Understanding the role of Asrij in hematopoietic stem cell aging

A Thesis submitted for the degree of

Master of Science

by

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Declaration

I hereby declare that this thesis entitled '**Understanding the role of Asrij in hematopoietic stem cell aging**' is an authentic record of research work carried out by me under the guidance of Professor Maneesha S. Inamdar at Laboratory of Stem Cell Biology and Vascular Biology, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore.

In keeping with the norm of reporting scientific observations, due acknowledgement has been made wherever work described here has been based on the findings of other investigations. Any omission owing to oversight or misjudgement is highly regretted.

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Certificate

This is to certify that the work described here in this thesis entitled '**Understanding the role of Asrij in hematopoietic stem cell aging**' is the result of investigations carried out by **Mr Tirath Raj Dwivedi** in Laboratory of Stem Cell Biology and Vascular Biology, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under my guidance and that the results presented here have not previously formed the basis for the award of any other diploma, degree or fellowship.

Maneesha S. Inamdar

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Date:

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Abbreviations

ATM	Ataxia-telangiectasia mutated
BM-HSPC	Bone marrow hematopoietic stem and progenitor cells
CSN	Constitutively photomorphogenic signalosome
DDR	DNA damage response
HSC	Hematopoietic stem cells
MDM2	Mouse Double Minute 2
mTOR	mammalian target of rapamycin
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase And Tensin Homolog
PUMA	P53 Up-Regulated Modulator Of Apoptosis
STAT	Signal transducer and activator of transcription
MDS	Myeloproliferative disease
PEV	Position effect variegation
ROS	Reactive oxygen species
AML	Acute myeloid leukemia
CML	Chronic myeloid leukemia
OCIAD	Ovarian Carcinoma Immunoreactive Antigen Domain
COSMIC	Catalogue of Somatic Mutations in Cancers
TCGA	The Cancer Genome Atlas
MDS	Myelodysplastic syndrome
LSK	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$
FACS	Flourescence activated cell sorter

Synopsis of thesis entitled

Understanding the role of Asrij in hematopoietic stem cell aging

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This study describes the role of Asrij in regulating molecular mechanisms governing the aging and lineage bias of bone marrow hematopoietic stem and progenitor cells (HSPCs: Lin$^-$ Sca1$^+$ c-Kit$^+$) using *asrij* conditional knockout mice (*asrij$^{fl/fl}$; Vav-iCre$^+$/ asrij^{CKO}*). Aging is a natural phenomenon, and as cells age they accumulate various inactivating/hyper activating mutations and also lose their ability to repair damage, eventually leading to senescence of the system. Although the p53 and Akt/mTOR pathways have been extensively studied in the process of tissue aging, mechanisms regulating bone marrow aging remain unclear. We find that Asrij expression decreases in wildtype HSPCs from 6 months of age and that *asrij* knockout (*asrij$^{fl/fl}$; CMV-Cre$^+$*) HSPCs show reduced levels of p53 and increased activation of the Akt/mTOR signalling pathway, suggesting that Asrij could be acting through the p53/mTOR pathway to regulate HSPC aging. To understand the role of Asrij, we characterized the *asrij CKO*. Characterization of these mutant mice showed premature aging phenotypes such as increased peripheral blood cells, HSPCs and myeloid differentiation at an early age and increased activation of Akt/mTOR in *asrij^{CKO}* HSPCs. The results of this study indicate a role for Asrij in regulating the signalling concerned with HSPC aging and will be applicable in understanding the development of adult hematopoietic system and its age-associated malignancies.

Hematopoiesis is the developmental process that regulates the formation of different components of blood. The hierarchal process of hematopoiesis, which involves different bone marrow subpopulations having varying proliferation and differentiation potencies, is widely studied to understand how blood stem cells maintain their ability to self-renew and undergo lineage-specific differentiation. Studying hematopoiesis not only helps understand mechanisms

regulating stem cell fate specification and lineage-specific differentiation but also aids in understanding mechanisms driving hematological malignancies.

The bone marrow comprises a heterogeneous population of stem cells, progenitors and differentiated cells growing in a matrix of stromal cells that acts as niche. With age, mutations and perturbation in tissue homeostasis make both the HSPCs and niche damage prone. The aging of HSCs is thought to get affected by both cell-intrinsic (transcription factors, signal transducers, epigenetic regulators) and -extrinsic mechanisms (niche-induced) leading to the characteristic loss of HSC quiescence. Functionally compromised stem cell having a reduced repopulation potential increase in number and their differentiation is skewed towards the myeloid lineage. There is an increased tendency to develop malignancies at a later stage.

Mammalian hematopoietic aging process is accompanied with a multitude of abnormalities in the self-renewal and differentiation capacity of HSCs and is marked by deregulation of cellular signal transduction. Signalling networks including the p53 mediated DNA damage response (DDR), insulin signaling and inflammatory cytokines have been implicated in regulation of aging process in HSCs. The mTOR pathway, which is known to play pivotal role in regulating senescence of various tissues when over-activated, can accelerate aging, whereas its inhibition can extend lifespan. While mice deficient in the negative regulators of mTOR (PTEN, Tsc1) showed reduced self-renewal and impaired reconstitution potential of HSCs, hyperactivation of mTOR has also been observed in chronologically aged HSCs. In favor of these findings it has been observed that Tsc1 deficiency induces aging phenotypes in young HSCs as well as rapamycin treatment restores HSC self-renewal capacity in old mice upon competitive transplantation. The tumor suppressor p53 is known to have high expression in the HSCs but its expression reduces upon aging. p53 plays an important role in deciding stem cell fate and negatively regulates mTOR activation to maintain HSC quiescence. Cells that lack p53 are not able to exercise inhibitory effects on mTOR, thereby leading to their senescence. Taken together, these studies raise important questions regarding the nature of molecular signals that regulate the p53-mTOR signalling in HSPCs and how perturbations in these signalling networks contributes to their functional decline leading to aging and development of leukemia.

Asrij is a conserved protein and plays an important role in regulating the balance between stemness and differentiation both in *Drosophila melanogaster* & *Mus musculus*. *Asrij* global knockout mice show defects in multiple signalling pathways including PI3K/Akt, Notch and JAK/STAT pathways, all of which are implicated in several kinds of hematological neoplasms. Knockout mice develop increased bone marrow HSPCs and splenomegaly from 6 months of age. Also, when exposed to stress like γ-irradiation or 5-FU, Asrij knockout mice show rapid exhaustion of the stem cell pool and reduced survival indicating diminished functionality of stem cells in the knockout mice.

Loss of p53 in mice leads to an aberrant increase in the number of HSPCs as observed in the old $p53^{+/-}$ mice (18-20 months) as compared to young mutants. Expectedly, the negative regulators of

p53 like MDM2 and MDM4 also play important roles in deciding HSC fate. In the mouse HSPCs, Asrij acts through CSN5, the fifth subunit of the COP9 signalosome to regulate p53 stability. Absence of Asrij leads to increased CSN5 levels, leading to increased ubiquitination of p53 and its resultant degradation making Asrij a novel regulator of p53. Moreover, increased activation of Akt is observed in the bone marrow of Asrij KO mice. Since, both p53 and Akt regulate activation of mTOR, it is possible that Asrij acts through controlled signalling pathways to regulate p53-mTOR axis during aging of HSPCs under normal and stress conditions. Based on these observations, I extended my analysis to check how loss of Asrij gives rise to premature aging phenotypes.

To understand how Asrij contributes to the aging of HSPCs and to avoid secondary effects that are possible as a result of global knockout of protein, we characterized Asrij conditional knockouts (*asrij*$^{fl/fl}$*; Vav-iCre*$^{+}$*)*. In Asrij conditional knockout mice, the expression of Asrij is confined only to the hematopoietic compartment. For a better understanding of mechanisms regulating HSPC aging and to understand the role of Asrij in governing the p53 and mTOR pathways involved in progression of age related phenotypes in HSPCs, we first checked if they showed aging phenotypes. We performed detailed bone marrow phenotypic characterization of different age groups (2, 4, 6, 8 and 10 months old) of these *asrij* mutants. We found significantly increased WBCs and platelets in the peripheral blood as well as increased bone marrow HSPCs and myeloid populations, indicating that deficiency of Asrij indeed results in the development of premature aging phenotypes, quite similar to the premature aging phenotypes observed in the global knockouts. Further, the tissue histopathology of these *asrij* mutant mice indicates development of lesions in the bone marrow. To further establish the role of Asrij in regulating HSPC aging, immunoblotting analysis of various signalling molecules involved in aging revealed that the PI3K-Akt-mTOR is activated more in *asrij*^{CKO} mice whereas p53 levels are reduced thus indicating priming of knockout HSPCs towards premature aging. Furthermore, Oncomine database mining indicates an association of the human *asrij* (*ociad1*) with old age myeloid leukemia's thus indicating loss of *asrij* could lead to development of myeloproliferative neoplasms upon aging.

In conclusion, this study asks how Asrij affects various signalling pathways having pre-established roles in aging of other tissue systems and helps in understanding the role of Asrij in regulation of aging of the hematopoietic compartment. A limitation of the study in its current form is that it does not provide mechanistic insights of how Asrij exercises regulation over the p53-mTOR axis and thus conclusions regarding the mechanistic interfaces are not possible. However, phenotypic characterization of *asrij* conditional mutant mice provide us insights into how perturbation of key regulators of signalling networks can ultimately lead to wide range of effects from development of hematological neoplasms to aging of the system.

Chapter 1: Introduction

The process of hematopoiesis serves as a blueprint for understanding stem cell biology, tissue aging and oncogenesis. Establishment of the blood system requires hematopoietic stem cells (HSCs), which are a small population of stem cells residing in the bone marrow. HSCs are capable of self-renewal and differentiation to various lineage committed cells. This process is regulated by cell-intrinsic pathways and the microenvironment in which HSCs reside [1] . Bone marrow homeostasis is maintained throughout the lifespan of an organism by HSCs which replenish multipotent progenitors and lineage precursors. These precursor cells give rise to individual differentiated cells like RBCs, which allow efficient transport of oxygen; platelets, which help in clotting of an injured blood vessel and leukocytes which are the key players of innate and adaptive immunity [2].

The HSPCs comprises of both the long-term (LT-) and short-term (ST-) HSCs marked by the cell surface markers **Lin$^-$ Sca1$^+$ c-Kit$^+$ (LSK).** HSPCs are identified based on flow cytometric analysis using antibodies designed against specific surface markers. Their precise characterization has been instrumental in providing a molecular framework to understand the signalling pathways that regulate stem cell properties. In the current view, HSPCs are classified as cells with varying engraftment potentials and their distinct bias towards the development of myeloid and lymphoid lineages [3]. HSPCs differ in their properties in a spatio-temporal manner. Cellular and molecular analysis of these cells helps us understand how their properties are lost or compromised over time leading to an overall aging of the hematopoietic system. Furthermore, accumulation of mutations in HSPCs over time and the loss of their ability to repair damage lead to ineffective hematopoiesis and development of various myeloproliferative disorders namely myelodysplastic syndrome (MDS) and chronic myeloid leukemia (CML) [4]. Currently, the research on HSPCs focuses on identification of molecular regulators and methods for *in vitro* and *in vivo* manipulation of these cells to make HSC-based therapy safe and promising.

HSCs become prone to damage as they age and develop several changes including a marked loss of stem cell quiescence, diminishing lymphoid potential and a differentiation bias towards the myeloid lineage.[5]. The aging process is regulated by various cell-intrinsic and extrinsic

processes, yet increasing evidence indicates a fundamental role of cell-intrinsic signal transduction in regulating HSC aging. Hence, we are interested in identifying signalling networks that regulate stem cell properties like quiescence and differentiation, which essentially get perturbed because of cellular senescence [6]. Numerous signalling networks have been reported to play an essential role in tissue aging, p53 mediated DNA damage regulation and mTOR pathway being the well-studied ones. It is reported that these signalling molecules exhibit an age-dependent change in expression which possibly explains how a few key regulatory molecules might regulate cell fate upon aging [7].

Asrij/OCIAD1 is a conserved protein reported to regulate stem cell properties like stemness and differentiation [8]. It is also known to be involved in maintaining blood cell homeostasis in both *Drosophila* and mice models. In mouse, at the molecular level, Asrij regulates p53 function via COP9 signalosome mediated ubiquitination and further inhibits its proteasomal degradation (Saloni Sinha MS thesis, 2015; Sinha et al., 2018 submitted). Perturbations in such closely-knit pathways where signalling molecules function in concert to catalyze a cellular change could lead to development of aberrant phenotypes as observed in aged cells. The current study tries to understand and elucidate importance of signalling networks that regulate cell fate and their role in preventing or promoting aging phenotypes in the murine hematopoietic system.

1.1 *Hematopoiesis*

Hematopoiesis is the developmental process that regulates formation of different components of blood. HSCs sit at the top of hematopoietic hierarchy and drive the process of continued blood production owing to their ability of pluripotency. HSCs are further classified as long-term reconstituting HSCs (LT-HSC) & short-term reconstituting HSCs (ST-HSCs). LT-HSCs are capable of self-renewal and differentiate throughout life, whereas the ST-HSCs have limited self-renewal ability and differentiate to give rise to multipotent progenitors (MPPs). MPPs are further capable of differentiating into common lymphoid (CLP) and common myeloid progenitors (CMP). All terminally differentiated blood cells are ultimately derived from CMP & CLP giving rise to two distinct hematopoietic lineages i.e. myeloid and lymphoid lineages each comprising of various kinds of cells with specialized functions [9] (Refer Figure 1.1).

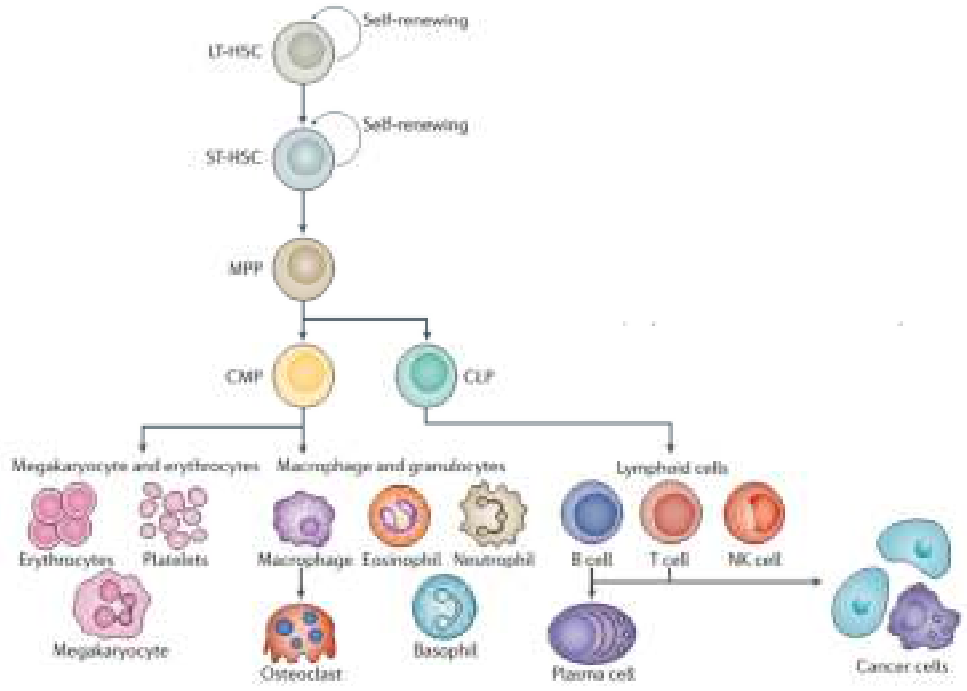

Figure 1.1: Bone marrow hematopoiesis. HSCs give rise to all mature blood cells. MPP, multipotent progenitors; CMP, common myeloid progenitors; CLP, common lymphoid progenitors; NK cell, natural killer cell (adapted from [9]).

Several models have been proposed to explain the hematopoietic hierarchy. With identification of cell-specific markers many previously undefined populations of cells are now being

incorporated into the hierarchy based on their characteristic differentiation potential. Previously, it has been well known that ST-HSCs differentiate and branch into myeloid and lymphoid lineages. Recently, it has been proposed that a pool of megakaryocyte-biased HSCs [10] (Refer Figure 1.2) directly gives rise to megakaryocytes. This gives rise to the hypothesis that there exists a pool of platelet-biased HSCs in the bone marrow which is higher in hierarchy as compared to the other progenitors. Such studies provide evidence for hematopoiesis being regulated at different levels in the hierarchy.

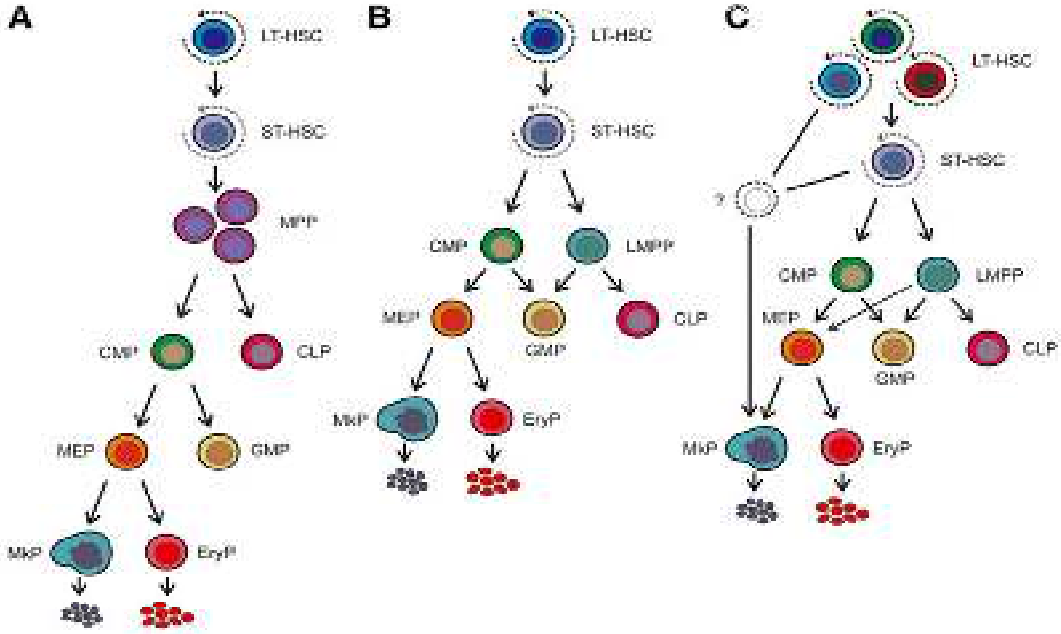

Figure 1.2: Different models of hematopoiesis. Hematopoietic hierarchy model have been modified over time marked by the inclusion of previously unidentified cell types (A) Classical model of hematopoiesis with a strict distinction between myeloid and lymphoid lineages (B) Model with lymphoid primed MPPs (LMPPs) incorporated (C) Model showing existence of platelet biased- HSCs (adapted from [10]).

1.1.1 *Murine Hematopoiesis*

Mouse (*Mus musculus*) has been widely used as a model organism to study the process of development. Over years, advancements in techniques like *in vitro* differentiation of bone marrow progenitors and bone marrow transplantation has made mouse an excellent model to understand mammalian hematopoiesis [11]. During development of the hematopoietic system, the sites of hematopoiesis shift in a sequential manner from yolk sac to aorta-gonad mesonephros

(AGM) to fetal liver and finally the bone marrow. Embryonic hematopoiesis can be divided into two waves. The initial wave called as primitive hematopoiesis takes place in the yolk sac at around embryonic day 7.5. Yolk sac associated development of blood cells is quite restricted to the erythroid lineage, known to mature rapidly. These cells do not express certain genes known to get expressed in later stages of embryonic development making them stand unique [12]. Subsequently, by E10.5, definitive hematopoiesis takes over where HSCs colonize fetal liver, spleen and ultimately bone marrow. This second wave of hematopoiesis gives rise to various kinds of multipotent, lymphoid and myeloid precursors [1]. Adult hematopoiesis takes over after birth with bone marrow being the major site of blood cell development (Refer Figure 1.3).

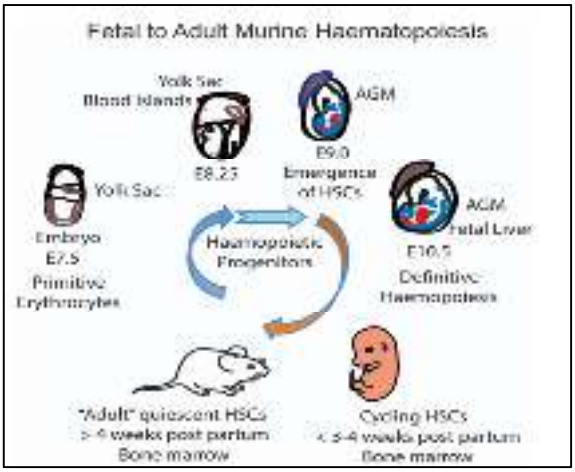

Figure 1.3: Development of mouse hematopoietic system. The shifting sites of hematopoiesis from embryonic to adult stage of mouse development (adapted from [13]).

1.2 *Transgenic mouse as a model to study hematopoiesis*

The development of transgenic mice having specific genes knocked out has helped improve our understanding of molecular mechanisms that regulate the complex process of hematopoiesis. The lox-Cre recombinase system has been one of the widely used protocols to generate both global and conditional mouse knockouts to identify specific roles of various genes and to dissect out signalling networks that regulate development of HSCs. Availability of specific gene manipulation tools like promoter Cre-drivers for specific tissues make mice amenable to detailed genetic and molecular analyses. This allows for a comprehensive analysis in mice to mimic human disease pathophysiology [14] (Refer Figure 1.4).

The major advantage one has while using tissue specific knockouts is being able to avoid any secondary effects brought about by global deletion of a gene. This ensures proper characterization of phenotypes observed as a result of gene knockout. Conditional knockouts often serve as a translation model to study human diseases including leukemia and lymphoma. To be an ideal disease model, the knockout should be able to replicate the genetic and molecular heterogeneity of blood cancers.

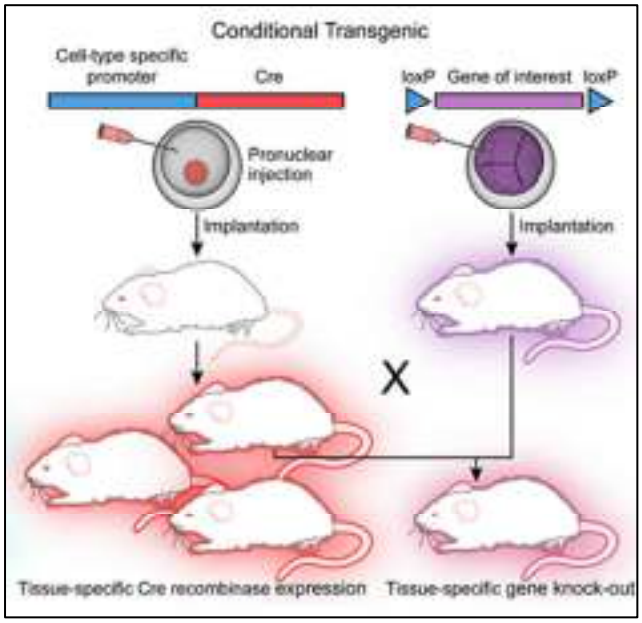

Figure 1.4: Generation of a conditional knockout. Tissue specific expression of Cre recombinase under the control of a promoter can generate conditional knockouts (adapted from [15]).

1.2.1 *Conditional knockouts of hematopoietic system*

The Cre recombinase system for tissue specific deletion of a target gene has been used widely to understand the process of hematopoiesis by making use of promoter driven Cre expression. One of the major set-back in usage of tissue specific deletion has been possibility of leaky expression i.e. expression of transgene happening outside the target cells or only in a proportion of cells. This phenomenon is also referred to as position effect variegation (PEV) which may further obscure the possible phenotypes and lead to misinterpretations [16]. To tightly regulate Cre expression, various strategies have been used including the usage of codon improved Cre (iCre). Transgenes encoding iCre optimize Cre expression through optimized codon usage, reduced CpG islands to limit epigenetic silencing and removed putative cryptic splice sites [17].

Depending upon the promoter driving Cre expression, the target gene can be deleted either in the entire hematopoietic lineage or in specific cell lineages:

1. ***Vav-iCre* transgenic mice**: The endogenous murine *vav* gene known to have inadvertent oncogenic activation during transfection experiments has a pan-hematopoietic expression and is known to drive transgene expression in all the different types of hematopoietic cells [18]. Outside hematopoietic compartment, *vav* can also drive expression in the endothelial lineages found in bone marrow as well as other hematopoietic tissues like spleen, lymph nodes and thymus. In spleen, the expression is limited to white pulp region specifically to the megakaryocyte lineage [19]. *Vav-iCre* has been shown to be capable of deleting the floxed target virtually in 100% of the cells. It proves to be a reliable model to understand effects of gene deletion on tissue specific regulation of hematopoietic signalling networks.
2. **hCD2 transgenic mice:** The hCD2 promoter driven knockout is used to generate a restricted pattern of transgene expression where the deletion occurs only in the B- & T-lymphocytes but not in the other hematopoietic cells. Furthermore, tight regulation of transgene expression via hCD2 helps negate the possibility of PEV [16].
3. ***Tek-Cre* transgenic mice:** also known as *Tie2-Cre* is a conditional knockout under the regulation of endothelial-specific receptor tyrosine kinase (*Tek*). It is useful in generating a gene deletion in the endothelial lineage to study the role of endothelial cell during embryogenesis and in adults specifically in development of the vascular niche of bone marrow. [20].

1.3 *Aging of the hematopoietic system: Phenotypes and mechanisms*

Stem cells, including HSCs, are capable of self-renewal and differentiation and for long have been exempted from effects of aging. Recent studies on how stem cell fate and function change with age have highlighted the fact that stem cells also are susceptible to aging like any other somatic cells. They lose their ability to self-renew and differentiate accompanied by derailment of cellular and molecular mechanisms that regulate stem cell properties [21],[22]. Understanding how stem cells age helps us understand how diseases develop as an individual ages and may help

devise therapeutic interventions for rejuvenating stem cells to improve and expand life span of an individual.

The hematopoietic system, comprising HSCs and various lineage committed cells, is also susceptible to aging. As HSC age they exhibit loss of quiescence, reduced regeneration potential, reduced ability to mount an adaptive immune response and an increased proportion of myeloid cells [23]. Recent studies evaluating HSCs at the clonal level, both in transplantation studies and during *in situ* hematopoiesis, have established that individual HSCs can exhibit lineage bias, giving rise to myeloid-biased, lymphoid-biased, or more balanced differentiation, with the proportion of myeloid biased HSCs increasing with age (Refer Figure 1.5). Lineage bias in old HSCs is also reported to be associated with old age hematological malignancies and age-associated inflammation (Elias et al., 2017) [24]. **With the onset of a demographic shift towards an older population the incidence of myeloid lineage associated leukemia is being reported to be higher in the elderly. There is a need for better understanding of how the hematopoietic system ages. Identifying molecular regulators of aging and developing therapies to counteract the harmful effects of stem cell aging may improve health span and overall life expectancy.**

At an evolutionary level, the gradual decline in lymphoid populations with age has been well supported by various theories on hematopoietic aging. An early peak in lymphocyte production is supported by natural selection and furthermore decline accompanied by expansion of myeloid population is inevitable to help in immune remodeling. The reduced lymphoid output helps conserve energy by minimizing energy consumption required for the process of antigen receptor rearrangement during lymphocyte development. This also minimizes the chance of developing mutations or translocations during genetic rearrangement process in an aged bone marrow [25].

HSC aging has been associated with changes in various cell-intrinsic and extrinsic mechanisms that affect properties like quiescence and lineage differentiation. These changes are marked by accumulation of DNA damage, shifts in cell polarity, increased ROS levels and mitochondrial dysfunction and deregulation of epigenetic landscapes.

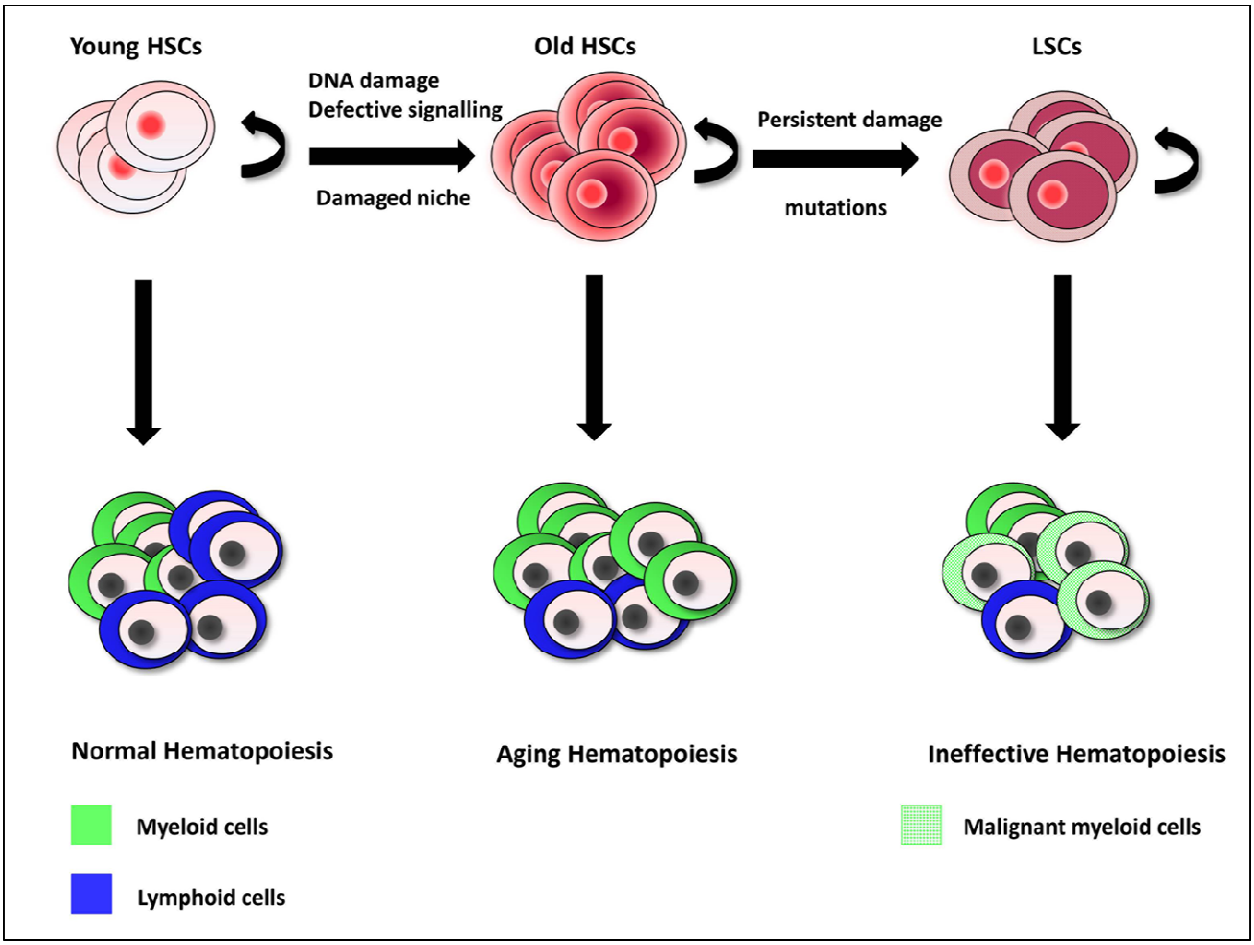

Figure 1.5: Hematopoietic aging and development of myeloid leukemia. With age, HSCs accumulate damage leading to expansion of HSC pool with reduced repopulation ability and an increased bias towards the myeloid lineage. Persistent DNA damage transforms aged HSCs into leukemic stem cells (LSCs) leading to development of myeloid leukemia (modified from [4]).

1.3.1 *Cell intrinsic mechanisms of HSC aging*

Hematopoietic aging can be contributed to various cell autonomous changes which occur in HSCs and the lineage cells leading to deregulation of stem cell fate specification and differentiation. The studies involving transplantation of old HSCs into lethally irradiated young mice show functional impairment of HSCs thus suggesting that HSC aging is largely a consequence of aberrations in cell intrinsic mechanisms. The defects may partially be supported by the defective and aged niche/microenvironment as a damaged niche does lead to development of leukemia [26], [27], [6].

1.3.1.1 *DNA damage, telomere shortening and repair*

Genotoxic stress has been long reported to be involved in HSC aging leading to accumulation of DNA damage in HSCs and erosion of telomeres from the end of chromosomes. Furthermore, the development of myeloid malignancies with age has been shown to be associated with accumulation of DNA damage inducing mutations in genes that regulate hematopoiesis. Shortening of telomere or a deficiency of telomerase, an enzyme that maintains their length leads to premature aging. However, negligible amount of telomere shortening has been reported in case of aged HSCs [22]. Other factors that could induce premature aging in the hematopoietic system include replicative errors, oxidative stress and other environmental insults. The presence of DNA damage in the aged HSCs is marked by the damage signature known as γH2AX foci which also represents a replicative stall or inhibition of ribogenesis [5, 28]. Accumulation of DNA damage as HSCs age accompanied by shortening of the protective telomeres could give rise to aging phenotypes due to mutations and deregulation of DNA damage response (DDR) genes. Several genes are known to be involved in maintaining DDR including the well-known tumor suppressor p53, cyclin-dependent kinase inhibitor 2A (CDKN2A) and genes involved in cell cycle arrest and apoptosis [29].

Signalling pathways that govern DNA Damage Response (DDR) including the classical ATM mediated non-homologous end joining (NHEJ) [30] as well as the p53-p21/PUMA pathway are known to play role in determining HSPC fate upon DNA damage. The choice of DNA repair mechanism made by HSCs depends largely upon the type of damage. The NHEJ repair is considered to get activated in response to double strand breakage (DSB) and is a low fidelity repair choice. Normally aged HSCs still exhibit damage induced γH2AX mark indicating loss of repair mechanism in the quiescent HSCs, whereas if stimulated for proliferation these HSCs are then capable of repairing damage better. Once in the cell cycle, HSCs switch to homologus recombination (HR) which is considered to be more stringent to maintain genomic integrity. However, early entry into cell cycle to repair DNA damage can exhaust HSCs; thus, a faithful repair mechanism is required to maintain stem cell identity [31].

The p53 pathway plays an important role in HSC fate determination and regulating DDR signalling. In response to DNA damage, p53 can engage in various downstream cellular events including cell cycle arrest mediated by the p53-p21 branch and cell death mediated by the p53-

PUMA branch leading to HSC elimination [32]. Taken together, these studies raise important questions regarding the nature of molecular signals that regulate DDR in HSPCs and how genotoxic damage contributes to their functional decline.

1.3.1.2 *Perturbations of the mTOR pathway*

The mTOR pathway regulates cellular metabolism and integrates various intra- and extracellular signalling pathways to maintain stem cells in quiescence [33]. It has been reported that mTOR promotes both transcription and translation in cells by phosphorylation of 4ebp1 (eukaryotic initiation factor 4E- binding protein-1) and S6K1 (p70 ribosomal S6 kinase-1). Deletion of the negative regulators of mTOR [Tsc (tuberous sclerosis), PTEN (phosphatase and tensin homologue)] promotes its activity, causes increased mitochondrial biogenesis and accumulation of ROS, leading to loss of cellular quiescence [34]. On the other hand, constitutive activation of mTOR leads to aging phenotypes in HSCs including loss of quiescence and development of lineage bias. Rapamycin treatment of the Tsc-mTOR mice mutants restores the aberrant hematopoietic defects and increases life-span [35]. Modulating the mTOR pathway using small molecular inhibitors assures therapeutic interventions to counteract effects of HSC aging.

1.3.1.3 *Loss of cell polarity*

The ability of HSCs to adhere properly to the stromal cells helps decide their homing and engraftment capacity during a transplantation experiment. It has been observed that old HSCs have reduced adherence and exhibit increased polar distribution of microtubules. This leads to a decline in repopulation capacity and an altered lineage bias with increased myeloid output. The small Rho GTPase Cdc42 (cell division control protein) is known to regulate cell polarity, elevated levels of which induce changes in the distribution of proteins important for maintaining polarity. A switch from the canonical to non-canonical Wnt signalling has been reported to mediate an increase in levels of Cdc42 in aged HSCs, thus, causing them to lose polarity and develop aberrant aging phenotypes [36].

1.3.1.4 *Mitochondrial dysfunction and ROS production*

Mitochondrion, the powerhouse of cell regulates cellular respiration and metabolism which is essential for maintaining HSC quiescence. Aging of cells has been linked to an increased

production of reactive oxygen species (ROS) and their effects on the mitochondrial DNA (mtDNA) and metabolism. In aged HSCs, increased ROS levels lead to mutations in the mtDNA owing to the proximity of ROS to mitochondria as compared to nuclear DNA. These mutations further create a loop where damaged and stressed mitochondria induce increase in ROS levels. Such changes affect cellular metabolism and leads to development of premature aging phenotypes [37]. Notably, increased ROS levels are also linked to increased activation of mitogen activated protein kinase (MAPK), mammalian target of rapamycin (mTOR) pathway that affect the downstream effector forkhead (FOXO) transcription family (namely foxo3) reported to be one of the human longevity factor [38],[39]. Furthermore, impairment of autophagy in aged HSCs which is important for cycling of damaged organelles, contributes to mitochondrial dysfunction and hematopoietic defects in the elderly [40].

1.3.1.5 *Impairments in the lineage specification*

Transcriptome analysis of purified HSPCs (isolated from 10 months old C57BL/6 mice) show deregulation of various lineage specification genes which regulate a fine balance between the lymphoid and myeloid cell development. The IL7 (Interleukin-7) mediated Akt-STAT5 pathway is reported to be important in lymphoid specification. Perturbations in this signalling axis leads to down-regulation of lymphoid specification genes like Notch1 and Gata3 (GATA binding factor 3) along with an increased expression of myeloid promoting genes such as Runx1 (runt-related transcription facor-1), thus promoting myeloid bias in aged HSCs [41]. Furthermore, during the progression of myeloid leukemia such as CML and systemic mastocytosis, involvement of tyrosine kinases like Akt and STAT5 appears crucial. Constitutive activation of Akt and STAT5 induce drug resistance ; whereas inhibiting their phosphorylation proves to be effective in treatment and necessitates development of specific and potent molecular inhibitors of tyrosine kinases [42].

1.3.2 *Cell extrinsic mechanisms of HSC aging*

As discussed above, the mechanisms that regulate HSC aging are thought to be primarily restricted to the stem cell compartment, but recent studies highlight involvement of the microenvironment/niche in which HSCs reside. The cytokines released by niche undergo

alterations in their expression with age which manifests in the form of a systemic low-grade inflammation or inflammaging. The process of inflammaging is marked by increased levels of circulating inflammatory cytokines which is also associated with other phenotypes of aging like HSC function loss and myeloid bias [43]. The effect of niche has been elucidated using competitive transplantation assays where the aged niche cells are transplanted into young mice and vice-versa. Although not much is known about the differences between young and old HSCs but a few reports indicate that upon aging the niche undergoes changes in its composition and function. This includes increased adipogenesis, reduced bone formation and changes in the ECM [44] .

1.3.2.1 *Inflammatory cytokines*

Inflammatory cytokines like the toll-like receptors (TLRs), Interferon's (IFNs) and SH2B adaptor protein 3 (LNK) are reported to be capable of inducing phenotypes similar to HSC aging when activated constitutively by negatively regulating self-renewal of HSCs. Inflammatory cytokines do so either by limiting the cytokine signalling or by abrogating among others TPO/JAK2/c-Kit signalling [45]. Knockout of LNK results in superior HSC re-population ability and a restored lymphoid potential. Molecular analysis reveals repression of the cell-cycle genes in LNK mutants leading to induction of HSC quiescence that partially explains the observed phenotypes [46].

Recently it has been shown that the niche-secreted factor, RANTES/Ccl-5, contributes towards development of myeloid skewing in aged bone marrow by regulating mTOR activation, indicating that niche can regulate the differentiation potential of HSCs [47]. In summary, these studies highlight the importance of cross-talk between cell-intrinsic and extrinsic pathways in driving HSC aging.

1.4 *Role of p53 in aging of HSCs*

The aging process is associated with damage to the building blocks of life (DNA, RNA and proteins). While the damaged proteins are cleared off from the system by processes like ubiquitin-proteasome pathway or autophagy, an arsenal of complex DNA damage response

machinery works to remove the lesions, thus maintaining genomic integrity. The tumor suppressor p53 is known to play central role in maintaining DNA damage where it orchestrates the DDR factors as an adaptive response [48]. Mutations in the genome stability factors along with a natural decline in repair ability of cells upon aging in regenerative tissues like the hematopoietic system render them vulnerable to damage and lead to aberrant cell division and differentiation. Depending upon the severity of damage, p53 can act through various signalling pathways to either induce cell cycle arrest or senescence or complete elimination of damaged cells from the system. p53 is regulated by a plethora of negative (MDM2, COP1) and positive regulators (ARF, NPM, PTEN) which fine tune expression of p53 in response to damage. They do so by either causing ubiquitination of p53 and priming it towards proteasomal degradation or by post-translational modifications that stabilize p53, thus maintaining a balance between tumor suppression and aging [49], [50], [51], [52], [53] (Refer Figure 1.6).

The p21 induced cell cycle arrest helps cells gain time to repair damage without letting damage get replicated and propagated into daughter cells. If the damage is too severe then p53 activates proteins like Puma and Noxa leading to cell death via apoptosis. Failure to act through these pathways as the cells accumulate DNA damage can lead to accumulation of mutations that can cause cells to become cancerous and age prematurely ([54], [55]). Furthermore, in response to damage, cells usually inhibit mechanisms that promote cell growth e.g. The IGF (Insulin growth factor) network that regulates various longevity factors including Hif1α and FOXO transcription family. In summary, the cell responds to aging associated damage and assures longevity by: **A.** activation of repair mechanisms that delay accumulation of damage. **B.** regulation of longevity factors which decide threshold of DNA damage tolerance.

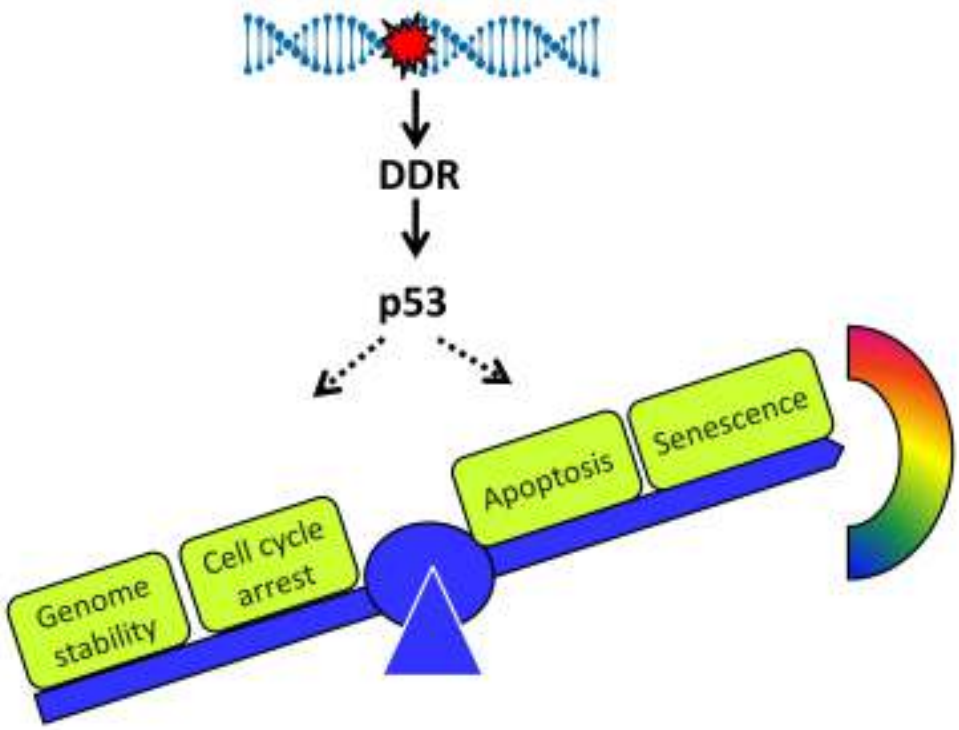

Figure 1.6: p53 maintains balance between aging and health. In response to damage, the DNA damage response machinery gets activated where p53 fine tunes a balance between healthspan, tumor suppression and aging by exerting control over mechanisms that regulate cell fate. (Adapted from [48]).

1.5 *Importance of the p53-mTOR axis in aging of HSCs*

mTOR is a serine/threonine protein kinase of the phosphatidylinositol-3-OH kinase (PI(3)K)-related family that regulates cellular metabolism in response to nutrients and hormonal cues [56]. It is highly regulated and activated by various cell surface receptors, specifically the insulin receptor. Upon activation of the insulin signalling pathway, phosphoinositide-dependent kinase l (PDK1) and PI3K get activated, which further activate mTOR by activating Akt via phosphorylation of residues T308 and S473. mTOR regulates cellular function through its action on S6K1 & 4EBP. In the recent years, mTOR has been shown to be linked with tissue aging where it regulates various signalling networks and longevity factors, as observed in flies and mice treated with Rapamycin (mTORC1 inhibitor)[57]. There are various cell processes which are affected by mTOR during the aging process including mRNA translation, mitochondrial metabolism, autophagy, stem cell maintenance[58]. As cells age, a balance needs to be

maintained between stress response and cell proliferation to delay and avoid accumulation of damage. While mTOR regulates cell proliferation, p53 is known to regulate stress response. It has been reported that this balance is maintained by auto-regulatory feedback loops which work in between p53 and mTOR. p53 is capable of inhibiting mTOR by exerting action on negative regulators of mTOR (Tsc-1, PTEN), thus maintaining cells in a growth arrested state while damage is repaired. Conversely, PI3K-Akt pathway is capable of inhibiting p53-mediated transcription and apoptosis. Akt being a positive regulator of MdM2 is capable of inhibiting p53 by priming it for proteasomal degradation. Therefore, a fine balance between p53 and mTOR signalling networks is capable of regulating various processes including cell cycle arrest, apoptosis, senescence and autophagy. These cellular processes further regulate the balance between cell proliferation and cell death [59], [60] (Refer Figure 1.7). The activity of mTOR is important in regulating HSCs and is reported to be involved in the aging process where activation of mTOR is increased as HSCs age. mTOR activation is further exacerbated by a decrease in the damage repair ability of p53 network giving rise to aberrant hematopoietic phenotypes and leads to development of leukemia. The signalling components upstream of mTOR are known to get mutated in leukemia with constitutive activation of the PI3K/Akt/mTOR pathway detectable in 50-80% of AML patients and thus play an important role in how mTOR affects development of disease[61].

1.6 *Role of Asrij as a molecular regulator of hematopoiesis*

Asrij/OCIAD1 belongs to the Ovarian Carcinoma Immunoactive antigen (OCIA) - domain containing family of proteins. It is expressed in the mouse yolk sac, embryonic blood islands and blood vessels [8]. Asrij is a conserved protein, localizes to endosomes and mitochondria (The Human Protein Atlas) and regulates stem cell properties like self-renewal and differentiation [62], [63]. Studies in *Drosophila* & mouse revealed a novel function of Asrij in maintaining blood homeostasis. Studies on the global knockout of *asrij* (*asrij*$^{fl/fl}$*; CMV-Cre*$^{+}$) revealed that these mice develop aberrant hematopoietic phenotypes like loss of HSC quiescence and increased myeloid differentiation (Saloni Sinha, MS Thesis, 2015). Although these mutants show increased peripheral blood counts at an early age, bone marrow defects arise by the age of 6 months.

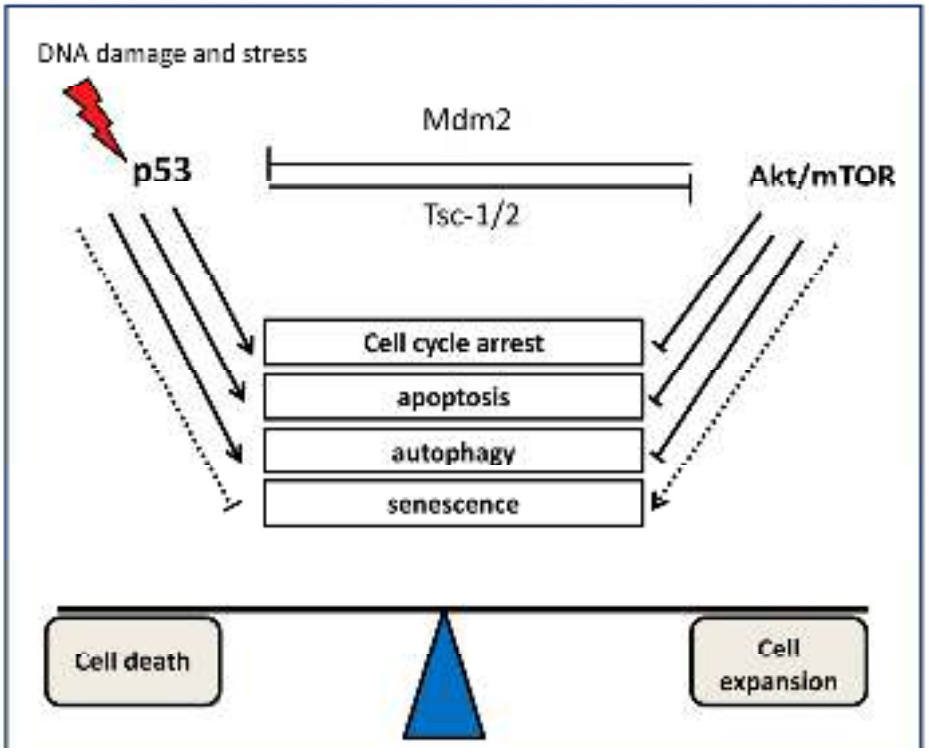

Figure 1.7: p53 and mTOR maintain a balance between cell expansion and death. p53 and mTOR inhibit each other depending upon the cell's response to stress and maintain processes that regulate cellular aging (Adapted from [64]).

This indicates that deletion of *asrij* has an age-dependent effect on the process of hematopoiesis. At the molecular level, Asrij facilitates endosomal activation of STAT3 and regulates various signalling pathways including JAK-STAT pathway, Notch pathway and p53 network. Detailed analysis of *asrij*$^{fl/fl}$*; CMV-Cre*$^{+}$ mice revealed that absence of Asrij leads to reduced expression of p53 at an early age of 2 months indicating that the molecular defects accumulate gradually over age giving rise to phenotypic changes in bone marrow. At molecular level, Asrij interacts with CSN5, sub-unit of COP9 signalosome and possible aids in destabilizing the interaction between p53 and its negative regulator Mdm2 in blood stem cells. This is evident from the observation that absence of Asrij leads to increased levels of Mdm2 (in the *asrij* KO HSPCs) which further ubiquitinates p53 leading to its degradation. Treatment of knockout mice with Nutlin-3 (inhibitor of p53-Mdm2 interaction) activated p53 levels thus maintaining stem cell counts (Sinha et al., unpublished data) (Refer Figure 1.8). Furthermore, preliminary analysis of the global knockout mice also showed increased activation of the Akt-mTOR axis. Since Asrij controls p53 and

mTOR levels and that its depletion leads to premature aging, knockout mice could be used to understand the process of hematopoietic aging.

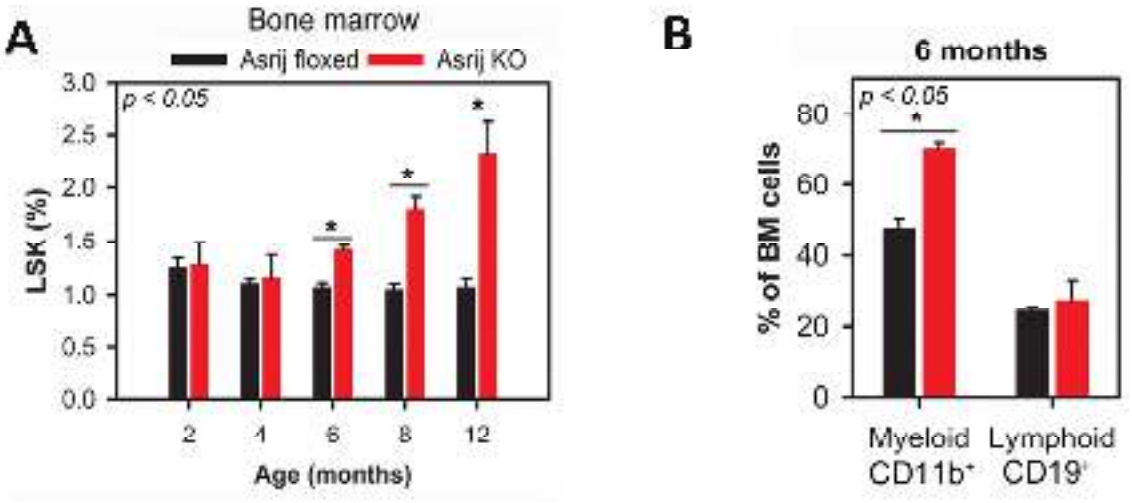

Figure 1.8: Asrij global knockout mice exhibit defective hematopoiesis. A. Increased HSPCs (LSK %) in the knockout mice from the age of 6 months. **B.** Increased myeloid differentiation in the knockout mice. (Adapted from Sinha et al., unpublished data).

1.7 *Aims of the present study*

The primary aim of the present study is to understand how Asrij regulates the process of hematopoietic aging and how its absence leads to the development of age-associated blood disorders.

The specific aims of this study were:

1.) **To establish a role for Asrij in hematopoietic aging.**
2.) **To characterize conditional mutants of Asrij.**
3.) **To understand how tissue specific regulation of p53-mTOR axis affects hematopoietic aging.**

Chapter 2: Association of Asrij with aging of the hematopoietic system

2.1 *Introduction*

The process of hematopoiesis is tightly regulated by signalling networks at various stages of development. With age, hematopoietic stem cells (HSCs) lose their property of self-renewal and differentiation giving rise to aberrant hematopoietic phenotypes and an increased risk of development of blood disorders. In recent years, p53 signalling network has been shown to play a crucial role in deciding health-span of HSCs. Also studies on mammalian target of rapamycin (mTOR), another important molecule known to regulate tissue aging indicates that p53 and mTOR work in concert to maintain a balance between cell death and proliferation [64] .Age-dependent expression of various genes is known to have effects on how the properties of stem cells change over time. As mentioned earlier, both p53 and mTOR have an age-dependent expression, where p53 expression reduces and mTOR activation increases as HSCs age, leading to loss of quiescence [7]. Since the p53-mTOR axis regulates HSC quiescence and is important in the development of aging phenotypes, identification of regulators upstream to the p53-mTOR axis will help understand how these molecules get perturbed over time and lead to impairments in stem cell properties.

Asrij/OCIAD1 is reported to have high expression in the hematopoietic cells, including the HSPCs (hematopoietic stem and progenitor cells) and plays an important role in maintenance of stem cell properties. Deletion of *asrij* leads to late onset of aberrant hematopoietic phenotypes, including loss of HSC quiescence and myeloid bias, thus making the Asrij mutants a reliable model to study HSC aging (Sinha et al., unpublished data).

2.2. *Materials and methods*

2.2.1 *Database mining to look for association of Asrij with age related blood disorders*

To establish a link between Asrij and age related blood disorders, patient sample bioinformatics databases were mined to check for gene mutations and perturbations in expression of Asrij. Since myeloid leukemia is usually old age associated, databases with information on leukemia were specifically mined. The databases used for analysis include **C**atalogue of **S**omatic **M**utations in **C**ancers (COSMIC, https://cancer.sanger.ac.uk/cosmic),**T**he **C**ancer **G**enome **A**tlas (TCGA) and Oncomine (https://www.oncomine.org). The datasets in these repositories are composed of patient samples represented as DNA sequence data and microarray data measuring either mRNA expression or DNA copy number on primary tumors and cell lines, usually from published research. The data is collected from public repositories such as Gene Expression Omnibus (GEO). Statistical analysis is done either manually or computationally to check for significance of the distributions obtained from data analysis.

The data presented in these datasets was selected for hematopoietic tissue distribution and further categorization of patient data was done on the basis of age, gender and mutations present. The data retrieved from the COSMIC database (GrCh38 COSMIC version 38) represents number of samples containing mutations in a given loci and samples where the gene of interest (OCIAD1) is misexpressed. To retrieve patient data pertaining to OCIAD1, the gene name was searched for in the search bar on the COSMIC homepage. The data obtained was further selected for the hematopoietic and lymphoid tissue under the tissue distribution table. Out of 3516 samples, a total of 74 patient samples which had patient details (ICGC-AML-KR Study (COSU544), Landau DA et al., 2013) were selected and analysed for distribution across age and cancer types. The results obtained were further analysed for statistical significance. The data were analysed using chi-square test to check if the observed ratio of patient samples across age is a true representative of the selected dataset. Furthermore, gene mutations found in the patient samples were modelled using RaptorX (http://raptorx.uchicago.edu) to check for any changes in the structure of the human OCIAD1 protein.

In Oncomine database, the data analysis provides results that rank genes by their significant differential expression in disease samples when compared to normal samples. The database

makes use of outlier analysis or Cancer Outlier Profile Analysis (COPA) to identify expression of an oncogene where high gene expression is observed in the given dataset within a cancer type and furthermore validated using t-test. The gene of interest (OCIAD1) was searched for in the oncomine search bar, the data obtained was further filtered for the cancer type under primary filters tab. The data was selected for leukemia and patient details from the AML category were analysed. For further analysis the TCGA leukemia dataset (Reporter ID: 223010_s_at) was chosen as it contains patient details categorised by age and type of leukaemia's. The selected dataset contained details of 197 patient samples, which were then grouped by age and analysed using the outlier analysis at 95th percentile.

2.2.2 *Isolation of bone marrow and LSK (Lin$^-$ Sca1$^+$ c-Kit$^+$)*

Bone marrow was flushed out from the femur and tibia of 2, 6 and 12 months old mice (C57BL6/J) by centrifuging the bones at 6000 x g for 2 minutes.

For isolation of LSK, mouse bone marrow was flushed using sterile PBS from femur and tibia and single cell suspension was obtained by passing it through 70 μm cell strainer to avoid clumps. Filtered bone marrow cell suspension was centrifuged at 1000x g for 2 minutes and resuspended in 1 ml of sterile PBS. Cell viability was assessed by 7-amino-actinomycin D (7-AAD) dye efflux activity. Fluorescently conjugated antibodies used for sorting of HSPCs were lineage cocktail (CD3-T-cell marker, CD45R (B220)-B-cell marker, Ly6C and Ly6G (GR-1)-granulocyte marker, CD-11b (Mac-1)-Macrophage marker and TER 119-RBC marker), PE-Cy7-labelled Sca-1, PE-labelled c-Kit (CD117) and FITC-labelled CD34. (Catalogue No.: BD560492, Mouse hematopoietic stem cell isolation kit, BD Biosciences). These experiments were performed with the help of Saloni Sinha.

2.2.3 *Sample preparation*

Cells were re-suspended in ice-cold lysis buffer consisting of 1% Nonidet-P-40, 150mM NaCl, 50mM Tris/HCl (pH 7.5), 100mM DTT, Na3VO4, and PI Cocktail (Catalogue No. P8340-5 ml, Protease Inhibitor Cocktail, SIGMA), incubated at 4°C for 2 hours with intermittent vortexing

and then they lysates were centrifuged at 13,000 rpm for 10 minutes and supernatant was collected into fresh vials and Bradford's assay (Catalogue No. 500-0006, protein assay dye reagent concentrate, 450 ml, Bio-Rad) was done to estimate protein concentration before proceeding for Western Blotting.

2.2.4 *Western Blotting*

The protein lysates were checked for protein quality using Coomassie Brilliant blue staining (Catalogue No. 42655, SIGMA) and then 40 µg of the protein lysate was loaded on 12% PAGE gel consisting of: Polyacrylamide (Catalogue No. 1610154, Biorad), Tris/HCl (pH 8.8 for resolving gel, pH 6.8 for stacking gel), 20% SDS (Catalogue No.L3771, SIGMA), 10% Ammonium persulphate (Catalogue No.A3678, SIGMA), TEMED (Catalogue No. 17919, Thermo Fisher Scientific). The gel was run at 100V for 2 hours, and transfer was set-up on nitrocellulose membrane using transfer buffer containing Tris Base, Glycine (Catalogue No. 24755, Fischer Scientific), 100% methanol and 20% SDS at 100V for 2 hours. The membrane was blocked using 1% BSA (Catalogue No. TC194, Himedia) and then incubated in the primary antibodies. Antibodies and dilutions used for the analysis:

Protein	**Name of the antibody**	**Company and catalogue number**	**Dilution**
Asrij	Anti-OCIAD1 Rabbit polyclonal	Abcam; ab91574	1:4000
p53	P53 antibody Rabbit-mAb	CST; 9282	1:500
Akt	Akt (pan) Rabbit-mAb	CST; 4691	1:2000
pAkt	Phospho-Akt (Thr 308) Rabbit mAb	CST; 13038	1:1000
mTOR	Anti-mTOR antibody	Abcam; ab2732	1:2000
pmTOR	Anti-mTOR (phosphor S2448) Rabbit mAb	Abcam; ab109268	1:5000
GAPDH	Anti- GAPDH Rabbit antibody	SIGMA; G9545	1:4000
Vinculin	Monoclonal Anti-Vinculin mouse mAb	SIGMA; V4505	1:1000

Table 2.1: Details of primary antibodies used for Western Blotting.

Lysates were normalised with respect to GAPDH, Vinculin expression levels. Secondary antibody used was HRP-conjugated (Goat anti-rabbit IgG-HRP antibody, 1ml, GeNei, Catalogue No.: 621140380011730, dilution 1:2000; Rabbit anti-mouse IgG-HRP antibody, 1ml, GeNei

Catalogue No.: 52127) and blots were developed using ECL chemiluminescence kit (BioRad, Catalogue No. : 1705062, Biorad)

2.2.5 *Densitometry analysis*

To quantitatively determine the fold change in protein and phospho-protien levels (e.g. pAkt/Akt ratio) in respective protien lysates, we measured the density and pixel counts for each band using ImageJ (version 1.4.3.67) software and normalized the values to loading control for plotting.

2.2.6 *Statistical analysis*

For COSMIC data analysis chi-square test was used for each category of leukemia represented in the database. The ratio of number of patients per age group was checked for statistical significance.

Formula used for chi-square test: $\sum(O-E)^2/E$ [where, O: Observed value, E:Expected value]

COPA- Cancer Outlier Profile analysis [65] was developed to identify transcripts up-regulated only in a small subset of cancers where the mean across samples is scaled to 0.0 and the absolute deviation is scaled to 1.0. Features are then ranked based on their values at the 75^{th}, 90^{th} and 95^{th} percentile. (Specific details of the statistical analysis can be found from Rhodes et al., 2007)

The data presented in the Western Blot graphs represent fold change (mean ± SEM). Statistical significance of fold change in protein levels across different age groups was calculated using single factor ANOVA.

2.3. *Results*

2.3.1 *Database mining indicates increased association of Asrij/OCIAD1 with old age leukemia*

Analysis of the patient data obtained from COSMIC showed increased number of patients with misexpressed *asrij/ociad1* in the old age group (50-89 years age) and in the old age associated leukemia such as Blast-phase CML, AML (Acute myeloid leukemia), CML (Chronic myeloid leukemia). Although *ociad1* was misexpressed in leukemia of lymphoid lineage, such as marginal zone lymphoma and B-cell lymphoma but was not associated with old age (Refer Table 2.2 and Figure 2.1). Furthermore, there were two unique *ociad1* mutations found to be associated with haematological malignancies (Refer Table 2.3 and Figure 2.2).

Leukemia type (number of samples)	Number of patients			Chi-square value	Degree of freedom (n-1)	*p*-value
	Young age (1-29)	Middle age (30-49)	Old age (50-89)			
AML (16)	3	1	12	12.875	2	0.01
CML (18)	0	0	18	13.5	2	0.01
Blast phase CML (8)	2	4	2	1	2	>0.5
Marginal zone	0	3	9	10.5	2	0.01
B-cell lymphoma (10)	1	6	3	3.8	2	>0.05

Table 2.2: Distribution of patient samples across age with perturbations in *ociad1*.

Using RaptorX software, OCIAD1 wildtype and mutant proteins could be modelled and homology modelling of the gene mutations found in the database was found to have effects on the conformation of protein as depicted in Figure 2.2.

The Oncomine database representing distribution of AML patient samples having Asrij/OCIAD1 under-expressed (1.3-1.5 log2 fold-change) across different age groups indicated higher number of elderly patients presenting with the disease as compared to younger age groups (Refer Figure 2.1 (B)).

Mutation	**Leukemia type**
133T>C (F45L) (OCIA domain)	Chronic lymphocytic leukemia (CLL)
356C>A (A119E) (N-terminal)	Diffused large B-cell lymphoma

Table 2.3: *Ociad1* mutations reported in leukemia.

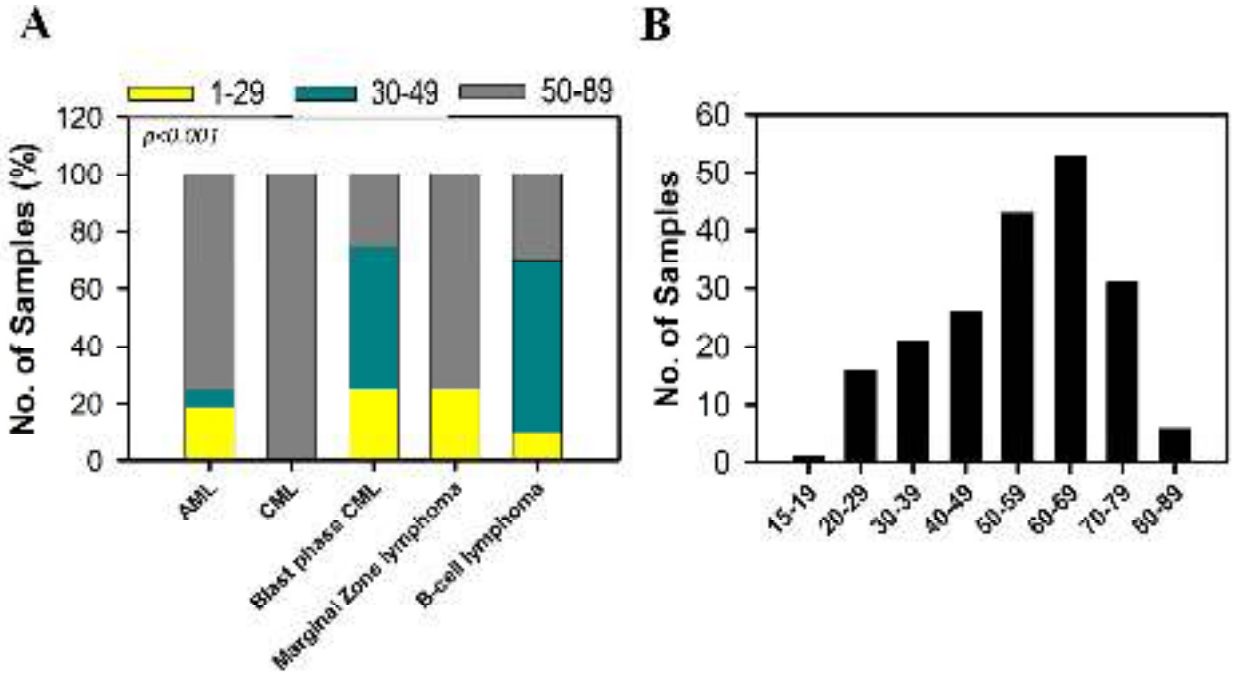

Figure 2.1: COSMIC and Oncomine database analysis shows association of Asrij with old age leukemia. **A.** Asrij/OCIAD1 is misexpressed in the different types of old age associated myeloid leukemia ($p<0.001$) but not associated with lymphomas in an age-dependent manner (NS) (n=74) (data were analysed using chi-square test). **B.** Distribution of AML patients across age show higher number of old age patients with Asrij/OCIAD1 under-expressed (n=197) (data were analysed using t-test).

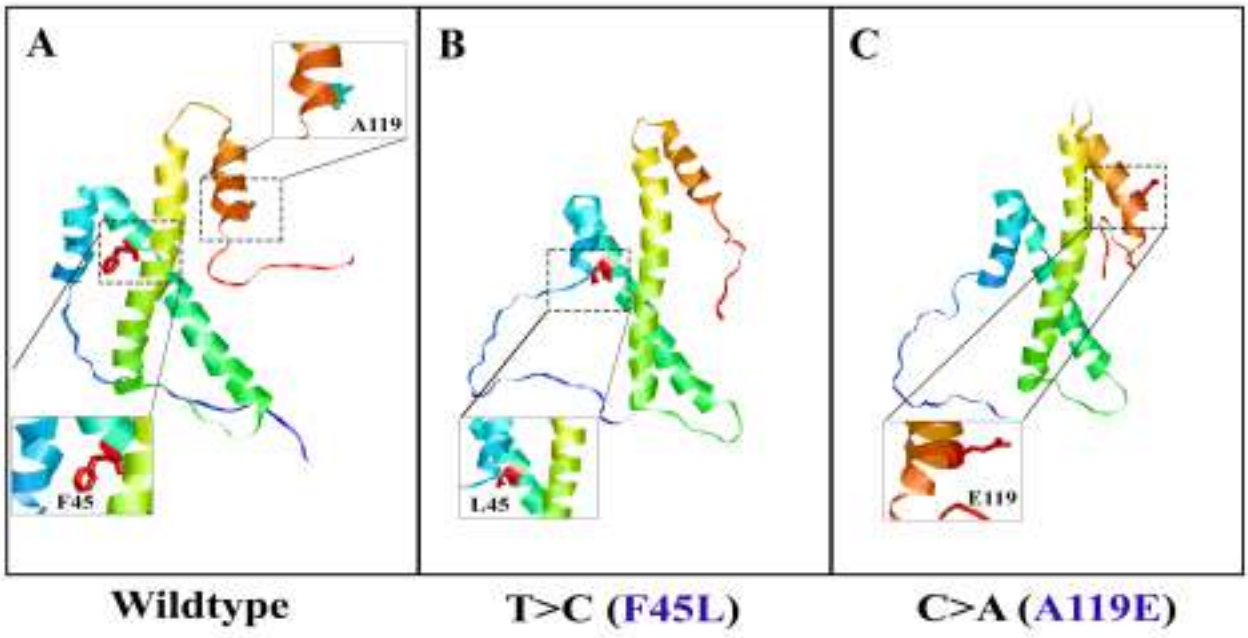

Figure 2.2: Structure prediction suggests that mutations in *ociad1* in patients having hematopoietic

tumors alter protein conformation. A. Predicted structure of wildtype OCIAD1 (source: RaptorX) **B.** OCIAD1 mutant with a substitution (133T>C) of phenylalanine 45 to leucine. **C.** OCIAD1 mutant with a substitution (356C>A) of alanine 119 to glutamic acid. (N to C terminus are represented in rainbow color scheme, inset represents substituted amino acids in stick mode).

2.3.2 *Age-dependent expression of p53, mTOR and Akt in BM cells*

Transcriptomic and RNA-Seq analysis of mouse bone marrow (Chambers, Nilsson) has been reported to have reduced levels of p53 and high levels of mTOR activation as the mice age. We confirmed these observations at protein level by performing Western Blot analysis on bone marrow isolated from 2, 6 and 12 months old wildtype mice (C57BL6/J) to check for levels of various signalling molecules across age. We found that expression of p53 reduces in the bone marrow as mice age and activation of Akt-mTOR goes up with age (Refer Figure 2.3) confirming age-dependent perturbations in critical signalling networks.

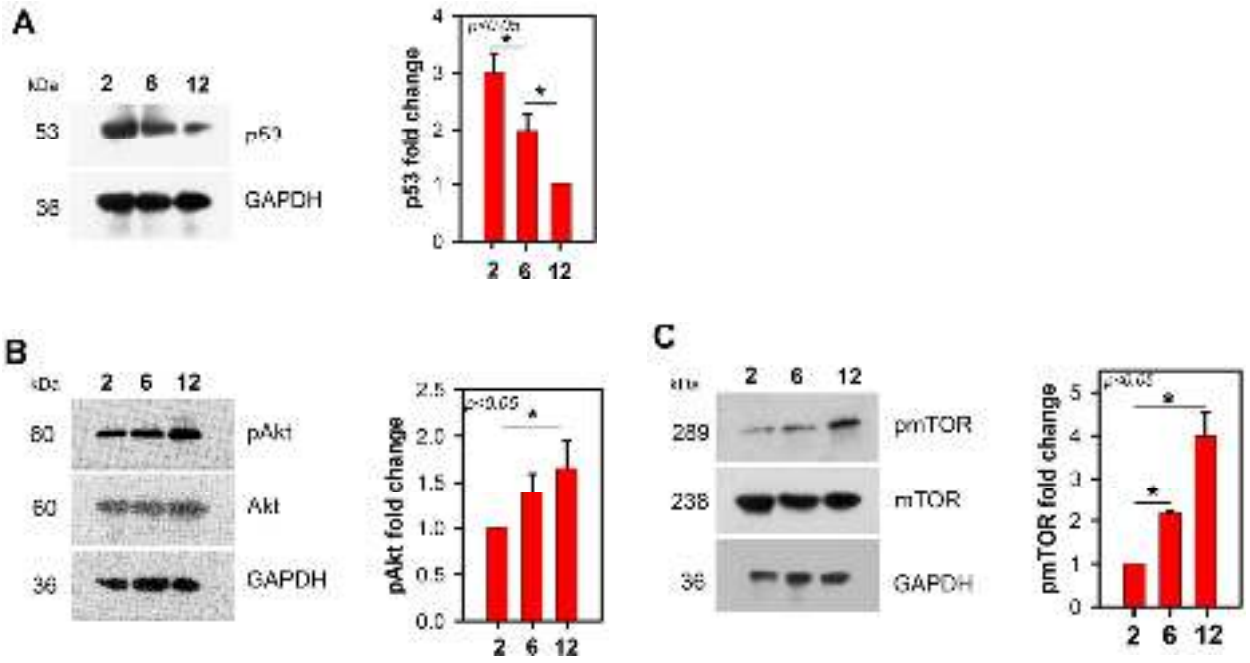

Figure 2.3: Age-dependent expression of signalling molecules. A. Western Blot of 2, 6 and 12 months old C57BL6/J mice showing decrease in expression of p53 as the mice age (N=3, n=2), **B.** Increased activation of the Akt with age (N=3, n=2), **C.** Increased activation of the mTOR with age (N=3, n=1). The graphs represent mean ± SEM and were checked for statistical significance using repeated measures ANOVA (*$p<0.05$).

2.3.3 *Age-dependent expression of Asrij in BM and stem-cell enriched population*

Findings from characterization of *asrij* global KO mice and database analysis reveal that loss of Asrij has an age-dependent effect on development of disease in the hematopoietic system. To check if Asrij exhibits an age-dependent expression Western Blotting of the whole bone marrow and HSPCs isolated from 2, 6 and 12 months old C57BL6/J mice revealed that expression of Asrij goes down with age in the bone marrow with a drastic decline by the age of 12 months (Refer Figure 2.4).

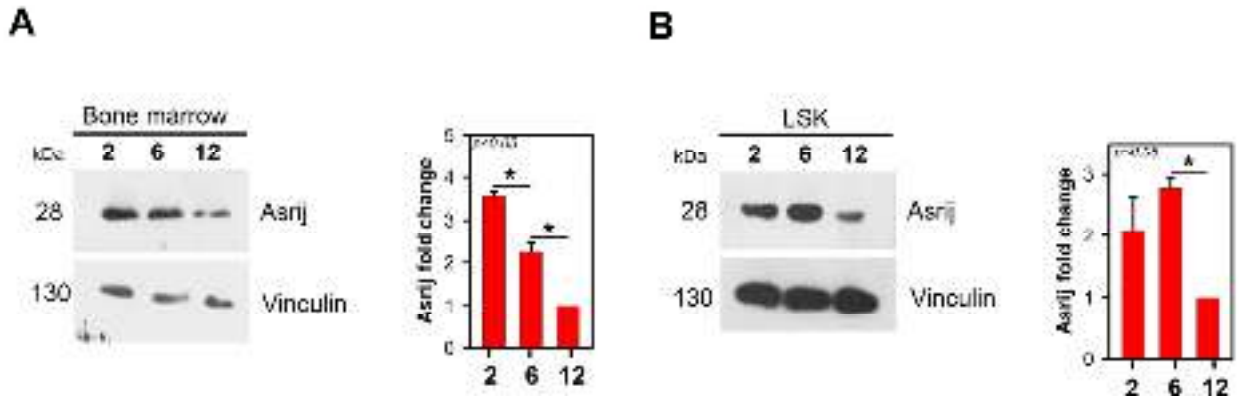

Figure 2.4 Age-dependent expression of Asrij. A. Western Blot of 2, 6 and 12 months old C57BL6/J mice show reduction in levels of Asrij with age in the bone marrow (N=3, n=2). **B.** Reduced levels of Asrij in 12 months old HSPC (LSK) population (N=3, n=2). The graphs represent mean ± SEM and were checked for statistical significance using repeated measures ANOVA (*$p<0.05$).

2.3. *Discussion*

Aging of the blood system gives rise to many diseases categorised as malignant (AML, myelodysplastic syndrome (MDS), lymphomas) and non-malignant (anemia, platelet disorders) diseases. Damage to blood stem cells can lead to premature aging in children, causing leukemia of the myeloid lineage. Understanding the signalling mechanisms and their key regulators which regulate the gradual process of HSC aging can help understand disease pathophysiology and help reduce risk of developing cancer in old people by serving as a biomarker.

Database mining of the gene *asrij*/*ociad1* for disease association showed an increase in number of patients having *asrij* misexpressed at an older age and was also linked with old age leukemia specifically myeloid leukemia like AML and MDS suggesting that *asrij* is more likely to get mutated or misexpressed as an individual ages. Furthermore, unique mutations were found to be

present in *ociad1* gene in case of leukemia patients. Although this study indicates that Asrij expression is perturbed in an age-dependent manner, an in-depth analysis of patient samples with blood disorders using techniques like DNA sequencing and RT-PCRs will help us understand how Asrij is affected during the development of these disorders.

Signalling networks known to regulate mouse HSC aging are reported to have an age-dependent expression that affects HSC properties. Confirming these observations, Western Blot analysis of a few of these molecules revealed that p53 expression is reduced as mice age along with an increase in the activation of Akt-mTOR axis. Remarkably, expression of Asrij was shown to be reduced as the mice aged, suggesting that Asrij may affect downstream target gene expression during aging.

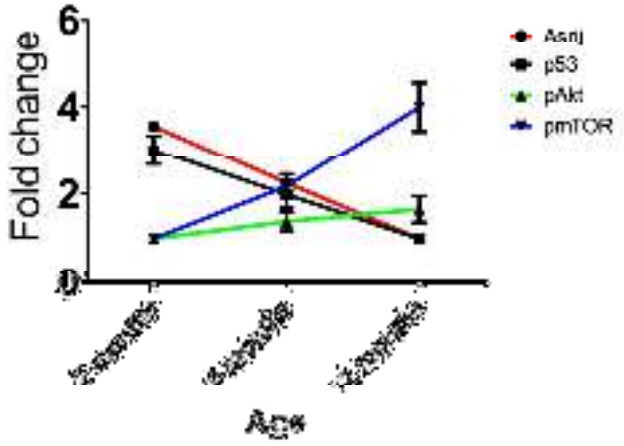

Figure 2.5: Age-dependent expression of various signalling molecules in the mouse bone marrow**.**

Changes in the expression of different signalling molecules like Asrij, p53, mTOR over time during the natural aging process of mice suggests that a decrease in expression of Asrij could lead to perturbations of other signalling networks controlled by it. These include the p53 mediated DNA damage response (DDR) and the mTOR pathway. The aging process could possibly get accelerated if Asrij is knocked out completely giving rise to aberrant hematopoietic phenotypes at an early age as compared to the wild type mice. To better understand the role of Asrij is regulating the process of HSC aging, characterisation of mice having the gene knocked out only in the hematopoietic lineage will prove helpful and also serve as a model to understand disease progression in case of old age leukemias.

Chapter 3: Functional role of Asrij in the aging of mouse hematopoietic system

3.1 *Introduction*

The process of hematopoietic aging is regulated by signalling molecules, which are important in deciding stem cell fate and function. Previous studies on *asrij* global knockout mice **(*asrij*$^{fl/fl}$; *CMV-Cre*$^{+}$)** show that these mice develop loss of HSC (hematopoietic stem cells) quiescence and myeloid bias as they age (*asrij*$^{fl/fl}$*; CMV-Cre*$^{+}$*;* Saloni Sinha MS Thesis, 2015). Furthermore, expression of Asrij was found to be age-dependent. Hence, to study the role of Asrij and signalling networks regulated by it during HSC aging and to avoid any secondary effects of global deletion, we decided to generate conditional knockouts (CKO) of Asrij **(*asrij*$^{fl/fl}$*; Vav-iCre*$^{+}$**).** This was achieved using tissue specific Vav-iCre recombinase system. This chapter describes generation and validation of the conditional mutants and their phenotypic characterization to check for the effect of deletion of *asrij* in the hematopoietic lineage and its correlation with the aging process.

3.2 *Materials and methods*

3.2.1 *Generation of asrij*$^{fl/fl}$*; Vav-iCre*$^{+}$ *mice*

asrij$^{fl/fl}$ mice bearing the loxP sites on either side of exon 6 of *asrij* (generated earlier in the laboratory in collaboration with the RIKEN Centre for Developmental Biology, Kobe, Japan) were mated with *Vav-iCre*$^{+/-}$ (Jackson Laboratories, USA) to generate *asrij*$^{fl/+}$*; Vav-iCre*$^{+/-}$ mice in the F1 generation. These progeny were then self-crossed to generate both the knockout mice (*asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$*)* and control mice *(asrij*$^{fl/fl}$*; Vav-iCre*$^{-/-}$*)* (Refer Figure 3.1). The mice thus generated were genotyped using a combination of primers to check for presence of Vav-iCre and excision of the gene of interest. Mice were maintained in accordance with Institutional Animal Ethics Committee (IEAC) guidelines.

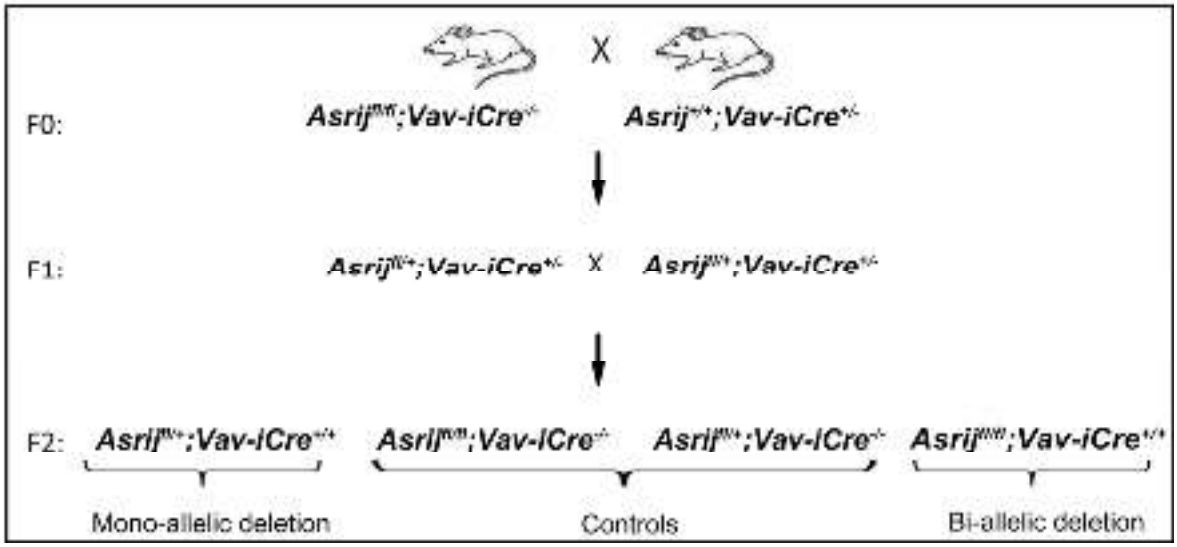

Figure 3.1: Schematic representation of the step-wise generation of *asrij* conditional mutants.

3.2.2 *Validation of asrij$^{fl/fl}$; Vav-iCre$^{+/+}$ mice*

The specific gene identification of conditional mutant mice was done using a combination of different types of Polymerase Chain Reaction (PCR). Genomic DNA was extracted from tail clippings and bone marrow of mice (Catalogue No.: G1N70-1KT, GenElute Mammalian Genomic DNA Miniprep Kit, Sigma) and was analysed by PCR. Primers used for checking the presence of Vav-iCre were: forward -5'-AGATGCCAGGACATCAGGAACCTG -3' and reverse -5'- ATCAGCCACACCAGACACAGAGATC -3'; to amplify 236 bp of the Vav-iCre locus. To identify the wild type (608 bp) and floxed locus (681 bp): forward- 5'-GGAGAATTGCGGCGCTCTTCTCC -3' and reverse- 5'- CCATCCATCCCTCTCCACTGG -3' and also to identify the excised copy (535 bp): forward- 5'-ATGAAGCAGTGTCTTGGGATTGC -3' and reverse- 5'- CCATCCATCCCTCTCCACTGG -3' were used (Refer Figure 3.2).

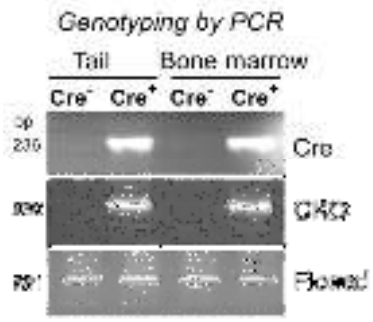

Figure 3.2: Genotyping PCRs for identification of *asrij*$^{fl/fl}$*; Vav-iCre*$^{+}$ mice.

3.2.3 *RNA isolation*

The mouse was sacrificed humanely and the body surface was cleaned properly with Diethyl Pyrocarbonate (DEPC) (Catalogue No. : 159220, SIGMA) treated water. The femur and tibia were dissected out using DEPC treated surgical instruments. Mouse bone marrow was flushed out using sterile PBS from femur and tibia and single cell suspension was prepared and mixed with 150µl Trizol reagent (Catalogue No. : 93289, SIGMA), the samples were pipetted vigorously to prepare a homogenous mixture at room temperature for 5 minutes. 40µl Chloroform (Catalogue No.: C2432, SIGMA) was then added to the tube, mixed and incubated for 10 minutes at room temperature. The tubes were then spun at 12,000 rpm for 10 min at 4^{o}C and the aqueous phase was transferred to a fresh 1.5 ml tube. 100µl Isopropanol (Catalogue No. : DD7F670852, Merck) was added to the samples, mixed and incubated for 5 minutes at room temperature. After incubation the tubes were spun at 12,000 rpm for 10 min at 4^{o}C. The pellet was then washed in 75% ethanol at 10,000 rpm, 4^{o}C. The pellet was air dried and dissolved in 10-20µl of DEPC-water.

The isolated RNA was further processed for DNase treatment. RNA solution was incubated with DNase (2.5µl/ 10µg RNA) (Catalogue No. : EN0525, ThermoFisher) and RNase free DNase Buffer (RDDB) (Catalogue No. : B43, ThermoFisher) in a total reaction volume of 100µl, this was incubated at 37^{o}C for 30 minutes. Equal volumes of Phenol: Chloroform was added to the reaction mixture and spun at 12,000 rpm, 4°C for 10 minutes. 0.3 M sodium acetate was added to the reaction mixture and absolute ethanol was added to make up the volume then the tubes were incubated at -20°C for 2 hours. The tubes were spun at 12,000 rpm for 10 minutes and the pellet thus obtained was washed with 70% ethanol. The pellet was air dried and dissolved in 20µl of

DEPC-water. The RNA samples were further quantitated and run on agarose gel to check for the quality of RNA.

3.2.4 *RT-PCRs*

cDNA was prepared from the isolated RNA in a reaction mix containing oligoDT, 5X RT-Buffer, 0.1M dTT, RNase inhibitor, 2mM dNTPs, Superscript Reverse transcriptase (RT) in a reaction volume of 20µl. The reaction mix was incubated at 42ºC for 50 minutes and then at 70ºC for 15 minutes. The cDNA thus prepared was then carried forward for RT-PCRs.

RT-PCR was set-up in a reaction volume of 10µl using cDNA (100ng/reaction), EvaGreen mixture, and the pair of forward and reverse primers: *asrij* forward 5'-GGAGATCTCGAGATGAATGGGAGGGCTGATTTTC-3' and reverse 5'-GTGGTGGCGGCCGGCTCACTCATCCCAAGTATCTCC -3' to amplify a region of 735 bp; *gapdh* forward 5'- TGCCCCCATGTTTGTGATG -3' and reverse 5'-TGTGGTCATGAGCCCTTCC -3' to amplify a region of 189 bp.

3.2.5 *Western Blotting*

Western Blotting to check for Asrij expression at protein level was done as described previously in 2.2.3.3. Antibodies used for Western Blotting:

Protein	**Name of the antibody**	**Company and catalogue number**	**Dilution**
Asrij	Anti-OCIAD1 Rabbit polyclonal	Abcam; ab91574	1:4000
α-tubulin	Anti –alpha tubulin antibody Rb	Abcam; ab18251	1;1000

Table 3.1: Details of primary antibodies used for Western Blotting.

3.2.6 *Immunostaining of bone marrow cells*

Mouse bone marrow cells were flushed out using sterile PBS from femur and tibia and single cell suspension obtained was passed through a 70 μm cell strainer. Filtered bone marrow cell suspension was then centrifuged at 1000 g for 2 minutes and the pellet obtained was resuspended in PBS. Cells were fixed in 4% paraformaldehyde solution (Merck, Millipore, USA) for 20 minutes at room temperature. After fixation, cells were washed with PBS and permeabilized with 0.1% Triton X-100 (SIGMA, Chemical CO., USA) in PBS for 20 minutes at room temperature. Cells were blocked with 3% FBS (GIBCO, Invitrogen, Carlsband, USA) for 1 hour at room temperature and then incubated with primary antibodies for 1 hour at 4ºC, followed by 2 washes with PBS. 1: 400 dilution of appropriate secondary antibody conjugated either to Alexa 488 or Alexa 568 (Molecular Probes, Carlsband, CA) in PBS was added and incubated for 1 hour at room temperature. Cells were then washed twice with PBS and analysed using flow cytometer. The bone marrow cells were first gated for $CD45^+$ cells which mark all the hematopoietic cells and then selected for $Asrij^+$ population. Primary antibodies used were: rabbit anti-OCIAD1 antibody (Catalogue No.: ab91574, Abcam) in the dilution of 1:100 and mouse anti-CD45 antibody (Catalogue No.: 550566, BD Pharmingen) in the dilution of 1:40.

3.2.7 *Complete blood cell counts*

Blood counts were taken across age groups from 2 to 10 months. Mouse peripheral blood was collected from punctured retro-orbital venous plexus into vials containing 2% EDTA (anticoagulant) solution (Catalogue No.: TC038, Himedia Laboratories). The blood cell counts were determined using an automated hematoanalyzer (Sysmex XP-100).

3.2.8 *Giemsa staining of blood and bone marrow*

Mouse peripheral blood was collected in 1.5 ml tubes containing EDTA. 6-8μl of blood was then spotted on a clean glass slide. Smear was prepared by quickly moving edge of another glass slide over the blood spot at an acute angle. The smear was air dried and fixed in methanol for 10 minutes. The slides were air dried and stained with diluted Giemsa stain (1:20 with PBS pH7.2)

for 10 minutes. The excess stain was removed by rinsing the slides in deionized water. Slides were air dried and evaluated at 100X magnification under IX-70 inverted microscope (Olympus, Tokyo, Japan).

3.2.9 *Flow cytometry analysis of bone marrow compartment*

Bone marrow counts were taken for 2, 4 and 6 months old mice. The HSPC (LSK) pool was stained for and analysed as mentioned in 2.2.3.2. Multipotent progenitors (MPP) were stained using CD135 (PE Rat anti-mouse CD135, Catalogue No. 561068). Bone marrow cells were also stained for the various hematopoietic lineages using CD11b (Purified Rat anti-mouse CD11b, Catalogue No. 553308) and Gr-1 (APC Rat anti-mouse Ly6-G and Ly6-C, Catalogue No. 561083) for myeloid and CD19 (FITC Rat anti-mouse CD19, Catalogue No. 553785) and CD-3 (FITC Rat anti-mouse CD3 molecular complex, Catalogue No. 555274) for the lymphoid lineage. After staining, the HSPCs and lineage cells were analysed by flow cytometry (FACS Aria II, BD Biosciences). The flow cytometry data was analysed using FlowJo v10 software. These experiments were done with the help of Saloni Sinha.

3.2.10 *Statistical analysis*

Statistical comparison of blood counts of *asrij$^{fl/fl}$; Vav-iCre$^{-/-}$ and asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* across different age groups was done using repeated measures single factor ANOVA. The bone marrow counts for different hematopoietic sub-populations were analysed using single factor ANOVA and the graphs plotted represent mean ± SEM.

3.3 *Results*

3.3.1 Expression analysis of Asrij in the hematopoietic lineage of conditional knockout (*asrij$^{fl/fl}$; Vav-iCre$^{+/+}$*) mice

The genotyped mice were further validated for Asrij expression in the bone marrow at the level of RNA and protein. RT-PCR and Western Blotting for Asrij in the bone marrow revealed that

its expression is reduced in the conditional knockout (*asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$*)* as compared to the control (*asrij*$^{fl/fl}$*; Vav-iCre*$^{-/-}$*)* mice. Since bone marrow comprises a heterogeneous population of cells, including the stromal cells, where Vav cannot drive gene deletion, hence we observed that Asrij expression is not completely lost in the conditional mutants. To further validate that Asrij is deleted only in the hematopoietic linage, bone marrow was immunostained with CD45 (pan-hematopoietic marker) and Asrij. We could not detect any staining for Asrij in case of the mutants (*asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$*),* thus confirming hematopoietic deletion of Asrij (Figure 3.3).

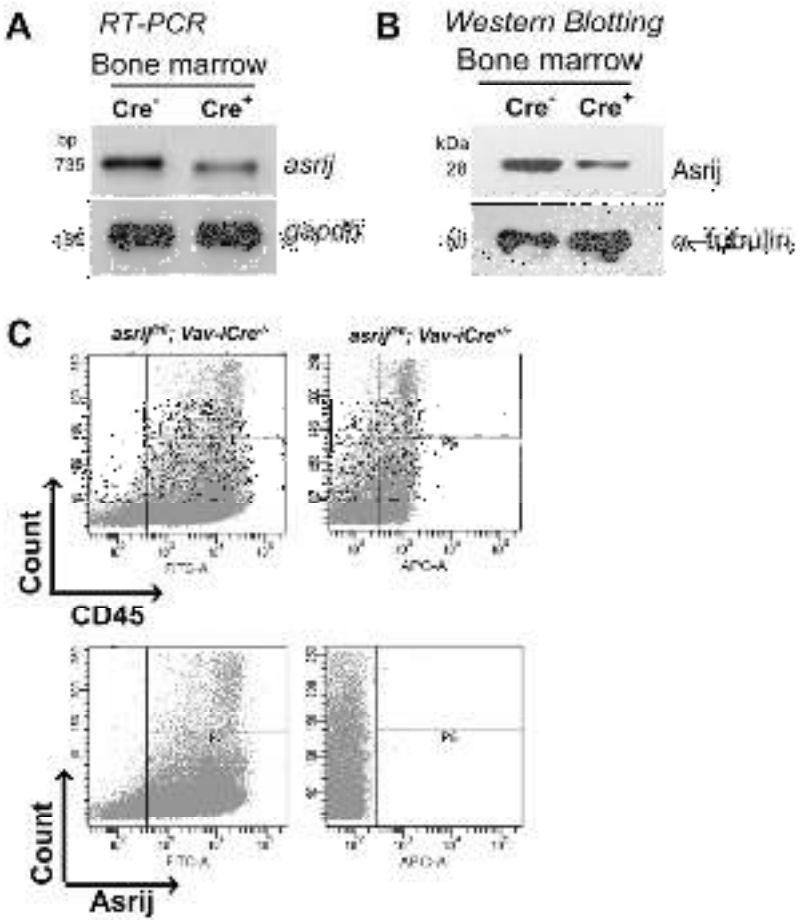

Figure 3.3: Expression analysis of *asrij* in the hematopoietic cells of *asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$ knockout mice. A. RT-PCR for asrij shows reduced expression in the mutants. **B.** Western Blotting of mutant bone marrow shows reduced protein levels of Asrij. **C.** Bone marrow cells isolated from the *asrij*$^{fl/fl}$ and *asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$ mice stained for CD45 (a pan-hematopoietic marker) show no staining for Asrij in case of the mutants (n=2).

3.3.2 Deletion of Asrij in the hematopoietic lineage affects steady state hematopoiesis

Analysis of peripheral blood counts of 2, 4, 6, 8 and 10 months old *asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$ mice revealed increased counts of RBCs, WBCs ($p<0.05$ for both) as the CKO mice age and consistently elevated platelet counts across age groups ($p<0.05$). Increase in the platelet counts is indicative of development of a myeloproliferative disorder upon conditional knockout of *asrij*. Furthermore, Giemsa stained blood shows defects in the myeloid lineage as observed from presence of platelet clumps and large platelets in the blood smears of mutant mice (Refer Figure 3.4).

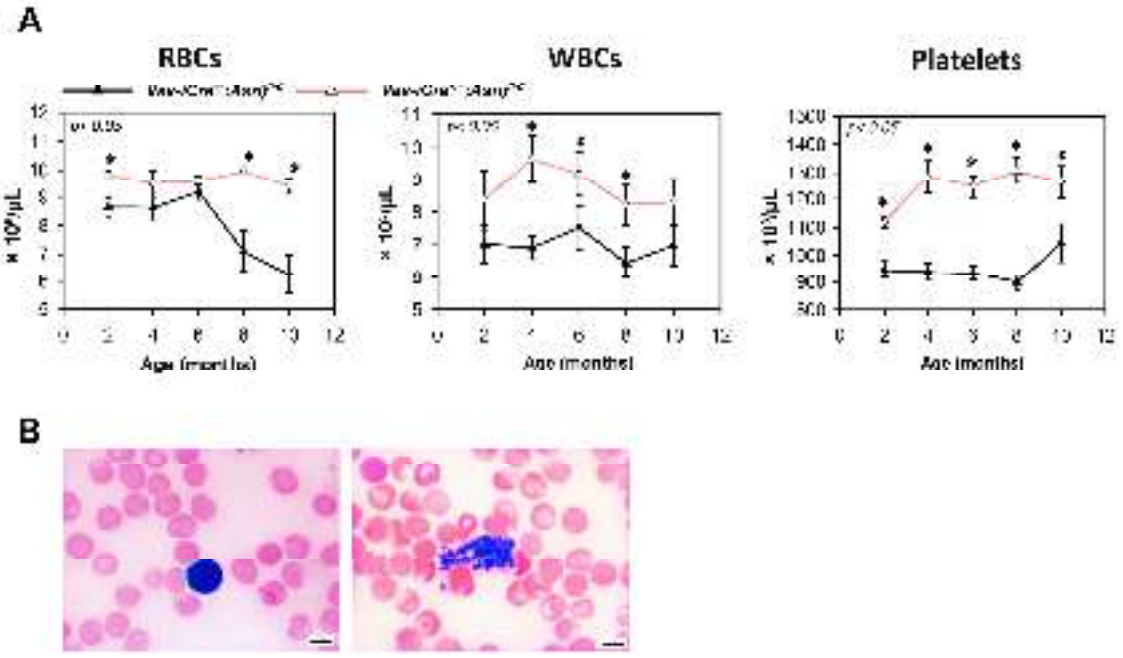

Figure 3.4 Increased blood cell counts in the *asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$mice across age groups. A. Increased RBCs, WBCs and platelet counts in the conditional mutants as compared to the control mice (n=10 each age group) ($p<0.05$). **B.** Presence of large platelets (panel 1) and platelet clumps (panel 2) observed in the mutants (n=2) (scale bar = 50µm). The blood count graphs represent mean ± SEM and were checked for statistical significance using repeated measures ANOVA (*$p<0.05$).

3.3.3 *asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$ *mice exhibit loss of HSPC quiescence with age*

The global knockout mice (*asrij*$^{fl/fl}$*; CMV-Cre*$^{+/+}$) exhibit an increased pool of HSCs by the age of 6 months indicating role of Asrij in maintaining their quiescence. To further investigate

whether the same holds true for the conditional mutants *(asrij$^{fl/fl}$; Vav-iCre$^{+/+}$)* and to understand role of Asrij in regulating stem cell properties, we performed bone marrow immunophenotyping using flow cytometry to profile various sub-populations. When counted using hemocytometer, an increase in the total bone marrow cell count was observed in the mutants. Furthermore, the HSPCs were identified as LSK (Lin$^-$ Sca-1$^+$ c-Kit$^+$) sub-population on the FACS plot and were further gated for LT-HSCs (LSK CD34$^-$) and ST-HSCs (LSK CD34$^+$). The analysis done on 2, 4 and 6 months old mice (n=3 each) revealed that there is no change in the number of HSCs at the age of 2 months but there is gradual increase in HSPC counts as the mice age indicating an age-dependent loss of HSC quiescence in the conditional mutants (Refer Figure 3.5). The defects observed in the CKO mice are pre-mature when compared to normal aging process. Thus, *asrij* CKO mice could serve as a model to understand aging at an early age or accelerated aging and how it is involved in the development of blood disorders.

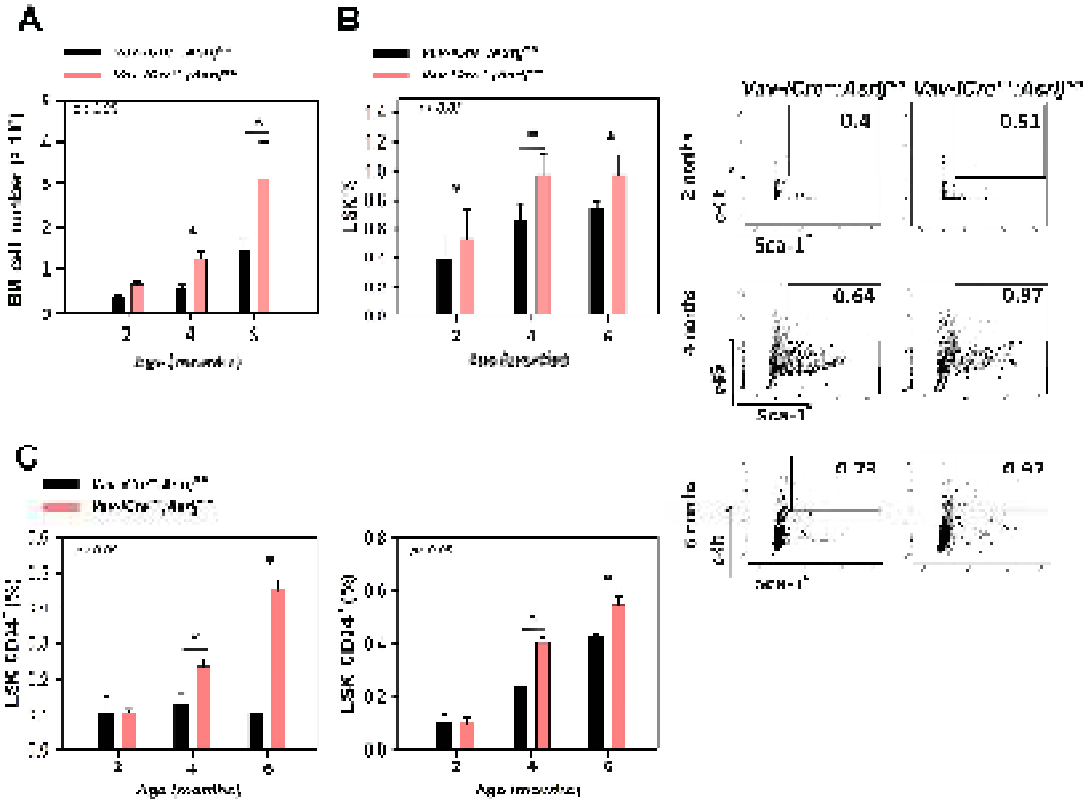

Figure 3.5 Loss of HSC quiescence in *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$*. A. *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* exhibit increase in the bone cell number as they age (n=3). **B.** FACS plots representing increase in HSPC (LSK) as mice age (n=3). **C.** Increase in LSK CD34^{-ve} and LSK CD34^{+ve} of the conditional mutants from the age of 4 months (n=3). Graphs represent mean ± SEM and were checked for statistical significance using single factor ANOVA (*$p<0.05$).

3.3.4 *Bone marrow flow cytometry analysis of asrij$^{fl/fl}$; Vav-iCre$^{+/+}$ mice shows bias towards the myeloid lineage*

Bone marrow of *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* mice was further analysed for different progenitors and differentiated cells to check for any defect in lineage differentiation potential of the mutant HSPCs. For analysis of multipotent progenitors (MPP), bone marrow cells were stained with CD135 (a marker of MPP). Myeloid and lymphoid populations were identified using markers CD11b and Gr-1; and CD19 and CD-3, respectively. No change was observed in the MPP counts upon conditional deletion of *asrij,* however, analysis of 2, 4, and 6 months old mice revealed that the conditional mutants had increased myeloid cells (CD11b$^+$ and Gr-1$^+$), but did not show any consistent difference in the lymphoid pool (CD19$^+$ and CD3$^+$) (Refer Figure 3.6).

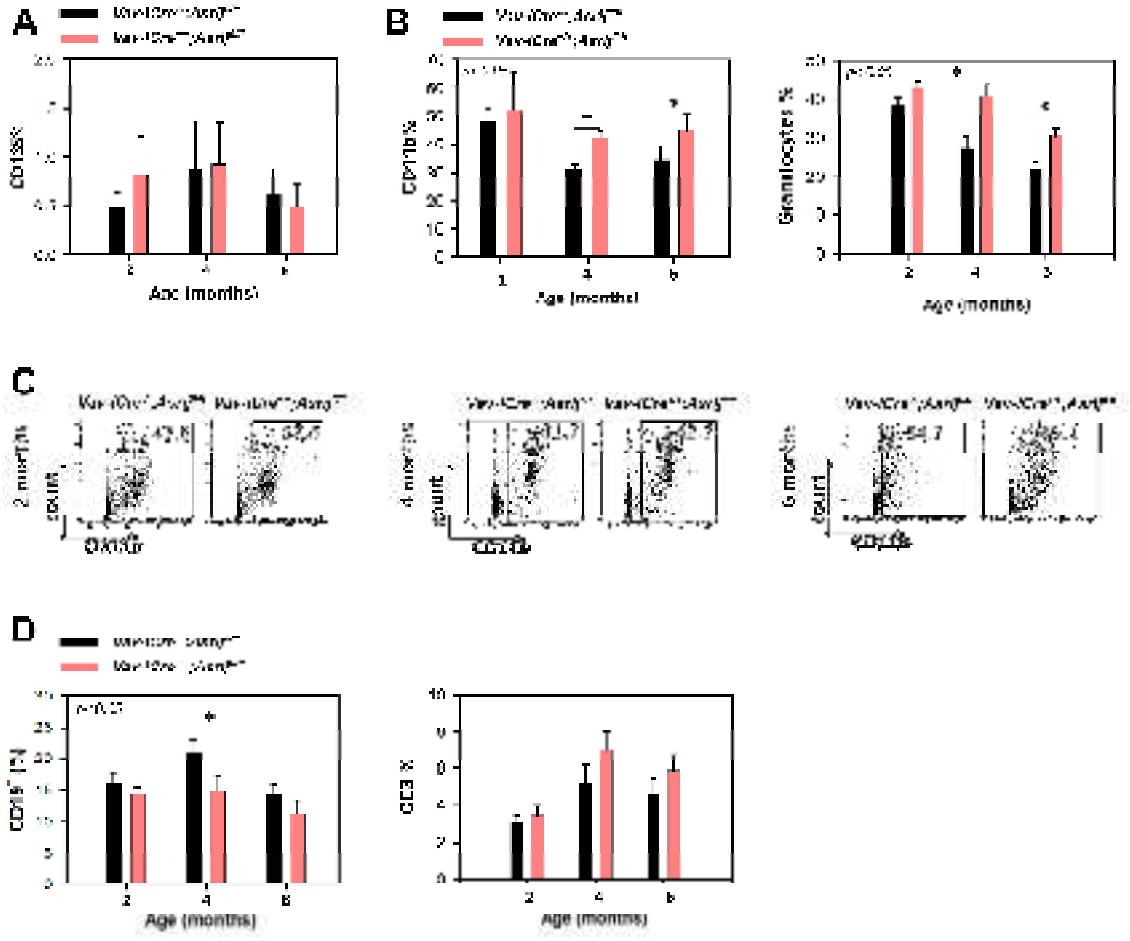

Figure 3.6 Conditional Deletion of Asrij affects lineage differentiation. A. No change in MPP counts (n=3). **B.** Increase in the myeloid populations (CD11b$^+$ and Gr-1$^+$) with age (n=3). **C.** Scatter plots representing increase in CD11b$^+$ cells in the CKO mice across age. **D.** No change in lymphoid counts (CD19$^+$ and CD-3$^+$) (n=3). Graphs represent mean ± SEM and tested for significance using single factor ANOVA (*$p<0.05$).

3.4 *Discussion*

The previously characterized global knockouts (*asrij*$^{fl/fl}$*; CMV-iCre*$^{+/+}$) of *asrij* showed aberrant hematopoietic phenotypes. To further confirm role of *asrij* in maintaining HSPC quiescence, we generated conditional knockouts (*asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$). Genetic identification of the conditional knockout mice was done using a combination of different PCR-based genotyping. Furthermore, kncokout was confirmed at the level of RNA and protein and also by flow cytometry.

Conditional knockouts of *asrij* were viable and fertile and suggest that Asrij is dispensable for embryonic development. To check whether tissue specific deletion of *asrij* has an effect on HSPC quiescence and differentiation, we analysed the blood and bone marrow of the mutants. Increased levels of blood counts and histological anomalies in the blood smears indicate role of Asrij in maintaining steady state hematopoiesis. Moreover, Asrij deficiency leads to expansion of HSPC pool including both LSK CD34^{+} and LSK CD34^{-} cells along with an increased myeloid pool as the *asrij* CKO mice age. Thus, in agreement with previously reported phenotypes of global knockouts of *asrij*, the conditional mutants also show defective hematopoiesis. Taken together, these results suggest that Asrij regulates proliferation and differentiation of HSPCs and when knocked out it initiates an accelerated aging like phenotype which gradually develops into a myeloproliferative disorder. Understanding molecular mechanisms and signalling pathways involved in aging in the context of *asrij* CKO would help us understand how Asrij maintains HSPC quiescence. Furhermore, these mice models help us understand role of Asrij in regulating signalling networks in a tissue specific manner and how its absence accelerates hematopoietic aging.

Chapter 4: Molecular regulation of hematopoietic aging by Asrij

4.1 *Introduction*

Cell intrinsic signalling networks play a crucial role in the emergence of hematopoietic stem cell (HSC) aging phenotypes leading to development of various blood related disorders. Previous studies on the global knockout mice (***asrij$^{fl/fl}$; CMV-iCre^{+}***) of *asrij* reveal that these mice develop aging-like phenotypes in their bone marrow and show defects in various signalling networks including COP9 (Constitutively photomorphogenic signalosome) mediated p53 degradation (Sinha et al., Unpublished) and Akt mediated mTOR (mammalian target of rapamycin) activation (data not shown). Furthermore, the conditional knockouts of Asrij (***asrij$^{fl/fl}$; Vav-iCre^{+}***) also exhibit similar aging phenotypes. To further elaborate on the tissue-specific role of Asrij in regulating these signalling networks, we performed molecular analysis of various signalling networks known to be involved in bone marrow aging using conditional mutants and Asrij overexpression cell lines. This chapter describes the molecular characterization of conditional mutants to check for the effect of deletion of Asrij in the hematopoietic lineage and its correlation with the aging process.

4.2 *Materials and methods*

4.2.1 *Sorting of the LSK (Lin^{-} Sca1^{+} c-Kit^{+}) population*

HSPCs (LSK) were isolated from 6 month old conditional mutants (*asrij$^{fl/fl}$; Vav-iCre$^{+/+}$*) as described in section 2.2.3.3.

4.2.2 *Western Blotting*

Western Blotting was done using bone marrow isolated from 6 month old mice as described previously in 2.2.3.3. Antibodies used for Western Blotting are as follows:

Protein	Name of the antibody	Company and catalogue number	Dilution
CSN5	COPS5 anyibody rabbit	CST; 6895	1:1000
γH2AX	Phosphor-histone H2A.X (Ser 139) rabbit mAb	CST;9718	1:1000
H3	H3 Rabbit polyclonal Ab	Abcam; ab46765	1:1000
STAT5	Anti-STAT5 rabbit antibody	CST; 9363S	1:1000
pSTAT5	Anti-pSTAT5 rabbit antibody	CST; 9359S	1:1000
PI3K	Anti-PI3 Kinase p85 alpha Rb mAb	Abcam; ab191606	1:500
pPI3K	Anti-PI3 Kinase p85 alpha (phospho Y607) Rb mAb	Abcam; ab182651	1:250
β-Actin	Monoclonal anti-beta actin mouse mAb	SIGMA; A5316	1:1000

Table 4.1: Details of primary antibodies used for Western Blotting.

4.2.3 *Culture and transfection of HEK293 cells*

HEK293 cells were seeded in 60mm petri dishes and the culture was incubated at 37°C for 48 hours. When the cells reached desired confluency (40-50%), they were transfected with 5µg DNA using Calcium Chloride method. The transfected cells were grown for 48 hours and harvested using centrifugation. The cell pellet thus obtained was lysed using IP-lysis buffer and further proceeded for Western Blotting.

The plasmid clones used for this experiment were: pCMVTag2A (vector) and pCMVmArjTag2B (full length Asrij cloned between EcoRI and XhoI in pCMVTag2B with Flag-tag towards N-terminal of Asrij). The clone preparation and cell culture work was done by Rajarshi Batabyal.

4.2.4 *Densitometry analysis*

Radiographs obtained from the Western Blotting experiments were scanned and processed using ImageJ (version 1.4.3.67) and graphs were plotted using SigmaPlot (version 11.0).

4.2.5 *Statistical analysis*

The data presented in the Western Blot graphs represent fold change (mean ± SEM). Statistical significance of fold change in protein levels across different age groups was calculated using single factor ANOVA.

4.3 *Results*

4.3.1 *Impairment of p53 mediated DNA damage repair and CSN5 observed in asrij$^{fl/fl}$; Vav-iCre$^{+/+}$*

The p53 signalling network is important in regulating cell fate upon aging. p53 regulates the fine balance between healthy and accelerated aging by regulating cellular senescence. p53 is also known to maintain HSCs in their quiescent state. Hence, a perturbation in this network can have effects on HSC properties (Pant et al., 2012). Previously analysis of global knockout of *asrij* (Saloni Sinha, MS thesis 2015) revealed that Asrij interacts with the CSN5 subunit of COP9 signalosome to regulate interaction between p53 and Mdm2, thereby maintaining the stability of p53. We further tested for effects of *asrij* deletion in the hematopoietic lineage by checking the levels of proteins like CSN5 and p53 in the bone marrow of *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* mice. Western Blotting analysis revealed reduced p53 and increased CSN5 levels in the bone marrow of conditional knockout mice as compared to the control mice. This confirms the observation that Asrij does regulate p53 stability and CSN5 expression and that in its absence mice develop aberrant hematopoietic phenotypes. Furthermore, we sorted out HSPC population from the bone marrow of both the control and knockout mice and probed for DNA damage using antibodies against γH2AX (marker of DNA damage). It was observed that *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* mice had increased levels of γH2AX indicative of elevated DNA damage in CKO HSPCs (Refer Figure 4.1). Taken together, these results highlight that loss of Asrij leads to impairment of the DNA damage repair machinery thus making the mutant HSPCs prone to detrimental effects of aging. Hence, this could be a causative factor in premature loss of HSPC quiescence as reported in *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* mice.

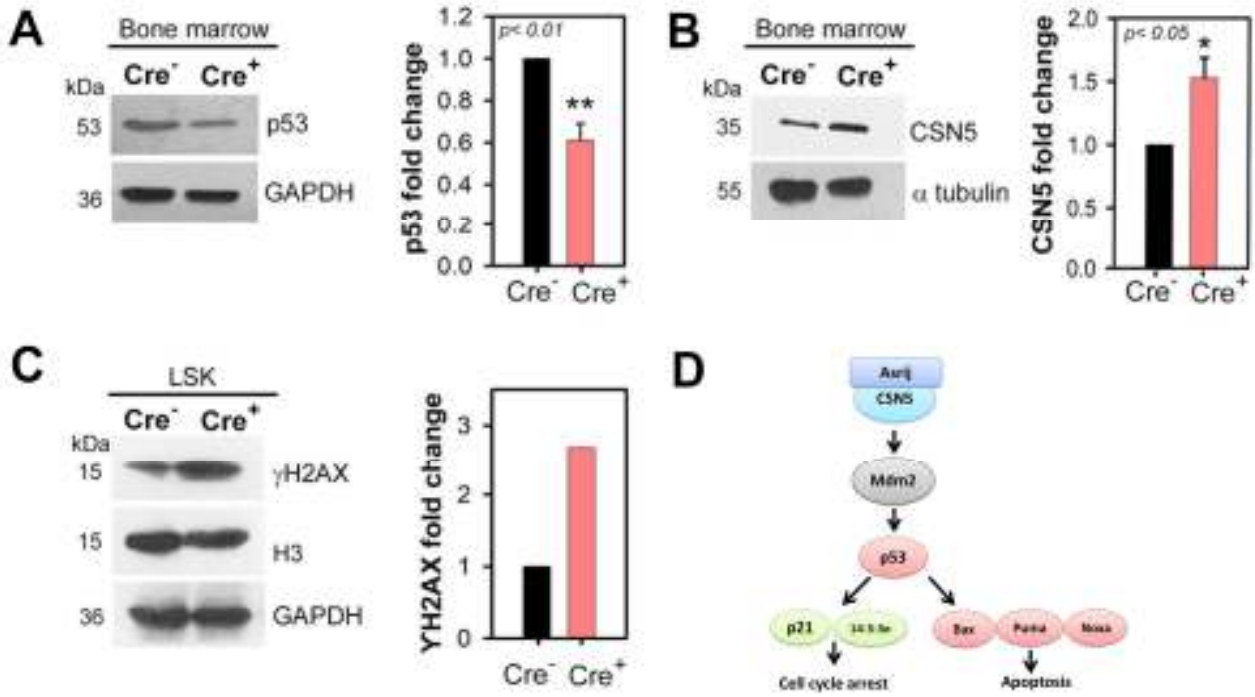

Figure 4.1: Impairment of DNA damage in the *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* mice. **A.** Reduced levels of p53 in the conditional knockouts (Cre^{+}) (N=3, n=3) **B.** Increased levels of CSN5 observed in the knockouts (N=3, n=3) **C.** High levels of γH2AX in the knockout HSPCs indicate elevated DNA damage (N=2, n=2). Graphs represent fold change (mean ± SEM) (*$p<0,05$, **$p<0.01$) **D.** Schematic representation showing interaction of Asrij with DNA repair mechanism in the bone marrow.

4.3.2 *Loss of Asrij leads to increased activation of Akt-STAT5 signalling*

The IL-7 mediated Akt-STAT5 signalling has been reported to be essential in lineage specification of HSCs. Constitutive activation of Akt and STAT5 is reported to be a hallmark of myeloid leukemia where parallel over-activation of these signalling pathways leads to increased production of the myeloid cells. Western Blotting revealed increased levels of activated Akt and STAT5 in the bone marrow of CKO mice as compared to the controls (Refer Figure 4.2). These results indicate a possible involvement of increased Akt and STAT5 signalling in inducing myeloid bias in the CKO mice as shown previously. Further analysis of the target genes of Akt and STAT5 will help us discern whether the observed phenotypes develop in a lineage restricted manner.

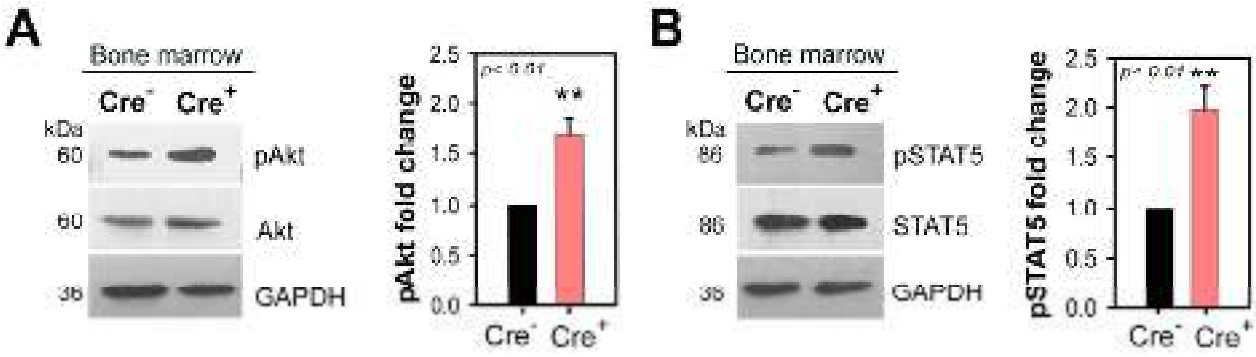

Figure 4.2: Increased Akt and STAT5 activation in the *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* mice. Increased activation of **A.** Akt (N=3, n=3) and **B.** STAT5 observed in the conditional mutants (N=3, n=2). Graphs represent fold change (mean ± SEM) (**p<0.01*).

4.3.3 *Loss of Asrij leads to increased activation of PI3K-Akt-mTOR signalling*

The PI3K-Akt-mTOR pathway is known to regulate various processes inside a cell by regulating metabolism and cell fate. Upon aging there occurs an increased activation of mTOR thus leading to deregulation of processes controlled by it. The observation that both p53 and Akt are affected in *asrij$^{fl/fl}$; Vav-iCre$^{+/+}$* mice led us to investigate mTOR activation. Western Blotting analysis revealed that conditional mutants exhibit increased activation of all three components of PI3K-Akt pathway i.e. PI3K, Akt and mTOR (Refer Figure 4.3). This indicates that mTOR activation could either be increased due to loss of its inhibitor (p53) or due to an overall increase in the activation of PI3K-Akt signalling. This also indicates that Asrij keeps a check on the activation/ phosphorylation event of these kinases, either directly or indirectly, thus exerting its effect on the maintenance of HSPC quiescence and lineage specification. Decrease in expression of Asrij could possibly give rise to HSPC aging phenotypes. A detailed analysis of the molecules targeted by mTOR would help us understand how its altered activity regulates cell fate decision upon aging in the CKO mice.

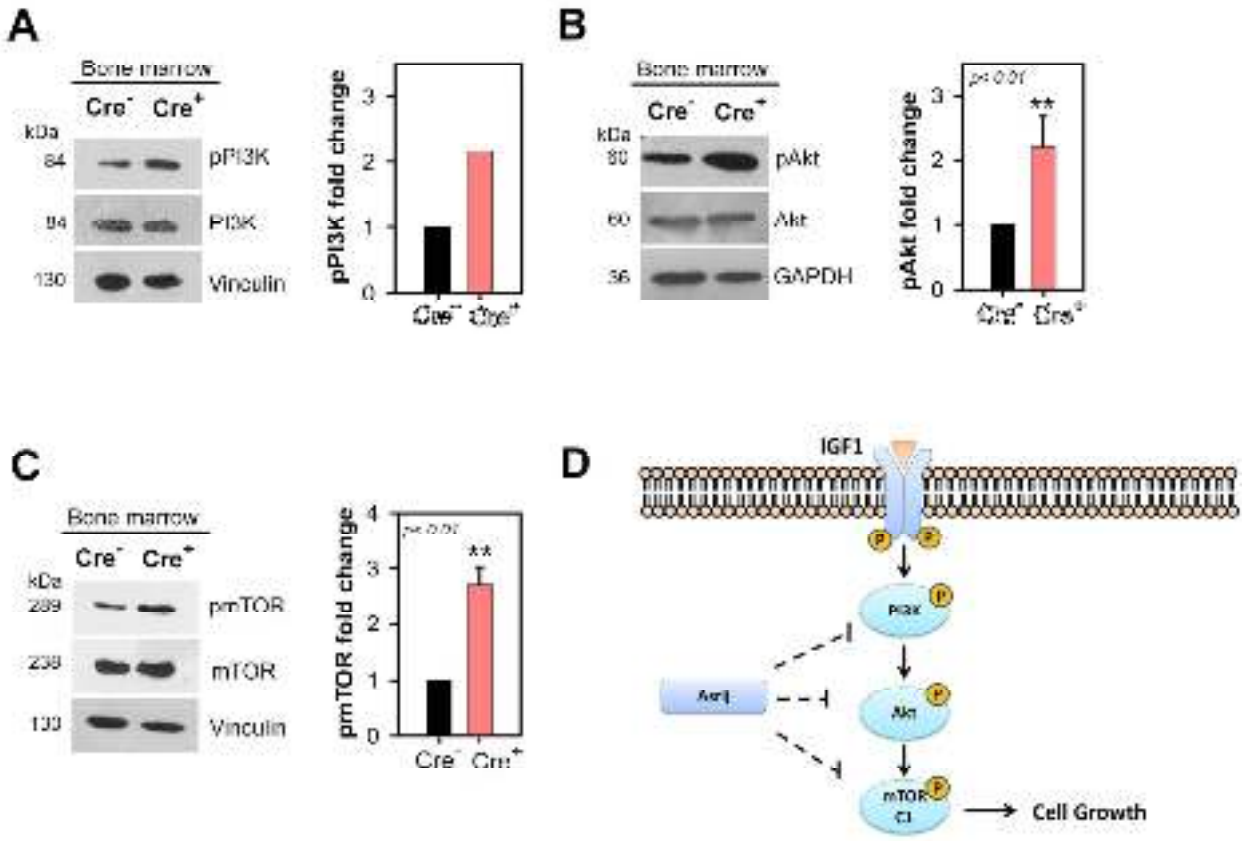

Figure 4.3: Increased activation of PI3K-Akt-mTOR axis in the *asrij*$^{fl/fl}$; *Vav-iCre*$^{+/+}$ mice. A. Increased levels of phospho-PI3K in the CKO mice (N=2, n=2) **B.** increased levels of phospho-Akt (N=3, n=3) **C.** increased activation of mTOR (N=3, n=2). Graphs represent fold change (mean ± SEM) (***p<0.01*) **D.** Schematic representation showing interaction of Asrij with PI3K-Akt-mTOR pathway in the bone marrow.

4.3.4 *Overexpression of Asrij leads to reduced activation of PI3K-Akt-mTOR signalling*

To check the effect of Asrij overexpression on the activation of PI3K-Akt-mTOR signalling, HEK293 cells were transfected with plasmid containing full length *asrij* fragment. Western Blotting analysis revealed that Asrij overexpression leads to reduced activation of the PI3K-Akt-mTOR (Refer Figure 4.4) axis. This indicates that perturbation of Asrij expression affects activation of kinases that regulate mTOR pathway and that Asrij could possibly act as a true regulator of the kinase activation.

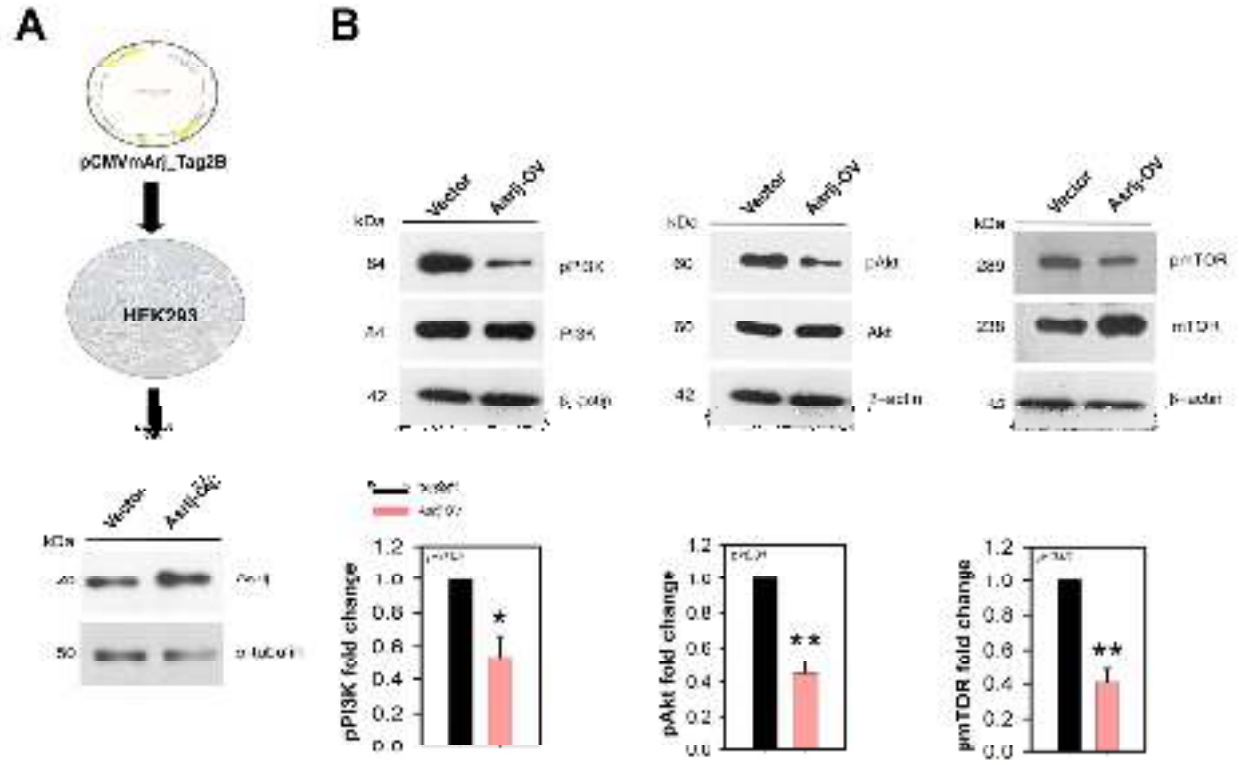

Figure 4.4: Reduced activation of PI3K-Akt-mTOR axis upon Asrij overexpression. A. Generation and validation of the overexpression cell line (done by Rajarshi Batabyal) **B.** Reduced levels of phospho-PI3K-Akt-mTOR axis in the Asrij OV cells (N=3, n=2). Graphs represent fold change (mean ± SEM) (***p<0.01*).

4.4 *Discussion*

Phenotypic characterization of the *asrij* conditional mutants (***asrij*$^{fl/fl}$*; Vav-iCre*$^{+/+}$*)** revealed early bone marrow aging phenotpyes. To understand the molecular mechanisms, we performed Western Blotting analysis in bone marrow and HSPCs and found that depletion of Asrij in the hematopoietic lineage leads to impairment of the p53 mediated DNA damage repair. Reduced p53, increased CSN5 (earlier shown to be a regulator of p53 stability) along with high levels of γH2AX foci were observed in the CKO HSPCs. Since p53 maintains HSC quiescence, we propose that reduction in p53 activity could impede the DNA repair pathways leading to an uncontrolled proliferation of HSCs, even when they are damaged. Furthermore, CKO mice also show defects in the activation of Akt and STAT5 both of which are known to regulate expression of lineage specification genes (e.g. Runx1). This could lead to biased differentiation towards one

lineage (myeloid) at an early age. These defects could further give rise to a myeloproliferative disorder as mice age. Finally, we also report that the conditional mutants show increased activation of mTOR signalling pathway and that overexpressing Asrij in HEK393 cells reverses this activation. This indicates that perturbation of Asrij could lead to emergence of aging phenotypes due to defects in signalling pathways required for maintaining a balance between cell proliferation and cell death. The increase in mTOR activation could either be facilitated because of reduction in the levels of tumor suppressor p53 (which normally inhibits mTOR in response to damage and stress), or because of an overall increase in the phosphorylation of the kinases involved in mTOR activation (Refer Figure 4.5). Therefore, this study highlights the function of Asrij in regulating both the p53 network and the mTOR pathway in the process of bone marrow aging. It also highlights function of Asrij as tissue specific molecular regulator of signalling networks. This study also provides a characterization of mammalian model which can be used for drug screening for leukemia and to understand the process of hematopoietic aging.

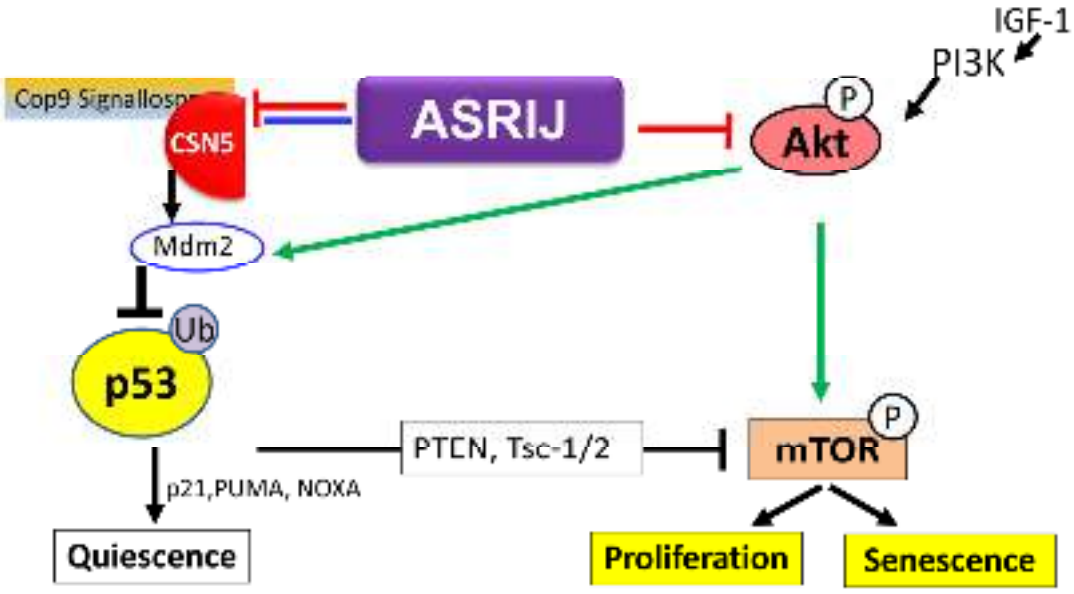

Figure 4.5: Asrij regulates p53-mTOR axis during bone marrow stem cell aging. Asrij maintains p53 stability via CSN5 mediated control of Mdm2 and also regulates the activation of the mTOR pathway consequently leading to loss of HSC quiescence and cellular senescence.

To further understand molecular mechanisms by which Asrij regulates the p53/mTOR pathways, making use of cell culture system is feasible to study interactions between Asrij and the signalling molecule of interest. Also, Asrij can regulate activation of STAT3 on endosomes. Therefore, it could be possible that Asrij facilities the activation of kinases like PI3K, Akt and mTOR on endosomes, thereby exerting its control over these signalling pathways as an endosome mediated regulation. We could also check for a phenotype rescue by using various small molecule inhibitors like Wortmannin (inhibitor of PI3K) or Rapamycin (inhibitor of mTOR) to confirm that the observed phenotypes are truly due to perturbation of these pathways. Furthermore, in recent years, mTOR signalling has been linked to metabolic quiescence and production of reactive oxygen species (ROS) in mitochondria of HSCs and is important in maintaining HSC stemness [34]. Reports form our lab indicate that in the mouse embryonic stem cells (mESCs), Asrij is capable of maintaining pluripotency [63]. Therefore, regulation of mTOR by Asrij could have an effect on mitochondrial metabolism and quiescence thereby playing a role in maintenance of HSC stemness. These studies would further elaborate on role of Asrij in aging of blood stem cells and development of hematological disorders.

Chapter 5: Discussion

Aging research has experienced an unprecedented advance over recent years, particularly with the discovery that the rate of aging is controlled, at least to some extent, by conserved genetic pathways. Aging of the hematopoietic system is characterised by loss of HSC (hematopoietic stem cells) quiescence and skewed differentiation towards the myeloid lineage. Recent studies have highlighted the importance of cell intrinsic signalling pathways in the development of aging phenotypes. Defects in signalling networks accompanied by accumulation of DNA damage leads to aberrant hematopoiesis with age increasing incidence of cancer in the elderly. This study highlights the role of Asrij/OCIAD1 in regulation of signalling networks that govern cell proliferation and differentiation in the hematopoietic system using conditional knockout mice of *asrij.* We observe that expression of Asrij goes down with age in the bone marrow and HSPCs (hematopoietic stem and progenitor cells) indicating a functional decline. Understanding signalling mechanisms and their key regulators which control the gradual process of HSPC aging can help understand how aging leads to aberrant hematopoiesis.

5.1 *Aging of hematopoietic system is accompanied by perturbations in Asrij*

Aging of HSPCs is linked with development of myeloid leukemia in old people (Elias et al., 2017) [24]. Perturbations in various signalling network is well-associated with functional decline in aged HSCs (Haan and Lazare., 2018) [6]. This gives rise to a need for identification and characterization of molecular regulators of HSPC aging. Asrij is important for maintenance of stem cell properties. Analysis of *asrij* global KO (*asrij*$^{fl/fl}$*; CMV-iCre*$^{+/+}$) mice revealed that Asrij is important for HSPC proliferation and lineage differentiation. We checked for association of Asrij with old age blood disorders using *in-silico* approach. Mining of patient databases such as COSMIC, TCGA and Oncomine revealed that incidence of Asrij being perturbed in leukemia increases with age*;* this could partially be explained because of mutations in the OCIA domain as observed in a few patients. Analysis of C57BL6/J mice bone marrow revealed that various signalling molecules (p53, mTOR) implicated in aging of HSPCs show an age-dependent expression. Remarkably, Asrij expression changed in a similar age-dependent manner. Detailed

analysis of leukemia patient samples would help us understand how *asrij* is implicated in myeloproliferative neoplasms.

5.2 *Knockout of Asrij in the hematopoietic system exhibit increased HSC counts and myeloid differentiation*

Understanding the role of a gene of interest using conditional knockouts helps negate secondary effects of gene deletion in other tissues. We generated conditional knockouts of *asrij (asrij$^{fl/fl}$; Vav-iCre$^{+/+}$).* Analysis of different age groups of *asrij* CKO (conditional knockouts) mice revealed that they develop increased HSPC counts as they age. Analysis of peripheral blood revealed an increase in RBC, WBC and platelet counts along with presence of platelet clumps and premature cells in blood. Furthermore, analysis of the bone marrow sub-populations revealed that *asrij* CKO mice exhibit an increase in the myeloid populations, indicating a possible role for Asrij in maintaining differentiation of stem cells into different lineages.

5.3 *Asrij modulates signalling pathways associated with aging of hematopoietic system*

Cell intrinsic signalling is known to play an important role in deciding stem cell fate upon aging of bone marrow. We checked for defects in signalling networks implicated in HSPC aging. Analysis of 2 months old *asrij* CKO mice revealed that multiple signalling molecules including the CSN5-p53 and PI3K/Akt/mTOR axis were perturbed. The p53 pathway regulates DNA damage in response to stress. Reduction in p53 levels indicate reduced damage repair ability of *asrij* CKO bone marrow cells. Furthermore, we observe increased activation of Akt-STAT5 indicative of increased myeloid differentiation. Finally, we also observe increased activation of the PI3K/Akt/mTOR axis.

The implication of activated mTOR is well known in aging of the hematopoietic system. Defects in signalling networks highlight importance of Asrij in regulating various signalling molecules in the hematopoietic system. Both p53 and mTOR regulate each other via their downstream effectors (e.g. Tsc-1, Mdm2) in response to various stressors (DNA damage, UV response), thereby regulating important cellular processes like apoptosis, autophagy and senescence.

Impairment of the p53 axis as reported from studies on both the global and conditional KO of *asrij* further indicate that activation of mTOR might be under control of p53. Also, the increased degradation of p53 could possibly be mediated by Akt mediated MDM2 (mouse double minute 2) up-regulation. *In-vitro* analysis of interactions between Asrij and signalling molecules would help understand how the activation of multiple kinases in a signalling cascade is regulated by Asrij.

5.4 *Asrij knockout mouse as a model for HSPC aging*

Hematopoietic aging is a complex phenomenon regulated by signal transduction and interactions between the bone marrow hematopoietic cells and their niche. Understanding molecular mechanisms and signalling pathways involved in aging using *asrij* CKO help us understand how Asrij maintains HSPC frequency by regulating signalling networks in the bone marrow and how its absence gives rise to aging hematopoiesis. This study also provides characterization of a mammalian model which can be used for drug screening for leukemia. To further understand the process of hematopoietic aging, regulation of mitochondrial metabolism and quiescence via Asrij mediated mTOR signalling would help explain how the interplay between multiple signalling networks help in maintenance of HSC stemness. Also, analysis of the stem cell enriched pool of bone marrow would further confirm molecular perturbations as observed in the mutant bone marrow. The findings of this report help in elucidating role of Asrij in aging of blood stem cells and its effects on development of hematological disorders, making *asrij* CKO an ideal model to study aging of blood system.

5.5 *Conclusions*

The process of hematopoiesis is maintained by signalling networks operating inside the hematopoietic cells and niche factors secreted by the BM microenvironment. Cellular aging leads to changes in the bone marrow composition including alterations in molecular circuitry of HSPCs. We report that defects in the signalling networks important for cell fate regulation can lead to aging of bone marrow prematurely. The *asrij* CKO mice exhibit premature aging

phenotypes as observed from cellular and molecular analysis of the mutant bone marrow. Our findings suggest a role for Asrij in regulating p53 degradation and mTOR activation in bone marrow as depletion of Asrij leads to perturbations in these pathways. Role of Asrij in maintaining bone marrow niche upon aging warrants further investigation. This study also supports the previously reported importance of intrinsic factors in bone marrow aging. Further investigation of how phosphorylation events are regulated inside the cell would help us understand molecular dynamics of HSPC aging.

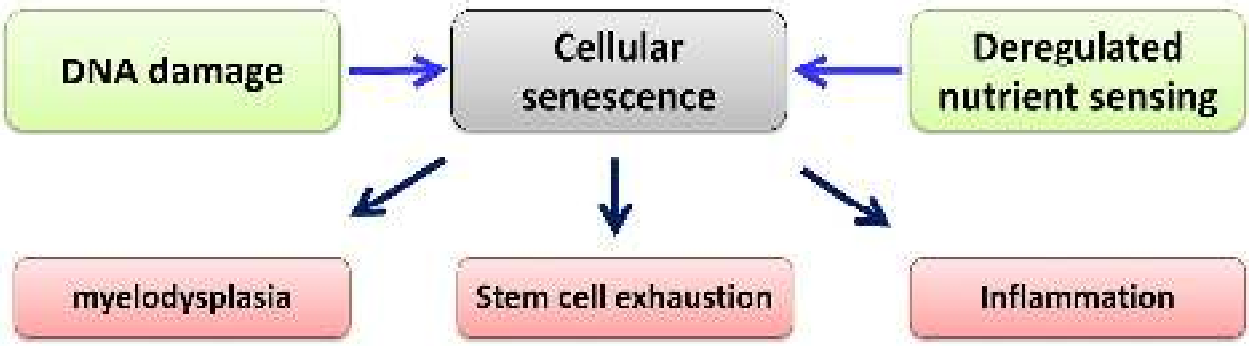

Figure 5.1: Age-associated hematopoietic defects observed in the conditional knockouts of *asrij*. Depletion of Asrij in the bone marrow leads to cellular senescence due to accumulation of DNA damage and deregulation of nutrient sensing pathways. Premature aging is further characterised by stem cell exhaustion, inflammation and myelodysplasia.

Hematopoietic aging is associated with changes in the stem cell properties. Understanding how signalling networks regulate HSPCs aging would help recognize key regulators of stem cell fate specification and differentiation. Furthermore, the activation of PI3K/Akt axis is linked to MDM2 stabilization and is implicated in AML (acute myeloid leukemia) with non-mutational p53 inactivation (Prokocimer et al., 2017) [66]. Making use of mouse knockouts helps us understand these signalling axes and serves as tool to design therapeutic drugs against aging associated myeloproliferative diseases.

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