The spiders at the center of the web
Csde1 and strap control translation in erythropoiesis
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Citation for published version (APA):
Moore, K. S. (2018). The spiders at the center of the web: Csde1 and strap control translation in erythropoiesis.
Chapter 1

General Introduction

An adapted version of this chapter has been published as:

RNA-binding proteins and regulation of mRNA translation in erythropoiesis

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In the human body, the production of red blood cells (erythropoiesis) is a delicate balance between supply and demand. The body must maintain a population of erythrocytes that is large enough to adequately oxygenate tissue, while avoiding elevated risk of hypertension, clotting and stroke when the erythrocyte population is too large. This chapter presents an overview of the molecular mechanisms which control gene expression during erythropoiesis, with particular emphasis on the translation of mRNA transcripts to protein, and the consequences when translational control is disrupted.

### 1.1 HEMATOPOIESIS & ERYTHROPOIESIS

Hematopoiesis is the process by which new blood cells (red and white) are formed. The process is governed by the maintenance of hematopoietic stem cells (HSCs) in the bone marrow, which undergo asymmetric division into one daughter HSC and a lineage specific progenitor [1]. HSCs and their immediate daughter cells are characterized by the presence of cell surface markers Sca1 and c-kit, and lack of markers associated with cells committed to a specific branch of differentiation [2]. Hence, these cells at the top of the hematopoietic hierarchy are referred to as the LSK (lineage-/Sca1+/c-kit+) compartment [3]. Long-term hematopoietic stem cells (LT-HSCs) are mostly quiescent and can sustain lifelong hematopoiesis, whereas short-term hematopoietic stem cells (ST-HSCs) divide more frequently but have a limited lifespan [4–6]. LT-HSCs are negative for cell surface markers CD34 and Flt3, while ST-HSCs are Flt3-positive. A third member of the LSK compartment are multipotent progenitors (MPPs), which are positive for both CD34 and Flt3 and form the proliferating compartment that actively replenishes all the blood cell lineages with the required number of progenitors. The hematopoietic tree branches at this point into the lymphoid and myeloid common progenitors (CLP and CMP, respectively). CLPs differentiate into B, T, and NK immune cells, whereas CMPs split further into granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs) [3]. MEPs subsequently differentiate into megakaryocytic and erythroid precursors (Figure 1A).

From CMP to mature erythrocyte, the erythropoietic lineage is characterized by morphology and colony-forming capacity (Figure 1B). The CMP is described as colony-forming unit- granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) [7,8]. A CFU-GEMM gives rise to a burst-forming unit-erythroid (BFU-E) containing multiple thousands of cells expressing the erythropoietin receptor (EpoR) at low levels [9]. EpoR expression is increased during the subsequent stage of differentiation, during which a smaller, hemoglobin-containing colony-forming unit-erythroid (CFU-E) is formed. Further differentiation towards the mature erythrocyte occurs in multiple morphologically recognizable stages [10]. The proerythroblast contains a large nucleus
with multiple observable nucleoli. The basophilic erythroblast is smaller in diameter with a proportionally smaller nucleus : cytoplasm ratio and granular cytoplasm. The polychromatophilic erythroblast displays initial condensation of the nucleus without visible nucleoli and a regionally acidophilic cytoplasm. The orthochromatic erythroblast contains a darker nucleus with a further reduction in nuclear volume relative to the cytoplasm and a visible reddish tint due to increased hemoglobin concentration. By the reticulocytic stage, the cell has lost its nucleus. Upon subsequent loss of the ribosomes and mitochondria, the erythrocyte has fully matured.

Figure 1. Schematic overview of the hematopoiesis and erythropoiesis. (A) Self-renewing cells in the LSK compartment differentiate via the CLP (common lymphoid progenitor) into lymphocytes (B: B-cell, T: T-cell, NK: natural killer cell) or the CMP (common myeloid progenitor) and the GMP (granulocyte-macrophage progenitor) into granulocytes (G) and macrophages (M). The MEP (megakaryocyte-erythroid progenitor) yields megakaryocytes (MK) or progress down the erythroid lineage. (B) Erythroid-committed cells display characteristic morphology. Maturation progresses through BFU-E (burst-forming unit-erythroid), CFU-E (colony-forming unit-erythroid), Pro-EB (pro-erythroblast), Baso-EB (basophilic erythroblast), PC-EB (polychromatophilic erythroblast), Ortho-EB (orthochromatic erythroblast). The cell loses the nucleus to become the reticulocyte and finally, loss of the mitochondria and ribosomes mark the mature erythrocyte.
1.1.1 Transcription factors controlling erythropoiesis

Erythrocytes circulate through human peripheral blood for approximately 120 days. Every day, the human body produces $10^{11}$ new erythrocytes, which requires a tight balance between progenitor proliferation and maturation. An excess of erythrocytes poses a severe risk of thrombosis, whereas a paucity of erythrocytes causes anemia with a risk of ischemic damage in the tissues. Maintenance of this balance requires tightly controlled regulation of erythropoietic homeostasis, enacted by a cascade of transcription factors and signaling pathways that control erythropoiesis. Several transcription factors are essential for determining the erythroid program of gene expression. Gata1 functions as the master regulator of erythropoiesis, inducing the expression of EpoR [11–13] and commitment to the erythroid lineage. Together with cofactor Fog1, it promotes the transcription of $\beta$-globin [14]. Nfe2 controls globin synthesis via chromatin remodeling and by binding directly to the promoters of both $\alpha$- and $\beta$-globin as well as ferrochelatase, a heme biosynthetic enzyme [15]. Also of importance in terminal erythroid differentiation is Klf1 (Kruppel-like factor 1), a transcription factor essential for the synthesis of $\beta$-globin [16]. Haploinsufficiency in Klf1 causes hereditary persistence of fetal hemoglobin (HPFH) [17]. Furthermore, Klf1 is involved in many cellular changes required for the maturation of erythroblasts to erythrocytes [18].

1.1.2 Signaling in erythropoiesis

Signaling cascades allow for the adaption of erythropoietic expansion and differentiation upon demand. For instance, during hypoxic stress, Epo production in the kidneys is dramatically increased, allowing the body to counteract tissue hypoxia via the expansion of erythrocyte production [16]. The binding of Epo to EpoR triggers a conformational change that activates the cytoplasmic tyrosine kinase Jak2 [10]. In turn, Jak2 triggers a downstream signaling cascade via the Stat5, Akt and PI3 kinase pathways to support the proliferation and differentiation of erythropoietic progenitors [19–21]. Stat5 induces the expression of anti-apoptotic Bcl-xL to maintain viability during terminal maturation [11]. In the absence of Bcl-xL, erythropoiesis is negatively regulated primarily via caspase-mediated apoptosis. The activation of caspases 8 and 9 activates downstream effector caspases (such as caspase 3), resulting in the cleavage of Gata1 [10]. This molecular safety mechanism prevents the overproduction of erythrocytes, which can lead to hyperviscosity and ischemic stroke. In summary, low levels of Epo cause cell death by apoptosis, intermediate levels prevent differentiation, and high levels of Epo allow the production of mature erythrocytes [10]. Epo therefore functions as a dosage-dependent regulator of erythropoiesis in response to physiological demand.

Stem cell factor (Scf), the ligand for the c-Kit receptor, acts in conjunction with Epo to repress differentiation and maintain the population of erythroid progenitors [22]. Scf
signaling is enacted via the phosphoinositide-3-kinase (Pi3k) pathway, resulting in the activation of protein kinase B (Pkb) [16]. Pi3k/Pkb signaling has two major effector pathways in erythroblasts. On the one hand, it prevents nuclear localization of the Foxo3a transcription factor that otherwise induces erythroid differentiation [23]. Concurrently, it activates mammalian target of rapamycin (mTOR), which interacts with translation initiation factors to promote the translation of a specific set of transcripts [24].

Increasingly, it is understood that erythropoietic signaling pathways enact not only transcriptional, but also translational control of gene expression. In particular, tight regulation of the initiation of translation is critical for the balance between proliferation and differentiation. The mechanisms which govern the initiation of translation in the context of erythropoiesis is discussed in the next section. This includes an overview of cap-dependent translation, which is enhanced by Epo/Scf signaling and inhibited by heme deficiency or oxidative stress, as well as features within the 5’UTR which determine transcript sensitivity to regulatory pathways.

### 1.2 CAP-DEPENDENT TRANSLATION INITIATION

According to the central dogma of molecular biology, genetic information encoded in DNA is transcribed into mRNA in the nucleus, which is exported into the cytoplasm and translated into proteins. Studies on gene expression have historically focused on transcription as the primary regulatory mechanism. However, there is increasing evidence to suggest that translation is another major determinant of protein abundance in the cell [25]. In other words, transcription determines whether a given gene is expressed, but translation determines the level of expression. Control of translation is mediated by a complex web of overlapping mechanisms, including, but not limited to, regulation of translation initiation, the length of the poly(A) tail and the presence of short upstream open reading frames (uORFs). Below, a brief overview of these topics as pertains to erythropoiesis is presented.

Canonically, translation in higher eukaryotes is initiated via an interaction between the 5’ cap structure of the mRNA and a family of translation initiation factors, which cooperate to recruit the ribosome [26]. Briefly summarized, a ternary complex (TC) consisting of Met-tRNA, eIF2 and GTP associates with the 40S ribosomal subunit and with eIFs 1, 1A, 3 and 5 to form the 43S pre-initiation complex (PIC). At the same time, cap-binding factor eIF4E associates with scaffolding protein eIF4G and the RNA helicase eIF4A to form the eIF4F complex [27]. eIF4F interacts with poly-A binding protein (Pabp), forming a closed-loop structure between the 5’ and 3’ end of the mRNA. The recruitment complex is subsequently formed via an interaction between eIF4G in the closed-loop structure and eIF3 in the PIC. Upon recruitment, the complex is referred
to as the 48S initiation complex. The preinitiation scanning complex scans the bound mRNA until encountering an initiation codon, at which point recognition of the initiation codon triggers the hydrolysis of eIF2-bound GTP by eIF5 and the recruitment of the 60S large ribosomal subunit, marking the end of initiation phase and the beginning of elongation (Figure 2).

Availability of GTP-loaded eIF2 is the limiting factor of translation initiation [28]. Once translation initiation has occurred, hydrolysis of GTP to GDP ensures that eIF2 must be reloaded before a new round of translational initiation can begin. This is accomplished via GDP-GTP exchange factor eIF2B, allowing the pre-initiation complex containing eIF2-GTP and Met-tRNA to reform. This mechanism is subject to control by phosphorylation (for example, by HRI, see section 1.2.2: eIF2 phosphorylation in erythropoiesis) of the \( \alpha \) subunit of eIF2, which prevents the GDP-GTP exchange, resulting in the inhibition of cap-dependent translation (Figure 3A).

![Figure 2. Cap-dependent translation initiation.](image)

Cap-dependent translation begins when the ternary complex (consisting of Met-tRNA, eIF2 (2) and GTP) associates with the 40S ribosomal subunit and eukaryotic initiation factors (eIFs) 1, 1A, 3 and 5 (labelled numerically in the figure). This assembly is referred to as that 43S pre-initiation complex (PIC). The PIC interacts with mRNA in a closed-loop formation, enacted by the binding of eIF4E (4E) to the 5' cap and binding of PABP to the poly(A) tail, with scaffolding protein eIF4G (4G) bridging the two. RNA helicase eIF4A (4A) promotes a configuration conducive to ribosomal landing. The heterotrimer consisting of eIF4A, eIF4E and eIF4G is referred to as the eIF4F complex. After docking with the ribosome, the 48S pre-initiation complex (PIC) is formed, and the transcript is scanned until encountering a start codon, upon which eIF2-bound GTP is hydrolyzed and the 60S ribosomal subunit is recruited. At this stage, initiation of translation is complete, and translational elongation begins. Figure adapted from Kong and Lasko, 2012 (with permission).
1.2.1 Epo and Scf control translational initiation in erythroblasts

Stem cell factor (Scf) cooperates with erythropoietin (Epo) to expand erythroblast numbers in vivo and in vitro, whereas erythroblasts mature to erythrocytes in presence of Epo only. The crucial pathway activated by Scf is mTOR-dependent release of the cap binding factor eIF4E from its binding protein, 4EBP [29] (Figure 3B). Overexpression of eIF4E inhibits erythroid differentiation. Upon release from 4EBP, eIF4E can once again bind the scaffold protein eIF4G, allowing the formation of the pre-initiation scanning complex [27,30]. Epo signaling overlaps with Scf signaling on the mTOR pathway. The result of Epo signaling is a partial phosphorylation of 4EBP, which requires synergy with Scf stimulation for the phosphorylation of all 4EBP phosphorylation sites to sustain erythroid proliferation [29]. The primary antagonist of mTOR-mediated 4EBP phosphorylation is protein phosphatase 2A (Pp2a), a ubiquitously expressed phosphatase that changes target specificity based upon complex formation with regulatory proteins [31].

Although the availability of eIF4E controls overall translation initiation, transcripts with a TOP (terminal oligopyrimidin tract) or secondary structures are hypersensitive to the available eIF4E concentration [32,33]. One such transcript is immunoglobulin binding protein 1 (Igbp1), the mRNA of which is constitutively expressed in erythroblasts, but selectively translated only upon Scf-induced eIF4E release [24]. Igbp1 is more suitably known as the alpha4 subunit of Pp2a. As a regulatory subunit, it inhibits the catalytic activity of Pp2a on 4EBP. Expression of Igbp1/alpha4 thereby enhances the effect of low levels mTOR activation on mRNA translation. Scf-induced expression of Igbp1 acts as a positive feedback mechanism in the polysome recruitment of multiple eIF4E-sensitive mRNAs, resulting in the attenuation of erythroid differentiation.

1.2.2 eIF2 phosphorylation in erythropoiesis

Regulation of translation initiation is of particular importance in hemoglobin synthesis. Erythrocytes carry approximately 250 million hemoglobin molecules, each consisting of 4 globin peptides and 4 iron-containing heme molecules. This huge iron reservoir has a large oxidative, damaging potential. Iron deficiency reduces heme availability and presents a risk of cell damage from the accumulation of free α- and β-globins that form toxic precipitates known as Heinz bodies [34]. Therefore, in- and export of iron in erythroblasts, and the synthesis of heme and globin needs to be tightly coupled. This process is controlled by several mRNA translation mechanisms [35]. Heme acts as a signaling molecule which binds to eIF2 associated kinase (eIF2ak1), also called HRI (Heme Regulated Inhibitor) [36]. During heme deficiency, HRI phosphorylates eIF2 alpha, preventing the exchange of eIF2-bound GDP for GTP by eIF2B and therefore also preventing the reassociation of the preinitiation scanning complex (Figure 3A) [37,38]. This results in a global inhibition of protein synthesis [39]. This mechanism
Figure 3. Molecular regulation of cap-dependent translation in erythropoiesis. (A) eIF2 phosphorylation inhibits cap-dependent translation under stress conditions. After a round of translation initiation, eIF2 must be reloaded with new GTP by eIF2B in order for the ternary complex to be reformed and for the 60S ribosomal subunit to be recruited in subsequent cycles of translational initiation. Under stress conditions, eIF2 is phosphorylated by, for example, HRI (heme regulated inhibitor, during heme scarcity), PKR (protein kinase R, upon recognition of viral dsRNA), PERK (PRK-like endoplasmic reticulum kinase, upon aggregation of misfolded proteins), or GCN (General control nonderepressible, during amino acid starvation), preventing eIF2B from exchanging eIF2-bound GDP for GTP. The lack of unphosphorylated eIF2 leads to translational arrest. (B) 4E-BP phosphorylation regulates cap-dependent translation. Scf signaling, and, to a lesser extent, Epo signaling, activates the mTOR pathway, which leads to the phosphorylation of 4E-BP and the release of cap-binding protein eIF4E. Increased availability of eIF4E stimulates translation globally, but has a pronounced effect on transcripts containing structured 5' UTRs, including Igbp1. Ibp1 inhibits the dephosphorylation of 4E-BP by Pp2a, strengthening the effect of mTOR activation.
is similar to eIF2 alpha phosphorylation resulting from detection of (viral) double-stranded RNA via eIF2ak2 (PKR), ER-stress via eIF2ak3 (PERK), and amino acid starvation via eIF2ak4 (GCN2) [40]. In addition to heme deficiency, HRI is activated both by oxidative stress and by denatured cytoplasmic proteins [35].

### 1.2.3 Upstream open reading frames

Sensitivity to eIF2 phosphorylation is predicted by the presence of upstream open reading frames (uORFs) in the 5' UTR of the transcript [27,41]. uORFs render mRNA transcripts hypersensitive to translation initiation factors because their translation requires reassociation of eIF2:GTP and tRNA\textsubscript{i}m\textsuperscript{et} with the scanning complex (Figure 2). uORFs may also inhibit the translation of select transcripts when they overlap with the coding sequence (CDS), in which case, the start codon is mostly in a less favorable Kozak consensus sequence. Multiple uORFs in the 5′UTR can impose a complex regulation of translation of both the uORFs and the CDS to suppress protein expression. The *thrombopoietin* (*TPO*) transcript contains seven uORFs which negatively inhibit translation, the seventh of which overlaps with the protein-coding open-reading frame [42]. Although translation is generally inhibited upon eIF2 phosphorylation, the translation of specific transcripts may be enhanced under these conditions. One such transcript is activating transcriptional factor 4 (Atf4), which is essential for erythroid differentiation and for reduction of oxidative stress during the basophilic erythroblast stage [43]. Atf4 contains two uORFs, the second of which overlaps with the CDS [44]. Translation of the second uORF inhibits Atf4 protein expression by overwriting the CDS [45]. Decreased abundancy of unphosphorylated eIF2 due to HRI activity allows for a sufficient delay in ribosomal reassociation to select for the CDS instead of the inhibitory uORF, providing a mechanism for Atf4 expression under stress conditions. Other uORF-containing transcripts may, instead, be hypersensitive to eIF2 phosphorylation and translation may be impaired more than average upon eIF2 phosphorylation. These transcripts include Pabpc1 and Csde1 [46], the latter of which will be discussed in detail in section 1.6.

### 1.2.4 Translational control of iron homeostasis

Iron homeostasis is achieved via a balanced regulation of iron import via transferrin receptor 1 (Tfr1), storage in ferritin and export via ferroportin [47]. Post-transcriptional control over these proteins is enacted by iron regulatory proteins (IRPs). IRPs are recruited to ferritin mRNA via a conserved sequence that forms a hairpin structure in the 5′UTR [48,49]. Binding of the IRP to the iron responsive element (IRE) prevents the association of the 43S preinitiation complex to the mRNA transcript [50]. The presence of iron blocks the IRE-IRP interaction, allowing translation of the formerly repressed ferritin transcript. A similar mechanism governs the translation of *ferroportin* [51].
Interestingly, a splice variant of ferroportin lacking the IRE is expressed in duodenum and erythroid cells permits the escape of IRP-mediated translational control [52]. By contrast, the Tfr1 transcript possesses 5 IREs in the 3’UTR rather than the 5’UTR [53,54]. Binding of IRPs to Tfr1 confers increased mRNA stability to the transcript, resulting in an inverse relationship between Tfr1 protein expression and iron abundancy [55,56].

Both IRPs cooperate to control iron homeostasis, yet they are regulated via different mechanisms. Irp1 is a bifunctional protein with both enzymatic and RNA-binding activity. It possesses the capacity to act as an aconitase in the catalyzation of citrate to isocitrate [57]. The catalytic activity is dependent upon the assimilation of an additional iron atom in the active site, with the result that Irp1 functions as an enzyme when iron is abundant, and as an RNA-binding protein when iron is depleted [58–60]. Because the formation of the iron-sulphur cluster in the active site requires an oxygen-free environment [61], the RNA-binding form is preferentially induced in the presence of the vasodilating agent nitrous oxide [62].

Although Irp2 is 57% homologous with Irp1 in humans, it does not function as an aconitase under iron-rich conditions [63]. Unlike Irp1, Irp2 is rapidly degraded by the proteasome when iron and oxygen levels are high [63,64]. Degradation of Irp2 is prevented by low oxygen pressure [65]. Taken together, Irp1 and Irp2 are capable of controlling iron homeostasis under both low and high iron and oxygen supply, allowing a proportional response to environmental stimuli [47].

### 1.3 IRES-DEPENDENT TRANSLATION IN HEMATOPOIESIS

In addition to cap-dependent translation, ribosomes can associate on a subset of transcripts carrying an internal ribosomal entry site (IRES) [66]. While there is no consensus sequence or universal structural motifs for IRESs, they typically contain complex structural elements which include stem loops and pseudoknots [67,68]. The majority of IRESs are found within the 5’UTR directly upstream of the initiation codon, though they can also exist within the coding region, causing synthesis of a truncated protein [69,70]. IRES-mediated translation is preferred under stress conditions, which are characterized by decreased availability of cap-dependent translation [71,72], due, for example, to eIF2 phosphorylation [73,74] or cleavage of scaffolding protein eIF4G [67]. This includes, but is not limited to, viral infection, hypoxia, nutrient starvation, ER stress and cell differentiation [67,75].

Translation via an IRES requires the binding of IRES transactivating factors (ITAFs) such as Polypyrimidine Tract Binding protein (Ptb) [76,77] and Csde1 [78–80]. IRES-mediated translation initiation is less competitive in ribosome recruitment. It is probably for that reason that primarily IRES-dependent translation initiation is suppressed.
when fewer ribosomes are present, which is a hallmark of Diamond Blackfan Anemia (DBA, see section 1.5.1 Diamond-Blackfan Anemia) [81]. The induction of severe anemia due to loss of ribosomes in this latter disease indicates that IRES-mediated translation is of particular importance in erythropoiesis.

Several genes involved in hematopoiesis are subject to IRES-mediated translation, among which are Bag1 and Runx1. Bag1, an Hsp70 cochaperone, is required for terminal differentiation of erythroblasts [81]. All three Bag1 isoforms are produced from a single transcript, dependent upon the involvement of either cap-dependent or IRES-mediated translation [82]. Two ITAFs are involved in this process: Pcbp1, which remodels the RNA to allow ribosomal entry, and Ptb, which is necessary for the recruitment of the ribosome to the Bag1 transcript [83]. In hematopoiesis, Bag1 deficiency is lethal at day E13.5 in mice with pronounced defects in brain and liver tissue [84]. shRNA-mediated knockdown of Bag1 in erythroblasts results in the production of fewer hemoglobinated daughter cells from erythroblasts cultured under differentiation conditions. Bag1 is implicated in DBA, wherein haploinsufficiency of ribosomal proteins results in the loss of Bag1 and other transcripts from polysomes [81]. In a DBA model, reduced expression of Rps19/Rpl11 resulted in diminished IRES-driven translation of Bag1 in a bicistronic reporter construct, indicating a possible deregulation of IRES-mediated translation in DBA.

Another example of IRES-mediated translation is Runx1 (also called Aml1), a transcription factor essential for fetal liver hematopoiesis [85], which contains an IRES proximally upstream to one of its two promoters [86,87]. The absence of the IRES in Runx1 causes embryonic fatality due to pericyte development and disordered proliferation and differentiation of hematopoietic cells in the fetal liver [88].

### 1.4 The Role of the Poly(A) Tail in mRNA Stability and Translation

The mRNA poly(A) tail protects the transcript from degradation, but the length of the poly(A) tail also affects translation initiation. The length of the poly(A) tail is determined by the recruitment of polyadenylation proteins. In the nucleus, there are four sequence elements and their binding proteins involved in selecting the site of cleavage and polyadenylation [89]. Upon cleavage site recognition, a nuclear poly(A) polymerase is recruited to synthesize the poly(A) tail. Typically this is carried out by poly(A) polymerase alpha, but depending on the cell type and specific transcript, another non-canonical polymerase may be involved [89].

Upon export to the cytoplasm, interaction with the poly(A) tail may enhance or repress translation of the mRNA transcript. A broad array of RNA-binding proteins
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governs this process. Here, an overview of some of the larger families of poly(A)-
interacting proteins and their influence on hematopoiesis will be presented.

1.4.1 PABPs promote translational initiation and stabilize transcripts via
poly(A) binding
Essential to the initiation of translation is the binding of Poly(A) Binding Proteins
(Pabp) to the poly(A) tail. PABPs directly interact with the eIF4G scaffold protein of
the eIF4F cap-binding complex. This brings the mRNA tail close to the cap, and forms
a circular mRNA conformation that is believed to optimize recycling of translation
initiation and elongating factors [90]. The interaction of Pabp with eIF4G stabilizes
pre-initiation scanning complexes on the 5’UTR [91]. A longer poly(A) tail increases
Pabp affinity for target transcripts, which enhances the stabilization of multiple pre-
initiation scanning complexes and thereby enhances translation initiation efficiency.
Additionally, the binding of Pabp to the poly(A) tail protects the transcript from dead-
enylation [92], a process which is discussed in more detail below.

The most common Pabp is Pabpc1. In erythroid cells, however, there is a prominent
role for Pabpc4 [93]. Pabpc4 binds to a specific subset of transcripts with short poly(A)
tails containing an AU-rich motif, including α-globin, and protects them from further
degradation. Depletion of Pabpc4 blocks induced terminal differentiation of mouse
erthroblast leukemia (MEL) cells by altering the expression of five genes associated
with erythroid maturation. Receptor tyrosine kinase c-Kit is strongly upregulated in
Pabpc4-depleted MEL cells. This activity is specific to Pabpc4 and is not redundant
with Pabpc1. Given that downregulation of c-Kit is essential for cell cycle arrest prior
to terminal differentiation [16,94], the rescue of c-Kit expression is likely responsible
for the inability of Pabpc4-depleted MEL cells to form mature erythrocytes [93]. Also
induced were c-Myb, c-Myc, CD44, and Stat5a, all well-studied genes which promote
erthroblast maintenance at the expense of differentiation [95].

1.4.2 CPEBs control poly(A) tail length
In the cytoplasm, the length of the poly(A) tail may undergo additional processing
to control translation of target transcripts. Critical to this process are the Cytoplas-
mic Polyadenylation Element Binding proteins (CPEBs). CPEBs recruit cytoplasmic
poly(A) polymerases to promote translation, the most well-characterized of which is
Gld-2 (Germ line development 2) [96]. Depending on the cell type, a cytoplasmic vari-
ant of the classic nuclear poly(A) polymerase may be recruited instead [97–99]. Cpeb1
is known to increase mRNA stability by binding to Pabpc1 and Pabpc1L (also called
Epab) [100,101]. The process is further regulated by the involvement of additional RNA-
binding proteins, such as Pumilio, which may stabilize the binding of CPEBs but also
may function as a deadenylation factor and translational repressor [89]. CPEBs can
also recruit the deadenylating CCR4/NOT complex, which cooperates with Pumilio to deadenylate target transcripts. The CCR4/NOT complex is composed of several Cnot subunits, which individually have varying roles in a myriad of physiological processes [102]. In particular, Cnot9 was identified as an erythropoietin-responsive gene, indicating a role for the complex in erythropoiesis [103]. Deadenylation initially represses translation, because less Pabp can bind and connect to eIF4G proteins in preinitiation complexes. Ultimately, deadenylation results in silencing of mRNA via 3'-5' degradation by the exosome, or 5'-3' degradation by Xrn1 after decapping by Dcp1-Dcp2 [102].

Of the CPEB family members, Cpeb4 is specifically induced during erythroid differentiation [104], via the transcription factors Gata1 and Tal1 [95,105]. Cpeb4 not only promotes deadenylation, but also represses translation via binding to the eIF3 complex [104]. Cpeb4 is additionally capable of binding to and repressing its own mRNA, forming a feedback loop that maintains Cpeb4 levels within a range required for terminal erythropoiesis.

### 1.4.3 Musashi-mediated translational control in hematopoiesis

In addition to CPEBs and PABPs, cytoplasmic polyadenylation is regulated by Musashi-1 and -2 (Msi1, Msi2), which interact with mRNA via the MSI-binding element (MBE) [89]. The MBE is known to confer cytoplasmic polyadenylation in the absence of CPEB activity, and knockdown of Msi1 prevents polyadenylation of Mos kinase, a regulator of meiosis during oocyte maturation [106]. Strikingly, however, no interactions between MSI proteins and the polyadenylation machinery have been described [89].

Although it is unclear how Msi proteins influence polyadenylation, there is a significant body of research detailing their function as RNA-binding proteins. Msi proteins are oppositely regulated from the Auf1/HnrpD family of AUBPs (see section 1.4.4: AUBPs in hematopoiesis), indicating their role as global translational regulators [107]. The majority of studies done on Musashi-mediated translational repression have been done with Msi1. Msi1 represses the translation of target transcripts by competing with eIF4G for binding with Pabp, preventing the formation of the 80S ribosome subunit [108]. Transcripts silenced by Msi1 include cell cycle regulators such as Numb, an inhibitor of the Notch pathway and p21, and cyclin-dependent kinase inhibitor [109,110]. RNA binding domains between Msi1 and Msi2 are largely homologous (85-95%), but Msi2 has no Pabp-binding domain [111]. However, there is evidence to suggest that Msi2 alters Notch localization and upregulates Hes1, a Notch reporter protein [112], suggesting that Msi1 and Msi2 overlap in regulating common targets.

Msi2 is abundantly expressed in primitive LSK cells of the hematopoietic lineage, where Msi1 expression is nearly absent [112–114]. The expression of Msi2 is subsequently downregulated during differentiation. Downregulation of Msi2 alters the
balance between self-renewal and differentiation of HSCs via Notch pathway interaction [112,114]. This effect is achieved without influencing apoptotic rates or homing behavior. A mouse line [Msi2^Gt/Gt] expressing a truncated Msi2 gene results in a marked decrease in short term hematopoietic stem cells (ST-HSCs) and multipotent progenitors (MPPs) while the effect on long term hematopoietic stem cells (LT-HSCs) was nominal [113]. In contrast to RNA interference experiments, Msi2-defective LSKs from the mouse model display impaired proliferation. A non-competitive bone marrow transplantation experiment showed a decrease in the LT-HSC population [112]. This is in contrast with findings under steady state conditions. In addition, a doxycycline-inducible Msi2 transgenic mouse model observed an increase in ST-HSC/MPP populations and a decrease in LT-HSC, whereas Msi2 overexpression increased LT-HSC self-renewal in transplanted mice. Furthermore, Msi2 was found to be significantly downregulated in erythroblasts derived from the CD34+ hematopoietic stem cells of β-thalassemia patients relative to those derived from healthy controls [115]. Taken together, these findings suggest that Msi2 is of particular importance during stress hematopoiesis as well as self-renewal and stem cell homeostasis. Studies on Msi2 in hematopoiesis have been largely functional in focus and do not mechanistically investigate mRNA polyadenylation via Msi2. Although the direct molecular targets of Msi2 in hematopoiesis are unknown, gene expression profiling indicates a regulatory function for pathways involved in HSC proliferation, including Meis1, HoxA9, HoxA10 [114], Ras, MAPK, cyclin D1, and Myc [112,113]. The role of Msi2 in hematopoiesis has been extensively reviewed in de Andrés-Aguayo et al., 2012.

1.4.4 AUBPs in hematopoiesis

AU-rich elements (AREs) are sequence motifs (typically AUUUA) in the 3’UTR which recruit a large family of AU-binding proteins (AUBPs) that regulate translation. Translational regulation via AUBPs can occur via a number of mechanisms, namely via deadenylation, but also via transcript sequestration to P-bodies and stress granules [116]. AUBPs key to erythropoiesis include the Tristetraprolin (TTP) family members (Zfp36, Zfp36l1, Zfp36l2) and HuR/Elav1.

TTP family members interact with Not1 to promote the rapid deadenylation by the CCR4/NOT complex [117]. Interestingly, Zfp36l1 and Zfp36l2 demonstrate opposite regulation in erythropoiesis. Zfp36l1 downregulates Stat5b expression, reducing the formation of erythroid colonies [118]. By contrast, Zfp36l2 is required for burst-forming unit-erythrocyte (BFU-E) renewal [119]. Therefore, maintenance of erythrocyte homeostasis requires simultaneous downregulation of Zfp36l1 and upregulation of Zfp36l2.

Elav1 is a ubiquitously expressed AUBP with thousands of direct and functional targets [120,121]. Relevant hematopoietic roles for ELAV1 in humans include the
stabilization of mRNAs for BCL2, MCL1, cyclin A, cyclin B1, cyclin D1, lymphotoxin-α, GM-CSF, IL4, VEGF, CD3, CD95L, GATA-3, XIAP, and survivin, and destabilization of mRNAs for AML1/RUNX1, CD2, VAV1, NFκBIE, CD3ε, TNFa, and STAT3 (reviewed in [116]). ELAV1 additionally enhances the translation of mRNAs encoding p53, cytochrome c, XIAP, and BCL2 while suppressing translation of p27, MYC, and WNT5 (reviewed in [116]). Interestingly, ZFP36l1 is a functional target of ELAV1 in human cells exposed to oxidative stress, wherein ionizing radiation decreases ZFP36L1 transcript binding by ELAV1, resulting in a decreased recruitment of ZFP36L1 to polysomes [122]. This suggests that Elav1 may synergize with Zfp36l1 in regulating erythropoiesis under some conditions.

1.5 ABERRANT TRANSLATION AND HEMATOPOIETIC DISEASE

Disruption of translation has serious physiological consequences. Specifically, mutations in ribosomal proteins can cause a family of diseases known as ribosomopathies that result in abnormal hematopoiesis. Among these are Diamond-Blackfan Anemia (DBA), Shwachman-Diamond Syndrome (SDS), and Dyskeratosis Congenita (DC). It is important to note that all of these hematopoietic ribosomopathies present with clinically diverse symptoms, suggesting that dysregulation of ribosomal protein expression results in complex defects with organ-specific effects. It remains unclear why ribosomal mutations result in diverse clinical outcomes, instead of translational deregulation in an organism-wide, embryonically lethal manner.

1.5.1 Diamond-Blackfan Anemia

DBA is an inherited anemia that presents with severe red cell aplasia in infant patients (<1 year of age) while all other blood lineages remain normal [123]. The primary feature of DBA is a reduction of erythroid precursors in the bone marrow [124], but the DBA phenotype can vary widely in severity, with approximately half of patients afflicted with skeletal and growth abnormalities, such as craniofacial deformities, diminished stature, abnormalities of the thumb and the presence of a cleft palate [125]. Genetically, DBA is characterized by a haploinsufficiency of ribosomal proteins [126]. Loss of ribosome functionality can be the result of one or more mutated genes, most prominently RPS19 (25% of patients) [127], but mutations in both the small ribosomal subunit (RPS7, RPS10, RPS17, RPS24, RPS26, RPS29) and the large ribosomal subunit (RPL5, RPL11, RPL15, RPL26, RPL27, RPL35A) have been observed [126,128,129]. An imbalance of ribosome synthesis inhibits cell proliferation via p53 activation [130]. However, in one third of patients, the underlying mutation is yet to be identified, though there are many ribosomal proteins that remain unscreened [123].
In atypical cases, DBA is associated with mutations of erythroid transcription factor GATA1 [131,132]. GATA1 mutations resulting in DBA may alter the splice donor site of exon 2 or the start codon of the GATA1 open reading frame, resulting in reduced expression of GATA1 [132,133]. In this case, it is possible that GATA1 causes the red cell aplasia of DBA via a signaling defect during erythropoiesis rather than behaving as a ribosomopathy [123]. However, GATA1 binds to the region upstream of the promoter for several ribosomal proteins, including RPS19, suggesting that it may also play a role in the ribosome abnormalities found in DBA patients [134].

1.5.2 Shwachman-Diamond Syndrome

SDS is an autosomal recessive syndrome characterized by bone marrow failure, neutropenia, decreased red cell/platelet counts, deficient pancreatic functioning, and an increased risk of leukemia (reviewed in [135]). SDS is caused by homozygous mutations in the Shwachman Bodian Diamond syndrome (SBDS) gene [136]. SBDS is involved in the regulation of RNA metabolism and ribosome functioning [137,138]. The ortholog of SBDS in S. cerevisiae, Sdo1p, regulates assembly of the 60S ribosomal subunit via assembly protein TIF6P and also regulates the interaction of the 60S subunit with the 40S subunit, playing a critical role in the formation of the complete ribosome [139]. Cells derived from SDS patients are unusually sensitive to rRNA transcription inhibition [135,140,141]. Comparisons between yeast models for DBA (RPL33A) and SDS (SDO1) show decreased formation of the 60S ribosomal subunit and an increase in polysomes containing stalled 48S initiation complexes [142]. However, in the SDS model, these incompletely assembled ribosomal precursors accumulate in the nucleus, whereas in the DBA model, the 60S precursors are rapidly degraded. This suggests that the defect in ribosome assembly observed in SDS is distal to the ribosomal defects observed in DBA [135].

1.5.3 Dyskeratosis Congenita

DC is both clinically and genetically heterogeneous (reviewed in [143]). Physiologically, it is characterized by mucocutaneous aberrations, bone marrow failure, anemia, and an increased vulnerability to carcinogenesis [144]. There are three recognized genetic subtypes of DC. The X-linked recessive version is caused by mutations in dyskerin [145], a component of small nucleolar RNA ribonucleoproteins (snoRNPs) involved in splicing that is also a telomerase component. The autosomal dominant version of DC has heterozygous mutations in either the RNA component (TERC) [146] or the enzymatic component (TERT) [147,148] of the telomerase complex. There is also an autosomal recessive version of DC, wherein the genetic cause remains unclear. The pathology of DC when caused by dyskerin, TERT or TERC mutations is believed to be a consequence of chromosome instability due to defective telomerase activity [143]. Pa-
tients with dyskerin mutations display additional defects in rRNA synthesis, ribosome biogenesis, and mRNA splicing, suggesting that translational control is paramount to causing the DC phenotype in this genetic subtype [149–151].

In this chapter, the importance of both cap-dependent and cap-independent translation in determining gene expression programming during erythropoiesis has been demonstrated. Ribosomopathies indicate that mutations in the broader translational machinery may, surprisingly, have tissue-specific consequences. At the intersection of these concepts is a protein known as Cold Shock Domain-containing E1 (Csde1), an ITAF implicated in DBA [81]. The next section summarizes the known functions of Csde1 in translational regulation and erythropoiesis.

1.6 CSDE1 IS AT THE CROSSROADS OF TRANSLATIONAL REGULATION

Csde1, originally called Unr (upstream of N-ras) is an RNA-binding protein with five cold-shock domains [152]. The first evidence for Csde1’s biological function involved the silencing of Msl (male sex lethal) during sex determination of Drosophila melanogaster via binding to the 3’UTR [153–158]. Repression of Msl-2 occurs via a direct interaction between Csde1 and 3’UTR-bound Pabp, resulting in the prevention of Pabp-mediated recruitment of the 43S PIC [159]. Paradoxically, the Csde1-Pabp complex has also been reported to stimulate translation by stabilizing the interaction between Pabp and eIF4G [160].

1.6.1 Csde1 and IRES-mediated translation

Csde1 is implicated in both endogenous and viral IRES-mediated translation in mammalian cells. The presence of viral double-stranded RNA causes eIF2 phosphorylation via PKR, resulting in a preferential advantage for cap-independent translation via IRESs. Both rhinovirus and poliovirus contain IRES elements that require an interaction between Csde1 and Ptb [79,161]. Early studies indicated that all five of Csde1’s cold shock domains were necessary for maintenance of Csde1’s affinity for rhinovirus IRESs, whereas cold shock domains 1 and 2 had the most impact on Csde1’s binding to Msl-2 [156,162], and cold shock domains 2 and 4 were the only required elements for stimulation of translation via Pabp [160].

The cooperation between Csde1 and Ptb in regulating IRES activity extends to cellular transcripts. The Csde1 transcript contains an IRES within the 5’UTR which, when bound by Ptb and Csde1 itself, downregulates IRES-dependent translation of Csde1 [78,163,164]. Pathways such as mitosis and apoptosis specifically promote IRES-mediated translation at the expense of cap-dependent translation via, among other mechanisms, phosphorylation of eIF2 [67,73]. Csde1 IRES activity is strongly
upregulated during mitosis due to increased binding of hnRNP C1/C2 proteins with simultaneous release of Csde1 and Ptb [163]. The resultant increase in Csde1 expression during the G2/M phase of the cell cycle facilitates the IRES-mediated translation of cyclin-dependent PITSLRE kinases, which are essential for centrosome maturation and mitotic spindle formation [73,165]. During apoptosis, Csde1 and Ptb upregulate Apaf-1 (apoptotic protease-activating factor 1) via binding to the IRES within the 5’UTR [166]. Binding of Csde1 and Ptb changes the conformation of the IRES of Apaf-1 to a single-stranded region, granting access for ribosomal recruitment and thereby permitting the translation of the transcript [80].

1.6.2 Csde1 and the poly(A) tail

Csde1 is also implicated as a regulator of mRNA stability via poly(A) deadenylation. Csde1 binds to c-fos major protein-coding determinant of instability (mCRD) motifs in conjunction with Pabp [167]. In contrast to the stabilizing influence of Pabp when interacting with CPEBs, the Csde1-Pabp complex promotes transcript degradation via recruitment of the deadenylase Ccr4. Given that binding of Pabp and Csde1 to the poly(A) tail can also increase transcript stability, Chang et al., (2004) propose a model in which the Csde1-Pabp complex initially protects the poly(A) tail from deadenylation by Ccr4 prior to initiation of translation. Upon ribosomal transit of the mCRD, a conformational change is triggered that forms a landing pad for Ccr4, resulting in decreased expression as a consequence of transcript deadenylation and subsequent degradation.

1.6.3 Csde1 and 4E-T

Yet another role for Csde1 in the regulation of translation is apparent in its interaction with the 4E-Transporter (4E-T), itself a translational regulator with a wide breadth of functions. 4E-T competitively binds to cap-binding protein eIF4E, preventing its association with scaffolding protein eIF4G [168] while simultaneously reducing ribosomal access to the 5’ cap via interaction with RNA-binding proteins at the 3’UTR [169,170]. Furthermore, 4E-T is a component of the CPEB translation repressor complex [171], the CCR4-NOT complex [172], and enhances decay of transcripts containing AU-rich elements [173–175]. Finally, 4E-T is involved in shuttling between the cytoplasm and the mRNA triage and degradation centers known as Processing Bodies (P-bodies) [176,177]. 4E-T is directly bound by Csde1 and indirectly bound by Strap (also known as Unrip, Unr-interacting protein) through Csde1 [178]. Kamenska et al., (2016) shows that Csde1 binds to Cnot4 in manner mutually exclusive with binding of Cnot4 to 4E-T, theoretically abrogating 4E-Ts role as a bridge between Cnot1 and Cnot4 complex subunits. Due to the complexity of the overlapping pathways associated with 4E-T, it is unclear precisely how Csde1 cooperates with 4E-T in the regulation of translation. The
authors suggest that Csde1 and Ddx6, an RNA helicase component of the CPEB repressor complex, simultaneously bind 4E-T to either redundantly repress translation or to selectively affect specific translational stages. Another possibility is that Csde1 acts as a competitive inhibitor of 4E-T binding to other, unknown cofactors/repressors, disruption of which unravels a network of interactions necessary for translational repression.

1.6.4 Csde1 in DBA

CSDE1 is implicated in Diamond-Blackfan Anemia (DBA). A study by Horos et al., (2012) employed the knockdown of Rps19 and Rpl11 to simulate the DBA phenotype in Trp53 -/- mouse erythroblasts. Polysomal and subpolysomal mRNA was isolated via a sucrose gradient and hybridized separately to microarrays to identify which transcripts were susceptible to diminished polysome recruitment in the DBA model. Among the transcripts lost was Csde1. Protein expression levels were correspondingly decreased after Rps19/Rpl11 knockdown, whereas mRNA expression was unaltered. These results were reproducible in erythroblast derived directly from DBA patients and in p53 competent cells, making the negative inhibition of CSDE1 translation p53-independent. Expression of Csde1 steadily increases during progression through the hematopoietic lineage, with levels 250x higher in erythroblasts versus LSKs, CMPs, and GMPs. Additionally, knockdown of Csde1 results in impairment of erythroblast proliferation and differentiation. The known functions of Csde1 are summarized in Figure 4.

1.7 SCOPE OF THE THESIS

Erythropoiesis needs to be tightly regulated to maintain the number of erythrocytes in peripheral blood between narrow limits. At the molecular and cellular level, this requires a transcriptional program that is fine-tuned at the level of mRNA translation and protein stability. Given that Csde1 is an essential protein that is abundantly expressed in erythroid cells, it stands to reason that Csde1 is a critical regulator of translation during erythropoiesis. We aimed to unravel the mechanism of Csde1-dependent translational regulation in erythropoiesis, via identification of not just Csde1’s target transcripts, but also its protein binding partners, and whether protein complex formation alters Csde1 function. We examined the phenotypic differences in erythroblasts after reduction of Csde1 expression and after removal of a select RNA binding domain (Chapter 2). We subsequently investigated the role of Strap, a Csde1-binding partner, on the translation of Csde1-bound transcripts and identified a possible role for Strap during hypoxic stress (Chapter 3). More globally, we quantified the effect of eIF2 phos-
Figure 4. Hierarchical overview of Csde1-mediated translational regulation. Csde1 has been empirically demonstrated to regulate both cap-dependent and IRES-mediated translation, as well as directly affecting the stability of select mRNA transcripts. It may either promote or repress cap-dependent translation, as its interaction with PABP can either facilitate or inhibit ribosomal recruitment. The role of Csde1 in IRES-mediated translation is generally stimulatory, as seen in the increased IRES activity of Apaf-1 under apoptotic conditions. IRES-driven translation of PITSLRE kinase and of Csde1’s own transcript is enhanced during the cell-cycle. The same holds true for viral IRESs. Csde1 may additionally both promote or inhibit mRNA degradation via transient interaction with the CCR4-NOT complex. Interaction with PABP contributes to the protection of the poly(A) tail from CCR4-NOT. Finally, Csde1 interacts with 4-ET, which shuttles transcripts between the cytoplasm and P-bodies, where they are sequestered and degraded. As 4-ET also functions as part of the CPEB repressor complex and the CCR4-NOT complex, Csde1 likely cooperates with 4-ET to influence translational stability as part of a highly complex and overlapping group of regulatory pathways.

phorylation on ribosome occupancy as a mechanism for assaying which transcripts are differentially translated under stress conditions (Chapter 4).
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