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The Role of p38 α in Stress Erythropoiesis

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Abstract

During mammalian erythropoiesis, hematopoietic stem cells develop into erythroblasts, which form blood islands with macrophages and further extrude the nucleus during terminal differentiation into erythrocytes. The production of erythrocytes dramatically increases in stress erythropoiesis, such as during fetal development or anemia. There is increasing knowledge about hormonal signaling regulating steady state and stress erythropoiesis and new insights into molecular pathways essential for erythroblast differentiation. However, the molecular pathways involved in erythroblast differentiation, especially under stress conditions are still largely unknown.

It was shown previously that mice lacking *p38α* in embryonic tissues or specifically in hematopoietic cells show increased numbers of nucleated erythrocytes in the fetal liver and newborn peripheral blood. Moreover, phenylhydrazine (PHZ)-induced anemia increases the number of nucleated erythrocytes in the spleen and peripheral blood of mice with hematopoietic deletion of *p38α*. Additionally, erythroblasts lacking *p38α* almost completely fail to enucleate during *in vitro* differentiation, suggesting that the function of *p38α* in enucleation is cell-autonomous.

Here I will describe my attempts to identify substrates of *p38α*, which are essential for erythroblast enucleation. Furthermore, I will demonstrate that the function of *p38α* in enucleation of erythroblasts is cell-autonomous and that *p38α* is a key mediator of erythroid differentiation under stress conditions. The impaired enucleation phenotype is recapitulated in mice lacking *p38α* specifically in the erythroid lineage, but not in mice with macrophage-specific deletion of *p38α*. Moreover, *p38α*-deficient erythroblasts show aberrant expression of genes related to erythroid differentiation, such as *Gata1* and different hemoglobin isoforms. The

impaired enucleation phenotype of *p38 α* -deficient erythroblasts is similar to that of erythroblasts lacking Rb. Importantly, negative regulators of Rb, such as p53, p21 and p27 were down-regulated and the level of hyperphosphorylated Rb is increased in *p38 α* -deficient erythroblasts. Gene expression profiling analysis also reveals that target genes of E2F-1, which is repressed by Rb, are up-regulated, such as Cyclin E, Cdk2 and Pcna. These data indicate that *p38 α* is essential for enucleation of erythroblasts in a cell-autonomous manner and for the regulation of differentiation-related gene expression under stress erythropoiesis, likely through activating the Rb pathway.

Zusammenfassung

Während der Erythropoese von Säugetieren entwickeln sich hämatopoetische Stammzellen zu Erythroblasten, welche zusammen mit Makrophagen Blutinseln bilden und in der terminalen Differenzierung zu Erythrozyten schließlich den Nukleus ausstoßen. In der Stresserythropoese, wie zum Beispiel während fötaler Entwicklung oder Anämie, nimmt die Produktion von Erythrozyten dramatisch zu. Es gibt ein stetig wachsendes Wissen über hormonelle Regulierung von Basal- und Stresserythropoese und neue Einblicke in molekulare Netzwerke, welche essentiell für die Erythroblastendifferenzierung sind. Die molekularen Netzwerke der Erythroblastendifferenzierung, besonders in Stresssituationen, sind jedoch noch immer größtenteils unbekannt.

Zuvor wurde gezeigt, dass Mäuse denen $p38\alpha$ im embryonalen Gewebe oder in hämatopoetischen Zellen fehlt, eine erhöhte Anzahl an nukleierten Erythrozyten in der fötalen Leber und im peripheren Blut von Neugeborenen aufweisen. Darüberhinaus wird die Anzahl von nukleierten Erythrozyten in der Milz und dem peripheren Blut von Mäusen mit spezifischer Deletion von $p38\alpha$ in hämatopoetischen Zellen durch Phenylhydrazin induzierte Anämie erhöht. Zusätzlich wurde gezeigt, dass $p38\alpha$ -defiziente Erythroblasten während der *in vitro* Differenzierung fast komplett versagen zu enukleieren. Dies legt nahe, dass die Funktion von $p38\alpha$ in der E nukleierung zellautonom ist.

In der hier vorliegenden Arbeit beschreibe ich meine Versuche Substrate von $p38\alpha$ zu bestimmen, welche essentiell für die E nukleierung von Erythroblasten sind. Weiteres zeige ich, daß die Funktion von $p38\alpha$ in der E nukleierung von Erthroblasten zellautonom ist und daß $p38\alpha$ eine Schlüsselfunktion in der erythroiden Differenzierung in Stresssituationen hat. Der Phänotyp der gestörten E nukleierung wird in Mäusen, denen $p38\alpha$ speziell in der erythroiden Linie fehlt,

aber nicht in Mäusen mit spezifischer Deletion von $p38\alpha$ in Makrophagen rekapituliert. Zusätzlich haben $p38\alpha$ -defiziente Erythroblasten abweichende Expression von Genen, welche mit der erythroiden Differenzierung in Zusammenhang stehen, wie zum Beispiel Gata1 und diverse Hämoglobinisoformen. Der Phänotyp der gestörten E nukleierung ähnelt dem von Rb -defizienten Erythroblasten. Wichtig ist, daß $p38\alpha$ -defiziente Erythroblasten ein reduziertes Maß an negativen Regulatoren von Rb, und zwar p53, p21 und p27, und ein erhöhtes Maß an hyperphosphorylierten Rb aufweisen. Ebenso hat eine Analyse des Genexpressionsprofils ergeben, dass Zielgene von E2F-1, welcher von Rb reprimiert wird, hochreguliert sind; zum Beispiel Cyclin E, Cdk2 und PcnA. Diese Daten zeigen, daß die Rolle von $p38\alpha$ in der E nukleierung von Erythroblasten während der Stresserythropoese zellautonom ist und $p38\alpha$ essentiell ist für die Regulierung von differenzierungsbezogener Genexpression. Darüberhinaus vermute ich, daß $p38\alpha$ seine Funktionen durch die Aktivierung des Rb Singalweges ausübt.

1. Introduction

1.1. Mammalian erythropoiesis: from multipotent progenitors to mature erythrocytes

1.1.1. Ontogeny and origins of erythropoiesis in mammals

Erythropoiesis is the process in which multipotent stem cells develop into erythroblasts, which next form mature erythrocytes. During mammalian erythropoiesis erythroblasts extrude the nucleus during terminal differentiation into erythrocytes, which is characteristic for mammals and almost unique in the animal kingdom [2, 3]. In mammals erythrocytes circulate in cardiovascular system and are crucial for oxygen supply to the entire organism. A cardiovascular system including functional erythrocytes is essential for development, growth and survival of an organism from the beginning of embryonic post-implantation period throughout its whole lifespan [4]. Hence, the onset of erythropoiesis is required in early stages of embryonic development. Until today the ontogeny and developmental origins of erythropoiesis in mammals have been studied intensively and reviewed comprehensively (reviewed in refs. [1, 5]).

During embryonic development the initial population of erythrocytes circulating in the capillary network consists of primitive erythrocytes. It is characteristic for primitive erythrocytes to mature intravascular and to retain the nucleus. Additionally, primitive erythrocytes accumulate fetal hemoglobin and have a relatively large size (Fig.1) [1]. Contradictory to a long hold opinion, it has been shown that primitive erythrocytes are capable to enucleate later in development [6]. Primitive erythrocytes derive from a transient population of primitive erythroid-colony forming cells (EryP-CFC). EryP-CFCs, which are located in the yolk sac and

are observable in the murine embryo at E7.25 earliest [7]. At E7.5 EryP-CFCs rapidly give rise to primitive erythroid cells in the mesoderm layer of the yolk sac. The arising primitive erythroid cells form pools, which are termed “blood islands” [1, 8]. Subsequently, the blood islands become enveloped by endothelial cells, which also form the initial vascular plexus of the yolk sac, due primitive erythrocytes mature intravascular [9]. The primitive erythrocytes start circulating and enter the embryo proper concurrently with the onset of cardiac function at E8.25 [10]. Up to E12 only primitive erythrocytes circulate in the embryo. Therefore, the formation of primitive erythrocytes is essential for accurate oxygen supply and survival of the embryo at early stages of fetal development [11].

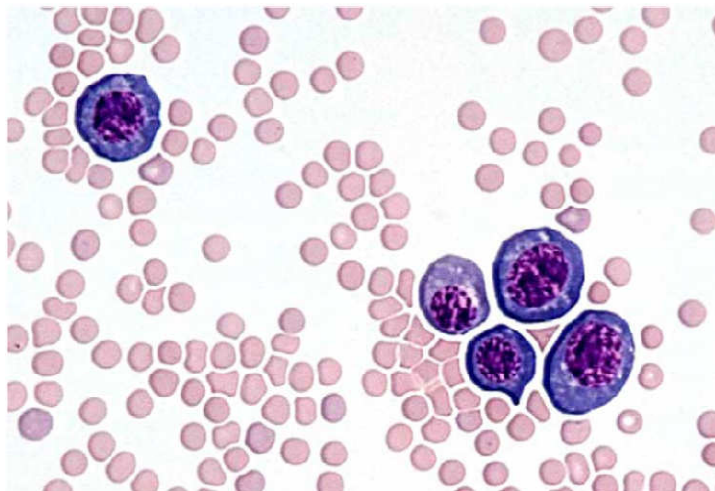


Figure 1. Morphological differences of primitive and adult definitive erythroid cells in the mouse. Peripheral blood from E9.5 embryos and adult mice were mixed together, cytospun, and stained with Wright-Giemsa. The primitive erythroid cells are nucleated and at the basophilic stage of maturation. On the other hand, definitive erythrocytes are enucleated and markedly smaller. (Figure was adapted from *McGrath*, 2008 (ref. [1]))

With the beginning of E12 circulating primitive erythrocytes are successively replaced by fetal definitive erythrocytes released from the fetal liver. Fetal definitive erythrocytes are defined by a relative small size in comparison to primitive erythrocytes, by accumulation of adult hemoglobin and most prominently by the absence of a nucleus (Fig. 1) [1]. However, fetal definitive erythrocytes are twice as large and accumulate twice as much hemoglobin as adult definitive erythrocytes [6]. Noteworthy, definitive erythrocytes undergo enucleation and terminal

differentiation before entering the blood stream [1]. Progenitors of early fetal definitive erythropoiesis are yolk sac-derived burst forming unit erythroid (BFU-E) cells. BFU-Es emerge in the yolk sac at E8.25 and start circulation with the onset of cardiac function [12]. These yolk sac-derived BFU-Es populate the newly formed fetal liver at E10 and subsequently generate fetal definitive erythrocytes. At E12 the first mature fetal definitive erythrocytes start the circulation [7].

After that, it is assumed that yolk sac-derived BFU-Es in the fetal liver are successively replaced by BFU-Es derived from long term hematopoietic stem cells (HSC). HSCs begin to populate the fetal liver at E11 and give rise to diverse progenitors of the hematopoietic system including BFU-Es. HSC-derived BFU-Es are responsible for continued fetal definitive erythropoiesis in the fetal liver till birth and for adult definitive erythropoiesis in the bone marrow as well. After birth definitive erythropoiesis in the fetal liver ceases rapidly and adult definitive erythropoiesis takes place in the bone marrow [1].

Because of the different populations of progenitors for primitive and definitive erythropoiesis, it is reasonable that these progenitors derive from various developmental origins. It is assumed that erythroid progenitors originate from two distinct lineages. The progenitors for primitive and early fetal definitive erythropoiesis, EryP-CFCs and BFU-E, arise in the yolk sac and are considered to share a common progenitor; the hemangioblast precursor. The hemangioblast precursors contain potential to give rise to hematopoietic and epithelial cells. Hemangioblast precursors undergo an early commitment to hematopoietic or vascular lineage and therefore are not found in the yolk sac themselves. Importantly, it has been shown that hemangioblast precursors contain potential for primitive and definitive erythroid cells. Hence it is reasonable

that primitive erythroid and first definitive erythroid cells originate from hemangioblast precursors.

As mentioned before, the progenitors of late fetal and adult definitive erythropoiesis derive from long term HSCs. In contrast to yolk sac-derived progenitors, HSCs originate from intraembryonic source. HSCs arise at the paraaortic splanchnopleura region at E8.5-10 [1]. Incidentally, the paraaortic splanchnopleura region is termed aorta-gonad mesonephros region later in development [13]. During development HSCs are distributed over the blood stream and populate the thymus at E10.5, the fetal liver at E11, the splenic rudiment at E12.5 and the bone marrow at E16 [5, 14]. HSCs give rise to erythroid progenitors in the fetal liver and bone marrow and thereby drive the late fetal and adult definitive erythropoiesis.

Taken together the ontogeny of erythropoiesis in mammalian embryos can be simplified in a model of three succeeding waves as described by McGrath [1]. The first wave encompasses the primitive erythropoiesis, which consists of EryP-CFCs that give rise to primitive erythrocytes. The second wave encompasses the early definitive erythropoiesis, which is located in the fetal liver and consists of yolk sac-derived BFU-Es that give rise to fetal definitive erythrocytes. The third and final wave consists of HSC-derived BFU-Es, which are responsible for continued definitive erythropoiesis in the fetal liver up to birth and ultimately for adult definitive erythropoiesis in the bone marrow (Fig. 2) [1].

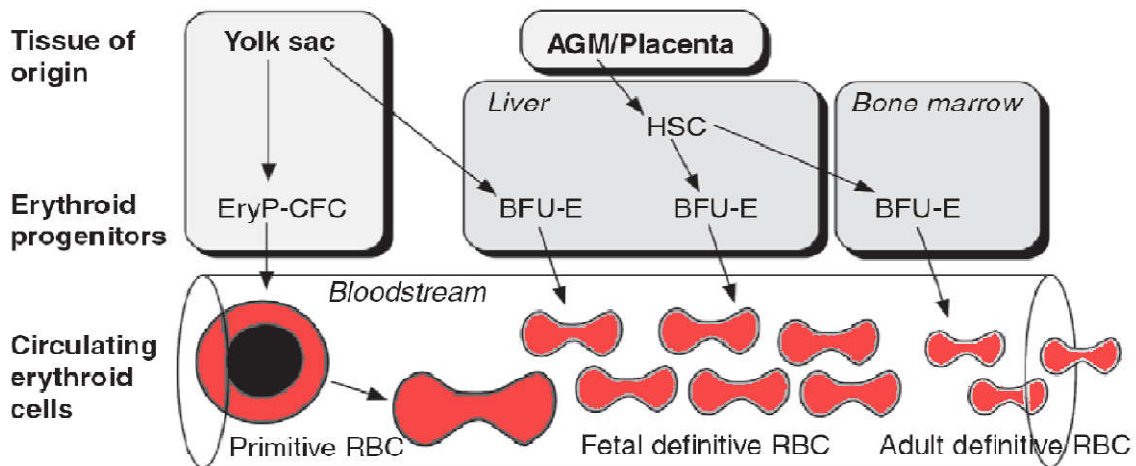


Figure 2. Three wave model of erythroid ontogeny in the mammalian embryo. The model is described in the text. (Figure was adapted from McGrath, 2008 (ref. [1]))

1.1.2. Adult definitive erythropoiesis

At normal physiological conditions 27×10^{12} erythrocytes circulate in blood stream of adult humans [15]. The total life span of an erythrocyte endures 120 days. Since life span of erythrocytes is restricted, the pool of erythrocytes has to be renewed constantly. The definitive erythropoiesis in adults has evolved as a steady state process in which the formation and degradation of erythrocyte balance each other [15]. For humans it has been calculated that a total number of 2×10^6 erythrocytes have to emerge per second [15].

At normal physiological conditions erythropoiesis takes place in the bone marrow and is maintained by continuous generation of erythroid progenitors derived from HSCs [16]. HSCs are capable to give rise to all types of blood cells, such as lymphocytes, granulocytes, neutrophils, monocytes, macrophages, osteoclasts, thrombocytes and also erythrocytes (Fig. 3A) [17]. According to this HSCs are multipotent stem cells, which have to undergo several steps of commitment and differentiation in order to generate mature progenies.

The first unipotential progenitors for the erythroid lineage are BFU-Es [18, 19]. The next stage, downstream of BFU-Es, consists of colony forming unit-erythroid (CFU-E) cells. BFU-Es and CFU-Es are defined by their colony-forming potential *in vitro* using semisolid medium containing EPO. Human BFU-Es are defined by the ability to give rise to an erythroid “burst” of approximately 1000 erythroid cells *in vitro*. In contrast, CFU-Es give rise to colonies consistent of up to 64 erythroid cells [19, 20].

The following steps of erythroid differentiation downstream of CFU-Es consist of rapidly maturing erythroblasts. Those erythroblasts have limited potential of self renewal and pass defined stages of maturation. The most immature erythroblasts are proerythroblasts. These proerythroblasts differentiate into basophilic erythroblasts under continuous cell proliferation [21]. Next, basophilic erythroblasts progress to polychromatophilic erythroblasts while proliferation ceases [22]. Afterwards polychromatophilic erythroblasts differentiate into orthochromatophilic erythroblasts. Relevantly, during these stages of maturation erythroblasts undergo rapid morphological changes. Differentiating erythroblasts continuously decrease their size, accumulate hemoglobin, and undergo progressive nuclear condensation [23].

During the terminal steps of erythroid differentiation orthochromatophilic erythroblasts extrude the nucleus and form reticulocytes. Besides, the extruded nucleus forms the transient pyrenocyte. Next, these reticulocytes degrade ribosomes and expel organelles. Finally, reticulocytes become biconcave discoid shaped in order to form mature erythrocytes, which enter the blood stream (Fig. 3B) [16].

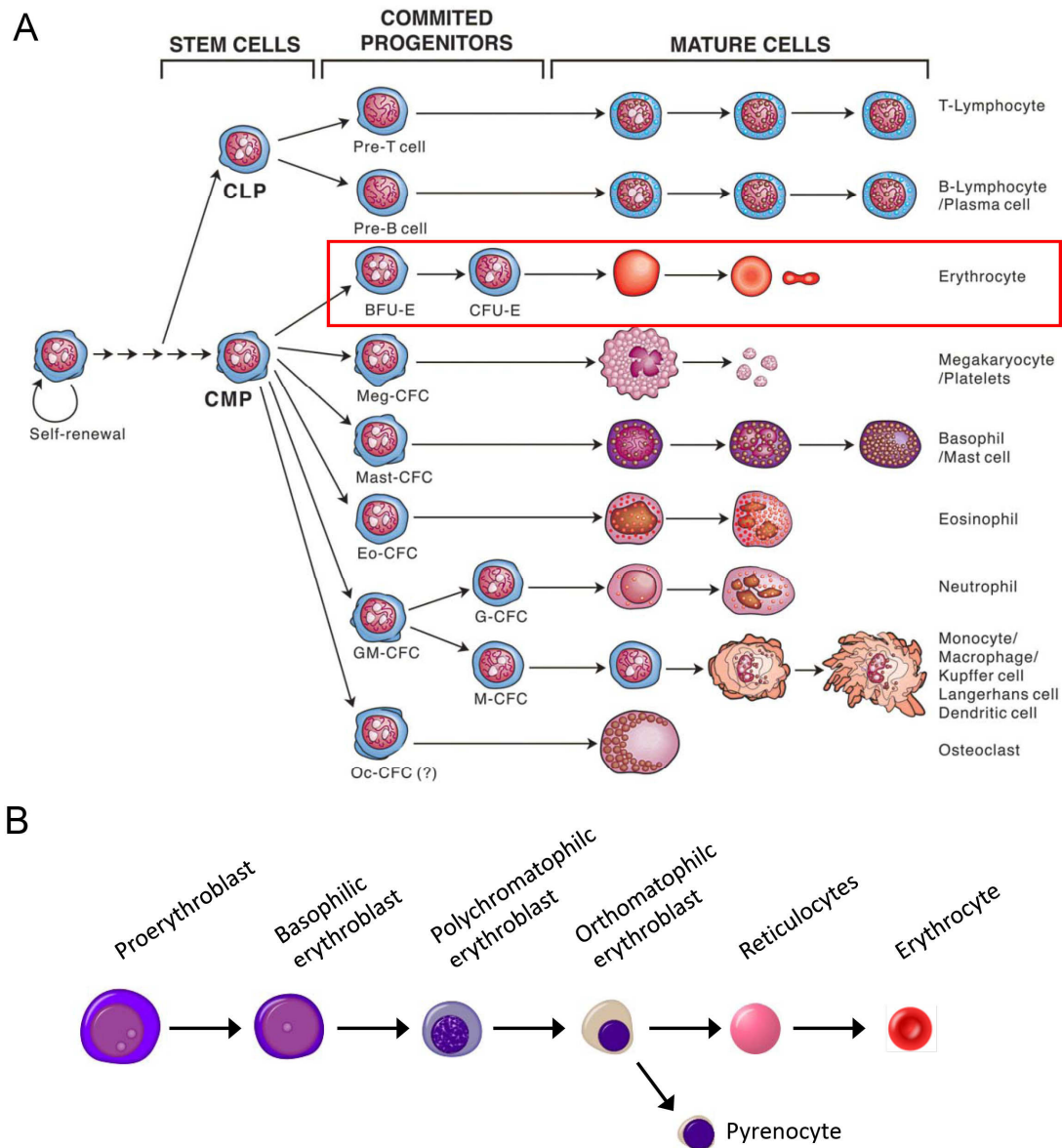


Figure 3: HSCs undergo several stages of differentiation and commitment to form mature progenies. (A) Schematic overview of hematopoiesis and its diverse progenies. The erythroid lineage is bordered in red. (B) Depiction of maturation stages of the erythroid lineage; from proerythroblast to erythrocyte. (Figure 3A was adapted from Metclaf D., *Blood lines*, 2007; AlphaMed Press. Figure 3B was adapted from Xira X, available at [http://en.wikipedia.org/wiki/File:Hematopoiesis_\(human\)_diagram.png](http://en.wikipedia.org/wiki/File:Hematopoiesis_(human)_diagram.png), update 05/22/2007.)

Noteworthy, the number of erythroid progenitors is significantly higher as it is demanded for the production of erythrocytes required under normal physiological conditions. To avoid overproduction of erythrocytes during steady state erythropoiesis, surplus erythroid progenitors undergo apoptosis. However, the overproduction of erythroblasts serves as a reservoir to increase the production of erythrocytes immediately in situation of higher demands, such as severe blood loss. The activation of the erythroblasts reservoir is regulated by a process termed as stress erythropoiesis, which is described in more detailed in the following paragraph [23].

1.1.3. Stress erythropoiesis

Under normal physiological conditions adult erythropoiesis is a steady state process in order to constantly renew the pool of erythrocytes. Beyond normal physiological conditions adult erythropoiesis acts as a highly dynamic process that responds to altered demands quickly. Severe blood loss, anemia of multiple etiologies and therapeutic procedures such as chemotherapy, generally result in tissue hypoxia. In response to tissue hypoxia, the production rate of erythrocytes is increased immediately by stress erythropoiesis. In contrast to steady state erythropoiesis, which is located in the bone marrow, stress erythropoiesis additionally takes place in the spleen. Stress erythropoiesis is stimulated by a 1000-fold increase in erythropoietin (Epo) levels, glucocorticoids, bone morphogenic proteins (BMP) and stem cell factors. These factors enhance the proliferation, reduce the doubling time and promote survival of erythroid progenitors [23]. Furthermore, erythroid differentiation is accelerated during stress erythropoiesis as well (Fig. 4) [24, 25].

The primarily function of Epo is to promote survival of erythroid progenitors during steady state as well as stress erythropoiesis by antiapoptotic effects [26]. The increased Epo level during

stress erythropoiesis results directly in an increased survival of erythroid progenitors. For instance, Epo antagonizes Fas-mediated apoptosis of erythroblasts by down-regulating expression of Fas and FasL, which subsequently results in reduced levels of Fas and FasL molecules on surface of proerythroblasts and basophilic erythroblasts [23]. In addition, Epo-signaling activates STAT5, which subsequently up-regulates the antiapoptotic gene bcl-x [27]. Both, antagonizing Fas / FasL signaling and up-regulation of bcl-x enhance erythroblasts survival and contributes to increased erythrocyte production.

Glucocorticoid-signaling during stress erythropoiesis promotes proliferation of early erythroid progenitors, which leads to increased production of erythrocytes as well. *In vitro*, glucocorticoids are capable to inhibit differentiation and promote self-renewal of CFU-Es [28]. Notably, overexpression of dominant negative glucocorticoid receptor mutant in mice abrogates the expansion of CFU-Es in response to acute anemia [29].

Additionally, during the recovery from acute anemia a distinct population of BFU-Es is found in the spleen, which is termed stress BFU-Es. In contrast to BFU-Es from steady state erythropoiesis, stress BFU-Es have reduced doubling time and only require the addition of Epo to form colonies *in vitro*. The BMP4/Smad5 signaling pathway was shown to be essential for the mobilization of stress BFU-Es in the spleen [24]. Noteworthy, erythrocytes produced during adult stress erythropoiesis, are increased in size and also contain fetal hemoglobin [30].

During fetal development the rapid growth of the embryo demands a likewise rapid expansion of the blood volume, which is compensated by expansive erythropoiesis. As a result the total mass of erythrocytes is increased by 70-fold during fetal development. However, the embryo constantly exhibits critical hypoxia [1, 31]. The expansive manner and hypoxic background of fetal erythropoiesis suggests that it is similar to adult stress erythropoiesis. A striking indication

for the similarity is the requirement of BMP4/Smad5 signaling pathway for the expansion of erythroid progenitors during fetal definitive and adult stress erythropoiesis. Intriguingly, it has been proposed that adult stress erythropoiesis is a reactivation of mechanisms from fetal definitive erythropoiesis, since erythrocytes derived from adult stress erythropoiesis exhibit similar morphology to fetal definitive erythrocytes and accumulate fetal hemoglobin as well [1].

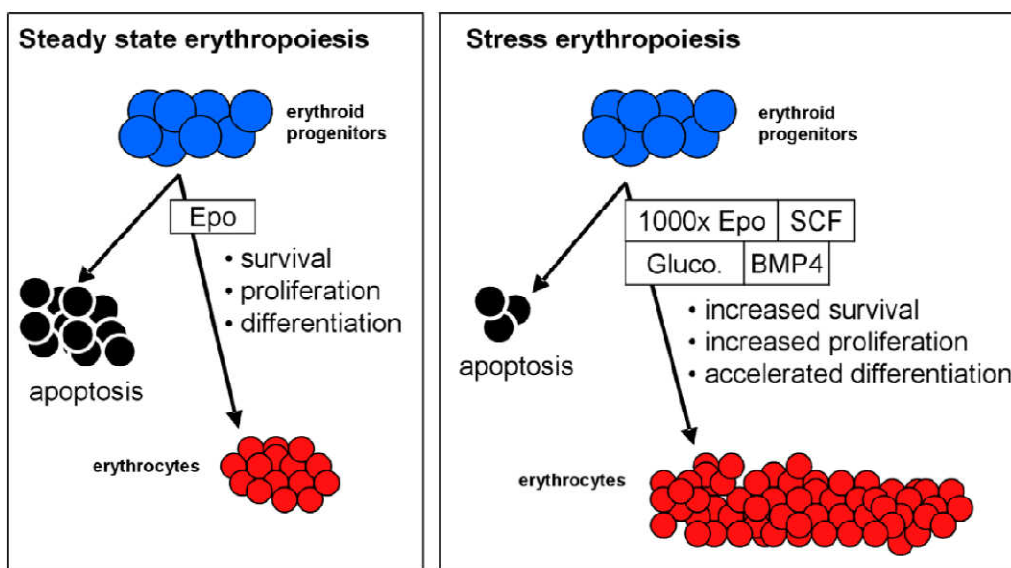


Figure 4. The erythroid lineage is differentially regulated during steady state and stress erythropoiesis. Different stimuli and their effects to the erythroid lineage during steady state and stress erythropoiesis are illustrated. Note, during stress erythropoiesis the production of erythrocytes is enhanced by increased survival, increased proliferation and accelerated differentiation of erythroid progenitor cells.

1.1.4. The erythroblastic island

During erythropoiesis differentiating erythroblasts associate with macrophages and form erythroblastic islands (Fig. 5A). An erythroblastic island consists of a central macrophage surrounded by maturing erythroblasts, including proerythroblasts through to young

reticulocytes (Fig. 5B). The number of erythroblasts associated to a macrophage is variable and differs between species. For instance, erythroblastic islands of rats contain 5 to 10 erythroblasts, whereas erythroblastic islands of humans contain up to 30 erythroblasts [32]. The function of macrophages in erythroblastic islands includes the promotion of erythroblasts survival as well as regulation of proliferation and maturation [33]. In general, macrophages implement their regulatory function to erythroblasts in three distinct ways; by direct cell contact via surface molecules, by secretion of cytokines and also by promoting erythroblast / erythroblast interaction.

Among others, erythroblast / macrophage interaction is mediated by the erythroblast macrophage protein (EMP) [33, 34]. EMP is a surface molecule, which is expressed in erythroblasts and macrophages and mediates interaction via homophilic binding. Remarkable, erythroblasts which are co-cultured with macrophages in the presence of anti EMP antibody show a significant decrease in proliferation, maturation and enucleation accompanied by a six fold increase in apoptosis. The rates of erythroblasts enucleation, maturation and apoptosis during EMP inhibition are similar to those of differentiating erythroblasts in the absence of macrophages [34, 35]. This result underlines the importance of cellular interaction between erythroblasts and macrophages mediated by EMP.

On the other hand, macrophages secrete cytokines such as IGF-1, TNF α and IL6, which have regulatory effects on erythroid cells. For instance, the presence of IGF-1 promotes proliferation of BFU-Es and CFU-Es, whereas TNF α is capable to inhibit erythropoiesis [32].

In addition, macrophages mediate erythroblasts / erythroblasts interaction by spatial organization. It has been shown that homotypic signaling between erythroblasts within erythroblastic islands is crucial for regulating GATA-1 activity and therefore is essential for

proper erythroblast differentiation [32]. In conclusion, these studies show that the regulatory functions of macrophages are essential for proper erythropoiesis *in vivo* and erythroid differentiation *in vitro*.

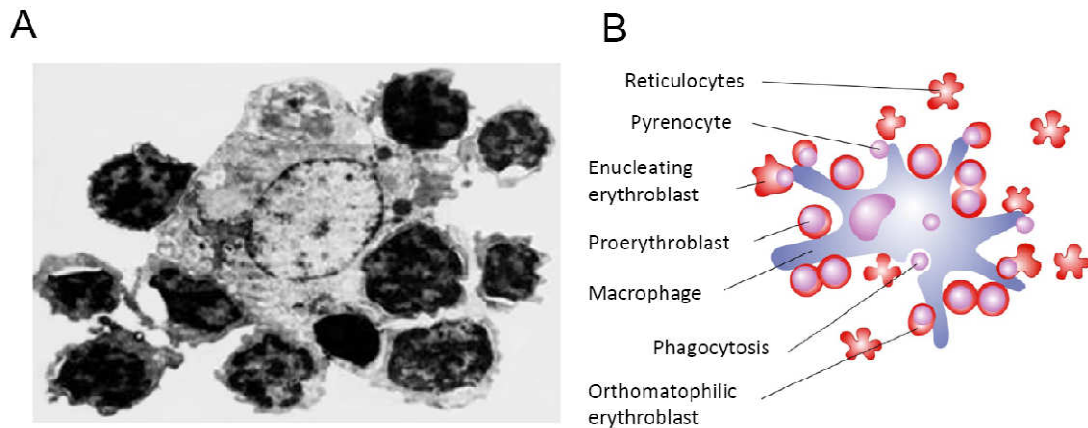


Figure 5. The erythroblastic island is a complex of erythroblasts associated to a central macrophage. (A) Transmission electron micrograph of an erythroblastic island isolated from rat bone marrow. Note the intensive cell contact between erythroblasts and the central macrophage. (B) Depiction of an erythroblastic island containing a central macrophage and associated erythroblasts. The maturation stages of associated erythroblasts range from proerythroblasts through to young reticulocytes. (Figures were adapted from *Chasis*, 2008 (ref. [32]))

1.1.5. Enucleation of erythroblasts

In mammals, three different cell types have been identified which lose their nucleus during differentiation; namely lens fibers, keratinocytes and erythrocytes [36]. In the erythroid lineage, enucleation is a hallmark of terminal differentiation. In humans approximately 2×10^6 mature erythrocytes arise per second [15]. Consequently, the process of erythroblast enucleation occurs very frequently and is also important for erythroid homeostasis. Among others, macrophages contribute essentially to erythroblast enucleation [33]. Since enucleation occurs in pure erythroblast *in vitro* cultures as well, it is reasonable that enucleation of erythroblasts is a cell-autonomous process [37]. However, in the absence of supporting cells the enucleation of

erythroblasts is less efficient and also partially blocked. The distribution and remodeling of cytoskeleton components in erythroblasts during enucleation have been described many years ago [38]. Despite that, little is known about genes and signaling pathways which initiate and regulate erythroblast enucleation in a cell-autonomous manner. It was proposed that enucleation is a kind of asymmetric cell division, but this assumption remains to be verified [39-41].

In recent years, a few studies described molecular mechanisms that were required for enucleation of erythroblasts in a cell-autonomous manner. In line with the assumption that enucleation of erythroblasts is a cytokinesis-like process, it has been shown that during enucleation a contractile actin ring (CAR) is formed between nascent reticulocyte and the nucleus during enucleation [38]. The Rho-GTPases, Rac1 and Rac2, were identified to be essential for CAR formation during terminal differentiation as well as enucleation of erythroblasts [42]. Furthermore, it was demonstrated that mDia2 is the major effector of Rac1 and Rac2 in CAR formation and enucleation. This study revealed genes essential for regulating erythroblast enucleation in the last steps of erythropoiesis in a cell-autonomous manner [42].

The tumor suppressor protein retinoblastoma (Rb) was shown to be essential for enucleation of erythroblasts in a cell-autonomous manner as well [43]. The authors showed that the function of Rb in enucleation of erythroblasts is only required during erythropoiesis under stress conditions, such as fetal development or anemia-induced stress erythropoiesis [43]. These results additionally demonstrate that enucleation of erythroblasts is differentially regulated during steady state and stress erythropoiesis. Despite recent findings about genes involved in enucleation of erythroblasts the entire process, especially during stress erythropoiesis, remains largely unknown.

1.2. Regulation and function of mitogen-activated protein kinases (MAPKs)

2.2.1. Upstream signaling of MAPKs

In multicellular organisms each cell is regulated by hierarchical structured systems to organize fundamental processes, such as development, homeostasis and survival. Therefore it is required that each cell is able to recognize and respond to regulative signals. Major components of cellular mechanisms for recognition and transduction of signals are MAPKs. The regulation and function of MAPKs is highly conserved in eukaryotes from yeast through to mammals [44, 45]. In vertebrates five subfamilies of MAPKs have been described including extracellular signal-regulated kinases ERK1/2, c-Jun N-terminal kinases JNK1/2/3, p38 α / β / δ / γ , ERK5 and ERK7. Extracellular stimuli such as growth factors, cytokines and toxins as well as environmental changes including cell adherence, osmolarity and temperature result in phosphorylation and activation of distinct MAPKs [46, 47]. Upon activation MAPKs regulate cellular processes, such as metabolism, proliferation, differentiation, survival and apoptosis by phosphorylating definite substrates [48].

The activation of MAPKs is regulated within MAPK signaling cascades. MAPK signaling cascades are integrated kinase networks and their central parts are structured in three regulative levels [46]. The first level consists of at least 20 MAPK kinase kinases (MKKK) and the second level consists of seven MAPK kinases (MKK). The third level encompasses eleven MAPKs. In addition, several splice variants are described for many MAPK genes. In general, extracellular stimuli are recognized by G-protein coupled receptors, which transduce the signals to small G-proteins. Next, these small G-proteins activate MKKKs. Subsequently to their activation each

MKKK phosphorylates and activate a specific subset of MKKs. These MKKs further activate definite members of the MAPK families (Fig. 6). Notably, the duration and cellular localization of kinase activity of each MAPK is dependent on its phosphorylating upstream kinases. Furthermore, activity of MAPKs is modulated by protein phosphatases and scaffold proteins. Thus, combinatory effects within MAPK signaling cascades, modulation of kinase activity and most importantly different properties of each MAPK enable a precise cellular response [46].

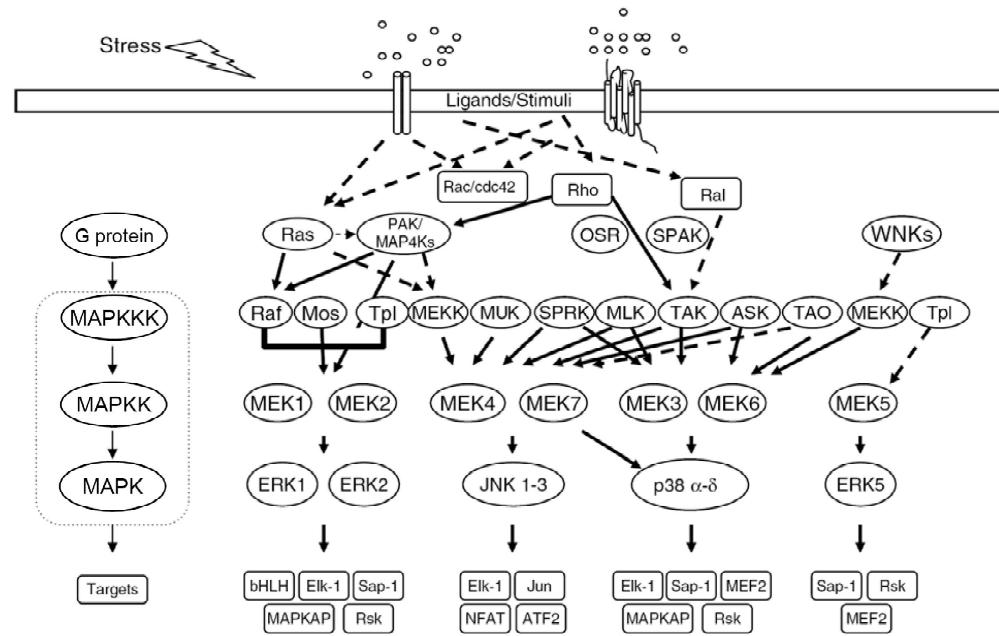


Figure 6. The MAPK signaling cascades are major components of cellular signal transduction. Illustration of the MAPK signaling cascades including ERK1, ERK2, JNK, p38 and ERK5 MAPK families with common upstream activators and prominent substrates. (Figure was adapted from *Ramman, 2007 (ref. [49])*)

1.2.2. The ERK, JNK and p38 MAPK families

MAPKs are grouped in 5 families and are encoded by at least eleven genes, which have additional splice variants [47]. ERK, JNK and p38 MAPK are the best studied MAPK families, which might also reflect their biological significance.

The genes of ERK1 and ERK2 are evolutionary conserved and share 83% identity [50]. Their main products, 44 kDa ERK1 and 42 kDa ERK2 are ubiquitously expressed. ERK1/2 regulate cellular processes such as proliferation, differentiation and survival. ERK1/2 are activated in response to growth factors, ligands for G-protein coupled receptors and cytokines. Among others, the upstream signaling cascade activating ERK1/2 includes small G-protein of the Ras-family, MKKKs of the Raf-family and the MKKs MEK1 and MEK2. Substrates regulated by ERK1 and ERK2 encompass protein kinases such as RSK, proteins involved in cell attachment such as paxillin and transcription factors including Elk1, c-Fos and c-Myc [50]. Notably, perturbed regulation of ERK1/2 by mutational activation of upstream regulators plays an important role in oncogenic transformation. Mutational activation of EGFR, Ras and Raf is found in multiple human cancers, such as lung and pancreatic cancers as well as melanomas [51].

The JNK MAPKs are also referred to as stress-activated protein kinase (SAPK) and include three JNK genes; *Jnk1*, *Jnk2* and *Jnk3* [52]. The main products of JNK genes are expressed in a short (46 kDa) and a long form (54 kDa). Whereas JNK1 and JNK2 are ubiquitously expressed, JNK3 expression is restricted to brain, heart and testis. JNKs regulate cytokine production, inflammatory response, programmed and stress-induced apoptosis as well as cell transformation and metabolism. JNKs are activated during inflammation and environmental stresses, such as heat shock and radiation. At least 14 of the described 20 MKKKs are capable to activate either

MEK4 or MEK7, which subsequently activate JNKs. Prominent substrates of JNKs are the transcription factors c-JUN, ATF-2, p53 and Elk-1 [52]. It has been shown that JNKs play different roles in multiple tumors. For instance, in 7,12-dimethylbenz(*a*)anthracene / 12-O-tetradecanoylphorbol-13-acetate (DMBA / TPA)-induced skin cancer model JNK1 is capable to suppress tumor development, whereas JNK2 promotes papilloma formation. On the other hand, it has been shown recently that JNK1 but not JNK2 promotes liver cancer development by down-regulation of p21 and up-regulation of c-myc. It remains to be determined, whether differential regulation of ERK, AKT and AP1 activity could explain the cell context-dependent functions of JNKs observed in skin and liver tumorigenesis [45].

The p38 MAPK subfamily, termed SAPK as well, includes four isoforms; p38 α , p38 β , p38 δ and p38 γ . Notably, p38 isoforms share up to 40% identity with other MAPKs and only 60% of identity among each other. Hence, it is reasonable that the p38 isoforms have highly diverse functions [49]. The isoforms p38 α and p38 β are expressed ubiquitously. In contrast, p38 γ is most prominently expressed in skeletal muscles and p38 δ is mainly found in testis, pancreas, kidney and small intestine [53]. p38 α is the best studied isoform among the p38 MAPKs and will be discussed more detailed later (see below). Members of p38 MAPK subfamily have been shown to regulate cellular process such as inflammatory response, cell cycle progression, proliferation and differentiation. p38 MAPKs are activated in response to pro-inflammatory cytokines, UV radiation, osmotic shock and hypoxia. The signaling cascade activating p38 MAPKs include small GTP-binding proteins of the Rho-family such as Rac1, Cdc42 and Rho which activate MKKKs such as MLKs, ASK1, TAK1 and members of the MEKK family. Next, these MKKKs activate MKK3 and MKK6, which specifically activate p38 MAPKs [53]. Additionally, p38 α has been shown to be activated by autophosphorylation upon TAB1 interaction [49]. Among others,

prominent substrates of p38 MAPKs are transcription factors p53 and STAT1, protein kinases MAPKAP2 and MNK1 and diverse proteins such as NHE1, cdc25 and TAU [53]. Recently, the relative contribution of the p38 isoforms (p38 β , p38 γ and p38 δ) has been studied in myofiber growth and regeneration of skeletal muscle in adult mice [54]. It has been shown that p38 β , p38 γ and p38 δ are dispensable for adult muscle formation and muscle regeneration after injury. However, chemical inhibition of p38 α in either p38 β , p38 γ or p38 δ -deficient adult primary myoblasts abrogated their differentiation, suggesting that p38 α is the essential isoform in adult myogenesis [54].

1.2.3. Signal integration and cross-talks of MAPK signaling cascades

As described before, MAPK signaling cascades are highly specific and exhibit linear signal transduction, which is modulated by scaffold proteins and phosphatases. Signal integration provides an additional way to modulate signal transduction and allows further specification of the cellular response. Diverse cross-talks of MAPK signaling cascades were found among each other as well as with other signaling pathways, such as PI3K/AKT pathway [45]. These cross-talks are generally mediated via upstream regulators or downstream targets of MAPKs and can have either antagonistic or supportive effects on the signal transduction and cellular response.

Recently, JNK- and p38-MAPK signaling pathways were found to have antagonistic effects on cell fate decisions [45, 55]. Whereas the MKK7-JNK pathway promotes proliferation, transformation, regeneration and prevents premature senescence; the MKK3/6-p38-MAPK pathway antagonizes these processes [55]. For instance, *mkk7*-deficient mouse embryonic fibroblasts (MEF) have impaired JNK activation, reduced cell proliferation, enhanced stress sensitivity and exhibit premature senescence [55, 56]. On the other hand, chemical and genetical

inhibition of p38-MAPK in MEFs leads to increased proliferation and prevents premature senescence [55, 57]. Interestingly, Wada *et al.* have shown that inhibition of p38-MAPK in *mkk7*-deficient MEFs could rescue the observed proliferation defects [55]. Moreover, stress induced premature senescence of *mkk7*-deficient MEFs was abolished by chemical inhibition of p38 α / β [55]. This study nicely demonstrates the properties and cross-talks of JNK- and p38-MAPK signaling pathway and is a good example for signal integration of MAPK signaling cascades.

On the other hand MAPK signaling cascades are also linked to other signaling pathways. For instance, The RAS/MEK/ERK pathway intimately interacts with PI3K/AKT pathway. Strikingly, both pathways are regulated by RAS [58]. The interaction between RAS/MEK/ERK and PI3K/AKT pathway is very prominent in regulation of apoptosis. The function of RAS/MEK/ERK pathway to suppress apoptosis is occasionally dependent on PI3K-dependent signaling as demonstrated in different leukemia model cell lines [59]. Additionally, AKT has been reported to trigger activation of RAF1/MEK/ERK pathway by a PKC-dependent, but RAS-independent mechanism. This mechanism was shown to be essential for the antiapoptotic effect of AKT in leukemia model cell lines [60].

In consideration of the diversity of MAPK signaling cascades and the complexity of signal integration, there is a very high number of possible interactions. This suggests that, despite being intensively studied, there are still many unknown properties and functions of MAPKs.

1.2.4. The function of p38 α in diverse cellular processes

p38 α is the best studied MAPK of the p38 MAPK family. p38 α is activated in response to pro-inflammatory cytokines and cellular stresses, such as radiation or osmotic shocks [53]. The

cellular processes regulated by substrates of p38 α encompass chromatin remodeling, transcription, mRNA stability, cytoskeleton remodeling as well as metabolism and cell cycle progression. Impaired regulation of p38 α function implicates pathological effects such as inflammatory diseases, neurodegenerative disorders, cardiovascular dysfunctions, cancer and developmental defects (Fig. 7) [45].

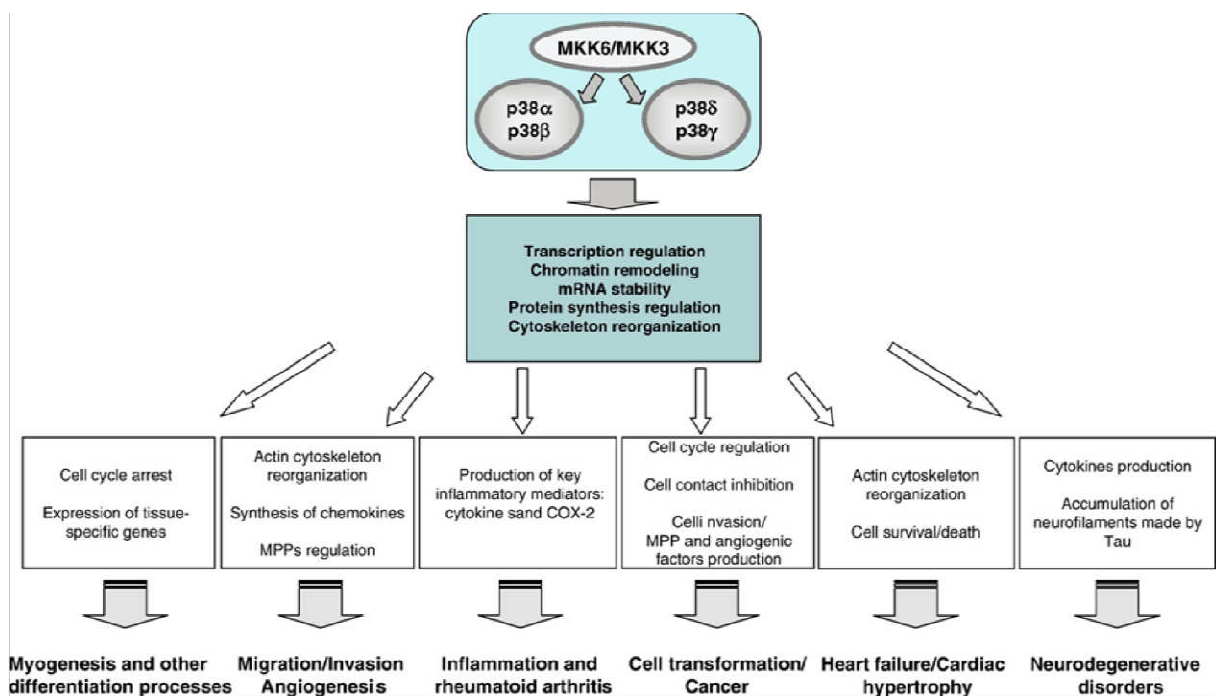


Figure 7. p38 MAPKs are required to regulate diverse physiological processes. p38 MAPKs are essentially involved in the regulation of diverse physiological processes. Loss p38 MAPKs leads to deregulation and pathological implications of these processes. Consider that p38 α was described to have a crucial role in most of the shown processes. (Figure was adapted from Cuenda, 2007 (ref.[53]))

The role of p38 α in inflammatory process has been studied intensively and is subject of recent clinical trials [61]. During inflammatory processes p38 α is activated in response to endotoxins such as liposaccharide and pro-inflammatory cytokines including TNF α and IL-6. Subsequently

to its activation p38 α promotes the production of cytokines such as TNF α , Il-6 and Il-8 (Fig. 8A). Furthermore, p38 α induces key inflammatory enzymes like COX2 and iNOS [61]. Noteworthy, p38 α promotes the production of cytokines, which have been shown to activate p38 α as well. Hence it is reasonable that p38 α is part of a positive feed back loop in regulating cytokine production [62]. In general, p38 α promotes cytokine production by transcriptional activation and mRNA stabilization [63, 64]. For instance, it has been shown recently that p38 α is required for stabilization of TNF α mRNA upon *Streptococcus pneumoniae* infection [64]. Current clinical trials examine the therapeutic potential of chemical inhibitors of p38 α to treat excessive inflammatory responses and chronic inflammation, such as rheumatic diseases and autoimmune diseases [61]. However, several studies demonstrate the necessity of p38 α to negatively regulate cell proliferation. Thus chemical inhibition of p38 α could also lead to unwanted proliferation and possibly promotes cancer development [48, 65].

A striking example for the requirement of p38 α in regulating cellular proliferation is the role of p38 α in liver cancer development [65]. Mice with liver-specific deletion of p38 α (*p38 α ^{Ali}*) show enhanced liver cancer development using diethylnitrosamine (DEN)-induced hepatocellular carcinoma model. Upon DEN-treatment *p38 α ^{Ali}* mice developed a higher number of liver tumors, which have increased size and augmented proliferation of tumor cells (Fig. 8B). Moreover, in this context it has been shown that p38 α mediates its antiproliferative effect by antagonizing the JNK/c-jun pathway [65].

On the other hand, p38 α plays a pivotal role during development and in differentiation of diverse stem and progenitor cells. Notably, straight knock-out of p38 α (*p38 α ^{KO}*) are embryonic lethal, which underlines the importance of p38 α during fetal development. Embryonic lethality of *p38 α ^{KO}* mice starts with the beginning of E10.5 and is thought to be a result of impaired placental

vascularization [66]. Nevertheless, tissue-specific deletion of *p38α* allows characterizing the function of *p38α* in late embryonic and postnatal development as well as in adult mice. For instance, mice with specific deletion of *p38α* in the embryo proper (*p38α^{fl/fl}*) were used to circumvent death due to placental defects [65]. At birth *p38α^{fl/fl}* mice have similar size compared to their littermates. However, soon after birth *p38α^{fl/fl}* mice are retarded in growth and virtually all of them die within in the first week (Fig. 8C). The cause of death is most likely lung dysfunction [65].

Defective differentiation of stem and progenitor cells lacking *p38α* has been described in bronchioalveolar stem cells, myoblasts, neuroblasts, adipoblasts and immune cells [67-71]. In adult mice *p38α*-deficiency in the lung leads to an increased number of stem and progenitor cells and an accumulation of immature progenies. In this context it has been shown that *p38α* is essential to inhibit proliferation by blocking EGFR and to promote differentiation by up-regulating *C/EBPα* and *HNF3β* [72]. In addition, *p38α^{fl/fl}* mice exhibit increased proliferation of myoblasts as well as delayed growth and maturation of myofibers in the neonatal period [68]. Remarkable, *p38α*-deficient myoblasts fail to form multinucleated myotubes *in vitro* (Fig. 8D). In addition, *p38α*-deficient myoblasts have reduced expression of muscle differentiation-specific genes and show impaired cell cycle exit during differentiation [68]. These studies show, that *p38α* is required to negatively regulate proliferation and to promote differentiation in both, maturing bronchioalveolar stem cells and myoblasts. Hence it is reasonable to assume that this might also be true for progenitor cells of other tissues. However, the molecular mechanisms regulated by *p38α* during differentiation are largely unknown and most likely cell type specific. Additionally, it is a reasonable hypothesis that impaired development in *p38α*-deficient mice is a result of tissue-specific differentiation defects rather than a lack of morphogenic signaling.

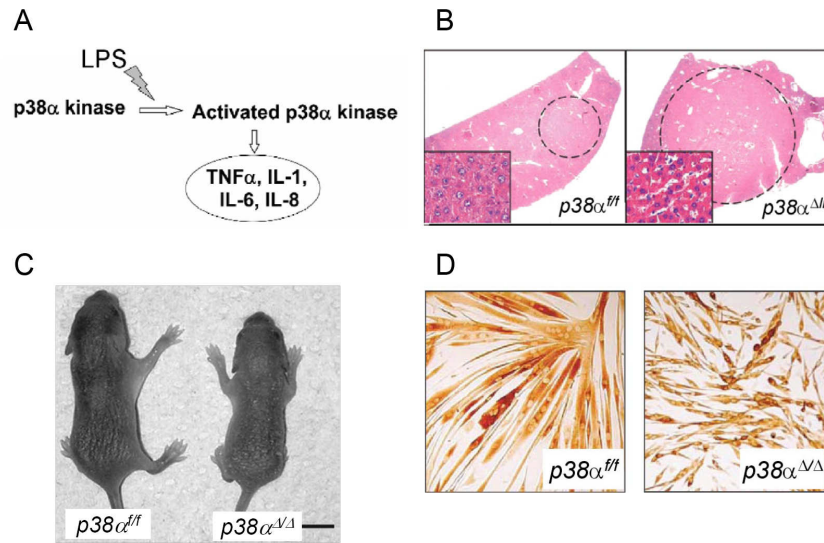


Figure 8. p38 α is involved in inflammation, tumorigenesis, development and differentiation. (A) Illustration of the regulatory function of p38 α in cytokine expression. (B) Loss of p38 α results in enhanced liver cancer development. (C) Deletion of p38 α in the embryo proper leads to growth retardation in the neonatal period. (D) p38 α -deficient myoblasts fail to form multinucleated myotubes *in vitro*. (Figure 8A was adapted from Schindler, 2007 (ref. [61]). Figure 8B was adapted from Hui, 2007 (ref. [48]). Figure 8C was adapted from Hui, 2007 (ref. [65]). Figure 8D was adapted from Perdiguero, 2007 (ref. [68])

1.3. p38 α is required for enucleation of erythroblasts under stress conditions

Survival, proliferation and differentiation of erythroid cells is primarily regulated by Epo. Epo interacts with erythropoietin-receptor (EpoR), which subsequently activates diverse signaling pathways, such as JAK-STAT and MAPK pathways [73, 74]. Advancement in methodology, especially in *in vitro* culture systems of erythroid cells, enabled to characterize signaling pathways that were activated by Epo and other stimuli and to analyze their function in erythroid proliferation and differentiation [37].

JAK2 is activated by Epo and promotes proliferation of erythroid cells [75]. The role of JAK2 in erythroid proliferation is underlined by the *JAK2* V617F mutation. This mutation causes constitutive activation of JAK2, leads to uncontrolled proliferation of erythroid cells and is very likely responsible for development of polychemia vera in humans [76]. ERK1/2, which is also activated by Epo, promotes proliferation of erythroid cells as well [77]. Constitutive activation of ERK1/2 in erythroid cells has been shown to cause prolonged Epo-independent proliferation [77]. Additionally, activated ERK1/2 blocks differentiation and suppresses apoptosis in erythroid cells [77, 78]. On the other hand, it was shown that inhibition of ERK1/2 resulted in increased apoptosis similar to Epo deprivation [78]. Epo signaling also leads to activation of JNK and p38 α [79-81]. Inhibition of JNK in primary murine bone marrow cells inhibited proliferation of BFU-Es, but did not affect the more differentiated CFU-Es [82]. Therefore, JNK activity was supposed to be important for proliferation of early erythroid progenitors [82]. Several studies describe a critical role of p38 α in erythropoiesis [83-86]. It was proposed that p38 α together with JNK suppresses ERK during erythroid differentiation, but this assumption and its effect remains to be verified [84].

It has been previously shown that mice lacking *p38 α* in the embryonic tissue (*p38 α ^{EA}*) or specifically in the hematopoietic system (*p38 α ^{Ablood}*) have impaired enucleation of erythroblasts under stress conditions [Wagner lab, unpublished]. During fetal development *p38 α ^{EA}* and *p38 α ^{Ablood}* mice exhibited nucleated erythrocytes in the peripheral blood (Fig. 9A, B). Interestingly, within one week after birth nucleated erythrocytes in the peripheral blood disappeared and were not observed in healthy adult *p38 α ^{Ablood}* mice as well (Fig. 9C). However, phenylhydrazine-(PHZ) induced hemolytic anemia in *p38 α ^{Ablood}* adult mice led to the reappearance of nucleated erythrocytes in the peripheral blood and in the spleen (Fig. 9D, E).

These mice also showed a dramatically reduced population of mature erythroid cells in spleen upon PHZ-treatment (Fig. 9F). Moreover, preliminary data suggested that erythroblasts enucleate normally in adult mice with macrophage-specific deletion of *p38α* upon PHZ-treatment [Wagner lab, unpublished].

To further analyze the role of *p38α* in differentiation of erythroblasts, *in vitro* culture system of fetal liver-derived erythroid progenitors (FLEP) was used. Noteworthy, *p38α^{Δ/Δ}* FLEPs almost completely failed to enucleate during *in vitro* differentiation (Fig. 9G). Furthermore, *p38α^{Δ/Δ}* FLEPs did not up-regulate surface markers related to terminal differentiation and exhibited reduced amount of hemoglobin [Wagner lab, unpublished]. These data suggest that the impaired enucleation of *p38α^{Δ/Δ}* FLEPs is accompanied by differentiation defects. Taken together, it has been shown that *p38α* is required for enucleation of erythroblasts during fetal development and adult stress erythropoiesis. Moreover, it is suggested from data from the Wagner lab that the function of *p38α* in enucleation of erythroblasts is cell-autonomous.

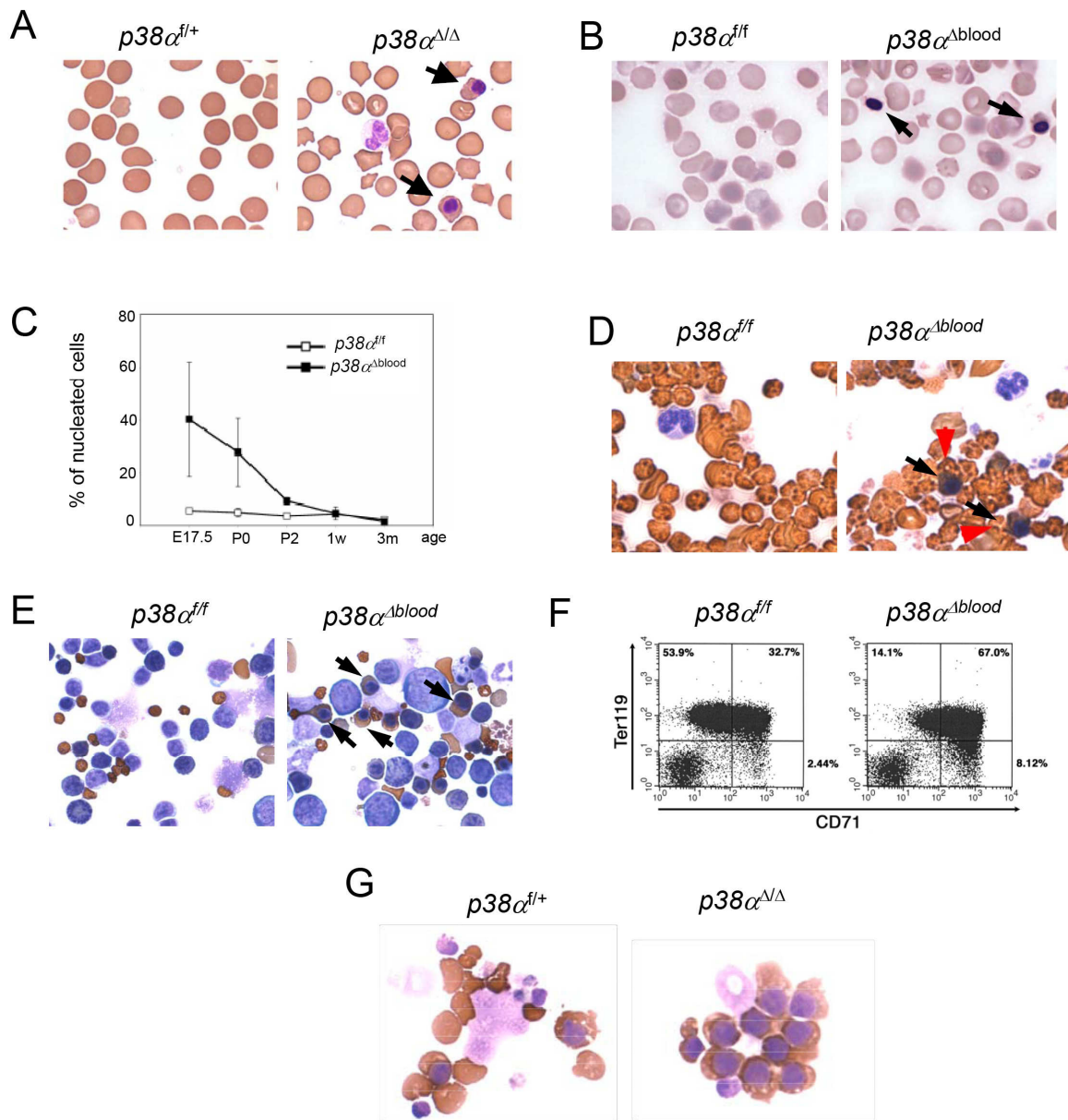


Figure 9. Loss of $p38\alpha$ affects enucleation of erythroblasts during stress erythropoiesis. (A, B) Blood smears of peripheral blood from $p38\alpha^{\Delta/\Delta}$, $p38\alpha^{Ablood}$ and control newborn mice. (C) Amount of nucleated blood cells in the peripheral blood of $p38\alpha^{Ablood}$ and control mice. (D, E) Peripheral blood smears and splenocyte cytopins from adult $p38\alpha^{Ablood}$ and control mice after PHZ-treatment. (F) TER119 / CD71 surface marker labeling of splenocytes from $p38\alpha^{Ablood}$ and control mice after PHZ-treatment analyzed by FACS. (G) Cytopined $p38\alpha^{fl/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEPs at 48h of differentiation. Nucleated erythrocytes are indicated by arrows. (Figures were obtained from Lijian Hui, Wagner lab, unpublished).

1.4. Goal of the diploma thesis

The ontogeny and origins of mammalian erythropoiesis have been studied intensively and lead to comprehensive models. Until today there is increasing knowledge about hormonal signaling regulating steady state and stress erythropoiesis and new insights into molecular pathways essential for erythroblast differentiation. However, the molecular pathways involved in erythroblast differentiation, especially under stress conditions are still largely unknown.

The goal of my diploma thesis work was to define substrates of p38 α essential for erythroblast differentiation, to provide convincing evidence for the cell-autonomous function of p38 α in erythroblast differentiation under stress conditions and to characterize by which molecular mechanism p38 α regulates erythroblast enucleation. I will describe my attempts to identify substrates of p38 α essential for erythroblast enucleation. Furthermore, I will demonstrate that the function of p38 α in enucleation of erythroblasts is cell-autonomous using mice with tissue-specific deletion of *p38 α* . In addition, I demonstrate that p38 α is required for regulating gene expression, which is characteristic for erythroid differentiation using the FLEP *in vitro* culture system. Moreover, there is aberrant activation of the retinoblastoma protein (Rb) pathway in *p38 α ^{Δ/Δ}* FLEPs. Noteworthy, the impaired differentiation of *p38 α* -deficient erythroblasts is similar to that of erythroblasts lacking Rb, suggesting that activation of the Rb pathway by p38 α is a key event for erythroblast differentiation, especially for enucleation under stress conditions.

2. Results

2.1. Identification of p38 α binding partners during erythroblast differentiation

The analysis of p38 α and its protein complex in differentiated erythroblasts should provide information about binding partners and substrates of p38 α , which are likely involved in erythroblast enucleation. Therefore, I performed co-immunoprecipitation of phosphorylated, activated p38 α (p-p38 α) and its protein complex from protein extracts of differentiated erythroblasts. p-p38 α antibodies were coupled to protein A-beads (p-p38 α -beads) and incubated with protein extracts from p38 $\alpha^{f/+}$ FLEPs differentiated for 24 and 48 hours. Protein extracts from p38 $\alpha^{4/4}$ FLEPs were used as negative controls. p-p38 α was efficiently immunoprecipitated, as shown by the depletion of p-p38 α protein levels after co-immunoprecipitation (Fig. 10).

Next, proteins bound to p-p38 α -beads were eluted, digested in solution and analyzed by mass spectroscopy. Histidine ammonia lyase, beta-globin, annexin A2, S1 protein C2, junction plakoglobin, Ribosomal protein L37a, Ribosomal protein 29 (Rpl29) and Cathepsin G were identified to immunoprecipitate solely with p-p38 α (Table 1). However, none of the identified proteins have been described to be substrates of p38 α or to be involved in erythropoiesis. The proteins Rpl29, junction plakoglobin and annexin A2 were shown to be involved in cell proliferation, differentiation and cytoskeleton remodeling [87-90] and may be of interest to be further characterized in erythroblast differentiation.

The FLEP *in vitro* culture system provided only a limited amount of protein extracts, which might also be reflected by the low number of detected peptides per protein. Thus, this experiment does not provide convincing data on p38 α substrates and binding partners. To obtain more protein extract of differentiated erythroblasts, I planed to establish an *in vitro* culture system of embryonic stem (ES) cell-derived erythroid progenitors (ESEP).

Gene symbol	Gene name	MW (kDa)	<i>p38α^{f/+}</i>		<i>p38α^{Δ/Δ}</i>	
			24h	48h	24h	48h
Mapk14	p38α	41	3	12	0	0
HaI	Histidine ammonia lyase	72	5	0	0	0
Hbb-bI	Beta-globin	16	4	0	0	0
Anxa2	Annexin A2	39	3	0	0	0
Hnrnpab	S1 protein C2	36	2	0	0	0
Jup	Junction plakoglobin	82	2	0	0	0
Rpl37a	Ribosomal protein L37a	11	0	2	0	0
Rpl29	Ribosomal protein 29	18	0	2	0	0
Ctsg	Cathepsin G	29	0	2	0	0

Table 1. Proteins immunoprecipitated with p-p38α. p-p38α and its protein complex were immunoprecipitated from protein extracts of *p38α^{f/+}* differentiated for 24h and 48h. *p38α^{Δ/Δ}* FLEPs were used as negative controls. The immunoprecipitate was digested in solution and analyzed by mass spectroscopy. The “numbers” describe the number of different peptides detected per protein. MW, molecular weight.

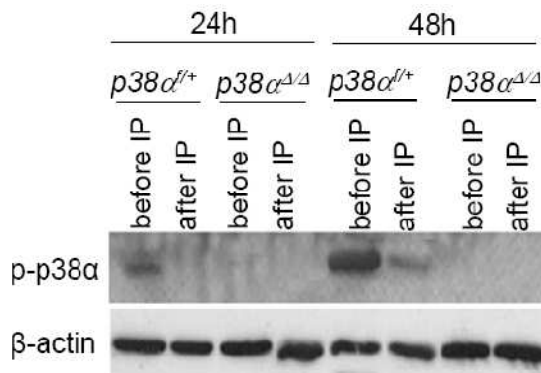


Figure 10. Protein levels of p-p38α are decreased after co-immunoprecipitation. Western Blot analysis of p-p38α protein levels in *p38α^{f/+}* and *p38α^{Δ/Δ}* FLEP protein extracts before and after immunoprecipitation of p-p38α; β-actin was used as a loading control.

2.2. Establishment of ESEP *in vitro* culture system for mass cultivation of erythroid cells

The *in vitro* culture system of FLEPs is an excellent tool to study differentiation of erythroblasts up to mature erythrocytes. However, the expansion of FLEPs is limited and the low cell number hindered the analysis of substrates and binding partners of p38 α by co-immunoprecipitation. Previously it was shown, that the ES-derived, ESEP *in vitro* culture system provides virtually unlimited supply of erythroid cells [91]. Therefore, I next tried to establish an *in vitro* culture system of wild-type and p38 α -deficient ESEPs.

2.2.1. Differentiation of p38 α ^{+/+} and p38 α ^{-/-} ES cells into ESEPs

To obtain wild-type and p38 α -deficient ESEPs, p38 α ^{+/+} and p38 α ^{-/-} ES cells were subjected to a two step protocol for directed differentiation as described [91]. Briefly, differentiation of ES cells was induced by withdrawal of leukemia inhibitory factor (LIF) and addition of differentiation promoting factors, such as human transferrin. Simultaneously, ES cells were plated on non-coated Petri dishes to allow formation of embryoid bodies (EBs). At day 6 of differentiation, EBs were dissociated into single cells and cultivated in erythroblast proliferation medium to expand erythroid progenitors.

After induction of differentiation both, p38 α ^{+/+} and p38 α ^{-/-} ES cells, formed EBs at day 2 to 3, which continuously increased in size up to day 6. The p38 α ^{+/+} EBs appeared to be roundish with a smooth surface. At day 5 the inner cell mass of p38 α ^{+/+} EBs turned brown and slightly reddish (Fig. 11A). In contrast, p38 α ^{-/-} EBs had irregular oval-shape and outgrowing cells on the surface, which detached during pipetting and reappeared on the following day. From day 4 onwards, the

inner cell mass of $p38\alpha^{-/-}$ EBs appeared to be black (Fig. 11A). Next, these EBs were dissociated into single cells and cultivated in erythroblast proliferation medium. After 7 hours of cultivation in erythroblast proliferation medium the culture of dissociated $p38\alpha^{+/+}$ EBs showed adherent cells morphologically resembling neurons, myocytes and fibroblasts as well as erythroblasts in suspension (Fig. 11B). From this culture, a pure $p38\alpha^{+/+}$ ESEPs culture was obtained after 4 days. However, the culture of dissociated $p38\alpha^{-/-}$ EBs showed no adherent cells and mainly cell clumps with only few erythroblast-like cells in suspension (Fig. 11B). Furthermore, it was not possible to obtain a $p38\alpha^{-/-}$ ESEP culture from these cells.

Previously, $p38\alpha$ has been described to be involved in differentiation of different cell types, such as myoblasts and lung stem cells [72, 92]. The lack of adherent cells and the impaired expansion of erythroid progenitors suggested that loss of $p38\alpha$ affected ES cell differentiation *in vitro*. Therefore, I analyzed the erythroid lineage in $p38\alpha^{+/+}$ and $p38\alpha^{-/-}$ EBs at day 6 of differentiation. Interestingly, FACS analysis showed a markedly increased population of immature c-KIT^{high} (49.4%) and CD71^{high} (79.2%) cells in $p38\alpha^{-/-}$ EBs compared to $p38\alpha^{+/+}$ EBs (36% and 68%, respectively) (Fig. 12A, B). TER119, a surface marker of mature erythroid cells, was not detected in $p38\alpha^{+/+}$ and $p38\alpha^{-/-}$ EBs (Fig. 12B). These data indicate that $p38\alpha^{-/-}$ EBs appear to have differentiation defects of erythroid progenitors. Moreover, the complete lack of adherent cells in the culture of dissociated $p38\alpha^{-/-}$ EBs suggests that $p38\alpha$ has an essential role in the differentiation of ES cells into the erythroid lineage *in vitro*.

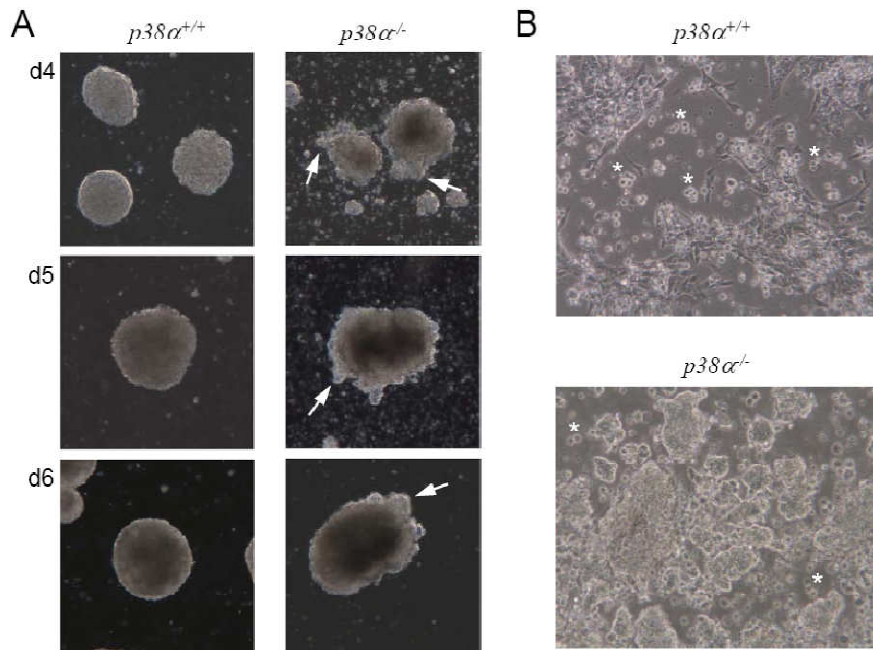


Figure 11. $p38\alpha^{-/-}$ ES cells form EBs with aberrant morphology. (A) $p38\alpha^{+/+}$ and $p38\alpha^{-/-}$ ES cells differentiated into EBs for 4, 5 and 6 days. Arrows indicate outgrowing cells on the surface of $p38\alpha^{-/-}$ EBs. (B) Culture from dissociated $p38\alpha^{+/+}$ and $p38\alpha^{-/-}$ EBs cultivated in erythroblast proliferation medium for 7 hours. Erythroblasts are indicated as “*”. Note that mainly cell clumps, few erythroblast-like cells and no adherent cells were observed in the $p38\alpha^{-/-}$ culture.

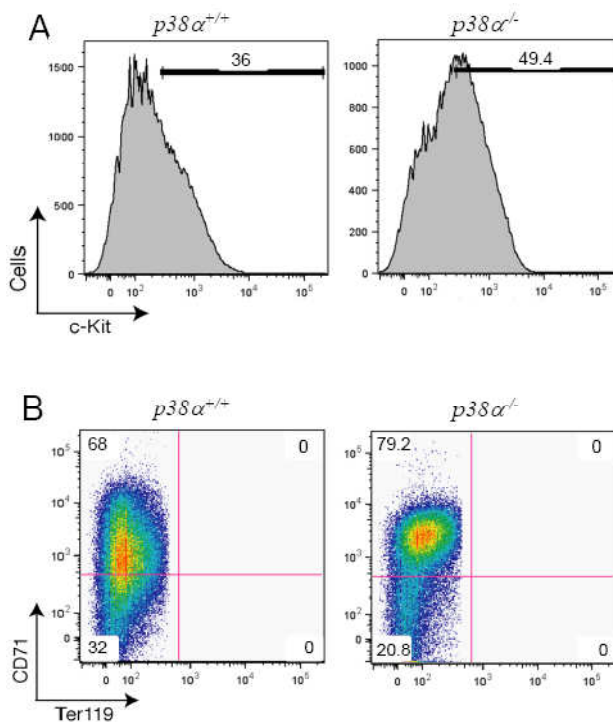


Figure 12. $p38\alpha^{-/-}$ EBs show an increased population of immature erythroid cells. (A, B) Surface markers of cells from dissociated $p38\alpha^{+/+}$ and $p38\alpha^{-/-}$ EBs were labeled with c-KIT (A) and CD71 / TER119 (B); labeled erythroid cells were analyzed by FACS.

2.2.2. Deletion of *p38α* floxed alleles in ESEPs

p38α^{-/-} ES cells showed impaired erythroid differentiation, which is likely due to the lack of *p38α*. To circumvent defective ES cell differentiation caused by loss of *p38α*, I generated ES cells with *p38α* floxed alleles (*p38α*^{fl/fl}). I planed to differentiate *p38α*^{fl/fl} ES cells into ESEPs and subsequently delete *p38α* floxed alleles in ESEPs. I initialized two approaches for the deletion of *p38α* floxed alleles, which are an inducible system using CreER(T2) and lentiviral-mediated expression of iCre [93, 94].

Deletion using CreER(T2): This gene encodes a Cre recombinase (Cre) fused to a mutant estrogen ligand-binding domain (ER(T2)). Recombinase activity of Cre is blocked by ER(T2), but is restored by blocking the inhibitory function of ER(T2) with tamoxifen [93]. I planed to generate a stable *p38α*^{fl/fl}/*CreER(T2)* ES cell line, differentiate these cells into ESEPs and delete *p38α* floxed alleles in ESEPs by tamoxifen treatment. I constructed a lentiviral vector containing *CreER(T2)* and a puromycin resistance gene; both genes under the control of hPGK promoters (pL4.hPGK.CreER(T2).hPGK.puro) (Fig. 13A). *p38α*^{fl/fl} ES cells were infected with pL4.hPGK.CreER(T2).hPGK.puro lentiviral particles for two days. Afterwards, *p38α*^{fl/fl} ES cells were selected for positive infection by addition of 1.5 μg/ml puromycin to the medium for 3 days. A *p38α*^{fl/fl}/*CreER(T2)* ES cell line was established from a single clone. To characterize the deletion efficiency of CreER(T2), *p38α*^{fl/fl}/*CreER(T2)* ES cells were treated with 1 μmol/ml tamoxifen for 2 days. PCR analysis confirmed efficient integration of *CreER(T2)* into the genome of *p38α*^{fl/fl} ES cells (Fig. 13B). However, no deletion of the *p38α* floxed alleles were detected (Fig. 13C). These data unfortunately imply that CreER(T2) did not exert recombinase activity upon tamoxifen treatment, likely due to insufficient expression levels or gene mutations.

Deletion using iCre: For the second approach I tried to delete the $p38\alpha$ floxed alleles in ESEPs by lentiviral-mediated iCre expression. I was able to infect wild-type ESEPs efficiently using pRRLSIN.cttp.hPGK.GFP lentiviral particles (Fig. 14A). Next I had to replace *GFP* in pRRLSIN.cttp.hPGK.GFP by *IRES.GFP* (pSS.hPGK.IRES.GFP) and insert *iCre* afterwards (pSS.hPGK.iCre.IRES.GFP) (Fig. 14B). To obtain $p38\alpha^{ff}$ ESEPs, $p38\alpha^{ff}$ ES cells were subjected to directed differentiation as shown before. There was no obvious phenotype during $p38\alpha^{ff}$ ES cell differentiation and a pure $p38\alpha^{ff}$ ESEP culture was established. The $p38\alpha^{ff}$ ESEPs showed properties similar to wild-type ESEPs and were not further characterized. For deletion of the $p38\alpha$ floxed alleles, ESEPs were infected with pSS.hPGK.iCre.IRES.GFP lentiviral particles; pSS.hPGK.IRES.GFP lentiviral particles were used as control. The infection efficiency of ESEPs was approximately ~70%, as determined by fluorescence microscopy. PCR analyses confirmed integration of *iCre* into the genome of $p38\alpha^{ff}$ ESEPs (Fig. 14C). Moreover, there was efficient deletion of the $p38\alpha$ floxed alleles (Fig. 14C). However, $p38\alpha^{ff}$ ESEP cultures expressing iCre died within a few days after infection, whereas the control culture could be expanded. These data suggest that lentiviral-mediated expression of iCre had toxic effects in ESEP cultures. It was shown previously that high expression levels of Cre can lead to genotoxic effects in proliferating mammalian cells [95]. Taken together, it was not possible for me to obtain $p38\alpha$ -deficient ESEPs from $p38\alpha^{-/-}$ and $p38\alpha^{ff}$ ES cells.

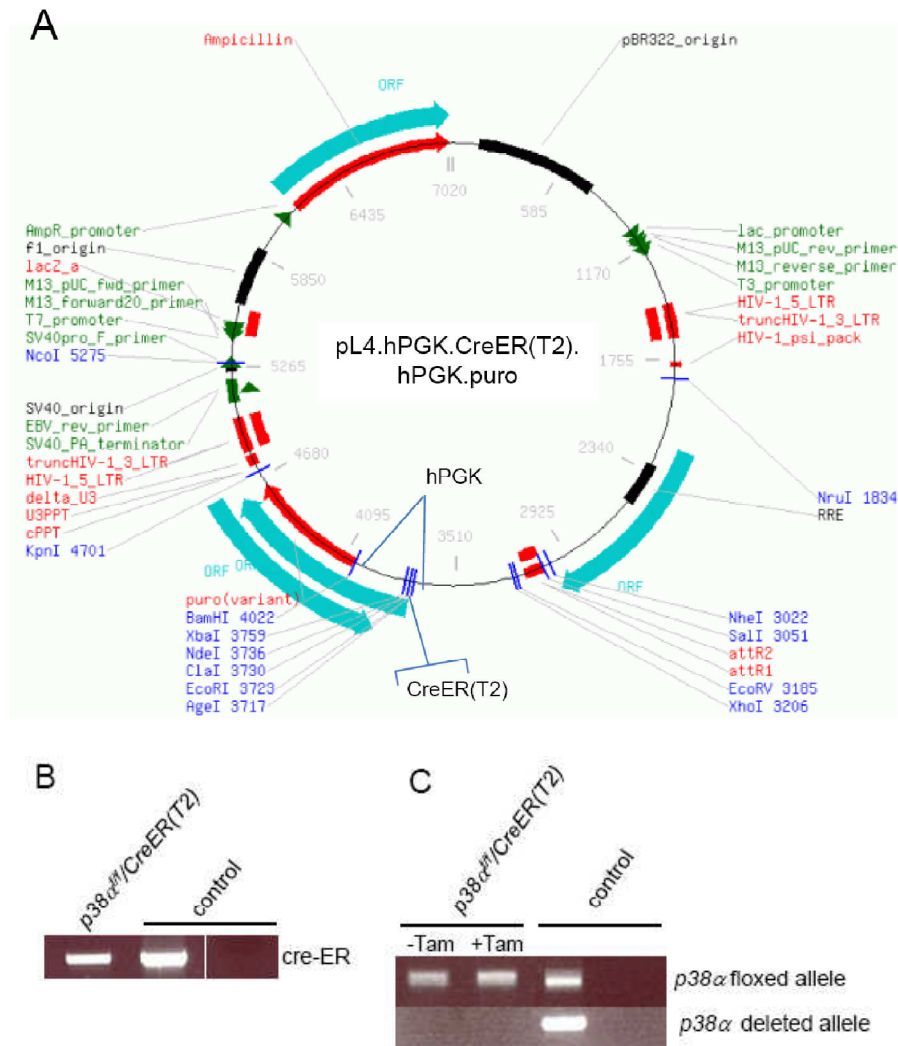


Figure 13. CreER(T2) does not exert recombinase activity in *p38α^{fl/fl}/CreER(T2)* ES cells. (A) Vector map illustrates pL4.hPGK.CreER(T2).hPGK.puro lentivirus vector. (B) PCR analysis of *CreER(T2)* in *p38α^{fl/fl}/CreER(T2)* ES cells. (C) *p38α* floxed and *p38α* deleted alleles in *p38α^{fl/fl}/CreER(T2)* ES cells upon Tamoxifen (Tam) treatment were analyzed by PCR. pL4.hPGK.CreER(T2).hPGK.puro plasmids and mouse genomic DNA with *p38α* floxed and deleted allele were used as positive controls, H₂O was used as negative control.

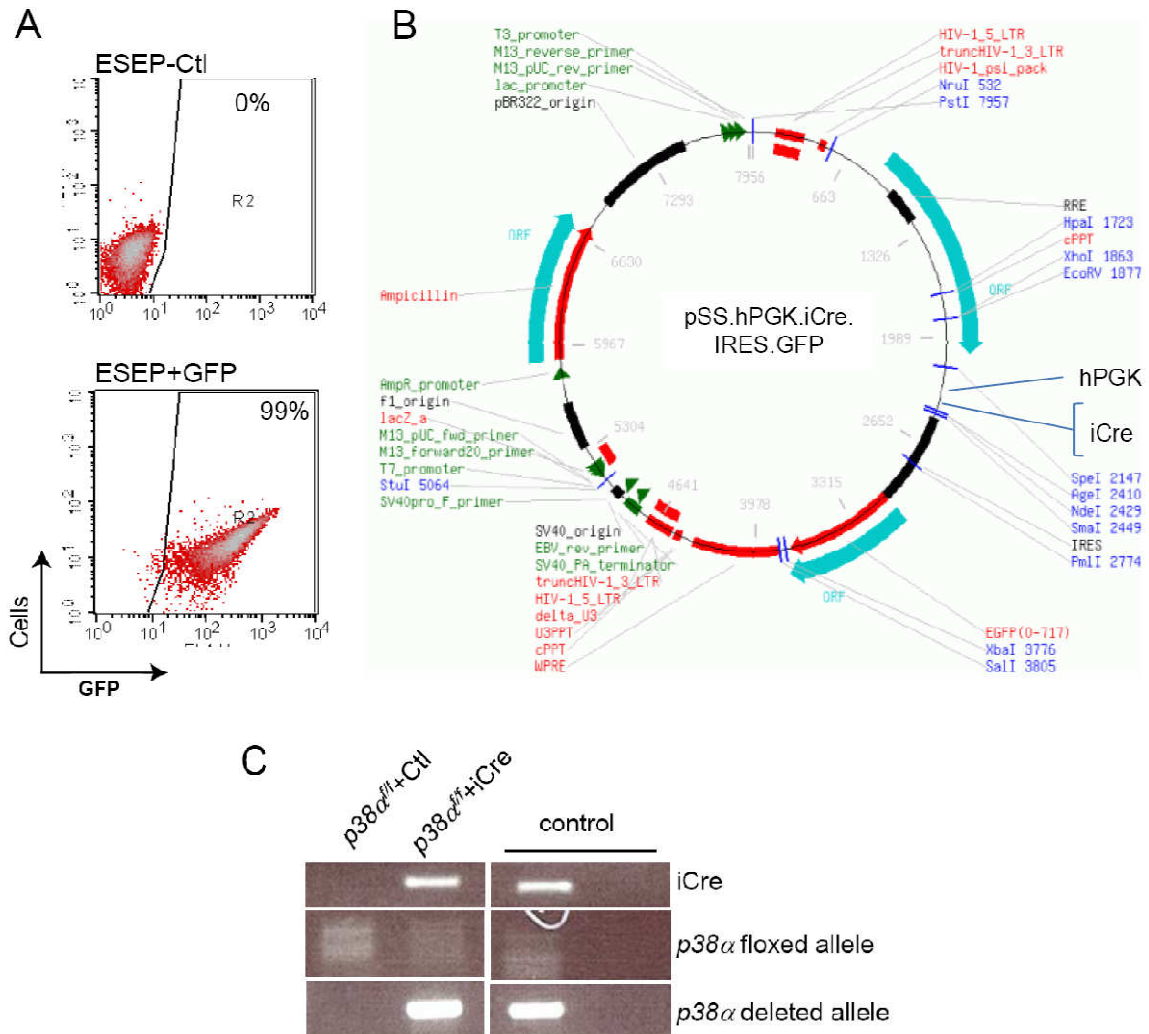
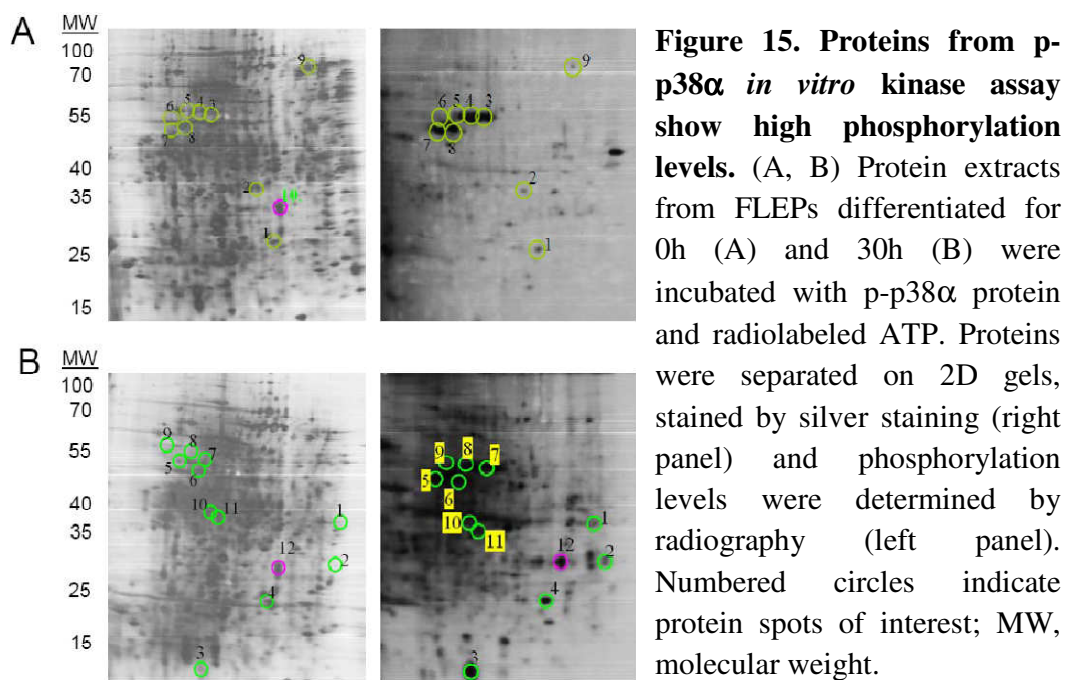


Figure 14. iCre deletes *p38α* floxed alleles in *p38α^{ff}* ESEPs. (A) GFP expression of wt ESEPs infected with pRRLSIN.cttp.hPGK.GFP lentiviral particles (ESEP+GFP) analyzed by FACS. Non-infected ESEPs were used as control (ESEP-Ctl) (B) Vector map illustrates pSS.hPGK.iCre.IRES.GFP lentivirus vector. (C) iCre, *p38α* floxed and *p38α* deleted alleles in *p38α^{ff}* ESEPs infected with pSS.hPGK.IRES.GFP (*p38α^{ff}*+Ctl) and pSS.hPGK.iCre.IRES.GFP (*p38α^{ff}*+iCre) lentiviral particles were analyzed by PCR. pSS.hPGK.iCre.IRES.GFP plasmids and mouse genomic DNA with *p38α* floxed and deleted allele were used as positive controls, H₂O was used as negative control.

2.3. Identification of p38 α substrates by p-p38 α in vitro kinase assay

A p-p38 α *in vitro* kinase assay was initiated previously in the Wagner lab by Lijian Hui in collaboration with Eleanor Coffey; *Centre for Biotechnology, Turku* to identify substrates of p38 α during erythroblast differentiation. Proteins extracts from FLEPs cultured in differentiation medium for 0h and 30h were incubated with purified p-p38 α and radiolabeled ATP. The proteins were separated on a 2D gel and visualized by silver staining. Phosphorylation levels of proteins were detected by radiography (Fig. 15). Highly phosphorylated protein spots were analyzed by mass spectroscopy. Potential substrates of p38 α among the proteins identified by mass spectroscopy analyses were assigned by Eleanor Coffey Lab and are listed in Table 2A, B. The *nuclear distribution gene C* (NUDC) is described to be activated upon phosphorylation and involved in cytokinesis [96, 97]. As enucleation of erythroblasts is supposed to resemble asymmetric cytokinesis, NUDC was an attractive potential substrate of p38 α that might play a role in erythroblast enucleation [39-41].



A

Spot	Symbol	Gene name
1	STMN1	Stathmin
2	NUDT5	ADP-sugar pyrophosphatase
2	CLIC1	Chloride intracellular channel protein 1
2	PSB4	Proteasome subunit beta type 4 precursor
3	GALK 1	Galactokinase
4		no significant hits
5	NUCKS	Nuclear ubiquitous casein and cyclin dependent kinases substrate
6		no significant hits
7	NPM1	Nucleophosmin
8	NPM1	Nucleophosmin
8	EF1D	Elongation factor 1 delta
9	CCT5	TCPE T-complex protein 1 subunit epsilon
10	ATP5H	ATP synthase D chain, mitochondrial

B

Spot	Symbol	Gene name
1	ALB	Albumin
2		no significant hits
3		no significant hits
4	STMN1	Stathmin
5		no significant hits
6	NPM1	Nucleophosmin
6	EF1D	Elongation factor 1 delta
7	GALK1	Galactokinase
7	TMOD3	Tropomodulin 3
7	NUDC	Nuclear distribution gene C
7	NUCKS	Nuclear ubiquitous casein and cyclin dependent kinases substrate
8	PRDX2	Peroxiredoxin-2
8	STRAP	Serine- threonine kinase receptor associated protein
8	GALK 1	Galactokinase
9		no significant hits
10	PSM6	26S protease regulatory subunit 6A
11	HSP90B	Heat shock protein 90, isoform b
12	ATP5H	ATP synthase D chain , mitochondrial
12	LYSCP	Lysozyme C type P precursor
12	G3BP1	ras GTPase activating protein binding protein 1

Table 2. Proteins identified as potential p38 α substrates by p-p38 α *in vitro* kinase assay. p-p38 α *in vitro* kinase assay was performed with protein extracts from FLEPs differentiated for 0h (A) and 30h (B). Highly phosphorylated protein spots were analyzed by mass spectroscopy and potential substrates of p38 α are listed.

2.4. Knock-down of *NUDC* did not affect erythroblast enucleation

NUDC was identified as a potential substrate of p38 α by the p-p38 α *in vitro* kinase assay using protein extract from differentiated FLEPs. Previously *NUDC* was shown to be activated upon phosphorylation and to play multiple roles in mitosis and cytokinesis in cultured mammalian cells [96, 97]. Hence, it was of interest to further characterize the function of *NUDC* in erythroblast enucleation. I applied shRNA-mediated knock-down of *NUDC* in wild-type FLEPs (FLEP sh-*NUDC*). Non-targeting shRNA was used as control (FLEP sh-Ctl). The efficiency of *NUDC* knock-down was shown by reduced *NUDC* protein level (Fig. 16A). FLEP sh-*NUDC* and FLEP sh-Ctl cells were subjected to differentiation and erythroblast enucleation was determined at 48 hours of differentiation. Interestingly, *NUDC* knock-down had no negative effects on the enucleation of erythroblasts (Fig. 16B). These data imply that *NUDC* is likely dispensable in erythroblast enucleation. Additionally, there were no obvious differentiation defects observed in *NUDC*-deficient FLEPs.

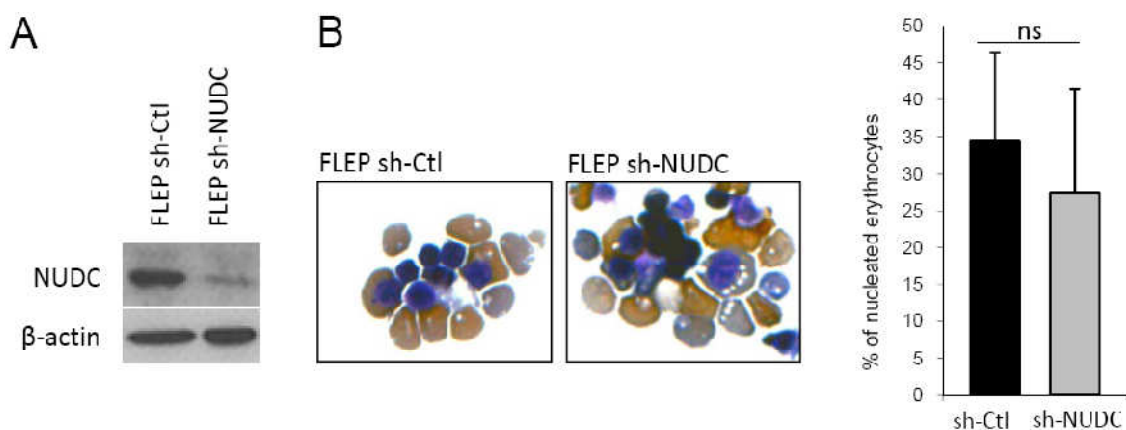


Figure 16. *NUDC* knock-down apparently does not affect erythroblast enucleation. (A) Western blot analysis of *NUDC* protein level in FLEP sh-Ctl and FLEP sh-*NUDC* cells; β -actin was used as loading control. (B) Benzidine stained cytopins of FLEP sh-Ctl and FLEP sh-*NUDC* at 48h of differentiation. (C) Amount of nucleated erythrocytes was quantified. * $P < 0.05$, Student's t test. Data are expressed as mean \pm SD. ns, non significant.

2.5. *p38α* promotes enucleation of erythroblasts in a cell-autonomous manner

During differentiation erythroblasts form blood islands together with macrophages, which facilitates the maturation and enucleation of erythroblasts [33]. It has been shown that perturbed function of macrophages results in defective erythropoiesis including impaired enucleation [33, 34, 98]. Hence it is crucial to specify whether the function of *p38α* in erythroblast enucleation during fetal development and stress erythropoiesis is required either in erythroblasts or macrophages.

To this end I crossed *p38α^{ff}* mice with *ErGFPcre* mice for specific deletion of *p38α* in the erythroid lineage (*p38α^{ff}/ErGFPcre; p38α^{Aery}*). I analyzed *p38α^{Aery}* mice in comparison to mice with specific deletion of *p38α* in macrophages (*p38α^{ff}/Lysm-cre; p38α^{ΔmΦ}*).

The specificity and efficiency of Lysm-cre-mediated deletion of *p38α* floxed alleles was characterized by Lijian Hui in our lab [Wagner lab, unpublished]. To characterize the function of *ErGFPcre*, I isolated bone marrow cells from *p38α^{Aery}* mice. The bone marrow cells were fractionized into erythroblasts, macrophages and undefined cells by FACS sorting using surface marker labeling specifically for erythroid cells and macrophages (TER119 and CD71 and MAC1, respectively). The specificity and efficiency of *ErGFPcre*-mediated deletion of *p38α* floxed alleles in the erythroid lineage is shown by PCR analysis (Fig. 17).

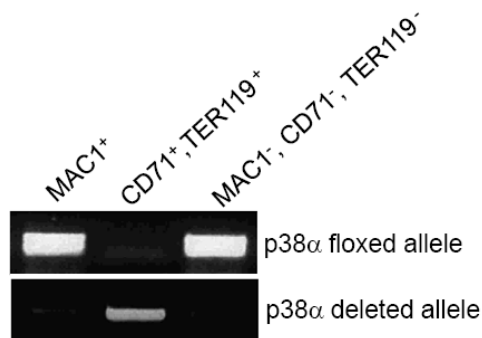


Figure 17. $p38\alpha$ floxed allele is specifically and efficiently deleted in the erythroid lineage of $p38\alpha^{Aery}$ mice. Surface marker of bone marrow cells from $p38\alpha^{Aery}$ mice were labeled with MAC1 and CD71/TER119. Bone marrow cells were fractionized into macrophages, erythroid and undefined cells by FACS sorting (MAC1⁺, CD71⁺/TER119⁺ and MAC1⁻, CD71⁻, TER119⁻, respectively). $p38\alpha$ floxed and $p38\alpha$ deleted allele were analyzed by PCR.

Both $p38\alpha^{Aery}$ and $p38\alpha^{Am\Phi}$ newborn mice were viable and did not exhibit obvious phenotypes, such as inflammatory disease or anemia; the adult mice were apparently healthy as well. During fetal development the rapid growth of the embryo requires expansive erythropoiesis, which is similar to adult stress erythropoiesis [24, 99]. Thus, effects of erythropoiesis under stress conditions can be examined in newborn mice. I analyzed peripheral blood from newborn mice at postnatal day 1 (P1). Interestingly, $p38\alpha^{Aery}$, but not $p38\alpha^{Am\Phi}$ newborn mice exhibited nucleated erythrocytes in the peripheral blood (Fig.18 A, D). These initial observations suggested that the function of $p38\alpha$ is cell-autonomous.

Previously it has been shown, that healthy adult $p38\alpha^{Ablood}$ mice do not exhibit nucleated erythrocytes in the peripheral blood. However, PHZ-induced anemic stress erythropoiesis in adult $p38\alpha^{Ablood}$ mice implicated defective enucleation of erythroblasts accompanied by a dramatically reduced population of mature CD71^{low}/TER119^{high} erythroid cells in the spleen [Wagner lab, unpublished]. I asked whether this phenotype is also observed in either $p38\alpha^{Aery}$ or $p38\alpha^{Am\Phi}$ adult mice. Neither $p38\alpha^{Aery}$ nor $p38\alpha^{Am\Phi}$ healthy adult mice showed nucleated erythrocytes in the peripheral blood. However, there was a reappearance of nucleated erythrocytes in the peripheral blood of adult $p38\alpha^{Aery}$ mice upon PHZ treatment (Fig. 18B). Additionally, up to 30% of the erythrocytes in the spleen were nucleated in these mice (Fig. 18C,

G). In contrast, these effects of PHZ-induced anemia were not observed in $p38\alpha^{Am\Phi}$ mice, which showed stress erythropoiesis similar to control mice (Fig. 18D, E, F, H). Interestingly, the significantly reduced population of mature $CD71^{low}/TER119^{high}$ erythroid cells in the spleen of $p38\alpha^{Ablood}$ mice after PHZ treatment could not be seen in $p38\alpha^{Aery}$ and $p38\alpha^{Am\Phi}$ mice. This cell population was also reduced in control mice after PHZ treatment compared to previous findings (Fig. 19A, B; Fig. 9F, respectively) [Wagner lab, unpublished]. The differences observed in control mice could be explained by variations in the experimental procedure and the genetic background.

Taken together, these data show that $p38\alpha$ promotes erythroblast enucleation during fetal development and stress erythropoiesis in a cell-autonomous manner. Moreover, these data suggest that the function of $p38\alpha$ to promote erythroblast enucleation is dispensable in macrophages.

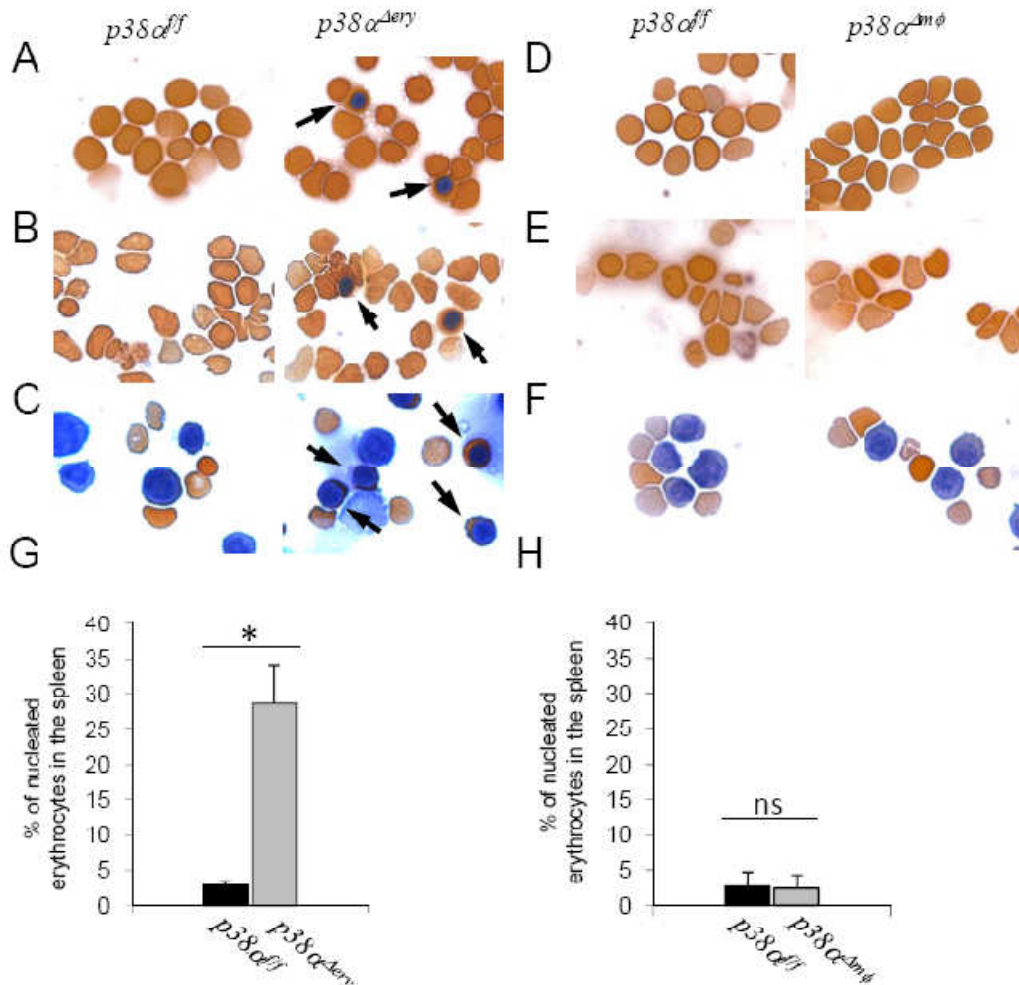


Figure 18. $p38\alpha$ promotes enucleation of erythroblasts during stress erythropoiesis in a cell-autonomous manner. (A, B, D, E) Peripheral blood smears of $p38\alpha^{Aery}$ (A) and $p38\alpha^{Am\phi}$ (D) newborns at P1, $p38\alpha^{Aery}$ (B) and $p38\alpha^{Am\phi}$ (E) mice at day 6 after PHZ treatment and control mice were benzidine stained. Nucleated erythrocytes are indicated by arrows. (C, F) Benzidine stained splenocyte cytopins of $p38\alpha^{Aery}$ (C) and $p38\alpha^{Am\phi}$ (F) and control mice at day 6 after PHZ treatment. (G, H) Nucleated erythrocytes in the spleen of $p38\alpha^{Aery}$ (G) and $p38\alpha^{Am\phi}$ (H) and control mice after PHZ treatment were quantified. * $P < 0.05$, Student's t test. Data are expressed as mean \pm SD.

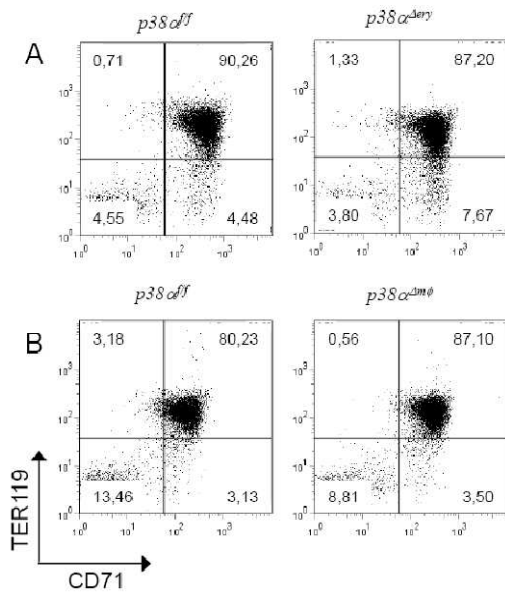


Figure 19. Similar maturation stages of splenic erythroid cells in $p38\alpha^{Aery}$ and $p38\alpha^{Am\phi}$ mice after PHZ-treatment. (A, B) Surface marker of splenocytes from $p38\alpha^{Aery}$ (A) and $p38\alpha^{Am\phi}$ (B) and control mice at day 6 after PHZ treatment were labeled with TER119 / CD71. Labeled splenocytes were analyzed by FACS.

2.6. Loss of $p38\alpha$ and chemical inhibition blocks enucleation of erythroblasts *in vitro*

As described before, $p38\alpha^{4/4}$ fetal liver-derived erythroid progenitors (FLEPs) almost completely fail to enucleate during *in vitro* differentiation [Wagner lab, unpublished]. To further confirm the role of $p38\alpha$ in enucleation of erythroblasts, I used ESEP *in vitro* culture system [91]. I applied shRNA-mediated knock-down of $p38\alpha$ in wild-type ESEPs (ESEP sh- $p38\alpha$). Non-targeting shRNA was used as a control (ESEP sh-Ctl). The efficiency of $p38\alpha$ knock-down was shown by decreased $p38\alpha$ protein level (Fig. 20A). As expected, the knock-down of $p38\alpha$ led to a significant increase of nucleated erythrocytes (91.8%) at 48h of differentiation, compared to ESEP sh-Ctl (66.7%) (Fig. 20B, C).

I also examined whether chemical inhibition of $p38\alpha$ kinase activity is sufficient to block enucleation of erythroblasts. Addition of 1 μ M SB202190 inhibitor to differentiating wild-type ESEPs from either 0h to 48h or 24h to 48h of differentiation resulted in a substantial increase of nucleated erythrocytes (96% and 76%, respectively) compared to untreated wild-type ESEPs

(53.5%) (Fig. 20D, E). These results further demonstrate that loss of p38 α and its kinase activity blocks enucleation of erythroblasts *in vitro*. Moreover, the data imply that the function of p38 α to promote erythroblast enucleation appears to be required at late stages of differentiation, since inhibiting the kinase activity of p38 α after 24h of differentiation is sufficient to block enucleation.

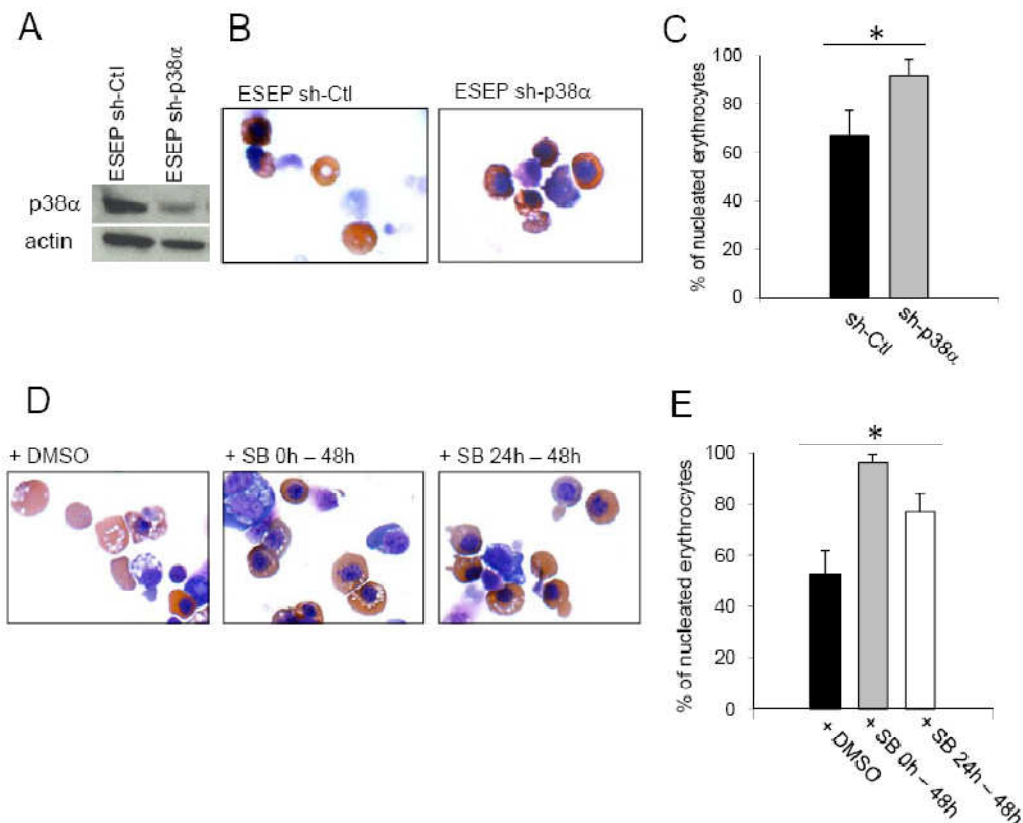


Figure 20. Enucleation of ESEPs is blocked by p38 α -knock-down and inhibition of p38 α kinase activity *in vitro*. (A) Western blot analysis of p38 α protein level in ESEP sh-Ctl and ESEP sh-p38 α cells. β -actin was used as loading control. (B) Benzidine stained cytopins of ESEP sh-Ctl and ESEP sh-p38 α at 48h of differentiation. (C) Amount of nucleated erythrocytes was quantified. (D, E, F) Benzidine stained cytopins of wild-type ESEPs treated with DMSO (D), 1 μ M SB202190 from 0h to 48h (E) and 1 μ M SB202190 from 24h to 48h. (F) Amount of nucleated erythrocytes was quantified. *P < 0.05, Student's t test. Data are expressed as mean \pm SD.

2.7. Expression analysis of $p38\alpha$ -dependent genes regulating erythropoiesis

Myoblasts lacking $p38\alpha$ exhibit impaired expression of differentiation-related genes during *in vitro* differentiation [68]. To verify whether $p38\alpha$ is also involved in regulating gene expression during erythroblast differentiation, I analyzed microarray data using “Gene Set Enrichment Analysis” (GSEA) software and performed qRT-PCR of differentiating $p38\alpha^{f/+}$ and $p38\alpha^{Δ/Δ}$ FLEPs.

GSEA analyses gene expression profiles and determines whether defined sets of genes are differently expressed between two biological states [100, 101]. Here I analyzed, whether distinct sets of genes are either up- or down-regulated in $p38\alpha^{Δ/Δ}$ FLEPs compared to $p38\alpha^{f/+}$ FLEPs at 36h of differentiation. GSEA analysis revealed that a set of genes that has been shown to be enriched in early hematopoietic progenitor cells, including *c-Myb* and *Parp1* was significantly up-regulated in $p38\alpha^{Δ/Δ}$ FLEPs (Fig. 21A). Accordingly, genes that have been shown to be enriched in mature hematopoietic cells, such as *Tmpo* and *Nix* were down-regulated in $p38\alpha^{Δ/Δ}$ FLEPs at the same point of time (Fig. 21B) [102]. Moreover, there was reduced expression of predicted *Gata1* and experimentally proven *C/EBP* target genes in $p38\alpha^{Δ/Δ}$ FLEPs (Fig. 21C, D) [103, 104]. To further determine the expression of genes indicating erythroid differentiation, I analyzed expression levels of hemoglobins using qRT-PCR. Indeed, $p38\alpha^{Δ/Δ}$ FLEPs exhibited reduced expression of primitive and definitive hemoglobin isoforms *Hba-a1*, *Hba-ax*, *Hbb-b1*, *Hbb-b2*, *Hbb-bh1*, and *Hbb-by* at 48h of differentiation (Fig. 21E).

Next, I asked whether the reduced expression of differentiation-related genes was due to deregulated expression of transcription factors regulating erythroid differentiation. Intriguingly, mRNA levels of the transcription factors *Gata1*, *C/EBP α* and *Gfi-1*, which are generally up-

regulated during erythroid differentiation, were reduced $p38\alpha^{A/A}$ FLEPs during differentiation (Fig. 22A, B, C). On the other hand, Gata2 and Fli-1, which are normally expressed in early erythroid progenitors and down-regulated during differentiation, were increased in $p38\alpha^{A/A}$ FLEPs during differentiation (Fig 22D, E). In conclusion, these data demonstrate that p38 α is required for regulating gene expression characteristic for erythroid differentiation. Furthermore, these data show that p38 α has a key function in regulating erythroid differentiation under stress conditions.

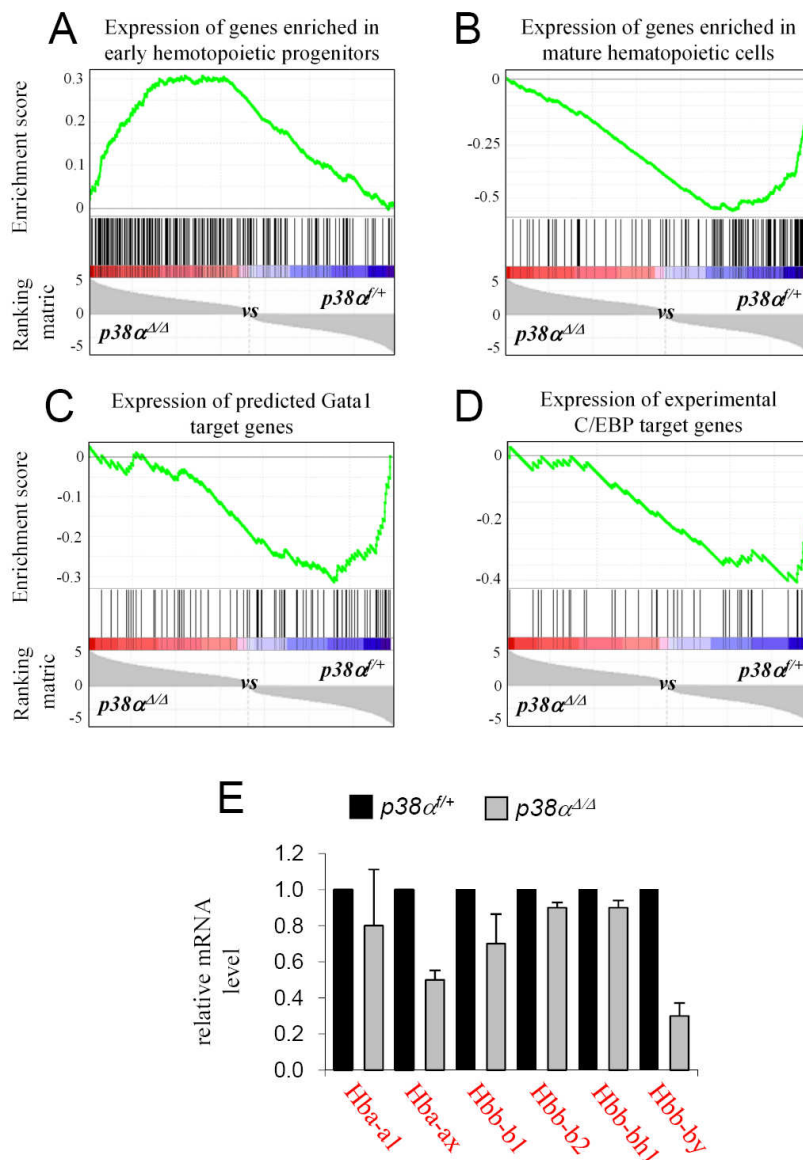


Figure 21. p38 α is involved in regulating the expression of differentiation-related genes.

(A-D) GSEA profiles of microarray data from $p38\alpha^{+/+}$ versus $p38\alpha^{A/A}$ FLEPs at 36h of differentiation demonstrating relative expression of genes enriched in early hematopoietic progenitors (A), enriched in mature hematopoietic cells (B), predicted Gata1 (C) and experimental C/EBP (D) target genes. (E) Relative mRNA level of Hbb-b1, Hbb-b2, Hbb-bh1, Hbb-by, Hba-a1 and Hba-ax in $p38\alpha^{+/+}$ and $p38\alpha^{A/A}$ FLEPs at 48h of differentiation was quantified by qRT-PCR. Data are expressed as mean \pm SD.

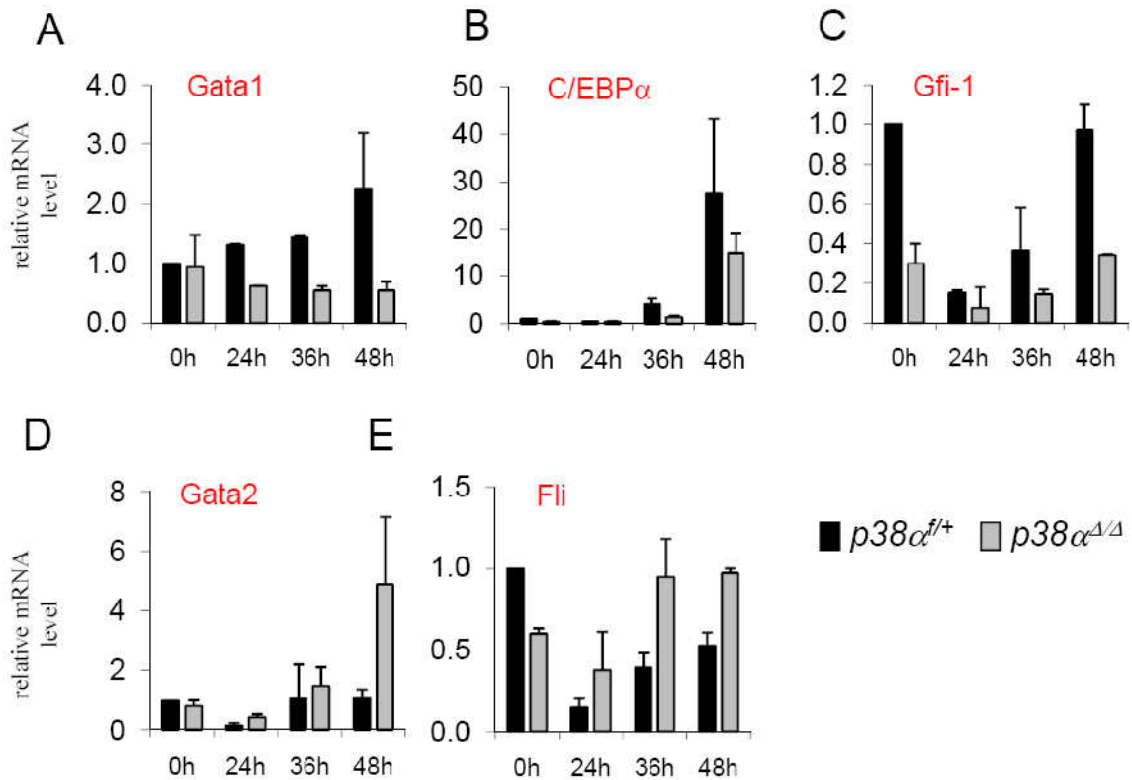


Figure 22. Transcription factors regulating erythroid differentiation are differently expressed in $p38\alpha^{\Delta/\Delta}$ FLEPs. (A-E) qRT-PCR analysis to quantify relative mRNA expression of Gata1 (A), C/EBP α (B), Gfi-1 (E), Gata2 (D) and Fli-1 (E) in $p38\alpha^{+/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEPs during differentiation. Data are expressed as mean \pm SD.

2.8. Defective activation of the Rb pathway in $p38\alpha$ -deficient erythroblasts

The impaired enucleation phenotype of $p38\alpha$ -deficient erythroblasts is similar to that of erythroblasts lacking Rb [43]. Therefore, I investigated whether $p38\alpha$ is required to regulate the Rb pathway during erythroid differentiation.

GSEA analysis of microarray data showed, that genes which are repressed by Rb and p16 were significantly up-regulated in *p38 α ^{Δ/Δ}* FLEPs at 36h of differentiation (Fig. 23A) [105]. Importantly, Western blot analysis of total Rb-protein showed an increased level of hyperphosphorylated and thereby inactivated Rb-protein in *p38 α ^{Δ/Δ}* FLEPs at 24h of differentiation (Fig. 23B). Furthermore, a prominent function of Rb is to repress the activity of E2F transcription factors [106]. Accordingly, E2F-1 target genes such as Cyclin E1, Cdk2 and PcnA are expected to be up-regulated in case of impaired activation of the Rb pathway [107]. Indeed, target genes of E2F-1 were up-regulated in *p38 α ^{Δ/Δ}* FLEPs at 36h of differentiation (Fig. 23C).

Next I asked whether upstream regulators of Rb were also affected by *p38 α* -deficiency during erythroblast differentiation. p53, p21 and p27 are upstream regulators of Rb [108, 109]. Indeed, mRNA and protein levels of p53, p21 and p27 were reduced in differentiating *p38 α ^{Δ/Δ}* FLEPs (Fig. 23D, E). Moreover, mRNA levels of p21 and mdm2, target genes of p53 were dramatically reduced, suggesting a diminished activity of p53 in *p38 α ^{Δ/Δ}* FLEPs during differentiation (Fig. 23E). These data show that *p38 α* is required for specific activation of the Rb pathway during erythroid differentiation. Moreover, the phenotypic similarities to *Rb*-deficient erythroblasts suggest that activation of the Rb pathway appears to be a major function of *p38 α* in regulating erythroblast differentiation, especially enucleation.

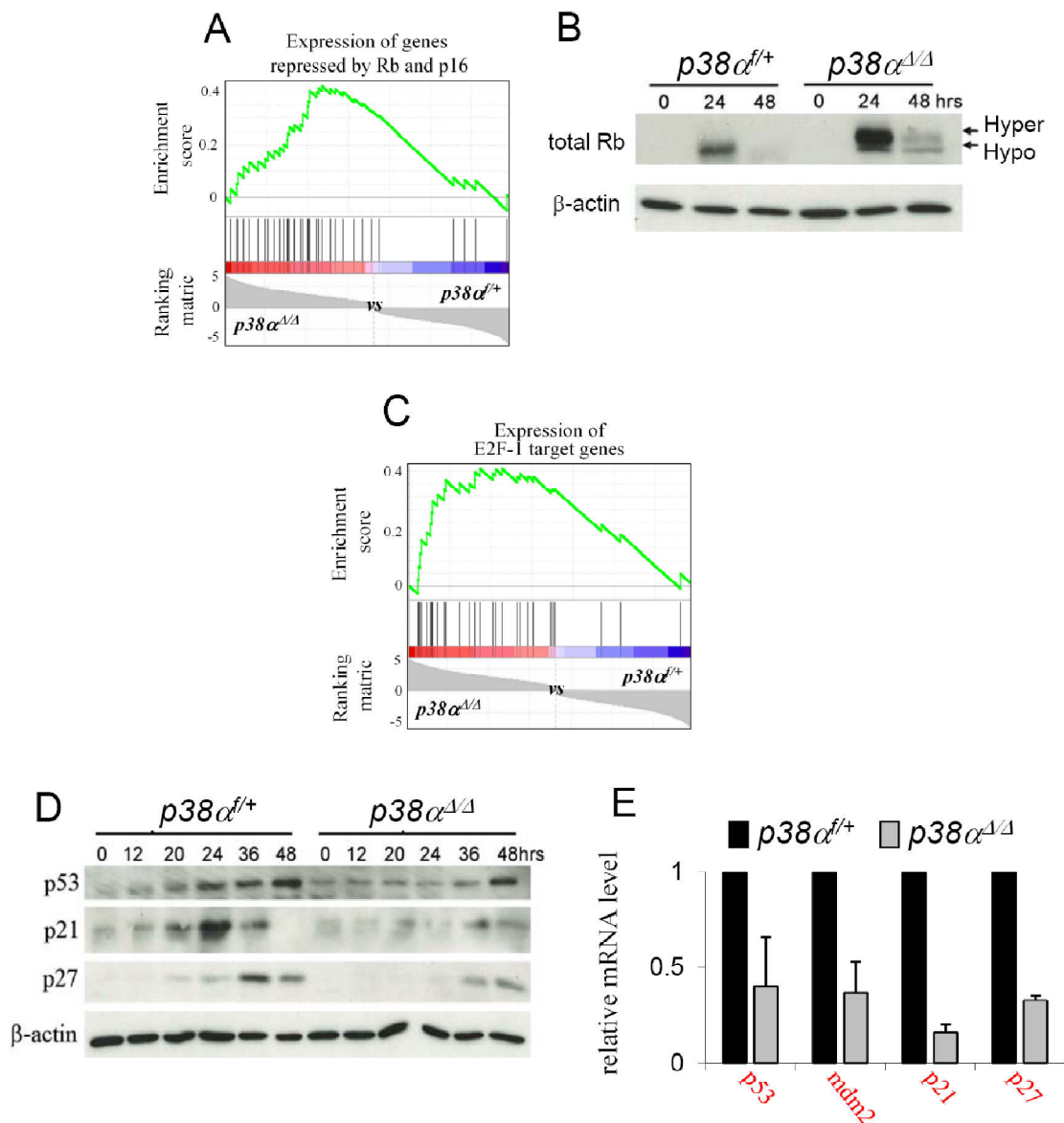


Figure 23. Defective activation of the Rb pathway in $p38\alpha^{\Delta/\Delta}$ FLEPs during differentiation. (A, B) Relative expression of genes repressed by Rb and p16 (A) and experimental E2F target genes (B) in $p38\alpha^{f/+}$ compared to $p38\alpha^{\Delta/\Delta}$ FLEPs at 36hrs of differentiation analyzed by GSEA. (C) Western blot analysis to quantify protein and phosphorylation level of total Rb in $p38\alpha^{f/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEPs during differentiation. β -actin was used as loading control. (D) Protein levels of p53, p21 and p27 in $p38\alpha^{f/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEPs during differentiation were quantified by Western blot analysis. β -actin was used as loading control. (E) Relative mRNA expression levels of p53, mdm2, p21, and p27 in $p38\alpha^{f/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEPs at 36h of differentiation quantified by qRT-PCR. Data are expressed as mean \pm SD.

2.9. *p38 α -deficient erythroblasts have delayed cell cycle exit*

During terminal erythroid differentiation, erythroblasts undergo cell cycle exit and accumulate in G0/G1 phase [110]. A basic function of Rb is to regulate cell cycle progression [111]. The aberrant activation of the Rb pathway implicates that *p38 α -deficient* erythroblasts may have impaired cell cycle exit during terminal differentiation. Therefore, I analyzed the cell cycle distribution of *p38 α ^{f/+}* and *p38 α ^{Δ/Δ}* FLEPs during differentiation using bromodeoxyuridine (BrdU) / propidium iodide (PI) double staining analyzed by FACS.

Indeed, *p38 α ^{Δ/Δ}* FLEPs had increased BrdU incorporation at 36h of differentiation, whereas *p38 α ^{f/+}* FLEPs already exhibited highly reduced BrdU incorporation and accumulated in G0/G1 phase (Fig. 24). However, after 48h of differentiation *p38 α ^{Δ/Δ}* FLEPs stopped BrdU incorporation and accumulated in G0/G1 phase as well (Fig. 24). The final accumulation of *p38 α ^{Δ/Δ}* FLEPs in G0/G1 phase indicates that *p38 α -deficiency* results in delayed cell cycle exit.

In conclusion, these results demonstrate that *p38 α* is required for regulating cell cycle exit during terminal differentiation and further confirm the attenuated activity of the Rb pathway in *p38 α ^{Δ/Δ}* FLEPs.

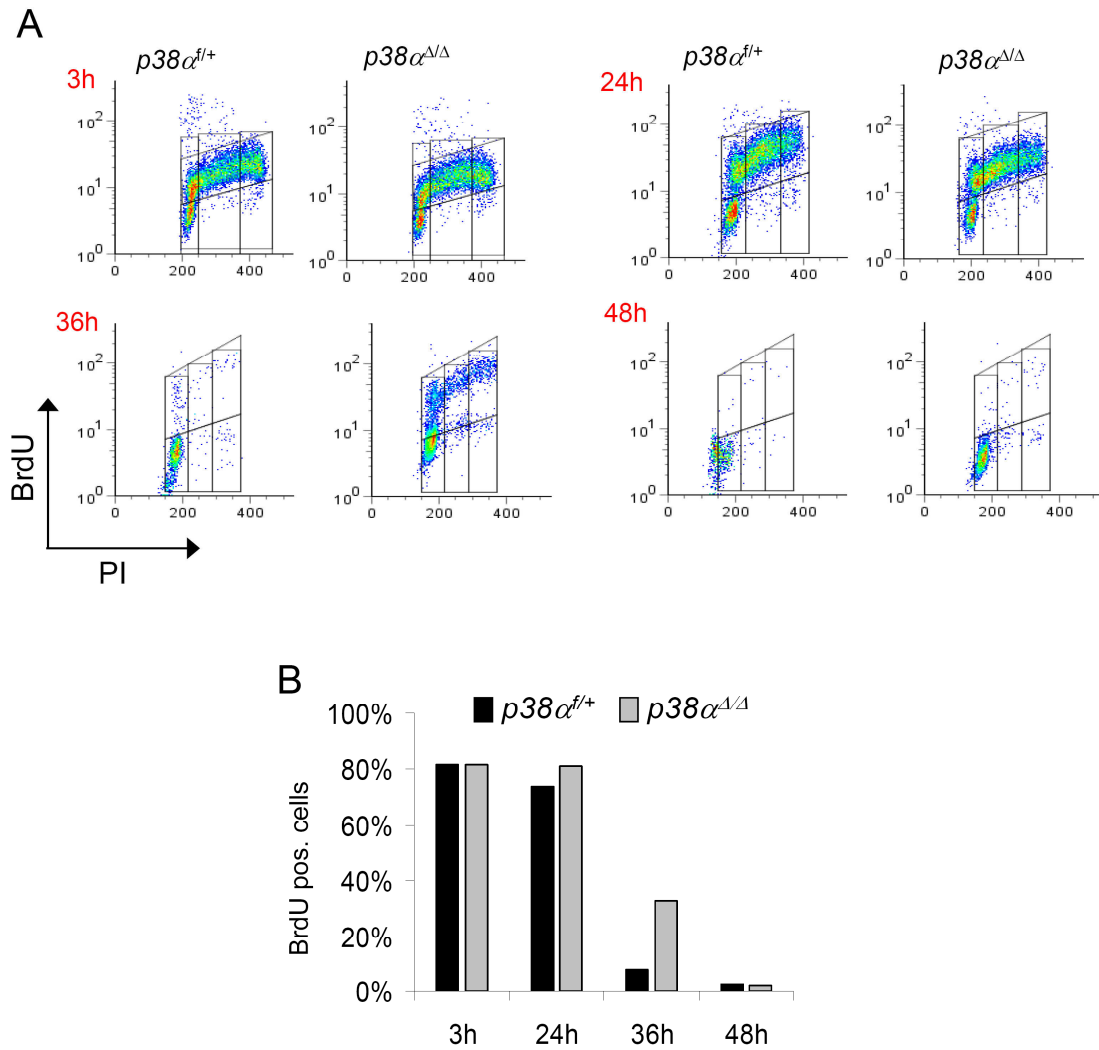


Figure 24. $p38\alpha^{\Delta/\Delta}$ FLEPs show delayed cell cycle exit. (A) BrdU / PI double staining of $p38\alpha^{f/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEPs at 3, 24, 36 and 48h of differentiation. BrdU / PI double stained FLEPs were analyzed by FACS. (B) Percentage of BrdU positive $p38\alpha^{f/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEPs at 3, 24, 36 and 48h.

3. Discussion

This is the first study describing the specific role of p38 α in murine erythropoiesis and erythroblast enucleation employing 4 different genetic mouse models as well as *in vitro* culture systems using erythroblast differentiation into mature erythrocytes. It was recently reported that p38 α plays a role in erythroid maturation *in vivo* and *in vitro* [85, 86, 112] and that p38 α is required for enucleation of erythroblasts during fetal development and adult stress erythropoiesis [Wagner lab, unpublished]. Preliminary unpublished data suggested that the function of p38 α is likely cell-autonomous. Differentiation defects such as reduced hemoglobinization and aberrant expression of TER119 and CD71 surface marker have been identified in *p38 α ^{Δ/Δ}* FLEPs, suggesting that p38 α has a function in regulating erythroblast differentiation [Wagner lab, unpublished]. Here, I showed that the function of p38 α in enucleation of erythroblasts is cell-autonomous using mice with deletion of *p38 α* specifically in the erythroid lineage. In addition, I was able to demonstrate that *p38 α* -deficient erythroblasts exhibited diminished differentiation due to deregulated gene expression. Moreover, *p38 α* -deficient erythroblasts have an attenuated activation of the Rb pathway likely due to reduced protein levels of p53, p21 and p27. The delayed cell cycle exit during terminal differentiation of *p38 α* -deficient erythroblasts is likely caused by aberrant activation of the Rb pathway.

Previously it was shown that *Rb*-deficient erythroblasts have impaired differentiation, defective cell cycle exit and, most strikingly, blocked enucleation under stress conditions [43]. These data suggest that p38 α is required to activate the Rb pathway to promote erythroblast differentiation, cell cycle exit and enucleation during stress erythropoiesis (Fig.25).

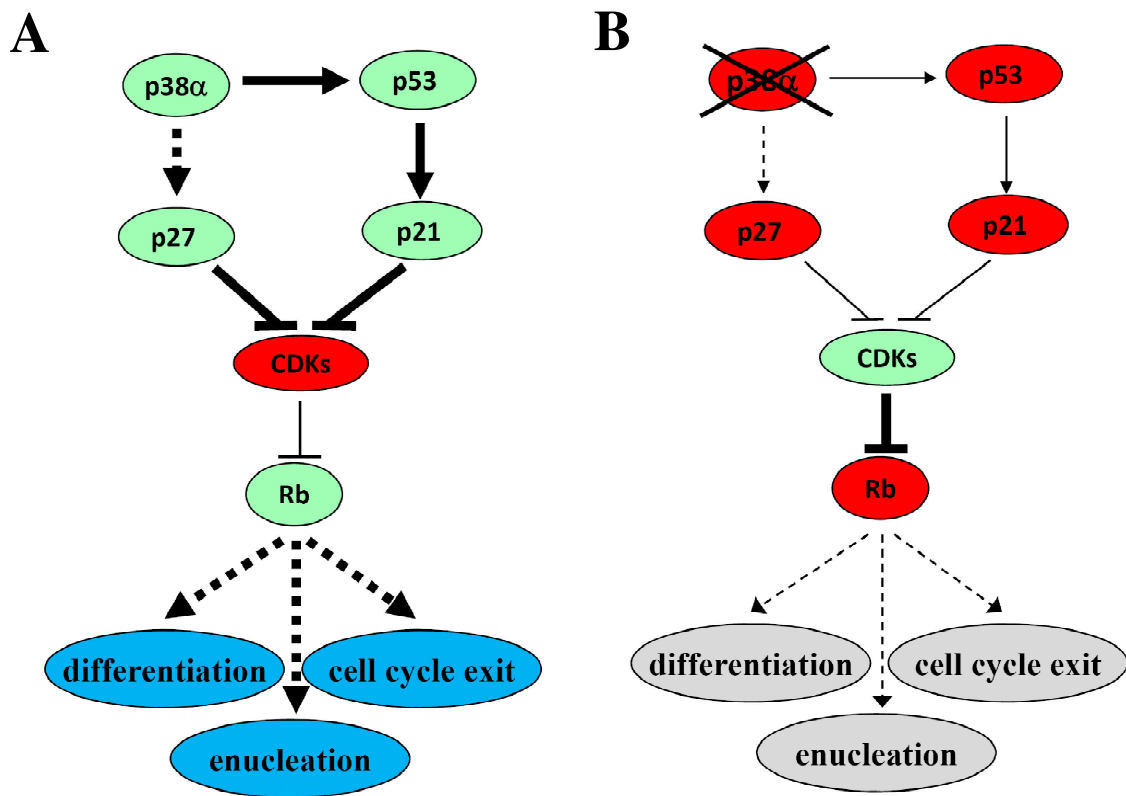


Figure 25. Proposed model of p38 α -dependent regulation of differentiation, cell cycle exit and enucleation of erythroblasts under stress conditions. (A) p38 α positively regulates p53, p21 and p27 resulting in inhibition of CDKs and subsequently in activation of Rb; activated Rb promotes differentiation, cell cycle exit and enucleation of erythroblasts under stress conditions. (B) In the absence of p38 α I found down-regulation of p53, p21 and p27, which leads to increased CDK activity as shown by hyperphosphorylated and inactivated Rb. Lack of Rb activity results in diminished differentiation, impaired cell cycle exit and blocked enucleation of erythroblasts under stress conditions. “green”, active protein; “red”, inactive protein; “blue”, processes promoted; “grey” processes not promoted.

3.1. Analysis of p38 α binding partners and substrates

To characterize binding partners and substrates of active p38 α during erythroblast differentiation, I performed co-immunoprecipitation of p-p38 α . p-p38 α was efficiently immunoprecipitated and the lack of p-p38 α in the negative control confirmed it. Mass spectroscopy identified proteins, which solely precipitated with p-p38 α . However, there were

also proteins identified, which solely precipitated with p-p38 α -beads in the negative control. None of the identified proteins were described to be substrates of p38 α or to be involved in erythropoiesis. Moreover, the number of peptides identified per protein was quite low, which is very likely a reflection of the limited amount of protein extracts derived from FLEP cultures. Thus, this experiment did not provide convincing results. To obtain more protein extract from differentiated erythroblasts, I established the ESEP *in vitro* culture system.

3.2. Establishment of p38 α -deficient ESEP culture

The ESEP *in vitro* culture was shown to provide virtually unlimited amount of erythroid cells [91]. To exclude false positive results from biochemical experiments, such as p-p38 α co-immunoprecipitation, it is crucial to generate a pure p38 α -deficient ESEP culture as a negative control. The differentiation of p38 $\alpha^{+/+}$ and p38 α^{ff} ES cells to ESEPs was successful. The obtained p38 $\alpha^{+/+}$ and p38 α^{ff} ESEP cultures showed properties similar to independently generated wild-type ESEPs, such as proliferation rate and spontaneous differentiation. However, it was not possible to generate p38 α -deficient ESEPs.

The first approach was to obtain p38 α -deficient ESEPs from p38 $\alpha^{-/-}$ ES cells. Interestingly, EBs derived from p38 $\alpha^{-/-}$ ES cells showed aberrant morphology. Whereas cultures from dissociated p38 $\alpha^{+/+}$ EBs showed adherent cell morphologically resembling neurons, myocytes and fibroblasts, there were no adherent cells in cultures from dissociated p38 $\alpha^{-/-}$ EBs. Cultures from dissociated p38 $\alpha^{-/-}$ EBs mainly showed cell clumps in suspension and it was not possible to obtain p38 $\alpha^{-/-}$ ESEPs from these cells. FACS analysis showed an increased population of immature erythroid cells in p38 $\alpha^{-/-}$ EBs, indicating differentiation defects in the erythroid

lineage. These data imply an essential role of p38 α in ES cell differentiation into the erythroid lineage *in vitro*. To further characterize the role of p38 α in ES cells differentiation *in vitro*, it would be necessary to analyze independent p38 α -deficient ES cells to exclude cell line specific effects.

To circumvent differentiation defects which might be caused by loss of p38 α , I generated p38 α^{ff} ES cells. p38 α^{ff} ES cells efficiently differentiated into ESEPs. However, it was not possible to delete the p38 α floxed alleles in ESEPs. The first approach was to establish an p38 $\alpha^{ff}/CreER(T2)$ ES cell line, for inducible deletion of the p38 α floxed alleles in ESEPs. CreER(T2) efficiently integrated into the genome of p38 α^{ff} ES cells, but there was no deletion of the p38 α floxed alleles detected after inducing recombinase activity by Tamoxifen treatment. These data unfortunately imply that CreER(T2) did not exert recombinase activity, likely due to insufficient expression levels or gene mutations.

The second approach was to delete the p38 α floxed alleles in ESEPs by lentiviral-mediated expression of iCre. The infection of ESEPs and deletion of the p38 α floxed alleles was successful. However, p38 α^{ff} ESEP cultures expressing iCre died within a few days after infection. It was shown before that high expression level of Cre recombinase has genotoxic effects in proliferating mammalian cells, suggesting that lentiviral-mediated expression of iCre has toxic effects on ESEPs [95].

All my attempts to generate p38 α -deficient ESEPs failed and therefore, it was not possible to obtain sufficient amounts of protein extracts of differentiated erythroblasts for further analyses of p38 α binding partners and substrates by co-immunoprecipitation.

3.3. Identification of p38 α substrates by *in vitro* kinase assay

In vitro kinase assay is a commonly used tool to screen for kinase substrates. To identify p38 α substrates in erythroblasts a p-p38 α *in vitro* kinase assay with protein extracts from differentiated erythroblasts was initiated previously in the Wagner lab by Lijian Hui in collaboration with Eleanor Coffey, *Centre for Biotechnology, Turku*. Potential substrates of p38 α identified by mass spectroscopy analysis were assigned by Eleanor Coffey. However, raw data from mass spectroscopy analysis showed a high number of proteins identified per analyzed spots. Occasionally there were discrepancy between the migration distance of identified proteins and their molecular weight. Moreover, there was no proper control to exclude false positive results. These conditions suggest a low specificity of the performed p-p38 α *in vitro* kinase assay. It would have been possible to increase the specificity of this assay and exclude false positive results by use of protein extracts from p38 α -deficient erythroblasts incubated with kinase dead p38 α protein as negative control. Nevertheless, the identified protein NUDC was shown to be activated upon phosphorylation and to be involved in nuclear migration during cytokinesis [96, 97]. Hence NUDC was an attractive candidate for further characterization.

3.4. The role of NUDC in erythroblast enucleation

It was shown previously that the activity of NUDC is controlled by phosphorylation and that NUDC has an essential role in cytokinesis. Knock-down as well as overexpression of *NUDC* in mammalian cell lines, resulted in multinucleated cells and blocked cytokinesis in midbody stage [96, 97]. Noticeable, enucleation of erythroblasts is supposed to resemble asymmetric cytokinesis [39-41]. It was shown that genes involved in cytokinesis, such as Rac1 and mDIA2

are essential for erythroblast enucleation [42]. As NUDC is activated by phosphorylation I hypothesized, that loss of $p38\alpha$ leads to impaired activation of NUDC, which subsequently blocks enucleation of erythroblasts. To test this hypothesis I applied shRNA-mediated knock-down of *NUDC* to wild-type FLEPs. Interestingly, knock-down of *NUDC* did not affect the enucleation of erythroblasts. Moreover, there were no obvious differentiation defects observed in NUDC-deficient erythroblasts. These data imply that NUDC is not involved in differentiation and enucleation of erythroblasts, although these data do not exclude that NUDC is a potential substrate of $p38\alpha$.

3.5. Cell-autonomous function of $p38\alpha$ in erythroblast enucleation

Erythroblast differentiation is facilitated by stromal cells and macrophages *in vivo* [32, 113]. Perturbed function of these cells results in impaired erythropoiesis as well. For instance, macrophages of the erythroblastic islands from *DNase II^{-/-}* mice show defective degradation of phagocytosed erythroblast nuclei. Subsequently, this defect results in impaired enucleation of erythroblasts [98]. Hence it is crucial to verify whether $p38\alpha$ functions in enucleation of erythroblasts cell-autonomously or not. Previous findings showed that the impaired enucleation phenotype of $p38\alpha^{\Delta/\Delta}$ during fetal development is recapitulated in newborn $p38\alpha^{Ablood}$ mice and also in adult $p38\alpha^{Ablood}$ mice upon anemia-induced stress erythropoiesis [Wagner lab, unpublished]. These findings showed that the function of $p38\alpha$ to promote enucleation of erythroblasts is dispensable in stromal cells. Preliminary data from the Wagner lab also showed that $p38\alpha^{Am\Phi}$ mice have normal enucleation of erythroblasts during stress erythropoiesis. Moreover, enucleation of $p38\alpha^{\Delta/\Delta}$ FLEPs was partially rescued in co-culture with wild-type or $p38\alpha$ -deficient macrophages. Thus, macrophages lacking $p38\alpha$ appeared capable to facilitate

enucleation of erythroblasts as well [Wagner lab, unpublished]. These data implicated that the function of p38 α to promote enucleation of erythroblasts is cell autonomous. To prove this assumption I analyzed erythroblast enucleation in mice specifically lacking p38 α in the erythroid lineage (p38 α^{Aery}) compared to mice specifically lacking p38 α in macrophages (p38 $\alpha^{Am\Phi}$). Noteworthy, defective enucleation of erythroblasts during fetal development and upon anemia-induced stress erythropoiesis in adult mice was only observed in p38 α^{Aery} and not in p38 $\alpha^{Am\Phi}$ mice. Thus, the recapitulation of the phenotype in p38 α^{Aery} , but not in p38 $\alpha^{Am\Phi}$ mice clearly shows that the function of p38 α in enucleation of erythroblasts is cell-autonomous.

3.6. *In vitro* culture to study the role of p38 α in erythroblasts

Previous studies that describe the function of p38 α in erythroid differentiation *in vitro* used rather artificial and unspecific methods, such as induced erythroleukemia models or chemical inhibition of p38 α [86, 112]. Using an *in vitro* culture system it was shown that p38 α -deficient erythroblasts undergo differentiation comparable to wild-type erythroblasts, but almost completely failed to enucleate during terminal differentiation. In contrast, mice with p38 α -deficient erythroblasts do not show completely blocked erythroblast enucleation under stress conditions. The intensified enucleation defect *in vitro* might be explained by enhanced stress conditions as well as the lack of macrophages, which facilitate enucleation of erythroblasts. I have shown that the knock-down of p38 α resulted in dramatically increased numbers of nucleated erythrocytes. Moreover, chemical inhibition of p38 α kinase activity in wild-type ESEPs showed similar effects. These results further confirm the role of p38 α in enucleation of

erythroblasts. Recapitulation of the phenotype in *p38α*-deficient FLEPs and ESEPs allows further characterization of the role of *p38α* using these *in vitro* culture systems.

3.7. *p38α* promotes erythroblast differentiation

The analysis of *p38α*^{Δ/Δ} FLEPs revealed differentiation defects in addition to blocked enucleation. It was previously shown that *p38α*-deficient erythroblasts display surface marker expression related to immature erythroid cells *in vivo* and *in vitro* [Wagner lab, unpublished]. Additionally, it was shown that erythroblasts have aberrant levels of transcription factors essential for erythroid differentiation upon treatment with *p38α/β*-inhibitor *in vitro* [112]. These data suggest that *p38α* has a function in erythroblast differentiation under stress conditions beyond regulating enucleation. Likewise, *p38α* is essential for up-regulation of muscle-specific genes during myogenesis [68]. Here I showed that decreased hemoglobinization is the result of reduced expression of several hemoglobin isoforms. Moreover, transcription factors which regulate erythropoiesis were aberrantly expressed in *p38α*^{Δ/Δ} FLEPs. In general, Gata1 and C/EBPα are up-regulated, whereas Gata2 and Fli-1 are down-regulated during progression of erythroblast differentiation [114, 115]. Indeed, this pattern was observed in *p38α*^{f/+} FLEPs. However, in *p38α*^{Δ/Δ} FLEPs the expression of GATA1 and C/EBPα was reduced and that of GATA2 and Fli-1 up-regulated. The attenuated expression of GATA1 and C/EBPα was further confirmed by reduced expression of their target genes as shown by GSEA analysis. There was aberrant expression of the transcription factor Gfi-1, although the function of Gfi-1 in erythroblast differentiation is not yet characterized. Correspondingly, *p38α*^{Δ/Δ} FLEPs exhibited an expression pattern related to early hematopoietic progenitors at late stage differentiation,

which is most likely the result of the aberrant expression of transcription factors essential for erythroblast differentiation. These data show that p38 α is essential for regulating gene expression during erythroblast differentiation under stress conditions. Consequently, the loss of p38 α leads to deregulated and diminished maturation. However, it remains to be determined whether these defects are also found *in vivo*. Noteworthy, impaired maturation was also found in lung and muscle epithelium lacking p38 α [68, 72].

3.8. p38 α is essential for proper activation of the Rb pathway

Rb has been shown to be an essential regulatory factor in erythropoiesis under stress conditions [43]. *Rb*-deficient erythroblasts have impaired enucleation of erythroblasts during fetal development and adult stress erythropoiesis. *In vitro* *Rb*-deficient erythroblasts completely fail to enucleate, display expression of surface marker genes related to immature erythroid cells and have impaired cell cycle exit [43]. It has been shown that the function of Rb in erythroblast differentiation is cell-autonomous, although the results are contradictory [43, 116]. These defects are very similar to those found in p38 α -deficient erythroblasts. Therefore, it is very likely that there is a connection between the function of p38 α and Rb in erythroblast differentiation under stress conditions. Indeed, gene expression profiling and Western blot analysis showed an attenuated activation of the Rb pathway in p38 $\alpha^{A/A}$ FLEPs. In addition, the delayed cell cycle exit found in p38 $\alpha^{A/A}$ FLEPs is most likely a direct result of attenuated activation of the Rb pathway. Whereas p38 $\alpha^{A/A}$ FLEPs have delayed cell cycle exit and finally accumulate in G0/G1 phase, *Rb*-deficient erythroblasts were described to have defective cell cycle exit and therefore accumulate in G2/M phase [43]. This difference might be explained by partially activated Rb in p38 $\alpha^{A/A}$

FLEPs via alternative or compensating pathways. Nevertheless, this result further confirms an attenuated activation of the Rb pathway in $p38\alpha^{A/A}$ FLEPs. Recent studies describe several pathways by which p38 α activity can cause Rb activation [68, 117, 118]. p38 α very likely regulates the Rb pathway through different molecular pathways in a cell type and context-specific manner. Here, I was able to show that $p38\alpha^{A/A}$ FLEPs have decreased expression levels of major cell cycle regulators, such as p53, p21 and p27. In addition, reduced expression of p21 and mdm2, both targets of p53, further confirm the attenuated p53 activity. It was shown that p53 can be directly phosphorylated and activated by p38 α [119]. p53 was found to be essential to inhibit proliferation of erythroid progenitors during stress erythropoiesis [120]. Moreover, p21 and p27 were described to accumulate during erythroblast differentiation [110]. Whereas p27 is supposed to play a major role in cell cycle regulation during terminal erythroid differentiation, the role of p21 is not yet fully characterized. These data suggest that p38 α activates Rb during terminal erythroid differentiation most likely through activation of p53 and up-regulation of p21 and p27. It remains to be determined by which mechanisms p38 α up-regulates these cell cycle regulators and whether one of these plays a major role.

In conclusion, these data show that activation of the Rb pathway during terminal erythroid differentiation under stress conditions is dependent on p38 α . As the phenotype of *Rb*-deficient erythroblasts is similar to that of $p38\alpha$ -deficient erythroblasts, I hypothesize that under stress conditions the major function of p38 α for regulating erythroblast differentiation and enucleation is to activate the Rb pathway. Intriguingly, similarities in differentiation defects were also described in other progenitor cells lacking either p38 α or Rb, such as myoblasts [68, 121]. To prove this hypothesis it is necessary to rescue the differentiation defects, most importantly the

blocked enucleation of $p38\alpha$ -deficient erythroblasts by endogenous or exogenous activation of the Rb pathway. Unfortunately until today my experimental approaches to activate the Rb pathway failed due to technical limitations of the methods used.

4. Material & Methods

4.1. Mice

Mice with $p38\alpha$ floxed alleles ($p38\alpha^{ff}$) have been described previously [65, 122]. To obtain $p38\alpha^{AA}$ mice, heterozygote *MORE-cre* / $p38\alpha^{ff/+}$ mice were crossed to $p38\alpha^{ff}$ mice as described by Hui et al [65]. *Lysm-cre* mice have been described previously [123]. *ErGFPcre* mice were a kind gift from U. Klingmüller, *Max-Planck-Institute Freiburg* and have been described previously [124]. The *LysM-cre* / $p38\alpha^{ff}$ ($p38\alpha^{AM\Phi}$) and *ErGFPcre* / $p38\alpha^{ff}$ ($p38\alpha^{Aery}$) mice were generated by crossing $p38\alpha^{ff}$ mice to *LysM-cre* and *ErGFPcre* mice, respectively. The genetic background of the intercross was C57Bl6/J x 129sv. All animal experiments were performed according to permissions from Austrian authorities.

4.2. Phenylhydrazine-induced hemolytic anemia

Phenylhydrazine (PHZ) (#P26252) was purchased from Sigma-Aldrich. 6 weeks old mice were injected intraperitoneally with 60 mg/kg PHZ at day 0 and day 1 as described [125]. Control mice were injected intraperitoneally with phosphate-buffered saline (PBS). On day 6 blood

samples were collected in EDTA-treated tubes from the tail vein. Blood smears were prepared using standard protocol. Mice were sacrificed by cervical dislocation and spleen and bone marrow were isolated. To obtain single cell suspension, spleens were squeezed through cell strainer and bone marrow cells were squeezed six times through a 27 gauge needle. Splenocytes and bone marrow cells were cytopspined.

4.3. Cell culture

$p38\alpha^{+/+}$ and $p38\alpha^{-/-}$ mouse embryonic stem (ES) cells were a kind gift of B. Binétruy, *University of Marseille* [126]. For generation of $p38\alpha^{ff}$ ES cells, $p38\alpha^{ff}$ mice were intercrossed. Blastocytes were collected at E3.5 and cultures established as described previously [127]. To obtain feeder independent ES cell culture, ES cells were cultured on gelatin coated plates without mouse embryonic fibroblast at the beginning of passage 4. Differentiation of the ES cells to ESEPs was performed as described by *Carotta et al.* [91]. To induce EB differentiation, 15 000 ES cells/ml were plated on non-coated Petri dishes (Greiner Bio-One, Kremsmünster, Austria) containing EB differentiation medium. EB differentiation medium was prepared as followed: IMDM, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (all from Gibco/BRL), 15% FCS (Euroclone), 50% methylcellulose, 50 μ g/mL ascorbic acid, 300 μ g/mL iron-saturated human transferrin (all from Sigma), 5% protein-free hybridoma medium (PFHM-II; Gibco/BRL) and 4×10^{-4} M MTG. At day 5 of differentiation, EBs were harvested by centrifugation and replated into fresh EB differentiation medium and further cultivated for 1 day. At day 6, EBs were harvested by centrifugation and were incubated in 1 x Trypsin (Gibco/BRL) at 37° C for 2 minutes. FCS was added and EB dissociated by repeated pipetting.

FLEPs were obtained from fetal livers isolated at E12.5 to 13.5 as reviewed by *Dolzning et al* [37]. Methods for expansion and differentiation of FLEPs and ESEPs were reviewed by Dolzning et al [37]. In brief, FLEPs and ESEPs expansive cultures were daily adjusted to a concentration of 2×10^6 cells / ml in serum-free medium (StemPro34 plus nutrient supplement; Gibco/BRL) plus human recombinant erythropoietin (Epo; 2 U/ml; Erypo; Janssen-Cilag AG, Baar, Switzerland), murine recombinant Kit-ligand (KL or stem cell factor [SCF]; 100 ng/ml; R&D Systems, Minneapolis, MN), 10^{-6} M dexamethasone (Sigma), and 40 ng/ml insulin-like growth factor 1 (IGF-1; Promega, Madison, WI). For differentiation of FLEPs and ESEPs, dead and spontaneous differentiated cells were removed by Ficoll (lymphocyte separation medium, 1078 g/cm^3 , Eurobio, France) purification and washed twice with phosphate-buffered saline (PBS). Purified erythroblasts were cultivated at 2×10^6 cells/ml in serum-free medium (StemPro34 plus nutrient supplement) supplemented with 10 U/ml Epo, 10 ng/ml insulin (Actrapid HM; Novo Nordisk), 3×10^{-6} M glucocorticoid receptor antagonist ZK112.993, and one mg/ml iron-saturated human transferrin. Cell number and size was determined using an electronic cell counter (CASY-1; Schärfe-System, Reutlingen, Germany). [37].

4.4. Benzidine staining

Blood smears and cytopins were benzidine stained as described [37]. Cells aliquots were spun onto glass slides using a cytopsin centrifuge at 150g for 7 min. Cytopins were dried with hairdryer. Cytopins were incubated for 4 min in methanol, 2 min in 1% benzidine solution, 1.5 min in H_2O_2 solution, washed with H_2O for 0.5 min, stained 4 min with Diff Quick Red, 40 sec with Diff Quick Blue, washed thoroughly with H_2O and dried with hairdryer [37]. Diff Quick Red (#130 834) and Diff Quick Blue (#130 835) were purchased from Dade Behring, Austria.

4.5. FACS analysis

Surface marker staining of primary cells was performed as described before [37]. In brief, approximately 0.5×10^6 cells were incubated in 500 μ l of 1% FCS/PBS for 30 min on ice in the dark with the respective antibodies directly coupled to fluorochromes at a final concentration of 1:100. Cells were washed twice in 1% FCS/PBS and analyzed directly. TER119-PE (#553673), CD71-APC (#h553266), CD117 (c-Kit)-FITC (#553354) and Mac1-FITC (#553310) monoclonal antibodies were purchased from BD Biosciences. Bromodeoxyuridine (BrdU) and propidium iodide (PI) double staining was performed as followed. 100 μ M BrdU was added to differentiation culture 3h before designated points of time. 2×10^6 cells were wash twice in 1% BSA/PBS and resuspended in 0,9% NaCL. For fixation cells were added in drops to 5 ml 70% Ethanol (ice cold) while vortexing and incubated on ice for 45 minutes. Afterwards, cells were pelleted, add to 1ml 2N HCl/0,5% Triton X-100 and incubated for 30 min at room temperature (RT) for permealization. Cells were pelleted, resuspend in 1ml 0,1M Sodium Tetraborate, pelleted and resuspend in 50 μ l 0,5% Tween/1%BSA/PBS again. For Brdu-labeling, 20 μ l α -BrdU were added and cells were incubated for 20 min in the dark at RT. Finally, cells were pelleted, resuspended in 1ml PI- Buffer (50 μ g/ml PI, 10mM Tris pH 7.5, 5mM MgCl₂ and 200 μ g/ml RNase A) and analyzed directly. Cells were pelleted by 10 min centrifugation at 1500 rpm at 4°C.

4.6. Immunoprecipitation of p-p38 α

Phospho-p38 MAPK (Thr180/Tyr182) antibodies (#9211) from Cell Signaling were chemical cross linked to Affi-Prep Protein A Support (#156-0006) beads from BioRad. Cross linking and immunoprecipitation of p-p38 α were performed as described previously [128, 129].

4.7. Vectors, lentiviral particle production and infection of ES cells and ESEPs

pRRLSIN.cPPT.PGK-GFP.WPRE lentiviral vector (Plasmid #12252) was obtained from Addgene. IRES-GFP was used from pIRES2-AcGFP1 (#632435) from Clontech. Plasmids containing CreER(T2), iCre and p14.blockit lentiviral vector were a kind gift from A. Souabni and M. Richter / *IMP Vienna*, respectively. Lentiviral vectors were cloned according to the maps shown before (Fig. 13A; Fig. 14B).

Lentiviral vectors for *NUDC* knock-down (#NM_010948), *p38 α* knock-down (#NM_011951) and plko.1-control vectors (#SHC001) were purchased from Sigma-Aldrich. All vectors were purified with the Jetstar Kit purchased from Genomed following manufactures instructions. Lentiviral particles were produced as described before using Lipofectamine and Plus Reagent for transfection following manufactures instructions [130]. Lipofetamine and Plus Reagent (#15338-100) were purchased from Invitrogen. For infection, at day 0 ESEPs were pelleted and resuspendend in viral supernatant containing 8 μ g / ml polybrene at a concentration of 4 x 10⁶ cells / ml. To enhance infection rate, ESEPs with viral supernatant were spun for 30 min at 1200 rpm in round bottom tubes. ESEPs were resuspended and adjusted to 2 x 10⁶ cells / ml with erythroblasts proliferation medium. At day1 ESEPs were adjusted to 4 x 10⁶ cells / ml using same volume of viral supernatant as used at day 0 and then diluted to 2 x 10⁶ cells / ml with erythroblasts proliferation medium. From day 2 to day 3 ESEPs were selected for positive infection by addition of 1 μ g / ml puromycin to the erythroblasts proliferation medium. ES cells for infected by lentivirus by addition of 30% viral supernatant of original volume and 4 μ g/ml polybrene to normal ES cell medium. ES cells were selected for positive infection by addition of 1 μ g/ml puromycin to ES cell medium for 3 days.

4.8. Protein blot analysis

Protein blot analyses were performed using standard protocols (Amersham Biosciences). We used antibodies against the following proteins: p38 α (#9212), p-p38 α (#9211), p53 (#2524) (Cell Signaling), p21 (#556431), Rb (#554136) (BD Bioscience), p27 (#sc-528) and NUDC (#sc-100794) (Santa Cruz).

4.9. Gene-Chip hybridizations and statistical analysis of data

The mouse microarray contained a set of 17,000 verified mouse cDNA clones printed onto polylysine-coated slides. Approximately 5 mg of total RNA from *p38 α ^{f/+}* and *p38 α ^{$\Delta\Delta$}* FLEP cultures at 0, 24, 36 hours of differentiation was primed in water with 1 μ l oligo Target Amp T7-oligo(dT) primer at 65°C for 5 min. Reverse transcription was performed in a mixture containing first-strand reaction buffer provided with SuperScriptII (Invitrogen) for 30 minutes at 50°C. Second strand DNA Synthesis was carried out using Taq Polymerase (Fermentas) at 65°C for 10 minutes. Amplification of RNA and Amino Allyl incorporation was performed using Amino Allyl MessageAmp aRNA Amplification kit (Ambion). cDNA was generated by incubation for 2 hours with SuperscriptII at 42°C. Remaining RNA was digested using RNase H. cDNA was purified (Qiaquick-kit) dried in speedvac and probes were labeled with a reactive fluorescent dye from Molecular Probes (Alexa 555 and 647). The Cy3-dUTP and Labelled probes were pooled and precipitated with blocking solution containing mouse Cot1 DNA at 80°C for 20 min. The probe was then denatured at 94°C for 1 min followed by prehybridization at 50°C for 1 h. It was then added to the microarray slides and incubated at 50°C overnight. After the hybridization, the slides were scanned with a Gene Pix 4060 scanner. Analysis was performed using Gene Pix Pro

4.1. Raw expression data were normalized to $p38\alpha^{f/+}$ at 0 hour of differentiation. Fold changes and P values were calculated (ANOVA, $P < 0.05$). The hybridization and part of the analyses was performed by A. Mairhofer as part of the IMP-IMBA micro-array service facility. These data were sorted for P -value < 0.05 and technical repeats were averaged. Processed data were analyzed by “Gene set enrichment analysis” (GSEA) software [100, 101].

4.10. Quantitative real-time PCR.

Total RNA was isolated from FLEPs using TRIZOL (Invitrogen) following manufacturers instructions. cDNA synthesis was performed with the Ready-To-Go You-Prime-It First-Strand Beads (Amersham Biosciences). qRT-PCR reactions were performed using SYBR Green (Molecular Probes) on an Opticon2 Monitor Fluorescence Thermocycler (MJ Research). Following primers were used: p21 (for: ACCCGGGTCCTTCTTGTTGTTTC, rev: CGTTTTTCGGCCCTGAGATGTTTC), p27 (for: GGTGGACCAAATGCCTGACT, rev: GCCCTTTTGTGGCGAAGA), p53 (for: AACCGCCGACCTATCCTTACCATC, rev: AGGCCCCACTTTCTTGACCATTGT), mdm2 (for: CAGAGACGCCCTCGCATC, rev: CTGAATCCTGATCCAGGCAATC), Hbb-b1 (for: GACCCAGCGGTACTTTGATAGC, rev: TGAGGCTGTCCAAGTGATTCA), Hbb-b2 (for: CCTGGGCAGGTTGGTATCC, rev: GGTTTTTCAGGCCCTCGTTAAAG), Hbb-bh1 (for: GAAACCCCCGGATTAGAGCC, rev: GAGCAAAGGTCTCCTTGAGGT), Hbb-y (for: TGGCCTGTGGAGTAAGGTCAA, rev: GAAGCAGAGGACAAGTTCCCA), Hba-a1 (for: CACCACCAAGACCTACTTTCC, rev: CAGTGGCTCAGGAGCTTGA), Hba-X (for: CTACCCCCAGACGAAGACCTA, rev: CTTAACCGCATCCCCTACGG), Gata1 (for: GAAGCGAATGATTGTCAGCA, rev: TTCCTCGTCTGGATTCCATC), Gata2 (for: TGCATGCAAGAGAAGTCACC, rev:

AGACTGGAGGAAGGGTGGAT), CEBP/ α (for: GCTGGAGTTGACCAGTGACA, rev:
AAACCATCCTCTGGGTCTCC), Gfi-1 (for: AGGAACGCAGCTTTGACTGT, rev:
CCTGTGTGGATGAAGGTGTG), Fli-1 (for: CAACCAGCCAGTGAGAGTCA, rev:
GCCCACCAGCTTGTTACATT) and Tubulin (for: CCTGCTGGGAGCTCTACT, rev:
GGTTCCAGGTCTACGAA).

5. References

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