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Function of the Inhibitor of Nuclear Factor kappa B Kinase alpha (IKKalpha) for the Nuclear Receptor Mediated Regulation of Cellular Differentiation

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Degree project in biology, Master of science (2 years), 2008

Examensarbete i biologi 30 hp till masterexamen, 2008

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List of abbreviations:

ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
APL	acute promyelocytic leukaemia
AR	androgen receptor
ATRA	all- <i>trans</i> retinoic acid
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CREB	cAMP response element binding
DBD	DNA-binding domain
ER	oestrogen receptor
ERR	oestrogen response region
GR	glucocorticoid receptor
H3K9	histone 3 lysine 9
HP-1	heterochromatin protein 1
IKK	I κ B kinase
I κ B	inhibitor of kappa-light-chain-enhancer of activated B cells
LBD	ligand-binding domain
LXR	liver X receptor
NEMO	NF κ B essential modulator
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NPM	nucleophosmin
NR	nuclear receptors
NUMA	nuclear mitotic apparatus
PBS	phosphate buffered saline
PCAF	p300/CBP-associated factor
PLA	proximity ligation assay
PLZF	promyelocytic leukaemia zink finger
PML	promyelocytic leukaemia
PPAR	peroxisome proliferator activated receptor
PKA	protein kinase A
PKC	protein kinase C
RAR	retinoic acid receptor
RARE	retinoic acid responsive element
RHD	Rel-homology domain
RXR	retinoic X receptor
RXRE	retinoic X responsive element
SBE	Smad-binding elements
SCCs	squamous cell carcinomas
SRC3	steroid receptor co-activator 3
STAT	signal transducers and activators of transcription
TBS	Tris-buffered saline
TNF α	tumour necrosis factor alfa
TR	thyroid hormone receptor

Summary

The differentiation therapy of cancer is based on the concept that malignant cells become arrested in early stages of their maturation, which leads to uncontrolled proliferation. Retinoids were the first differentiation agents to be used in the clinics. The retinoic acid treatment of acute promyelocytic leukemia (APL) has dramatically increased the survival of patients. Still the mechanisms behind the retinoid-induced differentiation remain not fully understood. A link between the retinoic acid effects on APL cells and the NF κ B pathway has been suggested, but the exact role remains to be elucidated. In addition, the inhibitor of nuclear factor κ B kinase α (IKK α) has been shown to regulate directly retinoid induced transcription, where its exact function is still debated. My report aimed to investigate in depth the involvement of IKK α in the transcriptional regulation at the retinoic acid receptor. Using *in situ* proximity ligation assay (PLA), I found IKK α recruitment to the retinoic acid receptor (RAR) in a ligand-dependent manner. Additionally, using western blot analysis I showed that targeting IKK α with small hairpin RNA (shRNA) led to the simultaneous down-regulation of IKK β and I κ B α protein levels, which increased the turnover rate for the NF κ B transcription factor subunit p50. Taken together my results suggested that formation of the IKK complex protects its components from degradation, and that knocking-down one of the proteins results in destabilization of the other subunits in human myeloid cell lines. Furthermore, I found that reduced levels of IKK α , IKK β and I κ B α did not impair the induction of retinoic acid-target genes and the cell cycle arrest caused by all-*trans* retinoic acid (ATRA). In contrast to these I showed that the IKK inhibitor BMS-345541 potently inhibits ATRA-induced transcription even when IKK α and IKK β protein levels are drastically reduced. As a conclusion, though my results are contradictory they suggest a possible function of IKK α for the induction of gene transcription caused by ATRA.

Introduction

Regulation of gene expression

Tight regulation of gene expression is what makes possible the development of a complex organism from a single cell zygote. The more complex the organism, the more strict regulation is required. In mammals, gene regulation is exerted at several interconnected levels starting from the transcriptional regulation. When talking about genes it is important to outline what a gene is. A simple definition of a gene is hard to find, as it has dramatically evolved during the past half-century (Brosius 2009). Nevertheless, a gene can be defined as the entire nucleic acid sequence, necessary for the synthesis of a functional product (Scherrer and Jost 2007). Beside the coding region, different regulatory DNA sequences are required for the tight and controlled synthesis of a particular RNA.

Low levels of basal transcription in eukaryotic cells are driven by the RNA polymerase II (RNA Pol II). To do this the RNA Pol II binds to a regulatory DNA sequence termed promoter. The promoter of an eukaryotic gene extends around 40 bp up- and downstream from the transcriptional start site and contains highly diverse and gene specific elements. In addition to the promoter, the transcription of individual genes is regulated in response to various stimuli through further *cis*-acting regulatory DNA sequences to which *trans*-acting proteins or transcription factors are binding. These *cis*-acting control elements can be located at the transcription start site or up to tens of thousands of base pairs either upstream or downstream (Alberts 2002). Due to such complex arrangement, where transcription from a single promoter can be regulated by multiple transcription factors binding to alternative control elements, gene expression programmes can be finely tuned in response to diverse signals.

Nuclear receptors

Nuclear receptors (NRs) are a class of DNA-binding transcription factors involved in the regulation of gene expression in response to various intracellular and extracellular stimuli. NRs bind DNA through a conserved DNA-binding domain (DBD). Additionally their function is regulated by binding of small molecules termed ligands to a conserved ligand-binding domain (LBD). The activation of nuclear receptors can subsequently lead to both activation and repression of gene expression, which is dependent on the recruitment of additional co-regulatory proteins, co-activators and co-repressors. Often the ligand binding induces conformational changes in the receptor, which lead to the dissociation of co-repressors, recruitment of co-activators and initiation of transcription. However, several nuclear receptors can repress transcription in ligand-dependent manner, either by binding co-repressors or by negatively regulating other transcription factors, termed gene transrepression. Thus, the actual function of a co-regulatory protein is hard to predict, as it is context dependent (Figure 1). (Perissi and Rosenfeld 2005)

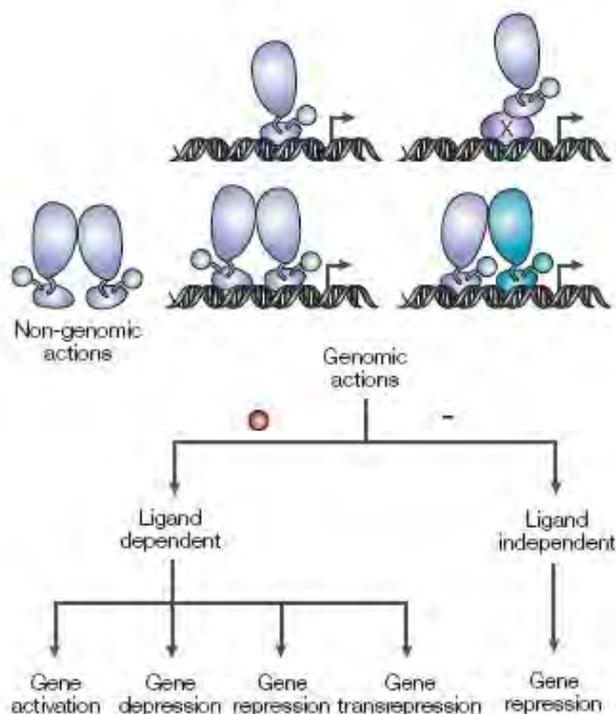


Figure 1. Mechanisms of regulation of transcription by nuclear receptors. Nuclear receptors can function as monomers, homo- or heterodimers, which bind DNA directly or through other transcription factors, “X”. They can exert both genomic and non-genomic actions, which can be ligand dependent or independent and can modulate transcription through gene activation, gene repression or gene transrepression (the negative regulation of transcription through the regulation of other proteins). Adopted from Perissi and Rosenfeld, 2005; with the permission and copyright of the Nature Publishing Group.

Ligand binding to nuclear receptors also regulates the exchange of receptors and co-activators on DNA. The liganded receptor or the associated co-activator are rapidly targeted for degradation, which represents an important feedback loop for the transcriptional regulation in response to diverse stimuli (Salghetti et al. 2000).

The mammalian nuclear receptor family comprises more than 45 transcription factors, which include receptors for steroid hormones, e.g. the estrogen receptor (ER), the androgen receptor (AR) and the glucocorticoid receptor (GR); receptors for non-steroidal ligands, such as the thyroid hormone receptor (TR) and retinoic acid receptor (RAR) and other receptors, which bind ligands from the lipid metabolism, as peroxisome proliferator activated receptors (PPARs) and liver X receptors (LXRs). For many nuclear receptors, called orphan receptors, ligands have still not been identified.

Nuclear retinoic acid receptors

Six different retinoid receptors have been identified so far, RAR α , β and γ ; and retinoic X receptor (RXR) α , β and γ . They have different functions and are expressed in different cell types. The ligands for the retinoid receptors are Vitamin A (all-*trans* retinol) derivatives. All-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid act as high-affinity ligands for RARs. For RXRs only 9-*cis* retinoic acid has a high affinity (Soprano et al. 2004). Since it remains

debated if 9-*cis* retinoic acid has a physiological function, the most important physiological ligand for the RAR-RXR heterodimer is ATRA.

RARs function as heterodimers with RXRs, where RXRs exist as homodimers and can additionally form heterodimers with other nuclear receptors, such as the peroxisome proliferator activated receptors (PPARs) and the liver X receptors (LXRs). Retinoid receptors are bound to two direct repeats of the consensus half-site DNA sequence “(a/g)g(g/t)tca”, which are separated by two or five “spacer” nucleotide direct repeats (DR2 and DR5, respectively) for the retinoic acid responsive elements (RAREs), or with one nucleotide (DR1) for the retinoic X response elements (RXREs) (Balmer and Blomhoff 2005).

In the literature, the list of genes reported as regulatory targets of retinoic acid is constantly expanding. However, for a major part of these genes, RAREs or RXREs have not been identified and the mode of gene regulation is considered dependent on intermediate transcription factors, non-classical association of receptors and more distant mechanisms (Blomhoff and Blomhoff 2006).

Additionally the function of the RARs and RXRs is regulated by phosphorylation. The N-terminal ligand binding domain and the DNA binding domain at the C-terminus are phosphorylated by protein kinase A (PKA) and protein kinase C (PKC), respectively, as well as a number of cyclin-dependent kinases, mitogen-activated kinases and Jun N-terminal kinases (Rochette-Egly et al. 1997; Bastien et al. 2000; Gianni et al. 2002). This leads to signalling integration from several different pathways at the RAR/RXR heterodimer, as a consequence of which different co-activator and co-repressor molecules can be recruited to determine the final outcome on the transcriptional regulation of retinoid-target genes.

Leukemia

Cancer is currently considered to be a genetic disease resulting from the accumulation of multiple mutations that abrogate the function of genes, which affect the fine balance of cell proliferation, differentiation and apoptosis (Vogelstein and Kinzler 1993). The Cancer Genome Project (<http://www.sanger.ac.uk/genetics/CGP/Census/>) assigns more than 1% of the human genome as predisposing to cancer via different mutations. For several reasons, e.g. access to pure tumour cells and often less complicated karyotypes than in solid tumours, about 70% of these genes have been associated with leukaemia and lymphoma (Futreal et al. 2004).

Leukaemia and lymphoma are blood cancers. A result of leukaemia is the accumulation of aberrant blast cells (progenitor blood cells), which are characterized by their proliferative capacity and lack of ability to differentiate. Leukaemia often arises from chromosomal translocations that involve the immunoglobulin heavy-chain locus and lead to protein over-expression or translocations generating fusion genes resulting in proteins with novel abnormal functions. (Ikeda et al. 2006) The most frequent players in the translocations appear to be transcription factor genes, which lead to the deregulation of their targets and the abrogation of the cellular pathways they control (Rabbitts 1994, Look 1997, Greaves and Wiemels 2003).

Acute leukemia is defined as a rapidly progressing blood cancer. Acute leukemias include AML (acute myeloid leukemia) and ALL (acute lymphocytic leukemia), which are

distinguished based on the haematopoietic cell lineage from which the leukemic clone is derived (Figure 2).

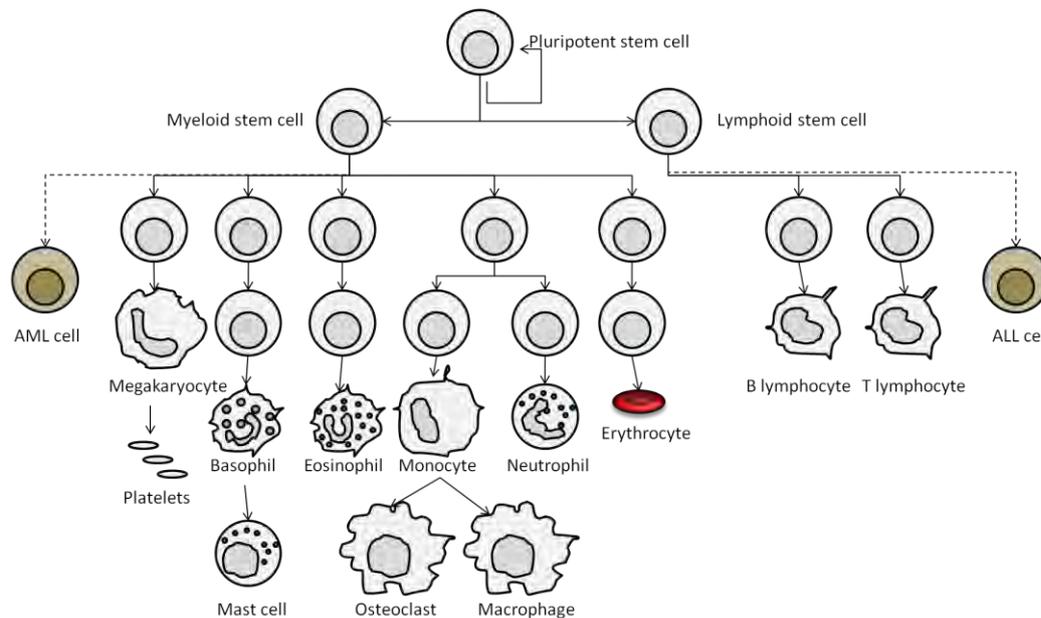


Figure 2. Blood cell differentiation and lineage specific development of acute leukaemia. In light grey – white blood cells (leukocytes), in red – red blood cells (erythrocytes). Abbreviations: AML – acute myeloid leukaemia, ALL – acute lymphocytic leukaemia. The figure is a property of Kalushkova A. after http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_Ch09/fig9_21.jpg.

Acute promyelocytic leukemia

Acute myeloid leukemia (AML) is a common term for all leukaemias of a myeloid origin. About 10% of all AML develops from cells arrested in their promyelocytic stage of the differentiation and thus is called acute promyelocytic leukemia (APL). APL affects young adults and both sexes equally. Symptoms include fatigue, anemia, infections, tendency to bleed and low levels of granulocytes, monocytes and platelets (Groopman and Ellman 1979).

The most common cause of APL is the balanced chromosomal translocation $t(15;17)(q22;q21)$. This generates a fusion between the promyelocytic leukaemia (PML) gene, which codes for a growth suppressing transcription factor, and the retinoic acid receptor- α (RAR α) gene, coding for a nuclear receptor involved in the regulation of myeloid differentiation (Kakizuka et al. 1991). The PML-RAR α fusion de-localizes PML, but retains the DNA binding capacity of RAR α at retinoic acid target genes. The fusion protein also tends to oligomerize, which ultimately leads to deregulation of both PML and RAR α targets. The oligomerized PML-RAR α has higher affinity for histone deacetylases (HDAC3) and in addition recruits the histone H3K9 methylase SUV39H1 (Minucci and Pelicci 2006) and DNA-methyl transferases to RAR α target genes (Di Croce et al. 2002).

Other translocations in APL involve alternative partners of RAR α such as the promyelocytic leukemia zinc finger protein in the generation of PLZF-RAR α ($t(11;17)$) (Chen et al. 1993), nucleophosmin (NPM), nuclear mitotic apparatus (NUMA) and STAT5b (Redner 2002). An additional abnormality observed is trisomy of chromosome 8 in 16% of the cases (Grimwade et al. 2000).

For the first time in the early 1980's a vitamin A derivative (13-*cis* retinoic acid) was clinically applied as a differentiation agent for the treatment of APL (Flynn et al. 1983; Fontana et al. 1986). In 1988 the physiological isomer all-*trans* retinoic acid (ATRA) was introduced (Huang et al. 1988). In clinical treatment, ATRA quickly substituted the 13-*cis* isomer inducing differentiation of APL-blasts 10-fold more efficiently (Chomienne et al. 1990). About 90% of patients with primary APL achieve complete hematological remission in 4-5 weeks when treated with pharmacological doses of ATRA alone (45 mg per m² body surface area per day) (Huang et al. 1988; Castaigne et al. 1990). However, patients in relapse develop resistance to ATRA, so that ATRA alone is not able to induce a second remission (Warrell 1993). In such cases different combinations of ATRA and additional chemotherapy have been proven effective (Kazunori 2007). To induce differentiation, all-*trans* retinoic acid is thought to activate the RAR/RXR-dependent transcription, which allows the cell to turn on their differentiation programmes. Previously an early lethal disease, APL is currently one of the AML subtypes with the best curing rate.

The nuclear factor κ B pathway

Twenty years of research and about 30 000 published articles outline the involvement of the nuclear factor κ B (NF κ B) pathway in the control of numerous physiological responses and, ultimately, the cell fate.

In mammals, there are five distinct NF κ B transcription factor members, RelA (p65), RelB, c-Rel, p50 (from the precursor p105) (NF κ B1) and p52 (from the precursor p100) (NF κ B2), which function as homo- or heterodimers and relate to each other through a highly conserved Rel homology domain (RHD). The RHD contains the nuclear localization signal, mediates dimerization and the binding to the DNA κ B elements in the promoters of target genes. (Häcker and Karin 2006, Perkins 2007, Hayden and Ghosh 2008)

In the absence of NF κ B activation, the NF κ B transcription factors are retained in the cytoplasm through the association with a member of the I κ B family (I κ B α , I κ B β , I κ B ϵ , I κ B γ and Bcl-3) of inhibitory proteins, or inhibition through an integral part of the transcription factor (p105 and p100), which masks the nuclear localization and DNA-binding sequence. Upon NF κ B activation the I κ B kinase (IKK) complex phosphorylates serine 32 and serine 36 (S32 and S36) on I κ B proteins, which targets them for proteasomal degradation. The release of the NF κ B transcription factors from the I κ B inhibitor unmasks their nuclear localization signal and leads to immediate NF κ B nuclear translocation and binding to promoters of target genes. (Häcker and Karin 2006, Perkins 2007, Hayden and Ghosh 2008)

The IKK complex is composed of two catalytic subunits IKK α and IKK β , and a regulatory subunit NF κ B essential modulator (NEMO, also called IKK γ). IKK α and IKK β share 52% of primary sequence identity and a highly conserved (65%) catalytic domain near the N-terminus. NEMO lacks a kinase domain and is not related to IKK α and IKK β . IKK α and IKK β form heterodimers and both can directly phosphorylate S32 and S36 on I κ B α . NEMO has a structural and regulatory role and it is supposed to mediate interactions with upstream activators of IKK. (Perkins 2007)

Initially the NF κ B pathway was reported as a mediator of the immune responses, since it is activated by bacteria and viruses and NF κ B target genes code for inflammatory cytokines,

chemokines, immune receptors and cell surface adhesion molecules. However, the list of physiological responses controlled by the NF κ B has continued to grow and currently NF κ B target genes are implicated in apoptosis, numerous stress conditions, cell adhesion, proliferation, immune responses and tissue remodelling. The activation of the NF κ B pathway is now considered as a common way for cancer cells to escape apoptosis (Hayden and Ghosh 2008). Deregulation of the NF κ B pathway is also observed in chronic inflammatory diseases and atherosclerosis (Baeuerle and Baltimore 1996, Perkins 2007).

In contrast to the NF κ B ability to promote proliferation, ATRA treatment in an APL-derived cell line leading to cell cycle arrest and differentiation has been reported to result in increased NF κ B-regulated gene expression (Altucci et al. 2001). In the same model, the ATRA induced NF κ B pathway was suggested to inhibit apoptosis and prolong the life span of the differentiated cells (Mathieu et al. 2005). Additionally, TNF α , a major inducer of the NF κ B, and ATRA show a complex synergy, which is able to overcome the differentiation block also in ATRA-resistant APL cells (Witcher et al. 2003; Witcher et al. 2004)

The Inhibitor of nuclear factor κ B kinase α

Despite their high structural similarity, IKK α and IKK β appear to have nonredundant function. While both IKK α and IKK β are crucial for I κ B phosphorylation (Adli et al. 2010), IKK α alone has distinct nuclear functions.

IKK α was first shown to accumulate in the nucleus and bind to the promoter regions of NF κ B-regulated genes upon stimulation with TNF α , in order to promote NF κ B-directed gene expression and thus survival, by Anest et al. 2003 and Yamamoto et al. 2003. They reported histone H3 on serine 10 as a novel substrate for IKK α phosphorylation, which placed IKK α among the molecules able to modify chromatin. IKK α phosphorylation of histone H3 on serine 10 leads to the removal of the HP-1 repressor and the recruitment of the CREB-binding protein (CBP) and RelA on NF κ B-responsive promoters to acetylate histone H3 on lysine 14 and promote transcription. Another study indicates that IKK α phosphorylates the nuclear receptor co-repressor SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), which targets it for degradation and paves the way for the recruitment of transcriptional activators such as RelA/p65, p300 and RNA polymerase II on NF κ B-regulated promoters (Hoberg et al. 2004; Hoberg et al. 2006)

Furthermore, IKK α is needed for the estrogen-regulated gene expression and thus for the estrogen-driven cell cycle progression through cyclin D1 and Myc. IKK α was shown to form a complex with ER α and the steroid receptor co-activator 3 (SRC3) on ER α -responsive promoters and phosphorylate them in response to estrogen to increase their transcriptional activity (Park et al. 2005). Recently a novel mechanism for IKK α regulated estrogen-induced cell cycle progression also has been identified. Upon estrogen stimulation, IKK α regulates the transcription from the transcription factor E2F-responsive promoters through recruitment of transcription factors and co-activators to E2F and the estrogen response region (ERR) to modulate the response to estrogen. IKK α also potentiates p300/CBP-associated factor (PCAF) -mediated acetylation of E2F1. Thus, it appears that IKK α regulates the estrogen-induced cell cycle at multiple levels (Tu et al. 2006).

Further recent studies have reported IKK α acting as an oncogene through the canonical NF κ B pathway in both liver and breast cancer (Merkhofer et al. 2009; Jiang et al. 2010). In promoting tumorigenesis, IKK α was also suggested to act through the non-canonical NF κ B pathway as a CBP kinase which upon TNF α stimulation phosphorylates the co-activator, resulting in its dissociation from the tumour suppressor p53 and binding to p65 (Huang et al. 2007).

The studies described so far all suggest that IKK α is needed for the cell cycle progression in response to diverse stimuli. In contrast, some recent reports point towards IKK α as being a tumour suppressor needed for the cell cycle exit and differentiation.

Four groups report mice knock-out studies suggesting a NF κ B-independent IKK α function in the development and differentiation of the epidermis, development of the mammary gland and B-cell maturation. Moreover, IKK α function for the terminal differentiation of keratinocytes seems to be independent of its kinase activity. (Hu et al. 1999; Li et al. 1999; Takeda et al. 1999; Sil et al. 2004) IKK α was suggested to regulate retinoic acid-induced transcription in keratinocytes needed for the appropriate epidermal formation by binding to promoters of RA-target genes (Gareus et al. 2007). Recently, IKK α was also suggested to serve as a nuclear co-factor for the TGF β -regulated transcription factors Smad2/3 in the epidermis, where it associates with Smad2/3 at Smad-binding elements (SBEs) of target genes. In contrast to the involvement of IKK α in ER signalling, IKK α here was found to positively control the expression of Myc negative regulators induced by TGF β (Descargues et al. 2008).

Reduced expression levels and mutations in the IKK α gene in aggressive cutaneous squamous cell carcinomas (SCCs) of human and mice have been identified recently, and IKK α was suggested to play a crucial role in regulating the G₂/M cell cycle checkpoint. In this study IKK α was shown to regulate transcription in an epigenetic fashion and independent from its kinase activity, shielding gene promoters from hypermethylation (Park et al. 2007; Zhu et al. 2007).

Taken together, the exact function of IKK α in controlling the cell fate still remains unclear, but what is now evident is that IKK α has a pivotal role in regulating complex cellular responses in both normal development and malignancy.

Protein detection by proximity ligation

The method of proximity ligation takes advantage of the more accessible and well developed DNA analysis to locate and further analyse proteins. In this method antibodies are used as affinity probes to detect the protein or proteins of interest. Furthermore, oligonucleotide probes are attached to the antibodies and through ligation of their free ends by DNA ligase, protein detection is converted to easier DNA detection (Gustafsdottir et al. 2005). During the last few years the proximity ligation technique has been modified for protein detection in various conditions and the DNA recognition can be carried out through several different well established methods, such as quantitative real-time PCR and *in situ* hybridization (Gustafsdottir et al. 2005; Söderberg et al. 2006).

Using *in situ* proximity ligation assay (*in situ* PLA) one can detect proteins in their native form in the cell, to catch them at their site of function. In this form of the proximity ligation method the oligonucleotide probes are ligated with an additional connector DNA to create a circular DNA molecule. This circular DNA molecule is then amplified using a proximity probe sequence as a primer with a special DNA polymerase through rolling circle amplification (RCA) to generate a blob of multiple copies of complementary DNA, which can subsequently be detected by *in situ* hybridization (Figure 3, Söderberg et al. 2006).

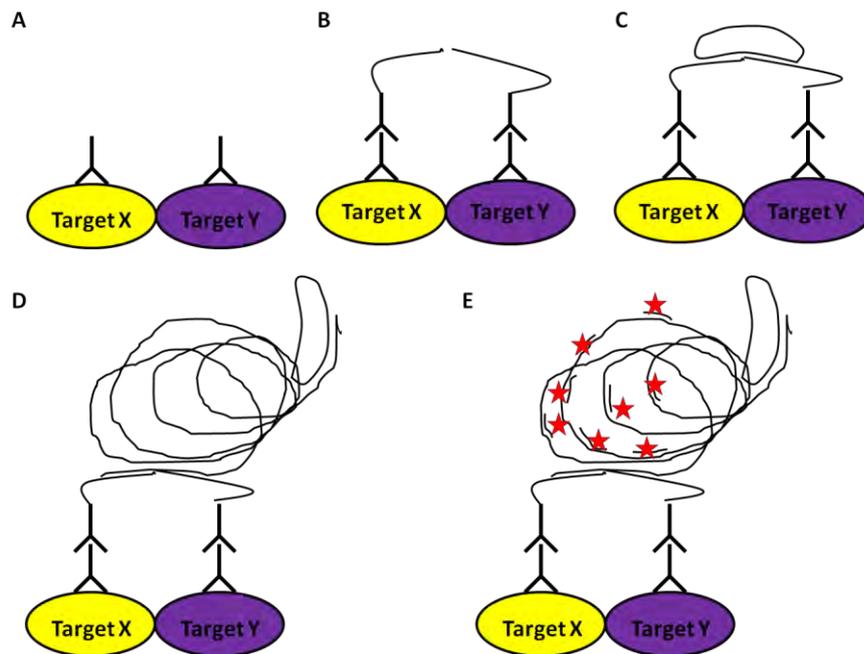


Figure 3. Schematic representation of the proximity ligation assay (PLA). A: The endogenous interacting target proteins (target X and target Y) are detected by primary antibody recognition. B: The primary antibodies are then detected by corresponding secondary PLA probes with attached oligonucleotides. C: When the two oligonucleotides are in close proximity they are enzymatically ligated to a subsequently added connector probe. D: Rolling circle amplification (RCA) is then initiated using one of the oligonucleotide probes as a primer and a specific DNA polymerase. E: The generated blob of DNA is detected by hybridization of fluorescently labelled complementary oligonucleotides. The figure is a property of Kalushkova A. after Söderberg et al. 2006.

RNA interference

RNA interference (RNAi) is a system for downregulation of gene expression that is governed by sequence complementarity to mRNA (Agrawal et al. 2003). Gene regulation by the RNAi system is very complex. In brief, endogenous microRNAs are transcribed by RNA polymerase II as capped and polyadenylated stemloop primary transcripts (pri-miRNAs). If these are artificially delivered into the cell and transcribed from a plasmid, the stemloop transcripts are called small-hairpin RNAs (shRNAs). The stemloop structure is then recognized and cut by two enzymes with endonuclease activity, Drosha and Dicer, which generates double-stranded approximately 22 nt long RNA duplexes (small interfering RNAs, siRNAs or micro RNAs, miRNAs). One of the two strands of the double-stranded RNA duplex is integrated into an RNA-induced silencing complex (RISC) and guides it to its complementary mRNA sequence, which ultimately leads to gene expression silencing (Figure 4, Cullen 2005). The silencing can be induced by cleavage of the mRNA by the Argonaut proteins, which are a part of the RISC complex and possess endonuclease activity. Additionally, silencing can occur through translational block caused by the RISC complex.

Complementarity of the siRNA to a genomic sequence may additionally cause heterochromatin formation and pre-transcriptional gene silencing (Matzke and Birchler 2005). The RNAi has been successfully used in the past years to define function of genes in cell lines where knock-down experiments are difficult to perform. In cell lines the shRNAs targeting desired gene sequences can be expressed from viral vectors and subsequently processed by the endogenous cellular RNAi system. Another way is to transfect cells with siRNAs targeting specific genes, which are then integrated into the RISC complex (Agrawal et al. 2003).

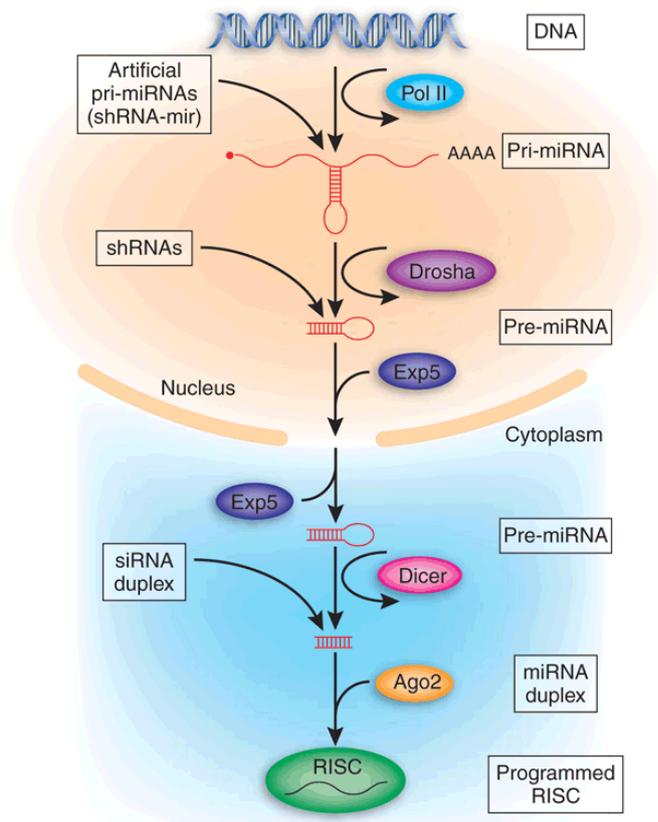


Figure 4. Schematic representation of the RNA interference (RNAi) pathway. The stemloop structure of microRNAs transcribed from microRNA genes in the human genome or artificially delivered to the cell are recognized and cut by two enzymes with endonuclease activity, Dicer and Drosha. This generates double-stranded approximately 22 nt long RNA duplexes, which are integrated into the RNA-induced silencing complex (RISC) and guide it to its complementary mRNA sequence, which ultimately leads to gene expression silencing. Adopted from Cullen 2005; with the permission and copyright of the Nature Publishing Group.

Tetracycline-controlled transcriptional activation

Tetracycline-controlled transcriptional activation is a method for regulation of gene expression by the antibiotic tetracycline or its derivative doxycycline. For this to be possible expression of certain factors from viral vectors introduced into the cell is needed. The tetracycline transactivator (tTA) fusion protein binds to tetracycline response element on DNA at tetracycline operator sequence, which when situated in a gene promoter will regulate its expression. The promoter is off (“tet-off” system) when tetracycline or doxycycline binds to the tTA protein preventing its binding to the *tet*-operator. In the opposite manner the tetracycline transactivator can be modified (called rtTA) so that it binds to the *tet*-operator only in the presence of tetracycline or doxycycline. This system is used to activate gene

expression and is called tet-on (Sprengel and Hasan 2007). When the tet-on system is used to induce the expression of a shRNA from a viral vector introduced into the cell, it will ultimately lead to the downregulation of the endogenous gene to which the shRNA is complementary.

SYBR Green real-time quantitative polymerase chain reaction principle

Polymerase chain reaction (PCR) is a sensitive method in which as little as one copy of sequence can be detected using specific primers. The real-time quantitative PCR is highly reliable and reproducible PCR in which the PCR product generated during each cycle of the amplification is measured as being directly proportional to the amount of initial template (Arya et al. 2005).

The SYBR Green real-time quantitative PCR is a PCR method using the specific chemistry of the DNA-binding dye called SYBR Green. SYBR Green binds to the minor groove of double stranded DNA in a nonsequence-specific manner. When bound to DNA SYBR Green is fluorogenic. In this way, with each cycle of DNA amplification more SYBR Green becomes bound to the newly synthesized DNA product and the fluorescence increases proportionally to the increased amount of DNA (Arya et al. 2005). When the template is amplified an amplification plot is generated (Figure 5), which integrates the detected fluorescence at each amplification cycle. The cycle where the amplification plot of a sample is exponential is considered as a threshold of the amplification efficiency and is called sample ct (cycle threshold) value. The ct values of the different samples can then be used to calculate template concentration. Since template with low concentration will generate enough product to reach the threshold at the later cycles of amplification, higher ct values correspond to lower template concentration. One of the most often used methods to interpret qPCR results is the delta delta ct method ($\Delta\Delta ct$). This method is based on the assumption that the target gene and the reference gene are amplified with the same efficiency, and the treatment used does not alter the expression of the reference gene, but only the target (Scheffe et al. 2006). The $\Delta\Delta ct$ is calculated as following: $\Delta ct \text{ target gene (for each treatment)} - \Delta ct \text{ reference gene (each treatment)} = \Delta ct \text{ target gene treated sample} - \Delta ct \text{ target gene untreated sample} = \Delta\Delta ct$. The final value to be plotted in a graph is calculated as a relative fold-change = $2^{-\Delta\Delta ct}$.

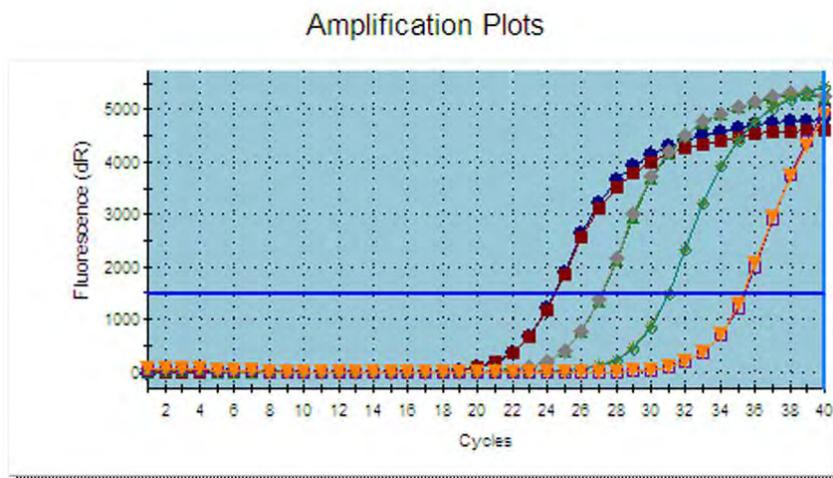


Figure 5. An example of real-time quantitative PCR amplification plots using SYBR green of samples with different initial template concentration run in duplicates. The blue line represents the threshold where the amplification of all samples is in its exponential range. The cycle where the amplification plot of a sample crosses the threshold line is called sample ct (cycle threshold) value and is subsequently used to calculate

template concentration. Since template with low concentration will generate enough product to reach the threshold at the later cycles of amplification, higher ct values correspond to lower template concentration. The Figure represents personal data.

Cell cycle analysis using propidium iodide

Propidium iodide (PI) is fluorescent dye that intercalates in double-stranded DNA and RNA and is an analogue of ethidium bromide. It is excited by 488 nm laser light and emits fluorescence at 562-588 nm. PI increases its fluorescence when bound to DNA or RNA. Cells incubated in a hypotonic solution are easily stained by PI without losing the integrity of their nuclei. When stained with PI, the cellular DNA content of each cell can be quantified using fluorescence-activated cell sorting (FACS) and percentage of cells in the different phases of the cell cycle calculated (Krishan 1975, Nunez 2001).

The FACS analysis is based on the flow cytometry technique in which cells in suspension are passed through a very thin fluid column one by one, so that they can be subsequently detected by an electronic apparatus and sorted according to different characteristics. In the case of fluorescence-activated cell sorting (FACS), cells are characterized based upon light detection of the fluorescence they emit. Various techniques for cell staining using fluorescently labelled antibodies or DNA incorporating dyes, such as PI, exist (Cunningham 2010).

Aim of the project

The overall aim of this project was to assess the importance of the IKK α activity for the RARE mediated transcription upon induction with retinoic acid. For this purpose I specifically aimed to determine whether IKK α is recruited to RAR/RXR heterodimer *in vivo*. Secondly, to generate IKK α knock-down myeloid sub-lines using shRNA to be able to evaluate the impact of IKK α suppression for the differentiation and cell cycle arrest of myeloid cell lines upon ATRA stimulation. And last, I aimed to evaluate the mechanisms behind the IKK α function on retinoic acid regulated promoters.

Results

IKK α is recruited to the RAR/RXR heterodimer after ATRA stimulation

According to previous studies IKK α is recruited to the promoters of RA-target genes after 15 – 30 min of ATRA treatment (Tshuikina M. personal communication). Considering this I aimed to investigate in depth the interaction between the kinase and the nuclear receptor heterodimer RAR/RXR. I used *in situ* PLA to detect IKK α recruitment to the RAR/RXR receptor heterodimer in a ligand dependent manner. Human fibroblast U3A cells were treated with 10^{-6} M ATRA for 15 min and independently with 4 μ M of the IKK inhibitor BMS-345541 for 15 min and 10^{-6} M ATRA for additional 15 min. After fixation, protein interactions were detected with primary antibodies against IKK α + RAR α , IKK α + RXR β , IKK α + IKK β and RAR α + RXR β (Figure 6). The results showed that IKK α was recruited to RAR α after 15 min of ATRA treatment (Figures 6D and 6E) and the subsequent treatment with BMS-345541 inhibited this interaction (Figure 6F). As a confirmation to this, an

interaction between IKK α and the RAR α heterodimer RXR β was observed in a similar manner and the results were evaluated quantitatively (Figure 7A and 7B). To control the assay specificity a cytoplasmic interaction between IKK α and IKK β (Figure 6A) and a nuclear interaction between RAR α and RXR β (Figure 6B) were used as positive controls. As a negative control, the assay was performed in the absence of primary antibodies (Figure 6C). Taken together these results indicate a ligand dependent interaction between the kinase IKK α and the nuclear retinoic acid receptor heterodimer RAR α /RXR β .

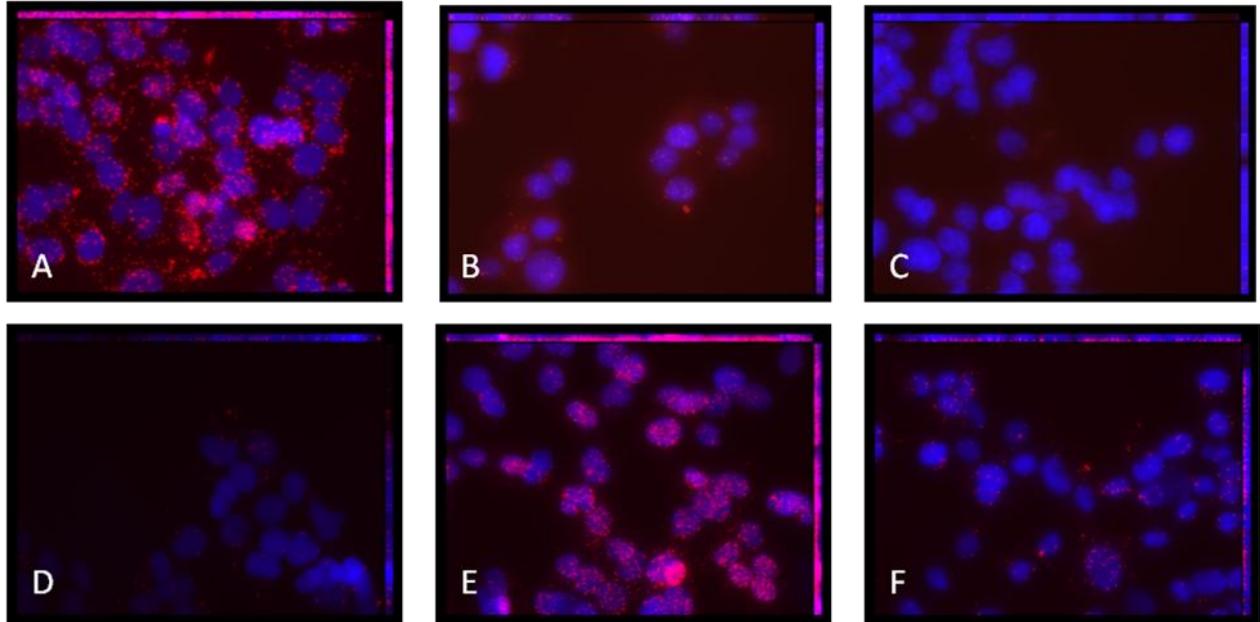


Figure 6. *In situ* detection of protein-protein interactions by proximity ligation assay. Human fibroblast U3A cells were treated with 10^{-6} M ATRA for 15 min and independently with 4 μ M of the IKK inhibitor BMS-345541 for 15 min and 10^{-6} M ATRA for additional 15 min. Upper panels (A, B and C) consist of control experiments. A: antibodies against IKK α and IKK β ; B: antibodies against RAR α and RXR β ; C: negative control without primary antibodies. Lower panels (D, E and F) show analysis of IKK α recruitment to RAR α . D: antibodies against IKK α and RAR α in untreated cells; E: antibodies against IKK α and RAR α after 15 min of 1 μ M ATRA treatment; F: antibodies against IKK α and RAR α when cells are treated first with 1 μ M BMS-345541 for 30 min and then 15 min with 1 μ M ATRA.

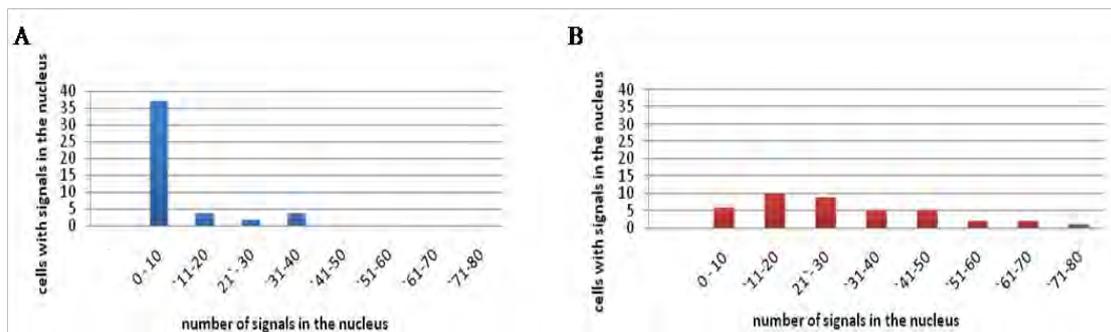


Figure 7. Quantification of *in situ* detection of protein-protein interactions by proximity ligation assay. Human fibroblast U3A cells were (A): left untreated or (B): treated with 10^{-6} M ATRA for 15 min. In both (A) and (B) antibodies against IKK α and RXR β were used. The rolling circle amplification (RCA) products were manually quantified after the *in situ* PLA.

Establishment of IKK α knock-down sub-lines

The efficiency of different small hairpin RNAs (shRNAs) to down-regulate the human IKK α protein levels was previously evaluated (Kalushkova A., Course report in Advanced techniques in molecular medicine, 2007, Uppsala University). Based on the previous results I selected one shRNA construct (number 18) for the establishment of sub-lines. Furthermore, the studies on the IKK α knock-down by Hu et al. (1999) and Takeda et al. (1999) suggested that the lack of IKK α would not be deleterious for cells in culture and that establishment of stable cell lines lacking IKK α should be possible.

For the establishment of IKK α sub-lines I used the U-937 cell line. The U-937 cells are myeloid cells arrested in an early stage of the monoblastic differentiation, originally derived from a patient with histocytic lymphoma. The U-937 cells are used as a differentiation model since they have the capacity to differentiate terminally upon treatment with ATRA, VitD3 (cholecalciferol) and TPA (12-O-tetradecanoylphorbol-13-acetate) (Oberg et al. 1993, Botling et al. 1995, Dimberg and Oberg 2003).

Cells were transfected using Amaxa™ electroporation with the pTMP (pSIN-TREmir30-PIG) vector, expressing the IKK α -targeting shRNA 18 or an empty vector. Since I assumed that a higher frequency of stable integration in the genome would be observed when using linear DNA, both vectors were linearized prior to the transfection. Under the selection pressure of 0.8 μ g/ml puromycin, resistant clones started appearing between two to three weeks after the transfection.

The obtained sub-lines were maintained under a constant selection pressure and evaluated for IKK α protein down-regulation by western blot. In the present study, the expression of the shRNA was driven by doxycyclin in a tet-on system, but was found to be active even in the absence of doxycyclin, which led to the establishment of constitutive knock-down sub-lines. The IKK α protein was detected with a monoclonal antibody against IKK α , to avoid a possible cross-detection of IKK β , which could appear with polyclonal antibody due to their close sequence similarity (Figure 8).

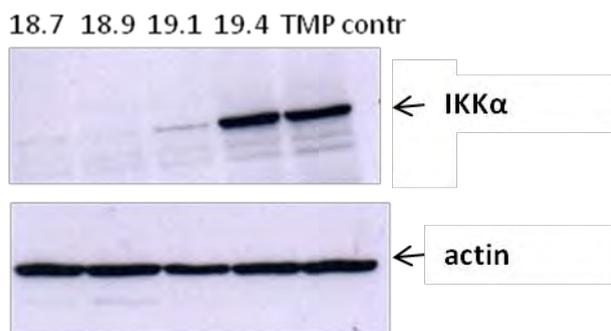


Figure 8. Western blot analysis of IKK α protein levels in whole-cell lysates from the generated U-937 sub-lines. The numbers 18 and 19 refer to the shRNA transfected, the number after the decimal refers to clone number. The TMP control was transfected with an empty vector. Actin was used as a reference protein. Antibodies used were anti-IKK α (sc-7606) and anti-actin (sc-1616).

As expected, the control sub-lines carrying the empty TMP vector did not show a significant difference in the IKK α expression, when compared with the expression in the wild type U-937 cells. On the other hand, different levels of IKK α down-regulation were observed in the sub-lines carrying the IKK α -targeting shRNA, even in the absence of doxycyclin (Figure 8).

Based on these results I selected the 18.7 and 18.9 sub-lines for further studies, as well as one control TMP sub-line.

I furthermore decided to assess the relative levels of *IKK α* mRNA. Surprisingly, I did not find any reduction of *IKK α* mRNA in the sub-lines compared to the U-937 paternal cell line (Figure 9).

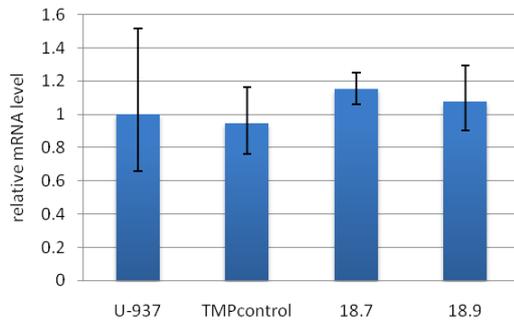


Figure 9. Relative quantification of *IKK α* mRNA levels in the generated sub-lines compared to the paternal U-937 cell line. Quantitative-RT-PCR was performed on cDNA from the U-937 cells, the control clone carrying empty TMP vector (TMPcontrol) and the clones expressing shRNA targeting *IKK α* (18.7 and 18.9). Actin was used as a reference gene. Error bars represent standard error of the mean of three measurements.

In conclusion, I generated stable *IKK α* knock-down U-937 sub-lines in which a strong *IKK α* protein knock-down was not consistent with the lack of reduction in the *IKK α* mRNA level.

IKK β protein levels are reduced in the absence of *IKK α*

I furthermore assayed *IKK β* protein (Figure 10) and relative mRNA (Figure 11) levels in the generated sub-lines. The antibody used for the western blot analysis of *IKK β* gave a few unspecific bands of lower molecular weight, when compared to the *IKK β* protein (87 kDa) (Figure 10). When determining the relative mRNA levels, I found twice higher level of *IKK β* in the empty TMP control vector than in the paternal U-937 cells, but no difference between the TMP control and the generated sub-lines (Figure 11).

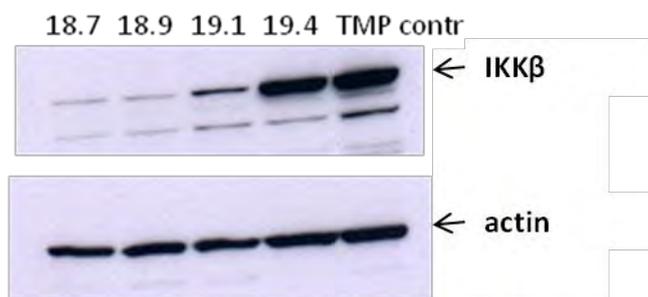


Figure 10. Western blot analysis of *IKK β* protein levels in whole-cell lysates from the generated U-937 sub-lines. The numbers 18 and 19 refer to the shRNA transfected, the number after the decimal refers to clone number. The TMP control was transfected with an empty vector. Actin was used as a reference protein. Antibodies used were anti-*IKK β* and anti-actin (sc-1616).

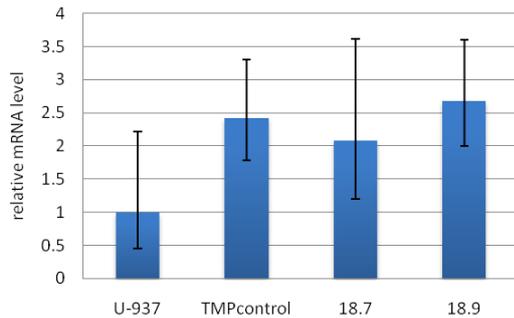


Figure 11. Relative quantification of IKK β mRNA levels in the generated sub-lines compared to the paternal U-937 cell line. Quantitative-RT-PCR was performed on cDNA from the U-937 cells, the control clone carrying empty TMP vector (TMPcontrol) and the clones expressing shRNA targeting IKK α (18.7 and 18.9). Actin was used as a reference gene. Error bars represent standard error of the mean of three measurements.

In conclusion, I found drastically reduced levels of IKK β protein coinciding with the reduction of IKK α protein, and no difference in the relative mRNA levels when compared with the sub-line carrying the empty TMP vector.

Reduced levels of both IKK α and IKK β lead to loss of I κ B α

Since the most prominent function of IKK β is the phosphorylation of I κ B α to target it for subsequent degradation upon TNF α signalling, I decided to evaluate protein levels of I κ B α in the sub-lines having drastically reduced levels of IKK β . Cells were treated with 100 ng per ml TNF α for 1 hour and protein levels were evaluated on western blot (Figure 12). I failed to observe I κ B α degradation upon TNF α stimulation in the paternal U-937 cell line. In contrast, I observed drastically reduced levels of I κ B α protein in the sub-lines 18.7 and 18.9, independent of the TNF α treatment. Normal protein levels of I κ B α were observed in both the U-937 cells and the control TMP clone. However, the clarity of my results was compromised by the quality of the antibody against I κ B α , which gave several bands around the expected size of 39 kDa. This bands might be caused by different modifications on the I κ B α protein or be unspecific. Using another antibody against I κ B α or qRT-PCR is needed to determine this. Actin protein reference is also needed to exclude a possible loading errors or general protein degradation.

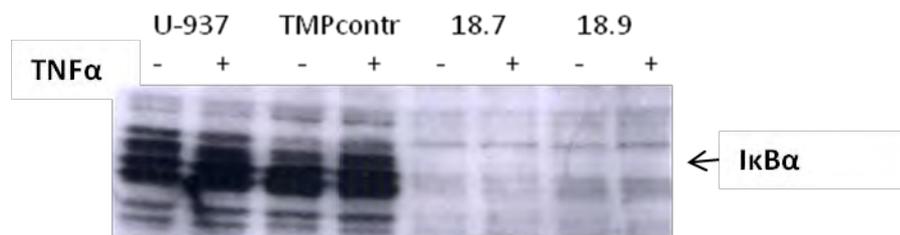


Figure 12. Western blot analysis of I κ B α protein levels in the generated IKK α knock-down U-937 sub-lines. The numbers 18 and 19 refer to the shRNA transfected, the number after the decimal refers to clone number. The TMP control was transfected with an empty vector. The parental U-937 cells were used as a reference. Antibody used was anti-I κ B α (sc-371). + TNF α indicates 1 hour of TNF α treatment, - TNF α – untreated cells.

In conclusion, reduced levels of the I κ B α protein were observed in the sub-lines 18.7 and 18.9, independent of the TNF α treatment. However, the protein detection seems to be unspecific and the experiment lacks the actin control for equal loading.

The NF κ B pathway is constitutively active in the absence of the inhibitor I κ B α

Since the IKK α shRNA knock-down sub-lines of U-937 showed reduced levels of IKK α as well as IKK β and I κ B α proteins. The p50 protein is most commonly associated with I κ B α , the role of which is to retain the p65/p50 heterodimers in the cytoplasm (Häcker and Karin 2006, Perkins 2007, Hayden and Ghosh 2008). Thus, I decided to evaluate the protein levels of the NF κ B subunit p50 (Figure 13). I observed lack of the p105 precursor of p50 in the generated sub-lines, when compared to the parental U-937 and the TMP control cells. This suggests increased levels of p105 to p50 turn over and constitutively active NF κ B in the absence of I κ B α .

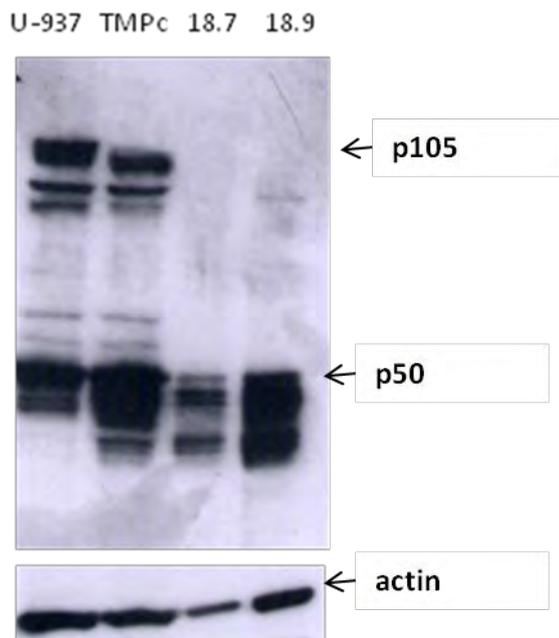


Figure 13. Western blot analysis of p105/p50 protein levels in the generated IKK α knock-down U-937 sub-lines. The numbers 18 and 19 refer to the shRNA transfected, the number after the decimal refers to clone number. The TMP control was transfected with an empty vector. The parental U-937 cells were used as a reference. Actin was used as a control for equal loading. Antibodies used were anti-p50 (sc-7178) and anti-actin (sc-1616).

Reduced levels of both IKK α and IKK β do not impair the ATRA- and VitD3-induced cell cycle arrest

Myeloid cells treated with all-*trans* retinoic acid (ATRA) differentiate into granulocytes. This process is characterized by cell cycle arrest in the G₀/G₁ phase, up-regulation of specific surface-markers such as CD11c and G-CSFR, and acquisition of a mature phenotype. Additionally, VitD3 treatment of myeloid cells induces a monocytic phenotype (Oberg et al. 1993, Botling et al. 1995, Dimberg and Oberg 2003). Thus, if IKK α is involved in the transcription initiation by retinoic acid, the IKK α knock-down would abolish the cell cycle

arrest induced by ATRA and VitD3. Considering this, I decided to test one of the sub-lines (18.9) for their ability to arrest in the G_0/G_1 phase of the cell cycle. Cells were induced with 1 μM ATRA and 0.1 μM VitD3 for 72 hours and cell cycle arrest assessed using propidium iodide (PI) staining of nuclei (Figure 14).

In conclusion, in contrast to my expectations, I did not observe any impairment in the cell cycle arrest of the generated $\text{IKK}\alpha$ knock-down cells.

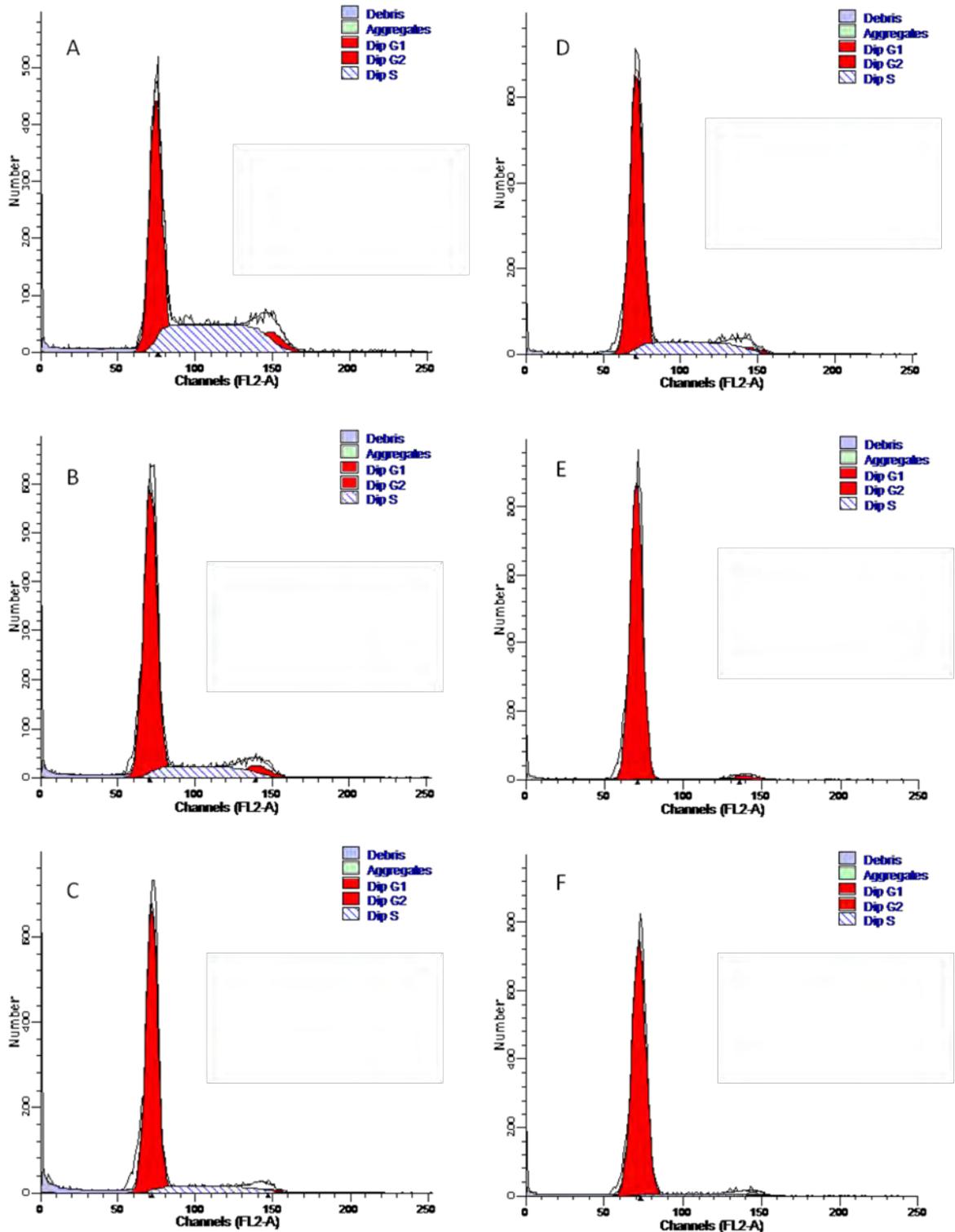


Figure 14. Cell cycle analysis using propidium iodide (PI) staining of cell nuclei after ATRA and VitD3 treatment. Left panels with U-937 cells, A: untreated control; B: 72 hours with 1 μ M ATRA; C: 72 hours with 0.1 μ M VitD3. Right panels with sub-line 18.9, D: untreated control; E: 72 hours with 1 μ M ATRA; F: 72 hours with 0.1 μ M VitD3. Cell debris are presented in blue, cell aggregates in green, diploid cells in the G1 or G2 phase of the cell cycle are in red, diploid cells in the S phase are presented with striped pattern. Percentage of cells present in each phase of the cell cycle is given in the corresponding panel for each treatment. Abbreviations: Dip G1 – diploid cells in G1, Dip G2 – diploid cells in G2, Dip S – diploid cells in S, On the x-axis is the intensity of the PI staining as representative of the DNA amount, on the y-axis the number of cells.

Reduced levels of both IKK kinases do not impair the retinoic acid-and VitD3-target gene response

This study was based on the hypothesis that IKK α is important for the transcription initiation at retinoic acid target genes (Gareus et al. 2007). Thus, the suppression of IKK α would inhibit the induction of retinoic acid-target genes. In the literature there is a growing list of genes that are induced by ATRA, either directly or indirectly (Blomhoff and Blomhoff 2006).

CD38 is a leukocyte differentiation antigen expressed during early stages of myeloid differentiation. CD38 has been shown to be specifically and strongly up-regulated in response to ATRA and VitD3 in human promyelocytic leukemia cells (Drach et al. 1994, Georgii-Hemming unpublished). RetSDR1 codes for a short chain dehydrogenase/reductase widely expressed in human tissues, thought to regenerate retinol in the retinol metabolism. RetSDR1 expression is tightly regulated by retinoids and gene deletion is suggested to play a role in retinol insensitivity and cancer progression (Cerignoli et al. 2002). Based on these observations I decided to evaluate the expression of *CD38* and *RetSDR1* in the sub-lines after ATRA induction. Cells were induced with therapeutic concentrations (10^{-6} M) of ATRA for 6 hours, after which RNA was isolated and relative mRNA levels evaluated by quantitative reverse transcriptase PCR (qRT-PCR) (Figure 15). I observed stronger induction of the *RetSDR1* (Figure 15A) gene in the TMP control sub-lines and even stronger in the IKK α knock-down sublines. The induction of transcription of *CD38* (Figure 15B) by ATRA was stronger in the generated sub-lines compared to the paternal U-937 cells. Whereas, VitD3 induced *CD38* transcription to a lesser extent when compared to ATRA and to a similar level in all cells.

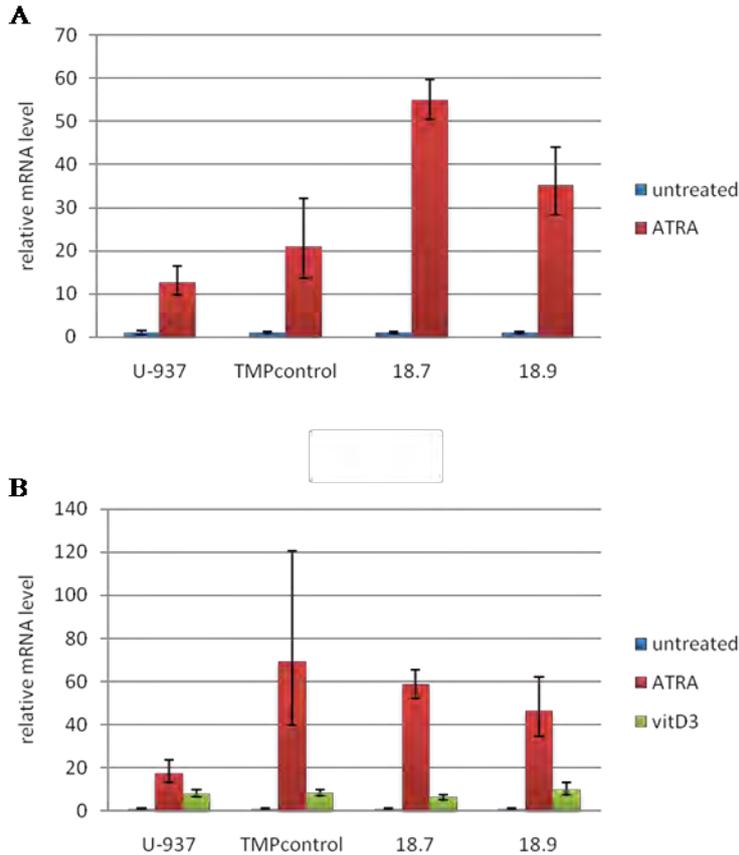


Figure 15. Quantification of *RetSDR1* and *CD38* mRNA after ATRA and VitD3 treatment relative to the untreated cells. A: *RetSDR1* mRNA levels after ATRA treatment. B: *CD38* mRNA levels after ATRA and VitD3 treatment. Quantitative-RT-PCR performed on cDNA from the paternal U-937 cell line, the control clone carrying empty TMP vector (TMPcontrol) and the clones expressing shRNA targeting *IKK α* (18.7 and 18.9). Actin was used as a reference gene. Error bars represent standard error of the mean of three measurements.

In conclusion, despite the simultaneous loss of both *IKK α* and *IKK β* in the generated sub-lines, 10^{-6} M ATRA induced the mRNA from both *CD38* and *RetSDR1* genes.

The IKK inhibitor BMS-345541 inhibits RA-target gene induction in the absence of both *IKK α* and *IKK β*

BMS-345541 (4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline) is identified as a catalytic inhibitor of IKKs (Burke et al. 2003). As such BMS-345541 inhibits the NF κ B-dependent transcription and has proven to be an important tool for investigating further the IKK function. At a concentration of BMS-345541 that inhibits *IKK α* (4 μ M, Burke et al. 2003), it drastically inhibits the RA-target gene induction (Kårehed, K., Tshuikina, M., Nilsson, K. and Öberg, F, personal communication). Consistent with that, I hypothesized that the reduced protein levels of both IKKs in the 18.7 and 18.9 sub-lines would prevent BMS-345541 from exerting its transcriptional block on the RA target genes *CD38* and *RetSDR1*. Cells were pre-treated with 4 μ M BMS-345541 for 30 min and induced with 10^{-6} M ATRA for 6 hours, after which RNA was isolated and relative mRNA levels evaluated in qRT-PCR (Figure 16). As expected, the transcription of both genes was induced after ATRA treatment compared to the transcription of these genes in the untreated cells. A strong difference was observed in the levels in induction between the paternal U-937 cells and the generated sub-

lines, where the induction was much stronger. This suggests contributing factors of the cell selection, rather than a specific involvement of $IKK\alpha$. BMS-345541 alone did not alter the gene expression of $CD38$ and $RetSDR1$, whereas the combination of BMS-345541 and ATRA inhibited the gene induction caused by ATRA alone.

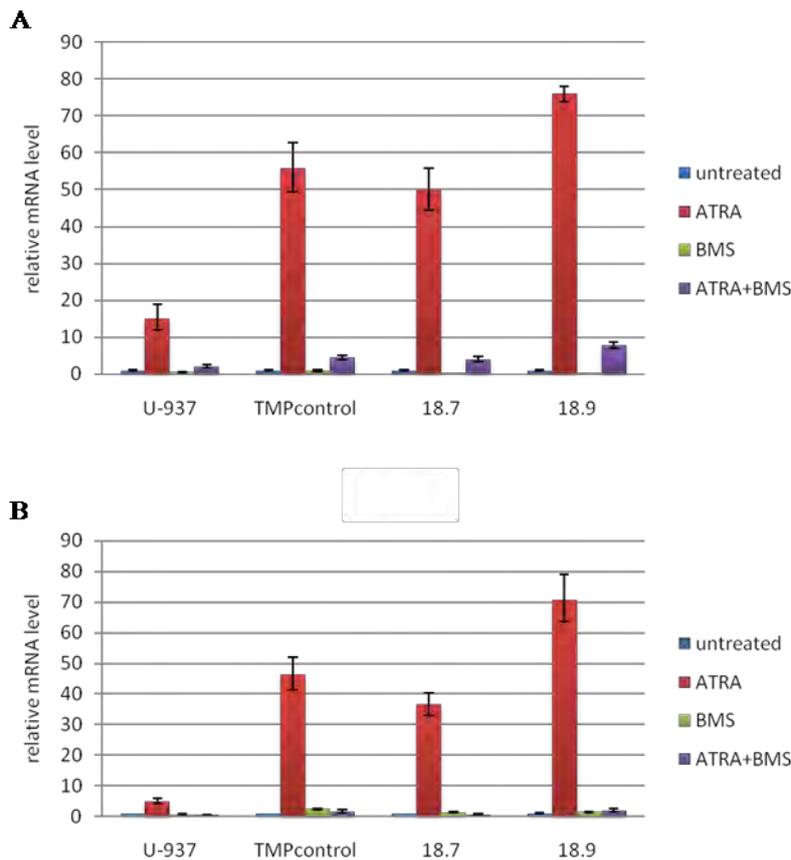


Figure 16. Quantification of $RetSDR1$ and $CD38$ mRNA after ATRA, BMS-345541 and combined treatment with ATRA and BMS BMS-345541, relative to the untreated cells. A: $RetSDR1$ mRNA level. B: $CD38$ mRNA levels. Quantitative-RT-PCR performed on cDNA from the paternal U-937 cell line, the control clone carrying empty TMP vector (TMPcontrol) and the clones expressing shRNA targeting $IKK\alpha$ (18.7 and 18.9). Actin was used as a reference gene. Error bars represent standard error of the mean of three measurements.

In conclusion, the results showed that BMS-345541 inhibited the RA-driven transcription in the presence of both $IKK\alpha$ and $IKK\beta$, but also when cells were seriously compromised in their IKK kinases protein levels.

Discussion

The block of terminal differentiation is a common feature of cancer, especially of haematopoietic malignancies. Though retinoic acid has been known to induce myeloid differentiation for decades, the exact mechanisms behind its function still remain to be identified. What is known is that retinoids are able to modulate gene expression programmes, which ultimately allow the cell to resume a process of maturation (Drumea et al. 2008). However, the precise mechanisms needed to govern the retinoic induced cell differentiation remain not fully understood (Huang et al. 2009). Identifying new transcriptional networks and mechanisms that collaborate in the final goal of setting a malignant cell to exit the cell cycle, gives hope to fight the APL resistance to ATRA and extend retinoid and differentiation treatment to other diseases.

IKK α role at the nuclear retinoic acid receptor (RAR)

The present investigation on the function of the inhibitor of nuclear factor κ B kinase α (IKK α) at the RAR/RXR heterodimer in myeloid cells started from previous results (Kårehed, K., Tshuikina, M., Nilsson, K. and Öberg, F, personal communication) suggesting that the inhibition of the NF κ B pathway delays the ATRA-induced cell cycle exit in the monocytic cell line U-937. Additionally, the RARE-driven transcription was inhibited by the IKK inhibitor BMS-345541 (Kårehed, K., Tshuikina, M., Nilsson, K. and Öberg, F, personal communication). Recently, IKK α is reported to associate to promoters of retinoic acid responsive genes after ATRA stimulation (Gareus et al. 2007). Taken together these results strongly suggested that the IKK complex and in particular IKK α is needed for the retinoic acid-induced gene transcription, which plays a role in the cellular differentiation. Previous results have confirmed IKK α recruitment to RA-responsive promoters in U-937 cells upon 15-30 min of ATRA stimulation (Tshuikina M. personal communication). Thereafter, considering the growing list of retinoic acid responsive genes, IKK α recruitment to a large number of genes and regulatory elements governing complex physiological responses in response to retinoic acid was suggested. To show such a global association of IKK α to the retinoic acid receptor I employed the *in situ* proximity ligation assay (*in situ* PLA), which is a novel cutting-edge technique allowing for direct observation of protein interactions. Using *in situ* PLA I showed that after 15 min of ATRA stimulation IKK α associates with the retinoic acid receptor-alpha (RAR α) and the retinoic X receptor-beta (RXR β). Furthermore, in the same PLA experiment I showed that BMS-345541 could inhibit the interaction between IKK α and RAR α . These results strongly encouraged me to further investigate the role of IKK α for the RA-induced transcription and myeloid differentiation. Additionally, the association of IKK α to RXR β raised an interesting question about IKK α function at other nuclear receptors associating with RXRs, such as the peroxisome proliferator activated receptors (PPARs) and the liver X receptors (LXRs), and a possible nuclear receptor programme crosstalk.

IKK α knock-down

Since gene knock-down probably is the best method to study gene function in mammalian cells, I generated IKK α knock-down sub-lines of the monocytic cell line U-937. U-937 cells

have been used extensively as a differentiation model that responds to different stimuli. All-*trans* retinoic acid induces granulocytic differentiation in U-937, whereas VitD3 induces a monocytic phenotype (Oberg et al. 1993, Botling et al. 1995, Dimberg and Oberg 2003).

IKK α knock-down U-937 sub-lines were generated using small hairpin RNA (shRNA) targeting IKK α , which was expressed from a doxycyclin-inducible promoter. Surprisingly I found out that IKK α protein levels were drastically down-regulated even in the absence of doxycyclin. This effect can be explained by the fact that in such stable cell lines DNA constructs are integrated randomly in the genome and their expression is strongly dependent on the chromatin structure at this site. It is also known that a tet-on system, where expression is activated by tetracycline or doxycyclin, often low basal expression levels due to traces of antibiotics in the sera added to the media of the growing cells. IKK α mRNA levels in the knock-down cell lines were not significantly different from those in the parental U-937 cell line. Protein down-regulation by RNA interference (RNAi) is suggested to function through three distinct mechanisms, target mRNA degradation, translational repression and miRNA-mediated mRNA decay. The actual mode of action is governed by the complementarity between the miRNA and the target mRNA. The target mRNA is suggested to get degraded when it is recognised by a perfectly complementary miRNA incorporated into RISC (RNA-induced silencing complex), which possesses nuclease activity. The endogenous microRNAs in animals are suggested to act mainly through a different mechanism, which does not require a complete complementarity to the target mRNA, but leads to a translational silencing by sequestering the ribosome (Zhang et al. 2007). Therefore, my results suggested that, though fully complementary to the IKK α mRNA, the shRNA used in this study leads to a translational silencing rather than mRNA degradation.

IKK α and IKK β protect each other from degradation

The IKK complex has two catalytic subunits IKK α and IKK β , which have a significant degree of sequence similarity (Hayden and Gosh 2008). To be able to discriminate between the different functions of the two kinases, I had to ensure the IKK β expression in the sub-lines. As expected, IKK β mRNA levels were stable, whereas I observed a dramatic decrease in IKK β protein levels coinciding with the loss of IKK α . Considering this I performed further bioinformatic analysis, which confirmed that the shRNA chosen for this study targets only the IKK α mRNA. As it is widely known (Perkins 2007) and as I have showed with the proximity ligation assay, IKK α and IKK β associate in a protein complex. Since gene silencing by RNAi became a widely used technique, recent reports suggest that proteins acting in complexes confer mutual protection from degradation to each other. This has been reported for the Bcl-2 family members, the anti-apoptotic Mcl-1 that binds to the pro-apoptotic Bim at the mitochondrial membrane to block the mitochondrial apoptosis cascade. When silencing each with shRNA, the other member is targeted for ubiquitin-proteasome degradation (Wuilleme-Toumi et al. 2007). Additionally, siRNA targeting of each of the PRC2 (Polycomb-repressive complex 2) components (EZH2, EED or SUZ12) was shown to lead to a post-transcriptional silencing of all three (Fiskus et al. 2006, Tan et al. 2007). Furthermore, a recent report on the newly identified partner in the PRC2 complex Jarid2 prompts towards the same mode of protection for all proteins in the complex (Li et al. 2010). I thus favour the idea that a similar mechanism of mutual protection from degradation is valid for IKK α and IKK β in human myeloid cell lines. However, mice IKK α gene knock-out studies and RNAi silencing, and IKK α RNAi silencing in other human cell lines (e.g. the

human breast cancer cell line MCF7) do not prompt towards the same mechanism (Gareus et al. 2006, Zhu et al. 2007, Tu et al. 2006).

Constantly active NFκB classical pathway in the absence of the IKKα

In the classical NFκB pathway, the main role of IKKα and IKKβ is to phosphorylate the NFκB inhibitor IκBα (Adli et al. 2010), which ultimately leads to the IκBα degradation and nuclear translocation of the transcription factor p65/p50. Additionally, p50 is the processed form of the precursor p105. The p105 precursor appears to be mainly processed constitutively, but it also shares structural and functional similarity with IκB proteins, and as such can be regulated by IKKβ phosphorylation. The modes of degradation appear to differ between IκBα and p105, where the former depends on ubiquitination and the second does not (Hayden and Gosh 2008). I thus decided to evaluate the protein levels of IκBα and p50. Surprisingly, I observed drastically low IκBα protein levels in both TNFα treated and untreated sub-lines having reduced levels of IKKα and IKKβ. IκBα is a known NFκB-inducible gene, generating an important negative feedback loop, which prevents NFκB overactivation. Considering these data, I decided to evaluate the NFκB subunit p105/p50 protein levels and I observed increased levels of p105 turn over to p50 and nearly undetectable levels of p105 precursor, concomitant with the down-regulation of IKKα, IKKβ and IκBα. The p65/p50 transcription factor heterodimer is the primary target for IκBα, where degradation of IκBα allows for rapid processing of p105 to p50 and the nuclear localization of the heterodimer. Mice IκBα gene knock-out studies have previously indicated an irreplaceable function of the inhibitor IκBα for NFκB termination and prolonged NFκB p65/p50 nuclear localization in the absence of it (Beg et al. 1995, Klement et al. 1996). Thus, I suggested that the NFκB classical pathway is constantly active in the absence of the IκBα inhibitor, where p105 is being constitutively processed to p50. To confirm this, further analysis on the activation of NFκB p65/p50-target genes would be needed.

Reduced protein levels of both IKKα and IKKβ do not abrogate the cell cycle arrest caused by ATRA

The inhibition of the NFκB pathway by the expression of the super repressor IκBα, in which the two serine amino acids at positions 32 and 36 are substituted with alanine (S32A/S36A) in the U-937 cells delays the cell cycle arrest and differentiation in response to ATRA. Additionally, the IKK inhibitor BMS-345541 inhibits of the expression of a RARE-driven luciferase reporter (Kårehed, K., Tshuikina, M., Nilsson, K. and Öberg, F, personal communication). Taken together, these results suggest that the IKK complex in the NFκB pathway plays a role for the ATRA-induced transcriptional regulation at the RAR/RXR and the cell cycle exit. Controversially, I did not observe any deviations in the ability to arrest the cell cycle in U-937 sub-lines having drastically reduced protein levels of both IKKα and IKKβ. To evaluate the importance of IKKα for the retinoic acid-induced transcription, I decided to use the 18.7 and 18.9 sub-lines to determine the induction of two RA-target genes. Two genes were selected, *CD38*, which is expressed in the early stages of the leukocyte differentiation and it is strongly induced by retinoic acid and *RetSDRI*, widely expressed in human tissues and regulated by RA as a feed-back loop in the retinoid metabolism (Drach et al. 1994, Georgii-Hemming, personal communication, Cerignoli et al. 2002). Consistent with the cell cycle arrest experiments, I found no abrogation in the ability of ATRA to induce these two genes in U-937 sub-lines having reduced IKKα and IKKβ protein levels. I currently

cannot exclude the involvement of the third IKK subunit, NEMO (IKK γ), in the regulation of the cell cycle. In fact, several IKK-independent functions of NEMO have been suggested. NEMO was found to translocate into the nucleus and enhance transcriptional activity through recruiting the CBP co-activator (Bracken et al. 2005, Perkins 2007). Moreover, I currently cannot exclude that targeting IKK α by RNAi, does not lead to NEMO protein destabilization, as it was suggested for IKK β reduced protein levels in the sub-lines. Further experiments need to be performed to determine the effect of the IKK α silencing for the other IKK component, NEMO (IKK γ) and the mode of down-regulation of IKK β . If IKK β is targeted for proteasomal degradation in the absence of IKK α , treating the sub-lines with a proteasomal inhibitor, would restore IKK β protein levels, but should not have an effect on IKK α down-regulation.

A recent study on ATRA-induced cell differentiation showed that different sets of genes are responsible for cell cycle arrest and cellular differentiation (Huang et al. 2009). This suggests that deeper investigation of the ability of the IKK α knock-down sub-lines to differentiate towards a specific cell lineage might be needed in order to prove a role for IKK α in the overall process of cell differentiation.

The IKK inhibitor BMS-345541 represses the ATRA-induced gene activation

Controversially to the strong down-regulation of both IKK catalytic subunits I found that the IKK inhibitor BMS-345541 could repress the ATRA-induced activation of *CD38* and *RetSDRI* in the 18.7 and 18.9 sub-lines. I consider several possible explanations. First, protein targeting by RNAi is currently widely employed method to study protein function, but it has the disadvantage that it does not lead to complete protein removal. It is possible that residual levels of IKK α continue to function for the transcriptional regulation in the nucleus. In fact, the phenotype of DNMT1 protein down-regulation compared to the gene knock-out varies significantly, where the pivotal role of DNMT1 in regulating global genomic stability is retained even at reduced protein levels, and that gene knock-out is deleterious for the cell (Brown and Robertson 2007). Second, I do not exclude the possibility that BMS-345541 targets another kinase or protein that functions at the RAR/RXR heterodimer. In fact, the suggested mode of function for BMS-345541 is through binding to allosteric sites on IKK α and IKK β , which leads to conformational changes and subsequently affects the active site, rather than competition for the ATP-binding (Burke et al. 2003). It is thus possible that BMS-345541 affects not only the kinase activity, but the stability of the components of the IKK complex. Considering this, further experiments are needed to determine the action of the IKK complex at the retinoic acid receptor. Additionally, a recent report shows that BMS-345541 treatment disrupts mitotic cell cycle transitions and indirectly blocks several cell cycle kinases (Blazkova et al. 2007). Taken together, these results prompt towards a new still unidentified role of the IKK complex in the regulation of the cell cycle.

Materials and Methods

Biological materials

The human monoblastic U-937 (Sundstrom and Nilsson 1976) cells and the generated sub-lines (See Stable transfection using Amaxa™ electroporation bellow) were maintained in RPMI 1640 medium (Sigma-Aldrich Sweden AB) and the human fibroblast U3A (McKendry et al. 1991) cells were maintained in Dulbecco's Modified Eagles Medium (DMEM, Sigma). Both media were supplemented with 10% foetal bovine serum (FBS, Sigma), 1% L-glutamine (Sigma) and 1% penicillin – streptomycin (Sigma). The medium for the stable U-937 sub-lines was in addition supplemented with 0.8 µg/ml puromycin.

To generate IKK α knock-down sub-lines I used short hairpin (shRNA) construct targeting the human IKK α gene identified at the RNAi Codex at <http://cancan.cshl.edu/cgi-bin/Codex/Codex.cgi>, which lists clones from the Hannon-Elledge shRNA-libraries (mouse and human). The shRNA selected for this study has an accession number NM_001278, Hairpin ID 1:v2HS_113018 and mature sequence product ccagatactttcttacta. The construct was later referred with the last two numbers of its ID, given in bold. The efficiency of the shRNA down-regulating IKK α protein levels was confirmed prior to this project (Kalushkova A. Course report in advanced techniques in molecular medicine, 2007, Uppsala University). The construct, which was purchased through Open Biosystems was initially integrated into a pSM2 (pSHAG-MAGIC 2) vector. To obtain regulation over the shRNA expression I excised the shRNA cassette from the pSM2 vector using XhoI and EcoRI restriction endonucleases and cloned it into a TMP (SIN-TREmir30-PIG) vector (Open Biosystems), the expression of which is driven by a doxycyclin-inducible promoter (Table 1).

Table 1. Plasmids.

Vector	Restriction sites used to clone the shRNA cassette	Promoter
pSM2 (pSHAG-MAGIC 2)	XhoI and EcoRI	U6 Pol III promoter
pTMP (pSIN-TREmir30-PIG)	XhoI and EcoRI	tetracycline-regulated Pol II promoter (TRE-CMV)

Plasmids were propagated in DH5 α competent bacteria (Subcloning efficiency DH5 α , Invitrogen), according to manufacturers' recommendations. Bacteria were grown on Luria-Bertani (LB)-agar plates or in LB liquid cultures containing 10% bacto tryptone, 5% yeast extract, 5% NaCl and 15% bacto agar (only for the LB-agar plates) in deionised water including 50 µg/ml chloramphenicol and 50 µg/ml kanamycin for the pSM2 (pSHAG-MAGIC 2) plasmid, or 100 µg/ml ampicillin for the pTMP (pSIN-TREmir30-PIG) plasmid. All bacteria growth incubations were at 37°C, liquid cultures before plasmid preparation were grown at light shaking. Plasmids were prepared using EndoFree Plasmid Maxi kit (QIAGEN) according to the manufacturer's protocol.

Cell cycle arrest and target gene expression was induced with 1 µM all-*trans* retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) or 0.1 µM 1 α ,25(OH) $_2$ -vitamin D3 (Hoffman-La Roche, Switzerland) on exponentially growing cells. The activation of the NF κ B pathway

was induced with 100 ng/ml TNF α . IKK complex inhibition was induced with 4 μ M BMS-345541.

Stable transfection using Amaxa™ electroporation

The pTMP (pSIN-TREmir30-PIG) vector was linearized with 1U per μ g of the restriction endonuclease ApaLI for increasing the efficiency of integration in the genome. About 1×10^6 U-937 cells were transfected with 0.5 μ g of the vector using Amaxa™ electroporation according to the manufacturer's protocol. Briefly, 1×10^6 cells were harvested at 490 x g for 5 min. The vector DNA and 100 μ l of Nucleofector V solution (Amaxa Biosystems) were added and cells were electroporated as recommended. After the transfection cells were maintained in RPMI 1640 medium (Sigma) plus 10% FBS, 1% L-glutamine, 1% penicillin – streptomycin antibiotics. Selection by 0.8 μ g/ml puromycin was applied to the cells 24 hours after the transfection.

Protein extraction

Cells were harvested at 490 x g for 5 min, washed once in 1 x PBS (phosphate buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ at a pH of 7.4) and lysed for 10-20 min in cell lysis buffer containing 0.1 M Tris-HCl (pH 8.0), 0.15 M NaCl, 1% nonyl phenoxyethylpolyethoxyethanol (NP-40), 2 x complete™ EDTA-free protease inhibitor (Roche), 0.1 mM NaVO₃, 10 mM NaF, 1 mM phenylmethanesulphonylfluoride (PMSF), 1 mM ZnCl₂, 50 μ M Na₂MoO₄ and 1 mM dithiothreitol (DTT). Protein lysates were separated from the cell debris at 16000 x g at 4°C for 10 min. Protein concentrations were measured using Bio-Rad Protein assay kit according to the manufacturer's protocol and self-prepared standards of albumin protein diluted at 1, 2, 5, 8, 10, 15, 20 and 25 μ g/ml (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

From each sample 10 μ g of protein was fractionated on pre-cast 10% Bis-Tris denaturing gels (NuPAGE® Novex, Invitrogen, Carlsbad, CA, USA). The protein contents were transferred to nitrocellulose membrane Hybond-C Extra (Amersham Biosciences) and blocked with 5% dry milk in 1 x TBS (Tris-buffered saline, 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) and 0.1% Tween. After the blocking, primary antibodies diluted 1:1000 in 5% dry milk blocking solution (as previously described) were added to the protein containing membranes. The antibodies used are presented in Table 3. The membranes were incubated with the primary antibodies (Table 2) over-night at 4°C. After the incubation the membranes were washed with 1 x TBS containing 0.1% Tween for 5 x 5 min. After the wash, secondary HRP (horseradish peroxidase)-linked antibodies against the primary antibodies species (Table 2) diluted 1:5000 in 5% dry milk blocking solution were added to the membranes and incubated for 1 hour at room temperature. After the incubation the membranes were washed as previously described. Protein target recognition was detected using the ECL plus Western Blotting Detection System (Amersham Biotech, Uppsala, Sweden) according to the manufacturer's protocol.

Table 2. Antibodies used in western blot and PLA analysis.

Antibody against	Type	Species	Manufacturer	Application
IKK α	Primary	Mouse monoclonal	Santa Cruz Biotechnology inc., Santa Cruz, CA, USA (sc-7606)	Western blot, PLA
actin	Primary	Goat polyclonal	Santa Cruz Biotech. (sc-1616)	Western blot
IKK β	Primary	Mouse monoclonal	Abgent, CA, USA (AM8109a)	Western blot, PLA
I κ B α	Primary	Rabbit polyclonal	Santa Cruz Biotech. (sc-371)	Western blot
p50	Primary	Rabbit polyclonal	Santa Cruz Biotech. (sc-7178)	Western blot
RAR α	Primary	Rabbit polyclonal	Santa Cruz Biotech. (sc-551)	PLA
RXR β	Primary	Mouse monoclonal	Santa Cruz Biotech. (sc-741)	PLA
Goat HRP-linked	Secondary	Rabbit polyclonal	DAKO	Western blot
Rabbit HRP-linked	Secondary	Donkey polyclonal	GE Healthcare Limited, Little Chalfont Buckinghamshire, UK	Western blot
Mouse HRP-linked	Secondary	Sheep polyclonal	GE Healthcare	Western blot

FACS analysis

For the cell cycle arrest 1×10^6 cells at concentration 0.2×10^6 cells/ml were induced with 10^{-6} M all-trans retinoic acid (ATRA) for 72 hours. Cell nuclei for the analysis of the DNA content were prepared according to the method previously described by Vindelov (Vindelov et al. 1983). Briefly, after the indicated incubation time, cells were washed once in 1 x PBS and subjected to 0.03 mg/ml trypsin (Sigma) for 10 min. Trypsinization was stopped by adding 0.5 mg/ml trypsin inhibitor (Sigma) and 0.08 mg/ml RNase A (Sigma) to the samples and incubation for 10 min. The prepared nuclei were stained with 0.2 mg/ml propidium iodine (Sigma) for 10-20 min. All incubations were carried out at room temperature. The DNA content per nucleus was analyzed using a FACScan instrument and the Modfit Software (Becton Dickinson, Mountain View, CA, USA). PI was excited at 488 nm laser light and detected through 562-588 nm band pass filter.

RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol[®] (Invitrogen) according to the manufacturer's protocol. RNA was resuspended in sterile Milli-Q water and DNase treatment was performed using DNase treatment and removal reagents – DNA-free[™] (Ambion), for 1 hour at 37°C. RNA concentration was determined using NanoDrop spectrophotometer according to the manufacturer's recommendations (Thermo Fisher Scientific Inc.). Reverse transcription using random primers (Invitrogen) was performed on 1 μ g of total RNA using SuperScript[™] III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol.

Quantitative Real-Time RT-PCR

Each independent quantitative real-time reverse transcriptase PCR (qRT-PCR) reaction contained 5 ng of cDNA, 12.5 μ l Platinum[®] SYBR[®] Green qPCR SuperMix UDG with Rox (Invitrogen) and 0.3 mM of each forward and reverse primers in a total volume of 25 μ l. All reactions were performed in triplicates. The sequence of the primers used is given in Table 3. The qRT-PCR conditions were as follows: 95°C for 2 min followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. The run and the analysis were performed using a Mx3000P instrument and software (Stratagene).

Table 3. Primers^a used in this study for the Quantitative Real-Time RT-PCR.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
<i>CD38</i>	cgggatccattgagcate	atcaatctgccagact
<i>RetSDR1</i>	atttcaagagtggccaggtg	ggccgagcaatacaggaat
<i>β-actin</i>	actggaacggtgaaggtgacag	ggtggcttttaggatggcaag

^a Primers were designed using the free on-line available Primer3 program at <http://frodo.wi.mit.edu/>.

***In situ* proximity ligation assay**

For the *in situ* proximity ligation assay (*in situ* PLA) adherent U3A cells were plated at a concentration of 4×10^4 cells/well in 8 chamber polystyrene tissue culture class slides on the day before the experiment (BD Falcon, BD Biosciences, Erembodegen, Belgium). On the day of the experiment the cells were induced with 10^{-6} M all-trans retinoic acid (ATRA) for 15 min and independently with 4 μ M BMS-345541 (4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline) for 15 min and 10^{-6} M ATRA for an additional 15 min, washed with 1xTBS (Tris-buffered saline) for 5 min and fixed using Zn-fix (0.1 M Tris-HCl containing 0.5 mg/ml Ca-acetate, 5 mg/ml Zn-acetate, 5 mg/ml ZnCl₂ and 0.2% Triton-X-100) for 15 min on room temperature. After the fixation, glass slides with cells were washed again with 1xTBS, dipped in 70% ethanol and dried. The *in situ* proximity ligation assay was then performed according to manufacturer's protocol (Duolink[™] *in situ* PLA, Olink Biosciences, Uppsala, Sweden). In brief, slides were blocked with blocking solution provided by the manufacturer or 3% BSA (bovine serum albumin) for 60 min at 37°C. The primary antibodies (described in Table 3) were diluted in the Duolink[™] antibody diluent at the desired concentration and incubated over night at 4°C. On the next day the slides were washed 3 x 5 min with 1xTBS including 0.1% Tween under gentle agitation at room temperature, and the PLA anti-mouse and anti-rabbit probes were applied and the samples were incubated for 2 hours at 37°C. After the incubation the slides were washed 3 x 5 min with 1xTBS + 0.1% Tween under gentle agitation at room temperature. Ligation was performed with a T4 ligase for 30 min at 37°C and slides were washed 2 x 5 min with 1xTBS + 0,1% Tween. The rolling circle amplification (RCA) was performed for 90 min at 37°C and slides were washed 2 x 5 min with 1xTBS + 0,1% Tween. Hybridization with labelled oligos was performed for 60 min at 37°C protected by light. Final washing was performed protected by light at room temperature as follows, 2 x saline sodium citrate (SSC; 1 x SSC: 3M sodium chloride and 300 mM trisodium citrate, pH 7) + 0.05% Tween 20 for 2 min, 1 x SSC + 0.05% Tween 20 for 2 min, 0.2 x SSC for 2 min, 0.02 x SSC for 2 min and 70% ethanol for 5 min. All the steps after the primary antibodies were performed with the Duolink[™] *in situ* PLA kit kindly provided by Olink Biosciences. Slides were mounted with *SlowFade*[®] Gold antifade reagent (Molecular Probes[™], Invitrogen, Eugene, Oregon, USA). Fluorescence was detected on an Epi-fluorescent Carl Zeiss microscope using AxioVision Rel. 4.6 software. The RCA products were counted using the BlobFinder free software available at <http://www.cb.uu.se/~amin/BlobFinder/>.

Acknowledgements

This thesis started long time ago, to turn into an incredible experience and struggle, in which I hope I have grown and learned some essential lessons. My sincere gratitude goes most of all to two people whom I own the possibility for doing this:

My thesis co-ordinator, Karin Carlson, for her enormous patience with me, in which she never lost her temper, though I honestly deserved it! I sincerely appreciate this!!! For her grand dedication to her work and her students, which is truly impressive!

The best supervisor, Fredrik Öberg, for accepting me in his group long time ago, for his patience, support, knowledge all the way through here and further down the PhD path!!! More than four years after I started, I enjoy my work so incredibly much! I hope that the IKK α story will turn into our big success!!!

I would further like to thank,
Inger and all the rest of the past and present people in our lab for turning hard work into great time!

All my friends in Sweden for making me feel truly at home!!!

My family for their great support and trust!

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