Regulation and function of the NFE2L3 (NRF3) transcription factor in hematopoietic cells

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ABSTRACT

Cap'n'collar (CNC) basic leucine zipper transcription factors are essential for regulating the activity of antioxidant and detoxification enzymes. Of particular interest is the last member of the family to be identified, NFE2L3 (Nuclear Factor, Erythroid 2 Like 3), also known as NRF3. Accumulating evidence suggests that NFE2L3 may play a role in cellular processes other than stress responses such as differentiation, inflammation and cell cycle control. Moreover, NFE2L3 expression is upregulated in many cancers, including hematopoietic malignancies. However, the function and the regulation of NFE2L3 in hematopoietic cells still remain elusive.

In the first part of the thesis, we addressed the question of whether NFE2L3 plays an oncogenic or a tumor suppressive role in hematopoietic cells by examining the mutations found in diffuse large B cell lymphoma (DLBCL) patient samples, one of the cancers in which NFE2L3 is highly expressed. We showed that the mutations found in DLBCL do not alter the transactivation capacity of NFE2L3 but database analysis suggests it would be worthwhile to examine the effect of NFE2L3 overexpression in cancer.

We also investigated the role of NFE2L3 in hematopoiesis using a *Nfe2l3*-deficient (*Nfe2l3*$^{-/-}$) mouse model. Examination of fully differentiated hematopoietic cells between the knockout and the wildtype mice revealed that the absence of NFE2L3 does not lead to major abnormalities, yet some minimal differences in erythroid-related parameters are noted.

Lastly, we studied the regulation of NFE2L3 in hematopoietic cells by various inflammatory agents. We report here the modulation of NFE2L3 expression by phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor alpha (TNFα), suggesting the possible implication of NFE2L3 in the immune response. Furthermore, we identified NF-κB as a potential regulator of NFE2L3.

Collectively, our studies shed light onto the unknown function and regulation of NFE2L3 in hematopoietic cells and provide the basis for future research.

RÉSUMÉ

Les facteurs de transcription à leucine zipper basique CNC (Cap'n'collar) sont connus pour avoir un rôle essentiel dans la réponse au stress oxydatif par la régulation de l'activité des enzymes de détoxication ainsi que des antioxydants. Le dernier membre découvert de la famille, NFE2L3 (Nuclear Factor, Erythroid 2 Like 3), aussi connu sous le nom de NRF3, se révèle être d'un intérêt particulier. En effet, plusieurs études suggèrent que NFE2L3 pourrait jouer un rôle dans différents processus cellulaires en dehors de la réponse au stress, comme par exemple la différenciation cellulaire, l'inflammation, ou encore le contrôle du cycle cellulaire. De plus, NFE2L3 est surexprimé dans plusieurs types de cancer, dont les cancers des cellules du sang. Malgré ces différents indices, la fonction et la régulation de NFE2L3 restent imprécises.

Dans la première partie de la thèse, nous nous intéressons au possible rôle de NFE2L3 comme oncogène ou suppresseur de tumeur dans les cancers des cellules du sang. Pour cela, notre étude compare les mutations retrouvées chez des patients atteints de lymphome de type B (DLBCL), l'un des cancers étant caractérisé par une surexpression de NFE2L3. Nos résultats ont montré que les mutations présentes dans le DLBCL n'altèrent pas le pouvoir transcriptionnel de NFE2L3 mais l'analyse de base de données suggère qu'il serait intéressant d'approfondir l'étude de la surexpression de NFE2L3 dans le cancer.

Ensuite, nous étudions le rôle de NFE2L3 durant l'hématopoïèse en utilisant un modèle de souris déficientes pour NFE2L3. L'analyse comparée des cellules hématopoïétiques différenciées entre nos souris déficientes pour NFE2L3 et leurs relatifs sauvages a indiqué que l'absence de NFE2L3

ne conduit à aucune anomalie majeure, bien que quelques différences minimes aient été notées au niveau des érythroïdes.

Enfin, nous avons étudié la régulation de NFE2L3 chez les cellules hématopoïétiques. Nos résultats indiquent que l'expression de NFE2L3 peut être régulée par différents agents impliqués dans l'inflammation tels que le phorbol 12-myristate 13-acetate (PMA) et le facteur de nécrose tumorale alpha (TNFα). Ces résultats suggèrent une possible implication de NFE2L3 durant la réponse immunitaire. De plus, la voie de signalisation cellulaire NF-κB a été identifiée comme un régulateur potentiel de NFE2L3.

L'ensemble de ces études nous permet donc d'éclairer nos connaissances sur les fonctions et la régulation de NFE2L3 dans les cellules hématopoïétiques. Les résultats obtenus durant cette maîtrise fournissent ainsi une base très intéressante pour permettre l'approfondissement de nos connaissances dans des recherches futures.

PREFACE & CONTRIBUTION OF AUTHORS

Preface:

In accordance with the McGill University guidelines for thesis preparation, the candidate chose to submit the thesis in the standard format. The thesis starts with a detailed review of the current literature in chapter 1, followed by materials and methods section in chapter 2. Results are presented in chapter 3, and a three-part discussion along with concluding remarks in chapter 4.

Contribution of authors:

All of the experiments presented in chapter 3 were performed by the candidate, with the exception of RNA-sequencing data analysis of DLBCL patient samples which was conducted by Dr. Ryan Morin.

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LIST OF ABBREVIATIONS

ARE	Antioxidant response element
BACH1	BTB and CNC homology 1
BACH2	BTB and CNC homology 2
BHT	Butylated hydroxytoluene
bZIP	Basic leucine zipper
CBC	Complete blood count
CNC	Cap ‘n’ Collar
DAG	Diacylglycerol
DLBCL	Diffuse large B cell lymphoma
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated Kinase
FBXW7	F-Box/WD Repeat-Containing Protein 7
GRA	Granulocyte
GSH	Glutathione
GST	Glutathione *S*-transferase
HCT	Hematocrit
HGB	Hemoglobin concentration
HO-1	Heme Oxygenase 1
IκB	Inhibitor of NF-κB
IL	Interleukin
INF-γ	Interferon gamma

KEAP1	Kelch Like ECH Associated Protein 1
LPS	Lipopolysaccharide
LYM	Lymphocyte
MAF	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-Activated Protein Kinase
MARE	Maf recognition element
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MON	Monocyte
NADPH	Nicotinamide adenine dinucleotide phosphate
NFE2	Nuclear Factor, Erythroid 2
NFE2L1	Nuclear Factor, Erythroid 2 Like 1 (or NRF1)
NFE2L2	Nuclear Factor, Erythroid 2 Like 2 (or NRF2)
NFE2L3	Nuclear Factor, Erythroid 2 Like 3 (or NRF3)
NF-κB	Nuclear Factor Kappa B
NHB	N-terminal homology box
NQO1	NAD(P)H Quinone Dehydrogenase 1
NSC	Neural stem cell
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PRDX6	Peroxiredoxin 6
RBC	Red blood cell

RCAN1-4	Regulator of calcineurin 1, isoform 4
RDW	Red cell distribution width
ROS	Reactive oxygen species
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SMC	Smooth muscle cell
t-BHQ	Tert-butylhydroquinone
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
UV	Ultraviolet
WBC	White blood cell

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CHAPTER 1. LITERATURE REVIEW

1.1 Cap ‘n’ Collar (CNC) transcription factors

Cap ‘n’ Collar (CNC) proteins belong to the basic leucine zipper (bZIP) transcription factor family comprising a region rich in basic residues required for DNA binding and a leucine-zipper motif required for dimerization [1]. CNC transcription factors are characterized by the CNC domain located N-terminally to the DNA binding domain which confer their DNA-binding specificity [2]. The CNC domain recognizes NFE2 (Nuclear Factor, Erythroid 2)-, MARE (Maf recognition element)-, ARE (antioxidant response element)- and StrE (stress-response element)/EpRE (electrophile response element)-type DNA binding sites [3-6].

These elements are present in the promoter or the enhancer of many genes involved in cellular responses to oxidative stress and xenobiotic stress. Indeed, transcriptional control of genes coding for antioxidant and detoxifying enzymes has been the focus of many laboratories studying CNC proteins function. ARE-containing genes which have been identified as the targets of CNC transcription factors code for detoxifying enzymes such as NAD(P)H quinone dehydrogenase 1 (NQO1) and heme oxygenase 1 (HO-1), antioxidant protein glutathione (GSH) and the enzymes that are involved in its production, as well as metabolic enzymes such as cytochrome P450 (CYPs) [7, 8].

In addition to the regulation of antioxidant and cytoprotective genes, CNC transcription factors are involved in development and homeostasis of various factors and are conserved across many

species [9]. Worms and flies have one CNC protein that can fulfill both functions: *Caenorhabditis elegans* Skn-1 [10] and *Drosophila* Cnc [11]. On the other hand, vertebrates express several CNC proteins with overlapping and distinct functions: NFE2 (previously known as p45 NFE2) [12, 13], its related factors Nuclear Factor, Erythroid 2 Like 1 (NFE2L1 or NRF1) [14], NFE2L2 (or NRF2) [15] and NFE2L3 (or NRF3) [16, 17] as well as the more distantly related family members, BTB and CNC homology 1 (BACH1) and BACH2 [18].

The members of CNC family require dimerization with other bZIP proteins such as small MAF (musculoaponeurotic fibrosarcoma) proteins like MAFF, MAFG and MAFK [19-23] or JUN proteins [3, 24] for DNA binding. It has been suggested that the abundance of small MAF proteins can influence the efficiency of dimerization [25] and that different combinations of heterodimer can result in different transcriptional activities [26, 27], thus, adding another layer of complexity to the poorly understood functions of CNC factors.

1.1.1 NFE2

With the discovery that its expression is mainly restricted to myeloid cells such as erythroid cells, mast cells and megakaryocytes [12, 28, 29], NFE2 was originally identified as an essential transcriptional activator of globin gene expression [30-32]. However, *Nfe2* knockout mice displayed only mild erythroid abnormalities but suffered severely from thrombocytopenia as a consequence of an arrest in the late stages of megakaryocyte maturation [33], suggesting that NFE2 is important in megakaryocyte differentiation and platelet production but is rather dispensable for hemoglobin synthesis [34-36].

More recently, a closer examination of *Nfe2*-null mouse embryonic megakaryocytes revealed that not only does NFE2 promote platelet genes expression during megakaryocyte maturation, but it also promotes intracellular accumulation of reactive oxygen species (ROS) [37], a crucial component of megakaryotic differentiation [38]. The authors proposed that the less potent NFE2 competes with the more potent NFE2L2 to regulate cytoprotective genes, thereby, increasing ROS signaling [37]. The study connects the function of NFE2 as a transcriptional regulator of antioxidant genes and its established role in megakaryocyte differentiation, and hints at the complex interplay that exists among CNC family members.

Equally important are the functions of NFE2 in non-myeloid cells. Recent studies report the presence of NFE2 expression in non-hematopoietic cells and highlight its importance in various cellular processes ranging from syncytiotrophoblast formation to bone formation [39-41].

1.1.2 NFE2L1

NFE2L1 exists as several different isoforms in a cell with various sizes ranging from 25 kDa to 120 kDa [42] (Figure 1). The 120 kDa NFE2L1 is a glycosylated full-length protein bound to the endoplasmic reticulum (ER) and is considered transcriptionally inactive. The shorter NFE2L1s are active isoforms localized in the nucleus [43, 44]. How the active forms are generated and translocated to the nucleus is one of the current research topics concerning NFE2L1. Studies suggest that 120 kDa NFE2L1 is deglycosylated into active 95 kDa isoform which can be further cleaved to give rise to shorter isoforms [42, 45-48]. It has also been postulated that alternative translation start sites give rise to 65 kDa NFE2L1 [14]. NFE2L1 isoforms are tightly regulated by proteasomal degradation by E3 ubiquitin ligase complexes. FBXW7 and VCP-HRD1

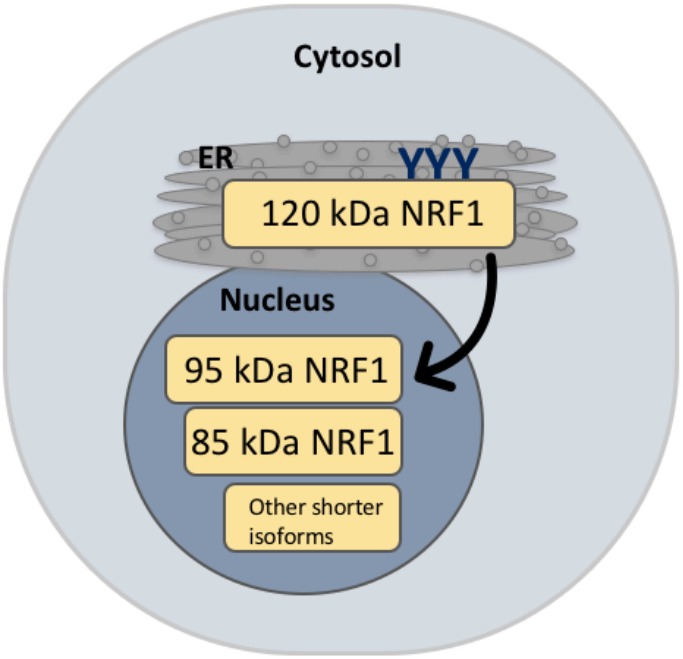


Figure 1. Intracellular localization of NFE2L1 isoforms

The full-length glycosylated 120 kDa NFE2L1 (or NRF1) is an inactive form that is sequestered in the endoplasmic reticulum (ER). The shorter active isoforms including 95 kDa and 85 kDa NFE2L1 are localized in the nucleus. It is hypothesized that the deglycosylation of 120 kDa isoform is required to generate 95 kDa isoform which is subjected to proteolytic cleavage to produce other smaller isoforms. The exact mechanisms of isoforms generation and their nuclear translocation are currently under investigation.

complex have been found to mediate the degradation of cytoplasmic isoforms while SKP1-β-TRCP complex controls the degradation of nuclear isoforms [49, 50].

As *Nfe2l1*-null (*Nfe2l1*$^{-/-}$) mice are embryonically lethal [51], the first evidence of NFE2L1 function in mediating antioxidant responses came from a study using mouse embryonic fibroblasts. The study showed that *Nfe2l1*$^{-/-}$ fibroblasts exhibited higher susceptibility to the toxicity of oxidants and showed decreased levels of the antioxidant glutathione (GSH) as well as the genes involved in its synthesis, suggesting the importance of NFE2L1 in GSH synthesis [52].

Transcriptionally, NFE2L1 has been shown to activate genes identified as its unique targets such as MT1 [53] but also the classic NFE2L2 targets like NQO1 [54], indicating that NFE2L1 and NFE2L2 have some distinct but also some overlapping functions. Indeed, *Nfe2l1*$^{-/-}$ and *Nfe2l2*$^{-/-}$ mouse embryonic fibroblasts showed similar reduction in the endogenous levels of antioxidant genes. Moreover, the loss of both genes resulted in the complete abolition of the expression of these target genes, implying the combinative roles of both genes in regulating antioxidant responses [54]. The study also showed that the induction of ARE-containing genes was much more impaired in *Nfe2l2*$^{-/-}$ fibroblasts, suggesting that NFE2L1 may not be as crucial as NFE2L2 in inducing antioxidant genes expression in response to external stimuli.

The overlapping ARE-containing targets between NFE2L1 and NFE2L2, together with the differences in their transactivation capacity prompted the question as to whether they would have opposing effects: as a less efficacious activator, NFE2L1 would compete with NFE2L2. In support of this speculation, an inducible liver-specific *Nfe2l1* knockout mouse model which

develops steatohepatitis revealed that *Slc7a11*, a gene that is positively regulated by NFE2L2, was significantly upregulated in the absence of NFE2L1, suggesting a negative regulation by NFE2L1 under normal conditions [55]. Based on this finding, the authors proposed a model where NFE2L1 may serve to limit *Slc7a11* gene expression under normal conditions and be displaced by NFE2L2 upon adequate stimulation, contributing to the fine tuning of the antioxidant responses. Consistent with this observation, another study showed that *Nfe2l1* deficiency in mouse livers led to an upregulation of basal expression levels of typical NFE2L2 target genes [53]. On the other hand, the 65 kDa NFE2L1 was reported to act as a dominant negative regulator of ARE-containing genes, suppressing NFE2L2-mediated gene induction *in vitro* [56].

Taken together, different isoforms of NFE2L1 may result in different transcriptional activities. The cross-talk between NFE2L1 and NFE2L2 is most likely be dependent on target genes, cell type and context. Hence, it remains to be determined under which circumstances NFE2L1 acts as an activator or as a suppressor and how its activities interact with that of NFE2L2. Nonetheless, it is becoming clear that the functions of NFE2L1 is not only limited to redox homeostasis. Emerging evidence shows that NFE2L1 is an important regulator of proteostasis [57] and cholesterol homeostasis [58].

1.1.3 NFE2L2

Unlike NFE2L1 and NFE2L3, NFE2L2 has only one form that is present in the cytosol. Under normal conditions, NFE2L2 is bound by Kelch-like EHC binding protein 1 (KEAP1), an adaptor protein for E3 ubiquitin ligase Cullin 3 (CUL3) and is thus constantly degraded through

ubiquitin-proteasome pathway (UPP) [59-61]. Upon the exposure to electrophiles or to oxidants, KEAP1 releases NFE2L2, allowing its entry to the nucleus, where it activates ARE-mediated genes [62] (Figure 2).

NFE2L2 is extensively studied and its role in regulating antioxidant genes is well established in the literature. Indeed, NFE2L2 is recognized as *de facto* activator of stress-responsive genes. Among the 200 genes identified as NFE2L2 targets are notably phase I and II drug detoxification enzymes, phase III drug transporters, GSH- and TXN-related antioxidants as well as their common cofactor, NADPH and NADPH-generating enzymes [7]. Reduced induction of antioxidant and detoxification enzymes and the concomitant increase in the sensitivity of *Nfe2l2*-deficient mice to drug-induced toxicity demonstrate the cytoprotective role of NFE2L2 that is effective in various organs including liver [63], skin [64] and respiratory system [65].

In the context of cancer, NFE2L2 has had, for a while, a good reputation for its crucial role in preventing carcinogenesis. This reputation was mostly built on various experiments demonstrating that the anti-carcinogenic activity of chemoprotective drugs such as oltipraz and sulforaphane are reduced if not impaired in *Nfe2l2*-deficient mice [66, 67]. However, accumulating data suggests that the cytoprotective abilities of NFE2L2 confer survival advantages to cancer cells via stress adaptation [68], chemoresistance [69] and metabolic reprogramming [70, 71]. Clinically, constitutive activation of NFE2L2 found in many cancers has been associated with poor prognosis in patients [72]. Taken together, NFE2L2 is a double-edged sword as a pharmaceutical target and thus requires serious consideration of the context-dependent nature of its activity.

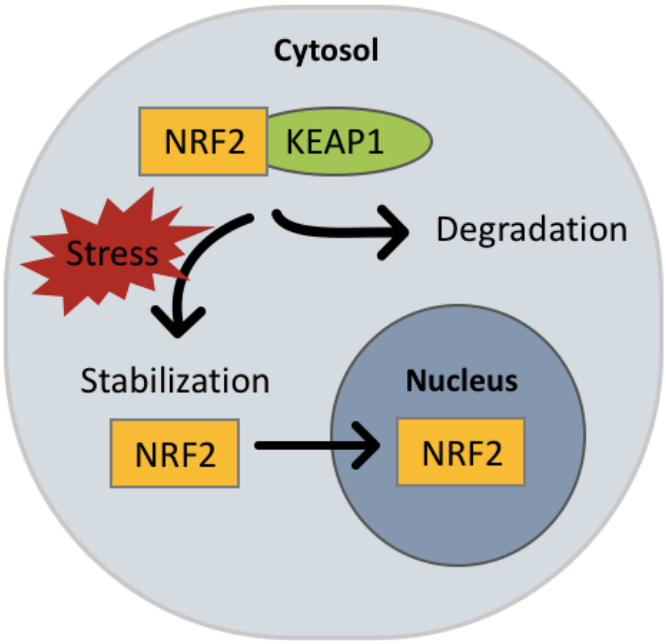


Figure 2. Intracellular regulation of NFE2L2

Under unstimulated conditions, NFE2L2 (or NRF2) is sequestered in the cytosol by KEAP1 which targets it for proteasomal degradation. Upon oxidative stress, KEAP1 undergoes a conformational change that releases NFE2L2. This allows NFE2L2 to enter the nucleus and to drive target genes expression by heterodimerizing with other bZIP factors.

1.1.4 BACH1 and BACH2

BACH1 and its paralog BACH2 differ from other CNC family members in that they gained additional protein interaction domain called BTB. BACH proteins interact with small MAFs to function mainly as a transcription repressor of ARE- and MARE-regulated genes [18, 73, 74]. Despite their structural similarity, BACH proteins manifest different tissue expression pattern and seem to have distinct functions. BACH1 is ubiquitously expressed [18] with relatively high expression in hematopoietic cells [75], while BACH2 expression is considered to be hematopoietic and neuronal cell-specific [76].

BACH1 has been well documented to repress HO-1 and NQO1 transcription [73, 74, 77]. Investigation into the interaction between BACH1 and NFE2L2 revealed that the inactivation of BACH1 was necessary for NFE2L2-induced NQO1 promoter activity in response to oxidative stress [74], suggesting that BACH1 repression is dominant over NFE2L2 induction of their common targets. Moreover, the removal of BACH1 repression alone was sufficient for the basal level of NFE2L2 to activate gene transcription, suggesting that BACH1 repression is present under normal conditions. In line with this data, BACH1 was shown to exit the nucleus within the first hours of antioxidant treatment [78], perhaps allowing the transcriptional activities of NFE2L2. BACH1 levels in the nucleus returned to the baseline level 4 hours later. Another study showed that the nuclear accumulation of BACH1 upon oxidative stress is delayed relative to NFE2L2 activation [79], suggesting that BACH1 may serve as a negative feedback loop to terminate NFE2L2-mediated antioxidant signaling. Furthermore, it has been shown that BACH1 transcript levels are induced by NFE2L2, further supporting the notion of BACH1 as a negative feedback loop [80]. Taken together, these results indicate that BACH1 is an important “off

switch" button for antioxidant responses. Moreover, new evidence suggests that BACH1 may also be implicated in cell cycle and subcellular transport processes [81].

The unique function of BACH2 in the context of oxidative stress is its ability to induce apoptosis [82, 83]. Overexpression of BACH2 has been shown to increase the sensitivity to numerous anticancer drugs with oxidative stress activities in B cells [84, 85]. However, BACH2 is more recognized for its roles in innate and adaptive immunity [86, 87]. In fact, BACH2 was identified as a crucial regulator of B cell antibody response as it mediates antibody class switching, somatic hypermutation of immunoglobin genes as well as B cell development [82, 88]. Recent data highlighted that BACH2, along with BACH1, favors B cell differentiation by repressing myeloid-related genes in common lymphoid progenitors (CLPs) [89]. Furthermore, being identified as the most prominent T cell super enhancer (SE), BACH2 has been found to be critical for maintaining T cell homeostasis [90]. Given the involvement of BACH2 in diverse immunologic processes, it is not surprising that BACH2 is considered a B cell-specific tumor suppressor [91-94].

Despite all these data, research on BACH1 and BACH2 is only at the beginning stage, with a lot more to discover in order to gain complete understanding of their functions.

1.2 NFE2L3

NFE2L3 was the last member of the CNC family to be identified. Although NFE2L3 represents a close homolog of NFE2L1 and NFE2L2, the regulation and functions of NFE2L3 remain largely

unknown to this date. This is partly due to the absence of any obvious aberrant phenotypes in *Nfe2l3*-deficient mice [60, 95]. In fact, these animals develop normally and are fertile, indicating that NFE2L3 is dispensable for mouse development. Furthermore, two double knockout mouse models, *Nfe2l3*$^{-/-}$/*Nfe2l2*$^{-/-}$ and *Nfe2l3*$^{-/-}$/*p45*$^{-/-}$ revealed that the absence of NFE2L3 causes no additional lethality [95], highlighting the functional redundancy that NFE2L3 shares with the other members of CNC family. Nevertheless, research over the past decade has begun to slowly unveil its role and regulation.

1.2.1 Structure and biochemical properties of NFE2L3

With an open reading frame of 694 amino acids, human NFE2L3 comprises domains that are conserved among all CNC factors (Figure 3): the CNC, basic DNA-binding and leucine zipper dimerization domains [16, 17]. Luciferase assays involving the fusion of different fragments of NFE2L3 to GAL4 DNA binding domain localized the transactivation domain to be between amino acids 298 and 399 [17]. NFE2L3 also contains N-terminal homology box 1 (NHB1) and NHB2 sequences, which are highly conserved between NFE2L1 and NFE2L3. NHB1 and its neighboring amino acids have been shown to target murine NFE2L1 and NFE2L3 to the endoplasmic reticulum (ER), where they are glycosylated [44, 96]. Seven potential N-glycosylation sites have been identified in NFE2L3 [97]. The function of NHB2 domain remains yet to be uncovered. In addition, a PEST sequence has been identified within NFE2L3 structure [97]. A PEST motif is a peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) and is associated with rapid intracellular degradation of proteins [98]. N-terminal regions containing this signal peptide were shown to negatively regulate NFE2L3 activity in both human and mouse orthologues, possibly by directing its proteolysis [44].

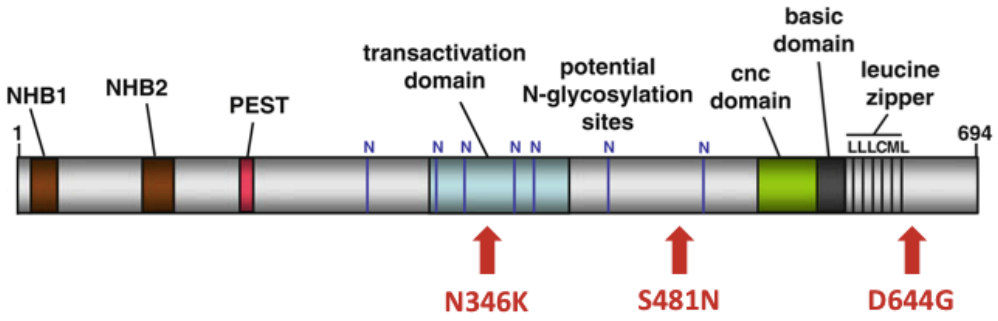


Figure 3. Structure of the human NFE2L3 protein and NFE2L3 mutation sites identified in diffuse large B cell lymphoma (DLBCL) patient samples

The CNC basic leucine zipper (bZIP) transcription factor NFE2L3 comprises a CNC homology, basic DNA-binding and leucine zipper dimerization domains. The transactivation domain is located between amino acid 298 and 399 as defined by Gal4-luciferase reporter studies [17]. NFE2L3 also contains confirmed and putative regulatory regions, N-terminal homology box 1 and 2 (NHB1 and NHB2), PEST motif as well as potential N-glycosylation sites (N). The position of NFE2L3 mutations identified in DLBCL patient samples are indicated. Figure adapted from [8].

1.2.2 Intracellular regulation of NFE2L3

NFE2L3 exists in three different forms in a cell (Figure 4) denoted as 'A,' 'B' and 'C' form [97]. The 'A' form is localized in the ER where it is glycosylated, while 'B' form is a non-glycosylated form found in the cytoplasm [99]. 'C' form is a N-terminally truncated form that is primarily present in the nucleus.

Cycloheximide chase assay showed that NFE2L3 has a short half-life of approximately 20 to 40 minutes, with C form being the most stable and B form being the most unstable form [97, 99]. The rapid turnover of NFE2L3 suggests that it may be subjected to ubiquitin-mediated proteolysis. Indeed, proteasome inhibitors such as MG-132, epoxomicin and *clasto*-lactacystin β-lactone stabilized NFE2L3 [97], suggesting that the turnover of all three forms is regulated by ubiquitin-proteasome pathway (UPP).

Further analysis revealed that the degradation of NFE2L3 is mediated by FBXW7 E3 ligase and is dependent on the phosphorylation by GSK3β kinase [99]. Overexpression of FBXW7 abolished NFE2L3 transcriptional activity, confirming the negative regulation of NFE2L3 activity via degradation. Another study showed that, analogous to NFE2L1 [50], the cytoplasmic and nuclear NFE2L3 is degraded by two distinct E3 ligase complexes VCP-HRD1 and SKP1-b-TRCP, respectively [100]. Collectively, the studies suggest that multiple proteolytic systems govern NFE2L3 regulation.

Owed to the stringent control by ER sequestration and ubiquitin-mediated protein degradation, NFE2L3 is present at a very low level under normal cellular conditions. However, it is

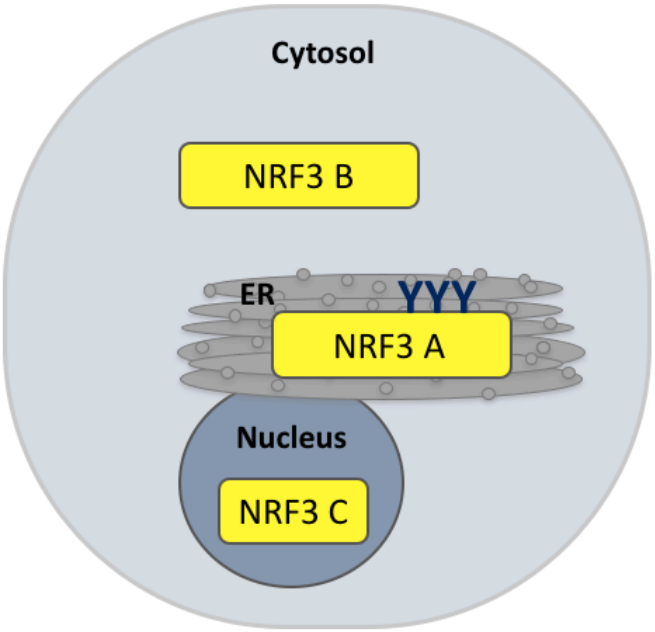


Figure 4. Intracellular localization of three NFE2L3 forms

NFE2L3 (or NRF3) cDNA gives rise to three different forms: a full-length glycosylated 'A' form, a full-length non-glycosylated 'B' form and a N-terminally cleaved 'C' form. They are localized in distinct cellular compartments as illustrated in the schematic.

hypothesized that in response to some unidentified stimuli, NFE2L3 is processed into the C form and subsequently translocated into the nucleus where it drives its target gene expression [97]. It has been shown that the processing and the translocation of nuclear NFE2L3 require proteolytic cleavage [44, 97, 100], and that this cleavage seems to be mediated by the aspartic protease DDI2 at the NHB2 domain [100], a mechanism that has also been reported for the cleavage of NFE2L1 [47].

1.2.3 Expression profile of NFE2L3 in tissues and cells

Contrary to NFE2L1 and NFE2L2 which are constitutively expressed in a wide range of tissues [14, 15], NFE2L3 is only expressed in a limited number of tissues [16, 97]. NFE2L3 expression has been detected at relatively low levels in many human tissues including heart, brain, lung, pancreas, colon, spleen and thymus, with remarkably high expression in placenta. Further examination of NFE2L3 placental expression revealed that its expression is restricted to cytotrophoblasts [17]. In line with this data, human choriocarcinoma cell lines BeWo and JAR, both of which are derived from malignant transformation of cytotrophoblasts, expressed high levels of NFE2L3, indicating a possible implication of NFE2L3 in the function and/or differentiation of cytotrophoblasts. Unlike human NFE2L3, mouse NFE2L3 is expressed at high levels in many tissues, including brain, thymus, testis and placenta [95]. Murine NFE2L3 was also observed in lung, stomach and uterus whereas no expression was detected in heart, liver, spleen, kidney and ovary. Different expression patterns between mouse and human NFE2L3 suggests that the protein may hold distinct functions in the respective organisms.

1.2.4 Molecular functions of NFE2L3

Like other CNC proteins, NFE2L3 requires heterodimerization with the members of small MAF transcription factor family, MAFF, MAFG and MAFK, in order to regulate its target gene expression [16, 17, 101]. The resulting complex has been shown to bind the *cis*-acting enhancer sequences that are typically recognized by other bZIP transcription factors [16]. These DNA binding sites comprise the Maf recognition element (MARE) and antioxidant response element (ARE), which govern the regulation of genes involved in cellular stress response and detoxification [16, 17, 44, 101].

Mouse NFE2L3 has been shown to induce gene expression driven by chicken β-globin MARE [16] and human NQO1 ARE [44]. Both studies showed that the transactivation capacity of NFE2L3 was lower than that of NFE2L1, and significantly lower when compared to NFE2L2. On the other hand, mouse NFE2L3 has also been reported to repress human NQO1 ARE promoter activity [101]. More recent study demonstrated that human NFE2L3 inhibits human Peroxiredoxin 6 (PRDX6) ARE promoter activity [102]. These conflicting results may be due to the differences in the promoter sequences used in the experiments or cell type variability, or both. Differences in human and mouse biology should also be considered.

Intriguingly, recent studies identified unique targets of NFE2L3 and reported the binding of NFE2L3 to the gene promoters that lack ARE- or MARE-like sequences [103, 104], suggesting that NFE2L3 may have functions other than cellular stress response.

1.2.5 Physiological functions of NFE2L3: lessons from *in vitro* and transgenic mouse models

Human and mouse NFE2L3 share 68% of their coding sequence identity, whereas other CNC family members show significantly higher homology between the two species: 89% for NFE2, 97% for NFE2L1 and 80% for NFE2L2 [16]. This discrepancy may translate into functional differences between human and mouse NFE2L3. However, despite the relatively low sequence similarity, transgenic mouse models contributed enormously to the investigation of the biological functions of NFE2L3 along with *in vitro* experiments.

1.2.5.1 Antioxidant responses

Despite the controversy regarding the nature of NFE2L3 transcriptional regulation, accumulating data suggests NFE2L3 as a transcriptional suppressor of antioxidant enzymes. NFE2L3 overexpression led to decreased basal and induced levels of NQO1 in response to tert-butylhydroquinone (*t*-BHQ) antioxidant treatment, whereas siRNA-mediated NFE2L3 silencing resulted in the increased NQO1 transcript and protein level in a dose-dependent manner [101]. Consistent with this data, NFE2L3 was also shown to negatively regulate ARE-regulated gene PRDX6 in A549 cells [102]. Given that PRDX6 is an antioxidant enzyme that reduces phospholipid hydroperoxides, thereby preventing lipid peroxidation and cell damage [105-107], this result suggests that NFE2L3 may mediate the repression of ARE-mediated antioxidant enzymes in this particular context.

However, the extent to which NFE2L3 regulation contributes to the homeostasis of antioxidant responses is open to debate. In fact, no difference was observed between NFE2L2

overexpression and co-transfection with NFE2L3 with respect to the induction of ARE-containing gene in response to oxidative stress [101], suggesting that NFE2L3 had no effect on NFE2L2 transcriptional activity. Another study using a mouse model showed that NQO1 induction by butylated hydroxyanisole (BHA), a NFE2L2/ARE pathway agonist [108], did not differ between wildtype and *Nfe2l3*-null mice [60]. In the same study, the comparison between *Nfe2l2*$^{-/-}$ and *Nfe2l3*$^{-/-}$/*Nfe2l2*$^{-/-}$ mouse models revealed that NFE2L3 did not contribute to the residual induction of NQO1 observed in *Nfe2l2*$^{-/-}$ mice, suggesting that NFE2L3 does not modulate NQO1 gene expression under this circumstance. Further investigation is required to fully understand under which conditions the effect of NFE2L3 regulation, alone or together with other homeostatic mechanisms, can overcome NFE2L2 activity.

1.2.5.2 Differentiation

Recent studies suggest that NFE2L3 is important in cellular differentiation. Examination of mouse embryonic stem cells (mESCs) undergoing smooth muscle cell (SMC) differentiation on collagen IV revealed that NFE2L3 was responsible for the induction of smooth muscle-specific genes such as SMαA, SM22α as well as myocardin [103], a master regulator of SMC markers [109]. Further analysis showed that NFE2L3 overexpression significantly increased NADPH oxidase activity and consequently increased superoxide production, a factor shown to be required to induce SMC differentiation [110], suggesting that NFE2L3 most likely promotes SMC differentiation by increasing ROS signaling. Moreover, NFE2L3 overexpression led to a dose-dependent downregulation of antioxidants genes NFE2L2, NQO1 and PRDX6 and an upregulation of NOX4 (NADPH oxidase homolog 4), a gene identified as the source of ROS

[111, 112], suggesting that NFE2L3-related ROS generation is partly mediated by NOX4. A subsequent study identified another gene to be responsible for NFE2L3-associated superoxide generation during SMC differentiation: PLA2G7, a novel target of NFE2L3 [104]. Interestingly, NFE2L3 silencing by siRNA inhibited ER stressor-induced SMC differentiation, suggesting that pro-myogenic activity of NFE2L3 may be triggered by ER stress [103].

Furthermore, NFE2L3 was also found to play an important role in mouse neural stem cell (NSC) differentiation [113]. Early upregulation of NFE2L3 mRNA was observed following NSC differentiation and its expression was highly correlated with that of myelin-specific genes including CNPase and myelin basic protein (MBP). NFE2L3 silencing by siRNA in NSCs and oligodendrocyte progenitor cells (OPCs) resulted in decreased number of progenitor cells and (pre-)myelinating oligodendrocytes, respectively, without impairing cell viability. These results suggest that NFE2L3 promotes neural stem cell differentiation into OPCs and the subsequent maturation into myelinating oligodendrocytes. However, the molecular mechanism of NFE2L3 in enhancing NSC differentiation remains to be elucidated.

Together, the data strongly suggests that NFE2L3 is an important regulator of differentiation. Moreover, NFE2L3 relies on its pro-oxidant functions to promote differentiation, at least in the context of SMC differentiation.

1.2.5.3 Cell adhesion

As the most outer tissue of a body, skin is subjected to frequent environmental challenges such as ultraviolet (UV) radiation. Many of these environmental insults can contribute to the production of ROS directly or indirectly, thereby increasing oxidative stress [114]. The importance of NFE2L2 in UV protection in keratinocytes by enhancing ROS detoxification has been verified in numerous *in vitro* and *in vivo* studies [115, 116]. In contrast to the anti-apoptotic function of NFE2L2, *Nfe2l3*$^{-/-}$ mice exhibited significantly less apoptosis in response to UV irradiation, oxidative stress and hyperosmotic stress [117]. Comparison between UV-challenged wildtype and *Nfe2l3*$^{-/-}$ cells revealed no differences in DNA damage and repair, intracellular ROS levels as well as the expression of cytoprotective NFE2L2 target genes, suggesting that the pro-apoptotic effect of NFE2L3 does not involve NFE2L2 antagonism. On the other hand, cell-cell and cell-matrix adhesion were significantly increased under both basal and UV-challenged conditions, indicating a novel role of NFE2L3 in cellular detachment. Indeed, *Nfe2l3*-deficient keratinocytes were highly sensitive to EDTA/EGTA and accutase treatments that interfere with cell adhesion. Further analysis revealed higher cell surface integrin levels and increased activity of focal adhesion kinase (FAK), a protein shown to promote adhesion-dependent cell survival [118, 119]. However, additional mechanisms might be in play since integrin levels were unaltered in non-challenged *Nfe2l3*-deficient cells in spite of the enhanced cell adhesion [117]. Together, the data suggests that NFE2L3 may promote apoptosis under challenging circumstances by disrupting cell adhesion, thereby, preventing the accumulation of damaged and possibly mutated cells. Loss of adhesion to extracellular matrix is a key feature of carcinoma *in situ* acquiring metastatic potential [120]. Inevitably, the importance of NFE2L3 in cell adhesion is connected to cancer cell invasion and it will be discussed later.

1.2.5.4 Inflammation and immune response

During skin wound healing, *Nfe2l2*$^{-/-}$ mice healed at a normal rate despite the significant reduction in cytokine mRNA expression and prolonged inflammation [121]. While NFE2L1 expression did not alter, NFE2L3 was highly upregulated in these mice, suggesting that NFE2L3 functionally compensated for the lack of NFE2L2 in mediating skin inflammatory response. Even though NFE2L3 was dispensable for skin wound repair [117], UV-irradiated *Nfe2l3*$^{-/-}$ mice had a reduced neutrophil infiltration and an attenuated induction of proinflammatory cytokines IL-1β and IL-6, suggesting a possible role of NFE2L3 in inflammation.

In fact, some other studies link NFE2L3 to inflammatory responses. NFE2L3 has been found to be induced by various inflammatory cytokines such as TNFα [17] and IFN-γ [122]. TNFα and IFN-γ are both proinflammatory cytokines released by various activated immune cells during inflammation and infection [123, 124]. One of the most important roles of TNFα is to trigger innate immune response [125]. This cytokine triggers downstream signaling cascades that can activate multiple transcription factors such as AP-1, NF-κB, MAPK and PI3K, mediating the important decision between survival and apoptosis [123, 125, 126]. IFN-γ is a type II interferon that is involved in both innate and adaptive immune responses and is mainly known for its antiviral activity [127]. It acts primarily through the JAK-STAT signaling pathway [128]. Paradoxically, TNFα and IFN-γ are now recognized for their disease-promoting role in various illness including chronic inflammation, autoimmunity and cancer [125, 129]. Collectively, these studies hint at the possible involvement of NFE2L3 in immunity.

In the study by Chevillard et al. [130], *Nfe2l3*$^{-/-}$ mice were administered a single dose of butylated hydroxytoluene (BHT) which is known to cause acute lung injury in mice [131]. While *Nfe2l2* deficiency rendered the mice highly sensitive to BHT-related toxicity [132], BHT-treated *Nfe2l3*$^{-/-}$ mice showed no additional morbidity compared to wildtype animals, with the exception of enhanced weight loss [130]. Further investigation revealed that the BHT-induced reduction of catalase activity was significantly less pronounced in the knockouts and NFE2L2 induction was abrogated, hinting at a possible regulation of catalase genes and NFE2L2 by NFE2L3. More interestingly, the endogenous and BHT-induced levels of inflammatory gene *Ptgs2* were attenuated in *Nfe2l3*$^{-/-}$ mice, suggesting that NFE2L3 may regulate immune responses.

1.2.5.5 Cancer

NFE2L3 is highly expressed in many cancer types and cancer cell lines [8]. Particularly high expression of NFE2L3 is found in testicular germ cell cancer, colorectal adenocarcinoma and diffuse large B cell lymphoma (DLBCL) [133, 134]. The amplification of NFE2L3 is a common genetic alteration found in cancer and is associated with the increase in its expression. Indeed, recurring somatic copy number gains and the concomitant upregulation of NFE2L3 were observed in testicular cancer [135] and lung adenocarcinoma [136]. NFE2L3 has also been reported to be upregulated in ductal carcinoma *in situ* (DCIS) and invasive breast carcinoma cell lines MCF10 [137], suggesting that NFE2L3 may play a role in tumor progression. Furthermore, cancer-specific DNA methylation was identified at the promoter of NFE2L3 in glioblastoma [138]. Although accumulating data alludes to the possible role of NFE2L3 in cancer, its

functions in the context of cancer biology remain unresolved. Nonetheless, recent findings on NFE2L3 shed some light on this issue.

Proliferation

A recent study by Chowdhury et al. [100] demonstrated that NFE2L3 promotes cancer cell proliferation. NFE2L3 downregulation using siRNA led to a reduced proliferation of DLD-1 cells of colorectal adenocarcinoma as demonstrated by cell counting. Further analysis revealed that the anti-proliferative effect of NFE2L3 knockdown is mediated by the reduced activity of UHMK1, a serine/threonine protein kinase which normally promotes cell cycle progression through G1 by repressing cyclin-dependent kinase (CDK) inhibitor, p27Kip1 [139]. Consistent with this data, cell cycle analysis of NFE2L3$^{-/-}$ cells confirmed a cell cycle arrest at G0/G1 with reduced populations at G2/M and S, suggesting that NFE2L3 may be a crucial oncogenic cell cycle regulator. Moreover, NFE2L3 has been identified as a potential target of miR-361-3p, a miRNA inhibitor which decreases cell survival by activating caspase-3/7-dependent apoptotic pathways and inducing cell cycle arrest in S phase in lung cancer [140], further suggesting NFE2L3 as a cell cycle promoter.

Invasion

In addition to its oncogenic role in cell proliferation, NFE2L3 has recently emerged as a new regulator of tumor invasion. Wang et al. [141] first showed that NFE2L3 was upregulated at both transcript and protein levels upon shRNA-mediated knockdown of RCAN1-4 (regulator of calcineurin 1, isoform 4), a protein previously identified as a metastasis suppressor in thyroid cancer [142]. The authors subsequently demonstrated that the loss of NFE2L3 in RCAN1-4$^{-/-}$

human thyroid cancer cells decreased spheroid formation and growth, and reduced invasiveness compared to the control, suggesting that NFE2L3 mediates the proliferation and invasion associated with RCAN1-4 knockdown [141]. Furthermore, NFE2L3 overexpression alone was sufficient to significantly increase cell invasion as demonstrated by Matrigel invasion assay without altering cell proliferation, suggesting that NFE2L3 is a crucial promoter of cell motility and metastasis in cancer biology. Consistent with these results, NFE2L3 expression was found to be the lowest in normal tissues and the highest in the invasive fronts and metastatic lesions of thyroid cancer patient tissue samples.

Carcinogenesis

Benzo[a]pyrene (B[a]P) is a chemical compound found in the cigarette smoke and is a potent environmental carcinogen which has been shown to alter DNA methylation [143]. Chevillard et al. [144] discovered that *Nfe2l3*$^{-/-}$ mice were more susceptible to the toxicity of B[a]P, exhibiting higher incidence of cancer development and concomitantly higher morbidity and mortality when compared to the wildtype animals, suggesting that NFE2L3 may be involved in carcinogen detoxification. Closer examination revealed that the majority of *Nfe2l3*$^{-/-}$ mice developed a distinct subtype of lymphoma originating from T cells, suggesting a T-cell specific function of NFE2L3 in mice. Together, the data suggests that NFE2L3 is tumor suppressive in carcinogen-induced lymphoma initiation. However, the molecular mechanisms underlying this phenomenon have not been understood yet.

1.3 NFE2L3: its place in the CNC family

Each member of the CNC family plays distinct role in antioxidant signaling. NFE2L2 is the main inducer of ARE-containing antioxidant and detoxification genes in response to cellular stress [7]. NFE2 may compete with NFE2L2 to increase ROS signaling required during megakaryocyte differentiation, while concomitantly increasing the expression of megakaryocyte-specific genes which have been identified as its unique targets [37]. NFE2L1 is believed to be a "fine-tuning tool" for NFE2L2-mediated antioxidant responses through co-inducing common targets [54], competing with NFE2L2 as a less potent activator [55], or by repressing common targets [56]. BACH1 is a repressive "guardian" responsible for deactivating the antioxidant signaling and maintaining the basal level under unstimulated conditions [74, 78-80]. Delayed induction of BACH1 in response to stress stimuli relative to NFE2L2 induction seems to be the key to their coordinated activities [79]. Along with BACH1, BACH2 is well known for its roles in various immunological processes, including B cell maturation [89].

As for NFE2L3, the protein is highly comparable to NFE2L1 with respect to its structure. Current literature presents two opposing views regarding the transcriptional control of NFE2L3 over ARE-containing genes: a transcriptional activator or repressor. If we consider evidence supporting the former view, NFE2L3 is the least potent activator and is among the many homeostatic mechanisms promoting the rapid recovery of induced antioxidant enzymes to their basal levels and maintaining the basal ROS level [101]. If we consider data that supports the later view, NFE2L3 is a repressor that seems to have a biologically relevant effect under limited circumstances. This is based on the observations that NFE2L3-mediated repression cannot

overcome gene induction by NFE2L2 in response to *t*-BHQ [101], whereas the repressive activity of NFE2L3 seems to play a crucial role in SMC differentiation [103]. In fact, NFE2L2 seems to be responsible for the maintenance of self-renewal potency of stem cells and its activity is downregulated during differentiation [145, 146]. These data suggest that NFE2L2 inhibition may be necessary for the efficacious transcriptional repression of NFE2L3. However, despite the accumulating interest in NFE2L3, whether NFE2L3 is a transcriptional activator or repressor is still controversial. Perhaps, it takes different roles in a context-dependent manner. Hence, a more careful analysis is needed when it comes to linking different studies. Similarly, comparing the transcriptional activities among CNC factors requires consideration of different factors such as binding affinity and protein processing in addition to cell type differences.

Intriguingly, a novel function of NFE2L3 that does not involve NFE2L2 antagonism has been discovered. While NFE2L2 promotes the survival of UV-irradiated suprabasal keratinocytes through increased detoxification and GSH synthesis [115], NFE2L3 deficiency was associated with increased survival, without any changes in the induction of NFE2L2 or its target genes in UV-irradiated basal keratinocytes [117]. Further analysis demonstrated an enhanced cell adhesion, suggesting that NFE2L3 may promote apoptosis through its unique function in cellular detachment. Consistent with this data, NFE2L3 expression was positively correlated with metastasis in thyroid cancer [142].

In spite of its oncogenic properties in cell invasion, the role of NFE2L3 in cancer is debatable. While NFE2L3 has been shown to prevent carcinogen-induced lymphomagenesis [144],

NFE2L3 knockdown decreased cell proliferation in colorectal cancer [100]. It may be speculated that the role of NFE2L3 in cancer may be context-dependent.

Last but not least, the complex interplay and functional redundancy among the CNC family members as well as their interactions with small MAF proteins should not be neglected in order to gain full insights. Moreover, the possibility that NFE2L3 may possess non-transcriptional functions or that ER-bound NFE2L3 may have an important role should not be overlooked.

Current literature highlights the role of NFE2L3 in differentiation, cell adhesion, immune responses as well as tumor initiation and progression. Despite the blooming research on NFE2L3 over the past decade, the functions of NFE2L3 and the detailed molecular mechanisms still remain elusive. The difficulties associated with identifying the functions of NFE2L3 seem to be partly due to the fact that its role is most likely to have an effect under specific circumstances. Nevertheless, recent data hints at the unique functions of NFE2L3 that are yet to be discovered.

1.4 Rationales and research hypotheses

Accumulating evidence suggests that NFE2L3 may play an important role in cancer. NFE2L3 has been reported to be upregulated in a variety of cancers and especially in blood cancer cell lines [16]. However, NFE2L3 has never been studied in the context of hematopoietic cells and malignancies. Therefore, the present thesis focuses on the investigation regarding the *in vivo* and *in vitro* functions and regulation of NFE2L3 in hematopoietic cells and lymphoma.

1.4.1 Investigating NFE2L3 mutations present in diffuse large B cell lymphoma (DLBCL)

Our previous study demonstrated that mice deficient in *Nfe2l3* were highly susceptible to carcinogen-induced lymphomagenesis, notably T cell lymphoblastic lymphoma [144], suggesting NFE2L3 as a tumor suppressor. However, NFE2L3 is overexpressed in many blood cancer cell lines [16], especially in diffuse large B cell lymphoma (DLBCL) [133, 134, 210]. Taking both observations into account and given that NFE2L3 has been reported to be significantly mutated in cancer [147], we hypothesize that the highly expressed NFE2L3 in DLBCL may harbor mutations that render it dysfunctional, thereby, hindering its tumor-suppressive activity.

1.4.2 Investigating the role of NFE2L3 in hematopoiesis

Reactive oxygen species (ROS) serve as important signaling molecules in cellular differentiation [148]. Several CNC transcription factors, including NFE2L3, have been shown to be implicated in the regulation of cellular differentiation [37, 89, 103, 113, 146]. Based on the studies demonstrating the crucial role of NFE2L3 in promoting smooth muscle cell maturation and neural stem cell differentiation [103, 113], we speculate that NFE2L3 may also promote hematopoietic differentiation. We hypothesize that *Nfe2l3* deficiency in mice will result in the impaired hematopoiesis, leading to the aberrant absolute or relative number of fully differentiated blood cells, or both.

1.4.3 Investigating the regulation of NFE2L3 levels in hematopoietic cells

Research on NFE2L3 has been unsuccessful in addressing the critical question of how NFE2L3 is activated. The identification of NFE2L3 stimuli will help us gain insights into its poorly

understood functions. Hence, my objective is to investigate the regulatory signals of NFE2L3 in hematopoietic cells. Since NFE2L3 has been reported to be induced by some proinflammatory cytokines [17, 122], we hypothesize that NFE2L3 may be involved in mediating immune responses and thus expect the stimuli which induce immune responses to modulate NFE2L3 expression in hematopoietic cells.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell culture and treatments

2.1.1 Cell lines and cell culturing

All cell lines were purchased from the American Type Culture Collection (ATCC). Human embryonic kidney cell line 293T (HEK293T) and human breast adenocarcinoma cell line MCF7 were grown in Dulbecco's Modified Eagle medium (DMEM) (Invitrogen). Human acute T cell leukemia cell line Jurkat and human Burkitt's lymphoma cell lines Raji and Namalwa were cultured in RPMI 1640 medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin (Invitrogen). In addition to these supplements, the medium for Namalwa cells contained 10mM HEPES (Invitrogen). Cells were incubated at 37˚C, 5% CO_2.

2.1.2 Reagents and treatments

Cells were treated as indicated with PMA (Sigma), ionomycin (Thermo Fisher), TNFα (Invitrogen), BAY11-7082 (Santa Cruz), JSH-23 (Cederlane), and LPS (Sigma).

2.1.3 Plasmids, transfections and luciferase reporter assay

Site-directed mutagenesis was performed using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer's instructions. MCF7 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were collected at 24 hours post-transfection. A

dual luciferase assay (Promega) was performed according to the manufacturer's instructions and the readings were normalized to renilla activity.

2.2 Protein extraction and immunoblot analysis

2.2.1 Whole cell extraction

Adherent cells were collected using 0.25% trypsin (Invitrogen) whereas suspension cells were collected by centrifugation. Cells were washed with PBS and lysed on ice for 20 minutes with whole-cell lysis buffer (10mM Tris-HCl pH 8.0, 420mM NaCl, 250mM sucrose, 2mM $MgCl_2$, 1mM $CaCl_2$, 1% Triton-X-100) supplemented with 1/10 phosphatase inhibitor PhosSTOP™ (Roche), 1/25 protease inhibitor cocktail cOmplete™ (Roche), 0.2mM PMSF and 1mM sodium orthovanadate. Lysates were cleared of debris by centrifugation at 13,000rpm for 10 minutes at 4˚C. Protein concentration was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad) according to the manufacturer's instructions. The samples were prepared using NuPAGE™ LDS Sample Buffer (Thermo Fisher) and NuPAGE™ Sample Reducing Agent (Thermo Fisher), then heated at 70˚C for 10 minutes.

2.2.2 Immunoblot

Protein extracts were separated on precast NuPAGE Novex (Invitrogen) or Criterion™ XT (Bio-Rad) 4-12% Bis-Tris gradient gels. SeeBlue™ Plus2 pre-stained protein standard (Invitrogen) was run with the samples. Proteins were transferred onto the PVDF membrane (Immobilon®-P, Millipore). The membrane was blocked for 1 hour at room temperature in 5% skim milk in TBS-T (50mM Tris-HCl pH 7.6, 200mM NaCl, 0.05 % Tween 20) and incubated with primary

antibody at 4˚C for overnight. After three 10-minute washes with TBS-T, the membrane was incubated with secondary antibody for 1 hour at room temperature followed by six 10-minute washes. The proteins were detected using Immobilon™ Western Chemiluminescent HRP substrate (Millipore) according to the manufacturer's instructions.

2.2.3 Antibodies

The antibodies used in the immunoblot analysis were anti-α-Tubulin (Sigma, T6074), anti-IκBα (Cell Signaling Technology, 9242) and anti-phospho-IκBα (Cell Signaling Technology, 2859). Horseradish peroxidase (HRP) – conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Thermo Scientific) were used at a dilution of 1:25,000.

2.2.4 Preparation of a polyclonal antibody against NFE2L3

For NFE2L3, a polyclonal antibody raised against human NFE2L3 (amino acids 220-243; KENSLQQNDDDENKIAEKPDWEAE) was generated as described previously (Chenais et al., 2005). Briefly, the peptide was coupled to keyhole limpet hemocyanin (KLH) and was used to immunize rabbits (Pocono Rabbit Farm & Laboratory). Antisera were tested in parallel with pre-immune serum to confirm the specificity to human NFE2L3. The serum was purified using the peptide coupled to Affi-Gel 15 (Bio-Rad) according to the manufacturer's instructions.

2.3 Hematology analysis using animal model

2.3.1 Mice

All animal procedures were approved by the McGill Animal Care Committee and conducted in accordance with the Canadian Council on Animal Care guidelines. The sample population consisted of C57BL/6 wildtype mice (WT; 8 males and 6 females) and *Nfe2l3* knockout mice (*Nfe2l3*$^{-/-}$; 9 males and 6 females). Mice were housed under standard housing conditions in a sterile facility.

2.3.2 Complete blood count (CBC) analysis

Starting from week 6, fresh blood samples were collected from the lateral saphenous vein every 2 weeks over the period of 18 weeks. Blood was mixed with ethylene diamine tetraacetic acid (EDTA) to prevent coagulation and analyzed by scil Vet ABC hematology analyzer (scil animal care). The following hematology variables were measured: red blood cell (RBC) and white blood cell (WBC) count, hemoglobin concentration (HGB), hematocrit (HCT), platelet concentration (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), mean platelet volume (MPV), as well as the 3-part differential white blood cell count which consists of the absolute and relative counts of lymphocytes (LYM), monocytes (MON) and granulocytes (GRA). The CBC data was analyzed using Excel and JMP software.

2.4 Flow cytometry

2.4.1 Cell staining

Male whole blood samples collected for CBC analysis were also used for flow cytometry analysis. After the CBC analysis, the samples were kept on ice until the cell staining procedure. Samples were processed within 2 hours of the blood being drawn. 30µl of blood was used to prepare each sample. The rest of the blood was pooled together and used for unstained control, compensation controls and fluorescence minus one (FMO) controls for each fluorophore. Cell viability control was prepared by subjecting cells to heat shock. Staining was performed according to the manufacturer's instructions (eBioscience): Viability Staining (Protocol C), Staining Cell Surface Antigens for Flow Cytometry (Protocol B) and RBC Lysis (Protocol B). Briefly, cells were stained with Fixable Viability Dye eFluor 506 (eBioscience) to label dead cells. 1% (v/v) BSA in PBS was used as buffer. Cells were then incubated with anti-mouse CD16/CD32 (eBioscience) to block Fc receptors. Subsequently, cells were stained with fluorophore-coupled primary antibodies and lysed using 1-step Fix/Lyse Solution (eBioscience). Samples were washed and resuspended in 300µl of buffer shortly before the flow cytometric analysis.

2.4.2 Fluorophore-conjugated antibodies

The following antibodies were used for flow cytometry: eFluor 450-conjugated anti-mouse CD19 (eBio1D3), PE-conjugated anti-mouse NK1.1 (PK136), Alexa Fluor 488-conjugated anti-mouse CD3e (145-2C11) and APC-eFluor 780-conjugated anti-mouse CD45 (30-F11). All antibodies were purchased from eBioscience.

2.4.3 Flow cytometry analysis

Data was acquired on a BD LSR Fortessa (BD Bioscience) and analyzed with FlowJo software. Lymphocytes were identified with CD45 antibodies. Using CD45 positive cell population, B and T cells were stained with antibodies to CD19 and CD3, respectively. Natural killer (NK) cells were identified as $CD19^{-}$ $CD3^{-}$ $NK1.1^{+}$. Fixable viability dye was used for live-dead discrimination. Flow cytometry was performed with male mouse blood samples only.

2.5 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) unless indicated otherwise. Results are representative of at least three independent experiments. Statistical differences between the two groups were examined with Student's *t*-test. *P*-values < 0.05 were considered statistically significant. The levels of statistical significance were: $*p < 0.05$, $** p < 0.01$, $*** p < 0.001$.

CHAPTER 3. RESULTS

3.1 Investigating NFE2L3 mutations present in diffuse large B cell lymphoma (DLBCL) patient samples

Given the overexpression of NFE2L3 in DLBCL tissue samples [133, 134, 210] and its protective role against lymphoma formation [144], we hypothesized that the highly expressed NFE2L3 found in DLBCL carries mutations that impede with the tumor-suppressive activity, contributing to the malignancies.

To verify this hypothesis, we collaborated with Dr. Ryan Morin [149] to analyze RNA-sequencing data of DLBCL patient samples. The analysis revealed that 8.85% (10/113) of the patients carried mutations in NFE2L3 transcripts, making NFE2L3 the most frequently mutated gene among the members of CNC factors and small MAF proteins (Table 1).

Each of these patients carried different mutations. In order to eliminate non-somatic mutations, the candidate mutations were mapped against 40 different whole genome shotgun (WGS) sequencing data. None of the mutations were present in the examined cases, implying that the mutations are most likely to be somatic. Next, a stringent database filtering was applied to remove single nucleotide variants (SNVs) that have been previously observed in normal samples. Data after the filtering revealed 3 highly likely true somatic mutations of NFE2L3: N346K, S481N and D644G.

Gene	Number of mutations (out of 113 patients)
NFE2	0/113 (0%)
NFE2L1	3/113 (2.65%)
NFE2L2	1/113 (0.88%)
NFE2L3	10/113 (8.85%)
BACH1	1/113 (0.88%)
BACH2	3/113 (2.65%)
MafF	0/113 (0%)
MafG	0/113 (0%)
MafK	2/113 (1.77%)

Table 1. Comparison of number of mutations found in CNC and small MAF gene transcripts from RNA-sequencing analysis of 113 DLBCL patients

The N346K mutation was located in the transactivation domain, while S481N and D644G mutations were found near one of the potential N-glycosylation sites and the leucine zipper dimerization domain, respectively (Figure 3). Based on their positions within NFE2L3 protein structure, we speculated that N346K, S481N and D644G may impair transactivation ability, intracellular regulation and dimerization ability of NFE2L3, respectively and that these mutations would result in the impediment of NFE2L3 activity.

We therefore performed a luciferase reporter assay to determine the effect of these mutations on the transcriptional capacity of NFE2L3. The three mutant NFE2L3s increased HO-1 ARE-mediated gene expression by approximately 6-fold compared to mock control, similar to the wildtype protein (Figure 5). Based on this data, we concluded that N346K, S481N and D644G mutations found in DLBCL cells do not affect the transactivation capacity of NFE2L3.

3.2 Identifying the role of NFE2L3 in hematopoiesis: our *in vivo* study

Since NFE2L3 has been identified as a crucial promoter of smooth muscle cell and neural stem cell differentiation [103, 113], we asked whether the absence of NFE2L3 would alter the differentiation of hematopoietic stem cells (HSCs). We addressed this question by examining blood parameters and fully differentiated lymphocytes in wildtype and *Nfe2l3*-null mice using complete blood count (CBC) and flow cytometry. The data for male and female mice were analyzed separately as previous studies report gender differences in the baseline levels of blood parameters even for the age-matched groups [150, 151]. Unfortunately, data for week 20 are unavailable due to an unidentified technical issue.

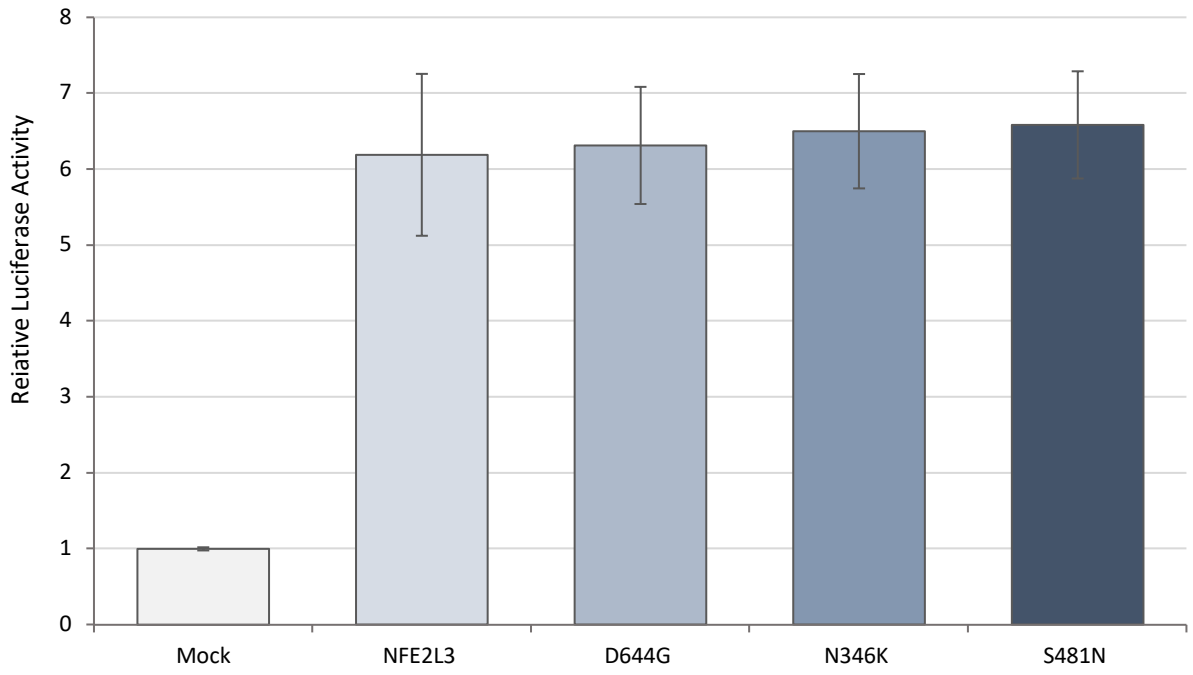


Figure 5. NFE2L3 mutations present in DLBCL patient samples do not modulate transactivation capacity.

MCF-7 cells were transiently transfected with pcDNA3.1^{+} Hygro control vector or the vector expressing wildtype or mutant NFE2L3 along with luciferase vector under the control of HO-1 promoter containing ARE region. Luciferase activities were normalized to renilla and to the promoter activity of the pcDNA3.1^{+} Hygro mock vector (set to a value of 1). Student *t*-test was used for statistical analysis.

3.2.1 Hematology analysis

Male sample population

CBC analysis for male mice (Figure 6A) revealed that *Nfe2l3*$^{-/-}$ mice showed reduced red blood cell count (RBC) and hematocrit (HCT) with higher mean corpuscular hemoglobin concentration (MCHC) at week 6. Subsequently, at weeks 10 and 14, they displayed lower red blood cell distribution width (RDW) when compared to the wildtype mice. We also observed higher absolute count of lymphocytes (#LYM) in *Nfe2l3*$^{-/-}$ mice.

Female sample population

Female mice exhibited a slightly different profile: more variables were found to be statistically different between control and knockout groups (Figure 6B). We noted the differences in erythroid-related variables in female mice at week 6. *Nfe2l3*$^{-/-}$ mice showed significantly higher hemoglobin concentration (HGB) and mean corpuscular hemoglobin (MCH), but also higher hematocrit (HCT) when compared to the control group. However, these observations were only sporadic. Indeed, despite the lack of statistical significance, *Nfe2l3*$^{-/-}$ mice continuously displayed lower values for all three variables than the wildtype animals for at least 8 weeks. The most consistent pattern was observed with mean corpuscular volume (MCV), where *Nfe2l3*$^{-/-}$ mice showed lower values in comparison with their wildtype counterparts', persisting for 10 weeks. Unlike the male mice, the 3-part differential white blood cell count revealed significant differences in the relative counts of lymphocytes, monocytes and granulocytes (%LYM, %MON and %GRA) as well as higher absolute number of granulocytes (#GRA) in the knockouts

compared to the controls at later time points (week 16, 18, 22 and 24). However, only the pattern observed with #GRA remained consistent over time.

3.2.2 Flow cytometry analysis

To closely examine the effect of NFE2L3 on lymphoid lineage differentiation, we performed a flow cytometry analysis on male blood samples. No significant differences were found between control and *Nfe2l3*-null mice with respect to the frequency and the absolute number of T, B and natural killer (NK) cells (Figure 7).

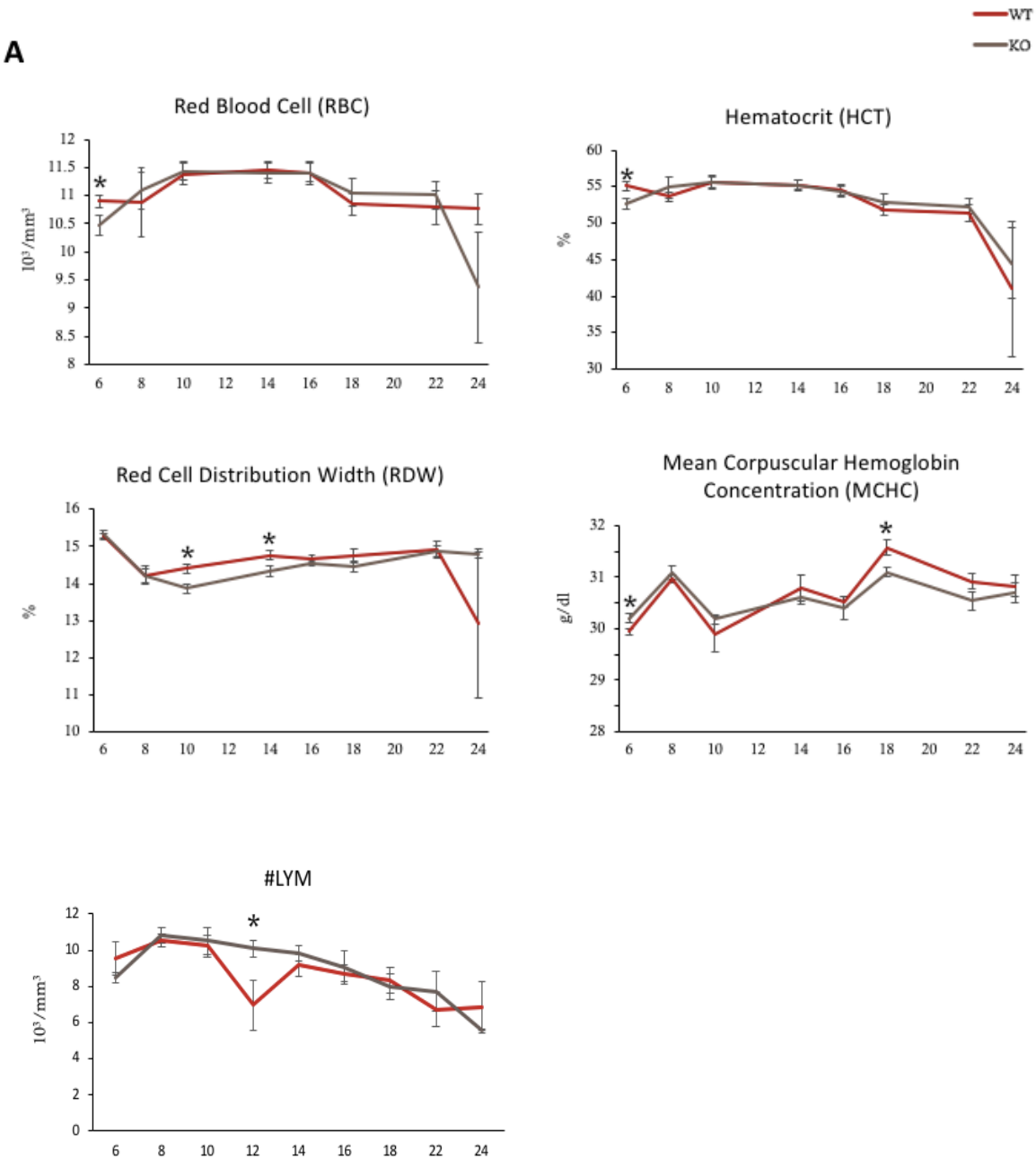
3.3 Cytokine regulation of NFE2L3 in hematopoietic cells

Previous studies showed that NFE2L3 can be induced by cytokines [17, 122]. We therefore sought out to determine whether NFE2L3 is regulated by the inflammatory stimuli in hematopoietic cells and whether the transcription factor is involved in inflammatory responses.

3.3.1 Phorbol 12-myristate 13-acetate (PMA) and ionomycin

In order to address our question, we first examined phorbol 12-myristate 13-acetate (PMA) and ionomycin since they are widely used to activate immune cells to produce cytokines [152, 153]. We speculated that if NFE2L3 is implicated in inflammation, its expression may be controlled by these compounds.

Human T lymphocyte Jurkat and human B lymphocyte Namalwa cell lines were stimulated with PMA (200ng/ml) alone or with ionomycin (1μM) for 2 hours (Figure 8). PMA treatment resulted


A
WT
KO
Red Blood Cell (RBC)
$10^3/mm^3$
Hematocrit (HCT)
%
Red Cell Distribution Width (RDW)
%
Mean Corpuscular Hemoglobin Concentration (MCHC)
g/dl
#LYM
$10^3/mm^3$

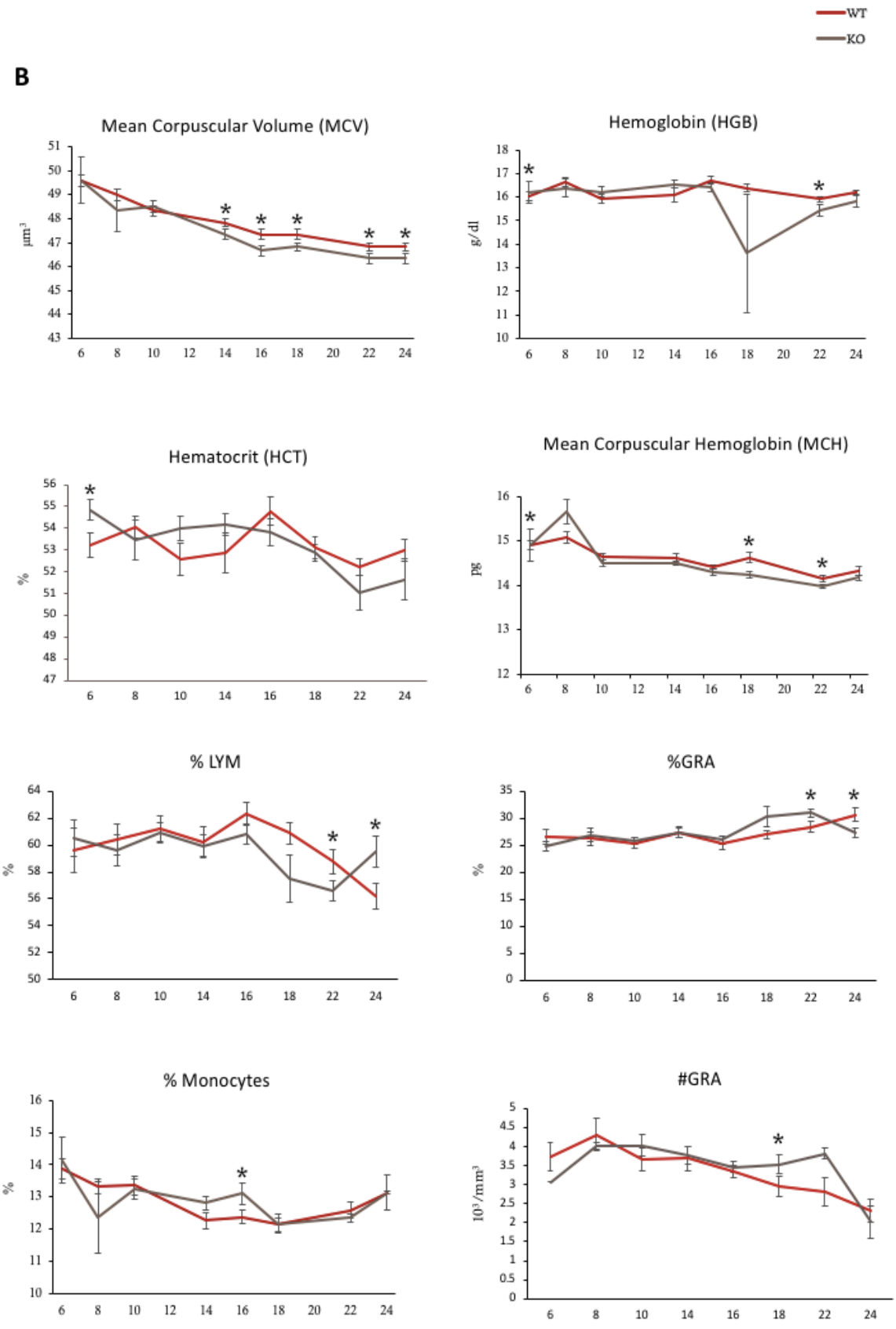

WT
KO
B
Mean Corpuscular Volume (MCV)
µm³
Hemoglobin (HGB)
g/dl
Hematocrit (HCT)
%
Mean Corpuscular Hemoglobin (MCH)
pg
% LYM
%
%GRA
%
% Monocytes
%
#GRA
10³/mm³

Figure 6. Nfe2l3-deficient mice show sporadic changes in blood parameters.

(A-B) Blood cells collected from wildtype (WT) and *Nfe2l3* knockout (KO) mice were examined for various blood parameters using CBC analysis. Data are presented as mean ± SD over time (weeks). *Nfe2l3*-deficiency resulted in short-lived statistical significance in some blood parameters in both males (A) and females (B). X-axis represents the mouse age (in weeks).

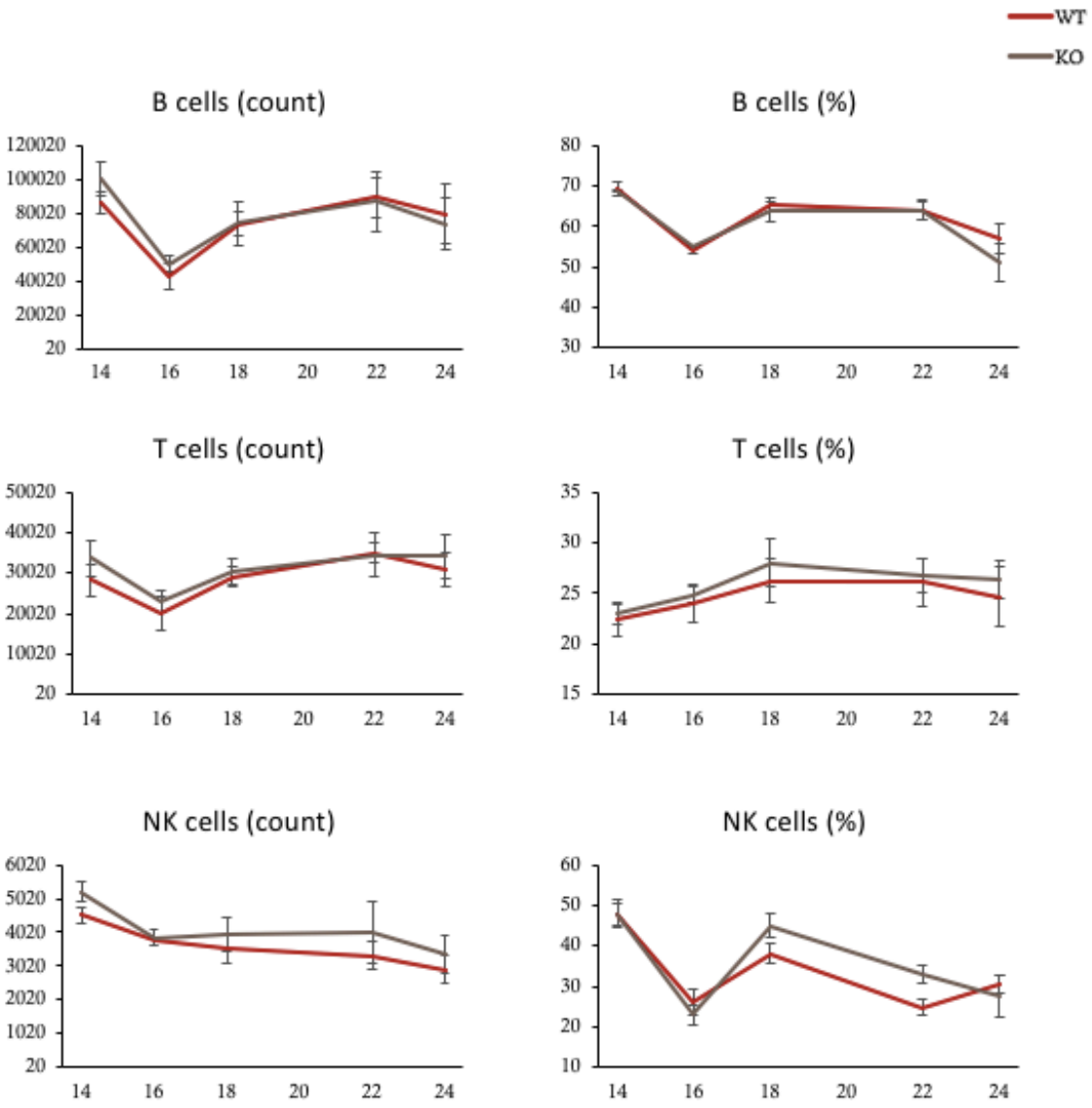


Figure 7. NFE2L3-deficiency has no effect on the composition of lymphocytes in blood.

Whole blood samples were collected from male wildtype (WT) and *Nfe2l3* knockout (KO) mice and the absolute and relative counts of B, T and natural killer (NK) cells were measured by flow cytometry. Time course analysis of the lymphocytes composition in blood shows no significant differences between the two genotypes. X-axis represents the mouse age (in weeks).

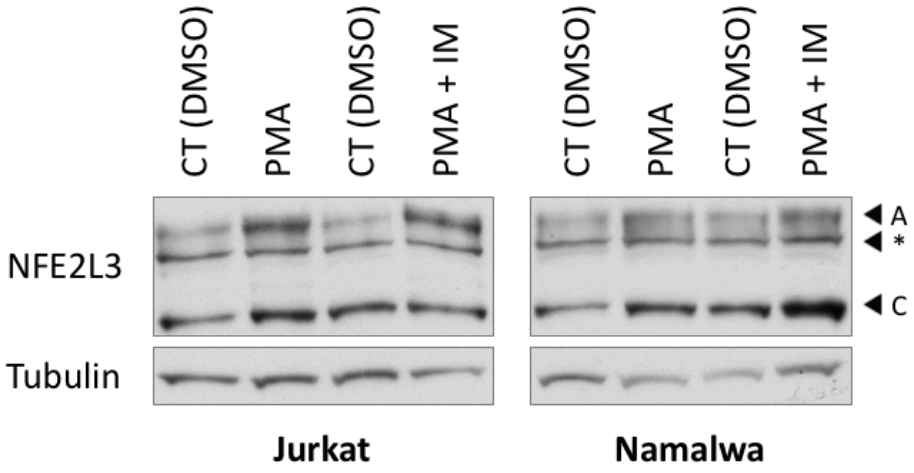


Figure 8. PMA induces NFE2L3 expression.

Jurkat and Namalwa were stimulated with PMA (200ng/ml) alone or with ionomycin (IM) (1μM) for 2 hours. Tubulin was used as a loading control. Asterisk indicates an unspecific band.

in an increase of NFE2L3 protein levels in both cell lines. However, co-treatment with ionomycin did not further enhance this NFE2L3 induction.

3.3.2 Tumor necrosis factor alpha (TNFα)

Next, we investigated whether NFE2L3 can be induced by the proinflammatory cytokine TNFα in hematopoietic cells since it has been shown to increase NFE2L3 expression in JAR choriocarcinoma cell line [17]. To answer this question, we stimulated Jurkat T lymphocytes as well as two B lymphocyte cell lines, Namalwa and Raji, with TNFα (20ng/ml) for 6 hours. NFE2L3 protein levels were upregulated in Jurkat and Namalwa, but not in Raji cells (Figure 9A). To verify whether the pattern of NFE2L3 induction by TNFα is similar between Namalwa and Jurkat, we conducted a time course analysis of TNFα treatment. Immunoblot studies revealed that NFE2L3 induction was detectable starting from 2 hours, reaching the maximal induction at 4 – 6 hours in both cell lines (Figure 9B).

As NF-κB is a key component of TNFα signaling [154], we wanted to examine whether NF-κB can regulate NFE2L3 expression. To answer this question, we pre-treated cells with one of the two NF-κB inhibitors, BAY-117082 (0.5μM, 2h) or JSH-23 (50μM, 16h), followed by TNFα treatment (20ng/ml, 6h). As expected, TNFα increased the protein levels of NFE2L3 as well as the phosphorylation of IκBα in both Jurkat and Namalwa cells (Figure 9C). Treatment with BAY-117082 effectively inhibited IκBα phosphorylation and led to a decrease in NFE2L3 induction by TNFα. Consistent with this observation, JSH-23 also abolished the effect of TNFα on NFE2L3 in Namalwa (Figure 9D). Jurkat cells were highly sensitive to JSH-23 and significant cell death was observed at any concentration above 1μM, so the effect of this

inhibitor could not be analyzed in this cell line. Of note, BAY-117082 and JSH-23 also diminished the basal expression levels of NFE2L3.

3.3.3 Lipopolysaccharide (LPS)

To determine whether NFE2L3 is involved in immune response to bacteria, we examined NFE2L3 modulation by lipopolysaccharide (LPS), a major component of the outer cell membrane of Gram-negative bacteria. No obvious change in NFE2L3 protein levels was observed in Jurkat and Namalwa cells with LPS stimulation at 10, 20 or 30μg/ml for 16 hours (Figure 10A). Since prior works reported using much lower concentrations for much shorter time duration [155-158], we conducted LPS stimulation using 1μg/ml for 30 or 60 minutes. In parallel, we also examined the effect of LPS under serum starvation in an attempt to eliminate any crosstalk with other signal pathways. However, NFE2L3 protein levels were modulated by none of these conditions (Figure 10B). We then analyzed higher LPS concentrations: 1, 2 and 5μg/ml for 30 or 60 minutes. NFE2L3 protein levels remained unaffected with LPS stimulation, whereas TNFα markedly induced its expression (Figure 10C).

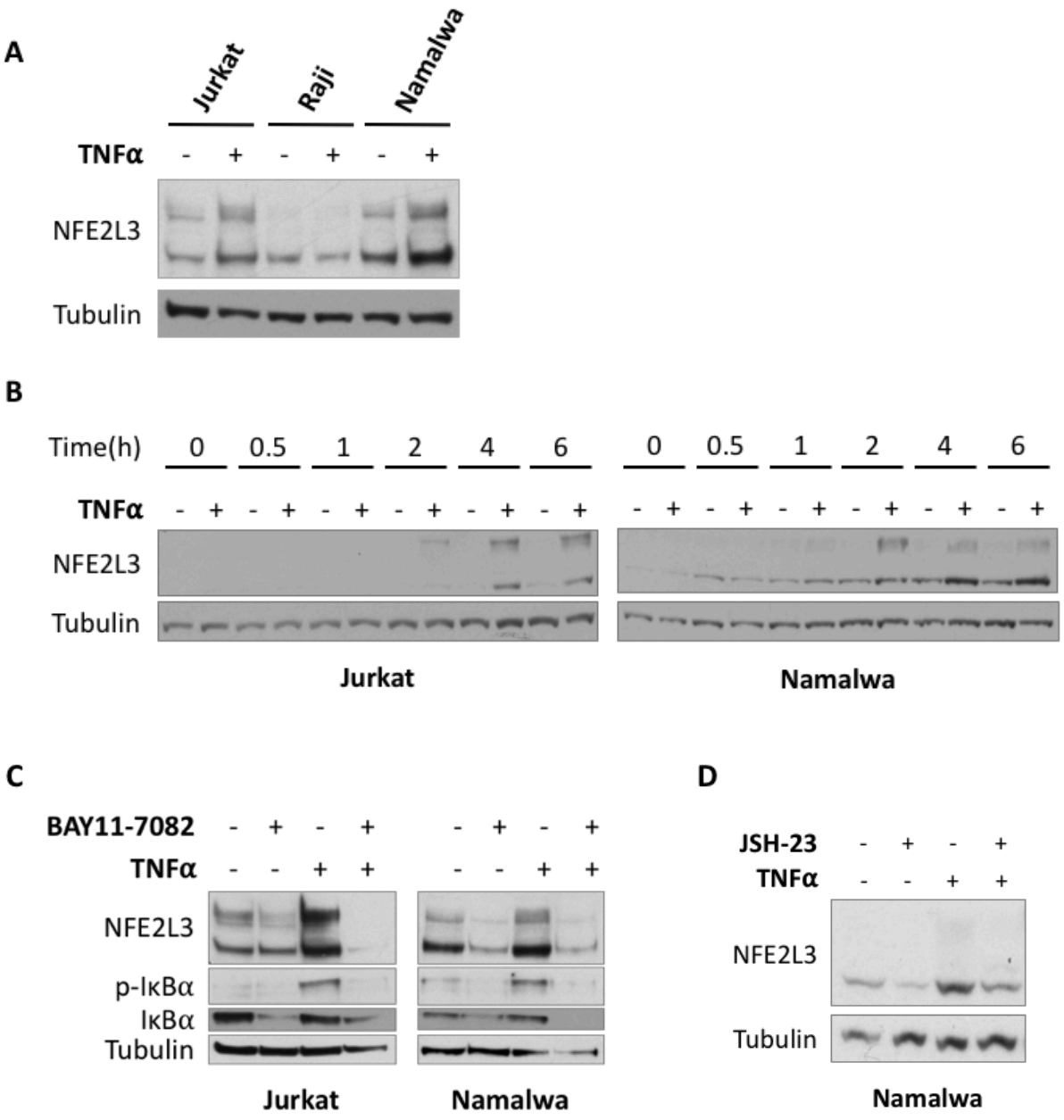


Figure 9. TNFα may induce NFE2L3 levels via the NF-κB pathway.

(A) Jurkat, Raji and Namalwa cells were seeded at a concentration of 5×10^5/ml, then incubated untreated or treated with TNFα (20ng/ml) for 6 hours. NFE2L3 is induced in response to the treatment in Jurkat and Namalwa cell lines, but not in the Raji cell line.

(B) Jurkat and Namalwa were incubated untreated or treated with TNFα (20ng/ml) at different time points. Maximal induction of NFE2L3 occurs 4-6 hours post-stimulation.

(C) Cells were incubated untreated or treated with TNFα (20ng/ml) with or without BAY11-7082 (0.5μM, 2 hrs) pre-treatment. TNFα induces NFE2L3 protein levels and leads to increased phosphorylation of IκBα. This induction is blocked by BAY11-7082.

(D) Namalwa cells were left untreated or treated with TNFα treatment (20 ng/ml, 6 hrs) with or without JSH-23 (50μM, 16hrs) pre-treatment. JSH-23 abolished TNFα-induced NFE2L3 expression.

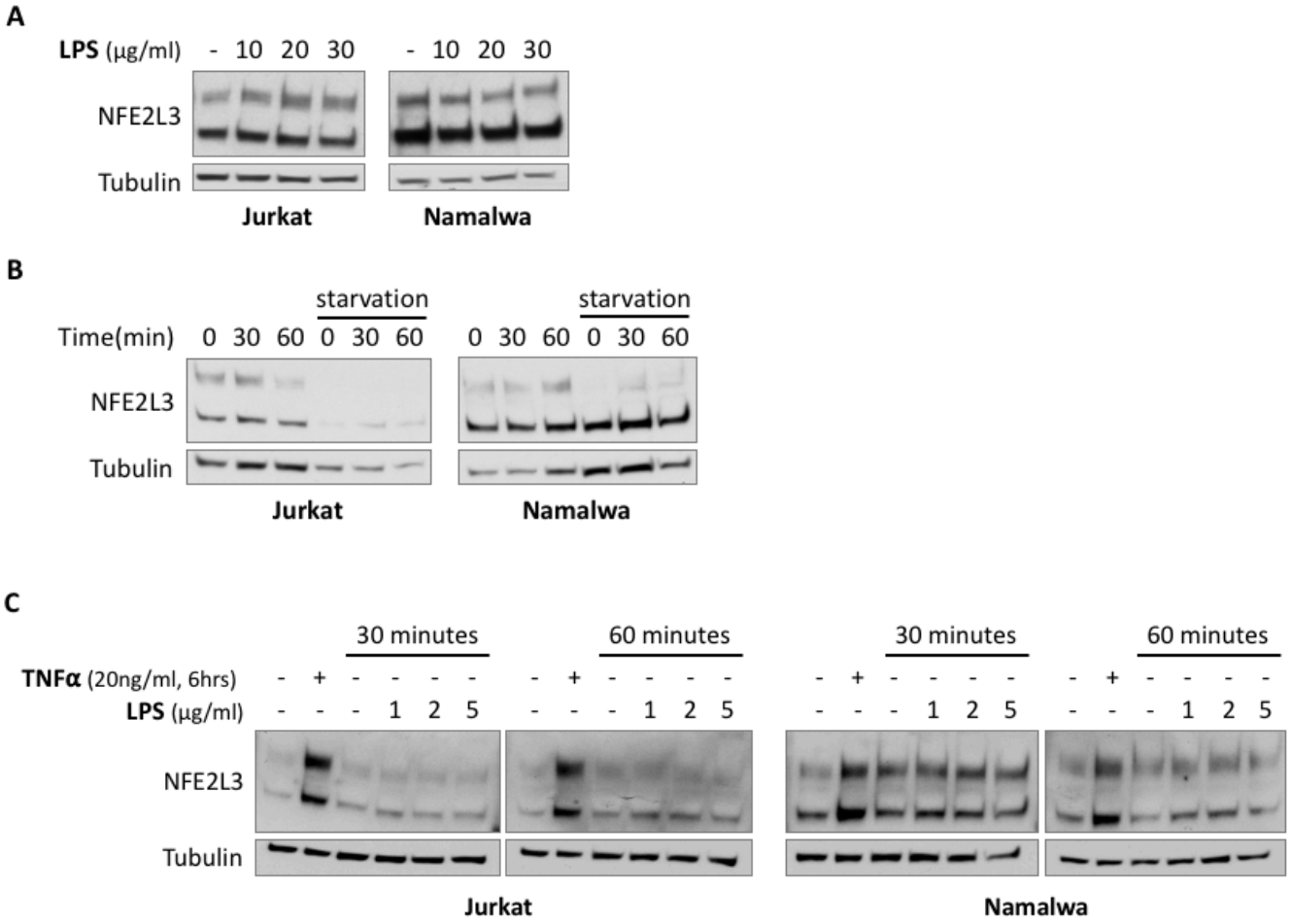


Figure 10. LPS does not modulate NFE2L3 protein expression.

(A-C) Jurkat and Namalwa cells were left untreated or treated with 10μg/ml, 20μg/ml, 30μg/ml LPS for 16 hours (A), or treated with 1μg/ml LPS for 0, 30 or 10 minutes either in normal or in starvation media (B) or treated with 1μg/ml, 2μg/ml or 5μg/ml LPS for 30 or 60 minutes (C). TNFα (20ng/ml, 6hrs)-treated cells were loaded together for comparison.

CHAPTER 4. DISCUSSION

4.1 Investigating NFE2L3 mutations present in DLBCL

Given the protective role of NFE2L3 against carcinogen-induced lymphomagenesis [144], the high expression of NFE2L3 in cancer prompted us to examine its mutations. Our luciferase reporter assay showed no differences in HO-1 ARE promoter activity between different NFE2L3 mutants and the wildtype protein, indicating that the 3 mutations identified in DLBCL patient samples do not alter the transactivation capacity of NFE2L3. However, because the assay was performed in a breast adenocarcinoma cell line MCF7, it is possible that the effect of mutations would have been masked if it is cell-type specific. Another possibility is that the mutations might affect the ability of NFE2L3 to regulate target genes that are not under the control of ARE.

Diffuse large B cell lymphoma (DLBCL) is a malignant transformation of mature B cells that is characterized by high heterogeneity in regard to the underlying molecular mechanisms and clinical manifestations [159]. This is not surprising given the fact that somatic hypermutation (SHM) and class-switch recombination (CSR), the key features of B cell development and functions, introduce genomic mutations in the germinal center (GC), where B cells proliferate extensively [160]. This inherent genomic instability is thought to be one of the key mechanisms hijacked by cancerous B cells to acquire mutations that confer survival advantages, termed driver mutations [161-163]. But there exists another type of mutations termed passenger mutations that emerge and persist without oncogenic properties simply due to the very nature of genomic instability, or because they happen to be a secondary effect of the oncogenic pathways, hence the

surname “hitchhikers” [162, 163]. Based on our result, it seems highly likely that the three somatic mutations of NFE2L3 in DLBCL are passenger mutations, representing one of the many accumulated mutations in the transformed cells with no major effect on cancer cell growth and survival.

While NFE2L3 mutations may have no effect on the activity, the aberrant overexpression of NFE2L3 in DLBCL merits attention. The human NFE2L3 locus is located on chromosome 7 whose gain has been observed in DLBCL [159, 164-166], although its pathological relevance in DLBCL is still under investigation [165, 167]. Consistent with this data, cBioportal database reports the most common mutations associated with NFE2L3 to be amplification [133, 134]. Therefore, it would be of interest to examine the effect of NFE2L3 overexpression in DLBCL.

4.2 Investigating the role of NFE2L3 in hematopoiesis

With recent studies suggesting a crucial role of NFE2L3 in stem cell differentiation [103, 113], we sought to identify the effect of *Nfe2l3* deficiency on blood stem cell maturation. If NFE2L3 plays a role in hematopoietic stem cell differentiation, we expect to see an accumulation of immature blasts and, as a consequence, a decrease of fully differentiated blood cells.

We found the main differences between *Nfe2l3*-deficient mice and the wildtype controls in both male and female mice to be erythroid parameters. We observed lower count and percentage of red blood cells with *Nfe2l3* deficiency in male mice at week 6, but this pattern did not persist over time. Although *Nfe2l3*$^{-/-}$ female mice displayed higher proportion of red blood cells in the

blood when compared to the wildtype animals at week 6, data over the last 9 weeks showed the opposite pattern but with no statistical significance. It is difficult to conclude based on these results whether NFE2L3 plays a role in erythropoiesis, but it would be interesting to investigate the role of *Nfe2l3* in erythropoiesis and its biological significance.

The phenotype we observed in male mice at week 6, with an increase in hemoglobin concentration per red blood cell, accompanied by a reduction in the number of red blood cells, is a condition termed hyperchromic anemia in humans. Additionally, *Nfe2l3*-deficiency was associated with lower red blood cell distribution width (RDW) in male mice, whereas hemoglobin concentration (HGB), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) were found to be acutely altered in female *Nfe2l3*-deficient mice. Together, the data suggest a possible involvement of NFE2L3 in erythrocyte-associated functions.

Interestingly, studies on other CNC transcription factors also report anemia and other erythroid-related phenotypes. Indeed, one of the main phenotypes of viable *Nfe2l1*$^{-/-}$ mouse embryos is the remarkably reduced hematocrit (HCT) values due to the apparent impairment in liver erythropoiesis [51]. Also, NFE2 has been shown to activate globin gene expression, although its role in hemoglobin synthesis may not be crucial [30-32]. Moreover, of the most commonly used ARE-containing genes in studying oxidative stress-induced signaling is the well-known target of the CNC transcription factors, *HO-1* [168]. Mice lacking *Ho-1* exhibited symptoms of anemia [169], while human patients with *HO-1* deficiency, although extremely rare, have been reported to suffer from dysregulated iron homeostasis and anemia [170, 171]. The involvement of other CNC transcription factors in the differentiation and the function of red blood cells further

supports the idea that NFE2L3 may be involved in these processes as well. However, this body of literature also reflects the possible functional redundancy among the CNC factors and, thus, may explain the reason why we do not see a major phenotype with *Nfe2l3* deficiency.

Nevertheless, female *Nfe2l3*$^{-/-}$ mice exhibited consistently lower MCV compared to wildtype animals over an extended period of time. While the biological significance may be questioned as the values only differ by less than 1mm^3, the data seems to suggest that *Nfe2l3* deficiency in female mice is associated with variability within MCV measures. In support of this idea, genome wide quantitative trait locus (QTL) analysis of multiple mouse strains showed that chromosome 7 on which NFE2L3 is located, along with chromosome 14, was highly associated with the MCV parameter [172], suggesting a strong genetic effect on the variations of MCV.

No difference was found between male wildtype and *Nfe2l3*$^{-/-}$ mice with respect to the lymphocyte composition as measured by flow cytometry, suggesting that NFE2L3 has no effect on lymphoid differentiation. We observed a higher total number of granulocytes in female *Nfe2l3*$^{-/-}$ mice at weeks 18 and 22. This was reflected in the ratio among lymphocytes, monocytes and granulocytes. However, this pattern did not persist at week 24, at which we saw an abrupt decrease in the granulocyte population and concomitant increase in the lymphocyte population. The reason for this sudden change is unknown but taking into consideration the slight decrease in red blood cell populations, it would be worthwhile to investigate whether NFE2L3 participates in the regulation of myeloid lineage differentiation.

Overall, our data collectively suggest that NFE2L3 is unlikely to be a crucial promoter of hematopoiesis but may have an effect on erythrocyte-related phenotypes and myeloid differentiation. Considering the roles of other CNC family factors in blood cell differentiation [37, 51, 89], it is possible that the NFE2L3 knockdown alone would not have been sufficient to induce a detrimental phenotype. Appropriate challenges may be required to understand the function of NFE2L3 in hematopoiesis. Perhaps, a better way to answer the question would be to examine the early phases of hematopoiesis as the accumulation of the blasts would be a better indication of the perturbed differentiation. Hence, it would be of interest to determine the number of hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) upon NFE2L3 knockout using flow cytometry [173]. Progenitor assays and transplantation studies can be performed to further support or reject the hypothesis [174, 175].

4.3 Investigating the regulation of NFE2L3 levels in hematopoietic cells

For the past decades, protein kinase C (PKC) has emerged as an important player in adaptive immunity notably through its control of nuclear factor-κB (NF-κB), another crucial player of inflammation and immunity [176]. PKC is a family of serine/threonine kinase enzymes involved in various cellular processes [177]. PKC activity requires an allosteric regulation by the effectors. The well-known cofactors of PKC are phosphatidylserine, diacylglycerol (DAG) and calcium ions (Ca^{2+}) [178], although Ca^{2+} is not crucial for PKC activation. PMA, an analog of DAG, along with ionomycin, a calcium ionophor, are widely used to study PKC signaling pathway. Here, we showed that the protein level of NFE2L3 is induced by PMA, but not by ionomycin.

At least 12 different isoforms of PKC have been identified in mammalian system [179]. PKC isoforms are categorized into 3 major classes, each of which exhibits different pattern of sensitivity to DAG and Ca^{2+} and are thought to be involved in different cellular functions [178, 179]. The classical isoforms (PKC-α, PKC-βI, PKC-βII and PKC-γ) are stimulated by both compounds, while the novel isoforms (PKC-δ, PKC-ε, PKC-θ and PKC-η) are activated by DAG only. On the other hand, the atypical class (PKC-ζ, PKM-ζ and PKC-ι/λ) does not respond to any of them. Based on these differential responses of PKC isoforms to the secondary messengers, we speculate that NFE2L3 activation by PMA is most likely mediated by a novel PKC(s). As PKC-θ has been shown to play a role in T cell activation and proliferation [180], it would be interesting to subsequently assess the effect of NFE2L3 activity on T lymphocyte functions.

Several studies provide evidence for PKC regulation on the transcriptional activity of NFE2L3 homologs. PKC inhibitor staurosporine inhibited NFE2L1 activity [181], suggesting NFE2L1 regulation by PKC. Other studies found that PKC phosphorylates NFE2L2 at Ser-40 and that this is important for the dissociation of NFE2L2 from its cytoplasmic inhibitor KEAP1 [182, 183]. To date, an atypical PKC has been identified to phosphorylate NFE2L2 [184].

However, phorbol esters like PMA activate targets other than PKCs such as MAPK (ERK1/2 and p38) [185, 186], and NFE2L2 activity was shown to be modulated by MAPK. In fact, MAPK ERK1/2 increased NFE2L2-mediated transcription of ARE-containing genes, suggesting the regulation by these MAPK pathways [187, 188] although the impact of such regulation was questioned [189]. The role of p38 MAPK in regulating NFE2L2 activity is debatable since it has been shown to induce and to repress NFE2L2 activity [188, 190, 191]. Interestingly, the

treatment with PKC inhibitors abolished, if not hindered, the induction of NFE2L2 signaling by PMA or *t*-BHQ, whereas this induction remained unaffected by MEK and p38 kinases inhibitors, highlighting the unique involvement of PKC in mediating antioxidant-induced NFE2L2 activity [182, 183]. As for NFE2L1, a study showed that its induction by low-dose radiation is reduced when treated with ERK1/2 inhibitor PD98059, suggesting the positive regulation by ERK1/2 [192]. Therefore, the cross-talk between PMA-induced signaling pathways must be considered when interpreting our data.

In addition to PMA, we also showed that TNFα induces NFE2L3, which is consistent with previous studies in choriocarcinoma cells [17]. NFE2L3 induction at both transcript and protein levels suggests a transcriptional upregulation or increased mRNA stability in response to the treatment. Furthermore, by using two different NF-κB inhibitors, BAY11-7082 and JSH-23, we showed that this induction is likely mediated by NF-κB.

NF-κB family of transcription factors comprise canonical members NF-κB1 p50, RELA (p65), c-Rel and non-canonical members NF-κB2 p52 and RELB [193]. NF-κB denotes a dimeric protein they form and is normally bound by its inhibitor IκB in the cytoplasm. Only when IκB kinase (IKK) phosphorylates IκB for degradation can NF-κB translocate into nucleus to drive its target gene expression. This is known as the canonical, or classical NF-κB pathway. Activation of the non-canonical pathway requires the phosphorylation of p100 by NF-κB-inducing kinase (NIK) and the processing of p100 into p52. BAY11-7082 inhibits NF-κB activation though IκBα phosphorylation inhibition [194], whereas JSH-23 blocks NF-κB nuclear translocation [195]. Based on this information, the observation that both inhibitors impeded NFE2L3 induction by

TNFα suggests that this regulation is likely mediated through the canonical pathway. In the context of immune responses, the canonical pathway is activated by proinflammatory signals and results in the activation of inflammatory genes [196]. On the other hand, the non-canonical pathway is triggered by developmental cues, leading to immune cell differentiation. Taken together, our result suggests that NFE2L3 may be involved in inflammatory and immune responses.

Surprisingly, TNFα induced NFE2L3 protein levels in Jurkat and Namalwa, but not in Raji, hinting at the differential regulation by TNFα between two Burkitt's lymphoma cell lines. A previous work reported the minimal presence of TNF receptor in Raji and the concomitant lack of NF-κB activation in response to ^{125}1-TNF treatment [197], suggesting that the unresponsiveness in Raji cell line may be attributable to the absence of NF-κB activity. Additionally, we observed that the inhibitors of NF-κB decreased the basal NFE2L3 expression in Jurkat and Namalwa, suggesting a constitutive activation of NFE2L3 through NF-κB pathway. Indeed, NF-κB has been shown to be constitutively activated in various hematopoietic malignancies and to contribute to cell survival and proliferation [198].

NF-κB is one of the primary targets of TNFα [199], but is also an important target of PKC signaling pathways in T cell receptor (TCR)-mediated activation of T lymphocytes [200] and B cell receptor (BCR)-mediated activation and survival of B lymphocytes [176, 201-203]. Therefore, it would be interesting to see whether there is a cross-talk between these pathways in regulating NFE2L3. However, since it has been suggested that BAY11-7082 may generally affect the ubiquitin system rather than being NF-κB-specific [204], further analyses such as

shRNA-mediated knockdown of NF-κB subunits are required to validate NF-κB as the upstream regulator of NFE2L3.

In order to further investigate the possible role of NFE2L3 in immune response, we examined NFE2L3 modulation in response to lipopolysaccharide (LPS) stimulation. LPS is a major building block of Gram-negative bacteria wall [158]. This compound binds Toll-like receptor 4 (TLR4) to induce innate immune responses in mammalian cells and can induce sepsis in humans. Despite the various range of LPS concentrations, stimulation time and conditions tested, NFE2L3 expression was not modulated by LPS.

The unresponsiveness observed in Namalwa cells may be explained by the considerably low surface expression of TLR4 in human B lymphocytes [205, 206] in contrast to its high expression in myeloid-originating cells [207]. Indeed, while TLR4 expression can be induced by activating B cells [208], LPS has been shown to be relatively ineffective in stimulating human primary B cells [158, 206]. On the other hand, TLR4 expression has been reported to be upregulated in various B cell malignancies [209]. Together, the data suggests that the LPS stimulation we used to be insufficient to stimulate Namalwa cells, indicating that the lack of NFE2L3 modulation may thus not be used to infer its role in LPS-mediated immune responses. Accordingly, we argue that the presence of TLR4 receptors needs to be confirmed first on each cell line in order to conclude whether NFE2L3 participates in LPS-mediated microbial inflammation.

Although further investigation is required to understand the role of NFE2L3 in inflammation and immune responses, our data show that NFE2L3 can be induced by proinflammatory cytokines, suggesting the possible implication of NFE2L3 in immune responses.

4.4 Concluding remarks

NFE2L3 is a transcription factor whose regulation and function remain largely unknown. To our knowledge, the role of NFE2L3 has not been fully investigated in the context of hematopoietic cells. In the present work, we show that the NFE2L3 mutations identified in DLBCL patient samples do not affect the transcriptional capacity of the protein, but we suggest studying the effect of NFE2L3 overexpression. Our *in vivo* data suggests that NFE2L3 may be involved in erythrocyte-related phenotypes. We also report here the modulation of NFE2L3 levels by PMA and TNFα and further identify NF-κB as a potential upstream regulator. Together, our data provide a rationale for future research deciphering the significance of NFE2L3 in hematopoietic malignancies and immune responses.

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