EpoR stimulates rapid cycling and larger red cells during mouse and human erythropoiesis

Daniel Hidalgo, Jacob Bejder, Ramona Pop, Kyle Gellatly, Yung Hwang, S. Maxwell Scalf, Anna E. Eastman, Jane-Jane Chen, Lihua Julie Zhu, Jules A. A. C. Heuberger, Shangqin Guo, Mark J. Koury, Nikolai Bastrup Nordsborg, and Merav Socolovsky.

The erythroid terminal differentiation program couples sequential cell divisions with progressive reductions in cell size. The erythropoietin receptor (EpoR) is essential for erythroblast survival, but its other functions are not well characterized. Here we use Epor−/− mouse erythroblasts endowed with survival signaling to identify novel non-redundant EpoR functions. We find that, paradoxically, EpoR signaling increases red cell size while also increasing the number and speed of erythroblast cell cycles. EpoR-regulation of cell size is independent of established red cell size regulation by iron. High erythropoietin (Epo) increases red cell size in wild-type mice and in human volunteers. The increase in mean corpuscular volume (MCV) outlasts the duration of Epo treatment and is not the result of increased reticulocyte number. Our work shows that EpoR signaling alters the relationship between cycling and cell size. Further, diagnostic interpretations of increased MCV should now include high Epo levels and hypoxic stress.
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d-cell formation (erythropoiesis) is continuous throughout life, replenishing senescent red cells and responding to increased demand during anemia, bleeding, or hypoxic stress. Anemia resulting from nutritional deficiencies, malaria, chronic disease, cancer, or hereditary hemoglobinopathies, accounts for 8.8% of all disabilities globally. Erythropoietin (Epo) is the principal and essential regulator of definitive (adult-type) erythropoiesis, regulating erythropoietic rate in the basal state and during the stress response. Epo acts through its receptor, EpoR, a transmembrane type I cytokine receptor, first expressed in the earliest erythroid-committed progenitors. EpoR expression peaks in colony-forming-unit-erythroid (CFU-e) progenitors (Supplementary Fig. 1) with the onset of erythroid terminal differentiation (ETD), a process that starts with the induction of erythroid gene transcription. During ETD, erythroblasts undergo 3–5 maturational cell divisions in which they become smaller, express hemoglobin, and enucleate to form reticulocytes. EpoR rescues proerythroblasts and basophilic erythroblasts (here collectively termed ‘early erythroblasts’) and CFU-e from apoptosis, a principal mechanism of erythropoietic rate regulation. EpoR is downregulated in late erythroblasts, which no longer depend on its signaling for survival. Chronic disease, cancer, or hereditary hemoglobinopathies, pared their ensuing differentiation with that of EpoR−/−. Thus, our initial analysis showed that, when rescued from apoptosis by Bcl-xL−/− erythroblasts with EpoR, contained hemoglobinized cells by 36 h post transduction, while EpoR−/− erythroblasts without Epo did not (Fig. 1e). However, differentiation of Bcl-xL−/− erythroblasts appeared to be accelerated, with cultures containing smaller and morphologically more mature erythroblasts, including many enucleated cells; there were few if any enucleated cells in cultures of EpoR−/− erythroblasts at this time (Fig. 1e).

Differentiation abnormalities of Bcl-xL−/− erythroblasts were also evident from flow cytometric analysis. In wild-type progenitors, the transition from the CFU-e stage to ETD is marked by sharp upregulation of CD71 (encoded by the transferrin receptor, Tfrc), followed by upregulation of Ter119, and upregulation of Epo−/− progenitors arrest in development prior to CD71 upregulation (the small number of Ter119+ cells in Epo−/− fetal liver are yolk-sac-derived erythroblasts). Transduction of Epo−/− fetal liver cells with EpoR allowed them to resume the expected sequence of cell surface marker expression, upregulating CD71 by 18 h and Ter119 by 36 h (Fig. 1g). By contrast, Bcl-xL−/− cells failed to upregulate CD71 at any point of the culture although they did upregulate Ter119 (Fig. 1g).

Thus, our initial analysis showed that, when rescued from apoptosis by Bcl-xL−/− progenitors can differentiate into hemoglobinized, enucleated red cells in the absence of additional EpoR signals. However, their ETD is abnormal, failing to upregulate CD71, and differentiating prematurely into fewer and smaller red cells.

Erythroblasts undergo fewer and slower cell cycles in the absence of EpoR signaling. CFU-e express the receptor tyrosine

**Results**

**Non-survival EpoR signals are essential for normal erythroid differentiation.** Erythroid differentiation in Epo−/− fetal liver is arrested at the CFU-e stage. Here we asked whether transducing Epo−/− CFU-e with Bcl-xL−/− an anti-apoptotic transcriptional target of EpoR signaling, would be sufficient to support erythroid differentiation. As control, we transduced EpoR−/− cells from the same fetal livers with the EpoR. The use of bicistronic retroviral expression vectors allowed us to track transduced cells (Fig. 1a). As expected, EpoR−/− cells transduced with ‘empty’ vector failed to give rise to CFU-e-derived colonies in semi-solid medium, whereas EpoR-transduced EpoR−/− cells (EpoR−/−/EpoR) generated CFU-e colonies in an Epo-dependent manner. Bcl-xL−/− transduced EpoR−/− cells (Bcl-xL−/−EpoR−/−) failed to give rise to CFU-e colonies of the usual size and appearance (Fig. 1b). Instead, they generated a similar number of much smaller colonies with fewer cells (colony areas were 439 ± 208 μm² versus 217 ± 106 μm², mean ± SD, for EpoR−/−/v. Bcl-xL−/−EpoR−/−, p = 3.6 × 10−13, Fig. 1c, d). Co-transduction of EpoR−/− cells with both Bcl-xL and a constitutively active form of Stat5, an EpoR-activated transcription factor, was also not sufficient to support the formation of normally-sized EpoR−/− CFU-e colonies (Fig. 1b).

Liquid cultures of Bcl-xL−/−EpoR−/− in the presence or absence of Epo, and of EpoR−/−EpoR−/− erythroblasts with Epo, contained hemoglobinized cells by 36 h post transduction, while EpoR−/− erythroblasts without Epo did not (Fig. 1e). However, differentiation of Bcl-xL−/−EpoR−/− erythroblasts appeared to be accelerated, with cultures containing smaller and morphologically more mature erythroblasts, including many enucleated cells; there were few if any enucleated cells in cultures of EpoR−/− erythroblasts at this time (Fig. 1e).
kinase Kit and the Interleukin-3 (IL3) receptor. Addition of stem cell factor (SCF, the Kit ligand) and IL3 to the media increased the overall yield of transduced Epor−/− fetal liver cells, but the difference in cell number between Bcl-xL-Epor−/− and EpoR-Epor−/− erythroblasts remained (Supplementary Fig. 2a). We modified our transduction protocol to make use of this improvement in yield, culturing freshly transduced Epor−/− progenitors for 15 h in SCF and IL3 before transitioning the cells to an Epo-containing medium for the remainder of differentiation. Since SCF and IL3 also promote the growth of myeloid cells, all analysis was performed on cells that were both negative for non-erythroid lineage markers and positive for reporters of transduction (hCD4 and/or GFP, Supplementary Fig. 2b, Fig. 2a).

Fig. 1 Abnormal ETD in the absence of EpoR signaling. a Experimental design. E12.5 Epor−/− fetal livers were transduced with bicistronic retroviral vectors encoding either Bcl-xL or EpoR, linked by an internal ribosomal entry site (IRES) to human CD4 (hCD4) or GFP reporters. Transduced cells differentiated in vitro into red cells over the ensuing 72 h. b Epor−/− CFU-e colonies, scored 48 h following transduction with either EpoR or Bcl-xL. Epo was added to the medium where indicated. Epor−/− fetal liver cells were also transduced with retroviral vectors encoding the following: ‘empty’ vector (‘V’), constitutively active Stat5 (Stat5 1*6), or doubly transduced with both Bcl-xL and Stat5 1*6. Data pooled from 3 independent experiments. Data are means ± SD. Only CFU-e colonies of a size comparable to those of wild-type colonies were scored. c Representative colonies from an experiment as in (b). d Colony area occupied by each of 75 colonies for each genotype (EpoR−/− or Bcl-xL−/−). Data pooled from 3 independent experiments as in (b). Two-sided t test, unequal variance. e Cytospin preparations of transduced Epor−/− fetal liver cells cultured in liquid medium for 36 h in the presence or absence of Epo as indicated. Cells were stained for hemoglobin with diaminobenzidine (brown stain, arrowheads) and counter-stained with Giemsa. Representative of 4 independent experiments. Double-headed arrows point at enucleated red cells; arrows point at pyrenocytes (extruded nuclei). The micrograph in the bottom panel is representative of cultures both in the presence or absence of Epo. f, g Flow-cytometric CD71/Ter119 profiles of freshly harvested Epor−/− and wild-type littermate fetal livers (f), and of Epor−/− fetal liver cells 18 and 36 h post transduction and culture in Epo-containing medium (g).
Pre-incubation with SCF and IL3 did not ameliorate the abnormalities of Bcl-xL-Epor<sup>−/−</sup> erythroblast differentiation. In particular, these cells failed to upregulate CD71 (Fig. 2a, b). We examined the possibility that these abnormalities were the result of overexpression of Bcl-xL, rather than the absence of EpoR signaling, by co-transducing Epor<sup>−/−</sup> progenitors with both EpoR and Bcl-xL, each linked to a distinct reporter (Supplementary Fig. 2c). The doubly transduced progenitors were indistinguishable from cells transduced with only the EpoR, indicating that the lower cell number and failure to upregulate CD71 were not the result of Bcl-xL overexpression, but rather, of absent EpoR signaling (Supplementary Fig. 2d-g).

The transferrin receptor is critical for iron import into erythroid cells. Iron deficiency leads to microcytic anemia. We therefore examined whether iron deficiency might account for the abnormal differentiation of Bcl-xL-Epor<sup>−/−</sup> erythroblasts, by co-transducing Epor<sup>−/−</sup> progenitors with Tfrc, in addition to either Bcl-xL or EpoR (Fig. 2c-e). In an alternative approach, we added iron-loaded ferric-salicilaldehyde isonicotinoyl hydrazone (Fe-SIH) to the culture medium of both Bcl-xL-Epor<sup>−/−</sup> and EpoR-Epor<sup>−/−</sup> erythroblasts.

**Fig. 2** EpoR stimulates cell-cycle shortening in early erythroblasts in vitro and in vivo. a The EpoR is required for CD71 upregulation. Epor<sup>−/−</sup> fetal livers were transduced with either EpoR or Bcl-xL retroviral vectors carrying the hCD4 reporter (see Supplementary Fig. 2b for experimental design). Transduced cells (hCD4<sup>+</sup>Lin<sup>−</sup>) were examined for expression of CD71 and Ter119. b Time course of CD71 expression following transduction as in (a). MFI; median fluorescent intensity, relative to t = 0; n = 2 independent experiments. c Growth of Epor<sup>−/−</sup> fetal liver cells transduced with either Bcl-xL or EpoR. Viable hCD4<sup>+</sup>Lin<sup>−</sup> cells were counted at the indicated times. Fe-SIH (10 μM) or deoxynucleosides (dN, 0.7 μM) were added to the medium as indicated. "Tfrc" cells were doubly transduced with both Tfrc, and either Epor or Bcl-xL. Data, pooled from n = 4 independent experiments and expressed relative to cell number at t = 0, were fit with exponential curves (R² values 0.8–0.94, least-squares fit). d Trypan blue negative cells, for the set of experiments in (c). e Cell doubling times ±95% confidence intervals, calculated from the fitting of exponential growth curves to the data in (e). f Cell-cycle shortening in early erythroblasts in vivo. Mice transgenic for the chimeric histone H2B-fluorescence-timer protein (H2B-FT) were injected with either saline or Epo (100 U) at 0 and 24 h. Bone marrow was analyzed at 36 h. H2B-FT fluoresces blue (B') for 1–2 h immediately following synthesis, and matures into a red fluorescent protein (R'). The ratio of blue to total fluorescence (B/(B + R)) is a function of cell-cycle length<sup>27</sup>. Shown are histograms of B/(B + R) in EryA erythroblasts (Ter119<sup>med</sup>CD71<sup>hi</sup>FSC<sup>hi</sup>). Histogram overlays are for n = 2 mice injected with either saline or Epo. g Relative cell-cycle lengths for the 4 mice analyzed in (f), for each of the indicated erythroblast maturation stages: ProE (Ter119<sup>med</sup>CD71<sup>hi</sup>Fsc<sup>hi</sup>), EryA (Ter119<sup>hi</sup>CD71<sup>hi</sup>FSC<sup>hi</sup>), EryB (Ter119<sup>hi</sup>CD71<sup>hi</sup>FSC<sup>hi</sup>), EryC (Ter119<sup>hi</sup>CD71<sup>hi</sup>FSC<sup>hi</sup>), p-value is for a two-tailed paired t-test, pairing Epo-injected and Saline-injected mice for each erythroblast stage (ProE and EryA/B/C). Late erythroblasts (EryC) may divide, but their cell cycle is no longer sensitive to Epo concentration.
SIH is a cell-membrane-permeable synthetic iron chelate, which, when pre-loaded with iron, will deliver iron intracellularly for heme synthesis, bypassing defects in Tfrc iron transport in erythroid cells43. Neither of these approaches altered the proliferative defect of Bcl-xL−/− erythroblasts (Fig. 2c, e). The viability of all erythroblasts was high with no significant difference between Bcl-xL−/− and EpoR−/− erythroblasts (Fig. 2d), suggesting that the proliferative defect of Bcl-xL−/− erythroblasts is the result of fewer cell divisions. In the first 26 h of culture, there was a substantial difference in doubling time (6.1 h vs. 8.6 h for EpoR−/−/− vs. Bcl-xL−/− erythroblasts, Fig. 2c, e). The doubling time of 6 h for EpoR−/−/− is in good agreement with our recent finding of a 6 h cell cycle in wild-type early erythroblasts in vivo26, and with the finding that early erythroblasts have the shortest cell cycle amongst bone-marrow hematopoietic progenitors47.

Iron may affect cell growth by acting as a cofactor in ribonucleotide reductase (RNR) catalysis of deoxyribonucleotide synthesis44. However, supplementation of the culture medium with deoxynucleobases (dN), which bypass RNR via the deoxyribonucleoside kinase salvage pathway45, had little effect on the proliferative defect (Fig. 2c, e). Taken together, in the absence of EpoR signaling, erythroblasts fail to upregulate CD71 and also undergo fewer and longer cell divisions. Supplementation with iron or deoxynucleobases does not rescue these deficits.

**Epo administration shortens cell-cycle duration in early erythroblasts in vivo.** To test whether EpoR stimulation alters cell-cycle length in vivo, we used a mouse transgenic for histone H2B fluorescent protein27. The ratio of blue fluorescent to total fluorescence (red + blue) is an indicator of cell-cycle length27. Fig. 3). These data confirm that Epo/EpoR signaling increases cell-cycle speed in wild-type erythroblasts in vivo.

**EpoR shortens both G1 and S phase through an iron-independent mechanism.** The onset of ETD is associated with cell-cycle shortening, from ~15 h in CFU-e, to 6 h in early erythroblasts4,26,40, including a shortened, 4-h-long S phase26. We asked whether the cell-cycle shortening effect of EpoR (Fig. 2e, g) is exerted in G1 or in S phase. The shortening of G1 by cytokine receptor signaling is well documented46, and we hypothesized that EpoR signaling, we doubly transduced Bcl-xL−/− erythroblasts with both EpoR and Bcl-xL were similar in size to those transduced with EpoR−/− and Bcl-xL−/− erythroblasts, respectively, mean ± sem, p = 0.001, t = 46 h). Although Bcl-xL−/− erythroblasts express significantly lower CD71 (Fig. 2a, b), the addition of Fe-SIH to the culture did not alter their smaller cell or nuclear size (Fig. 2b).

We asked whether the smaller size of Bcl-xL−/− erythroblasts could reflect an accelerated process of differentiation. If at any given time of the culture Bcl-xL−/− erythroblasts were smaller only as a result of being at a more advanced maturation stage, they should give rise to normally-sized enucleated reticulocytes, albeit at an earlier time. However, imaging flow cytometry showed that Bcl-xL−/− reticulocytes were significantly smaller (5.6 ± 0.5 μm vs. 4.5 ± 0.15 μm for EpoR−/− vs. Bcl-xL−/−, mean ± sem, p = 0.002, Fig. 4c, d).

To assess whether the smaller size of Bcl-xL−/− erythroblasts is the result of overexpression of Bcl-xL rather than absent EpoR signaling, we doubly transduced EpoR−/−/− fetal liver cells with both EpoR and Bcl-xL. We used the Bcl-xL-linked GFP and the EpoR-linked hCD4 fluorescence reporters to quantify expression and ensured that all comparisons were made between cells expressing similar levels of each retroviral vector (Supplementary Fig. 4b–d). We found that erythroblasts and reticulocytes transduced with both EpoR and Bcl-xL were similar in size to those transduced with only the EpoR, and significantly larger than those transduced with only Bcl-xL (Fig. 4c; Supplementary Fig. 4c, d). Therefore, Bcl-xL overexpression is not the cause of the smaller size of Bcl-xL−/− erythroblasts and reticulocytes.

The level of EpoR expression in transduced EpoR−/− cells positively correlated with erythroblast cell diameter (Supplementary Fig. 5). The relationship follows classical dose/response kinetics (Spearman correlation = 0.97, p-value = 0.004). By contrast, there was no correlation between Bcl-xL expression and cell diameter.

**EpoR regulation of red-cell size is independent of HRI.** HRI is activated by iron and heme deficiency and mediates the formation of smaller, hypochromic red cells, by inhibiting translation52. Bcl-xL−/− erythroblasts failed to upregulate CD71 (Tfrc), the principal iron transporter. Although iron supplementation
Fig. 3 EpoR regulates the speed of S phase. a Cell-cycle analysis of Epor−/− fetal liver cells transduced with either EpoR or Bcl-xL and cultured as in Supplementary Fig. 2b. Cells were pulsed with BrdU for 30 min at t = 9 h and were immediately harvested for analysis. The fraction (%) of erythroblasts (Lin−CD41−Lin−) in S phase is indicated, as is S phase speed, measured as the intra-S phase rate of BrdU incorporation (BrdU MFI within the S phase gate). b Summary of cell-cycle status and S phase speed, as measured by intra-S phase BrdU incorporation in EpoR or Bcl-xL-transduced Epor−/− fetal liver cells. Data is pooled from 6 independent experiments similar to (a). In all cases, cells were pulsed with BrdU for 30 min prior to harvesting for analysis. Data are mean ± sem. Intra-S phase BrdU (MFI) is expressed as the ratio to BrdU MFI of Bcl-xL-transduced fetal liver cells at t = 0 in each experiment. Significance p-values are two-tailed paired t test, pairing EpoR and Bcl-xL-transduced cells for each time point across all experiments (upper panel), and for t = 9 and t = 19 h in all experiments (middle and lower panels). c Effect of the cell-permeable iron carrier, Fe-SIH (10 μM) on S phase speed. Experiment and cell-cycle analysis as in (b). Cells were harvested at t = 9 h. d, e Summary of S phase speed (d) and cell-cycle status (e) in EpoR-transduced Epor−/− fetal liver cells at t = 9 h, experimental design as in Fig. 2b, and (a) to (e) above. S phase speed is expressed relative to the speed at t = 0 in each experiment. Shown are the effects of addition of Fe-SIH or dN to the medium, or of doubly transducing cells with both Bcl-xL and Tfrc. Data are mean ± sem for n = 4 independent experiments each for Fe-SIH and dN, and n = 3 for Tfrc. All experiments also had EpoR−/− fetal liver cells transduced with EpoR and with Bcl-xL without additional additives or transductions. P-value is for a two-sided t test, unequal variance.

did not rescue the smaller size of Bcl-xL−Epor−/− erythroblasts (Fig. 4b), it remained possible that intracellular iron delivery was somehow incomplete.

To determine definitively the relevance of the iron/heme/HRI pathway to cell size regulation by EpoR, we generated Epor−/−;Hri−/− mice (Fig. 5a). Similar to Epor−/− mice, Epor−/−;Hri−/− mice died at mid-gestation with severe anemia. We rescued both Epor−/− and Epor−/−;Hri−/− fetal liver cells in parallel, by transduction with either Bcl-xL or EpoR (Fig. 5b–e). In agreement with the known role of HRI as a negative regulator of erythroblast size, both Bcl-xL-transduced and EpoR-transduced erythroblasts were larger on the Epor−/−;Hri−/− genetic background than on the Epor−/− background. Importantly, for a given genetic background, either Epor−/−;Hri−/− or Epor−/−, the difference in size between Bcl-xL and EpoR-rescued cells remained (Fig. 5b–e).
**Fig. 4** Smaller erythroblasts that differentiate into smaller reticulocytes in the absence of EpoR. 

**a** Cell and nuclear diameter of hCD4^+Lin^- erythroblasts, measured by imaging flow cytometry. Experiment as in Fig. 2b. Polystyrene beads of known diameters were used for calibration (see "Methods", Supplementary Fig. 4a). Datapoints are population medians for individual samples, with 50,000 cells imaged per sample. Box and whiskers mark the 25th to 75th percentiles and min to max values, respectively, with the median indicated. Data pooled from 7 independent experiments, p-values are from two-tailed paired t-tests, pairing EpoR and Bcl-xL-transduced cells in each experiment. 

**b** A representative experiment as in (a), showing individual sample contour plots overlaid on scatter plots (each dot is one cell), of nuclear diameter vs. cell diameter. Red dots indicate distributions' medians. The effect of adding Fe-SIH to the culture medium is also shown. Data are hCD4^+Lin^- erythroblasts at 48 h post transduction. 

**c** Distinguishing erythroblasts from reticulocytes using imaging flow cytometry, with the nuclear dye Draq5. The analysis was performed on Ter119^+ cells. Representative images of 3 independent experiments are shown, from cultures of Epor^-/- fetal liver cells that were doubly transduced with bicistronic retroviral vectors encoding GFP and hCD4 reporters (see Supplementary Fig. 2c), at 48 h post transduction. Scale bar = 10 µm. 

**d** Reticulocyte cell diameter in cultures of Epor^-/- or Bcl-xL^-Epor^-/- at 48 h post transduction, identified as in (c). Data are population medians from 5 independent experiments. Box and whiskers as in (a). Two-tailed paired t-tests. 

**e** Reticulocyte diameters in cultures of Epor^-/- fetal liver cells that were doubly transduced with bicistronic vectors carrying GFP and hCD4 reporters (Supplementary Fig. 2c). These vectors were either 'empty' (VGFP, VhCD4) or encoded either Bcl-xL or EpoR (Bcl-xL_GFP, EpoR_hCD4). Violin lines mark the 25th, 50th, and 75th percentile with a white circle marking the mean. Representative of two independent experiments.
These results clearly show that EpoR signaling regulates cell size independently of the HRI pathway, since, even in the absence of HRI, EpoR signaling promotes the formation of larger erythroblasts (Fig. 5b–d) and reticulocytes (Fig. 5e).

Accelerated maturation in the absence of EpoR, assessed independently of cell size. Cell size is frequently used as indicator of erythroid maturational stage. Our initial impression was that Bcl-xL-Epor−/− erythroblasts completed their maturation sooner than EpoR-Epor−/− erythroblasts (Fig. 1e, g). However, the finding that Bcl-xL-Epor−/− erythroblasts are smaller throughout maturation makes cell size an unreliable indicator of maturational stage in these cells. We therefore assessed maturation using two alternative measures.

First, the cell surface marker Ter119, whose expression increases with maturation, reached significantly higher levels in Bcl-xL-Epor−/− erythroblasts than in EpoR-Epor−/− erythroblasts at 48 h.
EpoR signaling delays induction of p27KIP1, leading to increased number of cell cycles. To investigate the molecular mechanisms underlying EpoR-regulated functions, we compared gene expression in differentiating EpoR−/− and Bcl-xL−/− erythroblasts, using RT-qPCR (Supplementary Fig. 10). ETD markers Slc4a1 (Band3) and Hbb1 were induced similarly in both cell types. There were no significant differences in transcription factor expression, with the exception of Tal1, whose levels were 30% lower in Bcl-xL−/− (p < 0.005). Tal1 was previously linked to cell-cycle regulation in hematopoietic cells.

Among cell-cycle regulators, the CDK inhibitor p27KIP1 (Cdkn1b) was induced prematurely in Bcl-xL−/−, reminiscent of its premature expression in EpoR−/− primitive erythroblasts. A second member of the CIP/KIP family, p57KIP2 (Cdkn1c), was also expressed at somewhat higher levels. The induction of p27KIP1 toward the end of ETD in wild-type erythroblasts contributes to mitotic exit.

To determine the effect of its premature induction, we transduced wild-type fetal liver S1 cells (CD71highTer119mid) with either p27KIP1 or empty vector (Supplementary Fig. 11a). p27KIP1−/− transduced cells showed reduced proliferation, without affecting cell viability, suggesting they underwent fewer cell cycles (Supplementary Fig. 11b, c). Unlike Bcl-xL−/− erythroblasts, however, p27KIP1−/− transduced cells were larger, and slower to undergo maturation, as judged by lower nuclear offset (Supplementary Fig. 11d–f). Therefore, while the EpoR-mediated negative regulation of p27KIP1 increases cell-cycle number, its regulation of cell size and maturation rate are mediated by other pathways. Addition of the phosphatidylinositol 3-kinase (PI3K) inhibitor, LY2940023, to wild-type erythroblasts resulted in premature induction of p27KIP1 mRNA (Supplementary Fig. 12), suggesting that negative regulation of p27KIP1 by EpoR is mediated via PI3K.

Several EpoR signaling pathways are implicated in the regulation of cell size. EpoR activates three principal signaling pathways:

1. **Cytokine signaling**: EpoR signaling can activate STAT3, JAK2, and MEK/ERK pathways. STAT3 activation can promote cell survival and proliferation, while JAK2 activation can increase cell size and affect cell cycle progression. MEK/ERK signaling can promote cell proliferation and survival.
2. **PI3K/AKT/mTORC1 pathway**: EpoR signaling can activate PI3K, which in turn activates AKT and mTORC1. This pathway is involved in cell survival, proliferation, and metabolism.
3. **ERK signaling**: EpoR signaling can activate ERK, which can regulate cell cycle progression and cell size.

In addition, EpoR signaling can also affect cell cycle regulators, such as p27KIP1, which is involved in the inhibition of cell cycle progression and cell maturation. EpoR signaling can also affect stress signaling pathways, such as the unfolded protein response (UPR), which is involved in the regulation of cell survival and metabolism.

**Fig. 9b**: EpoR signaling delays induction of p27KIP1, leading to increased number of cell cycles. To investigate the molecular mechanisms underlying EpoR-regulated functions, we compared gene expression in differentiating EpoR−/− and Bcl-xL−/− erythroblasts, using RT-qPCR (Supplementary Fig. 10). ETD markers Slc4a1 (Band3) and Hbb1 were induced similarly in both cell types. There were no significant differences in transcription factor expression, with the exception of Tal1, whose levels were 30% lower in Bcl-xL−/− (p < 0.005). Tal1 was previously linked to cell-cycle regulation in hematopoietic cells.
pathways: ras/MAP kinase, Stat5, and PI3K. Neonatal mice hypomorphic for Stat5 have microcytic anemia. Here we found that, similarly, circulating red cells from E13.5 Stat5-deficient embryos are smaller than those of wild-type littermates (Supplementary Fig. 13a, b). Using U0126, a MEK1- and MEK2-specific inhibitor, and the PI3K inhibitor LY294002, we examined the role of these pathways in the regulation of cell size. PI3K inhibition significantly decreased the size of early (‘S2’) and late (‘S3’) erythroblasts and reticulocytes, but MEK1/2 inhibition had no consistently significant effect (Supplementary Fig. 13c, d). Therefore, it is likely that cell size regulation by EpoR is the integrated result of multiple signaling pathways.

Fig. 6 Red-cell size regulated by Epo concentration in mice and humans. a, b Epo regulates erythroblast diameter. CFU-e progenitors (‘S0’) enriched from wild-type fetal livers were differentiated in vitro in a range of Epo concentrations and analyzed at 48 h. Representative of two independent experiments. a S0 cells differentiate into the S1, S2, and S3 subsets by 48 h. b Cell diameter distributions for each erythroblast subset and Epo concentration. Violin lines are 25th, 50th, and 75th percentile. White circles mark the mean. c Nuclear offset measures nuclear eccentricity, independently of cell size. It is the ratio of the delta centroid (distance between the centers of the cell and the nucleus, \( \Delta \)), and cell diameter. Nuclear offset increases during erythroid morphological maturation. d-g Mice were injected with saline (n = 2) or Epo (n = 2 mice with either 5 U, 20 U, or 100 U). Bone marrow analyzed at 48 h. d Nuclear offset quintiles for Ter119+ erythroblasts in saline-injected mice. e CD71/forward-scatter (FSC) histograms for the nuclear offset quintiles in (d) of Ter119+ erythroblasts. Quintile values that were determined for saline-injected mice were also applied to the Epo-injected mice. Sequential quintiles are seen to contain increasingly mature erythroblasts. f Cell diameter in each nuclear offset quintile in (e), for each Epo dose. Violin lines are 25th, 50th, and 75th percentile, white circle marks the mean. Data representative of n = 2 mice per Epo dose. g Median cell diameter and median nuclear offset values in each nuclear offset quintile, for mice injected with Epo (100 U) or Saline. Data points are individual mice. h Human intervention studies. Epo was administered during the period indicated. In study #1, n = 25 subjects were treated with Epo and n = 9 subjects with placebo. In study #2, n = 24 each for placebo and Epo. Data is fractional change relative to the baseline values of each participant. Additional hematological parameters and data for placebo groups are in Supplementary Figs. 14, 15 and Supplementary statistical analysis. MCV, mean corpuscular volume; Retics, reticulocyte count. i RDW_SD and reticulocyte counts for human intervention study #1 described in panel (h).
Epo administration increases red-cell size (MCV) and size variation (RDW) in human volunteers. We examined the effect of Epo on red-cell size in healthy volunteers in three intervention studies. Participants were either given Epo (studies #1 and #2, Fig. 6h, Supplementary Figs. 14 and 15), or subjected to phlebotomy (study #3, Supplementary Fig. 16). In studies #1 and #2, the effect of Epo on athletic performance was examined, and will be either reported elsewhere (study #1) or was previously reported (study #2). Here we present the detailed blood parameters associated with these studies.

In study #1 (Fig. 6h, Supplementary Fig. 14), baseline parameters were established during four weekly blood samplings, followed by injection with Epo (20 IU/kg every other day, 25 subjects) or placebo (9 subjects) for 3 weeks. On average, hemoglobin increased by 5% over baseline values in the Epo group during the treatment period. Blood sampling continued for an additional 5 weeks following cessation of treatment. In study #2 (Fig. 6h, Supplementary Fig. 15), baseline measurements were followed by weekly dosing with Epo (24 subjects) or placebo (24 subjects) for 7 weeks, with Epo dosing adjusted to achieve an increase of 10–15% in hemoglobin. Follow-up continued for a month after cessation of treatment. In study #3 (Supplementary Fig. 16), 21 subjects participated in a randomized double-blind placebo-controlled crossover study in which 900 ml of whole blood was withdrawn from the treatment group by venipuncture.

Subjects were then followed for 25 days. In all three studies, there was a significant increase in MCV in the treatment groups compared with baseline values and with the placebo group, which persisted well beyond the treatment period (Supplementary Figs. 14–16, Supplementary statistical analysis). There was no correlation between MCV and the reticulocyte count, whose time courses were clearly divergent (r < 0.1 between MCV and reticulocyte count in all three studies, Pearson’s product–moment correlation, Supplementary statistical analysis).

In studies #1 and #2, the reticulocyte count increased during Epo treatment, but declined sharply below baseline values as soon as Epo treatment ceased. By contrast, MCV values remained high (Fig. 6h). Similarly, in study #3, MCV values continued to climb at a time when the reticulocyte count was declining (Supplementary Fig. 16). Thus, the increase in MCV is not the result of an increase in the number of reticulocytes. Together with the increase in MCV, there was an increase in red-cell distribution width (RDW-SD, Fig. 6i, Supplementary Figs. 14 and 15; no RDW is available for study #3). There was a significant, positive correlation between MCV and RDW-SD (r = 0.51, p = 2 × 10−28 for study #1; r = 0.52, 2 × 10−24 for study #2).

Red-cell volume declines continuously as red cells age. Therefore, we considered the possibility that the persistently elevated MCV following Epo administration might be the result of the expected increase in the relative number of younger red cells, rather than an increase in their size. To address this, we simulated the expected increase in MCV that would arise only from an increase in the proportion of younger red cells, assuming no effect of Epo signaling on red-cell size (Supplementary analysis: ‘Simulation of MCV’). This simulation indicated that an increased proportion of younger red cells cannot fully account for the extent or duration of the observed increase in MCV following Epo administration, consistent with a direct role for Epo signaling in the regulation of cell size.

Discussion

Using a genetic model in which we provide Epor−/− erythroblasts with exogenous survival signaling, we identified novel non-redundant functions for EpoR during ETD. EpoR signaling determines the number and speed of cell divisions and duration of terminal differentiation. While it has little effect on the broad ETD transcriptional program, it drives the formation of qualitatively different, larger red cells. In wild-type erythroblasts, EpoR signaling increases cell size in an Epo dose-dependent manner at every stage of erythroid terminal differentiation (ETD), leading to the production of larger reticulocytes and RGs. Human intervention studies are consistent with a similar effect of EpoR signaling on red-cell size in human erythropoiesis. In the discussion below we integrate the apparently disparate EpoR functions into a coherent model (Fig. 7).

We also discuss previously unexplained instances of macrocytic and heterogeneously-sized red cells, now interpretable as the result of increased EpoR signaling during hypoxic stress.

The ETD is a time of rapid change in many aspects of the cell. Our results support a model in which ETD has two phases: an early, Epo-dependent phase, and an Epo-independent late phase (Fig. 7). EpoR expression peaks in early erythroblasts, which are highly dependent on EpoR signaling for survival and proliferation. By contrast, late erythroblasts downregulate EpoR and are relatively resistant to apoptosis. The functions we identified here for EpoR signaling in ETD are similarly focused on early erythroblasts. In addition to EpoR signaling, ETD is also supported by the erythropoietic island niche, an area that was not addressed in our model.

We identified five key non-survival functions of EpoR signaling, in Epor−/− and in wild-type erythroblasts, in vitro and in vivo: (1) EpoR prolongs ETD, as determined by delayed expression of Ter119 and delayed increase in nuclear offset; (2) it increases the number of cell cycles; (3) it skews the distribution of developing erythroblasts in favor of earlier erythroblasts; (4) it increases cell-cycle speed; and (5) it increases cell size throughout ETD, generating larger and more heterogeneous red cells. The prolongation of ETD is consistent with the increase in the number of cycles. Neither informs us directly regarding the stage(s) of ETD that are being prolonged. However, the skewed distribution in favor of early erythroblasts indicates, based on the ergodic principle, see the “Methods” section, that EpoR signaling prolongs early ETD relative to the late ETD phase. Together, these observations suggest that EpoR prolongs the early phase of ETD by increasing the number of early ETD cell cycles. This conclusion is consistent with our data, showing the largest differences in cell-cycle number in response to EpoR occur in the first 24 h of ETD, and with the known responsiveness of early ETD to EpoR signaling. In addition, it explains the observation that EpoR increases cell-cycle speed, since early ETD cell cycles are unusually fast, and much faster than cycles in late ETD. Our observations show that EpoR signaling regulates the speed of these unique cycles.

Therefore, of the five EpoR functions, the first four are outcomes of an EpoR-driven increase in the number and speed of early ETD cell cycles (Fig. 7). One of the factors known to regulate the onset of late ETD is p27KIP1, whose induction promotes slower cycling and cell-cycle exit. Here we found that EpoR signaling increases cell-cycle number by inhibiting p27KIP1 mRNA induction through the PI3K pathway, which was previously reported to also lead to p27KIP1 promotosomal degradation. A similar role for EpoR, delaying p27KIP1 induction and morphological maturation, was noted in primitive yolk sac erythroblasts. The converse was found in Klf1−/−/− erythroblasts, which fail to induce p27KIP1 and fail to undergo cell-cycle exit. Here we found that exogenous premature expression of p27KIP1 in wild-type erythroblasts reduced their cycling, but did not accelerate maturation, and like other factors that reduce cycling, resulted in larger erythroblasts. Therefore, the effects of EpoR signaling on erythroblast maturation rate and cell size are unrelated to its suppression of p27KIP1.
The most surprising of our findings was the effect of EpoR signaling on cell size. We found that erythroblasts differentiating in the absence of EpoR gave rise to smaller red cells, in spite of undergoing fewer cell cycles. Further, in wild-type fetal liver erythroblasts, cell size was sensitive to Epo concentration within the physiological and stress range. These findings appear contrary to the well-established link between the loss in cell size and the number of cell divisions during ETD. Thus, deletions of E2F4, cyclin D3, CDK2, or CDK4 each reduce the number of cell divisions during ETD and result in macrocytic red cells. Similarly, macrocytic red cells are seen when nucleotide pools limit DNA synthesis rate, as in patients treated with hydroxyurea, or in B12 or folate deficiencies. The EpoR effect on red-cell size was also independent of a second established pathway, in which red-cell size is regulated by iron status via HRI. Neither iron supplementation nor deletion of HRI corrected the cell size deficit of Epor−/− erythroblasts. While these experiments do not exclude an interaction between HRI and EpoR signaling, they show conclusively that EpoR stimulation of larger red-cell size is independent of HRI.

Our data therefore suggest that EpoR regulates red-cell size through a novel mechanism. The finding that the EpoR-driven increase in cell size begins in early erythroblasts suggests that it takes place in the very same cells in which EpoR signaling also induces additional rapid cycles. We propose that the well-established coupling of cell size loss with cell divisions is a default state, seen in cells where EpoR signaling is weak or absent. We further suggest that strong EpoR signaling, as may occur in early erythroblasts, can override this default state, simultaneously increasing rapid cycling while maintaining cell size. As consequence, high-Epo levels increase the duration of the early ETD phase, increase the relative frequency of early erythroblasts, and also increase erythroblast cell size at every maturation stage, giving rise to larger red cells. In high Epo, red-cell size is also more heterogeneous, a result of the varying sensitivities of early erythroblasts to Epo. Erythroblasts with low sensitivity to Epo, here represented as cells expressing low levels of EpoR, receive only weak EpoR signals even in the presence of high Epo, giving rise to smaller red cells.

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**Fig. 7 EpoR signaling promotes rapid cycling while maintaining cell size in early erythroblasts.** Proposed model explaining EpoR-dependent functions during ETD. EpoR expression is limited to early erythroblasts, which are sensitive to EpoR signaling. When EpoR signaling is weak or absent, as in late erythroblasts, or in early erythroblasts in the presence of low Epo, cell divisions lead to cell size reductions. In contrast, strong EpoR signaling, as seen in Epo-sensitive early erythroblasts, can override this default state, simultaneously increasing rapid cycling while maintaining cell size. As consequence, high-Epo levels increase the duration of the early ETD phase, increase the relative frequency of early erythroblasts, and also increase erythroblast cell size at every maturation stage, giving rise to larger red cells. In high Epo, red-cell size is also more heterogeneous, a result of the varying sensitivities of early erythroblasts to Epo. Erythroblasts with low sensitivity to Epo, here represented as cells expressing low levels of EpoR, receive only weak EpoR signals even in the presence of high Epo, giving rise to smaller red cells.
levels, which generate only weak signaling and therefore relatively uniform small cells. High-Epo levels might be expected to support the survival of erythroblasts with varying Epo sensitivities3,10, in which the strength of EpoR signaling may vary, giving rise to a range of red-cell sizes (Fig. 7).

The relationship between high MCV, high RDW, and high levels of Epo may have been overlooked previously by being attributed to an increase in reticulocytes. We have excluded this possibility, finding no correlation between reticulocyte numbers and MCV. We also found that the extent and duration of increase in MCV following Epo administration cannot be accounted for solely by the skewing in the age distribution of circulating red cells in favor of younger cells (see Supplementary Analysis: Simulation of MCV). Indeed, our mouse data show increased cell size throughout terminal differentiation, including larger than normal reticulocytes, and not simply more numerous reticulocytes.

Recent GWAS and other studies have linked multiple genomic loci to the regulation of MCV97−99. These include Epo, Epor, and Lnk, all expected to alter EpoR signaling strength91. An Epo-mediated increase in MCV in clinical settings might be tempered by iron status or by pathology affecting terminal differentiation. Nevertheless, our work predicts that in the absence of erythroid pathology or nutritional deficiencies, Epo levels might be a key determinant of MCV. Indeed, an increase in Epo might account for the unexplained macrocytosis in hypoxicemic patients with chronic obstructive pulmonary disease92,93 and in iron-replete pregnancy94,95. An increase in RDW was recently proposed as a potential longer-term biomarker for brief hypoxicemic episodes in conditions such as acute respiratory distress, sepsis, or congestive heart failure96,97. Indeed, clinically, the RDW may prove to be a more sensitive marker of Epo signaling than the MCV. The regulation of MCV by Epo also clarifies unexplained changes in red-cell volume associated with Kit function. Kit regulates the proliferation of early erythroid progenitors but is downregulated with entry into ETD. Gain of function Kit mutations in mice lead to erythrocytosis as a result of excess progenitors entering ETD; the red cells are microcytic98, presumably in response to a compensatory decrease in Epo. Conversely, loss of function Kit mutations are associated with an increased MCV, which is in proportion to the severity of anemia98,99 and can be now be explained by a paucity of progenitors entering ETD and the expected compensatory increase in Epo100. Transgenic expression of Epo rescues the lethal c-KitV/V mutation, also resulting in macrocytic red cells99. Given the persistence of higher MCV and RDW beyond the period in which Epo is elevated, these markers may be useful additions to a panel of diagnostic markers for detecting hypoxic stress in the clinic as well as Epo doping by athletes.

The adaptive value, if any, of a higher MCV in erythropoietic stress is not yet clear. Surprisingly, the increase in MCV in our human intervention studies was not associated with increased corpuscular hemoglobin (MCH). On the contrary, we found a statistically significant decrease in mean corpuscular hemoglobin concentration (MCHC) in both Epo intervention studies, though not in the phlebotomy intervention. Interestingly, a lower MCHC may enhance the action of 2, 3, diphosphoglycerate (2,3-DPG), an allosteric regulator that binds hemoglobin and lowers its affinity for oxygen. Red-cell 2,3-DPG increases in response to anemia or hypoxia, improving oxygen unloading in tissues101,102. The affinity of 2,3-DPG to hemoglobin increases significantly at lower MCHC103. A lower MCHC may therefore improve the 2,3-DPG-dependent unloading of oxygen. Indeed, a lower MCHC is also an HRI-regulated outcome characteristic of microcytic iron-deficiency anemia, possibly for similar reasons. The EpoR-regulated increase in MCV might therefore provide a mechanism for lowering MCHC and improving oxygen unloading in tissues during hypoxic stress.

**Methods**

**Explanation of the ergodic principle.** The ergodic principle can be applied in biology to a multi-stage process in the steady state (e.g., steady-state differentiation in tissue, or the cell cycle90,92). It suggests that in a snapshot in time of cells undergoing the process, the number of cells at each stage is inversely proportional to the length of time that cells spend at that stage. Hence, finding that a differentiation stage contains many cells suggests that cells spend a longer period of time in that stage; conversely, if a differentiation stage is sparsely populated, this would suggest that transit through that stage is fast. Therefore, as applied here, finding that EpoR signaling skews the erythroblast population in favor of early erythroblasts suggests that cells are spending proportionally more time in the early erythroblast stage.

**Mice.** Study Epo+/− mice were obtained from Dr. Lothar Hennighausen (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). EpoR−/− mice were obtained from the Lodish laboratory, Whitehead Institute for Biomedical Research, Cambridge, MA. Balb/C mice were obtained from the Charles River Laboratories, Wilmington, MA. The Epo/Saline injection experiment on adult mice was conducted on male C57BL6 fluorescence timer (FT) transgenic mice. Mice were housed at a dedicated facility, with regulated temperature (range 20−26 °C), a 12 h/12 h light/dark cycle, and 30−70% humidity. Mice were fed on Iso Pro 3000 irradiated rodent diet #5P76. All experiments were conducted in accordance with animal protocol A-1586 approved by the University of Massachusetts Chan Medical School Institutional Animal Care and Use Committee.

**Culture medium and growth factors.** Fetal liver cells were cultured in IMDM with added L-glutamine and 25 mM HEPEPS (Gibco), 1% fetal calf serum (HyClone), 1% penicillin/streptomycin (ThermoFisher Scientific), 2 × 10−4 M 2,3-DPG (Sigma), supplemented when indicated with 0.5 IU/ml Epo (Procr, Amgen; 1 IU/ml = 1 ng/ml and 100 ng/ml SCF (Peprotech), and 10 ng/ml IL3 (Peprotech).

**Isolation of mouse erythroid progenitors.** To isolate wild-type 50 cells, fetal liver cells were depleted of lineage-positive cells by labeling with biotin-conjugated CD71, Ter119, Gr1, Mac1, and CD41 antibodies followed by magnetic separation (Dynabeads; BioLegend) according to the manufacturers’ instructions.

**Flow cytometry.** Fetal liver cells were analyzed on LSRII (BD Biosciences) cytometers using DIVA software (BD Biosciences). Dead cells were excluded using either ExcSep beads (a StemCell Technologies) or MoJoSort™ Streptavidin Nanobeads (BioLegend) according to the manufacturers’ instructions.

**Antibodies used.** PE Mouse Anti-Human CD4 (RPA-T4) (BD Biosciences) dilution 1:50 PE/Cy7 Rat Anti-Human CD71 (RI7217) (BioLegend) dilution 1:100 APC/Cyanine7 Rat Anti-Mouse Ter119 (Tet199) (BioLegend) dilution 1:100 PE Rat Anti-Mouse Ter119 (Ter119) (BD Biosciences) dilution 1:100 APC Rat Anti-Mouse Ter119 (Tet199) (BD Biosciences) dilution 1:100 PE Rat Anti-Mouse CD71 (C2) (BD Biosciences) dilution 1:100 PE Rat Anti-Mouse Ly-6G and Ly-6C/Gr1 (RB6-8C5) (BD Biosciences) dilution 1:100 PE Rat Anti-Mouse CD11b/Mac1 (M1/70) (BD Biosciences) dilution 1:100 PE Rat Anti-Mouse CD41 (MWReg30) (Thermo Scientific) dilution 1:100 FITC Rat Anti-Mouse Ly-6G and Ly-6C/Gr1 (RB6-8C5) (BD Biosciences) dilution 1:100 FITC Rat Anti-Mouse CD11b/Mac1 (M1/70) (BD Biosciences) dilution 1:100 FITC Rat Anti-Mouse CD41 (MWReg30) (BD Biosciences) dilution 1:100 FITC Rat Anti-Mouse CD45/RB20 (RA3-6B2) (BD Biosciences) dilution 1:100 FITC Hamster Anti-Mouse CD3e (145-2C11) (BD Biosciences) dilution 1:100 PE Rat Anti-Mouse Ly-6G and Ly-6C/Gr1 (RB6-8C5) (BioLegend) dilution 1:100 PE Rat Anti-Mouse CD11b/Mac1 (M1/70) (BioLegend) dilution 1:100 PE Rat Anti-Mouse CD41 (MWReg30) (BD Biosciences) dilution 1:100 PE Rat Anti-Mouse CD45/RB20 (RA3-6B2) (BD Biosciences) dilution 1:100 PE Hamster Anti-Mouse CD3e (500A2) (BioLegend) dilution 1:100

**Imaging flow cytometry.** Imaging flow cytometry was used to analyze cell fluorescence in conjunction with morphological parameters. It was performed on an Amnis Flowcytometer (Luminex Corporation, TX) using INSPIRE software v6.5 (Luminex Corporation, TX). Live nuclear diameter was measured using the cell-permeable far-red fluorescent DNA dye, DRAQ5® (Cell Signaling). Amnis data was analyzed using IDEAS software v6.0 (Luminex Corporation, TX). New mask functions were generated to analyze bright-field cell area (Definition: Object (M01, Ch01, Tight)) as well as DRAQ5 fluorescence nuclear area (Definition:
Morphology (M11, Ch11)). Raw mean Draq5 pixel intensity feature was generated using Drag5 Morphology mask for nuclear area. Raw flow cytometric feature data were exported and analyzed in R programing language.

Calibration of nuclear and cell diameters measured by imaging flow cytometry. Imaging flow cytometry was performed on standardized bead sizes, 2.0µ, 3.4µ, 5.1µ, 7.4µ, 9.96µ, and 14.3µ (Spherotech Inc.). IDEAS bright-field cell area mask (Definition: Object(M01, Ch01, Tigh)) was fitted to the bead image acquisition. The data were analyzed using R, and within each bead group, values that lie greater or less than 3 standard deviations from the mean were removed (0.9% of events were removed with this threshold). To correct biases in the cell area values calculated by the Amnis software, we fit a linear model (polynomial curve) using the manufactured bead diameter as a predictor for the Amnis calculated cell area (Stats, base R, degree = 2), with an R² value of 0.97. This model was then used to predict cell diameters from experimental cell areas.

Analysis of imaging flow-cytometry data. Further analysis of exported imaging flow-cytometry data was done using RStudio Version 1.2.1335, RStudio, Inc. Population distributions were log normalized. Population dataset were filtered by removing outliers that are 3 or more standard deviations from the mean.

Fluorescence quantile analysis (Supplementary Fig. 4). Events whose cell areas were 3 standard deviations from the mean were removed. For CD4 and GFP intensities, quantiles were calculated across all samples using the quantile function (Stats, base R). To visualize the data, a density plot was drawn using ggplot2 (geometry density) and colors chosen from viridis. Each event was then categorized by which bin it fell into (for CD4 and GFP respectively). After this, each event had 2 associated values, which quantile of GFP and which quantile of CD4 that it belonged to. For each of the 3 samples (Bc-xL-GFP, V-CD4, V-GFP = EpoRCD4, and Bc-xL-GFP, V-CD4 = EpoRCD4 using V-GFP = EpoRCD4 as a reference. These data were plotted as a heatmap using ggplot2 and the geom_tile function. To show the distributions of cell diameters for all samples within each composite bin, example density plots were drawn using ggplot2 (geom_density). Example insets were colored by sample, and separate panels were drawn for each composite quantile bin.

Nuclear offset. The intensity weighted delta centroid XY feature was used to measure the distance between the centroid features of two images: CD71 fluorescence for the cell image and DRAQ5 fluorescence for the nucleus. To calculate the cell diameter, first the correlation between CD71 area feature and the bright-field-based area feature was obtained by plotting both values for each event. This allowed us to assign a bright-field area value to each event based on the CD71 area, and then use this value, in combination with the bead calibration curve (see “Calibration of nuclear and cell diameters”) above, to calculate cell diameter. Nuclear offset was then calculated by dividing the delta centroid by cell diameter.

Identification of enucleated reticulocytes. Cells were selected by gating on focused, single cell, live, lineage (Gr1, Mac1, CD41, B220, CD3e) negative, hCD4 and GFP positive and Ter119 (BD Biosciences 553673). BrdU cell diameter, then use this value, in combination with the bead calibration curve (see Calibration of nuclear and cell diameters) above, to calculate cell diameter. Nuclear offset was then calculated by dividing the delta centroid by cell diameter.

Cytospins. Cells were spun onto coated Shandon™ Cytodiscs (Thermo Scientific) using a Shandon™ Cytospin at 800 rpm for 5 minutes. The slides were dried, fixed and stained. Cytospin preparations were examined using a Zeiss Axioskop 40 microscope using a SPOT Flex Camera (Diagnostic Instruments, Inc.) and imaged using SPOT v.5.6 software (SPOT Imaging).

Cell-cycle analysis. Cell-cycle status and S phase speed were analyzed using BrdU incorporation. Briefly, cells were pulsed at a final concentration of 33 μM BrdU for 30 min. Cells were immediately labeled with the LIVE/DEAD Kit (Invitrogen L25105), fixed, and permeabilized. Erthyroid subsets were identified using anti-CD71 (BD Biosciences 113812) and anti-Ter119 (BD Biosciences 553673). BrdU incorporation was measured by biotin-conjugated anti-BrdU (MOBU-1, Biolegend) followed by a secondary stain with Brilliant Violet 421™ Streptavidin (BioLegend). DNA content was measured by 7AAD (BD Biosciences).

Retroviral Transduction and in vitro differentiation of fetal liver cells. Epor, Bc-xL, and Tfrc were subcloned into MSCV-IRES-hCD4 retroviral vector. Bc-xL and Tfrc were also subcloned into MSIG-IRES-GFP retroviral vector (MSIG 1.1 SK). High-titer viral supernatants were prepared by co-transfecting the pCL-Eco packaging vector and desired plasmid into Phoenix cells using Lipofectamine 2000 transfection reagent (THERMOFISHER SCIENTIFIC). High-titer virus was collected in 'erythroid medium': IMDM (L-glutamine, 25 mM HEPES) (Gibco), 20% fetal calf serum, 1% penicillin/streptomycin, 10-5 M β-Mercaptoethanol.

Retroviral transduction was done by spin infection of EpoR/-/- or Epor+/- Hrt+/- fetal liver cells at 2000 rpm, 30 °C for 1 h on 10 µg/ml fibronectin (GIBCO) coated dishes in 4 µg/ml polybrene (Sigma), supplemented in some experiments with 0.5 µM Epo (Amgen). Transduced cells were incubated for 15 h with 100 ng/ml SCF and 10 ng/ml IL3 (Peprotech). Cells were then transferred to differentiation medium: IMDM (L-glutamine, 25 mM HEPES) (Gibco), 20% fetal calf serum, 1% penicillin/streptomycin, 10-5 M β-Mercaptoethanol, and 0.5 µM Epo (Amgen) for the indicated times. In the case of experiments that include EpoR/-/- or EpoR+/-, the media was also supplemented with 1 mg/ml iron-saturated human transferrin (Sigma). Diverse subsets were also supplemented with Fe-loaded salicylaldehyde isonicotinoyl hydrazone (Fe-SIH, 10 µM), a lipophilic iron chelator, a gift from the late Dr. Prem Ponka (McGill University, Montréal, Québec, Canada), with 0.7 µM deoxoerythronucleosides (3'-Deoxythymidine, 2'-Deoxoyguanosine monohydrate, 2'-Deoxyadenosine monohydrate, 2'-Deoxythymidine, Sigma).

In vitro differentiation of fetal liver cells with PI3K and MEK1/MEK2 inhibitors. Isolated wild-type 50 cells were cultured in differentiation media (Epo 0.5 µ/ ml) with 1 µM or 10 µM PI3K inhibitor, LY294002 (EMD Millipore) or MEK1/ MEK2 inhibitor, U0126 (EMD Millipore). Inhibitors were replenished every 24 h.

Colony-formation assays in methyleneblue. Retroviral transduction was done by spin infection of EpoR+/- fetal liver cells as described above. From each transduced sample (4 h post infection), 200,000 cells were mixed with 1 ml Metho Cult (M3254, STEMCELL TECHNOLOGIES) supplemented with 2 µM Epo (Amgen) and 1 µg/ml Methyleneblue (CFU-e) for 2 hours. Colonies were then stained with crystal violet and counted manually.

Expression of hemoglobin in erythroid colonies was confirmed by staining with diamino benzidine (Sigma) in situ before scoring. Colony area was measured using ImageJ version: 2.0.0-r-54/1.51h.

Quantitative RT-PCR assay. Total RNA was isolated from in vitro cultured fetal liver cells using the AllPrep DNA/RNA Micro Kit (Qiagen) and quantified by Quant-iT Ribogreen RNA reagent kit (Thermo Scientific) on the 3300 NanoDrop Fluorospectrometer. Reverse transcription was done using the SuperScript III first-strand synthesis system (Invitrogen) with random hexamer primers. Quantitative PCR was performed using the ABI 7300 sequence detection system with TaqMan reagents and TaqMan MGB probes (Applied Biosystems). Each reaction was carried out on a dilution series of the template cDNA to ensure linearity of signal. 

In vitro differentiation of fetal liver cells with PI3K and MEK1/MEK2 inhibitors. Isolated wild-type 50 cells were cultured in differentiation media (Epo 0.5 µ/ ml) with 1 µM or 10 µM PI3K inhibitor, LY294002 (EMD Millipore) or MEK1/ MEK2 inhibitor, U0126 (EMD Millipore). Inhibitors were replenished every 24 h.

Human intervention studies. Human intervention studies 1 and 3 were performed at the University of Copenhagen. In intervention study 1, subjects received recombinant human erythropoietin (rhEPO). In intervention study 3, participants were subjected to phlebotomy. Thirty-four healthy non-smoking males (n = 19) and females (n = 15) of European descent (age 29 ± 6 years, 184 ± 7 cm, and 77 ± 8 kg) participated in the erythropoietin treatment intervention. Thirty-two healthy non-smoking males (n = 25) received Epo, n = 9 received Placebo. Another 21 healthy non-smoking male subjects of European descent (age 29 ± 6 years, 184 ± 7 cm, and 77 ± 8 kg) participated in the phlebotomy intervention. No participant had donated blood for at least three months prior to the start of the study or been exposed to high altitude (<1000 m) for at least two months.

The human studies were conducted in Copenhagen, Denmark according to all applicable national and international rules and regulations including the Helsinki II declaration. Ethics approval for the studies (protocol numbers H-2-2014-109 and H-17036662, enclosed with the Supplementary files information; registration numbers: Efaces registration H2-2015-27765 for Study #1) were granted by the Regional Branch (Copenhagen Region) of the Danish National Committee on Health Research Ethics (https://en.nvk.dk/). Both studies aimed to identify novel
biodemakers following either phlebotomy (Study #3) or Epo administration (Study #1). All participants were informed both orally and in writing of potential risks and discomforts associated with participation before written consent was obtained. Participants were compensated for their participation (Study #1: sports equipment equivalent to ~5500 Danish kroner; Study #2, 5000 Danish kroner). Participants were recruited via advertising on social media, dedicated web-pages, and flyers. There is a potential selection bias toward healthier than average participants since the studies examined the effect of Epo on athletic performance. This appears unlikely to influence the results.

**Experimental design.** rEPO treatment: the study was used a randomized single-blinded placebo-controlled design. After weekly baseline collection of venous blood for 4 weeks, the participants received eleven intravenous injections of 20 IU·kg bw placebo-controlled design. After weekly baseline collection of venous blood for 5 weeks following treatment.

**Phlebotomy:** The intervention applied a randomized single-blinded placebo-controlled crossover design. The week before phlebotomy, two baseline venous blood samples were collected with 4 days apart. Next, the participants were phlebotomized of two whole-blood units, corresponding to 900 ml or sham-phlebotomized followed by venous blood collection 3, 14, and 25 days later. A recovery period of ~4 months was applied before the participants crossed over and repeated the experiment.

**Blood sample analysis.** All venous blood samples were collected in 2 ml EDTA-anticoagulated vacutainers (Becton Dickinson, New Jersey, USA) after at least 10 min of rest in a seated position and with <30 s use of tourniquet. In the rEPO trial, samples were analyzed for a complete blood count and a Sysmex NX-450 (Sysmex, Kobe, Japan) including mean cell volume, hemoglobin concentration, reticulocyte count, reticulocyte percentage, and red-cell distribution width. In the phlebotomy trial, samples were stored at 4 °C and analyzed within 2 h of collection for mean cell volume, hemoglobin concentration, reticulocyte count, and reticulocyte percentage using a Sysmex XE-2100 (Sysmex, Kobe, Japan).

Human intervention study 2 was performed at the Centre for Human Drug Research, Leiden, Netherlands. This study was reported elsewhere110, but reporting did not include MCV and RDW information. Briefly, non-professional well-trained male cyclists ages 28–30 were randomly assigned to placebo or recombinant human Epo (epoetin β) groups. Baseline measurements were followed by weekly dosing with Epo (24 subjects) or placebo (24 subjects) for 7 weeks. Epo dosing (5000–10,000 IU) was adjusted for each subject, to achieve an increase of 10–15% in hemoglobin over baseline. Follow-up continued for a month after cessation of treatment.

**Statistics.** For the human studies, we computed baseline-corrected values at each post-baseline time point for each subject by subtracting the corresponding subject-level mean baseline measurement, which was used to fit linear mixed-effect models using the nlme package112. For intervention studies 1 and 2, the model includes subject as random effect, treatment, time, and the interaction of treatment by time as fixed effects. To test whether Epo treatment and placebo differ significantly at each post-baseline time point, a set of pre-defined contrasts were performed using the multcomp package113 followed by multiplicity adjustment using Benjamini–Hochberg procedure114. For intervention study 3, each post-baseline time point was analyzed separately with the model that includes subject as random effect, treatment, time, and the interaction of treatment by time as fixed effects. See Supplementary Information.

For mouse and in vitro experiments, we used both parametric and non-parametric statistical significance tests for sample comparisons as indicated in each figure legend.

**Data availability**

Complete blood-count source data for the human studies are provided in the Supplementary statistical analysis of human intervention studies’ in the Supplementary Information file. Additional flow-cytometry data is available upon request. Source data are provided with this paper.

**Code availability**

The Supplementary MCV simulation python script is deposited in https://github.com/socolov/Simulation-of-MCV/.

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**References**

1. Kassebaum, N. J. et al. A systematic analysis of global anemia burden from 1990 to 2010. Blood 123, 615–624 (2014).
2. D’Andrea, A. D., Fasman, G. D. & Lodish, H. F. Erythropoietin receptor and interleukin-2 receptor b chain: a new receptor family. Cell 58, 1023–1024 (1989).
3. Stephenson, J. R., Axelrad, A. A., McLeod, D. L. & Shreve, M. M. Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc. Natl. Acad. Sci. USA 68, 1542–1546 (1971).
4. Tusi, B. K. et al. Population snapshots predict early haematopoietic and erythroid hierarchies. Nature 555, 54–60 (2018).
5. Wu, H., Liu, X., Jaenisch, R. & Lodish, H. F. Generation of committed erythroblast lineage progenitors does not require erythropoietin or the erythropoietin receptor. Cell 59, 59–67 (1994).
6. Koury, M. J., Bondurant, M. C., Graber, S. E. & Sawyer, S. T. Erythropoietin messenger RNA levels in developing mice and transfer of 125I-erythropoietin by the placenta. J. Clin. Invest. 82, 154–159 (1988).
7. Koury, M. J. & Bondurant, M. C. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 248, 378–381 (1990).
8. Koury, M. J. & Bondurant, M. C. The molecular mechanism of erythropoietin action. Eur. J. Biochem. 210, 649–663 (1992).
9. Koulmis, M., Poprigila, E., Hidalgo, D. & Socolovsky, M. In A Systems Biology Approach to Blood Vol. 844 (eds Corey, S. J., Kimmel, M. & Leonard, J. N.) 37–58 (Springer New York, 2014).
10. Wickrema, A., Bondurant, M. C. & Kranta, S. B. Abundance and stability of erythropoietin receptor mRNA in mouse erythroid progenitor cells. Blood 78, 2269–2275 (1991).
11. Broudy, V. C., Lin, N., Brice, M., Nakamoto, B. & Papayannopoulou, T. Erythropoietin receptor characteristics on primary human erythroid cells. Blood 77, 2583–2590 (1991).
12. Zhang, J., Socolovsky, M., Gross, A. W. & Lodish, H. F. Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. Blood 102, 3938–3946 (2003).
13. Kieran, M. W., Perkins, A., Orkin, S. Z. & Zon, L. Thrombopoietin-responses in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. Proc. Natl. Acad. Sci. USA 93, 9126–9131 (1996).
14. Lin, C. S., Lim, S. K., D’Agati, V. & Costantini, F. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. Genes Dev. 10, 154–164 (1996).
15. Iscove, N. N. The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. Cell Tissue Kinet. 10, 323–334 (1977).
16. Fang, J. et al. EPO modulation of cell-cycle regulatory genes, and cell division, in primary bone marrow erythroblasts. Blood 110, 2361–2370 (2007).
17. Ferro, F. J., Kozak, S. L., Hoatlin, M. E. & Kabat, D. Cell surface site for mitogenic interaction of erythropoietin receptors with the membrane glycoprotein encoded by Friend erythroleukemia virus. J. Biol. Chem. 268, 5741–5747 (1993).
18. Spivak, J. L. et al. Cell cycle-specific behavior of erythropoietin. Exp. Hematol. 24, 141–150 (1996).
19. von Lindern, M. et al. The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro. Blood 94, 550–559 (1999).
20. Malik, J., Kim, A. R., Tyre, K. A., Cherukuri, A. R. & Palis, J. Erythropoietin critically regulates the terminal maturation of murine and human primitive erythroblasts. Haematologica 98, 1778–1787 (2013).
21. Socolovsky, M., Dusanser-Fourt, I. & Lodish, H. F. The Prolactin receptor, as well as severely truncated erythropoietin receptors support differentiation of erythroid progenitors. J. Biol. Chem. 272, 14009–14013 (1997).
22. Socolovsky, M., Fallon, A. E. J. & Lodish, H. F. The prolactin receptor rescues EpoR−/− erythroid progenitors and replaces EpoR in a synergistic interaction with c-kit. Blood 92, 1491–1496 (1998).
23. Socolovsky, M., Lodish, H. F. & Daley, G. Q. Control of hematopoietic differentiation: lack of specificity in signaling by cytokine receptors. Proc. Natl. Acad. Sci. USA 95, 6573–6575 (1998).
24. Briskien, C., Socolovsky, M., Lodish, H. F. & Weinberg, R. The signaling domain of the erythropoietin receptor rescues prolactin receptor-mutant mammary epithelium. PNAS 99, 14241–14245 (2002).
25. Kadri, Z. et al. Phosphatidylinositol 3-kinase/Akt induced by erythropoietin renders the erythroid differentiation factor GATA-1 competent for TIMP-1 gene transcription. Mol. Cell Biol. 25, 7412–7422 (2005).
26. Hwang, Y. et al. Global increase in replication fork speed during a p57KIP2-regulated erythroblast cell fate switch. Sci. Adv. 3, e1700298 (2017).
27. Eastman, A. E. et al. Resolving cell cycle speed in one snapshot with a live-cell fluorescent reporter. Cell Rep. 31, 107840 (2020).
Hwang, Y., Hidalgo, D. & Socolovsky, M. The shifting shape and functional specializations of the cell cycle during lineage development. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 13, e1504 (2020).

Humbert, P. O. et al. E2F4 is essential for normal erythroid maturation and neonatal survival. *Mol. Cell* 6, 281–291 (2000).

Sankaran, V. G. et al. Cyclin D3 coordinates the cell cycle during differentiation to regulate erythrocyte size and number. *Genes Dev.* 26, 1725–1737 (2012).

Jayapal, S. R. et al. Hematopoiesis specific loss of Cdk2 and Cdk4 results in increased erythrocyte size and delayed platelet recovery following stress. *Haematologica* 100, 431–438 (2015).

Burns, E. R., Reed, L. J. & Wenz, B. Volumetric erythrocyte macrocytosis induced by hydroxyurea. *Am. J. Clin. Pathol.* 85, 337–341 (1986).

Suragani, R. N. et al. Heme-regulated eIF2alpha kinase activated Atf4 signaling pathway in oxidative stress and erythropoiesis. *Blood* 119, 5276–5284 (2012).

Chen, K. et al. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. *Nat. Rev. Syst. Biol.* 20, 725–735 (2015).

Hsieh, F. F. et al. Cell cycle exit during terminal erythrocyte differentiation is associated with accumulation of p27(Kip1) and inactivation of cdk2 kinase. *Blood* 96, 2746–2754 (2000).

Bylski, M. et al. GATA1-mediated proliferation arrest during erythroid maturation. *Mol. Cell. Biol.* 23, 5031–5042 (2003).

Bouscary, D. et al. Critical role for PI 3-kine in the control of erythropoietin-induced erythroid proliferator proliferation. *Blood* 101, 3436–3443 (2003).

Gnanapragasam, M. N. et al. EKLF/ELF1-regulated cell cycle exit is essential for erythroblast enucleation. *Blood* 128, 1631–1641 (2016).

Vlahos, C. J., Matter, W. F., Hui, K. Y. & Brown, R. F. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-

- benzopyran-4-one (LY294002). *Blood* 99, 5241–5248 (1994).

Porpiglia, E., Hidalgo, D., Koulmis, M., Tafazrifi, A. R. & Socolovsky, M. Stat5 signaling specifies basal versus stress erythropoietic responses through distinct and graded binary dimensions. *PLoS Biol.* 10, e1001383 (2012).

Khaled, A. R. et al. Cytokine-driven cell cycling is mediated through Cdc25A. *Proc. Natl. Acad. Sci. USA* 104, 1228–1235 (2007).

Gifford, S. C., Derganc, J., Shevkoplyas, S. S., Yoshida, T. & Bitensky, M. W. A role for Erk2 in erythroid maturation in vivo. *Blood* 119, 2288–2295 (2012).

Pop, R. et al. A key commitment step in erythropoiesis is synchronized with the cell cycle clock through mutual inhibition between PU.1 and S-phase progression. *PLoS Biol.* 8, e1000484 (2010).

von Lindern, M., Schmidt, U. & Beug, H. Control of erythropoiesis by erythropoietin and stem cell factor: a novel role for Bruton’s tyrosine kinase. *Cell Cycle* 3, 876–879 (2004).

Umemura, T., al-Khatti, A., Donahue, R. E., Papayannopoulou, T. & Stamatoyannopoulos, G. Effects of interleukin-3 and erythropoietin on in vivo erythropoiesis and F-cell formation in primates. *Blood* 74, 1571–1576 (1989).

Garrick, L. M. et al. Ferric-salicyldihydroxyisonicotinyl hydroxyl, a synthetic iron chelate, alleviates defective iron utilization by reticulocytes of the belgrade rat. *J. Cell Physiol.* 146, 460–465 (1991).

Nyholt, S. et al. Role of ribonucleotide reductase in inhibition of mammalian cell growth by potent iron chelators. *J. Biol. Chem.* 268, 26200–26205 (1993).

Eriksson, S., Munck-Petersen, B., Johansson, K. & Ecklund, H. Structure and function of cellular deoxuryridinucleoside kinases. *Cell. Mol. Life Sci. CMLS* 59, 113–123 (2002).

Zhu, L. & Skoultchi, A. I. Coordinating cell proliferation and differentiation. *Curr. Opin. Genet Dev.* 9, 11–17 (1999).

Daft, B. J. & Wojcikiewicz, D. M. Emerging EPO and EPO receptor regulators and signal transducers. *Blood* 125, 3536–3541 (2015).

Lodish, H. F., Ghaffari, S., Socolovsky, M., Tong, W. & Zhang, J. In *Erythropoietins, Erythropoietic Factors, and Erythropoiesis: Molecular, Cellular, Preclinical, and Clinical Biology* (eds. Elliott, S. G., Foote, M. & Molineux, G.) 155–174 (Birkhäuser, Basel, 2009).

Socolovsky, M. et al. Ineffective erythropoiesis in Stat5a–/– mice due to decreased survival of early erythroblasts. *Blood* 98, 3261–3273 (2001).

Favata, M. F. et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273, 18623–18632 (1998).

Heuberger, J. et al. Effects of erythropoietin on cycling performance of well trained cyclic: a double-blind, randomised, placebo-controlled trial. *Lancet Haematol.* 4, e374–e386 (2017).

Bosch, F. H. et al. Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll separation. *Blood* 79, 254–260 (1992).

Willekens, F. L. et al. Hemoglobin loss from erythrocytes in vivo results from spleen-facilitated vesiculation. *Blood* 101, 747–751 (2003).

Gifford, S. C., Derganc, J., Shevkoplyas, S. S., Yoshida, T. & Bitskeny, M. W. A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence. *Br. J. Haematol.* 135, 494–404 (2006).

Franco, R. S. et al. Changes in the properties of normal human red blood cells during in vivo aging. *Am. J. Hematol.* 88, 44–51 (2013).

d’Onofrio, G. et al. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. *Blood* 85, 818–823 (1995).

Socolovsky, M. et al. Negative autoregulation by FAS mediates robust fetal erythropoiesis. *PLoS Biol.* 5, e232 (2007).

Reichlin, S. Making sense of snapshot data: ergodic principle for clonal cell populations. *J. R. Soc. Interface* 14, 20170467 (2017).

Shearstone, J. R. et al. Global DNA demethylation during mouse erythropoiesis in vivo. *Science* 334, 799–802 (2011).

Panzenböck, B., Bartunek, P., Mapara, M. Y. & Zenke, M. Growth and differentiation of human stem cell factor/erythropoietin-dependent erythroid progenitor cells in vitro. *Blood* 92, 3658–3668 (1998).

Gnanapragasam, M. N. & Bieker, J. I. Orchestration of late events in erythropoiesis by KLF1/EKLF. *Curr. Opin. Hematol.* 24, 183–190 (2017).

Ginzberg, M. B., Kafri, R. & Kirschen, M. On being the right (cell) size. *Science* 348, 1245075 (2015).

Björklund, M. Cell size homeostasis: Metabolic control of growth and cell division. *Biochimica et Biophysica Acta (BBA) - Mol. Cell Res.* 1866, 409–417 (2019).

Dolzinger, M., Greißen, F., Sauer, T., Beug, H. & Müllner, E. W. Evidence for a size-sensing mechanism in animal cells. *Nat. Cell Biol.* 6, 899–905 (2004).

Narla, A. & Ebert, B. L. Ribosomopathies: human disorders of ribosome dysfunction. *Blood* 115, 3196–3205 (2010).

Kelley, L. P. et al. Survival or death of individual proerythroblasts results from differing erythropoietin sensitivities: a mechanism for controlled rates of erythrocyte production. *Blood* 82, 2340–2352 (1993).

Ludwig, L. S. et al. Transcriptional states and chromatin accessibility underlying human erythropoiesis. *Cell Rep.* 27, 3228–3240.e3227 (2019).
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Author contributions

M.S. conceived, designed, and supervised all of the mouse-based experiments and wrote the Supplementary MCV simulation script. N.B.N. conceived, designed, and supervised human studies #1 and #2. J.J.C. designed and performed human studies #1 and #3. J.A.A.C.H. designed and performed human studies and R.G. contributed analysis and code for the mouse experiments. J.B. designed and performed human studies #1 and #3. A.E.E. conceived, designed and supervised all of the mouse-based experiments and wrote the Supplementary MCV simulation script. All of the authors contributed to manuscript preparation.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information

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Correspondence

Correspondence and requests for materials should be addressed to Nikolai Baastrup Nordsborg or Merav Socolovsky.

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