Dissertation

Translation Initiation Factor 4E Binding Protein 1,2 (4E-BP1,2) in Hematopoiesis And Stress Erythropoiesis

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Zusammenfassung

Das ″Eukaryotische-Initiationsfaktor-4E Bindungsprotein″ (4E-BP) ist ein Inhibitor der Translationsinitiation. Nicht-phosphoryliertes 4E-BP bindet an den eukaryotischen Initiationsfaktor 4E (eIF4E). Diese Bindung blockiert die Rekrutierung des Initiationskomplexes eIF4F an die Cap-Struktur des 5´Endes von eukaryotischen zellulären mRNAs, was die Initiation der Translation verhindert. Phosphorylierung von 4E-BP durch die mTOR Kinase führt zur Dissoziation des 4E-BP/eIF4E Komplexes und erhöht die Verfügbarkeit von eIF4E, dies wird mit Zellproliferation assoziiert. Die Aktivität von eIF4E wird nicht nur von 4E-BP, sondern auch durch Phosporylierung reguliert, welche wiederum durch die ″MAP-Kinase-Interacting-Protein-Kinase″ (MNK) reguliert wird.

Drei Isoformen von 4E-BP sind bekannt: 4E-BP1, 4E-BP2 and 4E-BP3. 4E-BP1 und 4E-BP2 sind an oxidativem und adipogenetischen Stress beteiligt. Beide Proteine werden im hämatopoetischen System gleich exprimiert, wohingegen 4E-BP3 nicht detektiert wird. 4E-BP1 wird während der Erythroblasten-Proliferation phosphoryliert. Aus diesem Grund habe ich die Hämatopoese und die durch Phenylhydrazine (PHZ) induzierte Stress-Erythropoese in 4E-BP1 und 4E-BP2 Knock-Out Mäusen und 4E-BP1,2 Doppel-Knock-Out Mäusen analysiert. Ich konnte zeigen, dass die Hämatopoese in 4E-BPs defizienten Mäusen nicht beeinflusst wird. Allerdings zeigten 4E-BP1,2$^{-/-}$ und 4E-BP2$^{-/-}$ Mäuse eine verspätete Antwort auf Phenylhydrazin (PHZ) induzierten erythropoetischen Stress. Gleichzeitig war die mRNA Translation von GATA-1, ein essentieller erythropoetischer Transkriptionsfaktor in Erythroblasten runterreguliert. Die Signaltransduktionswege mTOR und MNK1 waren bei erythropoetischen Stress aktiviert. Diese Daten zeigen, dass 4E-BP2, aber nicht 4E-BP1, notwendig ist um auf erythropoetischen Stress zu reagieren und deuten an, dass die 4E-BP gesteuerte translations-regulierende Maschinerie eine Rolle in der Stress-Erythropoese spielt.

Schlagwörter:

Translationale Kontrolle, 4E-BP, Stress-Erythropoese, mTOR, Hämatopoese

Abstract

Translational regulation allows an organism to generate fast responses to environmental changes quickly. Eukaryotic initiation factor 4E binding protein (4E-BP) is an inhibitor of translation initiation. Unphosphorylated 4E-BP binds to eukaryotic initiation factor 4E (eIF4E) blocking recruitment of the initiation complex eIF4F to the cap structure at the 5' terminus of eukaryotic cellular mRNAs. Thus initiation of translation is blocked. Phosphorylation of 4E-BP by the mTOR kinase causes disassociation of the 4E-BP/eIF4E complex and increases the availability of eIF4E. EIF4E activity is not only regulated by 4E-BP, but also phosphorylation which is regulated by MAP kinase - interacting protein kinase (MNK).

Three isoforms of 4E-BP are known, termed 4E-BP1, 4E-BP2 and 4E-BP3. 4E-BP1 and 4E-BP2 are involved in oxidative and adipogenetic stresses in vivo. They are equally expressed in hematopoietic system, whereas 4E-BP3 is not detected. 4E-BP1 is phosphorylated during erythroblast proliferation. Erythroid differentiation is blocked by overexpresssion of eIF4E in tissue culture. These studies implied that 4E-BPs might play role in response to erythropoietic stress. I examined hematopoiesis and phenylhydrazine (PHZ) induced stress erythropoiesis in 4E-BP1 and 4E-BP2 individual knock out mice and 4E-BP1,2 compound knock out mice. I found that the hematopoiesis of 4E-BPs deficient mice were unaffected. However, 4E-BP1,2$^{-/-}$ and 4E-BP2$^{-/-}$ mice showed delayed response to phenylhydrazine (PHZ) induced erythropoietic stress. Simultaneously, the mRNA translation of GATA-1, which is the essential erythroid transcription factor, was downregulated in their erythroblasts. The signaling pathways through the mTOR and MNK1 were activated in erythropoietic stress. These data showed that 4E-BP2 but not 4E-BP1 was required for the response to erythropoietic stress and suggested that 4E-BP related translation regulatory machinery played a role in stress erythropoiesis.

Keywords:

translational regulation, 4E-BP, stress erythropoiesis, mTOR, hematopoiesis

Contents

1 Introduction

The regulation of translation rate plays a critical role in many fundamental biological processes, including cell growth, development and stress response. Translation is divided into three phases — initiation, elongation and termination. Translation initiation is the rate-limiting step and occurs in cap-dependent manner. This process requires a large number of translation initiation factors. Translation initiation factor 4F (eIF4F) complex consists of a mRNA cap structure binding protein — eIF4E, a large modular scaffolding protein performing a bridging function between the ribosome and mRNA — eIF4G and a helicase — eIF4A. EIF4E activity is tightly regulated by its inhibitor— eIF4E binding proteins (4E-BPs) and phosphorylation.

1.1 Cap-dependent translation initiation

The Cap structure m^7GpppN (where N is any nucleotide) is located at the 5'terminus of cellular eukaryotic mRNA molecules (except those in organelles). The basic model of the translation initiation process is as follows: The Methionyl-initiator tRNA (Met-tRNA $_i^{Met}$), GTP and eIF2 form a ternary complex. This ternary complex binds to 40S ribosomal subunit, which associates with eIF3 and eIF1A, to form 43S pre-initiation complex. EIF3, eIF1A and possibly also eIF5B stimulate this reaction. The resulting 43S pre-initiation complex binds to mRNA to form the 48S complex, in a reaction promoted by eIF4F complex (which includes eIF4E, eIF4A and eIF4G), eIF4B and eIF4H. The 48S complex then scans the mRNA until initiation codon AUG is recognized. This triggers eIF5 to hydrolyze GTP, the eIFs then dissociate and the 60S ribosomal subunit joins in assembling the fully functional 80S ribosome that is ready to begin peptide synthesis — elongation phase (Figure 1).

1.2 4E-BPs

So far three 4E-BP proteins are known, termed 4E-BP1, 4E-BP2 (Pause, Belsham et al. 1994) and 4E-BP3 (Poulin, Gingras et al. 1998). Mouse 4E-BP1 consists of 117 amino acids and shares 97.4% identity to rat 4E-BP1 (PHAS-I) (Hu, Pang et al. 1994; Lin, Kong et al. 1994; Lin, Kong et al. 1995) and 91.5% identity to human 4E-BP1. 4E-BP2 consists of 120 amino acids and shares 95% identity to human 4E-BP2 and 56% identity to 4E-BP1. 4E-BP3 consists of 101 amino acid and shares 57% and 59% identities to 4E-BP1 and 4E-BP2, respectively. The three genes comprise three exons and two introns. Drosophila possesses a single *4ebp* gene encoding d4E-BP (Poulin, Brueschke et al. 2003).

1.3 Expression patterns of 4E-BPs in mouse tissues

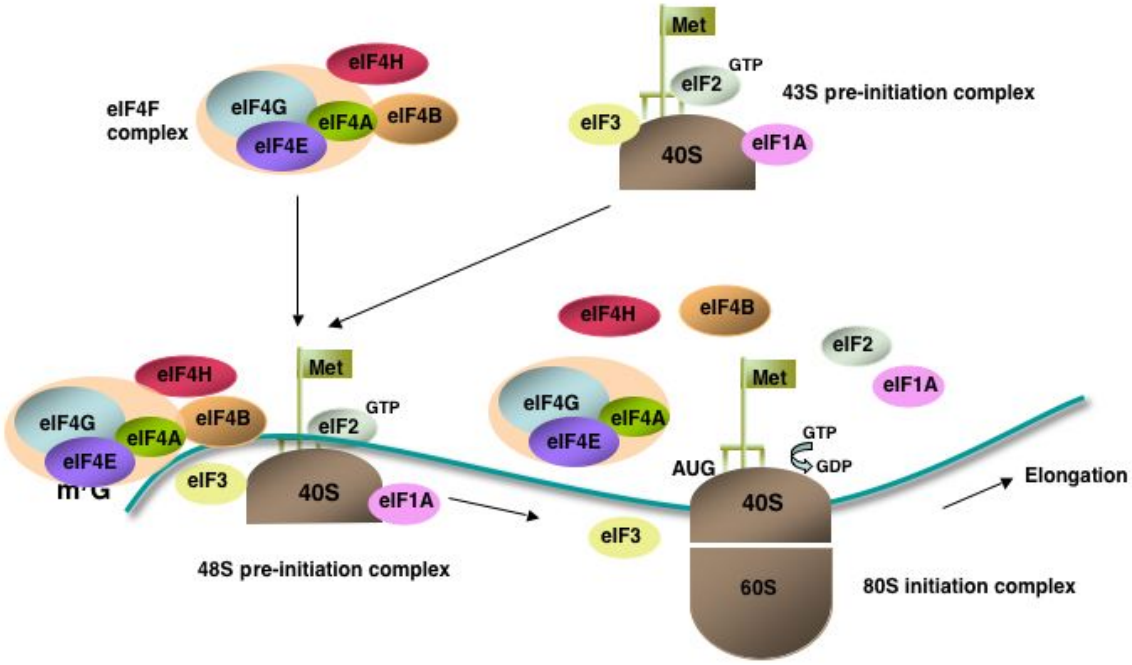


Fig.1. Cap-dependent translation initiation — rate-limiting step of translation Eukaryotic initiation factors (eIFs) are depicted as coloured, oval shapes in the figure. The Met-tRNA $_i^{Met}$, GTP and eIF2 form a ternary complex. This ternary complex binds to 40S ribosomal subunit, which associates with eIF3 and eIF1A, to form 43S pre-initiation complex. The resulting 43S pre-initiation complex binds to mRNA to form the 48S complex, in a reaction promoted by eIF4F complex (which includes eIF4E, eIF4A and eIF4G), eIF4B and eIF4H. The 48S complex then scans the mRNA until the initiation codon AUG is recognized. This triggers eIF5 to hydrolyze GTP, the eIFs then dissociate and the 60S ribosomal subunit joins in assembling the fully functional 80S ribosome that is ready to begin peptide synthesis — elongation phase.

4E-BPs are expressed at different levels in different tissues. In mouse 4E-BP1 is predominantly expressed in adipose tissue, pancreas, skeletal muscle and heart. 4E-BP2 is predominantly expressed in brain. 4E-BP3 is highly expressed in kidney. However, in testis and spleen, 4E-BP1 and 4E-BP2 are equally expressed, whereas three 4E-BP proteins can be detected in liver and colon. The expression variations of three 4E-BP proteins in different tissues may imply that each of the three 4E-BP proteins has its own function which is tissue specific (Figure 2).

1.4 Biological function of 4E-BPs

1.4.1 4E-BPs associate with eIF4E inhibiting eIF4F complex formation

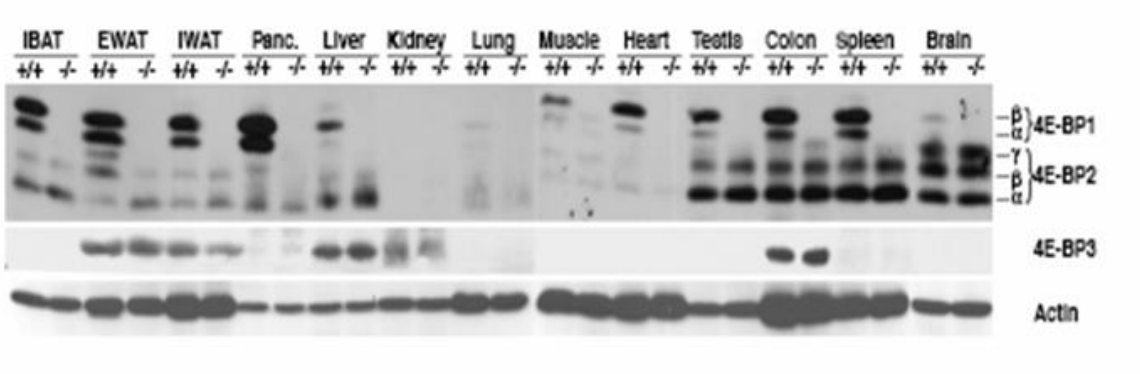


Fig.2. Western-blot analysis of 4E-BPs in tissues from wild type and homozygous *eif4ebp1* mice. IBAT, interscapular brown adipose tissue; EWAT, epididymal white adipose tissue; IWAT, inguinal WAT; Panc., pancreas. β and γ identify 4E-BP1 and 4E-BP2 phosphorylation isoforms that exhibit different electrophoretic mobilities in SDS-PAGE. (figure from Kyoko Tsukiyama-Kohara, et al. Nature Medicine 7,1128-1132(2001))

4E-BPs prevent eIF4E from associating with eIF4G to form eIF4F complex, resulting in the inhibition of 43S pre-initiation complex binding to mRNA (Haghighat, Mader et al. 1995; Mader, Lee et al. 1995). The interface of eIF4G interacting with eIF4E is a conserved 15-amino acid motif shared by 4E-BP proteins in all species studied to date (Figure 4). It contains the "core" sequence YXXXXLΦ in which X is any amino acid and Φ is a residue possessing an aliphatic portion, most often L, but sometimes M or F. Thus

4E-BPs compete with eIF4G to bind to eIF4E preventing eIF4G from associating with eIF4E to assemble functional eIF4F complex. Deletion of this sequence or mutation of the tyrosine or leucine residues to alanine(s) abolishes eIF4E binding. In addition, a 20-amino acid peptide derived from the eIF4E binding site of the mammalian 4E-BP1 or eIF4GII significantly inhibits cap-dependent translation (Fletcher and Wagner 1998; Marcotrigiano, Gingras et al. 1999). Crystallographic analysis demonstrates eIF4E binding peptides derived from either 4E-BP1 or eIF4GII exhibit an L-shaped α- helical conformation and bind to the convex dorsal surface of eIF4E. Direct and water-mediated hydrogen bonds, van der Waals, and hydrophobic interactions mediate the binding (Marcotrigiano, Gingras et al. 1997). The residues in eIF4G and 4E-BP have been demonstrated by mutagenesis studies to be crucial for the interaction with eIF4E. Replacement of the invariant Tyr(0)→Ala or double mutation Leu(5)→Ala/Leu(6) →Ala in human eIF4G abolishes interactions with eIF4E in vitro. Point

mutants of Tyr(0) →Ala and Leu(5) →Ala in 4E-BP3 showed reduced eIF4E binding both in vivo and in vitro. Changing Trp73→Ala in murine eIF4E prevents its interactions with 4E-BP1 and human eIF4GI (Marcotrigiano, Gingras, et al. 1999). Taken together, a highly efficient mechanism for the regulation of eIF4F formation has evolved in mammals. In this mechanism the inhibitory 4E-BPs act as molecular mimics of the eIF4E binding motif in the eIF4G proteins (Figure 3).

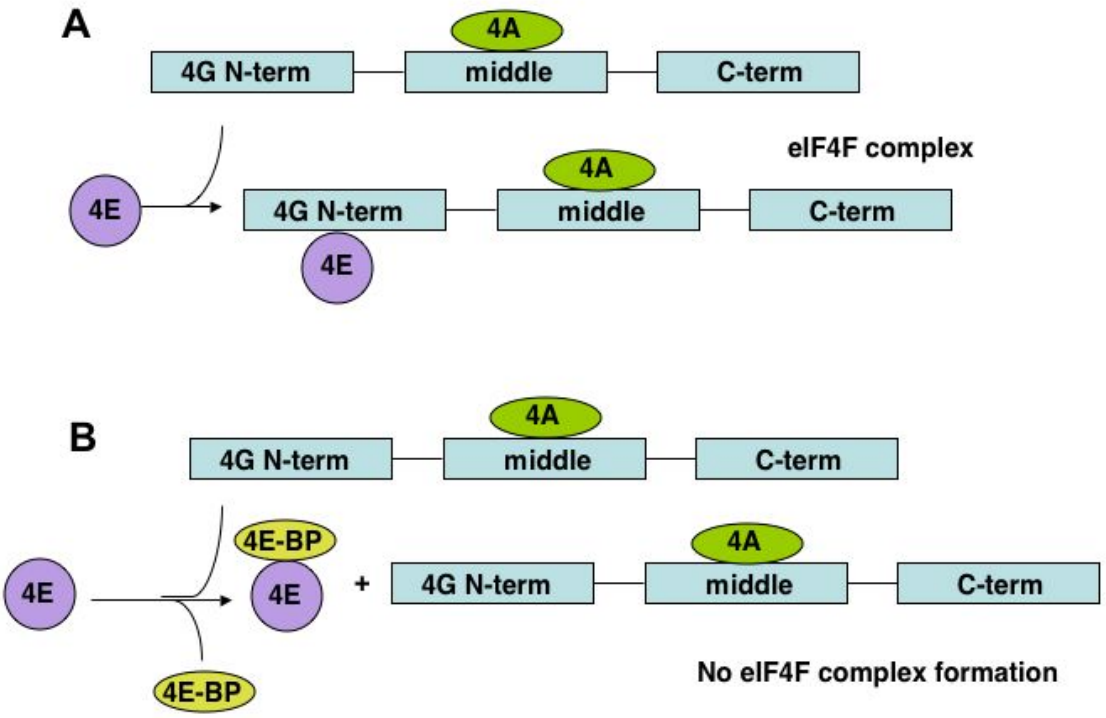


Fig.3. Regulation of eIF4F formation by 4E-BPs. (A) eIF4E binds to eIF4G which associates with eIF4A to form eIF4F complex. (B) 4E-BP and eIF4G compete for a common binding site on eIF4E to inhibit eIF4F complex formation (figure from Sonenberg, N., et al. Translation control of gene expression, Cold Spring Harbour Laboratory press, 2000).

1.4.2 Biological function of 4E-BPs

4E-BP proteins are important regulators of physiological functions and stress response. In Drosophila, there is only one 4E-BP (d4E-BP). Under starvation, d4E-BP deficient flies burn fat stores faster than wild type ones. Rapamycin (the inhibitor of mTOR, see 1.5.3) treatment, which increases 4E-BP activity, increases fat accumulation and life span under starvation condition in the wild type flies, whereas d4E-BP deficient flies accumulate less fat and have shorter life span than wild-type flies (Teleman, Chen et al. 2005). 4E-BP1 knock out and 4E-BP2 knock out mice have greater body weights and accumulate more fat compared to control mice. 4E-BP1,2 compound knock out mice which are fed with high fat diet (HFD) showed

significant increase in serum glucose, insulin, cholesterol, HDL-cholesterol level, accumulated more fat and had reduced metabolic rate and insulin sensitivity (Le Bacquer, Petroulakis et al. 2007) (Table 1). These observations imply that mTOR regulates 4E-BP to response to fat, glucose metabolic stress.

In mouse brain, 4E-BP2 is expressed higher than 4E-BP1 and 4E-BP3 (Figure2). 4E-BP2 knock out mice shows altered hippocampal long-term potentiation (LTP) and hippocampus–dependent memory deficits and enhanced eIF4F formation in the hippocamal area CA1 regions with increased amount of stimulation delivery (Banko, Poulin et al. 2005). In Drosophila, the d4E-BP activity is critical for survival under oxidative stress. Aging is a complicated physiological process that is characteristic of decreased physiological function and stress response ability. D4E-BP null mutation causes a significant decrease in lifespan (Teleman, Chen et al. 2005; Tettweiler, Miron et al. 2005). Upregulating 4E-BP activity by partial inhibition of TOR in yeast, worms and Drosophila results in a significant increase in the life spans of these organisms (Kaeberlein, Powers et al. 2005; Martin and Hall 2005) (Table 1).

1.5 The regulation of 4E-BPs

1.5.1 Phosphorylation of 4E-BPs

4E-BP activity is regulated at both transcriptional and post-translational level. 4E-BP1 was highly phosphorylated in response to insulin or growth factor stimulation of rat adipocytes or murine Swiss 3T3L1 adipocytes. Six Ser/Thr phosphorylation sites have been identified in the mammalian 4E-BP1 protein (Fadden, Haystead et al. 1997). Two phosphorylation residues, Thr-37 and Thr-46, lie on amino-terminal side of the eIF-4E binding motif (located at aa 54-60), and four phosphorylated residues, Ser-65, Thr-70, Ser-83 and Ser112, have been identified on the carboxy-terminal side of the eIF4E binding motif. 4E-BP phosphorylation is a highly ordered process. Phosphorylation of Thr-37 and Thr-46 acts as a priming step for Ser-65 and Thr-70 phosphorylation (Gingras, Gygi et al. 1999). Alignment of mammalian 4E-BPs reveals that all of the phosphorylated residues in 4E-BP1 are conserved in 4E-BP2 and 4E-BP3, except for Ser-112. However, two-dimensional gel analysis and tryptic phosphopeptide mapping only indicate that 4E-BP2 is also phosphorylated on Thr-37 and Thr-46. Moreover, the response to insulin treatment is weaker for 4E-BP2 phosphorylation compared to that of 4E-BP1 (Figure 4).

1.5.2 Biological function of 4E-BPs phosphorylation

The affinity of 4E-BPs to eIF4E is regulated by phosphorylation. Dephosphorylated 4E-BPs bind to eIF4E, reducing the availability of eIF4E to form eIF4F complex. Phosphorylated 4E-BPs release eIF4E, increasing the availability of eIF4E for eIF4F formation.

Fig.4. Alignment of the mammalian 4E-BPs through the eIF4E-binding site. The conserved eIF4E-binding motif is in red. Phosphorylated residues in 4E-BPs are in green (figure from Sonenberg, N., et al. Translation control of gene expression, Cold Spring Harbour Laboratory press, 2000)

1.5.2.1 4E-BPs phosphorylation and cell growth

Extracellular stimuli like hormones, growth factors and abundant nutrients activate certain signalling pathways (see 1.5.3), promote cell growth and proliferation and stimulate translation initiation and protein synthesis. These effects often combine with the increase of 4E-BP phosphorylation resulting in the disassociation of eIF4E with the 4E-BPs and thus increasing availability of eIF4E (Kleijn and Proud 2000) (Lynch, Fox et al. 2000) (Anthony, Anthony et al. 2001).

1.5.2.2 4E-BPs phosphorylation and apoptosis

Some stimuli, especially those which can ultimately result in apoptosis, usually inhibit translation initiation and abrogate 4E-BP phosphorylation, for example, starvation for essential nutrients (Fox, Kimball et al. 1998; Yoshizawa, Kimball et al. 1998; Kimball, Shantz et al. 1999; Vary, Jefferson et al. 1999; van Sluijters, Dubbelhuis et al. 2000), ischaemia, hypoxia (Tinton and Buc-Calderon 1999; Martin, Munoz et al. 2000), ethanol toxicity (Lang, Frost et al. 2000), growth factor deprivation (Kleijn and Proud 2000), strenuous exercise (Gautsch, Anthony et al. 1998), exposure to glucocorticoids (Shah, Kimball et al. 2000; Shah, Kimball et al. 2000) and infection (Vary and Kimball 2000). 4E-BP dephosphorylation and increased association of 4E-BP1 with eIF4E have been observed in response to all apoptotic inducers so far studied (Bushell, Wood et al. 2000). Several studies indicate that rapamycin induces apoptosis and dephosphorylation of 4E-P1 in B-cell lymphoma cell line BKS-2 (Muthukkumar, Ramesh et al. 1995), rheumatoid synovial cells (Muthukkumar, Ramesh et al. 1995) and human rhabdomyosarcoma cells (Hosoi, Dilling et al. 1999).

1.5.2.3 4E-BPs and transformation

4E-BP dephosphorylation during apoptosis induction implies its negative regulator activity in transformation. Overexpression of 4E-BPs in eIF4E and v-src transformed cells causes a significant reversion of the transformed phenotype (Rousseau, Gingras et al. 1996). Imatinib mesylate, the inhibitor of oncogene Bcr-Abl, induces translation initiation complex eIF4F formation and 4E-BP1 dephosphorylation in Bcr-Abl-expressing cell line and primary chronic myelogenous leukaemia (CML) cells (Prabhu, Saadat et al. 2007). During the differentiation of human papillomavirus-containing cells, 4E-BP1 is hyperphosphorylated combining with enhanced translation of oncoprotein E7 mRNAs (Oh, Kalinina et al. 2006). These studies suggest that regulation of 4E-BP phosphorylation is an important regulatory mechanism of 4E-BP function in cellular transformation.

1.5.3 The target of rapamycin (mTOR) signaling pathway-upstream of 4E-BPs

The phosphorylation of 4E-BPs is regulated by mTOR signaling pathway. Activation of mTOR pathway phosphorylates 4E-BPs. In response to different extracellular stimuli, mTOR integrates various signaling pathways to play a role in gene transcription, translation (regulate S6 kinase and 4E-BPs), ribosome biogenesis and macroautophagy (Figure 5).

1.5.3.1 Rapamycin and mTOR

Rapamycin is a macrocyclic lactone purified from the metabolite of bacterial strain Streptomyces hygroscopicus which was found in a soil sample from the Easter Island (known as Rapa Nui in the local language). Later rapamycin was found to inhibit proliferation of mammalian cells and to possess immunosuppressive properties. The mode of action of rapamycin is conserved from yeast to mammals. Upon entrance into the cell, rapamycin forms a complex with peptidyl-prolyl cis/trans isomerase FKBP12, which presumably assists in protein folding. This complex then binds to and inhibits its target—TOR (the target of rapamycin) (Abraham and Wiederrecht 1996; Thomas and Hall 1997).

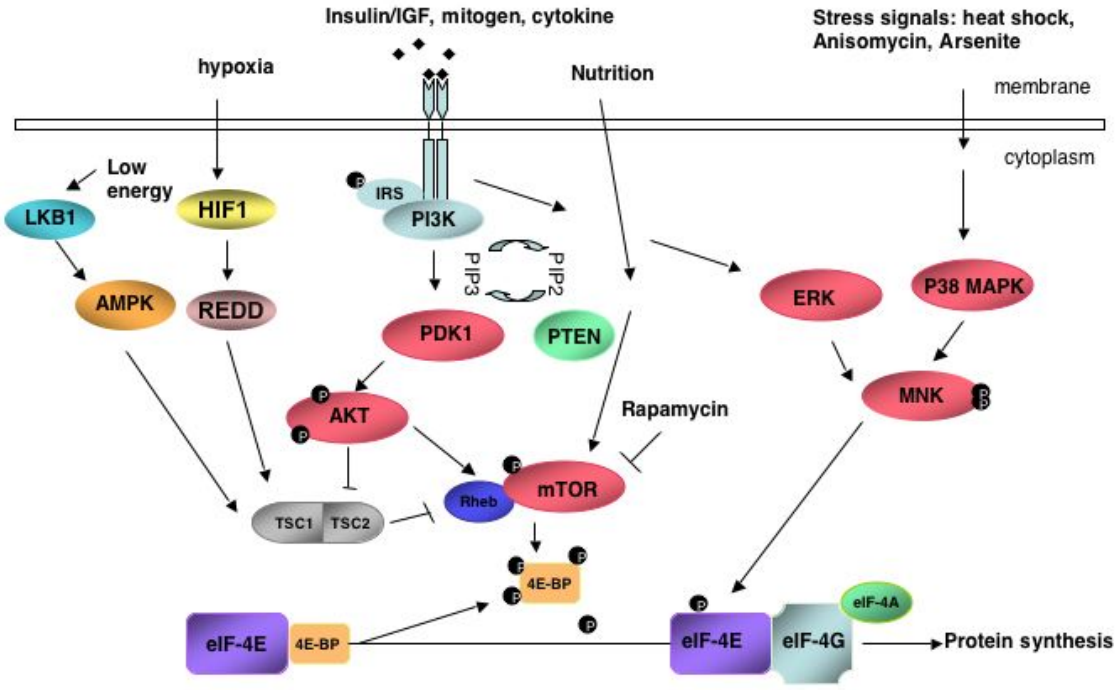


Fig.5. mTOR signaling and eIF4E phosphorylation regulation. EIF4E activity is regulated by 4E-BPs and phosphorylation. Activated mTOR phosphorylates 4E-BPs resulting in eIF4E release to interact with eIF4G, which combines with eIF4A to form eIF4F. mTOR integrates into several upstream signaling pathways according to different stimuli. Growth factors (insulin/IGF), mitogen, cytokines and nutrition activate mTOR. However, low energy and hypoxia inactivate mTOR. Extracellular stimuli activate ERK or p38MAPK signalling pathway to activate MNK. Activated MNK phosphorylates eIF4E to regulate eIF4F formation. Arrows represent activation. Bars represent inhibition (figure from www. cellsignaling.com).

All eukaryotic genomes examined so far (including yeasts, algae, slime mold, plants, worms, flies, and mammals) contain a *tor* gene. Unlike yeast, which in some cases possesses two *tor*

genes, higher eukaryotes possess only a single *tor* gene. Therefore two TOR proteins (TOR1 and TOR2) are present in S. cerevisiae, but only TOR1 is present in metazoans, including D. melanogaster (dTOR) and mammals (mTOR). Human *tor* gene has been mapped to chromosome 1p36.2. Mouse *tor* gene has been mapped to chromosome 4. The TOR proteins are highly evolutionarily conserved and share 40%-60% identity in different species. They belong to the phosphatidylinositol kinase-related kinase (PIKK) family. PIKK family members contain a carboxy-terminal serine/threonine protein kinase domain that resembles the catalytic domain of phosphatidylinositol 3-kinases (PI3Ks) and PI4Ks.

1.5.3.2 Insulin/IGF-PI3K-TOR

The TOR pathway and the insulin/IGF-PI3K signaling pathway are functionally connected. Important insights on the cross talk between these two pathways came from studies on the TSC1-TSC2 heterodimer (tuberous sclerosis complex) and the small GTPase Rheb (Ras-homolog enriched in brain) which binds directly to the kinase domain in TOR and activates TOR in Drosophila and mammalian cells.

Binding of insulin or insulin-like growth factors (IGFs) to their receptors leads to recruitment and phosphorylation of the insulin receptor substrate (IRS), and subsequent recruitment of PI3K. PI3K converts phosphatidylinositol-4,5-phosphate (PIP2) in the cell membrane to phosphatidylinositol-3,4,5-phosphate (PIP3). PIP3 accumulation is antagonized by the lipid phosphatase — phosphatase with tensin homolog (PTEN). PIP3 corecruits PtdIns-dependent protein kinase (PDK1) and protein kinase B (Akt) to the membrane, resulting in the phosphorylation and activation of Akt by PDK1. TOR1 is wired to the PI3K pathway through the tuberous sclerosis proteins hamartin (TSC1) and tuberin (TSC2). TSC1 and TSC2 form a heterodimer which prevents Rheb from binding to TOR, thus blocks TOR signaling. TSC2 is phosphorylated and functionally inactivated by Akt in response to insulin (Manning 2004) (Figure 5).

1.5.3.3 Nutrients

Amino acid starvation, in particular the absence of leucine, results in a rapid dephosphorylation of the TOR effectors S6K1 and 4E-BP1, whereas re-addition of amino acids restores S6K1 and 4E-BP1 phosphorylation in an TOR-dependent manner (Hay and Sonenberg 2004)(Figure 5).

1.5.3.4 Energy and hypoxia

AMP-activated protein kinase (AMPK) is activated in response to low cellular energy (high AMP/ATP ratio). Activated AMPK directly phosphorylates TSC2, leading to the inhibition of

TOR signaling (Inoki, Corradetti et al. 2005). The tumor suppressor serine/threonine kinase 11 (STK11/ LKB1) has been identified as an upstream kinase of AMPK, suggesting that LKB1 is linked to the TSC-TOR1 signaling pathway. *Lkb1* mutant cells exhibit hyperactive mTOR signaling (Corradetti, Inoki et al. 2004; Shaw, Bardeesy et al. 2004). Thus, upon energy deprivation, LKB1 in conjunction with AMP activates AMPK, which in turn phosphorylates and activates TSC2, resulting in the inhibition of mTOR. The regulation of mTOR in hypoxia is investigated in mammalian cells and Drosophila. During hypoxia, the transcription factor hypoxia-inducible factor 1 (HIF1) upregulates REDD (regulated in development and DNA damage responses; Scylla/ Charybdis in Drosophila), which acts upstream of TCS1/2 to inhibit TOR signaling (S6K and 4E-BP1 hypophosphorylation). However, in contrast to energy depletion, TOR inhibition by hypoxia requires neither AMPK nor LKB1, but the relationship between prolonged hypoxia and LKB1-AMPK pathway can not be excluded (Brugarolas, Lei et al. 2004; Reiling and Hafen 2004)(Figure 5).

Taken together, various extracelluar stimuli activate/inhibit mTOR through different signaling pathways to further regulate target genes expression.

1.5.4 Transcriptional regulation of 4E-BPs

Besides post-translational modification of 4E-BP by phosphorylation, 4E-BP is transcriptionally upregulated by forkhead-related transcription factor (FOXO) as well. FOXO binds directly to 4E-BP promoter. FOXO is inactivated by phosphorylation (Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999; Alvarez, Martinez et al. 2001). The insulin receptor phosphorylates FOXO through PI3K/dAKT signalling pathway. Except slight wing-size reduction, dFOXO-null flies do not show growth defect. However, they are hypersensitive to oxidative stress (Junger, Rintelen et al. 2003; Puig, Marr et al. 2003).

1.6 eIF4E phosphorylation and activity

1.6.1 Biochemical research

In addition to 4E-BPs, the activity of eIF4E is regulated by phosphorylation as well. However the effect of eIF4E phosphorylation on translation rate is not clear. The affinity of phosphorylated eIF4E to mRNA cap structure is not clear. Minish et al (Minich, Balasta et al. 1994) found an increase in 5' cap structure binding affinity of phosphorylated eIF4E compared with unphosphorylated eIF4E. Marcotrigiano et al (Marcotrigiano, Gingras et al. 1997; Marcotrigiano, Gingras et al. 1997) proposed the "clamping" model based on the crystallographic structure of the murine eIF4F complex with m^7GDP. It postulated the formation of a salt bridge between the phosphorylated Ser209 (the phosphorylation site of

mammalian eIF4E) and Lys159, which was situated across the entrance to the cap structure binding slot. Such a clamp could stabilize the mRNA chain at the protein surface. In controversy, Sheper et al and Zubereck J et al (Scheper, van Kollenburg et al. 2002; Zuberek, Wyslouch-Cieszynska et al. 2003) reported that phosphorylated eIF4E attenuated its interaction with the cap structure analogs or capped mRNA. Sheper, et al proposed two possible mechanisms based on the increase in the dissociation rate that was observed upon phosphorylation of eIF4E. One mechanism was that by decreasing the affinity for cap structure, phosphorylation of eIF4E could facilitate the release of tethered eIF4F from the 5'end of mRNA to promote ribosome scanning. Another mechanism was that the phosphorylation of eIF4E might be "reprogramming" of the translational machinery by the release of initiation factors from existing translational complex, allowing other mRNAs to become translated.

1.6.2 Biological function of eIF4E phosphorylation

Mammalian eIF4E is phosphorylated at Ser209 in response to mitogen, polypeptide hormones, growth factors, oxidative stress and cytokines which cause an increase in translation initiation and protein synthesis (Kleijn, Scheper et al. 1998; Gingras, Raught et al. 1999; Raught and Gingras 1999). Increased eIF4E phosphorylation seems not always related to increased translation rate. For example, an increase in eIF4E phosphorylation is observed in response to some types of cellular stress, including exposure to anisomycin, arsenite (Morley and McKendrick 1997), tumor necrosis factor-α and interleukin-1β, even though translation rates actually decrease in these situations. MAP kinase –interacting protein kinase (MNK) is the only known eIF4E kinase so far. There are two MNKs termed MNK1 and MNK2. MNK1 binds to the eIF4G family proteins rather than interact with eIF4E directly. This interaction is required for eIF4E phosphorylation (Morley and McKendrick 1997; Wang, Flynn et al. 1998). Expression of active mutants of MNK1 and MNK2 in 293 cells diminished cap-dependent translation in a transient reporter assay. The same effect on cap-dependent translation is observed when MNK1 is activated by the Erk or p38 pathway. In line with these findings, addition of recombinant active MNK1 to rabbit reticulocyte lysate results in a reduced protein synthesis in vitro. Overexpression of MNK2 causes a decrease in protein synthesis rate in 293 cells. By using CGP57380, a novel low-molecular weight kinase inhibitor of MNK1, it is found that eIF4E phosphorylation is not crucial to the formation of the initiation complex, mitogen-stimulated increase in cap-dependent translation and cell proliferation. This result suggests that the kinase activity of MNK may serve as a limit of cap-dependent translation (Knauf, Tschopp et al. 2001).

In Drosophila, the phosphorylation of eIF4E decreases upon heat shock concomitant with a decrease in translation rate. Drosophila eIF4E is phosphorylated at Ser251 that corresponds to extracellular signals. The flies in which eIF4E can no longer be phosphorylated (Ser ^{Ser251Ala}) have development defect and are smaller in size when compared to control. Adult eyes of the Ser ^{Ser251Ala} mutants have smaller and fewer ommatidia. Wing imaginal disk cells are smaller than those in wild type flies (Lachance, Miron et al. 2002)(Table 1). In the mixed genetic background (129SvJ1BALB/C) 4E-BP1 knock out mice, eIF4E phosphorylation increases in MEF combined with a increase in metabolic rate and UCP1 and PGC1 expression, which are adipose tissue metabolism related genes (Tsukiyama-Kohara, Poulin et al. 2001)(Table 1).

These studies suggest that eIF4E is regulated by phosphorylation during cell growth, development and stress response. However, so far there is no direct evidence of how eIF4E phosphorylation affects translation initiation rate.

1.6.3 Signaling pathway for eIF4E phosphorylation

Mitogen and stress induce eIF4E phosphorylation mediated by activation of the extracellular signal-regulated protein kinases (ERKs) and p38 mitogen-activated protein (MAP) kinases, respectively. These two pathways activate the common eIF4E kinase MNK to phosphorylate eIF4E (Figure 5).

1.7 eIF4E and transformation

Early studies show that stable expression of eIF4E in NIH3T3 and CHO cells enhances cellular proliferation, induces transformed morphology (spindle-shape, refractile cells) and promotes growth in soft agar (Lazaris-Karatzas, Montine et al. 1990). EIF4E cooperated with v-myc or E1A transforming primary rodent fibroblasts (Lazaris-Karatzas and Sonenberg 1992). Reduction of eIF4E decreases the malignancy of ras-transformed cloned rat embryo fibroblasts (Lazaris-Karatzas, Smith et al. 1992). EIF4E is overexpressed in many solid tumors and tumor cell lines. The list includes cancers of the colon, breast, bladder, lung, prostate, gastrointestinal tract, head and neck, Hodgkin's lymphoma and neuroblastomas (Kerekatte, Smiley et al. 1995; Anthony, Carter et al. 1996; De Benedetti and Harris 1999; Nathan, Franklin et al. 1999; Rosenwald, Chen et al. 1999; Wang, Rosenwald et al. 1999; Rosenwald, Hutzler et al. 2001; Rosenwald, Pechet et al. 2001; Wang, Lloyd et al. 2001). EIF4E transgenic mice develop tumors (16% B cell lymphomy, 13% angiosarcomas, 21% lung adenocarcinomas, 21% hepatocellular adenomas) beginning at 16 months of age (Table 1). EIF4E transgenic mice are crossed with c-Myc transgenic mice (develop B cell lymphoma by an average age of 3-4 months), the onset of lymphoma in eIF4E and c-Myc double transgenic mice are accelerated to less than one month of age. This tumor model suggests

eIF4E directly triggers tumor formation in vivo in tissues of various histological origins and the crucial contribution of the genetic cooperation between eIF4E and c-Myc toward lymphomagenesis (Ruggero, Montanaro et al. 2004).

1.8 eIF4E in ageing and stress

In Caenorhabditis elegans, loss of IFE-2, a specific eIF4E isoform that functions in somatic tissues, reduces global protein synthesis, protects from oxidative stress and extends lifespan. Knock down of mTOR further increases the lifespan of *ife-2* mutants. This observation combining with the studies in mice and Drosophila imply that mTOR-4E-BP-eIF4E translation machinery is involved in ageing and stress response (Syntichaki, Troulinaki et al. 2007)(Table 1).

1.9 Aim of the study

4E-BP1 and 4E-BP2 are equally and dominantly expressed in hematopoietic system. In HL-60 (promyelocytic leukaemia cell line) and U-937 (monoblastic cell line) cells, the translation rate is decreased when cells are induced to differentiate along the monocytic / macrophage pathway or along the granulocytic pathway. Induction of differentiation into monocytes/ macrophage in these two cell lines results in dephosphorylation of 4E-BP1 but it does not affect 4E-BP2. Induction of HL-60 into granulocytes results in increased expression of 4E-BP2 and decreased expression of 4E-BP1 (Grolleau, Sonenberg et al. 1999). In addition, activation of primary human immature $CD4^+CD8^+$ double positive thymocytes with anti-CD3 and anti-CD4 results in decrease of eIF4E phosphorylation and protein synthesis, whereas the eIF4E phosphorylation and protein synthesis are increased in mature $CD4^+$ or $CD8^+$ thymocytes following anti-CD3 and anti-CD4 stimulation. The expression of 4E-BP2 is higher in immature $CD4^+CD8^+$ thymocytes but lower in mature $CD4^+$ or $CD8^+$ thymocytes (Beretta, Singer et al. 1998). Finally, mTOR regulates both proliferation of megakaryocyte progenitors and differentiation of late stages megakaryocytes. Phosphorylations of mTOR, p70S6K1, and 4E-BP1 are diminished in thrombopoietin-cultured human megakaryocytes in response to rapamycin treatment. Rapamycin also induces a delay in the expression of megakaryocyte markers and prevents the generation of proplatelet megakaryocytes (Raslova, Baccini et al. 2006). These observations suggest that 4E-BPs related translation machinery is functional in the proliferation and differentiation of hematopoietic lineages. 4E-BP1 is phosphorylated during erythroblast proliferation. Other studies show that overexpression of eIF4E blocks erythroblasts differentiation in tissue culture. These studies imply that 4E-BPs might play role in response to the erythropoietic stress. The aim of the study was to investigate the function of 4E-BP1 and 4E-BP2 in hematopoiesis and stress erythropoiesis

using 4E-BP1 and 4E-BP2 individual knock out mice and 4E-BP1,2 compound knock out mice.

2 Materials

2.1 Animals

4E-BP1, 4E-BP2 individual knock out mice (4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$) and 4E-BP1,2 compound knock out mice (4E-BP1,2$^{-/-}$) with 129SvJ1BALB/C mixed genetic background (From Prof. Nahum Sonenberg, Department of Biochemistry, McGill University, Canada)

2.2 Chemicals and reagents

7-amino-actinomycin D (7-AAD)	Pharmingen
20× L- Glutamine	Gibco
100% ethanol	Merk
BSA	Sigma-Aldrich
Chemiluminescent HRP Substrate (Immobilon^{TM} Western)	Millipore
Dexamethasone	Sigma-Aldrich
dNTP	Amersham Biosciences
DNA Taq polymerase II	Amersham Biosciences
EDTA	Calbiochem
erythroid lysing buffer (BD Pharmlyse ^{TM})	BD Bioscience
Fetal bovine serum	Gibco
Ficoll-Paque Plus	Amersham Biosciences
HBSS	Gibco

HEPES	Merk
Isopropanol	Merk
NaN_3	Merk
NaCl	Merk
Non-fat dry milk powder	Merk
NP-40	Fluka Chemie
Phenylhydrazine hydrochoride	Sigma-Aldrich
phenol/choloroform	Roche
proteinase K	Sigma-Aldrich
protase inhibitor cocktail	Roche
random primers	Promega
reticulocyte stain	Sigma-Aldrich
sodium orthovanadate	Sigma-Aldrich
Sodium Dodecyl Sulfate	Roche
Superscript II reverse transcriptase	Invitrogen
Trypan Blue	Sigma-Aldrich
Tween-20	Sigma

2.3 Medium

MethoCult®3334	StemCell Technologies
MethoCult®3434	StemCell Technologies
StemPro-34 medium	Gibco

2.4 Cytokine

human recombinant erythropoietin (hEPO),	R&D System
insulin-like growth factor1 (IGF-1)	R&D System
murine recombinant stem cell factor (mSCF)	obtained from Prof.Hartmut Beug

2.5 Kits

Platinum® SYBR® Green qPCR SuperMix UDG	invitrogen
RNeasy Mini Kit	Qiagen

2.6 Antibodies

2.6.1 Fluorochrome-conjugated antibodies

FITC-anti-Ter119	Pharmingen
FITC-anti-B220	Pharmingen
FITC-anti-CD8	Pharmingen
FITC-anti-Gr-1	Pharmingen
PE-anti-CD71	Pharmingen
PE-anti-CD11b	Pharmingen
PE-anti-CD4	Pharmingen

2.6.2 Other antibodies

2.6.2.1 Primary antibodies

4E-BP1	Cell Signaling Technology
4E-BP2	Cell Signaling Technology
anti-Ter119-biotin	Pharmingen
α-Tubulin (B-7)	Santa Cruz
eIF-4E	BD Biosicence
GATA-1(C-20,sc-1233)	Santa Cruz
Mnk1(Thr197/202)	Cell Signaling Technology
Phospho-eIF4E (Ser209)	Cell Signaling Technology
Phospho-mTor (Ser2481)	Cell Signaling Technology

2.6.2.2 Horseradish peroxidase-conjugated secondary antibody

donkey anti-rabbit	Amersham Biosciences
donkey anti-goat	Santa Cruz

sheep anti-mouse	Amersham Biosciences

2.7 Appliances

Animal Blood Counter	Scil animal care company
Bradford protein analysis	Bio-Rad
FACSCalibur flow cyctometer	BD Bioscience
Light Cycler 3.5	Roche
MACS separater	Miltenyi Biotec
Western Blotting apparatus	Biometra

2.8 Consumables

1ml syringe	Omnifix-f, BRAUN
24 gauge needle	Sterican, BRAUN
100-µ strainer	BD Bioscience
Anti-biotin magnetic bead	Miltenyi Biotec
EDTA coated microvette	Sarstedt, Germany
MACS pre-sep filter	Miltenyi Biotec
MACS cell separation column	Miltenyi Biotec
PVDF membrane (Immobilon-P transfer membrane)	Millipore
X-ray film	Kodak

2.9 Software

CellQuest	BD Bioscience
Light Cycler software version 3.5.3	Roche Applied Science

3 Methods

3.1 Mice

4E-BP1$^{-/-}$ and 4E-BP2$^{-/-}$ mice were kindly provided by Dr. Nahum Sonenberg (Dapartment of Biochemistry and McGill Cancer Centre, McGill University, Montreal, Quebec H3G 1Y6, Canada). All the mice were maintained according to the procedure approved by the Max Delbrück Center (Germany) institutional animal care committee. 4-6 months old mice were analyzed (results of peripheral blood parameters obtained from 2-12 months old mice).

3.2 Phenylhydrazine induces hemolytic anemia

3.2.1 Phenylhydrazine ($C_6H_5NHNH_2$)

Phenylhydrazine, as an oxidant, is known to be an effective inducer of hemolysis when administered to normal animals. Its effect is mediated by production of phenylhydrayl radicals, H_2O_2, O_2^-, phenyldiazene and phenyl radicals produced by the interaction of the drug with oxyhemoglobin. As a consequence of the production of these free radicals hemoglobin is first oxidized to methemoglobin and then to hemicrome. At the same time, the membrane proteins, lipid, potassium and calcium permeability, band 3 clustering, autologous IgG binding are modified (Magnani, Rossi et al. 1988).

3.2.2 Induction of hemolytic anemia

Mice were intraperitoneally injected on day 0, 1 with 60mg/kg phenylhydrazine hydrochloride in phosphate-buffer saline (PBS).

3.3 Extraction of DNA from mouse tail

DNA lysis buffer:

EDTA 50mM

NaCl	100mM
Proteinase K	200μg/ml
SDS	0.2%
Tris	100mM (pH 8.0)

2-5mm mouse tail tip was cut off and put into 1.5ml microcentrifuge tube. 200μl DNA lysis buffer was added. The sample was shaken at 800rpm incubated at 55°C overnight. On the second day, the tube was incubated at 95 °C for 10 minutes to inactivate the proteinase K. The pellet was spun down and the supernatant was transferred into new tube. One volume of phenol/choloroform was added, mixed gently with the sample and centrifuged for 5minutes at 13000rpm. The aqueous phase was transferred into a new tube and 1/10 volume of 3M NaAc pH5.2 and 2 volumes of 100% ethanol were added. The tube was inverted and swirled to mix and precipitate DNA. The DNA was spun down at 14000rpm for 5 minutes at room temperature. The supernatant was aspired and washed once with 70% ethanol. The pellet was briefly air-dried at room temperature and was then dissolved in 20-30 μl TE buffer.

3.4 PCR-based genotyping

Genotyping of mice was performed by polymerase chain reaction (PCR) analysis of tail DNA. Primer sequences were as follows:

4e-bp1: forward primer (5'-GATGGAGTGTCGGAACTCACC-3'),

reverse primer (5'-GACCTGGACAGGACTCACCGC-3');

4e-bp2: forward primer (5'-GGTGGGACTGTCGGTCTTCTG -3') ,

reverse primer (5'-CAGCACCTGGTCATAGCCGTG-3')

neo: forward primer (5'-GCATCGAGCGAGCACGTACTC-3').

The PCR programme were as follows: 1 cycle at 94°C for 5 minutes, 30 cycles at 94°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute with a final cycle at 72°C for 5 minutes.

3.5 Acquirement of peripheral blood

Peripheral blood was obtained by puncturing the ventricle of the heart with a 21G needle. 100μl peripheral blood was placed in an EDTA coated microvette.

3.6 Isolation of bone marrow cell

Mice were sacrificed by CO_2 asphyxiation. Femurs were put in a sterilized Petri dish with PBS on ice. A 1ml-syringe with a 24 gauge needle was used to flush bone marrow from femur with cold PBS or HBSS and the cell suspension was passed through the needle several times and was filtered through MACS pre-sep filter to make single-cell suspension.

3.7 Isolation of spleen cell

Spleen was mechanically dissociated by pushing with a syringe plunger through a 100-µm strainer in PBS or HBSS. The cell suspension was passed through 24 gauge needle several times and was filtered through MACS pre-sep filter.

3.8 Measurement of haematological blood parameters

Peripheral blood was obtained from the heart with a 21G needle. 30µl of blood was put into an EDTA-coated microvette. 30µl of blood and 20µl reticulocyte stain were mixed well and incubated at room temperature for 15 minutes. The blood smears were made on the microscope slides, dried in air and evaluated under the oil immersion on a light microscope. Under normal erythropoiesis, 1000 cells per slide were counted. Under phenylhydrazine induced stress erythropoiesis (48 hours after second injection), 500 cells per slide were counted.

3.9 Cultivation of mouse erythroblasts

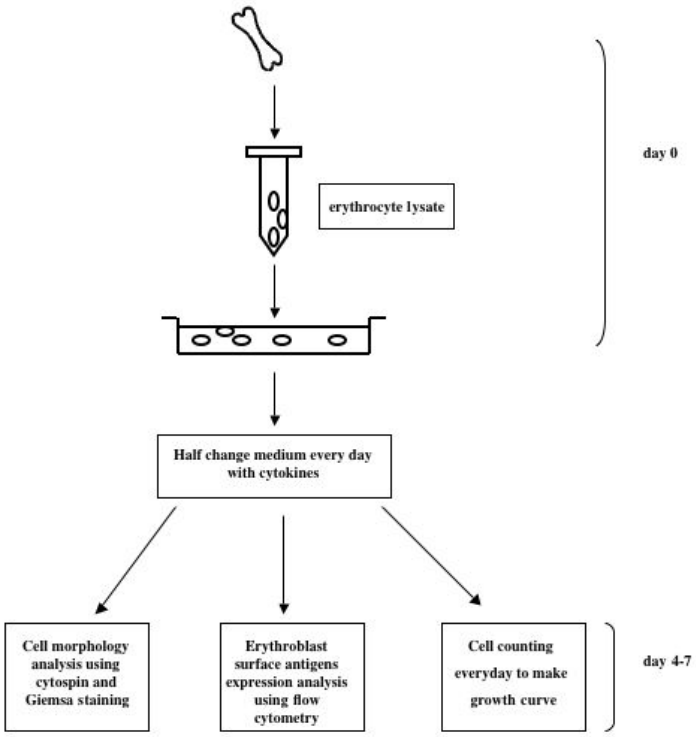
3.9.1 Procedure of erythroblasts cultivation

Bone marrow cells were isolated from one femur. The cells were suspended in 5ml erythroid lysis buffer for 8 minutes, centrifuged at 1000rpm for 5 minutes at room temperature and the supernatant was aspirated. The cells were washed twice with HBSS and passed through the 100µm Nylon cell strainer. 2-4 $\times 10^6$ / ml of mononuclear cells were seeded in StemPro-34 medium supplemented with nutritions, 2mM L-glutamine, 2U/ml human recombinant erythropoietin (hEPO), murine recombinant stem cell factor (SCF, 1:100 dilution), 10^{-6}M dexamethasone and 40ng/ml insulin-like growth factor (IGF-1). The mass cultures of erythroblast were subjected to daily half medium changes and addition of fresh factors. Cell density was maintained at 2-4 $\times 10^6$ / ml. In general, erythoblasts start to proliferate 4 to 6 days after seeding. About every 5-6 days, the cells were density gradient purified by ficoll centrifugation for 30 minutes at 700× g, 20°C to remove dead cells and differentiated cells. The cells were washed at least twice with HBSS to get rid of remaining ficoll before they

were re-suspended in the medium. The number of living cells was determined on the indicated day by trypan blue exclusion.

3.9.2 Determination the percentage of erythroblasts in the cultivated cells

To examine whether the cultivated cells were erythroblasts, we checked the expression of different lineages specific surface antigens using flow cytometry. I found that around 90% cells were erythroblasts, which expressed erythroid lineage surface antigens CD71, C-Kit, Ter119 (Figure 8A); 0.44% cells expressed B lymphocyte surface antigen B220 (Figure 8B); 2.12% cells expressed myeloid surface antigens CD11b, Gr-1(Figure 8C); 9.22% cells expressed megakaryocyte surface antigen CD41(Figure 8D).



3.9.3 Morphological analysis of cultivated cells

Morphological analysis of cultivated cells showed that most cells were erythroblasts (Figure9)

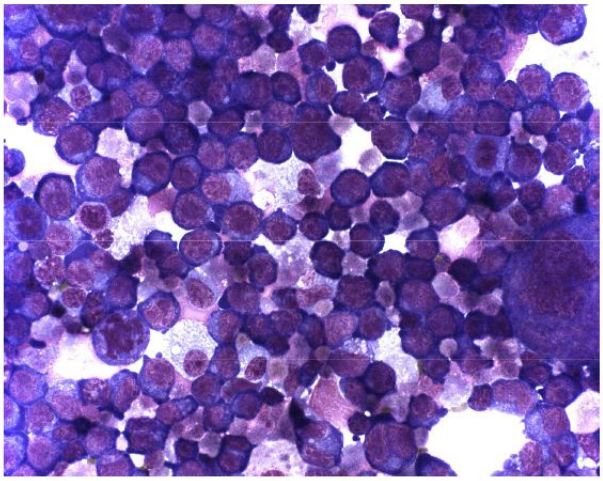


Fig.9. Morphological analysis of cultivated cells . Cultivated cells were harvested and subjected to cytospin and Giemsa staining.

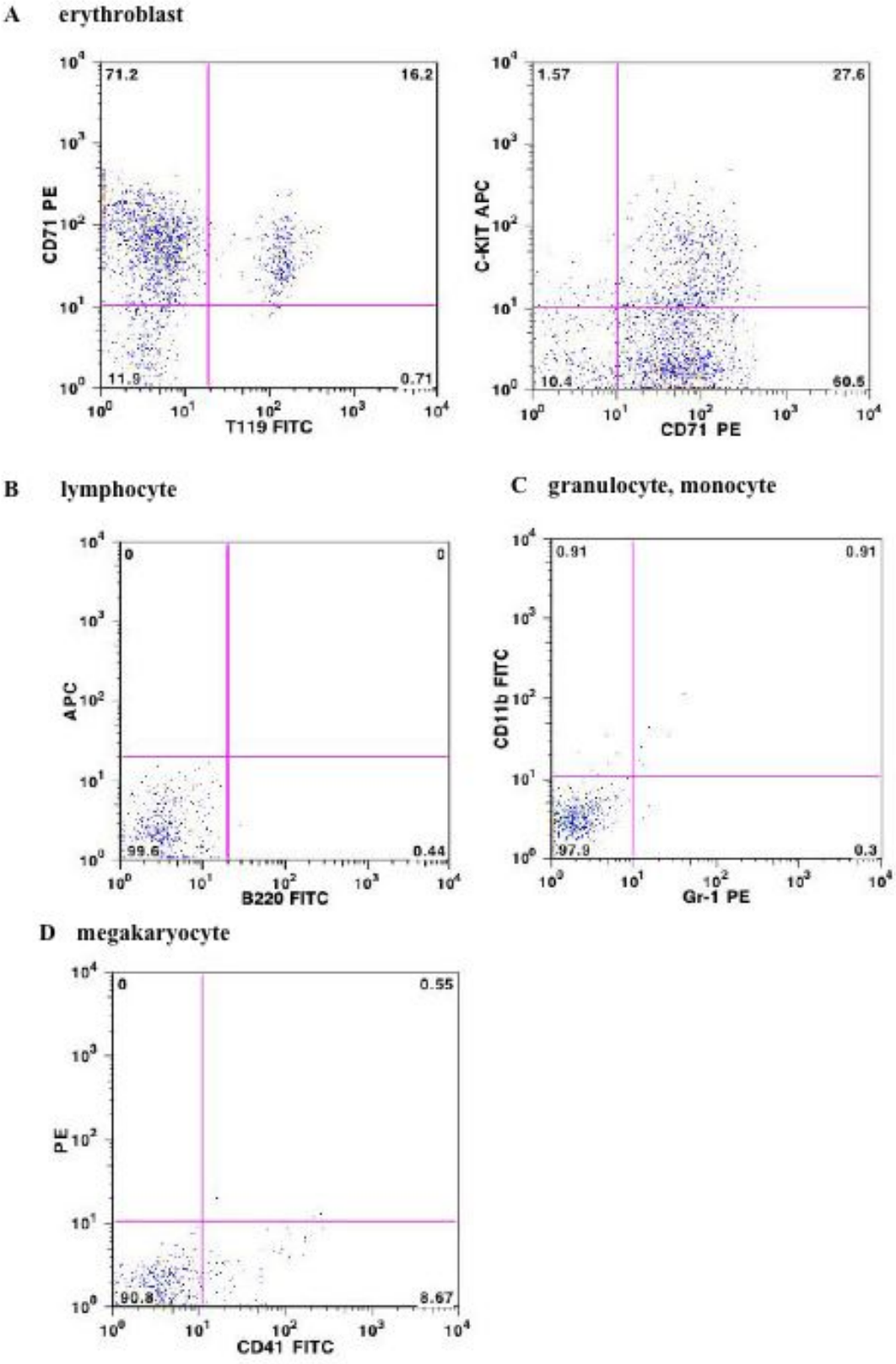


Fig.8. Expression of lineages specific surface antigens on cultivated cells using flow cytometry. Cells were labeled with fluorochrome-conjugated antibodies recognizing different lineage specific surface antigens: (A) erythrobalsts (CD71, C-KIT, TER119), numbers in the quadrants (upper left, upper and lower right) indicated the percentages of erythroblasts; (B) B lymphocytes (B220), numbers in the quadrants (upper and lower right) indicated the percentages of B lymphocytes; (C) Granulocytes/Monocytes (Gr-1, CD11b), numbers in the quadrants (upper left, upper and lower right) indicated the percentages of granulocytes and monocytes; (D)

Megakaryocytes (CD41), numbers in the quadrants (upper and lower right) indicated the percentages of megakaryocytes.

3.10 Flow cytometry

FACS buffer:

EDTA 2mM

FCS 2%

NaN_3 0.1%

In PBS

Single-cell suspension was treated with lysis buffer to lysate mature erythrocyte. The cells were then washed in FACS buffer and re-suspended in FACS buffer. Freshly isolated spleen cells, bone marrow cells or harvested cultivated erythroblasts were incubated with corresponding fluorochrome-conjugated antibodies. The immunostaining procedure was performed according to standard protocol: Briefly, about 10^6 cells were suspended in 100µl FACS buffer containing diluted fluorochrome-conjugated antibodies in FACS tubes and incubated for 15-20 minutes in the dark at 4°C. After washing once with cold FACS buffer, the cells were stained with 7-amino-actinomycin D (7-AAD) (1:100) for 2 minutes to exclude dead cells from analysis. After adding 200µl of FACS buffer, flow cytometry analysis was carried out on a Becton Dickinson FACSCalibur.

3.11 Colony forming assay

The cells from the spleen of control mice were suspended in 2ml HBSS. The cells from the spleen of PHZ treated mice were suspended in 3ml HBSS. 10µl cell suspension was mixed with 1ml 3% acetic acid and reacted for 8 minutes at room temperature to eliminate mature erythrocytes. Then mononuclear cells concentration of the cell suspension was determined. To detected CFU-E colonies, 300µl cell suspension that contains 2×10^5 spleen mononuclear cells was properly mixed with 3ml methylcellulose medium on the roller for 20 minutes. Finally 1ml mixture was plated in triplated in 33mm dish (6×10^4 cells/dish). The colonies were counted after 2 days in culture. To detected BFU-E colonies, 300µl cell suspension which contains 6×10^5 was mixed with 3ml methylcellulose medium. The colonies were counted after 9 days in culture. The cells from bone marrow were suspended in 2ml HBSS. The same procedure was used to detect CFU-E, BFU-E and CFU-GM colonies. 200µl cell suspension that contains 4×10^4 bone marrow mononuclear cells was mixed with 2ml methylcellulose medium and plated in duplicated in 33mm dish (2×10^4 cells/dish).

3.12 Enrichment of ter119 positive splenocytes

Splenocytes were treated with erythrocyte lysis buffer and washed twice with cold running buffer. Cells were then incubated with 10 µl of anti-Ter119-biotin primary antibody in 1ml of running buffer for 15 minutes at 4°C. The cells were washed once with cold running buffer. Cell pellet was re-suspended in 180µl of running buffer with 20µl of anti-biotin beads, and the reaction mixture were incubated in dark at 4°C for 15 minutes, followed by washing once with cold buffer. Cell pellet was re-suspended in 500µl of running buffer and cell separation was carried out using MACS cell separation column according to the manufacturer's instruction.

Rinsing buffer:

EDTA 2mM

in PBS

Running buffer:

BSA 0.5%

EDTA 2mM

in PBS

Cleaning buffer:

70% ethanol diluted from 100% ethanol solution

3.13 Preparation of protein extract

Lysis buffer

EDTA 1mM

HEPES 50mM pH7.0

NaCl 250mM

NP-40 0.1%

PMSF 100µg/ml

Protase inhibitor cocktail 1×

Sodium orthovanadate 0.5mM

Cells were incubated on ice for 30 minutes in lysis buffer. After centrifugating for 10 minutes at 10,000×g, total protein concentration of the supernatant was determined by Bradford protein assay.

3.14 Western blotting analysis

Same amount of protein of samples were separated on 8% to 15% SDS-PAGE and blotted onto PVDF membrane. After blocking the membrane in PBS containing 5% non-fat milk and 0.1%Tween-20 for 1 hour at room temperature, the membrane was incubated with the primary antibody in 5% non-fat milk at 4°C overnight. The following primary antibodies were used: Phospho-eIF4E (Ser209, 1:1000 dilution), Phospho-mTor (Ser2481, 1:1000 dilution), Phospho-Mnk1 (Thr197/202, 1:1000 dilution), 4E-BP1(1:1000 dilution), 4E-BP2 (1:1000 dilution); GATA-1 (C-20, 1:400 dilution), α-Tubulin (B-7, 1:1000 dilution). Next day, the membrane was washed three times with PBS containing 0.1% Tween-20 and incubated with corresponding secondary antibodies at room temperature for 1 hour. Horseradish peroxidase-conjugated secondary antibodies were used: donkey anti-rabbit (1:5000 dilution), sheep anti-mouse (1:5000 dilution), donkey anti-goat (1:5000 dilution). The immunoreactive bands were visualized by enhanced chemiluminiscence.

3.15 Real-time PCR

Total RNA was isolated from 5 x 10^5 purified Ter119 $^+$ erythroblasts of spleen using RNeasy Mini Kit and digested with DNase I to remove contaminating genomic DNA. Total RNA was denatured at 65°C for 10 minutes and reverse-transcribed by using Superscript II reverse transcriptase and random primers. Real-time polymerase chain reaction (PCR) was carried out using SYBR green fluorescent DNA labeling with the Light Cycler 3.5 instrument and the software version 3.5.3. *gapdh* was used as endogenous control. The PCR condition was as follows: initial denaturation at 95° for 30 seconds, then 45 cycles of the following were carried out (20 seconds at 95°C, 20 seconds at 60°C for GAPDH or at 56°C for mouse *gata-1*, 20 seconds at 72°C). PCR products were analyzed by agarose gel electrophoresis. Quantification was performed using comparative CT method. Standard curve was obtained from serial dilutions of a cDNA control and the relative expression levels were normalized to those of GAPDH.

The following primer sequences were used:

mouse *gata1:* forward: ACTGGCCTACTACAGAGAAGC

reverse: GTAGAGTGCCGTCTTGCCATA .

mouse *gapdh:* forward: AATGTGTCCGTCGTGGATCTGA,

reverse: GATGCCTGCTTCACCACCTTCT

3.16 Statistics

Statistical analysis was performed by 2-tailed, paired and unpaired Student's t-test. A *P* value less than 0.05 was considered significant.

4 Results

I investigated the role of 4E-BP1 and 4E-BP2 in hematopoiesis using 4E-BP1 and 4E-BP2 individual knock out mice (4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$) and 4E-BP1,2 compound knock out mice (4E-BP1,2$^{-/-}$).

4.1 Unaffected hematopoiesis of 4E-BPsKO mice

4.1.1 Adult hematopoiesis

The adult hematopoietic cell lineages are developed from hematopoietic stem cells (HSCs) which possess self-renewal ability and mainly maintain in bone marrow. HSCs give rise to multipotent progenitors (MPPs), which have lost the ability to self-renew, retaining their multi-lineage developmental potential. MPPs generate common lymphoid progenitors (CLP) and myeloid progenitors (CMP). Common progenitors further differentiate into lineage-restricted precursors, finally into mature blood cells including erythrocyte, granulocyte, monocyte/macrophage, megakaryocyte, platelets as well as B and T lymphoid cells (Schwarzenberger, Kolls et al. 2002) (Shizuru, Negrin et al. 2005) (Figure 10).

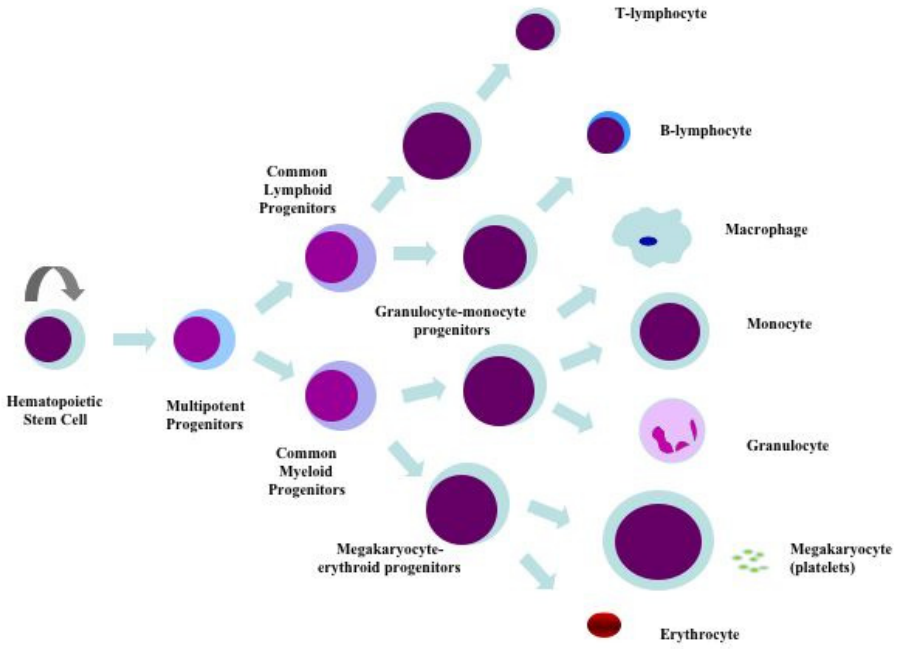


Fig.10. Adult hematopoiesis

HSCs, which possess self-renewal ability and multi-lineage differentiation potential, give rise to multipotent progenitors (MPPs), which have lost the ability to self-renew, retaining their multi-lineage developmental potential. MPPs differentiate into CLP and CMP. Common progenitors further differentiate into lineage-restricted precursors, finally to mature blood cells including erythrocyte, granulocyte, monocyte/macrophage, megakaryocayte as well as B and T lymphoid cells.

4.1.2 Unaffected peripheral blood parameters of different lineages in 4E-BpsKO mice

I examined hematopoiesis of 4E-BPsKO mice. First I found that the peripheral blood parameters of myeloid lineage and lymphoid lineage of 4E-BPs KO mice were unaffected including erythroid lineage parameters, which were normal as well compared to those of control mice (Table 2,3).

Tab. 3 peripheral blood erythroid parameters

	WT	4E-BP1,2$^{-/-}$	4E-BP2$^{-/-}$	4E-BP1$^{-/-}$
Parameters	n=17	n=20	n=13	n=13
RBC(× 10^6/mm^3)	10,54 ± 0,20	10,91 ± 0,19	10,83 ± 0,16	10,88 ± 0,33
Hemoglobin(g/dl)	16,71 ± 0,33	16,88 ± 0,32	17,19 ± 0,29	17,25 ± 0,48
Hematocrit(%)	56,38 ± 1,31	58,76 ± 1,21	58,35 ± 1,01	58,12 ± 1,96
MCV(µm3)	53,41 ± 0,52	53,80 ± 0,41	54,00 ± 0,32	53,31 ± 0,38
MCH(pg)	15,87 ± 0,15	15,46 ± 0,11	15,86 ± 0,11	15,88 ± 0,23
MCHC(g/dl)	29,67 ± 0,22	28,77 ± 0,22	29,44 ± 0,20	29,78 ± 0,43
Reticulocytes(%)	2,16 ± 0,29(n=9)	2,37 ± 0,18(n=15)	2,53 ± 0,24(n=11)	2,13 ± 0,36(n=5)
P value	-	> 0.05	> 0.05	> 0.05

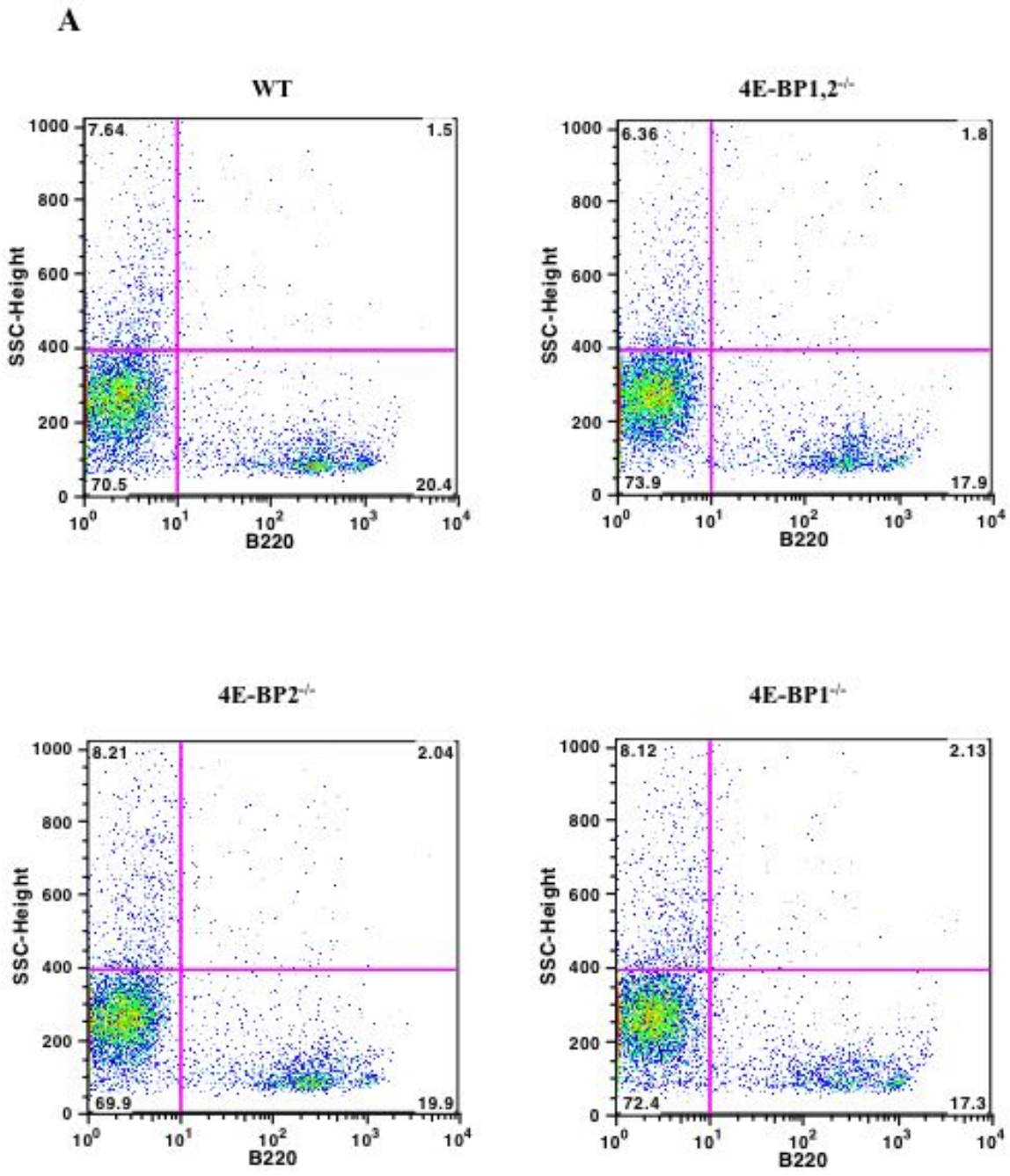
P value represented Student's t-test of the peripheral blood erythroid parameters of 4E-BPsKO mice compared to control mice; erythrocyte/red blood cell (RBC); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); mice were 2-12 months old.

These results indicated that disruption of 4E-BPs did not affect mature blood cells in peripheral blood. Next we examined the precursors and progenitors of myeloid and lymphoid lineages in the bone marrow of 4E-BPsKO mice.

4.1.3 Unaffected B lymphocyte frequencies in the bone marrow of 4E-BPsKO mice

B220, which is also known as CD45R, is expressed on all B lymphocytes, resting and activated, as early as the pro-B cell stage of differentiation. As a cell surface receptor, the B220 antibody is used as a lineage marker of B lymphocytes. I examined B lymphocytes in bone marrow of 4E-BPsKO mice monitored by the lineage surface antigen B220 using flow

cytometry. I found that disruption of 4E-BPs did not affect B lymphocytes in 4E-BPsKO mice ($P > 0.05$) (Figure 11).



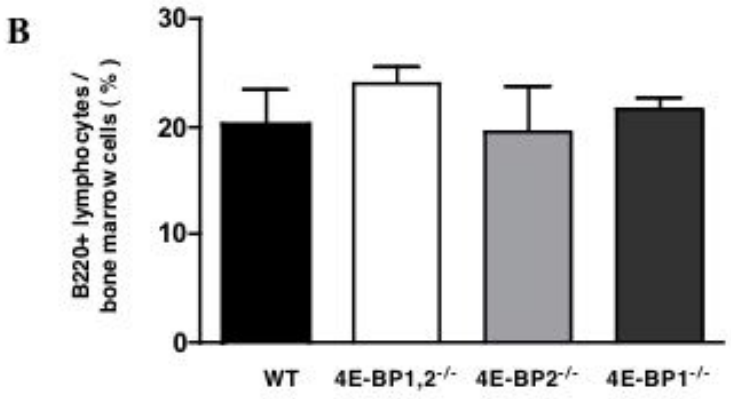
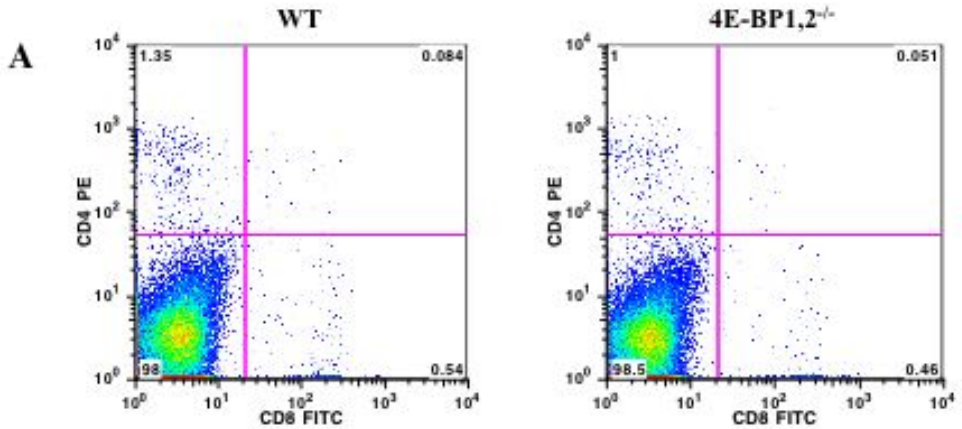
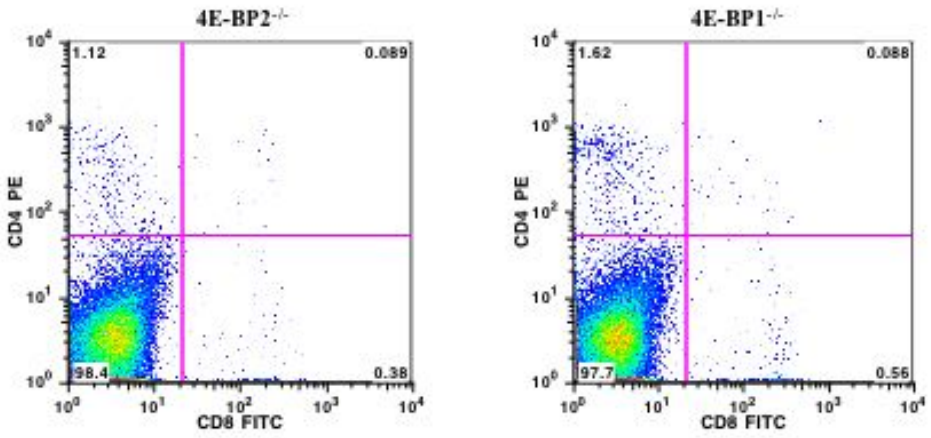


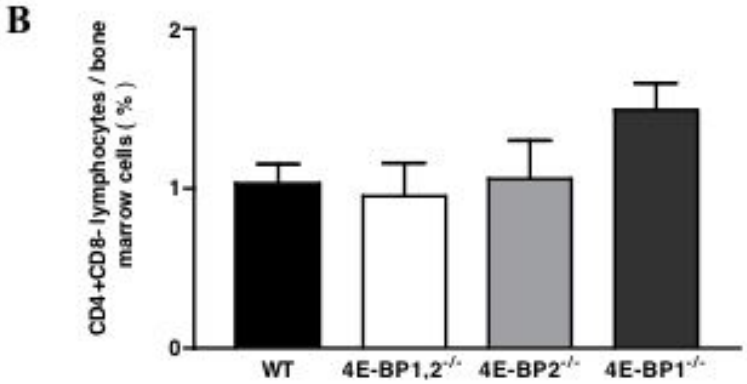
Fig.11. Unaffected B220 positive lymphocytes frequencies in the bone marrow of 4E-BPsKO mice. (A) An example of bone marrow B220 positive lymphocytes flow cytometry analysis of 4E-BPsKO and control mice. Numbers in quadrants (bottom) indicated percentages of B220 positive lymphocytes (upper and lower right) in bone marrow cells. (B) Results (mean ± s.e.m) obtained from 4E-BPsKO mice (n = 5) and control mice (n = 5).

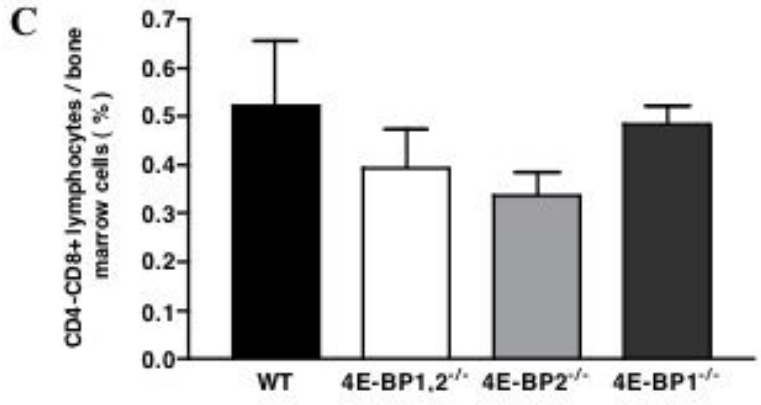
4.1.4 Unaffected T lymphocyte differentiation in the bone marrow of 4E-BPsKO mice

T lymphocyte is another subpopulation of lymphocyte. CD4 and CD8 are expressed on the T lymphocytes. CD4+CD8+ double positive cells are the late stage immature T lymphocytes. CD4+ cells are the MHC class II — restricted T lymphocytes, including most T helper cells and immunosuppressive regulatory T cells and a subset of NK-T cells. CD8+ cells are MHC class I — restricted T lymphocytes, including cytotoxic T cells. I examined T lymphocytes in the bone marrow of 4E-BPsKO mice monitored by the lineage surface antigens CD4 and CD8 using flow cytometry. I found unaffected T lymphocyte differentiation in 4E-BPsKO mice (Figure 12)




4E-BP2-/-
4E-BP1-/-
CD4 PE
CD8 FITC
1.12
0.089
98.4
0.38
1.62
0.088
97.7
0.56


B
CD4+CD8- lymphocytes / bone marrow cells (%)
WT
4E-BP1,2-/-
4E-BP2-/-
4E-BP1-/-


C
CD4-CD8+ lymphocytes / bone marrow cells (%)
WT
4E-BP1,2-/-
4E-BP2-/-
4E-BP1-/-

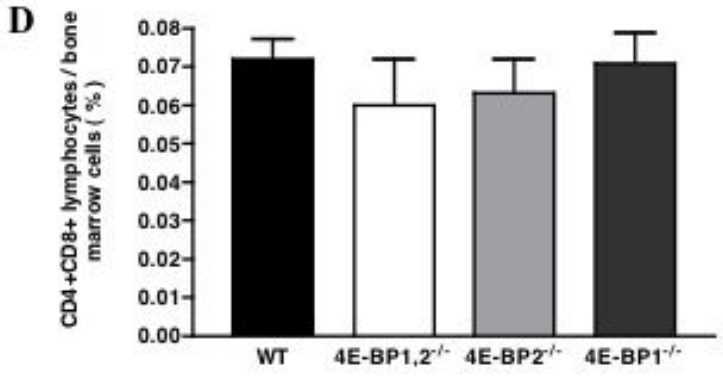
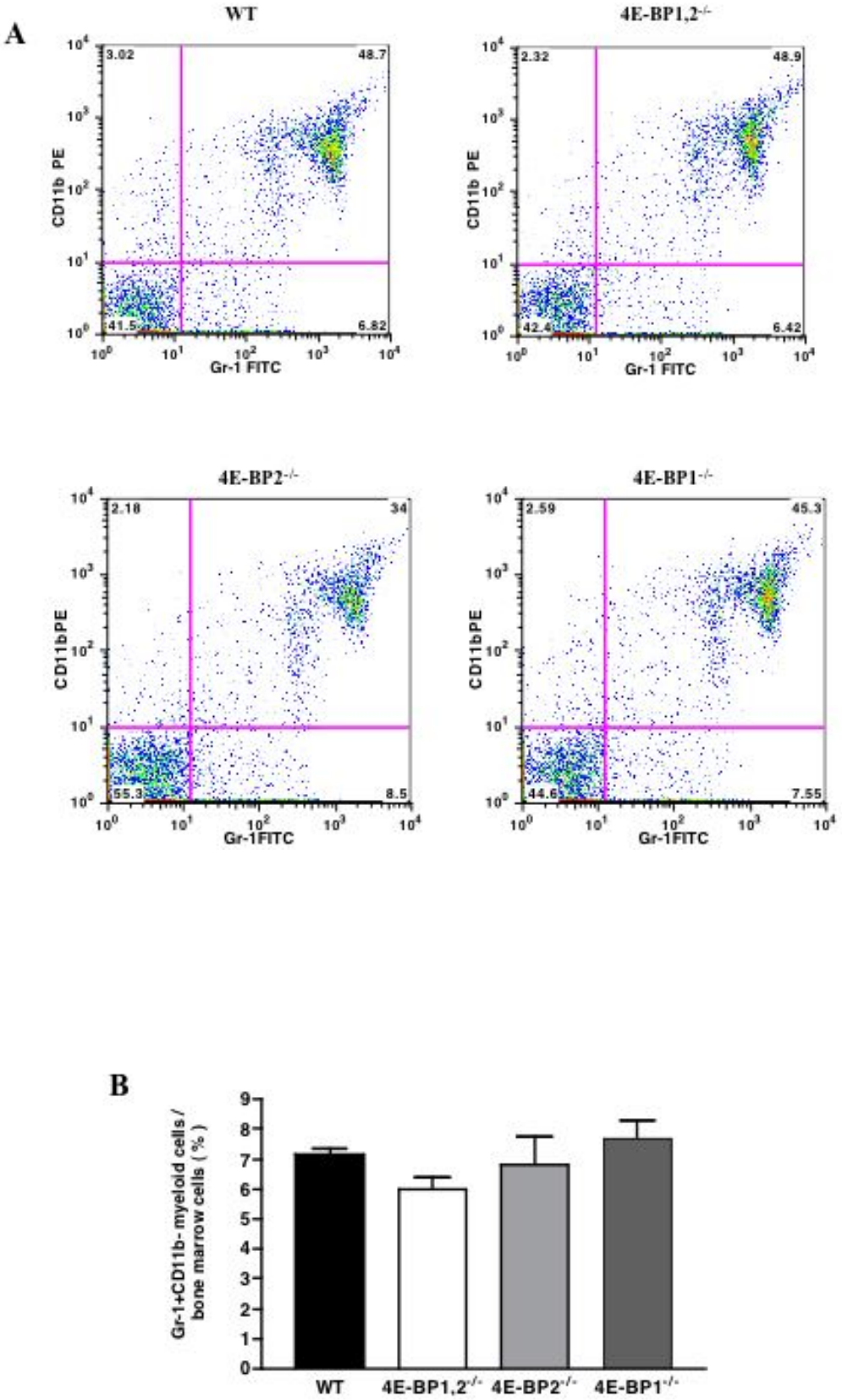


Fig.12. Unaffected T lymphocytes differentiation in the bone arrow of 4E-BPsKO mice.(A) An example of bone marrow T lymphocytes flow cytometry analysis of 4E-BPsKO and control mice. Numbers in quadrants (bottom) indicated percentages of CD4+CD8- T lymphocytes (upper left); CD4+CD8+ T lymphocytes (upper right); CD4-CD8+ T lymphocytes (lower right) in bone marrow cells. (B),(C),(D) Results (mean ± s.e.m) obtained from 4E-BPsKO mice (n = 4) and control mice (n = 5). (B) the percentages of CD4+CD8- T lymphocytes in bone marrow cells of 4E-BPsKO and control mice. (C) the percentages of CD4-CD8+ T lymphocytes in bone marrow cells of 4E-BPsKO and control mice.(D) the percentages of CD4+CD8+T lymphocytes in bone marrow cells of 4E-BPsKO and control mice.

4.1.5 Unaffected myeloid precursors in the bone marrow of 4E-BPsKO mice

Gr-1 (Ly6G) is expressed on myeloid precursor cells, granulocytes, and transiently on monocytes. The CD 11b receptor (Mac-1) is $\alpha M\beta_2$ integrin that is expressed on the surface of monocyte/macrophages, dendritic cells (DC) and granulocytes. CD11b+Gr-1+ cells display features of undifferentiated myeloid cells and contain precursors of different myeloid cell subsets. These cells have been termed immature myeloid cells. I examined myeloid precursors and mature myeloid cells in the bone marrow of 4E-BPsKO mice monitored by the lineage surface antigens CD11b and Gr-1 using flow cytometry. I found unaffected myeloid cells differentiation in 4E-BPsKO mice compared to the control mice (Figure 13)


A
WT
4E-BP1,2-/-
4E-BP2-/-
4E-BP1-/-
CD11b PE
Gr-1 FITC
3.02
48.7
41.5
6.82
2.32
48.9
42.4
6.42
2.18
34
55.3
8.5
2.59
45.3
44.6
7.55
B
Gr-1+CD11b- myeloid cells / bone marrow cells (%)
WT
4E-BP1,2-/-
4E-BP2-/-
4E-BP1-/-

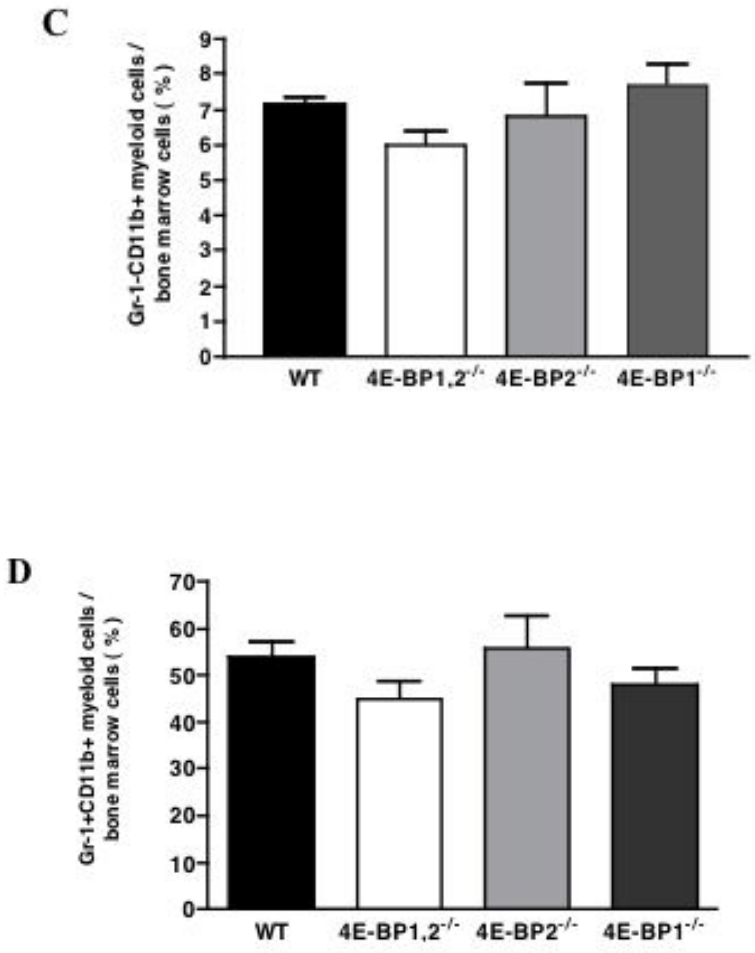


Fig.13. Unaffected myeloid cells differentiation in the bone marrow of 4E-BPsKO mice. (A) An example of bone marrow myeloid cells flow cytometry analysis of 4E-BPsKO and control mice. Numbers in quadrants (bottom) indicated percentages of CD11b+Gr-1- myeloid cells (upper left); CD11b+Gr-1+ myeloid cells (upper right); CD11b-Gr-1+ myeloid cells (lower right) in bone marrow cells. (B),(C),(D) Results (mean ± s.e.m) obtained from 4E-BPsKO mice (n = 5) and control mice (n = 5). (B) the percentage of Gr-1+CD11b- myeloid cells in the bone marrow of 4E-BPsKO and control mice. (C) the percentage of Gr-1-CD11b+ myeloid cells in the bone marrow of 4E-BPsKO and control mice. (D) the percentage of Gr-1+CD11b+ myeloid cells in the bone marrow of 4E-BPsKO and control mice.

4.1.6 Unaffected myeloid colony forming unit granulocyte/monocyte (CFU-GM) in the bone marrow of 4E-BPsKO mice

Next I performed colony forming assay to examine the functions of myeloid lineage progenitors. I found that CFU-GM (CFU-GM, CFU-G, CFU-M) frequencies in bone marrow of 4E-BPsKO mice were similar to those of control mice (Figure 14).

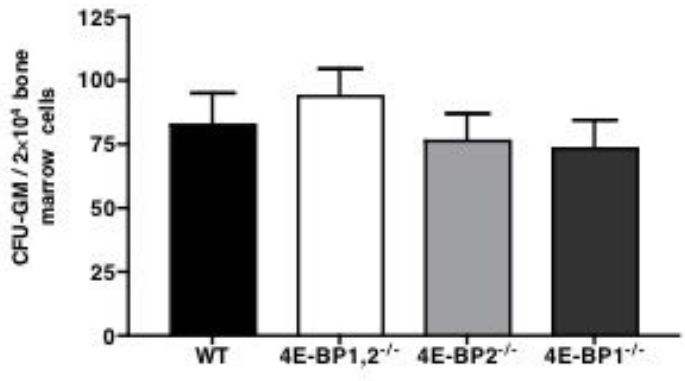


Fig.14. Unaffected CFU-GM frequencies in the bone marrow of 4E-BPsKO mice. The results of CFU-GM frequencies (mean ± s.e.m) in the bone marrow of 4E-BPKO and control mice obtained from three mice of each genotype (n = 3).

4.1.7 Erythropoiesis

The earliest erythroid-committed progenitor identified ex vivo -- the burst-forming erythroid unit (BFU-E). BFU-E cells divide and further differentiate into rapidly dividing erythroid colony-forming units (CFU-Es). Both of these 2 types of progenitors are identified by colony forming assays. BFU-E colonies take 15 days (human) or 7 to 10 days (mouse) to form in culture, whereas CFU-E colonies take 7 days (human) or 2 days (mouse). CFU-E progenitors undergo 3 to 5 divisions as they differentiate through proerythroblasts to erythroblasts that can be monitored by expression of the cell surface markers CD71 and TER119. Finally, the erythroblasts extrude their nuclei (enucleation) and become reticulocytes, which further expel all organelles and detach from their microenvironment to form mature circulating erythrocytes (Figure 15).

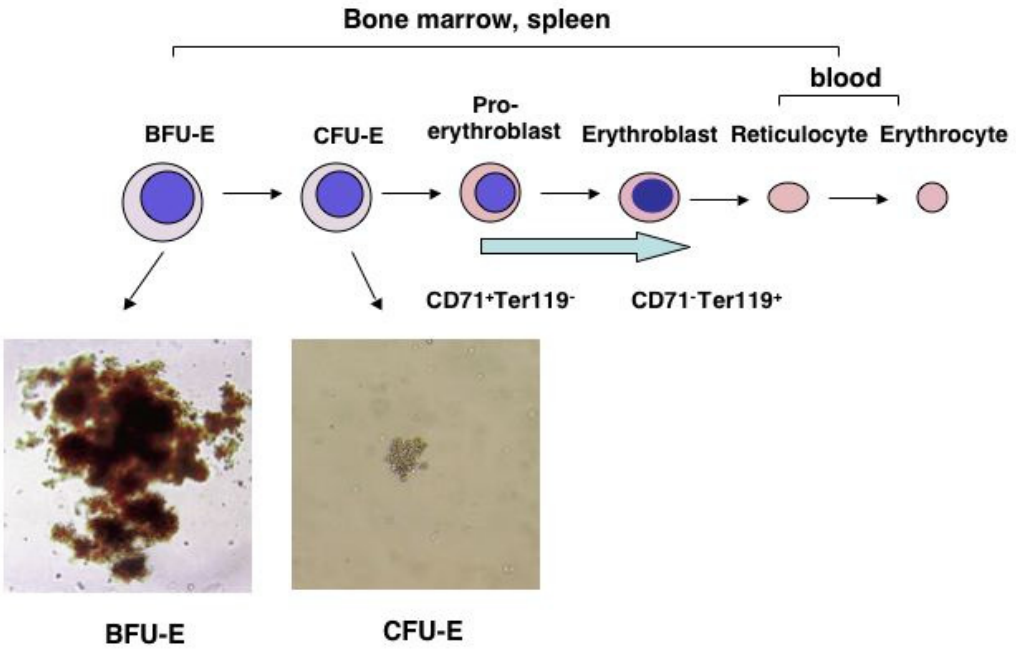
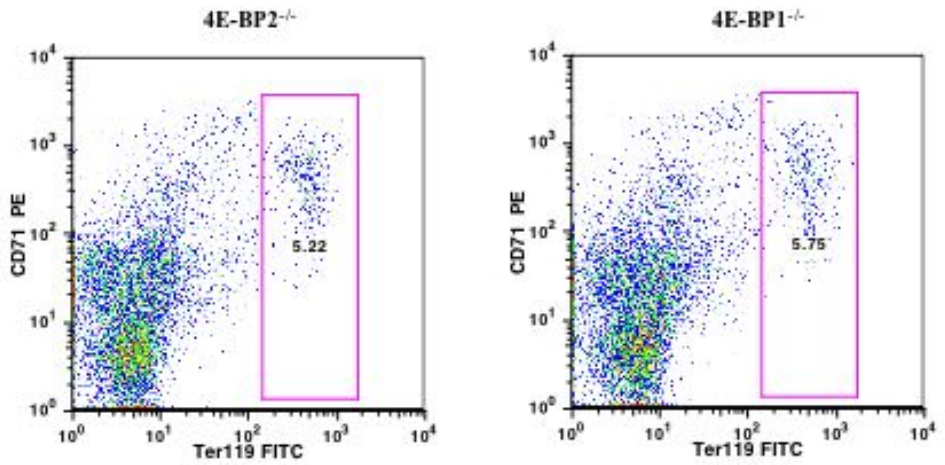


Fig.15. Adult Erythropoiesis. The earliest erythroid-committed progenitor identified ex vivo is the BFU-E. BFU-E cells divide and further differentiate into CFU-E (photos represent BFU-E and CFU-E forming in methylcellulose medium). CFU-E progenitors undergo 3 to 5 divisions as they differentiate through proerythroblasts to erythroblasts that can be monitored by expression of the cell surface markers CD71 and TER119. Finally, the erythroblasts extrude their nuclei (enucleation) and become reticulocytes, which further expel all organelles and detach from their microenvironment to form mature circulating erythrocytes.



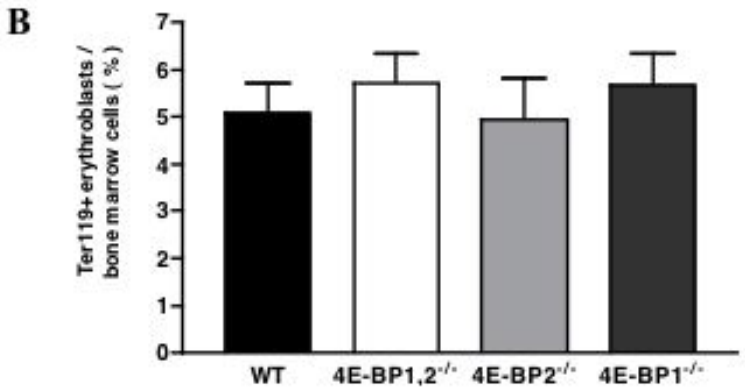


Fig.16. Unaffected erythroblasts in bone arrow of 4E-BPsKO. (A) An example of bone marrow erythroblasts flow cytometry analysis of 4E-BPsKO and control mice. Numbers in the boxed areas indicated percentages of gated Ter119 positive erythroblasts in bone marrow cells.(B) Results (mean ± s.e.m) of 4E-BPsKO and control mice obtained from 5 mice of each genotypes (n = 5).

4.1.8 Unaffected erythroblasts in spleen of 4E-BPsKO mice

Erythropoiesis occurs not only in bone marrow but also in spleen. Next I examined spleen erythroblasts monitored by CD71 and Ter119 with flow cytometry analysis and defined four populations (R1-R4) with specific staining characteristic: R1, pro-erythroblasts ($Ter119^{low}CD71^{hi}$); R2, early basophilic erythroblast ($Ter119^{hi}CD71^{hi}$); R3 and R4, late erythroblast ($Ter119^{hi}CD71^{med}$ and $Ter119^{hi}CD71^{low}$) (Socolovsky, Nam et al. 2001). I found that spleen erythroblasts differentiation was unaffected compared with control mice as well. (Table 4, Figure 17).

Tab. 4 Differentiation of spleen erythroblasts

	WT	4E-BP1,2$^{-/-}$	*P* value	4E-BP2$^{-/-}$	*P* value	4E-BP1$^{-/-}$	*P* value
	(n=5)	(n=5)		(n=5)		(n=4)	
R1	0,05±0,01	0,04±0,01	>0.05	0,04±0,01	>0.05	0,05±0,01	>0.05
R2	3,61±0,57	3,73±0,45	>0.05	3,88±0,90	>0.05	3,62±0,61	>0.05
R3	6,17±0,54	6,06±0,17	>0.05	6,36±0,53	>0.05	6,80±0,85	>0.05
R4	25,78±3,41	25,78±3,13	>0.05	26,96±2,91	>0.05	23,50±1,67	>0.05

P value represented Student's test of erythroblasts frequencies in splenocytes of 4E-BPsKO mice compared to control mic

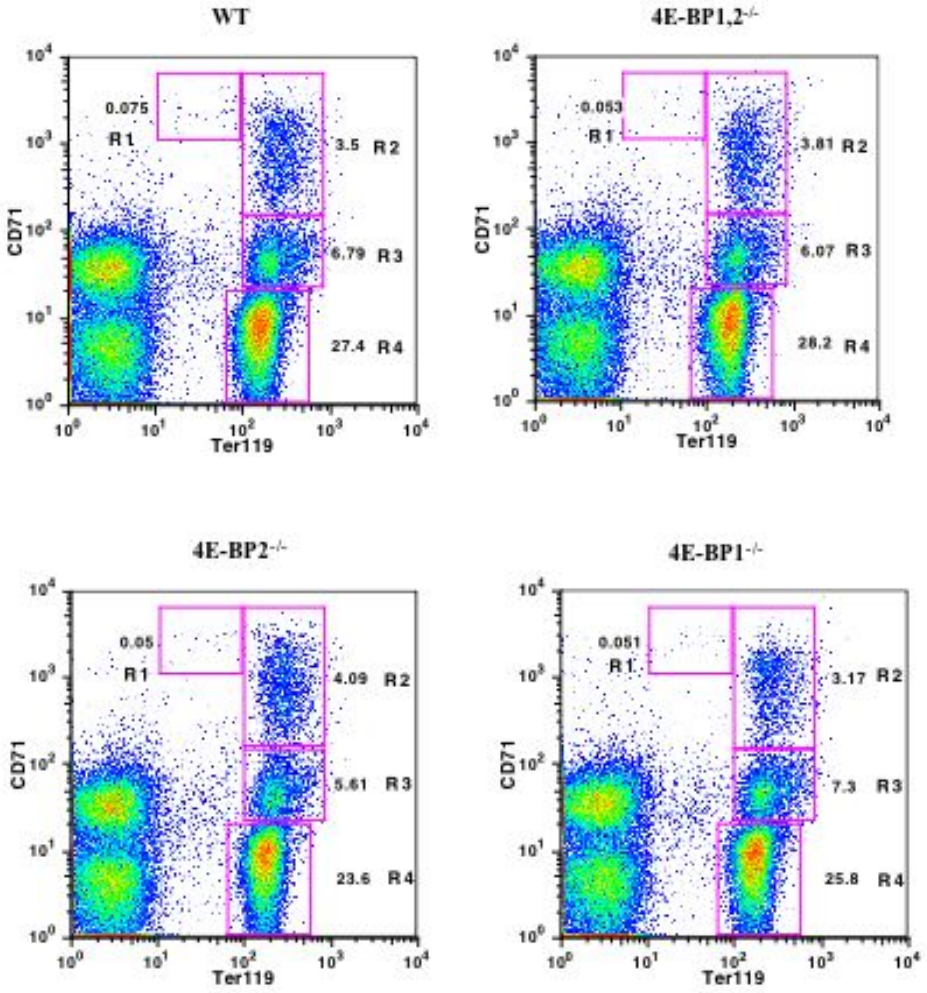


Fig.17. Unaffected spleen erythroblasts in 4E-BPsKO mice. This figure is an example of spleen erythroblasts flow cytometry of 4E-BPsKO and control mice. Numbers in the boxed areas indicated percentages of gated cells in each region (R1-R4).

4.1.9 Unaffected frequencies of erythroid progenitors CFU-E and BFU-E in the bone marrow and spleen of 4E-BPsKO mice

Next I examined the function of erythroid progenitors in the bone marrow of 4E-BPsKO mice. I found that erythroid committed progenitors CFU-E and BFU-E frequencies in 4E-BPsKO mice were similar to those of control mice (Figure 18,19).

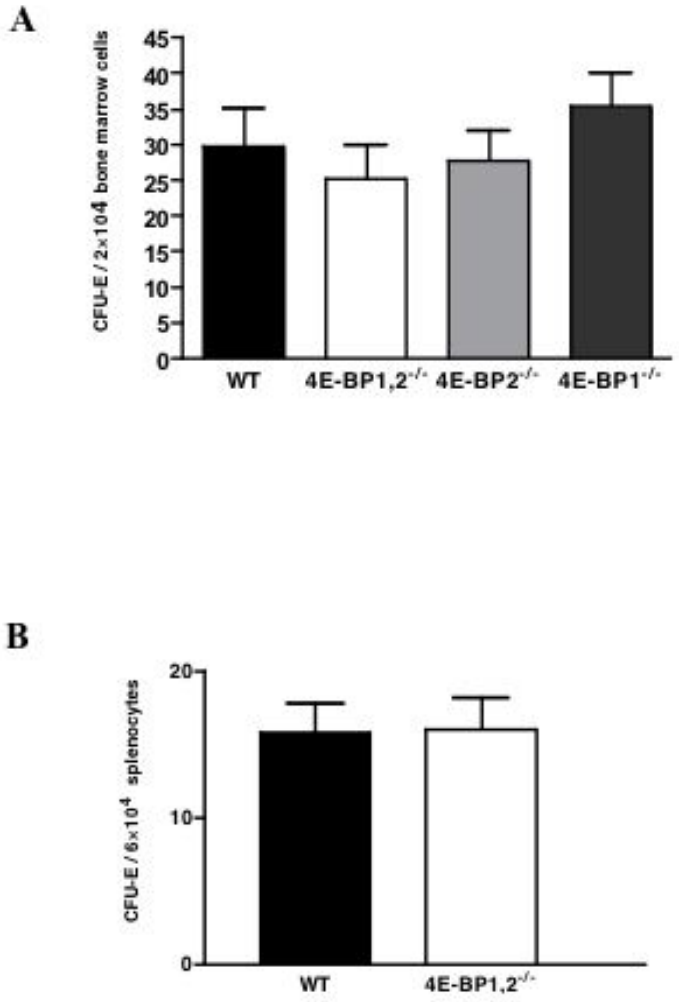
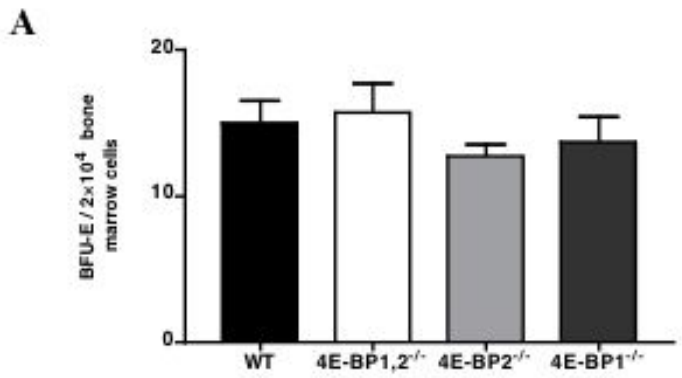


Fig.18. Unaffected frequencies of erythroid progenitors CFU-E in 4E-BPsKO mice.(A) The results of CFU-E frequencies (mean ± s.e.m) in bone marrow of 4E-BPsKOand control mice obtained from three mice of each genotype (n = 3). (B) The results of CFU-E frequencies (mean ± s.e.m) in the spleens of 4E-BP1,2$^{-/-}$ and control mice obtained from five mice of each genotype (n = 5).



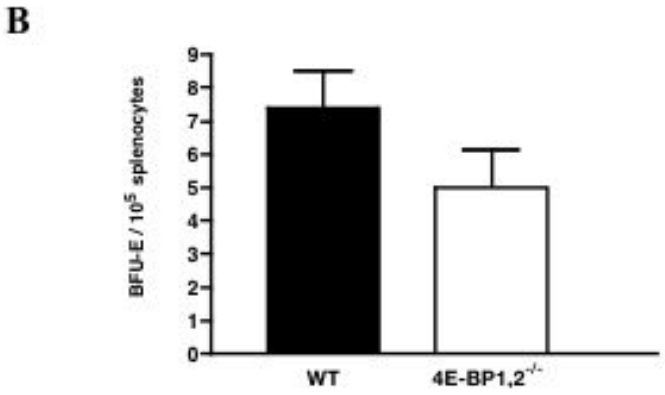


Fig.19. Unaffected frequencies of erythroid progenitors BFU-E in 4E-BPsKO mice. (A) The results of BFU-E frequencies (mean ± s.e.m) in the bone marrow of 4E-BPsKO and control mice obtained from three mice of each genotype (n = 3). (B) The results of BFU-E frequencies (mean ± s.e.m) in the spleens of 4E-BP1,2$^{-/-}$ and control mice obtained from five mice of each genotype (n = 5).

The results of peripheral blood analysis, flow cytometry analysis of bone marrow, spleen restricted lineages precursors and colony forming assay of bone marrow, spleen progenitors suggested that disruption of 4E-BPs did not affect hematopoiesis.

4E-BPs have been suggested to be involved in stress response. Although disruption of 4E-BPs did not affect hematopoiesis, especially erythropoiesis, 4E-BPs might be involved in stress erythropoiesis. 90% of adult murine erythroid progenitors are in the bone marrow and 10% in the spleen. However, during erythropoietic stress, erythropoiesis occurs mainly in the spleen (Richmond, Chohan et al. 2005; Ney 2006).

4.2 Upregulation of 4E-BP1, 4E-BP2 expression and 4E-BP1 phosphorylation in spleen erythroblasts in response to PHZ treatment

Phenylhydrazine (PHZ) induced erythropoietic stress is used to study the stress erythropoiesis. Therefore, I challenged the mice with PHZ that caused acute hemolytic anemia. In response to the challenge, erythroid progenitors expansion would occur in spleen and the expansion peaks at 36 to 48 hours after anemia induction.

First I examined whether and when PHZ treatment affected 4E-BP1, 4E-BP2 protein expression. I found that 1 hour after PHZ treatment, 4E-BP1, 4E-BP2 were upregulated in erythroblasts. At 3-hour, 6-hour and 9-hour, the upregulation was continued (Figure 20A). The phosphorylation of 4E-BP1 was upregulated 3 hours after PHZ treatment as well. I further examined 4E-BP1 and 4E-BP2 expressions in 4E-BPs mutants. I found that expression of 4E-

BP1 in 4E-BP2$^{-/-}$ erythroblasts and 4E-BP2 in 4E-BP1$^{-/-}$ erythroblasts were upregulated in response to PHZ treatment. However, the expression level of 4E-BP1 in 4E-BP2$^{-/-}$ erythroblasts and that of 4E-BP2 in 4E-BP1$^{-/-}$ erythroblasts were similar to those of control erythroblasts in both normal and stress erythropoiesis (Figure 20B).

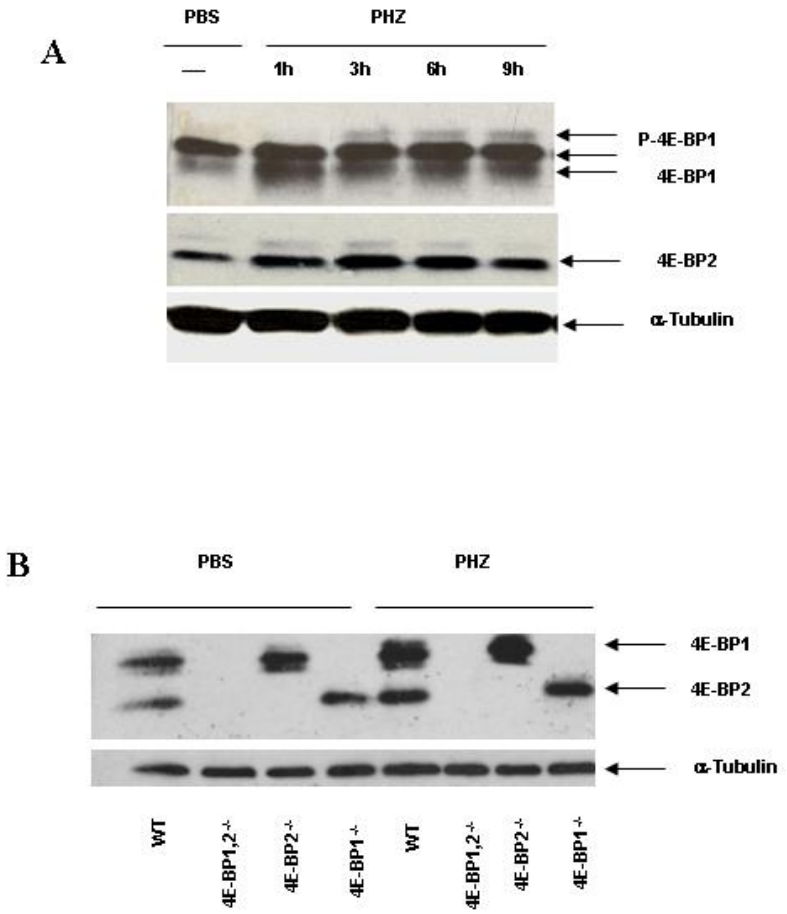


Fig.20. Upregulation of 4E-BP1, 4E-BP2 expression and 4E-BP1 phosphorylation in spleen erythroblasts in response to PHZ treatment.(A) Expression of 4E-BP1, 4E-BP2 and 4E-BP1 phosphorylation in response to PHZ treatment in time-dependent manner in control erythroblasts. γ indicated the unphosphorylated 4E-BP1; α,β were the phosphorylated 4E-BP1. α-Tubulin was the loading control. The erythroblasts from 3 to 5 wild-type mice were pooled for each time point. (B) Expression of 4E-BP1 and 4E-BP2 in 4E-BPsKO and control erythroblasts in both normal erythropoiesis and stress erythropoiesis. α-Tubulin was the loading control. Data obtained from two independent experiments. The erythroblasts from 3 to 5 mice were pooled for each genotype in each experiment.

4.3 Activation of kinase mTOR in spleen erythroblasts in response to PHZ treatment

MTOR signaling pathway regulates translation through 4E-BPs and S6K. I examined whether and when mTOR signaling pathway was activated by PHZ treatment. I found that 1 hour after PHZ treatment, mTOR phosphorylation was upregulated in erythroblasts (Figure 21), which upregulated 4E-BP1 phosphorylation (Figure 20).

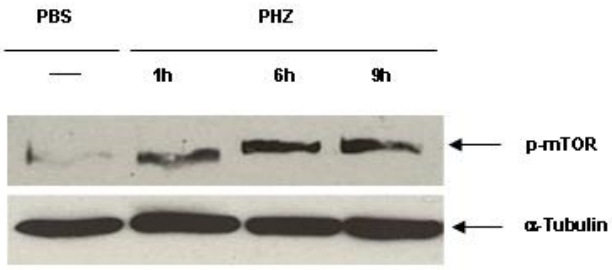


Fig.21. Activation of mTOR in spleen erythroblasts in response to PHZ treatment. Kinetics of mTOR phosphorylation in response to PHZ treatment in control spleen erythroblasts. α-Tubulin was the loading control. The erythroblasts from 3 to 5 wild-type mice were pooled for each time point.

These results indicated the translation regulatory machinery mTOR-4E-BPs was activated in response to PHZ induced erythropoietic stress.

4.4 Reduction of proliferation rate of 4E-BP1,2$^{-/-}$ erythroblasts ex vivo

To address whether 4E-BPs were crucial for erythroblast proliferation, I cultivated erythroblasts obtained from the bone marrow of 4E-BP1,2$^{-/-}$ and control mice in serum- free medium plus cytokines including stem cell factor (SCF), human recombinant erythropoietin (hEPO), insulin-like growth factor (IGF-1) and dexamethasone (DEX) to examine their proliferation rate under erythropoietic stress like condition (see method " cultivation of mouse erythroblasts"). I found that erythroblasts of 4E-BP1,2$^{-/-}$ proliferated 12.3 folds from days 4 to 7, whereas erythroblasts of control proliferated 15,5 folds (Figure 22). This result indicated that 4E-BP1 and 4E-BP2 were required for erythroblasts proliferation ex vivo.

4.5 Disrupted erythropoietic stress response of 4E-BP1,2$^{-/-}$ mice

4.5.1 Reduction of reticulocyte percentages in peripheral blood of 4E-BP1,2$^{-/-}$ mice 48 hours after PHZ treatment

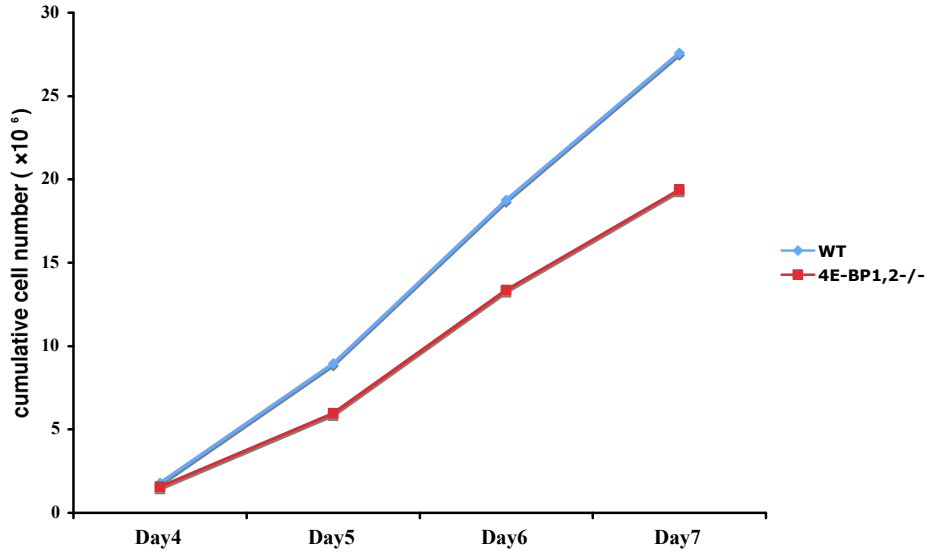


Fig.22. Reduction of proliferation rate of 4E-BP1,2 $^{-/-}$ erythroblasts ex vivo. Erythroblasts of 4E-BP1,2$^{-/-}$ and control mice were cultivated in serum-free medium supplemented with cytokines SCF, hEPO, IGF-1and DEX. Cells were counted daily from days 4 to 7 and cumulative cell numbers were determined. Data was one experiment of two independent experiments.

population is used to assess erythopoietic rate. I found that 48 hours after PHZ treatment, the percentages of reticulocytes in blood of both mutant and control mice were increased compared with normal situation (2%-3%). However, the percentage of reticulocytes of 4E-BP1,2$^{-/-}$ mice were lower: 4E-BP1,2$^{-/-}$, 27.86% ± 1.28%, versus control , 40.69% ± 3.42% ($P < 0.05$) (Figure 23). This result indicated that 4E-BP1 and 4E-BP2 were required for the proliferation of reticulocytes in stress erythropoiesis.

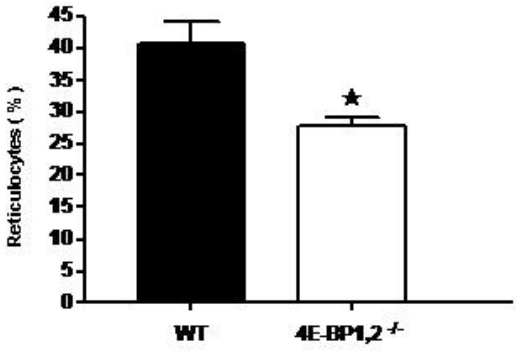
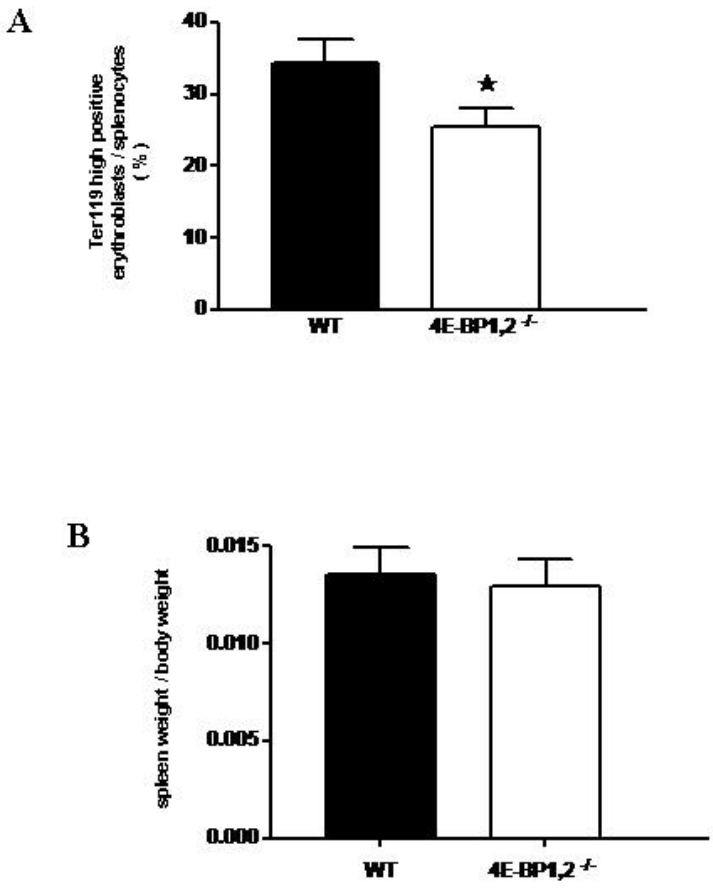


Fig.23. Reduction of reticulocyte percentages in peripheral blood of 4E-BP1,2$^{-/-}$ mice 48 hours after PHZ treatment. Results of reticulocyte percentages (mean ± s.e.m) in peripheral blood were from 4E-BP1,2$^{-/-}$ mice (n = 15) and control mice (n = 9). Data obtained from the pool of 4 independent

4.5.2 Reduction of spleen Ter119 ^{hi} erythroblasts percentages of 4E-BP1,2$^{-/-}$ mice 48 hours after PHZ treatment

I further quantitatively assessed erythroblasts in spleen using flow cytometry assay. The erythroblasts were stained with the transferrin receptor CD71 and erythroid specific cell surface antigen Ter119. I found that Ter119 high expression erythroblasts in 4E-BP1,2$^{-/-}$ were less than in control mice: 4E-BP1,2$^{-/-}$, 25.38% ± 2.73% versus control, 34.41%± 3.16%, ($P < 0.05$). No difference in size of spleen was observed between 4E-BP1,2$^{-/-}$ and control mice. Weight ratio of spleen to body of 4E-BP1,2$^{-/-}$, 0.0129 ± 0.0014, versus control, 0.0136 ± 0.0013 ($P > 0.05$) (Figure 24). These results indicated that loss of 4E-BP1 and 4E-BP2 inhibited the proliferation of erythroblasts in response to erythropoietic stress.



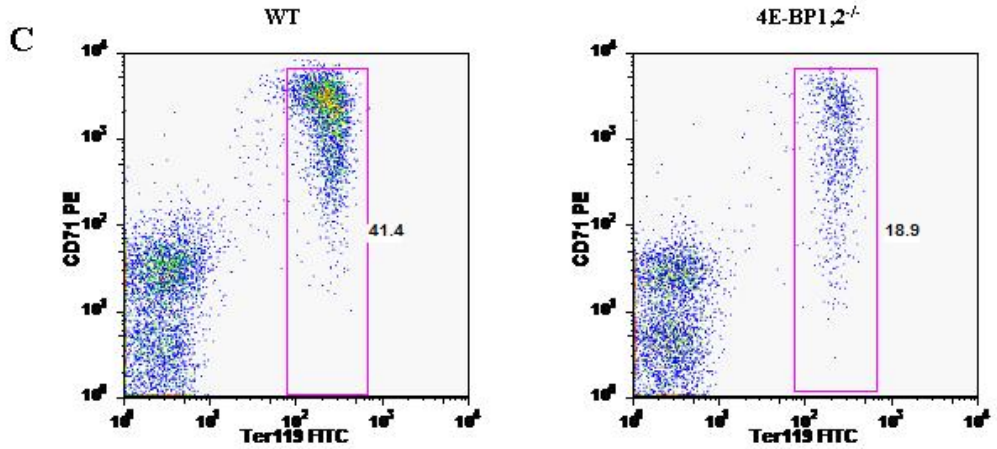


Fig.24. Reduction of spleen Ter119 ^{hi} erythroblasts percentages of 4E-BP1,2$^{-/-}$ mice 48 hours after PHZ treatment. (A) Erythroblasts results (mean ± s.e.m) were from 4E-BP1,2$^{-/-}$ mice (n = 12) and control mice (n = 9). Data obtained from the pool of 5 independent experiments. (B) Weight ratio of spleen to body of 4E-BP1,2$^{-/-}$ and control mice was similar at same time point. Results (mean ± s.e.m) were from 4E-BP1,2$^{-/-}$ mice (n = 9) and control mice (n = 8). Data obtained from the pool of 2 independent experiments. (C) An example of spleen erythroblasts flow cytometry assay of 4E-BP1,2$^{-/-}$ and control mice. Numbers in the boxed area indicated the percentages of Ter119 ^{hi} erythroblasts in splenocytes.

4.5.3 Reduction of spleen CFU-E frequencies of 4E-BP1,2$^{-/-}$ mice 48 hours after PHZ treatment

I further examined whether spleen erythroid progenitors were affected by loss of 4E-BPs in response to erythropoietic stress using colony forming assay. I found that 48 hours after PHZ treatment, the CFU-E frequency of 4E-BP1,2$^{-/-}$ was lower than that of control: 4E-BP1,2$^{-/-}$, 176.80± 31.09 , versus control, 391.70 ± 49.58 ($P < 0.05$). The BFU-E frequency of mutant was similar as that of the control: 4E-BP1,2$^{-/-}$, 18.8 ± 1.11, versus control, 19,89 ± 1.38 ($P > 0.05$) (Figure 25). These results indicated that 4E-BP1 and 4E-BP2 were required for the proliferation of erythroid progenitors CFU-E, but not BFU-E, in response to erythropoietic stress.

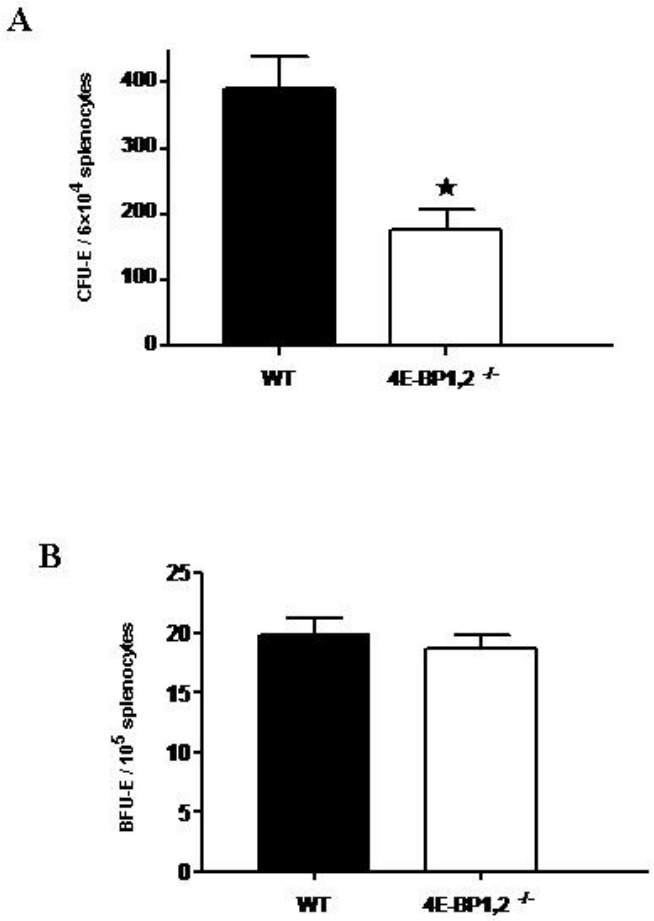


Fig.25. Reduction of spleen CFU-E frequencies in 4E-BP1,2$^{-/-}$ mice 48 hours after PHZ treatment. (A) The results of CFU-E frequencies (mean ± s.e.m) were from 4E-BP1,2$^{-/-}$ mice (n = 6) and control mice (n = 9). Data obtained from the pool of 6 independent experiments. (B) Unaffected spleen BFU-E of 4E-BP1,2$^{-/-}$ mice 48 hours after PHZ treatment. Results (mean ± s.e.m) were from 4E-BP1,2$^{-/-}$ mice (n = 5) and control mice (n = 9). Data obtained from the pool of 4 independent experiments.

Taken together, these results indicated loss of 4E-BPs led to inefficient proliferation of erythroid progenitor CFU-E and erythroblasts ex vivo and in vivo, suggesting 4E-BPs were required for stress erythropoiesis. 4E-BP1 and 4E-BP2 are equally expressed in splenocytes. I induced hemolytic anemia in 4E-BP1$^{-/-}$ and 4E-BP2$^{-/-}$ mice using the same method to explore the individual function of 4E-BPs in stress erythropoiesis.

4.6 Disrupted erythropoietic stress response of 4E-BP2$^{-/-}$ mice

4.6.1 Reduction of proliferation rate of 4E-BP2$^{-/-}$ erythroblasts ex vivo, but not of 4E-BP1$^{-/-}$ erythroblasts

Our ex vivo data showed that 4E-BP2$^{-/-}$ erythroblasts proliferated 12.8 folds from days 4 to 7, which was lower than that of control erythroblasts (15,5 folds), whereas 4E-BP1$^{-/-}$ erythroblasts proliferated 15.5 folds showing the same proliferation rate as that of control

erythroblasts (Figure 26). This result indicated that 4E-BP2 was required for the proliferation of erythroblasts ex vivo.

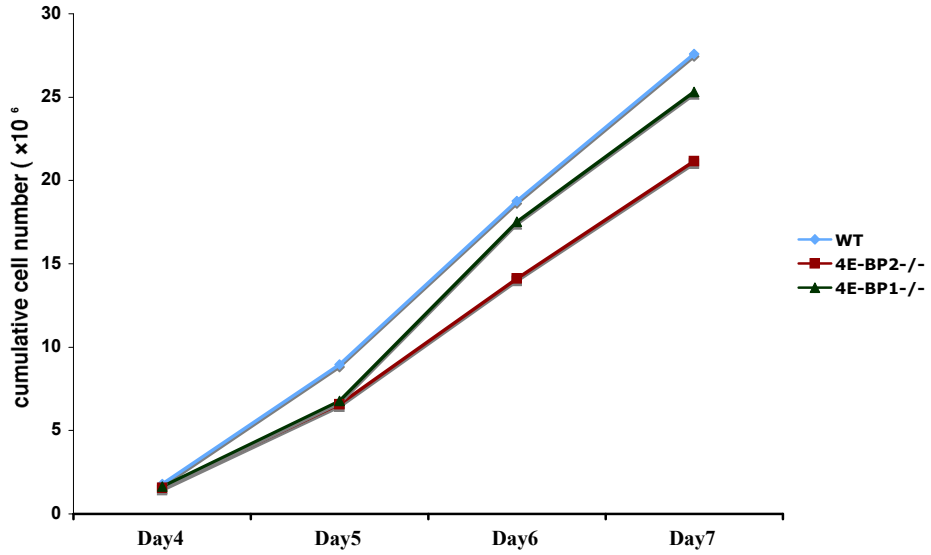


Fig.26. Reduction of proliferation rate of 4E-BP2$^{-/-}$erythroblasts, but not of 4E-BP1$^{-/-}$ erythroblasts. Erythroblasts of 4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$ and control mice were cultivated in serum-free medium supplemented with cytokines SCF, hEPO, IGF-1 and DEX. Cells were counted daily from day 4 to 7 and cumulative cell numbers were determined. Data was one experiment of two independent experiments.

4.6.2 Reduction of reticulocyte percentages in peripheral blood of 4E-BP2$^{-/-}$ mice 48 hours after PHZ treatment

Next I investigated the erythropoietic stress response of 4E-BP1$^{-/-}$ and 4E-BP2$^{-/-}$ mice in vivo. First I found that 48 hours after PHZ treatment, the reticulocyte percentages in the blood of 4E-BP2$^{-/-}$ mice were lower than those of control mice. In contrast, the reticulocyte percentages in the blood of 4E-BP1$^{-/-}$ mice were similar to those of control mice: 4E-BP2$^{-/-}$, 26.53% ± 1,77% ($P < 0.05$), 4E-BP1$^{-/-}$, 37.07% ± 3,29% ($P > 0.05$) , versus control , 40.69% ± 3.42% (Figure 27). This result indicated that 4E-BP2 was required for the proliferation of reticulocytes in response to erythropoietic stress.

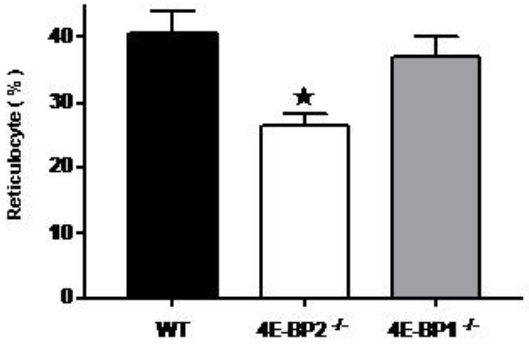
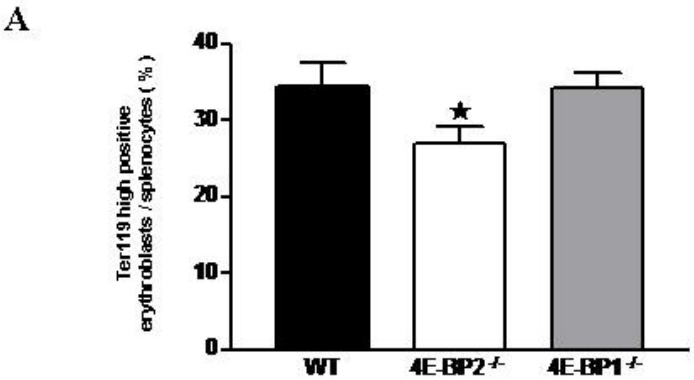


Fig.27. Reduction of reticulocyte percentages in 4E-BP2$^{-/-}$ peripheral blood, but not in 4E-BP1$^{-/-}$ mice 48 hours after PHZ treatment. The results of reticulocyte percentages (mean ± s.e.m) were from 4E-BP2$^{-/-}$ (n = 12), 4E-BP1$^{-/-}$ (n = 9) and control mice (n = 9). Data obtained from the pool of 4 independent experiments.

4.6.3 Reduction of spleen Ter119 ^{hi} erythroblasts percentages of 4E-BP2$^{-/-}$ mice 48 hours after PHZ treatment

Next I examined the spleen erythroblasts of 4E-BP1$^{-/-}$ and 4E-BP2$^{-/-}$ mice. We found that Ter119 high expression erythroblasts in 4E-BP2$^{-/-}$ mice were less than those in control: 4E-BP2$^{-/-}$, 26.99% ± 2.24% ($P < 0.05$), versus control, 34.41% ± 3.16% . There was no significant difference between 4E-BP1$^{-/-}$ and control mice. 4E-BP1$^{-/-}$, 34.29% ± 2.02% ($P > 0.05$). Weight ratio of spleen weight to body of 4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$ and control mice did not show significant difference. 4E-BP2$^{-/-}$, 0.0127 ± 0.0013 ($P > 0.05$), 4E-BP1$^{-/-}$, 0.0136 ± 0.0011 ($P > 0.05$), versus control, 0.0136 ± 0.0013 (Figure 28). These results indicated that loss of 4E-BP2 inhibited the proliferation of erythroblasts in stress erythropoiesis.



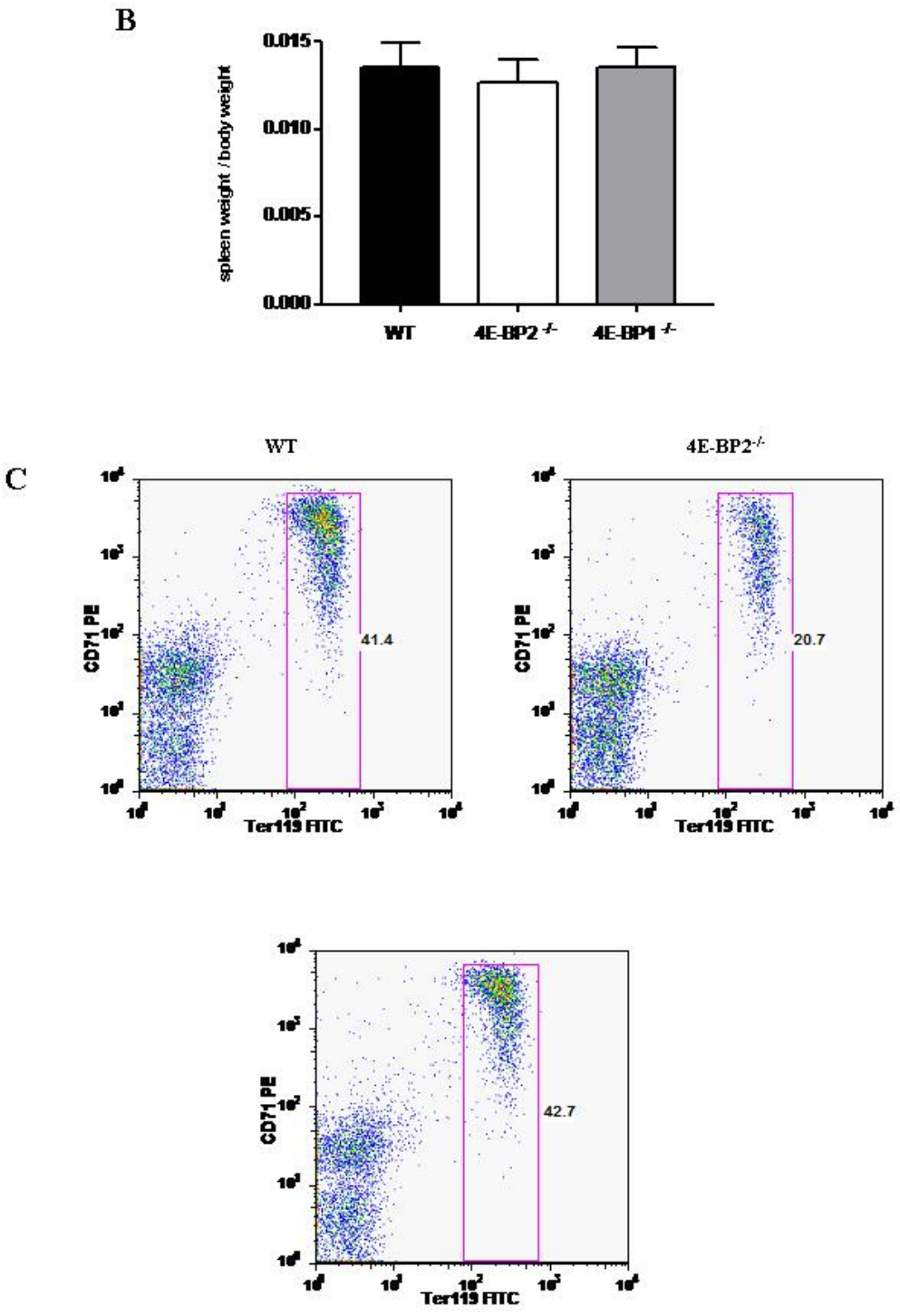


Fig.28. Reduced percentages of spleen Ter119 ^{hi} erythroblasts of 4E-BP2$^{-/-}$ mice, but not of 4E-BP1$^{-/-}$ mice, 48 hours after PHZ treatment. (A) The results of Ter119 high positive erythroblasts (mean ± s.e.m) were from 4E-BP2$^{-/-}$ (n = 14), 4E-BP1$^{-/-}$ (n = 14) and control mice (n = 9). Data obtained from the pool of 5 independent experiments. (B) Similar weight ratio of spleen to body of 4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$ and control mice at same time point. Results (mean ± s.e.m) were from 4E-BP2$^{-/-}$ (n = 9), 4E-BP1$^{-/-}$ (n = 9) and control mice (n = 8). Data obtained from the pool of 2 independent experiments. (C) An example of spleen erythroblasts flow cytometry assay of 4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$ and control mice. Numbers in the boxed area indicated the percentages of Ter119^{hi} erythroblasts in splenocytes.

4.6.4 Reduction of spleen CFU-E frequencies of 4E-BP2$^{-/-}$ mice 48 hours after PHZ treatment

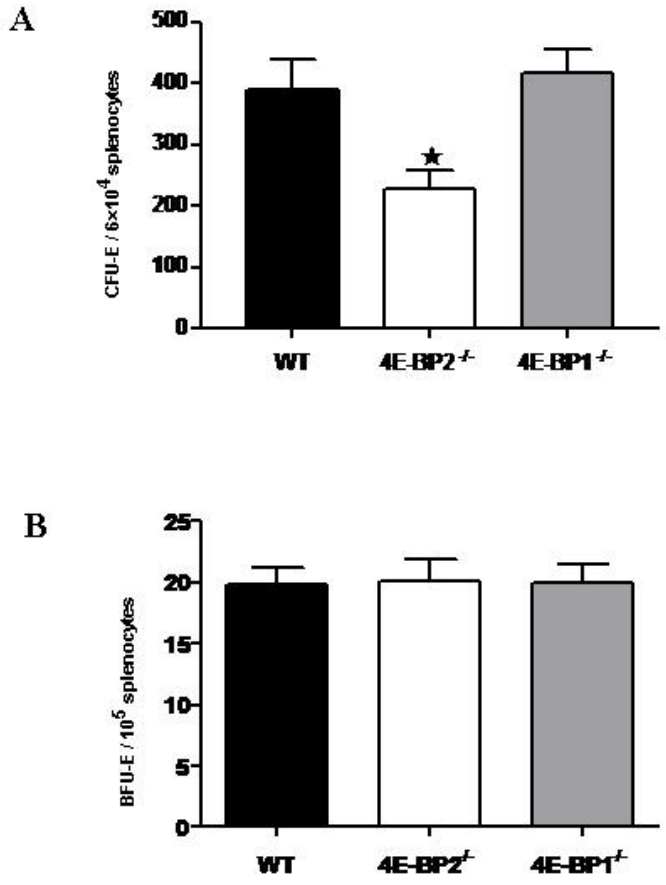


Fig.29. Reduction of spleen CFU-E frequencies of 4E-BP2$^{-/-}$ mice 48 hours after PHZ treatment. (A) The results of CFU-E frequencies (mean ± s.e.m) were from 4E-BP1$^{-/-}$ (n = 5), 4E-BP2$^{-/-}$ (n = 5) and control mice (n = 9). Data obtained from the pool of 2 independent experiments. (B) Spleen BFU-E frequencies of 4E-BP1$^{-/-}$ and 4E-BP2$^{-/-}$ mice were unaffected 48 hours after PHZ treatment. The results of BFU-E frequencies (mean ± s.e.m) were from 4E-BP1$^{-/-}$ (n = 5), 4E-BP2$^{-/-}$ (n = 5) and control mice (n = 9). Data obtained from the pool of 2 independent experiments.

Taken together, these results indicated that loss of 4E-BP2 led to inefficient erythropoiesis which was analogous to 4E-BP1,2$^{-/-}$. Remaining 4E-BP1 did not rescue the delayed response to erythropoietic stress of 4E-BP2$^{-/-}$ mice. However, 4E-BP1$^{-/-}$ mice showed normal response to erythropoietic stress, suggesting 4E-BP2 was required for stress erythropoiesis.

4.7 Downregulated protein expression of GATA-1 in 4E-BP1,2$^{-/-}$ and 4E-BP2$^{-/-}$ spleen erythroblasts 48 hours after PHZ treatment

4E-BP1,2$^{-/-}$ and 4E-BP2$^{-/-}$ mice showed proliferation delay at late stage of erythroid lineage in response to erythropoietic stress. GATA-1 is the central transcription factor of erythroid

proliferation and terminal differentiation and two GATA-1 proteins are detected in human K562 and mouse MEL erythroid cell lines resulting from alternative translation initiation site usage (Calligaris, Bottardi et al. 1995). I examined GATA-1 expression in spleen erythroblasts under erythropoietic stress. Attenuated GATA-1 expression was found in 4E-BP1,2$^{-/-}$ and 4E-BP2$^{-/-}$ erythroblasts. The attenuation was at the protein level, as the mRNA level was unaffected. These results suggested that loss of 4E-BP2 led to deregulation of GATA-1 expression at protein level, indicated that the translation control mechanism is involved in the regulation of GATA-1 expression (Figure 30).

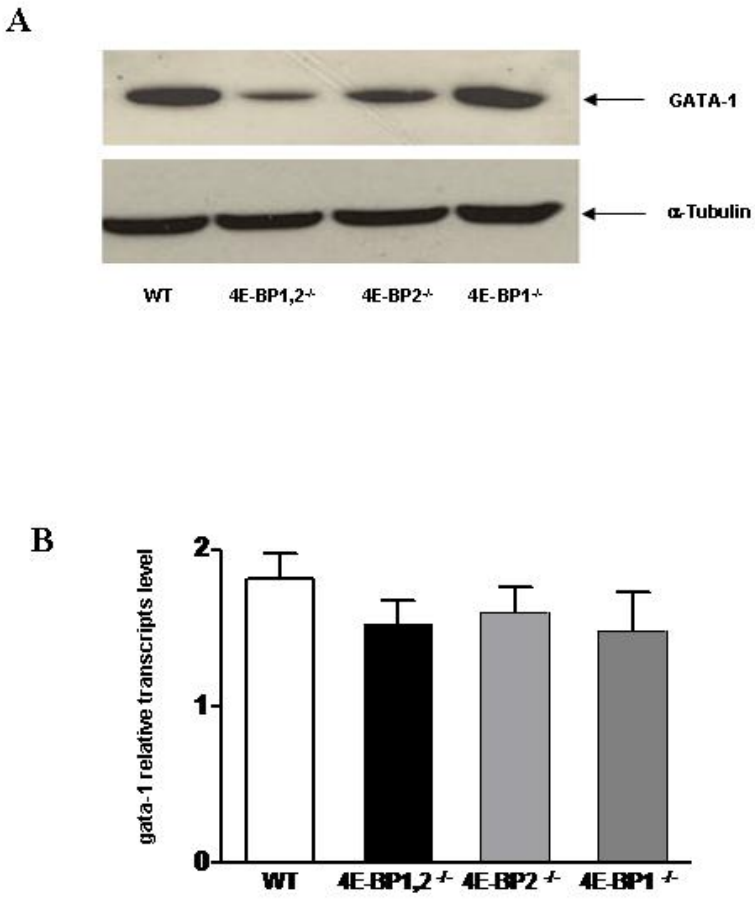


Fig.30. Deregulated protein expression of GATA-1 in 4E-BP1,2$^{-/-}$ and 4E-BP2$^{-/-}$ spleen erythroblasts 48 hours after PHZ treatment. (A) GATA-1 expression in 4E-BPsKO and control spleen erythrobasts under erythropoietic stress detected by Western Blot analysis. α-Tubulin was loading control. The erythroblasts from 3 to 5 mice were pooled for each genotype in each experiment. (B) *gata-1* transcripts level in 4E-BPsKO and control spleen erythroblasts under erythropoietic stress detected by real-time PCR. Values were relative to GAPDH transcripts. Results were from 5 mice for each genotype.

4.8 Activation of MNK1 and upregulation of eIF4E phosphorylation 48 hours after PHZ treatment

The activity of eIF4E is regulated by 4E-BPs and phosphorylation. Deletion of 4E-BPs will increase the availability of translation factor eIF4E, which is then amenable phosphorylation. I examined the phosphorylation state of eIF4E. I found that eIF4E phosphorylation was enhanced in 4E-BPsKO erythroblasts in both normal erythropoiesis and stress erythropoiesis (Figure 31A). MNK is the only known eIF4E kinase in mammals. I examined its activation. I found that MNK1 phosphorylation was enhanced 1 hour after PHZ treatment. Until 9 hours, the phosphorylation was further enhanced gradually. These results implied that the activation of the MAP kinase signaling pathway upregulated eIF4E phosphorylation in PHZ induced erythropoietic stress (Figure 31B).

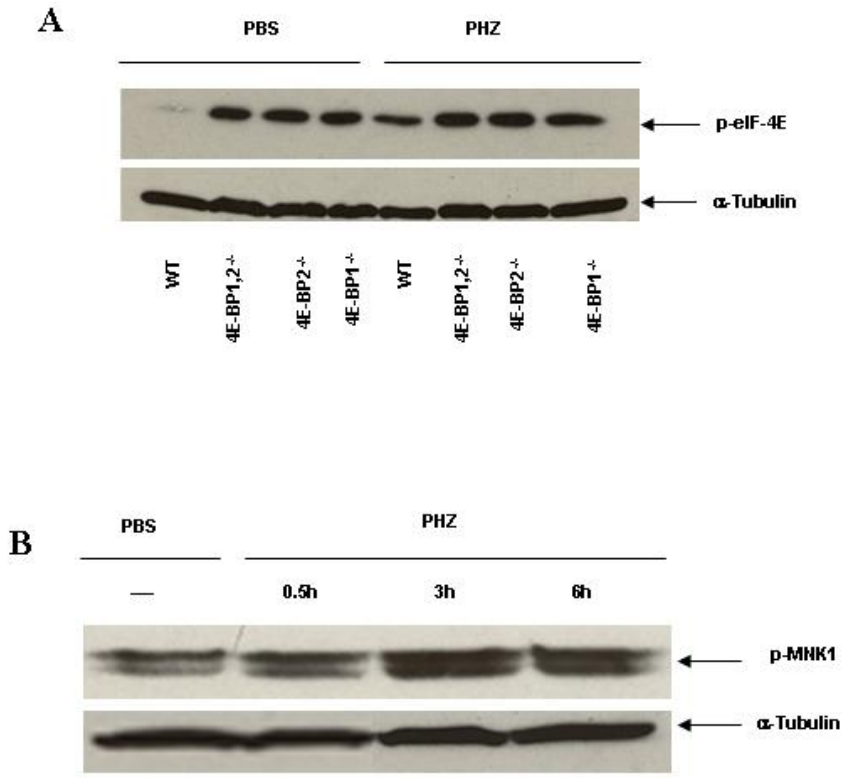


Fig.31. Activation of MNK1 and upregulation of eIF4E phosphorylation 48 hours after PHZ treatment.(A) eIF-4E phosphorylation in 4E-BPsKO and control erythroblasts in normal erythropoiesis and stress erythropoiesis. α-Tubulin was used as loading control. Data obtained from three independent experiments. The erythroblasts from 3 to 5 mice were pooled for each genotype in each experiment. (B) Kinetics of MNK1 phosphorylation in response to PHZ treatment in control erythroblasts. α-Tubulin was used as loading control. The erythroblasts from 3 to 5 wild-type mice were pooled for each time point.

5 Discussion and Outlook

I used 4E-BP1 knock out, 4E-BP2 knock out and 4E-BP1,2 compound knock out mice to investigate the functions of 4E-BPs in hematopoiesis and stress erythropoiesis. I found that disruption of 4E-BP1 and 4E-BP2 did not affect hematopoisis. However, the response to phenylhydrazine induced erythropoietic stress of 4E-BP1,2 compound and 4E-BP2 knock out mice was delayed. Thus, 4E-BP2 is required for stress erythropoiesis, although 4E-BP1 and 4E-BP2 are both predominantly expressed in the hematopoietic system. Our data not only addressed that 4E-BPs related translation regulation machinery were involved in the response to erythropoietic stress, but also indicates functional difference between 4E-BP1 and 4E-BP2.

The role of 4E-BPs as the mediators of stress response has been investigated in vivo. 4E-BP1,2 compound knock out mice and d4E-BP null Drosophila show deficient adipogenesis in response to adipogenetic stress (Le Bacquer, Petroulakis et al. 2007) (Teleman, Chen et al. 2005). 4E-BP2 is predominantly expressed in brain. 4E-BP2 knock out mice display impaired spatial learning, memory function (Banko, Poulin et al. 2005). I did not find hematopoiesis defect in 4E-BPs knock out mice. However, I observed that the expression levels of 4E-BP1 and 4E-BP2 were upregulated rapidly starting from one hour after phenylhydrazine treatment. MTOR, the upstream regulator of 4E-BPs, was activated as well. Under stress situations, for example, hypoxia, nutrition starvation and low energy, mTOR integrates various signaling pathways to regulate gene expression. Our results indicated that mTOR-4E-BPs signaling pathway was involved in the response to phenylhydrazine induced acute hemolytic anemia. The response to phenylhydrazine induced hemolytic anemia requires rapid proliferation of late erythroid progenitors that is mediated by the glucocorticoid receptor (Wessely, Deiner et al. 1997) (Bauer, Tronche et al. 1999). I found that 4E-BP1,2 compound knock out mice showed reduced erythroid progenitors (CFU-E) and precursors (erythroblasts) in spleen as well as reduced peripheral blood reticulocytes proportion in response to phenylhydrazine induced erythropoietic stress. Reduced proliferation rate of erythroblasts, which were cultivated in erythropoietic stress analogue medium in vitro, was observed as well. I observed mild stress erythropoiesis defect. The reason could be that the effect of 4E-BP3 could not be absolutely excluded although 4E-BP1 and 4E-BP2 were the dominant 4E-BP proteins in hematopoietic sytem (Tsukiyama-Kohara, Poulin et al. 2001). It might play compensatory role in hemolytic anemia. A 4E-BP2 and 4E-BP3 compound knock out mice might exhibit a more severe phenotype. On the other hand, the alternate factors that are not known could partly compensate for the functions of 4E-BPs in erythropoiesis. These data implied that 4E-BPs play a role in the proliferation of erythroid lineage cells under stress situation in vivo.

Although 4E-BP1 and 4E-BP2 share the same conserved 15 amino acids eIF4E interaction motif and 56% identity of amino acids (Tsukiyama-Kohara, Vidal et al. 1996), the tissue expression discrepancy implies their functional difference (Tsukiyama-Kohara, Poulin et al. 2001). To assess the contribution of 4E-BP1 and 4E-BP2 in stress erythropoiesis, I used the same method to challenge the 4E-BP1 knock out mice and 4E-BP2 knock out mice. I found that 4E-BP2 knock out mice exhibited similar defect of stress erythropoiesis in vivo and lower erythroblasts proliferation rate in vitro as those of 4E-BP1,2 compound knock out mice. However the response of 4E-BP1 knock out mice to erythropoietic stress and their erythroblast proliferation rate in vitro were similar as those of control mice. The reason could be that 4E-BP1 has been suggested to work as a metabolic brake that is activated to control fat metabolism in stress adipogenesis. Fat, glucose metabolic and oxidative stress resistance defect of 4E-BP null flies and 4E-BP1,2 compound knock out mice imply 4E-BP's "energy brake" function. Based on our results, 4E-BP2 is the major regulator in the hemolytic anemia, thus the existence of 4E-BP2 in 4E-BP1 knock out mice let them to respond to erythropoietic stress properly. These results suggested 4E-BP2 was required for stress erythropoiesis and implied that in addition to a general translation regulation, 4E-BP1 or 4E-BP2 could confer specific downstream gene translation regulation individually.

It would be very interesting to further investigate the regulatory mechanism of the tissue specificities of 4E-BP1 and 4E-BP2. Additionally, 4E-BPs related translation regulatory machinery might play roles in embryonic hematopoiesis as well. The functions of 4E-BPs in erythroid lineage differentiation are not clear at the moment. Epo-induced cultivated erythroblast differentiation may supply a good experimental system to address this question and new erythroid lineage specific regulators may be identified.

GATA-1 plays a central role in erythropoiesis. It belongs to a family of transcription factors with two conservative zinc finger DNA-binding motifs (Ko and Engel 1993). Loss of GATA-1 results in fetal embryonic anemia. In vitro colony forming assay and differentiation studies of GATA-1 deficient embryonic stem cellls have indicated that GATA-1 deficiency results in differentiation arrest at pro-erythroblast stage and undergo rapid apoptosis (Pevny, Simon et al. 1991; Weiss, Keller et al. 1994; Weiss and Orkin 1995; Fujiwara, Browne et al. 1996). Two GATA-1 proteins are detected in human K562 and mouse MEL erythroid cell lines resulting from alternative translation initiation site usage. Full-length protein is not detected in E8.5 mouse embryo, whereas the low molecular weight GATA-1 isoform is detected. Both proteins are detected in fetal liver (Calligaris, Bottardi et al. 1995). This study suggests that two GATA-1 proteins play different functions in embryonic hematopoiesis. So far no evidence

indicates the existence of regulatory elements in untranslated region of GATA-1 mRNA. I only found decreased full-length GATA-1 expression in erythroblasts of 4E-BP1,2 compound knock out and 4E-BP2 knock out mice. This abrogated expression was at the protein level. Short form of GATA- 1 was not detected in both mutant and control erythroblasts (data not shown). I did not find reduced GATA-1 transcripts level in 4E-BP1,2 compound knock out and 4E-BP2 knock out erythroblasts. Therefore, my result suggested that disruption of 4E-BP2 resulted in deregulation of GATA-1 mRNA translation, leading to the reduction of proliferation rate of erythroid progenitors and precursors, thus implied that translation regulation was involved in GATA-1 expression regulation. It will be reasonable to investigate whether regulatory elements exist in untranslated region of GATA-1 mRNA.

4E-BPs regulate translation initiation rate through regulating the activity of mRNA cap structure binding protein eIF4E. The activity of eIF4E is regulated by the phosphorylation through MAP kinase–interacting protein kinase 1/2 (MNK1/2) as well. I found upregulated eIF4E phosphorylation in erythropoietic stress compared to in normal erythropoiesis in control mice. The reason could be that MNK1 was activated. I did find increased MNK1 phosphorylation after phenylhydrazine treatment. Increased eIF4E and MNK1 phosphorylation are found in mouse hippocampus as well when it is stimulated with increased amount of stimulation delivery (Banko, Poulin et al. 2005). My results implied that the signaling through kinase MNK1 was activated resulting in the upregulation of eIF4E phosphorylation in phenylhydrazine induced erythropoietic stress.

The phosphorylation of eIF4E in 4E-BPKO erythroblasts was higher than that of control erythroblasts. The reason could be that deletion of 4E-BPs released more eIF4E that was then amendable for phosphorylation. The effect of phosphorylated eIF4E on translation rate is not clear. Mammalian eIF4E is phosphorylated in response to extracellular stimuli and stress that enhance or abrogate translation rate and protein synthesis (Kleijn, Scheper et al. 1998; Gingras, Raught et al. 1999; Raught and Gingras 1999; Tsukiyama-Kohara, Poulin et al. 2001; Banko, Poulin et al. 2005; Morley and McKendrick 1997). Phosphorylation of eIF4E is required for the development of Drosophila (Lachance, Miron et al. 2002). Data presented here indicated that enhanced eIF4E phosphorylation could be responsible for lower GATA-1 expression, however, whether this is a direct effect still need to explore.

4E-BP and ageing related HSC function

Besides the function of 4E-BPs in stress erythropoiesis, I observed reduced HSCs in the ageing 4E-BP1,2 knock out mice compared to control mice as well. HSCs exhaustion is

related to ageing. The mechanism by which the HSCs maintain activity of cell production throughout the life of the animal and escape from the risk of damage from long replicative histories is assumed to be “clonal succession”. The majority of the stem cells are maintained in a quiescent state. One or at most several stem cells supply differentiated cells simultaneously. In this way stem cells would replicate only when they assume that the role of an active clone is exhausted. Thus, the strategy of clonal succession supposes that the stem cell population will decrease with age when the numbers of used clones are increased. Crises during a lifetime, which require increased generation of differentiated cells, accelerate the rate of succession (Cudkowicz, Upton et al. 1964; Kay 1965; Harrison 1975; Bell and Van Zant 2004).

4E-BP and eIF4E are involved in the lifespan of Drosophila and C. elegans (Table 1). Upstream open reading frame (uORF) has been suggested to be involved in promoting translation at alternative downstream start sites to express truncated C/EBPα, C/EBPβ and stem cell leukemia factor (SCL). EIF4E and eIF-2 enhance truncated C/EBPα, C/EBPβ expression through uORF. Rapamycin inhibit the expression of truncated C/EBPα, C/EBPβ (Calkhoven, Muller et al. 2003). The uORF of SCL mutant mice showed enhanced self-renewal function of HSC (Mo et al. unpublished). PTEN is a negative regulator of the signaling through PI3K. Pten deletion causes the generation of transplantable leukemia-initiating cells and deletion of normal HSCs in mice. Rapamycin not only depletes leukemia-initiating cells, but also restores normal HSC function (Yilmaz, Valdez et al. 2006). These studies imply that translation regulation is involved in HSC function.

Thus we assumed that the defect of 4E-BPs might affect the pool of HSC. Strikingly, using flow cytometry we found that HSC number in the bone marrow of old (> 9m) 4E-BP1,2 compound knock out mice was decreased compared to that of control mice. However, in young mice (< 9m), the number of HSC in 4E-BP1,2 compound knock out mice was similar as that of control mice (data not shown). Although this observation need to be further explored, at least, it implied that translation control might be involved in ageing related HSC exhaustion.

Anemia in a significant proportion of the human population is not easily explained by the factors such as iron or folate deficiencies (Balducci and Carreca 2003; Ershler, Nifontova et al. 2003; Rothstein 2003). Unexplained anemia accounts for nearly half of those seen in elder population as well (Penninx, Guralnik et al. 2003; Cesari, Pahor et al. 2005). My results suggested that the mutation analysis of *4ebp* and *eif4e* in patients who suffered from unexplained anemia might provide a possible explanation.

In conclusion, I have found that deletion of 4E-BP2 deregulated erythroid specific transcription factor GATA-1 expression, leading to stress erythropoiesis defect in 4E-BP2 knock out and 4E-BP1,2 compound knock out mice. These results indicated that 4E-BP2 was required for stress erythropoiesis and suggested that 4E-BP2 related translation regulation machinery was involved in hematopoietic response to environmental stress.

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Abbreviations

Akt	protein kinase B
AMPK	AMP-activated protein kinase
APC	allophycocyanin
BFU-E	burst-forming erythroid unit
BSA	bovine serum albumin
CFU-E	erythroid colony-forming units
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
eIF4E	eukaryotic initiation factor 4E
EMCV	encephalomyocarditis virus
ERKs	extracellular signal-regulated protein kinases
EWAT	epididymal white adipose tissue
FITC	fluorescein isothiocyanate
FOXO	forkhead-related transcription factor
FRAP	FKBP-12-rapamycin associated protein
HBSS	Hank's Buffered Salt Solution
hEPO	human recombinant erythropoietin
HDL-cholesterol	high density lipoprotein-cholesterol
HFD	high fat diet
HIF1	hypoxia-inducible factor 1
IBAT	interscapular brown adipose tissue
IGF	insulin like growth factor
IGF-1	insulin-like growth factor
IRS	insulin receptor substrate

IWAT	inguinal white adipose tissue
LTP	hippocampal long-term potentiation
MAP kinase	p38 mitogen-activated protein kinase
MEP	megakaryocyte-erythroid progenitor
Met-tRNA $_i^{Met}$	methionyl-initiator Trna
MNK1	MARP kinase –interacting protein kinase 1
MPP	multipotent progenitors
mSCF	murine recombinant stem cell factor
Panc	pancreas
PBS	phosphate-buffered saline
PE	phycoerythrin
PHZ	phenlhydrazine
PIKK	phosphatidylinositol kinase-related kinase
PI3K	phosphatidylinositol 3-kinases
PIP3	phosphatidylinositol-3,4,5-phosphate
PTEN	lipid phosphatase — phosphatase with tensin homolog
REDD	regulated in development and DNA damage responses
Rheb	Ras-homolog enriched in brain
S6K	regulate S6 kinase
SCL	stem cell leukemia factor
STK11/ LKB1	serine/threonine kinase 11
TOR	the target of rapamycin
TSC1	hamartin
TSC2	tuberin
uORF	upstream open reading frame

4E-BP	eukaryotic initiation factor 4E binding protein
7AAD	7-Amino-Actinomycin D

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Erklärung

Ich versichere hiermit, dass die von mir vorgelegte Dissertation selbständig angefertigt wurde und ich die Stellen der Arbeit, die anderen Werken in Wortlaut oder Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe. Diese Dissertation wurde noch keiner anderen Fakultät zur Prüfung vorgelegt.

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Publikationen

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