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In vitro studies on the regulation of Erythropoiesis by Erythropoietin and Stem cell factor

Emile van den Akker

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In vitro studies betreffende de regulatie van erythropoiesis door erythropoietin en stam cel factor

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Promotor:
Prof. dr. B. Löwenberg

Overige leden:
Prof. dr. I. P. Touw
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Copromotor:
Dr. M. von Lindern

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Chapter 1

Epo and SCF cooperate to induce expansion of erythroid progenitors: An introduction

1.1 Growth factors, receptors and signal transduction, a general introduction

Both in higher eukaryotes as well as in bacteria, cells have to find a way to sense their surroundings to be able to respond to different circumstances. Sensing light, nutrition gradients, osmotic differences or chemical concentrations are examples of environmental input crucial for bacteria or a lower single cell eukaryotes to survive. A multi-cellular organism consists of various different cell types with different functions and at different locations. Some cell types can only find a niche in a specific organ with tight boundaries like the liver, spleen etc. Other cell types are found all over the body like macrophages or dendritic, antigen presenting, cells. Also for these cells it is of crucial importance to scan the environment to make proper adjustments and adaptations to function within the organism. Erythroid progenitors (the precursors of red blood cells), for instance, can sense a multitude of chemicals and peptides of which two are of crucial importance, Stem cell factor (SCF) and Erythropoietin (Epo). Interestingly, these factors elicit a different response when sensed in combination or when sensed on their own. In general the factors, ligands, bind to their specific protein counterpart, receptors, present on or in the recipient cell. Binding of a ligand to the receptor induces a signal transduction cascade in which a multitude of proteins is involved resulting in a cellular response. In the case of the erythroid progenitor the combination of SCF and Epo plus glucocorticoids induces expansion or "renewal" of progenitor cells, while Epo alone causes maturation into an erythrocyte. One important factor determining the strength of signal transduction is the concentration of the ligand present in the environment. For instance, at steady state conditions, the concentration of Epo is sub-optimal and more than 20% of the erythroid progenitors in the bone marrow fail to undergo maturation and die via apoptosis. A slight increase of Epo leads to enhanced survival of erythroid progenitors and hence to an increase in red blood cells. The concentration of Epo is dependent on the oxygen pressure in blood as sensed by the kidney and thus directly related to the environment the organism is living in. In general there are ligand concentration gradients present in the body as a result of dilution: i) a high concentration at the source of ligand production that decreases with increasing distance from the source in the case of local production and ii) a systemic production of hormones present with overall similar concentrations in the serum but resulting in concentration gradients in tissues. In addition to soluble factors, cell-cell contact can induce signalling events and, importantly, membrane bound ligands, like SCF, or ligand-presenting molecules like proteoglycans, can initiate signal transduction with different dynamics compared to the soluble counterpart. This way highly complex microenvironments are created in which a multitude of ligands is present at various concentrations or in membrane bound states that affect the strength and duration of signal transduction and cellular responses, probably at a slightly different level for each cell. To understand the cues that determine the fate of erythroid progenitors it is important to know which proteins are involved in the signal transduction pathways and how these pathways of various receptors and combinations of receptors can have a profoundly different outcome with respect to proliferation, differentiation and survival.

1.2 The hematopoietic system

A typical example of a complex microenvironment is the hematopoietic birth chamber: the bone marrow. All hematopoietic cells derive from one cell type; the stem cells. Stem cells are able to undergo constant self-renewal, while they also develop into the different hematopoietic progenitors that finally differentiate into the different functional blood cells (figure 1). Because of the limited life span of functional blood cells, constant replenishing of cells needed.

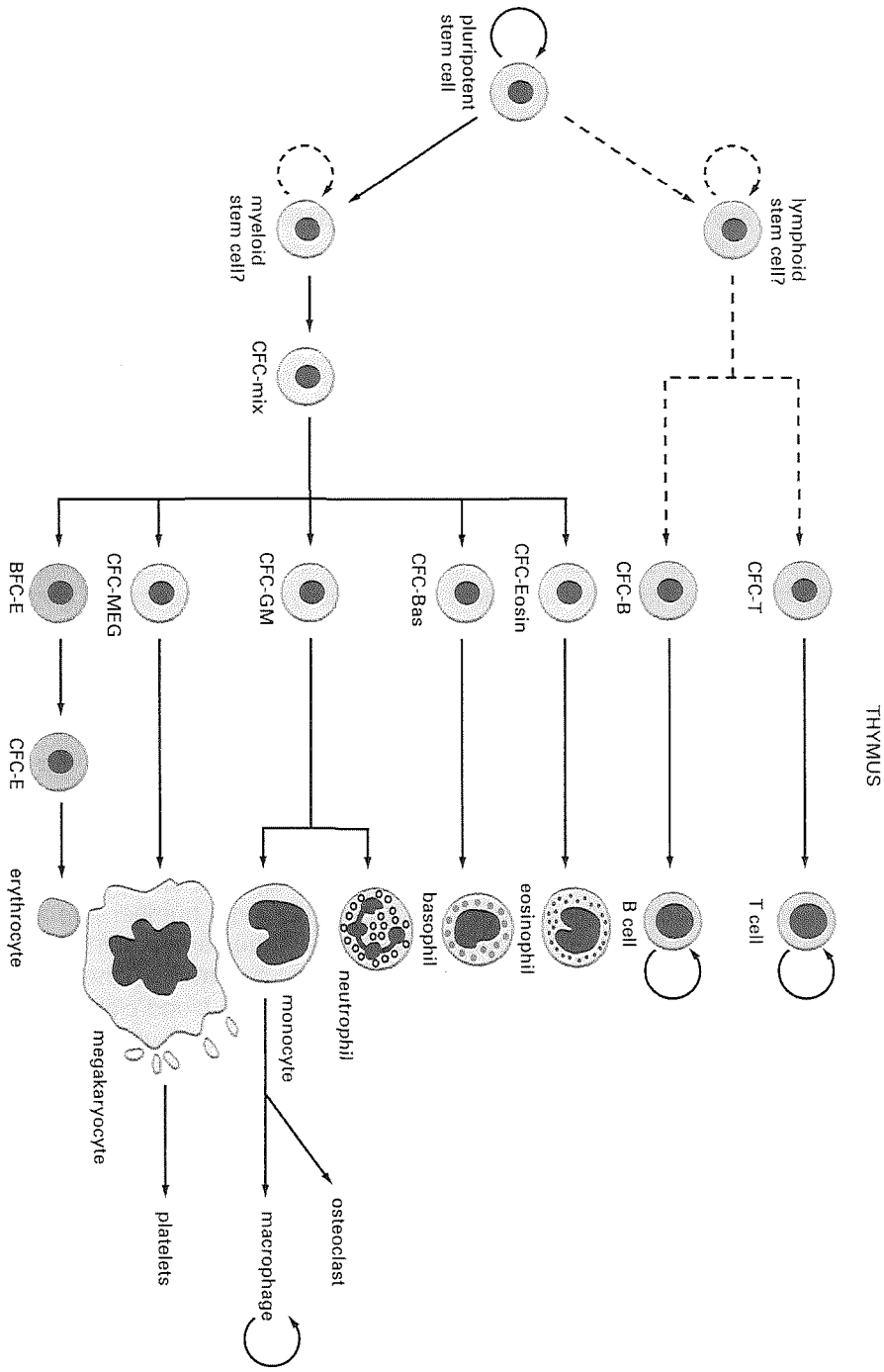

Figure 1. Hematopoiesis. The process of hematopoieis from the hematopoietic stem cell to the differentiated functional unit.

To cope with the dynamic requirements of the organism for certain blood cell types, progenitors are constantly sensing varying concentrations of a set of growth factors. According to the "instructive model", these different growth factor gradients influence the decision to which lineage the progenitor becomes committed and hence controls the number of cells entering the various lineages. In contrast, in the "permissive" model hematopoiesis is defined as a stochastic process in which the different growth factors play a role in the permission of lineages and in control of the total number of cells created within a lineage but not the actual lineage commitment decision itself. Nevertheless, both models dictate an enormous plasticity within hematopoietic system. Ultimate prove for one or the other model has not yet been obtained. As expected, a large number of growth factors, cytokines and hormones act on the hematopoietic system to control survival and a proper balance between expansion and maturation of cells at various stages in the distinct lineages of the hematopoietic system. Partly, these factors target different progenitors but in various combinations they also act synergistically on the same target cell. Some receptors may be lineage specific antennae for survival, proliferation or differentiation, like the receptor for erythropoietin (Epo) on the erythroid progenitors or granulocyte-colony stimulating factor (G-CSF) receptor on neutrophilic progenitors. In addition, the signalling molecules present in a specific cell type may determine their signalling capacities. Other receptors are expressed more widely in the hematopoietic system like the receptor for stem cell factor (SCF), or interleukin 3 (IL-3). Their signalling properties may be determined by the cellular context and the activation of cooperating receptors. Our knowledge on general signalling pathways used by distinct receptors has increased largely over the recent years. However, we still understand little of the specificity in signalling, nor of the integration mechanism of signals induced by cooperating receptors.

1.3 Erythropoiesis

1.3.1 The erythrocyte

45% of the blood volume consists of erythrocytes (red blood cells). Human mature erythrocytes have an average life span of 120 days (compared to 55 days in mice). Non-functional red blood cells are removed through phagocytosis by macrophages in the spleen and liver [1]. Since 10^{11} senescent erythrocytes are removed each day, the same number has to be produced to maintain proper red blood cell numbers. Epo levels in the serum mainly control this balance. Tight control is crucial as tiny fluctuations in red blood cell levels in the blood cause the blood to thicken in case of over production, which could lead to blood clotting, thrombosis, stroke etc. while a decreased production results in low oxygen pressure in tissues.

1.3.2 Developmental stages in erythropoiesis

Erythroid colony formation from total bone marrow has been studied extensively and has given insight into the cytokines and other growth factors necessary throughout erythropoiesis but has also into morphological changes and progenitor expansion kinetics at various stages of the erythroid development. .

This way a crude separation has been made between early and late committed progenitors. The burst forming units-erythroid or BFU-E, is a committed early progenitor able to generate a colony of up to 5000 erythrocytes (12 cell divisions) when grown in semi-solid media in presence of Epo and SCF [2,3]. The colony forming units-erythroid, the CFU-E, is a late progenitor able to generate up to 60 erythrocytes when grown in semisolid medium in presence of Epo (6 cell divisions). The BFU-E is mainly dependent on SCF and subsequently matures into a CFU-E, which is dependent on Epo for its expansion and survival during differentiation.

In addition to this classification by colony formation, erythroid progenitors can be classified from CFU-E to the maturated erythrocyte by morphology and the expression of stage-specific antigens. A CFU-E is morphologically defined as a pro-erythroblast, that develops into the basophilic erythroblast, the polychromatic erythroblast, the orthochromatic erythroblast and then to the erythrocyte. This process is accompanied by i) cell size reduction due to cell divisions with loss of size control ii) condensation of chromatin in the nucleus followed by enucleation and iii) increasing hemoglobin production. The enucleated reticulocyte still contains mitochondria, ribosomes and a golgi apparatus, which is lost within 24h during of further maturation into the erythrocyte.
Further characterization of erythroid differentiation is facilitated by suspension cultures in which pro-erythroblasts can be induced to undergo up to 20 'renewal divisions' in expansion medium (serum free medium supplemented with Epo, SCF and glucocorticoids), while they will synchronously differentiate to enucleated erythrocytes when expansion medium is replaced by differentiation medium (medium supplemented with Epo, Insulin and transferring). Differentiation entails 3 cell divisions without size control [4,5]. The large amounts of pure erythroid progenitors in a synchronous stage allows for extensive biochemical studies. An advantage of these suspension cultures of erythroid progenitors is the ease with which the erythroid progenitor is immortalized in absence of p53 without disrupting the differentiation capacity. This allows the establishment of cell lines from genetically modified mice p53 ([128], Schmidt in press)

1.3.3 Stress and fetal liver erythropoiesis

Upon blood loss, hemolysis, oxygen deprivation (high altitudes) or impaired erythrocyte function more red blood cells have to be made to sustain a proper oxygen tension in the body. This stress-induced erythropoiesis takes place in the spleen of mice and in the bone marrow in man. The necessary increase in red blood cells is accomplished by increasing the number of cell divisions an erythroid progenitor makes without entering terminal differentiation. This is further referred to as the "renewal" capability of erythroid progenitors. While basal conditions only require to replace the senescent loss (2.5% of total red blood cell population per day), stress induces a 10-fold increase in erythropoiesis. Several lines of evidence indicate that cooperation between the Epo-receptor, c-Kit and glucocorticoids is essential for stress-induced expansion of erythroid progenitors. Interestingly, it has been proposed that fetal erythropoiesis resembles stress erythropoiesis in its mission to sustain an adequate increase of erythrocytes through enhanced self-renewal. In fact, in the early embryo adult hematocrit levels have to be reached within only a few days, imposing huge demands on the erythropoietic system. This requires the erythroid progenitors to expand rigorously resembling the stress-erythropoiesis situation. The liquid cultures described in 1.3.2. exploit this self-renewal capability of the erythroid progenitor to obtain large amounts of erythroid progenitor. These cultures hence resemble a stress erythropoiesis model.

1.4 Cytokines and growth factors in erythropoiesis

Although Epo plays an important role in erythropoiesis it is not the only factor involved. A variety of other factors play a role in erythropoiesis. The major other factor is SCF, while in addition, ligands of nuclear hormone receptors (glucocorticoids, retinoic acid), transforming growth factor beta family members (TGFb, activin), insulin and insulin-like growth Ffactor-I (IGF-I), apoptosis inducers like the tumor necrosis factors (TNF), the interferon family and also ligands of G-protein coupled receptors (thrombin, nucleosides) have a role in mainte-

nance and control of erythroid cell numbers. Below the growth factors relevant to this thesis namely are outlined, followed by a short description of factors negatively influencing erythropoiesis.

1.4.1 Erythropoietin

The importance of Epo in erythropoiesis has been shown in knockout studies. Mice deficient in Epo or the EpoR die at day 12,5 of gestation with a lack of fetal liver erythrocytes [6]. It has long remained unclear whether Epo is required for every aspect of erythropoiesis including lineage determination, expansion, survival and differentiation. Epo is not required for lineage commitment since Epo-/- fetal livers do contain CFU-E progenitors that can be rescued *in vitro* by exogenous Epo [6,7]. In addition, over-expression of the EpoR does not result in increased commitment to the erythroid lineage [8]. In contrast, Epo is a limiting factor for expansion and survival of erythroid progenitors during onset of differentiation.

Epo is mainly produced in the kidney in response to the oxygen-pressure. However very low levels of Epo are produced independently of oxygen-levels by early erythroid progenitors (BFU-E) [9,10]. Due to the limiting concentrations of Epo in plasma, it is estimated that 20% of erythroblasts in the bone marrow undergo apoptosis [11,12]. However, this number could be an underestimation, due to rapid phagocytosis of apoptotic erythroid cells by macrophages *in vivo*. Elevation of Epo results in survival of progenitors during the differentiation process and therefore in increased red blood cell production [13-15].

In discussions on the permissive versus instructive role of lineage specific growth factors and cytokines, it is often claimed that permitting cell survival is the only function of Epo signalling. This hypothesis is supported by the finding that the prolactin receptor, GM-CSF, GCSF or the GH-receptor expression in erythroid progenitors can rescue erythroid colony formation of fetal liver cells from Epo-receptor (EpoR) -/- mice [16] or in absence of Epo during culturing [17,18]. In addition, BFU-E are committed to erythropoiesis but do not respond to Epo. These data indicate that the Epo/EpoR is not involved in the lineage decision. Notably, the receptors used to replace the EpoR are of the same class and recruit largely similar signalling pathways (e.g. Jak2, STAT5, PI3K). The apparent "lineage incompatible receptor switch rescue" phenotype may indicate that receptors of the same class, largely similar in signal transduction, but having a different activating ligand are evolutionary developed only to ensure lineage specificity. However, in most of these experiments the colony-forming ability of erythroid progenitors was scored without reporting proper differentiation parameters like hemoglobinization and enucleation. It is also not clear from these experiments whether the receptors for GM-CSF, GH and prolactin can synergize with the SCF receptor c-Kit. Nevertheless, the experiments show that induction of survival is vital during differentiation. Along the same lines, over-expression of the anti-apoptotic protein $BclX^L$, a STAT5 target induced late in erythroid differentiation, is sufficient for proper, Epo-independent differentiation of erythroid cells underscoring the necessity to prevent apoptosis [19]. It is possible that specific features of the EpoR are required to interact and cooperate with additional receptors under conditions that require expansion and its feedback control like stress, while steady state erythropoiesis in healthy mice mainly requires the survival function of the EpoR.

1.4.2 Stem cell factor is required for expansion of erythroid cells

While Epo acts exclusively on erythroid cells in the hematopoietic system, its effects are modulated by the more generally expressed growth factor SCF. SCF is required for the development of multiple cellular lineages. It is crucial for the migration of stem cells from the neural

crest to the reproductive system (giving rise to germ cells), the skin (giving rise to melanocytes) and the bone marrow [20]. Absence of SCF or c-Kit protein results in death in utero or in the perinatal period with severe macrocytic anemia. SCF is produced in various cell types including endothelial cells, fibroblasts, hematopoietic progenitors, and marrow stromal cells both as a soluble factor and as a membrane bound factor [21]. Steel Dicky (Sld) is a mutation in SCF that disrupts membrane association but allows synthesis of soluble SCF [22]. Mice carrying this mutation are viable, but lack skin pigmentation, are sterile and have severe anemia. The marrow of Sl/Sld mice contains strongly reduced numbers of CFU-granulocyte-macrophage (CFU-GM), CFU-spleen (CFU-S), BFU-E and CFU-E, indicating that membrane bound SCF has a crucial role in the proliferation of early hematopoietic progenitors [23]. It must be emphasized that the hematopoietic system of these mice is severely perturbed in all lineages underscoring a defect in early stem cells rather than in the specific lineages. Interestingly, injection of wt mice with antibodies directed against c-Kit, preventing ligand-induced c-Kit activation, had no effect on steady state erythropoiesis. However, upon induction of anemia no stress erythropoiesis was observed in the spleen of c-kit antibody treated mice [24]. This indicates i) that SCF is crucial for stress erythropoiesis but appears to be non-essential during steady state erythropoiesis and ii) that the anemia in observed in the Sl/Sld mice is probably caused by stem cell aberrations.
Analysis of transgenic mice expressing various forms of SCF showed that some cells (e.g. the hematopoietic precursor) can only benefit from membrane bound SCF, while other cells (e.g. myeloid progenitors) require soluble SCF [25]. In *in vitro* assays, soluble SCF is biologically active in erythropoiesis when used at relatively high concentrations (100 ng/ml; [4]). Addition of SCF in conjunction with Epo yields large size erythroid colonies in colony assays. In liquid cultures, expansion of erythroid progenitors also requires the combined action of Epo and SCF [4]. Although SCF delays differentiation and promotes renewal divisions of cells grown in culture, it does not completely block differentiation. As a result it yields enhanced numbers of mature erythrocytes. It has been suggested that the maturation of erythroid cells in presence of high levels of SCF may be due to SCF-induced down-modulation of its receptor c-Kit [26,27]. However, constitutive expression of wt c-Kit did not block renewal divisions of erythroid cells either and also yielded increased numbers mature erythrocytes [28].
Epo-induced differentiation of erythroid progenitors involves loss of c-Kit and relocalization of erythroid cells from stromal cells to macrophages, where enucleation takes place [29]. Macrophages are not required for enucleation, but they do phagocyte the nuclei. Interestingly, the effect of SCF on erythroid progenitors is exclusively measured in combination with Epo (note: although there has been one report of partial rescue of EpoR-/- progenitors by the combination of SCF/TPO and IL-6 [30]). When added in absence of Epo the erythroid progenitors undergo apoptosis [4,31,32]. From these *in vivo* and *in vitro* experiments it is clear that Epo and SCF cooperate to induce expansion of erythroblasts.

1.4.3 Glucocorticoids and stress erythropoiesis

One other factor found to be important in this cooperation are glucocorticoids (GC). Indeed, insufficient glucocorticoid levels are associated with anemia in Morbus Addison disease while overproduction, in patients with Cushing's syndrome, results in increased red blood cell, hemoglobin and hematocrit levels. GC-receptor (GR)-deficient mice die at birth due to respiratory failure. In addition the mice present with development failures (E15.5 onward) in multiple organs like liver and adrenal glands [33]. Isolated fetal liver erythroid progenitors from these GR-/- mice at E14.5 fail to proliferate in expansion medium (Epo, SCF and the artificial

glucocorticoid ligand Dexamethasone or Dex), indicating that Epo and SCF signalling requires functional GR to induce expansion. Replacing the normal GR with one deficient in DNA-binding in mice (GRdim/dim) results in viable mice that show impaired regulation of GR-dependent genes (which indicates that GR function in development does not rest on the DNA-binding capacity but rather on its association with other (transcription) protein complexes that can either be inducing or suppressing, though precise description exceeds the topic of this thesis [34,35]. These mice have normal adult erythropoiesis but do not show stress erythropoiesis when hypoxia is induced. While the number of splenic CFU-E in normal mice showed a 6-fold increase upon hypoxia induction, spleen CFU-E numbers did not change following hypoxia induction of GRdim/dim mice [5]. *Wt* fetal liver cells transplantated in sublethally irradiated GRdim/dim mice responded to hypoxic conditions with a normal stress response i.e. increase of spleen CFU-E. Thus the requirement for an intact GR in stress erythropoiesis is intrinsic to the erythroid progenitors. Importantly, the data show that the GR is not crucial for normal erythropoiesis but only in the specific cases of fetal liver and stress erythropoiesis. Moreover, the data suggest that erythroid progenitor expansion during fetal and stress erythropoiesis relies on similar mechanisms. The molecular mechanism of the cooperation between the GR and the Epor/cKit -in expansion of erythroid progenitors remains to be elucidated. It is been suggested that GR modulates the balance between differentiation and proliferation through regulation of the expression of transcription factors such as c-Myb [36,37] and its binding to GATA-1 [38,39]. Moreover, the GR has been found to associate with STAT5 and AP-1 [40,41].

A growing number of (transcription) factors downstream of Epo and SCF signalling, like E2f4, Stat5a/b (phosphorylated after Epo stmulation), ATF4 (upregulated by SCF) and Sfxn1 (flexed tail a protein involved in iron metabolism), seem to be essential for fetal liver and stress erythropoiesis rather than for steady state [42-45]; personal observations). This opens the possibility that Epo and SCF signalling cooperates with the GR in activating or suppressing specific Epo-, SCF-, GC-controlled genes. Studies have been done and are on the way to identify these and GC specific genes in erythroid progenitors [35]; unpublished results). *In vivo* GCs are also described to regulate Epo production in the kidney [46] via an unknown mechanism. This could also play an additional role in the observed absence of stress erythropoiesis. However, the combination of *in vitro* and *in vivo* experiments indicate a direct requirement of GCs in expansion of erythroid progenitors in response to hypoxia.

In conclusion, the cooperation of SCF and GCs with Epo appears to be to be redundant in normal erythropoiesis. The necessity for progenitor expansion and the observed synergy or cooperation probably is fetal liver and stress erythropoiesis specific to enable the maximum capacity of red cell production required at that particular stage. Taken together, expansion of the erythroid compartment involves renewal divisions of the erythroid progenitor (late BFU-E and CFU-E) for which SCF and glucocorticoids need to cooperate with Epo, while Epo is sufficient to allow differentiation.

1.4.4. Death receptors and erythropoiesis

Negative regulators of erythropoiesis include interferon gamma (IFN-γ), members of the tumor necrosis factor-alfa (TNF-α, TRAIL-R), Fas-ligand and transforming growth factor beta (TGF-β). The first three act mainly by inducing apoptosis, although they also may accelerate differentiation due to the activation of caspases. Cleavage of the capsases 1, 3 and 9 has been shown to be accelerating differentiation [150]. TGF-β inhibits progenitor proliferation and promotes differentiation via unknown mechanisms [47-51]. Due to limiting Epo levels, apoptosis

of erythroblasts in the bone marrow during erythropoiesis is quite significant (20%). Control of apoptosis may be very important to maintain proper red blood cells levels. Loss of control could cause enhanced erythroblasts survival resulting in erythrocytosis or anemia.

1.5 Signal transduction in erythroid progenitors.

To begin to understand why cells undergo renewal divisions in presence of Epo, SCF and GC, while they mature to erythrocytes in presence of Epo only, we need to get insight into how these growth factors elicit their effect on cells. Cooperation between Epo and SCF begins at the level of receptor activation and leads to activation of a myriad of signaling intermediates that control cellular processes such as gene expression, mRNA translation and protein trafficking. Although much is already known about the downstream signals of cytokine receptors, the specificity of each pathway with respect to differentiation, proliferation and survival is not clear. A main aim of this work was to analyse signal transduction by Epo and/or SCF to map the proteins responsible for erythroid progenitor expansion (cooperation between Epo and SCF) or differentiation (Epo only).

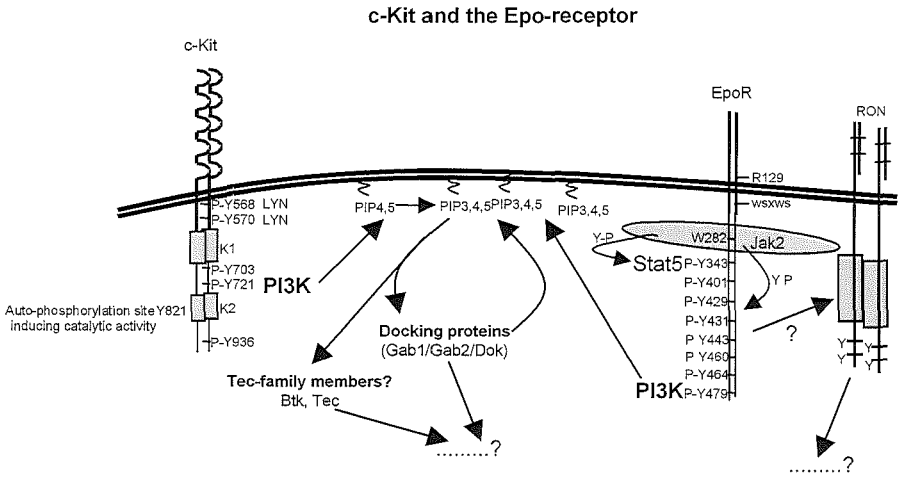

Figure 2. The SCF/Epo receptor complex. SCF and Epo induce tyrosine phosphorylation of c-Kit and the EpoR respectively. The phosphorylated c-terminal tails recruit and activate various signalling intermediates resulting in signal transduction and biological responses (see text for details)

1.5.1 *Epo downstream signaling; the Epo-receptor.*

The functional EpoR is a homodimer without tyrosine kinase activity, which is constitutively associated with the cytoplasmic tyrosine kinase Jak2 through the domain containing amino acid residue W282 ([52]; EvdA chapter 5, figure 2). Both the EpoR knockout and the Jak2 knockout are embryonic lethal due to absence of functional erythrocytes [6,7,53]. The EpoR belongs to the hematopoietic type 1 receptor superfamily, also including c-MPL, GM-CSFR, GCSFR, IL-2R, IL-7R, IL-9R, IL11R, IL12R, GHR and LIFR [54,55]. This family is characterized by a 20 amino acids stretch called the cytokine receptor homology region (CRH). This motif is located in the extracellular part near the transmembrane domain and contains four conserved cysteine residues and the so-called WSXWS motif. In addition, most family members share two intracellular motifs near the transmembrane: box1 (10aa) and box2 (10aa) [56-63].

Upon ligand binding, the conformation of the EpoR changes bringing the Jak2 kinases together [64,65]. This induces phosphorylation and activation of Jak2 and subsequent phosphorylation of 8 tyrosines in the intracellular part of the EpoR. These serve as docking sites for a large number of signalling intermediates that are activated in turn [66,67]. Below some examples of recruited protein to the EpoR are given, ordered from the transmembrane region of the Epo-receptor intracellular part to the c-terminus. The transcription factor STAT5 is recruited to Y343 and Y403 [68-70]. Doppler et al. [71] showed that STAT5 is able to bind to the GR and that this interaction leads to enhanced and synergistic transcription of the STAT5 target beta-casein but could also lead to transcription of specific GR-Stat5 responsive genes of which transcription is not induced by GCs or Stat5 alone. STAT5 knockout mice are not deficient in expansion but show significant increase in apoptosis during differentiation [43,72]. The role of STAT5 has thus been linked to survival during erythropoiesis and the interaction with GR could relate to this function. The tyrosine phosphatase SHP2 Is recruited to Y403, the same site as STAT5 73. SHP2 is thought to remove inhibitory signals, thereby activating signalling e.g. by Jak2 and Grb2 [74]. In contrast, the tyrosine phosphatase SHP1, docking to Y431/429 [75-77], decreases signalling by dephosphorylation of the EpoR and Jak2. Interestingly, in rare cases of familial erythrocytosis a truncated Epo-receptor that lacks the recruitment site for SHP1 is expressed. These people are hyper-responsive to Epo and show prolonged Jak2 activation [151]. The Src kinase Lyn docks with its SH2 domain to Y464 and Y479 [78]. The p85 subunit of phosphoinositide 3-kinase (PI3K) binds to Y481 [75,79,80] and the bridging molecule Grb2 docks to Y464 [66] and may subsequently recruit several other molecules such as Gab1, Gab2 and Shc [81-84], all phosphorylated upon EpoR activation [85,86].
Confusingly, C-terminal truncations do not hamper the mitogenic properties of the EpoR. Notably, even an EpoR with a C-terminal deletion that lacks all 8 tyrosines but still binds Jak2, can mediate cell survival and proliferation in factor dependent cell lines [87]. Expression of this truncated EpoR or a truncated EpoR lacking 7 of the 8 tyrosines can restore erythroid colony formation in fetal liver cells that lack the EpoR [16,87]. In summary, the fact that truncation mutants are not deficient in mitogenic signalling suggests i) that the c-terminal part and its signalling properties are not important for steady state erythropoiesis and fetal liver erythropoiesis and/or ii) the presence of compensating mechanism activated by Epo but independent of the C-terminal part of the EpoR, for instance through the actions of large docking molecules.

Several docking proteins are tyrosine phosphorylated after Epo stimulation among which are IRS2, Gab1 and Gab2 [85,88,89]. These docking proteins are not recruited by the Epo-receptor but the presence of a PH-domain recruits them to phosphatidylinositol-3,4,5-triphosphate (PI3K activity)in the vicinity of a receptor [90,91]. These docking proteins recruit PI3K them selves leading to increased PI3K activity, functioning as a positive feedback loop [92,93]. Indeed, over-expression of Gab1 potentates the activation of PKB (a protein downstream of PI3K), while expressing a Gab1 mutant lacking the PI3K recruitment site results in decreased PKB activation [94]. Besides PI3K, these docking molecules recruit a growing list of other signaling mediators, like phospholipase C (PLCs), Shc, Grb2, Crkl, Shp2, Shp1 and SHIP, plugging into several important signalling events (for review see [95,96]). For instance, Shp2 recruitment has been shown to be important for the activation of the Erk-MAPK route via stimulation with different stimuli resulting in mitotic responses [82,95,97], while the activation of the PLC-gamma family may be a way to control calcium release and diacylglycerol production (and PKC activation, [98]). Potential kinases responsible for the phosphorylation of Gab proteins have been found and include members of the Src-kinase family but also the tyrosine kinase receptor c-

Met [99,100]. Interestingly, Gab1 comprises a unique domain with which it docks to c-Met called the Met-binding-sequence (MBS). Absence of this domain results in abrogation of Gab1 tyrosine phosphorylation, while including the MBS sequence in Gab2 (normally not a c-Met substrate) results in Gab2 tyrosine phosphorylation by c-Met [101]. In erythroid progenitors a homologue of c-Met is expressed. This tyrosine kinase receptor named RON (or Stk in mice) is the susceptibility gene for spleen-focus-forming-virus-induced erythroleukemia. Comparable to its binding to c-Met, Gab1 is also a substrate of RON/Stk and Gab1 binding is mediated via the same MBS sequence (chapter 5). Gab1 deficiency leads to embryonic death due to liver defects and resembles the phenotype of HGF and c-Met knockout mice [99,102,103]. In contrast, the Gab2 and Gab3 deficient mice are viable and have only mild phenotypes [104,105]. Gab1 is more ubiquitously expressed, while Gab2 and Gab3 are tissue specific. The absence of phenotype in the Gab2 and Gab3 knockout mice may be due to compensation via Gab1.
In conclusion, these docking proteins, when activated, become signalling platforms with overlapping downstream signalling events and recruited proteins when compared to the proteins recruited by the EpoR or c-Kit themselves.

1.5.2 The RON/Stk tyrosine kinase

The tyrosine kinase receptor RON/STK was identified as the Fv2 Friend virus resistance locus [106,107]. Friends disease, existing only in mice, was first characterized by Charlotte Friend [152] and represents a multi-step carcinogenesis disease inflicted by spleen focus forming virus (SFFV; reviewed by [107]. There are two forms of SFFV, namely a polycythemic (SFFV-P) and an anemic (SFFV-A) strain, reflecting the effects of virus infection. Upon infection of erythroid progenitors with SFFV-P the viral gp55 protein interacts with the transmembrane domain of the Epo-receptor [108]. This interaction depends on residue S238 of the murine Epo-receptor (absent in the human EpoR) and M390 of gp55 [108]. Interestingly, susceptibility to SFFV depends on the presence of a short form of the tyrosine kinase receptor RON, suggesting a link between the EpoR/gp55 complex to RON. Indeed, RONsf and the EpoR interact when over-expressed in COS cells [107] (chapter 5) and gp55 has been shown to bind to RONsf resulting in constitutive activation of RONsf [109]. The EpoR-gp55 interaction induces constitutive phosphorylation of several Epo-downstream targets like the MAPK route, Gab1/2 and PI3K resulting in erythroid polycythemia. Subsequent genetic events (inactivation of p53 and upregulation of PU-1), result in a differentiation block and the final disease state: erythroid leukemia. Notably, over-expression of a family member of RON, v-Sea, in chicken erythroblasts results in PI3K and MAPK dependent transformation [110]. Moreover, signal transduction by c-Met, another family member of RON, depends on the activation of the Gab docking proteins and the subsequent activation of the MAPK route and PI3K. The constitutive activation of RONsf in SFFV transformed erythroblasts may lead to the constitutive activation of the Gab proteins, PI3K and the MAPK route though direct evidence for this as not been found. In both humans and mice, RON is expressed in erythroblasts as a long and a short form, originating from the same gene. An additional promoter containing a GATA-1 site in intron 10 creates a transcription initiation site for the short form of RON (RONsf), which lacks the extracellular part but retains the trans-membrane and intracellular domains [111]. C57/B6 mice harbour a mutation in the GATA1 site and consequently lack the short form of RON and are resistant to SFFV infections. Apart from its ability to phosphorylate Gab1, the expression of RON in erythroid progenitors and the implication of RONsf in constitutive activation of EpoR by SFFV-gp55 prompted us to study the role of RONfl in erythroid proliferation and differentiation and in Epo-induced signal transduction in particular (chapter 5). We found that RON

is a downstream effector of EpoR signalling responsible for recruitment and Epo-induced phosphorylation of Gab1.

1.5.3 The SCF-receptor: c-Kit

c-Kit is a receptor tyrosine kinase that belongs to the family of so called split kinase receptors [112]. This subfamily includes FLT3 and the receptors for CSF-1/M-CSF and PDGF, and is characterized by the separation of the ATP-binding region and the catalytic region of the kinase domain by an interkinase domain [113]. Ligand binding induces receptor oligomerization and subsequent auto/transphosphorylation, followed by activation of cytoplasmic target proteins. Members of the Src-family of tyrosine kinases, Lyn in particular, associate with phosphotyrosines 567 and 569 in the juxtamembrane region of murine c-Kit (corresponding to Y568 and Y570 in human c-Kit, respectively; [114]. This interaction is important for the phosphorylation of adapter molecule Shc and activation of Ras and Erk1/2, and correlated with SCF-induced mitogenicity in aortic endothelial cells [115]. Binding and activation of PI3K is mediated via phosphotyrosine 719 (Y721 in human c-Kit) in the inter kinase domain [116]. Bone marrow derived mast cells expressing KitY719F have a reduced mitogenic and survival response, while SCF-induced secretion, adhesion and ruffling are completely absent [116,117]. In addition, tyrosine residues 703 and 936 in human c-Kit bind the adapter proteins Grb2 and Grb7 [118]. Grb2 may link c-Kit to Sos, SHP2, or Cbl, while no binding partners of Grb7 are currently identified. The tyrosine kinase Jak2 and its targets STAT1 and STAT5 were reported to be activated by c-Kit [119,120], although this is likely to be cell dependent, as we have never found any evidence for SCF mediated JAK-STAT activation in erythroid progenitors (unpublished results).

Although c-Kit contains multiple tyrosine residues that could serve as SH2-domain docking sites, only few have been implicated in signal transduction. Instead, the activation of Lyn and PI3K appears to orchestrate multiple pathways in concert (figure 2; [117]. Therefore, we focussed on proteins downstream of SCF-induced PI3K and Lyn. In chapter 2 we describe that after SCF stimulation the tyrosine kinase Tec is activated downstream of PI3K and Lyn and that this activation of Tec leads to the phosphorylation of the docking protein Dok-1. Dok-1 can recruit multiple different proteins, and over-expression of Dok-1 is reported to lead to prolonged signalling [121].

1.6 Epo and SCF cooperation; intracellular mechanisms

On the level of progenitor expansion or 'renewal' the cooperation between Epo, and SCF is evident. However, our current knowledge of the actual intracellular fundaments of this cooperation is surprisingly poor. FRET-analysis showed that the EpoR and c-Kit are in close proximity [24]. Indeed, the EpoR and c-Kit interact irrespective of stimulation and cross-phosphorylation has been reported [122-124] (personal observations). Stimulation with SCF and remarkably also Epo results in clustering of c-Kit in the membrane again proving that c-Kit and the EpoR can be situated in the same membrane fraction/complex [122]. Nevertheless, it is not known if all c-Kit or EpoR is associated in the larger complexes or whether single c-Kit/EpoR combinations are present in the membrane. Neither is it known if there is a qualitative or quantitative difference between the c-Kit/EpoR complex or c-Kit, EpoR single receptor signalling at the same stage. So, the actual significance of this interaction with respect to the observed cooperation remains elusive.

During signal transduction induced by Epo and SCF co-stimulation, protein complexes can be formed or activated which, compared to the stimulation of the single receptors are i)

unique to co-stimulation and signaling (the activation of a protein not activated by either receptor when stimulated on its own); ii) more sustained or robust in strength due to activation of similar pathways giving additive effects or iii) inhibited upon co-stimulation and signaling. Evidence for the first and the third explanation has not been found until now, which justifies the search for new signal transduction pathways induced by Epo and SCF. We and others have found evidence for the second explanation. Upon co-stimulation of erythroid progenitors with Epo and SCF an additive effect can be seen with respect to duration and level of PKB and ERK phosphorylation [4]. We have found that inhibition of PI3K but not the MAPK route results in differentiation of erythroid progenitors under expansion conditions (Epo and SCF) [125,126]. In agreement with this, p85α deficient mice have transient fetal anemia and decreased numbers of erythroid progenitors as assessed by counting the number of CFU-Es and BFU-Es [127]. These data justify a thorough investigation of PI3K downstream pathways as these are potentially involved in the decision between self-renewal and differentiation of erythroid progenitors.

1.6.1 PI3K downstream events

Activation of PI3K yields PIP3 in the cell membrane, which subsequently recruits molecules with a Pleckstrin Homology (PH) domain like threonine/serine kinase (e.g. PDK and PKB; [128]), docking proteins (e.g. Dok, Gab or IRS family members) and tyrosine kinases (e.g. the Tec family). These proteins, when recruited and activated, control major cellular processes like mRNA translation and transcription, for instance through the de-activation of the PI3K dependent forkheads that play a role in cell cycle control and growth arrest during differentiation [126]. However, we focussed on signalling intermediates that could play a role in the immediate control or initiation of receptor signalling strength and duration.

Among these proteins are the Tec-family kinases of which Btk has been shown to be highly expressed in avian erythroblasts [129]. Tec family kinases have been shown to be implicated in signal amplification in response to different receptors [130,131]. The high expression of Btk in erythroblasts suggests a function for this kinase or a related family member in erythropoiesis. However, the downstream substrates of this family of kinases in erythroid progenitors are unknown. We were interested to see whether the Tec-kinase family plays a role in SCF or Epo signal transduction and whether these proteins could be important in the decision between expansion and differentiation. The function and downstream effectors of Btk and its family member Tec have been addressed in chapters 2, 3 and 4.

PH-domain containing docking proteins are also potentially interesting PI3K dependent targets. Similar like the Tec-family kinases, the phosphorylation of docking proteins has also been shown to result in signal enhancement. This is due to their ability to recruit similar signalling intermediates as the studied receptors (for review see [132]). The level of docking-molecule-phosphorylation may thus directly correlate with increased downstream signalling (as is observed after co-stimulation with Epo and SCF) and this could be a crucial mechanism for receptor cooperation. We observed a clear additive effect on the activation of PI3K and the MAPK route during Epo and SCF co-stimulation. Both PI3K and the MAPK route are downstream events of the docking proteins Gab1 and Gab2 (for review see [95]). Indeed, expression of only the PH domain of Gab1, thereby preventing normal Gab1 recruitment, results in attenuation of MAPK and PI3K activation after EGF stimulation [133]. In chapter 5 we addressed the function of these docking proteins in erythroid progenitors. In this light, we were also interested in the kinases responsible for Gab family phosphorylation and activation. In chapter five we found that the Epo-induced phosphorylation of Gab1 can accomplished by two kinases,

the Src kinase Lyn and the tyrosine kinase receptor RON/STK, while Gab2 is exclusively phosphorylated by Lyn. Its function in Gab1 phosphorylation/activation could make RON an important player in signal amplification. This has been addressed in chapter 5.

1.7. Erythropoiesis and disease: Fanconi anemia

A disbalance of red blood cell levels resulting in anemia or erythrocytosis is a major cause of disease related morbidity and mortality worldwide. In many cases anemia is secondary to diseases of other systems and hence may be indirect of nature, like low Epo levels in diabetes related renal failure or malaria. Major erythroid diseases in which anemia is caused by primary defects in the maturation of erythrocytes are sickle cell disease and thalassemias. Other diseases in which anemia is a prominent feature are myelodysplastic syndrome (MDS) and Fanconi disease. It is suggested that the anemia in these diseases is caused by aberrant events at the multi-potent progenitor level rather than an effect on the erythroid progenitor itself. Administration of Epo to Fanconi or MDS patients is not beneficial and in addition, cytokine signalling has been reported to be aberrant in both diseases while BFU-E and CFU-E numbers are severely decreased. Therefore, an aberrant erythroid progenitor phenotype leading to anemia may not be excluded.

Fanconi anemia (FA) is an autosomal recessive disease present in all races and ethnics groups (incidence 3 in 1 million). Patients with FA present in the clinic at early age (median 7 years) and have various symptoms among which decreased platelet counts and aplastic anemia. Before the age of 40, 81% of cases progresses to total bone marrow failure or other hematological abnormalities, which is also the major cause of death (for a review see 134. The actual cause of bone marrow failure and the observed anaemia are not fully understood. FA, at present, has eleven complementation groups: A, B, C, D1, D2, E, F, G , I, J and L most of them defined by cell fusion analysis [135-139]. The core proteins FANCA, B, C, E, F, G and L form a complex in the nucleus upon activation by certain cytokines (TNF-α treatment; [140] or by DNA-crosslinkers, and have a function in DNA (miss-match) repair and chromosomal stability [141,142]; for review see [143]. FANCL, the only Fanc protein with recognizable intramolecular structures (WD40-repeats and a ring finger), is responsible for the mono-ubiquitination of FancD2. Conform the role of the fanconi complex in DNA miss-match repair, the mono-ubiquitinated FANCD2 preferentially associates with chromatin and nuclear matrix, whereas non-ubiquitinated FANCD2 largely resides in the soluble nuclear fraction [144]. Malfunction of this complex is believed to result in chromosomal instability due to accumulation of DNA-damage. From the recently identified FANCI and FANCJ the functions are relatively unknown, though it was reported that FANCI acts upstream and FANCJ downstream of FANCD2 ubiquitination [145].

Apart from its role in DNA-repair and chromosomal stability, a positive role for FANCC has been suggested in activation and recruitment of Stat proteins to the IFN-γ receptor and related to this Fancc knockout mice have increased responses to IFN-γ, due to aberrant feedback control [146,147]. Interestingly, Fanconi deficient cells are highly sensitive to oxidative stress [148,149]. The production of ROS has been shown to influence the activity of phosphatases and may be another way to regulate cytokine receptor signalling. A higher sensitivity to IFN-γ-induced apoptosis due to ROS levels or due to aberrant Stat recruitment may give an alternative explanation to the observed bone marrow failure in fanconi patients. The absence of Fanconi proteins may thus affect cytokine or growth factor receptor signalling. We have examined the erythroid progenitors of Fanconi deficient mice for cytokine signal transduction potential and for their ability to expand and differentiate (chapter 6). This issue has been addressed in

chapter 6.

1.8 Main aim of the thesis

In this thesis we set out to unravel the components of the EpoR/c-Kit signalling complex to gain insight into the intracellular proteins involved in Epo and SCF regulated differentiation, survival and expansion. At present the proteins responsible for these processes are poorly described though recent data indicates that PI3K dependent signal transduction drives the expansion of erythroid progenitors. Absence of these signals results in accelerated differentiation. The signal transducing mediators downstream of PI3K may thus be part of an important switch, responsible for the regulation of the amounts of erythrocytes produced. Due to their described role in signal amplification and mitogenic signalling we focussed on PI3K regulated Tec-family kinases and docking molecules activated by SCF and/or Epo.

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Chapter 2

Stem cell factor induces PI3-kinase-dependent Lyn/Tec/Dok-1 complex formation in hematopoietic cells

Thamar B. van Dijk[1], Emile van den Akker[1], Martine Parren-van Amelsvoort[1], Hiroyuki Mano[2], Bob Löwenberg[1], and Marieke von Lindern[1]

1 Institute of Hematology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands
2 Department of Molecular Biology, Jichi Medical School, Kawachi-gun, Tochigi 329-0498, Japan

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Abstract

Stem cell factor (SCF) has an important role in the proliferation, differentiation, survival, and migration of hematopoietic cells. SCF exerts its effects by binding to c-Kit, a receptor with intrinsic tyrosine kinase activity. Activation of phosphatidylinositol 3'-kinase (PI3-K) by c-Kit was previously shown to contribute to many SCF-induced cellular responses. Therefore we investigated PI3-K-dependent signaling pathways activated by SCF. Here we report the PI3-K-dependent activation and phosphorylation of the tyrosine kinase Tec and the adapter molecule p62Dok-1. We show that Tec and Dok-1 form a stable complex with Lyn and two unidentified phosphoproteins of 56 and 140 kD. Both the Tec homology- and SH2-domain of Tec were identified as being required for the interaction with Dok-1, while two domains in Dok-1 appeared to mediate the association with Tec. In addition, Tec and Lyn were shown to phosphorylate Dok-1, while phosphorylated Dok-1 was demonstrated to bind to the SH2 domains of several signaling molecules activated by SCF, including Abl, CrkL, SHIP, and PLCg-1, but not those of Vav and Shc. These findings suggest that p62Dok-1 may function as an important scaffold molecule in c-Kit-mediated signaling.

Introduction

Stem cell factor (SCF; also called steel factor, mast cell growth factor, or Kit ligand) is an important growth factor for multiple cell types, including hematopoietic progenitor cells. SCF exerts its effects by binding to the product of the c-Kit proto-oncogene[1]. c-Kit is a receptor with intrinsic tyrosine kinase activity, structurally-related to Flt3 and the receptors for colony-stimulating factor-1 (CSF-1) and platelet-derived growth factor (PDGF)[2]. Loss-of-function mutations in the loci for murine c-Kit, White Spotting (W), or SCF, Steel[3-6], lead to macrocytic anemia and to mast cell deficiency, as well as a series of non hematological defects[1,7,8]. Conversely, mutations that render c-Kit constitutively active have been found in mastocytoma and myeloproliferative disease[9,10].

Within the hematopoietic compartment, SCF can support the survival and renewal of the earliest multilineage progenitors and regulates proliferation and differentiation of mast cell precursors. In combination with lineage-restricted cytokines, SCF delays differentiation and enhances the expansion of already committed progenitors of all lineages[1]. This phenomenon is particularly apparent in erythropoiesis, where the co-operation of c-Kit with the erythropoietin receptor (EpoR) is crucial for the proliferation of erythroblasts[11,12]. It has been shown that c-Kit and the EpoR are closely associated in erythroid cells[13], but the molecular basis for the synergy between c-Kit and the EpoR or other cytokine receptors has not been resolved.

The profound effects of SCF on proliferation and survival of different progenitor cell types has raised considerable interest in the identification of critical signal transduction pathways activated by c-Kit. SCF binding to c-Kit results in dimerization/oligomerization and subsequent transient tyrosine phosphorylation of the receptor[14]. As a result, proteins of the p21Ras-MAPK pathway, the phosphatidylinositol 3'-kinase (PI3-K), the tyrosine kinases Src/Fyn/Lyn and Tec, phospholipase Cg-1 (PLCg-1), Vav, Cbl, and p62Dok-1 are recruited to the c-Kit signaling complex and subsequently activated/phosphorylated[15-22]. However, the mechanism by which these molecules are recruited and activated has remained largely unresolved. Although human and murine c-Kit contain 20 intracellular tyrosine residues, only a few were described as docking sites for signaling molecules, when phosphorylated. Tyrosine's 703 and 936 in human c-Kit bind the adapter molecules Grb2 and Grb7, while Src-kinases

bind to tyrosine's 567 and 569 in the membrane proximal region, and p85 binds to tyrosine 719 in the kinase-insert domain of murine c-Kit[16,23,24]. A concerted action of Src kinases and PI3-K appears to be required for the (indirect) activation of the small G-protein Rac and the protein kinase JNK, which is an important step in mast cell proliferation[24].

In vitro studies have shown that activation of PI3-K contributes to survival, mitogenesis, chemokinesis, and differentiation induced by SCF[16,24-26]. Introduction of c-Kit mutants unable to activate PI3-K into c-Kit deficient mast cells does not restore SCF-induced cell adhesion, and only partially restores SCF-induced proliferation[16]. Furthermore, we recently observed that the PI3-K inhibitor LY294002 suppressed the biological effect of SCF on erythroid progenitors (manuscript in preparation). PI3-K yields PIP3 in the cell membrane, thereby creating binding sites for pleckstrin homology (PH)-domain containing signaling molecules[27]. The tyrosine kinase Tec is one such PH-domain containing protein activated by c-Kit[18]. Tec is also the founding member of a family of tyrosine kinases that includes Brutons' tyrosine kinase (Btk), Bmx, Itk/Tsk/Emt, and Rlk/Txk[28,29]. These kinases are characterized by a PH- and a Tec homology (TH)-domain in their amino-terminus, followed by SH3-, SH2-, and kinase domains[30]. In contrast to Src-kinases, they are devoid of a membrane-targeting myristylation site, and yet are rapidly recruited to the plasma membrane after stimulation of various cytokine receptors, the T and B cell receptors, and CD28[31-35]. The interaction between PIP3 and the PH-domain of Btk is critical for this translocation[36]. In the plasma membrane, Tec members are thought to be activated by Lyn or other members of the Src family[37,38]. Other studies have shown that the TH-domain of Tec is essential for the (in)direct interaction with c-Kit, but also for its association with Lyn and Vav[33,39].

In this study we show that Tec is activated by SCF in erythroid and megakaryocytic cell lines and that, when activated, it forms a stable complex with various proteins, including the recently cloned docking protein p62Dok-1. We found that both the activation of Tec and the phosphorylation of Dok-1 require PI3-K activity. We show that the TH- and SH2-domains of Tec mediate the interaction with Dok-1, while two separate domains in Dok-1 are involved in the interaction with Tec. We further show that Tec, and also Lyn, can phosphorylate Dok-1. Dok-1 contains 15 tyrosine residues, suggesting an important role for Dok-1 in recruiting SH2-domain containing proteins to the c-Kit signaling complex. In support of this hypothesis, we provide evidence that phosphorylated Dok-1 binds the SH2-domains of multiple signaling molecules.

Materials and methods

Cells, plasmids, and oligonucleotides

The human erythroleukemia cell lines F36P and TF-1, and the megakaryocytic cell line Mo7e, were maintained in RPMI 1640 medium (Life Technologies; Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Life Technologies) and recombinant human GM-CSF (5 ng/ml; Immunex; Seattle, WA). COS, 293, and amphotropic Phoenix cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FCS.

The complete cDNAs of murine Tec, human c-Kit, and human Dok-1 (a kind gift of Nick Carpino, St. Jude Children's Hospital, Memphis, Tennessee) were cloned in the expression vector pSG5 (Stratagene, La Jolla, CA) and the retroviral vector pBabe[40]. The expression vectors encoding Tec mutants and Δp85 have been described previously[41,42]. Constructs DokΔY1

and DokΔY5 were generated by PCR using the oligonucleotides Dok-F (5'-CGG AAT TCC CGG GGG CCA TGG ACG GA-3'), Dok-ΔY1 (5'-AAA ACT GCA GGT TAA CGG CTC TAC AAG GAG TTC TCC AGC-3'), and Dok-ΔY5 (5'-AAA ACT GCA GGT TAA CCA GCC TAC AGG GCT GGG GGA CTG-3') and the Expand High Fidelity PCR System (Roche; Basel, Switzerland). Fragments were cloned in the *EcoRI/PstI* sites of a modified pSG5. Constructs tagged with the hemagglutinin (HA) epitope, HA-Dok, HA-DokΔY5, and HA-DokΔY1 respectively, were generated by subcloning PCR products into pMT2SM-HA vector. The *PstI* fragments of pMT2HA-Dok and pMTHA-DokΔY5 were subcloned into pBABE to generate pBHA-Dok and pBHA-DokΔY1, respectively.

Transient transfection and viral transduction

For transfection experiments, COS cells were cultured in six-well dishes (Costar; Corning, NY), and 293 cells were cultured in 20 cm^2 dishes (Becton Dickinson, Franklin Lakes, NJ). After 24 hours, the cells were transfected with 6-12 mg of supercoiled plasmid DNA as described previously[43]. 16-20 hours after transfection, medium was refreshed and cells were harvested 24 hours later.

For viral transduction experiments, amphotropic Phoenix cells were cultured in six-well dishes and, 3 hours later, the cells were transfected using calcium phosphate. After 48 hours, cells were treated with mitomycin C (10 mg/ml) for 1 hour, washed three times and washed three more times 4 hours later. F36P cells ($2x10^5$/ml) were added and co-cultured for 20-24 hours in RPMI/DMEM medium (50/50%). F36P cells were removed carefully from the Phoenix cells and cultured in RPMI 1640 medium. To select for stable transfected cells, puromycin (2 mg/ml) was added 48 hours later.

Immunoprecipitations, western blotting, and antibodies

After serum starvation for 16 hours, F36P, Mo7e, and TF-1 cells (30x 106/ml) were stimulated with SCF (100 ng/ml; a generous gift of Amgen; Thousend Oakes, CA), EPO (5 U/ml; a generous gift of Janssen-Cilag; Tilburg, The Netherlands), or GM-CSF (50 ng/ml), or left unstimulated for 5 (SCF) or 10 (EPO and GM-CSF) minutes at 37°C. Reactions were stopped by adding ice-cold phosphate-buffered saline (PBS). Cells were lyzed in lysisbuffer (1% NP-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10 mM EDTA, and 10% glycerol, supplemented with Complete protease inhibitor mix (Roche), Pefablock (Merck; Darmstadt, Germany), and 1 mM orthovanadate) on ice for 15 minutes and centrifuged at 4°C for 10 minutes at 15000 rpm. Lysates of 15 x 10^6 cells were pre-cleared with protein G beads (Sigma; St. Louis, MO), incubated with the appropriate antibody (1 mg) for 90 minutes at 4°C and protein G beads were added for an additional hour. Precipitates were washed 3 times with lysis buffer, subjected to SDS-polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose membrane (Schleicher & Schuell; Dassel, Germany). Membranes were blocked in 0.6% bovine serum albumine (BSA), incubated with appropriate antibodies, and developed utilizing enhanced chemoluminescence (ECL, NEN; Boston, MA).

The following antibodies were used in this study: anti-Tec (06-561, Upstate Biotechnology; Lake Placid, NY), anti-TecSH331, anti-Dok-1 (M19, Santa Cruz (SC); Santa Cruz, CA), anti-phosphotyrosine (PY99, SC), anti-HA (F-7, SC), anti-Lyn (Transduction Laboratories; Lexington, KY), anti-phospho-PKB (Ser473; New England BioLabs (NEB); Beverly, MA), anti-PKB (NEB), anti- phospho-ERK1,2 (Thr202/Tyr204; E10, NEB), and anti-ERK1,2 (K-23, SC).

GST-pull down experiments

For SH2-domain binding studies, SCF-stimulated Mo7e cells were lyzed in immunoprecipitation buffer. Cleared lysates were incubated for 90 minutes with beads coupled to 10 mg of GST-SH2 fusion protein or to GST alone. Beads were washes three times with lysis buffer and resuspended in sample buffer. Proteins were separated by SDS-PAGE and blotted with anti-phosphotyrosine antibody. The GST-fusions with SH2 domains of the following proteins were used in this study: cAbl, CrkL, Fgr, Fps, GAP (both N- and C-terminal SH2 domains (N+C)), Grb14, Hck, Lyn, p85 subunit of PI3-K (SH2 N+C), PLCγ-1 (SH2 N+C), Shc, SHIP, Src, Syk (SH2 N+C), Vav, and Yes.

Results

Tec is activated in erythroid cells and complexes with various proteins following SCF addition
Tec has previously been shown to be activated by SCF in the human megakaryocytic cell line Mo7e[18]. To investigate whether Tec is specifically activated upon c-Kit activation and to identify associating proteins, Tec was immunoprecipitated from the human erythroid progenitor cell line F36P stimulated with SCF, EPO, or GM-CSF, from Mo7e cells stimulated with SCF or GM-CSF, and from human erythroleukemic TF-1 cells stimulated with SCF. Subsequently, Tec and co-precipitating proteins were immunoblotted with anti-phosphotyrosine antibody (PY99). As shown in figure 1a, phosphorylation of Tec in F36P cells is only detected following SCF stimulation, but not following incubation of the cells with EPO or GM-CSF, even though these cytokines induced tyrosine phosphorylation of multiple proteins (figure 1a, lower panel). An in vitro kinase assay confirmed that the tyrosine phosphorylation of Tec correlated with enhanced kinase activity of Tec (not shown). Interestingly, a prominant phosphorylated protein of 62 kD as well as proteins of 145-150, 140, and 56 kD were precipitated with Tec after SCF induction. The proteins of 145-150 kD were identified as c-Kit by Western Blot

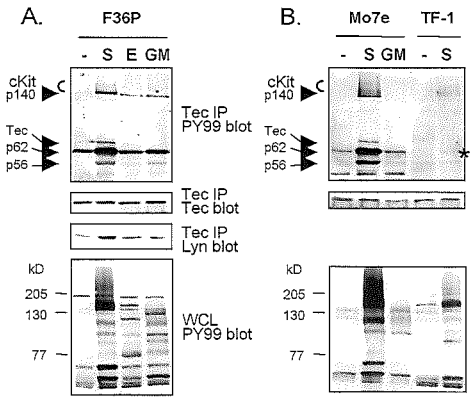

Figure 1. Tec is activated in erythroid cells and complexes with various proteins following SCF addition.
A and B. Tec was immunoprecipitated from F36P, Mo7e, and TF-1 cells incubated with SCF (S; 5 minutes), EPO, or GM-CSF (E, GM; 10 minutes) at 37°C. The immunoprecipitates (15 x 10^6 cells each) were analyzed by Western blotting with anti-phosphotyrosine (PY99; top panels) and anti-Tec (middle panels). Tec-precipitates from F36P cells were also analyzed with anti-Lyn (middle panel). As a control, whole cell lysates were analyzed with anti-phosphotyrosine (lower panels). Tec is specifically phosphorylated by SCF and complexes with c-Kit and proteins of 56, 62, and 140 kD following SCF addition (indicated by arrows).

analysis (not shown). Since p56Lyn is known to associate with Tec39, we tested whether the co-precipitating phosphoprotein of 56 kD was Lyn. Although the lower panel indeed shows that SCF treatment results in enhanced Tec-Lyn association, Lyn had a slightly faster mobility than p56. Therefore, the identities of p56 and p140 remain unknown.
Tec is also selectively phosphorylated by SCF in Mo7e cells (figure 1b). In these cells, a similar pattern of co-precipitating proteins is observed, indicating that the same signaling complex is formed after c-Kit activation in F36P and Mo7e cells. Although less clear, c-Kit and p62 (marked with an asterisk) were also detected in the Tec precipitates of SCF-stimulated TF-1 cells. The signal in these cells is less intensive, most likely due to the lower level of activated c-Kit when compared with F36P and Mo7e, as is apparent from the whole cell lysate controls.

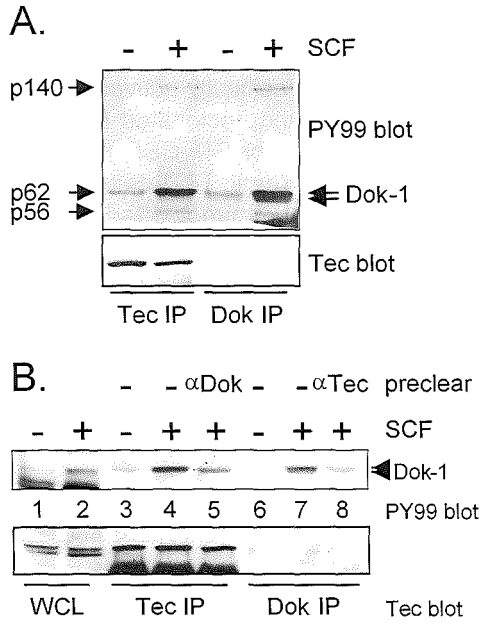

Figure 2. The p62 co-precipitating with Tec is p62Dok-1.
A. Tec and Dok-1 were immunoprecipitated from whole cell lysates of non-stimulated and SCF-incubated F36P cells and analyzed by Western blotting with anti-phosphotyrosine (PY99; top panel) and anti-Tec (lower panel). Two species of Dok-1 are phosphorylated by SCF (indicated by arrows), of which the upper one co-migrates with Tec-associated p62. Proteins of 56 and 140 kD are observed in both Tec and Dok-1 precipitates of SCF-treated cells (indicated by arrows).
B. Lysates of SCF-stimulated F36P cells were precleared with anti-Dok-1, followed by Tec immunoprecipitation, or vice versa. The lysates were analyzed by Western blotting with anti-phosphotyrosine (top panel) and anti-Tec (lower panel). Pretreatment of the lysates reduces the amount of phosphorylated p62/Dok-1, indicating that p62 is identical to the slower migrating species of Dok-1.

The p62 co-precipitating with Tec is p62Dok-1
The most prominent tyrosine phosphorylated protein in Tec precipitates is a protein of 62 kD. Recently, Dok-1, a docking protein of 62 kD, was cloned from chronic myelogenous leukemia progenitor cells and from v-Abl-transformed B cells22,44. In these cells, Dok-1 is constitutively phosphorylated. To examine whether Tec-associated p62 is identical to Dok-1, both Tec and Dok-1 were precipitated from SCF-stimulated F36P cells, blotted, and incubated with

PY99. Figure 2a shows that p62 exactly co-migrates with the slower migrating species of Dok-1. Strikingly, proteins of 140 and 56 kD co-precipitate with Dok-1, similar to what is observed when Tec is immunoprecipitated. These results suggest that (partially) identical complexes are precipitated with anti-Tec and anti-Dok sera. Reprobing of the blot with anti-Tec serum showed that Tec was present in the Tec-immunoprecipitations (figure 2a, lower panel). However, Tec was never detected in Dok-1 precipitations, possibly due to sterical hindrance of the antibody. Therefore, we assume that the co-precipitating p56 and p140 associate with Dok-1 rather than with Tec.

The anti-Dok-1 antibody could not be used in Western Blot analysis, but worked well in immunoprecipitations. To demonstrate that p62 is indeed Dok-1, lysates of SCF-stimulated F36P cells were first cleared with anti-Dok-1, followed by a Tec precipitation, and vice versa. If Tec and Dok-1 associate, the signal of p62/Dok-1 should be decreased in Tec- and Dok-1 precleared precipitates. In fact, significantly less p62 co-precipitates with Tec in lysates precleared from Dok-1 (figure 2b, lanes 4 and 5), while there is almost no p62Dok remaining left in a Dok-1 precipitate after the lysate was precleared with anti-Tec (figure 2b, lanes 7 and 8).Together, these data are consistent with the notion that the Tec interacting protein of 62 kD is identical to Dok-1.

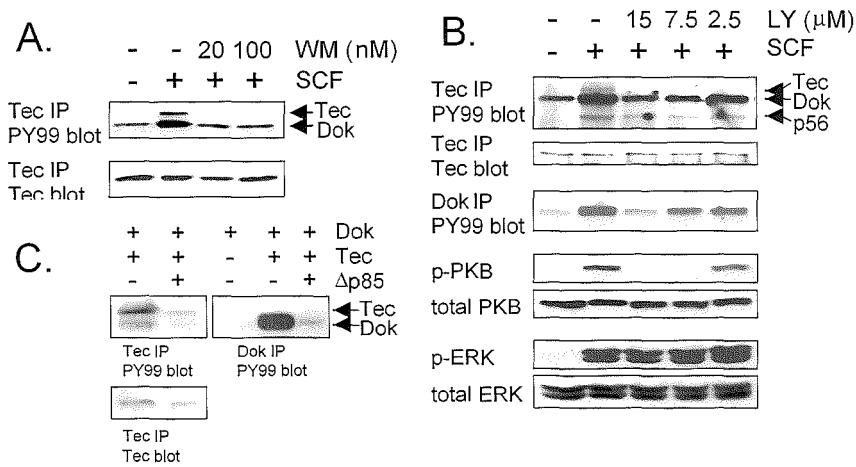

Figure 3. Tec and Dok phosphorylation is dependent on PI3-K activity.
F36P cells were incubated with different concentrations of the PI3-K inhibitors wortmanin (WM; 20 minutes; fig. A) or LY294002 (LY; 60 minutes; fig. B) at 37°C, after which the cells were incubated with SCF for 5 minutes.
A. Tec immuneprecipitates were analyzed by Western blotting with anti-phosphotyrosine (top panel) and anti-Tec (lower panel). WM clearly inhibits Tec and Dok-1 phosphorylation.
B. Lysates were incubated with anti-Tec and anti-Dok-1. Tec immune-precipitates were analyzed by Western blotting with anti-phosphotyrosine and anti-Tec, Dok-precipitates were analyzed with anti-phosphotyrosine. LY clearly inhibits Tec and Dok-1 phosphorylation. Whole cell lysates (1 x 10^6 cells) were also analyzed with anti-phospho-PKB and anti-phospho-ERK as control for the specificity of LY. LY inhibits PKB phosphorylation but not ERK phosphorylation.
C. 293 cells were transfected with expression vectors encoding Tec, Dok-1, or Δp85, or with empty vector (-). Tec and Dok-1 immuneprecipitates were analyzed by Western blotting with anti-phosphotyrosine (top panels) and anti-Tec (lower left panel). Tyrosine phosphorylation of Tec and Dok-1 can be blocked by co-expression of dominant negative PI3-K (Δp85).

Tec and Dok phosphorylation is dependent on PI3-K activity

Like Tec, Dok-1 also contains a PH-domain at its N-terminus, suggesting that recruitment of both proteins to the c-Kit signaling complex could depend on PI3-K activation. To inhibit PI3-K activity both the fungal metabolite wortmannin (WM) and the synthetic inhibitor LY294002 (LY) were employed. Pretreatment of the cells with 20 nM WM (20 minutes) or 15 μM LY (60 minutes) was sufficient to almost completely block the SCF-induced phosphorylation of Tec and the co-precipitating Dok-1 (figures 3a and 3b). As controls, phosphorylation of the classical PI3-K target, protein kinase B (PKB), and ERK were assessed. At 15 μM LY, phosphorylation of PKB was completely blocked as determined with specific antibodies against phospho-PKB (Ser473) (figure 3b) and phospho-PKB (Thr308) (not shown). In contrast, this concentration of LY had no effect on the SCF-induced activation of Erk1/2 (lower panel). These results demonstrate the specificity of the PI3-K inhibitors and suggest that PI3-K activation has to precede Tec/Dok phosphorylation, most likely to recruit these proteins to the signaling complex. In addition, since Erk1/2 phosphorylation is not affected by LY, it appears that SCF-induced activation of the Ras-MAP kinase pathway is independent of Dok-1 phosphorylation. In addition to pharmacological inhibitors, a dominant negative PI3-K construct (Δp85) was used to determine the role of PI3-K in the phosphorylation of Tec and Dok-1. Tec, Dok-1, and Δp85 were transiently co-expressed in various combinations in 293 cells. Figure 3c shows that both Tec and Dok-1 are heavily phosphorylated on tyrosine residues when co-expressed. Interestingly, the phosphorylation of both Tec and co-precipitated Dok-1 is reduced when also Δp85 is transfected (lanes 1 and 2). Furthermore, immuneprecipitation of Dok-1 shows that also the phosphorylation of Dok can be blocked by co-transfection of Δp85 (lanes 4 and 5). This indicates that PI3-K activity is required for the phosphorylation of Tec and Dok-1 in 293 cells.

Dok-1 is a substrate of Tec and Lyn in 293 cells

To study the interaction of Tec and Dok-1, transient transfection experiments were performed in 293 and COS cells. As shown in figure 4a, immunoprecipitation of Tec from transfected cells results in the detection of a single phosphorylated protein, corresponding to Tec (lane 2). When Dok-1 is co-expressed (lane 3), a 62 kD phosphoprotein is co-precipitated, indicating that Tec and Dok-1 can form a complex in an overexpression system. However, relatively little phosphorylated Dok-1 is precipitated with Tec in this system when compared to F36P and Mo7e cells. Notably, co-expression of Dok-1 reproducibly enhanced the phosphotyrosine content of Tec (compare lanes 2 and 3), suggesting that Dok-1 enhances the auto- or cross-phosphorylation of Tec, or recruits an endogenous kinase that can phosphorylate Tec. The tyrosine kinase Lyn has been shown to phosphorylate Tec37 and it was detected in a complex of Tec and Dok-1 in F36P cells (figure 1a). Therefore, Lyn was coexpressed with Tec and Dok-1 in 293 cells. Lyn increased Tec phosphorylation (figure 4a, compare lanes 2 and 4) and enhanced the intensity of Dok-1 detected by anti-phosphotyrosine antibodies in a Tec immunoprecipitation (compare lanes 3 and 5). The latter may be due to Lyn-induced increased Dok-1 phosphorylation and/or increased stability of the Tec/Dok-1 complex. To examine an effect of Lyn on the Tec/Dok-1 interaction, Tec was coexpressed with a Dok-1 tagged at the N-terminus with the hemagglutinin (HA) epitope, which allowed detection of total Dok-1 on Western Blots. More HA-Dok was co-precipitated with Tec when Lyn was co-transfected (figure 4b, lanes 3 and 4), suggesting that Lyn stabilizes the Tec/Dok-1 interaction. To analyze whether the association of Tec and Dok-1 is similarly enhanced through SCF-signaling, Tec was immunoprecipitated from F36P cells stably transfected with HA-Dok.

Activation of c-Kit indeed increased the association of Dok-1 and Tec (figure 4c). These data suggest that Lyn plays an important role in SCF-induced Tec/Dok-1 complex formation, which could be due to phosphorylation

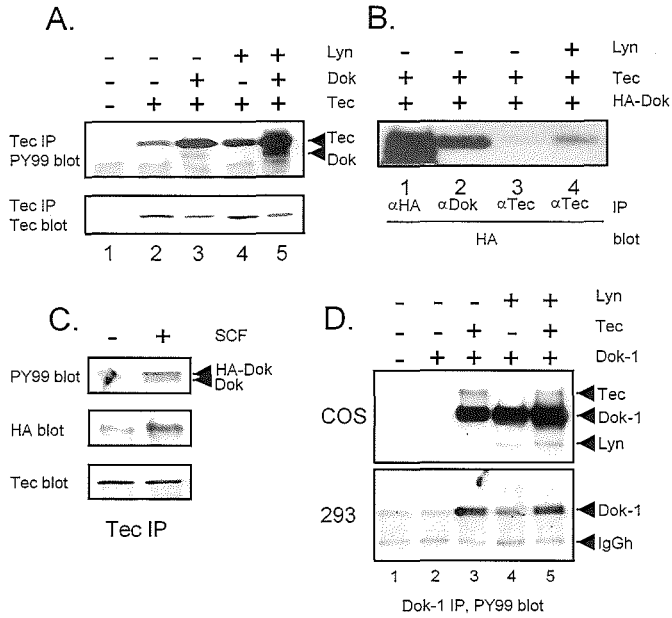

Figure 4. Dok-1 is a substrate of Tec and Lyn in 293 cells.
A. 293 cells were transfected with expression vectors encoding Tec, Lyn, or Dok-1, or with empty vector (-). Tec immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine (top panel) and anti-Tec (lower panel). Dok-1 co-precipitates with Tec, especially in the presence of Lyn.
B. 293 cells were co-transfected with HA-tagged Dok-1 and Tec, together with (+) or without (-) Lyn. Lysates were incubated with anti-HA, anti-Dok-1, or anti-Tec, after which the precipitates were analyzed with anti-HA. Lyn stabilizes the interaction between Tec and HA-Dok-1.
C. F36P cells were stably transfected with HA-Dok-1. Tec was precipitated from lysates of non-stimulated (-) or SCF-treated (+) cells, after which the precipitates were analyzed with anti-phosphotyrosine (upper panel), anti-HA (middle panel), and anti-Tec (lower panel). More HA-tagged Dok-1 is precipitated after SCF stimulation, indicating that the association of Tec and Dok-1 is stabilized within the c-Kit signaling complex.
D. COS cells (upper panel) and 293 cells (lower panel) were transfected with empty vector (-) or a Dok-1 expression plasmid (+), or co-transfected with Tec and/or Lyn. Dok-1 immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine (top panel). In COS cells, both Tec and Lyn can phosphorylate Dok-1, while in 293 cells Dok-1 is a better substrate for Tec than for Lyn.

of Dok-1 allowing interaction of the Tec SH2-domain with the phoshotyrosines of Dok-1.
To examine whether Lyn is able to phosphorylate Dok-1 directly, Lyn and Tec were coexpressed with Dok-1 in COS and 293 cells. In COS cells, both Tec and Lyn induced massive Dok-1 phosphorylation, while they had an additive effect when co-transfected (figure 4d, upper panel). Under these conditions, both Tec and Lyn co-precipitated with Dok-1 (indicated with arrows). In 293 cells, the SV40-driven promoter on the pSG5 plasmid is less active, resulting in a more modest expression of Tec and Lyn. In these cells, Dok-1 is a better substrate for Tec than for Lyn (figure 4d, lower panel, lanes 3 and 4). Moreover, Lyn had no additive effect on Tec-mediated Dok phosphorylation (lane 5). Thus, although Lyn can phospho

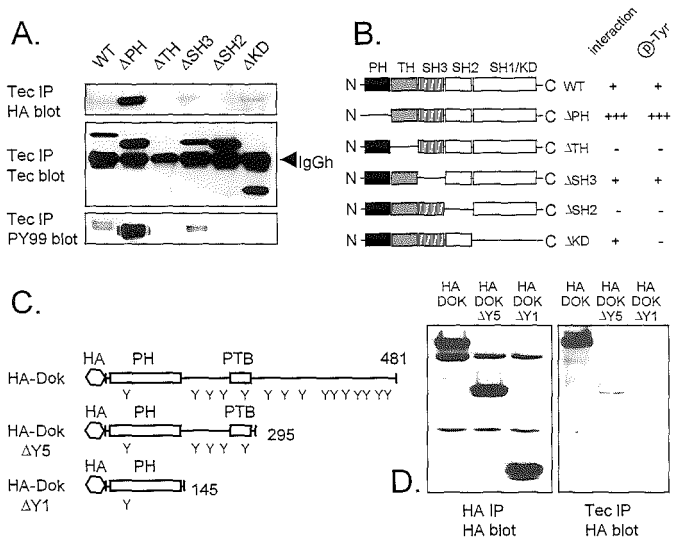

Figure 5. Domains involved in Tec/Dok association.
A. 293 cells were co-transfected with HA-Dok-1 and different Tec mutants. Tec immuneprecipitates were analyzed by Western blotting with anti-HA (top panel), anti-Tec (middle panel), and anti-phosphotyrosine (lower panel). The TH- and SH2 domains of Tec are crucial for the direct interaction with HA-Dok-1.
B. Schematic representation of the Tec mutants used and their Dok-1 interaction- and phosphorylation properties.
C. Schematic representation of full length HA-Dok-1, HA-DokΔY5, and HA-DokΔY1. Tyrosine residues (Y), the PH- and PTB domains, and total number of amino acids are indicated.
D. 293 cells were co-transfected with Tec and different HA-Dok-1 deletion constructs. Lysates were incubated with anti-HA for expression control and with anti-Tec. Precipitates were analyzed with anti-HA. Although the HA-Dok deletion constructs are equally expressed (left panel), only full length HA-Dok and a reduced amount of HA-DokΔY5 are detected in Tec precipitates (right panel), indicating that two domains in Dok-1 are involved in the binding to Tec.

rylate Dok-1, its tyrosine phosphorylation appears to depend mainly on Tec activity.
In conclusion, activation of c-Kit stabilizes the interaction between Tec and Dok-1, possibly by Lyn mediated Dok-1 phosphorylation. Subsequently, Tec phosphorylates Dok-1 to yield highly tyrosine phosphorylated Dok-1.

Domains involved in Tec/Dok association
Given the interaction observed between Tec and Dok-1, we next examined which domains were involved. Wild type (wt) Tec and Tec deletion constructs (see figure 5b for a schematic representation) were co-expressed with HA-Dok-1 in 293 cells. Subsequently, Tec precipitates were blotted and probed with anti-HA- and anti-phosphotyrosine antibodies. Wt Tec, TecDSH3, and TecDKD bind equal amounts of HA-Dok-1 (figure 5a, upper panel). In contrast, TecDPH is much more powerful in HA-Dok-1 binding, while no HA-Dok-1 is precipitated with TecDTH and TecDSH2. Similar results are obtained when the blot is probed with PY99, i.e., an equal Dok-1 phosphorylation by wt Tec and TecDSH3, enhanced phosphorylation with TecDPH, and no phosphorylation with TecDTH, TecDSH2. The only difference concerned TecDKD (no kinase activity), which failed in Dok-1 phosphorylation even though it precipitated HA-Dok-1, showing that indeed Tec kinase activity is responsible for the phosphorylation of HA-Dok-1. The detection of high levels of phosphorylated Dok-1 by TecDPH is most likely due to enhanced Dok-1 binding (upper panel). Expression of Tec-constructs was controled with an antibody against the TH-domain; therefore, TecDTH could not be detected (figure 5a).

Staining the blot with an antibody against the SH3 domain of Tec showed that TecDTH was expressed comparable to wt Tec (not shown). These data show that two independent domains of Tec, the TH and the SH2 domain, interact with Dok?1.

Dok-1 is a docking protein that contains a PH domain, a phosphotyrosine binding (PTB) domain, 15 tyrosine residues, and 10 proline motifs (PXXP). The phosphorylated tyrosines can act as SH2-binding sites, while the PXXP motifs mediate binding to SH3 domains. To roughly map the region(s) of Dok-1 that associate with Tec, two progressive deletion constructs were created (see figure 5c). In HA-DokDY5 193 amino acids were deleted at the C-terminus, which removes 10 tyrosine residues and 5 PXXP motifs. HA-DokDY1 consists of only the PH domain, one tyrosine and two PXXP motifs. The HA-Dok constructs were co-transfected with Tec in 293 cells. Lysates were incubated with anti-HA antibody as control (figure 5d, left panel), and with anti-Tec to detect the Tec/Dok interaction (right panel). The interaction of Tec with full-length Dok-1 is easily detected, while no HA?DokDY1 is observed in Tec precipitates. HA?DokDY5 also associates with Tec, although at a low level, suggesting that two domains in Dok-1 cooperate in binding to Tec. Alternatively, multiple phosphotyrosines of Dok-1 are able to bind the SH2 domain of Tec.

SH2 domains of distinct signaling molecules bind to phosphorylated Dok-1. We demonstrated that SCF induces the complex formation of Dok-1 with Tec and Lyn, and that Dok-1 is subsequently phosphorylated on tyrosine residues. Phosphorylated tyrosines act as binding sites for SH2-containing proteins. To determine which signaling intermediates could bind to phosphorylated Dok-1, glutathione S-transferase (GST) fusion proteins containing the SH2 domains from a range of signaling molecules were incubated with lysate from SCF-stimulated Mo7e cells.

The SH2 domains of the Src-family members Src, Fgr, Hck, and Yes, the tyrosine kinase cAbl, the adapter CrkL, rasGAP(SH2 N+C), the p85 subunit of PI3-K(SH2 N+C), PLCg(SH2 N+C), and SHIP bind efficiently to phospho-Dok-1 (figure 6). However, the SH2 domains of Fps, Grb14, Shc, Syk(SH2 N+C), and Vav show little or no association with Dok-1, illustrating a

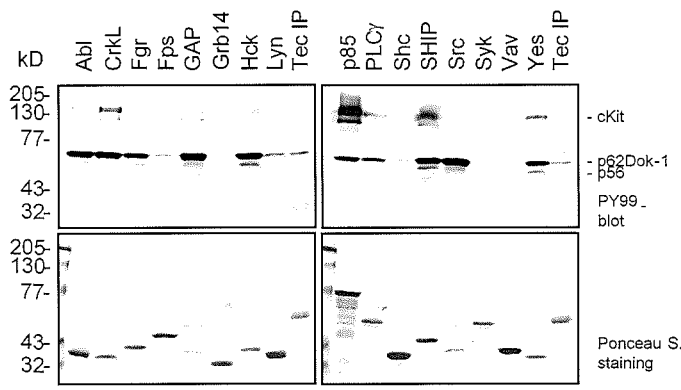

Figure 6. SH2 domains of distinct signaling molecules bind to phosphorylated Dok-1.
GST-fusion proteins containing the SH2-domains of several signaling molecules were incubated with lysates of SCF-induced Mo7e cells. As a reference for p62Dok-1, a Tec immunoprecipitate was included. The precipitates were analyzed by Western blot analysis with an antiphosphotyrosine antibody (upper panels). The blots were stained with Ponceau S. as a control for the amount of added GST-fusion protein (lower panels). Phosphorylated Dok-1 binds to the SH2 domains of various signaling intermediates.

high level of substrate-specificity. Precipitates that contain Dok-1 also include proteins of 145-150, 140, and 56 kD, although with different ratio's. It cannot be excluded that some of the GST-SH2 fusion proteins directly bind to c-Kit and thereby indirectly precipitate Dok-1. However, these results strongly suggests that Dok-1 can recruit a variety of signaling proteins and therefore that Dok-1 may play an important role in SCF-mediated signaling.

Discussion

Various studies have shown that SCF-mediated proliferation, survival, adhesion, migration, and differentiation depend on PI3-K activity[16,24-26]. PI3-K yields PIP3 in the cell membrane, which can subsequently recruit PH-domain containing signaling molecules[27]. One such protein activated by SCF is the tyrosine kinase Tec. We found that activation of c-Kit induces both Tec activation and the formation of a complex of Tec with c-Kit, Lyn, p62Dok-1, and two unidentified proteins of 56 and 140 kD, respectively. By using pharmacological inhibitors and overexpression of Δp85, a dominant negative PI3-K construct, we determined that both complex formation and phosphorylation require PI3-K activity. The Δp85 transfection was done in a transient assay, since we failed to generate cell lines that stably expressed Δp85 or c-KitY721F, a c-Kit mutant unable to activate PI3-K. We propose a model for Dok-1 in SCF signaling as shown in figure 7. This model involves the activation of PI-3K and Src-family members (e.g. Lyn) following c-Kit stimulation. The PH-domain-containing proteins Tec and Dok-1 are recruited to the plasma membrane where they co-localize with activated Lyn. Lyn associates with Tec and promotes its kinase activity. Subsequently, Tec and/or Lyn phosphorylate Dok-1, creating binding sites for SH2-containing proteins. It is possible that such Dok-1-interacting proteins are phosphorylated by Tec and/or Lyn as well.

Tec and Dok-1 are partially associated in non-stimulated serum-starved F36P and Mo7e cells (figure 1), indicating that the interaction is relatively stable in vivo. In co-transfection studies, however, the Tec/Dok-1 association is rather weak, although Dok-1 is heavily phosphorylated by Tec when co-expressed (figure 4a and 4d, respectively). Using an HA-tagged Dok-1 construct it was shown that c-Kit stimulation stabilizes the association between Tec and Dok (figure 4c). This implies that additional proteins are necessary to allow the formation of a stable complex. Our results suggest that Lyn is such a protein (figure 4b). Furthermore, Lyn also phosphorylates Dok-1 (figure 4d), while the SH2 domain of Tec is crucial for its association with Dok-1 (figure 5a). One possible mechanism explaining these results is that Lyn phosphorylates a subset of tyrosines in Dok?1, including the one(s) that mediate(s) the interaction with Tec. In turn, Tec may phosphorylate another subset of tyrosines of Dok-1. To further clarify the role of Tec in the phosphorylation of Dok-1 in vivo, we tried to stably transfect F36P cells with a Tec construct lacking its kinase domain. However, in all clones obtained the expression level of this mutant was too low to act as a dominant negative. It can therefore not be excluded that Src-family members fully account for the phosphorylation of Dok-1 in vivo. During our studies, Tec was identified as a possible kinase for CD28-mediated Dok-1 phosphorylation, while a Dok-related protein (Dok-R or p56Dok-2) has been reported as a direct target of Lyn[35,45]. These data are consistent with the conclusion that Dok-1 and Dok-related proteins are substrates for both the Tec- and Src-families of tyrosine kinases.

Dok-1 was first identified as a tyrosine phosphorylated protein of 62 kD associated with p120-RasGAP in fibroblasts transfected with v-Src46. In BCR-Abl transformed cells, Dok-1 also binds to RasGAP in a tyrosine phosphorylation-dependent manner. However, RasGAP was

not detected in Tec- and Dok-1-precipitates, although RasGAP was detectable in RasGAP precipitates and in whole cell lysate controls (data not shown). In contrast, we detected a tyrosine-phosphorylated protein of 140 kD in Tec or Dok-1 precipitates of SCF-stimulated cells. Interestingly, a protein of about the same size was shown to bind to Dok-R in EGF-stimulated cells[45]. A protein that is activated by SCF and has a molecular weight of 150 kD is the inositol (data not shown). In contrast, we detected a tyrosine-phosphorylated protein of 140

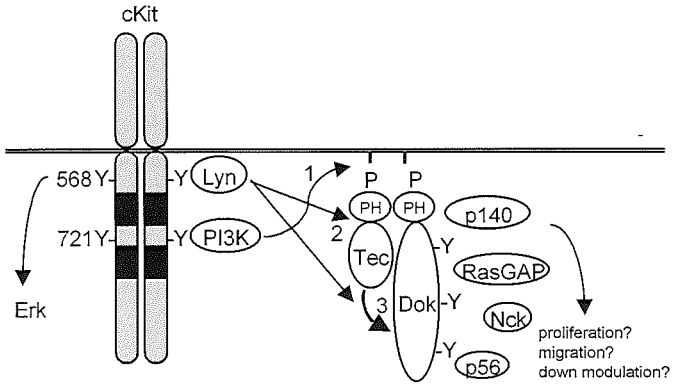

Figure 7. Model for Tec/Dok-1 phosphorylation in SCF-induced signaling.
See Discussion for details.

kD in Tec or Dok-1 precipitates of SCF-stimulated cells. Interestingly, a protein of about the same size was shown to bind to Dok-R in EGF-stimulated cells[45]. A protein that is activated by SCF and has a molecular weight of 150 kD is the inositol phosphatase SHIP-1. Therefore, we are currently investigating the role of this protein in the Dok-1 complex. In addition, the identity of p56 remains to be defined. Both p56Lyn and p52Shc are known to bind to Tec, but have another electrophoretic mobility than Dok-bound p56 (data not shown).

Still little is known about the physiological role of Dok-1. Dok-1 is directly associated with v-Abl and constitutively phosphorylated in chronic myelogenous leukemia cells[44,47]. Furthermore, the extent of tyrosine phosphorylation of Dok-1 has been shown to correlate with the transforming capacities of a number of different oncogenes, including v-Src, v-Fps, v-Fms, and v-Abl[46]. Because of the frequently noted correlation between constitutive tyrosine phosphorylation of Dok-1 and cellular transformation it has been suggested that Dok-1 plays an important role in mitogenic signaling. This idea is in agreement with the observations that Dok-1 is phosphorylated in response to SCF and that SCF primarily serves as a potent proliferation factor in hematopoietic progenitor cells. The results of other studies have suggested that Dok-1 may also play a role in cellular migration responses. Very recently, Noguchi et al showed that overexpression of wt Dok-1 enhanced insulin-induced migration, while this was not observed with DokY361F, a Dok-1 mutant unable to bind Nck[48]. Nck is an adapter molecule that links receptors to p21cdc42/Rac-activated kinase (PAK) and the Wiskott-Aldrich Syndrome protein (WASP)-WIP (WASP-interacting protein) complex, all of which contribute to changes in the actin cytoskeleton[49,50]. Furthermore, Rac has been shown to be activated by c-Kit via a Src- and PI3-K-dependent mechanism and plays an important role in SCF-induced proliferation of bone marrow derived mast cells[24]. One may speculate that the Tec/Dok-1 complex recruits and activates the components that regulate the (de) polymeriza-

tion of actin filaments, thereby regulating cell migration.
Dok-1 contains a phosphotyrosine binding (PTB) domain, 15 tyrosine residues, and 10 proline motifs (PXXP) and has an overall structure related to the insulin receptor substrates (IRS) 1-4 and GAB, all of which serve as docking molecules[51]. By recruiting subsets of signaling molecules into an activated receptor-complex, docking molecules play a key role in the coordination of the cellular response. Others have shown that p120RasGAP and Nck directly bind to Dok-1[22,48], while our results indicate that Tec as well as at least two unidentified proteins, associate to Dok-1. We further showed that many SH2 domains of proteins functioning in different signaling routes form a complex with Dok-1. These include the SH2 domains of Abl, CrkL, PLCγ-1, SHIP, and the p85 subunit of PI3-K, all proteins known to be activated by SCF[16,19,21,52]. Since CrkL is a direct target of BCR-Abl[53], it is possible that Dok-1 plays a role in the recruitment of kinases (Tec, Lyn, Abl) and their substrates. With the notion that the pleiotropic effects of SCF are largely dependent on PI3-K, as is the phosphorylation of Dok-1, we postulate that Dok-1 (and its phosphorylation control) is an important regulator of SCF-induced signal transduction.

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Chapter 3

Btk is required for an efficient response to erythropoietin and for SCF-controlled protection against TRAIL in erythroid progenitors.

Emile van den Akker[1]§*, Uwe Schmidt[2]§, Martine Parren-van Amelsvoort[1], Gabi Litos[2], Marella de Bruijn[3]#, Laura Gutiérrez[3], Rudi W. Hendriks[4], Wilfried Ellmeier[5], Bob Löwenberg[1], Hartmut Beug[2]§, Marieke von Lindern[1]§

1. Dept. Hematology, Erasmus MC, Rotterdam, The Netherlands
2. Institute of Molecular Pathology, Vienna, Austria
3. Dept. Cell Biology and Genetics, Erasmus MC, Rotterdam, The Netherlands
4. Dept. of Immunology, Erasmus MC, Rotterdam, The Netherlands
5. Institute of Immunology, University of Vienna, Vienna, Austria

present address: MRC Molecular Haematology Unit, Oxford, U.K.
* present address: Institute of Immunology, University of Vienna, Vienna, Austria
§ both authors contributed equally

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Abstract

Regulation of survival, expansion and differentiation of erythroid progenitors requires the well-controlled activity of signaling pathways induced by erythropoietin (Epo) and stem cell factor (SCF). In addition to qualitative regulation of signaling pathways, quantitative control may be essential to control appropriate cell numbers in peripheral blood. We demonstrate that Bruton´s tyrosine kinase (Btk) is able to associate with the Epo-Receptor (EpoR) and Jak2, and is a substrate of Jak2. Deficiency of Btk results in reduced and delayed phosphorylation of the EpoR, Jak2 and downstream signaling molecules like Stat5 and PLCγ1 as well as in decreased responsiveness to Epo. As a result, expansion of erythroid progenitors lacking Btk is impaired at limiting concentrations of Epo and SCF. In addition, lack of Btk results in increased sensitivity to TNF-related-apoptosis-inducing-ligand (TRAIL)-induced apoptosis and we show that SCF induces Btk to interact with TRAIL Receptor1. Taken together, our results indicate Btk to be a novel, quantitative regulator of Epo/SCF-dependent expansion and survival in erythropoiesis.

Introduction

Signals emanating from the receptors for erythropoietin (EpoR) and stem cell factor (c-Kit) are crucial to positively regulate erythropoiesis. Epo or EpoR knockout mice die at day 12.5 of embryonic development with a lack of erythrocytes [1], while mice carrying mutations in c-Kit or SCF are severely anemic [2]. *In vivo*, physiological Epo concentrations are low, leading to apoptosis of about 20% of the erythroid cells present in bone marrow [3]. This apoptotic process may be further enhanced upon activation of 'death receptors' [4] of which Fas and receptors for TNFa and TRAIL are expressed in erythroid progenitors [5-7]. The observed apoptosis is probably caused by an interplay of apoptotic and survival factors. For instance, in myelodysplastic syndrome (MDS) the lack of erythrocytes has been attributed to a failure to respond to Epo as well as by enhanced sensitivity to death ligands [8,9].

Ex vivo, erythroid progenitors can be expanded in presence of Epo, SCF and glucocorticoids, while Epo induces differentiation to erythrocytes [10-12]. Activation of the EpoR and c-Kit results in phosphorylation of the receptors and activation of multiple signaling pathways [13-15]. Common signaling pathways involve Src-like kinases, PI3-Kinase, PLCγ and MAPK, while Stat5 phosphorylation is only induced by Epo (for review see [16]). Although the molecular mechanisms of Epo/SCF synergy in progenitor expansion are not known, it may involve the specific activation of co-operating downstream effector molecules, or changes in signaling strength and/or duration. For instance, PI3-Kinase activation of PKB/Akt is significantly higher after SCF stimulation and persists longer than Epo-induced PKB/Akt phosphorylation [10], unpublished data). Inhibition of PI3-Kinase abrogates expansion of erythroid progenitors and induces differentiation instead [10].

PI3-Kinase activity generates phosphatidylinositol-3-phosphates (e.g. PIP3), which targets proteins with a pleckstrin homology (PH) domain to the plasma membrane. This includes members of the Tec family of cytoplasmatic tyrosine kinases i.e., Bmx/Etk, Btk, Itk, and Tec [17]. Tec kinases are primarily expressed in hematopoietic lineages [18]. Btk represents the most extensively studied member of the family. Btk mutations or deficiency cause XLA (X-linked agammaglobulinemia) in humans and a block in B-cell development, accompanied by the milder XID phenotype (X-linked immuno-deficiency) in mice [19-21]. Btk controls B-cell receptor

signaling and sensitivity to apoptosis induction. In DT40 cells, Btk associates with Fas and inhibits Fas-induced apoptosis [22], while Btk is required in the same cells for radiation-induced apoptosis [23]. Activation of Tec family kinases is largely dependent on the synergistic action of PI3-Kinase and a Src-like-kinase activity [24], although alternative pathways have been suggested [25]. The PH-domain of Tec kinases imposes membrane recruitment [26], which results in phosphorylation on a tyrosine residue in the kinase domain (e.g. Y551 in Btk). The subsequent conformational change releases intramolecular inhibitory interactions and allows autophosphorylation within the SH3 domain (e.g. Y223 in Btk) and maximum kinase activity [27-29]. Tec, as well as Btk, was also shown to associate with the non-Src-like-kinases Jak1 and Jak2 in COS cells and with Syk in B-cells [30-32]. Tec and Jak2 were shown to cross-phosphorylate each other [30,31], but the physiological significance of this interaction has not been investigated.

Expression of Btk and Tec in erythroid cells has been described [33,34], but so far only the role of Tec in c-Kit signaling has been studied in more detail [35]. The analysis of erythroid progenitors derived from *wt* and Btk deficient (Btk-) mice showed that Btk is required for progenitor expansion at low (i.e. physiological) growth factor concentrations. At the molecular level Btk enhanced Jak2 activity and loss of Btk resulted in reduced Epo-induced phosphorylation of the EpoR, Jak2, Stat5a/b and PLCγ. In addition, Btk- progenitors are highly sensitive to TRAIL-induced apoptosis as they lack SCF-induced association of Btk with TRAILR1.

Material and methods

Antibodies, growth factors and plasmids

Rabbit antisera against Jak2, a mouse monoclonal antibody recognizing phospho-Stat5a/b (Tyr694-Stat5a; Tyr 699-Stat5b) and antibodies for TRAILR1/2 were obtained from Upstate Biotechnology. Rabbit antisera recognizing the mouse EpoR, p44/p42 ERK1/2, PLCγ1, Btk, c-Kit and the anti-phosphotyrosine mouse monoclonal antibody PY99 were from Santa Cruz Biotechnology. Mouse monoclonals recognizing phospho-p44/p42 MAP kinase (Thr202/Tyr204) were from New England Biolabs. Phospho-Btk(Tyr223) was obtained from Cell Signalling. Anti-actin antibody was obtained from Sigma. Recombinant human Epo was a gift from Ortho-Biotech, Tilburg, The Netherlands, dexamethasone was purchased from Sigma and Mouse Stem Cell Factor was a kind gift from Amgen. Recombinant Trail was obtained from R&D Systems. The complete open reading frame of Btk and Btk kinase dead (K430R, a kind gift of Dr. J. Borst, NKI, Amsterdam), Tec, Jak2, Lyn, c-Kit and the EpoR were cloned into the mammalian expression vector pSG5 (Stratagene). Additionally, Btk was cloned into the retroviral vector pBabe-puro.

Generation of p53-deficient, Btk-deficient cell lines from bone marrow

Btk-deficient mice [21] and p53 +/- mice [36], both on a C57B6 background, were bred to obtain p53-/-, Btk- animals, which were genotyped by PCR [37]. Bone marrow was isolated from 6 week old animals to be cultured in StemPro medium (Life Technologies) supplemented with Epo (2U/ml), SCF (100ng/ml) and dexamethasone (50μM). Erythroid progenitors were expanded comparable to expansion of human erythroid progenitors from bone marrow [10]. Progenitors could be expanded for ~14 days, before the cells in the p53 *wt* culture started to differentiate or die. In the p53-deficient cultures, a part of the progenitors continued to divide as blasts, similar to what was previously observed for erythroid progenitors expanded from

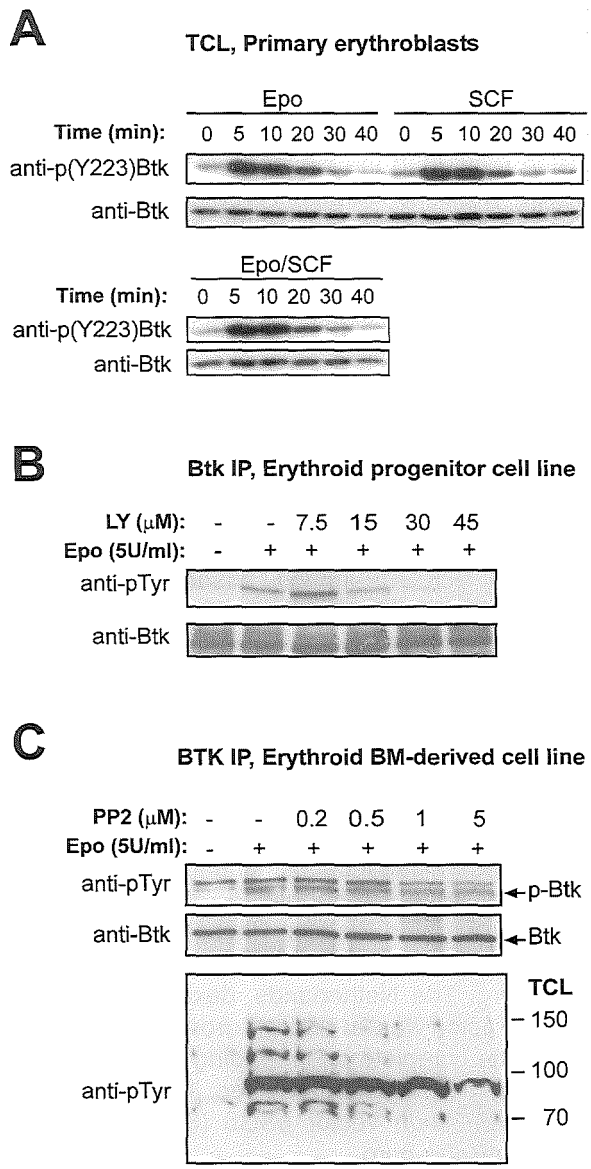

Figure 1, Epo and SCF induce Btk tyrosine phosphorylation. A: Primary, fetal liver derived progenitors were factor deprived and re-stimulated with Epo, SCF or Epo+SCF as indicated. Stimulation time is indicated in minutes. Btk phosphorylation was analysed using an anti-Y223 phospho-specific antibody. Total Btk was stained to confirm equal loading. (**B, C**) The immortalized, bone marrow derived, *wt* erythroid progenitor cell line 2B6 was factor deprived in presence of various concentrations of the PI3K inhibitor LY294002 (**B**) or the Src kinase inhibitor PP2 (**C**) as indicated and re-stimulated with Epo. Btk immune-precipitates were analyzed for phosphorylation using anti-phosphotyrosine antibodies (PY99; p-Btk is lower band) and stained for total Btk (**B**) or for phosphorylated proteins in total cell lysates (TCL) as a control (**C**).

p53-deficient mouse fetal liver [38]. The expanding erythroid progenitors were seeded in limiting dilution and several clones were isolated and examined for their differentiation potential and factor-dependence. All clones were strictly dependent on Epo, SCF and Dex to maintain renewal-divisions. When cells were washed and re-seeded in erythroid differentiation medium (StemPro supplemented with 5U/ml Epo and 1 mg/ml transferrin) all clones differentiated into hemoglobinised, enucleated erythrocytes within 72 hours as indicated by cell morphology and hemoglobin measurement. The p53-deficient, Btk *wt* clones 2B4 and 2C6 and the p53-deficient, Btk- clones 3G4 and 3E8 were used for all experiments described.

Cell lines, primary erythroid cultures, transfections and viral transductions
COS and ecotropic Phoenix (φE) cells were maintained in Dulbecco modified Eagle medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum (FCS, Life Technologies). For COS transfection experiments, 10^6 cells were seeded in 60mm dishes, transfected with 12μg DNA for 3-4hrs, washed and harvested after 48hrs as previously described [35]. Primary fetal liver and bone marrow erythroid progenitors as well as erythroid bone marrow cell lines were cultured in StemPro-34TM medium (Life Technologies) supplemented with Epo (2U/ml), SCF (100ng/ml) and Dex (50μM), unless indicated differently. Cell cultures were maintained at 1.5-3x10^6 cells/ml. To induce terminal erythroid differentiation, cells were washed in PBS and reseeded at 2-3 x 10^6 cells/ml in StemPro-34TM medium containing insulin (Ins, 4x10^{-4} IE/ml, Actrapid HM), the Dex antagonist ZK-112993 (3x10^{-6} M; [39], 1 mg/ml iron saturated human transferrin (Sigma) and various concentrations of Epo as indicated.
Viral transduction of the Btk- erythroid bone marrow cell line 3G4 was done as follows: φE cells were transfected as described for COS cells. After 48hrs of transfection cells were treated with mitomycin C (10μg/ml; Kyowa) for 1h, washed 3 times with PBS, left untreated for 4hrs, washed and 3G4 cells were added (0.5x10^6 cells per transfection) to be co-cultured in StemPro-34TM medium supplemented with growth factors as described above. After 48hrs, the erythroid cells were transferred from the φE cell layer to a new dish and selected for stable transfectants with puromycine (2μg/ml; Sigma). To obtain single cell derived clones, cells were seeded in semisolid Stempro-34TM medium supplemented with Epo, SCF and dexamethasone plus puromycin (2μg/ml).

Immune-precipitations and Western blotting
Primary erythroid cells and the erythroid bone marrow cell lines were factor deprived in plain IMDM (Life Technology) for 4hrs at 5x10^6 and re-stimulated for 10' at 37°C (40-80x10^6/ml) with SCF (500ng/ml), Epo (5U/ml), TRAIL (200ng/ml), or combinations of factors at concentrations indicated in the figure legends. To stop the reaction, 10 volumes of ice-cold PBS were added. Cell lysis, immune-precipitation, SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously [35]. Membranes were stripped in 63mM Tris-HCl pH 6.1, 2% SDS and 100mM β-mercaptoethanol for 30' at 50°C after which they were reblocked and re-stained.

Hemoglobin content determination and cell morphology
Small aliquots of the cultures were removed and analyzed for hemoglobin content by photometry as described earlier [40]. The values described were the average of triplicate measurements after normalization for cell number and mean single cell volume. For histological analysis erythroid cells at various stages of cell culture were cytocentrifuged onto glass slides and

subsequently stained with histological dyes and neutral benzidine for hemoglobin as described [41]. Nucleated and enucleated erythrocytes (brown or yellow stained, small cells), partially mature or immature cells (larger cells with grey or blue cytoplasm) and dead cells (fragmented/condensed nuclei, disintegrated cells) were counted under a microscope, evaluating >600 cells on multiple, randomly selected fields per sample (see also [49, 51]). Images were taken using a CCD camera and were processed with Adobe Photoshop.

Membrane expression of various receptors

Flow cytometric analysis was performed essentially as described previously [21]. Antibodies recognizing c-Kit, Ter119 and integrinε4 were obtained from Pharmingen. Biotynilated Epo (Bio-Epo) was made using the biotin labeling kit (Roche; #1418165) following the manufacturer´s protocol.

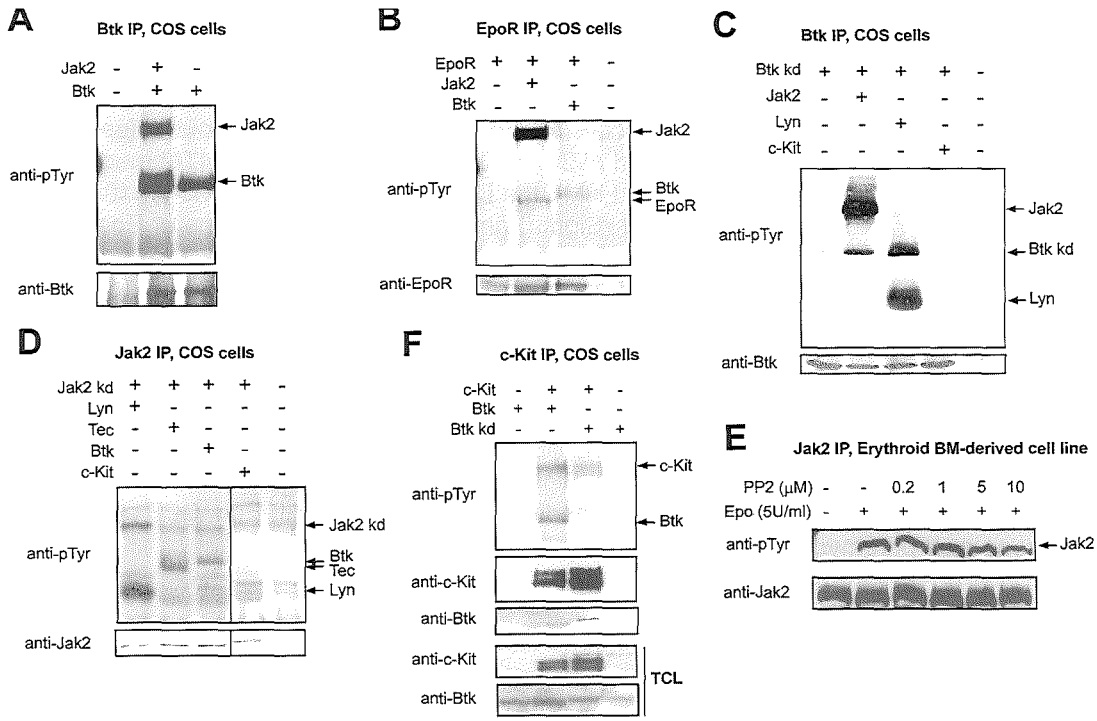

Figure 2, Btk co-immune-precipitates with and is a substrate of Jak2. A-D: COS cells were transfected with pSG5-based expression constructs encoding the indicated proteins. Expression of all proteins was verified in Western blots using specific antibodies (data not shown). After 48hrs cells were stimulated with 5U/ml Epo (**B**) or 500ng/ml SCF (lane 4, **C**; lane 4, **D** and **E**), harvested, lysed and Btk (**A**, **C**), EpoR (**B**), Jak2 (**D**) and c-Kit (**F**) were immune-precipitated. Upper panels represent immunoblots stained with anti-phosphotyrosine antibodies (PY99). The blots were re-stained with antibodies recognising the immune-precipitated proteins to check for equal loading. Arrows indicate the position of the immune-precipitated and co-immune-precipitating proteins. **E**: The erythroid cell line 2B6 was factor deprived in absence or presence of the Src -kinase family inhibitor PP2 as indicated and stimulated with Epo. Epo-induced Jak2 phosphorylation was assayed on immunoblots using PY99 (upper panel). Lower panels represent blots re-stained with anti-Jak2 to confirm equal loading. (**F**) c-Kit immuneprecipitates were stained with anti-phosphotyrosine antibodies (PY99) and blots were restained with anti-c-Kit and anti-Btk antibodies, while total cell lysates were stained with anti-c-Kit and anti-Btk to check expression levels.

Results

Btk is phosphorylated in response to Epo and SCF

To address whether Btk has a functional role in Epo/SCF-dependent erythropoiesis, we tested if Btk is tyrosine phosphorylated in response to Epo or SCF. Erythroid progenitors expanded from E12.5 mouse fetal liver were factor-deprived and re-stimulated with Epo and SCF. Epo as well as SCF induced tyrosine phosphorylation of the Btk autophosphorylation site Y223 with similar kinetics and intensity while co-stimulation with both Epo and SCF slightly enhanced the initial intensity of Btk phosphorylation (Figure 1A). Phosphorylation of Btk was also detected with the anti-phosphotyrosine antibody PY99 (data not shown). Since activation of Btk in B-cells is dependent on PI3-Kinase and Src-like-kinases, we examined the presence of a similar mechanism in erythroid cells. R1 immortalized erythroid progenitors were factor deprived and re-stimulated in presence of increasing inhibitor concentrations suppressing PI3-Kinase (LY294002; LY) or Src-like-kinase (PP2). Btk tyrosine phosphorylation was dose-dependently inhibited by LY with complete inhibition at 30-45μM (Figure 1B). Inhibition of Src-family-kinases with PP2 (0.1-5μM) did not result in an inhibition of Btk tyrosine phosphorylation (lower band, top panel Figure 1C). Inhibition of (unknown) tyrosine-phosphorylated proteins in total cell lysates by PP2 indicated that the inhibitor was functional. Unfortunately, the Src-kinase inhibitors can not be employed to examine whether SCF-induced Btk phosphorylation is dependent on Src-kinases, since they directly inhibit c-Kit kinase activity ([42]; data not shown). In conclusion, Epo induces PI3-Kinase-dependent and Src-kinase-independent activation of Btk in erythroid progenitors.

Btk interacts with the EpoR and Jak2 and is tyrosine phosphorylated by Jak2

The Epo-dependent activation of Btk suggests that Btk may interact with the EpoR and/or Jak2. Btk was expressed in COS cells together with Jak2 and/or the EpoR. Jak2 efficiently co-immune-precipitated with Btk (Figure 2A). Btk also co-immune-precipitated with the EpoR, but was not able to phosphorylate the EpoR in the absence of Jak2 (Figure 2B). Since Epo-induced Btk phosphorylation is independent of Src-kinases, we tested whether Jak2 or other kinases associated with the EpoR-complex are able to phosphorylate Btk. A kinase-dead mutant of Btk(K430R) was expressed in COS cells together with Jak2, Lyn or c-Kit. Jak2 and Lyn co-immune-precipitated with Btk and phosphorylated Btk on tyrosine (Figure 2C). To analyze whether, in reverse, Btk can phosphorylate Jak2, a kinase-dead mutant of Jak2 (K1114M) was expressed in COS cells together with Lyn, Btk, Tec, or c-Kit. While Lyn efficiently phosphorylated Jak2, Jak2 was not phosphorylated by Btk (Figure 2D). In contrast to reports by others [30,31], Tec was also not able to phosphorylate Jak2 in COS cells. In conclusion, Btk can co-immune-precipitate with the EpoR and Jak2, and Btk can serve as a substrate for Jak2. This may explain the PP2-resistent, Epo-induced Btk phosphorylation since PP2 did not affect Epo-induced Jak2 tyrosine phosphorylation (Figure 2E). To test whether c-Kit directly associates with and phosphorylates Btk, we co-expressed c-Kit with either Btk or Btk(K430R). While c-Kit co-immune-precipitated with *wt* Btk, this interaction required Btk-activity, since c-Kit did not associate with Btk(K430R) and was inable to phosphorylate Btk(K430R) (Figure 2F). This suggests that SCF-induced phosphorylation of Btk employs additional kinases.

Btk- erythroid progenitors are defective in proliferation, but not in differentiation

Since we found Btk to be a substrate of Epo/SCF signaling, we compared factor-dependent

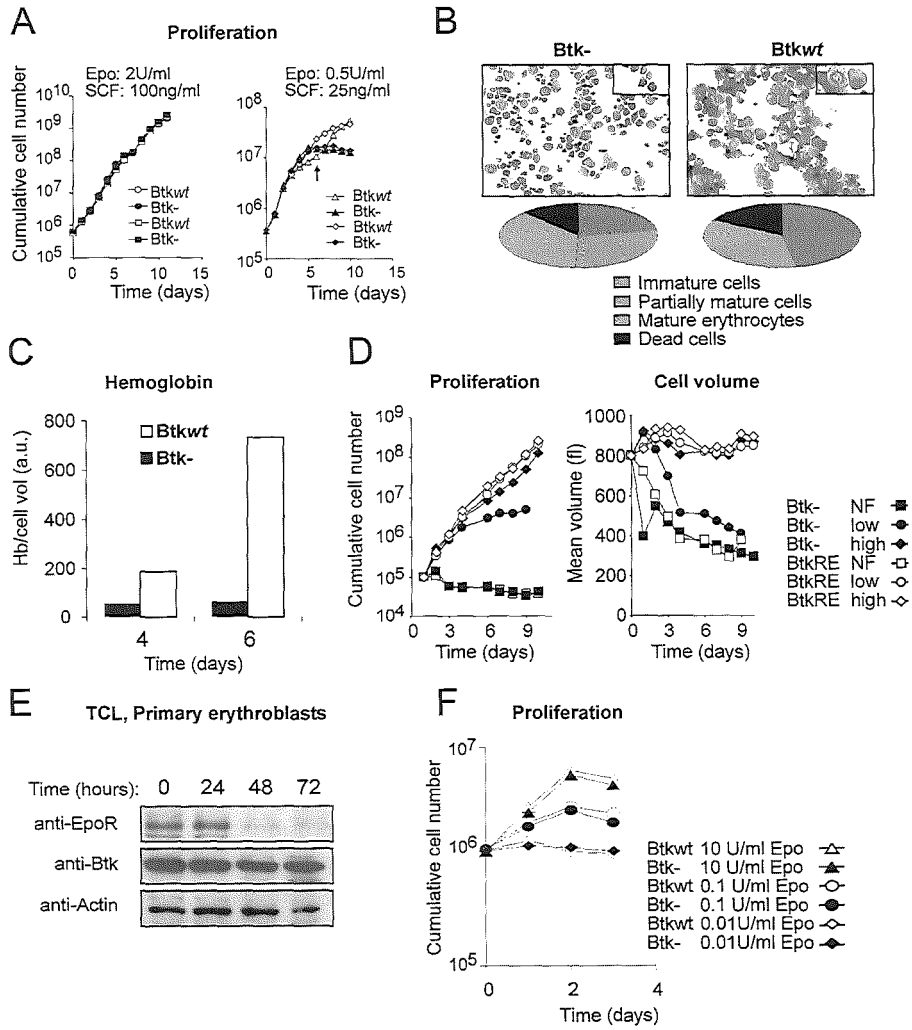

Figure 3. Btk- erythroid progenitors show enhanced differentiation at the expense of renewal. A: Btk- primary fetal liver progenitors were seeded in medium containing high (2U/ml Epo; 100ng/ml SCF) or low (0.5U/ml Epo; 25ng/ml SCF) concentrations of SCF and Epo, maintaining normal concentrations of Dex, and cumulative cell numbers were determined. The different symbols represent two independent experiments using *wt* and Btk- erythroid progenitors as indicated. **B**: Histological analysis of *wt* and Btk- cells described under **A** at day 6 (arrow in **A**). The percentages of erythroid cells at different stages of maturity (as indicated) were scored for *wt* and Btk- cultures and plotted in a pie chart. **C**: Hemoglobin content (Hb / cell volume in arbitrary units) of cells as described under **A** was measured at days 4 and 6. **D**: Btk- bone marrow cell lines ectopically re-expressing Btk (BtkRE) and Btk- cell lines (Btk-) were treated as described in **A**. Open symbols represent BtkRE and closed symbols represent Btk- cells cultured on low, high or no growth factors (NF; see **A**). **E**: Primary fetal liver progenitors were seeded in differentiation medium. Expression of Btk and the EpoR during differentiation was checked by Western blotting using Btk and EpoR antibodies. Equal loading was checked using an actin antibody. **F**: Cells were seeded in differentiation conditions at 10, 0.1 and 0.01 U Epo/ml. Cumulative cell numbers were determined. Symbols indicate the different growth conditions as well as *wt* and Btk- cultures.

survival, expansion and differentiation of *wt* and Btk- progenitors. Fetal liver cells from E12.5 Btk- and *wt* littermates were cultured in medium supplemented with Epo, SCF and Dex. In both cultures, progenitors proliferated with similar kinetics (Figure 3A). Since standard growth factor concentrations (2U/ml Epo; 100 ng/ml SCF) exceed physiological concentrations to ensure long-term expansion of progenitors at maximal proliferation rate, we also examined expansion of *wt* and Btk- cells at lower concentrations of Epo (0.5 U/ml) and SCF (25 ng/ml). While *wt* progenitors could be expanded for at least 10 days at reduced growth factor concentrations, cultures of Btk- progenitors became stationary at day 6 (Figure 3A). Analysis of cell morphology and hemoglobin staining of Btk- cultures at day 6 showed an increase in mature erythrocytes at the expense of erythroid progenitors, as compared to *wt* cells (Figure 3B). The same was evident by the much smaller average cell size exhibited by Btk- cells compared to *wt* cells, determined using an electronic cell counter (data not shown). Concordantly, Btk- cultures showed an increase in hemoglobin levels compared to *wt* cultures (3-fold at day 4; 11-fold at day 6; Figure 3C). In conclusion, Btk-deficiency abrogates the expansion capacity of erythroid progenitors exposed to limiting concentrations of Epo and SCF, favoring terminal differentiation at the expense of erythroid progenitor expansion.

To study Btk- cells in detail, including biochemical assays, we established immortal cultures of p53-deficient Btk- and Btk*wt* bone marrow derived erythroid progenitors (see material and methods). At standard concentrations of Epo and SCF Btk*wt* (2C6) and Btk- (3E8) immortal erythroid cultures could be similarly expanded and retained a cell volume of ~800fl. In contrast, decreased concentrations (see above) of Epo and SCF caused rapid cell size reduction and growth arrest in the Btk- progenitors. Re-expression of Btk in Btk- cells restored the expansion capacity and blast-like cell size at reduced growth factor concentrations (Figure 3D).

The observation that progenitor expansion was impaired in Btk- cells, while progenitors underwent terminal differentiation instead, suggests that Btk is not essential for differentiation. However, Btk expression is maintained during terminal differentiation while EpoR expression decreases during the later stages of differentiation (Figure 3E). To investigate whether Btk is required during Epo-induced terminal erythroid differentiation, fetal liver derived, primary *wt* and Btk- progenitors were seeded in differentiation medium containing varying concentrations of Epo (10-0.01 U/ml). Although proliferation during differentation showed an expected dependence on the Epo-concentration, no differences between differentiating *wt* and Btk- cultures were observed with respect to proliferation kinetics, hemoglobin content or cell morphology (Figure 3E and data not shown).

	CFU-E		BFU-E (100ng/ml SCF)		BFU-E (25ng/ml SCF)	
	wt	Btk-	*wt*	Btk-	*wt*	Btk-
Exp1	701±32	1848 ±170	761±32	789±32	481±48	567±74
Exp2	509±89	580±47	386±107	324±29	293±72	287±45
Exp3	512±67	574±48	534±16	523±15	360±8	374±27

colony numbers are per 10^5 mononuclear cells
all values shown represent mean values and standard deviation from 3 individual animals.

Table 1, Btk deficiency does not influence CFU-E and BFU-E formation. Fetal livers were isolated at E12.5 and wt and Btk- erythroid progenitors were assessed for their ability to induce BFU-Es and CFU-Es (as described in material en methods).

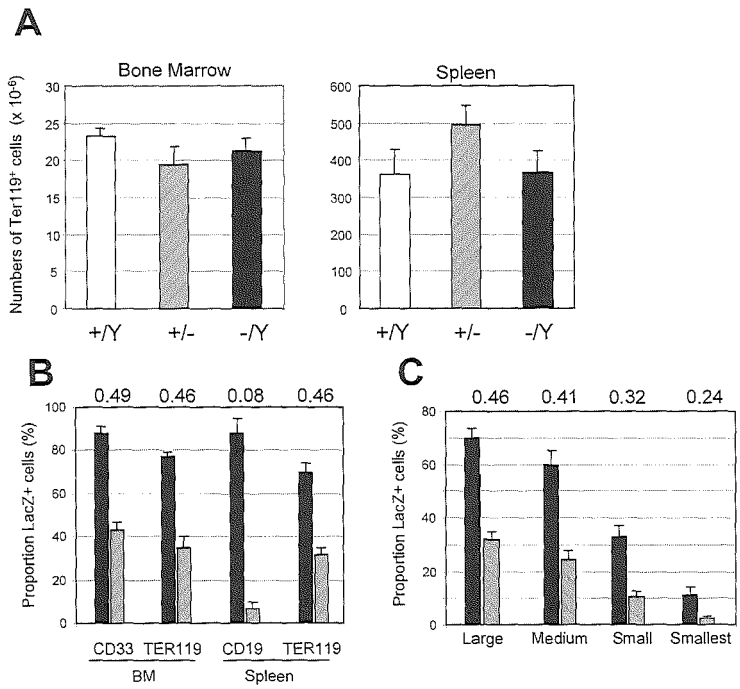

Figure 4, Btk-deficient cells contribute to stress erythropoiesis. (A) Total numbers of TER119 positive cells were determined in bone marrow and spleen of 4 Btk+/Y (open bar), 6 Btk+/- (hatched bar) and 4 Btk-/Y (black bar) PHZ-treated mice. (B) The ratio of LacZ+ cells in the myeloid (ER-MP20 (Ly-6C)high) and erythroid (TER119) compartment of the bone marrow (BM), and in the B-lymphoid (B220) and erythroid (TER119) compartment of the spleen was determined in 4 Btk-/Y (black bars) and 6 Btk+/- (hatched bars) PHZ-treated mice (blast fraction only, i.e. FSC high). The numbers above the graph indicate the ratio of LacZ+ cells in the Btk+/- mice compared to the Btk-/Y mice. (C) The ratio of LacZ+ cells in the erythroid compartment (TER119) of anemic spleens was determined in different cell size fractions (large to small, i.e. with decreasing FSC and increasing SSC) in 4 Btk-/Y (black bars) and 6 Btk+/- (hatched bars). The numbers above the graph indicate the ratio of LacZ+ cells in the Btk+/- mice compared to the Btk-/Y mice.

Erythropoiesis in Btk-deficient mice is not compromised

We next wanted to know whether Btk- mice display any erythroid phenotype during normal or stress erythropoiesis. Btk- and *wt* mice had similar numbers of circulating erythrocytes (8.6 ± 0.6 x10^6/ml and 8.2 ± 0.8 x10^6/ml) and reticulocytes (3.0 ± 0.3% and 3.1 ± 1.0%; n=4). Serum levels of Epo were below the detection threshold of 20U/l, corresponding to normal physiological levels as can be expected when circulating red cells are normal. The number of erythroid progenitors (BFU-E and CFU-E) was determined in E12.5 fetal livers. We did not observe consistent differences between BFU-E and CFU-E in three subsequent experiments, using 3 Btk-deficient and 3 *wt* animals in each experiment (Table I).

Btk-deficient cells contribute to stress erythropoiesis

To investigate the contribution of Btk-deficient cells to stress erythropoiesis, 4 Btk+ males, 4 Btk- males and 6 Btk+/- female mice (11 to 13 weeks of age) were injected with 0.4% phenylhydrazine (PHZ; 0.16ml/20g). PHZ was injected 3 times with 12hrs intervals and 72hrs after

the last injected the animals were sacrificed. Spleens and bone marrow (BM) were harvested and total cell numbers determined. As expected, PHZ-treatment led to similarly enlarged spleens in all respective mice (data not shown). The absolute numbers of TER119-positive cells in both BM and spleen from Btk+ and Btk- males were similar (Figure 4). Since, in the Btk-deficient mice, the Btk gene was inactivated by a targeted in-frame insertion of a *lacZ* reporter [21], Btk expression can be monitored using the *lacZ* gene activity under the control of the endogenous Btk promoter plus regulatory elements. Btk is located on the X-chromosome. In Btk+/- females, X-inactivation therefore either disables the LacZ-targeted allele or the *wt* Btk allele, which allows *in vivo* competition experiments between cells expressing or lacking Btk within a single animal.
We determined the number of LacZ+ cells in the TER119+ erythroid compartment in the BM and the anemic spleens, by loading cells with fluorescein-di-D-galactopyranoside substrate (Molecular Probes Europe) as previously described 21. As controls, we analyzed the myeloid (ER-MP20 (Ly-6C) high) compartment in the BM, in which there is no known selective disadvantage for Btk- cells, and the B220+ B cell compartment in the spleen, in which Btk- cells are expected to be essentially absent [21]. We found that in Btk- males ~90% of myeloid cells in the BM and B cells in the spleen were lacZ+. In Btk+/- animals the proportions of LacZ+ cells in the myeloid were half of those of Btk- males (46-49%), and LacZ+/Btk- cells did not contribute significantly to the B cell population in the spleen (figure 4B), consistent with our previous findings [21].
Within the total erythroid population in BM and spleen, the proportions of lacZ+ cells in Btk+/- females were close to half (~46%) of those found in the Btk- males, indicating that Btk is not critical for expansion or differentiation in the erythoid lineage. To quantify the contribution of Btk- cells to subsequent stages of erythropoiesis in detail, we determined the percentage of LacZ+ cells within the Ter119 positive population in 4 size classes, corresponding to increasing maturation of erythroid cells. In Btk- males, the percentage of LacZ+ cells in the Ter119+ population decreased with increasing maturity, reflecting an apparent decrease in Btk expression levels of Btk during erythroid maturation. Strikingly, however, the number of LacZ+ cells decreased significantly further in Btk+/- females (from 46 to 24%; figure 4C), indicating that Btk- cells are impaired in expansion or survival when competing with Btk+/- cells during terminal differentiation *in vivo*. The same phenomenon has been observed in bone marrow from PHZ treated and untreated mice (data not shown).

EpoR signaling is perturbed in Btk- erythroid progenitors
To unravel how Btk-deficiency could impair Epo/SCF-dependent progenitor expansion, we analyzed EpoR and c-Kit signaling. Btk*wt* (2C6) and Btk- (3E8) immortalized progenitors were factor-deprived and restimulated with Epo (5U/ml). Epo-induced phosphorylation of PLCγ1 was completely abrogated in Btk- cells. Surprisingly, Epo-induced phosphorylation of the EpoR, Jak2 and Stat5, was also significantly reduced in the Btk- cells compared to *wt* cells (Figure 5A). Epo-induced Stat5 phosphorylation was similarly reduced in primary progenitors (Figure 5B, data not shown). Re-expression of Btk in Btk- progenitors restored the phosphorylation levels of the EpoR, Jak2 and Stat5 to those in *wt* cells (Figure 5C). While Btk was also phosphorylated in response to SCF, SCF-induced phosphorylation of c-Kit was not impaired in absence of Btk (Figure 5D). To rule out that the impaired Epo-induced signal transduction in Btk- cells was due to decreased cell surface expression of the EpoR, expression of the EpoR and c-Kit was determined by flow cytometry of non-permeabilized cells. Surface expression of the EpoR and c-Kit was identical between *wt* and Btk- erythroid pro

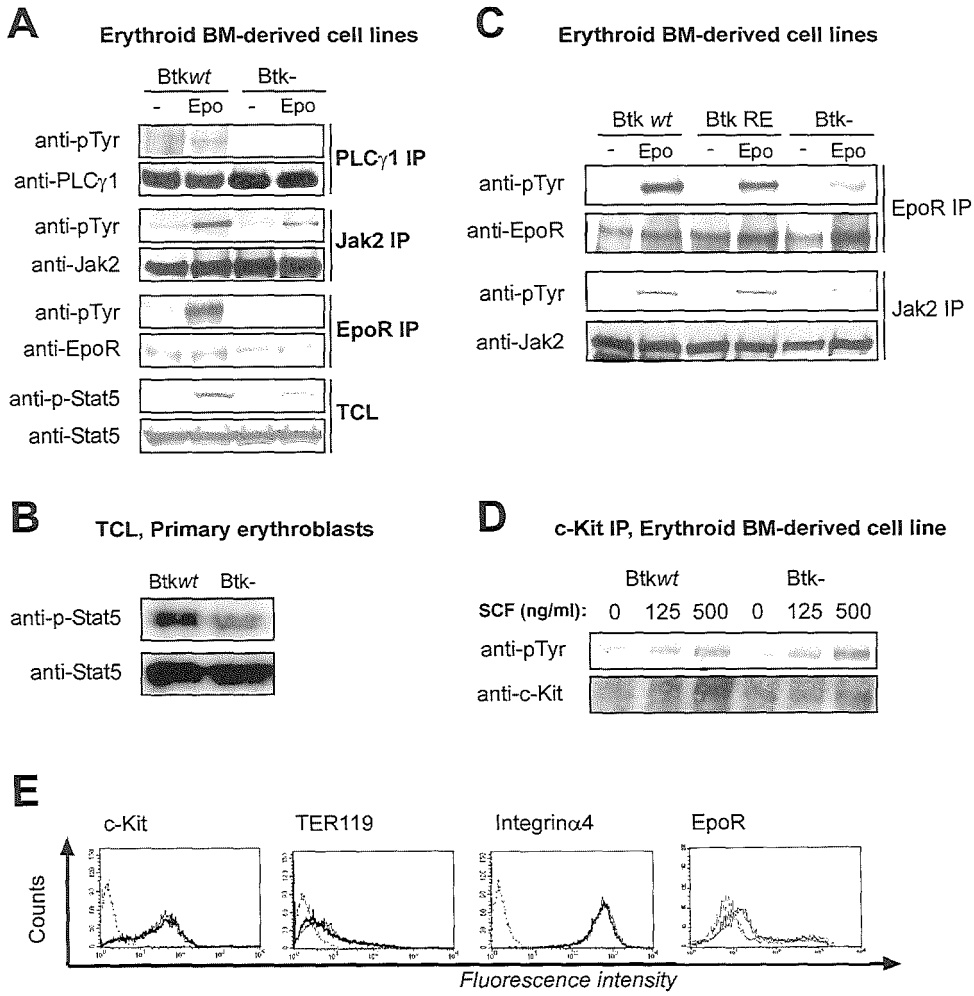

Figure 5, Btk- cells are perturbed in Epo-induced signaling. A-D: *wt* (Btk*wt*), Btk- (Btk-) and Btk re-expressing (BtkRE) bone marrow derived erythroid cell lines (**A**, **C**, **D**) and primary *wt* and Btk- fetal liver progenitors (**B**) were factor deprived and re-stimulated with Epo or SCF as indicated. Cells were lysed and the immune-precipitated proteins as well as total cell lysates (TCL) were analyzed by Western blotting. Upper panels represent blots stained with the anti-phosphotyrosine antibody PY99, except for the TCL which was stained with anti-phospho-Tyr694/699 Stat5a/b. Lower panels represent the same blot re-stained with antibodies recognizing the immune-precipitated protein as indicated or with anti-total-Stat5a/b for the TCL. **E**: Flow cytometric analysis of cell surface expression of c-Kit, Ter119, integrina4 by specific antibodies and EpoR by biotinylated Epo in *wt* (thick black lines) and Btk- (thin black lines) erythroid progenitors. Expression levels are shown as histograms depicting also control stainings (dotted lines) and addition of excess Epo (grey line) for EpoR detection. Mean fluorescence values varied less then 10% for each staining in three independent experiments indicating that *wt* and Btk- erythroid progenitors express similar levels or the respective cell surface markers.

genitors, using the erythroid markers Ter119 and integrinα4 as controls (Figure 5E).

Btk controls signaling kinetics.
To examine whether the reduced response to Epo in Btk- cells could be caused by altered signaling kinetics, the immortalized Btk*wt* (2C6), Btk- (3E8) and Btk re-expressing (RE) erythroid cells were factor deprived, re-stimulated with Epo for 5, 10, 60 and 120 minutes, and Stat5 phosphorylation was assessed. Consistently, Stat5 was rapidly phosphorylated in response to Epo in *wt* and Btk re-expressing cells, while phosphorylation in Btk- cells was largely impaired. However, at late time-points similar levels of Stat5 phosphorylation were observed in all cell types (Figure 6A). Since Jak2 phosphorylates Stat5, Jak2 was immunoprecipitated from cell lysates generated at increasing time intervals following Epo stimulation of *wt* (2C6) and Btk- (3E8) cells. Maximal phosphorylation of Jak2 was observed at 10 - 20 minutes following Epo stimulation in *wt* cells and only at 40 minutes in Btk- cells (Figure 6B; note that exposure of the blots was adapted to show (de)activation kinetics for Btk- and *wt* cells, requiring longer exposure of the PY-blots in the Btk- panel). Also phosphorylation of the EpoR, though drastically reduced in Btk- cells, is less rapidly downregulated (Figure 6C; requiring again different PY-exposures of Btk- and *wt* panels). Analysis of Epo-induced Stat5 phosphorylation kinetics in primary erythroid progenitors confirmed the results obtained with the cell lines: Stat5 phosphorylation decreased after 20 minutes in *wt* cells, while it increased up to 90 minutes following Epo-stimulation in Btk- cells (Figure 6D).

Btk regulates the ligand responsiveness of the EpoR
Since Btk controlled the efficiency of EpoR signaling in time, it may also control the responsiveness to critical Epo concentrations. Immortalized Btk*wt* (2C6), Btk- (3E8) and Btk re-expressing (RE) cells were factor-deprived and re-stimulated with Epo at concentrations ranging from 0.5 to 10 U/ml. Stat5 phosphorylation became detectable in *wt* and Btk re-expressing cells at 0.5 U/ml Epo, while similar levels were reached in Btk- cells using 5U/ml. With increasing levels of Epo, the decrease in Epo-induced Stat5a/b phosphorylation in Btk- cells became markedly less apparent (Figure 6E). Together the data show that Btk may have an important role in the regulation of signaling efficiency both in time as well as in response to suboptimal, physiological concentrations of Epo.

Expansion of Btk- erythroid progenitors is inhibited by TRAIL
Reduced sensitivity for Epo and hypersensitivity for 'death-ligands' is associated in several pathological conditions [9]. Notably, loss of Btk has been shown to render B-cells more sensitive to Fas-induced apoptosis [22,43]. To examine whether the reduced responsiveness to Epo in Btk- cells affects 'death-ligand'-induced apoptosis in primary erythroid progenitors, we expanded primary *wt* and Btk- erythroid progenitors from fetal liver in presence of various death inducing ligands. INFγ killed both *wt* and Btk- progenitors with identical kinetics while the addition of FasL (20ng/ml) or TNFα (10ng/ml) under optimal and suboptimal proliferation conditions had no effect on *wt* or Btk- cells (data not shown). However, under optimal proliferation conditions the addition of 20ng/ml TRAIL impaired the expansion of Btk- progenitors whereas *wt* control cells remained unaffected (Figure 7A). Morphological analysis indicates the presence of pycnotic cells (Figure 7B). On Western blots, Btk*wt* and Btk- cells express similar levels of TRAILR1 indicating that increased receptor expression is not the cause of the enhanced TRAIL sensitivity (Figure 7C). Expression of TRAILR2 was not observed in erythroid progenitors (Figure 7C).

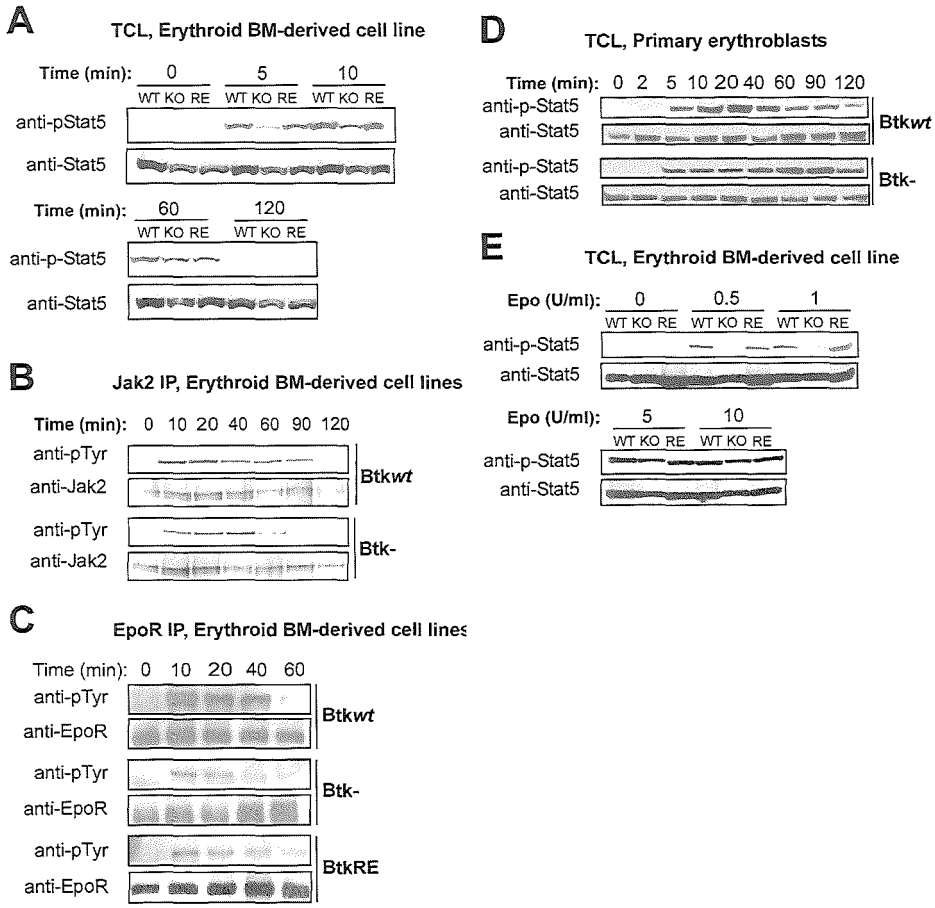

Figure 6, Btk- erythroid progenitors have a delayed Epo-induced Jak2 and Stat5 phosphorylation. A-E: *wt* (Btk*wt*), Btk- (Btk-) and Btk re-expressing (BtkRE) erythroid cell lines (**A-C**) or primary fetal liver progenitors (**D**) were factor deprived and re-stimulated with Epo for the indicated times. Cells were lysed and Jak2 (**B**) or the EpoR (**C**) was immune-precipitated. The immune-precipitations as well as total cell lysates (TCL) were analyzed by Western blotting. **A, D**: Blots were stained with anti-phospho-Tyr694/699 Stat5a/b (upper panels) and re-stained with anti-total Stat5a/b to check for equal loading (lower panels). **B, C**: Upper panels represent blots stained with anti-phosphotyrosine PY99, while lower panels represent the same blot re-stained with anti-Jak2 or anti-EpoR to confirm equal loading. PY-staining of these proteins in Btk- cells is much lower that in *wt* cells, Btk- panels were exposed much longer than *wt* panels, to enable proper comparison of kinetics of (de)activation. **E**: Erythroid progenitors were re-stimulated with increasing concentrations of Epo as indicated. Upper panels represent blots stained with anti-phospho-Tyr694/699 Stat5a/b and lower panels represent the same blot re-stained with anti-total Stat5a/b.

It has been reported previously that Btk can interact with Fas [22], while the Tec family member Bmx/Etk undergoes ligand-independent interaction with TNFaR2 [44]. These interactions protected the cells from death ligand induced apoptosis and allowed TNFα induced cellular migration and tube formation. Therefore, we tested whether Btk can interact with TRAILR1 and whether this interaction is dependent on either Epo or SCF. Progenitors expanded from *wt* fetal livers were factor-deprived and subsequently re-stimulated with Epo, SCF and/or TRAIL. Btk immune-precipitates were analyzed for the presence of TRAILR1. Both SCF and

TRAIL, but not Epo, induced an interaction of Btk and TRAILR1. In addition, co-stimulation of SCF and TRAIL resulted in a significantly enhanced TRAILR1 recruitment to Btk compared to SCF or TRAIL alone (Figure 7D). This suggests that SCF may inhibit TRAIL-induced apoptosis through enhancement of an interaction between the TRAILR1 and Btk, which may cooperate with reduced Epo-responsiveness.

Discussion

Of all circulating blood cells, the maintenance of circulating erythrocytes at constant, optimal numbers is most important. The major erythroid survival and expansion factor erythropoietin (Epo) is available at suboptimal levels (<30 U/l) and only part of the progenitors survives in the bone marrow to produce mature erythrocytes. In this study we show that the cytoplasmic tyrosine kinase Btk is required for an optimal response of erythroid progenitors to Epo, and for SCF-mediated survival in presence of TRAIL.

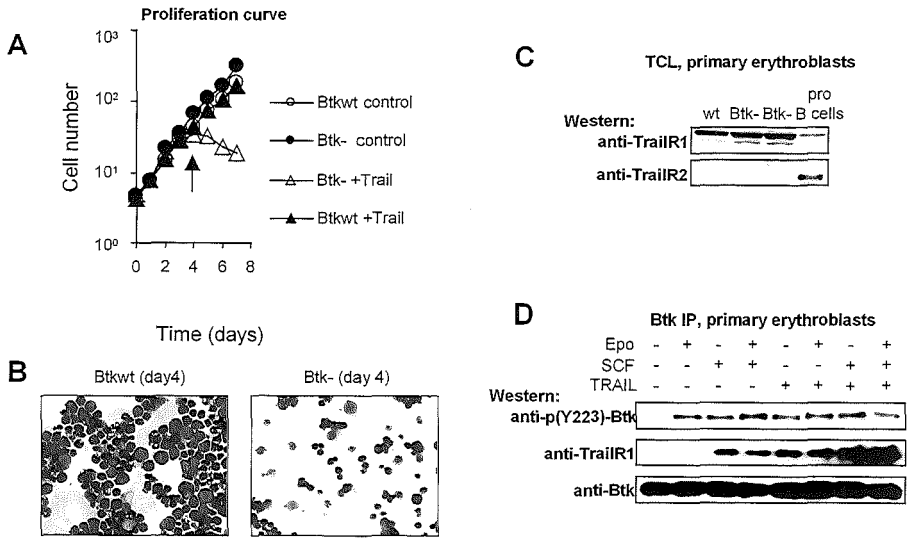

Figure 7. Btk- erythroid progenitors are no longer protected by SCF from TRAIL-induced apoptosis. A: *wt* and Btk- fetal liver cells were seeded in medium containing Epo, SCF and Dex, in presence and absence of TRAIL (20ng/ml) and cumulative cell numbers determined. The data for both *wt* and Btk- progenitors under the different culture conditions were plotted using different symbols as indicated **B**: Cytospins were prepared from day 4 cultures of *wt* and Btk- cells in presence of TRAIL (arrow in A) and stained with neutral benzidine plus histological dyes. **C**: Western blots from cell lysates of *wt* and Btk- primary fetal liver erythroid progenitors and primary pro B-cells (kind gift of M. Busslinger, IMP) were performed using anti-TRAILR1 and anti-TRAILR2 antibodies as indicated. **D**: *wt* primary fetal liver progenitors were factor deprived and re-stimulated with Epo, SCF and/or TRAIL (200 ng/ml) as indicated. Btk was immune-precipitated from cell lysates and analyzed by Western blotting. Btk tyrosine phosphorylation was analyzed using an anti-phospho-Tyr223-Btk antibody (upper panel), while co-immune-precipitation of TRAILR1 was checked with anti-TRAILR1 (middle panel). Equal loading was confirmed by re-staining the same blot with anti-total Btk (lower panel).

Btk is a target of Jak2 and controls the efficacy of EpoR signaling

Stimulation of erythroid progenitors with Epo and SCF induced phosphorylation of Btk on its autophosphorylation site Y223, indicating that catalytic activity of Btk is enhanced after Epo and SCF stimulation. Interestingly, Jak2 and LYN could phosphorylate Btk. Similarly, the Btk family member Tec is mainly phosphorylated by Lyn, but can also be phosphorylated by Jak1, Jak2 or Syk [30-32]. Since the Src kinase inhibitors PP1 and PP2 directly inhibit c-Kit catalytic activity [42]; data not shown), we did not investigate whether SCF-mediated Btk activation requires Lyn. This is, however, likely since c-Kit was not able to associate with kinase-deficient Btk or to phosphorylate Btk directly (Figure 2), while c-Kit is constitutively associated with Lyn in erythroid cells [35]. Notably, Tec is phosphorylated by Lyn in response to SCF and the same mechanism may hold for Btk.

Btk was expected to be required for the phosphorylation and activation of selected Epo-dependent signaling intermediates like PLCγ, a known downstream target of Tec-kinases. However, the role of Btk in the control of Epo responsiveness was surprising. In the absence of Btk, Epo-dependent phosphorylation of Jak2 and the EpoR required increased levels of Epo or prolonged exposure to Epo. Activation
of the SCF receptor, in contrast, was not perturbed. These data suggest that Btk may activate a signaling pathway that acts as an enhancer of Jak2 activity. Such pathways have not been described to date, but Btk does control a number signaling pathways that could be candidates for such a function. First, Btk is required to activate PLCγ,, whose main effectors are classical PKCs of which only PKCα is expressed in erythroid progenitors [45]. We previously showed that inhibition of these classical PKCs does severely impair Epo-induced signal transduction similar to what we observe in Btk- cells [45]. To test whether the potential absence of a PLC-PKC positive feedback loop in Btk- cells could be responsible for their impaired EpoR signaling, we examined whether direct activation of PKC could restore a *wt*-like Epo-responsiveness of EpoR signaling in these cells. Both bryostatin and phorbol-ester (PMA) did enhance Epo-induced phosphorylation of Jak2 and the EpoR in Btk- cells, but the degree of enhancement was not significantly stronger than in *wt* cells and did not fully restore the Btk-signaling defect. Second, since Btk associates with Jak2, it may directly affect the Jak2 associated protein complex. The Jak2-pseudokinase domain (Jak-homology region 2) is important in the regulation of Jak2 kinase activity [46]. Its presence is required for inhibition of Jak2 in absence of cytokines and for cytokine inducibility of Jak2 activity and Stat5 phosphorylation. The exact mechanism of this regulation, however, has not been elucidated. In conclusion, quantitative regulation of cytokine-associated Jak2 may play a role in the Epo-responsiveness of erythroid progenitors and may be controlled by distinct mechanisms dependent on either PKC or Btk.

Association of Btk with the TRAILR1 may regulate sensitivity to TRAIL-induced apoptosis

Regulation of the apoptosis threshold is important during erythropoiesis and several apoptosis inducers have been implicated in erythropoiesis like TNFα, FasL or IFNγ [9,47-49]. In erythroid progenitors, caspase activation in absence of EpoR activation results in Gata-1 cleavage and apoptosis [50]. The cytoplasmic serine kinase Raf is required to prevent caspase activation in immature cells [51]. However, constitutive inhibition of caspases blocks erythroid differentiation [51] as caspase activity is required in late stages of erythroid differentiation to cleave proteins involved in nuclear integrity and chromatin condensation 52. We show that Btk can associate with the TRAILR1 in response to SCF, which may protect against TRAIL-induced apoptosis. Similarly, Btk has been shown to inhibit Fas-induced apoptosis in DT40 B-cells, which was

dependent on association of Btk with Fas [22]. We suggest that Btk mediated protection from apoptosis might involve a similar mechanism in both cell types, i.e. interaction of the respective death receptor with Btk. What remains puzzling is the observation that both Epo and SCF induce phosphorylation of Btk, while only SCF induces association of Btk with TRAILR1. This could be explained by spatial constraints, which would imply that the TRAILR1 and c-Kit are located in close proximity. More likely, the association of Btk with either the TRAILR1 or Fas requires an additional bridging molecule that is regulated in response to SCF, but not in response to Epo. Such a 'bridging' molecule could also impose specificity of Btk for distinct 'death-receptors', like Fas or TRAILR1, in lymphoid or erythroid cells. In addition, the intact kinase domain of Btk is required for its association with Fas and probably also for its interaction with TRAILR1 [22]. Since *wt* and Btk- progenitors express similar levels of TRAILR1, the increased apoptosis induction in Btk-erythroblasts cannot be due to different 'death receptor' levels.

Btk-deficiency impairs renewal and enhances differentiation at limiting cytokine levels
Expansion of erythroid progenitors in the presence of Epo, SCF and Dex is abrogated in Btk- progenitors at limiting concentrations of Epo and SCF. Although the attenuated EpoR signaling and increased TRAIL-sensitivity of Btk- progenitors would have predicted increased apoptosis at limiting cytokine concentrations, we observed enhanced differentiation at the expense of renewal. Our recent results indicate that the role of EpoR signaling in early progenitors and maturing cells may be distinct in function. In early erythroblasts, signaling pathways triggered via intracellular P-Tyr docking sites in the EpoR contribute mostly to progenitor expansion, since erythroblasts from mice expressing a respective truncated EpoR undergo normal differentiation, but are defective for expansion of erythoblasts (H. Beug et al, unpublished). These pathways include Epo-induced phosphorylation of Ron/Gab1 and activation of PI3-Kinase and MAPkinases (van den Akker, manuscript submitted). Furthermore, Epo-induced STAT5 activation is required for survival during erythroid differentiation (upregulating Bcl-X_L), while lack of Stat5 in expanding progenitors causes differentiation at the expense of expansion in a Bcl-X_L -independent fashion (H. Beug et al, unpublished). Moreover, since activated caspases contribute to execution of the late erythroid differentiation program where apoptosis is prevented by upregulation of Bcl-XL [51,53], impaired protection against caspase activation in Btk- cells could contribute to differentiation rather than to apoptosis. This suggests that the lack of Btk may enhance differentiation utilizing both Epo- and SCF-activated signaling pathways.
Human XLA patients or Btk- mice are not anemic and the mice do not present with increased spleen size (a hallmark for stress erythropoiesis) as do Stat5-/- mice [54]. Importantly, we analyzed Btk- mice for their response to hypoxia, which was similar to *wt* littermates. *In vivo*, Btk-deficient cells showed a disadvantage over *wt* cells very late in maturation, but peripheral erythrocyte numbers and size were similar in *wt* and Btk- animals. Accordingly, we also found no indications for increased Epo-levels. Finally, numbers of early (BFU-E) and late (CFU-E) progenitors were similar in fetal livers of *wt* and Btk- animals.

In conclusion, Btk appears to be involved in the control of expansion, survival and differentiation of erythroid progenitors via two distinct mechanisms: i) via the regulation of Epo-induced signal transduction pathways with proliferation as the cellular readout and ii) via control of TRAIL-induced apoptosis, which may involve SCF-dependent association of Btk with TRAILR1. Interestingly, the combination of a reduced Epo-response and enhanced sensitivi-

ty to 'death-ligands' is a known pathological condition and occurs e.g. in myelodysplastic syndrome (MDS; [3,8,55]). MDS constitutes a very heterogeneous hematologic disease group that occurs mainly in the elderly. Since a reduced response to Epo and 'death-ligand' sensitivity are common, this condition may be necessary but not sufficient to cause overt disease. Rather, additional genetic aberrations may be required for full development of MDS or even AML. Therefore, it will be interesting to examine whether Btk- mice are prone to develop leukemia upon further genetic challenges e.g. by retroviral insertion mutagenesis.

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Chapter 4

The BTK Inhibitor LFM-A13 is a Potent Inhibitor of JAK2 Kinase Activity.

Emile van den Akker[1], Thamar B. van Dijk[1], Uwe Schmidt[2], Hartmut Beug[2], Bob Löwenberg[1] and Marieke von Lindern[1]

1 Institute of Hematology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands
2 Institute of Molecular Pathology, Dr. Bohrgasse 7, A-1030 Vienna, Austria

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Abstract

LFM-A13, or α-cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propenamide, was shown to inhibit Bruton's Tyrosine Kinase (Btk). We show that LFM-A13 efficiently inhibits Epo-induced phosphorylation of the erythropoietin receptor, Janus kinase 2 (Jak2) and downstream signalling molecules. However, the tyrosine kinase activity of immune-precipitated or *in vitro* translated Btk and Jak2 was equally inhibited by LFM-A13 in *in vitro* kinase assays. Finally, Epo-induced signal transduction was also inhibited in cells lacking Btk. Taken together we conclude that LFM-A13 is a potent inhibitor of Jak2 and cannot be used as a specific tyrosine kinase inhibitor to study the role of Btk in Jak2-dependent cytokine signalling.

Introduction

Cytokine receptors are devoid of intrinsic kinase activity and require the association with cytoplasmic tyrosine kinases to transmit signals upon ligand binding. Janus tyrosine kinase 2 (Jak2) activity is crucial for most cytokine receptors including the erythropoietin receptor (EpoR; [1]). Epo binding to the EpoR induces a conformational change which juxtaposes the associated Jak2 kinases resulting in cross-phosphorylation, activation and subsequent phosphorylation of the EpoR and induction of multiple signalling intermediates (for review see [2]). These include Stat5, protein kinase B (PKB), the Mitogen Activating Protein Kinase (Erk1/2) pathways but also tyrosine kinases like Lyn, a member of the Src-family, and Bruton's Tyrosine Kinase (Btk), a member of the Tec-family ([3]; Schmidt et al., in press).
Tec-family members harbour an N-terminal pleckstrin homology (PH) domain, a proline rich (PR) domain, a SRC homology 3 (SH3) domain, a SRC homology 2 (SH2) domain and a kinase domain (SH1). Upon membrane recruitment via its PH domain, activation of Tec-family members occurs through phosphorylation of tyrosine residues in the kinase domain by Src kinases (amino acid 551 in Btk; [4]). This phosphorylation event is believed to release the intramolecular interaction between the PR and the SH3 domain releasing the kinase domain from structural restraints [5,6]. Trans-phosphorylation results in activation of the kinase and autophosphorylation of a tyrosine within the SH3 domain (amino acid 223 in Btk; [4]). Both membrane recruitment and kinase activity are of paramount importance for Btk to function [7-9]. Btk has been shown to play a role in PhospholipaseC-γ1/2 activation [10-12] and cytoskeleton organisation via WASP [13]. Furthermore, it has been shown that Tec and Jak1 are able to cross-phosphorylate each other, indicating that Tec family kinases may have a modulatory effect on Jak kinases or vica versa [14,15].

Results and discussion

To analyse the role of Btk in EpoR signalling, we used the chemical compound α-cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propenamide or LFM-A13. This inhibitor was reported to bind specifically to the catalytic pocket of the Btk kinase domain and not of other related tyrosine kinases like Jak1, Jak3 and Src kinases [16]. Erythroid progenitors (R10 cell line) were factor deprived in presence and absence of 100μM LFM-A13, the recommended concentration for this inhibitor [16], and subsequently stimulated with Epo or left unstimulated. Cells treated with LFM-A13 during starvation did not show any abnormalities with respect to cell

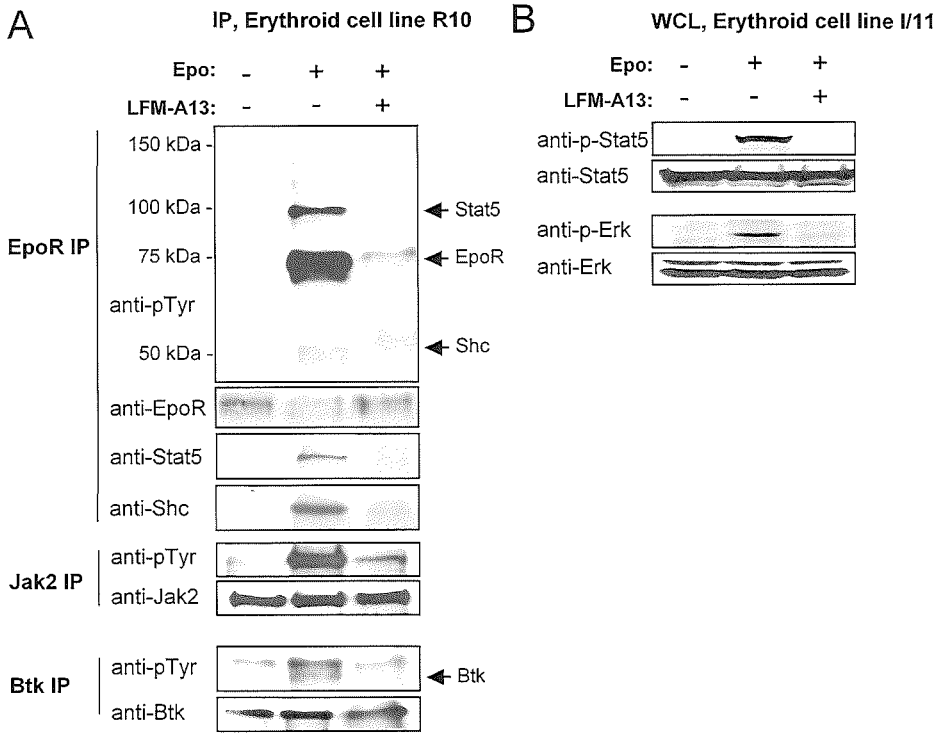

Fig. 1. Epo-induced phosphorylation of EpoR, Jak2, Btk, Stat5 and Erk1/2 is inhibited when cells are pre-treated with 100μM LFM-A13.
R10 cells were factor deprived in plain Iscoves medium (Invitrogen) for 4h in presence or absence of LFM-A13 (100μM) and subsequently stimulated with Epo (5U/ml; 10 min; 40-80x10^{6}cells/ml) at 37°C. Ten volumes ice-cold PBS were added to stop the reaction. Preparation of cell extracts, immune-precipitations, SDS-polyacrylamide gel electrophoresis and Western blots were performed as described by [17]. (**A**) The EpoR, Jak2 and Btk were immune-precipitated to analyse Epo-induced phosphorylation on Western blots. Upper panels represent anti-phospho-tyrosine stained blots; lower panels represent the same blots re-probed with the antibody used in the immune-precipitation (anti-EpoR, anti-Jak2 and anti-Btk) as indicated. In the EpoR immune-precipitate the position of size markers is indicated and arrows indicate the EpoR and known proteins co-immune-precipitating. Btk (lower band) is indicated with an arrow in the Btk immune-precipitate. (**B**) In the upper panels, Stat5 and Erk1/2 phosphorylation was detected with phospho-specific antibodies in whole cell lysates, lower panels represent the total amount of Stat5 and Erk1/2 detected with anti-Stat5 and anti-Erk1/2, respectively. Antibodies against the murine EpoR (#SC-697), Erk1/2 (#SC-94), Stat5 (#SC-836) and phospho-tyrosine (PY99, #SC-7020) were purchased from Santa Cruz, phospho-specific Erk1/2 (#9106L) from Cell signalling, antibodies against mouse Jak2 (#06-255), phospho-specific Stat5 (#05-495) from Upstate Biotechnology. Recombinant human Epo was a kind gift from Ortho Biotech (Tilburg, The Netherlands). LFM-A13 was manufactured by Boehringer Ingelheim as described by [16].

shape and viability compared to untreated cells (data not shown). While Epo efficiently induced phosphorylation of the EpoR, Jak2, Btk, Stat5 and Erk1/2, this was significantly reduced in the presence of 100μM LFM-A13 (Figure 1A, B). The co-immune-precipitating bands in the EpoR immune-precipitate representing Stat5 and Shc (Figure 1A; identified by specific antibodies) were absent when cells were pre-treated with LFM-A13. Thus LFM-A13 severely impairs EpoR phosphorylation and recruitment of proteins. Since we found that Btk is required for efficient signal transduction by the ligand-activated EpoR (Schmidt et al., in

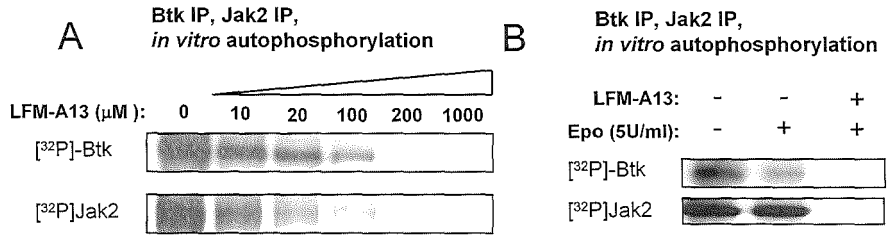

Fig. 2. LFM-A13 inhibits *in vitro* auto-phosphorylation of Btk and Jak2.
(**A**). Jak2 and Btk were immune-precipitated from factor depleted erythroid progenitors (R10) and tested for auto-phosphorylation in an *in vitro* kinase assay. Sepharose G beads (Sigma) with immune-precipitated Jak2 and Btk complexes were split into 6 equal portions and subsequently submitted to an *in vitro* kinase assays as described by 18) in presence of increasing concentrations LFM-A13 as indicated on the top. (**B**) Jak2 and Btk were immune-precipitated from cells non-stimulated (-) or Epo-stimulated (+; 5U/ml) and similarly submitted to an *in vitro* kinase assay. 100μM LFM-A13 was added to precipitates from stimulated cells.

press), we did expect LFM-A13 to inhibit Epo-induced signal transduction. However, the strong effect of LFM-A13 on Epo-induced signalling could also be due to direct inhibition of Jak2 by LFM-A13. To investigate whether LFM-A13 could act directly on Jak2, Btk and Jak2 were immune-precipitated from lysates of factor deprived cells and subjected to *in vitro* kinase assays in the presence of increasing amounts of LFM-A13. Surprisingly, the kinase activity of Btk and Jak2 (auto-phosphorylation) appeared to be inhibited with similar dose-response curves. Both kinases were already inhibited at 10μM LFM-A13, while complete inhibition occurred at 200μM LFM-A13 (Figure 2A). The same results were obtained when Btk and Jak2 were immune-precipitated from Epo-stimulated cells (Figure 2B). Epo stimulation leads to Btk and Jak2 phosphorylation resulting in a lower pool of non-phosphorylated Btk or Jak2, hence explaining the lower auto-phosphorylation in the Epo-stimulated lanes. The data suggest that LFM-A13 directly inhibits Jak2 kinase activity.
However, it could still be possible that the effect of LFM-A13 on Jak2 is mediated by Btk. If both proteins interact with each other, the presence of Btk in the Jak2 immune-precipitate could affect Jak2 auto-phosphorylation. To rule out Btk interference in the *in vitro* kinase assay, we used *in vitro* transcription/translation (ITT) to synthesise recombinant Jak2 that was subsequently subjected to an *in vitro* kinase assay in presence or absence of LFM-A13.

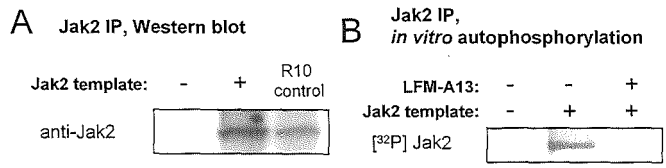

Fig. 3. LFM-A13 inhibits auto-phosphorylation of recombinant Jak2 made via *in vitro* transcription/translation (ITT).
The *in vitro* transcription/translation was carried out using the TnT® T7 coupled reticulocyte lysate system (#L4611; Promega) according to the manufacturer protocol. (**A**) Jak2 was immune-precipitated from ITT reactions in absence (lane1) or presence (lane2) of Jak2 template or from the erythroid progenitor cell line R10 and analysed for their presence on western blot using an anti-Jak2 antibody. (**B**) To test the effect of LFM-A13 on recombinant Jak2, immune-precipitated ITT Jak2 was subjected to an *in vitro* kinase assay in absence and presence of 100μM LFM-A13 (lane 2 and 3). Lane 1 represents Jak2 immune-precipitate on ITT -lysate with no Jak2 template present.

Expression of Jak2 was detected by immunoblotting using anti-Jak2 antibodies. Jak2 was only present when the template was added to the ITT and the mobility of recombinant Jak2 was the same as immune-precipitated Jak2 when compared to the erythroid progenitor cell line R10 (Figure 3A). Auto-phosphorylation of *in vitro* synthesised Jak2 in an *in vitro* kinase assay was fully inhibited by 100μM LFM-A13 (Figure 3B). This proves that LFM-A13 directly inhibits Jak2 kinase activity. Finally, we tested the effect of LFM-A13 in cells devoid of Btk. Immortalised cultures of erythroid progenitors established from Btk- or Btk*wt*, p53-deficient bone marrow, were factor deprived in presence or absence 100μM LFM-A13 and stimulated with Epo or left unstimulated. Epo-induced phosphorylation of the EpoR is impaired in Btk-deficient cells (Schmidt et al., in press; Figure 4A). However, the remaining Epo-induced phosphorylation of the EpoR is still blocked by LFM-A13. Importantly, Epo-induced phosphorylation of Jak2, Stat5 and Erk1/2, was clearly inhibited by 100μM LFM-A13 in Btk-deficient cells (Figure 4A, B), giving further prove that the effect of LFM-A13 is directly on Jak2 and independent of Btk. Finally we tested whether LFM-A13 still showed some of its alleged specificity. COS cells were transfected with the kinases Jak2, Tec, Btk and Lyn. Half of the cells was treated with LFM-A13, the other half left untreated. Cell lysates were harvested and examined for autophosphorylated kinase on Western blot. LFM-A13 inihibited autophosphorylation of Jak2, Tec and Btk, but it did not affect Lyn kinase autophosphorylation (Figure 5).

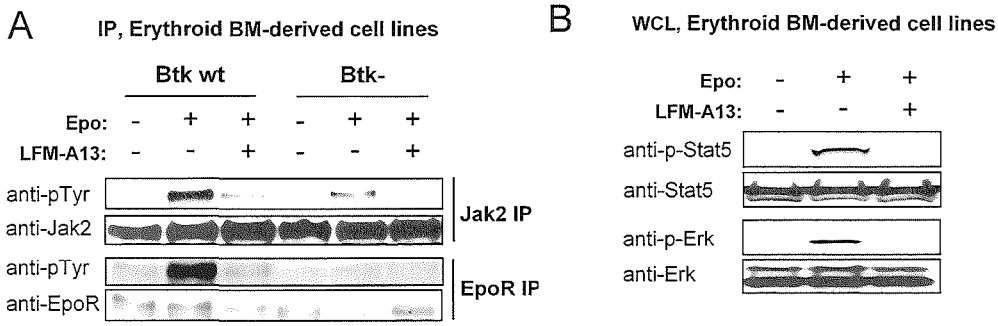

Fig. 4. LFM-A13 inhibits Jak2, Stat5 and Erk1/2 phosphorylation in Btk-deficient erythroid progenitor cells. The murine erythroid cell lines 2B6 (Btk*wt*) and 3G4 (Btk-) were established from p53-deficient mouse bone marrow of *wt* and Btk-deficient mice respectively as previously described [18]; Schmidt et al., in press). These Btk-deficient cells were factor-depleted in presence and absence of 100μM LFM-A13 as indicated and stimulated with Epo (5U/ml; 10 min.) when indicated. (**A**) The EpoR and Jak2 were immune-precipitated and assayed for tyrosine phosphorylation (PY99; upper panels) and for the presence of the EpoR and Jak2 by specific antibodies. (**B**) Lysates of the Btk- cells (3G4) were assayed on Western blots using phospho-specific antibodies for Stat5 or Erk1/2. Lower panels indicate the same blots re-probed with anti-Stat5 or anti-Erk1/2 to check for equal loading.

Thus, LFM-A13 does not inhibit the activity of all tyrosine kinases.
Both Jak2 and TEC-family kinases, among which Btk, are prominent cytoplasmic tyrosine kinases in hematopoietic cells and they co-operate in signalling pathways activated by e.g. cytokine receptors, the B-cell receptor and the Fcε receptor[19]. Moreover, Jak1 and Tec can associate and cross-phosphorylate each other [14] and we recently demonstrated that Btk similarly associates with Jak2 (Schmidt et al.,in press). Since lack of Btk impairs EpoR signalling, the effect of LFM-A13 on Epo-induced signal transduction was initially expected. However, since LFM-A13 inhibited intrinsic auto-phosphorylation of cellular or recombinant Jak2 in *in vitro* kinase assays and severely decreased Epo-induced Jak2 phosphorylation in cells lack-

ing Btk, we conclude that LFM-A13 directly inhibits Jak2 phosphorylation independent of its effect on Btk.

It was previously reported that the kinase activity of Jak1 and Jak3, close homologues of Jak2, was not inhibited by high concentration (139 to 278 μM) of LFM-A13 in *in vitro* kinase assays [16]. Importantly, the sequences of the kinase domains of Jak kinases are highly conserved. LFM-A13 incorporation into the catalytic site of Btk requires hydrogen bonds between the side chain of LFM-A13 and Arg525 and Asp539 of Btk and a hydrophobic pocket for the aromatic ring. The kinase domains of Btk and Jak kinases both contain Arginine- and Aspartic acid residues at comparable positions but there are several differences with respect to the hydrophobic pocket trapping the aromatic ring of LFMA-13. [16] discussed the consequences of these differences with respect to LFM-A13 binding affinity, but total understanding of this inhibition is still elusive. Jak1 and Jak3 kinase activity was shown to be unaffected by LFM-A13 [16] but no data was shown for Jak2, leaving the possibility that Jak2 is the exception in the Jak kinase family with respect to LFM-A13 sensitivity.

In conclusion, we show that LFM-A13 is not specific for Btk but also inhibits Jak2 kinase activity *in vitro* and *in vivo*. LFM-A13 is proposed to be a useful anti-leukemic and anti-thrombotic agent [20]. Although the successful use of STI571 to treat chronic myelogenous leukaemia proves that an inhibitor does not need to be specific to be effective[21], it is important that its specificity is known. Using LFM-A13 to elucidate the role of Btk in signal transduction of Jak2 dependent cytokine receptors could result in wrong conclusions due to simultaneous inhibition of Jak2 and Btk by LFM-A13.

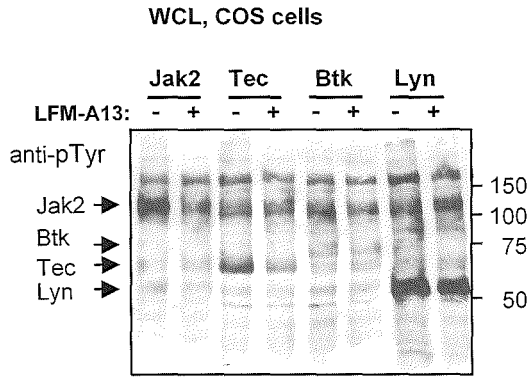

Fig.5. LFM-A13 inihibits Jak2, Tec and Btk, but not Lyn autophosphorylation in COS cells. COS cells were transfected with pSG5-based expression plasmids containing Jak2, Tec, Btk or Lyn, using calcium-phosphate precipitates as previously described [17]. Two dishes were used for each construct and LFM-A13 (100μM) was added 24 h after transfection to one of the dishes. 48 h after transfection cells were lysed and cell lysates were examined for phosphorylated proteins on a Western blot using the anti-phosphotyrosine antibody PY99. The position of the kinases is indicated by arrows. The position and of size markers is indicated at the right hand side (molecular weight in kDa).

Acknowledgements

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Chapter 5

The tyrosine kinase receptor RON functions downstream the erythropoietin receptor to induce expansion of erythroblasts

Emile van den Akker[1], Thamar van Dijk[1], Martine Parren-van Amelsvoort[1], Katja S. Grossmann[2], Ute Schaeper[2], Kenya Toney-Earley[3], Susan E. Waltz[3], Bob Löwenberg[1], Marieke von Lindern[1]

1. Department of Hematology, Erasmus MC, Rotterdam, The Netherlands
2. Max Delbruck Center , Berlin, Germany
3. Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, OH, USA.

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Abstract

Erythropoietin (Epo) is required for cell survival during differentiation and for progenitor expansion during stress and normal erythropoiesis. While signaling pathways may couple directly to docking sites on the Epo-receptor, additional docking molecules expand the signaling platform of the receptor. We studied the role of the docking molecules Gab1 and Gab2 in Epo-induced signal transduction and erythropoiesis. Inhibitors of phosphatidylinositide-3-kinase and Src-kinases suppressed Epo-dependent phosphorylation of Gab2. In contrast, Gab1 activation is dependent on recruitment and phosphorylation by the tyrosine kinase receptor RON, with which it is constitutively associated. Activation of RON induces phosphorylation of Gab1, MAPK and PKB, but not Stat5. Activation of RON was sufficient to replace Epo in progenitor expansion, but not in differentiation. In conclusion, we elucidated a novel mechanism specifically involved in expansion of erythroblasts, involving RON as a downstream target of the EpoR.

Introduction

Erythropoietin (Epo) is required for survival, proliferation and differentiation of erythroblasts. It acts together with other growth factors and hormones to balance expansion and terminal differentiation of the erythroid compartment. For instance, stem cell factor (SCF, ligand of c-Kit) cooperates with Epo to delay differentiation and enhance the number of progenitors[1,2]. The EpoR is a homodimer constitutively associated with Janus tyrosine kinase 2 (Jak2). Ligand binding induces a conformational change of the EpoR-dimer[3,4], resulting in activation of Jak2, phosphorylation of the EpoR on eight tyrosine residues and recruitment of multiple signaling molecules[5-7]. Mice deficient in Epo, the EpoR or Jak2 lack definitive erythropoiesis[8-11]. While this demonstrates a crucial role for Epo-induced Jak2 activation in erythropoiesis, the contribution of individual downstream signaling pathways to proliferation, differentiation and survival of erythroblasts is less clear. Notably, mice expressing a truncated EpoR, lacking all tyrosine residues are not anemic[12]. This may implicate that additional proteins, like docking molecules, can form a complex with the EpoR to create a signaling platform with sufficient specificity to regulate the balance of erythroid expansion and differentiation.
Candidates for this function are the Grb2-associated-binder (Gab) family members. At present three Gab family members have been identified, Gab1, Gab2 and Gab3[13,14]. Gab1 is ubiquitously expressed, while expression of Gab2 and Gab3 is tissue specific. In the hematopoietic system, Gab3 is expressed in B cells, T-cells as well as in the myeloid compartment, whereas Gab2 is expressed in all hematopoietic cells except for T-cells. Gab1-deficiency is embryonic lethal due to placental defects, while Gab1-/- embryos are also characterized by reduced size of the liver and defects in the migration of muscle precursor cells[15,16]. In contrast, Gab2 deficiency results only in mild defects in mast cell and macrophage responses[17,18] and Gab3-deficient mice show normal hematopoiesis[19]. Gab2 and Gab3 lack the c-Met binding sequence (MBS) of Gab1 and may therefore not complement Gab1 in c-Met signaling. Furthermore, the high homology between family members suggests that complementation by the ubiquitously expressed Gab1 may occur in Gab2/3 knockouts. Upon tyrosine phosphorylation, Gab proteins recruit signaling intermediates among which Shc (Src-homology containing protein), Shp2 (Src-homology domain containing phosphatase), the p85 subunit of PI3K (phosphatidylinositol-3-kinase) and PLCγ1/2 (phospholipase C)[20,21]. Gab1 is phosphorylated

following stimulation of the EpoR 22. However, it has not been examined whether Jak2 is directly involved in this process or whether other kinases could be required for Gab1 phosphorylation.

Gab1 is a direct substrate of the receptor tyrosine kinase c-Met[23]. RON (mouse Stk), a close homologue of c-Met, is expressed in erythroblasts as both a full length (RONfl) and a short form (RONsf), of which RONsf lacks the extracellular domain. Notably, susceptibility for Friend virus-induced murine erythroid leukemia is fully dependent on the presence of RONsf expression[24]. Friend virus induces Epo-independent growth of erythroblasts, via a mechanism that has not been completely resolved, but which involves binding of the viral protein gp55 to the EpoR and RONsf[25,26]. RON and c-Met are highly homologous at the multiple docking site of Gab1 interaction, which suggests a role for Gab1 in RON signaling. Activation of RON results in activation of PI3K and the mitogen activated protein kinase (MAPK) route[27,28], pathways implicated in expansion of erythroblasts[29] and downstream effectors of Gab proteins[22].

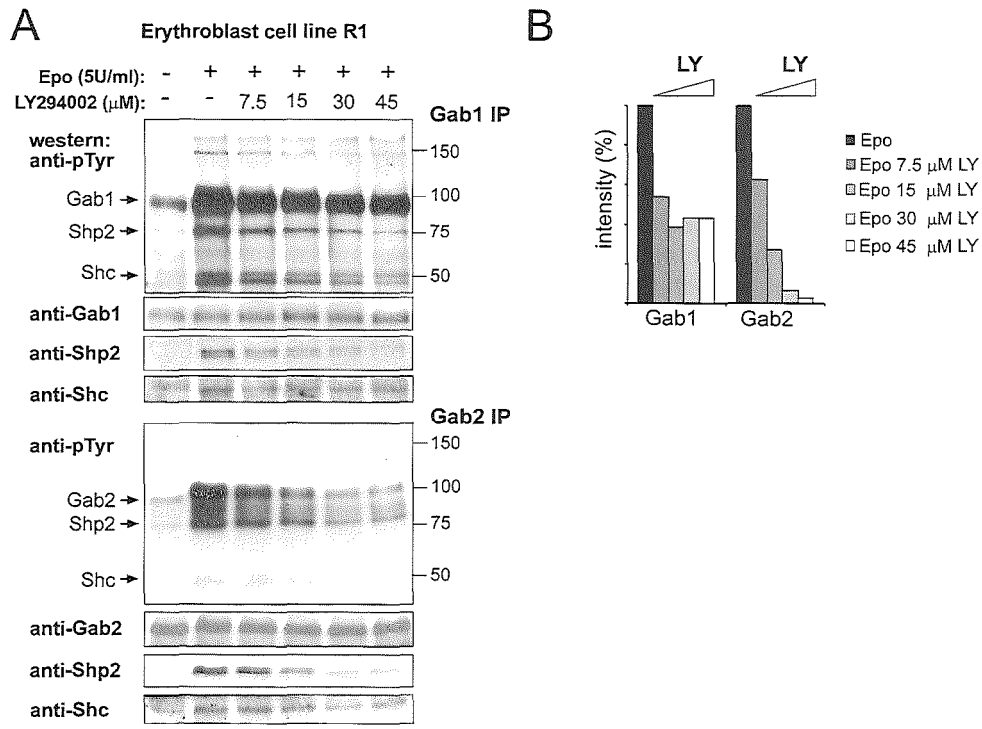

Figure 1. Epo-induced phosphorylation of Gab2, but not Gab1 is dependent on PI3K activity. A: The erythroblast cell line R1 was factor deprived (4hrs), left untreated (lane 1) or restimulated with Epo (5U/ml; 10') in presence of increasing amounts of the PI3K inhibitor LY294002. Gab1 and Gab2 were immune-precipitated from cell lysates and analyzed for tyrosine phosphorylation on Western blot. The same blots were stained with anti-Gab1 and anti-Gab2 to control equal loading. Co-immune-precipitated Shp2 and Shc were detected by specific antibodies. Size markers (kDa) as well as co-immune-precipitating proteins are indicated next to the panels. **B**: Tyrosine phosphorylation of Gab1 and Gab2 in presence of increasing concentrations LY294002 was quantified by densitometric analysis of the appropriate panels shown in (**A**). Intensity in absence of LY294002 was set at 100%.

We examined how Gab proteins function in Epo-induced signaling. We show that Gab2 is phosphorylated in response to Epo by a mechanism requiring a Src-like kinase and PI3K. In contrast, Epo-induced Gab1 phosphorylation occurs via a mechanism involving Epo-dependent activation of RON. Direct activation of RON, using a TrkA-RON fusion receptor, is sufficient to substitute Epo in progenitor expansion, while it cannot substitute for Epo to sustain differentiation to mature erythrocytes.

Results

Epo-induced phosphorylation of Gab1 and Gab2 is differentially dependent on PI3K activity
In accordance with previous observations, Gab1 and Gab2 are tyrosine phosphorylated in response to Epo in the erythroid cell line R1 (Figure 1A)[20,21]. Several phosphorylated proteins co-immune-precipitated with Gab1 and Gab2. Reprobing the same blots with specific antibodies identified the adaptor protein Shc (50kDa) and the tyrosine phosphatase Shp2 (75 kDa; Figure 1A). Epo-induced Gab1 and Gab2 phosphorylation was also detected in erythroblasts cultured from primary bone marrow and from fetal liver (data not shown). As an N-terminal Pleckstrin Homology (PH) domain characterizes Gab-proteins, their recruitment and phosphorylation is expected to be dependent on PI3K-activity. R1 cells were factor deprived and restimulated with Epo in presence of increasing concentrations of the PI3K-inhibitor LY294002 (7.5 - 45 μM; Figure 1A). While Epo-induced Gab2 phosphorylation was dose-dependently inhibited in presence of LY294002, Gab1 phosphorylation was to a large extent (>40%) resistant to inhibition by LY294002 (Figure 1A, B). Similarly, co-immune-precipitation of Shc with Gab2, but not with Gab1, was fully inhibited by LY294002. The level of phosphorylated Shp2 detected in Gab1 immune-precipitates was reduced at the high concentrations of LY294002, which may be due to phosphorylation of Shp2 by LY294002-sensitive kinases. Together the data suggest that alternative mechanisms for Gab1 and Gab2 phosphorylation may exist.

Gab1 is a substrate of RON and Lyn, Gab2 of Lyn only
To investigate what kinases are involved in the phosphorylation of Gab1 and Gab2, we expressed Gab1 or Gab2 in COS cells together with various kinases involved in EpoR and c-Kit signaling among which Jak2, Tec, Btk, Lyn, RONfl and RONsf. Expression of the various kinases was checked on a phosphotyrosine Western blot (Figure 2A). Next, Gab1 and Gab2 were immune-precipitated and analyzed for phosphorylation (Figure 2B, C). Expression of the Src-kinase family member Lyn resulted in phosphorylation of both Gab1 and Gab2. In addition, Gab1, but not Gab2 appeared to be a good substrate for RON. Moreover, RONfl and RONsf co-immune-precipitated with Gab1, but not with Gab2. Jak2, Tec and Btk did not efficienctly phosphorylate Gab1 or Gab2, although lack of Gab-phosphorylation by Tec may be due to its low autophosphorylation signal (Figure 2A). To investigate whether Src-kinases are required for Gab1/2 phosphorylation in erythroblasts, we analyzed Epo-induced phosphorylation of Gab1 and Gab2 in immortal (R1) and primary erythroblasts (cultured for 5 days from fetal livers) pre-incubated with the Src-kinase inhibitor PP2 (5μM). Epo-induced Gab1 phosphorylation appeared resistant to the Src-kinase inhibitor PP2, while Epo-induced Gab2 phosphorylation and co-immune-precipitation of Gab2-associated proteins was completely inhibited (Figure 2D, E).
The insensitivity of Epo-induced Gab1 phosphorylation to inhibitors of Src-kinases and PI3K

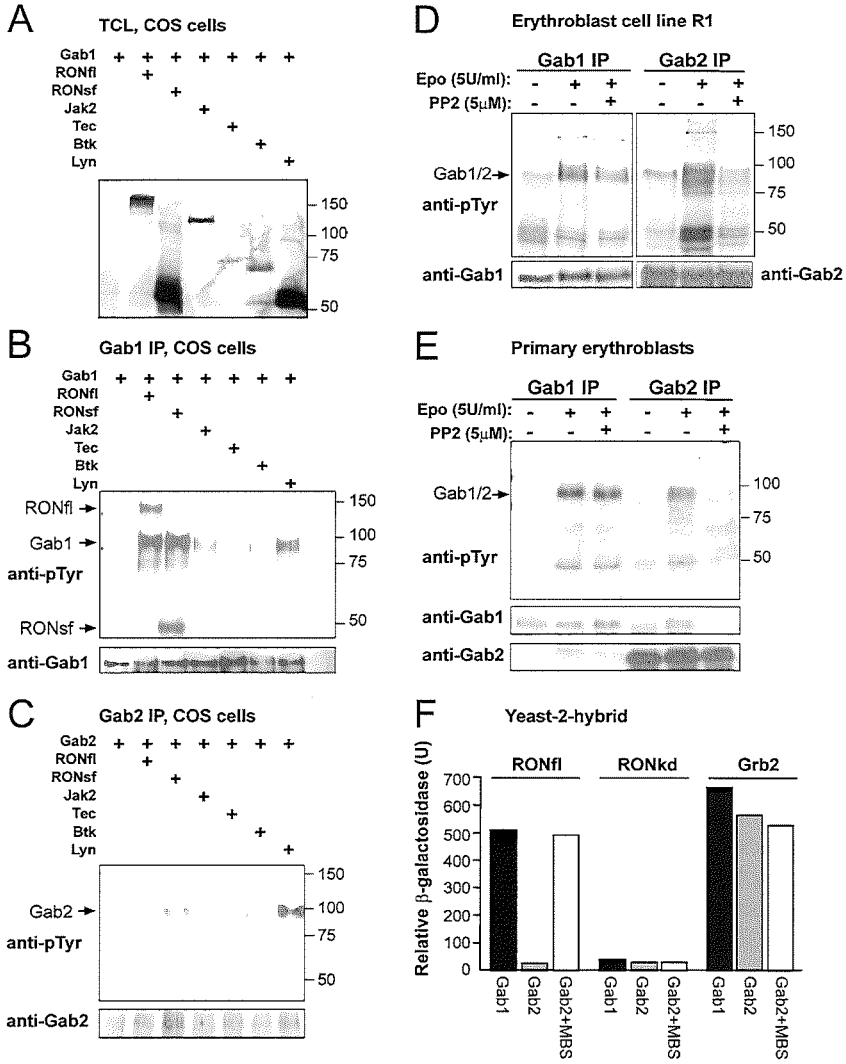

Figure 2. Distinct regulation of Gab1 and Gab2 tyrosine phosphorylation. A, B, C: COS cells were transfected with pSG5-based expression constructs encoding Gab1, Gab2 and various kinases as indicated. After 48hrs cells were harvested. (**A**) Phosphorylation status of transfected kinases was detected on Western blots by antiphosphotyrosine antibodies (PY99). Similarly, Gab1 (**B**) or Gab2 (**C**) immune-precipitates were analyzed for tyrosine phosphorylation. Arrows indicate the position of Gab1, Gab2, RONsf and RONfl. Size markers are indicated in kDa. Blots were restained with anti-Gab1 or anti-Gab2 as indicated. **D, E**: R1 erythroblasts (**D**) and fetal liver erythroblasts (**E**) were factor deprived for 4hrs in absence or presence of the Src-kinase family inhibitor PP2 (5μM) as indicated. Cells were left unstimulated or were stimulated with Epo (5U/ml: 10'). Epo-induced Gab1 or Gab2 phosphorylation was assayed on immunoblots using PY99 and equal loading was controlled by staining with anti-Gab1 or anti-Gab2. Size markers are indicated in kDa. **F**: Gab 1 associates directly with RON in yeast two-hybrid assays. Gab proteins were coexpressed with RON, kinase dead RON (RONkd) and Grb2 in the yeast reporter strain L40. Gab2+MBS encodes Gab2 protein with an insertion of the c-Met binding site (MBS). Associations were quantified by β-galactosidase liquid assay.

may result from the observed association of Gab1 and RON and phosphorylation of Gab1 by RON. To investigate whether Gab1 can interact with RON as it does with the RON family member c-MET, we carried out yeast two-hybrid (Y2H) interaction experiments[23]. The cytoplasmic domain of kinase active RON or kinase dead RON receptor (RON kd; K1114M) were expressed as bait together with full size Gab1 or Gab2 prey vector. Gab1, but not Gab2 associates efficiently with activated RON, while both Gab1 and Gab2 bind the known interaction partner Grb2 (Figure 2F). The association with RON in yeast is phosphorylation dependent since we did not detect the binding with kinase dead bait. A 13 amino acid c-Met binding sequence (MBS) is responsible for the direct association of Gab1 with the c-Met receptor[23]. A Gab2 construct in which the MBS of Gab1 is inserted can interact efficiently with RON indicating that the direct association of Gab1 with activated RON is mediated via the MBS of Gab1. In conclusion, RON is able to phsophorylate Gab1, while Src-like kinases are able to phosphorylate Gab1 and Gab2. The ability of RON to recruit and phosphorylate Gab1 may be responsible for PI3K- and Src-family-independent phosphorylation of Gab1 in erythroblasts.

Epo induces tyrosine phosphorylation of RON

If RON mediates Epo-induced Gab1 phosphorylation, it is expected to be a downstream target of Epo-signaling. The natural ligand for RON is MSP, although ligand-independent phosphorylation and activation has also been described (see discussion). To investigate factor-dependent RON tyrosine phosphorylation, R1 erythroblasts were factor deprived and stimulated with MSP or Epo. RON immune-precipitates were assayed for tyrosine-phosphorylated proteins. MSP failed to induce tyrosine phosphorylation of RON (Figure 3A). In contrast, RON was tyrosine phosphorylated in response to Epo. Tyrosine phosphorylated proteins of 100kDa, 70kDa and 50kDa were detected in the RON-immunoprecipitates from Epo-stimulated cells.The lack of response to MSP was not due to an inactive batch of MSP, since MSP induced phosphorylation of transiently expressed RONfl in HEK293 cells (Figure 3B). Note that Epo did not induce RON phosphorylation in cells lacking endogenous EpoR. To analyze factor-dependent recruitment of Gab1 to RON in erythroblasts, Gab1 was immune-precipitated from Epo-stimulated and non-stimulated lysates of primary and immortalized (R1) erythroblasts (Figure 3C, D).

In contrast to the data obtained by Y2H, RON co-immune-precipitated with Gab1 in all conditions tested (see discussion). We were not able to detect Gab1 in RON immune-precipitates, since a strong background band interfered with specific detection of Gab1 (data not shown). To check whether Gab1 is dependent on RON for its phosphorylation, we analyzed Epo-dependent Gab1 and Gab2 phosphorylation in erythroblasts expand
ed from E13.5 fetal livers of mice in which the intracellular part of RON, including the kinase domain and Gab1-docking domain was deleted (RON TK-/-)[30]. While Gab1 is phosphorylated in response to Epo in wt erythroblasts, it is not phosphorylated in RON TK-/- cells, despite the fact that Gab1 expression in RON TK-/- cells is comparable to wt cells (Figure 3E). In contrast, Epo-dependent phosphorylation of Gab2 is increased in RON TK-/- erythroblasts compared to wt cells (Figure 3F). In conclusion, the tyrosine kinase receptor RON is a target of EpoR signaling and the exclusive kinase required for recruitment and phosphorylation of Gab1.

RON is phosphorylated by Jak2 and associates with the EpoR

The EpoR activates several tyrosine kinases among which Jak2 and Lyn[31,32]. To identify kinas

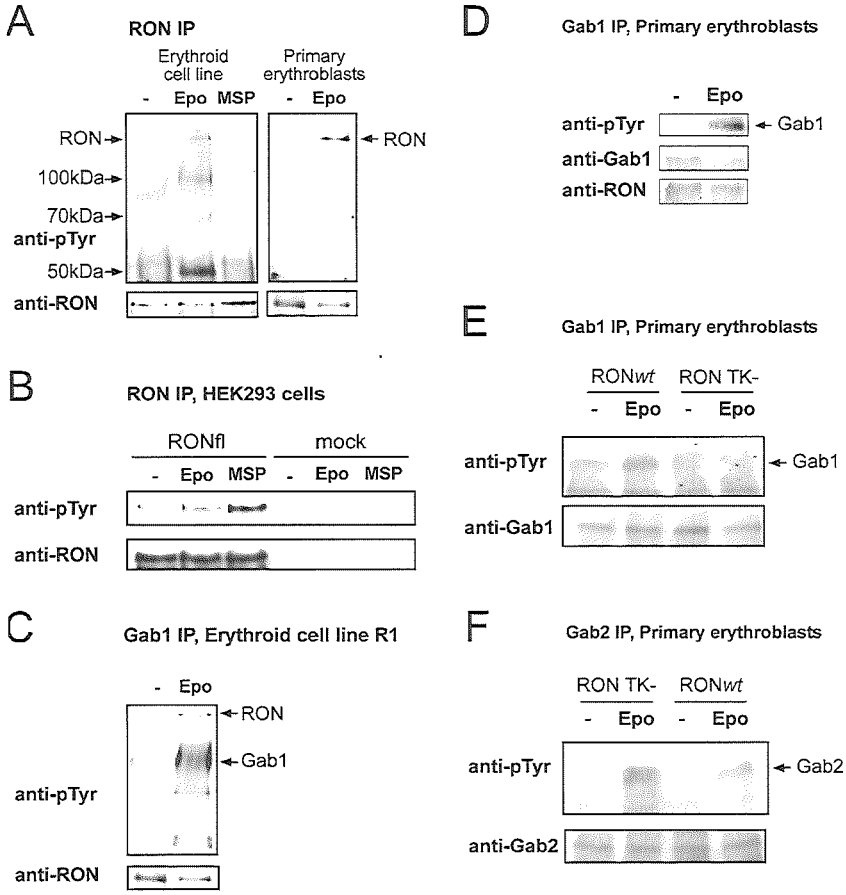

Figure 3. Gab1 is an interaction partner and substrate of RON in erythroblasts. A: Immortalised (R1) and primary fetal liver derived erythroblasts were factor-deprived and subsequently stimulated with Epo (5U/ml; 10') or MSP (100ng/ml; 10'). Anti-RON immune-precipitates were analyzed for proteins phosphorylated on tyrosine using monoclonal PY99. Blots were stained with anti-RON to control that the phosphorylated protein comigrates with RON and to control loading. Arrows indicate the position of RON and the position plus molecular weight of phosphorylated co-immune-precipitated proteins. Anti-RON did not stain a 150 kDa protein in immune-precipitates obtained with unrelated antibodies (e.g. anti-Btk; data not shown), indicating that the 150 kDa band is specific for the anti-RON immunoprecipitate. **B**: HEK293 cells were mock transfected or transfected with pSG5-RONfl, serum-deprived and stimulated with Epo (5U/ml; 10') or MSP (100ng/ml; 10'). Anti-RON immune-precipitates were tested for RON phosphorylation as described for (**A**). The erythroblast cell line R1 (**C**) and fetal liver derived erythroblasts (**D**) were factor-deprived and restimulated with Epo (5U/ml; 10'). Gab1 was immune-precipitated and blots were stained with anti-phoshotyrosine antibodies (PY99) and antibodies against Gab1 and RON as indicated. **E, F**: Fetal liver-derived erythroblasts derived from RON TK-/- embryos and wt littermates were factor deprived and restimulated with Epo (5U/ml; 10'). Western blots containing Gab1 and Gab2 immune-precipitates were stained with anti-phosphotyrosine and with anti-Gab1 or anti-Gab2 to check equal loading.

es with the potential to tyrosine phosphorylate RON, we expressed a kinase-dead RON (RONkd) in COS cells together with the tyrosine kinases Lyn, Tec and Jak2. Lyn, Tec and Jak2 all co-immunoprecipitated with wt RONfl (figure 4A), while the RON-specific antibodies did not immune-precipitate these kinases from COS cells when RON was not co-expressed (data not shown). Kinase-dead RONfl was only phosphorylated in presence of Jak2 (Figure 4B). Taking all other experiments into account this suggests, but not necessarily implies, that Jak2 also activates RON.
It has been shown that RONsf associates with the EpoR, but no data were published for RONfl[33]. Therefore, the EpoR, RONfl and Jak2 were expressed in COS cells, which were stimulated with Epo before harvesting. Both Jak2 and RON co-immune-precipitated with the EpoR, both in presence and absence of Epo-stimulation (Figure 4C, data not shown).

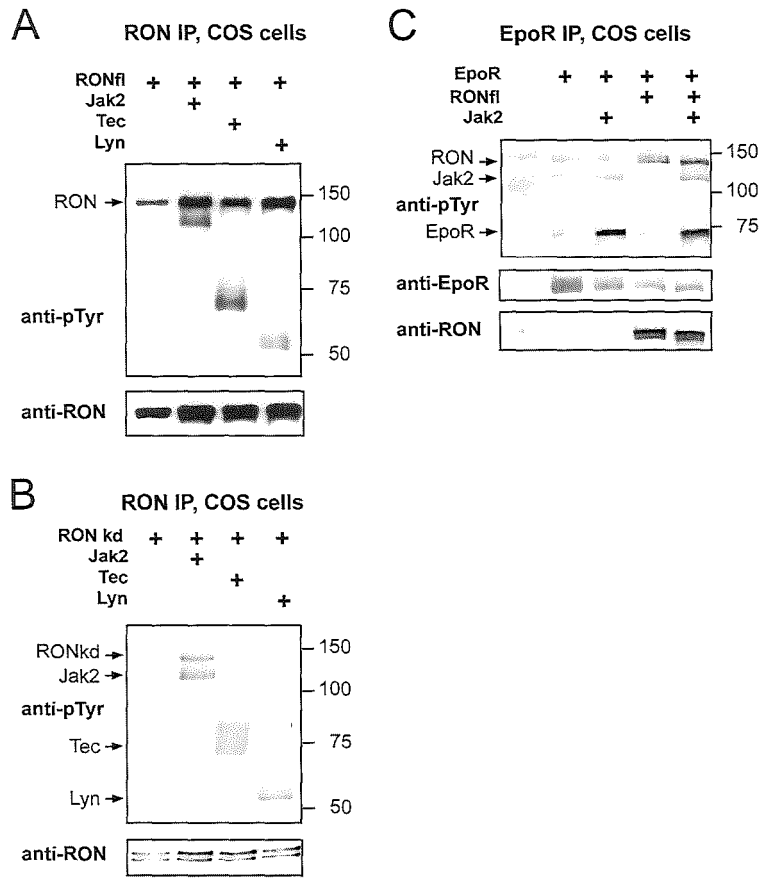

Figure 4. RON forms a complex with the EpoR and is a substrate of Jak2. A, B: COS cells were transfected with pSG5-based expression constructs encoding RONwt (**A**) or kinase deficient RON (RON kd, **B**) together with Lyn, Tec and Jak2. Cells were harvested without factor stimulation. **C**: COS cells were transfected with RONwt together with the EpoR and Jak2, and stimulated with Epo (5U/ml) 10' before cell harvest. **A, B, C**: Two days after transfection the COS cells were lysed and RON **(A, B)** or the EpoR (**C**) was immune-precipitated. The immune-precipitated proteins were subjected to SDS-PAGE and immunoblotting. Size markers are indicated in kDa. Upper panels represent blots stained with anti-phosphotyrosine antibodies (PY99), lower panels blots stained with anti-RON and/or anti-EpoR when appropriate. Arrows indicate the position of the expressed proteins.

However, Jak2 but not RON induced Epo-dependent EpoR phosphorylation. Co-expression of RON and Jak2 did not affect phosphorylation of the EpoR by Jak2, nor the association of RON and Jak2 with the EpoR.

RON phosphorylates Gab1 and activates the PI3K/PKB and Erk1/2 pathway, but not Stat5
We next examined the role of RON and Gab1 in expansion, differentiation and survival of erythroblasts. Since RON was not activated by MSP in erythroid cells, we constructed a Nerve Growth Factor (NGF)-responsive TrkA-RON fusion-receptor (Figure 5A), which was expressed in immortalized erythroblasts (R1). TrkA-RON was equally well expressed in a large number of clones and NGF stimulation resulted in phosphorylation of TrkA-RON (data not shown).
While Gab1, but not Gab2, was a substrate of RON in COS cells, NGF-induced acti
vation of TrkA-RON in R1 cells resulted in Gab1 and Gab2 tyrosine phosphorylation. NGF did not induce Gab1/2 phosphorylation in control clones, while Epo induced Gab1 and Gab2 phosphorylation in all cultures (Figure 5B). This implies that in the natural context of erythroblasts both Gab1 and Gab2 are downstream tar
gets of RON signaling. Since i) RON was able to associate with Lyn (Figure 4A, B) and ii) inhibition of Src-kinases abrogated Epo-induced Gab2 phosphorylation in erythroblasts (Figure 2D, E), it is possible that RON-mediated Gab2 phosphorylation occurs via Lyn. The RON homologue c-Met was similarly shown to associate with Lyn upon activation by HGF34. Both NGF and Epo induced phosphorylation and association of 120 and 150kDa unknown proteins with Gab1 in TrkA-RON expressing cells (Figure 5B). Finally, we tested whether activation of TrkA-RON induced some of the major signaling pathways activated by the EpoR. NGF, like Epo, induced phosphorylation of Erk1/2 and Protein Kinase B (PKB). In contrast, NGF failed to induce Stat5 (Figure 5C) or Stat3 tyrosine phosphorylation (data not shown).

Activation of RON can substitute Epo in induction of progenitor expansion
Expansion of erythroblasts, including the erythroblast cell line R1, is dependent on Epo, SCF and dexamethasone (Dex), while Epo induces differentiation into mature erythrocytes in absence of SCF and Dex. Exposure of erythroblasts to SCF and Dex in absence of Epo is unable to sustain renewal divisions[29]. To examine the role of RON signaling in Epo-dependent expansion and differentiation of erythroblasts, TrkA-RON expressing erythroblasts and control erythroblasts were seeded in medium with Epo, SCF and Dex or in medium supplemented with NGF, SCF and Dex. Vector-transduced control clones cultured in presence of NGF, SCF and Dex became pycnotic indicating that NGF does not exert any biological effect in control erythroblasts. In contrast, TrkA-RON expressing erythroblasts proliferated equally well in presence of NGF, SCF and Dex, compared to Epo, SCF and Dex (Figure 6B), and retained a blast morphology under both conditions (Figure 6C). Both the TrkA-RON clones and the empty vector are still factor dependent as absence of factors resulted in no growth and rapid cell death (Figure 6C). This showed that activation of RON can replace the ligand-activated EpoR in its function to enhance expansion of erythroblasts. Although we showed that RON can associate with the EpoR (Figure 4), Epo-independent proliferation of TrkA-RON expressing progenitors is not due to NGF-induced phosphorylation of the EpoR (Figure 6D).

Activation of RON delays Epo-induced differentiation.
We next examined whether activation of RON is able to support erythroid differentiation. TrkA-RON expressing- and control clones were seeded in differentiation medium without fac-

tors, supplemented with Epo (2U/ml) or with NGF (100ng/ml). Cumulative cell number, cell size, hemoglobin accumulation and cell morphology was determined at regular intervals. While Epo induced transient proliferation, a gradual decrease in cell size and accumulation

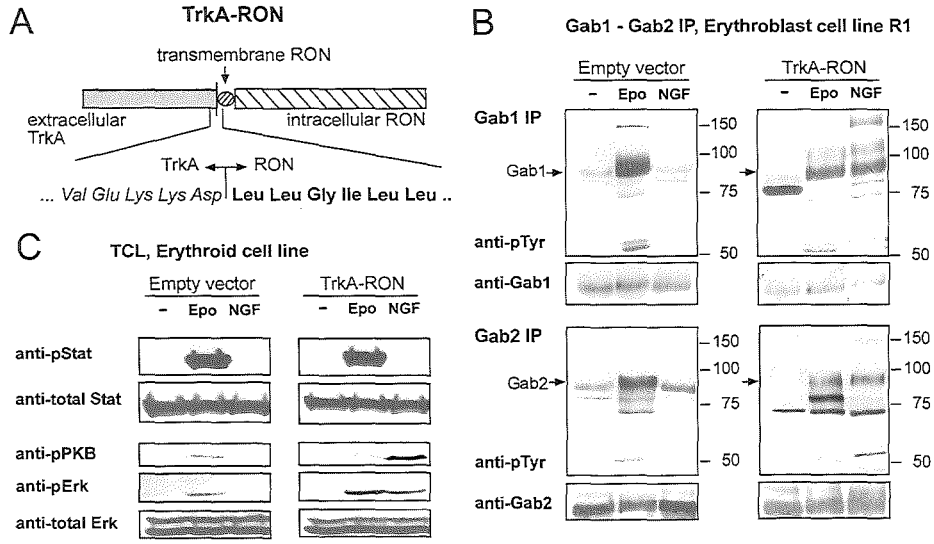

Figure 5. Activation of TrkA-RON induces phosphorylation of multiple downstream targets A: A representation of the TrkA-RON fusion protein transduced into erythroblasts. The extracellular part of TrkA (amino acids in italics) was fused to the total transmembrane domain and intracellular tail of RON (amino acids in bold). **B,C**: TrkA-RON-expressing and empty vector control cells were factor deprived and restimulated with Epo (5U/ml, 10') or NGF (100ng/ml, 10') as indicated. **B**: Western blots containing Gab1 and Gab2 immune-precipitates were stained with anti-phoshotyrosine antibodies and antibodies against Gab1 or Gab2. The position Gab1 and Gab2 is indicated by arrows, the position of size markers is indicated in kDa. C: Western blots containing whole cell lysates were stained for Ser473-phosphorylated PKB, Thr202/Tyr204-phosphorylated ERK1/2 and Tyr694/699-phosphorylated Stat5. To control for equal loading, the blot was restained with anti-ERK1/2 and anti-Stat5.

of hemoglobin, NGF completely failed to do so both in the control and in the TrkA-RON expressing cells (Figure 7A-C). TrkA-RON expressing cells rapidly died both in presence and absence of NGF (data not shown). Thus, activation of RON cannot substitute Epo in differentiation. Since activation of TrkA-RON can replace Epo during self-renewal we examined how the proliferative signals from TrkA-RON compare to Epo-induced differentiation signals. We cultured TrkA-RON clones with Epo, or with Epo and NGF and assayed proliferation, cell size and hemoglobinisation. The exposure of TrkA-RON expressing erythroblasts to NGF and Epo caused prolonged proliferation, delayed hemoglobinisation and blastoid cell size during the first 4 days of culture (Figure 7D-F). The data indicate that signals from TrkA-RON dominantly promote erythroid 'renewal divisions' but fail to sustain differentiation of erythroblasts (Figure 7G). Finally we examined whether the tyrosine kinase activity of RON and the activation of Gab1 are essential for the expansion of erythroblasts. Erythroid progenitors were derived
from E13,5 fetal livers of RON TK-/-[30] and Gab1-deficient[16] mouse embryos and control littermates and seeded in medium supplemented with Epo, SCF and Dex. All cultures expanded with similar kinetics for 14 days as previously shown for primary cells[29,35]. Progenitors cultured for 5 days from RON TK-/- livers and wt littermates, or from Gab-deficient livers and wt litter

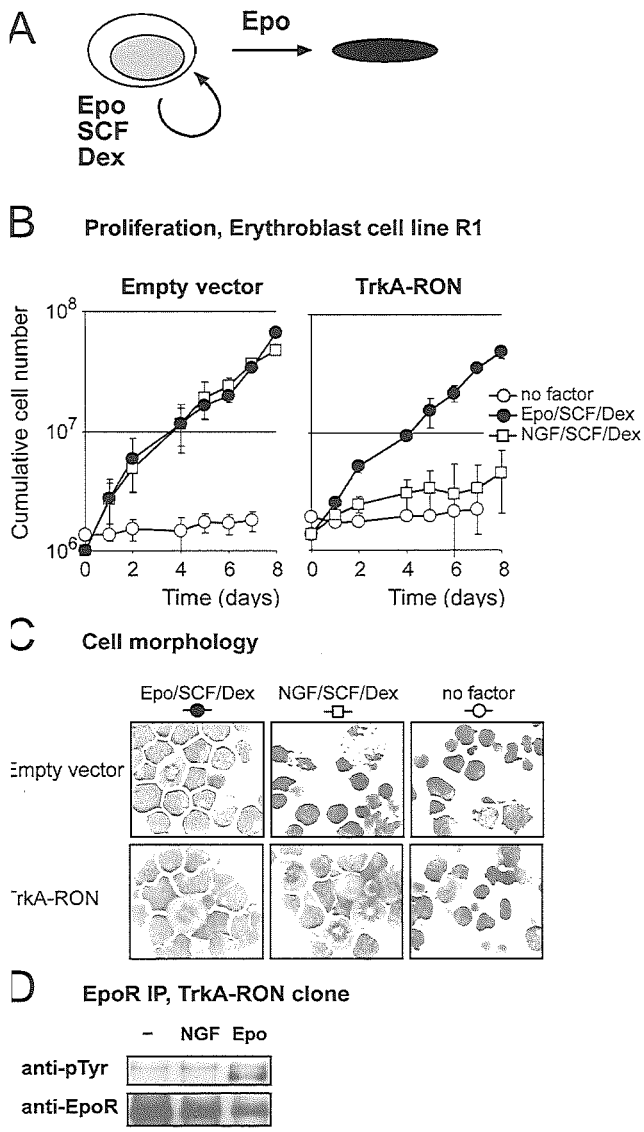

Figure 6. Activation of TrkA-RON can replace Epo in expansion of erythroblasts. A: Erythroblasts undergo renewal divisions in presence of Epo, SCF and Dex, while they differentiate to mature erythroblasts in presence of Epo. **B**: Empty vector (EV) and TrkA-RON transduced erythroblasts were seeded in medium containing no factors (O) or SCF and Dex supplemented with either Epo (2U/ml; ●) or NGF (10ng/ml; □). Cells were counted daily and cell densities were maintained between 1.5-3x10^6 cells/ml. Cumulative cell numbers were calculated. **C**: The morphology of the cells in the cultures described under **B** was examined at day 6. Both cultures contain proliferating blasts in presence of Epo/SCF/Dex. In presence of NGF/SCF/Dex control cultures (empty vector) contain pycnotic cells, while TrkA-RON expressing cultures contain proliferating blasts. In absence of factors both cultures contain pycnotic cells. **D**: TrkA-RON expressing cells were factor deprived (-) and stimulated with NGF (100ng/ml) or Epo (5U/ml). The EpoR was immune precipitated from cell lysates and analyzed on Western blot with anti-phosphotyrosine antibodies or specific antibodies to check for equal loading.

mates were seeded in differentiation conditions (5U/ml Epo, no SCF/Dex) and cell number, cell size and hemoglobin accumulation were detemined at 8 h intervals. In all cultures cells enucleated in 48 h as previously described[35] and no differences in cell proliferation, cell size or hemoglobin accumulation were detected (data not shown). Thus, activation of RON and Gab1 appears not to be crucial, which may be explained by the increased Epo-induced Gab2 phosphorylation observed in these cells (Figure 3F and data not shown).

Discussion

In the present study, we identified Gab1 and Gab2 as important players in Epo-induced "progenitor expansion signaling", each being recruited and phosphorylated by a distinct mechanism. Recruitment and phosphorylation of Gab2 is dependent
on the activation of PI3K and Src-like kinase respectively, while Gab1 phosphorylation is fully dependent on the tyrosine kinase receptor RON, which appeared to be a specific target of EpoR signaling.

Gab docking proteins are activated by distinct mechanisms
As can be expected from proteins carrying a PH-domain, Gab2 is recruited to the PIP3-rich pockets in the membrane around the activated receptor. Gab2 can interact with Grb2, which in turn associates with tyrosine residue Y464 of the EpoR[36]. However, the requirement of Grb2 to recruit Gab2 to the EpoR has not been analyzed. Possibly this interaction facilitates localization of Gab2 close to Lyn, reported to associate with tyrosine residues 464 and 479 of the EpoR[37]. The observation that the Src-kinase inhibitor PP2 suppressed Gab2 phosphorylation and the reported role for Lyn in EpoR signaling[38,39], make Lyn a likely kinase responsible for Gab2 phosphorylation. In contrast, Gab1 phosphorylation is only partially affected by inhibitors of PI3K or Src-kinase and Gab1 appeared to be recruited by the tyrosine kinase receptor RON. The observation that Epo-induced Gab1 phosphorylation is absent in erythroblasts expressing a RON receptor lacking the intracellular part indicated that RON is absolutely required for Epo-induced Gab1 tyrosine phosphorylation. The partial dependence of Gab1-phosphorylation on PI3K activity suggests that recruitment of Gab1 to RON can be facilitated by the interaction between the PH-domain of Gab1 with local PIP3. Alternatively, RON may phosphorylate only part of the Gab1 tyrosines, while other tyrosine residues may be phosphorylated by PI3K-dependent kinases like Tec or Btk. We did observe some discrepancies in the interaction between RON and Gab molecules in erythroblasts and in overexpression systems. First, the interaction between RON and Gab1 required RON kinase activity in Y2H experiments, while the interaction appeared constitutive in erythroblasts. Second, Gab2 was no substrate for RON upon overexpression in COS cells, while Gab2 was phosphorylated in response to TrkA-RON activation. To us these data represent the intricacy of the EpoR-signaling complex. RON can interact with Lyn (Figure 4A). In erythroblasts Lyn is abundantly expressed and present in the EpoR complex. Apparently Gab2 can be phosphorylated by Lyn associated with either the EpoR or RON. Concordantly, the RON homologue c-MET also associates with Src-kinases and its activation similarly induces phosphorylation of Gab1 and Gab2[40]. In addition, the interaction between RON and Gab1 may not only depend on interactions between phosphorylated tyrosine residues with the MBS and Grb2, but may be stabilized by additional scaffolding proteins.
The question arises whether the distinct regulation of Epo-induced Gab1 and Gab2 is asso

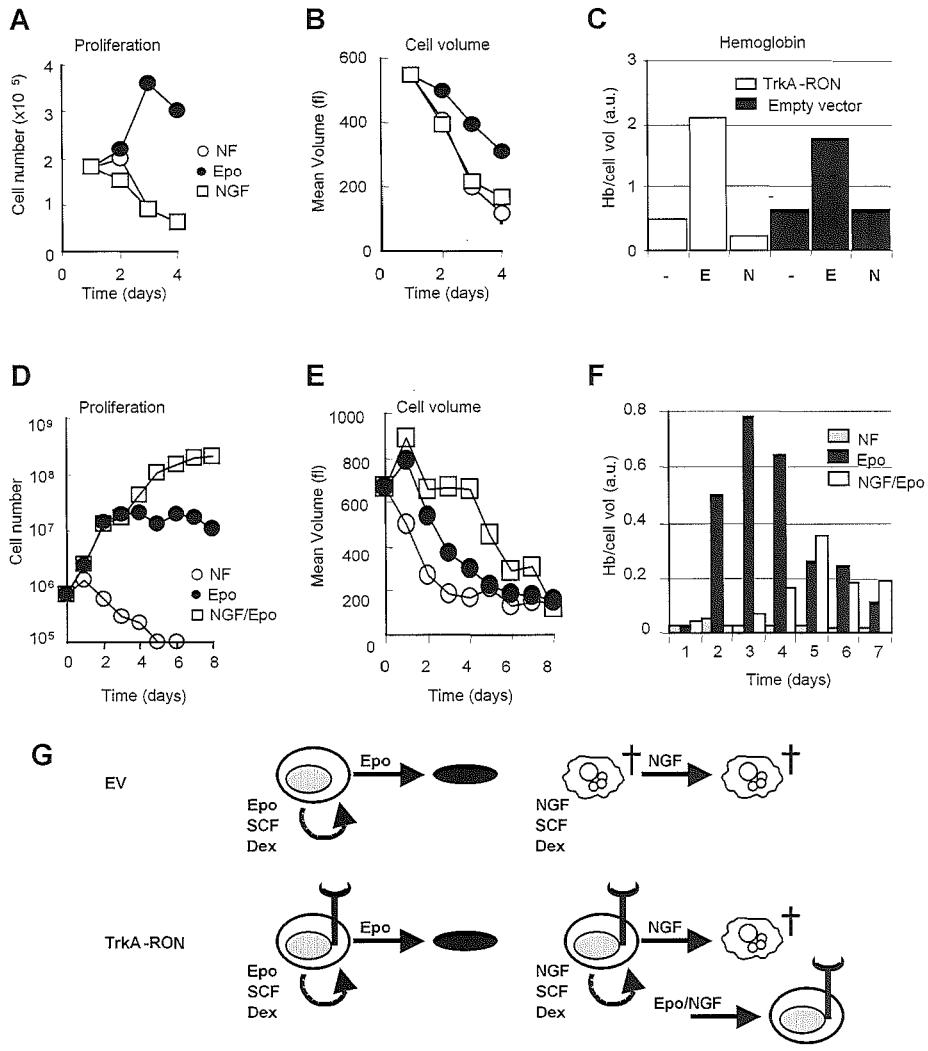

Figure 7. Activation of TrkA-RON fails to support erythroid differentiation and delays differentiation in presence of Epo. A, B: TrkA-RON expressing erythroblasts were cultured in medium containing iron-loaded transferrin without additional factor (NF; O), supplemented with Epo (2U/ml,●) or with NGF (10ng/ml, □). Total cell numbers (**A**) and mean cell volume (fl; **B**) are depicted. **C**: At day 4 of the experiment the hemoglobin content was measured (Hb/cell volume in arbitrary units) in empty vector transduced cultures and TrkA-RON expressing cultures seeded in absence of factor (-), or in presence of Epo or NGF. **D-F**: TrkA-RON expressing erythroblasts were seeded in differentiation medium lacking growth factor (NF; O), or medium supplemented with Epo (2U/ml, ●) or Epo (2U/ml) plus NGF (10ng/ml, □). Cumulative cell number (**D**), cell volume (fl, **E**) and hemoglobin accumulation (Hb/cell volume, in arbitrary units; **F**) were determined at daily intervals. **G**: Schematic representation of the results. Empty vector transduced cultures as well as TrkA-RON expressing cultures can be expanded in presence of Epo/SCF/Dex and differentiate in presence of Epo. When Epo is substituted by NGF, TrkA-RON-expressing cultures can be expanded, while control cells become pycnotic. However, NGF fails to support differentiation of TrkA-RON-expressing erythroblasts. In combination with Epo, NGF delays differentiation of TrkA-RON-expressing erythroblasts.

ciated with a specific function. Gab1-deficient mice die at day 14 to 16 of development due to placental defects[16]. At this time the liver is reduced in size. No obvious erythroid defect can be detected in Gab1-/- mice, but the liver defects exclude in vivo assessment of normal adult and stress erythropoiesis. We observed no difference in the in vitro expansion and differentiation capacity of erythroblasts derived from E13 Gab1-/- fetal livers, nor in erythroblasts from RON kinase-deficient mice that fail to phosphorylate Gab1 (data not shown). However, Gab1-/- as well as RON TK-/- erythroblasts did show enhanced Epo-induced tyrosine phosphorylation of Gab2. In all other cells, Gab-proteins can complement each other, resulting in healthy mice lacking Gab2 and Gab3. Rather than independent functions, the EpoR appears to have two mechanisms that assure phosphorylation of either Gab1 or Gab2 and that may be tuned to ascertain different levels of signaling activity.

RON is part of the EpoR signaling complex

Although RON can be activated via its ligand (MSP), increasing evidence suggests that RON can also be activated by MSP-independent mechanisms through heterodimerisation. RON has been shown to interact with various receptors like c-Met, integrins and the common beta chain of the IL5, IL3 and GM-CSF receptor leading to cross-activation[24,25,41-43]. Furthermore, hyaluronoglucosaminidase-2 (HYAL2) was found to be a functional inhibitor of RON. Sequestering of HYAL2 by the viral envelope protein of Jaagsiekte sheep retrovirus results in constitutive RON activity in absence of ligand[44]. In this study we observed that MSP does not induce RONfl phosphorylation or downstream signaling in erythroblasts, while Epo stimulation efficiently induced RON phosphorylation in the same cells. This occurred both in two independent cell lines derived from distinct genetic backgrounds and in erythroblasts expanded from wt fetal livers of either CBA or 129P mice (data not shown). Thus, ligand binding is apparently not a prerequisite for RON activation in erythroblasts. Also upon over-expression of RONfl in the erythroblast cell line R1, tyrosine phosphorylation of RON was induced by Epo but not by MSP (data not shown), suggesting that there may be an inhibitory mechanism active in these cells as previously described for epithelial cells[44].

Our data show that Jak2 can phosphorylate RON in COS cells. Since Gab1 phosphorylation is dependent on Epo stimulation in erythroid cells and on RON-kinase activity in COS cells, it suggests that Epo-induced Jak2 activation not only leads to phosphorylation of RON but also to its activation. Furthermore, the data suggest that the EpoR, Jak2 and RON are part of one complex as RON is a substrate of Jak2. While mice deficient in Epo, its receptor and the associated kinase Jak2 die at day 13 of gestation due to a lack of erythrocytes, mice in which the EpoR lacks all tyrosine-docking sites in the EpoR C-terminal tail develop normally without aberrations in erythropoiesis[12]. Previously it was suggested that signaling intermediates like Shc, Shp2 and PI3K could dock to Jak2 upon deletion of the C-terminal tail of the EpoR containing the eight phosphorylated tyrosine residues. However, since Epo-induced activation of Jak2 results in RON activation, which subsequently results in phosphorylation of Gab1 (directly) and Gab2 (via Src-kinases), these docking molecules may be responsible for the recruitment of several signaling intermediates like Shc, Ship2 and PI3K independent of an intact EpoR C-terminus.

RON is involved in renewal induction

The ability of ligand-activated TrkA-RON to replace Epo stimulation in expansion of progenitors but not in differentiation is in line with the role of the truncated avian homologue v-SEA, which also induces expansion of erythroblasts but not their differentiation[45]. However, while

expression of v-SEA is sufficient to induce renewal divisions in avian erythroblasts, signals emanating from an activated RON in mouse erythroblasts require cooperative signaling by c-Kit and the glucocorticoid receptor to induce expansion. As RON is a downstream target of EpoR signaling, the mechanism of cooperation between NGF, SCF and dexamethasone will most likely be similar to cooperative signaling between Epo, SCF and dexamethasone but remains to be elucidated. TrkA-RON expressing erythroblasts exposed to NGF alone rapidly die indicating that RON signal transduction via Gab1/2, PI3K, Pkb and the MAPK route is not sufficient for these cells to survive. Exogenous (over)expression of a kinase always warrants a careful interpretation of the results since its regulation may not be subject to normal controls. However, the fact that activation of TrkA-RON alone is not sufficient for survival or proliferation of erythroblasts, but requires cooperation with SCF suggests that its function closely mimics that of endogenous RON.

While activation of RON can substitute EpoR-activation to maintain renewal divisions in cooperation with SCF and dexamethasone, it also transiently sustained renewal divisions under differentiation conditions in presence of Epo. Thus, the EpoR apparently activates both renewal- and differentiation-specific signaling pathways, which need to be carefully balanced to maintain the proper number of red blood cells. When this balance is disrupted by e.g. increased RON activity, a polycythemic or leukemic phenotype may arise, as it occurs in the SFFV infected mice. As shown, Epo induces several alternative routes to activate common targets like PI3K and the MAPK route. So far it is unclear how these complementary pathways contribute to EpoR signaling and how cellular output is affected upon changes in the balance of these integrated cascades.

The data are consistent with the observation that RONsf is required for SFFV to induce erythroleukemia in mice. gp55 protein targets RONsf[33] resulting in constitutive phosphorylation and activation of RON, and Epo-independent expansion of progenitors. Similar to the TrkA-RON clones, SFFV-induced erythroleukemia still requires SCF for renewal-induction[46] and permits Epo-induced differentiation to functional erythrocytes. One of the major Epo-induced signaling molecules that is not activated by RON is Stat5. The inability of ligand-activated TrkA-RON to support in vitro terminal differentiation of erythroblasts could be due to lack of Stat5 phosphorylation. Stat5-deficient erythroblasts, cultured in vitro in serum free medium, are deficient in Epo-induced differentiation due to the inability to activate the anti-apoptotic protein $BclX_L$[35]. Concordantly, mice deficient in Stat5 are impaired in their response to hypoxia. Upon hypoxia induction, Stat5-deficient mice suffer from anemia accompanied by apoptosis of erythroblasts[47]. Yet, these mice have an enlarged spleen and expansion of erythroblasts in the spleen, suggesting impaired differentiation rather than a defect in expansion. These data suggest that Stat5 activation is essential for erythroid differentiation but not for expansion. The fact that RON does not activate Stat5 may be reflected in its inability to induce differentiation and survival during the onset of differentiation.

Materials and methods

Antibodies and growth factors

Rabbit antisera recognizing Jak2 or Gab1 and a mouse monoclonal antibody recognizing phospho-Stat5 (Y694-Stat5A/Y699-Stat5B; #05-495) were obtained from Upstate Biotechnology (Lake Placid, NY); rabbit antisera recognizing the mouse EpoR, RON, TrkA or p44/p42 Erk1/2 and the anti-phosphotyrosine mouse monoclonal antibody PY99 from Santa

Cruz Biotechnology (Santa Cruz, CA; sc-697; sc-322; sc-14024; sc-94; sc-7020 respectively); rabbit antisera recognising phospho-PKB (Ser473; #9271) and mouse monoclonal antibodies recognising phospho-p44/p42 Erk1/2 (Thr202/Tyr204; #9101) from Cell Signaling Technology (Beverly, MA). Rabbit antiserum recognizing Gab2 was a kind gift from Dr. T. Hirano (Osaka University, Japan). Human MSP was obtained from R&D systems, recombinant human Epo was a gift from Ortho-Biotech (Tilburg, The Netherlands), dexamethasone was purchased from Sigma (Zwijndrecht, The Netherlands) and SCF was obtained from the supernatant of producer CHO-cells.

Plasmids and oligonucleotides

The complete open reading frame of RONfl (a kind gift of Dr. Breathnach, Institut de Biologie-CHR, Nantes, France), Tec, Jak2, Lyn, EpoR, Gab1 and Gab2 were cloned into the mammalian expression vector pSG5 (Stratagene, La Jolla, CA) and the retroviral vector pBabepuro when indicated. The kinase dead form of RON, K1114M, was generated using the QuickchangeTM Site-directed Mutagenesis Kit (Stratagene) with the following oligonucleotides: 5'-CCA ATG TGC CAT CGA TTC ACT AAG TCG CA-3 and 5'-GCT CTC CAG CGC CGT CCA CTT CAC A-3'. To construct TrkA-RON the extracellular domain of the TrkA-receptor was expanded via PCR (from TrkA-Met[48]) using forward primer 5'- GCG AAT TCA TGC TGC GAG GCG GAC GGC GC-3' and reverse 5'-GCG ATA TCC TTC TTC TCC ACC GGG TCT CC-3' after which the fragment was cut with *EcoRI* and *EcoRV*. The RON transmembrane/intracellular domain PCR product was amplified using forward primer 5'-GCG ATA TCC TCC TTG GTA TCC TGC TGC CT-3' and reverse primer 5'-CGC TCG AGC TAA GCA GGT CCA GCC CAA GA-3' and digested with *EcoRV* and *XhoI*. Both fragments were simultaneously ligated into the *EcoRI/SalI* site of the retroviral vector pBabepuro creating the TrkA-RON fusion receptor (Figure 5A). The prey plasmids VP16-Gab1, VP16-Gab2 and VP16-Gab2+MBS have been described[23].

Yeast two-hybrid analysis

The Yeast strain L40 was used for the Yeast-two-Hybrid transformation assays[49]. It contains *his3* and *lacZ* as reporter genes. Bait plasmids BTM-RON and BTM-RONkd code for the kinase active and kinase inactive cytoplasmic part of the human receptor tyrosine kinase RON, respectively. This cytoplasmic part was fused to the LexA DNA-binding and dimerization domain. Sequences encoding amino acids 983 to 1400 of wt RON or RON K1114M were amplified by PCR (using 5`-GGA ATT CCG GAG GAA GCA GCT AGT TCT TCC-3` and 5`-AAA AAA GTC GAC TCA AGT GGG CCG AGG AGG CTC-3`) and cloned into pBMT as *EcoRI-SalI* fragments. The β-galactosidase assay measures *LacZ* reporter gene activity to quantify the interaction of bait and prey proteins. Yeast was grown in liquid culture and optical density measured at λ=600 nm, pelleted and cracked open by a freeze-thaw-technique in liquid nitrogen. b-galactosidase activity was determined colorimetrically using the compound ONPG (o-Nitrophenyl-β-D-Galactopyranosid) as a substrate (λ=420 nm). The following formula was used to calculate relative b-galactosidase activity: 1000 X OD 420 /(t X V X OD 600). t = time (min), V = Volume (ml).

Cell lines, primary cells, transfections and viral transductions

COS, HEK293 and ecotropic Phoenix (φE) cells were maintained in Dulbecco modified Eagle medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Life Technologies). For COS and HEK293 transfection experiments, 1 million cells

were seeded in 60mm^2 dishes and after 3-4hrs (COS cells) or 24hrs (293 cells) transfected with 12μg DNA as previously described[50]. After 24hrs the cells were washed once with PBS (phosphate buffered saline) and new medium was added. Cells were harvested 48hrs after transfection. Primary fetal liver erythroblasts as well as the murine erythroblast cell line R1 (generated as described before)[29] were cultured in StemPro-34TM medium (Life Technologies) supplemented with Epo (0.5U/ml), SCF (100ng/ml) and Dexamethasone (50μM). Cell cultures were maintained at 1.5-3x10^6 cells/ml. Viral transduction of the erythroblast cell line R1 was performed as described by Bakker et al.[51]. To obtain single cell derived clones, cells were seeded in semisolid Stempro-34TM medium supplemented with Epo, SCF and dexamethasone as described above plus puromycin (2μg/ml). To induce differentiation erythroid cells were washed two times with PBS and reseeded in StemPro-34TM medium supplemented with 5U/ml Epo and 0.5mg/ml iron-saturated human transferrin (Intergene). During differentiation cells were kept at 2-5x10^6 cells/ml

Hemoglobin content determination and cell morphology
To measure the hemoglobin content in cells, 3 x 50 μl aliquots of the cultures were taken and processed for photometric determination of hemoglobin as described before[51]. To analyze cell morphology 50μl samples were taken from culture, cytocentrifuged onto slides and stained with histological dyes and neutral benzidine for hemoglobin[52]. Images were taken using a CCD camera and processed with Adobe Photoshop.

Immune-precipitations and Western blotting
Primary erythroid fetal liver cells and the erythroblast cell line R1 were growth factor deprived for 4hrs in plain IMDM (Life Technology), while HEK293 cells were serum deprived for 24hrs in plain DMEM. The erythroblasts (40-80x10^6/ml) were stimulated at 37°C with SCF (1μg/ml; 5'), Epo (5U/ml; 10'), MSP (100ng/ml; 10') or NGF (100ng/ml; 10') as indicated, the HEK293 cells and COS cells were stimulated for 10' with 100 ng/ml MSP or Epo (5U/ml). Reactions were stopped with excess ice-cold PBS. Cells were washed twice with ice-cold PBS, lysed in ice-cold lysis buffer[53], incubated on ice for 10', nitrogen frozen and stored at -80°C until usage. Lysates were thawed on ice cleared by centrifugation at 4°C at 15,000rpm. Immune-precipitations, SDS-polyacrylamide gel electrophoresis and Western blots were performed as described previously 53. Membranes were stripped in 63mM Tris-HCl pH 6.1, 2% SDS and 100mM β-mercapto-ethanol for 30' at 50°C after which they were re-blocked and re-stained.

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CHAPTER 6

Reduced progenitor expansion and impaired signal transduction characterizes *in vitro* erythropoiesis of Fanca- and Fancg-deficient mice

Emile van den Akker[1], Henri J. van de Vrugt[2], Godfrey Grech[1], Bob Löwenberg[1], Fré Arwert[2], Marieke von Lindern[1]

1 Inst. Hematology, Erasmus MC, Rotterdam
2 Dept. of Clinical Genetics and Human Genetics, VU University medical center, Amsterdam, The Netherlands

In preperation

Abstract

Fanconi anemia (FA) is an autosomal recessive disorder in which the mechanisms underlying the disease are only partially understood. FA is commonly accompanied with anemia due to bone marrow hypoplasia. To analyze cytokine-dependent survival, expansion and differentiation of hematopoietic progenitors lacking Fanconi proteins, we compared erythropoietin (Epo) signaling in erythroid progenitors derived from mice lacking Fanca or Fancg, or from *wt* littermates. Epo-induced phosphorylation of Stat5 and Erk1/2 was severely reduced and delayed in progenitors lacking Fanca or Fancg. As a consequence, Epo-induced up- or down-regulation of target genes like p21WAF, GILZ, SOCS3, Spi2.1 and $BclX_L$ was reduced. In contrast to these downstream signaling intermediates, Epo-induced phosphorylation of the Epo-receptor and its associated kinase Jak2 were not affected. Stem Cell Factor-induced c-Kit activation and downstream Erk1/2 phosphorylation was equally efficient in *wt* or Fanca or Fancg-deficient cells. The expansion capacity of erythroid progenitors lacking Fanca or Fancg proteins was reduced, but only after prolonged culture (>7 days). Although mice lacking Fanconi proteins are healthy, the observed mechanism could contribute to a predisposition for anemia in FA, that may become apparent in the context of additional mutations.

Introduction

Fanconi anemia (FA) is an autosomal recessive disease characterized by developmental abnormalities, bone marrow failure and cancer predisposition. Patients with FA present at the clinic at early age (median 7 years) with decreased platelet counts and aplastic anemia. Before the age of 40, 81% of cases further progresses to total bone marrow failure or other hematological abnormalities, which is the major cause of death. FA cells show a large range of abnormalities including hypersensitivity to clastogenic and growth inhibiting effects of DNA cross-linking agents like mitomycin C, spontaneous chromosome breakage and an increased sensitivity to apoptosis inducers TNFα and IFNγ[1].

FA is a heterogeneous disease and so far eleven complementation groups have been identified[2,3]. FANCA, C, E, F and G may partly reside in the cytoplasm, but they aggregate to form a nuclear core complex together with the biotin ligase FANCL [4,542]. This complex recruits and ubiquitinates FANCD2, a process that requires FANCI[3939]. Mono-ubiquitination of FANCD2 is critical for its interaction with BRCA1 and affects DNA repair and S-phase checkpoint response [6-8]. FANCJ has a unknown role late in this process. As is evident from mice lacking Fanca, Fancc or Fancg and from *in vitro* studies on cell lines derived from FA patients, the hypersensitivity to DNA crosslinkers results from the inability to form a Fanconi core-complex in the nucleus[9]. The Fanc-deficient mice do not have bone marrow failure or an enlarged risk of acquiring solid tumors as described for FA progression. However, upon additional loss of p53 fancc-deficient mice develop a large variety of tumors, including leukemia[43]. To date the relation between genomic instability and the molecular mechanism of (aplastic) anemia is not clear. Bone marrow transplantation experiments revealed a reduced repopulation capacity of Fancc-deficient stem cells [10] and growth factor-induced proliferation and differentiation of Fancc-deficient bone marrow cells is reduced compared to *wt* littermates [11,12]. Although IFNg-induced gene expression and apoptosis is enhanced in FA lymphoid and fibroblastoid cell lines [13-15], IFNγ-induced STAT1 tyrosine phosphorylation is severely reduced in FANCC-deficient cells [16] and IFNγ treatment of Fancc-deficient mice did not lead to bone marrow failure

[17]. Therefore, increased sensitivity to IFNγ remains controversial and the cause of bone marrow failure remains elusive.
Both Fancc- and Fanca-deficient hematopoietic progenitors have a high rate of spontaneous apoptosis [18,19]. Epo-receptor (EpoR) activation suppresses apoptosis and allows differentiation of erythroid progenitors. The EpoR, like INFγR, is a member of the cytokine family and signals via the Jak-Stat pathway. FA patients presenting with anemia have high Epo serum levels that fail to rescue anemia. [20,21]. We found that Epo-induced Stat5 and Erk1/2 phosphorylation in Fanca- and Fancg-deficient erythroid progenitors was severely reduced. Epo/SCF-dependent expansion of Fanca- and Fancg-deficient mouse erythroid progenitors was initially normal, but an increased differentiation rate abrogated progenitor expansion significantly earlier than the expansion of respective *wt* progenitors.

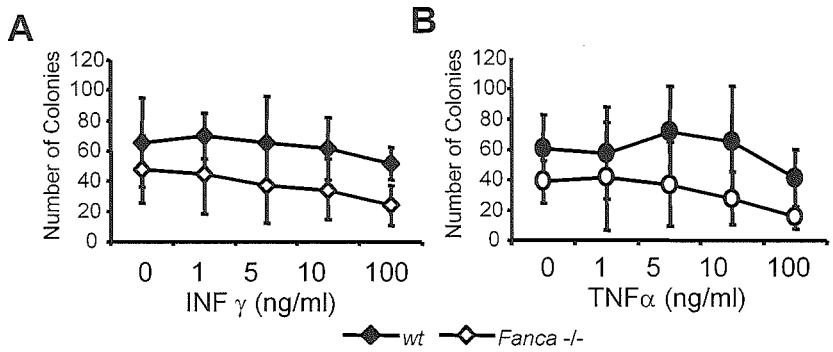

Figure 1. Colony formation of Fanca-deficient progenitors is not decreased in number nor hypersensitive to the apoptosis inducers IFN-g and TNF-a. Fanca-deficient and *wt* bone marrow cells were seeded in semisolid medium supplemented with SCF, IL-3, IL-6 and Epo in absence or presence of various concentrations of the apoptosis inducers TNF-α (A) or IFN-γ (B). The depicted colonies numbers represent mainly CFU-GM with an insignificant contribution of BFU-Es. Values represent mean and standard deviation of three experiments.

Results

Bone marrow of Fanca-deficient cells is not hypersensitive to IFNγ and TNFα.
FANCC deficient lymphoblastic cell lines are significantly more sensitive to IFNg- and TNFα-induced apoptosis. Furthermore, FA bone marrow shows increased spontaneous apoptosis. To examine whether bone marrow of Fanca-deficient cells is hypocellular and/or hypersensitive to inflammation factors, we isolated bone marrow from week 8 Fanca-deficient mice and *wt* littermates. Colony numbers were determined in semi-solid medium supplemented with SCF, IL-3, IL-6 and Epo (see M&M). In addition, increasing concentrations of IFNγ and TNFα were added. Although progenitor numbers in Fanca-deficient animals were consistently lower than in *wt* animals, the difference was not significant. In addition, no significant difference was observed between *wt* and Fanca-deficient mice with respect to IFNγand TNFα sensitivity of progenitors (Figure 1).

Fanca- and Fancg-deficient erythroid progenitors have a lower expansion capacity.
Mice deficient in Fanca or Fancg do not develop spontaneous anemia. However, since Fancc-deficient stem cells were shown to have a reduced capacity to sustain hematopoiesis in serial transplantations or upon inflicting long term hematopoietic stress [10,12], the expansion, survival or differentiation kinetics of hematopoietic stem cells may be impaired under stress

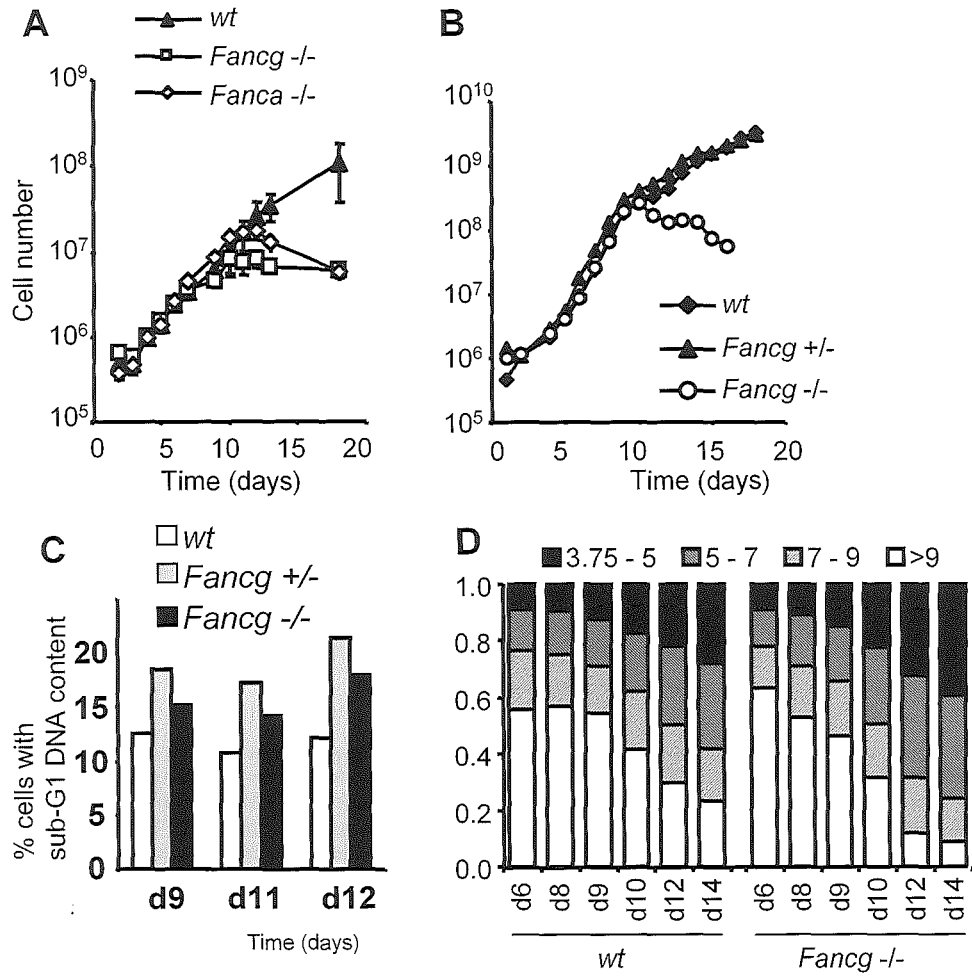

Figure 2. The expansion capacity of Fanca- and Fancg-deficient erythroid progenitors is significantly lower than in *wt* cells. **A**: *Wt* (black triangles), Fanca (white diamonds) or Fancg (white squares) deficient fetal liver erythroid progenitors were seeded in medium containing SCF, Dex and Epo. Cells were counted daily and cell densities were maintained between 1.5-3 million cells/ml. Cumulative cell numbers were calculated. **B**: An independent experiments using *wt* (black diamons), Fang+/- (black triangles) and Fancg-/- (white squares) fetal liver cells. **C**: Aliquots of the experiment described in **B** were taken on the indicated time points. The cells were fixed and stained with propidium Iodine to distinguish life from dead cell populations. Cells with subG1 DNA content *wt* (+/+), heterozygous (+/-) and Fancg-deficient (-/-) cultures are depicted as a percentage of the total population. **D**: Cell numbers of the cultures depicted in **B** were differentiated for size: 3.75-5 μm roughly corresponds to erythrocytes, 5-7 μm to late erythroblasts, 7-9 μm to early erythroblasts and 9-12 μm to erythroid progenitors (pro-erythroblasts)

conditions. Compensatory physiological processes may veil such defects *in vivo*. Therefore we checked *in vitro* expansion of erythroid progenitors as a measure of stress-erythropoiesis [22,23]. Synchronous differentiation of expanded progenitors can reveal subtle differences in differentiation kinetics. Fetal liver cells from E13 Fanca-/-, Fancg-/- and *wt* littermates were isolated and seeded in StemPro medium supplemented with Epo, SCF and dexamethasone as described [23]. Although erythroid progenitors from *wt* and deficient mice initially expand with

similar kinetics, expansion of the Fanca or Fancg-deficient erythroid progenitors stopped remarkably earlier than their *wt* counterparts (figure 2A, B). We did not detect increased cell death in Fanc-deficient cells (figure 2C). Instead, the relative number of cells reduced in size, corresponding to maturing cells that do not contribute to expansion, increased (Figure 2D and morphological analysis not shown). This shows that the expansion capacity of Fanca- and Fancg-deficient progenitors is exhausted significantly sooner compared to *wt* progenitors due to increased differentiation at the expense of renewal divisions. Upon induction of differentiation in presence of Epo, we did not observe any difference in proliferation, cell size regulation, hemoglobinization and cell morphology at various Epo-concentrations between Fanca or Fancg-deficient and *wt* cells (data not shown).

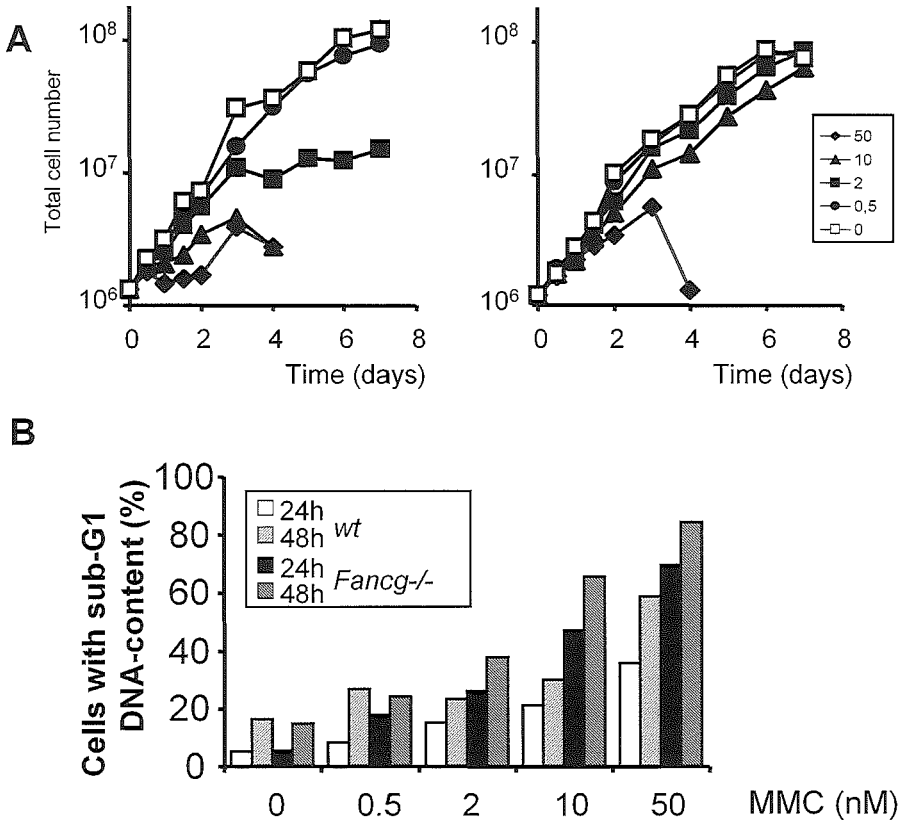

Figure 3. Fancg-deficient erythroid progenitors are hypersensitive to MMC. A: Erythroid progenitors from Fancg-deficient and *wt* fetal livers were expanded in presence of increasing concentrations of MMC as indicated (in nM) and total numbers of blasts (>8.5 μm) were calculated. **B**: 12 and 24 hours following addition of MMC cell aliquots were stained with PI and the percentage of cells with a subG1 DNA-content was determined.

Erythroid progenitors expanded from Fancg-/- deficient mice are MMC-sensitive
To examine the sensitivity of expanded erythroid progenitors for apoptosis-inducers, we added increasing concentrations of FAS-ligand, TRAIL, IFNα and mitomycin C (MMC) to day3 expanding cultures. Fancg-deficient cells and their *wt* counterparts were similarly insensitive to FASligand, TNFα and IFNα. In a 5 day assay, concentrations of 100ng/ml FAS-ligand, 20 mg/ml TRAIL and 104U/ml IFNα did not suppress progenitor expansion (data not shown) in concordance with previously obtained results for *wt* cells (Schmidt et al, in press). The same concentrations did inhibit cell growth of susceptible control cells. However, expansion of Fancg-deficient progenitors was affected at 2nM MMC (figure 3A), while *wt* cells were only inhibited at 50nM MMC (figure 3B). Cell death was measured by flow cytometry of propidium iodine (PI) stained cells. Reduced expansion was paralleled by an increase of cells with a sub-G1 DNA-content (figure 3C). In conclusion, loss of Fancg rendered the cells at least 10 times more sensitive to MMC induced cell death.

Stat5 and Erk1/2 phosphorylation is severely decreased in Fancg and Fanca knockout erythroid progenitors
Impaired activation of Stat-proteins has been described previously in Fancc-deficient mice and in FANCC deficient human cell lines. We analyzed Epo-induced phosphorylation of Stat5 and other signaling intermediates like Erk1/2. Fetal liver cells from Fancg-/- and *wt* littermates were expanded for 4 days to a homogenous population of erythroid progenitors. Cells expanded from a *wt* and 2 Fancg-deficient livers were starved and re-stimulated with Epo for increasing times. Stat5 phosphorylation was analyzed on Western blot. Epo induced STAT5 phosphorylation in *wt* and Fancg-/- cells, but phosphorylation in the Fancg-/- cells is severely reduced and delayed (figure 4A). As direct comparison of band intensities between two different blots/gels is impossible we performed second experiment in which samples are arranged differently to allow direct comparison at each time point. At each moment following Epo-stimulation of the cells Stat5 and Erk1/2 phosphorylation are clearly suppressed in both Fancg-deficient (figure 4B) and Fanca-deficient erythroid progenitors (figure 4C). Stat5 expression seems to be lower in the knockout mice versus the *wt*, but not to an extent that could explain reduced phosphorylation. This is not due to unequal loading, as the Erk1/2 protein levels, detected on the same blot, are not changed.

Reduced Stat5 and Erk1/2 phosphorylation is maintained at lower Epo concentration levels
The concentration of Epo used during stimulation is unphysiologically high. The question arises whether the observed drop in phosphorylation of Stat5 and Erk1/2 is also observed with lower, more physiological, concentrations of Epo. Erythroid progenitors expanded from fetal livers were factor deprived and re-stimulated with increasing amounts of Epo (0-30 U/ml) after which Stat5 and Erk1/2 phosphorylation was assessed in cell lysates. Stat5 and Erk1/2 phosphorylation were decreased in the Fanca-deficient cells compared to *wt* erythroid progenitors at all concentrations of Epo (figure 4D). Moreover, a plateau of maximum phosphorylation was reached in the knockout cells with 1-3 U/ml Epo while phosphorylation of Stat5 and Erk1/2 still increased with higher Epo concentration in *wt* cells. A plateau in *wt* cells was not observed within the concentration range used. The same results were obtained using Fancg-deficient erythroid progenitors (data not shown). This shows the response of erythroid progenitors to Epo is also aberrant at lower more physiological Epo concentrations.

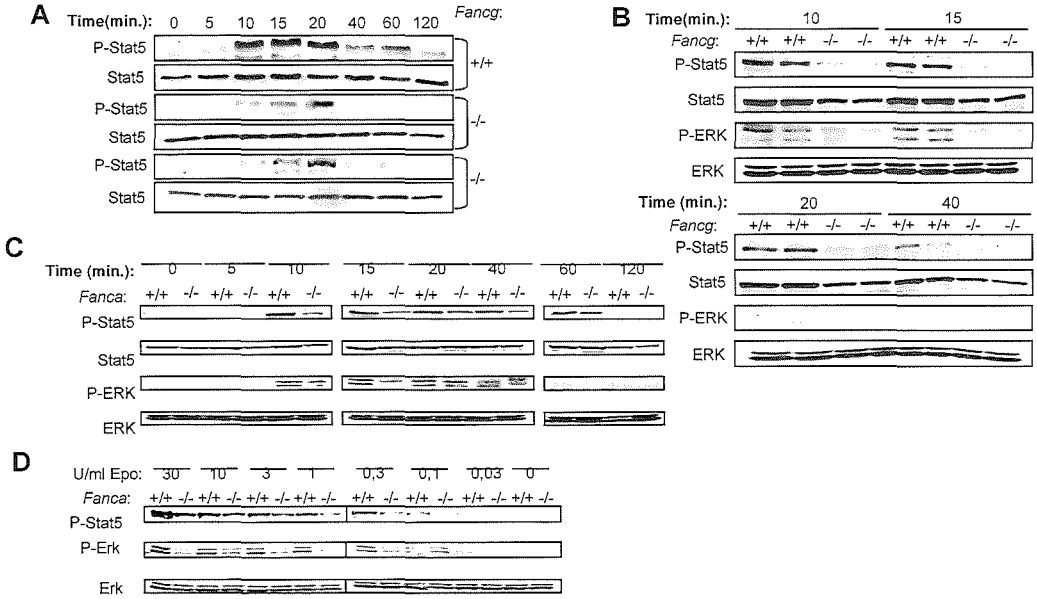

Figure 4. Stat5 and Erk1/2 phosphorylation is reduced in Fanca and Fancg deficient erythroid progenitors. **A-C**: *wt* (+/+) and Fancg (**A, B**) or Fanca (**C**) deficient (-/-) fetal liver erythroid progenitors were factor deprived for 4 hrs and re-stimulated with Epo (5U/ml) for the indicated time points. Cells were lysed and whole cell lysates (WCL) were analyzed by Western blotting. **D**: Wt (+/+) and Fanca deficient (-/-) fetal liver erythroid progenitors were factor deprived for 4hrs and re-stimulated with various concentrations of Epo (0-30U/ml) for 10'. Cells were lysed and whole cell lysates were analyzed by Western blotting. The Blots were stained with anti-phopho-Tyr694/699-Stat5a/b or anti-phospho-Thr202/Tyr204-Erk1/2. As a loading control the same blots were re-stained with anti-total-Stat5a/b or anti-total-Erk1/2 as indicated.

DNA damage causes reduced EpoR signaling

To examine whether reduced Epo-induced Stat5 phosphorylation is an artefact of *in vitro* expansion of Fanc-deficient erythroid progenitors, we stimulated freshly isolated bone marrow cells from *wt* and KO mice with Epo and examined Stat5 activation in an EMSA assay. Surprisingly, Stat5 activation in Fancg-deficient cells was similarly efficient as in *wt* littermates (figure 5A). This suggests that reduced signaling efficiency is due to cell culture conditions. To examine whether accumulation of DNA damage in cell culture could be responsible for the observed signaling defects, we treated *wt* erythroid progenitors expanded from fetal liver with sublethal doses of MMC for 24 hours and subsequently factor deprived the cells (4hrs) and restimulated the cells with Epo. While the total expression levels of Erk1/2 and Stat5 were constant, Epo-induced phosphorylation of Stat5 and Erk1/2 was impaired after treatment of erythroid cells with MMC concentrations of 10 and 25nM (Figure 5B). Thus, DNA-damage may be directly responsible for decreased Epo-responsiveness of erythroid progenitors in Fanc-deficient erythroid progenitors.

Jak2 phosphorylation, EpoR phosphorylation and membrane expression is unchanged.

Jak2, the cytoplasmic tyrosine kinase constitutively associated with the EpoR, has a crucial role in Epo-induced signaling. The EpoR is one of its prime targets, enabling Stat5 recruitment

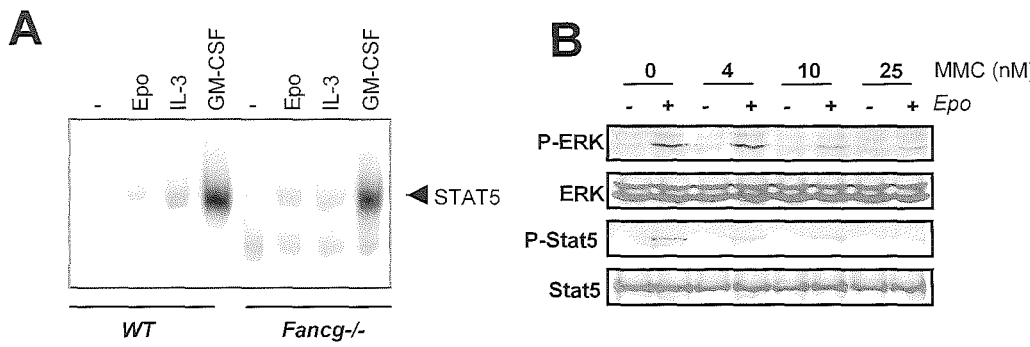

Figure 5. Impaired signaling is induced upon *in vitro* culture of Fanc-deficient cells or upon induction of sub-lethal DNA damage in wt cells. (**A**) Bone marrow cells were isolated from Fancg-deficient mice and *wt* littermates, seeded in medium serum-free medium without factors for 4hrs and stimulated with indicated cytokines for 10'. Nuclear extracts were assayed in a Stat5 EMSA, using a bcasein probe. Only the shifted band is shown. (**B**) Erythroid progenitors expanded from *wt* fetal liver were exposed to MMC as indicated for 24hrs in presence of fators as required for progenitor expansion. Cells were factor deprived (4hrs) and restimulated with Epo (10'). Western blots with cell lysates were probed with phospho-specific antibodies for Erk1/2 and Stat5 and for total Erk and Stat5.

and subsequent phosphorylation of Stat5 by Jak2. Therefore, we checked the phosphorylation status of Jak2 and the EpoR. Erythroid progenitors expanded from fetal livers were factor-deprived and re-stimulated with Epo. The EpoR and Jak2 were immune-precipitated from cell lysates and analyzed for phosphorylation of tyrosine residues. Progenitors expanded from Fanca-/-, Fancg-/- and *wt* fetal livers showed equally efficient Epo-induced phosphorylation of Jak2 and the EpoR (figure 6A). Erythroid progenitors, *wt* or deficient in Fanca or Fancg, were similarly restimulated with SCF to analyze signal transduction by SCF. Both the efficiency of SCF-induced c-Kit phosphorylation and SCF-induced Erk1/2 phosphorylation was equally efficient in all cell types (figure 6B). This indicates that Fanca- or Fancg-deficiency does not impose a general deficiency to activate e.g. Erk1/2 and Stat5 in erythroid progenitors but that it is specific for EpoR signal transduction.
Impaired signal transduction could be due to low cell membrane expression of the EpoR. EpoR membrane expression was estimated by the amount of Epo-binding sites on the membrane measured by flow cytometry using biotinylated Epo. EpoR membrane expression was similar in *wt*, Fanca-/- and Fancg-/- cells (figure 6C), indicating that the reduced Stat5 and Erk1/2 phosphorylation by Epo is not caused by a reduced EpoR expression on the membrane. The signal was specific for the EpoR as an excess of non-biotinylated Epo resulted in abrogation of the fluorescent signal.

Stat5 induced gene transcription is disregulated in Fanca and Fancg
Next we checked whether the reduced phosphorylation/activation of Stat5 and Erk1/2 affected Epo-induced gene transcription. The mRNA levels of known or previously established Epo target genes in erythroid progenitors, *p21WAF*, *Socs3*, *Spi2.1*, *BclX_L* and *GILZ* were analyzed by real-time PCR following factor deprivation and subsequent re-stimulation with Epo for 1 and 3hrs in *wt* and Fanca-deficient cells. Epo-induced regulation of these target genes was severely reduced or totally abrogated in Fanca-deficient erythroid progenitors (figure 7). Expression of ribonuclease-inhibitor (RI) served as a control. A second control, αtubulin, remained constant indicating proper expression levels of the controls. In conclusion, reduced

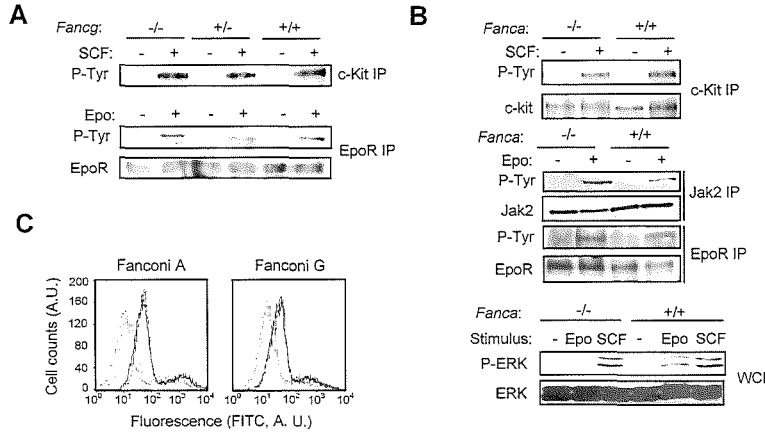

Figure 6. EpoR, Jak2 and c-Kit tyrosine phosphorylation after Epo or SCF stimulation is unchanged in Fanca and Fancg deficient fetal erythroid progenitors. A, B: *wt* (+/+) and Fancg (**A**) or Fanca (**B**) deficient (-/-) fetal liver erythroid progenitors were factor deprived for 4 hrs and stimulated with Epo (5U/ml; 10') or SCF (500ng/ml; 5') as indicated. Cells were lysed and the EpoR, Jak2 and c-Kit were immune-precipitated. Immune-precipitates were analyzed through Western blotting. Upper panels represent blots stained with anti-phospho-tyrosine (PY99), lower panels represent the same blot stained for the immune-precipitated protein as a loading control. **C**: EpoR membrane expression of fetal liver erythroid progenitors was checked by screening Epo binding sites on the membrane using biotinylated Epo. EpoR expression in *wt* cells (dashed line), expression in Fanca or Fancg deficient cells (undashed line). Black lines depict cells treated with biotinylated Epo, while the grey lines depict cells treated with biotinlylated Epo in presence of 10 times excess of non-biotinylated Epo to ensure specificity.

and delayed activation of Epo-induced signaling molecules in Fanca-deficient erythroid progenitors is mirrored by impaired activation of target genes.

Discussion

One of the early symptoms of Fanconi disease is anemia. Here we provide evidence that Fanca- and Fancg-deficient erythroid progenitors have an attenuated *in vitro* expansion capacity and that Epo signaling is disturbed in erythroid progenitors expanded from Fanca- and Fancg-deficient fetal livers. This reduced signaling efficiency is reflected in reduced mRNA expression of Epo-induced target genes. Among those are known targets of Stat5 like $BclX_L$, Socs3 and p21waf but also Epo-downregulated targets like GILZ.

Reduced expansion capacity and increased sensitivity to MMC.
Fanca- or Fancg-deficient erythroid progenitors are initially not affected in their expansion rate. However, the ability of the erythroid progenitors to undergo renewal divisions in response to Epo, SCF and Dex [23] is abrogated after circa 7 days, while *wt* cells can be expanded for more than two weeks. Analysis of the cell population revealed that reduced progenitor expansion *in vitro* was associated with enhanced differentiation and not with increased apoptosis. *In vivo*, total bone marrow progenitor numbers are reduced in Fanca-deficient mice, suggesting that the number of intrinsic divisions a hematopoietic progenitor

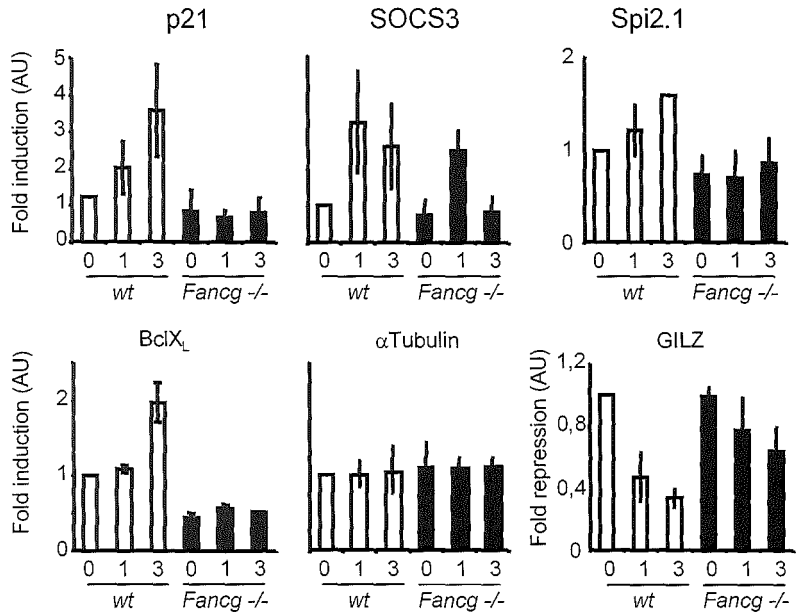

Figure 7. Epo-induced downstream transcription targets are reduced in Fanca and Fancg deficient erythroid progenitors. *Wt* and Fanca or Fancg deficient erythroid progenitors were factor deprived for 4hrs and re-stimulated with Epo for 3hrs. Values represent maen and standard deviation of at least 4 experiments. At the indicated time-points (0, 1, 3hrs) aliquots were taken from the stimulated cells for RNA isolation. mRNA levels of p21, SOCS3, Spi2.1, BclX$_L$, Tubulina, Ribonuclease Inhibitor (RI) and GILZ were determined by real-time-PCR. Fold induction of Epo-induced genes was normalized against the unchanged mRNA's in time of RI and is depicted as a function of the specific RNA levels of non-stimulated *wt* cells.

can make is reduced. Depletion of progenitors and stem cell populations has been reported in relation with Fanc deficiency [12,24,25].

FA is characterized by genomic instability and the inability to cope with oxidative stress as it occurs at the atmospheric O_2-tension. DNA-damage and oxidative stress, phenomena that go hand in hand, could be directly responsible for the observed loss of 'renewal capacity'. They activate stress responses like increased ATM and ATX activity and increased expression of p53. Enhanced levels of p53 associated with loss of Fanc-protein have been reported [26]. Increased p53 levels stop cell cycle progression in order to enable DNA repair. Subsequently, cells become senescent or apoptotic, when p53 is increased over a prolonged period of time at moderate or high levels. Hematopoietic progenitors can undergo apoptosis, but they do not undergo senescence and appear to undergo differentiation instead. In the erythroid lineage, p53 was shown to enhance differentiation and to abrogate proliferation of erythroid progenitors [28,29], while loss of p53 is a crucial step in Friend virus-induced erytholeukemia [27]. Notably, lack of p53 abrogates the hypersensitivity of Fancc-deficient cells for MMC, but also for TNFα and IFNγ, suggesting that the observed increased levels of p53 may be involved in bone marrow aplasia in FA [26].

Reduced EpoR signalling in Fanca and Fancg deficient erythroid progenitors

Both Stat5 and Erk1/2 are downstream targets of the EpoR. Stat5 is a direct substrate of Jak2, while ERK requires Jak2-dependent activation of signaling intermediates like Ras and Raf. We found that tyrosine phosphorylation of Jak2 and the EpoR as well as EpoR membrane expression was unaffected in erythroid progenitors deficient in Fanca or Fancg. Thus, the Fanc-proteins do not control receptor function per se, but rather the recruitment or activation of downstream signaling intermediates. This is not unique for the EpoR as it was previously reported that Stat1 activation by IFN-γ is impaired in FANCC lymphoblastoid cell lines [16,31]. Since SCF-induced ERK-phosphorylation is not affected, the signalling defect in Fanc-deficient hematopoietic cells may be specific for Janus-kinase-dependent cytokine receptors. It has been suggested that Fancc is directly involved in recruitment of Stat1 to the receptor. Fanca, Fancc and Fancg interact to form one complex in the cytosol [4,32]. Possibly, this complex is involved in recruitment and activation of Stat-proteins upon IFN-γ or Epo activation. However, Stat5 activation induced by Epo, IL3 or GM-CSF was not affected in freshly isolated bone marrow cells, only in expanded progenitors. Since Epo-induced activation of Stat5 and Erk1/2 was also impaired in *wt* cells upon induction of sublethal DNA damage, it is more likely that DNA-damage accumulated during culture and possibly oxidative stress are responsible for reduced signaling. Reactive oxygen species inactivate phosphatases by oxidation of cysteins in the reactive center. As a result, an increase of ROS mostly enhances signal transduction. However, other signaling routes, like Rac-mediated Rho activation and reorganization of the cytoskeleton is inhibited by ROS. Notably, induction of ROS induces hyporesponsiveness of T-cells by changing the structure of the T-cell receptor [35].

The observation that Epo-induced Stat5 activation is not impaired in freshly isolated bone marrow cells suggests that the reduced phosphorylation of Stat5 and Erk in response to Epo in erythroid progenitors expanded from fanca- and fancg-deficient fetal livers is a cell culture artifact. However, it may be a very relevant artifact since the high atmospheric oxygen tension in which the cells are cultured impose a significant increase in DNA-damage that fanc-deficient cells are unable to cope with. Laboratory mice that are not exposed to DNA-damage inducing agents probably never accumulate genomic stress comparable to expanded erythroid progenitors, while human individuals develop hematopoietic defects during the first or second decade of life.

In addition, the absence of anemia, in mice but also in the first years in human, may be due to the cooperation of several regulatory or complementary mechanisms. First, reduced Stat5 activation resulted in reduced expression of CIS and Socs3 and by consequence in attenuated negative feed-back regulation. Second, reduced Epo-signalling may be compensated by other factors. For instance, Mice lacking the C-terminal part of the EpoR are not affected in *in vivo* erythropoiesis [36], which was shown to be due to cooperation of Epo with unknown serum factors [37]. Finally, we showed that phosphorylation of Stat5 and Erk1/2 at 1 U/ml in *wt* cells is comparable to the phosphorylation induced by 30 U/ml in Fanca- or Fancg-deficient cells. FA-patients have increased Epo-serum levels, however, it is questionable whether mice that are not anemic have increased Epo-levels, since Epo production is driven by the O_2-tension in blood. We analysed Epo-levels in several wt and Fanca- and Fancg-deficient mice, which was always below the detection level of 20 U/l (data not shown), indicating that Epo levels in seemingly healthy Fanc-deficient mice are not increased above normal physiological levels.

In conclusion, we observed a decreased potential of progenitor expansion and impaired Epo-induced signal transduction in Fanca and Fancg deficient erythroid progenitors, but these phenomena do not impair erythropoiesis in mice. The data suggest that the observed defects develop during cell culture due to the inability to cope with the environment (i.e. mutagens and elevated oxygen tension). However, the process that may have been accelerated during *in vitro* culture may ultimately also take place *in vivo*. Notably, in humans, anemia may only occur in the second decade of life, which suggests that additional events are required in addition to the observed congenital defects.

Material and methods

Antibodies and growth factors
Rabbit antisera recognising Jak2 and a mouse monoclonal antibody recognising phospho-Stat5 (Tyr694-Stat5A; Tyr 699-Stat5B) were obtained from Upstate Biotechnology; rabbit antisera recognising the mouse EpoR, c-Kit, p44/p42 MAP kinase and the anti-phosphotyrosine mouse monoclonal antibody PY99 from Santa Cruz Biotechnology; mouse monoclonal antibodies recognising phospho-p44/p42 MAP kinase (Thr202/Tyr204) from New England Biolabs. Recombinant human Epo was a gift from Ortho-Biotech (Tilburg, The Netherlands), dexamethasone was purchased from Sigma (Zwijndrecht, The Netherlands) and SCF was a kind gift of Amgen. TNF-α and IFN-γ were purchased from R&D laboratories. MitomycinC from Kyowa Hakko Kogyo, Tokyo, Japan.

Primary erythroblast cultures
Primary fetal liver erythroblasts were obtained from day E12-13 fetal livers and cultured to a homogenous erythroid progenitor pool in serum free conditions using StemPro-34TM medium (Life Technologies) supplemented with Epo (0.5U/ml), SCF (100ng/ml) and Dex (50μM) 23. Usage of different concentrations of growth factors is indicated in the figure legends. Cell cultures were maintained at 1.5-3x10^6 cells/ml and a homogenous erythroblast culture was obtained within 4 days. To induce differentiation the erythroblasts were washed twice with PBS and reseeded in StemPro-34TM medium supplemented with 5U/ml Epo and 0.5mg/ml iron-saturated human transferrin (Intergen). During differentiation cells were kept at 2-5x10^6 cells/ml.

Bone marrow colony formation
Bone marrow cells were isolated from 8 weeks old *wt* or Fanca deficient mice via bilateral aspiration of femurs. Cells were resuspended in Iscove's medium supplemented with 20% fetal bovine serum as described by Koomen et al.[40]. Progenitor colony-forming assays were performed in triplicate, plating 2×10^4 cells/35-mm well according to manufacturer's instructions in methylcellulose media (Methocult GF M3434; Stem Cell Technologies). The cultures were done with or without various concentrations of recombinant murine TNF-α (0-100ng/ml) or IFN-γ (0-100ng/ml). Colonies were counted after 7 days.

Immune-precipitations and Western blotting
Primary erythroid fetal liver cells were growth factor deprived for 4hrs in plain IMDM (Life Technology). The erythroid progenitors (40-80x10^6/ml) were stimulated at 37°C with SCF (1μg/ml; 5'), or Epo (5U/ml; 10') as indicated. To stop the reaction, 10 ice-cold volumes of

PBS were added. Cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (20mM Tris-HCl pH 8.0, 137mM NaCl, 10mM EDTA, 50nM NaF, 1% (vol/vol) NonidetP-40, 10% (v/v) glycerol, 2mM Na3Vo4, 1mM Pefabloc (Roche), 50μg/ml aprotinin, 50μg/ml leupeptin, 50μg/ml bacitracin and 50μg/ml iodoacetamide) on ice for 10', nitrogen frozen and stored at -80°C until usage. Lysates were thawed on ice cleared by centrifugation at 4°C at 15,000rpm. Immune-precipitations, SDS-polyacrylamide gel electrophoresis and Western blots were performed as described previously [38]. Membranes were stripped in 63mM Tris-HCl pH 6.1, 2% SDS and 100mM b-mercaptoethanol for 30' at 50°C after which they were re-blocked and re-stained.

Flow cytometry

To measure EpoR expression on the erythroblast membrane, Epo was biotinylated following the biotin labeling kit protocol from Roche (#1418165). Cells were starved for 4 hours in plain IMDM (life technology) and washed twice with ice cold PSA (Phosphate buffered saline supplemented with 0.05% Sodium-azide and 1% Fetal calve serum; 2 min., 1800rpm). Erythroblasts, $1.0x10^6$ per sample, were subsequently incubated with 0,5U BioEpo in 50ml, supplemented with 5U/ml non-biotinylated Epo when appropriate, for 30 min at 4°C, washed with 1ml ice cold PSA, incubated in 1.5 mg in 50ml streptavidin-phycoerythrin conjugate at 4°C, washed with 1ml PSA and supplemented in 0.5ml PBS. Fluoresence was measured by flow cytometry.

To distinguish between life and dead cells, the DNA content was determined. 0.5 to $1.0x10^6$ cells were fixed and permeabilised with ice-cold methanol (0.5ml; 30') washed 2 times with PBS and incubated for 30' with 0.5% w/v RNAse A in PBS under constant shaking. DNA was stained with propidium Iodine (50μg/ml in PBS) and Fluoresence was measured by flow cytometry.

Real time PCR and Nothern blotting

Cells were factor deprived for 4hrs in plain IMDM and re-stimulated with Epo (5U/ml) for 0, 1 and 3hrs, cells were lysed in lysisbuffer (0.5% NP40, 10mM Tris-HCl pH7.5, 140mM NaCl, 1.5mM MgCl2, 20mM dithiothreitol (DTT), 40U/ml RNAsin) and nuclei were removed by centrifugation (6000 rpm for 3 min. at 4°C). Supernatant was adjusted to 1% SDS, 10mM EDTA and 400μg/ml ProteinaseK and incubated for 30 min. at 37°C. Total RNA was extracted with phenol/chloroform/isoamylalcolhol (25:24:1) and precipitated with isopropanol. 15μg RNA was separated on formaldehyde-containing agarose gels. To generate cDNA, 1μg total RNA was denatured and annealed to 2μg random hexamers (Amersham Pharmacia) at 65°C for 5', followed by 10' at room temperature. After addition of first-strand buffer (250mM Tris-HCl, pH8.3, 375mM KCl, 15mM MgCl2), 40μM dNTPs, 200mM DTT, 40U RNasin and 200U Superscript II RNase H- reverse transcriptase (Invitrogen), the reaction was incubated at 42°C for 2hrs, heated to 95°C for 3' and then quick-chilled on ice. The cDNA was diluted 1:10 to 1:200 prior to PCR amplification. The primer-sequences used for the amplification (5' to 3' order; forward, reverse): p21: ACC AGA GGG AGC CTG AAG ACT, ACC AGA GGG AGC CTG AAG ACT; BclXl: TGG TCG ACT TTC TCT CCT AC, TCA CTA CCT GCT CAA AGC TC; SOCS3: TCA AGA CCT TCA GCT CCA A, TCT TGA CGC TCA ACG TGA AG; GILZ: TGC GGA GTA CCT CAC AGA G, TCC TTG TCA GCG AAA GTA CCA; Spi2.1: ACT GCC TTG GCC CTC CTG TC, TGC CTG TGC TGA TCT GTA CC; Tubulin: TGC AGC GTG CTG TGT GCA TG, TCC TCT CGA GCC TCA GAG AA; Ribonuclease Inhibitor (RI): TCC AGT GTG AGC AGC TGA G, TGC AGG CAC TGA AGC ACC A. The real-time PCR was done with

TaqMan technology (PE Aplied Biosystems Model 7700 or 7900 sequence detector). The reactions were performed in a volume of 25µl of a mixture containing 4µl of the respective cDNA dilution, primers at 5µM and 12.5µl of 2X SYBR green PCR Master mix (PE Biosystems) containing Amplitaq Gold® DNA polymerase, reaction buffer, dNTP mix with dUTP, passive reference and the double stranded DNA (dsDNA)-specific fluorescence dye SYBR green I. The amplification program consisted of 1 cycle of 50°C with 2' hold, 1 cycle of 95°C with 10' hold, followed by 40 cycles of denaturation at 95°C for 15", annealing at 62°C for 30" and extension at 62°C for 30". The CT-values of RI and tubulin were used to normalize the values.

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Chapter 7

General discussion

Introduction

In this thesis I describe the identification and characterisation of proteins that function downstream of the receptors for erythropoietin (Epo) and stem cell factor (SCF), to gain insight into mechanisms that control Epo- and SCF-controlled expansion, survival and differentiation of erythroid progenitors. In particular, I describe (1) how the responsiveness to Epo may be controlled, which can be relevant to the occurence of anemia, and (2) factors that control progenitor expansion, which may be relevant to leukaemia. in line with this I characterized several Epo and/or SCF downstream targets with a regulatory role in Epo/SCF signalling, among which the kinases RON (Epo), Btk (Epo, SCF) and Tec (SCF) and the docking molecules Dok-1 (SCF), Gab1 (Epo) and Gab2 (Epo, SCF). We found that a functional Btk kinase as well as the Fanconi-pathway are required for efficient signal transduction upon EpoR ligand binding.
In addition, we elucidated a novel Epo-induced mechanism specifically involved in expansion but not in differentiation of erythroblasts, involving the tyrosine kinase receptor RON as a downstream target of the EpoR. We show, directly and indirectly, that the activation of PH-domain containing proteins like the docking molecules DOK-1, Gab1 and Gab2 or the Tec-family kinases Tec and Btk play a role in control of expansion but not differentiation. Activation of these proteins (Gab1, Gab2 by RON) or DOK-1 (by SCF) cannot sustain differentiation while deficiency of Btk, Gab1 or Gab2 results in normal differentiation. As stated PI3K is crucial for expansion (see introduction), the data in this thesis suggests that the PI3K downstream activated proteins Tec, Btk, DOK1 and Gab1/2 are important mediators of this PI3K-induced expansion of erythroid progenitors.

Epo-responsiveness: the modulators.

We and others have found proteins, like PKC, that can modify the amplitude of EpoR activation, [1,2]. It was found that EpoR, Jak2 tyrosine phosphorylation as well as downstream signalling was decreased in presence of inhibitors of PKC and PI3K. The responsible downstream events for this modulation were not identified. Nevertheless, it shows that i) EpoR responsiveness to Epo can be modulated at the level of initial receptor activation and ii) that Epo binding to the EpoR is not the only initiating event in EpoR activation. This creates a subtle switch that may specifically regulate erythroid progenitor numbers (expansion or differentiation). We found several other proteins that modulate Epo responsiveness (e.g. Btk, Fanconi A and Fanconi G in chapters 3 and 6 respectively).
Deficiency of Fanca or Fancg results a reduction of Epo-induced Stat5 and ERK1/2 activation, however, a reduction of Jak2 or EpoR tyrosine phosphorylation is not observed. In contrasts, Btk deficiency, similar to inhibition of PKC, results in severe reduction of Epo-induced signal transduction. Interestingly, PKC activators could not rescue Epo-responsiveness in Btk deficient erythroid progenitors, suggesting that PKC and Btk may have distinct modes of action on the Epo-responsiveness of the erythroid progenitor. On the cellular level, the lower Epo-responsiveness in cells deficient in Btk or active PKC caused a decrease or total absence of erythroid progenitor expansion [1] (chapter 3), confirming that these proteins may function as a molecular switch regulating erythropoiesis. Fanca and Fancg absence causes specific Epo-downstream pathways to be aberrantly activated (Stat5 and Erk1/2), but does not interfere with Jak2 or EpoR activation. Although the reduction in Stat5 and Erk is significant, no effect of Fanca and Fancg deficiency on the process of erythropoiesis *in vivo* was observed (chapter 6). However, *in vitro* self-renewal capability of the erythroid progenitors

was depleted significantly earlier. Therefore, the modulatory role of Fanc proteins may become visible *in vivo* during stress conditions like hypoxia, which is currently persued in the laboratory.

Receptor activation is regulated by several mechanisms, which could in principle be the targets of the modulators we described. First of all, the targets of these modulators could be phosphatases regulating Jak2 kinase activity like SHP1, SHP2 or membrane spanning phosphatases like PTP-1B [3] and proteins regulating the levels of reactive oxygen species that affect phosphatase activity. The regulation of phosphatase activity and ROS production has been found to be inversely correlated in the regulation of EGF-receptor kinase activity. Lowering the phosphatase activity as well as inducing ROS production resulted in lower threshold for EGF-receptor activation [4]. A similar mechanism may be present in erythroid progenitors. However, upon addition of the broad phosphatase inhibitor pervanadate during starvation and subsequent Epo stimulation, no prolonged or enhanced Epo-induced signalling was observed (data not shown). Nevertheless, It would still be interesting to see the effects of ROS levels on EpoR responsiveness, especially as one of the genes regulated by Epo and GCs is VDUP1, which is a negative regulator of the antioxidant TRX[5]. Second, modulators of EpoR responsiveness may recruit and/or activate proteins in the receptor complex important for proper organisation of the complex. Family members of the multi-PDZ domain proteins like MUPP1 could associate with c-Kit or the EpoR in erythroid progenitors to recruit many additional proteins. Furthermore, The Stat Interacting Protein 1 (StIP1) interacts with unphosphorylated Stat proteins and Jak2, which may pre-associate Stats with cytokine receptor complexes. With its 12 WD40 domains, StIP1 may also recruit numerous other proteins [6]. It will be important to isolate and characterize these and similar proteins and their interacting proteins to understand how the activity of PKC and Btk or events downstream of Fanconi proteins alter their function. Third, regulation of internalisation and degradation of the receptor may influence receptor signalling. Cell membrane expression of the EpoR was unchanfed under all conditions studied that showed reduced EpoR signalling (decreased PKC activity, Btk activity; absence of Fanconi proteins). Therefore we speculate that differences in the EpoR complex as it is present in the cell membrane may be the priming sequence to the EpoR signalling attenuation. To understand what these differences are we need to know the EpoR membrane complex composition and compare this with the composition upon interference with the modulators.

In conclusion, the process of erythropoiesis can be fine-tuned by various modulators that influence the responsiveness of the erythroid progenitors to Epo, suggesting a complex regulatory mechanism controlling the total number of erythrocytes produced.

Docking molecules

The Epo-receptor as well as c-Kit are tyrosine phosphorylated upon stimulation by their respective ligands. This enables recruitment of various proteins with Src-homology-2 domains (SH2-domains) and phospho-tyrosine-binding domains (PTB) that bind to specific phosphorylated tyrosines in the receptor, which is one of the initiation steps for ligand-induced signal transduction in the cell. Among these recruited proteins are proteins that recruit docking molecules that themselves again contain tyrosines that are phosphorylated leading to additional recruitment of proteins creating a protein network. How these docking molecules participate in erythropoiesis is at present unknown. Many interaction partners and downstream events have been described for the docking molecules studied here (DOK-1; chapter 2, Gab1/2; chapter 5) or reported in literature (IRS-1 [7]). Interestingly there is quite some over-

lap between EpoR/c-Kit directly recruited proteins and Gab or DOK family member recruited proteins. For instance recruitment of adaptors like Grb2, Shc, CRKL, CRK, PLC-gamma kinases like PI3K, LYN, Tec family members, Yes, Abl and phosphatases like SHP2 have been reported to be recruited by both c-kit and the EpoR as well as the docking molecules Dok, Gab1 and Gab2 (chapter 2; for review see [8-10]) . Thus alternative routes to the recruitment and activation of for instance PI3K and MAPK routes are present. The function of these alternative activation routes is not clear. They could be important for signal enhancement through the recruitment of additional signalling intermediates. In addition, they could play a role in signal maintenance and duration through differences in downregulation/degradation/internalisation between the receptor and the docking molecules, though no knowledge to this extent has been obtained yet. Interestingly though, deleting all the c-terminal tyrosines (8) in the Epo-receptor (which is still capable of binding Jak2) does not interfere with *in vivo* erythropoiesis [11], while Jak2 deficient mice fail to generate mature erythrocytes. This suggests that i) recruitment of specific proteins to the c-terminal part is not crucial for essential signal transduction pathways or that compensation via other pathways is possible and ii) that Jak2 binding and activation is the crucial event in erythropoiesis. Although it is known that this truncated EpoR (EpoR-H) does not activate Stat5, the effect on other signal transduction pathways, like PI3K and MAPK, in erythroid progenitors is unknown. BFU-E and CFU-E formation is normal in EpoR-H mice. However, the erythroid progenitors have a reduced expansion in serum-free conditions that can be restored by adding serum from EpoR-H mice [12]. Hence, unknown serum factors cooperate with Epo that modulate EpoR signalling. Interestingly, erythroid expansion is not absent in these progenitors indicating that there is a way to circumvent the necessity of the EpoR c-terminal tail, which could be activation of docking proteins that recruit more or less the same signalling intermediates as the receptor. We found a Jak2 dependent, but EpoR c-terminal tail independent, pathway that activates via RON (Stk) the docking molecules Gab1 and Gab2, both do not bind to the c-terminal tail of the EpoR. This alternative route leading to signalling intermediate recruitment may suffice to enable erythropoiesis in the EpoR-H mice. We found that erythroid progenitors lacking the RON/Stk-kinase domain and Gab1 phosphorylation are not affected in expansion of erythroid progenitors. It would be interseting to cross the RON-kinase deficient mice with EpoR-H mice to see whether this abolishes mutual complementation.
It was shown in this thesis that the homologous proteins Gab1 and Gab2 are differentially recruited to the EpoR/cKit signalling complex (chapter 5). This creates robustness of the system as similar responses can be generated via distinct signals that may complement each other. Notably, Epo-induced Gab2 phosphorylation was increased in Gab1- and RON-kinase deficient cells (chapter 5). It has not been investigated whether the distinct pathways also induce different phosphorylation patterns of Gab1 and Gab2. For functional analysis, the expression of multiple homologues in a single cell means that both have to be inactivated, e.g. by crossing both KO mice, by using combined siRNA approaches or by the use of dominant negatives. Gab1-/-;Gab2-/- double knockout cells are being generated, but already the Gab2+/-,Gab1-/- mice die too early to examine definitive erythropoiesis (U. Schaeper, pers. comm). As a potential dominant-negative Gab-molecule, we generated a truncated Gab1 protein that only retained the PH-domain and the MET-binding domain. Preliminary experiments showed that interference with all Gabs abrogates the ability of the cell to undergo renewal divisions in response to Epo and SCF.

PI3K and PH domain proteins are keys to expansion

The main challenge of studying signal transduction by a receptor complex or signalosome is to unravel the complexity, the integration of signals and the regulation of responsiveness and specificity of signal transduction occurring at any level during growth factor stimulation, and to relate this to functional output. A good example of a signalling molecule that is crucial for Epo/SCF-induced progenitor expansion and of which the regulation is complex and at the same time robust is PI3K. PI3K kinase docks to Y719 of c-Kit [13] to Y481 of the EpoR [14,15] but also to several tyrosines in Gab2, Gab1 and the IRS family [7,8]. PI3K has also been reported to bind to c-Cbl [16], which can interact with the EpoR, c-Kit as well as Dok-1 [16,17] (chapter 2). Thus, it appears that Epo and SCF-induced PI3K activity can be regulated via various proteins. Why are there so many pathways that can activate and recruit PI3K. Are the additional pathways necessary for signal augmentation in order to reach a threshold activity needed for distinct biological output, or do we look at evolutionary complementation and/or redundancy functioning as a "backup plan". Indeed, the enormous plasticity of signalling complexes becomes evident as loss of one PI3K binding site, for instance Gab1, in the signalosome is absorbed by shifting the balance to another site, like Gab2, with minimal effects on the process of erythropoiesis [11,13,18] (chapter 5). This suggests a binding site equilibrium state probably dependent on different variables like post-translation modifications, conformational changes, local protein concentration etc. that can be maintained due to the many pathways leading to PI3K activation and downstream signalling. This leads to a robust system capable of coping with changes in this system by changing from one PI3K-activating protein to the other PI3K-activation protein maintaining PI3K activation levels (Chapter5, [11,13,18]). Moreover, with respect to the levels of PI3K activation we showed that Epo and SCF co-stimulation results in a longer and enhanced PKB activation suggested to be the result of enhanced PI3K activation.

The level of PI3K activation appeared to be a critical event in expansion and delay of differentiation. P85α deficient mice have transient fetal anemia and decreased numbers of erythroid progenitors (both CFU-E and BFU-E). Furthermore, expansion on Epo and SCF was severely reduced compared to *wt* cells[19]. As apoptosis was not observed, the anemia is explained by the lack of Epo, SCF, GR-induced progenitor expansion in favour of differentiation. P85-/- adult mice are not anemic, which supports the hypothesis that this expansion of the erythroid progenitor is not required for steady state erythropoiesis (see also chapter 1). Instead, it is only required when erythropoiesis has to produce maximal numbers of erythrocytes for the body to function, like in the fetal liver and during hypoxic stress. Hence, no difference was detected upon comparing differentiation of p85-/- fetal liver erythroid progenitors on Epo. This is in agreement with the data in chapter 3 where deficiency of the PI3K downstream target Btk results in abrogated expansion, while differentiation is normal. The observations in p85-/- mice also show that low PI3K activity does not affect differentiation. Concordantly, we found that inhibition of PI3K with 15µM LY294004 abrogates Epo/SCF-induced expansion of human and mouse erythroid progenitors in favour of differentiation [20]. This, combined with the p85-/- data, indicates that the level of PI3K activation is of paramount importance for Epo/SCF-induced erythroid progenitor expansion.

As PI3K is crucial for expansion it is important to know its regulation and downstream effectors. PI3K activity results in PIP3 generation leading to recruitment of PH-domain containing proteins to the signalosome. These PH-domain proteins may subsequently recruit more PI3K leading to more PI3K activation, as shown for the Gab family in various cell lines [3,21,22]. In this thesis we describe that PI3K activity is the important event prior to Epo- and SCF-induced activation of the PH domain containing proteins of the Tec-family members, Gab family mem-

bers, Dok-1 and PKB (via PDKs; chapter 2, 3 and 5). We prove that Tec family member Btk deficiency results in abrogation of expansion during stringent renewal conditions (chapter 3). The phenotype could only be observed with limiting growth factor concentrations and is less severe when compared to PI3K inhibition with LY 294002, indicating that Btk modulates expansion but that it is not the only decisive factor downstream of PI3K that regulates expansion. As described in Chapter 5, Epo-signalling through RON is sufficient for expansion but not for differentiation. RON activates both Gab1 and Gab2, which both promote PI3K-recruitment and activation. Our group showed that activation of PKB, an important kinase downstream of PI3K, ensures the nuclear exclusion of FOXO-family members and enhances translation via eIF4E, both important pathways to sustain progenitor expansion([39]; Godfrey Grech, manuscript in preparation), making PKB an important inhibitor of differentiation.
The data in this thesis in conjunction with other data show that the cooperation between Epo and SCF depends on the recruitment of PH-domain containing proteins regulated by the levels of activated PI3K. Expansion of erythroid progenitors depends on different proteins downstream of PI3K which may be Btk, the Gab family, DOK-1 and PKB. We speculate that levels of PI3K activation by Epo or SCF alone are not sufficient for this expansion and that a certain threshold of PI3K activity must be reached. Whether this PI3K-activity threshold for progenitor expansion is required for a specific downstream pathway or for a number of them is not known. We showed that lack of one of these pathways impairs the ability of erythroid progenitors to expand. To understand the process of expansion and the contribution of these proteins, it is of importance to investigate the individual functionality of PI3K downstream signalling components and target genes in erythroid progenitors in order to discriminate the essential players. This is currently done in our laboratory at the transcriptional, translational and signal transduction level.

Stat5 and erythropoiesis.
An important signalling protein specifically activated by Epo but not by SCF is Stat5. We found that RON can cooperate with SCF to replace Epo during expansion but is not able to induce differentiation alone. This difference could be lack of Stat5 activation by RON. How does Stat5 regulate the process of erythropoiesis? Control of cell death is an important mediator of erythropoiesis (see also chapter 3, 5 and 6) and the most important role for Epo in erythropoiesis may be prevention of apoptosis through activation of Stat5 leading either directly or indirectly to up-regulation of anti-apoptotic proteins like $BclX_L$ or Bcl2 [23-25]. Indeed, inhibiting apoptosis through ectopic expression of BclXl results in Epo-independent differentiation in serum free conditions. The data favour the model that differentiation is a default, autonomous path [24] in which control of apoptosis is the regulatory event. Prevention of apoptosis can occur at several levels, first of all through the upregulation of anti-apoptotic proteins like Bcl2 and $BclX_L$ The cellular equilibrium between these anti-apoptotic proteins and pro-apoptotic proteins like Bax and related proteins is a decisive step in the induction of apoptosis. Indeed, selective deletion of $BclX_L$ in erythroid progenitors results in haemolytic anemia and profound splenomegaly due to apoptosis of differentiating erythroblasts[40]. $BclX_L$ and Bcl-2 are target genes of Stat5 both in erythroid system as well as in other hematopoietic lineages[41,42,43,44,] though no Stat5 binding sites were identified in the enhancer[45]. Indeed, Stat5 deficient erythroid progenitors have lower $BclX_L$ protein levels [23]. In line with this, Stat5 deficient mice have fetal anemia due to apoptosis of differentiating erythroblasts and show increased spleen weights, a hallmark of stress erythropoiesis and an indication that the organism tries

to compensate the lack of erythrocytes by increasing the numbers of erythroid progenitors (expansion/renewal [23]). This compensation ensures that the adult mice are not severely anemic and proves that the Stat5-/- erythroid progenitors, like BclX$_L$ deficient mice, retain their intrinsic ability to expand and to differentiate. The role of Stat5 during erythropoiesis can thus be linked to survival thereby allowing differentiation. Furthermore, the data suggest that Stat5 has no role in expansion or the actual process of differentiation. Stat5 deficiency does not totally abrogate erythroid progenitor survival as the adult Stat5-/- are not severely anemic, indicating that compensation through stress erythropoiesis is successful. Furthermore, it indicates the presence of other Epo-induced survival routes. However, the fact that i) Stat5 deficient erythroid progenitors are inhibited in expansion when cultured in serum free conditions, ii) that Stat5 interacts with GR to induce genes specifically expressed under expansion conditions, suggests a role for Stat5 in expansion ([46,47]; personal communication (beug)). The precise role of Stats in erythropoiesis is still elusive. It would be interesting to cross the Stat5-/- mice with mice expressing BclX$_L$ throughout erythropoiesis in order to see whether Stat5 is only needed for erythroid progenitor survival. Co-stimulation of erythroid progenitors with serum and Epo induces Stat3 activation, while Epo alone does not ([26]; personal observation), suggesting that compensation via Stat3 can rescue erythroid progenitors from apoptosis to some extent. Indeed, serum can augment erythroid progenitor expansion of cells with truncated EpoR to *wt* levels [12] indicating that additional serum components cooperate with the EpoR. Interestingly, constitutive Stat1 and Stat3 activation has been found in SFFV-P infected cells [27] that differentiate in absence of Epo, underlining a potential role for these proteins in survival. Combining Stat5 deficiency with a deficiency in other Stat family members may shed light on this putative compensation. Moreover, it was shown that additional Stat5 independent BclX$_L$ and Bcl2 up-regulating pathways exits in the Stat5 deficient mice during differentiation [23]. It would be interesting to cross the Stat5 deficient mice with mice disregulated in erythroid expansion (like ATF-1, GRdim/dim, p85a-/- or Btk-) as these mice probably lack the compensatory mechanism that rescues the Stat5 deficient mice from severe anemia.
An additional mechanism to regulate the number of erythrocytes is by modulating cell death via death receptors. In chapter 3, erythropoiesis is normal in the Btk deficient (Btk-) erythroblasts. However, the apoptotic agent TRAIL, increased sensitivity to apoptosis in the Btk- erythroid progenitors. This uncovers that Btk controls TRAIL-induced apoptosis in erythroid progenitors. Notably, it is SCF that controls association of Btk with the TRAIL-R1, which shows that also SCF has a role in surivival of erythroid progenitors. This introduces a second SCF-induced anti-apoptotic mechanism in erythroid progenitors, together with SCF-induced inhibition of caspase activity through raf-1 [5]. The anti-apoptotic mechanisms of Btk appears to be similar to the anti-apoptotic role of Btk in B-cells, where it binds and inhibits the FasL death receptor [28].

Friends virus induced erythroleukemia and RON(Stk)

Spleen focus forming virus (SFFV) or Friend virus is present in two forms SFFV-A and SFFV-P. Susceptibility to both viruses depends on the presence of a short form of Stk (Mouse homologue of RON). The two forms of RON are normally expressed in mouse and man [29,30] and are involved in erythroid expansion signalling (chapter 5). Infection with either form of the virus results in Epo-independent erythroid progenitor expansion due to the activation of the short form of RON by the viral protein GP55 31. We observe that activation of RON can substitute for Epo signalling in expansion, indicating that GP55 activated RON can be a primary event

in the observed Epo-independent expansion of erythroid progenitors. RON activation results in Gab1, Gab2 and subsequent PI3K and MAPK activation, but not in Stat3 or Stat5 phosphorylation. Normally, Epo induces Stat5 activation and not Stat1 and Stat3 (in serum free conditions). GP55-A and P introduction have been shown to result in Stat3 and Stat1 phosphorylation independent [27] or dependent of Epo [32] suggesting activation of one or more Jak family members. Although the RON family member, c-Met, has been shown to activate Stat3 in several cell lines [33,34], we do not find Stat3, Stat5 or Jak2 phosphorylation after RON stimulation (chapter 5, data not shown). However, we did not check whether RON induces the phosphorylation of Stat 1 or other Jak family members like Tyk, Jak1 or Jak3. Furthermore, gp55 activated RONsf/Stksf may be different from RON/Stk full length. In addition, these experiments were all done in presence of serum factors, while our experiments were done in serum free conditions. Indeed, GP55-P induces constitutive activation of Jak1 [35,36]. Concordantly, both GP55 proteins probably activate one or more members of the Jak family resulting in the observed Stat phosphorylation. Although the difference between GP55-P and GP55-A induced signalling is poorly understood, it is clear that GP55-A infected erythroid progenitors still require Epo to differentiate while GP55-P erythroid progenitors differentiate Epo independent. GP55-P, but not GP55-A, interacts with the EpoR through the transmembrane region, which is speculated to result in constitutive EpoR signalling [37]. We found that activation of RON is not sufficient to allow differentiation as cells go into apoptosis (chapter 5). This proves that GP55-P induces some additional signalling event required for survival of the erythroid progenitor during differentiation. Though this signalling event is presently unknown, it

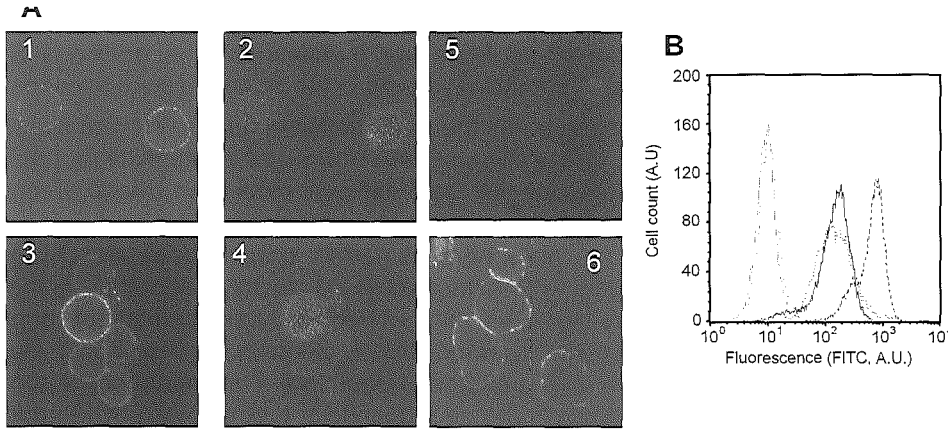

Figure 1, c-Kit is distributed equally on the cell membrane in small clusters. A-B: The erythroid progenitor cell line I/11 was factor deprived for 4 hours in plain medium after which aliquots ($1.0x10^6$ cells) were taken to analyze c-Kit, Transferrin and CD49e membrane expression. Cells were fixed, incubated with antibodies recognising the extracellular parts of c-Kit (2B6), Transferrin and CD49e as indicated and re-incubated with a FITC-labelled secondary antibody. **A**: $1.0x10^5$ cells were spotted on slides through centrifugation and fluorescence (membrane expression) was analyzed using Confocal microscopy. 1-2: anti-c-kit; 3-4: anti-integrinCD49e, 5: secondary antibody only control, 6: transferrin. **B**: the rest of the cells were analyzed by flow cytometri analysis to control proper staining. The black line represents c-kit, the dashed line integrin CD49e, the spotted line transferrin and the grey line the secondary antibody only control.

could include the observed Stat activation. However, as both GP55-A and GP55-P induce Stat family member phosphorylation, the studies must concentrate on which members of the Stat family are transcriptionally active and which Jak kinases are activated. Furthermore, these and novel signalling events of GP55-A and GP55-P need to elucidated and compared. For instance it would be interesting to study the levels of $BclX_L$ in GP55-P versus GP55-A as upregulation of this anti-apoptotic protein is crucial for survival during onset of differentiation. Interestingly, expansion of erythroid progenitors ectopically expressing $BclX_L$ still requires Epo. Combining both RON constitutive activation (resulting in Epo-independent expansion) and $BclX_L$ upregulation could suffice for GP55-P total Epo-independence and may explain the difference between GP55-A and P.

Future directions: the signalosome

The work presented in this thesis elucidate new signalling components necessary for proper Epo and SCF-induced survival and expansion or "self-renewal" of erythroid progenitors (chapter 2, 3, 5, 6).

It is still unclear whether the EpoR and c-Kit are present in one complex or whether they are in distinct functional complexes. Preliminary data show that c-Kit is distributed in numerous clusters on the membrane of erythroid progenitors (figure 1). Whether c-Kit, RON, the EpoR or TRAILR1 distribution is similar and if this distribution changes with varying conditions is at present unknown. Indeed shuttling of receptors in the membrane has been observed[48], which is another potential regulatory mechanism.

Knowing all this, it becomes important to describe the composition of the c-Kit and EpoR complex in the membrane setting during different conditions. Membrane complex isolation could be used to identify new proteins but also to compare the complex between the normal and aberrant state, for instance in Fanconi, MDS, specific knockouts or after incubation with inhibitors. In an attempt to obtain these complexes, we recently applied "an old" technique for new purposes namely the detergent-free lysis of cells through nitrogen cavitation (described by [49]). Antibodies recognising the extracellular part of the receptor whose complex one wishes to study are bound to magnetic beads. These antibody-bound-beads (ABBs) are subsequently bound to the cell. Proper binding of the ABBs to cells can be assessed with light microscopy and fluctuated for c-Kit-ABBs between 1-3 beads/cell (data not shown). Cells are disrupted by rapid nitrogen release after decompression from a 60bar environment incubation stage. Preliminary data shows that lysis via nitrogen cavitation creates sufficient enough particles to isolate distinct complexes (data not shown) but also that there is a clear enrichement in precipitated proteins compared to detergent based precipitation (figure 2). The proteins within this complex may be identified with mass spectrometry.

Advantages of N2-cavitation are (i) that only plasma-membrane associated complexes are precipitated, which avoids dilution by intracellular located receptors (which can be quite significant as for instance the EpoR is for 95% located intracellularly),(ii) that the complex is studied as situated in the membrane, where signal transduction starts, iii) that not only direct binding partners of this receptor are isolated but also proteins which are not directly bound but present in the complex, iv) in addition, the technique may be suitable to isolate proteins that need the membrane for stable association and which are lost with detergent-mediated lysis. Nevertheless, as we isolate a certain size of membrane fragments. This is adjustable by varying the nitrogen uptake of the cell through increasing or decreasing the pressure. Proteins which do not have a function in this complex are also isolated, particularly proteins

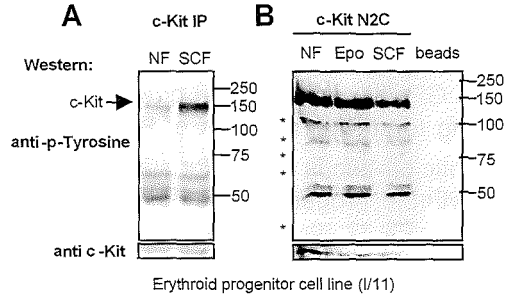

Figure 2, c-kit isolation via nitrogen cavitation leads to an enrichement of precipitated proteins when compared to detergent based c-kit isolation. A: I/11 cells were factor deprived for 4 hours and consequently re-stimulated with SCF as indicated. Cells were lysed via mild NP-40 based lysis buffer, c-Kit was immune-precipiated and subjected to SDS-PAGE and Western blotting. **B**: The erythroid progenitor cell line I/11 was factor deprived for 4 hours in plain medium after which c-Kit-ABBs or unbound beads were added and incubated for 10 min. (4°C). Cells were re-stimulated with SCF (5min.) or Epo (10 min.) at 37 °C as indicated and lysed via N2C. Lysates were subjected to SDS-PAGE and Western blotting. **A, B**: Upper panels represent blots stained with anti-phospho-tyrosine antibodies (PY99), the lower panels represents the same blots stained with anti-c-kit to check equal loading. Size markers are indicated in kDa, asterisks indicate co-immune-precipitating proteins, the arrow marks the size of c-Kit and the bands at 50kDa are antibody heavy chains.

that are abundantly present in the cell membrane. The number of common, non-receptor related proteins in part depends on the size of the isolated membrane fragments, the cell line used and/or the present developmental stage of the cell. To define and exclude contaminating proteins, additional receptor complexes that are not associated with the EpoR or cKit will be isolated by the same method from the same cells and the proteins present in these non-related receptor isolates will be compared. We assessed that the integrin receptor complex CD49e (VLA-4) is a good candidate to function as a non-related control and total protein stains of c-Kit and CD49e membrane-receptor complexes, precipitated onmagnetic beads, showed largely dissimilar patterns of proteins when separated by size (data not shown).
We hypothesize that receptors function in a so-called signalling complex or a 'signalosome', with the potential to influence each other's function. This is underlined by several obeservations: i) we and others [38] found that the EpoR and c-kit are associated in primary erythroid progenitors (data not shown), ii) Epo induced the activation of the tyrosine kinase receptor RON, which was found to interact with he EpoR (chapter 5), iii) SCF induces the interaction between Btk and the death receptor TRAILR1 thereby suggesting that TRAILR1 may also be part of this signalosome (chapter 3), iv) both SCF and Epo induce the recruitment and phosphorylation of docking molecules like Gab1, Gab2 (chapter 5) and Dok1 (chapter 2), v) these docking molecules themselves are able to bind numerous proteins again (chapter 2, 5). Furthermore, in the light of receptor synergy, cooperation or inhibition, future experiments should be directed on studying the cellular behaviour and signalling properties of a cell in relation to the different stimuli simultaneously received. Indeed, the contribution of other, at present unknown, signals *in vivo* may be crucial in understanding aberrant responses to growth factors. For instance, the observation that TRAIL induces cell death in Btk- cells but not in *wt* cells is indicative of the necessity to find the remaining components of the signalling platform. It further shows that single receptor studies may not reveal the true function or aberration of a certain cell type in which this receptor is expressed.

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Summary

Epo and SCF are essential growth factors during erythropoiesis. Epo is crucial for differentiation but also required in cooperation with SCF to induce expansion of erythroid progenitors. This expansion is regulated via PI3K dependent mechanisms. This thesis focuses on the molecular events downstream of PI3K necessary for appropriate activation of receptor complexes and downstream signalling pathways to control expansion, differentiation and/or survival of erythroid progenitors.
Chapter 1 states our current knowledge about the process of erythropoiesis, starting with a general introduction describing the importance of growth factors/receptors and a description of the hematopoetic system. It gives an overview of the various growth factors important at various stages of erythroid development and generally focuses on the EpoR/c-kit signalling complex, its signal transduction and the consequences of signal transduction for differentiation, expansion and survival or apoptosis of erythroid progenitors.

The importance of PI3K prompted us to investigate the role of TEC-family kinases. This kinase family harbours a PH-domain that imposes PI3K-dependent membrane recruitment. Chapter 2 describes the role of the founding member of the Tec kinase family namely TEC in c-Kit signalling. In erythroid cells, Tec is tyrosine phosphorylated after SCF but not with Epo stimulation in a PI3K dependent manner. Tec, together with LYN, is responsible for the phosphorylation of DOK-1 a multi-tyrosine containing docking protein of 60kDa. DOK-1 can potentially interact with Abl, CrkL, SHIP, and PLCγ-1. p62Dok-1 may function as an important scaffold molecule in c-Kit-mediated signaling. The activation of this docking protein, a PI3K dependent event, is speculated to be involved in signal enhancement.
Chapter 3 deals with a different Tec-family member, Bruton's Tyrosine Kinase (Btk), which appeared to be a novel target of Epo and SCF signalling. Btk is shown to be tyrosine phosphorylated after Epo and SCF stimulation and this phosphorylation event is PI3K dependent. Btk-deficient primary fetal liver erythroid progenitors as well as bone marrow and fetal liver-derived erythroid progenitor cell lines show reduced Epo-induced signal transduction, which could be rescued by re-expressing Btk. The reduced signaling results in reduced expansion in sub-optimal growth factor conditions and hypersensitivity to TRAIL-induced apoptosis. However, if the attenuation of signal transduction is linked to the observed phenotype in Btk deficient erythroblasts and how Btk and its downstream effectors mediate part of the Epo-responsiveness and SCF-mediated apoptosis is sill elusive.
Chapter 4 describes the characterisation of the alleged Btk inhibitor LFM-A13, presently used as an anti-leukemic drug in mouse studies. We prove that LFM-A13 not only inhibits Btk, but also efficiently inhibits JAK2. Jak2 and Tec-family members are important kinases in many hematopoietic cell systems as integral parts of cytokine signaling. Although LFM-A13 may be a useful inhibitor in some experiments, it is not suitable to examine the role of Tec-kinases in Jak2 dependent cytokine receptor systems.

The requirement of PI3K for expansion of erythroid progenitors also prompted us to examine docking molecules recruited in response to PI3K-activity, since they can mediate activation of numerous signalling pathways that will all be PI3K-dependent.
Chapter 5 describes the differential recruitment and phosphorylation of Gab1 and Gab2 upon stimulation of the EpoR. Recruitment of Gab2 is PI3K-dependent and Src-kinase inhibitors prevent its phosphorylation. In contrast, Gab1 is recruited and phosphorylated by the tyrosine

kinases receptor RON. RON tyrosine phosphorylation is found to be a downstream event of Epo but not SCF signaling. Since RON associates with Src-like kinases, activation of RON results in the phosphorylation of the docking proteins Gab1 and Gab2 and subsequent activation of PKB and the MAPK route. RON signalling can replace Epo signalling during expansion of erythroid progenitors but was not able to sustain differentiation on its own. Epo-induced differentiation was delayed upon co-activation of exogenous expressed RON. We prove that RON is a normal player in the EpoR signalosome and the kinase responsible for Epo-induced Gab1 phosphorylation.

Finally we examined Epo-induced signal transduction and Epo/SCF-dependent expansion plus Epo-dependent differentiation in erythroid progenitors deficient in Fanconi-proteins. Fanconi proteins have been implicated in DNA-repair, which has been linked to decreased cytokine signaling. In chapter 6 we investigated the effect of Fanca and Fancg deficiency on Epo and SCF signaling. We found that deficiency results in attenuated Epo-induced Stat5 and Erk signalling, while EpoR and Jak2 phosphorylation as well as c-Kit signal transduction remains untouched. As expected, lower Stat5 and Erk activation results in flawed Epo-regulated target gene expression. In addition, we observed reduced capacity of the erythroid progenitor to expand. We speculate that both the reduced signaling and the expansion defect are a product of hypersensitivity to (oxygen) ROS leading to enhanced DNA damage and impaired receptor signaling.

In Chapter 7 I discuss the data in the context of normal and aberrant erythropoiesis that expansion is dependent on the level of PI3K activity and the subsequent recruitment of PH-domain proteins. Most likely there is a threshold value that controles the balance between differentiation and expansion. Next, the role of Stat5 and the putative role of RON are discussed in the light of differentiation and Friend-virus induced erythroleukemia respectively. Since all experiments elude to the existence of a large receptor complex in which the EpoR, the SCF-receptor c-Kit, but also RON, docking molecules and additional kinases are associated, we set out to isolate and characterise the EpoR/c-Kit receptor complex. The aim of these studies is the identification and characterisation of proteins involved in the regulation of receptor responsiveness and signalling specificity as described in this thesis. Preliminary data and future plans are presented. Receptor complexes were isolated in their membrane context using N2-cavitation instead of detergents to disrupt cells. This technique allows for specific isolation of receptor complexes at the outer cell membrane without severe interruption of membrane structure

Samenvatting

Epo en SCF zijn de belangrijkste groei factoren tijdens de erythropoiesis. Epo is cruciaal voor de differentiatie van de erythroide voorlopercel tot rode bloedcel, terwijl Epo in samenwerking met SCF leidt tot expansie van diezelfde voorlopercel. De expansie is gereguleerd door PI3K afhankelijk mechanismen. De thesis spitst zich toe op die PI3K-afhankelijk processen welke belangrijk zijn om Epo en SCF de juiste signalen af te laten geven in erythroide voorlopercellen (e.g. expansie, differentiatie of overleving).
Hoofdstuk 1 geeft de huidige stand van kennis betreffende erythropoiesis weer. Een algemene introductie op groeifactoren en op de ontwikkeling van de rode bloedcel vanuit voorlopercellen in het beenmerg wordt gevolgd door een overzicht van verschillende groeifactoren en hun effect op erythropoiese. De nadruk wordt vooral gelegd op het EpoR/c-Kit complex en de signaaltransductie veroorzaakt door Epo en SCF binding aan de respectievelijke receptoren. De link tussen signaaltransductie en cellulaire respons wordt gemaakt, met de kanttekening dat hier nog verrassend weinig van bekend is. In dit licht wordt ook de coöperatie tussen de verschillende receptoren besproken.
Hoofdstuk 2 beschrijft de rol van de tyrosine kinase Tec in c-Kit signaaltransductie. Tec is de naamgever is van de Tec-familie van tyrosine kinases. In erythroïde cellen wordt Tec PI3K-afhankelijk gefosforyleerd op tyrosine residuen na stimulatie van de cellen met SCF. Tec is samen met Lyn verantwoordelijk voor de tyrosine fosforylatie van Dok-1, wat een multi-tyrosine docking eiwit is. Dok-1 kan vervolgens een groot aantal verschillende eiwitten binden, waaronder: Abl, Crkl, SHIP en PLCγ-1. Dok-1 zou dus een belangrijk eiwit kunnen zijn voor SCF geïnduceerde signaaltransductie en zou eventueel ook de amplitude en duur van SCF-geinduceerde signaal transductie kunnen versterken (een signaal versterker).
Hoofdstuk 3 beschrijft de functie van een ander lid van de Tec-familie in erythroide voorlopercellen, namelijk Btk. Btk wordt zowel na SCF en na Epo stimulatie van erythroide voorlopercellen op tyrosine gefosforyleerd op een PI3K-afhankelijke manier. De Epo geinduceerde signaaltransductie is sterk verzwakt in erythroide voorlopers die het Btk-gen missen, maar kan worden hersteld door Btk weer tot expressie te brengen in deze cellen. De gereduceerde signaaltransductie resulteert in verminderde expansie (versnelde differentiatie) van erythroïde voorlopercellen en een overgevoeligheid voor TRAIL geïnduceerde apoptose. Btk blijkt dus een rol te spelen bij de preventie van apoptose door SCF en heeft een positieve werking op Epo-geïnduceerde signaal transductie, via nog onbekende wegen.
Hoofdstuk 4 beschrijft dat de Btk remmer LFM-A13 niet alleen specifiek Btk remt maar ook de tyrosine kinase Jak2. In dit hoofdstuk laten we zien dat LFM-A13 de Jak2 kinase activiteit net zo efficiënt remt als de Btk kinase activiteit. Jak2 en Tec-familie-leden zijn belangrijke kinases in veel verschillende hematopoietische cellen en functioneren als integrale eenheden van cytokine signaal transductie. Het feit dat Jak2 ook geremd wordt door LFM-A13 impliceert dat deze inhibitor slechts beperkt gebruikt kan worden om de rol van Tec-family-leden te onderzoeken in de signaaltransductie van Jak2 afhankelijke cytokine receptoren.
Hoofdstuk 5 geeft de rol van de tyrosine kinase receptor RON weer in erythroide voorlopercellen. Fosforylatie van RON op tyrosine volgt op activatie van de EpoR en het daaraan gebonden Jak2 kinase. Fosforylatie en activatie van RON resulteert in de fosforylatie van Gab1 en Gab2, bekende docking moleculen (signaaltransductie versterkers) in Epo-geïnduceerde signaaltransductie. Directe activatie van RON kan Epo vervangen om samen met SCF expansie van voorlopercellen te induceren, maar activatie van Ron is niet voldoende om de erythroide voorlopercellen te laten uitrijpen tot rode bloedcellen. In combinatie met Epo

zorgt RON voor vertraagde differentiatie wat een specifieke rol van RON in expansie bevestigt.
Naast een karakterisatie van componenten van de Epo- en SCF-geïnduceerde signaaltransductie hebben we Epo-geïnduceerde signaaltransductie onderzocht in een model voor Fanconi Anemie. Fanconi eiwitten zijn betrokken bij DNA-herstel en beschermt de cell tegen oxidatieve stress. In hoofdstuk 6 beschrijven we dat deficientie van Fanca en Fancg leidt tot sterk verminderde fosforylatie van Stat5 en Erk in respons op Epo. Activatie van de EpoR en Jak2 in respons op Epo en van c-Kit in respons op SCF is echter niet aangetast. Zoals verwacht leidt deze verzwakte Epo signaal transductie tot verlaagde expressie van Epo-gereguleerde genen. Deficientie van Fanca en Fancg leidt ook tot een verlaagde capaciteit van de erythroide voorloper cellen om te expanderen. In discussie speculeren we dat zowel de verzwakte signaal transductie als het defect in expansie een gevolg kunnen zijn van toenemende DNA schade.
Hoofdstuk 7 bevat een integrale discussie van de verzamelde data. We introduceren de hypothese dat er een drempelwaarde aan PI3K activiteit nodig is voor expansie van voorlopercellen. Veel PI3K activiteit geeft expansie terwijl lage PI3K activiteit niet voldoende is om differentiatie uit te stellen. Verder geven we aan hoe de verschillende eiwitten beschreven in de voorgaande hoofdstukken hierin een rol kunnen spelen. Aan het einde wordt de hypothese geopperd dat receptoren in complexen voorkomen en dat deze signaal platvormen opgebouwd zijn uit een dynamische verzameling verschillende receptoren en effectoren. Een nieuwe manier om deze complexen te onderzoeken wordt geïntroduceerd.

Abbreviations

Btk	Brutons Tyrosine Kinase
CIS	Cytokine Induced SH2 containing protein
Dex	Dexamethasone
Dok	Downstream Of tyrosine Kinases
Epo	Erythropoietin
EpoR	Erythropoietin Receptor
Erk	Extracellular signal Regulated Kinase
FA	Fanconi Anemia
Fanc	Fanconi
Gab	Grb2 Associated Binder
GILZ	Glucocorticoid Induced Leucine Zipper
GP55	GlycoProtein 55
Grb	Growth Receptor Binder
IFN	Interferon
IL	Interleucine
Jak	Janus kinase or Just Another Kinase
LFM-A13	a-cyano-b-hydroxy-b-methyl-N-(2,5-dibromophenyl)propenamide
N2C	Nitrogen Cavitation
NGF	Nerve Growth Factor
PI3K	Phospho-Inositol-3-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	PhosphoLipase C
Ron	
SCF	Stem Cell Factor
SFFV	Spleen Focus Forming Virus
Shc	Src Homology 2 domain Containing protein
Shp	Src Homology domain containing Phosphatase
SOCS	Suppressor Of Cytokine Signalling
Stat	Signal transducer and Activator of Transcription
Stk	Stem-cell derived Tyrosine Kinase
Tec	Tyrosine kinase Expressed in hepatocellular Carcinoma
TNF	Tumor Necrosis Factor
TRAIL	TNF-a Related Apoptosis Inducing Ligand

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Curriculum vitae

Emile van den Akker werd geboren op 29 juli 1973 te Heerenveen in Friesland. Na het doorlopen van de MAVO, HAVO en VWO, afgesloten met de respectievelijke diploma's, is de schrijver na een jaar sterrenkunde (1993/1994) overgestapt naar de studie biologie (1994) aan de Rijksuniversiteit Groningen. In 1999 haalde hij zijn doctoraal examen met als hoofdrichting moleculaire biologie. Tijdens zijn eerste doctoraal onderzoek deed hij, onder begeleiding van Dr. G.N. Moll, Prof. dr. W. N. Konings en Prof. dr. A. J. Driessen (afdeling Moleculaire Microbiologie, Rijksuniversiteit Groningen), onderzoek naar de antibacteriële werking van twee plantaricinen EF en JK geproduceerd door *Lactobacillus Plantarum*. Het tweede doctoraale onderzoek werd gedaan onder begeleiding van Dr. A. K. Boer, Dr. A. L. Drayer en Prof. Dr. E. Vellenga (afdeling Hematologie, Academisch ziekenhuis Groningen) en betrof de karakterisering van verschillende SHIP mutanten op *in vitro* erythropoiesis. Aansluitend het afronden van de studie Biologie, begon hij op de afdeling Hematologie op de Erasmus universiteit Rotterdam onder leiding van Prof. dr. B. Löwenberg en Dr. M. Von Lindern aan het werk besloten met dit proefschrift. Heden ten dage werkt hij bij het Curie Instituut te Orsay (Ile de France) in de vakgroep "Régulations cellulaires et oncogenèse" geleid door Dr. J. Ghysdael, alwaar hij een onderzoek doet naar de binding partners van FLI-1 en de betekenis van deze interacties voor de transformerende eigenschappen van FLI-1 wanneer tot expressie gebracht in erythroide voorloper cellen.