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Abstract

Aging and autophagy in hematopoietic stem cells

Theodore Terence Ho

Aging is the greatest risk factor for a wide range of diseases, including cardiovascular diseases, cancers, neurodegenerative disorders, and diabetes. Yet why this is happening mechanistically remains largely unknown. Stem cells are responsible for the lifelong generation, maintenance, and repair of tissue and organ systems, and are therefore are both useful models to study cellular aging and attractive candidates for causing tissue and organismal aging. While stem cell aging has not been definitively linked to organismal aging, a decline in tissue function with age often correlates with a decline in stem cell function. Aging is also often accompanied by a decline in stem cell function, often correlating with degenerative diseases. In particular, during aging, hematopoietic stem cells (HSCs) lose their ability to regenerate the blood system, and promote disease development. Autophagy is critical for protecting HSCs from metabolic stress and has long been associated with health and longevity. Here, we show that loss of autophagy in HSCs causes accumulation of mitochondria and an activated metabolic state, which drives accelerated myeloid differentiation mainly through epigenetic deregulations, and impairs HSC self-renewal activity and regenerative potential. Strikingly, the majority of HSCs in aged mice share these altered metabolic and functional features. However, ~ 1/3 of aged HSCs exhibit high autophagy levels and maintain a low metabolic state with robust long-term regeneration potential similar to healthy young HSCs. Our results demonstrate that autophagy actively suppresses HSC metabolism by clearing active, healthy mitochondria to maintain quiescence and stemness, and becomes increasingly necessary with age to preserve the regenerative capacity of old HSCs.

Contributions to Presented Work

Chapter 2 of this dissertation contains previously published material:

> Chandel, Navdeep S., et al. "Metabolic regulation of stem cell function in tissue homeostasis and organismal ageing." *Nature cell biology* 18.8 (2016): 823-832.

I helped write sections of this review under the guidance of Emmanuelle Passegué (PhD).

Chapter 4 of this dissertation contains previously published material:

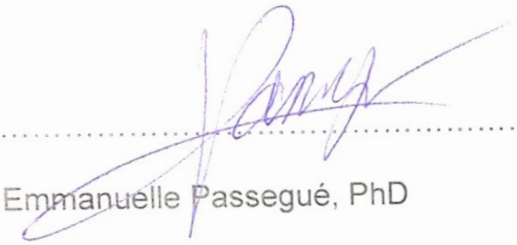
> Ho, Theodore T., et al. "Autophagy maintains the metabolism and function of young and old (hematopoietic) stem cells." *Nature* 543.7644 (2017):, 205.

I performed all of the experiments with help from Matthew Warr (PhD) for the initial Atg12cKO mice analyses, Emmalee.Adelman and Maria Figueroa (PhD) for DNA methylation studies, Olivia Lansinger and Evgenia.Verovskaya (PhD) for technical assistance, and Johanna Flach (PhD) for O-propargyl-puromycin experiments. I designed the experiments and interpreted the results with E.P., and with M.W. for Atg12cKO mice analyses. I wrote the manuscript with E.P.

Chapter 7 of this dissertation contains unpublished material that is currently in preparation.

Statement from research advisor

I directed and supervised the work presented in this dissertation. These publications and manuscript form a body of knowledge that makes original contributions to scientific knowledge. As such, this dissertation meets the standard for a doctoral dissertation at the University of California, San Francisco.



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Emmanuelle Passegué, PhD

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CHAPTER 1

General introduction

Aging

Aging is the greatest risk factor for a wide range of diseases, including cardiovascular diseases, cancers, neurodegenerative disorders, and diabetes (Niccoli and Partridge, 2012). Yet why this is mechanistically remains largely unknown. The heterogeneity of phenotypical aging due to different genetics, environments, and unknown factors, in addition to the requirements for long—often life-long—studies and large sample sizes make aging research particularly difficult, expensive, and lengthy. Furthermore, experiments, research, and discoveries originally made in model organisms such as *C. Elegans* worms, *Drosophila* flies, and mice are often not possible to repeat, let alone to reproduce, in higher animals such as non-human primates and humans. And due to the lack of an actual "aging" disease designation by the FDA for possible drug approval, public and private funding, clinical advancement, and commercialization of aging research has lagged far behind the more acute and devastating, but aging-associated, disease areas such as cancers and neurodegenerative diseases. Finally, aging itself is often viewed as an inevitable, acceptable process and not a health concern or disease to be studied and treated. Therefore because of these challenges, the field of aging research has often gone overlooked and underserved.

However, understanding the biology of aging holds immense promise to make great insights into disease origin, mechanism, and development of the innumerable age-related diseases. The potential impact on such a wide variety of diseases and on human health in general is incalculable. Furthermore, the possibility to begin to address and to treat diseases before their development and onset would fundamentally alter the idea of preventative medicine and the current approaches to health and disease management and could extend human healthspan (the years of healthy life) and possibly even lifespan.

Over the past few decades many advances have been made in identifying hallmarks of aging and cellular dysfunction (Lopez-Otin et al., 2013). However, the actual molecular causes of cellular aging are still hotly debated and under study. Many theories on the evolutionary development and the origin of biologically conserved aging, as well as its mechanisms, have been proposed over the years. In particular, metabolic rate, reactive oxygen species (ROS), DNA damage, telomere attrition, cellular senescence, inflammation, proteostasis, and stem cell exhaustion appear to be particularly intriguing, but clearly widely diverse, potential contributors to molecular, cellular, and organismal aging. Many of these insights have been made in genetic studies in model organisms such as yeast, flies, and worms to identify key genes important for organismal lifespan. Yet, in-depth studies on the biology of aging in individual cells, on its effects at the higher order of whole tissues, and in higher mammalian organisms are still lacking.

Stem cells

The idea that stem cells may play a crucial role in regulating tissue and organismal aging is appealing, as stem cells are responsible for the lifelong generation, maintenance, and repair of tissue and organ systems, and are therefore are both useful models to study cellular aging and attractive candidates for causing tissue and organismal aging. While stem cell aging has not been definitively linked to organismal aging, a decline in tissue function with age often correlates with a decline in stem cell activity (Lopez-Otin et al., 2013). Furthermore, stem cell function as well as numbers often decline during aging (Rando, 2006). Stem cells can be valuable models for studying cellular aging, as they are some of the only cell types that persist and function over the majority of an organism's lifetime, therefore able to accumulate features of cellular aging and to

be studied over time. Yet surprisingly, a recent study showed that muscle stem cells (MuSCs) were not actually essential for muscle maintenance during aging (Keefe et al., 2015), indicating that MuSCs at least may not be required for organismal health and longevity under steady state conditions.

Different adult tissue-specific stem cell populations vary widely in characteristics, and MuSCs are largely inactive and serve as emergency reserves for a mostly post-mitotic tissue, as opposed to more proliferative stem cell compartments that are essential to perform active tissue maintenance activities. However, MuSCs are still essential during injury, and old MuSCs have far less regenerative capacity than young, and muscle mass and health also decline with age (Gopinath et al., 2008). Many other stem cell compartments such as neural, skin, intestinal, and hematopoietic stem cells similarly decline in function with age, and are often depleted in number resulting in poor tissue repair and regeneration (Liu and Rando, 2011). Therefore while the link between stem cells and organismal lifespan remains unclear, decline of stem cell and tissue function are common features of aging.

Hematopoietic stem cells

In contrast to MuSCs, hematopoietic stem cells (HSCs) are absolutely essential for the highly proliferative blood system, and organismal health, throughout life. Maintenance of blood production is critical for homeostasis, and defective or impaired blood production is associated with adverse blood and health conditions. Proper blood maintenance depends on the unique ability of rare multipotent HSCs to self-renew and differentiate into all types of mature blood cells (Orkin and Zon, 2008). Thus HSCs are central to the regulated production of blood cells

throughout the lifetime of the organism, and also serve as a critical reservoir to repopulate the blood system in stress and regenerative conditions. HSCs are a particularly tractable system to study stem cells, as they are amenable to powerful techniques such as prospective isolation by fluorescence-activated cell sorting (FACS) and *in vivo* functional testing by transplantation.

HSCs self-renew for life, thereby making them one of the few blood cells that truly age. With age, the HSC pool becomes increasingly heterogeneous and loses its regenerative abilities, leading to a decline in immune responses, and increased rates of hematopoietic cancers, anemia, autoimmune diseases and inflammation (Rossi et al., 2008). Surprisingly, HSCs numerically expand with age, but paradoxically the functional activity of old HSCs on a per cell basis declines over time, leading to impaired engraftment and decreased blood regeneration capacity following transplantation (Rossi et al., 2008). Increasing evidence points to age-associated inflammation as a primary driver of aging of many organ systems (Franceschi and Campisi, 2014). Furthermore, blood and its contained factors are critical for both promoting aging and rejuvenating many aged tissues (Conboy et al., 2015). However, while many studies have thoroughly characterized the aging features of old HSCs, few definitive cellular, molecular, or genomic causes of HSC aging have been found (Chandel et al., 2016).

Metabolism

Metabolism and metabolic rate have long been linked to the rate of aging. Interestingly, smaller species with faster metabolisms have much shorter lifespans than larger species with slower metabolisms, and yet smaller individuals within a species often live longer than larger individuals (Speakman, 2005). Furthermore, growth factor signaling in particular has been found

to be crucial in the regulation of aging and lifespan, as loss of function mutations in growth factor signaling often lead to smaller, but longer-lived mutants in model organisms (Barbieri et al., 2003). Furthermore, interventions that decrease metabolic rate and/or growth factor signaling such as calorie restriction (CR) and rapamycin treatment have long been identified as robust lifespan extending conditions (Fontana et al., 2010). Furthermore, more recent studies in various organisms have revealed a central role for metabolic pathways in regulating stem cell function, and overactive growth factor signaling can lead to stem cell exhaustion and depletion (Chandel et al., 2016). Therefore, understanding the metabolic control of stem cell function and how it changes during aging is important to understand stem cell aging in general.

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CHAPTER 2

Energy metabolism and regulation of stem cell function in tissue homeostasis and organismal aging

Metabolic regulation of stem cell function in tissue homeostasis and organismal ageing

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Abstract

Many tissues and organ systems in metazoans have an intrinsic capacity to regenerate, which is driven and maintained largely by tissue-resident somatic stem cell populations. Ageing is accompanied by a deregulation of stem cell function and a decline in regenerative capacity, often resulting in degenerative diseases. The identification of strategies to maintain stem cell function and regulation is therefore a promising avenue to allay a wide range of age-related diseases. Studies in various organisms have revealed a central role for metabolic pathways in the regulation of stem cell function. Ageing is associated with extensive metabolic changes, and interventions that influence the cellular metabolism have long been recognized as robust lifespan-extending conditions. In this Review, we discuss recent advances in our understanding

of the metabolic control of stem cell function, and how stem cell metabolism relates to homeostasis and ageing.

Introduction

Metabolism is the sum of two processes known as anabolism and catabolism[1]. Anabolic pathways require energy from either adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide phosphate (NADPH) to generate macromolecules such as lipids and nucleotides. Catabolic pathways break down molecules to produce the energy required for anabolism. Stem cells engage in anabolic and catabolic pathways to varying degrees, depending on their functional state and the nature of the cell divisions they undergo[2]. Asymmetric divisions, which generate one stem cell and one differentiated cell per division, are necessary to maintain the stem cell pool and to provide differentiated progeny during homeostasis. Stem cells can also divide symmetrically and give rise to two identical progeny cells, to expand either the stem cell pool, as during embryonic development, or the progenitor pool in response to injury. Historically, metabolism was believed to primarily sustain the basic needs of stem cells and has therefore long been relegated as a consequence of a given functional state, as signalling pathways often activate the appropriate metabolic pathways[3]. However, evidence now suggests that metabolism can also dictate the functional states of stem cells, thereby directly controlling stem cell fate[3-5]. In this Review, we discuss metabolic adaptation to changing cellular states, the influence of nutrient-sensing pathways and redox states on stem cell metabolism, and conclude with assessing the role

of metabolism in stem cell ageing. We also focus on human pluripotent stem cells (PSCs), mouse haematopoietic stem cells (HSCs) and *Drosophila* intestinal stem cells (ISCs), for which the majority of data has been generated so far. We acknowledge that this strategy neglects the diversity of many other stem cell types, especially since HSCs and ISCs are among the most regenerative somatic stem cell populations and reside in highly specialized microenvironments.

Metabolic control of stem cell function

The following section provides insights into the metabolic regulation of pluripotency and quiescence in different types of stem cells (**Figure 1**), and how these metabolic strategies adapt stem cell function to the needs of the organism

Metabolism and pluripotency

PSCs have the ability to self-renew and differentiate into any tissue derived from the three germ layers of the developing organism[6]. PSCs are derived and maintained *in vitro* as self-renewing populations of embryonic stem cells (ESC) or induced PSCs (iPSCs), and can be directed to differentiate towards specific lineages through manipulation of the culture conditions. Previous studies have implicated cell metabolism as a key regulator of both PSC pluripotency and differentiation[6,7]. PSCs use glycolysis to generate ATP and demonstrate elevated levels of hexokinase II and uncoupling protein 2 (UCP2), as well as decreased activity of pyruvate dehydrogenase, which converts pyruvate into mitochondrial acetyl coenzyme A (acetyl-CoA)[8]. By contrast, during differentiation, ATP production shifts to the mitochondria and oxidative phosphorylation (OXPHOS)[9-11]. High expression of UCP2 in undifferentiated human PSCs

shunts pyruvate away from the mitochondria, thereby promoting glycolysis and maintaining pluripotency[12], although the benefit of a focus on glycolysis for maintaining pluripotency in proliferating PSCs is not yet fully understood. By contrast, the decrease in UCP2 protein levels and resulting increase in mitochondrial respiration is directly associated with differentiation[12], as human PSC differentiation can be actively suppressed following ectopic expression of UCP2. For these reasons, stimulation or inhibition of glycolysis directly influences PSC fate either through promotion or prevention of pluripotency (**Figure 1A**). Despite the reliance on glycolysis, a functional mitochondrial electron transport chain (ETC) is essential for maintaining pluripotency in PSCs[13], and a disruption of mitochondrial dynamics can directly impact on reprogramming efficiency[14-17]. The underlying mechanism by which mitochondrial respiration controls pluripotency remains elusive, especially as the bulk of ATP in PSCs is provided by glycolysis. Respiration might be required for the regeneration of NAD+ and flavin adenine dinucleotide to maintain tricarboxylic acid (TCA) cycle activity. Mitochondrial TCA cycle metabolites such as citrate and oxaloacetate are used for the *de novo* synthesis of lipids and nucleotides, respectively[1]. Acetyl-CoA and α-ketoglutarate (α-KG) are also required for the function of histone acetyltransferases (HATs) and Jumonji C domain histone demethylases (HDMs), respectively[18]. In addition, both murine ESCs and human PSCs utilize the mitochondrial threonine catabolism to synthesize S-adenosylmethionine[19,20], which is necessary to maintain their pluripotent state through the trimethylation of histone H3 lysine 4. For these reasons, the mitochondrial TCA cycle might directly regulate the epigenetic machinery to maintain pluripotency in proliferating PSCs, in addition to enabling appropriate biosynthesis.

Metabolism and quiescence

Under homeostatic conditions many tissue-resident somatic stem cells are quiescent, a state of growth cessation that limits unnecessary stem cell proliferation and allows for dynamic induction of tissue regeneration in adult organisms[21]. Extrinsic signals in the microenvironment activate quiescent stem cells to resume proliferation after stress or injury. However, over-activation often leads to stem cell exhaustion, loss of tissue function and eventually oncogenic transformations, features that are particularly accentuated during ageing[21-25].

Much of our understanding of the metabolic regulation of stem cell quiescence comes from studies on mouse HSCs. At any given time, over 95% of HSCs are quiescent with low division rates of about once a month for the most proliferative subset and once every 4-5 months for the most quiescent subset[26]. Many changes in the bone marrow niche, including increased production of growth-promoting or mobilizing cytokines, decreased levels of quiescence-enforcing factors, or direct transplantation procedures, stimulate HSCs to proliferate and differentiate to replenish the blood system. Similarly to PSCs, HSCs maintain a low mitochondrial metabolic activity and rely on glycolysis during quiescence, but depend on OXPHOS for activation and differentiation (**Figure 1B**)[27-30]. One key activator of glycolysis in quiescent HSCs is the transcription factor hypoxia-inducible factor 1 alpha (HIF-1α), which senses low oxygen levels of the bone marrow niche[27,31], and is broadly used to regulate stem cell fate in hypoxic conditions[32-34]. HIF-1α promotes glycolysis by increasing glycolytic genes such as lactate dehydrogenase A and pyruvate dehydrogenase kinase (PDK), thereby limiting mitochondrial respiration and elevating glycolytic flux by inhibiting pyruvate dehydrogenase and preventing the entry of pyruvate into the TCA cycle. In HSCs, HIF-1α promotes glycolysis largely by activating PDK2 and PDK4[28]. More differentiated blood cells exhibit reduced PDK2/4 levels and increased OXPHOS, ATP production, and elevated levels of reactive oxygen species

(ROS) produced as by-products of mitochondrial respiration. Importantly, loss of PDK2/4[28], deletion of HIF-1α[31] or its associated Cripto/GRP78 signalling molecule[35], and inactivation of the homeobox gene Meis1[36], which transcriptionally increases HIF-1α levels, all prevent the maintenance of HSC quiescence, promote differentiation and HSC exhaustion, mainly owing to increased ROS levels. Similarly, OXPHOS activation[29,30], a gain in mitochondrial mass[37], or increasing exposure to oxygen[38] also result in loss of quiescence, HSC exhaustion and impaired regenerative capacity due to enhanced differentiation. Conversely, OXPHOS reduction prevents differentiation and increases glycolytic flux, causing the accumulation of dysfunctional HSCs that have lost their regenerative capacity due to the inability to differentiate[28]. However, whether the decrease in mitochondrial respiration or other impaired mitochondrial functions are primarily responsible for the loss of HSC differentiation still remains unclear. In this context it is interesting to note that deletion of the 5' AMP-activated protein kinase in HSCs, which also alters mitochondrial activity, does not lead to any obvious differentiation defects[39]. By contrast, deletion of mitofusin 2, which directly disrupts mitochondrial fusion and tethering to the endoplasmic reticulum, impairs HSC lymphoid differentiation potential[40]. Hence, a common feature of the metabolic pathways that control HSC transition from quiescence to activation is their ability to influence the intracellular redox state. Besides HIF-1α, many other proteins that regulate quiescence also affect redox homeostasis and HSC function[4].

Mitochondrial fatty acid oxidation (FAO) plays an important part in HSC proliferation by regulating asymmetric versus symmetric differentiation[41]. Loss of the promyelocytic leukemia tumor suppressor gene or the peroxisome proliferator-activated receptor delta (PPARδ) gene, which are both required for beta-oxidation of fatty acids, results in reduced asymmetric divisions and increased symmetric commitment towards differentiated progeny, thus promoting HSC

exhaustion[41]. This effect can be reproduced by pharmacological inhibition of mitochondrial FAO using etomoxir. To date, the role of FAO in HSC asymmetric divisions has not been elucidated. Rapid proliferation might require mitochondrial FAO to replenish acetyl-CoA in support of the mitochondrial TCA cycle, as the pool of mitochondrial acetyl-CoA is diminished in resting HSCs due to increased PDK2/4 expression. As noted previously for PSCs, the maintenance of TCA cycle metabolites is probably required for epigenetic regulation of pluripotency, which might be important to preserve an undifferentiated state in the replenished daughter HSC. FAO might also aid in reducing ROS levels by increasing NADPH levels and for re-establishing quiescence in the replenished daughter HSC. Indirect support for the importance of mitochondria in mammalian asymmetric stem cell divisions comes from the intriguing observation that daughter cells that receive fewer aged mitochondria better maintain stem cell characteristics[42].

Metabolic adaptation of stem cell function

The regulation of stem cell function is often linked to the requirements of the tissue or organism, as in the *Drosophila* intestinal epithelium. ISCs constitute the vast majority of cells in the posterior midgut epithelium in adult *Drosophila* that are competent to undergo mitosis[43]. In young flies, ISCs are mostly quiescent, with only a low proliferation rate that allows replacement of the intestinal epithelium about once a week. In response to tissue damage or infection, ISCs become highly proliferative and undergo asymmetric cell divisions. ISCs can also undergo symmetric self-renewing divisions upon feeding, allowing the stem cell pool to be scaled according to the needs of the tissue during adaptive resizing, with insulin signalling dynamically controlling this switch from asymmetric to symmetric divisions[44]. The ability of ISCs to be activated and mount an acute, transient regenerative response is critical for fly health, and its

disruption results in metabolic defects and renders animals vulnerable to bacterial infections[43,45-50]. In ageing flies, ISCs hyperproliferate and initiate intestinal epithelial dysplasia that is associated with oxidative stress originating from age-related inflammatory processes in the gut, disruption of intestinal epithelial function, and a shortening of the lifespan[43,48-50]. Limiting ROS, increasing mitochondrial activity, modulating ETC efficiency, or reducing growth factor signalling in ISCs are all efficient strategies to promote ISC quiescence, and as a consequence limit age-related epithelial dysplasia and extend the Drosophila lifespan[43,49-52].

Collectively, these observations stress the conserved importance of metabolic regulation in the control of stem cell functions across different tissues, conditions and organisms. However, it should be noted that substantial differences exist between stem cell populations. For instance, while a gain in mitochondrial mass increases proliferative activity in mouse HSCs[37], it promotes quiescence in fly ISCs[50]. Furthermore, mesenchymal stem cells resemble HSCs in that they depend on glycolysis for multipotency and switch to OXPHOS during differentiation[53], whereas muscle stem cells (MuSCs) activate glycolysis and downregulate FAO during activation and differentiation[54,55]. By contrast, neural stem and progenitors cells seem to rely on lipogenesis for proliferation[56]. Whether these differences can be attributed to intrinsic variations within stem cells, or are consequences of different microenvironments or experimental paradigms remains to be established. The diversity among various somatic stem cell populations will need to be fully integrated to further distil the general principles of metabolic control of stem cell function.

Nutrient-sensing pathways in fate control

The changes in cellular metabolism observed during stem cell activation occur within a larger context of activating signals that include growth factors and inflammatory cytokines[1]. The response of stem cells to these signals is modulated by the dietary condition of the animal, presumably allowing stem cells to adjust their metabolic needs to the energetic reality of the tissue. Interestingly, two of the most conserved and effective methods of health and lifespan extension, calorie restriction and rapamycin (sirolimus) treatment[57,58], exert their health benefits through a direct effect on the cellular metabolism and likely by improving stem cell function. The effects of calorie restriction and rapamycin converge on the modulation of the mechanistic target of rapamycin (mTOR), which is a master sensor that integrates cues from nutrients and growth factors to regulate many processes, such as protein translation, mitochondrial biogenesis and autophagy[59]. Generally, mTOR is activated by the phosphatidylinositol 3-kinase (PI3K) signalling cascade and downstream activation of the serine/threonine protein kinase B (AKT), and inhibited by the tumour suppressor phosphatase and tensin homolog (PTEN), which negatively regulates the PI3K pathway. AKT phosphorylates and inactivates the tuberous sclerosis proteins 1 and 2 (TSC1/2) complex, a negative regulator of mTOR activity[60]. The TSC1/2 complex is regulated by a wide range of growth factors and stress signals, including the insulin/IGF signalling (IIS) pathway, and represents a critical node in signalling networks that arbitrate stem cell proliferation in response to nutritional conditions[60]. In fact, increasing TSC1/2 expression or suppressing mTOR activity is sufficient to extend the lifespan in both flies and mice[61,62].

In flies, the IIS and mTOR pathways directly regulate stem cell proliferation and maintenance in the intestine. For instance, mTOR signalling serves as a critical determinant of cell identity within the ISC lineage, and regulates ISC differentiation in a nutrient-dependent

manner[63]. Correspondingly, repression of mTOR signalling in ISCs renders them refractory to diet-induced differentiation. ISCs typically contain high levels of TSC2, which contribute to their resistance to dietary changes and promote long-term maintenance[63-66]. In animals exposed to high calorie diets, TSC2-deficient ISCs show increased differentiation resulting in the accelerated loss of ISC lineages. These observations point to interesting avenues for intervention, as ISC maintenance might be improved in conditions of chronically low IIS and TOR activity. Such an effect of TOR signalling on stem cell maintenance is consistent with the lifespan extension observed after suppression of TOR activity, through rapamycin or genetic means[61-63,65]. Interestingly, studies in the mouse intestine have highlighted the complex role of nutrient signals in stem cell function and homeostasis. Under dietary restriction, mTOR activity is reduced in ISC-supporting cells, so-called Paneth cells, eliciting the secretion of factors that promote ISC maintenance and proliferation[67]. A high-fat diet, however, promotes stemness and increases tumour formation in the mouse ISC lineage by stimulating the PPARδ gene[68].

The concept that suppression of mTOR activity by genetic, environmental or pharmacological treatments promotes stem cell quiescence, whereas activation of mTOR stimulates stem cell proliferation and differentiation, is gaining acceptance[69-72]. In human ESCs, activation of ribosomal S6 kinase downstream of mTOR directly triggers differentiation[71]. In MuSCs, mTOR activation after injury promotes the transition from a quiescent state to a newly defined ‘alert’ state, which stimulates a size increase and prepares MuSCs for the engagement of a proliferation program[73]. These mTOR-induced differentiation effects could be mediated by mitochondrial changes, as mTOR promotes mitochondrial biogenesis and OXPHOS in MuSCs, HSCs, and other stem cell populations[9,74]. Interestingly, differentiation is often accompanied by an increase in mitochondrial biogenesis, oxygen consumption, and ROS levels, with

mitochondrial fusion being required for stem cell differentiation into cardiomyocytes[10,75]. By contrast, mTOR over-activation increases mitochondria mass in HSCs and results in phenotypes nearly identical to overactive OXPHOS with loss of HSC function[72,76,77]. Similarly, loss of the *Dlk1-Gtl2* locus and associated non-coding RNA, which suppresses many components of the PI3K-mTOR pathway, lead to deregulated mitochondrial biogenesis and metabolic deregulations associated with HSC engraftment defects[78]. Many of these defects can be ameliorated by mTOR inhibition with rapamycin or through treatment with the anti-oxidant and ROS scavenger N-acetyl cysteine. Conversely, AKT1/2-deficient HSCs exhibit decreased ROS levels and are arrested in quiescence, unable to differentiate and regenerate the blood system, hence resembling HSCs with inhibited OXPHOS and overactive glycolysis[79]. Importantly, pharmacological ROS induction can rescue this differentiation block. The mechanisms by which suppression of mTOR activity promotes stem cell quiescence might largely revolve around mitochondrial regulation, although suppression of MYC activity has also been implied[80]. In addition, mTOR inhibits autophagy, which is the main proteostasis mechanism that can eliminate mitochondria through mitophagy[59]. During development, loss of autophagy in foetal HSCs resembles overactive OXPHOS, with mitochondrial accumulation, increased ROS production and loss of HSC function associated with HSC exhaustion[81]. In adult HSCs, loss of autophagy impairs HSC ability to survive metabolic stress[82], and it remains to be established whether mitochondrial biogenesis and metabolic regulation are also affected.

Altogether, these results indicate that diet and the mTOR pathway control stem cell fates and functions, possibly by regulating mitochondrial metabolic rates and/or oxidative states. These studies illustrate the breadth of developmental strategies used by various stem cell

populations to keep mitochondrial biogenesis and metabolic regulation in check, and to fine-tune stem cell function at the organismal level in response to nutrient conditions.

Redox regulation and stem cell function

The importance of controlled redox state changes for the maintenance and regulation of stem cell function has now been validated in many model systems and tissues, and ROS is emerging as a key regulator of stem cell fate decisions. Increasing ROS levels are an essential trigger of differentiation in various quiescent stem cell populations, including MuSCs, ISCs and HSCs[79,83,84]. Correspondingly, loss of mitochondrial DNA (mtDNA) and the associated decrease in ROS production are sufficient to suppress basal epidermal stem cell differentiation and deregulate skin homeostasis[85]. Nonetheless, maintenance of self-renewal activity in spermatogonial stem cells, neuronal stem cells, tracheal stem cells and HSCs strictly requires limiting ROS levels[4,86-88]. A unifying concept might be that low levels of mitochondrial ROS are required for stem cell quiescence and self-renewal, whereas a 'physiological' increase in ROS production is necessary for normal stem cell proliferation and differentiation. By contrast, aberrantly high ROS levels can cause DNA damage, promote stem cell exhaustion and tissue deterioration, and accelerate stem cell ageing[25,89]. The following sections discuss ROS regulation of stem cell function based on findings from studies in mouse HSCs and fly ISCs (**Figure 2**).

Redox regulation of HSCs

Increasing ROS levels induce differentiation of *Drosophila* haematopoietic progenitors[90]. This effect is retarded by the expression of antioxidant scavenger proteins, which suggests a role

for developmentally regulated ROS levels in priming progenitor cells for differentiation[90]. Increased ROS levels are also associated with proliferation and differentiation of mouse HSCs, primarily via modulation of p38 mitogen-activated protein kinase (MAPK) and the forkhead box O (FOXO) family of transcription factors[91,92]. ROS might also be essential for the mobilization, homing and lodging of HSCs in the bone marrow microenvironment[93,94]. HSCs with the lowest ROS levels have the highest self-renewal potential and repeated HSC serial transplantations, which increase ROS levels, are associated with a loss of regenerative capacity[27,95]. Similarly, many of the mutations that lead to aberrantly high ROS levels in HSCs also result in an impairment of quiescence and decreased HSC regenerative potential owing to enhanced differentiation at the expense of self-renewal[4]. The bone marrow niche, in turn, is essential for limiting ROS levels in quiescent HSCs, as it maintain low oxygen levels[27], secretes molecules that stimulate anti-oxidant pathways in HSCs[96,98] and favor the transfer ROS molecules from HSCs to stromal cells[99]. Precise localization within specific niches might be particularly important, as HSCs found near permeable sinusoidal blood vessels with higher exposure to blood demonstrate high ROS levels and are more migratory and less functional than HSCs with low ROS levels near less-permeable arterioles[101]. In addition, both human and mouse HSCs harvested under high O_2 conditions, as opposed to hypoxic conditions, exhibit oxygen-mediated stress, higher ROS levels, enhanced differentiation and impaired regenerative potential[38]. Antioxidant defence mechanisms including the sirtuin (SIRT) family of proteins, some of which promote mitochondrial biogenesis and metabolism by deacetylation of mitochondrial proteins, also influence HSC fate through the regulation of ROS levels. Loss of SIRT1 in young HSCs and SIRT3 in ageing HSCs causes increased differentiation and a loss of regenerative potential, similar to the HSC phenotypes elicited by elevated OXPHOS[102-106]. Furthermore, overexpression

of SIRT3, which normally declines with age, can reduce ROS levels and improve the functionality of HSCs from old mice[106]. SIRT1 activates FOXO3 in HSCs, whereas AKT signalling and the microRNA-212/132 cluster (Mirc19) inactivate FOXO3[92,102-106]. Loss of FOXO3, or the entire FOXO family, increases ROS levels and causes HSC exhaustion, both phenotypes that might be exacerbated with old age[92,107,108]. In HSCs, FOXO3 promotes autophagy, which is necessary for the survival of HSCs from old mice, perhaps as an adaptive response to constitutive metabolic stress from an ageing bone marrow niche[82]. For this reason, FOXO3-mediated autophagy and/or antioxidant effects during ageing could be important for both the regulation of ROS levels through mitophagy, and the supply of amino acids to maintain homeostasis.

Redox regulation of ISCs

Further evidence supporting the role of ROS in stem cell proliferation comes from the observation that the cap'n'collar CncC gene, the *Drosophila* homologue of the transcription factor nuclear factor-like 2 (NRF2), controls the proliferative activity of fly ISCs, hence safeguarding intestinal homeostasis[51]. In flies and vertebrates, NRF2/CncC regulates genes encoding key antioxidant enzymes and is negatively regulated by the cytoplasmic repressor Kelch-like ECH-associated protein 1 (KEAP1)[109]. Accordingly, loss of KEAP1 results in a gain of NRF2/CncC function, and a reduction in KEAP1 activity extends the lifespan and increases tolerance for oxidative stress in flies[110]. NRF2/CncC is constitutively active in ISCs, and repression of NRF2/CncC by KEAP1 is required for ISC proliferation[51]. As a result, NRF2/CncC-deficiency in ISCs elicits accumulation of ROS and excessive ISC proliferation. These findings, in combination with other signaling studies[47], suggest the presence of a network

of redox sensors and regulators that collectively maintain redox homeostasis and proliferative activity in ISCs.

Metabolism and stem cell ageing

The finding that a reduction in tissue function correlates with a reduction in stem cell activity has been proposed as a hallmark of ageing[22-25]. Stem cell ageing and associated tissue dysfunction primarily seem to result from a failure to maintain appropriate mitochondrial and metabolic regulations. Over 50 years ago, the "free radical theory of ageing" postulated that mitochondrial ROS contribute to ageing by damaging proteins, lipids, and DNA[111]. The accumulation of mutations in mtDNA during ageing is an attractive idea given that mtDNA lacks protective histones and is located in close proximity to the mitochondrial ETC, a major ROS source[112]. Evidence in support of this notion comes from studies of mtDNA 'mutator' mice, which express a proofreading-deficient mtDNA polymerase[113,114] and exhibit a range of degenerative phenotypes, indicative of premature ageing, and self-renewal defects in both HSCs and neural stem cells[115-117]. Although haematological malignancies commonly associated with ageing are evident in mutator mice, the transcriptional profile of mutator HSCs show minimal similarity with ageing HSCs[118], suggesting that ageing might not directly incur severe intrinsic mitochondrial defects. However, deregulated mitochondrial activity is associated with ageing, as shown by decreased SIRT7 expression in ageing HSCs[37]. An emerging revised theory is that mitochondrial dysfunction and associated deregulations in mechanisms that maintain stem cell self-renewal instead are causal for stem cell ageing. In the following section, we focus on the link between epigenetic deregulations and HSC ageing (**Figure 3**), and discuss rejuvenation strategies in the context of metabolic restoration.

Metabolic control of epigenetic changes

Epigenetic modifications can be passed on from stem cells to their progeny and allow fate specification and the maintenance of cellular identity. Chromatin modifiers are known to directly regulate ageing and longevity[119], and metabolic regulations have been linked to epigenetic regulations[18,120,121]. Nutrient conditions and metabolic states can drive epigenetic modifications, as metabolic intermediates and by-products of the TCA cycle serve as cofactors and substrates for epigenetic enzymes and many nutrient signalling pathways regulate both metabolism and epigenetics. Strikingly, nutrient deficiencies during development can cause epigenetic changes maintained throughout adulthood and, at least in *C. elegans*, parents have been reported to pass on the resulting epigenetic marks through multiple generations of descendants[122]. Despite a current lack of studies that specifically evaluate the metabolism of stem cells isolated from aged organisms, it is logical that metabolic changes acquired during ageing induce epigenetic alterations, and vice versa, that alterations in the metabolism can potentially exacerbate, and/or reinforce ageing-associated epigenetic changes.

DNA and histone methylation often promote stem cell self-renewal, whereas demethylation results in stem cell activation and differentiation[123]. Hypoxia reduces α-KG and increases succinate, thus inhibiting HDMs[124] and preventing HIF-1α degradation[125]. Consequently, the hypoxic bone marrow niche might aid in the maintenance of HSC quiescence and self-renewal by preserving methylation marks. By contrast, in activated HSCs, OXPHOS might lead to elevated α-KG production and decreased succinate levels, thereby activating HDMs and enabling or even driving proliferation and differentiation. Augmenting HSC proliferation through serial replicative challenges triggers a stepwise decline in DNA methylation[126]. With age, the processing of both α-KG and succinate deteriorates, which could

result in either demethylation or hypermethylation[127], and could explain the variable deregulation of epigenetics observed in ageing HSCs[128,129]. Although a slight global hypermethylation has been reported, no direct correlations with gene expression alterations have been observed[128], and many of the epigenetically affected genes are not expressed by HSCs but rather by downstream progenitors, thus supporting the hypothesis that age-associated changes in methylation contributes to the skewed lineage differentiation of ageing HSCs[123]. They could also affect the expression of OXPHOS genes or mtDNA, although their influence on the metabolism of ageing HSCs has not yet been investigated. Importantly, mutations in epigenetic enzymes that provide competitive advantages to HSCs and promote clonal haematopoietic expansion, such as DNMT3a[130], are often associated with the development of leukaemia and also commonly observed in elderly individuals[123].

HATs require cytoplasmic acetyl-CoA, which is largely generated from pyruvate through glycolysis rather than FAO, whereas histone deacetylases (HDACs) such as NAD+-dependent Sirtuin family Class III HDACs are regulated by the NAD+/NADH ratio[131]. HATs and HDACs also directly influence metabolic genes, particularly in the insulin and glucose pathways, and acetylate or deacetylate proteins important for mitochondrial biogenesis and metabolic regulations such as FOXOs or HIF1α. Loss of HATs or HDACs perturbs many aspects of stem cell function, and HATs in particular seem necessary for stem cell self-renewal[123]. In quiescent glycolytic stem cells such as HSCs, a high acetyl-CoA/low NAD+ ratio may therefore maintain acetylation and self-renewal activity, whereas in activated OXPHOS-driven stem cells deacetylation may instead contribute to differentiation. With age, a decline in NAD+ levels is observed in somatic tissues[132], as is a change in stem cell histone acetylation, with downregulation of HDAC1/5/6 and SIRT3/7, but not SIRT2 expression, in ageing HSCs[37,104,128].

However, glucose uptake is decreased in ageing HSCs, which could reduce acetylation levels[83]. In particular, a reduction of H4K16ac is evident in ageing HSCs, which is linked to increased CDC42 Rho-GTPase activity and loss of cell polarity[133]. Obesity, high fat diets, and specific fatty acids reportedly also alter HDAC activity[127] and cause a HSC dysfunction that resembles ageing[134,135].

Rejuvenation strategies and metabolic corrections

Interventions that influence the cellular metabolism, such as rapamycin treatment and calorie restriction, have long been acknowledged to extend the lifespan of model organisms and mammals[57,58]. Both methods reduce the signalling activity of the mTOR and other growth factor pathways, induce autophagy, and are reported to improve stem cell function in various tissues including the blood system[74,136]. Short-term fasting conveys a similar effect through inhibition of protein kinase A, which improves haematopoiesis and reverses the myeloid bias of ageing HSCs[137]. Surprisingly, this effect was associated with accelerated HSC proliferation, but also increased HSC function in transplantation experiments, two phenotypes that are usually inversely correlated[138]. Long-term calorie restriction exerts varying effects on HSC function in middle-aged mice, increasing quiescence and improving HSC regenerative capacity, but also further exacerbating of the decrease in lymphoid cell production that is characteristic of ageing[139]. Calorie restriction directly induces SIRT and antioxidant defences against ROS, triggers FAO and improves OXPHOS efficiency by reducing oxidative damage[127,140,141]. As a consequence, while calorie restriction in mice increases mitochondria numbers and OXHPOS, it also activates SIRT1 and FOXO3 and directly improves MuSC numbers and muscle regeneration[142]. Although N-acetyl cysteine can successfully rescue certain HSC defects associated with deregulated ROS

levels, antioxidants treatments have so far shown little ability to extend the health or lifespan in model organisms[143]. These results suggest that mitochondrial regulation and mitigation of the redox status, rather than an adjustment of ROS levels or metabolic pathways, could be an effective strategy for stem cell rejuvenation approaches. It has also been speculated that the life-extending effects of rapamycin or calorie restriction act through induction of autophagy[59], thereby mitigating ROS levels by removing dysfunctional or damaged mitochondria. Such a mechanism would be of particular relevance in the ageing organism, as mitochondrial quality control declines with age[37]. Finally, the possibility that these interventions improve cellular health through a lasting reversal of age-associated epigenetic changes should also be taken into account[144,145].

Conclusions

Moving forward, many areas remain unexplored regarding metabolic influences on the maintenance of pluripotency and the regulation of the switch between quiescent and activated stem cell states. One question is the contribution of subsidiary pathways emanating from different glycolytic intermediates, such as the pentose phosphate pathway and one-carbon metabolism[1,146]. Glucose 6-phosphate can enter the pentose phosphate pathway to generate NADPH and ribose that drive anabolic reactions and nucleotide synthesis. In addition, the glycolytic intermediate 3-phosphoglycerate can initiate the one-carbon metabolism to ultimately generate NADPH and folate intermediates for redox balance and nucleotide synthesis. The one-carbon metabolism also produces the S-adenosylmethionine necessary for methylation reactions[147]. Future studies should investigate how these different metabolic pathways dictate or influence stem cell fate. Comprehensive analyses will have to delineate and integrate the redox

modulations and antioxidant pathways that jointly regulate ROS levels and protein targets required for stem cell renewal and differentiation. Further advancing the study of metabolic control of epigenetic regulation will be imperative, as these processes likely not only regulate stem cell function during homeostasis, but also stem cell dysfunction during ageing and stem cell rejuvenation upon life-extending interventions. A deeper understanding of the metabolic regulation of stem cells could identify new and effective strategies to preserve or even rejuvenate the regenerative capacity of many tissues, which would help combat ageing at the organismal level.

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Figures and Figure Legends

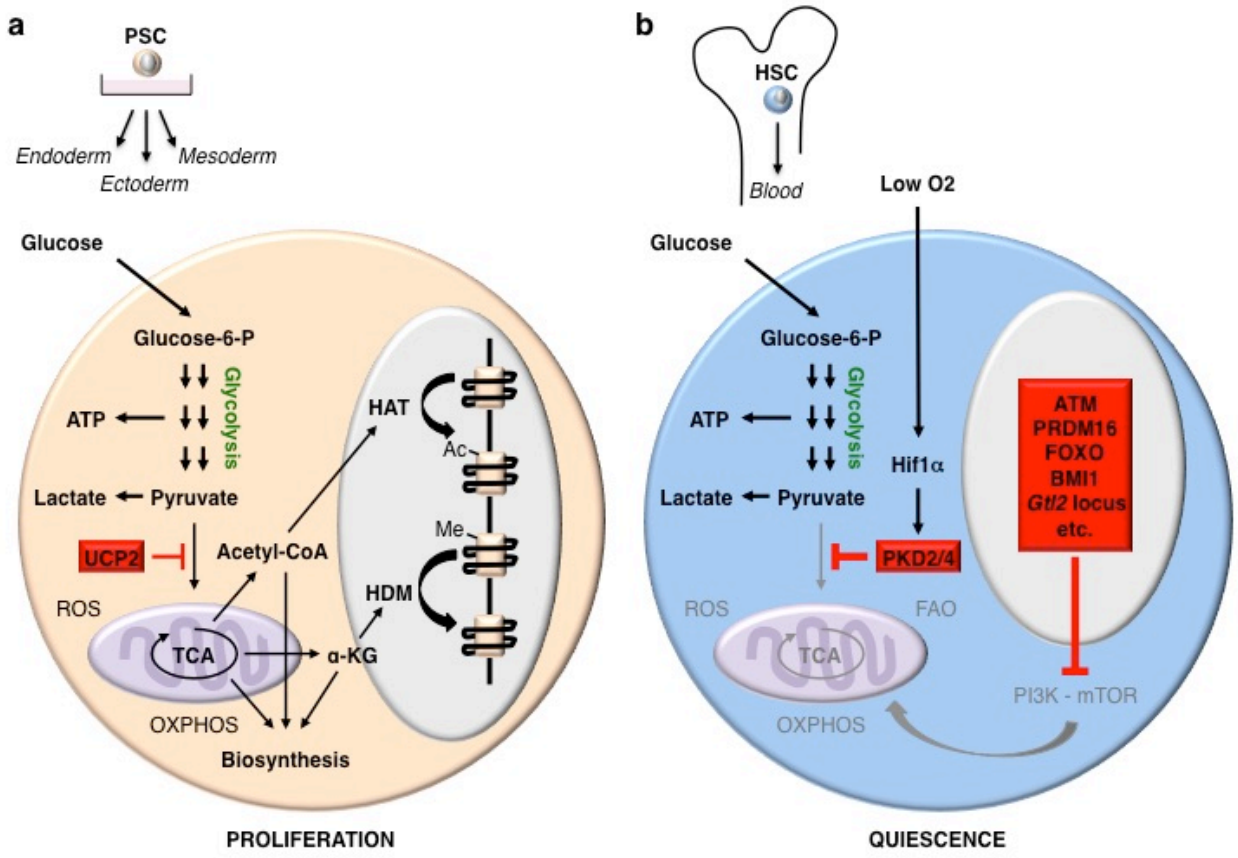

Figure 1| Metabolic regulation of stem cell pluripotency and quiescence.

(**a**) Human PSCs rely on high glycolytic flux to maintain pluripotency when proliferating. High levels of UCP2 in PSCs suppress the channelling of pyruvate into the mitochondrial TCA cycle, allowing its conversion to lactate and ATP production. However, mitochondrial activity is still important for pluripotency as mitochondrial TCA cycle metabolites such as acetyl-CoA and α-KG can also regulate the epigenetic machinery and the function of HATs and HDMs, respectively, in addition to their contribution to biosynthesis in proliferating PSCs.

(**b**) Mouse HSCs rely on high glycolytic flux to maintain their quiescence in the bone marrow niche. Low oxygen levels stimulate HIF-1α to activate PDK2/4, which suppress the channelling of pyruvate into the mitochondrial TCA cycle. In parallel, other important regulators of quiescence act in concert to repress the PI3K-mTOR pathway and mitochondria biogenesis, which altogether prevent OXPHOS, ROS production and FAO activation in quiescent HSCs.

Acetyl-CoA, acetyl coenzyme A; α-KG, α-ketoglutarate; ATP, adenosine triphosphate; BMI1, polycomb ring finger**;** FAO, fatty acid oxidation; FOXO, forkhead box O; HAT, histone acetyltransferases; HDM, histone demethylases; HIF-1α, hypoxia-inducible factor 1 alpha; HSC, haematopoietic stem cell; Me, methyl; mTOR, mechanistic target of rapamycin; O2, oxygen; OXPHOS, oxidative phosphorylation; PDK, pyruvate dehydrogenase kinase; PI3K, phosphatidylinositol 3-kinase; PRDM16, PR domain containing 16; PSC, pluripotent stem cell; ROS, reactive oxygen species; TCA, tricarboxylic acid; UCP2, uncoupling protein 2.

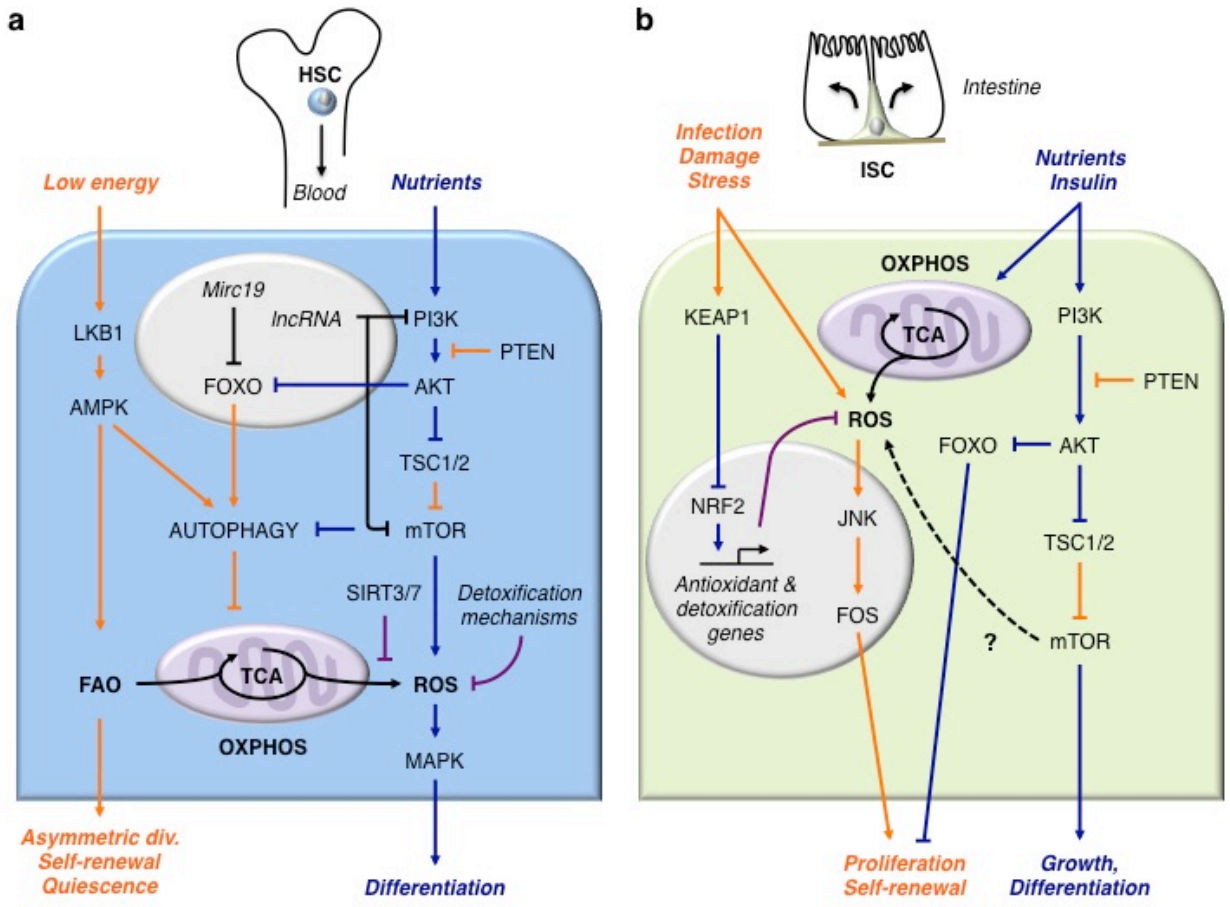

Figure 2| Redox regulation of stem cell function.

(**a**) In mouse HSCs, activation of the PI3K-mTOR pathway triggers differentiation by increasing OXPHOS and ROS production. By contrast, decreasing ROS levels through activation of detoxification mechanisms, repression of the PI3K-mTOR pathway and OXPHOS, and mitochondrial removal preserve HSC quiescence and allow self-renewal activity in part via activation of FAO.

(**b**) In Fly ISCs, self-renewal and proliferation require an increase in ROS production. NRF2/CncC is constitutively active in quiescent ISCs, but is inhibited in response to stress, allowing increased ROS levels to trigger proliferation by stimulating JNK/FOS signalling. TOR signalling is kept low in ISCs by high TSC2 expression levels. Activation of the PI3K-mTOR

pathway promotes differentiation into enterocytes, resulting in ISC loss. A role for mTOR-mediated ROS production in nutrient-induced proliferation of ISCs has not yet been described.

Yellow: low energy and stress pathways; blue: nutrients and insulin pathways; purple: detoxification pathways;

AMPK, AMP-activated protein kinase; AKT; serine/threonine protein kinase B; div.: division; FAO, fatty acid oxidation; FOS, FBJ murine osteosarcoma viral oncogene homolog; FOXO, forkhead box O; HSC, haematopoietic stem cell; ISC, intestinal stem cell; JNK, Jun-N-terminal Kinase; KEAP, Kelch-like ECH-associated protein 1; lncRNA: long non-coding RNA; MAPK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin; NRF2/CncC, nuclear factor-like 2/cap'n'collar; OXPHOS, oxidative phosphorylation; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; SIRT, sirtuin; TSC, tuberous sclerosis protein.

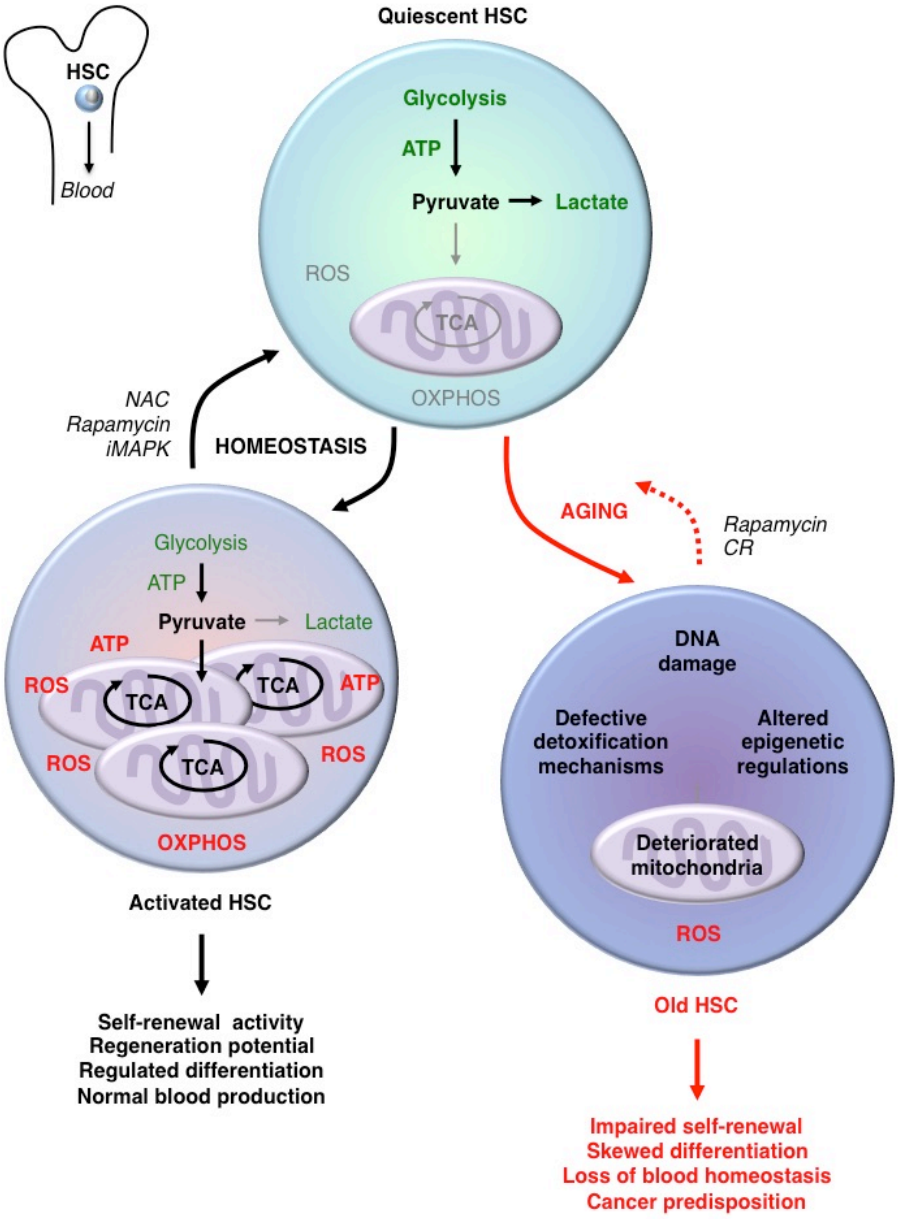

Figure 3| Mitochondrial dysfunction and stem cell ageing.

During homeostasis, HSCs alternate between a glycolytic quiescent state needed for self-renewal and blood regeneration, and an OXPHOS-driven activated state required for differentiation and blood production. Mutant HSCs with constitutive OXPHOS activation differentiate aberrantly and can be largely rescued by treatment with redox mitigators. This metabolic reversibility

preserves HSC function in a young organism. With age, HSCs display many faulty regulations, which are largely caused by deteriorated mitochondria activity, and contribute to the functional impairment of ageing HSCs and loss of blood homeostasis. Mutant HSCs with similar ageing phenotypes can be partially corrected by life-extension interventions that affect cellular metabolism.

ATP, adenosine triphosphate; CR, calorie restriction; HSC, haematopoietic stem cell; iMAPK, mitogen-activated protein kinase inhibitor; NAC, N-acetyl cysteine; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA, tricarboxylic acid.

CHAPTER 3

Autophagy background

Autophagy

Autophagy is a cellular recycling mechanism essential for cellular health (Cuervo, 2008). During autophagy, various cellular materials are targeted to double-membraned autophagosomes that subsequently fuse with lysosomes into autolysosomes, and are then degraded with nutrients released back into the cytoplasm to be recycled. Autophagy is important for maintaining homeostatic cellular health and integrity by regulating quality and number of various components such as organelles and proteins.

Autophagy also acts as a major stress response pathway after various types of stress, such as starvation or metabolic stress to recycle nutrients and small molecules to generate energy (Rubinsztein et al., 2011). Therefore autophagy is intrinsically linked to metabolic state and growth factor signaling, as starvation or inhibition of growth factor signaling activates autophagy to allow cells to survive. Autophagy is also responsible for some of the beneficial effects of hormesis, which is when low levels of stress induce stress responses that promote health effects (Rubinsztein et al., 2011).

Autophagy has also long been linked to organismal healthspan, lifespan and longevity in model organisms (Cuervo, 2008). It is necessary for health of many tissues, is induced by healthspan and lifespan extending treatments, and furthermore is necessary for longevity in lifespan-extending treatments and some long-lived genetic mutants (Rubinsztein, 2011). However, autophagy has long been widely reported to decline during aging in the few reported tissues such as rat muscle and liver, brain, and most recently even in muscle stem cells (Cuervo, 2008; Garcia-prat et al., 2016). However, most reports use whole tissue lysates, and/or use simple correlative readouts for autophagy such as gene expression or snapshots of levels of

single proteins that are extremely difficult to properly interpret without additional controls and conditions. Yet, while well known to be beneficial for cellular and organismal health and longevity, exactly how autophagy regulates cells, tissues, and organisms to keep them youthful and healthy during aging is still under study.

Autophagy in HSCs

Autophagy has been studied in the context of few stem cell compartments. In purported mesenchymal stem cells, various roles have been reported such as stress response and differentiation (Guan et al., 2013). In muscle stem cells, it was found to be important to generate extra energy before activation and proliferation (Tang et al., 2014), and most recently to be important to prevent muscle stem cell senescence during aging (Prat-diaz et al., 2016).

In the blood system, multiple studies have examined the role of autophagy in mature blood cells with varying results (Riffelmacher et al., 2017). Largely, autophagy appears important for lymphoid cells, but not myeloid cells. Autophagy was also found to be necessary for the early development of the blood system and fetal HSC maturation as shown by the perinatal lethality and deregulated fetal hematopoiesis observed in mice lacking the essential autophagy genes *Atg7* and *Fip200* (Mortensen et al., 2011; Liu et al., 2010). Additional studies have reported various roles of autophagy in HSCs, such as its necessity for HSC mobilization, degradation of Notch, DNA damage repair, cell cycle, ROS, and monocyte-macrophage differentiation (Leveque-El Mouttie et al., 2015; Cao et al., 2015a; Lin et al., 2015; Cao et al., 2015b; Cao et al., 2016; Cao et al., 2015c; Zhang et al., 2012).

Furthermore, our lab found that autophagy plays a critical role by protecting adult HSCs from metabolic stress (Warr et al., 2013). We used both genetic mouse models and pharmacological means to examine the autophagy response of HSCs following *ex vivo* cytokine withdrawal or *in vivo* starvation. We found that compared to more mature blood cells, HSCs are uniquely poised to mount a robust protective autophagy response to survive such stress that causes cell death in other more mature cell types (Warr et al., 2013). Moreover, we demonstrated that the longevity-associated transcription factor FOXO3A is essential to maintain a pro-autophagy gene program that poises HSCs for rapid autophagy induction (Warr et al., 2013). The *C. Elegans* homolog of FOXO3A was one of the first pro-longevity genes discovered, and FOXO3A is one of the few genes found to be enriched in human centenarians (Willcox et al., 2008). These results demonstrated that HSCs are uniquely wired to use autophagy as an adaptive stress response mechanism. However, the role of autophagy in steady state and regenerative adult HSC function and blood production, and how it changes with age and furthermore affects aging, remained to be established.

Autophagy regulation

Several regulators can trigger autophagy in response to metabolic stress (**Fig. 1**). Decreased nutrients, growth factors or cytokines can reduce receptor tyrosine kinase (RTK) signaling, inactivating the PI3K/AKT pathway, inhibiting the mammalian target of rapamycin (mTOR), and thereby inducing autophagy (He and Klionsky, 2009). mTOR Complex 1 (mTORC1) blocks

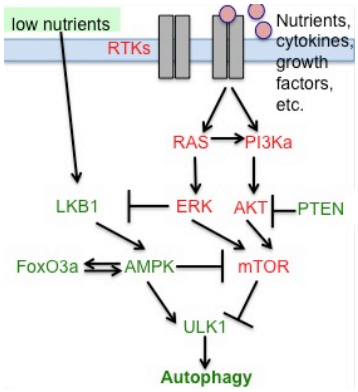

***Figure 1**. Autophagy regulation*

autophagy by inactivating Unc-51-like kinase 1 (ULK-1). 5' AMP-activated protein kinase (AMPK) can also sense metabolic stress and inhibit mTOR and directly activate ULK1. We and others have also shown that both catalytic subunits of AMPK (PRKAA1 and PRKAA2) are highly expressed in HSCs (Warr et al., 2013; Nakada et al., 2010), with *Prkaa2* being directly regulated by FOXO3a**.** This suggests that HSCs may be particularly poised to activate AMPK. Since many genes involved in growth and health such as insulin/IGF signaling, FOXO transcription factors, and AMPK converge on mTOR (Laplante and Sabatini, 2012), it is likely that the PI3K/AKT/mTOR pathway plays an important role in sensing metabolic stress and triggering autophagy in HSCs. Furthermore, mouse models deficient in *Foxo* family members (Tothova et al., 2007) or AMPK activator *Lkb1* (Nakada et al., 2010), or that display constitutive *Akt* (Kharas et al., 2010) or mTORC1 activation due to *Tsc1* (Chen et al., 2008; Gan et al., 2008) or *Pten* (Yilmaz et al., 2006) deletion, all result in HSC depletion and loss of function, which suggest that the functional defects of these mutant HSCs may be due to an inability to induce autophagy. Interestingly, these mouse models have similar phenotypes to those with over-active mitochondrial metabolism (Takubo et al., 2013). However, how HSCs sense metabolic stress and regulate autophagy is still undetermined.

Autophagy and aging

While HSCs isolated from young mice exhibit very low levels of autophagy under steady state conditions, in contrast we found evidence of high basal levels of autophagy in old HSCs isolated from aged mice (Warr et al., 2013). We found that old HSCs are less efficient at taking up nutrients, may be constitutively metabolically stressed, and therefore may rely on high basal

levels of autophagy for their continued survival. However, old HSCs are still able to respond further to acute metabolic stress by inducing an even greater level of autophagy upon cytokine withdrawal (Warr et al., 2013). These results indicate that autophagy not only preserves HSCs from starvation in a young organism, but also supports an old HSC compartment that faces unique metabolic challenges and may be required to maintain an aging, frail blood system. However, how autophagy is constitutively activated in old HSCs and whether it plays a beneficial effect by allowing a few healthy old HSCs to survive an adverse aging environment, or a detrimental effect by promoting the accumulation and expansion of dysfunctional old HSCs remained to be elucidated.

Interestingly, caloric restriction (CR), which induces autophagy, improves health, delays aging, and extends lifespan in many model organisms in an autophagy-dependent manner (Yang and Ming, 2012). Its mimetic drug rapamycin, which inhibits mTOR and induces autophagy, promotes health and inhibits age-related weight gain in mice (North and Sinclair, 2012). CR has also been reported to ameliorate HSC loss of function with age (Ertl et al., 2008), and rapamycin in old mice was reported to improve HSC self-renewal and function, and immunity (Chen et al., 2009). Therefore mTOR may be a crucial molecular intersection of metabolism, disease, aging, and HSC function in blood production, and the beneficial effects of CR and mTOR inhibition may work through induction of autophagy.

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CHAPTER 4

Autophagy maintains the metabolism and function of young and old stem cells

Introduction

We have previously found that FOX3A-driven autophagy is essential for hematopoietic stem cells (HSCs) to survive under metabolic starvation (Warr et al., 2013). Furthermore, we found evidence of increased activated basal autophagy in HSCs isolated from old mice, which was not present in HSCs from young mice. However, the role of autophagy in normal, steady-state adult hematopoiesis and HSC function was unknown, and furthermore the role and significance of altered autophagy activity during aging was unclear. Here, to inactivate autophagy in adult HSCs, we used our *Atg12*$^{flox/flox}$*:Mx1-Cre* conditional knockout (*Atg12*^{cKO}) mouse model to delete *Atg12* in the adult blood system. We utilized cellular and metabolic assays, electron microscopy, gene expression and DNA methylation analyses, and HSC transplantations to show that loss of autophagy in HSCs causes accumulation of mitochondria and an activated metabolic state, which drives accelerated myeloid differentiation mainly through epigenetic deregulations, and impairs HSC self-renewal activity and regenerative potential. We used *Gfp-Lc3* autophagy-reporter mice and other autophagy assays to show that strikingly, the majority of old HSCs in aged mice have nearly identical altered metabolic and functional features. However, ~ 1/3 of aged HSCs exhibit high autophagy levels and maintain a low metabolic state with robust long-term regeneration potential similar to healthy young HSCs. Our results demonstrate that autophagy actively suppresses HSC metabolism by clearing active, healthy mitochondria to maintain quiescence and stemness, and becomes increasingly necessary with age to preserve the regenerative capacity of old HSCs.

Autophagy maintains the metabolism and function of young and old stem cells

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With age, haematopoietic stem cells lose their ability to regenerate the blood system, and promote disease development. Autophagy is associated with health and longevity, and is critical for protecting haematopoietic stem cells from metabolic stress. Here we show that loss of autophagy in haematopoietic stem cells causes accumulation of mitochondria and an activated metabolic state, which drives accelerated myeloid differentiation mainly through epigenetic deregulations, and impairs haematopoietic stem-cell self-renewal activity and regenerative potential. Strikingly, most haematopoietic stem cells in aged mice share these altered metabolic and functional features. However, approximately one-third of aged haematopoietic stem cells exhibit high autophagy levels and maintain a low metabolic state with robust long-term regeneration potential similar to healthy young haematopoietic stem cells. Our results demonstrate that autophagy actively suppresses haematopoietic stem–cell metabolism by clearing active, healthy mitochondria to maintain quiescence and stemness, and becomes increasingly necessary with age to preserve the regenerative capacity of old haematopoietic stem cells.

Ageing is the greatest risk factor for many pathological conditions including cancers, neurodegenerative disorders, cardiovascular diseases, and diabetes[1]. Physiological ageing is a complex and multi-factorial process that is regulated by both genetic and environmental factors[2]. Although tissues across the body are seemingly affected in different ways, one emerging hallmark of ageing is that reduction in tissue function usually correlates with a reduction in stem cell activity[3]. The blood system is critical for many aspects of organismal health, and proper

maintenance of blood production relies on the ability of haematopoietic stem cells (HSCs) to self-renew and differentiate into all lineages of mature blood cells[4]. In adults, HSCs are rare and reside in specialized niches in the bone marrow cavity, where they are kept in a low metabolic, mainly glycolytic, quiescent state unless called upon to regenerate the blood system[5]. With age, HSCs lose their regenerative abilities, but their overall expansion maintains blood production in old organisms, albeit with typical features of blood ageing such as anaemia, immunosenescence, increased production of myeloid cells, and higher predisposition to haematological cancers[6]. Yet, how old HSCs (oHSCs) retain some functional abilities in an adverse ageing bone marrow microenvironment[7,8] remains largely unknown.

Macroautophagy (hereafter called autophagy) is an essential proteostasis and stress response mechanism that maintains cellular health by regulating the quantity and quality of organelles and macromolecules through lysosomal degradation, which is activated in response to nutrient deprivation and other stressors to generate energy and allow survival[9]. Autophagy is controlled by a series of autophagy-related genes (Atg) such as the ATG12 conjugation system (Atg12–Atg5–Atg16) that are essential for the formation of double-membrane autophagosomes. Autophagy is regulated by important nutrient-sensing pathways including the mechanistic target of rapamycin (mTOR) and 5′ AMP-activated protein kinase (AMPK), which inhibits and activates autophagy, respectively. Autophagy is critical for the proper development of the blood system[10,11], for HSC mobilization[12], and to allow adult HSCs to survive acute metabolic stress[13]. Autophagy declines with age in many tissues[14], and reduced autophagy levels in muscle stem cells directly contribute to decreased regenerative potential and muscle atrophy[15]. In contrast, we previously found increased basal autophagy in oHSCs[13]. Here, we set out to identify how autophagy controls HSC function, and how changes in autophagy levels affect HSC ageing.

Aging phenotypes upon loss of autophagy

To inactivate autophagy in adult HSCs, we used our previously described Atg12flox/flox:Mx1-Cre conditional knockout (Atg12cKO) mouse model[13], and deleted Atg12 with poly(I:C) (pIC) at 4 weeks of age (Fig. 1a). Surprisingly, the blood system of Atg12cKO mice remained largely healthy, with no persisting anaemia or lymphopenia observed over time as reported in other contexts[10,11,16] (Extended Data Fig. 1a). Atg12cKO mice showed increased cellularity in the peripheral blood and spleen, and, similar to autophagy inactivation in fetal HSCs[11], had a skewed ratio of circulating myeloid versus lymphoid cells, thus resembling the myeloid-bias observed in old mice (Fig. 1b and Extended Data Fig. 1b–f). In contrast, Atg12cKO mice maintained normal numbers of phenotypic HSCs (Lin−/c-Kit+/Sca-1+/Flk2−/CD48−/CD150+) over time, with expanded multipotent progenitor (MPP) and granulocyte/ macrophage progenitor (GMP) compartments contributing to the myeloid expansion (Extended Data Fig. 1g–k). These phenotypes were conserved in a distinct Atg5flox/flox:Mx1-Cre conditional knockout (Atg5cKO) model (Extended Data Fig. 2a–e), and demonstrated features of premature blood ageing in adult autophagy-deficient mice.

To investigate the regenerative capacity of Atg12cKO HSCs, we first performed classic transplantation experiments with purified HSCs to directly measure their self-renewal and multilineage reconstitution activity (Extended Data Fig. 2f). Transplantation of 250 Atg12cKO HSCs into lethally irradiated recipients led to significantly impaired engraftment, with reduced overall chimaerism, myeloid-biased lineage distribution, and decreased numbers of regenerated HSCs (Fig. 1c). These features were further exacerbated upon secondary transplantation of 500 re-isolated Atg12cKO HSCs, and directly demonstrated defective self-renewal activity in autophagy-deficient HSCs that closely resembled the functional impairment of oHSCs

(Extended Data Fig. 2g). To address whether the need for autophagy changed with age, we next transplanted 2 × 106 bone marrow cells from non-pIC-treated animals into lethally irradiated mice, induced Atg12 deletion 2 months after transplantation, and followed the recipients for up to 16 months after pIC treatment (Extended Data Fig. 2h). Of note, Atg12cKO mice could not be aged past 8 months after pIC owing to hepatomegaly from off-target deletion in the liver. Importantly, haematopoietic-specific deletion of Atg12 in transplanted mice led to a progressive age-related decline in donor chimaerism and myeloid-biased lineage distribution (Fig. 1d), thus confirming the cell-intrinsic nature of these defects. These ageing features were further exacerbated upon deletion of autophagy in mice transplanted with bone marrow cells from 24-month old animals (Extended Data Fig. 2i). Collectively, these results indicate striking similarities between autophagy-deficient HSCs and oHSCs with defective self-renewal activity and myeloid-biased differentiation potential, and demonstrate that autophagy is most critical for HSC function during ageing and in conditions of intense regenerative stress such as transplantation.

Autophagy regulates HSC metabolism

We next investigated how loss of autophagy affects HSC function. Electron microscopy analyses revealed increased numbers of total and elongated, fused mitochondria in Atg12cKO HSCs, which was directly confirmed by immunofluorescence staining for the mitochondrial protein TOM20 and flow cytometry measurements of mitochondrial mass with Mitotracker Green (MTG) (Fig. 2a–c and Extended Data Fig. 3a). Atg12cKO HSCs also had expanded endoplasmic reticulum (ER) and Golgi compartments, and increased numbers of small vesicles and lysosomes, confirmed by immunofluorescence staining and flow cytometry dye measurements (Extended Data Fig. 3b–e). These cellular features, together with elevated levels

of p62 (Extended Data Fig. 3f), confirmed the loss of bulk autophagy in Atg12cKO HSCs, and suggest activation of alternative mechanisms of cellular recycling[17] to allow HSC maintenance at steady state. Autophagy is known to degrade mitochondria through several pathways including the PINK1/PARK2 stress mitophagy pathway[18], which specifically clears damaged mitochondria. HSCs from Park2−/− mice[19] showed decreased levels of mitochondrial membrane potential dye tetramethylrhodamine-ethyl-ester (TMRE), and TMRE/MTG ratio, probably reflecting damaged mitochondria (Extended Data Fig. 3g). In contrast, Atg12cKO HSCs had increased TMRE levels and TMRE/MTG ratio, indicating more active mitochondria (Fig. 2d, e). Further characterization of Park2−/− mice also revealed no similarities to the phenotypes of Atg12cKO mice, and normal function of Park2−/− HSCs in transplantation experiments (Extended Data Fig. 3h–n). These results indicate that autophagy is most important for the clearance of healthy, active mitochondria in HSCs, as opposed to stress-induced specific removal of damaged mitochondria via mitophagy as recently studied[20,21].

To understand the link between autophagy regulation and mitochondrial activity, we next grew wild-type HSCs or HSCs isolated from autophagy-reporter Gfp–Lc3 mice[22] in cytokine-rich (+cyt) conditions to force them out of quiescence and investigate their activated state before their first cell division (Fig. 2f). As expected, mTOR was rapidly activated in +cyt conditions, whereas it remained inactive in cytokine-starved (−cyt) conditions, while AMPK was transiently induced (Fig. 2g, h and Extended Data Fig. 4a). This was accompanied by a rapid reduction of GFP–LC3 levels reflecting autophagy activation in –cyt conditions, and increased GFP–LC3 and p62 levels indicating autophagy inhibition in +cyt conditions (Fig. 2i and Extended Data Fig. 4b, c). Furthermore, pharmacological mTOR inhibition or AMPK activation directly induced autophagy in Gfp–Lc3 HSCs grown in +cyt conditions, while AMPK inhibition reduced

autophagy induction in −cyt conditions (Extended Data Fig. 4d). In +cyt conditions, we also found a steady increase in mitochondrial mass and membrane potential, reflecting mitochondrial activation, and cell size, NADH levels and glucose uptake, suggesting increased metabolic activity (Fig. 2j). Seahorse metabolic flux analyses measuring oxygen consumption rates confirmed markedly increased oxidative phosphorylation (OXPHOS) levels in activated HSCs (aHSCs) grown for 21 h in +cyt conditions compared with freshly isolated, quiescent HSCs (qHSCs) (Fig. 3a). Collectively, these results demonstrate that qHSCs switch from a normally low OXPHOS state to a high mitochondria-driven OXPHOS state during activation.

Strikingly, Atg12cKO HSCs also exhibited these features of an aHSC state, with increased cell size, NADH, ATP, and glucose uptake (Fig. 3b, c and Extended Data Fig. 4e). Furthermore, freshly isolated HSC-enriched Atg12cKO LSK cells (Lin−/c-Kit+/Sca-1+) displayed increased OXPHOS levels, specifically maximum capacity, with unchanged glycolysis levels measured by extracellular acidification rate (Fig. 3d and Extended Data Fig. 4f). Similar to Atg12cKO LSKs, oHSCs also exhibited increased cell size, NADH and ATP levels, and elevated OXPHOS with decreased glycolysis (Fig. 3c, e and Extended Data Fig. 4e, g), suggesting an overactive oxidative metabolism. However, in contrast to Atg12cKO HSCs, oHSCs had decreased TMRE levels and TMRE/MTG ratios (Extended Data Fig. 4h), which could indicate the presence of some damaged mitochondria[23]. Collectively, these results demonstrate that HSC activation is directly associated with metabolic activation and increased mitochondrial OXPHOS, and that, in the absence of autophagy, HSCs are kept in an activated state reminiscent of the alert state recently described for muscle stem cells[24]. They also show that oHSCs, like autophagy-deficient HSCs, are metabolically more active than young HSCs (yHSC).

Loss of autophagy alters HSC fate

We then explored the consequences of increased oxidative metabolism for Atg12cKO HSC fate decisions. Similar to aHSCs, Atg12cKO HSCs showed increased levels of reactive oxygen species (ROS), which did not cause DNA damage or apoptosis (Extended Data Figs 4i–k and 5a). As expected for more metabolically activated cells, Atg12cKO HSCs also had elevated protein synthesis rates and increased cell cycle activity (Extended Data Fig. 5b, c). Notably, oHSCs showed reduced ROS levels and decreased protein synthesis rates, which contrasted with their OXPHOS-activated status, but is probably influenced by their replication stress features[25] (Extended Data Fig. 5d, e). However, both Atg12cKO HSCs and oHSCs[25] displayed a similar loss of quiescence and pro-myeloid differentiation associated with increased unipotent mature colonies and decreased multipotent immature colonies formed in methylcellulose (Fig. 4a, b). Strikingly, yHSCs treated with the autophagy inhibitor bafilomycin A (BafA) and untreated oHSCs showed a matching reduction in multipotent colonies, which was greatly exacerbated in BafA-treated oHSCs (Fig. 4c). Interestingly, treatment with the antioxidant N-acetylcysteine (NAC), which is known to ameliorate many ROS-mediated phenotypes[26], did not rescue the precocious myeloid differentiation of Atg12cKO HSCs in methylcellulose, nor limit the myeloid expansion in Atg12cKO mice treated in vivo (Extended Data Fig. 5f–i). These data demonstrate that the non-cytotoxic increase in ROS levels observed in metabolically activated Atg12cKO HSCs has no major role in driving precocious myeloid differentiation in autophagy-deficient HSCs, in contrast to what has been shown in myeloid progenitors in flies[27]. Altogether, these results indicate that the overactive metabolic state caused by loss of autophagy results in loss of quiescence and accelerated myeloid differentiation, which closely mirrors the deregulations observed in oHSCs.

Loss of autophagy perturbs HSC epigenetic poising

To gain a better understanding of the mechanisms affected by the loss of autophagy, we performed gene expression microarray analyses on HSCs and GMPs. While significant transcriptional differences were observed in Atg12cKO HSCs using a P value <0.01, surprisingly, apart from Atg12, no individual genes were significantly differentially expressed using a false discovery rate of 0.05 (Supplementary Table 1 and Extended Data Fig. 6a, b). However, gene set enrichment analyses revealed reduced expression of HSC-identity genes28 and increased expression of both myeloid genes[28] and genes elevated in oHSCs[25] (Extended Data Fig. 6c). Complementary Fluidigm qRT– PCR analyses confirmed limited transcriptional changes in Atg12cKO HSCs, but also identified a small set of significantly downregulated genes that render them more similar to downstream MPPs (Fig. 4d and Supplementary Tables 2 and 3). DAVID analyses also showed changes in mitochondrial and other metabolic terms in Atg12cKO GMPs, confirming more significant gene expression alterations in progenitor cells (Extended Data Fig. 6d and Supplementary Table 1). Since metabolism has become increasingly linked to epigenetic regulation[26], we also performed enhanced reduced-representation bisulfite sequencing (ERRBS) analyses of DNA methylation. Strikingly, Atg12cKO HSCs showed a significantly altered DNA methylation profile, with 162 hypermethylated and 783 hypomethylated differentially methylated regions (DMRs) (Fig. 4e). DAVID analyses identified phosphoproteins as the most enriched class of genes in hypomethylated DMRs (Supplementary Table 4). Taken together, these results are consistent with the idea that changes in fate decisions observed at steady state in autophagy-deficient HSCs are the consequence of an epigenetic reprogramming that begins to alter gene expression in HSCs, with amplification of transcriptional changes in downstream progenitors.

Metabolic-driven epigenetic remodeling

To gain a better understanding of the link between metabolic activation, epigenetic reprograming and control of HSC fate decisions, we conducted a more extensive analysis of 21h +cyt aHSCs versus freshly isolated qHSCs, and performed both ERRBS and Fluidigm qRT–PCR analyses. Strikingly, we also observed an altered DNA methylation profile, which was more restricted than in Atg12cKO HSCs, but also mainly consisted of hypomethylation events with 38 hypomethylated and 17 hyermethylated DMRs (Fig. 4f). Gene Ontology analyses indicated enrichment for Stat3 regulation (Supplementary Table 5). Moreover, we found a clear overlap between the changes in gene expression in aHSCs and Atg12cKO HSCs, suggesting activation of similar mechanisms of fate decision (Fig. 4d and Supplementary Table 2). To directly test the importance of metabolic-driven epigenetic remodelling in aHSCs, we added α-ketoglutarate (αKG), a necessary co-factor for many demethylases[29], S-adenosylmethionine (SAM), a methyl donor cosubstrate for methylases[30], and metformin (Mtf), an inhibitor of mitochondrial complex I[31], to differentiating HSCs grown in +cyt conditions for 3 days, and analysed lineage commitment. Strikingly, addition of αKG enhanced myeloid differentiation, while addition of SAM or Mtf preserved stemness in +cyt HSCs (Fig. 4g). These results indicate that even in the context of strong differentiation stimuli, the epigenetic remodelling associated with metabolic activation directly affects HSC fate decisions. Finally, we measured SAM and αKG cellular levels (Extended Data Fig. 6e, f). While only trending in Atg12cKO HSCs, we observed a strong reduction in SAM levels in aHSCs, and found a large increase in αKG levels in both Atg12cKO and 21h +cyt c-Kit-enriched bone marrow cells. These results suggest that epigenetic remodelling and DNA demethylation are early consequences of increased oxidative metabolism, and play a direct role in HSC loss of stemness and accelerated myeloid differentiation.

Collectively, they demonstrate an essential role for autophagy in clearing metabolically activated mitochondria and allowing HSCs to maintain a mostly glycolytic, quiescent state.

Autophagy levels define distinct subsets of old HSCs

While oHSCs share many deregulated features with Atg12cKO HSCs, we previously reported increased basal autophagy in oHSCs[13]. To address this discrepancy and to determine its significance, we profiled GFP–LC3 expression in various haematopoietic populations of young and old Gfp–Lc3 mice as a surrogate of autophagy levels. In unfractionated bone marrow cells, mature B and T cells, and most of the progenitor populations including GMPs and MPP4, GFP–LC3 levels were increased with age, indicating an overall decrease in autophagy activity, as already reported for T cells[32] (Fig. 5a, b). Interestingly, this corresponded to populations that were either unchanged or decreased in number in old mice (Extended Data Fig. 7a). In contrast, expanded myeloid cells did not have altered GFP–LC3 levels, and the enlarged MPP2/3 and HSC compartments both contained subsets with decreased GFP– LC3 expression. While the average GFP–LC3 expression and overall mTOR activity did not significantly change between yHSCs and oHSCs (Extended Data Fig. 7b), careful examination of oHSCs revealed an increase in cells with both lower and higher GFP–LC3 levels, reflecting the appearance of subsets with either high autophagy (AThi) or low autophagy (ATlo), respectively (Fig. 5c). Re-analysis of electron microscopy pictures13 (Fig. 5d) and measurement of autophagy with Cyto-ID dye (Extended Data Fig. 7c, d) also indicated that approximately one-third of oHSCs had autophagosomes and activated autophagy. Thus, while most of the blood cells including most of

the HSC compartment show no activation or a decline in autophagy with age, a fraction of oHSCs have increased autophagy levels.

To study the differences between AThi and ATlo oHSCs, we isolated cells with the 33% lowest and 33% highest GFP–LC3 levels, respectively (Extended Data Fig. 7e). This sub-fractionation revealed no changes in nuclear FoxO3a, but uncovered decreased mTOR activity specifically in AThi oHSCs (Fig. 6a and Extended Data Fig. 7f), thus identifying the cause for autophagy induction in this subset. By electron microscopy analyses, oHSCs with autophagosomes displayed normal youthful organelle biology, while oHSCs without autophagosomes showed almost identical features to Atg12cKO HSCs, with expanded ER, Golgi, and small vesicle compartments, and increased numbers of total and elongated mitochondria, which was confirmed by TOM20 immunofluorescence staining in ATlo oHSCs (Fig. 6b and Extended Data Fig. 7g, h). ATlo oHSCs also had increased cell size and elevated TMRE, NADH, ATP, glucose uptake, and ROS levels (Fig. 6c–f and Extended Data Fig. 7i–k), remarkably similar to the overactive metabolic features of Atg12cKO HSCs. This resulted in increased cell cycling, over-proliferation in self-renewal conditions, and pro-myeloid differentiation specifically in ATlo oHSCs (Fig. 6g and Extended Data Fig. 7l, m). Strikingly, nucleolar γH2AX foci that are hallmarks of replication stress in oHSCs[25] were almost exclusively found in ATlo oHSCs (Fig. 6h). In contrast, AThi oHSCs closely resembled healthy yHSCs for all of these characteristics. Taken together, these results indicate that by preventing entry into an activated state, autophagy preserves a subset of oHSCs from replication stress and associated cellular ageing. This is actually very different from the functions of autophagy in muscle stem-cell activation[33] and prevention of senescence[15].

Autophagy activation maintains healthier old HSCs

We next analysed the functionality of these subsets and transplanted 250 ATlo or AThi oHSCs into lethally irradiated recipients (Extended Data Fig. 8a). While both subsets initially engrafted at similar levels, ATlo oHSCs rapidly declined and were unable to maintain efficient long-term reconstitution and HSC numbers (Fig. 6i). In contrast, AThi oHSCs exhibited surprisingly robust long-term reconstitution and HSC regeneration potential. These functional differences persisted with a more restrictive 15% GFP–LC3 AThi/lo oHSC cutoff and upon secondary transplantation, although they became attenuated, perhaps owing to normalization of autophagy levels in re-isolated oHSC subsets, potentially as a consequence of repeated exposure to a young bone marrow microenvironment (Extended Data Fig. 8b, c). They were also specific to oHSCs, as ATlo and AThi yHSCs isolated with the same 33% GFP–LC3 cutoffs had no differences in regenerative abilities (Extended Data Fig. 8d). Collectively, these results demonstrate that ATlo and AThi oHSCs are functionally distinct subsets, with autophagy-activated oHSCs being the fittest aged stem cells responsible for the majority of the repopulation potential, and autophagy-inactivated oHSCs driving most of the blood ageing phenotypes. We also investigated the reversibility between ATlo and AThi oHSC subsets. Strikingly, there were no discernible differences in CD150 expression between these two subsets, and oHSCs were almost identical to yHSCs for both mTOR activation in +cyt conditions and pharmacological modulation of autophagy levels (Extended Data Fig. 9a–c). In fact, ATlo and AThi oHSCs, despite initial differences in GFP–LC3 levels, were able to repress and activate autophagy to similar extents in ±cyt conditions, and upon glutamine starvation (Extended Data Fig. 9d–g). Furthermore, both ATlo and AThi oHSCs had equivalent autophagy flux, which was comparable to yHSCs (Extended Data Fig. 9f). These results demonstrate that while subsets of oHSCs with different

mTOR and autophagy levels exist in vivo, they both maintain the ability to upregulate and downregulate autophagy upon strong stimulation.

Discussion

Our results demonstrate an essential function for autophagy in removing activated mitochondria and controlling oxidative metabolism, thereby maintaining HSC stemness and regenerative potential (Extended Data Fig. 10). They link metabolic reprogramming with epigenetic modifications in the control of HSC fate, and establish autophagy as one of the essential gatekeepers of HSC quiescence. This role becomes even more important during ageing as the inability of the majority of oHSCs to activate autophagy in vivo results in an overactive OXPHOS metabolism that drives most of the ageing blood phenotypes, including impaired engraftment and replication stress. While all oHSCs remain competent for autophagy induction, only about one-third of them activate autophagy in the ageing bone marrow microenvironment[7,8], and maintain a low metabolic state with robust regeneration potential akin to yHSCs. Our findings have exciting implications for rejuvenation therapies as they identify a cellular characteristic that can be directly targeted to improve oHSC function and preserve the health of an ageing blood system. In this context, understanding why some old stem cells activate autophagy and others do not, as well as identifying the environmental drivers for this differential adaptive response, will help further our understanding of cellular ageing, and develop more targeted approaches for improving organismal health during ageing.

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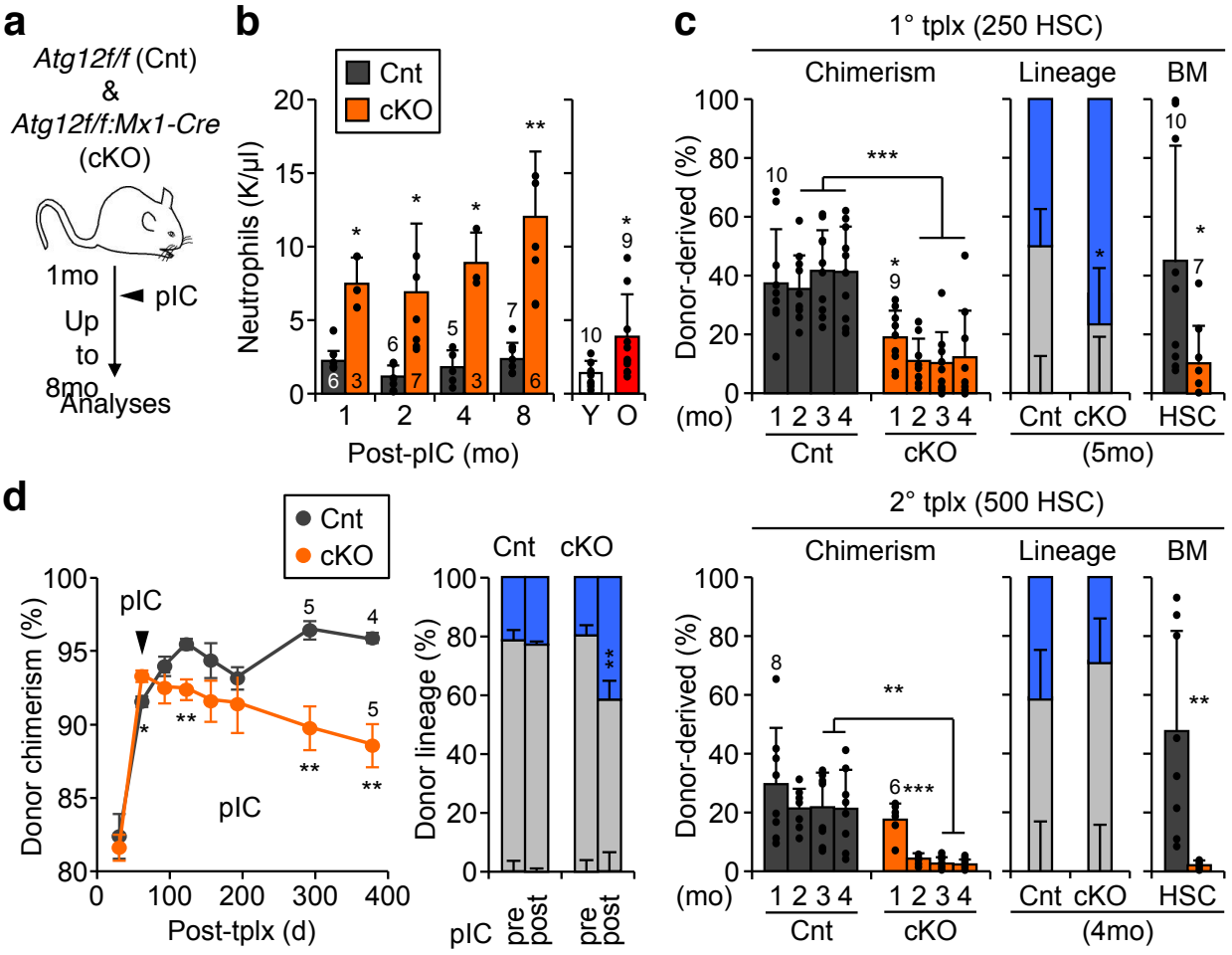


Figure 1 | Accelerated blood aging phenotypes in autophagy-deficient mice. a, Scheme for deleting *Atg12* in the adult blood system. **b**, Neutrophil counts in peripheral blood (PB) of control (Cnt) and *Atg12*^{cKO} (cKO) mice post-pIC (left), and young (Y) and old (O) mice (right); mo: month. **c**, Serial transplantations (tplx) of Cnt and cKO HSCs showing donor chimerism (left) and lineage distribution (center) in PB, and HSC chimerism (right) at the indicated times post-tplx in primary (top row) and secondary (bottom row) recipients. **d**, *Atg12* deletion in recipients transplanted with 2x10^6 BM cells from 2mo-old non-pIC treated Cnt and cKO donors

showing donor chimerism in PB post-pIC (left; ± S.EM.) and lineage distribution at 61d (pre-pIC) and 291d (post-pIC) post-tplx (right). Data are mean ± S.D. except when indicated. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

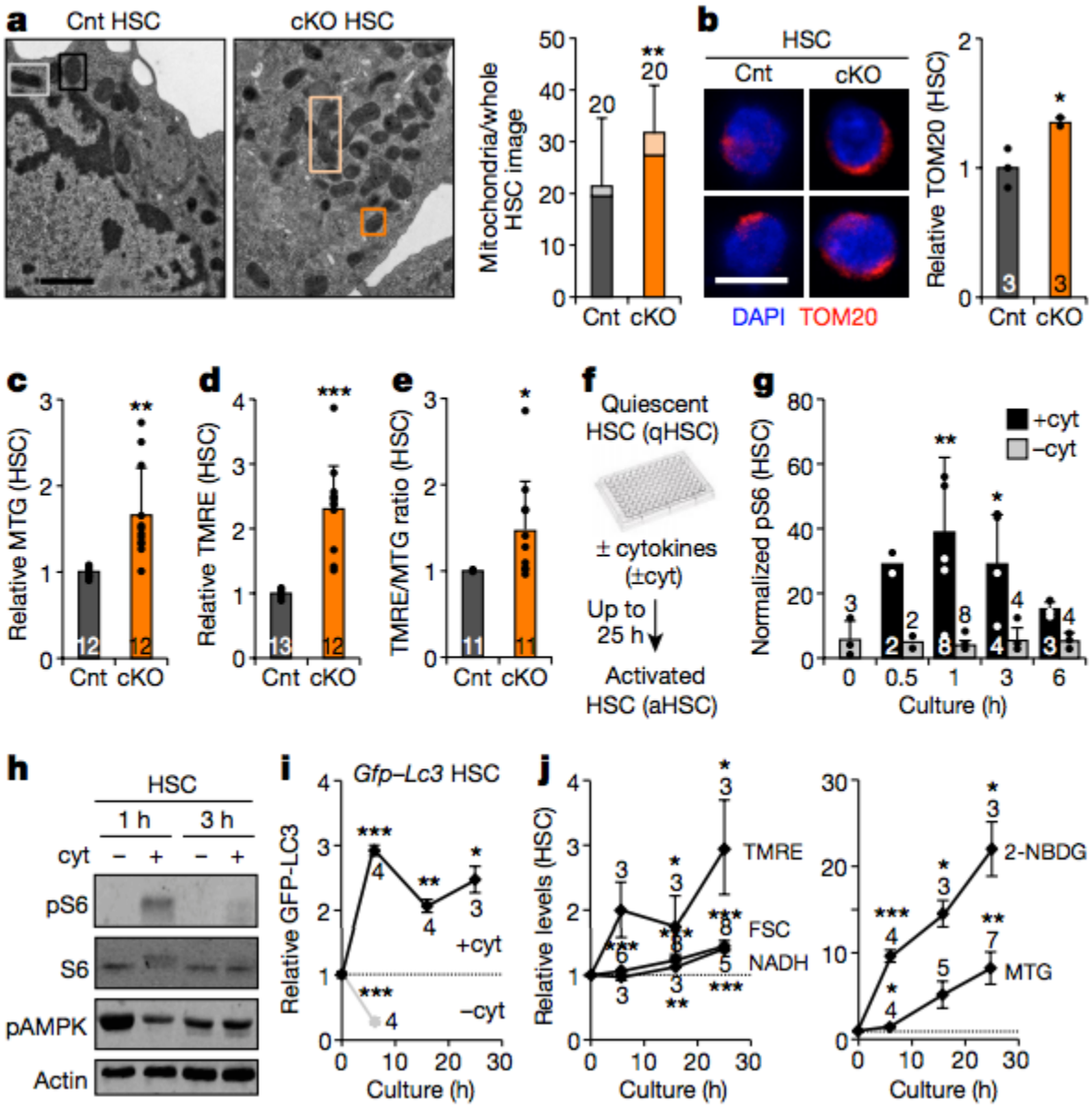


Figure 2 | Mitochondrial characteristics in autophagy-deficient and activated HSCs. a, Representative EM micrographs and quantification of total, normal (dark box) and elongated (light box) mitochondria in Cnt and cKO HSCs; scale bar, 1 μm. Statistical significance is for all three parameters, n indicate cell numbers. **b**, Representative TOM20 IF staining and quantification of mitochondria in Cnt and cKO HSCs. **c-e**, Mitochondria parameters in Cnt and cKO HSCs: (c) MTG levels, (d) TMRE levels, and (e) TMRE/MTG ratio. **f-j**, *in vitro* HSC activation: (f) experimental scheme (hr: hour); (g) pS6 levels measured by flow cytometry (results are normalized to IgG levels); (h) pS6, total S6, and pAMPK levels measured by Western blot (actin is used as loading control); (i) GFP-LC3 levels (n = 6 for 0hr; ± S.E.M.); and (j) mitochondrial parameters (n = 6 (TMRE), 10 (FSC), 7 (NADH), 4 (2-DG) and 7 (MTG) at 0hr; ±S.E.M.). Data are mean ± S.D. except when indicated, and are expressed relative to Cnt HSC (a-e) or 0hr HSC (g, i, j) levels. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

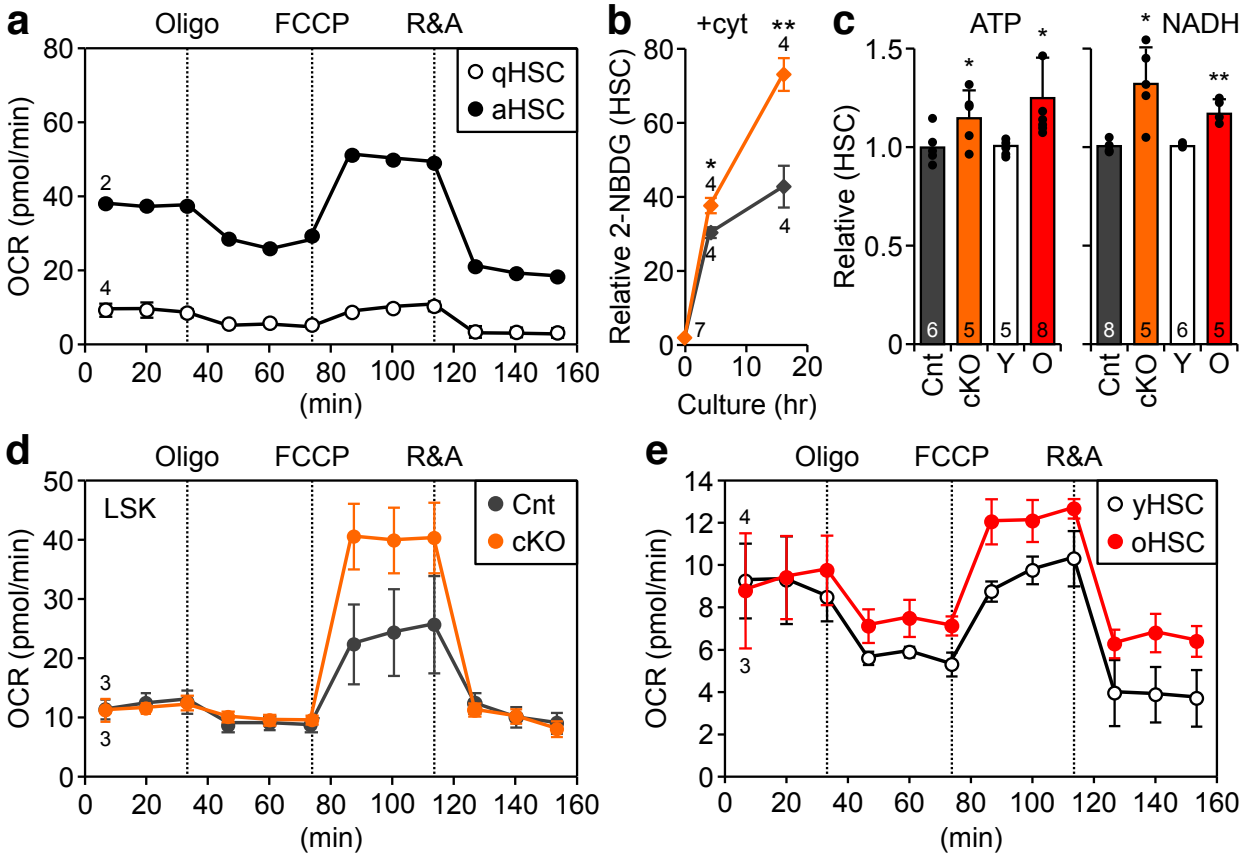


Figure 3 | Loss of autophagy and aging cause metabolic activation in HSCs. a, OXPHOS levels measured by oxygen consumption rates (OCR) in freshly isolated, quiescent HSCs (qHSC, same as yHSC in e) or activated HSCs (aHSC; 21hr +cyt culture); oligo: oligomycin; R&A: rotenone and antimycin; min: minute. **b**, glucose uptake in Cnt and cKO HSCs cultured as indicated. Results are expressed relative to 0hr Cnt HSC levels. **c**, ATP and NADH levels in the indicated HSC populations (± S.D.). **d**, OXPHOS levels in Cnt and cKO LSK cells. **e**, OXPHOS levels in yHSCs and oHSCs. Data are mean ± S.E.M. except when indicated. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

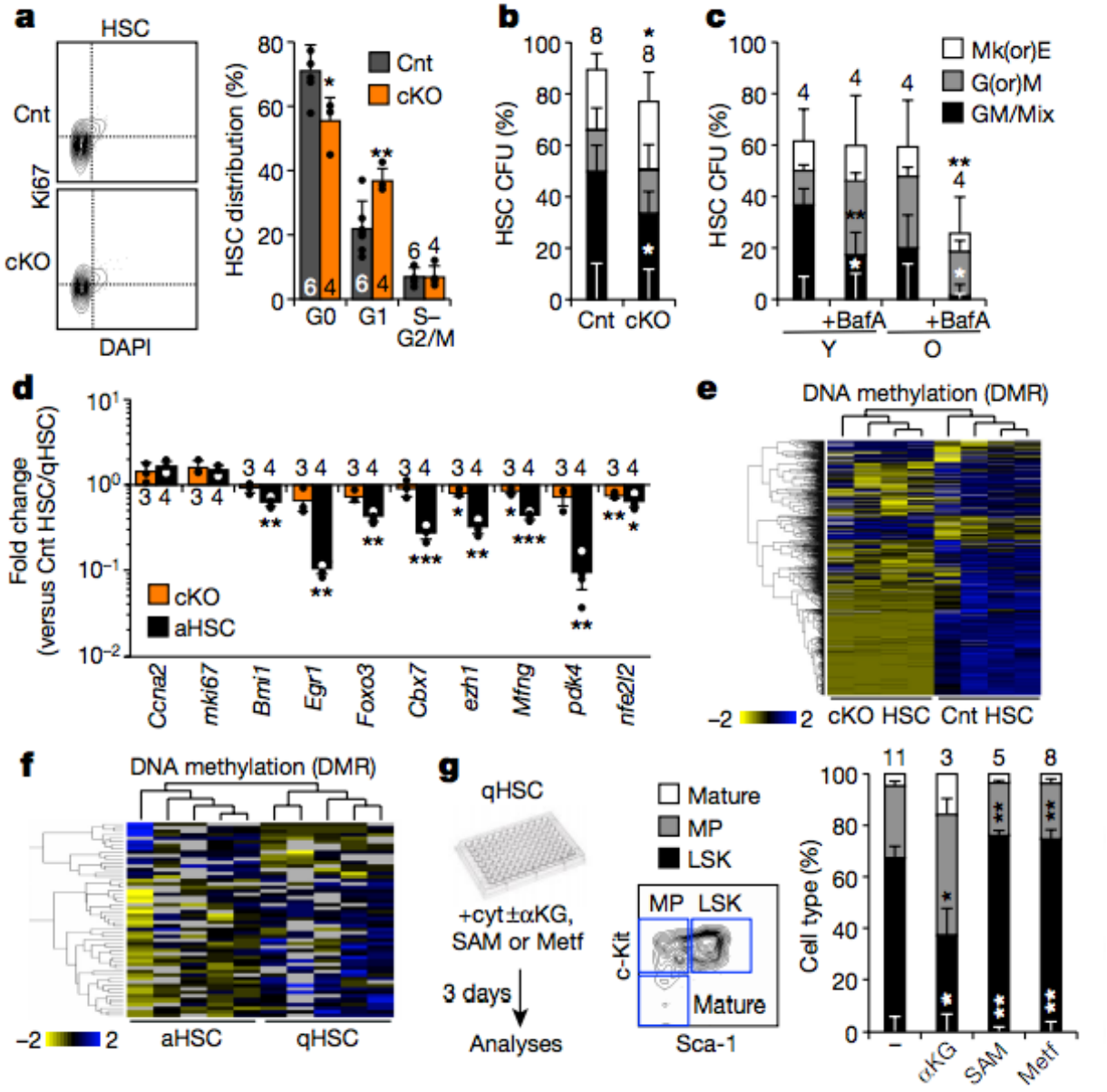


Figure 4 | Loss of autophagy affects HSC fate through epigenetic deregulation. a, Representative FACS plots and quantification of cell cycle distribution in Cnt and cKO HSCs. **b**, **c**, Colony formation in methylcellulose from (b) Cnt and cKO HSCs, and (c) yHSCs and oHSCs ± BafA; CFU: colony-forming unit; Mk(or)E and G(or)M: mature megakaryocyte, erythroid, granulocyte or macrophage colonies; GM/Mix: immature GM or GMMkE colonies. Results are

expressed as percent of plated cells. **d**, Selected genes from Fluidigm analyses of cKO HSCs and aHSCs. Results are expressed as fold change compared to levels in Cnt HSCs and qHSCs, respectively (3 technical pools of 100 cells are averaged per biological replicate). **e**, **f**, Heatmap of differentially methylated regions (DMRs) in (e) cKO vs. Cnt HSCs (n = 4) and (j) aHSCs vs. qHSCs (n = 5) ERBBS analyses. **g**, Impact of αKG, SAM and metformin (Metf) on HSC differentiation with scheme for *in vitro* treatment (left), gating strategy (middle) and quantification after 3 days culture. Data are means ± S.D. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

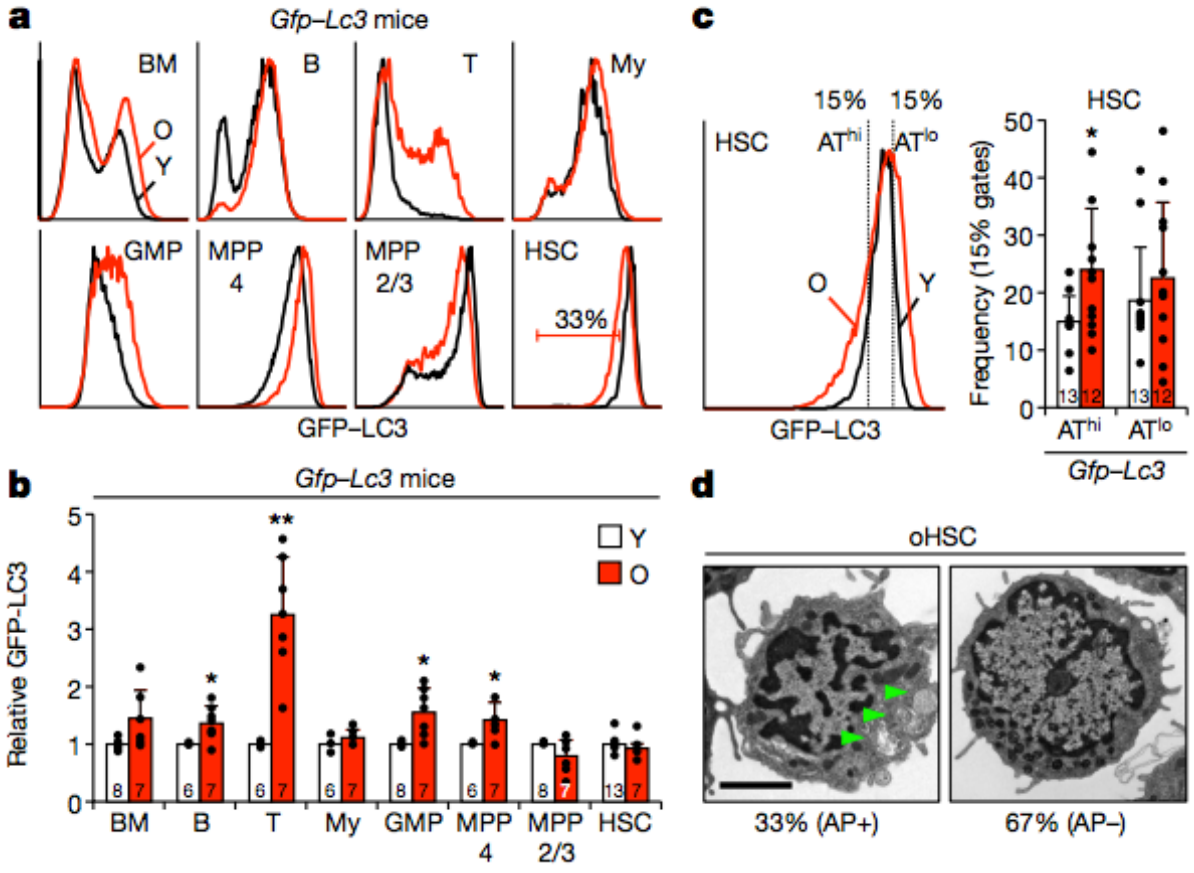


Figure 5 | Different autophagy activity in aged hematopoietic populations. a, **b**, Autophagy levels measured in young and old *Gfp-Lc3* mice: (a) representative FACS plots, and (b) quantification of GFP-LC3 expression. Results are expressed relative to the respective young populations. **c**, Representative FACS plots and frequency of young and old HSCs within the 15% GFP-LC3 low/autophagy high (AT^{hi}) and 15% GFP-LC3 high/autophagy low (AT^{lo}) yHSC gates. **d**, Representative EM micrographs and percent of oHSCs ± autophagosomes (AP, arrowheads) (58 cells total); scale bar, 2 μm. Data are means ± S.D. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

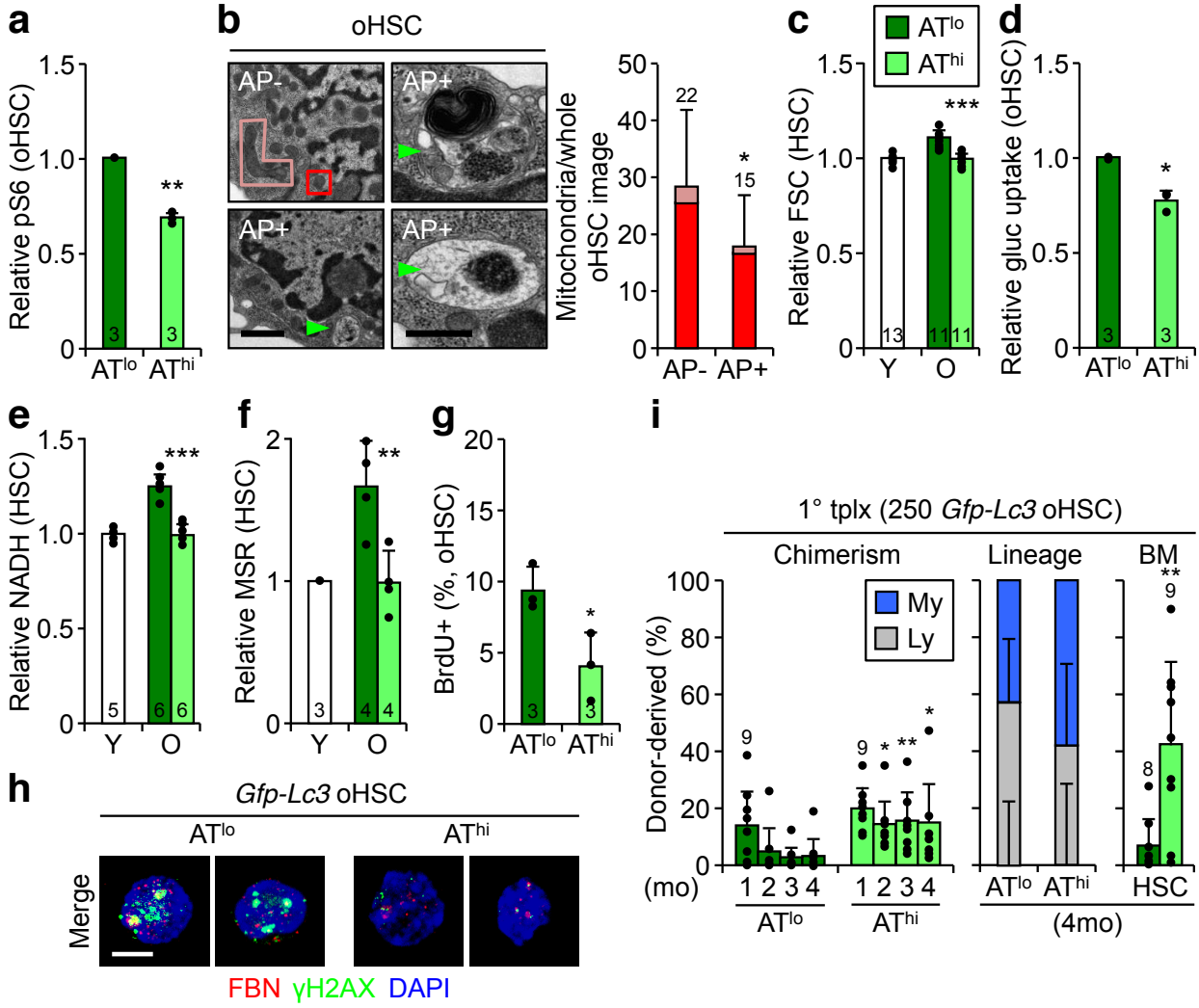
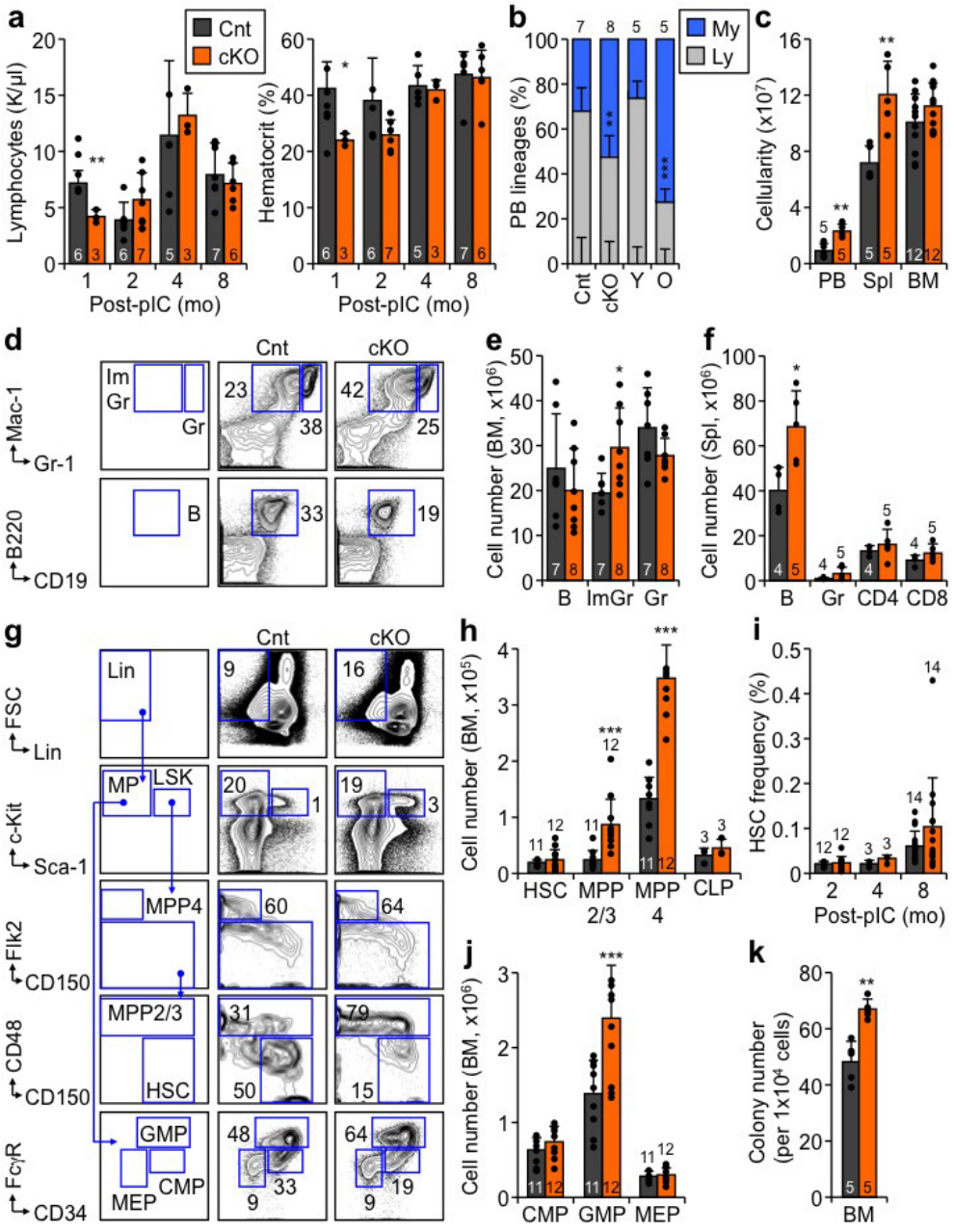


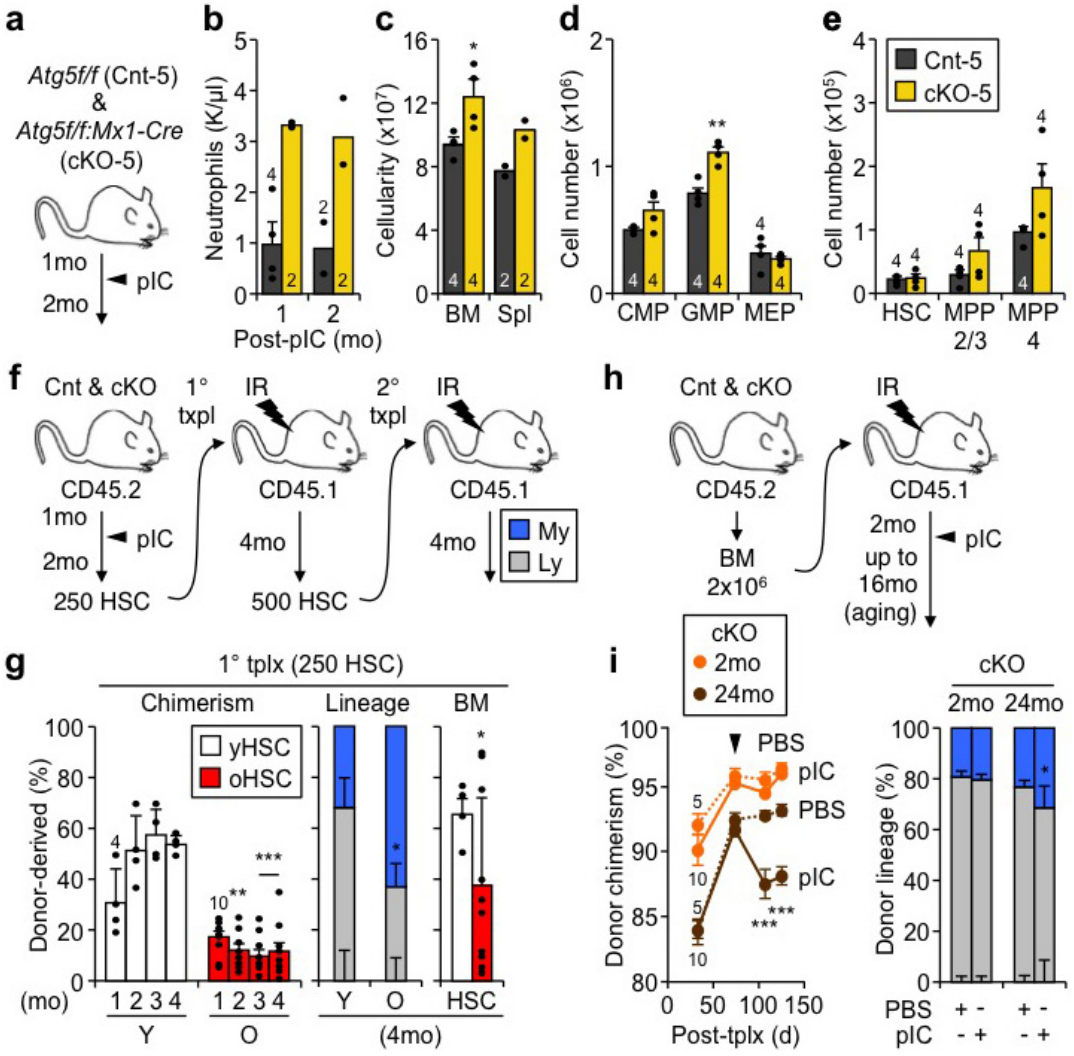
Figure 6 | Autophagy-activated old HSCs are healthier stem cells. a, pS6 levels in AT^{lo} and AT^{hi} oHSCs. **b**, Representative EM micrographs and quantification of total, normal (dark box) and elongated (light box) mitochondria in oHSCs ± autophagosomes (AP, arrowheads); scale bars, 1 µm. Statistical significance is for all three parameters, n indicate cell numbers. **c-f**, Metabolic parameters in AT^{lo} and AT^{hi} oHSCs: (c) cell size, (d) glucose uptake, (e) NADH and (f) ROS levels. **g**, Cycling activity in AT^{lo} and AT^{hi} oHSCs. **h**, Representative examples of 3

independent experiments showing IF images of fibrillarin (FBN)/γH2AX replication stress foci in AT^{lo} and AT^{hi} oHSCs; scale bar, 5 μm. **i**, Transplantation of AT^{lo} and AT^{hi} oHSC subsets with donor chimerism (left) and lineage distribution (center) in PB, and HSC chimerism (right) at the indicated times in primary recipients. Data are mean ± S.D., and are expressed relative to AT^{lo} oHSCs (a, d, g) or yHSCs (c, e, f). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.



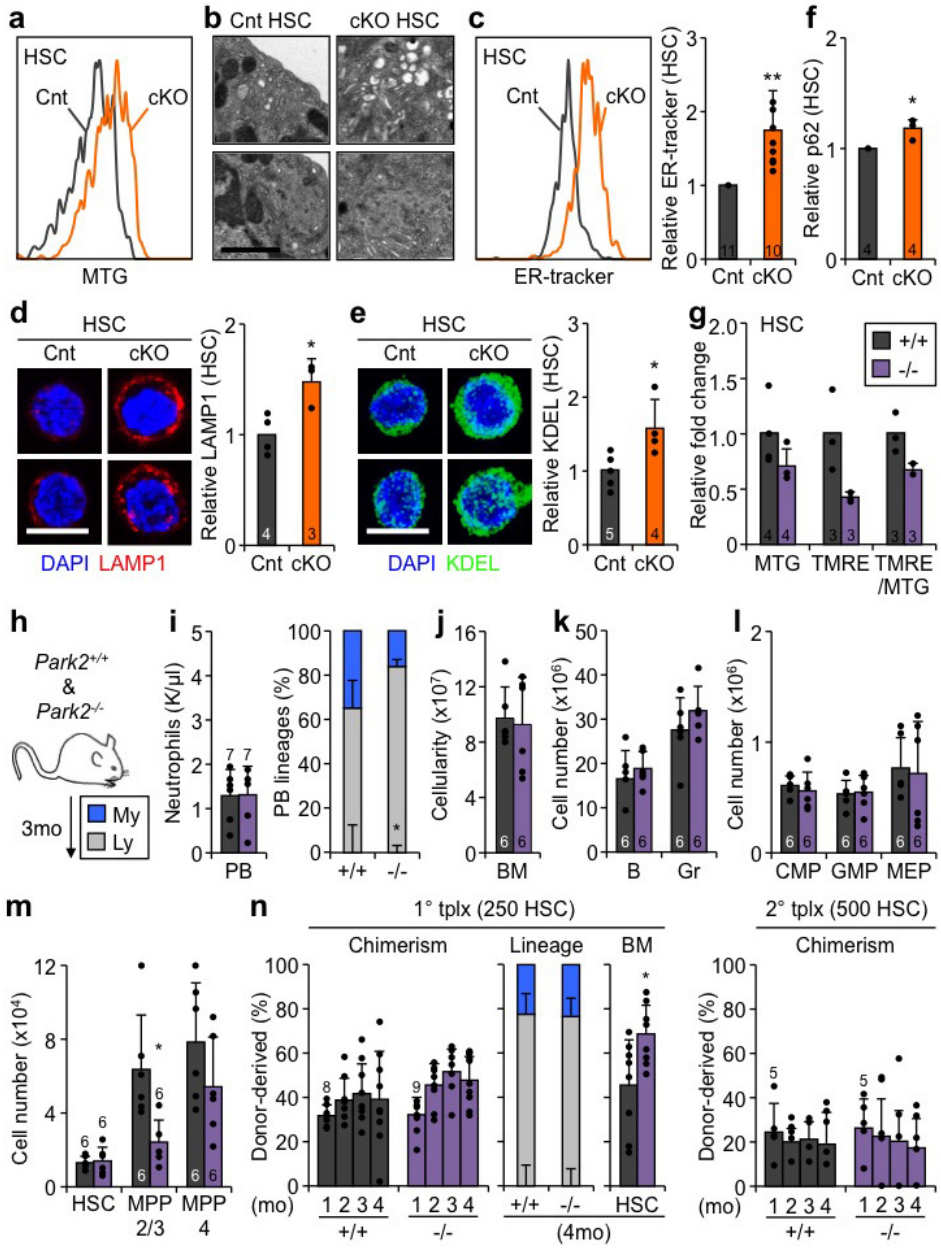
Extended Data Fig. 1 | Characterization of autophagy-deficient *Atg12*^{cKO} mice. a, CBC analyses of lymphocytes and hematocrit in control (Cnt) and *Atg12*^{cKO} (cKO) mice post-pIC treatment. **b**, Lineage distribution in PB of Ctrl and cKO mice at 2 months post-pIC, and in young and old mice; My: myeloid; Ly: lymphoid. **c**, Total cell numbers in PB, spleen (Spl), and BM of Ctrl and cKO mice at 2 months post-pIC. **d**, Gating strategy for mature populations;

ImGr: imature granulocytes/monocytes; Gr: granulocyte; B: B cells. **e**, **f**, Mature populations in (e) BM and (f) spleen of Ctrl and cKO mice at 2 months post-pIC. **g**, Gating strategy for imature BM populations; Lin: lineage negative; MP: myeloid progenitors; CMP: common myeloid progenitor; MEP: megakaryocyte/erythrocyte progenitor. **h**, Quantification of immature BM populations in Ctrl and cKO mice at 2 months post-pIC; CLP: common lymphoid progenitor. **i,** HSC frequency over time in Ctrl and cKO mice post-pIC. **j**, Quantification of MP BM populations in Ctrl and cKO mice at 2 months post-pIC. **k**, Colony formation in methylcellulose from Cnt and cKO BM at 2 months post-pIC. Data are mean ± S.D.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

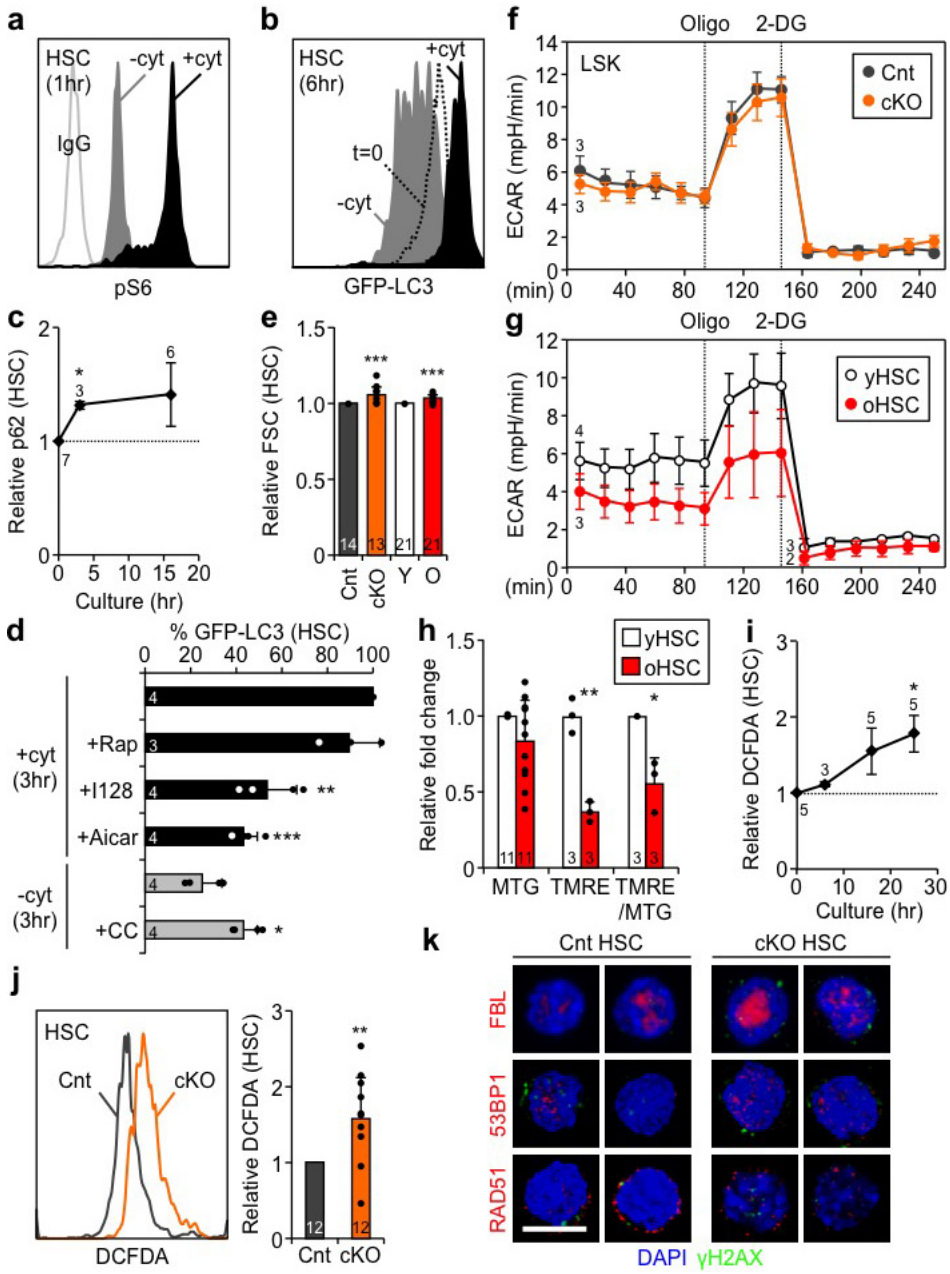


Extended Data Fig. 2 | Characterization of autophagy-deficient *Atg5*^{cKO} mice and regenerative capacity of *Atg12*^{cKO} HSCs. a-e, Hematopoietic features of control (Cnt-5) and *Atg5*^{cKO} (cKO-5) mice post-pIC treatment**:** (a) scheme for deleting A*tg5* in the adult blood system, and (b) neutrophil counts in PB, (c) total cell numbers in BM and spleen, and quantification of (d) MP and (e) immature BM populations at 2 months post-pIC. **f**, Scheme for Cnt and cKO HSC primary and secondary transplantation (tplx). **g**, Engraftment of young (Y) and old (O) HSCs with donor chimerism in PB over time (left), and lineage distribution in PB (center) with HSC chimerism (right) at the indicated time post-tplx. **h**, Scheme for aging

recipients transplanted with non-pIC treated Cnt and cKO BM cells and subsequently deleted for *Atg12*. **i**, *Atg12* deletion in recipients transplanted with $2x10^6$ BM cells from 2mo or 24mo-old non-pIC treated cKO donors with donor chimerism in PB post-PBS or pIC (left, ± S.E.M), and lineage distribution in PB at 125d post-tplx (right). Data are mean ± S.D. except where indicated. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

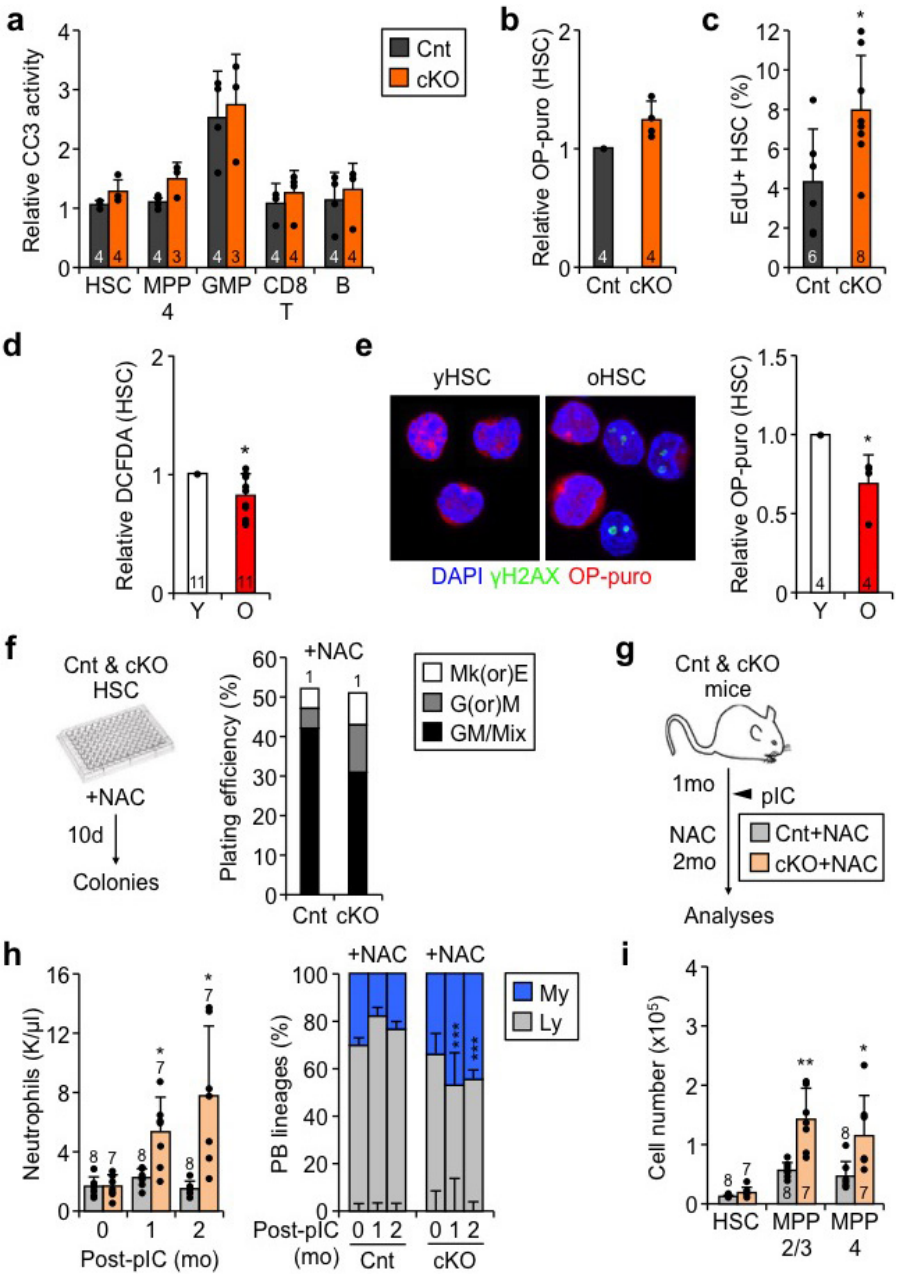


Extended Data Fig. 3 | Altered biology of *Atg12*^{cKO} HSCs and characterization of *Park2*$^{-/-}$ mice. a, Representative FACS plot of MTG staining in Cnt and cKO HSCs. **b**, Representative EM micrographs depicting expanded small vesicles (top) and ER/Golgi (bottom) in Cnt and cKO HSCs; scale bar, 1 μm. **c**, Representative FACS plot and quantification of ER mass measured by ER-tracker FC staining in Cnt and cKO HSCs. **d**, **e**, Representative IF images and quantification of (d) LAMP1 and (e) KDEL staining in Cnt and cKO HSCs; scale bars, 10 μm. **f**, p62 levels in Cnt and cKO HSCs. **g,** Mitochondria parameters in *Park2*$^{+/+}$ and *Park2*$^{-/-}$ HSCs. **h-m**, Characterization of *Park2*$^{-/-}$ mice: (h) Scheme for analyses, (i) CBC parameters (left) and lineage distribution in PB (right), (j) BM total cell numbers, (k) BM mature populations and (l, m) BM immature populations. **n**, Transplantation (tplx) of *Park2*$^{-/-}$ HSCs showing donor chimerism (left) and lineage distribution (center) in PB, and HSC chimerism (right) at the indicated time post-tplx in primary and secondary recipients. Data are mean ± S.D. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.



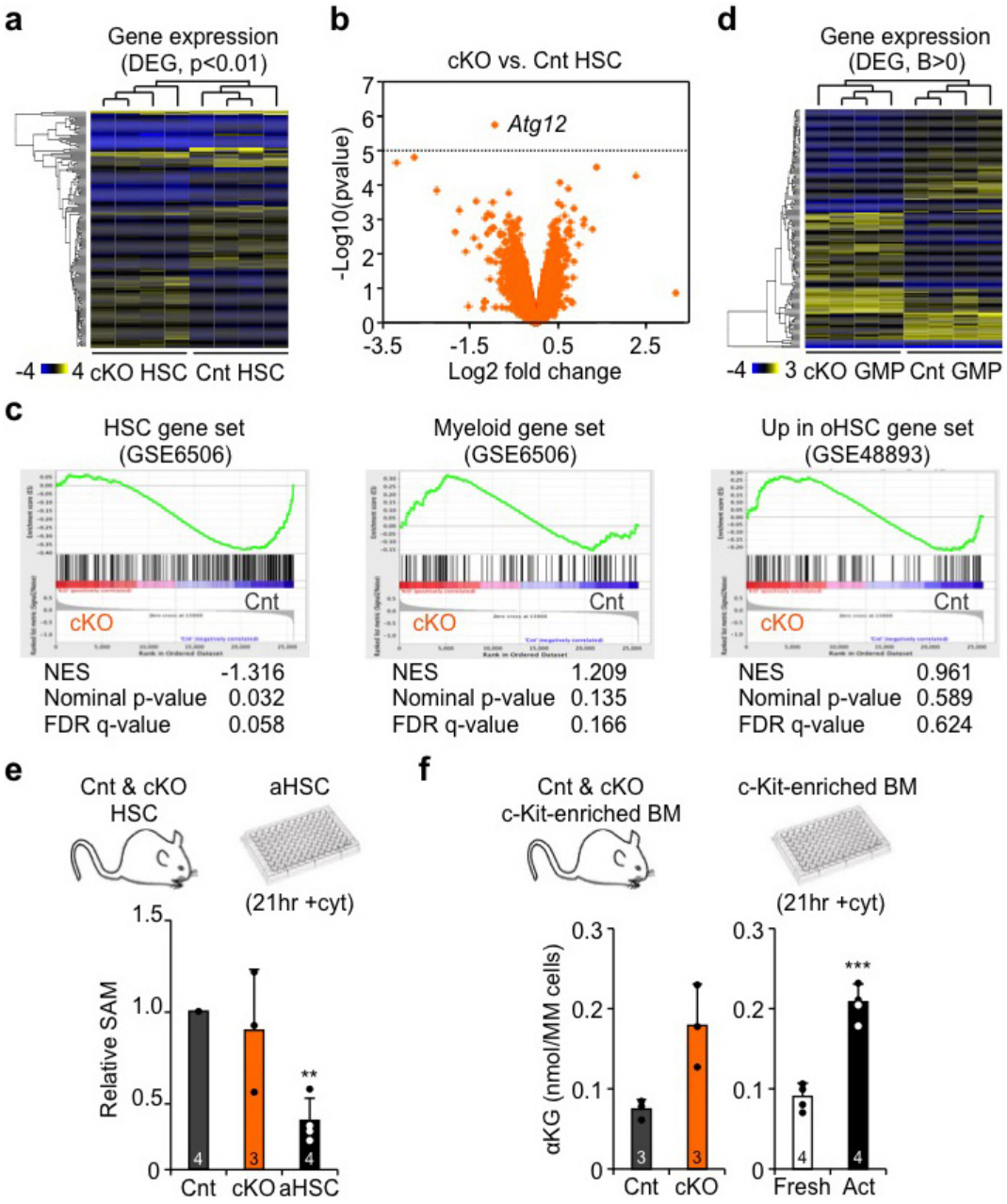
Extended Data Fig. 4 | Comparison between activated HSCs, *Atg12*^{cKO} HSCs and old HSCs. **a**, Representative FACS plot of pS6 levels in HSC cultured for 1 hour (hr) ± cytokines (cyt). **b**, Representative FACS plot of GFP-LC3 in freshly isolated HSCs (t=0) and HSCs cultured for 6hr ±cyt. **c**, Inactivation of autophagy in +cyt measured by p62 levels. **d**, Drug modulation of autophagy levels in *Gfp–Lc3* HSCs cultured for 3hr ±cyt; Rap: rapamycin; I128: Ink128; CC:

compound-C. Results are expressed as percent GFP-LC3 levels upon 3hr culture in +cyt conditions. **e**, Cell size measured by forward scatter (FSC) in Cnt and cKO HSCs, and young and old HSCs. **f**, **g**, Glycolysis activity measured by extracellular acidification rate (ECAR) in (f) Cnt and cKO LSKs, and (g) young and old HSCs; Oligo: oligomicyn; 2-DG: 2-deoxy-d-glucose. **h**, Mitochondria parameters in young and old HSCs. **i,** ROS levels in activated HSCs. **j**, Representative FACS plot and quantification of ROS levels in Cnt and cKO HSCs. **k**, Representative examples of 3 independent experiments showing IF co-staining of γH2AX with fibrillarin (FBN), 53BP1 and RAD51 in Cnt and cKO HSCs; scale bar: 10 μm. Data are mean ± S.D. except for line graphs, and are expressed relative to 0hr HSCs (c, i), Cnt HSC (e, j), or young HSC (e, h) levels.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.



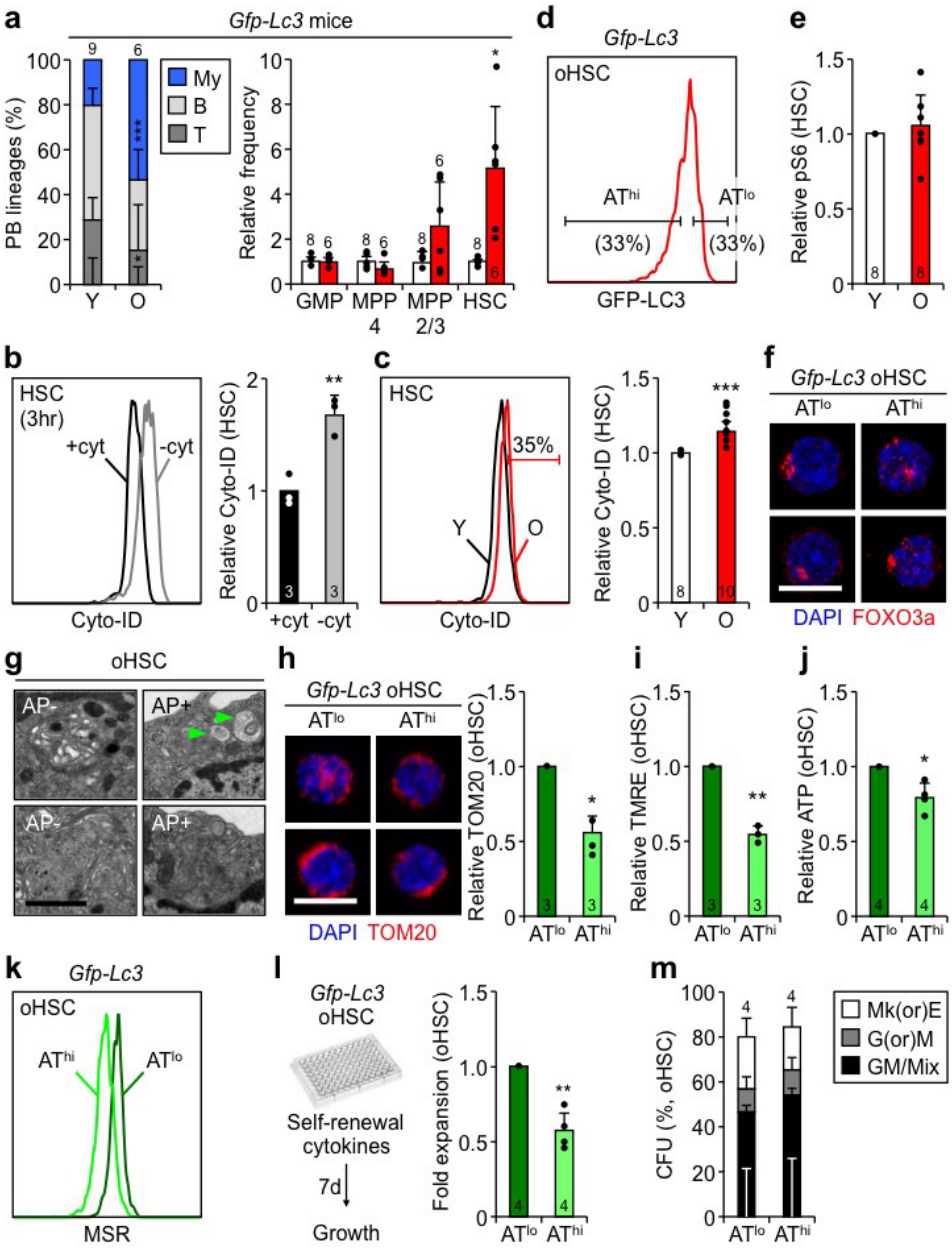
Extended Data Fig. 5 | Properties of autophagy-deficient and old HSCs, and effect of ROS scavenging in *Atg12*^{cKO} mice. a-c, Characteristics of Cnt and cKO HSCs: (a) apoptosis measured by cleaved caspase 3 (CC3) activity, (b) protein synthesis measured by O-propargyl-puromycin (OP-puro) IF staining, and (c) cycling activity measured by EdU incorporation. **d**, **e**, Characteristics of young and old HSCs: (d) ROS levels, and (e) protein synthesis with

representative OP-puro IF staining (left) and quantification (right). **f**, Scheme for N-acetylcysteine (NAC) *in vitro* treatment and representative example of colony formation in methylcellulose from NAC-treated Cnt and cKO HSCs; CFU: colony-forming unit; Mk(or)E and G(or)M: mature megakaryocyte, erythroid, granulocyte or macrophage colonies; GM/Mix: immature GM or GMMkE colonies. Results are expressed as percent of plated cells. **g-i**, Scavenging ROS levels in $Atg12^{cKO}$ mice: (g) scheme for NAC *in vivo* treatment after pIC deletion of *Atg12*, (h) neutrophil counts (left) and lineage distribution (right) in PB, and (i) quantification of immature BM populations. Data are mean ± S.D., and are expressed relative to Cnt HSC (a, b), or young HSC (d, e) levels.*$p \leq 0.05$, **$p \leq 0.01$.

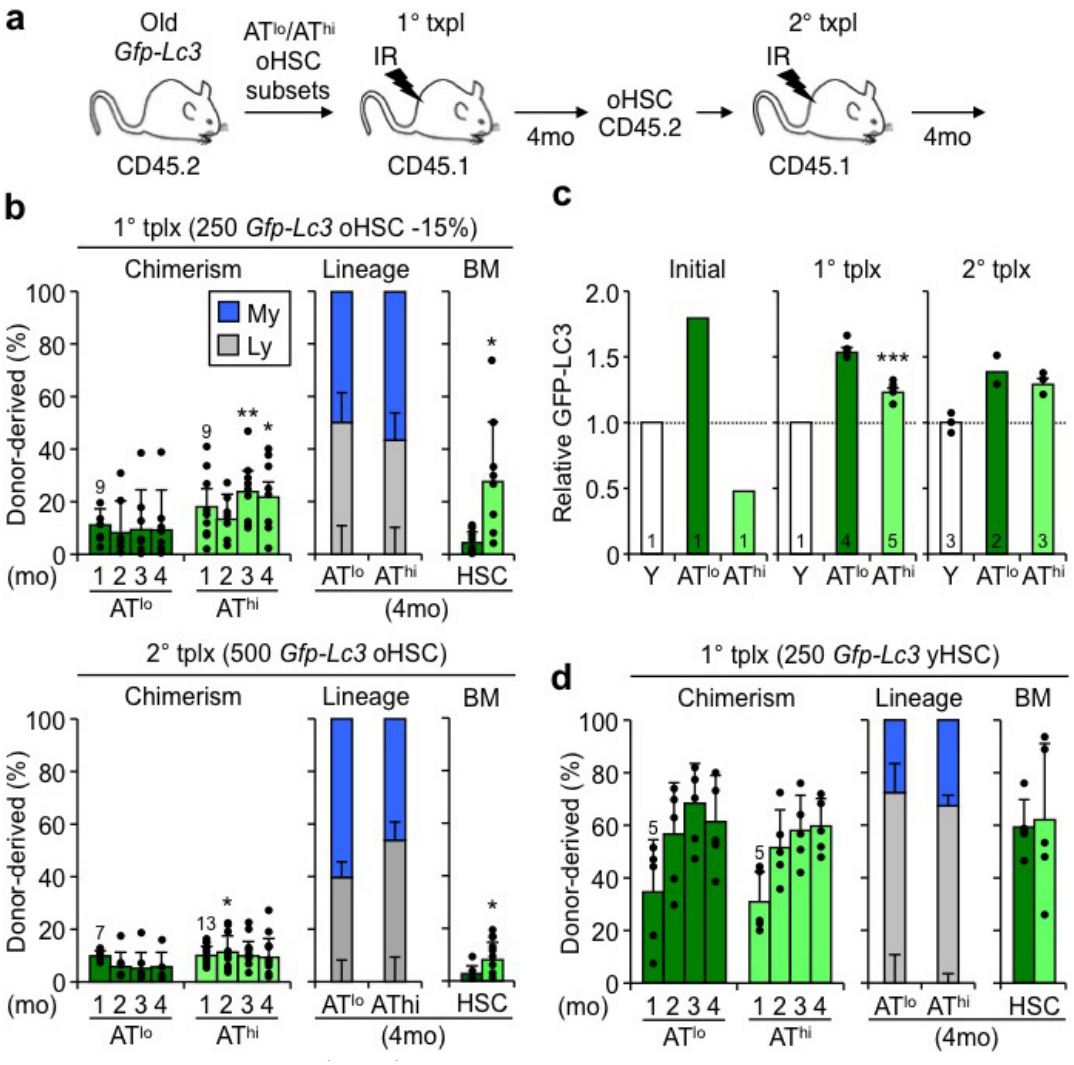


Extended Data Fig. 6 | Differential gene expression in *Atg12*^{cKO} HSCs and GMPs, and regulation of DNA methylation in HSCs. a, Heatmap of differentially expressed genes (DEG) in cKO vs. Cnt HSC microarrays (n = 4). **b**, Volcano plot of DEGs in cKO vs. Cnt HSCs. **c**, Gene set enrichment analyses (GSEA) of DEGs in cKO vs Cnt HSCs: NES: normalized enrichment score. **d**, Heatmap of DEGs in cKO vs. Cnt GMP microarrays (n = 4). **e**, SAM levels

in Cnt and cKO HSCs, and activated HSCs. **f**, aKG levels in c-kit-enriched Cnt and cKO BM (left), and freshly isolated and activated c-kit-enriched BM (right). Data are means ± S.D. *p ≤ 0.05, ***p ≤ 0.001.

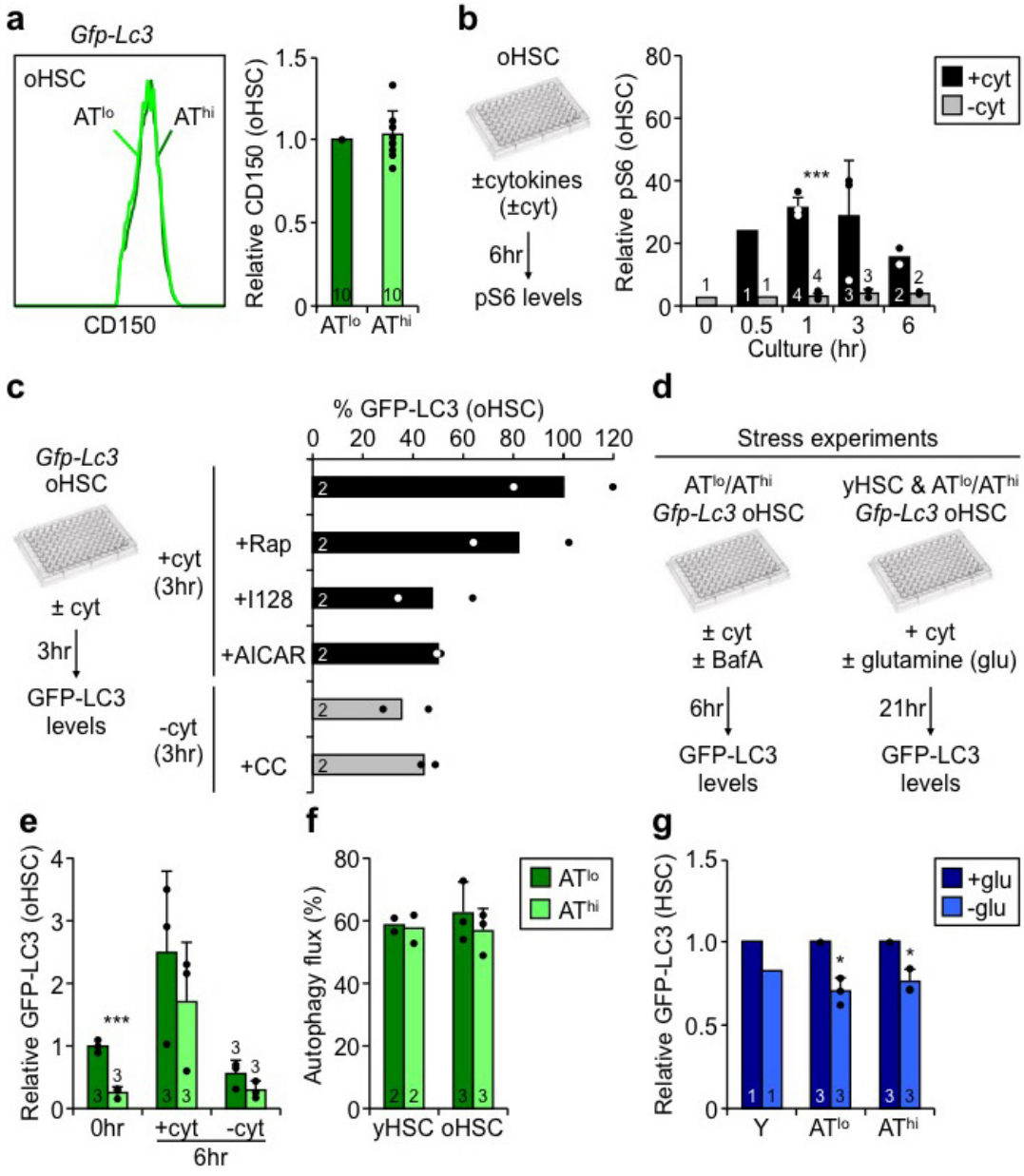


Extended Data Fig. 7 | Additional analyses of old *Gfp-Lc3* HSCs. a, Lineage distribution in PB (left) and frequency of the indicated populations (right) in young and old *Gfp-Lc3* mice (related to Fig. 5a, b). **b**, pS6 levels in young and old HSCs. **c**, **d**, Representative FACS plots and quantification of Cyto-ID dye levels in (c) HSCs cultured for 3hr ± cyt, and (d) young and old HSCs. **e**, Representative FACS plots of autophagy low (AT^{lo}) and autophagy high (AT^{hi}) oHSC subsets. **f**, Representative examples of 3 independent experiments showing IF staining of FOXO3a in AT^{lo} and AT^{hi} oHSCs; scale bar, 10 μm. **g**, Representative EM micrographs of oHSCs ± autophagosomes (AP) showing vesicles (top) and ER/Golgi (bottom) compartments; scale bar, 1 μm. **h,** Representative IF staining and quantification of TOM20 in AT^{lo} and AT^{hi} oHSCs; scale bar, 10 μm**. i-j**, Characteristics of AT^{lo} and AT^{hi} oHSCs: (i) TMRE levels, (j) ATP levels, and (k) ROS levels measured by Mitosox Red (MSR) staining. **l**, Expansion of AT^{lo} and AT^{hi} oHSCs in self-renewal culture conditions. **m**, Colony formation in methylcellulose from AT^{lo} and AT^{hi} oHSCs; CFU: colony-forming unit; Mk(or)E and G(or)M: mature megakaryocyte, erythroid, granulocyte or macrophage colonies; GM/Mix: immature GM or GMMkE colonies. Results are expressed as percent of 100 plated cells. Data are mean ± S.D., and are expressed relative to +cyt HSC (b), yHSC (c, e), or AT^{lo} oHSC (h-j, l) levels. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

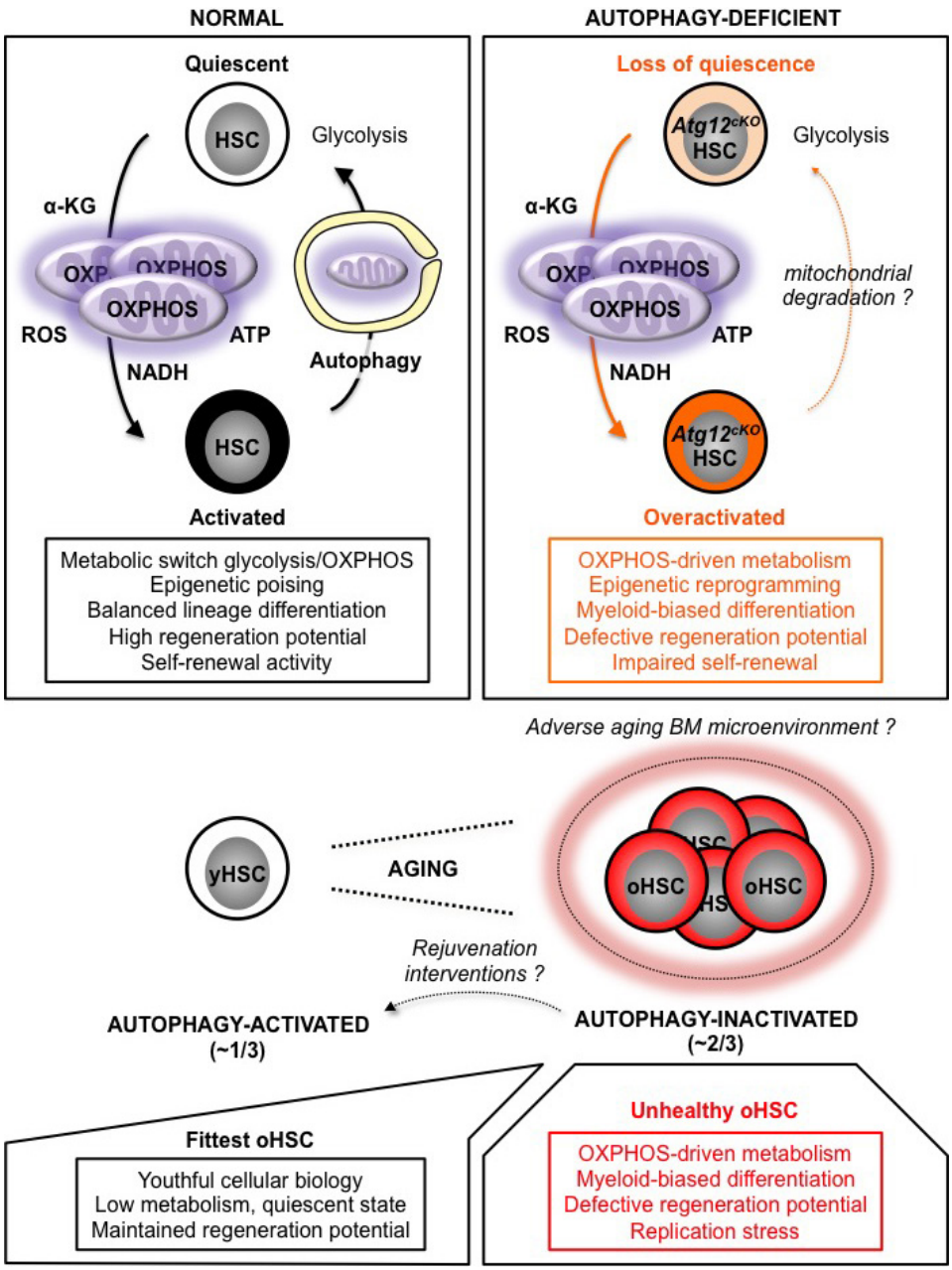


Extended Data Fig. 8 | Functionality of AT^{lo} and AT^{hi} old HSC subsets. a, Scheme for primary and secondary transplantations (tplx) of AT^{lo} or AT^{hi} young and old HSCs. **b**, Transplantation of AT^{lo} and AT^{hi} oHSC subsets with 15% GFP-LC3 high/low expression cutoff showing donor chimerism (left) and lineage distribution (center) in PB, and HSC chimerism (right) at the indicated times post-tplx in primary (top row) and secondary (bottom row) recipients. **c**, GFP-LC3 levels in AT^{lo} and AT^{hi} oHSCs before transplantation, and in primary and secondary recipients. Results are expressed relative to GFP-LC3 levels in young HSCs (± S.E.M.). **d**, Transplantation of AT^{lo} and AT^{hi} yHSC subsets with 33% GFP-LC3 high/low

expression cutoff showing donor chimerism (left) and lineage distribution (center) in PB, and HSC chimerism (right) at the indicated times post-tplx in primary recipients. Data are mean ± S.D. except when indicated. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.



Extended Data Fig. 9 | Autophagy capability of AT^{lo} and AT^{hi} old HSC subsets. a, Representative FACS plot and quantification of CD150 levels in AT^{lo} and AT^{hi} oHSCs. **b**, pS6 levels measured by FC in HSCs cultured ±cyt for the indicated times. Results are normalized to IgG levels. **c**, Drug modulation of autophagy levels in *Gfp–Lc3* oHSCs cultured for 3hr ±cyt; Rap: rapamycin; I128: Ink128; CC: compound-C. Results are expressed as percent GFP-LC3 levels upon 3hr culture in +cyt conditions. **d**, Scheme for the stress experiments assessing the autophagy capability of AT^{lo} and AT^{hi} oHSCs. **e**, **f**, Response to cytokine starvation with (e) GFP-LC3 levels in freshly isolated (t=0) and upon 6hr culture in ±cyt conditions, and (f) autophagy flux after 6hr culture in ±cyt ±BafA conditions. Percent of flux is calculated as [100 x (1 - (-BafA:+BafA))]. **g**, Response to glutamine (glu) deprivation. Data are means ± S.D. (a, b, e, f, g) or ± S.E.M. (c), and are expressed relative to AT^{lo} oHSC (a, e) or freshly isolated (t=0) oHSC (b) levels, or relative to +glu conditions (g). ***$p \leq 0.001$.



Extended Data Fig. 10 | Model for the role of autophagy in HSC function and HSC aging. HSC activation is accompanied by mitochondria activation and a shift in metabolic activity from glycolysis to OXPHOS, which provides energy and increases the production of mitochondrial metabolites such as α-ketoglutarate (α-KG) that act as substrates/co-factors for epigenetic

enzymes. Metabolically activated HSCs are poised to undergo lineage priming and produce differentiated progeny to regenerate the blood system. However, activated HSCs must also return to quiescence to maintain the stem cell pool. In this context, autophagy plays an essential role by clearing activate mitochondria to allow OXPHOS-driven HSCs to efficiently revert to a mostly glycolysis-based metabolic quiescence. Without autophagy, HSCs display an overactive OXPHOS-driven metabolism that promotes myeloid-biased differentiation and loss of stemness as a consequence of epigenetic reprogramming. Other mechanisms of mitochondria elimination likely allow some autophagy-deficient HSCs to return to quiescence during homeostasis, but they do not substitute for autophagy in maintaining HSC function in conditions of intense regeneration stress like transplantation. This role of autophagy becomes even more important with age as the inability of ~ 2/3 of oHSCs to activate autophagy results in an overactive OXPHOS metabolism that impairs self-renewal, promotes proliferation and myeloid differentiation, and contributes to replication stress. These unhealthy oHSCs drive most of the aging blood phenotypes. In contrast, ~ 1/3 of oHSCs activate autophagy, control their metabolic activity, and are the fittest old stem cells that retain functional abilities in an adverse aging BM microenvironment. As all oHSCs remain competent for autophagy induction, it will be exciting to test whether rejuvenation interventions aimed at activating autophagy in unhealthy autophagy-inactivated oHSCs will improve the health of the aging blood system.

Online Methods

Mice. Young (6-12 weeks) and old (24-28 months) wild type C57Bl/6 mice of both genders were either bred and aged in house, or obtained from the National Institute on Aging (NIA) aged rodent colonies. *Mx1-Cre*[34], *Atg12*$^{flox/flox}$*:Mx1-Cre* [13], *Atg5*$^{flox/flox}$ [35], *Park2*$^{-/-}$ [19] and *Gfp-*

Lc3 [22] and were all on a pure C57Bl/6 background and have been described previously. *Atg5*$^{flox/flox}$*:Mx1-Cre* mice were generated by crossing *Atg5*$^{flox/flox}$ with *Mx1-Cre* mice. Old (24-31 months) *Gfp-Lc3* mice were aged in house. For *Mx1-Cre*-mediated deletion, 4-week-old mice were injected intraperitoneally (i.p.) three times 2 days apart with 125 µg poly(I/C) (pIC, GE Healthcare) in 100 µl PBS. pIC-injected *Atg12*$^{flox/flox}$ or *Atg5*$^{flox/flox}$ mice were used as controls (Cnt). pIC-injected *Atg12*$^{flox/flox}$*:Mx1-Cre* and *Atg5*$^{flox/flox}$*:Mx1-Cre* conditional knock-out (cKO) mice were used 2-3 months post-pIC injection, unless otherwise indicated. For deletion in transplanted mice, recipients were injected 2 months after transplantation with pIC as described above. For mouse studies, no specific randomization or blinding protocol was used, animals of both genders were utilized, and all experiments were performed in accordance with UCSF IACUC approved protocols.

***In vivo* assays.** For 5-fluorouracil (5-FU; Sigma-Aldrich) treatment, mice were injected i.p. with 150 mg/kg 5-FU or vehicle (PBS) for four times once a month and analysed for blood parameters by regular bleeding every 3-5 days. For *in vivo* starvation experiments, mice were deprived of food for 24hr with free access to water. For transplantations experiments, 8-12 week old CD45.1 C57Bl/6-Boy/J recipient mice were lethally irradiated (11Gy, delivered in split doses 3hr apart) using a Cs^{137} source (J.L. Shepherd), and injected retro-orbitally with either 250-1000 purified CD45.2 HSCs delivered together with 250,000 Sca-1-depleted helper CD45.1 BM cells, or just with $2x10^6$ unfractionated CD45.2 BM cells. Transplanted mice were kept on antibiotic-containing water for 4 weeks, and analyzed for donor-derived chimerism by monthly bleeding. Peripheral blood (PB) was obtained via retro-orbital bleeding, and collected in 4 ml of ACK (150 mM NH_4Cl/10 mM $KHCO_3$) containing 10 mM EDTA for flow cytometry analyses, or in EDTA-coated tubes (Becton Dickenson) for complete blood counts (CBC). CBC analyses were

performed using a Hemavet hematology system (Drew Scientific). For EdU incorporation, mice were injected with 100 µl of 1 mg/ml 5-ethynyl-2 deoxyuridine (EdU, Thermo Fisher Scientific, A00144) 3hr before sacrifice. For N-acetylcysteine (NAC) treatments, mice were given water containing 1 mg/ml NAC (Sigma-Aldrich, A7250-100G) for 8 weeks starting on the day of the final pIC treatment.

Flow cytometry. Hematopoietic stem and progenitor cells were analysed and/or isolated as described[36]. BM cells were obtained by crushing leg, arm and pelvic bones (with eventually sternum and spines for some experiments) in Hanks' Buffered Saline Solution (HBSS) containing 2% heat-inactivated FBS (staining media, SM), and single cell suspensions of splenocytes by mechanical dissociation between two slides of whole spleens. Erythrocytes were removed by ACK lysis, and single cell suspensions of BM cells were purified on a Ficoll gradient (Histopaque 1119, Sigma-Aldrich). BM cells were then enriched for c-Kit$^+$ cells using c-Kit microbeads (Miltenyi Biotec, 130-091-224) and an AutoMACS cell separator (Miltenyi Biotec) or MACS Separation LS Columns (Miltenyi Biotec, 130-042-401). For cell analyses, unfractionated BM cells were incubated with purified rat anti-mouse lineage antibodies (CD3 from BioLegend, and CD4, CD5, CD8, B220, Ter119, Mac-1 and Gr-1 from eBioscience) followed by goat anti-rat-PE-Cy5 (Invitrogen, A10691) and subsequently blocked with purified rat IgG (Sigma-Aldrich). Cells were then stained with c-Kit-APC-eFluor780 (eBioscience, 47-1171-82), Sca-1-PB (BioLegend, 108120), CD48-A647 (BioLegend, 103416), CD150-PE (BioLegend, 115904) and Flk2-bio (eBioscience, 13-1351-82) followed by either SA-PeCy7 (eBioscience, 25-4317-82) or SA-Qdot605 (Invitrogen, Q10101MP) for HSC staining, or together with CD34-FITC (eBioscience, 11-0341-85) and FcγR-PerCP-eFluor710 (eBioscience, 46-0161-82) for combined HSC/myeloid progenitor staining. For *Gfp-Lc3* mice analyses and dye

staining requiring FITC and/or PE channels, HSCs were stained instead with Lin/PE-Cy5, Sca-1-PE-Cy7 (BioLegend, 108114), CD48-PB (BioLegend, 1103418), CD150-APC (BioLegend, 1115910), and Flk2-Bio followed by SA-PeCy7. For myeloid progenitor analyses in *Gfp-Lc3* mice, cells were separately stained with Lin/PE-Cy5, c-Kit-APC-eFluor780, Sca-1-PB, FcγR-PerCP-eFluor710 and CD34-Bio (Biolegend, 119304) followed by SA-Qdot605. For HSC chimerism analyses, HSCs were stained with Lin/PE-Cy5, c-Kit-APC-eFluor780, Sca-1-PB, CD48-A647, CD150-PE, and Flk2-bio followed by SA-Qdot605, together with CD45.2-FITC (eBioscience, 11-0454-85) and CD45.1-PE-Cy7 (eBioscience, 25-0453-82). For mature cell analyses, unfractionated BM cells were stained with Mac-1-PE-Cy7 (eBioscience, 25-0112-82), Gr-1-PB (eBioscience, 57-5931-82), B220-APC-Cy7 (eBioscience, 47-0452-82), CD19-PE (eBioscience, 12-0193-82), CD4-FITC (BD Biosciences, 553729) and CD8-PE (Biolegend, 100908). For PB chimerism analyses, cells were stained with Mac-1-PE-Cy7, Gr-1-PB, B220-APC-Cy7, CD3-APC (eBioscience, 17-0032-82) and Ter-119-PE-Cy5 (eBioscience, 15-5921-83) together with either CD45.2-FITC or CD45.2-Bio follows by SA-Qdot605 and CD45.1-PE (eBioscience, 12-0453). Stained cells were re-suspended in SM with 1 μg/ml propidium iodide (PI) to exclude dead cells. Sorted or gated HSCs were analysed for cell size and NADH autofluorescence using forward scatter (FSC) and UV450/50 channels, respectively. For ROS, mitochondrial mass and ER analyses, sorted HSCs were washed with HBSS, incubated for 15 min at 37°C with dichlorofluorescin diacetate (DCFDA, 10 μM, Invitrogen, C400), Mitosox Red (MTR, 5uM, Thermo Fisher Scientific, M36008), Mitotracker Green (MTG, 100 nM, Thermo Fisher Scientific, M7514), or ER-Tracker Green (100 nM, Thermo Fisher Scientific, E34251) in HBSS. For mitochondrial membrane potential or autophagy analyses, sorted HSCs were incubated for 15 min at 37°C with tetramethylrhodamine-ethyl-ester (TMRE, 200 nM, Enzo,

enz-52309) or Cyto-ID (1:1000, Enzo, ENZ-51031-0050) in SM. HSCs were then washed with SM, re-suspended in SM containing 1 μg/ml PI, and analyzed for dye fluorescence in the FITC or PE channels. For intracellular staining, sorted HSCs were washed in PBS, fixed in Cytofix/Cytoperm buffer (BD Biosciences), washed in PermWash (BD Biosciences), permeabilized with CytoPerm Plus (BD Biosciences) for 10 min at room temperature (RT), re-fixed in Cytofix/Cytoperm buffer for 10 min at 4°C, washed in PermWash and incubated in PermWash overnight at 4°C with the following primary antibodies: rabbit anti-phospho-S6 (Cell Signaling, 2211) or anti-phospho-S6 A488 (Cell Signaling, 4854), and guinea pig anti-p62 (Progen, GP62-C). Cells were then washed in PermWash and incubated in PermWash for 2hr at 4°C with the appropriate secondary antibody: anti-rabbit A488 (Invitrogen, A21206) or A594 (Invitrogen, A11037) and anti-guinea pig A488 (Invitrogen, A11073) or A647 (Abcam, ab150187). Cells were washed once more in PermWash and re-suspended in PermWash for analyses. For intracellular bromodeoxyuridine (BrdU) staining, Cytofix/Cytoperm treated HSCs were incubated for 30 min with 0.5 U/μl DNAseI in 3% BSA/0.2x PBS/5mM MgCl2/2mM CaCl2 at RT, washed in PermWash, incubated with mouse anti-BrdU primary antibody for 30 min at RT, washed in PermWash, incubated with APC-conjugated goat anti-mouse (Thermo Fisher Scientific, A21241) secondary antibody for 30 min at RT, washed once more in PermWash and re-suspended for analyses. For intracellular Ki67 and 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, 32670-5MG-F) staining, unfractionated BM cells were first stained with Lin/PE-Cy5, c-Kit-APC-eFluor780, Sca-1-PE-Cy7, CD150-PE and CD48-A647 as described above, and then stained with anti-Ki67 (eBioscience, 11-5698-80) in PermWash for 2hr at 4°C. Cells were then washed with PermWash, re-suspended in PBS/3% FBS containing 1 μg/ml DAPI, and incubated for, 20 min prior to analysis. Cell isolation was performed on a

FACSAria III (Becton Dickenson) using double sorting, and cell analyses were performed using a FACSAria or FACS LSR II using DIVA software (Becton Dickenson).

Cell culture. All cultures were performed at 37°C in a 5% CO_2 water jacket incubator (Thermo Fisher Scientific). Cells were grown in StemPro34 medium (Invitrogen) supplemented with penicillin (50 U/ml)/streptomycin (50 μg/ml) and L-glutamine (2 mM), and with or without the following cytokines (±cyt): SCF (25 ng/ml), Flt3L (25 ng/ml), IL-11 (25 ng/ml), IL-3 (10 ng/ml), GM-CSF (10 ng/ml), Epo (4 U/ml) and Tpo (25 ng/ml) (Peprotech). For self-renewal cultures, only SCF, IL11, and Flt3L were used. Bafilomycin A (BafA, 5 nM, Sigma, B1793-2UG), rapamycin (Rapa, 200 nM, EMD Millipore, CAS 53123-88-9), Ink128 (200 nM, gift from Dr. D. Ruggero), AICAR (5mM, Sigma, A9978), Compound C (CC, 40 μM, EMD Millipore, 171260) or corresponding vehicles (i.e., DMSO or water) were added to the ±cyt media when indicated. For glutamine starvation experiments, L-glutamine was omitted from the +cyt medium. For apoptosis and ATP assays, HSCs (400 cells per well) were sorted directly into 40 μl of SM media in 384-well solid white luminescence plates and 40 μl of either Caspase-Glo 3/7 (Promega, G8091) or CellTiter-Glo (Promega, G7570) was then directly added to each well. Both assays were performed according to manufacturer's instructions and read on a luminometer (Synergy2, BioTek). For colony forming unit (CFU) assays, unfractionated BM cells (10,000 cells per 1 ml in 3 cm dish) or sorted HSCs (100 cells per 1 ml in 3 cm dish, or 1 cell in 100 μl per well of a 96-well plate) were cultured in methylcellulose (Stem Cell Technologies, M3231) supplemented with L-Glutamine, Penicillin/Streptomycin and the cytokines described above, together with BafA when indicated. Three 3 cm dishes or 60-72 individual cells in 96-well plate were scored per condition after 8 to 12 days of culture. For measurement of glucose uptake, HSCs (400-2000 cells) were grown in +cyt media containing 100 μM 2-(n-(7-nitrobenz-2-oxa-

1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBD glucose, Invitrogen, N13195). Cells were then washed once in SM, re-suspended in SM containing 1 μg/ml PI, and analyzed for 2-NBD glucose fluorescence in the FITC channel. For measurement of glucose uptake in GFP-LC3 HSCs, 6000 HSCs were cultured with 100 μM 2-deoxyglucose (2-DG) for 4hr and then processed according to manufacturer's instructions (Glucose Uptake-Glo Assay, Promega, J1341). For BrdU incorporation assays, HSCs (1000-2000 cells) were directly sorted into 96-well plates, cultured for 3hr with 60 μM BrdU (Sigma, B5002) and analysed by flow cytometry as described above. For measurement of nascent protein synthesis, HSCs were cultured with, 20 μM O-propargyl-puromycin (OP-puro, Thermo Fisher Scientific, C10457) for 30 min in +cyt media, processed according to the manufacturer's instructions, and analysed by immunofluorescence staining as described below. For replication stress assays, cells were cultured with the same cocktail of cytokines and aphidicolin (50 ng/ml, Sigma, A4487) in Iscove's modified Dulbecco's media (IMDM) supplemented with 5% FBS (StemCell Technology, 06200), 1x penicillin/streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 50 μM 2-mercaptoethanol.

Immunofluorescence staining. HSCs (400-1500 cells) were sorted directly onto poly-lysine coated slides (VWR International, P-4981) and fixed in PBS/4% PFA for 10 min at RT. Slides were permeabilized in PBS/0.15% Triton-X100 for 2 min at RT and blocked in PBS/1% BSA for 1hr at RT. Slides were then incubated in PBS/1% BSA with either rabbit anti-FoxO3a (Millipore, 07-1719), mouse anti-phospho-H2AX (Ser139) (Millipore, 05-636), rabbit anti-53BP1 (Novus Biologicals, NB100-904), rabbit anti-FBL (Cell Signaling, 2639), mouse anti-RAD51 (Abcam, ab1837), mouse anti-LAMP1 (Developmental Studies Hybridoma Bank, 1D4B), mouse anti-KDEL (Abcam, ab12223) or rabbit anti-TOM20 (Santa Cruz Biotechnology, sc-11415) for 1hr at

37°C. Slides were then washed three times in PBS and incubated for 1hr at 37°C in PBS/1% BSA with appropriate secondary antibodies (all from Life Technologies): A488-conjugated goat anti-mouse (A-11029), A594-conjugated goat anti-rabbit (A-11037), A594- or A488-conjugated goat anti-rat (A-11007, A-10528), and A488-conjugated donkey anti-mouse (A-21202). Slides were finally washed three times in PBS and mounted using VectaShield (Vector Laboratories) containing 1 μg/ml DAPI. EdU incorporation was detected using A594-labeled azide click chemistry according to the manufacturer's instruction (Life Technologies, Click-iT® EdU imaging assay, C10339). For OP-puro analysis, after processing, slides were stained with anti-phospho-H2AX and then A488-conjugated goat anti-mouse. Cells were imaged on a SP5 Leica Upright Confocal Microscope (20x or 63x objective), and images were processed using Volocity software (v.4.4, Improvision, Waltham, MA, USA) and ImageJ. Between 5 to 8 z-stacks were taken per image, and total fluorescence per cell was measured using Volocity on ≥, 20 cells per condition.

***In vitro* assays.** For Western blot analyses, sorted HSCs (~30,000-cells per condition) were lysed in RIPA buffer, run on standard 12% SDS-page gels, transferred onto nitrocellulose membranes (Bio-Rad, 162-0232) and blocked with 5% bovine serum albumin (BSA) in blocking buffer (Li-COR Biosciences, 927-40000) containing 0.1% Tween (BBT) for 1hr at RT according to standard protocols. Membranes were incubated for overnight at 4°C in BBT with primary antibodies in BBT, washed once with BBT, incubated for 1hr at 4°C in BBT with appropriate secondary antibodies, and detected on an Odyssey Infrared Imaging System (Li-COR Biosciences). Membranes were eventually stripped with NewBlot Stripping Buffer Nitro (Li-COR Biosciences, 928-40030) according to manufacturer's instructions before being re-incubated with another primary antibody. Rabbit anti-phospho-AMPKα (Cell Signalling, 2535),

rabbit anti-phospho-S6 (Cell Signalling, 2215), mouse anti-S6 (Cell Signalling, 2317), and rabbit anti-actin (Sigma-Aldrich, A2066) were used as primary antibodies and IRDye 800CW Goat Anti-Rabbit IgG (Li-COR Biosciences, 926-32211) or IRDye 800CW Goat Anti-Mouse IgG (Li-COR Biosciences, 926-32210) as secondary antibodies. For Seahorse metabolic flux experiments, oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using a 96-well Seahorse Bioanalyzer XF 96 according to manufacturer's instructions (Agilent Technologies). In brief, HSCs or LSK cells (75,000 cells per well) were sorted directly into 96-well plates pre-coated for 3hr with poly-lysine (Sigma-Aldrich, P4707) and containing cyt+ media. Plates were then centrifuged for 5 min at 1200 rpm and media was replaced with 175 µl of temperature/CO2 pre-adjusted +cyt media. Plates were either immediately analyzed or cultured for 22hr prior analysis done according to manufacturer's instructions with the following exceptions. For glycolysis assays, the starting +cyt media already contained glucose and 2 µM Oligoymycin followed by 1 M 2-DG were used. For OXPHOS assays, 1 µM Oligomycin followed by 2 µM FCCP were used. For both assays, mix and read times were also extended to 7 min and 4 min, respectively. For electron microscopy (EM) analyses, cells (40,000-75,000 per condition) were pelleted for 5 min at 4°C at 600 g, fixed on ice for 30 min in 0.1M NaCacodylate, pH 7.4, containing 2% Glutaraldehyde and 1% PFA, and pelleted at 3000 g for 10 min at 4°C. Samples were then submitted to the Gladstone Institute (UCSF) Electron Microscopy Core Facility for standard transmission electron microscopy ultrastructural analyses. Mitochondria numbers and morphology were visually scored. For aKG measurements, 10^6 c-kit-enriched BM cells were processed according to manufacturer's instructions (Biovision, K677-100). For SAM measurements, 15,000 HSCs were processed according to manufacturer's instructions (Mediomics, 1-1-1004).

Gene expression analyses. For microarray analyses, RNA was purified from sorted HSCs and GMPs (7,000-20,000 cells obtained from individual mice) using the Arcturus PicoPure RNA Isolation Kit, amplified using Ovation Pico WTA System V2 (NuGEN, 3302-12), fragmented and biotinylated using the Encore Biotin Module (NuGEN, 4200-12), and hybridized on Affymetrix Gene ST 1.0 microarrays according to manufacturers' instructions. Transcriptome profiling was performed at the Gladstone Institute Genomics Core (UCSF). Gene expression was first measured at the probe set level (n=241,619) using the RMA (Robust Multi-array Average) methodology followed by quantile normalization[37]. Quality of the data was assessed using principal component analysis (PCA) as well as unsupervised hierarchical clustering. Probe set annotation for the MoGene1.0st v1 array was downloaded from Affymetrix's website and the 241,619 probe sets were mapped to the 28,853 main transcript set (excluding controls). This data set was used for all analyses. We used a family of generalized, linear models implemented with the R package, LIMMA, to assess differential expression between groups of interest[38]. Nominal *p*-values were corrected for multiple comparisons using a false discovery rate (FDR) cutoff of 0.05. Gene Set Enrichment Analyses (GSEA) were performed according to standard procedures on curated gene sets from previous publications. Normalized expression values for each replicate were used to generate an expression data set file, and GSEA was run using the weighted enrichment score. DAVID was used to functionally annotate the differentially expressed or methylated genes to GO terms. For Fluidigm analyses, the Fluidigm 96.96 Dynamic Array IFC were performed as previously described[36]. Briefly, HSCs or MPPs (100 cells/well) were directly sorted into 96 well-plates containing CellsDirect lysis buffer (Invitrogen, 11753-100), reverse-transcribed and pre-amplified for 18 cycles using Superscript III Platinum Taq DNA polymerase (Invitrogen, 18080-044) using a custom-made set of 96 proprietary target-specific primers

(Fluidigm). The resulting cDNAs were analyzed on a Biomark system (Fluidigm) using EvaGreen Sybr dye (Bio-Rad, 172-5211). Data were collected with Biomark Data Collection Software (Fluidigm) and analyzed using Biomark qPCR analysis software with a quality threshold of 0.65 and linear baseline correction. Melt curves and Tm values for each assay reaction were checked individually, and reactions with melt curves showing multiple peaks or poor quality were discarded, leaving 69 genes for further analyses. For gene expression quantification, data were exported as an Excel .csv file and analyzed by the ΔΔCt method using *Actb* normalization.

Methylation analyses. DNA was isolated from sorted HSCs (4,000-24,000 cells obtained from individual mice) using the Allprep DNA/RNA Mini Kit (Qiagen, 80204) and used for library preparation. Enhanced reduced-representation bisulfite sequencing (ERRBS) was performed as previously described[39], except for gel size selection as only fragments of 150-450 bp were excised due to the low input amount of starting DNA. Libraries were sequenced on an Illumina HiSeq 2500. Reads were aligned against a bisulfite-converted mouse genome (mm10) using Bowtie and Bismark (versions 2.2.6 and 0.14.5, respectively). After filtering and normalizing by coverage, differentially methylated cytosines (DMC) were identified using MethylKit (version 0.9.4) and R statistical software (version 3.2.1)[40]. Significant differentially methylated regions (DMR) were identified with eDMR (version 0.6.3.1) using the following parameters ≥ 2 DMC, ≥ 3 CpG, mean methylation difference ≥, 20, and DMR q-value ≥ 0.05. DMRs were annotated to the RefSeq genes as previously described[41]. DAVID was used to functionally annotate the differentially methylated genes to GO terms, SP_PIR_KEYWORDS, and KEGG Pathways with an FDR cutoff of 0.1. ChIP-enrich was used to functionally annotate the differentially methylated

genes to GO terms and KEGG Pathways with an FDR cutoff of 0.05. Maximum gene set size was limited to 500, and DMR were annotated to the nearest gene

Statistics. All experiments were repeated as indicated. *N* indicates the numbers of independent biological repeats. Numbers of independent experiments are reported in the supplementary data. Data were expressed as means ± standard deviation (S.D.), or standard error of the mean (S.E.M.) as indicated when a group within an experiment had less than 3 independent repeats, and for all line graph visualization of results. Mice for transplantation were randomized, samples were alternated whenever possible, and no blinding protocol was used. No statistical method was used to predetermine sample size. *P*-values were generated using two-tailed student's t-test, and were considered significant when ≤ 0.05.

Data availability. Datasets that support the findings of this study have been deposited online with the GEO accession numbers GSE81721 and GSE81721. Source data for all the figures are provided with the paper.

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Supplementary Information

Autophagy maintains the metabolism and function of young and old stem cells. Ho, T.T., Warr, M.R., Adelman, E., Lansinger, O.M., Flach, J., Verovskaya, E.V., Figueroa, M.E.& Passegué, E.

Supplementary Table 1 | Functional annotation of autophagy-deficient HSC and GMP microarray gene expression analyses.

HSCs

Term	Count	Fold Enrichment	Benjamini	FDR
Downregulated DEGs				
Negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	5	43.4	2.6E-03	7.0E-03
Antigen processing and presentation	6	15.7	2.7E-03	3.3E-02
Immune response	8	8.2	1.2E-02	6.3E-02
Upregulated DEGs				
Response to virus	8	38.8	3.3E-07	1.5E-06
Defense response to virus	8	19.6	1.9E-05	1.7E-04
Double-stranded RNA binding	6	36.6	4.8E-05	5.7E-04

GMPs

Term	Count	Fold Enrichment	Benjamini	FDR
Downregulated DEGs				
Acetylation	70	2.80	4.1E-13	4.6E-18
Phosphoprotein	67	2.77	1.2E-12	5.9E-13
Nucleus	67	2.78	1.6E-12	8.0E-12
Nucleus	107	2.09	1.6E-11	6.3E-11
Cytoplasm	29	4.40	5.8E-09	5.3E-06
Mitochondrion	63	2.24	5.3E-08	7.5E-06
Mitochondrion	48	2.56	1.5E-07	7.1E-05
rRNA processing	132	1.57	2.2E-06	2.3E-04
Nucleolus	17	6.78	5.6E-06	2.5E-04
Cytoplasm	18	5.87	8.0E-06	3.1E-04
Ribosome biogenesis	72	1.76	3.4E-05	8.2E-04

Ribosome biogenesis	72	1.76	3.4E-05	4.3E-03
Nucleoplasm	28	2.85	5.8E-05	6.2E-03
Poly(A) RNA binding	30	2.69	6.3E-05	9.5E-03
Redox-active center	41	2.36	8.3E-05	5.9E-02
rRNA processing	19	4.20	3.3E-04	8.1E-02
Hydrolase	28	2.43	7.8E-04	8.3E-02
Upregulated DEGs				
Immunity	33	4.3	3.3E-09	1.6E-08
Immune system process	32	4.3	3.2E-08	3.2E-08
Response to virus	16	9.6	7.1E-08	1.4E-07
Innate immunity	24	5.3	3.1E-08	3.1E-07
Defense response to virus	19	5.8	2.8E-06	8.6E-06
Antiviral defense	15	7.8	6.7E-07	1.0E-05
Innate immune response	26	3.3	1.5E-04	6.1E-04
Staphylococcus aureus infection	8	7.5	1.6E-02	9.9E-02

DAVID analyses of downregulated and upregulated (FDR < 0.1) differentially expressed genes (DEG) with a p-value < 0.01 between Cnt and *Atg12*^{cKO} HSCS or GMPs (n = 4).

Supplementary Table 2 | Fluidigm qRT-PCR measurements of gene expression changes in autophagy-deficient and activated HSCs.

Genes	***Atg12*^{cKO} HSC**	**aHSC**
Axin2	1.72 ± 0.13*	0.43 ± 0.10
Bax	1.35 ± 0.37	2.78 ± 0.48*
Bbc3	n.d.	0.30 ± 0.13*
Bcl2	1.10 ± 0.20	0.58 ± 0.03
Bcl2l1	1.13 ± 0.21	1.46 ± 0.19***
Birc2	0.91 ± 0.07	0.39 ± 0.04***
Bmi1	0.92 ± 0.13	0.63 ± 0.07**
Cbx7	0.89 ± 0.20	0.27 ± 0.05***
Ccl3	0.73 ± 0.54	0.09 ± 0.04**
Ccna2	1.42 ± 0.37	1.64 ± 0.22
Ccnb1	1.04 ± 0.21	0.43 ± 0.09***
Ccnd1	1.49 ± 0.17	0.92 ± 0.18
Ccne1	1.24 ± 0.89	2.04 ± 0.35
Cd34	1.28 ± 0.45	0.59 ± 0.06*
Cd48	1.45 ± 0.64	3.59 ± 0.87*
Cdc20	1.04 ± 0.23	0.35 ± 0.1**
Cdk2	0.26 ± 0.09	3.47 ± 0.49
Cdkn1b	0.89 ± 0.18	0.36 ± 0.06**
Cebpa	0.85 ± 0.23	0.93 ± 0.52
Csf1r	0.56 ± 0.28	0.28 ± 0.13*
Csf2ra	0.89 ± 0.30	0.29 ± 0.08**
Csf3r	0.76 ± 0.04**	0.74 ± 0.24
Dnmt3a	0.87 ± 0.10	0.45 ± 0.05**
Egr1	0.65 ± 0.21	0.10 ± 0.01**
Epor	0.91 ± 0.06	1.05 ± 0.19
Ezh1	0.79 ± 0.06*	0.32 ± 0.06**
Ezh2	1.33 ± 0.20	0.94 ± 0.11
Flt3	1.44 ± 0.48	0.36 ± 0.05**
Fn1	1.62 ± 1.85	4.28 ± 0.77*
Foxo3	0.72 ± 0.11	0.42 ± 0.06**
Fzd2	6.97 ± 5.02	0.13 ± 0.05*
Gata2	0.98 ± 0.22	0.50 ± 0.02***
Gfi1	0.66 ± 0.08	0.29 ± 0.16*
Gfi1b	1.10 ± 0.45	0.63 ± 0.11
Hes1	1.57 ± 0.55	0.05 ± 0.04*
Hes5	1.27 ± 0.28	0.79 ± 0.06
Hhip	n.d.	n.d.
Hif1a	0.85 ± 0.15	1.08 ± 0.12
Id1	0.47 ± 0.21	1.73 ± 0.37
Ikzf1	0.98 ± 0.18	0.75 ± 0.07
Il6	0.55 ± 0.29	0.09 ± 0.03***
Il7r	0.60 ± 0.26	0.12 ± 0.13**

Irf8	0.68 ± 0.17	0.35 ± 0.17*
Jun	1.00 ± 0.08	0.02 ± 0.00***
Lrp5	0.80 ± 0.10	0.95 ± 0.10
Mcl1	0.89 ± 0.22	1.02 ± 0.11
Mfng	0.84 ± 0.04*	0.44 ± 0.05***
Mki67	1.58 ± 0.31	1.49 ± 0.18
Mpl	0.74 ± 0.12	0.40 ± 0.05***
Myc	1.14 ± 0.32	0.65 ± 0.08*
Nfe2l2	0.74 ± 0.04**	0.63 ± 0.10*
Nfkbia	0.87 ± 0.07	0.32 ± 0.06**
Pax5	1.03 ± 0.43	0.11 ± 0.03*
Pdk4	0.71 ± 0.19	0.09 ± 0.05**
Prkdc	1.15 ± 0.01	0.58 ± 0.06*
Ptch1	0.82 ± 0.05*	0.72 ± 0.06**
Rad51	1.30 ± 0.26	2.06 ± 0.41
Rpa1	1.16 ± 0.22	1.56 ± 0.10*
Runx1	0.96 ± 0.25	0.84 ± 0.07
Sfpi1	0.92 ± 0.11	0.90 ± 0.15
Slamf1	0.95 ± 0.22	0.80 ± 0.14
Smad7	0.71 ± 0.07	0.39 ± 0.13*
Xiap	0.94 ± 0.09	0.53 ± 0.06**
Xrcc5	0.98 ± 0.10	0.75 ± 0.09
Xrcc6	0.97 ± 0.09	0.77 ± 0.06
Zfpm1	1.05 ± 0.39	0.29 ± 0.02**

HSCs isolated from either control (Cnt) or *Atg12*^{cKO} mice (n = 3), and freshly isolated wild type quiescent HSCs (qHSC) or HSCs activated (aHSC) by 21hr +cyt *in vitro* culture (n = 4) were used for Fluidigm gene expression analyses (3 technical pools of 100 cells/mouse). Results are mean ± SD and are expressed as fold changes compared to levels in Cnt HSCs for *Atg12*^{cKO} HSCs, and qHSCs for aHSCs; n.d., not detected, *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001.

Supplementary Table 3 | Fluidigm qRT-PCR measurements of gene expression changes in autophagy-deficient MPPs.

	MPP2		MPP3		MPP4	
Genes	**Cnt**	***Atg12^{cKO}***	**Cnt**	***Atg12^{cKO}***	**Cnt**	***Atg12^{cKO}***
Axin2	2.69 ± 0.99	3.09 ± 0.83	1.41 ± 0.32	1.27 ± 0.84	1.00 ± 0.62	0.76 ± 0.25
Bax	0.76 ± 0.14	0.84 ± 0.16	1.23 ± 0.11	1.06 ± 0.20	0.61 ± 0.04	0.71 ± 0.11
Bbc3	0.23 ± 0.21	0.55 ± 0.23	n.d.	n.d.	0.54 ± 0.39	n.d.
Bcl2	0.85 ± 0.12	0.96 ± 0.13**	0.58 ± 0.05	0.82 ± 0.14*	1.22 ± 0.06	1.46 ± 0.32
Bcl2l1	0.50 ± 0.10	0.71 ± 0.12	0.97 ± 0.20	0.71 ± 0.06	0.44 ± 0.11	0.45 ± 0.02
Birc2	0.47 ± 0.09	0.49 ± 0.09	0.40 ± 0.14	0.40 ± 0.06	0.62 ± 0.22	0.48 ± 0.02
Bmi1	0.46 ± 0.06	0.59 ± 0.07*	0.46 ± 0.06	0.48 ± 0.05	0.50 ± 0.06	0.48 ± 0.00
Cbx7	0.34 ± 0.05	0.41 ± 0.06	0.11 ± 0.04	0.13 ± 0.05	0.55 ± 0.24	0.37 ± 0.03
Ccl3	0.95 ± 0.23	0.94 ± 0.27	1.52 ± 0.51	1.55 ± 0.66	5.23 ± 2.21	5.27 ± 2.05
Ccna2	1.50 ± 0.56	2.24 ± 0.28	1.69 ± 0.13	1.49 ± 0.13	1.24 ± 0.29	1.40 ± 0.08
Ccnb1	1.76 ± 0.22	1.75 ± 0.11	2.18 ± 0.34	2.09 ± 0.26	2.40 ± 0.31	2.79 ± 0.31
Ccnd1	0.26 ± 0.03	0.41 ± 0.09*	0.12 ± 0.05	0.25 ± 0.17	0.82 ± 0.21	0.84 ± 0.32
Ccne1	1.63 ± 0.39	1.89 ± 0.08	1.44 ± 0.16	1.29 ± 0.10	1.01 ± 0.17	1.00 ± 0.05
Cd34	0.75 ± 0.29	1.23 ± 0.19	1.35 ± 0.33	1.57 ± 0.68	2.72 ± 0.19	2.88 ± 0.23
Cd48	23.3 ± 6.88	32.4 ± 9.99*	47.9 ± 10.1	46.5 ± 5.24	30.7 ± 11.3	46.3 ± 1.74
Cdc20	1.66 ± 0.33	1.7 ± 0.27	1.73 ± 0.24	2.13 ± 0.36	2.93 ± 0.55	3.38 ± 0.50
Cdk2	0.44 ± 0.37	0.16 ± 0.10*	0.40 ± 0.25	0.12 ± 0.08**	0.22 ± 0.12	0.17 ± 0.04
Cdkn1b	0.59 ± 0.12	0.74 ± 0.15	0.37 ± 0.07	0.46 ± 0.1**	0.57 ± 0.07	0.55 ± 0.04
Cebpa	8.74 ± 3.31	8.93 ± 3.4	34.2 ± 4.13	32.9 ± 0.18	15.8 ± 0.73	15.7 ± 3.42
Csf1r	2.49 ± 0.94	1.69 ± 0.53	4.14 ± 0.54	3.75 ± 0.51	4.33 ± 1.02	5.21 ± 1.25
Csf2ra	0.53 ± 0.21	0.67 ± 0.13	0.57 ± 0.17	0.69 ± 0.15	0.49 ± 0.07	0.48 ± 0.05
Csf3r	0.40 ± 0.01	0.49 ± 0.07	1.45 ± 0.46	1.35 ± 0.24	1.39 ± 0.33	1.22 ± 0.13
Dnmt3a	0.39 ± 0.08	0.56 ± 0.16	0.43 ± 0.10	0.49 ± 0.05	0.76 ± 0.07	0.67 ± 0.08
Egr1	0.42 ± 0.44	0.55 ± 0.39	0.05 ± 0.03	0.09 ± 0.06	0.19 ± 0.17	0.15 ± 0.12
Epor	2.01 ± 1.08	1.80 ± 0.45	0.14 ± 0.11	0.10 ± 0.07	n.d.	n.d.
Ezh1	0.27 ± 0.08	0.34 ± 0.02	0.33 ± 0.07	0.34 ± 0.07	0.55 ± 0.17	0.52 ± 0.08
Ezh2	1.65 ± 0.33	1.64 ± 0.08	1.44 ± 0.3	1.26 ± 0.06	1.32 ± 0.36	1.18 ± 0.07
Flt3	0.43 ± 0.21	1.00 ± 0.38	0.81 ± 0.42	1.32 ± 0.71	15.9 ± 3.27	17.0 ± 3.87
Fn1	0.81 ± 0.91	1.07 ± 0.72	0.22 ± 0.06	0.21 ± 0.13	0.18 ± 0.11	0.29 ± 0.21
Foxo3	0.42 ± 0.22	0.63 ± 0.07	0.23 ± 0.02	0.30 ± 0.12	0.57 ± 0.21	0.52 ± 0.02
Fzd2	12.2 ± 4.94	9.16 ± 8.06	7.86 ± 2.89	11.8 ± 1.04	49.9 ± 23.7	33.6 ± 3.27
Gata2	0.68 ± 0.22	0.96 ± 0.29	0.14 ± 0.02	0.21 ± 0.11	0.11 ± 0.03	0.11 ± 0.00
Gfi1	0.93 ± 0.21	0.56 ± 0.46	6.39 ± 0.68	5.98 ± 0.88	2.48 ± 0.40	2.67 ± 0.38
Gfi1b	0.82 ± 0.26	1.09 ± 0.32	0.16 ± 0.08	0.21 ± 0.08	0.19 ± 0.06	0.15 ± 0.02
Hes1	0.39 ± 0.36	0.49 ± 0.2	n.d.	0.62 ± 0.64	0.17 ± 0.17	n.d.
Hes5	0.70 ± 0.27	0.56 ± 0.28	0.36 ± 0.16	0.44 ± 0.07	2.85 ± 1.19	1.96 ± 0.61
Hhip	0.79 ± 0.20	0.54 ± 0.31	1.56 ± 0.78	1.22 ± 0.64	1.39 ± 0.36	2.77 ± 0.46
Hif1a	0.67 ± 0.09	0.57 ± 0.04	1.16 ± 0.41	0.96 ± 0.17	0.64 ± 0.16	0.61 ± 0.06

Id1	1.28 ± 0.52	1.43 ± 0.51	1.22 ± 0.46	1.90 ± 0.49*	3.46 ± 2.04	2.86 ± 0.67
Ikzf1	0.98 ± 0.40	1.06 ± 0.16	1.20 ± 0.07	1.10 ± 0.07	1.25 ± 0.13	1.23 ± 0.08
Il6	1.44 ± 0.36	1.33 ± 0.39	1.48 ± 0.32	1.73 ± 0.32	4.03 ± 2.10	3.18 ± 0.54
Il7r	1.98 ± 0.41	1.44 ± 0.24	1.93 ± 0.53	2.30 ± 0.21	4.16 ± 0.92	4.69 ± 1.16
Irf8	7.35 ± 3.42	4.23 ± 4.33	8.99 ± 5.57	11.2 ± 0.87	6.65 ± 3.11	9.99 ± 2.98
Jun	0.97 ± 0.52	1.39 ± 0.53	0.36 ± 0.13	0.44 ± 0.10	0.53 ± 0.18	0.55 ± 0.11
Lrp5	0.43 ± 0.09	0.53 ± 0.08	0.52 ± 0.06	0.50 ± 0.04	1.07 ± 0.26	1.11 ± 0.13
Mcl1	0.36 ± 0.10	0.49 ± 0.12	0.50 ± 0.05	0.52 ± 0.08	0.35 ± 0.01	0.35 ± 0.05
Mfng	0.25 ± 0.04	0.30 ± 0.04	0.25 ± 0.04	0.27 ± 0.03	0.57 ± 0.18	0.49 ± 0.06
Mki67	2.26 ± 0.11	2.38 ± 0.30	1.67 ± 0.44	1.43 ± 0.33	1.26 ± 0.05	1.17 ± 0.18
Mpl	0.17 ± 0.04	0.23 ± 0.04	0.11 ± 0.00	0.14 ± 0.02	0.28 ± 0.10	0.25 ± 0.04
Myc	1.13 ± 0.07	1.16 ± 0.24	1.52 ± 0.59	1.35 ± 0.37	1.46 ± 0.30	1.54 ± 0.46
Nfe2l2	0.60 ± 0.08	0.60 ± 0.03	0.67 ± 0.06	0.63 ± 0.03	0.79 ± 0.21	0.71 ± 0.14
Nfkbia	0.58 ± 0.14	0.57 ± 0.07	0.78 ± 0.27	0.62 ± 0.04	0.68 ± 0.20	0.66 ± 0.06
Pax5	0.32 ± 0.12	0.48 ± 0.32	0.38 ± 0.11	n.d,	0.65 ± 0.57	0.65 ± 0.18
Pdk4	1.44 ± 0.35	1.55 ± 0.53	1.15 ± 0.21	1.43 ± 0.07	3.28 ± 1.32	2.82 ± 0.10
Prkdc	0.63 ± 0.31	1.04 ± 0.04	0.62 ± 0.04	0.63 ± 0.08	0.77 ± 0.17	0.78 ± 0.03
Ptch1	0.42 ± 0.12	0.52 ± 0.07	0.32 ± 0.04	0.33 ± 0.08	0.61 ± 0.10	0.55 ± 0.03
Rad51	1.50 ± 0.46	1.98 ± 0.39	1.61 ± 0.18	1.48 ± 0.31	0.99 ± 0.19	0.87 ± 0.03
Rpa1	0.78 ± 0.27	1.04 ± 0.04	0.86 ± 0.01	0.83 ± 0.02*	0.69 ± 0.09	0.68 ± 0.08
Runx1	0.56 ± 0.22	0.74 ± 0.13	0.29 ± 0.07	0.37 ± 0.12	0.48 ± 0.13	0.51 ± 0.12
Sfpi1	0.89 ± 0.32	0.98 ± 0.13	3.04 ± 0.28	2.66 ± 0.06	1.25 ± 0.29	1.45 ± 0.05
Slamf1	0.23 ± 0.03	0.33 ± 0.07	0.00 ± 0.00	0.01 ± 0.00	n.d.	0.00 ± 0.00
Smad7	0.42 ± 0.16	0.40 ± 0.19	0.28 ± 0.06	0.25 ± 0.08	0.64 ± 0.19	0.54 ± 0.06
Xiap	0.45 ± 0.04	0.58 ± 0.12	0.50 ± 0.10	0.46 ± 0.08	0.48 ± 0.06	0.42 ± 0.04
Xrcc5	0.53 ± 0.09	0.63 ± 0.05	0.34 ± 0.06	0.34 ± 0.06	0.68 ± 0.26	0.60 ± 0.28
Xrcc6	0.85 ± 0.11	0.86 ± 0.02	1.01 ± 0.20	0.91 ± 0.11	1.16 ± 0.30	1.18 ± 0.17
Zfpm1	1.06 ± 0.14	1.19 ± 0.42	0.05 ± 0.01	0.09 ± 0.05	0.20 ± 0.03	0.16 ± 0.01

MPPs isolated from either Cnt or *Atg12*^{cKO} mice (n = 3) were used for Fluidigm gene expression analyses (3 technical pools of 100 cells/mouse). Results are mean ± SD and are expressed as fold changes compared to levels in Cnt HSCs; n.d., not detected, *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$.

Supplementary Table 4 | Functional annotation of autophagy-deficient HSC ERRBS results

Hypomethylated DMRs		
Term	Count	FDR
Phosphoprotein	380	8.8E-18
Nucleus	222	1.3E-06
Armadillo repeat-containing domain	8	2.9E-05
Transcription regulation	103	1.5E-04
Transcription	105	2.1E-04
Protein binding	205	2.6E-04
Alternative splicing	220	2.9E-04
Ubl conjugation	89	2.6E-03
Cadherin, N-terminal	13	7.0E-03
Membrane	310	8.6E-03
Nucleus	274	9.2E-03
Cytoplasm	296	1.2E-02
Cytoplasm	196	1.4E-02
Homophilic cell adhesion via plasma membrane adhesion molecules	20	2.0E-02
Transcription, DNA-templated	106	3.1E-02
Cell adhesion	34	5.2E-02
DNA-binding	84	8.2E-02
Receptor activity	19	9.0E-02
Hypermethylated DMRs		
Term	Count	FDR
Nucleus	56	1.5
Phosphoprotein	84	1.6

DAVID analyses of hypomethylated (FDR < .1) and hypermethylated (FDR < 2) differentially methylated regions (DMR) between Cnt and *Atg12*^{cKO} HSCs (n = 4).

Supplementary Table 5 | Functional annotation of activated HSCs ERRBS results

Description	p-value	FDR
Spongiotrophoblast layer development	3.2E-08	1.0E-04
Cell differentiation involved in embryonic placenta development	7.2E-08	1.0E-04
Positive regulation of tyrosine phosphorylation of Stat3 protein	4.3E-07	5.0E-04
Negative regulation of transcription factor import into nucleus	7.0E-07	7.0E-04
Regulation of tyrosine phosphorylation of Stat3 protein	1.0E-06	8.0E-04
Tyrosine phosphorylation of Stat3 protein	2.3E-06	1.4E-03
Regulation of gliogenesis	3.9E-06	2.1E-03
Regulation of astrocyte differentiation	8.8E-06	4.1E-03
Gliogenesis	1.5E-05	6.1E-03
Positive regulation of tyrosine phosphorylation of STAT protein	1.7E-05	6.4E-03
Negative regulation of protein import into nucleus	2.3E-05	7.9E-03
Microtubule binding	1.1E-05	8.8E-03
Negative regulation of gliogenesis	3.2E-05	9.8E-03
Negative regulation of phosphorus metabolic process	4.0E-05	1.1E-02
Negative regulation of nucleocytoplasmic transport	4.0E-05	1.1E-02
Regulation of tyrosine phosphorylation of STAT protein	4.3E-05	1.1E-02
Visual learning	5.0E-05	1.2E-02
Negative regulation of transmembrane transport	7.0E-05	1.3E-02
Visual behavior	7.0E-05	1.3E-02
Negative regulation of intracellular protein kinase cascade	7.3E-05	1.3E-02
Regulation of transcription factor import into nucleus	7.4E-05	1.3E-02
Tyrosine phosphorylation of STAT protein	7.4E-05	1.3E-02
Negative regulation of intracellular protein transport	8.1E-05	1.3E-02
Positive regulation of JAK-STAT cascade	8.2E-05	1.3E-02
Myelin sheath	5.8E-05	1.4E-02
Basement membrane	6.6E-05	1.4E-02
Xenobiotic catabolic process	9.7E-05	1.5E-02
Transcription factor import into nucleus	1.1E-04	1.7E-02
Negative regulation of intracellular transport	1.3E-04	1.8E-02
Astrocyte differentiation	1.3E-04	1.8E-02

GO pathway analyses by ChIP-enrich of both hypomethylated and hypermethylated DMRs between qHSCs and aHSCs (FDR < 0.02) (n = 5).

CHAPTER 5

Discussion

I. Autophagy regulates HSC fate through metabolic and epigenetic regulation

Requirement for autophagy across different ages

Because previous studies had found that autophagy in the blood system was so critical for organismal survival during fetal development, it was surprising that conditional loss of autophagy in the adult blood system resulted in no severe health problems. Furthermore, aside from expansion of myeloid cells, the blood system was remarkably unremarkable, with no severe lymphopenia or anemia that is seen in fetal deletion, and normal HSC numbers, compared to the complete HSC depletion after fetal deletion. This highlights the importance of using conditional deletion after full development, as fetal HSCs are extremely different in function as well as in characteristics, as they are rapidly proliferating and responsible for generation of the entire blood system, compared to the largely quiescent and metabolically inactive adult HSCs. Therefore there is a far greater requirement for autophagy in fetal HSCs compared to steady state adult HSCs. Yet it is still surprising that the blood system is largely functional with a loss of autophagy.

However, under strong regenerative stress conditions of HSC transplantation, the lack of autophagy resulted in severe defective regenerative ability of *Atg12* cKO HSCs, and HSC exhaustion and depletion after 5 months, which was drastically exacerbated in secondary transplantation, which now resembles what is observed in fetal HSCs. Therefore the need for autophagy in HSCs may not have to do with the fetal age or development per se, but rather with the functions and highly replicative state. While *Atg12* cKO HSCs initially engraft and produce blood, they rapidly decline over the following months, likely due to an over-proliferation and

differentiation and subsequent exhaustion of the stem cell compartment. This would be similar to the original *Atg12* cKO mice which exhibit an overall increase in blood cells in the peripheral blood, spleen, and bone marrow, as well as expanded MPP, and GMP numbers, effectively demonstrating an overall over-proliferation and differentiation all the way down the hierarchy towards mature myeloid cells. This aligns perfectly with the *in vitro* proliferation and differentiation data and the overall model of precocious myeloid differentiation due to the loss of ability to maintain HSC quiescence. Therefore while autophagy is not necessary for adult HSCs under homeostatic steady state conditions, it becomes extremely essential during times of intensive regenerative stress and to maintain the quiescent HSC reserves.

Interestingly, compared to fetal or young adult HSCs, old HSCs appear to reside somewhere in between in terms of their dependence on autophagy. While the myeloid bias and functional deficits of young *Atg12* cKO HSCs greatly resembled those of wildtype old HSCs, the phenotypes of *Atg12* cKO HSCS were even further exacerbated over the course of aging, with even further accelerated myeloid bias and failure to maintain the blood. In the aging of *Atg12* cKO BM, this may perhaps reflect the total regenerative stress that has slowly accumulated over the lifetime of the animals, resulting in an accumulation of over-active old HSCs that precociously proliferate and differentiate with reduced ability to maintain quiescence, which simply has not had the time to happen in young animals. In the acute deletion of *Atg12* in old BM, it was clear that old HSCs depend more on autophagy than young HSCs. This may be due to an activated, inflammatory environment or circulating factors generated and maintained by old blood cells, which may increase the need for autophagy to maintain quiescence (Ergen et al., 2012; Itkin et al., 2016). But together, these data demonstrate that old HSCs have a greater need for autophagy than young HSCs even under steady state, non-regenerative conditions. This is

interesting from a broader perspective, as autophagy has been observed to decline in other tissues, yet is required for many lifespan-extending interventions and genetic mutations, some of which even induce autophagy (Rubinsztein et al., 2011). Therefore an increased need for autophagy during aging, for regulation of metabolism and stem cell control, for proteostasis quality control, or for other functions could help explain these observations.

Requirement for autophagy across different cell types

However, what remains unclear is why loss of autophagy specifically results in overproduction of and bias towards myeloid cells, as opposed to other mature blood cells. Various studies report conflicting results on the role and necessity of autophagy in mature blood cells (Riffelmacher et al., 2017). While seemingly necessary for clearance of organelles during maturation of erythrocytes (Sandoval et al., 2008), as we similarly found, clearly mice with autophagy loss in HSCs and the blood system are still somehow able to produce enough, and even normal amounts of red blood cells as we found. Studies conflict on the importance of autophagy in lymphocytes, but many report that autophagy is necessary for their formation or proper maturation (Riffelmacher et al., 2017). In contrast, most studies find that myeloid cell production does not require autophagy, which would explain the extreme myeloid bias in autophagy knockout models. However, one study reports that it is essential for monocyte-macrophage differentiation (Zhang et al., 2012). Interestingly, a more recent study reported that myeloid cells, but not other mature cells or HSCs, utilize an alternative form of autophagy that does not require traditional *Atg* genes, which allows them to survive and persist in traditional autophagy deletion mouse models (Cao et al., 2015). A comprehensive profiling of autophagy

activity and role across many types of blood cells had not been done, but our analyses of GFP-LC3 levels show that there are vastly different levels of GFP-LC3 protein present in different cell types, suggesting very different levels of autophagy activity and dependence. This would make sense when considering the widely varying functions and activities of different cell types. Therefore the skewed blood production seen in autophagy knockout models may be due to differing dependencies on autophagy of individual mature cell types for their production and/or survival, as opposed to direct effects on differentiation proclivities of HSCs. The specific role and necessity of autophagy in these various cell types warrants further research.

This becomes particularly interesting in the context of aging, where we found widely varying age-associated changes in autophagy levels in different blood cell populations, such as evidence of massively decreased autophagy in old T cells, which are depleted in old mice. Therefore in addition to the ability to maintain high autophagy levels, ability to utilize alternative forms of autophagy may also be important for maintaining certain blood cells during aging.

Cellular compensation for loss of autophagy

In fact, young HSCs themselves likely utilize some other form of cellular recycling or even an alternative form of autophagy to maintain their homeostasis when bulk macroautophagy is deleted, as the accumulated ER, Golgi, vesicles, and lysosomes in *Atg12* cKO HSCs may reflect some sort of protein stress, ER stress, or alternative recycling process in response to or to compensate for the lack of macroautophagy. This might explain the ability of HSCs and blood cells to survive and function adequately during homeostasis despite complete loss of traditional macroautophagy.

Fascinatingly, old HSCs that do not exhibit autophagosomes looked strikingly similar to young *Atg12* cKO HSCs, with expanded ER, Golgi, and vesicles or lysosomes. Therefore, such aberrant cell biology occurs naturally during aging, and utilization of different cellular recycling mechanisms may result from loss of autophagy activity in AT^{lo} old HSCs, and may contribute to their dysfunction during aging. Importantly, mitochondrial UPR and general UPR have been recently found to be important during aging of HSCs (Mohrin et al., 2015). So while we have focused on aberrant mitochondrial biology, alternative forms of autophagy and the cellular compensation response to loss of autophagy merit further study.

Mitophagy

While increased mitochondria as a result of loss of autophagy is not surprising, it is surprising that the expanded mitochondria of *Atg12* cKO HSCs actually had increased membrane potential and activity, as most autophagy knockouts which result in accumulation of dysfunctional or damaged mitochondria that are not cleared by mitophagy, similar to what we found in our stress-mitophagy *Park2* knockout model. This demonstrates that steady state autophagy in HSCs is responsible for regulating metabolic state and clearing normal, active, healthy mitochondria as opposed to damaged mitochondria.

It was even more surprising that *Park2* KO HSCs exhibited a completely normal blood system with perfectly functioning HSCs, especially in light of recent reports of the importance of stress-induced mitophagy for HSCs (Vannini et al., 2016; Ito et al., 2016). While Ito et al claim that mitophagy is essential for self-renewing expansion of HSCs, they only examine the PINK/PARKIN stress-mitophagy pathway, and not the many other mitophagy pathways or even

mitophagy through bulk macroautophagy. Furthermore, the data are largely correlative and not mechanistic as they show that fatty acid oxidation is important for HSCs and stimulates mitophagy, but not that mitophagy plays any specific role. They used siRNA against *Park2*, which in fact actually did not affect cell division or growth at all *in vitro*. They reported reduced transplantation success of a shParkin silenced subset of Tie2 HSCs, but transduction protocols are challenging in HSCs, which differentiate rapidly during this process, and therefore any effects may be on the culture environment and not on normal *in vivo* HSC function/maintenance. They also report reduced transplantation success using a *Pink1* KO model, but *Pink1* has multiple other roles in mitochondrial fission, mitochondrial motility, and even ROS removal, any of which may be even more important for HSC function in transplantation than stress-mitophagy, an argument which is buoyed by the fact that we saw absolutely no effects on primary or secondary transplantation in a full *Park2* KO model, in which even stronger effects would be expected.

Vannini et al. show that the mitochondrial membrane potential uncoupler FCCP induces mitophagy in HSCs, which it is already commonly used for, and separately show that FCCP preserves HSC stemness in proliferative *in vitro* culture conditions. They claim that the beneficial effects of FCCP therefore work through mitophagy, but do not show this. This

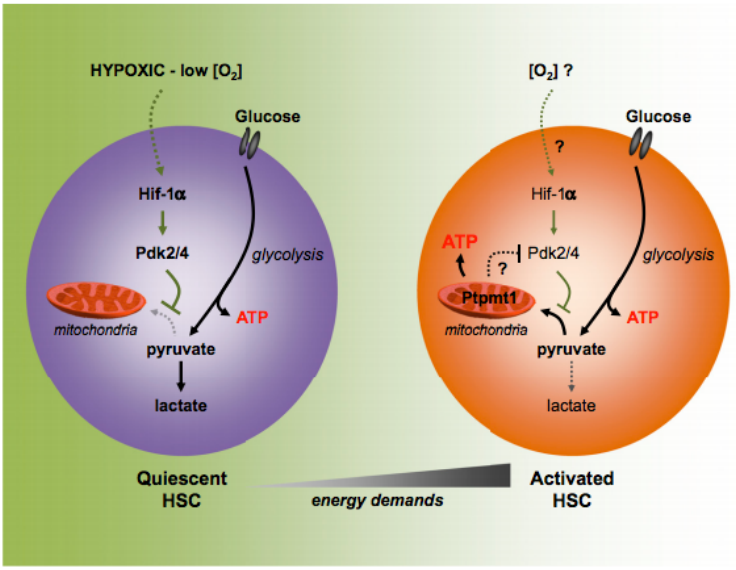

Figure. 1. *Metabolism in HSCs (Warr and Passegué, 2013).*

hypothesis would support our findings and model where mitochondrial levels and activity regulate and drive HSC proliferation and differentiation. We discovered that during growth conditions *in vitro,* HSCs turn off autophagy and rapidly increase mitochondrial content and membrane potential in preparation for proliferation and differentiation, and ETC inhibitor metformin could preserve HSC stemness. We also found that and that loss of autophagy *in vivo* promoted HSC proliferation and differentiation. Therefore FCCP may indeed similarly preserve HSC stemness by inducing mitophagy and reducing mitochondrial content and activity, and/or even simply by reducing mitochondrial metabolism by uncoupling mitochondrial membrane potential. Therefore, based on our data, bulk autophagic clearance of healthy mitochondria is crucial for regulating the energetic and metabolic state of HSCs, but not stress-induced mitophagy of damaged mitochondria.

HSC metabolism

We hypothesized that the increased and overactive mitochondria may directly cause the overactive HSC proliferation, differentiation and exhaustion we observed. Based on three studies of metabolism genes in HSCs that primarily used knockout models discussed above (Takubo et al., 2013; Simsek et al., 2010; Yu et al., 2013), the general model in the field proposes that quiescent HSCs rely on glycolysis for low metabolic activity, but switch to an OXPHOS-dependent high metabolic rate for activation, proliferation, and differentiation into progenitors (Warr and Passegué, 2013).These studies found that mutations to inhibit mitochondrial metabolism resulted in aberrantly high stemness and HSC maintenance with loss of ability to differentiate (Yu et al., 2013), whereas loss of glyocolysis resulted in over-proliferation and

exhaustion (Takubo et al., 2013). However, actual direct measurements of OXPHOS or glycolysis in quiescent versus activated HSCs had never been reported, and no studies have directly examined metabolism at all in activated HSCs, let alone the transition from one to another. Furthermore, how these different types of metabolism and metabolic activity are generally regulated in HSCs, and how they themselves may regulate HSCs, was largely unknown.

Therefore, to understand the biological relevance of the aberrant mitochondrial biology of *Atg12* cKO HSC relative to naturally occurring HSC states, we first conducted comprehensive analyses of metabolically activated HSCs compared to quiescent HSCs. Here for the first time we actually tracked and measured the metabolic transition of quiescent HSCs to activated HSCs *in vitro* and indeed showed that mitochondrial mass and membrane potential increase steadily and significantly. Metabolic measurements in rare and purified stem cell compartments such as HSCs are extremely difficult due to very few cells per mouse, as well as small cell size and low metabolic activity compared to the more abundant, large, and active cell lines or cancer cells for which metabolic assays are often developed and optimized. After significant optimization and with very large numbers of mice and 70,000 HSCs per well, we detected massively increased OXPHOS in activated HSCs compared to quiescent HSCs, definitively showing how mitochondrial metabolism changes during HSC activation.

Furthermore, as might be expected, during activation HSCs grew in size, NADH levels, and glucose uptake, ROS levels, and mTOR is rapidly activated. Interestingly, aligned with activation of mTOR, a well characterized inhibitor of autophagy, autophagy was downregulated during this metabolic and cellular activation, perhaps to prevent mitochondria and other pro-growth contents from being cleared. This is in contrast to muscle stem cells (MuSCs), where

mTOR is also activated during cellular activation (Rodgers et al., 2015), but autophagy is somehow actually also upregulated to provide extra energy during the growth period (Tang et al., 2014). However, MuSCs utilize metabolic pathways very differently than HSCs and actually upregulate glycolysis during activation. But perhaps despite their differences, autophagy may in fact play a similar role in removing mitochondria and remodeling the cells to rebalance metabolic usage, but for activation as opposed to for quiescence.

Autophagic control of metabolism and cell fate

Interestingly, genetic KO of autophagy resulted in strikingly similar results with increased NADH, cell size, glucose uptake, OXPHOS, ROS levels, and nascent protein synthesis. Therefore, loss of autophagy results in HSC phenotypes mirroring the natural reduction in autophagy and metabolic activation of HSCs before proliferation and differentiation, two very activities which *Atg12* cKO HSCs precociously exhibit in the primary mice. Furthermore, direct analysis of cell cycle activity in *Atg12* cKO HSCs indeed revealed a loss of quiescence and increased cycling *in vivo*, as well as increased differentiation at the cost of stemness and self-renewal ability *in vitro*, by genetic or pharmacological autophagy inhibition. These data again correspond to the precocious over-proliferation and myeloid differentiation found in the primary *Atg12* cKO mice. Therefore, *Atg12* cKO HSCs have the cellular and metabolic characteristics that resemble those of normal HSCs in the early stages of cellular activation in preparation for proliferation and differentiation, and subsequently have an aberrant propensity for precocious proliferation and differentiation both *in vitro* and *in vivo*. These data cohesively demonstrate that autophagy acts as a gatekeeper of HSC metabolism, quiescence, and

differentiation, and that without autophagy, HSC mitochondrial metabolism goes unchecked, quiescence cannot be maintained, and precocious proliferation and differentiation ensues.

Reactive oxygen species

Increased ROS production is often a byproduct of increased mitochondrial membrane potential and activity, is believed to increase during aging and contribute to cellular aging, and often has deleterious effects such as causing DNA damage (Finkel and Holbrook, 2000). While ROS levels in *Atg12* cKO HSCs were indeed elevated, surprisingly they did not result in any appreciable DNA damage or apoptosis. ROS have also been found to promote myeloid progenitor differentiation in flies (Owusu-Ansah and Banerjee, 2009), and have been implicated in multiple mouse models with similar precocious myeloid proliferation and differentiation and HSC exhaustion (Chandel et al., 2016). Therefore, we hypothesized that the increased ROS in *Atg12* cKO HSCs may serve as a signaling trigger from the increased mitochondrial metabolism to promote differentiation. Reducing ROS levels with the antioxidant ROS-scavenger NAC has successfully rescued over-proliferative defects in HSCs from multiple other mouse models (Chandel et al., 2016). Yet surprisingly, NAC was unable to ameliorate precocious myeloid differentiation of *Atg12* cKO HSCs *in vitro* or *in vivo*, with myeloid cell and MPP expansion still prominent. Therefore ROS is not a key mediator of the phenotypes in autophagy deficient HSCs.

Metabolic regulation of epigenetics

Because metabolism can directly affect and regulate epigenetic modifications and regulation, as metabolites and metabolic byproducts serve as cofactors and substrates for many epigenetic enzymes, we hypothesized that perhaps the overactive mitochondrial metabolism in *Atg12* cKO HSCs may result in altered epigenetic marks that promote proliferation and differentiation. In particular, aKG is produced during the TCA cycle, and is an essential co-factor for DNA and histone demethylases. Indeed, we found extremely elevated levels of aKG in *Atg12* cKO bone marrow, as well as very comparably elevated levels in activated BM, demonstrating that activated OXPHOS indeed results in elevated aKG levels. Correspondingly, we found significant DNA hypomethylation in 783 regions in *Atg12* cKO HSCs. In HSCs that had been activated for 21 hours, we also observed significant hypomethylation, but at far fewer regions, perhaps due to insufficient time. Therefore mitochondrial-driven metabolic activation of HSCs, either due to loss of autophagy or during normal growth activation, may promote DNA hypomethylation.

To determine what genes may be affected by this DNA hypomethylation, we conducted microarray analyses on *Atg12* cKO HSCs for gene expression. Yet surprisingly, we found no individual significantly differentially expressed genes besides Atg12, likely due to subtle changes and insufficient sample size for statistical power. However, when analyzing sets of related genes with GSEA, we found enrichment for myeloid genes and genes that are upregulated during aging, and loss of HSC-identity genes, corresponding to our biological data and phenotypes. In addition, microarray analysis of *Atg12* cKO GMPs revealed far more significant gene changes, enriched for mitochondrial and metabolic terms. This indicates that epigenetic changes in HSCs may not greatly affect gene expression in HSCs, but rather the gene

expression of downstream progenitor progeny, and/or that loss of autophagy may have even greater direct effects in progenitor populations.

Additional, more precise, multiplexed microfluidic qRT-PCR analyses confirmed few and small gene expression changes, and we conducted similar analyses of activated HSCs compared to quiescent HSCs. Interestingly, in *Atg12* cKO HSCs compared to control, there were trends towards increased cell cycle activator genes and decreased self-renewal, lymphoid, and cell cycle inhibitor genes, corresponding to our cellular phenotypes. Of note, PDK4, which shunts pyruvate from OXPHOS into glycolysis, trended towards decreased expression in *Atg12* cKO HSCs and was significantly decreased in activated HSCs. Almost all of these trends and genes were similarly but even further changed in activated HSCs as well as MPP2s. These data indicate that while loss of autophagy and metabolic activation may have only subtle effects on gene expression in HSCs, their gene expression begins to resemble that of activated HSCs and more mature MPP2s.

Therefore the metabolic and epigenetic reprogramming of autophagy-deficient HSCs results in small gene expression changes that render them similar to activated HSCs, MPPs, and old HSCs instead of quiescent HSCs, with increased myeloid gene expression, and this reprogramming is perhaps amplified and manifested in further gene expression changes in downstream in progenitor populations.

Metabolic and epigenetic regulation of cell fate

Finally, to directly test the mechanistic role of such metabolic and epigenetic remodeling in HSC cell fate decisions, we added demethylase co-factor aKG, methyl donor SAM, or

OXPHOS inhibitor metformin to activating HSCs *in vitro.* Even under strong activating culture conditions, aKG promoted even faster differentiation, while SAM and metformin slowed differentiation and maintained stemness. This indicates that the increased levels of aKG from OXPHOS in autophagy-deficient or activated HSCs is sufficient to promote rapid differentiation, while inhibiting OXPHOS or maintaining methylation is sufficient to preserve stemness and prevent differentiation.

As mentioned above, this aligns with a report that uncoupling mitochondria and lowering membrane potential with FCCP similarly prevents HSC differentiation *in vitro* (Vannini et al., 2016). Interestingly, SAM was previously found to similarly inhibit myoblast proliferation and differentiation (Fuso et al., 2001), and recently aKG was likewise found to promote differentiation of human pluripotent stem cells while its counterpart succinate delayed differentiation (TeSlaa et al., 2016). In contrast, aKG promotes self-renewal in ES cells, while succinate promotes differentiation (Carey et al., 2015). Therefore the same important co-factors for epigenetic enzymes can directly regulate stem cell fate in different ways, depending on the type of stem cell.

Autophagy regulates HSC fate through metabolic and epigenetic regulation

Altogether, we have directly linked mitochondrial activation and metabolism to methylation and to regulation of HSC cell fate and differentiation during HSC activation, and furthermore have identified autophagy as a critical gatekeeper of this regulation. While under homeostatic steady state conditions, without autophagy the blood system is capable of maintaining relatively healthy, functional blood production, it results in a precocious cellular and

metabolic activation of HSCs which results in precocious proliferation and differentiation and loss of their regenerative capacity.

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II. Autophagy defines distinct subsets of old HSCs

Old HSCs resemble autophagy-deficient HSCs

Old HSCs share many phenotypes with *Atg12* cKO HSCs such as significant and nearly identical loss of regenerative ability and significant myeloid bias. We also found that old HSCs closely resembled metabolically activated autophagy-deficient HSCs in many cellular characteristics, with increased cell size, NADH, and ATP. Furthermore, with the first metabolic data on old stem cells ever, we found increased OXPHOS, similar to *Atg12* cKO LSK cells, and decreased glycolysis in old HSCs. Our lab previously also observed a loss of quiescence in old HSCs (Flach et al., 2014) nearly identical to *Atg12* cKO HSCs, and in CFU assays old HSCs remarkably resembled young *Atg12* cKO HSCs or WT HSCs treated with BafA to inhibit autophagy, with reduced stemness and increased differentiation. Furthermore, BafA addition to old HSCs strikingly even further exacerbated the phenotypes with an overall loss of regenerative ability, greatly reduced stemness and almost complete differentiation. Therefore again, the need for autophagy drastically increases with age. In addition, the subtle gene expression changes in autophagy-deficient HSCs resembled those that change with age in WT HSCs. Altogether, loss of autophagy causes many premature aging phenotypes, rendering autophagy-deficient HSCs very similar to old HSCs.

Because of these similarities, our thorough cellular analysis of autophagy-deficient HSCs has also provided insight into the biology of naturally aged, dysfunctional old HSCs. While there are very few known mechanisms of HSC aging, altered metabolic regulation and activity may be an important driver of HSC age-associated dysfunction. Based on our data and previous studies,

a proper balance of high glycolytic and low OXPHOS activity are important for maintaining the proper balance of HSC self-renewal and HSC proliferation and differentiation. In the same way that altering this metabolic balance in genetic models can profoundly reduce HSC functional ability, natural skewing during aging may have similar effects. Old HSCs resemble both autophagy-deficient HSCs and activated HSCs, and the altered metabolism in all three may similarly cause their activated state, loss of quiescence, and increased differentiation observed in all three. This may help explain their reduced regenerative capacity, as cycling HSCs are known to be far less functional than quiescent HSCs (Passegué et al., 2005). Why exactly this is remains unclear, perhaps due to cellular remodeling in preparation for proliferation and differentiation at the expense of self-renewal ability. While we did not examine epigenetic profiles of old HSCs, their altered metabolism may also remodel their epigenetic state to impart their classical aging phenotypes such as myeloid bias. Altogether these data suggest that the deregulated metabolism and the activated state we have discovered in old HSCs contributes to their age-associated dysfunction.

However, some characteristics of old HSCs did not resemble autophagy-deficient *Atg12* cKO HSCs. *Atg12* cKO HSCs did not expand in number prematurely, or at an accelerated rate during aging, nor did they have yH2AX foci indicating replication stress. And old HSCs on average did not have increased mitochondrial mass by Mitotracker green dye, and surprisingly had significantly decreased mitochondrial membrane potential. This was particularly surprising considering their increased OXPHOS activity, although the increase was primarily the maximum capacity as opposed to steady state OXPHOS. Alternatively, the increased OXPHOS as measured by increased oxygen consumption may reflect reduced efficiency or coupling of the electron transport chain, explaining increased readings yet reduced mitochondrial membrane

potential. This may indicate that it is in fact the actual process or byproducts of attempted mitochondrial metabolism that play such important roles in HSC biology, as opposed to the actual energy produced. In that case, the inefficiency of mitochondrial metabolism in old HSCs may explain some of their dysfunction. This reduction in membrane potential may be due to unhealthy mitochondria with mitochondrial protein folding stress observed in old HSCs (Mohrin et al., 2015), in contrast to the healthy mitochondria that accumulate in young autophagy-deficient HSCs. In line with reduced mitochondrial membrane potential, we actually observed decreased levels of ROS. This was very surprising in light of the prevailing idea that ROS increases with age generally, and in HSCs (Porto et al., 2015). Furthermore, we found an average decrease in nascent protein synthesis in contrast to autophagy-deficient HSCs. Finally, when we compared the differentially methylated regions of DNA from *Atg12* cKO HSCs compared to previously published data on old wildtype HSCs (Sun et al., 2014), we found very little overlap. Therefore, while old and *Atg12* cKO HSCs were similar in many regards, in certain important characteristics they were not, thus it was essential to determine exactly how autophagy changed with age in old HSCs to mediate these varying similar and disparate metabolic and cellular changes.

Autophagy levels during aging

While old HSCs largely resembled autophagy-deficient HSCs, our lab had previously observed evidence of aberrantly activated basal autophagy in old HSCs that was absent in young HSCs (Warr et al., 2013). Our previous study utilized electron microscopy to detect large autophagosomes present in some old HSCs but not young, yet this was a qualitative comparison.

Therefore to begin to address these questions, we first set out to quantitatively profile autophagy activity across the blood system of young and old mice. We utilized Gfp-Lc3 mice to measure autophagy levels, as Lc3 protein is degraded during autophagic flux, and so its levels are usually inversely correlated to autophagy activity. This is not necessarily always the case as a snapshot measurement of Lc3 reflects not only the rate of flux through autophagosomes, but also the baseline levels as well as generation of new Lc3 protein. Therefore additional measurements after blockade of autophagic flux are often also required, and so we used multiple methods to measure autophagy in HSCs to confirm that Gfp-Lc3 levels indeed inversely correlated to autophagy activity, such as flux assays with BafA, *in vitro* starvation experiments, *in vivo* starvation experiments, the autophagy dye CytoID, and electron microscopy after starvation.

Autophagy levels and activity are widely reported to decline with age in all of the few other tissues that have been studied (Cuervo, 2008). However many of these studies use whole unfractionated tissue lysates without resolution of individual cell populations. Indeed, when we measured Gfp-Lc3 levels in unfractionated total whole bone marrow, we found increased Gfp-Lc3 in old animals indicating decreased autophagy. Furthermore, the highly abundant mature B and T cells also exhibited increased Gfp-Lc3 representing decreased autophagy, and T cells had been previously reported to reduce autophagy levels with age (Puleston et al., 2014). Furthermore, some of the more abundant and mature progenitor populations such as GMPs and MPP4s appeared to have decreased autophagy as well. Therefore at a whole tissue-level and in the more abundant mature populations, it seems that autophagy declines during aging, similar to reports in other tissues.

However in contrast, the most rare, immature, and important HSC and MPP2/3 stem and progenitor compartments exhibited decreased Gfp-Lc3 indicating increased autophagy in old

mice. The increased autophagy in old MPP2/3 populations was even more pronounced than in HSCs, and future experiments should examine its significance, especially since MPPs are even more important for steady state day-to-day maintenance of the blood system than HSCs. However, the HSCs from old mice were highly variable, usually exhibiting increased autophagy, but sometimes also a wider distribution with cells with decreased autophagy as well. Therefore averaging the mean fluorescence intensities of Gfp-Lc3 old HSCs obscured the changes at the extremes and failed to accurately reflect the increased autophagy. However when gating cells based on the highest and lowest levels of autophagy, it was evident that there was a significant increase in autophagy in a subset of about 1/3rd of old HSCs, and decreased autophagy in a fraction as well. To confirm that these cells truly had activated autophagy, we re-examined our previous electron microscopy images of old HSCs and found that again, roughly 1/3 exhibited clear autophagosomes, while the rest did not. Similarly, measuring autophagy levels with an autophagy-sensitive CytoID dye, approximately 33% of old HSCs had increased autophagy levels compared to young HSCs.

Therefore to reconcile the previously seemingly conflicting data, we found that old HSCs do indeed exhibit increased basal autophagy compared to young HSCs, but actually only in a small subset of about 1/3rd of the cells, while the majority exhibit the same or even decreased levels. And while bulk analyses of unfractionated total bone marrow displayed an overall decrease in autophagy with age, the most important stem and progenitor cells were revealed to have increased autophagy with age by precise isolation of these rare cell compartments. This highlights the perils of analyzing bulk tissues without cellular granularity.

Interestingly, populations that decline with age such as B and T cells and MPP4s, exhibited reduced autophagy with age, while those that stayed constant or expanded like myeloid

cells or HSCs had the same or increased autophagy. This raises the intriguing possibility that autophagy is protective of blood cells during aging to allow them to survive an adverse aging environment and to maintain or expand in number, while loss of autophagy activity results in depletion of certain populations. Therefore ability to utilize autophagy during aging may contribute to the shift in numbers and ratios of blood cells during aging, such as the progressive myeloid bias and HSC expansion.

We next set out to determine how these increased autophagy levels were regulated and maintained. mTOR is the best characterized inhibitor of autophagy, and we had found that mTOR activation coincided with reduction of autophagy during HSC activation. Therefore we hypothesized that perhaps decreased levels of mTOR activity during aging promoted increased autophagy. However in contrast, a previous study reported significantly increased mTOR activity in old HSCs compared to young. Surprisingly, in multiple independent experiments we consistently found absolutely no difference in mTOR activity in old HSCs compared to young HSCs, neither decreased or increased.

Identification of subsets of old HSCs

Having discovered the heterogeneity of autophagy levels in old HSCs, we realized the possibility that analyses of total unfractionated HSCs would average out the data, obscuring possible differences between the old subsets and comparisons to young HSCs. Furthermore, the subsets with higher and lower autophagy levels could explain why some data on unfractionated old HSCs resembled autophagy-deficient HSCs, while others did not. Therefore, we were next determined to utilize this Gfp-Lc3 reporter system to resolve this heterogeneity to deconstruct

the biology of old HSCs. Why a subset of old HSCs aberrantly activate autophagy whereas others and young HSCs do not was unclear. And was the activated autophagy in these high autophagy old HSCs a stress response mark of the most damaged and dysfunctional HSCs, or perhaps a sign of a healthy response that played a protective role?

Therefore we fractionated the total HSC population and sorted out the 1/3rd of old HSCs with activated autophagy (AThi) and compared them to the 1/3rd with the lowest autophagy (ATlo) in order to decipher the differences in their underlying biology and answer these important questions. Indeed, separating AThi from ATlo old HSCs now revealed a significant difference in mTOR activity, with AThi old HSCs having reduced levels. Furthermore, we showed that as in young HSCs, mTOR inhibition was sufficient to inhibit autophagy activity in old HSCs as well. Therefore while total old HSCs did not have increased or decreased mTOR activity compared to young HSCs, within the population mTOR activity is heterogeneous and thus explains the differing levels of autophagy in the AThi and ATlo subpopulations.

High autophagy preserves youthful cell biology

As mentioned above, old HSCs without autophagosomes also resembled *Atg12* cKO HSCs in their altered cellular morphology with increased small vesicles, lysosomes, ER, and Golgi, while old HSCs with autophagosomes looked just like young healthy HSCs. While unfractionated old HSCs had no significant difference in mitochondria levels compared to young, we found significantly increased mitochondria in ATlo old HSCs compared to AThi. Furthermore, analysis of EM images likewise revealed that old HSCs that did not exhibit autophagosomes had increased total and elongated mitochondria numbers, in fact almost

identical to young *Atg12* cKO HSCs. In contrast, old HSCs with visible autophagosomes had decreased mitochondria numbers, strikingly almost identical to young control HSCs. Indeed, ATlo old HSCs also had higher mitochondrial membrane potential compared to AThi, albeit still presumably lower than young HSCs, and higher ATP levels and glucose uptake. Furthermore, ATlo old HSCs specifically had increased cell size and NADH levels, with even larger increases compared to young HSCs than unfractionated old HSCs, while AThi old HSCs were small and had less NADH almost identical to young HSCs. In addition we found other characteristics of a mitochondrial-driven over-activated HSC such as increased cell cycle and reduced self-renewal ability. Thus, even the phenotypes of unfractionated old HSCs that perceptibly resembled young autophagy-deficient HSCs were revealed to be further exacerbated specifically in ATlo old HSCs. In summary, the phenotypes of unfractionated old HSCs that resemble young autophagy-deficient HSCs are driven by ATlo old HSCs that have similar characteristics, and not AThi old HSCs that largely resemble young healthy HSCs.

Resolving the heterogeneity of the old HSCs also revealed other features that that provide additional novel insight into the mechanisms of stem cell aging. While we originally detected no increase in ROS in unfractionated old HSCs compared to young, we now discovered increased mitochondrial ROS specifically in ATlo old HSCs but not in AThi old HSCs. So in fact ROS may play a role in HSC aging, but specifically only in ATlo old HSCs. We also found that the yH2AX foci distinctive of replication stress in old HSCs (Flach et al, 2014) were strikingly exclusively only in ATlo old HSCs, but not AThi. Furthermore, we have since found that only ATlo old HSCs are sensitive to additional exogenous replication stress, whereas AThi more resistant like young HSCs. We also found that the reduced overall nascent protein synthesis in

old HSCs appeared to be specific to ATlo old HSCs with large yH2AX foci and not AThi old HSCs.

Therefore these data indicate that the aberrantly high basal levels of autophagy in the subset of AThi old HSCs actually maintain seemingly youthful and healthy cell biology, while low autophagy in ATlo old HSCs results in phenotypes nearly identical to autophagy KO in young HSCs, and these ATlo old HSCs drive many classical and novel phenotypes of old HSCs that we have discovered. We have discovered a novel axis and mechanism of stem cell aging in which low autophagy levels allow aberrant metabolic and cellular activation that promotes cellular aging in some old HSCs and reduces their functional capabilities.

AThi old HSCs are functionally superior to ATlo old HSCs

Finally, to determine if these AThi old HSCs that appear young were actually functionally youthful and superior to ATlo old HSCs, we conducted transplantations into lethally-irradiated young recipient mice. While initially both ATlo and AThi old HSCs engrafted to similar levels, ATlo old HSCs rapidly lost their ability to maintain the blood system, and were almost entirely exhausted and depleted after 4 months, again very similar to young autophagy-deficient HSCs. In contrast, AThi old HSCs maintained surprisingly robust, long-term ability to maintain the blood and a persisting HSC compartment, remarkably even superior to normal unfractionated old HSCs. Transplantation of unfractionated old HSCs in the same experiment resulted in intermediate results between ATlo and AThi old HSCs. This was reproduced in multiple independent experiments, also using different cell numbers, and also using more restrictive cutoffs. Interestingly, there were absolutely no differences between young HSCs with

higher and lower levels of Gfp-Lc3 isolated and transplanted similarly, indicating that this heterogeneity and necessity of high autophagy in HSCs is unique to old and not young HSCs.

Therefore autophagy levels directly and positively correspond to functionality of old HSCs, and old HSCs with high basal autophagy activity not only maintain youthful cell biology characteristics of a young healthy HSC, but also superior functional regenerative ability compared to old HSCs with low autophagy, which resemble autophagy-deficient HSCs with overactive mitochondrial metabolism and an overactive state with severely compromised regenerative abilities.

AThi and ATlo old HSCs are distinct subsets

Due to the relatively Gaussian distribution of Gfp-Lc3 in old HSCs, we set out to determine if autophagy levels truly define distinct, biological subsets of old HSCs, or if they are a random snapshot of a dynamic continuum of HSCs. First, we examined the levels of Gfp-Lc3 in ATlo and AThi old HSCs before and after sorting and even after short-term culture to see if they had equilibrated or returned to a normal distribution. Interestingly, we found that these populations maintained their very distinct levels of autophagy after these short periods. Next, we compared the original levels of Gfp-Lc3 in old ATlo and AThi HSCs before transplantation into recipients to the levels 4 months after transplantation. Strikingly, ATlo HSCs still maintained significantly lower levels of autophagy than AThi, demonstrating that they are truly distinct populations that actively maintain different levels of autophagy, as opposed to a random continuum. However, after the rigors of transplantation and 4 months of residency in a young bone marrow niche, the disparity of autophagy levels between the two populations was

attenuated. Furthermore, we next conducted secondary transplantations of the same re-isolated HSCs from the primary transplants. After an additional 4 months in young secondary recipients, ATlo and AThi old HSCs still maintained slightly different levels of autophagy, yet now further equilibrated to similar levels. Therefore, old HSCs maintain distinct levels of autophagy in an old BM niche and continue to maintain distinct levels even after transplanted into a young BM niche. However, over time these distinct populations are amenable to modulating autophagy levels. It is also important to note the possibility of a selection bias created when re-sorting transplanted HSCs, as perhaps of all of the ATlo old HSCs, the healthiest with the highest autophagy activity survived 4 months and were re-isolated.

Interestingly, the absolute levels of Gfp-Lc3 in both ATlo and AThi old HSCs after transplantation greatly increased, indicating decreased autophagy, relative to untransplanted young control HSCs. In fact, both populations now displayed higher Gfp-Lc3 indicating lower autophagy than the young control HSCs. This may suggest that old HSCs have increased levels of autophagy specifically due to an aged BM niche and microenvironment, whereas once in a young BM niche, all old HSCs utilize lower basal levels of autophagy than young HSCs. However, this may also be due to the strong perturbation of irradiation on the recipient mice and therefore BM niche, and/or due to the stress of transplantation. These concepts could be directly addressed in the future by conducting competitive transplantations of both young and old HSCs with different markers together into the same recipient mice, and/or by conducting transplantations without irradiation using alternative methods such as valine depletion (Taya et al., 2016) or antibody depletion (Czechowicz et al., 2007). Furthermore, transplantations of old AThi and ATlo HSCs into old mice, and young HSCs into old mice, would provide further insight into the role of the aged BM niche in HSC autophagy regulation. Altogether, these data

indicate that ATlo and AThi old HSCs are indeed distinct populations that maintain different levels of autophagy, but which may be cell-extrinsically regulated and modulated by a young microenvironment.

Furthermore, in secondary transplantations, AThi old HSCs still performed slightly better than ATlo, although with far less disparity than in primary transplantations. This suggests that while the distinct functional difference between these populations is in fact cell-intrinsic and maintained over time, perhaps even the aged cell biology and reduced functional capacity of ATlo old HSCs is amenable to rejuvenation by a young niche as well as by autophagy levels. In addition, AThi old HSCs regenerated slightly more lymphoid cells and less myeloid cells, which they did not in the primary transplantations. These data indicate that a young BM niche may be able to rejuvenate some of the aging phenotypes and dysfunction of old HSCs.

Autophagic capabilities of AThi and ATlo old HSCs

Due to the semi-permanent difference in autophagy that was maintained *in vivo* in ATlo and AThi old HSCs, we next wanted to determine if these populations had different intrinsic ability to activate and utilize autophagy. Surprisingly, when put into strong pro-growth *in vitro* culture conditions, both populations were able to rapidly turn off autophagy to similar degrees, and when starved of cytokines or glutamine *in vitro* both were able to activate autophagy to similar levels, unexpectedly even equivalent to young HSCs. This was highly surprising due to their maintenance of different autophagy levels *in vivo* at steady state and even after transplantation into young mice. This demonstrates that all old HSCs still preserve the capacity

to both turn autophagy on and off in response to strong stimuli, and that old HSCs do not actually lose autophagic ability with age as has been reported in other tissues (Cuervo, 2008).

Maintenance of autophagy levels in old HSCs

Yet despite equivalent autophagic capacity, ATlo and AThi old HSCs actively maintain different steady state levels of autophagy *in vivo*. While we found that decreased mTOR activity could explain the high levels of autophagy in AThi old HSCs, why mTOR activity was decreased in these old HSCs, and why specifically in AThi has yet to be determined. The bone marrow niche is known to change with age (Kusumbe et al., 2016), and levels of important factors change in the bone marrow, such as reduction in important cytokines and increased inflammatory factors (Ergen et al., 2012). Furthermore, our lab previously discovered reduced glucose uptake by old HSCs compared to young, perhaps due to reduced cytokine and growth factor signaling. Therefore, perhaps a low cytokine, low nutrient, aging environment stimulates autophagy, and promotes a hormesis stress response that is beneficial to AThi old HSCs.

However, ATlo old HSCs do not activate autophagy despite their preserved autophagic ability. This suggests that they may reside in different micro-niches where specific factors or interactions may regulate and help maintain different autophagy levels. Going forwards it would be interesting to utilized *in situ* imaging to determine exactly where these distinct subsets reside in the BM. Recent studies have found differing properties of HSCs based on location and proximity to blood vessels (Itkin et al., 2016), and megakaryocytes and bone are similarly important for HSC maintenance (Bruns et al., 2014; Morrison and Scadden, 2014). In particular, a very recent study used *in vitro* assays to expose HSCs to plasma and proposed a model in

which HSCs near more permeable blood vessels that received more exposure to blood had increased activation and ROS, promoting migration and differentiation but compromising regenerative and survival abilities (Itkin et al., 2016). These characteristics of HSCs near permeable vessels sound strikingly similar to those of ATlo old HSCs. We also found increased vascular leakiness in bone marrow of old mice, which may increase the number of such activated or ATlo HSCs in old mice which are less abundant or detectable in young mice. HSCs also reside in a highly hypoxic niche, and hypoxia is known to induce autophagy. Therefore the high oxygenation and supply of blood born factors such as pro-growth factors may activate mTOR and inhibit autophagy, resulting in metabolic remodeling and HSC activation. In contrast, perhaps AThi old HSCs reside in separate micro-niches further away from blood flow, and can maintain a high autophagy, low metabolic state.

Role of autophagy in other stem cells

Importantly, our results may have even broader impact beyond the hematopoiesis field. We examined autophagy in the distinct but related tissue of the bone marrow stroma. Unfractionated stromal cells, like most mature blood cells, showed decreased autophagy with age. However, old endothelial cells (EC) and stromal progenitors such as osteoblastic lineage cells (OBC) and stem cell-like mesenchymal stromal cells (MSC) all displayed increased autophagy levels. Furthermore, although no differences were observed between young ATlo and AThi MSCs and OBCs, or between old OBC progenitors, AThi old stem cell-like MSCs had higher colony formation activity than ATlo old MSCs, indicating increased need for autophagy specifically during aging and specifically in the most rare, important stem cell compartments. In

addition, our collaborators have similarly examined autophagy in young and old neural stem cells and likewise have found strikingly parallel results (Leeman et al., in press). Collectively, these findings indicate that autophagy is essential for a variety of types of stem cells and becomes increasingly important during aging to maintain the most healthy and youthful old stem cells, and that autophagy activation may be a general adaptive response of the healthier old stem cells to an adverse aging environment.

Yet there are some tissue-type differences in the role of autophagy in other stem cell populations, in particular muscle stem cells (MuSCs). HSCs and MuSCs differ in a variety of ways, such as using inverse types of metabolism during quiescence versus activation (Chandel et al., 2016; Ryall et al., 2015). In line with this, a recent study found that autophagy was activated and was essential for MuSCs to generate energy during cellular activation (Tang et al., 2014), as opposed to being down-regulated during HSC activation and being essential for HSC quiescence as we found. Similarly, autophagy was reported to be important for preventing MuSC senescence during aging (Garcia-Prat et al., 2016). While mitochondrial levels increased with loss of autophagy in MuSCs, their overall membrane potential decreased and ROS increased, causing senescence. Therefore the role of autophagy in MuSCs is very different than in HSCs. In line with this, autophagy declined with age in MuSCs, as reported widely in other bulk tissues, as opposed to the increased levels in old HSCs. However, despite these differing functions, autophagy is still a beneficial function essential for old MuSCs, and there remains the intriguing possibility that fractionation of old MuSCs based on autophagy levels would similarly distinguish different populations of healthier and more dysfunctional old MuSCs.

Conclusion

This work shows for the first time that autophagy actively suppresses metabolism to maintain stemness, and directly links OXPHOS to DNA methylation in the control of stem cell fate, an axis which could be manipulated for translational applications. It provides the first evidence of increased need for autophagy during aging in stem cells and the first evidence of a subset of healthy HSCs in old mice. In our discovery of ATlo and AThi old HSCs, we established an unprecedented method to prospectively separate healthy from un-healthy old HSCs based on autophagy levels. This enabled us to identify biology causing functional aging, separating it from general chronological aging phenotypes, to discover of a novel mechanism of stem cell aging in which low autophagy enables precocious metabolic and cellular activation that causes cellular aging in the majority of old HSCs and renders them functionally impaired.

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CHAPTER 6

Current work and future directions

I. Causes and consequences of autophagy activation in old HSCs

Dissociating functional from chronological aging to identify new mechanisms of aging

Our discovery of AThi old HSCs may have revealed a more pure and potent population of true HSCs, specifically during aging. The number of phenotypical HSCs defined by the traditional marker strategies increases dramatically with age, by over 10-fold by 24 months of age. However, the function of individual old HSCs on average is significantly reduced, and the pool becomes significantly more variable with age. Young HSCs in contrast are far more homogenous, and we found that separation based on autophagy levels provides no further distinction or purification. A common debate in the field is whether true HSCs should indeed be defined by markers as opposed to their original definition of a single HSC having the ability to reconstitute a lethally-irradiated recipient mouse with multi-lineage engraftment over a long-term period. Therefore although they have identical traditional cell-surface markers, perhaps only AThi old HSCs are true HSCs and do not expand as much with age, and ATlo old HSCs are actually short-term HSCs or MPPs that acquire classical HSC markers.

This heterogeneity of highly variable unfractionated old HSCs may have confounded previous studies, as surprisingly few molecular differences have been detected between young and unfractionated old HSCs in a variety of studies (Flach et al., 2014; Sun et al., 2014; Beerman et al., 2013), likely due to averaging of the disparate AThi and ATlo populations. Our new ability to prospectively purify the most "aged" AT^{lo} and most "youthful" AT^{hi} subsets of old HSCs for the first time enables resolution of this heterogeneity in old HSCs by filtering out the "noise" of the more youthful AThi old HSCs in order to decipher the biology of aging in the truly

dysfunctional subset of ATlo old HSCs that drive the majority of aging phenotypes. We will be able to identify molecular drivers of HSC functional aging by dissociating them from chronological age. We have already discovered that autophagy preserves a subset of old HSCs and identified low autophagy and aberrant metabolic and cellular activation as new mechanisms of HSC aging. Now we will utilize our system to identifying additional novel molecular causes of aging.

We have recently conducted RNAseq on old AT^{lo} , old AT^{hi} , and young HSCs. Preliminary analyses reveal many striking differences between AT^{lo} and AT^{hi} old HSCs, and as hypothesized, the transcriptomes of old AT^{hi} HSCs resemble those of young HSCs far more than old AT^{lo} HSCs. The ability to specifically purify the truly dysfunctional old HSCs reveals drastic changes that were only barely detectable in previous analyses comparing unfractionated old HSCs to young HSCs. Now we can use the power enabled by this approach to identify actual drivers of HSC aging and dysfunction from chronological age and passenger aging-associated changes. We will identify candidate aging drivers that have similar expression patterns in young HSCs and youthful AT^{hi} old HSCs compared to dysfunctional AT^{lo} old HSCs, whereas genes changed similarly in both AT^{hi} and AT^{lo} HSCs will represent passenger chronological changes.

Replication stress and genomic integrity

Recent clonal analysis studies of hematopoiesis have demonstrated that large expansion of few clones is an aging phenotype, and is associated with increased mutations in cancer-related genes and increased risk of cancers and mortality in humans, in contrast to young humans who have an even balance of many small clones (Jaiswal et al., 2014). Yet how this originates at a

cellular and molecular level in individual stem cells is entirely unknown. Cancer incidence increases greatly with age, and in addition to promoting longevity and health, autophagy has been found to have preventative roles against cancer development (Cuervo, 2008). Furthermore, we discovered increased replication stress in AT^{lo} but not AT^{hi} old HSCs, implicating them as potential upstream sources of clonal hematopoiesis and blood cancers. It is possible that they have acquired mutations that will, or will lead to additional mutations that will, eventually drive clonal expansion such as MLL-fusions or other events associated with acute transformation. Examples of such mutations that increase HSC competitive fitness and precede leukemic transformations have already been described, such as mutations in the epigenetic enzymes Dnmt3a and Tet2 (Jaiswal et al., 2014).

Leveraging our identification of the functional and molecular heterogeneity in old HSCs, we will study how autophagy and this heterogeneity of old HSCs contribute to accumulation of mutations, clonal hematopoiesis, and cancer genesis and progression. This could provide valuable insight into this process and cancer onset during aging. We have started conducting whole exome sequencing on AThi and ATlo old HSCs to measure the incidence of mutations in each subset. To assess for major chromosomal rearrangements, we will perform spectral karyotyping (SKY) analyses on AThi and ATlo old HSCs. In the future we will utilize emerging clonal tracking technologies to directly investigate the clonal contribution of subsets of old HSCs with different levels of autophagy. By using a novel dual barcoding and fluorescent viral labeling technique (Cornils et al., 2014) in AThi and ATlo old HSCs, we will be able to isolate specific clones for cellular and functional analyses and to directly determine if acquired mutations in individual clones result in pre-leukemic competitive fitness, malignant transformation, or both, in individual HSC clones. Such approaches will determine how autophagy levels affect DNA

damage and mutations in old HSCs and elucidate the molecular origin of aging-associated clonal hematopoiesis and its association to cancer incidence and mortality in the elderly.

II. Targeting autophagy for HSC rejuvenation

Determining the effects of organismal lifespan interventions on stem cell aging

Our work demonstrates that all old HSCs have the *ability* to activate autophagy, but only some actively utilize this ability under homeostatic conditions in an old mouse. Our transplantation and *in vitro* autophagy studies demonstrate that autophagy can be directly manipulated and activated in old HSCs by a young BM niche, starvation, or drugs. This raises the intriguing and important prospect of therapeutically activating autophagy to preserve and even rejuvenate old HSCs.

However there remain important questions about the timeframe of the regulation of HSC fitness by autophagy. We found that autophagy is necessary for HSC function, becomes increasingly important during aging, and that high levels denote the healthiest old HSCs while low levels denote the least functional. We could rapidly turn autophagy on and off within minutes *in vitro*, but how quickly can ATlo and AThi old HSCs actually functionally and permanently convert to the other, if at all?

Would inhibiting or turning off autophagy in AThi old HSCs immediately remove their youthful biology? In fact, when we deleted autophagy in the blood system of young transplanted mice, during aging we observed loss of blood maintenance and accelerated and exacerbated

aging phenotypes such as myeloid bias. Similarly, acute deletion of autophagy in old BM transplanted into young mice resulted in an immediate loss of blood maintenance and increased myeloid bias. Therefore autophagy may be needed constantly as a gatekeeper of HSC activation. However, these effects were still not as severe as might be expected from a complete loss of autophagy in old HSCs, likely due to the steady state system of an unchallenged blood system lacking immediate need for HSC function. Further experiments are needed to determine if such complete conversion to autophagy-deficient old HSCs results in drastic HSC and blood failure under regenerative conditions, as might be observed after injury, traumatic blood loss, infections, etc. The effects of loss of autophagy under such conditions would likely be even worse than those seen in regenerating fetal and young adult blood systems with autophagy deletion.

Conversely, for how long must autophagy be elevated to confer youthful cell biology and robust regenerative function? Is quickly activating autophagy in an ATlo old HSC enough to convert it to an AThi old HSC, to remodel its cellular makeup and restore its regenerative capabilities? Perhaps a short autophagy pulse may clear mitochondria, reduce mitochondrial metabolism, and restore a glycolysis-based quiescent state. But due to the many different aspects of the cellular biology of old HSCs, such as accumulated replication stress, it may be difficult to truly convert all old HSCs to AThi HSCs. And would an ATlo old HSC simply revert to low autophagy levels after the autophagy activator was removed? If their autophagic state is maintained by a physical micro-niche, then perhaps. Lasting rejuvenation may require longer, more chronic, constant maintenance of high autophagy levels, or perhaps even treatments from a younger or middle age to prevent or slow development of cellular aging from the outset.

Many of the treatments known to activate autophagy also have been shown to improve organismal healthspan and lifespan in model organisms, such as rapamycin treatment, starvation,

calorie restriction, and even exercise regimens. As stem cells are integral for maintenance and function of tissues and organ systems, we sought to determine whether these interventions may work through rejuvenation of stem cells. Therefore, we have conducted experiments to determine not only if these treatments indeed activate autophagy in and rejuvenate old HSCs, but also if they effect beneficial effects independently of autophagy.

Rapamycin treatment

Previous studies may also shed some light on these possibilities. One study reported extremely increased levels of mTOR activation in old HSCs compared to young, and reported that rapamycin treatment in old mice rejuvenated the function of old HSCs (Chen et al., 2009). This might fit with our model of dysfunctional old HSCs with lower autophagy activity and aberrantly increased metabolic and cellular activation, and we found that HSCs undergoing cellular activation rapidly activate mTOR and simultaneously lose HSC identity and functional abilities. However this does not account for the subset of AThi old HSCs with increased autophagy levels and lower mTOR activation. However, in many independent experiments, we found nearly identical levels of mTOR activity in old HSCs and young HSCs. This previous report may be due to artifacts from distorted antibody staining due to fixation on unpurified populations of cells. However, we did find that ATlo old HSCs specifically have increased mTOR levels compared to AThi, which would indicate that mTOR inhibition may at least benefit the ATlo subset. Yet while this study never showed actual effects of rapamycin on mTOR activity in old HSCs, the idea that rapamycin and mTOR inhibition may activate autophagy to convert more old HSCs to AThi HSCs is intriguing. Alternatively its effects may

work indirectly through niche cells or other cells affecting bone marrow fluid and/or the BM niche.

However, while we found that mTOR was an important inhibitor and AMPK an important activator of autophagy in HSCs *in vitro*, interestingly rapamycin was an extremely weak activator of autophagy despite strong inhibition of mTOR, while mTOR inhibitor Ink128 or AMPK activator AICAR were far more effective. Furthermore, we treated old mice with rapamycin for 3 months (**Fig. 1a**) and observed no induction of autophagy in HSCs, almost no effects on the cellular composition of blood and bone marrow (**Fig. 1b, c**), and surprisingly no signs of rejuvenation or improved function in old HSCs (**Fig. 1d-j**). Therefore rapamycin is a weak inducer of autophagy in HSCs and does not rejuvenate old HSCs. Longer or lifelong treatments may prove more effective, and future experiments should study the effects of stronger and more specific mTOR inhibitors such as Ink128.

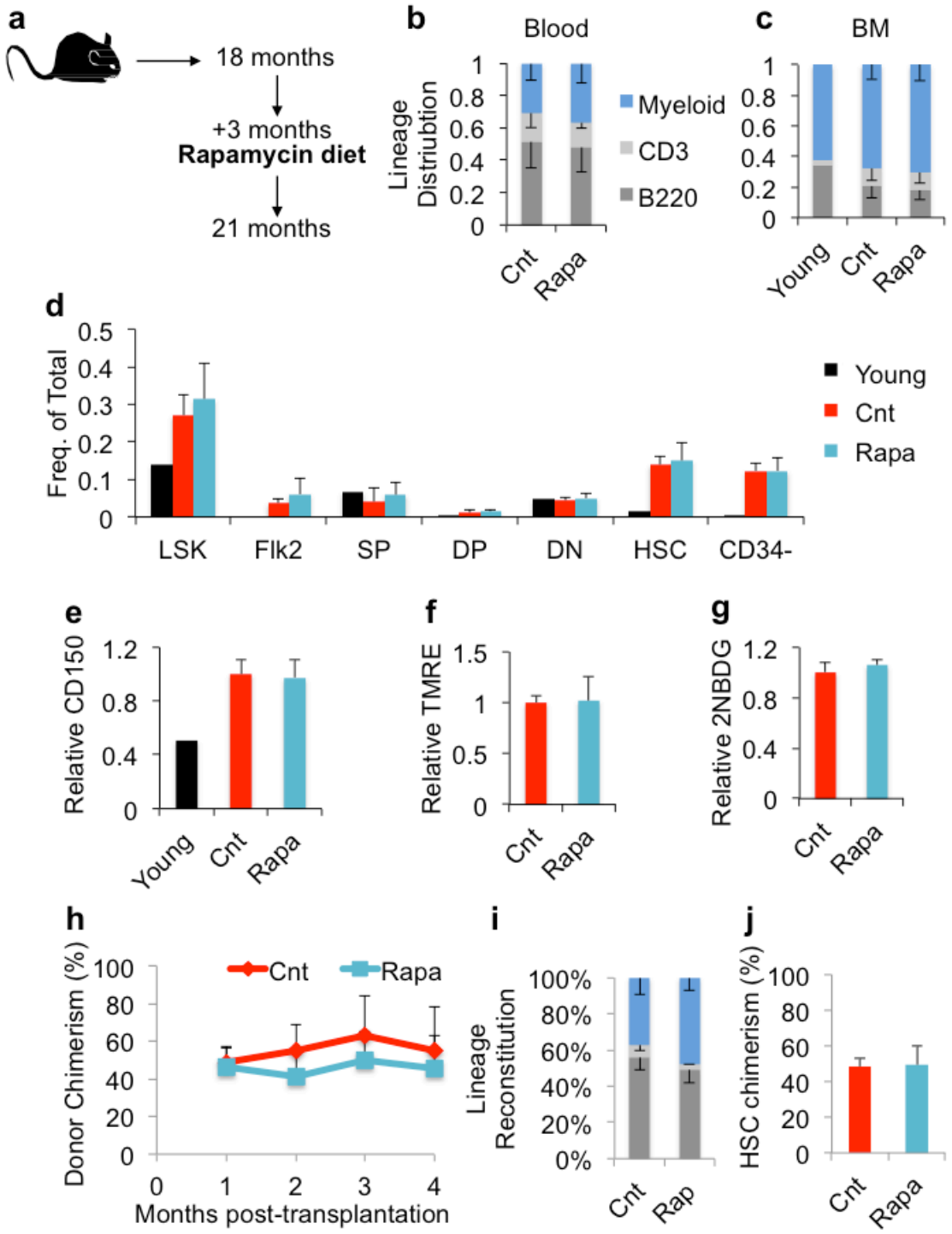

Figure 1. Rapamycin treatment in old mice. ***a)*** *Scheme.* ***b, c)*** *Lineage distribution in blood and bone marrow.* ***d)*** *Frequency of stem and progenitor populations.* ***e)*** *CD150 levels.* ***f)*** *Mitochondrial membrane potential.* ***g)*** *Glucose uptake.* ***h)*** *Blood donor chimerism,* ***i)*** *lineage reconstitution, and* ***j)*** *HSC chimerism 4 months after HSC transplantation. n=1, 5, 7 for Young, control, rapamycin (b-g), or n=5 (h-j). Error bars = S.D.*

Starvation

Starvation is a classic and robust method to induce an autophagy stress response. A recent study used multiple rounds of starvation called "prolonged fasting" to improve blood regeneration after chemotherapy (Cheng et al., 2014). Prior studies have found that autophagy is induced by radiation and can be radioprotective, although in particular being used by cancer cells to survive radiation (Lin et al., 2015; Apel et al., 2008). While autophagy was not examined, the effects of prolonged fasting may work by activating autophagy in HSCs and converting them into more functional and fit AThi HSCs. This may reduce metabolic activity and promote quiescence, thus rendering the HSCs less susceptible to such replication-dependent damage and cell death. More recently, autophagy was reported to specifically protect the blood system from radiation by promoting DNA damage repair (Lin et al., 2015). This aligns with our discovery of reduced replication stress in AThi old HSCs compared to ATlo, and reinforces the idea that autophagy protects HSCs and maintains their health. It is also interesting to note that similar to our results, autophagy is particularly essential in young HSCs again only under strong stress conditions, and one that induces regeneration.

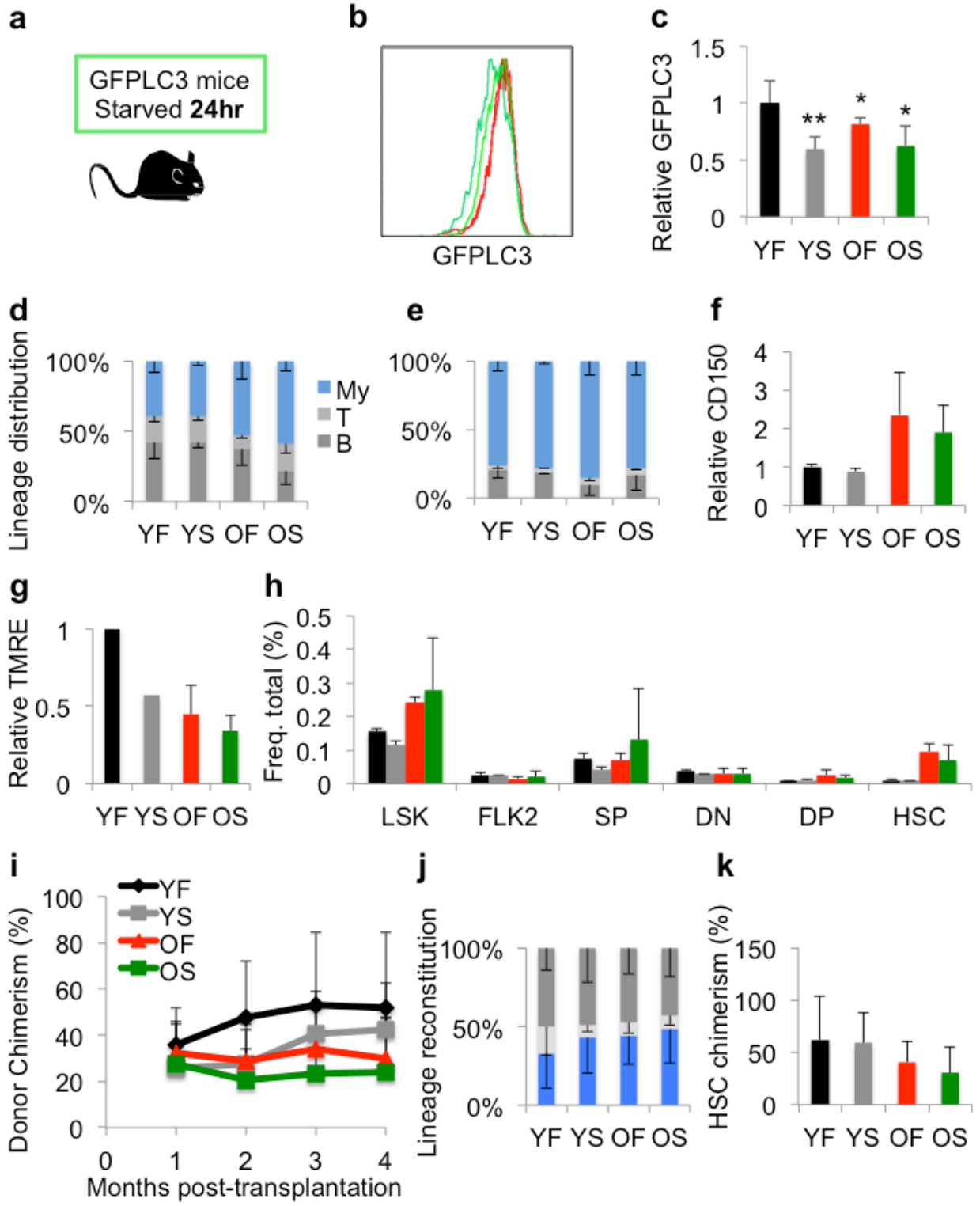

Figure 2. Starvation in old mice. ***a)*** *Scheme.* ***b, c)*** *Autophagy induction measured by GFPLC3 levels.* ***d,e)*** *Lineage distribution in blood and bone marrow.* ***f)*** *CD150 levels.* ***g)*** *Mitochondrial membrane potential.* ***h)*** *Frequency of stem and progenitor populations.* ***i)*** *Blood donor chimerism,* ***j)*** *lineage reconstitution, and* ***k)*** *HSC chimerism 4 months after HSC transplantation. n=8, 3, 4, 4 (b-f, h), or 2, 1, 6, 4 (g), or 4, 5, 15 18 (i-k). Error bars = S.D.*

Furthermore, Cheng et al. (2014) found that extended prolonged fasting with 8 cycles reduced the myeloid bias in old mice untreated with chemotherapy, although they did not report any functional or transplantation data. These reports again raise the intriguing possibility that even short-term induction of autophagy may be able to improve old HSC function, possibly by converting ATlo old HSCs to AThi.

Therefore, we conducted starvation experiments with old mice to determine if strong and acute activation of autophagy can convert ATlo old HSCs to AThi and rejuvenate old HSCs and an old blood system (**Fig. 2a**). 24 hours of starvation indeed induced a strong, acute autophagy response in HSCs and increased the number and percentage of old HSCs with high levels of autophagy (**Fig. 2b, c**), but it did not result in any signs of rejuvenation or improved function (**Fig. 2d-k**). Therefore such acute induction of autophagy is insufficient to permanently convert ATlo old HSCs into true, highly functional AThi HSCs. It is also possible that other negative effects of strong starvation on the mice may overwhelm any potential rejuvenation of HSCs. Alternatively, perhaps ATlo old HSCs cannot survive such stressful starvation and undergo cell death, instead of converting to AThi HSCs, although our *in vitro* data indicates that ATlo HSCs can still readily activate autophagy when starved.

Calorie restriction

Calorie restriction (CR) may also be able to stimulate autophagy and improve old HSC function. CR has long been shown to improve healthspan and lifespan of model organisms, often requiring autophagy (Rubinsztein et al., 2011). Two previous studies reported conflicting results of calorie restriction on old (22-25 months) HSCs, finding rejuvenation in BALBc mice but not

in C57BL/6J (B6), although these studies used outdated transplantation procedures (Chen et al., 2003, Ertl et al., 2008). Two very recent studies have utilized lifelong CR regimens in B6 middle-aged (12 months) (Tang et al., 2016) and old mice (Lazare et al., 2017) and examined HSC function. While CR was reported to improve regenerative capacity of middle-aged HSCs, it had no effects in the second study of 24-month-old mice. Interestingly, CR was reported to increase quiescence of middle-aged HSCs, perhaps an effect from activating autophagy and converting over-active ATlo old HSCs to AThi. Therefore whether CR can rejuvenate old HSCs was still unclear, and whether it induced autophagy in HSCs had not been investigated.

We conducted multiple independent experiments and found that in old mice who had been undergone lifelong CR (**Fig. 3a**), the hematopoietic system was largely unaffected (**Fig. 3b, c**). While there was a slight reduction in the expansion of progenitor cells, there was no significant reduction in the classical HSC expansion (**Fig. 3d**). Furthermore, markers of HSC aging (Fig. 3e, 3f) and loss of function with age (**Fig. 3g-i**) were unchanged.

Therefore perhaps lifelong treatment may result in compensatory responses or other negative side effects. More acute treatments in late life may be able to provide a more appropriate amount of stress to induce hormesis effects and induce rejuvenation. CR did not perceptibly induce autophagy in old HSCs, although it remains possible that it induces autophagy at earlier times for shorter durations or to smaller degrees below the limit of detection.

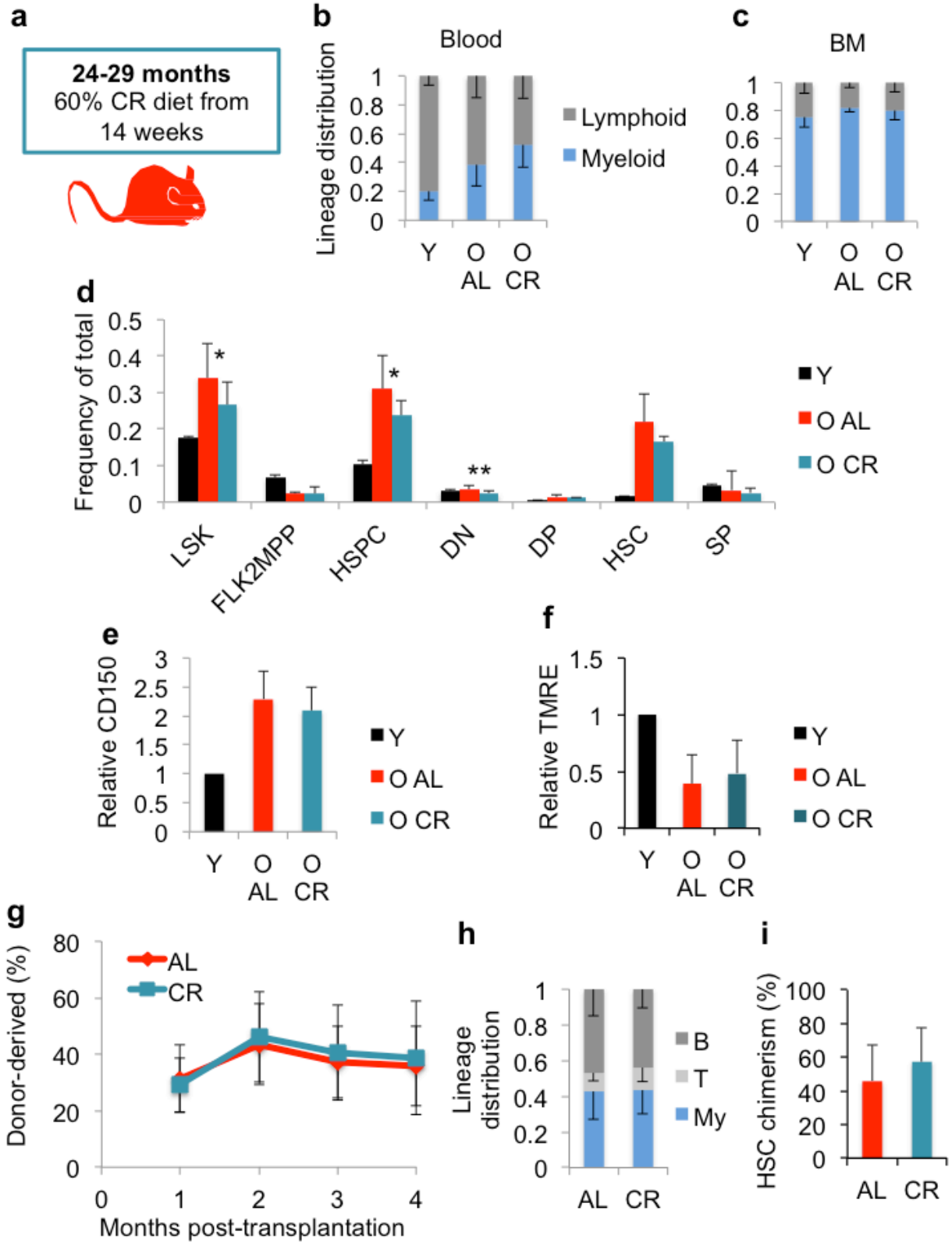

Figure 3. Calorie restriction in old mice. ***a)*** *Design.* ***b, c)*** *Lineage distribution in blood and bone marrow.* ***d)*** *Frequency of stem and progenitor populations.* ***e)*** *CD150 levels.* ***f)*** *Mitochondrial membrane potential.* ***g)*** *Blood donor chimerism,* ***h)*** *lineage reconstitution, and* ***i)*** *HSC chimerism 4 months after HSC transplantation. n=6, 10, 10 (b), or 10 (c-e), or 6, 9 9 (f), or 25 (g-i). Error bars = S.D.*

Exercise

We also examined the effects of exercise on old HSC function. Exercise is known to provide great health benefits and even to induce autophagy in some tissues (He et al., 2012), although its effects on HSCs and potential ability to rejuvenate the hematopoietic system have not been studied. However, it had almost no effects on the blood system in either young or old mice (**Fig. 4a-d**), and did not ameliorate HSC expansion in old mice. Furthermore, the age-associated increase in CD150 levels on old HSCs was not reduced, and HSC regenerative capacity was not affected in young or old HSCs in transplantation studies (**Fig. 4f-h**). Therefore while exercise provides many health benefits, it does not provide rejuvenation benefits in the blood system or to old HSCs.

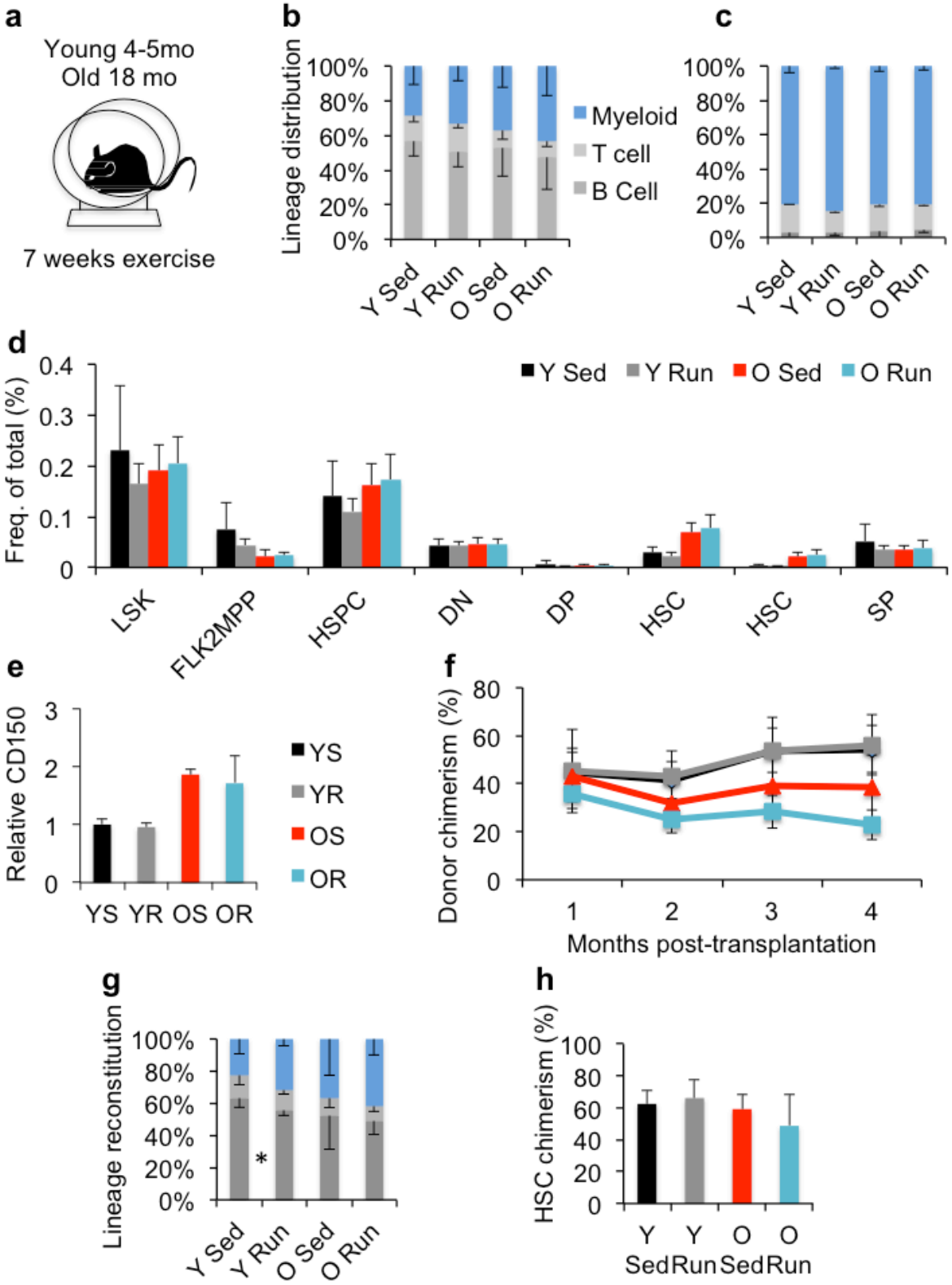

***Figure 4. Exercise in old mice**. **a**) Design. **b, c**) Lineage distribution in blood and bone marrow. **d**) Frequency of stem and progenitor populations. **e**) CD150 levels. **f**) Blood donor chimerism, **g**) lineage reconstitution, and **h**) HSC chimerism 4 months after HSC transplantation. n=9, 3, 9, 8 (b), or 4, 4, 4, 6 (c-e), or 5 (f-h). Error bars = S.D.*

Exposure to young blood

Exposure of old mice to circulating blood factors in young mice has shown remarkable ability to rejuvenate many tissue systems, almost every one that has been studied, including brain, muscle, and heart (Conboy et al., 2015). However, the effects of such youthful factors on aged hematopoietic stem cells and the aged blood system itself that carries such factors has not been studied. Parabiosis is a procedure where the blood system of two animals are linked by surgically stitching their peritoneum and skin together. Therefore, we used parabiosis or plasma injections to understand the effects of age-regulated systemic factors on the hematopoietic system (**Fig.5a**). Interestingly, while we found ~40% crossover of circulating blood cells in isochronic and heterochronic parabionts (**Fig. 5b**), very few bone marrow cells transferred to their parabionts (**Fig. 5c**), with the exception of a highly preferential relocation of old HSCs specifically into young bone marrow (**Fig. 5d**). This was not a characteristic of HSCs versus other bone marrow cells, but rather was specific to old HSCs, as young HSCs did not crossover to old bone marrow. However, surprisingly we found that joint circulatory systems had neither a rejuvenating nor aging effect on characteristic phenotypes of old or young blood systems, respectively (**Fig. 5e-h**). Furthermore, neither exposure to young blood, nor even relocation to a young bone marrow niche by some old HSCs, improved functional ability of old HSCs (**Fig. 5i**). Therefore the hematopoietic system that carries factors with rejuvenation abilities for many other tissues is in fact itself impervious to those factors.

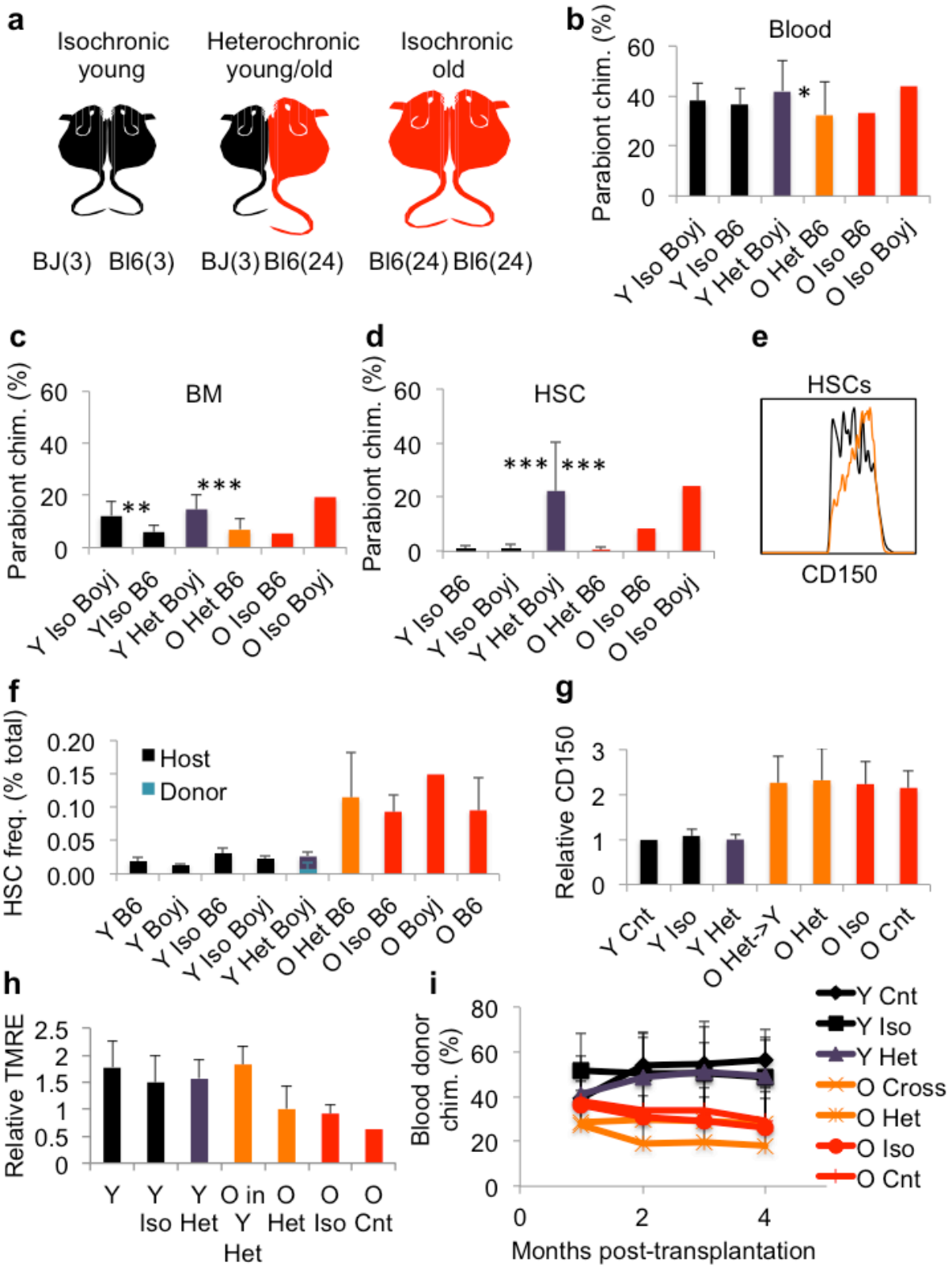

Figure 5. Hematopoietic analysis of parabiosed mice. ***a)*** *Design.* ***b, c, d)*** *Chimerism of parabiont pair cells in blood, bone marrow, and HSCs.* ***e, g)*** *CD150 levels in HSCs.* ***f)*** *Frequency of HSCs.* ***h)*** *Mitochondrial membrane potential.* ***i)*** *blood donor chimerism 4 months after HSC transplantation. n=8, 9, 16, 16, 1, 1 (b), or 10, 10, 15, 16, 1, 1 (c, d), or 4, 5, 10, 10, 15, 16, 8, 1, 4 (f), or 9, 20, 15, 14, 16, 10, 5 (g), or 4, 4, 5, 2, 4, 3, 1 (h), or 5, 10, 15, 10, 10, 9, 4 (i). Error bars = S.D.*

Outlook

Altogether it appears that well-established healthspan and lifespan treatments have few effects on old HSCs and largely do not work through autophagy, or induce HSC rejuvenation. These interventions are systemic treatments that undoubtedly have many effects of varying degrees of benefit or detriment on different organ systems and cell types. Perhaps more specific autophagy inducers may be more effective with fewer side effects, such as recently identified SMER28 (Doulatov et al., 2017), or the Tat-beclin 1 peptide (Shoji-Kawata et al., 2013).

Other studies have reported limited HSC rejuvenation effects to varying degrees. Inhibition of Cdc42 by CASIN restored youthful polarity to old HSCs, and was reported to rejuvenate their function as well (Florian et al., 2012). However, functional improvement was only seen in secondary and not primary transplants, and treatments were done *in vitro* for 16 hours, long after the metabolic and cellular activation and loss of function of HSCs. Therefore the effects may simply have worked through the inhibition of activation as opposed to actual rejuvenation. Such treatments and studies must be done *in vivo* or minimally in comparison to uncultured controls to be properly evaluated. Recently, treatment of old mice with the drug ABT263 that depletes senescent cells was reported to rejuvenate regenerative function of old HSCs, although it seems unlikely that its effects worked directly on HSCs, which do not senesce themselves, but more likely through beneficial systemic effects of depleting senescent cells in other tissues (Chang et al., 2016). Another very recent study reported that osteopontin is important for HSCs and that treatment of old HSCs with osteopontin restored youthful HSC polarity and slightly improved regenerative functions (Guidi et al., 2017). Therefore there is precedent of a few reports of rejuvenation of some aspects of old HSC function, yet clearly very little relative to other stem cell populations. In particular, MuSCs and neural stem and

progenitors seem to be readily rejuvenated by many different interventions, such as rapamycin treatment, CR, exercise, parabiosis, and others (Rando and Chang, 2012).

This may be due a limited replicative lifespan of HSCs, a theory developed from prior studies demonstrating that HSCs can only divide a limited number of times before exhaustion. While an old study using outdated techniques initially reported that HSCs were able to persist and function many times longer than the lifetime of a mouse after serial transplantations over many years (Harrison et al., 1978), more recent studies have shown that HSCs are exhausted after only 4-6 serial transplantations (Ito et al., 2006; Kamminga et al., 2005). Indeed, as others and we have shown, replication stress is a key feature of HSC dysfunction and aging (Passegué et al., 2005; Beerman et al., 2013; Flach et al., 2014; Walter et al., 2015).

If this is the case, then autophagy levels may in a sense be a marker of HSC health by way of their divisional history and replicative age, as opposed to a dynamic determinant of HSC fitness. High autophagy levels may be a characteristic of the subset of the healthiest old HSCs that has regulated their functional state by enforcing low metabolism and quiescence, ensuring very few cell divisions and a young replicative age, thus maintaining their regenerative capacity. In contrast, HSCs that maintain low levels of autophagy maintain a more active metabolic rate, leading to more divisions over their lifetime, more replication stress, and faster replicative aging, resulting in the classical dysfunctional aging phenotypes of an old HSC. This would also explain the equal function of ATlo and AThi young HSCs, as both subsets have accumulated very few divisions, whereas with age the disparity in replicative age and functional fitness widens. In this case, short-term changes in autophagy would not truly convert AThi and ATlo old HSCs, and long-term activation of increased levels of autophagy would be required from a young age to maintain more healthy old HSCs. Tracking autophagy levels as well as divisional history of

individual HSCs over a lifetime with a combination of autophagy reporter mice and H2B-labeled mice (Foudi et al., 2009) could address this model.

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