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DANKE

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Zusammenfassung

Signal Transducer and Activator of Transcription 5 (STAT5) ist ein wesentlicher Effektor der Erythropoietin (EPO) Signaltransduktion in der erythroiden Differenzierung. Erstmals wurde eine vollständige phänotypische Analyse im erythroiden Kompartiment von Mäusen durchgeführt, denen beide Stat5 Isoformen (STAT5a und STAT5b) komplett fehlen. Der beobachtete Phänotyp der hypochromen mikrocytären Anämie und die daraus resultierende perinatale Letalität führten nicht nur zur Bestätigung von bekannten STAT5 Funktionen, wie Schutz vor Apoptose, sondern auch zur Entdeckung neuer STAT5 Rollen in der Eisen-Aufnahme und Häm-Biosynthese.

Anämie von Stat5 defizienten Mäusen konnte auf erhöhte Apoptose im Erythron durch verringerte Expression des anti-apoptotischen Proteins BCL-X_L und Verlust des anti-apoptotischen Proteins MCL1 zurückgeführt werden. Da jedoch Apoptose allein nicht zu hypochromen oder mikrocytären Phänotypen führt, zeigte die Suche nach weiteren Defekten Eisenmangel in Stat5 defizienten erythroiden Zellen. Der Mangel an zellulärem Eisen konnte durch reduzierte Expression von Transferrin-Rezeptor 1 (TFR1), der wichtigsten Komponente der zellulären Eisen-Aufnahme, erklärt werden. TFR1 Expression wird sowohl auf transkriptioneller als auch auf posttranskriptioneller Ebene gestört. Einerseits ist *Tfr1* ein direktes Ziel-Gen von STAT5, was zu eingeschränkter transkriptioneller Aktivität in der Knock-out-Situation führt. Andererseits wird im Stat5 Knockout *Tfr1* mRNA-Stabilität durch verringerte Expression des Iron Regulatory Protein 2 (IRP2) vermindert, das bestimmte Sequenzen im 3 'UTR der *Tfr1* mRNA bindet, was Schutz vor Degradation bietet, wenn zelluläres Eisen rar ist. Reduzierte *Tfr1* mRNA Transkription und Stabilität führen zu verringerter TFR1 Oberflächenexpression, verringerter zellulärer Eisen-Aufnahme und schließlich zu Problemen in der Häm-Synthese, jenem Prozess mit dem höchsten Eisen Bedarf in differenzierenden erythroiden Zellen. Drastisch reduzierte Häm-Synthese führt wiederum durch die langsame Hämoglobin Akkumulation zu hypochromer microcytärer Anämie.

In-vitro-Differenzierung von Stat5-Knockout Erythroblasten kann nicht durch direkte Eisenzugabe unter Umgehung des TFR1 Defekts gerettet werden. Daher müssen auch andere Mechanismen betroffen sein, die Hämoglobin Akkumulation behindern. Es konnte gezeigt werden, dass vier der acht Häm-Biosynthese Enzyme in Abwesenheit von STAT5 herunter reguliert waren. Vor allem die Expression von δ-Amino-Levulinic Acid Synthase 2 (ALAS2), dem geschwindigkeitsbestimmenden Enzym im Syntheseweg, war sowohl auf mRNA als auch auf Protein Ebene stark vermindert. Da sich alle vier enzymatischen Gene als unwahrscheinliche direkte STAT5 Ziele erwiesen, wurde der Status des gesamten hämatopoetischen transkriptionellen Netzwerks in *Stat5*$^{-/-}$ Erythroblasten ermittelt. Knockout Erythroblasten hatten stark erhöhte *Ldb1* und *Gata2*

mRNA Niveaus. Von beiden Genen ist bekannt, dass sie bei Überexpression erythroide Differenzierung durch Interferenz mit GATA-1-Aktivität negativ beeinflussen. Da in den Promotoren von allen vier herunter regulierten Häm-Biosynthese Enzymen GATA-1 Bindungsstellen gefunden wurden, kann der Mangel an Häm-Synthese auf die erhöhte Expression von LDB1 und GATA2 zurückgeführt werden.

Im Vergleich zu Erythropoetin und Erythropoetin-Rezeptor (*EpoR*) Knockouts, hat Verlust von *Stat5* einen milderen Phänotyp. Dieser Unterschied könnte aus partieller Kompensation durch andere STAT-Familienmitglieder mit sehr ähnlichen Ziel DNA-Sequenzen, wie STAT3, resultieren. Erhöhte Phosphorylierungsniveaus von STAT3 wurden in *Stat*-defizienten Erythroblasten gemessen, in denen dieser Effekt durch Verlust von Cytokine-Inducible-SH2 Protein (CIS), Suppressor Of Cytokine Signaling 1 (SOCS1) und Suppressor Of Cytokine Signaling 3 (SOCS3), die wichtige Negativregulatoren der EPOR Signaltransduktion sind, erklärt werden konnte. Im Gegensatz dazu zeigt eine anderen Familie von Negativregulatoren, Protein Inhibitors of Activated STAT (PIAS Proteins), deutlich erhöhte mRNA-Niveaus, was, mit hoher Wahrscheinlichkeit, erhöhter STAT Aktivität infolge des Verlusts von SOCS-Proteinen entgegenwirkt. Obwohl weitere funktionelle Experimente nötig sind, um kompensatorische Wirkungen zu untersuchen, können diese Ergebnisse die nicht notwendigerweise funktionelle, erhöhte Phosphorylierung von STAT3 in *Stat5* defizienten Erythroblasten und anderen Geweben erklären.

Abstract

Signal transducer and activator of transcription 5 (STAT5) is an essential downstream effector of erythropoietin (EPO) mediated signaling in erythroid differentiation. We conducted the first full phenotypical analysis in the erythroid compartment of mice completely lacking both *Stat5* isoforms (*Stat5a* and *Stat5b*). The observed phenotype of hypochromic microcytic anemia and perinatal lethality led to the confirmation of known STAT5 functions, like apoptosis protection, but also to the discovery of novel STAT5 roles in iron uptake and heme biosynthesis.

Anemia of Stat5 deficient mice could be attributed to increased apoptosis in the erythron caused by decreased expression of the anti-apoptotic protein BCL-X_L and loss of the anti-apoptotic protein MCL1. Since apoptosis alone does not explain the emergence of hypochromic or microcytic erythrocytes additional defects were suspected. This led to the finding of iron deficiency in *Stat5* deficient erythroid cells. Lack of cellular iron could be traced back to reduced expression of transferrin receptor 1 (TFR1), the main player in cellular iron uptake. TFR1 regulation was found to be disturbed both at transcriptional and posttranscriptional level. On the one hand *Tfr1* is a direct STAT5 target with reduced transcriptional activity in the knockout situation. *Tfr1* mRNA stability, on the other hand, is impaired in knockouts due to decreased expression of iron regulatory protein 2 (IRP2), which binds several sequence elements in the 3' UTR of *Tfr1* mRNA to protect it from degradation, when cellular iron is scarce. Reduced *Tfr1* mRNA transcription and stability leads to decreased TFR1 surface expression, diminished cellular iron uptake and finally to problems in heme synthesis, the process with the highest iron demand in differentiating erythroid cells. Drastically reduced heme synthesis carries over to diminished hemoglobin accumulation, which in turn causes hypochromic microcytic anemia.

In vitro differentiation of *Stat5* knockout erythroblasts cannot be rescued by supplying cells with iron complexes bypassing their TFR1 defect. Therefore additional mechanisms must be affected by loss of *Stat5* that hinder hemoglobin accumulation. Indeed, four out of eight enzymes involved in heme biosynthesis were found to be downregulated in the absence of STAT5. Most importantly δ-aminolevulinic acid synthase 2 (ALAS2), the rate limiting enzyme in the pathway, displayed severely reduced mRNA and protein levels. Since all four genes were unlikely to be direct STAT5 targets, the status of the hematopoietic transcriptional network in $Stat5^{-/-}$ erythroblasts was surveyed further. Knockout erythroblasts had highly elevated levels of *Ldb1* and *Gata2* mRNA, both known to interfere with erythroid differentiation by negatively affecting GATA1 activity. Since GATA1 binding sites are present in the promoters of all four downregulated heme biosynthetic enzymes, the lack of heme synthesis may be linked to increased expression of LDB1 and GATA2.

In comparison to ablation of erythropoietin (*Epo*) and erythropoietin receptor (*EpoR*) knockouts, loss of *Stat5* has a milder phenotype. This difference may be attributed to partial rescue by other STAT family members with similar DNA-binding consensus sequences like STAT3. Higher levels of phosphorylated STAT3 were indeed observed in *Stat5* deficient erythroblasts, which could be traced back to loss of cytokine-inducible-SH2-containing protein (CIS), suppressor of cytokine signaling 1 (SOCS1) and suppressor of cytokine signaling 3 (SOCS3), which constitute important negative regulators of EPOR signaling. In contrast another family of negative regulators, protein inhibitors of activated STAT (PIAS) proteins, displayed significantly elevated mRNA levels, counteracting increased STAT activity due to loss of SOCS proteins. Although further functional experiments are needed to clarify compensatory effects, these findings may help to explain the elevated levels of STAT3 phosphorylation found in erythroblasts and other tissues lacking *Stat5*.

Introduction

Hematopoiesis

Hematopoiesis, the generation of blood‘s cellular components, is an essential process for all higher eukaryotes. Mature blood cells are involved in e.g. defense against pathogens, wound sealing, bone restructuring and gas exchange. Despite their diverse functions all these mature cell pools are replenished by a single type of somatic stem cell, the hematopoietic stem cell (HSC). The hematopoietic system is commonly accepted as the prime example for hierarchical multi-lineage differentiation from one common stem cell type via multipotent progenitors and committed precursors to mature cells.

Hematopoietic Stem Cells

Ontogeny

During vertebrate development at least four different regions are involved in hematopoiesis. In sequential order the mammalian sites of hematopoiesis are yolk sac, aorta-gonad mesonephros (AGM) region, fetal liver and bone marrow (Orkin and Zon 2008) (see Figure 1). Recently the placenta has been proposed as another hematopoietic region and possible origin of hematopoietic stem cells, since significant numbers of HSCs have been found there (Gekas, Dieterlen-Lievre et al. 2005; Ottersbach and Dzierzak 2005). For the last years it has been unclear if placental HSCs are generated *de novo* or colonize the placenta via circulation. A very recent experiment was able to provide evidence for *de novo* generation of HSCs, as *Ncx1* knockout embryos, which lack hearbeat and therefore circulation, still developed HSCs in their placentas (Rhodes, Gekas et al. 2008).

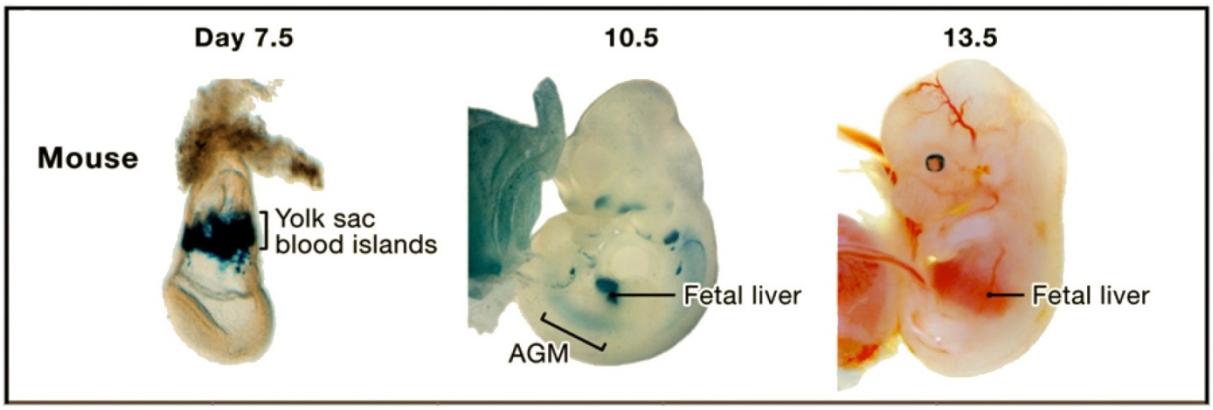

Figure 1: Hematopoiesis occurs first in yolk sac blood islands and later at the aorta-gonad mesonephros (AGM) region, placenta, and fetal liver. Yolk sac blood islands are visualized by LacZ staining of transgenic embryo expression GATA1-driven LacZ. AGM and FL are stained by LacZ in Runx1-LacZ knockin mice. (Orkin and Zon 2008)

Hematopoietic activity in different regions is temporally overlapping but distinct in characteristics. In the mouse hematopoiesis is independently initiated in the yolk sac, which creates primitive erythrocytes, macrophages and progenitors of definitive erythroid, megakaryocyte and mast cell lineage, as well as in the para-aortic splanchnopleura, which gives rise to the AGM region later. The hemangioblast, a controversial cell that originates both hematopoietic and vascular tissues (Choi, Kennedy et al. 1998), is believed to be the source of yolk sac derived blood cells, as the region displayed little, if any, HSC potential in early engraftment experiments (Murry and Keller 2008). Nevertheless recent data suggests that yolk sac cells may seed HSCs in the fetal liver (Samokhvalov, Samokhvalova et al. 2007). Hematopoiesis in the AGM region generates cells of myeloid, lymphoid and definitive erythroid lineage derived from primordial HSCs. These primordial or primitive HSCs arise from the ventral wall of the single aorta tube within the AGM region between day 10 and 11 of mouse embryonic development.

At embryonic day 9.5 the newly formed fetal liver is colonized by external hematopoietic elements, which are probably of yolk-sac origin (Palis 2008). Subsequent migration of AGM region derived hematopoietic cells into the fetal liver may lead to emergence of adult-repopulating capability. Niches that support fetal HSCs in the fetal liver similar to adult HSC niches in the bone marrow have not yet been found. This is not entirely surprising, since fetal HSCs do not share all properties with adult ones. In contrast to adult HSCs, fetal HSCs are not quiescent but steadily cycling, which would demand a difference in fetal niche architecture (Orkin and Zon 2008).

In birds and mammals the last stage on the way to adult hematopoiesis is the bone marrow. It is generally assumed that fetal HSCs colonize the bone marrow via circulation (Laird, von Andrian et al. 2008), but there are indications that fetal liver and bone marrow may be seeded at similar times during development (Delassus and Cumano 1996). For a detailed temporal and spatial overview of hematopoiesis in ontogeny see

Figure 2. It will be necessary to directly track cellular migration in the embryo to finally clarify the interconnections between the four hematopoietic sites and their contribution to adult hematopoiesis.

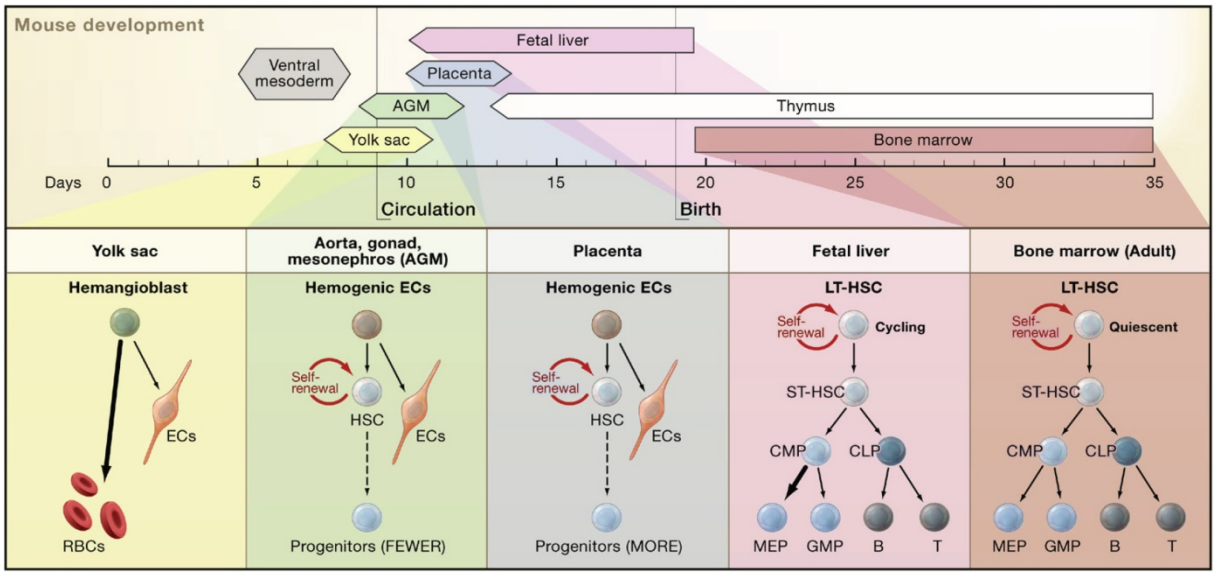

Figure 2: The yolk sac hemangioblast is proposed to give rise to both blood and endothelial cells (ECs). The next region of hematopoiesis is the AGM. It has been proposed that the AGM forms hemogenic ECs in the ventral wall of the aorta that bud off HSCs. Placental HSCs could arise through de novo generation or colonization upon circulation, or both. The relative contribution of each of the above sites to the final pool of adult HSCs remains largely unknown. Subsequent definitive hematopoiesis involves the colonization of the fetal liver, thymus, spleen, and ultimately the bone marrow. long-term HSC (LT-HSC); short-term HSC (ST-HSC); common myeloid progenitor (CMP); common lymphoid progenitor (CLP); megakaryocyte/erythroid progenitor (MEP); granulocyte/macrophage progenitor (GMP) (Orkin and Zon 2008)

Niche

HSCs give rise to at least eight different lineages (Orkin and Zon 2008) while maintaining their stable abundance by self-renewal. To succeed in both tasks HSCs need to undergo asymmetric cell division, where one daughter cell differentiates further and the other retains its "stemness", or have to employ a form of postmitotic, extrinsic decision process. Although asymmetric cell division of HSCs and progenitors has already been postulated in 1984 (Suda, Suda et al. 1984), definite evidence for this model has not yet been found. Recent observations in human HSCs argue in favor of the asymmetric division model, as four proteins (CD53, CD62L/L-selectin, CD63/lamp3, and CD71/transferrin receptor) have been found to segregate differentially during division. (Beckmann, Scheitza et al. 2007) Nevertheless alternatives to the asymmetric division model have been brought up. The obvious heterogeneity in HSC fate can be explained by reversible expression of functional stem cell potentials controlled by niche-induced developmental asymmetries. (Stumpf, Waskow et al. 2006) Therefore the model suggests that cells divide symmetrically into equal daughter cells, although their further

fate differs depending on extrinsic cues in form of cell-cell and cell-microenviroment interactions.

The specific microenviroment for stem cell maintenance is usually referred to as niche. The hematopoietic niche has been subject to extensive research for the last decade. The bone-marrow endosteum is believed to provide the necessary signals to HSCs that enable their maintenance. The endosteal surface is covered by bone-lining cells, often imprecisely termed osteblasts in the literature. Bone-lining cells are heterogenous in respect of differentiation level and ability to synthesize bone, but contain only a minority of true osteoblasts (Kiel and Morrison 2008). Additionally both osteoclasts and vascular cells reside within the endosteum and both may have important roles in regulating the fate of hematopoietic stem cells and progenitors. So far seven factors have been found to be genetically necessary for normal HSC maintenance *in vivo*: Angiopoietin (Arai, Hirao et al. 2004), Ca^{2+} ions (Adams, Chabner et al. 2006), CXCL12 (Zou, Kottmann et al. 1998; Ara, Tokoyoda et al. 2003), sonic hedgehog homologue (SSH) (Trowbridge, Scott et al. 2006), osteopontin (Nilsson, Johnston et al. 2005; Stier, Ko et al. 2005), stem-cell factor (SCF) (Barker 1994; Kiel and Morrison 2006) and thrombopoietin (Kimura, Roberts et al. 1998; Yoshihara, Arai et al. 2007). The physiologically relevant sources for those factors have not yet been determined, although angiopoietin, thrombopoietin and CXCL12 are thought to be secreted by osteoblasts (Petit, Szyper-Kravitz et al. 2002; Arai, Hirao et al. 2004; Yoshihara, Arai et al. 2007). Alternatively angiopoietin is also secreted by megakaryocytes, CXCL12 by reticular cells and thrombopoietin by liver and kidney. (Kiel and Morrison 2008) Clarification of the importance of osteoblastic secretion can only be achieved once those factors have been conditionally deleted in osteoblasts. Interestingly all of the factors mentioned above are soluble, so direct cell-cell contact of HSCs with niche cells may not be necessary. It has been suggested that HSCs depend on homotypic adhesion to osteoblasts via N-cadherin (Zhang, Niu et al. 2003; Wilson, Murphy et al. 2004), but this finding has recently been profoundly challenged (Kiel, Radice et al. 2007), as cells with HSC activity do not express N-cadherin and N-cadherin deficiency has no effect on adult HSC maintenance.

In addition to the endosteum a second niche for HSCs has been suggested, the perivascular niche. During embryonic and fetal development HSCs reside primarily in vascular or perivascular niches (Gekas, Dieterlen-Lievre et al. 2005; Ottersbach and Dzierzak 2005; Mikkola and Orkin 2006). It is therefore reasonable to assume that endothelial cells have the potential to maintain hematopoietic stem cells in the adult organism as well. A potential perivascular niche was first revealed when adult bone marrow was stained for signaling lymphocyte activation molecule (SLAM) markers, a marker combination which detects HSCs with high specifity. 60% of HSCs were found adjacent to sinusoids, whereas only 20% were associated with the endosteum. (Kiel, Yilmaz et al. 2005) This observation can be explained by various models. A) The

perivascular niche offers HSCs necessary cell-cell contacts, whereas the endosteum contributes soluble factors, resulting in HSCs residing in the perivascular niche. B) HSCs reside in the the endosteal niche, but constant trafficking between niche and circulation brings a high percentage of stem cells close to blood vessels, especially if entry into the vessel is a time consuming part of migration. C) HSCs reside in distinct endosteal and perivascular niches that are either functionally redundant or differ in HSC regulation.

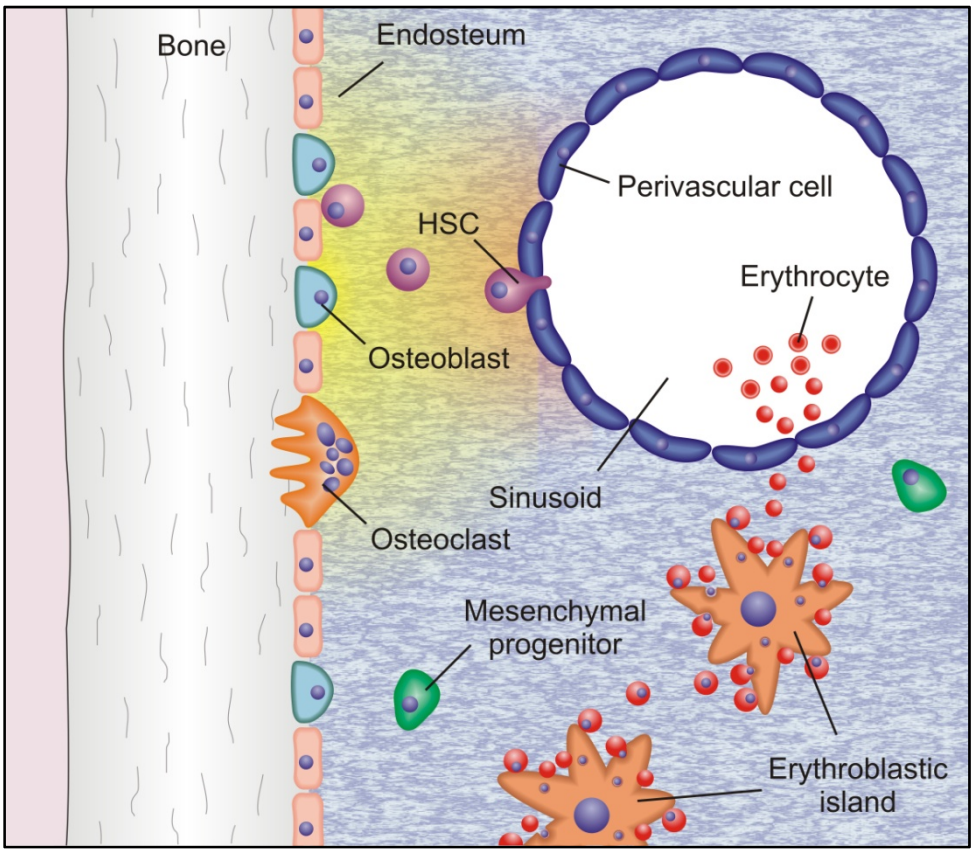

Figure 3: Bone marrow niche. Endosteal and perivascular cells are both believed to be involved in HSC maintenance. It is yet unknown if they form separate niches that may or may not be functionally redundant or contribute to a single common niche.

Although endosteal, vascular and perivascular cells are believed to be the main players in HSC maintenance, it still cannot be excluded that other cell types play important roles or even form extramedullary niches. Adipocytes, for example, have been shown to excrete adiponectin, a factor that increases HSC proliferation while retaining them in an immature state. (DiMascio, Voermans et al. 2007) The generation of tissue-specific knockouts of HSC maintenance factors will shed new light on contributions of different cell types and will hopefully improve our understanding of niche anatomy in mammals.

Heterogeneity

Recent findings demonstrated that the HSC population is far less homogenous than anticipated. Analysis of long term repopulation on a single cell level revealed three different types of HSCs that vary in self-renewal capacity, but more importantly in the proportions of their progenies' lineage commitment. It could be shown that HSCs can be roughly divided into three different populations. Balanced HSCs give rise to lymphoid and myeloid cells in a 85:15 ratio. Myeloid biased (My-Bi) HSCs generate lower levels of lymphoid cells and lymphoid biased (Ly-Bi) HSCs produce lower levels of myeloid cells (Muller-Sieburg, Cho et al. 2004). Interestingly this lineage bias is heritable and maintained in serial transplantations (Sieburg, Cho et al. 2006; Dykstra, Kent et al. 2007). Although different subpopulations among HSCs have been convincingly highlighted, how or when responsible epigenetic imprintings occur is not yet known.

Multipotent Progenitors

The process of commitment from pluripotent HSC to lineage restricted progenitor is subject to extensive controversy. Multipotent progenitors are distinguished by their surface markers and defined by their ability to reconstitutue certain cell types *in vivo* and to generate colonies of certain lineages in vitro. Based on the work of Irvin Weissman's group multipotent progenitors have been assembled into strict hierarchies of steadily narrowing lineage potential (Reya, Morrison et al. 2001). The first descendant of the hematopoietic stem cell (often called long term HSC) is improperly called short term HSC (ST-HSC). The name is misleading, as the ST-HSC is no true stem cell but a multipotent progenitor and has no ability to maintain itself. Upon transplantation ST-HSCs reconstitute all hematopoietic lineages, but only for limited time until depletion of the transplanted cells (Uchida, Jerabek et al. 1996). Downstream of the ST-HSC lies the multipotent progenitor (MPP), the last cell type to give rise to all hematopoietic lineages. The MPP branches into common myeloid progenitor (CMP) (Akashi, Traver et al. 2000) and common lymphoid progenitor (CLP) (Kondo, Weissman et al. 1997). The CMP differentiates further into megakaryocyte/erythroid progenitors (MEP) and granulocyte/macrophage Progenitors and has lost any ability to contribute to the lymphoid lineage. In contrast the CLP is restricted to generation of B- and T-cell progenitors (see Figure 4).

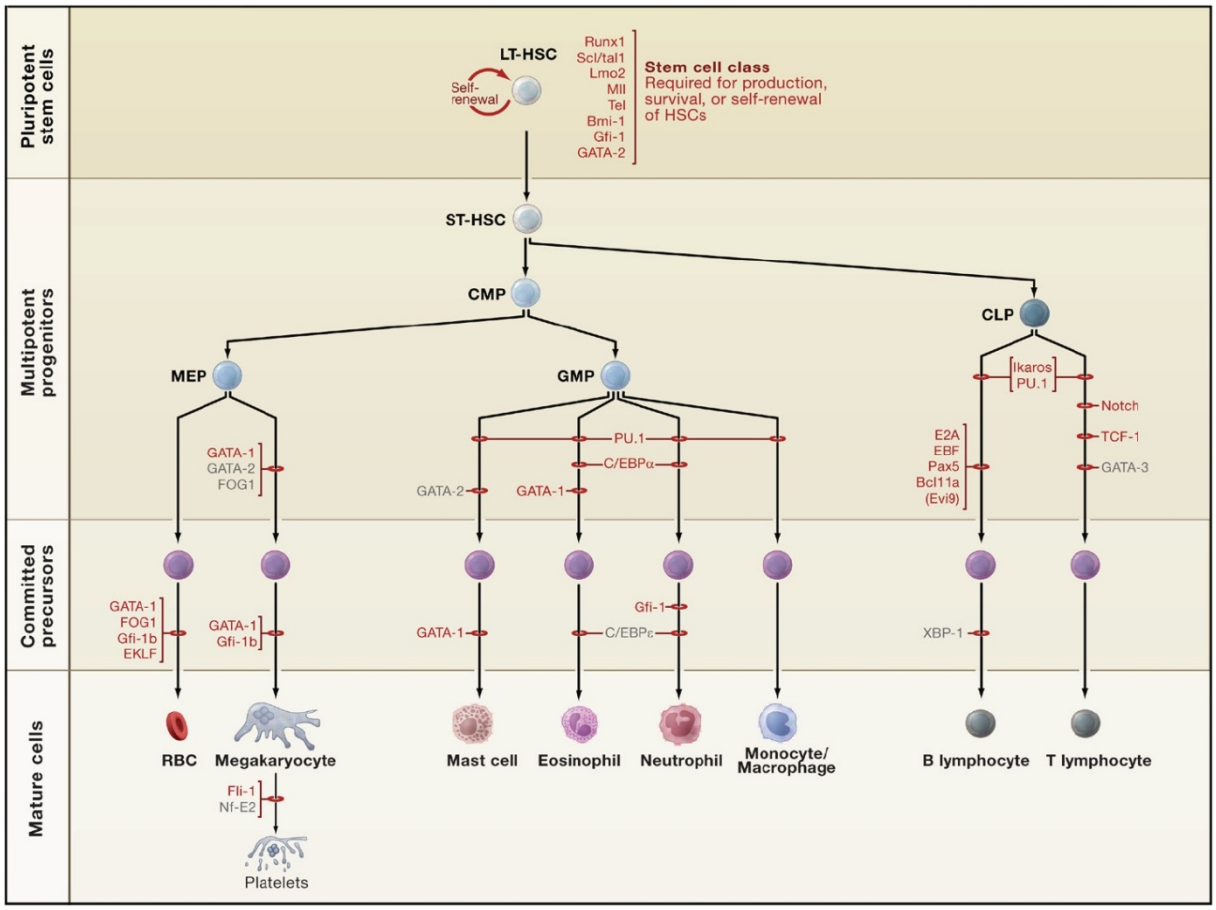

Figure 4: The hematopoietic lineage tree and its most important transcription factors. The stages at which hematopoietic development is blocked in the absence of a given transcription factor, as determined through conventional gene knockouts, are indicated by red loops. The factors depicted in red have been associated with oncogenesis. (Orkin and Zon 2008)

In recent years the dogma of strict hierarchical lineage determination in hematopoiesis was called into question as further multipotent progenitor populations with granulocyte/macrophage and lymphoid potential have been found (Luc, Buza-Vidas et al. 2007). This lymphoid-primed multipotent progenitor (LMPP) hints to higher dynamics in the HSC commitment process than originally anticipated. The model of binary lineage restriction decisions at strict branching points got interesting competition in explaining observed phenomena. A gradual loss of lineage potential, for example, can be very well predicted with older models based on stochastic HSC commitment (Till, McCulloch et al. 1964; Suda, Suda et al. 1984). CMP, CLP, MEP and GMP may be milestones on heavily trafficked highways, but it cannot be excluded that a great variety of minor roads may lead to the same destinations.

Committed Precursors and Mature Cells

The greatest expansion in cell numbers occurs during transition from committed progenitors to mature cells. At the committed precursor level the final eight lineages

arise. The GMP originates all myeloid cells, specifically basophil, eosinophil and neutrophil granulocytes, mast cells and monocytes. The CLP creates all lymphoid cells by differentiation into both T-cell and B-cell precursors. The MEP spawns both the megakaryocyte lineage, which results in the generation of platelets, and the erythroid lineage, which leads to red blood cell formation. Detailed description of all mature hematopoietic cells in property and function would exceed the scope of this introduction. Therefore, given the underlying projects, emphasis will be laid on erythropoiesis.

Erythropoiesis

Erythropoiesis, the generation of red blood cells, is a fascinating tightly regulated process, whose numerical facts alone are awe inspiring. Under steady state conditions the human body releases 2 x 10^6 reticulocytes into the bloodstream every second, with the capability to increase this output 15- to 20-fold under stress conditions (Chasis and Mohandas 2008). In the blood stream reticulocytes mature to erythrocytes gaining the ability to facilitate gas exchange even in the most remote tissues. Due to its special membrane properties the red blood cell can even enter capillaries, whose diameter is 25% smaller than the cell's. Together with the nucleus erythrocytes have lost all other organelles and therefore belong to the few cell types that rely on glycolysis alone. After approximately 120 days aged human red blood cells are phagocytosed by macrophages and degraded. In the following I want to introduce mammalian erythroid ontogeny, differentiation, important transcription factors and the erythroid niche.

Ontogeny

In the mouse the first primitive erythroblasts, which inhabited the blood islands of the yolk sac since E7.25, enter the newly established circulation at E8.25 (McGrath, Koniski et al. 2003; Lucitti, Jones et al. 2007). In the course of the following 8 days these erythroblasts undergo synchronous maturation in circulation resembling differentiation of definitive erythroblasts. Since *in vitro* studies suggested that primitive erythroblasts do not enucleate spontaneously, it was assumed that the first wave of erythrocytes in the embryo stays nucleated (Palis 2008). Recent findings have shown though that primitive erythroblasts do enucleate, but probably only upon interaction with macrophages (McGrath, Kingsley et al. 2008). In contrast to its definitive counterpart the primitive erythrocyte expresses not only α1-globin, α2-globin, β1-globin and β2-globin, but also ζ-globin, βH1-globin and εγ-globin (Trimborn, Gribnau et al. 1999). This mixed globin content results from a maturation-dependent globin-gene expression switch, where primordial ζ-globin and βH1-globin gene transcription is gradually superseded by α1-

globin, α2-globin and εγ-globin gene transcription at the transition to the reticulocyte stage (Kingsley, Malik et al. 2006). At E9.5 the second wave of erythropoiesis starts when BFU-E and CFU-E cells from the yolk sac colonize the newly formed fetal liver. These progenitors give rise to the first definitive erythoid cells in the embryo even before adult-repopulating hematopoietic stem cells can be found in the fetal liver (Palis 2008). The third and last wave of erythropoiesis occurs towards the end of gestation in both the late fetal liver and the emerging bone marrow, which is colonized by fetal liver HSCs (see Figure 5). Third wave erythroblasts are direct descendents of HSCs and resemble their adult steady-state counterparts in all aspects, except for their greater and more rapid proliferation capacity and higher responsiveness to EPO (Rich and Kubanek 1976; Migliaccio and Migliaccio 1988; Emerson, Thomas et al. 1989).

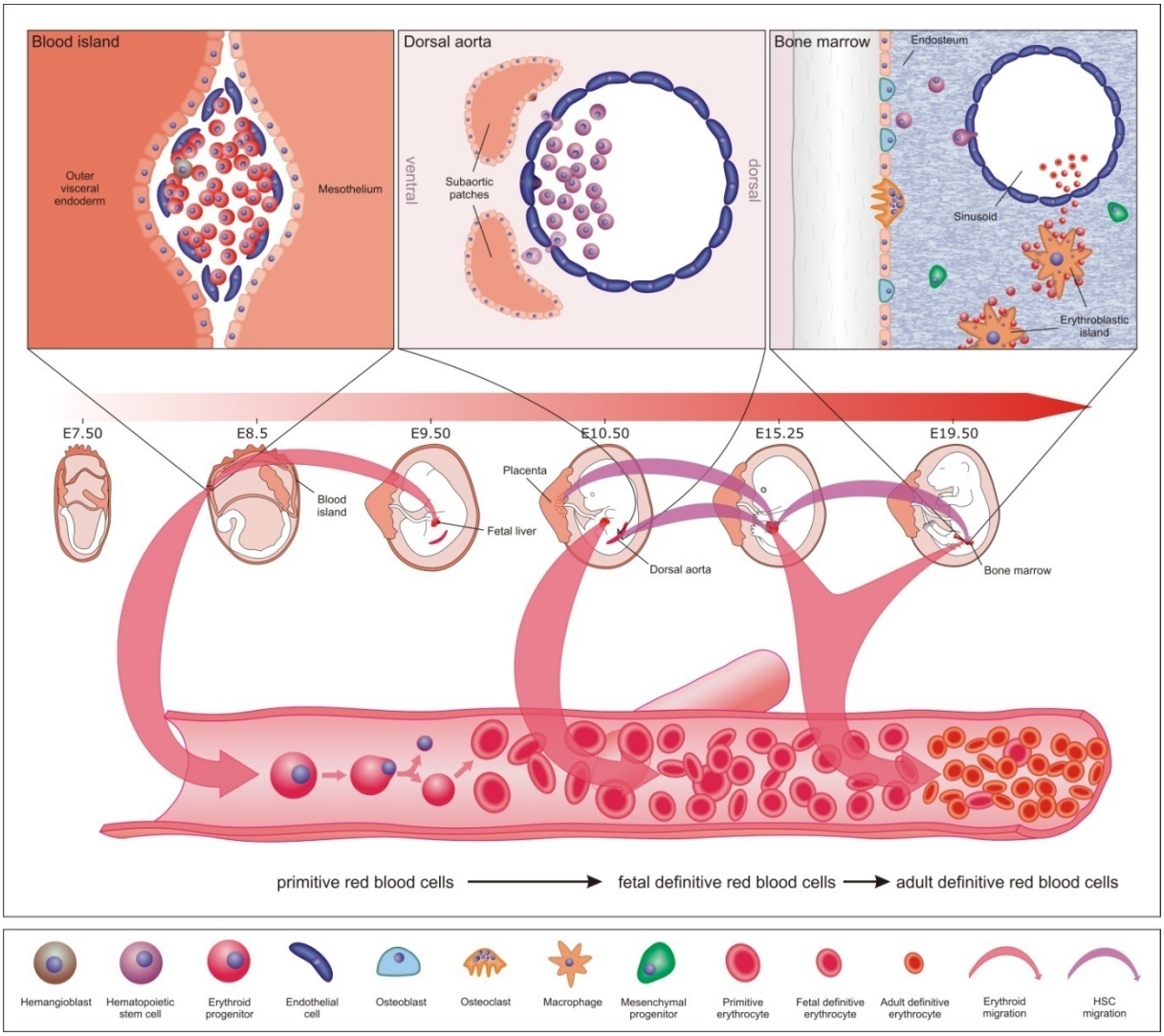

Figure 5: Hematopoietic ontogeny and the three waves of erythropoiesis. The first wave consists of primitive erythroid progenitors that originate in the yolk sac blood islands from hemangioblasts and generate a synchronous cohort of primitive erythroid precursors that mature in the bloodstream and enucleate to form reticulocytes and pyrenocytes. The second wave consists of a transient wave of definitive erythroid progenitors (BFU-E) that emerge from the blood islands and seed the fetal liver. There they generate maturing definitive erythroid precursors that enucleate to become the first circulating definitive erythrocytes of the fetus. At the same time adult-repopulating hematopoietic stem cells (HSCs) arise independently in the placenta and dorsal aorta. After migration into the fetal liver these HSCs are the source of the third

wave of erythropoiesis which consists of a continuous stream of adult definitive erythrocytes. In late gestation the focus of red blood cell generation is gradually shifted from the fetal liver to the bone marrow, which was seeded before by fetal liver HSCs. Unlike primitive erythroid cells, definitive erythroid precursors mature extravascularly within erythroblast islands.

Lineage committment

In steady-state adult erythropoiesis the first lineage decision leading to generation of erythrocytes occurs at the MPP stage. The decision in CMP or CLP commitment has recently been shown to involve the antagonistic action of the transcription factors GATA1 and PU.1, mimicking the long-known downstream lineage decision at the CMP level (Arinobu, Mizuno et al. 2007). Antagonism is a common theme in lineage commitment, where transcription factors do not only promote their own lineage, but also actively counteract any other (Orkin and Zon 2008)(see Figure 6). Depending on initial expression levels of particular transcription factors differentiation is pushed into a single direction with self-augmenting certainty. PU.1, the transcription factor driving maturation into mast cell/basophil/eosinophil/neutrophil/monocyte lineage, has been shown to bind to his erythroid/megakaryocytic counterpart GATA1 thereby abolishing its ability to bind to DNA (Galloway, Wingert et al. 2005; Rhodes, Hagen et al. 2005; Liew, Rand et al. 2006). Additionally PU.1 antagonizes CBP/p300 mediated acetylation of GATA1, which leads to disturbed GATA1 chromatin occupancy (Hong, Kim et al. 2002; Lamonica, Vakoc et al. 2006). In certain contexts PU.1 can even recruit corepressor complexes consisting of pRb, SUV39H and heterochromatin protein 1α to GATA1 target genes (Stopka, Amanatullah et al. 2005).

The erythroid cell/megakaryocyte decision at the MEP level, the last lineage choice in erythropoiesis, is made by another pair of functional cross-antagonizing transcription factors, EKLF and FLI1. The megakaryocytic transcription factor FLI1 is able to downregulate target genes of its erythroid counterpart EKLF via action of its ETS domain and recruitment to erythroid promoters by association with EKLF. EKLF can in return actively repress FLI1 target genes like GPIX (Starck, Cohet et al. 2003). Thus the final maturation of progenitors to erythrocytes is controlled by a delicate balance of multiple transcription factors.

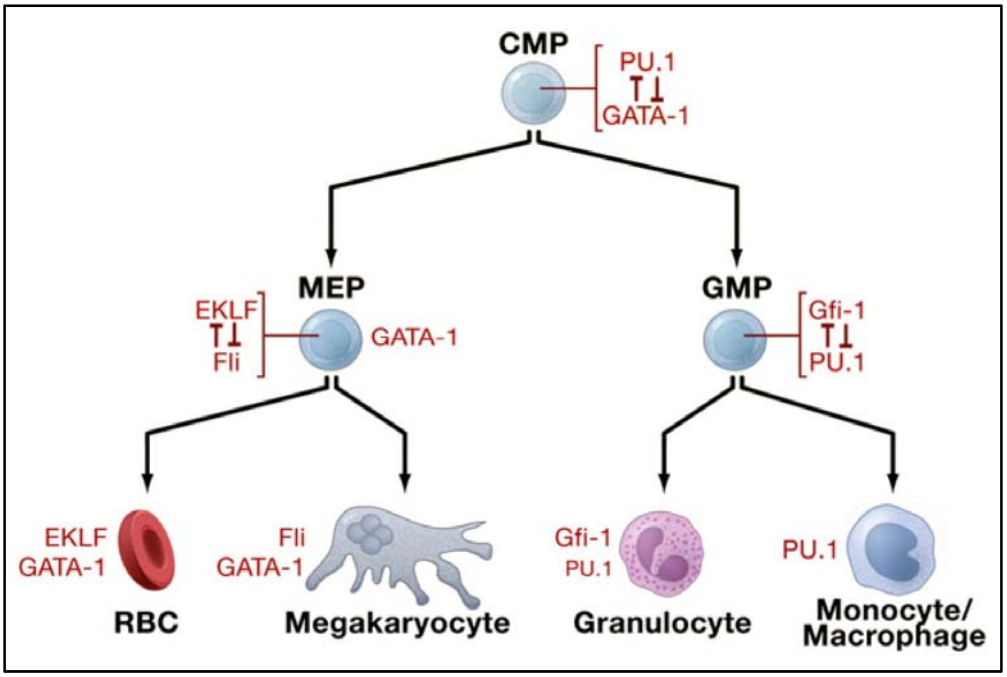

Figure 6: Transcription factor antagonism in lineage determination (Orkin and Zon 2008)

Maturation Stages

Based on classic colony assays the first committed erythroid progenitors are known as BFU-e (burst forming unit – erythroid) and CFU-e (colony forming unit – erythroid). Downstream of these rather undefined populations lies the proerythroblast, the basophilic erythroblast, the polychromic erythroblast and finally the orthochromatic erythroblast (Socolovsky 2007). The different blast stages are distinguished by morphology and cover the transition from a large cell, whose cytoplasm appears deeply royal blue with a reddish nucleus upon Wright-Giemsa staining, to a small cell with colorless cytoplasm and a deep blue compacted nucleus (see Figure 7). The conversion to the next stage is marked by ejection of the compacted nucleus. This process gives rise to the pyrenocyte, which consists of the cell membrane enclosed nucleus, and the reticulocyte, which contains the majority of cytoplasm. As further translation is mandatory for sufficient hemoglobinization at the final stages of maturation, reticulocytes contain high levels of erythroid transcripts and are therefore light-blue upon Wright-Giemsa staining. Reticulocytes enter the bloodstream via sinusoids in the bone marrow and mature to erythrocytes in circulation.

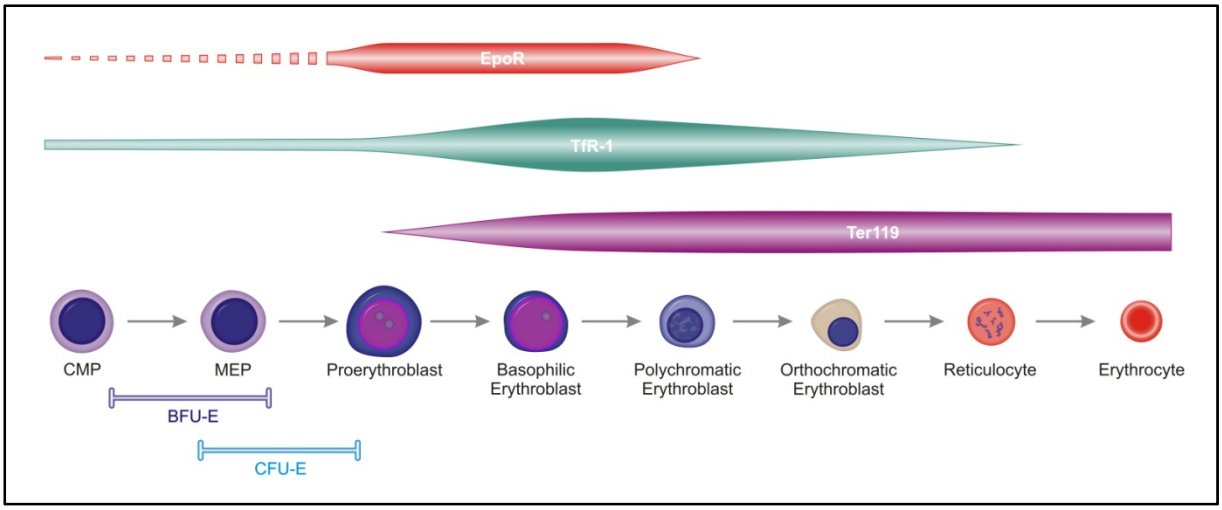

Figure 7: Erythroid maturation stages and surface markers. The broken EpoR expression line is result of various reports of EpoR expression on cells upstream of the proerythroblast. Cells are drawn as characteristically seen in Wright's, May-Giemsa or May-Grünwald-Giemsa stain, with the exception of the reticulocyte which is depicted when characteristically stained with methylene blue or Azure B. BFU-E and CFU-E marks the range of detected cells with the respective colony assays.

Important transcription factors

GATA1

The dual zinc-finger GATA transcription factor family member GATA1 is the main regulator of erythropoiesis, as most erythroid specific genes feature at least one GATA motif [(A/T)GATA(A/G)] (Evans, Reitman et al. 1988; Reitman and Felsenfeld 1988; Merika and Orkin 1993). Targeted disruption of *Gata1* has been shown to lead to total ablation of erythropoiesis in mice (Tsai, Keller et al. 1994), which is at least in part due to absent induction of the anti-apoptotic gene *Bcl-xL* in erythroid progenitors (Weiss and Orkin 1995). The exact effect of GATA1 is strongly dependent on both expression level and developmental stage of erythroid cells. Inducible GATA1 expression leads to proliferation arrest via downregulation of *c-Myc* and erythroid maturation in committed erythroid progenitors (Rylski, Welch et al. 2003). In more immature progenitors, however, GATA1 does not act as cell cycle blocker, but influences cell-fate commitment decisions towards erythroid, eosinophil and megakaryocytic lineages (Kulessa, Frampton et al. 1995; Heyworth, Pearson et al. 2002). The observed plethora of GATA1 effects is a direct result of the multitude of known interaction partners. FOG1, TRAP220, CBP/p300, PU.1, SP1 and EKLF have all been shown to interact with the GATA1 (Merika and Orkin 1995; Blobel, Nakajima et al. 1998; Crispino, Lodish et al. 1999; Rekhtman, Radparvar et al. 1999; Stumpf, Waskow et al. 2006). Beyond simple protein-protein interaction GATA family members were reported to nucleate multi-protein complexes (Rodriguez, Bonte et al. 2005), which adds a further layer of complexity to its function.

GATA2

A close homologue to GATA1, GATA2, is expressed earlier in hematopoiesis and is crucial for survival and proliferation of HSCs (Tsai, Keller et al. 1994; Tsai and Orkin 1997; Minegishi, Ohta et al. 1999). The so-called GATA switch, where increasing levels of GATA1 displace GATA2 from its own promoter coupled with *Gata2* repression (Grass, Boyer et al. 2003), is an important process in erythroid maturation. Experiments using an estrogen-inducible GATA2-ER fusion protein have shown that continued GATA2 expression promotes proliferation but blocks terminal differentiation of primary erythroblasts (Kriegel 1993). Although GATA1 and GATA2 occupy largely the same chromosomal loci in the genome, they are thought to interact with different partners to facilitate expression or repression of target genes.

FOG1

Friend of GATA1 (FOG1) is an important interaction partner and coregulator of GATA1 that facilitates its chromatin occupancy and GATA switches (Pal, Cantor et al. 2004). *Fog1* knockout mice die during mid-embryonic development, probably due to severe anemia (Tsang, Fujiwara et al. 1998). Cells devoid of FOG1 are unable to repress GATA2, which suggests that FOG1 might be an important link between GATA1 and chromatin remodeling complexes that enforce silencing on GATA2 and other loci. Interaction of FOG1 with the nucleosome remodeling and histone deacetylase (NuRD) complex, which induces transcriptional repression and structural changes in chromatin, has been shown in MEL cell nuclear extracts (Hong, Nakazawa et al. 2005).

EKLF

Erythroid Kruppel-like transcription factor (EKLF) is essential for erythropoiesis and adult globin gene expression (Nuez, Michalovich et al. 1995; Perkins, Sharpe et al. 1995). Although EKLF was initially believed to be solely a regulator of definitive erythropoiesis, recent years saw additional roles ascribed to this transcription factor in primitive erythropoiesis (Hodge, Coghill et al. 2006). Heme biosynthetic enzymes, cytoskeletal proteins and most prominently β-like globin are direct or indirect targets of EKLF (Drissen, von Lindern et al. 2005; Nilson, Sabatino et al. 2006; Pilon, Nilson et al. 2006). EKLF is considered to be an important factor in hemoglobin switching, the process that leads to increasing amounts of adult globins at the expense of the earlier fetal globins in peripheral blood (Donze, Townes et al. 1995; Perkins, Gaensler et al. 1996).

The switch itself is suggested to be a result of increased levels of EKLF in definitive versus primitive cells (Zhou, Pawlik et al. 2006).

Niche

The niche for erythroblastic development, the erythroblastic island, consists of central macrophage surrounded by erythroblasts in various developmental stages (Bessis 1958)(see Figure 8). Erythroid islands localize primarily to regions throughout the bone marrow, but can also be found in fetal liver and spleen. Interestingly there is a gradient of erythroblastic differentiation towards sinusoids, where significantly more proerythroblasts are associated with nonadjacent macrophages and more mature erythroblasts occupy islands neighboring sinusoids (Yokoyama, Etoh et al. 2003). This finding raises the possibility that erythroblastic islands may either be motile structures or that erythroblasts move in the course of differentiation in direction of blood vessels by detaching and reattaching to macrophages (Chasis and Mohandas 2008).

Role

It has been suggested that central macrophages phagocytose extruded nuclei and provide ferritin as an iron source to maturing erythroblasts (Bessis and Breton-Gorius 1962; Leimberg, Prus et al. 2008). Pyrenocytes can be detected and subsequently engulfed by macrophages due to rapid loss of phosphatidylserine asymmetry in their membrane, giving them a resemblance to apoptotic cells (Yoshida, Kawane et al. 2005). The importance of erythroblastic islands has been a matter of dispute for several years, since erythroblasts can be terminally differentiated *in vitro* in the presence of erythropoietin but absence of macrophages (Dolznig, Boulme et al. 2001; Carotta, Pilat et al. 2004; Leberbauer, Boulme et al. 2005). Nevertheless it has been convincingly shown that the abundance of erythroblastic islands correlate directly with erythropoietic activity in mice (Chasis and Mohandas 2008). Additionally a controversial publication has shown that absence of enucleation and abnormal differentiation of erythroblasts in *Rb* knockout mice may be, at least in part, attributed to differentiation defects of *Rb* null macrophages (Iavarone, King et al. 2004). Furthermore knockout of the cytoskeletal-associated protein palladin, which is expressed in central macrophages, leads to disorganization of erythroblastic islands, erythroid differentiation defects, increased erythroblast apoptosis, anemia and finally to death at E15.5 (Liu, Li et al. 2007). Based on those findings one can say that erythroblast differentiation does not depend on instructive input from macrophages, but there is mounting evidence for central macrophages to act in a supportive and possibly regulative manner.

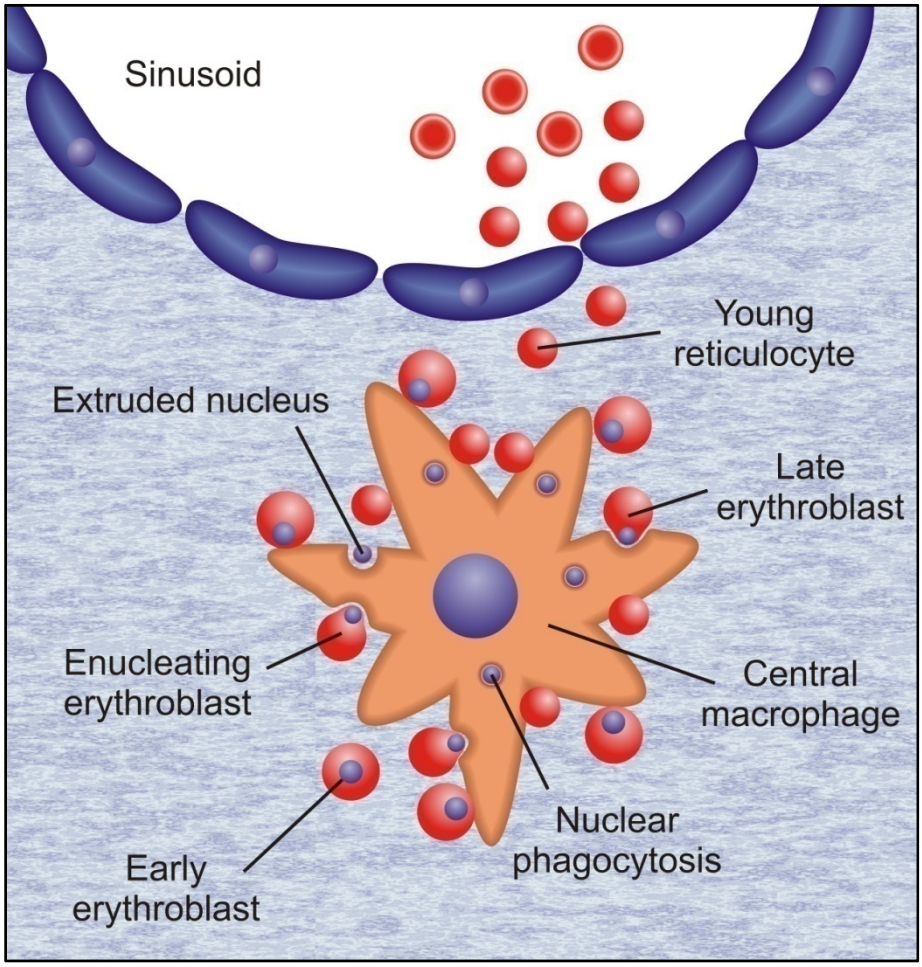

Figure 8: Proliferation and differentiation processes occurring within the erythroid niche. Early-stage erythroblasts are larger cells with centrally located nuclei. In the course of differentiation erythroblasts reduce their size and position their nucleus adjacent to the plasma membrane. Expelled nuclei undergo phagocytosis by central macrophage. Young reticulocytes detach from the macrophage and enter the circulation, where they mature to erythrocytes.

Cell-cell interactions in the niche

Cell-cell interactions between erythroblasts and macrophages have been studied for the last 15 years. The erythroblast macrophage protein (EMP) is expressed on both erythroblasts as well as on macrophages and connects both cell types via homophilic association (Hanspal and Hanspal 1994). The importance of this interaction is stressed by the phenotype of *Emp* null fetuses, who die perinatally and suffer from severe anemia (Soni, Bala et al. 2006). Additionally *in vitro* erythroblast cultures showed increases in apoptosis coupled with decreased proliferation, maturation and enucleation when grown either without macrophages or cultivated in presence of anti-Emp antibody (Hanspal, Smockova et al. 1998).

Also the $\alpha_4\beta_1$ integrin–VCAM-1-based erythroblast–macrophage interaction plays an important role in erythroblastic island integrity, since antibodies against either protein disrupt their structure (Sadahira, Yoshino et al. 1995). Similarly interference with binding of the intercellular adhesion molecule 4 (ICAM4) on erythroblasts with its binding partner α_V integrin on macrophages leads to a striking decrease in erythroid niche formation (Lee, Lo et al. 2006). Interestingly a soluble isoform of ICAM4, ICAM4S, is upregulated later in terminal differentiation, which may help young reticulocytes to detach from macrophages and enter circulation (Lee, Spring et al. 2003; Chasis and Mohandas 2008).

Direct erythroblast macrophage contact has been shown to increase erythroid proliferation by a factor of 3 (Rhodes, Kopsombut et al. 2008). Nevertheless it is not yet clear which niche interaction promotes enhanced proliferation. In addition to the already mentioned adhesion factors two other candidates have been identified. Ephrin 2 (HTK-ligand) is presented on the surface of macrophages, whereas erythroblasts express its receptor EphB4 (HTK), whose activation may be responsible for faster cycling (Inada, Iwama et al. 1997; Suenobu, Takakura et al. 2002). Another candidate for decreased transit time in the G0/G1 phase is KIT ligand (stem cell factor, SCF) on macrophages binding to KIT on erythroid progenitors (Muta, Krantz et al. 1995). Although the existence of a positive proliferation effect within the niche is well documented, the exact nature of the respective signals still remains elusive.

Not only macrophage–erythroblast, but also erythroblast–erythroblast interactions apparently can influence erythropoiesis. The binding of FAS-Ligand, expressed late in differentiation, to FAS, expressed continuously on human erythroblasts, causes receptor clustering and induces apoptosis in the FAS expressing cells (De Maria, Zeuner et al. 1999). Interestingly FAS crosslinking induces cell death only in early erythroblasts and even these can be protected against apoptosis by high levels of erythropoietin as they occur under anemic conditions. The result is a flexible self adjusting system that restricts erythropoiesis under homeostatic circumstances, but unleashes its full potential in times of need. In fetal mice similar observations have been made, although in contrast to the human situation FAS-mediated cell death is induced by interactions within the population of early erythroblasts (Socolovsky, Murrell et al. 2007).

Soluble signals in the niche

In addition to cell–cell mediated signal transduction pathways a plethora of soluble factors influence erythropoiesis. Macrophages secrete cytokines like insulin-like-growth-factor-1 (IGF1), which stimulate growth of both CFU-E and BFU-E (Kurtz, Hartl et al. 1985; Sawada, Krantz et al. 1989). Additionally erythroblasts have been shown to release the soluble protein GAS6 upon EPO stimulation, which enhances EPO receptor

signaling (Angelillo-Scherrer, Burnier et al. 2008). In the course of differentiation erythroblasts secrete the angiogenic factors vascular-endothelial-growth-factor-A (VEGF-A) and placenta growth factor (PIGF), although they do not express receptors for these factors (Tordjman, Delaire et al. 2001). A likely explanation would be that VEGF-A and PIGF act as paracrine effectors on macrophages controlling island structure, localization or stimulating further cross-talk.

A variety of cytokines, chemokines and interleukins, most of which can be associated with inflammation, have negative effects on erythropoiesis (Means 2004). The modes of suppressive action are multifaceted. Tumor necrosis factor α (TNFα) can retard erythropoiesis either by facilitating cleavage of GATA1 via caspases (De Maria, Zeuner et al. 1999) or by impeding differentiation in a caspase-independent way (Dai, Chung et al. 2003). Interleukin 6 (IL6) interferes on another level, as it upregulates hepcidin expression, which in turn blocks iron export from macrophages by stimulating ferroportin internalization and degradation (see below for details). This leads to reduced iron availability in the erythroid niche and hampers further maturation (Nemeth and Ganz 2006). In conclusion a plethora of positive and negative signals regulate erythropoiesis in the niche, whose individual contributions and hierarchies are still to be fully understood. Improved understanding of the complex interplay within the erythroid niche may very well be of medical relevance, as it may lead to therapies for those anemias that neither respond to iron nor EPO treatment.

Heme biosynthesis

Oxygen transport is of vital importance for all multicellular organisms. In vertebrates specialized cells, the erythrocytes, are the key transporters involved in oxygen delivery and removal of carbon dioxide from the periphery. High levels of the metalloprotein hemoglobin within erythrocytes enable them to fulfill this specialized function. Hemoglobin molecules consist of four α-helical globular protein subunits, each with a non-covalently bound Heme group that can associate with O_2 (Perutz 1964). Heme itself is a porphyrin, a heterocyclic, planar, organic ring with a central iron atom. In the non-oxygenated state iron resides as Fe^{2+} within heme's planar ring system, which coordinates the ion at four positions. The fifth coordination site is occupied by a nitrogen atom from a histidine side chain of the globin protein, while the sixth coordination site is free to interact with 0_2 (see Figure 9a). In the oxygenated state iron donates one electron to oxygen, formally becoming Fe^{3+}, which joins the complex as superoxide anion ($^{\cdot}OO^{-}$) (Wittenberg, Wittenberg et al. 1970). In the periphery oxygen is released due to the combined effect of reduced oxygen partial pressure and decreased pH (Bohr effect) as result of higher amounts of solute CO_2 in the plasma (see Figure 9b).

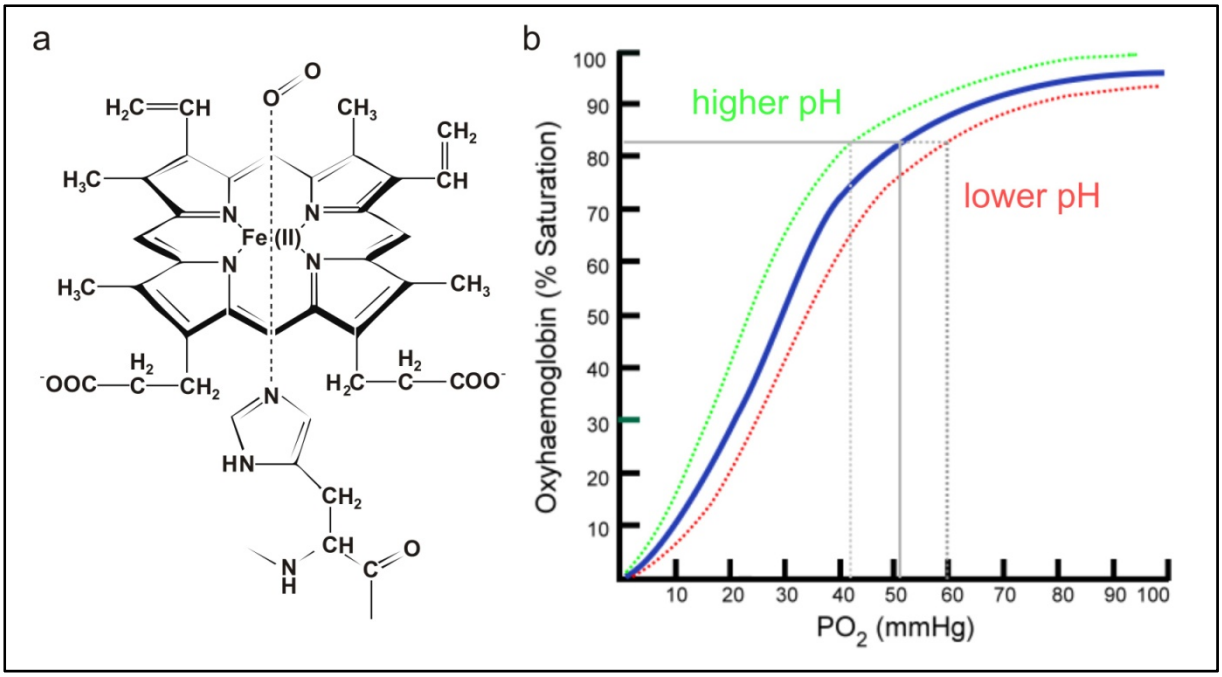

Figure 9: Oxygen coordination in hemoglobin. (a) In oxyhemoglobin iron is coordinated at four points by the porphyrin ring, on one point by a globin histidine sidechain and binds one oxygen molecule with its last coordination site. (b) Hemoglobin saturation in dependence of oxygen partial pressure (pO_2). The Bohr effect leads to increased oxygen release in tissues with high metabolic activity due to local acidification caused by solute CO_2 and lactic acid.

In mammals the generation of heme from glycine and succinyl-CoA is a tightly regulated eight step process (Ajioka, Phillips et al. 2006) (see Figure 10). Strict control is necessary as disruption of the delicate balance between heme and globin levels leads to cytotoxicity due to excess globin (Thalassemia) or accumulation of heme precursors (Porphyria). Function and regulation of the heme biosynthesis pathway has been subject to research since the early 1980s. The following will summarize the major findings up to today:

5-aminolevulinic acid synthase (ALAS)

In the first step of heme biosynthesis glycine and succinyl-CoA are condensated into 5-aminolevulinic acid (ALA). This reaction is catalyzed within mitochondria by two different enzymes, ALA synthase 1 (ALAS1) and ALA synthase 2 (ALAS2), which are the rate limiting enzymes of heme synthesis. ALAS1 is ubiquitously expressed, whereas ALAS2 is specific for erythroid precursors (Ajioka, Phillips et al. 2006). At the transcriptional level important differences in *ALAS1* and *ALAS2* regulation were reported. *ALAS1* transcription was shown to be upregulated by the peroxisome proliferator-activated coactivator (PCG1α) (Handschin, Lin et al. 2005), whose effects are mediated by interaction with nuclear regulatory factor 1 (NRF1) and FOXO1 (a forkhead family member) (Virbasius and Scarpulla 1994; Wu, Puigserver et al. 1999). Transcriptional control of *ALAS2* is less

well documented, but believed to be regulated by erythroid specific transcription factors including GATA1 (Srivastava, Borthwick et al. 1988). At the post-transcriptional level the major splice variant of *ALAS1* mRNA is destabilized in the presence of heme, whereas the minor splice variant, which contains exon 1B, is not subject to heme mediated decay (Cable, Miller et al. 2000; Roberts and Elder 2001). Post-transcriptional regulation of *ALAS2* is mediated by interaction of IRE binding proteins (IRP) with iron responsive elements (IRE) in the 5' untranslated Region of *ALAS2* mRNA. Under low iron conditions IRPs block the translation of the *ALAS2* mRNA linking heme synthesis with iron availability (Napier, Ponka et al. 2005; Rouault and Tong 2005; Wingert, Galloway et al. 2005). At the post-translational regulation level both ALAS1 and ALAS2 have heme binding motifs (HRMs) embedded within their mitochondrial targeting sequences (Lathrop and Timko 1993). Binding of heme to these HRMs blocks mitochondrial import, leading to post-translational end-product feedback inhibition. Interestingly the HRM-mediated import inhibition seems only to be functional for ALAS1, as ALAS2 was still imported under the same experimental conditions (Munakata, Sun et al. 2004).

5-aminolevulinic acid dehydratase (ALAD)

ALA molecules exit the mitochondrion via an unknown mechanism and are further condensed in the cytoplasm by 5-aminolevulinic acid dehydratase (ALAD) forming porphobilinogen (PBG). In humans the *ALAD* gene consists of two alternatively spliced non-coding and eleven coding exons (Kaya, Plewinska et al. 1994). Transcription is regulated by two different promoters, a "housekeeping" one and a erythroid specific promoter. The latter contains binding sites for erythroid transcription factors, among them again GATA1 (Kaya, Plewinska et al. 1994).

Porphobilinogen deaminase (PBGD)

Catalyzed by porphobilinogen deaminase (PBGD) four molecules of PBG form the unstable tetrapyrrole hydroxymethylbilane (HMB). Like *ALAD* the human *PBGD* gene is driven by a "housekeeping" and an erythroid-specific promoter. Depending on the promoter used, two splice variants occur starting either with exon 1 joined to exon 3 (housekeeping) or exon 2 joined to exon 3 (erythroid). The erythroid promoter closely resembles that of ALAD and other erythroid promoters (Mignotte, Eleouet et al. 1989).

Uroporphyrinogen III synthase (URO3S)

Uroporphyrinogen III synthase (URO3S) catalyzes the conversion of HMB into uroporphyrinogen III. Again the single *URO3S* gene is driven by two different promoters. The "housekeeping" promoter features SP1, NF1, AP1, OCT1 and NRF2 binding sites, whereas among others the erythroid promoter contains 8 GATA1 binding sites. Although only the erythroid transcript contains all of exon 2, both transcripts produce identical proteins (Aizencang, Bishop et al. 2000).

Uroporphyrinogen III decarboxylase (UROD)

Uroporphyrinogen III is decarboxylated four times in a clock wise manner by Urophorphyrinogen III decarboxylase (UROD) (Smith and Francis 1979) to form coproporpyhrinogen III. The *UROD* gene has no erythroid-specific promoter (Romana, Dubart et al. 1987). *UROD* mRNA levels are nevertheless significantly increased in erythroid tissues by an unknown mechanism (Romeo, Raich et al. 1986).

Coproporphyrinogen oxidase (CPO)

The propionate groups of two neighboring pyrrole rings are oxidatively decarboxylated by coproporphyrinogen oxidase (CPO) giving rise to protoporphyrinogen IX. In mammals this step takes place in the mitochondrial intermembrane space. Although an oxygen independent form of the enzyme exists, it is not utilized by higher eukaryotes (Yoshinaga and Sano 1980). Correct localization of CPO in the mitochondrial intermembrane space depends on a unusually long leader sequence and may be mediated by peripheral-type benzodiazepine receptors (Taketani, Kohno et al. 1995; Dailey, Woodruff et al. 2005). The human *CPO* promoter features a single GATA site, an SP1 like element and a not yet characterized regulatory element (Takahashi, Taketani et al. 1998).

Protoporphyrinogen oxidase (PPO)

Oxidation of protoporphyrinogen IX catalyzed by protoporphyrinogen oxidase (PPO) leads to formation of protoporphyrin IX. PPO is located on the outer surface of the inner mitochondrial membrane and depends therefore on mitochondrial import. The promoter of the human *PPO* gene contains a GATA1 binding site, which implies possible erythroid-specific regulation (Taketani, Inazawa et al. 1995).

Ferrochelatase (FeCH)

On the inner surface of the inner mitochondrial membrane ferrochelatase (FeCH) catalyzes the last step of heme biosynthesis, i.e. the insertion of iron into protoporphyrin IX. Each monomer of the dimeric FeCH contains a nitric oxide-sensitive 2Fe-2S cluster (Burden, Wu et al. 1999) and is thought to interact with PPO through the inner mitochondrial membrane (Koch, Breithaupt et al. 2004), although such direct interaction is apparently not necessary for heme synthesis (Proulx, Woodard et al. 1993). The human FeCH gene is driven by a single promoter, which contains binding sites for SP1, NFE2 and GATA1 (Taketani, Inazawa et al. 1992) and is believed to interact with hypoxia inducible factor 1 (HIF1) (Liu, Ang et al. 2004). Intracellular iron levels regulate FeCH expression probably via availability of iron-sulfur clusters (Taketani, Adachi et al. 2000). Like the activity of ALAS2, FeCH is tightly regulated by feedback mechanisms since especially metal-free protopoporphyrin IX can induce massive damage to cell components due to its ability to facilitate generation of reactive oxygen species.

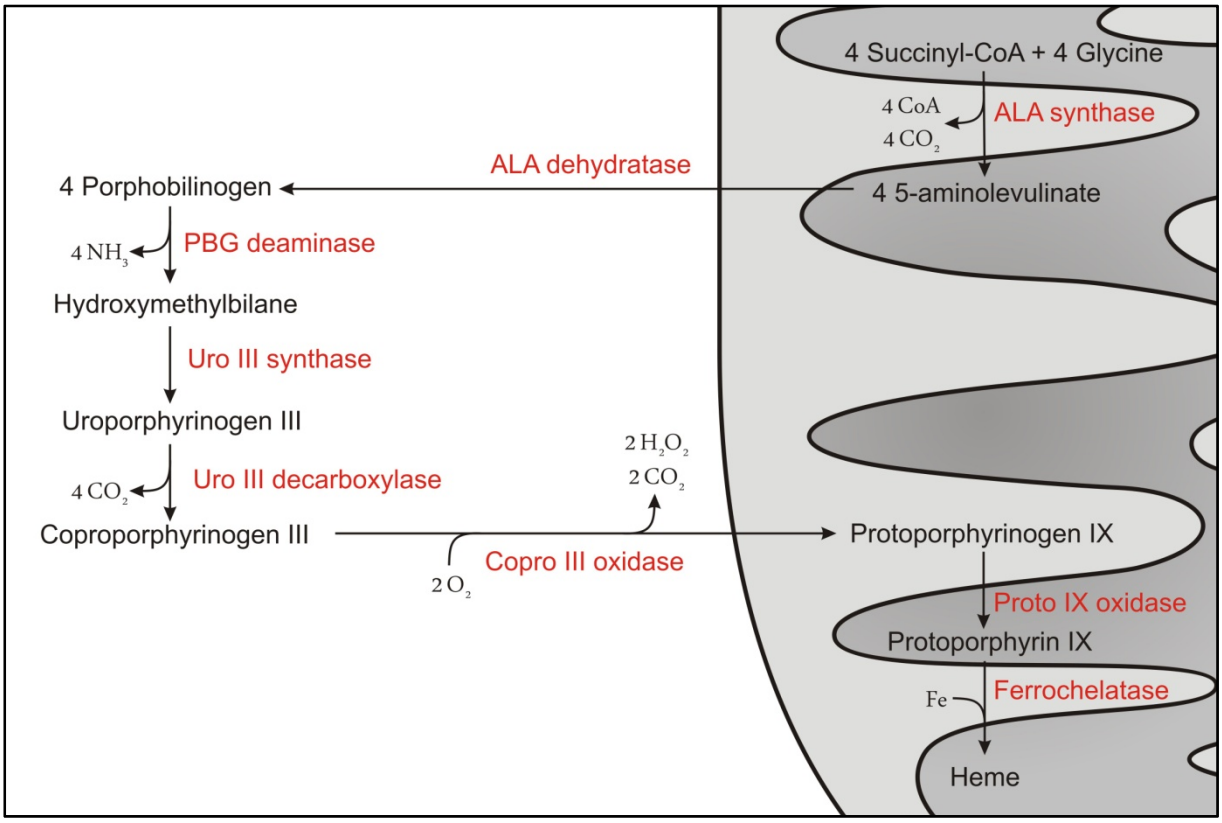

Figure 10: Heme biosynthesis. For details see text.

Balancing globin levels

It is essential that all three components of hemoglobin, α-globin, β-globin and heme are synthesized in an exact 2:2:4 ratio, since accumulation of any single component is cytotoxic. Regulation mechanisms exist both at the transcriptional and at the translational level. Transcriptionally heme modulates globin synthesis by binding to the transcription factor BTB and CNC homology 1 (BACH1) during erythroid differentiation (Taketani 2005; Igarashi and Sun 2006). More importantly heme controls protein synthesis in general and globin synthesis in particular via one of the eIF2α kinases termed heme regulatory inhibitor (HRI). When HRI is synthesized, it incorporates a single heme molecule into its N-terminal domain and dimerizes rapidly. Subsequently both HRI molecules can phosphorylate each other in three stages. The first and second lead to complex stabilization and open binding sites for two further heme molecules. If heme is abundant, it binds to the HRI dimer and prevents any further phosphorylation forcing the regulator into an inactive state. In heme deficiency, however, the last irreversible phosphorylation step occurs and the dimer gains eIF2α kinase activity (Chen 2007)(see Figure 11). eIF2 is a heterotrimeric protein, which binds GTP to facilitate the assembly of the 43S preinitiation complex on mRNAs. GTP is hydrolyzed to GDP in the course of translation and has to be replaced by another GTP for a further round of initiation. eIF2B is the GDP/GTP exchange factor responsible for this task. When the α subunit of eIF2 is phosphorylated, eIF2B is tightly bound by eIF2 and can no longer exchange GDP for GTP. As a result no new initiation can occur once eIF2B has been outtitrated by phosphorylated eIF2α (Krishnamoorthy, Pavitt et al. 2001). It is still unclear, whether special mechanisms exist that lead to predominant shut-down of globin translation over other proteins. It is due to the action of HRI that many different types of anemias, e.g. iron deficiency anemia, erythropoietic protoporphyria or β-thalassemia, combine the phenotypes of hypochromia with microcytosis (Chen 2007).

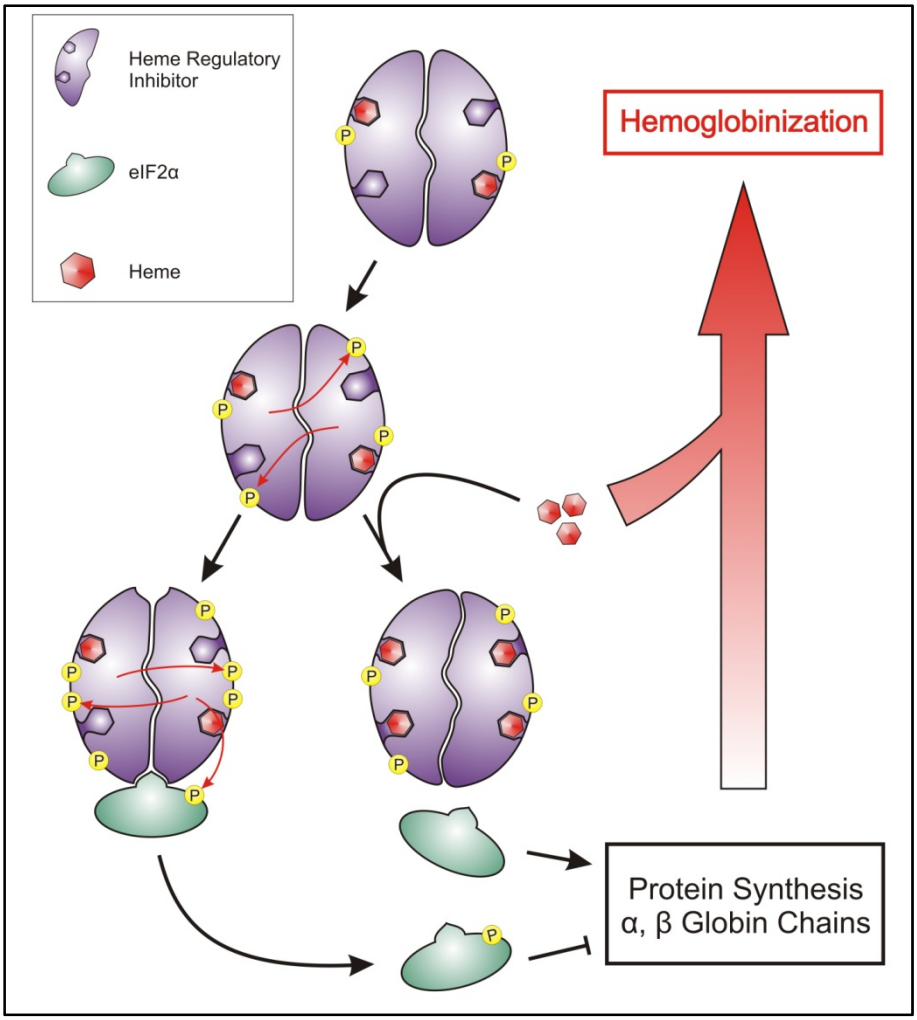

Figure 11: Heme regulatory inhibitor regulation. Activation of HRI occurs in three autophosphorylative steps. The first step stabilizes the dimer, whereas the second opens additional heme binding sites. The third and final irreversible activation step is antagonized by binding of two more heme molecules.

Iron metabolism

The ability of Fe^{3+} to accept and Fe^{2+} to donate electrons is essential in a plethora of biological processes. Metal ions and especially ferric and ferrous Fe-ions are preferably used when catalyzing electrochemical exchanges, due to their electrochemical potential ideally suited for biochemical redox processes. The same reactivity that makes Fe-ions crucial in metabolism renders them highly dangerous when left unattended. Upon encountering H_2O_2 both Fe^{2+} and Fe^{3+} can generate highly reactive oxygen radicals via the Fenton reaction, which can cause severe tissue and DNA damage. As a result complex safe-guard mechanisms have evolved, enabling utilization of iron's reactive properties, while averting most of its detrimental effects.

Iron is incorporated into cytochromes, myoglobin, a multitude of enzymes, but most notably into hemoglobin. Usually humans have 3-4 grams of iron within their body,

~2.5 g of which are contained in hemoglobin. Iron in hemoglobin is used to bind oxygen, which can in consequence be transported within erythrocytes and distributed throughout the body. Although roughly 20-30 mg of iron are used every day in heme synthesis, we do not need to take up more than 1-2 mg of dietary iron per day thanks to efficicent re-utilization and absence of dedicated iron excretion mechanisms (see Figure 12). In the following the complex regulation mechanisms that control iron homeostasis will be introduced.

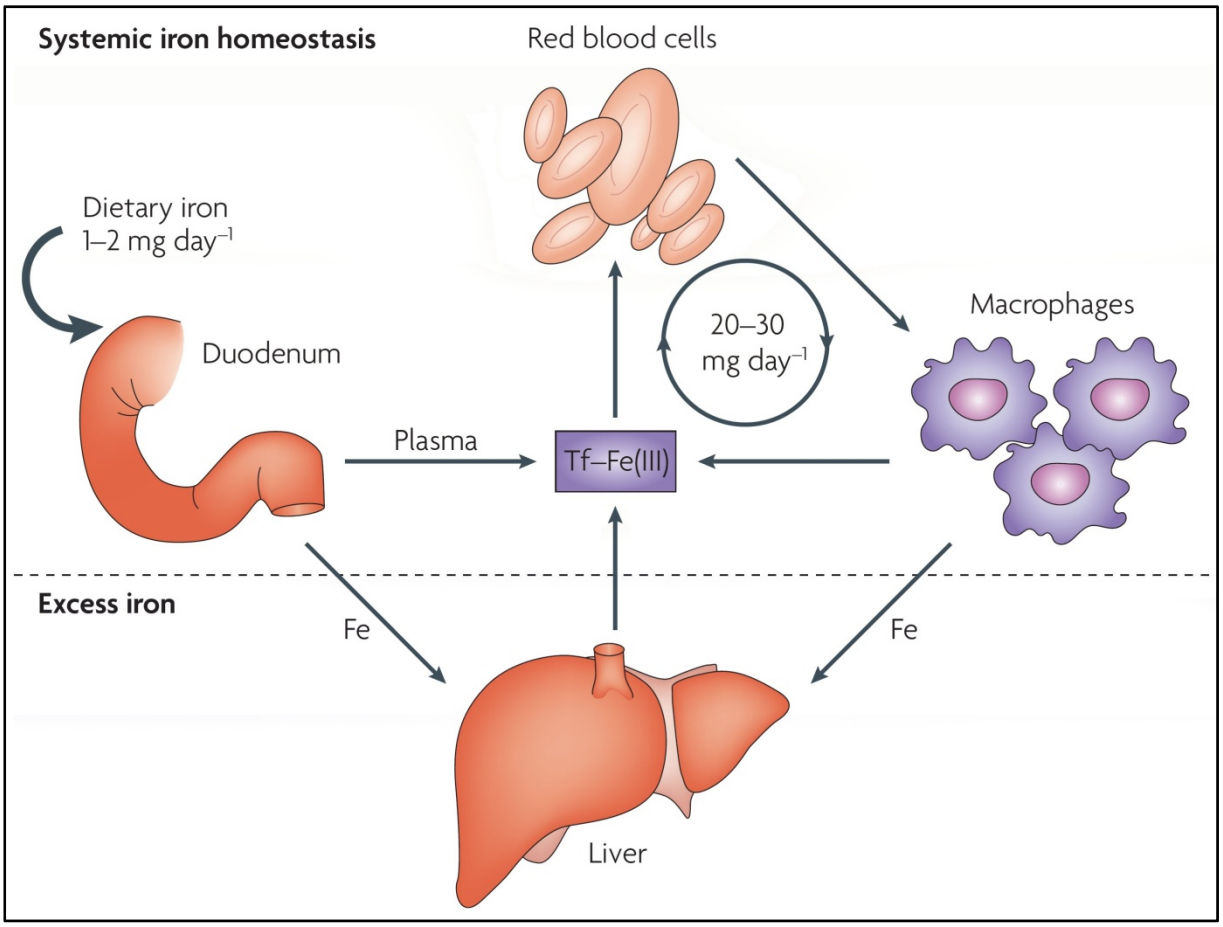

Figure 12: Systemic iron homeostasis. Dietary iron (1–2 mg day^{-1}) is absorbed by cells of the duodenum before being exported into plasma where it binds to transferrin (Tf). Transferrin-bound iron is delivered to tissues and cells (primarily to reticulocytes) where it is incorporated in heme. Old erythrocytes are phagocytosed by macrophages, which degrade hemoglobin and recycle iron back into plasma (20–30 mg day^{-1}) where it again binds transferrin. If iron is absorbed or released into the plasma at a higher level than the iron-binding capacity of transferrin, then the excess, nontransferrin-bound iron is deposited in parenchymal tissues (such as the liver). (De Domenico, McVey Ward et al. 2008)

Dietary iron uptake

Mechanism

Uptake of dietary iron occurs primarily in the duodenum and upper jejunum (De Domenico, McVey Ward et al. 2008). To reach the plasma iron has to pass through both

the apical and basolateral membrane of epithelial cells (see Figure 13). Dietary iron is either still bound to heme or occurs in its ferric Fe^{3+} form, which is soluble in the acidic environment of the intestinal lumen. To traverse the apical membrane ferric iron has to be converted to ferrous (Fe^{2+}) iron. This is catalyzed by a ferrireductase, most probably DCYTB, which facilitates the transmembrane transport of electrons from cytosolic NADPH to extracellular ferric iron (De Domenico, McVey Ward et al. 2008). Since *Dcytb* knockout mice display no obvious defect in iron uptake, future research may uncover further ferrireductases acting on the apical surface of the duodenal endothelium (Gunshin, Starr et al. 2005). The actual iron uptake is carried out by apical mucosal divalent metal transporter 1 (known as DMT1, NRAMP2 or DCT1), a highly conserved protein that symports ferrous iron (or other divalent metal ions) and protons into the cell utilizing the H^+ gradient created by the acidic duodenal lumen (Andrews 2008). DMT1 is the only known iron importer in the intestine but it is ubiquitous in the endosomes of all cell types. Although *DMT1* knockout mice are severely anemic, they are not entirely iron deficient in all tissues and develop to term (Gunshin, Fujiwara et al. 2005). It has been suggested that materno-fetal iron transport works independently of DTM1 and that at least non-hematopoietic tissues may have alternative enzymes or pathways compensating for the loss of DMT1. This interpretation is corroborated by studies describing non-transferrin bound iron uptake by a multitude of cultured cells (Sturrock, Alexander et al. 1990; Kaplan, Jordan et al. 1991; Inman, Coughlan et al. 1994; Randell, Parkes et al. 1994; Barisani, Berg et al. 1995; Parkes, Randell et al. 1995).

Fe^{2+} brought into a gut cell by DMT1 can either be stored by ferritin, a cytosolic iron-storage molecule, or exported into plasma by ferroportin, the only known cellular iron exporter (Donovan, Brownlie et al. 2000), for further utilization by other tissues. Ferritin forms a multimeric spheric protein consisting of 24 subunits, which can incorporate up to 4500 Fe^{3+} ions into its core. The complex is comprised of L (for light or liver) and H (for heavy or heart) subunits, of which the H subunit acts as ferroxidase converting Fe^{2+} into Fe^{3+} upon storage in the ferritin shell (De Domenico, McVey Ward et al. 2008). As expression of ferroportin leads to depletion of ferritin-iron and subsequent ferritin degradation (De Domenico, Vaughn et al. 2006), it can be argued that iron storage in ferritin is a default pathway only used in the absence of cellular iron export.

Ferroportin (also called IREG1, MTP1, SLC39A1 or SLC40A1) is expressed on duodenal mucosa, placenta, hepatocytes and on macrophages, who are involved in recycling of effete erythrocytes (De Domenico, Vaughn et al. 2007; Andrews 2008). The early embryonic lethal phenotype of *Ireg1* null mice proves the transporter's importance in iron transfer from mother to fetus (Donovan, Lima et al. 2005). Tissue specific deletion of *Ireg1* leads to iron accumulation in macrophages and hepatocytes, substantiating the assumption that there are no functionally compensating homologues (De Domenico, McVey Ward et al. 2008). Ferroportin is thought to transport iron across the membrane

in its ferrous form. The transporter is nevertheless intrinsically linked with a group of multicopper oxidases including ceruloplasmin (CP) and hephaestin (HEPH), which oxidize Fe^{2+} to Fe^{3+} (Osaki, Johnson et al. 1966; Roeser, Lee et al. 1970). Mutations or total deficiency of *CP* in mice leads to increased iron storage and reduced or ablated ability to export iron through ferroportin (Harris, Durley et al. 1999; Jeong and David 2003; De Domenico, Ward et al. 2007). Mutations in *HEPH* (expressed in intestinal cells and retina) likewise cause impeded iron export via ferroportin (Vulpe, Kuo et al. 1999). Both ceruloplasmin and hephaestin act beyond the cellular membrane, outside the cell. It is therefore reasonable to assume that multicopper-oxidases create a chemical gradient by converting exported Fe^{2+} into Fe^{3+} that is then the driving force behind iron transport via ferroportin.

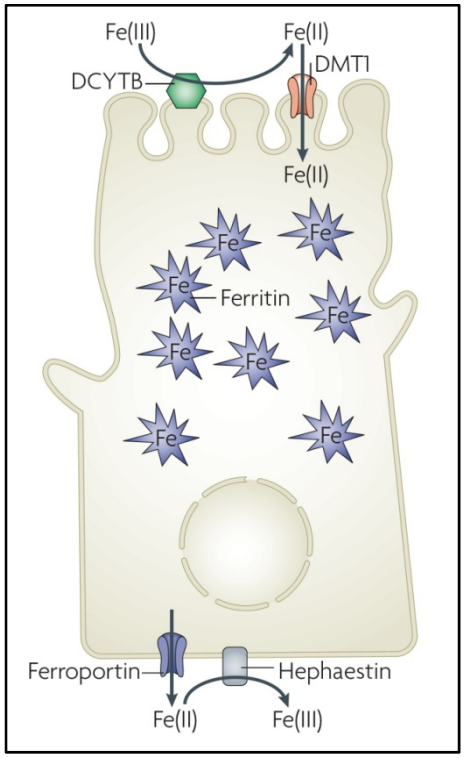

Figure 13: Iron transport across the enterocyte. Ferric iron (Fe(III)) in the diet is converted to ferrous iron (Fe(II)) by a ferroreductase (DCYTB) that is located on the apical surface of enterocytes of the duodenal mucosa. Fe(II) is then transported into enterocytes through the divalent metal transporter DMT1. Fe(II) in enterocytes can be incorporated into the cytosolic iron-storage molecule ferritin or can be transported across the basolateral surface of enterocytes into the plasma by ferroportin. Fe(II) is subsequently converted to Fe(III) by a membraneassociated ferroxidase, hephaestin. (De Domenico, McVey Ward et al. 2008)

Regulation

Intestinal iron absorption, macrophage hemoglobin recycling and hepatocyte iron storage are controlled systemically. The coordinator for iron homeostasis is a defensin-like peptide hormone called hepcidin. Biologically active hepcidin is generated after furin mediated cleavage of the larger prohepcidin peptide, which is synthesized in hepatocytes. Although mice have two hepcidin genes, only one of them, *Hamp*, is active in iron regulation (De Domenico, McVey Ward et al. 2008). *Hamp* knockout leads to most severe juvenile hemochromatosis (excessive absorption of dietary iron resulting in a pathological increase in body iron) (Nicolas, Bennoun et al. 2001; Roetto, Papanikolaou et al. 2003),

whereas overexpression of hepcidin causes iron-limited anemia, i.e. pathologically reduced hemoglobin formation due to lacking iron availability (Nicolas, Bennoun et al. 2002; Weinstein, Roy et al. 2002). Hepcidin binds to ferroportin and causes the transporter's tyrosine phosphorylation. Upon this ferroportin becomes subsequently internalized, dephosphorylated, ubiquitinylated and finally degraded in lysosomes (De Domenico, Ward et al. 2007). This removes ferroportin from the plasma membrane and results in inhibition of iron export. On a systemic level hepcidin expression causes reduced dietary iron uptake in the intestine and blocks iron release of macrophages of the reticuloendothelial system that recycle effete erythrocytes. Generally hepcidin expression leads to reduced iron levels in the plasma (Andrews 2008).

Due to its small size hepcidin is probably excreted by the kidney on its first pass. This allows for a fast and dynamic response to changing iron needs. On the other hand this demands strict regulation and constant production of the peptide hormone. Proteolytic cleavage of the prohormone by furin has been shown to be rather unregulated (Valore and Ganz 2008), which suggests hepcidin regulation to occur at the transcriptional or translational level. The finding that *Hamp* mRNA levels are severely elevated in response to iron overload (Pigeon, Ilyin et al. 2001) and reduced as result of iron deficiency (Weinstein, Roy et al. 2002), hints to regulation by transcriptional control. Additional studies could show that *Hamp* mRNA levels are decreased under hypoxic conditions and when dealing with ineffective erythropoiesis (Nicolas, Chauvet et al. 2002; Adamsky, Weizer et al. 2004). Inflammation, on the contrary, leads to increased hepcidin levels to deprive invading pathogens of essential iron and augment innate immune response (Ganz 2006).

Transcriptional control of *Hamp* is primarily exerted by bone morphogenic protein (BMP)/SMAD signaling. This surprising discovery was made when hepatocyte specific *Smad4* knockout mice were diagnosed with severe hemochromatosis, resembling the hepcidin knockout phenotype (Wang, Li et al. 2005). In parallel hemojuvelin (HJV or HFE2), which is mutated in patients with severe early onset hemochromatosis, was found to act as BMP coreceptor stimulating hepcidin transcription (Babitt, Huang et al. 2006). HJV is coupled to the membrane of hepaotcytes and muscle cells by a GPI anchor. When membrane associated HJV binds BMP and facilitates signaling through the BMP receptor *Hamp* transcription is stimulated. HJV can be cleaved from the surface of muscle cells and acts as competitive antagonist of membrane-bound HJV due to its association with BMP in the plasma (Lin, Goldberg et al. 2005). Recent findings suggest that presence of iron-saturated transferrin prevents the shedding of HJV by muscle cells (Zhang, Anderson et al. 2007), which would make an interesting mechanism how muscle can influence systemic iron levels in times of increased myoglobin need. Activation of the *Hamp* gene in inflammation is controlled by the interleukin 6 (IL6)-signal transducer and activator of transcription 3 (STAT3) axis (Wrighting and Andrews 2006; Verga Falzacappa, Vujic

Spasic et al. 2007). Interestingly STAT3 mediated hepcidin activation seems to rely on the presence of SMAD4, since no gene induction can be observed in its absence.

Hepcidin expression is negatively regulated by the hypoxia-inducible-factor (HIF) family of transcription factors. Under normoxia HIF is hydroxylated by a prolyl-hydroxylase and ubiquitinylated by von-Hippel-Lindau (VHL) factor which leads to subsequent HIF degradation (Kaelin 2005). In the absence of oxygen HIFs are stabilized and act as transcription factors that inhibit hepcidin expression (De Domenico, McVey Ward et al. 2008).

A recent study suggests that another negative signal acts on hepcidin expression to ensure sufficient iron supply in times of increased erythropoiesis. Hepcidin is downregulated by high levels of EPO, which usually occur in stress erythropoiesis following blood loss or under hypoxia (Pinto, Ribeiro et al. 2008). This effect was EPO receptor-dependent and associated with repressive actions of C/EBPα, which binds to the *Hamp* promoter and consecutively raises plasma iron levels.

Once Fe^{3+} has reached the plasma it is bound by transferrin, a glycoprotein with two homologous iron binding domains. Transferrin is synthesized in the liver, retina, testis and brain and binds ferric iron with very high affinity in presence of carbonate anions (De Domenico, McVey Ward et al. 2008). Under physiological conditions 20-30% of circulating transferrin is iron loaded. The abundance of iron-free apotransferrin and its high affinity to Fe^{3+} ensure that hardly any iron ions get the opportunity to form toxic oxygen radicals. Additionally transferrin serves as vehicle to bring iron specifically into those cells that need it, as described in more detail in the following section.

Cellular iron uptake

Mechanism

Transferrin receptor 1 (TFR1) is expressed by almost all dividing cells (Andrews 2008). Its importance is stressed by the lethality of its knockout at E12.5 (Levy, Jin et al. 1999). TFR1 forms a homodimer connected by disulphide bonds at the cell surface, which specifically binds one molecule of differic transferrin per subunit. Once the transferrin-transferrin receptor complex is formed, it is rapidly internalized by endocytosis via clathrin-coated pits. The resulting endosome is subsequently acidified by action of an ATP-dependent proton pump that lowers the vesicle's pH to ~5.5 (De Domenico, McVey Ward et al. 2008). At this pH condition both transferrin and transferrin receptor undergo conformational changes to release Fe^{3+} (Bali, Zak et al. 1991; Sipe and Murphy 1991). Still inside the endosome free ferric iron is converted into ferrous iron by the ferrireductase STEAP3. This step is very important for further iron transport to the

cytosol, as evident from *Steap3* mutations, which cause microcytic anemia due to defects in iron delivery to reticulocytes (Ohgami, Campagna et al. 2005). DMT1, the very same Fe^{2+}-H^+ symporter moving iron across the apical membrane duodenal epithelial cells, transports the metal out of the endosome into the cytoplasm. There it is either directly used (e.g. for heme synthesis after import into mitochondria by mitoferrin) or stored in ferritin for later usage or detoxification. Owing to the acidic endosomal pH apotransferrin does not dissociate from TFR1 and the complex recycles to the cell surface. Once in neutral pH apotransferrin detaches and is free again to bind ferric iron in the plasma (van Renswoude, Bridges et al. 1982; Dautry-Varsat, Ciechanover et al. 1983).

Interestingly most tissues, with exception of erythroid, lymphoid and neuroepithilial lineages, develop normally in the absence of *Tfr1* (Levy, Jin et al. 1999). Therefore another non-*Tfr1* dependent mechanism has to exist that can supply other tissues with iron. The first logical candidate would be TFR2, a close homologue to TFR1 with reduced affinity for transferrin. TFR2 is expressed on hepatocytes and erythroid precursors and has rather been associated with hepcidin regulation via detection of differric transferrin levels, than with iron uptake (Frazer and Anderson 2003). Supported by studies of non-transferrin-bound iron uptake by cultured cells (Kaplan, Jordan et al. 1991; Inman, Coughlan et al. 1994; Barisani, Berg et al. 1995) other candidates like L-type calcium channels (Oudit, Sun et al. 2003) or lipocalin-2-mediated siderophore-like iron uptake (Devireddy, Gazin et al. 2005) have been named, but proof for their physiological relevance is not yet compelling.

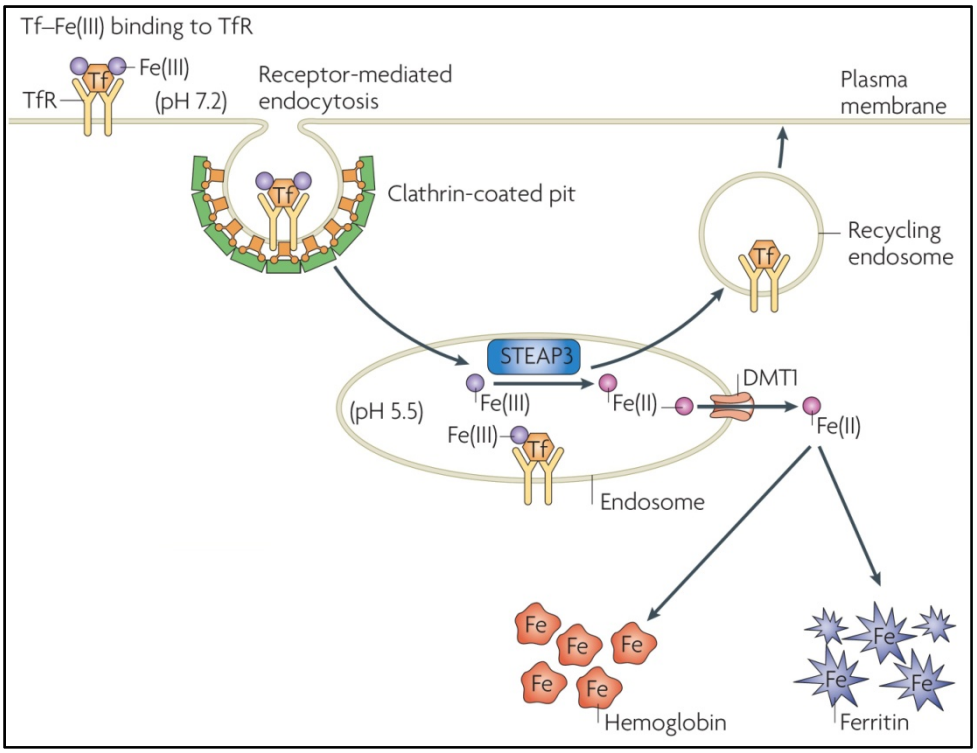

Figure 14: Endocytosis of the complex of iron, transferrin and the transferring receptor. The plasma protein transferrin (Tf) binds Fe(III) with high affinity. At the neutral pH (7.2) in plasma, Tf–

Fe(III) binds to the transferrin receptor (TfR) on the cell surface from where it is internalized by receptor-mediated endocytosis through clathrin-coated pits. The internalized vesicle (an endosome) becomes acidified (pH 5.5) by the action of an H+ ATPase (not shown). As the pH of the endosome decreases, the structure of the Tf–TfR complex changes and Fe(III) is released from Tf–Fe(III). Fe(III) is converted to Fe(II) by the endosomal reductase STEAP3 and is then transported out of the endosome into the cytosol by divalent metal transporter-1 (DMT1). Fe(II) can be stored in ferritin in nonerythroid cells or incorporated into hemoglobin in erythroid cells. The Tf–TfR complex is exocytosed by a recycling endosome. (De Domenico, McVey Ward et al. 2008)

Regulation

Both iron deficiency and iron overload have detrimental effects on cells. It is therefore necessary to employ a highly responsive regulatory system to control intracellular iron homeostasis. Transcriptional as well as post-transcriptional mechanisms have been identified that upkeep this delicate balance. Expression of ferritin and *Tfr1* mRNA is controlled by a number of cytokines and differentiation factors (Kwak, Larochelle et al. 1995; Ponka 1997), though post-transcriptional control is believed to be the pivotal regulation point for these proteins. Both H- and L-ferritin mRNAs were found to harbor highly conserved sequences in their 5' untranslated regions (UTRs), which block their expression in absence of iron (Aziz and Munro 1987; Hentze, Rouault et al. 1987). In parallel highly similar sequences in the 3' UTR of *Tfr1* were shown to increase mRNA stability ensuing protein expression under low iron conditions (Mullner and Kuhn 1988). According to thermodynamic predictions these sequences form RNA stem loop structures termed iron responsive elements (IREs) (Casey, Hentze et al. 1988). Two proteins binding to IREs were identified and named iron regulatory protein 1 (IRP1) and iron regulatory protein 2 (IRP2) (Hentze, Caughman et al. 1987; Caughman, Hentze et al. 1988; Leibold and Munro 1988; Rouault, Hentze et al. 1988; Mullner, Neupert et al. 1989). IRP1 bears strong resemblance to the mitochondrial enzyme aconitase, and even displays aconitase activity under high iron conditions (Kaptain, Downey et al. 1991), but is enzymatically inactive when iron is scarce. The IRE-binding activity is inversely regulated. IRP1 cannot bind RNA when iron is abundant, but associates with IREs when iron concentration is low. Iron sensing of IRP1 occurs via its iron-sulphur cluster ($4Fe{\cdot}4S$), which can only form at high iron levels. IRP2, however, does not contain an iron-sulphur cluster and has no known enzymatic activity. IRP2 IRE-binding activity is controlled at the level of protein stability. Hence it accumulates under low iron conditions, but is quickly oxidized, ubiquitinylated and degraded when iron is abundant (Guo, Phillips et al. 1995; Iwai, Klausner et al. 1995; Yamanaka, Ishikawa et al. 2003).

In addition to ferritin and transferrin mRNAs other transcripts have been found that contain IREs. Ferroportin and aminlevulinic acid synthase 2 have 5' IREs, whereas *DMT1* has 3' IREs. All these mRNAs share a common regulation mechanism that controls their expression at the posttranscriptional level (see Figure 15).

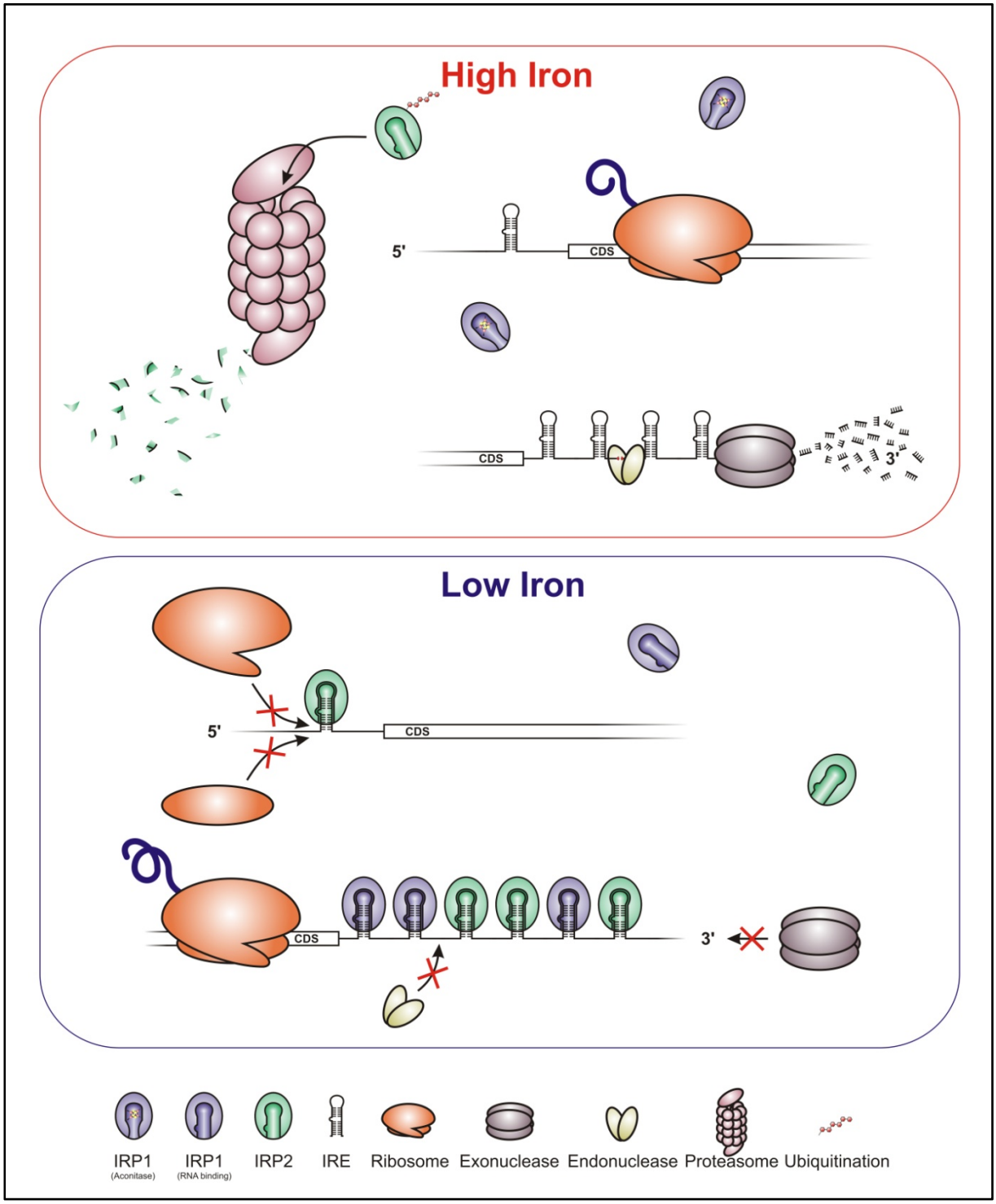

Figure 15: Posttranscriptional regulation via IREs. mRNAs with IREs in their 5' or 3' UTR are inversely regulated by iron availability. Under high iron conditions IRPs do not bind to IREs, since IRP1 contains an iron-sulphur cluster and acts as aconitase, whereas IRP2 is ubiquitinylated and degraded. As a result mRNAs with 5' IREs can be translated and those with 3' IREs are destabilized and degraded by combined endo- and exonucleolytic activity. Under low iron conditions mRNA associated IRPs block translation of 5' IRE mRNAs and stabilize 3' IRE mRNAs, which leads to increased translation of 3' IRE genes.

EPO signaling

Erythropoietin (EPO), also called hematopoietin or hemopoietin, is a glycoprotein regulating red blood cell production. EPO secretion takes place in the kidney and is modulated by oxygen tension via hypoxia inducible factors (HIFs). Serum EPO levels vary over a wide dynamic range depending on the needs of the organism, ranging from a basal 15 U/l to values of 10.000 U/l in anemic humans (Jelkmann 2004). EPO confers essential growth, differentiation and survival signals to erythroid progenitors in the fetal liver, bone marrow and spleen. A variety of pathways is influenced by EPO , among them signaling via STATs, mitogen activated protein (MAP) kinase, phosphatidylinositol 3 kinase (PI3K), LYN kinase and Bruton's tyrosine kinase (BTK). In the following the different ways how EPO-dependent signals influence erythropoiesis will be outlined.

Erythropoietin Receptor and Janus Kinase 2

The initial recipient of the EPO signal is EPO receptor (EPOR), a cytokine receptor superfamily member expressed on erythroid progenitors, myocytes, cortical neurons and prostatic, breast and ovarian epithelia (Richmond, Chohan et al. 2005). On the cell surface EPOR occurs as preformed dimer that undergoes conformational changes upon binding of a single EPO molecule. This change in the extracellular fibronectin type II domains is carried over to the cytoplasmatic tail, where it results in approximation and subsequent activation of a prebound pair of Janus kinases 2 (JAK2). In turn the JAKs tyrosine-phosphorylate each other and eight tyrosines in the cytoplasmatic tail of EPOR to generate docking sites for Src homology 2 (SH2) domain carrying proteins. Interestingly it has been shown that deletion of all but one tyrosine (Y343) in the EPOR cytoplasmatic tail (*EpoR H* mutant) has no detrimental effect on erythropoiesis in mice, and even additional mutation of Y343 (*EpoR HM* mutant) causes only minor delays in differentiation and slightly increased apoptosis (Zang, Sato et al. 2001). At first glance these data indicate that EPOR-STAT signaling, which is supposed to depend on phosphorylated tyrosine residues on the receptor, is not mandatory in erythropoiesis. Other studies, however, have shown that STAT5 can be activated in the absence of phosphoryrosine residues in the EPOR cytoplasmatic tail (Damen, Wakao et al. 1995). A low level of STAT5 activity due to random encounters of inactive STAT5 dimers with activated JAK2 might explain the mild phenotype of *EpoR HM* mice and reopens the question of the necessity of STAT5 mediated EPO signaling in erythropoiesis.

Epo or *EpoR*-deficient mice die in mid-embryonic development (E12.5–E13.5) at a time of massive erythroid expansion to satisfy the growing oxygen need of the

developing embryo (Wu, Liu et al. 1995). Lethality due to hematopoietic defects has been proven by rescuing viability of *EpoR* deficient mice by reintroduction of *EpoR* under the control of the hematopoietic *Gata1* promoter (Suzuki, Ohneda et al. 2002). *Jak2* knockout mice have a very similar phenotype, but die one day earlier, probably due to the involvement of JAK2 in other signaling pathways (Neubauer, Cumano et al. 1998).

Signal transducer and activator of transcription (STATs)

STATs are the most common targets of JAKs. Seven distinct, but highly conserved members of the STAT family are expressed in mammals. All members share a common structure with an N-terminal domain, an α-helical coiled-coil DNA binding domain and a C-terminal domain. Both N- and C-terminal domain are connected to the DNA-binding domain via flexible linkers and allow for conformational changes during activation. STAT C-terminal domains contain both a SRC homology domain to bind phosphorylated tyrosines and a tyrosine residue that can be phosphorylated by JAKs. The transactivation domain is also located at the C-terminus, while the tasks of the N-terminus are less clear. It is believed to be important for tetramerization on the promoters of various target genes (John, Vinkemeier et al. 1999) and antiparallel preassociation of inactive STAT dimers in the cytoplasm (Wenta, Strauss et al. 2008). These antiparallel dimers bind to phosphorylated tyrosines on the cytoplasmatic tail of cytokine receptors (e.g. EPOR) via their SH2 domains. Once in spatial proximity to receptor-associated janus kinases, the tyrosine residues in the STAT C-terminal domains are phosphorylated. Subsequently the dimers detach from the receptor, the conformation changes and both dimer subunits bind their partner's phosphorylated tyrosine with their own SH2 domain. Afterwards the activated STAT dimer is imported into the nucleus, where it binds DNA and acts as transcription factor. The exact mechanism and dynamics of nuclear import and export of STATs is not yet fully understood and may vary between STAT family members. Nevertheless there are indications that phosphorylation and dimerisation rather lead to decreased nuclear export than increased nuclear import probably due to masking of a nuclear export signal when the activated dimer binds to DNA (Meyer and Vinkemeier 2004; Reich 2007).

In vitro studies have shown that EPO is able to stimulate STAT1, STAT3 and STAT5a/b. The erythroid phenotype of *Stat1* deficient mice is not striking. The animals are viable and exhibit no serious developmental defects, but fail, as expected, to respond to IFNα and IFNγ. They display a partial shift of erythropoiesis from bone marrow to spleen and have slightly reduced numbers of erythroid progenitors (Halupa, Bailey et al. 2005). *Stat3* deficiency has not been analyzed for erythroid defects due to the knockout's early lethality. Crossing vav-Cre on available *Stat3* floxed mouse lines should soon allow

evaluation of *Stat3* contribution to erythropoiesis. The first *Stat5a/b* double knockout approach was performed by targeting N-terminal domains of both Stat5a and Stat5b (STAT5$^{\Delta N/\Delta N}$) (Teglund, McKay et al. 1998). *Stat5*$^{\Delta N/\Delta N}$ adult mice had normal hematocrits and displayed no problems in steady state hematopoiesis, but were slower to recover from hemolytic stress. *STAT5*$^{\Delta N/\Delta N}$ embryos, however, were anemic, had lower numbers of erythroid progenitors and showed increased apoptosis due to reduced expression of *Bcl-X*$_L$ (Socolovsky, Fallon et al. 1999). Unfortunately it turned out that the N-terminal targeting approach did not fully ablate STAT5 function, but created a truncated protein that was still able to dimerize, bind DNA and activate target genes (Hoelbl, Kovacic et al. 2006; Yao, Cui et al. 2006). In 2004 the first bona fide *Stat5a/b* double knockout (*Stat5*$^{-/-}$) was reported (Cui, Riedlinger et al. 2004). In contrast to the *Stat5*$^{\Delta N/\Delta N}$ situation, *Stat5*$^{-/-}$ mice die perinatally or at latest shortly after birth. Although severe defects in all lymphoid lineages were shown (Hoelbl, Kovacic et al. 2006; Yao, Cui et al. 2006; Yao, Kanno et al. 2007), bona fide *Stat5* double knockout mice had not been analyzed for erythroid phenotypes.

Mitogen activated protein kinase pathway

When EPO binds to EPOR subsequent events lead to phosphorylation of multiple tyrosines in the cytoplasmatic tail of EPOR. One of these, Y464, acts as docking site for growth factor receptor-bound protein 2 (GRB2), an SH2 domain containing adaptor protein that links activation of various cytokine receptors with MAP kinase signaling (Barber, Corless et al. 1997). Additionally GRB2 can associate indirectly with EPOR by binding to SHIP1 or SHP2 (Tauchi, Damen et al. 1996; Mason, Beattie et al. 2000). In turn GRB2 binds son of sevenless (SOS) a guanine nucleotide exchange factor that can act on Ras-GTPases and activates the canonical MAP kinase cascade (Haq, Halupa et al. 2002). This culminates in the activation of ERK1/2, a kinase commonly associated with proliferation. Additionally both p38 and SAPK/JNK are targets of EPO stimulation, both of which had initially been described as integrators of stress response. Signaling culminating in ERK1/2 and p38 activation on the one hand and SAPK/JNK activation on the other may originate from different regions of the EPOR (Haq, Halupa et al. 2002). While the former uses the proximal, the latter uses the distal region of the cytoplasmatic tail, which lacks tyrosine residues (Richmond, Chohan et al. 2005).

Different p38 isoforms play an important role in erythropoiesis and show different expression patterns in the course of maturation. *p38δ* is only expressed in late erythroblastst differentiation (Uddin, Ah-Kang et al. 2004), whereas *p38α* displays continously high expression. *p38α* deficiency leads to fatal fetal anemia, which could be

traced back to diminished *Epo* expression due to decrease stability of its mRNA (Tamura, Sudo et al. 2000).

Phosphatidylinositol-3-kinase signaling

Similar to MAP kinase signaling there are direct and indirect ways to activate PI3K signaling via EPO stimulation. Phosphorylated Y479 on the EPOR cytoplasmatic tail recruits PI3K via its regulatory subunit p85 to the membrane. Alternatively p85 can be attracted to the EPOR by binding mediating proteins like Casitas B lymphoma (CBL), GRB2-associated binding protein 1 (GAB1), GAB2, insulin-receptor substrate 2 (IRS2) and the guanine nucleotide exchange factor VAV (Wojchowski, Gregory et al. 1999). The p110 catalytic subunit of PI3K phosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP2) to create phosphatidylinositol-(3,4,5)-triphosphate (PIP3). PIP3 acts as docking site for proteins with plekstrin homology (PH) domains, most prominently 3-phosphoinositide-dependent protein kinase 1 (PDPK1) and AKT (also known as protein kinase B, PKB, or AKT1). PDPK1 phosphorylates AKT on a single threonine, which is necessary for its activation. An additional second modification on AKT S473 is required, but the responsible kinase is still unknown. Biphosphorylated AKT acts on a great number of downstream targets, among them forkhead box O3A (FOXO3a) transcription factor (Kashii, Uchida et al. 2000). FOXO3a transcriptional activity is inhibited by phosphorylation and plays an important role in balancing erythroid self renewal versus erythroid maturation via differential target gene activation (Bouscary, Pene et al. 2003; Bakker, van Dijk et al. 2007). *Foxo3a*-deficient mice are anemic and have enhanced numbers of reticulocytes (Castrillon, Miao et al. 2003). B cell translocation gene 1 (*Btg1*) was recently identified as Foxo3a target (Bakker, Blazquez-Domingo et al. 2004). BTG1 is believed to have anti-proliferative functions and may drive erythroid cells into differentiation. $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, is another important anti proliferative target of FOXO3a that is antagonized by PI3K signaling (Bouscary, Pene et al. 2003). Mice with deleted PI3K regulatory subunit p85α display diminished erythropoiesis and reduced BFU-E and CFU-E progenitor numbers, which were traced back to reduced proliferation along the erythroid lineage (Huddleston, Tan et al. 2003). Therefore one can assume that PI3K activation gives important cues to erythroid cells balancing their self-renewal versus terminal differentiation.

Additionally PI3K activation was suggested to play a major role in apoptosis protection of erythroid cells. Studies in apoptosis-resistant erythroid cell lines, however, revealed that PI3K signaling is necessary but not sufficient for apoptosis protection (Bao, Jacobs-Helber et al. 1999). The importance of anti-apoptotic PI3K signaling and other

roles in erythropoiesis will become clearer when more AKT targets in erythroid cells have been elucidated.

LYN

Although the activation of LYN and other SRC family tyrosine kinases has been associated with EPO stimulation, the exact mechanisms are largely unknown. An *in vitro* study in the 32D/EpoR-Wt and F36E hematopoietic cell lines demonstrated physical association of Lyn with EPOR (Chin, Arai et al. 1998). Nevertheless it remains unclear, whether Lyn binds to already phosphorylated EPOR via its SH2 domain, or preassociates with EPOR via its tyrosine kinase domain and phosphorylates the receptor by its own action. Lyn deficiency has been shown to impair erythroid differentiation in the human erythroid cell line J2E-NR (Tilbrook, Ingley et al. 1997). *Lyn* knockout mice express reduced levels of GATA1, EKLF and STAT5a/b in erythroblasts, have increased extramedullary hematopoiesis in the spleen and develop increasing anemia with age (Ingley, McCarthy et al. 2005). Unfortunately fetal hematopoiesis was not analyzed in this study leaving possible earlier effects unexplored.

Bruton's tyrosine kinase

Bruton's tyrosine kinase (BTK), a member of the tec tyrosine kinase family, was reported to be subject to EPO stimulation. BTK features a PH domain and therefore binds to PIP3 at the membrane. The tyrosine kinase is likely to be phosphorylated and thereby activated by JAK2 upon EPO stimulation. Activated BTK can recruit phosphatidylinositol-4-phosphate 5-kinase (PIP5K) to the membrane, which synthesizes phosphatidylinositol-(4,5)-biphosphate (PIP2), the substrate of both PI3K and phospholipase-C-γ 1 (PLCγ1) (von Lindern, Schmidt et al. 2004). PLCγ1, which binds to PIP3 with its own PH domain, is directly phosphorylated by BTK and thus activated. PLCγ cleaves PIP2 and generates inositol-1,4,5-triphosphate (IP3), an important signaling molecule, which leads to mobilization of intracellular calcium and protein kinase C (PKC) activation. Therefore the interplay between BTK activity, which provides PIP2, PI3K activity catalyzing the conversion from PIP2 to PIP3, and PLCγ1 activity, which competes with PI3K for the same substrate, controls the availability of binding sites for all PH-domain dependent enzymes associated with EPOR function.

Further evidence exists that BTK acts as an enhancer for other EPOR signaling pathways. Although *Btk* deficient mice have no obvious erythroid phenotypes, their erythroblasts are more prone to undergo differentiation when cultivated under self-renewal conditions *in vitro* (Schmidt, van den Akker et al. 2004). This is corroborated by

the fact that tyrosine phosphorylation of EPOR and JAK2 is diminished and STAT5 phosphorylation delayed in *Btk-null* erythroid cell lines. Additional studies will be needed to elucidate the exact mechanisms how BTK is able to enhance EPO signaling.

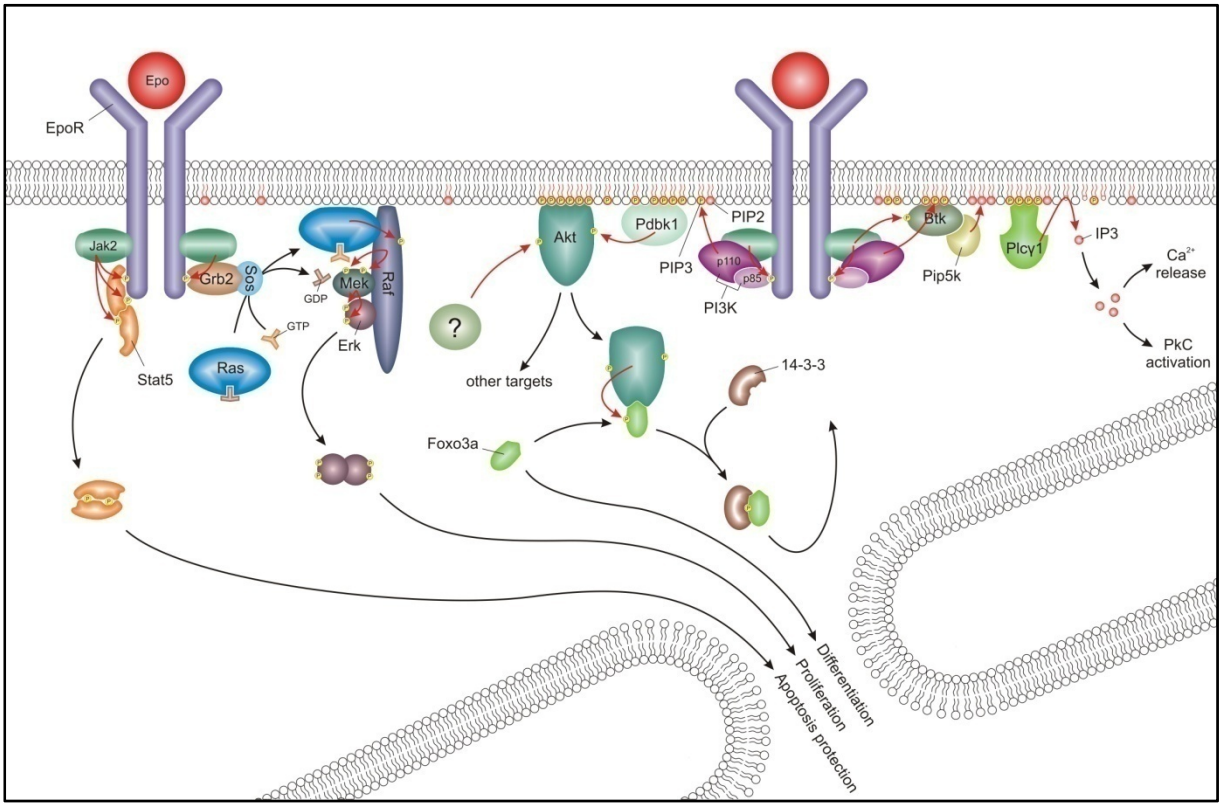

Figure 16: EPO signal transduction. The main players of EPO-dependent JAK-STAT, MAPK, PI3K and BTK signaling. For details see text.

Negative Regulation

Although there are multiple ways of EPO signaling negative regulation, many of which include tyrosine phosphatases like SHP1, SHP2 or CD45, I want to introduce two particular protein families that antagonize EPO signaling by different modes of action.

Suppressors of Cytokine Signaling (SOCS)

Suppressors of cytokine signaling are important negative regulators of STAT signaling also involved in suppression of other pathways like Toll-like-receptor signaling or NFκB signaling. The SOCS protein family consists of eight members (CIS, SOCS1-7) with high degrees of homology. The proteins contain a C-terminal SOCS box, a central SH2 domain and a unique N-terminal region. The SOCS box interacts with a number of other proteins, among them elongin B, elongin C, cullin-5 and RING-box-2 (RBX2), which can recruit E2-type ubiquitin transferases (Kamura, Maenaka et al. 2004). Therefore it is assumed that SOCS proteins can act as E3 ubiquitin ligases and lead to the degradation

of interaction partners bound to their N-terminal domain (Yoshimura, Naka et al. 2007). SOCS genes have been shown to respond to EPO stimulation and are supposed to feed back on EPO-signaling pathways as immediate early targets (Starr, Willson et al. 1997; Richmond, Chohan et al. 2005). SOCS proteins have three different ways of action to interfere with cytokine signals in general and STAT signaling in particular: 1) By their SH2 domain they can compete with downstream factors for phosphorylated tyrosines on cytokine receptors. 2) Via their kinase inhibitor domain they can downregulate JAK activity. 3) Acting as E3 ubiquitin ligases they can mark components or whole receptor complexes for degradation (see Figure 17).

CIS and SOCS3 have been shown to bind directly to Y401 of EPOR, thereby competing for binding with STAT5a/b, SHP2 and SHIP1 (Sasaki, Yasukawa et al. 2000; Hortner, Nielsch et al. 2002; Ketteler, Moghraby et al. 2003). SOCS1 binds to the activation loop of JAK2 and induces subsequently its ubiquitination and degradation (Ungureanu, Saharinen et al. 2002). Links between other SOCS proteins and EPO signaling have not yet been established. CIS knockout mice are viable and show no obvious phenotype (Marine, McKay et al. 1999). SOCS1 knockouts on the other hand display erythroid defects in the form of CFU-E hyper-responsiveness to EPO, a differentiation delay and reduced hematocrit (Metcalf, Alexander et al. 1999). A first study with SOCS3 knockout embryos reported increased numbers of erythroid progenitors in SOCS3 knockout embryos with increased proliferative capacity *in vitro* and led to severe erythrocytosis (Marine, McKay et al. 1999). Unfortunately these results could not be reproduced by another group, who attributed the observed early lethality to placental defects due to defective leukemia inhibitory factor (LIF) signaling (Roberts, Robb et al. 2001). In the end a conditional knockout of SOCS3 will be necessary to assess its contribution to the erythroid lineage.

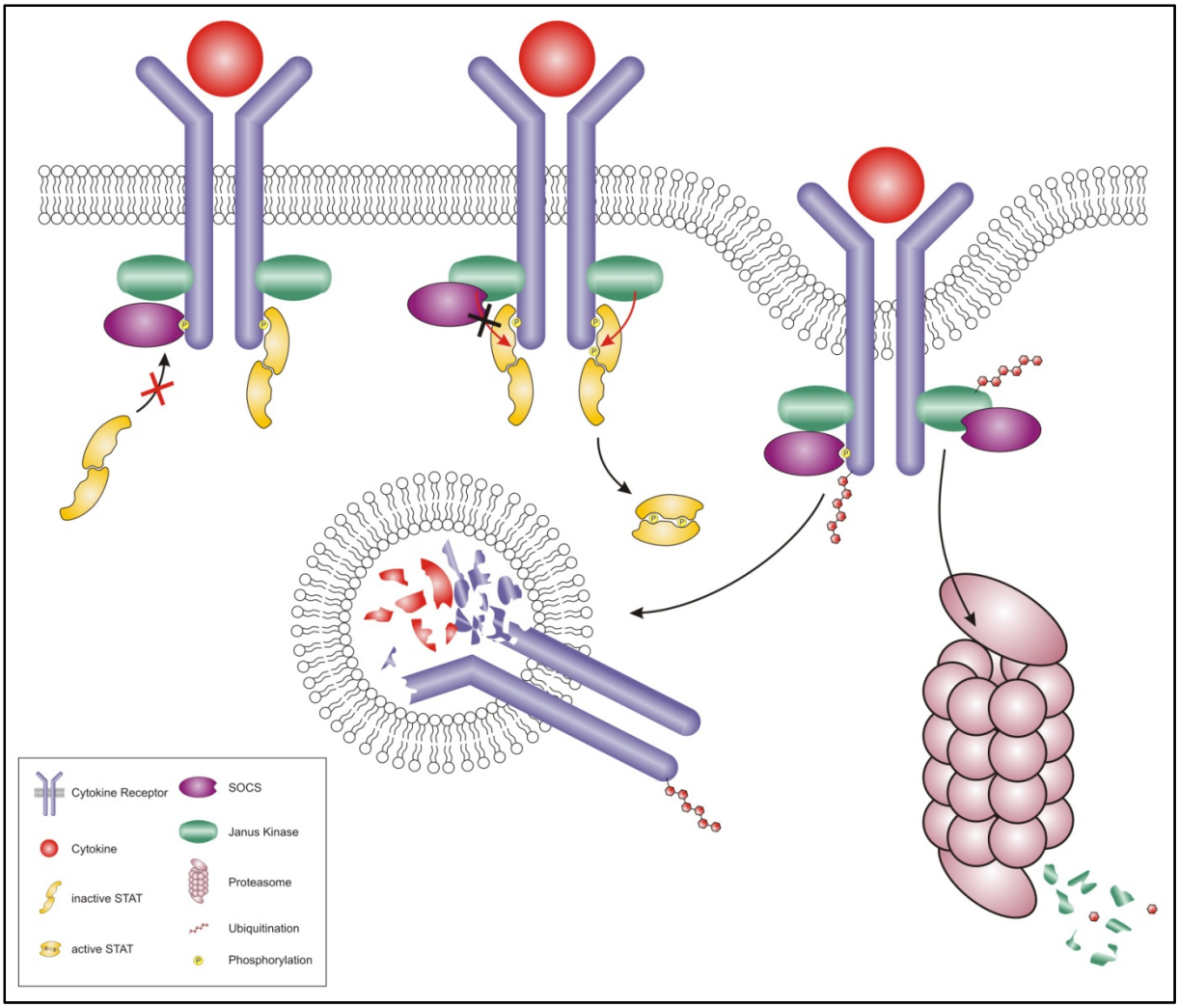

Figure 17: Mechanisms of SOCS protein signal suppression. SOCS proteins can interfere with binding of downstream factors by competing for phosphotyrosine binding site on cytokine receptors. Alternatively SOCS proteins can antagonize activity of associated kinases, like JAKs. Additionally their ubiquitin ligase function can lead to degradation of signalosome components, or even of the whole signaling complex.

Protein inhibitors of activated STAT (PIAS)

Contrary to their name protein inhibitors of activated STAT (PIAS) regulate not only STATs, but also SMADs, NFκB, p53, JUN, MYB, LEF and a variety of other transcription factors. Unlike SOCS proteins PIAS proteins exert their regulative function not in the cytoplasm, but in the nucleus. The mammalian PIAS protein gene family is characterized by a high overall degree of sequence identity (more than 40%) in a number of conserved domains and motifs (Shuai and Liu 2005). The N-termini of PIAS proteins feature a SAP domain (SAFA and SAFB, apoptotic chromatin-condensation inducer in the nucleus and PIAS domain), which binds to A+T rich sequences in scaffold-attachment-regions (SARs). These are usually found near gene enhancers and may therefore be important for PIAS targeting. A central conserved CCCHCCCC-motif-type RING-finger-like zinc-binding domain (RLD) is necessary for the SUMO-E3-ligase activity

of PIAS proteins. The greatest variance among PIAS proteins resides within their C-terminal domain, which contains a putative SUMO1-interaction motif (SIM) not fully conserved throughout the family. The importance of this motif is not yet clear, as even PIAS proteins without SIMs are functional in promoting conjugation of SUMO to proteins.

PIAS family members have four different ways to repress or stimulate transcriptional activity of their target transcription factors. 1) They can inhibit transcription factor binding to DNA by association with the factor in the nucleus. 2) PIAS proteins can either act as corepressors by recruiting histone deacetylases (HDACs) when bound to activated transcription factors on DNA, or as coactivators when attracting p300 or CBP in the same situation. 3) They can mediate sumoylation of target transcription factors with either activating or repressing consequences for the respective factor. 4) Using its SAP domain a PIAS family member can sequester transcription factors to distinct subnuclear structures, thereby reducing the pool of factors available for activating target genes (see Figure 18).

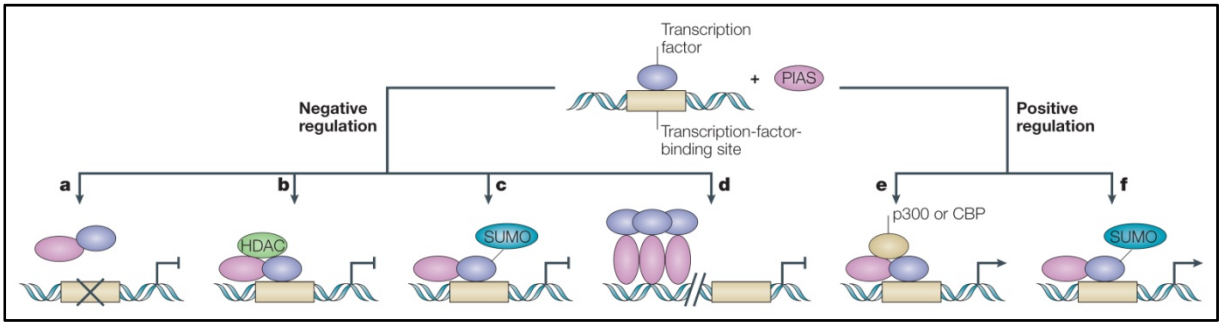

Figure 18: Proposed mechanisms of PIAS-mediated transcriptional regulation. Protein inhibitor of activated STAT proteins (PIAS proteins) can regulate transcription either positively or negatively. Four mechanisms have been proposed for PIAS-mediated transcriptional repression. a) PIAS proteins block the DNA-binding activity of a transcription factor. b) PIAS proteins function as transcriptional corepressors by recruiting histone deacetylase (HDAC) to inhibit transcription. c) PIAS proteins promote sumoylation of the transcription factor, which inhibits its transcriptional activity. d) PIAS proteins inhibit transcription by sequestering transcription factors in distinct subnuclear structures. PIAS proteins have also been implicated in transcriptional activation through two mechanisms. e) PIAS proteins recruit the transcriptional co-activators p300 or CBP (cyclic-AMP-responsive-element-binding protein (CREB)-binding protein) to enhance transcription. f) PIAS proteins promote sumoylation of the transcription factor, which positively regulates its transcriptional activity. (Shuai and Liu 2005)

PIAS proteins demonstrate remarkably high regulation specificity. PIAS3 modulates STAT5 and STAT3 activity by blocking DNA binding of activated STAT dimers (Chung, Liao et al. 1997; Rodel, Tavassoli et al. 2000; Sonnenblick, Levy et al. 2004). The implications of PIAS protein deficiency or overexpression in the erythroid compartment have not yet been assessed. *Pias1* (regulator of STAT1) knockouts, however, showed increased expression of STAT1 and NFκB targets, hypersensivity to LPS-induced endotoxic shock, growth retardation and partial perinatal lethality (Liu, Mink et al. 2004; Liu, Yang et al. 2005). Therefore it would certainly be worthwhile to analyze the effects of *Pias3* deficiency on development and hematopoiesis.

Projects

STAT5 regulates cellular iron uptake of erythroid cells via IRP2 and TfR1

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Stat 5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1

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Stat 5 Regulates Cellular Iron Uptake of Erythroid Cells via IRP-2 and TfR-1

Running title: Stat5 regulates iron uptake into erythroid cells

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Keywords: Stat5, erythropoietin, iron, apoptosis, iron regulatory protein, transferrin receptor

Abbreviations: Bcl-x_L, B-cell leukemia/lymphoma-x_L; Epo, erythropoietin; EpoR, erythropoietin receptor; Hct, hematocrit; Hgb, hemoglobin content; IRP-1&2, iron regulatory protein 1&2; Jak2, Janus kinase 2; MCH, mean corpuscular hemoglobin; Mcl-1, myeloid cell leukemia-1; MCV, mean corpuscular volume; qPCR, quantitative PCR; RBC; red blood cell count; Stat5, signal transducer and activator of transcription 5; TfR-1, transferrin receptor 1;

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Abstract

Erythropoiesis strictly depends on signal transduction through the erythropoietin receptor (EpoR) – Janus kinase 2 (Jak2) – signal transducer and activator of transcription 5 (Stat5) axis, regulating proliferation, differentiation and survival. The exact role of the transcription factor Stat5 in erythropoiesis remained puzzling, however, since the first Stat5-deficient mice carried a hypomorphic *Stat5* allele, impeding full phenotypical analysis. Using mice completely lacking *Stat5* – displaying early lethality – we demonstrate that these animals suffer from microcytic anemia due to reduced expression of the anti-apoptotic proteins Bcl-x_L and Mcl-1 followed by enhanced apoptosis. Moreover, transferrin receptor-1 (TfR-1) cell surface levels on erythroid cells were >2-fold decreased on erythroid cells of $Stat5^{-/-}$ animals. This reduction could be attributed to reduced transcription of TfR-1 mRNA and iron regulatory protein 2 (IRP-2), the major translational regulator of TfR-1 mRNA stability in erythroid cells. Both genes were demonstrated to be direct transcriptional targets of Stat5. This establishes an unexpected mechanistic link between EpoR/Jak/Stat signaling and iron metabolism, processes absolutely essential for erythropoiesis and life.

Introduction

Erythroid cell formation needs to be tightly regulated to maintain proper tissue oxygenation. Although in humans, about 10^{11} red cells are produced every day, total red cell numbers are kept within a narrow range in bone marrow, spleen and fetal liver. While early erythroid lineage commitment is controlled by numerous transcription factors and their binding partners (like GATA-1, FOG-1 and EKLF-1)[1], later stage differentiation from erythroblasts to mature erythrocytes is strictly regulated by erythropoietin (Epo)[2].

Epo induces dimerization of erythropoietin receptor (EpoR), which results in juxtaposition and activation of the receptor-associated Janus Kinase 2 (Jak2). Jak2 subsequently phosphorylates several tyrosine residues in the cytoplasmic tail of EpoR, which in turn acts as docking sites for signaling molecules such as PI3-K[3], MAPK[4], PKC[5] and PLC-gamma[6]. A central pathway of EpoR signaling is the activation of the transcription factor Signal Transducer and Activator of Transcription 5 (Stat5)[7,8]. Upon EpoR phosphorylation, Stat5 molecules are tyrosine-phosphorylated by Jak2 and translocate to the nucleus. This leads to activation of Stat5 target genes such as *Pim-1, c-myc, Bcl-x_L, D-type cyclins, oncostatin M* and *SOCS* members that play important roles in differentiation, proliferation, apoptosis and other processes[9-15].

The importance of Epo signaling is evidenced by the phenotypes of $Epo^{-/-}$, $EpoR^{-/-}$ and $Jak2^{-/-}$ mice, which die *in utero* at embryonic day E13.5 due to defects in erythropoiesis. Fetal livers of $Jak2^{-/-}$ animals completely lack BFU-E (burst forming unit-erythroid) and CFU-E (colony forming unit-erythroid) progenitors. In line with this, Epo- and EpoR-deficient embryos also fail to develop mature erythroid cells *in vivo*[16-18]. Interestingly, mice expressing a truncated form of EpoR ($EpoR_H$), which solely activates Stat5 and lacks all critical phosphotyrosine sites required to activate other signaling pathways, exhibited no strong erythroid phenotype[15,19], suggesting Stat5 as a critical component of the EpoR signaling pathway. Un-

expectedly, however, mice expressing an EpoR mutant additionally lacking the binding site for Stat5 ($EpoR_{HM}$), displayed no overt erythroid phenotype except under stress conditions[15,19]. Moreover, the mice initially reported to be deficient for Stat5a and Stat5b were viable and had a surprisingly mild erythroid phenotype[7,20,21]. Although showing fetal anemia, adult animals only displayed mild defects in recovery from phenylhydrazine-induced erythrolytic stress. This was explained by increased apoptosis in early erythroid progenitors, due to lack of Stat5-induced expression of the anti-apoptotic gene *Bcl-*x_L[21]. Later, however, these initial Stat5 knockout animals – now referred to as $Stat5^{\Delta N/\Delta N}$ – were found to express a N-terminally truncated protein able to bind DNA and to activate a subset of Stat5 target genes[14,22-25]. In contrast to $Stat5^{\Delta N/\Delta N}$, the recently described *bona fide* Stat5a/b null knockout mice (referred to as $Stat5^{-/-}$)[8] die during gestation or at latest (<2%) shortly after birth. Complete ablation of Stat5 resulted in severe defects in generation of all lymphoid lineages[23,24,26]. However, an accurate analysis of the erythroid compartment from these mice is still missing.

Maturing erythroid progenitors depend on large amounts of bio-available iron (humans require 20mg iron complexed to transferrin per day) to enable efficient heme synthesis. Cellular uptake of iron-loaded transferrin is mediated by transferrin receptor-1 (TfR-1, also called CD71)[27], followed by receptor internalization. Accordingly, maturing erythroid cells express high levels of TfR-1. However, as excess iron would lead to oxidative damage, expression of proteins involved in iron uptake, storage and utilization is tightly controlled. In case of TfR-1, this occurs primarily at the level of TfR-1 mRNA stability[28] and to a lesser extent at the transcriptional level[29]. At low iron conditions, trans-acting iron regulatory proteins (IRP1+2) bind to conserved hairpin structures (iron responsive elements; IREs) in the 3´-untranslated region (UTR) of TfR-1 mRNA, which selectively stabilizes this mRNA and ensures proper TfR-1 cell surface expression and iron uptake[30-32]. Upon iron excess, IRP-1 is converted to a cytosolic aconitase catalyzing isomerization of citrate to isocitrate[33] while IRP-2 is degraded by the proteasome[34]. Thus, both proteins no longer bind to IREs, resulting in

strongly reduced TfR-1 mRNA stability which leads to reduced TfR-1 cell surface expression and iron uptake[35-37]. Recently generated knockout mouse models for IRP-1 and IRP-2 suggested IRP-2 as the master regulator of IRE-regulated mRNAs, as ablation of IRP-2 led to a decrease in TfR-1 expression and microcytic anemia while IRP-1 knockout animals had no overt phenotype[38,39].

Here we show that Stat5$^{-/-}$ embryos suffer from severe microcytic anemia, a disease mostly associated with iron deficiency and characterized by small-sized red blood cells. In Stat5-deficent animals this anemia had two causes: Firstly, fetal livers were much smaller in knockout embryos, due to a strong increase of apoptosis in the erythron. We demonstrate that the anti-apoptotic proteins Mcl-1 and Bcl-x_L were largely absent in Stat5$^{-/-}$ cells, but ectopic expression of Mcl-1 complemented the survival defect of Stat5$^{-/-}$ erythroid cells. Secondly and more importantly, we demonstrate for the first time a direct link between Stat5 and iron metabolism. In the absence of Stat5, IRP-2 expression was strongly decreased, resulting in >2-fold lower cell surface expression of TfR-1 and thus strongly reduced iron uptake in erythroid cells. Together, the high levels of apoptosis and impaired iron uptake caused severe microcytic anemia and probably contributed to the death of Stat5$^{-/-}$ embryos.

Materials and Methods

Cell culture and retroviral infections

Stat5$^{+/-}$ mice[8] were maintained under pathogen-free conditions and bred on a mixed background (C57Bl/6xSv129F1) to obtain Stat5$^{-/-}$ embryos. For the determination of blood indices heparinized blood was measured in a Vet animal blood counter (Scil Animal Care). Serum Epo-levels were determined using a *Quantikine* mouse-erythropoietin ELISA-kit (R&D Systems) according to the manufactures protocol, measured on a Victor^{3}V 1420 multilabel counter (Perkin Elmer). All animal experiments described in our manuscript were performed in accordance with Austrian and European laws and under approval of the ethical and animal protection committees of the Veterinary University of Vienna.

Fetal livers of E13.5 mouse embryos (Stat5$^{-/-}$ and wild type; =wt) were resuspended in serum-free medium (StemPro-34^{TM}, Invitrogen). In brief, for self-renewal[40], cells were seeded into medium containing 2U/mL human recombinant Epo (Erypo, Cilag AG), murine recombinant stem cell factor (SCF; 100ng/mL, R&D Systems), 10^{-6}M dexamethasone (Dex, Sigma), and insulin-like growth factor 1 (IGF-1; 40ng/mL, Promega). The resulting erythroblast cultures were expanded by daily partial medium changes and addition of fresh factors, keeping cell densities between 2-4x10^6 cells/mL. Proliferation kinetics and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System)[40]. For retroviral infections, fetal liver erythroblasts were co-cultured for 72h with retrovirus-producing fibroblast cell lines selected for high virus production[14]. Infection efficiency was 75-95%, as measured by flow cytometry for GFP expression. Photomicrographs were taken using an Axiovert 10 microscope (Zeiss) equipped with a 63x oil-immersion objective lens (numerical aperture 44-07-61). Images are presented at 630x magnification.

Flow cytometry

Cultured erythroblasts or single cell suspensions of freshly isolated fetal livers were stained with fluorescence-conjugated antibodies (all Becton Dickinson Biosciences; =BD) against Ter-119 (PE-conjugated) and TfR-1 (biotinylated) for *in vivo* erythroid development analyses. Annexin V (APC-conjugated) staining was performed according to the manufacturers' instructions. For *in vivo* proliferation assays, pregnant mice were injected with 80mg 5-bromo-2-deoxyuridine (BrdU) per kg body mass. After 1h, embryos were isolated, fetal liver cells fixed, and stained with anti-BrdU-FITC plus Ter119-PE, following the manufacturer's protocol (BrdU flow kit, BD). Samples were analyzed on a FACS-Calibur flow cytometer (BD). Where indicated, $Ter119^+$ cells were isolated by magnetic cell sorting using AutoMACS (Miltenyi Biotech).

Western blot analysis

Antibodies used for Western blotting: anti-mouse ERK1/2, anti-horse ferritin H, anti-mouse actin (all Sigma), anti-mouse TfR-1 (BioSource), anti-mouse Bcl-$x_{L,}$ anti-mouse PCNA, anti-mouse Stat5 (all BD Transduction Laboratories), anti-mouse Mcl-1 (Abcam), anti-mouse eIF4E, anti-mouse eIF2-alpha , pSer-eIF2-alpha (all Cell Signaling), anti-rat IRP-1[41], anti IRP-2[39].

Chromatin immuno-precipitation

Chromatin immunoprecipitation was performed as in[42]. $2x10^7$ primary erythroblasts were stimulated with 10U Epo/mL for 30min. Cells were fixed with 1% formaldehyde for 30min. DNA was sonicated using a Bandelin Sonoplus GM70 sonicator (cycle count 30%; power 45%; 6x30 sec). DNA fragments were recovered using anti-mouse Stat5ab (C20, Santa Cruz) or anti-mouse Stat5 (N20, Santa Cruz). Recovered DNA fragments were directly used for PCR analysis.

Quantitative Real Time PCR

RNA was isolated using RNeasy Mini Kit (Quiagen). RNA integrity was checked with the Agilent Bioanalyzer (Agilent). 2.5 µg RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Real Time PCR was performed on an Eppendorf Mastercycler RealPlex using RealMasterMix (Eppendorf) and SYBR Green. Results were quantified using the "Delta Delta C(T) method"[43].

Statistical analyses

Statistical analyses were performed using Excel 2004 (Microsoft). Student's t-test was used to calculate p-values (two-tailed). p-values: $p \leq 0.05$, one asterisk; $p \leq 0.01$, two asterisks. Data are presented as mean values ± standard deviation.

Results

Stat5$^{-/-}$ embryos are severely anemic

Embryos lacking the entire *Stat5* locus (i.e. *Stat5a/b*) die during gestation or at latest perinatally (~99%) with severe defects in diverse hematopoietic lineages[23,24]. The previously demonstrated pivotal role of Stat5 in Epo-signaling[19-22] prompted us to analyze the function of Stat5 in erythropoiesis in detail. E13.5 Stat5$^{-/-}$ embryos and newborn Stat5$^{-/-}$ animals appeared paler than their wt littermates, particularly in the fetal liver region (Figure 1A). The relative abundance of erythroid cells in Stat5$^{-/-}$ fetal livers (~80% of all cells) remained unchanged, as determined by staining for the pan-erythroid marker Ter119 followed by flow cytometry (Figure 1B, left). Yet, the size of Stat5$^{-/-}$ fetal livers in E13.5 was visibly reduced and total fetal liver cellularity was decreased by 50% (n=6), corresponding to a similar reduction in the total number of erythroid cells (Figure 1B, right). Since anemia causes elevation of Epo levels[44] to counteract hypoxia, sera from E16.5 or newborn wt and Stat5$^{-/-}$ animals were analyzed for Epo levels. These were highly elevated in Stat5$^{-/-}$ versus wt embryos (3.8±0.6-fold; n=5), newborn animals showed an even higher elevation (35.2±5.1-fold; n=5; Figure 1C). This strongly suggested that Stat5-deficient animals suffer from severe anemia. To determine the specific type of anemia, blood from E16.5 wt versus Stat5$^{-/-}$ animals was analyzed (Table 1). Red blood cell counts of E16.5 Stat5$^{-/-}$ embryos was lowered to 1,0±0.3x10^6/mm^3, in contrast to 2,4±0,4x10^6/mm^3 in wt embryos. In line with this, hematocrit (Hct) of E16.5 Stat5$^{-/-}$ embryos was reduced to 9,8±0,7% as compared to 31.4±1,8% in wt embryos. Likewise, mean corpuscular volume (MCV), hemoglobin content (Hgb) and mean corpuscular hemoglobin (MCH) of E16.5 Stat5$^{-/-}$ blood was strongly reduced. These effects also were clearly visible in blood smears, showing hypochromic microcytic erythrocytes (Supplementary Figure 1A+B).

An additional cause for early lethality and high serum Epo levels could have been a lung defect leading to reduction in red cell oxygenation. Analysis of tissue sections from wt

and Stat5$^{-/-}$ newborn animals, however, did not reveal any histological differences (not shown). Taken together, Stat5$^{-/-}$ mice suffer from microcytic anemia.

Loss of Stat5 causes enhanced apoptosis in the fetal liver

Hypomorphic Stat5$^{\Delta N/\Delta N}$ mice displayed enhanced erythroid cell death, attributed to reduced expression of the anti-apoptotic protein Bcl-x_L[20,21]. To determine apoptosis in Stat5$^{-/-}$ mice, freshly isolated fetal liver cells were stained for Ter119 and apoptosis assessed by Annexin V staining. In Stat5$^{-/-}$ embryos, the frequency of Annexin V-positive cells was >2-fold enhanced, regardless of developmental stage (Figure 2A). In line with this, Stat5$^{-/-}$ fetal liver cells showed a >6-fold reduction of erythroid colony numbers in CFU-E assays irrespective of Epo concentrations, supporting the notion that lack of functional Stat5 reduces cell survival (Figure 2B). In contrast, no significant alterations were observed in BFU-E assays (Supplementary Figure 1C).

To assess potential differences in viability of Stat5-deficient erythroid cells in an adult (bone marrow) versus an embryonic (fetal liver) microenvironment, short term transplantation experiments were performed. Equal amounts of sorted GFP-expressing wt- or Stat5$^{-/-}$ pro-erythroblasts cultured from fetal livers (c-Kit^{+}/TfR-1^{high}/Ter119^{-} cells[15]) were injected into lethally irradiated wt recipients (Figure 2C). This setup was chosen to circumvent any influence of the well-known re-population defect of Stat5-deficient hematopoietic stem cells[45-47]. Three days after transplantation, bone marrow cells were harvested and scored for GFP-positive mature Ter119^{+} erythroid cells, as previously reported[48]. In line with the preceding experiments, a 5-fold reduction in abundance of transplanted mature Stat5$^{-/-}$ versus wt cells was determined in the adult microenvironment (bone marrow; Figure 2D). Moreover, these data indicated a cell-autonomous survival defect of Stat5$^{-/-}$ erythroid cells.

Stat5-deficiency leads to reduced expression of anti-apoptotic proteins

Consistent with the apoptotic phenotype described above, $Ter119^{+}$ cells from $Stat5^{-/-}$ fetal livers showed decreased levels of Bcl-x_L as compared to wt cells (Figure 3A, left). Nevertheless, Bcl-x_L protein levels in $Stat5^{-/-}$ cells were still ~50% of wt. This prompted us to analyze the expression of other anti-apoptotic *Bcl-2* family members. We focused on Mcl-1 for three reasons: (i) Mcl-1 is up-regulated during early erythroid commitment in human cells[49], (ii) its bone marrow-specific ablation reduces blood formation[50] and (iii) it appears to be regulated by Stat5[51-53]. Indeed, Mcl-1 protein and mRNA expression were drastically reduced in Stat5-deficient cells (Figure 3A, right; Figure 3B). To assess if *Mcl-1* is an Epo-inducible Stat5-regulated gene, primary wt and $Stat5^{-/-}$ erythroblasts were factor-deprived for 2h and subsequently re-stimulated with Epo for 30min. Quantitative PCR (qPCR) revealed a 3.5-fold increase in Mcl-1 mRNA in Epo-stimulated cells which was abrogated in Stat5-deficient cells (Figure 3C).

To test if exogenous Mcl-1 provides protection against apoptosis to erythroid cells, primary wt or $Stat5^{-/-}$ fetal liver cells were transduced with retroviral constructs encoding *GFP*, *Bcl-x_L* or *Mcl-1*. Erythroblasts were cultivated for 48h in the presence or absence of Epo and apoptosis was determined by flow cytometry. Ectopic expression of either Mcl-1 or Bcl-x_L completely prevented apoptosis of wt as well as Stat5 knockout erythroblasts upon Epo withdrawal (Figure 3D).

The decrease of fetal liver size and cellularity in $Stat5^{-/-}$ embryos could also have been due to reduced proliferation of erythroid cells, as suggested by the known ability of Stat5 to enhance expression of proliferation-promoting genes such as *c-Myc, Cyclin D2* and *D3 or oncostatin M*[14,23,54,55]. Cell division kinetics of erythroid cells *in vivo* and *in vitro*, however, were similar in $Stat5^{-/-}$ and wt cells (Supplementary Figure 2, Supplementary Text).

Taken together, Sta5-deficient fetal liver erythroid cells were massively apoptotic. This effect could be attributed to reduction of Bcl-x_L levels together with complete loss of Mcl-1, translating into massive decrease of fetal liver cellularity.

TfR-1 expression is strongly reduced in Stat5$^{-/-}$ erythroid cells

To analyze if Stat5-deficient mice had a defect in erythroid lineage commitment, wt and Stat5$^{-/-}$ fetal livers were analyzed for the presence of Megakaryocytic-Erythroid Progenitors[56,57] (MEP), the first erythroid-committed progenitor detectable by flow cytometry. Interestingly, we observed a two-fold increase of the MEP compartment in Stat5-deficient fetal livers (Supplementary Figure 3), suggesting a compensatory attempt to counteract the increased erythroid cell death during definitive erythropoiesis.

To determine whether the anemia in Stat5$^{-/-}$ embryos was due to a defect in erythroid differentiation, fetal liver cells were analyzed for erythroid markers Ter119 and TfR-1. This combination allows staging of maturing erythroid cells from immature progenitors (TfR-1^{low} Ter119^{low}) over an intermediate stage (TfR-1^{high} Ter119^{high}) to late orthochromatophilic erythroblasts (TfR-1^{neg} Ter119^{high}; Figure 4A, left; gates R1 to R5, increasing maturity[58]). Stat5-deficient and wt fetal livers contained cells of all differentiation stages at indistinguishable frequencies (Figure 4A). For detailed morphological analysis, wt and Stat5$^{-/-}$ fetal liver cells were sorted according to their cell surface marker phenotype (R2-R5), spun onto glass slides and subsequently stained with either May-Grunwald Giemsa or Benzidine-Wright Giemsa (Supplementary Figure 4). No apparent morphological differences in maturity between wt and Stat5$^{-/-}$ cells were observed. Thus, the reduction in fetal liver cellularity (Figure 1B) was probably not due to differentiation arrest at a distinct step of maturation. We did, however, observe a reproducible decrease in TfR-1 cell surface expression, which prompted us to align the gating strategy accordingly.

Accumulation of hemoglobin is the hallmark of terminal erythropoiesis, requiring an enormous up-regulation of iron intake via increased expression of TfR-1. Quantification of TfR-1 levels in Stat5-deficicient versus wt cells by flow cytometry revealed a >2-fold reduction in knockout cells (Figure 4B). This was confirmed at the mRNA level (Figure 4C) and further corroborated by Western blot analysis of wt, $Stat5^{+/-}$ and $Stat5^{-/-}$ fetal liver cell lysates (Figure 4D+E). A recent report described functional Stat5 binding sites (GAS elements) in the first intron of the TfR-1 gene, using an erythroleukemic cell line expressing a constitutively active Stat5 variant [59]. To corroborate these data, we decided to analyze DNA binding of endogenous Stat5 to these elements in primary fetal liver erythroblasts after Epo-stimulation by chromatin immunoprecipitation (Figure 4F). Indeed, DNA binding of Stat5 to all three sites analyzed was confirmed and apparently resulted in an Epo-induced increase of TfR-1 mRNA as quantified by qPCR (Figure 4G). As expected from the well-known inverse relation in expression of TfR-1 to the iron-storage protein ferritin[30], $Stat5^{-/-}$ cells showed elevated levels of ferritin protein (Supplementary Figure 5).

As a direct consequence of reduced TfR-1 cell surface expression, we observed a significant reduction of intracellular iron (~40%) in freshly isolated $Stat5^{-/-}$ fetal livers as measured by atomic absorption spectrometry (Figure 4H), further supporting the idea of altered iron metabolism in $Stat5^{-/-}$ cells. Reduced iron availability leads to a drop in heme synthesis[60], known to result in activation of heme-regulated inhibitor (HRI)[61]. This kinase, via inactivation of translation initiation factor eIF2-alpha, throttles expression of globins to ensure that heme, alpha- and beta-globin are always synthesized at the appropriate ratio of 4:2:2[61]. To test if this regulatory circuit was disturbed in $Stat5^{-/-}$ erythroid cells, abundance of globin mRNAs in lysates from erythroid cells sorted out of wt or $Stat5^{-/-}$ fetal livers were analyzed by qPCR. Indeed, relative levels of both globin mRNAs were significantly reduced in $Stat5^{-/-}$ cells; moreover, eIF2-alpha showed the expected increase in phosphorylation (Supplementary Fig-

ure 6). In summary, these data demonstrated that Stat5$^{-/-}$ erythroid cells were severely iron-deficient.

IRP-2 expression and mRNA binding activity is reduced in Stat5-deficient cells

Stabilization of TfR-1 mRNA by binding of IRP-1 and IRP-2 is considered the pre-dominant mechanism to satisfy the iron demand of proliferating cells[30,31]. A possible activation of IRPs by Epo has been discussed[62,63]. Accordingly, Western blot analyses for IRP-1 and IRP-2 from lysates of wt and Stat5$^{-/-}$ primary erythroblasts revealed a striking, 5-fold down-regulation of IRP-2 in Stat5 knockout cells (Figure 5A+B, right), accompanied by a 2-fold up-regulation in IRP-1 expression. IRP-1+2 mRNA levels were similarly changed in Stat5-deficent cells (data not shown). Determination of IRP-2 RNA-binding in Stat5$^{-/-}$ cells using *in vitro* transcribed, radioactively labeled IRE probes in electrophoretic mobility shift assays (EMSA, Figure 5C) showed a similar decrease of IRP-2 activity (Figure 5D). Given the important role of IRP-2 in TfR-1 expression in erythroid cells[38,39], these data strongly suggested that the decrease of IRP-2 was an additional cause for reduction of TfR-1 cell surface expression.

IRP-2 is a direct transcriptional target of Stat5

To test a possible direct role of Stat5 in regulating *IRP-2* expression, we analyzed the *IRP-2* promoter in detail. A region 1030-1100bp upstream of the transcriptional start site contained one perfect Stat5 DNA-binding site ($TTCN_3GAA$)[64] plus two adjacent low-affinity Stat5 response elements with a mismatch in one half-site of the inverted repeat (Figure 6A). Annotated Stat5 sites[24,64] together with the IRP-2 sequence I suggested that the latter fulfilled bioinformatic criteria for perfect Stat5 binding (Figure 6B).

To test if there was a direct transcriptional induction of *IRP-2* by Stat5, the 2kb fragment of the *IRP-2* promoter upstream of the predicted transcription start site, comprising all three putative Stat5 response elements (REs), was inserted into a firefly-luciferase reporter

gene construct. 293T cells were co-transfected with Stat5a and EpoR cDNAs together with the respective reporter construct. Transfected cells were stimulated with Epo or left untreated. Epo-treated cells displayed a significant increase of luminescence over untreated controls (Figure 6C). Direct Epo-induced expression of endogenous *IRP-2* and *oncostatin M* (a bona-fide Stat5 target gene) was demonstrated in murine erythroid leukemia cells: Following 3h of factor deprivation, cells were re-stimulated for 1h with Epo and mRNA expression levels determined by qPCR. Epo stimulation induced expression of IRP-2 as well as oncostatin M about 3-fold (Figure 6D).

To further substantiate that IRP-2 is a direct target of Stat5, EMSAs were performed. 293T cells were co-transfected with constructs encoding EpoR and murine Stat5a. Transfected cells were Epo-stimulated or left untreated. Extracts were subsequently subjected to EMSAs using radiolabeled oligonucleotides encompassing either the newly identified Stat5-RE I of the IRP-2 promoter or a well-described Stat5-RE probe from the bovine β-casein promoter as positive control. For super-shifts, a serum directed against the C-terminus of Stat5 was added to the oligonucleotide/lysate mixture. Stat5-DNA complexes were clearly evident in Epo-stimulated extracts, using both, the IRP-2-I or the β-casein probe, as these complexes were readily super-shifted upon addition of anti-Stat5 serum (Figure 6E). Similar results were obtained using Epo-stimulated cells transfected with murine Stat5b (data not shown).

To test if Stat5 recognizes one of these putative DNA-binding sites *in vivo*, we finally performed chromatin-immunoprecipitation (ChIP) experiments using two different anti-sera directed against N- or C-terminal epitopes in Stat5. PCR analysis of immuno-precipititated Stat5-DNA complexes from Epo-stimulated primary wt erythroblasts yielded a PCR product representing Stat5-binding sites in the *IRP-2* promoter in both specific Stat5 ChIPs, but not in control IgG ChIP experiments (Figure 6F).

Together, these results indicated that Stat5 is directly involved in the control of *TfR-1* transcription as well as in the modulation of its mRNA stability by regulating expression of *IRP-2*.

Discussion

In this paper cooperating mechanisms underlying the erythroid defect leading to microcytic anemia in Stat5$^{-/-}$ mice were uncovered, demonstrating a novel direct link between the EpoR-Stat5 axis and regulation of iron metabolism *in vivo*. First, Stat5$^{-/-}$ fetal livers showed reduced cellularity due to massively enhanced apoptosis of maturing erythroid cells, apparently caused by defective expression of the anti-apoptotic genes *Mcl-1* and *Bcl-x$_L$*. Second, Stat5$^{-/-}$ erythroid cells exhibited reduced expression of IRP-2 and TfR-1, resulting in a large decrease of TfR-1 cell surface expression, iron uptake and globin synthesis. Together, these mechanisms appear to be sufficient to explain the severe anemia of Stat5$^{-/-}$ animals.

Complete ablation of Stat5 leads to early lethality

None of the conditional Stat5 knockout models created so far in multiple cell types such as hemangioblasts (Stat5fl/fl Tie2-Cre)[59] B-cells (CD19-Cre)[65], T-cells (CD4-Cre, Lck-Cre)[23,24], hepatocytes (albumin-Cre, albumin-alpha-fetoprotein-Cre)[25,66], pancreatic β-cells/hypothalamus (Rip-Cre)[67], endocrine/exocrine pancreas progenitors (Pdx1-Cre)[67], or skeletal-muscle (Myf5-Cre)[68] die during fetal development. In contrast, ablation of Stat5 in the entire organism resulted in mortality[8] during gestation or at latest shortly after birth. Since Epo$^{-/-}$, EpoR$^{-/-}$ and Jak2$^{-/-}$ mice all die *in utero* at E13.5 due to defects in definitive erythropoiesis and given the prominent role of Stat5 in EpoR-signaling, it was unexpected that a few Stat5$^{-/-}$ embryos developed to term.

There are several possible explanations for the discrepancy in phenotypes. First, we detected high levels of pY-Stat1 and pY-Stat3 in Stat5-deficient cultivated primary erythroblasts as well as in lysates from freshly isolated fetal livers but not in wt counterparts (MAK, FG, unpublished). This is in line with increased pY-Stat1 and pY-Stat3 levels found upon liver-specific Stat5 deletion[66,69,70]. Since Stat3 and Stat5 response elements are similar[64], increased activation of Stat3 might partially compensate for loss of Stat5. Second, the anemia of

Stat5$^{-/-}$ embryos led to a compensatory elevation of Epo levels in the serum, which was most pronounced in the few newborn animals. This might contribute to prolonged survival mediated by hyper-activation of Stat5-independent EpoR signaling. Third, Stat5-deficient erythroid cells exhibited elevated levels of phosphorylated eIF2-alpha, indicative for an active "integrated stress response" (ISR), presumably via the kinase heme-regulated inhibitor (HRI)[71]. In mouse models for the red blood cell disorders erythropoietic protoporphyria and beta-thalassemia, ablation of HRI exacerbated the phenotype of these diseases[71]. Thus, the modulation of translational efficiency to balance heme and globin production could represent another protective mechanism accounting for the "mild" erythroid phenotype of Stat5$^{-/-}$ animals. Nevertheless, the ultimate reason for the early death of the animals remains to be determined.

Stat5 is not essential for erythroid differentiation

Earlier studies addressing the role of Stat5 (i.e. Stat5a+b) in erythropoiesis were performed with *Stat5*$^{\Delta N/\Delta N}$ animals. These mice are born, viable and show only a mild erythroid phenotype[20,21]. *Stat5*$^{\Delta N/\Delta N}$ animals express a N-terminally-truncated Stat5, which still activates target genes[22]. Here we used Stat5$^{-/-}$ mice lacking the entire *Stat5a/b*-locus[8]. Animals lacking other components of Epo signaling upstream of Stat5 (Epo, EpoR or Jak2)[2], all die *in utero* around E13.5, due to a block in definitive erythropoiesis. If Stat5 was the only crucial target of this pathway, full Stat5 knockout animals should show a similarly severe phenotype. Indeed, Stat5-deficient animals display erythroid defects and die at latest after birth. Although Stat5 is essential for differentiation of other hematopoietic lineages like maturation of pre-pro- to pro-B cells[23,24], or in formation of FoxP3^{+} regulatory T-cells[26], the observed block in erythroid maturation *in vivo* was not complete. The presence of erythroid cells at all developmental stages in Stat5$^{-/-}$ embryos strongly argued against an absolutely essential function of Stat5 in erythroid development. Nevertheless, there were defects in hemoglobinization of Stat5$^{-/-}$

erythroid cells, which may have several causes. For instance, it could decrease through direct defects in the erythroid differentiation program and/or through a secondary response to iron deficiency.

Involvement of Stat5 in iron metabolism

The most striking observation in the peripheral blood morphology of Stat5$^{-/-}$ animals was an apparent microcytic hypochromic anemia. This type of anemia, characterized by decreased mean corpuscular volume and reduced mean cell hemoglobin, is frequently associated with iron deficiency. Thus it was tempting to investigate the molecular players involved.

The normal adaptive response to low iron is the well-characterized feedback regulation that increases TfR-1 mRNA stability upon binding of IRPs to its 3'-UTR (Figure 6). In Stat5$^{-/-}$ cells, this response apparently was impeded as delineated from the reduced expression of TfR-1, which in turn was the direct result of decreased IRP-2 protein levels. This mechanistic link was further substantiated by the measured reduction in total intracellular iron in Stat5$^{-/-}$ fetal livers, finally resulting in decreased globin mRNA expression. It remained unclear, however, whether a connection between Stat5 and IRP-2 expression existed. The promoter region of the *IRP-2* gene contains three adjacent potential binding sites for Stat5, which indeed turned out to be functional. Moreover, qPCR showed reduced IRP-2 mRNA abundance in the absence of Stat5. Thus, one could envision a chain of events in Stat5-deficient erythroid cells, starting with decreased IRP-2 and TfR-1 expression, resulting in a net decrease of TfR-1 mRNA stability and abundance, followed by diminished TfR-1 surface expression. The consequence would be insufficient iron uptake (even in iron-depleted cells), ultimately leading to decreased heme synthesis, activation of the integrated stress response pathway and reduced globin mRNA translation. Interestingly, no functional compensation for low IRP-2 levels by the highly homologous IRP-1 protein was observed. This is in line with *in vivo* data from corresponding IRP-1 or IRP-2 knockout animals[38,39], which indicated that IRP-2 is the predomi-

nant regulatory factor modulating TfR-1 mRNA stability. While ablation of *IRP-1* produced no overt phenotype, loss of *IRP-2* resulted in hypochromic microcytic anemia due to reduced TfR-1 expression, a phenotype reminiscent to the one of Stat5 knockout animals described here. Accordingly, lowering the expression of TfR-1 by 50% led to a similar phenotype, as TfR-$1^{+/-}$ mice also displayed the same type of anemia[74]. It should be mentioned, however, also other conditions are known to result in microcytosis, including ablation of the genuine Stat5-target Pim-1[9].

Involvement of Stats in iron metabolism might even be a more general mechanism. Hepcidin[75,76], the dominant regulator of dietary iron absorption in enterocytes and iron release from macrophages, is a direct Stat3 target gene[75,76]: Upon infection, the inflammatory cytokine IL-6 promotes hepcidin expression via Stat3, trapping iron in macrophages, resulting in decreased plasma iron concentrations. Hepcidin expression is decreased by hypoxia and anemia, directly responding to increased Epo levels[75-77]. Thus its regulation in the anemia resulting from Stat5-deficiency may be of interest in future studies.

Stat5$^{-/-}$ fetal liver cells exhibit high levels of apoptosis

In erythropoiesis, up-regulation of Bcl-x_L was found to be defective in Stat5$^{\Delta N/\Delta N}$ erythroid cells[20,21]. Other studies, however, suggested that Bcl-x_L prevents apoptosis only of late stage erythroblasts[11,78] but not directly via EpoR[78]. Upon re-addressing this question in mice that are fully devoid of Stat5, we observed a reduction in Bcl-x_L levels of about 50% in fetal liver erythroid cells. Furthermore, Mcl-1 expression in Stat5$^{-/-}$ erythroid cells was analyzed, based on the finding that this *Bcl-2* gene family member could be a Stat5 target gene[52,53,79]. Indeed, Mcl-1 was completely absent in Stat5$^{-/-}$ fetal liver cells whereas Epo-stimulation of wt primary erythroblasts led to a 3.5-fold increase of Mcl-1 mRNA. Furthermore, re-introduction of Mcl-1 or Bcl-x_L completely prevented apoptosis of wt as well as Stat5 knockout erythroblasts upon Epo withdrawal.

Besides the finding that Mcl-1 is a Stat5-dependent Epo target gene, down-regulation of Mcl-1 in Stat5$^{-/-}$ erythroid cells could also occur through an additional mechanism, which is induced through iron deficiency in Stat5 knockout animals. Recently it was suggested that Mcl-1 levels decrease after activation of the phospho-eIF2-alpha-mediated ISR pathway already mentioned above[80-82]. eIF2-alpha-phosphorylation can be induced by different kinases in response to several stress stimuli[83], including HRI. Iron deficiency activates HRI, which in turn phosphorylates eIF2-alpha on its inhibitory Ser51, resulting in global reduction of mRNA translation[61], which immediately affects Mcl-1, as it is a highly unstable protein[84]. The observed reduced iron levels, together with elevated eIF2-alpha phosphorylation in Stat5$^{-/-}$ primary erythroblasts suggested an active ISR in Stat5$^{-/-}$ cells. Hence, loss of Stat5 could lead to a direct decrease of Mcl-1 mRNA, but alternatively also to a down-regulation of Mcl-1 protein due to iron deficiency-induced ISR. Taken together, the apoptosis in Stat5$^{-/-}$ fetal livers most probably reflects a composite effect of reduced levels of Bcl-x_L and loss of Mcl-1.

This contribution should help to clarify the long-discussed role of Stat5 in erythropoiesis *in vivo*: We identify Stat5 as a key factor regulating erythroid iron metabolism *in vivo* and, additionally, link the anti-apoptotic machinery of erythroid cells with their iron uptake system.

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Author Contribution

MAK, FG, HG, MS, MA and BK conducted experiments. MAK and EWM designed experiments and interpreted results. HB contributed essential reagents and worked on the draft of the manuscript. MAK, RM and EWM wrote the paper.

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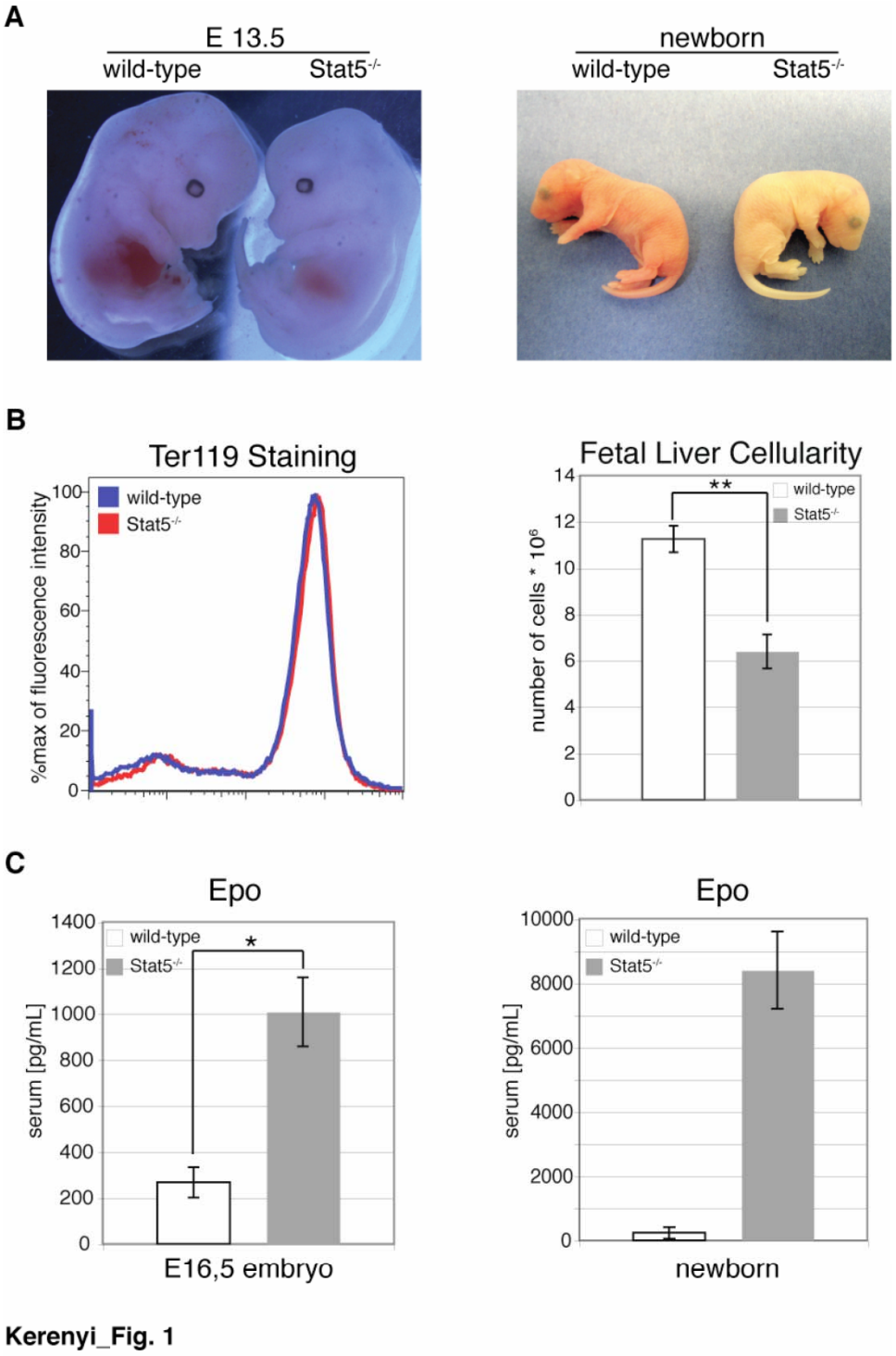
E 16.5 embryos	wild-type	Stat5-/-
RBC (10^6/mm^3)	2.4±0.4	1.0±0.3**
Hct (%)	31.4±1.8	9.8±0.7**
Hgb (g/dL)	9.1±1.2	2.3±0.7**
MCV (μm^3)	128.2±3.7	95.9±5.2**
MCH (pg)	37.5±0.3	23.1±0.7**

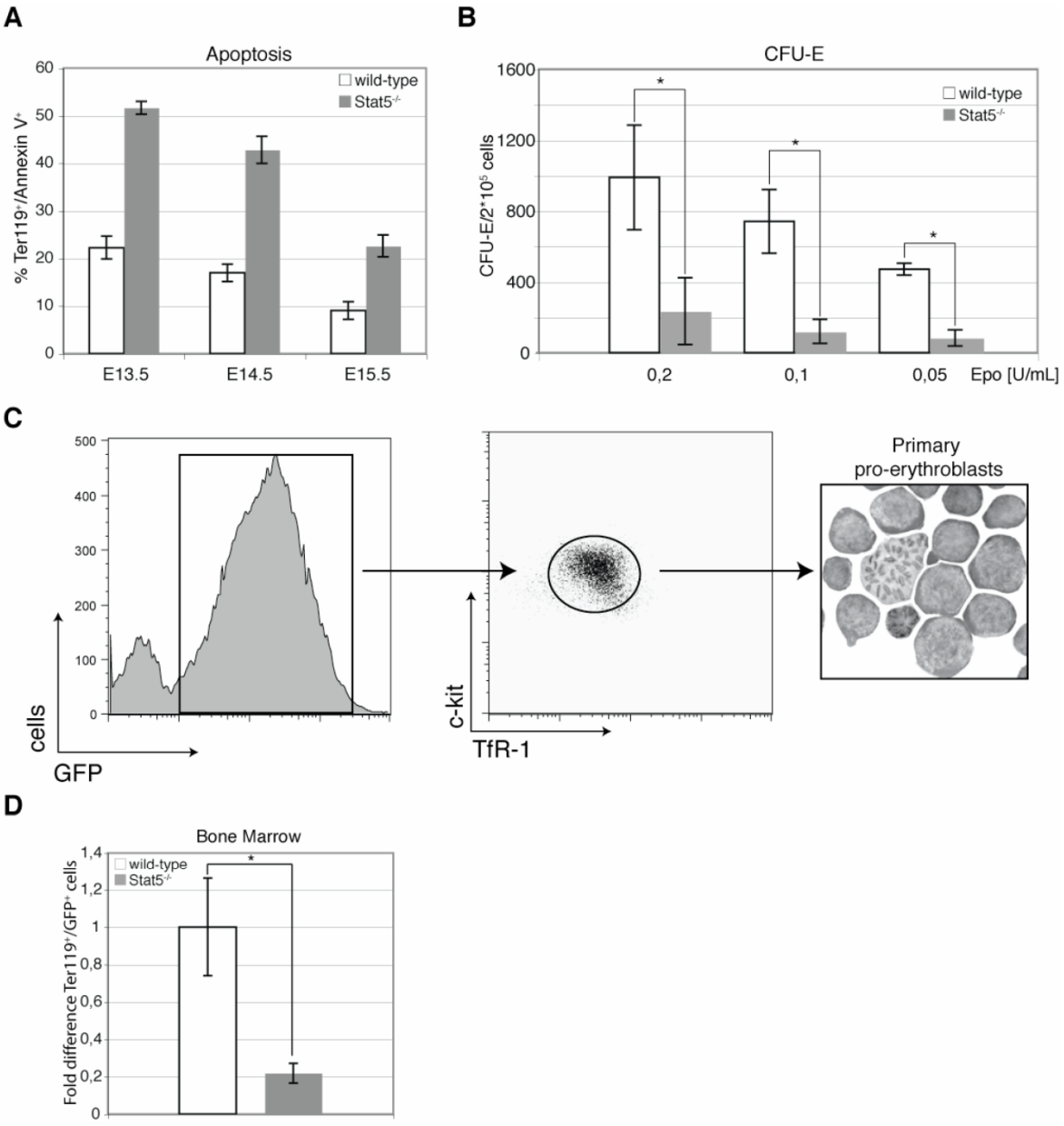
Table 1. Stat5$^{-/-}$ embryos mice display severe microcytosis. Blood indices of E16.5 Stat5$^{-/-}$ embryos (data are presented as mean ± SEM; n=15 each genotype). RBC, red blood cell count; Hct, hematocrit; Hgb, hemoglobin content; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

Figures and Figure Legends



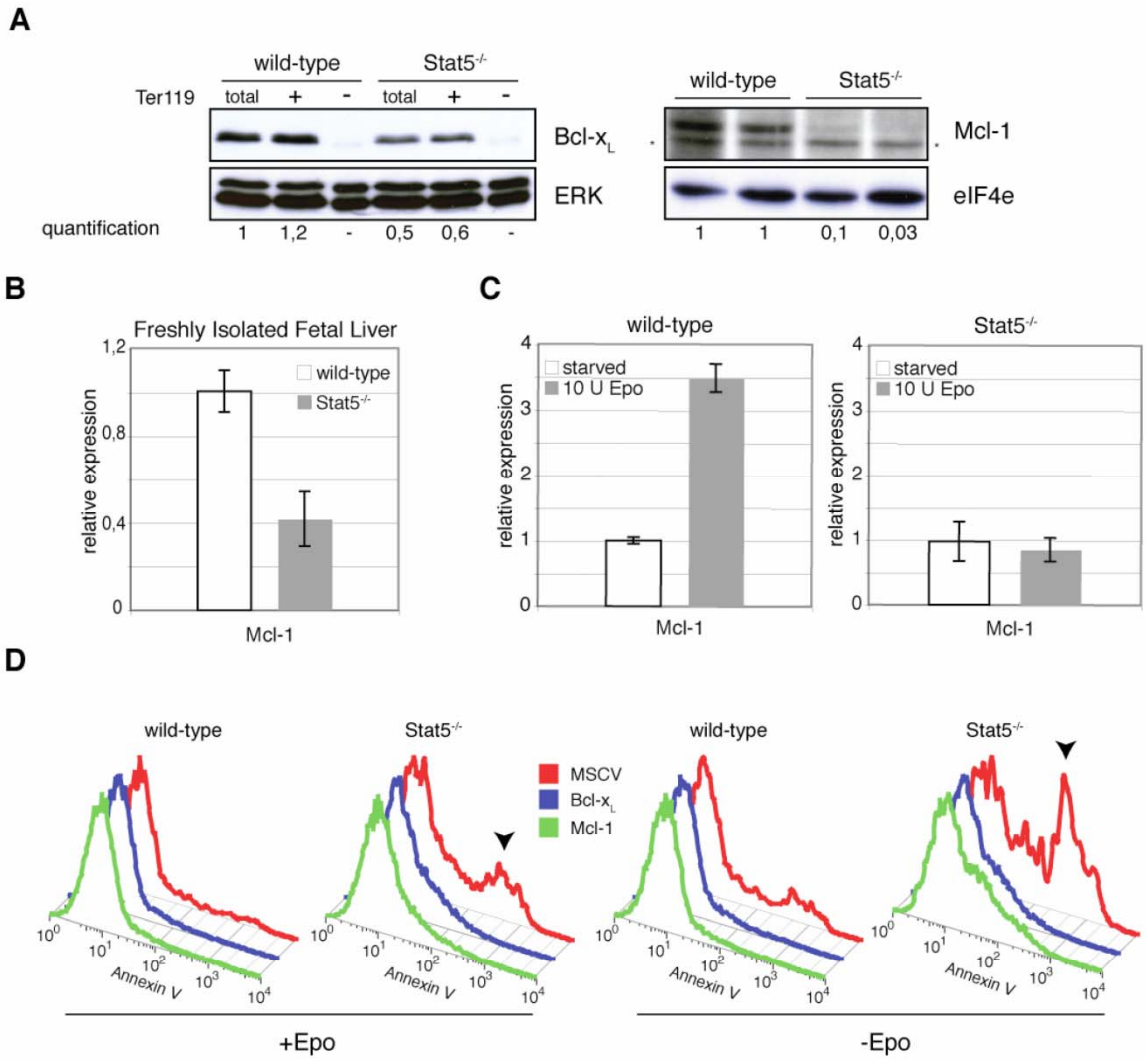
Kerenyi_Fig. 1

Figure 1. Stat5$^{-/-}$ embryos are severely anemic. (A) Wt and Stat5$^{-/-}$ E13.5 embryos (left) and newborn animals (right). (B) Ter119-positive erythroid cells (left) and fetal liver cellularity (right; data are presented as mean ± SD; n=6) of wt versus Stat5$^{-/-}$ fetal livers. (C) ELISA for Epo from serum of wt and knockout E16.5 embryos and newborns (data are presented as mean ± SD; n=5).



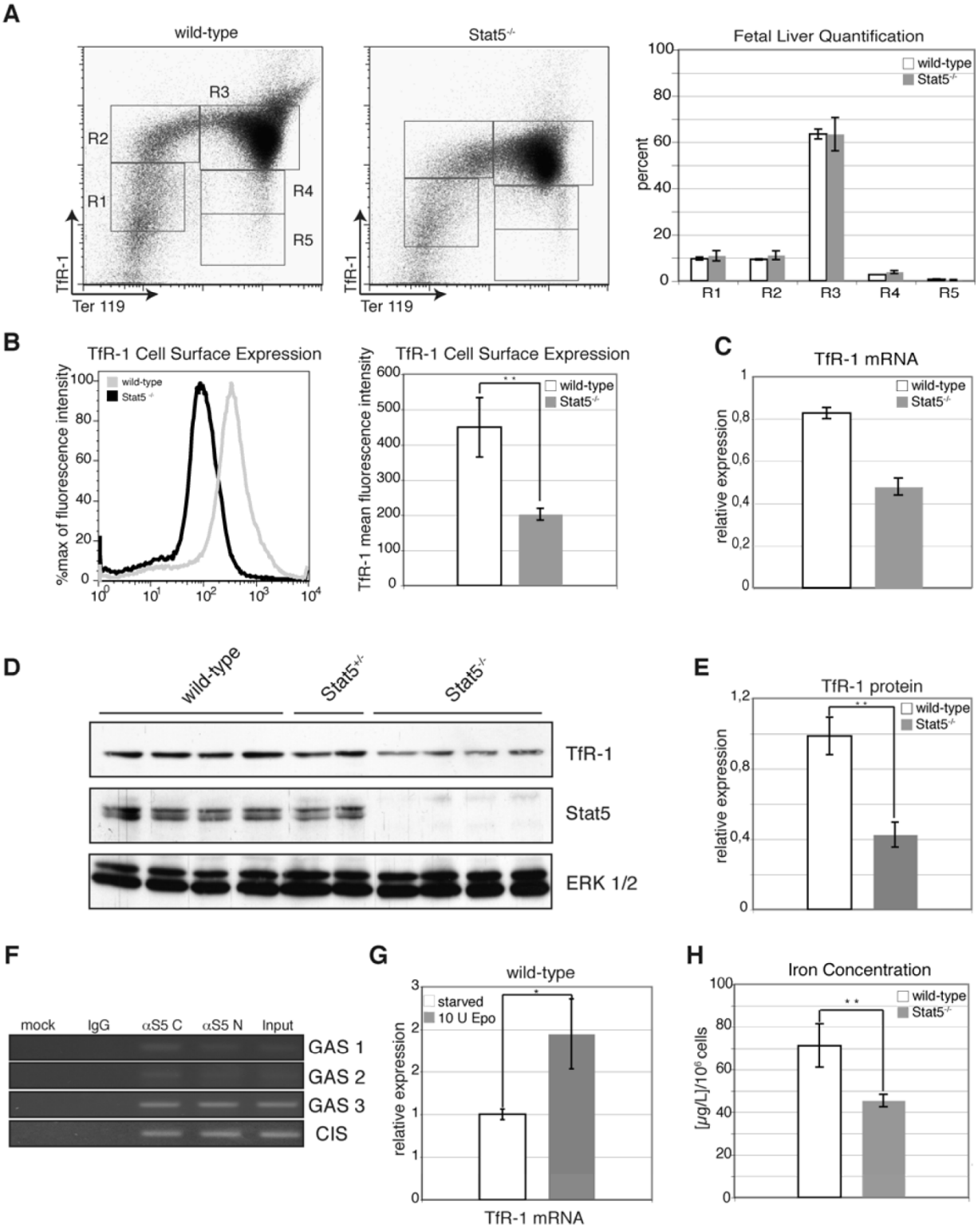
Kerenyi_Fig. 2

Figure 2. Loss of Stat5 results in increased levels of apoptosis in fetal liver cells. (A) Freshly isolated fetal livers from E13.5-E15.5 were stained for Ter119 and Annexin V to determine rates of apoptosis (data are presented as mean ± SD; n=3) for each genotype and time point. (B) CFU-E colonies derived from wt or Stat5$^{-/-}$ fetal liver cells using the indicated Epo concentrations (data are presented as mean ± SD; n=4). (C) E13.5 fetal liver cells of wt and Stat5$^{-/-}$ embryos were infected with a retrovirus encoding GFP. TfR-1^{high}/c-Kit^{+}/GFP^{+} cells were isolated by FACS after seven days under self-renewal conditions (cytospin, right panel). (D) 1.5x10^{7} of TfR-1^{high}/c-Kit^{+}/GFP^{+} cells were injected into the tail vein of lethally irradiated mice (950 rads) and Ter119^{+}/GFP^{+} bone marrow cells scored three days later (mean ± SD; n=4).



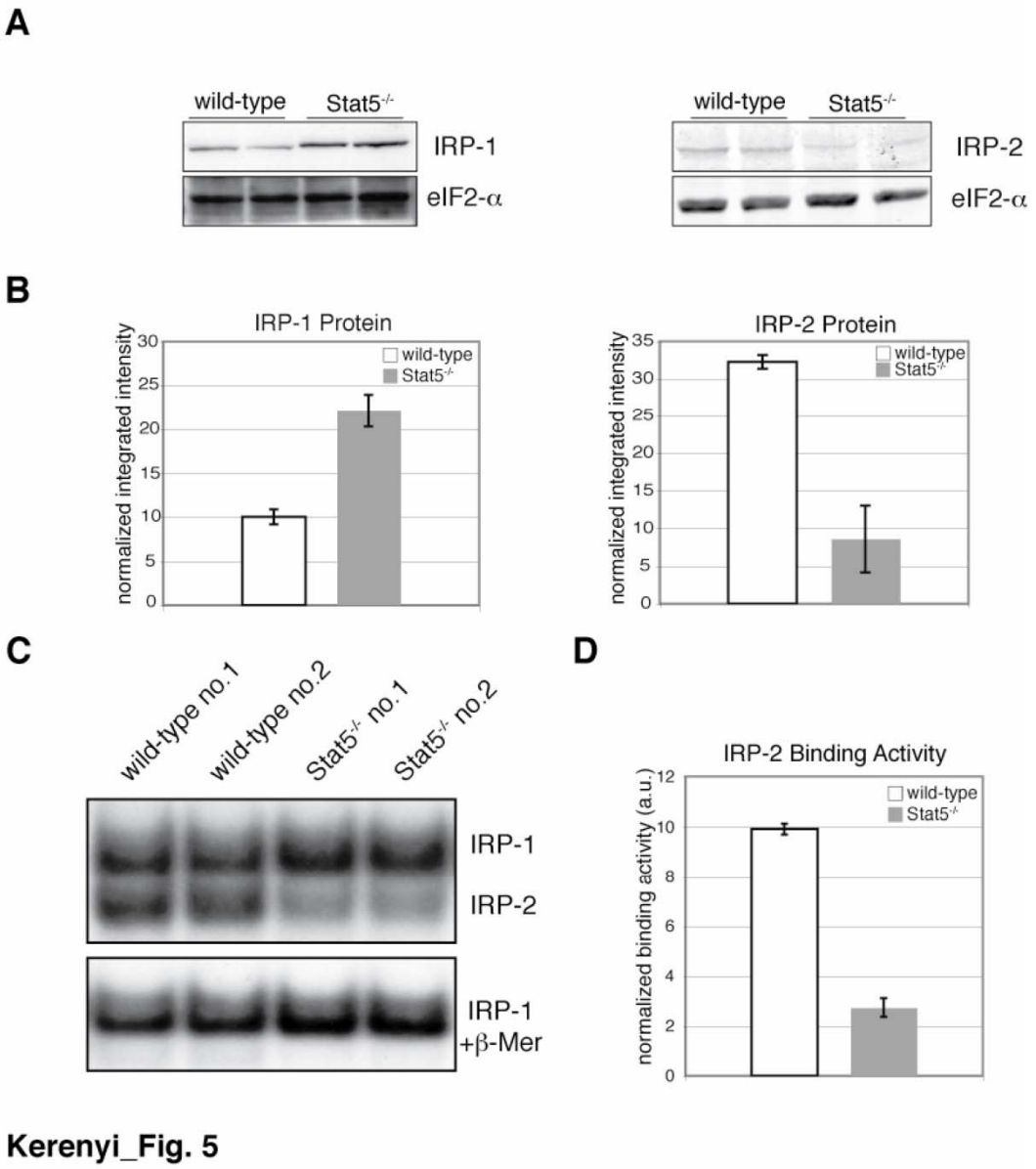
Kerenyi_Fig. 3

Figure 3. Loss of the anti-apoptotic protein Mcl1 in Stat5$^{-/-}$ fetal liver. (A) Western blot of lysates from freshly isolated fetal livers or Ter119^{+} and Ter119^{-} sub-fractions (separated using magnetic beads; see Material and Methods) for Bcl-x_L. (left; ERK, loading control). Western blot for Mcl-1 of two individual freshly isolated fetal livers of each genotype (right; eIF4E, loading control). (B) Quantitative PCR analysis for Mcl-1 mRNA isolated from the Ter119^{+} sub-fraction of freshly isolated fetal liver cells (data are presented as mean ± SD; n=3) (C) Wt and Stat5$^{-/-}$ primary erythroblasts expanded for 5 days under self renewal conditions (see Material and Methods) were deprived of factors for 3h followed by a 30min re-stimulation with 10U/mL Epo. qPCR analysis for Mcl-1 (representative experiment; error-bars are SD of experimental triplicates) (D) Wt or Stat5$^{-/-}$ fetal liver cells were infected with retroviruses encoding GFP alone (MSCV), or Bcl-x_L plus GFP, or Mcl-1 plus GFP from bi-cistronic constructs. After retroviral infection (72h), primary erythroblasts were cultivated for another 48h under self-renewal conditions (see Methods) in the presence or absence of Epo. Rates of apoptosis were determined by flow cytometry for Annexin V. One representative set of histograms from three independently performed experiments of GFP-gated Annexin V positive cells at 48 hours of treatment is depicted. Arrowheads indicate increased levels of apoptosis.



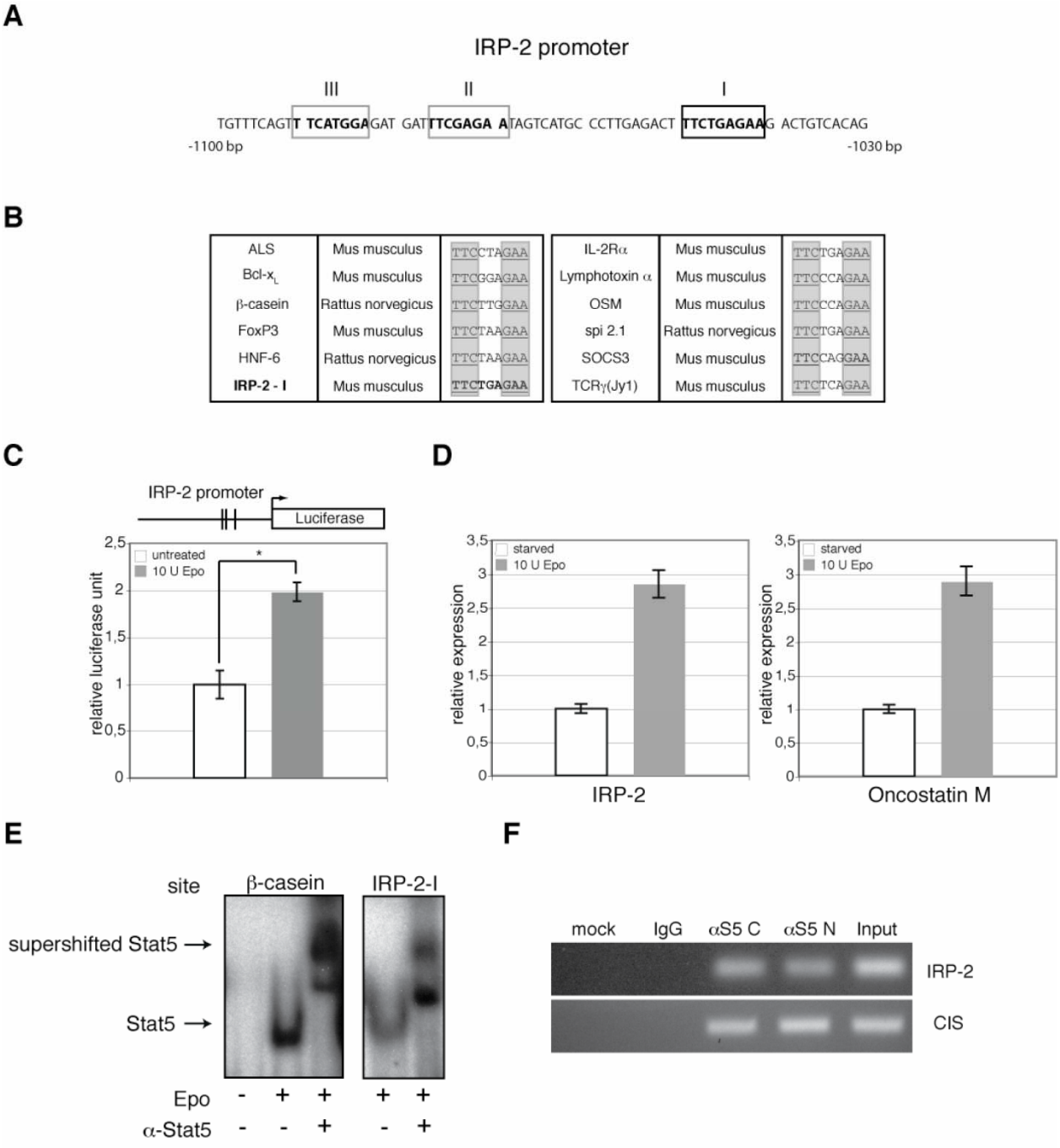
Kerenyi_Fig. 4

Figure 4. Cell surface expression of TfR-1 is strongly reduced in Stat5$^{-/-}$ erythroid progenitors. (A) Representative flow cytometry histograms of E13.5 wt and Stat5$^{-/-}$ fetal liver cells stained for the erythroid markers TfR-1 and Ter119 (left). The sequence from gate R1 (TfR-1^{low} Ter119^{low}) to gate R5 (TfR-1^{-} Ter119^{high}) represents development from the most immature erythroid progenitors (late BFU-E; CFU-E) to mature erythroid cells (orthochromatic erythroblasts; reticulocytes)[58]. Quantification of gates R1-R5 (data are presented as mean ± SD; n=4) (right). (B) Cell surface expression of TfR-1 of Terr119^{high} gated wt (blue line) or Stat5$^{-/-}$ (red line) fetal liver cells (left). Quantification of TfR-1 cell surface expression of wt and Stat5$^{-/-}$ fetal livers (right; data are presented as mean ± SD; n=4). (C) Expression of TfR-1 mRNA from lysates of freshly isolated wt or Stat5$^{-/-}$ fetal liver cells (data are presented as mean ± SD; n=3). Expression was normalized on HPRT levels. (D) Western blot analysis of freshly isolated wt and Stat5$^{-/-}$ fetal liver cell lysates for TfR-1. ERK was used as loading control. (E) Densitometric quantification of TfR-1 Western blot in 3D. (F) Primary wt fetal liver erythroblasts were stimulated with Epo for 30min and ChIP for Stat5 was performed. DNA from Epo-stimulated primary wt erythroblasts was recovered using two different anti-sera directed against N- or C-terminal epitopes (alphaS5 C, alphaS5 N). Specific PCR products from Stat5-binding sites GAS 1, GAS 2, and GAS 3 in *TfR-1* intron 1[59] were only obtained with Stat5-specific antibodies but not with control IgGs. PCR for the genuine Stat5 site in the *CIS* promoter was used as positive control. (G) Primary wt fetal liver-derived erythroblasts were factor depleted for 2.5h followed by a 1.5h of Epo stimulation (10U/mL). TfR-1 mRNA expression was scored by qPCR normalized on HPRT (data are presented as mean ± SD; n=4). (H) Iron concentration in freshly isolated fetal liver lysates determined via atomic absorption spectrometry (data are presented as mean ± SD; n=4; see Material and Methods).



Kerenyi_Fig. 5

Figure 5. Stat5-deficient erythroid cells display reduced *IRP-2* expression and mRNA binding activity. (A) Western blot analysis of primary wt and Stat5$^{-/-}$ erythroblast lysates for IRP-1 (left) and IRP-2 (right) (B) Quantification of Western blot analysis from (A) (data are presented as mean ± SD). Samples were normalized on eIF4E as levels and quantified using the Odyssey infrared imaging system. (C) Two representative lysates each, of wt and Stat5$^{-/-}$ erythroblasts, were subjected to RNA-EMSAs for IRP-1 and IRP-2 using an IRE-RNA probe corresponding to the IRE of mouse ferritin heavy chain[85] (for experimental details see Supplementary Methods). (D) Quantification of IRP-2 binding activity (data are presented as mean ± SD; n=4).



Kerenyi_ Fig. 6

Figure 6. Loss of Stat5 directly decreases IRP-2 gene expression. (A) Sequence of the IRP-2 promoter -1030bp to –1100bp upstream of the transcription start, showing one perfect GAS site (boxed in black) and two GAS sites with one mismatch (grey) (B) Multiple perfect Stat5 sites taken from[26,64], together with the IRP-2-I. (C) Luciferase reporter assay using a DNA fragment ranging from 2kb immediately upstream of the predicted IRP-2 transcription start site. Vertical lines indicate the approximate positions of the putative Stat5 response elements. 293T cells were co-transfected with constructs encoding IRP-2-firefly-luciferase, renilla-luciferase, Stat5a and EpoR. Cells were treated with 10U/mL Epo or left untreated, and luminescence scored 3h later. Transfection efficiencies were normalized to renilla-luciferase activity. (D) Epo-dependent induction of endogenous *IRP-2* and *oncostatin M* analyzed via quantitative PCR in murine erythroid leukemia cells serum deprived for 3h followed by stimulation with 10U/mL Epo (1h). Quantitative PCR was normalized on HPRT. (E) 293T cells were co-transfected with constructs for EpoR and wt Stat5a, followed by 30min stimulation with 10U Epo/mL. Whole cell extracts of these cells were subjected to EMSAs using either the IRP-2-I oligonucleotide (left), or β-casein oligonucleotide as positive control (right). Respective arrows indicate Stat5 DNA complexes and Stat5 DNA complex super-shifts. (F) Primary wt fetal liver erythroblasts were stimulated with Epo for 30min and ChIP for Stat5 was performed. Recovered DNA was analyzed for the presence of promoters of IRP-2 and CIS (positive control) by PCR.

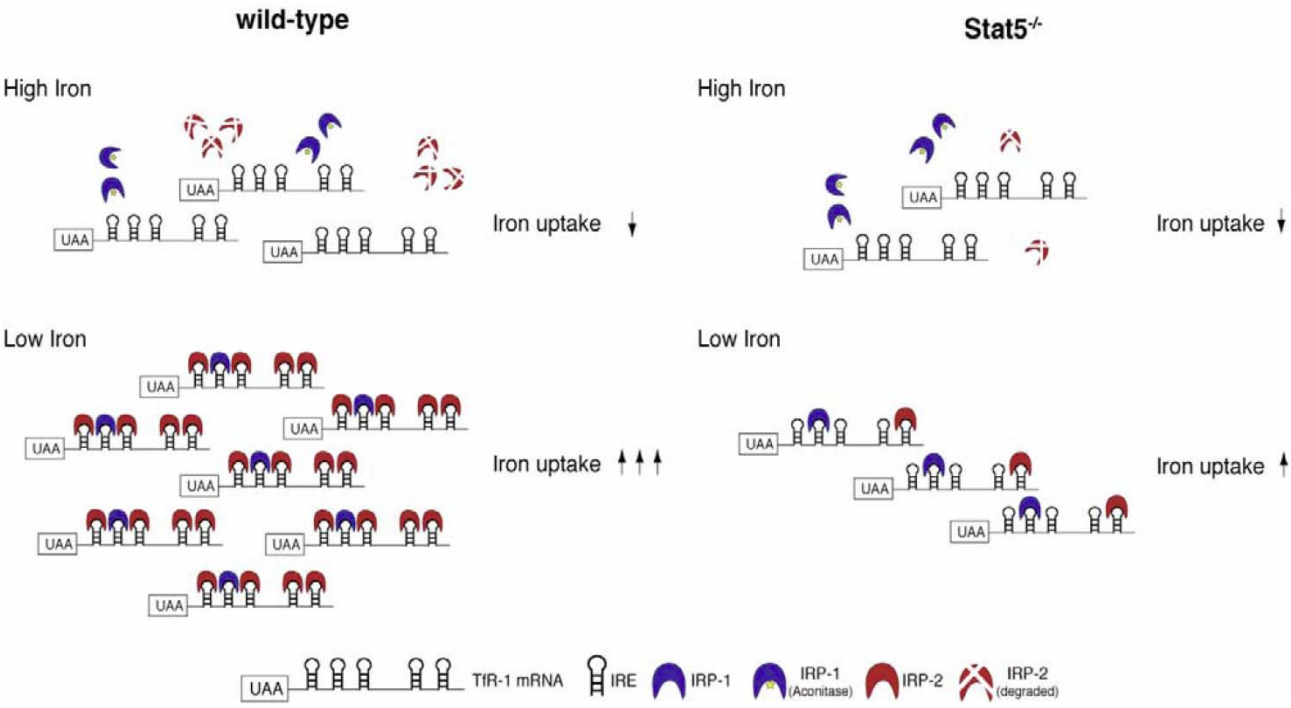


Figure 7. Model for involvement of Stat5 in iron uptake. (wild-type) In iron replete cells, IRP-1 is converted into cytosolic aconitase (catalyzes isomerization of citrate to isocitrate in the citric acid cycle and exhibits no mRNA binding affinity; yellow asterisk) and IRP-2 is degraded. Therefore both cannot bind to IREs in the 3´UTR of TfR-1 mRNA. Free unprotected IREs in turn enhance degradation rates of TfR-1 mRNA, resulting in reduced iron uptake. In iron depleted cells, IRP-1+2 bind to the respective IREs, thereby stabilizing TfR-1 mRNA, resulting in increased iron uptake. (Stat5$^{-/-}$) Due to lack of Stat5, basal TfR-1 transcript abundance is reduced in comparison to wild type cells. In addition, Stat5 deficiency further results in decreased levels of IRP-2 and, in consequence, a reduction of binding to IREs in the 3´UTR of TfR-1 mRNA and decreased transcript stabilization. Together, this constitutes a double-negative effect on erythroid iron uptake even in a situation of high iron demand, as in iron deficiency anemia.

Consequences of Stat5 knockout for heme biosynthesis

Recently it was described that *Stat5* deficient mice die during gestation or at latest perinatally due to severe hypochromic microcytic anemia (Kerenyi, Grebien et al. 2008). This phenotype could be traced to the combined effects of increased apoptosis and decreased iron uptake into erythroid cells. Lack of heme in erythroid differentiation is accompanied by phosphorylation of the translation initiation factor eIF2α via heme-regulated inhibitor (HRI) (Chen 2007). The resulting block in translation hinders further globin synthesis and buys time for heme synthesis to restore equilibrium. Although eIF2α can also be phosphorylated by other integrated stress response kinases, like the double-stranded RNA responsive kinase PKR, it can be assumed that eIF2α phosphorylation in erythroid differentiation is mainly caused by HRI activity. Phosphorylated eIF2α can therefore serve as indirect evidence for insufficient heme levels in erythroid cells. Cultured *Stat5*$^{-/-}$ erythroblasts indeed displayed increased levels of phosphorylated eIF2α (Figure 1) consistent with the reported iron deficiency in those cells.

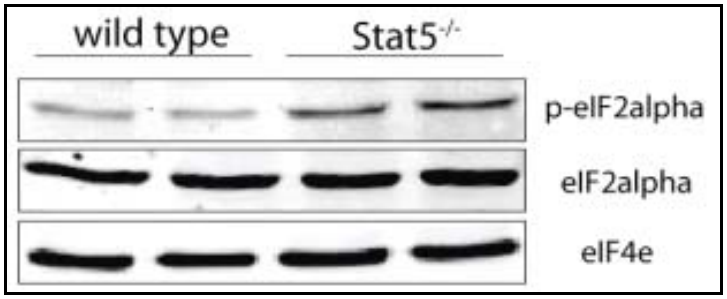


Figure 1: Increased eIF2α phosphorylation in *Stat5*$^{-/-}$ erythroblasts.

Although reduced iron levels do affect heme biosynthesis due to limited availability for incorporation into protoporphyrin IX, one cannot rule out direct or indirect transcriptional consequences on enzyme levels caused by absence of STAT5. This possibility was corroborated by the observation that hemoglobin synthesis during in vitro differentiation of *Stat5*$^{-/-}$ erythroblast could not be rescued by the addition of ferric ammonium citrate (data not shown), which increases cellular iron concentration bypassing the TF/TFR1 pathway (Goto, Paterson et al. 1983; Kaplan, Jordan et al. 1991).

Materials and Methods

Erythroblast cell culture, stimulation and differentiation

Stat5$^{+/-}$ mice (Cui, Riedlinger et al. 2004) were bred on a mixed background (C57Bl/6xSv129F1) to obtain *Stat5*$^{-/-}$ embryos. Fetal livers of E13.5 mouse embryos (*Stat5*$^{-/-}$ and wild type) were isolated and resuspended in serum-free medium (StemPro-34^{TM}, Invitrogen). For self-renewal (Grebien, Kerenyi et al. 2008) cells were seeded into medium containing 2U/ml human recombinant Epo (Erypo, Cilag AG), murine recombinant stem cell factor (SCF; 100ng/mL, R&D Systems), 10^{-6}M dexamethasone (Dex, Sigma) and insulin-like growth factor 1 (IGF1; 40 ng/ml, Promega). The resulting erythroblast cultures were expanded by daily partial medium changes and addition of fresh factors, keeping cell densities between 2-4 x 10^6 cells/ml. Proliferation kinetics and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System). EPO stimulation was done by starving erythroblasts (8 days in culture) for 3 hours in serum-free medium without additional factors and stimulation for 30 min to 2 hours with 10 U/ml Epo. For differentiation induction continuously proliferating erythroblasts were seeded at 2–3 × 10^6 cells/ml in differentiation medium containing StemPro-34TM with 10 U/ml Epo, Insulin (4 × 10^{-4} IE/ml, Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark), the Dex antagonist ZK-112993 (3 × 10^{-6} M), and iron-saturated human transferrin (Sigma) at 1mg/ml. Differentiating erythroblasts were maintained at densities between 2-4 × 10^6 cells/ml. For photometric hemoglobin determination 50 µl aliquots were removed from the cultures and analyzed as described earlier (Kowenz, Leutz et al. 1987). The values are the average of triplicate measurements after normalization for cell number and volume.

Quantitative real time PCR

RNA was isolated using RNeasy Mini Kit (Quiagen) with on column DNA digestion. RNA integrity was checked with the a Bioanalyzer (Agilent). 2.5 µg RNA were reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Quantitative real time PCR was performed on a Mastercycler RealPlex (Eppendorf) using RealMasterMix (Eppendorf) and SYBR Green. Results were quantified using the "Delta Delta C(T) method" (Livak and Schmittgen 2001) standardized on *Hprt* expression.

Gene	Forward Primer	Reverse Primer
Alas2	TTCAGACACAATGACCCAGGC	CCACATCACACAATTCCTCCAG
Alad	CTACTTTCACCCACTGCTTCGG	GGCTGGACATCATCAGGAACA
Ap1	TGAAAGCGCAAAACTCCGAG	GCACCCACTGTTAACGTGGTTC
bKlf	AGCCTTGTTGCAAGAGAACCA	TGCACCCATCATAGTCGCAT
Brg1	CCAAACTCCGTGCAACGAA	CTGCTTTTGTGGTTCCATCAAG
cMyb	TCCTCAAAGCCTTTACCGTACC	CCGTCATCTGGTCCTCTGTCTT
Cp1	TAGCCGACGAAGTGATCGAGTC	GCAATGCGAGGACATCACTCA
Cpo	ATGCTGTCCATTTCCACCGTAC	CCCCGCGGTGAACTATAAAGA
E2a	ACTTCAGCATGATGTTCCCGCT	ACCTTCGCTGTATGTCCGGCTA
Eklf	GAACTTTGGCACCTAAGAGGCA	AGGAGCAGGCATAAGGCTTCTC
Elf1	TTGCAAGTAACGGCATGGAG	TCCACACAAGCTAGACCAGCAT
Ets1	GCCGACTCTCACCATCATCAA	CATTTCTTTGCTGCTCGGAGTT
FeCh	CCACAGTATAGCTGCTCCACCA	TGTCGATTGTGCTCCACTTCA
Fli1	CAGACCAGTCCTCACGACTGAA	GGAGAGGACTTTTGTTGAGGCC
Fog1	ATCCCCTGAGAGAGAAGAACCG	GACAAGGCGCACATATAGCAGT
Gata1	TGAACTGTGGAGCAACGGCTA	GCCCGTTTGCTGACAATCAT
Gata2	AGATGAATGGACAGAACCGGC	CTTCTTCATGGTCAGTGGCCTG
Hprt	TGATTAGCGATGATGAACCAGG	CCTTCATGACATCTCGAGCAAG
Ldb1	CAAACGGCTACAGAACTGGACA	GGTCCGGCCAATGGTATATCT
Lmo2	ACGGAAATTGTGCAGGAGAGAC	ACCCGCATCGTCATCTCATAG
Nfe1	ACCTGGCATTGACCTCTCAGA	TCCCTGAACATCTTTGTGCAG
Nfe2	GAACAGGTTATCACAGCTGCCT	CTTGGAACATTTAGACCCTGCA
Pbgd	CCTGGAAAAAAACGAAGTGGAC	GGTTTTCCCGTTTGCAGATG
Ppo	GGATTGGCCGCAAGTTATCAT	TCGGATTGAGCGGATCCAT
Pu.1	AGGCGTGCAAAATGGAAGG	CCCAGTAGTGATCGCTATGGCT
Runx1	CAGGTAGCGAGATTCAACGACC	GCAACTTGTGGCGGATTTGT
Scl	GATGCCTTCCCCATGTTCA	ATTCACATTCTGCTGCCGC
Sp1	CAGGCCTCCAGACCATTAACCT	CCATCACCACCAGATCCATGA
Sp3	GTAGCTTGCACCTGTCCCAACT	GCAGGTGTGCTCTCAGATGTGA
Uro3s	CTGCGATTGTGTGGCCTAGAA	TCCTCCAAAGCCTTCAGGATG
Urod	TGGACAGTGGCTCCAAAGAAA	CCGATCTCTTCCTCAGATGCAT

Table 1: qPCR primer pairs

Western blot

Antibodies used for Western blotting: anti-mouse ERK1/2 (Sigma M5670), anti-mouse ALAS2 (Santa Cruz sc-32334)

Statistical analysis

Statistical analysis was performed using Excel2007 (Microsoft). Data are presented as mean values ± standard deviation.

Results

Expression of ALAS2 is downregulated in Stat5 deficient erythroblasts

Eight enzymatic steps lead to the generation of heme from four molecules of glycine and succinyl-CoA (Table 2). The most important enzyme is δ-aminolevulinic acid synthase (ALAS). It catalyzes the rate limiting step and is therefore subject to dynamic transcriptional and posttranscriptional regulation. The erythroid-specific *Alas2* gene is regulated by a variety of erythroid transcription factors (e.g. GATA1) (Srivastava, Borthwick et al. 1988) and posttranscriptionally controlled via IRPs binding to the single IRE in the 5' UTR of its mRNA (Wingert, Galloway et al. 2005). Since levels of IRP2, the major IRP in erythroid cells (Cooperman, Meyron-Holtz et al. 2005), are reduced in *Stat5*-deficient erythroblasts, one would anticipate an increase in ALAS2 expression in such cells. Unexpectedly cultured fetal *Stat5* knockout erythroblasts as well as whole fetal liver extracts exhibited severely reduced levels of *Alas2* mRNA and protein (Figure 2). This helps to explain the strongly reduced hemoglobinization of *Stat5*$^{-/-}$ erythroblasts during *in vitro* differentiation and may contribute to the severe hypochromic microcytic anemia of *Stat5*$^{-/-}$ embryos. The observed changes in *Alas2* mRNA and protein levels might indicate that STAT5 has a direct or indirect effect on transcription of the *Alas2* gene.

abbr.	short name	full name
ALAS2	ALA synthase	δ-aminolevulinate Synthase
ALAD	ALA dehydratase	Porphobilinogen Synthase
PBGD	PBG deaminase	Porphobilinogen Deaminase
URO3S	Uro III synthase	Uroporphyrinogen III Synthase
UROD	Uro III decarboxylase	Uroporphyrinogen Decarboxylase
CPO	Copro III oxidase	Coproporphyrinogen Oxidase
PPO	Proto IX oxidase	Protoporphyrinogen Oxidase
FeCH	Ferrochelatase	Ferrochelatase

Table 2: Abbrevation, short and full names of the eight enzymes in the heme biosynthetic pathway.

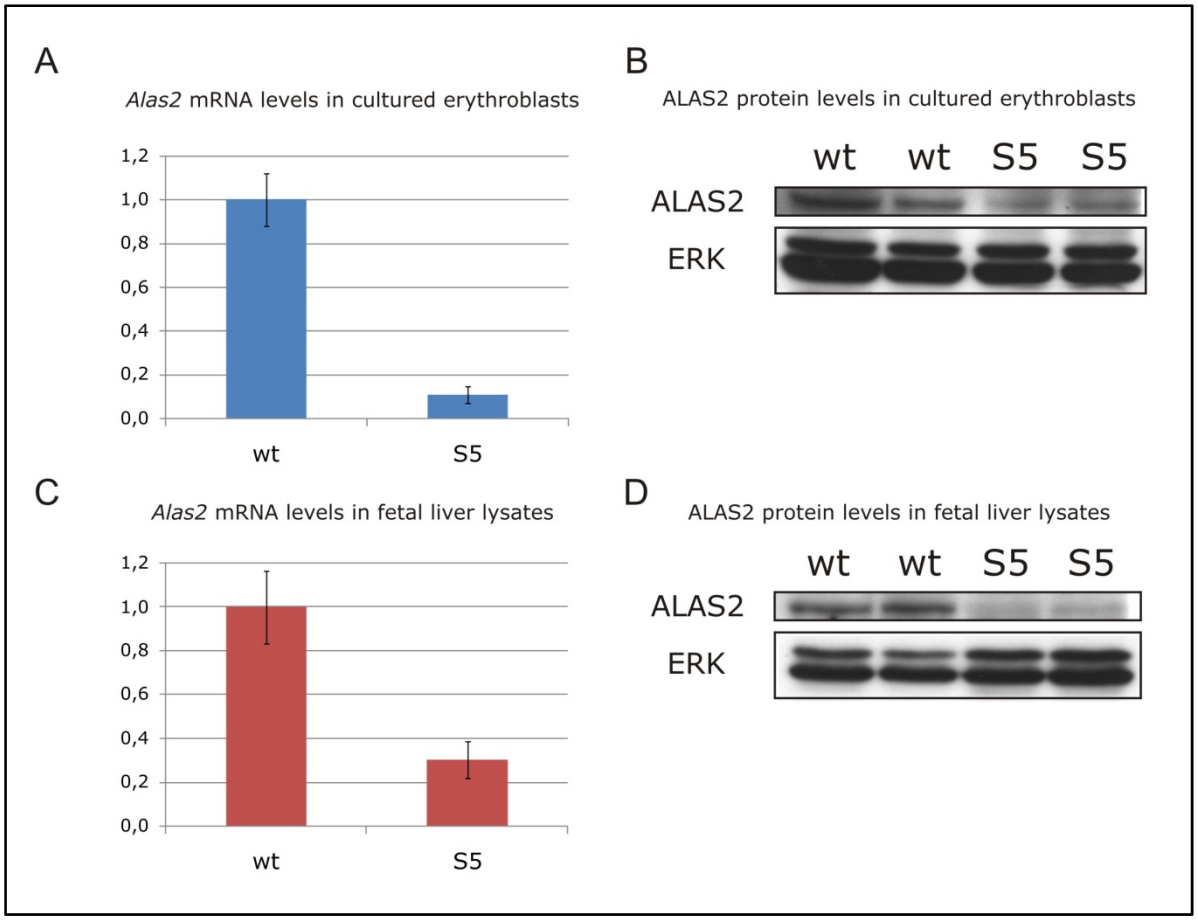


Figure 2: Alas2 expression in wt and *Stat5*$^{-/-}$ (S5) cultured erythroblasts and whole fetal liver extracts. A) *Alas2* mRNA levels in cultured erythroblasts. B) ALAS2 protein abundance in cultured erytrhoblasts. C) *Alas2* mRNA expression in fetal liver extracts. D) ALAS2 protein amounts in fetal liver extracts. *Alas2* is downregulated on mRNA and protein level in *Stat5*$^{-/-}$ cells both *in vivo* and *in vitro*.

Bypassing ALAS2 does not rescue in vitro differentiation of Stat5$^{-/-}$ *erythroblasts*

ALAS2 catalyzes the condensation of glycine and succinyl-CoA into 5-aminolevulinic acid (ALA). If soley reduced ALAS2 expression was responsible for lack of hemoglobinization in *Stat5*$^{-/-}$ erythroblasts during *in vitro* differentiation, the effect should be mitigated by direct supply of the reaction product ALA to the cells. No differences could be seen in differentiation with or without exogenous ALA addition (Figure 3) demonstrating that loss of *Stat5* must have further detrimental effects on hemoglobin production.

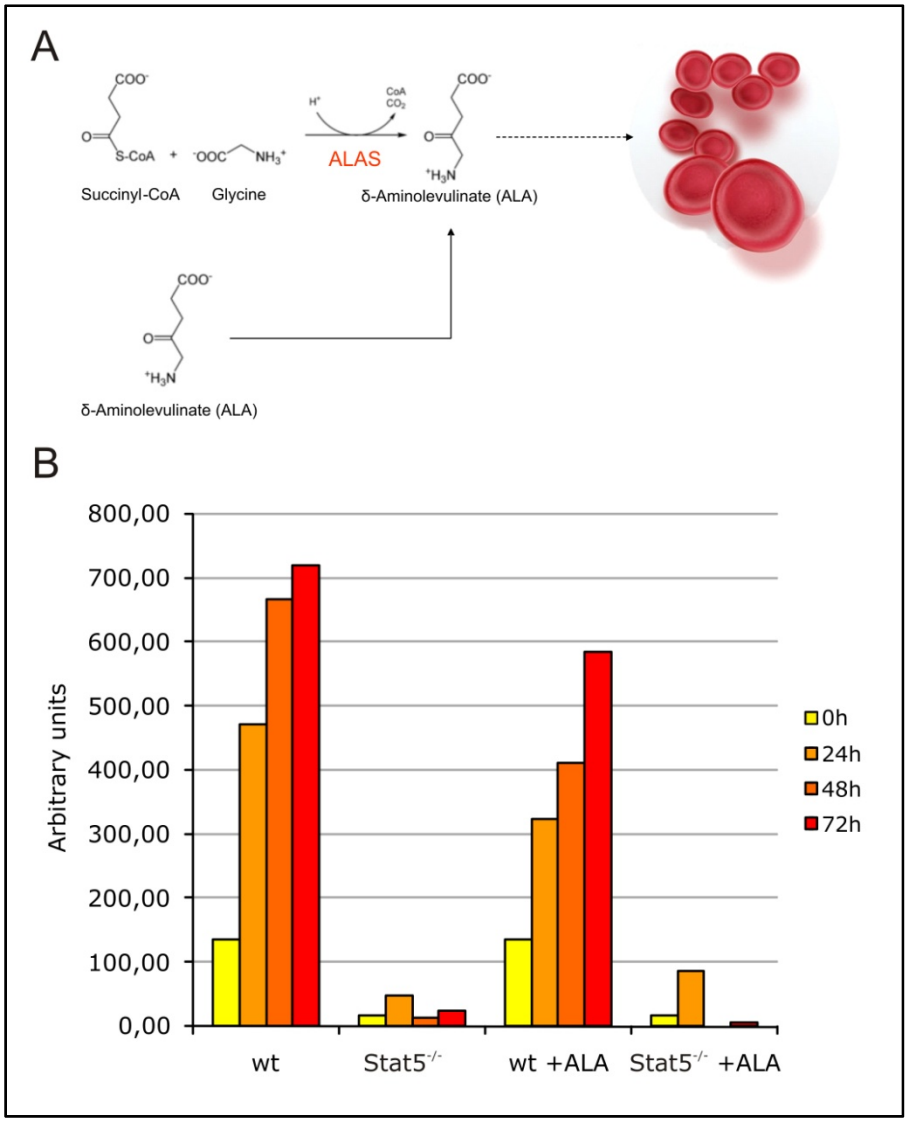


Figure 3: A) Bypassing ALAS via supplementation of δ-aminolevulinate. B) Photometric heme assay with our without ALA supplementation. *Stat5*$^{-/-}$ erythroblasts completely fail to hemoglobinize with or without addition of δ-aminolevulinate.

Reduced expression of heme biosynthetic enzymes in cultured Stat5$^{-/-}$ *erythroblasts*

Since circumvention of ALAS2 did not rescue the defective *in vitro* differentiation of *Stat5* deficient erythroblasts, relative expression levels of all other enzymes of the heme biosynthetic pathway were determined in cultured erythroblasts. *Alas2*, *Uro3s*, *Cpo* and *FeCh* exhibited significantly reduced mRNA abundance in cultured *Stat5*$^{-/-}$ erythroblasts in comparison to wt cells (Figure 4A). Besides Alas2, FeCh is probably the most interesting gene amongst those found to be down regulated. Although it is not rate limiting for heme biosynthesis, it has been shown to be regulated both at the transcriptional level (via e.g. GATA1, HIF1, SP1) (Taketani, Inazawa et al. 1992), but more importantly at the posttranslational level (by the availability of Fe-S clusters) (Burden, Wu et al. 1999; Taketani, Adachi et al. 2000). These regulation mechanisms suggest that the rate of

heme synthesis in erythroid cells does not only depend on ALAS2, but also on an iron-related control over FECH activity.

Reduced expression of heme biosynthetic enzymes in d13.5 Stat5$^{-/-}$ fetal livers

To exclude that reduction of heme biosynthetic enzyme expression in cultured *Stat5*$^{-/-}$ erythroblasts was a cell culture artifact, a similar set of experiments was conducted with RNA freshly isolated from whole fetal liver lysates (Figure 4B). The same four enzymes as outlined above showed significant reduction in mRNA levels corroborating the initial observations.

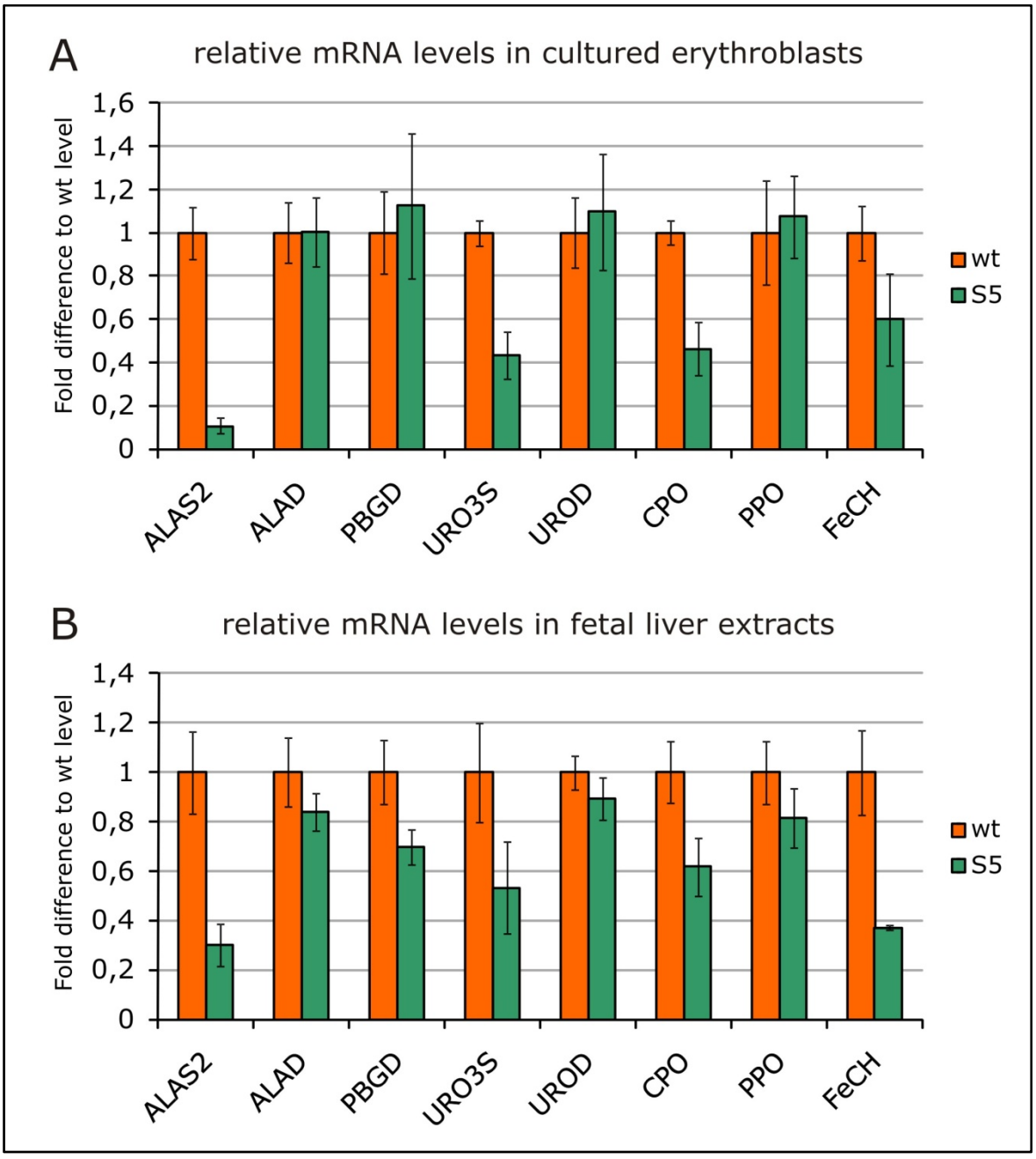


Figure 4: mRNA levels of heme biosynthetic enzymes in A) cultured erythroblasts and B) fetal liver extracts. *Alas2*, *Uro3s*, *Cpo* and *FeCh* are downregulated both *in vitro* and *in vivo*. ALAS2 = δ-aminolevulinate synthase, ALAD = porphobilinogen synthase, PBGD = porphobilinogen deaminase, URO3S = uroporphyrinogen III synthase, UROD = uroporphyrinogen decarboxylase, CPO = coproporphyrinogen oxidase, PPO = protoporphyrinogen oxidase, FeCH = ferrochelatase

mRNA levels of ALAS2, URO3S, CPO and FeCH are elevated upon Epo stimulation

To clarify if the enzymes with reduced expression in STAT5-deficient erythroblasts are targets of erythropoietin (Epo) signaling, cultured erythroblasts were kept in plain medium for 3 hours to blunt growth factor-signaling and subsequently stimulated with EPO for 2 hours (Figure 5). All enzymes tested exhibited a significant increase in mRNA upon stimulation. *Cpo* and *FeCh* were clearly upregulated by EPO in wt, but only slightly in *Stat5*$^{-/-}$ cells. Interestingly this was not the case for *Alas2* and *Uro3s*, where a prominent stimulation was observed both in wt and Stat5$^{-/-}$ erythroblasts, albeit starting from a very low level in the knockout cells. It is well known that EPO activates several pathways including MAPK- and PI3K-signaling (Richmond, Chohan et al. 2005), which may provide the transcriptional signals in this case. Even though all four affected enzymes displayed EPO responsiveness, the results from stimulation of Stat5$^{-/-}$ erythroblasts demonstrated that the reduced mRNA levels of all four enzymes cannot be attributed to the absence of STAT5 as possible direct transcription factor alone.

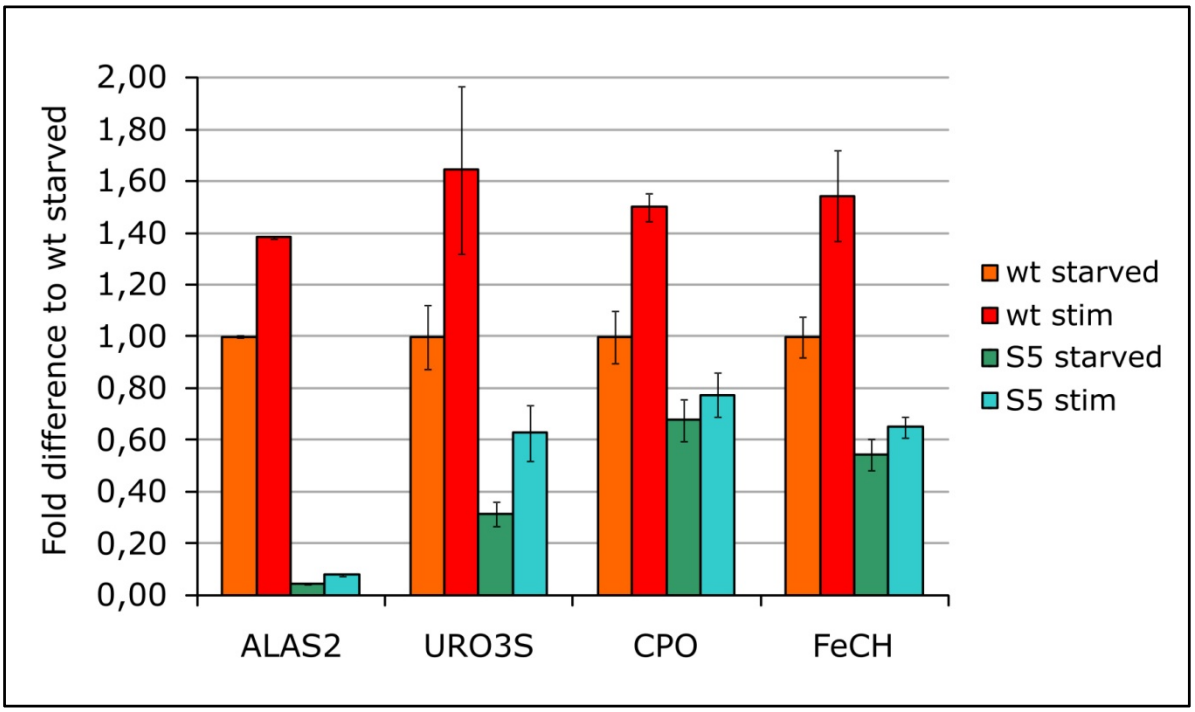


Figure 5: Changes in mRNA levels in cultured erythroblasts of four heme biosynthetic enzymes upon starvation and EPO stimulation. All four genes can be stimulated by addition of EPO in the wt situation, but *Cpo* and *FeCh* seem to lose their EPO responsiveness under *Stat5* deficiency (S5).

Promoter analysis of ALAS2, URO3S, CPO and FeCH

The promoter sequences of all four enzymes were screened for possible STAT5 binding sites (consensus sequence: TTCYNRGAA (Ehret, Reichenbach et al. 2001)) (Table 3). With exception of a single STAT5 binding site in the *Cpo* promoter there were no perfect matches in the tested regions. *Alas2* and *Uro3s* have several single mismatch STAT5 binding sites that may enable direct regulation by STAT5, but due to absence of perfect sites this is rather improbable. Therefore transcriptional downregulation of all four enzymes in *Stat5*$^{-/-}$ erythroblast is unlikely to be caused by loss of STAT5 as direct regulator alone. Promoters of human *ALAS2*, *URO3S*, *CPO* and *FeCH* have already been analyzed and the results published (Taketani, Inazawa et al. 1992; Surinya, Cox et al. 1997; Takahashi, Taketani et al. 1998; Aizencang, Bishop et al. 2000). The erythroid promoters contain binding sites for a multitude of transcription factors (Table 4), with the most prominent one being GATA1, which is present in promoters of all four genes.

	ALAS2	URO3S	CPO	FeCH
Perfect Stat5 binding site	-	-	1	-
single mismatch Stat5 binding site	4	3	1	-

Table 3: Perfect and single mismatch Stat5 binding sites in heme biosynthetic enzyme promoters

	ALAS2	URO3S	CPO	FeCH
AP1	-	2	-	-
CACCC	1	1	4	-
CCAAT	1	1	-	-
E47	-	2	-	-
Ets	1	-	-	-
GATA1	2	8	12	1
IL6RE	-	-	-	1
NFE2	1	1	-	1
SP1 like	-	-	1	4
vMYB	-	1	-	-

Table 4: Transcription factor binding sites in promoters of heme biosynthetic enzymes

GATA1 levels are unchanged in wt and Stat5$^{-/-}$ erythroblasts

As GATA1 was the most promising candidate to be affected by loss of *Stat5*, the abundance of its mRNA was analyzed in cultured wt and *Stat5*$^{-/-}$ erythroblasts (Figure 6). There was, however, no difference in mRNA amounts, ruling out decreased GATA1 levels as being responsible for reduced expression of heme biosynthesis enzymes in the *Stat5* knockout situation.

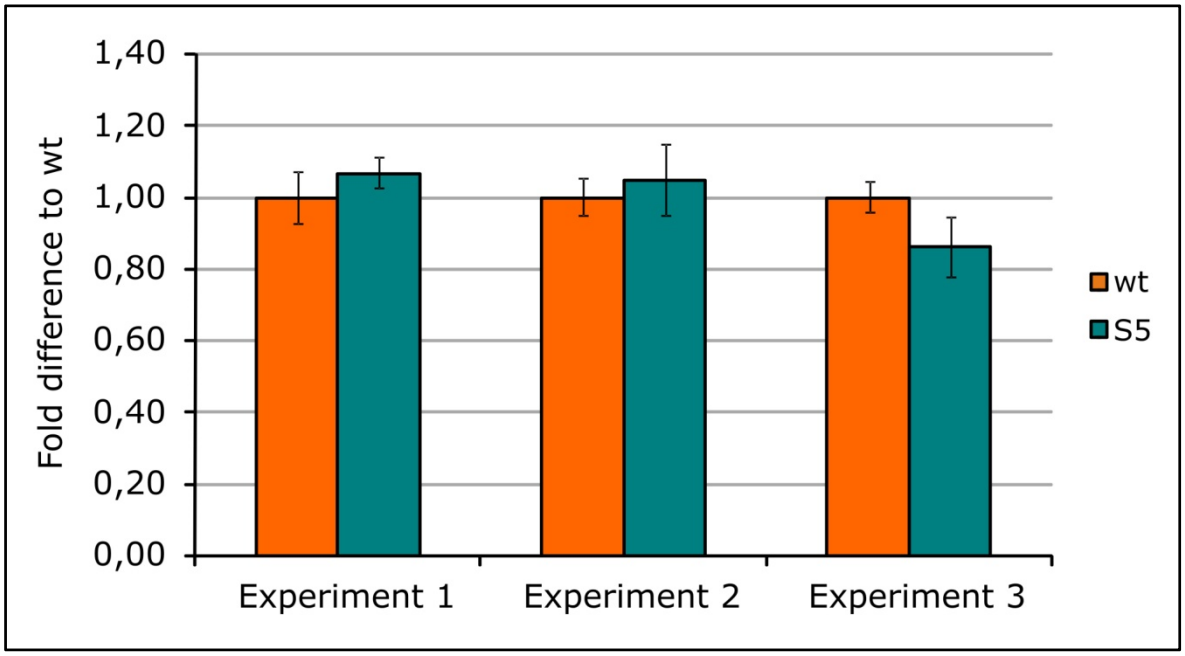


Figure 6: *Gata1* mRNA levels in cultured wt and *Stat5*$^{-/-}$ (S5) erythroblasts

Changes in the transcriptional network of Stat5$^{-/-}$ erythroblasts

Since all four of the enzymes downregulated by STAT5 deficiency have binding sites for a multitude of different transcription factors, mRNA levels of other known hematopoietic transcription factors were analyzed by qPCR in cultured wt and *Stat5* knockout erythroblasts to find other candidates that may account for the observed phenotype. Of 21 tested transcription factors 3 were more than 2 fold downregulated due to loss of STAT5. 12 did not show any significant change, but 6 were even massively upregulated (Figure 7 and Figure 8A). At first glance this was unexpected, as STAT5 has always been associated with transcriptional activation rather than repression. On the other hand these changes in transcription factor expression could be indirect effects of *Stat5* deficiency, e.g. caused by loss of STAT5 dependent transcriptional repressors.

PU.1 is downregulated in *Stat5* deficient erythroblasts and is known to be important in decisions between granulocyte versus macrophage lineage commitment. Lower levels of PU.1 should have no detrimental effects on erythropoiesis, since reduced abundance would only further promote development along the erythroid axis (Nerlov and Graf 1998). Likewise there is no known function for *Runx1* in erythropoiesis, but it is necessary for generation of fetal HSCs (Okuda, van Deursen et al. 1996). *SP1* deficiency, however, has been associated with delayed onset of definitive erythropoiesis. Although lack of SP1 may delay erythroid differentiation it does not explain the observed block in *in vitro* differentiation (Kruger, Vollmer et al. 2007).

Among the six upregulated factors three would indeed have known implications on erythropoiesis. *Cp1*, *Ets1* and *E2a* have roles in other hematopoietic lineages, like B- and T-cell development, but so far neither up- nor downregulation has been reported to affect red blood cell generation. *Eklf*, however, is known to promote β-globin expression (Asano and Stamatoyannopoulos 1998). Therefore its upregulation should facilitate erythroid differentiation rather than hampering it, making EKLF overexpression an unlikely explanation for the observed phenotype. *Ldb1* is essential for stem cell maintenance, and its loss causes severely reduced erythropoiesis. Overexpression of LDB1, however, has been shown to hinder erythroid *in vitro* differentiation (Visvader, Mao et al. 1997). It has been suggested that LDB1 is involved in formation of several transcriptional complexes. As LDB1 interacts with GATA1, the differentiation block may be a result of disrupted or altered GATA1 transcriptional complexes.

GATA2, the last transcription factor found to be upregulated, is required in HSCs and hematopoietic progenitors. Interestingly overexpression of GATA2 in proerythroblasts has been linked to enhanced proliferation and blocked differentiation in *in vitro* experiments. This phenotype has been attributed to failure of those cells to undergo the GATA switch properly (Figure 8B), where GATA1 displaces GATA2 from common binding sites in target gene promoters and represses further GATA2 expression.

The high expression of *Ldb1* and *Gata2* mRNA in *Stat5*$^{-/-}$ erythroblasts brings us back to the very first assumption that lack of GATA1 leads to the observed reductions in heme biosynthetic enzyme levels. In fact the data indicate that there is no deficiency in GATA1, but rather a deleterious surplus of LDB1 and GATA2 that may disrupt transcriptional complexes on the one hand and out-titrate GATA1 on chromosomal binding sites on the other, resulting in drastically decreased transcriptional activation of GATA1 target genes.

Figure 7: mRNA levels of hematopoietic transcription factors in wt and *Stat5* deficient (S5) erythroblasts.

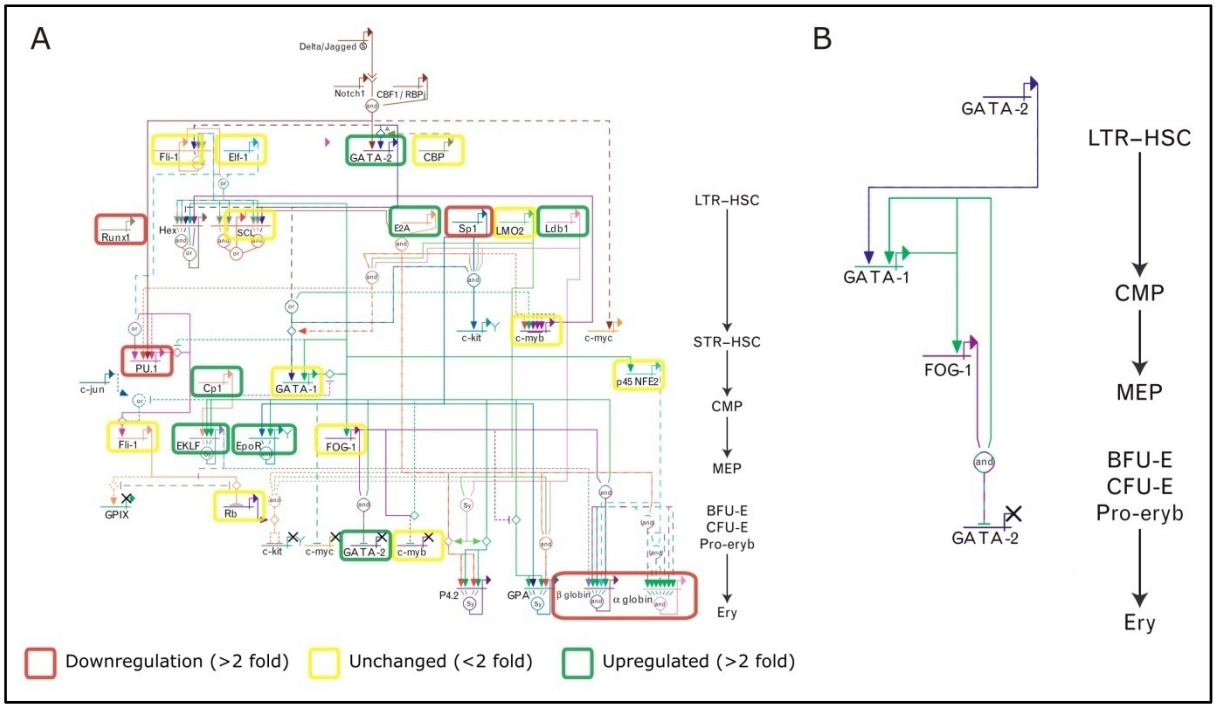


Figure 8: A) Transcriptional network of erythroid differentiation with annotated changes in *Stat5* knockout cells. (adapted from (Loose, Swiers et al. 2007) B) Schematic GATA switch visualization.

Discussion

Stat5$^{-/-}$ mice have been shown to suffer from hypochromic microcytic anemia due to defects in iron uptake (Kerenyi, Grebien et al. 2008). The knockout phenotype is even more severe in *in vitro* differentiation, probably due to the lack of compensatory *in vivo* signals. In culture *Stat5* knockout erythroblasts failed to hemoglobinize during differentiation. This finding cannot be attributed to iron deficiency alone, since supplementation of *Stat5* deficient erythroblasts with additional iron did not rescue the phenotype (data not shown). ALAS2, the rate limiting enzyme of heme biosynthesis, was found downregulated on both mRNA and protein level, but also bypassing the enzyme by adding its reaction product to the cells did not revert the phenotype. Three other heme biosynthetic enzymes, URO3S, CPO and FeCH had significantly reduced mRNA levels in *Stat5*$^{-/-}$ erythroblasts, which might fully explain the failed rescue by ALAS2 circumvention and in combination could aggravate the observed defect in iron utilization. GATA1, practically the most important specific transcription factor in eryrthoid differentiation, is known to stimulate transcription of all four downregulated heme biosynthesis genes. Although GATA1 mRNA levels were unchanged, its ability to promote transcription of target genes and drive erythroid progenitors into differentiation was probably severely hampered by a surplus of LDB1 and GATA2 that was detected in *Stat5* knockout cells.

Even though the current data point to the model outlined above there are still several key experiments to be done. First of all it is not yet known if the elevated *Ldb1*

and *Gata2* mRNA levels carry over to elevated levels of the corresponding protein. This question can be easily addressed by Western blotting with suitable antibodies. Even more important is the question whether knockdown of *Ldb1* and/or *Gata2* in erythroid cells will rescue the *in vitro* differentiation defect of *Stat5* deficient erythroblasts. Finally the mechanism by which loss of STAT5 interferes with *Ldb1* expression and the GATA switch needs to be elucidated. A possible molecular mechanism could involve components of the nucleosome remodeling and histone deacetylase (NuRD) complex. FOG1, an interaction partner of GATA1 essential for GATA switching, has been shown to interact with the NuRD complex to silence target genes (Hong, Nakazawa et al. 2005). It is assumed that binding of GATA1 – FOG1 complexes to the *Gata2* promotor recruits the NuRD complex, which then leads to silencing of the *Gata2* gene and a global change from GATA2 to GATA1 predominance. If components of the NuRD complex were STAT5 targets, their loss would lead to the observed disability to perform GATA switching. A link between *Stat5* and gene-repression potential was further corroborated by the fact that more tested transcription factors were found up- than downregulated upon loss of *Stat5* in erythroid cells. Lack of GATA switching, on the other hand, could also be a secondary effect of increased *Ldb1* expression. GATA1 binds to target sites in complexes with multiple proteins like FOG1, LMO2 and importantly LDB1 (Meier, Krpic et al. 2006). If the complex is in fact disrupted or influenced in its specifity by high levels of LDB1, GATA1 could not exert its repressive effect on the *Gata2* promoter.

Apart from problems in heme biosynthesis additional processes are apparently hampered by the loss of *Stat5* in erythroid *in vitro* maturation. Also globin expression was found to be significantly reduced in *Stat5* deficient cells (data not shown). It is unclear though, if this is direct Stat5 dependent transcriptional effect, an indirect effect due to reduced GATA1 activity or an attempt to balance globin with decreased heme levels.

Compensatory effects of *Stat3* upon loss of *Stat5*

One possible explanation for the milder phenotype of loss of *Stat5* (perinatally lethal) in comparison to *EpoR* (lethal E13.5), *or Epo* (lethal E13.5) knockout (Wu, Liu et al. 1995) would be partial compensation of lacking STAT5 signals by STAT3. STAT5 and STAT3 share highly similar binding sites and elevated STAT3 phosphorylation has in fact been observed in the absence of STAT5 in the liver (Cui, Hosui et al. 2007). It is still unclear, however, which mechanisms cause increased STAT3 phosphorylation and whether the effect is biologically significant.

Materials and methods

Erythroblast cell culture

Stat5$^{+/-}$ mice (Cui, Riedlinger et al. 2004) were bred on a mixed background (C57Bl/6xSv129F1) to obtain *Stat5*$^{-/-}$ embryos. Fetal livers of E13.5 mouse embryos (*Stat5*$^{-/-}$ and wild type) were isolated and resuspended in serum-free medium (StemPro-34TM, Invitrogen). For self-renewal (Grebien, Kerenyi et al. 2008) cells were seeded into medium containing 2U/mL human recombinant EPO (Erypo, Cilag AG), murine recombinant stem cell factor (SCF; 100ng/mL, R&D Systems), 10^{-6}M dexamethasone (Dex, Sigma) and insulin-like growth factor 1 (IGF1; 40ng/mL, Promega). The resulting erythroblast cultures were expanded by daily partial medium changes and addition of fresh factors, keeping cell densities within 2-4x10^6 cells/mL. Proliferation kinetics and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System). EPO stimulation was done by starving erythroblasts (8 days in culture) for 3 hours in serum-free medium without additional factors and stimulation for 30 min to 2 hours with 10 U/ml Epo or 20 ng/ml IL6 (RnD).

Quantitative real time PCR

RNA was isolated using RNeasy Mini Kit (Quiagen) with on column DNA digestion. RNA integrity was checked with the Agilent Bioanalyzer (Agilent). 2.5 µg RNA were reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Real time quantitative PCR (qPCR) was performed on an Eppendorf Mastercycler RealPlex using RealMasterMix (Eppendorf) and SYBR Green. Results were quantified using the "Delta Delta C(T) method" (Livak and Schmittgen 2001) standardized on *Hprt* expression.

Gene	Forward Primer	Reverse Primer
Cis	QuantiTect Mm_Cish_1_SG (Quiagen)	
Socs1	QuantiTect Mm_Socs1_1_SG (Quiagen)	
Socs2	CGAGCTCAGTCAAACAGGATGG	TAGTCGGTCCAGCTGACGTCTT
Socs3	TTTTCTTTGCCACCCACGG	TTCTCGCCCCCAGAATAGATG
Socs4	TCCACACCCAGATCGACTACGT	GCTTCGGCTGCATATTTGTCC
Socs5	CTGAGCCCTTCCAAGACCTTTT	GCACCCGAGTCACTGTCTTTCT
Socs6	CCTTGCTGGTGACTTCGTGAA	TCTTCCCTTTCTCATCCTCGC
Socs7	TGGAAAAGTGCGGCTGGTA	GAGGCTCAGGATGTAACGAGGA
Pias1	CAGTGCGGAACTAAAGCAAATG	AAGAAGTTCGTGTTTGCGTCC
Pias2	CTTTCCTTTGCCTGGCTATGC	TTGGCACAGCTGAAGACAACCT
Pias3	CGTGTACCTGGTGAGGCAATTG	TTTCTCCTTGATCAGTGCCCG
Pias4	GCTGGTGGAGGCCAAAAACAT	TGGTCACCAGTTCGTGCTTCAG
Hprt	TGATTAGCGATGATGAACCAGG	CCTTCATGACATCTCGAGCAAG

Table 1: qPCR primer pairs

Western blot

Antibodies used for Western blotting: phosphorylated STAT5 (Upstate Biotechnology 05-495); STAT5 (BD Transduction Laboratories 610191); phosphorylated STAT3 (Cell Signaling 9131); STAT3 (Santa Cruz sc-7179); actin (Cell signaling 4967)

Statistical analysis

Statistical analysis was performed using Excel2007 (Microsoft). Data are presented as mean values ± standard deviation.

Results

Increased phosphorylation of STAT3 in Stat5 knockout erythroblasts

Both wt and *Stat5* deficient erythroblasts were starved and subsequently stimulated with EPO, as activator of STAT5 dependent signaling, or IL6, a cytokine upstream of STAT3 phosphorylation (Figure 1). Epo stimulation led, as expected, to STAT5 phosphorylation in wt but not in knockout erythroblasts. IL6 stimulation caused, likewise expected, STAT3 phosphorylation in both wt and knockout erythroblasts. Interestingly EPO stimulation also led to increased STAT3 phosphorylation in *Stat5*$^{-/-}$ but not wt erythroblasts, which hints to a potential compensatory effect in the knockout situation.

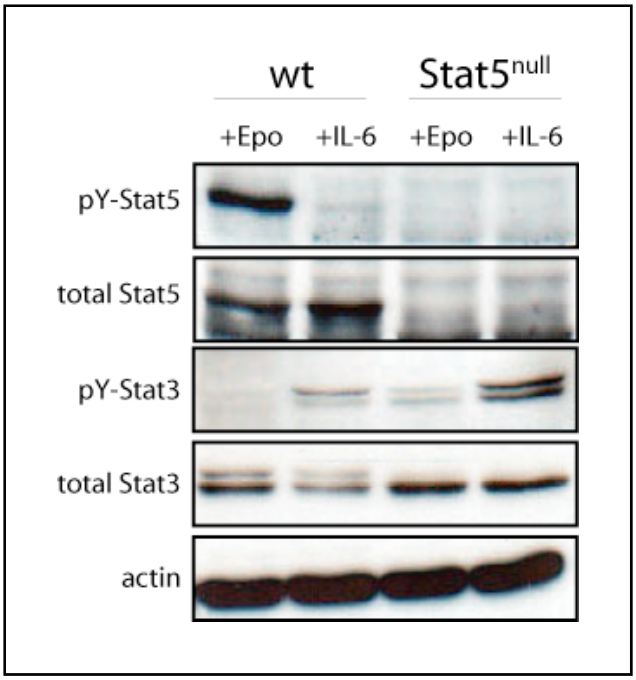


Figure 1: Phosphorylation of STAT5 and STAT3 in wt and *Stat5* knockout (Stat5^{null}) erythroblasts.

Loss of suppressor of activated cytokine signaling (SOCS) mRNA in Stat5$^{-/-}$ erythroblasts

SOCS proteins are among the most important negative regulators of JAK-STAT signaling. There are three different ways for interference of SOCS proteins with STAT activity: 1) Using their SH2 domain they can compete with downstream factors for phosphorylated tyrosines on cytokine receptors. CIS and SOCS3, for example, have been shown to bind directly to Y401 of EPOR competing with STAT5a/b (Sasaki, Yasukawa et al. 2000; Hortner, Nielsch et al. 2002). 2) Using their kinase inhibition domain they can downregulate JAK activity. 3) Acting as E3 ubiquitin ligases they can mark components or whole receptor complexes for degradation. In this context SOCS1 was reported to bind JAK2 at its activation loop inducing ubiquitination and subsequent degradation of the kinase (Ungureanu, Saharinen et al. 2002). Additionally *Socs* genes have been shown to respond to EPO stimulation and are supposed to feed back on EPO-signaling pathways as immediate early targets (Starr, Willson et al. 1997; Richmond, Chohan et al. 2005). So far only CIS, SOCS1 and SOCS3 have been associated with regulation of EPO signaling.

Loss of negative regulation of STAT signaling linked to the loss of Stat5 would explain an increase in phosphorylated STAT3. Therefore mRNA levels of all SOCS family members were analyzed in wt and *Stat5*$^{-/-}$ erythroblasts (Figure 2).

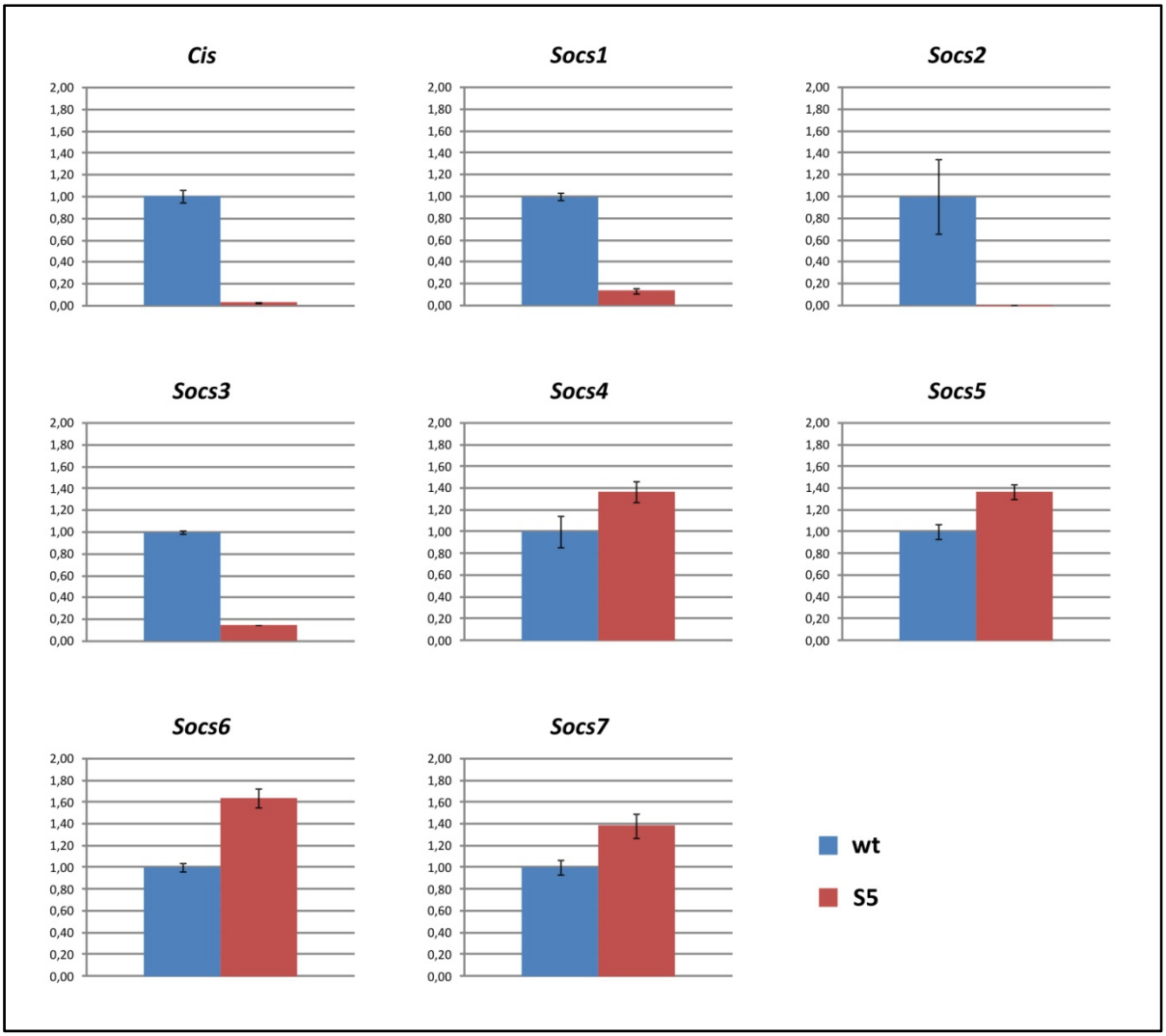


Figure 2: *Socs* gene family mRNA expression in wt versus *Stat5* knockout (S5) erythroblasts. *Cis*, *Socs1*, *Socs2* and *Socs3* mRNAs are almost absent in *Stat5* deficient erythroblasts. The transcripts of other *Socs* family members are slightly upregulated.

Cis, *Socs1*, *Socs2* and *Socs3* expression was practically absent in *Stat5* deficient erythroblasts. The mRNA of other family members were slightly upregulated, probably due to a compensatory mechanism. The magnitude of the observed effect makes all four downregulated genes likely targets of STAT5 in erythroid cells, which would corroborate their proposed role in feedback inhibition of EPO signaling. So far only CIS, SOCS1 and SOCS3 were reported to be involved in the regulation of the EPO/EPOR pathway. Loss of these three molecules is likely to cause overshooting EPO signaling due to increased availability of phosphorylated tyrosine binding sites on the EPOR and elevated STAT phosphorylation as result of decreased JAK degradation. This increase in signaling pressure may lead to phosphorylation of STAT3 in the course of EPOR activation, even if STAT3 would bind EPOR/JAK2 complexes with very low affinity.

Elevated levels of protein inhibitor of activated STAT (PIAS) proteins in Stat5$^{-/-}$ erythroblasts

Negative regulation of STAT signaling by PIAS proteins interferes with EPO signaling in the nucleus. PIAS family members have high individual specificity for their targets. PIAS3, for example, regulates both STAT5 and STAT3 mediated transcriptional activation by blocking the DNA-binding of activated STAT dimers (Chung, Liao et al. 1997; Rodel, Tavassoli et al. 2000; Sonnenblick, Levy et al. 2004). Actions of PIAS proteins meddle with the direct correlation between STAT phosphorylation and STAT target gene transcription. An increase in PIAS protein, especially PIAS3, would therefore mitigate or even abolish effects of increased STAT3 phosphorylation.

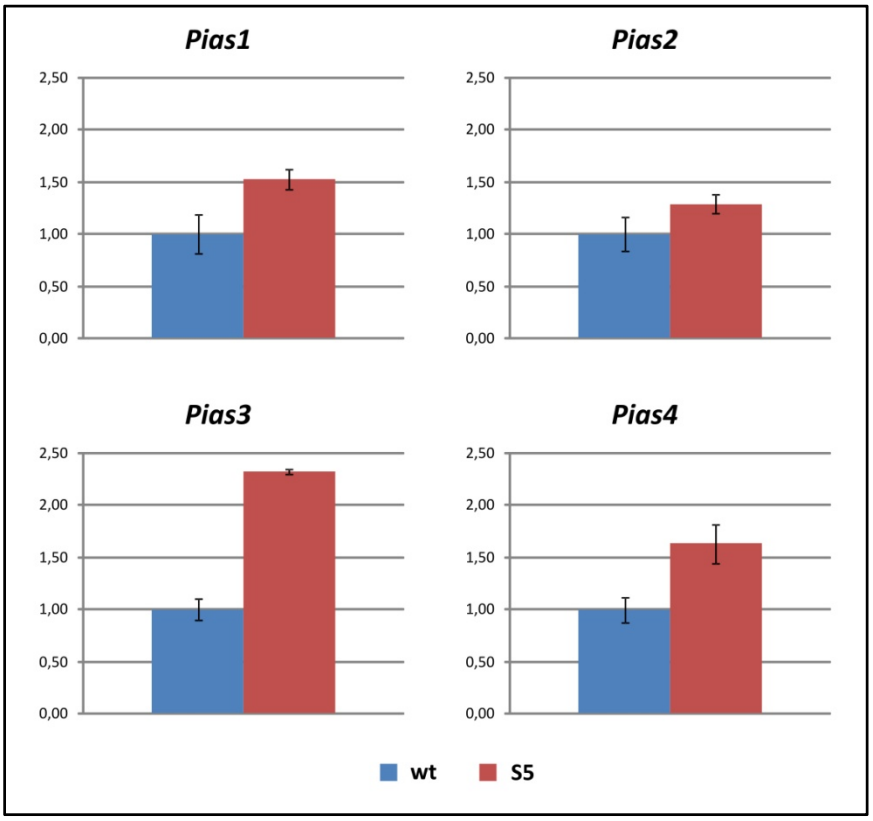


Figure 3: mRNA of PIAS family members in wt and *Stat5* deficient (S5) erythroblasts. *Pias1*, *Pias2* and *Pias4* are slightly upregulated, whereas *Pias3* shows significantly increased mRNA abundance.

Pias3 mRNA levels were found to be increased in erythroblasts in the absence of *Stat5*. This potential increase in PIAS3 protein should at least partially counteract the effects of decreased SOCS expression on STAT3 without affecting its phosphorylation status.

Discussion

Similar to previous findings in *Stat5* deficient liver (Cui, Hosui et al. 2007), STAT3 phosphorylation was fount to be increased in *Stat5* knockout erythroblasts. This enhanced phosphorylation was likely to be result of reduced expression of the potential STAT5 target genes *Cis*, *Socs1* and *Socs3*. Although STAT3 binds to the EPOR with much lower affinity than STAT5, the increase in phospho-tyrosine binding site availability and accessibility due to the absence of STAT5 and decreased JAK degradation leads to noticable STAT3 phosphorylation that may compensate partially for the loss of STAT5. The potential increase in PIAS3 protein, however, may counteract any elevated STAT3 activity in the nucleus by interfering with its ability to bind DNA. Although the degree of downregulation of *Socs* mRNAs levels was larger than the induction in *Pias3* transcripts, the actual quantitative contributions of both mechanisms are not yet known. Thus at present no conclusion can be drawn without further experiments to analyze STAT3 activity in *Stat5* knockout erythroblasts on a functional level.

Although a mechanistic foundation for STAT3 mediated compensation for loss of STAT5 has been laid, a series of experiments has to be conducted to extend the current observations and address their biological significance. First of all both SOCS and PIAS expression have to be verified on protein level. To assess STAT3 acitivity in the *Stat5*$^{-/-}$ situation, knockout erythroblasts should be transfected with *Stat3* reporter constructs and stimulated with EPO. Activation of the reporter by EPO would not fully prove but corroborate a possible STAT3 compensation. When this experiment gives a positive result, it still remains to be seen whether increased STAT3 activity provides a functional advantage for *Stat5* deficient erythroblasts. This can be tested by monitoring proliferation and apoptosis of *Stat5* knockout erythroblasts either treated with a small molecule STAT3 inhibitor (STAT3 Inhibitor V Stattic, Calbiochem) or subjected to lentiviral *Stat3* knockdown. *Stat5* knockout erythroblasts have been shown to suffer from increased apoptosis but displayed unchanged proliferation in comparison to wt cells (Kerenyi, Grebien et al. 2008). Absence of proliferation defects was rather unexpected since D-type cyclins have been reported to be STAT5 targets in hematopoietic cells (Matsumura, Kitamura et al. 1999). Likewise indications exist that STAT3 activity can drive D-type cyclin expression in other tissues (Sinibaldi, Wharton et al. 2000). Cyclin expression and closely linked cell proliferation may therefore be interesting candidates to assess STAT3-mediated compensation for loss of STAT5 on a biological basis. Similarily both STATs have been associated with apoptosis protection (Puthier, Derenne et al. 1999; Kerenyi, Grebien et al. 2008), which could lead to an even worse apoptosis phenotype in *Stat5* knockout erythroblasts with ablated STAT3 activity.

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Curriculum Vitae

Personal Information

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E-mail:	helmuth.gehart@imp.ac.at
Date of birth:	February 9th 1984 (Vienna)
Citizenship:	Austria

Education

since August 2007	Diploma thesis under joint supervision of Ernst W. Muellner and Hartmut Beug
July 2007	Completion of the first part of the second diploma thesis with high distinction
May 2006	Completion of the first diploma examination with high distinction
since October 2003	Study of Molecular Biology at the University of Vienna (Austria)
2002 - 2003	Military service as paramedic
2002	High school diploma with high distinction
1994 - 2002	High school in Laa a. d. Thaya (Austria)
1990 - 1994	Elementary school in Mistelbach (Austria)

Research Experience

Title: Analysis of CPK3 activation conditions
Group: Markus Teige (University of Vienna)
Type: Lab Rotation
Duration: 1 month

Calcium dependent kinases (CPKs) in Arabidopsis thaliana are supposed to be involved in hormone signalling, growth, development, carbon and nitrate metabolism, stress reactions and pathogen responses. Direct functional characterization of these proteins, however, is still in its infancy. The goal of my 1 month project was to gain insights into calcium dependency, autophosphorylation potential and stress-induced activation of AtCPK3. In the course of my work I was able to determine two Ca2+ concentration activation thresholds of CPK3 and prove its autophosphorylation. Additionally I demonstrated salt-stress dependent CPK3 activation in vivo while establishing an activation timecourse upon stress induction.

Title: Effects of loss of Stat5 on heme biosynthetic enzymes
Group: Ernst W. Muellner (Medical University of Vienna)
Type: Lab Rotation
Duration: 1 month

Mice deficient for signal transducer and activator of transcription 5 (Stat5) die perinatally due to a severe hypochromic mycrocytic anaemia. One of the possible reasons for the observed hypochromia is impairment of the heme biosynthetic pathway. The goal of this project was to elucidate whether loss of Stat5 had an effect on heme biosynthesis and if a potential effect would be direct or indirect. I analyzed expression levels of all heme biosynthetic enzymes in vivo and in cultured primary erythroblasts and could prove a significant downregulation of four enzymes in the pathway. Among them was the rate limiting enzyme Alas2. Additionally I could show that circumvention of Alas2 by supplying cells with its product 5-aminolevulinate was not sufficient to rescue erythroid differentiation in vitro. Direct stimulation of the affected enzymes using Epo in primary erythroblasts was successful, which does not prove but indicate that Stat5 acts as direct transcriptional activator of all four enzymes.

Title: Investigation on cellular effects of certain kinase inhibitors on tumour cell lines
Group: Ulrike Weyer-Czernilofsky (Boehringer Ingelheim Austria)
Type: Internship
Duration: 2 months

Most small molecule cancer drug candidates interfere with signalling pathways that are inseparably coupled to proliferation and survival. Boehringer Ingelheim has created large sets

of compounds blocking a specific kinase target. The aim of my project was to study and compare direct and indirect effects of the respective small molecule inhibitors on various signalling pathways, cell cycle progression and apoptosis. Furthermore it should be clarified if cell lines with higher endogenous activity of the kinase of interest were more susceptible to inhibition. During my work I was able to unravel different modes of action of the tested small molecule inhibitors depending both on compound and cell line. Additionally I demonstrated that cell lines with inherent higher activity of the target kinase are slightly, but not severely stronger affected by directed kinase inhibition.

Title: Establishment of cell proliferation assays for use in anti-cancer drug development
Group: Frank Hilberg (Boehringer Ingelheim Austria)
Type: Internship
Duration: 2 months

One of the main characteristics of any anti cancer drug is its ability to repress tumour cell proliferation. Today's pharmacological research focuses on high throughput screenings for inhibitors of certain targets. Therefore it is necessary for screening assays to be implementable at large scale. Generally this is much harder to achieve with cellular assays in comparison to biochemical tests, especially if a single method should be applicable to a variety of different tumour cell lines. Therefore the goal of my project was to establish an up scalable cell proliferation assay that can be used to analyze proliferation-impeding capabilities of small molecule inhibitors for a specific kinase target in a multitude of cell lines. I set up a multitude of different assays, ranging from biochemical DNA quantification to the use of Cellomics (automated high-throughput fluorescence microscopic imaging system), compared their readouts and tested their usability on more than 15 human tumour cell lines. The most reliable method I was able to establish is now Boehringer Ingelheim's standard method for testing kinase inhibitors.

Title: Stat5 in erythropoiesis and iron metabolism
Groups: Ernst W. Muellner (Medical University of Vienna)
Hartmut Beug (Research Institute of Molecular Pathology, IMP)
Type: Diploma thesis
Duration: 12 months

Signal transduction along the erythropoietin receptor (EpoR) – janus kinase 2 (Jak2) – signal transducer and activator of transcription 5 (Stat5) axis is essential for erythropoiesis as it regulates proliferation, differentiation and survival. Mice devoid of Stat5 die perinatally, which can be traced back to a severe hypochromic microcytic anaemia. The goal of my diploma thesis was to elucidate the reasons for the anaemic phenotype of Stat5-deficient mice. The results were three distinct, but interconnected ways through which loss of Stat5 affects erythropoiesis. First we established that anaemia in Stat5-/- mice is linked to enhanced

apoptosis caused by reduced expression of the anti-apoptotic proteins Bcl-x_L and Mcl-1. Additionally four out of eight heme biosynthetic enzymes displayed diminished expression in the Stat5 knockout situation, which could be linked to loss of Stat5 as transcriptional activator. Moreover cell surface levels of transferrin receptor 1 (TfR-1) were decreased to less than half on erythroid cells from Stat5-/- animals, leading to lowered iron availability in the respective cells. This reduction could be attributed to a combined effect of decreased TfR-1 mRNA transcription (as direct target of Stat5) and reduced mRNA stability as a result of low iron regulatory protein 2 (IRP-2) expression levels. Furthermore IRP-2 was demonstrated to be another direct transcriptional target of Stat5 resulting in a double negative effect on TfR-1 abundance. An upcoming paper, on which I was awarded co-authorship, describes this unexpected link between Jak-Stat signalling and iron metabolism and has recently been accepted for publication in "Blood".

Publications

Marc A. Kerenyi; Florian Grebien; Helmuth Gehart; Manfred Schifrer; Matthias Artaker; Boris Kovacic; Hartmut Beug; Richard Moriggl and Ernst W. Müllner (2008). "Stat5 regulates cellular iron uptake of erythroid cells via IRP2 and TfR-1." *Blood* – first edition

Honours and Scholarships

July 2007	Completion of the first part of the second diploma examination with high distinction
January 2007	Excellence scholarship*, University Vienna
May 2006	Completion of the first diploma examination with high distinction
January 2006	Excellence scholarship*, University Vienna
January 2005	Excellence scholarship*, University Vienna
January 2004	Excellence scholarship*, University Vienna

* The University Vienna awards excellence scholarships to students that display exceptional performances based on grades and achieved ECTS points.

Other

since 2005	Member of the Molecular Biological Society (www.mbsociety.org)
since 2006	Member of MBS Board of Directors
since 2007	Chairman of the Molecular Biological Society
Languages:	German (native speaker) English (fluent) Russian (basics)