An ERK-Dependent Feedback Mechanism Prevents Hematopoietic Stem Cell Exhaustion

Highlights
- MEK/ERK and AKT/mTORC1 are reversibly activated during hematopoiesis
- MEK1 prevents HSC exhaustion during stress hematopoiesis
- Feedback phosphorylation of MEK1 by ERK limits AKT/mTORC1 activation
- ERK-mediated MEK1 phosphorylation returns activated HSCs to quiescence

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In Brief
Baccarini and colleagues identify a cell-intrinsic feedback mechanism limiting the strength of MEK/ERK and AKT/mTORC1 signals during the activation of hematopoietic stem cells. The mechanism hinges on the negative feedback phosphorylation of MEK1 by activated ERK and is required to prevent HSC exhaustion.

Graphical Abstract
An ERK-Dependent Feedback Mechanism Prevents Hematopoietic Stem Cell Exhaustion

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SUMMARY

Hematopoietic stem cells (HSCs) sustain hematopoiesis throughout life. HSCs exit dormancy to restore hemostasis in response to stressful events, such as acute blood loss, and must return to a quiescent state to prevent their exhaustion and resulting bone marrow failure. HSC activation is driven in part through the phosphatidylinositol 3-kinase (PI3K)/AKT/mTORC1 signaling pathway, but less is known about the cell-intrinsic pathways that control HSC dormancy. Here, we delineate an ERK-dependent, rate-limiting feedback mechanism that controls HSC fitness and their re-entry into quiescence. We show that the MEK/ERK and PI3K pathways are synchronously activated in HSCs during emergency hematopoiesis and that feedback phosphorylation of MEK1 by activated ERK counterbalances AKT/mTORC1 activation. Genetic or chemical ablation of this feedback loop tilts the balance between HSC dormancy and activation, increasing differentiated cell output and accelerating HSC exhaustion. These results suggest that MEK inhibitors developed for cancer therapy may find additional utility in controlling HSC activation.

INTRODUCTION

The production of blood cells depends on the fitness of the hematopoietic stem cell (HSC) compartment, which contributes to multilineage hematopoiesis throughout life (Bernitz et al., 2016; Busch et al., 2015; Sawai et al., 2016; Sun et al., 2014). HSCs are mostly quiescent but can be reversibly activated to meet the increased demand imposed on the organism by stresses, such as infections, blood loss, or chemotherapy-induced myelotoxicity (Baldrige et al., 2010; Essers et al., 2009; Wilson et al., 2008). HSCs exist in at least three different states: a dormant state, characterized by quiescence and by the highest regenerative potential in the HSC cell compartment (Bernitz et al., 2016; Takizawa et al., 2011; Wilson et al., 2008); an active or homeostatic state, reached by gradual transition from the dormant state, in which the HSCs are still quiescent but “primed” for replication (Cabezas-Wallscheid et al., 2017; Wilson et al., 2008); and a fully activated state, characterized by active cycling and typically induced during emergency hematopoiesis. Metabolic changes play an important role in HSC activation. Quiescent HSCs rely primarily on anaerobic glycolysis. They contain low numbers of mitochondria, limited essentially by mitophagy (Ho et al., 2017; Ito et al., 2016; Warr et al., 2013), and low levels of reactive oxygen species (ROS). Conversely, HSC activation is characterized by a continuous increase in biosynthetic activity and correlates with mitochondrial expansion, increased reliance on oxidative phosphorylation, and increased ROS levels (Bigarella et al., 2014; Cabezas-Wallscheid et al., 2017; Chandeli et al., 2016; Schultz and Sinclair, 2016). The mTOR pathway, which stimulates mitochondrial biogenesis and counteracts autophagy, is a major player in HSC activation and differentiation (Chen et al., 2008; Kharas et al., 2010; Rodgers et al., 2014; Yilmaz et al., 2006); in contrast, retinoic acid-vitamin A signaling antagonizes HSC proliferation and promotes the return to quiescence after activation (Cabezas-Wallscheid et al., 2017).

The functionality of the HSC compartment hinges on the balance between quiescence and activation. A reduction in the ability of HSCs to exit quiescence will result in insufficient blood cell production, whereas an increased propensity to exit quiescence or failure to re-enter it will cause HSC exhaustion and bone marrow failure. The identity of the cell-intrinsic mechanism(s) required to terminate activation and re-enter dormancy and the signaling pathways that mediate this transition remain key unresolved questions in the field.

The MEK/ERK and phosphatidylinositol 3-kinase (PI3K) pathways are common targets of growth factors regulating hematopoiesis (Chung and Kondo, 2011; Polak and Buitenhuis, 2012). In mouse models, constitutive activation of these pathways promotes HSC proliferation at the expense of quiescence. Both pathways are activated downstream of RAS, and at least one of the RAS paralogs, KRAS, is required in HSCs for adult hematopoiesis (Dammsnawad et al., 2016); constitutively activated
KRASG12D sensitizes HSCs and progenitors to cytokines (Van Meter et al., 2007), whereas NRASG12D has a bimodal effect on HSCs, increasing regenerative potential in a slow-cycling pool and decreasing it in a fast-cycling pool (Li et al., 2013). In contrast, constitutive activation of the PI3K pathway by deletion of its antagonist PTEN decreases regenerative potential and causes HSC exhaustion, primarily via mTOR activation (Kalaitzidis et al., 2012; Kharas et al., 2010; Lee et al., 2010; Magee et al., 2012; Yilmaz et al., 2006; Zhang et al., 2006). However, mTORC1 activity is required for hematopoiesis (Guo et al., 2013; Hoshii et al., 2012; Kalaitzidis et al., 2012). The roles ascribed to the RAF/MEK/ERK in hematopoiesis so far are lineage specific. Oncogenic BRAFV600E promotes monocyte expansion (Kamata et al., 2005; Staser et al., 2013); in contrast, epiblast-restricted ablation of MEK1 causes extramedullary hematopoiesis and myeloproliferation (Zmajkovicova et al., 2013). Finally, RAF1 is required for erythroid differentiation (Kolbus et al., 2002; Rubiolo et al., 2006).

From all of the above, it is clear that efficient hematopoiesis requires the fine-tuning of the MEK/ERK and PI3K pathways. However, the mechanisms involved in this regulation are currently unknown. We have previously shown that activated ERK can dim the activation of both pathways by feedback phosphorylation of a specific residue on MEK1 (Catalanotti et al., 2009; Zmajkovicova et al., 2013), suggesting that these feedbacks may play a role in hematopoiesis.

RESULTS

MEK1 Prevents HSC Exhaustion during Emergency Hematopoiesis

To study the cell-autonomous effects of MEK1 in hematopoietic cells in detail, we used a conditional knockout (cKO) mouse model inducing hematopoietic system-restricted MEK1 deletion (Var1CRE;Map2klF/F mice, hereafter termed MEK1-cKO; de Boer et al., 2003). MEK1 was efficiently deleted in MEK1-cKO bone marrow (BM), whereas expression of the paralog MEK2 was unaffected (Figure S1A). One-year-old MEK1-cKO showed a moderate peripheral pancytopenia (Figure S1B), which correlated with reduced HSC numbers and loss of label-retaining cells (Figures S1C and S1D; for full HSC fluorescence-activated cell sorting [FACS] gating strategy, see Figure S1E). We next tested the regenerative capacity of MEK1-deficient HSCs by performing serial competitive reconstitution assays, in which CRE+, F/F, or cKO Ly5.2+onor BM cells (each containing 100 HSCs) were mixed with F/F Ly5.1+ competitor BM and injected into lethally irradiated Ly5.1+ recipient mice (Figure 1A). MEK1-cKO cells could contribute to all lineages but yielded low levels of peripheral blood, BM, and HSC chimerism (Figure 1B) and exhausted after the second round of transplantation (Figure 1C). Consistent with this defect in HSC regenerative capacity, MEK1 ablation suppressed chemotherapy-induced emergency hematopoiesis. Repetitive exposure to the myelotoxic drug 5-FU (Figure 1D) caused HSC expansion in F/F animals, whereas in MEK1-cKO mice, the HSC compartment contracted dramatically, leading to BM failure and premature death (Figures 1E and 1F). In the initial phases of the treatment, however, the output of differentiated cells in both BM and peripheral blood of MEK1-cKO animals was higher than that of controls (Figure 1E).

A more detailed analysis of the hematopoietic compartment showed that all other stem and precursor cell subsets analyzed behaved similarly to HSCs, with numbers indistinguishable from the F/F controls in young animals and significant contraction occurring in aging, chemotherapy, and transplantation (Mendeley Data, https://doi.org/10.17632/7rdg6mj5k6h.1).

The defect caused by MEK1 ablation was cell intrinsic and could be recapitulated in long-term cultures (LTCs) of HSCs seeded on F/F feeder layers. In these experiments, MEK1-cKO HSCs produced a significantly higher number of lineage+ cells than F/F cultures, whereas the number of cells capable of generating colony-forming units (CFUs) steadily decreased (Figure 1G). Increased output of differentiated cells and HSC exhaustion correlated with reduced numbers of HSCs in G0 in all the systems investigated (Figure 1H).

MEK1 Guards against HSC Exhaustion by Regulating the Expression of Genes Promoting Cell Cycle and Oxidative Phosphorylation

To gain further insight on the role of MEK1, we next focused on mice recovering from a single 5-FU injection, which promotes the reversible activation of virtually all dormant HSCs (Wilson et al., 2008). In this setup, the temporal correlation among contraction of the HSC compartment, increased HSC proliferation, and increased output of differentiated cells in MEK1-cKO mice was readily apparent (Figures 2A–2C). To explore the mechanisms driving HSC exhaustion in an unbiased manner, we performed transcriptome-wide RNA sequencing (RNA-seq) experiments comparing F/F and MEK1-cKO HSCs from untreated animals or from animals recovering from one single 5-FU injection administered 9 days before harvesting the cells, at the time of maximum HSC expansion in F/F BM. 483 genes were upregulated and 450 were downregulated in 5-FU-treated F/F HSCs (complete gene list in Table S1); among these genes, two groups were clearly impacted by MEK1 ablation (Figures 2D, 2E, and S2). The first group included genes connected with

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Figure 1. MEK1 Ablation Increases HSC Proliferation and Differentiation, Leading to HSC Exhaustion

(A) Serial transplantation protocol.

(B) Kaplan-Meier survival curve. Median survival time (MST): F/F = 84 days; MEK1-cKO = 39 days; p < 0.001 according to the log rank (Mantel-Cox) test.

(C) Colony-forming units (CFUs) and % lineage+ cells derived from HSCs in LTC.

(D) Kaplan-Meier survival curve. Median survival time (MST): F/F = 84 days; MEK1-cKO = 39 days; p < 0.001 according to the log rank (Mantel-Cox) test.

(E) HSCs per femur, lineage+ cells per femur, and peripheral blood parameters (Hb, hemoglobin; PLT, platelets; WBC, white blood cells) during repetitive 5-FU treatment.

(F) Cell cycle distribution of HSCs harvested 12 weeks after transplantation (Transplut), 12 days after the third 5-FU injections (rep 5-FU), or after 6 weeks in LTC. Error bars represent the SD of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001 comparing CRE+ or F/F to cKO. See also Figure S1.
Mitochondrial mass and mitochondrial membrane potential/cell did not significantly differ in F/F and cKO HSCs recovering from a single 5-FU injection (Figure S3G). However, mitochondrial mass was increased and membrane potential (Δψm) was decreased in MEK1-cKO HSCs and multipotent progenitors upon repetitive 5-FU stress, as well as in cKO HSCs in LTCs (Figures 3D, 3E, and S3F), suggesting an accumulation of damaged mitochondria in these models. Damaged mitochondria are cleared by PINK1/PARK2-mediated mitophagy (Pickrell and Youle, 2015), which supports HSC maintenance downstream of the PML/PPARδ pathway (ito et al., 2012, 2016). The PINK1 gene was downregulated in the cKO (Table S1), and PINK1 protein expression was reduced in MEK1-cKO HSCs from 5-FU-treated mice or from LTCs (Figure 3F). Confocal microscopy revealed reduced co-localization of mitochondria with lysosomes (labeled with Tom20 [translocase of outer membrane 20] and LAMP1 [lysosomal-associated membrane protein 1] antibodies, respectively) in cKO HSCs from 5-FU-treated mice or from LTCs (Figure 3G), suggesting decreased mitophagy.

**MEK/ERK and AKT/mTORC1 Are Reversibly Activated during Emergency Hematopoiesis**

The data above show that MEK1 dials down proliferation, OXPHOS, and the accumulation of ROS and damaged mitochondria in HSC stimulated to proliferate in vivo and in vitro. To elucidate the MEK1-dependent mechanisms governing these key parameters of reversible HSC activation, we combined surface marker and intracellular (phospho)protein FACS analysis and focused on MEK-related HSC signaling during the recovery from a single 5-FU injection (for antibody validation, see Mendenley Data, https://doi.org/10.17632/7rdg6mjk5h.1). We analyzed MEK phosphorylation on the RAF-dependent activation sites (S218/222) and MEK1 phosphorylation on T292, the ERK-dependent site required for MEK1/MEK2 inactivation and for efficient PTEN-mediated PIP3 turnover at the membrane (Catalanotti et al., 2009; Zmajkovicova et al., 2013). In addition, we analyzed the activation of AKT (defined as the phosphorylation of S473) and of its downstream target mTORC1, which is involved in HSC mobilization through ROS control and mitochondrial biogenesis (Chandel et al., 2016; Ito and Suda, 2014). Activation of mTORC1 was defined as phosphorylation of mTOR and of the mTORC1 target S6. In HSCs of 5-FU-treated mice, phosphorylation of MEK1, ERK, AKT, and mTORC1 paralleled HSC proliferation, output of differentiated cells, and ROS increase (compare Figure 4A with Figures 2A–2C and 3A). Activation of ERK, AKT, and mTORC1 was also significantly increased in MEK1-cKO HSCs isolated from 5-FU-treated or transplanted mice or from LTCs (Figure S4A). Furthermore, the transcription factor FOXO3A, which promotes HSC maintenance (Miyamoto et al., 2007; Warr

Figure 2. MEK1 Ablation Delays Return to Quiescence during Recovery from a Single 5-FU Injection and Promotes the Expression of Cell Cycle and OXPHOS Genes

(A–C) BM cellularity and HSCs per femur (A), HSC cell cycle distribution (B), and blood parameters (C) during the recovery from 5-FU injection. 
(D and E) Heatmap (D) and scatterplots (E) of genes differentially expressed in F/F and cKO HSCs from untreated or 5-FU-treated mice (9 days prior isolation). Blue, increased expression in F/F HSCs; red, increased expression in cKO HSCs. 
(F) qRT–PCR of selected cell cycle and OXPHOS genes in FAC-sorted HSCs. Data are normalized to expression levels in F/F HSCs from untreated mice (dotted line).

Error bars represent the SD of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001 comparing CRE+ or F/F to cKO. White asterisks, comparison of 5-FU-treated versus untreated animals of the same genotype. See also Figure S2 and Table S1 for a complete gene list.
et al., 2013) and is negatively regulated by ERK (Yang et al., 2008) and AKT (Brunet et al., 1999), was phosphorylated in HSC from 5-FU-treated mice and more robustly so in MEK1-cKO HSC in this and all other models tested (Figures 4B and S4B). On day 9 after 5-FU injection, FOXO3A was excluded from the nuclei of both F/F and cKO HSCs (Figure 4C) and the expression of its target genes Sod2 and Catalase (involved in limiting ROS levels), as well as of Pink1 (Ito and Suda, 2014; Ito et al., 2016; Mei et al., 2009; Miyamoto et al., 2007; Rimmelé et al., 2015; Tothova and Gilliland, 2007; Warr et al., 2013), was downregulated, particularly in the cKO (Figure 4D). Thus, coordinated activation of the ERK and AKT pathways coincides with increased proliferation, ROS production, and the control of mitophagy during the recovery from myelotoxic 5-FU treatment. In line with this, phosphorylation of the RAF-dependent and ERK-dependent sites on MEK1 as well as ERK and AKT phosphorylation were lowest in

Figure 3. MEK1 Ablation HSCs Promote Oxidative Stress and Decrease Mitophagy in HSCs
(A) ROS levels in HSCs recovering from 5-FU injection.
(B) Representative FACS histograms of ROS levels in HSCs exposed to chronic stress as in Figure 1. Values represent mean fluorescence intensity (MFI) ± SD.
(C) Kaplan-Meier survival curve of mice. MST: F/F = 93 days; cKO = 39 days; F/F + NAC = 92 days; cKO + NAC = 65 days. **p < 0.01 according to the log rank (Mantle-Cox) test; n = 10.
(D and E) Mitochondrial mass (Mitotracker Green, D, and ΔΨ per mitochondrial mass, E) in HSCs isolated from mice exposed to stress or grown in LTC.
(F) FACS analysis of PINK1 protein levels in HSCs.
(G) Representative confocal images and quantification of the co-localization of mitochondria (TOM20+) and lysosomes (LAMP1+) in HSCs exposed to a single 5-FU injection (9 days before isolation) and chronic stresses as defined in the legend to Figure 1.
Error bars represent the SD of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001. White asterisks, comparison of 5-FU-treated versus untreated animals of the same genotype. See also Figure S3.
HSCs and increased with differentiation in the BM of untreated F/F mice (Figures S4C and S4D).

Control of mTORC1-Dependent ROS Production by MEK1 Regulates HSC Activation and Regenerative Potential

To explore the relationship among HSC activation, OXPHOS and ROS increase, and ERK/AKT signaling, we silenced MEK1 or inhibited MEK in mouse or human HSC LTCs. It is important to note here that the MEK inhibitor was titrated to achieve fine-tuning rather than complete inhibition of ERK activation (about 50%; Figures 5C and 7C), which is not tolerated by the cells. Both treatments mimicked the defects induced by CRE-mediated MEK1 ablation, reducing the number of CFUs, increasing ROS levels and mitochondrial mass, and decreasing mitochondrial fitness (Figures S5A–S5D). Inhibition of OXPHOS and mTOR by metformin (Foretz et al., 2014) or addition of NAC increased CFU numbers in mouse and human LTCs, normalized ROS levels, and rescued the mitochondrial phenotype caused by MEK1 deficiency or MEK inhibition without affecting ERK or AKT activation (Figures S6A–S6F). Thus, increased ROS production is responsible for mitochondrial damage in our system and is the consequence, not the cause, of the increased ERK/AKT signaling induced by MEK1 silencing or inhibition. MEK1 down-regulation activated both the ERK and the AKT pathway, whereas chemical MEK inhibition prevented activation of ERK and phosphorylation of MEK1 on the ERK-dependent site T292 (Catalanotti et al., 2009; Zmajkovicova et al., 2013) and activated the AKT pathway exclusively (Figures S5E and S5F). These results strongly suggested that AKT pathway activation, rather than the sustained MEK2/ERK phosphorylation characteristic of MEK1-deficient cells, was responsible for the phenotypes...
observed. Indeed, inhibition of PI3K by LY294002 or of mTORC1 by rapamycin (Chiang and Abraham, 2005; Shimobayashi and Hall, 2014) fully rescued the biological phenotypes of MEK1-deficient mouse HSCs (Figures 5A and 5B, left) or MEK-inhibited human HSCs (Figures 5A and 5B, right). PI3K inhibition also selectively reduced the phosphorylation of mTOR and of FOXO3A on the AKT-dependent site and restored PINK1 protein expression (Figure 5C).

To determine whether the control of mTORC1 signaling, OXPHOS, and ROS by MEK1 is causally related to the increased HSC attrition observed in the MEK1-cKO mice, we treated animals exposed to single or multiple 5-FU injections with NAC or rapamycin. Both NAC and rapamycin reduced the rebound of HSC numbers as well as the percentage of actively cycling HSCs in control F/F mice recovering from a single 5-FU injection, indicating that the transient increase in mTORC1 signaling and ROS is causally related to the production of HSCs (Figure 6A). Importantly, both inhibitors reduced ROS production, demonstrating that mTORC1 is responsible for the transient increase in ROS during HSC activation (Figures S7A and S7B). In MEK1-cKO mice, NAC and rapamycin had the opposite effect; they reduced the percentage of cycling HSC, increased the numbers of BM HSCs (Figure 6A), and decreased the output of differentiated cells (Figures S7C and S7D), essentially erasing the difference in the reaction of MEK1-cKO and F/F mice to myelotoxic 5-FU treatment and rescuing the phenotype. NAC and rapamycin treatment also protected MEK1 cells from attrition during repetitive 5-FU chemotherapy (Figures 6B and S7E).

Most importantly, treatment of recipient mice with NAC and rapamycin fully rescued the reconstituting potential of MEK1-deficient HSCs in a competitive transplantation experiment (Figures 6C, 6D, S7G, and S7H).

ERK-Dependent Phosphorylation of MEK1 T292 Is the Linchpin of the Intracellular Negative Feedback Mechanism that Allows Reversible HSC Activation

To gain insight into the mechanism underlying the MEK1-dependent negative feedback, we attempted to rescue the phenotypes induced by MEK1 ablation in mouse HSCs (Figures 7A–7C, left) or by MEK1 inhibition in human HSCs (Figures 7A–7C, right) by expressing wild-type MEK1 and its phosphorylation site mutants T292D (mimicking ERK-dependent feedback phosphorylation) or T292A (preventing ERK-dependent feedback phosphorylation). T292D MEK1 rescued all biochemical and biological phenotypes induced by MEK1 ablation or MEK inhibition, whereas T292A MEK1 failed to do so. Wild-type MEK1, on the other hand, rescued the phenotypes induced by MEK1 deficiency, but not those due to MEK1 inhibition, which prevents T292 phosphorylation (Figures 7A–7C, compare the left with the right).

FOXO3A mutants that cannot be phosphorylated by AKT, but not those that cannot be phosphorylated by ERK, significantly increased the number of CFUs in MEK1-deficient mouse HSC LTCs as well as in MEK-inhibited human HSC LTCs (Figure 7A). This correlated with a decrease in damaged mitochondria (Δψ per mitochondrial mass; Figure 7B) and with restored expression of the FOXO3A target PINK1 (Figure 7C). ROS levels remained high in FOXO3A (AKT)-transduced HSCs (Figure 7B). Ectopic PINK1 expression also reduced the number of damaged mitochondria without affecting ROS production and increased the number of CFUs recovered from mouse MEK1-deficient LTCs (Figures 7A and 7B, left). In MEK-inhibitor-treated human HSC LTCs, on the other hand, PINK1 expression only marginally reduced damaged mitochondria and failed to restore CFU numbers (Figures 7A and 7B, right). Whereas the reason for this discrepancy is currently unknown, it is worth noting in this context that ERK inhibition can decrease PINK1 stability and PARK2 recruitment (Park et al., 2017).

DISCUSSION

Collectively, our data show that the ERK and the PI3K pathways are coordinately activated in HSCs and that a cell-intrinsic negative feedback signaling loop involving phosphorylation of MEK1 by activated ERK limits the strength and duration of PI3K/AKT/mTORC1 signaling to allow activated HSCs to return to quiescence. If this ERK-dependent feedback is disabled, HSCs shift from a dormant to an active or primed state (Cabezás-Wallscheid et al., 2017), which has been described as “GALERT,” interestingly induced by mTORC1 in other stem cell populations (Rodgers et al., 2014). The mechanism is at work during homeostasis, as shown by the contraction of the hematopoietic compartment in untreated, aged MEK1-cKO mice; however, its essential role in preventing HSC burnout becomes obvious when HSCs are induced to proliferate in vivo by chemotherapy or transplantation and in in vitro in LTCs. In all these models, exhaustion is driven by a combination of mTORC1-dependent ROS production, which damages the mitochondria, and decreased FOXO3A/PINK1-dependent clearance of these organelles (Figure 7D).

Our data exemplify the relevance of paralog-specific signaling, and more precisely of paralog-specific pathway cross-talk, in the ERK pathway. In the RAF/MEK/ERK cascade, multiple paralogs are able to carry out common function, consisting in MEK phosphorylation in the case of the three RAF kinases and in ERK phosphorylation in the case of the two MEK kinases. This raises the question of why so many paralogs have survived evolution. In evolutionary terms, the paralogs RAF1 and MEK1 can be considered “moonlighting proteins,” multifunctional proteins that have
evolved to carry out at least one essential function that is separate from their role within the ERK pathway. Specifically, RAF1 and MEK1 are required to regulate the activity of other pathways (RHO-dependent pathways in the case of RAF1; PI3K in the case of MEK1) via protein-protein interaction (Desideri et al., 2015; Dorard et al., 2017). Intriguingly, it is the activation of the core pathway (RAF/MEK/ERK) that generates the phosphorylated forms of RAF1 and MEK1 required for the cross-talk with the RHO and with PI3K pathway, respectively (Varga et al., 2017; Majkovicova et al., 2013). In the case of MEK1, T292 phosphorylation promotes the formation of a ternary complex (MEK1/MAGI100/PTEN) required for the translocation of PTEN to the membrane, where the phosphatase turns over PIP3 and dims signaling (Majkovicova et al., 2013). T292 first appears in amniota and is then conserved in this clade, hinting at an important role for this residue in the control of signal strength and diversification in complex organisms. In the specific context of HSC development, it is worth noting that the earliest fossil evidence of a hematopoietic bone marrow, found in a sarcopterygian fish with a close evolutionary relationship to the tetrapoda (Sanchez et al., 2014), precedes the appearance of MEK1 T292, making it tempting to speculate that this residue might have evolved to represent an advantage in this context. Much progress has been made in understanding the signals mobilizing HSC in homeostasis and in emergency hematopoiesis in the context of the bone marrow niche (Crane et al., 2017). The emerging picture is exceedingly complex, including signals delivered through soluble factors and through cell-cell contact. Our results show that

Figure 7. ERK-Dependent Phosphorylation of MEK1 T292 Feeds Back on AKT/mTORC1/FOXO3A Signaling to Balance HSC Quiescence and Differentiation

Effect of lentiviral expression of MEK1 and FOXO3A (wild-type or phosphosite mutants) and of PINK1 on mouse F/F and cKO HSCs or on IMEK-treated human HSCs.

(A–C) CFUs (A), ROS levels and Δψ per mitochondrial mass (B), and intracellular signaling (C) were determined after 6 weeks. Data show fold change relative to mouse cKO or human IMEK-treated HSCs (normalized as 1; dotted line; n = 3). Error bars represent the SD of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001, comparing vector-infected HSCs with HSCs infected with the indicated constructs.

(D) Model: differentiation-promoting events in red; see text for details.
these stimuli, whatever their nature, converge on the MEK/ERK and PI3K modules. T292 phosphorylation by activated ERK might have evolved as a robust system of checks and balances, ensuring that these two crucial pathways will be coordinately reset irrespectively of the stimulus that activated them; the only requirement is the presence of the protein–protein interaction partners mediating the feedback (i.e., MAGI100), which determines the tissue specificity of the phenotypes. In favor of this hypothesis, it is worth noting that the defective repopulating potential of both MEK1 cKO HSCs (Figure 6) and PTEN KO HSCs (Lee et al., 2010; Yilmaz et al., 2006) is caused by excessive mTORC1 activation as exemplified by the rescue of this function through rapamycin treatment of the recipients.

On a more translational note, the complexity of the signals that regulate hematopoiesis limits the design of interventions aimed at modulating the entry or exit of HSCs from quiescence, whereas the feedback mechanism described here is druggable. At least in LTCs, chemical inhibition of MEK mimics the effect of ablation, preventing the inactivation of the AKT/mTORC1 pathway and increasing HSC output. This suggests that targeting the MEK/ERK pathway could be developed into a strategy to fine-tune the HSC compartment, for instance by remobilizing dysfunctional HSCs with reduced differentiation potential, as observed in aging (Akunuru and Geiger, 2016). In the context of leukemia, oncogenic NRASG12D, the common activator of MEK and PI3K, is considered an early, predisposing mutation potentially promoting the expansion of pre-leukemic clones. NRASG12D promotes the proliferation and reduces the repopulating potential of a subset of HSCs while reducing the proliferation and increasing the repopulating potential of another subset. It is tempting to speculate that, in the latter subset, the strict feedback control of PI3K signaling promotes proliferation, preserving a pool of self-renewing clones, whereas in fast-proliferating HSC pools, reduced feedback control would tilt the balance toward sustained PI3P signaling, fast proliferation, and ultimately exhaustion. In line with this hypothesis, MEK1 ablation or inhibition restricts NRAS-driven disease (Nowacka et al., 2016; Wang et al., 2013) and mTOR activation suppresses leukaemogenesis in PTEN-deficient animals (Kalaitzidis et al., 2012; Lee et al., 2010); targeting MEK/ERK to modulate both pathways could promote the exit of malignant stem cells from quiescence to sensitize them to chemotherapy and/or stimulate their differentiation.

**STAR+METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and two tables and can be found with this article online at https://doi.org/10.1016/j.stem.2018.05.003.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, M.B., C. Bock, and V.S.; Methodology and Investigation, C. Baugmarter, S.T., I.F., M.F., and R.S.; Data Curation and Formal Analysis, C. Baumgartner and M.B.; Funding Acquisition and Supervision, M.B.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


## STAR METHODS

### KEY RESOURCES TABLE

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### Experimental Models: Cell Lines

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### Experimental Models: Organisms/Strains

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Manuela Baccarini (manuela.baccarini@univie.ac.at).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

To obtain conditional knockout (cKO) mice lacking MEK1 in the hematopoietic system, MEK1 F/F mice (Catalanotti et al., 2009) were crossed with Vav1-iCre transgenic mice (de Boer et al., 2003). Genotyping of MEK1 was performed by using 3 different primers: primer 1, 5'-GACGTGGTGAACAGGAAAGGGATTGGG-3'; primer 2, 5'-TGGAGCTGGAGTCACGGGTGGTTGA-3'; and primer 3, 5'-GCAGACTGGGACGGTGCACGGTG-3'. Primer pair 1 and 3 amplifies a 470-bp fragment of the endogenous Map2k1 allele and a 520-bp fragment of the floxed allele, whereas primer pair 1 and 2 amplifies a 600-bp fragment of the null Mek1 allele (Catalanotti et al., 2009). The following primers were used for Vav1-iCre PCR: forward 5'-GCCTGCCCTCCCTGGATGCCACCT-3' and reverse 5'-GTGGCAGAAGGGGCACCCACACCATT-3'. Both mouse strains (MEK1 F/F and MEK1 cKO) were on a pure C57/BL6 Ly5.2 (= CD45.2) background. Unless otherwise specified, all experiments were performed with 8-12 weeks old body weight-matched mice from both genders. For transplantation experiments, 12 weeks old Ly5.1 (= CD45.1) C57/BL6 recipient mice were used. All animals were maintained at the MFPL animal facility under specific pathogen-free conditions and all experiments were performed in accordance with a protocol authorized by the Austrian Ministry of Science and Communications. The health status was determined by using sentinel animals housed under the same conditions. Human bone marrow derived CD34+ progenitor cells from young healthy female and male donors were obtained from CellSystems.

METHOD DETAILS

5-FU treatment
To induce emergency hematopoiesis, 5-Fluorouracil (5-FU; Sigma) was dissolved in 0.9% NaCl for 15 min at 55°C and 150 mg/kg were injected intraperitoneally either as a single dose or as multiple doses (up to 7, once every 12 days; repetitive 5-FU).

N-acetyl-L-cysteine treatment in vivo
N-acetyl-L-cysteine (NAC; Sigma) was dissolved in PBS and 100 mg/kg was administered daily by subcutaneous injection. To study the effect of ROS on the recovery from a single 5-FU injection, animals were pre-treated with NAC for 7 days.

Rapamycin treatment in vivo
Rapamycin (RAPA; Enzo Life Science) was dissolved in absolute ethanol (10 mg/ml), further diluted in 5% PEG-400/5% Tween80/ PBS and 0.4 mg/kg were administered daily by intraperitoneal injection. To study the effect of mTOR signaling on the recovery from a single 5-FU injection, animals were pre-treated with RAPA for 7 days.

Serial competitive stem cell transplantation assay
A 1:1 mixture of Ly5.2+ donor (F/F, cKO, or CRE) and Ly5.1+ competitor BM cells (2 × 10^6 total BM cells containing 100 donor and 100 competitor HSCs) were prepared in HBSS and transplanted by tail-vein injection into lethally irradiated, two-three months old C57BL/6 Ly5.1 (= CD45.1) recipient mice. Starting three weeks after transplantation, peripheral blood was collected once per week in EDTA tubes and blood counts were performed on a V-Sight analyzer (Menarini Diagnostics, Italy). Donor chimerism in the peripheral blood was determined by FACS analysis. BM analysis was performed 12 weeks after transplantation. At this time point, 2 × 10^6 BM cells isolated from primary recipients were re-injected into lethally irradiated Ly5.1+ secondary recipient mice and monitored by the same protocol. In selected experiments, recipients were treated with NAC, RAPA, or the respective vehicle once per day starting from 2 weeks after transplantation.

5-bromodeoxyuridine label experiment
To determine the numbers of 5-bromodeoxyuridine (BrdU, Sigma) label-retaining cells, a single dose of BrdU (200 mg/kg i.p.) was administered to 5-month old animals, followed by 1 mg/ml BrdU in drinking water (14 days; BrdU water was exchanged every 3 days). After a chase period of 200 days, BM cells were isolated and stained with surface antigen markers for mouse HSPC characterization. BrdU incorporation was measured by flow cytometry using the FITC BrdU Flow Kit (BD Biosciences) according to the manufacturer’s instructions. BM cells from non-BrdU injected mice were used as a control.

In vitro long-term co-culture (LTC) assay
LTC assays were performed according to the protocol from StemCell Technologies (https://cdn.stemcell.com/media/files/manual/MA28412-Human_Long_Term_Culture_Initiating_Cell_Assay.pdf). For mouse HSCs (LSK CD150+ CD48-), Ly5.1 BM Stromal feeder layer cells were generated in 96-well plates (flat bottom, tissue culture treated) according to the protocol. The M2-10B4 mouse fibroblast cell line (ATCC® CRL-1972; ATCC) was used as stromal feeder layer for human HSCs (CD34+CD38- cells). After irradiation of the feeder layers, 100 HSCs per well were FACs-sorted directly onto the feeder layer and incubated for up to 10 weeks at
33°C (5% CO₂) in MyeloCult medium (M5300 for mouse cells or M5100 for human cells, StemCell Technologies, supplemented with 1μM hydrocortisone). Half of the medium was replaced with fresh medium once per week. In selected experiments, cells were treated with DMSO (1:10,000 dilution; Sigma) IMEK ( = U0126, 250 nM; Cell Signaling Technologies = CST), PI3K ( = LY294002, 1 μM; CST), Rapamycin (500 nM; Enzo Life Science), Metformin (500 μM; Sigma), and NAC (100 μM; Sigma), either alone or in different combinations.

Cells were harvested at different time points (2, 4, 6, 8, 10 weeks) and transferred to cytokine-supplemented methylcellulose medium (MethoCult GF M3434 or MethoCult H4435 Enriched, StemCell Technologies). Colony forming units (CFUs) were scored after 10 days. For further functional characterization after 6 weeks in co-culture, ROS levels, mitochondrial mass, mitochondrial membrane potential, and intracellular signaling in HSCs identified as Ly5.2+ LSK CD48- (mouse) or CD34+ CD38- cells (human) were determined by FACS.

HSC senescence was assessed by single-cell-sorting LSK CD150+ CD48- cells into cytokine-supplemented methylcellulose medium (MethoCult GF M3434) and counting the number of colonies derived from 100 sorted single-cells after 10 days of incubation.

**Flow cytometry analysis and sorting of mouse and human HSCs**

Mouse BM cells were recovered by crushing of femora and tibiae in 5 mL of 2% FCS/IMDM medium using a mortar and pestle. The cell suspension was filtered through a 70 μm cell strainer and washed twice in 2% FCS/IMDM. Viability staining was performed following red blood cell lysis (eBioscience) using the fixable viability dye eFluor 450 (eBioscience). Gating started with a FSC/SSC dot plot visualizing all acquired events. After doublet discrimination using FSC-A/FSC-W, singlets were gated for live cells. Live single cells were then plotted against surface/intracellular markers. The boundary between positive and negative cells was determined by fluorescence-minus-one (FMO) and isotype controls. For FACS analysis of SLAM-marker-defined mouse HSPCs, cells were stained with a cocktail of biotinylated lineage-specific antibodies against Gr-1 (RRID:AB_469757), CD5 (RRID:AB_466338), CD2 (RRID:AB_466314), CD3ε (RRID:AB_466320), Ter119 (RRID:AB_466796), CD8α (RRID:AB_466345), and B220 (RRID:AB_466450) (all from eBioscience), washed thrice in 1% FCS/PBS, and subsequently stained with streptavidin-PerCP (RRID:AB_2336918, BD Bioscience) together with fluorophore-labeled antibodies against Sca1 (RRID:AB_466085, eBioscience), c-KIT (RRID:AB_1272177, eBiosciences), CD150 (RRID:AB_493460, BioLegend) and CD48 (RRID:AB_1727501, BD Biosciences) for immature progenitors or fluorophore-labeled antibodies against Sca1, c-KIT, CD48 (RRID:AB_2016604 or RRID:AB_465020, eBioscience) and CD16/32 (RRID:AB_465568, eBioscience) for mature progenitors. For FAC-sorting, BM cells were first stained with the biotinylated lineage-cocktail followed by lineage-depletion using α-biotin beads and an AutoMACS cell separator (Miltenyi Biotec). Lineage-depleted BM cells were then labeled with a panel of antibodies to identify HSPCs (Sca1, c-KIT, CD150, CD48). Mouse HSPCs were defined as follows: lin+, lineage-positive (differentiated cells); lin- lineage-negative cells; LK, lin- Sca1- c-KIT+ (mature progenitors) = MEP, lin- Sca1+ c-KIT- CD34+ CD16/32+; CMP, lin- Sca1+ c-KIT- CD34+ CD16/32-; GMP, lin- Sca1+ c-KIT+ CD34- CD16/32-; MEP, lin- Sca1+ c-KIT+ CD34+ CD16/32+. B cells were identified as B220+, T cells as CD3ε+, and myeloid cells as Mac1/Gr1+. Differentiation was analyzed by staining with conjugated antibodies against specific surface markers as defined above plus α-Mac1 (RRID:AB_953560, eBiosciences), B cells were identified as B220+, T cells as CD3ε+, and myeloid cells as Mac1/Gr1+. In competitive transplantation experiments, peripheral blood chimera was assessed by co-staining with antibodies against Ly5.1 (RRID:AB_465059) and Ly5.2 (RRID:AB_657752; both from eBiosciences).

**Cell cycle analysis**

Surface marker stained BM cells were washed in phosphate-buffered saline (PBS; pH 7.4), fixed in Cytofix/Cytoperm buffer (BD Bioscience) for 30 min at room temperature (RT), washed twice with PermWash buffer (BD Bioscience), and incubated in PermWash buffer with Ki-67-FITC antibody (1:20, BD Bioscience) for 1h at RT. Cells were then washed twice with PermWash buffer, resuspended in 1% BSA/PBS containing 1μg/ml DAPI, and incubated for further 15 min before analysis.

**ROS, MitoTracker and mitochondrial membrane potential measurement**

Freshly isolated BM or cells from LTCs washed in PBS and incubated for 30 min at 37°C with CellROX Green or CellROX Deep Red (1 μM, Thermo Fisher Scientific), MitoTracker Green or MitoTracker Deep Red (20 nM, Thermo Fisher Scientific), or tetramethylrhodamine ethyl ester perchlorate (TMRE; 5 nM, Sigma) in 2% FCS/IMDM. After labeling, cells were washed three times with PBS and stained with cell surface markers for mouse or human HSCs. A minimum of 100 HSCs per condition were analyzed by FACS.

**FACS intracellular staining for (phospho)-proteins**

For intracellular staining, BM cells were stained with HSC surface markers, fixed for 20 min at RT in Cytofix/Cytoperm buffer (BD Bioscience), washed twice with PermWash buffer (BD Bioscience), and incubated for 1h at RT with the following primary antibodies: α-cleaved caspase 3 (RRID:AB_2070042; CST, 1:200), α-MEK1 (RRID:AB_10893788, CST, 1:200), α-pMEK1/2 S218/222
(RRID:AB_331648, CST; 1:100), α-MEK1/2 (RRID:AB_823567, CST; 1:100), α-pMEK1 T292 (RRID:AB_568845, Upstate; 1:100),
α-MEK2 (RRID:AB_2140641, CST; 1:100), α-ERK1/2 (RRID:AB_330744, CST; 1:200), α-pERK1/2 (RRID:AB_331646, CST; 1:200),
α-AKT (RRID:AB_329827, CST; 1:200), α-pAKT S473 (RRID:AB_329825, CST; 1:100), α-FOXO3a (RRID:AB_836876, CST; 1:200),
α-pFOXO3A S294 (RRID:AB_1086878, CST; 1:100), α-pFOXO3A S253 (RRID:AB_2106674, CST; 1:100), α-mTOR (RRID:
AB_2108622, CST; 1:250), α-p-mTOR S2448 (RRID:AB_10861552, CST; 1:100), α-S6 (RRID:AB_331355, CST; 1:200), α-pS6
(RRID:AB_916156, CST; 1:100), α-PINK1 (RRID:AB_447627, Abcam; 1:200) or α-rabbit-mAb-lgG-isotype (RRID:AB_1550038,
CST; concentration matched) and α-mouse-mAb-lgG-isotype (RRID:AB_10829607, CST; concentration matched). Cells were
washed thrice in PermWash buffer and subsequently incubated with the conjugated secondary antibodies Alexa488-goat α-rabbit-
goat α-mouse or Alexa647-goat α-rabbit/-goat α-mouse (all from Thermo Fisher Scientific) for 1h at RT. After washing 3 times
in PermWash buffer, cells were resuspended in 2%BSA/PBS and analyzed. All sorting and FACS analysis experiments were per-
formed using a BD FACS Aria III or BD LSM Fortessa analyzers. Data were analyzed with the FlowJo (10.2) software. A validation
of the antibodies used in this study is deposited in the Mendeley database (https://doi.org/10.17632/7rdg6mj5k5h.1).

Immunofluorescence staining
HSCs (500-1000 cells) were FAC-sorted directly onto poly-L-lysine coated coverslips, incubated for 2h, and fixed in PBS/4% PFA for
20 min at RT. Cells were permeabilized in PBS/0.1% Triton X-100 for 10 min at RT prior to blocking in PBS/0.1% Triton/5% BSA for
1h at RT. Cells were incubated in blocking solution with the following primary Abs, either for 2h at RT or at 4°C overnight:
rabbit α-FOXO3a (RRID:AB_836876; CST; 1:200), mouse α-TOMM20 (RRID:AB_945896, Abcam; 1:100), rabbit α-LAMP1
(RRID:AB_775978, Abcam; 1:150) or mouse α-phospho-Histone H2AX (S139 = anti-γH2AX; RRID:AB_309864; 1:200). After incu-
bation, cells were washed 3-5 times with PBS and incubated for 1h at RT in blocking solution with Alexa488-conjugated goat α-rabbit-
bit, Alexa488-conjugated goat α-mouse, or Alexa647-conjugated goat α-rabbit (all from Thermo Fisher Scientific). Cells were then
washed thrice in PBS and mounted in VectaShield with DAPI (Vector Laboratories). Slides were imaged on an inverse point scanning
confocal microscope (Zeiss LSM 710 Airyscan, 63X or 100X magnification), and images were processed using Zeiss imaging soft-
ware. For statistics, 100 cells per condition were scored on a Zeiss Axio Imager Z2 microscope.

RNA-seq library preparation and data analysis
100 HSCs per sample were FAC-sorted and incubated in 96 well plates containing lysis buffer. cDNA synthesis and enrichment were performed
according to the Smart-seq2 protocol as described previously (Picelli et al., 2014). ERCC spike-in RNA (Ambion) was added to
the lysis buffer in a dilution of 1:1,000,000. Library preparation was conducted on 1 ng of cDNA using the Nextera XT library prepa-
ration kit (Illumina) as described. Sequencing was performed by the Biomedical Sequencing Facility at CeMM using the 50 bp single-
read setup on the Illumina HiSeq 3000/4000 platform. To analyze the data, we first trimmed off sequencing adapters from the
reads generated, and then aligned the reads using Bowtie v 1.1.1 (Langmead et al., 2009) parameters: -q -p 6 -a -m 100–minins
0–maxins 5000–fr–sam–chunkmbs 200) to the cDNA reference transcriptome (mm10 cDNA sequences from Ensembl v 77). Next,
we removed duplicate reads before quantifying transcript levels with BitSeq v 1.12.0 (Glaus et al., 2012). The raw transcript counts
were loaded into R and collapsed by taking the expression level of the most strongly expressed transcript per gene. For statistical
analysis, we used these read counts as input for DESeq2 (Love et al., 2014) factoring in the date of library preparation and sequencing
as a covariate to reduce the effect of technical variation. For visualization, we used variance-stabilized data.

For knockdown experiments, predicted mouse shMEK1 (5′-TGCTTGTGACAGTGACGCA
CGACGTCGAACATCGAATAGTGAAAGCCCAAGATGTATTTACCTGACTGTACCTCGGCCTACTGCTCGGA-3′) and
human shMEK1 (5′-GAAGGTGATAT TGCTTGTGACAGTGACGCAACCTCTGTTGTATTTATATAGTTGAAGCCACAGTAGTATATA
GAATACACGACAGGTCGCCTACTGCTCGGACTCAAGGGGCTAG-3′) sequences were subcloned into the lentiviral SGEF
vector (containing GFP) using Xhol/EcoRI enzymes and Gibson cloning strategy (Sellmann et al., 2013). The same SGEF
vector was used for the overexpression of the following constructs: MEK1 WT, MEK1 T292D, MEK1 T292A, FOXO3A WT, FOXO3A
ERK-insensitive (S294A, S344A, S425A), FOXO3A AKT-insensitive (T32A, S253A, S315A), FOXO3A ERK/AKT-insensitive (combined
ERK and AKT point mutations), and PINK1. The genes of interest were inserted into the lentiviral SGEF vector downstream of the PGK
promoter using BveI/Csil enzymes and thus replaced the selection marker puromycin. All MEK1 constructs have been described pre-
viously (Catalanotti et al., 2009; Zmajkovicova et al., 2013). FOXO3a WT and FOXO3a ERK-insensitive constructs were a kind gift of

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Mien-Chie Hung's Lab. FOXO3a AKT-insensitive and FOXO3a ERK/AKT-insensitive constructs were generated by site-directed mutagenesis. The PINK1 construct was purchased from Addgene (pGEX5X.1 PINK1 WT, Plasmid #13321).

The lentiviral constructs encode the gene of interest and a fluorescent marker for FAC-sorting. Lentiviral particles were produced in 293T cells using a standard protocol. HSCs were infected for 48 hours with lentivirus-containing medium supplemented with SCF (10ng/ml; PeproTech) and TPO (10ng/ml; PeproTech) in the presence of 8μg/ml polybrene. After 48 hours, positively transduced cells (GFP-positive) were sorted by FACS.

QUANTIFICATION AND STATISTICAL ANALYSES

The experiment in Figures 2D, 2E, and S2 was performed once (n = 3-4 animals); the transplantation experiments in Figures 1B and 1C were performed twice; all other experiments were performed three times or more. In all Figs, n = number of biological replicates. In all LTC experiments n = 3, each assayed in three technical replicates. Unless otherwise specified, data are represented as mean ± standard deviation. Statistical analysis was performed using the two tailed Student’s t test, with the exception of the survival data (Figures 1F and 3C), which were analyzed using the Mantel–Cox log-rank test. In all cases, *p < 0.05; **p < 0.01; and ***p < 0.001 were considered statistically significant.

DATA AND SOFTWARE AVAILABILITY

The accession number for the preprocessed RNA-seq data of untreated and 5-FU stressed MEK1 F/F and cKO HSCs reported in this paper is GEO: GSE112842. A detailed description of data analysis and the software used can be found in Method Details. The analysis of the F/F and MEK1 cKO hematopoietic compartments in different stress models and the validation of the antibodies used in the phosflow experiments is deposited in the Mendeley database (https://doi.org/10.17632/7rdg6mjk5h.1).