The role of the inhibitor of \( \kappa \)B kinase alpha (IKK\( \alpha \)) in epigenetic changes mediated by RAR-RXR nuclear receptor-activated transcription

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To my family………

To the souls of my grandparents………

To my beloved people……….
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List of abbreviations

ATRA : All-Trans- retinoic acid
AF : Activation function
AIB1 : a steroid receptor co-activator amplified in breast and ovarian cancer
CBP : CREB binding protein
CREB : cAMP response element binding protein
ChIP : Chromatin immunoprecipitation
DBD : DNA binding domain
ERα : Estrogen receptor alpha
H3 : Histone 3
H3K9 : Histone 3 lysine 9
HATs : Histone acetyl transferases
HDACs : Histone deacetylases
IKKα : Inhibitor of NF-κB Kinase alpha
IκB : Inhibitor of NF-κB
IL-1 & IL-6 : Interleukin 1 and 6
LBD : Ligand binding domain
NCoR : Nuclear receptor co-repressor
NCoEx : Nuclear co-repressor exchange factors
NEMO : NF-κB essential modulator
NES : Nuclear export signal
NF-κB : Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS : Nuclear localization signal
NRs : Nuclear receptors
PCAF : P300/CBP associated factor
PIC : Pre-initiation complex
PTMs : Post-translational modifications
REs : Response elements
SMRT : Silencing mediator of retinoic acid and thyroid hormone
SRC : Steroid receptor co-activator
TAD : Trans-activation domain
TAFs : TBP associated factors
TBP : TATA box binding protein
TGFβ : Transforming growth factor β
Summary

The inhibitor of κB kinase alpha (IKKα) is a component of the inhibitor of κB kinase (IKK) complex in the nuclear factor (NF)-κB signaling pathway. IKKα is central to the activation of the NF-κB pathway in response to different stimuli. However, in addition to its role in the NF-κB pathway, several studies demonstrated that IKKα regulates gene expression in NF-κB dependent and independent manner. In this project, I studied the possible role of IKKα in epigenetic changes mediated by the retinoic acid receptor-retinoic x receptor (RAR-RXR) nuclear receptor heterodimers in the human monoblastic leukemia U937 cell line. I confirmed the results of previous works that all-trans-retinoic acid (ATRA) treatment leads to enhanced expression of RAR-RXR regulated genes ultimately resulting in cell cycle exit. Chromatin immunoprecipitation (ChIP) analysis demonstrated the enrichment of histone 3 lysine 14 acetylation (H3K14ac) and histone 3 serine 10 phosphorylation (H3S10p) at the retinoic acid response element (RARE) site of the ATRA-target gene CD38, but ATRA-induced changes were small and inconsistent with the strong induction of CD38 gene expression. IKKα knockdown did not affect the expression levels of ATRA target genes and the levels of H3K14ac as well as H3S10p at CD38 RARE site. This leaves the door open to investigate the levels of these two modifications at CD38 transcriptional start site (TSS), study the changes of other H3 modifications and analyze more ATRA-target genes. Taken together my results do not provide evidence to support a role for IKKα in the epigenetic changes mediated by the RAR-RXR nuclear receptor in the human monoblastic leukemia U937 cell line.
Introduction

Gene expression

Gene expression is the process by which information from DNA sequences (genes) is used to synthesize functional molecules (gene products), which can be either RNA or proteins. Gene expression takes place in two consecutive steps: transcription and translation. Transcription is the process at which the genetic information stored in the DNA sequence is copied into RNA sequence. Sometimes the RNA molecules represent the final products of gene expression and exert biological functions themselves, such as the RNA molecules in the splicing machinery and in the ribosome. However, RNA molecules function as carriers of the genetic information that cells need to produce proteins. Translation is the second step at which the language of RNA is converted into amino acid language to produce proteins. Gene expression can be regulated at different levels; transcriptional and post-transcriptional levels. However, regulation at the level of transcription is the most important control point since at this step cells take the decision to express a gene or not (Alberts. B. et al. 2008).

Transcriptional regulation requires the action of two systems; cis and trans-acting elements. The cis-acting elements are regulatory DNA sequences to which the trans-acting elements bind in order to modulate gene expression. The trans-acting elements are proteins that bind DNA, such as transcription factors, which can be either general or specific. In addition, some proteins regulate gene expression indirectly by binding to transcription factors themselves. These proteins are called co-regulators, which can activate or repress gene expression (Strachan & Read. 2004).

In multicellular organisms such as humans, the binding of transcription factors, activators, repressors and co-regulators to DNA is not straightforward. The binding of these proteins is impinged by the compact structure of eukaryotic chromatin. Therefore, the problem of DNA packaging into chromatin has to be solved in order to allow the binding of transcription factors to regulate gene expression (Alberts, B. et al. 2008). The nucleosome is the basic unit of eukaryotic chromatin, in which DNA (about 147 bp) is wrapped around a histone octamer. Two subunits of each of the four core histone proteins (H3, H4, H2A and H2B) form the histone octamer. Another histone protein called H1, which lies outside the nucleosome, functions in connecting nucleosome particles with each other and in establishing higher ordered chromatin structures. The core histones are globular proteins except for their N-terminal parts (N-terminal tails), which are unstructured. Histone tails protrude out from the nucleosomes and are targets for different posttranslational modifications (PTMs), which regulate chromatin structure, and hence gene expression (Lewin, B. 2008; Alberts, B. et al. 2008). The study of histone modifications is one part of a new growing field in molecular biology, which is called epigenetics.

Epigenetics and gene regulation

Epigenetic is defined as heritable changes in gene expression that are independent of DNA sequence. Epigenetic information is maintained by three mechanisms: DNA methylation, histone protein modifications and non-coding RNA. These mechanisms work synergistically to signal certain biological functions in response to different stimuli (Strachan & Read. 2004; Lewin, B. 2008; Alberts, B. et al. 2008). DNA methylation and histone PTMs are the widely
studied epigenetic mechanisms. However, I will focus on histone protein, especially H3 protein modifications.

Histone tail PTMs have been linked to many DNA-templated processes such as transcription, replication, recombination and repair. In addition, it is known that human chromatin is divided into two main domains: heterochromatin (closed chromatin) and euchromatin (open chromatin). The formation of these two distinct chromatin domains is also regulated by histone protein modifications (Kouzarides, T. 2007). This indicates that histone modifications are very important regulators of many cellular processes such as differentiation, proliferation and homeostasis. Aberrations in epigenetic mechanisms, including histone tail modifications, may have disastrous consequences, such as cellular transformation (formation of tumors) (Ducasse & Brown. 2006; Lennartsson & Ekwall. 2009).

Histone tails are subjected to different PTMs such as acetylation, methylation, phosphorylation, ubiquitination, and ribosylation. These modifications act together to regulate many DNA-dependent processes especially transcription (Strahl & Allis. 2000; Kouzarides, T. 2007; Latham & Dent. 2007). Histone tails acetylation, methylation and phosphorylation are widely studied modifications due to their crucial roles in regulating gene expression (Figure 1).

**Figure 1**: Histone 3 tail epigenetic posttranslational modifications. a) Different modifications of H3 tail which includes methylation (M), acetylation (A), phosphorylation (P). b) Different H3 modifications and their biological outcome (meaning). The figures show single letter amino acid code and their residue number. R: Arginine, K: lysine, S: Serine. ©2008 From Molecular Biology of the Cell 5E by Alberts et al. Reproduced with permission of Garland Science/Taylor and Francis LLC.

Histone acetylation occurs at lysine residues and it has been proved that histone lysine acetylation is enriched at active euchromatin regions; in other words, histone lysine acetylation is associated with active gene expression (Strahl & Allis. 2000; Latham & Dent. 2007; Berger, L. S. 2007). Histone lysine acetylation level is controlled by the activity of two different enzyme complexes; histone acetyl transferases (HATs) and histone deacetylases (HDACs). HATs catalyze the addition of acetyl group to lysine residues. HATs are parts of
larger protein complexes, called co-activators which are recruited to the promoter regions to enhance gene expression. HAT activity is counteracted by HDACs, which catalyze the removal of acetyl groups. HDACs are part of the co-repressor complexes that inhibit gene expression (Strahl & Allis. 2000; Berger, L. S. 2007).

Another widely studied histone modification is the addition of methyl groups at lysine and arginine residues mainly on H3 and H4. Lysine side chain can be mono-, di-, or trimethylated, while arginine can be mono- or dimethylated. Unlike histone acetylation, methylation is either associated with gene activation or repression depending on the modified residue and the state of methylation (mono-, di-, or trimethylated) (Martin & Zhang. 2005; Kouzarides, T. 2007). The methylation of H3K4, H3K36 and H3K79 is associated with gene activation, while methylation of H3K9, H3K27 and H4K20 signals transcriptional repression. Again specific enzymatic activities regulate the balance between the addition and removal of methyl groups to histone tails (Martin & Zhang. 2005).

Histone 3 phosphorylation has been shown to be associated with transcriptional activation (Latham & Dent. 2007). For example, phosphorylation of H3 at serine 10 (H3S10) promotes H3K14 acetylation, which results in activation of gene expression (Edmondson, G. D et al. 2002). In addition, phosphorylation of H3 at threonine 6 (H3T6) prevents demethylation of H3K4 at promoter regions resulting in enhanced transcriptional activity (Metzger, E. et al., 2010).

In summary, regulation of gene expression is a complex process that does not rely only on the binding of transcription factors and the formation of transcription initiation complex. Epigenetic PTMs of histone proteins play an important role in regulating gene expression. Histone tail modifications modulate the binding of protein complexes that either activate or repress gene expression. Acetylation of H3K9 and H3K14, methylation of H3K4 and H3K36, phosphorylation of H3T6 and H3S10 leads to the formation of an open chromatin complex (euchromatin), enhanced binding of transcription factors and subsequent gene expression. In contrast, H3K9 and H3K14 deacetylation, methylation of H3K9 and H3K27 leads to the binding of protein complexes that catalyze the formation of closed chromatin structure (heterochromatin) leading to gene repression. Fine control of epigenetic modifications is central to gene expression profiles, which regulate many cellular activities, such as proliferation and differentiation. Aberrations in epigenetic modifications disrupt normal gene expression, which might be deleterious to cells, leads to many diseases, cellular transformation and tumorigenesis.

Nuclear receptors

Nuclear receptors (NRs) are ligand-dependent sequence-specific transcription factors that form homo- and hetero-dimers, which are localized in the cytoplasm and/or the nucleus. NRs regulate (either activate or repress) the expression of many genes that play key roles in different cellular processes, such as cell proliferation, differentiation, metabolism, and development (Krauss, G. 2001). Sequencing the human genome has led to the identification of 48 NRs genes, highly indicating the importance of NRs in regulating gene expression (Maglich, M. J. et al, 2001). All NRs share the same structure, all contain a highly conserved DNA binding domain (DBD), a moderately conserved ligand binding and dimerization domain (LBD), two activation function domains (AF1 and AF2), and a variable region that contains the nuclear localization signal (Figure 2) (Krauss, G. 2001; Kishimoto. et al. 2006).
The natural ligands for NRs are fat soluble hormones, which includes the steroid hormones, thyroid T3 hormone, retinoids, vitamin A, D and their derivatives (Krauss, G. 2001; Perissi & Rosenfeld. 2005). NRs are divided into two main groups, based on their cellular localization and the type of their ligands. Type I NRs reside in both the cytoplasm and the nucleus as homodimers. They form the receptors for steroid hormones; hence they are called steroidogenic receptors. Type II NRs are localized in the nucleus as heterodimers and they bind to non-steroidogenic ligands such as thyroid T3 hormone, vitamin, D vitamin A and its retinoid derivatives (Krauss, G. 2001; Kishimoto. et al. 2006).

In principle, NRs ligands diffuse passively through the plasma membrane where they bind to their receptors in the cytoplasm, or utilize transport proteins to pass the cytoplasm reaching the nucleus where they bind to their receptors. In the cytoplasm, ligand binding induces a conformational change in NR’s structure that leads to their activation and subsequent nuclear translocation. In the nucleus, ligand bound NRs regulate the expression of target genes through binding to specific DNA sequences called response elements (REs) (Figure 3) (Krauss, G. 2001; Perissi & Rosenfeld. 2005; Kishimoto. et al.2006). NRs that are localized in the nucleus bind also to their REs in the absence of ligand, which often results in transcriptional inactivation. In general, ligand-free NRs act as repressors, and inhibit gene expression, while ligand-bound NRs act as activators and enhance gene expression (Krauss, G. 2001; Perissi & Rosenfeld. 2005; Kishimoto. et al.2006).

Figure 2: Domain structure of nuclear receptors showing the N and C termini. AF1: activation function domain 1, DBD: DNA binding domain, LBD: ligand binding domain, AF2: activation function domain 2, NLS: nuclear localization signal, NES: nuclear export signal (Nagy & Schwabe.2004). Reproduced with permission of Elsevier Ltd. All rights reserved.

Figure 3: Signalling through nuclear receptors. Nuclear receptor ligands diffuse through the plasma membrane to the cytoplasm, where they either bind to their cytoplasmic localized receptor or transported to the nucleus in order to bind their receptors there. Nuclear receptors regulate gene expression through interaction with the transcriptional machinery.HRE: hormone response element, NR: nuclear receptors, GTFs: general transcription factors, RNA pol II: RNA polymerase II (Kishimoto. et al.2006). Reproduced with permission of Endocrine Journal, the Japan Endocrine Society.
In addition, NRs can regulate gene expression indirectly by binding to other transcription factors. Furthermore, NRs have other non-genomic effects that do not involve gene expression. These reactions are quick, happen within minutes, and they affect signal transduction pathways (Perissi & Rosenfeld. 2005; Devlin, M. T. 2006).

Regulation of gene expression via nuclear receptors

As mentioned above, ligand binding modulates the activity of NRs and thereby gene expression. However, the conformational change induced by ligand binding does not fully explain how NRs regulate gene expression. Regulation of gene expression by NRs involves the recruitment of co-regulators. These enzyme complexes are divided into two main groups; co-activators and co-repressors, which induce and suppress gene expression, respectively (Jenster, G.1998; Kishimoto. et al.2006). Co-regulator enzyme complexes regulate gene expression by modifying chromatin structure through the addition of functional groups to histone proteins, which either enhance or repress transcription. Furthermore, histone modifications recruit other protein complexes, called ATP dependent chromatin remodeling complexes which are responsible for nucleosomal rearrangement (Figure 4).

![Figure 4](image)

Together; histone modifications and nucleosome remodeling regulate gene expression by controlling the accessibility of DNA to different transcription factors and RNA polymerases, preventing or allowing transcription to start (Jenster, G.1998; Kishimoto. et al.2006).

Ligand free NRs preferentially recruit co-repressors through their LXXI/HIXXXI/L consensus motifs in which L represents leucine, X represents any amino acid, I represents...
Isoleucine, and H represents Histidine. Co-repressors utilize these motifs to interact with NRs through the LBD (Jenster, G. 1998; Rosenfeld & Glass. 2001; Nagy & Schwabe. 2004; Perissi & Rosenfeld. 2005). Among the co-repressors recruited by ligand free NRs are the nuclear receptor co-repressor (NCoR) and the silencing mediator of retinoic acid and thyroid hormone (SMRT). These co-repressor complexes have in common a HDAC activity resulting in transcriptional inhibition. In contrast, ligand bound NRs recruit co-activator complexes. The co-activator complexes utilize their LXXLL consensus motifs to interact with activation domains (AF1 and AF2) on NRs. Among the co-activators recruited by NRs are the steroid receptor co-activator-1 (SRC-1), CREB binding protein (CBP) and the adenovirus E1A-binding protein (P300) (Jenster, G. 1998; Rosenfeld & Glass. 2001; Nagy & Schwabe. 2004; Perissi & Rosenfeld. 2005). Co-activator complexes contain HAT activity, leading to the formation of transcriptionally permissive chromatin.

The switch from co-repressors to co-activators is a complex process that does not rely only on ligand binding and the accompanied conformational change of NRs. This switch also involves the utilization of specific proteins, called nuclear co-repressor exchange factors (NCoEx), that function as adaptor proteins for the ubiquitin/19S proteasome complex, thereby mediating the proteasomal degradation of co-repressors and the recruitment of co-activators (Jenster, G. 1998; Kishimoto. et al. 2006).

In summary, NRs modulate gene expression in a ligand dependent manner. Ligand free NRs recruit co-repressors that deacetylate histone proteins leading to chromatin packaging and transcriptional repression. Ligand binding induces a conformational change on NRs leading to the release of co-repressors and the recruitment of co-activators. Co-activators possess a HAT activity that acetylates histone tails. Histone acetylation opens the chromatin structure and consequently leads to transcriptional activation. The switch from co-repressor to co-activators utilizes exchange factors and the activity of proteasomal complexes.

The Inhibitor of ɄB kinase alpha and the NF-ɄB signaling pathway

The inhibitor of ɄB kinase alpha (IKKa) is one component of the inhibitor of ɄB kinase (IKK) complex of the NF-ɄB signaling pathway. The IKK complex consists of three subunits: the IKKa, IKKβ and IKKγ (NF-κB essential modulator NEMO). The IKK complex is central for NF-κB pathway activation in response to different stimuli. IKKa and IKKβ have a high degree of sequence similarity and their kinase activity is needed for NF-κB pathway activation. IKKγ is different from IKKa and IKKβ because it has no kinase activity and it is considered to be a regulatory subunit (Hacker & Karin. 2006).

In normal, non-stimulated cells, the NF-κB is inactive due to the cytosolic sequestration of NF-κB transcription factors by binding to the inhibitor of ɄB (IκB) proteins. This prevents NF-κB transcription factors translocation to the nucleus, where they bind to specific DNA elements termed κB elements, activating gene expression. Upon stimulation, the IKK complex gets activated and phosphorylates the IκB proteins, targeting them for ubiquitinylation and proteasomal degradation. This liberates the NF-κB transcription factors leading to their nuclear translocation, enhancing the expression of target genes (Li & Verma. 2002; Hayden & Ghosh. 2008). The NF-κB pathway is activated in response to different stimuli, including pathogens, stress signals and pro-inflammatory cytokines, such as tumor-necrosis factor (TNF) and interleukin-1 (IL-1). The NF-κB transcription factors family regulates the
expression of many genes such as anti-microbial peptides, cytokines, chemokines, and anti-apoptotic which are central for immune responses (Li & Verma. 2002).

In addition to its essential role in the activation of innate and adaptive immune responses, the NF-κB pathway has been shown to regulate the expression of many genes such as those responsible for cell proliferation, migration, angiogenesis (formation of blood vessels), and survival. Aberrant activation of NF-κB promotes cancer formation (Perkins, D. N.2007; Lee & Hung.2008).

The Inhibitor of κB kinase alpha and regulation of gene expression

In addition to its role in activation of the NF-κB pathway, IKKα plays key roles in regulation of gene expression in NF-κB dependent and independent manners (Hayden & Ghosh. 2008; Lee & Hung.2008). Yamamoto et al (2003) and Anest et al (2003) demonstrated IKKα nuclear localization in mouse embryonic fibroblast (MEF) cells upon TNFα stimulation. Furthermore, they showed that IKKα is required to activate the expression of IκBα, IL-8 and IL-6 (NF-κB target genes) in a TNFα dependent manner, and IKKα gene knockout represses their expression. Both groups demonstrated that IKKα functions as a chromatin modifying enzyme by adding a phosphate group on H3 Ser 10 (H3S10), and IKKα absence abolishes H3S10 phosphorylation. This further substantiates the role of IKKα in inducing the expression of NF-κB responsive genes. Using chromatin immunoprecipitation (ChIP), both groups showed that the kinetics of H3S10 phosphorylation overlaps IKKα recruitment to the promoter regions of NF-κB target genes. Furthermore, Yamamoto et al. (2003) demonstrated that IKKα interacts with CBP leading to H3K14 acetylation, and IKKα knock out MEFs have significant decrease in H3S10 phosphorylation as well as H3K14 acetylation. Taken together, the results from these two studies indicate the crucial role of IKKα in transcriptional regulation of NF-κB target genes.

Furthermore, it has been shown that IKKα regulate the expression of NF-κB target genes by stimulating the exchange of co-repressors for co-activators. Hoberg, E. J. et al. (2004) demonstrated that IKKα enhances the expression of NF-κB target genes by phosphorylating SMRT. Phosphorylation of SMRT by IKKα is a prerequisite for the recruitment of 14-3-3ε molecular chaperon and E2 ubiquitin ligase, which stimulates SMRT nuclear export and proteasomal degradation. This leads to the loss of SMRT and its associated HDAC activity, co-activator recruitment, which subsequently results in transcriptional activation. Using ChIP analysis, they demonstrated an inverse recruitment of IKKα and SMRT to the promoter regions of NF-κB responsive genes. Knocking down IKKα abrogates gene expression due to the persistent SMRT occupancy of the promoter regions.

However, IKKα has been shown to regulate gene expression in NF-κB independent manner. For example, IKKα is required for normal epidermal differentiation. It functions as a cofactor for TGFβ-Smad2/3 signaling pathway independent of Smad4. IKKα function in this system is independent of its kinase activity. The TGFβ-Smad2/3-IKKα pathway regulates the expression of genes that promote cell cycle exit. This includes Mad1 and Ovol1, which encode negative regulators of c-Myc. Ablation of IKKα in keratinocytes leads to cell proliferation and carcinogenesis suggesting a tumor suppressor role of IKKα (Descargues, P. et al. 2008). Furthermore, it has been shown that IKKα controls epidermal barrier and tight junction formation in keratinocytes by regulating retinoic acid target genes expression.
Disruption of IKKα leads to enhanced trans-epidermal water loss, and malformation of tight junction (Gareus, R. et al. 2007).

Moreover, IKKα activates estrogen mediated gene expression independent of NF-κB components in breast cancer cell line, MCF7. IKKα forms a complex with estrogen receptor α (ERα) and AIB1/SRC-3 co-activator at the promoter regions of estrogen responsive genes such as the gene encoding cyclin-D1. IKKα phosphorylates ERα and AIB1/SRC-3 co-activator complex leading to transcriptional activation of cyclin-D1. Knocking down IKKα results in decreased expression of ERα controlled genes. Furthermore, ChIP assays showed a decrease in the levels of H3S10p and H3K14ac in MCF7 cells expressing short interfering RNA (siRNA) against IKKα compared to control MCF7 cells. This is consistent with the function of IKKα as H3S10 kinase, and activation of AIB1/SRC-3 co-activator HAT by IKKα (Park, J-K. et al. 2005).

In addition, it has been shown that IKKα regulates estrogen induced cell cycle progression by regulating the expression of the transcription factor E2F1 (Tu, Z. et al.2006). In this study, Tu. Z. et al. showed IKKα recruitment to E2F1 promoter in MCF7 cell line is estrogen dependent and it is associated with increased E2F1 expression. Knocking down IKKα abolishes E2F1 expression, thereby blocking the cell cycle progression from G1 to S phase. Furthermore, IKKα modulate E2F1 activity by direct protein-protein interaction. IKKα stimulates E2F1 activity by enhancing its acetylation. In this way, IKKα is involved in regulating the expression of E2F1 target genes.

Furthermore, it has been shown that IKKα is recruited in a ligand dependent manner to the promoter regions of all-Trans-retinoic acid (ATRA) target genes in U937 (human acute monoblastic leukemia) cell line. Upon ATRA treatment, IKKα is recruited directly to the RAR-RXR nuclear receptor heterodimers, which regulate the expression of myeloid specific genes in U937. This suggests a role for IKKα in regulating the expression of ATRA responsive genes during the differentiation therapy of the highly proliferative U937 leukemia cell line into terminally differentiated mature macrophages (Kalushkova, A.2008, Uppsala University, Master thesis in Biology).

Taken together, the exact mechanisms by which IKKα regulate gene expression need to be elucidated. From the studies mentioned above, it can be concluded that IKKα can directly modify the chromatin epigenetically by phosphorylating H3S10, which in turn enhances H3K14 acetylation leading to gene activation. Furthermore, IKKα can form complexes with other proteins that function either as transcription factors or co-activators modulating their activity and hence gene expression.
U937 Cell line

The U937 cell line is a human derived monoblastic leukemia cell line that has high proliferative activity and blocked in its differentiation at a pre-terminal stage. The U937 cell line is characterized by a population doubling time of 20-48 hours, and morphologically they resemble monocytes. The U937 cell line has been used for many decades as a model to study the mechanisms of cellular transformation, differentiation and therapy in order to understand the pathogenesis of leukemia (Sundström, C. & Nilsson, K. 1976; Harris & Ralph. 1985). The U937 cell line has been treated with retinoid acid (RA) and its derivative ATRA, cyclic adenosine 3′:5′- monophosphate and 1α,25-dihydroxycholecalciferol in order to induce terminal differentiation. Treatment of U937 cell line with these agents result in the acquisition of monocytic characteristics (terminal differentiation) and cell cycle arrest (Olsson & Breitman. 1982; Olsson, I. et al. 1983). This led to the emergence of new treatment regimes in hematology and the development of the differentiation therapy concept (Gullberg, U. et al. 1986; Petrie, K. et al. 2009). These discoveries have increased our understanding of leukemia, its molecular pathogenesis, treatment and improved survival rate of patients. However, the exact mechanisms by which RA, ATRA and other agents induce U937 differentiation are not well understood.

Quantitative Real Time PCR

Real-time PCR, also known as quantitative PCR (qPCR) is a reliable variation of PCR that is used to quantify the amount of double stranded DNA (dsDNA) generated during the PCR reaction, which is directly proportional to the amount of template DNA used as starting material (Ginzinger, D.G. 2002). qPCR has been used extensively in many applications in molecular biology for research and diagnostic purposes. For example, qPCR is used to study gene expression by measuring the level of mRNA in tissue samples, single nucleotide polymorphism (SNP) genotyping, bacterial and viral infection, and study DNA copy number variation (Syyänen, AC. 2001; Arya, M. et al. 2005). Real-time quantitative PCR can be divided into two main methods (variants) based on their chemistries: the fluorescent probe based method, also known as TaqMan method, and the DNA intercalating based (SYBR green) method (Arya, M. et al. 2005). In my project I used the SYBR green based method.

The intercalating-based method utilizes dyes, such as SYBR green (Figure 5). This method is based on the fact that SYBR green fluoresces (emits light signals), when it gets incorporated into dsDNA. The amount of fluorescent reflects the amount of dsDNA generated during PCR reaction. This method depends on the quality of the dsDNA generated during PCR and its main disadvantage is being nonspecific because the dye can bind to primer dimers, and nonspecific PCR products (Arya, M. et al. 2005). Fluorescent signals are detected in a real time by a sensitive camera, and computer software converts these signals into plots termed amplification plots. In order for a signal to be detected, it should pass a certain threshold called the Ct value. The Ct value reflects the number of PCR cycles at which the emitted signals are greater than the minimum detection level. Ct is inversely related to the amount of the starting template prior the reaction, and Ct values are essential to produce reliable, interpretable and reproducible data (Arya, M. et al. 2005). In every single qPCR reaction, housekeeping genes such as β-actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal RNA (rRNA) genes are used as reference to study the changes in the level of the DNA (or RNA) sequence of interest.
Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a widely used technique to study protein-DNA and protein-RNA interaction in vivo. The technique is based on the fact that formaldehyde treatment of living cells generates cross-links between proteins and DNA, RNA, and proteins that reside close to each other on chromatin (Figure 6). The cross-linked chromatin is sheared by sonication into small fragments of less than 1 kb (Svotelis, A. et al. 2009). These fragments are then incubated with antibodies against the protein of interest, such as transcription factors, histone proteins, modified form of the protein of interest (e.g., acetylated, methylated, or phosphorylated), or epitope tagged protein (Aparicio, O. et al. 2004). After immune selection for protein-DNA interaction, cross-linking is reversed by heating, releasing DNA sequences, which are only bound to the protein of interest. This DNA is used for further analysis such as measuring the percentage of protein bound DNA to a control DNA (protein free DNA) by quantitative real time (qRT-PCR), DNA sequencing (ChIP-Seq) to uncover the sequence that is bound by the protein and for genome-wide studies, and for microarray studies (ChIP-Chip) performing functional studies on the protein of interest (Aparicio, O. et al. 2004; Struhl, K. 2007). Using ChIP, we can detect sequence specific interaction, such as transcription factors-promoter interaction and sequence non-specific interaction, for example, co-activator and/or transcription factors-RNA polymerase interaction. In addition, ChIP aims to indentify the location of modified histone proteins in the genome, histone modifying enzymes, and proteins that bind to modified histones. Therefore, ChIP is the method of choice to study the dynamic protein-DNA interaction, protein-protein interaction on chromatin, and the kinetics of proteins recruitment to gene regulatory sequences during transcription.
Aim of the project

Previous preliminary experiments in our laboratory suggested that IKKα is recruited directly to the retinoic acid receptor-retinoic x receptor (RAR-RXR) nuclear receptor heterodimers in a ligand (ATRA) dependent manner in the U937 cell line. The overall aim of my project was to investigate the role of IKKα in epigenetic changes mediated by RAR-RXR nuclear receptors activated transcription in U937 cell line. To address this, firstly, I sought to describe the dynamic epigenetic changes at ATRA target genes in U937 cell line upon ATRA treatment and correlate this to the expression levels of ATRA target genes before and after ATRA treatment. Then, I wanted to investigate the role of IKKα in epigenetic changes by knocking down IKKα in U937 sublines transfected with lentiviral vectors expressing short hairpin RNA (shRNA) against IKKα. Finally, I wanted to study the effect of IKKα knock down on the expression levels of ATRA target genes in U937 sublines.
Results

Enhanced expression of retinoic acid receptor-retinoic X receptor (RAR-RXR) regulated genes upon ATRA treatment in U937 cell line

To assess the response of RAR-RXR regulated genes (ATRA target genes) to ATRA treatment, I used quantitative real-time PCR to ascertain the effect of ATRA treatment on the expression levels of two ATRA target genes; RetSDR1 and CD38. I treated the U937 cells with 1 µM ATRA for 24 hours, and then extracted the total RNA for reverse transcription synthesis of cDNA. Real-time PCR results revealed an enhanced expression (~100 fold increases) of RetSDR1 and CD38 level in ATRA treated U937 cells compared to the untreated control cells (Figure 7). Actin gene was used as a reference gene. Furthermore, the efficiency of ATRA treatment on induction cell cycle arrest of U937 cell line was confirmed by flow cytometry (FACS) analysis of the cell cycle using propidium iodide (PI) to stain the DNA (data not shown).

![Figure 7](image-url): Fold change in the level of CD38 mRNA (blue) and RetSDR1 mRNA (red) in U937 cell line in response for 24 hr treatment with 1 µM ATRA. Untreated U937 cell line was used as a control cell line. X-axis represents cell treatment with ATRA. The Y-axis represents the fold change in mRNA level. Actin was used as a reference gene. Error bars represent standard deviation of three measurements.

ATRA treatment causes slight increase in the levels of Histone 3 serine 10 phosphorylation (H3S10p) and Histone 3 lysine 14 acetylation (H3K14ac) at the retinoic acid response element (RARE) site of CD38 gene

The 100 fold increase in gene expression of ATRA target genes indicates active transcription, which is associated with epigenetic marks that enhance gene expression. To study the dynamic epigenetic changes of histone marks at ATRA target genes, I decided to run a ChIP experiment to investigate changes in the level of two transcription associated histone
modifications; Histone 3 serine 10 phosphorylation (H3S10p) and Histone 3 lysine 14 acetylation (H3K14ac) at the retinoic acid response element (RARE) of CD38 gene. I treated the U937 cell line with or without ATRA for 24 hrs, then I ran ChIP using specific antibodies against H3K14ac, H3S10p, total H3 as a positive control, and IgG as a negative control. ChIP assay revealed very small increase in the levels of H3K14ac as well as H3S10p at CD38 RARE site (Figure 8). Changes in H3K14ac and H3S10p levels were interpreted as a percent of input DNA.

![Figure 8](image)

**Figure 8**: Changes in the level of H3K14ac and H3S10p at the retinoic acid response element (RARE) of CD38 gene in response to 24 hrs ATRA treatment in U937 cell line (Red). Untreated U937 cell line (blue) was used as a control cell line. The X-axis represents the immunoprecipitation (IPs) using antibodies against H3K14ac, H3S10p, total H3 (positive control), and nonspecific IgG antibodies (negative control). Y-axis represents the enrichment in the levels of acetylation, phosphorylation and H3 levels as a percent of input. The input represents 1/10 of the total genomic DNA used in ChIP and it is used as a reference. Error bars represent the standard deviation of three measurements.

**Induction of IKKα knockdown**

To study the role of IKKα in epigenetic changes mediated by RAR-RXR nuclear receptor in the U937 cell line, I used two previously transfected U937 derived sublines; sublines 19.1 and 4. Subline 19.1 was transfected with lentiviral vector containing a doxycycline inducible shRNA that targets IKKα mRNA, while subline 4 was transfected with a control lentiviral vector, which has shRNA that does not target any known mRNA. I treated the sublines with or without 1µg/ml doxycycline over several time points to study the kinetics of IKKα knockdown (data not shown). Surprisingly, clear knockdown was detected at 168 and 216 hrs, which indicates a slow kinetics in IKKα knockdown. The efficiency of doxycycline induction was assessed by FACS analysis (data not shown). I extracted the proteins and ran a western
Western blot to assess IKKα knockdown. I used antibodies against IKKα, actin as a control, and red fluorescent protein as an internal control to further check doxycycline response. Western blot analysis demonstrated a clear knockdown of IKKα at both time points in the 19.1 subline (Figure 9 A and B). To further validate the knockdown, qPCR analysis was performed on cDNA samples representing RNA extracted at 168 hrs only (due to the loss of RNA during extraction at 216 hrs) to measure the levels of IKKα mRNA. However, qPCR did not reveal any difference in the levels of IKKα mRNA between in both sublines before and after doxycycline treatment (Figure 9 C).

Figure 9: Western blot analysis of IKKα knockdown, in subline 19.1 compared to the control subline 4 treated with or without 1µg/ml Doxycycline (Dox) for (A) 168 hrs and (B) 216 hrs. (C) QPCR analysis of IKKα mRNA levels in treated vs. untreated subline 19.1 (blue). Subline 4 (red) was used as a control subline. X-axis represents subline treatment with doxycycline, while Y-axis represents fold change in mRNA levels. Error bars represent standard deviation of three measurements.
IKKα knockdown did not result in any changes in the levels of H3K14ac and H3S10p at the RARE site of CD38 gene in the silencing 19.1 subline

To investigate the possible role of IKKα on epigenetic H3 posttranslational modifications at ATRA target genes, I studied the effect of IKKα knockdown on the levels of H3K14ac and H3S10p at the RARE site of the CD38 gene. I cultured both 19.1 (the silencing) subline and subline 4 (control subline) with or without 1 µg/ml doxycycline for 168 hrs, and then I cultured each treated subline with or without 1 µM ATRA for 24 hrs. On these samples, I ran ChIP assay to assess the levels of H3K14ac and H3S10p. Total histone 3 was used as a positive control, while nonspecific IgG antibodies were used as a negative control. ChIP assay did not detect any changes in the levels of H3K14ac as well as H3S10p in the silencing 19.1 subline in any combinations of treatment. However, there was a small increase in the levels of H3K14ac in ATRA treated control subline 4 compared to ATRA untreated control (Figure 10).

![Figure 10](image)

**Figure 10**: Effects of IKKα knockdown on the levels of H3K14ac and H3S10p in the silencing subline 19.1. Subline 4 was used as a control subline. Sublines treatments can be uncovered from the figure legend to the right. The X-axis represents the immunoprecipitation (IPs) using antibodies against H3K14ac, H3S10p, total H3 as positive control, and nonspecific IgG as a negative control. The Y-axis represents enrichment relative to the input. Input was 1/10 of the total genomic DNA used in IPs for ChIP. Error bars represent standard deviation of three measurements.

IKKα knockdown did not affect the expression level of ATRA target genes

To evaluate the effect of IKKα on the expression of ATRA target genes in the U937 cell line, I ascertained the expression levels of CD38 and RetSDR1 by qPCR in the silencing subline 19.1 and the control subline 4. I treated the sublines with and without 1 µg/ml doxycycline for 168 hrs, and then I further treated them with and without 1 µM ATRA for 24 hrs. I extracted total RNA for reverse transcription synthesis of cDNA. Quantitative real-time PCR analysis
did not reveal any effect of IKKα knockdown on the expression levels of CD38 and RetSDR1 mRNA in subline 19.1 that expresses silencing shRNA against IKKα. However, subline 4 showed great reduction in levels of CD38 and RetSDR1 mRNA in combined treatment (Figure 11 A and B). Actin was used as a control gene.

A.

B.

Figure 11: The effect of IKKα knockdown on the expression of ATRA target genes (A) CD38 and (B) RetSDR1 in subline 19.1 (blue) and subline 4 (red). Subline 4 was used as a control subline. X-axis represents cell line treatment, and Y-axis represents fold change in mRNA level. Error bars represent standard error of the mean of three measurements.
Discussion

Epigenetic posttranslational modifications of histone proteins have been connected to many DNA-dependent processes, such as transcription, replication, recombination and repair. Histone protein modifications regulate gene expression by promoting the formation of open or closed chromatin structure that activate or repress gene expression, respectively (Kouzarides, T. 2007). Histone proteins posttranslational modifications are carried out by a group of enzyme complexes, called co-regulators, which interact with transcription factors, activators or repressors (Strachan & Read. 2004). Nuclear Receptors is a family of transcription factors that regulate gene expression in a ligand dependent manner. NRs regulate (either activate or suppress) gene expression using several mechanisms which includes: chromatin remodeling (nucleosomal rearrangement), direct interaction with transcriptional pre-initiation complex (PIC), and by recruiting transcription co-regulators that mediate the interaction between the NRs and PIC (Jenster, G.1998). Ligand free nuclear receptors recruit co-repressors that have HDAC activity leading to the formation of closed chromatin structure, thereby inhibiting transcription. Ligand binding stimulates the exchange of co-repressors for co-activators, which have HAT activity that is required for the formation of transcriptionally permissive chromatin (Jenster, G.1998; Kishimoto. et al.2006).

Effect of ATRA treatment on the expression levels of CD38 and RetSDR1 mRNA and Histone 3 posttranslational epigenetic modifications in the U937 cell line

The present study is investigating the role of IKK\(\alpha\) in epigenetic changes mediated by RAR-RXR heterodimers in the human monoblastic leukemia U937 cell line. RAR-RXR heterodimers are well known NRs that respond to ATRA treatment and the genes they regulate are called ATRA target genes (Krauss, G. 2001). Therefore, I decided to start this investigation studying the effect of ATRA treatment on the expression levels of two ATRA target genes; RetSDR1 and CD38 in U937 cell line. ATRA treatment resulted in 100 fold induction in the expression of both genes compared to their levels in untreated control U937 cells. It is known that treatment of the U937 cell line with retinoic acid and ATRA lead to cell cycle arrest and subsequent differentiation into mature monocytes (Olsson, L. I. & Breitman, R. T.1982; Petrie, K. et al. 2009). Cell cycle analysis by FACS revealed that treatment of the U937 cell line with ATRA lead to cell cycle arrest (data not shown).

The great induction in gene expression and cell cycle exit upon ATRA treatment, led me to investigate the dynamic epigenetic changes at the retinoic acid response elements (RARE) of CD38 gene. I was interested in investigating two H3 posttranslational modifications; H3K14ac and H3S10p, which are known to be increased during active transcription (Latham & Dent.2007). I chose these two modifications due to two main reasons; first, histone acetylation and phosphorylation destabilize histone protein-DNA interaction due to the loss of the positive charge of the lysine side chain by acetylation, and the repelling effect of the negatively charged phosphate group (Berger, L. S. 2007; Kouzarides, T. 2007). Therefore, histone acetylation and phosphorylation open up the chromatin leading to enhanced binding of transcription factors, activators, and co-activators, which ultimately result in transcription activation. Second, it has been shown that IKK\(\alpha\) functions as a chromatin modifying enzyme by phosphorylating H3S10 (Yamamoto, Y. et al. 2003; Anest, V. et al.2003) and H3S10p promotes H3K14ac (Edmondson, G. D. et al. 2002). Therefore, these two histone modifications are the best candidates to study the role of IKK\(\alpha\) in my system.
ChIP assay revealed a very small increase in the levels of H3K14ac as well as H3S10p at RARE site in 24 hrs ATRA treated U937 cells compared to untreated cells. These results are not consistent with the great induction (~100 fold) in CD38 gene expression upon ATRA treatment. I speculate that these modifications are needed for basal level of transcription because analysis of the qPCR results (Ct values) can speculate that CD38 gene is expressed at very low levels in ATRA untreated U937 cell. Therefore, enhanced CD38 expression might be associated with other transcriptionally enhancing histone modifications. For example, H3T6p has been recently shown to enhance androgen-dependent gene expression by preventing H3K4 demethylation (Metzger, E. et al. 2010). Consequently, active genes are characterized by increased levels of H3T6p, H3K4me2, and H3K4me3. In addition, it has been shown that H3K14ac is always present at the promoter regions of estrogen responsive genes and the levels of H3K14ac do not change after estrogen treatment. However, the levels H3K18ac, H3K23ac, and H3R17me are increased upon estrogen treatment, which may indicate similar mechanisms with RAR-RXR nuclear receptor (Daujat, S. et al. 2002).

Another possibility is that the basal level of gene expression is maintained by the balance between transcriptional activating and repressing histone marks, and ATRA induced gene expression is associated with the removal (great decrease) in histone repressive marks, such as H3K9me3 and H3K27me3. Therefore, assessment the changes in histone repressive marks should be done. Moreover, ChIP analysis did not include the epigenetic modifications at CD38 transcriptional start site (TSS); therefore, further analysis of histone 3 posttranslational modifications at the TSS should be performed. Furthermore, I think studying the epigenetic mechanisms before and after ATRA treatment requires genes that are not transcribed (switched off) in the absence of ATRA, and only induced (switched on) upon ATRA treatment, which is lacking in my study.

Effect of IKKα knockdown on epigenetic modifications of H3 at the RARE site of CD38 gene

Although ATRA treatment did not lead to a great increase in the levels of H3K14ac and H3S10p at CD38 RARE site in the U937 cell line, I sought to investigate the effect of IKKα on the levels of both modifications hoping to see any difference in the levels of both modifications in the silencing subline 19.1 compared to the control non-silencing subline 4. Studying the IKKα knockdown revealed a slow kinetics in the knockdown because clear knockdown of IKKα at the protein level was detected at 168 hrs induction with doxycycline. In contrast, the levels of IKKα mRNA were similar in both sublines, which indicates that shRNA against IKKα resulted in translational inhibition of the protein, but not mRNA degradation as mentioned in Bartel, P. D. (2004).

ChIP analysis on the silencing subline 19.1 using antibodies against H3K14ac and H3S10p did not detect any differences in the levels of both modifications before and after 24 hrs treatment with 1µM ATRA. Again, I reasoned that this is due to the fact that I am looking at modifications that are required for basal gene expression, but not ATRA induced gene expression. Furthermore, analysis of CD38 TSS and genes that only transcribed upon ATRA treatment should be performed. Therefore, these results cannot rule out the possible role of IKKα in epigenetic changes mediated by RAR-RXR heterodimers. In my study IKKα, which is known to interact directly with the RAR-RXR heterodimers through the LXXLL consensus motifs (Kalushkova, A. personal communication), may not function as H3S10 kinase, but might affect epigenetic changes by regulating the activity of RAR-RXR heterodimers,
enhancing the switch of co-repressor for co-activators (Hoberg, E. J. et al. 2004; Park, J-K. et al. 2005), which necessitate the investigation of other histone marks.

In addition, ChIP assay showed a small increase in H3K14ac in the non-silencing subline 4 upon 24 hrs treatment with 1 µM ATRA compared to the U937 cell line treated similarly with ATRA. This could suggest that the transfection of U937 with the non-silencing lentiviral vector generating subline 4 could have some effects that have made the cells respond differently to ATRA treatment.

Effect of IKKa knockdown on the expression level of ATRA target genes

To evaluate the effect of IKKa knockdown on the expression levels of ATRA target genes, I ascertained the expression of CD38 and RetSDR1 by qPCR in 19.1 and 4 sublines. Analysis of CD38 and RetSDR1 expression levels did not show any difference between the two combined treatments (-Dox +ATRA, and +Dox +ATRA) in subline 19.1. This is may be due to that cells have adapted the knockdown and developed compensatory mechanisms to activate gene expression. These results cannot rule out the involvement IKKa in regulation the expression of ATRA target genes. However, subline 4 which has non-silencing shRNA showed a great decrease in the levels of CD38 and RetSDR1 mRNA in combined treatment, which might be due to doxycycline effects.

Conclusion

In this investigation I confirmed previous works, which demonstrated that ATRA treatment lead to enhanced expression of RAR-RXR regulated genes, which culminates in cell cycle exit and differentiation of U937 cell line. ChIP analysis reported an enrichment of H3K14ac and H3S10Pp at the RARE site at least for the ATRA target gene CD38. However, ATRA induced changes were not so big to explain the great enhancement in gene expression upon ATRA treatment. This necessitates looking at other transcription enhancing histone marks, decrease in the levels of repressive histone marks, analyze CD38 TSS, and more ATRA target genes. Moreover, IKKa did not affect the levels H3K14ac as well as H3S10p at the CD38 RARE site, and also the expression of ATRA target genes. This is may be due to the slow kinetics of IKKa knockdown, which have made the cells adapt the knockdown and developing compensatory mechanisms to enhance gene expression. Overall, the present investigation does not support or exclude the possible role for IKKa in regulating the expression of ATRA target genes in the U937 cell line.
**Materials and methods**

**Cell lines, culture, and treatment**

U937-1 Cell line; a human monoblastic cell line was cultured in RMPI-1640 medium (Sigma-Aldrich, St. Louis, USA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich Cheme, Taulkirchen, Germany), 1% stable L-glutamine, Glutmax, (Invitrogen, CA, USA), and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, USA) in a humidified atmosphere containing 5% CO₂ at 37 ºC. Cell line U937-1 was transfected (electroporation, Amaxa™ Biosystems) with two different linearized lentiviral constructs (Expression Arrest™ TRIPZ™ lentiviral inducible shRNAmir, Open Biosystems) generating two U937-1 sublines, called 19.1 and 4. The vector was engineered in a way that it fuses the red fluorescent protein (RFP) open reading frame with the small hairpin RNA (shRNA) targeting IKKα mRNA. RFP is used as an internal control to check induction efficiency. In addition, the vector contains a puromycin selection marker. Subline 19.1 was transfected with lentiviral vector that expressing a doxycycline inducible shRNA targeting IKKα mRNA. Subline 4 was transfected with a vector (control vector) contains shRNA that does not target any known mRNA sequence. Sublines were maintained in RMPI-1640 medium (Sigma-Aldrich, St. Louis, USA) containing 10% FBS (Sigma-Aldrich, St. Louis, USA), 1% stable L-glutamine, Glutmax, (Invitrogen, CA, USA), 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, USA) and 0.8 µg/ml puromycin (Invitrogen, CA, USA). IKKα knockdown was performed by inducing the expression of siRNA with 1µg/ml doxycycline (Sigma-Aldrich, St. Louis, USA) treatment. Induction of cell differentiation was done by treating cells with 1µM all-Trans-retinoic acid (ATRA) (Sigma-Aldrich, St. Louis, USA).

**Protein extraction and western blot**

Total protein was extracted from cells harvested at 1500 rpm for 5 minutes (min) at room temperature (RT). Cells were washed one time with 1x cold phosphate buffer saline (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl) of pH 7.4, and then centrifuged at 2500 rpm for 5 min at 4 ºC. Cells were lysed on ice for 30 min in a cell lysis buffer (0.1 M Tris-HCl of pH 8.0, 0.15 M NaCl, 1% nonyl phenoxypolyethoxylethanol- NP-40) containing (2x complete EDTA-free protease inhibitor (Roche, Mannheim, Germany), 10 mM NaF, 1mM phenylmethanesulphonylfluoride (PMSF), 1 µM ZnCl₂, 50 µM Na₂MoO₄, 0.1 mM Na₃VO₄ and 1 mM of (DTT ) dithiothreitol). Proteins were separated from cell debris at 12000 rpm for 15 min at 4 ºC. Protein concentration was measured using Bio-Rad protein assay kit following the manufacturer’s protocol (Bio-Rad laboratories, CA, USA). For western blot, 20 µg of protein were loaded into 10% Bis-Tris NuPAGE® Gel (Invitrogen, Persey, UK), then ran for 60 min at 200 Volt in 1x MOPS buffer (50 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 3.5 mM Tris-base and 1.025 mM EDTA) of pH 7.7. Proteins were transferred to a nitrocellulose membrane at 20 Volt for 13 min using iBlot™ dry blotting system (Invitrogen, CA, USA). Membrane was blocked in 5% dry milk solution dissolved in 1%TTBS buffer (25 mM Tris, 150 mM NaCl, 2 mM KCl and 0.1 % Tween) of pH 7.4. Membranes were incubated at 4 ºC overnight with primary antibodies in 5% dry milk against IKKα (Rabbit antibodies, Cell signaling Technology, Inc), RFB (rabbit antibodies, Evrogen JSC, Moscow, Russia) and actin (goat antibodies, Sanat Cruz Biotechnology, CA, USA). Proteins were detected using secondary HRP (horseradish peroxidase) labeled antibodies and the ECL plus western blotting detection system (Amersham Biosciences, Uppsala, Sweden) following the manufacturer’s protocol.
RNA extraction and cDNA synthesis

Total RNA was extracted from whole cells using TRIzol® method (Invitrogen, CA, USA) following the manufacturer’s protocol. RNA samples were resuspended in sterile DEPC pyrogen treated, RNase/DNase free water (Invitrogen, CA, USA). RNA concentration was measured using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Reverse transcription synthesis of cDNA was done on 2 µg of total RNA using SuperScript™ III reverse transcriptase with random primers (Invitrogen, CA, USA) following the manufacturer’s protocol.

Quantitative Real-Time PCR

cDNA (5 ng) was used for quantitative reverse transcriptase real-time PCR (qRT-PCR) of 25 µl total volume. Each independent PCR reaction contains 12.5 µl of Platinum® SYBER® Green qPCR SuperMix-UDG with Rox (Invitrogen, CA, USA), and 0.3 µM of each primer. The qRT-PCR conditions were as follows: 95 ºC for 30 2 min followed by 40 cycles of 95 ºC for 30 sec and 60 ºC for 1 min. The run and analysis were performed on Mx3000P instrument and software (Stratagene, AH diagnostic AB, Skarholmen, Sweden). Primer sequences for CD38 gene: F 5’-CGG GAT CCA TTG AGC AT C-3’ and R 5’-ATC AAT CTT GCC CAG ACT-3’. Primer sequences for RetSDR1 gene: F 5’-ATT TCA AGA GTG GCC AGG TG-3’ and R 5’-GGC CGA GCA ATA CAG GAA T-3’. Primer sequences for IKKĮ gene: F 5’- TTC TTC AGG AGG TGG A-3’ and R 5’-TGC AGT GTT CAA AAG A-3’. Q-PCR values were normalized to actin gene: F 5’-ACT GGA ACG GTG AAG GTG ACA G-3’ and R 5’-GGT GGC TTT TAG GAT GGC AAG-3’.

Chromatin Immunoprecipitation assay

Chromatin immunoprecipitation analysis was conducted using the OneDay ChIP Kit™ (Diagenode, Belgium) following the manufacturer’s protocol. Briefly, U937-1 cells were treated with 0.37% formaldehyde at room temperature for 10 min to crosslink histones to DNA. The cross linking reaction was stopped by adding glycin of 0.125M at room temperature for 5 min. Cells were washed with 1x cold PBS, then resuspended in cell lysis buffer (10mM Tris-HCl pH 8.0; 10mM NaCl; 0.2% NP-40) with protease inhibitors (as in western blot) and nuclei were purified by centrifugation at 2500 rpm for 5 min at 4 ºC. Nuclei were lysed on ice for 10 min using nuclei lysis buffer (MOPS (free acid); 0.5 M EDTA Ph 8.0; NaCl; 1% SDS; 10% NP-40; 10% DOC) with protease inhibitors (concentration as in western blot). Chromatin was sonicated at maximum amplitude for 15 min x 3 times using Bioruptor™ (Diagenode, Belgium) producing fragments of 200-1000 bp length. Chromatin was purified, divided into several fractions and incubated with antibodies at 4 ºC overnight. Antibodies used were: H3K14ac (Millipore™, CA, USA), H3S10p (Millipore™, CA, USA), H3 (Abcam®, Cambridge, UK), and IgG (Diagenode, Belgium) as a negative control. Pure DNA lacking antibodies was used as the input sample (input DNA was 10 times less in volume than the one used for immunoprecipitation). Changes in chromatin modifications were done on DNA by real time qPCR for CD38 gene. The primer pair was designed to span the retinoic acid receptor response element (RARE): F 5’-AGG GTG TCG CAT TTT C-3’ and R 5’-CTA CAC CCT CCC TAC C-3’. PCR conditions were as previously mentioned in qPCR section. Analysis of fold induction over input was calculated using the 2-Delta Delta CT (2-∆∆CT) method (Livak & Schmittgen,2002).
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