Role of p45 NF-E2 and TXNIP

in terminal erythroid differentiation

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Abstract

Red blood cell pathologies are among the most prevalent causes of mortality and morbidity worldwide, highlighting the need to gain more understanding of the normal erythropoiesis process and to find novel targets to efficiently modulate it. In particular, there are still many open questions with respect to the regulation of terminal erythroid differentiation, therefore we investigated the role of the proteins p45 NF-E2 and TXNIP in this process.

In vitro studies have demonstrated that absence of p45 NF-E2 leads to abrogation of α- and β-globin production, while its reintroduction rescues globin expression. *In vivo*, the majority of p45 NF-E2 knockout mice die shortly after birth due to hemorrhage. Neonates display severe anemia, while surviving adults present splenomegaly, severe thrombocytopenia and mild erythroid abnormalities, namely reduced hemoglobin content per cell, reticulocytosis and hypochromia. We investigated the role of p45 NF-E2 in terminal erythroid differentiation, since little is known about its function in red cells *in vivo*. We showed that deletion of p45 NF-E2 affects the serum level of erythropoietin, which is upregulated by over two-fold in knockout mice. Loss of p45 NF-E2 was associated with an increase in splenic erythropoiesis, as evidenced by an accumulation of early precursors. These observations are consistent with a stress erythropoiesis phenotype. In the bone marrow, we observed a different distribution of precursor populations compared to wild-type mice, suggesting a partial differentiation block. Therefore, the spleen is likely compensating for ineffective erythropoiesis in the bone marrow. Analysis of bone marrow samples revealed increased GATA1 levels in p45 NF-E2$^{-/-}$ mice as well as an increased proportion of erythroid cells arrested at the G0/G1 stage of cell cycle. Those results are

consistent with studies linking GATA-1 to cell cycle regulation and provide mechanistic insights into the role of p45 NF-E2 in erythroid differentiation.

TXNIP is a ubiquitous protein involved in various cellular processes including differentiation, growth, apoptosis and redox control. Knockout mice display a metabolic defect of impaired fatty acid utilization. With respect to hematopoiesis, TXNIP has been shown to play roles in natural killer cells and hematopoietic stem cells. Our results are the first that suggest a role for TXNIP in erythropoiesis. We observed a robust increase of TXNIP in mouse erythroleukemia (MEL) cells treated with DMSO or HMBA, known inducers of erythroid differentiation. This upregulation was present at the transcript and protein levels and detected early. The increase of TXNIP was not abrogated by the addition of the antioxidant N-acetylcysteine. The upregulation of TXNIP was confirmed in another model of erythroid differentiation, G1E-ER cells, which undergo differentiation upon activation of the GATA1 transcription factor. In addition, we showed that TXNIP levels were induced following inhibition of the MAP kinases p38 or JNK, while blocking ERK1/2 did not have an effect on TXNIP. TXNIP is also linked to iron homeostasis, since we observed increased iron uptake and decreased transferrin receptor in an overexpressing clone. *In vivo*, our results with $TXNIP^{-/-}$ mice revealed a new phenotype of impaired splenic erythropoiesis with a partial block between basophilic and late basophilic/polychromatic erythroblasts.

In summary, our data demonstrated that p45 NF-E2 and TXNIP are both involved in the process of terminal differentiation of erythroid precursors.

Abrégé

Les pathologies des globules rouges sont parmi les causes les plus prévalentes de mortalité et de morbidité, d'où la nécessité de comprendre mieux le processus d'érythropoïèse et de trouver de nouvelles cibles pour le moduler. Plusieurs questions demeurent en suspens quant à la régulation de la différentiation érythroïde terminale, de sorte que nous avons investigué le role des protéines p45 NF-E2 et TXNIP dans ce processus.

Des études *in vitro* ont démontré que l'absence de p45 NF-E2 mène à l'abrogation des globines α et β, alors que son réintroduction permet de recouvrer leur expression. *In vivo*, la majorité des souris knockout pour p45 NF-E2 meurent peu après la naissance suite à des hémorragies. Les nouveau-nés démontrent une anémie sévère, alors que les adultes survivants présentent une splénomégalie, une sévère thrombopénie ainsi que des anomalies légères des globules rouges, telles une concentration réduite d'hémoglobine par cellule, de la réticulocytose et de l'hypochromie. Nous avons étudié le role de p45 NF-E2 dans la différentiation érythroïde terminale, car peu de choses sont connues quant à sa fonction *in vivo*. Nous avons montré que l'absence de p45 NF-E2 affecte les niveaux d'érythropoïétine dans le sérum, qui se trouvent doublés dans les souris knockout. La perte de p45 NF-E2 est associée à une augmentation de l'érythropoïèse dans la rate, comme démontré par une accumulation des précurseurs plus précoces. Ces observations dénotent un phénotype d'érythropoïèse de stress. Dans la moelle osseuse nous avons observé une distribution altérée des populations de précurseurs, comparé aux souris de type sauvage, suggérant un blocage partiel de la différentiation. Ainsi, la rate compense pour une érythropoïèse inefficace dans la moelle osseuse. L'analyse des échantillons de moelle

osseuse a révélé une augmentation des niveaux de GATA1 chez les souris p45 NF-E2$^{-/-}$, ainsi qu'une proportion accrue des cellules arrêtées à l'étape G0/G1 du cycle cellulaire. Ces résultats sont consistants avec des études associant GATA1 à la régulation du cycle cellulaire et procurent un élément de mécanisme quant au role de p45 NF-E2 dans la différentiation érythroïde.

TXNIP est une protéine ubiquitaire impliquée dans plusieurs processus cellulaires, tels la différenciation, la croissance, l'apoptose et le contrôle redox. Les souris knockout présentent des défauts dans l'utilisation des acides gras. Par rapport à l'hématopoïèse, TXNIP a été démontré comme jouant un rôle dans les cellules tueuses naturelles et les cellules souches hématopoïétiques. Nos résultats sont les premiers à suggérer un rôle pour TXNIP dans l'érythropoïèse. Nous avons observé une augmentation robuste de TXNIP dans les cellules d'érythroleucémie murine traitées avec le DMSO ou le HMBA, des inducteurs connus de différentiation érythroïde. Cet accroissement au niveau des transcrits et de la protéine est observé tôt. L'augmentation de TXNIP n'est pas abrogée par l'ajout de l'antioxidant N-acétylcystéine. L'accroissement de TXNIP a été confirmé dans un second modèle de différentiation érythroïde, les cellules G1E-ER, qui se différencient suite à l'activation du facteur de transcription GATA1. De plus, nous avons montré que les niveaux de TXNIP sont induits suite à l'inactivation des MAP kinases p38 et JNK, alors que bloquer ERK1/2 n'a pas d'effet sur TXNIP. TXNIP est aussi lié à l'homéostasie du fer, puisque nous avons observé une augmentation de l'internalisation du fer et une diminution des niveaux du récepteur de transferrine dans un clone qui surexprime cette protéine. *In vivo*, nos résultats avec les souris TXNIP$^{-/-}$ révèlent un nouveau phénotype lié à l'érythropoïèse dans la rate, soit un blocage dans la transition entre les érythroblastes basophiles et les érythroblastes basophiles tardifs et polychromatiques.

En résumé, nos données démontrent que p45 NF-E2 et TXNIP sont tous deux impliqués dans la différentiation terminale des précurseurs érythroïdes.

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I am very grateful to my family who has continuously supported me during my graduate studies. Thank you to my husband Sergey Tikhonov whose optimism has kept me motivated in moments

of doubt and encouraged to persevere. To a new addition to my family, my son Viktor, whose presence in that last year has brought many rays of sun to my life. I am also forever indebted to my parents for encouraging my curiosity and interest in learning and being the first ones to teach me how to face challenging situations. I am very lucky to share my life with those remarkable people.

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Preface

This thesis is submitted in a traditional format and follows the "Guidelines for Thesis Preparation" outlined by Graduate and Postdoctoral Studies at McGill. The studies presented in this dissertation were conducted by the candidate under the supervision of Dr. Volker Blank. Chapter I is a general introduction. Materials and methods are described in Chapter II, while results are outlined in Chapter III and discussed in Chapter IV. Sections from Chapters II and IV and figures from section A of Chapter III appear in a peer reviewed paper in which the candidate was the first author:

1. **Abnormal differentiation of erythroid precursors in p45 NF-E2(-/-) mice.** Jadwiga J. Gasiorek, Zaynab Nouhi and Volker Blank. Experimental Hematology. 2012 May; 40(5): 393-400.

Sections from Chapter II and IV and figures from section B of Chapter III are included in a manuscript currently in preparation:

2. **TXNIP regulates the differentiation of murine erythroid precursors.** Jadwiga J. Gasiorek, Marc R. Mikhael, Daniel Garcia-Santos, Simon T. Hui, Prem Ponka and Volker Blank **(manuscript in preparation)**

Contribution of authors

All research presented in the results chapter, which includes all figures of the two papers mentioned in the previous section, was performed by the candidate with the exception of the iron uptake assay in figure 25. For the results presented in figure 25, the candidate has carried out the cell treatment and prepared the figure, while the iron uptake assays were performed by Dr. Marc R. Mikhael with the contribution of Dr. Daniel Garcia-Santos, both from the laboratory of Dr. Prem Ponka. Dr. Prem Ponka has provided advice with the iron studies. Dr. Simon T. Hui provided the $TXNIP^{-/-}$ mice. Dr. Volker Blank conceived the study and corrected the manuscripts and the thesis. Dr. Zaynab Nouhi has helped with the maintenance and genotyping of p45 $NF\text{-}E2^{-/-}$ mice.

Other contributions

The candidate contributed to a peer-reviewed paper not included in this thesis, by performing the experiments presented in figure 5 of that paper and preparing that figure:

1. **Antagonistic roles of the ERK and p38 MAPK signalling pathways in globin expression, haem biosynthesis and iron uptake.** Louay Mardini, Jadwiga Gasiorek, Lucie Carrière, Matthias Schranzhofer, Barry H. Paw, Prem Ponka and Volker Blank. Biochem J. 2010 Nov 15; 432(1): 145-51.

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List of Designations and Abbreviations

Designations

The designations used in this thesis follow the **"Guidelines for human Gene Nomenclature"**. Hester M. Wain, Elspeth A. Bruford, Ruth C. Lovering, Michael J. Lush, Mathew W. Wright and Sue Povey. Genomics 79(4): 464-470 (2002)

Mouse

Gene, cDNA, RNA	First letter upper case. Word italicized (e.g. *Txnip)*
Protein	All upper case letters. (e.g. TXNIP)

Human

Gene, cDNA, RNA	All upper case letters. Word italicized (e.g. *TXNIP*)
Protein	All upper case letters. (e.g. TXNIP)

Abbreviations

ACH	Active chromatin hub
AGM	Aorta-gonad-mesonephros
AKT	V-akt murine thymoma viral oncogene homolog 1
ALA	Delta-aminolevulinic acid
ALAS	Delta-aminolevulinic acid synthase
AML1	Acute myeloid leukemia 1

AMPK	AMP-activated protein kinase
AP-1	Activator protein-1
ARE	Antioxidant response element
ASK-1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
BACH1	BTB and CNC homology 1
BACH2	BTB and CNC homology 2
BasoE	Basophilic erythroblast
BAX	BCL2 associated X protein
BCL-2	B-cell CLL/lymphoma 2
BCL-6	B-cell lymphoma 6
Bcl-xL	B-cell lymphoma-extra large
BFU-E	Burst-forming unit-erythroid
bZIP	Basic leucine zipper
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
c-Fos	FBJ murine osteosarcoma viral oncogene homolog
ChoRE	Carbohydrate response element
ChoREBP	Carbohydrate response element binding protein
c-Jun	Jun oncogene
CBP	CREB-binding protein
CREB	Cyclic AMP response element-binding protein
CFU-E	Colony-forming unit-erythroid

CFU-Mk	Colony-forming unit-megakaryocyte
ChIP-Seq	Chromatin immunoprecipitation sequencing
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNC	Cap'n'collar
CXCR4	Chemokine (C-X-C) motif receptor 4
DMSO	Dimethyl sulfoxide
DMT1	Divalent metal transporter 1
DNase	Deoxyribonuclease
eIF2α	Eukaryotic translation initiation factor 2, subunit 1 alpha
eIF4e	Eukaryotic translation initiation factor 4e
ECD	Ecdysoneless homolog
EKLF	Erythroid krüppel-like factor
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ER	Endoplasmic reticulum
ERK	Extracellular-regulated kinase
EryP	Primitive erythroid progenitor
ES	Embryonic stem
FCHL	Familial combined hyperlipidemia
FOG	Friend of GATA
FOXO	Forkhead box, subgroup O

FSC	Forward scatter
GATA1	GATA binding protein 1
GATA2	GATA binding protein 2
GC	Glucocorticoids
GCM1	Glial cells missing homolog 1
GLUT1	Glucose transporter 1
GMP	Granulocyte-myeloid progenitor
GRX	Glutaredoxine
GSH	Glutathione
GTPase	Guanosine triphosphatase
HCC	Hepatocellular carcinoma
HCT	Hematocrit
HGB	Hemoglobin
HIF-1	Hypoxia inducible factor-1
HIF-2	Hypoxia inducible factor-2
HMBA	Hexamethylene bisacetamide
HO1	Heme oxygenase 1
HRE	Hypoxia response element
HRM	Heme regulatory motif
HS2	DNase hypersensitive site 2
HSC	Hematopoietic stem cell
HSF	Heat shock factor
HTLV	Human T-lymphotrophic virus

IL-1β	Interleukin-1β
IRE1α	Endoplasmic reticulum to nucleus signalling 1 alpha
ITCH	Itchy E3 ubiquitin protein ligase
IUGR	Intrauterine growth restriction
JAK2	Janus kinase 2
JNK	C-Jun N-terminal kinase
LCR	Locus control region
LIMS1	LIM and senescent cell antigen-like-containing domain protein 1
LMPP	Lymphoid-primed multipotent progenitor
LPS	Lipopolysaccharide
LT-HSC	Long-term hematopoietic stem cell
MAF	Musculoaponeurotic fibrosarcoma oncogene
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MEL	Mouse erythroleukemia
MEP	Megakaryocytic/erythroid progenitor
MLX	MAX dimerization protein
MONDOA	MLX interacting protein
MPN	Myeloproliferative neoplasm
MPP	Multipotent progenitor
MPV	Mean platelet volume

mtHSP70	Mitochondrial heat shock protein 70
mTOR	Mammalian target of rapamycin
MZF1	Myeloid zinc finger 1
NAC	N-acetylcysteine
NF-E2	Nuclear factor, erythroid-derived 2
NFE2L1	Nuclear factor, erythroid 2-like 1
NFE2L2	Nuclear factor, erythroid 2-like 2
NFE2L3	Nuclear factor, erythroid 2-like 3
NF-ĸB	Nuclear factor-kappa B
NF-Y	Nuclear factor Y
NK	Natural killer
NLRP3	NOD-like receptor family, pyrin domain containing 3
NLS	Nuclear localisation signal
OrthoE	Orthochromatic erythroblast
p45 NF-E2	Transcription factor NF-E2 45 kDa subunit
PARP1	Poly (ADP-ribose) polymerase 1
PBGD	Porphobilinogen deaminase
PBMC	Peripheral blood mononucleocyte
PDGF	Platelet-derived growth factor
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PI3K	Phosphatidylinositol 3 kinase
PKA	Cyclic AMP-dependent protein kinase A
PLC	Phospholipase C

PLT	Platelets
PolyE	Polychromatic erythroblast
PPAR	Peroxisome proliferator-activated receptor
ProE	Proerythroblast
PTEN	Phosphatase and tensin homolog
PV	Polycythemia vera
RAS	Ras viral oncogene homolog
RB	Retinoblastoma-associated protein
RBC	Red blood cells
RDW	Red cell distribution width
REDD1	Protein regulated in development and DNA damage response 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAHA	Suberoyl anilide hydroxamic acid
Sca-1	Stem cell antigen-1
SCF	Stem cell factor
SCL	Stem cell ligand
SDF-1	Stromal cell-derived factor-1
SFFV	Spleen focus-forming virus
SKN-1	Skin antigen-1
SOD	Superoxide dismutase
SOX6	Sex determining region Y-box 6
SPI1	Spleen focus forming vius (SFFV) proviral integration oncogene

STAT5	Signal transducer and activator of transcription 5
ST-HSC	Short-term hematopoietic stem cells
SUMO	Small ubiquitin-related modifier
TAL1	T-cell acute lymphocytic leukemia 1
TBP	TATA-binding protein
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TRX	Thioredoxin
TXNIP	Thioredoxin-interacting protein
UPR	Unfolded protein response
USF	Upstream stimulatory factor
UVA	Ultraviolet A
VEGFR2	Vascular endothelial growth factor receptor 2
VHL	Von Hippel-Lindau

Chapter I: Introduction

1. Rationale to study terminal erythroid differentiation

Anemia is a widespread condition affecting populations worldwide. The most commonly occurring is iron-deficiency anemia, since about a fifth of the world population is afflicted with this disease [1]. Other nutrition associated anemias include folate and vitamin B12 deficiencies.

The second most prevalent is the anemia of inflammation, also known as anemia of chronic disease, as it represents nearly 20% of anemia cases in older adults [2]. Its definition is broad, encompassing patients who present chronic infections, as well as illnesses such as cancer, renal failure or chronic inflammatory diseases like rheumatoid arthritis [3]. Given the ageing of the population, the number of people afflicted by this type of anemia will continue to be on the rise.

An additional category in this disease is hemolytic anemia, which is caused by premature destruction of red blood cells. It is observed in sickle cell disease, where a mutation in the β chain of globin leads to polymerization of hemoglobin and formation of misshaped red blood cells which become destroyed. Another genetically transmitted type of anemia are thalassemias, recessive disorders characterised by a defective synthesis of either alpha or beta globin chains, leading to an imbalance that prevents proper hemoglobin formation and causes toxicity. In 2005 it was estimated that 900 000 individuals would be born with thalassemia worldwide in the following 20 years [4].

Although anemias are common and their classification can be quite extensive, therapeutic options are limited. Current treatments for red blood cell disorders include hydroxyurea, which acts by reactivating fetal hemoglobin. It has been used in patients with thalassemia and sickle

cell anemia, but its downsides are important adverse effects and a lack of response in some patients [5]. A common therapy is the erythropoietin (EPO), but again some patients are not responsive or acquire resistance over time. In addition, some studies suggest that EPO given to cancer patients to correct their chronic anemia could stimulate cancer growth [6, 7]. For patients who cannot be treated with drugs other options are transfusions or allogenic stem cells transplant from a related donor.

Therefore, a better understanding of the mechanisms involved in terminal erythroid differentiation and identification of new targets to modulate this process may provide much needed novel therapy options for anemic patients. Moreover, a better understanding of late-stage erythropoiesis could also help in the research for artificial blood, as blood from donors raises shortage issues and safety concerns [8].

2. General introduction to erythropoiesis

2.1. Sites of erythropoiesis

Erythropoiesis is established in sequential waves throughout different anatomical sites. The first primitive erythroid progenitors (EryP) arise in the yolk sac of the mouse embryo at embryonic day 7 [9]. In the human they have been detected at about 3-4 weeks of gestation [10]. Those primitive EryP are large cells that possess a nucleus and synthesize embryonic globin chains. In contrast, definitive erythroblasts are small, enucleated cells producing adult globins. Definitive erythropoiesis arises also in the yolk sac, then migrates to the aorta-gonad-mesonephros region (AGM) and is further transported into the fetal liver. Later, the major site of erythropoiesis becomes the bone marrow, which takes over around half-gestation in human and after birth in

mice. In the adult, extramedullary erythropoiesis exists outside the bone marrow, in organs such as the spleen and liver [11].

2.2. The erythroblastic islands

Definitive erythropoiesis occurs in niches called erythroblastic islands identified over 50 years ago using electron microscopy [12]. Those structures are formed by erythroblasts at different stages of maturation surrounding a central macrophage cell **(Figure 1).** Although erythroid cells can proliferate and differentiate in an autonomous manner *in vitro*, this process is fairly inefficient, especially the final step of enucleation [13]. It can be greatly enhanced when the erythroblasts are co-cultured with macrophages. The macrophage performs multiple functions including anchorage of erythroblasts [14], signaling for enhanced proliferation [13], additional source of iron for the synthesis of heme [15] and phagocytosis of the nucleus upon terminal differentiation [16].

2.3. From the HSC to the reticulocyte

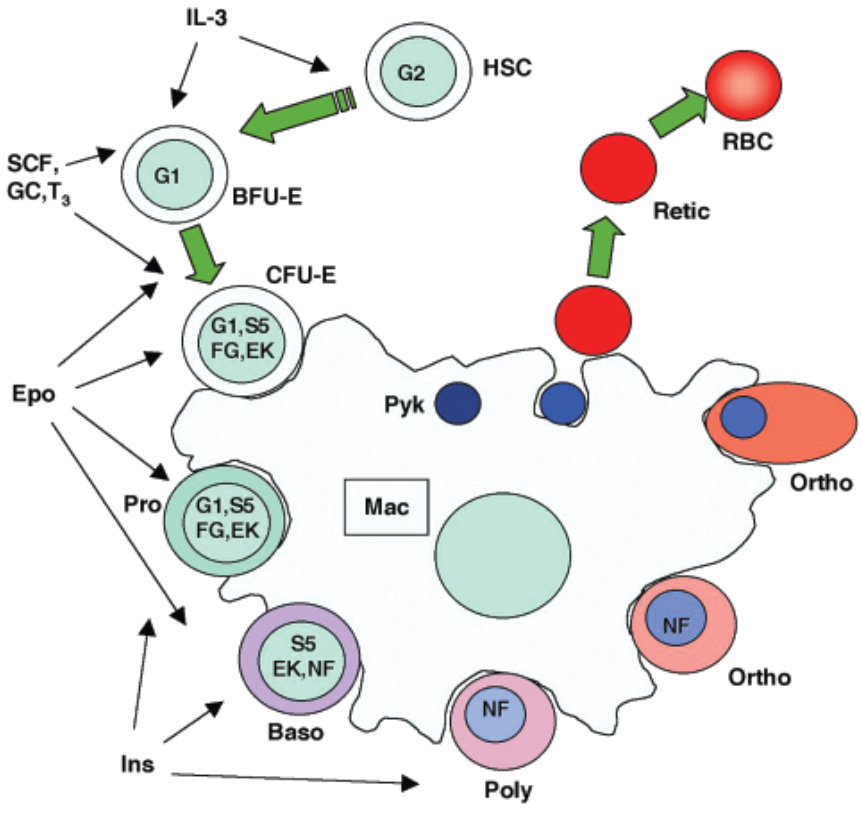
The average life span of erythrocytes is 120 days in humans, therefore they need to be continuously replaced. Erythropoiesis is characterised by two phases, the first one involving proliferation and commitment. The hierarchical process to generate mature erythrocytes starts, as for all blood cells, from the rare hematopoietic stem cells (HSCs). Those cells have been initially defined in the bone marrow of mice based on surface markers as cells with the phenotype Lin^- $Thy1.1^{lo}Sca\text{-}1^+$ [17] and are able to proliferate and self-renew. Long term repopulating HSCs (LT-HSCs) give rise to short-term repopulating HSCs (ST-HSCs), followed by multipotent

Figure 1. Representation of an erythroblastic island.

In the terminal stages of erythroid differentiation the erythroblasts surround a central macrophage, which will engulf the expelled nuclei. Some of the transcription factors and external factors that influence the maturation are illustrated.

HSC, hematopoietic stem cell; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; Pro, proerythroblast; Baso, basophilic erythroblast; Poly, polychromatic erythroblast; Ortho, orthochromatic erythroblast; Retic, reticulocyte; RBC, red blood cell; Mac, macrophage; Pyk, pyknotic nucleus; IL-3, interleukin-3; SCF, stem cell factor; GC, glucocorticoids, T_3, thyroid hormone; Epo, erythropoietin; Ins, insulin; G2, GATA2; G1, GATA1; S5, STAT5, FG, FOG; EK, EKLF; NF, NF-E2

Figure reprinted from [18] with permission.



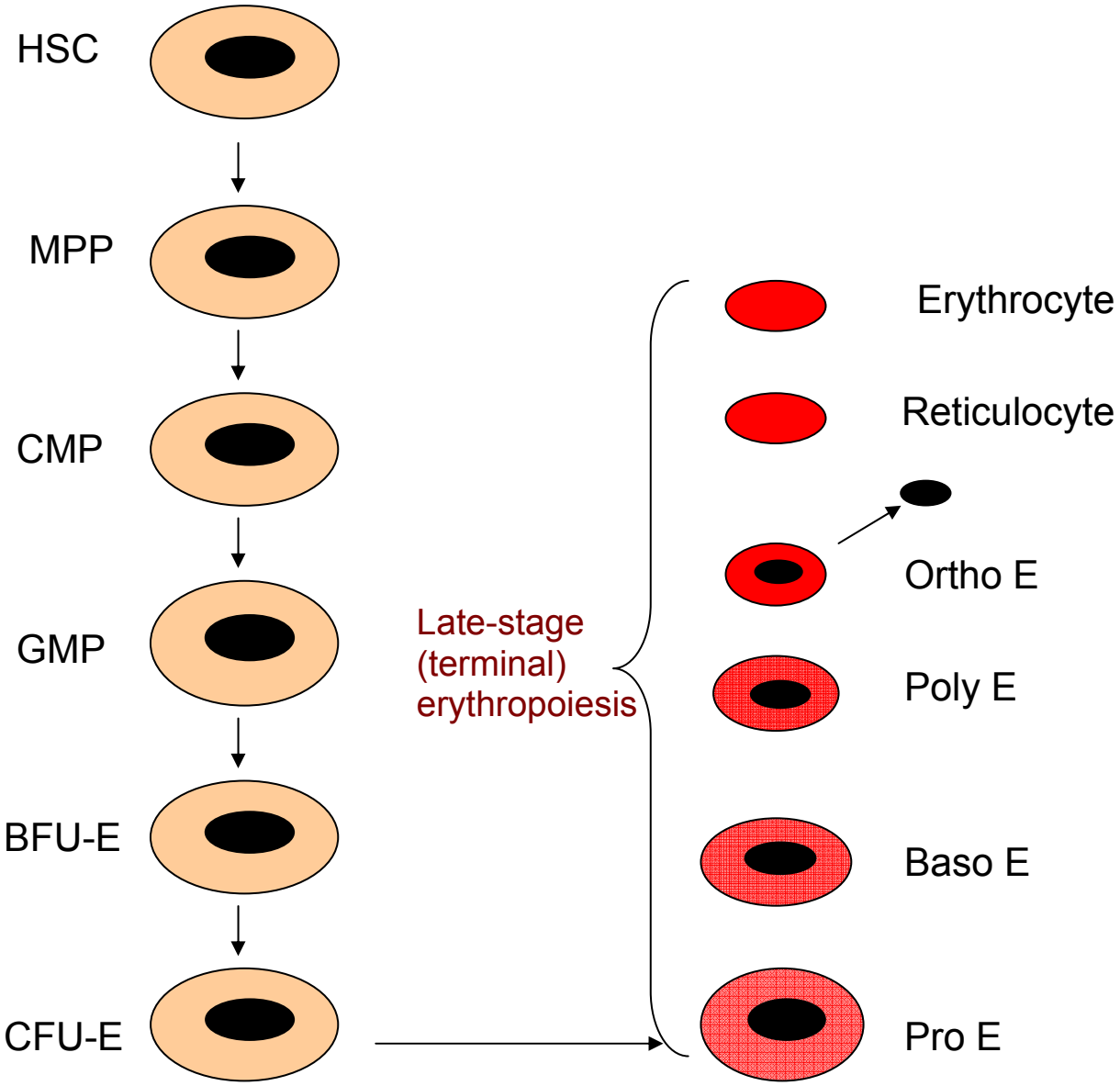
progenitors (MPPs) [19, 20]. When they commit to the differentiation pathway they give rise to lymphoid-primed multipotent progenitors (LMPPs), then common lymphoid progenitors (CLPs), which differentiate into lymphocytes and natural killer cells [21, 22]. On the other hand, MPPs will differentiate into common myeloid progenitors (CMPs) [23]. From the CMPs arise granulocyte-myeloid progenitors (GMPs) and megakaryocytic/erythroid progenitors (MEPs) [24]. The next steps involve the appearance of progenitors committed to the erythroid lineage [25]. Those are burst-forming units-erythroid (BFU-E), which form large colonies in methylcellulose medium, followed by colony forming units-erythroid (CFU-E), which form smaller colonies [26].

Following proliferation and commitment, arises the phase of terminal differentiation. From the CFU-E stage cells differentiate to the first morphologically recognizable precursor, the proerythroblast (ProE), which will undergo 3 to 4 mitosis along its differentiation pathway. This cell is further differentiated into the basophilic erythroblast (BasoE), polychromatic erythroblast (PolyE) and orthochromatic erythroblast (OrthoE). The orthochromatic erythroblast becomes a reticulocyte after it expels most of its organelles, including the nucleus and assumes its typical biconcave disc shape. The reticulocyte is released into the bloodstream, where it matures to become an erythrocyte and performs the essential task of providing oxygen to tissues while eliminating carbon dioxide **(Figure 2).**

Figure 2. Stages of erythropoiesis

Hematopoietic cells at different stages of erythroid differentiation: hematopoietic stem cell (HSC), multipotent progenitor (MPP), common myeloid progenitor (CMP), megakaryocytic/erythroid progenitor (MEP), burst-forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E), proerythroblast (ProE), basophilic erythroblast (BasoE), polychromatic erythroblast (PolyE), orthochromatic erythroblast (OrthoE).

Terminal (late-stage) erythroid differentiation is initiated at the proerythroblast stage.


HSC
MPP
CMP
GMP
BFU-E
CFU-E
Late-stage
(terminal)
erythropoiesis
Erythrocyte
Reticulocyte
Ortho E
Poly E
Baso E
Pro E

2.4. Hemoglobin

The transport of oxygen is performed by hemoglobin. The levels of this protein rise dramatically in terminal erythroid differentiation, starting at the proerythroblast stage, so to represent a third of the erythrocyte's volume. Production of functional hemoglobin requires the coordinated synthesis of globin chains and of heme, as well as efficient iron uptake.

2.4.1. Synthesis of globin chains

There has been extensive research on the globin gene, since it was the first gene to be cloned [27]. Adult hemoglobin comprises 2 α- and 2 β-globin chains, whose genes need to be tightly regulated at the transcriptional level, in order to be produced at the 1:1 ratio. The human β-globin gene cluster is present on chromosome 11 and consists of 5 types of globin genes (ε, Gγ, Aγ, δ and β), which are expressed sequentially during development, through a mechanism called hemoglobin switching. Globin promoters contain three important regulatory sequences, the TATA, CAAT and CACCC boxes [28]. This globin gene cluster is also regulated by distant elements, which form the locus control region (LCR), composed of 5 DNAse I hypersensitive sites **(Figure 3A)**. The α-globin gene cluster is present on chromosome 16 and comprises 3 genes (ζ2, α2 and α1) and some pseudogenes (ψζ1, ψζ2, ψα2, ψα1, θ1) **(Figure 3B)**.

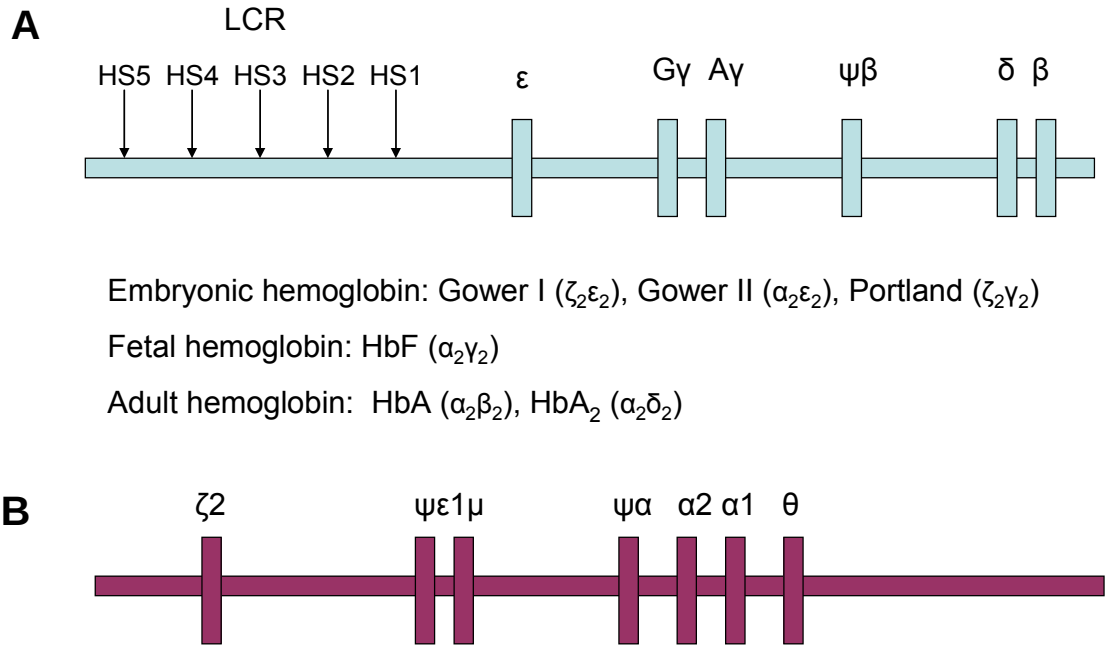
2.4.2. Synthesis of heme

The prosthetic group heme is present in a variety of proteins, such as cytochrome p450 enzymes, catalases, the eIF2α kinase and some transcription factors. It is also incorporated in each globin chain to form hemoglobin. The molecular structure of heme has been described as a ring of 4 pyrroles called protophorphyrin IX, containing a molecule of iron [29]. The synthesis of heme is

Figure 3. Structure of the globin gene clusters

A. Beta globin

B. Alpha globin



a multistep process, which begins in the mitochondria and continues in the cytosol, with the last steps occurring again in the mitochondria **(Figure 4)**. The initial step of this process involves the conversion of a glycine and a succinyl-CoA into delta-aminolevulinic acid (ALA) by the delta-aminolevulinic acid synthase (ALAS). This enzyme is present in two forms, the ubiquitous ALAS1 and the erythroid specific ALAS2. The final step catalyzed by ferrochelatase consists of the insertion of iron into protoporphyrin IX to form heme.

2.4.3. Iron uptake

In order to complete heme synthesis, there needs to be iron incorporation. Iron is transported in plasma by a protein called transferrin [30], which binds two molecules of iron and delivers it to cells, through transferrin receptors, which are internalized in clathrin-coated endosomes **(Figure 5)**. The task of importing iron to the mitochondria to be inserted by ferrochelatase into protophorphyrin IX is performed mainly by the transmembrane protein mitoferrin [31].

2.5. Role of transcription factors in terminal erythroid differentiation

The developmental program of terminal erythroid differentiation is tightly coordinated by specific transcription factors, which not only drive the expression of erythroid genes, but also suppress proliferation and inhibit apoptosis, in order to enable the differentiation pathway.

The transcription factor TAL1, a master regulator of hematopoietic cells, was shown to promote erythroid differentiation, since knocking it down in an *in vitro* model of erythroid differentiation, reduced globin and cell surface erythroid markers [32]. On the other hand, the transcription factor SPI1 (PU.1) plays a repressive role in erythroid differentiation in the erythroleukemia cell

Figure 4. Biosynthesis of heme

Cytosolic and mitochondrial steps of heme biosynthesis and enzymes involved.

Figure adapted from [33].

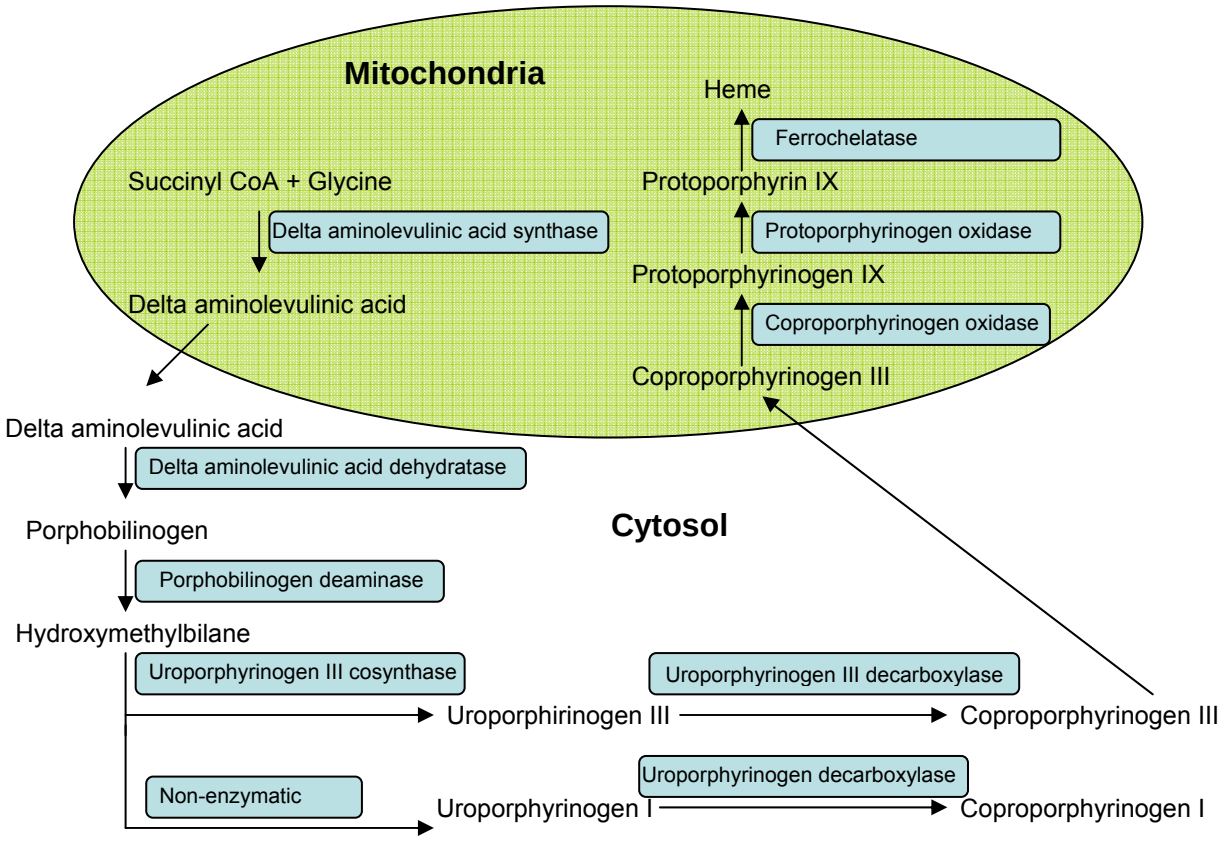
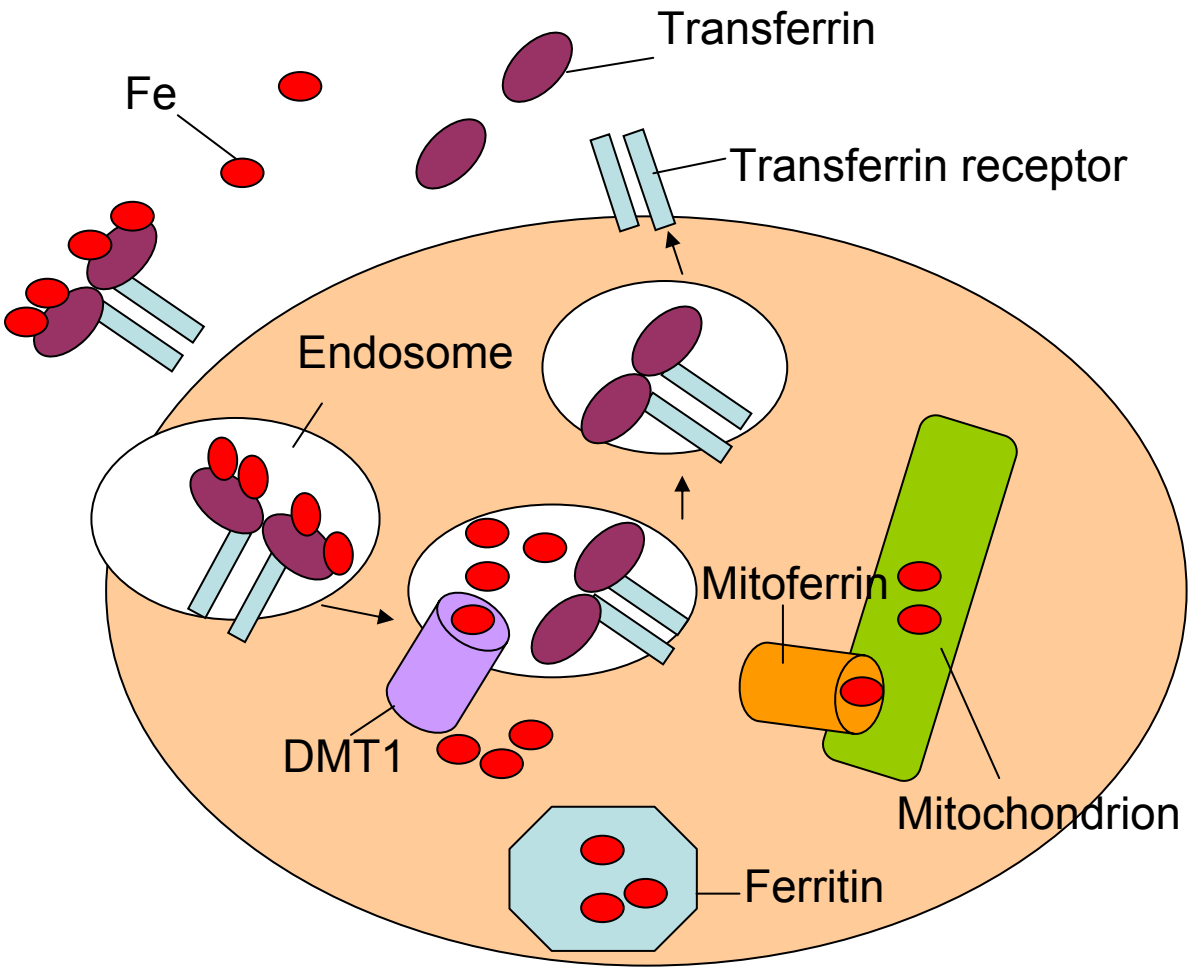


Figure 5. Iron uptake from transferrin.

The main source of iron for cells is assimilated through the transferrin pathway. Two atoms of Fe^{3+} associate with a transferrin molecule and bind to a dimeric transferrin receptor at the cell surface. The complex is then internalized in endosomes and following acidification, iron dissociates from transferrin, is reduced to Fe^{2+} and transported to the cytosol by DMT1. A significant amount is then transported into mitochondria through mitoferrin and excess can be stored in ferritin. The transferrin receptor is recycled back to the cell surface and transferrin is released.



line MEL cells. Knocking down PU.1 by siRNA allows cells to resume terminal differentiation and accumulate hemoglobin [34]. Similarly, downregulation of GATA2 is necessary to undergo terminal erythroid maturation [35]. The erythroid krüppel-like factor (EKLF) was shown to play important roles beyond the proerythroblast stage, including production of β-globin and heme synthesis enzymes [36].

The list of transcription factors involved in terminal erythroid differentiation is extensive, but GATA1 has emerged as a master regulator. *In vitro* differentiation studies showed the requirement of GATA1 for erythroid cells to mature beyond the proerythroblast stage [37]. In accordance with those observations, mice knockout for GATA1 die *in utero* around 10.5 and 11.5 days of gestation and display erythropoiesis arrested at the stage of the proerythroblast [38]. Impaired terminal erythroid maturation was also observed in adult mice, using conditional inducible knockouts [39]. Similarly, an *in vivo* knockdown of GATA1 generated by disrupting a cis regulatory element, that reduced GATA1 to 20-25% of its normal levels, also displayed impaired terminal erythroid differentiation [40].

GATA1 is involved in globin transcription, as it binds to regulatory globin sequences and recruits other transcription factors such as SCL and NF-E2 and ultimately leads to polymerase II recruitment late in maturation [41]. During differentiation GATA1 plays a role in promoting cell survival by increasing the levels of the anti-apoptotic Bcl-xL in conjunction with erythropoietin [42]. GATA1 is also associated with cell proliferation, inducing cell cycle arrest at the G1 stage, through a mechanism that involves transcriptional repression of c-myc [43] as well as transcriptional repression of c-kit and its downstream targets [44]. GATA1 was also shown to

upregulate cyclin-dependent kinase inhibitors p18 and p27 [43] and to regulate the cyclin-dependent kinase inhibitor p21 at the transcriptional level [45]. An additional mechanism through which GATA1 plays a role in cell cycle progression and apoptosis involves p53, which is also a regulator of those pathways. Indeed, GATA1 was shown to bind to p53 in a murine erythroleukemia cell line and overexpression of either of the two transcription factors had an inhibitory effect on the activity of the other [46]. GATA1 activity can be repressed by PU.1, which binds to DNA and to GATA1 and recruits the co-repressor pRB [47].

An important player in erythroid differentiation is also the transcription factor p45 NF-E2, to which the third section is dedicated, as it is one of the topics of this thesis. A brief overview of the erythroid transcription factors discussed in this section is presented in **Table 1**.

2.6. Function of erythropoietin in terminal erythroid differentiation

In addition to transcription factors, secreted factors also play an important role in erythroid differentiation. The list includes cytokines such as stem cell factor (SCF) or interleukin-11, as well as erythropoietin (EPO) [48]. EPO is an amply studied protein, with a well-defined role in the initial phases of late-stage erythropoiesis.

The primary site of EPO production is the kidney in the adult [49] and the liver in the fetus [50]. Normally expressed a low level, EPO is induced in response to hypoxia. Initially, transcriptional induction of EPO in response to hypoxia was identified to be mediated by binding of the transcription factor hypoxia-inducible factor-1 (HIF-1) to its enhancer [51]. More recent studies have implicated also HIF-2 in the regulation of EPO [52, 53]. Erythropoietin binds to its receptor

Table 1. Major erythroid transcription factors

Brief overview of major transcription factors implicated in erythropoiesis and their function.

TAL 1	Proliferation of early erythroid progenitors (BFU-Es)
PU.1	Blocking of erythroid differentiation by inhibition of GATA1
GATA-2	Inhibition of terminal erythroid differentiation
GATA-1	Promotion of terminal erythroid differentiation (beyond the proerythroblast stage)
EKLF	Erythroid lineage commitment, globin switching
NF-E2	Induction of globin genes

(EPOR) mainly present on erythroid cells and induces its dimerization [54]. This leads to the activation of the tyrosine kinase janus kinase 2 (JAK2), which is associated with the cytosolic domain of EPOR [55]. In turn, activated JAK2 initiates phosphorylation of EPOR and activation of STAT5, important for erythroid survival among others by increasing the transcription of the anti-apoptotic protein Bcl-xL [56]. Another cascade initiated by EPOR involves the activation of signalling mediated by phosphatidylinositol 3-kinase (PI3K) [57] **(Figure 6)**.

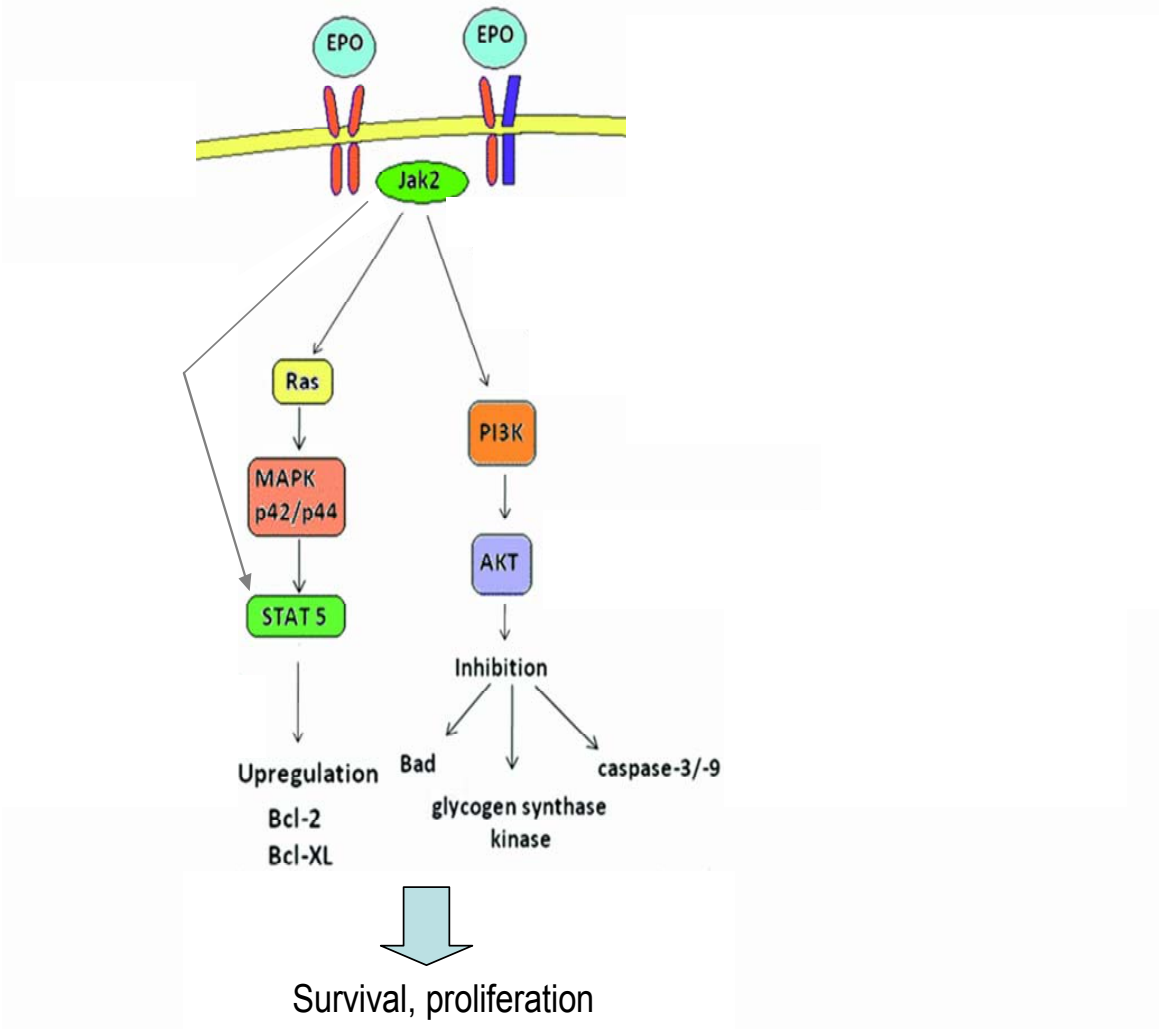
In vitro, EPO-mediated differentiation has been shown to involve signalling through reactive oxygen species (ROS), since it was inhibited by the use of anti-oxidants [58]. As well, a large transcriptome analysis demonstrated that EPO modulates factors involved in cell cycle regulation, such as p27 or Cyclin B1 [59].

Mice knockout for EPO and its receptor die at embryonic day 13 due to altered definitive erythropoiesis. The liver contains BFU-E and CFU-E colonies, although the survival and proliferation of CFU-Es and their downstream differentiation to proerythroblasts and beyond is compromised [60]. However primitive erythropoiesis in the yolk sac proceeds nearly normally, producing terminally differentiated cells, although with reduced size and proliferation rate [61]. Indeed it was shown that although EPOR is expressed in the yolk sac, EPO expression only starts with the initiation of definitive erythropoiesis in the embryo [62].

Figure 6. EPO signalling.

Binding of EPO to its receptor induces its dimerization and activation of JAK2, which engages pathways that will induce transcription of genes mediating cell survival and proliferation.

Figure modified from [63].



2.7. MAP kinases and terminal erythroid differentiation

Regulation of erythropoiesis depends as well on a family of proteins that are critical components of many signalling pathways, the mitogen-activated protein kinases (MAPKs), comprised of extracellular-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK).

In an EPO-dependent mouse erythroleukemia cell line this cytokine was shown to activate p38 and JNK and transiently ERK, their kinase activity being important for EPO-mediated proliferation and survival [64]. The importance of JNK activity has been further confirmed in human EPO-dependent cell lines, in which a synthetic inhibitor led to altered proliferation. In mouse progenitors inhibition of JNK decreased proliferation of BFU-Es without affecting CFU-Es or proerythroblasts [65]. In addition, the cytokine tumor necrosis factor-alpha (TNF-α) activated JNK to perform a role in proliferation, however this was restricted to erythroleukemia cells, not to cultured primary erythroid cells [66]. Erythroid leukemia cell lines independent of EPO, such as the ones infected with the spleen focus-forming virus (SFFV) displayed constitutive activation of JNK, highlighting the importance of this kinase in proliferation [67]. One erythroid target of activated JNK was shown to be the transcription factor p45 NF-E2, which is phosphorylated by this kinase, a signal leading to its ubiquitination and degradation. This was observed in uninduced mouse erythroleukemia (MEL) cells, while upon induction of differentiation by addition of DMSO, JNK was inactivated and p45 NF-E2 protected from degradation [68].

While JNK and p38 have been associated mostly with erythroid proliferation and survival signalling, ERK has been linked to the differentiation step. The erythroid cell line SKT6, which

can be induced to differentiate with EPO or DMSO, displayed increased differentiation when treated with an inhibitor of ERK, while the opposite effect was observed when ERK was constitutively activated [69]. Similarly, the erythroid EPO-responsive cell line ELM-I-1 exhibited increased β-globin transcripts when treated with an inhibitor of ERK, while they were decreased in the presence of an inhibitor of p38 [70]. In addition to increased globin transcripts, chemical inhibition of ERK has been associated with increased heme synthesis and iron uptake, while the opposite effects occured while inhibiting p38 in MEL cells induced to differentiate [71]. Moreover, ERK also plays a role in the survival of erythroid cells in response to EPO, since its inhibition reduced EPO-mediated upregulation of the antiapoptotic protein Bcl-xL [72]. ERK signalling in response to EPO has been shown as well to be blocked by the enzyme aconitase through a mechanism not fully understood, which involves an interaction between ERK and aconitase, the enzyme shown to be inactivated in iron deprivation conditions [73]. The mouse model knockout for the isoform ERK1 allowed the demonstration that this kinase is dispensable for bone marrow erythropoiesis. It is however a negative regulator of splenic erythropoiesis in steady state conditions, since the knockout mice displayed reduced apoptosis of splenic erythroid progenitors [74].

In the cell line SKT6, which differentiates in response to EPO, blocking p38 as well as JNK kinases, altered the differentiation, while an inhibitor of ERK did not. However, all three kinases were necessary for proliferation in the EPO-dependent cell line FD-EPO [75]. The latter cell line was able to differentiate independently of EPO following a short exposure to osmotic and heat stress, through a pathway that necessitated the activation of p38 and JNK [76]. Targeted disruption of the p38 alpha subunit in mice led to embryonic death and a phenotype that

indicated anemia, with most cells arrested at the proerythroblast stage and diminished levels of EPO in fetal liver [77]. In K562 cells which differentiate and produce hemoglobin in response to hydroxyurea, inhibition of ERK promoted differentiation, while blocking p38 inhibited differentiation [78]. The same antagonistic pattern of ERK and p38 effects has been observed in K562 cells induced to differentiate with activin A [79] or cyclosporine A [80]. The kinase p38 exists in four isoforms, namely alpha, beta, gamma and delta, and *in vitro* differentiation of CD34+ hematopoietic progenitors, revealed that mainly p38alpha and p38delta become activated in terminal erythroid differentiation [81]. In a mouse bone marrow culture system, using uncommitted stem cells selected with the Sca-1 marker and grown in a commercial media for erythroid differentiation, p38 inhibitors caused a delay in differentiation [82]. In an *in vitro* model using erythroid progenitors to produce conditions of stress erythropoiesis p38 was shown to be necessary for efficient enucleation by regulating targets p21 and retinoblastoma (RB) protein [83].

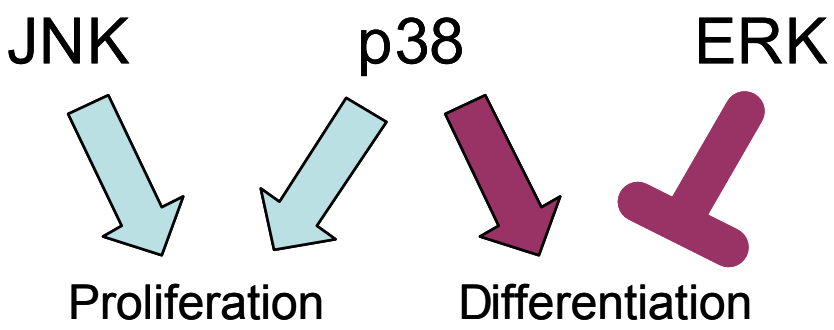
Although the effects observed can vary somehow depending on the cell model used, **figure 7** outlines the general effects of MAPKs on erythroid proliferation and differentiation.

2.8. Models to study terminal erythroid differentiation

Various models can be used to study terminal erythroid differentiation, but for the purpose of this thesis I will emphasize the ones used during my studies.

Figure 7. Effects of MAPK on erythropoiesis.

In general, JNK and p38 have been associated with erythroid proliferation and survival. ERK as well as p38 have been linked to erythroid differentiation, mediating opposite effects.



2.8.1. MEL cells

Mouse erythroleukemia (MEL) cells are virally transformed cell lines derived from the spleen of mice injected with Friend virus. These cells are erythroid precursors arrested at the stage of the proerythroblast [84]. A very small fraction, about 1% of those cells can differentiate spontaneously, but in order to undergo efficient terminal differentiation, they need to be exposed to chemical inducers, such as dimethyl sulfoxide (DMSO) or hexamethylene bisacetamide (HMBA) [85, 86]. Those treatments induce proliferation arrest and a commitment to differentiation, which translates into 4-5 divisions, up to the stage of orthochromatic erythroblast. In some particular cell culture conditions these cells can proceed to extrude their nuclei to form reticulocyte-like cells. MEL cells exhibit characteristic changes along differentiation, which mimic those of normal erythroid precursors. Those include a progressive decrease in cell size, changes in surface markers, nuclear condensation, production of high amounts of hemoglobin, transcriptional activation of hallmark genes, including *Gata1*, *Nf-e2*, *transferrin receptor* and *Alas2*, with a general transcriptional repression of many other genes [87]. However, undifferentiated MEL cells do not exhibit responsiveness to EPO, as normal proerythroblasts do, although it has been shown that they can develop a response to EPO, following a treatment with DMSO [88]. MEL cells can proliferate in the absence of EPO, as they display constitutive activation of the erythropoietin receptor, probably because of the gp55 protein of the Friend spleen focus-forming virus acting as a pseudoligand [89].

2.8.2. G1E-ER cells

Another cellular model used relies on the importance of GATA1 for terminal differentiation and the presence of its binding sites in a large subset of erythroid genes. Erythroid cells of mice

deficient for this transcription factor stop maturation at the proerythroblast stage, then undergo apoptosis. GATA1-deficient murine embryonic stem (ES) cells have been electroporated with a plasmid specific for erythroid expression, due to expression of the DNase hypersensitive site II of β-globin, and containing the cDNA for human anti-apoptotic protein Bcl-2. Then, the cells were differentiated *in vitro* in conditions to produce erythroid cells, which generated one clone, called G1E cells, that had the characteristics of a GATA1 null proerythroblast, but instead of undergoing apoptosis, continuously proliferated in culture [90]. Based on this parental cell line, G1E-ER cells were generated to restore GATA1 function. The G1E-ER cell line expresses a fusion of the ligand-binding domain of estrogen receptor and the coding sequence for GATA1, therefore GATA1 activity can be conditionally restored by the addition of β-estradiol [42].

2.8.3. Mouse models

Although *in vitro* models are interesting as they allow rapid generation of results through a variety of assays, experiments *in vivo* permit the observation of interactions in the context of a whole body. Recently developed protocols using surface markers allow the study of terminal erythroid differentiation directly in mice. They discriminate between the stages of proerythroblast through orthrochromatic erythroblast using the erythroid specific Ter119 membrane protein which starts to be expressed on proerythroblasts and transferrin receptor, whose expression decreases with terminal differentiation. In addition, forward scatter is used, since cells become smaller when they progress through differentiation [91, 92]. This method allows the analysis of changes in terminal erythroid maturation, due to the presence of a genetic mutation or treatment with specific reagents. Mouse models also allow the possibility to ablate the expression of specific genes by gene targeting, resulting in knockout animals.

3. The transcription factor p45 NF-E2

An important player in erythropoiesis, the transcription factor NF-E2 is a heterodimer formed by one of the ubiquitously expressed small MAF family proteins and the p45 NF-E2 subunit, a member of the CNC transcription factors.

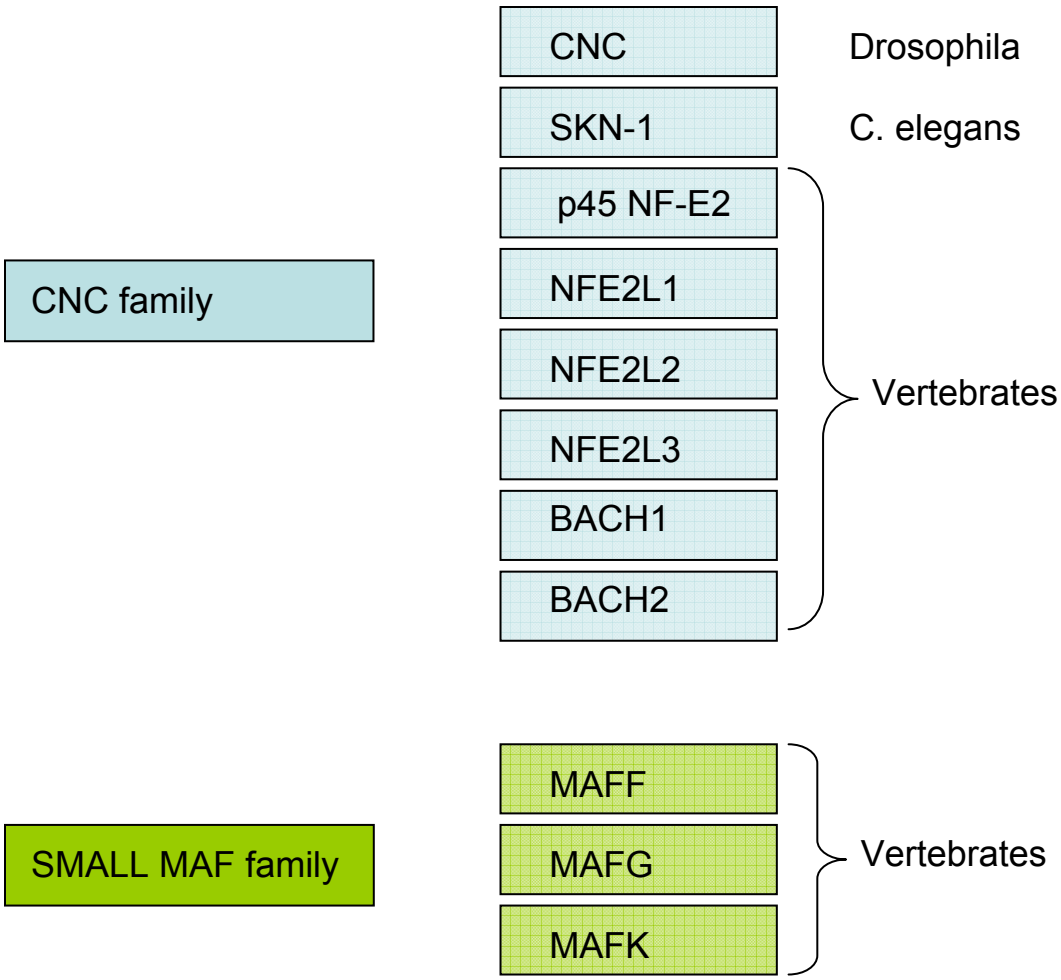
3.1. Cloning and characterisation of p45 NF-E2

The gene encoding the transcription factor *p45 NF-E2* localizes to chromosome 12 in human [93] and to chromosome 15 in mice [94]. Two transcripts are present with differences in the 5' non-coding region, one of them being more abundant in adult hematopoietic sites, while the other one dominates in the fetal liver [95]. The protein p45 NF-E2 is a member of the cap'n'collar (CNC) family of transcription factors [96] a classification that arose due to their homology to the drosophila CNC [97] and C. elegans SKN-1 [98], implicated in development. In vertebrates, other members include NFE2L1 (nuclear factor, erythroid 2-like 1), NFE2L2 (nuclear factor, erythroid 2-like 2), NFE2L3 (nuclear factor, erythroid 2-like 3), BACH1 (BTB and CNC homolog 1) and BACH2 (BTB and CNC homolog 2) [99] **(Figure 8)**.

The transcription factor p45 NF-E2 binds to DNA through a basic leucine zipper (bZip) domain, while forming a heterodimer with a small MAF transcription factor [93, 96, 100, 101]. The sequence recognized by the heterodimer is an extended AP-1 motif, which is (T/C)GCTGA(C/G)TCA(T/C) [96]. The binding partner of p45 NF-E2 has been identified as a member of the family of small MAFs [101], a family of proto-oncogenes ubiquitously expressed related to the viral v-maf [102], including MAFF, MAFG and MAFK proteins **(Figure 8).** Those proteins, possessing a bZIP binding domain but devoid of a transactivation domain, can act as

Figure 8. CNC and small MAF transcription factors.

List of members of the CNC and small MAF families of transcription factors.



repressors when homodimerizing between themselves or as activators when heterodimerizing with members of the CNC transcription factors [103]. *In vitro*, p45 NF-E2 has been found to interact with all three of the small MAFs [101]. Evidence *in vivo* suggests the major interaction in megakarocytes occurs with MAFG and MAFF [104], while in erythroid cells p45 NF-E2 interacts with MafG [105] and MafK [106]. For instance, it was shown, that at the β-globin locus in MEL cells, MafK forms a repressive complex with BACH1, but switches interacting partners with p45 NF-E2 upon induction of differentiation to form a complex that drives globin activation [106].

While the small MAFs are ubiquitous proteins, p45 NF-E2 exhibits tissue restriction. The major sites of p45 NF-E2 are hematopoietic cells, such as erythroid cells, megakaryocytes [107] and also mast cells [93, 96]. In mice it has been found restricted to hematopoietic tissues, including fetal liver and adult bone marrow or spleen [96]. Intriguingly, more recently p45 NF-E2 has been detected in trophoblastic cells, challenging the notion that it is a hematopoietic-restricted factor [108].

3.2. Regulation of p45 NF-E2

To exert its transcriptional activity p45 NF-E2 needs to be transported into the nucleus. This has been shown to be an active process necessitating an intact nuclear localisation signal (NLS) and importin-7. Mutations in the NLS reduced its nuclear translocation and transcriptional activity, leading to impaired platelet production in megakaryocytes [109]. Within the nucleus p45 NF-E2 is located within euchromatin, while its dimerizing partner MAFK is present in heterochromatin

regions in MEL cells. Upon differentiation, the small MAF subunit relocalises to euchromatin, therefore allowing p45 NF-E2 to exert its transactivation function [110].

As is the case with many proteins, p45 NF-E2 is subject to post-translational modifications, which by controlling its level or localisation can have an impact on its transcriptional activity. In human erythroleukemia K562 cells it has been shown to be SUMOylated at lysine 368, a modification that enhanced its DNA binding affinity as well as transactivation [111]. In contrast, ubiquitination of p45 NF-E2 by the E3 ligase Itch on lysine 63, diminished its transactivating capacity by retaining it in the cytoplasm [112]. The p45 NF-E2 transcription factor is also phosphorylated on serine 157 by Phospho-JNK in uninduced MEL cells, a modification that induces its ubiquitination and targeted degradation. This process was reversed in MEL cells induced to differentiate with DMSO when JNK was inactive [68]. Binding of p45 NF-E2 to DNA was diminished in the absence of cAMP-dependent protein kinase A (PKA), which can phosphorylate p45 NF-E2 *in vitro* [113]. Although it was not due to a direct effect on p45 NF-E2, but rather through an effect on its interaction with yet to be identified transcriptional regulators [114]. More recently, it has been demonstrated *in vitro* that SUMOylation of p45 NF-E2 was enhanced by phosphorylation by PKA [115]. Also, the transcriptional activity of p45 NF-E2 was increased by the acetylation of its binding partner MAFG mediated by CREB-binding protein [116]. In addition, p45 NF-E2 contains within its transactivation domain motifs termed PPXY that bind to WW domains of proteins, one of them being necessary for transactivation [117, 118]. More precisely, this PPXY motif in p45 NF-E2 is necessary for the protein to induce hyperacetylation at histone H3, methylation of K4 at histone H3 and recruitment of RNA polymerase II at the site of the β-globin genes [119].

There are two potential heme regulatory motifs (HRMs) present in p45 NF-E2, but heme was dispensable for globin gene induction in MEL cells [120]. On the contrary, BACH1 possesses 6 HRMs and heme was negatively regulating its DNA binding, by displacing it from its DNA bound complexes with small MAFs for the sake of p45 NF-E2. The structure of p45 NF-E2, including modifications is summarized in **Figure 9**.

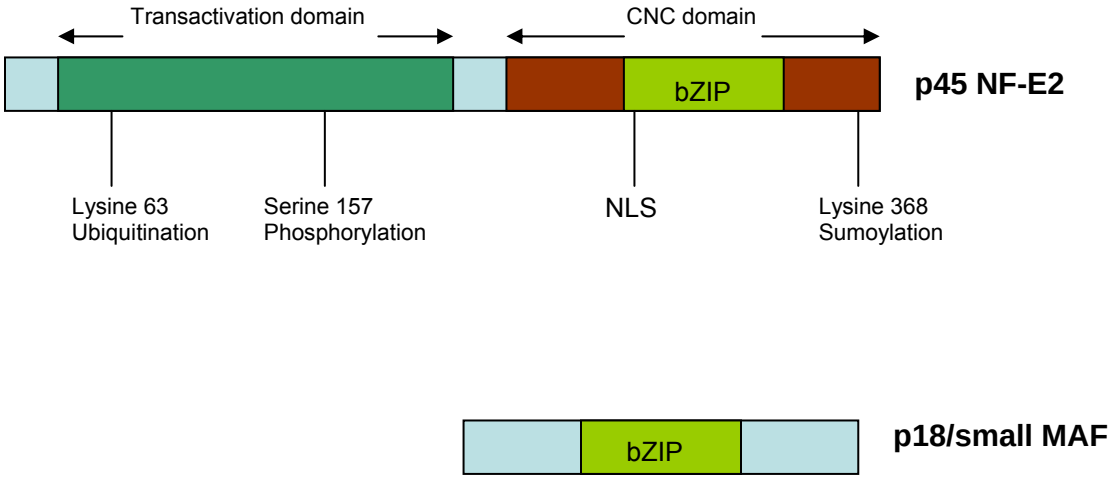
Human erythroleukemia cells transfected with NF-KB display a decrease in p45 NF-E2 transcripts and protein, indicating that this transcription factor exerts a negative regulation on p45 NF-E2, consistent with the high levels of NF-KB in early erythroid progenitors [121]. Another transcription factor that negatively affects p45 NF-E2 activity in erythroid cells is c-Jun, which forms a heterocomplex with the small maf subunit of NF-E2 that is inactive and inhibits globin genes transcription [122].

A different way of influencing p45 NF-E2 activity by some proteins is by modulating its levels. This is the case for phospholipase C (PLC) β1, whose nuclear overexpression diminished the protein levels of p45 NF-E2 in MEL cells [123]. Interleukin-4 has been shown to downregulate p45 NF-E2 mRNA and protein levels in megakaryocytes [124]. In addition, the levels of p45 NF-E2 were decreased by the histone deacetylase inhibitor Givinostat in cells of myeloproliferative neoplasms harboring a mutation in JAK2 [125]. In megakaryocytes, platelet-derived growth factor (PDGF) upregulated p45 NF-E2 transcripts and protein [126].

Figure 9. Structure of NF-E2.

Major structural domains and modifications of the two subunits of the NF-E2 transcription factor.

CNC: cap'n'collar NLS: nuclear localization signal bZIP: leucine zipper



3.3. Role of p45 NF-E2 in different cellular systems

The transcription factor p45 NF-E2 is an important regulator of erythroid and megakaryocytic function, with a series of reports about roles in other tissues and cell types. The phenotype of p45 NF-E2 knockout mice is summarized in **Table 2**.

3.3.1. Function of p45 NF-E2 in non-hematopoietic cells

A study has reported that mice deficient in p45 NF-E2 had increased bone mass specifically in bony sites of hematopoiesis, but not in other bones [127]. Those mice displayed increased numbers of highly proliferating osteoblasts and osteoclasts, filling their marrow cavity as they were aging. The mechanism is not completely understood, since cells of the osteoblast lineage do not express p45 NF-E2. However *in vitro* co-culture experiments suggested that an interaction between megakaryocytes and osteoblasts could be driving this increased proliferation [127]. Also, the fact that the bone phenotype was recapitulated by transferring spleen cells from p45 NF-E2 knockout mice into irradiated wild-type animals was indicative of the involvement of hematopoietic cells [128].

The absence of p45 NF-E2 in mice also leads to intrauterine growth restriction (IUGR) and a clue with respect to this phenotype has been recently revealed by the finding that this transcription factor is expressed in cells of the trophoblast. The p45 NF-E2 transcription factor has been found to regulate vascularisation of the placenta and embryonic growth through acetylation of glial cells missing homolog 1 (GCM1), an important player in syncytiotrophoblast formation [108].

Table 2. Phenotype of mice knockout for p45 NF-E2.

Effects observed in the absence of p45 NF-E2 in mice in hematopoietic and non-hematopoietic tissues.

Non-hematopoietic	- Increased bone mass in bony sites of hematopoiesis - Intrauterine growth restriction	[127] [108]
Hematopoietic	- Neonatal death of 90% of mice due to hemorrhage, lack of platelets caused by impaired maturation of megakaryocytes - Mild anemia in surviving adults, more pronounced in neonates, splenomegaly, extensive reticulocytosis	[129] [130]

3.3.2. Role of p45 NF-E2 in megakaryocytes

When mice deficient in p45 NF-E2 were first generated their most striking phenotype was the absence of platelets, which leads to the death of over 90% of pups by hemorrhage shortly after birth. It results from an arrest in maturation in the late-stages of megakaryocyte development, including reduced formation of cytoplasmic granules, but the cells still proliferate normally in response to thrombopoietin [129]. It has also been shown that the proportion of colony forming units-megakaryocytes (CFU-Mks) versus total cells is lower in fetal livers and adult spleens of knockout mice [131]. On the contrary, overexpression of p45 NF-E2 in mice bone marrow cells, gave rise to increased number of CFU-MKs, enhanced megakaryocyte maturation and higher release of platelets, indicating a role for this transcription factor not only in terminal megakaryocyte development, but also in its initial steps [132].

In megakaryocytes a critical binding partner of p45 NF-E2 is MAFG. However, MAFG not only mediates DNA binding through its bZip domain, but also targets the p45 NF-E2/MAFG heterodimer to a specific subnuclear localization, since the deletion of its C-terminal domain does not prevent DNA binding, but recapitulates the platelet phenotype observed in p45 NF-E2 knockouts [133].

The major role of p45 NF-E2 in megakaryocytes is transcriptional regulation of genes involved in their maturation and biogenesis of platelets. A first direct target of p45 NF-E2 in megakaryocytes has been identified as thromboxane synthase [134]. The p45 NF-E2 protein is also recruited to the promoter of RAB27B, a small GTPase localised in granules and implicated in platelet synthesis [135]. The megakaryocyte-specific β1-tubulin is also a target of p45 NF-E2,

as its expression is recovered upon restoration of this transcription factor [136]. The kinase adaptor protein LIMS1 is another target of p45 NF-E2, demonstrated using chromatin immunoprecipitation and transactivation experiments [137]. A more recent study combining whole wide-genome analysis by ChIP-seq and microarray in primary megakaryocytes has identified 49 genes whose regulatory regions could bind p45 NF-E2 and whose expression was modulated by this transcription factor, including 15 genes with established functions in platelet biogenesis [138].

In addition to transcriptional regulation of platelet genes, p45 NF-E2 contributes to megakaryocyte maturation by positively regulating intracellular reactive oxygen species (ROS) levels. The elimination of ROS is largely accomplished by induction of NFE2L2 targets and since p45 NF-E2 shows very similar binding specificity, it can compete with NFE2L2 for induction of proteins that eliminate ROS. There is an increase in ROS during megakaryocyte maturation, suggesting that they play an important role in signalling [133].

3.3.3. The erythroid cells and p45 NF-E2

In erythroid cells p45 NF-E2 regulates the three aspects of hemoglobin production, namely synthesis of globin chains, synthesis of heme and uptake of iron.

The transcription factor p45 NF-E2 has been initially identified as a protein binding to the promoter region of the porphobilinogen deaminase (PBGD) gene, coding for the third enzyme of the heme biosynthesis pathway [139, 140]. Another enzyme, ferrochelatase, catalyzing the last step of heme synthesis, also possesses a p45 NF-E2 binding site in its promoter [141] as does the

erythroid ALAS2 [142]. The enzyme heme oxygenase 1 (HO1) responsible for the catabolism of heme also has a binding sequence for p45 NF-E2 [143]. There is also binding of p45 NF-E2 to the promoter of alpha-spectrin, a protein that is found in the membrane of erythrocytes, as shown by reporter assays [144].

The transcription factor p45 NF-E2 is further necessary for the production of globin proteins in erythroleukemia cell lines. An erythroleukemia cell line, termed CB3, which is devoid of p45 NF-E2, due to the integration of Friend virus in one allele and the loss of the other allele has been a valuable model. Those cells fail to express high levels of α- and β-globin upon induction of differentiation, while reintroduction of p45 NF-E2 or a tethered p45 NF-E2 –small Maf dimer rescues globin expression [145, 146]. Furthermore, hemin (iron protoporphyrin IX) induces globin expression in human erythroleukemia K562 cells and induces the transcription of a reporter containing NF-E2 binding sites [147]. The regions necessary for p45 NF-E2 transactivation consist of two proline rich sequences within its transactivation and CNC domains [148]. It has been demonstrated that p45 NF-E2 in addition to GATA1 is necessary for the formation of the DNAse I hypersensitive site 4 present in the enhancer region of β-globin, which would allow the chromatin to assume a more "opened" conformation [149]. Similarly, p45 NF-E2 sites are also necessary for transactivation mediated by DNAse I hypersensitive sites 2 and 3 [150]. In fact, p45 NF-E2 exerts a chromatin remodelling action on the DNAse I hypersensitive site 2 (HS2) of β-globin, by disrupting nucleosomes in an ATP-dependent fashion and allowing subsequent binding of GATA1 [151]. There is evidence that the enhancer HS2 interacts with the promoter at the β-globin locus, since p45 NF-E2 binds to the HS2 and is also interacting through its N-terminal domain with a protein present at the promoter site, namely

TAFII130, which is associated with TATA binding protein [152]. A confirmation of *in vivo* binding of p45 NF-E2 to the HS2 site came from a study analyzing erythroleukemia cells and cells from mouse fetal liver using ChIP [153]. It has also been reported that p45 NF-E2 binds to the DNAse I HS2 site of β-globin, when the chromatin is still in the repressive state, before remodelling occurs [154]. However, in MEL cells the recruitment of p45 NF-E2 to the locus control region (LCR) and promoter of β-globin as shown by ChIP is greatly increased upon induction of differentiation. Interestingly, the promoter does not contain p45 NF-E2 binding sites, so its presence could be explained through binding to proteins associated with the promoter, or through DNA looping of regions of the LCR to the promoter [155]. In human multipotent progenitors p45 NF-E2 has been shown to be recruited to the LCR and promoter regions, in a fashion that is dependent on the transcription factor EKLF and that includes also the recruitment of cofactors TBP, CBP and BRG1 [156]. However, in contrast to GATA1 and EKLF, p45 NF-E2 seems dispensable for the formation of the active chromatin hub (ACH), a structure in which the distant LCR loops towards regions where globin genes are present. In p45 NF-E2 knockout mice there is increased binding of NFE2L2 at the LCR, but not to the promoter, suggesting a possible compensation through this transcription factor [157]. The transcription factor p45 NF-E2 also binds to and recruits the methyltransferase G9a at the β-globin locus, which plays an activating role for adult β-major chains, while repressing embryonic chains [158]. The recruitment of polymerase II to the promoter of the adult β-globin gene has been demonstrated to necessitate p45 NF-E2 and USF, a ubiquitous transcription factor shown to interact with p45 NF-E2 [159].

A role for p45 NF-E2 as a factor favoring erythroid maturation, has been suggested by experiments where enforced overexpression of this transcription factor in the monoblastoid M1 cell line yields erythroid and megakaryocytic colonies, while its overexpression in hematopoietic progenitors from fetal liver increases erythroid colonies [160]. Interestingly, in the erythroid J2E cell line, overexpression of p45 NF-E2 results in increased proliferation and morphological changes consistent with maturation, while the levels of hemoglobin are decreased [160].

A potential role for p45 NF-E2 in erythroid cells is protection against oxidative stress. Hence, red blood cells from p45 NF-E2-deficient mice have been shown to express higher levels of ROS at baseline, as well as markedly increased ROS when treated with H_2O_2 compared with those from wild-type mice. Those results translated *in vivo* into a higher sensitivity to the oxidative stress inducer phenylhydrazine, which caused a more severe drop in hematocrit and increased reticulocytosis in p45 NF-E2 knockout mice compared to wild-type counterparts. One postulated mechanism are reduced levels of catalase in red blood cells from knockout mice [161].

In vivo, the importance of p45 NF-E2 in erythroid cells was illustrated by the fact that mice generated with a deficiency in p45 NF-E2 display erythroid abnormalities and anemia, although milder than expected. Erythroid abnormalities are more pronounced in neonates, of which more than 90% die of hemorrhage due to lack of platelets. However, surviving adults still display hypochromia and presence of target cells, as well as slightly lower hematocrit and hemoglobin. It was speculated that this phenotype was caused by increased bleeding due to lack of platelets. Those mice also display extensive reticulocytosis and splenomegaly, possibly signs of increased compensatory erythropoiesis [130]. The relatively mild erythroid effect cannot be attributed to

functional compensation by the related factor NFE2L2, since mice deficient in both these transcription factors do not display more severe erythroid abnormalities than those deficient in p45 NF-E2 alone [162]. In addition, the absence of p45 NF-E2 is an important step in the progression of Friend virus induced erythroleukemia in mice, since heterozygous mice infected with the virus present higher tumor incidence and increased tumor size compared to their wild-type counterparts. Those results indicate that p45 NF-E2 functions as an inhibitor of erythroid proliferation *in vivo* [163].

In a clinical setting, overexpression of p45 NF-E2 has been identified in polycythemia vera (PV) patients, a disorder characterized by overproduction of erythroid cells and sometimes megakaryocytes and platelets. The severity of the symptoms correlated with the degree of upregulation of this transcription factor [164]. In addition, overexpression of p45 NF-E2 in CD34+ cells from healthy donors recapitulated the PV phenotype of increased EPO-independent erythroid differentiation and expansion of HSCs/CMPs, while silencing of p45 NF-E2 in cells from PV patients had the opposite effect [165]. In addition to PV, increased expression of p45 NF-E2 has also been observed in patients with other myeloproliferative neoplasms (MPNs), such as essential thrombocythemia and primary myelofibrosis, through a mechanism that may involve the transcriptional upregulation of p45 NF-E2 mediated by AML1 [166]. Furthermore, MPN patients displayed insertion and deletion mutations in p45 NF-E2, leading to the production of a truncated version, unable of DNA binding or transactivation on its own, but shown *in vitro* to increase the activity of wild-type p45 NF-E2 [167].

In addition to p45 NF-E2, about which not much is know in terminal erythroid differentiation, my research project also focused on TXNIP, yielding results that implicate this protein for the first time in erythropoiesis.

4. The alpha arrestin TXNIP

Thioredoxin-interacting protein (TXNIP) is a member of the alpha arrestin family of proteins, adaptors interacting with components of many signalling pathways, including G-protein coupled receptors, MAP kinases and tyrosine kinases [168, 169] **(Figure 10).** It is a ubiquitous protein present in many tissues and different cell compartments, including cytoplasm, nucleus, mitochondria and cellular membrane.

4.1. Characterization of TXNIP

TXNIP has been initially identified and cloned as a 46 kDa protein induced by 1,25-dihydroxyvitamin D3 in the promyelocytic leukemia cell line HL-60 [170]. The mouse gene has been mapped to chromosome 3, with a promoter containing multiple regulatory elements, including TATA and CCAAT sequences, as well as binding sites for transcription factors GATA1 and MZF1. In the mouse strain HcB-19, which displays a phenotype characteristic of familial combined hyperlipidemia (FCHL), the gene responsible for the disease has been initially mapped to chromosome 3 and later refined to be TXNIP harbouring a nonsense mutation [171, 172].

The promoter of TXNIP contains a series of regulatory elements **(Figure 11)** and is regulated by various transcription factors**.** For instance, a heat shock element, bound by heat shock factor

Figure 10. The arrestin family.

General structure and localisation of the members of the arrestin family.

Figure modified from [173].

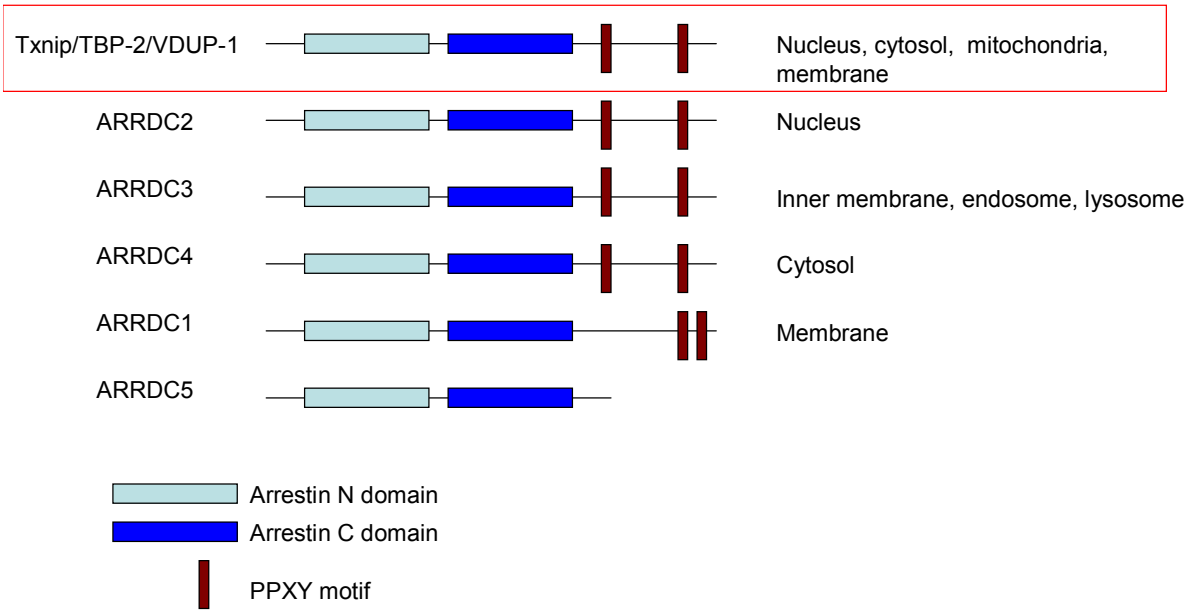
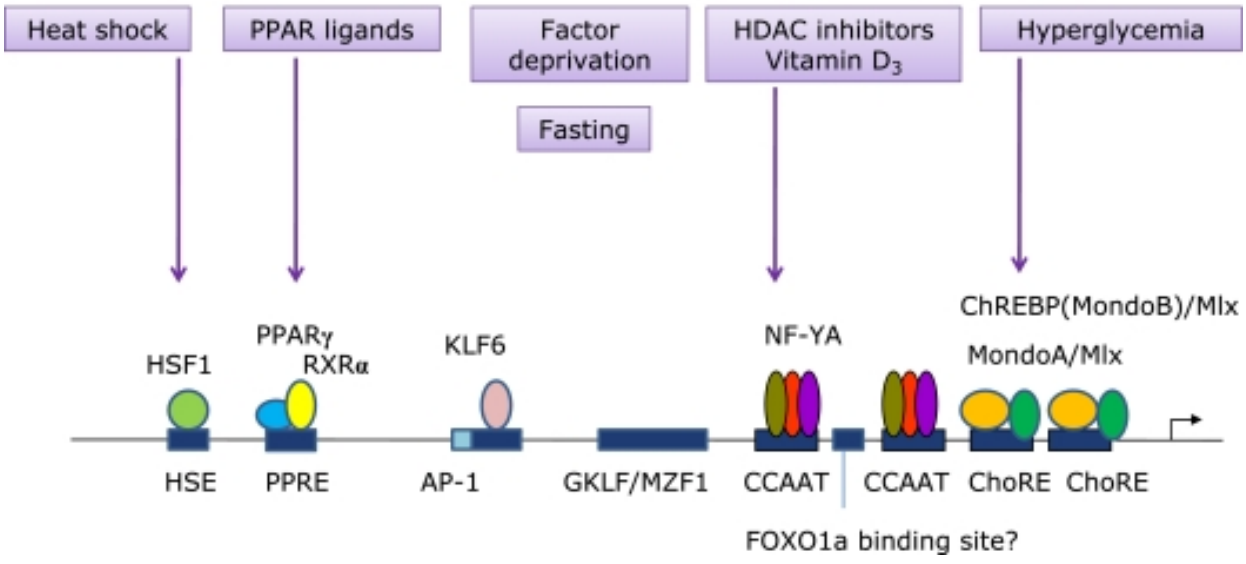


Figure 11. Regulatory sequences at the promoter of TXNIP.

Stimuli and transcription factors influencing expression of TXNIP. HSE: heat shock element, PPRE: peroxisome proliferator-activated receptor (PPAR) response element, AP-1: activator protein-1, GKLF: gut-enriched Krüppel-like factor, MZF1: myeloid zinc finger 1, putative FOXO1a-binding site, ChoRE: carbohydrate response element, HSF1: heat shock factor 1, RXRα: retinoid X receptor α, KLF6: Krüppel-like factor 6, NF-YA: nuclear transcription factor Y subunit α, ChREBP: carbohydrate response element-binding protein, Mlx: Max-like protein X.

Figure reprinted with permission from [173].



(HSF) is mediating its upregulation in conditions of stress, such as high cell density and serum deprivation [174]. Transcripts of *TXNIP* have also been shown to be induced by exposure of melanoma cells to UVA radiation [175].

Another transcription factor that induces TXNIP is hypoxia inducible factor 1 alpha (HIF1α), under hypoxic conditions in pancreatic cancer cells [176]. However in Hela cells, *TXNIP* is downregulated at the transcriptional level by hypoxia, through a mechanism that does not involve HIF1α, but reduced binding of the transcription factor MONDOA:MLX to its carbohydrate response elements [177]. In contrast, TXNIP levels have been shown to be downregulated in macrophages by PPARγ/RXRα through interference with binding of c-Jun/c-Fos to AP-1 sites [178].

TXNIP has also been shown to be positively regulated by the transcription factor FOXO1a in a human liver cell line [179] and by FOXO3a in fibroblasts [180]. Besides transcriptional regulation, the levels of TXNIP can be adjusted by its degradation. In the adipocyte cell line 3T3-L1 insulin caused the degradation of TXNIP through the proteasome pathway by a mechanism that necessitates PI3 kinase activity [181]. In cancer cells, it has been demonstrated that the ubiquitin ligase interacting with TXNIP and regulating its proteosomal degradation is ITCH [182].

In addition to being regulated by different transcription factors and stimuli, TXNIP has been shown to regulate proteins, often by directing their subcellular localisation. TXNIP can function as a shuttle protein and its overexpression has been associated with increased degradation of

HIF1α. It associated with the HIF1α/VHL complex to mediate the nuclear export of HIF1α necessary for its cytoplasmic degradation in conditions independent of hypoxia [183]. In line with those results, TXNIP has been shown to be a negative regulator of HIF1α-mediated transcription. In mouse lung epithelial cells, overexpression of TXNIP led to reduced signal of a luciferase reporter containing the hypoxia response element (HRE), both in hypoxic and normoxic conditions [184]. Nuclear localisation of TXNIP, dependent on importin α-1 has also been shown in the pancreatic β-cell line INS-1 in steady state conditions, while upon exposure to oxidative stress, induced by H_20_2 treatment, TXNIP shuttled to the mitochondria. When located in this compartment, it bound to mitochondrial thioredoxin2, releasing apoptosis signal-regulating kinase 1 (ASK-1) and leading to induction of apoptosis [185].

4.2. TXNIP in different processes

The majority of studies on TXNIP point to a role in oxidative stress, metabolism and cell growth with a few reports about its function in hematopoietic cells. The phenotype of mice deficient in TXNIP is summarized in **Table 3**.

4.2.1. ROS and TXNIP

4.2.1.1. ROS in hematopoietic cells

Reactive oxygen species (ROS) are free radicals resulting from a partial reduction of oxygen. Some frequently observed radicals are superoxide (O_2^-) or hydroxyl (OH^-) [186]. Besides extracellular sources of ROS, for instance UV radiation or pollutants, they can also be generated inside cells as part of normal cellular processes, such as the respiratory chain in the mitochondria or enzymatic reaction of NADPH oxidases [186, 187].

Table 3. Phenotype of mice deficient in TXNIP.

Studies with mice knockout for TXNIP or with HcB-19 mice, which present a naturally occurring mutation in TXNIP.

Metabolism	In fasting conditions: Hypoglycemia Increased insulin secretion Higher triglycerides Higher fatty acids in plasma	[188], [189]
Carcinogenesis	- Spontaneous hepatocellular carcinoma in 40% mice - Increased hepatocellular carcinoma and bladder cancer after treatment with chemicals	[190] [191], [192]
Hematopoiesis	- Impaired maturation of natural killer cells - Impaired function of dendritic cells - Abnormalities in hematopoietic stem cells	[193] [194] [195], [196]

Since excessive levels of ROS can be harmfull to nucleic acids, lipids or proteins, cells have acquired defense mechanisms. They include multiple antioxidant enzymes, for instance superoxide dismutase (SOD), glutathione (GSH), glutaredoxine (GRX) catalase or thioredoxin (TRX) as well as non-enzymatic components, for example vitamins C and E [197, 198].

More recently, it has emerged that ROS can also participate in signal transduction and gene expression in various cell types, including hematopoietic cells [199]. ROS levels in HSCs were shown to influence their self-renewal potential. Indeed, when two different populations of HSCs were identified, sharing the same membrane markers but different in terms of their ROS levels, the population with lower ROS displayed increased self-renewal capacity [200]. Although little is known about the mechanisms involved, AKT signalling as well as FOXO transcription factors have been implicated in regulation of ROS levels and self-renewal capacity of HSCs [201-203].

Besides regulating self-renewal and survival of HSCs, some reports implicate ROS in hematopoietic differentiation. In *Drosophila*, downmodulation of ROS was shown to impair the differentiation of cells equivalent to mammalian myeloid progenitors [204]. In mice, ROS were shown to promote megakaryocytic differentiation and release of platelets [133, 205]. As well, commitment to megakaryocytic differentiation *in vitro* from human HSCs was dependent on an increase in ROS [206]. Differentiation of a monocytic cell line into macrophages was also dependant on an increase in ROS levels, concomitant with a decrease in the antioxidant enzyme catalase [207]. Similarly, ROS were necessary for *in vitro* differentiation of human monocytes into dendritic cells [208]. With regards to red blood cells, the differentiation of the human myelocytic leukemia cell line K562 into erythroid cells with various chemical inducers was

inhibited by concomitant treatment with antioxidants [209]. Furthermore, in progenitors from mouse fetal liver induced to differentiate with EPO, the expression of erythroid markers was reduced upon addition of antioxidants [58].

4.2.1.2. Role of TXNIP in redox signalling

One of the methods that has led to TXNIP identification was through a yeast two-hybrid screen as a protein interacting with thioredoxin, a major component of the redox system, and binding of TXNIP had a negative effect on the expression and reducing activity of thioredoxin [210, 211]. The interaction with thioredoxin necessitates two cysteines present in TXNIP and its nature is a disulfide-linked complex [212]. However, the role of TXNIP in redox regulation is not only thioredoxin-dependent since the knockout mice display an increase in NADH without having an altered thioredoxin activity compared to wild-type animals [213].

Based on its interaction with thioredoxin, TXNIP participates in oxidative stress response, since its overexpression diminished the activity of thioredoxin as a reducer of reactive oxygen species and led to increased cell death of mouse fibroblasts [214]. On the other hand, reduced levels of TXNIP led to enhanced activity of thioredoxin [215]. In Muller retinal glia cells exposed to chronic hyperglycemia, such as in diabetes, there was an upregulation of TXNIP, which mediated increased oxidative stress, reduction in ATP and autophagy [216]. Inhibition of the ROS scavenging function of thioredoxin has also been observed in aortic smooth muscle cells in conditions of upregulation of TXNIP by hyperglycemia, an upregulation blocked by inhibiting the MAPK p38. This mechanism was believed to contribute to the detrimental effects of diabetes on the vasculature [217]. In a rat retinal capillary pericyte cell line decrease of TXNIP through

siRNA or the inhibitor AzaS diminished generation of ROS and caspase-3 activation mediated by high glucose [218]. Therefore, in general, upregulation of TXNIP leads to an increase in ROS and its downregulation decreases ROS levels.

Not only is TXNIP regulating ROS, but the redox status is also influencing the levels of TXNIP. Oxidative stress and high glucose have also been shown to induce TXNIP in neuronal cells [219]. In mouse renal mesangial cells defective in TXNIP, ROS were diminished not by increased neutralisation by thioredoxin but by a decrease in their generation in response to high glucose [220].

One of the master regulators of the oxidative response is NFE2L2. In mouse hearts the transcription factor NFE2L2 bound to an antioxidant response element (ARE) in the promoter of TXNIP to suppress its basal expression and induction by glucose, and also suppressed binding of transcription factor MONDOA, an activator of TXNIP.

Mainly through effects on redox status, TXNIP has also been associated with inflammation. Upon increase of ROS, TXNIP has been shown to be released from thioredoxin and to bind the NLRP3 inflammasome, participating in its activation [221]. In fibroblasts reduced TXNIP expression was also triggered by other proinflammatory molecules, such as interleukin-1β and tumor necrosis factor-α [222]. In hepatocytes exposed to high glucose, downregulation of TXNIP through siRNA decreased inflammation. Similar results were observed *in vivo* in rats treated for diabetes with quercetin or allopurinol, which display reduced inflammation, partly through downregulation of TXNIP [223].

Besides a role in apoptosis, ROS also function in different signalling pathways, including proliferation. Overexpression of TXNIP in aortic smooth muscle cells led to suppression of thioredoxin activity implicated in proliferation, such as evidenced by decreased DNA synthesis [224]. Similarly, increased expression of TXNIP in cardiomyocytes decreased protein synthesis following exposure to mechanical strain and reduced hypertrophy in response to aortic constriction [225].

Briefly, TXNIP levels are regulating redox status, mainly through thioredoxin and have an effect on functions influenced by ROS, such as apoptosis, inflammation or proliferation.

4.2.2. Function of TXNIP in metabolism

Transcripts and promoter activities of TXNIP have been shown to be upregulated by glucose in the prostate carcinoma cell line LNCaP as well as the hepatocellular carcinoma cell line HepG2, through a mechanism that required an intact carbohydrate response element (ChoRE) in the promoter of TXNIP [226]. The ChoRE has also been shown to be necessary and sufficient for TXNIP upregulation in response to glucose in pancreatic β-cells [227]. An additional ChoRE in the TXNIP promoter has been identified later and shown to be necessary for optimal induction by glucose, which also necessitated the recruitment of nuclear factor Y (NF-Y) to two CCAAT boxes, in order for the MONDOA:MLX complex to be recruited to the ChoREs [228]. In addition, upregulation of TXNIP through MONDOA also occured in the presence of non-glucose sugars, such as hexose, allose, 3-O-methylglucose and glucosamine [229]. In pancreatic cells ChoREBP overexpression upregulated *TXNIP* transcripts in response to glucose, while the opposite effect was observed upon ChoREBP knockdown. The mechanism involves recruitment

of ChoREBP to the TXNIP promoter, as well as binding of the coactivator histone acetyltransferase p300 [230]. Therefore, the complex MONDOA:MLX and ChoREBP appear as the two major regulators of TXNIP in conditions of hyperglycemia.

The levels of TXNIP were also induced by high glucose in human aortic cells, a phenomenon that was dependent on the transcription factor FOXO1 and the MAPK p38 [231]. On the contrary, the transcription factor FOXO1 downregulated *TXNIP* transcripts in pancreatic INS-1 cells, in addition to competing for binding at the ChoRE in the presence of glucose induction [232].

Some pathways have been shown to inhibit the upregulation of TXNIP in hyperglycemia. For instance, stimulation of signalling through cAMP reduced TXNIP induction by glucose and was associated with TXNIP ubiquitination and proteasomal degradation [233]. TXNIP induction in response to glucose was also blunted by inhibitors of mitochondrial oxidative phosphorylation [234]. Consistent with a downregulation of TXNIP in hypoglycemia, metformin, a drug used in diabetic patients to lower blood glucose has been shown to inhibit TXNIP at the transcriptional level [235].

TXNIP has also been demonstrated to regulate the uptake of glucose in adipocytes and skeletal muscle [236]. High intracellular glucose has been shown to upregulate TXNIP through the transcriptional dimeric complex formed by MONDOA:MLX, which in turn had a negative effect on glucose uptake [237]. The negative effect of TXNIP on glucose uptake was mediated through an effect on the glucose transporter GLUT1. TXNIP both reduced transcripts of GLUT1 through

an indirect effect and directly reduced membrane GLUT1 presence by binding to this transporter and inducing its internalisation [238]

The metabolic effects of TXNIP have been extensively studied in mice. When subjected to fasting, HcB-19 mice, the model with a naturally occurring mutation in TXNIP, increase their secretion of insulin and display hypoglycemia, as well as higher than normal triglycerides and ketone bodies. Gluconeogenesis was also affected in fasting conditions [188]. Increased hyperlipidemia in HcB-19 mice was characterized by higher triacylglycerol stores in the liver. The mechanisms contributing to this phenomenon were enhanced synthesis of fatty acids coupled to an increase in their esterification [239].

Another mouse model of TXNIP-deficiency generated by targeted mutation displayed a similar fasting phenotype of decreased glucose and higher fatty acids in plasma. In addition those mice presented increased fasting-induced death characterized by renal and hepatic dysfunctions and bleeding [189]. In response to fasting, TXNIP knockout mice also displayed decreased cAMP levels and increased glycogen, specifically in oxidative muscles, but not the glycolytic ones. TXNIP was inhibited by fatty acids by a mechanism that involved AMPK [240]. In addition, in conditions of energy stress AMPK has been shown to phosphorylate TXNIP, which led to its degradation [238]. TXNIP has also been shown to regulate the peroxisome proliferator activated receptor alpha (PPARα), an important player in the regulation of fatty acids consumption [241].

In conditions where TXNIP knockout mice were fed a regular diet, they still displayed major abnormalities in glucose and lipid metabolism, but without any effect on insulin expression

[213]. When exposed to a high-fat diet TXNIP knockout mice exhibited increased adipogenesis with enhanced expression of PPARγ target genes compared to their wild-type counterparts [242]. Obese mice (ob/ob) with disrupted TXNIP did not present an alteration in their obesity phenotype, but displayed decreased hyperglycemia and improved tolerance to glucose [243]. In ob/ob mice TXNIP has also been shown to regulate levels of the bioactive interleukin-1beta (IL-1β), a contributing player to insulin resistance [244]. In order to allow adipogenesis TXNIP needed to be degraded. In this regard, overexpression of thioredoxin stabilized TXNIP and led to inhibition of adipogenesis [245].

Mice knockout for TXNIP displayed impaired glucose production following a glucagon challenge and the phenotype affecting glucose production was observed also in mice with a targeted deletion of TXNIP in the liver, indicating an important metabolic role for this protein in another organ than the pancreas. Overexpression of intact TXNIP in hepatocytes from TXNIP-deficient mice increased the production of glucose, while this was not the case for TXNIP containing the mutation C247S, which abolished binding to thioredoxin. Interestingly, the pool of available thioredoxin was not changed in TXNIP-deficient mice [246]. Another study however has shown that the phenotype of general TXNIP-null mice was not observed in the liver-specific knockout model, but in the heart and muscle specific knockouts. In this context, the inappropriate metabolic response to fasting has been shown to involve the oxidative inactivation of PTEN, which led to the activation of AKT [247].

A role for TXNIP in metabolism has also been associated with its expression in the hypothalamus, in neurons that sense nutrients. While TXNIP was overexpressed in the

hypothalamus in the presence of an excess of nutrients, its downregulation in this region prevented obesity and insulin resistance induced by diet [248]. Overexpression of TXNIP in Agrp neurons increased obesity induced by diet and adiposity with no effect on food intake [249]. Also increased expression of Txnip was observed in the state of torpor, a condition characterized by slower metabolic rates, and this increase was detected in the hypothalamus, white and brown adipose tissue, as well as in the liver [250].

4.2.3. TXNIP and Apoptosis

The roles of TXNIP in metabolism and redox control presented in the previous sections are also related to its function in apoptosis. For instance, diabetes has been associated with cardiomyopathy. Interestingly, in mice cardiomyocytes increased glucose led to upregulation of TXNIP, increase in cleaved caspase-3 and induction of apoptosis, while treatment with beta-blockers reduced levels of TXNIP and those of cleaved caspase-3 as well as apoptosis. Additionally, in the hearts of HcB-19 mice (deficient in TXNIP) the levels of cleaved caspase-3 were reduced at baseline [251]. In cardiomyoblasts, downregulation of TXNIP decreased apoptosis caused by exposure to hydrogen peroxide and the mechanism involved ASK-1 [252]. On the contrary, overexpression of TXNIP in cardiomyocytes led to increased apoptosis in non-challenging conditions and following exposure to hydrogen peroxide [253].

Besides cardiac cells, the effect of TXNIP on apoptosis has been extensively studied in the pancreas. In pancreatic INS-1 cells it has been demonstrated that overexpression of TXNIP increased apoptosis, as assessed by a higher BAX/BCL2 ratio, enhanced caspase-3 levels and increased cleavage of caspase-9 [227]. High glucose in diabetes also mediated toxicity and

apoptosis of β-cells of the pancreas. This phenomenon was mediated by TXNIP, since HcB-19 mice (deficient in TXNIP) were protected, and the mechanism involved the mitochondrial apoptotic pathway, including cleavage of caspase-3 and release of cytochrome c [254]. HcB-19 mice, as well as mice with a β-cell-targeted deletion of TXNIP, displayed increased pancreatic cell mass and were protected against diabetes induced by streptozotocin. Absence of TXNIP inhibited the mitochondrial apoptosis pathway by inducing signalling through AKT and Bcl-xL [255]. Exposure in culture of β-pancreatic islets from HcB-19 mice to high glucose, did not lead to caspase-3 cleavage and induction of apoptosis, as it was the case for islets from wild-type mice, indicating a pro-apoptotic role for TXNIP [256]. In β-cells TXNIP deficiency had a protective effect on the glucose-induced apoptosis mediated through mitochondria, but not on the fatty acids-induced apoptosis mediated through ER stress [257].

Some reports have also implicated TXNIP in mediation of apoptosis in other cell types. In macrophages activation of peroxisome proliferator-activated receptor gamma (PPARγ) resulted in upregulation of TXNIP at the transcript and protein levels and was accompanied by increased activity of caspase-3 and increased apoptosis [258]. In T cells infected with HTLV, glucocorticoids (GC) increased TXNIP levels, which was mediating an apoptotic response, while in GC-resistant cells TXNIP expression was lost, along with their sensitivity to GC-induced apoptosis [259, 260]. In β-cells exposed to glucocorticoids (GCs) TXNIP has been shown to be overexpressed by a pathway that implicated the p38 MAPK and to mediate the apoptotic effect of GCs [261]. In mouse mesangial cells, downregulation of TXNIP through siRNA reduced apoptosis caused by high glucose, as assessed by reduced caspase-3 cleavage and ASK-1 activation [262]. The apoptotic pathway is commonly associated with TXNIP, since this protein

is competing with ASK-1 for binding to thioredoxin. Increased TXNIP levels lead to dissociation of ASK-1, which in turn activates the MAPK pathway and mediates apoptosis.

TXNIP has also been shown to be involved in apoptosis through its participation in the unfolded protein response (UPR), which originates from the accumulation of misfolded proteins in the ER. A key player of the UPR, IRE1α, induced TXNIP, which led to the activation of the NLRP inflammasome, causing release of proinflammatory cytokines and activation of caspases [263]. In fact, both the IRE1α and the PERK pathways of the ER stress response have been shown to upregulate TXNIP, leading to its promotion of inflammation and apoptosis in pancreatic β-cells [264].

4.2.4. Role of TXNIP in cell cycle and cancer

Downregulation of TXNIP has been observed in cancer tissues compared to normal tissues, including breast, lung and stomach cancer. The histone deacetylase inhibitor SAHA, an anti-cancer drug which leads to growth arrest and differentiation, caused an increase in the levels of TXNIP, suggesting this protein as one of its targets [265]. There is a reduction in TXNIP levels in acute myeloid leukemia (AML) and 3-Deazaneplanocin A, an agent that induces apoptosis in those cells, has been associated with an increase in TXNIP, decrease in thioredoxin activity and increased ROS [266]. Upregulation of TXNIP has been shown to exert a growth suppressive activity in the breast cancer cell line MCF-7. Overexpression of TXNIP in breast cancer cells as well as treatment with the anticancer drug SAHA led to accumulation of TXNIP mainly in the nucleus, by a mechanism that required importin α-1 [267]. Reduced levels of TXNIP have been associated with induced renal carcinoma compared to normal renal tissue and the decrease in

TXNIP was necessary for increased proliferation [268].

When fibroblasts were maintained in a quiescent state by serum deprivation, transcriptional and translational inhibition of TXNIP was necessary for the first cycle of entry into the G1 phase after serum addition. The mechanism involved the RAS-MAPK pathway [269]. The role of TXNIP in cell cycle progression is also important for its tumor suppressor function. In fact, overexpression of TXNIP through transfection had a growth inhibiting effect on tumor cells, as it arrested these in the G0/G1 phase of cell cycle [270]. Similarly, fibroblasts deficient in TXNIP displayed increased proliferation, with decreased cell cycle progression. This process was associated with reduced levels of the cyclin-dependent kinase inhibitor p27, caused by a decrease in stability due to translocation from nucleus to the cytoplasm mediated by JAB1. In contrast, overexpression of TXNIP blocked this translocation, thus increasing levels of p27, leading to cell cycle progression [271]. In the hepatocellular carcinoma cell line Huh-7 D-allose has been shown to induce cell cycle arrest, by a mechanism that implicated induction of TXNIP, which had a stabilizing effect on p27, the regulator of cell cycle transition between G1 and S [272].

Aside from its well-characterised role in cell cycle regulation, TXNIP is involved in invasion and cancer progression. In fact, p21 has been shown to bind to the promoter of *TXNIP* and to repress its transcription in breast cancer MCF-7 cells, which led to increased thioredoxin activity and promotion of invasion and angiogenesis [273]. Similarly, the epithelial to mesenchymal transition, a crucial step in invasion was increased in the lung cancer cell line A549 in the absence of TXNIP. One of the mechanisms implicated was increased transcriptional activity of TGF-β, as assessed by a luciferase reporter and transcript upregulation of TGF-β targets [274].

The vast majority of studies suggest a negative effect of TXNIP on proliferation and cancer progression and point to a role as tumor suppressor, although a few reports demonstrate the opposite effects, which might be cell or context-dependent. For instance, mice knockout for TXNIP displayed accelerated regeneration of the liver following a partial hepatectomy, caused by increased cell proliferation [275]. Also, melanoma cells treated with anti-sense TXNIP and injected into mice caused reduced tumor growth. Melanoma cells transfected with TXNIP siRNA also displayed decreased ROS and Fas ligand [276]. Increased expression of TXNIP also promoted transendothelial migration of human SK-MEL-28 melanoma cells, which is an *in vitro* assay to represent intravasion, a step in the establishment of metastasis. The mechanism of action of TXNIP in this process has been shown to be redox-sensitive, as it is inhibited by the antioxidant N-acetylcysteine [175].

In vivo, HcB-19 mice (which are deficient in TXNIP) developed spontaneously hepatocellular carcinoma (HCC) with a prevalence of about 40%, which started to appear around 8 months. Those mice displayed increased levels of alpha-fetoprotein and p53, markers of HCC [190]. In addition, $TXNIP^{-/-}$ mice exhibited increased HCC after treatment with the chemical diethylnitrosamine. The mechanism implicated increased proliferation and upregulation of the cytokine TNF-α, which led to activation of NF-κB [191]. Similarly, mice knockout for TXNIP were more sensitive to bladder carcinogenesis induced by N-butyl-N-(4-hydroxybutyl) nitrosamine. The mechanism involved increased activation of the ERK kinase, through stimulation of the CXCR4 receptor by stromal cell-derived factor-1 (SDF-1) [192]. Hence, all studies in mice suggest a role for TXNIP as a tumor suppressor.

The non-hematopoietic functions of TXNIP presented in the previous paragraphs are summarized in **Figure 12**.

4.2.5 TXNIP and hematopoiesis

Not much is known about the role of TXNIP in hematopoiesis. Only a handful of studies have been reported and their results are summarized in **Figure 13**.

The absence of TXNIP in mice did not have a significant effect on B and T lymphocytes numbers, although *in vitro* assays showed increased proliferation with some treatments, for instance IL-2. However, mice deficient in TXNIP displayed greatly reduced numbers of natural killer (NK) cells in the spleen, bone marrow and lung. In addition, those NK cells had decreased levels of CD122, the receptor for IL-2β and an important marker of their development. The reduction in CD122 was observed both *in vivo* analyzing cell populations in mice, as well as *in vitro* inducing differentiation of HSCs into NK cells. In addition, NK cells from $TXNIP^{-/-}$ mice exhibited decreased survival and differentiation when treated with IL-15 compared to their wild-type counterparts [193].

Moreover, dendritic cells devoid of TXNIP were not affected in their maturation and expression of membrane markers, but produced less IL-6 and IL-12 cytokines when exposed to LPS in culture. Also, dendritic cells derived from $TXNIP^{-/-}$ mice were not as efficient in priming T cells and induced less efficient production of cytokines in alloreactive $CD4^{+}$ T cells. In terms of immune response, following immunization $TXNIP^{-/-}$ mice displayed reduced delayed-type hypersensitivity, but no differences in terms of antibodies production [194]. Mice devoid of

Figure 12. Non-hematopoietic roles of TXNIP.

Illustration of TXNIP functions in pathways of redox control, apoptosis, cell cycle and metabolism.

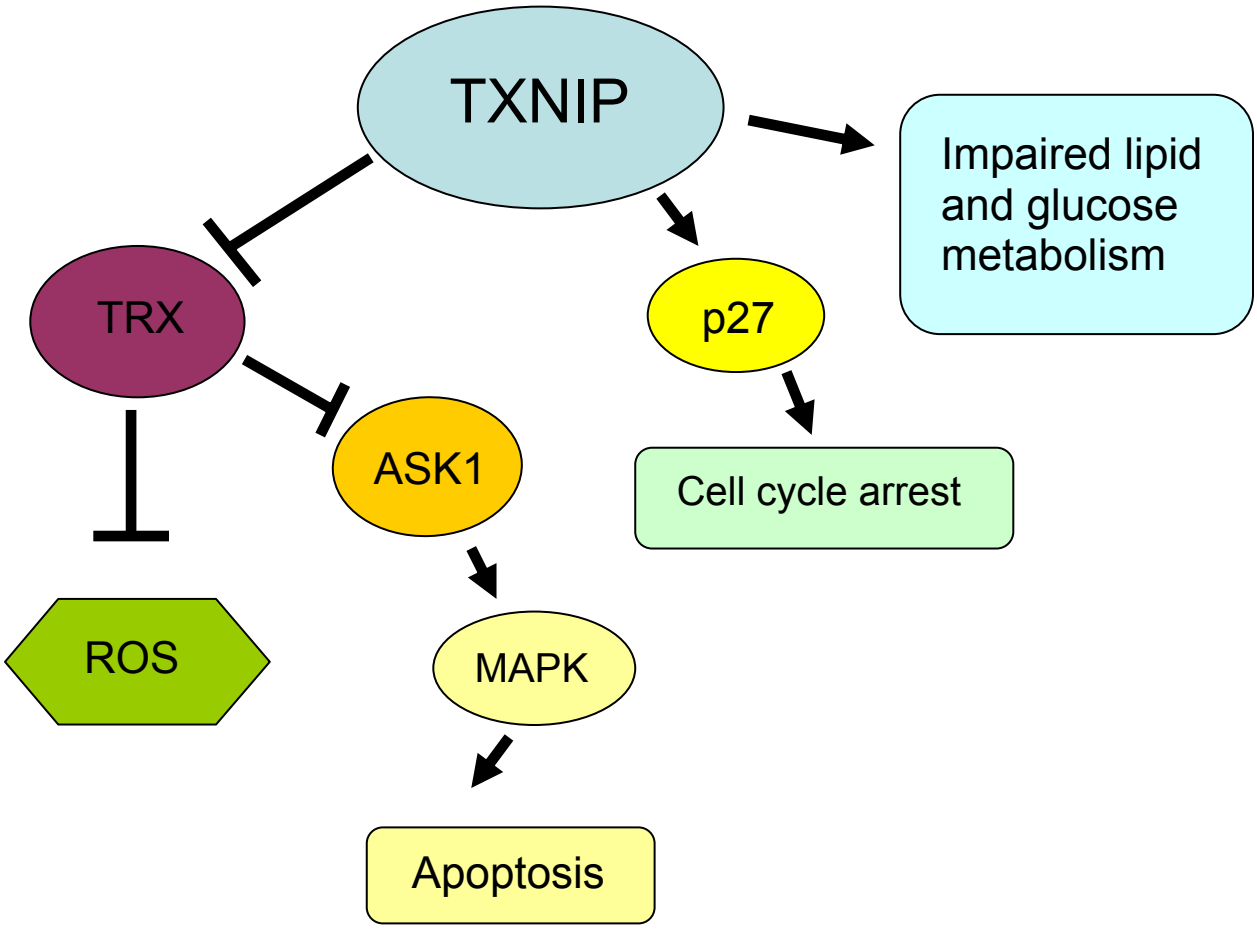
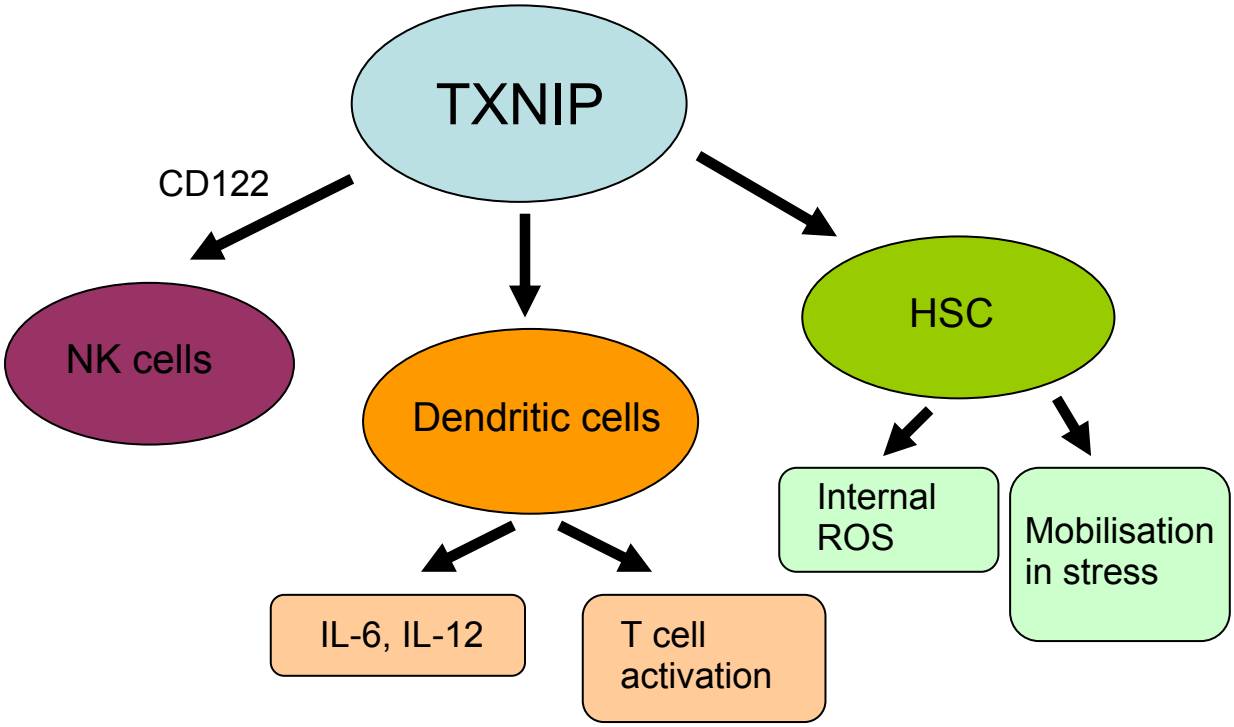


Figure 13. Roles of TXNIP in the hematopoietic compartment.

Schematic representation of functions of TXNIP in natural killer, dentritic and hematopoietic stem cells.



TXNIP also displayed a higher population of B cells in germinal centers following immunization, probably due to increased proliferation. Furthermore, TXNIP was shown to decrease the expression of BCL-6, a protein involved in formation of germinal centers [277].

TXNIP also plays a role in hematopoietic stem cells (HSCs). Its levels were shown to be high in long-term HSCs (LT-HSCs) and they decreased when those cells differentiated into short-term HSCs (ST-HSCs) and then to multipotent progenitors (MPPs). Aged TXNIP$^{-/-}$ mice displayed reduced LT-HSCs in the bone marrow compared to their wild-type counterparts. In addition, TXNIP$^{-/-}$ mice showed a significant reduction in the LT-HSC pool after treatment with fluorouracil (5-FU) along with a longer period of recovery of hematopoietic cell populations. The stress induced by 5-FU increased mobilization of HSCs in TXNIP$^{-/-}$ mice and reduced the proportion of quiescent LT-HSCs [195].

TXNIP$^{-/-}$ mice were also shown to display increased ROS levels in hematopoietic cells, suggesting an antioxidant function of TXNIP in this cellular type. These mice were also more sensitive to oxidative stress induced by paraquat treatment. The regulation of the oxidative stress response was mediated through interaction of TXNIP with p53 increasing its stability and transcriptional activity [196].

Specific research aims

The objectives of the research presented in this dissertation were to analyze the roles of a p45 NF-E2 and TXNIP in terminal erythroid differentiation. Many aspects of the role of p45 NF-E2 in late-stage erythroid differentiation remain elusive, while no studies have been reported with regards to TXNIP in this process. We have pursued our goal by:

A. Analyzing the phenotype of p45 NF-E2 knockout mice with regards to the distribution of specific erythroid cell populations and regulation of erythroid target genes.
B. Investigating the role of TXNIP in cellular models of erythroid differentiation and analyzing the erythroid phenotype of TXNIP knockout mice.

Chapter II: Materials and methods

1. Cells and culture conditions

1.1. MEL cells

Mouse erythroleukemia (MEL cells- clone 745 GM86) were maintained in Dulbecco's modified Eagle's medium with low glucose (DMEM low glucose) (Invitrogen) to which were added 10% fetal bovine serum (FBS) (Invitrogen), 100 units/mL of penicillin (Invitrogen) and 100 μg/mL of streptomycin (Invitrogen).

1.2. G1E and G1E-ER cells

G1E (parental) and G1E-ER cells were a kind gift from Dr. Mitchell Weiss [42]. Those cells were maintained in IMDM supplemented with 15% FBS (Invitrogen), 100 units/mL of penicillin (Invitrogen) and 100 μg/mL of streptomycin (Invitrogen). In addition, a 500 mL bottle of media was supplemented with 6.2μL in thioglycerol (Sigma), 2U/mL of EPO (Pharmacy) and conditioned media from CHO cells producing kit ligand.

For all experiments, MEL cells or G1E-ER cells were seeded at a density of 2×10^5 cells/mL.

2. Treatments

2.1. Induction of differentiation

MEL cells were induced to differentiate by treatment with 1.8% dimethyl sulfoxide (DMSO) (Sigma) or 4mM hexamethylene bisacetamide (HMBA) (Aldrich). G1E-ER cells were treated with 10nM β-estradiol (Sigma) to induce activation of the GATA-1/ER fusion protein and differentiation.

2.2. Inhibition of MAPKs

One hour before induction with 4 mM HMBA (Aldrich), MEL cells were treated with the following inhibitors of MAP kinases: 5μM of the Erk1/2 inhibitor UO126 (Promega), 3μM of the p38α/β MAPK inhibitor SB202190 (Calbiochem) or with 10μM of the JNK inhibitor SP600125 (Millipore).

2.3. NAC treatment

MEL cells were treated with the antioxidant N-acetylcysteine (NAC) (Sigma-Aldrich) at concentrations of 0.1nM, 1nM and 10nM for 6 hours concomitantly with 4mM HMBA (Sigma-Aldrich).

3. Plasmid construction and transfections

3.1. *Txnip* plasmids

Prior to cloning into pCRBluntII vector (Clontech) *Txnip* was amplified from total RNA from MEL cells using the following primers (Sigma):

Forward: 5'-ATCATGGTGATGTTCAAGAA-3'

Reverse: 5'- GGCTCACTGCACGTTGTTGT-3'

pCRBluntII-*Txnip* in the Sp6 orientation was then digested with SpeI and EcoRV, and the *Txnip* fragment was subcloned into pTRE2Hygro (Clontech) digested with NheI and EcoRV, generating the pTRE2-Txnip vector.

3.2. Generation of a stable MEL cell clone

MEL cells were transfected with the Tet-Off vector (Clontech) by electroporation and G418 (Multicell) was used in the selection media. Stable clones were selected and tested for inducibility by luciferase assay after transfection with pTRE2Luciferase vector (Clontech) and addition or not of Doxycyclin (Clontech). A clone with a good induction (termed Tet-OffC) was selected to be transfected with the pTRE2-Txnip vector and Hygromycin (Multicell), G418 (Multicell) and Doxycyclin (Clontech) were used in the selection media. Cells were screened for inducibility. We identified 2 stably transfected clones that induce *Txnip* in the absence of Dox.

4. Analysis of RNA and protein cell extracts

4.1. RNA isolation, reverse-transcription and real-time PCR

MEL cells treated or not with HMBA were collected in Trizol (Ambion) and RNA was isolated according to the manufacturer's protocol. RNA was digested with the enzyme DNAse I (Roche) and reverse transcription was performed with the Transcriptor First Strand cDNA synthesis kit (Roche) using random hexamers and following the instructions of the manufacturer. Quantitative

real-time PCR was performed using the Lightcycler Faststard DNA Master SYBR Green I kit (Roche). The following primers were used for *Txnip* and the 18S ribosomal subunit:

Txnip forward: 5'-ATCCCAGATACCCCAGAAGC-3'

Txnip reverse: 5'-TGAGAGTCGTCCACATCGTC-3'

18S forward: 5'-CTCAACACGGGAAACCTCAC-3'

18S reverse: 5'-CGCTCCACCAACTAAGAACG-3'

4.2. Protein isolation and immunoblotting

Cell pellets were resuspended in Laemmli sample buffer (0.16 M Tris-HCl [pH 6.8], 10% glycerol, 4% sodium dodecyl sulfate, and 5% β-mercaptoethanol). Protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were incubated overnight at 4°C with the primary antibodies. The antibodies used are directed against p45 NF-E2, GATA1 (Santa Cruz Biotechnology), TXNIP (MBL), transferrin receptor (Invitrogen), ferritin (Sigma), mtHsp70 (ABR), eIF4e (Cell signalling) or β-actin (Sigma). The secondary horseradish peroxidase−conjugated antibodies used were anti-rabbit, anti-mouse (Pierce/Thermo Fisher Scientific) or anti-rat (Santa Cruz Biotechnology).

5. Iron uptake and incorporation into heme

TXNIP overexpressing clone S2 was used to measure iron uptake. Doxycycline was removed or not from the media for 48h to induce Txnip overexpression. Then cells were incubated with HMBA for 72h. ^{59}Fe2-labelled Tf was prepared from ^{59}FeCl3 (PerkinElmer) and apo-Tf, as described previously [278]. Cells were incubated with ^{59}Fe for the last 24 h of the HMBA treatment. Then, cells were collected, lysed in water and total protein levels were determined. Measurement of ^{59}Fe in heme and non-heme fractions was carried out as described previously [279, 280]. Briefly, cells where collected by centrifugation, washed in ice-cold PBS, lysed in water and boiled in 1 ml of 0.2 M HCl. The samples were then transferred to an ice-bath and ^{59}Fe-labelled heme containing proteins were precipitated with ice-cold 7% TCA trichloroacetic acid (TCA), then collected by centrifugation. Precipitated proteins were washed with 7% TCA post-centrifugation; supernatants contained non-heme^{59}Fe. Radiolabelled iron measurements of ^{59}Fe in haem and non-haem fractions were carried out on a Packard Cobra γ-counter (PerkinElmer).

6. Mice

Mice deficient in p45 NF-E2 on a mixed 129S4/S6 background were a kind gift from Dr. Ramesh A. Shivdasani (Dana-Faber Cancer Institute, Harvard Medical School) [129]. Mice knockout for TXNIP on a pure C57/BL6 background were provided by Dr. Simon Hui (UCLA). Mice were kept at 22°C with equal periods of light and darkness. Water and food were available ad libitum. All procedures were conducted according to McGill University guidelines, which are set by the Canadian Council on Animal Care.

7. Blood analysis and EPO treatment

For blood analysis the collection was done from the sephanous vein. The samples were run immediately on a VET ABC machine (Vet Novations). For the EPO treatment, mice were injected intraperitoneally with 3000 units/kg of EPO (EPREX) 3 times a week for 3 weeks.

8. EPO assay

Blood from WT and p45 NF-E2$^{-/-}$ mice was collected by cheek bleed. After overnight coagulation at 4°C, blood was spun down at 1000*g* for 20 minutes at 4°C and the supernatant was collected as serum and stored at −80°C until further analysis. EPO concentration in the serum was determined by enzyme-linked immunosorbent assay (ELISA) using a mouse/rat EPO immunoassay (R&D Systems) following manufacturer's instructions.

9. Flow cytometry

Cells were collected from bone marrow through flushing with a 26-gauge needle and spleen by mechanical dissociation before passing through a 100-μm cell strainer (BD Biosciences). Single-cell suspensions were then blocked with a CD16/CD32 antibody (1 μg/mL) before staining with a fluorescein isothiocyanate–conjugated antibody against CD71 (1 μg/mL) and a phycoerythrin-conjugated antibody against Ter119 (1 μg/mL) (BD Biosciences). 7-Aminoactinomycin D (BD Biosciences) was used for the exclusion of dead cells. Samples were analyzed on a FACSCalibur (BD Biosciences).

10. Cell cycle analysis

Cells were collected from the bone marrow of 6- to 7-week-old mice and passed through a 100-μm cell strainer. Cell surface staining for CD71 and Ter119 was performed as described above. After a wash in 1× phosphate-buffered saline/2% fetal bovine serum cells were resuspended in 500 μL of a solution of phosphate-buffered saline/2% fetal bovine serum/0.03% saponin, with the addition of 5 μL of a 7-aminoactinomycin D solution (BD Biosciences). After 30 minutes incubation at 37°C, cells were kept on ice and analyzed using a FACSCalibur (BD Biosciences). Data were interpreted using Flowjo 7.6.1 software (Tree Star Inc.).

11. Cell enrichment with magnetic beads

Cells from bone marrow of 6- to 7-week-old mice were incubated with anti-Ter119 antibodies conjugated to magnetic beads (BD Biosciences) and isolated according to manufacturer's instructions. For separation, we used the MagneSphere Magnetic Separation Stand (Promega).

12. Gel quantification and statistics

Gels were quantified using the Image Lab software. Where applicable, data from experiments are expressed as mean ± standard error of the mean. Analysis was performed using Student's two-tailed *t* test and $p < 0.05$ was considered significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Chapter III: Results

A. Novel role of p45 NF-E2 in erythroid differentiation

A.1. Terminal erythroid differentiation in spleen of wild-type and p45 NF-E2$^{-/-}$ mice

Under normal conditions, red blood cells are mainly produced by the bone marrow of mice, but the spleen can function as a secondary organ in situation of increased demand or anemic stress [281]. Studies from other laboratories have revealed splenomegaly in p45 NF-E2$^{-/-}$ mice [130]. We analyzed cells from spleens of 6- to 7-week and 3- to 4-month-old mice using flow cytometry. We identified erythroid precursors at the final stages of differentiation with the cell surface markers Ter119 (erythroid marker) and CD71 (transferrin receptor), as well as cell size (forward scatter [FSC]) using a previously described method [56]. This approach allows classifying Ter119^{+} cells into four subpopulations, namely, proerythroblasts (ProE) (Ter119medCD71highFSChigh), basophilic erythroblasts (EryA) (Ter119highCD71highFSChigh), late basophilic and polychromatic erythroblasts (EryB) (Ter119highCD71highFSClow), and orthochromatic erythroblasts and erythrocytes (EryC) (Ter119highCD71lowFSClow). We observed a different distribution of erythroid precursors when comparing the spleens of WT and p45 NF-E2$^{-/-}$ mice **(Figure 14)**. Compiling data from a series of mice, we observed an increase in the proportion of early Ter119^{+} populations, which was statistically significant for the EryB population and a proportional decrease in the most mature EryC population **(Figure 15A)**. In addition, the proportion of Ter119^{+} cells on total cells in the spleen was increased by 1.4-fold in 6- to 7-week-old and by 1.3-fold in 3- to 4-month-old knockout mice **(Figure 15B)**. We conclude that p45 NF-E2$^{-/-}$ mice display altered splenic erythropoiesis in steady-state conditions.

Figure 14. Altered splenic erythropoiesis in p45 NF-E2$^{-/-}$ mice.

Representative flow cytometry analysis of the erythroid precursor populations isolated from the spleen of a WT and a p45 NF-E2$^{-/-}$ mouse (3- to 4-month old). CD71 (transferrin receptor) and Ter119 are markers for different stages of erythroid cell precursors. The Ter119-high population was further analyzed for forward scatter (FSC) and CD71 expression.

EryA. basophilic erythroblasts; EryB. late basophilic and polychromatic erythroblasts; EryC. orthochromatic erythroblasts and erythrocytes; ProE. proerythroblasts.

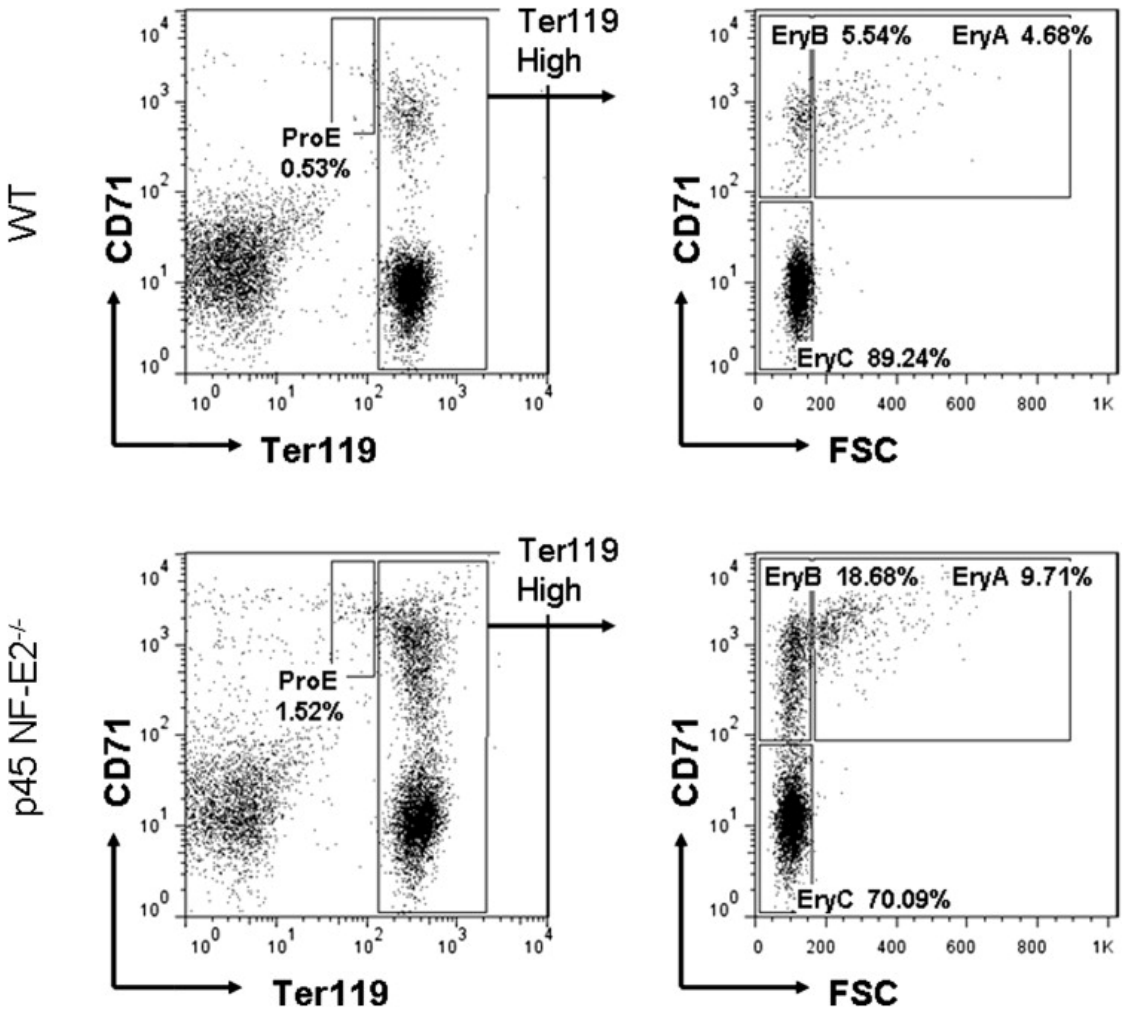
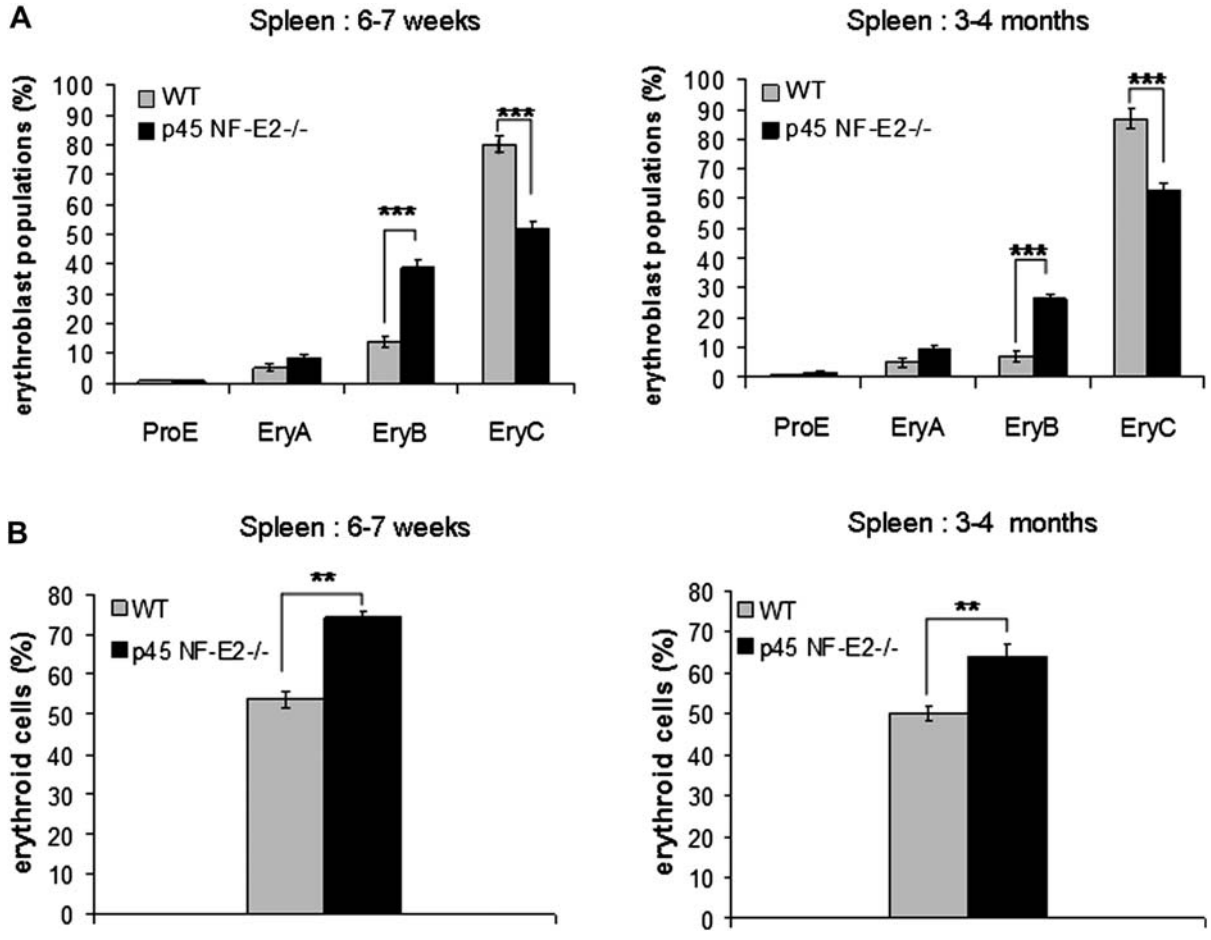

WT
p45 NF-E2-/-
CD71
Ter119
Ter119 High
ProE 0.53%
EryB 5.54%
EryA 4.68%
EryC 89.24%
ProE 1.52%
EryB 18.68%
EryA 9.71%
EryC 70.09%
FSC

Figure 15. Increase in the proportion of the EryB population in p45 NF-E2$^{-/-}$ mice.

Spleen cells from wild-type (WT) and p45 NF-E2$^{-/-}$ mice were stained with antibodies against CD71 and Ter119. Cells were analyzed by flow cytometry. (**A**) Percentage of cells in the different erythroblast populations in spleen of WT and p45 NF-E2$^{-/-}$ mice. Average from spleens of 6- to 7-week-old mice (WT n = 11, p45 NF-E2$^{-/-}$ n = 10) and 3- to 4-month-old mice (WT n = 5, p45 NF-E2$^{-/-}$ n = 6). (**B**) Percentage of erythroid cells (ProE, EryA, EryB, and EryC) on total cell number. Average from spleens of 6- to 7-week-old mice (WT n = 11, p45 NF-E2$^{-/-}$ n = 10) and 3- to 4-month-old mice (WT n = 5, p45 NF-E2$^{-/-}$ n = 6). Student's *t* test: $^{**}p < 0.01$; $^{***}p < 0.001$.

EryA. basophilic erythroblasts; EryB. late basophilic and polychromatic erythroblasts; EryC. orthochromatic erythroblasts and erythrocytes; ProE. proerythroblasts.

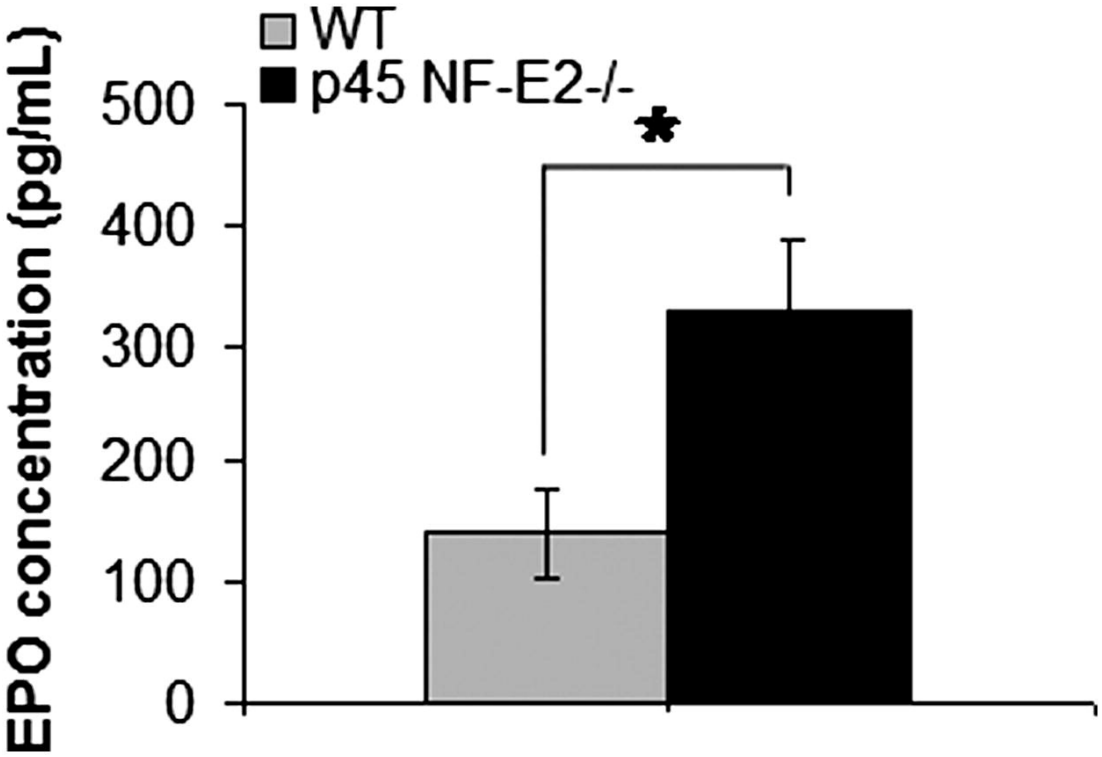

A
Spleen : 6-7 weeks
erythroblast populations (%)
WT
p45 NF-E2-/-
***
***
ProE
EryA
EryB
EryC
Spleen : 3-4 months
erythroblast populations (%)
WT
p45 NF-E2-/-
***
***
ProE
EryA
EryB
EryC
B
Spleen : 6-7 weeks
erythroid cells (%)
WT
p45 NF-E2-/-
**
Spleen : 3-4 months
erythroid cells (%)
WT
p45 NF-E2-/-
**

A.2. Erythropoietin in p45 NF-E2$^{-/-}$ mice

EPO is a 34-kDa glycoprotein, which is a major regulator of erythropoiesis in steady-state conditions and in the presence of anemia. EPO levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit specifically designed for the mouse protein in order to increase accuracy and sensitivity. We observed a more than twofold increase in serum erythropoietin levels in p45 NF-E2$^{-/-}$ mice compared to wild-type mice **(Figure 16)**.

Figure 16. Increased EPO levels in the serum of p45 NF-E2$^{-/-}$ mice.

EPO levels were measured by enzyme-linked immunosorbent assay (ELISA) in the serum of 3- to 4-month-old wild-type (WT) and p45 NF-E2$^{-/-}$ mice; WT n = 9 and p45 NF-E2$^{-/-}$ n = 9. Student's *t* test: $^{*}p < 0.05$.

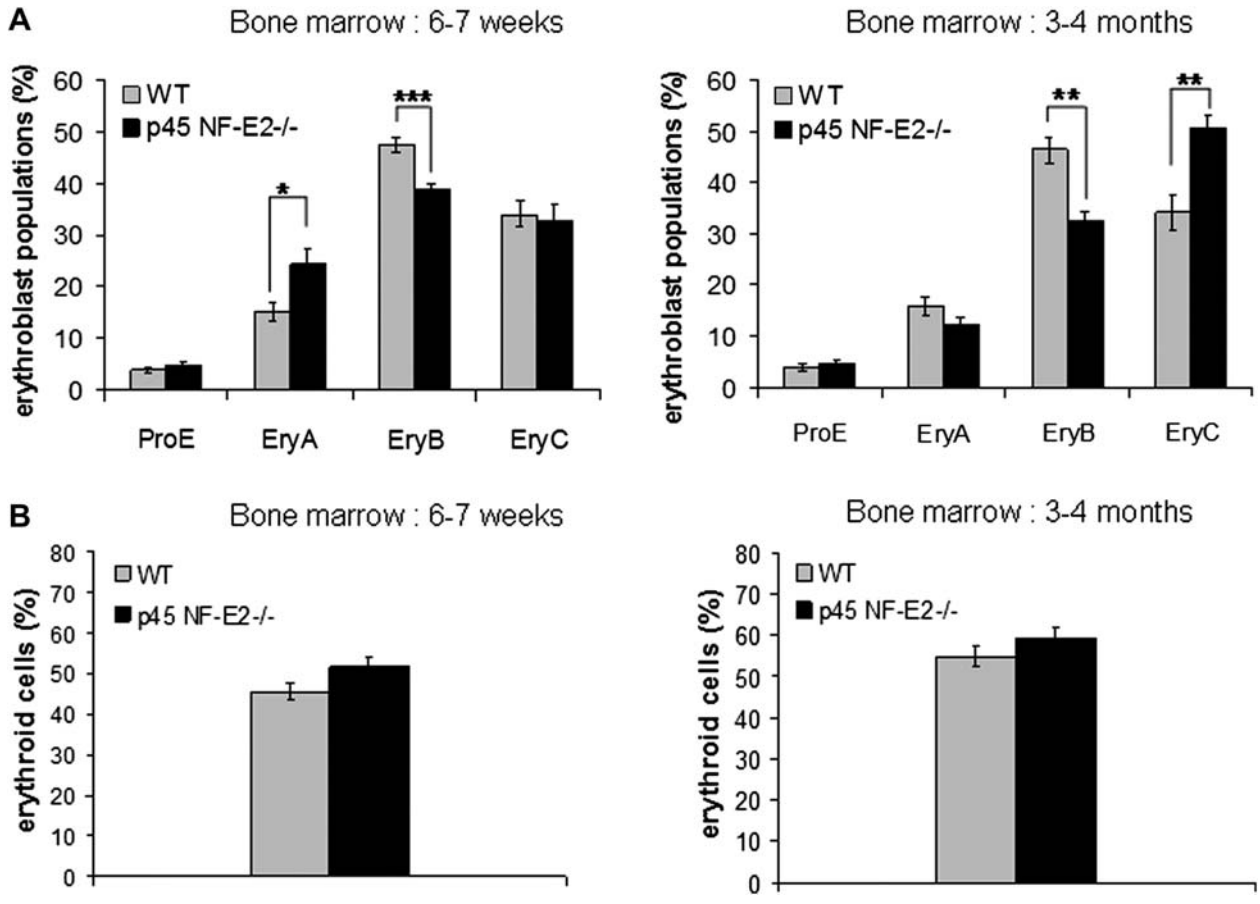

WT
p45 NF-E2-/-
EPO concentration (pg/mL)
500
400
300
200
100
0
*

A. 3. Bone marrow erythropoiesis in p45 NF-E2 knockout mice

Under normal physiological conditions, the majority of erythropoiesis takes place in the bone marrow and we sought to investigate this hematopoietic compartment. We observed significant differences in the distribution of erythroid precursors in the bone marrow when comparing age-matched p45 NF-E2$^{-/-}$ and WT animals. In 3- to 4-month-old mice, we detected a decrease in the EryB population and a proportional increase in the more mature EryC population **(Figure 17A)**. At that age, the mice displayed increased bone mass in the femur, which has been reported previously [127]. Cellularity of the bone marrow in p45 NF-E2$^{-/-}$ mice was greatly reduced due to calcification [127]. Therefore, we analyzed younger mice before the onset of calcification to eliminate the differences that might arise from the change in bone composition. Analysis of 6- to 7-week-old mice revealed an increase in the proportion of EryA cells and a decrease in the proportion of EryB cells **(Figure 17A)**, suggesting a partial differentiation block between these two stages in the bone marrow compartment. On the other hand, the proportion of erythroid cells to total cell number was not significantly modulated in the bone marrow of p45 NF-E2$^{-/-}$ mice **(Figure 17B)**.

Figure 17. Modified erythroid precursor distribution in bone marrow of p45 NF-E2$^{-/-}$ mice.

Bone marrow cells from wild-type (WT) and p45 NF-E2$^{-/-}$ mice were stained with antibodies against CD71 and Ter119. Cells were analyzed by flow cytometry. (**A**) Percentage of cells in the different erythroblast populations in bone marrow of WT and p45 NF-E2$^{-/-}$ mice. Average from bone marrow of 6- to 7-week-old mice (WT n = 11, p45 NF-E2$^{-/-}$ n = 10) and 3- to 4-month-old mice (WT n = 5, p45 NF-E2$^{-/-}$ n = 6). (**B**) Percentage of erythroid cells (ProE, EryA, EryB, and EryC) on total cell number. Average from bone marrow of 6- to 7-week-old mice (WT n = 11, p45 NF-E2$^{-/-}$ n = 10) and 3- to 4-month-old mice (WT n = 5, p45 NF-E2$^{-/-}$ n = 6). Student's *t* test: $^{**}p < 0.01$. EryA. basophilic erythroblasts; EryB. late basophilic and polychromatic erythroblasts; EryC. orthochromatic erythroblasts and erythrocytes; ProE. proerythroblasts.


A
Bone marrow : 6-7 weeks
Bone marrow : 3-4 months
erythroblast populations (%)
WT
p45 NF-E2-/-
ProE
EryA
EryB
EryC
*
***
**
**
B
Bone marrow : 6-7 weeks
Bone marrow : 3-4 months
erythroid cells (%)
WT
p45 NF-E2-/-

A.4. GATA1 expression and cell cycle in p45 NF-E2$^{-/-}$ mice

To define the mechanism of altered erythroid differentiation in p45 NF-E2$^{-/-}$ mice, we analyzed protein extracts from total bone marrow cells of knockout animals and observed an increase in the levels of GATA1 transcription factor, one of the master regulators of erythropoiesis **(Figure 18A)**. To determine whether this increase was specific to erythroid precursor cells, we performed magnetic cell sorting using the Ter119 surface marker. The Ter119^{+} population of p45 NF-E2$^{-/-}$ mice displayed higher GATA1 protein levels compared to WT mice **(Figure 18B)**.

Because in previous studies overexpression of GATA1 has been associated with cell cycle arrest at the G_1 phase [43], and because cell cycle control is an important step in differentiation, we analyzed the cell cycle profiles of erythroid cells. We examined bone marrow cells from 6- to 7-week-old WT and p45 NF-E2$^{-/-}$ mice. We performed cell-surface staining of erythroid precursors using the surface markers Ter119 and CD71. We then permeabilized the cells with saponin, allowing the labeling of live nonfixed cells, and stained DNA using 7-aminoactinomycin D. For p45 NF-E2$^{-/-}$ mice, we observed an increase in the erythroid precursors arrested at the G_0/G_1 phase of cell cycle **(Figure 19)**.

Figure 18. Increased GATA1 expression in the absence of p45 NF-E2.

(**A**) Protein levels of GATA1 were analyzed in bone marrow whole cell extracts from different 6- to 7-week-old wild-type (WT) and p45 NF-E2$^{-/-}$ mice. Actin was used as loading control. (**B**) Protein levels of GATA1 were analyzed in extracts from Ter119^{+} cells magnetically sorted from bone marrow of different WT and p45 NF-E2$^{-/-}$ mice. Actin was used as loading control.

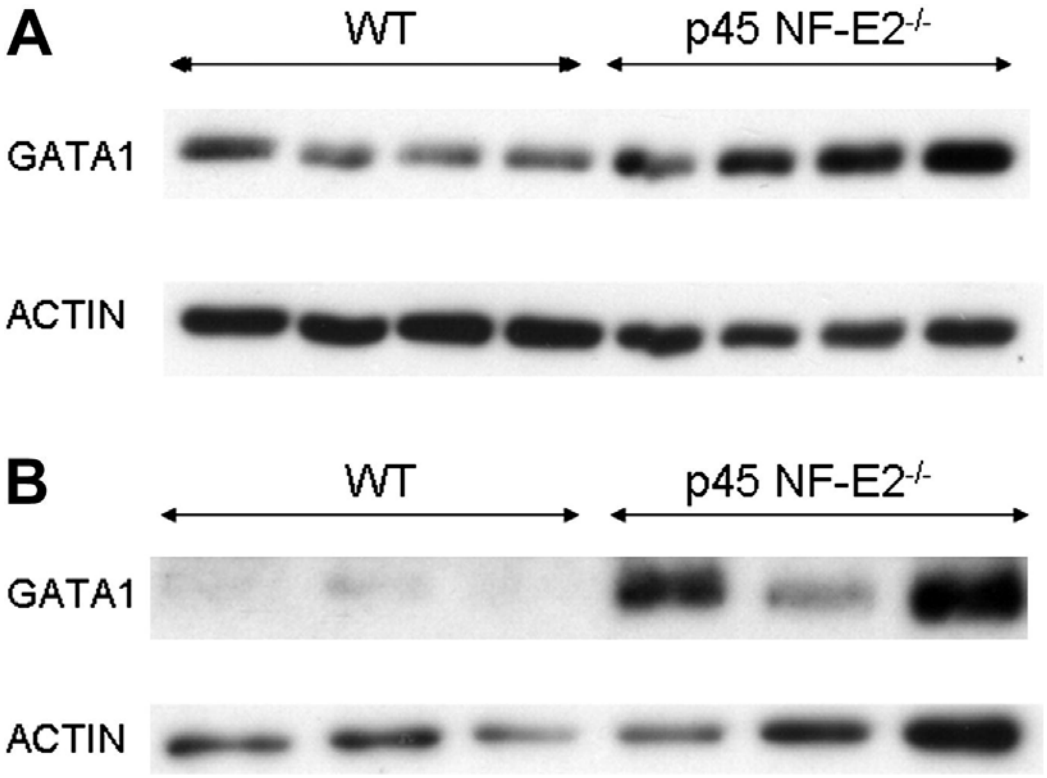
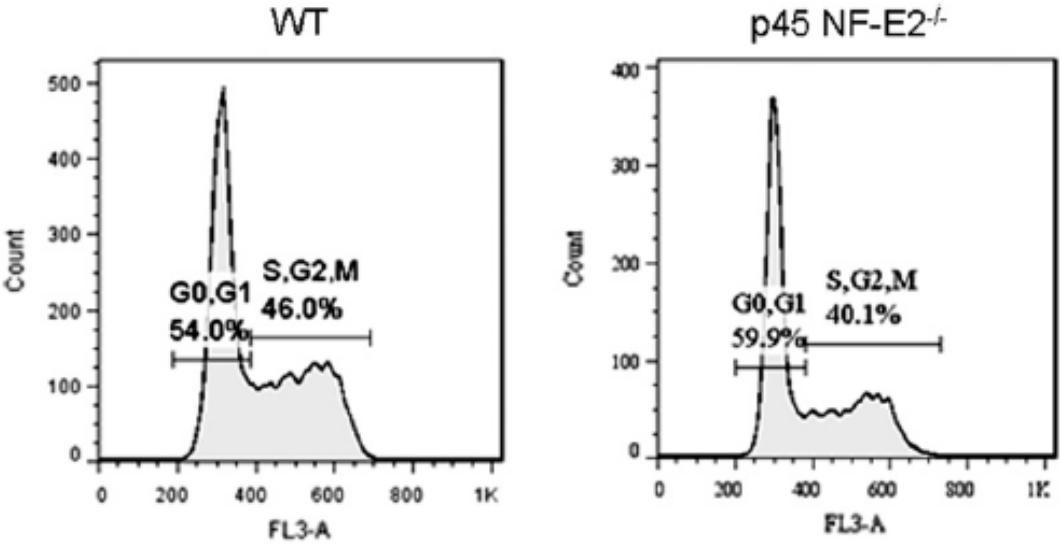

A
WT
p45 NF-E2-/-
GATA1
ACTIN
B
WT
p45 NF-E2-/-
GATA1
ACTIN

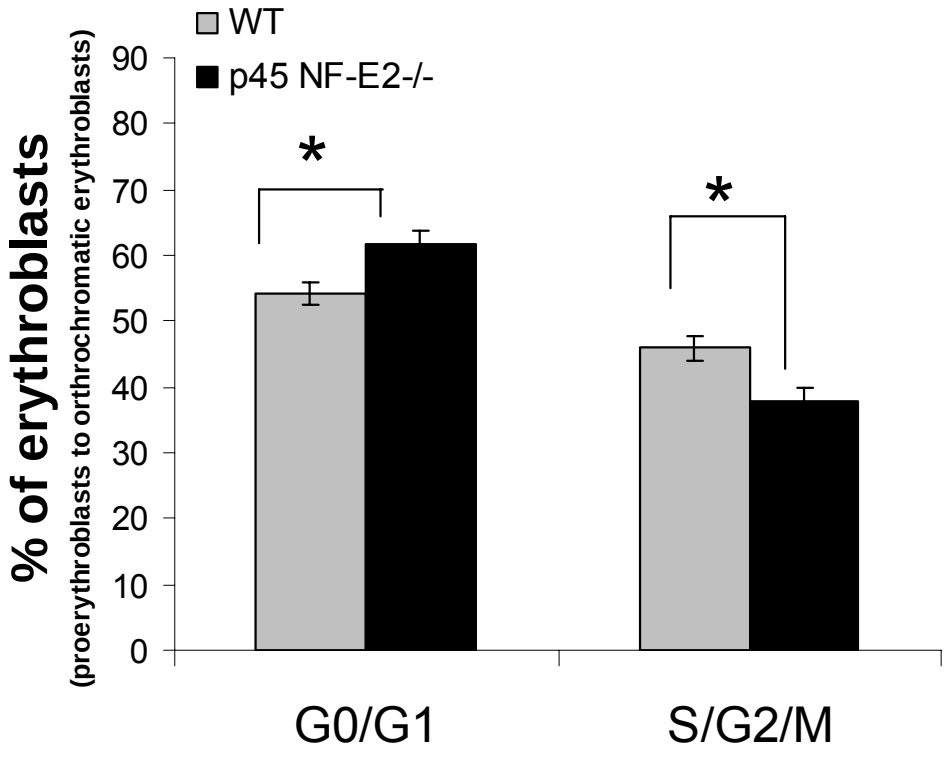
Figure 19. Modified cell cycle profile in the absence of p45 NF-E2.

Cells from bone marrow of wild-type (WT) and p45 NF-E2$^{-/-}$ mice were stained for the erythroid precursor markers CD71 and Ter119, then permeabilized and stained with 7-aminoactinomycin D for DNA content. (**A**) Typical cell cycle profile of a WT and p45 NF-E2$^{-/-}$ mouse for all erythroblasts (proerythoblasts to orthochromatic erythroblasts). (**B**) Analysis of cell cycle in erythroblasts of WT (n = 5) and p45 NF-E2$^{-/-}$ (n = 5) mice. Student's *t* test: $^{*}p < 0.05$.

A


WT
p45 NF-E2-/-
Count
G0,G1
54.0%
S,G2,M
46.0%
G0,G1
59.9%
S,G2,M
40.1%
FL3-A

B


WT
p45 NF-E2-/-
% of erythroblasts
(proerythroblasts to orthrochromatic erythroblasts)
*
*
G0/G1
S/G2/M

B. TXNIP as a novel erythroid regulator

B.1. TXNIP expression in erythroid differentiation.

In the quest for novel regulators of erythroid differentiation, we analyzed the arrestin protein TXNIP, one of the transcripts shown to be upregulated in MEL cells upon differentiation with DMSO in a microarray performed previously in the lab. MEL cells induced to differentiate by treatment with the polar compound HMBA displayed highly increased levels of *Txnip* transcripts as assessed by real-time PCR **(Figure 20)**. The increase of transcripts was accompanied by an induction of TXNIP protein, which was observed quickly, a few hours following the start of treatment **(Figure 21)**. Similarly, the differentiating agent DMSO also led to an increase in TXNIP protein in MEL cells **(Figure 21)**. The upregulation of TXNIP protein was visible as soon as 4 hours following treatment and peak levels were observed after 6 hours of treatment **(Figure 21)**. At later time-points, although levels of TXNIP tend to decrease in both control and induced MEL cells, they remain higher in cells treated with inducing agents compared to control cells for each time-point analyzed **(Figure 21)**.

The induction of TXNIP was also confirmed at the protein level in a different model of erythroid differentiation. The G1E-ER cells that can be treated with estradiol to activate GATA1 and to induce differentiation also displayed increased TXNIP protein following induction. On the other hand, the parental cell line G1E devoid of GATA1 and unable to differentiate did not exhibit a change in TXNIP levels, confirming that the increase of TXNIP in G1E-ER cells was not caused by an unspecific effect of estradiol **(Figure 22)**.

Figure 20. Induction of *Txnip* transcripts in differentiating MEL cells.

Real-time analysis of *Txnip* transcript levels in extracts form MEL cells treated with 4mM HMBA for 6, 24 and 48 hours. For each time point of treatment the levels of *Txnip* transcripts in control cells have been set to 1. Transcripts for the 18S ribosomal subunit have been used as control. The results are an average of 3 independent experiments. Student's *t* test: ** $p < 0.01$, *$p < 0.05$.

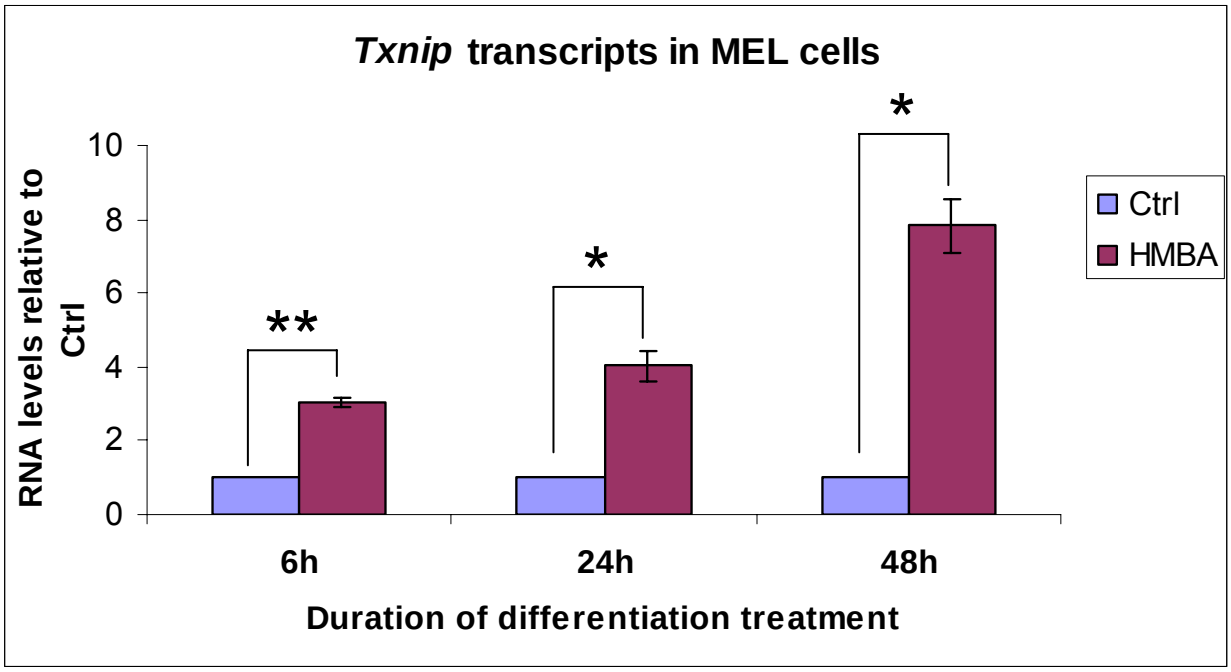
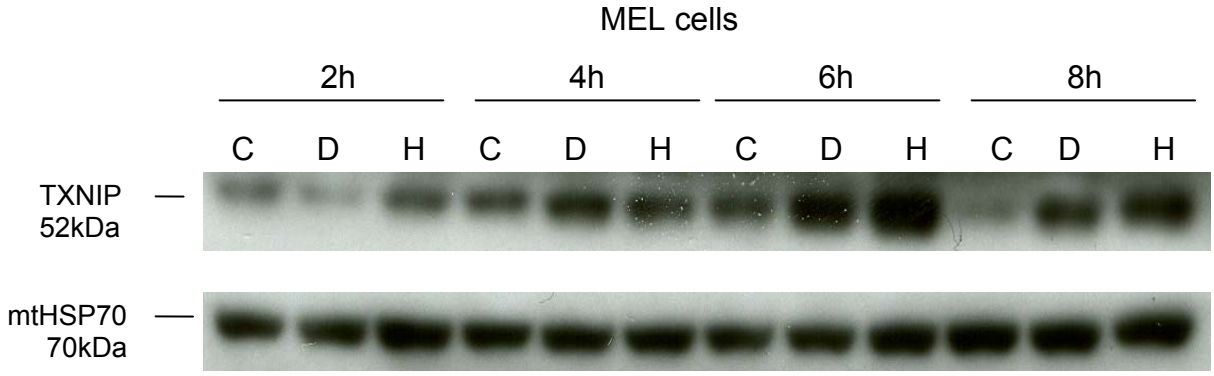

Txnip transcripts in MEL cells
RNA levels relative to Ctrl
10
8
6
4
2
0
**
*
*
6h
24h
48h
Duration of differentiation treatment
Ctrl
HMBA

Figure 21. TXNIP protein levels are increased in differentiating MEL cells.

Immunoblot analysis of whole cell extracts of MEL cells induced or not with 1.8% DMSO or 4mM HMBA for 2, 4, 6, 8, 12, 24 and 48 hours. Mitochondrial HSP70 (mtHSP70) has been used as loading control.

C: Control, D: DMSO, H: HMBA


MEL cells
2h
4h
6h
8h
C D H C D H C D H C D H
TXNIP
52kDa
mtHSP70
70kDa

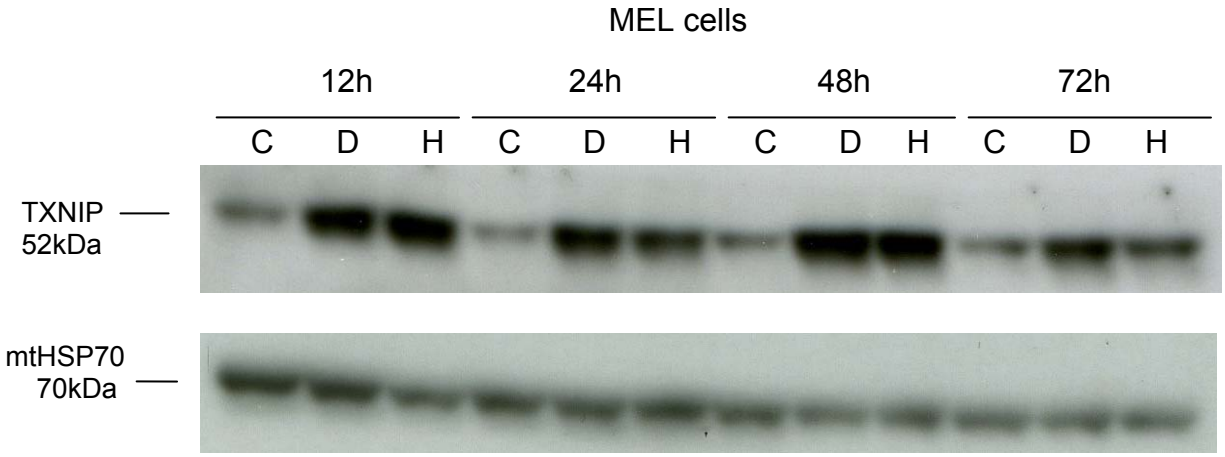
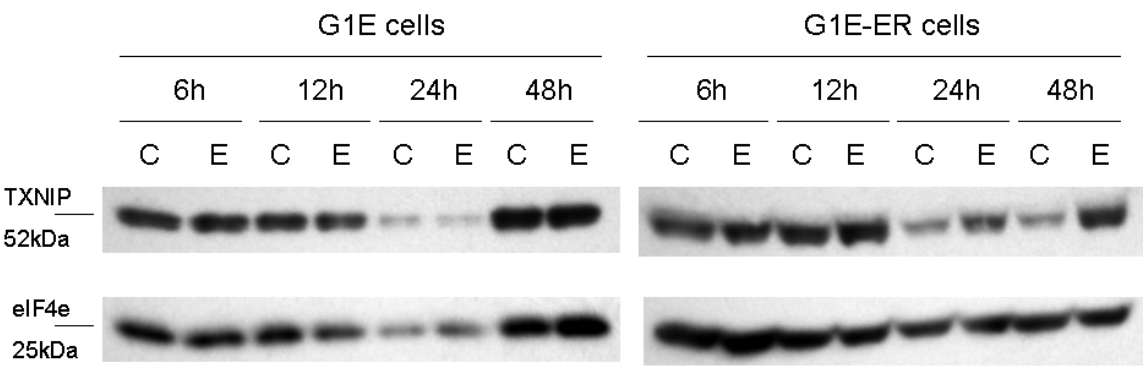

MEL cells
12h
24h
48h
72h
C D H C D H C D H C D H
TXNIP
52kDa
mtHSP70
70kDa

Figure 22. TXNIP protein levels are increased in differentiating G1E-ER cells.

Immunoblot analysis of whole cell extracts of parental G1E and G1E-ER cells, which have been induced or not with β-estradiol (10^{-8}M) for 6, 12, 24 and 48 hours. Levels of eIF4e protein have been used as a loading control. The blot is representative of at least 3 experiments.

C: Control, E: Estradiol


G1E cells
G1E-ER cells
6h
12h
24h
48h
6h
12h
24h
48h
C E C E C E C E C E C E C E C E
TXNIP
52kDa
eIF4e
25kDa

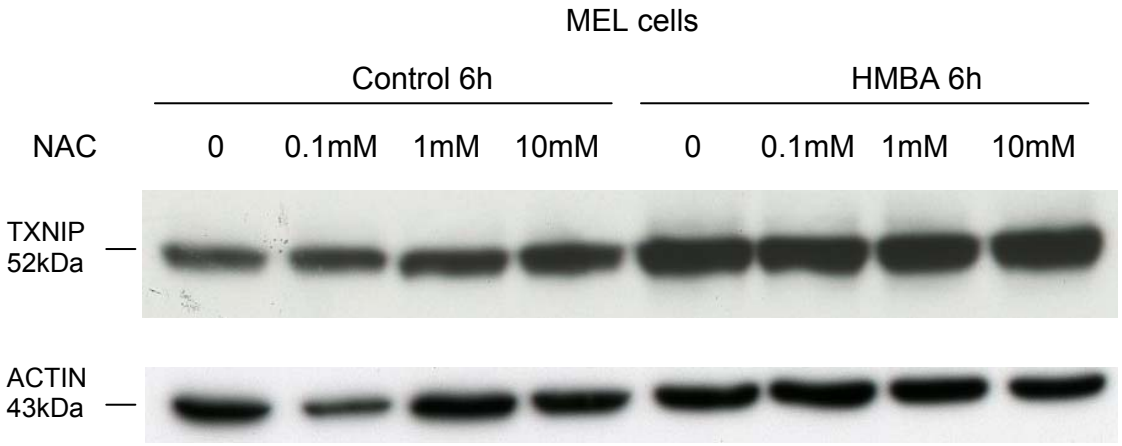
B.2. Effect of ROS on TXNIP induction in differentiating MEL cells.

Since TXNIP is a known partner of thioredoxin, a major component of the ROS scavenging system, we tested whether the upregulation of TXNIP in the presence of HMBA was due to the fact that this inducer generates ROS. In control cells, the use of N-acetylcysteine (NAC), an antioxidant, to reduce ROS, slightly upregulated TXNIP protein. However, the significant induction of TXNIP observed with HMBA treatment after 6 hours was not altered by the addition of NAC **(Figure 23)**.

Figure 23. TXNIP upregulation in the presence of the ROS scavenger NAC.

Immunoblot analysis of whole cell extracts of MEL cells induced or not with 4mM HMBA for 6 hours in the presence of NAC (0.1mM, 1mM and 10mM). Actin was used as loading control. The blot is representative of at least 3 independent experiments.

NAC: N-acetylcysteine


MEL cells
Control 6h
HMBA 6h
NAC
0
0.1mM
1mM
10mM
0
0.1mM
1mM
10mM
TXNIP
52kDa
ACTIN
43kDa

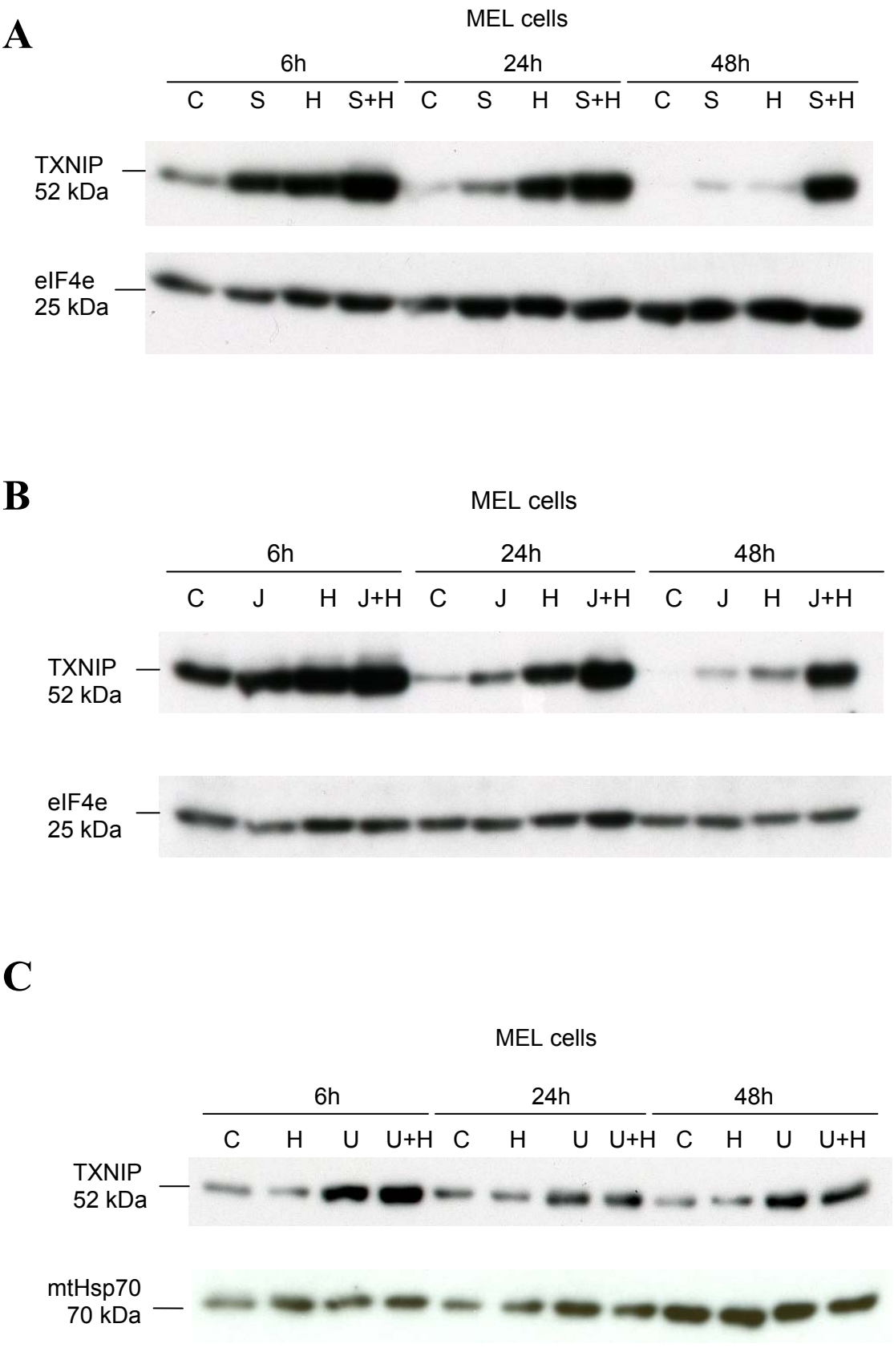
B.3. Regulation of TXNIP by MAP kinases in MEL cells.

Since previous studies in other cell types have shown that TXNIP levels are controlled by the p38 MAPK [231], we sought to investigate whether TXNIP was regulated by MAP kinases in MEL cells. The inhibition of p38 MAPK and JNK led to an increase in TXNIP protein levels and acted synergistically with HMBA to yield an even greater upregulation **(Figure 24A&B)**. In contrast, inhibiting ERK1/2 MAPKs did not alter the levels of TXNIP protein **(Figure 24C)**. Our data suggest that in MEL cells, p38 MAPKs and JNK act as negative regulators of TXNIP, while ERK MAPKs are not involved in the regulation of the expression of this protein.

Figure 24. TXNIP protein is upregulated in MEL cells treated with inhibitors of the MAPKs p38 and JNK, but not ERK.

Immunoblot analysis of whole cell extracts of MEL cells induced or not with 4mM HMBA for 6, 24 and 48 hours. Where indicated, cells have been treated with the **(A)** p38 inhibitor SB202190 (5μM) or **(B)** JNK inhibitor SP600125 (10μm) or **(C)** ERK inhibitor UO126 (3μM) 1h prior to the HMBA induction. The results are representative of at least 3 experiments. Mitochondrial Hsp70 (mtHsp70) or eIF4e were used as loading controls.

C: Control, H: HMBA, S: SB202190 (p38 inhibitor), J: SP600125 (JNK inhibitor), U: UO126 (ERK inhibitor)


A
MEL cells
6h
24h
48h
C S H S+H C S H S+H C S H S+H
TXNIP
52 kDa
eIF4e
25 kDa
B
MEL cells
6h
24h
48h
C J H J+H C J H J+H C J H J+H
TXNIP
52 kDa
eIF4e
25 kDa
C
MEL cells
6h
24h
48h
C H U U+H C H U U+H C H U U+H
TXNIP
52 kDa
mtHsp70
70 kDa

B.4. Regulation of iron uptake by TXNIP

We next analyzed whether TXNIP could play a role with regards to iron uptake or heme incorporation in MEL cells. Using a stable clone, in the presence of overexpression of TXNIP we observed a 1.2-fold increased iron uptake into cells treated with HMBA, while the percentage of incorporation of iron into heme remained unchanged **(Figure 25A)**. The protein levels of the transferrin receptor were downregulated upon overexpression of TXNIP in HMBA treated cells **(Figure 25B)**.

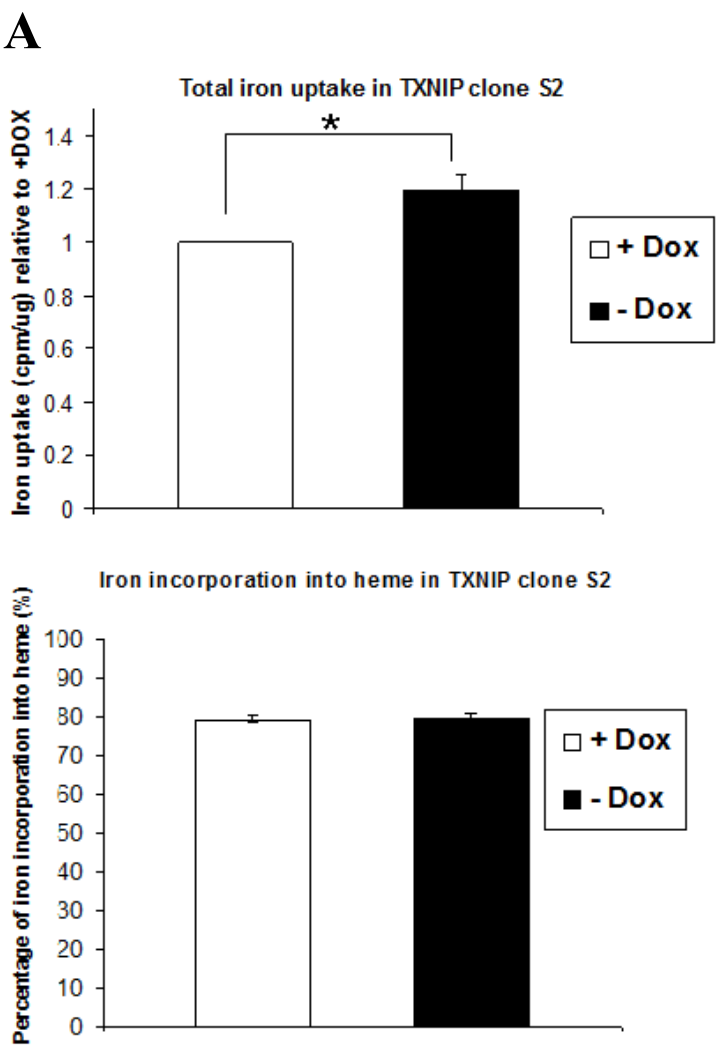
Figure 25. TXNIP overexpression increases iron uptake and decreases TFR expression.

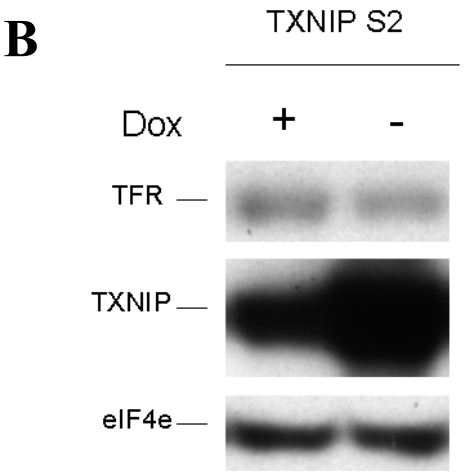
A) MEL TXNIP stable clone S2 was induced to overexpress TXNIP by removal of DOX (-DOX) (Tet-Off system). Subsequently, cells were treated with 4 mM HMBA for 72 hours. Radioactive iron uptake and incorporation into heme in the last 24 hours of the treatment were measured. Results are an average ± SEM of 4 independent experiments. Obtained counts (CPM) were normalized to μg of protein and expressed relative to the values in the absence of overexpression (+DOX). n = 4; *p-value < 0,05

CPM. Counts per minute

B) MEL TXNIP stable clone S2 was induced to overexpress TXNIP by removal of DOX (-DOX) (Tet-Off system). Subsequently, cells were treated with 4 mM HMBA for 72 hours. Immunoblot of whole cell extracts was performed. The blot is representative of at least 3 independent experiments. The loading control used was eIF4e.

Figure 25.


A
Total iron uptake in TXNIP clone S2
Iron uptake (cpm/ug) relative to +DOX
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+ Dox
- Dox
Iron incorporation into heme in TXNIP clone S2
Percentage of iron incorporation into heme (%)
100
90
80
70
60
50
40
30
20
10
0
+ Dox
- Dox


B
TXNIP S2
Dox
+
-
TFR
TXNIP
eIF4e

B.5. Erythroid phenotype of TXNIP $^{-/-}$ mice.

Since our results in cellular models suggested a modulation of TXNIP in erythroid cells, we sought to investigate the phenotype of TXNIP knockout mice (provided by Dr. Hui from UCLA). It has been reported previously that one year old TXNIP$^{-/-}$ mice display splenomegaly [195]. This condition can also be observed in anemia or reduced erythropoiesis, when the spleen expands and acts as a secondary organ of erythropoiesis to compensate. We confirmed increased spleen mass in TXNIP$^{-/-}$ mice. At the age of 6 months, the spleen represented 0.257% of total body mass in wild-type mice and 0.327% in TXNIP$^{-/-}$ mice **(Figure 26A)**. Blood analysis of those mice did not reveal significant differences in major erythroid parameters, such as red blood cell counts, hematocrit or hemoglobin levels **(Figure 26B)**. A small but statistically significant increase was observed for the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) parameters in TXNIP$^{-/-}$ mice at 6 months **(Figure 26B)**, although not recapitulated in older one year old mice (data not shown).

Analysis of terminal erythroid differentiation using a flow cytometry protocol revealed that TXNIP$^{-/-}$ mice present a greatly reduced proportion of EryB cells. In the spleen, this population represented 11.1% of total erythroid precursors (Ter119+cells) in wild-type mice, while it decreased to 6.7% in TXNIP$^{-/-}$ mice. This suggests a partial block in the transition between the EryA, enriched in basophilic erythroblasts, and EryB, enriched in late basophilic and polychromatic erythroblast cells. A similar block was observed in the bone marrow, although to a lesser extent and not statistically significant **(Figure 27)**.

Based on a previously reported study, which showed that treatment of mice with EPO is reducing the blood glucose [282] and given that TXNIP is highly upregulated in response to high glucose, we investigated the response of the knockout mice in response to EPO (EPREX) injected 3 times a week for three weeks at a dose of 3000U/kg. Red blood cells parameters, such as the hematocrit or hemoglobin, were similarly induced in response to EPO treatment in wild-type and $TXNIP^{-/-}$ mice, only a mild difference in the mean corpuscular volume was observed **(Table 4)**.

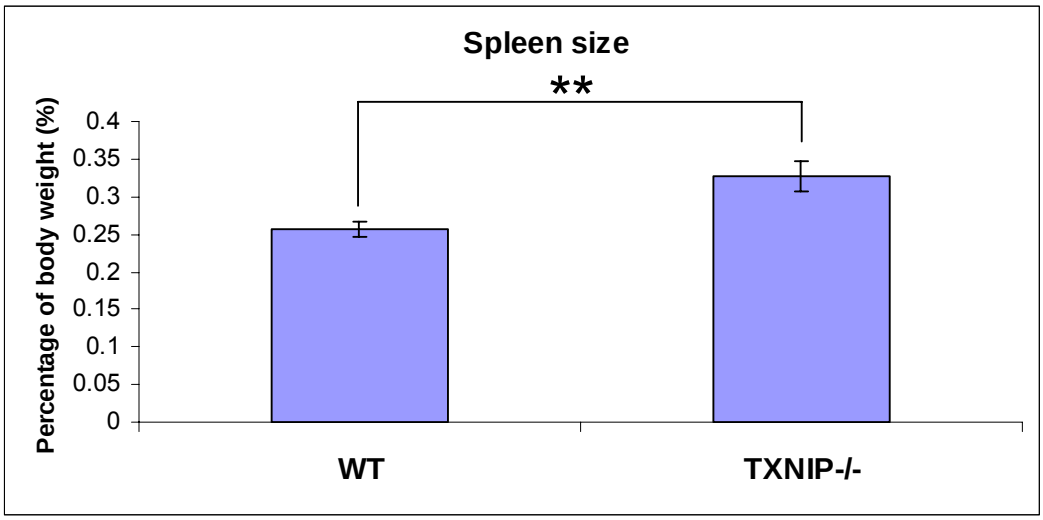
Figure 26. Spleen size and blood parameters of wild-type and TXNIP$^{-/-}$ mice.

A) The size of the spleen of 6 month-old wild-type (WT) and TXNIP$^{-/-}$ males expressed as a percentage of total body weight. n=5 **p-value < 0.01

B) Blood parameters of 6 month old wild-type (WT) and TXNIP$^{-/-}$ males. n=5 *p-value < 0.05

Legend: RBC. Red blood cells; HGB. Hemoglobin; HCT. Hematocrit; PLT. Platelets; MCV. Mean corpuscular volume; MCH. Mean corpuscular hemoglobin; MCHC. Mean corpuscular hemoglobin concentration; RDW. Red cell distribution width; MPV. Mean platelet volume

A



B

	WT		TXNIP-/-		
	Average	*SEM*	*Average*	*SEM*	*p-value*
RBC	11.08	0.29	11.05	0.41	0.963
HGB	16.86	0.50	17.78	0.43	0.203
HCT	51.42	1.37	53.30	1.57	0.393
PLT	763.60	149.25	544.60	109.16	0.275
MCV	46.60	0.24	48.20	0.49	* 0.027
MCH	15.22	0.18	16.12	0.22	* 0.014
MCHC	32.74	0.23	33.36	0.22	0.089
RDW	15.06	0.13	14.70	0.14	0.097
MPV	5.38	0.15	5.70	0.24	0.288

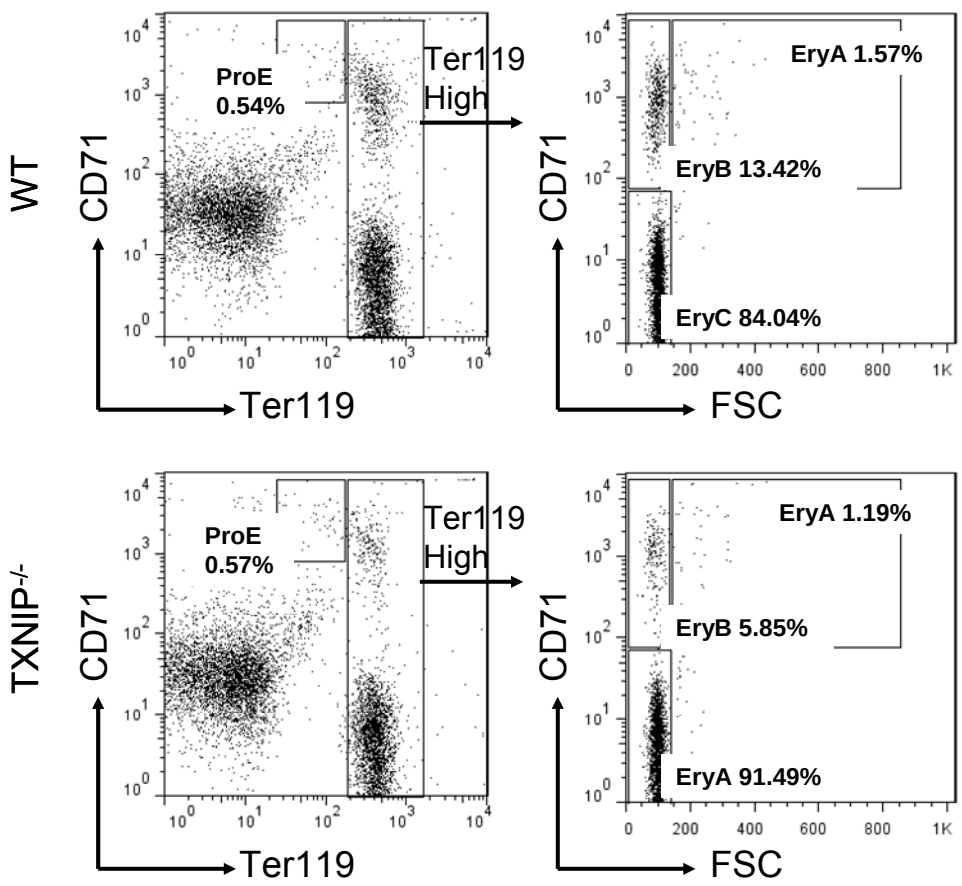
Figure 27. Distribution of erythroid precursors in the spleen and bone marrow of wild-type and TXNIP$^{-/-}$ mice.

A) Representative flow cytometry analysis of the erythroid precursor populations isolated from the spleen of a 6 month-old wild-type and a TXNIP$^{-/-}$ mouse. CD71 (transferrin receptor) and Ter119 were used as markers for different stages of erythroid cell precursors. The Ter119-high population was further analyzed for forward scatter and CD71 expression.

B) Percentage of cells in the different erythroblast populations in spleen and bone marrow of wild-type and TXNIP$^{-/-}$ mice. Average from spleens of 6 month-old mice (wt n=13, TXNIP$^{-/-}$ n=14).

EryA. basophilic erythroblasts; EryB. late basophilic and polychromatic erythroblasts; EryC. orthochromatic erythroblasts and erythrocytes; ProE. proerythroblasts.

A



B

Spleen	**WT**		**TXNIP$^{-/-}$**		
	Average	***SEM***	***Average***	***SEM***	***p-value***
ProE	1.78	0.35	2.37	0.37	0.2580
EryA	2.99	0.63	2.53	0.48	0.5640
EryB	**11.08**	**1.24**	**6.72**	**0.64**	* **0.0057**
EryC	84.16	1.61	88.38	1.36	0.0563
%Ter119+ cells	47.57	1.87	45.17	2.19	0.4130
Bone marrow	**WT**		**TXNIP$^{-/-}$**		
	Average	***SEM***	***Average***	***SEM***	***p-value***
ProE	6.40	0.91	7.57	0.69	0.315
EryA	14.90	0.82	14.18	0.82	0.546
EryB	25.85	1.48	21.83	1.44	0.063
EryC	52.85	2.39	56.68	2.20	0.251
%Ter119+ cells	44.59	2.60	42.97	2.01	0.628

Table 4. Blood parameters of wild-type and TXNIP$^{-/-}$ mice treated with EPO.

Blood parameters of 6 month old wild-type (WT) and TXNIP$^{-/-}$ males treated with EPO at a concentration of 3000 units/kg, 3 times a week for 3 weeks n=4 *p-value < 0.05

Legend: RBC. Red blood cells; HGB. Hemoglobin; HCT. Hematocrit; PLT. Platelets; MCV. Mean corpuscular volume; MCH. Mean corpuscular hemoglobin; MCHC. Mean corpuscular hemoglobin concentration; RDW. Red cell distribution width; MPV. Mean platelet volume

	WT EPO n=4		TXNIP-/- EPO n=4		
	Average	***SEM***	***Average***	***SEM***	***p-value***
RBC	14.20	0.74	13.76	0.40	0.628
HGB	21.40	1.34	22.35	1.09	0.602
HCT	67.13	3.12	70.08	2.64	0.498
PLT	575.00	17.01	605.50	84.87	0.748
MCV	48.25	0.63	52.00	1.00	* 0.025
MCH	15.08	0.28	16.20	0.44	0.084
MCHC	31.05	0.21	31.23	0.29	0.646
RDW	17.73	0.22	19.33	0.57	0.058
MPV	6.85	0.48	6.65	0.21	0.723

Chapter IV: Discussion

A. Novel role of p45 NF-E2 in erythroid differentiation

Previous studies, which used cellular models of erythroid differentiation have identified p45 NF-E2 as an important player in the control of α- and β-globin gene expression [105, 120, 145, 146]. It was therefore surprising that blood analysis of surviving adult mice knockout for p45 NF-E2 revealed only a mild anemia [130]. Further studies of those mice are lacking, which caught our interest, since recent methods in flow cytometry enable to assess differentiation of erythroid precursors from the proerythroblast to the erythrocyte stages [91, 92].

Mice knockout for p45 NF-E2 have been reported to display a 6-fold increase in spleen mass [130] and we also observed this splenomegaly. Based on the erythroid surface marker Ter119, our data revealed a higher proportion of erythroid cells per total cell number in spleens of knockout mice. Additionally, p45 NF-E2$^{-/-}$ mice exhibited altered splenic erythropoiesis, displaying an increase in early precursor populations. In steady-state conditions, bone marrow is the predominant site of red blood cell production, with the spleen playing only a marginal role. Indeed flow cytometry results from wild-type mice showed that 80 to 90 percent of erythroid cells in the spleen belong to the EryC population, which represents orthochromatic erythroblasts and erythrocytes. In the case of wild-type mice these cells are likely mature erythrocytes found in the blood circulation system of the spleen, including those at the end of their life-span, which return to the spleen to be recycled and phagocytosed by macrophages. However, the flow cytometry profile of the spleen of p45 NF-E2$^{-/-}$ mice changed drastically, as the most mature erythroid population (EryC) represented only 50 to 60 percent of total erythroid cells. This difference was accounted for by an increase in early precursors. The proportion of proerythroblasts (ProE) and basophilic erythroblasts (EryA) showed a tendency to be higher in

knockout mice, while the proportion of late basophilic and polychromatic erythroblasts (EryB) was significantly increased by more than 3-fold. Therefore the fact that the spleen is highly enlarged in p45 NF-E2$^{-/-}$ mice and actively producing erythrocytes indicates that an important proportion of red blood cells in circulation originate from the spleen in addition to the bone marrow, while wild-type mice rely highly on bone marrow. In addition, enhanced splenic erythropoiesis was also illustrated by the fact that the proportion of erythroid cells that are positive for Ter119 is increased in the spleen of p45 NF-E2$^{-/-}$ mice. The compensatory splenic erythropoiesis in p45 NF-E2$^{-/-}$ mice is a manifestation often observed when anemia needs to be corrected, indicating a possible defect in bone marrow erythropoiesis. In summary, our data confirmed a compensatory splenic erythropoiesis, suggested by the initial study on the erythroid phenotype of p45 NF-E2$^{-/-}$ mice that reported splenomegaly and increased reticulocytosis [130]. Our results indicated that a great proportion of erythropoieisis was ongoing in the spleen of p45 NF-E2$^{-/-}$ mice, although a published study did not report increased morbidity when those mice were subjected to splenectomy [131]. In line with those results, we hypothesize that extramedullary erythropoiesis could be transported to other sites in the absence of the spleen, for instance to the liver, as has been observed previously [283].

Another evidence for anemia is the fact that p45 NF-E2$^{-/-}$ mice display a 2-fold increase in the levels of erythropoietin, the major cytokine that controls erythropoiesis. Although ELISA kits designed for human EPO are often used for mice, we analyzed our samples using a kit specifically designed for mice, to allow more accurate and sensitive measurements of smaller differences. Indeed, over 10 fold increases in EPO levels have been observed, but these high concentrations are linked to acute and severe erythroid stress, such as lysis of red blood cells by

injecting the hemolytic agent phenylhydrazine [284]. More modest increases are expected to arise in conditions of chronic anemia, for instance the 1.83-fold increase in EPO levels in mice devoid of Sox6 [284] is in line with our observations in chronically anemic p45 NF-E2$^{-/-}$ mice. Earlier studies showed that increased levels of EPO and its signalling are associated with enhanced splenic erythropoiesis in mice [285], which correlates well with our results. Indeed, data in the literature showed that exposure of early splenic erythroblasts to increased EPO in conditions of erythroid stress, led to the downregulation of the death receptor Fas and Fas ligand expressed on their surface, which resulted in increased survival and enhanced erythropoiesis [285]. Therefore, measuring EPO levels allowed us to associate the increase in splenic erythropoiesis to a systemic effect of EPO, rather than a local effect of p45 NF-E2 on erythroid precursors in the spleen. In addition, EPO signalling has been shown to activate the transcription factor STAT5, which upregulated the anti-apoptotic protein BcL-xL to promote survival of early erythroblasts [56]. It would be of interest to investigate whether one of those regulatory mechanisms occurs in p45 NF-E2$^{-/-}$ mice.

Both the enhanced splenic erythropoiesis and increase in serum EPO suggested defective erythropoiesis in the bone marrow. However, the possibility remained that bone marrow terminal erythroid differentiation would not be modified, since in some situations, such as in ERK1 knockout mice, there is enhanced splenic erythropoiesis, but no change in bone marrow erythroid populations [74]. In the case of p45 NF-E2$^{-/-}$ mice, we observed altered bone marrow late-stage erythropoiesis. Mice aged from 3 to 4 months have a decrease in the late basophilic and polychromatic population (EryB) and a proportional increase in the most mature orthochromatic and erythrocyte population (EryC). It has been reported that p45 NF-E2$^{-/-}$ mice of this age present

increased bone mass in bony sites of hematopoiesis [127]. In accordance with those observations, we observed greatly reduced bone marrow in these mice, as assessed by decreased cell number recovered from the femur. Therefore, although the proportion of erythroid cells in the bone marrow did not differ between wild-type and p45 NF-E2$^{-/-}$ mice, the total number of those cells was greatly reduced, explaining the observed anemia. As to the accumulation of the most mature erythroid population (EryC), it is possible that the increased calcification in bone marrow is impeding the release of erythrocytes into circulation. At this stage it was not possible to say if p45 NF-E2 had an intrinsic function in erythroid precursors in the bone marrow or if the effect was due to increased calcification [127]. To circumvent this problem, we analyzed younger mice, aged 6 to 7 weeks, prior to the onset of calcification. At that age, we observed a partial block in the progression from the basophilic (EryA) to late basophilic/polychromatic (EryB) erythroblasts in the bone marrow of p45 NF-E2$^{-/-}$ mice. Our results suggest a role for p45 NF-E2 *in vivo* in the bone marrow at this transition step. Of note, the initial analysis of p45 NF-E2$^{-/-}$ mice has reported decreased hemoglobin content per mature erythrocyte [130]. This study may be relevant for our results that hint at a transition block resulting in the accumulation of a population that is less differentiated, hence contains less hemoglobin.

We further investigated the effect of dysregulated erythropoiesis in p45 NF-E2$^{-/-}$ mice by studying the master regulator of terminal erythropoiesis, the transcription factor GATA1. Immunoblot of extracts from total bone marrow cells suggested an increase of GATA1 protein in the absence of p45 NF-E2. We obtained a similar result in our analysis of protein extracts derived from bone marrow erythroid precursors, isolated using the surface marker Ter119. GATA1 is known to be highly expressed in proerythroblasts and then decrease in more

differentiated populations [286], while in parallel the levels of p45 NF-E2 are increasing [41]. Future studies may determine whether p45 NF-E2 can act as a transcriptional repressor of GATA1 during the progression of terminal erythroid differentiation, in a similar way that GATA1 has been shown to repress GATA2 in earlier steps of erythropoiesis [287]. It would be of interest to know whether the increase in GATA1 protein in p45 NF-E2$^{-/-}$ mice is secondary to a transcriptional upregulation or an increase in protein stability. In the case of a difference at the RNA level, chromatin immunoprecipitation experiments would indicate if p45 NF-E2 occupies GATA1 promoter or enhancer regions. Alternatively, since GATA1 was shown to regulate p45 NF-E2 transcription [288], the absence of p45 NF-E2 may lead to increased GATA1 levels in order for the cells to rescue p45 NF-E2 expression. Besides a direct or indirect effect of p45 NF-E2 on GATA1 levels or vice versa, there is also the possibility that the increase of GATA1 expression in the bone marrow of p45 NF-E2 knockout mice is due to the higher numbers of earlier erythroid precursors, which express it at higher levels.

Differentiation is also tightly linked to cell cycle control, as this process usually results in cell cycle arrest. In the case of erythroid terminal differentiation, the situation is slightly different, since cells still undergo a few divisions from the proerythroblast to the mature erythrocyte stage, as they become more differentiated and smaller in size. However, the cell cycle is profoundly modified as *in vitro* models showed that the G1 stage is reduced from 11 hours to 5 hours and the control of cell size is lost allowing cells to become smaller [289]. Our studies showed an increased proportion of erythroid cells, positive for the Ter119 marker, arrested at the G0/G1 stage of the cell cycle in bone marrow of p45 NF-E2$^{-/-}$ mice. Failure to progress in the cell cycle correlated with our flow cytometry results in bone marrow, which showed an accumulation of

the earlier (EryA) population. Those results were also consistent with the increase in GATA1 levels, since previous studies have associated upregulation of GATA1 to an arrest at the G1 cell cycle stage. Different pathways have been implicated in this phenomenon, for instance binding of GATA1 to the *c-Myc* or *c-Kit* promoters, and the repression of those targets was shown to lead to cell cycle arrest [43, 290]. On the other side, GATA1 was shown to be a direct activator of p21, a protein regulating the cell cycle and inducing erythroid differentiation [45]. Future experiments on p45 NF-E2$^{-/-}$ mice may be based on these previous studies, to analyze which components of cell cycle progression are affected in the cells of knockout mice.

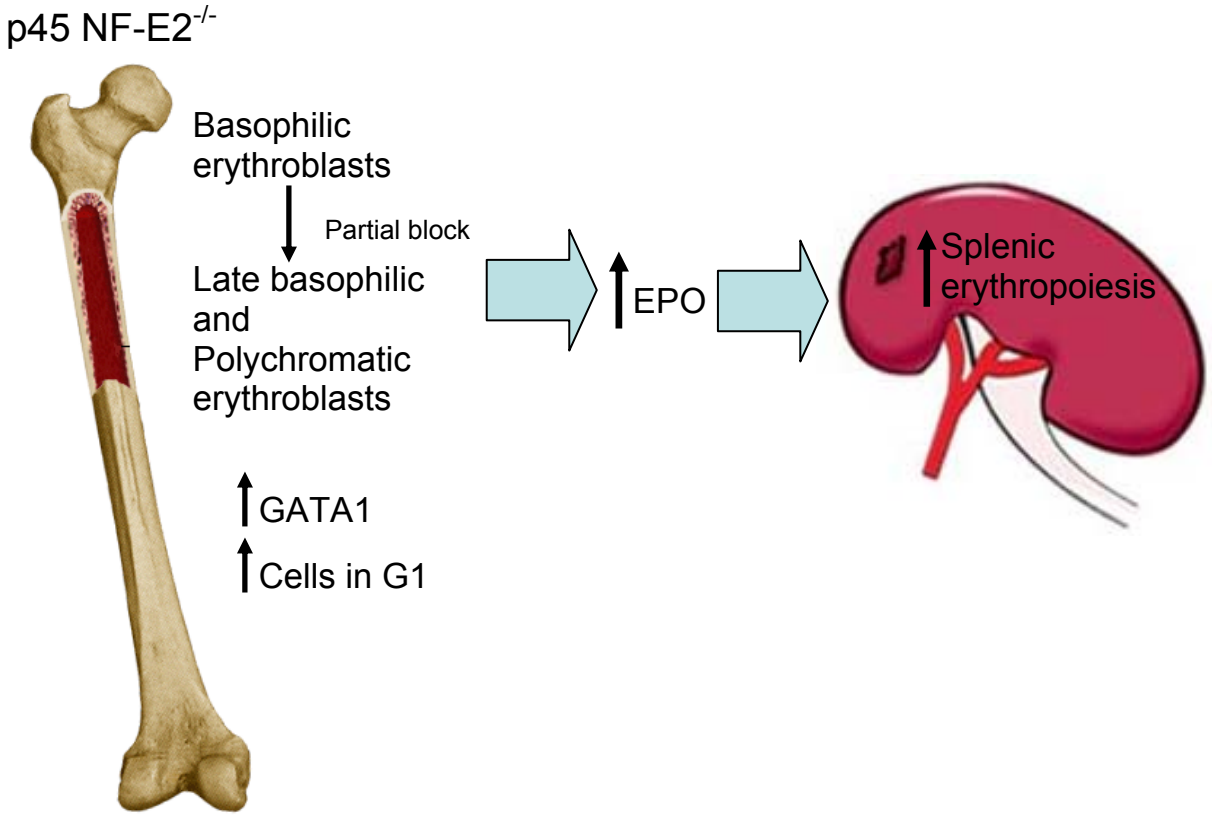
The interpretation of our results as to a new role of p45 NF-E2 in erythropoiesis *in vivo* is summarized in a model in **Figure 28**. We propose that the absence of p45 NF-E2 *in vivo* causes a partial differentiation block of erythroid precursors in the bone marrow, with increased GATA1 expression and inhibition of cell cycle progression. This causes anemia that leads to upregulation of EPO, which drives increased splenic erythropoiesis.

Many aspects as to the role of p45 NF-E2 in erythropoiesis *in vivo* remain elusive. One of the major ones is the divergence between its vital function in globin expression in mouse erythroleukemia cells [105, 120, 145, 146] and its relatively mild phenotype in p45 NF-E2$^{-/-}$ mice *in vivo*, including a lack of difference in transcripts of globin genes in fetal livers and adult reticulocytes [130]. It has been suggested that other members of the CNC transcription factors could be compensating for the role of p45 NF-E2 in mice erythroid cells, although transcripts of NFE2L1 and NFE2L2 as well as protein levels of NFE2L1 were shown to be indistinguishable in fetal livers of wild-type and p45 NF-E2$^{-/-}$ mice [130]. It would however be interesting to

analyze adult mouse erythroid tissues for this compensation, since the phenotype is milder in surviving adults than in the fetuses and neonates. Published studies have shown that the phenotype of p45 NF-E2 and NFEL2 double knockout mice does not increase the anomalies observed for p45 NF-E2 alone [291], and we observe similar results in compound p45 NF-E2 and NFEL3 mice (data not shown). In contrast, NFE2L1 knockout mice are embryonic lethal and display anemia [292], making this CNC member the most likely to compensate. It would be of interest to analyse the erythroid phenotype of mice knockout for both p45 NF-E2 and conditional knockouts for NFE2L1.

Figure 28. Novel role of p45 NF-E2 in erythroid differentiation *in vivo*

Absence of p45 NF-E2 in mice affects differentiation of bone marrow erythroid precursors, leading to an increase in EPO and compensatory splenic erythropoiesis.



B. TXNIP as a novel regulator of erythroid differentiation

Many aspects of terminal erythropoiesis remain to be uncovered, including the participation of new players in this process. In quest for novel regulators, we identified TXNIP, based on an Affymetrix genechip expression array screen with differentiating MEL cells, previously performed in the laboratory.

Our experiments revealed a robust upregulation of *Txnip* transcripts in MEL cells induced to differentiate with HMBA. Indeed, after 6 hours of treatment the difference between control and treated cells is a 3-fold increase, while we observed a 4-fold and 8-fold increase after 24 and 48 hours, respectively. The fact that the upregulation of *Txnip* was initiated earlier than changes characteristic of erythroid differentiation such as increased globin or iron uptake, which happen at 24 and especially at 48 hours, suggests that the upregulation of this protein could be a factor driving erythroid maturation, rather than an indirect effect in cells that are already differentiated. A recent study based on isolation of human peripheral blood mononucleocytes (PBMCs), their differentiation in culture to yield CFU-Es, proerythroblasts, intermediate and late erythroblasts and their isolation using flow cytometry gave rise to a database of transcripts differentially regulated in the different populations using Affymetrix microarrays [293]. Using this database we observed that the transcripts of *Txnip* were highly upregulated, the more the cells were differentiated, indicating that the pattern we observed in mouse cells could also be at play in human cells. It would be interesting to investigate, which proteins are responsible for the increase of *Txnip*, with a focus on transcription factors that have been associated with erythroid differentiation. In human endothelial aortic cells, *Txnip* has been shown to be regulated by

FOXO1 [231]. Since the FOXO family member that has been associated with erythropoiesis is FOXO3a [294], it would be of interest to examine whether *Txnip* can be regulated by this family member in the context of erythroid cells. In addition, the promoter of *Txnip* contains a binding site for GATA1, the master regulator of erythroid differentiation [295]. In future studies, we will analyze a possible transcriptional regulation of *Txnip* by GATA1. Alternatively, the increase in *Txnip* transcripts could also arise from enhanced mRNA stability, which would be measured using actinomycin D treatment to inhibit transcription [296].

The strong increase in *Txnip* transcripts translated into an upregulation of TXNIP protein levels in MEL cells induced to differentiate with both HMBA and DMSO. An important induction in treated versus control cells was observed after 6 hours of treatment and persisted throughout the 72 hours. Of note, the levels of TXNIP relative to whole protein extracts were higher in the early time points both in control and treated cells, while they decrease with longer treatments. It is possible that TXNIP needs to be upregulated to peak amounts early in differentiation, while kept at lower levels at later steps of differentiation, although still higher in induced than in control cells. While we observed a transcriptional regulation it is not excluded that increased TXNIP protein levels arise in part also from enhanced stability of the protein. Approaches including the treatments with cyclohexamide, inhibiting protein synthesis or proteasome inhibitors would help to sort out the contribution of these two mechanisms.

TXNIP has been largely involved in redox homeostasis, mainly through the control of its binding partner thioredoxin, a major antioxidant and ROS scavenger [210, 211, 214]. Concomitantly, TXNIP has been shown to be induced by oxidative stress, for instance in neuronal cells [219].

We therefore investigated if TXNIP upregulation was dependent on ROS, by adding the antioxidant NAC to cells treated with HMBA for 6 hours. The antioxidant NAC had a minor effect on TXNIP levels in control cells and did not impede the robust TXNIP upregulation observed in the presence of HMBA. It would be of interest to assess whether this effect can be replicated using other antioxidants, such as glutathione. Our results suggest that TXNIP is not regulated by ROS in erythroid cells, but they do not exclude that the induction of TXNIP has an effect on ROS levels. Future experiments will be designed to measure if TXNIP upregulation has an effect on ROS levels in MEL cells. These experiments will be pertinent since with regards to erythroid differentiation, ROS have been shown to perform important roles in EPO signalling and in the induction of erythroid differentiation [58].

Another model of erythroid differentiation used in this research project to investigate the regulation of TXNIP was the G1E-ER cell line, which is EPO-dependent and differentiates in response to activation of the transcription factor GATA1. We showed an upregulation of TXNIP protein levels in these cells upon induction of differentiation, although not as early as 6 hours, but rather at 12, 24 and especially 48 hours. Alternatively, additional models may be used to assess the status of TXNIP along the progression in erythroid differentiation, such as *in vitro* differentiation of fetal liver cells from mice [297] or of human CD34+ cells [298], cultured in a media with specific cytokines and recombinant growth factors.

The next phase of my project was the characterisation of the signalling pathways that control TXNIP expression during erythroid differentiation. Previously, we have studied the role of MAPK in erythroid differentiation in the MEL cells model and identified p38 as a positive

regulator of globin production, heme synthesis and iron uptake, while ERK had the opposite effect [71]. Studies performed on human aortic endothelial cells have demonstrated that the upregulation of TXNIP by glucose is dependent on p38 MAPK [231]. The MAPK p38 and ERK have also been shown to be necessary for induction of TXNIP by high glucose in rat mesangial cells [299].

Using inhibitors of MAP kinases, our results showed that in MEL cells TXNIP levels are not regulated by ERK1/2. In contrast, inhibiting p38 with SB202190 increased TXNIP levels in control cells, while there was a synergistic increase in cells treated with both the differentiation inducer HMBA and the p38 inhibitor, especially after 48 hours of treatment. This synergy may be explained by the fact that TXNIP itself can stimulate its own expression through a positive feedback loop mechanism [300]. In addition, the levels of TXNIP were very high in cells treated with HMBA and the p38 inhibitor after 48 hours of treatment, the time-point at which we observed a decrease in markers of erythroid differentiation in our previous study [71]. It is possible that a very robust induction of TXNIP is necessary early in differentiation, but that maintaining these levels high later may inhibit the differentiation process. In addition, our results demonstrated that JNK kinase regulates TXNIP in a similar way as p38 MAPK in MEL cells. Inhibition of JNK with SP600125 led to increased TXNIP protein levels in MEL cells and we observed a synergistic upregulation in cells treated with both the differentiation inducer HMBA and the JNK inhibitor. It will be of interest to examine if the modulation of TXNIP by MAPKs is also observed at the transcriptional level in erythroid cells. In addition to being regulated by MAPKs, future experiments will analyze whether TXNIP itself is modulating the activity of the MAP kinases p38, JNK and ERK. For instance, TXNIP has been shown to regulate the

phosphorylation of ERK in bladder cancer cells [192]. Also phosphorylation of JNK and p38 MAPKs induced by the tumor-suppressor lipid ceramide is dependent on TXNIP [301].

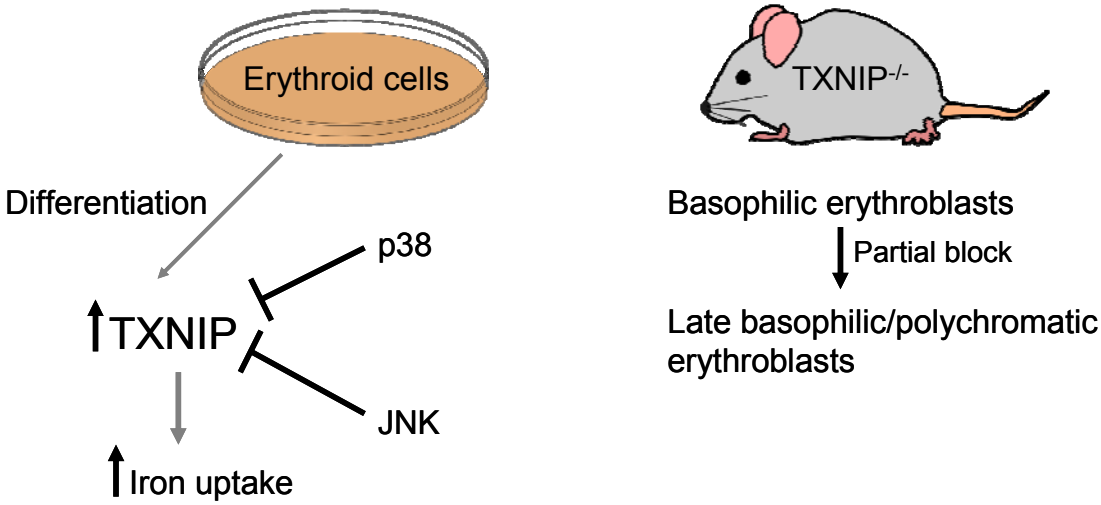
Since our *in vitro* results have suggested a role for TXNIP in erythroid differentiation, we were interested to analyze the phenotype of TXNIP$^{-/-}$ mice with regards to their erythroid phenotype. Besides its well-documented role in metabolism, a few recent studies have provided some evidence as to a possible role of this protein in the hematopoietic compartment. TXNIP has been implicated in the proper functioning or maturation of hematopoietic stem cells, dendritic cells and natural killer cells [193-196]. Blood analysis of mice up to 12 months of age did not indicate a difference in erythroid parameters, such as red blood cell counts, hematocrit and hemoglobin. Similarly, red blood cell parameters in mice treated with EPO were induced to a similar extent in both wild-type and TXNIP$^{-/-}$ mice, suggesting this protein is dispensible for EPO signalling in erythropoiesis. The EPO dosage chosen was based on a study where decreased glucose levels were observed (ASH conference 2010, poster 4232), since TXNIP is known to be regulated by glucose. It would be of interest to test if a lower dose, more representative of the one given to patients with renal failure, would show a detectable difference in EPO sensitivity between wild-type and TXNIP$^{-/-}$ mice. In steady-state conditions we observed an increase in the spleen size from 0.257% of body mass to 0.327% already at 6 months of age. A splenomegaly has been reported previously for one year old mice and for younger mice treated with 5-FU, partly because of increased mobilization of HSCs [195]. Our analysis of the erythroid precursor populations in the spleen by flow cytometry does not suggest increased splenic erythropoiesis, as we do not observe increase in the proportion of the early erythroid populations. However, we detect altered splenic erythropoiesis, illustrated by a partial block between the EryA and EryB

stages, namely between basophilic and late basophilic/polychromatic erythroblasts. Many possibilities arise as to the role of TXNIP at this transition step, since this is a step involving robust globin induction, iron uptake and heme synthesis, implicating changes in multiple signalling pathways. A recent study suggests the presence of TXNIP on the cell membrane and a function in mediating the internalization of the GLUT1 receptor, in experiments using transferrin receptor as a control for internalization through clathrin-coated pits [238]. It prompted us to analyze whether TXNIP could be implicated in iron uptake, as it is a very important pathway at the transition from basophilic to polychromatic erythroblasts, the step where we observe a block *in vivo*. Our data *in vitro* in MEL cells suggest that overexpression of TXNIP results in increased iron uptake and decreased protein levels of the transferrin receptor. Therefore, TXNIP acts to enhance iron uptake, but not through upregulation of transferrin receptor. It is possible that TXNIP participates in a signalling pathway that leads to more efficient trafficking of transferrin. In turn, more iron in cells could act to decrease the levels of transferrin receptor that we observe, possibly through and mechanism mediated by iron regulated proteins (IRPs). Alternatively TXNIP has been mainly implicated in cell cycle control in other cell types, therefore analysing markers of cell cycle transition such as p21, p27 or cyclin D1 will help in determining whether the differentiation block observed *in vivo* results from impairment of cell cycle control.

In summary our results provide the first indication of a role of TXNIP in erythroid differentiation and are depicted in **Figure 29**.

Figure 29. Role of TXNIP in erythroid differentiation.

I*n vitro* and *in vivo* evidence of a role of TXNIP in erythroid differentiation.



Many aspects of terminal erythroid differentiation are still unknown and their elucidation would lead to the identification of therapeutic targets for disorders or red blood cells and generation of artificial blood substitutes. The results presented in this dissertation provide novel insights into the roles of p45 NF-E2 and TXNIP, for which little is known with regards to erythropoiesis *in vivo*. Both proteins are regulated in erythroid cells by MAP kinases, essential components of signalling in erythroid differentiation. The transcription factor p45 NF-E2 has been shown to be phosphorylated by JNK [68], while our results indicate a repression of TXNIP by p38 and JNK. In addition both proteins are necessary for efficient differentiation of erythroid precursors in the spleen in mice, while p45 NF-E2 also affects the distribution of erythroid precursors in the bone marrow. Overall our results provide additional information about terminal erythropoiesis with regards to the roles of p45 NF-E2 *in vivo* and the novel erythroid regulator TXNIP.

Contribution to original knowledge

The results of this dissertation bring additional knowledge as to the role of p45 NF-E2 in erythroid differentiation. They provide as well the first report of a function for TXNIP in terminal erythroid differentiation. The contribution of the candidate to original knowledge is the following:

1. The candidate was the first to identify increased proportion of early erythroid precursors in the spleen of p45 NF-E2$^{-/-}$ mice and increased serum EPO. In addition, she was the first to demonstrate a partial block in terminal erythropoiesis in the bone marrow of p45 NF-E2$^{-/-}$ mice, accompanied by an increase in GATA1 levels and an altered cell cycle.

2. The candidate was the first to provide evidence of the implication of TXNIP in the erythroid differentiation program. She demonstrated for the first time increased TXNIP levels upon induction of differentiation in different cellular models and its regulation by MAP kinases in the erythroid lineage. She also provided the first evidence of altered differentiation of erythroid precursors in TXNIP$^{-/-}$ mice and its implication in iron uptake.

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