Highlights

- Unperturbed hematopoiesis can be sustained long-term while the HSC pool is depleted
- CITED2 promotes HSC survival but not quiescence under homeostatic conditions
- CITED2 maintains the HSC pool by controlling *Mcl1* and *Pten* expression

TOC blurb

Kranc, Guitart and colleagues demonstrate that *Cited2* deletion causes a progressive HSC loss under steady-state conditions without perturbing normal haematopoiesis. The authors show that CITED2 maintains HSCs by regulating the expression of *Mcl1* and *Pten*. Finally, they indicate that CITED2 promotes multiple pathways necessary for HSC maintenance and suppresses those detrimental to HSC integrity to coordinate HSC function.

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TITLE

CITED2 coordinates key haematopoietic regulatory pathways to maintain the HSC pool in both steady-state haematopoiesis and transplantation

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SUMMARY

Haematopoietic stem cells (HSCs) reside at the apex of the haematopoietic differentiation hierarchy and sustain multilineage haematopoiesis. Here, we show that the transcriptional regulator CITED2 is essential for life-long HSC maintenance. While haematopoietic-specific *Cited2* deletion has a minor impact on steady-state haematopoiesis, *Cited2*-deficient HSCs are severely depleted in young mice and fail to expand upon ageing. Moreover, although they home normally to the bone marrow, they fail to reconstitute haematopoiesis upon transplantation. Mechanistically, CITED2 is required for expression of key HSC regulators, including GATA2, MCL-1 and PTEN. Haematopoietic-specific expression of anti-apoptotic MCL-1 partially rescues the *Cited2*-deficient HSC pool and restores their reconstitution potential. To interrogate the Cited2→Pten pathway in HSCs, we generated *Cited2*;*Pten* compound heterozygous mice, which had a decreased number of HSCs that failed to reconstitute the HSC compartment. Additionally, CITED2 represses multiple pathways whose elevated activity causes HSC exhaustion. Thus, CITED2 promotes pathways necessary for HSC maintenance and suppresses those detrimental to HSC integrity.

INTRODUCTION

Haematopoiesis is a dynamic and essential process, with the capacity to meet a large demand for differentiated blood cells (~10^{11} cells per day in humans) (Sender and Milo, 2021). Haematopoiesis critically depends on a pool of bone marrow (BM)-resident adult haematopoietic stem cells (HSCs) at the apex of the haematopoietic differentiation hierarchy, which possess unique self-renewal capacity and multilineage differentiation potential (McCracken et al., 2016). The strict regulation of survival, quiescence, self-renewal and differentiation in HSCs is essential for life-long maintenance of their pool. While much progress has been made in identifying individual pathways which suppress or promote these fates, the key regulators which coordinate these pathways to maintain the HSC pool both in steady-state and under conditions of physiologic stress remain poorly understood.

CITED2 (CBP/p300-interacting-transactivator-with-an ED-rich-tail 2) is a transcriptional regulator which co-activates or represses multiple transcription factors, including AP-2 (Bamforth et al., 2001), HIF-1alpha (Bhattacharya et al., 1999), PPAR-α (Tien et al., 2004), SMAD2/3 (Chou and Yang, 2006), and c-MYC (Chou et al., 2012) to regulate fundamental cellular processes such as proliferation, metabolism, differentiation, migration and autophagy. Consistent with its ubiquitous expression and pleiotropic impact on diverse transcription factors, CITED2 is essential for embryonic development, including foetal liver haematopoiesis (Bamforth et al., 2001; Bamforth et al., 2004; Chen et al., 2007; Weninger et al., 2005; Withington et al., 2006; Yin et al., 2002), ES cell biology (Kranc et al., 2015; Li et al., 2012), adult tissue functions (Kim et al., 2018; Lee et al., 2009; Liu et al., 2019), cellular proliferation (Kranc et al., 2003), and cancer progression (Fernandes et al., 2020). Thus, CITED2 is an important regulator of diverse molecular, cellular and developmental processes.

A growing body of evidence indicates that CITED2 is a key regulator of adult HSC biology (Du et al., 2012; Du et al., 2014; Korthuis et al., 2015; Kranc et al., 2009). We previously reported that inducible *Mx1-Cre*-mediated deletion of *Cited2* (in which *Mx1-Cre* is induced by poly(I:C)-stimulated IFN-α production) results in a rapid loss of HSCs via apoptosis and a resultant BM failure (Kranc et al., 2009). In this context, the significant loss of the HSC pool upon inducible *Cited2* deletion is at least in part caused by upregulation of the $p19^{ARF}$-p53 pathway, as genetic ablation of *Cdkn2a* (encoding $p16^{INK4A}$ and $p19^{ARF}$) or *Trp53* (encoding p53) rescues depletion of *Cited2*-deficient HSCs. Another study (Du et al., 2012) employing a different *Cited2* floxed allele, also demonstrated that poly(I:C)-inducible *Mx1-Cre*-mediated *Cited2* deletion results in loss of HSCs (by affecting their quiescence and survival), compromises their reconstitution potential, and

leads to a rapid BM failure upon myelotoxic stress. In this study, loss of quiescence, but not increased apoptosis, upon inducible *Cited2* deletion is mediated at least in part by HIF-1alpha, as *Hif1a* deletion partially restores impaired quiescence of HSCs lacking *Cited2* and improves their ability to reconstitute the HSC compartment upon transplantation (Du et al., 2012). Additional analyses of *Cited2*-deficient HSCs indicated alterations in HSC metabolism, namely a decrease in glycolytic flux and an increase in mitochondrial activity (Du et al., 2014), a state associated with a decline in HSC function (Lawson et al., 2021; Wang et al., 2014). However, given that the previous studies outlined above involved poly(I:C) administration, which is known to induce interferon response, pro-inflammatory pathways and subsequent over-proliferation in HSCs, the functional significance of CITED2 in maintenance of the HSC pool under steady-state conditions is yet to be investigated.

Here, we reveal that under steady-state conditions CITED2 is largely dispensable for unperturbed long-term multilineage haematopoiesis, but is critically required for the maintenance of the HSC pool and HSC function post-transplantation. We found that CITED2 represses pathways that inhibit HSC maintenance and promotes pathways required for HSC integrity. Notably, our functional genetic approaches found that key regulators of HSC maintenance, namely MCL-1 and PTEN, act downstream of CITED2 and mediate, at least in part, its critical role in regulating the HSC pool. Taken together, we propose that CITED2 coordinates multiple fundamental stem cell regulatory pathways to promote the maintenance of the HSC pool under steady-state conditions and upon transplantation.

RESULTS

Cited2 deletion does not derail normal steady-state haematopoiesis

To determine the expression of *Cited2* at different levels of the haematopoietic hierarchy, we sorted $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells, $LSKCD48^{-}CD150^{+}$ HSCs, $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$ (LK) myeloid progenitors and more mature Lin^{-} and differentiated Lin^{+} haematopoietic cell populations, and performed qRT-PCR. *Cited2* was expressed in all compartments, with significantly higher expression in the HSC population compared to myeloid progenitors and more mature haematopoietic cell populations (**Figure 1A**). Furthermore, to compare the expression of *Cited2* in long-term-HSCs ($LSKCD34^{-}CD135^{-}$), MPP1 ($LSKCD34^{+}CD135^{-}CD150^{+}CD48^{-}$), MPP2 ($LSKCD34^{+}CD135^{-}CD150^{+}CD48^{+}$), MPP3 ($LSKCD34^{+}CD135^{-}CD150^{-}CD48^{+}$) populations, lymphoid-primed multipotent progenitors ($LSKCD34^{+}CD135^{+}$) which correspond to the MPP4 population, and CMP ($LKCD34^{+}FcγRII/III^{low}$), GMP ($LKCD34^{+}FcγRII/III^{high}$) and MEP ($LKCD34^{-}FcγRII/III^{low}$) compartments, we analysed our SMART2-seq single-cell expression data in these

populations (Nestorowa et al., 2016). *Cited2* was rather uniformly expressed across these populations (**Figure 1B**), with the highest expression in the HSC, GMP and CMP compartments, and lowest in the MEP population. Moreover, we employed our 10x Genomics single-cell RNA-seq data set (Dahlin et al., 2018) to compare *Cited2* expression between HSCs and committed progenitor cell compartments. *Cited2* was expressed highly in HSCs and committed megakaryocytic progenitors, but decreased in committed lymphoid, basophilic, neutrophilic, and erythroid progenitor cells (**Figure 1C**). Thus, *Cited2* is ubiquitously expressed in haematopoiesis, with robust high expression at the apex of the haematopoietic hierarchy, and lower expression in more differentiated cells.

Given that the functional significance of *Cited2* in unperturbed haematopoiesis remains poorly understood, we combined the *Cited2*^{fl} allele (Kranc et al., 2009; MacDonald et al., 2008) with *Vav-iCre* (de Boer et al., 2003) to generate *Cited2*$^{fl/fl}$;*Vav-iCre* (*Cited2*^{CKO}) mice (**Figure 1D**), where *Cited2* is deleted specifically from the haematopoietic system shortly after the emergence of HSCs. Consequently, *Cited2* expression was completely lost in BM c-Kit^{+} cells isolated from *Cited2*^{CKO} mice (**Figure 1E**). Surprisingly, *Cited2* deletion had no impact on animal viability, and all animals survived to adulthood without any obvious defects. Peripheral blood (PB) analyses of 8-12-week-old *Cited2*^{CKO} mice revealed unaffected WBC counts, with mild anaemia and thrombocythemia (**Figure 1F**). Furthermore, *Cited2*^{CKO} mice displayed normal BM cellularity (**Figure 1G**) and unaffected numbers of differentiated myeloid and B lymphoid cells in the BM and spleens (**Figure 1H**), as well as normal distribution of T cells in the thymi and spleens (**Figure S1** and **Figure 1I**). *Cited2*^{CKO} mice also had normal myeloid and megakaryocytic progenitor cell numbers (**Figure 1J**). Notably, consistent with mild anaemia **(Figure 1F**), mice lacking *Cited2* had significantly decreased numbers of BM erythroid progenitors (**Figure 1K**). Finally, CFC assays showed normal differentiation potential of *Cited2*^{CKO} BM cells (**Figure 1L** and **1M**). Importantly, however, *Cited2* deficient cells failed to form secondary colonies after re-plating, suggesting that *Cited2* is required for propagation or self-renewal of progenitor cells (**Figure 1M**). Taken together, while *Cited2* deletion compromises erythroid progenitors and causes mild anaemia, it is otherwise not essential for normal steady-state multilineage haematopoiesis in young adult mice.

Cited2 is required for the maintenance of the HSC pool under steady-state conditions and its expansion upon ageing

We next determined the impact of *Cited2* deletion on HSCs and primitive progenitor cells. We found that the total number of LSK cells (the compartment comprising HSCs and functionally distinct lineage-biased multipotent progenitors (MPP1-4)) was not affected in young 8-12-week-

old *Cited2*^{CKO} mice (**Figure 2A**). Markedly, however, mice lacking *Cited2* displayed significant depletion of HSCs and the most primitive progenitors (i.e. MPP1 population), with an increase in MPP2 population and unchanged numbers of the MPP3-4 populations. Thus, despite a select reduction in absolute HSC and MPP1 cell numbers, *Cited2*^{CKO} mice sustain largely unaffected unperturbed steady-state multilineage haematopoiesis.

Next, we investigated the impact of prolonged *Cited2* deficiency on long-term HSC maintenance during unperturbed haematopoiesis. We aged mouse cohorts for 52 weeks and found that *Cited2* deficiency had no impact on mouse survival (data not shown). Consistent with physiological HSC ageing, during which the HSC pool undergoes expansion (Geiger et al., 2013), we found that ageing *Cited2*^{CTL} mice had approximately 5-fold more HSCs compared to 8-12-week-old *Cited2*^{CTL} mice (**Figure 2A-B**). Strikingly however, *Cited2*^{CKO} HSCs failed to expand upon ageing and remained severely depleted compared to *Cited2*^{CTL} HSCs (**Figure 2B**). Furthermore, while the MPP1 population also remained depleted, the numbers of MPP2 and MPP3/4 populations were increased in 52-week-old *Cited2*^{CKO} mice. Aging *Cited2*^{CKO} mice had normal BM cellularity and unaffected numbers of primitive and differentiated myeloid cells and T cells, while B cell numbers were decreased (**Figure 2C-D**). Apart from anaemia and a significant drop in B cells, PB analyses showed no other major abnormalities (**Figure 2D**-**E**). Taken together, *Cited2* is essential for both the maintenance of HSCs and their expansion during physiological aging, but remarkably, despite this, *Cited2* is not critical for long-term multilineage haematopoiesis. Notably, these data also reveal the requirement for CITED2 in the maintenance of the B cell lineage, meriting further investigations.

Cited2 is essential for post-transplantation HSC functions

Given that *Cited2*^{CKO} mice do not display any severe haematopoietic defects despite a substantial depletion of the HSC pool, we next investigated the multilineage reconstitution capacity of *Cited2*^{CKO} HSCs. We competitively transplanted 100 HSCs from 8-12-week-old *Cited2*^{CKO} and *Cited2*^{CTL} mice into lethally irradiated recipients (**Figure 3A**). *Cited2*-deficient HSCs failed to reconstitute haematopoiesis (**Figure 3B**), and were unable to contribute to the LSK pool of recipient mice (**Figure 3C**). Given that HSCs lacking *Cited2* have no reconstitution activity but *Cited2*^{CKO} mice are able to sustain haematopoiesis, we next asked whether the reconstitution activity is contained within the LSK compartment. We competitively transplanted LSK cells from 8-12-week-old *Cited2*^{CKO} and *Cited2*^{CTL} mice (together with 200,000 unfractionated CD45.1^{+} BM cells) and found that they also dramatically failed to reconstitute haematopoiesis (**Figure 3D**), and did not contribute to the LSK pool of the recipients (**Figure 3E**). Given that both *Cited2*-deficient

HSCs and LSK cells fail to reconstitute haematopoiesis, we asked whether *Cited2* loss impacts on the ability of HSCs to home to the BM. We transplanted LSK cells from *Cited2*^{CKO} and *Cited2*^{CTL} mice into irradiated recipients (**Figure 3F**), and found equal numbers of control and *Cited2*-deficient CD45.2^{+} cells 18 hours after injection, indicating that LSK cells lacking *Cited2* are able to home to the BM as efficiently as their *Cited2*^{CTL} counterparts (**Figure 3G**). Therefore, although multilineage steady-state haematopoiesis is maintained in *Cited2*^{CKO} mice, neither HSC nor LSK populations lacking *Cited2* have the ability to repopulate haematopoiesis upon transplantation. Thus, although *Cited2*-deficient HSCs home to the BM, they critically require *Cited2* to contribute to and sustain haematopoiesis upon transplantation.

HSCs lacking *Cited2* remain quiescent but display increased apoptotic rate

HSC depletion and their failure to sustain haematopoiesis upon serial transplantation frequently result from a loss of HSC quiescence or increased apoptosis (Rossi et al., 2012). Thus, we investigated whether the reduction of HSCs and their reconstitution failure upon *Cited2* deletion is associated with changes in these HSC fates. To determine the cell-cycle status of *Cited2*-deficient HSCs, we employed Ki67 and DAPI staining, which showed no differences in quiescence between *Cited2*^{CTL} and *Cited2*^{CKO} HSCs (**Figure 3H**). Notably, however, the percentage of actively cycling HSCs (i.e. those in S/G2/M phases) was decreased in the absence of *Cited2* (**Figure 3H**). Furthermore, to determine the rate of cell death in *Cited2*-deficient HSCs, we used Annexin V staining. *Cited2*-deficient HSCs, but not primitive progenitors, displayed a significantly increased rate of apoptosis compared to their *Cited2*^{CTL} counterparts (**Figure 3I**). Therefore, *Cited2*-deficient HSC depletion, their inability to expand over time and reconstitute haematopoiesis upon transplantation likely result, at least in part, from a decrease in HSC cycling and an increase in their apoptosis.

***Cited2* maintains HSCs by regulating *Mcl1* expression**

To understand the mechanisms through which *Cited2* loss depletes the HSC pool in *Cited2*^{CKO} mice and compromises HSC functions upon transplantation, we performed gene expression analyses in HSCs sorted from *Cited2*^{CKO} and *Cited2*^{CTL} mice. Interestingly, we found that the expression of key HSC regulators, including *Mcl1*, *Pten* and *Gata2* (Menendez-Gonzalez et al., 2019; Opferman et al., 2005; Yilmaz et al., 2006) was decreased in *Cited2*-deficient HSCs (**Figure 4A**), suggesting that CITED2 may control several pathways important for HSC maintenance.

Myeloid cell leukaemia 1 (MCL-1) is a pro-survival BCL-2 protein family member, whose *Mx1-Cre*-mediated deletion results in severe loss of HSCs and BM failure (Opferman et al., 2005), thus resembling the phenotype resulting from *Mx1-Cre*-mediated *Cited2* deletion (Kranc et al.,

2009). Moreover, MCL-1 is also required for self-renewal of human HSCs (Campbell et al., 2010a). Given that *Mcl1* expression was decreased in *Cited2*-deficient HSCs, we sought to determine whether MCL-1 can rescue HSC defects resulting from *Cited2* deletion *in vivo*. We bred *Cited2*^{CKO} mice to *VavP-Mcl1* transgenic mice (Campbell et al., 2010b), which over-expresses *Mcl1* specifically within haematopoietic system, under the control of *Vav* regulatory elements (**Figure 4B**). Interestingly, *Mcl1* over-expression in *Cited2*^{CKO} mice (i.e. *Cited2*^{CKO};*Mcl1* mice) resulted in a partial rescue of the HSC and MPP1 cell pools compared to *Cited2*^{CKO} mice (**Figure 4C**). To investigate if *Mcl1* over-expression can support *Cited2*-deficient HSCs to reconstitute haematopoiesis, we competitively transplanted 100 HSCs from *Cited2*^{CTL}*, Cited2*^{CTL}*;Mcl1, Cited2*^{CKO} and *Cited2*^{CKO};*Mcl1* mice into lethally irradiated recipient mice (**Figure 4D**). Significantly, while *Cited2*-deficient HSCs failed to reconstitute recipient mice, HSCs from *Cited2*^{CKO};*Mcl1* mice successfully repopulated recipients, comparable to the reconstitution potential of HSCs from *Cited2*^{CTL} and *Cited2*^{CTL}*;Mcl1* mice (**Figure 4E-F**). No statistically significant differences were found in repopulation potential between HSCs from *Cited2*^{CTL} and *Cited2*^{CTL}*;Mcl1* mice (**Figure 4E-F**). Consistent with these data, HSCs from *Cited2*^{CKO};*Mcl1* mice contributed significantly more to the total BM and HSC compartments of recipient mice compared to *Cited2*^{CKO} HSCs which failed to efficiently reconstitute recipients (**Figure 4G**). Finally, BM cells from *Cited2*^{CTL}*, Cited2*^{CTL}*;Mcl1, Cited2*^{CKO} and *Cited2*^{CKO};*Mcl1* mice efficiently generated primary colonies (**Figure 4H**), and while *Cited2*-deficient cells failed to replate secondary colonies, strikingly, *Cited2*^{CKO};*Mcl1* cells efficiently produced comparable colony numbers to control cells (**Figure 4I**). Therefore, *Mcl1* acts downstream of *Cited2 in vivo* and at least in part mediates CITED2 functions in sustaining the HSC pool under steady-state haematopoiesis and promoting their long-term reconstitution capacity.

Cited2 regulates *Pten* to maintain the HSC pool

PTEN is required for cell-autonomous HSC maintenance (Yilmaz et al., 2006; Zhang et al., 2006) and its deletion results in loss of adult HSC function through activation of mTORC2-dependent signalling (Magee et al., 2012). Given that *Pten* expression was decreased in *Cited2*-deficient HSCs (**Figure 4A**), we sought to genetically interrogate the putative CITED2→PTEN axis. We took advantage of the principle that if genes act in common pathways they should genetically interact, i.e. the compound heterozygosity should generate phenotypes not observed in mice heterozygous for a single gene of interest (Vidal et al., 2011). We investigated whether combined *Pten* and *Cited2* heterozygosity causes loss of HSC functions compared to *Pten* or *Cited2* heterozygosity alone. We generated *Cited2*$^{+/fl}$;*Pten*$^{+/fl}$*;Vav-iCre* (*Cited2*^{Het};*Pten*^{Het}), *Cited2*^{Het}, *Pten*^{Het} and control mice (**Figure S2A**). We found that *Cited2*^{Het} and *Pten*^{Het} mice had normal BM

cellularity and largely unaffected numbers of Lin$^-$, LK and LSK cells within the BM (**Figure S2B-C**). However, while control, *Cited2*^{Het} and *Pten*^{Het} mice had comparable numbers of HSCs, *Cited2*^{Het};*Pten*^{Het} mice displayed subtly but significantly decreased numbers of HSCs. To assess the repopulation capacity of *Cited2*^{Het};*Pten*^{Het} HSCs, we competitively transplanted HSCs of all relevant genotypes into lethally irradiated recipients (**Figure S2D**). While HSCs of all genotypes gave equal overall long-term reconstitution capacity in the PB compartment (**Figure S2E**), *Cited2*^{Het};*Pten*^{Het} HSCs had a slightly decreased capacity to contribute to the BM reconstitution of the recipient mice (**Figure S2F**). Moreover, *Cited2*^{Het};*Pten*^{Het} HSCs failed to contribute to the HSC compartments of the recipients (**Figure S2G**). Therefore, given that *Cited2*^{Het};*Pten*^{Het} HSCs sustain haematopoiesis upon transplantation, but fail to efficiently repopulate the HSC compartment of the recipients, the CITED2→PTEN axis may contribute to long-term HSC maintenance but is unlikely to be essential for the reconstitution potential of HSCs.

Cited2 deletion dysregulates multiple pathways whose strict control is required for HSC integrity

To further understand why *Cited2*-deficient HSCs undergo depletion and fail upon transplantation, we examined global gene expression in *Cited2*-deficient HSCs by RNA-seq. This analysis identified a number of dysregulated genes, with 119 upregulated and 143 downregulated genes (**Figure 5A**). Gene set enrichment analysis (GSEA) indicated a broad dysregulation of multiple pathways and processes, which was compatible with the loss of HSC function. Notably, we found that *Cited2*-deficient HSCs displayed activation of proinflammatory pathways, c-MYC and E2F targets, K-RAS and mTORC1 signalling, whose elevated activity is known to lead to HSC exhaustion or loss of their reconstitution potential (Kim et al., 2017; Pietras, 2017; Sasine et al., 2018; Wilson et al., 2004; Yilmaz et al., 2006) (**Figure 5B-C**). Consistent with upregulation of E2F and c-MYC targets, analysis of proximal promoters of upregulated genes revealed the presence of E2F and c-MYC motifs suggesting that CITED2 may repress E2Fs and c-MYC (**Figure 5D**). We also found that promoters of the upregulated genes were enriched for the motif of RUNX1 (**Figure 5D**), whose increased expression in adult HSCs results in loss of their reconstitution potential (Ichikawa et al., 2008).

Given that we observed activation of the mTORC1 signalling signature upon *Cited2* deletion in HSCs (**Figure 5B-C**), we asked whether rapamycin, a known mTORC1 inhibitor (Guertin and Sabatini, 2007), can rescue the re-plating defect resulting from *Cited2* deficiency. We serially plated BM cells from *Cited2*^{CTL} and *Cited2*^{CKO} mice into CFC assays, in the presence or absence of rapamycin. As expected, *Cited2*^{CTL} and *Cited2*^{CKO} cells generated primary colonies (**Figure**

S3A). However, while *Cited2*^{CTL} cultures efficiently produced secondary colonies, *Cited2*^{CKO} cells displayed a replating defect, regardless of the presence or absence of rapamycin (**Figure S3A**). As such, we conclude that upregulation of the mTORC1 pathway alone in *Cited2*-deficient cells is insufficient to elicit the observed phenotypes. The identification and dissection of upregulated pathways upon *Cited2* deletion, which act together to cause HSC depletion merit future investigation.

We next focused on pathways which were down-regulated in *Cited2*^{CKO} HSCs. Interestingly, *Cited2* loss led to an overall downregulation in genes whose expression is a hallmark of long-term HSC signature (Ivanova et al., 2002) (**Figure 5B** and **5E**). *Cited2* loss also led to a decreased signature of the p53 pathway (**Figure 5B** and **5E**), whose inhibition is detrimental to HSC integrity and function (Liu et al., 2009). *Cited2*^{CKO} HSCs displayed down-regulation of the hypoxic signature (**Figure 5B** and **5E**), implying their intrinsic inability to adapt to the physiologically hypoxic BM microenvironment, where HSCs reside (Spencer et al., 2014). Moreover, we found that the reactive oxygen species (ROS) defence pathway was downregulated (**Figure 5B**). However, ROS levels were not elevated in *Cited2*^{CKO} HSCs (**Figure S3B**), and treatment of *Cited2*^{CKO} mice with the antioxidant N-acetyl-L-cysteine (NAC) did not rescue HSC depletion (**Figure S3C-D**), suggesting that ROS is unlikely to be responsible for the observed effects in HSCs lacking *Cited2*. Furthermore, in addition to the GSEA, our data revealed that several genes essential for HSC functions, including *Prdm16*, *Men1*, *Rnh1* and *Akt2* were down-regulated in *Cited2*^{CKO} HSCs (**Figure S4**) (Andina et al., 2019; Gudmundsson et al., 2020; Juntilla et al., 2010; Maillard et al., 2009). Finally, further interrogation of promoters of the downregulated genes revealed the presence of motifs for STAT3, STAT4, E2A and ZFX transcription factors (**Figure 5F**), all of which are required for HSC maintenance and function (Galan-Caridad et al., 2007; Holmfeldt et al., 2016; Mantel et al., 2012; Semerad et al., 2009). Thus, CITED2 controls multiple fundamental pathways in HSCs to safeguard their integrity.

DISCUSSION

Given that the functional role of CITED2 in HSC biology under unperturbed conditions remains poorly understood, here we conditionally deleted *Cited2* specifically from the haematopoietic system. While steady-state haematopoiesis in young and ageing mice lacking *Cited2* was largely unaffected, HSCs were significantly depleted in young mice, at least in part via increased apoptosis, and failed to expand upon physiological ageing. Importantly, these findings imply that HSCs under steady-state conditions require *Cited2* to regulate the size of their pool but not to sustain long-term multilineage haematopoiesis. Furthermore, while *Cited2*-deficient HSCs

successfully homed to the BM upon transplantation, they completely failed to reconstitute haematopoiesis, indicating that HSCs critically require *Cited2* to function under stressful conditions imposed by transplantation. Finally, significantly, phenotypes elicited by *Cited2* deficiency demonstrate that unperturbed multilineage haematopoiesis can be sustained long-term while the HSC pool is drastically depleted, thus underscoring the remarkable plasticity of the haematopoietic system under steady-state conditions.

Previous studies using poly(I:C)-inducible *Mx1-Cre* concluded that CITED2 regulates HSC survival and quiescence by repressing INK4A/ARF and HIF-1alpha, respectively (Du et al., 2012; Kranc et al., 2009). However, cellular and molecular mechanisms via which CITED2 controls the HSC pool under steady-state conditions and HSC reconstitution potential upon transplantation have remained largely unexplored. Here we found that under steady-state conditions *Cited2*-deficient HSCs maintain their quiescent state but undergo increased apoptosis. Notably, *Cited2* is required for the expression of HSC survival and self-renewal regulator *Mcl1* (Campbell et al., 2010b; Opferman et al., 2005), whose haematopoiesis-specific overexpression partially rescued depletion of *Cited2*-deficient HSCs, and their ability to reconstitute long-term multilineage haematopoiesis. We therefore propose that the CITED2→MCL-1 axis protects the integrity of the HSC pool by promoting HSC survival under the steady-state conditions and upon transplantation. Furthermore, *Cited2* was necessary for normal expression of *Pten*, whose deletion results in a cell-autonomous HSC depletion and defective reconstitution potential (Yilmaz et al., 2006). Consistent with *Pten* downregulation, *Cited2*-deficient HSCs displayed increased signatures of PI3K/AKT and mTOR signalling, whose suppression by PTEN is essential for HSC maintenance (Lee et al., 2010; Yilmaz et al., 2006). These data are in concordance with the previous demonstration that *Cited2* deletion in HSCs leads to an increased AKT activity (Du et al., 2014). Indeed, given that PTEN is a negative regulator of AKT, our results help to explain increased AKT activation upon *Cited2* loss. Given that *Cited2*^{Het};*Pten*^{Het} HSCs displayed normal multilineage reconstitution potential but poorly contributed to the HSC pool upon transplantation, and that rapamycin did not rescue defects resulting from *Cited2* deficiency, it is likely that the CITED2→PTEN/PI3K/AKT/mTOR axis contributes to long-term HSC maintenance but is not solely critical for HSC functions.

Poly(I:C)-inducible *Mx1-Cre* deletion of *Cited2* results in loss of quiescence of HSCs, a phenotype which is partially mediated by HIF-1alpha (Du et al., 2012; Du et al., 2014). Notably, however, our data indicate that *Vav-iCre*-mediated deletion of *Cited2* under homeostasis has no impact on HSC quiescence. Furthermore, our GSEA analyses showed that hypoxia signature was in fact

downregulated in *Cited2*-deficient HSCs. The discrepancies between our findings and those by Du et al. (Du et al., 2012; Du et al., 2014) may be explained by different strategies of gene deletion, as poly(I:C) (used to induce *Mx1-Cre*-driven gene ablation) is known to transiently alter HSCs (i.e. induce cycling, increase frequency and alter phenotype), unlike *Vav-iCre*, which is constitutively expressed, and as such more accurately allows for gene deletion under steady-state conditions. Thus, it is possible that concurrent poly(I:C) administration and *Cited2* deletion in the *Mx1-Cre*-mediated model may exhibit exacerbated phenotypes not seen in the *Vav-iCre* model.

While in this study we focused on functional interrogation of the CITED2→MCL-1 and CITED2→PTEN axes, our work suggests that CITED2 is also likely to control other diverse pathways to coordinate HSC biology. Our analyses indicate that CITED2 represses multiple pathways downstream of c-MYC, E2F and RUNX1 transcription factors and K-RAS and pro-inflammatory signalling pathways, whose upregulation has detrimental consequences for HSC integrity (Ichikawa et al., 2008; Kim et al., 2017; Pietras, 2017; Sasine et al., 2018; Wilson et al., 2004; Yilmaz et al., 2006). Finally, CITED2 is necessary for the expression of genes regulated by STAT3, STAT4, E2A and ZFX transcription factors, which are essential for HSC maintenance (Galan-Caridad et al., 2007; Holmfeldt et al., 2016; Mantel et al., 2012; Semerad et al., 2009). Thus, given its dual action in gene transcription, we propose that CITED2 functions at the centre of the transcriptional regulatory network to repress pathways detrimental to HSC integrity, and promote those necessary for HSC maintenance.

EXPERIMENTAL PROCEDURES

Mice

All mice were on the C57BL/6J genetic background. *Cited2*$^{fl/fl}$ (Kranc et al., 2009; MacDonald et al., 2008), *VavP-Mcl1* (Campbell et al., 2010b), *Pten*$^{fl/fl}$ (Yilmaz et al., 2006) and *Vav-iCre* (de Boer et al., 2003) mice were described previously. All transgenic and knockout mice were CD45.2^{+}. Recipient mice were CD45.1^{+}/CD45.2^{+}. All experiments on animals were performed under UK Home Office authorisation.

Flow cytometry

BM, spleen and PB samples were stained and analysed as described previously (Guitart et al., 2017). FACS analyses were performed using a LSRFortessa (BD). Cell sorting was performed on a FACSAria Fusion (BD). Data were analysed using FlowJo.

A combination of anti-mouse antibodies purchased from BD Biosciences, Biolegend and Life Technologies was used. The following BD Biosciences antibodies were used: Fc block

(Cat#553142), anti-CD4; biotin conjugated (Cat#553649), anti-CD5; biotin conjugated (Cat#553019), anti-CD8a; biotin conjugated (Cat#553029), anti-CD11b; biotin conjugated (Cat#553309), anti-CD45R/B220; biotin conjugated (Cat#553086), anti-Ter119; biotin conjugated (Cat#553672), anti-Gr-1/Ly-6G/C; biotin conjugated (Cat#553125), anti-CD34; FITC conjugated (Cat#553733), and streptavidin; BV421 conjugated (Cat#563259). Biolegend antibodies used were anti-c-Kit/CD117; APC conjugated (Cat#105812), anti-c-Kit/CD117; APC-Cy7 conjugated (Cat#105826), anti-Sca-1; APC-Cy7 conjugated (Cat#122520), anti-Sca-1; PB conjugated (Cat#108125), anti-CD48; PE conjugated (Cat#103406), anti-CD150; PE-Cy7 conjugated (Cat#115914), anti-CD135; APC conjugated (Cat#135310), anti-CD135; PE conjugated (Cat#135305), anti-CD16/32; APC-Cy7 conjugated (Cat#101328), anti-CD41; APC conjugated (Cat#133914), anti-CD105; PE conjugated (Cat#120408), anti-CD127; BV421 conjugated (Cat#135023), anti-TER-119; FITC conjugated (Cat#116206), streptavidin; PerCp conjugated (Cat#405213), anti-CD19; APC-Cy7 conjugated (Cat#115530), anti-CD45R/B220; APCCy7 conjugated (Cat#103224), anti-CD11b; APC conjugated (Cat#101211), anti-Gr-1/Ly-6G/C; PE-Cy7 conjugated (Cat#108416), anti-CD4; PE conjugated (Cat#130310), anti-CD8a; PE conjugated (Cat#100708), anti-CD45.1; FITC conjugated (Cat#110706), anti-CD45.2; PB conjugated (Cat#109820), anti-Ki67; FITC conjugated (Cat#652410). Annexin V; FITC conjugated (Cat#640906) and 7-AAD (Cat#420403) was purchased form Biolegend. DAPI was purchased from Life Technologies (Cat#D1306).

Administration of NAC

Mice received 30 mg/mL of NAC (Sigma) in drinking water for 4 weeks. The water bottles containing NAC were changed twice a week.

ROS detection

For detection of mitochondrial super oxide, $3x10^6$ BM cells stained first for LSK were resuspended in X-Vivo 15 medium (without phenol red) supplemented with 10% FCS, loaded with 5 μM MitoSox™ red (Invitrogen) for 20min at 37°C and analysed using FACS.

Colony forming cells (CFC) assays

CFC assays were performed using MethoCult™ M3434 (STEMCELL Technologies). Colonies were tallied at day 10. For CFC replating, CFC1 cells were washed with IMDM then seeded in M3434.

Transplantation assays

Lethal irradiation of CD45.1$^+$/CD45.2$^+$ recipient mice was achieved using a split dose of 11 Gy (two doses of 5.5 Gy administered 4 hours apart) at an average rate of 0.58 Gy/min using a Cesium 137 GammaCell 40 irradiator. For transplantations 100 HSCs or 2,000 LSK sorted from BM of 8-10 weeks adult mice mixed with 200,000 support CD45.1$^+$ wild-type BM cells were injected into lethally irradiated CD45.1$^+$/CD45.2$^+$ recipient mice.

Homing assay

LSK cells sorted from CD45.2$^+$ BM were injected into CD45.1$^+$ lethally irradiated recipients (160,000 cells per mouse). After 18h, recipients were sacrificed and BM CD45.2$^+$ chimerism was analysed by FACS.

qRT-PCR

Gene expression analyses were performed as described previously (Guitart et al., 2017). Differences in input cDNA were normalised with *Actb* expression.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. P values were calculated using a Mann-Whitney U test.

RNA-seq, GSEA and DNA motif analysis

RNA-seq was performed on sorted HSCs from *Cited2*^{CKO} and *Cited2*^{CTL} animals (n=5 per genotype). On average, 6200 cells per sample were collected and 53.7 million single-ended 85-bp reads per sample were sequenced. Reads were aligned to the GCRm38 mouse genome using HISAT2 v2.1.0 (Kim et al., 2015), and read counts were assessed per gene using the Rsubread package v2.0.1 in R. Differential expression analysis was further performed using the Wald test in DESeq2 v1.24, and genes were ranked according to moderated *t* statistics computed by DESeq2 for GSEA. The ranked gene list was compared to gene lists in the Hallmark subset of the MSigDb database v7.0 using the GSEA software tool v3.0 (Subramanian et al., 2005).

For DNA motif analysis of proximal promoter regions, lists of up- and down-regulated genes (FDR<0.05, fold change>20%) were mapped to gene loci using the basic set of ENCODE genomic annotation from Ensembl v91. Next, proximal promoters were defined as the region from 200 bp upstream to 100 bp downstream of the transcription start site. Proximal promoter coordinates were shuffled within chromosomes using the bedtools shuffle tool with the –chrom flag to generate control DNA regions for motif analysis. Finally, DNA motifs overrepresented in promoters compared to control regions were identified by Homer v4.10.

DATA AND CODE AVAILABILITY

The RNA-seq data are deposited in NCBI's Gene Expression Omnibus (accession GSE175372).

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AUTHOR CONTRIBUTIONS

K.R.K., A.V.G. and H.L. designed experiments and wrote the paper. A.V.G. and H.L. performed *in vivo* experiments and data analyses and interpretation. L.N.L. and M.B. analysed gene expression. K.J.C. and S.C. produced *VavP-Mcl1* transgenic mice. A.T., J.D., A.V., A.B., C.M., E.G., C.M-C., C.S., L.A., and J.C. helped with *in vivo* experiments, FACS, and data analyses. D.O., B.G and N.R. provided significant expertise to this study. K.R.K. and A.V.G. contributed equally to this work.

DECLARATINON OF INTERESTS

The authors have no competing interests to declare.

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FIGURE TITLES AND LEGENDS

Figure 1. Haematopoiesis-specific *Cited2* deletion does not derail multilineage haematopoiesis. (**A**) *Cited2* mRNA levels in cells isolated from 8-week-old C57BL/6 mice (n=4). (**B**) *Cited2* expression in different haematopoietic compartments determined using single-cell SMART2-seq. **(C)** *Cited2* expression in HSCs and committed progenitor cell compartments determined by 10x Genomics single-cell RNA-seq. (**D**) *Cited2*$^{fl/fl}$ mice were bred to *Vav-iCre* mice to generate *Cited2*$^{fl/fl}$*;Vav-iCre* (*Cited2*^{CKO}) mice. *Cited2*$^{fl/fl}$ mice were used as controls (*Cited2*^{CTL}). (**E**) *Cited2* expression in c-Kit^{+} cells from BM of *Cited2*^{CTL} and *Cited2*^{CKO} mice (n=4). (**F**) PB counts of 8-10-week-old mice (n=14). (**G**) Total BM cellularity (n=6-9). (**H**) Total number of differentiated myeloid (Gr-1^{+}Mac-1^{+}) and B cells (CD19^{+}B220^{+}) in BM and spleen (Spl) (n=6-9). (**I**) Total number of mature CD4^{+} and CD8^{+} T cells in spleens (n=6-9). (**J**) Total number of Lin^{-} cells, LK cells, pre-GM (LKCD41^{-}FcγRII/III^{-}CD150^{-}CD105^{-}), GMP (LKCD41^{-}FcγRII/III^{+}), Pre-MegE (LKCD41^{-}FcγRII/III^{-}CD150^{-}CD105^{+}), MkP (LKCD41^{+}CD150^{+}) cells (n=5). (**K**) Total number of Pre-CFU-E (LKCD41^{-}FcγRII/III^{-}CD150^{+}CD105^{+}), CFU-E (LKCD41^{-}FcγRII/III^{-}CD150^{+}CD105^{+}CD71^{+}Ter119^{-}) and Pro Ery (LKCD41^{-}FcγRII/III^{-}CD150^{+}CD105^{+}CD71^{+}Ter119^{+}) cells (n=5). (**L**) Colony forming unit (CFU) assay performed with 10^4 BM cells from 8-10-week-old mice. CFU-megakaryocyte (CFU-Mk), CFU-erythroid (CFU-E), Burst Forming Unit (BFU-E), CFU-granulocyte (CFU-G), CFU-monocyte/macrophage (CFU-M), CFU–granulocyte and monocyte/macrophage (CFU-GM); at least three lineages (CFU-Mix), CFU with all 4 lineages, granulocyte, erythroid, monocyte/macrophage and megakaryocyte (CFU-GEMM) (n=7). (**M**) Colony counts of primary and secondary cultures (n=7). For B-C and F-M, data are mean ± SEM. *, P<0.05; **, P<0.01

Figure 2. *Cited2* is required for the maintenance of the HSC pool and its expansion upon ageing. (**A**) Total number of LSK cells, LSKCD48^{-}CD150^{+}Flt3^{-}CD34^{-} HSCs, LSKCD48^{-}CD150^{+}Flt3^{-}CD34^{+} MPP1, LSKCD48^{+}CD150^{+}Flt3^{-} MPP2, LSKCD48^{+}CD150^{-}Flt3^{-} MPP3 and LSKCD48^{+}CD150^{-}Flt3^{+} MPP4 populations in BM of 8-10-week-old mice (n=6-9). (**B**) Total number of LSK cells, HSCs, MPP1, MPP2, MPP3/MPP4 and LK cells in BM in 52-week-old mice (n=6). (**C**) BM cellularity in 52-week-old mice (n=6-9). (**D**) Total number of differentiated myeloid cells (Gr-1^{+}Mac-1^{+}) and B cells (CD19^{+}B220^{+}) in BM, spleen (Spl) and PB in 52-week-old mice (n=6-9). (**E**) PB counts in 52-week-old *Cited2*^{CTL} and *Cited2*^{CKO} mice (n=6-9). For A-E, data are mean ± SEM. *, P<0.05; **, P<0.01.

Figure 3. HSCs critically require *Cited2* to reconstitute haematopoiesis upon transplantation. (**A**) Transplantation assay: 100 HSCs or 2,000 LSK cells sorted from *Cited2*^{CTL} and *Cited2*^{CKO} mice were transplanted into lethally irradiated recipients together with 200,000 support CD45.1^{+} total BM cells. (**B-C**) Percentage of donor-derived CD45.2^{+} cells in (**B**) PB and (**C**) the LSK cell compartment of recipient mice following transplantation of 100 HSCs (n=5 recipients per donor; 2 donors per genotype). (**D-E**) Percentage of donor-derived CD45.2^{+} cells in (**D**) PB and (**E**) the LSK compartment of the recipient mice following transplantation of 2,000 LSK cells (n=4 recipients per donor; 4 donors per genotype). (**F**) Homing assay: 160,000 LSK cells were injected into irradiated CD45.1^{+} recipient mice and analysed 18hrs later. (**G**) Percentage of donor-derived CD45.2^{+} cells in BM (n=4-5). (**H**) Percentage of HSCs from 8- to 10-week-old *Cited2*^{CKO} and *Cited2*^{CTL} mice in the G_0 (DAPI^{-}Ki67^{-}), G_1 (DAPI^{-}Ki67^{+}), and S/G_2/M (DAPI^{+}Ki67^{+}) phases of the cell cycle (n=3). (**I**) Percentage of Annexin V^{+} cells in the HSC, MPP, HPC-1 and HPC-2 cell compartments in BM of *Cited2*^{CKO} and *Cited2*^{CTL} mice (n=3). For B-E and G-I, data are mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

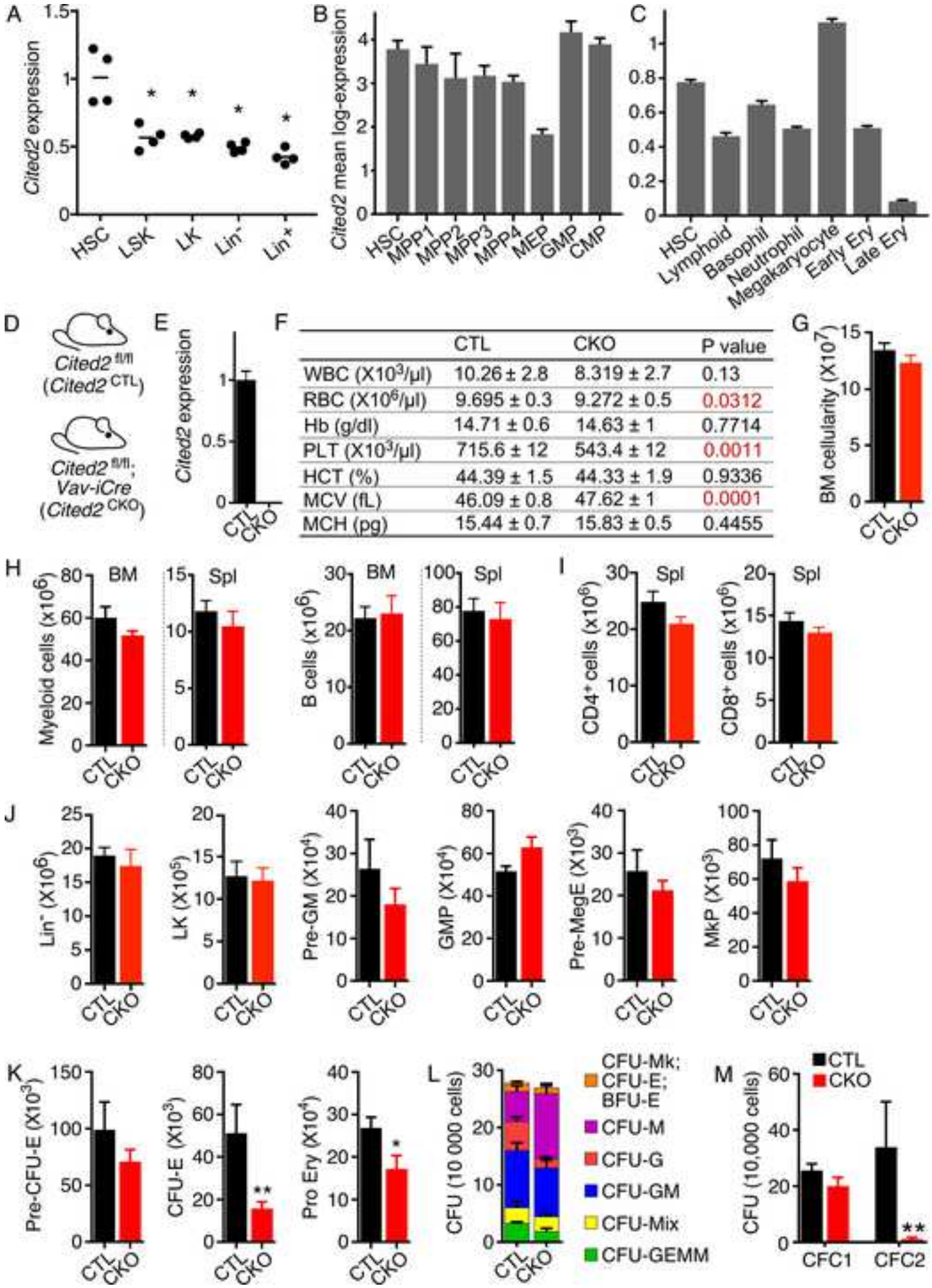
Figure 4. *Mcl1* partially restores normal HSC maintenance and function of *Cited2*-deficient HSCs. (**A**) Expression of *Cited2, Mcl1, Pten*, *Gata1*, *Gata2*, *Foxo3a* and *Bmi1* in LSK cells sorted from 8-week-old *Cited2*^{CTL} and *Cited2*^{CKO} mice (n=4). (**B**) Schematic representation of experimental mouse cohorts; *Cited2*^{CTL}, *Cited2*^{CTL}*;Mcl1*, *Cited2*^{CKO} and *Cited2*^{CKO}*;Mcl1*. (**C**) Total number of HSC, MPP1, MPP2 and MPP3/MPP4 cell populations in BM of 8 to 10-week-old mice (n=4-6 mice per genotype). (**D**) Transplantation assay: 100 HSCs were transplanted into lethally irradiated recipients together with 200,000 unfractionated CD45.1^{+} BM cells. (**E**) Percentage of donor-derived CD45.2^{+} cells in PB following transplantation (n=4 recipients per donor; n=2-3 donors). (**F**) Percentage of donor-derived CD45.2^{+} cells overall in the monocyte, granulocyte, B cell, and T cell compartments of PB. (**G**) Percentage of donor-derived CD45.2^{+} cells in total BM and HSC compartments of the recipient mice. (**H**) CFU assay performed with 10^4 BM cells from 8 to 10-week-old mice (n=5). (**I**) Total CFC counts of primary and secondary cultures (n=5). For A, C and E-I, data are mean ± SEM. *, P<0.05; **, P<0.01.

Figure 5. *Cited2*-deficient HSCs exhibit molecular signatures of functional HSC decline. (**A**) Expression scatter-plot of *Cited2*^{CTL} vs *Cited2*^{CKO} HSCs from 8-12-week-old mice (n=5). Transcripts significantly up- (red) and down-regulated (blue) in *Cited2*^{CKO} are highlighted (FDR<0.05 and FC>20%). (**B**) GSEA showing hallmark pathways up- and down-regulated in *Cited2*^{CKO} HSCs. (**C**) GSEA plots showing up-regulated pathways in *Cited2*^{CKO} HSCs. (**D**) DNA motifs of transcription factors enriched in proximal promoters (from -200bp to +100 bp from

transcription start site) of genes up-regulated in *Cited2*^{CKO} HSCs. (**E**) GSEA plots showing down-regulated pathways in *Cited2*^{CKO} HSCs. (**F**) DNA motif enrichments in proximal promoters of genes that are down-regulated in *Cited2*^{CKO} HSCs.

Figure 1

Click here to access/download;Figure;Figure 1.jpg



	CTL	CKO	P value
WBC (X10^3/µl)	10.26 ± 2.8	8.319 ± 2.7	0.13
RBC (X10^6/µl)	9.695 ± 0.3	9.272 ± 0.5	0.0312
Hb (g/dl)	14.71 ± 0.6	14.63 ± 1	0.7714
PLT (X10^3/µl)	715.6 ± 12	543.4 ± 12	0.0011
HCT (%)	44.39 ± 1.5	44.33 ± 1.9	0.9336
MCV (fL)	46.09 ± 0.8	47.62 ± 1	0.0001
MCH (pg)	15.44 ± 0.7	15.83 ± 0.5	0.4455

Figure 2

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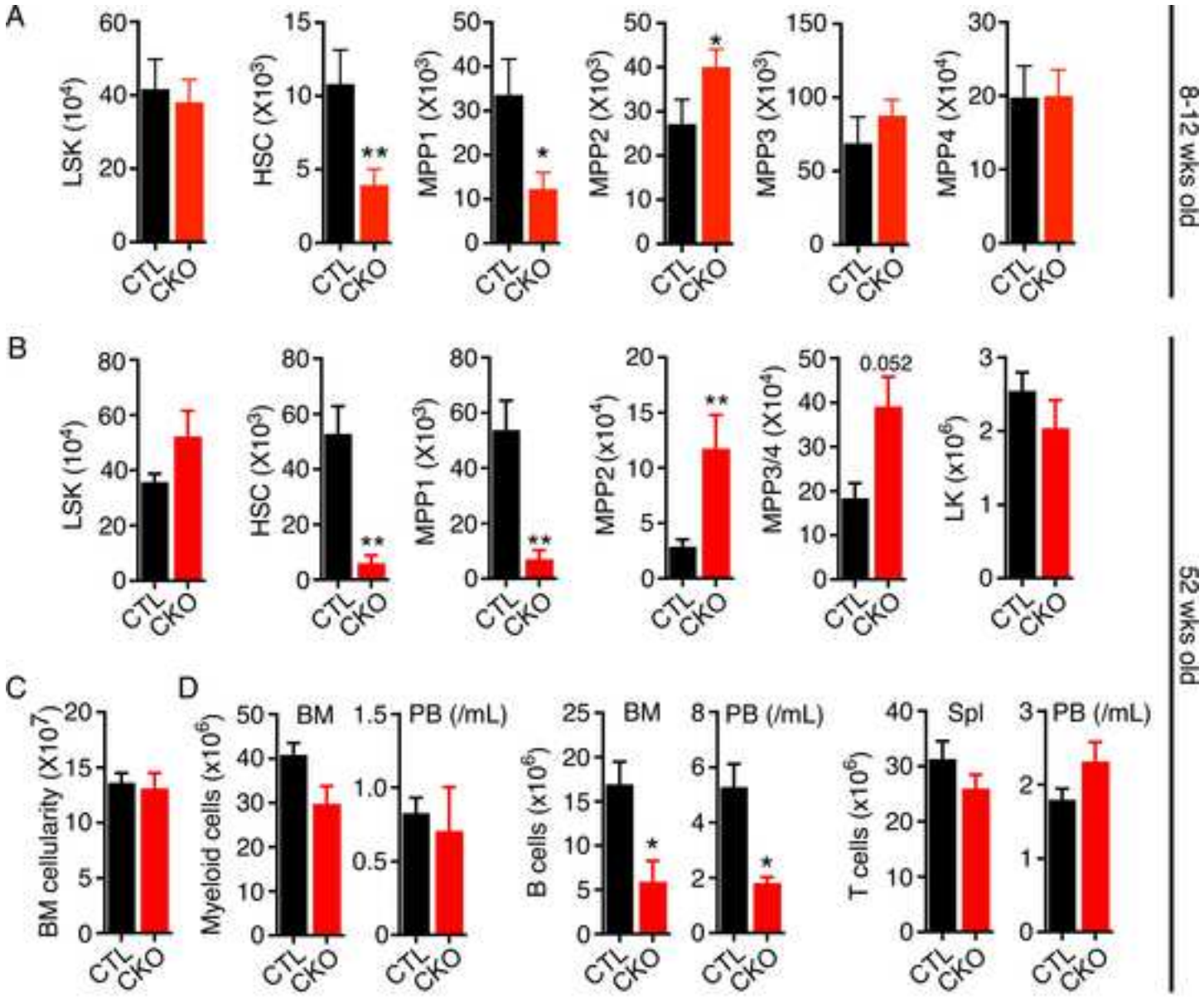

E

	CTL	CKO	P value
WBC (X10^3/μl)	9.893±3.881	5.952± 2.348	0.1522
RBC (X10^6/μl)	9.829± 0.96	7.997± 1.463	0.0307
Hb (g/dl)	14.21±1.131	11.94±2.188	0.0542
PLT (X10^3/μl)	1049±257	693± 281	0.0640
HCT (%)	41.6±6.504	34.40±9.762	0.4135

Figure 3

Click here to access/download;Figure;Figure 3.jpg

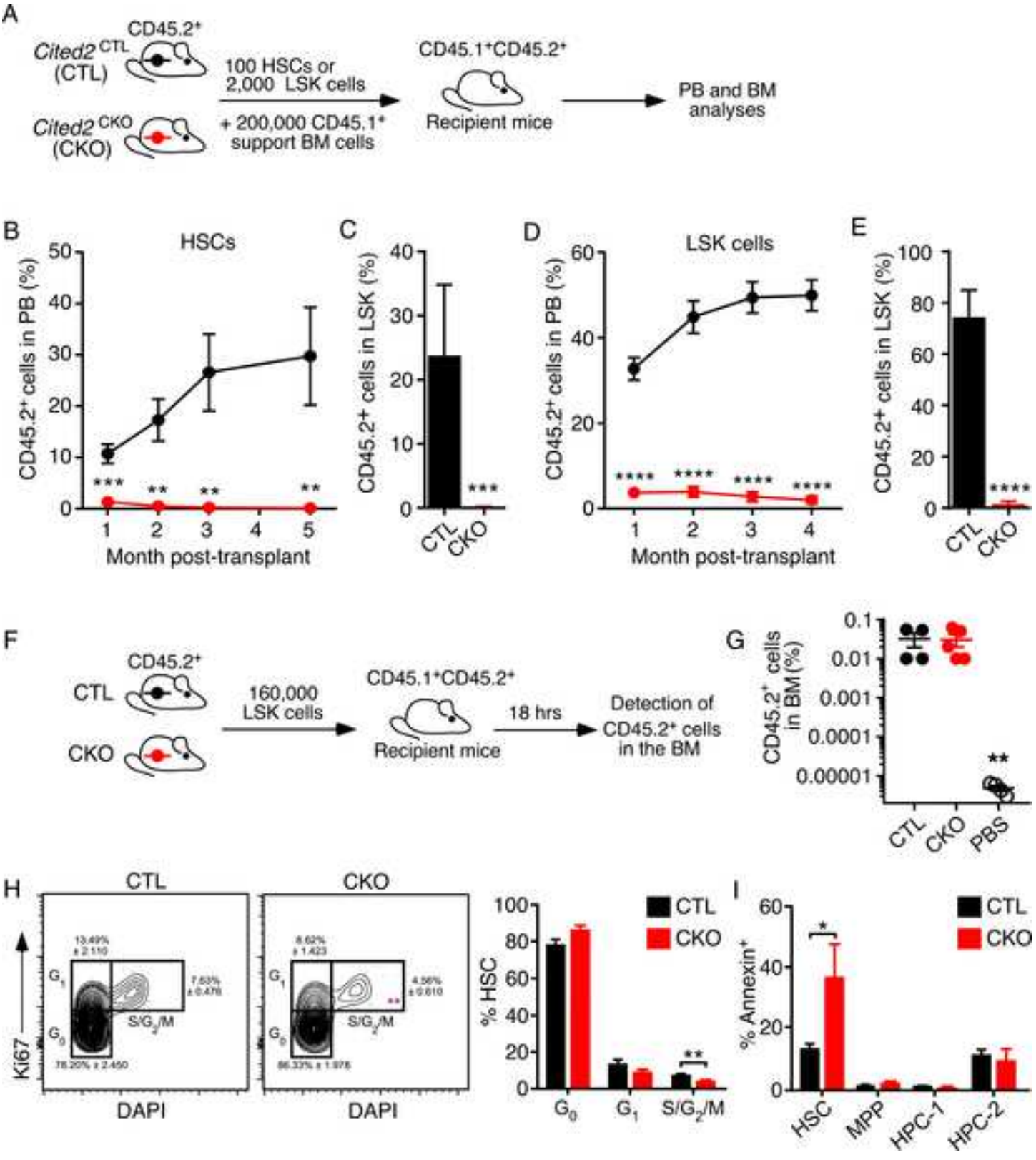

Figure 4

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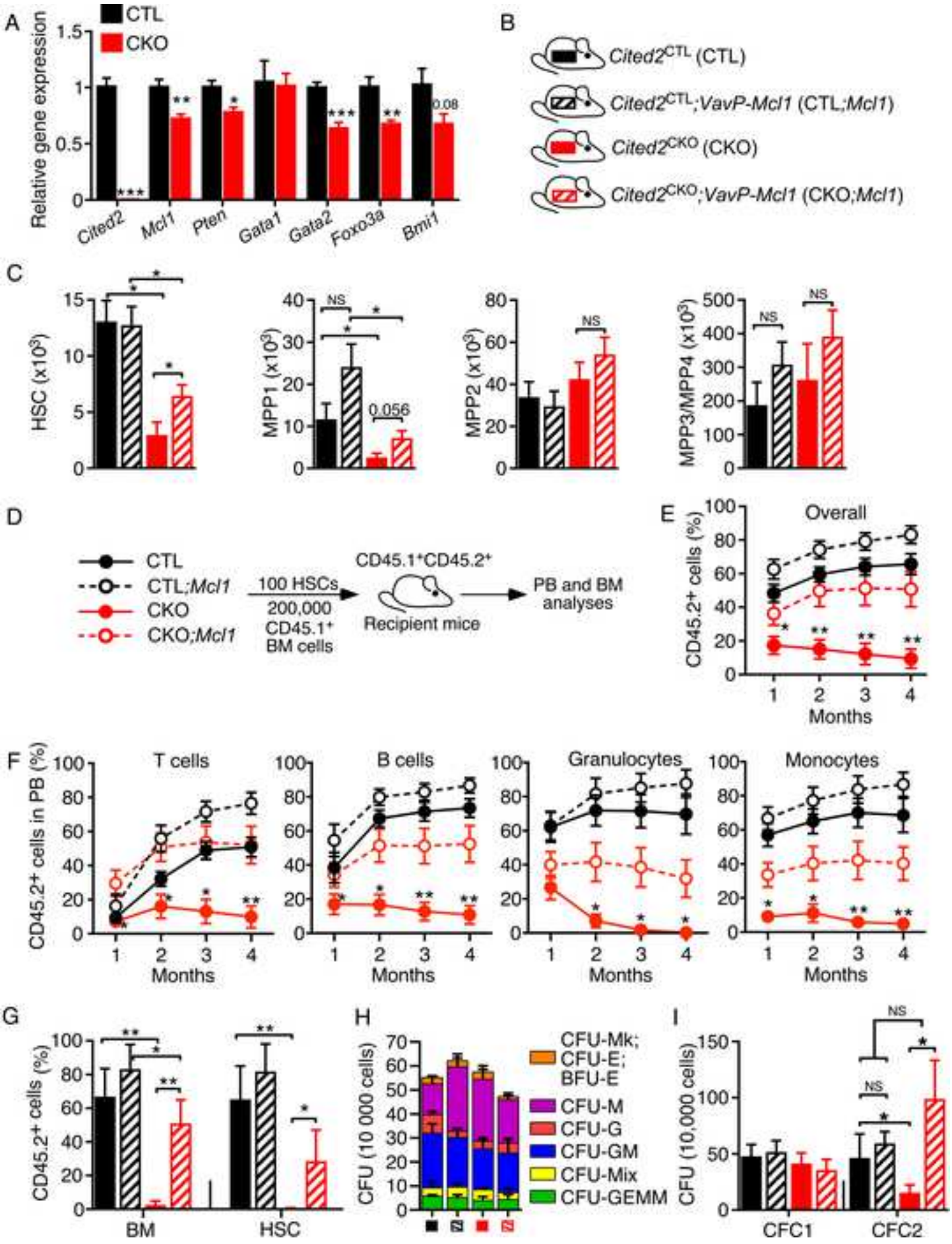

Figure 5

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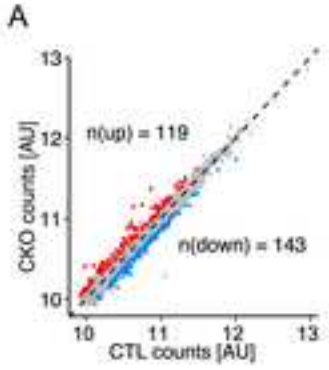
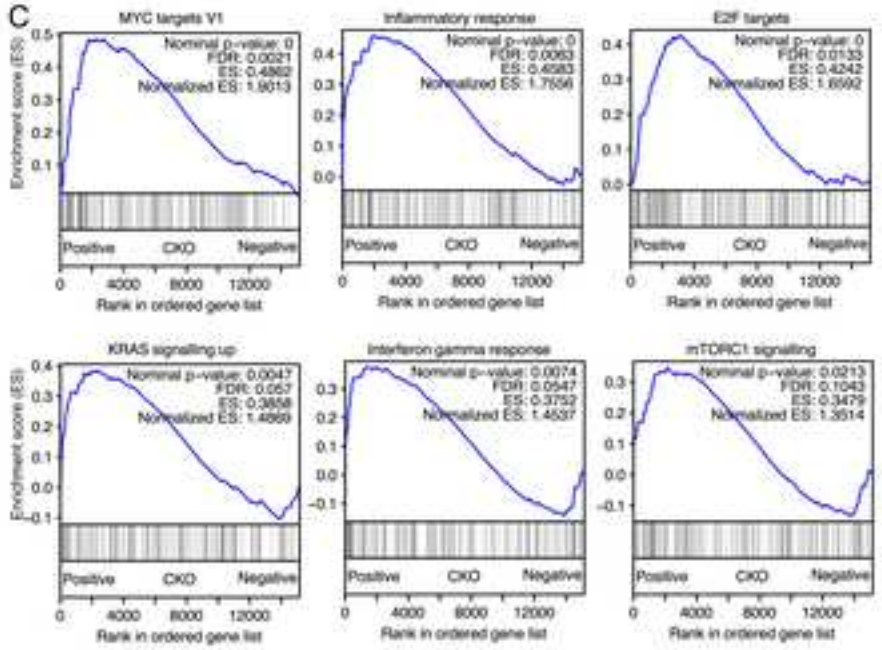


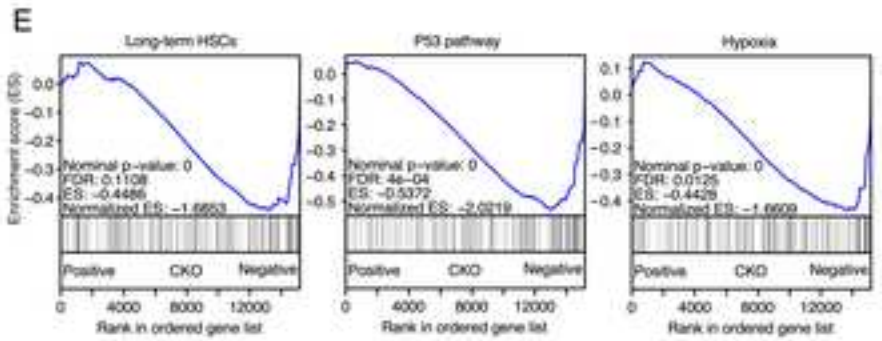
B

GSEA Category	NES	P-val	FDR	
MYC targets V1	1.9	0	0.002	Upregulated in CKO
G2M checkpoint	1.83	0	0.004	
Inflammatory response	1.76	0	0.006	
E2F targets	1.66	0	0.013	
KRAS signalling up	1.49	0.005	0.057	
Interferon gamma response	1.45	0.007	0.055	
mTORC1 signalling	1.35	0.021	0.10	
P53 pathway	-2.02	0	0.0004	Downregulated in CKO
Reactive oxygen species	-1.85	0	0.002	
Long-term HSCs (Ivanova et al.)	-1.68	0	0.11	
Hypoxia	-1.66	0	0.013	
Xenobiotic metabolism	-1.61	0	0.018	
Estrogen response early	-1.51	0.007	0.034	
Adipogenesis	-1.5	0.003	0.033	
Heme metabolism	-1.47	0.003	0.043	



D

Motif	Motif name	P-val	% of targets with motif
	E2F3	1e-42	21.62%
	E2F4	1e-24	16.99%
	E2F1	1e-10	8.11%
	c-MYC	1e-15	10.42%
	n-MYC	1e-9	12.74%
	MAX	1e-8	14.29%
	RUNX1	1e-7	12.74%



F

Motif	Motif name	P-val	% of targets with motif
	STAT3	1e-32	17.62%
	STAT4	1e-25	14.94%
	E2A	1e-19	20.69%
	ZFX	1e-28	25.29%