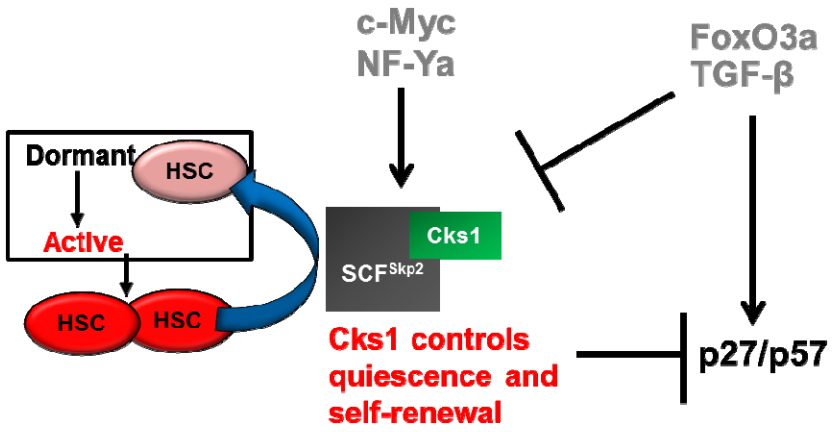


Figure 34: Hypothetical model representing possible regulators of *Cks1* in early hematopoiesis.

5.6. Cks1 is an essential regulator in cancer cells

The balance between HSC quiescence and activation is not only important for regeneration but also impacts the accumulation of oncogenic mutations. Indeed, it has been shown that prolonged activation of HSC can lead to malignant transformation [225]. Since the level of Cks1 is shown to be a critical determinant in the balance between HSC quiescence and activation (Chapter 4.2), one would predict that decreased Cks1 would favor quiescence, and

increased Cks1 expression promotes HSC activation. Thus, it would be likely that Cks1 levels were increased in stem cell cancers. In favor of this hypothesis, it was shown (Chapter 4.4.1.), that Cks1 transcription levels were increased in patients with CML, a classical hematopoietic stem cell disorder [186], and that treatment of the disease results in decrease of the Cks1 transcription levels. Furthermore, BCR-ABL expression in *Cks1*-/- LSK resulted in decreased colony forming capability of these cells (Chapter 4.4.2.). In addition, in various other malignancies elevated levels of Cks1 transcript and protein are associated with high proliferation and poor prognosis [218, 226, 227], indicating that oncogene-induced Cks1 overexpression contributes to aggressive disease and treatment resistance. Indeed, Myc overexpression or activation that leads to induction of Cks1 expression [148] results in suppression of p27 levels and resistance to the Abl inhibitor imatinib in CML [228]. Further data that link SCF^{Skp2} to fate decisions in cancer-initiating cells come from successful pharmacological inactivation of Skp2 as a novel therapeutic means [229]. Since not every Skp2-mediated substrate recognition requires the presence of Cks1 [218], it is currently not clear whether for some cancer types the isolate inhibition of the Cks1-Skp2 physical interaction could be a more specific and/or less toxic approach as compared to ubiquitous SCF^{Skp2} inhibition. In this context it also has to be noted that loss of Cks1, but not loss of Skp2, results in a significant delay in tumorigenesis in Myc lymphoma model [148, 230], and that Cks1 has cell cycle regulatory functions independent of p27 suppression (this study and [206]).

5.7. Conclusion

In the present study, Cks1 is identified as an essential regulator of HPC/HSC fate operating upstream of CDK. p27 is shown to be an essential downstream target of the SCF^{Skp2}-Cks1 complex in the regulation of lineage distribution at stress hematopoiesis. Cks1 loss prominently affects CD150+ LSK by inhibiting exit from quiescence and cell cycling and protecting the more mature HPC from apoptosis. Accumulation of SCF^{Skp2}-Cks1 targets, the CKI p21, p27, p57 and the Rb family member p130 in absence of Cks1 in the earliest phenotypic hematopoietic population (CD150+ LSK) suggest that the E3 ligase SCF^{Skp2} is specifically active in these early cells, uniquely dependent on Cks1 and critically

important in the control of HSC fate. Referring to current studies, it is most likely that all Cks1 CKI targets and p130 are involved in the HSC control.

In CML, Cks1 is overexpressed and Cks1 loss results in impaired oncogene-induced growth factor independence. Therefore Cks1 is proposed to be a central intermediate for normal and oncogene-induced cell cycle regulation in hematopoiesis and a therapeutic target in hematologic malignancies.

6. List of tables and figures

7. Abbreviations

5-FU	5-Fluorouracile
7-AAD	7-Amino-Actinomycin D
A	Adenine
β-catenin	Beta-catenin
ACK	Ammonium-Chloride-Potassium
Ang	Angiopoetin
APC/C	Anaphase promoting complex/cyclosome
APS	Ammoniumpersulfate
Asb2	Ankyrin repeat and SOCS box protein 2
BCR-ABL	breakpoint cluster region-abelson
BM	Bone marrow
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C	Cytosine
c-Cbl	Casitas B-cell lymphoma
CD	Cluster of differentiation
CDE	cell cycle-dependent element
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
CFU	Colony forming unit
CHR	cell cycle genes homology region
CKI	CDK inhibitor
c-Kit	Stem cell factor receptor
Cks	Cyclin dependent kinase subunit
CKS1B	CDC28-protein kinase regulatory subunit 1B
CLP	Common lymphoid progenitor
cm	Centimeter
CML	Common myeloid leukemia
CMP	Common myeloid progenitor
Cul1	Culin 1

DKO	double knockout
DMEM	Dulbecco’s modified Eagle's medium
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitole
E2F	E2 promoter binding factor
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FANKL	Fanconi anemia, complementation group L
Fbxw7	F-box and WD40 domain protein 7
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FL	Fetal liver
Flk2	Fetal liver kinase 2
FLT3L	Fms-like tyrosine kinase 3- ligand
FoxM1	Forkhead box M1
FoxO3	Forkhead box 03
G	Guanine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Granulocyte-monocyte-progenitor
Gy	Gray
HBSS	Hank's buffered salt solution
HCl	Hydrochloride
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hgb	Hemoglobin
HIF-1a	Hypoxia-inducible factor-1alpha
HRP	Horse radish peroxidase
HPS	Hematopoietic progenitor cells
HSC	Hematopoietic stem cells
Ig	Immunoglobulin
IkB	Ikappa-B
IL	Interleukin

IL7Ra	Interleukin receptor 7a
i.p.	intraperitoneal
kB	Kilo Base
kDa	Kilo Dalton
KCl	Potassium chloride
Lin-	Lineage negative
LT-HSC	Long-term repopulating hematopoietic stem cell
M-CSF	Macrophage colony-stimulating factor
MEP	Megakaryocyte-erythrocyte progenitor
min	Minutes
MPP	Multipotent progenitor
Myc	Myelocytomatosis oncogene
mRNA	Messenger ribonucleic acid
MSC	mesenchymal stromal cells
NaF	Sodium fluoride
NF-kB	Nuclear factor-kappaB
NF-Y	Nuclear factor-Y
NP40	Tergitol-type NP-40
PBS	Dulbecco's phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PIK3	Phosphatidylinositol 3-kinase
PLT	Platelets
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidyl serine
Pten	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
Rb	Retinoblastoma
RBL2	Retinoblastoma-like 2 (p130)
RNase	Ribonuclease
RT PCR	Real Time PCR
Sca-1	Stem cell antigen-1
SCF^{Skp2}	Skp1-Cullin-F-box-Skp2 complex

SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sek	Sekonds
SEM	Standard error of the mean
Skp	S-phase kinase-associated protein
SLAM	Signaling Lymphocytic Activation Molecule
SOCS	Suppressor of cytokine signalling
ST-HSC	Short-term repopulating hematopoietic stem cells
TGF	Transforming growth factor
Suc1	suppressor of *Cdc2* mutation
T	Thymine
Tris	Tris(hydroxymethyl)aminomethane
TPO	Thromopoietin
Ub	Ubiquitin
VHL	Von Hippel-Lindau protein
Vs.	Versus
WBC	White blood cells
WT	Wildtype

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9. Publications

1. Höglund A, Nilsson LM, Muralidharan SV, Hasvold LA, Merta P, Rudelius M, **Nikolova V**, Keller U, Nilsson JA (2011) *"Therapeutic implications for the induced levels of Chk1 in Myc-expressing cancer cells"*. Clin Cancer Res; 17 (22):7067-79.

2. Kratzat S, **Nikolova V**, Miething C, Hoellein A, Schoeffmann S, Gorka O, Pietschmann E, Illert AL, Ruland J, Peschel C, Nilsson J, Duyster J, Keller U (2012) *"Cks1 is required for tumor cell proliferation but not sufficient to induce hematopoietic malignancies."* PLos ONE; 7(5):e37433.

3. Baumann U, Fernández-Sáiz V, Lemeer S, Rudelius M, Engel K, **Tomiatti V**, Targosz B, Knorn A, Braun M, Perner S, Klapper S, Dreyling M, Miething C, Leitges M, Peschel C, Keller U, Kuster B and Florian Bassermann *"PKCδ directs SCFFbxo25 mediated degradation of Hax-1 to regulate cell survival and suppress lymphomagenesis"* Submitted Manuscript.

4. **Tomiatti V**, Pietschmann E, Kratzat S, Hoellein A, Quintanilla-Fend L, von Bubnoff N, Peschel C, Oostendorp R, and Keller U *"Cks1 is a critical regulator of hematopoietic stem cell cycling, quiescence and homeostasis, operating upstream of Cdk inhibitors"* Submitted Manuscript.

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