NOVEL FUNCTIONS OF ERYTHROPOIETIN RECEPTOR SIGNALING

A Dissertation Presented

By

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Cancer Biology Program
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Abstract

Erythroid terminal differentiation 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. I used $Epor^{-/-}$ mouse erythroblasts endowed with survival signaling to identify novel non-redundant EpoR functions. I found that, paradoxically, EpoR signaling increases red cell size while also increasing the number and speed of erythroblast cell cycles. Specifically, I found that high levels of EpoR signaling increase the size and shorten the cycle of early erythroblasts, which are amongst the fastest cycling somatic cells. I confirmed the effect of erythropoietin (Epo) on red cell size in human volunteers, whose mean corpuscular volume (MCV) increases following Epo administration. Our work shows that EpoR signaling alters the expected inverse relationship between cell cycle length and cell size. Further, diagnostic interpretations of increased MCV should now include high Epo levels and hypoxic stress.

The ability of EpoR signaling to increase cell size in rapidly cycling early erythroblasts suggests that these cells have exceptionally efficient EpoR-driven mechanisms for growth. I found evidence for this in ongoing work, where $Epor^{-/-}$ and $Stat5^{-/-}$ single-cell transcriptomes show dysregulated expression of ribosomal proteins and rRNA transcription and processing genes. Global rates of ribosomal rRNA transcription and protein synthesis increase in an EpoR-
dependent manner during a narrow developmental window in early ETD, coincident with the time of cell cycle shortening. Our work therefore suggests EpoR-driven regulation of ribosome biogenesis and translation orchestrating rapid cycling and cell growth during early ETD.
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Preface

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CHAPTER I: INTRODUCTION

The Mammalian Erythropoiesis.

Red blood cells (RBCs) or erythrocytes, are the most abundant cells in the body, transporting oxygen from the lung or placenta to body tissues (Kuhn, Diederich et al. 2017). The volume of packed RBC as a fraction of blood volume is known as the hematocrit, and in a healthy human and mice at sea level accounts for approximately half of all blood volume. RBCs circulate for 100-120 days in humans and for 45 days in mice before undergoing senescence (Koury 2014). Mammalian erythropoiesis is the process of RBC formation from hematopoietic stem cells (HSCs) through erythroid progenitor and precursor intermediates. The rate of RBC production is stable throughout life, mainly replenishing senescent RBCs but also can be dynamic to provide an immediate response to stress conditions such as hypoxia (Koulnis, Porpiglia et al. 2014). Abnormal erythropoiesis may be the result of nutritional deficiencies, genetic disorders or infections and may result in anemia. Malignant disorders such as myelodysplastic syndrome or myeloproliferative diseases can also present with anemia (Koury 2014).

During development, there are two distinct lineages of mammalian erythropoiesis: primitive erythropoiesis and definitive (adult-type) erythropoiesis. Primitive erythropoiesis is the formation of nucleated erythrocytes in the extra-embryonic yolk sac, which populate the blood during the initial six weeks of gestation in humans, or in the initial 12 days of gestation in mice (Palis and Segel
Primitive erythropoiesis is replaced by definitive erythropoiesis during the embryonic stage (Palis, Robertson et al. 1999). Definitive erythropoiesis originates from stem and progenitor cells in the walls of the great arteries in the aorta/gonad/mesonephros region (the AGM region), which migrate to the fetal liver where they undergo expansion. This lineage gives rise to enucleated erythrocytes, initially in the fetal liver during mid-gestation and later in the postnatal bone marrow and spleen (Palis and Segel 1998, Wolber, Leonard et al. 2002, Yumine, Fraser et al. 2017).

Definitive erythroid differentiation has two broad phases, termed early erythropoiesis and erythroid terminal differentiation (ETD) (Tusi, Wolock et al. 2018). During early erythropoiesis, HSCs give rise to early- and late-burst-forming-unit erythroid (BFU-e) progenitors, which differentiate into colony-forming-unit erythroid (CFU-e) progenitors (Gregory and Eaves 1977, Gregory and Eaves 1978). These early erythroid progenitors traditionally have been identified by their in vitro colony-forming potential in semi-solid medium (Stephenson, Axelrad et al. 1971, Gregory and Eaves 1977, Gregory and Eaves 1978). CFU-e progenitors are capable of extensive self-renewal cell divisions prior to transitioning to the ETD stage (von Lindern, Zauner et al. 1999, England, McGrath et al. 2011). During ETD, erythroblasts activate the terminal transcriptional program including expression of hemoglobin. They undergo 3–5 maturational cell divisions that are accompanied by cell size loss, and then enucleate to form reticulocytes. Reticulocytes lose intracellular organelles and
undergo further morphological change to form biconcave RBCs (Moras, Lefevre et al. 2017).

**Studying Mammalian Erythropoiesis.**

Mammalian erythropoiesis is a dynamic, complex, multi-step developmental process (Orkin and Zon 2008). Several mammalian and non-mammalian model organisms and culture systems have been used to study erythropoiesis, including mice, zebra fish, xenopus, avian models, and *in vitro* differentiation cultures of human embryonic stem cells (HuESCs) (Tsiftsoglou, Vizirianakis et al. 2009).

Classic methods for identifying early erythroid progenitors involve colony-formation potential in semi-solid medium. Early erythroid progenitors have been staged as either BFU-e or CFU-e. Mouse BFU-e progenitors have a high proliferative potential and can differentiate to form 64 to 1000 RBCs in “burst-like” colonies within 4 to 10 days in culture (Axelrad, McLeod et al. 1974). Mouse CFU-e progenitors have a lower proliferative capacity, forming colonies of 8 to 32 RBCs in 48 to 72 hours in culture (Stephenson, Axelrad et al. 1971). Erythroid precursors (also known as erythroblasts or normoblasts) are morphologically identifiable, and were therefore defined based on morphological criteria. These include cell size, which decreases with erythroblast maturation, basophilic staining in early erythroblasts (the result of high levels of transcription and translation), mixed coloration or ‘polychromatic’ later erythroblasts (a result of
both basophilic staining and increasing hemoglobin expression) and ‘orthochromatic’ or uniform staining of late erythroblasts (a result of high hemoglobin concentration and loss of cellular ribosomes and RNA). Erythroblast maturation also entails gradual nuclear condensation.

Traditional colony-formation assays and morphological criteria cannot be used to isolate erythroid progenitors and precursors prospectively, and therefore do not allow for differentiation-stage-specific molecular analysis. To address this limitation, flow cytometric approaches were developed by our laboratory and by others. These approaches distinguish sequential developmental stages of mouse erythroblasts during ETD (Socolovsky, Nam et al. 2001, Zhang, Socolovsky et al. 2003, Liu, Pop et al. 2006, Chen, Liu et al. 2009, Pop, Shearstone et al. 2010). The first such approach used two cell surface markers, the transferrin receptor (CD71), which is rapidly upregulated at the onset of ETD and downregulated in late ETD; and the erythroid-specific epitope Ter119, upregulated in CD71-high erythroblasts during ETD. These were combined with the forward scatter (FSC) parameter, which indicates cell size, to provide a snap shot in vivo of the ETD developmental process (Socolovsky, Nam et al. 2001, Zhang, Socolovsky et al. 2003, Liu, Pop et al. 2006, Pop, Shearstone et al. 2010, Koulnis, Pop et al. 2011). An alternative labeling scheme makes use of CD44 in place of CD71, retaining the FSC and Ter119 parameters (Chen, Liu et al. 2009). Dual staining with CD71 and Ter119 in the murine fetal liver allows for visualization of sequential erythroid differentiation stages termed S0 (least mature) to S5 (most
mature) (Fig. 1.1). The upregulation of CD71 from medium to high levels in Ter119-negative cells defines the transition from flow cytometric subset S0 \((CD71^{\text{low/med}}\text{-Ter}119^{-})\) to flow cytometric subset S1 \((CD71^{\text{high}}\text{-Ter}119^{-})\). The S0 subset is heterogeneous, containing HSCs, multipotential progenitors as well as BFU-e and CFU-e progenitors. The transition from S0 to S1 marks the commitment to ETD, is S phase-dependent, and takes place during S phase of the last CFU-e generation (Pop, Shearstone et al. 2010). The subsequent transition from S1 to S2 is defined by the upregulation of Ter119; The remaining subsets (S3-S5) contain increasingly more mature erythroid precursors completing ETD (Pop, Shearstone et al. 2010). In adult bone marrow and spleen, erythroid precursors (ProE [least mature], EryA, EryB, and EryC [most mature]) can be similarly identified and isolated based on expression of CD71, Ter119 and FSC (Fig. 1.2)(Liu, Pop et al. 2006, Koulnis, Pop et al. 2011).
Figure 1.1. Flow cytometric analysis of mammalian definitive erythropoiesis.

E14.5 fetal liver cells were labeled for CD71 and Ter119; subsets S0 (early progenitors) to S5 (late precursors) form an erythroid developmental sequence. Cytospin preparations with Giemsa and diaminobenzidine staining from each developmental subset (right panel).
Figure 1.2. Flow cytometric identification of erythroid precursors.

Gating strategy: Spleen cells were processed and labeled with antibodies directed at CD71, Ter119. The ProE gate contains CD71\textsuperscript{high}Ter119\textsubscript{intermediate} cells. Ter119\textsuperscript{high} cells are further analyzed; here, CD71\textsuperscript{high} cells are subdivided into less mature, large ‘EryA’ erythroblasts (CD71\textsuperscript{high}Ter119\textsuperscript{high}FSC\textsuperscript{high}) and smaller, more mature ‘EryB’ erythroblasts (CD71\textsuperscript{high}Ter119\textsuperscript{high}FSC\textsuperscript{low}). The most mature erythroblast subset is ‘EryC’ (CD71\textsuperscript{low}Ter119\textsuperscript{high}FSC\textsuperscript{low}).
The advent of imaging flow cytometry has allowed combined cell-surface marker analysis of ETD with a quantitative analysis of traditional morphological criteria, including cell size, nuclear size and eccentric positioning of the nucleus as erythroblast mature (Kalfa and McGrath 2018).

More recently, our laboratory used single cell RNA-sequencing (scRNA-seq) data to develop a flow cytometric approach that allows prospective isolation of early erythroid progenitors, the BFU-e and CFU-e, from mouse adult bone marrow, using the cell surface markers Kit, CD55, CD105, CD49f, CD41, CD71, and CD150 (Fig. 1.3) (Tusi, Wolock et al. 2018).
Figure 1.3. A novel flow cytometric labeling scheme isolates erythroid progenitors in adult bone marrow.

Kit+CD55+ bone marrow cells were labeled using CD49f (encoded by Itga6) and megakaryocytic and erythroid markers. Gates P1 and P2 pertain to CFU-e and BFU-e progenitors respectively. Gates P3, P4, and P5 pertain to basophils, megakaryocytes, and multi-potential progenitors (MPP) respectively (Tusi, Wolock et al. 2018).
Feedback regulation of erythropoietic rate in the basal state and during stress.

A negative feedback loop regulating tissue oxygen tension.

Mammalian erythropoiesis is dependent on erythropoietin (Epo), a glycoprotein hormone that signals via its receptor, EpoR, a transmembrane type I cytokine receptor (D’Andrea, Fasman et al. 1989). The production of RBCs is regulated through a negative feedback loop where low tissue oxygen tension triggers Epo secretion that activates the EpoR on the surface of erythroid progenitors and precursors, promoting an increase in RBC production (Koulnis, Porpiglia et al. 2014). Epo is secreted mainly by the kidney in the adult, and by the fetal liver, in response to low tissue oxygen tension. Oxygen sensing is through the prolyl hydroxylase enzymes (PHD1-3), which require oxygen to hydroxylate the alpha subunit of the heterodimeric transcription factor Hypoxia Inducible Factor (HIF). Prolyl-hydroxylated HIF increases the affinity of the HIF alpha subunits (HIF1a in fetal liver and HIF2a in adults), for the E3 ubiquitin ligase VHL, resulting in their proteosomal degradation. Tissue hypoxia decreases prolyl hydroxylation, stabilizing HIF2a /HIF1a and leading to HIF transcriptional activation. HIF binds to the Hypoxia Response Element (HRE) in the 3’ end of the Epo gene, inducing its expression (Semenza 2009).

High levels of Epo secretion trigger the erythropoietic stress response, where Epo-activated EpoR signaling expands erythroid progenitor and precursor pools. This increase in erythropoietic rate can be measured as an increase in the
number of circulating reticulocytes, indicating the number of new RBCs produced within the preceding 24-hour period (Brugnara 1998, Socolovsky 2007, Brugnara, 1994 #567). The dynamic range of the Epo/pO$_2$ negative feedback loop is seen in anemic patients, where Epo increases exponentially with decreasing hematocrit, and may reach levels 1000-fold higher than basal levels (Erslev and Caro 1987).

**B EpoR pathways regulating erythropoietic rate.**

Erythroid progenitors and precursors vary in their sensitivity to Epo, based on their developmental stage; late CFU-e and early erythroblasts have the highest number of EpoR on their surface and are the most sensitive to Epo signaling (Zhang, Socolovsky et al. 2003). Within a given developmental stage, there is further heterogeneity to Epo signaling. This heterogeneity is believed to be the basis for the graded response in erythropoietic rate to increasing Epo levels (Kelley, Koury et al. 1993, Socolovsky, Murrell et al. 2007).

A landmark paper in 1990 showed that a principal function of EpoR signaling in erythroblasts is to rescue these cells from apoptosis (Koury and Bondurant 1990). Several EpoR-activated survival pathways have been identified since, including the induction of the anti-apoptotic protein Bcl-x$_L$ (Socolovsky, Fallon et al. 1999, Koulnis, Porpiglia et al. 2012), and suppression of pro-apoptotic proteins, Fas and Bim; these pathways were found to contribute directly to erythropoietic rate regulation in vivo (Liu, Pop et al. 2006, Socolovsky, Murrell et al. 2007, Koulnis, Liu et al. 2011, Koulnis, Porpiglia et al. 2012).
Whether EpoR signaling increases erythropoietic rate by regulating the cell cycle of erythroid progenitors was unclear, and is one of the areas addressed by my thesis work (Chapter 2).

**Cell cycle mechanisms in erythropoiesis.**

In all lineages, differentiation proceeds in parallel with cell division. Stem cells are quiescent, and re-enter the cycle when stimulated to differentiate or self-renew. Fully differentiated cells undergo mitotic exit. But beyond these specific interactions between differentiation state and the cycle, cell division is generally regarded as a generic process whose function is to increase cell number.

Our recent work in the erythroid lineage showed, however, that there are several key interactions between the cell cycle and specific developmental stages in erythropoiesis, and that the cycle undergoes substantial re-modeling in lock-step with developmental transitions (Fig. 1.4). Most notably, the Socolovsky lab found that the transition from the CFU-e stage to ETD is an S-phase dependent transcriptional switch; a pause in S phase progression blocks the CFU-e to ETD switch, which resumes as soon as S phase is allowed to proceed. The reverse is also true: a pause in differentiation at this stage also arrests the cycle (Pop, Shearstone et al. 2010, Khoramian Tusi and Socolovsky 2018). The cycle corresponding to the CFU-e/ETD switch is extremely short, lasting only 6 h, including an S phase that is shorter and faster than S phase in preceding cycles, a result of globally-faster replication forks (Pop, Shearstone et al. 2010,
Shearstone, Pop et al. 2011, Hwang, Futran et al. 2017). The precise role and mechanism of this extremely short cycle is not fully known. However, we found that it is a result, in part, of downregulation of the CDK inhibitor p57KIP2 (Hwang, Futran et al. 2017), and that the fast S phase is required for a process of global DNA demethylation, which accelerates ETD gene induction (Shearstone, Pop et al. 2011).

Recently, we also found that, in the period leading up to the CFU-e/ETD transition, erythroid progenitors undergo gradual shortening in their G1 phase. Further, following the CFU-e/ETD transition, the cycle again gradually lengthens, ultimately leading to mitotic exit and enucleation.
Figure 1.4. CFU-e to ETD transition is a cell cycle synchronized switch.

Developing committed erythroid progenitors spend an increasing fraction of their time in S phase, as a result of G1 shortening. The CFU-e to ETD transition takes place in S phase of a single cell cycle, and is dependent on S phase progression. This cell cycle is unusually short, in part as a result of S phase shortening, from 7 h in preceding cycles, to only 4 hours. Over the course of 3 to 5 ETD cell divisions, erythroblast cell cycle length increases due to an S-phase and G1 lengthening (Pop, Shearstone et al. 2010, Shearstone, Pop et al. 2011, Hwang, Futran et al. 2017).
The role and mechanisms of EpoR and Stat5 signaling in erythropoiesis.

A. Intracellular signaling by EpoR.

The EpoR, together with the IL-2 receptor, are the founding members of the cytokine receptor superfamily (D'Andrea, Fasman et al. 1989, D'Andrea, Lodish et al. 1989), which today is known to comprise over 3 dozen receptors for multiple ligands including hematopoietic growth factors such as thrombopoietin, G-CSF, IL-3 and GM-CSF as well as factors that regulate non-hematopoietic tissue including growth hormone, leptin, LIF and prolactin (Watowich, Wu et al. 1996, Robb 2007). These receptors do not have an enzymatic activity in their cytoplasmic domain. Instead, Box1 and Box2 motifs in the cytoplasmic domains of the receptors' signaling chains associate with a cytoplasmic tyrosine kinase from the Janus Kinase (JAK) family (Ihle and Kerr 1995, Watowich, Wu et al. 1996, Robb 2007). The extracellular domains are characterized by a Fibronectin III-like subdomain and a WSXWS motif that plays a role in protein folding (Constantinescu, Ghaffari et al. 1999).

Cytokine receptors are classified into subfamilies based on the oligomeric complexes that they form. The EpoR subfamily, which also includes the growth hormone receptor, the prolactin receptor and the thrombopoietin receptor, are all homodimeric receptors (Zhang, Radhakrishnan et al. 2009). The inactive receptors are expressed as pre-existing homodimers on the cell surface, with each receptor chain pre-associated with Jak2. Ligand binding results in a conformational change that brings the cytoplasmic domains of the receptor
chains closer, allowing trans-phosphorylation and activation of associated Jak2, which in turn phosphorylates up to eight conserved tyrosines on the receptor's cytoplasmic domain (Constantinescu, Ghafrar et al. 1999, Lodish, Ghaffari et al. 2009). These phosphorylated tyrosines act as docking sites for intracellular adaptor and signaling molecules. Some of these signaling molecules, including Stat5, themselves become phosphorylated and activated. Signaling pathways activated besides Stat5 include Ras/MAPK, and PI3K/Akt1. Feedback inhibitors of Jak2 signaling are also activated (Fig. 1.5)(Constantinescu, Ghafrar et al. 1999, Lodish, Ghaffari et al. 2009).
Figure 1.5. EpoR cytosolic domains and signaling molecules.

Schematic of the eight conserved tyrosine residues in the cytosolic domain of the EpoR, which are phosphorylated by Jak2 upon ligand binding. The phosphorylated tyrosines act as docking sites for signaling molecules that are essential for survival, proliferation and differentiation (Lodish, Ghaffari et al. 2009).
B. **Signaling by Stat5.**

Signal Transducer and Activator of Transcription (STAT) proteins were first discovered as mediators of interferon signaling (Levy and Darnell 2002). There are seven members of the STAT protein family, Stat1, 2, 3, 4, 5a, 5b & 6. Of these, Stat3 and Stat5a/b have pleiotropic functions downstream of multiple cytokine receptors. Stat5a and Stat5b are >90% identical in their sequence and are here collectively referred to as 'Stat5'. Both are expressed in erythroid cells and are the principal Stat signaling mediators downstream of the EpoR.

Stat5 has several conserved domains: amino-terminal and coiled coil domains that play roles in its dimerization and protein interactions; a DNA binding domain; a conserved linker; an SH2 domain and a carboxy terminal transactivation domain. The SH2 domain docks on phosphorylated tyrosines in the cytoplasmic domains of activated receptors. In the case of the EpoR these are in a conserved YXXL sequence context (Y343, Y401, Y429 and Y431 on the mouse EpoR cytoplasmic domain) (Klingmuller, Bergelson et al. 1996, Paffett-Lugassy, Hsia et al. 2007). Once docked on the EpoR, Y694 in the Stat5 tyrosine activation domain becomes phosphorylated. This modification changes the distribution of Stat5 from primarily cytosolic to nuclear, leading to transcriptional activation (Vinkemeier 2004).

The Stat DNA-binding domain recognizes a family of palindromic ‘GAS’ enhancers of the general sequence TTTCCNGGAAA. The optimal sequence for
both Stat5a and Stat5b is TTC(C/T)N(G/a)GAA (Soldaini, John et al. 2000). Stat5 optimally binds to two tandem GAS enhancers, separated by 6 base-pairs. Several such Stat5 binding sites have been identified in known erythroid Stat5 target genes, including Bcl-xL and pim1 (Fig. 1.6)(Socolovsky, Fallon et al. 1999, Soldaini, John et al. 2000).
Figure 1.6. Cytosolic Stat5 activation and translocation to the nucleus for erythroid transcriptional activation.

Epo (ligand) binding to EpoR (cytokine receptor) allows for trans-phosphorylation and activation of associated Jak2, which phosphorylates eight conserved EpoR cytoplasmic tyrosines that serve as docking sites for SH2 domain-containing signaling molecules such as Stat5. Active Stat5 then enters the nucleus and begins transcription of erythroid specific genes.
C. **The functional roles of EpoR and Stat5 in erythroid progenitors.**

*Epo<sup>−/−</sup>, Ep<sub>or</sub><sup>−/−</sup> and Stat5<sup>−/−</sup> embryos all die at mid-gestation, corresponding to the developmental stage when definitive erythropoiesis becomes dominant over primitive erythropoiesis (Wu, Liu et al. 1995, Socolovsky, Fallon et al. 1999, Cui, Riedlinger et al. 2004). All three genotypes die as a result of severe anemia. Specifically, the fetal livers of *Epo<sup>−/−</sup>* and *Ep<sub>or</sub><sup>−/−</sup>* embryos contain early erythroid BFU-e and CFU-e progenitors, but completely lack erythroblasts undergoing terminal differentiation (Wu, Liu et al. 1995) indicating that the transition from the CFU-e stage to ETD coincides with the onset of absolute dependence on EpoR signaling. Flow cytometric analysis of the *Ep<sub>or</sub><sup>−/−</sup>* fetal liver shows developmental arrest at the S0 to S1 transition, corresponding to the CFU-e to ETD transcriptional switch (Fig. 1.7)(Pop, Shearstone et al. 2010, Tusi, Wolock et al. 2018).
Figure 1.7. Developmental arrest at the onset of ETD in EpoR⁻/⁻ embryos

CD71/Ter119 profiles for E12.5 EpoR⁻/⁻ and wild-type littermate fetal livers. Upregulation of CD71 coincides with the onset of EpoR dependence. Erythroid differentiation of EpoR⁻/⁻ cells is blocked at the transition from S0 to S1, which marks the onset of ETD. Ter119⁺ cells in EpoR⁻/⁻ liver are nucleated yolk-sac erythrocytes (Pop, Shearstone et al. 2010).
Unlike the \(Epor^{-/-}\) fetal liver, the \(Stat5^{-/-}\) fetal liver does contain erythroblasts, but they are fewer, and developmentally delayed, compared with fetal livers of wild-type littermate embryos (Socolovsky, Fallon et al. 1999, Koulnis, Porpiglia et al. 2012).

The absolute dependence of ETD on EpoR is the result of essential EpoR survival signaling, including the induction of Bcl-x\(_L\) via Stat5 activation, and suppression of Fas and Bim (Koury and Bondurant 1990, Socolovsky, Fallon et al. 1999, Koulnis, Porpiglia et al. 2012). In addition, EpoR/Stat5 signaling contributes to the upregulation of CD71 (the transferrin receptor) at the onset of ETD (Zhu, McLaughlin et al. 2008), critical for iron import for heme synthesis in erythroblasts.

Although EpoR becomes essential at the point in erythroid differentiation when the ETD transcriptional program is activated, it does not determine this program. This was shown in \(Epor^{-/-}\) CFU-e, which can differentiate into mature red cells, if transduced with the prolactin receptor, which belongs to the same subfamily of cytokine receptors as the EpoR, and activates similar signaling pathways (Socolovsky, Dusanter-Fourt et al. 1997, Socolovsky, Fallon et al. 1998). Conversely, the EpoR can rescue the differentiation of prolactin-receptor deficient mammary epithelium (Brisken, Socolovsky et al. 2002). Other similar experiments suggest that the role of cytokine receptor signaling in lineage differentiation is permissive, rather than deterministic (Socolovsky, Lodish et al. 1998). The erythroid transcriptional program is specified by a combination of
general and lineage-specific transcription factors, that primarily include GATA2 and Gata1, Klf1, Tal1/SCL, Myc, Myb & E2F4 (Orkin and Zon 2008).

**Open questions regarding EpoR and Stat5 signaling.**

Taken together, EpoR signaling, in part through Stat5, becomes critical for erythropoiesis at the transition from the early progenitor stage (CFU-e) to the erythroblast stage (ETD). EpoR does not specify the ETD transcriptional program. Instead, it exerts essential *permissive* functions during ETD, including suppression of erythroblast apoptosis, a mechanism that, in addition to facilitating erythroid differentiation, regulates the erythropoietic stress response.

It is not clear, however, whether EpoR’s permissive functions extend beyond survival signaling. In particular, it is not known if EpoR signaling plays a role in activation of the ETD transcriptional program. Gata1 and the other erythroid transcription factors that specify the ETD program are expressed in CFU-e, well before ETD (Tusi, Wolock et al. 2018). Therefore, expression of these transcription factors, by itself, is not sufficient to activate the ETD program. In my thesis work, I used a genetic approach to ask whether EpoR signaling somehow participates in or is required for, this function.

It is also unclear whether EpoR regulates the erythroblast cell cycle. Our laboratory showed that the CFU-e to ETD transcriptional switch is dependent on S phase, and takes place during a unique cycle that is shorter, including a shorter S phase, than preceding CFU-e cycles (Pop, Shearstone et al. 2010,
Shearstone, Pop et al. 2011, Hwang, Futran et al. 2017). In part, cell cycle shortening at this time is regulated through suppression of the CDK inhibitor p57\textsuperscript{KIP2} (Hwang, Futran et al. 2017). In my thesis, I used a genetic approach to test the possibility that cell cycle shortening at the onset of ETD, as well as cell cycle number, are also regulated by EpoR.

Finally, in addition to addressing specific questions regarding EpoR’s role in ETD, I took an unbiased approach to the discovery of novel EpoR functions, using single-cell RNA sequencing (scRNAseq). Our laboratory recently used scRNAseq to delineate the incremental transcriptional changes that take place during the process of erythropoiesis, starting with multi-potential progenitors, through early erythrocytic/basophil-mast cell/megakaryocytic progenitors, to early committed erythroid progenitors and finally to ETD (Tusi, Wolock et al. 2018). In my thesis and in work that is ongoing, I undertook scRNAseq of fetal livers from mutant EpoR and Stat5 embryos, to determine the effect of EpoR signaling on transcriptional pseudotime. This analysis led me to discover an unexpected role for EpoR and Stat5 in ribosome biogenesis and global protein translation.
CHAPTER II:

EPOR STIMULATES RAPID CYCLING AND LARGER RED CELLS DURING MOUSE AND HUMAN ERYTHROPOIESIS

Abstract

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.
Introduction

Red-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 (Kassebaum, Jasrasaria et al. 2014). 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 (D'Andrea, Fasman et al. 1989), first expressed in the earliest erythroid-committed progenitors. EpoR expression peaks in colony-forming-unit-erythroid (CFU-e) progenitors (Stephenson, Axelrad et al. 1971, Tusi, Wolock et al. 2018) (Fig. 2.1) with the onset of erythroid terminal differentiation (ETD) (Wu, Liu et al. 1995), a process that starts with the induction of erythroid gene transcription (Tusi, Wolock et al. 2018). 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 progenitors from apoptosis (Koury, Bondurant et al. 1988, Koury and Bondurant 1990), a principal mechanism of erythropoietic rate regulation (Koury and Bondurant 1992, Koulnis, Porpiglia et al. 2014). EpoR is downregulated in late erythroblasts, which no longer depend on its signaling for survival (Broudy, Lin et

The absolute dependence of definitive early erythroblasts on EpoR signaling for survival makes it challenging to identify other essential functions of EpoR in these cells. Key open questions include a role for EpoR in cell-cycle regulation. Although early reports suggested that Epo does not alter the erythroblast cell cycle (Iscove 1977), EpoR signaling induces cell-cycle genes in these cells (Fang, Menon et al. 2007), and is essential for the cycling of Epo-dependent cell lines (Ferro, Kozak et al. 1993, Spivak, Ferris et al. 1996) and cultured CFU-e (von Lindern, Zauner et al. 1999). EpoR also promotes cycling in yolk-sac derived primitive erythroblasts during early embryonic development (Malik, Kim et al. 2013). Therefore, EpoR may also be required for the cycling of adult-type erythroblasts, a function that may contribute to the erythropoietic stress response.

A second open question is whether EpoR is required for induction of erythroid genes. EpoR and similar cytokine receptors do not instruct lineage choice and are instead required for essential permissive functions (Socolovsky, Dusanter-Fourt et al. 1997, Socolovsky, Fallon et al. 1998, Socolovsky, Lodish et
(Al 1998, Braken, Socolovsky et al. 2002). It is not clear, however, whether these include signals that facilitate erythroid gene transcription. EpoR signaling was shown to phosphorylate GATA1, a key erythroid transcriptional regulator, but the broad impact of this phosphorylation on GATA1 function is not clear (Kadri, Maouche-Chretien et al. 2005).

To address these gaps, we developed a genetic system that identifies essential non-survival functions of EpoR signaling. We rescued mouse Epor\(^{-/-}\) fetal liver progenitors from apoptosis by transduction with the anti-apoptotic protein Bcl-x\(_L\), and compared their ensuing differentiation with that of Epor\(^{-/-}\) progenitors that were rescued by re-introduction of the EpoR. We found that the Bcl-x\(_L\) survival signal, in the absence of any EpoR signaling, supported expression of the erythroid transcriptional program and formation of enucleated red cells. However, key ETD features were abnormal. First, erythroblasts underwent slower and fewer cell cycles, suggesting a cell-cycle role for EpoR. We confirmed this role in adult mice in vivo, finding that Epo administration shortened the cycle of early erythroblasts, cells that are already amongst the fastest cycling cells in the bone marrow (Hwang, Futran et al. 2017, Eastman, Chen et al. 2020, Hwang, Hidalgo et al. 2020). Second, we found that, unexpectedly, despite stimulating rapid cycling, EpoR signaling increases cell size in both erythroblasts and red cells. This contrasts with the well-established inverse relationship between the number of erythroblast cell divisions and red-cell size (Burns, Reed et al. 1986, Humbert, Rogers et al. 2000, Sankaran,
Ludwig et al. 2012, Jayapal, Wang et al. 2015). Using mice doubly deleted for both EpoR and heme-regulated elF2a kinase (HRI), we found that EpoR regulation of red-cell size is also independent of the well-described iron and heme-regulated pathway (Suragani, Zachariah et al. 2012, Zhang, Macias-Garcia et al. 2018, Chen and Zhang 2019). We confirmed these findings in healthy human volunteers that were administered Epo, finding an increased MCV that persisted long after Epo and reticulocyte levels returned to baseline (Hidalgo, Bejder et al. 2021). Our work reveals novel EpoR functions, and suggests hypoxia, anemia, and other high-Epo syndromes as new diagnostic interpretations of increased red-cell size in the clinic.
FACS subsets, adult bone marrow: ProE  EryA  EryB  EryC

FACS subsets, fetal liver: SO, S1  S2  S3  S4, S5
**Figure 2.1. Developmental stages of erythropoiesis when EpoR is expressed or essential.**

The principal stages of the erythroid trajectory (Tusi, Wolock et al. 2018). EpoR expression is induced at the BFU-e stage, and is downregulated in late erythroblasts. It is essential for the survival of early erythroblasts. FACS subsets used in the analysis of mouse fetal liver or adult bone marrow are also indicated; their horizontal position corresponds to their approximate developmental stage.

MPP = Multipotential progenitors; EBMP = Erythroid, Basophil/mast cell, Megakaryocytic progenitors; BFU-e = Burst-forming unit erythroid; CFU-e = Colony-forming unit erythroid
Results

Non-survival EpoR signals are essential for normal erythroid differentiation.

Erythroid differentiation in Epor−/− fetal liver is arrested at the CFU-e stage (Wu, Liu et al. 1995, Kieran, Perkins et al. 1996, Lin, Lim et al. 1996, Malik, Kim et al. 2013). Epor−/− CFU-e can be rescued in vitro by transduction with EpoR or a similar cytokine receptor (Wu, Liu et al. 1995, Socolovsky, Dusanter-Fourt et al. 1997). Here we asked whether transducing Epor−/− CFU-e with Bcl-xL, an anti-apoptotic transcriptional target of EpoR signaling (Silva, Grillot et al. 1996, Motoyama, Kimura et al. 1999, Socolovsky, Fallon et al. 1999, Koulnis, Porpiglia et al. 2012), 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. 2.2).

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. 2.2b). 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-Epor−/− v. Bcl-xL-Epor−/−, p=3.6 × 10−13; Fig. 2.2c, 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. 2.2b).

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 hours post-transduction, while EpoR-Epor−/− erythroblasts without Epo did not (Fig. 2.2e). 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-Epor−/− erythroblasts at this time (Fig. 2.2e).

Differentiation abnormalities of Bcl-xL-Epor−/− 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, Tfr), followed by upregulation of Ter119 (Pop, Shearstone et al. 2010, Hwang, Futran et al. 2017, Tusi, Wolock et al. 2018). Epor−/− progenitors arrest in development prior to CD71 upregulation (the small number of Ter119+ cells in Epor−/− fetal liver are yolk-sac-derived erythroblasts (Pop, Shearstone et al. 2010), (Fig. 2.2f). Transduction of Epor−/− fetal liver cells with EpoR allowed them to resume the expected sequence of cell surface marker expression, upregulating CD71 by 18 hours and Ter119 by 36 hours (Fig. 2.2g). By contrast, Bcl-xL-Epor−/− cells failed to upregulate CD71 at any point of the culture although they did upregulate Ter119 (Fig. 2.2g).
Thus, our initial analysis showed that, when rescued from apoptosis by Bcl-x<sub>L</sub>, Epo<sup>−/−</sup> 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.
Figure 2.2. Abnormal ETD in the absence of EpoR signaling.

a Experimental design. E12.5 Epor<sup>−/−</sup> fetal livers were transduced with bicistronic retroviral vectors encoding either Bcl-x<sub>L</sub> 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 hours.

b Epor<sup>−/−</sup> CFU-e colonies, scored 48 hours following transduction with either EpoR or Bcl-x<sub>L</sub>. Epo was added to the medium where indicated. Epor<sup>−/−</sup> 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-x<sub>L</sub> and Stat5 1*6. Data pooled from 3 independent experiments. 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-Epor<sup>−/−</sup> or Bcl-x<sub>L</sub>- Epor<sup>−/−</sup>). Data pooled from 3 independent experiments as in ‘b’.

e Cytospin preparations of transduced Epor<sup>−/−</sup> fetal liver cells cultured in liquid medium for 36 hours, 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.
Flow cytometric CD71/Ter119 profiles of freshly harvested Epor<sup>-/-</sup> and wild-type littermate fetal livers (f), and of Epor<sup>-/-</sup> fetal liver cells 18 and 36 hours post transduction and culture in Epo-containing medium (g).
Erythroblasts undergo fewer and slower cell cycles in the absence of EpoR signaling.

CFU-e express the receptor tyrosine kinase Kit and the Interleukin-3 (IL3) receptor (Umemura, al-Khatti et al. 1989, Socolovsky, Fallon et al. 1998, von Lindern, Schmidt et al. 2004). 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-x\(_L\)-Epor\(^{-/-}\) and EpoR-Epor\(^{-/-}\) erythroblasts remained (Fig. 2.3a). We modified our transduction protocol to make use of this improvement in yield, culturing freshly transduced Epor\(^{-/-}\) progenitors for 15 hours 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, Fig. 2.3b, Fig. 2.4a).

Pre-incubation with SCF and IL3 did not ameliorate the abnormalities of Bcl-x\(_L\)-Epor\(^{-/-}\) erythroblast differentiation. In particular, these cells failed to upregulate CD71 (Fig. 2.4a, b). We examined the possibility that these abnormalities were the result of overexpression of Bcl-x\(_L\), rather than the absence of EpoR signaling, by co-transducing Epor\(^{-/-}\) progenitors with both EpoR and Bcl-x\(_L\), each linked to a distinct reporter (Fig. 2.3c). 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 (Fig. 2.3d-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−/− erythroblasts, by co-transducing Epor−/− progenitors with Tfrc, in addition to either Bcl-xL or EpoR (Fig. 2.4c–e). In an alternative approach, we added iron loaded ferricsalicylaldehyde isonicotinoyl hydrazone (Fe-SIH) to the culture medium of both Bcl-xL-Epor−/− and EpoR-Epor−/− erythroblasts. 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 cells (Garrick, Gniecko et al. 1991). Neither of these approaches altered the proliferative defect of Bcl-xL-Epor−/− erythroblasts (Fig. 2.4c, e). The viability of all erythroblasts was high with no significant difference between Bcl-xL-Epor−/− and EpoR-Epor−/− erythroblasts (Fig. 2.4d), suggesting that the proliferative deficit of Bcl-xL-Epor−/− erythroblasts is the result of fewer cell divisions. In the first 26 hours of culture, there was a substantial difference in doubling time (6.1 hours v. 8.6 hours for EpoR-Epor−/− v. BclxL-Epor−/− erythroblasts, Fig. 2.4c, e). The doubling time of 6 hours for EpoR-Epor−/− is in good agreement with our recent finding of a 6 hour cell cycle in wild-type early erythroblasts in vivo (Hwang, Futran et al. 2017), and with the finding that early erythroblasts have the
shortest cell cycle amongst bone-marrow hematopoietic progenitors (Eastman, Chen et al. 2020).

Iron may affect cell growth by acting as a cofactor in ribonucleotide reductase (RNR) catalysis of deoxyribonucleotide synthesis (Nyholm, Mann et al. 1993). However, supplementation of the culture medium with deoxyribonucleosides (dN), which bypass RNR via the deoxyribonucleoside kinase salvage pathway (Eriksson, Munch-Petersen et al. 2002), had little effect on the proliferative defect (Fig. 2.4c, 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 deoxyribonucleosides 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 fused to a fluorescent timer protein (H2B-FT, Fig. 2.4f), which fluoresces blue when first synthesized but matures over 1–2 hours into a red fluorescent protein (Eastman, Chen et al. 2020). The ratio of blue fluorescence to total fluorescence (red + blue) is an indicator of cell-cycle length (Eastman, Chen et al. 2020). Administration of Epo (100 U) once daily resulted in a clear shift in the ratio of blue to total fluorescence at 36 hours, in all bone-marrow early erythroblast subsets (Fig. 2.4f, g and Fig. 2.5). These data
confirm that Epo/EpoR signaling increases cell-cycle speed in wild-type erythroblasts \textit{in vivo}. 
Figure 2.3. Reduced growth of Bcl-xL- *Epor<sup>-/-</sup>* erythroblasts and their failure to upregulate CD71 persists in SCF and is not the result of Bcl-xL over-expression.

a Effect of IL-3 and SCF on viable cell number. *Epor<sup>-/-</sup>* fetal livers were transduced as in Fig 1a, and cultured for 19 hours, in the presence of Epo, and either in the presence or absence of IL-3 and SCF. Trypan-blue negative cells were scored. Data points are independent cultures pooled from three independent experiments. Box is 25th to 75th percentiles with the median marked; whiskers are min to max values. 2-tailed t-test, unequal variance.

b Modified experimental design, used in all subsequent experiments unless otherwise indicated. *Epor<sup>-/-</sup>* fetal livers were transduced at t = -15 h with bicistronic retroviral vectors encoding Bcl-x<sub>L</sub> or EpoR and the hCD4 reporter and cultured in the presence of IL-3 and SCF for 15 hours. At t = 0 h, they were switched to an Epo containing medium, in the presence of additional factors as indicated in each experiment. Analysis was performed on transduced cells expressing hCD4 and negative for non-erythroid lineage markers (CD41, Mac1, Gr1, B220, CD3e).

c Experimental design: *Epor<sup>-/-</sup>* fetal livers were doubly transduced with bicistronic retroviral vectors, in the three shown combinations. Vectors encoded Bcl-x<sub>L</sub>, EpoR or ‘empty vector’ (V); reporter constructs (GFP or hCD4) for each vector are shown as a superscript. Transduced cells were cultured in IL-3 and SCF for 15 hours, and switched to an Epo containing medium at t=0. Analysis was performed on cells that were doubly positive for both hCD4 and GFP, and
negative for non-erythroid lineage markers (hCD4^+GFP^+Lin^-, where Lin = CD41, Mac1, Gr1, B220, CD3e).

d  Flow cytometric analysis of Epor^-/- hCD4^+GFP^-Lin^- erythroblasts, showing failure to upregulate CD71 in cells lacking the EpoR, but not in cells doubly transduced with both EpoR and Bcl-xL.

e  Growth curves of doubly transduced Epor^-/- fetal liver cells as in ‘a’. Viable hCD4^+Lin^- cells were counted at the indicated time points. Data are representative of two independent experiments, expressed relative to t=0.

f  Cell viability, expressed as the fraction (%) of trypan blue negative cells, for the same set of samples shown in ‘c’.

g  Relative cell number of doubly transduced erythroblasts (Lin^-hCD4^+GFP^+), for each of the indicated retroviral combinations at 48 and 72 hours of differentiation.
**Figure 2.4.** EpoR stimulates cell cycle shortening in early erythroblasts *in vitro* and *in vivo*.

**a** The EpoR is required for CD71 upregulation. *Epor−/−* fetal livers were transduced with either EpoR or Bcl-x<sub>L</sub> retroviral vectors carrying the hCD4 reporter (see Extended Data Fig 2b for experimental design). Transduced erythroid cells (hCD4<sup>+</sup>Lin<sup>−</sup>) were examined for expression of CD71 and Ter119.

**b** Time course of CD71 expression following EpoR or Bcl-x<sub>L</sub> retroviral transduction as in ‘a’. MFI; median fluorescence intensity, expressed relative to t=0. Data from two independent experiments.

**c** Growth of *Epor−/−* fetal liver transduced with either Bcl-x<sub>L</sub> 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 are pooled for each set of conditions from n=4 independent experiments (‘control’ growth curves in which cells were transduced with either EpoR or Bcl-x<sub>L</sub> but without additional manipulation of iron or dN were included in every experiment). Data are expressed relative to cell number at t=0, and were fit with exponential curves (R<sup>2</sup> values ranging between 0.8 and 0.94, least squares fit).

**d** Cell viability, expressed as the fraction (%) of trypan blue negative cells, for the same set of experiments shown in ‘c’.

**e** Cell doubling times ± 95% confidence intervals, calculated from the fitting of exponential growth curves to the data in ‘c’.
Cell cycle shortening in early erythroblasts \textit{in vivo}. Mice transgenic for a fusion of histone H2B and a 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 long-lived red fluorescent protein (‘R’). The blue to total fluorescence ratio in the cell (expressed as $B/(B+R)$) is a function of cell cycle length (Eastman, Chen et al. 2020). Shown are histograms of $B/(B+R)$ in live EryA erythroblasts (Ter119$^{\text{high}}$CD71$^{\text{high}}$FSC$^{\text{high}}$). Histogram overlays are for 2 mice injected with saline and 2 mice injected with Epo.

Relative cell cycle lengths for the 4 mice analyzed in ‘f’, for each of the indicated erythroblast maturation stages: ProE (Ter119$^{\text{med}}$CD71), EryA (Ter119$^{\text{high}}$CD71$^{\text{high}}$FSC$^{\text{high}}$), EryB (Ter119$^{\text{high}}$CD71$^{\text{high}}$FSC$^{\text{low}}$), EryC (Ter119$^{\text{high}}$CD71$^{\text{low}}$FSC$^{\text{low}}$). p-value is for a paired $t$ test, pairing the average values for Epo-injected and Saline injected mice for each of the early erythroblast stages (ProE and EryA/B). Cell cycle length was calculated as R/B. Late erythroblasts continue to divide, but their cell cycle is no longer sensitive to Epo concentration. This conclusion is evident in lack of response of EryC erythroblasts to Epo injection, and in their longer cycle.
Figure 2.5. EpoR stimulates cell cycle shortening in vivo.

a, b  Mice transgenic for the fluorescent timer protein fusion H2B-FT were injected with either saline or Epo (100 U) and analyzed at 36 h (see Figure 2f for additional detail).

a  Representative flow cytometric profiles of bone marrow erythroblasts

b  Histograms of $B/(B+R)$ in live cells, for each of the erythroblast subsets gated in ‘a’, and for non-erythroid lineage-positive cells in the same bone-marrow sample (‘Lin+’). Histogram overlays are for 2 mice injected with saline and 2 mice injected with Epo.
**EpoR shortens both G1 and S phase through an iron-independent mechanism.**

The onset of ETD is associated with cell-cycle shortening, from ~15 hours in CFU-e, to 6 hours in early erythroblasts (Pop, Shearstone et al. 2010, Hwang, Futran et al. 2017, Tusi, Wolock et al. 2018), including a shortened, 4-hour-long S phase (Hwang, Futran et al. 2017). We asked whether the cell-cycle shortening effect of EpoR (Fig. 2.4e, g) is exerted in G1 or in S phase. The shortening of G1 by cytokine receptor signaling is well documented (Matsumura, Kitamura et al. 1999, Zhu and Skoultchi 2001, Khaled, Bulavin et al. 2005, Quelle 2007, Dalton 2015). However, to our knowledge, there are no reports of cytokine signaling altering S phase speed.

To examine this possibility, we pulsed cultures of EpoR or Bcl-xL-transduced Epor<sup>−/−</sup> erythroblasts with bromodeoxyuridine (BrdU), a nucleoside analog that is incorporated into DNA during S phase, and analyzed the cells 30 minutes following the pulse. The fraction of cells that are labeled with an anti-BrdU antibody indicates the proportion of cells in S phase at the time of the pulse. Further, the amount of BrdU incorporated into S phase cells during the 30 minute pulse, as measured by the BrdU mean fluorescence intensity (MFI) of S phase cells, indicates the intra-S phase rate of DNA synthesis, which is inversely related to S phase duration (Hwang, Futran et al. 2017). We found that, in the first 10 hours of ETD, BrdU MFI in S phase cells was 50% higher in EpoR-
Epor\(^{-/-}\), compared with Bcl-x\(_L\)-Epor\(^{-/-}\) erythroblasts, suggesting that EpoR signaling increases S phase speed (Fig. 2.6a, b).

If the slowing of S phase alone could account for the increased cell-cycle length of Bcl-x\(_L\)-Epor\(^{-/-}\) erythroblasts, S phase would constitute a larger fraction of total cell-cycle duration. However, the fraction of Bcl-x\(_L\)-Epor\(^{-/-}\) erythroblasts in S phase was actually somewhat lower, with a corresponding increase in the fraction of cells in G1 (Fig. 2.6b). These observations suggest that, in the absence of EpoR signaling, both S and G1 phases lengthen.

Supplementing the culture medium with Fe-SIH increased S phase speed modestly in all Epor\(^{-/-}\) erythroblasts (Fig. 2.6c). There was no rescue of S phase speed in Bcl-x\(_L\)-Epor\(^{-/-}\) erythroblasts by either the addition of deoxyribonucleosides or double transduction with both Bcl-x\(_L\) and Tfrc (Fig. 2.6d), although there was a small increase in the number of cells in S phase in the latter (Fig. 2.6e). Taken together, these results indicate that EpoR is essential for accelerating both G1 and S phases of the cycle in early ETD, via mechanisms that are largely independent of iron and the nucleotide pool.
Figure 2.6. EpoR regulates the speed of S phase.

a  Cell cycle analysis of *Epor*<sup>−/−</sup> fetal liver cells transduced with either EpoR or Bcl-x<sub>L</sub> and cultured as in Extended Data Fig 2b. Cells were pulsed with BrdU for 30 min at t=9 h and were immediately harvested for analysis. The fraction (%) of erythroblasts (hCD4<sup>+</sup>Lin<sup>−</sup>) 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-x<sub>L</sub>-transduced *Epor*<sup>−/−</sup> fetal liver cells. Data is pooled from 6 independent experiments similar to ‘a’. In all cases, cells were pulsed with BrdU for 30 minutes prior to harvesting for analysis. Data are mean ± sem. Intra-S phase BrdU (MFI) is expressed as the ratio to BrdU MFI of Bcl-x<sub>L</sub>-transduced fetal liver cells at t=0 in each experiment. Significance p values are paired t test, pairing EpoR and Bcl-x<sub>L</sub>-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 mM) 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 and Bcl-x<sub>L</sub>-transduced *Epor*<sup>−/−</sup> fetal liver cells at t=9 h, experimental design as in Fig 2b, and ‘a’ to ‘c’ above. S phase speed is expressed relative to the speed at t=0 in
each experiment. Shown are the effects of adding 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<sup>−/−</sup> fetal liver cells transduced with EpoR and with Bcl-x<sub>L</sub> without additional additives or transductions.
Imaging flow cytometry shows $Epor^{−/−}$ erythroblasts and reticulocytes are smaller.

Nutritional deficiencies, drugs, or genetic perturbations that reduce the number of cell divisions lead to the formation of larger red cells (macrocytosis (Burns, Reed et al. 1986, Humbert, Rogers et al. 2000, Sankaran, Ludwig et al. 2012, Jayapal, Wang et al. 2015, Nagao and Hirokawa 2017)). Therefore, we expected that the fewer cell divisions of $Bcl_{xL}-Epor^{−/−}$ erythroblasts would result in larger size for these cells. Instead, they appeared to be smaller (Fig. 2.2e). To address this question quantitatively, we measured cell and nuclear size in EpoR-$Epor^{−/−}$ and $Bcl_{xL}-Epor^{−/−}$ erythroblasts by imaging flow cytometry (Fig. 2.7a, b). We calibrated the measured cell areas by comparing them with beads of known diameter (Fig. 2.8a). We found that both cell and nuclear size were significantly smaller in the absence of EpoR (7.5 ± 0.6 µm and 6.7 ± 0.7 µm for EpoR-$Epor^{−/−}$ and $Bcl_{xL}-Epor^{−/−}$ erythroblasts, respectively, mean ± sem, p=0.001, t=46 hours). Although $Bcl_{xL}-Epor^{−/−}$ erythroblasts express significantly lower CD71 (Fig. 2.4a, b), the addition of Fe-SIH to the culture did not alter their smaller cell or nuclear size (Fig. 2.7b).

We asked whether the smaller size of $Bcl_{xL}-Epor^{−/−}$ erythroblasts could reflect an accelerated process of differentiation. If at any given time of the culture $Bcl_{xL}-Epor^{−/−}$ 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-Epor−/− reticulocytes were significantly smaller (5.6 ± 0.5 µm vs. 4.5 ± 0.15 µm for of EpoR-Epor−/− vs. Bcl-xL-Epor−/−, mean ± sem, p=0.002, Fig. 2.7c, d).

To assess whether the smaller size of Bcl-xL-Epor−/− 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 (Fig. 2.8b–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. 2.7e; Fig. 2.8c, d). Therefore, Bcl-xL overexpression is not the cause of the smaller size of Bcl-xL-Epor−/− erythroblasts and reticulocytes.

The level of EpoR expression in transduced Epor−/− cells positively correlated with erythroblast cell diameter (Fig. 2.9). 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.
a

Time (h)

Cell diameter (μm)

Nuclear diameter (μm)

p=0.01

p=0.001

p=0.04

p=0.005

b

Time (h)

Cell diameter (μm)

Nuclear diameter (μm)

(6.9, 1.7)

(7.2, 1.8)

(6.1, 1.4)

(6.0, 1.4)

c

Erythroblasts

(Red)

Bcl-xL

GFP

Draq5

Ter119

Overlay

Erythroblasts

(nucleated)

Reticulocytes

(enucleated)

d

Reticulocyte diameter (μm)

p=0.002

EpoR

Bcl-xL

e

Retroviral vectors:

EpoR

Bcl-xL

EpoR + Fe-SIH

EpoR + Fe-SIH

EpoR + Fe-SIH

Time (h)

48 72

48 72

48 72

48 72
Figure 2.7. 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, Figure 2.8a). Data points 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 2-tailed paired t-tests, pairing EpoR^- and Bcl-x_L^- 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 48h 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 are shown from cultures of Epor^-/- fetal liver cells that were doubly transduced with bicistronic retroviral vectors encoding GFP and hCD4 reporters (see Fig 2.3c) at 48 hours post transduction.
d Reticulocyte cell diameter in cultures of EpoR-Epor<sup>−/−</sup> or Bcl-x<sub>L</sub>-Epor<sup>−/−</sup> at 48 h post transduction, identified as in ‘c’. Data are population medians from 5 independent experiments. Box and whiskers as in ‘a’.

e Reticulocyte diameters in cultures of Epor<sup>−/−</sup> fetal liver cells that were doubly-transduced with bicistronic vectors carrying GFP and hCD4 reporters (Fig 2.3c). These vectors were either ‘empty’ (V<sup>GFP</sup>, V<sup>hCD4</sup>) or encoded either Bcl-x<sub>L</sub> or EpoR (Bcl-x<sub>L</sub><sup>GFP</sup>, EpoR<sup>hCD4</sup>). Violin lines mark the 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentile with a white circle marking the mean. The data is representative of two independent experiments.
Figure 2.8. A smaller cell size in the absence of EpoR is not the result of Bcl-x\textsubscript{L} over-expression.

\textbf{a} Calibration of the cell diameter measurement, representative of 8 independent experiments. Imaging flow cytometry was used to measure the area of polystyrene beads (Spherotech) of known diameter (indicated in legend, in micrometers). Data were fitted with a polynomial curve. The box and whiskers mark the 25\textsuperscript{th} to 75\textsuperscript{th} percentiles and min to max values, respectively, for all data-points with the median indicate. Calibration was performed for each imaging flow cytometry experiment.

\textbf{b - c} Epor\textsuperscript{-/-} fetal livers were doubly transduced with bicistronic retroviral vectors, in three combinations, as described in Figure 2.3: EpoR-IRES-hCD4 + ‘empty’ GFP vector (EpoR\textsuperscript{hCD4} + V\textsuperscript{GFP}); Bcl-x\textsubscript{L}-IRES-GFP + ‘empty’ hCD4 vector (Bcl-x\textsubscript{L}\textsuperscript{GFP} + V\textsuperscript{hCD4}); or with Bcl-x\textsubscript{L}-IRES-GFP + EpoR-IRES-hCD4 (Bcl-x\textsubscript{L}\textsuperscript{GFP} + EpoR\textsuperscript{hCD4}). Transduced cells were cultured in IL-3 and SCF for 15 hours, and switched to an Epo containing medium at t = 0 h. Analysis was performed on cells that were doubly positive for both hCD4 and GFP, and negative for non-erythroid lineage markers (hCD4\textsuperscript{+}GFP\textsuperscript{+}Lin\textsuperscript{-}, where Lin = CD41, Mac1, Gr1, B220, CD3e). The analysis below was performed at t = 48 h.

\textbf{b} In order to compare cells with similar expression of each of the retroviral constructs, we divided the erythroblast population (hCD4\textsuperscript{+}GFP\textsuperscript{+}Lin\textsuperscript{-}) into five fluorescence intensity quantiles for each of the GFP and hCD4 channels. The dashed line indicates the baseline fluorescence of cells that are not transduced.
with the corresponding vector (that is, cells transduced with only a GFP vector when examining the hCD4 channel, and vice versa).

c Erythroblast (upper panel) and reticulocyte (lower panel) cell diameters for cells expressing Bcl-x<sub>L</sub><sup>GFP</sup> + V<sup>hCD4</sup>, compared with cells expressing Bcl-x<sub>L</sub><sup>GFP</sup> + EpoR<sup>hCD4</sup>. The comparison is done separately for each quantile combination, and is depicted relative to the diameter of cells expressing EpoR<sup>hCD4</sup> + V<sup>GFP</sup> in the same quantile combination. For example, the diameters of cells transduced with Bcl-x<sub>L</sub><sup>GFP</sup> + V<sup>hCD4</sup> in quantile combination 10 (containing cells in the 5<sup>th</sup> quantile of the GFP channel and 2<sup>nd</sup> quantile of the hCD4 channel), are compared with diameters in quantile combination 10 of cells transduced with Bcl-x<sub>L</sub><sup>GFP</sup> + EpoR<sup>hCD4</sup>, and expressed as a ratio to the diameters in quantile combination 10 of cells transduced with EpoR<sup>hCD4</sup> + V<sup>GFP</sup>. Quantile combinations containing fewer than 10 cells were excluded.

d Reticulocyte diameters for each quantile combination, represented as histograms. Data as described in ‘c’.
Figure 2.9. Cell surface expression of EpoR regulates erythroblast cell size.

Epor⁻/⁻ fetal liver cells were doubly transduced with bicistronic retroviral vectors and cultured for 48 hours in Epo = 0.5 U/ml (experiment as in Fig 2.3c).

a Cell diameter is plotted against EpoR expression for cells transduced with EpoR⁹CD⁴, or against Bcl-xL expression for cells transduced with Bcl-xL⁹GFP. Transduced erythroblasts were divided into quintiles based on their expression of either GFP or hCD4. Median cell diameter is plotted against the median fluorescence for cells in each quintile. Left panels shows contour plots for all cells in each quintile, with median value is shown in white. Right panels show median values for each quintile, fitted with a dose/response curve of the form EC₅₀/(1+ (EC₅₀/x)). Spearman correlations and associated p-values are shown on each graph.

b Variability in the cell diameter measurement within each quintile, and as a function of EpoR expression levels in transduced cells, in the same dataset as in 'a'.
**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 translation (Han, Yu et al. 2001). Bcl-\(x_L\)-Epor\(^{-/-}\) erythroblasts failed to upregulate CD71 (Tfrc), the principal iron transporter. Although iron supplementation did not rescue the smaller size of Bcl-\(x_L\)-Epor\(^{-/-}\) erythroblasts (Fig. 2.7b), 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\(^{-/-}\)Hn\(^{-/-}\) mice (Fig. 2.10a). Similar to Epor\(^{-/-}\) mice, Epor\(^{-/-}\)Hn\(^{-/-}\) mice died at mid-gestation with severe anemia. We rescued both Epor\(^{-/-}\) and Epor\(^{-/-}\)Hn\(^{-/-}\)-fetal liver cells in parallel, by transduction with either Bcl-\(x_L\) or EpoR (Fig. 2.10b–e). In agreement with the known role of HRI as a negative regulator of erythroblast size, both Bcl-\(x_L\)-transduced and EpoR-transduced erythroblasts were larger on the Epor\(^{-/-}\)Hn\(^{-/-}\) genetic background than on the Epor\(^{-/-}\) background. Importantly, for a given genetic background, either Epor\(^{-/-}\)Hn\(^{-/-}\) or Epor\(^{-/-}\), the difference in size between Bcl-\(x_L\) and EpoR-rescued cells remained (Fig. 2.10b–e). 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. 2.10b–d) and reticulocytes (Fig. 2.10e).
Figure 2.10. EpoR regulates cell size independently of HRI.

a  
*Epor*^−/−^ and doubly-deleted *Epor*^−/−^*Hri*^−/−^ E12.5 embryos with wild-type littermates

b  
Cell and nuclear diameters in fetal livers from either *Epor*^−/−^ or *Epor*^−/−^*Hri*^−/−^ embryos, transduced with either EpoR or Bcl-x<sub>L</sub>, at t=48h post transduction. Individual sample contour plots are overlaid on scatter plots (each dot is one cell). Red dots indicate the distributions’ medians.

c  
Summary data for cell and nuclear area, for two independent experiments as in ‘b’, each containing all 4 genotype/retrovirus combinations. Data are mean ± SD for each cell population. Each transduced population consisted of pooled fetal liver cells from either *Epor*^−/−^ or *Epor*^−/−^*Hri*^−/−^ embryos. Cell diameter data for all genotypes is significantly different (p = 0.0008, one-way ANOVA between population means). On the *Hri*^−/−^ *Epor*^−/−^ background, cell diameter is significantly different between Bcl-x<sub>L</sub>- and EpoR-transduced cells (p=0.019, one-way ANOVA from two independent experiments).

d  
Imaging flow cytometry of representative Lin<sup>−</sup>hCD4<sup>+</sup>Ter119<sup>+</sup> erythroblasts from each of the genotype/retroviral combinations at t = 48 h.

e  
*Epor*^−/−^ and *Epor*^−/−^*Hri*^−/−^ Reticulocyte cell diameter, from cultures transduced with either EpoR or Bcl-x<sub>L</sub>. Representative of 2 experiments. Violin lines mark the 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentile with a white circle marking the mean.
Accelerated maturation in the absence of EpoR, assessed independently of cell size.

Cell size is frequently used as indicator of erythroid maturational stage (Liu, Pop et al. 2006, McGrath, Bushnell et al. 2008, Chen, Liu et al. 2009, Kalfa and McGrath 2018). Our initial impression was that Bcl-xL-Epor^−/− erythroblasts completed their maturation sooner than EpoR-Epor^−/− erythroblasts (Fig. 2.2e, 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 (Liu, Pop et al. 2006, McGrath, Bushnell et al. 2008, Chen, Liu et al. 2009, Kalfa and McGrath 2018), reached significantly higher levels in Bcl-xL-Epor^−/− erythroblasts than in EpoR-Epor^−/− erythroblasts at 48 hours (Fig. 2.11a).

Second, we looked at nuclear offset, a quantitative measure of nuclear eccentricity that is independent of cell size (McGrath, Bushnell et al. 2008) (Fig. 2.11b–d). The nuclear offset is the ratio of the delta centroid (the distance between the geometrical centers of the cell and the nucleus) to the cell diameter (Fig. 2.11b). Nuclear offset increased continuously throughout ETD, but did so earlier in Bcl-xL-Epor^−/− erythroblasts, with the difference between Bcl-xL-Epor^−/− and EpoR-Epor^−/− erythroblasts peaking at 48 hours (p=0.02) (Fig. 2.11c, d). Taken together, both Ter119 expression and nuclear offset suggest that EpoR signaling prolongs erythroblast maturation.
a

$$p = 0.002$$

Erythroblast Ter119 (relative to control)

48 Time (h)

$$\Delta$$ centroid

cell nucleus

diameter

$$Nuclear\ offset = \frac{\Delta centroid}{diameter}$$

d

$$p = 0.02$$

Nuclear offset (relative to control)

24, 48, 72 Time (h)
Figure 2.11. EpoR signaling prolongs the ETD stage.

a Ter119 intensity in Epor−/− erythroblasts (Lin− hCD4+) transduced with either Bcl-xL or EpoR, at 48 h. Data are mean Ter119 intensities from 9 independent experiments. Box and error bars are mean ± SD. p values are for a 2-sided paired t test.

b Explanation of the nuclear offset measurement. The geometrical centers (=centroids) of the cell and the nucleus are indicated by green and blue solid circles. The distance between the centroid is the delta centroid (red dashed line, also known as the X,Y delta centroid). The nuclear offset is a dimensionless ratio of the delta centroid to the cell diameter.

c Representative nuclear offset measurements of Epor−/− erythroblasts (Lin− hCD4+) transduced with either Bcl-xL or EpoR, at the indicated time points. Violin lines mark the 25th, 50th and 75th percentile with a white circle marking the mean.

d Nuclear offset measurements of Epor−/− erythroblasts (Lin− hCD4+) transduced with either Bcl-xL or EpoR. Individual datapoints are population medians pooled from 9 independent experiments. Box and error bars are mean± SD. p values are for a 2-sided paired t test.
The differences between EpoR-transduced and Bcl-x<sub>L</sub>-transduced Epor<sup>−/−</sup> erythroblasts are maintained across a wide range of EpoR expression levels.

The Epor<sup>−/−</sup> transduction model allows confident identification of essential non-survival functions of the EpoR. This model suffers, however, from two drawbacks. First, it is in vitro. We therefore tested whether our conclusions hold in vivo (Fig. 2.4f, Fig. 2.5, and below). Second, retroviral expression of EpoR and Bcl-x<sub>L</sub> may differ in timing or expression level from their physiological profiles. We have shown above that the slower cycles, lower CD71 expression and smaller cell size of Bcl-x<sub>L</sub>-Epor<sup>−/−</sup> erythroblasts result from absence of EpoR signaling rather than Bcl-x<sub>L</sub> overexpression (Figs. 2.3, 2.8). To investigate the potential effect of EpoR overexpression, we transduced Epor<sup>−/−</sup> erythroblasts with high-titer, undiluted retroviral supernatant, resulting in a ~3.5-fold higher expression of EpoR, compared with physiological expression in fresh or cultured erythroblasts (Fig. 2.12a). We then transduced Epor<sup>−/−</sup> fetal liver cells with either undiluted, high-titer retroviral supernatant or with five-fold and ten-fold dilutions of the same supernatant. EpoR expression decreased 8-fold by qRT-PCR in cells transduced with a 10-fold diluted supernatant. We found that the differences between EpoR-transduced and Bcl-x<sub>L</sub>-transduced Epor<sup>−/−</sup> erythroblasts, in CD71 expression, cell number, maturation rate, and cell size, were all maintained regardless of retroviral titer (Fig. 2.12b–f). Therefore, EpoR functions identified with the Epor<sup>−/−</sup> transduction model are not narrowly dependent on EpoR or Bcl-x<sub>L</sub> expression.
levels. Further, the viability of EpoR or Bcl-xL-transduced $Epor^{-/-}$ erythroblasts is comparable to that of wild-type erythroblasts (Fig. 2.13).
Figure 2.12. Functional differences between EpoR-transduced and Bcl-x-transduced Epor\(^{-}\) erythroblasts are maintained across a wide range of EpoR expression levels.

a  *Epor* gene expression in *Epor*-transduced *Epor\(^{-}\)/* fetal liver cells cultured for 15h, compared to *Epor* gene expression in similarly cultured wild-type fetal liver cells or in freshly isolated wild-type fetal liver subsets (Ter119\(^{-}\)Lin\(^{-}\), S0, S1, S2). mRNA was measured by quantitative RT-PCR, normalized to the β-actin mRNA, and expressed relative to the S0 subset. Data are 3 (for S0 and S1) or 2 (EpoR-transduced *Epor\(^{-}\)/* fetal liver cells) independent experiments and 2 (whole fetal liver cells and Ter119\(^{-}\)Lin\(^{-}\) t=0h) or 3 (Ter119\(^{-}\) Lin\(^{-}\) SCF/IL3, Ter119\(^{-}\) Lin\(^{-}\) SCF/IL3/Epo t=15h) technical replicates.

b  Effect of retroviral titres on CD71 expression. *Epor\(^{-}\)/* fetal livers were transduced with retroviral supernatants (‘SN’) which were either undiluted (‘1’), or diluted by 1:5 or 1:10 as indicated. Retroviral vectors encoded Bcl-x\(_L\) or EpoR and the hCD4 reporter. Expression of Epor mRNA was 8-fold lower in cells transduced with the ten-fold dilution of retroviral SN. Analysis was performed on hCD4\(^{+}\) Lin\(^{-}\) cells. Violin lines mark the 25\(^{th}\), 50\(^{th}\) and 75\(^{th}\) percentile with a white circle marking the mean.

c,d  Nuclear offset and cell diameter measurements at t=48h following EpoR or Bcl-x\(_L\) retroviral transduction as in ‘b’. Violin lines mark the 25\(^{th}\), 50\(^{th}\) and 75\(^{th}\) percentile with a white circle marking the mean.
e  Viable cell number following EpoR or Bcl-xL retroviral transduction as in ‘b’.

f  Cell viability, expressed as the fraction (%) of trypan blue negative cells, for the cultures shown in ‘e’.
Figure 2.13. EpoR-transduced \( Epor^{-/-} \) erythroblasts have similar viability to that of wild-type fetal liver cells.

Viability of wild-type and EpoR-transduced fetal liver cells during in vitro culture, measured as the fraction of cells that are trypan- blue negative. \( Epor^{-/-} \) fetal livers were transduced with bicistronic retroviral vector encoding EpoR and the hCD4 reporter. Wild-type fetal liver cells and EpoR- \( Epor^{-/-} \) transduced cells were cultured in the presence of IL-3 and SCF for 15 hours. At \( t=0 \) h, they were switched to an Epo containing medium and differentiated \textit{in vitro} for 72 hours. Data are 2 independent experiments.
Epo increases cell size and prolongs maturation of wild-type erythroblasts 

*in vitro and in vivo.*

To test our conclusions outside the *Epor*−/− transduction model, we asked whether Epo concentration affects cell size and maturation rate in wild-type erythroblasts in culture, and during Epo administration to mice *in vivo.* We differentiated wild-type fetal liver CFU-e (‘S0’ in Fig. 2.14a (Pop, Shearstone et al. 2010)) *in vitro* in the presence of a range of Epo concentrations. Cell size increased in an Epo-concentration-dependent manner, at every stage of differentiation, including reticulocytes (Fig. 2.14b, Fig. 2.15a). The Epo-concentration range affecting cell size, from 0.01 to 10 Units/ml, corresponds to the entirety of the physiological and stress range *in vivo* (Erslev, Wilson et al. 1987, Kojima, Matsuyama et al. 1995). Higher Epo also increased reticulocyte size heterogeneity (Fig. 2.15b).

Epo also caused a dose-dependent delay in maturation. As expected, higher Epo resulted in higher cell number at all stages of differentiation (Fig. 2.15c). However, the distribution of erythroblasts at higher Epo concentrations was increasingly skewed in favor of earlier differentiation subsets (Fig. 2.15d). Similarly, the intensity of Ter119 expression decreased at higher Epo concentrations (Fig. 2.15e). We further assessed cell maturation by measuring the nuclear offset, which decreased with increasing Epo concentration at all flow-cytometric stages (Fig. 2.15f–g). Together these findings show that Epo prolongs ETD in a dose-dependent manner.
We also assessed the effect of Epo on erythroblast cell size in vivo. We injected mice with a range of Epo doses and used nuclear offset as a size-independent measure of maturational stage (Fig. 2.14c). We divided the nuclear offset distribution of all Ter119+ bone-marrow erythroblasts from saline-injected mice into quintiles (Fig. 2.14d). Increasing nuclear offset quintiles corresponded to increasingly mature erythroblast subsets as judged by the established criteria of decreasing CD71 and cell area, confirming the utility of this approach (Fig. 2.14e). We then used the nuclear offset quintiles values from these control mice to classify Ter119+ erythroblasts from Epo-injected mice into five maturational stages. We found that for a given nuclear offset-defined maturational stage, there was an Epo dose-dependent increase in cell diameter. This effect was particularly striking in erythroblasts that corresponded to the two most mature quintiles (Fig. 2.14f, g), confirming that Epo dose regulates erythroblast cell size.

Taken together, graded increases in Epo/EpoR signaling result in graded increases in cell size, shown by varying either the ligand concentration in wild-type erythroblasts (Fig. 2.14b, Fig. 2.15a) or receptor expression in Epor−/− erythroblasts (Fig. 2.9a).
Figure 2.14. Red cell size is sensitive to Epo concentration in both mice and humans.

**a, b** Epo concentration effect on ETD. Wild-type fetal liver cells were enriched for CFU-e progenitors ('S0') and differentiated *in vitro*, in a range of Epo concentrations between 0.01 and 20 U/ml. Cultures were analyzed at 48 h. Data is representative of two independent experiments.

**a** S0 cells upregulate cell surface markers CD71 and Ter119 during differentiation. At 48h cells are distributed between erythroblast subsets S1, S2 and S3. The shown example is from a culture in the presence of Epo at 0.1 U/ml.

**b** Cell diameter distributions of S0 cells at t=0h, and of erythroblast subsets S1 to S3 at 48 h, for cultures in the presence of the indicated Epo concentrations. Violin lines mark the 25th, 50th and 75th percentile with a white circle marking the mean.

**c** Nuclear offset is the ratio of the delta centroid (distance between the centers of the cell and the nucleus, D) and the cell diameter. It is a measure of nuclear eccentricity that is independent of cell size. The cartoon indicates how nuclear offset can be used to measure the increasing nuclear eccentricity during erythroid morphological maturation.

**d-g** Mice were injected with either saline (n = 2) or Epo (5 U, 20 U or 100 U, n=2 for each Epo dose), and bone marrow was analyzed at 48 h.

**d** Ter119+ bone marrow erythroblasts in saline-injected mice were divided into 5 maturational stages, by dividing the nuclear offset distribution into quintiles.
e  CD71/forward-scatter (FSC) histograms for Ter119+ erythroblasts in each of the nuclear offset quintiles. For mice injected with Epo, cells were divided into 5 maturational stages based on the nuclear offset values defined by the control (saline) nuclear offset quintiles.

f  Cell diameter for each of the nuclear offset quintiles in ‘d, e’, for each injected Epo dose. Violin lines mark the 25th, 50th and 75th percentile with a white circle marking the mean. Data are representative from one of two mice injected for each Epo dose.

g  Median cell diameter and median nuclear offset values for cells in each nuclear offset quintile, for mice injected with either Epo (100 U) or Saline. Each data point is for one mouse.

h  MCV and reticulocyte count in two independent human intervention studies. Epo was administered during the period indicated. Because of variability between participants in hematological parameters, data is represented as fractional change relative to the baseline values of each participant (Hidalgo, Bejder et al. 2021). MCV, mean corpuscular volume; Retics, reticulocyte count.

i  RDW_SD and reticulocyte counts for human intervention study #1 (Hidalgo, Bejder et al. 2021).
Figure 2.15. Multiple ETD parameters are sensitive to Epo concentration.

**a - g** Experiment as in Figure 2.14a-b. Wild-type fetal liver cells were enriched for CFU-e progenitors (‘S0’) and differentiated *in vitro*, in a range of Epo concentrations between 0.01 and 20 U/ml. Cultures were analyzed at 48 h. Data are pooled from two independent experiments.

**a** Cell diameter (population medians) of erythroblast subsets S1 to S3 and of reticulocytes.

**b** Heterogeneity in reticulocyte diameter. CV, coefficient of variation.

**c, d** Viable cell number (‘c’) and cell frequency in the culture (‘d’) for each erythroblast subset.

**e** Ter119 intensity of all nucleated erythroblasts in the culture, for each of the indicated Epo concentrations at 48h.

**f, g** Nuclear offset at each Epo concentration and for each erythroblast subset. Violins (‘e’) are representative of two independent experiments. Data medians from both experiments are plotted in ‘f’. Violin lines are the 25th, 50th and 75th percentile with a white circle marking the mean.
EpoR signaling delays induction of p27<sup>KIP1</sup>, leading to increased number of cell cycles.

To investigate the molecular mechanisms underlying EpoR-regulated functions, we compared gene expression in differentiating EpoR-<i>Epor</i>−/− and Bcl-x<sub>L</sub>-<i>Epor</i>−/− erythroblasts, using RT-qPCR (Fig. 2.16). ETD markers <i>Slc4a1</i> (Band3) and <i>Hbb1</i> 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-x<sub>L</sub>-<i>Epor</i>−/− (p < 0.005). Tal1 was previously linked to cell-cycle regulation in hematopoietic cells (Dey, Curtis et al. 2010, Chagraoui, Kassouf et al. 2011).

Among cell-cycle regulators, the CDK inhibitor p27<sup>KIP1</sup> (Cdkn1b) was induced prematurely in Bcl-x<sub>L</sub>-<i>Epor</i>−/−, reminiscent of its premature expression in <i>Epor</i>−/− primitive erythroblasts (Malik, Kim et al. 2013). A second member of the CIP/KIP family, p57<sup>KIP2</sup> (Cdkn1c), was also expressed at somewhat higher levels. The induction of p27<sup>KIP1</sup> toward the end of ETD in wild-type erythroblasts contributes to mitotic exit (Hsieh, Barnett et al. 2000, Bouscary, Pene et al. 2003, Rylski, Welch et al. 2003, Gnanapragasam, McGrath et al. 2016). To determine the effect of its premature induction, we transduced wild-type fetal liver S1 cells (CD71<sup>high</sup>-Ter119<sup>neg</sup>) with either p27<sup>KIP1</sup> or ‘empty vector’ (Fig. 2.17a). p27<sup>KIP1</sup>-transduced cells showed reduced proliferation, without affecting cell viability, suggesting they underwent fewer cell cycles (Fig. 2.17b, c). Unlike Bcl-x<sub>L</sub>-<i>Epor</i>−/− erythroblasts, however, p27<sup>KIP1</sup>-transduced cells were larger, and slower to
undergo maturation, as judged by lower nuclear offset (Fig. 2.17d–f). Therefore, while the EpoR-mediated negative regulation of p27^KIP1 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, LY294002 (Vlahos, Matter et al. 1994), to wild-type erythroblasts resulted in premature induction of p27^KIP1 mRNA (Fig. 2.18), suggesting that negative regulation of p27^KIP1 by EpoR is mediated via PI3K.
Figure 2.16. Premature expression of p27\textsuperscript{KIP1} in the absence of EpoR.

Gene expression in Bcl-x\textsubscript{L} or EpoR-transduced Epor\textsuperscript{−/−} fetal liver cells during their differentiation in vitro. mRNAs were quantitated with RT-qPCR and are expressed relative to expression of β-actin in the same cells.

In each experiment, RT-qPCR was carried out using a dilution series of the cDNA. Data for each gene is mean± SD pooled from a number of independent as follows: Ccnd1, n=6; Ccnd2, n=7; Ccnd3, n=6; E2F4, n=5; Ccne1, n=5; Ccne2, n=6; Ccna1, n=5; Cdk4, n=3; Cdk6, n=3; Cdk2, n=3; Cdkn1c (p57\textsuperscript{KIP2}), n=7; Cdkn1b (p27\textsuperscript{KIP1}), n=7; Cdkn1a (p21\textsuperscript{cip1}), n=2; Slc4a1(Band3), n=6; Hbb1, n=7; Tfrc, n=6; Lmo2, n=6; Bcl-x\textsubscript{L}, a representative experiment of 3; Gata1, n=7; Spi1 (PU.1), n=7; Tal1, n=6; Nfe2, n=6. p values are for a 2-sided paired t test.
Figure 2.17. Premature expression of p27 only partly reproduces the Bcl-xL-Epor⁻/⁻ phenotype.

a  p27<sup>KIP1</sup> gene expression in differentiating wild-type fetal liver cells transduced with either p27<sup>KIP1</sup> or with ‘empty vector’; the bicistronic vectors also expressed the hCD4 reporter. S1 subset cells (CD71<sup>high</sup>Ter119<sup>neg</sup>) were FACS sorted from E13.5 fetal liver cells, transduced with retroviral vectors and cultured in IL-3, SCF, and Epo-containing (0.5U/mL) medium <i>in vitro</i> for 72 hours. mRNA was measured by qRT-PCR, normalized to the β-actin mRNA, and expressed relative to that in control (empty vector)-transduced cells at t=24h. Data are from two independent experiments.

b  Viable cell number (hCD4<sup>+</sup> Annexin V<sup>-</sup>) following p27<sup>KIP1</sup> or ‘empty vector’ retroviral transduction for the experiments in ‘a’.

c  Cell viability (% trypan blue negative cells), for the experiments in ‘a’.

d-f  Nuclear offset (‘d’), cell diameter (‘e’) and nuclear diameter (‘f’) following p27<sup>KIP1</sup> or ‘empty vector’ retroviral transduction for the experiments in ‘a’. Violin lines mark the 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentile with a white circle marking the mean. Colors as in ‘a’, grey marks t=0.
(a) Cdkn1b (p27\textsuperscript{kip1})

Relative mRNA levels

Time (h)

- Vehicle
- LY 294002 1\textmu M
- LY 294002 10\textmu M

(b) Cell number

Time (h)

- Vehicle
- LY 294002 1\textmu M
- LY 294002 10\textmu M

(c) Cell viability (%)

Time (h)
Figure 2.18. PI3-kinase inhibition results in premature induction of p27\textsuperscript{KIP1} and in decreased number of differentiating erythroblasts.

a p27\textsuperscript{KIP1} gene expression in wild type fetal liver cells treated with LY 294002. Ter119\textsuperscript{Lin}\textsuperscript{−} cells were sorted from E13.5 wild-type fetal liver and cultured in the presence of either vehicle (DMSO) or the PI3 kinase inhibitor LY 294002 at the indicated concentrations. mRNA was measured by qRT-PCR, normalized to the β-actin mRNA, and expressed relative to vehicle. Data is median of 4 technical replicates.

b Viable cell number for the experiment in ‘a’. Data represents 3 technical replicates.

c Cell viability (% of trypan blue negative cells) for the experiment in ‘a’.
Several EpoR signaling pathways are implicated in the regulation of cell size.

EpoR activates three principal signaling pathways: ras/MAP kinase, Stat5 (Koulnis, Porpiglia et al. 2012, Porpiglia, Hidalgo et al. 2012), and PI3K (Lodish, Ghaffari et al. 2009, Kuhrt and Wojchowski 2015). Neonatal mice hypomorphic for Stat5 have microcytic anemia (Socolovsky, Nam et al. 2001). Here we found that, similarly, circulating red cells from E13.5 Stat5-deficient embryos are smaller than those of wild-type littermates (Fig. 2.19a, b). Using U0126, a MEK1- and MEK2-specific inhibitor (Favata, Horiuchi et al. 1998), 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 (Fig. 2.19c, d). Therefore, it is likely that cell size regulation by EpoR is the integrated result of multiple signaling pathways.
Cell diameter (μm)

- Litter A
- Litter B
- Litter C
- Litter D

Wild-type Stat5^+/− Stat5^-/-

ANOVA p=0.036

Drug (μM)

- Vehicle
- LY 294002
- U0126

Reticulocyte diameter (μm)

- Cell diameter (relative to vehicle control)

p=0.001
p=0.008
p=0.001
p=0.0003
p=0.01
Figure 2.19. EpoR regulates cell size via multiple signaling pathways.

a  Cell diameters of circulating red blood cells in E13.5 littermate embryos from matings of Stat5<sup>+/−</sup> x Stat5<sup>+/−</sup> mice. Violin lines mark the 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentile with a white circle marking the mean.

b  Median cell diameters of circulating red blood cells from panel ‘a’, by genotype. The box and whiskers mark the 25<sup>th</sup> to 75<sup>th</sup> percentiles and min to max values, respectively with the median indicated. p value is one-way ANOVA. The data is pooled from a total of n = 5 wild type, n = 7 Stat5<sup>−/−</sup>, and n = 15 Stat5<sup>+/−</sup> embryos, derived from n = 4 litters, as shown in panel ‘a’.

c  Effect of inhibiting either PI3 kinase (with LY294002) or MEK1/MEK2 (U0126) on the cell diameter of S2 and S3 erythroblasts and on reticulocytes. S0 cells were isolated from E13.5 wild-type fetal liver and differentiated in vitro in the presence of either vehicle (DMSO) or with inhibitors at the indicated concentrations. Diameters were measured at 48 h. Data are population medians from 3-5 experiments for each inhibitor concentration. Box and whiskers mark the 25<sup>th</sup> to 75<sup>th</sup> percentiles and min to max values respectively, with the median indicated. p values are for unpaired 2-tailed t-test. p value for one-way ANOVA is <0.0001.

d  Violin plot of reticulocyte diameters for a representative experiment from ‘c’, inhibitor concentrations were each 10mM. Violin lines mark the 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentile with a white circle marking the mean. p value is for unpaired t test. One-way ANOVA p value for all samples is <0.0001.
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. 2.14h, (Hidalgo, Bejder et al. 2021)), or subjected to phlebotomy (study #3, (Hidalgo, Bejder et al. 2021)). 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 (Heuberger, Rotmans et al. 2017)). Here we present the detailed blood parameters associated with these studies.

In study #1 (Fig. 2.14h, (Hidalgo, Bejder et al. 2021)), 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. 2.14h, (Hidalgo, Bejder et al. 2021)), 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 (Hidalgo, Bejder et al. 2021), 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 (Hidalgo, Bejder et al. 2021). 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, (Hidalgo, Bejder et al. 2021)). 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. 2.14h). Similarly, in study #3, MCV values continued to climb at a time when the reticulocyte count was declining (Hidalgo, Bejder et al. 2021). 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. 2.14i, (Hidalgo, Bejder et al. 2021); no RDW is available for study #3). There was a significant, positive correlation between MCV and RDW-SD ($r = 0.51$, $p = 2 \times 10^{-28}$ for study #1; $r = 0.52$, $2 \times 10^{-24}$ for study #2).

Red-cell volume declines continuously as red cells age (Bosch, Werre et al. 1992, d’Onofrio, Chirillo et al. 1995, Willekens, Roerdinkholder-Stoelwinder et al. 2003, Gifford, Derganc et al. 2006, Franco, Puchulu-Campanella et al. 2013). 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 EpoR signaling on red-cell size (Hidalgo, Bejder et al. 2021). 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 EpoR 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 RCs. Human intervention studies are consistent with a similar effect of EpoR signaling on red-cell size in human erythropoiesis (Hidalgo, Bejder et al. 2021). In the discussion below we integrate the apparently disparate EpoR functions into a coherent model (Fig. 2.20). 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 (Koulnis, Porpiglia et al. 2012) (Fig. 2.20). EpoR expression peaks in early erythroblasts, which are highly dependent on EpoR signaling for survival (Koury, Bondurant et al. 1988, Liu, Pop et al. 2006, Socolovsky, Murrell et al. 2007), and exquisitely sensitive to Epo, as judged by Stat5 phosphorylation (Porpiglia, Hidalgo et al. 2012). By contrast, late
erythroblasts downregulate EpoR (Zhang, Socolovsky et al. 2003) and are relatively resistant to apoptosis (Liu, Pop et al. 2006, Socolovsky, Murrell et al. 2007). 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 erythroblastic 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 (Thomas 2017) (see the “Materials and 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 hours 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 (Hwang, Futran et al. 2017, Eastman, Chen et al. 2020), and much faster than cycles in late ETD (Shearstone, Pop et al. 2011, Hwang, Futran et al. 2017, Eastman, Chen et al. 2020); 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. 2.20). One of the factors known to regulate the onset of late ETD is p27KIP1, whose induction promotes slower cycling and cell-cycle exit (Panzenböck, Bartunek et al. 1998, Hsieh, Barnett et al. 2000, Gnanapragasam and Bieker 2017). 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 proteosomal degradation (Bouscary, Pene et al. 2003). A similar role for EpoR, delaying p27KIP1 induction and morphological maturation, was noted in primitive yolk-sac erythroblasts (Malik, Kim et al. 2013). The converse was found in Klf1−/− erythroblasts, which fail to induce p27KIP1 and fail to undergo cell-cycle exit (Gnanapragasam, McGrath et al. 2016). 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 p27^{kip1}.

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 (Humbert, Rogers et al. 2000), cyclin D3 (Sankaran, Ludwig et al. 2012), CDK2, or CDK4 (Jayapal, Wang et al. 2015) 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 (Burns, Reed et al. 1986), 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 (Han, Yu et al. 2001, Chen and Zhang 2019). 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 (Zhang, Macias-Garcia et al. 2018), 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 (Porpiglia, Hidalgo et al. 2012), can override this default state and maintain cell size in spite of rapid cycling (Fig. 2.20). The maintenance of cell size in dividing cells is the norm in most tissues (Ginzberg, Kafri et al. 2015, Björklund 2019) and so it is possible that EpoR signaling permits early erythroblasts to employ similar pathways of size control as those found outside ETD. The mechanisms that determine the characteristic size of a cell and that maintain it through cell divisions are not fully understood, but are thought to depend on strong growth factor signaling to promote the metabolic pathways required for building biomass (Björklund 2019). To maintain their size, cells must attain a size threshold before committing to cell division; in avian erythroblasts and other cell types, a larger size correlates with a longer G1 phase (Dolznig, Grebien et al. 2004, Ginzberg, Kafri et al. 2015). The ability of EpoR signaling to increase cell size in early erythroblasts, which are some of the most rapidly dividing cells in vivo (Hwang, Futran et al. 2017, Eastman, Chen et al. 2020), predicts that these cells have exceptionally efficient mechanisms for growth. Conversely, this also implies that impairments in growth pathways would have a specifically deleterious effect, potentially contributing to
the selective damage of ribosomopathies in the erythroid lineage (Narla and Ebert 2010).

Together with an increase in cell size, high Epo also increased cell size heterogeneity, in mouse and human. Unlike low Epo 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 sensitivities (Kelley, Koury et al. 1993, Liu, Pop et al. 2006), in which the strength of EpoR signaling may vary, giving rise to a range of red-cell sizes (Fig. 2.20).

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 (Hidalgo, Bejder et al. 2021). 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 MCV (Seiki, Naito et al. 2018, Ludwig, Lareau et al. 2019, Read, Schlauch et al. 2019, Timmer, Tanck et al. 2019). These include Epo, Epor, and Lnk, all expected to alter EpoR signaling strength (Tumburu and Thein 2017). 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 hypoxemic patients with chronic obstructive pulmonary disease (Pavlović-Kentera, Bogdanović et al. 1977, Tsantes, Papadhimitriou et al. 2004) and in iron-replete pregnancy (Chanarin, McFadyen et al. 1977, Hoffbrand and Provan 1997). An increase in RDW was recently proposed as a potential longer-term biomarker for brief hypoxemic episodes in conditions such as acute respiratory distress, sepsis, or congestive heart failure (Yčas, Horrow et al. 2015, Schepens, De Dooy et al. 2017). Indeed, clinically, the RDW may prove to be a more sensitive marker of EpoR 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 microcytic (Geissler, McFarland et al. 1981), 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 anemia (Geissler, McFarland et al. 1981, Waskow, Terszowski et al. 2004), and can be now be explained by a paucity of progenitors entering ETD and the expected compensatory increase in Epo (Kabay, Akiyama et al. 1995). Transgenic expression of Epo rescues the lethal c-Kit<sup>W/W</sup> mutation,
also resulting in macrocytic red cells (Waskow, Terszowski et al. 2004). 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 (Hidalgo, Bejder et al. 2021). 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 tissues (Benesch and Benesch 1967, Bunn 1981). The affinity of 2,3-DPG to hemoglobin increases significantly at lower MCHC (Garby and De Verdier 1971). 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.
**Strong EpoR signaling**
Increased cell cycle number & speed, cell size maintained

**Weak EpoR signaling**
Fewer & slower cell cycles, decreasing cell size

- **High Epo**
  - **Early ETD (Epo-dependent)**
  - **Late ETD (Epo-independent)**
  - **Strong EpoR signaling**
  - **Weak EpoR signaling**

- **Low Epo**
  - **Higher MCV, increased RDW**
  - **Lower MCV, lower RDW**
Figure 2.20. EpoR signaling promotes rapid cycling while maintaining cell size in early erythroblasts.

Proposed model explaining EpoR-dependent functions during ETD. Only early erythroblasts express appreciable cell surface EpoR and 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 a loss in cell size. 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 result in larger erythroblasts 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, are expected to attain only weak EpoR signaling even in the presence of high Epo, giving rise to smaller red cells.
Materials and 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 cycle (Ginzberg, Kafri et al. 2015, Hu, Eastman et al. 2019)). 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.

Stat5−/− 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 dark/light 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 25mM HEPES (Gibco), 20% fetal calf serum (Hyclone), 1% penicillin/streptomycin (ThermoFisher Scientific), 2 × 10^{-4}M β-Mercaptoethanol (Sigma), supplemented when indicated with 0.5 IU/ml Epo (Procrit, Amgen; 1 IU/ml = 1.2 ng/ml) and 100 ng/ml SCF (Peprotech), and 10 ng/ml IL3 (Peprotech).

**Isolation of mouse erythroid progenitors.**

To isolate wild-type S0 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 using either EasySep beads a (StemCell Technologies) or MojoSortTM Streptavidin Nanobeads (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 DAPI (Roche). FACS data were analyzed using FlowJo software (Tree Star Inc., CA).

Antibodies used:

- PE Mouse Anti-Human CD4 (RPA-T4) (BD Biosciences) dilution 1:50
- PE/Cy7 Rat Anti-Mouse CD71 (RI7217) (BioLegend) dilution 1:100
- APC/Cyanine7 Rat Anti-Mouse Ter119 (Ter119) (BioLegend) dilution 1:100
- PE Rat Anti-Mouse Ter119 (Ter119) (BD Biosciences) dilution 1:100
- APC Rat Anti-Mouse Ter119 (Ter119) (BD Biosciences) dilution 1:100
- biotin Rat Anti-Mouse CD71 (C2) (BD Biosciences) dilution 1:100
- biotin Rat Anti-Mouse Ter119 (BD Biosciences) dilution 1:100
- biotin Rat Anti-Mouse Ly-6G and Ly-6C/Gr1 (RB6-8C5) (BD Biosciences) dilution 1:100
- biotin Rat Anti-Mouse CD11b/Mac1 (M1/70) (BD Biosciences) dilution 1:100
- biotin 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 CD45R/B220 (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 CD45R/B220 (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 Flowsight cytometer (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 Draq5 Morphology mask for nuclear area. Raw flow cytometric feature data were exported and analyzed in R programming 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, Tight)) 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 manufacturer bead sizes as a predictor for the Amnis calculated cell area (Stats, base R, degree = 2), with an $R^2$ 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 (Fig. 2.8).

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 (geom_density) (Wickham 2016) and colors chosen from viridis (Garnier 2018). 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 (Bcl-x\textsubscript{L} \textsuperscript{GFP} + V\textsuperscript{hCD4}, V\textsuperscript{GFP} + EpoR\textsuperscript{hCD4}, and Bcl-x\textsubscript{L} \textsuperscript{GFP} + EpoR\textsuperscript{hCD4}), the mean cell diameter was then calculated within each of these composite bins (i.e., the mean cell diameter in Sample X, for GFP quantile Y and CD4 quantile Z). Next, the ratio of the mean cell diameters within each CD4/GFP bin were calculated between Bcl-x\textsubscript{L} \textsuperscript{GFP} + V\textsuperscript{hCD4} and Bcl-x\textsubscript{L} \textsuperscript{GFP} + EpoR\textsuperscript{hCD4} using V\textsuperscript{GFP} + EpoR\textsuperscript{hCD4} 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 positive events. The raw mean pixel intensity of Draq5 (nuclei) was plotted against the total Draq5 intensity (nuclei), giving two clearly distinct populations. We visually confirmed lack of Draq5 signal in the enucleated reticulocyte population.

Cytospins.

Cells were spun onto coated ShandonTM Cytoslides (Thermo Scientific) using a ShandonTM Cytospin3 at 800 rpm for 5 minutes. The slides were dried, fixed and stained (Pop, Shearstone et al. 2010). 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 (Hwang, Futran et al. 2017). Briefly, cells were pulsed at a final concentration of 33 µM BrdU for 30 minutes. Cells were immediately labeled with the LIVE/DEAD
Kit (Invitrogen L23105), fixed, and permeabilized. Erythroid 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 (Bio-Legend). DNA content was measured by 7AAD (BD Biosciences).

**Retroviral Transduction and in vitro differentiation of fetal liver cells.**

Epor, Bcl-xL, and Tfrc were subcloned into MSCV-IRES-hCD4 retroviral vector. Bcl-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, 25mM HEPES) (Gibco), 20% fetal calf serum, 1% penicillin/streptomycin, 10^{-4}M β-Mercaptoethanol.

Retroviral transduction was done by spin infection of Epor^{-/-} or Epor^{-/-} Hnf^{-/-} fetal liver cells at 2000 rpm, 30 °C for 1 hour on 50 µg/ml fibronectin (GIBCO) coated dishes in 4 µg/ml polybrene (Sigma), supplemented in some experiments with 0.5 U/ml Epo (Amgen). Transduced cells were incubated for 15 hours with 100 ng/ml SCF and 10 ng/ml IL3 (Peprotech). Cells were then transferred to differentiation medium: IMDM (L-glutamine, 25mM HEPES)
(Gibco), 20% fetal calf serum, 1% penicillin/streptomycin, $10^{-4}$M β-Mercaptoethanol, and 0.5 U/ml Epo (Amgen) for the indicated times. In the case of experiments that include $Epor^{-/-}Hri^{-/-}$, the media was also supplemented with 1 mg/ml iron-saturated human transferrin (Sigma). Where indicated, liquid cultures 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 deoxyribonucleosides (3'-'Deoxythymidine, 2'-Deoxyguanosine monohydrate, 2'-Deoxyadenosine monohydrate, 2'-Deoxycytidine, Sigma).

**In vitro differentiation of fetal liver cells with PI3K and MEK1/MEK2 inhibitors.**

Isolated wild-type S0 cells were cultured in differentiation media (Epo 0.5 U/ml) with 1 µM or 10 µM PI3K inhibitor, LY294002 (EMD Millipore) or MEK1/MEK2 inhibitor, U0126 (EMD Millipore). Inhibitors were replenished every 24 hours.

**Colony-formation assays in methylcellulose.**

Retroviral transduction was done by spin infection of $Epor^{-/-}$ fetal liver cells as described above. From each transduced sample (4 hours post infection), 200,000 cells were mixed with 1 ml MethoCult (M3234, STEMCELL Technologies) supplemented with 2 U/ml Epo (Amgen). Erythroid (CFU-e) was
scored from duplicate plates on day 3 of culture. Expression of hemoglobin in erythroid colonies was confirmed by staining with diaminobenzidine (Sigma) in situ before scoring. Colony area was measured using ImageJ version: 2.0.0-r-54/1.51 h.

**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.

**TaqMan MGB probes used:** β-actin (Mm02619580_g1), PU.1 (Mm00488140_m1), GATA1 (Mm01352636_m1), GATA-2 (Mm00492300_m1), Alas2 (Mm01260713_m1), Band3 (Mm01245920_g1), β-globin (Mm01611268_g1), p21 (Mm00432448_m1), p27 (Mm00438168_m1), p57 (Mm01272135_g1), p16ink4a (Mm01257348_m1), p15ink4b (Mm00483241_m1), p18ink4c (Mm00483243_m1), p19ink4d (Mm00486943_m1), CCND1 (Mm00432359_m1), CCND2 (Mm00438071_m1), CCND3 (Mm01612362_m1),
CCNE1 (Mm00432367_m1), CCNE2 (Mm00438077_m1), CCNA2 (Mm00438063_m1), CCNA1 (Mm00432337_m1), E2F2 (Mm00624964_m1), E2F4 (Mm00514160_m1), Tfrc (Mm00441950_m1), Bcl-xL (Mm00437783_m1), DNMT1 (Dnmt100599784), Ifitm1 (Mm01279023_m1), Ifitm3 (Mm00847057_s1), Tal1 (Mm00441665_m1), NFE2 (Mm00801891_m1), LMO2 (Mm00493153_m1), cdk6 (Mm00438163_m1), cdk6 (Mm01311342_m1), cdk4 (Mm00726334_s1), cdk2 (Mm00443947_m1), cdc25a (Mm00483162_m1), cdc25b (Mm00499136_m1), cdc25c (Mm00486880_m1), Klf1 (Mm00516096_m1).

**Epo stimulation in vivo.**

Epo (Epoetin alfa; Amgen) was injected subcutaneously in a total volume of 300 µL in sterile isotonic saline, at the indicated doses and frequencies.

**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 $25 \pm 3$ years, height $179 \pm 10$ cm and weight $70 \pm 10$ kg) participated in the erythropoietin treatment intervention: $n = 25$ received Epo, $n = 9$ received Placebo. Another 21 healthy non-smoking male subjects of European descent (age $29 \pm 6$ years, $184 \pm 7$ cm, and $77 \pm 8$ 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 letters for the studies (protocol numbers H-2-2014-109 & H-17036662, enclosed with the Supplementary Information files; registration number, registration number NCT04227665 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 aim to identify novel biomarkers 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. rhEPO treatment:**

The study 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⁻¹ epoetin alpha (Eprex, Janssen, Birkerød, Denmark) (rhEPO group, 25 participants; 13 male and 12 female) or saline (placebo group, 9 participants; 6 male and 3 female) every second day. Venous blood samples were collected weekly during the treatment and for 5 weeks following treatment.

**Experimental design. 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 minutes of rest in a seated position and with <30 seconds use of tourniquet. In the rhEPO
trial, samples were immediately analyzed for a complete blood count using a Sysmex XN-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 hours 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 elsewhere71, but reporting did not include MCV and RDW information. Briefly, non-professional well trained male cyclists ages 28–50 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 package (Lindstrom and Bates 1988). 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 package (Hothorn, Bretz et al. 2008) followed by multiplicity adjustment using Benjamini–Hochberg procedure (Benjamini and Hochberg 1995). For intervention study 3, each post-baseline time point was analyzed separately with the model that includes subject as random effect, treatment, period, and sequence of treatments as fixed effects (supplementary statistical analysis of human studies).

For mouse and in vitro experiments, we used both parametric and nonparametric statistical significance tests for sample comparisons as indicated in each figure legend.
CHAPTER III:
EPOR AND STAT5 SIGNALING ACCELERATE RIBOSOME BIOGENESIS
AND PROTEIN SYNTHESIS RATES IN EARLY ERYTHROBLASTS

Abstract

We recently found that EpoR signaling promotes rapid cycling of early erythroblasts, while simultaneously increasing their cell size, resulting in the formation of larger and more numerous red cells, in both mice and humans. These findings suggest that EpoR signaling re-sets the relationship between cell division and cell growth during erythroid terminal differentiation. To investigate this possibility, we undertook single cell RNA sequencing (scRNA-seq) of mouse fetal livers deleted for either Epor or one of its downstream signaling mediators, Stat5. This analysis showed that EpoR and Stat5 signaling regulate expression of multiple genes involved in ribosomal RNA (rRNA) transcription and processing as well as regulators of translation. Further, functional analysis showed an EpoR and Stat5-dependent spike in the rates of rRNA transcription, global protein synthesis, and the rate of growth in cell size, centered on a narrow developmental window at the onset of erythroid terminal differentiation. This unusual spike in growth rate correlates with a spike in the speed of the cell cycle within the same developmental window. Our findings suggest novel EpoR/Stat5 pathways that accelerates growth in cell biomass through regulation of ribosome
biogenesis and protein translation rates, potentially explaining the sensitivity of the erythroid lineage to ribosomal protein mutations.
Introduction

We recently found that EpoR signaling increases the number and speed of early erythroblast cell cycles, including the speed of S phase. EpoR signaling also increases early erythroblast cell size, in both fetal liver and adult tissue (chapter II, (Hidalgo, Bejder et al. 2021)). These findings suggest that EpoR signaling increases the rate of growth in biomass, since early erythroblasts are either maintaining or growing in size while their cycle becomes shorter. Given that early erythroblasts have some of the shortest somatic cycles ((Hwang, Futran et al. 2017, Eastman, Chen et al. 2020), the mechanisms that make this possible are of special interest.

The mechanisms underlying the regulation of cycling and growth by EpoR are not clear. Growth factors promote progression through the G1 phase by activating G1 cyclin/CDK complexes via cMyc activation and inhibition of CDK inhibitors (Wang 2021). Stat5 was shown to induce all three D-cyclins in various tissues (Matsumura, Kitamura et al. 1999, Martino, Holmes et al. 2001, Brockman, Schroeder et al. 2002, Fernández de Mattos, Essafi et al. 2004, Fang, Menon et al. 2007, Sakamoto, Creamer et al. 2007, Mao, Li et al. 2011, Gupta, Li et al. 2019, Zhao, Xu et al. 2019), so it is likely that EpoR/Stat5 signaling shortens G1 via this mechanism. However, little is known regarding growth factor regulation of S phase speed, an effect exerted by EpoR in early erythroblasts (Chapter II).
In addition to promoting cycling, growth factors promote cell growth, through pathways that include PI3K and MAPK/Erk activating mTor, a central regulator of ribosome biogenesis. In both yeast and mammals, Tor/mTor stimulates all three RNA polymerases to increase pre-rRNA, 5S rRNA and ribosomal protein mRNA transcription as well as ribosomal protein translation (Mayer and Grummt 2006, Saba, Liakath-Ali et al. 2021). Growth factor signaling also activates c-Myc (Kelly, Cochran et al. 1983, Kelly, Cochran et al. 1984), which induces multiple genes controlling multiple aspects of cell growth and proliferation (Dang, O'Donnell et al. 2006), including direct regulation of multiple steps in ribosome biogenesis (van Riggelen, Yetil et al. 2010). EpoR is therefore likely to promote cell growth via the stimulation of mTor and c-Myc. Stat5 was recently found to bind to the c-Myc super-enhancer (Pinz, Unser et al. 2016)

In this chapter we began to investigate the mechanisms that allow EpoR to promote fast cycling and exceptionally fast growth in erythroblasts. We began by undertaking single-cell RNA-sequencing of Epor<sup>−/−</sup> and of Stat5<sup>−/−</sup> fetal liver, in order to identify dysregulated pathways and determine the effect of cell transcriptomes. To our surprise, we found that genes implicated in ribosomal biogenesis and protein translation were dysregulated in both these models. Measurement of ribosomal biogenesis and protein synthesis rates in these mutant fetal livers (FLs) confirms a unique role for EpoR and Stat5 signaling in these functions.
Results


To investigate the roles of EpoR and Stat5 signaling in early erythroid progenitors, we undertook single-cell RNA sequencing (scRNA-seq) of fetal livers (FLs) from mid-gestation Epor−/− and Stat5−/− embryos using the inDrops platform (Klein, Mazutis et al. 2015). In each case, we sequenced cells from 3 mutant FLs along with 3 matching wild-type littermate controls (Fig. 3.1). We filtered out cell transcriptomes with low reads, high fraction of mitochondrial transcripts (dead/dying cells), and cell doublets/multiplets. At least 4300 cells passed these quality control measures for each of the genotypes (range: 4300 to 6937 cells, Fig. 3.1. and Fig. 3.2.).

Projection of the data onto 2 dimensions using Uniform Manifold Approximation and Projection (UMAP (Becht, McInnes et al. 2018)) graphs show that Epor−/− and Stat5−/− fetal livers have far fewer cells expressing the ETD program, marked by genes like beta-globin (Fig. 3.1. and Fig. 3.2.). We further projected the transcriptomes of each fetal liver onto a reference dataset of wild-type fetal liver cells from our previous scRNA-seq analysis (Tusi, Wolock et al. 2018). This approach allowed us to identify cell transcriptomes that were part of the erythroid trajectory, and to find their location on the reference erythroid pseudotime, in which cells that are considered part of the erythroid trajectory are ordered along a linear axis based on the Population Balance Analysis (PBA) algorithm (Tusi, Wolock et al. 2018) (Fig. 3.2 a, c).
We previously divided the erythroid pseudotime into five segments, based on functional and transcriptomic criteria. These developmentally sequential segments correspond to multipotential progenitors (MPP), erythroid/basophil or mast cell/ megakaryocytic progenitors (EBMP), early erythroid progenitors (EEP, functionally BFU-e), committed erythroid progenitors (CEP, functionally CFU-e), and precursors undergoing erythroid terminal differentiation (ETD). For both Epor<sup>-/-</sup> and Stat5<sup>-/-</sup> fetal livers, the distribution and number of cell transcriptomes prior to the ETD stage is not significantly different from that of wild-type; but there is a sharp drop in both cell number and cell density during ETD (Fig. 3.2, Table 3.1).
Figure 3.1. Cells in erythroid terminal differentiation are absent in $\text{Epor}^{-/-}$ and $\text{Stat5}^{-/-}$ fetal livers.

UMAP of 3 $\text{Epor}^{-/-}$ (E12.5, left panels), 3 $\text{Stat5}^{-/-}$ (E13.5, right panels), and 3 matching wild-type littermate controls for each genotype. Mutant FLs have fewer cells in the ETD program (as seen in Hbb-bs UMAP) and over-express cKit.
Figure 3.2. *Epor<sup>−/−</sup>* and *Stat5<sup>−/−</sup>* erythroid trajectory cells ordered along a linear ‘pseudotime’ have fewer cells and lower cell density in ETD.

(a,c) Transcriptomes of 3 *Epor<sup>−/−</sup>* (E12.5), 3 *Stat5<sup>−/−</sup>* (E13.5), and 3 matching wild-type littermate controls for each genotype were projected onto a reference wild-type dataset from a previous scRNA-seq analysis allowing for identification of their location on a reference erythroid pseudotime (Tusi, Wolock et al. 2018).

(b,d) Cell number quantification for mutant and wild-type FLs throughout the erythroid pseudotime. Mutant FLs display a normal cellular distribution prior to the ETD stage but significantly lower cell number and density during ETD (left panels, see Table 3.1).
Table 3.1. Mutant (*Epor* /− and *Stat5* /−) and wild-type fetal liver cell distribution throughout an erythroid pseudotime.

Quantification of *Epor* /− (top panel), *Stat5* /− (bottom panel) and matching wild-type littermate cell number and cell density during several developmental stages using an erythroid pseudotime (n=3 for each genotype). Statistical values are for an unpaired *t* test.
Transcriptional changes to key erythroid regulators in the Epor⁻/⁻ and Stat5⁻/⁻ fetal liver.

Full analysis of differentially expressed genes will be documented elsewhere. Here we note that for both the Stat5⁻/⁻ and Epor⁻/⁻ datasets, there was failure to upregulate erythroid terminal differentiation genes including globin genes and the transferrin receptor (Tfrc) (Fig. 3.3). In the case of Tfrc, this is consistent with well-described Stat5 transcriptional regulation (Kerenyi, Grebien et al. 2008, Zhu, McLaughlin et al. 2008). We also noted that key erythroid transcriptional regulators including Klf1, Gata1 and Tal1 were expressed at significantly lower levels than in wild-type controls, throughout erythroid pseudotime, which may in part be responsible for the ETD differentiation arrest (in Epor⁻/⁻ FLs) or delay (in Stat5⁻/⁻ FLs) (Fig. 3.3). By contrast, Kit, encoding a tyrosine kinase receptor expressed in hematopoietic stem cells that is gradually downregulated with differentiation, was expressed at significantly higher levels throughout erythroid pseudotime, in both Epor⁻/⁻ and Stat5⁻/⁻ fetal livers (Fig. 3.3). Also of interest, the transcription factor Myc was expressed at normal levels for most of erythroid pseudotime but failed to decrease in ETD, remaining significantly higher than in wild-type. These observations might be linked: Kit over-expression in the Epor and Stat5-deleted FLs may be responsible for both higher Myc and lower levels of erythroid transcriptional regulators (Haas, Riedt et al. 2015, Dahlin, Hamey et al. 2018).
a  Epor⁻/⁻ v wild-type

Erythroid pseudotime

b  Stat5⁻/⁻ v wild-type

Erythroid pseudotime
Figure 3.3. $Epor^{−/−}$ and $Stat5^{−/−}$ fetal livers display transcriptional changes in important erythroid regulators.

Gene expression changes throughout the erythroid pseudotime for specific erythroid regulators and erythroid terminal differentiation genes in (a) $Epor^{−/−}$ (blue), (b) $Stat5^{−/−}$ (blue) and matching wild-type littermate (magenta) fetal livers (n=3 for each genotype).
Cell-cycle gene dysregulation in Epor<sup>−/−</sup> and Stat5<sup>−/−</sup> fetal livers.

We found expression changes in cell cycle genes that are consistent with our observations in Chapter II, where we showed that EpoR signaling accelerates S phase, shortens the cycle and increases the number of cell divisions in early erythroblasts. Positive regulators of the cycle were expressed at significantly lower levels. These include G1 and S-phase cyclins (Ccnd2, Ccnd3, Ccne1, Ccna2), G1 CDKs (Cdk4, Cdk6), S-phase transcription factor E2f4, and the regulatory subunit of ribonucleotide reductase (Rrm2) (Fig. 3.4). Conversely, some negative regulators of the cycle were over-expressed, including the CDK inhibitors Cdkn2c and Cdkn2d. Of note, for some genes (e.g. Ccnd2), the deficit in expression was more severe in the Stat5<sup>−/−</sup> fetal livers than in Epor<sup>−/−</sup>, possibly reflecting mouse strain differences, or alternatively, Stat5 activation by other receptors, e.g. Kit, in the Epor<sup>−/−</sup> fetal liver.
a) Epor\(^{-/-}\) v wild-type: cell cycle genes

- 

b) Stat5\(^{-/-}\) v wild-type: cell cycle genes

-
Figure 3.4. *Epor*\(^{-/-}\) and *Stat5*\(^{-/-}\) fetal livers display cell cycle gene changes throughout the erythroid pseudotime.

Gene expression changes throughout the erythroid pseudotime for specific cell cycle genes in (a) *Epor*\(^{-/-}\) (blue), (b) *Stat5*\(^{-/-}\) (blue) and matching wild-type littermate (magenta) fetal livers (n=3 for each genotype).
Dysregulation of ribosome biogenesis and translation regulation genes.

We found multiple ribosomal protein genes among the most highly differentially expressed genes in both Epor<sup>−/−</sup> and Stat5<sup>−/−</sup> FLs (Fig. 3.5a, Fig. 3.6a). Translation elongation factors Eef1g, Eef1b2, Eef1a1, were also highly expressed. Conversely, multiple genes that are involved in ribosomal RNA transcription and processing during ribosome biogenesis were downregulated in both the Stat5<sup>−/−</sup> and Epor<sup>−/−</sup> datasets (Fig. 3.5 and Fig. 3.6), as were translation initiation factors Eif1, Eif1ad, Eif4e.

Ribosome biogenesis is a complex, energetically expensive process that involves all three RNA polymerases and multiple ribosomal biogenesis factors (RBFs) many of which are themselves multi-gene ribonucleoprotein (RNP) complexes (Pederson 2011, Bohnsack and Bohnsack 2019). The process begins in the nucleolus with the transcription of the pre-ribosomal RNA (pre-rRNA) 47S transcript by RNA polymerase I. Import of 80 ribosomal proteins from the cytoplasm to the nucleolus leads to assembly of the pre-ribosomal 90S particle, in which the pre-rRNA is extensively modified by RBFs, including a central cleavage event that gives rise to the precursors of the large (60S) and small (40S) ribosomal subunits. Following export to the cytoplasm the ribosome subunits undergo additional processing and maturation. Fig. 3.5b and Fig. 3.6b show several rRNA transcription and processing genes that are downregulated in the Epor<sup>−/−</sup> and Stat5<sup>−/−</sup> FLs: Ddx21 (an RNA helicase, constituent of the UTP-B complex forming part of the small subunit processome (Bohnsack and Bohnsack
2019)); Snu13 (constituent of the U3 small nucleolar (sno)RNP, also part of the processome); PnoI (plays a role in the maturation of the decoding center during 40S biogenesis); Rrs1 (facilitates the integration of the 5S RNP into the pre-60S complex); Nkrf (constituent of the XND complex that remodels pre-rRNA transcript prior to its initial cleavage); Nop9 (a nucleolar protein); Gar1 (part of a pseudouridylation complex that modifies rRNAs); Ttf1 (Transcription termination factor 1, required for termination and re-initiation of the rRNA transcription cycle by RNA polymerase I). In addition, expression of multiple subunits of RNA polymerase I (transcribing rRNA), II (ribosomal protein transcription) and III (5S transcription) are also downregulated in the Stat5−/− FLs (Fig. 3.7, which documents expression of all the subunits for all three polymerases).

The upregulation of ribosomal protein transcripts, coupled with downregulation of transcripts encoding regulators of rRNA transcription, appeared paradoxical, since it might be expected that these two processes would be positively correlated and closely coupled. To look at this issue further, we asked whether we could determine rRNA transcript levels in the scRNA-seq data. Although the inDrops platform enriches for polyadenylated mRNAs, approximately 20% of transcripts are derived from non-polyadenylated products, either rRNA or pre-mRNAs. We examined our scRNA-seq datasets in collaboration with Dr. Robert Beagrie and Jennifer Herrmann (Weatherall Institute of Molecular Medicine, University of Oxford) and they indeed found that both Epor−/− and Stat5−/− FLs expressed significantly fewer rRNA transcripts than
wild-type FL controls, using two different approaches for aligning rRNA transcripts to the genome (personal communication). By itself, this finding is associated with methodological uncertainties: the selection for polyadenylated mRNA and away from rRNA, and the challenges of aligning repetitive rRNA to the genome. Nevertheless, this early result motivated us to pursue this question further.
a Epor⁻/⁻ v wild-type: ribosomal proteins, translation elongation
Figure 3.5. *Epor<sup>−/−</sup>* fetal livers display an upregulation of ribosomal protein genes and translation elongation factors while displaying downregulation of rRNA processing genes and of translation initiation factors.

(a) 3 *Epor<sup>−/−</sup>* (blue) and 3 wild-type littermate (magenta) fetal liver gene expression profiles for representative ribosomal protein and translation elongation factor genes throughout erythroid pseudotime, which are upregulated in mutant FLs. (b) Representative rRNA processing and translation initiation factor genes, which are downregulated in mutant FLs.
Stat5\(-/-\) v wild-type: ribosomal proteins, translation elongation

Transcripts per cell (normalized)

Erythroid pseudotime
Figure 3.6. *Stat5*\(^{-/-}\) fetal livers display an upregulation of ribosomal protein genes and translation elongation factors while displaying downregulation of rRNA processing genes and of translation initiation factors. 

(a) 3 *Stat5*\(^{-/-}\) (blue) and 3 wild-type littermate (magenta) fetal liver gene expression profiles for representative ribosomal protein and translation elongation factor genes throughout an erythroid pseudotime, which are upregulated in mutant FLs. (b) Representative rRNA processing and translation initiation factor genes, which are downregulated in mutant FLs.
Expression of RNA Polymerases I, II and III subunits in the Stat5^{-/-} FLs
Figure 3.7. *Stat5*<sup>−/−</sup> fetal livers display dysregulation of multiple subunits of RNA polymerase I, II, and III.

Expression of all the subunits for RNA polymerase I, II, and III in *Stat5*<sup>−/−</sup> (blue) and wild-type littermate (magenta) fetal livers (n=3 for each genotype). Multiple subunits of all three polymerases are downregulated in *Stat5*<sup>−/−</sup> FLs.
Western blot and qPCR analysis of selected ribosomal proteins in Epor<sup>−/−</sup> and Stat5<sup>−/−</sup> FLs.

We examined a number of ribosomal proteins in FACS-sorted FL subsets (S0, S1, S3) using western blotting. This experiment showed no significant difference, for any given developmental stage between the global levels of ribosomal proteins in Epor<sup>−/−</sup> FLs and wild-type controls, though it did show the expected higher levels of ribosomal proteins in earlier erythroblasts (S1, Fig. 3.8). We also examined the global levels of the mature rRNA transcripts, 28S and 18S, using qPCR (Fig. 3.8).

These measurements of ribosomal proteins and mature rRNA transcripts reflect the total number of ribosomes per cell, rather than their biogenesis rate. There is evidence that ribosomal proteins made in excess are rapidly degraded (Lam, Lamond et al. 2007, Yasuda, 2020 #3956, Sung, Reitsma et al. 2016). We therefore next sought to determine the dynamics of ribosome biogenesis.
a

**Relative mRNA levels**

- **Wild-type S0**
- **Wild-type S1**
- **Epor-/- S0**

![Bar charts showing relative mRNA levels for different genes](chart_a)

b

**Experiment 1**

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![Western Blots showing protein levels](chart_b)

c

**Expression relative to β-Actin**

- **Epor-/- S0-Med**
- **Wild-type S0-Med**

![Expression relative to β-Actin for different genes](chart_c)

d

**Expression relative to β-Actin**

- **S0-Low**
- **S0-Med**
- **S1**
- **S3**

![Expression relative to β-Actin for different genes](chart_d)
Figure 3.8. *Epor<sup>-/-</sup>* fetal liver display normal total ribosomal proteins and mature rRNA transcripts per cell.

(a) Gene expression in sorted S0 *Epor<sup>-/-</sup>* and S0/S1 wild-type littermate fetal liver cells. mRNAs were quantitated with RT-qPCR and are expressed relative to expression of β-actin in the same cells. In each experiment, RT-qPCR was carried out using a dilution series of the cDNA. Data for each gene is mean± SD pooled from 2 independent experiments. (b) Western blot and (c,d) quantification by densitometry showing no significant difference between *Epor<sup>-/-</sup>* S0-med and wild-type S0-med total ribosomal protein but there is an increase in total ribosomal protein content from S0-med to S1. Western blot quantifications are representative of two independent experiments. Data for each ribosomal protein is mean ± SD.
Measurement of rRNA synthesis rate with 5-Ethynyl uridine (EU).

We measured total RNA synthesis by subjecting freshly harvested FLs to a brief pulse of 5-ethynyl uridine (EU), a uridine analog that incorporates into newly synthesized RNA, and that can be quantified following cell fixation using click chemistry. To obtain the rRNA fraction of this signal, we ultimately plan to use imaging flow cytometry, which should allow us to quantify the EU signal that is specific to nucleoli (Bryant, McCool et al. 2022). We are in the process of developing this assay, which requires a high-resolution imaging flow cytometer (not available at UMASS Medical School). As an interim measure, we assumed that the majority of the EU signal reflects rRNA synthesis, since rRNA accounts for nearly all (80-95%) of total cellular RNA, and is also likely to account for the majority of new RNA synthesis in rapidly cycling cells.

We harvested Epor\textsuperscript{-/} and Stat5\textsuperscript{-/} FLs and matched littermate controls, and following 3 hour incubation \textit{in vitro} in the presence of Epo, we pulsed them with EU (1mM) for 2, 5, 10, and 20 minutes. We then labeled the FL cells with cell surface markers that will distinguish erythroid developmental subsets, followed by a viability stain, fixation, permeabilization and click chemistry. We expressed EU incorporation relative to the incorporation by S0-low cells in each embryo. EU incorporation rate was linear between $t= 5$ and $t= 20$ min, and so we chose to pulse cells for 10 min in all subsequent experiments.

In wild-type FLs, EU incorporation rate showed a marked, developmental-stage-dependent dynamic profile. It increased sharply with initial differentiation,
peaking in S1, where it reached 2 fold the rate of S0-Low (Fig. 3.9a, Table 3.2). Further differentiation resulted in a rapid decline in EU incorporation rate, to 1.2 fold in S2, with a further decline to 0.6 in S3. EU incorporation in Epor\(^{-/-}\) FLs was consistently lower (for S0-Med, EU incorporation was 1.7 ± 0.05 v 1.3 ± 0.04 in WT v Epor\(^{-/-}\), p = 5 x 10\(^{-5}\), unpaired t-test with unequal variance, n = 4 biological replicates for Epor\(^{-/-}\) v n = 5 biological replicates for wild-type).

EU incorporation was also slower in the Stat5\(^{-/-}\) FLs in both S0-Med and in S1, where it reached 2.8 ± 0.20 v 2.2 ± 0.25 in WT v Stat5\(^{-/-}\) (p = 0.04, n = 3 biological replicates for Stat5\(^{-/-}\) v n = 5 biological replicates for wild-type). The peak of EU incorporation rate in the Stat5\(^{-/-}\) was lower but broader, persisting into later developmental stages (S2), before declining (Fig. 3.9b).
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Figure 3.9. Changes in global protein synthesis rate and rRNA transcription rate within developmental subsets in mutant and wild-type fetal livers. (a-d) rRNA transcription rate following a 10 min 5-ethynyl-uridine (EU, 1mM) pulse and (b) global protein synthesis rate following a short O-Propargyl-Puromycin (OPP, 25μM) pulse in Epor⁻/⁻ (left panels, blue), Stat5⁻/⁻ (right panels, blue) and wild-type littermate (orange) fetal liver cells (see 5-Ethynyl Uridine (EU) and O-Propargyl-Puromycin (OP-Puro) in Materials and Methods). There is a lower rRNA transcription and global translation rate in mutant FLs leading up to S1. However, in Stat5⁻/⁻ FLs, the lower peak of rRNA transcription and global translation rate persists into later developmental stages. (e, f) Erythroid differentiation assessed via Ter119 signal. Delayed differentiation (lower Ter119) is observed at later developmental stages in Stat5⁻/⁻ FLs. (g, h) Cell frequency assessed at each developmental stage. Statistical analysis for rRNA transcription and global translation rates between mutant FL and wild-type controls (lower panel, unpaired t test with unequal variance). Data for three independent experiments. EU assay: Epor⁻/⁻ n=4 pooled biological replicates, Epor⁺/⁺ n=5 biological replicates, Stat5⁻/⁻ n=3 biological replicates, Stat5⁺/⁺ n=5 biological replicates (see Table 3.2.). OPP assay: Epor⁻/⁻ n=4 pooled biological replicates, Epor⁺/⁺ n=5 biological replicates, Stat5⁻/⁻ n=4 biological replicates, Stat5⁺/⁺ n=4 biological replicates (see Table 3.2.)
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Table 3.2. Descriptive statistics for rRNA transcription and global translation rates in mutant and wild-type fetal livers.
Measurement of rRNA synthesis rate using Fluorescence in-situ hybridization (FISH).

In collaboration with the laboratory of Dr. Vikram Paralkar (U.Penn), we are undertaking a novel assay developed by his lab (Antony, George et al. 2022) that measures rRNA synthesis rate using flow-cytometry-based FISH (FISH-Flow), specifically detecting the first pre-rRNA 47S transcript. Fifteen different fluorescent probes are hybridized to the unique 5' ETS of the 47S transcript. Because the 47S transcript is transient, the signal generated by this FISH assay reflects rRNA synthesis rate.

Initial pilot analysis of sorted S0 and S1 cells from Epor\(^{-/-}\) and wild-type FLs shows substantially lower levels of the 47S transcript in Epor\(^{-/-}\) FLs (Fig. 3.10). In ongoing work, we are developing this protocol further, to allow simultaneous labeling of cell-surface markers with intracellular FISH. We are also planning to develop a FISH-Flow assay for the mature, 28S rRNA, which would allow us to assess total ribosome content of the cell.
DNA (DAPI)

47S (FISH)

WT / Ter119^-/-

Epor^-/-

p=0.0003

p=0.04

p=0.0005

b
Figure 3.10. *Epor*<sup>−/−</sup> fetal livers display lower pre-rRNA 47S transcript.

(a) Flow cytometric analysis of 47S transcript in *Epor*<sup>−/−</sup> (top panel, left) and wild-type Ter119<sup>neg</sup> (top panel, right) FLs. (lower panel) Histogram of 47S transcript for *Epor*<sup>−/−</sup> (blue) and wild-type Ter119<sup>neg</sup> (orange) FLs. *Epor*<sup>−/−</sup> n=2 biological replicates, *Epor*<sup>+/+</sup> n=2 biological replicates. (b) Lower levels of the 47S transcript in *Epor*<sup>−/−</sup> FLs. Summary of two independent experiments and three biological replicates for each genotype.
Measurement of global protein synthesis rate using a puromycin analog.

Puromycin is a translation inhibitor, an analog of aminoacyl-tRNA (charged tRNA bound to its cognate amino acid). It incorporates into the C-terminal end of nascent peptides during the ribosomal elongation cycle, resulting in their 'puromyclation', interrupting further elongation (Hidalgo San Jose and Signer 2019). A brief pulse of puromycin will therefore label nascent peptides but not other cellular protein, providing a measure of global protein synthesis rate. A puromycin derivative containing a terminal alkyne, O-propargyl-puromycin (OPP) allows measurement of puromylated nascent peptides using click chemistry (Liu, Xu et al. 2012, Hidalgo San Jose and Signer 2019).

Similarly to the strategy we used when pulsing cells with EU, we harvested fresh Epor⁻/⁻ and Stat5⁻/⁻ FLs, and following a 3 hour incubation period with Epo in vitro, we pulsed them with OPP (25 µM) for 2, 5, 10, or 20 min. We labeled the cells with cell-surface markers that will distinguish developmental stage, and following fixation, used click chemistry to determine OPP incorporation. We found that the OPP incorporation increased linearly for the first 5 min but started to decline by 10 min. We used 5 min OPP pulses in all subsequent experiments.

OPP incorporation profile was similar to that of EU, increasing sharply with the developmental transition from S0-Low to S0-Med, and peaking in S1, where it reached 3 to 4 fold the rate of S0-Low (Fig. 3.9c, d, Table 3.2). Further differentiation resulted in a rapid decline in OPP incorporation rate. OPP
incorporation was significantly lower in both $Epor^{−/−}$ and $Stat5^{−/−}$ FLs (for WT v $Epor^{−/−}$ S0-Med, $2 ± 0.2$ v $1.4 ± 0.1 p = 0.0013$; for $Stat5^{−/−}$ v WT S1, $3.1 ±0.30$ v $1.8 ± 0.35$, $p = 0.001$). Similar to EU, the OPP peak in the $Stat5^{−/−}$ FL was lower but broader.

**Peak protein synthesis rates in S1 cells correlate with a peak in cell cycle speed but not with ETD gene induction.**

Our results show an EpoR and Stat5-dependent peak in ribosome biogenesis and protein synthesis rates in S1, which precedes the phase of rapid ETD gene expression (as seen by Ter119 expression, Fig. 3.9 e, f; globin synthesis rate is maximal in S3 (Oudelaar, Beagrie et al. 2020)) and also precedes the rapid increase in erythroblast cell number (between S2 and S3, Fig. 3.9 g, h). Therefore, the S1 peak in ribosome biogenesis and protein synthesis rates is unlikely to be directly linked either to cell division *per se* or to erythroid gene induction.

S1 cells are characterized by an exceptionally short cycle and fast S phase (Pop, Shearstone et al. 2010, Shearstone, Pop et al. 2011, Tusi, Wolock et al. 2018, Hwang, Hidalgo et al. 2020). The S1 cycle was recently found to be the shortest of all hematopoietic cells (Eastman, Chen et al. 2020). Further, our work in Chapter II shows that the fast cell cycle speed in S1 is in part the result of EpoR and Stat5 signaling, and that, in spite of increasing cell cycle speed, EpoR signaling also increases cell size in these cells. We therefore began to examine
the relationship between ribosome biogenesis rates, proteins synthesis rates, and cell cycle speed.

The fraction of cells in each phase of the cycle was not significantly different between Stat5−/− and WT FLs (Fig. 3.11a). Nevertheless, S phase speed (BrdU incorporation rate in S phase cells) was substantially lower in all Stat5−/− FL developmental subsets (Fig. 3.11b). We combined our S phase speed measurement with the measurement of the fraction of cells in each cell cycle phase to calculate relative cell cycle lengths, and the lengths of each cell cycle phase. Our analysis shows that cell cycle duration fails to decrease in Stat5−/− S1 cells, and remains longer in all subsequent Stat5−/− FL developmental subsets (Fig. 3.11c, left panel). The major contributor to the longer cell cycle duration is the longer S phase of Stat5−/− cells (Fig. 3.11c).

Further, we found that S phase and cell cycle speeds closely correlated with protein synthesis rates across all WT developmental subsets (Fig. 3.12 a, b, left panels). This correlation is lost (for S phase speed) or is much weaker (for cell cycle speed) in the Stat5−/− FL (Fig. 3.12 a, b, right panels). These data suggest a Stat5-mediated causal link between cell speeds and protein synthesis rates during erythropoiesis; in particular, they suggest that the striking peak in protein synthesis rate in S1 cells allows these cells to maintain biomass in the face of an extremely short cycle.
Figure 3.11. Slower S phase speed and longer cell cycle in the absence of Stat5 signaling.

(a) Cell cycle phase frequency for various erythroid developmental stages. Cell cycle phase distribution remains normal throughout erythroid development in the absence of Stat5. (b) Slower S phase speed (BrdU incorporation rate in S phase cells) at all developmental stages in the absence of Stat5 signaling (see Cell Cycle in Materials and Methods). (c) Cell cycle length and cell cycle phase lengths for G1, S, and G2/M for Stat5−/− and wild-type littermate FLs. Longer cell cycle length (left panel) in the absence of Stat5 signaling due a longer S phase length (slower S-phase as seen in (b)). Stat5−/− n=4 biological replicates, Stat5+/+ n=2 biological replicates.
Figure 3.12. S phase and cell cycle speed correlate with protein synthesis rate during erythroid differentiation.

(a) Correlation between S phase speed and global protein synthesis rate (OPP incorporation) during erythroid development (left panel) and loss of correlation in Stat5\textsuperscript{−/−} FLs (right panel). (b) Correlation between cell cycle speed (the inverse of cell cycle length) and global translation rate (OPP incorporation). Stat5\textsuperscript{−/−} n=4 biological replicates, Stat5\textsuperscript{+/−} n=2 biological replicates.
The rate of increase in cell size peaks in S1 cells, correlating with peak protein synthesis rate.

We next asked how protein synthesis rate was related to growth in cell size. We measured cell size using the flow cytometric forward-scatter (FSC) parameter. For each developmental subset, we determined cell size in each cell cycle phase (Fig. 3.13a), and then calculated the increase in cell size during an average cycle, by subtracting cell size in G1 from cell size in G2M. This approach showed that Stat5\(^{-/-}\) progenitors in S0 grew to larger sizes than WT in a given cycle, but that in subsets S1 to S3, cell size was very similar in both genotypes (Fig. 3.13b, middle panel). We then calculated, for each subset, the rate of growth in cell size, by dividing the increase in cell size in a given cycle by the duration of that cycle (cycle duration plotted in Fig. 3.13b, left panel). The calculated rate of growth in cell size has a dynamic profile that peaks in S1 (Fig. 3.13b, right panel), closely resembling the dynamics of protein synthesis and ribosome biogenesis rates. Further, as was the case for protein synthesis rate, growth rate in cell size was significantly lower in the Stat5\(^{-/-}\) FLs (Fig. 3.13b, right panel). Across all WT FL subsets, there was a strong correlation between the rate of growth in cell size and protein synthesis rate (Pearson r = 0.87). The two processes also correlated in Stat5\(^{-/-}\) FLs but the correlation was weaker (Fig. 3.13c).
Figure 3.13. The rate of cell size growth across ETD subsets tracks closely with protein synthesis rate.

(a) Increase in cell size (assessed via forward scatter (FSC) parameter) through the cell cycle for each developmental subset in Stat5−/− (blue) and wild-type FL (orange). (b) (left panel) Longer cell cycle length in the absence of Stat5 signaling throughout all developmental stages. (middle panel) Cell size growth per cycle for each developmental subset. Actual cell size growth per cycle in Stat5−/− is the same in ETD (S1–S3) in spite of much slower growth rate, as a result of a much longer cycle. (right panel) The rate of cell size growth across ETD subsets closely relates with the OP-puro incorporation rate, both spiking in S1. (c) Correlation between the rate of increase in cell size (FSC) and global translation rate (OPP incorporation). Stat5−/− n=4 biological replicates, Stat5+/+ n=2 biological replicates.
Discussion

In this chapter, we found that EpoR/Stat5 signaling mediates a sharp increase in ribosome biogenesis (Ribi) and protein synthesis (PS) rates. Both peak during a narrow developmental window in early ETD (S1 cells). Ribi and PS rates increase 2 to 2.5 fold and 3 to 4 fold, respectively, compared with earlier progenitors (S0-Low) or with later erythroblasts (S3). Although hemoglobin synthesis begins in S1 cells, it reaches maximal speed only in S3 (Oudelaar, Beagrie et al. 2020). The increase in erythroblast cell number persists throughout ETD. Therefore, neither cell division per se nor hemoglobin synthesis rates correlate in their timing with the peaks in Ribi/PS rates.

By contrast, we found that Ribi/PS rates throughout ETD correlate closely with the speed of S phase, the speed of the cycle as a whole, and, most important, with the rate of growth in cell size. These correlations are lost in Stat5−/− erythroblasts, suggesting that EpoR/Stat5 signaling is critical for activating pathway(s) that match the growth in cell size to cell cycle speed. The peak in growth rates for cell size in S1, presumably a consequence of peak rates for Ribi/PS, are therefore likely a mechanism that maintains or increases cell size in S1 cells given their extremely short cycle (Shearstone, Pop et al. 2011, Hwang, Futran et al. 2017).

Cell size in Stat5+/− S1 to S3 erythroblasts is similar to wild-type, in spite of much slower cycles and slower Ribi/PS rates. We therefore speculate that during this developmental period, cell size may be a regulated parameter, such that cell
cycle speed and Ribi/PS rates are adjusted to keep it constant. Currently, it's not clear if the primary deficit in Stat5$^-$ erythroblasts is in ramping up S phase and cycle speeds, resulting in a compensatory slowing of Ribi/PS, or whether the primary failure is in accelerating Ribi/PS rates, with the cycle slowing in response. Direct interaction between Ribi and the cell cycle machinery was identified recently, with specific ribosomal proteins binding and regulating CDK inhibitors and cyclin/CDK complexes (Bury, Le Calvé et al. 2021). Further, a recent RNAi screen identified inhibitors of RNA polymerase I transcription, with consequent slowing S and G2M phases of the cycle (Ogawa, Buhagiar et al. 2021). In ongoing experiments, we are investigating the causal relationships between EpoR/Stat5 signaling, cell cycle speed and Ribi/PS rates. This can be done with acute perturbations using drugs, slowing or arresting the cycle and examining the consequences for Ribi/PS rates, and by inhibiting Ribi/PS, to see consequences for cell cycle speeds. We are also planning to examine genetic mouse models in which erythroblast cell cycle speeds are abnormal (S phase speed is increased in p57KIP2$^-$/ (Hwang, Futran et al. 2017) and decreased in E2F4$^-$) , and in which ribosomal protein haploinsufficiency slows Ribi/PS rates.

Cell cycle speed in early erythroblasts is principally regulated by S phase speed, since S phase occupies the largest fraction of the S1 cycle. scRNA-seq analysis shows that, in addition to regulating cyclin D levels, which would impact G1 phase progression, EpoR and Stat5 also increase the transcript levels of cyclins E and A as well as E2F4, Mcm helicase subunits, and ribonucleotide
reductase, all S phase regulators. These findings are consistent with our data in chapter II, where EpoR was required for a fast S phase speed in S1 cells, and where Epo administration to mice decreased cell cycle duration in early erythroblasts. Together these data suggest that, in addition to regulating G1 length, a function they share with many growth factor and cytokine receptors (Matsumura, Kitamura et al. 1999, Zhu and Skoultchi 2001, Khaled, Bulavin et al. 2005, Quelle 2007, Dalton 2015, Wang 2021), EpoR and Stat5 also regulate the speed of S phase, a function that is much less well reported or understood.

The mechanisms that allow EpoR and Stat5 to regulate Ribi and PS rates are not clear. The picture that emerges from our scRNA-seq analysis appears paradoxical: ribosomal protein transcripts are increased, but transcripts for genes that participate in rRNA transcription and processing are decreased. The deficit in rRNA transcription is confirmed by our EU pulse experiments and by the initial 47S FISH analysis, which makes the increase in ribosomal protein transcripts particularly unusual. The picture is also split in the case of regulation of PS: translation initiation factors (e.g. Eif4e, Eif4g2, Eif5a) are decreased, while translation elongation factors (Eef1a1, Eef1b2, Eef1g) are increased.

Ribosomal protein synthesis and rRNA transcription are thought to be coordinated (de la Cruz, Gómez-Herreros et al. 2018). Excess ribosomal proteins are degraded rapidly by the ubiquitin-proteosomal system (Sung, Reitsma et al. 2016). ‘Nucleolar stress’, an excessive increase in either uncomplexed RPs or in rRNA, results in stabilization of p53 and cell cycle arrest. This process is
mediated via binding of 5S RNP (consisting of 5S RNA, Rpl5 and Rpl11) to Mdm2, interfering with its E3 ubiquitin ligase activity towards p53 (Budde and Grummt 1999, Macias, Jin et al. 2010, Donati, Bertoni et al. 2011). Through this mechanism, p53 is activated by the skewing in the ribosomal protein pool in ribosomal haploinsufficiencies, e.g. Diamond Blackfan Anemia, contributing to disease mechanisms (Jaako, Debnath et al. 2015, Kamio, Gu et al. 2016). We do not, however, see evidence of a p53 signature in our Epor−/− and Stat5−/− transcriptomes.

We hypothesize that the increase in ribosomal protein transcripts results from over-expression and activation of Kit, which we see in both the Epor−/− and Stat5−/− FLs. Kit signaling might be expected to activate the canonical pathways that lead to Ribi via mTor and c-Myc. In the absence of EpoR or Stat5, it may promote a compensatory increase in Ribi, successfully increasing ribosomal protein transcripts but failing to support sufficient rRNA synthesis. If so, this would suggest a non-redundant function for EpoR/Stat5 signaling specifically in rRNA transcription and processing in early ETD, potentially via a novel pathway that is not accessible to Kit. We are testing this hypothesis genetically, in Epor−/− FLs on a W41 background, in which Kit bears a hypomorphic mutation (Nocka, Tan et al. 1990). We are also planning to undertake scRNA-seq of W41/Epor−/− FLs, in order to help distinguish transcriptional consequences of Epor deletion from those of compensatory Kit over-expression.
Taken together, data in this chapter shows that EpoR and Stat5 signaling play a critical role in supporting an unusual, developmental-stage-specific increase in Ribi and PS rates in early ETD. This increase matches the unusually short cycle of these cells. These data explain our earlier work, in which we found that EpoR signaling paradoxically promoted both faster cycling and a larger cell size in early erythroblasts, leading to the formation of larger red cells. The molecular pathways that allow EpoR and Stat5 to support exceptionally fast growth rates are not yet clear.
Materials and Methods

Mice.

Stat5−/− 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. Mice were housed at a dedicated facility, with regulated temperature (range 20–26 °C), a 12 h/12 h dark/light 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 25mM HEPES (Gibco), 20% fetal calf serum (Hyclone), 1% penicillin/streptomycin (ThermoFisher Scientific), 2 × 10−4M ß- Mercaptoethanol (Sigma), supplemented when indicated with 0.5 IU/ml Epo (Procrit, Amgen; 1 IU/ml = 1.2 ng/ml) and 100 ng/ml SCF (Peprotech), and 10 ng/ml IL3 (Peprotech).
Isolation of mouse erythroid progenitors.

To isolate wild-type S0, S1 cells, fetal liver cells were sorted on a FACS Aria and FACS Aria Fusion (BD Biosciences).

Western blot analysis.

Sorted fetal liver cells or fetal liver cells from expansion and differentiation cultures were incubated in lysis buffer [1% NP-40, 50 mM tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol. Lysis buffer was supplemented with fresh protease inhibitors, sodium fluoride (NaF) [Sigma, 201154], sodium orthovanadate [Sigma, 450243], Phenylmethanesulfonyl fluoride (PMSF) [Sigma, P7626], cOmplete Mini, EDTA-free [Roche, 11836170001] and rotated at 4°C for 30 min. Supernatants were prepared by centrifugation at 4°C for 15 min and quantified by the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, 23227). Protein electrophoresis was carried out using the NuPAGE Novex Bis-Tris Gel System (Invitrogen). Polyvinylidene difluoride membranes were probed with antibodies against RPS3 (Cell Signaling, 9538S), RPS6 (Cell Signaling, 2217S), RPL28 (Abcam, ab138125), RPS15a (Bethyl Laboratories, A304-990A-M), and β-actin (Abcam ab8227). Target protein bands were detected by ChemiDoc XRS+ system (Bio-Rad) and quantified using Image Lab software (Bio-Rad).
Flow cytometry.

Fetal liver cells were analyzed on the Cytek Biosciences Aurora cytometer using SpectroFlo software (Cytek Biosciences). Dead cells were excluded using LIVE/DEAD Kit (Invitrogen L23105). FACS data were analyzed using FlowJo software (Tree Star Inc., CA).

Antibodies used:

AF647 Rat Anti-Mouse CD71 (C2) (BD Biosciences, 563504) dilution 1:100
BUV395 Rat Anti-Mouse Ter119 (TER-119) (BD Biosciences, 563827) dilution 1:100
Biotin Rat Anti-Mouse Ter119 (BD Biosciences, 553672) dilution 1:100
APC-H7 Rat Anti-Mouse cKit (2B8) (BD Biosciences, 560185) dilution 1:200
AF700 Rat Anti-Mouse Ly-6G and Ly-6C/Gr1 (RB6-8C5) (BioLegend, 108422) dilution 1:100
AF700 Rat Anti-Mouse CD11b/Mac1 (M1/70) (BioLegend, 101222) dilution 1:100
AF700 Rat Anti-Mouse F4/80 (BM8) (BioLegend, 123130) dilution 1:100
AF700 Rat Anti-Mouse CD41 (MWReg30) (BioLegend, 133926) dilution 1:100
AF700 Rat Anti-Mouse CD45R/B220 (RA3-6B2) (BioLegend, 103232) dilution 1:100
AF700 Hamster Anti-Mouse CD3e (500A2) (BioLegend, 152316) dilution 1:100
AF700 Rat Anti-Mouse CD19 (6D5) (BioLegend, 115528) dilution 1:100
AF700 Rat Anti-Mouse CD4 (RM4-4) (BioLegend, 116022) dilution 1:100
**Cell-cycle analysis.**

Cell-cycle status and S phase speed were analyzed using BrdU incorporation (Hwang, Futran et al. 2017). Briefly, cells were pulsed at a final concentration of 33 µM BrdU for 30 minutes. Erythroid subsets were stained using anti-CD71 (BD Biosciences, 563504), anti-Ter119 (BD Biosciences, 563827), anti-cKit (BD Biosciences, 560185). Non-erythroid subsets were stained using a lineage cocktail (Gr1, Mac1, F4/80, CD19, CD4, CD41, B220, CD3e, see flow cytometry). Cells were immediately labeled with the LIVE/DEAD Kit (Invitrogen L23105), fixed, and permeabilized. BrdU incorporation was measured by biotin-conjugated anti-BrdU (PRB-1, EMD Millipore, MAB3262B) followed by a secondary stain with Brilliant Violet 421™ Streptavidin (Bio-Legend). DNA content was measured by 7AAD (BD Biosciences).

**Quantitative RT-PCR assay.**

Total RNA was isolated from sorted fetal liver cells using the RNeasy Plus 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.
TaqMan MGB probes used: β-actin (Mm02619580_g1), Rpl23 (Mm00787512_s1), Rps3 (Mm00656272_m1), Eef1a1 (Mm01973893_g1), Rps24 (Mm01623058_s1), Rps2 (Mm01971861_g1), Rpl37a (Mm01546394_s1), Rn28s1Rn45s (Mm03682676_s1), Rn18sRn45s (Mm03928990_g1).

5-Ethynyl Uridine (EU) and O-Propargyl-Puromycin (OP-Puro).

E12.5 (Epor) and E13.5 (Stat5) fetal liver cells were cultured (see culture medium and growth factors) for 3 hours at 37°C. Cultured fetal liver cells were pulsed with either 1mM EU (ThermoFisher Scientific, E10345) for 10 min or 25µM OP-Puro (MedChem Express, HY-15680) for 5 min. As controls, DMSO was used for No EU control, while addition of 10µg/ml cyclohexamide (Sigma, C7698) 1 hour prior to pulsing cultured cells with OP-Puro was used for No OP-Puro control. Cells were collected on ice and stained for CD71, Ter119, cKit, and non-erythroid lineage markers (see Flow cytometry) for 30 minutes on ice. Cells were immediately labeled with the LIVE/DEAD Kit (Invitrogen L23105), fixed, and permeabilized. EU incorporation was detected using an Alexa Fluor 488 azide (ThermoFisher Scientific, A10266) and Click-iT Cell Reaction Buffer Kit (ThermoFisher Scientific, C10269) according to the manufacturers’ instructions.
**RNA FISH Flow.**

Fetal livers were harvested from midgestation mouse embryos (E12.5) and mechanically dissociated. Wild-type S0, S1 cells and Epor<sup>−/−</sup> S0 cells (E12.5) were obtained by either sorting by flow cytometry as described (Pop, Shearstone et al. 2010, Koulnis, Pop et al. 2011) in a FACSAnia Fusion (BD Biosciences) using a 100-µm nozzle or by magnetic separation. For the latter, wild-type S0 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 using MojoSort<sup>TM</sup> Streptavidin Nanobeads (BioLegend) according to the manufacturers’ instructions. Epor<sup>−/−</sup> fetal liver cells were depleted of lineage-positive cells by labeling with biotin-conjugated Ter119, Gr1, Mac1, and CD41 antibodies followed by magnetic separation (Epor<sup>−/−</sup> FLCs are all developmentally arrested in S0, see Fig. 1.7). Wild-type S0, S1 and Epor<sup>−/−</sup> S0 cells were cultured in differentiation media (Epo 0.5U/ml) (see **Culture medium and growth factors**) and processed as described (Antony, George et al. 2022).

**Cell preparation for scRNA-seq.**

**Tissue collection.**

Fetal livers were prepared by mechanical dissociation in staining buffer (1X PBS, 0.2% BSA, 0.08% Glucose).
**Density gradient centrifugation.**

Dead cells and debris were removed from the fetal liver samples using density centrifugation in OptiPrep density gradient medium as previously described (Tusi, Wolock et al. 2018) with some modifications (Sigma, D1556). Fetal liver cells were re-suspended in 0.9 ml staining buffer, mixed with 2 mls of 40% of OptiPrep in 1X PBS, and placed in a 15-ml centrifuge tube. The cell suspension was carefully over-layered with 4 ml of 20% OptiPrep solution, and 2 ml of 5% OptiPrep solution, and centrifuged at 800g for 15 min (centrifuge break off). The top visible cell band that formed during centrifugation contained the live cells that were used directly in the inDrops (Klein, Mazutis et al. 2015) platform.

For *Stat5*\(^{+/+}\) and *Stat5*\(^{-/-}\) inDrops samples, an initial Ter119 negative enrichment was performed by labeling fetal liver cells with biotin-conjugated Ter119 (BD Biosciences) antibody followed by magnetic separation using EasySep beads (StemCell Technologies) according to the manufacturers’ instructions. After enrichment, 5% of pre-enriched fetal liver cells were re-introduced to the enriched sample assuring a complete erythroid differentiation trajectory. Afterwards, dead cells and debris were removed from the fetal liver samples as previously described.

**Single-cell transcriptome droplet microfluidic barcoding using inDrops.**

For scRNA-seq, we used inDrops (Klein, Mazutis et al. 2015) following a previously described protocol (Zilionis, Nainys et al. 2017, Tusi, Wolock et al.
inDrops samples were prepared from 3 $Epor^{+/+}$ and 3 $Stat5^{+/+}$ biological replicates and 3 $Epor^{-/-}$ and 3 $Stat5^{-/-}$ biological replicates. Following droplet barcoding reverse transcription, emulsions were split into aliquots of approximately 3000 single-cell transcriptomes and frozen at $-80^\circ$C. Twelve libraries were prepared.

**Sequencing.**

The libraries were sequenced on NextSeq 500 runs using a NextSeq High Output flow cell (75 cycles $\sim$400 million reads).

**Single cell RNA-seq analysis.**

Raw sequencing data (FASTQ files) were processed using the inDrops.py bioinformatics pipeline (available at https://github.com/indrops/indrops) (Zilionis, Nainys et al. 2017). All ambiguously mapped reads were excluded from analysis. Reads were aligned to the Ensemble release 81 mouse mm10 cDNA reference.

The raw counts matrices for each sample were analyzed separately using the SCANPY package (Wolf, Angerer et al. 2018). Cell transcriptomes were filtered on the basis of low reads, high fraction of mitochondrial gene transcripts (>10%) and doublets or multiplets using Scrublet (Wolock, Lopez et al. 2019), and were subjected to total counts normalization excluding highly variable genes. Following initial visualization of the data using UMAP (Becht, McInnes et al. 2018), clusters
that were not contiguous with the principal hematopoietic cluster were excluded (these contained yolk-sac lineage cells or non-hematopoietic cells including hepatocytes and endothelial cells, based on marker gene expression, (Tusi, Wolock et al. 2018)). PCA analysis was used to project each sample onto a reference feta liver dataset, and each cell transcriptome acquired the erythroid pseudotime parameters of the nearest cell in the reference dataset, as described (Tusi, Wolock et al. 2018).
CHAPTER IV:

DISCUSSION

In this thesis, I discovered new functions of EpoR and Stat5 signaling in erythropoiesis. Previously, the best documented function of EpoR signaling was its anti-apoptotic effect, which becomes essential in ETD, and which was thought to be the principal mechanism through which an increase in Epo drives an increase in erythropoietic rate. My work expands the known functions of EpoR, showing that it plays critical roles in regulating cell cycle number and speed, and erythroblast and red cell size. These functions are clearly relevant to the Epo-mediated erythropoietic stress response. They also have broader implications outside physiological erythropoiesis.

The transition of CFU-e progenitors to ETD is an S-phase-dependent transcriptional switch, which takes place during an unusually short cycle with a fast S phase (Shearstone, Pop et al. 2011, Hwang, Futran et al. 2017, Tusi, Wolock et al. 2018). Here I showed that EpoR and Stat5 signaling are required for the short cell cycle and fast S phase at the CFU-e/ ETD transition. Both functional and transcriptomic analysis suggest that EpoR/Stat5 signaling accelerate the cycle not only through classic pathways that act on G1 cyclin/CDK complexes, but by shortening S phase, a novel function with potential relevance to signaling by other receptors.
Further, I found that, in response to high Epo, there is an increase in cell size in erythroblasts of all stages, leading to the formation of larger red cells. This observation is also supported by data from human volunteers. I further showed that cell size control by EpoR signaling is distinct from previously known pathways involving iron status or the number of cell divisions during ETD. My recent work confirms a critical role for EpoR and Stat5 signaling in the control of cell size, showing that they promote exceptionally fast ribosome biogenesis and protein synthesis rates specifically in cells in which cell cycle speed is high, at the onset of ETD. These findings raise a number of questions and suggest future research directions.

First, what is the selective advantage of larger red cells with lower hemoglobin concentration during tissue hypoxia? Older reports suggest that the affinity of 2,3-DPG to hemoglobin increases at low hemoglobin concentrations, shifting the oxygen dissociation curve to the right, which would improve oxygen unloading in tissues (Garby and De Verdier 1971). Of interest, in iron deficiency, red cells are both smaller and "hypochromic", that is, have lower hemoglobin concentrations. The precise relationship between red cell size, hemoglobin concentration, the oxygen dissociation curve and Epo/hypoxia are testable with newer technologies.

Second, the dynamics of both ribosome biogenesis and protein synthesis during ETD is especially interesting, since both peak in S1 cells, known to have the shortest cell cycle in ETD. This suggests an interaction between cell cycle
speed and the rate of growth in biomass. There appears to be a unique requirement for EpoR/Stat5 signaling for rapid growth, since Kit signaling, which is also active in these cells cannot fully compensate in their absence, in spite of its upregulation in Stat5-/- and EpoR-/- embryos. These findings suggest potentially new through which cytokine receptors control the rate of growth in biomass.

Third, the regulation of growth in biomass in mammalian cells is not well understood, and is an area that is especially relevant to cancer. The prevalent model suggests that there is a 'cell size checkpoint' in G1 phase of the cycle, and that cells must attain a permissive size in order to pass this checkpoint and enter S phase (Ginzberg, Kafri et al. 2015, Zatulovskiy and Skotheim 2020). Clearly this model is not sufficient to explain the control of biomass in early erythroblasts, where G1 is extremely short, and where we found that most of cell size growth takes place during S phase and G2-M. Further study of the regulation of biomass in early erythroblasts may have implications for other cells, including cancer cells, where Stat5 is active.

Finally, my work uncovers novel characteristics of S1 cells. These unique cells undergo an S-phase-dependent sharp transcriptional switch, as they transition from a CFU-e cell state to ETD (Tusi, Wolock et al. 2018). In addition to their unusually fast cycle, we now find that they have unusually fast growth rates. What is the functional significance of these processes? Faster growth, coupled with a fast cycle, might accelerate dilution of protein regulators, helping
to accelerate a 'proteome switch', from the CFU-e proteome to an ETD proteome. Whether or not this is the case, the exceptionally fast ribosome biogenesis rate required by S1 cells could become an Achilles heel in ribosome haploinsufficiency syndromes such as Diamond Blackfan Anemia (DBA). It may explain the unusual sensitivity of the erythroid lineage to these syndromes. A better understanding of the mechanisms involved in regulating ribosome biogenesis specifically at this stage of erythropoiesis may suggest new therapeutic avenues in ribosomopathies.

Summary and a model for cell growth and cell cycle regulation in early erythroblasts

The model proposed in Figure 4.1 outlines the canonical pathways that regulate cell cycling and cell growth, and adds novel pathways suggested by our work. The two principal growth factor receptors regulating cell cycling and cell growth in early erythroblasts belong to different receptor families. Whereas EpoR is a cytokine receptor, Kit is a receptor tyrosine kinase, resembling the insulin and insulin-like growth factor I receptors, the two best studied receptors in connection with growth and biomass signaling. Both Kit and EpoR activate ras/raf/MAPK and PI3-kinase (Lev, Yarden et al. 1992, Haseyama, Sawada et al. 1999, Pircher, Geiger et al. 2001, Ratajczak, Majka et al. 2001, Khaled, Bulavin et al. 2005, Haas, Riedt et al. 2015, Kuhrt and Wojchowski 2015). EpoR, but not Kit, also activates Stat5 (Socolovsky, Nam et al. 2001, Dolznig, Grebien et al.

However, our results cannot be explained by these known pathways alone. Specifically, we find that EpoR signaling, in addition to accelerating G1, also shortens S phase, at least in part through Stat5 activation, and the transcriptional induction of E2F4, cyclins E and A (Chapter III; (Hidalgo, Bejder et al. 2021)). To our knowledge, EpoR/Stat5 regulation of S phase speed is a novel mechanism of cell cycle regulation by growth factor signaling, with potential implications for other cytokine receptors that signal through the Jak/Stat pathways. It will be interesting to fully elucidate the mechanisms involved in this process as well as the lineages and developmental stages in which it is relevant. In the case of the erythroid lineage, the extremely fast cycle and S phase of S1
cells coincides with a cell-state switch. A fast S phase is required for loss of DNA methylation (Shearstone, Pop et al. 2011) and potentially of other epigenetic marks, which may causally link fast replication to a switch in cell state. Faster replication is also clearly relevant to the erythropoietic stress response, increasing cell division number with minimal prolongation of the process of red cell production.

Coincident with the fast S phase of S1 cells, we found a spike in the rates of rRNA transcription, protein synthesis and cell size growth. The spike in protein synthesis is almost entirely lost in Epor−/− and Stat5−/− mice, while the spike in rRNA transcription is significantly attenuated. These results suggest that Stat5 is a novel regulator of rRNA transcription and protein synthesis, although the downstream mechanisms are not yet known. Published Stat5 binding sites suggested by ChIP-seq analysis in erythroid cell lines (Gillinder, Tuckey et al. 2017) do not correspond to the rRNA transcription and processing genes or to the translation initiation factors that we identified as differentially downregulated in the Epor−/− and Stat5−/− fetal livers. This may be due to failure of cell lines to mimic the very narrow developmental window (the transient S1 cell state) in which the Stat5-mediated spike in growth takes place. We will examine this possibility by undertaking CUT&RUN for Stat5 in primary fetal liver cells. Alternatively, Stat5 may exert its effects by transcriptionally regulating an intermediate, such as Myc (Schuringa, Chung et al. 2004) or mTor. Although there is no strong evidence for Stat5 regulation of mTor, a recent report suggests
that Stat5 transcriptionally regulates c-Myc by binding the c-Myc superenhancer (Pinz, Unser et al. 2016). Stat5 may also regulate chromatin indirectly, potentially as part of a transcriptional complex with other DNA binding factors.

Taken together, our work suggests novel pathways for accelerating both cell cycling and cell growth, uniquely regulated by EpoR and Stat5 signaling during a developmental cell fate switch. Elucidating the underlying mechanisms of these processes could have implications for other lineages and for specific anemia syndromes, particularly those secondary to ribosomal haploinsufficiency.

Finally, the mechanisms underlying the close correlation between S phase speed and the rate of cell growth are not known. This correlation may be the result of co-regulation of both of these processes by similar upstream pathways. Alternatively, there may be direct interaction between the cell cycle and cell growth. Known pathways that link these two processes include the activation of p53 in response to nucleolar stress (Budde and Grummt 1999, Macias, Jin et al. 2010, Donati, Bertoni et al. 2011), leading to cell cycle arrest; and the transition from G1 to S phase once cell size reaches a permissive size (Ginzberg, Kafri et al. 2015, Zatulovskiy and Skotheim 2020). Neither of these pathways explains the tight correlation that we found between S phase speed and cell growth in ETD. There may therefore be additional mechanisms that allow S phase speed to be sensed by cell growth pathways, a possibility that will be addressed in future work.
Figure 4.1. Novel EpoR functions regulating cell growth and cell cycle in early erythroblasts.

Proposed model explaining novel EpoR-dependent functions during canonical protein synthesis and cell cycle pathways. Both Kit and EpoR activate ras/raf/MAPK and PI3-kinase; however, Stat5 is activated only by EpoR signaling. PI3-kinase and MAPK activate mTor complexes and the Myc transcription factor, which are involved in cell growth but also promote cell cycling, principally by cyclin D transcription. EpoR signaling also promotes cyclin D transcription via Stat5 and in doing so, accelerates G1 to S phase transition. EpoR signaling, in addition to accelerating G1, also shortens S phase in part through Stat5 activation and also increases the rates of rRNA transcription, protein synthesis and cell size growth.
Bibliography


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