Control of mRNA Translation in Erythropoiesis

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Controle van mRNA translatie in de rode bloedcelvorming

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Cover designed by family Grech and family Spiteri

Thesis printed by Littera Picta

to Claudine
to Bethany Ann
in memory of my dad
to all the family

TABLE OF CONTENTS

Introduction

CHAPTER 1

Section 1 Hematopoiesis

1.1 Development and commitment of the hematopoietic stem cell

Hematopoietic stem cells (HSCs) replenish the stem cell compartment, and give rise to oligolineage progenitors. These progenitors expand to maintain the hematopoietic compartment and differentiate into various blood lineage progenitors. Lineage positive progenitors are committed for differentiation into mature blood cells.

Developmentally there are two temporally separate hematopoietic processes. Primitive hematopoiesis occurs in the blood islands of the yolk sac around 7.5 days post coitum (d.p.c.) in the mouse, giving rise to large, nucleated erythrocytes. It precedes the advent of definitive hematopoiesis with stem cells originating in the AGM (aorta, gonads, and mesonephros) during 9.5 to 11.5 d.p.c. in mouse and 30 to 37 days of gestation in man (Medvinsky and Dzierzak 1996). Definitive HSCs first expand in the AGM and then migrate and colonise the foetal liver and spleen where they differentiate into their progeny (heterogeneous population of early and late hematopoeitic progenitors). After birth, definitive hematopoiesis is primarily confined to bone marrow.

Decisions needed for development and commitment of hematopoietic stem cells and their progeny is orchestrated by the transcriptional program.

1.2 Regulation of Hematopoiesis by transcription factors

Transcription factors have a pivotal role in hematopoiesis and regulate HSC early development, survival, proliferation and lineage commitment. Transcription factors, among which Runx1, SCL, Gata-2 and ALL-1 maintain a gene expression program unique to HSCs (Phillips et al. 2000). Also the maintenance of HSC self-renewal requires specific transcription factors among which Notch1 (Varnum-Finney et al. 2000) and Bmi-1 (Park et al. 2003).

HSC commit to either the common myeloid precursor (CMP) or the common lymphoid precursor (CLP). The upregulation of both PU.1 and Gata-1 marks the commitment to CMPs (Scott et al. 1994). The CMPs undergo further lineage divergence into megakaryocytic/erythroid progenitors (MEPs) and granulocytic/monocytic progenitors (GMPs) upon Gata-1 and PU.1 mutual exclusive expression, respectively. Commitment to the erythroid lineage is characterized by the expression of erythroid specific transcription factors Gata-1, Eklf and Nfe2 determining the erythroid program. C/EBPα is required for the generation of the GMP compartment and its expression also denotes selectivity in differential commitment to monocytic lineage (Wang et al. 2006). In addition to the auto- and cross-regulatory effects of these factors at the transcription level, increasing evidence supports the importance of expressing different isoforms that modulate lineage commitment in hematopoietic cells. The expression of truncated forms of the transcription factor, Stem Cell Leukaemia (Scl) is regulated by differential initiation of translation (Calkhoven et al. 2003) and results in erythroid lineage differentiation. Expression of functional isoforms due to differential translation has been described for other hematopoietic transcription factors (Calligaris et al. 1995; Calkhoven et al. 2000) and disruption of isoform ratios are implicated in disease (Pabst et al. 2001; Cleaves et al. 2004).

1.3 Hematopoietic Signals: the microenvironment

The HSC compartment undergoes self renewal at a constant low rate to maintain the HSC population while preventing stem cell exhaustion throughout life. Proliferation of HSCs is orchestrated by Notch (Varnum-Finney et al. 2000), sonic hedgehog (Bhardwaj et al. 2001) and Wnt (Reya et al. 2003) signalling. Wnt-mediated maintenance of undifferentiated HSCs requires Notch1 (Reya et al. 2003) and the upregulation of HoxB4 by Wnt signalling supports self-renewal of HSCs (Antonchuk et al. 2002). The same pathways are activated in response to mitogenic stimuli to induce proliferation of cells. The production of TGF-β1, within the quiescent micro-environment, safeguards HSC from exhaustion by downmodulating cell surface expression of mitogenic cytokine receptors (cKit, Flt3, IL-6R, Mpl) (Fortunel et al. 2003).

Wnt signalling has been implicated in both maintenance of the early progenitor compartment and in lineage commitment (Brandon et al. 2000; Reya et al. 2003). Upon commitment (section 1.2), lineage specific cytokines and growth factors regulate maintenance and differentiation of progenitor cells. Stem Cell Factor (SCF) supports proliferation of various hematopoietic compartments. In erythropoeisis the lineage-specific cytokine erythropoietin (Epo) works in concert with SCF to regulate the balance between proliferation and differentiation of erythroid progenitors. Through different mechanisms, both Epo and SCF protect erythroid progenitors fromTRAIL-induced apoptosis (Schmidt et al. 2004; Mirandola et al. 2006), which exemplifies the role of signalling mechanisms in regulating cellular sensitivity to the microenvironment.

1.4 Disregulation of Hematopoiesis

The balance between proliferation and differentiation of committed progenitors is under tight control, to maintain the progenitor pool and ensure maturation in response to physiological demand. The production of increased numbers of mature blood cells during stress, requires higher progenitor proliferation rates. Concurrently, feedback mechanisms must be closely coordinated to repress progenitor proliferation and to restore physiological cell numbers when the stress is over (Vattem and Wek 2004). Deregulation of this balance will result in disease. Myelo-Proliferative Disorders (MPD) and anaemia originate from hyperproliferative potential that can be sustained or can result in bone marrow exhaustion, respectively. Constitutive active tyrosine kinase receptors (FLT-3, cKit), activated Jak2 and mutant PTEN promote proliferation and/or survival in committed progenitors that are still capable to differentiate. This results in hyperproliferative phenotype (Vainchenker and Constantinescu 2005; Kelly et al. 2002b; Gilliland and Griffin 2002; Zhang et al. 2006). In addition to hyperproliferation, a perturbed program blocking terminal differentiation gives rise to leukaemia. Translocations t(8:21) and t(15;17) give rise to the fusion proteins AML1/RUNX1 and PML-RARα, both inhibiting hematopoietic differentiation by recruiting repression complexes to target genes of AML1 and RARα respectively (Kelly et al. 2002a; Tallman et al. 2002). Expression of these fusion proteins in hematopoietic progenitors, confers propagation in serial murine transplantation models (Grisolano et al. 1997; Higuchi et al. 2002) but is not sufficient to induce leukaemia. Cooperation

events exemplified by the complementation of Flt3-ITD mutant to PML-RARα transgenic bone marrow cells results in 100% penetrance of an APL-like disease when transplanted to secondary recipients (Kelly et al. 2002a).

To study the balance between expansion and differentiation of erythroid progenitors we use an in vitro system in which erythroid progenitors can be expanded in the presence of Epo, SCF and dexamethasone, while they maintain the capacity to differentiate when cultured in Epo alone. Signals emanating from extrinsic factors modulate the gene expression program at various levels, including transcription, transcript maturation, stability and translation.

Section 2 Erythropoiesis

2.1 Regulation of erythropoeisis

The continuous demand of an organism for mature circulating blood cells requires a tight balance between maintenance of progenitor compartments versus terminal differentiation to satisfy the demand and ensure prevention from exhaustion. The human bone marrow must replace 10^{11} erythrocytes daily under normal physiological erythropoiesis. The main regulator of erythropoiesis is the glycoprotein hormone Erythropoietin (Epo), produced in the kidney in response to oxygen tension in the blood. The function of Epo initiates from the specific interaction to its cell surface receptor (EpoR). The expression of the EpoR is dependent on the transcription factor Gata-1 (Zon et al. 1991) and increases on progenitor cells as they mature from early BFU-E (Burst-forming unit erythroid) to late CFU-E (Colony-forming unit erythroid) (Erslev and Caro 1986; Sawada et al. 1990). Hence, generation of BFU-E and CFU-E progenitors is Epo-independent (Wu et al. 1995), while Epo is required for terminal differentiation into erythrocytes. This is supported by the lack of definitive erythropoiesis in Epo knockout mice that die at day 12.5 of gestation and the *in vitro* rescue of Epo-/- foetal liver cells by addition of exogenous Epo (Lin et al. 1996). Interestingly, definitive erythropoiesis is normal in mice expressing an EpoR mutant ($EpoR_H$) capable to recruit Jak2 but lacking signal transducer binding sites (Zang et al. 2001), suggesting that the definitive erythroid differentiation is not a result of an instructive program by Epo signalling.

The role of Epo is to sense erythropoietic demand during hypoxia. In stress erythropoiesis, Stem Cell factor (cKit ligand) and glucocorticoids (GR) work in concert with Epo to induce expansion of progenitors in the mouse spleen (Broudy et al. 1996; Bauer et al. 1999; Wessely et al. 1999). The requirement for SCF in acute erythroid expansion was demonstrated by the observation that antibodies preventing c-Kit activation abolished splenic hematopoiesis upon induction of haemolytic anaemia in mice, while the antibodies had no effect on steady state erythropoiesis (Broudy et al. 1996).

During terminal differentiation to enucleated, haemoglobinised erythrocytes, the cell undergoes drastic changes in the cytoskeleton leading to enucleation, and accumulates haeme and globin peptides. To accomplish these changes, the intrinsic

gene expression program is modulated at the level of transcription factor expression (section 1.2), alternative mRNA splicing and regulation at translational level. Interestingly, the erythroid protein 4.1R isoforms selectively include a cytoskeletal membrane binding domain as required during late erythropoiesis (Pinder et al. 1993). Alternative 5' exons that increase complexity within the N-terminal regions of transcripts were found in 35% of erythroid genes studied (Tan et al. 2006). This suggests that erythroid specific promoter usage and alternative splicing might offer a temporal and cell specific transcriptional modulation of critical genes and their products.

The enormous increase in haeme synthesis during haemoglobinisation of erythroblasts, requires tight coordination between iron uptake and storage of iron with tight coordination to the production of globins. This is mainly accomplished by translational control that supports the erythroid program by sensing metabolite levels and drive quick responses to maintain cellular equilibrium state. At low iron level, iron responsive proteins (IRPs) bind to and stabilize specific RNA structures (Iron regulatory elements) found in the 5'UTR of ferritin and 3'UTR of the transferrin receptor (*TfR1*) transcripts (Thomson et al. 1999). This results in stabilization of the *TfR1* transcript accompanied by enhanced receptor expression and iron uptake. Conversely the IRE/IRP complex in ferritin results in inhibition of translation initiation resulting in decreased storage (Cazzola and Skoda 2000). Collectively, iron is made available for enhanced haeme synthesis under tight control of iron. In addition, globin production is attenuated by HRI (kinase of eIF2α) in response to the lack of haeme (Han et al. 2001). During iron deficiency, haeme levels are low, resulting in phosphorylation of eIF2α and inhibition of the translation machinery.

2.2 Role of SCF and Epo signalling in expansion / differentiation of erythroid progenitors

Proliferation and differentiation require different signals from the EpoR. The EpoR has no intrinsic kinase activity, but is constitutively associated with the tyrosine kinase Jak2. The EpoR/Jak2 complex activates a complex signalling network including the Stat5 and Map kinase pathways and signalling through phosphoinositide-3 kinase (PI3K). Activation of the EpoR results in phosphorylation and activation of the tyrosine kinase receptor Ron (Recepteur d'origine Nantese, also named Stk: Stem cell tyrosine kinase), which is associated with the adaptor protein Gab1 (Grb2 associated binder). Gab1, in turn, is able to activate PI3K and the Ras-Map kinase pathway. Interestingly, direct activation of Ron is able to replace Epo in renewal but not in differentiation of erythroid progenitors. The observation that Stat5 is not phosphorylated upon activation of Ron suggests that Stat5 is not involved in proliferation of erythroid progenitors but required for their differentiation (van den Akker et al. 2004). One of the targets of Stat5 in differentiation may be expression of the anti-apoptotic $BclX_L$ protein (Dolznig et al. 2002). Various attempts have been made to study the importance of Stat5 in erythroid differentiation. Mice constructed to be *Stat5ab*-deficient have normal steady state erythropoiesis except for a mild anaemia (Teglund et al. 1998), although foetal erythropoiesis is compromised (Socolovsky et al. 2001). These results unexpectedly suggest that

Stat5 is not required for steady state erythropoiesis, although it is worth mentioning that these mice are Stat5 hypomorph due to the presence of an in frame CUG codon giving rise to expression of a truncated Stat5 protein in erythroid progenitors. This truncated Stat5 protein fails to form tetramers, required on most promoters, but it can function as a dimer. Conversely, Stat5 dominant negative mutants results in loss of terminal differentiation in vitro and *Stat5ab-/-* derived cells showed compensatory upregulation of other Stat-family members and underwent apoptosis upon induction of terminal differentiation in vitro (Dolznig et al. 2006). Exogenous Bcl-X_L completely rescued this phenotype (Dolznig et al. 2002), supporting the central role of Stat5/ BclX_L in Epo-induced survival during terminal differentiation.

Similar to Epo, SCF and glucocorticoid dependent expansion of erythroid progenitors in the spleen of hypoxic mice, foetal liver derived primary erythroid progenitors or ES-derived erythroblasts can be expanded in the presence of Epo, SCF and glucocorticoids, retaining the capacity to differentiate in mature erythrocytes in the presence of Epo alone (Figure 1.1) (Dolznig et al. 2001; von Lindern et al. 2001; Carotta et al. 2004). This cellular system has proven extremely powerful to study the effect of key players in erythroid progenitor expansion versus differentiation.

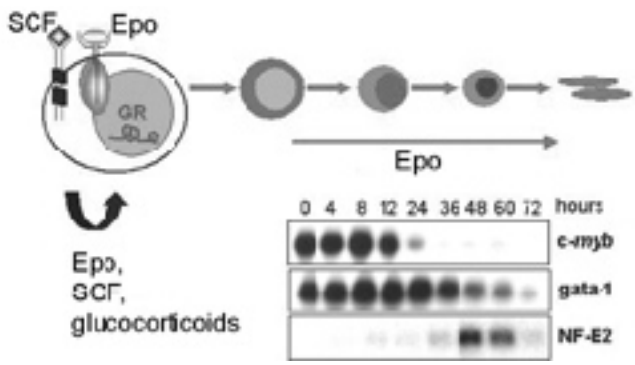


Figure 1.1 In the model system we use, the cooperation of Epo, SCF and glucocorticoids induces renewal of erythroid progenitors, whereas the cells undergo terminal differentiation in absence of SCF and glucocorticoids. Terminal differentiation requires Epo and insulin as survival factors and is characterised by 3-4 cell divisions with cell size reduction, haemoglobin accumulation and enucleation. Expression of transcription factors c-myb, Gata-1 and Nfe2 indicate maturity of terminally differentiating erythroid progenitors. Adapted from Kolbus et al. (2003). (GR;Glucocorticoid receptor)

Epo and SCF transduce signals via multiple cooperating pathways in erythroid progenitors (von Lindern et al. 1999; Wessely et al. 1999; Dolznig et al. 2002; von Lindern et al. 2004). Activation of PI3K generates PIP3, which serves as an anchor for PH-domain containing proteins, both adaptor molecules such as Gab2 and Dok1 and kinases such as Tec, Btk, PDK1 and PKB (Tang et al. 1994; Stokoe et al. 1997; Leevers et al. 1999; Zhao et al. 1999; Saito et al. 2001). Although both Epo and SCF induce activation of PI3K in erythroid progenitors, the efficiency with which downstream signalling pathways are activated shows large differences (Bakker et al. 2004; Blazquez-Domingo et al. 2005), suggesting differential susceptibility to feedback pathways. Particularly the activation of PKB is much more responsive to SCF compared to Epo in *in vitro* cultured erythroid progenitors (von Lindern et al. 2001). Active PKB phosphorylates Foxo3a, which results in its cytoplasmic retention and inhibits transcriptional activation of p27, p130Rb2, Btg1 and cyclin G2 that all inhibit cell cycle progression (Bakker et al. 2004). PKB, also activates the mTOR/ eIF4E pathway (Blazquez-Domingo et al. 2005) resulting in enhanced translation

efficiency of structured mRNAs due to increased levels of eIF4F and in increased expression of factors involved in ribosome biosynthesis. PI3K/mTOR activation keeps the balance towards proliferation of progenitors and upon downregulation of PI3K, Foxo3a signals execution of terminal differentiation.

Although the requirement for glucocorticoids *in vitro* is well proven, its role *in vivo* seems restricted to stress erythropoiesis (Bauer et al. 1999). Glucocorticoids upregulate cell cycle inhibitors and proapoptotic genes in erythroid progenitors as well as in lymphoid cells where glucocorticoids induce apoptosis. However, most of these genes are counterregulated by SCF. Glucocorticoids also induce genes such as *c-Kit* and *Jumonji*, known to be critical in hematopoiesis (Kitajima et al. 1999). To date, however, it is unclear which glucocorticoid target genes could contribute to sustained renewal of erythroid progenitors (Kolbus et al. 2003).

2.3 Growth factor sensitivity in disease

The high demand for erythrocytes in circulation is satisfied by continuous high level of erythroid progenitor expansion and differentiation in the bone marrow. Hence, negative feedback of EpoR signalling is required to prevent erythrocytosis. EpoR mutations resulting in truncations in the C-terminal region lack recruitment of the phosphatase SHP-1 resulting in constitutive proliferative signals and hypersensitivity to Epo (Klingmuller et al. 1995). These mutants are associated with Primary familial polycythemia (Furukawa et al. 1997) with normal cell maturation and increased blood cell production. Hypersensitivity can be a result of loss of negative feedback but also be caused by activating mutations resulting in enhanced proliferation and/or survival. For instance the V617F *JAK2* mutation (James et al. 2005) is found predominantly in Polycythemia Vera (PV) patients, a myeloproliferative disorder (MPD) characterized by massive erythrocytosis. The mutation resides in the kinase inhibitory domain, resulting in constitutive JAK2 kinase activity. The role of constitutive active cytokine receptor mutants in MPD and cooperative events that lead to leukaemia has been described in Section 1.4. In human disease, constitutive activating mutations in *FLT3* are found in 30 to 35% of adult AML, *N-RAS* and *K-RAS* mutations in 20% and *cKIT* mutations account for 5% of cases (Beghini et al. 2000; Gilliland and Griffin 2002), supporting that tyrosine kinase receptor mutations represent collaborative events in leukaemogenesis following loss or gain of function mutations in hematopoietic transcription factors such as fusion proteins, C/EBPα and PU1.

Conversely, in myelodysplastic syndrome (MDS) lack of circulating erythrocytes occurs due to impaired responsiveness to Epo (Backx et al. 1992; Hoefsloot et al. 1997) or aberrant response to inhibitory cytokines. Epo stimulation of erythroid progenitors derived from MDS bone marrow fail to induce Stat5 DNA-binding (Hoefsloot et al. 1997), suggesting that dysplastic cells result from maturation commitment without the capacity to drive the terminal differentiation program. In addition, genomic instability driven scenescence give rise to progenitors with impaired Epo signalling as shown in DNA repair deficient mouse models (M. von Lindern, unpublished), suggesting another plausible mechanism to impaired responsiveness to cytokines. Protection to inhibitory cytokines, such as tumour necrosis factor α (TNF-α), TNF-β, interferon-α (IFN-α), IFN-β, IFN-γ and transforming growth factor-β (TGF-β) (Majka et al. 2000;

Giron-Michel et al. 2002; Verma et al. 2002; Chung et al. 2003) (Section 1.3) is compromised due to the impaired signalling. This results in increased susceptibility to apoptosis (Fontenay-Roupie et al. 1999) resulting in ineffective hematopoiesis.

Although many signalling pathways have been unravelled, their effect on gene expression program is very fragmentary. We undertook large-scale gene expression profiling to examine modulation of gene expression at the level of transcript translation in response to growth factor signalling. The results are discussed in Chapter 3 and 4.

Section 3 Translation Initiation

3.1 Translation Initiation mechanism

Gene expression is regulated at the transcription level, producing a cell-specific mRNA pool, that undergoes fine tuned translation regulation in response to environmental and developmental cues. Regulation of translation (*i*) permits fast cellular responses to growth factors, inducing specific proteins to be expressed, and (*ii*) enables expression of pro-apoptotic proteins when the transcription program is inhibited. Translation Initiation is an important level of translation control. There are two limiting steps in the formation of the initiation complex, namely release of initiation factor eIF4E to recruit the eIF4F complex to the cap structure and the binding of GTP to initiation factor eIF2.

Protein synthesis is controlled by mTOR that is responsive to growth factor stimulation, amino acid uptake and ATP levels. PI3K/PKB activates mTOR (mammalian target of rapamycin, also named Frap, Raft1 or Rapt) through phosphorylation of the tumour suppressor complex Tsc1/Tsc2 (tuberous sclerosis protein). Tsc1/Tsc2 releases Rheb (RAS-homologue enriched in brain), a small GTPase that positively modulates mTOR function (Figure 1.2) (Inoki et al. 2003). Amino acid availability is sensed through Tsc1/Tsc2. The tumour suppressor complex Tsc1/Tsc2 also integrates signals from energy levels through the AMP-kinase.

Activation of mTOR results in phosphorylation and activation of S6K (Rps6kb1; p70S6 kinase) and hierarchical phosphorylation of 4EBP (4E-Binding Protein) (Wang et al. 2005). Since rapamycin and Wortmannin inhibit both phosphorylation of S6K and 4EBP, the PI3K/PKB/mTOR pathway is considered to be a direct signalling cascade. However, a rapamycin resistant S6K1 is still sensitive to wortmannin indicating that the PI3K and mTOR signals to S6K1 can be disconnected (Cheatham et al. 1995; Weng et al. 1995). Phosphorylation of 4EBP occurs at multiple sites primed by phosphorylation at threonine 37 and 46 (Gingras et al. 1999; Gingras et al. 2001). The modest mitogen-stimulated phosphorylation of these sites is resistant to rapamycin, indicating activity of other mitogen-dependent kinases (Gingras et al. 2001). Rapamycin inhibits phosphorylation of serine 65 and threonine 70 (Gingras et al. 2001), indicating that mTOR actively maintains these phosphorylated 4EBP sites. The kinase responsible for the phosphorylation event that it is modulated by mTOR is unknown (Wang et al. 2005). Inhibiting protein phosphatases type 1 and 2 prevents

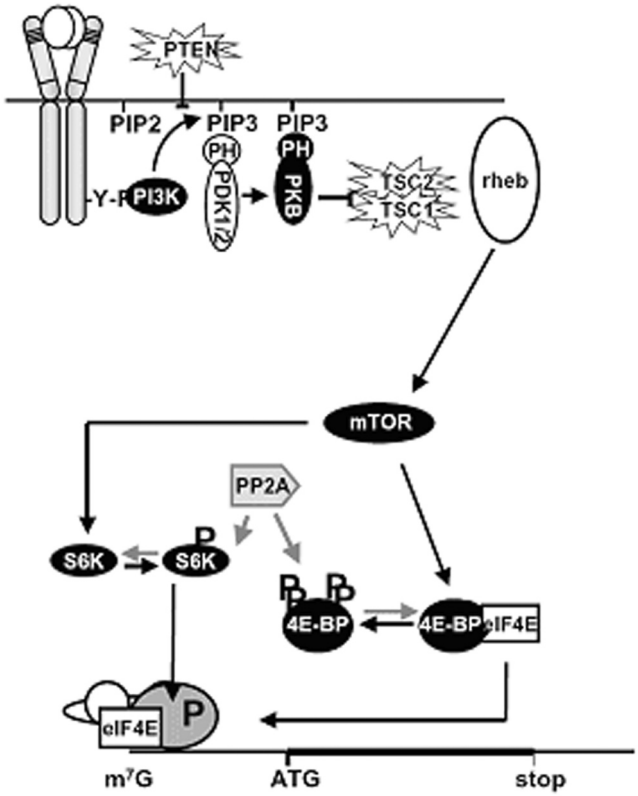


Figure 1.2 ***The PI3K/PKB/mTOR pathway controls mRNA translation.*** SCF-receptor activation results in recruitment of PI3K to the receptor, which generates phosphorylates membrane lipids (PIP3) that form an anchor for the PH-domain containing kinases PDK1 and PKB. PIP3 is dephosphorylated by the tumour suppressor PTEN, which silences the PI3K-pathway. At the membrane PDK1 phosphorylates PKB, which phosphorylates the tuberous sclerosis tumour suppressor genes Tsc1 and Tsc2. Upon phosphorylation these genes release the GTPase Rheb to activate mTOR. Activation of mTOR results in phosphorylation of p70S6 kinase (S6K) and eIF4E-binding protein (4E-BP). Upon phosphorylation, 4E-BP releases the cap-binding translation initiation factor 4E (eIF4E), which allows for association of eIF4E with the proteins that form the eIF4F scanning complex and with the 40S ribosomal subunit (see Figure 1.3).

rapamycin-induced dephosphorylation of 4EBP (Peterson et al. 1999), suggesting that mTOR inhibition releases a phosphatase to act on its downstream targets. Hence, the mechanism by which mTOR controls 4EBP phosphorylation may entail both regulation of a 4EBP-kinase and of the serine/threonine phosphatase PP2A (Figure 1.2) (Di Como and Arndt 1996; Murata et al. 1997; Inui et al. 1998; Wang et al. 2005). PP2A is the main phosphatase acting on S6K and 4EBP1. PP2A exists in various complexes that shift target specificity depending on the binding of regulatory components. Immunoglobulin binding protein 1 (Igbp1, also named $\alpha 4$) binds to and sequestrates PP2A, inhibiting dephosphorylation of 4EBP and S6K. The PP2A-α4 complex formation is modulated by mTOR, offering release of negative feedback from its own downstream targets 4EBP and S6K (Inui et al. 1998; Kong et al. 2004); (Chung et al. 1999).

4EBP hyperphosphorylation results in the release of the mRNA cap-binding factor eIF4E (eukaryotic Initiation Factor 4E) (Murakami et al. 2004). Subsequently, eIF4E can bind the scaffold protein eIF4G, which enables the formation of an eIF4F scanning complex containing eIF4E, eIF4G and the RNA helicase eIF4A. eIF4F associates with several other translation factors and the small subunit of the ribosome. This complex scans the 5'UTR for the first AUG codon in an appropriate sequence context (Kozak 2005), where the complete ribosome associates with

the methionine-tRNA and all translation factors required for protein synthesis (Pestova et al. 2001). The cap-binding eIF4E protein is the rate limiting factor in the scanning process (Sonenberg and Gingras 1998) and therefore its release upon phosphorylation of 4EBP is a crucial control mechanism in polysome recruitment of mRNAs. In addition, eIF4E is phosphorylated by MAP-kinase signal-integrating kinases Mnk1 and Mnk2 (Flynn and Proud 1995; Joshi et al. 1995) in response to insulin and stress (Waskiewicz et al. 1997). The role of eIF4E phosphorylation is still controversial. Studies show contradictory affinity kinetics to the cap structure upon eIF4E phosphorylation on Ser209 (Minich et al. 1994; Scheper et al. 2002). Reduced eIF4E affinity to the cap, might implicate modification needed for re-initiation of translation, or inhibition of cap-dependent translation (Scheper et al. 2002).

The eIF2 complex consists of three peptide chaines (eIF2α, β and γ), and binds GTP and methionine-loaded tRNA to form the eIF2·GTP/Met-tRNA$_i^{Met}$ ternary complex. Once the proper AUG is selected methionine is delivered to the translation start site at the expense of energy and eIF2·GDP is released. eIF2·GDP is recycled by eIF2B. eIF2α can be phosphorylated at Serine 51, which inhibits the nucleotide exchange in eIF2 by eIF2B (Figure 1.3) (Clemens 2001).

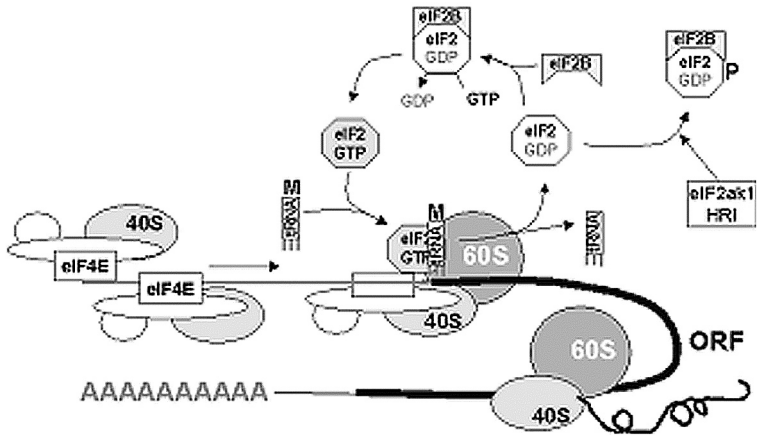


Figure 1.3 When an AUG in an appropriate Kozak-sequence is reached, eIF2:GTP associated with a methionine loaded tRNA can position this first amino acid and facilitate recruitment of the 60S ribosomal subunit to start protein synthesis. This delivery of methionine requires energy and eIF2:GDP leaves the complex to be recharged to eIF2:GTP by eIF2B. When eIF2 is phosphorylated by any of the 4 stress eIF2 associated kinases (eIF2ak1-4) such as haem-responsive inhibitor (HRI), P-eIF2 still associates with eIF2B, but eIF2B can no longer recharge eIF2:GDP to eIF2:GTP, which precludes loading with methionine-tRNA.

Phosphorylation of eIF2α plays an important role in translation regulation by various physiological cell stress conditions. The eIF2α kinases HRI, PKR, PEK and GCN2 phosphorylate and inhibit eIF2α in response to haeme levels, viral infection, ER stress and low nutrients respectively (Figure 1.4) (Wek et al. 2006). In erythrocytes, the haeme-regulated eIF2α kinase (HRI) phosphorylates and inhibits eIF2α in response to low haeme levels to prevent production of excess globin peptides (Han et al. 2001).

The regulation of translation initiation complex formation and assembly to

RNA transcripts offers a possibility to control translation at a global level and at the level of specific transcripts. Whereas global levels are relatively mildly regulated, specific transcripts can fully depend on regulatiuon by translation as a function of regulatory elements (Figure 1.4) (Graff et al. 1997; Clemens and Bommer 1999; Graff and Zimmer 2003).

Because eIF4E is under direct control of the PI3K/mTOR pathway, we examined the role of eIF4E on erythropoiesis (chapter 2).

3.2 Translation Initiation sensitivity to eIFs

3.2.1 Regulation of eIF4F-sensitive transcripts

Overexpression of the limiting translation initiation factor eIF4E can transform fibroblasts (Lazaris-Karatzas et al. 1990). Our results indicated that overexpression of eIF4E inhibited erythroid differentiation (Blazquez-Domingo et al. 2005). Sequestering eIF4E by overexpressing an mTOR-insensitive 4E-BP1 results in cell cycle inhibition (Fingar et al. 2004). These observations indicate that free, active eIF4E attributes to transformation capacity. Although eIF4E and its associated factors are general translation factors, they bind and scan mRNAs with a short and simple 5' Untranslated Region (UTR) much more efficiently than mRNAs with a long and structured 5'UTR (De Benedetti and Graff 2004). Secondary structures in the 5'UTRs of transcripts can result in structural hindrance that inhibits ribosomal scanning (Manzella and Blackshear 1990). Translation block can be released by removal of the structural elements or by overexpression of eIF4E (Manzella et al. 1991; Shantz et al. 1996; Koromilas et al. 1992). eIF4E sensitivity implies a direct role of eIF4E in melting complex 5'UTR sequences (Methot et al. 1996). Interestingly, S6K1 phosphorylates eIF4B, which is important for the recruitment of the helicase complex to the pre-initiation complex (Holz et al. 2005). In addition to structures with high enthalpy other variables such as the relative distance of hairpin loop structures from the 5' methyl G cap (Babendure et al. 2006) and association of structures with specific proteins can dictate translation efficiency. Structured mRNAs encode for growth promoting proteins such as VEGF, ODC, Igbp1 and Uhmk1 (De Benedetti and Harris 1999; Graff and Zimmer 2003; Manzella and Blackshear 1990; Godfrey Grech submitted).

A particular type of structured mRNA are TOP-mRNAs. The TOP (terminal oligopyrimidine tract) sequence confers selective translation to a given mRNA as it renders polysome recruitment dependent on mTOR activation (Jefferies et al. 1997). TOP sequences are present in mRNAs encoding ribosomal proteins and elongation factors (Terada et al. 1994) to render the energy consuming process of ribosome biogenesis dependent on the presence of mitogenic factors and nutrients. The involvement of S6K as a downstream effector of mTOR in the regulation of TOP mRNAs has been challenged by analysis of S6K knockout mice (Barth-Baus et al. 2002). This strongly suggests that additional factors are involved. One preliminary study suggests that a TOP-sequence binds an inhibitory factor that can be depleted from cell extracts (Biberman and Meyuhas 1999).

3.2.2 eIF2α phosphorylation and AUG selection

Recognition of an AUG codon depends on the sequence context. The better an initiation codon resembles the consensus sequence (A/GnnAUGG), the more efficiently it will be recognised by the eIF2-ternary complex (Kozak). The initiation codon of the protein coding ORF mostly lies in a perfect Kozak sequence. The 5'UTR may contain additional upstream AUGs (uAUG) resulting in upstream open reading frames (uORF). These usually inhibit translation efficiency. First, ribosome dissociation at the end of the uORF may end transcript scanning. Second, the peptide encoded by the uORF may actively interfere with the pre-initiation scanning complex. Activation of the eIF2 associated kinases e.g. by lack of haeme or amino acids, reduces available ternary complex and restricts AUG recognition to codons within a Kozak sequence. Phosphorylation and inhibition of eIF2α limits the formation of initiation complex at a potential AUG. However, it also alters the relative expression of uORF and encoded protein because it alters the efficiency with which different Kozak sites are recognised. A particular condition that has been found in several transcripts in an uORF out of frame with the protein coding ORF, but overlapping the initiation codon of the protein coding ORF. This implies that the uORF has to be skipped to allow protein expression, indicated as leaky scanning. Leaky scanning of AUG codons is involved in translation control of GCN4 (Hinnebusch 1993), ATF4 (Blais et al. 2004; Vattem and Wek 2004), C/EBP (Calkhoven et al. 2000) and Scl/Tal (Calkhoven et al. 2003). Interestingly, recovery from translation initiation inhibition during low eIf2 availability requires leaky scanning of an inhibitory uORF in activating transcription factor 4 (ATF4) transcript (Wek et al. 2006) promoting translation of ATF4 and transcription of its target Gadd34. This results in dephosphorylation of eIF2α (Patterson et al. 2006).

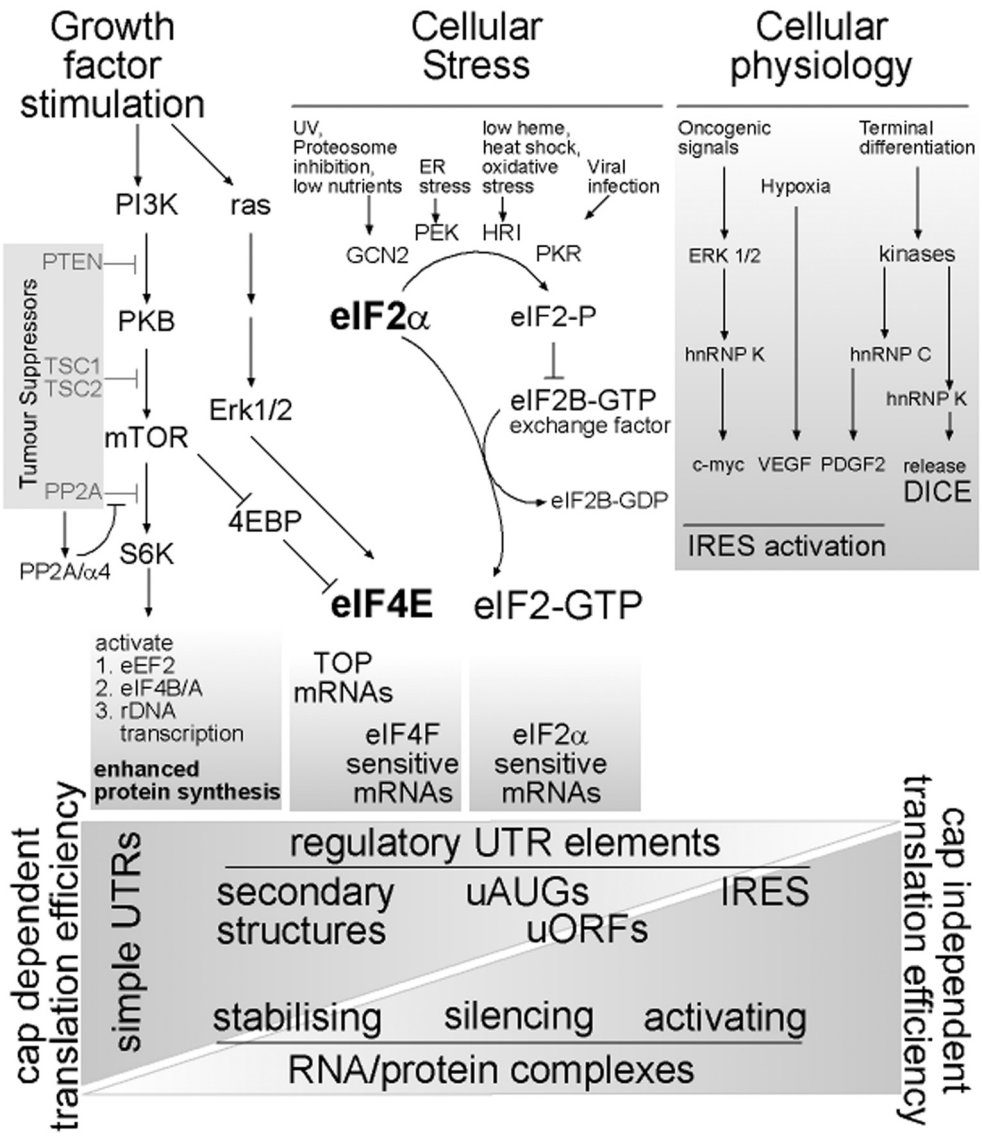
Differential expression of functional isoforms of the hematopoietic transcription factors is also under control of selective translation initiation site recognition. An alternative AUG in the open reading frame (ORF) of Gata1 is preferred at low eIF2 activity, resulting in a truncated protein with similar binding activity to form heterodimers, but a different transactivation potential (Calligaris et al. 1995). The transcripts of several Ets-family members, Scl/Tal and C/EBPα and β contain an uORF that, out of frame, overlaps the AUG start codon of the full length isoform of the transcription factor. (Calkhoven et al. 2000; Calkhoven et al. 2003). Because the uAUG of the overlapping uORF starts with an uAUG in a suboptimal Kozak consensus, it is only at enhanced availability of eIF2α:GTP and eIF4E, that the uORF is translated, the initiation codon of the full length protein is skipped and a downstream AUG codon is selected. This results in a truncated transcription factor protein that acts as a dominant negative isoform.

The importance of the relative abundance of C/EBPα isoforms is evidenced by the occurrence of mutations in acute myeloid leukaemia (AML) cases that inhibit translation of the full-length C/EBPα protein (Pabst et al. 2001). The functional 30kDa truncated protein expressed in these patients was shown to inhibit G-CSF receptor in 32Dcl3 cells induced to differentiate into neutrophils (Cleaves et al. 2004). Introducing the full length Scl in mouse bone marrow cells favoured megakaryocytic development over erythroid commitment of the megakaryocytic/erythroid progenitor (Calkhoven et al. 2003) (Figure 1.5).

The list of transcripts regulated at the level of alternative AUG usage in

hematopoietic regulation is increasing (Liu et al. 1999; Liu et al. 2005). This leaves us with the question of what portion of the mRNA pool is controlled at the level of translation. The incomplete information on full length 5'UTR sequences is still a hurdle in these types of analysis. Using oligo-capped cDNA libraries (Suzuki et al. 2000), 954 full length 5'UTRs were determined and analysed for the occurrence of upstream AUGs. 29% of the UTRs contained at least one AUG upstream of the proper initiation codon, represented by 569 uAUGs. These were further classified on the basis of being in frame or out of frame with the coding sequence and for having (uORF) or not a terminator codon. 41% of uAUGs were defined as uORFs potentially regulating translation efficiency and selection of AUG usage to drive isoforms, 7% without a termination codon and being in frame suggesting prolonged N-termini and 12% without a stop codon and being out of frame. Interestingly 359 uAUGs in 211 UTRs adequately satisfy Kozak's consensus. These percentages indicate that translation initiation control by uAUG and uORFs may represent a regulatory event that is more common than expected. Translation initiation inhibition of growth regulatory proteins (growth factors, cytokines, oncogenes, repressors of tumour suppressor inhibitors and others) is a known phenomenon (Kozak 1991) and this may be extended to regulatory proteins that attenuate cellular terminal differentiation.

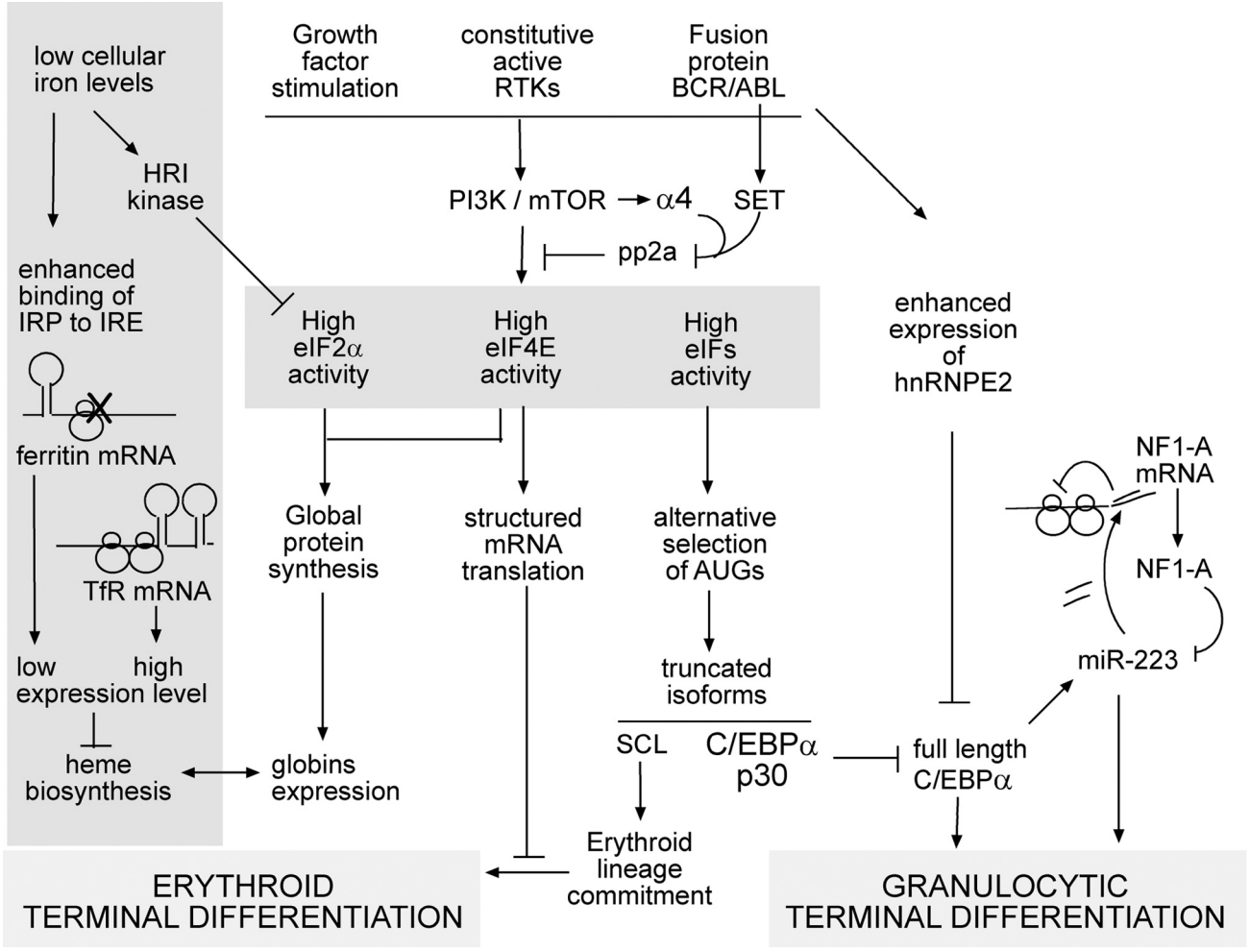
Figure 1.4 **Translation Initiation control during Growth Factor stimulation, cellular stress and cellular physiology.** Growth factor addition activates the PI3K/PKB/mTOR pathway releasing the limiting translation initiation factor 4E (eIF4E) from a repression complex with 4EBP and activating S6K resulting in enhanced cap-dependent translation efficiency of structured mRNAs, and ribogenesis. Interestingly, the tumour suppressor proteins PTEN, Tsc1/2 and Pp2a are involved in attenuating this pathway. Another limiting initiation factor, eIF2α is involved in providing methionine-tRNA in a complex with the 60S ribosome subunit to start peptide synthesis once the proper AUG is recognised. eIF2 is phosphorylated by GCN2, PEK, HRI or PRK in response to various stress conditions. Low levels of eIF4E and eIF2-GTP as a result of 4EBP repression or stress-induced eIF2 phosphorylation respectively, repress cap-dependent translation. These conditions are optimal for translation initiation from Internal Ribosomal Entry Sites (IRES). The levels of eIFs modulate translation initiation and this depends on the codes offered by the transcripts. Some transcripts are ideal to be translated under stress conditions having IRES structures in their 5'UTRs, others have secondary structures that are difficult to melt and hence hinder the scanning process. The presence of uORFs, attenuates translation initiation and also have a role in protein isoform formation. RNA-binding proteins modulate specific mRNAs by stabilising, silencing or activating the transcripts. These RNA/protein complexes (RNPs) have an important role in cellular physiology. Some RNPs respond to oncogenic signals, while others are covalently modified and drive translation in response to terminal differentiation signals as in the case of the DICE elements. [Translation Initiation factors in bold]


Growth factor stimulation
Cellular Stress
Cellular physiology
PI3K
ras
PTEN
PKB
TSC1
TSC2
mTOR
Erk1/2
PP2A
PP2A/α4
S6K
4EBP
Tumour Suppressors
UV, Proteosome inhibition, low nutrients
ER stress
low heme, heat shock, oxidative stress
Viral infection
PEK
HRI
GCN2
PKR
eIF2α
eIF2-P
eIF2B-GTP exchange factor
eIF2B-GDP
eIF4E
eIF2-GTP
Oncogenic signals
Terminal differentiation
Hypoxia
ERK 1/2
kinases
hnRNP K
hnRNP C
hnRNP K
c-myc
VEGF
PDGF2
release DICE
IRES activation
activate
1. eEF2
2. eIF4B/A
3. rDNA transcription
enhanced protein synthesis
TOP mRNAs
eIF4F sensitive mRNAs
eIF2α sensitive mRNAs
cap dependent translation efficiency
simple UTRs
regulatory UTR elements
secondary structures
uAUGs
uORFs
IRES
stabilising
silencing
activating
RNA/protein complexes
cap independent translation efficiency

3.2.3 Cap Independent translation

Regulatory elements at the 5'UTR of mature transcripts render translation dependent on signalling or other environmental conditions such as iron availability. Some transcripts however have a highly structured 5'UTR meant to completely block cap-dependent translation initiation. Translation initiation reverts to internal ribosome entry sites (IRES). The structural complexity of IRES elements argue in favour of their role as translation inhibitors, although it is more correct to define these structures as modulators of translation. For instance although the 5'UTR of platelet-derived growth factor (*PDGF2*) is long (1022bp), structured and contains upstream ORFs, it is efficiently translated during megakaryocytic differentiation via binding and activation of the IRES by hnRNP C (Figure 1.4) (Bernstein et al. 1995; Sella et al. 1999). Hence, specificity of IRES-mediated gene expression is determined by IRES trans-acting factors (ITAFs), present in a particular cellular state.

Figure 1.5 **Translation initiation control relays signals to erythroid and granulocytic differentiation.** SCF binds c-kit, a Receptor Tyrosine Kinases (RTK), activating PI3K/mTOR pathway in the same way as constitutive active mutant RTKs and kinase active fusion protein, BCR/ABL. mTOR downstream effector proteins are maintained active by attenuating the phosphatase Pp2a which is inhibited by SCF-driven Igbp1/alpha4 expression and enhanced expression of SET in response to BCR/ABL. High activity of translation initiation factors enhances polysome recruitment of structured mRNAs and delays erythroid terminal differentiation. During erythroid terminal differentiation the balance between globin synthesis and haeme biosynthesis is under the tight control of translation initiation. Iron Responsive Element (IRE) in the UTRs of ferritin and transferin modulate iron uptake and storage in accordance to demand of haeme. Low cellular iron levels triggers phosphorylation of eIF2α to reduce the production of globin proteins. High eIFs levels also regulate commitment to the erythroid or megakaryocytic lineage by selective usage of AUGs in the SCL transcript driving different isoform production. The same mechanism is used to produce truncated isoforms of the transcription factor C/EBPα that acts as a dominant negative form of the full length and hence inhibits granulocytic terminal differentiation. Another form of translation control is involved in regulation of C/EBPα transcription activity. Full length C/EBPα enhances transcription of micro RNA 223 (mRNA-223), an inhibitor of NFI-A translation. NFI-A is a competitor for binding C/EBPα DNA sites and hence its inhibition results in a positive feedback loop driving granulocytic differentiation. In addition to transcription inhibition of full length C/EBPα driven by selective AUG usage or translation silencing of competitors, the role of RNA-binding proteins is important in modulating terminal differentiation. BCR/ABL enhance the expression of hnRNPE2 that binds the UTR of C/EBPα transcript and inhibits translation.


low cellular iron levels
HRI kinase
enhanced binding of IRP to IRE
ferritin mRNA
TfR mRNA
low expression
high level
heme biosynthesis
Growth factor stimulation
constitutive active RTKs
Fusion protein BCR/ABL
PI3K / mTOR
α4
SET
pp2a
High eIF2α activity
High eIF4E activity
High eIFs activity
Global protein synthesis
structured mRNA translation
alternative selection of AUGs
truncated isoforms
SCL
C/EBPα p30
globins expression
Erythroid lineage commitment
ERYTHROID TERMINAL DIFFERENTIATION
enhanced expression of hnRNPE2
full length C/EBPα
NF1-A mRNA
NF1-A
miR-223
GRANULOCYTIC TERMINAL DIFFERENTIATION

Section 4 Control of Translation beyond the Initiation stage

4.1 Translation elongation regulation

Whilst translation initiation can regulate specific transcripts depending on transcript structure and the physiological state of the cell, general translation can be modulated via regulation of peptide elongation. The elongation factor, eEF1A recruits amino acyl-tRNA at the expense of GTP (Sheu and Traugh 1997) and eEF2 is required for ribosomal translocation during elongation (Ryazanov and Davydova 1989). Interestingly, eEF2-dependent ribosome translocation is inhibited upon phosphorylation by eEF2 kinase (Redpath et al. 1993). S6K phosphorylates and inactivates eEF2 kinase enhancing efficiency of general translation elongation in an mTOR-dependent manner (Wang et al. 2001). Response to signals to attenuate translation machinery is physiologically relevant to minimize the utilization of energy in unnecessary processes and in the case of elongation to retain translational fidelity (Carr-Schmid et al. 1999).

4.2 RNA-binding proteins: transcript stability and translation control

mRNA translation is regulated by activities of the translation machinery components as described above but also via regulation of proteins that bind to specific mRNAs. RNA binding protein complexes attenuate expression by modulating stability, degradation, cellular localisation and silencing of specific RNAs or subgroups of mRNAs. The most studied RNA-binding proteins present in ribonucleoprotein (RNP) particles are the heterogeneous nuclear ribonucleoproteins (hnRNPs) that recognize AU-rich elements (ARE) and coordinate expression of mRNAs at the level of nuclear-cytoplasmic shuttling (Veyrune et al. 1996), cytoplasmic mRNA turnover (Shaw and Kamen 1986) and silencing of cell state- and type-specific mRNAs (Ostareck et al. 1997; Notari et al. 2006). The ARE is located in the 3' untranslated region of many short-lived transcripts from cytokines, proto-oncogenes, growth factors or cell cycle regulators (Bakheet et al. 2001). Interestingly, Tristetraprolin (TTP) and Butyrate response factor (BRF1) belong to the same protein family and both promote ARE-dependent mRNA decay. Mice lacking TTP suffer from systemic inflammatory syndrome as a consequence of enhanced secretion of TNFα and GM-CSF, the products of two ARE-containing mRNAs (Taylor et al. 1996). Mice lacking BRF1 showed embryonic lethality due to chorioallantoic fusion defects (Stumpo et al. 2004). This indicates that the proteins have preferential substrates. Different classes of ARE are defined, Class I contains dispersed AU motifs, present in nuclear transcription factors and proto-oncogenes; Class II contains tandem AU motifs present in inhibitory cytokines, and Class III contains U-rich sequence but lack the canonical AUUUA motif. In contrast to TTP and Brf1, members of the ELAV family of RNA-binding proteins (eg. HuR), bind and stabilize ARE-containing transcripts (Ford et al. 1999). Interestingly, in the context of a closed loop model of translated eukaryotic mRNAs, the recruitment of HuR and other RNA binding proteins to 3'UTR elements results in the formation of complexes between HuR and the scanning ribosome at the 5'UTR. This stabilises the transcript and may facilitate translation initiation at the proper AUG in transcripts with a structured 5'UTR(Mehta et al. 2006)

Interactions between RNA-protein complexes at the 3'UTR the pre-initiation scanning complex may also allow repositioning of ribosomes across inhibitory structures (Figure 1.3). Thus sequences in the 3'UTR may cooperate with translational control elements the 5'UTR. In addition, modification of RNA binding-proteins by signalling can be another level of regulating translation efficiency at the proper AUG. Silencing of transcripts by binding of hnRNP K and hnRNP E1 to differentiation control element (DICE) in the 3' UTR of 15-lipoxygenase (*LOX*) mRNA transcript (Ostareck et al. 1997), can be released during terminal erythroid differentiation by phosphorylation of hnRNP K (Habelhah et al. 2001; Ostareck-Lederer et al. 2002).

Several other examples show that phosphorylation of RNA-binding proteins modulates transcript stability and/or translation, but the exact mechanism is mostly unknown. For example: *(i)* the activity of BRF1 to promote ARE-dependent decay is strongly reduced by protein kinase B (PKB) phosphorylation at serine 92 of BRF1 (Schmidlin et al. 2004), and *(ii)* p38 stabilises AU-rich mRNA transcripts that are involved in apoptosis (Dean et al. 2004). TPA (12-O-tetradecanoylphorbol-13-acetate)-induced monocytic differentiation results in enhanced IRES-driven translation of p27 (Kip1) suggesting modulation of cell cycle and differentiation by internal ribosome recruitment (Cho et al. 2005). Induction of megakaryocytic differentiation in K562 cells, results in PKR-dependent IRES-activation of platelet-derived growth factor (Gerlitz et al. 2002).

Interestingly, phosphorylation of ribosomal protein L13a by Interferon-gamma results in dissociation of L13a from the 60S ribosome subunit and recruitment of an Rpl13-containing protein complex to a structural element in the 3'UTR of ceruloplasmin (Mazumder et al. 2003) resulting in translation repression. This mechanism incorporates 2 novel issues. First, the ribosome is able to present signalling sensitive factors that can be released to attenuate translation of specific transcripts without affecting global synthesis rates. Second, regulatory elements in the 3'UTR recruit protein complexes within the circular mature transcripts and interact with scanning complexes in the 5'UTR, hence modulating translation initiation efficiency.

Organisation of RNAs into functional subgroups that are responsive to extrinsic and intrinsic factors (Keene and Lager 2005) underlines a relatively unexplored aspect of gene expression modulation that might drive cell fate in the same manner as regulation of the transcriptome by transcription factors. This is exemplified for transcripts that contain internal ribosome entry site (IRES) that are modulated by IRES trans-acting factors (ITAFs) as described in section 3.2.3.

The identification of a large number of transcripts subject to growth factor dependent translation made it possible to investigate which RNA structures and sequences may contribute to this translational control (chapter 5 and discussion).

4.3 Translation silencing by specific miRNAs

MicroRNAs (miRNAs) are endogenous duplexes of 19-22 nucleotides that regulate gene expression by targeting repression complexes to selected transcripts, inducing either degradation or translation repression (Bartel 2004). Primary transcripts of miRNAs are transcribed by polymerase II as long, capped, polyadenylated and

spliced RNAs (Lee et al. 2004). To produce mature miRNAs, the pre-RNAs are processed by nuclear and cytosolic RNaseIII-type endonucleases, namely Drosha and Dicer. The resulting miRNA duplexes are loaded into RNA-induced silencing complex (RISC) where one of the strands is engaged in imperfect base pairing with selected sequences in target mRNAs (Bartel 2004). Sufficient complementarity to the target mRNA triggers cleavage and degradation of the mRNA, or the transcript is translationally repressed if suitable homology permits formation of the complex on the mRNA (Zeng and Cullen 2003). Studies on translation repression of lin-14 by lin-4 miRNA in the nematode C. elegans showed that polysome loading is retained suggesting that repression occurs independently of translation initiation. One possible mechanism is to stall or slow ribosome on the transcript, although degradation of nascent peptide cannot be ruled out (Olsen and Ambros 1999). In mammalian cells premature dissociation of ribosomes accounts for miRNA silencing (Petersen et al. 2006).

miRNAs constitute a new level of gene expression regulation with a role in maintenance of multiple cellular functions in a temporal and cell-specific manner. miRNAs that are preferentially expressed in murine hematopoietic tissues may target specific effectors to modulate normal hematopoiesis. In fact, miR-181 is strongly expressed in the thymus, miR-223 in the bone marrow and miR-142 in all hematopoietic compartments (Chen et al. 2004). In the same study ectopic expression of miR-181 in murine multipotent progenitors enhanced B lineage, giving evidence of miRNA involvement in hematopoietic lineage commitment.

Interestingly, retinoic acid-induced granulocytic differentiation results in increased expression of miR-223. miR-223 represses the transcription factor NFI-A, releasing C/EBPα DNA-binding sites (Figure 1.5). The presence of C/EBPα binding sites in the miRNA-223 promoter region, represents an auto-regulatory circuitry. A 2-fold induction in granulocytic commitment was observed upon overexpression of miR-223 in NB4 cells (Fazi et al. 2005). The high expression of miR-221 and miR-222 in human cord blood-derived CD34+ progenitor cells decreased during unilineage erythroid commitment. Forced expression of these miRNAs in CD34+ derived erythroid culture inhibited cell proliferation concomitant with reduced levels of c-kit protein levels (Felli et al. 2005).

Section 5 Biology of Abnormal Hematopoiesis – the role of translation control

Much progress has been made in understanding the role of transcription factors (Tenen et al. 1997; Friedman 2002) and dynamic protein complexes that regulate gene transcription (Dang et al. 2000; Westman et al. 2002; Suzuki et al. 2003; Rodriguez et al. 2005). Selective recruitment of transcription complexes that specify the repression or activation of transcription, does not only require recognition of responsive elements in the DNA, but also post-translational modification of transcription factors that influences their interaction and activity (Tootle and Rebay 2005). The most common modifications are phosphorylation, acetylation, methylation, ubiquitination (Yang 2005; Gregoire et al. 2006). In addition, modifications of histones controls not only the activity of polymerases, but also the accessibility to chromatin (Freiman

and Tjian 2003). Attention to transcription regulatory complexes in hematopoietic progenitors has been accelerated by the observation that transcription factors are frequently involved in translocations associated with AML (Tenen 2003). In addition, studies show that the dynamics of transcription factor recruitment to promoter sites determines the onset of terminal differentiation as opposed to cell proliferation (Goardon et al. 2006).

Transcription control and protein modification is only part of the molecular biology of haematological disease. It is well accepted that mitogenic factors control ribosome biosynthesis and mRNA translation efficiency, which is required to ensure that cell growth (i.e. cell mass production) is coupled to cell proliferation (Alvarez et al. 2003). In addition to this simple view, translation control offers an efficient and rapid mechanism to acquire gene expression profiles that promote tumorigenesis and cancer progression (Audic and Hartley 2004; De Benedetti and Graff 2004). However, standard RNA expression profiles have not given insight into regulation at this level of gene expression indicating that the role of translation control in haematological disease has been greatly underestimated.

5.1 Translation attenuation and isoform formation

Molecular lesions associated with disturbed translational control of cytokines and disturbance of transcription factor isoforms ratios has been implicated in haematological diseases. Translation attenuation of cytokines is exemplified by mutations found in Hereditary thrombocythemia (HT). Thrombopoeitin (TPO) is a potent cytokine driving megakaryopoiesis and platelet production, which is under translation initiation repression by the use of seven uAUGs driving five short uORFs (Ghilardi et al. 1998). A G526T transversion in the untranslated region of TPO creates a stop codon, shortening the inhibitory uORF and resulting in enhanced ribosome re-initiation at the proper AUG (Ghilardi et al. 1999). A mutation identified within the splice donor of intron 3 results in alternative splicing omitting inhibitory uORFs from the 5'UTR of TPO (Wiestner et al. 1998).

As described previously (section 3.2.2), the relative abundance of C/EBPα isoforms is perturbed in some AML by the occurrence of mutations that inhibit translation of the full-length C/EBPα protein (Pabst et al. 2001). From studies on children with trisomy 21, acquired mutations in *GATA1* account for the exclusive production of a short isoform, Gata1s, as a result of initiation at a downstream AUG relative to the proper start codon. Hence the short isoform lacks the N-terminal domain resulting in decreased transactivating potential (Calligaris et al. 1995). All cases develop transient myeloproliferative disorder (TMD) and acute megakaryocytic leukaemia (AMKL) (Calligaris et al. 1995; Wechsler et al. 2002; Gurbuxani et al. 2004). The occurrence of preferential megakaryocytic lineage commitment in these disorders suggest that in normal hematopoiesis a non-mutated *GATA1* mRNA can be regulated at the level of alternate AUG usage to express differential isoforms (Calligaris et al. 1995) as exemplified by SCL isoform ratio in erythroid vs megakaryocytic commitment (Calkhoven et al. 2003).

5.2 Signalling deregulation in leukaemia transformation

Mutations that enhance the translation machinery also play a central role in enhanced aggressiveness of various human cancers including AML (Longley et al. 2001). The D816V mutation in the kinase domain of cKit activates the PI3K/PKB/mTOR pathway conferring sensitivity to rapamycin (Gabillot-Carre et al. 2006). Interestingly, rapamycin induces cell cycle arrest and apoptosis in patient-derived neoplastic mast cells harbouring the D816V *cKIT*, but not in normal human cord-blood derived mast cells (Gabillot-Carre et al. 2006). This implies that inhibitors targeting translation initiation regulators are therapeutic candidates in the treatment of aggressive systemic mastocytosis (associated with *cKIT* D816V) and AML harbouring the D816V *cKIT* mutant that is present in 10 to 40% of core-binding factor leukaemia (Beghini et al. 2000). Another line of interest for therapeutic approaches is the use of IC87114, a specific inhibitor of PI3K Class 1 p110δ. p110δ is constitutively expressed at high levels in blast cells of AML (Billottet et al. 2006) and IC87114 induces death of leukaemic blasts with no affect on normal haematopoietic progenitor cells (Sujobert et al. 2005; Billottet et al. 2006). The frequency of active PI3K is higher than the incidence of mutations in *RAS* or in the receptor tyrosine kinases (RTK) *FLT3* and *cKIT* (Cornillet-Lefebvre et al. 2006), suggesting the need for targeting PI3K downstream effectors. Phosphatases that attenuate kinase activity are eligible for targeting. The phosphatases, PTEN and Pp2a attenuate the PI3K/mTOR pathway, directly effecting ribosome biosynthesis and translation initiation (Podsypanina et al. 2001; Backman et al. 2002; Blazquez-Domingo et al. 2005). Pharmacological inactivation of mTOR with rapamycin reduces neoplastic proliferation (Podsypanina et al. 2001) in PTEN deficient mice, and reverses tumour growth in cancer cells characterised by activated PKB (Neshat et al. 2001). This suggests a central role of enhanced protein synthesis in neoplastic transformation. In addition, the leukaemic potential of BCR/ABL-expressing cells can be inhibited by pharmacological activation of the phosphatase Pp2a (Neviani et al. 2005). This suggests a central role of deregulated PI3K/mTOR/translation machinery in CML (Ly et al. 2003; Gingras et al. 2004).

Interestingly, the expression of RNA-binding proteins that attenuate translation of specific subsets of mRNAs (Perrotti et al. 2005), has been implicated in the transition from chronic CML to blast crisis events (Perrotti et al. 2003; Perrotti and Calabretta 2004) by suppressing differentiation (Perrotti et al. 2002) and increasing resistance to apoptosis (Trotta et al. 2003). For instance, ectopic expression of hnRNP E2, an RNA-binding protein upregulated during blast crisis of CML, resulted in downregulation of C/EBPα and G-CSFR in myeloid progenitor cells, inhibiting granulocytic differentiation (Perrotti et al. 2002).

In conclusion, modulation of signalling directed to the PI3K/mTOR/eIF4E pathway via activating mutants in RTKs and repression of phosphatases predisposes to hyperproliferation (Neviani et al. 2005) and block of differentiation (Blazquez-Domingo et al. 2005). The concomitant modulation of transcription factor isoforms, in response to translation initiation factor levels, strengthens the importance of translation control as a pharmaceutical target in haematological disease.

Scope of the thesis

Erythroid progenitors can be induced to undergo renewal divisions in presence of Epo, SCF and glucocorticoids, whereas the same cells mature to erythrocytes in presence of Epo. This model has proved to be useful to study how SCF can sustain proliferation and inhibit differentiation of progenitor cells. The main objective of the study described in this thesis is to understand how Epo/SCF-controlled gene expression regulates the balance between proliferation and differentiation of erythroid progenitors. To achieve this goal we performed gene expression profiling using polysome bound mRNA from cells that are deprived from and re-stimulated with Epo plus SCF, and from cells at different stages of differentiation. Interestingly, we observed that Stem Cell Factor (SCF) transduced signals are not only capable to regulate gene expression at the level of gene transcription, but also at the level of polysome recruitment, hence translation initiation. The cap-binding translation initiation factor 4E (eIF4E) is an important target of the PI3K/PKB/mTOR signalling pathway. Overexpression of eIF4E inhibited differentiation of erythroid progenitors, similar to addition of SCF. This observation prompted us to identify eIF4E sensitive transcripts that are recruited to polysomes in response to SCF. Through comparison of Epo/SCF-controlled gene expression in polysome bound RNA versus total mRNA, we were able to identify a unique list of genes encoding transcripts with signalling-dependent polysome loading that are potentially involved in SCF-repression of erythroid differentiation. Nine genes with a predicted function in signal transduction or gene expression regulation were selected to study their expression regulation in more detail and to examine their biological role in erythropoiesis. These studies revealed that Igbp1 (Immunoglobulin binding factor 1) blocked erythroid differentiation. Igbp1 is one of the regulatory subunits of the phosphatase Pp2a (also designated α4) and sequestrates the active subunits from dephosphorylating mTOR targets, hence maintaining activity resulting in enhanced translation initiation and ribogenesis.

Finally, we addressed mechanisms of translation control of these transcripts. We screened for regulatory elements within the untranslated regions (UTRs) of these transcripts, and initiated functional assays.

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Translation initiation factor eIF4E inhibits differentiation of erythroid progenitors.

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CHAPTER 2

ABSTRACT

Stem cell factor (SCF) delays differentiation and enhances expansion of erythroid progenitors. Previously, we performed expression profiling experiments to link signaling pathways to target genes using polysome-bound mRNA. SCF-induced phosphoinositide-3-kinase (PI3K) appeared to control polysome recruitment of specific mRNAs associated with neoplastic transformation. To evaluate the role of mRNA translation in the regulation of expansion versus differentiation of erythroid progenitors, we examined the function of the eukaryote initiation factor 4E (eIF4E) in these cells. SCF induced a rapid and complete phosphorylation of eIF4E-binding protein (4E-BP). Overexpression of eIF4E did not induce factor-independent growth, but specifically impaired differentiation into mature erythrocytes. Overexpression of eIF4E rendered polysome recruitment of mRNAs with structured 5'-untranslated regions largely independent of growth factor and resistant to the PI3K inhibitor LY294002. In addition, overexpression of eIF4E rendered progenitors insensitive to the differentiation inducing effect of LY294002, indicating that control of mRNA translation is a major pathway downstream of PI3K in the regulation of progenitor expansion.

INTRODUCTION

Leukemia may arise through mechanisms that enable proliferation and survival of normal cells when expansion of the progenitor pool is required. Control of the balance between expansion and maturation by cytokines and growth factors is such a mechanism. While erythroid progenitors require erythropoietin (Epo) to mature into erythrocytes, the progenitor pool can be expanded both *in vivo* and *in vitro* in response to the cooperative action of Epo, stem cell factor (SCF) and glucocorticoids (Broudy et al. 1996; Bauer et al. 1999; Dolznig et al. 2001; von Lindern et al. 2001). SCF cooperates with Epo to suppress differentiation and sustain renewal divisions of erythroid progenitors. Cooperation of SCF and cytokines is also observed in other hematopoietic progenitors (Broxmeyer et al. 1991). A mutated SCF receptor (v-Kit) was initially identified as a viral oncogene in a feline leukemia virus (Qiu et al. 1988). Mutations in c-Kit that induce constitutive activity are found specifically in t(8;21) and inv(16) leukemia, but autocrine loops activating c-Kit are also reported in other types of leukemia (Beghini et al. 2000; Zheng et al. 2004). In an avian model system, SCF cooperates with oncogenic MLL fusion genes to transform lympho-myeloid multipotent progenitors (Schulte et al. 2002). Furthermore, activating c-Kit mutations are common in gastrointestinal stromal tumors (GIST; Kim et al. 2004) and in bilateral testicular germ-cell tumors (Looijenga et al. 2003). This suggests that inhibition of erythroid differentiation by SCF may exemplify a more general role of SCF in the regulation of cell growth and differentiation.

The expansion of erythroid progenitors in the presence of SCF is abrogated by the phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002 (von Lindern et al. 2001), resulting in terminal differentiation instead. An important effector of PI3K is Protein kinase B (PKB), which controls cellular processes such as cell cycle progression, apoptosis and mRNA translation through phosphorylation of e.g. Forkhead transcription factors, the proapoptotic protein Bad and the mammalian target of rapamycin (mTOR) (Datta et al. 1999; Gingras et al. 2001; Proud 2002; Alvarez et al. 2003; Bakker et al. 2004; Fingar et al. 2004). To identify critical pathways and targets downstream of SCF-signaling, we performed profiling experiments using polysome-bound mRNA to detect those mRNAs that are expressed and translated into protein. Expression of the putative oncogene, *nucleoside diphosphate kinase B* (*Ndpk-B* also known as *Nm23-M2 or Nme2),* appeared to be regulated by SCF. Regulation did not occur at the level of gene transcription, but exclusively through the recruitment of its mRNA into polysomes, which was fully dependent on PI3K activity (Joosten et al. 2004). As selective recruitment of mRNAs into polysomes appears to be an important regulatory mechanism in cell growth control and tumorigenesis (Rajasekhar et al. 2003; Bader and Vogt 2004), we examined the role of mRNA translation in PI3K-dependent control of expansion and differentiation of erythroid progenitors.

The mRNA cap-binding eukaryote Initiation Factor 4E (eIF4E) recruits the scaffolding protein eIF4G, which associates among others with the mRNA helicase eIF4A and the small subunit of the ribosome. This complex scans the 5' untranslated region (5'UTR) of mRNA until an appropriate AUG is recognized. In binding to the limiting factor eIF4E, eIF4G has to compete with the 4E-binding proteins (4E-BP). Unphosphorylated 4E-BP binds and inhibits eIF4E, but eIF4E is released upon

phosphorylation of 4E-BP by the mTOR kinase (Gingras et al. 1999a). The increased availability of eIF4E is associated with cell proliferation (Fingar et al. 2004). Increased levels of eIF4E are detected in a number of solid tumors, especially in breast, colon and head/neck tumors (De Benedetti and Harris 1999). Interference with translation initiation via overexpression of a mTOR-insensitive 4E-BP1 results in the inhibition of the cell cycle progression (Fingar et al. 2004). These observations suggest that the availability of eIF4E in translation initiation attributes to neoplastic transformation. In addition, it has also been suggested that eIF4E enhances nucleocytoplasmic transport of specific transcripts. Aberrant regulation of eIF4E-dependent mRNA transport contributed to leukemogenesis as it impaired granulocytic and monocytic differentiation (Topisirovic et al. 2003).

Although eIF4E and its associated factors are general translation factors, they bind and scan mRNAs with a short and simple 5'UTR much more efficiently than mRNAs with a long and structured 5'UTR. The 5'UTR of the *Ndpk-B* transcript, that is strictly dependent on Epo- and SCF-induced PI3K activity, begins with a terminal oligopyrimidine (TOP) sequence and contains an inverted repeat (Joosten et al. 2004). The TOP sequence confers selective translation to a given mRNA as polysome recruitment becomes dependent on mTOR activation (Jefferies et al. 1997). TOP sequences are present in mRNAs encoding ribosomal proteins and elongation factors to render the energy consuming process of ribosome biogenesis dependent on the presence of mitogenic factors and nutrients. Additional secondary structures within the 5'UTR may also control mRNA translation such as the iron response element that renders translation of the transferrin receptor dependent on the availability of iron (Thomson et al. 1999).

In this manuscript we show that control of mRNA translation is an important PI3K-dependent pathway regulating progenitor expansion. We demonstrate that SCF induces rapid PI3K-dependent phosphorylation of 4E-BP and that overexpression of eIF4E in erythroid progenitors delays differentiation and enhances renewal divisions in the absence of SCF. We identified mRNAs upregulated by SCF-induced polysome recruitment. These mRNAs contain a long and structured 5'UTR (Y-box binding protein-1), a short 5'UTR starting with a TOP sequence (the splicing factor U2-Snrpb") or both a TOP sequence and an inverted repeat (*Ndpk-B*). Polysome recruitment of all three mRNAs was strongly enhanced by eIF4E overexpression in cells that were factor deprived, while eIF4E overexpression did not affect polysome association in the presence of SCF.

RESULTS

Epo and SCF control eIF4E levels available for mRNA translation.

Since 4E-BP sequester eIF4E in the absence of growth factors, we examined 4E-BP phosphorylation in erythroid progenitors in response to Epo and SCF. Upon factor deprivation only unphosphorylated 4E-BP are detected. Epo induced partial phosphorylation of 4E-BP while only SCF induced complete phosphorylation of 4E-BP (Figure 1A). Comparison of phospho-specific and total 4E-BP staining showed that migration of 4E-BP is directly related to its phosphorylation state. An unphosphorylated α-isoform, a partially phosphorylated β- and a fully

phosphorylated γ-isoform can be discerned in agreement with the proposed two-step phosphorylation mechanism (Gingras et al. 1999a). Importantly, SCF but not Epo is able to induce phosphorylation of Ser64, the last step in a cascade involving the sequential phosphorylation of T36/T45, T69 and S64 to release eIF4E (Figure 1B, Gingras et al. 2001). Epo/SCF-induced phosphorylation of 4E-BP was abrogated in the presence of the PI3K-inhibitor LY294002 and the mTOR inhibitor rapamycin (Figure 1B). The MEK1-inhibitor U0126 partially inhibited the hyperphosphorylation of 4E-BP (Figure 1C), which may indicate that part of the TSC/mTOR pathway is controlled through MEK1 (Tee et al. 2003; Naegele and Morley 2004). However, the major effect of LY294002 and the minor effect of U0126 are in accordance with the observation that inhibition of PI3K but not Mek1 abolished polysome association of *NDPK-B* (Joosten et al. 2004).

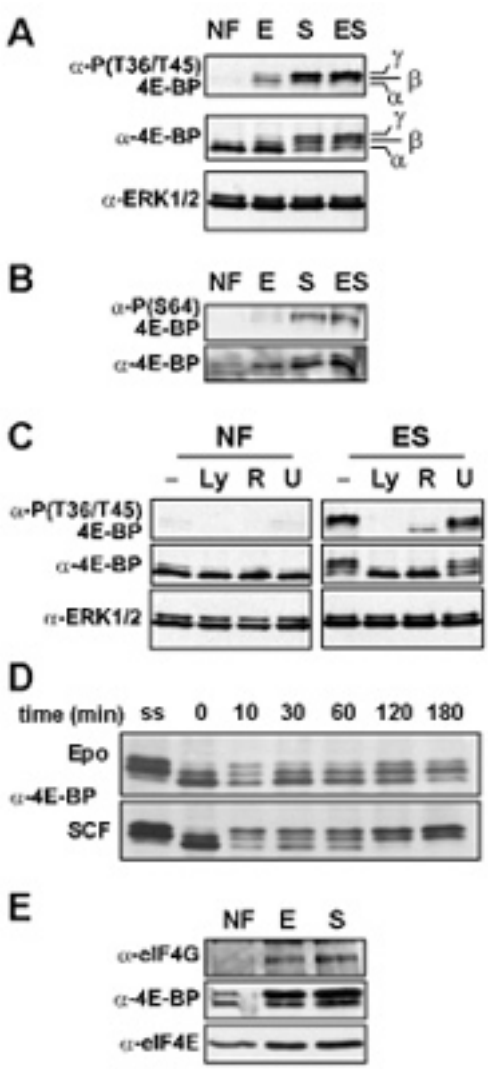

Figure 1 **Phosphorylation of 4E-BP is controlled by SCF and Epo in erythroid progenitors**. **A/B**: I/11 cells were factor deprived for 4 h, stimulated with Epo (E, 5U/ml), SCF (S, 100 ng/ml) or Epo plus SCF (ES) for 10 min, or left untreated (NF). **C**: I/11 cells were factor deprived in presence of 10μM LY294002 (LY), 10 nM rapamycin (R) or 20 μM U0126 (U) and stimulated for 10 min with Epo and SCF (ES) in presence of the inhibitors. Western blots with total cell lysates (**A/C**) or with 4E-BP immunoprecipitates (**B**) were stained with antibodies recognizing T36/T45 phosphorylated 4E-BP (α-P(T36/T45)-4E-BP), S64 phosphorylated 4E-BP (α-P(S64)-4E-BP) or total 4E-BP (α-4E-BP). ERK1/2 staining was used as sample loading control (α-ERK1/2). The unphosphorylated α-isoform, a partially phosphorylated β-isoform and a fully phosphorylated γ-isoform of 4E-BP are indicated in panel A. **D:** Samples were taken at various time points following addition of Epo or SCF (ss, steady state, cells not subjected to factor deprivation). Western blots were stained with antibodies recognizing 4E-BP. **E**: I/11 cells were factor deprived (NF) or restimulated as indicated in panels A/B and eIF4E was precipitated with m^7GTPsepharose. Precipitates were stained for eIF4G, 4E-BP and eIF4E on Western blots.

Since only SCF induced full phosphorylation of 4E-BP during 10' stimulation, we examined the kinetics of 4E-BP phosphorylation to exclude that the difference was due to different kinetics of induction and feedback modulation. Even after prolonged stimulation of erythroid progenitors with Epo, phosphorylation of 4E-BP was partial and only low amounts of the fully phosphorylated γ-isoform were detected. In contrast, SCF rapidly induced complete phosphorylation of 4E-BP, which was not dephosphorylated in continuous presence of the factor (Figure 1D). Subsequently, we tested whether phosphorylation of 4E-BP actually resulted in reduced 4E-BP binding to eIF4E and increased association of eIF4E with the scaffolding protein eIF4G. m^7GTP-sepharose was used to precipitate eIF4E from extract of $15x10^6$ cells that were factor deprived or restimulated with Epo and/or SCF. Surprisingly the amount of 4E-BP pulled down from restimulated cells increased, concomitant with increased precipitation of eIF4E. However, also eIF4G binding to eIF4E increased (Figure 1E). This indicates that 4E-BP is in excess, but that stimulation of erythroid progenitors with SCF does induce eIF4F complexes.

The level of eIF4E available for mRNA translation initiation is regulated by 4E-BP phosphorylation, but we also observed factor-induced upregulation of eIF4E expression in mRNA profiling experiments (Kolbus et al. 2003). Real-time quantitative PCR showed that eIF4E mRNA increased approximately 2-fold in I/11 erythroblasts following a 2h Epo, SCF or Epo/SCF exposure compared to factor-deprivation of I/11 cells (Figure 2A). This upregulation was due to an increase in total transcript because the relative distribution between subpolysomal and polysomal associated mRNA did not change in response to factor-deprivation and restimulation (Figure 2B). Increased mRNA expression results in increased protein expression (Figure 2C,D), which is in accordance with the data shown in Figure 1E. During terminal erythroid differentiation eIF4E mRNA expression was downmodulated approximately 4-fold in 72 hours (Figure 2E).

Together, the data show that the level of eIF4E available for mRNA translation is controlled by SCF at least at two levels: phosphorylation of 4E-BP allowing increased eIF4G binding, and increased expression.

eIF4E overexpression in I/11 erythroid progenitor cells impairs differentiation and enhances cell renewal

If regulation of eIF4E is a major effector of SCF-induced PI3K, overexpression of eIF4E is expected to affect the PI3K-dependent balance between expansion and differentiation of erythroid progenitors (von Lindern et al. 2001). Using retroviral expression vectors, we established multiple clones expressing myc-tagged eIF4E (eIF4E-myc; Figure 3A). The eIF4E-myc could efficiently bind 4E-BP, which enabled the exogenously expressed eIF4E-myc to titrate the inhibitory function of 4E-BP (Figure 3B). Using m7GTP-sepharose we pulled down eIF4E from control and eIF4E overexpressing cells that were factor-deprived and restimulated. In contrast to control cells, factor-deprived eIF4E overexpressing cells contained eIF4E-eIF4G complexes (Figure 3C). More eIF4G was recruited by eIF4E in the eIF4E overexpressing erythroid progenitors under all conditions (Figure 3C,D). Since all unphosphorylated 4E-BP was bound by the excess of eIF4E, induction of 4E-BP phosphorylation upon stimulation of the cells with SCF resulted in a more pronounced decrease in 4E-BP

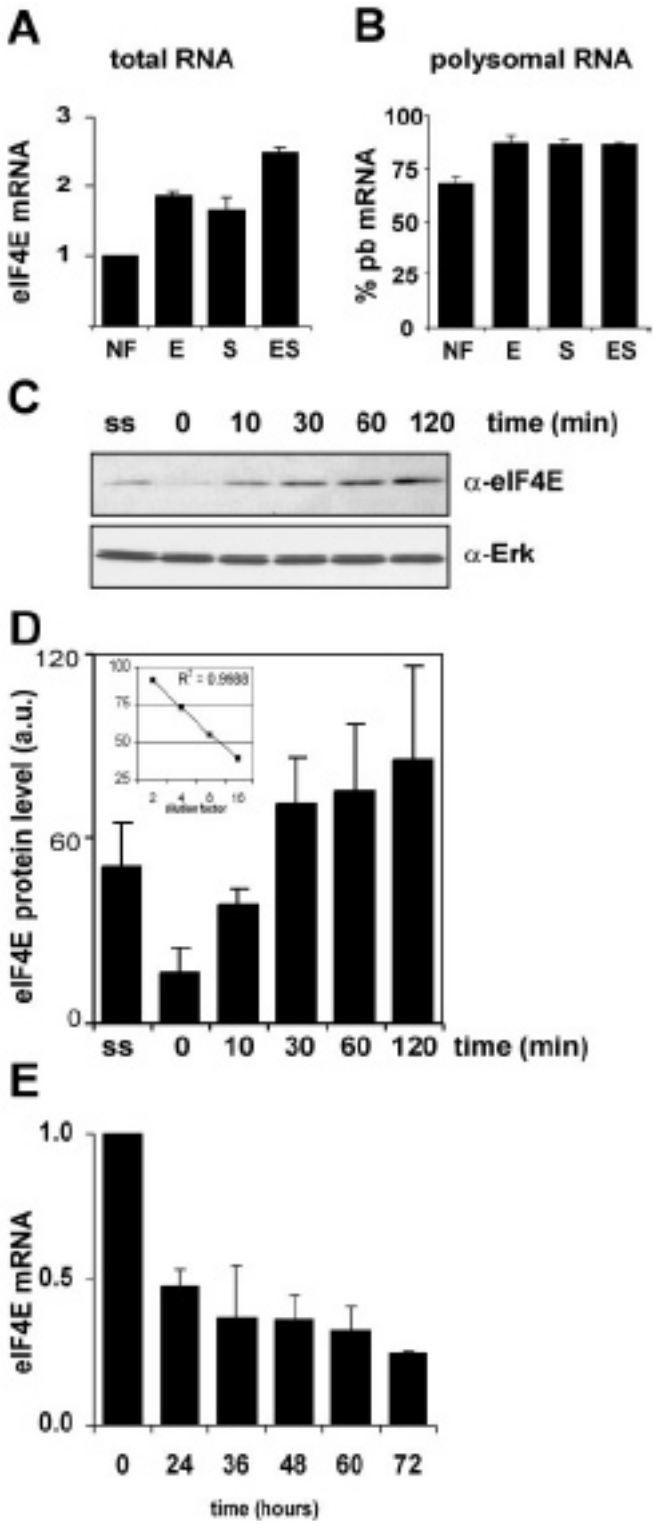

Figure 2 **eIF4E expression is upregulated upon growth factor stimulation in l/11 cells**. **A, B:** l/11 cells were factor deprived for 4 h and stimulated with Epo (E), SCF (S) or Epo plus SCF (ES) or left untreated (NF) for 2 h. Total (A), free and polysome associated mRNA (B) was isolated and eIF4E mRNA was quantified using real-time PCR. **A**: eIF4E expression in factor-stimulated cells is given as fold-change ratio compared to untreated cells. **B**: The percentage of eIF4E mRNA present in the polysome-bound (pb) fraction is calculated. **C**: l/11 cells were factor deprived and restimulated with Epo plus SCF for various time intervals as indicated. Protein samples were analyzed on Western blot for eIF4E expression. The Western blot was stained with α-actin to control for equal loading. **D:** quantification of eIF4E intensities. Bars indicate the average of 3 independent experiments, a representative of which is shown in 2C. Error bars indicate SD. The insert demonstrates the linearity of the eIF4E antibody used (r^2=0.9988). Two-fold dilutions of total cell lysate are tested for eIF4E expression. Expression is given in arbitrary units (a.u.) **E**: l/11 cells were induced to differentiate and samples were harvested for mRNA isolation at the indicated time points. Expression of eIF-4E was examined by real-time RT-PCR and is given as fold-change ratio compared to expression in expanding l/11 cells (t0).

binding than observed in control cells (compare Figures 1E and 3D). In conclusion, although the level of myc-eIF4E overexpression detected in Figure 3A is modest compared to endogenous eIF4E, the increase in eIF4G-association indicates that the expression of myc-eIF4E significantly enhances the level of free eIF4E (not bound to 4E-BP).

Using immunofluorescence we analyzed the subcellular localization of endogenous eIF4E and of eIF4E-myc. Previously it has been shown that eIF4E may accumulate in nuclear bodies to prevent cytoplasmic translocation of specific mRNAs (Topisirovic et al. 2003). A small fraction of endogenous eIF4E appeared in nuclear bodies, but eIF4E-myc appeared to be exclusively cytoplasmic (Figure 3E). Thus, it is not likely that eIF4E-myc in erythroid progenitors functions through a non-translational mechanism such as retaining specific mRNAs in the nucleus. Finally, we also expressed an eIF4E mutant in which tryptophan-73 was mutated to alanine (W73A mutant) in I/11 cells. The W73A mutant is unable to bind the scaffolding protein eIF4G or 4E-BP. A low level of eIF4E(W73A) expression was detected in mass cultures within a week after transduction, but expression of this mutant was rapidly lost and we were unable to establish single cell derived clones expressing the W73A mutant (data not shown). This suggests that eIF4E may not only bind 4E-BP or the scaffold eIF4G, but that positive factors can also be competed for by eIF4E-myc overexpression.

To examine the effect of eIF4E overexpression on the balance between expansion and differentiation of erythroid progenitors, we exposed vector control cells and eIF4E-myc expressing clones to conditions inducing expansion (Epo, SCF, Dex), delayed differentiation (Epo, SCF), differentiation (Epo) or apoptosis (no factor). Both control and eIF4E-myc expressing cells fail to proliferate or mature in absence of growth factors (Figure 4A,E). Under differentiation conditions, i.e. in presence of Epo only, control cells undergo prompt differentiation, characterized by transient proliferation, decrease in cell size and accumulation of hemoglobin (Figure 4A-C). Notably, this differentiation is markedly delayed in eIF4E-myc expressing cells indicated by prolonged expansion, maintenance of a blast-like cell size and lack of hemoglobin accumulation (Figure 4E-G). In presence of Epo plus SCF, control cells were delayed in differentiation, but e.g. cell size reduction starts almost as rapidly as in the presence of Epo only. Under these conditions, differentiation of eIF4E-myc expressing cells was essentially absent. The delay of differentiation in eIF4E-myc expressing cells is also evident from the cell morphology of the distinct cultures after 4 days exposure to Epo (Figure 4D,H).

The effect of eIF4E-myc expression on Epo-dependent differentiation was analyzed for multiple independent clones, which showed consistent inhibition of differentiation (Figure 5).

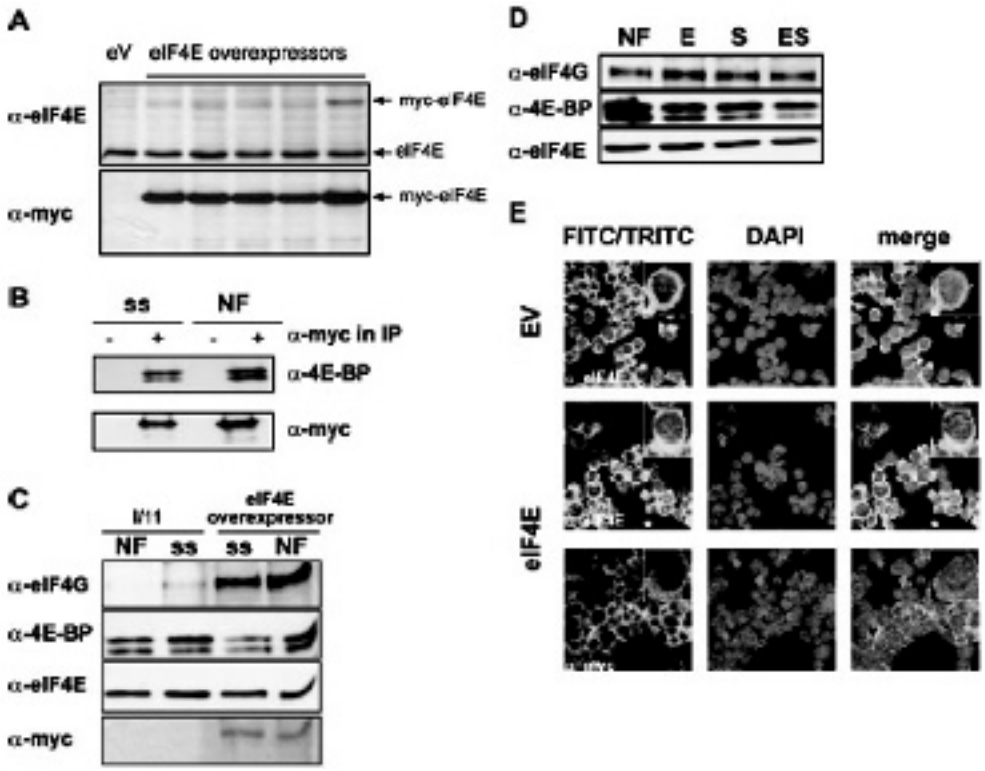

Figure 3 **Ectopic myc-eIF4E protein is able to interact and titrate out endogenous 4E-BP and is mainly localized in the cytoplasm of I/11 cells**. **A**: Lysates of an empty vector transduced clone (eV) and various myc-eIF4E transduced clones were tested for eIF4E expression on Western blots using antibodies recognizing eIF4E (top panel) or the myc-tag (lower panel). The myc-eIF4E and endogenous eIF4E proteins have different mobility and are indicated by arrows. **B**: myc-eIF4E expressing I/11 cells expanding in presence of Epo, SCF and Dex (ss) or factor deprived for 4 h (NF) were lysed and myc-eIF4E was immunoprecipitated with anti-myc antibody (α-myc, + lanes). Mock immunoprecipitations using sepharose beads without antibody (- lanes) served as controls. Western blots were stained for 4E-BP (top) or the c-myc tag (bottom). Unphosphorylated and hypophosphorylated 4E-BP (α- and β-form) were co-immunoprecipitated with myc-eIF4E. The myc-tagged eIF4E could efficiently bind to endogenous 4E-BP. **C/D**: eIF4E was precipitated by m^7GTPsepharose and tested for eIF4G and 4E-BP association on Western blots. Endogenous eIF4E was detected by specific antibody, myc-eIF4E was detected by anti-myc antibody. Lysates were prepared from myc-eIF4E expressing and control I/11 cells that were steady state expanding (ss) or factor deprived (NF) (**C**) or factor-deprived (NF) and restimulated with Epo (E; 5U/ml, 60'), SCF (S; 100ng/ml; 60') or Epo plus SCF (ES) (**D**). **E**: Cytospins of empty vector (upper panel) and myc-eIF4E (middle/lower panel) expressing I/11 cells are fixed and stained for eIF4E (upper, middle left panel) or the c-myc tag (lower left panel) using FITC- and TRITC labeled second antibodies respectively. In addition nuclei of all cells are stained by DAPI (middle panels). The right panels represent the overlay of eIF4E or c-myc with DAPI. A small fraction of endogenous eIF4E appeared in nuclear bodies, whereas myc-eIF4E seems to be exclusively cytoplasmic.

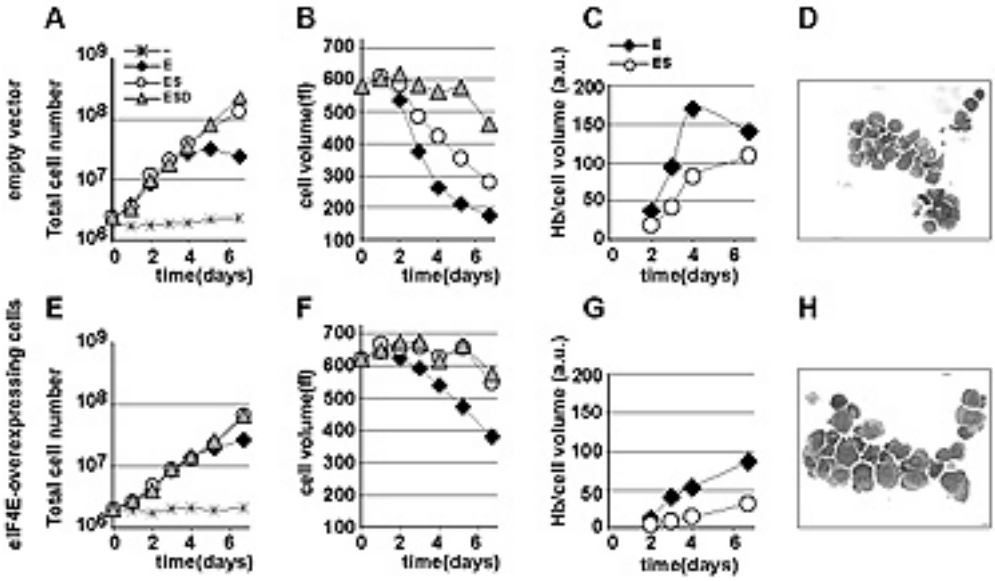

Figure 4 **Overexpression of eIF4E impairs differentiation of I/11 erythroid progenitors**. I/11 cells transduced with an empty control vector (**A-D**) or with an eIF4E expression vector (**E-H**) were seeded in medium without factor (-), or supplemented with Epo (E, 2U/ml), Epo plus SCF (ES, 100ng/ml SCF) or Epo, SCF and dexamethasone (ESD, 10^{-6}M dexamethasone). (**A, E**) Cumulative cell number, (**B, F**) cell size and (**C, G**) and hemoglobin content per cell volume (arbitrary unites; a.u.) were analyzed at regular intervals for 7 days. At day 4, cells seeded in Epo were harvested for cytospins and stained for hemoglobin and histological dyes (**D, H)**. Hemoglobinised and enucleated erythrocytes are present in control cells, while eIF4E expressing cells contain mainly blasts.

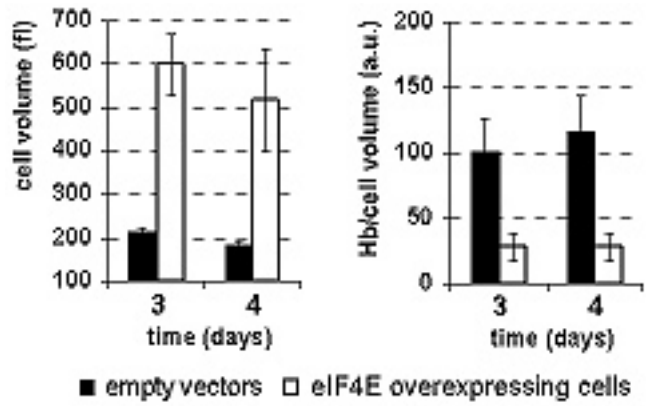

Figure 5 **Overexpression of eIF4E consistently impairs differentiation of I/11 erythroid progenitors**. **A, B:** Vector and myc-eIF4E transduced clones were subjected to differentiation as described for Figure 4. The average cell volume (**A**) and hemoglobin content (**B**) as measured 3 and 4 days following induction of differentiation is shown for 6 clones each. Error bars represent standard deviation.

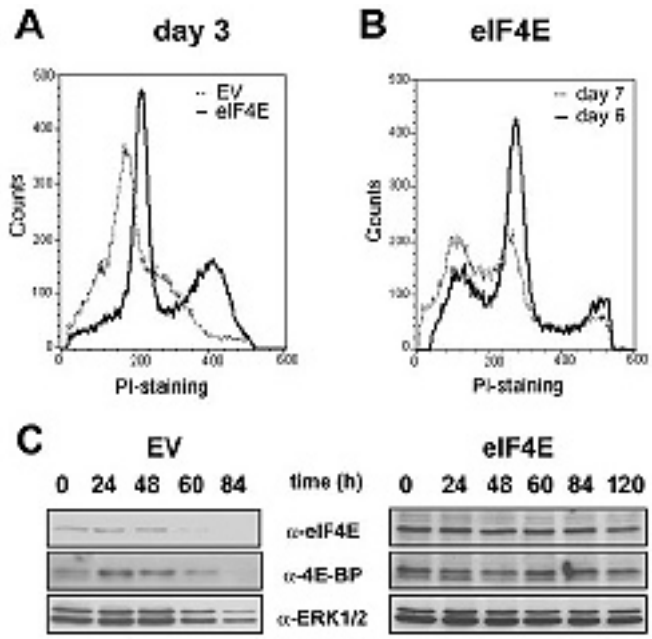

Figure 6 **eIF4E overexpression in I/11 cells impairs differentiation but fails to prolong renewal in presence of Epo**. **A, B**: Empty vector (EV) control clones and *myc*-eIF4E expressing cells (eIF4E) were fixed and permeabilized, stained with propidium-iodine (PI) and analyzed by flow cytometry. **A**: Three days following reseeding of the cells in differentiation medium (supplemented with Epo only), the EV control cells were predominantly arrested in the G1 phase of the cell cycle, the G2 peak is absent. The PI-staining is relatively low because the nuclei are condensed and bind less PI. A prominent peak representing the G2 phase of the cell cycle is present in eIF4E overexpressing cells. **B**: At day 6 and day 7 following seeding in differentiation medium, the eIF4E overexpressing cells are still in cycle as demonstrated by the G2 peak, but in time the number of cells with a sub-G1 DNA content (indicating dead cells) accumulates. **C**: Protein lysates of empty vector (EV) control clones and *myc*-eIF4E expressing cells (eIF4E) were harvested at increasing intervals following reseeding of the cells in differentiation medium. Western blots were stained with antibodies recognizing endogenous eIF4E, 4E-BP or ERK1/2 as indicated. Erk served as a loading control.

Measuring the DNA content by flow cytometry at day 3 following reseeding in differentiation medium confirmed that control cells arrest in the G1-phase of the cell cycle, while eIF4E-myc cells can be detected in all phases of the cell cycle (Figure 6A; the G1-peak of control cells is shifted to the left because the highly condensed DNA of late erythroblasts binds less propidium iodine; no significant number of apoptotic cells is detected by annexin staining or Tunnel assay). However, eIF4E-myc expressing cells are not transformed and fail to undergo prolonged renewal in presence of Epo. Seven days following reseeding in differentiation conditions the cultures of eIF4E-myc expressing cells accumulate cells with a sub-G1 DNA-content and annexin positive staining (Figure 6B and data not shown), suggesting increased apoptosis. In the control cultures all cells have enucleated. These results were confirmed in 4 control clones and 6 eIF4E-myc expressing clones. The failure to undergo differentiation could be due to the failure to downregulate eIF4E (Figure 2C). Indeed, expression of eIF4E protein decreased during differentiation of control cells, followed by decreased expression of 4E-BP. However, expression of endogenous eIF4E remained high in eIF4E-myc progenitors (Figure 6C).

PI3kinase inhibition does not abrogate cell expansion of l/11 eIF4E-overexpressing cells.

We showed that PI3K-activity rather than MEK1/ERK-activation is essential to maintain cell renewal divisions in erythroid progenitors (von Lindern et al. 2001). Since phosphorylation of 4E-BP and release of eIF4E is one of the PI3K effectors, we analyzed whether constitutive expression of eIF4E maintains cell renewal in presence of the PI3K-inhibitor LY294002. Addition of LY294002 to renewal conditions decreased expansion of control cultures and accelerated cell differentiation evidenced by increased hemoglobinization. In contrast, eIF4E-myc expressing cells did not alter cellular renewal or differentiation in response to LY294002 (Figure 7). This indicates that control of translation initiation is a major target of PI3kinase in erythroid development.

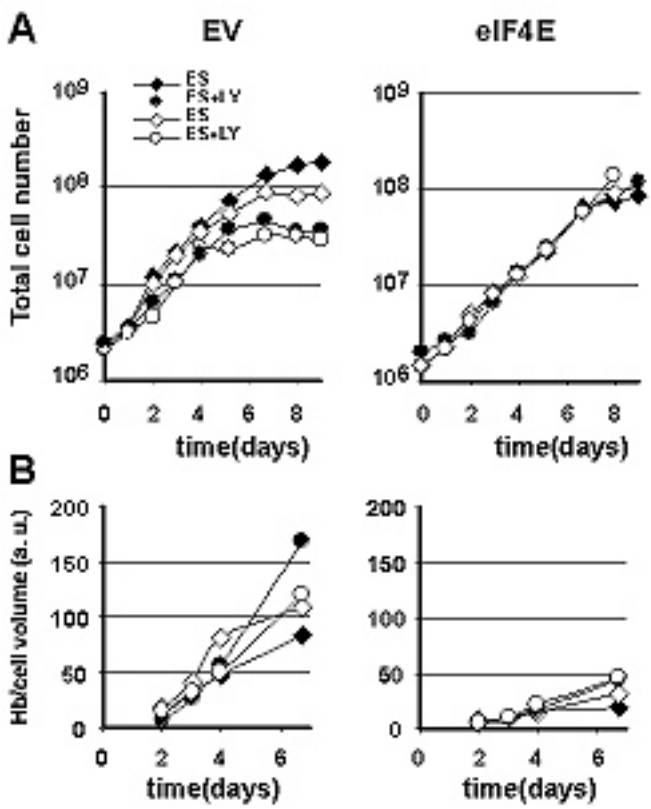

Figure 7 **Overexpression of eIF4E renders cells insensitive to the PI3K inhibitor LY294002**. Two empty vector control clones and two eIF4E overexpressing clones (black and white symbols indicate separate clones) were seeded in medium supplemented with Epo and SCF (ES) in absence (diamantes) or presence (circles) of LY294002 (LY; 10 μM). (**A**) Total cell number and (**B**) hemoglobin (Hb) content per cell volume (in arbitrary units; a.u.) were monitored daily.

Polysome association of Ndpk-B, U2-Snrpb" and YB-1 transcripts depends on PI3K activation or eIF4E expression.

Recently we screened for Epo- and SCF-induced genes in a profiling assay using polysome bound mRNA. Expression of *Nucleoside diphosphate kinase-B* (*Ndpk-B*) appeared to be controlled specifically at the level of polysome recruitment by a mechanism involving SCF-activated PI3K activity (Joosten et al. 2004). Since other targets upregulated by SCF may be regulated by the same mechanism, the

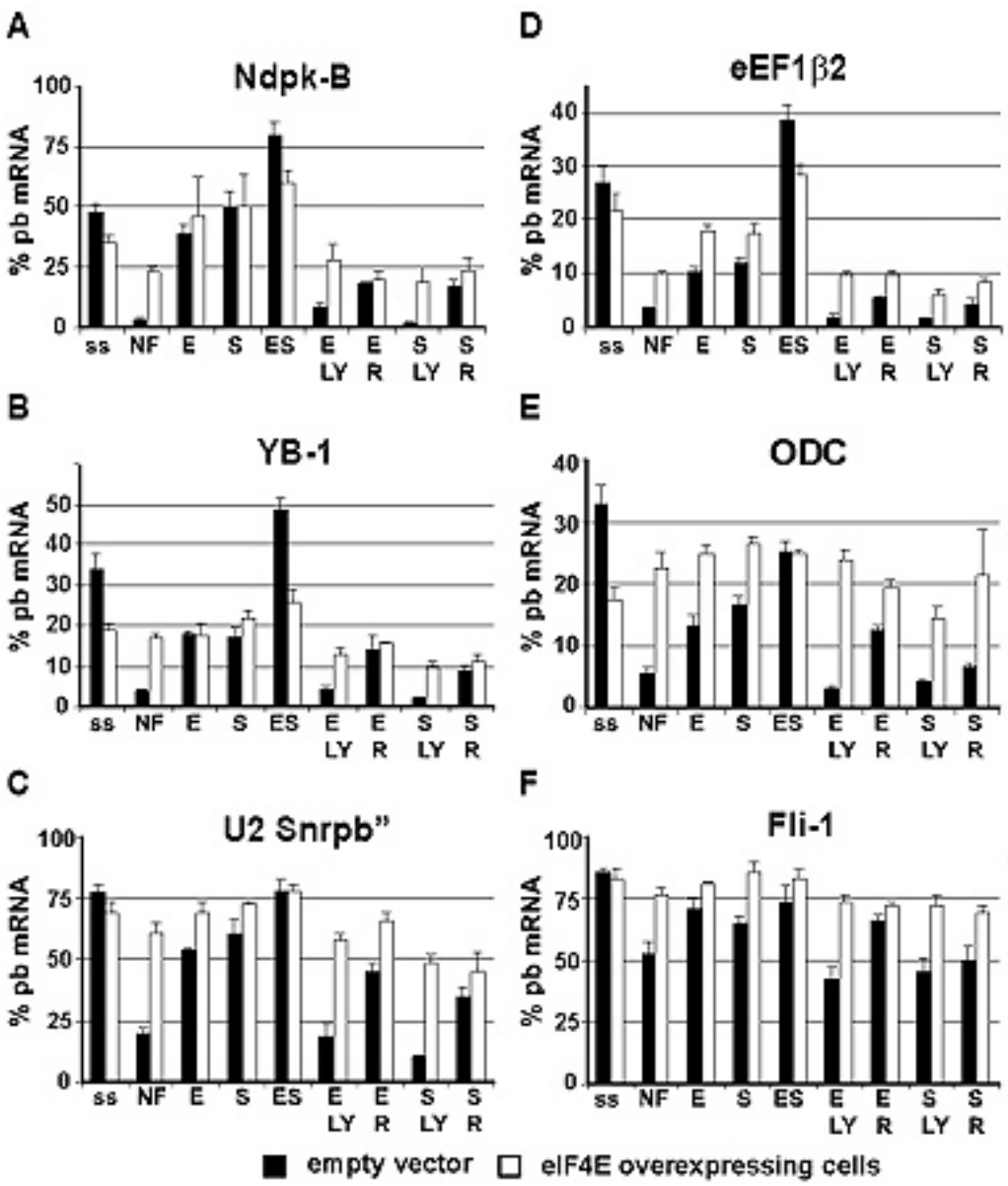

Figure 8 **eIF4E overexpression increases polysome association of structured mRNAs** in absence of growth factors or in presence of growth factors and the PI3K inhibitor LY294002. I/11 control cells (black bars) and eIF4E overexpressing cells (white bars) were factor deprived in absence or presence of LY294002 (LY; 10 μM) or rapamycin (R; 10nM) and restimulated for 2 h with Epo (E, 5U/ml), SCF (S, 100 ng/ml), Epo plus SCF (ES). In addition, cells were left untreated (NF) or were harvested from steady state proliferating cultures (ss). Free and polysome bound mRNA was isolated and assayed for the expression of (**A**) *NdpkB*, (**B**) *YB-1*, (**C**) *U2-Snrpb"*, (**D**) *eIF1β2*, (**E**) *ODC* and (**F**) Fli-1. The percentage of mRNA associated with polysomes (pb-mRNA) was calculated for the different genes under the different conditions.

expression of Epo- and SCF-upregulated genes was examined by quantitative RT-PCR in total, free and polysome-associated mRNA fractions (supplemental data). In addition to *Ndpk-B*, we found expression of the splicing factor *U2-Snrpb"* and the *Y-box binding protein-1* (*YB-1*) to be regulated by polysome association (supplemental data and Figure 8). In absence of growth factor, less than 10, 5 and 25% of *Ndpk-B*, *YB-1* and *U2-Snrpb"* mRNAs, respectively, were detected in the polysomal fractions. Upon stimulation with Epo and SCF, 75% of the *Ndpk-B*, 80% of the *U2-Snrpb"* and 50% of the *YB-1* messengers were found in the polysomal fractions and polysome association was dependent on PI3K activation (Figure 8A, B, C, black bars).

Whereas the 5'UTR of *Ndpk-B* starts with a TOP tract and contains an inverted repeat, the 5'UTR of *U2-Snrpb"* is short and contains a TOP sequence (AA146248), whereas the 5'UTR of *YB-1* is long, highly structured and lacks a TOP sequence (Fukuda et al. 2004; X57621). Thus, these three genes represent different types of mRNAs and may be a representative panel to study the pathways involved in gene-specific mRNA recruitment to polysomes. We examined the effect of eIF4E overexpression on factor-dependent polysome association of *Ndpk-B*, *U2-Snrpb"* and *YB-1* mRNA. Polysome recruitment of *Fli-1* mRNA was analyzed as a negative control to assess the general effect of LY294002 and rapamycin on polysome formation. Polysome association of *eukaryote elongation factor 1*β (*eEF1*β) and *ornithine decarboxylase* (*ODC*) was examined as a positive control. They contain a TOP-sequence and a highly structured 5'UTR, respectively. Enhanced eIF4E expression increased polysome association of *Ndpk-B*, *YB-1* and *U2-Snrpb"* mRNA in factor deprived cells and it rendered factor-induced polysome association resistant to the PI3K-inhibitor LY294002 (Figure 8, white bars). The polysome association of the mRNAs in Epo or SCF stimulated cells was not different between control and eIF4E overexpressing cells.

Both control mRNAs, *eEF1*β and *ODC*, known to be regulated by polysome association showed the same level of regulation as *Ndpk-B*, *YB-1* and *U2-Snrpb"*. In contrast, at least half of all *Fli-1* mRNA remained associated with polysomes independent of PI3K activity. Upon factor deprivation and inhibition of PI3K, 20 - 25% of the *Fli-1* mRNA was lost from polysomes. This can be considered the general effect of growth factor stimulation on mRNA translation initiation, which is much lower than the effect observed for structured mRNAs.

DISCUSSION

Control of expansion versus maturation of hematopoietic progenitors requires a tight regulation of the gene expression program. It becomes increasingly evident that the expression of genes critical for progenitor renewal and tumorigenesis is not only regulated at the level of gene transcription, but also by control of mRNA translation (De Benedetti and Graff 2004). Expansion of erythroid progenitors is critically dependent on SCF-induced PI3K activity. SCF-induced PI3K/PKB activity controls the level of eIF4E available for translation initiation and we show that overexpression of eIF4E in erythroid progenitors impairs their differentiation and enhances renewal divisions in absence of SCF. Notably, inhibition of PI3K did not lead to differentiation of eIF4E overexpressing erythroid progenitors as it does in control cells indicating that control of eIF4E is a pathway of major importance downstream of PI3K in expanding erythroid progenitors. We observed that either SCF-induced signal transduction or eIF4E overexpression resulted in a significant increase in the polysome recruitment of a specific set of mRNAs with a structured 5'UTR such as *Ndpk-B*, *YB-1* and *U2-Snrpb*". This supports the notion that the expression of not only ribosomal proteins and translation factors but also of other proteins with an important role in cellular metabolism and proliferation are controlled at the level of polysome recruitment. Therefore, we suggest that gene-specific recruitment of mRNAs into polysomes by SCF-induced PI3K/PKB activity may contribute to the control of expansion and maturation of erythroid progenitors.

Release of eIF4E is a major pathway downstream PI3K in erythroid progenitors

Overexpression of eIF4E specifically inhibits differentiation. Notably, induction of erythroid maturation does not require inhibition of cell cycle progression, but involves 3-4 cell divisions with loss of cell size control, resulting in 8-16 erythrocytes (4μm in mice) from a single proerythroblast (12μm) (Dolznig et al. 2001; von Lindern et al. 2001). In consequence, maintenance of cell size is crucial in renewal divisions, which requires a proper balance between protein synthesis and G1 progression through the restriction point in the cell cycle (Zetterberg et al. 1995; Dolznig et al. 2004). This indicates an important role for protein synthesis, and for a limiting factor in mRNA translation such as eIF4E in particular, in the control of expansion and differentiation of erythroid progenitors.

We previously showed that inhibition of PI3K abrogates the ability of erythroid progenitors to maintain a renewal program (von Lindern et al. 2001). Because overexpression of eIF4E rendered expansion of erythroid progenitors insensitive to the PI3K inhibitor LY294002, selective protein synthesis appears to be a major pathway downstream of SCF-induced progenitor expansion. However, we also demonstrated that SCF-induced activation of the PI3K/PKB pathway results in phosphorylation of Foxo3, which results in its cytoplasmic retention and inhibition of its transcriptional activity (Bakker et al. 2004). Among the Foxo3 targets are p27, $p130^{Rb2}$, Btg1 and cyclin G2 that all inhibit cell cycle progression (Bakker et al. 2004; Bakker submitted). These genes are upregulated when PI3K activity is inhibited in control cells. Yet, erythroid progenitors overexpressing eIF4E undergo normal renewal divisions in absence of PI3K activity. This seeming contradiction may

have two explanations. First, expression of Foxo-family members is low in erythroid progenitors and Foxo3 expression is markedly increased during differentiation, resulting in Foxo3 activity 48 h after induction of differentiation when cells arrest in G1. The low levels of Foxo3 protein in erythroid progenitors may result in insufficient expression of the cell cycle inhibitors to arrest cells when all other signals are activated. Second, preliminary analysis of translationally controlled genes suggests that increased eIF4E expression results in the enhanced expression of proteins that inhibit the cell cycle inhibitors such as kinase interacting with stathmin (Kis, unpublished data). Thus, control of eIF4E availability is a major pathway downstream of PI3K to maintain renewal divisions of erythroid progenitors. Upon downregulation of PI3K other pathways such as Foxo3 activation are activated to execute the differentiation program.

Constitutive eIF4E expression does not render erythroid progenitors independent of growth factors

It has been shown that the PI3K/PKB/mTOR pathway is required for the tumorigenic phenotype of various tumors and sufficient for oncogenic transformation of chicken embryo fibroblasts (De Benedetti and Harris 1999; Aoki et al. 2001; Avdulov et al. 2004). In breast tumor-derived cell lines and eIF4E-transformed fibroblasts, increased expression of eIF4E and phosphorylation of 4E-BP prevent apoptosis upon factor deprivation. Erythroid progenitors overexpressing eIF4E are not factor-independent for either renewal or differentiation and are not prevented from apoptosis in absence of growth factors. Moreover, eIF4E expression impairs differentiation, but is not able to sustain long-term renewal. Upon moderate overexpression the cells undergo delayed differentiation but high levels of eIF4E eventually induce erythroid progenitors to die under differentiation conditions. Whereas eIF4E availability may be the crucial pathway downstream of PI3K signaling, it is not sufficient for sustained expansion or differentiation of erythroid progenitors. The distinct effects of eIF4E overexpression in different cell types, i.e. control of apoptosis versus control of differentiation, is in accordance with the different function of PI3K and Foxo transcription factors in these cells. Whereas PI3K is mainly known as a survival factor in fibroblastoid cells (Franke et al. 1997), it controls the balance between expansion and differentiation of erythroid progenitors (Bakker et al. 2004; van den Akker et al. 2004). The tyrosine kinase receptor RON/Stk is a downstream target of the EpoR (van den Akker et al. 2004). Direct activation of RON, using a NGF-inducible TrkA-RON fusion induced phosphorylation of Gab1 and Gab2 and strong PI3K activation but it was not able to induce renewal divisions in absence of SCF, or differentiation in absence of Epo. Thus, expansion requires additional signaling pathways initiated by the SCF-receptor cKit and differentiation requires Epo-induced Stat5 activation (Dolznig et al. 2002; van den Akker et al. 2004). Whether the biological effect of eIF4E is rescue from apoptosis or impaired differentiation, in both cases it contributes to enhanced expansion of progenitors and to neoplastic transformation.

Increased eIF4E expression recruits specific RNA transcripts to polysomes

Although eIF4E is predominantly a translation initiation factor, overexpression of eIF4E in myeloid progenitors was suggested to contribute to leukemogenesis by increased nuclear export of cyclinD1 (Topisirovic et al. 2003). The proline-rich

homeodomain protein, PRH/Hex, disrupts eIF4E nuclear bodies and reduces export of D-cyclins (Topisirovic et al. 2003). We did observe nuclear bodies in control cells using antibodies against endogenous eIF4E, but upon overexpression of eIF4E these nuclear bodies disappeared. Interestingly, we found that PRH/Hex is upregulated in response to SCF. This involved both transcriptional and translational control as the 28bp long 5'UTR of the PRH transcript contains a TOP sequence (Grech, unpublished results). Possibly, the increased expression of PRH/Hex suppressed nuclear localization of eIF4E.

In consequence, delayed differentiation of erythroid progenitors upon overexpression of eIF4E must be due to the effects of eIF4E on mRNA translation. Increased availability of eIF4E allows more eIF4F, harboring the eIF4A helicase, to bind and scan structured mRNAs, i.e. to recruit the structured mRNAs into polysomes (Gingras et al. 1999b). Until recently, PI3K/PKB/mTOR-dependent polysome recruitment was mainly described for mRNAs containing a TOP sequence and encoding ribosomal proteins and translation factors (Meyuhas 2000; Stolovich et al. 2002). Few other structured mRNAs were shown to be regulated at the level of translation initiation such as *c-Myc* and *ornithine decarboxylase* (*ODC*; (De Benedetti and Graff 2004)). In this manuscript we describe PI3K and eIF4E-dependent polysome recruitment of *Ndpk-B*, *YB-1* and *U2-Snrpb"*. Notably, both *Ndpk-B* and *YB-1* are suggested to be involved in tumor progression (Postel 1998; Janz et al. 2002). *Ndpk-B* 5'-UTR contains a TOP-tract and an inverted repeat predicted to form a stem-loop structure (Joosten et al. 2004). Removal of the *Ndpk-B* 5'-UTR caused loss of *Ndpk-B* translational control. *YB-1* mRNA contains a highly structured 5'UTR and lacks a TOP tract, whereas *U2-Snrpb"* has a short 5'UTR starting with a TOP sequence. All three mRNAs as well as the controls *EF1*β (TOP mRNA) and *ODC* (structured mRNA) were hardly or not associated with polysomes in the absence of growth factors. Of unstructured mRNAs, such as Fli-1 mRNA, at least 50% of the mRNA remains associated with polysomes in absence of growth factors. Overexpression of eIF4E increased polysome association of *Ndpk-B*, *YB-1* and *U2-Snrpb"* in the absence of growth factors, but did not completely rescue polysome association compared to SCF-induced polysome association. This suggests that additional signaling still plays a role in mRNA translation, possibly by phosphorylation of proteins that stabilize structures in the mRNAs.

Besides increased recruitment of structured mRNAs to polysomes, increased expression of eIF4E may act via other mechanisms as well and such additional mechanisms may contribute to the observed phenotype of eIF4E overexpressing erythroid progenitors. In conjunction with other initiation factors (eIF2 and eIF3) eIF4E affects selection of the ATG-initiation codon. Under conditions of suboptimal growth factors, protein synthesis will start at the first ATG start codon that is embedded in an optimal Kozak sequence (Kozak 1989). However, when translation initiation factors are abundantly available, less optimal ATG codons can be selected to start protein synthesis while it concurrently enables initiation factors to continue mRNA scanning beyond a first open reading frame (ORF) to re-initiate at the next ATG. Short ORFs in the 5'UTR can serve to attenuate translation of the functional ORF (e.g. thrombopoietin; Ghilardi et al. 1998), but upstream ORFs can also affect the choice of the start codon for the functional protein and thereby the translation of antagonistic proteins from the same mRNA (e.g. cEBPβ, SCL; Calkhoven et al. 2000; Calkhoven et al. 2003).

MATERIALS AND METHODS

Cells

l/11 cells were cultivated in StemPro-34™ medium (Life Technologies) as described (von Lindern et al. 2001). For expansion, the medium was supplemented with 0.5U/ml Epo, (kind gift from Ortho-Biotech, Tilburg, The Netherlands), 100ng/ml SCF (supernatant of CHO producer cells) and 10^{-6}M dexametasone (Dex, Sigma-Aldrich). To induce differentiation, cells were cultivated in StemPro-34™ medium supplemented with 5U/ml Epo and 0.5mg/ml iron-loaded transferring (Intergene). Cell numbers and cell size distribution were determined using an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany). LY294002 and rapamycin were obtained from Alexis (Schwitzerland).

Hemoglobin content determination and cell morphology

Small aliquots of the cultures were removed and analyzed for hemoglobin content by photometry as described earlier (Kowenz et al. 1987; Bakker et al. 2004). The values obtained were the average of triplicate measurements after normalization for cell number and mean single cell volume. Cell morphology was analyzed in cytospins stained with histological dyes and neutral benzidine (Beug et al. 1982), using an OlympusBx40 microscope (40x objective, NA 0.65), an OlympusDp50 CCD camera and Viewfinder Lite 1.0 acquisition software. Images were cropped using Adobe photoshop 6.0.

SDS-PAGE, western blotting, immunoprecipitation and antibodies

For acute stimulation with growth factors, proliferating l/11 cells were washed twice with phosphate-buffered saline (PBS) and seeded at 4 x 10^6 cells/ml in plain Iscove's modified Dulbecco's medium (IMDM, Life Technology). After 4 h factor deprivation, cells were stimulated at 37°C with SCF (100ng/ml) or Epo (5U/ml). Cells were harvested after the indicated time points by addition of ice-cold PBS.

Cell lysates, SDS-PAGE, immunoprecipitation and Western blotting were performed as described previously (van Dijk et al. 2000). To analyze eIF4E and 4E-BP, 10 μl of protein extract (≈1x10^6 cells) was loaded onto a 15% polyacrylamide gel. The antibodies used were: α-eIF4E, α-4E-BP1, α-Phospho-4E-BP1(Thr37/46), α-Phospho-4E-BP1(Ser65) (Cell Signaling Technology, Inc.), α-ERK1(K-23) and α- Myc (Santa Cruz Biotechnology, Inc.). For immunoprecipitation, Myc-eIF4E was immunoprecipitated from the lysate of 15x10^6 cells by an overnight incubation at 4°C with the monoclonal α-myc antibodies (1 μg antibody/15x10^6 cells), followed by an hour incubation at 4°C with 15 μl of a 50% solution of protein G-Sepharose beads (Pharmacia LKB).

m^7GTP-Sepharose affinity chromatography

For the isolation of eIF4E and associated proteins, 15x10^6 cells were lysed in buffer C (50mM MOPS/KOH (pH7,2); 0.5mM EDTA; 0.5mM EGTA; 100mM KCl; 14mM 2-mercaptoethanol; 50mM NaF; 100mM GTP and protease inhibitor cocktail) and subjected to m^7GTP-Sepharose chromatography as described previously

(Morley and Pain 1995). Briefly, the lysed cells were incubated at 4°C with 25ml of equilibrated sepharose resin (7-Methyl GTP-Separose 4B; Amersham Biosciences) for 1 hour. The resin was washed three times with buffer C and recovered proteins eluted directly into sample buffer for SDS/PAGE analysis.

Generation of myc-eIF4E expressing l/11 clones

The *myc*-eIF4E cDNA (NCBI accession number M61731) containing six *myc*-tags at the 5' start of the coding sequence was isolated from the pCS3MT vector and inserted into the eukaryotic retroviral expression vector pBabe using *EcoRI* and *BamHI* restriction sites. Retroviral transduction was performed as described (Bakker et al. 2004). Briefly, $0.5x10^6$ ecotropic Phoenix cells were transfected with 12 μg plasmid DNA (myc-eIF4E-pBabe) using calcium-phosphate coprecipitation assay. After 40 h, cells were treated with 10μg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) for 1 h and washed 3 times with PBS. $2x10^6$ l/11 cells were added in 4ml StemPro-34™ medium supplemented with Epo, SCF and Dex and co-cultured for 24 h. Subsequently, l/11 cells were removed from the Phoenix cells and cultured in semisolid medium (Methocel-containing StemPro-34TM, supplemented with factors) containing 2 μg/ml puromycin (Sigma). After 7 days well-separated colonies were picked, expanded and analyzed for myc-eIF4E expression.

Immunofluorescence microscopy

Cells (eIF4E overexpressing and empty vector clones) were spun onto a microscope slide. The cells were fixed in 4% paraformaldehyde and permeabilized for 30 min with 0.2% TritonX. After blocking for 1h in PBS containing 1% BSA and 0.05% Tween, the fixed cells were incubated for 1 h at *rt* with anti-eIF4E antibody (Cell Signaling Technology; #9742), and with anti-myc-antibodies (Santa Cruz; 9E10) for the eIF4E-overexpressing cells. The slides were washed and incubated for 1h at *rt* with FITC anti-rabbit or TRITC anti-mouse secondary antibody (DakoCytomation), respectively. Cover slips were mounted with a drop Vector Shield (Vector laboratories Inc; H-1000), including DAPI (0.3ng/μl). Imaging of the cells was done with 543nm, 488nm, 405nm excitation provided by an argon laser and a 63 x 1.4 NA apochromat objective lense (Carl Zeiss MicroImaging) for FITC, TRITC and DAPA respectively. Zeiss AIM software version 3.2 was used for merging the images.

Flow cytometry

To distinguish between live and dead cells, the DNA content was determined. 0.5 to $1.0x10^6$ cells were fixed and permeabilized 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 iodide (50mg/ml in PBS) and fluorescence was measured by flow cytometry.

RNA isolation and cDNA synthesis

Total RNA was isolated using the Trizol reagent (Life Technologies) as recommended in the manufacturer's protocol. Isolation of polysomal RNA by sucrose gradient fractionation was performed as described (Mullner 1997; Joosten et al. 2004). Cell extracts were layered on a 4ml linear sucrose gradient (15–40% sucrose [w/v]) and 8 fractions were collected. Northern blotting indicated that fractions 1-4 contain nonpolysomal and subpolysomal mRNA, while fractions 5-8 consisted of polysome-bound RNA. These fractions were pooled to generate subpolysomal and polysomal mRNA of each sample. RNA was quantified by UV-absorbance. Poly(A)$^+$ mRNA was purified and cDNA was generated as described (Joosten et al. 2004)

Real-time PCR

The real-time PCR assay involved TaqMan technology (PE Applied Biosystems Model 7700 sequence detector), using the double stranded DNA-specific fluorescence dye SYBR green I to detect PCR product as previously described (Kolbus et al. 2003). The amplification program consisted of 1 cycle of 50°C with 2' hold (AmpErase UNG incubation), 1 cycle of 95°C with 10' hold (AmpliTaq Gold Activation), followed by 40 cycles of denaturation at 95°C for 15", annealing at 62°C for 30" and extension at 72°C for 30". All the different primer pairs had similar optimal PCR annealing temperatures. Acquisition of the fluorescence signal from the samples was carried out at the end of the elongation step. To confirm amplification specificity, the PCR products from each primer pair were subjected to agarose gel electrophoresis and the dissociation curve was checked at the end of each run. Gene-specific primers corresponding to nucleoside diphosphate kinase B (Ndpk-B, X68193), eukaryotic translation elongation factor eEF-1β2 (MGC:6763), eukaryotic translation initiation factor eIF-4E (M61731), Fli-1 (X59421), ornithine decarboxylase (ODC, M12330), mammalian ribonuclease inhibitor (IMAGE:1366946), U2 splicing factor Snrpb" (AA146248) and Y-box binding protein 1 (YB-1, X57621) were obtained from Invitrogen Life Technologies or Sigma-Genosys Ltd. The sequences of the primers used for the amplification are listed in Table I.

Table I. Primer sequences used for real-time PCR amplification.

Name	Forward Primer Sequence	Reverse Primer Sequence
Ndpk-B	5'ATG GGA TTC GGA GAC CTG AA3'	5'TCA GCA GGT GGT GGA CCA GA3'
eEF-1b2	5'ATG GGA TTC GGA GAC CTG AA3'	5'TCA GCA GGT GGT GGA CCA GA3'
eIF4E	5'TCT AAT CAG GAG GTT GCT AAC3'	5'TAG ACA ACT GGA TAT GGT TGTA3'
Fli-1	5'TGC AGC CAC ATC CAA CAG AG3'	5'TGA AGG CAC GTG GGT GTT AG3'
ODC	5'TG ACG TCA TTG GTG TGA GC3'	5'TAT CAA GCA GAT GCA TGC TGT3'
RI	5'TCC AGT GTG AGC AGC TGA G3'	5'TGC AGG CAC TGA AGC ACC A3'
U2 Snrpb"	5'TCA GTT TGG ACA CGT GGT AG3'	5'TCC TTG TCA GCG AAA GTA CCA3'
YB-1	5'TGC AGG AGA GCA AGG TAG AC3'	5'TGG TGG ATC GGC TGC TTT TG3'

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SCF-dependent polysome recruitment of specific transcripts controls the balance between renewal and differentiation in erythroid progenitors.

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Short title: Igbp1/α4 inhibits erythroid differentiation

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CHAPTER 3

ABSTRACT

SCF-induced activation of phosphoinositide-3-kinase is required for erythroid progenitors to undergo renewal. The activation of mTOR (Target of Rapamycin) and subsequent release of the cap-binding translation initiation factor eIF4E plays an important role in the regulation of proliferation versus maturation of erythroid progenitors. To study the contribution of selective polysome recruitment of mRNAs, we compared SCF-dependent gene expression between total and polysome bound mRNA. This yielded a list of 115 genes subject to major regulation by translation initiation. Detailed analysis showed that SCF-induced polysome recruitment exceeded 5-fold regulation, and was PI3K-dependent and eIF4F-sensitive for 9 out of 10 genes. One of the targets, Igbp1/α4, binds and inhibits the serine/threonine phosphatase Pp2a that functions as an mTOR antagonist in translation initiation. Constitutive expression of Igbp1/α4 strongly impaired erythroid differentiation, maintained 4EBP and p70Sk phosphorylation and enhanced polysome recruitment of structured mRNAs. This showed that PI3K-dependent polysome recruitment of Igbp1/α4 acts as a strong positive feedback mechanism on translation initiation and underscores the important regulatory role of selective polysome recruitment.

INTRODUCTION

The balance between expansion and differentiation of hematopoietic progenitor cells is controlled by cytokines and growth factors. In erythropoiesis, stem cell factor (SCF), the ligand for cKit, cooperates with glucocorticoids and erythropoietin (Epo) to suppress differentiation and sustain renewal divisions of erythroid progenitors *in vitro* (Dolznig et al., 2001; von Lindern et al., 2001) as well as *in vivo* in response to hypoxia (Bauer et al., 1999; Broudy et al., 1996). Activation of PI3K (phosphotidylinositol-3 kinase) is required for proliferation of erythroid progenitors (Haseyama et al., 1999; Klingmuller et al., 1997; Nishigaki et al., 2000; Sui et al., 2000; von Lindern et al., 2001). Activation of PI3K generates PIP3, which serves as an anchor for PH-domain containing proteins, of which protein kinase B (PKB) is a major PI3K effector. Although both Epo and SCF induce activation of PI3K in erythroid progenitors, the efficiency with which downstream signalling pathways are activated shows large differences (Bakker et al., 2004; Blazquez-Domingo et al., 2005). In *in vitro* cultured erythroid progenitors the activation of PKB is much more responsive to SCF compared to Epo (von Lindern et al., 2001, Bakker et al., 2004). PKB activates mTOR (mammalian target of rapamycin, also named Frap1) through phosphorylation of the tumour suppressor complex Tsc1/Tsc2 (tuberous sclerosis protein), which releases Rheb (RAS-homolog enriched in brain) to phosphorylate mTOR (Inoki et al., 2003; Tee et al., 2003). Activation of mTOR results in phosphorylation and activation of p70S6 kinase (Rps6kb1; Dufner and Thomas, 1999), and hierarchical phosphorylation of 4EBP (4E-Binding Protein; Gingras et al., 1999a) resulting in release of the mRNA cap-binding factor eIF4E (eukaryotic Initiation Factor 4E; Gingras et al., 2001). Subsequently, eIF4E can bind the scaffold protein eIF4G to the site otherwise occupied by 4EBP, which enables the formation of a eIF4F scanning complex containing eIF4E, eIF4G and the RNA helicase eIF4A (Gingras et al., 1999b). eIF4F associates with several other translation factors and the small subunit of the ribosome. This complex scans the 5'UTR for the first AUG codon in an appropriate sequence context (Kozak, 2005), where the complete ribosome recruits methionine-tRNA and all translation factors required for protein synthesis (Pestova et al., 2001). The cap-binding eIF4E protein is the rate-limiting factor in the scanning process (Duncan et al., 1987; Sonenberg and Gingras, 1998) and therefore its release upon phosphorylation of 4EBP is a crucial control mechanism in polysome recruitment of mRNAs. Importantly, transcripts with a short and simple 5'UTR show a limited sensitivity to 4EBP phosphorylation, whereas transcripts with a long and structured 5'UTR or with a terminal oligopyrimidine tract (TOP) are highly sensitive to the concentration of eIF4F complexes in the cell (Jefferies et al., 1997; Koromilas et al., 1992; Kozak, 2005).

The mechanism by which mTOR controls 4EBP phosphorylation may entail both regulation of a 4EBP-kinase and of the serine/threonine phosphatase Pp2a (Di Como and Arndt, 1996; Inui et al., 1998; Murata et al., 1997; Wang et al., 2005). Pp2a is the main phosphatase acting on p70S6K and 4EBP1, thereby suppressing translation initiation. Pp2a exists in various complexes that shift target specificity depending on the binding of regulatory components. mTOR modulates the formation of the Pp2a-α4 complex sequestrating the phosphatase activity away from its own downstream targets 4EBP and p70S6 kinase (Inui et al., 1998; Kong et al., 2004).

We showed previously that 4EBP is rapidly and strongly phosphorylated by SCF (Blazquez-Domingo et al., 2005), and identified transcripts that require SCF-induced PI3K/mTOR activation to be recruited to polysomes, while their transcript levels in the cells are unchanged (Blazquez-Domingo et al., 2005). The 5' untranslated regions (UTR) of *Nm23*, *Ybx1* and *Snrpb"* include stable hairpin loops, long complex sequences and TOP structures respectively, structures that impose gene expression control at the translation level.

Overexpression of eIF4E increased the levels of eIF4F complexes and suppressed erythroid differentiation in the absence of SCF, indicating an important role for mechanisms that control eIF4F formation in regulating the balance between expansion and differentiation in erythropoiesis (Blazquez-Domingo et al., 2005). This is in line with reports stating that increased expression of eIF4E in tumour samples is associated with increased malignancy (Bader and Vogt, 2004; De Benedetti and Graff, 2004; De Benedetti and Harris, 1999; Topisirovic et al., 2003). It suggests a major role for selective translation of specific mRNAs, but an overview of mRNAs subject to factor-dependent polysome recruitment is missing.

In this study we compared factor-dependent mRNA expression in total and polysome bound mRNA and identified a list of 115 transcripts that require PI3K or increased eIF4F levels for polysome recruitment. From these we selected 9 genes suspected to be involved in signal transduction or gene expression and analysed their expression regulation and biological function in erythroid progenitors. Except for one gene, Grwd1, that was regulated by gene transcription and mRNA translation, the selected genes were strictly regulated by SCF-induced activation of PI3K and eIF4F. Strikingly, we identified the Pp2a-associated protein Igbp1/α4 as a target of SCF-dependent polysome recruitment. Constitutive expression of Igbp1/α4 in erythroid progenitors enhanced the mTOR-dependent phosphorylation of S6K and 4EBP, releasing eIF4E. This resulted in impaired differentiation of erythroid progenitors, and enhanced polysome recruitment of other targets identified in this screen.

RESULTS

Transcripts dependent on Epo/SCF-induced polysome recruitment

To identify mRNA transcripts that are selectively recruited to polysomes upon growth factor stimulation of erythroid progenitors, we compared factor-induced gene expression at the level of total and polysome bound (pb) RNA using mRNA profiling. Erythroid progenitors were factor deprived (4h) and restimulated (2h) with Epo plus SCF, or left unstimulated. Four biologically independent replicates were prepared using two established *p53*$^{-/-}$ erythroblast cultures with different genetic background (I/11 and R10; Schmidt et al., 2004; von Lindern et al., 2001). Total and pb RNA were isolated twice from each culture, cRNA was generated and hybridized to Affymetrix oligonucleotide arrays. Rosetta Resolver software was used to normalize and analyse the intensity data. The ratio of gene expression in Epo/SCF stimulated samples (ES) versus factor-deprived cells (NF) was calculated for total and pb mRNA by Rosetta Resolver software. To identify genes differentially regulated in total versus polysome bound RNA, we applied a two step selection (for strategy see supplemental Figure S1). First, the variance between the ES/NF ratio's for total and pb RNA was derived by 'Analysis of Variance' (ANOVA; p=0.01). Second, selected

genes had to be differentially expressed in presence or absence of Epo/SCF in total or polysome bound mRNA in both I/11 or both R10 hybridisations ($p=0{,}001^2$) This resulted in 115 probe sets, representing 111 transcripts, subject to Epo/SCF-controlled polysome recruitment (supplementary Table S-II). To relate differential polysome recruitment to gene expression during differentiation, we derived pb mRNA from steady state expanding and differentiating progenitors (48 and 60 h following differentiation induction). Following hybridization of oligonucleotide arrays we used Rosetta Resolver to calculate the gene expression ratio of differentiation over steady state renewal. Subsequent cluster analysis of the gene expression ratio's resulted in a matrix that groups the selected probe sets into 5 separate clusters (Figure 1; supplementary Table S-II).

To gain insight into regulation of gene expression in the various clusters, we plotted the normalized intensity data obtained from the array hybridisation with total and pb RNA, from factor-deprived and Epo/SCF-restimulated cells (weighted average of the 4 hybridisations; Supplementary Figure S2). Genes in cluster 1, 3 and 5 are subject to Epo/SCF-enhanced polysome recruitment, and decreased expression during differentiation. Cluster 1 represents constitutive expression in total RNA and Epo/SCF-dependent polysome recruitment, cluster 3 represents factor-dependent increased transcript level, but a much larger stronger increase of Epo/SCF-induced polysome recruitment, cluster 5 represents Epo/SCF-induced repression of expression in total mRNA with maintained, constant levels in pb RNA, which, in effect, indicates increased Epo/SCF-induced polysome recruitment from a smaller transcript pool. In contrast, genes present in cluster 2 and 4 are subject to enhanced polysome recruitment following factor deprivation, which is abrogated by Epo/SCF restimulation (supplementary Figure S2). Only genes represented in cluster 4 are upregulated during differentiation.

Polysome recruitment of selected transcripts depends on the PI3K/mTOR/eIF4E pathway

Next, 9 genes were selected that (*i*) require Epo/SCF for polysome recruitment, (*ii*) are downregulated in differentiation, and (*iii*) are suggested to function in signal transduction or gene expression (Table I). Quantitative RT-PCR (Q-PCR) was used to analyse whether polysome recruitment was always dependent on PI3K and mTOR, and on eIF4E expression.

First, we examined how signalling-dependent expression in total and pb RNA correlates between array-data and Q-PCR. We determined transcript levels in subpolysomal and pb RNA by Q-PCR and calculated the percentage polysome recruitment. *Nm23-M2* and *Ybx1* were tested as positive controls. A control gene, *Fli-1* (Blazquez-Domingo et al., 2005), that is not regulated at the level of RNA-specific polysome recruitment, showed at most 2-fold difference in polysome recruitment in response to Epo or SCF (Table I, Figure 2C,F). In contrast, 8 out of 9 of the selected genes showed at least a 5-fold increase in polysome recruitment in response to Epo or SCF (Table I, Figure 2A,B,D,E). As shown previously for *Nm23-M2* and *Ybx1*, SCF-induced increase in polysome recruitment exceeded Epo-induced poysome recruitment (Joosten et al., 2004). Cluster 1 genes (*Igbp1, mEd2, Rnf138, Nap1l1, Cnih, Nubp1*) were almost exclusively upregulated in pb but not in total mRNA; cluster 5 genes (*Uhmk1, Hnrpa1*) were downregulated in total mRNA in accordance with the

array data (Table I). In presence of the PI3K-inhibitor LY294002 or the mTOR inhibitor rapamycin, polysome recruitment of the control gene Fli-1 was approximately 2-fold decreased, but all genes that require Epo/SCF for polysome recruitment, dissociate from polysomes in presence of PI3K and mTOR inhibitors (Table I; Figure 2D-F).

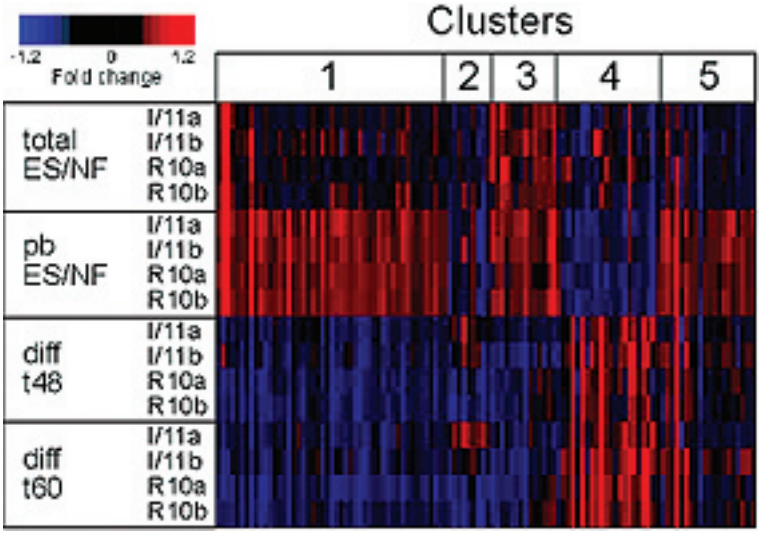
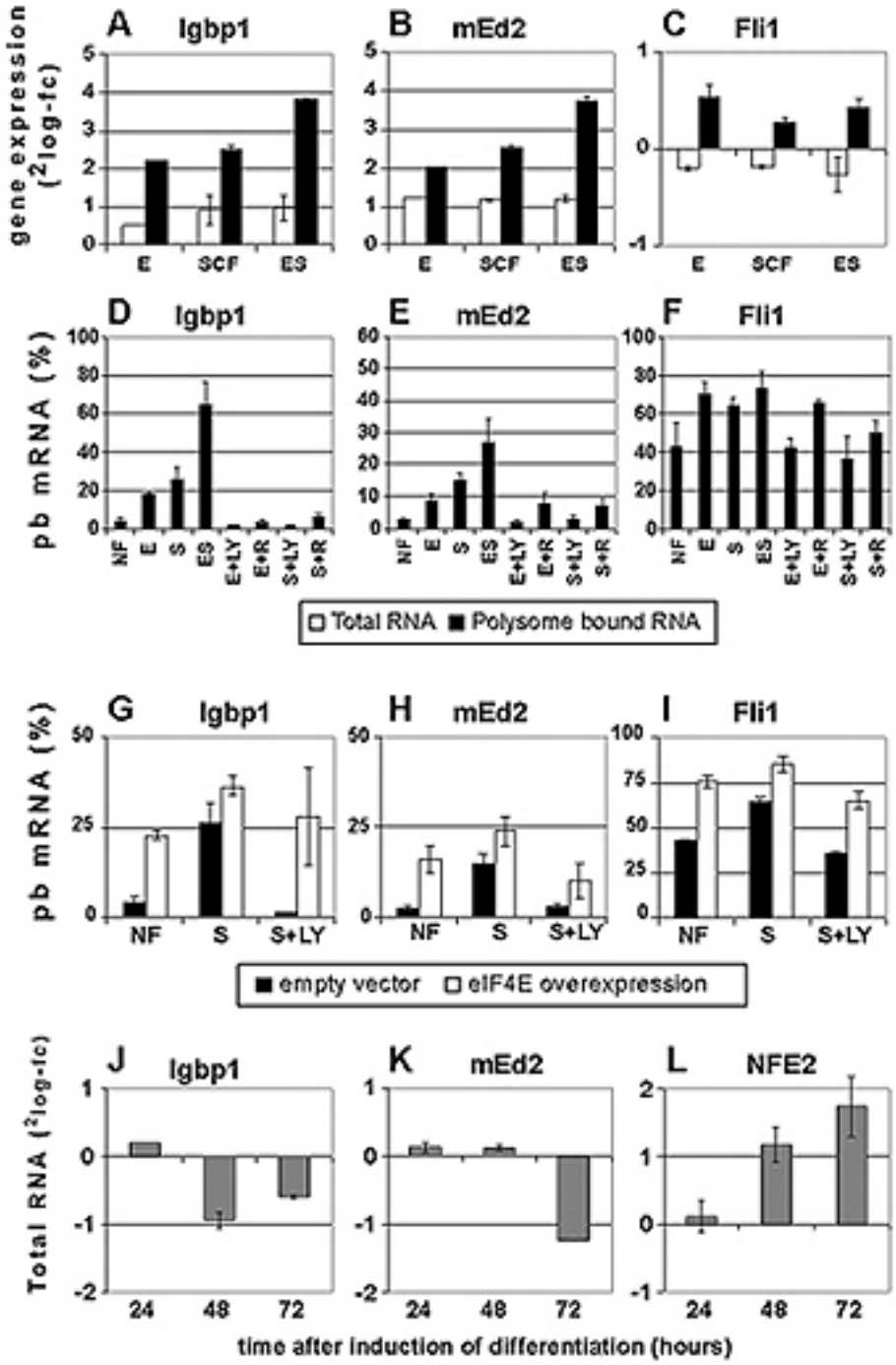


Figure 1 **Cluster analysis of genes subject to regulation by Epo/SCF-controlled mRNA polysome recruitment and their regulation during differentiation**. I/11 cells and R10 cells were factor deprived for 4h and stimulated with Epo plus SCF (ES), or left untreated (NF) for 2h. Total RNA and polysome bound (pb) mRNA was isolated and used for RNA profiling on MG_U74Av2 Affymetrix oligonucleotide arrays. For each of two biologically independent experiments (I/11a and I/11b; R10a and R10b) the ES/NF ratio of intensity data was calculated. Significant variance between ES/NF ratios obtained with total and pb RNA was calculated using ANOVA (pvalue 0.01), and differential expression in at least 2 single experiments had to be significant ($p=0.001^2$). This yielded the 115 genes plotted in this heat map. In two independent experiments I/11 and R10 cells were differentiated for 48h and 60h to generate pb RNA that was profiled on the same Affymetrix oligonucleotide arrays. Expression ratios were clustered using Rosetta software and Pearson correlation. A bar in the left upper corner indicates how up- and down-regulation correlate with the intensity of red and blue respectively on a ^{10}log-scale.

Figure 2 **Polysome recruitment of *Igbp1* and *mEd2* is PI3K and eIF4E sensitive**. I/11 cells were factor-deprived for 4h and subsequently restimulated with Epo (E, 2U/ml), SCF (S, 100 ng/ml) or both for 2h. Total and pb mRNA were isolated and relative amounts of *Igbp1* (**A**), *mEd2* (**B**) and *Fli1* (**C**) mRNA measured by semi-quantitative RT-PCR. Expression ratio's in restimulated versus factor deprived cells is calculated and shown in panels **A-C**. In addition, free and polysome bound mRNA were isolated from I/11 cells that were factor deprived and restimulated in absence or presence of LY294002 (LY; 10 μM) or rapamycin (R; 10nM) and restimulated for 2 h with Epo (E, 2U/ml) or SCF (S, 100 ng/ml). The percentage of mRNA associated with polysomes (pb-mRNA) was calculated for the different genes under the different conditions (**D-F**). I/11 cells transduced with an empty control vector (black bars) or with an eIF4E expression vector (eIF4E overexpressing cells; white bars) were factor deprived in absence or presence of LY294002 (LY; 10 μM) and restimulated for 2 h with SCF (S, 100 ng/ml). In addition, cells were left untreated (NF). Free and polysome bound mRNA was isolated and assayed for the expression of (**G**) *Igbp1* (**H**) *mEd2*, and (**I**) *Fli-1*. The percentage of mRNA associated with polysomes (pb-mRNA) was calculated for the different genes under the different conditions. I11 cells were induced to differentiate and total RNA isolated from time point 0hours, 24hours (t24), 48hours (t48) and 72 hours (t72). The relative amount to time point 0 is plotted (**J-L**; fold change 2 log value). The error bars represent 3 replicates each from 2 different RNA batches.


A Igbp1
B mEd2
C Fli1
gene expression (^{2}log-fc)
E
SCF
ES
D Igbp1
E mEd2
F Fli1
pb mRNA (%)
NF
E
S
ES
E+LY
E+R
S+LY
S+R
Total RNA
Polysome bound RNA
G Igbp1
H mEd2
I Fli1
pb mRNA (%)
NF
S
S+LY
empty vector
eIF4E overexpression
J Igbp1
K mEd2
L NFE2
Total RNA (^{2}log-fc)
24
48
72
time after induction of differentiation (hours)

		ANOVA selected - enhanced polysome recruitment upon ES stimulation																		Controls					
		PI3K dependent																PI3K indepen		positive				negative	
		overexpressed						no clones																	
		phenotype				no phenotype										no protein									
		Igbp1		mED2		Hnrpa1		Rnf138		Nap1l1		kist		Cnih		Nubp1		Grwd		Nm23		Yb1		Fli1	
Empty vector	ss	50.64	6.45	30.15	6.29	41.02	9.64	26.57	9.18	43.28	7.81	47.41	1.63	15.97	3.83	31.84	1.21	79.31	8.19	47.53	3.38	34.06	3.91	85.31	2.02
	NF	4.10	1.54	2.71	0.59	6.73	1.43	3.15	2.10	2.56	1.43	10.20	2.35	0.97	0.24	5.85	1.59	45.52	2.83	3.07	0.86	3.77	0.48	42.97	11.78
	E	17.97	1.32	8.73	2.23	16.31	0.22	37.30	7.00	14.15	5.96	14.51	1.11	7.50	2.76	29.93	7.91	44.54	8.45	38.94	3.43	17.97	0.76	70.68	5.30
	S	26.13	5.69	15.05	2.41	33.14	3.09	40.57	12.51	35.34	6.18	36.53	2.88	8.11	0.19	28.95	4.00	76.46	8.03	49.96	6.31	17.17	2.11	64.43	3.81
	ES	64.77	12.29	26.74	7.76	61.21	7.62	54.05	22.85	49.70	23.32	38.43	1.09	19.23	4.87	44.83	11.42	75.35	4.85	79.31	6.20	48.81	3.07	72.86	9.07
	S+LY	1.55	0.11	3.14	0.65	2.26	2.75	N.D.		10.79	5.91	11.67	3.03	N.D.		N.D.		49.14	5.71	1.97	0.43	2.09	0.31	36.30	11.65
	S+rap	5.93	1.99	7.38	2.56	13.55	0.45	34.64	22.93	24.44	18.26	18.90	5.77	N.D.		12.00	3.94	58.96	1.87	16.78	2.68	8.84	1.27	50.15	6.02
	E+LY	1.66	0.24	1.82	0.75	3.53	0.24	N.D.		3.82	4.52	4.59	1.63	0.56	0.12	26.14	0.49	22.91	2.96	8.44	1.44	4.36	0.86	42.30	5.61
	E+rap	3.64	1.24	7.80	3.36	16.42	0.62	5.56	3.37	16.18	5.55	15.66	4.93	7.87	2.75	18.23	7.81	65.45	2.28	18.14	0.65	14.24	3.28	65.61	2.39
eIF4E overexpression	NF	22.80	1.32	16.07	3.80	30.32	4.31	28.81	7.28	22.73	5.87	72.14	1.06	25.89	8.99	17.64	2.30	64.07	11.60	20.78	2.23	16.95	1.49	75.67	3.51
	S	36.53	2.88	23.93	4.19	59.73	8.01	50.86	2.10	55.03	12.56	60.72	3.48	47.41	7.49	63.54	0.52	92.22	14.84	53.54	15.34	21.75	2.22	85.09	5.24
	S+LY	28.04	13.62	10.07	4.94	32.19	5.68	12.59	6.31	12.48	0.24	60.98	1.73	17.41	14.77	38.06	9.44	79.38	5.63	20.28	8.12	9.54	1.71	65.10	8.84
Total RNA norm to NF	E	1.59	0.45	2.36	0.05	0.45	0.22	2.78	0.31	1.09	0.03	1.23	0.07	1.23	0.40	2.60	0.24	7.00	1.68	1.48	0.30	1.10	0.23	0.71	0.14
	S	2.06	0.49	2.26	0.13	0.56	0.03	2.20	0.32	1.27	0.11	1.31	0.06	1.31	0.30	2.52	0.32	5.75	0.11	1.29	0.10	1.31	0.13	0.81	0.07
	ES	2.19	0.64	2.31	0.15	0.49	0.02	2.46	0.01	1.78	0.18	1.86	0.07	1.94	0.11	3.15	0.43	7.23	0.34	2.08	0.06	1.44	0.17	0.80	0.10
pb RNA norm to NF	E	4.70	0.36	4.04	0.15	2.62	0.54	3.04	0.36	6.97	2.10	4.59	0.32	4.95	1.03	3.56	0.90	1.01	0.02	8.08	1.10	5.01	0.46	1.45	0.13
	S	5.69	0.10	5.80	0.52	5.11	0.61	9.12	0.88	12.03	5.10	6.04	0.24	5.98	1.72	6.44	1.36	1.76	0.05	11.53	0.66	5.05	0.23	1.21	0.04
	ES	14.89	2.56	13.47	1.53	9.74	2.40	21.54	1.14	9.21	2.50	17.27	0.02	17.32	2.34	16.19	2.73	1.71	0.04	14.49	3.20	11.68	0.33	1.34	0.09
Total RNA norm to ss	diff t24	1.16	0.12	1.09	0.06	1.13	0.13	3.35	0.09	0.51	0.08	1.33	0.13	1.33	0.02	0.64	0.27	1.66	0.15	0.77	0.13	0.79	0.23	1.20	0.04
	diff t48	0.53	0.03	1.08	0.03	0.94	0.10	1.67	0.06	0.51	0.10	1.30	0.24	0.81	0.05	0.37	0.20	0.77	0.10	0.51	0.02	0.50	0.09	1.12	0.08
	diff t72	0.66	0.01	0.43	0.01	0.52	0.06	0.55	0.02	0.34	0.16	0.38	0.14	0.74	0.01	0.39	0.01	0.27	0.02	0.36	0.10	0.32	0.06	1.36	0.07

Table 1. Percent polysome recruitment (right column gives standard deviation for triplicates of two independent cDNAs) measured in response to growth factor addition and the dependence on PI3K and mTOR pathway, and on eIF4E expression. Results obtained using Quantitative RT-PCR (Q-PCR). The various conditions tested are abbreviated as described in Figure 2.

Because the PI3K/mTOR pathway controls phosphorylation of 4EBP, which releases eIF4E and increases the availability of the eIF4F scanning complex, overexpression of eIF4E is expected to render the selected transcripts less sensitive to Epo/SCF-induced PI3K activity (Blazquez-Domingo et al., 2005). Indeed, PI3K-dependent polysome recruitment of all transcripts became constitutive in cells overexpressing eIF4E (Table I, Figure 2G,H). The effect on the *Fli-1* control is again 2-fold maximum (Figure 2I).

We previously showed that PI3K activity is required for the expansion of erythroblast cultures, and overexpression of eIF4E impaired differentiation (Blazquez-Domingo et al., 2005; von Lindern et al., 2001). Therefore, proteins whose expression is controlled by PI3K-dependent mRNA polysome recruitment may be mainly required during erythroblast proliferation. Pb mRNA was isolated from I/11 cells induced to differentiate and Q-PCR showed that the selected genes were all downregulated during differentiation (Table I, Figure 2J,K). The erythroid transcription factor *Nfe2* is known to be upregulated during differentiation and is shown as a control (Figure 2L).

Epo/SCF-dependent protein expression of Igbp1 and Uhmk1

To examine whether protein expression of selected genes is faithfully represented by polysome recruitment, we analysed protein expression of Igbp1 and Uhmk1 on Western blot and compared factor-dependent expression with Fli-1. To verify that expression of Igbp1 and Uhmk1 is primarily controlled at the level of mRNA translation, we analysed protein expression during steady state proliferation, following inhibition of gene transcription by Actinomycin D (ActD) and upon inhibition of mRNA translation by cycloheximide (CHX). In contrast to Fli-1, expression of Igbp1 and Uhmk1 was more sensitive to inhibition of translation compared to inhibition of transcription (Figure 3A). Factor-deprivation decreased Igbp1, but not Uhmk1 protein levels (Figure 3B), which is in accordance with differential polysome recruitment, because Igbp1 showed constant transcript levels and strict factor-dependent polysome recruitment, whereas Uhmk1 showed decreased transcript levels with sustained polysome recruitment upon factor-deprivation (Supplemental Figure S2H). Restimulation rapidly increased Igbp1 and Uhmk1 protein expression which was inhibited by rapamycin as much as with CHX. Fli-1 expression, however, was inhibited by CHX, but not by rapamycin (Figure 5B). Thus, factor-dependent Igbp1 and Uhmk1 protein expression was appropriately reflected by polysome-bound RNA levels, and not by total mRNA levels.

Functional analysis of target genes

Since SCF-induced, PI3K-dependent pathways sustain expansion and delay differentiation of erythroid progenitor cultures, we next investigated whether the proteins that require SCF-induced signalling to be synthesised have a major role in this process. Selective polysome recruitment, in general, depends on the 5' structured UTR of the transcripts. From the 9 selected genes, 8 showed PI3K-dependent polysome recruitment and 7 of these 8 genes could be expressed from retroviral expression vectors in which we cloned the ORF downstream of sequences encoding the myc-epitope (for unclear reasons Nubp1 resisted cloning in pBabe). Expression of selected genes in the Phoenix cells was analysed on Western Blots (data not shown)

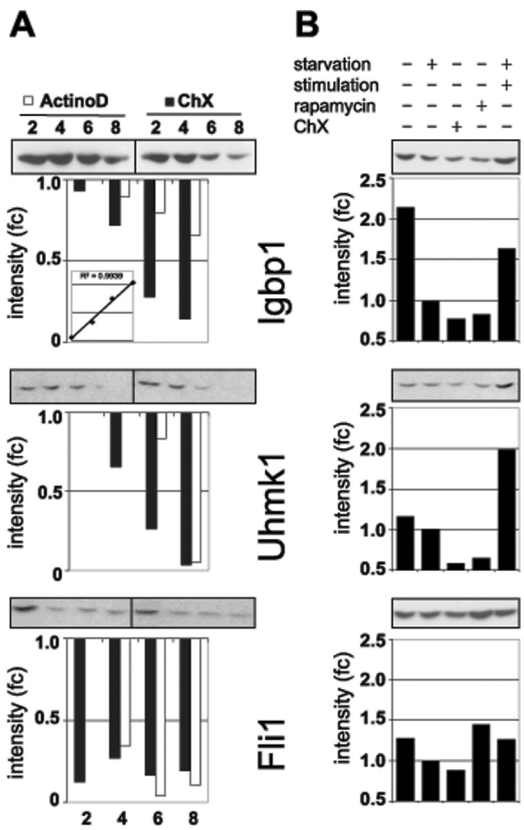


Figure 3 **Signalling dependent expression of Igbp1 and Uhmk1 protein**. **A:** Inhibitors of translation (cycloheximide 50μg/ml; ChX) and transcription (Actinomycin D 10μg/ml; ActinoD) were added for 2, 4, 6 and 8 h to proliferating I/11 cells. The insert shows the linearity of Igbp1 detection in subsequent 2-fold dilutions of total cell lysate (r^2=0.9939). Bars for ChX (black) and ActinoD (white) indicate fold change (fc) expression compared to ActinoD 2hr expression. Compared to Fli-I, expression of Igbp1 and Uhmk1 is more sensitive to translation inhibition. **B:** I/11 cells were factor deprived for 4 h and restimulated with Epo plus SCF for 2 h (stimulation) or left untreated (starvation). A steady state (ss) sample is also included. Inhibitors of mTOR (rapamycin 40ng/ml; rapa) and translation elongation (ChX) were added during factor deprivation and subsequent restimulation. Bars indicate fold change of protein expression compared to expression after factor deprivation.

and its cellular distribution assessed by Immunofluorescence (Supplementary Figure S3). The expression vectors were transduced into I/11 cells and single cell-derived clones were established. In every experiment aimed to establish clones expressing selected genes, empty vector control clones were established as well.

Four of the eight genes, *Uhmk1*/*Kis, Cnih, Rnf138, and Nap1l1* could be expressed transiently in Phoenix cells (Supplementary data Figure S3), but we failed to establish I/11 clones stably expressing these genes. *Hnrpa1***,** *Igbp1* and *mEd2* **(***2010315L10Rik***)** were expressed in Phoenix and in I/11 cells and several I/11, single cell-derived clones were established. To analyse how individual target genes contribute to SCF-induced suppression of differentiation, these clones were subjected to differentiation conditions. Differentiation parameters including cell number, mean cell volume (Figure 4A), haemoglobin per cell volume (Figure 4B) and cell morphology at various time points (Figure 4C) were analysed. Upon induction of differentiation, empty vector transduced control cells rapidly became smaller and accumulated haemoglobin (Figure 4A,B). After 72 hours they mostly showed a mature morphology (Figure 4C). Constitutive expression of *Hnrp1* showed essentially the same differentiation kinetics. Constitutive expression of *Igbp1* and *mEd2***,** however, clearly impaired differentiation. Erythroblasts constitutively expressing *Igbp1* remained large, failed to accumulate haemoglobin and kept a blast like morphology (Figure 4A-C). Erythroblasts constitutively expressing *mEd2* were partially impaired

in differentiation. The cells became smaller, but did not accumulate haemoglobin and showed a partially differentiated morphology (Figure 4A-C). In these experiments we used the *mEd2* ORF published to enhance an ERK-dependent reporter construct (Matsuda et al., 2003). In a later stage longer cDNAs became available that express the ORF recently described to encode a novel Q-SNARE protein D12 (Okumura et al., 2006). Similar to *Uhmk1, Rnf138* and *Cnih*, we could express D12 in Phoenix cells, but we failed to establish stable expression in I/11. Thus, constitutive expression of a full length protein seems incompatible with proliferation of erythroid progenitors while expression of a truncated protein inhibits differentiation.

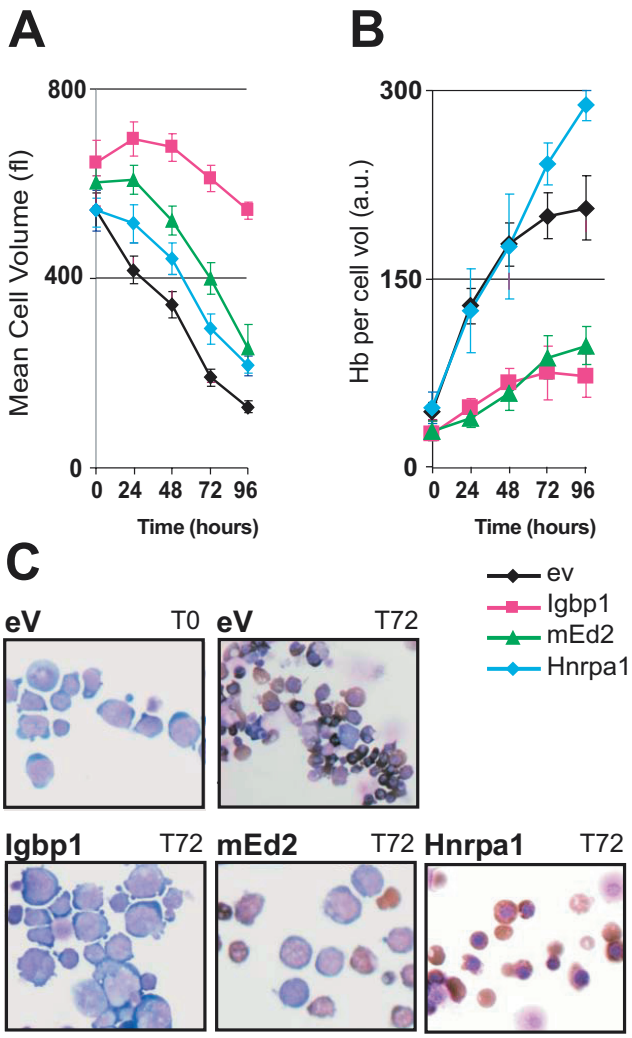


Figure 4 **Overexpression of *Igbp1* and *mEd2* impairs differentiation of I/11 erythroid progenitors**. I/11 cells transduced with an empty control vector or with an *Igbp1*, *mEd2* and *Hnrpa1* expression vectors were seeded in differentiation medium supplemented with Epo (E, 20U/ml). **A:** Mean Cell volume (fl) and **B:** hemoglobin content per cell volume (arbitrary unites; a.u.) were analyzed at regular intervals for 4 days. **C:** At the start of the assay and at day 3, cells were harvested for cytospins and stained for hemoglobin (brown color) and histological dyes. Hemoglobinised and enucleated erythrocytes are present in control cells and cells constitutively expressing *Hnrpa1*, while *Igbp1* and *mEd2* expressing cells contain mainly blasts.

Igbp1 affects 4EBP and p70S6k phosphorylation

Igbp1 is able to associate with the serine/threonine phosphatase Pp2a and modifies Pp2a phosphatase activity (Inui et al., 1998; Kong et al., 2004). Interestingly, Pp2a is the major phosphatase of 4EBP and p70S6k and Igbp1 inhibits Pp2a-activity on these targets. Therefore we analysed Epo- and SCF-dependent 4EBP and p70S6k phosphorylation in control and Igbp1 expressing erythroblasts. As we have shown previously, SCF but not Epo is able to induce full phosphorylation of 4EBP in control cells (Blazquez-Domingo et al., 2005). Upon constitutive expression of Igbp1, however, stimulation of erythroblasts by Epo is sufficient to induce hyperphosphorylation of 4EBP (Figure 5A). Similarly, constitutive expression of Igbp1 increased Epo-induced p70S6k to levels only obtained with Epo plus SCF in control cells (Figure 5B).

Constitutive expression of alpha4 enhances polysome recruitment of structured mRNAs

Because phosphorylation levels of 4EBP and p70S6k affect translation of structured mRNAs, we tested polysome recruitment of mRNAs in steady state expanding cells, in factor-deprived and Epo-restimulated cells. Expression levels of various genes were measured by Q-PCR in subpolysomal and polysome-bound mRNA fractions isolated from empty vector-transduced control erythroblasts and erythroblasts constitutively expressing Igbp1. *Igbp1* itself showed increased polysome recruitment upon factor-deprivation and Epo restimulation (Figure 5D), which is not surprising as the expressed construct lacks the regulatory sequences responsible for factor-dependent translation. Polysome recruitment of *Fli-1*, which is not subject to factor-dependent translation, is also not affected by Igbp1 expression (Figure 5C). However, two transcripts with a terminal oligopyrimidine tract, *eIF1β* and *Rps4* as well as two transcripts with a highly structured 5'UTR, *mEd2* and *Nm23-M2*, show increased polysome recruitment (Figure 5E-H). Together these data indicate that translational control of Igbp1 is an important positive feedback signal to enhance polysome recruitment of structured mRNAs.

DISCUSSION

Overexpression of eIF4E rendered proliferation of erythroid progenitors independent of SCF-induced PI3K activation and indicated that activation of the PI3K-mTOR-eIF4E pathway is an important mechanism by which SCF delays differentiation and sustains proliferation of erythroid progenitors (Blazquez-Domingo et al., 2005). This suggests that selective polysome recruitment of structured mRNAs is crucial to erythropoiesis. However, only few such transcripts are known. In this study we identified a unique set of genes that are hardly regulated at the level of gene transcription and are recruited to polysomes by an SCF-induced, PI3K-dependent, increase in eIF4F complexes. Functional analysis revealed that constitutive expression of one of these genes, Immunoglobulin binding protein 1 (*Igbp1, also named* α4 subunit of Pp2a), strongly inhibited terminal differentiation of erythroid progenitors. Whereas Pp2a dephosphorylates the mTOR targets 4EBP and p70S6K, constitutive expression of Igbp1/α4 maintained phosphorylation of 4EBP and p70S6K, particularly in presence

of Epo and absence of SCF. Igbp1/α4 appeared to function in a positive feed-back loop of mTOR regulated polysome recruitment of structured mRNAs. The complete inhibition of differentiation induced by constitutive Igbp1/α4 expression emphasises the important role of selective polysome recruitment in control of gene expression and cell fate determination.

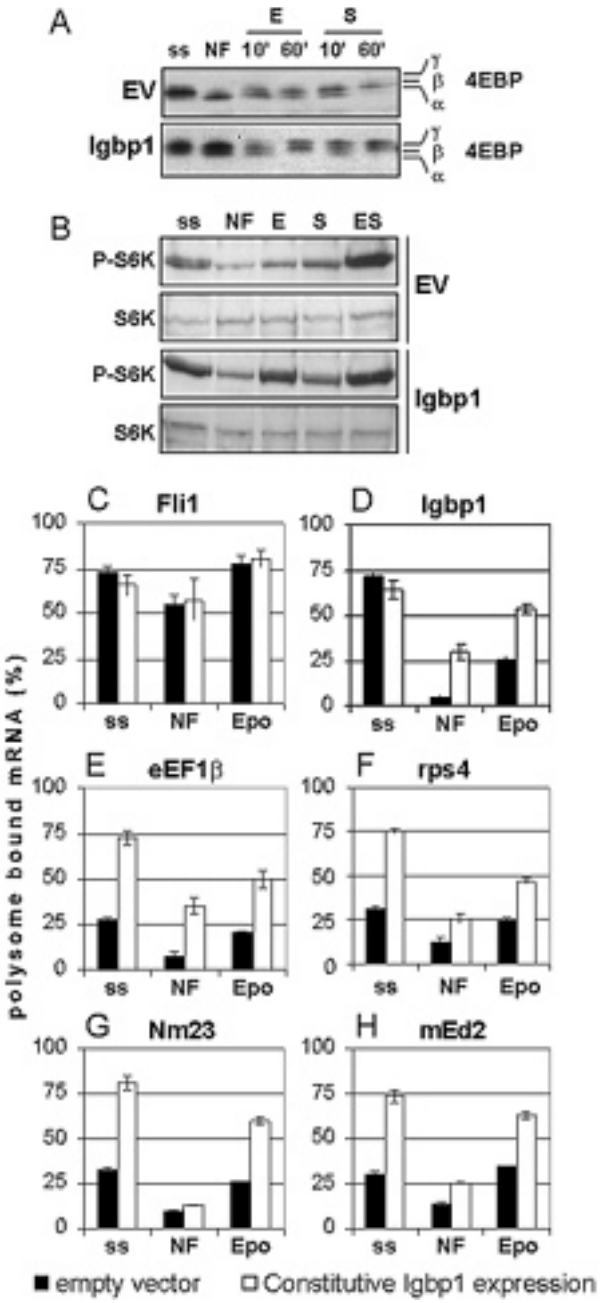


Figure 5 **Constitutive Igbp1 expression increases phosphorylation of 4EBP and S6K and enhances Epo induced polysome recruitment of structured transcripts**. **A-B**: I/11 cells transduced with an empty control vector (ev) or with an *Igbp1* expression vector were factor deprived for 4 h, stimulated with Epo (E, 5U/ml) or SCF (S, 100 ng/ml) or Epo plus SCF (ES), as indicated or left untreated (NF). Expanding I/11 cells in the presence of Epo, SCF and dexamethasone are denoted as ss (steady state). **A:** Western blots with total cell lysates were stained with antibodies recognizing total 4EBP (4EBP Ab). The non-phosphorylated, hypo and hyper-phosphorylated proteins can be discriminated by their distinct electrophoretic mobility as α, β and γ-isoforms respectively. **B:** Western blots with samples stimulated for 10 minutes were stained with a phospho-specific antibody against p70S6K (P-S6K) and counterstained for total S6K to control for equal loading. **C-H:** I/11 empty vector control (black bars) or constitutive *Igbp1* expressing cells (white bars) were factor deprived and restimulated for 2 h with Erythropoietin (Epo, 2U/ml). In addition, cells were left untreated (NF). Free and polysome bound mRNA was isolated and assayed for the expression of *Fli-1* (**C**), *Igbp1* (**D**), *eEF1β* (**E**), *rps4* (**F**), *Nm23* (**G**), and *mEd2* (**H**). The percentage of mRNA associated with polysomes (pb-mRNA) was calculated for the different genes under the different conditions. Constiutive *Igbp1* expression enhances polysome recruitment of translationally controlled transcripts under Epo conditions alone.

Specific transcripts recruited to polysomes by SCF signalling

It is well recognised that transcript levels determined by total mRNA profiling differ from protein expression levels. Polysome bound mRNA profiling integrates control at the level of transcription, mRNA nuclear export and polysome recruitment, getting results closer to proteomics (Pradet-Balade et al., 2001). Recently, Rajasekhar and collegues demonstrated PI3K- and Mek1-dependent, selective polysome recruitment of mRNA in v-Ras/v-Akt transformed glioblastoma cells (Rajasekhar et al., 2003). We found little overlap between our studies, which may be due to the use of a different cell type and a different experimental approach addressing quick responses to stimuli in our study as opposed to the effect of genetically perturbed pathways.

Both studies indicate that gene expression profiling using polysome bound mRNA (pb RNA) will reveal differentially expressed genes that would not be detected at the level of total RNA. However, the advantage of using pb mRNA may depend on the conditions and samples used. Conventional profiling with total mRNA may be preferred to find targets of transcription factors, or when structural, noncoding RNAs have to be included in the targets. In conclusion, the distribution of Epo/SCF-controlled gene expression detected using total and pb mRNA indicates that polysome recruitment is an important level at which signalling-dependent gene expression is regulated and that the use of pb RNA in RNA profiling studies can increase the sensitivity with which alterations in gene expression can be detected.

Selective polysome recruitment depends on structured UTRs

SCF signalling stimulates cap-dependent translation and is expected to identify transcripts that require increased levels of the eIF4F complex and enhanced p70S6K activation. It is broadly accepted that these transcripts are characterized by structured 5'UTRs (Koromilas et al., 1992, De Benedetti et al, 2004). Our list of translationally regulated genes contained ribosomal proteins and some translation factors known to contain a TOP sequence (Levy et al., 1991) (mainly present in clusters 1 and 5; Table S-I). The difference between factor-induced expression (array data) in total versus pb RNA of these genes, however, was mostly less than 1,5-fold. The genes selected for functional analysis showed a more extensive control of expression at the level of mRNA polysome recruitment. None of them contained a TOP sequence, but the structures that contribute to control of translation initiation are not easily discernable. Cross-species comparisons and comparisons between genomic and coding sequences present in genome databases suggest that most of these structured 5'UTRs are incompletely represented. However, even when the 5'UTR is known, the structural configuration that controls polysome recruitment occurs in combination with short sequences that are on their own not discriminative as is shown by the limited recognition site of the iron response element stem-loop element (Thomson et al., 1999) or the consensus pseudoknot structure bound by Fragile-X mental retardation protein (Frm1; Darnell et al., 2005). In addition to hairpin and pseudoknot structures, upstream AUGs (uAUG) may affect polysome recruitment. Translation of upstream open reading frames (uORF) can attenuate translation of the proper ORF as a result of premature dissociation of ribosomes (Child et al., 1999; Kozak, 1987) and/or stalling of the scanning complex by interaction of the nascent peptide with components of the complex (Ruan et al., 1996). Although the transcription start site

of Igbp1/α4 and mEd2 is not yet fully known, both seem to contain potential hairpin structures and uAUGs.

Signalling mRNA translation; role of Pp2a

The activity of the central regulator of protein synthesis, mTOR, is modulated by a variety of signals (Gingras et al., 1999b; Gingras et al., 2001; Inoki et al., 2003). Thus, polysome recruitment of transcripts that require activation of the PI3K/mTOR/eIF4E pathway is expected to be sensitive to amino acid starvation and lack of cAMP as well (Hara et al., 1998; Lawrence et al., 1997). However, activation of mTOR may not be sufficient to induce polysome recruitment, additional PI3K-dependent kinases may also be involved (Dennis et al., 1996; Peterson et al., 2000; Wang et al., 2005; Weng et al., 1995). The possible requirement for additional kinases may also explain why erythroblasts are only dependent on PI3K activation, while the glioblastoma model requires cooperation between v-Ras/Mek1 and PI3K/mTOR signalling (Rajasekhar et al., 2003).

The major antagonist of mTOR and/or cooperating kinases is the serine/threonine phosphatase Pp2a (Kloeker et al., 2003). Functional Pp2a consists of a catalytic subunit, a structural subunit ($Pp2a_A$) and a variable regulatory subunit ($Pp2a_B$; Kloeker et al., 2003). Among the regulatory subunits is Igbp1/α4. Pp2a was already implied in mTOR regulated polysome recruitment as addition of rapamycin disrupts the complex of Pp2a and Igbp1 (Inui et al., 1998) sequestering Pp2a to other functions (Kamibayashi et al., 1994). Most of these studies employed ectopic expression studies, or factor-independent cell lines which precluded analysis of factor-dependent expression of Igbp1/α4. Constitutive expression of Igbp1/α4 potently inhibited differentiation of erythroblasts in presence of Epo. The interaction of Igbp1/α4 with Pp2a controls more cellular processes in addition to phosphorylation of 4EBP and p70 S6K (Liu et al., 2001). However, the observation that (*i*) Igbp1/α4 expression enhances phosphorylation of 4EBP and p70S6K in presence of Epo to levels normally reached by Epo plus SCF, plus (*ii*) the finding that polysome recruitment of previously identified genes is rendered independent of SCF by constitutive Igbp1/α4 expression (similar to eIF4E overexpression; Blazquez-Domingo et al., 2005), suggests that positive feedback in the polysome recruitment of structured mRNAs is a major role of SCF-induced expression of Igbp1/α4 in erythroid progenitors.

Interestingly, another inhibitor of Pp2a, the putative oncogene SET (von Lindern et al., 1992) is induced in CML (Neviani et al., 2005). Therapeutic activation and ectopic expression of Pp2a, inhibits SET activation by BCR/ABL and resulted in reduced proliferation and leukemogenic capacity in transplantation murine models (Neviani et al., 2005).

Genes regulated by selective polysome recruitment and their tumorogenic potential

Both polysome recruitment as a function of UTR structure and the release of mRNAs silenced in Ribonuclear protein (RNP)-complexes offer the possibility of rapid alteration of predisposed gene expression patterns in response to mitogen and constitute a target for tumorigenic deregulation (Audic and Hartley, 2004; De Benedetti and Harris, 1999).

It is of interest that we do not find many transcription factors to be regulated at the level of polysome recruitment, which confirms that this level of regulation may be specifically targeted to pre-disposed expression profiles for quick responses. This is consistent with data showing that transcripts for kinases, phosphatases and transcription factors are not shifted from polysomes upon rapamycin treatment. In the same study, the transcripts of RNA-binding proteins that are part of RNP-complexes were identified as transcripts dissociated from polysomes upon rapamycin treatment (Grolleau et al., 2002). Similarly, we also detected several RNA binding proteins that associated with polysomes upon growth factor addition such as Hnrpa1. From all targets tested, *Hnrpa1* was the only gene that could be constitutively expressed at abundant levels. It is known to be involved in the generation of correct splice variants encoding the erythrocyte membrane protein Band4.1 (Hou et al., 2002; Yang et al., 2005) and incorrect splicing has major consequences *in vivo* (Caceres and Kornblihtt, 2002).

For functional studies, we selected genes that were recruited to polysomes by growth factors addition and downmodulated in differentiation. We assumed that these could be constantly expressed during expansion of progenitors. However, most of the genes we tested could not be constitutively expressed. There may be a need to express these proteins only during a specific phase of the cell cycle. Notably, these genes included *Uhmk1*/*Kis* and *Cnih* which associate with and control stability of tubulins during mitosis (Maucuer et al., 1997; Roth et al., 1995). Igbp1/α4 has also been implicated in tubulin stability (Liu et al., 2001), possibly contributing to the slow growth and low expression of α4 in erythroid progenitors. Rnf138 contains protein domains for nuclear localisation (confirmed by immunofluorescence) and ubiquitin ligase function. Constitutive expression may result in degradation of nuclear proteins that otherwise need to be controlled as a response to a particular cellular state. mEd2 is also known as MAPK activating protein PM26 (Matsuda et al., 2003) and its human homologue is 'uncharacterised hematopoietic stem/progenitor cells protein MDS032'. Recently, mEd2 was shown to be a Q-SNARE protein, termed D12, involved in ER-trafficking (Okumura et al., 2006). Interestingly, reduced expression of D12 resulted in increased phosphorylation of eIF2a. This opens the interesting possibility that translational control of mEd2/D12, by surveillance of protein quality in the ER, also functions in a feed-back mechanism; a feedback from eIF4E activation to protection of eIF2a from inactivation by phosphorylation.

Finally, the strong inhibition of erythroid differentiation by overexpression of eIF4E or by constitutive expression of IGBP1/α4, the high levels of eIF4E found in several cancer types (De Benedetti and Graff, 2004) and the promising results of rapamycin as an anticancer drug (Bjornsti and Houghton, 2004; Hidalgo and Rowinsky, 2000) indicate that many translationally controlled genes await further characterisation with respect to function and regulation and that control of translation is not simply control of cell growth to reach 'start' in the cell cycle, but that it is an important and selective level of regulation of gene expression.

Constitutive activation of PI3K has frequently been found in both solid tumours and leukemia (Grandage et al., 2005; Luo et al., 2003; Min et al., 2003; Neshat et al., 2001; Sujobert et al., 2005; Vivanco and Sawyers, 2002). Currently, rapamycin homologues are tested as anticancer drugs in a large variety of tumours, yielding promising results (Bjornsti and Houghton, 2004; Hidalgo and Rowinsky, 2000;

Panwalkar et al., 2004). Although it is assumed that the anticancer effect of rapamycin and its analogues is due to a general inhibition of protein synthesis in proliferating cells, it is more likely that inhibition of mTOR specifically targets structured mRNAs. The promising antitumour effects of rapamycin and the finding that constitutive expression of IGBP1/α4 inhibited maturation and sustained expansion of progenitor cells, underline the importance to understand mechanisms of translation initiation. The genes identified in this study are a good starting point for such studies.

MATERIALS AND METHODS

Cells

I/11 cells were cultivated in StemPro-34™ medium (Life Technologies) as described (von Lindern et al., 2001). For expansion, the medium was supplemented with 0.5U/ml Epo, (kind gift from Ortho-Biotech, Tilburg, The Netherlands), 100ng/ml SCF (supernatant of CHO producer cells) and 10^{-6}M dexametasone (Dex, Sigma-Aldrich). To induce differentiation, cells were cultivated in StemPro-34™ medium supplemented with 5U/ml Epo and 0.5mg/ml iron-loaded transferrin (Intergene). Cell numbers and cell size distribution were determined using an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany). LY294002 (10μM in final volume) and rapamycin (40ng/ml) were obtained from Alexis (Schwitzerland), Actinomycin D (10μg/ml) and Cycloheximide (50μg/ml) from Sigma-Aldrich.

Haemoglobin content determination and cell morphology

Small aliquots of the cultures were removed and analyzed for hemoglobin content by photometry as described earlier (Bakker et al., 2004; Kowenz et al., 1987). The values obtained were the average of triplicate measurements after normalization for cell number and mean single cell volume. Cell morphology was analyzed in cytospins stained with histological dyes and neutral benzidine (Beug et al., 1982), using an OlympusBx40 microscope (40x objective, NA 0.65), an OlympusDp50 CCD camera and Viewfinder Lite 1.0 acquisition software. Images were cropped using Adobe photoshop 6.0.

Microarray hybridization and analysis

A MIAME compatible description of sample preparation and hybridization protocols is given in supplementary data. The data extraction strategy is described in supplementary Figure S1. Microarray data were normalised using the Rosetta Resolver ® system, as described in (Weng et al., 2006). Weighted averages were calculated using log error data extracted for each probe set. The error weight was calculated (log error / total log error of 4 hybridisations) and the average calculated integrating the error weight.

SDS-PAGE, western blotting and antibodies

For acute stimulation with growth factors, proliferating I/11 cells were washed twice with phosphate-buffered saline (PBS) and seeded at 4 x 10^6 cells/ml in plain Iscove's modified Dulbecco's medium (IMDM, Life Technology). After 4h factor deprivation, cells were stimulated at 37°C with SCF (100ng/ml) or Epo (5U/ml). Cells were

harvested after the indicated time points by addition of ice-cold PBS. Cell lysates, SDS-PAGE, immunoprecipitation and Western blotting were performed as described previously (van Dijk et al., 2000). 10 μl of protein extract (≈1x10^6 cells) was loaded onto a 15% polyacrylamide gel. The antibodies used were: α-4EBP1 (Cell Signaling Technology, Inc.), α-Uhmk1 (Gift from M Boehm NIH/NHLBI), α-alpha4 (Gift from D L Brautigan, Center for Cell Signaling, University of Virginia), S6K-P (Cell Signaling) α-Fli1 and α- Myc (Santa Cruz Biotechnology, Inc.).

Transduction of I/11 clones

The coding sequences (NCBI accession number NM_008784; NM_029768; NM_010447; NM_207623; NM_015781; NM_010633; NM_009919; NM_153419) were amplified with Pfu polymerase (Promega M7741) using primers designed to insert an EcoRI at the 5'end and a ClaI site at the 3' end of the PCR product. The PCR product was inserted in TA vector (Invitrogen KNM2040-01). The EcoRI/ClaI digestion product was inserted into the same sites of a pBlueScript vector. The PCR primer was designed to abolish the Kozak sequence and insert the ATG of the coding sequence in frame to a six *myc*-tag sequence at the EcoRI site of the pBlueScript vector. A BamHI/SalI digestion product containing the N-terminal myc-tag and the coding sequence was inserted into the eukaryotic retroviral expression vector pBabe. Retroviral transduction was performed as described (Bakker et al., 2004). Briefly, 0.5x10^6 ecotropic Phoenix cells were transfected with 12 μg plasmid DNA using calcium-phosphate coprecipitation assay. After 40 h, cells were treated with 10μg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) for 1 h and washed 3 times with PBS. 2x10^6 I/11 cells were added in 4ml StemPro-34^{TM} medium supplemented with Epo, SCF and Dex and co-cultured for 24 h. Subsequently, I/11 cells were removed from the Phoenix cells and cultured in semisolid medium (Methocel-containing StemPro-34TM, supplemented with factors) containing 2 μg/ml puromycin (Sigma). After 7 days well-separated colonies were picked, expanded and analyzed for myc-coding sequence expression.

Immunofluorescence microscopy

Phoenix cells were grown on microscope cover slips, placed at the bottom on 5ml pedri dishes. The cells were transfected with myc-tagged constructs used for the transductions (see above). The cells were fixed in 4% paraformaldehyde and permeabilized for 30 min with 0.2% TritonX. After blocking for 1h in PBS containing 1% BSA and 0.05% Tween, the fixed cells were incubated for 1 h at *rt* with anti-myc-antibodies (Santa Cruz; 9E10). The slides were washed and incubated for 1h at *rt* with TRITC anti-mouse secondary antibody (DakoCytomation). The Cover slips were mounted with a drop Vector Shield (Vector laboratories Inc; H-1000), including DAPI (0.3ng/μl). Imaging of the cells was done with 543nm, 488nm, 405nm excitation provided by an argon laser and a 63 x 1.4 NA apochromat objective lense (Carl Zeiss MicroImaging) for FITC, TRITC and DAPI respectively. Zeiss AIM software version 3.2 was used for merging the images.

RNA isolation and cDNA synthesis

Total RNA was isolated using the Trizol reagent (Life Technologies) as recommended in the manufacturer's protocol. Isolation of polysomal RNA by sucrose gradient

fractionation was performed as described (Joosten et al., 2004; Mullner, 1997). Cell extracts were layered on a 4ml linear sucrose gradient (15–40% sucrose [w/v]) and 8 fractions were collected. Northern blotting indicated that fractions 1-4 contain nonpolysomal and subpolysomal mRNA, while fractions 5-8 consisted of polysome-bound RNA. These fractions were pooled to generate subpolysomal and polysomal mRNA of each sample. RNA was quantified by UV-absorbance. Poly(A)$^+$ mRNA was purified and cDNA was generated as described (Joosten et al., 2004).

Real-time PCR

The real-time PCR assay involved TaqMan technology (PE Applied Biosystems Model 7700 sequence detector), using the double stranded DNA-specific fluorescence dye SYBR green I to detect PCR product as previously described (Kolbus et al., 2003). The amplification program consisted of 1 cycle of 50°C with 2' hold (AmpErase UNG incubation), 1 cycle of 95°C with 10' hold (AmpliTaq Gold Activation), followed by 40 cycles of denaturation at 95°C for 15", annealing at 62°C for 30" and extension at 72°C for 30". All the different primer pairs had similar optimal PCR annealing temperatures. Acquisition of the fluorescence signal from the samples was carried out at the end of the elongation step. To confirm amplification specificity, the PCR products from each primer pair were subjected to agarose gel electrophoresis and the dissociation curve was checked at the end of each run. Gene-specific primers corresponding to Igbp1 (NM_008784); mEd2 (NM_029768); Hnrpa1 (NM_010447); Rnf138 (NM_207623); Nap1l1 (NM_015781); Uhmk1 (NM_010633); Cnih (NM_009919); Nubp1 (NM_153419), Grwd1 (NM_153419), Pscd3 (NM_011182), Txnip (NM_023719), nucleoside diphosphate kinase B (Ndpk-B, 008705), eukaryotic translation elongation factor eEF-1β2 (NM_018796), ribosomal protein S4 (rps4, M73436), Fli-1 (NM_008026), mammalian ribonuclease inhibitor (IMAGE:1366946), Y-box binding protein 1 (YB-1, X57621), Nfe2 (NM_008685) were obtained from Invitrogen Life Technologies or Sigma-Genosys Ltd. Sequences are available in supplementary Table S-5.

Supplementary data

Supplementary figures 1 to 3 are found below, pages 88 and 89.

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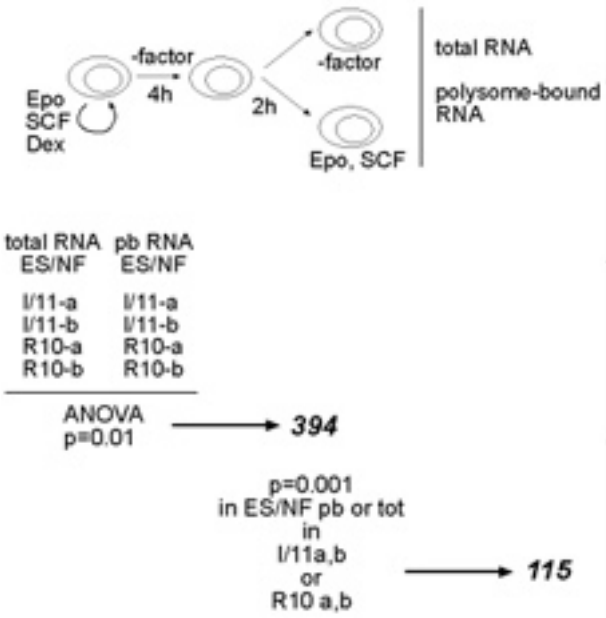
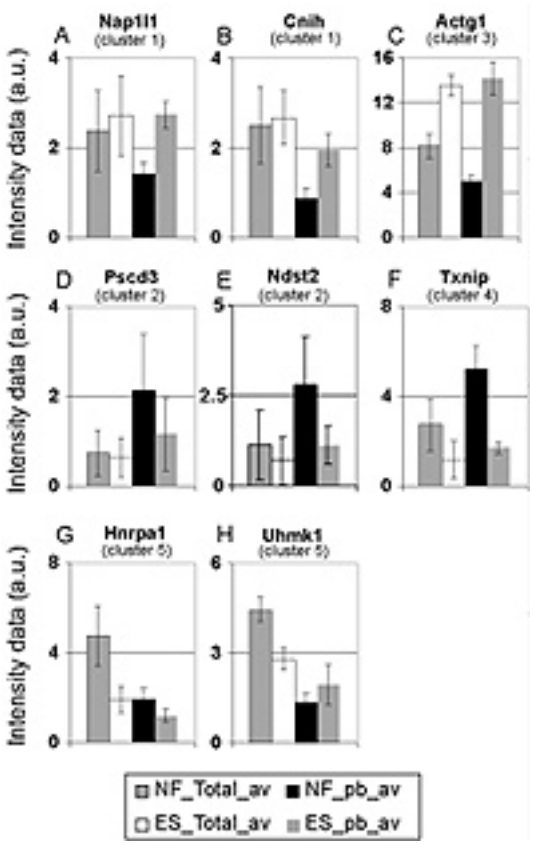
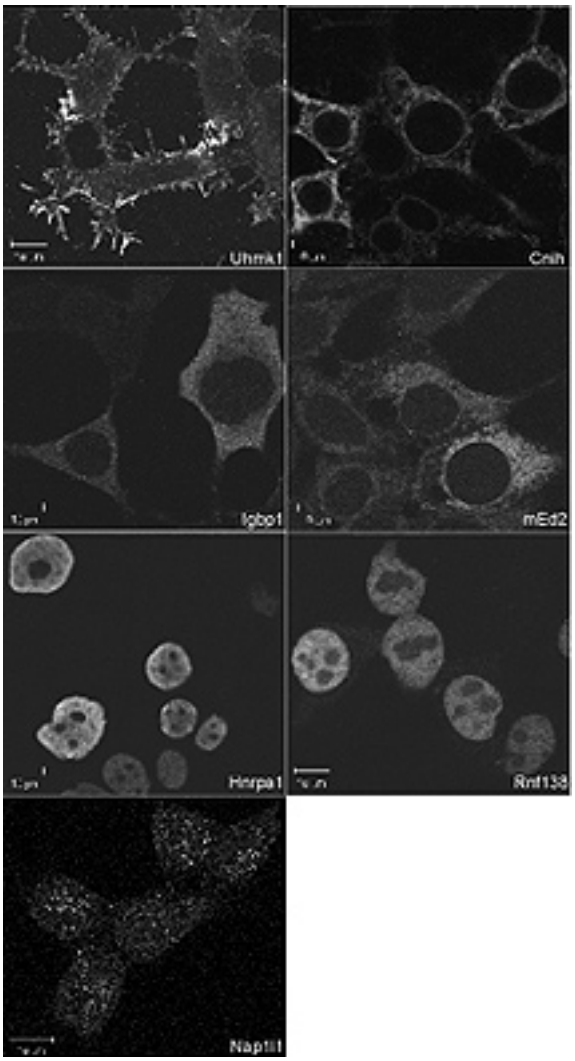


Supplementary Figure 1
The array data analysis strategy to identify the translationally controlled genes is depicted here and has been extensively explained in the text and referred to in Figure 1. I/11 cells and R10 cells were factor deprived for 4 hours and stimulated with ES, or left untreated (NF). For each condition 2 of each erythroid progenitor cell line were treated (I/11 denoted as I/11-a and I/11-b, while R10 denoted as R10-a and R10-b). This diagram gives an overview of the gene lists that have been extracted during the analysis and the statistical thresholds used.



Supplementary Figure 2.
Intensity data profiles of representative genes of various clusters show different polysome recruitment regulation upon stimulation as compared to total RNA. The bars represent the weighted average of Intensity data calculated from the 4 biological replicas for each condition. The error bars represent the standard error between the 4 intensity values. The intensity values plotted is the normalized geometric mean calculated by the Rosetta Resolver software. Heat Map cluster 1 represented by Nap1l1 and Cnih (A,B), cluster 3 by Actg1 (C); cluster 2 and 4 by Pscd3, Ndst2 and Txnip (D-F); and cluster 5 by Hnrpa1 and Uhmk1 (G, H).



Supplementary Figure 3
Phoenix cells were grown on microscope slides and myc-tagged coding sequences of transiently transfected. The cells were fixed and stained for myc tag FITC- labeled second antibodies. The expression of Uhmk1, Cnih, Igbp1, mEd2, Hnrpa1, Rnf138 and Nap1l1 is localized at microtubule active centers, endoplasmic reticulum associated with nuclear membrane, cytoplasmic, possible associated with reticulum, nuclear with higher concentrations at the nuclear circumference, nuclear and in discrete speckles through the cytoplasm and nucleus, respectively.

Factor-dependent regulation of gene expression during expansion and differentiation of erythroid progenitors: regulation of mRNA translation.

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Short title: Gene expression program

Manuscript in preparation

CHAPTER 4

ABSTRACT

SCF and glucocorticoids cooperate with Epo to induce renewal divisions of erythroid progenitors, whereas Epo is required for survival of erythroblasts during differentiation. Renewal is dependent on PKB activation, which enhances translation initiation efficiency and retains Foxo3a in an inactive state. To study the impact of signalling-controlled gene expression on the balance between renewal and differentiation of erythroid progenitors, we set up mRNA profiling experiments to identify genes sensitive to Epo, SCF and/or Dex, and regulated during erythroid differentiation. Most of the Epo-controlled genes were similarly regulated by Epo and SCF and counter regulated during differentiation. This underscores the role of Epo signalling in induction of progenitor renewal. In addition, we identified a small group of genes that are upregulated specifically by Epo and upregulated during differentiation. All known Stat5 target genes are comprised in this set of genes. The mRNA profiles were generated using polysome bound (pb) mRNA. Comparison of Epo/SCF-controlled gene expression between profiles of total and pb mRNA demonstrate a prominent effect of signalling on polysome recruitment. The prominent, and so far underestimated effect of signalling on mRNA translation prompted us to evaluate signalling dependent expression of translation factors and proteins involved in ribosome biosynthesis. Together, ribosome synthesis regulation and polysome recruitment ensure enhanced translation efficiency in presence of mitogenic factors.

INTRODUCTION

The number of circulating erythrocytes in the blood is tightly controlled, which requires a robust balance between expansion, survival and maturation of the erythroid progenitor population. Insight into mechanisms regulating this balance has been obtained from *in vivo* studies using genetically modified mice and from *in vitro* cultures of erythroid progenitors, that can be established in serum free medium supplemented with Epo, stem cell factor (SCF) and glucocorticoids (i.e. the artificial glucocorticoid dexamethasone; Dex). These studies showed that SCF and glucocorticoids cooperate with Epo to induce renewal divisions of erythroid progenitors both *in vitro* and *in vivo* (Broudy et al. 1996; Bauer et al. 1999; Wessely et al. 1999; von Lindern et al. 2001; Dolznig et al. 2006), whereas Epo is required for survival of erythroblasts during differentiation (Wu et al. 1995; Lin et al. 1996; Dolznig et al. 2002). Renewal or differentiation of erythroid progenitors requires the activation of distinct gene expression programs. Several erythroid specific transcription factors are known to control erythropoiesis such as Gata-1, Eklf and Nfe-2 (Scott et al. 1994). However, these transcription factors have mainly been studied in the context of haemoglobin regulation and very little is known about genes involved in the growth factor-controlled balance between renewal and differentiation. This study aims to get insight into gene expression programs controlled by Epo, SCF and glucocorticoids.

Mice lacking Epo or the Epo-receptor (EpoR) die at embryonic day 13,5 with a lack of mature definitive erythrocytes (Wu et al. 1995; Lin et al. 1996). Although Epo can induce a multitude of signalling pathways (for review see Richmond et al. 2005), the phosphorylation and transcriptional activation of Stat5 appeared to be crucial for Epo-dependent differentiation of erythroblasts (Socolovsky et al. 2001; Dolznig et al. 2002; Dolznig et al. 2006). Epo-induced Stat5 activation controls $BclX_L$ upregulation during erythroid differentiation, which appeared to be sufficient for survival and differentiation of erythroblasts *in vitro* (Dolznig et al. 2002; Dolznig et al. 2006). However, upregulation of $BclX_L$ is delayed compared to Stat5 phosphorylation and most likely involves intermediates. Notably, the enhancer involved in the massive upregulation of *BCLX* gene expression during erythroid differentiation does not contain STAT5 binding sites (Tian et al. 2003). Therefore, it remains unclear which genes activated by Stat5 are crucial in erythroid differentiation.

Whereas Epo-induced Stat5 activation is crucial for survival during differentiation (Dolznig et al. 2002), other Epo-induced pathways are required to induce renewal divisions (van den Akker et al. 2004) in cooperation with signals emanating from the SCF-receptor cKit. Activation of phosphoinositide 3-kinase (PI3K) appeared to be particularly important for Epo/SCF-induced renewal divisions of erythroid progenitors as inhibition of PI3K abrogates renewal and induces differentiation instead (von Lindern et al. 2001). Activation of PI3K generates PIP3, which serves as an anchor for PH-domain containing proteins, both adaptor molecules such as Gab2 and Dok1 and kinases such as Tec, Btk, PDK1 and PKB (Tang et al. 1994; Stokoe et al. 1997; Leevers et al. 1999; Zhao et al. 1999; Saito et al. 2001). Although both Epo and SCF induce activation of PI3K in erythroid progenitors, the efficiency with which downstream signalling pathways are activated shows large differences (Bakker et al. 2004; Blazquez-Domingo et al. 2005). In cultured erythroid progenitors the activation

of PKB is more responsive to SCF compared to Epo (von Lindern et al. 2001; Bakker et al. 2004). We found that two major pathways downstream of PKB control renewal versus differentiation of erythroid progenitors. First, PKB phosphorylates the class O Forkhead transcription factor 3a (Foxo3a), which retains Foxo3a in an inactive state in the cytoplasm. Foxo3a translocates to the nucleus upon factor-deprivation to activate genes involved in apoptosis and stress-responses (Greer and Brunet). Foxo3a is transcriptionally upregulated during erythroid differentiation, while PKB activation decreases, resulting in increased Foxo3a activity late in differentiation (Bakker et al. 2004). Thus, Epo/SCF-induced activation of PKB silences genes that are upregulated following factor-deprivation and differentiation induction. Second, PKB activates mTOR (mammalian target of rapamycin) (Inoki et al. 2003; Tee et al. 2003) modulating phosphorylation and activation of p70S6 kinase (Rps6kb1) (Dufner and Thomas 1999) and hierarchical phosphorylation of 4EBP (4E-Binding Protein) (Gingras et al. 1999), resulting in activation of the translation machinery (Wang et al. 2001; Raught et al. 2004) and release of the mRNA cap-binding factor eIF4E (eukaryotic Initiation Factor 4E) (Gingras et al. 2001), respectively. The cap-binding eIF4E protein is the rate limiting factor in the mRNA scanning process (Duncan et al. 1987; Sonenberg and Gingras 1998), particularly for structured mRNAs that are only translated at increased eIF4E levels. Overexpression of eIF4E increased the levels of eIF4F complexes and suppressed erythroid differentiation in the absence of SCF, indicating an important role for recruitment of structured mRNAs, potentially responsive to SCF in regulating the balance between expansion and differentiation in erythropoiesis (Blazquez-Domingo et al. 2005).

The aim of this study is to get insight into the impact of signalling-controlled gene expression on the balance between renewal and differentiation of erythroid progenitors. We set up profiling experiments to identify (*i*) how Epo, SCF and Dex control erythroid gene expression independent of each other, (*ii*) how they mutually affect regulation of gene expression under renewal conditions, and (*iii*) how and to what extent genes regulated during differentiation are controlled by signal transduction.

Increasingly, discrepancies between the transcriptome and the proteome are uncovered, indicating the importance of post-transcriptional regulation particularly in the incidence and progression of disease (Rajasekhar et al. 2003; Perrotti and Calabretta 2004). Our own results also indicate an important role for PI3K/mTOR controlled polysome recruitment of specific mRNAs (Blazquez-Domingo et al. 2005). Therefore we used polysome-bound mRNA to screen for growth factor-controlled gene expression on oligonucleotide arrays. For short-term stimulation by Epo, SCF and/or Dex we also used total RNA to generate the opportunity to identify transcripts selectively recruited to polysomes. Analysis of the data shows that growth factor induced polysome loading is an important level at which signalling-dependent gene expression is regulated and that the use of pb RNA in RNA profiling studies can increase the sensitivity with which alterations in gene expression can be detected. The prominent effect of polysome recruitment on signalling-induced gene expression profiles prompted us to evaluate signalling- and differentiation-dependent expression of genes involved in protein synthesis. Ribosome synthesis is generally known to be under control of mitogenic factors supposedly by selective translation of ribosomal proteins. However, we found only a modest regulation of the expression

of ribosomal proteins and a much more pronounced regulation of a large number of factors involved in expression and maturation of rRNA, in association of rRNA and ribosomal proteins to ribosomal subunits, and in export and trafficking of ribosomal subunits.

RESULTS and DISCUSSION

Establishing gene expression profiles

For the profiling studies we used four biologically independent replicates derived from two established *p53*$^{-/-}$ erythroblast cultures with different genetic background (I/11 and R10, Figure 1) (von Lindern et al. 2001; Schmidt et al. 2004). Two independent experiments were performed for each background. To assess factor dependent gene regulation, erythroid progenitors were factor deprived (4h) and restimulated (2h, 6hr and 18hr) with Epo, SCF or dex, with a combination thereof, or left unstimulated (NF). The combinations we tested represented delayed differentiation (Epo plus SCF) or sustained renewal (Epo, SCF, Dex Figure 1) (Dolznig et al. 2001; von Lindern et al. 2001). Because it has been reported that growth factor signalling may activate nuclear hormone receptors in absence of ligand (Weigel and Zhang 1998; Labriola et al. 2003), we included the glucocorticoid receptor antagonist ZK112,993 (ZK) in combination with Epo and SCF to address glucocorticoid-specific gene regulation. Polysome-bound RNA was isolated cRNA was generated and hybridized to Affymetrix oligonucleotide arrays. At 2h following factor stimulation, we also isolated cytoplasmic RNA, which was similarly processed to cRNA and hybridised to oligonucleotide arrays. Rosetta Resolver software was used to normalize and analyse the intensity data. The ratio of gene expression in growth factor stimulated samples versus factor-deprived cells (NF) was calculated by Rosetta Resolver software. Concurrently, erythroid progenitors were induced to undergo terminal differentiation by switching renewal conditions (Epo, SCF, Dex) to differentiation conditions (increased concentration of Epo plus iron-saturated transferrin). Polysome-bound RNA was generated from steady state growing progenitors and progenitors induced to differentiate for 6, 22, 48 or 60 hours (Figure 1), processed to cRNA and used to hybridise oligonucleotide arrays. Rosetta resolver was used to calculate the gene expression ratio of differentiated cells over steady state growing cells.

Gene expression regulated by Epo and SCF

To assess Epo and SCF dependent gene expression and the potential effects of these genes on the balance between renewal and differentiation of erythroid progenitors, we first identified genes regulated by Epo, SCF or Epo plus SCF by 'Analysis of Variance' (ANOVA, p=0.01). The ANOVA analysis was performed with a relatively low stringency. Concurrent quantitative PCR analysis of random targets indicated that increasing the probability (p-value) of the ANOVA comparison did not improve the selection procedure and excluded too many valid candidates (data not shown). Analysis of selected data showed that gene probes that are unjustly excluded at increased p-value, show a variation between the 4 biological replicates that exceeds the differential expression that consistently occurred within every single experiment. Therefore we decided to retain low stringency ANOVA (p=0.01) as a first step,

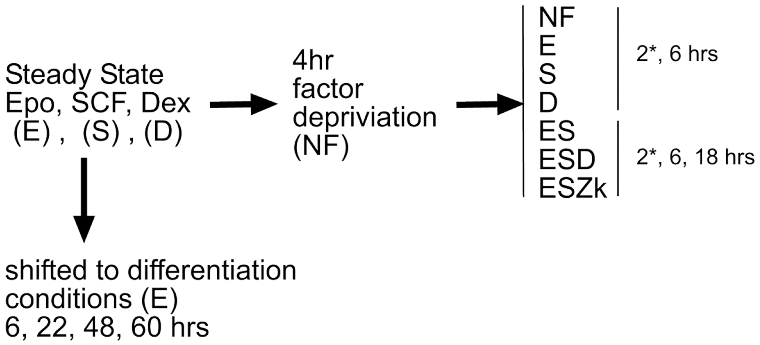

Figure 1 **The gene expression profiling strategy to identify genes differentially regulated by growth factor signalling and during erythroid differentiation**. I/11 cells and R10 cells were factor deprived for 4 hours and Epo, SCF, ES, ESD, ESZK or Dex were added for 2 hours, 6 hours or 18 hours or cells were left untreated (NF). For each condition we performed duplicate experiments for both erythroid progenitor cell lines (I/11 denoted as I11-a and I11-b; R10 denoted as R10-a and R10-b). For the 2 hour time points both Total and polysome bound (pb) mRNA were isolated, while for all the other conditions pb RNA was isolated. Cells (4 replicas as above) were induced to differentiate under Epo conditions. Cells were harvested at 6, 22, 48 and 60 hours and pb RNA isolated.

followed by more stringent selection of differential expression in response to Epo and/or SCF in at least both I/11 or both R10 samples (p=0.001 in two experiments, indicated as $p=0.001^2$). Comparisons were made between polysome-bound RNA profiles corresponding to factor-deprived cells and cells stimulated for 2h and 6h either with Epo, with SCF or with Epo plus SCF. These lists were combined to form the “signalling signature”. Next the signalling-induced and differentiation-induced expression ratio’s were clustered for these ‘signalling signature’ genes (K-means, Pearson; Figure 2, supplemental data Table S-II).

As observed before using total RNA, essentially all genes upregulated in response to SCF are also upregulated in response to Epo (cluster 4 and 5)(Kolbus et al. 2003). The vast majority of these genes is downregulated during differentiation, suggesting that among those genes we may find important regulators of progenitor renewal. Conversely, not all genes upregulated in response to Epo are also upregulated by SCF. Cluster 1 represents genes upregulated by Epo, not regulated by SCF and upregulated during differentiation (Figure 2). This cluster contains all known Stat5 target genes including *Cish, Pim1, Cdkn1a*/*p21*^{WAF}*, and TGF*β*2*. Cluster 1 is the only cluster containing genes that are upregulated in response to Epo and during differentiation (Figure 2). From previous studies on Foxo3a target genes we expected genes that are inversely regulated by Epo and SCF such as *Cited2* (Bakker et al, submitted). *Cited2* was also identified in cluster 1.

The Epo-controlled gene expression profile is in accordance with previous observations that most Epo-induced signalling pathways are required for expansion of the progenitor pool under conditions of hypoxic stress and not for erythroid differentiation. Only the activation of Stat5 is required for Epo dependent maturation of erythroid progenitors (Dolznig et al. 2002; van den Akker et al. 2004). Concordantly, it is only 1 small gene cluster containing Stat5 target genes that is upregulated by Epo and during differentiation. In contrast, the majority of Epo-induced genes is downregulated during differentiation, and many Epo-repressed genes are upregulated during differentiation. Although it remains to be shown which pathways drive expression of genes in cluster 1 versus clusters 4 and 5, the genes present in cluster 1 are strong candidates for Stat5 targets that may proof informative for erythroid differentiation induction and control of target genes during differentiation (eg *BclX*$_L$). Conversely, clusters 4 and 5 may harbour genes inhibiting differentiation in response to the cooperative signal of Epo and SCF.

Gene expression regulated by dexamethasone

To identify genes regulated by glucocorticoids, i.e. by dexamethasone, we used the same selection procedure (ANOVA between groups with a threshold of p=0.01, differential expression in at least both I/11 or both R10 experiments with a threshold of p=0.001[2]) for comparisons of (*i*) polysome-bound RNA profiles obtained after 2 or 6 h treatment with Epo, SCF and Dex (ESD) with profiles obtained 2 or 6 h following Epo, SCF, and Zk112,993 (ESZk) treatment, and (*ii*) polysome-bound RNA profiles of factor deprived cells and profiles obtained following 2 and 6 h treatment with Dex. These selected genes were combined and used to cluster Dex-induced, signalling-induced and differentiation-induced expression ratio's (Figure 3, supplemental Table S-III). Interestingly, gene regulation by Dex and by Epo plus SCF occurred in all possible combinations: (*i*) Genes in cluster D1 were upregulated in response to Dex both in absence and in presence of Epo plus SCF (cluster D1, Figure 3); (*ii*) some genes were only regulated by Dex in presence of Epo plus SCF (cluster D2) or Epo/SCF signalling enhanced Dex-induced gene expression (cluster D3), whereas (*iii*) other genes were upregulated in response to Dex, but repressed upon addition of Epo plus SCF to Dex (cluster D5 and D6, Figure 3), subsequent these genes could be upregulated (D2, D5) or repressed (D1, D3, D6) during differentiation.

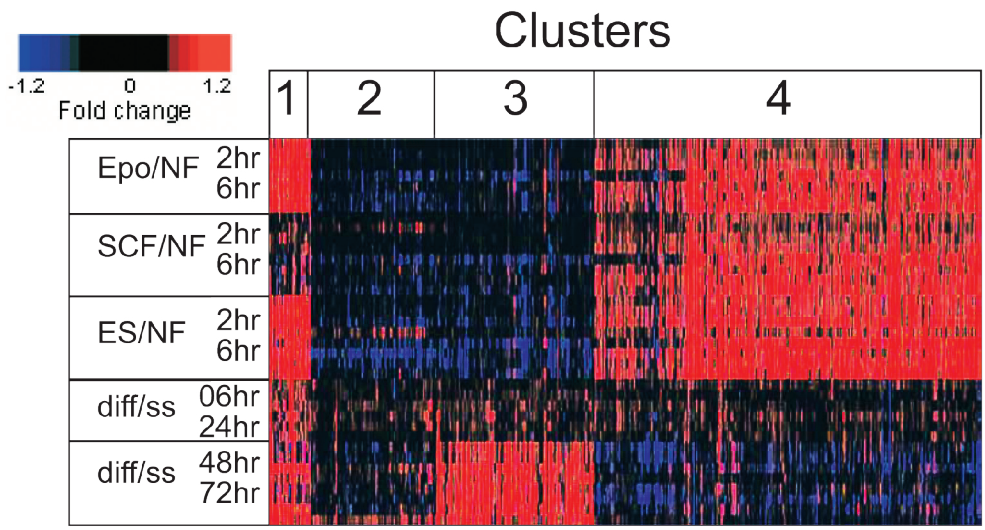

Figure 2 **Cluster analysis of genes that respond to growth factor stimulation and their regulation during differentiation**. Probe sets (Affymetrix MG_U74Av2) that are differentially expressed in response to growth factor stimulation were obtained by ANOVA comparing intensity data of hybridisations representing factor-deprived and restimulated cells (see text). These selected probe sets were clustered by gene expression ratios representing the effect of signal transduction and differentiation as indicated(differentiation time point/steady state; diff/ss). Rosetta resolver software was used for clustering (K-means and Pearson correlation). A bar in the left upper corner indicates how up- and down-regulation correlate with the intensity of red and blue respectively on a ^{10}log-scale.

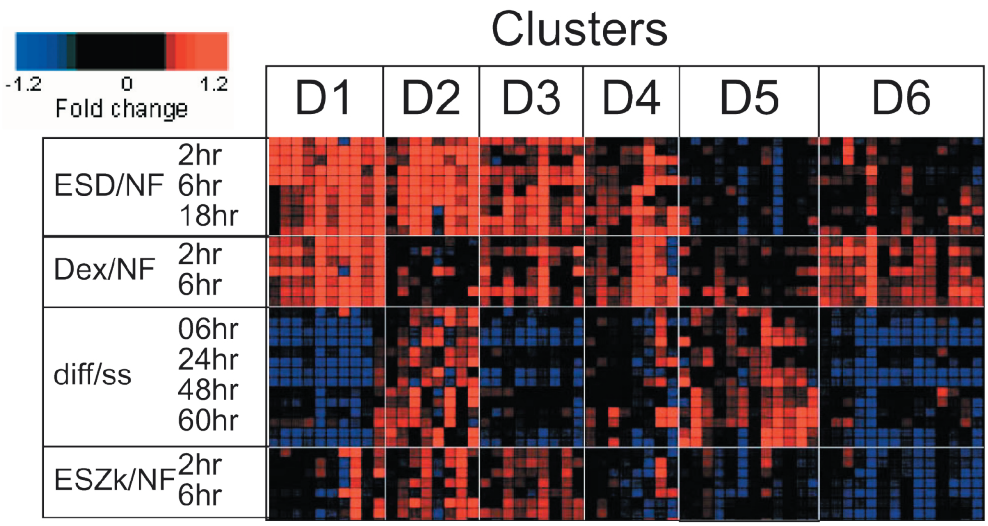

Figure 3 **Cluster analysis of genes that respond to Dexamethasone (Dex) and their regulation during renewal and differentiation conditions**. Probe sets (Affymetrix MG_U74Av2) that are differentially expressed upon addition of Dex were obtained by ANOVA, comparing intensity data of hybridisations representing ESD and ESZk conditions, combined with a comparison between Dex and NF (see text). The selected probe sets were clustered for regulation by Dex and differentiation conditions according to gene expression ratios as indicated (differentiation time point/steady state; diff/ss). Rosetta resolver software was used for clustering (K-means, Pearson correlation). A bar in the left upper corner indicates how up- and down-regulation correlate with the intensity of red and blue respectively on a ^{10}log-scale.

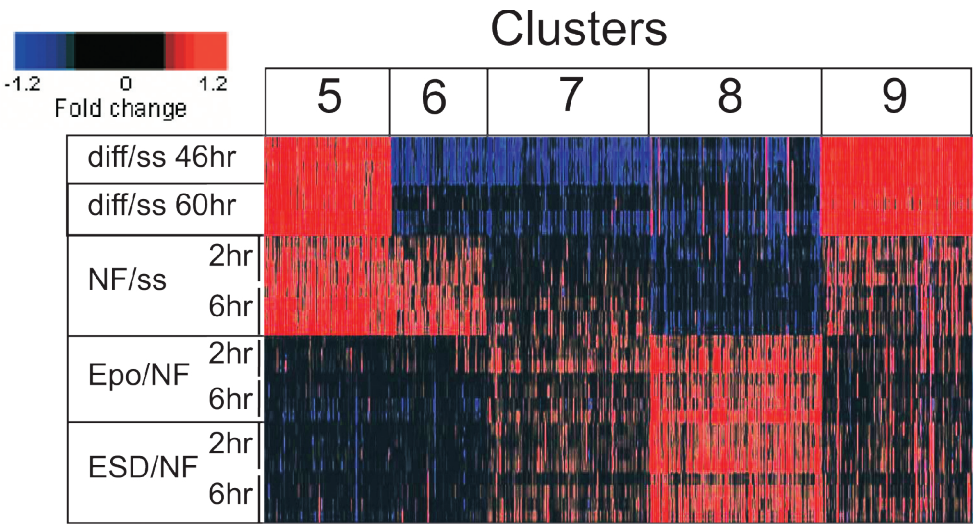

Figure 4 **Cluster analysis of genes that are regulated during differentiation and their expression during growth factor stimulation and deprivation conditions**. Probe sets (Affymetrix MG_U74Av2) that are differentially expressed upon differentiation induction were obtained by ANOVA, comparing intensity data of hybridisations representing differentiation time points and steady state cells. The selected probe sets were clustered for gene expression during differentiation, in response to growth factors and upon factor deprivation using gene expression ratios as indicated. Rosetta resolver software was used for clustering (K-means, Pearson correlation). A bar in the left upper corner indicates how up- and down-regulation correlate with the intensity of red and blue respectively on a ^{10}log-scale.

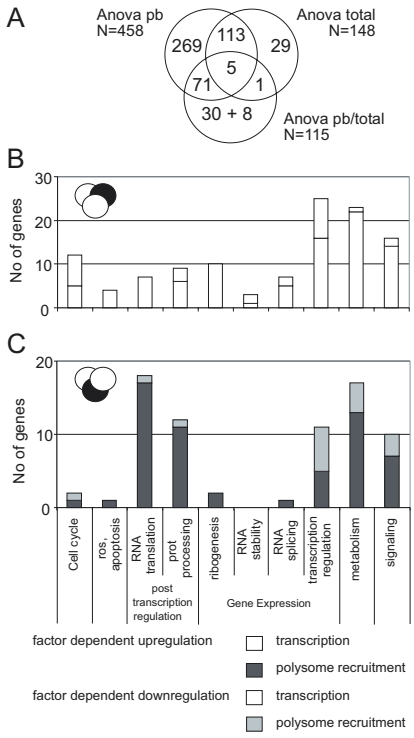

Figure 5 **Classification of selected genes according to regulation and function**. **A:** To analyse gene expression regulation in response to Epo/SCF ANOVA was used to compare intensity data of Epo/SCF treated (ES) versus untreated (NF) cells (see text). This yielded a selection of 458 probe sets that are regulated in pb RNA profiles (Anova pb) and 148 probe sets in total RNA profiles (Anova total) To select genes genes specifically regulated by polysome recruitment ANOVA was used to compare ES/NF ratios between pb and total RNA which selected for 115 probe sets (Anova pb/total)(see text). The Venn diagram shows the overlap of the ANOVA lists (Supplementary Table V, VI, VII). **B, C:** Signalling-controlled genes were classified according to function and according to regulation at at the level of transcription (total RNA level only [29 genes] or polysome association following transcription [113 genes] (**B**), or at the level of polysome recruitment (significant ratio pb/total and not transcriptionally regulated [109 genes])(**C**).

The different expression patterns are partly associated with the different nature of Dex target genes. They include genes known to be involved in glucocorticoid-induced apoptosis such as *Gpcr25* (*G-protein coupled receptor 25*; also known as *T-cell death associated gene 8 – Tdag8*) (Malone et al. 2004) and *Txnip* (*Thioredoxin-interacting protein) (Wang et al. 2006)*, but they also include genes known to be essential in hematopoietic development such as *jumonji, Jarid2 (AT rich interactive domain 2)* (Kitajima et al. 1999) and *cKit* (Broudy et al. 1996), or involved in cell cycle progression such as *Per1* (*Period homolog 1)* (Walisser and Bradfield 2006), *Nek2* and *Nek7* (*NIMA (never in mitosis gene a)-related expressed kinase 2* and *7)* (Quarmby and Mahjoub 2005), whereas induction of *Hpgd* (*hydroxyprostaglandin dehydrogenase)* suppresses prostaglandin-induced cAMP activity, which signals erythroid differentiation (Bakker et al, submitted). Notably Dex-induced expression of genes associated with apoptosis induction, such as *Gpcr25* (Tdag8), can also be further enhanced by SCF, which suggests that a G-protein coupled receptor that induces cell death in one cell type, may have a positive effect in another cell type. In addition, several Dex-induced genes are repressed by SCF, suggesting a protective effect of growth factor signalling on glucocorticoid-induced apoptosis and/or cell cycle inhibition. The expression of Dex-controlled genes in absence and presence of growth factors, during renewal and differentiation erythroid progenitors, may shed light not only on important processes in erythropoiesis, but also on the multifaceted role of glucocorticoids in lymphopoiesis, where glucocorticoids are mostly known as potent apoptosis inducers *(Webb et al. 2003)*.

Gene expression regulated during erythroid differentiation

Finally, we identified the genes that are up- or downregulated during differentiation to assess to what extent these genes are controlled by combinations of renewal or differentiation factors. Because the cluster analysis of the “signalling signature” genes indicated that the major changes in gene expression occur in samples taken late in differentiation, we compared polysome-bound RNA profiles of steady state growing erythroid progenitors with profiles of cells induced to differentiate for 48 and 60 h ($p=0.01$ for ANOVA between groups; $p=0.001^2$ for differential regulation in at least both I/11 or both R10 experiments). The selected genes are indicated as the “differentiation signature”. To asses the relation between gene regulation during differentiation and upon factor-deprivation and restimulation we clustered differential expression in differentiating compared to steady state growing progenitors with differential expression in factor-deprived compared to steady state growing, and in restimulated progenitors compared to factor-deprived progenitors. Restimulation involved renewal (Epo, SCF, Dex) or differentiation (Epo) factors. (Figure 4, supplemental Table S-IV). The first striking observation is the lack of discrimination between gene expression patterns induced upon restimulation of erythroid progenitors by differentiation (Epo) or renewal (Epo, SCF, Dex) factors for 2 or 6h. One third of genes downregulated during differentiation is upregulated by Epo- and Epo,SCF,Dex-induced signalling. This underlines again the ambiguous role of Epo, acting as a differentiation factor but most of all as a proliferation factor to increase the number of erythroid progenitors in response to hypoxia.

Epo/SCF-induced polysome recruitment

It is well recognised that transcript levels determined by total mRNA profiling differ from protein expression levels. Polysome bound mRNA profiling integrates control at the level of transcription, mRNA nuclear export and polysome recruitment, getting results closer to proteomics (Pradet-Balade et al. 2001). One of the factors controlling polysome recruitment in response to PI3K-activation is the cap-binding, eukaryotic translation initiation factor 4E (eIF4E) that is the limiting factor in the eIF4F pre-initiation scanning complex. We previously showed that overexpression of eIF4E blocked Epo-induced differentiation (Blazquez-Domingo et al. 2005), which indicates that translation initiation has a critical role in erythroid differentiation.

To estimate to what extent polysome recruitment contributes to Epo/SCF-controlled gene expression as a whole, we identified differential expression in response to Epo/SCF in total and pb RNA separately. First, we performed ANOVA ($p=0.01$) between the intensity data obtained with RNA from factor-deprived and Epo/SCF-restimulated cells. This yielded 470 probe sets for total RNA and 1051 for pb RNA. Further selection of differential expression in at least both I/11 or both R10 samples ($p=0.001^2$) reduced the number of probe sets to 148 for total RNA and to 458 for pb RNA (Figure 5A, supplemental Tables S-V, S-VI). The overlap between these sets (differential gene expression measured in total and pb RNA) is 118 probe sets (111 genes). This leaves 340 probe sets as being 'exclusively' regulated in polysome-bound mRNA.

To identify transcripts specifically subject to Epo/SCF-controlled polysome recruitment, we took another approach and directly compared ES/NF ratio's for total and pb RNA by ANOVA ($p=0.01$) plus differential expression in absence or presence of Epo/SCF in at least both I/11 or both R10 samples ($p=0.001^2$). To retain selective polysome recruitment with constant RNA expression as well as reduced RNA expression with maintenance of polysome binding, differential expression had to occur either in total or in pb RNA hybridisations . This reduced the number of differentially regulated genes between total and pb mRNA to 115 probe sets representing 111 genes (Grech, submitted, Table S-VII). Comparison of both methods indicates that only 62 of the 340 probe sets exclusively regulated by Epo/SCF in pb mRNA had been selected as differentially regulated in response to Epo/SCF between total and pb mRNA. The discrepancy is due to the fact that many genes are regulated at the level of total mRNA just below the probability threshold we set, while their regulation surpasses the threshold in pb mRNA (supplemental Table S-VI). In other words, transcription and translation initiation cooperate to enhance or reduce expression in response to Epo/SCF, but these genes are not specifically regulated by polysome recruitment. This phenomenon was also observed when higher or lower threshold were used. Importantly, however, approximately 20% of Epo/SCF-regulated genes was subject to major regulation at the level of polysome recruitment. This indicates that the use of pb mRNA to study differential gene expression in response to Epo, SCF and Dex or during differentiation (Figures 2-4) increased the sensitivity of our profiling study.

Cellular processes regulated by gene transcription or mRNA translation

To examine whether genes regulated specifically by polysome recruitment or regulated at the transcriptional level control different cellular processes, we classified

the genes controlled by Epo/SCF stimulation in total, or only in pb mRNA according to function (Figure 5B,C). The mRNAs encoding ribosomal proteins and some translation factors start with a terminal oligopyrimidine tract (TOP) that renders polysome recruitment sensitive to mTOR activation (Meyuhas 2000). These genes were mainly regulated in the pb mRNA fraction (Figure 3C), which validates our selection. Strikingly, also Epo/SCF-controlled expression of genes involved in protein modification and protein stability are mostly regulated by polysome recruitment. In contrast, genes involved in ribogenesis are mainly transcriptionally controlled and also Epo/SCF-controlled regulation of the cell cycle and gene transcription occurs mostly at the transcriptional level. Genes involved in metabolism are either translationally or transcriptionally controlled. Interestingly, this group contains 8 genes involved in lipid biosynthesis that are all regulated by gene transcription, and 6 genes involved in glucose metabolism that are all controlled by polysome recruitment.

Thus, certain cellular mechanisms are controlled by signalling-induced gene transcription, whereas other mechanisms are largely regulated at the level of translation initiation. The profiles established in this study enable us to analyse the expression of translation factors, including proteins involved in ribosome synthesis, that may drive translational control in the signalling- and differentiation signatures.

Regulation of translation factors:eIF2

Translation initiation is tightly regulated in response to cellular conditions. The two limiting translation factors regulated by environmental signals are (*i*) the cap-binding factor eIF4E that recruits all subunits of the pre-initiation scanning complex and (*ii*) eIF2, the prime factor of the eIF2·GTP/Met-tRNA$_i^{Met}$ ternary complex responsible for selection of the AUG start-codon (Clemens 2001; Pestova and Kolupaeva 2002). We have previously shown that activation of the PI3K/PKB/mTOR pathway and release of eIF4E is important for polysome recruitment of structured mRNAs (Blazquez-Domingo et al. 2005; chapter 3). Whereas eIF4E is regulated by mitogenic factors, eIF2 is phosphorylated and inhibited by 4 kinases that are activated in response to various stress conditions (Proud 2005; Wek et al. 2006). Interestingly, our data indicate that eIF2 function is positively regulated at the transcriptional level in response to growth factors (Table-I). This is of direct relevance to the profiles obtained with pb mRNA, because analyses of the 5'UTR of translationally regulated genes commonly revealed one or more uORF (chapter 6). The eIF2 level is the main determinant of uORF translation and its potential consequences for polysome recruitment (Morris and Geballe 2000).

The eIF2 is a trimeric complex existing of an α, β and γ chain (eIF2s1, eIF2s2 and eIF2s4). When the eIF2·GTP/Met-tRNA$_i^{Met}$ ternary complex (TC) has delivered methionine at the AUG start codon, eIF2·GTP is reduced to eIF2·GDP that is recycled to eIF2·GTP by eIF2B, consisting of 5 subunits. The eIF2α subunit can be phosphorylated on serine115. Phosphorylation does not affect the association of eIF2 with eIF2B, but it disables the reloading of eIF2 with GTP. The major eIF2 kinase in erythroid cells is eIF2ak1or haem-regulated kinase (HRI) (Han et al. 2001). The profiling results show that the α and β subunits of *eIF2* and the b1 subunit of *eIF2B* are upregulated in response to Epo/SCF signalling and downregulated during differentiation. In contrast, *HRI* is downregulated in response to Epo/SCF

and upregulated in differentiation (Table-I). Thus, Epo/SCF signalling increases the eIF2·GTP/Met-tRNA$_i^{Met}$ TC. This TC subsequently associates with the 40S ribosomal subunit which is mediated by a complex of translation initiation factors eIF1, eIF3 and eIF5 (Singh et al. 2005). These factors are involved in conformational changes of the complex when the AUG start codon is recognised. In addition, eIF5 binds eIF2·GDP, which may serve as a eIF2 reservoir or may compete for eIF2B/eIF2·GDP interaction and recovery of stable ternary complex (Singh et al. 2006). We observed that *eIF1* is upregulated by Epo/SCF and downregulated during differentiation, which is in agreement with increased availability of the TC. Surprisingly, *eIF5* is downregulated by signalling and clearly upregulated during differentiation (Table-I), suggesting that eIF5 may have a competitive role, rather than a supporting role in the function of the TC (Singh et al. 2006).

Regulation of translation factors: elongation and termination

Control of protein elongation and translation termination has a general effect on protein synthesis, but it may also affect the regulatory role of uORFs. Interestingly, we find downregulation of *elongation factor 1d* (*eEf1d)* and upregulation of its close homologue *Gtpbp2* during differentiation (Table-I), which is in accordance with the observation that these antagonistic factors have been found as a putative oncogene and tumorsuppressor gene respectively (De Bortoli et al. 2006; Mulholland et al. 2006). Micro-RNAs inhibit translation by inducing premature ribosome dissociation (Petersen et al., 2006). Both *Argonaut2*/*eIF2c2* and *Rck*/*Ddx6*, involved in translational repression via miRNAs (Chu and Rana 2006), are downregulated by Epo/SCF signalling (Table-I), indicating that growth factor signalling may repress miRNA function and increase the stability of translation elongation complexes. Finally, the translation termination factor *eTf1* is upregulated by Epo/SCF signalling, but its function in regulation of gene expression is unknown.

Proteins binding AU-rich elements

Proteins binding AU-rich elements (ARE) in the 3'UTR of transcripts recruit poly(A)-specific ribonuclease (Parn) and complete deadenylation results in degradation of the mRNA. But the length of the poly-A tail is also important for the amount of polyA binding protein (PABP) that can bind. PABP directly associates with the eIF4F complex and stabilizes pre-initiation scanning complexes (Mangus et al. 2003). In addition, ARE-binding proteins can directly recruit PABP to interact with the pre-initiation complexes. The ARE are present in the 3' untranslated region of many short-lived transcripts from cytokines, proto-oncogenes, growth factors or cell cycle regulators (Bakheet et al. 2001). Interestingly, the Tristetraprolin family members *Brf1 (Butyrate responsive factor 1 or Znf36-like 1)* and *Brf2 (Znf36l2)* known to promote ARE-dependent decay, are regulated by SCF signalling and during differentiation in an opposing way. Whereas *Brf1* is active in late, almost mature erythroid cells, *Brf2* expression is induced by renewal conditions (Table-I). Notably, *Brf2* is also strongly induced by glucocorticoids. Mice lacking *Brf1* or *Brf2* have different phenotypes (Ramos et al. 2004, Ciais et al., 2004), suggesting that these homologues have distinct targets; one of which is VEGF repressed by Brf1, which is accordance with *VEGF* upregulation in renewing erythroid progenitors and downregulation in

		E2	E6	S2	S6	ES2	ES6	ESD2	ESD6	D2	D6	diff-6	diff-22	diff-46	diff-60
rRNA gene transcription															
polymerase (RNA) I associated factor 1	Praf1	1.87	2.61	1.84	3.02	2.53	3.56	2.53	3.62	0.99	1.48	0.79	0.74	0.34	0.34
RNA polymerase 1-2	Rpo1-2	1.23	1.45	1.15	1.61	1.43	1.61	1.33	1.70	0.96	1.33	0.95	0.95	0.77	0.79
RNA polymerase 1-3	Rpo1-3	1.87	1.88	1.44	1.78	2.10	2.40	1.71	1.99	0.92	0.94	1.18	0.95	0.49	0.49
RRN3 RNA polymerase I transcription factor homolog	Rrn3	1.10	1.34	1.26	1.33	1.38	1.45	1.37	1.46	0.87	1.18	1.09	1.14	0.70	0.73
Processing 35S pre-RNA															
block of proliferation 1	Bop1	1.19	2.50	1.33	2.68	1.64	3.89	1.42	3.82	0.93	0.97	0.88	1.27	0.45	0.42
pescadillo homolog 1 (zebrafish)	Pes1	1.01	0.93	0.98	1.00	1.11	1.00	1.00	1.07	1.02	0.99	0.94	0.91	0.51	0.47
DEAD (Asp-Glu-Ala-Asp) box polypeptide 52	Ddx52	1.13	0.90	1.17	0.93	1.21	0.93	1.15	0.94	1.01	1.03	0.97	1.00	0.52	0.49
Processing 25 and 5.8S rRNA in 60S subunit															
brix domain containing 1	Bxdc1	1.62	1.76	1.50	1.76	1.99	2.16	1.69	2.03	0.77	1.10	0.89	0.92	0.52	0.63
brix domain containing 2	Bxdc2	1.16	1.19	1.16	1.36	1.36	1.34	1.32	1.27	0.98	0.94	0.95	1.16	0.58	0.61
peter pan homolog (Drosophila)	Ppan	1.38	1.10	1.50	1.15	1.60	1.36	1.52	1.54	0.76	1.06	1.09	0.96	0.49	0.52
exosome component 5	Exosc5	1.16	1.27	1.13	1.26	1.15	1.32	1.11	1.22	0.90	0.66	1.01	0.96	0.67	0.61
U3 snoRNP complex: trimming 18S															
UTP14, U3 snRNP homolog A (yeast)	Utp14a	1.50	1.43	1.29	1.60	1.80	1.87	1.70	2.35	1.04	1.45	0.81	0.47	0.05	0.22
IMP3, U3 snRNP, homolog (yeast)	Imp3	1.42	1.39	1.31	1.38	1.64	1.55	1.54	1.37	0.93	0.83	1.11	0.63	0.63	0.63
fibrillarin	Fbl	1.03	0.96	0.95	0.92	1.01	1.07	1.05	1.07	1.01	0.69	1.05	1.05	0.60	0.53
RNA terminal phosphate cyclase-like 1	Rcl1	1.07	1.34	1.29	1.72	1.32	1.62	1.08	1.52	0.82	1.16	0.99	0.87	0.49	0.52
EMG1 nucleolar protein homolog	emg1	1.22	1.20	1.07	1.19	1.18	1.37	1.20	1.20	1.04	0.80	1.04	0.90	0.74	0.70
nucleolar protein family A, member 2	Nola2	1.54	1.86	1.44	1.82	1.65	2.18	1.59	1.94	0.88	0.80	1.08	1.11	0.77	0.75
nucleolar protein 5	Nol5	1.33	1.45	1.31	1.52	1.51	1.77	1.38	1.74	0.89	0.92	0.96	0.82	0.44	0.48
rRNA modification															
FtsJ homolog 3 (E. coli)	Ftsj3	1.06	1.36	1.10	1.62	1.51	1.73	1.25	1.67	0.93	1.29	0.93	1.05	0.43	0.45
nucleolar protein 1	Nol1	1.68	1.92	1.75	2.26	2.18	2.58	2.15	2.65	0.92	1.23	0.96	0.99	0.73	0.60
nucleolar protein 5A	Nol5a	1.16	1.73	1.11	1.92	1.62	2.27	1.45	1.95	0.94	1.33	0.88	0.99	0.46	0.42
NOL1/NOP2/Sun domain family 2	Nsun2	1.25	1.25	1.07	1.30	1.25	1.45	1.15	1.34	0.93	0.84	0.90	0.87	0.58	0.61
NOL1/NOP2/Sun domain family 2	Nsun2	1.42	1.63	1.15	1.61	1.48	1.83	1.40	1.61	0.92	0.72	0.92	0.86	0.53	0.58
NOL1/NOP2/Sun domain family 2	Nsun2	1.26	1.35	1.11	1.40	1.32	1.53	1.23	1.36	0.96	0.85	0.94	0.90	0.58	0.61
nucleolar and coiled-body phosphoprotein 1	Nolc1	1.77	2.01	1.64	2.13	2.24	2.46	1.88	2.15	0.92	1.07	0.87	0.92	0.52	0.48
nucleolar protein family A	Nola1	1.06	1.04	1.01	1.09	1.07	1.12	1.02	1.07	1.01	0.94	0.94	0.52	0.74	0.68
60S assembly															
DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	Ddx18	1.17	1.44	1.22	1.49	1.59	1.73	1.31	1.67	0.92	1.03	0.90	0.78	0.56	0.55
DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	Ddx18	1.37	1.75	1.35	1.74	1.87	1.91	1.37	1.73	0.95	1.27	0.98	0.94	0.64	0.53
DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	Ddx21	1.30	1.63	1.29	1.67	1.60	1.59	1.41	1.58	1.03	1.07	0.97	0.92	0.64	0.61
DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	Ddx51	1.16	1.68	1.27	1.91	1.62	2.27	1.57	2.50	1.01	1.32	0.97	1.00	0.38	0.42
DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	Ddx54	0.98	1.05	0.96	1.01	1.07	1.13	1.06	1.20	0.91	0.92	1.02	0.94	0.53	0.49
nuclear import 7 homolog	Nip7	1.85	1.81	1.75	1.85	2.83	2.32	2.14	2.12	0.88	1.16	0.93	0.97	0.43	0.44
60S export and trafficking															
NMD3 homolog (S. cerevisiae)	Nmd3	2.01	1.34	1.76	1.47	2.69	1.61	2.17	1.43	0.87	0.80	0.99	1.12	0.64	0.82
GTP binding protein 4	Gtpbp4	2.07	1.83	1.47	1.58	2.26	1.92	2.12	1.84	0.97	1.00	0.99	0.89	0.84	0.92
RRS1 ribosome biogenesis regulator homolog (S. cerevisiae	Rrs1	2.01	1.66	1.86	2.01	2.69	2.05	2.17	2.02	0.87	0.98	1.00	0.89	0.44	0.50
40S export and trafficking															
nucleolar complex associated 4 homolog (S. cerevisiae)	Noc4l	1.52	1.96	1.23	1.53	1.76	2.20	1.60	2.06	0.97	1.03	0.99	1.04	0.71	0.67
rRNA degradation															
exosome component 8	Exosc8	0.89	0.93	0.86	0.95	0.82	0.83	0.89	0.82	1.00	1.26	1.12	1.57	4.26	3.44
nucleolar protein, function unknown															
guanine nucleotide binding protein-like 3	Gnl3	1.05	1.19	1.10	1.25	1.23	1.25	1.13	1.29	0.90	1.00	0.91	0.85	0.48	0.48
midnolin	Midn	1.68	1.50	2.17	1.98	2.76	1.56	2.39	1.62	0.86	1.36	0.68	0.59	0.69	0.87
Mki67 (FHA domain) interacting nucleolar phosphoprotein	Mki67ip	1.41	1.39	1.42	1.52	1.96	1.72	1.61	1.61	0.87	1.18	0.94	1.31	0.54	0.56
nucleophosmin 1	Npm1	1.25	1.20	1.21	1.18	1.29	1.24	1.22	1.19	0.92	0.81	0.98	0.90	0.67	0.71
nucleoplasmin 3	Npm3	1.25	1.32	1.03	1.25	1.37	1.80	1.14	1.48	0.90	0.82	0.87	0.79	0.59	0.42
proliferation-associated 2G4	Pa2g4	1.49	1.63	1.46	1.59	1.46	1.83	1.61	1.73	0.97	0.80	0.92	0.94	0.51	0.59

translation initiation factors															
eukaryotic translation initiation factor 1A	Eif1a	1.98	1.74	1.95	1.80	2.55	2.07	2.35	1.89	0.96	1.08	0.95	0.51	0.59	0.58
eukaryotic translation initiation factor 1A	Eif1a	2.09	1.85	2.17	1.90	2.65	2.24	2.46	2.26	0.91	1.03	1.09	0.93	0.34	0.39
eukaryotic translation initiation factor 5	Eif5	0.84	0.71	0.82	0.71	0.79	0.72	0.77	0.68	0.98	0.83	1.13	1.21	1.60	2.04
eukaryotic translation initiation factor 2 alpha kinase 1	Eif2ak1	0.89	0.74	0.82	0.67	0.65	0.55	0.78	0.61	1.11	1.20	0.99	1.18	3.27	3.96
eukaryotic translation initiation factor 2B, subunit 1 (alpha)	Eif2b1	1.25	1.42	1.29	1.40	1.57	1.65	1.37	1.62	0.90	0.93	1.08	1.06	0.44	0.45
eukaryotic translation initiation factor 2, subunit 1 alpha	Eif2s1	1.44	1.76	1.30	1.59	1.63	1.84	1.57	1.63	1.01	1.04	1.02	1.18	1.01	0.93
eukaryotic translation initiation factor 2, subunit 2 (beta)	Eif2s2	1.29	1.64	1.36	1.48	1.67	1.99	1.38	1.65	0.86	0.78	0.98	0.75	0.68	0.71
eukaryotic translation initiation factor 4B	Eif4b	1.18	0.97	1.17	1.10	1.17	1.05	1.21	1.03	1.08	0.81	0.97	0.78	0.63	0.58
translation elongation and termination															
eukaryotic translation elongation factor 1 delta	Eef1d	1.22	1.17	1.11	1.13	1.36	1.29	1.29	1.31	0.95	0.84	1.08	1.07	0.72	0.61
GTP binding protein 2	Gtpbp2	0.82	0.73	0.87	0.63	0.73	0.64	0.70	0.65	1.02	1.17	0.87	0.92	2.78	4.14
eukaryotic translation termination factor 1	Etf1	1.54	1.71	1.52	1.68	1.83	1.96	1.85	1.85	0.91	1.06	1.09	1.05	0.82	0.87
Eukaryotic translation termination factor 1 (Etf1), mRNA	Etf1	1.58	1.78	1.52	1.68	1.93	2.03	1.69	1.76	0.94	1.02	1.04	1.07	1.02	1.06
Eukaryotic translation termination factor 1 (Etf1), mRNA	Etf1	1.56	1.71	1.56	1.69	1.91	2.06	1.74	1.76	0.94	0.95	1.07	1.12	0.98	1.01
eukaryotic translation termination factor 1	Etf1	1.71	1.82	1.54	1.84	2.04	2.23	1.97	2.12	0.91	0.90	1.00	0.80	0.78	0.78
Hbs1-like (S. cerevisiae)	Hbs1l	0.93	1.01	1.12	1.13	1.04	1.05	1.05	1.05	1.06	1.14	0.97	1.00	1.12	1.23
RNA-binding proteins involved in selective mRNA translation															
fragile X mental retardation syndrome 1 homolog	Fmr1	1.02	1.09	1.21	1.19	1.15	1.06	1.10	1.08	0.83	0.83	1.09	0.94	0.47	0.49
poly A binding protein, cytoplasmic 4	Pabpc4	1.04	1.00	1.04	0.97	1.15	1.01	1.09	1.11	1.01	1.23	1.15	1.16	1.57	1.33
poly(rC) binding protein 2	Pcbp2	1.29	1.18	1.27	1.21	1.37	1.18	1.35	1.26	0.91	0.87	0.99	0.61	0.69	0.68
miRNA function															
eukaryotic translation initiation factor 2C, 2 (Argonaut)	Eif2c2	0.72	0.52	0.98	0.62	0.66	0.56	0.61	0.62	1.07	1.13	0.91	0.94	0.84	0.65
DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 (Rck)	Ddx6	0.86	0.62	1.08	0.46	0.71	0.34	0.77	0.41	1.23	1.05	1.19	0.94	1.19	0.92
ribosomal proteins															
ribosomal protein L10A	Rpl10a	1.17	1.22	1.14	1.16	1.27	1.23	1.20	1.17	0.96	0.86	1.03	0.97	0.88	0.89
ribosomal protein L13a	Rpl13a	1.23	1.40	1.16	1.44	1.65	1.66	1.48	1.42	0.96	1.04	0.89	0.71	0.88	0.81
ribosomal protein L18	Rpl18	1.16	1.27	1.14	1.21	1.33	1.33	1.25	1.32	0.95	1.16	0.99	1.20	0.79	0.71
ribosomal protein L19	Rpl19	1.01	1.11	1.10	1.15	1.02	1.28	1.00	1.10	1.06	0.95	1.11	1.28	1.65	1.70
ribosomal protein L24	Rpl24	1.34	1.38	1.27	1.41	1.56	1.64	1.41	1.52	0.89	0.92	1.02	1.16	1.04	1.15
ribosomal protein L27a	Rpl27a	1.35	1.34	1.06	1.36	1.77	1.76	1.56	1.47	0.86	0.71	1.12	1.20	1.15	1.09
ribosomal protein L28	Rpl28	1.35	1.20	1.22	1.17	1.64	1.49	1.47	1.53	0.84	0.83	1.13	1.16	1.02	0.94
ribosomal protein S10	Rps10	1.12	1.12	1.05	1.11	1.22	1.26	1.18	1.15	0.95	0.97	1.02	1.02	0.85	0.81
ribosomal L1 domain containing 1	Rsl1d1	1.27	1.39	1.26	1.45	1.61	1.58	1.48	1.49	0.94	0.87	0.95	1.04	0.53	0.55
RNA stability and translation															
TROVE domain family, member 2	Trove2	0.90	0.79	0.85	0.70	0.95	0.75	0.92	0.78	0.89	1.03	1.09	1.08	1.61	3.10
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1	Elavl1	1.17	1.34	1.25	1.32	1.33	1.64	1.34	1.72	0.98	0.85	0.99	0.94	0.76	0.73
zinc finger protein 36, C3H type-like 1	Zfp36l1	0.77	0.93	0.74	0.89	0.51	0.66	0.45	0.54	0.83	1.21	1.09	0.47	1.65	1.85
zinc finger protein 36, C3H type-like 2	Zfp36l2	4.50	1.26	19.85	4.62	24.96	2.61	15.50	3.42	6.06	5.95	0.48	1.22	0.19	0.12
Zinc finger protein 36, C3H type-like 2	Zfp36l2	0.92	0.79	1.39	1.44	1.18	0.87	1.71	1.52	1.57	1.60	0.68	1.36	0.33	0.39
nuclear cap binding protein subunit 1	Ncbp1	1.13	1.09	1.01	1.33	1.18	1.28	1.09	1.34	1.01	1.05	0.82	0.94	0.63	0.56

Table 1 **Genes involved in mRNA translation, regulated by signal transduction and/or during differentiation**. Expression data shown are weighted averages if fold changes; Epo/NF (E), SCF/NF (S), Epo+SCF/NF (ES), Epo+SCF+Dex (ESD), Dex/NF (D) and differentiation/steadystate (Diff). The number indicates hours of exposure.

differentiation (Table-SII). Also members of the ELAV family of RNA-binding proteins bind and stabilize ARE-containing transcripts, and promote their translation (Ford et al. 1999). *Elavl1* (human antigen R; HuR) is moderately upregulated under renewal conditions (Table-I). In contrast, the expression of *Trove-2* (Sjogren syndrome antigen B, Ssb) is upregulated during differentiation. *Trove-2* was identified as an auto-antigen in Lupus erythromatosis, and the protein was found to control polysome recruitment of specific mRNAs (Horton et al. 2001).

Control of mRNA translation: ribosome biosynthesis

Growth factor signalling limits the energy consuming protein synthesis to growth and proliferation of cells. Mitogenic signals are required to induce rRNA synthesis (Stefanovsky et al. 2001; James and Zomerdijk 2004) and to recruit transcripts encoding proto-oncoproteins (De Benedetti and Graff 2004), ribogenesis components (Jefferies et al. 1994) and translation factors (Terada et al. 1994) to polysomes. Mitogenic factors were assumed to control ribosome biosynthesis through factor-dependent translation of ribosomal proteins. The terminal oligopyrimidine (TOP) sequence immediately following the mRNA cap is supposed to render translation dependent on mTOR activation. However, we found that Epo/SCF-controlled translation of ribosomal proteins is modest (at most 1.5 fold). Instead, control of other key regulators may contribute to control of ribogenesis. Knowledge on ribosome biosynthesis has rapidly increased as ribosomes or protein complexes containing specific ribosomal proteins have been isolated from yeast to be analysed by mass spectroscopy (Fromont-Racine et al. 2003). Subsequent domain analysis in combination with genetic experiments has indicated that the generation of ribosomes requires a very complex biosynthesis machinery in which at least 170 nucleolar proteins are found to participate. Whereas the analysis of ribosome biosynthesis has revealed control mechanisms in yeast, to date little is know about the regulation in higher eukaryotes. Nevertheless, defects in ribosomal and nucleolar proteins underlie diseases such as Diamond Blackfan Anemia (DBA) and Swachman-Bodian-Diamond syndrome (SBDS)(Liu and Ellis 2006).

The initial step in the nucleolus is the generation of noncoding ribosomal RNA. The 35S pre-rRNA is transcribed by RNA polymerase I, a 5S rRNA is transcribed by RNA polymerase III. Expression of several proteins in both polymerase complexes (Rpo1-3, Rpo1-1, Rrn3, Polr3k) is upregulated in response to Epo/SCF (Table-I), but the major regulation is on *Polr1e* (polymerase I polypeptide E; at least 4-fold up by Epo/SCF and downregulated during differentiation), a transcription factor that associates with Ubtf (upstream binding transcription factor) to activate Polymerase I activity (Voit and Grummt 2001). Notably, this interaction requires PI3K-dependent phosphorylation of Ubtf (Drakas et al. 2004), which places activation of Pol-I by the Polr1e/Ubtf complex under tight control of Epo/SCF.

Next, the 35S pre-rRNA is processed in the 90S preribosome to produce the 18S rRNA integrated in the 40S ribosomal subunit, and 25 plus 5.8 rRNAs integrated in the 60S ribosomal subunit. The processing complex harbours Bop1 (block of proliferation 1) and Pes1 (pescadillo homolog 1) (Gratenstein et al. 2005). *Bop1* is strongly upregulated by Epo/SCF, and both *Bop1* and *Pes1* are downregulated during differentiation.

Following cleavage of the pre-RNA, the subunits are trimmed to their mature size. For the 25 and 5.8 rRNAs of the 60S ribosomal subunit, this requires brix-domain

containing proteins Bxdc1 and Bxdc2, and Ppan (Peter pan homologue) (Eisenhaber et al. 2001). Maturation of the 18S rRNA of the 40S subunit requires Imp3 and Imp4 associated with Mphosph10 in the U3-snoRNP complex (Granneman et al. 2003). Several Brix domain-containing proteins are upregulated 1,5 to 2-fold in response to Epo/SCF. In addition, various U3-snoRNP associated proteins are upregulated in response to Epo/SCF and downregulated in differentiation (Table-I), the exact role of which is not clear.

Maturation of the rRNA subunits also requires extensive modification of nucleotides, which mainly occurs in regions that do not associate with proteins and which may stabilize the conformation of the rRNA subunit. Nsun2 (Nol1/Nop2/Sun domain family 2), Nol1 (nucleolar protein 1), Nol5a and ftsj3 (FtsJ homolog E.coli) are all involved in rRNA methylation. *Nol1* and *Nol5a* are most prominently upregulated in response to Epo/SCF, whereas *Nol5a* and *Ftsj3* are downregulated during differentiation. Notably, expression of *Nol1* has been associated with many types of cancer (Busch et al. 1991; Husson et al. 2002). In addition, Nola1 and Nolc1 are involved in pseudo-uridinylation of rRNA and particularly Nolc1 is significantly upregulated in response to Epo/SCF (Table-I).

Proper folding of rRNAs and interaction with ribosomal proteins is promoted by RNA helicases of the Ddx-family (DEAD (Asp-Glu-Ala-Asp) box polypeptides). We found Ddx18, Ddx21, Ddx51 and Ddx54 that are all involved in assembly of the 60S preribosomeal subunit to be upregulated in response to Epo/SCF and downregulated during differentiation.

Finally the import of all proteins into the nucleolus and the export of the 60S ribosomal subunit to the cytoplasm also needs to be controlled. Among the genes induced in response to Epo/SCF is Nip7 (nucleolar import 7) and the export factors Rrs1 (ribosome biogenesis regulator homolog) and Nmd3 that both interact with Rpl11 and control trafficking of the large ribosomal subunit within the cell. Both factors are similarly upregulated in response to Epo/SCF (3-fold) but *Rrs1* is prominently downregulated in differentiation. Export of the 60S subunit requires several GTPases. Gtpbp4 (Nog1), which binds the trafficking factor Nmd3, is strongly upregulated by Epo/SCF, and known to be regulated by mTOR (Honma et al. 2006). Thus, cooperative signals emanating from the EpoR and cKit enhance ribosome biosynthesis at the level of rRNA synthesis, processing and modification, and by facilitating the interaction of mature rRNA with ribosomal proteins, and the export of ribosomal subunits.

Genetic experiments have predominantly been performed in yeast and deletion of most of the proteins mentioned above is lethal. In contrast, overexpression may contribute to tumorigenesis. However, disruption of normal expression may have cell type specific effects. For example, enhanced expression of pes1 or UBF in 32D cells did not render cells factor-independent nor did it interfere with differentiation, whereas both factors efficiently transformed mouse embryo fibroblasts (Prisco et al. 2004). Interestingly, the nuclear import factor Nip7 (KD93) was identified as a novel protein expressed in human hematopoietic stem/progenitor cells (Liu et al. 2004).
Together, the regulation of so many factors involved in ribosome synthesis strongly suggests that the number of ribosomes is tightly regulated. The question is what for? It is mostly assumed that proliferative signals have to enhance global and specific protein synthesis, which is repressed during terminal differentiation when

cell division stops (Hensold et al. 1996; Krichevsky et al. 1999; Kroll et al. 2001). However, signalling via mTOR is able to repress erythroid progenitor differentiation (Blazquez-Domingo et al. 2005; chapter 3). Just as the availability of translation factors specifically controls translation of structured transcripts, this may also be true for the availability of ribosomal subunits. Moreover, it becomes increasingly clear that ribosomal proteins may interact with specific structural elements in transcripts to act as a translational regulator (Remacha et al. 1995; Jefferies et al. 1997; Mazumder et al. 2003). The finding that the composition of ribosomes is cell- and cell state-specific with altered affinity for specific mRNAs supports that the ribosome is not only a general protein synthesis machine, but involved in selective transcript translation (Mauro and Edelman 2002). Therefore, this analysis of signalling-controlled ribosome synthesis is not only the analysis a random set of signalling targets, but should help to explain the difference between a signalling dependent gene expression profile obtained with total or polysome bound mRNA. We need to understand how signalling controls the protein synthesis machinery, to be able to unravel the mechanisms that regulate translation of specific transcripts.

MATERIALS AND METHODS

Cells

I/11 cells were cultivated in StemPro-34™ medium (Life Technologies) as described (von Lindern et al. 2001). For expansion, the medium was supplemented with 0.5U/ml Epo, (kind gift from Ortho-Biotech, Tilburg, The Netherlands), 100ng/ml SCF (supernatant of CHO producer cells) and 10^{-6}M dexametasone (Dex, Sigma-Aldrich). To induce differentiation, cells were cultivated in StemPro-34™ medium supplemented with 5U/ml Epo and 0.5mg/ml iron-loaded transferrin (Intergene). For factor deprivation and restimulation, cells were washed twice in Hank's buffered saline solution (HBSS; Life technologies) and seeded in IMDM supplemented with 0.2% purified BSA (Life Technologies). After 4h Epo (5U/ml), SCF (500ng/ml), Dex (10^{-6}M), ZK112,993 (10^{-6}M) were added as indicated for 2h, 6h or 18h. Cell numbers and cell size distribution were determined using an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany).

Microarray hybridization and analysis

A MIAME compatible description of sample preparation and hybridization protocols is given in supplementary data. The data extraction strategy is described in supplementary Figure S1. Microarray data were normalised using the Rosetta Resolver ® system, as described in (Weng et al. 2006). Weighted averages were calculated using log error data extracted for each probe set. The error weight was calculated (log error / total log error of 4 hybridisations) and the average calculated integrating the error weight.

Supplementary Data

Supplementary Tables are found online http://www1.erasmusmc.nl/hematologie/index.php?cId=73.

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Retroviral Insertion interferes in growth factor induced polysome recruitment of the putative oncogene Nm23-M2

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Short title: Distruption of translation control

Work in Progress

CHAPTER 5

ABSTRACT

The 5'UTR of the nucleotide diphosphate kinase Nm23-M2 renders translation of Nm23-M2 dependent on PI3K-induced polysome recruitment. Nm23-M2 is also a common viral integration site in MLV-induced mouse leukaemia. In this study we demonstrate that viral integrations 5' of the AUG start codon can perturb factor dependent translation of the targeted gene. A virus integration in the 5'UTR of Nm23-M2 in the murine leukaemia cell line NFS61 results in a LTR-Nm23 fusion transcript that disrupts a predicted stem-loop structure. This is associated with growth factor-independent polysome recruitment of Nm23-M2. To identify the transcription start site of Nm23, 5'RACE experiments were conducted on cDNA generated at 60°C to overcome secondary structure. This approach characterised a longer 5'UTR containing high enthalpy structures, upstream open reading frames and putative signaling responsive elements. To study the effect of these structures on control of mRNA translation, luciferase constructs flanked with *Nm23-M2* UTR sequences were expressed in factor-dependent cell lines to compare contribution of putative regulatory elements to PI3K-dependent polysome recruitment. Upstream ORFs in the distal part of the 5'UTR appeared to enhance polysome recruitment, while stem-loop elements in the proximal part of the 5'UTR inhibit translation. In contrast, upstream ORFs in the 5'UTR of the transcript *mEd2*, attenuate polysome recruitment. Thus, the position and interplay between regulatory elements within a UTR orchestrate translation control of a transcript. Proviral integration within complex 5'UTRs resulting in deletion of regulatory elements constitutes a novel mechanism of constitutive expression of potential oncogenes.

INTRODUCTION

Retroviral insertion mutagenesis has proven to be a powerful method to identify genes involved in leukaemia, particularly now that insertion sites can be combined with expression profiling in human AML (Erkeland et al. 2006). Various mechanisms may account for retrovirally induced malignancies (Jonkers and Berns 1996; Wolff 1997). Retroviral insertion can activate transcription when its long terminal repeat (LTR) acts as a promoter or as an enhancer of neighbouring genes. Insertion within the 3' untranslated region (3'UTR) of a gene may result in removal of destabilising elements and hence promotes stabilisation of the transcript. In contrast, retroviral insertions within the coding sequence of the gene may silence expression. A so far underestimated mechanism by which retroviral insertions may interfere with gene expression is through replacement of a structured 5'UTR that imposes translation control on the transcript.

Translation initiation requires binding of the eukaryotic translation initiation factor 4E (eIF4E) to the mRNA cap. eIF4E is a limiting factor kept in check by eIF4E binding protein (4E-BP). Phosphorylation of 4E-BP releases eIF4E to associate with the scaffold protein eIF4G and the RNA helicase eIF4A to form the eIF4F scanning complex which associates with the 40S small ribosomal subunit to scan the 5'UTR for the first AUG codon in an appropriate context (Kozak 1989). Another rate limiting factor in translation initiation is eIF2. GTP-bound eIF2 associates with methionine loaded tRNA to form the the eIF2·GTP/Met-tRNA$_i^{Met}$ ternary complex (TC), which also associates with the 40S ribosomal subunit and serves to recognise the AUG initiation codon. At the initiation codon, the 40S and 60S subunits associate to start protein synthesis.

Secondary structures and upstream open reading frames (uORF) can inhibit scanning of the 5'UTR and silence translation (Manzella and Blackshear 1990). Overexpression of eIF4E can release inhibition of translation from structured mRNA (Manzella et al. 1991; Shantz et al. 1996; Koromilas et al. 1992; Blazquez-Domingo et al. 2005), most likely because the pre-initiation scanning complex supplies RNA helicase activity to melt complex 5'UTR sequences. In addition to structures with high enthalpy other variables such as the relative distance of hairpin loop structures from the 5' methyl G cap (Babendure et al. 2006), association of specific proteins with stem-loop structures (Cazzola and Skoda 2000) and the presence of upstream Open Reading Frames (uORFs), attenuate translation initiation (Morris and Geballe 2000). Translation of uORFs can attenuate translation of the proper ORF as a result of premature dissociation of ribosomes (Kozak 1987; Child et al. 1999), stalling of the scanning complex by interaction of the nascent peptide with components of the complex (Ruan et al. 1996) or due to the presence of rare codons (Meijer and Thomas 2003). Conversely, uORFs can release translation silencing conferred by inhibitory elements within the 5'UTR. Once the transcript is engaged in peptide production, secondary structures are more easily unfolded.

Polysome recruitment of *Nm23-M2* is sensitive to growth factors and availability of the cap-binding translation initiation factor eIF4E (Blazquez-Domingo et al. 2005). Interestingly, *Nm23-M2* was found to be a common retroviral interation site in murine leukemia's induced by the CasBr-M Murine Leukemia Virus (MuLV) (Joosten et al. 2002). We here describe retroviral insertion within the 5'UTR of *Nm23-M2* that replaces the structured 5'UTR by viral LTR sequences. This fusion

transcript of proviral LTR-Nm23 has lost translation control (Figure 1C). We studied regulatory elements in *Nm23-M2* transcript that are lost upon retroviral insertion. Similar regulatory elements found in *mEd2* transcript, previously identified as a translationally controlled gene, were studied as a comparison. The elements in these two transcripts are differentially distributed along the 5'UTR. Using in vitro systems we dissected the UTRs to identify potential regulatory elements. Response to PI3K activity is assessed using mouse pre-B cell line, Ba/F3 and chicken erythroblasts, HD3. Surprisingly, the newly found uORFs in the distal part of the transcript relax translational control by the secondary structure in the proximal region of the *Nm23-M2* 5'UTR. The underlying mechanism is still unclear, although specific recognition and expression of uORFs might play a role in derepression of translation from the proper AUG. Finally, a high enthalpy structure and a putative signalling responsive element in *Nm23-M2* UTR have been assigned to the proximal region of the proper AUG. These elements are missing in the transcript isolated from the tumour-derived cell line harbouring the retroviral integration in *Nm23-M2*.

RESULTS

Viral integration results in a LTR-Nm23-M2 fusion transcript with simple 5'UTR

In CasBr-induced mouse leukemia, several retroviral integrations have been mapped 5' of the AUG start codon of *Nm23-M2*. (Joosten et al. 2002). Analysis of 20 cell lines derived from MLV-transformed murine leukemias indicated the presence of a retroviral integration 5' of *Nm23-M2* in the NF61 cell line. This offered the possibility to isolate RNA transcripts and identify a potential fusion transcript. Using oligonucleotide primers in the ORF of *Nm23* and in the CasBr LTR, we amplified a LTR-Nm23 fusion transcript (Figure 1). Interestingly, the integration is within the 5'UTR of *Nm23-M2* in between two inverted repeats and disrupts a high enthalpy stem-loop structure. Similarly the Terminal OligoPyrimidine (TOP) tract previously identified by Rapid Amplification cDNA Extension (RACE) is lost. Hence a complex 5'UTR is changed into a simple one, disrupting translation control. To test this possibility, we examined *Nm23-M2* polysome association in NFS61 and NFS33, a related cell line in which the *Nm23-M2* locus is intact, following factor deprivation and restimulation with IL-3. The NFS61 line will express the LTR-*Nm23-M2* fusion transcript from one allele and a normal *Nm23-M2* from the other allele. Therefore, factor-deprivation and restimulation still affects the overall polysome recruitment of total *Nm23-M2* transcripts. However, following factor deprivation, restimulation increases polysome association from 22 to 66% in a control cell line (NFS36), and from 61 to 73% in the NFS61 cell line (with LTR-Nm23 fusion transcript).

Translation modulation of luciferase using Nm23-M2 UTRs

To address the effect of the lost regulatory elements on Nm23-M2 expression, we used reporter assays. As a model system we used Ba/F3 cells that are suitable for transient transfections and respond to factor deprivation and restimulation, as measured by phosphorylation status of 4EBP protein (Figure 2A). For the reporter construct we amplified the endogenous promoter including the 5'UTR of *Nm23* up to the proper AUG and fused this to the ORF of the luciferase reporter. The 3'UTR of

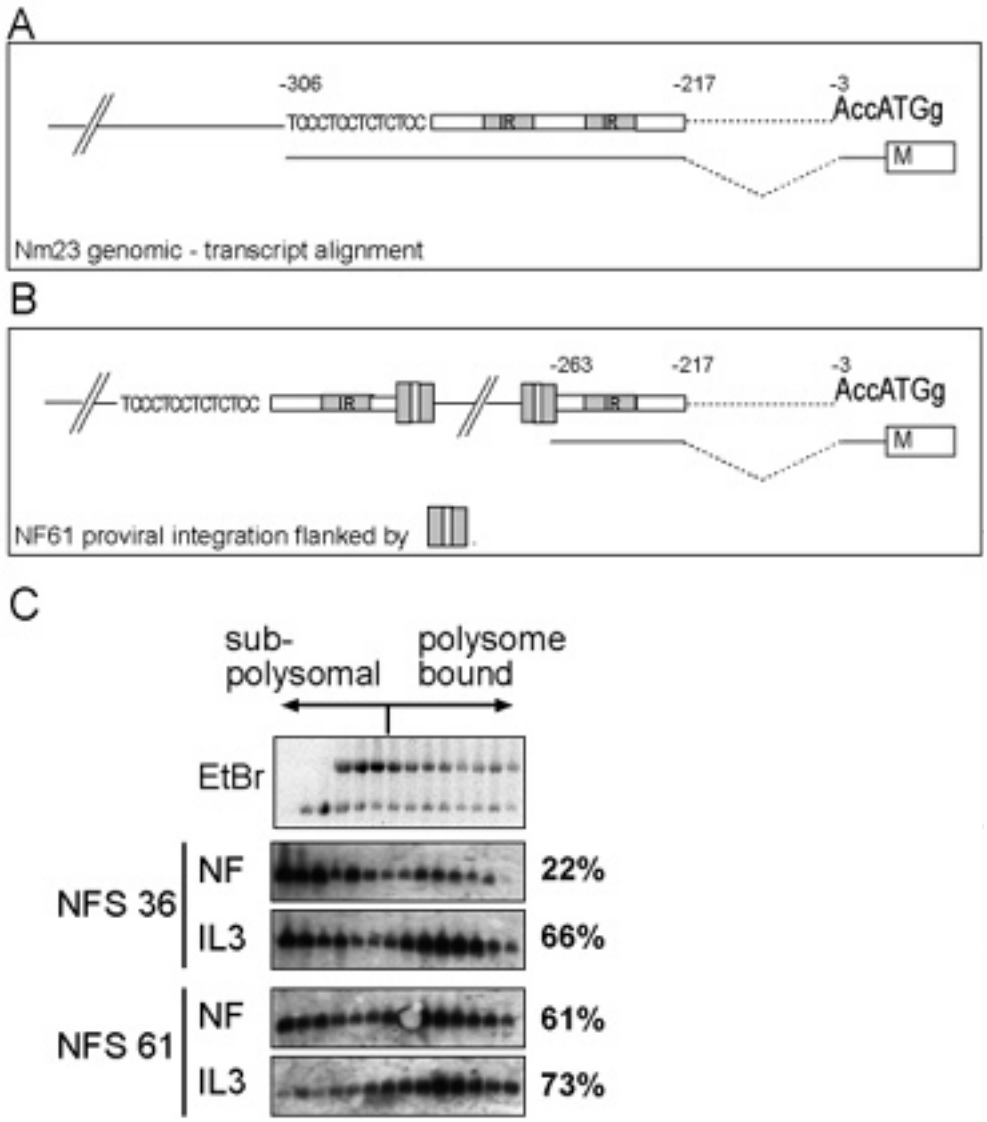

Figure 1 **Viral integration results in a LTR-*Nm23-M2* fusion transcript that lost translation control**. **A**: Representation of genomic sequence and the alignment with the resulting endogenous *Nm23-M2* transcript. The dotted line indicate the position of the 214bp intron starting from position –217 to –3 relative to A of ATG. The boxes indicate the untranslated exonic region. The grey boxes show the position of an inverted repeat (IR) within the UTR. The transcript starts with a tract of pyrimidines (TOP). **B**: The proviral sequence in the cell line NFS61 was found to be inserted at position –263 in between the inverted repeats. **C**: Tumour derived cell lines NFS36 and NFS61 were factor-deprived (NF) and subsequently restimulated with IL3 (10ng/ml). Polysome bound (pb) mRNA was isolated, and fractions were used for Northern analysis. A radio-labeled Nm23-M2 probe was used to detect the *Nm23-M2* transcript. Quantification by ImageQuant allowed to measure the percentage of mRNA associated with polysomes (pb-mRNA).

Nm23-M2 was fused to the stop codon of luciferase to retain all possible non-coding regulatory sequences. Using a full length endogenous promoter ensured that the proper transcription start site is taken independent of the correct transcription start site prediction of previous RACE experiments. To quantify reporter expression in response to factor deprivation and restimulation, a short half-life of the luciferase protein was imperative to ensure short depriviation time points prior to stimulation. Therefore we used the instable luciferase construct from pGL2 within *Nm23-M2* regulatory sequences and first measured luciferase activity following factor-deprivation (Figure 2B). Next, the luciferase activity of the *Nm23-M2* construct was measured following 6h factor deprivation and restimulation in presence and absence of inhibitors of protein translation (rapamycin) or gene transcription (Actinomycin D). This indicated that factor restimulation induced reporter expression dependent on translation, but independent of transcription (Figure 2C).

Regulatory elements in the 5'UTR of Nm23-M2

In previous studies we identified several transcripts subject to PI3K-dependent polysome recruitment in response to SCF restimulation of erythroid progenitors. Blast searches on EST-libraries identified 5'UTR sequences that extended beyond the 5'UTR of cDNA libraries available from the same sources (NCBI). RACE experiments to analyse the transcription start site and the most 5' sequence of these transcripts failed under standard conditions, most likely because of the presence of structural elements (data not shown). However, using a mutant RT-enzyme active at 60°C, we could resolve the 5'UTR of *mEd2*/ *D12* that contains a predicted structure with an enthalpy of –21kcal/mol over 10nt (Figure 3). Strikingly, also the RACE product for *Nm23-M2* extended beyond the previously described TOP element. Instead of 170bp, we could establish that *Nm23-M2* contains a 5'UTR of 439bp. RNA secondary structure analysis and scanning for consensus sequences of the 5'UTR sequence of *Nm23-M2* identified several putative regulatory elements, *(i)* A stretch of 76 nucleotides from -59 to -135 lacking adenosine, resulting in long stretches of GC and GU base pairing with UC-rich bulges in between (Figure 3). This high enthalpy structure is conserved between mouse and human transcripts, *(ii)* uORFs that are situated 5' to the high enthalpy region, and *(iii)* signalling-sensitive structures denoted as a Gamma interferon activated inhibitor (GAIT) element. This element was identified by RegRNA (http://regrna.mbc.nctu.edu.tw) and lies within the uORF initiating at an uAUG with perfect Kozak sequence. These GAIT elements are conserved in human and rat. Also the distribution of uORFs and secondary structures along the 5'UTR is conserved between human, rat and mouse.

Effect of Regulatory elements on expression

To assay to what extent the different structures contribute to factor dependent transcript translation, we made use of reporter constructs. To facilitate rapid screening in both *in vitro* transcription translation assays and in transient transfections in PI3K-dependent cell lines, we cloned a metallotheinine (MT)-T7 promoter combination in front of 5'UTR sequences of *Nm23-M2* and *mEd2*, which, in turn, were fused to the AUG startcodon of the pGL2-derived luciferase gene. Compared to the full length

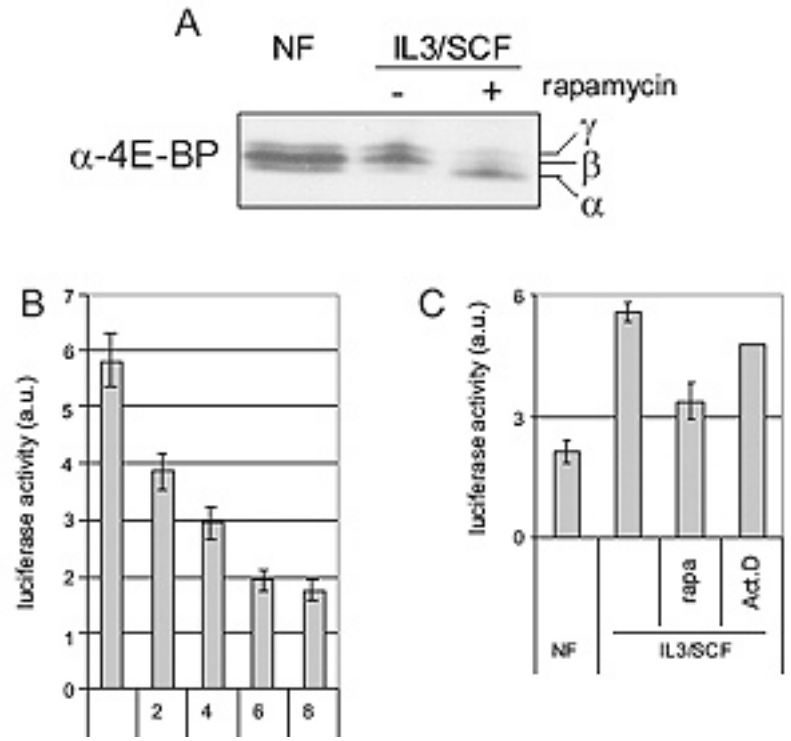

Figure 2 **The untranslated regions (UTRs) of *Nm23-M2* induce translation control when flanking the luciferase coding sequence**. **A**: BA/F3 cells were factor deprived for 6hours (NF) and stimulated with IL3 (10ng/ml) plus SCF (100ng/ml) for 2 hours in the absence or presence of rapamycin (inhibitor of mTOR). Western blots with total cell lysates were stained with antibodies recognising total 4E-BP (α-4E-BP). The unphosphorylated, fast moving α-isoform, a partially phosphorylated β-isoform, and a fully phosphorylated, slow moving γ-isoform are indicated. **B**: A reporter construct with the luciferase coding sequence flanked with the UTRs of *Nm23-M2* and the endogenous *Nm23-M2* promoter, was transfected into BA/F3 cells. The cells were factor deprived and the luciferase activity was measured at 2 hour time intervals, up to 8 hours. **C**: BA/F3 cells were transfected as in (B), factor deprived for 6 hours and restimulated with IL3 (10ng/ml) plus SCF (100ng/ml) for 2 hours in the presence of the translation inhibitor, rapamycin (10nM) or the transcription inhibitor, Actinomycin D (10μg/ml; ActinoD) or without inhibitors.

5'UTR, removal of the upstream ORFs in *mEd2* UTR resulted in an increase in luciferase readout following *in vitro* transcription/translation indicating that the uORFs attenuate translation efficiency. Surprisingly, the opposite effect was observed when the uORFs in *Nm23-M2* were deleted (Figure 4A). Transfection of the reporter constructs in HD3 or the murine BA/F3 cell line confirmed that the uORFs in *Nm23-M2* release the inhibitory effect in the proximal 5'UTR region. The presence of the uORFs increased the luciferase activity, whereas mRNA expression was similar for the full length and deletion construct (Figure 4B). Further mutational analysis will be designed to investigate the contribution of GAIT elements and uORFs. In addition, it is important to investigate the distribution of these elements relative to the complex inverted repeat regions, known to inhibit translation initiation.

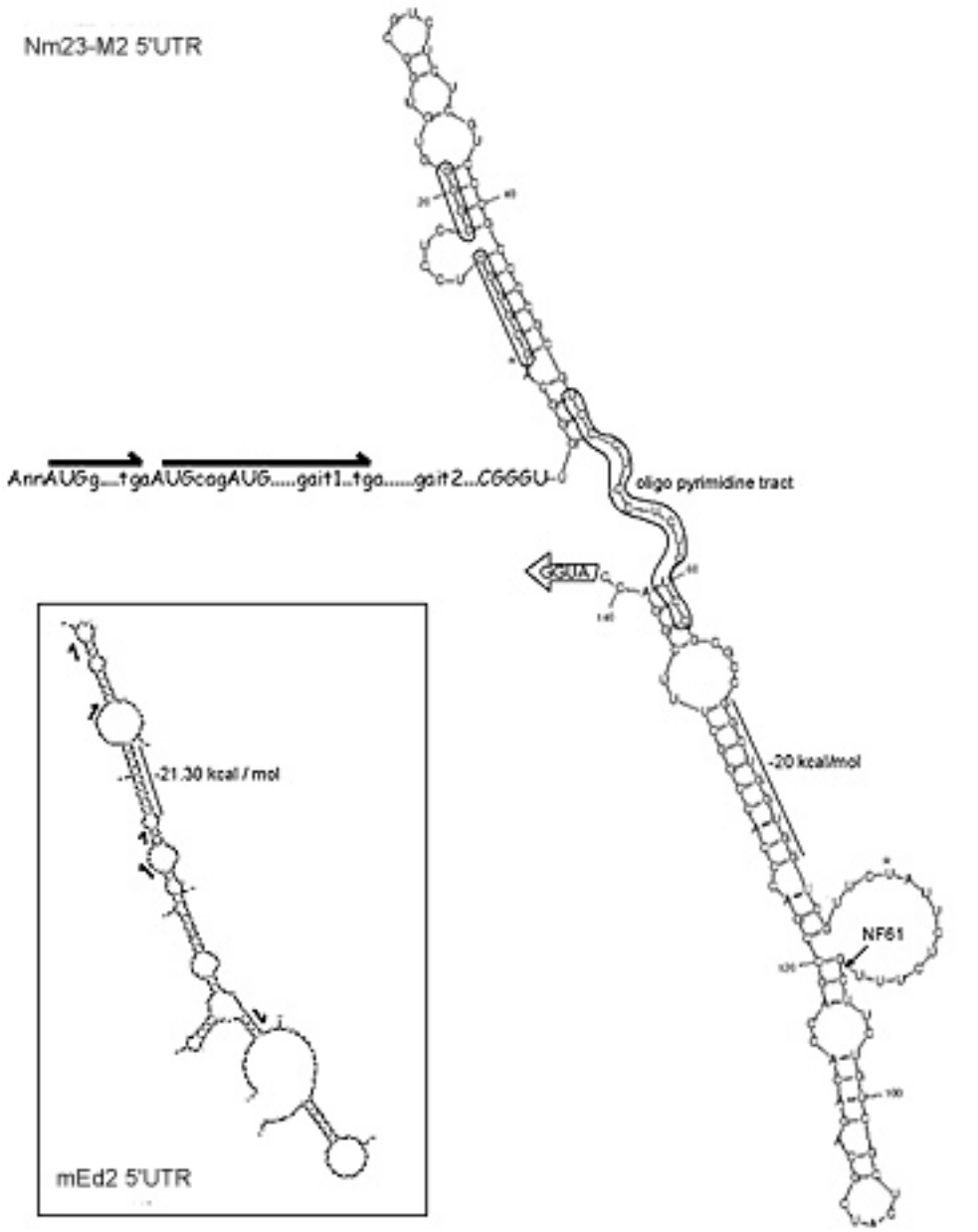

Figure 3 **Regulatory elements within extended *Nm23-M2* 5'UTR sequence as compared to elements in *mEd2***. An open arrow indicates the proper AUG of *Nm23-M2* transcript. Black arrows indicate the upstream open reading frames (uORFs). The region delimited by (*) do not contain A's and harbours the oligo pyrimidine tract thought to be the transcription start site in previous experiments. An inverted repeat with a high enthalpy (-20 kcal/mol) is indicated. The secondary structure was predicted by Mfold software (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi). The uORFs and the "gait" structure were predicted using the scanning program regRNA (http://regrna.mbc.nctu.edu.tw). The viral integration site occurs at the nucleotide denoted as NF61. The smaller representation of the *mEd2* 5' UTR structure shows the position of uAUGs and the high enthalpy inverted repeat.

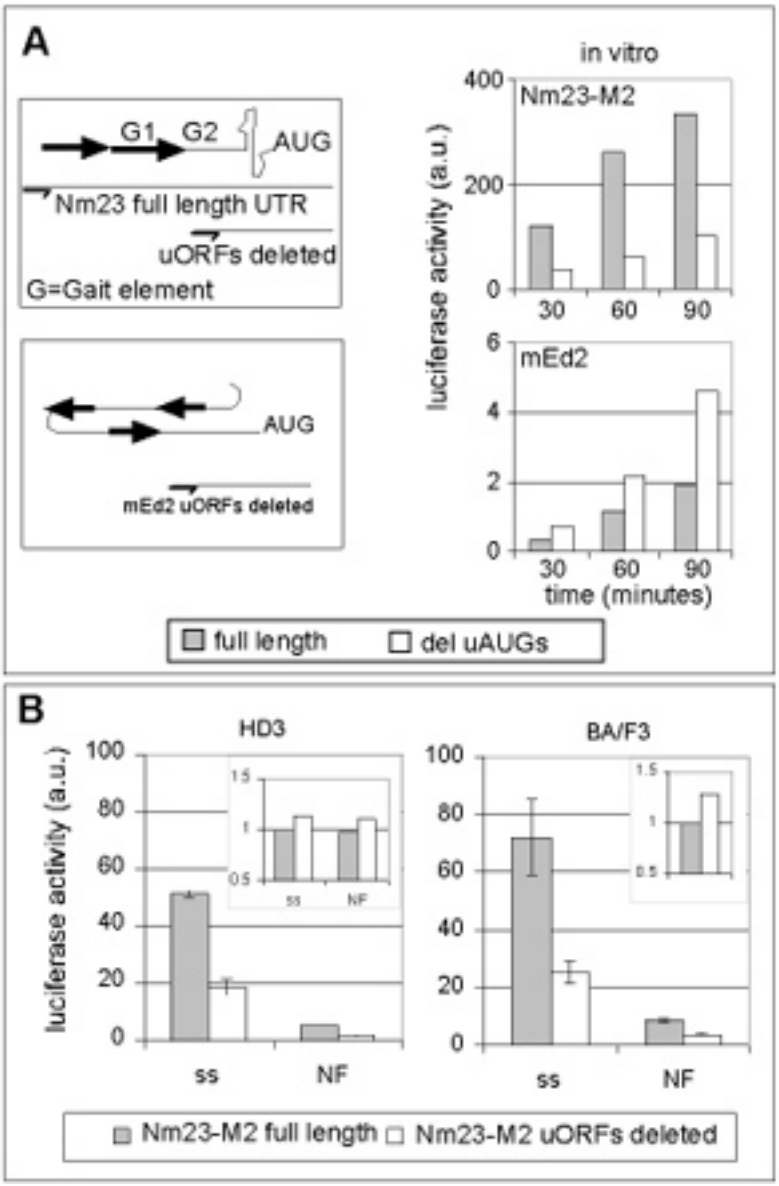

Figure 4 **The uORF region contributes to translation efficiency in both Nm23-M2 and mEd2**. **A**: The 5'UTRs of *Nm23-M2* and *mEd2* were introduced between a T7 promoter and the coding sequence of the luciferase ORF. The left panel indicates the primer positions for UTRs with [full length] or lacking the uORF region [del uAUGs]. The bold black arrow indicate uORFs; half arrows the position of the primer to amplify the UTR product; and G1/G2 the position of the Gait elements in *Nm23-M2*. The right graphs show the kinetics of in vitro transcribed/ translated luciferase activity. Lysate was harvested at increasing incubation times, showing linear increase of luciferase activity. **B**: The same constructs for *Nm23-M2* were transfected in BA/F3 cells and HD3 cells. Signalling was abrogated by growth factor removel (NF) and the luciferase activity compared to steady state (ss) conditions. The small graph inserts indicate the level of transcript in the cells quantified by Real Time PCR. Error bars indicate the variance from 3 independent experiments.

DISCUSSION

Nm23-M2 was identified as a common viral integration site in Cas-Br induced leukemias (Joosten et al. 2002). However, viral insertion upstream of the transcription start site did not result in enhanced expression. Instead, disruption of regulatory elements within the 5'UTR of *Nm23-M2* transcript permitted polysome recruitment and initiation of translation in the absence of growth factors (Joosten et al. 2004). Reanalysis of the 5'UTR of *Nm23-M2* showed a much more complex structure than initially anticipated. Interestingly, upstream ORFs in the distal part of the 5'UTR appeared to enhance polysome recruitment, while stem-loop elements in the proximal part of the 5'UTR inhibit translation. Together these regions are responsible for growth factor dependent polysome recruitment.

The role of growth factor sensitive structured UTR of Nm23-M2

Proviral integration within the *Nm23-M2* locus was detected in 56% of the primary leukemias (Joosten, unpublished data). The importance of *Nm23-M2* as a common viral integration site is supported by association of high expression of the human homolog, *NM23-H2* to a certain group of poor-risk myeloid leukemias (Yokoyama et al. 1996; Yokoyama et al. 1998). The growth factor-dependent expression of Nm23-M2 may explain why we could not establish erythroid progenitor clones constitutively expressing this protein. Notably, we were unable to induce constitutive expression of most transcripts subject to growth factor-dependent translation (chapter 3). This rendered the study of the oncogenic potential of Nm23-M2 in hematopoietic cells unconclusive.

Another approach to address the role of Nm23-M2 was a study on the loss of translation control by proviral integration. The oligoclonal nature of the MLC-induced tumours rendered the assessment of protein levels compared to transcript levels difficult to interpret and polysome gradients from tumour material was not feasible either. The NF61 cell line contains a proviral insertion within the 5'UTR of *Nm23*, resulting in a transcript without high enthalpy structures and lacking all the elements identified in this study. In addition the elements are replaced by the viral sequence allowing easy scanning and efficient polysome recruitment of the modified UTR of *Nm23-M2* fusion transcript and hence constitutive expression. This suggest that deletion of regulatory elements within non-coding regions of complex transcripts permits a novel mechanism of constitutive expression of potential oncogenes that are otherwise sensitive to growth factor-dependent translation initiation.

Characterisation of regulatory elements within the Nm23-M2 UTR

Despite all progress in the genome projects, it remains difficult to predict a transcriptional start site, leaving the identification of 5'UTR sequences to experimental approaches. Our own assays indicated that many structured 5'UTRs could only be reverse transcribed under conditions that destabilise secondary structures (e.g. RT at 65°C). We used both databases and experimental approaches to identify the longest transcripts for *Nm23-M2* and *mEd2*, in an effort to characterise regulatory elements within the UTR of *Nm23-M2* that are disrupted by viral insertions. *mEd2* was taken along since it was found to have similar elements in its 5'UTR, although

the distribution was different. A high enthalpy structure was found downstream of, or located within the uORFs in *Nm23-M2* and *mEd2* respectively (Figure 3). This presents an interesting interplay between selection of uAUGs and melting of structures.

Translation initiation efficiency is governed by ribosome recruitment, scanning, and selection of the proper AUG. Increased eIF4E availability upon growth factor signalling recruits the RNA helicase eIF4A to melt secondary structures (Koromilas et al. 1992). In addition, the availability of the ternary complex of GTP-bound eIF2 associated with methionine-bound tRNA (eIF2:GTP-Met-tRNA$_i$) determines the efficiency with which AUG startcodons are recognised (Morris and Geballe 2000). The uORF in the *Nm23-M2* 5'UTR starts with a perfect consensus sequence (AnnAUGG; -387) suggesting that regulation of eIF2 and differential selection of the uAUG is not the primary level of regulation. Instead, a first level of regulation may be the efficiency with which scanning continues beyond the uORF. It has been described before that an uORF with the uAUG before a stable secondary structure enhances scanning through that structure. It is supposed that the bulgy ribosome and the process of translation destabilises secondary structure. The surprising condition in the 5'UTR of Nm2-M2 is that the high enthalpy secondary structure is not within the uORF, but separated by at least 207 nucleotides. This may mean that the peptide encoded by the uORF affects the stability of the secondary structure, or the efficiency with which the pre-initiation scanning complexes can unfold the structure. Although the presence of an uORF upstream of secondary structure is conserved, the sequence of the encoded peptide is not conserved between mouse and man, which renders a function for the peptide less likely. The proximal structural element may be stabilised by proteins that are released following signalling. However, the fact that the structures block reverse transcription at 42°C on naked RNA indicates that their stability does not require protein binding. Instead signalling may stimulate protein-RNA interactions that destabilise the secondary structure.

In addition to the high enthalpy structures and uORFs, the *RegRNA* RNA-analysis program identified two GAIT elements. A GAIT element resembles the Iron Response element (Sampath et al. 2003), suggesting that such structures are candidates for protein/RNA interactions. In Ceruloplasmin transcript the GAIT element is present in the 3'UTR and binds RpL13a, which is released from the 60S ribosomal subunit upon phosphorylation by IFNγ. Binding results in a translationally inhibitory complex (Sampath et al. 2003) (Mazumder et al. 2003). The GAIT element in *Nm23-M2* may bind RpL13a, but it is also possible that other proteins are binding to control the pre-initiation scanning complex and polysome recruitment of *NM23-M2*. The role of the GAIT elements awaits mutational analysis.

MATERIALS AND METHODS

Cells

BA/F3 and tumour cell lines were cultured at a density of 0.2-1.0$\times 10^6$ cells per ml, in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Hyclone, PerBio) and 10ng/ml

murine IL-3 (supernatant). HD3 cells were cultured at a density of 1-2×10^6 per ml in S13 medium (ISCOVE medium supplemented with 12% Fetal Calf Serum; 0.05% chicken serum; 0.1% Bovine Serum Albumin; 0.02% bicarbonate and 1×10^{-4}M β-mercaptoethanol). Genomic DNA was isolated as described previously (Joosten et al. 2002).

RNA isolation, Northern blot and cDNA synthesis

Cell extracts were prepared by lysis at 4°C in extraction buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 1.5 mM MgCl2, 0.5% Nonidet-P40, 20 mM dithiothreitol, 150 _g/ml cycloheximide, 1 mM phenylmethylsulfonyl fluoride, and 500 units/ml RNasin), and nuclei were removed by centrifugation (1000 × g for 10 min at 4°C). The supernatant were centrifuged (12,000 × g for 5 min at 4 °C) to eliminate mitochondria. The supernatant was either loaded on sucrose gradients to isolate polysome bound mRNA as described in (Joosten et al. 2004) or taken as total RNA. The total supernatant was digested with 100 μg of proteinase K in 1% SDS and 10 mM EDTA (30 min at 37°C). RNAs were recovered by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. Similarly the fractions from sucrose gradients were digested and RNA extracted. RNA was quantified by UV-absorbance.

For Northern blot analysis, RNAs were loaded on denaturing 1.2% formaldehyde agarose gels and subsequent blotted to Hybond-N membranes (Amersham Biosciences). We used a 612-bp nm23-M2 cDNA fragment as a probe. After hybridization, filters were scanned on a PhosphorImager (Amersham Biosciences), and signals were quantified by phosphorimaging.

cDNA for Real-time PCR was generated as described (Joosten et al. 2004). For RACE experiments, 60ng of purified poly(A)$^+$ mRNA and 5'-TGAAGGTACGCTCGAGG-3' for *Nm23* and 5'-TGGATGCAAGATACAGATAGC -3' for mEd2, were used to synthesise cDNA at 60°C in accordance to manufacturer's protocol, using Roche Transcriptor Reverse Transcriptase (Roche 03531317001) supplied together with mRNA capture kit (Roche 117878960). First stand cDNA was purified using High pure purification columns (Roche 11732668001) and dA-tailed. The first PCR (15s at 94°C, 30s at 59 °C, 72 °C for 10cycles followed by 25 cycles of 15s at 94°C, 30s at 58 °C, 72 °C) was performed using the Nm23 primer (5'-TGCCTGGTCTTGCCAGTCG-3'), the mEd2 primer (5'- TCACAGCGACAAAGCAGC-3') and the dT-linker forward primer supplied by the kit. Nested PCR was performed using the Nm23 primer (5'- TGCACGCCCTCTCCTTGCAG-3'), the mEd2 primer (5'-TGAAGCAGAGTCTCTGTAGTCC -3') and the forward primer supplied in the kit.

Subsequently, the final products were cloned directly into pCR2.1 (Invitrogen) according to the instructions of the manufacturer. Nucleotide sequencing was carried out using a binding domain sequencing kit according to instructions from the provider (PE Biosystems). Sequencing was carried out on an ABI 310 automatic sequencer (PE Biosystems) using the M13 forward primer (5_-GTAAAACGACGGCCAGT-3_). All primers were obtained from Sigma-Genosys Ltd.

SDS-PAGE, western blotting and antibodies

For acute stimulation with growth factors and affect of rapamycin, proliferating BA/F3 cells were washed twice with phosphate-buffered saline (PBS) and seeded

at 4 x 10^6 cells/ml in plain RPMI (Invitrogen). After 6 h factor deprivation, cells were stimulated at 37°C with IL3 (10ng/ml) and SCF (100ng/ml) in the presence or absence of rapamycin (40ng/ml). Cells were harvested after the indicated time points by addition of ice-cold PBS. Cell lysates, SDS-PAGE, immunoprecipitation and Western blotting were performed as described previously (van Dijk et al. 2000). 10 μl of protein extract (≈1x106 cells) was loaded onto a 15% polyacrylamide gel. The antibodies used were: α-4E-BP1 (Cell Signaling Technology, Inc).

Cloning of Nm23 and mEd2 sequencies and Luciferase Reporter assays

The promoter including the 5'UTR of Nm23 was digested from a pGL3 construct described in (Joosten et al. 2004), and ligated into the pGl2 construct (Promega; NcoI site inserted at luciferase AUG and SnaBI site inserted 5' to the polyA tail, using Quickchange Site Directed Mutagenesis kit from Stratagene). The 3'UTR was amplified from a cDNA library and inserted to replace the poly tail of pGl2 vector by a SnaBI / BamHI digest. The resulting luciferase with endogenous UTRs of Nm23, was used to investigate signalling-dependent translation control.

The mouse *Nm23*, *mEd2* Untranslated regions (UTRs) were amplified from a cDNA library using the following primers, harbouring an XhoI site in the forward primer and an NcoI site in the reverse primer: forward Nm23 5'-CCGCTCGAGCGGTTAATACGACTCACTATAGGGAGAGGGCTGAATTTCGGAGA TAG-3', reverse Nm23 5'-CATGCCATGGTCCGAAAGCCGGTGGGTCGGCTG-3'; forward mEd2 5'-CCGCTCGAGCGGTTAATACGACTCACTATAGGGCAGGTCAAG GTTCCCGCTACAG-3', reverse mEd2 5'- AACTGCAGAACCAATGCATTGGAGAAA AGGGCGGAAGTGCCTGAGTGAGG -3', and the Expand High Fidelity PCR System (Roche). The transcription start site was taken at the 5' end of the longest RACE product identified in this study. The shorter forms of UTRs were amplified using the same reverse primers, and a forward primer downstream of the uAUGs 5'-CCGCTCGAGCGGTTAATACGACTCACTATAGGGACAGAGACGACTGGCAAGAC CAG-3' in Nm23 and 5'- CCGCTCGAGCGGTTAATACGACTCACTATAGGGAAGGC CAGGTATCAAGACCATC -3' in mEd2. The PCR products were ligated in pCR2.1 vector (Invitrogen). The fragment was digested out with XhoI and NcoI and cloned in pGL2-basic.

For reporter assays, 10×10^6 Ba/F3 cells were electroporated (0.28kV, capacitance 960μFD) with maximum 20μg of DNA. After recovery for several hours in normal media, cells were washed and grown overnight in the presence or absence of IL-3 and SCF, or cells were IL-3 deprived for 6 hours and stimulated for 2hrs with IL-3 and SCF in the presence or absence of rapamycin (Alexis, Switzerland). Luciferase activity was measured using the Steady-Glo system (Promega). Transfection efficiency was determined by cotransfecting lacZ and analyzing β-galactosidase activity. For comparison of mutated UTRs with full length UTR, the cells were washed and grown overnight in the presence or absence of IL-3 and SCF, or cells were IL-3 deprived for 6hours.

The luciferase assays were also performed in HD3 cells. A DEAE protocol was used to efficiently transfect these cells. In Brief, 20×10^6 cells were washed twice in 10ml TBS. 12.5μg DNA was dissolved in 50 μl TBS kept at 37°C. 75μl prewarmed DEAE-Dextran (5mg/ml) was mixed well and incubated for 30 min at room temperature (r.t.). 1.375 ml TBS were added to the DNA and mixed well, followed

by mixing the resulting DNA-solution to the cell pellet. After 30minutes incubation at RT, mixing at intervals of 10 minutes, 9ml TBS and 2ml S13 medium were added. The cells were washed with 4 ml of S13 medium and seeded at a density of $1.4x10^6$/ml. HD3 cells were straved at 42°C for 6hours followed by re-stimulation at 37oC for 3 hours. 3×10^6 cells were lysed for luciferase assay and 20×10^6 cells were lysed to isolate cytoplasmic mRNA and quantitate luciferase transcript by Real-time PCR.

Real-time PCR

The real-time PCR assay involved TaqMan technology (PE Applied Biosystems Model 7700 sequence detector), using the double stranded DNA-specific fluorescence dye SYBR green I to detect PCR product as previously described (Kolbus et al. 2003). The amplification program consisted of 1 cycle of 50°C with 2min hold (AmpErase UNG incubation), 1 cycle of 95°C with 10 min hold (AmpliTaq Gold Activation), followed by 40 cycles of denaturation at 95°C for 15s, annealing at 62°C for 30s and extension at 72°C for 30s. All the different primer pairs had similar optimal PCR annealing temperatures. Acquisition of the fluorescence signal from the samples was carried out at the end of the elongation step. To confirm amplification specificity, the PCR products from each primer pair were subjected to agarose gel electrophoresis and the dissociation curve was checked at the end of each run. Gene-specific primers flanking a 300bp product within the luciferase coding sequence, forward 5'-TCAGATTCTCGCATGCCAG-3' and reverse 5'-TGGTACTAGCAACGCAC-3' were obtained from Sigma-Genosys Ltd.

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Discussion

CHAPTER 6

Erythroid progenitors undergo renewal divisions in the presence of Epo, SCF and glucorticoids, retaining the capacity to differentiate in culture with medium containing Epo alone. The SCF driven renewal and repression of differentiation is PI3K dependent. The cellular system used in this study proved to be extremely powerful to study the importance of signalling pathways in the decision between expansion and differentiation of erythroid progenitors. The block of erythroid differentiation resulting from constitutive expression of eIF4E suggested an important role of SCF-driven availability of eIF4E in repression of differentiation. Increased eIF4F activity enhances the translation efficiency of complex mRNA transcripts. To identify transcripts that are recruited to polysomes upon SCF addition we hybridised polysome bound mRNA and total RNA from both factor deprived and re-stimulated erythroid progenitors to oligonucleotide arrays. Subtraction of regulation at total and polysome bound fractions characterized a unique list of transcripts that are regulated at the level of polysome recruitment and hence initiation of protein synthesis. Among the most regulated genes, Nm23 was also found as a common virus integration site with insertions deleting a high enthalpy structure in the 5' untranslated region of the mature transcript, suggesting a novel mechanism of proviral disruption of gene expression. Two genes, Igbp1/α4 and mEd2, repressed erythroid differentiation upon constitutive expression, indicating a critical role of these translationally controlled genes in the balance between expansion and differentiation. Interestingly Igbp1/α4 attenuates the negative regulation of mTOR targets by the S/T-phosphatase Pp2a. This prompted us to investigate the effect of constitutive Igbp1/α4 on the mTOR effectors 4EBP and S6K. Both were constitutively phosphorylated upon constitutive Igbp1/α4 expression. Surprisingly, Epo stimulation resulted in S6K phosphorylation when Igbp1/α4 was constitutively expressed, suggesting that in these clones both increased translation efficiency of complex transcripts and enhanced ribogenesis might drive expansion and repress terminal differentiation. The potential role of ribogenesis is supported by positive regulation of ribosomal factors and rDNA transcription regulation by SCF as shown in the profiling experiment.

6.1 Structural RNA elements dictating translational control

Translation regulation is one of the targets of constitutive active growth factor signalling. Rate limiting eIF2 and eIF4E play a critical role in translation initiation of a subset of mRNAs that have cis-regulatory elements such as uORFs (Calkhoven et al. 2000; Morris and Geballe 2000; Dever 2002; Calkhoven et al. 2003) and secondary structures that impair ribosomal scanning for the proper AUG (Graff et al. 1997; Clemens and Bommer 1999; Willis 1999; Graff and Zimmer 2003; De Benedetti and Graff 2004). Inhibition of ribosomal scanning through structural hindrance can silence translation (Manzella and Blackshear 1990). Release of translation block by removal of structural elements (Joosten et al. 2004) or overexpression of eIF4E (Manzella et al. 1991; Koromilas et al. 1992; Shantz et al. 1996; Blazquez-Domingo et al. 2005) implies a direct role of eIF4E in melting complex 5'UTR sequences. SCF signalling stimulates cap-dependent translation and is expected to enhance translation of transcripts that require increased levels of the eIF4F complex. These transcripts are generally characterized by structured 5'UTRs (Koromilas et al. 1992,

De Benedetti et al., 2004 or the presence of terminal oligopyrimidine tract (TOP) sequence (Jefferies et al. 1997).

6.1.1 A unique list of translationally controlled genes.

We set out to identify polysome recruitment as a target of SCF. Utilising polysome-bound RNA for array hybridization increases the value of the data by including information on transcripts from which peptides are being synthesized, and also increases the sensitivity for low abundant transcripts. This approach has been used previously to identify differential loading to polysomes upon inhibition of PI3K and ras pathway in glioma cells (Rajasekhar et al. 2003), and during hypoxia during which differential sensitivity to mRNA translation inhibition contribute to specific hypoxia-induced protein expression (Koritzinsky et al. 2005; Wouters et al. 2005).

A profiling study using polysome-bound mRNA derived from glioblastoma cells (Rajasekhar et al. 2003) identified hundreds of genes that were differentially expressed (>2-fold change) in cells upon transformation by v-Ras and/or v-Akt or in transformed cells treated with or without inhibitors of PI3K and MEK1. Instead, only 67 genes were found to be regulated >2-fold when total RNA was used for profiling (Rajasekhar et al. 2003). Taking the ratio of pb over total RNA into account 343 known genes were found to be regulated at the level of polysome recruitment dependent on v-Ras/v-Akt signalling. This is in accordance with our observation that the number of genes selected as being differentially regulated in response to growth factors is at least 3-fold increased in polysome bound RNA compared to total RNA at any choosen threshold. However, the overlap in identified genes in the v-Ras/v-Akt glioblastoma cells and Epo/SCF-stimulated erythroid progenitors was small, which may have been caused by the use of a different cell type and a different experimental approach addressing quick responses to stimuli in our study as opposed to the effect of genetically perturbed pathways. In addition, the use of 4 independent replicates in our study and a different statistical analysis may have rendered our study more stringent in the selection of genes that are predominantly regulated by selective polysome recruitment.

Both studies indicate that gene expression profiling using polysome-bound mRNA has major advantages. Polysome recruitment of specific transcripts responds to a wide variety of extracellular signals including growth factors but also nutrients and hypoxia, and intracellular signals including protein misfolding and apoptosis (Pain 1996; Gingras et al. 1999; Raught and Gingras 1999; Gingras et al. 2001; Lang et al. 2002; Stolovich et al. 2002; Rajasekhar et al. 2003; Blais et al. 2004; Blazquez-Domingo et al. 2005). This indicates that gene expression profiling of many different conditions including cancer could benefit from this approach. However, the advantage of using pb mRNA may depend on the conditions and samples used. Notably, different cellular processes are regulated at distinct levels, which is best exemplified by the observation that factor-dependent expression of genes involved in lipid metabolism were regulated at the level of gene transcription with concomitant polysome recruitment, whereas glucose metabolism was regulated largely at the level of polysome recruitment. Conventional profiling with total mRNA may be preferred to find targets of transcription factors, or when structural, noncoding RNAs have to be included in the targets.

6.1.2 Regulatory elements in the 5'UTR of translationally controlled genes

We examined the 5'UTR of transcripts of which translation was regulated by SCF-induced polysome recruitment. This list contained many ribosomal proteins and some translation factors (chapter 3) known to contain a TOP sequence (Levy et al. 1991). The difference between factor-induced expression (array data) in total versus polysome bound RNA of these genes, however, was mostly less than 1,5-fold. To evaluate the most regulated transcripts, we selected genes that were at average at least 1.5-fold more regulated in pb RNA compared to total RNA. This selection was made in addition to the probability of differential expression as described in chapter 4. This left us with 49 translationally controlled genes.

The UTR sequence of these transcripts were downloaded from the NCBI database and blasted to mouse EST database. For 27 genes the UTR sequence could be extended based on novel EST sequences that corresponded to flanking regions in the genome. We excluded 18 genes from our UTR analysis because the UTRs were too short or not unique in the genome. Transcripts with too short UTRs may possess structural elements that hinder extension using conventional cDNA synthesis, therefore further analysis is needed to ensure the proper length. The remaining 31 UTRs were analysed for length, GC content, presence of upstream AUGs and upstream open reading frames (uORFs), inverted repeats that increase the enthalpy by at least -20kcal/mol (Babendure et al. 2006) and other structures such as pseudoknots. The distance of such structures from the proper AUG and relative to uORFs was taken also in account (data not shown).

Two of the 31 selected transcripts contained a TOP sequence, while the other structures that contribute to control of translation initiation are not easily discernable. The bioinformatic tools, RegRNA (http://regrna.mbc.nctu.edu.tw) and UTRscan (http://bighost.area.ba.cnr.it/BIG/UTRScan/) were used to score for putative regulatory elements in the UTR sequences. First, 11 genes contain a putative IRES sequence in the 5'UTR. Because structures functioning as an IRES are ill-defined, these structures must be interpreted as extensive secondary structures with multiple potential functions. 12 UTRs contained a single or multiple uORFs and interestingly 7 UTRs scored for structures that are recognised as GAIT elements (Gamma interferon activated inhibitor of Ceruloplasmin mRNA translation). These elements occur as single or multiple elements with a total of 12 elements distributed in the 7 UTRs. Half of these elements were found within uORFs having a perfect Kozak sequence suggesting a regulation at the level of AUG selection. Interestingly, it has been shown in other studies that addition of IFNγ results in phosphorylation of ribosomal protein L13a which is released from the 60S ribosomal subunit and forms an inhibitory complex with the GAIT element (Mazumder et al. 2003; Sampath et al. 2003). The striking similarity to the Iron Responsive Element (IRE) suggests that such structures are candidates for protein/RNA interactions making them potentially responsive to signals. Interestingly, the GAIT element in Nm23 transcript is conserved between mouse, rat and human and its position within the uORF is conserved in rat.

So far, we have not examined the role of the 3'UTR in selective polysome recruitment. However, protein complexes that associate with both the 5' and 3' UTR may be critical to control mRNA translation (Figure 6.1).

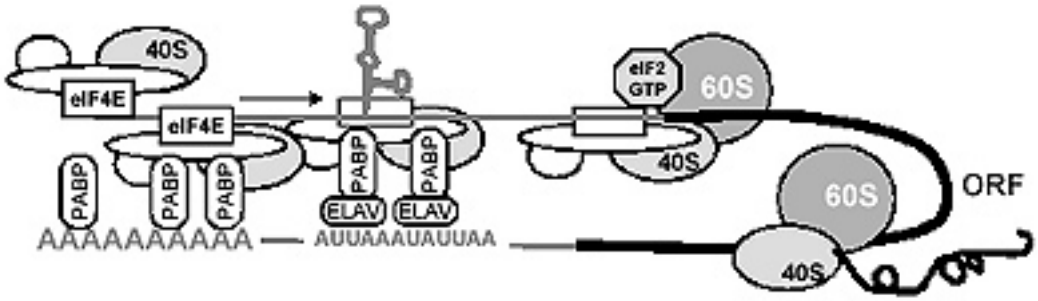

Figure 6.1 **Stabilisation of mRNA scanning complex**. Upon phosphorylation of 4E-BP, eIF4E can bind the scaffold protein eIF4G that recruits several other proteins and protein complexes among which the 40S ribosomal subunit. This complex scans along the 5'UTR to the first AUG in a proper sequence context. EIF4G also binds PolyA binding protein (PABP) which brings 5' and 3' UTR in close proximity and facilitates recycling of translation complexes, which enhances translation efficiency. Proteins that bind AU-rich repeat-like sequences such as ELAV1 (HuR) can also bind PABP. The stabilization of scanning complexes by PABP may facilitate scanning past structural elements such as stem-loop structures or uORFs. Small ribonuclear proteins may further stabilise the complex of ELAV and PABP. Together with base-pairing between repeats in the 3'UTR and the 18S rRNA in the 40S ribosomal subunit these interactions may reposition the scanning complex on the 5'UTR.

The most studied RNP particles are the hnRNPs that recognize AU-rich elements (ARE) and coordinate expression of mRNAs at the level of nuclear-cytoplasmic shuttling (Veyrune et al. 1996), cytoplasmic mRNA turnover (Shaw and Kamen 1986), and silencing of cell state- and type-specific mRNAs (Ostareck et al. 1997; Notari et al. 2006). The ARE is located in the 3' untranslated region of many short-lived transcripts from cytokines, proto-oncogenes, growth factors or cell cycle regulators (Bakheet et al. 2001). Interestingly, the family members BRF1 and BRF2 known to promote ARE-dependent decay, are regulated by SCF signaling in an opposing way and there expression is reverted upon induction or erythroid differentiation. Collective expression of predisposed transcript subgroups (Keene and Lager 2005) underlines a relatively unexplored gene expression modulation that might drive cell fate in the same manner as regulation of the transcriptome by transcription factors.

Future Experiments
The elements identified in the UTRs offer a unique possibility to analyse the mechanisms of signalling-controlled polysome recruitment. Mutagenesis of identified regulatory elements will be investigated in reporter assays to assess the contribution to translation control.
RNA-based electrophoretic mobility shift assays (RNA-EMSA) may prove useful to identify protein complexes on specific RNA molecules that shift in response to growth factor signaling. Affinity purification of protein complexes on RNA coupled to beads followed by mass spectroscopy is a powerful method to identify RNA-binding proteins that either stabilise or destabilise secondary structures.

An interesting additional experiment would be to immunoprecipitate specific mRNA binding proteins to identify mRNA subsets as described in (Tenenbaum et al. 2000).

In addition to structural elements also uORFs can have various functions in the regulation of polysome recruitment. An uORF can be located upstream of the main ORF as seen in mEd2, but an uORF can also be out of frame overlapping with the main ORF and in that way block translation from the protein-coding start-codon such as observed for Cnih. Interestingly, the uORF in the Cnih transcript encodes a cross species conserved peptide, which suggests that this peptide may have a particular function in the regulation of translation. In some transcripts, the encoded peptide of uORFs can interact with the 40S ribosomal subunit and cause stalling of the scanning complex. Such a mechanism has been extensively studied in the mRNA encoding S-adenosylmethionine decarboxylase (AdoMetDC). The AdoMetDC uORF codes for the hexapeptide, MAGDIS, that suppresses translation of AdoMetDC by interacting with a component of the translation machinery (Hill and Morris 1993).

Future Experiments
Reporter assays designed to investigate the effect of mutations directed to change amino acids within peptide of uORF in Cnih, would give indication of importance of the sequence.

6.1.3 Complexity and structures in Nm23, mEd2 and Igbp1/α4 mature transcripts
For further analysis we selected the transcripts of mEd2 and Igbp1/α4 because their constitutive expression impaired erythroid progenitor differentiation, and Nm23-M2 because it was found as a common virus integration site. Interestingly, a cell line derived from MLV induced tumours identified a proviral insertion that replaces the structured UTR of Nm23, with a simple sequence that looses translation control (Chapter 5).

6.1.3.1 Regulatory elements in Igbp1/α4 transcript
Highly structured 5'UTRs of translationally controlled transcripts may not unfold at the temperature used in standard reverse transcriptase reactions. The RACE strategy was modified to synthesis first strand cDNA at 65°C starting from polyA+ mRNA. Although we could extend the UTR sequence of other transcripts in our list, the RACE product of Igbp1/α4 indicated that the sequence is only 21bps. Interestingly, the human Igbp1/α4 5' UTR is 297bp long, and this sequence shows regions of high homology with the mouse genomic sequence. Northern blot of total RNA prepared from mouse and human cell lines, showed clearly that the mouse Igbp1/α4 transcript is as long as the human (Figure 6.2 A). Primers were designed within the homologous regions at position -161 to -192 relative to the mouse alpha4 AUG and 92bp upstream of the longest alpha4 EST (DV65442). PCR amplification yielded a 118bp product and sequencing confirmed the presence of a longer 5'UTR (Figure 6.2 B). This suggests that a high enthalpy structure hinders nested PCRs designed for the RACE protocol.

Future Experiments
RACE experiments will be done using nested primers designed upstream of the pseudoknot structure of alpha4, within the sequence that was confirmed present by PCR.

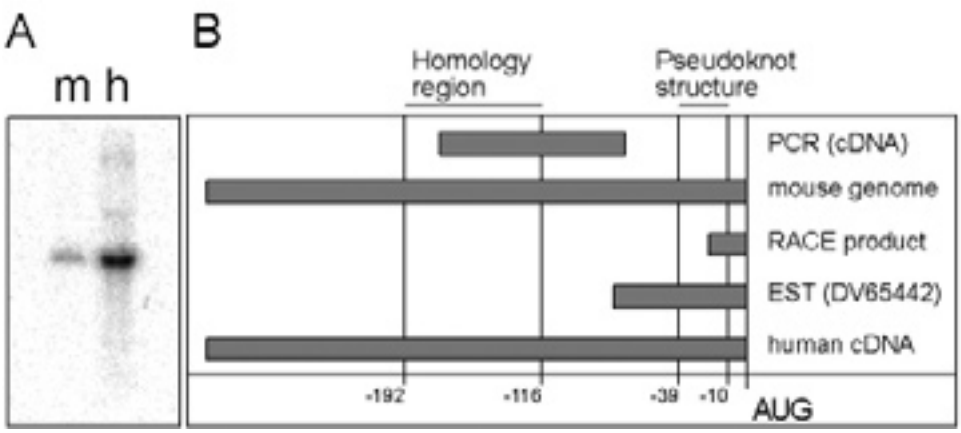

Figure 6.2 **Identification of 5'UTR of alpha4**. **A**: Northern blot of total RNA prepared from mouse and human cell lines. **B**: Alignment of different species with the mouse Igbp1/α4 cDNA and genomic sequence. The homology between mouse genome and human cDNA is indicated. In addition the position of the pseudoknot and the 118bp PCR product from mouse cDNA are annotated.

6.1.3.2 The leader sequence of mEd2

RACE experiments consistently showed that the 5' UTR of mEd2 corresponded to the sequence of the longest EST found in databases. Amplification of the whole length UTR of mEd2 resulted in 2 products. Sequencing of these products show the presence of an alternatively spliced intron of 180bp. Q-PCR suggests that the spliced form is 5-fold more abundant compared to the unspliced form. The unspliced isoform is not regulated during differentiation of erythroid progenitors (Figure 6.3 B), whereas the spliced isoform is downregulated (Figure 6.3 C) Interestingly, the AG of the acceptor splice sequence is immediate upstream of the proper AUG and splicing of the intron results in the abolishment of the perfect Kozak sequence at this site (Figure 6.3 A). In addition, a consensus uAUG is located 5' of the alternatively spliced intron. Splicing puts this upstream AUG in frame of the coding sequence. Thus the unspliced form contains a strong uAUG and associated uORF of 60nt that most likely affect translation, whereas that same consensus uAUG may be used as main AUG to produce a protein with 14additional amino acids at the N-terminal side *(*Figure 6.3 A*)*. This intriguing result suggests that regulation of the splicing isoforms also controls translation initiation.

Notably, the first predicted protein was even much smaller and started at the an AUG at position +157. This protein was described as MDS032. This protein was tested for an effect on signalling-controlled promoter activity and enhanced Erk-dependent reporter activity. It is this protein that inhibits erythroid differentiation when constitutively expressed. Recently, mEd2 was reported as the D12 Q-SNARE protein with a translational start at AUG at position 0. Despite several trials, we failed

to express this protein isoform in erythroid progenitors, suggesting that deregulated expression may be incompatible with proliferation of these cells. We have not yet tried to express the protein with an extended N-terminal part that is predicted to be encoded by the spliced transcript.

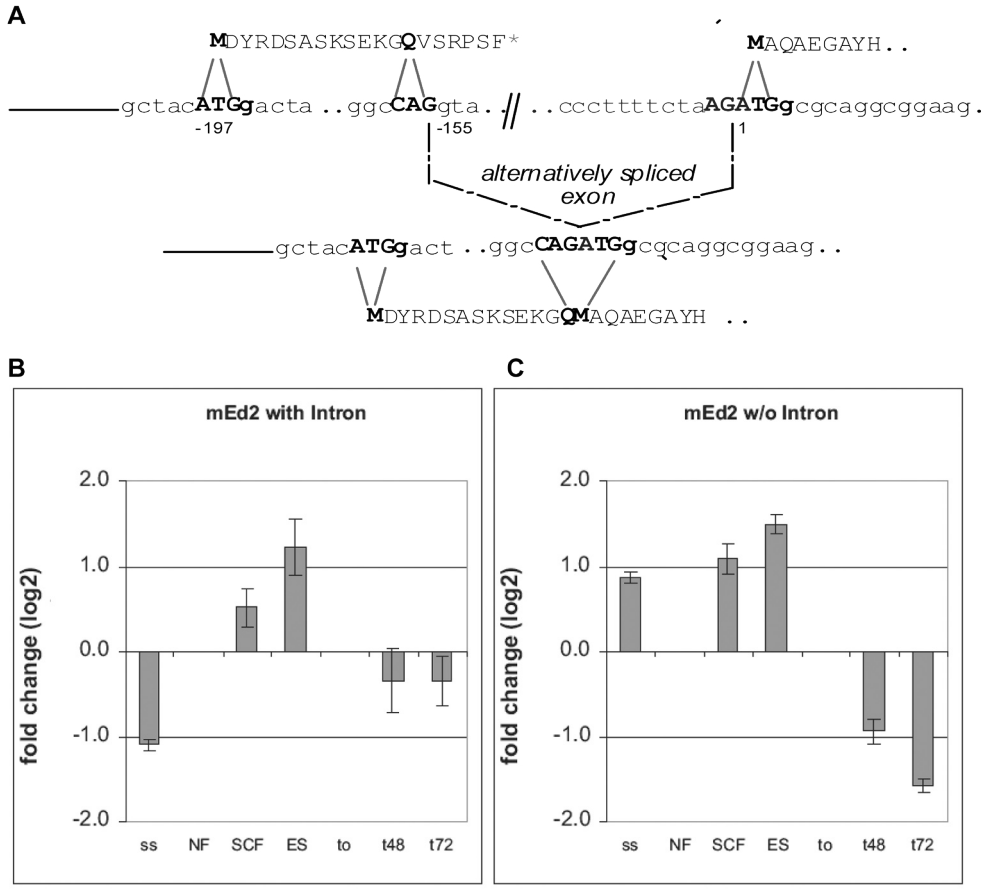

Figure 6.3 **Identification of 5'UTRs of mEd2**. **A**: Loss of Kozak sequence in spliced mEd2 transcript isoform. **B**: Quantitative PCR showing fold increase by growth factor signaling using primers specifically amplifying mEd2 isoform with intron as compared to mEd2 transcript without intron in (**C).**

6.1.3.3 The highly complex Nm23 UTR

We extended further the 5'UTR of Nm23 by 40bp upstream of the longest EST. Critical investigation of the newly identified UTR sequences of Nm23-M2 and mEd2 showed the presence of high enthalpy structures that are distributed differently in relation to the region containing the uORFs. Using luciferase assays we investigated the role of uORFs. Interestingly, the uORFs of mEd2 attenuate translation initiation while the uORFs in Nm23 release inhibition of the high enthalpy

structure proximal to the proper AUG. The mechanism responsible for this release is not easily discernable, and the possible explanation is that peptide synthesis at the uORFs change the conformation of the RNA transcript to allow opening of the high enthalpy structure. In addition, the secondary structure predication software (Mfold; http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) did not recognize the GAIT structure in the uORF of Nm23-M2, the sequence lies within a region of low enthalpy and hence conformation of this region is subject to change upon differential recruitment of RNA binding protein complexes.

6.2 Role of Igbp1/α4 and mEd2 in translational feed-back control

As already briefly indicated in chapter 3, both alpha4 and mEd2 may be involved in positive feed-back control of mRNA translation. Igbp1/alpha4 associates with the phosphatase Pp2a, which dephosphorylates 4E-BP and p70S6K and thus acts as a direct antagonist of TOR activation. Igbp1/α4 modifies the target specificity of Pp2a and inhibits dephosphorylation of TOR-targets. We showed that constitutive expression of Igbp1/α4 in erythroid progenitors sustains proliferation and inhibits differentiation in absence of SCF, enhances Epo-dependent phosphorylation of 4E-BP and p70S6K to levels that otherwise require Epo plus SCF, and enhances polysome recruitment of mRNAs, whose polysome association was previously shown to be strictly PI3K/mTOR dependent (Chapter 3). Thus, Igbp1/α4 acts in a positive feed-back loop on mTOR function, analogous to constitutive activation of kinase receptors.

The phosphatase Pp2a has been described to induce G1 arrest in epithelial cells by dephosphorylation of p70S6K upon TGFβ addition (Petritsch et al. 2000). Block of erythroid progenitor differentiation, by constitutive expression of alpha4, can be rescued by addition of TGFβ (Figure 6.4). However, the action of TGFβ to rescue differentiation block is still unresolved and in addition to modulation of Pp2a activity, the Smad pathway may also play a role. The release of differentiation block by TGFβ implies that Igbp1/α4-expressing cells are not clonal anomalies, but retain the capacity to differentiate if the strength of the differentiation signal is increased. Interestingly, another inhibitor of Pp2a, the putative oncogene SET [von Lindern et al., 1992 is induced in CML (Neviani et al. 2005). Therapeutic activation and ectopic expression of Pp2a inhibits SET activation by BCR/ABL and resulted in reduced proliferation and leukemogenic capacity in transplantation murine models (Neviani et al. 2005).

Future Experiments
Using a flag at an appropriate distance C-terminal of the start sites, we will first need to determine which AUG is really used *in vivo* in erythroid progenitors. Subsequently we will test how alternative splicing affects translation initiation.

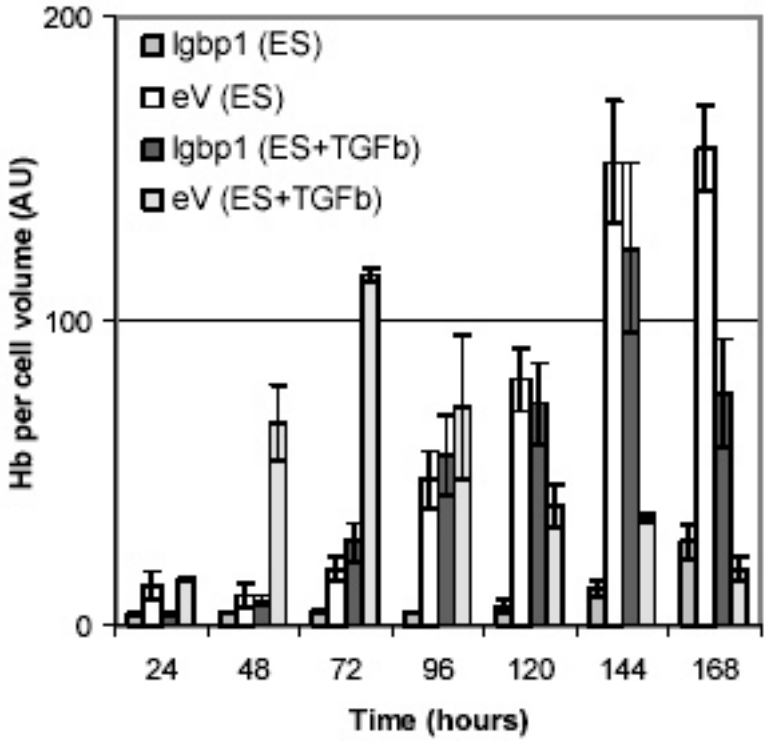

Figure 6.4 **TGFβ induces differentiation of clones constitutively expressing Igbp1/α4**. The addition of TGFβ to Epo plus SCF (ES) cultures induce differentiation of clones derived from I/11 cells transduced with an empty retroviral construct (eV) as shown by the peak of Hemoglobin at 72 hours. Single cell derived clones constitutively expressing Igbp1/α4 proliferate in ES conditions and do not accumulate hemoglobin. Upon addition of TGFβ the block in differentiation was released with a peak in hemoglobin at day 6 (144hrs).

Constitutive expression of the second gene, mEd2, also inhibited erythroid differentiation. Recently mEd2 was shown to be a SNARE protein (Q-SNARE protein D12) with quality control function in ER-lysosome trafficking. Interestingly, the expression level of mEd2/D12 correlated with eIF2 phosphorylation, suggesting that mEd2/D12 may act in a feedback loop signalling ER-stress to eIF2 phosphorylation, although the kinase responsible for the phosphorylation of eIF2 in response to unfolded proteins in the ER, PERK (Protein kinase R-like ER-localised eIF2α kinase), was not involved in this process (Figure 6.5).

Interestingly, the translationally controlled genes attributed to a block of erythroid differentiation are directly involved in feedback mechanisms to maintain mTOR activity and recover eIF2 activity after stress, hence both promoting translation efficiency. This suggest the importance of phosphatases in attenuation of signaling pathways in particular involved in cell fate decisions as in induction of differentiation.

Comparison of polysome recruited transcripts using microarray analysis on RNA isolated from clones with constitutive expression of eIF4E or Igbp1/α4 under Epo conditions, would give insight on crucial effectors in erythroid differentiation delay. Including other model cell lines for other hematopoietic lineages would increase the

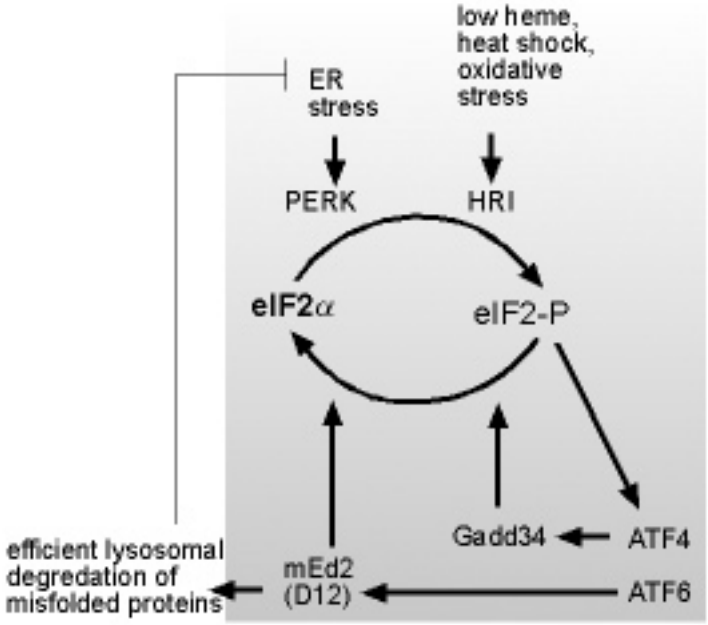

Figure 6.5 **Restoring translation efficiency following stress release**. Upon stress the eIF2α kinases PERK and Heme Regulated Inhibitor (HRI) phosphorylate eIF2α (eIF2-P). Inactivation of eIF2 enhance expression of the activated-trasncription factor 4 (ATF4) and ATF6. For ATF4 it has been shown that an inhibitory uORF is not selected at low eIF2 levels (Vattem and Wek 2004). Growth Arrest and DNA Damage-Inducible Protein, Gadd34 is a direct target of ATF4 and is involved in dephosphorylation of eIF2α. Similarly mEd2/D12 is involved in restoration of PERK induced stress response.

potential to identify critical mechanisms involved in the balance between proliferation and differentiation of hematopoietic progenitors.

In addition, to investigate if the block in erythroid differentiation can be rescued directly by interfering with pp2a activity, clones constitutively expressing Igbp1/α4 will be transduced with the active subunit of pp2a as in (Neviani et al. 2005).

6.3 Selective mRNA translation and cell fate determination

Pluripotent hematopoietic stem cells in the bone marrow of the adult renew to supply a pool of cells capable to diverge into different lineages. Extracellular factors play a critical role in lineage commitment, proliferation, survival and differentiation. Stem Cell Factor (SCF), interleukin 3 (IL3), and granulocyte-macrophage colony stimulating factor (GM-CSF) contribute to a wide range of lineages, while action of erythropoietin, G-CSF and thrombopoietin are confined to specific blood lineage. The issue of cytokine-controlled commitment being an instructive program as opposed to a stochastic event with a permissive role of cytokines is still a matter of debate. The lack of specificity in signaling by cytokine receptors (Socolovsky et al. 1998) in the different hematopoietic lineages suggest that the principal function is the expansion and survival of committed progenitors, as opposed to instructive programs for lineage specific gene expression which is laid down by key transcription factors functioning in a network with multiple layers of feedback control.

SCF-induced PI3K/PKB activity controls the level of eIF4E available for translation initiation and we show that overexpression of eIF4E in erythroid progenitors impairs their differentiation and enhances renewal divisions in absence of SCF. Now we find that control of selective polysome recruitment of structured

transcripts is a major pathway downstream of SCF required to sustain renewal divisions. This implies that the intrinsic transcription program must provide the transcripts of which signalling determines whether or not they are translated. As such, transcription could be seen as instructive and translation as the permissive level of commitment and differentiation. However, growth factors may control both levels. As discussed in Chapter 4, certain cellular mechanisms are controlled by signalling-induced gene transcription, whereas other mechanisms are largely regulated at the level of translation initiation. Interestingly, preliminary results indicate that target genes of the erythroid transcription factors GATA and EKLF are subject to regulation at the level of polysome recruitment. Hence signalling-induced enhanced polysome recruitment overlaps the transcriptional program of these key factors. Further analysis is needed to understand the contribution of such target genes in terminal erythroid differentiation.

6.4 Control of protein synthesis: Igbp1/α4 regulates more than eIF4E release

Constitutive eIF4E expression impairs differentiation, but is not able to sustain renewal beyond 7 days in the absence of SCF. Upon moderate overexpression the cells undergo delayed differentiation but high levels of eIF4E eventually induce erythroid progenitors to die under differentiation conditions.

Interestingly, constitutive expression of Igbp1/α4 results in maintained phosphorylation of both mTOR targets: 4EBP1 and S6K and it blocks erythroid differentiation (Chapter 3). In contrast to eIF4E, alpha4 was able to sustain renewal in absence of SCF for at least 10 days. Surprisingly, we observed that Epo addition, but not SCF was capable to phosphorylate S6K in the context of Igbp1/α4 constitutive expression. This suggests that Igbp1/α4 acts at the level of mTOR activity resulting in simultaneous increase in ribosomal capacity that accommodates enhanced recruitment of non competitive mRNAs. Although overexpression of eIF4E results in constitutive expression of Igbp1/α4, these levels of Igbp1/α4 may not reach the levels that are needed to attenuate phosphatase activity as in the case of constitutive activation of Igbp1/α4. Hence eIF4E overexpression is expected to be limiting in transcriptional activation of ribosomal units. In fact, the expression of Igbp1/α4 in eIF4E clones is comparable to Igbp1/α4 levels of Epo plus SCF stimulated erythroid progenitor cells, while in constitutive expressed Igbp1/α4 the ectopic protein is additional to normal expression (data not shown).

S6K is a key factor in the regulation of ribosome biosynthesis and a prominent target of Igbp1/α4. Ribogenesis is considered to be particularly important for cell growth (cell mass production and cell size control), which is intimately linked to cell cycle progression (Jorgensen et al. 2004). During hematopoietic differentiation induction cell growth and cell cycle are uncoupled to produce smaller mature blood cells. Both IL3 and GCSF stimulate cell cycle, but GCSF-induce neutrophilic differentiation is accompanied by a significant decrease in ribosome abundance and rDNA transcription (Kroll et al. 2001). A similar event occurs early in differentiation of murine erythroleukemic cells (Sherton and Kabat 1976) suggesting that ribosomal content is a common target of differentiation induction.

Strikingly, defects in genes that are critically involved in ribogenesis in yeast contribute to the aetiology of several diseases that are congenital or acquired.

Diamond Blackfan Anemia (DBA) is characterised by mutations in ribosomal protein S19, which is involved in association of the 40S pre-ribosome (Draptchinskaia et al. 1999; Ellis and Massey 2006). Dkc1, the gene affected in X-linked Dyskeratosis congenita (DC) is a pseudouridine synthetase involved in 35S rRNA modification (Heiss et al. 1998; Ruggero et al. 2003; Liu and Ellis 2006). Although the enzyme also affects the RNA component of the telomerase complex, mice with deficient enzyme activity suffer from decreased ribosome capacity well before a telomeric defect becomes apparent (Ruggero et al. 2003). Another inherited marrow failure syndrome, Shwachman-Diamond syndrome is associated with mutations in the SBDS gene encoding an RNA-binding protein localized throughout the cell and particularly in the nucleolus in a cell cycle dependent manner (Austin et al. 2005). It is noteworthy that the above mentioned defects in genes involved in ribogenesis are associated with anaemia, suggesting that either proper function or proper synthesis rates of ribosomes is important to control renewal, survival or differentiation of erythroid progenitors. Additional functions to supply of translational machinery have been attributed to ribosomes. Interestingly, the ribosome components are subject to signalling modifications resulting in releasable factors that attenuate translation of specific transcript (Mazumder et al. 2003). This suggests that modulation or loss of ribosomal constituents does not necessarily mean lowering of global protein synthesis rate, but might modulate specific RNA groups.

The ribosome-associated diseases mentioned above, are all associated with bone marrow failure and to understand the disease mechanism it will be important to understand how ribosome synthesis affects normal hematopoiesis, which has hardly been studied. The results may yield important insight into anaemia in the elderly, which is an increasingly occurring condition in the aging populations of the Western world.

Further investigation is required to examine whether ribogenesis only affects cell mass and cell cycle progression or whether it mainly affects the translation of selective mRNAs encoded by genes that control the balance between erythroid progenitor renewal and differentiation. It will be interesting to analyse whether transcripts regulated by eIF4E and eIF2 are also sensitive to the efficiency of ribosome biosynthesis.

6.5 Proliferation requires cell cycle progression and cell growth

Protein synthesis is generally perceived as an essential cellular process modulated in response to cell growth and cell cycle progression (Pardee 1989; Pyronnet and Sonenberg 2001) and its deregulation is a consequence rather than a cause of tumourogenesis. This is challenged by increasing evidence.

Myc is known as a tumour gene promoting cell cycle progression but only a few genes have been identified as targets for regulation by c-Myc complexes (Niki et al. 2000). Transcriptional upregulation of *eIF4E* is dependent on the transcription factor c-Myc (Jones et al. 1996). We found c-Myc to be upregulated by SCF, while Mxi and Mad, two factors competing for the common heterodimerisation partner Max, are downregulated in response to SCF. Moreover, we validated Myc single strand binding protein (MSSP/Rbms1), a factor enhancing the transforming capacity of c-Myc (Niki et al. 2000), as a target specifically regulated by SCF- and eIF4E-dependent translation initiation (polysome recruitment). The importance of cMyc-

regulated translation initiation factors has long been underestimated, while the search for targets that could explain the highly oncogenic potential of cMyc has been disappointing (Eisenman 2001). In addition, transcription of ribosomal proteins, ribosome assembly factors (Kim et al. 2000), and nucleolar proteins are myc targets genes (Boon et al. 2001; Menssen and Hermeking 2002). This suggests that the transforming capacity of myc could be attributed to enhanced translation initiation efficiency accompanied by enhanced translation machinery capacity.

Multiple tumour suppressors regulate ribosome production and translation initiation, underlining an important role in normal cell homeostasis (Fingar et al. 2004; Inoki et al. 2005). PTEN negatively regulates rpS6 phosphorylation and eIF4E availability by inhibiting the PI3K/mTOR pathway, directly effecting ribosome biosynthesis and translation initiation (Podsypanina et al. 2001; Backman et al. 2002; Blazquez-Domingo et al. 2005). Similarly, TSC1/2 and pp2a act at the level of mTOR activation (Inoki et al. 2002) and maintenance of active mTOR targets (Di Como and Arndt 1996; Gingras et al. 2001), respectively.

6.6 Selective mRNA translation and AML

Our findings proved to be extremely difficult to correlate with existing AML patient information derived from extensive microarray data, since this has been derived from total RNA hybridisations (Valk et al. 2004). For instance the expression of Igbp1/α4 is constant in all patients in the cluster analysis, while the levels of the protein varied between samples as expected for a translationally controlled gene.

In yeast, the homologue of Igbp1/α4 is Tap42, which is required for the TOR pathway to be active. The TOR pathway in yeast is blocked when Tip41 binds and sequesters Tap42. The human homologue of Tip41 is MGC3794. Although this protein has not been studied it is expected –in analogy with yeast- that it suppresses the mTOR pathway and may act as a tumour suppressor gene. It is not known whether this gene is also regulated at the level of mRNA translation or at the level of gene transcription. Interestingly, gene expression profiling and clustering in AML indicated that the Tip41 homologue MGC3794 is downregulated in a specific set of AML (cluster 5 in the study of Valk et al. 2004). This suggests the interesting possibility that this cluster has an increased activity of the mTOR pathway.

Preliminary trials were attempted using a number of approaches to quantify mTOR, S6K, eIF4E activity and alpha4 levels in patient material, but a high throughput investigation is needed to address the link between deregulation of Igbp1/α4 feedback and significance in disease.

Mechanisms that may control Pp2a activity in leukemia should be investigated. On the one hand, the role of deregulated Pp2a activity should be examined in leukemia models. In addition screening of AML samples for mutations deregulating Pp2a activity may shed light on the role of Pp2a in the regulation of proliferation and differentiation of hematopoietic progenitors. First, it is worth to study (*i*) the prognostic significance of the potential tumour suppressor Tip41 and (*ii*) specifically the activity of the mTOR pathway in these AML samples. Second, it has been found that mutations of Tyr307 and Leu309 in the catalytic subunit of Pp2a, favour association with the alpha4 subunit and promote protein synthesis (Chung et al. 1999). To investigate whether mutations in Pp2a are involved in leukemogenesis, it is important to screen at least for these mutations.

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Summary

Summary

The balance between proliferation and differentiation of committed hematopoietic progenitors is under tight control to maintain the progenitor pool and ensure maturation in response to physiological demand. Whereas development of the different hematopoietic lineages requires the coordinate expression of transcription factors, the balance between proliferation and differentiation is controlled by growth factors. These include cytokines such as erythropoietin (Epo) and granulocyte-colony stimulating factor (G-CSF) required for differentiation into specific lineages, as well as growth factors that enhance proliferation and delay differentiation of progenitors such as stem cell factor (SCF) and the ligand for FMS-like tyrosine kinase 3 (Flt3-ligand).

To investigate how the balance between expansion and differentiation of erythroid progenitors is controlled by growth factors, we used a model in which erythroid progenitors proliferate in the presence of Epo, SCF and glucocorticoids while they maintain the capacity to differentiate upon addition of Epo alone. SCF-driven proliferation and inhibition of differentiation is dependent on phosphoinositide-3-kinase (PI3K) activation. The main objective of the study described in this thesis is to understand how PI3K-controlled gene expression regulates the balance between proliferation and differentiation of erythroid progenitors. In particular, we studied the role of PI3K dependent control of mRNA translation.

An extensive literature review of the importance of translation control in the regulation of hematopoiesis, is given in **chapter 1**. In general, translation of mRNA depends on the two major factors: (1) the availability and activity of the translation machinery and (2) the structure of a messenger. Structural elements in the mRNA render translation of the transcript sensitive to the availability of translation factors, which affect the translation of simple transcripts far less.

The SCF-induced activation of PI3K controls the kinase mTOR (Target of Rapamycin), which releases eukaryote initiation factor 4E (eIF4E) from a repression complex to form an active pre-initiation scanning complex (eIF4F). We could demonstrate that increased expression of eIF4E enhanced the formation of the eIF4F scanning complex and enhanced the recruitment of structured mRNAS into polyribosomes. Importantly, enhanced expression of eIF4E impaired differentiation of erythroid progenitors into mature erythrocytes, indicating an important role of this pathway in the regulation of proliferation versus maturation of erythroid progenitors (**chapter 2**).

To identify transcripts that are subject to selective polysome recruitment in response to growth factors, we compared SCF-dependent gene expression between total and polysome bound mRNA (**chapter 3**). This yielded a list of 115 genes subject to major regulation by translation initiation. The transcripts of nine out of ten genes tested in more detail required PI3K or increased eIF4E expression to be associated with polyribosomes. Among these genes is *Igbp1* (*Immunoglobulin binding protein-1*) that binds and inhibits the serine/threonine phosphatase Pp2a. This phosphatase functions as an mTOR antagonist in translation initiation. Constitutive expression of Igbp1 strongly impaired erythroid differentiation, maintained phosophorylation of two important mTOR targets, eIF4E binding protein (4EBP) and S6-kinase (p70Sk) phosphorylation and enhanced polysome recruitment of structured mRNAs. Thus,

we found that PI3K-dependent polysome recruitment of Igbp1 acts as a strong positive feedback mechanism on translation initiation, which underscores the important regulatory role of selective polysome recruitment for erythropoiesis.

Next, we evaluated the importance of growth factor-induced polysome recruitment versus transcriptional regulation of gene expression. In addition, we analysed to what extent growth factor-controlled genes are differentially regulated during differentiation and *vice versa* (**chapter 4**). Surprisingly, we found a 2,5-fold increase in growth factor-controlled genes upon analysis of polysome-bound mRNA compared to total RNA. The prominent, and so far underestimated effect of signalling on mRNA translation prompted us to evaluate signalling dependent expression of translation factors and proteins involved in ribosome biosynthesis. Together, ribosome synthesis regulation and polysome recruitment ensure enhanced translation efficiency in presence of mitogenic factors as discussed in **chapter 4**.

Next, we studied which elements in the 5' untranslated region (5'UTR) of Nm23-M2 are responsible for growth factor dependent protein synthesis (**chapter 5**). Nm23-M2 was not only found to be subject to translational control, it is also a common virus integration site in retrovirally induced murine leukaemia. A virus integration in the 5'UTR of Nm23-M2 in the murine leukaemia cell line NFS61 results in a LTR-Nm23 fusion transcript that disrupts a predicted stem-loop structure and perturbs factor dependent translation. The 5'UTR of *Nm23-M2* contains high enthalpy structures, upstream open reading frames and putative signalling responsive elements. To study the effect of these structures on growth factor dependent mRNA translation, luciferase constructs flanked with *Nm23-M2* UTR sequences were expressed in factor-dependent cell lines. Upstream ORFs in the distal part of the 5'UTR appeared to enhance polysome recruitment, while stem-loop elements in the proximal part of the 5'UTR inhibit translation. A similar approach is being taken for two other translationally controlled genes that both impair erythroid differentiation when constitutively expressed: Igbp1 and mEd2. Both genes contain a complex 5'UTR which we identified by homology searches and 5'RACE (**chapter 6**).

In conclusion, the studies presented in this thesis demonstrated that polysome recruitment of structured mRNAs is a major target of SCF-induced PI3K activation, which is required for the expansion of the pool of erythroid progenitors. First, this mechanism of translational control involves the regulation of the cap-binding factor eIF4E. Also the growth factor dependent regulation of multiple other translation factors and ribosome synthesis factors is likely to contribute to enhanced mRNA translation, particular of highly structured transcripts. Second, we identified a number of genes that are subject to translational control. The analysis of their untranslated regions may shed light on the mechanism of transcript specific translational control.

Samenvatting

De balans tussen groei en uitrijping van bloedcelvoorlopers moet nauwkeurig gereguleerd worden om zowel de voorraad voorlopercellen als hun uitrijping aan te passen aan de vraag. Terwijl de ontwikkeling van de verschillende typen bloedcellen een gecoördineerde expressie van transcriptiefactoren vereist, wordt de vermeerdering van voorlopercellen vooral geregeld door groeifactoren. Hiertoe behoren cytokines die de uitrijping in specifieke celtypen controleren, zoals erythropoietine (Epo) en 'granulocyte colony stimulating factor' (G-CSF), en groeifactoren die celgroei stimuleren en de uitrijping remmen zoals stam cel factor (SCF) en de factor die de tyrosine kinase receptor Flt3 bindt (Flt3-ligand).

Voor ons onderzoek hebben we een model systeem gebruikt waarin voorlopercellen kunnen uitgroeien in aanwezigheid van Epo, SCF and glucocorticoïden, terwijl de cellen uitrijpen tot rode bloedcellen in aanwezigheid van alleen Epo. SCF-geïnduceerde groei en remming van het uitrijpen is afhankelijk van phosphoinositide-3-kinase (PI3K) activiteit. Het doel van de studies beschreven in dit proefschrift is om te begrijpen hoe PI3K afhankelijke regulatie van gen expressie de balans tussen groei en uitrijping van rode bloedcelvoorlopers controleert. In het bijzonder hebben we de rol van PI3K in het vertalen van mRNA bestudeerd.

Hoofdstuk 1 bevat een uitgebreid literatuur overzicht over de rol die controle van mRNA vertaling kan spelen in de bloedcelvorming. Het vertalen van een mRNA hangt af van twee factoren: (1) de beschikbaarheid en activiteit van de vertaalmachinerie, en (2) de structuur van het mRNA. Structuren in het mRNA kunnen ervoor zorgen dat het vertalen van een mRNA afhankelijk wordt van de beschikbaarheid van translatiefactoren, terwijl het vertalen van eenvoudige mRNAs hierdoor veel minder wordt bepaald.

PI3K reguleert het kinase mTOR (Target of rapamycin), waardoor de eukaryote translatie initiatie factor 4E (eIF4E) vrijkomt om een actief pre-initiatie scanning complex te vormen (eIF4F). Een matig verhoogde expressie van eIF4E bleek te resulteren in forse verhoging van het eIF4F scanning complex, en in het vertalen van transcripten met een complexe RNA structuur. Een verhoogde expressie van eIF4E blokkeerde ook de uitrijping van voorlopers tot rode bloedcellen wat erop duidt dat eIF4E afhankelijk translatie van mRNAs van belang is om de balans tussen groei en uitrijping van voorlopercellen te reguleren (**hoofdstuk 2**).

Om de transcripten te identificeren waarvan de vertaling afhangt van de aanwezigheid van groeifactoren, hebben we een vergelijking gemaakt tussen SCF-geïnduceerde gen expressie op het niveau van totaal RNA versus ribosoom-gebonden RNA (**hoofdstuk 3**). Zo vonden we 115 genen waarvan de expressie vooral gecontroleerd wordt door selectieve vertaling. Voor negen van de tien transcripten die we getest hebben, was de vertaling afhankelijk van PI3K activiteit en eIF4E expressie. Tot deze genen behoorde *Igbp1 (Immunoglobulin binding protein 1)*. Igbp1 bindt en remt het serine/treonine fosfatase Pp2a. Dit fosfatase functioneert als een antagonist van mTOR in de vertaling van mRNAs en daardoor kan Igbp1 de mRNA vertaling juist stimuleren. Een constante expressie van Igbp1 blokkeerde de uitrijping van voorlopercellen tot rijpe rode bloedcellen, handhaafde de fosforylatie van 4EBP en p70S6kinase door mTOR, en liet transcripten met een complexe structuur beter met de ribosomen associëren. De PI3K afhankelijke vertaling van

Igbp1 functioneert dus als een sterk positief terugkoppelingseffect op de vertaling van complex gestructureerde transcripten, wat het belang van dit proces voor rode bloedcel ontwikkeling onderstreept.

Vervolgens hebben we geanalyseerd hoe groot het belang van groeifactor afhankelijk betrekken van mRNAs in polyribosomen is ten opzichte van groeifactor afhankelijke gentranscriptie. Daarbij hebben we ook bekeken in welke mate de expressie van genen die gereguleerd worden door groeifactoren verandert tijdens de uitrijping tot rode bloedcellen (**hoofdstuk 4**). Tot onze verrassing identificeerden we 2,5 maal zoveel groeifactor gereguleerde genen in polysoomgebonden RNA als in totaal RNA. Het prominente en tot nu toe ondergewaardeerde effect van groeifactor-geïnduceerde vertaling van mRNAs was aanleiding om de factor afhankelijke expressie van translatie factoren en eiwitten betrokken bij ribosoom synthese nader te analyseren.

Vervolgens hebben we bestudeerd welke elementen in de 5' onvertaalde regio (5'UTR) van het Nm23-M2 transcript groeifactor afhankelijke vertaling bepalen (**hoofdstuk 5**). Nm23-M2 is niet alleen gevonden als een gen waarvan expressie wordt geregeld op het niveau van mRNA vertaling, het is ook doelwit van retrovirale integratie in muis leukemie. Een virus integratie in de 5'UTR van Nm23-M2 in de leukemie cellijn NF61 resulteert in een fusietranscript van virale sequenties met Nm23-M2 waardoor een stabiele stem-loop structuur verloren gaat en de regulatie verstoord wordt. Om het effect van verschillende structurele elementen in de 5'UTR van Nm23-M2 op de vertaling van het transcript te bestuderen hebben we ze gefuseerd met sequenties die coderen voor luciferase en tot expressie gebracht in groeifactor afhankelijke cellijnen. De uORFs bleken de vertaling van het fusietranscript te bevorderen, terwijl stem-loop elementen de vertaling remden. Eenzelfde aanpak wordt gevolgd voor twee andere genen die we geïdentificeerd hebben als zijnde gereguleerd op het niveau van mRNA vertaling, en in staat de uitrijping van rode bloedcelvoorlopers te remmen: Igbp1 en mEd2. Van beide genen hebben we de 5'UTR bepaald, die zeer complex blijkt te zijn (**hoofdstuk 6**).

De studies die zijn beschreven in dit proefschrift laten zien dat de vertaling van transcripten met een complexe RNA structuur een belangrijke signaalfunctie is van SCF-geïnduceerde PI3K activiteit, die noodzakelijk is om het aantal rode bloedcelvoorlopers te laten groeien. Het mechanisme omvat de regulatie van de translatie initiatie factor eIF4E, maar ook de expressie van een groot aantal andere translatie factoren, en eiwitten die de ribosoom aanmaak reguleren. We hebben een groot aantal genen geïdentificeerd die gereguleerd worden door groeifactor afhankelijke vertaling. Analyse van de onvertaalde stukken van het transcript zal hopelijk het mechanisme van groeifactor afhankelijke mRNA vertaling onthullen.

4EBP	eIF4E binding protein
ActD	Actinomycin D
AML	acute myeloid leukemia
ANOVA	Analysis of Variance
ARE	AU-rich element
BFUe	Burst-forming unit erythroid
CFU-e	Colony-forming unit erythroid
ChX	cycloheximide
CLP	Common lymphoid precursor
CML	chronic myeloid leukemia
CMP	Common myeloid precursor
Dex	dexamethasone
eEF	eukaryote elongation factor
eIF4E	eukaryote initiation factor 4E
Epo	Erythropoietin
EpoR	Erythropoietin Receptor
Flt3	fetal liver tyrosine kinase-3
GAIT	Gamma interfron activated inhibitor
GMP	granulocytic/monocytic progenitor
hnRNP	heterogenous nuclear ribonucleoprotein
HRI	haeme regulated eIF2alpha kinase
HSCs	Hematopoietic Stem Cells
Igbp1	Immunoglobulin binding protein 1
IRE	Iron Responsive Element
IRES	Internal Ribosome Entry Site
ITAF	IRES trans-acting factor
LTR	long terminal repeat
MDS	myelodysplastic syndrome
mEd2	mouse embryonic differentiation factor 2
MEP	Megakaryocytic/erythroid progenitor
miRNA	micro RNA
MPD	Myelo-Proliferative Disorder
mTOR	mammalian Target of Rapamycin
NF	No Factor added (depriviation)
ODC	ornithine decarboxylase
p70SK	S6 kinase (70kDa)
pbRNA	polysome bound RNA
PI3K	posphoinositide-3-kinase
PKB	Protein Kinase B
PV	polycythemia vera
RACE	Rapid Amplification cDNA Extension
Rheb	RAS-homologue enriched in brain
rRNA	ribosomal RNA
RTK	receptor tyrosine kinase

SCF	Stem Cell Factor
TGF	transforming growth factor
TNF	tumour necrosis factor
TOP	terminal oligopyrimidine tract
TPO	thrombopoeitin
Tsc	tuberous sclerosis protein
uAUG	upstream AUG
uORF	upstream open reading frame
UTR	untranslated region

Curriculum Vitae

Godfrey Grech was born on 9 December 1972 in Malta. He graduated at the University of Malta specialising in Biology and Chemistry in 1994. The first experience in medical research gained was at the Department of Pathology, University of Malta. The main purpose of the laboratory was to characterise β-thalassaemia in the Maltese population and implement screening techniques for other diseases. He pursued a Masters degree at the University of Malta with the objective to develop population screening for the metabolic disorder, glucose-6-phosphate dehydrogenase deficiency, using the infrastructure (samples, methodology) already available for screening hemoglobinopathies. A number of variants were identified and a new variant (G6PD Malta I) was characterised by sequencing the coding sequence of the gene (unpublished thesis). Allelic frequencies characterising the disease were calculated using more than 2000 samples.

Following this experience he moved on to work in the food industry gaining management skills by being responsible for the technical projects and implementation of quality systems. The main achievements in industry included an award for top performance in Licence examinations, which led him to become the technical manager with direct duties to the Board of Directors of the company. The direct responsibilities included the overall co-ordination of the production plant, management of projects, the documentation and implementation of quality and food safety standards and training of personnel.

His re-integration to the medical science research was possible by being appointed as an Assistant Lecturer at the Pathology department (University of Malta) to deliver lectures in Cell Biology and Molecular Biology to medical students and as a Research Laboratory Co-ordinator where he was involved in technology transfer to monitor treatment of thalassaemic patients undergoing clinical trials.

He moved to pursue his doctorate studies in the molecular control of erythropoiesis at the Department of Hematology (Erasmus MC). Currently he has been appointed as a Lecturer at the University of Malta, coordinating the Masters in Pathology course. He is attracted to research that addresses both basic mechanisms in biology and translation of results to medical applications.

PUBLICATIONS

Blazquez-Domingo, M.*, Grech, G.* and von Lindern, M. (2005) Translation Initiation Factor 4E Inhibits Differentiation of Erythroid Progenitors. Mol. Cell. Biol., 25, 8496-8506. * authors contributed equally

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G.Grech, A.E.Felice. 1996. Molecular Characterisation of Glucose-6-phosphate dehydrogenase deficient cases in the Maltese population. Thesis.

PRESENTATIONS

Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK
Invited by: Prof. Doug Higgs. November 23, 2005

University of Malta, Malta
Invited by: Dr. Pierre Schembri Wismayer. November 28, 2005

Istituto Superiore di Sanita`, Rome, Italy
Invited by: Prof Anna Rita Migliaccio. September 22-23, 2005

University of Malta, Malta
Invited by: Dr. Pierre Schembri Wismayer. November 15, 2004

Institute of Molecular Pathology, Vienna, Austria
Invited by: Prof. Hartmut Beug. July 27-29, 2004

Laboratoire de Biologie Moléculaire et Cellulaire, Lyon, France
Invited by: Prof. Jacques Samarut. January 16-17, 2004

PARTICIPATION IN CONFERENCES

European Hematology Association 2006 Oral Presentation
Amsterdam, the Netherlands; June 15-18,

American Society of Hematology 2005 Oral presentation
Atlanta, US; December 10-13,

2005Najaarsconferentie 2005 NVvH Oral presentation
Lunteren, The Netherlands; November 2-4,

Erasmus Workshop on Molecular Therapeutics in Acute Leukemia Poster
Rotterdam, The Netherlands; October 7-8, 2005,

American Society of Hematology 2004 Oral presentation
San Diego, US; December 4-7,

2004Najaarsconferentie 2004 NVvH Oral presentation
Lunteren, The Netherlands; November 3-5,

FEBS, Dubrovnik Signaling Course Poster
Dubrovnik, Croatia; May 21-27, 2004,

Erasmus Workshop on Molecular Therapeutics in Acute Leukemia Poster
Rotterdam, The Netherlands; September 12-13, 2003

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