Proliferation Drives Aging-Related Functional Decline in a Subpopulation of the Hematopoietic Stem Cell Compartment

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Young HSCs

JAK2V617F Proliferation

Old HSCs

~75%

"Young" transcriptome

~25%

JAK2/MAPK
Proliferation
p53
DNA Damage
Myeloid genes

+6%
Proliferation drives ageing related functional decline in a subpopulation of the haematopoietic stem cell compartment

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Abstract
Ageing of the haematopoietic stem cell (HSC) compartment is characterised by lineage bias and reduced stem cell function, the molecular basis of which is largely unknown. Using single-cell transcriptomics, we identified a distinct subpopulation of old HSCs carrying a p53 signature indicative of stem cell exhaust alongside pro-proliferative JAK/STAT signalling. To investigate the relationship between JAK/STAT and p53 signalling, we challenged HSCs with a constitutively active form of JAK2 (V617F) and observed an expansion of the p53 positive subpopulation in old mice. Our results reveal cellular heterogeneity in the onset of HSC ageing and implicate a role for JAK2V617F driven proliferation in the p53 mediated functional decline of old HSCs.
Highlights

- Single cell transcriptomics reveals gene signature associated with functional decline in old HSCs
- Functional decline is driven by prolonged proliferation and upregulation of p53 pathway
- Only a subpopulation of HSCs show ageing related features pointing to heterogeneity in the rate of ageing

Introduction

Organismal ageing is accompanied by a gradual decline in regenerative capacities. This decline has been associated with reduced stem cell function, where the ageing stem cell pool is unable to repopulate tissues upon cellular loss during physiological turnover or after tissue injury (Beerman et al. 2010). In the haematopoietic system, stem cell ageing is evident in a weakening of the adaptive immune response and a general decline of haematopoietic stem cell fitness (Beerman et al. 2010).

The weakening immune response has been attributed to a shift from a balanced lymphoid/myeloid output towards a myeloid skew with age (Rossi et al. 2005). Although HSCs showing a skew in their myeloid/lymphoid output can also be found in young mice, the aggregate output is balanced. In contrast, with age proportionally fewer lymphoid biased HSCs are found (Grover et al. 2016). In addition to the lineage skew, ageing of the haematopoietic system also results in reduced performance in blood reconstitution and engraftment, irrespective of lineage output (Dykstra et al. 2011). In addition, accumulation of DNA damage and upregulation of p53 in aged HSC populations is well documented (Dumble et al. 2007; Rossi et al. 2007). p53 is a key regulator of ageing in haematopoiesis with high levels of p53 leading to premature ageing features such as reduced engraftment (Dumble et al. 2007). However, while Grover and colleagues (Grover et al. 2016) were able to shed light on the molecular signature responsible for lineage skewing with age, little is known about the molecular basis of the functional decline of HSCs with age. It is for example unknown how uniformly the functional impairment is distributed within the HSC compartment, and it is unclear what factors and pathways are directly relevant to the decline.

Using an index sorting strategy and single cell assays for highly purified long-term HSCs (LT-HSCs), we identify HSC ageing as a heterogeneous process by characterising a HSC subpopulation marked through p53 activation in old mice. Further transcriptional description of the subcluster shows myeloid-bias, and JAK/STAT and MAPK driven pro-proliferative gene signatures, reminiscent of the proliferation driven cell cycle arrest in cellular senescence (Serrano et al. 1997). Moreover, expansion of this old-specific subpopulation could be triggered by constitutively activating Jak2. We propose a model whereby prolonged proliferation in HSCs driven by the JAK/STAT pathway leads to a functionally impaired HSC subpopulation defined by p53 pathway upregulation with age.

Results

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The long term haematopoietic stem cell (LT-HSC) compartment harbours a distinct subpopulation with age

To determine how the transcriptional heterogeneity in LT-HSC is associated with age we index-sorted single LT-HSCs using ESLAM markers (Fig.1A) from bone marrow of mice aged 4 months (n=192) and 18 months old (n=192). This approach resulted in a distinct HSC population evident through comparison with two published haematopoietic single cell transcriptome data sets of young and old HSCs (lineage negative Sca-1*, c-Kit*, CD150*, CD48) (Grover et al. 2016; Kowalczyk et al. 2015), projecting all data sets onto a haematopoietic stem cell expression atlas (Nestorowa et al. 2016)(Sup.Fig.1A). We obtained 119/192 old and 99/192 young cells after quality control (Sup. methods and Sup.Fig.1B) and used a k-means based consensus clustering approach for single cell transcriptomes (SC3)(Kiselev et al. 2017).

One cluster was entirely made up of old HSCs from replicate mice (referred to as "old specific" cluster) (Fig.1B) being well-defined as measured by silhouette index (Si= 0.92, Fig. 1x) and distinct. Marker genes driving cluster formation were calculated using SC3 (n=62, Sup.Table 1, Fig.1C). To investigate whether a similar cluster exists in young LT-HSCs, cells were clustered separately (Sup.Fig.1C), with no similar cluster detectable (Sup.Fig.1C). Re-clustering old LT-HSCs separately identified the old specific cluster with an identical subset of marker genes (Sup.Fig.1C, Fig.1D). To ensure that differences in cell type were not driving the clustering, we compared ESLAM markers from the index sort data with no difference in intensity of CD45 (p=0.8925), CD48 (p=0.4851), CD150 (p=0.7208) or EPCR (p=0.6472) expression (Fig.1E). To validate the marker genes, we used single cell differential expression (SCDE) (Kharchenko et al. 2014) to identify differentially expressed genes between the old specific subpopulation and the other HSCs. SCDE confirmed a subpopulation of old LT-HSCs expressing marker genes identified by SC3 (Fig.1F). In summary, we identified a transcriptionally distinct subpopulation old LT-HSCs.

The old specific cluster is characterised by anti- and pro-proliferative pathways

To characterise the old specific cluster, we characterised the marker genes underlying the clustering (Fig.1C and Sup.Table1). One of the top marker genes, the cell-cycle regulator Cdkn1a, is a well-known p53 target (Beckerman & Prives 2010), with p53 previously been implicated in regulating HSC ageing and quiescence (Dumble et al. 2007). To further test p53 regulation of the old specific cluster we used a list of senescence specific p53 genes (Kirschner et al. 2015). We identified a significant enrichment of marker genes as p53 related (6/68 marker genes, p=1e-06, Fig.2A, Sup.Table1), many of which are negative regulators of proliferation. We were unable to identify enrichment of p53 apoptosis (p=0.5) (Kirschner et al. 2015), autophagy (p=0.06) (Kenzelmann Broz et al. 2013) or checkpoint (p=0.12) (Kenzelmann Broz et al. 2013) targets in the marker genes (Fig.2A and Sup.Table1). No p53 targets were detected in the marker genes of the other five clusters in Fig.1C (see Sup.Table1).

In the marker gene list we noted multiple jun/fos transcription factors which are targets of JAK/STAT signalling in the haematopoietic system, where JAK/STAT stimulates proliferation and differentiation (Rawlings et al. 2004). To test for enrichment of JAK/STAT targets, we compared
the marker genes with ChIP-seq data from patient megakaryocytes, where the JAK/STAT pathway drives disease (Lau et al. 2015). We identified a significant enrichment of STAT targets in the marker genes (Lau et al. 2015) in our marker genes (green fields, Fig.2A, p=0.0005 for pStat3 targets 6/68, p=0.0006 for pStat5 targets 4/68, Sup.Table1). No Stat3 or Stat5 targets were detected in the other five clusters in Fig.1C (Sup.Table1). In summary, analysis of the marker genes suggested a co-activation of cell cycle repressing p53 and pro-proliferative JAK /STAT signalling in the old specific cluster.

Several lines of evidence are consistent with the concept that pro-proliferative and anti-proliferative stimuli exist in the same old HSC subpopulation. Firstly, unbiased KEGG pathway analysis of the marker genes (Kanehisa et al. 2012) revealed an enrichment for “Cell Cycle”, due to the presence of cell cycle inhibitors, p53 targets (red bars, Fig.2B) and the MAPK pathway (Fig.2B, green bar), which is driven by JAK activation in haematological cells (Vainchenker & Constantinescu 2013).

Secondly, we compared gene target lists for p53, STAT3 and STAT5 to marker genes for the old specific HSC cluster. Gene set enrichment analysis (GSEA) (Subramanian et al. 2005) highlighted a significant enrichment of senescence specific p53 (p53 ES=0.11, p= 0.009), STAT3 (ES=0.137, p<0.001) and STAT5 (ES=0.084, p=0.039) targets in those marker genes (Fig.2C). No enrichment of apoptosis (no values returned, data not shown) or checkpoint related p53 targets (p=0.44, Sup.Fig.2A) were detected. GSEA found no enrichment of p53 and STAT3/5 targets comparing pooled young and old HSCs (p=0.422 for p53 Apo p=0.65 for p53 RIS, p=0.44 for Stat5, p=0.357 Stat3, Sup.Fig.2B and data not shown).

Thirdly, we generated a JAK/STAT signature mimicking HSC behaviour, by stimulating a stem cell like cell line (HPC7) with thrombopoietin (TPO) for microarray analysis (Fig.2D) (Park et al. 2016). Binding of TPO to its receptor activates JAK2 and its downstream targets STAT1,3 and 5 (Rawlings et al. 2004). GSEA highlighted a strong enrichment of TPO regulated genes (top 100 genes, p< 9.81e-10) in the old specific cluster (Fig.2D).

Lastly, we confirmed the old specific cluster by indirect immunofluorescence (IF) in HSCs from young and old mice (n=3) for p53 phosphorylated at Serine15 (pp53Ser15) and FosB phosphorylated at Serine27 (pFosBSer27). Only old HSCs showed high levels of pp53Ser15 and pFosBSer27 (Sup.Fig.2C). p53 response and senescence are associated with persistent levels of DNA damage. We stained old HSCs for p53 for gH2AX by IF and blind scored cells with >5 foci gH2AX. We found an enrichment for gH2AX over p53 positive cells (Fig. 2E, Sup.Fig. 2D), providing a mechanism for p53 upregulation. We tested the p53 positive old HSCs for changes to proliferation by EdU injection into mice and co-staining for pp53Ser15 protein using IF. EdU incorporation was significantly lower in p53 positive cells, suggesting reduced proliferation (Fig.2F).

The old specific cluster is enriched for myeloid-biased HSCs and regulated by transcription factors controlling quiescence and proliferation

One feature of the ageing HSC compartment, the myeloid basis, has been described on a single cell basis (Grover et al. 2016). To identify lineage biases in our old specific cluster, we overlaid our marker gene list with gene sets for bi-potent granulocyte/macrophage progenitors (preGM),
megakaryocyte/erythroid progenitors (preMeg) and common lymphoid progenitors (CLP) using GSEA (Pronk et al. 2007). We identified an enrichment of preGM genes (ES=0.2, p<0.01), with no enrichment in preMeg or CLP genes (Fig.2G), suggesting a myeloid bias in our old specific HSC cluster. These results could explain the myeloid bias emerging with age where a subset of myeloid primed HSCs reconstitute the blood system more frequently over time followed by exhaust, leaving a large pool of myeloid primed progenitors in the system. We interrogated the marker genes specific to our old specific HSCs with respect to regulation of HSC function and found 8/12 transcription factors regulating quiescence and proliferation (Sup.Table 2, p=6.779747e-24). Nuclear receptor subfamily 4 group A member 1 (Nr4a1) has been reported to be upregulated on myeloid biased HSCs (Land et al. 2015), further supporting the myeloid bias. Perturbations of some marker genes have been reported and suggest an enrichment for quiescence regulators in the old specific subcluster (Sup.Table 3).

**Constitutive Jak2 activation increases contribution to age specific subpopulation of LT-HSCs**

The upregulation of the p53 pathway in Jak2 context has previously been reported in erythroblasts from patients with myeloproliferative disease (MPN) (Chen et al. 2014). In the majority of MPN Jak2 is constitutively active through a mutation at JAK2V617F (Baxter et al. n.d.) where p53 is thought to maintain genome stability in the chronic phase of disease (Chen et al. 2014). In this study, we do not observe activation of p53 in proliferating young HSCs (Fig.1C, Sup.Fig.1C). One explanation for the lack of p53 activation in young HSCs is that p53 upregulation correlates with number of replications, similar to the role of p53 in replicative senescence (Beauséjour et al. 2003). To test the association between JAK/STAT-driven proliferation and p53 activation, we used single cell approaches in homozygous JAK2V617F mice where JAK2V617F provides a constant proliferative stimulus (Li et al. 2014). We first characterised proliferation kinetics of single, young and old WT and JAK2V617F HSCs in vitro and found a significant increase in proliferation in young JAK2V617F HSCs after 72 hours in culture (p=0.002 1st division, p=0.0001 2nd division, p=0.0001 3rd division) (Fig.3A and Fig.3B). Consistent with our transcriptomic data (Fig.3C) we failed to detect p53 activation in young JAK2V617F HSCs by IF for pp53Ser15 (Fig. 3D), concluding that JAK2V617F increases proliferation without evoking a p53 response in young LT-HSCs. We tested if JAK2V617F exerts an effect with age through a prolonged, lifelong increase in proliferation. We found two lines of evidence agreeing with prolonged and not acutely enforced proliferation evoking the old associated p53 signature. Firstly, we characterised proliferation kinetics of single old JAK2V617F HSCs in vitro where old JAK2V617F HSCs show a loss of JAK2V617F driven increased proliferation as measured by numbers of division at 72 hours in culture (p=0.2117 1st division, p=0.0046 2nd division, p=0.0039 3rd division). In agreement with published data in heterozygous JAK2V617F context (Kent et al. 2013), old homozygous JAK2V617F HSCs show a significant decrease in average division when compared to old WT HSCs at 48 and 72 hours in culture (48 hrs: p=0.007 1st division, p= 0.0001 2nd division, p=0.106 3rd division; 72 hrs: p=0.0338 1st division, p=0.0004 2nd division, p=0.2142 3rd division, Fig.3A, Fig.3B). In summary, the JAK2V617F enhanced proliferative effect seen in young HSCs is lost in old HSCs.
Secondly, to establish causality for Jak2 driving the old specific cluster formation, we tested the effect of the long-term increase in proliferation on individual cells in the ageing HSC compartment using single cell transcriptomics. We generated single cell transcriptional profiles of young (70 cells) and old (139 cells) LT-HSCs homozygous for JAK2V617F (Li et al. 2014). Following clustering by SC3 (Kiselev et al. 2017) we identified the same old-specific cluster as in the WT cells, evidenced by an identical set of marker genes (Fig 3E). A careful comparison of the number of cells in the old specific cluster showed that it contained 26% and 33% of WT cells in replicates 1 and 2 respectively (Fig.3E and data not shown). In the Jak2V617V homozygous context, this contribution increased to 32% and 47% for replicates 1 and 2 respectively (Fig.3E and data not shown), a statistically non-significant increase (p=0.146). To optimise the number of cells included in our analysis, we scored 1463 cells from 3 old and 3 young WT and JAK2V617F mice (Fig. 3D), by positive pp53Ser15 and pFosB IF. In old WT LT-HSCs, we found an increase of 36% in double-positive HSCs when compared to young WT HSCs, an increase consistent with our scRNA-seq data. The old specific cluster expanded to 64% in JAK2V617F HSCs, consisting of significant increase compared to WT (p=0.0001). To pin point the onset of the p53 signature in LT-HSCs we generated single cell transcriptional profiles of 12 months old JAK2V617F (n=2, 130 cells) and WT mice (n=2, 144 cells) (Fig.3F). No overlap with the marker genes for the p53 cluster was found, suggesting that the onset of the p53 signature within LT-HSCs might be late in the mouse lifespan and a state switch-like, stochastic event. Taken together, our data suggest that pro-proliferating constitutive Jak2 activation expands the old-specific cluster, evoking a p53 response (Fig.3G). We hypothesise that enforced proliferation in young HSCs leads to stem cell exhaust and p53 activation with age.

Discussion

The regenerative capacity of a tissue declines with age. Whether this reduction of stemness is equally distributed between stem cells or affects only a subset of cells is not well understood. In this study, only a distinct subpopulation of HSCs carried a signature indicative of functional decline as characterised by p53 signalling, pointing towards functionally heterogeneous stem cell ageing. Whether this heterogeneous exhaust serves as a strategy to ensure stem cell function over an entire lifespan is unclear. A role for p53 in haematopoietic ageing has been established. In HSCs, increased p53 activity leads to reduced functionality and proliferation with age, whereas decreased p53 levels increase HSC proliferative capacity with age (Dumble et al. 2007), suggesting a direct link between p53 dosage and regenerative capacity in HSCs. Despite the wealth of phenotypical characterisation of p53 dosage on the HSC compartment as a whole, our single cell approach reveals how p53 activation is distributed within the HSC compartment and provides the missing deconvoluted transcriptional signatures of p53 activated and unactivated aged HSCs. Observations from studies in progeroid and hyperactivated p53 mouse models suggest a more generalised role for p53, possibly extending the relevance of our findings to non-hematopoietic tissues (Varela et al. 2005; Tyner et al. 2002). We found that p53 activated HSCs co-expressed cell cycle inhibitory and proliferative transcripts from MAPK and JAK/STAT signalling. This co-activation of pro-
anti-proliferative pathways has been described in senescent cells induced by hyperactivation of MAPK signalling (Serrano et al. 1997). A role for JAK2 signalling has previously been implicated in senescence induction of haematopoietic lineage negative Sca-1- Kit+ cells (Li et al. 2010) with accumulation of γH2Ax foci, reduced rates of proliferation and inhibition of apoptosis. These phenomena were only observed 26 weeks after JAK2V617F induction, suggesting a cumulative rather than an immediate effect of JAK2V617F on proliferation (Li et al. 2010). A link between enforced proliferation and stem cell exhaustion has been shown where loss of CDKN1A drives HSCs into hyperproliferation and subsequent exhaust (Cheng et al. 2000). A recent study showed number of cell divisions as a limiting factor in HSC regenerative capacity (Bernitz et al. 2016). Based on the co-activation of anti-proliferative pathways of p53 in individual HSCs, the opposing effects of JAK2V617F on HSC proliferation in young and old mice and the increase of p53 activated cells under prolonged but not acute JAK2V617F activation, we provide support for a link between cumulative proliferation and stem cell exhaustion with age. We identified a myeloid bias in the old specific HSC population which could not be detected in the other old HSCs (Fig. 2F). Myeloid bias is well documented in the literature (Rossi et al. 2005), but molecular mechanisms are thus far elusive. We speculate that myeloid biased HSCs proliferate more over the lifetime of a mouse leading to HSC exhaust at the same time as providing a pool of myeloid biased progenitors reconstituting the HSC compartment. This heterogeneity in HSC ageing might be mediated by the bone marrow niche where it has been shown that aged HSCs home further away from the endosteum compared to young HSCs, leading to decreased regenerative capacity (Geiger et al. 2013). However, this hypothesis awaits further investigation.

Experimental Procedures

Mice
Mice were generated as described (Li et al., 2014). All mice in this study were 4 to 18 months old. Mice were kept in specific pathogen free conditions and all experiments were performed according to the UK Home Office regulations.

Cell isolation and Flow Cytometry
Single cells were obtained from bone marrow suspension as described (Kent et al., 2013). ESLAM cells were isolated using CD45-FITC (Clone 30-F11), EPCR-PE (Clone RMEPCR1560), CD150-Pacific Blue (Clone TC15) and CD48-APC (Clone HM48-1).

Single cell cDNA and library preparation
cDNA from single cells was obtained as described (Picelli et al., 2013). Illumina Nextera reagents were used for library construction and sequenced on a HiSeq 2500 (125 bp PE reads).

Data analysis
Data analysis was performed using SC3 and SCDE tools (Kiselev et al. 2017; Kharchenko et al. 2014). For details see sup. methods.
**Immunofluorescence**

ESLAM cells were processed as described (Li et al., 2010) using anti-p-Serine15 p53 (clone 16G8) and anti-p-Serine27 FosB (ab 62433) antibodies. Cells were blinded and scored on an Axiolmager Z2 (Zeiss). A two-sided Fisher’s exact test was used to calculate p-values.

**Single ESLAM cultures**

Single ESLAM cells were cultured, blinded and counted as described (Kent et al., 2013). A two-sided Fisher’s exact test was used to calculate the p-values.

**HPC7 cell culture and microarray**

HPC7 cells were grown as described (Park et al. 2016). For microarray, cells were serum-starved and stimulated with TPO for 30 minutes. Total RNA was extracted with RNeasy Kit (Qiagen). Cambridge Genomic Services processed all samples using Illumina WG-6 BeadArrays. For data analysis see supplemental methods.

**Author contributions**

KK, TC, WR and ARG designed the study. KK, TC, ICM, HJP, JL, DGK and RK performed experiments. DFSC, VK, MH and TC performed bioinformatics analysis. KK analysed all other data. TLH and DCP helped with mouse experiments. KK, TC, WR and ARG wrote the manuscript.

**Accession numbers**

FACS data are deposited in FlowRepository under FR-FCM-ZY64. Microarray data are deposited in GEO under GSE87687. Single cell sequencing data can be found in GEO under GSE87631.

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**References**


Kenzelmann Broz, D. et al., 2013. Global genomic profiling reveals an extensive p53-


Figure legends

Figure 1: LT-HSCs display a distinct subpopulation with age
A) Sorting strategy for HSCs. B) SC3 clustering of young and old HSC transcriptomes. Replicates: purple and green bars. Age: orange (young) and turquoise (old) bars. Similarity between cells is indicated from blue to red (identical). C) Heatmap of top ten marker genes of cluster 1. Expression is shown from blue (low) to red (high). D) Silhouette plots for all clusters. The silhouette index (si) and the number of cells per cluster is given. xSi =average silhouette index. E) Boxplots for ESLAM marker intensity for old specific (blue) and other old HSCs (red). F) SCDE plots for marker genes of cluster 1 in young (blue) and old (orange) HSCs. Expression levels of individual cells are given by individual lines (see Sup.Methods).
Figure 2: Age specific cluster carries signature of pro-proliferative and anti-proliferative stimuli
A) Manual annotation of top 20 marker genes with Ras senescence (RIS), Apoptosis (Kirschner et al., 2015), pStat3 and pStat5 data sets (Lau et al., 2015). Red marks p53 and green marks pStat targets. B) KEGG pathway analysis of marker genes for old specific cluster. Selected pathways are shown as ratio of enrichment (Green, pro-proliferative; Red, anti-proliferative). C) GSEA for p53 RIS, Apoptosis, STAT3 and STAT5 targets. p-value and enrichment score are shown. D) Schematic of TPO regulated microarray experiment in HPC7. GSEA of TPO specific targets and marker gene list. E) Immunofluorescence quantification of old HSCs for p53Ser15 and/or gH2AX (n=3). F) Quantification of EdU incorporation, gH2AX and p53 in old HSCs (n=3; Error bars = SEM). G) GSEA for lineage markers (preGM, precursors to granulocyte and macrophage lineage; preMEG, precursors to megakaryocyte and erythroid lineage; CLP, common lymphoid progenitor). ref: Reference, exp: Experiment, #genes: number of genes, ChIP: Chromatin Immunoprecipitation, MA: microarray, ES: Enrichment score.

Figure 3: Constitutively active JAK2 increases cell contribution to the old specific subpopulation
A) Kinetics of JAK2V617F and WT HSCs. Ratios of JAK2V617F and WT for indicated divisions in young (black) and old (grey) HSCs. Black line denotes WT levels (n=3; Error bars = SEM). B) Kinetics data from young (upper) and old (lower) WT (blue) and JAK2V617F (red) HSCs. Percentage of all cells upon division (n=3; Error bars = SEM, Hrs= hours) C) SC3 clustering of HSC transcriptomes from young (upper orange bars) and old (upper turquoise bars) WT (lower orange bars) and Jak2V617F (lower turquoise bars) mice (Replicates: green, red). Similarity between cells is indicated from blue to red (identical). D) SC3 clustering of old HSC transcriptomes from WT and JAK2V617F mice (replicate 1). Overlap with marker genes is given as percentage. Cell number for old specific HSCs is given as percentage of all old HSCs. E) Immunofluorescent images of old WT and JAK2V617F HSCs stained for pSer15p53 (green), pSer27FosB (red) and DAPI (blue). Bar plots quantify the percentage of double positive HSCs from indicated mice (n=3; Error bars = SEM). F) SC3 clustering of one year old WT and JAK2V617F HSCs. Similarity between cells is indicated from blue to red (identical). Overlap with marker genes is shown in percent. G) Model for relation between proliferation and exhaust of HSCs. Compared are physiological (upper) against enforced proliferation through JAK2V617F (lower). Young HSCs in JAK2 condition expand faster (number of cells), but exhaust more readily upregulating p53 and cyclin-dependent kinase inhibitors (CDKNs)(shades of red). Arrow cycle= self-renewal.
Figure 1

A. WT young vs WT old

B. Heatmap showing expression levels of various markers in young and old cells.

C. Gene expression heatmap for single cells.

D. Clustering analysis showing expression levels across different clusters.

E. Box plots for gene expression levels of CD45, EPCR, CD48, and CD150.

F. Individual and joint posterior distributions for gene expression levels of Hes1, FosB, and Skil.
**Figure 2**

### A: Marker gene expression

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### B: KEGG pathways

- MAPK signalling
- Cell cycle
- TGF-Beta signalling
- p53 signalling

### C: Pathway activity

#### p53 RIS activated genes

- ES = 0.11
- p = 0.009

#### p53 Apo activated genes

- n.s.

#### STAT3 activated genes

- ES = 0.137
- p < 0.001

#### STAT5 activated genes

- ES = 0.084
- p = 0.039

### D: TPO activated genes

- ES = 0.373
- p < 0.001

### E: p53 and gH2AX

- % positive IF staining
- 0
- 30
- 60
- 80
- gH2AX and p53

### F: % positive IF staining

- old HSCs
- no staining
- gH2AX
- EdU

### G: Pre-Meg and CLP activation

- preMeg positive
- ES = 0.2
- p < 0.001

- CLP positive
- n.s.
Fig. 3

A. Division Mut/WT

B. % Cells after 1st division:
   - young
   - WT
   - old

C. WT Old
   - 26% old cluster
   - 100% Marker Gene overlap

D. pFosB, pP53Ser15, DAPI

E. JAK2V617F Old
   - 32% old cluster
   - 100% Marker Gene overlap

F. WT 12 months Hom 12 months
   - cluster size
   - % overlap of marker genes

G. Age: 0-18 months
   - WT: normal proliferation
   - JAK2V617F: proliferative stimulus
   - young exhausted p53/CDKNs