mTORC1 activity is essential for erythropoiesis and B cell lineage commitment

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Mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase that mediates phosphoinositide-3-kinase (PI3K)/AKT signalling. This pathway is involved in a plethora of cellular functions including protein and lipid synthesis, cell migration, cell proliferation and apoptosis. In this study, we proposed to delineate the role of mTORC1 in haemopoietic lineage commitment using knock out (KO) mouse and cell line models. Mx1-cre and Vav-cre expression systems were used to specifically target Raptorfl/fl (mTORC1), either in all tissues upon poly(I:C) inoculation, or specifically in haemopoietic stem cells, respectively. Assessment of the role of mTORC1 during the early stages of development in Vav-cre Raptorfl/fl mice, revealed that these mice do not survive post birth due to aberrations in erythropoiesis resulting from an arrest in development at the megakaryocyte-erythrocyte progenitor stage. Furthermore, Raptor-deficient mice exhibited a block in B cell lineage commitment. The essential role of Raptor (mTORC1) in erythrocyte and B lineage commitment was confirmed in adult Mx1-cre Raptorfl/fl mice upon cre-recombinase induction. These studies were supported by results showing that the expression of key lineage commitment regulators, GATA1, GATA2 and PAX5 were dysregulated in the absence of mTORC1-mediated signals. The regulatory role of mTOR during erythropoiesis was confirmed in vitro by demonstrating a reduction of K562 cell differentiation towards RBCs in the presence of established mTOR inhibitors. While mTORC1 plays a fundamental role in promoting RBC development, we showed that mTORC2 has an opposing role, as Rictor-deficient progenitor cells exhibited an elevation in RBC colony formation ex vivo. Collectively, our data demonstrate a critical role played by mTORC1 in regulating the haemopoietic cell lineage commitment.

Recent studies of erythroid differentiation have revealed differences in the lineage potential of distinct cellular subsets between fetal and adult progenitor populations, with a higher proportion of megakaryocyte-erythroid progenitors arising from multipotent and oligopotent progenitor subsets during fetal haemopoiesis, while adult erythroid-committed cells are derived predominantly from unipotent progenitors1. These studies suggest that the erythroid cell lineage may be differentially regulated during ontogeny. Multipotent progenitor (MPP) cells give rise to lymphoid-primed multipotent progenitors (LMPPs) and oligopotent common myeloid progenitors (CMPs). LMPPs have the capacity to differentiate towards common lymphoid progenitors (CLPs) mainly, with reduced megakaryocyte-erythroid progenitor (MEP) potential, while CMPs branch towards MEPs and granulocyte-macrophage progenitor (GMP) cells2. MEPs further commit to the erythroid lineage through formation of burst-forming unit-erythroid (BFU-E) and then colony-forming unit-erythroid (CFU-E) cells, which then progress through erythroblast stages, reticulocyte maturation and terminal differentiation into red blood cells (RBCs)3.

Erythropoiesis is governed by the complex regulation of key transcription factors (TFs), enabling a balance between self-renewal and differentiation of erythrocyte progenitors and terminal erythroblasts. The zinc-finger TFs GATA1 and GATA2 play critical and non-redundant roles during erythroid maturation. GATA2 is expressed in HSCs and early progenitor populations regulating the expression of self-renewal genes, and genes responsible for initiating GATA1 expression. GATA1 plays a vital role in erythroid differentiation, sustaining its own expression and suppressing GATA2 expression, a process called GATA factor switching. GATA1−/−

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murine embryos give rise to lymphoid cells and non-haemopoietic tissues, but not a mature erythroid population, resulting from a block at the pro-erythroblast stage due to increased apoptosis. Ikaros also plays a role in fetal and adult erythropoiensis and erythroid lineage commitment, with Ikaros gene silencing leading to an irreversible switch to the myeloid lineage. PU.1 TF is an established master regulator in haemopoiesis involved in primitive cell fate decisions. PU.1 expression levels determine myeloid and lymphoid cell fates: higher expression of PU.1 leads to myeloid cell fate while a lower expression to a lymphoid fate. The myeloid cell fate is not solely regulated by PU.1 expression, but also the inhibition of GATA1 and the expression of CEBPα, PU.1 and GATA1 physically interact to regulate lineage fate where upregulation of GATA1 inhibits PU.1 transcription and promotes erythroid lineage differentiation, while expression of PU.1 inhibits GATA1 expression promoting myeloid lineage fate. GATA1 binds to the promoter region of erythroid-krüppel-like factor (e-KLF/Klf1) which is vital for erythropoiensis, regulating MEP lineage fate decisions during erythrocyte development and globin switching from γ- to β-GLOBIN during erythrocyte maturation. In humans, β-GLOBIN is expressed when erythropoiesis moves to the bone marrow (BM). Initially, the yolk-sac expresses e-GLOBIN, followed by the expression of γ-GLOBIN in the fetal liver (FL) and spleen. Therefore, e-KLF mediated β-GLOBIN expression is vital for mature erythrocyte development. While e-KLF plays a role in erythropoiesis, KLF2 is involved in endothelial growth, vascular remodeling and inflammation responses, which is vital for embryonic development. Lower expression levels of PU.1 skew progenitors towards a lymphoid lineage enhancing E2A expression, a TF involved at the earliest stages of BC cell development. Lack of E2A leads to a block in B cell development at the pre-proB and pro-B stages. E2A drives the expression of early B cell factor 1 (EBF1), which together with E2A, regulate PAX5 and RAG genes responsible for B cell lineage commitment and V(D)J recombination to form the pre-B cell receptor complex on pre-B cells.

The mTOR/akt signaling pathway has been shown to play a vital role in haemopoietic lineage development and maturation. The mTOR pathway is activated by a variety of growth factor receptors and nutrients including glucose, iron and amino acids. mTOR forms two different complexes – mTORC1 and mTORC2. mTORC1 is composed of 6 proteins and mTORC2 of 7 proteins. Of these, mTOR kinase is common, along with G3L, DEPTOR, and the TTI1/TEL2 complex. The subunits that make the respective complexes unique are RAPTOR (rapamycin TOR-sensitive) and PRAS40 for mTORC1 and RICTOR (rapamycin TOR-insensitive), mSin1, and PROTOR1/2 for mTORC2. AKT lies upstream of mTORC1 and downstream of mTORC2 thus playing a crucial role in mTOR pathway regulation. Downstream of mTORC1, S6 kinase 1 (S6K1) inhibits its mTORC2 activity, thereby creating a negative feedback loop and another regulatory mechanism for this pathway.

A critical role of mTORC1 has been identified in erythropoiesis whereby mTORC1 is regulated by dietary iron and Raptor ablation at the haemopoietic stem cell (HSC) stage leads to perinatal lethality. Furthermore Raptor KO and overexpression leads to macrocytic and macrocytic anaemia respectively. However, discrepancies in the field remain, as there is research demonstrating that mTORC1 inhibition does not cause anaemia and that mTORC1 inhibition improves anaemia in a sickle cell disease model. In this study, we address the role of Raptor (mTORC1) in normal haemopoietic lineage commitment both in fetal and adult developmental stages, using the cre-LoxP system to excise Raptor (mTORC1) in adult mice (Mx1-cre) and at the HSC stage (Vav-cre) in embryos, as well as the K562 cell line, to address the role of mTOR-mediated signals during RBC differentiation.

**Results**

**mTORC1 plays a critical role in B cell and RBC lineage development in vivo.** Genotyping analyses of Vav-cre+/ Raptorfl/+ breeding revealed that the mice exhibiting the Vav-Raptor KO (Vav-cre+/ Raptorfl/fl) did not reach weaning age (4 wk), while being present at normal ratios during gestation, suggesting that Vav-cre+/ Raptorfl/fl mice died perinatally (Table 1). Analysis of embryos (E13–17) revealed a significant downregulation of Raptor expression in FL isolated from Vav-Raptor KO mice as expected, and the embryos were pale during gestation compared to Vav-cre+/ Raptorfl/fl (Vav-Raptor control) (Fig. 1A, B, & data not shown). Induction of Raptor excision with poly(I:C) in adult Mx1-Raptor cKO mice revealed a significant downregulation in Raptor expression in the BM and spleen (Fig. 1C) together with an increase in splenic weight and cellularity, and BM cellularity in Mx1-cre+/ Raptorfl/fl mice (Mx1-Raptor cKO) compared to Mx1-cre+/ Raptorfl/fl mice (Mx1-Raptor control) (Fig. 1D–G).

Analysis of the presence of selected haemopoietic lineages in these mouse models revealed a small but significant decrease in the percentage of Ter119+ erythroid populations in Vav-Raptor KO FL at E15 compared to Vav-Raptor control (Fig. 2A & Suppl Fig. 1A). Additionally, there was a trend in decrease in the percentage of Ter119+ erythroid populations in the BM, coupled with a significant increase in percentage of Ter119+

| Age | Medelian Ratio | Cre− Raptor+/+/R | Cre− Raptor+/−/R | Cre− Raptor−/+R | Cre− Raptor−/−R | Chi² Value | p Value |
|-----|---------------|-----------------|-----------------|-----------------|-----------------|------------|--------|
| Weaning | Expected | 6.5 | 6.5 | 6.5 | 14.92 | ** | * |
| | Actual | 13 | 9 | 4 | 0 | | |
| E13 | Expected | 12.25 | 12.25 | 12.25 | 12.25 | 2.92 | 0.50 |
| | Actual | 12 | 8 | 15 | 14 | | |

Table 1. Deletion of Raptor in the haemopoietic lineages leads to death prior to weaning. The expected mendelian ratios and actual genotyped ratios of Vav−cre+/ Raptor+/+ mouse matings are shown at weaning (4 wk; n = 26) and at embryonic day 13 (E13; n = 49). Chi tests values and statistical analyses are shown (**p ≤ 0.001). p value calculated by the Chi Test which determines p value based on degrees of freedom.
population in the spleen likely due to increased extramedullary haemopoiesis in Mx1-Raptor cKO mice compared to Mx1-Raptor control (Fig. 2B & Suppl Fig. 1B). Flow cytometric analysis of B lineage cells revealed a significant downregulation in the percentage of CD19+ B lineage cells in E17 Vav-V KO FL and Mx1-cKO BM, spleen and blood (Fig. 2C–E; Suppl. Fig. 1), coupled with a significant decrease in the number of CD19+ B lineage cells in the Mx1-Raptor cKO BM (Fig. 2F). These data suggest a vital role for mTORC1 in B cell development. Both the KO models showed a concomitant increase in the percentage of CD11b+Gr1− immature myeloid cells, together with a significant decrease in the percentage of CD11b+Gr1+ mature myeloid cells (Fig. 2G,H,I; Suppl. Fig. 1). These data were coupled with a significant increase in the number of CD11b+Gr1− myeloid cells in the Mx1-cKO BM (Fig. 2I), while the mature myeloid cell numbers were unaltered in the Mx1-cKO BM and spleen (Fig. 2K). These data suggest that myeloid lineage maturation is deregulated in the absence of Raptor expression. As it is well established that mice can survive in the absence of white blood cell populations, the absence of adult Vav-Raptor KO mice is likely due to the critical role of mTORC1 in erythropoiesis which results in the decrease in RBC populations and the pallor of the mice.

mTORC1 plays a critical role in B and RBC lineage commitment in vivo. To gain a deeper understanding of the stage at which Raptor-deficiency blocks lineage commitment/development we carried out flow cytometric analysis on the haemopoietic progenitor populations. Supporting the lack of CD19+ B cells, a significant reduction in the percentage of pre-proB and pro-B cells was noted in FL and BM isolated from Vav-Raptor KO and Mx1-Raptor cKO mice respectively, compared with control mice (Fig. 3A,B; Suppl. Fig. 2). This finding
was coupled with a significant elevation in the Lin−Sca-1+CD117hi (LSK) population, suggesting a block in B cell development prior to lineage commitment. Of note, no significant differences in LSK and progenitor B cell populations were observed between VavCrecre/+Raptorwt/wt and VavCrecre−/Raptorfl/fl mice, indicating that the phenotypic changes were due to the excision of Raptor (Fig. 3A). Analysis of the proportion of myeloid progenitors in E15

Figure 2. Mx1/Vav-cre+Raptorfl/fl mice exhibit a disruption in haemopoiesis in vivo. Graphs showing the percentage of haemopoietic lineage calculated from flow cytometry data gated as indicated in Supplemental Fig. 1. Average percentage of Ter119+ erythroid population in Vav-cre−Raptorwt/wt, Vav-Raptor control, and Vav-Raptor KO E15 FLs (n = 7) (A) and BM and spleen of Mx1-Raptor control and Mx1-Raptor cKO mice (n = 3) (B); Average percentage of CD19+ B cells in E15 (n = 6) (C) and E17 (n = 4) (D) FLs and BM, spleen and blood of Mx1-Raptor control and Mx1-Raptor cKO mice (n = 5) (E); Average number of CD19+ B cells in BM and spleen of Mx1-Raptor control and Mx1-Raptor cKO mice (n = 5) (F); Average percentage of CD11b+Gr1− (immature myeloid) and CD11b+Gr-1+ mature myeloid populations in Vav-control, and Vav-KO E15 FLs (n = 6) (G), and CD11b+Gr1− (immature myeloid) (H) and CD11b+Gr-1+ mature myeloid populations (I) in Mx1-Raptor control and Mx1-Raptor cKO BM and spleens (n = 5). Average number of CD11b+Gr1− (I) and CD11b+Gr-1+ myeloid populations (K) in Mx1-Raptor control and Mx1-Raptor cKO BM and spleens (n = 5). All Mx1-cre mice were given 4 doses of poly(I:C) and assessed 5 wk post inoculation. Light blue bars - cre−/Raptorwt/wt; blue - cre+/Raptorfl/fl; red bars - cre+/Raptorwt/wt, maroon bars - cre+/Raptorfl/fl as indicated. Data are expressed as mean ± SEM, one way ANOVA (p ≤ 0.05; ** ≤ 0.001; *** ≤ 0.0001; **** ≤ 0.00001).
FL isolated from Vav-Raptor KO mice revealed no significant changes in the Sca-1loCD117hi, CMP and GMP populations compared to the control mice (Fig. 3C; Suppl. Fig. 3A). An elevation in the percentage of MEPs in E13 and E15 FLs isolated from Vav-Raptor KO mice suggested a developmental arrest in erythropoiesis at the MEP stage in the absence of Raptor expression at the HSC stage in vivo (Fig. 3D). Interestingly, adult mice with induced Raptor-deficiency exhibited a significant decrease in Sca-1loCD117hi population and MEP population suggesting a block in erythropoiesis prior to the MEP stage in Mx1-Raptor cKO BM (Fig. 3E). Additionally, a significant decrease in GMP population was noted, suggesting that differentiation of CMPs towards MEPs is deregulated in the BM in Mx1-Raptor cre cKO mice. The difference in progenitor populations between the FL and BM with Raptor-deficiency highlights the potential for mTORC1 to play similar but not identical roles in lineage maintenance at different stages of development and ontogeny.

In support of our phenotypic analyses showing a block in B cell and RBC development, analysis of key genes responsible for enabling differential lineage commitment revealed a significant downregulation in the expression of B cell specific TFs Ebf1 and Pax5 in FLs isolated from Vav-Raptor KO mice, while Cebpα levels were unaltered (Fig. 3F, G). Assessing erythroid lineage commitment factors, Pu.1, β-Globin and Gata1 levels were significantly

**Figure 3.** Mx1 and Vav-creRaptorfl/fl mice exhibit a block in B and RBC lineage commitment at the LSK and MEP stage in vivo. Surface expression of Lin-Sca-1-CD117+ (LSK), pre-proB (CD117+CD220+CD19-) and proB (CD117+CD19+B220+) populations in E15 Vav-Raptor KO FL compared to Vav-cre Raptorfl/fl and Vav-creRaptorfl/fl FL controls (n = 6) (A) and in BM of Mx1-Raptor control (blue) compared to Mx1-Raptor cKO (red) mice (n = 6) (B); (C) Graph showing surface expression of S1loc-Kithi (Sca-1hiCD117lo) population in E15 FL in Vav-cre Raptorfl/fl (light blue) and Vav-Raptor control (blue) and Vav-creRaptorfl/fl (light red) and Vav-Raptor KO (dark red) (n = 6); (D) Surface expression of megakaryocyte erythrocyte progenitor (MEP) in E13 and E15 FL in Vav-KO mice compared to cre Raptorfl/fl and Vav-control (E13 n ≥ 3; E15 n ≥ 6); (E) BM of Mx1-control or Mx1-cKO mice showing surface expression of S1loc-Kithi, CMP, GMP and MEP populations (n ≥ 5); Gene expression of E17 FL demonstrating the fold change in Pu.1, early B cell factor 1 (Ebf1), and Pax5 gene expression (F), along with fold changes in gene expression of E15 FL Cebpα, Gata1, Gata2, β-Globin (G), and Kruppel-like factor 1 and 2 (Klf1 and Klf2) (H) in CD45+ sorted Vav-Raptor KO FL compared to Vav-Raptor control FL cells (n ≥ 4). All Mx1-cKO mice were given 4 doses of poly(I:C) and assessed 5 wk post inoculation. Data are expressed as mean ± SEM, one way ANOVA (p * ≤ 0.05; ** ≤ 0.001; *** ≤ 0.0001; **** ≤ 0.00001).
downregulated in Vav-Raptor KO mice with trends in an increase in Gata2 levels. Additionally, there was a significant downregulation in the expression of Klf1 and Klf2, TFs which play a role in embryonic erythropoiesis and development (Fig. 3H), indicating an aberration in erythropoiesis with Raptor deficiency in Vav-KO FLs.

Inhibition of mTORC1 blocks RBC differentiation in vitro. To further assess the role of mTORC1 in RBC differentiation in vitro, we made use of the human BCR-ABL+ erythroleukaemia cell line K562, that differentiates towards a RBC-like lineage when exposed to stress. Stress was induced by replacing glucose in complete media with galactose (Gal-media). A significant increase in the percentage of CD71+GlyA+ cells, an elevation of the erythroid marker CD71 surface expression and a reduction in cellular granularity was observed (Fig. 4A,B, Suppl. Fig. 4A) in K562 cells cultured in Gal-media, coupled with a significant increase in gene expression of β-GLOBIN, GATA1 and GATA2 (Fig. 4C). Treatment of K562 cells with either rapamycin (allosteric mTORC1 inhibitor) or AZD8055 (dual mTORC1/2 inhibitor) reduced erythroid differentiation in vitro, as indicated by a significant decrease in the generation of erythroid cells and a reduction in gene expression of erythroid markers, (Fig. 4B,C), indicating mTORC1 inhibition blocks RBC differentiation in vitro.

To assess erythroid colony formation capacity of HPCs in the absence of mTORC1 activity, HPCs were isolated from BM of Mx1-Raptor control or cKO mice and CFC assays were performed. Mx1-Raptor cKO mice lacked CFC activity, as indicated by the lack of colony-forming unit-erythroid (CFU-E), blast-forming unit-erythroid (BFU-E) or granulocyte-erythroid-megakaryocyte-macrophage (GEMM) colonies in the absence of Raptor expression, compared to cre- controls (Fig. 5A,B). Interestingly, myeloid progenitor CFC assays performed in Vav-cre- (Vav-Rictor control) and creRictor (Vav-Rictor KO) HPCs to assess the role of mTORC2 in early myeloid/erythroid colony formation demonstrated a significant increase in CFU-E colonies and a trend in increase in GEMM colonies in Vav-Rictor KO HPCs (Fig. 5C,D), suggesting a suppressive role of mTORC2 in erythropoiesis. Supporting these data, flow cytometric analysis of the CFCs generated showed a decrease in percentage of Ter119+ cells in Mx1-Raptor cKO HPCs, while an increase was noted in Vav-Rictor KO HPCs (Suppl. Fig. 5).

Discussion
Our data support previously published work demonstrating a critical role of mTORC1 during B cell and erythropoiesis in two mouse models of Raptor-deletion in vivo, and further demonstrate the importance of mTORC1 function in in vitro RBC differentiation models: CFC assays and human cell line K562.
Examining the role of mTORC1 in normal erythropoiesis at the HSC stage, in both fetal and adult mice, revealed subtle differences at the two developmental stages. *Raptor*-deficient embryos were paler than *Raptor*-control mice, characteristic of mice with RBC deficiency as noted in EPO/EPO-R mice. Knight *et al.*, demonstrated high mTORC1 activity in proE cells (reticulocytes) characterised by elevated phosphorylation of the downstream target S6 thereby identifying a critical role for mTORC1 in RBC development. Additionally, they showed a link between iron availability and haemoglobin (Hb) synthesis in RBC development with mTORC1 signalling, as RBCs have decreased mTORC1 signalling during iron deficiency (ID) *in vitro* and *in vivo*. We observed a significant decrease in Ter119+ erythrocytes, with an arrest at the MEP stage shown by a significant increase in the MEP population at E15, together with an increase in Sca-1lo CD117hi cells in the FL of Vav-Raptor KO. This is consistent with published data demonstrating Vav-Raptor KO FL exhibit microcytic anaemia with
begins before birth at E16.528. The block in development of the erythroid lineage was supported by a significant observed in FL at E13/E15. This is likely due to a transition in haemopoiesis from the FL to the BM, which between the controls and 

biogenesis from HSPCs to proerythroblasts by mTORC138. This study shows a unique requirement for mTORC1 translation at early stages in RBC development has been reported, demonstrating the regulation of mitochondrial downregulation in Gata1, β-Globin, Klf1 and Klf2 expression with Raptor-deficiency at the HSC stage, suggesting mTORC1-mediated signalling may aid in the regulation of GATA switching to enable erythropoiesis.

In contrast, induced Raptor-deficiency in adult mice (Mxl-Raptor cKO) is not lethal, and these mice displayed a significant decrease in Sca-1+CD117h and MEP populations in the BM. This may be due to the lineage potential of progenitors differing between fetal and adult mice, as FL HSCs possess a higher proliferative and metabolic capacity (increased oxidative phosphorylation) than BM-derived HSCs which could affect Sca-1+CD117h and MEP primitive populations29. Nevertheless, there is a trend in decrease in Ter19+ erythropoiesis in the BM of adult mice with Raptor-deficiency, as noted in the Vav-cre model, suggesting a disruption in erythropoiesis in the BM. Indeed, Guo et al., demonstrated that Mxl-Raptor cKO mice have reduced RBCs and Hb in the BM due to increased apoptosis of RBCs. Additionally, erythocyte development was blocked at the proE stage confirming our results that mTORC1 blocks RBC development. Mxl-Raptor cKO mice exhibited splenomegaly, with the spleen showing a significant increase in Ter19+ population, which could suggest a possible compensatory mechanism leading to the promotion of erythropoiesis due to a lack of erythrocytes, suggesting extramedullary haemopoiesis in the spleen30. Alternatively, as has been shown previously to be a main site for erythropagocytosis, it is possible that the function of these erythrocytes is compromised and are accumulated in the spleen for phagocytosis30. We observe splenomegaly and an increase in red pulp. As the red pulp is one of the major sites of erythrocyte destruction, it is possible that an accumulation of erythrocytes in the spleen indicates erythrocyte depletion32. The observed differences between the models may be due to the mechanism of Raptor-excision. While both models result in the deletion of Raptor in all haemopoietic lineages, the Mxl-Raptor cKO requires inoculation with poly(I:C), through induction of type 1 interferons, which may disrupt haemopoietic cell survival. Published studies indicate that the impact of poly(I:C) treatment on haemopoietic cells is transient, lasting for up to 48 hr33. Here, phenotypic analyses were carried out 5 wk after the last poly(I:C) inoculation, by which time the key poly(I:C) induced effect should be Raptor-excision. While this timeline may enable the outgrowth of undeleted alleles in Mxl-Raptor cKO mice, the optimisation of poly(I:C) induced Raptor-excision leading up to this study reduced the likelihood of this occurring in the timeframe of these experiments, and Raptor expression was consistently downregulated up to 8 wk post-poly(I:C) inoculation.

Using established mTOR inhibitors to block RBC differentiation in the K362 cell line provided support for the results presented from our mouse models, indicating that erythroid development is dependent on mTORC1 activity. Interestingly, a recent report shows that K562 cells express a distinct mTOR-containing complex, mTORC3 (mTOR associated with ETV7) that lacks other mTORC1/2 containing proteins, and is associated with rapamycin-resistance when upregulated in human cancer cells34. Our finding that K562 differentiation is inhibited by rapamycin treatment suggests that mTORC3 does not play a major role in this process. Supporting this, Ohyashiki et al. have previously shown that iron regulates mTORC1 signalling in RBCs as iron chelators greatly reduced mTORC1 signalling in K562 cells35. Overexpression of elf4E and sustained mTORC1 expression in erythroid progenitor cell line I/11 also improves differentiation36,37. An additional role of mTORC1 in initiation of translation at early stages in RBC development has been reported, demonstrating the regulation of mitochondrial biogenesis from HSPCs to proerythroblasts by mTORC134. This study shows a unique requirement for mTORC1 in RBC development. These data, together with our findings suggest that mTORC1, iron metabolism and erythropoiesis have an inter-dependent regulatory link.

Assessment of erythroid colony formation capacity revealed that Raptor-deficient BM lacked colony formation capacity. Consistent with this, Mxl-Raptor cKO cells have previously been shown to lack the ability to form CFU-E and BFU-E colonies30. Indeed, shRNA targeting S6K1 in murine BM cells significantly decreased CFU-E and BFU-E colony formation capacity demonstrating the importance of mTORC1-S6K axis is mediating erythropoiesis28. ER-Raptor cKO LSK cells also lack colony formation capacity which is rescued by the retroviral transduction of Raptor suggesting the importance of mTORC1 in haemopoiesis30. Our assessment of Rictor-deficient BM colony formation capacity revealed that Vav-Rictor KO BM formed a significantly higher number of erythroid colonies than their controls. This suggests a possible feedback system for erythroid regulation wherein mTORC1 drives erythroid formation, whereas mTORC2 suppresses erythropoiesis. Our study compared the Mxl-Raptor cKO and Vav-Rictor KO models. Vav-mediated KO models generate a constitutive KO, which can result in signalling pathway adaptation38. Indeed, crosstalk between ERK-MAPK and mTOR signalling pathways has recently been demonstrated, with ERK activity regulating mTORC1 activation to limit the promotion of HSC cycling in favour of quiescence. HSCs derived from MEK1-cKO mice exhibit exhaustion due to increased mTORC1-mediated ROS production resulting in increased mitochondrial damage32. While each model has a different timing for gene deletion, the cells placed in CFC assays were removed from Mxl-Raptor mice 5 wk after inoculation with poly(I:C) in vivo, thus inducing optimal Raptor excision.

Of note, publications also suggest that mTORC2 negatively regulates erythropoiesis, via heme-regulated elf2α kinase (HRI) and activation transcription factor 4 (ATF4), in a mouse model of ID. HRI is activated in response to heme deficiency, thereby stimulating ATF4 to regulate Hb levels via the translation of stress response genes. In mutant mice that lack phosphorylated elf2α (elf2αP) in the RBC lineage (eAAA)45, mTORC1 activity is elevated in erythroid precursors. This study demonstrated that elf2αP and ATF4 of HRI-integrated stress response are required in ID to repress mTORC1 signalling and mitigate ineffective erythropoiesis. As such, pharmacological
inhibition of mTORC1 significantly improved RBC production and differentiation, and increased Hb levels in the blood. Furthermore, constitutive activation of mTORC1 in haemopoietic lineages exhibited macrocytic hyperchromic anaemia with splenomegaly and ineffective erythropoiesis, similar to that seen in HRI−/− mice. However, although ATF4 is required to inhibit mTORC1 signalling, ATF4−/− mice develop microcytic hypochromic anaemia, unlike HRI−/− mice. These studies suggest that mTORC1 activity plays an inhibitory role in erythropoiesis during ID anaemia. Furthermore, Foxo3−/− mice (model with similar genotypic profile to β-thalassemia), exhibit increased phosphorylation of mTOR targets including AKT, S6, 4EBP1, suggesting aberrant mTOR signalling in diseased conditions. Treatment of Foxo3−/− mice with rapamycin increased RBC counts and Hb levels, with increased cell cycling in immature erythrocytes compared to untreated mice. Therefore, in contrast to normal erythropoiesis, where mTORC1 plays a fundamental role in RBC generation, diseased conditions alter mTORC1 signalling to disrupt erythropoiesis, which is overcome by mTORC1 inhibitors.

The early block in B cell lineage commitment and development in the FL and BM of both Raptor-deficient models, mirrored that described previously in Mx1-Raptor cKO mice. The observed increase in LSK populations, which normally give rise to pre-proB cells, in both FL and E15 FL suggests a block at the LSK stage during B cell development with Raptor-deficiency at the fetal and in adult stages of development. These findings were supported by the observed downregulation in Ebf1 and Pax5, TFs essential for B cell lineage commitment. A similar trend has been observed in the BM of mtor-cKO mice, and the spleen of Mx1-Raptor cKO mice, with the majority of LSK cells in S phase suggesting increased cell cycling due to perturbations in mTORC1 signalling. Iwata et al., demonstrate a block in B cell development at the pre-B cell stage within Mbl-Raptor cKO adult mice, which is not rescued by the introduction of an anti-apoptotic Bcl2l transgene suggesting that this block, caused by the lack of mTORC1, is independent of BCL2L44. Our model, which excises Raptor at the HSC stage, demonstrates a requirement for mTORC1 signalling for B cell lineage commitment. In ER-Raptor cKO BM there is an increase in LSK cells together with a decrease in phosphorylation of 4EBP1 and S6 and decrease in mature B cells indicating that the role of mTORC1 in B lymphopoiesis is not model specific. Indeed, Zheng et al. highlight the importance of mTORC1 specifically in B cell development and proliferation, but not survival, in an IL7R-mTORC1-Myc dependent and STAT5-independent manner, as the loss of mTORC1 leads to a block at the preB cell stage in CD2-mTOR and CD2-Raptor cKO models. Furthermore, Keating et al. demonstrated a critical role for mTORC1 in B cell class switching and anti-viral responses, with Rosa26-Raptor KO mice having reduced germinal centre formation in response to influenza infection, a finding that was replicated with rapamycin treatment. Interestingly, rapamycin treatment prevented B cell class-switching, yielding antibodies that mediated heterotypic protection, by targeting more conserved regions of hemagglutinin, a surface protein expressed on erythroid cells. However, although ATF4 is required to inhibit mTORC1 signalling, ATF4−/− mice develop microcytic hypochromic anaemia, unlike HRI−/− mice. These studies suggest that mTORC1 activity plays an inhibitory role in erythropoiesis during ID anaemia. Furthermore, Foxo3−/− mice (model with similar genotypic profile to β-thalassemia), exhibit increased phosphorylation of mTOR targets including AKT, S6, 4EBP1, suggesting aberrant mTOR signalling in diseased conditions. Treatment of Foxo3−/− mice with rapamycin increased RBC counts and Hb levels, with increased cell cycling in immature erythrocytes compared to untreated mice. Therefore, in contrast to normal erythropoiesis, where mTORC1 plays a fundamental role in RBC generation, diseased conditions alter mTORC1 signalling to disrupt erythropoiesis, which is overcome by mTORC1 inhibitors.

In agreement with previously published data, similar roles of mTORC1 in myeloid lineage development were observed in both fetal and adult mice with a significant increase in CD11b+Gr1+ populations and a concomitant decrease in CD11b−Gr1− populations in both Vav-Raptor KO FL and Mx1-Raptor cKO BM. This result has been recapitulated in ER-cre model suggesting that the role of mTORC1 in myeloid development is not model specific. We observed a decrease in GMP population leading to a decrease in mature myeloid population with Raptor-deficiency in Mx1-Raptor cKO mice. Indeed, a decrease in CMPs has previously been reported in Mx1-Raptor cKO mice supporting the decrease in mature myeloid lineage with Raptor-deficiency. However, the increase in CD11b+ population in both models also suggests that even though there is a block in early myeloid populations, it does not disrupt the ability of the progenitors to develop into immature myeloid cells. ER-Raptor cKO mice displayed an increase in GMP and MEP populations with a block in CD11b+Gr1− mature myeloid populations with no changes in P66, alluding to the phenotypic differences between KO models. The lack of change in pS6 expression in myeloid cells (with a significant decrease in pS6 in B cells) suggests a differential regulation of mTORC1 activity in different haemopoietic lineages. Nonetheless, mTORC1 plays a role in the maturation of myeloid cells in both adult and fetal models. While previous studies support a cell autonomous effect of Raptor-deletion in the development of haemopoietic lineages, mixed BM chimeras should be performed to confirm our results, as myeloid lineage expansion has the potential to inhibit erythroid and lymphoid lineages. In addition, deletion of Raptor in HSCs has been shown to affect osteoclast generation, which in turn would impact the BM microenvironment and subsequent haemopoiesis.

Taken together, we performed analyses in two different mTORC1 KO mouse models at different stages of development in vivo and two distinct RBC differentiation assays in vitro to tease out the role of mTORC1 in haemopoietic lineage development. We confirm the fundamental role of mTORC1 in haemopoiesis, during RBC and B cell lineage commitment both in fetal and adult mice (Fig. 3E).

Materials and Methods

Mice. Mice expressing floxed Raptor (Raptorfl/fl) or Rictor (Rictorfl/fl) constructs were obtained from Prof. Michael N. Hall (University of Basel, Switzerland). Prof. Tessa L. Holyoake (University of Glasgow, UK) provided transgenic mice expressing Mx1-cre and Vav-cre, which were bred and maintained at the Beatson Research Unit (Glasgow, UK). All experimental protocols were approved by the local AWERB committee and national home office, and all methods were carried out in accordance with standard animal housing conditions under local and home office regulations. Mx1-cre−/− or Vav-cre−/− transgenic mice were crossed with Raptorfl/fl or Rictorfl/fl to obtain the desired KO models. The Mx1 promoter was activated in Mx1-cre−/−.Raptorfl/fl or -Rictorfl/fl mice by inoculating the mice with 4 doses of 10 mg/kg polyinosinic:polycytidylic acid (poly(I:C); TL3R agonist) once every 2 days to induce a conditional KO (cKO). The mice were assessed 5 wk after the last inoculation. No significant differences were observed in organ cellularity, and B/myeloid lineage percentage and number when B6.SJL wild type mice and Mx1-cre−/−Rictorfl/fl mice were compared after treatment with poly(I:C); Supplemental Fig. 6). The Vav promoter is active at E13 in HSCs, thereby inducing a haemopoietic-restricted KO. Haemopoietic
lineage cells in a single cell suspension were isolated from BM, spleen and blood of transgenic mice, and filtered through a 70 µm nylon mesh (Fisher Scientific, Leicestershire, UK). The cells collected from the BM and spleen were enriched for haemopoietic lineage cells by density centrifugation using Lympholyte-Mammal (Cedarlane, Canada), centrifuging the cell suspension for 20 min at 625 g at RT. Thereafter the cells were washed in PBS, centrifuged at 500 g for 10 min at RT and counted.

Flow cytometry. Samples derived from the organs were prepared for flow cytometry as described previously. All antibodies were purchased from BD Biosciences (Oxford, UK), except Gr-1 (Clone RB6-8C5; eBiosciences) and Ter119 (Clone TER-119; BioLegend). K562 cells with/without treatment were harvested and 2 × 10^5 cells were stained with GlyA (Clone GAR2) and CD71 (Clone AC102) from Miltenyi Biotec. All samples were suspended in 100 µl PBS and acquired using the FACS Diva software package (BD Biosciences) on the FACSCantoII flow cytometer. The resultant data were analysed using FlowJo software (Tree Star Inc., OR).

qRT-PCR. RNA was extracted from fresh cells by following the RNAeasy Qiangen Kit protocol. cDNA was then made from the RNA using standard protocols provided by Invitrogen. Alternatively, CD45+ cells were sorted into the PCR tubes (300 cells/tube) containing the one-step PCR master mix of 5 µl (Cells direct 2x reaction (2.8 µl), 0.2x Primer mix (1.4 µl), RNase out (0.056 µl), Superscript III RT/Platinum Taq Mix (0.112 µl), TE Buffer (0.672 µl) (part of SuperScript™ III Platinum™ One-Step qRT-PCR Kit, Invitrogen)). Samples were run on a PCR with the following conditions: 50 °C for 15 min, 95 °C for 2 min followed by 20 cycles of 95 °C for 15 sec and 65 °C for 4 min. The samples were then held at 4 °C. After the completion of the PCR, the samples were diluted 10x with TE buffer (45 µl). qPCR was carried out by using 300 nM of forward and reverse primer for each gene. All reactions were performed in technical triplicates and at least three biological replicates using the 7900HT Fast Real-Time PCR system (Applied Biosystems, Warrington, UK), programmed to complete 40 cycles as follows: 50°C for 20 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The primers used are listed in Supplementary Table 1. The q/RT-PCR follows the MIQE guidelines.

Erythroid differentiation of K562 cells. For normal cell growth, K562 cells were cultured in RPMI-1640 supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-Glutamine (complete media). To induce erythroid differentiation, K562 cells were cultured in RPMI-1640 (no glucose) supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-Glutamine and 11 mM Galactose (Gal-media). For erythroid differentiation, K562 cells (2 × 10^5) were cultured in either complete media or Gal-media for up to 7 days and then analysed by flow cytometry or qPCR. For drug treatments, cells were treated with 10 nM rapamycin (allosteric mTORC1 inhibitor) or 100 nM AZD8055 (competitive dual mTORC1/2 inhibitor) or vehicle control (DMSO) as indicated.

Colony forming cell (CFC) assay. Bones from transgenic mice were crushed and enriched for haemopoietic lineages as described above. Haemopoietic progenitor cells (HPCs) were isolated using the MACS mouse CD117 MicroBeads according to the manufacturer’s protocol (Miltenyi Biotec, Surrey, UK). Up to 2 × 10^6 cells were then plated in MethoCult™ M3334 erythroid colony promoting methylcellulose or MethoCult™ GF M3434, in 6 cm dishes as per the manufacturer’s protocol (Stemcell Technologies, Grenoble, France). The dishes were monitored and colonies were counted.

Statistics. The Chi Test was used to assess whether there were statistical differences between the different genotypes generated from crossing Vav-cre−/−/Raptorfl/fl mice. Chi Test calculates deviations by chance if the expected ratios are known. Medelian ratios were the expected ratios, which were compared to the genotypes observed at weaning stage of mice. The statistical significance, if any, is calculated using the Chi Test taking into account the degrees of freedom. Statistical analyses were carried out between the data sets using GraphPad Prism 6 Software (San Diego, California, USA). Statistical analyses carried out were unpaired t tests, or for data with multiple comparisons one way ANOVA, where p ≤ 0.05 is considered significant. * ≤ 0.05; ** ≤ 0.001; *** ≤ 0.0001; **** ≤ 0.00001.

Data availability
No datasets were generated or analysed during the current study.

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Author contributions
N.M. designed/perform the majority of experiments, analyzed and interpreted the data, carried out statistical analysis and drafted the manuscript; J.C., J.H. and C.E. performed some experiments, assisted in experimental design and carried out data analysis; K.D. provided technical assistance with the in vivo models and analyzed the data; O.S. obtained funding for the study and supervised the studies; A.M.M. obtained funding for the study, designed the research, supervised the studies, analyzed and interpreted the data and wrote the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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